Bortezomib Sensitizes Pancreatic Cancer Cells to Endoplasmic Reticulum Stress-Mediated Apoptosis

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Abstract

Bortezomib (PS-341, Velcade) is a potent and selective inhibitor of the proteasome that is currently under investigation for the treatment of solid malignancies. We have shown previously that bortezomib has activity in pancreatic cancer models and that the drug induces endoplasmic reticulum (ER) stress but also suppresses the unfolded protein response (UPR). Because the UPR is an important cytoprotective mechanism, we hypothesized that bortezomib would sensitize pancreatic cancer cells to ER stress-mediated apoptosis. Here, we show that bortezomib promotes apoptosis triggered by classic ER stress inducers (tunicamycin and thapsigargin) via a c-Jun NH2-terminal kinase (JNK)–dependent mechanism. We also show that cisplatin stimulates ER stress and interacts with bortezomib to increase ER dilation, intracellular Ca2+ levels, and cell death. Importantly, combined therapy with bortezomib plus cisplatin induced JNK activation and apoptosis in orthotopic pancreatic tumors resulting in a reduction in tumor burden. Taken together, our data establish that bortezomib sensitizes pancreatic cancer cells to ER stress-induced apoptosis and show that bortezomib strongly enhances the anticancer activity of cisplatin.

Introduction

The proteasome is responsible for the degradation of most cellular proteins and is therefore key to the regulation of many processes, including cell cycle progression and apoptosis. The role of the proteasome in regulating the growth and survival of tumor cells makes it an attractive therapeutic target. Bortezomib, also known as PS-341 or Velcade, is a potent and selective inhibitor of the proteasome that is currently being evaluated for the treatment of various cancers (1). It has shown activity in multiple myeloma and recently received Food and Drug Administration approval for treatment of the disease. However, single-agent therapy produces responses in a minority (33%) of multiple myeloma patients and even lower activity in solid tumors (2, 3). Thus, there is currently strong interest in exploiting the unique mechanisms of action of bortezomib within the context of combination chemotherapy, but how to best do so remains unclear.

Disruptions in Ca2+ homeostasis, inhibition of protein glycosylation, and accumulation of misfolded proteins can all challenge the function of the endoplasmic reticulum (ER)-Golgi network, resulting in ER stress (4). The accumulation of unfolded proteins can be induced by agents, such as tunicamycin, which blocks N-linked protein glycosylation; brefeldin A, which inhibits ER to Golgi transport; or DTT, which impairs the formation of disulfide bonds (5). Another agent, thapsigargin, also induces ER stress via inhibition of the sarcoplasmic/endothoplasmic Ca2+-ATPase, which disrupts ER calcium homeostasis (6). Cells respond to ER stress via activation of a cytoprotective signaling pathway termed the unfolded protein response (UPR; ref. 7). The first effect of the UPR is to reduce the protein synthetic load by inhibiting bulk translation via PKR-like ER kinase phosphorylation of eif2α (8). Phosphorylation of eif2α redirects it to alternative transcriptional targets, including protein chaperones (GRP78/BiP) and proteasomal subunits (9). These effects cooperate to promote increased degradation of misfolded or aggregated proteins via the proteasome. However, if the UPR is overwhelmed, ER stress triggers a unique pathway of apoptosis that is mediated via activation of ER-resident caspases [caspase-12 in murine cells (10) and caspase-4 in human cells (11, 12)].

Recent work from our laboratory and others showed that bortezomib induces ER stress-mediated apoptosis in tumor cells (13–16). Interestingly, ER stress has also been implicated in the effects of at least two chemotherapeutic agents [cisplatin and geldanamycin/17-allyl-amino-geldanamycin (17-AAG); refs. 17–19]. Here, we report that bortezomib interferes with the UPR and sensitizes pancreatic cancer cells to apoptosis stimulated by the classic ER stress inducers tunicamycin and thapsigargin. Furthermore, we show that bortezomib enhances ER stress induced by cisplatin, resulting in increased antitumor activity in an orthotopic pancreatic cancer xenograft model. In contrast, our results suggest that 17-AAG interferes with bortezomib-induced c-Jun NH2-terminal kinase (JNK) activation and apoptosis. Taken together, our results suggest that bortezomib-mediated disruption of the UPR represents a novel strategy to enhance the antitumor activity of cisplatin and any other agent that induces cell death via a classic ER stress-dependent mechanism.

Materials and Methods

Animals and cell lines. Male nude mice (BALB/c background) were purchased from the American Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). L3.6pl human pancreatic cancer cells were established as described previously (20). The murine cell line L929 was kindly provided by L.J. Fincher (University of Texas, M.D. Anderson Cancer Center, Houston, TX). Cell lines were maintained in MEM supplemented with 10% fetal bovine serum (FBS) along with sodium pyruvate, nonessential amino acids, l-glutamine, vitamins, and antibiotics under conditions of 5% CO2 in air at 37°C.

Antibodies and chemicals. Antibodies were obtained from the following commercial sources: anti–cytochrome c, heat shock protein...
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Quantification of DNA fragmentation. DNA fragmentation was measured by PI staining and fluorescence-activated cell sorting (FACS) analysis as described previously (21). Following incubation with indicated concentrations of bortezomib, 1 μmol/L thapsigargin, 5 μg/mL tunicamycin, 40 μmol/L SP600125, 20 μmol/L cisplatin, 1 μmol/L 17-AAG, 1 μmol/L gencitabin, or combinations of the compounds, cells were harvested, pelleted by centrifugation, and resuspended in PBS containing 50 μg/mL PI, 0.1% Triton-X-100, and 0.1% sodium citrate. For the SP600125 studies, cells were preincubated with the inhibitor for 1 hour before exposing them to bortezomib. Cells were incubated with the PI solution and flow cytometric analysis of stained cells was done with a Becton Dickinson FACSCalibur (San Jose, CA).

Measurement of caspase-3 activity. Cells (1 × 10^6) were plated in 10-cm dishes with 10% FBS/MEM and allowed to attach for 24 hours. Cells were then incubated with various agents as described for DNA fragmentation analysis. Following incubation, cells were washed in PBS and resuspended in appropriate buffers as described in the FITC-conjugated monoclonal active caspase-3 antibody apoptosis kit (PharMin, San Jose, CA). Cells were fixed, permeabilized, and stained with FITC-conjugated caspase-3 antibody as directed by the kit manufacturer. Flow cytometric analysis of stained cells was done with a Becton Dickinson FACSCalibur (San Jose, CA).

Immunoblotting. Cells (1 × 10^6) were incubated with 100 mmol/L bortezomib unless otherwise stated, 40 μmol/L SP600125, 20 μmol/L cisplatin, 1 μmol/L 17-AAG, 5 μg/mL tunicamycin, 1 μmol/L gencitabin, or drug combinations. For the SP600125 studies, cells were pretreated for 1 hour before bortezomib treatment. Cells were collected using a cell scraper at 4°C and then lysed as described previously (22). Total cellular protein (~ 25 μg) from each sample was subjected to SDS-PAGE, proteins were transferred to nitrocellulose membranes, and the membranes were blocked with 5% nonfat milk in a TBS solution containing 0.1% Tween 20 for 1 hour. The blots were then probed with primary antibodies, washed, and probed with species-specific secondary antibodies coupled to horseradish peroxidase. Immunoreactive material was detected by enhanced chemiluminescence (West Pico, Pierce, Inc., Rockville, IL).

Preparation of cytosolic extracts for cytochrome c measurement. Cells were preincubated with SP600125 for 1 hour before exposing them to bortezomib for 24 hours. Cells were harvested, washed twice in PBS, resuspended in lysis buffer [20 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, and a protease inhibitor tablet], and incubated on ice for 15 minutes. Lysates were then centrifuged at 10,000 × g for 15 minutes at 4°C. Supernatants were collected and subjected to immunoblotting.

Transmission electron microscopy. Cells were exposed to drugs on six-well plates for 12 hours and then harvested. Cells were fixed with 3% glutaraldehyde and 2% paraformaldehyde dissolved in 0.1 mol/L sodium cacodylate. Transmission electron microscopy of cells was done as described previously (23). Briefly, sections were cut in a LKB Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a LKB Ultrostainer, and examined in a JEM 1010 transmission electron microscope (JEOL, Inc., Peabody, MA). Digital images were obtained using the AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA).

Clonogenic survival assays. Cells were treated with 10 or 100 mmol/L bortezomib with or without 40 μmol/L SP600125 for 12 hours. After drug treatment, 300 cells per well were plated into six-well plates with fresh medium for 14 days. The colonies were washed with PBS, fixed with methanol, and stained with crystal violet. The colonies were counted using a gel documentation system (Alpha Innotech, San Leandro, CA).

Small interfering RNA–mediated silencing of caspase-4 and IRE1α. Cells were transfected in six-well plates with specific or nontarget control small interfering RNA (siRNA) constructs (Dharmacon, Lafayette, CO) for 40 hours according to the manufacturer’s protocol. The constructs used were the siRNA SMARTPool IRE1α and caspase-4 siRNA sense 5′-GAGACUAUAGUGUGAAGUGAUAAU-3′ and antisense 5′-UACAUUCUCAGACUGUCCUU-3′. For control, siRNA directed against firefly luciferase was used. Cells were transfected with 200 nmol/L of the above siRNA using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Following silencing, cells were treated for 24 hours with 100 nmol/L bortezomib, 20 μmol/L cisplatin, or combination and DNA fragmentation was quantified by PI-FACS. Efficiency of RNA interference (RNAi) was measured by immunoblotting.

Measurement of intracellular Ca2+ levels. Cells were grown in medium with or without 100 nmol/L bortezomib, 20 μmol/L cisplatin, or combination for 12 hours. Cells were collected, washed in PBS, and incubated with 1 μmol/L calcium green-1-AM (Molecular Probes, Eugene, OR) for 30 minutes. Flow cytometric analysis of stained cells was done with a Becton Dickinson FACSCalibur.

Orthotopic implantation of tumor cells and treatment schedule. L3.6pl cells were harvested from culture flasks and transfected to serum-free HBSS. Male nude mice were anesthetized with methoxylurane, a small left abdominal flank incision was made, and tumor cells (1 × 10^3) were injected into the subcapsular region of the pancreas using a 30-gauge needle and a calibrated push button–controlled dispensing device (Hamilton Syringe Co., Reno, NV). To prevent leakage, a cotton swab was held cautiously for 1 minute over the site of injection. The abdominal wound was closed in one layer with wound clips (Autoclip; Clay Adams, Parsippany, NJ). Tumors were established for 14 days. Animals were then injected i.p. with 1 mg/kg bortezomib, 5 mg/kg cisplatin, or a combination of the drugs every 72 hours for 14 days. Mice were sacrificed and primary tumors in the pancreas were excised and weighed. For immunohistochemistry, tumor tissue was formalin fixed and paraffin embedded.

Quantification of phosphorylated c-Jun NH2-terminal kinase levels. Tumors were characterized using colorimetric immunohistochemistry to determine phosphorylated JNK levels. Paraffin sections were mounted on positively charged Superfrost slides (Fisher Scientific, Houston, TX) and dried overnight. Sections were deparaffinized in xylene followed by treatment with a graded series of alcohol [100%, 95%, and 80% ethanol/ double-distilled H2O (v/v)] and rehydrated in PBS (pH 7.5), treated with pepsin for 15 minutes at 37°C, and washed with PBS. Endogenous peroxidases were blocked with 3% H2O2 in methanol. Sections were stained overnight with an antibody to phosphorylated JNK followed by 1-hour incubation with horseradish peroxidase–conjugated secondary antibody. Positive reactions were visualized by incubating the slides with stable 3,3’-diaminobenzidine. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin (colorimetric development), and mounted with Universal Mount (Research Genetics, Birmingham, AL). Control samples exposed to secondary antibody alone showed no specific staining. Staining intensity was quantified by densitometric analysis of five random high-power fields containing viable tumor cells, and results correspond to the average absorbance.

Quantification of apoptosis in situ. Analysis of DNA fragmentation by fluorescent terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) was done using a commercial kit (Promega, Madison, WI) as described previously (24–26). All slides were mounted using Prolong Antifade reagent (Molecular Probes). Images were obtained using a Zeiss LSM510 confocal microscope (Oberkochen, Germany). Percentages of positive cells were then determined using a laser scanning cytometer (LSC) as described previously (27). For each treatment group, four independent fields were selected at random from different tumors so that the comparison among groups would involve roughly equivalent numbers of cells.
Statistical analyses. Statistical significance of differences observed in drug-treated and control samples were determined using the Tukey-Kramer comparison test. Differences were considered significant in all experiments at $P < 0.05$.

Results

Bortezomib interferes with induction of the unfolded protein response and sensitizes pancreatic cancer cells to endoplasmic reticulum stress-mediated apoptosis. Bortezomib treatment induces an abnormal accumulation of ubiquitin-conjugated proteins resulting in ER and/or cytoplasmic stress (13). Considering this, we investigated the effects of bortezomib on the UPR. A conventional ER stress agent (tunicamycin) induced the expression of the UPR target genes BiP (GRP78) and CHOP (GADD153) and induced eif2α phosphorylation (Fig. 1A). Bortezomib also induced UPR target genes to a limited extent but blocked the strong induction of BiP and CHOP and eif2α phosphorylation induced by tunicamycin at 24 hours (Fig. 1A) and in cells exposed to another conventional ER stress agent (thapsigargin) at all time points examined beginning at 1 hour (13), suggesting that bortezomib induced limited activation of the UPR. Because the UPR is a cytoprotective response (28), we speculated that bortezomib might interact with agents that activate the UPR more efficiently to increase ER stress-mediated apoptosis. To test this hypothesis, we measured JNK phosphorylation, caspase-3 activation, and DNA fragmentation in cells exposed to bortezomib, conventional ER stress agents (tunicamycin and thapsigargin), or combinations of bortezomib plus tunicamycin or thapsigargin.

Time-course analysis confirmed that bortezomib and tunicamycin induced increases in phosphorylated JNK within 4 hours (data not shown). Consistent with our hypothesis, levels of JNK and caspase-3 activation and DNA fragmentation were substantially higher in cells exposed to the combinations for 24 hours than they were in cells exposed to single agents (Fig. 1B and C). Because gemcitabine is the current frontline therapy for pancreatic cancer, we investigated whether the drug induced the UPR. Consistent with a previous study using the nucleoside analogue 5-hydroxymethyl-2'-deoxyuridine (29), gemcitabine did not induce the UPR genes CHOP or BiP or enhance bortezomib-induced apoptosis (Fig. 1D).

Bortezomib also promotes cisplatin-induced apoptosis. Conventional ER stress agents, such as tunicamycin and thapsigargin, have not been used clinically to treat cancer. However, recent studies concluded that two clinically relevant agents, cisplatin and the geldanamycin analogue, 17-AAG, increase ER

![Figure 1. Bortezomib suppresses the UPR and sensitizes pancreatic cancer cells to ER stress-mediated apoptosis.](image-url)
stress in cancer cells (18, 19). Therefore, we investigated the effects of bortezomib plus cisplatin or 17-AAG on apoptosis in L3.6pl cells. In a first series of experiments, we assessed the effects of bortezomib, cisplatin, or 17-AAG on the expression of proteins that are induced in association with the UPR by immunoblotting. Consistent with prior reports, all three agents increased the expression of the chaperone proteins, BiP and HSP70 (refs. 19, 30, 31; Fig. 2A). Similarly, bortezomib and cisplatin both activated JNK and caspase-4 in L3.6pl cells (Fig. 2A and promoted caspase-12 processing in murine L929 cells (Fig. 2B), consistent with the conclusion that they induce apoptosis via ER stress. Interestingly, however, 17-AAG did not activate JNK or caspase-4 in L3.6pl cells (Fig. 2A), nor did it promote the processing of caspase-12 in L929 cells (Fig. 2B). Therefore, 17-AAG does not seem to induce apoptosis via a classic ER stress-dependent mechanism.

We then assessed the effects of combinations of bortezomib plus cisplatin or 17-AAG on apoptosis in L3.6pl cells. As we had observed in cells exposed to bortezomib plus tunicamycin or thapsigargin, cells exposed to bortezomib plus cisplatin displayed higher levels of DNA fragmentation and caspase-3 activation (Fig. 2C) than cells exposed to either agent alone. In contrast, levels of DNA fragmentation and caspase-3 activity were significantly lower in L3.6pl cells exposed to 17-AAG plus bortezomib than they were in cells exposed to bortezomib alone and were similar to the levels observed in cells exposed to 17-AAG alone (Fig. 2C). Very similar results were obtained when the effects of the combinations on JNK phosphorylation were assessed. Levels of JNK phosphorylation were consistently higher in cells exposed to bortezomib plus cisplatin compared with the levels observed in cells exposed to either bortezomib or cisplatin alone (Fig. 2D). In contrast, 17-AAG blocked the JNK phosphorylation observed in L3.6pl cells exposed to bortezomib alone (Fig. 2D).

c-Jun NH2-terminal kinase activation is required for bortezomib-induced apoptosis. We next investigated whether JNK activation was essential for bortezomib-induced apoptosis. Dose-response studies indicated that JNK activation was associated with caspase-3 processing and DNA fragmentation (Fig. 3A). To more directly determine the importance of JNK activation to bortezomib-induced apoptosis, we exposed cells to bortezomib in the absence or presence of the chemical JNK inhibitor, SP600125, and measured downstream events associated with apoptosis. SP600125 blocked bortezomib-induced caspase-3 processing and DNA fragmentation (Fig. 3A). To further investigate the importance of JNK activation, we assessed the effects of combinations of bortezomib and 17-AAG on JNK activation in L3.6pl cells. As we had observed in cells exposed to bortezomib and 17-AAG alone, bortezomib increased JNK phosphorylation in the absence or presence of 17-AAG (Fig. 3B). In contrast, 17-AAG blocked the JNK phosphorylation observed in cells exposed to bortezomib alone (Fig. 3B).
Cisplatin enhances bortezomib-induced endoplasmic reticulum stress. To more directly assess the effects of bortezomib and cisplatin on ER stress, we examined their effects on ER morphology by transmission electron microscopy (Fig. 4A). The results confirmed that the drug combination promoted increased ER dilation in a manner that was consistent with what we observed in cells undergoing thapsigargin-induced ER stress (positive control). To obtain functional evidence for ER stress, we measured the effects of the combination on cytosolic Ca\(^{2+}\) levels, which are controlled by ER Ca\(^{2+}\) release. The results confirmed that exposure to either bortezomib or cisplatin led to an increase in the cytosolic Ca\(^{2+}\) level that was further enhanced in cells exposed to the combination (Fig. 4B). Chemical inhibitors of JNK or caspase-4 (Z-LEVD) inhibited apoptosis as did a broad-spectrum caspase inhibitor (z-VAD-fmk; Fig. 4C), strongly suggesting that ER stress was the major driving force for apoptosis in the cells. Consistent with the caspase-4 chemical inhibitor results, siRNA-mediated knockdown of caspase-4 also reduced DNA fragmentation induced by bortezomib, cisplatin, and the combination (Fig. 4C).

Small interfering RNA-mediated silencing of IRE1α enhances bortezomib-induced apoptosis. In response to classic ER stress, IRE1α dissociates from GRP78/BiP and recruits the adaptor protein TRAF2 to promote activation of JNK (33). To determine whether a similar mechanism was involved in bortezomib-mediated JNK activation, we measured the effects of bortezomib on JNK phosphorylation and apoptosis in cells transfected with an IRE1α-specific siRNA construct. Interestingly, siRNA-mediated silencing of IRE1α led to enhanced JNK activation (Fig. 5A) and apoptosis (Fig. 5B) following bortezomib treatment, indicating that bortezomib stimulates both processes via IRE1-independent mechanisms.

Effects of bortezomib and cisplatin in orthotopic L3.6pl tumors. In a final series of experiments, we examined the effects of the bortezomib plus cisplatin combination on the growth of human pancreatic tumors in vivo. L3.6pl cells were placed...
orthotopically in the pancreas glands of nude mice, and tumors were allowed to grow for 14 days. Therapy consisted of 1 mg/kg bortezomib, 5 mg/kg cisplatin, or both given by i.p. injection every 72 hours. Tumor growth was significantly inhibited by single-agent bortezomib or cisplatin treatment (Fig. 6A). However, therapy with the combination of bortezomib plus cisplatin produced a greater decrease in tumor weights compared with those observed in animals treated with either single agent (Fig. 6A). Together, our data confirm that mechanism-based combination therapy with bortezomib plus cisplatin leads to the desired effects on apoptosis and growth inhibition in a relevant preclinical model of human pancreatic cancer.

Discussion

We have shown previously that bortezomib has anticancer activity in human pancreatic cancer cells in vitro and in vivo (26, 34). We also showed recently that bortezomib induces apoptosis in pancreatic cancer cells via a process that seems to involve an unorthodox form of ER and/or cytoplasmic stress (13). The effects of bortezomib differ from those elicited by conventional ER stress inducers (tunicamycin and thapsigargin) in that they are not associated with activation of the UPR. Here, we show that bortezomib not only inhibits tunicamycin-induced eIF2α phosphorylation but also interferes with tunicamycin-induced BiP and CHOP induction, additional characteristics of the UPR.

Figure 4. Cisplatin enhances bortezomib-induced ER stress. A, combination of bortezomib and cisplatin induces increased ER dilation. L3.6pl cells were treated with 100 nmol/L bortezomib, 20 μmol/L cisplatin, combination, or 1 μmol/L thapsigargin for 12 hours. Cells were collected and visualized by electron microscopy as described in Materials and Methods. Arrows, individual ER. B, cisplatin and bortezomib treatment increase intracellular Ca2+ levels. L3.6pl cells were treated with 100 nmol/L bortezomib, 20 μmol/L cisplatin, or combination for 12 hours. Cells were harvested and intracellular Ca2+ levels were measured as described in Materials and Methods. Columns, mean (n = 3); bars, SD. *, significantly different from either single-agent treatment. C, inhibition of JNK, caspase-4, and caspases reduce bortezomib, cisplatin, and combination-induced apoptosis. L3.6pl cells were treated with 100 nmol/L bortezomib, 20 μmol/L cisplatin, or combination for 24 hours with or without chemical inhibitors (40 μmol/L SP600125, 10 μmol/L Z-LEVD, and 20 μmol/L Z-VAD). siRNA-mediated knockdown of caspase-4 (C-4 siRNA) was carried out as described in Materials and Methods. Immunoblotting was done to detect caspase-4 levels following RNAi. DNA fragmentation was measured by PI-FACS analysis. Columns, mean; bars, SD (n = 3). *, significantly different from DMSO-treated group.

These results are consistent with an earlier study in multiple myeloma cells that also concluded that bortezomib disrupts the UPR (14). The UPR plays a critical cytoprotective role during ER stress by activating pathways that attenuate translation, increase chaperone production, and enhance proteasomal degradation (9). Therefore, it might be possible to exploit the ability of bortezomib to attenuate the UPR by combining it with agents that induce a more classic UPR response in pancreatic cancer cells.

To test this hypothesis, we investigated the effects of bortezomib on apoptosis induced by tunicamycin or thapsigargin in L3.6pl pancreatic cancer cells. We found that JNK activation, caspase-3 activity, and DNA fragmentation were all higher in cells exposed to bortezomib plus tunicamycin or thapsigargin compared with the levels observed in cells exposed to any of the three components alone. Tunicamycin and thapsigargin are still used mainly as laboratory tools, although thapsigargin analogues are being developed for prostate cancer therapy (6). Clinically, gemcitabine is the most effective therapy for pancreatic cancer, being developed for prostate cancer therapy (6). Clinically, gemcitabine is the most effective therapy for pancreatic cancer, being developed for prostate cancer therapy (6).

Therefore, it might be possible to exploit the ability of bortezomib to attenuate the UPR by combining it with agents that induce a more classic UPR response in pancreatic cancer cells.

**Figure 5.** Knockdown of IRE1α enhances bortezomib-induced JNK activation and apoptosis. **A,** siRNA-mediated knockdown of IRE1α was carried out as described in Materials and Methods. Immunoblotting for IRE1α, phosphorylated JNK, JNK, and actin were done in untransfected, IRE1α siRNA-transfected, and luciferase (nontarget) siRNA-transfected cells treated with or without 100 nmol/L bortezomib. **B,** knockdown of IRE1α enhanced bortezomib-induced apoptosis. DNA fragmentation was measured by PI-FACS analysis. Columns, mean (n = 3); bars, SD. *, significantly different from DMSO-treated group.

Consistent with our hypothesis, we observed an increase in BiP and HSP70 chaperone expression and JNK activation, all markers of ER stress, in L3.6pl cells exposed to cisplatin. To more directly determine the relevance of ER stress to cisplatin-induced cell death, we investigated whether ER-resident caspases are activated by cisplatin. Caspase-12 is an ER-resident caspase that is processed in murine cells in response to ER stress and is required for ER stress-induced apoptosis (4). However, recent work showed that expression of functional human caspase-12 isrestricted to women of African descent, and caspase-4 may play a more important role in human cells (11, 12). Our results show that cisplatin stimulated caspase-12 processing in murine L929 cells and caspase-4 in human L3.6pl cells, and a peptide inhibitor or siRNA-mediated silencing of caspase-4 attenuated cell death, confirming that ER stress played an important role in cisplatin-induced apoptosis.

Finally, bortezomib enhanced cisplatin-induced ER dilation, ER Ca2+ release, JNK activation, caspase-3 activity, and DNA fragmentation, confirming the hypothesis that the drugs exacerbate the effects of one another on ER stress.

Molecular chaperones, including GRP78/BiP and HSP70, play important roles in the UPR by preventing the aggregation of misfolded proteins and shuttling them to the 20S proteasome for degradation (9). Other HSPs may also attenuate cellular stress caused by the accumulation of misfolded proteins as shown by the observation that down-regulation of HSP27 promotes bortezomib-induced apoptosis (38). It is therefore possible that HSP90 also limits bortezomib-induced protein aggregation and apoptosis. Consistent with this idea, a previous study showed that exposure of human MCF-7 breast cancer cells or E6/E7-transformed fibroblasts to 17-AAG plus bortezomib resulted in increased growth inhibition and cell death associated with phenotypic changes (ER vacuolization) characteristic of ER stress (17). However, our data indicate that 17-AAG interferes with bortezomib-induced JNK activation and apoptosis-associated caspase-3 activation and DNA fragmentation (Fig. 2). Consistent with this observation, 17-AAG also antagonized the action of cisplatin in colon cancer cells via inhibition of JNK activation (39). We strongly suspect that the apparent discrepancy between our results and those published previously (17) are reconcile by the fact that we used specific assays for apoptosis, whereas the other group used more general assays of cell proliferation and death [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium].
(40–43). It is therefore possible that one or more of these client proteins is required for bortezomib-mediated JNK activation and apoptotic cell death. Whether the enhanced nonapoptotic cell death induced by combinations of 17-AAG plus bortezomib (17) translates into enhanced in vivo activity without toxicity will require additional investigation. It will also be of interest to investigate how down-regulation of HSP70 influences apoptosis induced by either bortezomib or 17-AAG. If our model is correct, we would predict that it would enhance apoptosis induced by the former and inhibit apoptosis induced by the latter.

A recent report showed that proteasome inhibitors suppress the activity of IRE1α, but the underlying mechanisms involved remain unclear (14). Because IRE1α has been implicated in the JNK activation that occurs in response to conventional ER stress (33), we investigated whether IRE1 was also important for bortezomib-induced JNK activation. Strikingly, knockdown of IRE1α resulted in enhanced bortezomib-induced JNK activation and apoptosis. Therefore, although bortezomib has some effects on components of the UPR (BiP and CHOP), activates JNK, and induces ER stress-associated caspases (caspase-4 and caspase-12), it seems to do so in an atypical manner. Further impairment of the UPR by inhibiting IRE1α may therefore prove to be an effective strategy to enhance bortezomib-induced apoptosis.

Previous studies show that JNK translocates to the mitochondria where it is involved in cytochrome c and Smac release (44, 45). SP600125 blocked bortezomib-induced cytochrome c release and significantly reduced caspase-3 activity and DNA fragmentation. In addition, SP600125 also inhibited caspase-4 processing showing that JNK activation is an early event during ER stress-mediated apoptosis. The exact role of caspase-4 during apoptosis is not known, but it is tempting to speculate that it may be an initiator caspase linking the ER and mitochondrial pathways of apoptosis.
The strong anticancer activity of bortezomib in combination with cisplatin observed in vitro prompted us to evaluate its efficacy in an orthotopic pancreatic cancer mouse model. Consistent with our previous work (26, 34), single-agent bortezomib significantly reduced tumor burden and similar results were observed with cisplatin therapy. Therapy with a combination of the two agents led to a further decrease in tumor burden that was associated with enhanced JNK activation and apoptosis. Bortezomib is a potent inhibitor of tumor cell proliferation due to its ability to block cyclin-dependent kinase activity (34). However, the high level of TUNEL positivity observed suggests that the reduction in tumor burden was probably due to an increase in apoptosis. Importantly, no significant systemic toxicity was observed in our study. Cisplatin is known to induce peripheral neuropathy, and recent phase I trials have shown that patients who had previously received platinum analogues developed peripheral neuropathy when they were subsequently treated with bortezomib (1). Therefore, the possible toxicity of the bortezomib-cisplatin combination on ER stress, apoptosis, and tumor growth, our data support planned clinical trials to evaluate the efficacy of this combination in patients with pancreatic cancer and other solid malignancies.

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References

Correction: Article on Bortezomib Sensitizes Cells to ER Stress

In the article on how bortezomib sensitizes cells to ER stress in the December 15, 2005 issue of Cancer Research (1), there is an error in Fig. 6B. The corrected figure appears below.

![Figure 6](image)

Bortezomib Sensitizes Pancreatic Cancer Cells to Endoplasmic Reticulum Stress-Mediated Apoptosis

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