Bortezomib Inhibits PKR-Like Endoplasmic Reticulum (ER) Kinase and Induces Apoptosis via ER Stress in Human Pancreatic Cancer Cells

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Abstract

Bortezomib (Velcade, formerly known as PS-341) is a boronic acid dipeptide derivative that is a selective and potent inhibitor of the proteasome. We hypothesized that proteasome inhibition would lead to an accumulation of misfolded proteins in the cell resulting in endoplasmic reticulum (ER) stress. The ability of bortezomib to induce ER stress and the unfolded protein response was investigated in a human pancreatic cancer cell line, L3.6pl. Bortezomib increased expression of ER stress markers, CHOP and BiP, but inhibited PKR-like ER kinase and subsequent phosphorylation of eukaryotic initiation factor 2α (eif2α), both of which are key events in translational suppression. These effects resulted in an accumulation of ubiquitylated proteins leading to protein aggregation and proteotoxicity. Peptide inhibitor or small interfering RNA targeting ER-resident caspase-4 blocked DNA fragmentation, establishing a central role for caspase-4 in bortezomib-induced cell death. The translation inhibitor cycloheximide abrogated bortezomib-induced protein aggregation, caspase-4 processing, and all other characteristics of apoptosis. Because malignant cells have higher protein synthesis rates than normal cells, they may be more prone to protein aggregation and proteotoxicity and possess increased sensitivity to bortezomib-induced apoptosis. Taken together, the results show that bortezomib induces a unique type of ER stress compared with other ER stress agents characterized by an absence of eif2α phosphorylation, ubiquitylated protein accumulation, and proteotoxicity. (Cancer Res 2005; 65(24); 11510-9)

Introduction

The 26S proteasome is a primary component of the protein degradation machinery of the cell and is responsible for the destruction of >80% of all cellular proteins (1, 2). The rapid elimination of targeted proteins is key to the activation or repression of many cellular processes, including cell cycle progression and apoptosis. The roles of proteasome in regulating cell growth, survival, and metastasis of cancer cells make it an attractive therapeutic target (3–5). The first proteasome inhibitor, bortezomib (Velcade, formerly known as PS-341), recently received Food and Drug Administration (FDA) approval for the treatment of multiple myeloma and is being evaluated for the treatment of solid tumors (6). The antineoplastic effects of bortezomib have been attributed, in part, to inhibition of IκB degradation leading to inactivation of the prosurvival transcription factor, nuclear factor-κB (NF-κB). However, recent findings indicate that inhibition of NF-κB activity accounts for only a small fraction of the anticancer properties of bortezomib (7, 8), indicating that the mechanisms of action of the drug remain to be fully characterized.

Proteasome inhibition may prevent the clearance of misfolded proteins by the endoplasmic reticulum (ER)–associated degradation (ERAD) pathway, resulting in ER stress (9, 10). The ER serves several important functions, including post-translational modification, folding and assembly of newly synthesized proteins, and regulated calcium storage (11). These functions are essential to cell survival, such that their perturbation can result in cellular damage, ER stress, and apoptosis (11). ER stress may be induced by agents, such as thapsigargin, which inhibits the sarcoplasmic/endoplasmic Ca2+-ATPase resulting in ER Ca2+ depletion, or tunicamycin, which blocks N-linked protein glycosylation.

The ER has evolved highly specific signaling pathways to ensure that its protein-folding capacity is not overwhelmed (12). These pathways are collectively termed the unfolded protein response (UPR), and they are required for cell survival in the face of ER stress. Four functionally distinct components of the UPR have been identified (10, 13, 14). The first one involves up-regulation of ER chaperone proteins (BiP/GRP78 and GRP94) to increase protein-folding activity and to prevent protein aggregation. The second consists of translational attenuation to reduce the load of new protein synthesis and to prevent further accumulation of misfolded proteins. Inhibition of translation is accomplished by PKR-like ER kinase (PERK), which phosphorylates and inhibits the α subunit of eukaryotic initiation factor 2 (eif2α) in response to ER stress. The third component is an increase in the degradation of proteins misfolded in the ER by ERAD. The ERAD pathway signals to increase the expression of numerous genes involved in proteasomal-mediated protein degradation. The fourth element of the UPR involves induction of apoptosis, which occurs when cytotoxic functions of the ER are overwhelmed (15).

Pancreatic epithelial cells have a highly developed ER due to a heavy engagement in insulin and digestive enzyme secretion (15), and it has been suggested that they may be particularly sensitive to ER stress-induced apoptosis (16, 17). In previous studies, we showed that bortezomib potently stimulates apoptosis in pancreatic tumor cells (18, 19). Here, we tested the hypothesis that the cytotoxic effects of bortezomib result from ER stress in human pancreatic cancer cells. We showed that bortezomib induces ubiquitin-conjugated protein accumulation by blocking proteasomal degradation while...
allowing continued protein synthesis through inhibition of PERK activity. The large accumulation of ubiquitylated proteins leads to protein aggregation, which mediates the cytotoxic activity of bortezomib characterized by caspase-4 cleavage. Consistent with these data, inhibition of translation blocks bortezomib-induced protein accumulation and apoptosis. In addition, the high translation rate of malignant cells (compared with low translation rates of normal cells) may make them selectively sensitive to bortezomib-induced proteotoxicity and apoptosis.

Materials and Methods

Cell lines, antibodies, and chemicals. L3.6pl human pancreatic cancer cells were established as described previously (20). The human pancreatic cancer cell lines HS766t, MiaPaCa-2, Panc1, AsPC1, Capan-2, BxPC-3, and HpaF-2 were obtained from the American Type Culture Collection (Rockville, MD). The murine cell line L929 was kindly provided by Dr. I.J. Fidler (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, 1-glutamine, vitamins, and antibiotics under conditions of 5% CO2 in air at 37°C. The immortal pancreatic duct epithelium cell line HPDE6-E6-E7 was established and cultured as described previously (20, 21). Antibodies were obtained from the following commercial sources: anti–cytochrome c, anti-active caspase-3, and BIP/GPR78 (Transduction Laboratories, San Diego, CA); anti-eti2a, phosphorylated eti2a, phosphorylated e-Jun, e-Jun, e-Jun NH2-terminal kinase (JNK), caspase-12, and phosphorylated PERK (Cell Signaling, Beverly, MA); anti-CHOP/GADD153, ubiquitin, protein phosphatase 1 (PP1), PERK, and GADD34 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-actin (Sigma Chemical, St. Louis, MO); anti-caspase-4 and calreticulin (StressGen, Victoria, British Columbia, Canada); and anti-phosphorylated JNK (Biosource, Camarillo, CA). Alexa Fluor 488 goat anti-mouse, Texas red goat anti-rabbit, and To-Pro-3 were obtained from Molecular Probes (Eugene, OR). Bortezomib was kindly provided by Millennium Pharmaceuticals (Boston, MA). Propidium iodide (PI), thapsigargin, tunicamycin, staurosporine, and cycloheximide were obtained from Sigma. Z-VAD-fmk and Ac-LEVD-CHO were purchased from R&D Systems (Minneapolis, MN).

Reverse transcription-PCR. Total RNA was extracted from L3.6pl pancreatic cancer cells using the RNasey kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA using the Omniscript RT kit (Qiagen). Primer sequences used for PCR amplification are as follows: GADD153 sense 5'-ACTCATCGTGGTCAATGAG-3' and antisense 5'-TTCTTCTGTTCTGGAACTCTG-3' and antisense 5'-TCACCAGGTGCACTCCCC-3' and antisense 5'-TGACGAGCAGCATAGTC-3' and antisense 5'-GCGTGTCAGAAGCTTTCC-3' and gyceraldehyde-3-phosphate dehydrogenase antisense 5'-CGGAGTCAAGGATTGGC-3' and antisense 5'-GTGACGAGATGATCATGGAC-3'. Reactions were carried out in 50 μL volumes composed of 50 ng cDNA, 0.2 μmol/L each primer (Sigma-Genosys, The Woodlands, TX), 0.2 mmol/L each of deoxynucleotide triphosphates (Roche, Indianapolis, IN), 1.0× Gold PCR buffer, 1.5 mmol/L MgCl2, 2.5 units AmpliTaq Gold (Applied Biosystems, Foster City, CA), and nuclease-free H2O (Promega, Madison, WI). PCR amplification of all three genes was done in a Robocycler (Stratagene, La Jolla, CA) using the following cycling conditions: 1 cycle of 3 minutes at 94°C, 3 minutes at 54°C, and 3 minutes at 72°C followed by 35 cycles of 1 minute at each temperature. The final cycle was modified to allow for a 7-minute extension at 72°C. PCR products were electrophoresed on 100 V for 45 minutes on a 1.2% agarose gel and imaged using a gel documentation system (AlphaImager, San Leandro, CA). Equal quantities of input cDNA were used for all amplifications.

Immunoblotting. Cells (1 × 106) were incubated with 100 mmol/L bortezomib unless otherwise stated, 50 μmol/L cycloheximide, 1 μmol/L thapsigargin, 100 ng/mL rhTRAIL, 5 μg/mL tunicamycin, or drug combinations. For the combination studies, cells were pretreated with cycloheximide for 2 hours 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 overnight with relevant 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).

Measurement of intracellular Ca2+ levels. Cells were grown in medium with or without 100 mmol/L bortezomib for 12 hours. Cells were collected, washed in PBS, and incubated with 1 μmol/L calcium green-1 (Molecular Probes) for 30 minutes. Flow cytometric analysis of stained cells was done with a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA).

Quantification of DNA fragmentation. DNA fragmentation was measured by PI staining and fluorescence-activated cell sorting (FACS) analysis as described previously (23). Cells were plated in six-well plates (1 × 104 well). Following incubation with indicated drugs, 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 combination studies, cells were pretreated with cycloheximide for 2 hours before bortezomib treatment. Cells were incubated with the PI solution for 1 hour, and flow cytometric analysis of stained cells was done with a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA).

Caspase-3 assay. Cells (1 × 106) were plated in 10-cm dishes with 10% FBS/MEM and allowed to attach for 24 hours. Cells were treated as described for DNA fragmentation analysis above. Following treatment, cells were washed in PBS and resuspended in appropriate buffers as described in the FITC-conjugated monoclonal active caspase-3 antibody apoptosis kit (PharMingen, San Diego, 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 FACScan (Becton Dickinson, Mountain View, CA).

Confocal microscopy. Pancreatic cancer cells were treated on chamber slides. After drug exposure, cells were fixed with 4% paraformaldehyde, permeabilized using 0.2% Triton X-100, and incubated overnight with indicated primary antibodies. Fluorescent secondary antibodies were used to visualize protein localization. To-Pro-3 was used to counterstain the nucleus. Images were obtained using a Zeiss LSM510 confocal microscope (Zeiss, Oberkochen, Germany).

Preparation and transfection of small interfering RNAs. The annealed double-stranded caspase-4 small interfering RNAs (siRNA) were obtained from Dharmacon (Lafayette, CO): caspase-4 siRNA sense 5'-GGACUAUGUGUAUGUAUU-3' and antisense 5'-UCAUCUACUA- CUAUACUUU-3'. For control, siRNA directed against firefly luciferase was used. Cells were transfected with 100 nmol/L of the above siRNA using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Transfected cells were incubated at 37°C for 40 hours without changing the medium. Efficiency of RNA interference (RNAi) was measured by immunoblotting using an antibody to anti-caspase-4.

Transmission electron microscopy. Transmission electron microscopy of cells was done as described previously (24).

Metabolic labeling. To measure global translation, equal number of cells were plated in six-well plates, allowed to attach, washed with PBS, and placed in [35S]cysteine- and [35S]methionine-free medium containing 5% dialyzed FBS. Cells were pretreated with bortezomib, thapsigargin, or cycloheximide for the indicated times. The cells were then labeled with [35S]methionine and [35S]cysteine (Translabel, ICN, Irvine, CA) for the last 30 minutes of treatment. Cells were then harvested and lysed and protein was separated by SDS-PAGE followed by autoradiography to visualize radiolabeled proteins. In parallel, each lysate was subjected to trichloroacetic acid precipitation and the radioactivity was then counted. Cells were then labeled with [35S]methionine and [35S]cysteine incorporation into proteins.

Immunoprecipitation. Cells (1 × 106) were incubated on 100 × 20-mm plates with 100 mmol/L bortezomib for 24 hours. Cells were lysed at the plates on 4°C with a 1% Triton X-100 lysis buffer containing 150 mmol/L NaCl, 25 mmol/L Tris (pH 7.5), 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, 10 mmol/L β-glycerophosphate, and a Complete Mini

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Bortezomib Induces Proteotoxicity

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protease inhibitor tablet. After centrifugation, the clarified supernatant was incubated with anti-GADD34 antibody for 1 hour followed by incubation with protein A/G-Sepharose beads for 12 hours at 4°C. The immunocomplex was washed thrice with lysis buffer followed by addition of SDS sample buffer. Proteins were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated with the indicated primary antibody followed by secondary antibody coupled to hors eradish peroxidase and the signal was revealed by chemiluminescence.

Preparation of cytosol for cytochrome c measurement. Cells were treated with cycloheximide 2 hours before bortezomib treatment for 24 hours. Cells were harvested and 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. Cells were centrifuged at 10,000 × g for 15 minutes at 4°C. Supernatants were collected and subjected to immunoblotting.

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 (significantly different from untreated controls).

Results

Bortezomib induces endoplasmic reticulum stress-induced apoptosis. It seemed likely that proteasome inhibition might trigger ER stress. To investigate this possibility, we examined the effects of bortezomib on the expression of proteins that are modulated by classic inducers of ER stress in other model systems (12). In these experiments, the ER Ca2+-ATPase inhibitor thapsigargin and the glycosylation inhibitor tunicamycin served as positive controls for ER stress. Reverse transcription-PCR (RT-PCR) and immunoblotting analyses confirmed that, like thapsigargin, bortezomib induced CHOP/GADD153 and BiP/GRP78 at the mRNA and protein levels (Fig. 1A). Bortezomib also stimulated an increase in intracellular Ca2+ concentration (Fig. 1B) and JNK activation (Fig. 1C), which are also common consequences of ER stress (25). Bortezomib (100 nmol/L) induced significant DNA fragmentation in L3.6pl cells (Fig. 1C). Our previous work showed that 10 nmol/L bortezomib blocks proteasomal activity by 50% in these cells, whereas 100 nmol/L bortezomib inhibits proteasomal activity by 80% (18).

Caspase-12 mediates ER stress-induced apoptosis in murine cells (26), but most humans do not possess a functional caspase-12 gene (27). Caspase-12 was indeed activated by bortezomib in murine L929 cells (Fig. 1D). Because human caspase-4 is a potential orthologue of murine caspase-12 (48% sequence similarity), we investigated whether caspase-4 was involved in bortezomib-induced cell death. Caspase-4 colocalized with the ER resident protein, calreticulin (Fig. 2A), and it was cleaved to its active form in L3.6pl cells treated with bortezomib, thapsigargin, or tunicamycin but not rhTRAIL (Fig. 2A). The active caspase-4 cleavage fragment was formed in all bortezomib-sensitive pancreatic cancer cell lines but not in any of the cells that were resistant to the drug (Fig. 2B). Furthermore, a peptide inhibitor of caspase-4 (Ac-LEVD-CHO) prevented bortezomib- or tunicamycin-induced apoptosis but failed to inhibit DNA fragmentation induced by staurosporine and rhTRAIL (Fig. 2C). Finally, siRNA-mediated knockdown of caspase-4 also reduced bortezomib-induced apoptosis (Fig. 2D). Taken together, our results show that caspase-4 activation mediates bortezomib-induced apoptosis.

Bortezomib fails to inhibit translation. Surprisingly, and in contrast to thapsigargin, bortezomib did not induce phosphorylation of eIF2z (Fig. 3A), a key event of the UPR that mediates inhibition of translation (28). Therefore, we investigated whether bortezomib had any effect on protein synthesis. Bortezomib failed to suppress Figure 1. Bortezomib induces ER stress. A, RT-PCR and Western analysis of BiP and CHOP expression. L3.6pl cells were treated for 12 hours with 100 nmol/L bortezomib (BZ) or 1 μmol/L thapsigargin (TG) followed by RNA isolation and RT-PCR analysis as described in Materials and Methods. L3.6pl cells were treated with 100 nmol/L bortezomib or 1 μmol/L thapsigargin for 24 hours and immunoblotting was done as described in Materials and Methods. B, bortezomib increases intracellular calcium levels. L3.6pl cells were treated for 12 hours with 100 nmol/L bortezomib, stained with calcium green-1, and analyzed by flow cytometry. C, JNK activation correlates with bortezomib-induced apoptosis. L3.6pl cells were treated for 24 hours with indicated concentrations of bortezomib. Apoptosis was determined by PI-FACS analysis and phosphorylated JNK/JNK expression by immunoblotting. PI-FACS was done in triplicate. Bars, SD. *, significantly different from control. D, bortezomib induces caspase-12 cleavage. Murine L929 cells were treated with 1 μmol/L thapsigargin, 5 μg/mL tunicamycin (TM), or 100 nmol/L bortezomib for 24 hours followed by immunoblotting for caspase-12.
translation in cells pulsed with [35S]cysteine and [35S]methionine, whereas a more conventional inducer of ER stress (thapsigargin) dramatically reduced [35S]cysteine and [35S]methionine incorporation into proteins (Fig. 3B). The ability of bortezomib to inhibit proteasomal degradation while allowing steady protein synthesis resulted in an accumulation of ubiquitin-conjugated proteins. Analysis of bortezomib-treated cells by immunofluorescent anti-ubiquitin staining and confocal microscopy revealed the appearance of perinuclear ubiquitin-containing aggregate structures characterized previously as aggresomes (ref. 29; Fig. 3C). The protein aggregates were organized as electron dense particles in a central core when they were visualized by transmission electron microscopy (Fig. 3C).

Bortezomib inhibits PKR-like endoplasmic reticulum kinase activity and induces GADD34 expression. To further investigate the effects of bortezomib on the UPR, we examined the effects of the drug on PERK and GADD34, which regulate eif2α phosphorylation. Bortezomib decreased the low basal levels of phosphorylated eif2α observed in pancreatic cancer cells and induced the expression of GADD34, the regulatory subunit of an eif2α-specific holophosphatase complex (refs. 30–32; Fig. 3D). Coimmunoprecipitation analyses showed that GADD34 formed a complex with PP1 in bortezomib-treated cells (Fig. 3D). However, bortezomib also blocked thapsigargin-induced PERK and eif2α phosphorylation at a time point (1 hour) that preceded GADD34 accumulation (Fig. 3D). Consistent with these results, knockdown of GADD34 by RNAi did not attenuate translation or bortezomib-induced apoptosis (data not shown). Therefore, the ability of bortezomib to inhibit PERK activation itself seems to be critically involved.

Cycloheximide inhibits bortezomib-induced protein accumulation and apoptosis. To examine whether loss of translational control was essential for bortezomib-induced cell death, we examined the effects of the translation inhibitor, cycloheximide, on aggresome formation, ER stress, JNK activation, and apoptosis in L3.6pl cells. Cycloheximide blocked [35S]cysteine and [35S]methionine incorporation into proteins in cells exposed to bortezomib studied.
(Fig. 4A) and consequently prevented the accumulation of ubiquitylated proteins (Fig. 4B) and aggresome formation (Fig. 4C). Cycloheximide also restored eIF2α phosphorylation and blocked the bortezomib-induced increase in GADD34 expression (Fig. 4D). It also inhibited bortezomib-induced JNK phosphorylation and subsequent c-Jun phosphorylation (Fig. 5A). Due to the absence of protein aggregate accumulation, bortezomib-induced caspase-4 processing (Fig. 5B), cytochrome c release (Fig. 5C), caspase-3 activation (Fig. 5D), and DNA fragmentation (Fig. 5D) were inhibited by cycloheximide. These results show that the failure of bortezomib to induce eIF2α phosphorylation and inhibit translation play critical roles in its cytotoxic activity in pancreatic cancer cells.

Immortalized normal pancreatic epithelial cells have a lower translation rate and are resistant to bortezomib-induced apoptosis. The increased cell proliferation of aggressive cancers requires a general increase in protein synthesis and an increased

Figure 3. Bortezomib inhibits PERK resulting in active translation and the accumulation of ubiquitin-conjugated aggregates. A, bortezomib does not induce eIF2α phosphorylation. L3.6pl cells were treated with 100 nmol/L bortezomib or 1 μmol/L thapsigargin for 24 hours and immunoblotting was done as described in Materials and Methods. B, effects of bortezomib and thapsigargin treatment on [35S]cysteine/[35S]methionine (Cys/Met) incorporation. Cells were treated with 100 nmol/L bortezomib or 1 μmol/L thapsigargin for indicated times and pulsed with [35S]cysteine/[35S]methionine for 30 minutes. Lysates were electrophoresed on SDS-PAGE gels and radiolabeled polypeptides were visualized by autoradiography. Quantification of [35S]cysteine/[35S]methionine incorporation was measured using a scintillation counter. Representative of three independent experiments. C, bortezomib induces aggresome formation. L3.6pl cells were incubated for 24 hours with 100 nmol/L bortezomib, stained with an anti-ubiquitin antibody, and imaged using confocal microscopy as described in Materials and Methods. L3.6pl cells were fixed and prepared for electron microscopy as described in Materials and Methods. D, bortezomib induces GADD34 expression and inhibits PERK activity. Immunoblotting was done for GADD34 and phosphorylated eIF2α as described in Materials and Methods. GADD34 interacts with PP1. GADD34 and associated proteins were immunoprecipitated as described in Materials and Methods, resolved on SDS-PAGE gels, and transferred to nitrocellulose. Blots were then probed with either anti-PP1 or GADD34 antibodies. Additional lysates from untreated and treated cells were run on separate SDS-PAGE gels and probed with anti-PP1 antibody as a control. Bortezomib inhibits PERK activity. L3.6pl cells were treated with 100 nmol/L bortezomib, 1 μmol/L thapsigargin, or combination (BZ+TG) for 1 hour followed by immunoblotting with anti-phosphorylated eIF2α or anti-phosphorylated PERK antibody as described in Materials and Methods.
dependency on proteasome function to degrade superfluous proteins (33). Oncogenic activation of Ras leads to an up-regulation of translational machinery and is extremely prevalent in pancreatic neoplasms (34). To examine the potential role of increased translation in pancreatic cancers to the tumor selectivity of bortezomib, we measured protein synthesis and apoptosis in L3.6pl pancreatic cancer cells and in an immortalized normal human pancreatic epithelial cell line (HPDE6-E6E7). Importantly, this immortalized normal pancreatic epithelial cell line has a near-normal genotype and phenotype (20). We observed that global translation rates were much higher in the L3.6pl cells compared with the HPDE6-E6E7 (Fig. 6A). Consistent with our previous results, bortezomib did not attenuate translation in either cell line, whereas thapsigargin induced a marked decrease. Cycloheximide was used as a positive control for translational suppression. The level of protein synthesis correlated with apoptosis induced by bortezomib (Fig. 6B), suggesting that the higher translation rates of pancreatic cancer cells compared with normal cells may make them selectively sensitive to bortezomib-induced apoptosis.

Discussion

The proteasome mediates the degradation of many proteins involved in tumor cell proliferation and apoptosis, making it an attractive target for pharmacologic inhibition in cancer. Bortezomib is a potent and selective proteasome inhibitor that has displayed marginal toxicity (mostly peripheral neuropathy in patients who have received platinum analogues or other agents linked to this toxicity) in phase I clinical trials in a variety of different tumor types (35), and it recently received FDA approval for use in the therapy of multiple myeloma (36). Studies by our laboratory and others have shown that bortezomib also has promising antitumor activity in pancreatic cancer models (18, 19, 37, 38). Despite the recent success of bortezomib as an anticancer agent, its mechanism(s) of action remains to be fully characterized. We hypothesized that proteasome inhibitor treatment would result in the accumulation of unneeded proteins leading to protein aggregation and ER stress-induced apoptosis. Secretory cells (i.e., pancreatic cells) have extensively developed ERs that allow them to deal with their high protein...
synthetic load and may render them selectively sensitive to agents that induce ER stress. Indeed, the high immunoglobulin production displayed by plasma cells may underlie the activity of bortezomib in multiple myeloma (12).

In a first series of experiments, we characterized the effects of bortezomib on markers of ER stress in the L3.6pl pancreatic cancer cell line. Bortezomib induced BiP and CHOP expression and increased intracellular calcium levels, JNK activation, and caspase-12 processing, all of which are indicative of ER stress (15). A recent study has implicated the involvement of caspase-4 during ER stress-induced apoptosis (39). Therefore, we investigated whether caspase-4 was processed during bortezomib-induced apoptosis. Our data indicate that caspase-4 resides in the ER and is cleaved following bortezomib treatment and that its inhibition abrogates bortezomib-induced apoptosis. Consistent with a role of caspase-4 in bortezomib-induced apoptosis, its processing was only observed in cell lines that were sensitive to bortezomib-induced apoptosis. In addition, peptide- or siRNA-mediated inhibition of caspase-4 blocked bortezomib-induced apoptosis. Taken together, the data show that induction of ER stress-induced apoptosis by caspase-4 mediates bortezomib-induced apoptosis.

Interestingly, bortezomib treatment did not induce phosphorylation of eif2α, a key cytoprotective component of the UPR that is required for translational attenuation (40). This prompted us to more directly examine the effects of bortezomib on translation. Consistent with their effects on eif2α phosphorylation, thapsigargin attenuated protein synthesis, whereas bortezomib did not. A previous study reported that thapsigargin-treated NIH 3T3 cells resume translation after several hours (41). Interestingly, we did not observe translational recovery in L3.6pl cells that were exposed to thapsigargin continuously, but protein synthesis was restored after several hours when cells were washed and incubated in thapsigargin-free medium. Our findings suggest that bortezomib is uniquely effective in inducing ER stress by virtue of the fact that it does not inhibit protein synthesis.

Several reports have identified a negative feedback loop that relieves translational inhibition during ER stress via GADD34-mediated PPI activation (30–32). Our results showed a relationship between GADD34 induction and a decrease in eif2α phosphorylation in cells treated with bortezomib and we found that GADD34 directly interacted with PPI. However, we did not observe an initial decrease in translation followed by protein synthesis recovery, suggesting that bortezomib prevents translational attenuation before GADD4 induction. We showed that bortezomib inhibits eif2α phosphorylation before GADD4 induction by blocking PERK autophosphorylation. Regardless of the mechanism, the ability of bortezomib to inhibit eif2α phosphorylation allows protein synthesis in the face of proteasomal inhibition causing ubiquitin-conjugated protein accumulation and aggregation. This result is in contrast to two recent studies showing that proteasome inhibitors

![Figure 5](image_url)

**Figure 5.** Inhibition of translation blocks bortezomib-induced apoptosis. A, cycloheximide treatment inhibits bortezomib-induced JNK activation and subsequent c-Jun phosphorylation. L3.6pl cells were treated with 100 nM bortezomib, 50 µM cycloheximide, or combination for 24 hours. Immunoblotting was done as described in Materials and Methods. B, cycloheximide inhibits bortezomib-induced caspase-4 processing. Cells were treated as stated above and immunoblotting was done using an anti-caspase-4 antibody. C, cycloheximide inhibits bortezomib-induced cytochrome c release. L3.6pl cells were treated for 24 hours as described above. Cytosolic extracts were prepared and immunoblotting was done as described in Materials and Methods. Representative blots are shown. D, cycloheximide inhibits bortezomib-induced caspase-3 activity and DNA fragmentation. L3.6pl cells were treated with bortezomib or cycloheximide and apoptosis was measured by PI-FACS analysis or active caspase-3 activity assay after 24 hours as described in Materials and Methods. Columns, mean (n = 3); bars, SD. *, significantly different from bortezomib-treated cells.
lead to autophosphorylation of PERK in a head and neck squamous cell carcinoma model (42) and eIF2α phosphorylation in mouse embryonic fibroblasts (43). Although we agree with the authors in that bortezomib induces ER stress, we argue that eIF2α remains dephosphorylated due to PERK inhibition and/or GADD34 stabilization. Our results are consistent with a study in multiple myeloma showing that proteasome inhibitors disrupt the UPR (44). It is possible that the discrepancies between the studies lie in differences between the model systems.

Studies using PERK−/− cells have indicated that it plays an important role in cytoprotection, as these cells were more sensitive to ER stress due to the inability of the cells to regulate protein synthesis (28). Because PERK activation and translational suppression are critical for cell survival in the presence of ER stress, it is likely that bortezomib can synergize with other agents that induce ER stress. Consistent with this idea, the ER stress inducers thapsigargin and tunicamycin enhanced bortezomib-induced apoptosis in our model.6 Therefore, we investigated whether inhibition of translation would relieve the proteotoxicity and subsequent ER stress induced by bortezomib. The translation inhibitor cycloheximide prevented bortezomib-induced ubiquitylated protein accumulation, aggresome formation, and apoptosis.

Figure 6. L3.6pl pancreatic cancer cells have a higher translation rate and are more sensitive to bortezomib-induced apoptosis than immortalized pancreatic epithelial cells. A, equal numbers of L3.6pl and HPDE6-E6E7 cells were plated and allowed to attach followed by treatment with 100 nmol/L bortezomib, 1 μmol/L thapsigargin, and 50 μmol/L cycloheximide for 2 hours. [35S]cysteine/[35S]methionine was added during the last 30 minutes of treatment. Cells were harvested and prepared as described in Materials and Methods and radiolabeled [35S]cysteine/[35S]methionine incorporation into proteins was measured using a scintillation counter. Columns, mean (n = 3); bars, SD. B, immortalized normal human pancreatic epithelial cells are resistant to bortezomib-induced apoptosis. Cells were treated with 100 nmol/L bortezomib for 24 hours and apoptosis was measured by PI-FACS analysis as described in Materials and Methods. Columns, mean (n = 3); bars, SD. C, schematic of bortezomib-induced apoptosis. Bortezomib induces GADD34 expression and inhibits PERK activity resulting in dephosphorylated eIF2α. This allows protein synthesis despite the inhibition of proteasomal degradation creating a large accumulation of undegraded proteins. The high abundance of proteins tend to aggregate generating proteotoxicity and ER stress. Bortezomib-induced ER stress-mediated apoptosis requires caspase-4 processing and subsequent caspase-3 activation.

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dephosphorylation of eIF2α, and corresponding GADD34 induction. Furthermore, cycloheximide also blocked events associated with bortezomib-induced apoptosis, including JNK activation, cytochrome c release, caspase-4 and caspase-3 activation, and DNA fragmentation. In fact, cycloheximide similarly protected PERK−/− cells under ER stress conditions, indicating the importance of translational regulation to cell survival (28).

We have established that continued translation in the presence of proteasome inhibition leads to the formation of ubiquitin-conjugated protein aggregates and induction of ER stress in pancreatic cancer cells. The majority of pancreatic cancer cells package these protein aggregates into structures termed aggresomes. We determined that the majority of cells possessing aggresomes were void of ER stress and resistant to bortezomib-induced apoptosis.6 These observations are consistent with a recent report showing that aggresomes may be cytoprotective storage areas for large accumulations of undegraded proteins (45). Furthermore, disruption of aggresome formation by inhibiting histone deacetylase 6 greatly enhances bortezomib-induced ER stress and apoptosis (46).6 Another resistance mechanism to bortezomib-induced apoptosis is up-regulation of chaperone proteins, such as heat shock proteins 27 and 70, which both prevent protein aggregation (47, 48). Taken together, the ability of bortezomib to induce protein aggregation seems to be critical for the stimulation of ER stress and contributes to the cytotoxic activity of the drug.

Several reports have found that bortezomib has tumor-selective activity (49–51). However, the molecular explanation advanced to explain this phenomenon is that cycling cells might be generally more sensitive to proteasome inhibition than resting cells, perhaps because so many crucial cell cycle checkpoints are regulated by the proteasome. Our results provide an alternative explanation. We show here that pancreatic cancer cells display a higher baseline rate of translation compared with immortalized pancreatic epithelial cells, which should make them more vulnerable to the protein buildup and subsequent ER stress caused by proteasome inhibition. In parallel studies, we showed that normal murine pancreatic epithelial cells were also more resistant to bortezomib-induced aggresome formation and cell death than were adjacent cancer cells within orthotopic pancreatic tumors exposed to bortezomib in vivo, and we have obtained similar results in comparisons of the effects of bortezomib in EBV-transformed normal B lymphocytes and multiple myeloma cells.7 Therefore, the unique effects of bortezomib on the UPR may explain its selectivity for malignant cells, because elevated translation is probably a general feature of cancer. Clinical trials using bortezomib for the treatment of advanced solid tumors are currently under way. We have submitted a letter of intent to the Cancer Therapeutics Evaluation Program of the National Cancer Institute (NCI) to investigate bortezomib in combination with suberoylanilide hydroxamic acid.

In summary, our results show that bortezomib activates a novel pathway of ER stress-induced apoptosis characterized by unregulated translation leading to the accumulation of ubiquitin-conjugated, aggregated proteins (Fig. 6C). By targeting the ER, bortezomib may be an effective therapy for pancreatic cancer and other cancers derived from secretory cells, which may be hypersensitive to protein aggregation and subsequent ER stress-mediated apoptosis.

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