Aggresome Disruption: A Novel Strategy to Enhance Bortezomib-Induced Apoptosis in Pancreatic Cancer Cells


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Abstract
The proteasome inhibitor bortezomib (formerly known as PS-341) recently received Food and Drug Administration approval for the treatment of multiple myeloma, and its activity is currently being evaluated in solid tumors. Bortezomib triggers apoptosis in pancreatic cancer cells, but the mechanisms involved have not been fully elucidated. Here, we show that pancreatic cancer cells exposed to bortezomib formed aggregates of ubiquitin-conjugated proteins ("aggresomes") in vitro and in vivo. Bortezomib-induced aggresome formation was determined to be cytoprotective and could be disrupted using histone deacetylase (HDAC) 6 small interfering RNA or was determined to be cytoprotective and could be disrupted using histone deacetylase (HDAC) 6 small interfering RNA or chemical HDAC inhibitors, which resulted in endoplasmic reticulum stress and synergistic levels of apoptosis in vitro and in an orthotopic pancreatic cancer xenograft model in vivo. Interestingly, bortezomib did not induce aggresome formation in immortalized normal human pancreatic epithelial cells in vitro or in murine pancreatic epithelial cells in vivo. In addition, these cells did not undergo apoptosis following treatment with bortezomib, suberoylanilide hydroxamic acid, or the combination, showing tumor selectivity. Taken together, our study shows that inhibition of aggresome formation can strongly potentiate the efficacy of bortezomib and provides the foundation for clinical trials of bortezomib in combination with HDAC inhibitors for the treatment of pancreatic cancer.

Introduction
The 26S proteasome is responsible for the degradation of >80% of all cellular proteins (1, 2) and plays a key regulatory role in cell cycle progression and apoptosis, making it an attractive therapeutic target in cancer (3, 4). Bortezomib (Velcade; formerly known as PS-341) is a peptide boronate inhibitor of the proteasome that recently received Food and Drug Administration (FDA) approval for the treatment of multiple myeloma (5), and it is currently being evaluated for the treatment of solid tumors (6). The antineoplastic effects of bortezomib have been attributed, in part, to inhibition of NF-κB degradation leading to inactivation of the prosurvival transcription factor, nuclear factor-κB (NF-κB; ref. 7). However, recent findings showed that inhibition of NF-κB activity accounts for only a small fraction of the anticancer activity of bortezomib (8, 9), indicating that the mechanisms of action of the drug remain to be fully characterized.

We suspected that bortezomib induces cell death by causing a buildup of misfolded and otherwise damaged proteins, thereby triggering endoplasmic reticulum (ER) stress (10–13). The ER is especially vulnerable to the presence of misfolded proteins because of its involvement in post-translational modification, folding and assembly of newly synthesized proteins, and regulated calcium storage (14). Pancreatic epithelial cells possess a highly developed ER due to a heavy engagement in insulin and digestive enzyme secretion (15), and they seem to be particularly sensitive to ER stress-induced apoptosis (16, 17).

In previous studies, we showed that bortezomib stimulates apoptosis in approximately half of the tested human pancreatic cancer cell lines (18, 19). Here, we show that bortezomib causes the sequestration of ubiquitin-conjugated proteins into structures termed aggresomes in these cells. Aggresomes seem to participate in a cytoprotective response that is activated in response to proteasome inhibition perhaps by shuttling ubiquitylated proteins to lysosomes for degradation (20). Previous studies showed that bortezomib interacts synergistically with histone deacetylase (HDAC) inhibitors to induce apoptosis in multiple myeloma and chronic myelogenous leukemia (21–23). We show that these effects are closely associated with disruption of aggresome formation leading to enhanced ER stress. Together, the results strongly suggest that bortezomib-induced apoptosis is mediated via ER stress, and they identify a clinically viable strategy (disruption of aggresome formation) to overcome bortezomib resistance in a subset of human pancreatic cancer cells.

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 (NCI)-Frederick Cancer Research and Development Center (Frederick, MD). I.3.5p pl human pancreatic cancer cells were established as described previously (24). All other pancreatic cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). Human pancreatic cancer cell lines were maintained in MEM. The immortal pancreatic duct epithelium cell line HPDE6-E6E7 was established and cultured as described previously (25, 26).

Antibodies and Chemicals
Antibodies were obtained from the following commercial sources: anti-active caspase-3 (Transduction Laboratories, San Diego, CA); anti-HDAC6, HDAC7, and HDAC1 (Cell Signaling, Beverly, MA); anti-ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-calreticulin (StressGen, Cruz Biotechnology, Santa Cruz, CA).
Bortezomib and stained for detection of ubiquitin (aggresome formation). L3.6pl cells were incubated for 24 hours with 100 nmol/L antibody as described in Materials and Methods. Bortezomib for 24 hours. Immunoblotting was done with an anti-ubiquitin ubiquitylated proteins (Ub-proteins). L3.6pl cells were incubated with 100 nmol/L bortezomib (BZ) and calreticulin as described in Materials and Methods. Fluorescence was visualized by confocal microscopy.

**Transmission Electron Microscopy**

Transmission electron microscopy (TEM) of cells was done as described previously (27). Ultrathin sections were cut in a LKB Ultrasound microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a LKB Ultrastainer (Leica), and examined in a JEM 1010 TEM (JEOL, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using the AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA).

**Immunoblotting**

Cells (1 × 10^5) were incubated with 100 nmol/L bortezomib for 24 hours. Cells were collected using a cell scraper at 4°C and lysed as described previously (19). 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 HRP. Immunoreactive material was detected by enhanced chemiluminescence (West Pico Pierce, Inc., Rockville, IL).

**Measurement of Intracellular Ca^{2+} Levels**

Cells were grown in medium with or without 100 nmol/L bortezomib, 5 µmol/L SAHA, or a combination of the two drugs for 12 hours. Cells were collected, washed in PBS, and incubated with 1 µmol/L Calcium Green-1 acetoxyethyl ester (Molecular Probes) for 30 minutes. Flow cytometric analysis of stained cells was done with a Becton-Dickinson FACSCalibur (San Jose, CA).

**Preparation and Transfection of Small Interfering RNA**

The annealed double-stranded HDAC6 and luciferase small interfering RNA (siRNA) were obtained from Dharmacon (Lafayette, CO): HDAC6 siRNA sense 5'-GCAGUUAACGAUUUACUUAU-3' and antisense 5’-P-AUCAGAAUCUAUAAUGCUU-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 and immunocytochemistry using anti-HDAC6 antibodies.

**Quantification of DNA Fragmentation**

DNA fragmentation was measured by PI staining and fluorescence-activated cell sorting (FACS) analysis as described previously (19). Cells were plated in six-well plates (1 × 10^6 per well). Following drug incubation, 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. DNA fragmentation was quantified by flow cytometric analysis.

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**Figure 1.** Bortezomib treatment induces ubiquitin-conjugated protein accumulation and aggresome formation. **A**, bortezomib (BZ) stabilizes ubiquitylated proteins (Ub-proteins). L3.6pl cells were incubated with 100 nmol/L bortezomib for 24 hours. Immunoblotting was done with an anti-ubiquitin antibody as described in Materials and Methods. **B**, bortezomib induces aggresome formation. L3.6pl cells were incubated for 24 hours with 100 nmol/L bortezomib and stained for detection of ubiquitin (Ub) and calreticulin as described in Materials and Methods. Fluorescence was visualized by confocal microscopy. **C**, TEM reveals aggresome structure. L3.6pl cells were fixed and processed for TEM as described in Materials and Methods. **D**, bortezomib does not induce aggresome formation in immortalized normal human pancreatic epithelial cells (HPDE6-E6E7). Cells were incubated with 100 nmol/L bortezomib for 24 hours, and aggresome formation was assessed by anti-ubiquitin immunofluorescence as described above.

Victoria, British Columbia, Canada). Horseradish peroxidase (HRP)-conjugated secondary antibodies for immunoblotting were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Alexa Fluor 488 goat anti-mouse antibody and Texas red goat anti-rabbit antibody were obtained from Molecular Probes (Eugene, OR). Bortezomib was kindly provided by W.G. Bornmann (M. D. Anderson Cancer Center, Houston, TX).

**Confocal Microscopy**

Cell lines. Pancreatic cancer and normal pancreatic epithelial cells were plated on chamber slides before 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. ToPro-3 (Molecular Probes) was used to counterstain the nucleus. Images were obtained using a Zeiss LSM510 confocal microscope (Oberkochen, Germany).

**Tumor sections.** Paraffin sections were prepared as described previously (19). Sections were incubated overnight with anti-ubiquitin antibody followed by 1-hour incubation with Alexa Fluor 488 secondary antibody. Analysis of DNA fragmentation by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) was done using a commercial kit (Promega, Madison, WI). PI was used to counterstain the nucleus. All slides were mounted using Prolong anti-fade reagent (Molecular Probes). Images were obtained using confocal microscopy as described above. Percentages of TUNEL-positive cells were determined using a laser scanning cytometer (LSC) as described previously (19).
Quantification of Apoptotic Cells and Aggresome Formation

Aggresome formation was detected by immunofluorescence staining with an anti-ubiquitin antibody and confocal microscopy. The presence of a single perinuclear inclusion was considered indicative of aggresome formation, and the results were confirmed by TEM. Aggresomes were quantified manually by scoring ~200 cells as aggresome positive or aggresome negative in three separate fields by confocal microscopy. Cell death was determined in the same cell populations by immunofluorescence with an antibody specific for active caspase-3 or by fluorescent TUNEL, which were done simultaneously with visualization of aggresomes to distinguish aggresome-positive and aggresome-negative apoptotic cells. ToPro-3 was used to counterstain nuclei. To quantify apoptotic cells with or without aggresomes, ~200 apoptotic cells were counted and scored as aggresome positive (defined aggresome) or aggresome negative (no visual aggresome) using a confocal microscope, and scoring was repeated thrice.

Orthotopic Implantation of Tumor Cells and Treatment Schedule

L3.6pl pancreatic cancer cells were harvested from culture flasks after brief trypsinization and transferred to serum-free HBSS. Only single-cell suspensions of >90% viability determined by trypan blue exclusion were used. Male nude mice were anesthetized with methoxyflurane, a small left abdominal flank incision was made, and tumor cells (1 × 10⁶) 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). A successful subcapsular intrapancreatic injection of tumor cells was confirmed by the appearance of a fluid bubble without i.p. leakage. To further 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 before therapy. Animals were then injected i.p. with bortezomib at a dosage of 1 mg/kg every 72 hours, 50 mg/kg SAHA daily, or a combination of the two drugs for 21 days. Mice were killed by cervical dislocation, and primary tumors in the pancreas were excised and weighed. For immunohistochemistry, tumor tissue was formalin fixed and paraffin embedded.

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 stimulates aggresome formation in pancreatic cancer cells. Inhibition of the proteasome resulted in the accumulation of ubiquitylated proteins in human pancreatic cancer cells as assessed by immunoblotting (Fig. 1A). Analysis of the subcellular distribution of these ubiquitin conjugates by immunofluorescent anti-ubiquitin staining and confocal microscopy revealed that they formed large perinuclear structures consistent in appearance with aggresomes (Fig. 1B; ref. 13). TEM revealed that the aggresomes were organized as electron-dense particles in a central core (Fig. 1C). Although bortezomib induced aggresome accumulation in most cancer cells, it did not induce aggresomes in immortalized normal pancreatic epithelial cells (Fig. 1D).

Figure 2. Aggresome formation protects against bortezomib-induced apoptosis. A, bortezomib-treated L3.6pl cells that fail to form aggresomes exhibit ER dilation. Cells were prepared as described previously, and representative images were taken of the ER structure within the drug-treated cells. B and C, aggresome-positive cells are resistant to bortezomib-induced apoptosis. L3.6pl cells were treated with 100 nmol/L bortezomib for 24 hours. Cells were stained with anti-ubiquitin antibody to visualize aggresomes, and either anti-active caspase-3 antibody or TUNEL assay was used to distinguish apoptotic cells. ToPro-3 was used to counterstain the nucleus in (B). Representative images. D, quantification of apoptotic cells with or without aggresomes. Apoptotic cells (~200) were counted and scored as aggresome positive (single-defined aggresome) or aggresome negative (no visual aggresome) using a confocal microscope. Columns, mean (n = 3); bars, SD.
SAHA disrupts bortezomib-induced aggresome formation and sensitizes aggresome-positive cells to apoptosis. A. Majority (6 of 8) of pancreatic cancer cell lines form a significant percentage of aggresomes following bortezomib treatment, which can be inhibited by SAHA. Cells were treated with 100 nmol/L bortezomib with or without 5 μmol/L SAHA for 24 hours, stained with anti-ubiquitin antibody to visualize aggresomes, and quantified using confocal microscopy. Cells (~200) were counted and scored per cell line. Columns, mean (n = 3); bars, SD. B, kinetic analysis of aggresome formation. Panc1 and L3.6pl cells were treated with 100 nmol/L bortezomib with or without 5 μmol/L SAHA for the indicated times. The presence of a single perinuclear inclusion was scored as an aggresome. Quantification was done as described in Materials and Methods. C, HDAC inhibitors block aggresome formation. Cells were treated with 100 nmol/L bortezomib and 5 μmol/L SAHA. Green, ubiquitin-conjugated proteins were visualized using Alexa Fluor 488 secondary antibody; blue, nucleus was counterstained with ToPro-3. Image was generated by confocal microscopy and is representative of the treatment group. Similar results were obtained when trichostatin A was used instead of SAHA (data not shown). TEM of drug-exposed L3.6pl cells. Arrows, ubiquitin-conjugated proteins. Note the single perinuclear aggresome in bortezomib-treated cells and the dispersion of ubiquitin-conjugated proteins in cells exposed to bortezomib + SAHA. D, HDAC inhibitors enhance bortezomib-induced apoptosis in aggresome-forming cells. Left, cells were incubated with 100 nmol/L bortezomib, 500 nmol/L trichostatin A (TSA), 5 μmol/L SAHA, or combinations for 24 hours. Apoptosis was measured by PI-FACS analysis. Columns, mean (n = 3); bars, SD. Right, immortalized normal pancreatic epithelial cells (HPDE6-E6E7) are unaffected by the bortezomib and SAHA combination. Cells were treated with 100 nmol/L bortezomib, 5 μmol/L SAHA, or combination for 24 hours, and apoptosis was measured by PI-FACS analysis. Columns, mean (n = 3); bars, SD.
Aggresome formation limits bortezomib-induced apoptosis.

We next investigated the relationship between aggresome formation and bortezomib-induced ER stress and apoptosis. Visual examination of electron micrographs of bortezomib-treated L3.6pl cells indicated that aggresome-positive cells displayed no visible signs of ER stress, whereas aggresome-negative cells appeared more like cells exposed to thapsigargin (an ER Ca\(^{2+}\) ATPase inhibitor that is known to induce ER stress) and displayed significant dilation of the ER (Fig. 24). To further investigate the relationship between aggresome formation and cell death, we quantified the levels of apoptosis in aggresome-positive and aggresome-negative cells by confocal microscopy (Fig. 2B and C). Almost all of the aggresome-positive cells were TUNEL and active-caspase-3 negative, whereas aggresome-negative cells were TUNEL and active-caspase-3 positive (Fig. 2D). Thus, aggresomes seem to play a cytoprotective function that limits bortezomib-induced apoptosis.

**HDAC inhibition disrupts aggresomes and enhances bortezomib-induced apoptosis.** We next determined the prevalence of

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**Figure 4.** HDAC6 inhibition disrupts aggresome formation and sensitizes pancreatic cancer cells to bortezomib-induced apoptosis. A, HDAC6 knockdown disrupts aggresome formation. RNAi was done as described in Materials and Methods. Cells were stained with anti-ubiquitin antibody to visualize aggresomes and anti-HDAC6 antibody to examine expression levels. Images were obtained by confocal microscopy. Quantification of aggresome-positive cells. Cells (~200) were counted and scored as aggresome positive (single-defined aggresome) or aggresome negative (no visible aggresome) using a confocal microscope. Columns, mean (n = 3); bars, SD. B, knockdown of HDAC6 enhances bortezomib-induced apoptosis. L3.6pl cells were treated with 100 nmol/L bortezomib in the presence or absence of HDAC6 siRNA. Luciferase siRNA was used as an off-target control. Immunoblotting showed silencing of HDAC6 (H6) without change in HDAC7 (H7) or HDAC1 (H1) expression. Apoptosis was determined by PI-FACS. Columns, mean (n = 3); bars, SD. Asterisk, significant difference from bortezomib-treated untransfected (UN) and luciferase (LUC) siRNA-transfected cells.

**Figure 5.** Aggresome disruption sensitizes pancreatic cancer cells to ER stress. A, electron micrographs of ER morphology in cells exposed to 100 nmol/L bortezomib, 5 \(\mu\)mol/L SAHA, or both drugs. Black arrows, ER; red arrows, aggresome in bortezomib-treated cells or disrupted aggresome in combination-treated cells. B, exposure to bortezomib + SAHA disrupts intracellular Ca\(^{2+}\) homeostasis. L3.6pl cells were treated for 12 hours with 100 nmol/L bortezomib, 5 \(\mu\)mol/L SAHA, or both drugs, loaded with Calcium Green-1 acetoxymethyl ester, and analyzed by flow cytometry as described in Materials and Methods. Columns, mean (n = 3); bars, SD. Asterisk, significant difference from either bortezomib or SAHA single-agent treatment.
Bortezomib-induced aggresome formation in a panel of eight pancreatic cancer cell lines. Six of them, including two bortezomib-resistant cell lines, displayed significant levels of aggresome formation in >40% of cells, which could be inhibited by the HDAC inhibitor, SAHA (Fig. 3A). Interestingly, no aggresomes were detected in immortalized normal pancreatic epithelial cells (Fig. 1D; data not shown). Kinetic analysis of aggresome formation in Panc1 (bortezomib-resistant) and L3.6pl (bortezomib-sensitive) pancreatic cancer cells showed that 60% to 70% of cells possessed an aggresome by 12 hours after exposure to 100 nmol/L bortezomib.
bortezomib (Fig. 3B). The HDAC inhibitors SAHA and trichostatin A blocked bortezomib-induced aggresome formation (Fig. 3B and C) and dramatically sensitized aggresome-positive cells to bortezomib-induced apoptosis (Fig. 3D). Because the immortalized normal pancreatic epithelial cells did not form aggresomes, they were unaffected by the combination of bortezomib and SAHA (Fig. 3D). Because previous work implicated the cytosolic HDAC6 in proteasome inhibitor-mediated aggresome formation in other cellular models (28, 29), we tested the effects of silencing HDAC6 expression on aggresome formation and apoptosis. Consistent with the previous results (28, 29), silencing HDAC6 disrupted aggresome formation (Fig. 4A) and sensitized L3.6pl pancreatic cancer cells to bortezomib-induced apoptosis (Fig. 4B). Because aggresomes seemed to segregate with viable cells, we examined the effects of disruption on ER stress. The combination of bortezomib plus SAHA produced a significant increase in ER dilation (Fig. 5A) and ER Ca\(^{2+}\) release (Fig. 5B; ref. 30) compared with the levels observed in cells exposed to either single agent.

**Effects of bortezomib and SAHA in orthotopic human pancreatic tumors.** In a final series of experiments, we investigated the effects of bortezomib with or without SAHA on aggresome formation and cell death in orthotopic L3.6pl tumors. Established tumors were treated biweekly with 1 mg/kg bortezomib, daily with 50 mg/kg SAHA, or a combination of the two agents for 21 days, and the experiment was done twice. Tumors treated with bortezomib alone contained numerous aggresomes that were disrupted in tumors treated with bortezomib plus SAHA (Fig. 6A). Importantly, bortezomib did not induce aggresomes in adjacent normal murine pancreatic epithelial cells (Fig. 6A). Therapy with either bortezomib or SAHA alone stimulated moderate increases in tumor cell apoptosis as measured by TUNEL staining and LSC (tubacin) was isolated by Schreiber’s laboratory (39), and in a more recent study, Anderson’s group showed that it also synergized with bortezomib to induce apoptosis in multiple myeloma cells (29).

**Discussion**

The proteasome has become an attractive target for pharmacologic inhibition in cancer. Bortezomib is the first proteasome inhibitor evaluated in clinical trials, where it displayed manageable toxicity at target doses in a variety of different tumor types (31) and received FDA approval in 2003 for use in the treatment of multiple myeloma (5). *In vitro* and *in vivo* studies done by our laboratory and others have shown that bortezomib also has promising antitumor activity in pancreatic cancer models (18, 19, 32, 33). Bortezomib activates the mitochondrial (intrinsic) pathway of apoptosis (31, 34, 35), but recent evidence indicates that these effects may occur downstream of events initiated within the ER (36, 37).

Here, we show that pancreatic cancer cells exposed to bortezomib contain electron-dense structures consisting of ubiquitin-conjugated proteins that have been termed aggresomes (38). In pancreatic cancer cells, aggresomes seem to play a cytoprotective function presumably sequestering aggregated ubiquitylated proteins and perhaps targeting them for lysosomal degradation (20), thereby limiting ER stress (Fig. 5; ref. 13). Aggresome formation requires the expression of a cytosolic, cytoskeleton-associated HDAC (HDAC6) as shown by our observation that aggresomes are abolished in cells transfected with a HDAC6-specific siRNA construct (Fig. 4A). Our data are consistent with a previous study, which showed that HDAC6 silencing or expression of enzymatically inactive HDAC6 proteins prevented aggresome formation in a neuronal model system (24). Virtually all bortezomib-induced apoptosis was restricted to aggresome-negative cells, and agents that disrupted them (HDAC6 siRNA or chemical HDAC inhibitors) dramatically increased cell death, reversing bortezomib resistance in two of the cell lines (Figs. 3 and 4). Importantly, a more selective chemical inhibitor of HDAC6 (tubacin) was isolated by Schreiber’s laboratory (39), and in a more recent study, Anderson’s group showed that it also synergized with bortezomib to induce apoptosis in multiple myeloma cells (29).

**Table 1. Therapy of L3.6pl human pancreatic cancer cells implanted into the pancreas of nude mice**

<table>
<thead>
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<th>Experiment no.</th>
<th>Treatment group</th>
<th>Tumor incidence</th>
<th>Tumor weight (g)</th>
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<th>Body weight (g)</th>
<th>Mortality</th>
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NOTE: L3.6pl cells were injected into the pancreas of nude mice and established for 14 days. Mice were injected i.p. with DMSO and saline (control), bortezomib (1 mg/kg) every 72 hours, SAHA (50 mg/kg) daily, or both drugs for 21 days. Following 3 weeks of treatment, mice were sacrificed, and tumor weight, tumor volume, and body weight were recorded. All mice had pancreatic tumors. No mice mortality occurred during either experiment. Gastrointestinal toxicity indicates diarrhea as characterized by loose stools in the bowel.

*P < 0.05 versus control, bortezomib, and SAHA treatment groups.
Therefore, it will be of interest to compare tubacin to the more conventional pan-HDAC inhibitors with respect to its effects on bortezomib-induced aggresome formation and apoptosis in preclinical models of human pancreatic cancer.

Aggresomes have been observed in many pathologic states, including neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease (11, 40). A recent report showed that cells possessing aggresomes formed by α-synuclein and synphilin-1 are resistant to apoptosis (41), consistent with our conclusion that they inhibit (rather than stimulate) cell death. It is tempting to speculate that the peripheral neuropathy observed in patients treated with bortezomib (7) may be associated with aggresome formation and ER stress in peripheral neurons, which we plan to explore in future studies. Whether HDAC inhibitors exacerbate peripheral neuropathy will have to be watched closely in ongoing clinical trials of the combination.

To further address the therapeutic potential of the combination of bortezomib and HDAC inhibitors, we examined the anti-tumor effects of bortezomib and SAHA in an L3.6pl orthotopic pancreatic cancer model. Tumors exposed to the maximum tolerated dosage of bortezomib (1 mg/kg biweekly) displayed extensive aggresome formation (Fig. 6). Treatment with SAHA disrupted bortezomib-induced aggresome formation, decreased pancreatic tumor weight, and enhanced tumor cell apoptosis (Fig. 6). We are currently “recycling” (24) the bortezomib-resistant Panc1 cells to enhance their tumorigenic potential so that the effects of combination therapy on an aggresome-positive, bortezomib-resistant tumor can be evaluated. Importantly, bortezomib did not induce aggresomes in the normal human pancreatic epithelial cells in vitro nor in normal murine pancreatic epithelial cells in vivo, and the normal cells did not undergo apoptosis in response to single-agent or combination therapy (Fig. 3; data not shown). The tumor cell selectivity of bortezomib or HDAC inhibitors has been reported previously (42–44), and our data indicate that they also display tumor selectivity when they are combined. In addition to the immortalized normal pancreatic epithelial cells examined here, EBV-transformed normal B cells also failed to form aggresomes, whereas two multiple myeloma cell lines did so following bortezomib exposure.8

We do not have an explanation for why normal cells tend not to form aggresomes, but the question is currently under investigation. Increased cell proliferation is a hallmark of aggressive cancers and requires a general increase in protein synthesis and a heavy dependency on proteasomal degradation of aged, misfolded, or oxidized proteins. Several of the signal transduction pathways (Ras, phosphatidylinositol 3-kinase, mitogen-activated protein kinase, and mammalian target of rapamycin) implicated in solid tumor progression activate various components of translation machinery (45). We speculate that the lower translation rates exhibited by normal cells make them resistant to bortezomib-induced aggresome formation and subsequent proteotoxicity.

Although aggresomes seem to play important cytoprotective functions in cells exposed to bortezomib, aggresome formation was not the only mechanism of drug resistance we observed in our cell lines. Two of them (MiaPaCa-2 and Hs766T) remained relatively resistant to bortezomib when it was combined with trichostatin A or SAHA presumably because the cells did not form aggresomes in response to proteasome inhibition. Consistent with our in vitro observations, orthotopic implanted MiaPaCa-2 tumors in mice treated with bortezomib also did not form aggresomes (data not shown). Molecular chaperones interact with nonnative protein conformations to block protein aggregation and toxicity. It is possible that the failure of the MiaPaCa-2 and Hs766T cells to form aggresomes was due to high chaperone [heat-shock protein (Hsp)] levels.9 Previous studies showed that Hsp27 and Hsp70 promote resistance to proteasome inhibitor-induced apoptosis (46, 47), and they might also be expected to neutralize the cytotoxic effects of protein aggregates independently of aggresome formation. We are currently investigating the role that Hsps may play in resistance to bortezomib-induced apoptosis.

As noted above, other recent studies have implicated ER stress in the effects of bortezomib and other proteasome inhibitors in different tumor types (36, 37, 48). Our results confirm that cells undergoing apoptosis in response to bortezomib or bortezomib plus SAHA displayed alterations consistent with ER stress, including ER dilation, ER Ca2+ release, induction of ER stress-associated genes (GADD34, CHOP, and Grp78/BiP), and Jun N-terminal kinase activation (Figs. 2 and 5; refs. 49, 50). However, we believe that the strongest evidence for the involvement of ER stress in bortezomib-induced cell death comes from mechanistic studies that identified the caspases required for bortezomib-induced apoptosis. Pioneering work with knockout mice established that caspase-12 mediates ER stress-induced apoptosis in murine cells (51), and bortezomib-induced apoptosis is also associated with caspase-12 activation in the mouse (28, 30, 37, 49, 50). However, a recent study showed that expression of functional caspase-12 in humans is limited to females of African descent (52), strongly suggesting that another caspase plays a more central role in ER stress-induced apoptosis in humans. The caspase-12 orthologue, caspase-4, is an excellent candidate, and a recent study confirmed that it is involved in ER stress-induced and amyloid-β-induced apoptosis in human neuronal cells (53). Using immunofluorescence staining and confocal microscopy, we observed that caspase-4 localizes to the ER in pancreatic cancer cells and is cleaved to its active form in the four cell lines that undergo apoptosis in response to bortezomib (49). Furthermore, peptide-based or siRNA inhibitors of caspase-4 block bortezomib-induced apoptosis (49, 50). In ongoing work, we are investigating how bortezomib activates caspase-4 and how caspase-4 activation may initiate mitochondrial events to promote cell death.

Taken together, our findings implicate ER stress in apoptosis induced by bortezomib in human pancreatic cancer cells (outlined in Fig. 6C), and they provide the framework for clinical trials of bortezomib and SAHA or other HDAC inhibitors in patients with pancreatic cancer. We have submitted a letter of intent to the Cancer Therapeutics Evaluation Program, NCI [H. Xiong, D.J. McConkey, and J.L. Abbruzzese (coprincipal investigators)] to do such a trial.

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