Non-proteasomal targets of the proteasome inhibitors bortezomib and carfilzomib: a link to clinical adverse events

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Statement of Relevance to Translational Research

Bortezomib has validated the proteasome as a target for the treatment of myeloma, but is hindered by a painful peripheral neuropathy (PN) that may occur due to proteasome inhibition. Carfilzomib, a second in class proteasome inhibitor, is in late stage clinical trials and displays minimal signs of peripheral neuropathy. These observations suggest a proteasome independent mechanism (off-target activity) underlying PN.

In this study, we utilized an in vitro model of neurodegeneration to that, despite equivalent proteasome inhibition, only bortezomib induced neurite degeneration. Utilizing a multi-faceted approach to identify off-targets that included activity-based probe (ABP) profiling in cells and in silico database mining we found that bortezomib targets several serine hydrolases. We report the first demonstration of inhibition of a non-proteasomal target (cathepsin G) in bortezomib treated patients using ABPs. Also, bortezomib inhibits a pro-survival protease, HtrA2/Omi, in neuronal cells, supporting the hypothesis that bortezomib-induced PN results from off-target activity.
ABSTRACT

Purpose: Bortezomib (Velcade™), a dipeptide boronate 20S proteasome inhibitor and an approved treatment for multiple myeloma is associated with a treatment-emergent, painful peripheral neuropathy (PN) in >30% of patients. Carfilzomib, a tetrapeptide epoxyketone proteasome inhibitor, currently in clinical investigation in myeloma, is associated with low rates of PN. We sought to determine whether PN represents a target mediated adverse drug reaction (ADR).

Experimental Design: Neurodegenerative effects of proteasome inhibitors were assessed in an in vitro model utilizing a differentiated neuronal cell line. Secondary targets of both inhibitors were identified by a multi-faceted approach involving candidate screening, profiling with an activity-based probe and database mining. Secondary target activity was measured in rats and patients receiving both inhibitors.

Results: Despite equivalent levels of proteasome inhibition, only bortezomib reduced neurite length, suggesting a non-proteasomal mechanism. In cell lysates, bortezomib, but not carfilzomib, significantly inhibited the serine proteases cathepsin G (CatG), cathepsin A, chymase, dipeptidyl peptidase II, and HtrA2/Omi at potencies near or equivalent to that for the proteasome. Inhibition of CatG was detected in splenocytes of rats receiving bortezomib and in peripheral blood mononuclear cells derived from bortezomib-treated patients. Levels of HtrA2/Omi, which is known to be involved in neuronal survival, were upregulated in neuronal cells exposed to both proteasome inhibitors but was inhibited only by bortezomib exposure.
Conclusion: These data demonstrate that bortezomib-induced neurodegeneration in vitro occurs via a proteasome-independent mechanism and that bortezomib inhibits several non-proteasomal targets in vitro and in vivo, which may play a role in its clinical ADR profile.
INTRODUCTION

Poly-pharmacology is the study of identifying secondary targets for an active pharmaceutical agent (drug promiscuity). In some disease etiologies, targeting multiple proteins may improve the therapeutic efficacy, such as for antipsychotic drugs or kinase inhibitors with anti-cancer activity (1). However, off-target activity can lead to adverse drug reactions (ADR) such as the case with fenfluramine and torcetrapib, two agents halted in development due to fatal hypertension that was the consequence of secondary target inhibition (1-3). Highlighting the promiscuity of small molecule inhibitors, a recent in silico screening of 213 Roche compounds revealed an average of 6.3 target proteins per compound (1). The standard approach for detecting off-target activity consists of screening compounds against panels of targets, often involving recombinant proteins in cell-free systems, and in silico prediction tools (1;1;4). More recently, whole genome- and proteome-based analyses have been utilized to infer potential off-target activity, but these methods identify affected pathways rather than direct protein targets for a drug (3).

Bortezomib (Velcade™) is a dipeptide boronate proteasome inhibitor that has been approved for the treatment of multiple myeloma (MM) and mantle cell lymphoma (5). The proteasome is a multicatalytic protease whose active sites are unique N-terminal threonine proteases (6;7). Proteasome inhibition results in the modulation of many cellular activities, including alterations in signal transduction pathways, modifications in antigen presentation, and induction of an apoptotic response (5). Despite robust anti-tumor activity in the clinic, bortezomib therapy is hindered by a treatment-emergent peripheral neuropathy. In relapsed and/or refractory MM,
single agent bortezomib results in peripheral neuropathy in 35 – 52% of patients with Grade 3/4 rates ranging from 8 – 14% (8-11). The mechanism of this neuropathy is unknown but may represent the effects of proteasome inhibition in sensory neurons (12).

The clinical success of bortezomib has led to the development of several new proteasome inhibitors, including agents with chemical structures distinct from bortezomib (13;14). Of these, carfilzomib, a tetrapeptide epoxyketone, is the furthest in clinical development, with Phase 2 trials in myeloma and solid tumors ongoing (15). Recently, it was reported that treatment-emergent neuropathy occurred in 14.6% of 505 treated patients with 1.2% reaching a Grade 3/4 level(16;17). Carfilzomib and bortezomib display an equivalent potency for the chymotrypsin-like (CT-L) subunits of the proteasome but differ in the length of their peptide backbone (tetrapeptide vs. dipeptide) and in the reactive pharmacophore responsible for inhibition of proteasome active sites (13;14;18). The epoxyketone warhead of carfilzomib forms a dual covalent adduct that can only be formed with the side chain hydroxyl and free amine of the N-terminal threonine active site that is found exclusively within the proteasome. On the other hand, the boronate warhead of bortezomib forms a tetrahedral intermediate with the side chain hydroxyl group (13). This tetrahedral intermediate can also be formed with other classes of proteases, such as serine proteases. Indeed, cathepsin G (CatG) and chymase, two serine proteases, were previously demonstrated to be targets of bortezomib, but their inhibition in enzymatic assays using purified enzymes was reported to occur at concentrations 100-fold or more above that required for proteasome inhibition (19). However, the activity of bortezomib against non-proteasomal proteases in physiologic conditions (i.e., in cells or in vivo) has not been
determined and the contribution of on- and off-target effects in the induction of peripheral neuropathy remain an open question.

We report here that bortezomib induces neurotoxicity by a proteasome-independent mechanism in an in vitro model of neurodegeneration, which involves the quantitation of neurite length and cell survival in differentiated SH-SY5Y (neuroblastoma) cells. To investigate the propensity of bortezomib and carfilzomib to inhibit non-proteasomal targets, we pursued a multi-faceted approach utilizing a panel of purified enzymes, activity-based probe (ABP) profiling in cells and cell lysates, and in silico database mining. The results presented here are the first practical application of ABPs for profiling off-target activity of an FDA-approved drug. Using both purified enzymes and ABP, we were able to show that bortezomib targets several serine hydrolases, including CatG, cathepsin A (CatA), chymase, and dipeptidyl peptidase II (DPPII). By the ABP approach, which enabled the measurement of activity in cells and in vivo, we found that the potency of bortezomib against several serine proteases was higher than previously reported. Furthermore, we demonstrate inhibition of CatG in splenocytes from bortezomib-treated rats and in peripheral blood mononuclear cells (PBMC) from bortezomib-treated patients. Finally, through both database mining and substrate monitoring in SH-SY5Y cells, we found that bortezomib is a potent inhibitor of HtrA2/Omi, a stress-induced protease involved in neuronal cell survival. Interestingly, carfilzomib showed almost no inhibitory activity against serine proteases in vitro and in vivo. Taken together, these data support the hypothesis that peripheral neuropathy is not a class effect of proteasome inhibitors, and may involve off-target inhibition by bortezomib.
METHODS

Compounds
The biotinylated general serine hydrolase fluophosphonate probe (FP-biotin) was synthesized as previously described (20). Bortezomib (trade name Velcade®) was purchased from a pharmacy. Cbz-Leu-Leu-Leu-Boronic Acid was purchased from AG Scientific, Inc. (San Diego, CA). MG-132 (Cbz-Leu-Leu-Leu-Aldehyde) was purchased from Boston Biochem (Boston, MA). Carfilzomib was synthesized as previously described (US 07232818). Cbz-Leu-Phe-ketoepoxide and Cbz-Leu-Leu-Leu-ketoepoxide were synthesized as previously described (WO 2005111008) with >95% purity by HPLC and NMR methods. Cbz-Leu-Phe-aldehyde was synthesized as previously described (21) and the aldehyde version of carfilzomib was synthesized by the reduction of the Weinreb amide of carfilzomib to an aldehyde with lithium aluminum hydride in THF with >75% purity by HPLC and NMR methods.

Cells
Peripheral blood mononuclear cells (PBMC) from normal healthy volunteers were obtained from All Cells (Berkeley, CA). HepG2 (hepatocellular carcinoma), THP-1 (acute monocytic leukemia), 786-O (renal cell adenocarcinoma), and SH-SY5Y (neuroblastoma) cells were obtained from the American Type Culture Collection (ATCC) and were cultured in media recommended by the supplier at 37°C in 5% CO₂.

Animals
Male Sprague-Dawley rats (275-300g) were purchased from Charles River Laboratories (Hollister, CA) and housed for 1 week before experimentation. For all experiments, animals had access to food and water ad libitum. All experiments were done under protocols approved by an institutional animal care and use committee.

**SH-SY5Y neurodegeneration assay**

This assay was performed as previously described (22;23). Briefly, SH-SY5Y cells were differentiated in multiwell plates by culturing in the presence of retinoic acid for 5 days followed by exposure to brain derived neutrotrophic factor for 3 days. The cells were then treated with compound for 24, 48 and/or 72 hours, fixed and stained with Hoechst (blue) for identification of cell nuclei staining to identify live cells and neuronal-specific βIII-tubulin (green) for the cytoskeleton to visualize cell bodies and neurites. Endpoint analysis was performed by high content imaging and quantitative image analysis on an IN Cell Analyzer 1000 system (GE Healthcare, Piscataway, NJ), utilizing software designed to segment cellular morphological features based on size- and fluorescence intensity-related criteria.

**Purified enzyme assays**

Substrate-based assays were initiated by enzyme addition and monitored for aminoacyl 7-amino-4-methylcoumarin amide (MCA) and 7-amino-4-methylcoumarin (AMC) product formation with a plate-based spectrofluorometer (Tecan, San Jose, CA). Chymase (EMD Chemicals, San Diego, CA) activity was measured using Suc-Ala-Ala-Pro-Phe-AMC (MP Biomedicals, Solon OH) in assay buffer consisting of 200 mM HEPES, pH 8.0, 2 M NaCl, and 0.01% Triton X-100 at 27°C. CatG (EMD) activity was measured using Suc-Ala-Ala-Pro-Phe-AMC (MP
Biomedicals, Solon, OH) in assay buffer consisting of 50 mM NaOAc, pH 5.5, 2 mM EDTA, and 1 mM DTT at 30°C. CatA (R & D Systems, Minneapolis, MN) activity was measured using MCA-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp) in assay buffer consisting of 50 mM MES, pH 5.5, and 5 mM DTT at 27°C. Dipeptidyl peptidase II (DPPII; R & D Systems, Inc., Minneapolis, MN) activity was measured using Suc-Lys-Pro-AMC (MP Biomedicals) in assay buffer consisting of 25 mM MES, pH 6.0 at 27°C. IC50 values were determined based on the reaction velocity measured between 60 and 75 min using GraphPad Prism 4.01 (San Diego, CA). HtrA2/Omi (R & D Systems) activity was measured in a gel-based assay using β-casein (Sigma-Aldrich Corp., St. Louis, MO) as a substrate as described by the manufacturer. The processed proteins were resolved by SDS-PAGE and detected by silver staining (Invitrogen, Carlsbad, CA).

Detection of protease activity in lysates

PBMC lysate IC50 determinations: 2 – 3 x 10^6 cells were lysed per condition in 50 – 100 μl of lysis buffer (phosphate buffered saline (PBS) with 1% Nonidet P40 (NP40)) for 1 hr on ice. Lysates were cleared by centrifugation and treated with inhibitor at various concentrations for 30 min followed by 1 hr incubation with 10 μM FP-biotin at room temperature (RT). SDS was added to a final concentration of 1% (5 μl in a 50 μl reaction volume) and the samples were boiled for 5 min. The SDS was diluted to 0.1% with PBS (V_F = 500 μl) and incubated with rotating overnight with 20 μl of pre-washed Pierce® Streptavidin UltraLink® Resin (Thermo Scientific, Rockford, IL) at RT. FP-biotin bound proteome resin was washed three times in lysis buffer and after the final wash any remaining liquid was extracted with a 30 gauge needle. The resin was resuspended in 10 μl PBS, 20 μl 4X SDS-PAGE loading dye (Bio-Rad, Hercules, CA),
and 3 μl of 10X reducing agent (Bio-Rad). The FP-biotin bound proteome was eluted by 3 cycles of 5 min boiling and vortexing. 10 μl of the eluate was loaded onto each lane for Western blot analysis. The signal was quantitated by densitometry using Image J software (http://rsbweb.nih.gov/ij/) and IC₅₀ values were determined by GraphPad Prism 4.01. Antibodies used for western blotting were mouse monoclonal anti-human CatA and anti-human chymase (CMA1) obtained from R & D Systems, rabbit polyclonal anti-human CatG and anti-human DPPII purchased from Abcam (Cambridge, MA) and, horseradish peroxidase-conjugated (HRP) secondary antibodies obtained from Jackson ImmunoResearch Laboratories, Inc (Westgrove, PA). ImmunoPure® Streptavidin resin and HRP-streptavidin overlay were purchased from Thermo Scientific (Rockford, IL).

**Profiling tumor cell lines:** Tumor cells (1.5 – 3 x 10⁶ cells per condition) were lysed in lysis buffer for 30 min on ice and cleared by centrifugation. The lysates were then incubated with 10 μM bortezomib or carfilzomib for 30 min at RT and then probed with 5 μM of FP-biotin at RT for 1 hr. Proteins were separated by SDS-PAGE chromatography and FP-bound targets detected by western blotting with HRP-conjugated streptavidin.

**Ex vivo treatment of whole blood**

Whole blood (10 ml) was treated with 100 nM of bortezomib or carfilzomib for 40 min and transferred to BD Vacutainer cell preparation tubes (CPT; BD Biosciences, Franklin Lakes, NJ) to isolate PBMC. After centrifugation, PBMC were collected, erythrocytes were removed by hypotonic lysis (BD PharmLyse™, BD Biosciences), and the cells were lysed and cleared as described above. Cleared lysate was probed with 5 μM of FP-biotin at RT for 1 hr. CatG was detected as described above.
Pharmacodynamics *in vivo*

Rats received intravenous bolus administration of bortezomib at 0.3 mg/kg and carfilzomib at 7 mg/kg. Fifteen minutes after dosing, animals were sacrificed by CO₂ narcosis, and blood and spleens were collected and processed as described previously (24). Proteasome chymotrypsin-like activity was measured as described previously (24) and CatG activity in lysates from erythrocyte–depleted splenocytes was measured by FP-biotin probe binding as described above using an anti-rodent CatG antibody (Santa Cruz Biotechnologies, Santa Cruz, CA).

Whole blood (10 ml) was collected from consenting patients receiving 1.0 (N = 1), 1.3 (N = 2), or 1.6 mg/m² (N = 1) bortezomib into CPT to isolate PBMC as described above. Proteasome and CatG activity was measured as described above. The study was approved by the independent cantonal research ethics review board and performed in accordance with good clinical practice guidelines, the Declaration of Helsinki, and the applicable national law.

Western blotting for HtrA2 substrates

SH-SY5Y cells were seeded at 3 x 10⁵ cells/ml and cultured for 2 days. The cells were then treated for 12 hrs with carfilzomib or bortezomib (both at 100 nM) and harvested for Western blotting analysis. Cell pellets were lysed in PBS + 1% NP40 + protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) for 1 hr on ice and the lysates were cleared by centrifugation. Proteins were resolved by SDS-PAGE transferred to nitrocellulose and probed with antibodies for HtrA2, eIF4G1 and pyruvate dehydrogenase. All the antibodies were purchased from Abcam.
**Statistical analysis**

For comparisons of treatment groups, a one-way ANOVA followed by Bonferroni post-hoc analysis using GraphPad Prism (version 4.01) was done. Statistical significance was achieved when $P < 0.05$. 
RESULTS

Bortezomib induces neurite degeneration in vitro by a proteasome-independent mechanism

To investigate the role of proteasome inhibition in neurite degeneration, an in vitro assay was employed. This assay involved the differentiation of the human neuroblastoma cell line SH-SY5Y in the presence of retinoic acid and brain derived neurotrophic factor (BDNF), followed by exposure to proteasome inhibitors (22). Following treatment, cells were stained with Hoechst dye (blue) to detect nuclei and FITC anti-βIII-tubulin (green) to detect neurite outgrowth and analyzed using a high content image analysis software (Figure 1A). Exposure of differentiated cells to 10 nM bortezomib resulted in a 40% reduction in total and average cellular neurite length (Figure 1B) that was not a result of cell death (Figure 1C). Exposure to an equimolar concentration of carfilzomib did not affect neurite length or cell viability in this system. Both agents resulted in equivalent levels of proteasome inhibition (~60%) following 1 hr of exposure (Figure 1C). After 72 hr of drug exposure across a concentration range of 10μM – 1nM both agents caused neurite loss that was associated with cell death (data not shown). Even with these longer exposures, bortezomib was 5.6- and 11-fold more potent than carfilzomib at inducing cell death and inhibiting neurite length, respectively. The observation that carfilzomib does not alter neurite morphology at non-cytotoxic concentrations despite potent proteasome inhibition suggests that neurite degeneration induced by bortezomib is mediated by a mechanism that is independent of proteasome inhibition.

Bortezomib inhibits multiple serine proteases in vitro
Bortezomib has been demonstrated to inhibit the serine proteases chymotrypsin, catG and chymase, but not proteases with a preference for basic residues at P1, such as thrombin (19). We used a candidate approach to compare the activity of bortezomib and carfilzomib against a panel of twenty-one serine, cysteine, aspartyl and metallo proteases (See Supplemental Data section for the full list of proteases tested). At 10 μM, bortezomib inhibited serine proteases with a P1 selectivity of large hydrophobic or aromatic amino acids, (chymotrypsin, CatG and chymase,) by 95%, and elastase, rennin and angiotensin converting enzyme (ACE) by 30 – 40%. On the other hand, carfilzomib displayed modest inhibition of chymotrypsin (~40%) but had minimal to no activity against the other twenty proteases. These data demonstrate that bortezomib has a broader off-target activity profile against non-proteasomal proteases than was previously considered.

In order to broadly profile the activity of bortezomib as a inhibitor of serine hydrolases, we utilized an activity based probe (ABP) approach using a general serine hydrolase probe fluorophosphonate- (FP-) biotin (19;20); Supp Figure 2). Lysates from three peripheral blood mononuclear cell (PBMC) samples, an easily accessible primary human tissue that is a rich source of proteases (Supp Table 1), were exposed to both proteasome inhibitors prior to addition of the FP-biotin probe. Isolated streptavidin bound proteins were identified by liquid chromatography and tandem mass spectrometry (LC/MS/MS). As a validation of the method, we found that the broad-based protease inhibitors difluorophosphonate (DFP) and diisocoumarin (DCI) resulted in the inhibition of several serine hydrolases (Supp Table 1). Further validating this approach, CatG inhibition by bortezomib was detected. By the ABP approach, we also detected inhibition of cathepsin A (CatA) and dipeptidyl peptidase II (DPPII) by bortezomib, two
proteases that were not evaluated in the candidate screen (Supp Table 1). Carfilzomib did not result in significant inhibition of serine proteases in PBMC lysates as measured by ABP profiling.

We extended these findings by determining the relative potency of bortezomib using fluorescent substrate assays on purified CatG, CatA, chymase, and DPPII. Bortezomib had varying potencies against these purified enzymes, with 50% inhibitory concentration (IC$_{50}$) values ranging from 0.3 – 9 µM (Table 1). To investigate whether the activity of the enzymes might differ in a complex cellular milieu, we determined the IC$_{50}$ values using the FP-biotin probe and a gel-based assay (Table 1 & Figure 2A). In each case, bortezomib was more potent against serine proteases in cell lysates versus purified enzymes. For CatA and chymase, IC$_{50}$ values were equivalent to that against the proteasome. Carfilzomib did not result in significant inhibition of these serine proteases in either assay system. These data demonstrate potent inhibition of several serine protease targets of bortezomib in physiologically relevant conditions.

Next, we utilized the FP-biotin probe to detect protease inhibition in three tumor cell lines. Lysates of HepG2 (liver adenocarcinoma), THP-1 (monocytic leukemia), and 786-O (renal adenocarcinoma) were exposed to bortezomib or carfilzomib. In all samples, we detected one or more FP-biotin-reactive protein species whose binding to FP-biotin could be blocked by prior exposure to 10 µM bortezomib, but was not affected by carfilzomib (Figure 2B). We identified CatA as a bortezomib target in HepG2 lysates and CatG in THP-1 cells lysates (data
not shown). Taken together, these data demonstrate a broad and variable profile of non-proteasomal targets for bortezomib.

**Peptide boronic acids are potent serine protease inhibitors**

Two possible explanations for the striking difference in serine protease inhibition between bortezomib and carfilzomib are the peptide length (di- vs. tetrapeptide) and the reactive pharmacophore (boronate vs. epoxyketone). Indeed, peptide boronates have been described as potent serine protease inhibitors (25;26). To address the roles of peptide length and pharmacophore on off-target activity, we generated a matrix of nine compounds related to the peptide backbones of carfilzomib (hPhe-Leu-Phe-Leu), MG-132 (Leu-Leu-Leu), and bortezomib (Phe-Leu), where each of the peptide backbones were synthesized with each of the three pharmacophores: epoxyketone, aldehyde, and boronate (Figure 2C). PBMC lysates were exposed to each compound and probed with FP-biotin and Western blotting for four target proteases (CatG, CatA, DPPII and chymase). Boronate-containing compounds, regardless of the peptide backbone, were potent inhibitors of serine proteases, while none of the epoxyketone containing inhibitors resulted in detectable inhibition (Figure 2D). Aldehyde-based compounds were also unable to inhibit FP probe binding, further suggesting that serine protease inhibition is driven by the boronate pharmacophore. These data were further validated by fluorogenic substrate assays with CatG and the three warhead versions of Leu-Leu-Leu (compounds 4-6) where only the boronic acid analog (compound 6) was capable of inhibiting enzymatic activity (IC$_{50}$ = 78 nM).

**Bortezomib inhibits serine proteases in intact cells and in vivo**
Following exposure of intact PBMC to bortezomib, we could detect CatG inhibition via ABP profiling, suggesting that this approach could be applied to in vivo settings (data not shown). Subsequently, we incubated whole blood for 1 hr with 100 nM of bortezomib, a concentration/exposure period maintained in patients receiving a dose of 1.3 mg/m²(19;27;28), and observed potent inhibition of CatG by ABP detection in isolated PBMC. Carfilzomib, on the other hand, did not affect CatG activity, despite equivalent levels of proteasome inhibition to bortezomib (Figure 3A).

To determine if bortezomib results in serine protease inhibition in vivo, bortezomib or carfilzomib were administered intravenously (i.v.) to rats, and splenocytes were isolated 15 minutes after dosing and analyzed for inhibition of CatG and the proteasome. Bortezomib inhibited splenocyte CatG activity by 50% and proteasome activity by 70% (Figure 3B). On the other hand, carfilzomib resulted in ~85% inhibition of the proteasome but no inhibition of CatG activity. Interestingly, when splenocytes were isolated 1 hr after administration of bortezomib, CatG activity had recovered to basal levels, while proteasome inhibition remained at ~70% (data not shown).

Next, we investigated the inhibition of non-proteasomal proteases in PBMC derived from myeloma patients treated with either agent. We found a time- and dose-dependent inhibition of CatG in patients receiving 1.0, 1.3, and 1.6 mg/m² bortezomib (Figure 3C). One hour after administration of 1.6 mg/m², both CatG and proteasome CT-L and LMP2 activities were inhibited by 60 - 70% in PBMC samples (Figure 3C and Supplemental Figure 4). In the PBMC samples from patients treated with 20 mg/m² carfilzomib, no inhibition of CatG was detected in
PBMCs of two solid tumor patients treated with 20 mg/m² bolus administration of carfilzomib, despite proteasome inhibition of >75% (Supplemental Figure 3). These findings demonstrate that non-proteasomal proteases are inhibited in vivo following administration of bortezomib.

By Western blot analysis, we could not detect expression of the bortezomib-targeted proteases identified in PBMC (CatG, CatA, chymase and DPPII) in SH-SY5Y cells (data not shown). Furthermore, probing lysates from these cells with FP-biotin did not yield discernible proteins on streptavidin blots, indicating that target identification by LC/MS/MS would not be fruitful (data not shown). Therefore, we utilized a database mining approach to identify potential bortezomib targets that could be involved in the neurite degeneration in differentiated SH-SY5Y cells described previously (Figure 1). MEROPS (http://merops.sanger.ac.uk/) is a comprehensive database of all known proteases and gene sequences predicted to be proteases. Of the two hundred serine proteases in the database, one hundred are annotated for a P1 preference. Of these, fourteen proteases met our search criteria of having a P1 preference of Leu, Phe, or Tyr (Supp Table 2) with five of them already described above (CatG, CatA, chymase, and chymotrypsin B and C). We performed literature searches on the remaining nine candidates to determine if a role in neurodegeneration or neuronal cell survival had been reported. One of these proteases, HtrA2/Omi, a stress-inducible mitochondrial protease involved in neuronal cell survival (29), was identified as a potential target. We utilized a gel-based assay to measure processing of the substrate β-casein by purified enzyme and found that bortezomib was an extremely potent inhibitor of HtrA2/Omi (IC₅₀ = 3 nM). We could detect HtrA2/Omi in SH-SY5Y cells and expression increased following a 12 hr exposure to either proteasome inhibitor at 100 nM (Figure 3D). Interestingly, cleavage of the known substrates eIF4G1 and
pyruvate dehydrogenase was inhibited in cells exposed to bortezomib, but not carfilzomib (Figure 3D). For both substrates, the amounts of cleavage products were higher in carfilzomib-treated cells than DMSO-treated controls, correlating with the increase in expression of HtrA2/Omi. However, despite an increase in HtrA2 expression, there was no decrease in the cleavage of the two substrates in the bortezomib-treated cells, demonstrating inhibition of the enzyme in these cells.
DISCUSSION

Peripheral neuropathy has been described as the most important treatment-emergent adverse event associated with bortezomib therapy (8;9;19;30). Recently published results with carfilzomib, a chemically distinct but equipotent proteasome inhibitor, suggest that peripheral neuropathy is not a significant toxicity in treated MM patients (16;17;31;32). Using an in vitro model of neurodegeneration, we found that bortezomib, but not carfilzomib, resulted in reduced neurite length and neuronal cell survival despite equivalent levels of proteasome inhibition with both agents. These findings mirror those obtained in animals receiving chronic administration of bortezomib in which rats and monkeys showed peripheral nerve degeneration and necrosis that manifested in behavioral changes such as tremors, reduced activity and reduced sensory nerve conduction velocity (12;33). In contrast, repeat dose administration of carfilzomib to rats and monkeys did not affect neurobehavior or result in histomorphologic changes to peripheral nerves (31). Though a randomized, comparative clinical trial with both agents will be required to provide definitive evidence, these findings suggest that peripheral neuropathy is not a class effect of proteasome inhibitors, as has been proposed (34), but rather, is a specific ADR of bortezomib.

We utilized a candidate screen approach to determine specific protease families and target profiles for bortezomib. From this, we utilized ABP profiling with a general serine hydrolase probe to survey primary cells for other non-proteasomal targets of bortezomib. When converted to a quantitative assay, we were able to show that bortezomib results in inhibition of multiple serine proteases with to relative levels similar to the CT-L and LMP2 subunits of the proteasome. Importantly, we found that bortezomib administration to myeloma patients resulted in a potent
and dose-dependent inhibition of one of these proteases (CatG). Future studies will explore the onset and recovery of CatG and other serine proteases in patient tissue samples following bortezomib exposure.

The use of the ABP approach to target profiling reveals several new and clinically relevant findings. By measuring off-target activity in physiologically relevant conditions (i.e., in cells and cell lysates), we found that bortezomib potently inhibited several serine proteases at clinically relevant concentrations. This suggests that the utilization of purified proteins may underestimate a drug’s potency against secondary targets. Further, by using the ABP approach, we obtained an unbiased and broad survey for target profiling. The identification of DPPII, which has a strong preference for proline-containing dipeptides, as a target demonstrates that bortezomib is a potent and promiscuous inhibitor of multiple classes of proteases. Finally, we show that secondary target inhibition can be monitored in vivo by the ABP approach. Inhibition of CatG in the spleen of bortezomib-treated rats demonstrates tissue inhibition of non-proteasomal targets. Potent inhibition of CatG in PBMC of patients receiving bortezomib is the first demonstration of the use of ABP profiling to monitor off-target activity in patients with an approved drug.

We also applied the ABP approach to show that serine protease inhibition is a generalized property of peptide boronates. When boronates are added to the tri- and tetrapeptide backbone of MG-132 and carfilzomib, respectively, the resulting compounds are potent serine protease inhibitors. Peptide epoxyketones, even the epoxyketone version of bortezomib, had no activity against serine proteases. Inhibition of DPPII by the tri- and tetrapeptide boronates suggests that the promiscuity of boronic acid-containing compounds may be broader than originally
anticipated. However, we cannot rule out a role for the peptide backbone, as MG-132 could mediate potent inhibition of chymase as determined by the use of a fluorogenic substrate (data not shown). While the ABP approach enables the detection of multiple targets in a physiologically relevant condition, such as primary cells, it is limited by the relative abundance of the probe to individual targets. This may explain why chymase was not found in PBMC via LC/MS/MS target identification but was detectable in Western blot analysis of PBMC lysates. Another limitation to the assay is the cellular source of targets. Indeed, we found distinct patterns of non-proteasomal target inhibition in individual tumor cell lines exposed to bortezomib. Further exploration with the ABP approach will involve different cell types and new active site probes. However, by utilizing the P1 preference for bortezomib against serine proteases we determined using purified enzymes and ABP profiling, we were able to perform database mining as a screen for other proteases that may be potential targets of bortezomib in cells, but either lack affinity for the probe or are underexpressed in PBMC. From this approach, we found that HtrA2/Omi, a serine protease with weak affinity for the FP-biotin probe (data not shown) and whose expression is induced following cellular stress, is potently inhibited by bortezomib.

HtrA2/Omi is expressed as mitochondrial and endoplasmic reticulum forms, each with distinct sets of substrates (35-37). Mitochondrial HtrA2/Omi has been shown to play a protective role in both in vitro and in vivo models of neurodegeneration and lymphocyte survival (29;38-40). Following exposure to either bortezomib or carfilzomib, HtrA2/Omi expression increased in SH-SY5Y cells to levels similar to that reported for MG-132 (35), which may be the result of an unfolded protein response leading to oxidative stress. In these cells, processing of
two substrates of HtrA2/Omi, eIF4G1, a translation factor, and pyruvate dehydrogenase, a Kreb’s cycle regulatory enzyme involved in generating oxidative species, are inhibited by bortezomib but not carfilzomib. Since both of these substrates have been implicated in neurodegenerative diseases (35;41), inhibition of their processing may represent downstream mechanisms by which bortezomib alters neuronal cell function. Our data are consistent with a model in which bortezomib reduces neurite length by dual inhibition of the proteasome (resulting in oxidative and proteotoxic stress) and the neuronal pro-survival protease HtrA2/Omi.

In conclusion, we have utilized a multi-faceted approach, including the first use of an ABP technique in clinical samples, to show that two structurally and mechanistically distinct proteasome inhibitors, bortezomib and carfilzomib, have dramatically different profiles for non-proteasomal targets in vitro and in vivo. Together with data from our in vitro model of neurite length inhibition, we propose that a specific ADR of bortezomib, peripheral neuropathy, may be caused by distinct, off-target activities. Further exploration of the target profile for both agents by the approaches such as those described here is warranted.
FIGURE LEGENDS

Figure 1. Bortezomib induces neurite degeneration in vitro by a proteasome-independent mechanism. (A) Chemical structures of carfilzomib (1) and bortezomib (2). (B) SH-SY5Y cells were differentiated, treated in triplicate for 24 hrs with control (DMSO), 10 nM bortezomib or 10 nM carfilzomib and stained with Hoechst nuclear stain (blue) to quantify cell number and neuronal-specific FITC-beta-III tubulin (green) to visualize neurite lengths. Representative fluorescent images (20X) for each condition are shown. (C) Quantitative analysis of cell count, neurite length (μm), and the ratio of average neurite length/per field was performed using the fluorescent images and high content image analysis software containing algorithms designed for morphological segmentation and measurement. Twenty fields of view were analyzed per well. The experiment was performed twice with similar results. Proteasome activity was measured by a fluorescent substrate (Suc-LLVY-AMC) assay. * = P<0.05 by one-way ANOVA and Bonferroni post-hoc testing.

Figure 2. Peptide boronic acids are potent serine protease inhibitors. (A) PBMC lysates were treated with carfilzomib (CFZ) or bortezomib (BTZ) at concentrations ranging from 0.1 nM to 10 μM followed by FP-biotin probe binding, SDS-PAGE and Western blotting. Samples were probed with antibodies to cathepsin A (CatA), cathepsin G (CatG), chymase, and dipeptidyl peptidase II (DPPII). A representative blot of two replicates is shown and IC_{50} values are presented in Table 1. (B) Lysates from HepG2, THP-1, and 786-O cells were heat-inactivated for a negative control (lane a), or treated with 1% DMSO (lane b), 10 μM bortezomib (lane c) or
10 μM carfilzomib (lane d). Lysates were treated with CFZ and BTZ as in Panel (A) and blots were probed with streptavidin-HRP. Arrowheads indicate the FP-biotin reactive targets competed by BTZ. A representative experiment of two performed is shown. (C) Structures of the epoxyketone, aldehyde, and boronic analogs of peptide epoxyketone inhibitors. BTZ (3), MG-132 (5), and CFZ (7) are indicated. (D) PBMC lysates were treated with compounds 1-9 at 10 μM and analyzed as described for Panel A. C = DMSO control.

Figure 3. Bortezomib inhibits serine proteases in vivo. (A) Whole blood was incubated with bortezomib and carfilzomib for 40 min at 100 nM and PBMC were isolated. Lysates were exposed to FP-biotin and analyzed for the presence of CatG as described in Figure 2. Proteasome activity was measured as described in Figure 1. (B) Splenocytes were isolated from rats 15 minutes after receiving intravenous administration of vehicle, bortezomib (0.3 mg/kg) or carfilzomib (7 mg/kg). Lysates were probed with FP-biotin to detect CatG activity as described in Figure 2 or analyzed for proteasome activity as described in Figure 1. Values are presented as means (± S.E.M.) relative to vehicle controls (N = 3/group). The experiment was performed twice with similar results. (C) PBMC were isolated from whole blood samples taken at pre-dose, and 10 and 3060 minutes post-dose administration of 1.0, 1.3, or 1.6 mg/m² bortezomib on Day 1 of Cycle 1. All values are N=1 except the 10 min draw for the 1.3 mg/m² dose which represents an average (± S.E.M.) of 2 patient samples. Lysates were probed for CatG and proteasome activity as described in (B.). (D) Undifferentiated SH-SY5Y cells were treated for 12 hrs with DMSO, 100 nM bortezomib or 100 nM carfilzomib. Lysates were analyzed for levels of HtrA2/Omi, eIF4G1 and, pyruvate dehydrogenase, and Hax, a protein required for HtrA2...
activation was used as a control to demonstrate equal loading. Densitometry was performed and levels for each protein relative to DMSO-treated samples are indicated.
Table 1. IC$_{50}$ values of serine proteases in purified enzymes and PBMC lysates. 95% confidence intervals are shown within parenthesis.

<table>
<thead>
<tr>
<th>Target</th>
<th>P1 selectivity</th>
<th>Purified Enzyme</th>
<th>PBMC Lysate*</th>
<th>Purified Enzyme</th>
<th>PBMC Lysate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CatA</td>
<td>F, Y, V, E</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>2.5</td>
<td>0.0047</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.6 – 4.1)</td>
<td>(0.0022 – 0.0098)</td>
</tr>
<tr>
<td>CatG</td>
<td>L, F, H, Y, N, I, V</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>0.95</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.77 – 1.2)</td>
<td>(0.12 – 0.76)</td>
</tr>
<tr>
<td>Chymase</td>
<td>F, Y</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>0.28</td>
<td>0.0016</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.26 – 0.31)</td>
<td>(0.00071 – 0.0036)</td>
</tr>
<tr>
<td>DPPII</td>
<td>P, A, R</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>8.7</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(5.6 – 14)</td>
<td>(0.17 – 0.4)</td>
</tr>
<tr>
<td>HtrA2/Omi</td>
<td>L, V</td>
<td>&gt;10</td>
<td>N/A</td>
<td>0.003</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.001 – 0.009)</td>
<td></td>
</tr>
<tr>
<td>Proteasome (LMP7)</td>
<td>L,F</td>
<td>0.025</td>
<td>0.022</td>
<td>0.031</td>
<td>0.00099</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.015 – 0.041)</td>
<td>(0.016 – 0.030)</td>
<td>(0.021 – 0.047)</td>
<td>(0.00063 – 0.0016)</td>
</tr>
</tbody>
</table>

N/A denotes the value is not available. * Lysate IC$_{50}$ values were generated by using the ABP method except LMP7 activity which was measured using a fluorogenic substrate assay.
Acknowledgements

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Reference List


Ref Type: Generic

Ref Type: Generic


Figure 1

A. Bortezomib

B. Carfilzomib

Figure 1

B. Vehicle | Bortezomib | Carfilzomib

C. Neurite Length/Field | Cell Count/Field | Proteosome Activity

BTZ  | CFZ  | BTZ  | CFZ  | BTZ  | CFZ

***  | N.S.  | ***  | N.S.  | N.S.  | N.S.
Figure 2

A. CFZ DMSO BTZ

CatA CatG Chymase DPPII

B. HepG2 THP-1 786-O

a b c d a b c d a b c d

C. Peptide Sequence | Pharmaphacore
--- | ---
Leu-Phe | Ketoepoxide Aldehyde Boronic Acid
1 | [Chemical Structure]
2 | [Chemical Structure] 3: Bortezomib

Leu-Leu-Leu | 4 | [Chemical Structure] 5: MG-132

Leu-Phe-Leu-hPhe | 7: Carfilzomib

D. Peptide | CatA CatG chymase DPPII
--- | ---
1 | [Chemical Structure]
2 | [Chemical Structure]
3 | [Chemical Structure]
4 | [Chemical Structure]
5 | [Chemical Structure]
6 | [Chemical Structure]
7 | [Chemical Structure]
8 | [Chemical Structure]
9 | [Chemical Structure]
C | [Chemical Structure]
Non-proteasomal targets of the proteasome inhibitors bortezomib and carfilzomib: a link to clinical adverse events

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