Discovery of PI-1840, a novel non-covalent and rapidly reversible proteasome inhibitor with anti-tumor activity

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Running Title: PI-1840, a novel non-covalent proteasome inhibitor with anti-tumor activity

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Background: Food and Drug Administration (FDA)-approved proteasome inhibitors act covalently which hampers their safety.

Results: Structure Activity Relationship (SAR) studies, mass spectrometry and dialysis identified PI-1840 as a non-covalent proteasome inhibitor that sensitizes human cancer cells to p53 and Bcl2 antagonists.

Conclusion: Non-covalent proteasome inhibitors suppress in vivo tumor growth with little toxicity in mouse xenografts.

Significance: Discovery of non-covalent proteasome inhibitors warrants their development as anti cancer drugs.

Abstract

The proteasome inhibitor Bortezomib is effective in hematologic malignancies such as multiple myeloma but has little activity against solid tumors, act covalently and is associated with undesired side effects. Therefore, non-covalent inhibitors that are less toxic and more effective against solid tumors are desirable. Structure activity relationship studies led to the discovery of PI-1840, a potent and selective inhibitor for chymotrypsin-like (CT-L) (IC₅₀ value = 27 ± 0.14 nM) over Trypsin-Like (T-L) and peptidylglutamyl peptide hydrolyzing (PGPH) (IC₅₀ values >100 μM) activities of the proteasome. Furthermore, PI-1840 is over 100-fold more selective for the constitutive proteasome over the immunoproteasome. Mass-spectrometry (LC-MS/MS) and dialysis studies demonstrate that PI-1840 is a non-covalent and rapidly reversible CT-L inhibitor. In intact cancer cells, PI-1840 inhibits CT-L activity, induces the accumulation of proteasome substrates p27, Bax and IκB-α, inhibits survival pathways and viability, and induces apoptosis. Furthermore, PI-1840 sensitizes human cancer cells to the mdm2/p53 disruptor, nutlin, and to the pan Bcl-2 antagonist BH3-M6. Finally, in vivo, PI-1840 but not Bortezomib suppresses the growth in nude mice of human breast tumor xenografts. These results warrant further evaluation of non-covalent and rapidly reversible proteasome inhibitor as potential anticancer agents against solid tumors.

Introduction

Dysregulation of the catalytic processes mediated by the ubiquitin/proteasome system (UPS) contributes to the pathogenesis of many diseases, including cancer (1, 2). More than 80 % of cellular proteins are degraded by the UPS (3), including proteins that regulate cell cycle progression, DNA repair and apoptosis (4-6). Deregulation of various components of
the UPS resulting in increased degradation of cell cycle inhibitors or pro-apoptotic proteins (e.g. p21Cip1, p27Kip1, p53, Bax) contributes to malignant transformation (3, 7). The UPS has two distinct steps: recognition/ubiquitination and degradation (5, 8). The ubiquitin-protein ligase system results in the transfer of multiple ubiquitin molecules to the target protein (9). Degradation of such multi-ubiquitinated proteins occurs on a large 26S proteasome complex (5, 8) that contains three proteolytic enzymes, peptidylglutamyl peptide hydrolyzing (PGPH), trypsin-like (T-L), and chymotrypsin-like (CT-L) activities, residing in the β1, β2, and β5 catalytic subunits, respectively (3, 7).

In contrast to normal cells, cancer cells generally have higher levels of proteasome activity (3), and have acquired a series of mutations that render them dependent on strong activation of survival pathways (10). One of these is the phosphorylation-dependent recognition and subsequent degradation of cellular proteins by the UPS. Furthermore, compare to normal cells, cancer cells show higher sensitivity towards the pro-apoptotic effects of proteasome inhibition. Therefore, the UPS has become a promising target for anti-cancer strategies (3, 7, 11, 12).

Although two proteasome inhibitors, Bortezomib and Carfilzomib, are FDA approved and others are in clinical trials, they are all covalent inhibitors (13, 14). Covalent inhibitors have highly reactive and unstable chemical groups, and are therefore less specific (15). This is believed to be a major cause for toxicity to patients. Furthermore, Bortezomib is active against liquid but not solid tumors; and its covalent binding which would limit its widespread tissue distribution could be a possible reason. In contrast to covalent inhibitors, non-covalent inhibitors have the advantage of rapid binding and dissociation kinetics that would allow broader tissue distribution, reaching both liquid and solid tumors. Only very few non-covalent inhibitors have been identified and none have entered clinical trials (16, 17). It is important to point out that at present it is not known whether non-covalent inhibitors suffer from

Experimental Procedures:

Materials. DMEM, RPMI-1640, DMEM/Ham’s F-12, horse serum, penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Atlanta Biologicals (Atlanta, GA). Purified 20S proteasome (rabbit), purified 20S immunoproteasome (human), fluorogenic peptide substrates Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal CT-L activity), benzyloxy-carbonyl (Z-Leu-Leu-Glu-AMC (for the proteasomal PGPH activity) were purchased from Boston Biochem (Cambridge, MA). Fluorogenic peptide substrate Bz-Val-Gly-Arg-AMC (for the proteasomal T-L activity) was obtained from Biomol International (Plymouth Meeting, PA). Antibodies were obtained from the following suppliers: p27 Kip1 ( BD Biosciences, San Jose, CA), and β-actin (Sigma-Aldrich, St. Louis, MO); Phospho-Akt (S473), phospho-S6 Ribosomal Protein (Ser240/244), S6 Ribosomal Protein (5610), cleaved PARP (Asp214) (D64E10) XP, cleaved Caspase-3 (Asp175) (S1A1E) (Cell Signaling, Danvers, MA); Akt1/2 (N-19), survivin (FL-142), IKB-α (C-21), Bax (N20) (Santa Cruz Biotechnology, Santa Cruz, CA), MTT (Calbiochem, ). The pan Bcl-2 antagonist BH3-M6 and the proteasome inhibitors PI-1833 and PI-1840 were all synthesized in-house as reported previously (18, 19). Bortezomib was purchased from Selleckchem, Houston, TX. Nutlin 1 was purchased from Sigma-Aldrich. All other reagents were from Sigma-Aldrich unless otherwise noted.

Determination of CT-L, T-L and PGPH proteolytic activities.

These assays were performed exactly as described by us previously (20). Briefly, 1 nM of purified 20S rabbit proteasome or
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immunoproteasome was incubated with 20 µM Suc-Leu-Leu-Val-Tyr-AMC for the CT-L activity, Bz-Val-Gly-Arg-AMC for the T-L activity, and benzylxycarbonyl Z-Leu-Leu-Glu-AMC for the PGPH activity for 1 h at 37°C in 100 µl of assay buffer (50 mM Tris-HCl, pH 7.6) with or without compound, and the hydrolyzed 7-amido-4-methyl-coumarin (AMC) was measured using a WALLAC Victor2 Counter. To determine proteasome activity in whole cell extracts (5µg) from cultured cells, lysates were used instead of 20S rabbit proteasome. Whole cell extracts were prepared by homogenizing the cells in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40), centrifuging the lysates at 12,000 g, and collecting the supernatants as whole cell extracts as described previously by us (20).

Protein Digestion, Peptide Purification and LC-MS/MS Analysis.
These procedures were performed exactly as described by us previously (18). Briefly, after purified 20S Proteasome (rabbit) (1 nM) was incubated for 30 min with inhibitors in 50 mM Tris-Hcl, pH 7.6, acetonitrile and trypsin were added (4 hr, 37° C). The digest was concentrated and the peptides were extracted with C18 reversed phase pipette tip columns, and injected into mass spectrometer. To assess LC-MS/MS performance, tryptic peptides from horse apomyoglobin (25 fmol) were spiked in each LC-MS/MS analysis. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) peptide sequencing experiments were performed using a nanoflow liquid chromatograph (U3000, Dionex, Sunnyvale, CA) interfaced with an electrospray ion trap mass spectrometer in order to detect and localize modified peptides from the proteasome exactly as described by us previously (18).

Database Searching and Data Analysis.
The data searching and analysis was performed exactly as described by us previously (18). Briefly, the 22 rabbit proteasome protein sequences from the UniProt (http://www.uniprot.org) were searched using Sequest, (21) and the search results summarized in Scaffold 3.0 (www.proteomesoftware.com). For peptide quantification, the integrated peak areas were calculated from ion chromatograms using QuanBrowser from Xcalibur 2.0 (Restriction: m/z (+/- 0.02); retention time (120 seconds)). To insure proper sequence assignment, manual inspection of the accuracy of the m/z values and the fragmentation patterns of the target peptides was performed exactly as described by us (18).

Dialysis using purified rabbit 20S proteasome.
We used the same dialysis method that we used in our previous study (18) to determine the effect of dialysis on CT-L activity. Briefly, compounds PI-1840 (1 µM) and lactacystin (2.5µM) or vehicle (DMSO) were added to 20S proteasome (rabbit) at a final concentration of 1 nM in proteasome assay buffer (50 mM Tris-HCl, pH = 7.6) and incubated at room temperature for 30 min. Then, the proteasome-compound mixtures were added to mini dialysis units (3500 MWCO Thermo Scientific Slide-A-Lyzer) (Rockford, IL) and dialyzed against proteasome assay buffer. Immediately (t = 0) and at different time points (20 , 60, 120, 240, 480 and 1080 min) of dialysis at 4 ºC, samples were taken from the dialysis cassette and the CT-L activity of 20S proteasome was determined as described by us previously (18). CT-L activity was normalized against CT-L activity of DMSO control.

Cells, Cell culture and extract preparation.
MDA-MB-468 and MDA-MB-231 (human breast cancer cells), HCT-116, HCT-116-p53-/-, HCT-116-HKH2 (human colon cancer cells), PC-3 (human prostate cancer cells) were cultured in DMEM medium. DU145, LNCaP (human prostate cancer cells), RPMI-8226, U266 (human multiple myeloma cells), Colo357 (human pancreatic adenocarcinoma cells), RXF-397 (human renal carcinoma cells) were cultured in RPMI-1640 medium. All media were supplemented with 10% fetal bovine serum (FBS), and 1% PenStrep antibiotics. Normal immortalized MCF-10A
breast cells were cultured in DMEM/Ham’s F-12 containing 5% horse serum, 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 500 ng/ml hydrocortisone and 0.01 mg/ml insulin. Cells were maintained at 37ºC in a humidified incubator in an atmosphere of 5% CO₂.

**Western blot analysis.**
To prepare whole cell lysates, cells were washed with PBS twice, and lysed in 30 mM Hepes, pH 7.5, 10 mM NaCl, 5 mM MgCl₂, 25 mM NaF, 1 mM EGTA, 1% Triton-X-100, 10% glycerol, protease inhibitor cocktail, 2 mM PMSF, 2 mM Na₃VO₄, and 6.4 mg/ml p-nitrophenylphosphate. Lysates were cleared by centrifugation at 12,000 x g for 15 min, and the supernatants were collected as whole cell extracts. The protein concentration was determined by the Bradford assay. Cell lysates (50 µg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane, probed with specific antibodies, and signals were visualized by enhanced chemoluminescence (ECL, Amersham, Piscataway, NJ) according to the manufacturer's protocol.

**MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.**
Cancer cells or normal immortalized MCF-10A breast cells were plated in 96-well plates in 100 µl of above specified medium and allowed to attach overnight. Cells were then incubated for different time points with varying concentrations of drugs or appropriate controls. After that, media was aspirated and replaced with 100 µl complete media containing 1 mg/ml MTT and incubated for three hours at 37ºC in 5% CO₂ humidified incubator. After incubation, media was aspirated and DMSO was added. Cells were then incubated for 10 min at room temperature while shaking, and the absorbance was determined at 540 nm using a µQuant spectrophotometric plate reader (Bio-TEK, Winooski, VT).

**Antitumor study of human tumor xenografts in nude mice.**
Female nude mice (Charles River Laboratories, Wilmington, MA) were maintained and treated in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. Exponentially growing MDA-MB-231 cells were cultured via trypsinization, pelleted at 300 g for 5 min. Cells were re-suspended in 50% metrizig with DPBS (Invitrogen) at 10x10⁶ cells per 200 µl, and injected into right flank of mice. The tumor xenografts were monitored with an electronic caliper measurements and tumor volume (V) was calculated using the formula V= (W x L²)/2, where width is the largest diameter and length is the smallest diameter. When the tumors reached ~ 250 mm³, the animals were randomized and treatment schedules were implemented. Treatments consisted of intraperitoneal (i.p.) injections of vehicle control (30% HPCD) (n=5) or PI-1840 (n=5) at 150 mg/kg (everyday for 14 days) and Bortezomib (n=6) at 1 mg/kg (2x per week for 14 days).

**Results**

**HTS and Hit-to-Lead Optimization Identifies PI-1840 as a Potent, Non-covalent and Rapidly Reversible Proteasome CT-L Inhibitor.**
Our efforts to develop non-covalent proteasome inhibitors have recently resulted in the identification of the hit PI-1833 from the screening of a ChemBridge 50,000 compound library against the CT-L activity of purified 20S proteasome (IC₅₀ = 0.6 µM (Figure 1A)). Through extensive structure activity relationship (SAR) studies and hit-to-lead optimization, we found that replacing the methyl by a propyl in ring A and replacing the ring B tolyl by pyridyl resulted in PI-1840 (IC₅₀ = 27 nM) which is 22-fold more potent than the initial PI-1833 hit (IC₅₀ = 600 nM) (Figure 1A, right panel). The chemical synthesis of PI-1833, PI-1840 and analogs as well as the details of SAR and hit-to-lead optimizations studies have recently been published (18). The present manuscript describes the biological characterization of PI-
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PI-1840 from in vitro and cell culture studies to in vivo anti tumor efficacy studies. PI-1840 was selective for the CT-L over T-L (IC$_{50}$ value > 100µM) and PGPH-L (IC$_{50}$ value >100 µM) activities of the proteasome (Fig. 1A). Furthermore, Fig. 1B (left panel) shows that PI-1840 was 121-fold more selective for the constitutive 20S proteasome over the immunoproteasome (IC$_{50}$ 18 nM vs 2170 nM). In contrast, Bortezomib was 2-fold more selective for the immunoproteasome over the constitutive proteasome (IC$_{50}$ 4 nM vs 8 nM) (Fig. 1B, right panel). To determine whether PI-1840 inhibits the CT-L activity of the proteasome in a covalent or a non-covalent manner, we first used Liquid Chromatography Coupled Tandem Mass Spectrometry (LC-MS/MS) and then confirmed the results with dialysis as described under Experimental Procedures. Figures 2 A, B and C show the MS spectra of the tryptic digests of the 20S proteasome after incubation with vehicle, PI-1840 and lactacystin, respectively. Tryptic peptides from the 20S proteasome treated with vehicle contained unmodified protonated TTTLAFK (m/z 781.4504) (Figure 2A). TTTLAFK (structure shown in Figure 3A) corresponds to the N-terminal tryptic peptide of rabbit 20S proteasome subunit β type-5 with the first T in this peptide corresponding to threonine 1 of the active site of CT-L. A similar pattern was observed with tryptic peptides from the 20S treated with PI-1840 with the unmodified protonated TTTLAFK (m/z 781.4397) (Figure 2B). The Unmodified Thr-1-containing peptide was confirmed by both intact mass spectrum and tandem mass spectrum. In contrast, Figure 2C shows that tryptic peptides from the 20S proteasome treated with lactacystin (an known covalent and irreversible CT-L inhibitor (22, 23), contained a doubly charged, lactacystin-modified threonine peptide (m/z 497.7778) (structure shown in Figure 3B). The observation that only Thr-1 on β-5 was modified by lactacystin was documented by searches matching experimental data to peptides from the database of rabbit 20S proteasome β-5, β-1 and β-2 subunits. Similarly, searches matching experimental data (from vehicle and PI-1840 treated samples) to peptides from the 22 rabbit 20S proteasome sequences (UniProt data base (http://www.uniprot.org)) show no peptide modifications from the β-5, β-1 and β-2 subunits. Taken together, these results suggest that, unlike lactacystin, PI-1840 does not bind covalently to the proteasome.

We next determined the reversibility of binding of PI-1840 and lactacystin to CT-L by dialysis as described under Experimental Procedures. Figure 3C shows that the CT-L activity in the dialysis compartment from the sample that was treated with PI-1840 begins to recover within the first few minutes and recovered fully by 18 hours of dialysis. In contrast, the CT-L activity from the Lactacystin-treated sample remained potently inhibited even after 18 hours of dialysis (Figure 3C). These results are consistent with the LC-MS/MS results (Figures 2 B and C) that demonstrated that Lactacystin but not PI-1840 binds covalently to the active site Thr-1 of the CT-L subunit of the proteasome.

**PI-1840 is more potent than PI-1833 at inhibiting proteasome activity, accumulating proteasome substrates, inhibiting survival pathways and inducing apoptosis in human cancer cells.**

Figure 1 shows that PI-1840 is more potent than PI-1833 in vitro. We next determined whether PI-1833 and PI-1840 are cell-permeable and whether PI-1840 is more potent than PI-1833 at inhibiting the CT-L activity of the proteasome in intact cells. To this end, we treated MDA-MB-468 cells with PI-1833 and PI-1840 for two hours and determined the CT-L activity as described under Experimental Procedures. We found that both compounds inhibited CT-L activity in a dose-dependent manner and, consistent with our in vitro results, PI-1840 was 10-fold more potent (IC$_{50}$=1.55 µM) than PI-1833 (IC$_{50}$=1.60 µM). We next determined how fast PI-1840 can reach its target and if its inhibition of CT-L is selective over T-L and PGPH-L activities in intact cells. To this end, we first treated MDA-MB-468 cells with PI-1840 and measured the CT-L, T-L and PGPH activities over time as described under Experimental Procedures.
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Procedures. Figure 4A shows that PI-1840 reached its target within ten min. and inhibited CT-L activity. Consistent with in vitro results, PI-1840 did not inhibit T-L and PGPH activities in intact cells (Figure 4A). Inhibition of the CT-L activity of the proteasome is predicted to result in the accumulation of known CT-L substrates. Therefore, we next treated MDA-MB-468 cells with increasing concentrations of PI-1833 and PI-1840 for 48 hours and processed the cells for Western blotting as described under Experimental Procedures. Figure 4B shows that both PI-1833 and PI-1840 increased the accumulation of CT-L substrates p27, IκB-α and Bax but that PI-1840 was more potent than PI-1833, consistent with its more potent activity to inhibit CT-L in vitro and in MDA-MB-468 cells.

Proteasome inhibitors are known to affect many signal transduction pathways that are critical to tumor cell survival (24, 25). We therefore investigated the effects of PI-1833 and PI-1840 on these pathways as well as their ability to induce apoptosis. Figure 4C shows that PI-1840 was much more potent than PI-1833 at decreasing the levels of P-Akt, P-S6 and survivin, and resulted in induction of apoptosis as apparent from caspase-3 activation and PARP cleavage.

PI-1840 inhibits the viability of a broad spectrum of human cancer cell lines.

Figures 1 through 4 demonstrated that PI-1840 is a potent, selective, non-covalent and rapidly reversible proteasome inhibitor that induced the accumulation of CT-L substrates, inhibited tumor survival pathways and induced apoptosis. The cell culture studies were done in one cell line MDA-MB-468, and therefore we next determined the ability of PI-1840 to inhibit CT-L activity and viability in a broad spectrum of human cancer cell lines from different lineages including breast, colon, prostate, pancreatic, renal and lung cancers as well as multiple myeloma. To this end, we treated the various cell lines with 20 uM PI-1840 for 2 hours and analyzed the CT-L activity. We also treated the various cell lines with increasing concentrations of PI-1840 for 120 hours and analyzed viability by MTT assays as described under Experimental Procedures. Table 1 shows that PI-1840 reached its target in all the cell lines and inhibited CT-L activity by 45 to 90 % depending on the cell line. Furthermore, PI-1840 also inhibited the viability of all the human cancer cell lines with IC50 values from as low as 2.2 uM in MDA-MB-231 cells to as high as 45.2 uM in RXF-397. Interestingly, in the case of the 2 HCT-116 isogenic cell lines where the CT-L activities from both HCT-116 (p53 +/+ ) and HCT-116 (p53 -/- ) were inhibited equally (88.4 +/- 4.6 % and 89.9 +/- 3.0 %, respectively) (Table 1), PI-1840 was twice more potent at inhibiting viability in the former than in the latter (IC50 values of 8.7 +/- 1.0 and 16.0 +/- 1.3 uM, respectively) (Table 1), suggesting that p53 may contribute to the anti-proliferative activity of PI-1840. In contrast, in the 2 HCT-116 isogenic cell lines where the CT-L activities from both HCT-116-HKH-2 (mutant K-Ras) and HCT-116 (wild type K-Ras) were inhibited equally (87.5 +/- 5.6 % and 88.4 +/- 4.6 %, respectively) (Table 1), PI-1840 was also as effective in both cell lines at inhibiting viability (IC50 values of 6.3 +/- 0.8 uM and 8.7 +/- 1.0, respectively (Table 1). Moreover, LNCaP cells that are Bax (+/+ ) are 5.7-fold more sensitive to PI-1840 anti-proliferative effects than the Bax (-/-) DU-145 cells (Table 1). Finally and importantly, PI-1840 inhibited only weakly the viability of “normal”, non-transformed breast cells (MCF-10A; IC50 = 314.3 +/- 23.9 uM) and normal foreskin fibroblasts (HCA2; IC50 = 86 +/- 20 uM). (Table 1).

PI-1840 sensitizes human cancer cells to the mdm2 antagonist Nutlin.

Blocking the degradation of p53 with proteasome inhibitors may not be sufficient to induce apoptosis since the accumulated p53 can be inactivated by binding partners such as mdm2. Therefore, we next evaluated whether nutlin, which disrupts the binding of p53 to mdm2 (26) can sensitize cells to PI-1840. To this end, we treated HCT-116 cells with either nutlin alone or in combination with PI-1840...
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and determined their effects on viability by MTT as described under Experimental Procedures. Table 2 (upper panel) shows that in the absence of PI-1840, nutlin inhibited the viability of HCT-116 cells with an IC$_{50}$ value of 6.2 µM (average of 2 experiments). In contrast, in the presence of 5, 10 and 15 µM PI-1840, nutlin IC$_{50}$ values were 2.7, 2.0 and 1.4 µM. Taken together, these results demonstrate that PI-1840 sensitized HCT-116 to nutlin inhibition of viability by as much as 4.4 fold. To determine whether this sensitization is p53–dependent, we performed similar studies with the isogenic HCT-116 (p53 (-/-)) cells. Table 2 (upper panel) shows that in the absence of PI-1840, nutlin was 8.9-fold less effective at inhibiting the viability of HCT-116 (p53 +/-) cells (IC50 value of 55.1 µM) than it is at inhibiting that of its isogenic HCT-116 (p53+/+) counterpart (6.2 µM). Furthermore, PI-1840 did not sensitize HCT-116 (p53/-) cells to nutlin (IC50 values for vehicle, 5, 10 and 15 µM PI-1840 were 55.1, 59.8, 57.0 and 58.0, respectively).

PI-1840 sensitizes human cancer cells to the pan Bcl-2 antagonist BH3-M6.

Inhibition of the degradation of pro-apoptotic proteins such as Bax by proteasome inhibitors may not be sufficient to induce apoptosis since the accumulated Bax can be inactivated by binding partners such as Mcl-1 or BclxL. Therefore, we next evaluated whether BH3-M6, which disrupts the binding of anti-apoptotic proteins Bcl-xL, Mcl-1 and Bcl-2 to the pro-apoptotic proteins Bax, Bak and Bim (19) can sensitize cells to PI-1840. To this end, we treated LNCaP cells with either BH3-M6 alone or in combination with PI-1840 and determined their effects on viability by MTT as described under Experimental Procedures. Table 2 (lower panel) shows that in the absence of PI-1840, BH3-M6 inhibited the viability of LNCaP with an IC50 value of 16.9 µM (average of 2 experiments). In contrast, in the presence of 5, 10 and 15 µM PI-1840, BH3-M6 IC50 values were 13.0, 8.8 and 4.1 µM, respectively (Table 2, lower panel), demonstrating that PI-1840 sensitized LNCaP to BH3-M6 inhibition of viability by as much as 4-fold. To determine whether this sensitization is Bax–dependent, we performed similar studies with DU-145 cells, which unlike LNCaP cells, lack Bax. Table 2, lower panel shows that in the absence of PI-1840, BH3-M6 was 3.4-fold less effective to inhibit the viability of DU-145 cells (IC$_{50}$ value = 57.6 µM) than that of LNCaP (16.9 µM). More importantly, unlike in LNCaP cells, PI-1840 did not sensitize DU-145 cells to BH3-M6. Indeed, the BH3-M6 IC50 values for DU-145 treated with vehicle, 5, 10 and 15 µM PI-1840 were 57.6, 61.1, 55.4, and 53.8 µM, respectively (Table 2, lower panel).

**PI-1840 but not Bortezomib inhibits the growth of human breast tumor xenografts in nude mouse.**

We next evaluated the anti-tumor activities of PI-1840 and Bortezomib in nude mice bearing solid tumors. To this end, we implanted human breast cancer MDA-MB-231 cells s.c. in nude mice, and when tumors reached an average size of about 250 mm$^3$, the mice were treated either with vehicle (30% HPCD in H$_2$O), PI-1840 (150 µg/kg/day, i.p, daily) or Bortezomib (1 µg, 2x weekly, i.p.). Figure 5A shows representative examples of mice, treated with either vehicle, PI-1840 or Bortezomib. The tumor from the mouse treated with vehicle grew from an initial tumor volume of 279 mm$^3$ to 910 mm$^3$. Similarly, the tumor from the Bortezomib treated mouse grew from 239 to 852 mm$^3$. In contrast, the tumor from the PI-1840 treated mouse grew from 280 to only 374 mm$^3$ (Figure 5A). Thus, compare to vehicle, PI-1840 inhibited tumor growth by 85 %, whereas Bortezomib had little effect on tumor growth. Figure 5B shows the average tumor volume change of all mice treated. The volume of tumors from mice treated with vehicle increased on average by 288 +/- 91 % over the treatment period of 14 days. Similarly, the volume of tumors from mice treated with Bortezomib increased on average by 263 +/- 28 %. In contrast, the volume of tumors from mice treated with Bortezomib increased on average by 263 +/- 28 %. Therefore, PI-1840 inhibited the growth of MDA-MB-231 tumor xenografts by 76 % ((1-
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(69/288) x 100) (p=0.04) whereas Bortezomib inhibited tumor growth by only 8.7 % ((1- (263/288)) x 100) and this was not a statistically significant effect compared to vehicle treated mice (p=0.78). Furthermore, over the 14-day treatment period the body weight of the vehicle-treated mice increased on average by 4.34 %. In contrast, the Bortezomib-treated mice lost on average 6.21 % of their body weight. The body weight of the PI-1840-treated mice increased on average by 0.12 %.

Discussion
The approval by the FDA of Bortezomib further validated targeting the proteasome for the development of anticancer drugs. While this proteasome inhibitor has benefitted patients with Multiple Myeloma and Mantle Cell Lymphoma, it is ineffective against solid tumors and is associated with undesirable side effects. Carfilzomib, a more recently approved proteasome inhibitor, appears to also be more active against liquid tumors although it has not been tested as thoroughly as Bortezomib against solid tumors. These drawbacks could be due at least in part to the fact that Bortezomib inhibits the CT-L activity of the proteasome by binding covalently to threonine 1 of the beta 5 subunit of the proteasome. While this suggestion needs to be further supported with more direct evidence, the development of non-covalent proteasome inhibitors to determine whether they lack these drawbacks is highly desirable. In this manuscript we describe the discovery of a potent and selective proteasome inhibitor, PI-1840, that blocked selectively the CT-L activity in a non-covalent and rapidly reversible manner. Proteasome inhibitors that are approved by the FDA (Bortezomib and Carfilzomib) and those that are in clinical trials (i.e. MLN9708, NPI-0052 and CEP18770) all act covalently. There are only a few non-covalent proteasome inhibitors that have been reported, and these include the natural product cyclic peptide TMC-95 (27) and its linear peptide mimics (28) as well as capped peptides (noncyclic and isosteres peptides) (29) and hydroxyurea (30). Furthermore, most of the non-covalent inhibitors previously reported except for hydroxyurea are peptidic in nature whereas PI-1840 is a non-peptidic, small organic molecule that is unlikely to suffer from the peptidic compound liabilities such as peptide degradation and poor cellular uptake. Finally, PI-1840 is the only non-covalent proteasome inhibitor that has been evaluated in vivo and shown to have anti tumor activity against solid tumors. Importantly, in our in vivo studies, PI-1840 was compared head-to-head to Bortezomib, and only PI-1840 and not Bortezomib, was found to be active against solid tumors.

PI-1840 was able to inhibit the CT-L activity and cell viability in a wide spectrum of tumor types from several lineages including breast, colon, prostate, pancreatic and renal cancers as well multiple myeloma (See Table 1). The fact that PI-1840 was twice more potent at inhibiting cell viability in the HCT 116 (p53 +/-) than in HCT 116 (p53 -/-) suggested that the CT-L substrate p53 contributes at least in part to the ability of PI-1840 to inhibit viability. In contrast, the K-Ras mutation status in the same HCT-116 cell line appears to matter little. Although the 2 prostate cancer cell lines LNCaP and DU-145 are not isogenic, LNCaP cells that are Bax positive was several fold more sensitive to PI-1840 anti-viability effects than the Bax negative DU-145 suggesting that Bax may be critical. However, other genetic differences between the 2 cell lines and the fact that PI-1840 inhibited the CT-L activity of LNCaP more potently may also be contributing factors. Finally and importantly, PI-1840 was less active at inhibiting the viability of “normal”, cells. Although we do not know why these cells are less sensitive to PI-1840, this is an important finding that is consistent with the finding that in vivo PI-1840 is not toxic as judged grossly by little changes in the body weight of the mice.

While in about half of human cancers, p53 is inactivated by mutations, in the other half p53 is wild type and inactivated by various mechanisms (31, 32). One of the major antagonists of p53 is the E3 ligase mdm2 that
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binds and inactivates p53 at least in part by ubiquitinating p53 and consequently inducing its degradation by the proteasome (33-35). Nutlin is a small molecule that inhibits mdm2/p53 binding freeing up p53 to cause apoptosis (26). However, the ability of the freed up p53 protein to induce apoptosis could be hampered by its ubiquitination by other E3 ligases and subsequent degradation by the proteasome CT-L. Therefore, inhibiting the CT-L activity with PI-1840 may enhance the ability of Nutlin to inhibit survival and induce apoptosis. Indeed our combination studies demonstrated that PI-1840 sensitized greatly HCT-116 to nutlin inhibition of viability. Furthermore, PI-1840 did not sensitize HCT-116 (p53-/-) cells to nutlin, suggesting that the ability of PI-1840 to sensitize to nutlin is dependent on p53, and that free p53 may be required for PI-1840 to inhibit cell viability. Nutlin itself is much less effective at inhibiting the viability of HCT-116 (p53-/-) cells compared to its isogenic HCT-116 (p53+/+) cells, consistent with previous reports (36, 37). Taken together, these results warrant further evaluation of combination therapy of mdm2 antagonists such nutlin and non-covalent proteasome inhibitors such as PI-1840 in human tumors which express wild type p53.

Recently, we have reported on the development of a novel pan Bcl-2 antagonist, BH3-M6, which induces apoptosis by inhibiting the binding of the anti-apoptotic proteins Bcl-xL, Mcl-1 and Bcl-2 to the pro-apoptotic proteins Bax, Bak and Bim, freeing up the latter to cause apoptosis (19). However, the ability of the freed up pro-apoptotic proteins to induce apoptosis could be hampered by their degradation by the proteasome CT-L. We found PI-1840 to sensitize greatly LNCaP to BH3-M6 inhibition of viability. In DU-145 cells, that lack Bax, BH3-M6 was less effective consistent with our previously published data (19). Furthermore, unlike in LNCaP cells, PI-1840 did not sensitize DU-145 cells to BH3-M6, suggesting that the accumulation of free Bax or other pro-apoptotic proteins may contribute to the mechanism by which PI-1840 inhibits viability. Finally, the fact that LNCaP expresses wild type functional p53 may contribute to its higher sensitivity to PI-1840 since p53 is known to up-regulate Bax.

Our findings that PI-1840 but not Bortezomib inhibited the growth in mice of MDA-MB-231 breast tumors coupled with the fact that PI-1840 has little effect on mouse body weight supports the suggestion that non-covalent proteasome inhibitors may be less toxic and more active against solid tumors. The fact that PI-1840 demonstrated little toxicity in vivo is consistent with its lack of activity against the “normal” MCF-10A and HCA2 cells in cell culture studies. Furthermore, selective inhibition of the constitutive proteasome over the immunoproteasome by PI-1840 may be associated with less toxicity to cells of lymphoid origin where the immunoproteasome is selectively expressed (38). While immunoproteasome-specific inhibitors are believed to have great potential in immune-related diseases such as lupus erythematosis and inflammatory bowel disease, their potential for cancer therapy is not clear (38, 39). Since our interest is mainly in targeting solid tumors, the fact that PI-1840 does not inhibit the immunoproteasome is not a liability.

Finally, while the finding that PI-1840 is active in solid tumors that are resistant to Bortezomib is encouraging, further confirmation of this observation with non-covalent proteasome inhibitors other than PI-1840 is important. Furthermore, demonstrating that non-covalent inhibitors lack the drawbacks of covalent inhibitors in other solid tumors as well as in other animal models is also of paramount importance. If confirmed these findings could be translated into clinic where non-covalent proteasome inhibitors can be used either as single agents or in combination to treat a wider spectrum of human tumors including solid tumors.

Conflict of Interest
Authors declare no conflict of interest.

Acknowledgments
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PI-1840, a novel non-covalent proteasome inhibitor with anti-tumor activity

Biology Core as well as the Proteomics Core at the Moffitt Cancer Center for their cooperation and expertise.
References

PI-1840, a novel non-covalent proteasome inhibitor with anti-tumor activity

Figure Legends:

Figure 1. PI-1833 and its potent analogue PI-1840 are selective inhibitors for CT-L activity in vitro. (A) Chemical structures of PI-1833 and PI-1840 with their IC50 values against different proteasome activities. (B) Effects of PI-1840 and Bortezomib on the CT-L activities of the 20S constitutive rabbit proteasome and 20S human immunoproteasome. Data are representative of at least 2 independent experiments.

Figure 2. Lactacystin but not PI-1840 binds covalently to CT-L of the 20S proteasome. (A-C) LC-MS/MS analysis: Purified rabbit 20S Proteasome was incubated either with Vehicle (A), PI-1840 (B) or Lactacystin (C) and the tryptic digests analyzed by LC-MS/MS as described under Experimental Procedures. The b ions (red) and y ions (blue) designate the N-terminus and the C-terminus of the peptide, respectively. The number next to each ion represents the number of amino acids in that fragment (i.e. y4 = LAFK from C-terminus of the peptide).

Figure 3: (A) Unmodified TTTLAFK peptide (B) Clasto-Lactacystin modified TTTLAFK peptide. (C) Dialysis: Purified rabbit 20S proteasome was incubated with Vehicle control, 1 µM PI-1840 or 2.5 µM lactacystin and was subjected to dialysis at 4º C for different lengths of time as described under Experimental Procedures. Percentage of CT-L activity (relative to vehicle treated control samples) was then determined at different time points. Data are representative of 2 (LC/MS-MS) and 3 (dialysis) independent experiments.

Figure 4. Selective inhibition of the proteasomal CT-L activity in whole cells, accumulation of proteasome substrate proteins, inhibition of cell survival pathways, and induction of apoptosis. (A) Exponentially growing human breast cancer MDA-MB-468 cells were treated with 5 µM PI-1840 for the indicated time points, followed by measurement of CT-L, T-L, and PGPH activities in whole cell extracts as described under Experimental Procedures. (B & C) Human breast cancer MDA-MB-468 cells were treated with the indicated concentrations of PI-1833 and PI-1840 for 48 hrs, followed by Western blot assay using the indicated antibodies as
PI-1840, a novel non-covalent proteasome inhibitor with anti-tumor activity described under Experimental Procedures. Data are representative of at least 2 independent experiments.

Figure 5. Antitumor efficacy of PI-1840 and Bortezomib against human breast cancer MDA-MB-231 xenografts in nude mouse. Mice bearing MDA-MB-231 tumors were treated with vehicle (closed squares), PI-1840 (open circles) or Bortezomib (filled circles) as described under Experimental Procedures. (A) Representative tumor growth curves form vehicle-, PI-1840- and Bortezomib-treated mice. (B) Average percent change in tumor volumes from mice treated with vehicle, PI-1840 and Bortezomib. There were statistically significant differences between vehicle and PI-1840 in the average percent change in tumor volume on every day of measurement with p values of 0.006, 0.019, 0.009, 0.018, 0.039 and 0.045 on days 3, 5, 7, 10, 12 and 14 of treatment, respectively. In contrast, there were no statistically significant differences between vehicle and Bortezomib in the average percent change in volume with p values of 0.243, 0.751, 0.951, 0.842, 0.709 and 0.786 on days 3, 5, 7, 10, 12 and 14 of treatment, respectively.
**Table 1:** Effects of PI-1840 on CT-L activity and viability of human cancer cells and “normal” cells

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Cell lines</th>
<th>CT-L % inhibition at 20µM</th>
<th>Viability (IC50 (µM))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>MDA-MB-468</td>
<td>63.2 ± 5.4</td>
<td>4.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>59.0 ± 4.3</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>Colon</td>
<td>HCT-116</td>
<td>88.4 ± 4.6</td>
<td>8.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>HCT-116-p53/-</td>
<td>89.9 ± 3.0</td>
<td>16.0 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>HCT-116-HKH-2</td>
<td>87.5 ± 5.6</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>Prostate</td>
<td>DU-145</td>
<td>64.7 ± 5.0</td>
<td>28.0 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>LNCaP</td>
<td>88.8 ± 1.0</td>
<td>4.9 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>PC3</td>
<td>71.4 ± 4.1</td>
<td>15.0 ± 1.1</td>
</tr>
<tr>
<td>Multiple Myeloma</td>
<td>RPMI 8226</td>
<td>49.3 ± 2.5</td>
<td>26.0 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>U266</td>
<td>66.0, 58.0</td>
<td>15.6 ± 4.2</td>
</tr>
<tr>
<td>Pancreatic adenocarcinoma</td>
<td>Colo 357</td>
<td>45.2 ± 25.9</td>
<td>15.4 ± 2.4</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>RXF 397</td>
<td>53.8 ± 1.9</td>
<td>45.2 ± 1.0</td>
</tr>
<tr>
<td>Normal Foreskin Fibroblast</td>
<td>HCA2</td>
<td>41.0 ± 2.0</td>
<td>86.0 ± 20.0</td>
</tr>
<tr>
<td>Normal/immortalized breast cells</td>
<td>MCF-10A</td>
<td>63.2 ± 5.4</td>
<td>314.3 ± 23.9</td>
</tr>
</tbody>
</table>

Human cancer cells from various lineages and non-transformed MCF-10A and HCA2 cells were treated with 20 uM PI-1840 for 2 hours and analyzed for CT-L activity as described under Experimental Procedures. In separate experiments, the cells were treated with increasing concentrations of PI-1840 for 120 hours and analyzed for viability by MTT assays as described under Experimental Procedures.
Table 2.

<table>
<thead>
<tr>
<th></th>
<th>IC50 (µM)</th>
<th></th>
<th>HCT-116-p53+/+</th>
<th>HCT-116-p53/-/-</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
<td>Expt. 1</td>
<td>Expt. 1</td>
</tr>
<tr>
<td>Nutlin</td>
<td>5.24</td>
<td>7.15</td>
<td>55.12</td>
<td></td>
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<tr>
<td>Nutlin + PI-1840 (5µM)</td>
<td>2.59</td>
<td>2.89</td>
<td>59.77</td>
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<td>Nutlin + PI-1840 (10µM)</td>
<td>2.15</td>
<td>1.80</td>
<td>57.02</td>
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<tr>
<td>Nutlin + PI-1840 (15µM)</td>
<td>1.85</td>
<td>0.86</td>
<td>57.95</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>IC50 (µM)</th>
<th></th>
<th>LNCaP</th>
<th>DU-145</th>
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<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>BH3-M6</td>
<td>16.62</td>
<td>17.12</td>
<td>55.23</td>
<td>59.94</td>
</tr>
<tr>
<td>BH3-M6 + PI-1840 (5µM)</td>
<td>12.88</td>
<td>13.17</td>
<td>60.43</td>
<td>61.86</td>
</tr>
<tr>
<td>BH3-M6 + PI-1840 (10µM)</td>
<td>11.99</td>
<td>5.64</td>
<td>58.47</td>
<td>52.32</td>
</tr>
<tr>
<td>BH3-M6 + PI-1840 (15µM)</td>
<td>3.66</td>
<td>4.50</td>
<td>52.65</td>
<td>54.88</td>
</tr>
</tbody>
</table>

Upper Panel: PI-1840 sensitizes HCT-116-p53+/+, but not HCT-116-p53/-/- cells to Nutlin mediated inhibition of cell viability. The two HCT-116 cell lines were treated with either nutlin alone or in combination with PI-1840 and the effects on viability determined by MTT as described under Experimental Procedures. Lower Panel: PI-1840 sensitizes LNCaP, but not DU-145 cells to BH3-M6 mediated inhibition of cell viability. LNCaP and DU145 cells were treated with either BH3-M6 alone or in combination with PI-1840 and the effects on viability determined by MTT as described under Experimental Procedures.
Figure 1.

A.

IC<sub>50</sub>: CT-L 0.6 ± 0.18 µM; T-L >100 µM; PGPH >100 µM

B.

IC<sub>50</sub>: CT-L 27 ± 14 nM; T-L >100 µM; PGPH >100 µM
Figure 2.

A. Peptide from vehicle treated proteasome
Exp m/z: 781.4504
Error: 6.2 ppm

B. Peptide from PI-1840 treated proteasome
Exp m/z: 781.4397
Error: 7.5 ppm

C. Modified Peptide from Lactacystin-treated proteasome
Exp m/z: 497.7778
Error: 2.8 ppm
Figure 3.

A. clasto-Lactacystin

Chemical Formula: C_{36}H_{60}N_{8}O_{11}
Exact Mass: 780.4382

B. Lactacystin

Chemical Formula: C_{48}H_{75}N_{9}O_{15}
Exact Mass: 993.5383

C. Graph showing % CT-L activity over time (mins): PI-1840 and Lactacystin
Figure 4.

A. Graph showing the percentage activity over time for various treatments.

B. Western blots for different concentrations of PI-1833 and PI-1840 showing protein levels for p27, IKB-α, Bax, and Actin.

C. Western blots for different concentrations of PI-1833 and PI-1840 showing protein levels for pAkt, TAkt, pS6, TS6, Survivin, cleaved Casp. 3, and cleaved PARP.
Figure 5.

A. Tumor Volume (mm$^3$)

B. % Change in Tumor Volume
Discovery of PI-1840, a novel non-covalent and rapidly reversible proteasome inhibitor with anti-tumor activity
Aslamuzzaman Kazi, Sevil Ozcan, Awet Tecleab, Ying Sun, Harshani R. Lawrence and Said M. Sebti

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