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Chemical and Biological Evaluation of Dipeptidyl Boronic Acid Proteasome Inhibitors for Use in Prodrugs and Pro-Soft Drugs Targeting Solid Tumors

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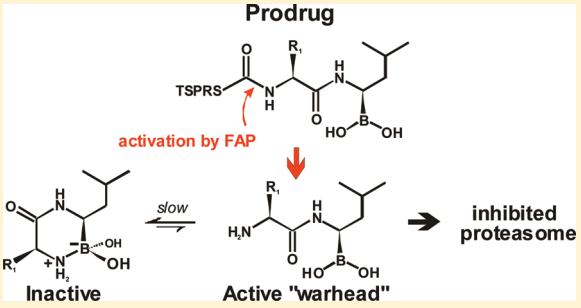
Supporting Information

ABSTRACT: Bortezomib, a dipeptidyl boronic acid and potent inhibitor of the 26S proteasome, is remarkably effective against multiple myeloma (MM) but not against solid tumors. Dose-limiting adverse effects from "on target" inhibition of the proteasome in normal cells and tissues appear to be a key obstacle. Achieving efficacy against solid tumors therefore is likely to require making the inhibitor more selective for tumor tissue over normal tissues. The simplest strategy that might provide such tissue specificity would be to employ a tumor specific protease to release an inhibitor from a larger, noninhibitory structure. However, such release would necessarily generate an inhibitor with a free N-terminal amino group, raising a key question: Can short peptide boronic acids with N-terminal amino groups have the requisite properties to serve as warheads in prodrugs? Here we show that dipeptides of boroLeu, the smallest plausible candidates for the task, can indeed be sufficiently potent, cell-penetrating, cytotoxic, and stable to degradation by cellular peptidases to serve in this capacity.



INTRODUCTION

Bortezomib (**1a**, Figure 1) is the dipeptidyl boronic acid Phe-boroLeu acylated at the N-terminus with a pyrazinoyl group and can be written as Pyz-Phe-boroLeu. It is a potent, reversible inhibitor of the proteolytic activity of the 26S proteasome, a multisubunit, multicatalytic complex with chymotrypsin-like (β5), trypsin-like (β2), and caspase-like (β1) enzymatic sites, so named for the preferred cleavages they mediate.[1−4] A functional proteasome is essential for intracellular protein homeostasis and cellular regulation.[5−10] Bortezomib gained FDA approval for the treatment of patients with multiple myeloma (MM) in 2003 and has since transformed their care. In 2006, it was approved for patients with relapsed mantle cell lymphoma (MCL).[11−15] The remarkable efficacy observed for this first-in-class agent against these hematological malignancies validated the proteasome as an anticancer target and stimulated efforts to use bortezomib to treat other types of cancers, especially solid tumors such as prostate, breast, and lung. While there have been some hints of activity against solid tumors, the hoped-for efficacy has not been realized.[16−19] Dose-limiting toxicities seem likely to be the major obstacle to success. Even in MM and MCL, where bortezomib is efficacious, adverse effects are a major problem. Peripheral neuropathy (PN) is perhaps the most important, as it often limits the dose and therefore the efficacy, and is the principal reason patients elect to stop treatment.[20]

Multiple groups have been searching for second-generation proteasome inhibitors with better efficacy and safety profiles, with the goal of lessening the PN in MM patients but also of achieving efficacy against solid tumors. Several second generation inhibitors have reached the clinic and are shown in Figure 1.[1,4,21] Compound **3** (CEP-18770, Figure 1), like bortezomib, is a boronic acid, and it has an inhibitory profile against the three proteasome active sites very similar to that of bortezomib.[20] The epoxy ketone inhibitor **4** (carfilzomib, Figure 1), structurally related to the natural product epoxomicin, is a potent, irreversible inhibitor that exhibits specificity for β5 over β2 and β1 and a reduced tendency to inhibit other nonproteasomal proteases.[20,22−24] Proteasome inhibitor **5** (NPI-0052 or salinosporamide A, Figure 1) is a β-lactone natural product isolated from the marine bacterium *Salinispora tropica*. Like **4**, it is an irreversible proteasome inhibitor. It differs from **4** and bortezomib in that it potently inhibits all three sites.[20,21,25−27] Second generation inhibitor **6** (MLN9708), the most recent entry into clinical trials, is another boronic acid and again has IC_{50} values very similar to those of bortezomib for all three sites. It differs from bortezomib in that it has a faster off-rate from the proteasome. It is orally available and rapidly hydrolyzes to yield **7** (MLN2238).[20,22,28] The faster off-rate of **6** has been proposed to result in better antitumor activity by permitting greater distribution of the drug into the tissues and thereby into the tumor. Higher levels of drug and proteasome inhibition (74.9% for **6** vs 44.8% for bortezomib) were

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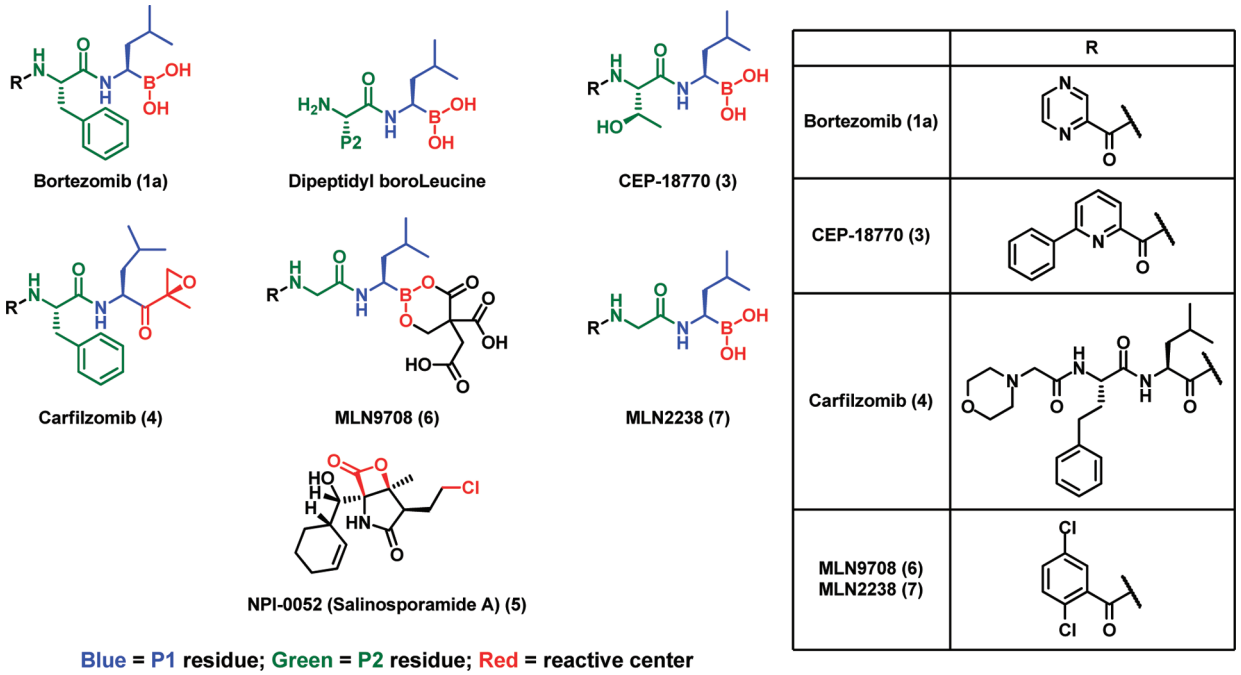

Figure 1. Structural comparison of dipeptidyl boronic acids, bortezomib (**1a**), and the second-generation proteasome inhibitors CEP-18770 (**3**), carfilzomib (**4**), salinosporamide A (**5**), MLN9708 (**6**), and MLN2238 (**7**).

observed in mouse tumor tissue relative to blood cells, supporting this idea.[20,29]

The above second-generation compounds are, like bortezomib, relatively potent inhibitors of the proteasome with IC_{50} values in the low nanomolar range. There are differences between them and bortezomib, mostly in the inhibitory profiles against the three proteasome active sites or in specificity for the proteasome over other proteases, that could lead to improvements in safety or efficacy. Thus far, however, the improvements appear to be incremental. The one that seems to show the most promise against solid tumors, **6**, is also the only rapidly reversible inhibitor, suggesting that potency and specificity for the proteasome may be less important in achieving efficacy against solid tumors than other factors such as distribution to the target tissues.[20,28]

Proteasome inhibition results in the buildup of misfolded and damaged intracellular proteins, leading to the disruption of multiple cellular signaling pathways and ultimately cell death.[30−34] Multiple myeloma cells seem to be especially sensitive to proteasome inhibition, quite likely because of their overproduction of protein and high protein turnover, resulting in greater need for proteasomal degradation and clearance.[3,11,12,14,15,19,35,36] Nevertheless, all cells, both normal and malignant, require a functioning proteasome. Different types of cells exhibit differences in sensitivity to proteasome inhibition, but proteasome inhibition will, at some level and duration, be lethal to essentially any cell. The adverse effects observed for bortezomib and other proteasome inhibitors are quite likely to arise from "on-target" inhibition in "off-target tissues" rather than off-target inhibition of nonproteasomal enzymes. Neuropathic pain, for example, which occurs to some degree with each of the above inhibitors, has been linked to the accumulation of ubiquitinated proteins in cells of the peripheral nervous system.[37]

Therefore, to achieve efficacy against solid tumors with a proteasome inhibitor may require improving inhibitor selectivity for tumor tissue over normal tissues rather than improving inhibitor potency for the proteasome or specificity for the proteasome over other enzymes. A tumor-activated prodrug could provide such tissue specificity. The simplest approach would be to design a longer boroLeu-based peptide that is itself not a protease inhibitor but is instead a substrate for a tumor-specific protease whose action releases a shorter peptide of boroLeu, which is a proteasome inhibitor (Figure 2). Proteases are particularly well suited for the "activation" role, and several "tumor specific" proteases have been identified. These include, for example, fibroblast activation protein (FAP), prostate specific antigen (PSA), and prostate specific membrane antigen (PSMA).[38−46]

Any peptidyl boronic acid released from such constructs would ipso facto have a free N-terminal amino group. This raises some concerns as the free amino group might be expected to limit potency, cell permeability, or stability, particularly as it might render the released inhibitors susceptible to aminopeptidase degradation. Another consideration is that it would be advantageous for the released inhibitor to be as small as possible to allow for the incorporation of tumor specificity into the larger peptide without it becoming too large and cumbersome, but smallness could also limit inhibitory potency. Dipeptidyl boronic acids are perhaps the smallest entities that have the potential to serve in this capacity, but they present an additional complication. On the basis of previous findings with dipeptidyl boroPro, they are expected to undergo a pH-dependent cyclization that could significantly attenuate their inhibitor potency at physiological pH. But this phenomenon could also prove useful when used as the warhead of a prodrug. The dipeptidyl boronic acid inhibitor would be released at the tumor site in the open-chain, enzyme inhibitor form. Cyclization subsequent to release would then help to attenuate systemic effects as excess inhibitor diffuses from the tumor site. Such defined and programmed loss of pharmacological activity with time is characteristic of the action of agents termed "soft drugs". Soft drugs need to be applied directly to the intended site of action.[47−50] Prodrugs of soft drugs have previously been termed "pro-soft" drugs, although there are very few working examples and little exploration of their

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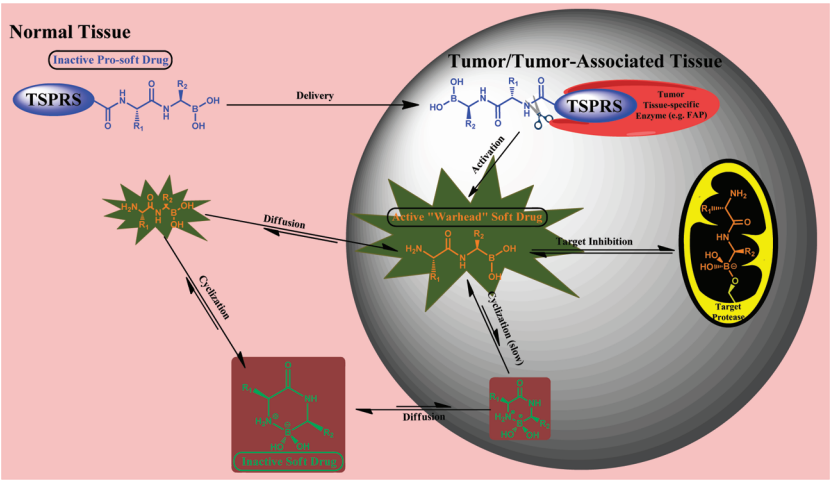

Figure 2. Dipeptidyl boroLeu can be attached to the C-terminus of tumor-specific protease recognition sequences (TSPRS) and thus activated by tumor tissue-specific proteases to release the active inhibitor within tumor and tumor-associated tissues. The active "warhead" is then available to inhibit a target protease like the proteasome in this tissue. This dipeptidyl boronic acid is inactivated through cyclization, a reaction that is ideally favored upon diffusion away from the intended target tissue.

potential.[51−53] However, we have recently reported on the design and characterization of a pro-soft derivative of Val-boroPro.[55]

Here we evaluate a series of dipeptidyl boronic acids with respect to proteasome inhibitory potency, cell permeability, cytotoxicity, susceptibility to degradation by cellular lysates, which would include aminopeptidase and other cellular peptidases, and intramolecular cyclization. The results show that although dipeptidyl boronic acids vary widely with respect to these factors, within this class there are molecules that would be suitable for use in constructing proteasome inhibitor-based prodrugs for the selective targeting and treatment of solid tumors.

RESULTS AND DISCUSSION

Proteasome Inhibitory Potency in Vitro and pH-Dependent Cyclization. Previous work has shown that dipeptidyl boronic acids containing boroPro or boroAla exist in a pH-dependent equilibrium (Figure 3) between an open-chain, enzyme inhibitory form (A) and a cyclic, inactive form (D). The open chain structure is favored at low pH, but as the pH is increased and the N-terminal ammonium ion deprotonates, the cyclic structure becomes increasingly favored.[55−61] For dipeptides of boroPro the cyclic structures are strongly favored over the open chain structures at physiological pH, by factors ranging from ∼100- to 2000-fold, depending on the P2 residue.[57,61] For dipeptides of boroAla, the cyclic structures are not as strongly favored. For example, Ala-boroAla has a ratio of cyclic to open chain of ∼5.0 at pH 7.4 and typically varies from ∼2 to 20.[56] One effect of the cyclization reaction is to attenuate the potency of these inhibitors at physiological pH as equilibrium is approached.[57,61]

Determining the proteasome inhibitor potency of a boroLeu dipeptide therefore also requires ascertaining its cyclization behavior, as cyclization can obscure intrinsic potency. Cyclization is a relatively slow process and can be conveniently measured by NMR or by comparing the inhibitory potency following preassay equilibration of the inhibitor for 24 h at pH 2.0 versus pH 7.6, using the pH 2.0 activity as representing that of the fully open chain species. The sensitivity and dynamic range of NMR limit its usefulness to measurement of cyclization indexes (CIs)

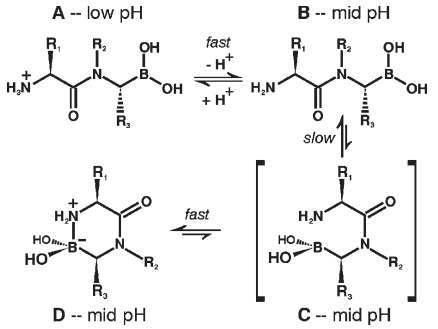

Figure 3. Cyclization reaction of dipeptide boronic acids.

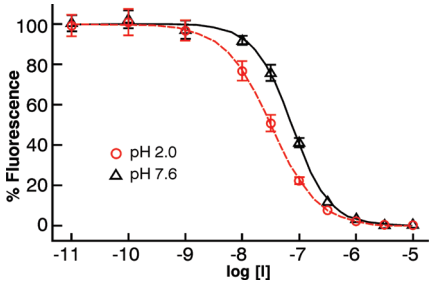

Figure 4. In vitro proteasome inhibition by **2c** is dependent on the pH of preassay incubation. The potency of the compound as an inhibitor of the proteasome is reduced when incubated at pH 7.6 for 24 h prior to assay.

that are <100, whereas the pH dependent enzyme assay can determine CIs that are much larger.

Phe-boroLeu, **2c**, differs from bortezomib only in that it lacks the N-terminal pyrazinoyl group. It has a free N-terminal amino group and therefore exhibits the expected pH-dependent inhibition (Figure 4). NMR spectroscopy was used to confirm that the pH-dependent effect is indeed due to cyclization and that it is fully reversible (Figures S1−S5). The CI for Phe-boroLeu is ∼3.7, far less than that of boroPro dipeptides but on par with those for boroAla dipeptides.[56]

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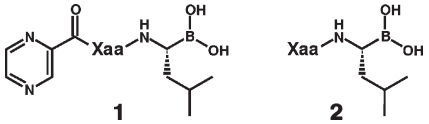
Table 1. Proteasome Inhibitor Potency, Cyclization Index, Cell Permeability, and Cytotoxity for Dipeptidyl boroLeu Families 1 and 2



		in vitro IC_{50} (nM)							
compd	Xaa, P2 amino acid	pH 2.0	pH 7.6	CI	IC-IC_{50} (nM)	IC-IC_{50}/IC_{50}	CyT_{50} (nM)	CyT_{50}/IC_{50}	CyT_{50}/IC-IC_{50}
				P2 Amino Acids with N-Terminal Acylation					
1a	Phe (bortezomib)	10 ± 1	14 ± 2	1.4 ± 0.2	27 ± 12	2.7 ± 1.2	4 ± 3	0.4 ± 0.3	0.16 ± 0.13
1b	Thr	12 ± 2	25 ± 3	2.1 ± 0.4	790 ± 140	65 ± 14	100 ± 50	8 ± 5	0.12 ± 0.07
1c	Ser	16 ± 1	18 ± 2	1.1 ± 0.2	2500 ± 300	150 ± 25	210 ± 20	13 ± 2	0.08 ± 0.01
1d	β-Me-Thr	34 ± 2	28 ± 2	0.8 ± 0.1	520 ± 130	15 ± 4			
1e	Gly	110 ± 10	180 ± 30	1.6 ± 0.3	1800 ± 500	16 ± 5			
				Natural P2 with Free N-Termini					
2a	Gly	21 ± 2	430 ± 60	20 ± 4	>10K	>470	670 ± 280	30 ± 10	<0.07
2b	Trp	29 ± 3	100 ± 10	3.6 ± 0.6	>1000	>30	400 ± 40	14 ± 2	<0.4
2c	Phe	37 ± 5	140 ± 20	3.7 ± 0.8	>10K	>270	90 ± 80	2.4 ± 2.3	<0.01
2d	Tyr	42 ± 4	120 ± 10	2.9 ± 0.4	>10K	>240	160 ± 50	4 ± 1	<0.02
2e	Ala	50 ± 7	360 ± 30	7 ± 1	>10K	>200	3200 ± 2800	64 ± 57	<0.3
2f	Asn	63 ± 7	770 ± 40	12 ± 2	>10K	>160			
2g	Leu	66 ± 3	170 ± 20	2.6 ± 0.3	>10K	>150			
2h	Thr	80 ± 9	450 ± 30	5.6 ± 0.7	>10K	>120	2500 ± 950	30 ± 10	<0.3
2i	Ser	190 ± 20	1100 ± 200	6 ± 2	>10K	>50	1700 ± 1500	8.9 ± 8.0	<0.2
2j	His	510 ± 130	5200 ± 1000	10 ± 3	>1000	>20			
				Non-Natural P2 with Free N-Termini					
2k	1-Nal[a]	26 ± 3	120 ± 10	4.4 ± 0.7	150 ± 30	6 ± 1	11 ± 1	0.4 ± 0.1	0.07 ± 0.01
2l	2-Nal[a]	33 ± 8	220 ± 20	7 ± 2	450 ± 70	13 ± 4			
2m	3,5-Df-Phe[a]	43 ± 5	100 ± 10	2.3 ± 0.5	790 ± 150	18 ± 4	47 ± 3	1.0 ± 0.2	0.06 ± 0.01
2n	Bpa[a]	50 ± 6	400 ± 50	7.9 ± 1.4	1400 ± 100	30 ± 5			
2o	4-F-Phe	60 ± 6	130 ± 20	2.3 ± 0.5	>10K	>170	79 ± 9	1.3 ± 0.2	<0.01
2p	Nle	75 ± 8	300 ± 90	4 ± 1	>10K	>130	950 ± 70	13 ± 2	<0.1
2q	2-F-Phe	76 ± 8	140 ± 10	1.8 ± 0.2	3100 ± 500	41 ± 8			
2r	3,4-Df-Phe[a]	81 ± 3	150 ± 10	1.9 ± 0.2	2100 ± 300	26 ± 4			
2s	3-F-Phe	82 ± 6	110 ± 10	1.3 ± 0.1	1300 ± 100	16 ± 2			
2t	β-Me-Thr	90 ± 10	150 ± 10	1.7 ± 0.2	>10K	>110			
2u	*t*-Bu-Gly	100 ± 10	150 ± 10	1.6 ± 0.2	>10K	>110	710 ± 50	7 ± 1	<0.07
2v	Et-Gly	110 ± 10	300 ± 30	2.8 ± 0.4	>10K	>90	1500 ± 300	14 ± 4	<0.2
2w	Dpa[a]	110 ± 10	350 ± 50	3.2 ± 0.5	1000 ± 100	9 ± 2			
2x	Nva	110 ± 10	540 ± 80	5 ± 1	>10K	>90	1100 ± 600	10 ± 6	<0.1
2y	Pf-Phe[a]	170 ± 40	470 ± 60	2.8 ± 0.7	3300 ± 900	20 ± 7			
2z	Chg	240 ± 30	900 ± 100	3.7 ± 0.7	>10K	>40			
2aa	Gla	600 ± 100	3000 ± 200	5 ± 1	>10K	>20			
				Natural P2 with N-Terminal Alkylation					
2ab	*N*,*N*-diMeGly	36 ± 2	610 ± 40	17 ± 1	>10K	>280			
2ac	Sar	39 ± 2	2200 ± 200	56 ± 7	>10K	>260	160 ± 140	4.1 ± 3.6	<0.07

[a] Nal = naphthylalanyl; Bpa = biphenylalanyl; Df-Phe = difluorophenylalanyl; Dpa = diphenylalanyl; Pf-Phe = (pentafluorophenyl)alanyl; Nva = norvalinyl; Nle = norleucyl; Chg = cyclohexylglycyl; Gla = γ-carboxyglutamyl; Sar = sarcosyl.

A series of boroLeu dipeptides were similarly examined, and their IC_{50} values following pre-equilibration at pH 2.0 and pH 7.6 are listed in Table 1 together with their corresponding cyclization indices. The compounds are listed in order of decreasing potency with respect to the pH 2.0 IC_{50} values because this value, always the smaller of the two, corresponds more closely to the intrinsic potency of the compound. Compounds **2a**−**j** are dipeptides of boroLeu with naturally occurring amino acids at P2, while compounds **2k** through **2aa** have non-naturally occurring amino acids at this position. The pH 2.0 IC_{50} values range from 21 to 620

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nM or from about 2- to 62-fold less potent than bortezomib. The pH 7.6 IC_{50} values are significantly higher, ranging from 100 to 2900 nM or from 10- to 290-fold less potent than bortezomib. The pH 7.6 values would be the more relevant set for use of the compounds as conventional proteasome inhibitors. But for use as warheads in proteasome inhibitor prodrugs, the pH 2.0 values are more relevant, as the open-chain form would be released from the prodrug at the target site. The CI values range from 1.3 (**2s**) to 20 (**2a**). A CI of 1.3 is too small to be of interest or concern. A factor of 20, however, is large enough to provide a useful soft drug effect. Interestingly, Gly-boroLeu (**2a**) has both the smallest IC_{50} and the largest CI of the boroLeu-based dipeptides having a free amino group.

Several dipeptides of boroLeu substituted on the N-terminus were also examined. Several of these, **1b**−**e**, are bortezomib-like in that they are pyrazinoylated on the N-terminus, and two, **2ab** and **2ac**, are alkylated. The pyrazinoylated compounds, as expected, do not cyclize, but the alkylated compounds do cyclize. In fact, with a CI of 56, **2ac** has the greatest CI of any of the boroLeu-based dipeptides examined. It is also a reasonably potent proteasome inhibitor with an IC_{50} of 39 nM, or only 4-fold less potent than bortezomib. The reason **1a**−**e** do not cyclize while **2ab** and **2ac** do is that the electron lone pair of the N-terminal nitrogen in the acylated dipeptides is delocalized in the amide π system and thus unavailable for nucleophilc attack on the boron atom, whereas in the alkylated dipeptides it is in a σ system and available for donation to the boron to form the boron−nitrogen dative bond of the cyclic structure.

Cell Permeability. A number of cancers express a tumor-specific protease on their cell surface or induce its expression on the surface of the surrounding stromal cells (e.g., FAP).[38−46] If one of these proteases is used as the activating enzyme, the released proteasome inhibitor would need to have cell-penetrating ability to be effective. The cell penetrating ability of the compounds listed in Table 1 was therefore assessed. This was done by determining their ability to inhibit the proteasome inside cells in culture using the proteasome sensor cell line (Clontech), which is an HEK 293 cell line stably transfected with a proteasome-sensitive fluorescent reporter. The reporter is a fusion protein of a green fluorescence protein with a degradation domain (ornithine decarboxylase), which targets the protein for degradation by the proteasome.[62,63]

Normally, the levels of fusion protein are low, owing to rapid degradation by the proteasome within the cell. However, when there is a decrease in proteasome activity, the fusion protein concentration increases and the cell emits green fluorescence. The amount of fluorescence is dependent upon the degree to which the proteasome has been inhibited within the cell, allowing a determination of an intracellular IC_{50} value (IC-IC_{50}) as listed in Table 1.[62,63] The ratio IC-IC_{50}/IC_{50} is a measure of cell permeability. A value of 1.0 would correspond, in essence, to unrestricted access or "100% permeability". The larger the ratio, the lower is the cell-penetrating ability of the inhibitor.

The IC-IC_{50}/IC_{50} for bortezomib, **1a**, is ∼2.7 indicating that almost 3-fold higher concentrations of bortezomib are needed in the extracellular medium to inhibit the proteasome within cells than would be needed if it were not sequestered within the cell. Nevertheless, this value indicates that bortezomib has significantly more cell-penetrating ability than the other "bortezomib-like" inhibitors (**1b**−**e**), which is perhaps not surprising because it has the aromatic and hydrophobic Phe in P2, while **1b**−**e** have more hydrophilic side chains in this position. The notion that compounds with polar or charged P2 side chains should have less cell-penetrating power than those with hydrophilic side chains appears to apply to the other compounds in Table 1.

Dipeptides of boroLeu with free N-terminal amino groups have significantly less cell-penetrating ability than their N-terminally blocked counterparts, as might be expected. This is illustrated when one compares **1a**, **1b**, **1d**, **1e** with **2c**, **2h**, **2t**, **2a**, respectively. But there are exceptions: **2i** appears to be 3-fold more cell-penetrating than **1c**. While none of the dipeptides of boroLeu are as cell penetrating as bortezomib, some are cell penetrating enough for use as warheads in prodrugs. For example, six (**2k**, **2l**, **2m**, **2s**, **2w**, **2y**) have IC-IC_{50}/IC_{50} values of 20 or less, and one (**2k**) with a value of ∼6.0 is almost within 2-fold of bortezomib's value of ∼2.7.

Cytotoxicity. Cytotoxicity was measured using the standard MTT cytotoxicity assay[64−66] on both HEK 293 cells and follicular small-cell lymphocytic leukemia (FSCLL) cells. HEK 293 PS cells seem to be quite resistant to proteasome inhibition-mediated cyctotoxicity, showing a CyT_{50} value of 347 nM for **1a** and values greater than 10 μM for the other compounds in Table 1 (data not shown). However, all of the tested compounds exhibit measurable cytotoxicity against FSCLL cells (Table 1). Perhaps not surprisingly, bortezomib (**1a**) is the most cytotoxic of the compounds tested, with a CyT_{50} of 4 nM. However, Phe-boroLeu (**2c**) was only about 20-fold less cytotoxic with a CyT_{50} value of 91 nM. A CyT_{50} of 91 nM is most likely sufficiently cytotoxic that Phe-boroLeu itself could be considered for use as the warhead in a prodrug. There are, however, several dipeptidyl boronic acids that are significantly more cytotoxic and therefore perhaps more promising than **2c**. These include **2k** and **2m** with CyT_{50} values of 11 and 47 nM, respectively.

The relationship between cytotoxicity and proteasome inhibitory potency in vitro and in cells are listed in Table 1 as the ratios CyT_{50}/IC_{50} and CyT_{50}/IC-IC_{50}, respectively. The CyT_{50}/IC-IC_{50} ratio is of special interest because it relates cytotoxicity to inhibition of the proteasome achieved within the cell. A value of 1.0 means by definition that the CyT_{50} and IC-IC_{50} are equal. Values greater than 1.0 would suggest that more than 50% inhibition is needed to kill 50% of the cells while values less than 1.0 would indicate the reverse. The ratio for bortezomib (0.16) suggests that less than 50% inhibition, perhaps around 20%, is all that is required to kill 50% of the cells. This is not too surprising, especially as it is not entirely out of line with in vivo PK/PD studies.[20,29,67−71]

However, the ratio for some of the dipeptidyl boronic acid inhibitors is even less, sometimes dramatically so, as for example for **2c**, **2o**, and **2m**, and these values do seem unreasonable; i.e., it is not likely that less than ∼5% inhibition of the proteasome could be sufficient to trigger cell death, even in cell culture where the inhibition is maintained over the 24 h period. What then is the explanation for these results? One possibility is that there is a different mechanism triggering cell death. Another is that IC-IC_{50} values are significantly under-reporting the actual degree of proteasome inhibition achieved in the cell for reasons that are not entirely clear. One way in which this could happen is if the inhibitors were degraded over time such that at 24 h, when the proteasome activity is measured, there is little of the inhibitor left, yet sufficient inhibition was attained over the first few hours to trigger cell death. We have ruled this out by showing that the inhibitors show no sign of decomposition after 24 h of incubation in the cell cultures.

The unreasonable values of CyT_{50}/IC-IC_{50} appear to occur for inhibitors where no in-cell proteasome inhibition was

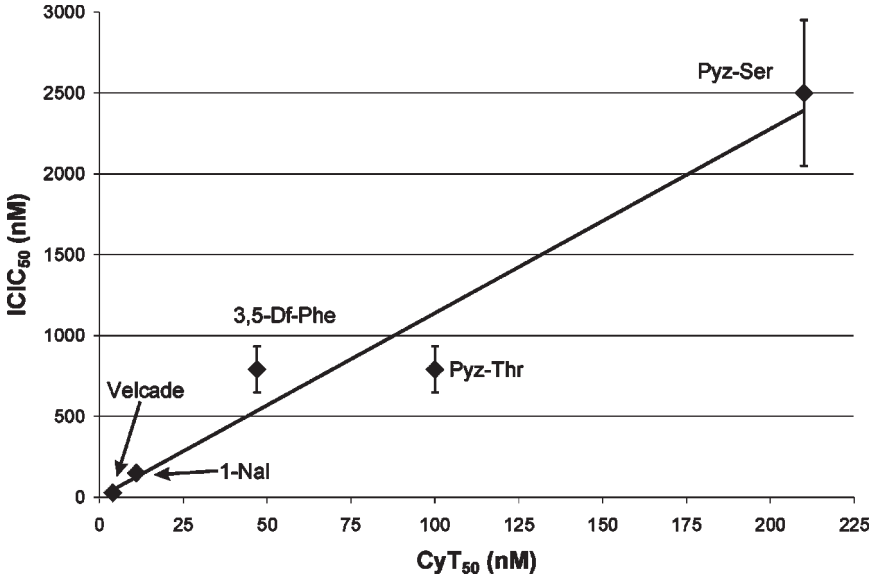


Figure 5. Correlation between cytotoxicity (CyT_{50}) and intracellular proteasome inhibitory potency as measured by the IC-IC_{50}.

detected and only cytotoxicity values could be measured. For those inhibitors where a clear IC-IC_{50} is observed, a reasonably good correlation exists between these values and the corresponding cytotoxicities (Figure 5). This strongly supports the notion that proteasome inhibition is the mechanism triggering cytotoxicity for most, if not all, of the compounds examined. Note that even for these more effective inhibitors, the IC-IC_{50} values are nevertheless still about 5−10 times larger than the CyT_{50}. We cannot therefore be sure whether fractional inhibition of the proteasome, about 10−30%, is sufficient to cause cell death or whether the assay is under-reporting the inhibition actually being achieved within the cell, at least with respect to one or more biologically relevant substrates.

Susceptibility to Aminopeptidase Degradation. A free N-terminal amino group might be expected to render the dipeptide of boroLeu susceptible to degradation by aminopeptidases. Such susceptibility could significantly affect the suitability of dipeptides of boroLeu as warheads in the design and construction of tumor-specific proteasome inhibitor prodrug. It could also affect some of the parameters determined here such as cytotoxicity, intracellular inhibitory potency, and cell permeability and perhaps account for some of the discrepancy between CyT_{50} and IC-IC_{50}.

To determine if degradation by aminopeptidases or other cellular enzymes is a factor, **2a**, **2c**, and **2e** were incubated with intact cells (either HEK 293 or FSCLL cells) and with cell lysates for 24 h at 10^{-4} M. At various time points, aliquots were removed and analyzed by LCMS. Peaks corresponding to the parent compound and degradation products were identified and their areas measured. No evidence of aminopeptidase degradation, or other degradation products, was detected for any of the dipeptidyl boronic acids (Figure S6a and Figure S6b). A tripeptidyl boronic acid, Leu-Phe-boroLeu, was also examined. In contrast with the dipeptidyl boronic acids, it was rapidly degraded by both HEK cell lysate and FSCLL cell lysate, as determined by the disappearance of the parent compound and corresponding appearance of the products of aminopeptidase degradation (Figure S6c). Thus, dipeptidyl boronic acids appear to be much more stable to aminopeptidases than tripeptidyl boronic acids, perhaps owing to the non-natural amino acid moiety, boroLeu acid, in P1′.

CONCLUSIONS

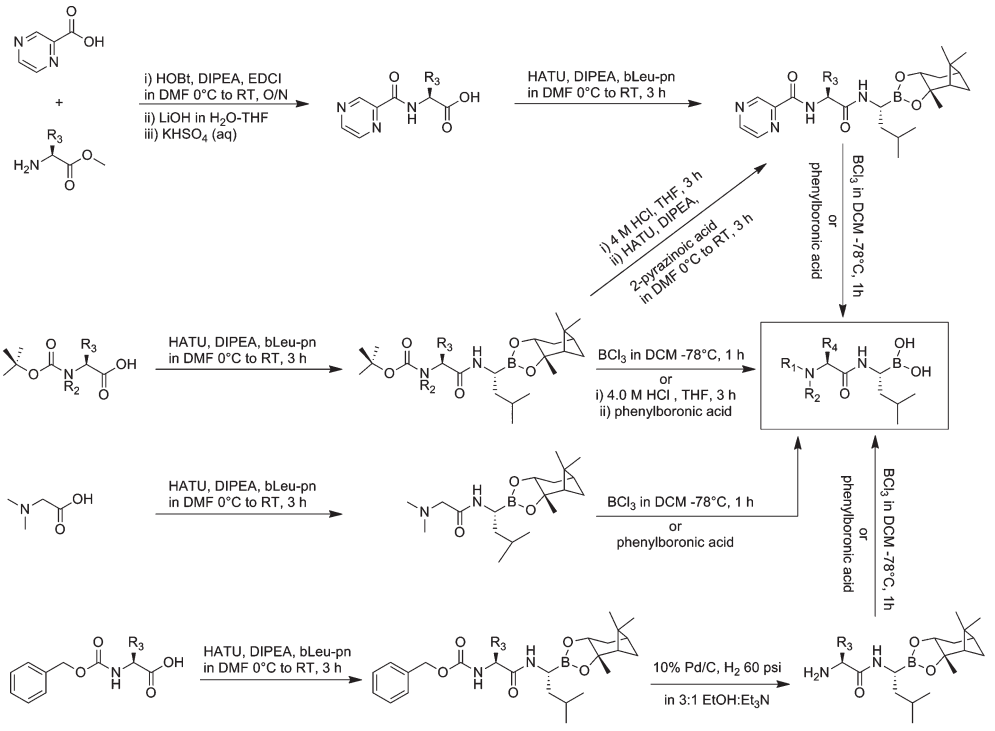
Despite being relatively small and having a free amino group, dipeptides of boroLeu can be sufficiently potent, cell penetrating, cytotoxic, and resistant to degradation by cellular proteases to serve as warheads in the construction of prodrugs designed to selectively target the proteasome in solid tumors. The candidate that best fulfills all of the desired properties is perhaps **2k**, but **2c**, **2m**, **2o**, and **2ac** might also be suitable. The dipeptides of boroLeu can also undergo a pH-dependent cyclization, which, although relatively modest compared to that exhibited by dipeptides of boroPro, might in some cases be sufficient to provide additional tissue specificity by limiting systemic exposure to the active proteasome inhibitor following activation at the tumor. This phenomenom could make **2a** one of the better candidates, as its true cytotoxicity could be 20-fold better than the values listed in Table 1 for a short time following release from a prodrug at the tumor.

EXPERIMENTAL SECTION

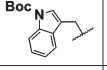
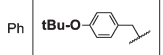
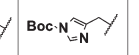
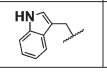
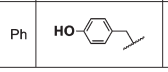
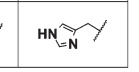
Synthesis of Dipeptidyl Boronic Acids. The (+)-pinanediol ester of leucineboronic acid starting material was synthesized using the methods described in the literature.[72−74] Dipeptidyl boronic acids were synthesized using standard methods, a brief description of which is given below.[72−80] Typical reaction scales were 2.0 mmol.

Synthesis of N-Pyrazinoylated Amino Acids. A solution of 1.0 equiv of 2-pyrazinoic acid and 1.3 equiv of the desired P2 amino acid C-terminal methyl ester containing side chain protecting groups as necessary (see Scheme 1) in 10 mL of DMF under argon atmosphere was cooled on an ice bath to 0 °C and allowed to stir for 10 min. To the cooled mixture, an amount of 1.3 equiv each of HOBt, EDC, and DIPEA was added. The mixture was stirred for 15 min, after which it was warmed to room temperature and stirred overnight. The mixture was diluted with 200 mL of an aqueous solution of either 10% (w/v) citric acid or 0.1 M $KHSO_4$ and extracted three times with 100 mL of ethyl acetate. The organic layers were pooled, dried over $MgSO_4$, filtered and solvent removed under vacuum. The resulting clear oil was purified by

Scheme 1. Synthesis of Dipeptidyl Boronic Acids and N-Pyrazinoylated Dipeptidyl Boronic Acids



1a - 1e: R_1 =  , R_2 = H **2a - 2j:** R_1, R_2 = H **2ab, 2ac:** R_3, R_4 = H

	1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	2f	2g	2h	2i	2j
R_3	Ph				H	H		Ph		Me		iBu			
R_4	Ph				H	H		Ph		Me		iBu			

	2ab	2ac
R_1	Me	Me
R_2	Me	H

2k - 2z: R_1, R_2 = H
2aa: R_1 = H, R_2 = Cbz

	2k	2l	2m	2n	2o	2p	2q	2r	2s	2t	2u	2v	2w	2x	2y	2z	2aa
R_3						*n*-Bu						Et		*n*Pr		Cy	
R_4						*n*-Bu						Et		*n*Pr		Cy	

Ph = Phenyl, *t*Bu = tertiary-butyl, Me = methyl, *i*Bu = isobutyl, Cbz = carbobeznzyloxy, *n*Bu = *n*-butyl, Et = ethyl, *n*Pr = *n*-propyl, Cy = cyclohexyl

flash chromatography using a mixture of hexanes and ethyl acetate as the mobile phase. The purified pyrazinoylated aminoacyl ester was dissolved in a mixture of 5 mL of THF and 5 mL of water and subsequently cooled on an ice bath to 0 °C. To the stirring solution, an amount of 2.0 equiv of LiOH was added. The mixture was allowed to stir at 0 °C for 3 h, after which it was neutralized with 200 mL of $KHSO_4$ and extracted three times with 100 mL of ethyl acetate. The aqueous phase was collected and lyophilized to yield the pyrazinoylated amino acid as a pale yellow powder.

Coupling to Leucineboronic Acid (+)-Pinanediol Ester. Pyrazinoylated, N-terminally Boc-protected, or N-terminally Cbz-protected amino acid was then dissolved in 10 mL of DMF, and 1.0 equiv of leucineboronic acid (+)-pinanediol ester hydrochloride (boroLeu-pn, bLeu-pn) was added. The mixture was cooled on an ice bath to 0 °C under argon atmosphere and stirred for 10 min. To the cooled mixture was added 2.2 equiv of DIPEA followed by 1.2 equiv of HATU. The mixture was stirred for 15 min, after which it was warmed to room temperature and stirred for an additional 3 h. The mixture was diluted with 200 mL of an aqueous solution of either 10% (w/v) citric acid or 0.1 M $KHSO_4$ and extracted three times with 100 mL of ethyl acetate. The organic layers were pooled, and solvent was removed under vacuum. The resulting pyrazinoylated, N-terminally Boc-protected or N-terminally Cbz-protected dipeptidyl boronic acid (+)-pinanediol esters as clear to pale yellow oils were purified by flash chromatography using a mixture of hexanes and ethyl acetate as the mobile phase.

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Synthesis of Pyrazinoylated Dipeptidyl Boronic Acid (+)-Pinanediol Esters. This was accomplised by one of two synthetic routes. In the first synthetic route, pyrazinoylated P2 amino acids are coupled to boroLeu-pn as described in the above section. The second method is briefly described here. The *N*-Boc-protected dipeptidyl boronic acid (+)-pinanediol ester was dissolved in 10 mL of a solution of 4 M HCl in THF. The mixture was allowed to stir for 3 h at room temperature, after which the resulting white precipitate was collected by vacuum filtration and washed 3× with cold THF, resulting in the deprotected dipeptidyl boronic acid (+)-pinanediol ester. The precipitate was dried under vacuum and redissolved in 10 mL of DMF. To the solution, an amount of 1.2 equiv of 2-pyrazinoic acid was added. The mixture was cooled on an ice bath to 0 °C, placed under an argon atmosphere. The mixture was allowed to stir for 10 min, after which 1.2 equiv of HATU followed by 2.2 equiv of DIPEA were added. The mixture was allowed to stir at 0 °C for an additional 15 min before allowing it to warm to room temperature, after which the mixture was allowed to stir for 3 h. The mixture was diluted with 200 mL of an aqueous solution of either 10% (w/v) citric acid or 0.1 M $KHSO_4$ and extracted three times with 100 mL of ethyl acetate. The organic layers were pooled, dried over $MgSO_4$, filtered, and solvent was removed under vacuum. The resulting pyrazinoylated dipeptidyl boronic acid (+)-pinanediol ester as a clear to pale yellow oil was purified by flash chromatography using a mixture of hexanes and ethyl acetate as the mobile phase.

Removal of the N-Terminal Cbz-Protecting Group. The N-terminally Cbz-protected dipeptidyl boronic acid (+)-pinanediol ester was dissolved in 10 mL of a 3:1 mixture of ethanol and triethylamine and placed in a hydrogenator flask. To the solution, 10% Pd/C was added and the reaction mixture placed under a hydrogen atmosphere at 60 psi and shaken overnight. The mixture was filtered and solvent removed under vacuum to yield the deprotected dipeptidyl boronic acid (+)-pinanediol ester.

Removal of the (+)-Pinanediol and P2 Amino Acid Side Chain Protecting Groups. This was accomplished using one of two methods. In the first method, 1.0 equiv of the (+)-pinanediol ester was dissolved in 10 mL of dichloromethane. The solution was then cooled on a dry ice/acetone bath to −78 °C under argon atmosphere. Once cooled, an amount of 2.0 equiv of 1 M BCl_3 in DCM was added dropwise over the course of 15 min, after which the mixture was stirred for 1 h at −78 °C. The mixture was then allowed to warm to room temperature, and solvent was removed by rotary evaporation. Next, an amount of 10 mL of anhydrous methanol was added to the flask, the mixture stirred for 15 min, and the methanol removed under vacuum. The addition of 10 mL of methanol, stirring for 15 min, and followed by removal under vacuum were repeated an additional five times. The resulting oil was dissolved in a minimal volume of water, frozen on dry ice, and lyophilized overnight to yield the crude dipeptidyl boronic acid. It is worth noting that any Boc and *t*-Bu protecting groups are also removed during this reaction.

In the second method Boc and *t*-Bu protecting groups present were removed first. This was accomplished by dissolving 1.0 equiv of the dipeptidyl boronic acid (+)-pinanediol ester dissolved in 10 mL of a solution of 4 M HCl in THF. The mixture was allowed to stir for 3 h at room temperature, after which the resulting white precipitate was collected by vacuum filtration and washed 3× with cold THF, resulting in the deprotected dipeptidyl boronic acid (+)-pinanediol ester. The deprotected dipeptidyl boronic acid (+)-pinanediol ester and 1.0 equiv of phenylboronic acid were dissolved in 100 mL of water adjusted to pH 3 with 2 N HCl. Then an amount of 100 mL of either hexane or diethyl ether was added and the reaction vigorously stirred for 30 min. The organic layer was removed and replaced with a fresh 100 mL. The mixture was stirred vigorously for 30 min. This procedure was repeated an additional three times. The organic layers were pooled, dried over $MgSO_4$, and reduced to quantitatively yield phenylboronic acid (+)-pinanediol ester for later recovery of (+)-pinanediol. The aqueous layer was reduced in volume and lyophilized to yield the crude deprotected dipeptidyl boronic acid.

The final dipeptidyl boronic acids and pyrazinoylated dipeptidyl boronic acids were purified using a Varian ProStar 210 HPLC, equipped with a C18 reverse-phase column (Supplico Analytical Discovery C18 25 cm × 21.2 mm, 5 μm particle size, catalog no. 569226) and UV/vis detector, using a linear gradient of increasing acetonitrile with a flow rate of 20 mL/min. Product yields were typically between 45% and 65% after purification. All compounds were demonstrated to have a purity of >95% using an Agilent (formerly HP) 1100 series LC/MSD equipped with an orthogonal electrospray mass spectometer (G2455A), autosampler/autoinjector (G1367A WPALS), thermostated column compartment (G1316A ColCom), sample chiller (G1330A ALSTherm), binary pump (G1312A BinPump), degasser (G1322A), and UV/vis detector (G1315B DAD). Samples were run over a C18 reverse-phase column (Agilent Eclipse Plus 2.1 mm × 50 mm, 3.5 μm particle size, catalog no. 959743-902) using linear gradients of increasing acetonitrile at a flow rate of 0.2 mL/min. The UV signals at 215 and 260 nm and the total ion chromatogram were monitored, and purity was evaluated by examining and integrating the resultant peaks.

NMR Characterization of Compounds. Chemical shift, coupling constants, and integration data are shown below. Spectra of the compounds with complete assignments can be found in the Supporting Information.

*((R)-3-Methyl-1-((S)-3-phenyl-2-(pyrazine-2-carboxamido)propanamido)butyl)boronic Acid (**1a**).* ^{1}H NMR (300 MHz, 80% D_2O + 20% CD_3CN) δ 9.17 (s, 1H), 8.88 (d, J = 2.4 Hz, 1H), 8.82 (s, 1H), 7.44 (dd, J = 10.6, 5.1 Hz, 5H), 5.02 (t, J = 7.6 Hz, 1H), 3.44−3.22 (m, 2H), 2.84 (dd, J = 8.9, 6.4 Hz, 1H), 2.16−2.07 (m, 1H), 1.39 (dd, J = 12.6, 5.8 Hz, 1H), 1.34−1.19 (m, 2H), 0.89 (dd, J = 6.1, 4.3 Hz, 6H).

*((R)-1-((2S,3R)-3-Hydroxy-2-(pyrazine-2-carboxamido)butanamido)-3-methylbutyl)boronic Acid (**1b**).* ^{1}H NMR (300 MHz, D_2O) δ 9.18 (s, 1H), 8.82 (d, J = 2.3 Hz, 1H), 8.77 (s, 1H), 4.46−4.32 (m, 1H), 2.80 (t, J = 7.7 Hz, 1H), 2.04 (s, 1H), 1.53 (td, J = 13.2, 6.5 Hz, 1H), 1.46−1.31 (m, 2H), 1.27 (d, J = 6.4 Hz, 3H), 0.85 (dd, J = 6.3, 3.1 Hz, 6H).

*((R)-1-((S)-3-hydroxy-2-(pyrazine-2-carboxamido)propanamido)-3-methylbutyl)boronic Acid (**1c**).* ^{1}H NMR (300 MHz, D_2O) δ 9.21 (s, 1H), 8.85 (d, J = 2.2 Hz, 1H), 8.79 (s, 1H), 4.84 (d, J = 5.1 Hz, 1H), 4.12−3.97 (m, 2H), 2.91−2.79 (m, 1H), 1.58 (dt, J = 13.3, 6.7 Hz, 1H), 1.49−1.21 (m, 2H), 0.89 (dd, J = 6.1, 4.5 Hz, 6H).

*((R)-1-((S)-3-Hydroxy-3-methyl-2-(pyrazine-2-carboxamido)butanamido)-3-methylbutyl)boronic Acid (**1d**).* ^{1}H NMR (300 MHz, D_2O) δ 9.19 (s, 1H), 8.83 (d, J = 2.3 Hz, 1H), 8.77 (s, 1H), 2.78 (dd, J = 9.0, 6.6 Hz, 1H), 1.55 (td, J = 13.2, 6.5 Hz, 1H), 1.38 (d, J = 8.1 Hz, 6H), 1.47−1.23 (m, 2H), 0.87 (dd, J = 6.4, 2.4 Hz, 6H).

*(R)-(3-Methyl-1-(2-(pyrazine-2-carboxamido)acetamido)butyl)-boronic Acid (**1e**).* ^{1}H NMR (300 MHz, D_2O) δ 9.18 (s, 1H), 8.81 (s, 1H), 8.75 (s, 1H), 4.29 (s, 2H), 2.82 (t, J = 7.2 Hz, 1H), 1.56 (dt, J = 18.7, 6.1 Hz, 1H), 1.48−1.18 (m, 2H), 1.02−0.62 (m, 6H).

*(R)-(1-(2-Aminoacetamido)-3-methylbutyl)boronic Acid (**2a**).* ^{1}H NMR (300 MHz, D_2O) δ 3.81 (s, 2H), 3.03 (dd, J = 8.7, 6.5 Hz, 1H), 1.70−1.51 (m, 1H), 1.52−1.43 (m, 1H), 1.43−1.26 (m, 1H), 0.88 (t, J = 6.6 Hz, 6H).

*((R)-1-((S)-2-Amino-3-(1H-indol-3-yl)propanamido)-3-methylbutyl)boronic Acid (**2b**).* ^{1}H NMR (300 MHz, D_2O) δ 7.59 (d, J = 7.7 Hz, 1H), 7.47 (d, J = 8.0 Hz, 1H), 7.23 (s, 1H), 7.20 (d, J = 8.1 Hz, 1H), 7.13 (t, J = 7.3 Hz, 1H), 4.26 (dd, J = 8.8, 6.6 Hz, 1H), 3.45−3.21 (m, 2H), 2.61 (dd, J = 9.6, 6.8 Hz, 1H), 0.93 (d, J = 7.3 Hz, 2H), 0.87 (s, 1H), 0.64 (dd, J = 15.7, 6.0 Hz, 6H).

*((R)-1-((S)-2-Amino-3-phenylpropanamido)-3-methylbutyl)boronic Acid (**2c**).* ^{1}H NMR (300 MHz, 90% H_2O + 10% D_2O) δ 8.63 (s, 1H), 7.50−7.32 (m, 3H), 7.32−7.19 (m, 2H), 4.21 (dd, J = 8.7, 6.7 Hz, 1H), 3.24 (dd, J = 13.6, 6.4 Hz, 1H), 3.13 (dd, J = 13.6, 9.0 Hz, 1H), 2.85−2.68 (m, 1H), 1.30−1.03 (m, 3H), 0.77 (dd, J = 8.3, 5.8 Hz, 6H).

*((R)-1-((S)-2-Amino-3-(4-hydroxyphenyl)propanamido)-3-methylbutyl)boronic Acid (**2d**).* ^{1}H NMR (300 MHz, D_2O) δ 7.07 (d, J = 8.3

Hz, 2H), 6.80 (d, J = 8.3 Hz, 2H), 4.09 (dd, J = 9.8, 6.1 Hz, 1H), 3.12 (dt, J = 17.2, 8.6 Hz, 1H), 2.95 (dd, J = 13.5, 10.0 Hz, 1H), 2.63 (dd, J = 10.3, 5.0 Hz, 1H), 1.17−0.83 (m, 3H), 0.68 (dd, J = 10.3, 6.1 Hz, 6H).

*((R)-1-((S)-2-Aminopropanamido)-3-methylbutyl)boronic Acid (**2e**).* ^{1}H NMR (300 MHz, 90% H_2O + 10% D_2O) δ 8.72 (s, 1H), 4.11 (q, J = 7.0 Hz, 1H), 3.04−2.84 (m, 1H), 1.69−1.42 (m, 2H), 1.53 (d, J = 7.1 Hz, 3H), 1.44−1.26 (m, 1H), 0.90 (t, J = 6.0 Hz, 6H).

*((R)-1-((S)-2,4-Diamino-4-oxobutanamido)-3-methylbutyl)boronic Acid (**2f**).* ^{1}H NMR (300 MHz, D_2O) δ 4.25 (t, J = 6.4 Hz, 1H), 3.03−2.74 (m, 3H), 1.58−1.35 (m, 2H), 1.26 (dt, J = 19.7, 7.1 Hz, 1H), 0.80 (t, J = 6.1 Hz, 6H).

*((R)-1-((S)-2-Amino-4-methylpentanamido)-3-methylbutyl)boronic Acid (**2g**).* ^{1}H NMR (300 MHz, DMSO) δ 4.08 (t, J = 7.3 Hz, 1H), 2.97 (dd, J = 9.3, 6.4 Hz, 1H), 1.92−1.72 (m, 2H), 1.64 (ddd, J = 19.1, 12.8, 5.8 Hz, 2H), 1.58−1.46 (m, 1H), 1.46−1.30 (m, 1H), 1.12−0.97 (m, 6H), 0.97−0.81 (m, 6H).

*((R)-1-((2S,3R)-2-Amino-3-hydroxybutanamido)-3-methylbutyl)boronic Acid (**2h**).* ^{1}H NMR (300 MHz, D_2O) δ 4.04 (p, J = 6.3 Hz, 1H), 3.75 (d, J = 6.8 Hz, 1H), 2.95−2.82 (m, 1H), 1.61−1.44 (m, 1H), 1.44−1.35 (m, 1H), 1.29 (dd, J = 13.9, 6.8 Hz, 1H), 1.21 (d, J = 6.4 Hz, 3H), 0.81 (t, J = 5.2 Hz, 6H).

*((R)-1-((S)-2-Amino-3-hydroxypropanamido)-3-methylbutyl)boronic Acid (**2i**).* ^{1}H NMR (300 MHz, D_2O) δ 4.12−4.01 (m, 1H), 3.88 (qd, J = 12.3, 5.2 Hz, 2H), 2.93 (dd, J = 9.0, 6.5 Hz, 1H), 1.62−1.36 (m, 2H), 1.36−1.21 (m, 1H), 0.82 (t, J = 5.9 Hz, 6H).

*((R)-1-((S)-2-Amino-3-(1H-imidazol-4-yl)propanamido)-3-methylbutyl)boronic Acid (**2j**).* ^{1}H NMR (300 MHz, D_2O) δ 8.70 (s, 1H), 7.42 (s, 1H), 4.23 (dd, J = 8.2, 6.6 Hz, 1H), 3.35 (dd, J = 7.2, 2.8 Hz, 2H), 2.97 (dd, J = 9.1, 5.5 Hz, 1H), 1.40−1.15 (m, 3H), 0.81 (t, J = 6.2 Hz, 6H).

*((R)-1-((S)-2-Amino-3-(naphthalen-1-yl)propanamido)-3-methylbutyl)boronic Acid (**2k**).* ^{1}H NMR (300 MHz, D_2O) δ 8.16−7.88 (m, 3H), 7.75−7.38 (m, 4H), 4.37 (dd, J = 10.4, 5.9 Hz, 1H), 3.77 (dd, J = 13.6, 5.8 Hz, 1H), 3.61 (dd, J = 14.2, 10.7 Hz, 1H), 2.59 (dd, J = 9.6, 5.3 Hz, 1H), 0.99−0.84 (m, 1H), 0.79 (dd, J = 17.0, 7.1 Hz, 2H), 0.64 (t, J = 7.1 Hz, 5H).

*((R)-1-((S)-2-Amino-3-(naphthalen-2-yl)propanamido)-3-methylbutyl)boronic Acid (**2l**).* ^{1}H NMR (300 MHz, D_2O) δ 7.94 (t, J = 9.6 Hz, 3H), 7.79 (s, 1H), 7.69−7.48 (m, 2H), 7.42 (d, J = 8.4 Hz, 1H), 4.34 (dd, J = 9.6, 6.0 Hz, 1H), 3.46 (dd, J = 13.4, 5.7 Hz, 1H), 3.26 (dd, J = 22.9, 9.7 Hz, 1H), 2.68 (t, J = 7.7 Hz, 1H), 0.97 (dd, J = 19.0, 11.6 Hz, 2H), 0.79 (tdd, J = 20.0, 13.0, 6.6 Hz, 2H), 0.58 (d, J = 6.3 Hz, 3H), 0.44 (d, J = 6.3 Hz, 3H), 0.34 (dd, J = 21.0, 14.4 Hz, 1H).

*((R)-1-((S)-2-Amino-3-(3,5-difluorophenyl)propanamido)-3-methylbutyl)boronic Acid (**2m**).* ^{1}H NMR (300 MHz, D_2O) δ 6.93 (dd, J = 15.4, 8.6 Hz, 3H), 4.21 (dd, J = 9.1, 6.4 Hz, 1H), 3.27 (dd, J = 13.6, 6.2 Hz, 1H), 3.13 (dd, J = 13.5, 9.5 Hz, 1H), 2.91−2.78 (m, 1H), 1.35−1.07 (m, 3H), 0.80 (t, J = 6.2 Hz, 6H).

*((R)-1-((S)-3-([1,1′-Biphenyl]-4-yl)-2-aminopropanamido)-3-methylbutyl)boronic Acid (**2n**).* ^{1}H NMR (300 MHz, D_2O) δ 7.73 (d, J = 5.6 Hz, 4H), 7.54 (t, J = 7.4 Hz, 2H), 7.42 (dd, J = 19.7, 7.6 Hz, 3H), 4.27 (dd, J = 9.8, 5.8 Hz, 1H), 3.35 (dd, J = 13.3, 5.8 Hz, 1H), 3.16 (dd, J = 13.1, 10.4 Hz, 1H), 2.73 (dd, J = 10.0, 5.3 Hz, 1H), 1.27−0.84 (m, 3H), 0.67 (dd, J = 13.7, 6.3 Hz, 6H).

*((R)-1-((S)-2-Amino-3-(4-fluorophenyl)propanamido)-3-methylbutyl)boronic Acid (**2o**).* ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.62 (s, 1H), 7.26−7.13 (m, 2H), 7.06 (t, J = 8.8 Hz, 2H), 4.14 (dd, J = 9.0, 6.4 Hz, 1H), 3.17 (dd, J = 13.7, 6.2 Hz, 1H), 3.05 (dd, J = 13.6, 9.5 Hz, 1H), 2.80−2.66 (m, 1H), 1.25−0.94 (m, 3H), 0.80−0.62 (m, 6H).

*((R)-1-((S)-2-Aminohexanamido)-3-methylbutyl)boronic Acid (**2p**).* ^{1}H NMR (300 MHz, D_2O) δ 3.92 (t, J = 6.7 Hz, 1H), 2.82 (dd, J = 9.4, 6.2 Hz, 1H), 1.78 (dd, J = 14.0, 7.3 Hz, 2H), 1.49 (ddd, J = 16.1, 11.4, 4.6 Hz, 1H), 1.38 (td, J = 9.3, 5.3 Hz, 1H), 1.32−1.15 (m, 5H), 0.79 (dd, J = 10.6, 5.4 Hz, 9H).

*((R)-1-((S)-2-Amino-3-(2-fluorophenyl)propanamido)-3-methylbutyl)boronic Acid (**2q**).* ^{1}H NMR (300 MHz, D_2O) δ 7.40 (dd, J = 13.3, 6.1 Hz, 1H), 7.34−7.08 (m, 3H), 4.24 (dd, J = 9.0, 6.3 Hz, 1H), 3.25 (qd, J = 13.7, 7.9 Hz, 2H), 2.88−2.73 (m, 1H), 1.34−1.02 (m, 3H), 0.78 (dd, J = 8.5, 5.9 Hz, 6H).

*((R)-1-((S)-2-Amino-3-(3,4-difluorophenyl)propanamido)-3-methylbutyl)boronic Acid (**2r**).* ^{1}H NMR (300 MHz, D_2O) δ 7.17 (ddd, J = 27.2, 18.2, 8.8 Hz, 2H), 7.00 (s, 1H), 4.13 (dd, J = 9.2, 6.3 Hz, 1H), 3.18 (dd, J = 13.7, 6.1 Hz, 1H), 3.04 (dd, J = 13.6, 9.5 Hz, 1H), 2.77 (dd, J = 9.4, 5.4 Hz, 1H), 1.30−0.96 (m, 3H), 0.72 (t, J = 6.3 Hz, 6H).

*((R)-1-((S)-2-Amino-3-(3-fluorophenyl)propanamido)-3-methylbutyl)-boronic Acid (**2s**).* ^{1}H NMR (300 MHz, D_2O) δ 7.33 (dd, J = 14.4, 7.6 Hz, 15H), 7.10−6.90 (m, 45H), 4.17 (dd, J = 9.0, 6.4 Hz, 7H), 3.20 (dd, J = 13.6, 6.3 Hz, 14H), 3.07 (dd, J = 13.5, 9.4 Hz, 15H), 2.83−2.66 (m, 14H), 1.30−0.95 (m, 48H), 0.71 (t, J = 6.4 Hz, 97H).

*((R)-1-((S)-2-Amino-3-hydroxy-3-methylbutanamido)-3-methylbutyl)-boronic Acid (**2t**).* ^{1}H NMR (300 MHz, D_2O) δ 8.90 (s, 1H), 3.87 (s, 1H), 2.94 (dd, J = 8.9, 6.7 Hz, 1H), 2.04 (s, 1H), 1.59 (ddd, J = 17.0, 13.0, 6.4 Hz, 1H), 1.51−1.18 (m, 2H), 1.39 (s, 3H), 1.29 (s, 3H), 0.88 (dd, J = 6.3, 3.5 Hz, 6H).

*((R)-1-((S)-2-Amino-3,3-dimethylbutanamido)-3-methylbutyl)boronic Acid (**2u**).* ^{1}H NMR (300 MHz, D_2O) δ 3.75 (s, 1H), 2.86 (dd, J = 9.0, 6.7 Hz, 1H), 1.57 (dq, J = 12.9, 6.5 Hz, 1H), 1.50−1.25 (m, 2H), 1.07 (s, 9H), 0.88 (dd, J = 6.5, 2.3 Hz, 6H).

*((R)-1-((S)-2-Aminobutanamido)-3-methylbutyl)boronic Acid (**2v**).* ^{1}H NMR ^{1}H NMR (300 MHz, D_2O) δ 3.90 (t, J = 6.6 Hz, 1H), 2.86 (dd, J = 9.2, 6.5 Hz, 1H), 1.85 (p, J = 7.4 Hz, 2H), 1.61−1.45 (m, 1H), 1.45−1.35 (m, 1H), 1.35−1.20 (m, 1H), 0.91 (t, J = 7.5 Hz, 3H), 0.83 (dd, J = 6.2, 4.9 Hz, 6H).

*((R)-1-((S)-2-Amino-3,3-diphenylpropanamido)-3-methylbutyl)-boronic Acid (**2w**).* ^{1}H NMR (300 MHz, D_2O) δ 7.59 (d, J = 7.3 Hz, 2H), 7.54−7.26 (m, 8H), 4.43 (d, J = 11.8 Hz, 1H), 2.69 (dd, J = 9.6, 5.6 Hz, 1H), 0.94 (ddd, J = 13.4, 9.3, 5.3 Hz, 2H), 0.86−0.75 (m, 1H), 0.68 (dd, J = 9.0, 6.2 Hz, 6H).

*((R)-1-((S)-2-Aminopentanamido)-3-methylbutyl)boronic Acid (**2x**).* ^{1}H NMR (300 MHz, D_2O) δ 3.95 (t, J = 6.8 Hz, 1H), 2.84 (dd, J = 9.3, 6.4 Hz, 1H), 1.78 (dt, J = 9.8, 5.6 Hz, 2H), 1.61−1.43 (m, 1H), 1.43−1.34 (m, 1H), 1.35−1.20 (m, 3H), 0.87 (t, J = 7.2 Hz, 3H), 0.83 (dd, J = 6.1, 5.0 Hz, 6H).

*((R)-1-((S)-2-Amino-3-(perfluorophenyl)propanamido)-3-methylbutyl)boronic Acid (**2y**).* ^{1}H NMR (300 MHz, D_2O) δ 4.19 (t, J = 7.5 Hz, 1H), 3.32 (d, J = 7.5 Hz, 2H), 2.99 (t, J = 7.0 Hz, 1H), 1.31 (dd, J = 12.5, 8.8 Hz, 4H), 0.83 (t, J = 6.0 Hz, 7H).

*((R)-1-((S)-2-Amino-2-cyclohexylacetamido)-3-methylbutyl)boronic Acid (**2z**).* ^{1}H NMR (300 MHz, H_2O + D_2O) δ 3.81 (d, J = 6.4 Hz, 1H), 2.89 (dd, J = 9.5, 6.3 Hz, 1H), 1.95−1.84 (m, 1H), 1.86−1.61 (m, 6H), 1.62−1.52 (m, 1H), 1.52−1.42 (m, 1H), 1.42−1.31 (m, 1H), 1.31−1.16 (m, 2H), 1.15−1.00 (m, 2H), 0.90 (dd, J = 6.5, 3.9 Hz, 6H).

*2-((S)-2-Amino-3-(((R)-1-borono-3-methylbutyl)amino)-3-oxopropyl)malonic Acid (**2aa**).* ^{1}H NMR (300 MHz, D_2O) δ 4.09 (t, J = 6.8 Hz, 1H), 3.00 (dd, J = 8.8, 6.4 Hz, 1H), 2.42 (d, J = 6.9 Hz, 2H), 1.52 (ddd, J = 15.1, 11.4, 6.1 Hz, 2H), 1.37 (dt, J = 14.2, 7.1 Hz, 1H), 0.88 (t, J = 6.0 Hz, 6H).

*(R)-(1-(2-(Dimethylamino)acetamido)-3-methylbutyl)boronic Acid (**2ab**).* ^{1}H NMR (300 MHz, D_2O) δ 3.92 (s, 2H), 3.01 (dd, J = 9.2, 6.2 Hz, 1H), 2.86 (s, 6H), 1.65−1.38 (m, 2H), 1.32 (dd, J = 13.6, 5.9 Hz, 1H), 0.82 (t, J = 6.8 Hz, 6H).

*(R)-(3-Methyl-1-(2-(methylamino)acetamido)butyl)boronic Acid (**2ac**).* ^{1}H NMR (300 MHz, D_2O) δ 3.80 (s, 2H), 2.95 (dd, J = 9.2, 6.1 Hz, 1H), 2.66 (s, 3H), 1.60−1.34 (m, 2H), 1.34−1.20 (m, 1H), 0.79 (t, J = 6.8 Hz, 6H).

Preparation of Inhibitors for Assay. Inhibitor was dissolved in 100% ethanol to a concentration of 0.1 M. The ethanol stock solution was then diluted 10- to 100-fold into either pH 2.0 buffer (0.148 M HCl, 0.052 M KCl) or pH 7.6 buffer (25 mM HEPES, 0.5 mM EDTA at pH 7.6) and allowed to stir and equilibrate overnight at room temperature. Further

dilutions were carried out just prior to assay in preassay incubation buffers at a concentration 10-fold higher than the final inhibitor concentration.

In Vitro Proteasome Inhibition Assays. Assays of the chymotrypsin-like proteolytic activity of the 20S proteasome using succinyl-leucyl-leucyl-valinyl-tyrosyl-7-amino-4-methylcoumarin (Suc-LLVY-AMC, Bachem) as the substrate have been previously described.[81] As boronic acids are slow, tight-binding inhibitors, enzyme solution was incubated with inhibitor for 10 min prior to substrate addition. The fluorescence for each well was read ($\lambda_{excitation}$ = 360 nm and $\lambda_{emission}$ = 460 nm) after an additional 30 min. Each condition and concentration of inhibitor was prepared in quadruplicate. Data were collected using Perkin-Elmer Wallac Vector3V 1420 multilabel counter 96- and 384-well plate reader equipped with a 355 nm excitation filter and a 460 nm emission filter and running Wallac 1420 Workstation Manager, version 3.0.0, revsion 4, component version 3.00.0.53 software. The data were then analyzed using Graph Prism 5.0.3, fit to a variable-slope sigmoidal equation, and reported as % fluorescence (normalized relative fluorescence).

Wells in which reaction buffer was added in place of enzyme solution and wells lacking SDS for enzymatic activation were used as negative controls. As a positive control, varying concentrations of bortezomib (**1a**) were used alongside each compound of interest. To ensure that the pH 2.0 samples were not affecting the assay pH, the pH was measured for a subset of wells for each compound and each assay in both pH 2.0 and pH 7.6 assay wells.

Cell Lines. The Living Colors HEK 293 ZsGreen proteasome sensor cell line (hereafter referred to as HEK 293 PS) was obtained from Clontech Laboratories, Inc. (catalog no. 6351535). These are HEK 293 cells that have been stably transfected with a vector that expresses a fusion protein between the ZsGreen fluorescent protein and the proteasome-targeting sequence of murine ornithine decarboxylase.[62,63] Cells were maintained in phenol red free DMEM (Gibco catalog no. 31053) with 10% fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine (Gibco catalog no. 25030), 4.5 g/L D-glucose, 1 mM sodium pyruvate (Gibco catalog no. 11360), 100 mg−100 U/L penicillin−streptomycin (Gibco catalog no.15140), and 0.2 mg/L G418 at 37 °C and 5% CO_2. The follicular small-cell lymphocytic leukemia (FSCLL) cell line was obtained from Dr. Jonathan Cheng, M.D., of the Fox Chase Cancer Center, Philadelphia, PA. Cells were maintained suspended in phenol red free RPMI-1640 (Gibco catalog no. 11835) medium with 10% fetal bovine serum, 4.5 g/L D-glucose, 100 mg−100 U/L penicillin−streptomycin, 10 mM HEPES, and 2.0 g/L sodium bicarbonate.

In-Cell Proteasome Inhibition Assays. Cells were plated at a density of 3.2 × 10^4 cells per well in 96-well flat-bottom sterile tissue-culture treated polystyrene plates (Costar catalog no. 3603) and incubated for 24 h at 37 °C and 5% CO_2. After 24 h, inhibitor dilutions were added to the plate with gentle mixing, yielding a total well volume of 100 μL. The cells were incubated at 37 °C and 5% CO_2 for 24 h. After 24 h with inhibitor, cells were viewed via fluorescent microscope. Cell medium was removed, and cells were lysed by adding 100 μL of cell lysis buffer (0.025 M HEPES, 0.5 mM EDTA, and 5% (v/v) TritonX-100) and gently shaking for 1 h. Plates were read on a 96-well fluorescence plate reader (λ_{ex} = 485 nm, λ_{em} = 535 nm). Each condition and concentration of inhibitor was prepared in quadruplicate. Data were collected on a Perkin-Elmer Wallac Vector3V 1420 multilabel counter 96- and 384-well plate reader equipped with a 485 nm excitation filter and a 535 nm emission filter and running Wallac 1420 Workstation Manager, version 3.0.0, revision 4, component version 3.00.0.53 software. The results were then analyzed using Graph Prism 5.0.3, fit to a variable-slope sigmoidal equation and reported as % fluorescence (normalized relative fluorescence). Wells containing cells and medium only and cells with medium and either pH 2 or pH 7.6 buffer only were included as negative controls. As a positive control, a plate with dilutions of bortezomib was included with each assay performed.

MTT Assays. MTT assays for cytotoxicity have been described.[64−66] Briefly, cells were plated at a density of 3.2 × 10^4 cells per well in 96-well black, flat clear-bottom sterile tissue-culture treated polystyrene plates (Costar catalog no. 3603) and incubated for 24 h at 37 °C and 5% CO_2. After 24 h, inhibitor dilutions were added to the plate, with gentle mixing, yielding a total well volume of 100 μL. The cells were incubated at 37 °C and 5% CO_2 for 24 h. After 24 h, 10 μL of MTT dye solution (5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in 1× PBS) was added to each well. Cells were incubated with dye for 3 h at 37 °C and 5% CO_2, after which an amount of 100 μL of MTT solubilization solution (10% (v/v) TritonX-100, 0.1 M HCl in isopropanol) was added to each well. The plates were sealed in parafilm and gently shaken overnight to dissolve the precipitated MTT metabolite. Absorbance values for each well were obtained by reading the plates on a 96-well plate reader at 570 nm. Each condition and concentration of inhibitor was prepared in quadruplicate. Data were collected using either a Perkin-Elmer Wallac Vector3V 1420 multilabel counter 96- and 384-well plate reader equipped with 570 and 670 nm absorbance filters and running Wallac 1420 Workstation Manager, version 3.0.0, revision 4, component version 3.00.0.53 software or a Molecular Devices Spectramax 340PC monochromator-based absorbance plate reader equipped with a peltier unit running SoftMax Pro, version 4.3 LS. The results were then analyzed using Graph Prism 5.0.3, fit to a variable-slope sigmoidal equation, and reported as % absorbance (normalized absorbance). Wells containing cells and medium only and cells with medium and either pH 2 or pH 7.6 buffer only were included as negative controls. As a positive control, a plate with dilutions of bortezomib was included with each assay performed.

Inhibitor Degradation Assays. *Intact Cells.* Cells were plated at 2 × 10^6 cells in 900 μL of medium in a sterile 24-well tissue-culture treated polystyrene plate (Costar no.3527) and incubated for 24 h at 37 °C + 5% CO_2. Aliquots of 45 μL were removed at each time point, and quenched with 5 μL of stop solution (100 μM Trp-Tyr, used as an internal standard for LCMS, in 1 M HCl).

Cell Lysate and Conditioned Medium. Cells were grown under the conditions described above. Medium was collected after 48 h, and cells were collected, washed with 1×x PBS, resuspended in 1× PBS + 25% glycerol, pH 7.2, at 10^7 cells/mL, and lysed using sonication. Inhibitor stocks were prepared in 1× PBS + 25% glycerol, pH 7.2, at 10^{-3} M and dilutions made in the same buffer. Cell lysate was warmed to 37 °C, and for each concentration of inhibitor, an amount of 20 μL was added to 180 μL aliquots of the warmed cell lysate, and the components were mixed. At each time point, an amount of 5 μL of the reaction was removed and added to a mixture of 45 μL of 50 mM sodium phosphate and 5 μL of stop solution (100 μM Trp-Tyr in 1 M HCl). Positive control samples included leucinyl-*p*-nitroanilide incubated with cell lysate or conditioned medium, and negative controls included leucinyl-*p*-nitroanilide or inhibitor incubated in 1× PBS + 25% glycerol.

LCMS Analysis. Samples were then run on a ThermoQuest Finnigan LCQ-Duo electrospray ionization LCMS equipped with a C18 reversed phase HPLC column (Agilent Zorbax Eclipse Plus C18 narrow bore RR 2.1 cm × 50 mm, 3.5 μm particle size, part no. 959743-902), autosampler, and UV/vis detector. We used the preprogrammed convex 10 gradient curve of acetonitrile from 2% to 98% over 7 min at a flow of 0.2 mL/min for the first 6.25 min. The mass spectrometer was set to run in full scan MS positive ion mode with a mass range of 50.00−380.00 amu.

■ ASSOCIATED CONTENT

Supporting Information. NMR structural assignments, 2D NOESY NMR spectra, in-cell stability data, and cyclization reversibility data. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

Chg, cyclohexyl(glycine); Gla, γ-carboxyglutamic acid; K_i, inhibition constant; IC_{50}, median inhibition concentration; CI, cyclization index; EC_{50}, median effective concentration

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