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Incorporation of Non-natural Amino Acids Improves Cell Permeability and Potency of Specific Inhibitors of Proteasome Trypsin-like Sites

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Abstract

Proteasomes degrade the majority of proteins in mammalian cells by a concerted action of three distinct pairs of active sites. The chymotrypsin-like sites are targets of antimyeloma agents bortezomib and carfilzomib. Inhibitors of the trypsin-like site sensitize multiple myeloma cells to these agents. Here we describe systematic effort to develop inhibitors with improved potency and cell permeability, yielding azido-Phe-Leu-Leu-4-aminomethyl-Phe-methyl vinyl sulfone (**4a**, LU-102), and a fluorescent activity-based probe for this site. X-ray structures of **4a** and related inhibitors complexed with yeast proteasomes revealed the structural basis for specificity. Nontoxic to myeloma cells when used as a single agent, **4a** sensitized them to bortezomib and carfilzomib. This sensitizing effect was much stronger than the synergistic effects of histone acetylase inhibitors or additive effects of doxorubicin and dexamethasone, raising the possibility that

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ASSOCIATED CONTENT

Supporting Information

X-ray data collection details and refinement statistics, dose–response curves for inhibition of purified 26S proteasome, yeast 20S proteasome, and proteasomes in RPMI-8226 cells, proteasome and cathepsins inhibition by **10**, effect of a combination of carfilzomib HDAC inhibitor panobinostat on RPMI-8226 cells, complete synthetic details and characterization of all compounds and synthetic intermediates, NMR spectra of compounds **4b–20**, and LC-MS traces of **4a**, **8**, and **12**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes

4INR (y20S-**4a**), 4INT (y20S-**12**), 4INU (y20S-**8**).

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combinations of inhibitors of the trypsin-like site with bortezomib or carfilzomib would have stronger antineoplastic activity than combinations currently used clinically.

INTRODUCTION

Proteasomes are proteolytic machines responsible for the turnover of the majority of proteins in mammalian cells. The proteasome inhibitors bortezomib and carfilzomib (PR-171)¹ are used for treatment of multiple myeloma (MM). Four second-generation proteasome inhibitors, marizomib (salinosporamide A, NPI-0052),² delanzomib (CEP-18770),³ ixazomib (MLN-9708),⁴ and oprozomib (ONX-0912, PR-047),⁵ are in clinical testing.

Proteasomes have three different types of active sites, namely the chymotrypsin-like (5), trypsin-like (2), and caspase-like (1). Cells of the immune system express γ -interferon-inducible immunoproteasomes, which have slightly different catalytic subunits, namely the 5i (LMP7), 2i (MECL1), and 1i (LMP2). Of these, the chymotrypsin-like sites (5 and 5i) have long been considered the only suitable targets for drug development. Bortezomib, carfilzomib, and all drugs presently undergoing trials were developed to target these sites.⁶ However, bortezomib, delanzomib, and ixazomib cotarget the caspase-like sites (1 and 1i),^{3,4,7} while marizomib cotargets the trypsin-like and caspase-like sites.² We have demonstrated that in most MM cell lines, cytotoxicity of inhibitors does not correlate with inhibition of the chymotrypsin-like sites but does correlate with the loss of specificity and onset of inhibition of either the caspase-like or the trypsin-like sites.⁸

Recently we have developed selective cell-permeable inhibitors of the trypsin-like site and demonstrated that they selectively sensitize MM cells to bortezomib and carfilzomib.⁹ Although these peptide epoxyketones are useful research tools, our attempts to demonstrate sensitization of solid tumor cells to bortezomib and carfilzomib were limited by variable cell permeabilities and low yields of the synthetic procedure. Thus, better inhibitors are needed.

In this study, we describe the development of more potent inhibitors of trypsin-like sites that contain non-natural amino acids, are easier to synthesize, have better cell permeability, and are as potent in sensitizing myeloma cells to carfilzomib and bortezomib as first-generation compounds. We also report on the X-ray structures of these inhibitors complexed with yeast proteasomes.

RESULTS

Design, Synthesis, and Initial Characterization of Inhibitors

Four compounds described in our previous work,⁹ NC-002 (**1a**), NC-012 (**2**), NC-022 (**3**), and az-NC-002 (**1b**), are N-terminally capped epoxyketones with an arginine in the P1 position (Figure 1A). The guanidino group of the arginine side chain may perform a nucleophilic attack on the epoxyketone electrophile, leading to cyclization and inactivation of the inhibitor. To improve the chemical stability of these inhibitors, we aimed to replace the guanidine by other functional groups, such as para-substituted phenylalanine derivatives

because these derivatives would not cyclize. These substitutions would also allow us to investigate the influence of the basicity and length of the side chain on the activity of the inhibitor. In the set of compounds described in this study, we used benzylamino ($pK_a = 9.3$), pyridyl ($pK_a = 5.2$), and aniline ($pK_a = 4.6$) groups as arginine mimics for the P1 side chain (Figure 1B). These substitutions were introduced on a modified **1a** (Ac-Leu-Leu) scaffold, in which the N-terminal acetyl group was replaced with an azido-phenyl group. The rationale for this replacement was the anticipation that the bulky phenyl group would bind in the P4 pocket and thus increase the inhibitors' potency. An azido group was added to provide opportunity for additional modification. Previously, we found that replacing the epoxyketone electrophile with a vinyl sulfone increases the specificity of inhibitors for the chymotrypsin-like sites.¹⁰ Therefore, we decided to test whether the same is true for the trypsin-like sites.

The initial evaluation of compounds was performed on extracts from HEK293 cells (Figure 2A) and purified 26S proteasomes from rabbit muscles (Table 1). In extracts, inhibition of the three active sites was assessed with activity-based probes. Inhibition of the active sites of the purified proteasome was measured with site-specific fluorogenic substrates (Table 2). Two trends emerged from these experiments. First, vinyl sulfones were clearly more potent and more trypsin-like site-selective inhibitors than epoxyketones with the same peptide sequence (e.g., compare compounds **4a** and **4b**, **5a** and **5b**). Second, potency and specificity for the trypsin-like sites decreased from benzylamine to aniline to pyridyl side chain. The vinyl sulfone with benzylamine side chain in the P1 position (compound **4a**) was the most potent and specific inhibitor of trypsin-like sites. Compound **5a** (anilino-vs) was an equipotent inhibitor of trypsin-like and caspase-like sites, and anilino-ek (**5b**) and pyridino-vs (**6**) residues in the P1 position generated inhibitors of the chymotrypsin-like sites. Notably, compound **4a** was more potent than any of the first-generation compounds (Figure 1A) or a vinyl sulfone with a natural lysine side chain in the P1 (compound **7**). In keeping with the tradition of naming compounds so that the last digits indicate the active site inhibited, we designated the most potent compound **4a** "LU-102," where LU stands for Leiden University.

Because vinyl sulfones were more potent and specific than epoxyketones, all other inhibitors were synthesized using a vinyl sulfone as the warhead. Previously, we have found that compound **3** is a more potent inhibitor of trypsin-like sites than **1a**. Therefore we next synthesized compound **8**, with the compound **3** scaffold, benzylamine as P1 substituent, and vinyl sulfone as the warhead (Figure 1C). Although compound **8** (LU-122) was more potent than **3**, it was less potent than **4a** (Figure 2, Table 2). Replacing benzylamino with an aniline side chain in the P1 position (compound **9**) generated an inhibitor of the chymotrypsin-like site.

Earlier work by Groll and Bogoy revealed that basic residues in the P1 and P3 positions are important elements of specific inhibitors of the trypsin-like sites.¹¹ Indeed, we found previously that the most potent inhibitor (compound **2**) has basic (Arg) residues in the P1 and P3 positions.⁹ Therefore, we replaced the aliphatic side chain in the P3 position with pyridyl (**10**), aniline (**11**), and benzylamine (**12**) side chains (Figure 1D). While the first two compounds had potency and specificity comparable to that of **4a** (Figure 2B, Table 3) compound **12** (LU-112) was more specific and potent than **4a** (Figure 2B). Replacing the P1 benzylamino side chain in **12** with an aniline side chain led to a loss of specificity (compound **13**). Replacing it with a pyridyl side chain led to further loss of selectivity and to loss of potency (compound **14**). However, these compounds still retained specificity for the trypsin-like site and were more potent than compounds **5a** and **6**, which had leucine in the P3 position and the same P1 residues. Thus, we confirm the important role of basic residues in the P3 position for the selectivity of inhibitors of trypsin-like sites.

To complete the structure–activity relationship studies, we investigated the effects of replacing basic P1 residues with leucine in the P1 position using para-substituted phenylalanines in the P3 position as scaffold. This led to an equipotent inhibitor of chymotrypsin-like and trypsin-like sites (compound **15**) when the P3 position was occupied by a benzylamine side chain (Figure 2B, Table 3). Peptide vinyl sulfones with aniline and pyridyl side chains in the P3 and Leu in P1 (compounds **16** and **17**) acted as inhibitors of chymotrypsin-like sites. Finally, we have generated a compound with unsubstituted phenylalanines in both P1 and P3 positions (compound **18**). It specifically inhibits the chymotrypsin-like sites. Thus, at least one strongly basic side chain in either P3 or P1 position is needed to target these inhibitors on the trypsin-like sites.

When comparing results obtained using activity assays in extracts of human cells (Figure 2) and in purified rabbit 26S proteasomes (Tables 1–3), we noticed that inhibition of purified 26S was stronger than inhibition of proteasome in extracts. We also noticed that **12** behaved as the most potent inhibitor in extracts but was a less potent inhibitor of purified proteasome than **4a** and **13**. Furthermore, certain inhibitors of chymotrypsin-like sites (e.g., compounds **6**, **16**, and **17**) inhibited these sites less potently and selectively in extracts than in purified proteasomes. The reasons for these differences are unknown. We can only speculate that a mixed population of proteasomes (e.g., presence of latent 20S¹² or of 20S particles activated by PA200 or PA28), post-translational modifications, or association with proteasome-interacting proteins in extracts could be responsible for these differences. Thus, conclusions about inhibitor specificity that are based on experiments with purified proteasomes should not be automatically extended to proteasomes in biological samples.

Finally, we used click chemistry to attach a BODIPY-FL dye to **3a** and **12** molecules and thus generate an activity-based probe for the 2 site (Figure 1E). However, this modification reduced the specificity of **12** (compound **20** (BODIPY-LU-112), Figure 3B) and led to complete loss of specificity of **4a** (compound **19** (BODIPY-LU-102), Figure 3A), as compound **19** reacted with 2 and 5 subunits (Figure 3) and equipotently inhibited trypsin- and chymotrypsin-like activities (not shown).

X-ray Structure of Inhibitors Complexed with Yeast 20S Proteasomes

To characterize the binding modes of inhibitors in the 20S proteasome, a structurally diverse set of the most potent inhibitors, **4a**, **8**, and **12**, were soaked in 20S yeast proteasome crystals. Data sets were collected for these three compounds and processed to 2.9, 2.7, and 3.1 Å with an R_{free} = 0.230, 0.231, and 0.238 for complexes with **4a**, **8**, and **12**, respectively (Table S1, Supporting Information). The refined crystal structures revealed clear electron densities of all three compounds in the trypsin- and chymotrypsin-like active sites (Figure 4). As expected, the vinyl sulfone formed a covalent bond with the Thr1 through a 1,4-Michael addition reaction, and the peptide backbone was shown to adopt an antiparallel β -sheet conformation typical for peptide-based inhibitors.

The benzylamine group in the P1 position of all three compounds protrudes into the spacious and hydrophilic S1 pocket of the trypsin-like site and is further stabilized through hydrogen bonding to the amine group of the Glu53 residue in 2 (Figure 4A). The P2 groups are solvent-exposed, whereas the P3 groups have a major effect on the binding profile. In the case of compound **12**, the benzylamino group forms a tight hydrogen bond with Asp120 and accommodates the aromatic group perfectly in the S3 pocket through a series of van der Waals interactions, thereby profoundly stabilizing this moiety in the trypsin-like site. For compounds **4a** and **8**, the P3 moiety also protrudes into the S3 side pocket, but there are no favorable interactions with the protein.

The aromatic capping group of **8** and the azide and phenyl groups of **4a** and **12** protrude nicely into the S4 pocket. These residues are stabilized by van der Waals interactions with Leu115 and Ile116. The azido group of **4a** and **8** was not clearly defined in the electron density, though weak hydrogen bonds with Gln22 in **2** and Asp114 in **3** most likely further stabilize this moiety.

To fully understand the molecular basis of specificity, interaction of inhibitors in the chymotrypsin-like site must be analyzed. The benzylamino group in P1 protrudes into the hydrophobic S1 pocket and is hydrogen bonded to Glu122 of the **6** subunit (Figure 4B). Similarly to the trypsin-like site, the P2 side chains of the ligands are solvent exposed. The S3 pocket in the chymotrypsin-like site is so large that the Val, Leu, and benzylamino groups in **4a**, **8**, and **12**, respectively, do not form significant interactions. The N₃ group in **4a** and **12** is well-defined in the electron density and forms strong hydrogen bonds with **6** Asp116, thus contributing to the stabilization of both inhibitors. The more flexible benzyl group of **4a** and **12** protrudes into the S4 pocket and fits better than the P4 group of **8**.

To better correlate these structures of inhibitor complexes with yeast proteasomes with our findings about the specificity of these compounds in mammalian proteasomes, a superposition of both bovine and mouse constitutive proteasome^{13,14} with all three yeast structures was performed (Figure 4C,D). Structures of bovine and mouse proteasomes were used because they are the only two available structures of mammalian constitutive proteasomes. Human proteasome subunits **2**, **3**, and **5**, **6**, which form the trypsin- and chymotrypsin-like sites, are 94–100% identical to their bovine and mouse counterparts. In yeast subunits, only 45–67% of residues are identical. The superposition revealed a similar conformation of the side chains but with some notable differences.

Most of these differences were found in the chymotrypsin-like site. The most notable is the replacement Glu122 in the **5** subunit of yeast proteasome (Figure 4D), which forms a hydrogen bond with the P1-benzylamine group, with Gln122 in bovine/mouse/human constitutive proteasome, which cannot form a tight bond to the amine group of the benzylamine. Furthermore, the azido group and phenyl ring fit adequately into the S4 pocket formed by His98 and Tyr98 of **6** in yeast, but in constitutive mouse, bovine, and human proteasomes His98 is replaced by a larger Tyr98 that repels the benzyl group and does not allow its correct positioning (Figure 4D). This leads to the prediction that all three inhibitors will be stronger inhibitors of chymotrypsin-like sites in the yeast proteasomes, resulting in lower selectivity for the trypsin-like sites. In fact, when we measured the inhibition of yeast proteasomes by these inhibitors (Table 4) we found that the ratio of IC₅₀(Chym-L sites)/IC₅₀(Try-L sites) was 400-fold higher for **4a** and 8-fold higher for **8** in mammalian 26S proteasomes than for yeast 20S particles.

12 was a more selective inhibitor of trypsin-like sites in yeast proteasomes than in their mammalian counterparts (Table 4). This higher selectivity is probably a consequence of a completely different binding pattern of the P3 side chain in the trypsin-like site. In particular, the hydrogen bonding that precisely stabilizes the P3 side chain in the yeast-**12** complex cannot be formed in mammalian proteasomes because Ala120 in yeast is replaced by Met120 (Figure 4C). However, the amine of the benzylamine group is stabilized by other residues, including Ser112, and Tyr136 of subunit **3**, which are more hydrophobic in yeast (Gly112 and Phe136, respectively). As a result, **12** is a >4000-fold more potent inhibitor of yeast trypsin-like sites than is **4a** (Table 4), while in mammalian proteasomes the potencies of **12** and **4a** are comparable (Table 3 and Figure 2B).

Effects of Compounds on Cells

We have tested the five most potent compounds for their ability to inhibit proteasomes in a variety of cell lines. The disadvantage of **3** is that its cell permeability varies from cell line to cell line.⁹ For example, its IC₅₀ for inhibition of trypsin-like activity in the MM cell lines NCI-H929 and RPMI-8226 varied 25-fold (Table 5). Therefore, we chose these two cell lines to evaluate proteasome inhibition by the five most potent and 2-specific inhibitors. All five inhibit proteasomes in MM cells (Table 5). Most importantly, the difference in proteasome inhibition between NCI-H929 and RPMI-8226 cells was only 3-fold for **4a**, 2.5-fold for **12**, and 5-fold for **11**. Only **8** showed greater than 10-fold difference between cell lines. However, when we expanded our analysis to HEK-293 cells, only **4a** showed good inhibition (IC₅₀ = 2.7 μM). Compounds with two basic side chains, i.e., **10**, **11**, and especially **12**, were much less permeable (Table 6).

Although the vinyl sulfones were clearly more potent than the epoxyketones (Figure 2, Table 1), they could potentially inhibit cathepsins.^{10a} To determine if this is the case, the activity of cathepsins was measured in NCI-H929 cells with the five most potent and specific vinyl sulfones (Figure 5A). For this purpose, we used Z-FR-amc, which is cleaved by most cellular cathepsins.¹⁵ Partial inhibition of cathepsins was observed in cells treated with **4a** (Figure 5A). Following an identical 6 h treatment of cells, cathepsin inhibition occurred at a higher concentration than inhibition of trypsin-like activity and approximately at the same concentration as inhibition of caspase-like and chymotrypsin-like sites. **12** was a slightly more potent inhibitor of cathepsins. **8** did not cause any inhibition of cathepsins. **11** behaved similarly to **4a** (Figure 5A). Cells treated with compound **10** showed partly reduced cathepsin activity but at concentrations lower than those that inhibit trypsin-like activity (Figure S4). We interpret these data as showing that **4a**, **12**, **11**, and **10** inhibit one or two yet-to-be identified cathepsins, that **4a** and **11** are less potent inhibitors of these enzymes than of proteasome trypsin-like sites, that **12** inhibits cathepsins and proteasomes with a similar potency, and that **10** is a more potent inhibitor of a yet-to-be-identified cathepsin.

On the basis of the potency, cell permeability, and selectivity, we have chosen **4a** as the lead compound for future work. It inactivates proteasome in RPMI-8226 cells at much faster rate than **3** (Figure 5B). In **4a**-treated cells, steady-state inhibition is achieved after 1 h treatment; in **3**-treated cells, it was not achieved even after 6 h (Figure 5B).

In our previous study, we have shown that **3** is not toxic to myeloma cells when used as a single agent but sensitizes them to specific inhibitors of the chymotrypsin-like sites and to the FDA-approved proteasome inhibitors bortezomib and carfilzomib.⁹ Similarly **4a** was not toxic to RPMI-8226 cells but sensitized them to bortezomib (Figure 6A). It was a slightly more potent sensitizer of these cells to carfilzomib than was **3** (Figure 6B). Inhibition of cathepsin(s) is unlikely to contribute to sensitization by **4a** because E-64d, an inhibitor of all intracellular thiol proteases, did not sensitize cells to carfilzomib and did not significantly increase sensitization by **3** (Figure 6C). E-64d was used at 10 μM concentration, which completely inhibits thiol proteases.^{10a} The sensitizing effect of **4a** on carfilzomib was stronger than the additive effects of doxorubicin (Figure 6D,F) and dexamethasone¹⁶ and the synergistic effects of carfilzomib with histone deacetylase (HDAC) inhibitors (Figure 6E, and Figure S5 of Supporting Information). Thus, a combination of an inhibitor of trypsin-like sites with bortezomib or carfilzomib could potentially have stronger therapeutic effect than the bortezomib combinations currently used clinically.

DISCUSSION

In the past, utilization of non-natural amino acids led to improved specificity of inhibitors of the chymotrypsin-like sites¹⁷ and to development of the orally bioavailable carfilzomib analogue oprozomib.⁵ Here we have described how replacing Arg with non-natural amino-substituted phenylalanine derivatives leads to a dramatically improved potency of inhibitors of the trypsin-like sites. Because the importance of basic residues in the P1 and P3 position has been demonstrated earlier,^{11,18} we focused on testing side chains with pK_a in the 4.5–5.0 and 9–10.5 ranges. We found that the most basic benzylamine side chain produced the most potent and specific inhibitor. It should be noted that a phenyl ring in P1 is also important for potency, because compound **7**, with Lys-vs in the P1 position, was less potent than **4a**, while it has a side chain with similar basicity. Future work directed toward testing additional non-natural amino acids in the P1 and P3 positions, with the side-chain pK_a covering the 5.5–9.0 range, will verify these conclusions and may improve cell permeability of compounds.

This work has also revealed differences in inhibitor active site specificity in yeast and mammalian proteasomes, which can be explained by comparison of the structures of inhibitor complexes with the yeast proteasome with those of the structures of bovine and murine particles,^{13,14} which are 97% identical to human. Two of the three inhibitors compared, **4a** and **8**, are 8–447-fold more trypsin-like site-specific in mammalian proteasomes than in yeast core particles (Table 4). Superimposing the structure of mammalian proteasomes with that of inhibitor complexes with yeast proteasomes provides the explanation. Specifically, Glu122 residue of the β_6 subunit located at the bottom of the S1 pocket of the trypsin-like site and forming a hydrogen bond with the P1 side chain is replaced by Gln122, which cannot form the hydrogen bond.

Another specificity difference between yeast and mammalian proteasomes is that the presence of basic residues in the P3 position appears to be more important for the yeast particles than for human. We base this conclusion on the observation that **12**, which has a benzylamino side chain in P3, stands out as the most potent and specific inhibitor of the yeast trypsin-like site (Table 4) but in mammalian proteasomes has similar potency to **4a** and is only slightly more specific (Figure 2B, Table 3). A possible explanation for these differences is that the hydrogen bond between Asp-120 of the β_3 subunit and the basic group of the side chain, which is clearly important for inhibitor binding to yeast proteasomes, cannot be formed in mammalian proteasomes (Figure 4E,F). Finally, differences in the structure of the S4 pockets of the chymotrypsin-like sites may also lead to higher trypsin-like specificity of **4a** and **8** in mammalian proteasomes. Thus many differences in inhibitor specificity between mammalian and yeast proteasomes arise.

Another important finding of the present study is that vinyl sulfones are more specific and more potent inhibitors of trypsin-like sites than epoxyketones. It expands our earlier findings that not only the peptide side chains but also the warhead influences the active site specificity of inhibitors of the chymotrypsin-like sites.¹⁰ The unintended consequence of switching from epoxyketone to peptide vinyl sulfone inhibitors was partial inhibition of cysteine cathepsins by the majority of the most potent compounds. Although elimination of this off-target effect will be one of the design goals of the next generation of compounds, it does not limit the usefulness of these reagents as potent and specific inhibitors of proteasome-trypsin-like sites, because contribution of cathepsin inhibition to any biological activity of **4a** can always be ruled out by using E-64d, as we did in the experiment shown in Figure 6C. As we continue developing **4a** as a sensitizer of myeloma cells to bortezomib and carfilzomib, its ability to inhibit cathepsins may even be beneficial, as cathepsins have been also implicated in tumor growth, migration, invasion, metastasis, and angiogenesis.¹⁹

Another advantage of **4a** over **3** is that the starting material in the warhead synthesis, Phe (Scheme 1), is substantially less expensive than Boc-Arg(Pbf), which we used in the synthesis of compound **3**.⁹ Furthermore, **4a**'s higher potency, better cell permeability, and fewer variations in cell permeability from cell line to cell line (Tables 5 and Table 6: we have successfully used it in many solid tumor cell lines) should make **4a** more useful for further studies to interrogate the biology of this site. Finally **4a**, unlike **3**, is capable of inhibiting trypsin-like sites in mice (manuscript in preparation).

In conclusion, **4a** represents an important addition to the palette of tools that allows us to individually down-regulate proteasome active sites to any desired extent in living cells and animals.

EXPERIMENTAL PROCEDURES

Synthetic Procedures

General Procedures—On the basis of the UV peak area in LC-MS (provided in the Supporting Information, pp S61–S63), the purity of **4a**, **12**, and **8** is 95%, 97%, and 90%. All other compounds tested except **18** are >95% pure on the basis of LC-MS and NMR. Compound **18** is ~80% pure by the same criteria. ¹H and ¹³C NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm ( ) relative to tetramethylsilane, CD₃OD, or CDCl₃ as internal standard. LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3 μm C18 50 mm × 4.60 mm column (detection simultaneously at 214 and 254 nm) coupled to a PE Sciex API 165 mass spectrometer with ESI or a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200–600 nm) coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. The applied buffers were H₂O, acetonitrile, and aq 1.0% TFA.

(S)-2-(((Benzyloxy)carbonyl)amino)-3-(4-((2,2,2-trichloroacetamido)methyl)phenyl)propanoic Acid (**22**).²⁰

L-Phenylalanine (8.26 g, 50.0 mmol) was added in portions to concentrated H₂SO₄ (35 mL) and the temperature maintained at 25 °C. *N*-(Hydroxymethyl)trichloroacetamide (1.05 equiv, 52.5 mmol, 10.1 g) was added in portions while the temperature was maintained at 20–25 °C. The cooling bath was removed, and the light-brown cloudy solution was stirred at room temperature for 1 h. The reaction mixture was added to ice (500 mL), and the pH was adjusted to pH 5.5 with aq 8 M NaOH solution while maintaining the quench temperature at 15–20 °C. The white solid was filtered off and washed with ice-cold H₂O. The residue was dissolved in a 1:1 mixture of H₂O/dioxane (100 mL), and the pH was adjusted to pH 9 by addition of Na₂CO₃. Next, benzyl chloroformate (7.32 mL, 50.0 mmol) was added and the mixture was stirred for 4 h. Concentrated aq HCl was added until pH 1, and the mixture was extracted twice with EtOAc. The combined organic layers were extracted with brine, dried (MgSO₄), and concentrated under reduced pressure. The resulting crude product was purified by column chromatography (25%–60% EtOAc/PE), and the title compound was obtained as a colorless solid (yield: 8.29 g, 17.5 mmol, 35%). ¹H NMR (400 MHz, CDCl₃): = 10.16 (s, 1H), 7.31–7.21 (m, 6H), 7.13 (d, *J* = 7.88 Hz, 2H), 7.09 (d, *J* = 7.97 Hz, 2H), 5.58 (d, *J* = 8.21 Hz, 1H), 5.05–4.97 (m, 2H), 4.60 (dd, *J* = 13.68, 6.42 Hz, 1H), 4.40 (d, *J* = 5.54 Hz, 2H), 3.14 (dd, *J* = 13.57, 4.65 Hz, 1H), 3.01 (dd, *J* = 13.81, 6.53 Hz, 1H) ppm. ¹³C NMR (100 MHz, CDCl₃): = 174.70, 162.06, 155.85, 135.68, 135.31, 135.15, 129.57, 128.31, 128.28, 127.76, 127.57, 92.30, 66.91, 54.41, 44.54, 36.99 ppm.

(S)-2-(((Benzyloxy)carbonyl)amino)-3-(4-(((*tert*-butoxycarbonyl)amino)methyl)phenyl)propanoic Acid (23)

Compound **22** (2.82 g, 5.94 mmol) was treated with 20% w/w NaOH in H₂O/EtOH (1:1) for 1 h, after which TLC analysis indicated complete conversion of starting material. Next, aq 3 M HCl was added until pH 7, and the mixture was concentrated under reduced pressure. The resulting crude compound was dissolved in THF (40 mL) and cooled to 0 °C. Boc₂O (1.5 equiv, 8.91 mmol, 2.0 g) was added, and the solution was basified by addition of Na₂CO₃ until pH 9. The mixture was stirred at room temperature for 3 h, after which it was acidified with aq 10% w/v HCl until pH 2 and extracted with EtOAc (3×). The combined organic layers were extracted with brine, dried over MgSO₄, and concentrated under reduced pressure. The resulting crude mixture was purified by column chromatography (20% 100% EtOAc/PE), and the title compound was obtained as a colorless solid (yield: 1.90 g, 4.45 mmol, 75%). ¹H NMR (400 MHz, CDCl₃): δ = 9.32 (s, 1H), 7.36–7.28 (m, 5H), 7.16–7.02 (m, 4H), 5.33 (d, *J* = 7.64 Hz, 1H), 5.09 (q, *J* = 12.32, 12.32, 12.29 Hz, 2H), 4.95 (s, 1H), 4.65 (d, *J* = 6.41 Hz, 1H), 4.26–4.19 (m, 2H), 3.20–3.04 (m, 2H), 1.45 (s, 9H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 174.79, 156.16, 155.77, 137.47, 136.15, 134.80, 129.61, 128.46, 128.14, 128.03, 127.69, 79.85, 66.99, 54.52, 44.31, 37.30, 28.36 ppm.

(S)-2-(((Benzyloxy)carbonyl)amino)-3-(4-(((*tert*-butoxycarbonyl)amino)methyl)phenyl)-*N*-methoxy-*N*-methyl-propionamide (24)

Carboxylic acid **23** (4.45 g, 10.4 mmol) was dissolved in DCM (75 mL). To this were added NH(Me)OMe·HCl (1.5 equiv, 15.6 mmol, 1.55 g), HCTU (1.5 equiv, 15.6 mmol, 6.45 g), and DiPEA (4.5 equiv, 46.7 mmol, 7.72 mL), and the mixture was stirred for 2 h until TLC analysis indicated a completed reaction. The solvent was evaporated under reduced pressure, and the residue was dissolved in EtOAc. This was extracted with aq 1 M HCl (2 ×), saturated aq Na₂CO₃ (2 ×), and brine, dried over MgSO₄, and concentrated under reduced pressure. The product was purified by column chromatography (10% 75% EtOAc/PE) and obtained as colorless oil (yield: 4.81 g, 10.2 mmol, 98%). ¹H NMR (400 MHz, CDCl₃): δ = 7.29–7.22 (m, 5H), 7.14 (d, *J* = 8.12 Hz, 2H), 7.09 (d, *J* = 8.17 Hz, 2H), 6.02 (d, *J* = 8.49 Hz, 1H), 5.35 (s, 1H), 5.00 (dd, *J* = 28.51, 12.34 Hz, 2H), 4.96–4.94 (m, 1H), 4.21 (d, *J* = 5.20 Hz, 2H), 3.62 (s, 3H), 3.10 (s, 3H), 3.02 (dd, *J* = 13.63, 5.63 Hz, 1H), 2.85 (dd, *J* = 13.27, 7.70 Hz, 1H), 1.43 (s, 9H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 171.54, 155.50, 137.23, 136.02, 135.04, 129.04, 127.92, 127.48, 127.42, 126.98, 78.64, 66.11, 61.01, 51.78, 43.76, 37.46, 31.52, 27.96 ppm. [α]_D²³ = +10.1 (*c* = 1, CHCl₃). HRMS: calcd for C₂₅H₃₃N₃O₆ 472.24421 [M + H]⁺; found 472.24402.

(S)-*tert*-Butyl 4-(3-(Methoxy(methyl)amino)-3-oxo-2-(tritylamino)propyl)benzylcarbamate (29)

Compound **24** (1.43 g, 3.04 mmol) was dissolved in a 50:1 mixture EtOH/AcOH (25 mL), and argon was bubbled through this solution for 15 min. Next, Pd/C (10% w/w, 0.1 g) was added, and hydrogen was bubbled through the mixture until TLC indicated complete consumption of starting material after 4 h. Argon was bubbled through for another 15 min, after which the mixture was filtered over Celite and the filtrate concentrated under reduced pressure. The deprotected amine (as AcOH salt) was obtained in a crude yield of 1.21 g (max. 3.04 mmol) and was subsequently dissolved in DCM (20 mL). To this were added Et₃N (2 equiv, 6.08 mmol, 0.85 mL), DMAP (0.1 g), and trityl chloride (1.5 equiv, 4.56 mmol, 1.30 g). The mixture was stirred for 6 h, after which it was concentrated under reduced pressure, redissolved in EtOAc, extracted with aq 10 mM HCl and brine, dried over MgSO₄, and concentrated under reduced pressure. The resulting mixture was purified by column chromatography (10% 50% EtOAc/PE), and the title compound was obtained as a colorless foam (yield: 0.68 g, 1.17 mmol, 38%). ¹H NMR (400 MHz, CDCl₃): δ = 7.47 (s,

1H), 7.34 (d, $J = 7.33$ Hz, 6H), 7.26–7.20 (m, 4H), 7.18–7.05 (m, 9H), 5.10 (s, 1H), 4.28 (s, 2H), 4.00 (t, $J = 5.60, 5.60$ Hz, 1H), 3.18 (s, 3H), 2.92 (dd, $J = 13.24, 5.63$ Hz, 1H), 2.77 (dd, $J = 12.93, 7.51$ Hz, 1H), 2.63 (s, 3H), 1.44 (s, 9H) ppm. ^{13}C NMR (100 MHz, CDCl_3): = 174.80, 155.74, 145.92, 137.18, 137.13, 130.25, 128.70, 127.33, 127.15, 125.86, 79.07, 70.59, 60.00, 54.09, 44.19, 41.86, 31.96, 28.20 ppm. $[\alpha]_{\text{D}}^{23} = +58.6$ ($c = 1$, CHCl_3).

(*S,E*)-tert-Butyl 4-(4-(Methylsulfonyl)-2-(tritylamino)but-3-en-1-yl)benzylcarbamate (30)

Weinreb amide **29** (0.65 g, 1.12 mmol) was dissolved in Et_2O (15 mL), put under an argon atmosphere, and cooled to 0 °C. LiAlH_4 (2 equiv, 2.25 mmol, 0.56 mL of a 4 M solution in Et_2O) was added slowly, and the mixture was stirred at 0 °C for 1 h, after which TLC analysis indicated complete conversion of the starting compound. Aqueous 0.1 M HCl (15 mL) was slowly added, and the layers were separated. The organic layer was extracted with aq 0.1 M HCl and brine, dried over MgSO_4 , and concentrated under reduced pressure. Diethyl ((methylsulfonyl)-methyl)phosphonate (1.5 equiv, 1.68 mmol, 0.39 g) was dissolved in THF (20 mL) and cooled to 0 °C under an argon atmosphere. NaH (1.5 equiv, 1.68 mmol, 67.2 mg, 60% w/w in mineral oil) was slowly added, and the mixture was stirred at 0 °C for 30 min. Next, the freshly obtained aldehyde (in THF (2 mL)) was slowly added, and the mixture was stirred for 2 h while slowly warming it to room temperature. After this time, TLC analysis indicated complete conversion of the aldehyde. EtOAc (20 mL) was added, and the mixture was extracted with aq 10 mM HCl (2×) and brine, dried over MgSO_4 , and concentrated under reduced pressure. The title compound was obtained after column chromatography (20%–50% EtOAc/PE) as a colorless foam (yield: 0.57 g, 0.95 mmol, 85%). ^1H NMR (400 MHz, CDCl_3): = 7.46 (d, $J = 7.6$ Hz, 6H), 7.28 (t, $J = 7.20, 6.80$ Hz, 6H), 7.20 (t, $J = 7.20, 7.20$ Hz, 3H), 7.13 (d, $J = 7.60$ Hz, 2H), 6.87 (d, $J = 8.00$ Hz, 2H), 6.57 (dd, $J = 14.80, 7.00$ Hz, 1H), 5.96 (d, $J = 14.80$ Hz, 1H), 4.80 (s, 1H), 4.24 (d, $J = 5.60$ Hz, 2H), 3.49 (q, $J = 6.00$ Hz, 1H), 2.61 (s, 3H), 2.54 (dd, $J = 13.20, 5.20$ Hz, 1H), 2.33 (dd, $J = 13.20, 8.20$ Hz, 1H), 1.44 (s, 9H) ppm. ^{13}C NMR (100 MHz, CDCl_3): = 155.59, 150.21, 145.74, 137.42, 135.28, 129.53, 128.35, 128.02, 127.70, 127.14, 126.44, 78.91, 71.05, 55.33, 43.79, 42.43, 41.86, 28.09 ppm. $[\alpha]_{\text{D}}^{23} = -21.3$ ($c = 1$, CHCl_3). HRMS: calcd for $\text{C}_{36}\text{H}_{40}\text{N}_2\text{O}_4\text{S}$ 619.26010 $[\text{M} + \text{Na}]^+$; found 619.26001.

(*S,E*)-tert-Butyl 4-(4-(Methylsulfonyl)-2-aminobut-3-en-1-yl)-benzylcarbamate (31)

Trityl-protected amine **30** (0.54 g, 0.90 mmol) was treated with 1% v/v TFA/DCM (15 mL) at rt. To this yellow solution was added H_2O (1 mL), which resulted in a colorless suspension. After the mixture was stirred for 30 min, aq 10 mM HCl (20 mL) was added and DCM was removed under reduced pressure. The aqueous layer was extracted with Et_2O (3×) and basified with NaHCO_3 until pH 9, after which it was extracted with DCM (3×). The latter combined organic layers were dried over MgSO_4 and concentrated under reduced pressure. The resulting deprotected amine proved to be pure on LC-MS analysis and was subjected to the next step without further purification.

General Procedure: Azide Couplings and Deprotection—Compounds **4–18** were prepared via azide coupling of properly protected tripeptide hydrazide (e.g., $\text{N}_3\text{Phe-Leu-Leu-NHNH}_2$ for compounds **4–8**) and properly protected vinyl sulfone amines and epoxyketone amines.²¹ The appropriate hydrazide was dissolved in 1:1 DMF:DCM (v/v) and cooled to –30 °C. $t\text{BuONO}$ (1.1 equiv) and HCl (4 M solution in 1,4-dioxane, 2.8 equiv) were added, and the mixture was stirred for 3 h at –30 °C, after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. The epoxyketone or vinyl sulfone as a free amine was added to the reaction mixture as a solution in DMF. DiPEA (5 equiv) was added to the reaction mixture, which was allowed to warm to room temperature slowly overnight. The mixture was diluted with EA and extracted with H_2O

(3×). The organic layer was dried over MgSO_4 and purified by flash column chromatography. For deprotection, the product was dissolved in DCM (2.5 mL/mmol). TFA (2.5 mL/mmol) was added, and the mixture was stirred for 30 min, after which it was concentrated under reduced pressure in the presence of toluene (3×). The obtained crude product was purified by RP-HPLC. Tripeptide hydrazide were prepared by hydrazinolysis of tripeptide methyl esters²¹ synthesized by standard procedures of solution peptide chemistry as described in Supporting Information.

N₃Phe-Leu-Leu-Phe(4-CH₂NH₂)VS TFA Salt (4a)

¹H NMR (400 MHz, CD₃OD): δ = 7.39–7.21 (m, 9H), 6.78 (dd, J = 15.20, 5.34 Hz, 1H), 6.55 (dd, J = 15.21, 1.52 Hz, 1H), 4.82–4.77 (m, 1H), 4.36–4.27 (m, 2H), 4.17 (dd, J = 8.61, 4.80 Hz, 1H), 4.07 (s, 2H), 3.19 (dd, J = 14.05, 4.75 Hz, 1H), 3.02–2.95 (m, 3H), 2.92 (s, 3H), 1.63–1.43 (m, 6H), 0.93 (t, J = 5.65, 5.65 Hz, 6H), 0.88 (d, J = 6.24 Hz, 6H) ppm. ¹³C NMR (100 MHz, CD₃OD): δ = 174.45, 174.27, 171.95, 146.65, 139.63, 137.85, 133.01, 131.90, 131.30, 130.47, 130.26, 129.67, 128.13, 65.56, 53.74, 53.49, 52.46, 44.11, 42.83, 41.80, 41.61, 40.29, 38.71, 25.95, 25.86, 23.47, 23.46, 21.96, 21.94 ppm. LC-MS: t_R (min): 6.99 (ESI-MS (m/z): 654.20 ($M + H^+$)). HRMS: calcd for C₃₃H₄₇N₇O₅S 654.34321 [$M + H$]⁺; found 654.34322.

Bodipy-triazole-Phe-Leu-Leu-Phe(4-CH₂NH₂)VS TFA Salt (19)

Compound **4a** (5.68 mg, 8.69 μmol) and BODIPY-FL-alkyne²¹ (1.5 equiv, 13.0 μmol , 4.28 mg) were dissolved in a 1:1:1 mixture of H₂O/*t*BuOH/Tol (1.5 mL), to which were added CuSO₄ (0.1 equiv, 0.87 μmol , 0.87 μL of a 1 M solution in H₂O) and sodium ascorbate (0.15 equiv, 1.3 μmol , 1.3 μL of a 1 M solution in H₂O). The reaction was stirred at 80 °C for 4 h. LC-MS analysis revealed complete consumption of the azide and formation of a single product (t_R (min): 10.41 (ESI-MS (m/z): 981.20 ($M + H^+$))), which was assigned to be the corresponding benzaldehyde. The mixture was concentrated under reduced pressure and dissolved in MeOH (1.5 mL). To this were added NH₄OAc (10 equiv, 70 μmol , 5.4 mg) and NaCNBH₄ (2 equiv, 15 μmol , 1.0 mg), and the reaction was stirred for 15 h, after which LC-MS analysis indicated a complete disappearance of the aldehyde peak. The reaction was quenched by addition of aqueous HCl (100 μL , 1M), and the mixture was concentrated under reduced pressure. The title compound was obtained after RP-HPLC purification (gradient: 30% → 70% ACN/aq 0.1% TFA) as a red/brown solid (yield: 2.1 mg, 2.14 μmol , 29%). ¹H NMR (400 MHz, CD₃OD): δ = 7.77 (s, 1H), 7.31 (d, J = 7.91 Hz, 2H), 7.25 (d, J = 8.08 Hz, 2H), 7.03–6.97 (m, 5H), 6.75 (dd, J = 15.17, 5.40 Hz, 1H), 6.50 (dd, J = 15.26, 1.28 Hz, 1H), 6.08 (s, 2H), 5.52 (dd, J = 10.52, 5.15 Hz, 1H), 4.85–4.81 (m, 1H), 4.29–4.22 (m, 2H), 3.95 (s, 2H), 3.37–3.34 (m, 2H), 2.97–2.91 (m, 4H), 2.87 (s, 3H), 2.72–2.67 (m, 2H), 2.40 (s, 6H), 2.33 (s, 6H), 1.88–1.79 (m, 2H), 1.64–1.41 (m, 8H), 0.93–0.75 (m, 12H) ppm. LC-MS: t_R (min): 8.42 (ESI-MS (m/z): 982.40 ($M + H^+$)). HRMS: calcd for C₅₂H₇₀BF₂N₉O₅S [$M + H$]⁺ 982.53545; found 982.53653.

Compounds **1–3** were synthesized as described.⁹ Synthetic procedures and analytical data for remaining compounds are provided in Supporting Information.

Biological and Structural Analysis

Materials—Bortezomib, varinostat, and panobinostat were purchased from LC laboratories, carfilzomib was from Selleck, doxorubicin was from Biomol (now acquired by ENZO), and E-64d was from Calbiochem. Suc-LLVY-amc, Z-LRR-amc Z-LLE-amc, and Z-FR-amc were purchased from Bachem, and Acn-LPnLD-amc and Ac-RLR-amc were custom synthesized by GenScript. Activity-based probes were synthesized as described previously.^{22,23}

Competition Assays in Cell Lysate—Lysates of HEK-293T cells were prepared by sonication in 3 volumes of lysis buffer containing 50 mM Tris pH 7.5, 1 mM DTT, 5 mM MgCl₂, 250 mM sucrose, 2 mM ATP, and 0.025% digitonin. Protein concentration was determined by the Bradford assay. Cell lysates (13.5–15 µg total protein) were exposed to the inhibitors for 1 h at 37 °C prior to incubation with BODIPY-TMR-Ahx₃L₃-vs (MV151)²² or BODIPY-TMR-epoxomicin²³ (0.5 µM each) for an additional 1 h at 37 °C, followed by 5 min boiling with a reducing gel-loading buffer and fractionation on 12.5% SDS-PAGE. In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on a Typhoon Variable Mode Imager (Amersham Biosciences) using the Cy3/Tamra settings (λ_{ex} 532 nm, λ_{em} 560 nm) to detect BODIPY-TMR-Ahx₃L₃-vs²² and BODIPY-TMR-epoxomicin²³ and Cy2/Fam settings (λ_{ex} 488 nm, λ_{em} 520 nm) to detect compounds **19** and **20**. Intensities of 2 and 5 bands were measured by fluorescent densitometry and divided by the intensity of bands in mock-treated extracts, and average values of three independent experiments were plotted against inhibitor concentrations. IC₅₀ (inhibitor concentrations giving 50% inhibition) values were calculated using GraphPad Prism software.

Inhibition of Purified 26S Proteasomes—Purified 26S proteasomes from rabbit muscles^{10a} was incubated with different concentrations of inhibitors for 30 min (final proteasome concentration 3 µg/mL) followed by 58-fold dilution in 100 µM solutions of fluorogenic substrates Suc-LLVY-amc, Acn-LPnLD-amc, and Ac-RLR-amc of chymotrypsin-like, caspase-like, and trypsin-like sites. Cleavage of peptides was recorded by monitoring the fluorescence of the released Amc continuously for 30 min (λ_{exc} = 380, λ_{em} = 460 nm) using a SpectraMaxM2 plate reader (Molecular Devices). The rate of reaction was determined from the slopes of reaction progress curves and expressed as the percentage of the rate by mock-treated (not inhibited) proteasome. The % inhibition was plotted against inhibitor concentration, and IC₅₀ values were determined using nonlinear, four-parameter, least-squares fit in GraphPad Prism software (Figure S1, Supporting Information).

Inhibition of Yeast 20S Core Particles—Enzyme activity of yeast 20S core particles was determined by continuously monitoring the hydrolysis of the fluorogenic substrate Suc-LLVY-amc, ZLRR-amc, and ZLLE-amc for chymotrypsin-, trypsin-, and caspase-like activity in 20 mM Tris, 0.01% (w/v) SDS, pH 7.5, for 1 h at room temperature ([E]₀ = 0.10 nM; [S]₀ = 250 µM). Proteasomes were preincubated with inhibitors for 15 min, and inhibitors were present during activity assays. Fluorescence was measured at λ_{exc} = 360, λ_{em} = 460 nm using a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies). IC₅₀ values were obtained by plotting the percent inhibition against inhibitor concentration (Figure S2B) and fitting the experimental data to equation: % inhibition = $100[I]_0/(IC_{50} + [I]_0)$.

Crystallization and Structure Determination—Crystals of the 20S proteasome from *S. cerevisiae* were grown in hanging drops at 24 °C as already described²⁴ and incubated for at least 12 h with the respective compounds. The protein concentration used for crystallization was 40 mg/mL in Tris-HCl (10 mM, pH 7.5) and EDTA (1 mM). The drops contained 3 µL of protein and 2 µL of the reservoir solution, which contained 30 mM of magnesium acetate (MgAc₂), 100 mM of MES (pH 6.8), and 10% of 2-methyl-2,4-pentanediol (MPD). Crystals were soaked in a cryoprotecting buffer (30% MPD, 20 mM MgAc₂, 100 mM MES pH 6.8) and cooled in a stream of liquid nitrogen gas at 90 K (Oxford Cryo Systems) for data collection. The space group of CP:inhibitor complexes belong to P2₁ with cell dimensions of about $a = 135$ Å, $b = 300$ Å, $c = 144$ Å, and $\beta = 113^\circ$ (ST1). Data sets were collected using synchrotron radiation with $\lambda = 1.0$ Å at the X06SA-

beamline in SLS/Villingen/Switzerland. X-ray intensities and data reduction were evaluated by using the XDS program package.²⁵ The anisotropy of diffraction was corrected by an overall anisotropic temperature factor by comparing observed and calculated structure amplitudes using the program CNS.²⁶ Electron density was improved by averaging and back transforming the reflections 10 times over the 2-fold noncrystallographic symmetry axis using the program package MAIN.²⁷ Conventional crystallographic rigid body, positional, and temperature factor refinements were carried out with CNS using coordinates of the yeast 20S proteasome structure as a starting model.²⁴ Topology and parameter files for the ligand were received by Powell minimization of their respective pdb files using the program Sybyl²⁸ and SKETCHER in CCP4 pack.²⁹ Model building of the ligand into the experimental electron density was performed using COOT.³⁰ Subsequent Translation/Libration/Screw (TLS) vibrational motion refinement was performed using the program REFMAC5 in CCP4 pack or CNS.^{26,31} Ramachandran plot analysis was executed using SFCHECK in CCP4 pack.³² Final graphic representations of molecules were completed using the programs MOLSCRIPT,³³ BOBSCRIPT,³⁴ and PyMOL.³⁵

Cell Culture and Viability Assay—RPMI-8226 and NCI-H929 were obtained from ATCC. They were cultured in RPMI-1640 media supplemented with 10% FBS, penicillin, streptomycin, and plasmocin in a 5% CO₂ humidified incubator. HEK-293T cells were cultured in DMEM containing 10% fetal calf serum, 10 units/mL penicillin, and 10 µg/mL streptomycin in a 7% CO₂ humidified incubator. Viable cells were determined with Alamar Blue mitochondrial dye conversion assay as described in our previous publications.^{8–10}

Inhibition of Proteasomes in Cultured Cells—Proteasome inhibition in MM cells was measured with luminogenic substrates using ProteasomeGlo assay (Promega). RPMI-8226 or NCI-H929 cells (5×10^5 /mL) were incubated with inhibitors for times indicated. An aliquot of culture was pelleted, and medium was removed and replaced with PBS. The resulting cell suspension was mixed with luminogenic substrates in the manufacturer-provided lysis/assay buffer. After 3 min mixing on an orbital shaker, the plate was incubated in the dark for additional 17 min, followed by the measurements of the luminescence, performed in triplicate for each substrate/data point. Data were analyzed as for purified proteasomes. Proteasome inhibition in HEK-293T cells was measured by a competition assay in living cells. Cells (1×10^6 per well of a six-well plate) were incubated with the inhibitors for 4 h at 37 °C, and this was followed by a 2 h incubation with 5 µM BODIPY-TMR-epoxomicin.²³ Next, the medium was removed, and the cells were washed with PBS and harvested. After being flash frozen in liquid nitrogen, the cells were resuspended in 4 volumes of homogenization buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl₂, 1 mM DTT, 2 mM ATP, 0.025% digitonin), sonicated (12 W, 1 min), and centrifuged at 16 000 rcf at 0 °C for 20 min. The supernatant was collected, and the protein concentration was determined by the Bradford assay and used to normalize gel loading. The samples were analyzed by SDS-PAGE and fluorescent densitometry as described for the competition experiment in cell lysates. IC₅₀ values were calculated from three independent experiments using GraphPad Prism software.

Cathepsins Activity Assay—Inhibitor-treated and mock-treated NCI-H929 cells were harvested, washed with PBS, and permeabilized on ice with 0.05% digitonin in 4–5 volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 250 mM sucrose, 5 mM MgCl₂, 1 mM DTT, 1 mM ATP, and 0.5 mM EDTA. Cytosol was squeezed out by centrifugation for 15 min at 20 000g at +4 °C, and the residual cell pellet was lysed with a buffer containing 50 mM Bis-Tris-HCl (pH 5.5), 10% glycerol, 5 mM MgCl₂, 1 mM EDTA, 2 mM DTT, and 0.5% CHAPS. The protein concentration was determined using a Pierce 600 nM assay and used to normalize activity assay. An aliquot of the thusly prepared cytosol-depleted acidic extracts

was added to 100 μ L of 40 μ M pan-cathepsin-substrate Z-FR-amc in 100 μ M phosphate buffer pH 6.0, 2 mM EDTA, 4 mM DTT.¹⁵ The increase of fluorescence from released amc was recorded continuously for 30 min, and the rate of reaction was calculated from the slopes of the linear reaction progress curves. As established previously, cleavage of this substrate was completely blocked in extracts of cells treated with 5 μ M E-64d.^{10a}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS USED

Ahx	-aminohexanoic acid
Amc	7-amino-4-methylcoumarin
amc	7-amino-4-methylcoumarin amide
BODIPY-FL	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene
CHAPS	3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
DiPEA	<i>N,N</i> -diisopropylethylamine
EA	ethyl acetate
ek	epoxyketone
HBTU	2-(1- <i>H</i> -benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCTU	2-(6-chloro-1- <i>H</i> -benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Hmb	3-hydroxy-2-methylbenzoyl
MES	morpholinoethanesulfonic acid
MM	multiple myeloma
MPD	2-methyl-2,4-pentanediol
PE	petroleum ether
Suc	succinyl
TBTA	tris[(1-benzyl-1- <i>H</i> -1,2,3-triazol-4-yl)methyl]-amine
Tol	toluene
vs	methyl vinyl sulfone
Z	benzyloxycarbonyl

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Figure 1.
Structures of compounds 1–20.

Figure 2.

Assays of compounds in HEK-293 lysates. Occupancy of active sites in extracts treated with inhibitors at indicated concentrations was probed with BODIPY-TMR-Ahx₃L₃-vs (A) or BODIPY-TMR-epoxomicin (B). Inset in the top right of panel A indicates the migration pattern of the catalytic subunits in the untreated extract.

Figure 3.

Activity-based probes derived from **4a** and **12**. Extracts of HEK-293T cells were incubated with probe **19** (A) or probe **20** (B), followed by labeling of residual active sites with BODIPY-TMR-Ahx₃L₃-vs (A) or BODIPY-TMR-epoxomicin (B). Chemical structures of compounds are presented on Figure 1E.

Figure 4.

Crystal structure of the yeast 20S proteasome, cocrystallized with inhibitors. (A, B) Superposition of all yeast 20S proteasome structures cocrystallized with **4a**, **8**, and **12**, depicted in cream, yellow, and salmon, respectively: (A) Trypsin-like and (B) chymotrypsin-like active sites, 2 and 5 are depicted in cartoon form in green and wheat, and 3 and 6 are colored in gray. (C, D) Structural superposition of the yeast and murine 20S proteasome in complex with **12**: (C) trypsin-like and (D) chymotrypsin-like active site, whereas the murine proteasome subunits is highlighted by more discrete colors. (E, F) Close-up view of the S3 pocket in the trypsin-like sites in the yeast proteasome inhibited by

12 (E) and **4a** (F), superposed with the murine 20S structure. Differences in amino acids involved in the substrate binding are highlighted as ball and stick models. Amino acid residues numbering is according to the sequence of the *Thermoplasma acidophilum* proteasome.³⁶ S1–S5 pockets of the proteasome are labeled.

Figure 5.

Inhibition of proteasome in multiple myeloma cells by most potent inhibitors. (A) Comparison of proteasome and cathepsin inhibition after 6 h treatment of NCI-H929 cells. Values are mean $\pm$ SEM of two to four independent measurements. (B) Inhibition of trypsin-like activity in RPMI-8226 cells by **4a** and **3**. See Figure S4 of Supporting Information for inhibition of all three activities by **3**, **4a**, **12**, and **11** following 6 h treatment of RPMI-8226 cells.

Figure 6.

4a sensitizes RPMI-8226 cells to bortezomib and carfilzomib. (A) Sensitization to bortezomib. Cells were treated with bortezomib (Bz) for 1 h, followed by 47 h treatment with **4a** (1 μM). Values are mean ± SEM of three biological replicates. (B) Comparison of sensitization to carfilzomib (Cfz) by **3** (27 μM) and **4a** (1 μM). Cells were treated by carfilzomib for 1 h and then by **3** or **4a** or mock-treated for 47 h. Values are mean ± SEM of two to seven biological replicates. (C) E-64d does not sensitize cells to carfilzomib. Cells were treated with carfilzomib for 1 h, followed by 48 h treatment with either E-64d (10 μM), or **3** (27 μM), or both. Values are mean ± SEM of two independent experiments, data for Cfz

only, Cfz+3 are from panel B. (D) Effect of combined treatment with bortezomib and doxorubicin (Dox). Cells were treated with 1 μ M doxorubicin (Dox) for 24 h and then with bortezomib for an additional 48 h in the absence of Dox. Values are mean $\pm$ SEM of two independent experiments. (E) Effect of combined treatment with carfilzomib and HDAC inhibitor varinostat. Cells were treated with Cfz for 1 h, followed by 48 h treatment with 1 μ M varinostat. One μ M varinostat reduced viability to $38.6 \pm 0.5\%$. Values are mean $\pm$ SEM of two independent experiments. Another HDAC inhibitor, panobinostat, had a similar synergistic effect (Figure S5). (F) Effect of combined treatment with doxorubicin and carfilzomib. Cells were treated with 1 μ M doxorubicin (Dox) for 24 h and then with carfilzomib for an additional 24 h. Values are mean $\pm$ SEM of three biological replicates. Numerical values in panels D–F are combination indexes (CIs), calculated with CalcuSyn software. CIs could not be calculated for data in panels A and B because **4a** and **3** had no effect on cells when used as a single agent. (G) Effect of single agent varinostat treatment. Cells were treated with varinostat for 48 h. Values are mean $\pm$ SEM of two to four independent experiments. (H) Effect of single agent doxorubicin treatment. Cells were treated with Dox for 24 h and then cultured in drug-free media for an additional 48 h. Values are mean $\pm$ SEM of two independent experiments.

Scheme 1.

Synthesis of Para-Substituted Benzylamino Phenylalanine Epoxyketone and Vinyl Sulfone Warheads^a

^aReagents and conditions: (a) (i) *N*-(hydroxymethyl)trichloroacetamide, H₂SO₄, H₂O; (ii) benzyl chloroformate, Na₂CO₃, H₂O, 1,4-dioxane, 35%; (b) (i) 20% NaOH, EtOH/H₂O 1:1; (ii) Boc₂O, Na₂CO₃, THF, H₂O, 75%; (c) NH(Me)OMe·HCl, HCTU, DiPEA, DCM, 98%; (d) 2-bromopropene, *t*BuLi, THF, -78 °C, 94%; (e) NaBH₄, CeCl₃·7H₂O, MeOH, 0 °C, 92%; (f) (i) *t*BuOOH, VO(Acac)₂, DCM, 0 °C; (ii) Dess-Martin periodinane, DCM, 56%; (g) H₂, Pd black, TFA, MeOH; (h) (i) H₂, Pd/C, AcOH, EtOH; (ii) TrCl, Et₃N, DMAP, DCM, 38%; (i) (i) LiAlH₄, Et₂O, 0 °C; (ii) diethyl ((methylsulfonyl)methyl)phosphonate, NaH, THF, 0 °C, 85%; (j) 1% TFA/DCM.

Table 1
Optimization of the P1 Position and the Warhead of the Inhibitor Based on the Scaffold of 1a

compd ^b	P1-electrophile	IC ₅₀ (μM) for the 26S proteasomes ^a			
		Tr-L	Chym-L	Casp-L	IC ₅₀ (Chym-L)/IC ₅₀ (Tr-L)
4a	benzylamino-vs	0.0038	1.7	16	447
4b	benzylamino-ek	0.25	2.7	11	10.8
5a	anilino-vs	0.11	0.04	23	0.36
5b	anilino-ek	1.8	0.01	>30	0.0056
6	pyridino-vs	0.42	<0.1	30	<0.23
7	Lys-vs	0.74	22	30	30
1a	Arg-ek	1	>30	>30	>30

^a 30, no inhibition at 30 μM; >30, inhibition between 10% and 50% at 30 μM.

^b Chemical structure of compounds are presented in Figure 1B.

Table 2

Inhibition of Purified 26S Proteasomes from Rabbit Muscle by Derivatives of **3** with Modified Phenylalanine Side Chains in the P1 Position

compd ^b	IC ₅₀ (μM) ^a			IC ₅₀ (Chym-L)/IC ₅₀ (Tr-L)
	Tr-L	Chym-L	Casp-L	
3	0.3	~80	~80	267
8	0.059	5.9	21	100
9	30	0.2	30	0.0067

^a 30, no inhibition at 30 μM; >30, inhibition between 10% and 50% at 30 μM.

^b Chemical structures of compounds are presented in Figure 1C.

Table 3

Optimization of the P3 Position in Vinyl Sulfones (Figure 1D) of the Inhibitors Using Purified 26S Proteasomes^{a,b}

Compound: Name	Side chains		IC ₅₀ (μM) for sites:			IC ₅₀ (Chym-L)/ IC ₅₀ (Tr-L)
	R3	R1	Tr-L	Chym-L	Casp-L	
4a			0.0038	1.7	16	447
10			0.012	1.05	30	87.5
11			0.030	3.4	30	113
12			0.0066	8.9	18	1348
13			0.0063	0.19	30	59
14			0.045	0.41	>30	9.1
15			0.18	0.01		0.057
16			24	<0.004	30	<0.00017
17			24	0.005	30	0.00021
18			30	1	30	n.d
2	Arg	Arg-ek	0.08	>30	>30	>375

^a 30, no inhibition at 30 μM; >30, inhibition between 10% and 50% at 30 μM.

^b Chemical structures of compounds are presented in Figure 1D.

Table 4

Inhibition of Yeast 20S Proteasomes by Selected Compounds^a

compd	IC ₅₀ (μM) for yeast 20S proteasomes			IC ₅₀ (Chym-L)/IC ₅₀ (Tr-L)	
	Tr-L	Chym-L	Casp-L	yeast	rabbit
4a	2.6	2.7	–	1.04	447
8	1.6	19.3		12.1	100
12	0.00055	6.1		11090	1348

^aNo inhibition of caspase-like sites was observed at 200 μM (Figure S2A, Supporting Information). Dose–response curves used for determination of IC₅₀ values are presented in Figure S2.

Table 5 Inhibition of Proteasomes in Multiple Myeloma Cells by Specific Inhibitors of Trypsin-like Sites^a

compd	MM cell line	IC ₅₀ (μM) ^b		
		Tr-L	Chym-L	Casp-L
3	NCI-H929	0.9	30	30
	RPMI-8226	24	30	>30
4a	NCI-H929	0.05	1.7	2.2
	RPMI-8226	0.17	1.2	1.6
8	NCI-H929	0.2	13	30
	RPMI-8226	3.2	15	>30
12	NCI-H929	0.17	10.5	17.4
	RPMI-8226	0.42	27	>30
11	NCI-H929	0.11	3.6	22
	RPMI-8226	0.58	7.2	>30
10	NCI-H929	0.27	10.5	>30

^a Cells were treated with inhibitors for 6 h followed by measurement of proteasome activities with ProteasomeGlo assay.^b 30, no inhibition at 30 μM; >30, inhibition between 10% and 50% at 30 μM. The dose–response curves used to calculate IC₅₀ are in Figure 5, and Figures S3 and S4 of Supporting Information.

Table 6 Inhibition of Proteasomes in HEK-293T Cells by Specific Inhibitors of Trypsin-like Sites^a

compd	IC ₅₀ (μM) ^b		
	Tr-L	Chym-L	Casp-L
4a	2.7	~50	50
12	50	>50	50
11	10	>50	50
10	27	>50	50

^a Cells were treated for 4 h, followed by assessment of inhibition with the activity-based probe.^b 50, no inhibition at 30 μM; >50, inhibition between 10% and 50% at 50 μM.