Review

Cell
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Proteasome Inhibitors: An Expanding Army Attacking a Unique Target

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DOI 10.1016/j.chembiol.2012.01.003

Proteasomes are large, multisubunit proteolytic complexes presenting multiple targets for therapeutic intervention. The 26S proteasome consists of a 20S proteolytic core and one or two 19S regulatory particles. The 20S core contains three types of active sites. Many structurally diverse inhibitors of these active sites, both natural product and synthetic, have been discovered in the last two decades. One, bortezomib, is used clinically for treatment of multiple myeloma, mantle cell lymphoma, and acute allograft rejection. Five more recently developed proteasome inhibitors are in trials for treatment of myeloma and other cancers. Proteasome inhibitors also have activity in animal models of autoimmune and inflammatory diseases, reperfusion injury, promote bone and hair growth, and can potentially be used as anti-infectives. In addition, inhibitors of ATPases and deubiquitinases of 19S regulatory particles have been discovered in the last decade.

It has been a decade since one of us reviewed the field of proteasome inhibitors in this journal (Kisselev and Goldberg, 2001) and almost that long since the US Food and Drug Administration (FDA) approved the proteasome inhibitor bortezomib (Velcade, PS-341) for treatment of multiple myeloma (MM) in 2003. During these years, proteasome inhibitors continued to serve as valuable tools for cell biologists and immunologists who used them to dissect the proteasome role in protein degradation and antigen presentation (see Kisselev and Goldberg, 2001, for detailed review). The field has seen many new developments since then. Bortezomib, initially approved as a third-line therapy for relapsed and refractory MM, is now approved as a frontline treatment for this disease. Five other proteasome inhibitors have entered clinical trials (Molineaux, 2012) and several new structural classes of proteasome inhibitors have been discovered. X-ray structures of all major structural classes have been solved, revealing the amazing diversity of mechanisms by which proteasomes can be inhibited (Groll and Huber, 2004). Specific inhibitors of individual active sites and numerous activity-based probes have been developed, and inhibitors of the enzymatic activities of the 19S regulatory particles have been discovered. Mechanisms of selective antineoplastic activity in MM cells of proteasome inhibitors are much better understood.

In this review, we first discuss the rationale for proteasome targeting in MM, then review the proteasome and its active sites. We then look at the different structural classes of proteasome inhibitors before introducing specific inhibitors of individual active sites and describing what they taught us about the relative roles of these sites as drug targets in cancer. We then focus on existing, experimental, and potential clinical applications of proteasome inhibitors beyond oncology. Finally, we review the newly discovered inhibitors of enzymatic activities of the 19S regulatory particles and their potential clinical applications.

Antineoplastic Activity of Proteasome Inhibitors and Development of Bortezomib for the Treatment of Myeloma

The ubiquitin-proteasome pathway is the major quality-control pathway for newly synthesized proteins in every eukaryotic cell (Coux et al., 1996; Hershko and Ciechanover, 1998). Furthermore, through specific targeted destruction of regulatory proteins, this pathway participates in the regulation of numerous cellular and physiological functions. For example, cell-cycle progression is impossible without timely degradation of cyclins and cyclin-dependent kinase inhibitors (cdk) by the ubiquitin-proteasome pathway (King et al., 1996). This finding suggested that proteasome inhibitors should block this process and so prevent malignant cells from proliferating. Although proteasome inhibitors were initially developed as anti-inflammatory agents (see Goldberg, 2010, for a detailed account of bortezomib development), when cultured cells derived from different cancers were treated with proteasome inhibitors, it was quickly discovered that this treatment caused rapid apoptosis. Furthermore, apoptosis was selective for transformed cells, reducing concerns that proteasome inhibitors would be too toxic due to inhibition of the protein quality control functions of the ubiquitin-proteasome pathway in normal cells (see for review Adams, 2004, and Kisselev and Goldberg, 2001).

Bortezomib was found to have a unique cytotoxicity pattern against an NCI panel of 60 cell lines derived from different cancers (Adams et al., 1999). In animal studies, bortezomib reduced the growth rate of xenograft tumors and showed a remarkable ability to block angiogenesis (LeBlanc et al., 2002) and reduce metastasis (Teicher et al., 1999), providing a rationale for clinical trials. Accordingly, phase I clinical trials were conducted on a variety of solid tumors (Aghajanian et al., 2002) and hematologic malignancies (Orlowski et al., 2002). Several responses were observed in patients with MM (Orlowski et al., 2002). This led to focused phase II trials and rapid FDA

Table 1. Proteasome Inhibitors Used Clinically or in Clinical Trials for the Treatment of Multiple Myeloma

Compound	Chemical nature	Adm. Route	Status	Developed by	Active Sites Targeted
bortezomib	boronate	IV	approved	Millennium	β5/β5i > β1/β1i > β2i
carfilzomib	epoxyketone	IV	Ph III/FDA-filed	ONYX	β5/β5i > > β2i∼β1i
marizomib	β-lactone	IV/oral	phase I	Nereus	β5/β5i > β2/β2i > β1/β1i
CEP-18770	boronate	IV/oral	phase I–II	Cephalon	β5/β5i > β1/β1i
MLN-9708	boronate	oral/IV	phase I–II	Millennium	β5/β5i > β1/β1i
ONX-0912	epoxyketone	oral/IV	phase I	ONYX	β5/β5i

approval based on the results of those trials (Richardson et al., 2003), initially (in 2003) as a third-line treatment for a relapsed and refractory disease and then (in 2008) as front-line treatment for a newly diagnosed MM patients.

For years it was not clear why MM is so responsive to bortezomib. Initially, it was thought that transcription factor NF-κB is its main target (Adams, 2004; Chauhan et al., 2005b). MM cells are transformed plasma cells residing in the bone marrow (BM), and NF-κB activity is important for the maintenance of interactions between MM and BM stromal cells. This factor regulates expression of IL-6 and IGF-1, which promote growth, survival, and chemoresistance of MM cells in the BM milieu (Chauhan et al., 2005b). Activation of NF-κB involves up to two proteasome-dependent steps (Palombella et al., 1994), so inhibition of NF-κB activation contributes to bortezomib activity in MM; however, this is not the major factor responsible for bortezomib's antineoplastic activity, and inhibition of NF-κB signaling has a much milder effect on myeloma cells than does inhibition of proteasomes (Hideshima et al., 2002). NF-κB plays an important role in the proliferation and chemo-resistance of many solid tumors. Bortezomib has no efficacy in these malignancies.

As already noted, a main function of the ubiquitin-proteasome pathway is quality control of newly synthesized proteins. MM cells are the most protein secretors of all cell types. They synthesize and secrete large amounts of IgG or IgA (Bianchi et al., 2009; Cenci et al., 2011), one of the most complex protein molecules to synthesize. IgG is a four-chain protein that contains multiple disulfide bonds. Individual IgG chains that fail to properly fold or assemble are degraded by proteasomes via the endoplasmic reticulum (ER)-degradation pathway, so a high rate of IgG biosynthesis in MM cells places an unusually high burden on the proteasomes. MM cells are therefore under permanent ER stress and can be easily induced, by proteasome inhibition, into the unfolded protein response (Obeng et al., 2006). Moreover, increased production of IgG by MM cells increases their sensitivity to proteasome inhibitors (Meister et al., 2007). As a result, partial inhibition of proteasomes in vivo by bortezomib, which is not toxic to patients' normal cells, is sufficient to kill MM cells. Proteasome inhibitor-induced apoptosis always involves upregulation of a proapoptotic BH3 only member of Bcl-2 family (Fennell et al., 2008), most frequently NOXA (Chen et al., 2010; Fernández et al., 2005; Qin et al., 2005). NOXA expression in hematologic malignancies is controlled by a transcription factor ATF3 (Chen et al., 2010; Wang et al., 2009), which is induced by ER stress.

The success of bortezomib has stimulated interest in proteasomes as targets in oncology, and today at least five other compounds—two peptide boronates, two peptide epoxyke-
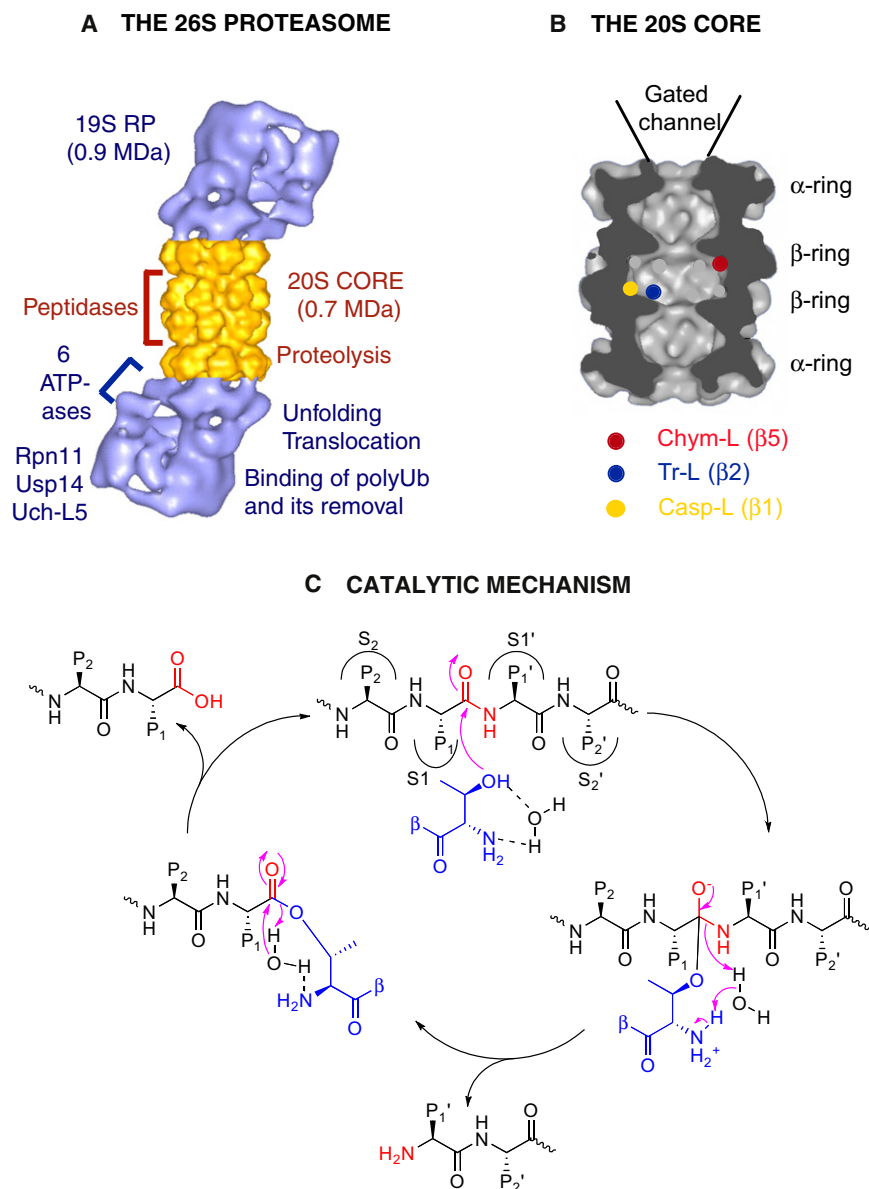
tones, and one marine natural product, β-lactone—are at various stages of clinical development (Table 1).

Structure and Active Sites of Proteasomes

The 26S proteasome is a large (2.5 MDa), multisubunit, ATP-dependent proteolytic complex that processively degrades proteins into small peptides. The proteasome is an unusual target because it contains many enzymatic active sites that are druggable, potentially allowing for the fine-tuning of pharmacological response. It consists of a hollow cylindrical 20S proteolytic core and one or two 19S regulatory particles (RPs, activators, Figure 1A). The 19S RP recognizes ubiquitylated substrates and prepares them for proteolysis, which occurs inside the 20S cores. The 20S cores are hollow cylindrical structures comprising 2 pairs of 14 different polypeptides arranged in 4 stacked rings (Figure 1B). Six subunits carry catalytic residues for the proteolytic sites: two are chymotrypsin-like (β5), two trypsin-like (β2), and two caspase-like (β1). These three types of sites are targeted by the majority of inhibitors discussed in this review.

In mammals, cells and tissues of the immune system also express the immunoproteasome. The core particle of the immunoproteasome contains different catalytic subunits: LMP2 (β1i), MECL (β2i), and LMP7 (β5i). Immunoproteasomes have a slightly altered substrate specificity and produce more peptides with hydrophobic and basic C termini and fewer peptides with acidic C termini to match the specificity of major histocompatibility class I molecules (Kloetzel and Ossendorp, 2004; Rock and Goldberg, 1999). Thymal cortical epithelial cells express thymoproteasome, a proteolytic particle closely related to the immunoproteasome but with β5i replaced by a unique subunit, β5t (Murata et al., 2007).

All active sites cleave peptide bonds by an unusual mechanism in which the hydroxyl group of N-terminal catalytic threonine serves as the catalytic nucleophile (Figure 1c) (Groll and Huber, 2004). The role of β1, β2, and β5 active sites in protein degradation and cell growth was first addressed by site-directed mutagenesis in the yeast *S. cerevisiae*. Inactivation of β5 sites by mutation of their catalytic threonine significantly retarded growth, increased sensitivity to conditions that increase production of abnormal proteins (e.g., heat and canavanine, an arginine analog whose incorporation causes production of misfolded proteins), and caused significant accumulation of all proteasome substrates tested (Chen and Hochstrasser, 1996; Heinemeyer et al., 1997). Similar mutations of the catalytic threonine of the β1 sites caused no phenotypic defects and did not lead to accumulation of substrates (Arendt and Hochstrasser, 1997; Heinemeyer et al., 1997). Inactivation of the β2 sites reduced

A   THE 26S PROTEASOME

19S RP
(0.9 MDa)

Peptidases

6
ATP-
ases

Rpn11
Usp14
Uch-L5

20S CORE
(0.7 MDa)

Proteolysis

Unfolding
Translocation

Binding of polyUb
and its removal

B   THE 20S CORE

Gated
channel

α-ring

β-ring

β-ring

α-ring

● Chym-L (β5)
● Tr-L (β2)
● Casp-L (β1)

C   CATALYTIC MECHANISM

Figure 1. The Proteasome
(A) The 26S particle. Location and functions of different subunits are indicated.
(B) Cross-section of the 20S proteolytic core showing location of the active sites.
(C) The catalytic mechanism of the proteasome. Proteasome is blue. Substrate is black except for scissile bond, which is red.

inhibit chymotrypsin-like sites. Later results of site-directed mutagenesis in yeast (see above) confirmed that this site is the most important target. These efforts to develop cell permeable inhibitors of chymotrypsin-like sites were aided by the ability of hydrophobic peptides to enter cells, as these sites cleave preferentially after hydrophobic residues (Kisselev and Goldberg, 2001). However, most β5 inhibitors also inhibit the caspase-like and/or trypsin-like sites at higher concentrations, usually by coincidence rather than design. For example, bortezomib was developed as an inhibitor of chymotrypsin-likes sites (Adams, 2004) but was later found to co-inhibit caspase-like sites (Altun et al., 2005; Berkers et al., 2005; Kisselev et al., 2006). Most second-generation boronates also coinhibit caspase-like sites.

Major Structural Classes of Inhibitors of Proteolytic Sites of the 20S Core

Proteasome inhibitors are structurally diverse, and can be divided into two large groups based on whether or not they form a covalent bond with the active site threonine. These two groups can be further subdivided into structural classes (Figure 2). All noncovalent inhibitors are reversible and so are some covalent inhibitors (aldehydes, glioxals, and to some extent, boronates). In addition, allosteric inhibitors that do not interact with active sites have been described.

Interestingly, of the eight major structural classes of inhibitors of eukaryotic proteasomes discussed here, five (aldehydes, β-lactones, epoxyketones, syrbactins, and cyclic peptides) were either discovered as natural products or have natural products among them (Figure 2). Clearly, microorganisms learned of the importance of the proteasome to their eukaryotic neighbors long before scientists discovered this fascinating particle. Although the chymotrypsin-like sites are the primary targets of all natural product proteasome inhibitors, these substances all coinhibit trypsin-like and caspase-like sites at higher concentrations, probably because complete or near complete inhibition of all three sites is needed to carry out the function for which they
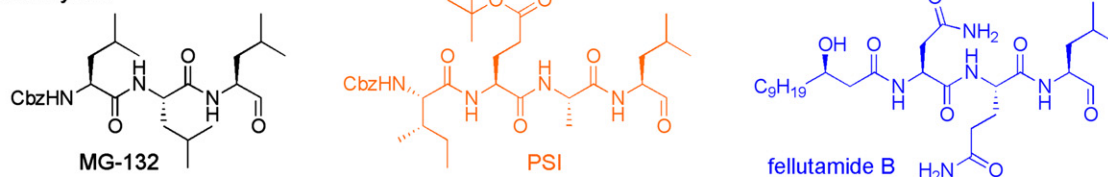
growth rates slightly and reduced the degradation rate of some model substrates (Arendt and Hochstrasser, 1997; Heinemeyer et al., 1997). A yeast strain in which the β1 and β2 sites were both inactive had a stronger growth defect than strains in which only β2 was inactive, but had fewer phenotypic defects than a strain lacking functional β5 sites (Heinemeyer et al., 1997). Thus, the β5 (chymotrypsin-like) sites were apparently the most important sites in protein breakdown, whereas the β1 (caspase-like) sites appeared to be functionally redundant, raising the interesting question of why the latter had evolved and been conserved.

The chymotrypsin-like site was the primary target of the very first peptide aldehyde inhibitors developed (Rock et al., 1994). These compounds inhibited protein degradation in cells. Because of this biological activity, future efforts to develop proteasome inhibitors focused on optimizing their capacity to
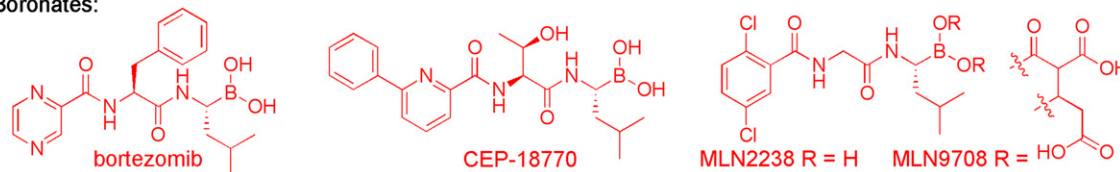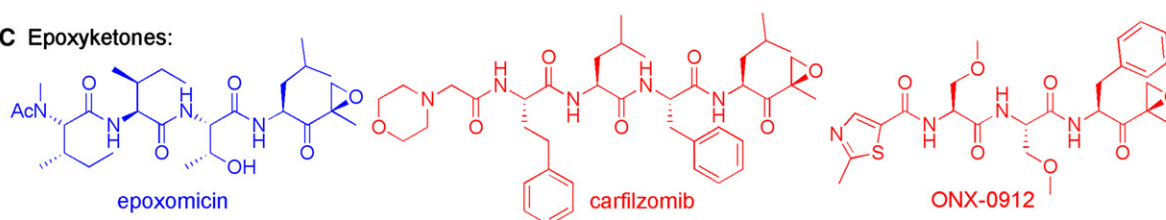
Figure 2. Representatives of the Major Classes of Covalent Proteasome Inhibitors
(A) Aldehydes; (B) boronates; (C) epoxyketones; (D) α-ketoaldehyde; (E) β-lactones; (F) vinyl-sulfones; (G) syrbactines; (H) bacteria-specific oxatiazol-2-ones.
Natural products are blue. Synthetic inhibitors used clinically for the treatment of cancer (FDA-approved or in clinical trials) are red; natural product in clinical trials
for the treatment of cancer is purple. Synthetic inhibitors that were tested clinically for other indications are orange. (Omuralide is a derivative of a natural product
lactacystin.)

evolved: to kill their natural neighbors by impairing their protein quality control pathways.

Inhibitors That Form Covalent Bonds with Active Sites

Covalent inhibitors usually consist of an electrophilic trap that interacts with the active site threonine and a peptide moiety. Based on the nature of electrophilic traps employed for these purposes, eight major classes of proteasome inhibitors can be distinguished (Figure 2).

Peptide Aldehydes. Peptide aldehydes (e.g., MG-132 [Adams et al., 1998; Palombella et al., 1994; Tsubuki et al., 1993], PSI [Figueiredo-Pereira et al., 1994]; Figure 2A) were the first inhibitors to be developed and, largely due to their low cost, are still the most widely used. These rapidly reversible, potent inhibitors block proteasomes by forming a hemiacetal with the hydroxyl of the active site threonines (Figure 3A). Most are synthetic, but several natural product peptide aldehydes have been discovered (e.g., tyropeptin A [Momose et al., 2001]), fellutamide B [Hines et al., 2008]). Aldehydes are well-known inhibitors of serine and cysteine proteases. Although MG-132 is a more potent inhibitor of proteasome than of cathepsins and calpains (Tsubuki et al., 1996), when using these inhibitors in cell culture, it is important to confirm the involvement of proteasomes in the physiological event that is the subject of the study by using more specific proteasome inhibitors (e.g., epoxomicin, bortezomib, lactacystin).

Aldehydes are oxidized rapidly in vivo and do not have systemic activity when used in mice (Lindsten et al., 2003). An interesting approach to circumventing this problem is to synthesize semicarbazone prodrugs. These have submicromolar potency (Leban et al., 2008) and delay tumor growth in xenograft models of glioma in mice, albeit at very high doses (150 mg/kg) (Roth et al., 2009). Bortezomib is active in vivo at 1 mg/kg (LeBlanc et al., 2002) and carfilzomib is active at 3–5 mg/kg (Demo et al., 2007). Therefore, substantial improvement in potency is needed before semicarbazones can be used as research tools or therapeutic agents.

Peptide Boronates. Peptide boronates (e.g., boronate analog of MG-132 MG-262, bortezomib, and two boronates in clinical trials, CEP-18770 and MLN2238; Figure 2B) are much more potent synthetic inhibitors of the proteasome than are the corresponding aldehydes (Adams et al., 1998). Boronates form tetrahedral adducts with active site threonines (Figure 3B), which are further stabilized by a hydrogen bond between the N-terminal amino group of the threonine and one of the hydroxyl groups of the boronic acid (Groll et al., 2006a). This hydrogen bond explains why boronates are more potent inhibitors of proteasomes than of serine proteases, a group of enzymes that they were originally developed to inhibit. Although inhibition of serine proteases by bortezomib was originally shown to be several orders of magnitude weaker than inhibition of proteasome (Adams et al., 1998), recent studies have revealed that bortezomib inhibits HtrA2/Omi, an ATP-dependent serine protease in mitochondria (Arastu-Kapur et al., 2011). HtrA2 protects neurons from apoptosis, and inhibition of HtrA2 is now believed to be the cause of peripheral neuropathy (Arastu-Kapur et al., 2011), the major dose-limiting toxicity of bortezomib in patients (Richardson et al., 2005). Boronic acid analog of MG132, MG262, inhibits ATP-dependent serine protease Lon from bacteria (Frase et al., 2006). Mammalian homolog of Lon, together with mammalian

homolog of another ATP-dependent bacterial serine protease, ClpXP, is involved in the protein quality control in the mitochondrial matrix. Peptidyl boronates are capable of inhibiting mammalian Lon and ClpXP proteases (Fishovitz et al., 2011), although inhibition of these proteases by bortezomib or two boronates in clinical trials has not been reported.

Although boronates are reversible inhibitors, boronate-proteasome adducts have much slower dissociation rates than do proteasome-aldehyde adducts. The off-rate of bortezomib is so slow that on the time scale of a typical cell culture experiment (a few hours to a day), proteasome inhibition by bortezomib is essentially irreversible. One of the clinical implications of bortezomib's slow off-rate is that once it is bound to the proteasome in red blood cells, it cannot be released. Taking this into consideration, scientists at Millennium Pharmaceuticals, Inc., have designed a second-generation boronate, MLN2238 (Figure 2B), to be a less potent inhibitor with a faster off-rate. As a result, MLN2238 has a much larger volume of distribution, presumably because drug initially bound to proteasome in blood is able to dissociate and penetrate into tissues (Kupperman et al., 2010). In addition, MLN2238 can achieve stronger inhibition of chymotrypsin-like activity in vivo (Kupperman et al., 2010) and does not inhibit HtrA2 (Chauhan et al., 2011). When formulated as a boronic ester prodrug, MLN9708, it is orally bioavailable.

Another independently developed, orally bioavailable boronate, CEP-18770 (Figure 2B), is undergoing clinical testing (Piva et al., 2008). Early results of clinical trials indicate that unlike with bortezomib, peripheral neuropathy is not a rate-limiting toxicity of CEP-18770 (Ruggeri et al., 2009). Like bortezomib, MLN2238 and CEP-18770 coinhibit caspase-like sites (Kupperman et al., 2010; Piva et al., 2008).

Peptide α',β'-Epoxyketones. Peptide α',β'-epoxyketones (Figure 2C) are the most specific and potent proteasome inhibitors known to date. In the decade-plus since the proteasome was identified as a target of the natural products epoxomicin and eponemycin (Meng et al., 1999a; Meng et al., 1999b), no off-target effects of these compounds have been found. The crystal structure of the yeast proteasome in complex with epoxomicin explains this exquisite specificity, revealing a six-membered morpholine ring formed by the N-terminal threonine and epoxyketone moiety of the inhibitor (Groll et al., 2000). This structure suggests that the catalytic hydroxyl first attacks the carbonyl group of the pharmacophore (Figure 3C). Then, the free α-amino group of the threonine opens up the epoxide and completes the formation of the morpholino adduct. Thus, epoxyketones take specific advantage of the unusual catalytic mechanism employed by the proteasome. Catalytic residues of serine and cysteine proteases do not have α-amino group and cannot form such an adduct. Potency, exquisite specificity, and relative ease of synthesis (in our hands, they are easier to synthesize than boronates) have made this natural product scaffold a popular choice for synthetic modifications, and hundreds of epoxyketones have been synthesized in the past decade. Modification of the peptide fragment has led to the development of many site-specific inhibitors and activity-based probes (Verdoes et al., 2010).

Two compounds in clinical trials for the treatment of cancers, carfilzomib (Demo et al., 2007) and ONX-0912 (Figure 2C), are epoxyketones. Of the five proteasome inhibitors undergoing
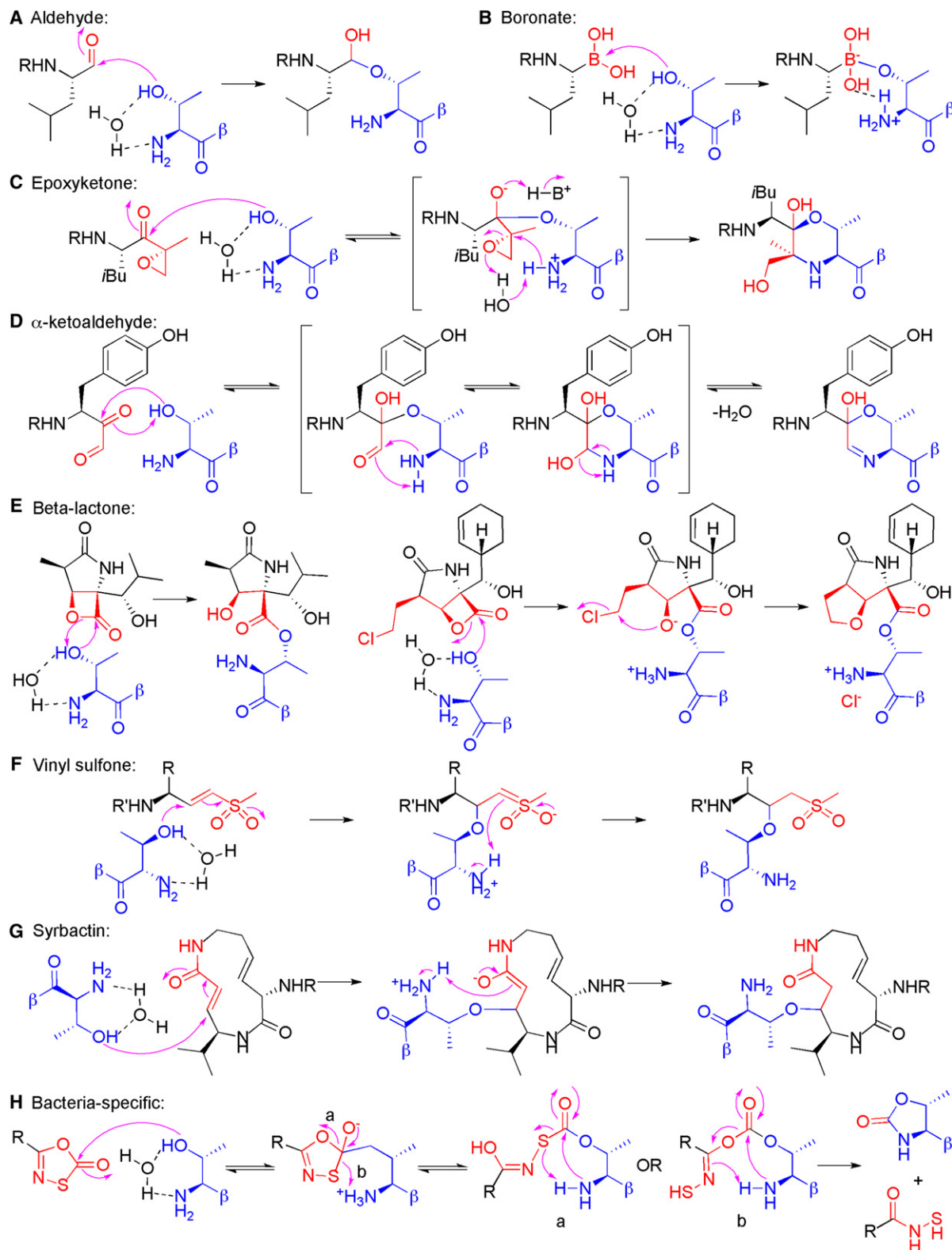
Figure 3. Mechanism of Proteasome Inhibition by Covalent Inhibitors
(A) Aldehydes; (B) boronates; (C) epoxyketones; (D) α-ketoaldehyde; (E) β-lactones; (F) vinyl-sulfones; (G) syrbactines; (H) bacteria-specific oxatiazol-2-ones.
Proteasome is blue. Inhibitors are black except for electrophiles, which are red.

clinical testing, carfilzomib is the most advanced. It causes stronger inhibition of the chymotrypsin-like activity of the proteasome in blood of patients than does bortezomib—88% at the highest dose used in phase I trial, where maximal tolerated dose has not been reached (O'Connor et al., 2009). Inhibition by bortezomib does not exceed 70% at maximal tolerated dose (Hamilton et al., 2005). In phase II trials, carfilzomib has achieved a remarkable 24% partial response rate in a heavily pretreated patient population (a median of five prior lines of multidrug therapy). Carfilzomib is undergoing Phase III trials for MM and will likely be approved by the FDA in 2012. Importantly, incidents of peripheral neuropathies are greatly reduced compared to bortezomib (Molineaux, 2012), consistent with neuropathies being an off-target effect due to inhibition of HtrA2 by bortezomib and with lack of inhibition by the more-specific epoxyketones (Arastu-Kapur et al., 2011). Intensive medicinal-chemistry efforts led to the development of an orally bioavailable analog PR-047 (ONX-0912, Figure 2C), a remarkable achievement considering that this compound is a tripeptide (Zhou et al., 2009).

Peptide Ketoaldehydes. Peptide ketoaldehydes (Figure 2D) were discovered in the 1990s (Lynas et al., 1998) but were largely ignored by the proteasome community because their benefits over other classes of inhibitors were not clear. The X-ray structure of a peptide ketoaldehyde in complex with yeast proteasomes reveals the formation of a unique six-membered ring with the N-terminal catalytic threonine (Gräwert et al., 2011). The ring contains a hemiketal and Schiff base, suggesting a mechanism of inhibition not unlike that exerted by epoxyketones (Figure 3D). Like epoxyketones, ketoaldehydes take specific advantage of the unique catalytic mechanism employed by proteasomes. The ring structure predicts that ketoaldehydes should have little or no off-target effects; in fact, they are 1000-fold more potent inhibitors of proteasome than of chymotrypsin and trypsin (Lynas et al., 1998). This specificity, in combination with the reversibility of the Schiff base, allows this class of inhibitors to occupy a unique niche as highly specific, reversible, covalent inhibitors, as all other classes are either irreversible (epoxyketones, β-lactones) or reversible but not specific (aldehydes). They should now be considered as candidates to replace the peptide aldehyde MG132 in experiments when both specificity and rapid reversibility of action are required.

β-Lactones. β-lactones (Figure 2E) are nonpeptide natural products originally represented by clasto-lactocystin-β-lactone (omuralide) (Craiu et al., 1997; Dick et al., 1997; Fenteany et al., 1995) and its synthetic analog PS-519 (Soucy et al., 1999). The latter (Figure 2E) was once undergoing clinical testing for the treatment of reperfusion injury (see below). Although much more specific than aldehydes, this class of compounds is not as specific as epoxyketones, and inhibition of certain serine proteases (cathepsin A and tripeptidyl peptidase II) by omuralide has been reported (see Kisselev and Goldberg, 2001, for review). Most importantly, omuralide was recently found to inhibit mitochondrial ATP-dependent protease Lon (Granot et al., 2007). Although β-lactones are potent inhibitors, they are not as potent as epoxyketones. In cell culture, omuralide is approximately five-fold less potent than epoxomicin (A.K., unpublished data).

Many β-lactone inhibitors have been discovered in the past decade (see Groll and Potts, 2011, for review). The most prom-inent member of this group is marizomib (NPI-0052, salinosporomide A; Figure 2E), a compound derived from a marine microorganism, *Salinispora tropica* (Chauhan et al., 2005a). Like omuralide, marizomib inactivates proteasomes by esterifying the catalytic threonine hydroxyl. Uniquely to marizomib, the opening of the β-lactone ring is followed by formation of a tetrahydrofuran ring as the result of nucleophilic displacement of the chloride atom of the inhibitor (Groll et al., 2006c) (Figure 3E). All β-lactone adducts are slowly hydrolyzed by water, resulting in reactivation of the proteasome (Dick et al., 1997). The tetrahydrofuran ring stabilizes the adduct, resulting in a more prolonged inhibition (Manam et al., 2008).

Marizomib is the most potent of all proteasome inhibitors presently undergoing clinical trials. It produces stronger (up to 100%) and longer-lasting inhibition of the chymotrypsin-like sites and also targets the trypsin-like and the caspase-like sites (Potts et al., 2011). It is now undergoing Phase I clinical trial for the treatment of multiple myeloma, leukemia, lymphomas, and solid tumors.

Another group of β-lactones, belactosins (Asai et al., 2004) (Figure 2E), is of interest because these inhibitors—unlike all others—bind to the so-called primed sites (i.e., mimic substrate-binding sites downstream of the scissile bonds (Groll et al., 2006d)).

The ability of so many microorganisms to generate proteasome inhibitors raises the question of how they themselves avoid the action of these substances. In many prokaryotes, the proteasome is not essential. In *Salinispora tropica,* where the proteasome is essential, the marizomib biosynthesis operon also encodes a different proteasome subunit, which is 50-fold less sensitive to marizomib than subunits encoded elsewhere in the genome (Kale et al., 2011).

Peptide vinyl sulfones are synthetic proteasome inhibitors first described by Bogyo et al. (1997) that covalently modify the proteasome's catalytic β-subunits (Figure 2F). The structure of the resulting covalent adduct shows that the hydroxyl group of the proteasome's catalytic threonine reacts with the double bond of the vinyl sulfone moiety in a Michael addition (Groll et al., 2002) (Figure 3F). Although peptide vinyl sulfones are easier to synthesize than epoxyketones, they are also less potent (Screen et al., 2010) and less specific. They do not inhibit serine proteases, but were first described as inhibitors of cysteine proteases (Palmer et al., 1995); selectivity of inhibition depends on the peptide portion of the inhibitor. However, they offer certain advantages in the development of site-specific inhibitors (see below), and many activity-based proteasome probes described in the literature are vinyl sulfones (Verdoes et al., 2006; Verdoes et al., 2010).

Syrbactins consist of a 12-membered lactam core linked to a peptide sequence (Figure 2G). The α, β-unsaturated amide in this lactam structure undergoes Michael-type 1,4-addition of the hydroxyl of the catalytic threonine to yield an irreversible ether band (Groll et al., 2008) (Figure 3G). This resembles mechanisms of inhibition by vinyl sulfones. One natural compound of this class, syringolin A (SylA), was discovered as virulence factor of the plant pathogen *Pseudomonas syringae* and shown to inhibit plant and yeast proteasomes (Groll et al., 2008). Another natural compound of this class, glidobactin A (GlbA), was discovered as an antitumor antibiotic (Oka et al., 1988). Its

cellular target was not identified until its structural similarity with SylA was noticed some 20 years later. It is not yet clear whether this class of inhibitors has any off-target effects. Given the similarity of their mechanism to that of vinyl sulfones, inhibition of cysteine proteases cannot be excluded.

Oxatiazol-2-Ones. Oxatiazol-2-ones (Figure 2H) inhibit mycobacterial proteasomes by irreversibly cyclocarbonylating them (Figure 3H) (Lin et al., 2009). This reaction causes large conformational changes in the enzyme, which are stabilized by the interactions outside of the active sites. Some critical residues that are needed to induce and stabilize these changes are different in human proteasomes, hence the selectivity of these compounds for mycobacterial proteasomes (see below for the potential therapeutic significance of these compounds).

Noncovalent Inhibitors
Cyclic Peptides. Cyclic peptides (Figure 4A): TMC-95 and its derivatives are conformationally constrained cyclic peptide natural compounds that bind tightly to all active sites of the proteasome and simply block access of substrate to the catalytic threonines (Groll et al., 2001). Total synthesis of TMC-95 has been accomplished, and several synthetic derivates with different degrees of active-site specificity have been prepared (Groll et al., 2006b). Argyrin A is another cyclic peptide natural-product proteasome inhibitor with antitumor activity (Nickeleit et al., 2008). Its binding mode to the active sites has not yet been elucidated by X-ray diffraction. Finally, recently discovered scytonemides A and B (Krunic et al., 2010) add to this growing class of natural-product proteasome inhibitors.

Noncyclic Peptides and Peptide Isosteres. The first case of proteasome inhibition by a peptide isostere was by the HIV protease inhibitor ritonavir (André et al., 1998). The related benzylstatine peptide (Figure 4B) was synthesized by investigators at Novartis in the course of an HIV protease inhibitor project. Optimization of this compound yielded a number of selective inhibitors of chymotrypsin-like site activity (García-Echeverría, 2002), ultimately leading to potent N- and C-terminally capped dipeptides (Furet et al., 2004); e.g., capped dipeptide 1 (Figure 4B). Independent efforts by scientists at Millennium led to a series of potent capped dipeptides with a similar structure (Ki for the $\beta5$ site in the $\sim$10-nM range) (Blackburn et al., 2010); e.g., capped dipeptide 2 (Figure 4B). X-ray diffraction studies revealed that the C-terminal cap binds in the S1 substrate-binding pocket, the amino acid residue side chains bind in the S2 and S3 pockets, the N-terminal cap binds in the S4 pocket, mimicking polypeptide chain upstream of the scissile bond; no contact to the active-site threonine is made (Blackburn et al., 2010). 5-methoxy-1-indanone-dipeptide benzamide (CVT-659), described a decade earlier (Lum et al., 1998), has a similar structure (Figure 4B). Thus, N- and C-terminally capped dipeptides can be considered an independently verified structural class of proteasome inhibitors. Interestingly, appending a β-lactam ring to a C-terminally capped inhibitor converts these reversible inhibitors into covalent irreversible inhibitors (Imbach et al., 2007).

Nonpeptide Inhibitors. PI-083 (Figure 4C) was identified by screening of chemical libraries (NCI diversity set) against purified proteasome (Kazi et al., 2009). It is predicted by molecular modeling to interact non-covalently with the active sites. Its most remarkable feature is that it inhibits proteasome selectively in transformed cells, targeting all three active sites. Proteasome in nontransformed cells is not inhibited. It also has much stronger activity in xenograft models of breast and lung cancer than does bortezomib.

Hydroxyureas, another class of inhibitors recently identified by screening, have been improved by subsequent chemical modification to generate a compound that inhibits chymotrypsin-like activity with a Ki in 30 nM range (Figure 4C). X-ray diffraction reveals that bulky hydrophobic groups occupy the S1 and S3 pockets but no direct contact with the active-site threonine is made (Gallastegui et al., 2012).

Nonspecific Proteasome Inhibitors
The long list of agents that inhibit the proteasome is not limited to specific proteasome inhibitors. For example, the proteasome is inhibited by the thiazole antibiotics thiostrepton and siomycin A, which block the translocation step of protein synthesis in bacteria by binding to the large ribosomal subunits (Pandit et al., 2011). Proteasomes are also reported to be inhibited by green-tea polyphenols (Nam et al., 2001), certain triterpenoids (Tiedemann et al., 2009), and many other electrophilic natural products (see Yang et al., 2010, for review). These compounds induce apoptosis in proliferating cells, but whether apoptosis is due to proteasome inhibition is not clear as these compounds may have dozens if not hundreds of other cellular targets (Liby et al., 2007; Yang et al., 2009).

Allosteric Inhibitors
PR-39 was discovered as an antibacterial 39 residue peptide in the porcine intestine. It was later shown to inhibit the proteolytic activities of the 20S proteasome allosterically by binding to the $\alpha7$ subunits (Gaczynska et al., 2003). In addition, it disrupts interaction of the 20S particles with the 19S regulatory complexes (Gaczynska et al., 2003). 5-amino-8-hydroxyquinoline is a low-micromolar noncompetitive allosteric inhibitor, shown by NMR to bind to the α subunits inside the proteasome's inner chamber (Li et al., 2010). It has the ability to overcome resistance to bortezomib in cultured cell lines.
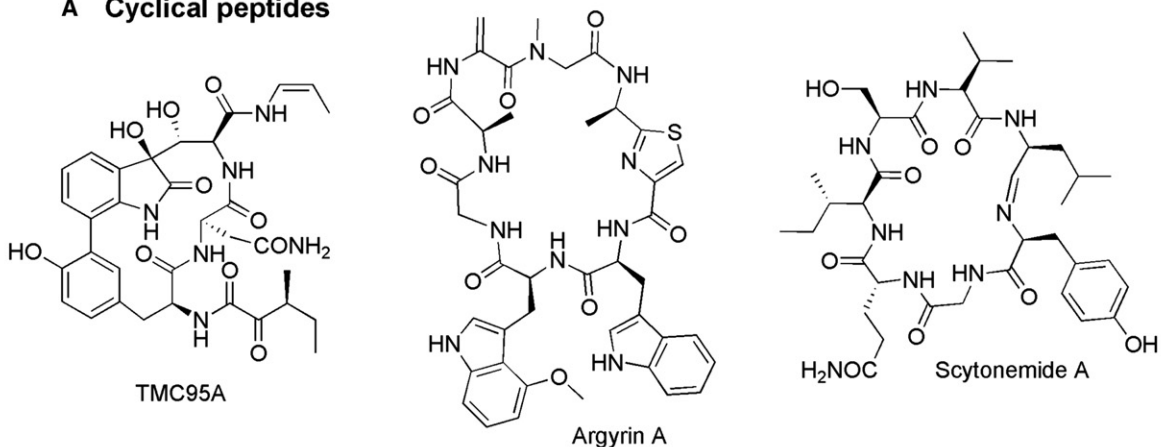
Site-Specific Inhibitors
Most inhibitors discussed above primarily block chymotrypsin-like sites but also coinhibit caspase-like and/or trypsin-like sites. Because chymotrypsin-like sites had been considered rate limiting in protein breakdown, trypsin-like and caspase-like sites were not considered drug targets until the surprising observation was made that inhibition of chymotrypsin-likes sites alone is not sufficient to block protein degradation in HeLa cells and that either caspase-like or trypsin-like sites need to be coinhibited (Kisselev et al., 2006). Because bortezomib coinhibits caspase-like sites, this observation raised the question of whether coinhibition of caspase-like sites is essential for its antineoplastic activity. This stimulated interest in the development of specific inhibitors of the individual active sites, and within a few years, cell-permeable inhibitors and active-site probes of all three activities had been developed (Figure 5).

The development of specific inhibitors of the chymotrypsin-like sites has challenged the common dogma that active-site specificity is determined by the peptide portion of the inhibitor but not by the active-site electrophile. It was found that replacing epoxyketone in NC-005 (Figure 5A) with a vinyl sulfone moiety increases specificity for the chymotrypsin-like sites (Screen et al., 2010;

A   Cyclical peptides

B   Capped dipeptides
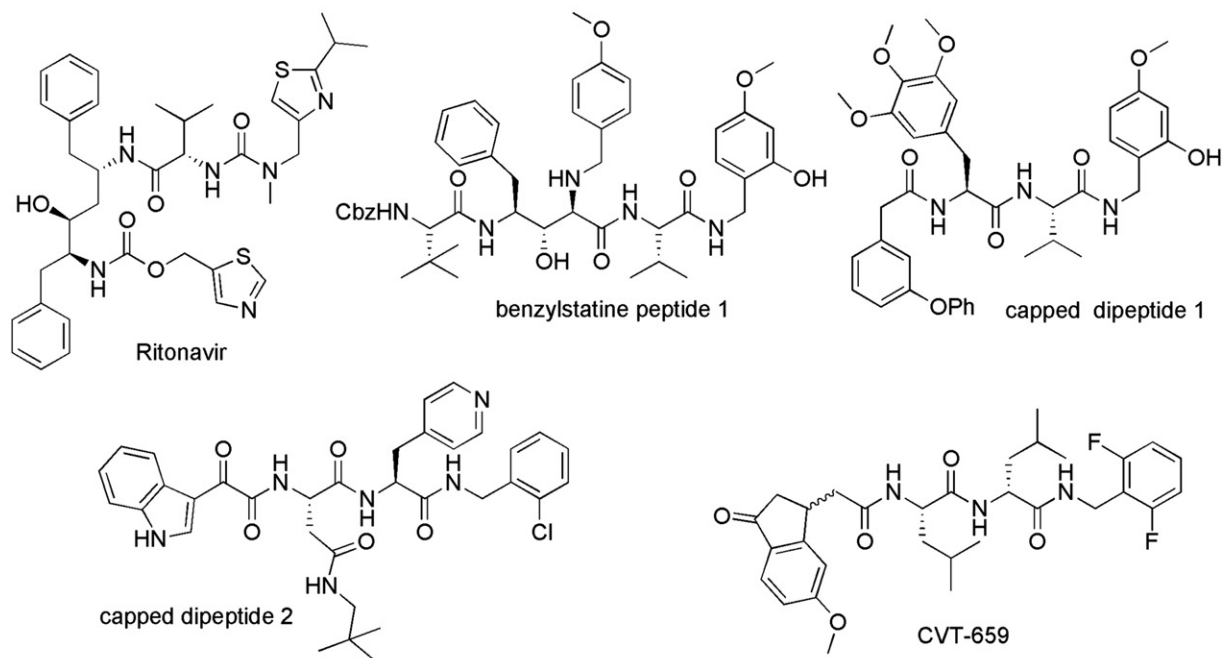
C   Others

Figure 4. Noncovalent Proteasome Inhibitors
(A) Cyclic peptides.
(B) N- and C-terminally capped dipeptides.
(C) Others.

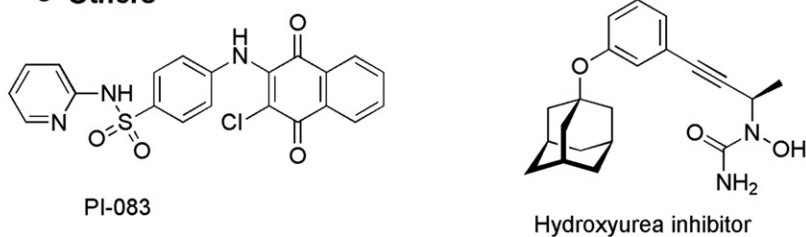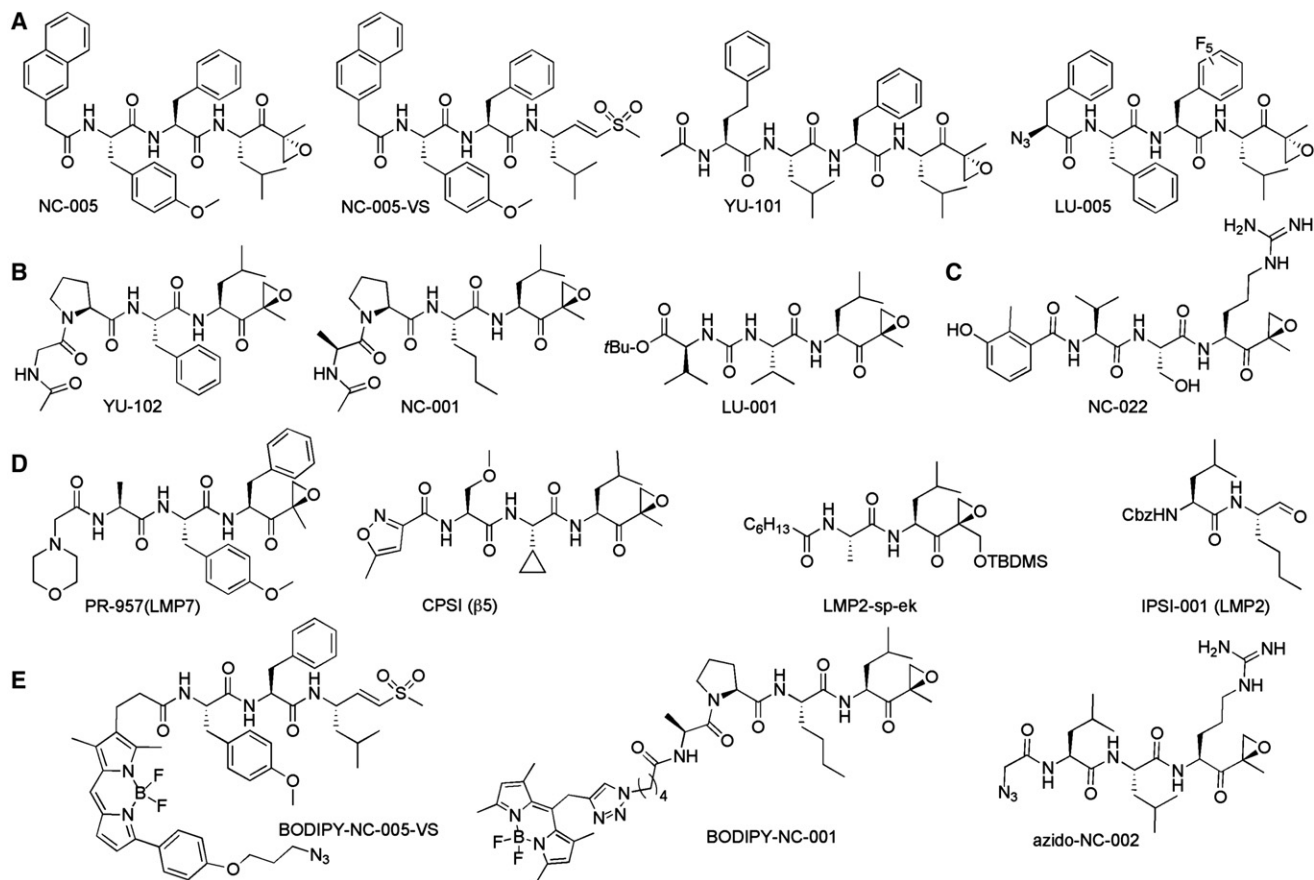**Figure 5. Site-Specific Inhibitors**
(A) Inhibitors of the chymotrypsin-like sites: YU-101 (Elofsson et al., 1999), NC-005 (Britton et al., 2009), NC-005-VS (Screen et al., 2010), and LU-005 (Geurink et al., 2010).
(B) Inhibitors of the caspase-like sites. YU-102 (Myung et al., 2001), NC-001 (Britton et al., 2009), and LU-001 (van der Linden et al., 2012) inhibit β1 and β1i sites.
(C) Inhibitor of the trypsin-like sites (Mirabella et al., 2011).
(D) Inhibitors with selectivity for immunoproteasome subunits over their constitutive counterparts and vice versa. PR-957 (Muchamuel et al., 2009) is β5i (LMP7)-selective, and CPSI (Parlati et al., 2009) is β5-selective. LMP2-sp-ek (Ho et al., 2007) and IPSI-001 (Kuhn et al., 2009) are β1i (LMP2)-selective.
(E) Activity-based probes (Mirabella et al., 2011; Verdoes et al., 2010). Azido-NC-002 requires subsequent modification by a biotinylated phospane in a Staudinger-Bertozzi ligation to reveal polypeptides modified by the probe.

Verdoes et al., 2010). Interestingly, increasing specificity dramatically decreased cytotoxicity for HeLa cells (Screen et al., 2010).

A bigger challenge has been the development of cell-permeable inhibitors of the trypsin-like sites. Several specific but cell-impermeable inhibitors of these sites were synthesized in the past decade (Loidl et al., 1999; Nazif and Bogyo, 2001). Another structural class, peptide vinyl esters (Marastoni et al., 2005), initially reported as cell-permeable inhibitors of the trypsin-like sites, did not have any inhibitory activity when resynthesized by another group (Screen et al., 2010). Finally two cell-permeable peptide epoxyketones were discovered last year (Mirabella et al., 2011) (Figure 5C). These inhibitors and also inhibitors of caspase-like sites (Figure 5B) (Britton et al., 2009) sensitize MM cells to inhibitors of the chymotrypsin-like sites. Furthermore, inhibitors of the trypsin-like sites selectively sensitize MM cells to bortezomib and carfilzomib (Mirabella et al., 2011). Thus, while the chymotrypsin-like sites are the major drug targets in cancer, co-targeting the caspase-like and trypsin-like sites increases cytotoxicity of proteasome inhibitors. Site-specific inhibitors can

now be used to define the active-site profile needed to achieve maximal cytotoxicity and best selectivity for malignant cells.

Given the subtle differences in specificity between constitutive and immunoproteasomes, the most impressive achievements of recent years were the developments of specific inhibitors (Figure 5D) of the chymotrypsin-like subunit of the immunoproteasome (β5i/LMP7) (Muchamuel et al., 2009) and its constitutive counterpart β5 (Parlati et al., 2009), as well as of the caspase-like subunit of the immunoproteasome (LMP2/β1i) (Ho et al., 2007; Kuhn et al., 2009). Taken together, all these inhibitors enable investigators to individually downregulate individual active sites to the desired extent in living cells and in some cases (e.g., using LMP7 inhibitor) in laboratory animals.

Potential Therapeutic Applications of Proteasome Inhibitors beyond Cancer
Treatment of Organ Transplant Patients
As discussed at the beginning of this review, production of large quantities of antibodies by MM cells make them exquisitely

Table 2. Activity of Proteasome Inhibitors in Rodent Models of Autoimmune and Inflammatory Diseases

Disease/model	Inhibitor	Reference
lupus nephritis	bortezomib	(Neubert et al., 2008)
lupus	PR-957	(Ichikawa et al., 2011)
myasthenia gravis	bortezomib	(Gomez et al., 2011)
multiple sclerosis	bortezomib	(Fissolo et al., 2008)
streptococcal cell-wall induced polyarthritis	bortezomib	(Palombella et al., 1998)
rheumatoid arthritis	PR-957	(Muchamuel et al., 2009)
irritant sensitivity	epoxomicin, YU-101	(Elofsson et al., 1999)
psoriasis	PS-519	(Elliott et al., 2003)
asthma	PS-519	(Elliott et al., 1999)
colitis	bortezomib	(Schmidt et al., 2010)
	PR-957	(Basler et al., 2010)

sensitive to proteasome inhibitors. Nonmalignant MM precursors—antibody-producing plasma cells—are also very sensitive to proteasome inhibitors (Bianchi et al., 2009; Cenci et al., 2006). This sensitivity is being explored therapeutically for treatment of acute allograft rejection in transplant patients (Everly, 2009; Trivedi et al., 2009). Although this treatment is not officially approved by the FDA, this is the second indication for which bortezomib is being used clinically and the first outside oncology.

Autoimmune Diseases

The same mechanism—selective destruction of antibody-producing plasma cells—is behind bortezomib activity in animal models of autoimmune diseases, including lupus nephritis, myastenia gravis, and others (Table 2). Although bortezomib is currently in phase IV trials for the treatment of lupus nephritis in humans, the less toxic second-generation inhibitors have a much better chance of being used clinically for this indication. An even better choice would be the LMP7-selective inhibitor PR-957 (ONX0914), which attenuates progression of autoimmune rheumatoid arthritis (Muchamuel et al., 2009) and lupus (Ichikawa et al., 2011) in the experimental murine models. The effect is observed at about 1/10 of maximal tolerated dose (MTD), while bortezomib and carfilzomib exert these effects at concentrations close to MTD. PR-957 not only decreases production of antibodies but also dramatically lowers levels of multiple proinflammatory cytokines by affecting a yet-to-be-defined pathway (Muchamuel et al., 2009). This effect is apparently NF-κB independent as no inhibition of NF-κB activity (see below) was observed at concentrations that blocked cytokine productions. It is immunoproteasome-specific as a specific inhibitor of the β5 subunit did not block cytokine production. This data suggest that functions of immunoproteasome extend beyond production of antigenic peptides.

Anti-Inflammatory Activity of Proteasome Inhibitors

The critical biochemical event in initiation of the inflammatory response is the rapid destruction of the IκB inhibitor of the transcription factor NF-κB (Palombella et al., 1994), which activates the expression of many genes encoding inflammatory mediators (e.g., TNF, IL-1, IL-6), enzymes (cyclooxygenase, NO synthase), and leukocytes adhesion molecules (ICAM, VCAM) (Pahl, 1999). In fact, bortezomib was initially pursued as an anti-inflammatory

agent (Goldberg, 2010). Anti-inflammatory effects of proteasome inhibitors have been demonstrated in animal models of arthritis, psoriasis, asthma, colitis, and other inflammatory conditions (Table 2). As discussed above, recent findings indicate that anti-inflammatory effects of proteasome inhibitors may not necessarily arise from inhibition of NF-κB activation (Muchamuel et al., 2009).

Treatment of Reperfusion Injury after Stroke

The ability of proteasome inhibitors to reduce inflammation provides the rationale for their development for treatment of ischemic stroke. When the site of ischemic brain injury in stroke patients is reperfused, inflammation occurs, exacerbating injury; treatments are needed to prevent this damage. Since 2000, numerous studies have been conducted using middle cerebral artery occlusion and reperfusion injury model in rats (reviewed in Williams et al., 2006). The β-lactone proteasome inhibitor PS-519 (Figure 2E) has been found to reduce activation of NF-κB, attenuate production of cytokines and cellular adhesion molecules, and reduce neutrophil and macrophage infiltration in rat brain (Williams et al., 2006). Proteasome inhibitors' ability to promote nerve growth-factor secretion (Hines et al., 2008) may be an additional factor contributing to neurologic recovery in animals treated with this compound. PS-519 successfully completed phase I clinical trials in humans (Shah et al., 2002). However, the anticipated high cost of further clinical trials and high failure rate of past trials in stroke impedes further development of this compound.

Stimulation of Bone and Hair Growth by Proteasome Inhibitors

The ability of proteasome inhibitors to promote bone growth was discovered during a cell-based screen to identify compounds that stimulate transcription from the bone morphogenic protein (BMP)-2 promoter. Peptide aldehyde proteasome inhibitor PSI (Figure 2A) was one of the compounds identified in this screen (Garrett et al., 2003). Other proteasome inhibitors (e.g., epoxomicin, lactacystin) had the same effect. Mechanism of activation involves inhibition of processing of the transcription factor Gli-3 of the Hedgehog signaling pathway into a truncated form that represses the BMP-2 promoter (Garrett et al., 2003). Interestingly, bortezomib has been associated with osteoblast activation in MM patients (Zangari et al., 2005).

The studies of effects of proteasome inhibitors on bone growth in mice (Garrett et al., 2003) involved subcutaneous injections of PSI. During these experiments, investigators noticed increased growth of new hair follicles around injection sites (Mundy et al., 2007). Further experiments showed that this effect can be obtained when the compound is applied topically and that the mechanism of increase also involves upregulation of the BMP-2 pathway. These experiments in mice led to successful phase I and II human trials of topical PSI for treatment of male pattern baldness. Small amounts that eventually got absorbed into blood were most likely rapidly oxidized, and systemic toxicity was avoided.

Proteasome Inhibitors as Anti-Infectives

Growing resistance of *M. tuberculosis* to antibiotics as well as an absolute requirement for proteasomes for pathogen persistence in mice (Gandotra et al., 2007) makes the bacterial proteasome an attractive target for novel therapeutics. Oxathiazol-2-one proteasome inhibitors (Figure 2H) are only the second class of
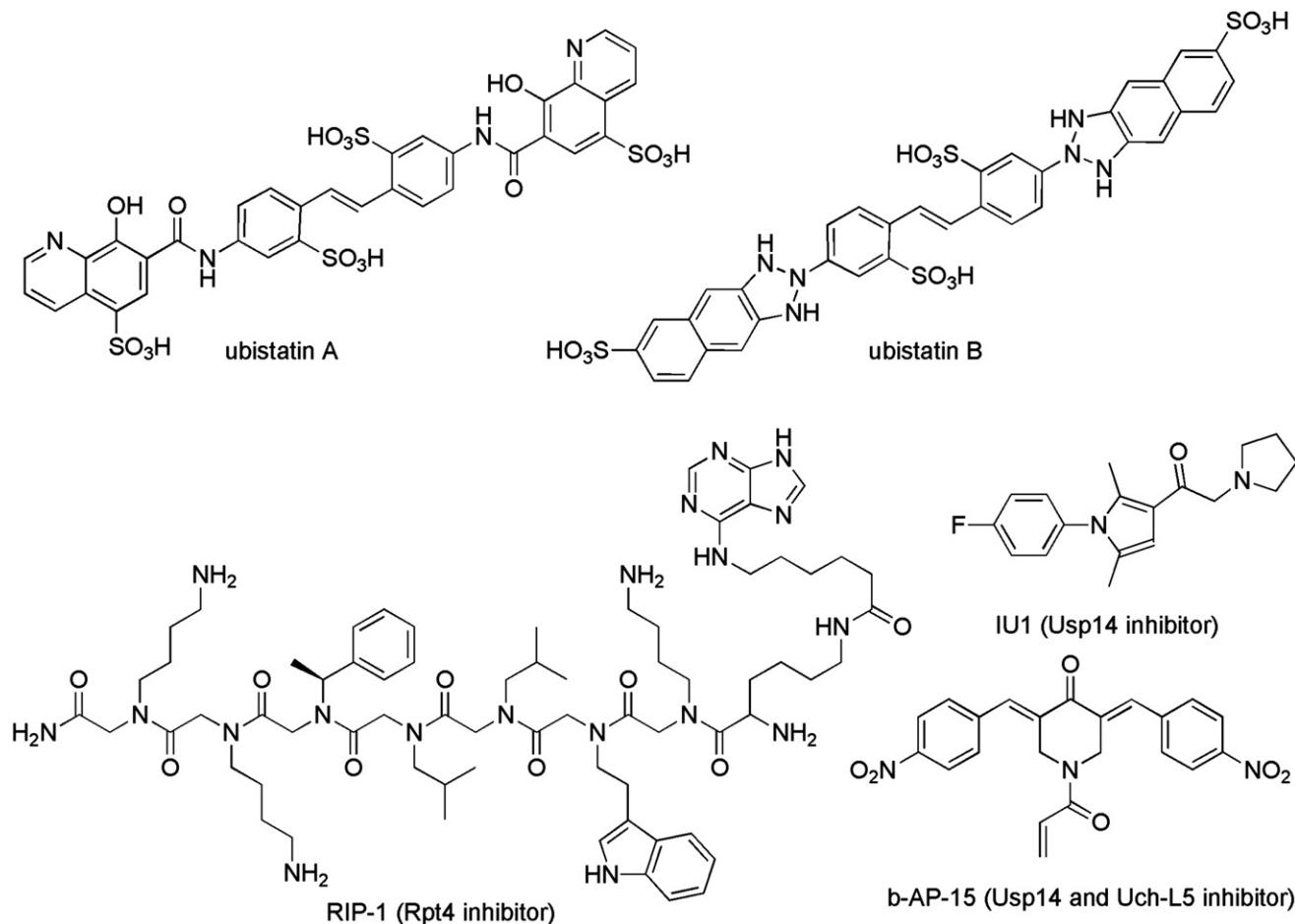
Figure 6. Inhibitors of 19S RP

compounds with significant ability to kill nonreplicating bacteria (Lin et al., 2009). Proteasome inhibitors can kill the malarial parasite *Plasmodium falciparum* at different stages of its life cycle (Czesny et al., 2009) and have trypanocidal activities (Steverding et al., 2005). However, it remains to be determined whether inhibitors selective for the proteasomes of these lower eukaryotes can be developed.

Inhibitors of the 19S Regulatory Particles and Their Potential Uses

19S regulatory particles (RP) contain at least 19 different polypeptides. RP recognize ubiquitylated proteins and unfold them, control access of substrates to the core, and recycle ubiquitin. This particle has been a subject of extensive investigations in the past decade (see Finley, 2009, for review). They revealed that ubiquitylated proteins bind to multiple receptors. Ubiquitin chain is removed and recycled. Substrates are unfolded and threaded into the proteolytic core through a narrow gated channel in the α ring of the 20S core (Figure 1B). The unfolding and translocation is carried out by 6 ATPases of AAA family that form a ring, interacting with the 20S core. Another function of these ATPases is to open the channel in the 20S core (Köhler et al., 2001).

Development of inhibitors of RP is lagging behind the inhibitors of 20S core. Potential drug targets in the RPs are ATPases, ubiq-

uitin receptors, and deubiquitylating enzymes (Figure 1A). The first inhibitors of the 19S RP to be reported were ubistatins (Figure 6), which blocked binding of ubiquitin chains to their receptors (Verma et al., 2004). However, in the 7 years since their discovery, no single study using these compounds has been published. One purine-capped peptoid inhibitor of the Rpt4 ATPase of the 19S RP has been reported (Lim et al., 2007). However, this compound has not been tested for off-target effects. Given the abundance of ATPases in the cell in general and specifically of the AAA family of ATPases, development of specific inhibitors of proteasomal ATPases is expected to be challenging.

The 19S RP contains three deubiquitylating enzymes, Rpn11, Usp14, and Uch37 (Uch-L5). Rpn11 is a metalloprotease. Rpn11-mediated removal of ubiquitin chains is associated with substrate degradation (Verma et al., 2002). Its activity is essential for substrate degradation. Inhibitors of Rpn11 are expected to exert biological effects similar to or even stronger than those of inhibitors of proteolytic sites. Usp14 (Ubp6 in yeast) and Uch-L5 are cysteine proteases. They mediate stepwise ubiquitin removal from the distal end of the chain. Usp14 activity antagonizes protein degradation (Lee et al., 2011). Two inhibitors of isopeptidases were discovered recently, which have opposite effects on protein degradation (D'Arcy et al., 2011; Lee et al.,

2010). In contrast to other proteasome inhibitors, specific Usp14 inhibitor IU-1 (Figure 6) stimulates protein degradation, including breakdown of oxidatively damaged proteins and of specific proteins implicated in neurodegenerative disease-associated proteotoxicity (Lee et al., 2010). Because of this, Usp14 is now being pursued as a target for treatment of neurodegenerative diseases where stimulation of proteasome by Usp14 inhibitors is expected to have a therapeutic benefit. Conversely, dual inhibitor of Usp14 and Uch-L5, b-AP-15 (Figure 6) has a biological effect similar to traditional proteasome inhibitors—accumulation of ubiquitylated protein, induction of apoptosis of malignant cells, and inhibition of growth of tumor cells (D'Arcy et al., 2011). The reasons between the opposite effects of these two different Usp14 inhibitors are not clear. One possibility is that Uch-L5 activity is required for protein degradation and that coinhibition of Uch-L5 overrides the stimulatory effects of Usp14 inhibition on proteolysis.

Future Directions

Ten years ago, we predicted the discovery of new natural product proteasome inhibitors. This has happened with the discovery of marizomib, syringolin A, fellutamide B, and others. While this trend may continue in the next decade, screening efforts by academic and industrial laboratories and subsequent modification of the hits will certainly generate more synthetic inhibitors as well. Synthetic efforts will focus more on site-specific inhibitors, especially for subunits for which no selective inhibitors are available (e.g., β2, β2i, β1, β5t). The next decade will also see further development of Usp14/Ubp6 inhibitors for treatment of neurodegenerative diseases. We may also witness the development of first-of-their-kind inhibitors of ATPases of 19S RPs and of Rpn11 inhibitors. We will see the introduction of second-generation proteasome inhibitors in clinical practice for the treatment of myeloma and perhaps also of other cancers. At the same time, development of inhibitors for therapeutic use outside oncology (e.g., in autoimmune disease) will continue, and we may also see FDA approval of such agents for these diseases. We anticipate that this trend will continue, and hope that ongoing efforts to discover new proteasome inhibitors and elucidate targets of natural products will further expand the therapeutic potential of proteasome inhibitors.

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