REVIEW

Novel Proteasome Inhibitors to Overcome Bortezomib Resistance

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The proteasome is an intracellular enzyme complex that degrades ubiquitin-tagged proteins and thereby regulates protein levels within the cell. Given this important role in maintaining cellular homeostasis, it is perhaps somewhat surprising that proteasome inhibitors have a therapeutic window. Proteasome inhibitors have demonstrated clinical efficacy in the treatment of multiple myeloma and mantle cell lymphoma and are under evaluation for the treatment of other malignancies. Bortezomib is the first and only Food and Drug Administration–approved proteasome inhibitor that inhibits this enzyme complex in a reversible fashion. Although bortezomib improves clinical outcomes when used as a single agent, most patients do not respond to this drug and those who do respond almost uniformly relapse. As such, efforts are underway to develop proteasome inhibitors that act through mechanisms distinct from that of bortezomib. Specifically, inhibitors that bind the active site of the proteasome and inhibit the complex irreversibly have been developed and are in advanced clinical trials. Inhibitors that act on sites of the proteasome outside of the catalytic center have also been identified and are in preclinical development. In this review, we discuss the structure and function of the proteasome. We then focus on the molecular biology, chemistry, and the preclinical and clinical efficacy of novel proteasome inhibitors as strategies to inhibit this target and overcome some forms of bortezomib resistance.

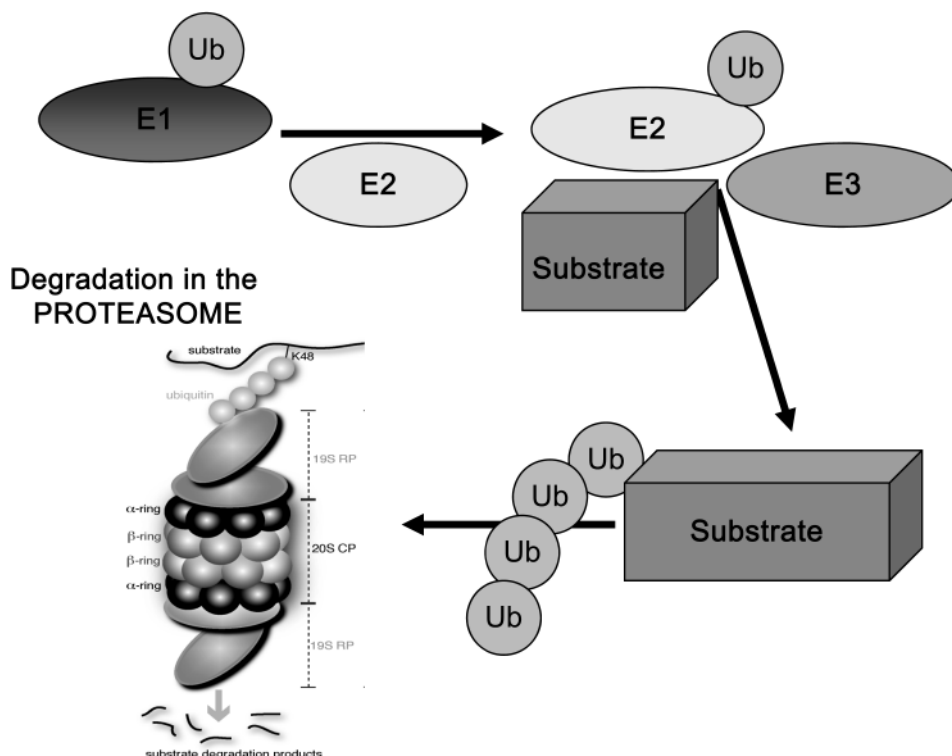## The Ubiquitin and Proteasome Pathway

The ubiquitination and proteasome degradation pathway is a multistep enzymatic cascade in eukaryotes in which ubiquitin is conjugated via a lysine residue at position 48 to target proteins for destruction. Proteins tagged with lysine 48–linked chains of ubiquitin are marked for degradation in the proteasome enzyme complex (Figure 1). Through this pathway, the cell rids itself of excess and misfolded proteins and regulates biological processes, including cell proliferation. In the first step of this cascade, a ubiquitin-activating enzyme (E1) hydrolyzes ATP and adenylates and binds a ubiquitin molecule. A second ubiquitin molecule is then adenylated and bound to a different site of the same E1 enzyme (1–3). The E1 enzyme then transfers the first adenylated ubiquitin molecule to a ubiquitin-conjugating enzyme (E2). In the final step, a ubiquitin ligase (E3) catalyzes the transfer of the ubiquitin from the E2 enzyme to a lysine residue on the target protein (4). This step is repeated to create a growing polyubiquitinated chain on the target protein. Proteins that are polyubiquitinated are then targeted for degradation by the proteasome (5). To date, only four E1-activating enzymes involved in transferring ubiquitin molecules to E2 ligases have been identified across multiple species: Uba1, Uba6, Ube1x, and Ube1y. Of these, Uba1 is the predominant isoform in humans and other species. In contrast, Ube1y is expressed only in the mouse testis (6). Uba6 and Uba1x are expressed in most tissues, but their functions and possible redundancy with Uba1 are not well understood (6). In contrast to the limited number of E1 enzymes, approximately 30 E2 enzymes, and

hundreds of E3 ligases have been identified. Through this diversity, the E2 and E3 ligases establish the specificity of the ubiquitin and proteasome pathway (7,8). Of note, proteins that are tagged with a single ubiquitin group or with lysine 63–linked chains of ubiquitin are not marked for proteasomal degradation. Rather, these forms of ubiquitination alter protein function or localization and thereby regulate processes such as receptor internalization (9), endocytosis (10), transcription (11), and DNA repair (12–14).

Proteasome Structure and Function

Detailed biophysical and biochemical studies have established that the 26S proteasome consists of a 20S core particle that is bound to one or two 19S regulatory particles (15–17) (Figure 2, A). The 20S core particle contains the active sites that hydrolyze substrate peptide bonds. The 19S regulatory particle is responsible for substrate recognition, deubiquitination, unfolding, and translocation into the core particle. Detailed x-ray structures of 20S proteasomes from the archaebacterium *Thermoplasma acidophilum* and yeast (18,19) indicate that core particles from different organisms are generally built from a common prototype comprising four stacked heptameric rings, which form a cylindrical-shaped hollow cavity that sequesters the active sites that catalyze the peptide bond hydrolysis reaction. Substrates enter the interior of each core particle cavity via a narrow (eg, 13-Å diameter in *T. acidophilum*) passageway known as the alpha-annulus, which is located at either end of the barrel-like structure of the 20S core particle. Entry into the

Figure 1. The ubiquitin–proteasome pathway. The ubiquitination and proteasome degradation pathway marks proteins with lysine 48–linked chains of ubiquitin for degradation in the proteasome enzyme complex. The ubiquitin E1 enzyme, Uba1, binds ubiquitin and transfers it the E2 enzyme. The E2 enzyme transfers the ubiquitin to the target protein (substrate) with the help of E3 ubiquitin ligase. Proteins that are polyubiquitinated are then targeted for degradation by the proteasome. CP = core particle; K48 = lysine 48; RP = regulatory particle; Ub = ubiquitin.
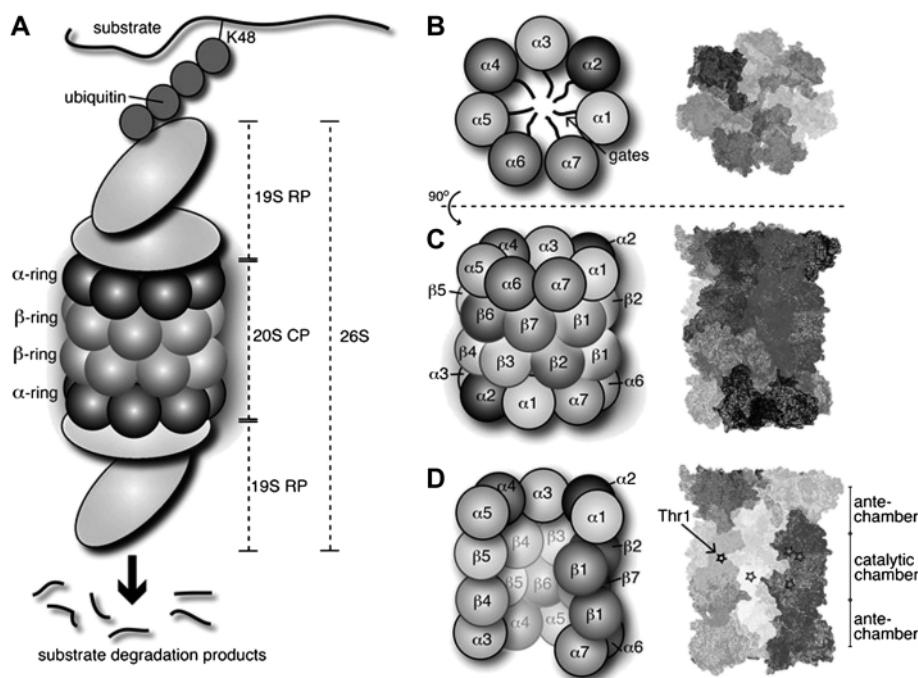
alpha-annulus is regulated by the N-termini of the alpha subunits, which act as "gates" (Figure 2, B) that are opened upon binding of the 19S regulatory particles (20). The 19S regulatory particle comprises: 1) a base, which contains both a hexameric ring of ATPases that binds to the 20S core particle and leads to gate opening, as well as four other proteins, including Rpn10 and Rpn13, which recognize ubiquitin, and UCH37 and Ubp6, which are C-terminal

ubiquitin hydrolases that process ubiquitin, and 2) a lid consisting of at least nine polypeptide chains including Rpn11, which removes ubiquitin from substrates (17,21).

High-resolution crystal structures are available for the 20S core particle from archaebacterial proteasomes, including *T. acidophilum* (18), as well as for bovine (*Bos taurus*) (22) and yeast (*Saccharomyces cerevisiae*) (19) eukaryotic proteasomes. They reveal that the heptameric

Figure 2. Structure of the proteasome. **A**) Topological arrangement of proteins in the 26S proteasome. The 26S proteasome consists of the 20S core particle (CP) (built from four stacked heptameric rings) and the 19S regulatory particle (RP). Substrates ubiquitinated with chains of ubiquitin linked at Lys48 (K48) are recognized by the 19S RP; substrates are subsequently deubiquitinated, unfolded, and passed into the 20S CP for degradation. **B**) Top view of the structure of the yeast proteasome showing the arrangement of the component α subunits in each α-ring in both a cartoon representation (**left**) and mesh representation based on the crystal structure (**right**). The amino termini of the α subunits form gates that block the approximately 13 -Å diameter α-annulus, through which substrates enter the 20S CP. The gate is shown closed in the crystal structure. **C**) Side view of the structure of the yeast proteasome showing the outside surface via the two types of representations as described in (**B**). **D**) Side view with two subunits removed per ring to reveal the inside of the 20S CP. Each interface between rings forms a chamber, with the antechamber at the interface between the α- and β-rings and the catalytic chamber at the interface between β-rings. **Stars** indicate the active sites in the β1, β2, and β5 subunits that correspond to the positions of the atoms that act as nucleophiles in the hydrolysis reaction. K48 = lysine 48.

rings of prokaryotic core particles, each of which are composed of seven identical α or β subunits, are stacked in a specific order ($\alpha_7\beta_7\beta_7\alpha_7$) such that the gates and active sites are located on the α and β rings, respectively (Figure 2, A). These rings generate three interconnected chambers: two outer antechambers that are formed by the adjacent α and β rings and a catalytic chamber that is formed by the two adjacent β rings. Eukaryotic proteasomes have a similar structure except that the α and β rings are each composed of seven related yet distinct subunits (ie, α1–α7 and β1–β7, respectively). In addition, the α and β subunits have a fixed topological arrangement within each ring (Figure 2, C and D), and they differ from each other by various insertion segments and amino- and carboxyl-terminal extensions that make contacts between adjacent subunits (19). However, whereas all 14 β subunits in each prokaryotic core particle form an active site capable of catalyzing peptide bond hydrolysis, only a subset of β subunits are catalytically active in eukaryotic core particles. For example, β1, β2, and β5 are catalytically active in both the yeast and bovine proteasomes (19,23,24), and the location of functional groups important to catalysis in the β7 subunit has led to the suggestion that it may also be active in the bovine proteasome (22). Substrate specificity pockets located in the vicinity of each active site bind to 10 amino acid stretches in the substrate that flank the peptide bond that is cleaved and thereby determine the cleaving preferences of the β1, β2, and β5 subunits (25,26). In particular, β1 has caspase-like activity and a limited preference for branched-chain amino acids (cleaving after acidic residues, as well as after Ile, Leu, and Val), β2 exhibits trypsin-like activity (cleaving after basic residues), and β5 has chymotrypsin-like activity (cleaving after hydrophobic residues, as well as a tendency to cleave after small neutral and branched side chains) (19,24,26–28).

Degradation of protein substrates by the proteasome requires the protein to traverse the regulatory gate of the proteasome and interact with the proteolytic enzymes in the catalytic core. Binding of 19S regulatory particles (also known as activators) to the α rings results in gate opening, which enable substrates to enter the 20S core particle, where they are degraded. The different regulatory particles (ie, 19S, 11S, Blm10, and their homologs in different organisms) appear to achieve a gate opening by a similar mechanism, whereby the carboxyl-terminal residues of the component activator subunits (eg, Rpt2 and Rpt5 of the 19S regulatory particle) bind to a pocket formed at the interface of adjacent α subunits in the α ring (29,30). Following gate opening, substrates translocate into the catalytic chamber of the core particle, where
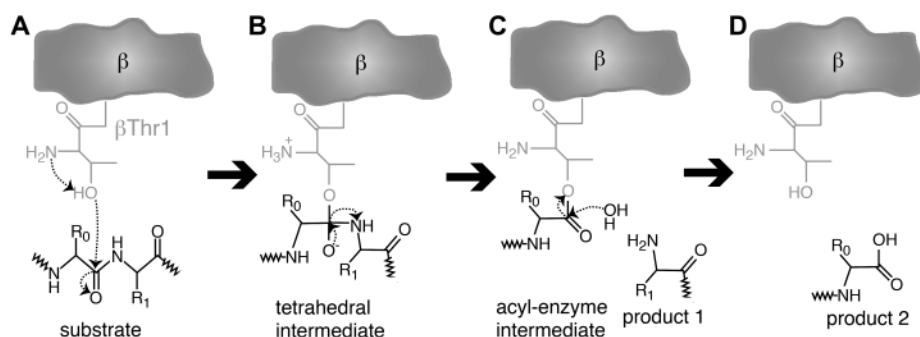
they are degraded into peptides that vary from 3 to 25 amino acids in length (average length distribution: 8–12 amino acids) (26,31,32). Degradation is processive, whereby each substrate is cleaved in multiple locations without release of partially hydrolyzed substrates from the core particle (32). The mechanism of degradation is conserved for all types of catalytically active β subunits (25,33) and is summarized in Figure 3. The β subunits are members of the N-terminal nucleophile (Ntn) hydrolyase family of proteins (34), in which the amino-terminal amino acid β threonine 1 (βThr1) acts as the catalytic nucleophile in the hydrolysis reaction. Specifically, the carbon atom in the substrate amide bond undergoes nucleophilic attack by the hydroxyl group of βThr1 (18) to yield a tetrahedral intermediate (Figure 3). The amino terminus of βThr1 facilitates this step by acting as a general base whereby it extracts the proton from the βThr1 hydroxyl group (18). Subsequently, the intermediate decomposes in a manner that results in breakage of the substrate peptide bond, which releases one substrate degradation product, whereas the other degradation product remains conjugated to the active site via an ester linkage to form the acyl-enzyme intermediate (Figure 3). Finally, water attacks the acyl-enzyme intermediate, resulting in a second tetrahedral intermediate that yields a second substrate cleavage product, with the active site of the proteasome regenerated for further catalytic cycles (25,33).

In eukaryotes, the 20S core particle components can change in response to biological stimuli. For example, stimulation of cells with interferon gamma induces the expression of three catalytically active β subunits. These subunits, along with a unique 11S regulatory particle, form a complex called the immunoproteasome. The immunoproteasome is involved in generating peptides for presentation to major histocompatibility complex class I molecules, but it also has classic proteolytic activity (35–37). Increased expression of the immunoproteasome complex has been reported in myeloma (38–41), where it may represent the predominant form of the proteasome. It is also noteworthy that relapsed myeloma may be associated with lower levels of the immunoproteasome and increased levels of the constitutive proteasome (41).

Proteasome Inhibitors

Proteasome inhibitors induce cell death in malignant cells in culture, which is not surprising given the important role of the proteasome in regulating levels of many proteins that are required for normal cellular functions. What is perhaps surprising is that

Figure 3. Mechanism of substrate degradation by the proteasome. **A**) The substrate amide group undergoes nucleophilic attack by the proteasome, where the amino terminus of β threonine 1 functions as a general base. **B**) The corresponding tetrahedral intermediate collapses to release one substrate degradation product, **C**) whereas the other product remains conjugated to the proteasome via an ester linkage . **D**) Water can attack this ester to result in a second tetrahedral intermediate that decomposes to yield the second substrate degradation product.

inhibition of this complex has a therapeutic window and is preferentially toxic to malignant cells and that proteasome inhibitors display preclinical activity in mouse models of malignancy and clinical efficacy in myeloma and mantle cell lymphoma.

Bortezomib is the first and, currently, the only proteasome inhibitor approved by the US Food and Drug Administration for the treatment of relapsed multiple myeloma and mantle cell lymphoma (42,43). Bortezomib is a competitive inhibitor of the proteasomal enzymes that reversibly binds the active site of the proteasome. Proteasome inhibitors with different mechanisms of action have been developed in an effort to overcome resistance to bortezomib and develop proteasome inhibitors with different toxicity profiles. These additional proteasome inhibitors include drugs that bind irreversibly to the active sites of the proteasome as well as molecules that allosterically inhibit the function of the proteasome by binding the complex outside of the active site. Bortezomib and other proteasome inhibitors will be discussed in detail below.

Although the preclinical and clinical efficacy of proteasome inhibitors as antitumor agents has been well described, the mechanism by which proteasome inhibitors lead to cell death of malignant cells in vitro and in vivo has not been fully elucidated. Proteasome inhibitor–induced cell death is associated with induction of endoplasmic reticulum (ER) stress and activation of the unfolded protein response, inhibition of the nuclear factor kappa B inflammatory pathway, activation of caspase-8 and apoptosis, and increased generation of reactive oxygen species (44–47). Although questions remain regarding the cell-based effects of proteasome inhibitors and the basis for their therapeutic window, clinical studies have demonstrated the efficacy of this class of therapeutics.

Competitive Proteasome Inhibitors

The nucleophilic nature of the active site Thr1 residue of each catalytic β subunit (see above) has been exploited in the design of
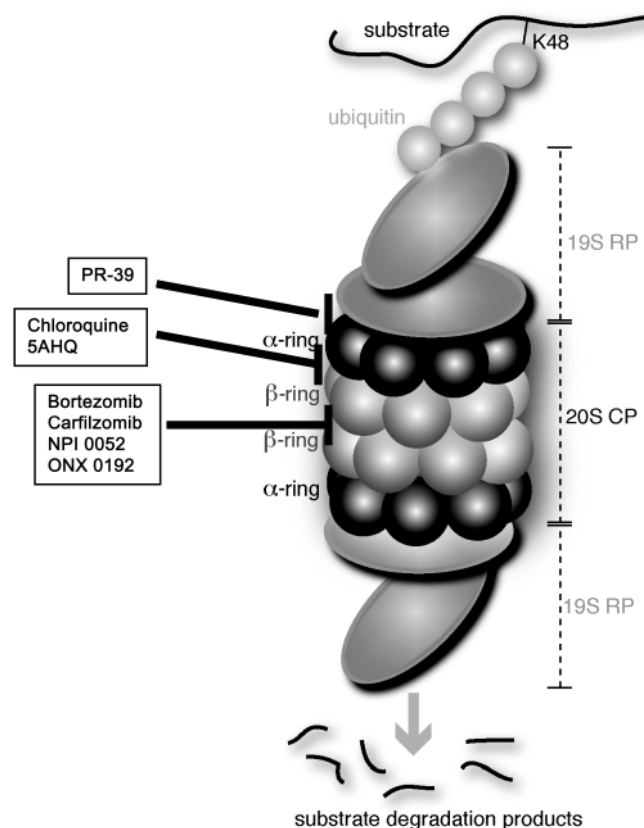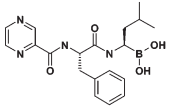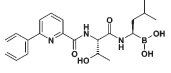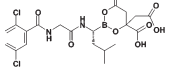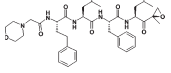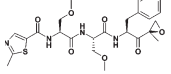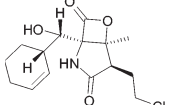

Figure 4. Sites of action of proteasome inhibitors. The reversible proteasome inhibitor bortezomib and the irreversible proteasome inhibitors carfilzomib, ONX 0912, and NPI 0052 bind the active site of the proteasome at the β5 subunits of the 20S core particle (CP). Chloroquine and 5AHQ interact with the proteasome at the interface between the α and the β subunits outside of the active site of the proteasome. The peptide PR-39 binds the α subunits, likely at the top of the CP, near the 19S subunit. 5AHQ = 5-amino-8-hydroxyquinoline; K48 = lysine 48; RP = regulatory particle.

Table 1. Reversible and irreversible proteasome inhibitors*

Chemical structure	Name	Type	Developer	Route of administration	Development status
	Bortezomib	Reversible	Millennium: The Takeda Oncology Company	Intravenous	FDA approved
	CEP-18770	Reversible	Cephalon	Intravenous, oral	Phase I
	MLN-9708	Reversible	Millennium: The Takeda Oncology Company	Intravenous, oral	Phase I
	Carfilzomib	Irreversible	Onyx	Intravenous	Phase IIb
	ONX 0912	Irreversible	Onyx	Oral	Phase I
	NPI-0052	Irreversible	Nereus	Intravenous	Phase I

* FDA = Food and Drug Administration.

proteasome inhibitors with electrophilic functional groups. Selectivity of these inhibitors is dictated by the composition of the substrate-binding pocket, which differs in the three catalytic β subunits (48,49). A wide range of specific peptidyl compounds and natural products have been developed that inhibit the enzymatic complex of the proteasome at low nanomolar concentrations (50–52).

Bortezomib. Bortezomib (N-acyl-pseudo dipeptidyl boronic acid) is a dipeptide that binds reversibly to the chymotrypsin-like β5 subunit of the catalytic chamber of the 20S proteasome (Figure 4, Table 1). Bortezomib is the first and, currently, the only proteasome inhibitor approved by the US Food and Drug Administration (42,43).

Clinical efficacy. Initial approval of bortezomib for patients with relapsed and refractory myeloma was based on data from a phase II study in which patients with relapsed and refractory myeloma received intravenous bortezomib (1.3 mg/m^2 on days 1, 4, 8, and 11 in 21-day cycles for up to eight cycles) (42). Twenty-eight percent of patients achieved a complete, near-complete, or partial response and 35% had some response. The median duration of the response among patients with any response to the drug was 12 months (range = 1.3–16.7 months). Toxic effects of this therapy included grade 3 thrombocytopenia (28% of patients) and grade 3 neuropathy (12% of patients). The incidence of neuropathy of any grade was 31%. In a phase III study (53), patients with relapsed myeloma were randomly assigned to receive intravenous bortezomib (1.3 mg/m^2 on days 1, 4, 8, and 11 in 21-day cycles for cycles 1–8) followed by intravenous bortezomib (1.3 mg/m^2 on days 1, 8, 15, and 22 in 35-day cycles for cycles 9–11) or high-dose oral dexamethasone (40 mg on days 1–4, 9–12, and 17–20 in 35-day cycles for cycles 1–4 and on days 1–4 in 28-day cycles for cycles 5–9). Patients who received bortezomib had better clinical outcomes compared with those who received dexamethasone, including higher complete and partial response rates (38% for bortezomib vs 18% for dexamethasone) and longer 1-year survival.

More recently, bortezomib has been used in front-line therapy for myeloma, either as a single agent or in combination with standard or emerging therapies. For example, in the randomized phase III Velcade as Initial Standard Therapy in Multiple Myeloma: Assessment with Melphalan and Prednisone (VISTA) trial, patients with newly diagnosed symptomatic myeloma who were not candidates for high-dose therapy and autologous bone marrow transplant were treated with melphalan (9 mg/m^2) and prednisone (60 mg/m^2) on days 1–4 with or without bortezomib (1.3 mg/m^2 on days 1, 4, 8, 11, 22, 25, 29, and 32 for cycles 1–4 and on days 1, 8, 22, and 29 for cycles 5–9) (54). This study of 682 patients demonstrated that the addition of bortezomib improved the time to progression (median time to progression with vs without bortezomib: 20.7 vs 15 months). The addition of bortezomib also improved overall survival.

On the basis of these encouraging results, bortezomib has also been added to upfront induction regimens for newly diagnosed multiple myeloma. For example, in a phase II study, patients with newly diagnosed myeloma received eight 3-week cycles of bortezomib, lenalidomide, and dexamethasone (55). At the phase II doses, 100% of patients responded and 57% of patients achieved a complete response. Toxic effects included painful neuropathy in 32% of patients. Other trials in newly diagnosed patients with myeloma

have evaluated bortezomib in combination with other agents such as melphalan, prednisone, and thalidomide (56,57) or dexamethasone, cyclophosphamide, and lenalidomide (56,57). Thus, the trend in clinical trials with newly diagnosed patients with myeloma is to combine agents that are known to be active against this disease, including bortezomib, to improve the response rates.

It is noteworthy that despite its efficacy, bortezomib has a very narrow therapeutic window, which is potentially in keeping with the important normal functions of the proteasome. The therapeutic dose of bortezomib is 1.3 mg/m^2, whereas 1.5 mg/m^2 produced dose-limiting toxic effects in phase I studies (58). As studies are completed with proteasome inhibitors that target this complex through different mechanisms or specificities, it will be interesting to see whether this narrow therapeutic window is a characteristic of this entire class of therapeutic agents.

Mechanisms of resistance. Despite the clinical success of bortezomib in myeloma and mantle cell lymphoma (43,53), resistance to this drug remains a clinically significant problem. For example, in studies of bortezomib in relapsed refractory patients (42), almost all responding patients ultimately progressed. Even when bortezomib was used as a single agent in newly diagnosed patients, 52% did not achieve a partial response or better (59). Moreover, the clinical response to bortezomib in other hematologic malignancies and solid tumors remain low (58).

These results indicate that either inherent or acquired resistance to the specific proteasome inhibitor bortezomib, and this general class of therapeutics is an important clinical problem. Resistance to proteasome inhibitors has been examined in cell-based studies, and these results highlight potential clinical mechanisms of bortezomib resistance. In studies of cultured cells, resistance to proteasome inhibitors can be either at the level of the proteasome or downstream of this enzymatic complex.

Resistance at the level of the proteasome can render cells resistant to bortezomib. For example, Oerlemans et al. (60) treated human leukemia THP1 cells with increasing concentrations of bortezomib in a stepwise fashion to generate clones that were up to 500-fold more resistant to the drug compared with the parental cell line. Subsequent genetic and proteomic analyses revealed that the bortezomib-resistant cells overexpressed the β5 subunit (PSMB5) and had a mutation in the binding site for bortezomib. A similar approach was used to generate bortezomib-resistant clones of human T-cell leukemia Jurkat cells with mutations in the β5 subunits (61). Likewise, human leukemia K562 cells inherently overexpress the β5 subunit of the proteasome and are also more resistant to bortezomib compared with other leukemia and myeloma cell lines (62).

Currently, it is unclear whether mutations in or overexpression of the β5 subunit are responsible for clinical resistance to bortezomib. In a case report, Politou et al. (63) described a myeloma patient who developed resistance to bortezomib. The patient's myeloma cells were isolated at the time of resistance, and the coding region of the *PSMB5* gene was sequenced. No mutations in the gene were noted. However, the authors did not assess whether β5 was overexpressed in the relapsed myeloma sample. Moreover, no cell-based studies were conducted to determine the cells' response to bortezomib in culture. Clearly, larger studies are needed to define the clinical relevance of de novo and acquired β5

mutations and overexpression in patients with myeloma and other malignancies.

Studies in cultured cells have revealed that, in addition to β5 mutation and overexpression, factors downstream of the proteasome enzymatic complex can mediate resistance to bortezomib. For example, Zhang et al. (64) generated and characterized a bortezomib-resistant mesothelioma cell line that was almost 20-fold more resistant to bortezomib-induced cell death compared with the bortezomib-sensitive parental cell line. However, they found that bortezomib continued to inhibit the enzymatic activity of the proteasome in this resistant line as it did in the parental line, indicating that resistance was downstream of the enzymatic complex. The resistant cells displayed increased expression of the mitochondrial chaperone protein Bip and the proapoptotic transcriptional factor CCAAT/enhancer-binding protein homologous protein, which appeared to protect them from bortezomib-mediated ER stress and the unfolded protein response. Likewise, Chauhan et al. (65) found that overexpression of heat shock protein 27, which aids in the regulation of protein folding, also rendered lymphoma cells resistant to bortezomib. However, the clinical relevance of these defects downstream of the proteasome complex in the clinical setting is uncertain.

Understanding the molecular basis of resistance to proteasome inhibitors in patients with myeloma and other malignancies will aid in the development of therapeutic strategies to overcome bortezomib resistance. When resistance is due to defects at the level of the proteasome, novel proteasome inhibitors that are more potent than bortezomib or that target this complex at sites distinct from those targeted by bortezomib may overcome bortezomib resistance. Conversely, when resistance to bortezomib is due to defects downstream of the proteasome, novel proteasome inhibitors may not be effective. Of course, clinical resistance may also be mediated by pharmacokinetic factors that affect the stability, metabolism, and tissue delivery of bortezomib. In such cases, the development of proteasome inhibitors with different pharmacokinetic properties may also be clinically useful for some patients.

MLN9074 and CEP-18770. Additional reversible orally available proteasome inhibitors have been developed and advanced into phase I clinical trials, including MLN9708, which is hydrolyzed to the active form MLN2238 (66), and CEP-18770 (67,68). Overall, the preclinical studies in cultured cells and mouse models have demonstrated that these drug candidates produce anticancer effects similar to those produced by bortezomib. In addition, synergistic cell death was observed when CEP-18770 was combined with doxorubicin, melphalan, and arsenic trioxide in myeloma cell lines and with melphalan in a myeloma mouse model, including a statistically significant reduction in tumor growth compared with melphalan alone (67). Because the mechanism of action of these drugs is similar to that of bortezomib, it is unlikely that they will overcome bortezomib resistance. Rather, their advantage is their oral route of administration, which offers convenience for the patient and dose flexibility.

Irreversible Proteasome Inhibitors

In an effort to overcome bortezomib resistance, novel proteasome inhibitors have been developed that act through mechanisms distinct from bortezomib (Table 1). Because they differ in terms of their chemical structure and mechanism, these newer proteasome inhibitors may also possess side-effect profiles that are distinct from that of bortezomib. Examples include proteasome inhibitors that are based on the epoxyketone pharmacophore, such as carfilzomib. Such compounds form a unique morpholino ring with Thr1 of the active catalytic sites of the proteasome in a two-step process. First, the oxygen of the Thr1 hydroxyl group acts as a nucleophile to attack the carbonyl carbon atom of the epoxyketone, producing a hemiacetal. Then, a second nucleophilic attack by the Thr1 α-amino nitrogen on the C2 carbon of the epoxide ring results in the formation of the morpholino adduct.

Carfilzomib. Carfilzomib (previously known as PR-171) is a tetrapeptide epoxyketone–based, irreversible proteasome inhibitor (Figure 4, Table 1). As an irreversible inhibitor, carfilzomib produces more sustained inhibition of the proteasome compared with bortezomib because synthesis of new proteasome complexes is required to reverse the effects of carfilzomib. Compared with bortezomib, carfilzomib is a more potent and more selective inhibitor of the chymotrypsin-like activity of the proteasome and the immunoproteasome (41,69). Moreover, compared with bortezomib, carfilzomib has little or no off-target activity outside of the proteasome, making it superior to bortezomib in this regard (70). Compared with bortezomib, carfilzomib more potently induces cell death in malignant cells and is more effective in xenograft models, which is consistent with its higher affinity for the proteasome (69). In addition, carfilzomib remains cytotoxic to some cells that are resistant to bortezomib. For example, carfilzomib induced cell death in bortezomib-resistant HT-29 human colorectal adenocarcinoma cells (70), as well as in CD138-positive multiple myeloma cells from bortezomib-refractory patients (69).

Carfilzomib has also been evaluated in clinical trials. In a phase I clinical trial of intravenous administration of escalating doses of carfilzomib in patients with refractory hematologic malignancies, the half-life of the drug was exceptionally short (<30 minutes) (71). However, within 1 hour after administration of carfilzomib, proteasome activity in normal peripheral blood mononuclear cells was reduced by more than 75%. Recovery of proteasome activity was observed within 4 days of stopping the drug. The dose-limiting toxic effects of carfilzomib in this study were neutropenia and thrombocytopenia. No instances of grade 3 or 4 peripheral neuropathy were observed with carfilzomib. Carfilzomib has also demonstrated clinical efficacy in some patients who have relapsed after bortezomib treatment. In a phase II study (72), patients with relapsed and refractory myeloma were treated with carfilzomib. Twenty-four percent of patients achieved an overall response of partial response or better, and the median duration of response was 7.4 months. Importantly, responses were observed in patients who had disease progression after receiving bortezomib. Ongoing studies are evaluating this drug in combination with lenalidomide and low-dose dexamethasone in patients with relapsed myeloma.

ONX 0912. Although carfilzomib is a useful therapeutic agent for overcoming some forms of bortezomib resistance, it, like bortezomib, is administered intravenously because its oral bioavailability is poor. Therefore, the development of orally bioavailable proteasome inhibitors would provide more flexible dosing and better convenience for patients. A systematic structure–affinity relationship

study led to the discovery and development of ONX 0912 (73,74), an orally bioavailable epoxyketone-based proteasome inhibitor, which is a truncated derivative of carfilzomib that maintains the potency, selectivity, and antitumor activity of carfilzomib (Figure 4, Table 1). For example, in recent preclinical studies (74), ONX 0192, like carfilzomib, inhibited the chymotrypsin-like activity of the proteasome and induced cell death when added to cultures of myeloma cell lines or primary cells from patients. By contrast, ONX 0192 was not cytotoxic to normal hematopoietic cells. In addition, oral ONX 0192 delayed tumor growth in a myeloma xenograft with efficacy similar to intravenous carfilzomib (74).

Because of their peptidic structure, proteasome inhibitors such as carfilzomib and ONX0192 can be degraded by endogenous proteases and peptidases in the plasma, which limits their efficacy. Thus, there is interest in developing nonpeptidic proteasome inhibitors, which, theoretically, should have better bioavailability than the peptidic proteasome inhibitors. A series of such compounds are the omuralide derivatives, which include the clinical candidate NPI-0052.

NPI-0052. NPI-0052 is another irreversible proteasome inhibitor that binds the active site of the proteasome. NPI-0052 is a secondary metabolite of the marine actinomycete *Salinispora tropica* (75) and a highly potent and selective proteasome inhibitor (47) (Figure 4, Table 1). By crystal structure analysis, NPI-0052 covalently binds to the active sites of the proteasome through a highly stable acyl ester bond (49).

Unlike bortezomib and carfilzomib, which are selective for the chymotrypsin-like activity of the proteasome, NPI-0052 inhibits all three enzymatic activities of the proteasome but displays preferential activity against the chymotrypsin-like and trypsin-like proteasome enzymes (47). NPI-0052 induces cell death in leukemia and myeloma cell lines and delays tumor growth in myeloma mouse xenografts models (47,76,77). In a phase I clinical trial, patients with relapsed and/or refractory hematologic malignancies including myeloma were treated with increasing doses of NPI-0052 (78). Target inhibition was demonstrated as measured by a reduction in the proteasome activity in red cells. The drug was well tolerated, and the only dose-limiting toxic effect was hallucinations. In the reports to date, patients have achieved stable disease after NPI-0052 treatment, and further evaluation of efficacy is ongoing. It is noteworthy that the half-life of NPI-0052 is less than 5 minutes, so a single daily dose may not provide enough drug exposure to achieve clinical benefit.

Thus, when taken together, irreversible proteasome inhibitors show potential efficacy in the treatment of myeloma and demonstrate an ability to overcome some forms of bortezomib resistance. Further laboratory-based mechanistic studies that define the causes of bortezomib resistance would be useful in identifying subgroups of patients that are likely respond to irreversible proteasome inhibitors. One might predict that cell lines and patients with resistance to bortezomib because of defects downstream of the proteasome would remain resistant to irreversible proteasome inhibitors. However, the newer irreversible and more potent inhibitors may overcome resistance to bortezomib due to increased levels of the β5 subunits of the 20S proteasome. Given that irreversible proteasome inhibitors are showing efficacy in the setting of relapsed disease (72), future studies will also likely examine these irreversible inhibitors in the upfront treatment setting. In addition,

studies of irreversible proteasome inhibitors in combination with other chemotherapeutic agents may identify strategies to further enhance efficacy or decrease toxic effects.

Allosteric Proteasome Inhibitors

It is conceivable (but, to our knowledge, not yet tested in cell culture studies or clinical trials) that mutations in the β5 subunit would render cells resistant to irreversible proteasome inhibitors. Thus, proteasome inhibitors that target the proteasome by binding outside of the active site might be a useful therapeutic strategy to overcome some forms of bortezomib resistance. To date, both peptide and chemical allosteric inhibitors of the proteasome have been developed and tested in vitro and/or in vivo (Table 1, Figure 4). However, none has yet to be advanced into clinical trial.

Peptide Proteasome Inhibitors

PR-39 is a 39-amino acid peptide that was initially isolated from pig intestine and has been shown to function as an antimicrobial agent (79). Subsequent studies have demonstrated that this peptide inhibits the enzymatic activity of the proteasome when added to either isolated proteasomes or cell extracts (80,81). Kinetic studies demonstrated that PR-39 has a noncompetitive mode of inhibition (80,81). Biochemical studies demonstrated that PR-39 disrupts the assembly of the 19S and 20S particles, thereby preventing formation of the full 26S proteasome complex (80,81). Available data suggest that this peptide interacts with the α7 subunit in the regulatory region of the 20S proteasome, thereby preventing assembly of the complete proteasome (80,81). Moreover, the interaction between PR-39 and the α7 subunit (80,81) produces conformational changes in the 20S subunit that interfere with the enzymatic activity of the complex (80,81). Although the biochemical data for PR-39 are convincing, this peptide is unlikely to advance beyond a chemical probe to understand the effects of allosteric inhibition of the proteasome. Advancing this therapy to the clinic would be challenging. For example, the long chain length of this peptide might make this compound unstable after systemic administration and as such limit its use in vivo as a proteasome inhibitor.
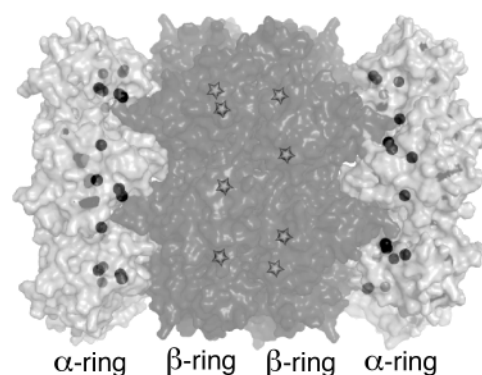

Figure 5. Interaction between chloroquine and the proteasome. The chloroquine-binding site, which is located at the interface between the α- and β-rings, as determined by methyl transverse relaxation optimized spectroscopy–based nuclear magnetic resonance experiments in this transparent surface representation of the structure of the *Thermoplasma acidophilum* proteasome. **Spheres** indicate the locations of methyl groups that changed in response to chloroquine. **Stars** indicate the positions of the active sites.

Nonetheless, this work highlights a potential target site for the development of novel proteasome inhibitors.

Chemical Proteasome Inhibitors

Chloroquine. Allosteric chemical inhibitors that target the proteasome outside of the active site have also been developed and, compared with PR-39, might be better candidates for clinical development. For example, Sprangers et al. (82) showed that the antimalarial drug chloroquine inhibits the enzymatic activity of the proteasome when added to eukaryotic cell extracts or purified 20S archaeal proteasome from *T. acidophilum*. Detailed enzymatic kinetic studies demonstrated that chloroquine inhibits the proteasome via a noncompetitive mechanism. Nuclear magnetic resonance spectroscopy revealed that chloroquine binds reversibly to the 20S archaeal proteasome between the α and β subunits of the $\alpha_7\beta_7\beta_7\alpha_7$ barrel-like structure, approximately 20 Å from the proteolytic active sites (Figure 5). Moreover, nuclear magnetic resonance spectroscopy also revealed that the reversible proteasome inhibitor MG132, which binds the catalytic site of the proteasome, can simultaneously bind the proteasome along with chloroquine, further supporting an allosteric mechanism of action for chloroquine. This study was the first, to our knowledge, to describe a chemical noncompetitive proteasome inhibitor that binds outside of the active site of the proteasome. Moreover, it established nuclear magnetic resonance spectroscopy as a platform through which to develop allosteric proteasome inhibitors.

5-amino-8-hydroxyquinoline (5AHQ). Despite the intriguing nuclear magnetic resonance spectroscopy results with chloroquine, the affinity of chloroquine for the proteasome is relatively low, and the concentration of chloroquine required to inhibit the enzymatic activity of the proteasome is not likely to be pharmacologically achievable in humans. Thus, it is noteworthy that more potent allosteric proteasome inhibitors with a chemical structure similar to chloroquine have also been described and may be more clinically relevant (62). For example, the structurally related compound 5AHQ was recently shown to inhibit the enzymatic activity of isolated proteasome in a noncompetitive fashion (62). Nuclear magnetic resonance spectroscopy revealed that 5AHQ interacts with the α subunits of *T. acidophilium* proteasome at a site distant from the catalytic site. 5AHQ also induced cell death in myeloma and leukemia cell lines and preferentially induced cell death in primary myeloma and leukemia cells compared with normal hematopoietic cells (62). In mouse models of leukemia, orally administered 5AHQ delayed tumor growth and inhibited proteasome activity (62). 5AHQ was also well tolerated in mice at doses up to sixfold higher than the dose required for antitumor effects. These results support a potential therapeutic window for 5AHQ and highlight this compound as a lead for a new series of proteasome inhibitors.

The true clinical value of allosteric proteasome inhibitors may lie in their ability to overcome some forms of resistance to active site inhibitors such as bortezomib. To explore this possibility, the cytotoxicity of 5AHQ was tested in human leukemia THP1 cells that were selected for their resistance to increasing concentrations of bortezomib and which overexpress a mutant β5 subunit and in human leukemia K562 cells, which naturally overexpress the β5 proteasome subunit (62). The findings—that 5AHQ is cytotoxic

and inhibits the proteasome in these bortezomib-resistant cells—are consistent with the hypothesis that allosteric proteasome inhibitors can overcome bortezomib resistance due to mutation or overexpression of the β5 subunit. In addition, these findings support the notion that 5AHQ inhibits the proteasome through a mechanism distinct from that of bortezomib. Although these results are promising and support the development of molecules similar to 5AHQ as novel proteasome inhibitors, more work is needed before 5AHQ can be advanced to clinical trials. For example, x-ray structural analysis of the 5AHQ–proteasome complex is needed to better discern the mechanism by which 5AHQ inhibits the proteasome. Moreover, additional pharmacokinetic and toxicology studies are required.

Clioquinol. The antiparasitic agent clioquinol is another quinoline compound that is structurally related to 5AHQ and has been shown to inhibit the proteasome through a unique mechanism. Clioquinol is an oral antimicrobial drug that was prescribed from the 1950s to 1970s to treat and prevent intestinal parasitic disease (83,84). To our knowledge, the mechanism of action of clioquinol as an antiparasitic agent remains unknown. In the 1970s, reports emerged in Japan about an association between clioquinol and subacute myelo-optic neuropathy (85,86). Because of this association with neurological toxicity, clioquinol was removed from the market. However, essentially no neurotoxicity was reported outside of Japan despite the use of more than 500 million doses, suggesting that the toxicity was related to the Japanese formulation or features specific to the Japanese population. More recently, clioquinol has been shown to have anticancer efficacy in preclinical in vitro and in vivo models (87,88). At least part of the anticancer activity of clioquinol is due to its ability to inhibit the proteasome. Clioquinol inhibits the proteasome through multiple mechanisms that are dependent on and independent of its ability to bind metals such as copper and zinc (87). As a metal-dependent proteasome inhibitor, clioquinol may act as a metal ionophore to increase intracellular levels of metals, including copper and zinc, by transporting the metal ions from the extracellular environment into the cell or by mobilizing weakly bound intracellular metal stores (87,89,90). Given its moderate affinity for copper, clioquinol may bind this metal in regions of high concentrations and release it in regions of lower copper concentrations (91). Alternatively, copper binding and dissociation may be dependent on pH, with dissociation occurring in intracellular regions with lower pH (91). Thus, through these mechanisms, clioquinol may deliver metal ions such as copper to the proteasome.

The exact mechanism by which copper inhibits the proteasome has not been fully defined. Copper—either in the Cu(I) state or during reduction from Cu(II) to Cu(I)—could interact with thiol and amino groups outside the active site of the proteasome, thereby inducing changes in the conformation of the proteasome, leading to a decrease in proteasome function. The metal-dependent mechanism by which clioquinol inhibits the proteasome may explain its ability to induce cell death preferentially in malignant cells compared with normal cells. For example, patients with hematologic malignancies have higher levels of copper in their plasma and malignant cells compared with plasma samples and normal cells from healthy volunteers (92–94). Thus, clioquinol

may preferentially facilitate metal-dependent inhibition of the proteasome in malignant cells.

Clioquinol also inhibits the proteasome complex through metal-independent mechanisms. For example, at higher concentrations that do not appear pharmacologically achievable, clioquinol directly inhibits the enzymatic activity of the *T. acidophilum* proteasome in the absence of heavy metal ions, including copper (87). However, the mechanism of this inhibition has not been fully characterized.

In preclinical studies, clioquinol induced cell death in malignant cells over normal cells and delayed tumor growth in mouse models of solid tumor and hematologic malignancies (87,88). Given the antitumor activity of clioquinol in vitro and in vivo, this drug could be rapidly advanced into clinical trials as a proteasome inhibitor, given its prior safety in non-Asian populations. In fact, a phase I study of escalating doses of clioquinol is currently underway in non-Asian patients with relapsed and refractory hematologic malignancies (ClinicalTrials.gov Identifier: NCT00963495).

Conclusions

The proteasome is an enzymatic complex that degrades ubiquitin-tagged proteins via its chymotrypsin-, trypsin-, and caspase-like enzymatic activities. Bortezomib is a reversible competitive inhibitor of this complex that has been shown to improve clinical outcome of patients with multiple myeloma and mantle cell lymphoma when used as a single agent and in combination with other chemotherapeutic agents. Despite the success with bortezomib, most patients do not respond to this drug when it is used as a single agent, and the majority of responders ultimately relapse. Thus, strategies that overcome bortezomib resistance are needed for these patients. To date, most efforts have focused on developing proteasome inhibitors that act on the proteasome through mechanisms that are distinct from that of bortezomib. For example, carfilzomib is an irreversible proteasome inhibitor that appears to have clinical efficacy in some patients who have relapsed after bortezomib treatment. Other compounds that are still in preclinical development act outside the active site of the proteasome and can overcome bortezomib resistance in cell lines with mutant β5 subunits or that overexpress the catalytically active β5 subunits.

Another emerging and potentially interesting strategy to overcome bortezomib resistance is to target the ubiquitination pathway at the level of the E1 enzyme Uba1. Inhibiting UbaI with chemical compounds that block transfer of ubiquitin from Uba1 to the E2 enzymes prevents protein ubiquitination and leads to the accumulation of excess intracellular proteins (95). E1 enzyme inhibitors induce cell death through a mechanism related to ER stress that is somewhat similar to the mechanism of proteasome inhibitors. In addition, preliminary data indicate that E1 inhibitors are also effective at inducing cell death in some cells that are resistant to bortezomib (95). Future studies with second-generation E1 inhibitors with more drug-like properties will better clarify the potential clinical utility of this strategy in overcoming bortezomib resistance.

In summary, competitive inhibition of the proteasome by targeting the active site has led to the development of bortezomib, which is currently approved for the treatment of myeloma and mantle cell lymphoma. This success has spawned the development of second-generation intravenous and oral proteasome inhibitors. The optimal clinical use of these inhibitors in relation to bortezomib (ie, as first- or second-line agents) remains to be determined.

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