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Proteasome Inhibitor Drugs

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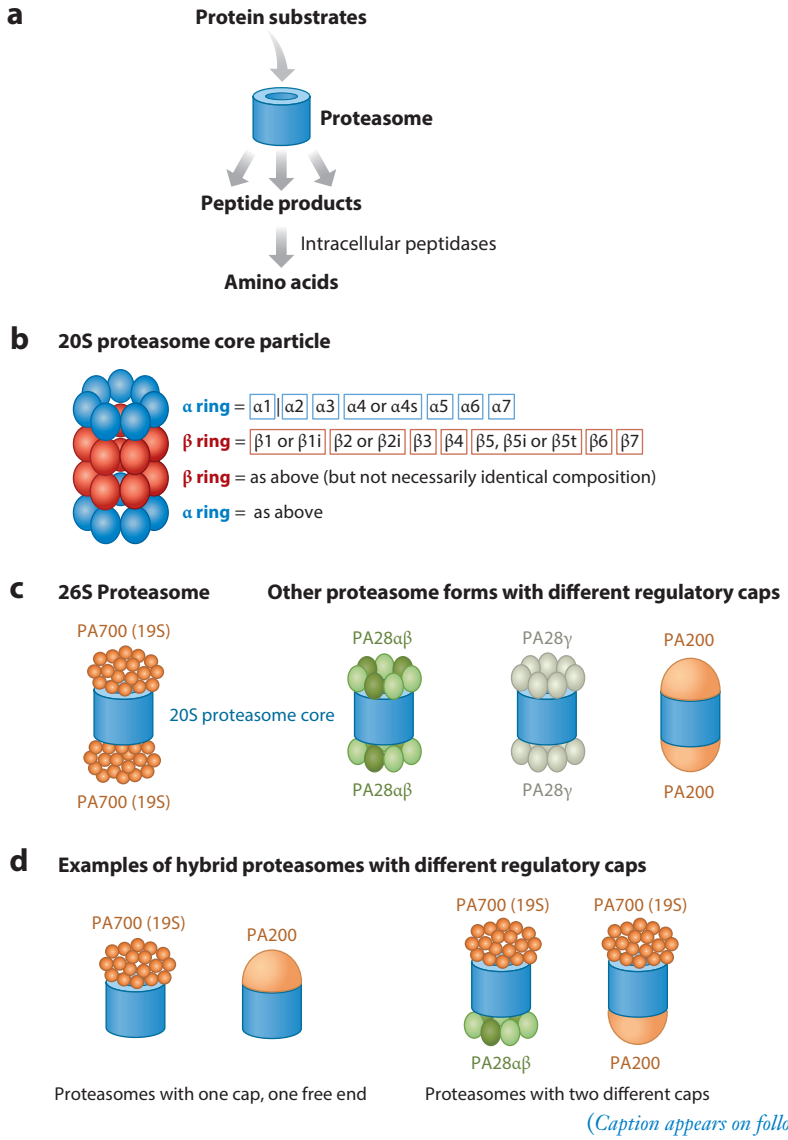
Abstract

Proteasomes are large, multicatalytic protein complexes that cleave cellular proteins into peptides. There are many distinct forms of proteasomes that differ in catalytically active subunits, regulatory subunits, and associated proteins. Proteasome inhibitors are an important class of drugs for the treatment of multiple myeloma and mantle cell lymphoma, and they are being investigated for other diseases. Bortezomib (Velcade) was the first proteasome inhibitor to be approved by the US Food and Drug Administration. Carfilzomib (Kyprolis) and ixazomib (Ninlaro) have recently been approved, and more drugs are in development. While the primary mechanism of action is inhibition of the proteasome, the downstream events that lead to selective cell death are not entirely clear. Proteasome inhibitors have been found to affect protein turnover but at concentrations that are much higher than those achieved clinically, raising the possibility that some of the effects of proteasome inhibitors are mediated by other mechanisms.

BACKGROUND AND SCOPE

The proteasome is a large, multicatalytic protein complex that degrades many cellular proteins (**Figure 1**). Proteasome inhibitors are an important new class of drugs for the treatment of multiple myeloma and mantle cell lymphoma, and they are currently in clinical trials for additional types of cancer (1–5). Proteasome inhibitors also function as immunosuppressants (6–8), inhibit bone resorption (9, 10), and may have other applications (11, 12). Bortezomib was the first proteasome inhibitor to be US Food and Drug Administration (FDA)-approved in 2003, followed by carfilzomib in 2012 and ixazomib in 2015—other compounds are currently in clinical trials (1, 5, 13). While the primary mechanism of action of these drugs is the inhibition of catalytically active subunits, the downstream events that lead to selective cell death are not clear. The current dogma



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

The proteasome core structure and diverse forms. (*a*) The overall scheme in which proteins are degraded is a multistep process where the proteasome first converts the substrate protein into peptides, and these peptides are subsequently digested into amino acids by peptidases. (*b*) The composition of the 20S proteasome core consists of four rings, with each ring containing seven different proteins. The two outer rings are the α rings, composed of α1–7 subunits. The proteasome in sperm uses a different α4 protein (α4s) than the α4 subunits found in other tissues. The two inner rings are the β rings, composed of β1–7 subunits. Four of these subunits are not catalytically active (β3, β4, β6, and β7). The three catalytically active β subunits are each encoded by multiple genes and, depending on the cell type, one or more of these alternative subunits may be incorporated into the basic 20S core, thereby altering the catalytic properties of the proteasome. The composition of the two β rings is independent, leading to mixed proteasomes. (*c*) Regulatory caps can bind to the 20S core particle, thereby affecting access to the active sites within the core. PA700 is also known as 19S; PA28 is also known as PA26, 11S, and REG; and PA200 is also known as Blm10. The examples shown here have the same regulatory subunits on both sides of the core. (*d*) Examples of proteasomes with a single regulatory subunit or with two different regulatory subunits on the different sides of the 20S core. These are representative examples, and additional combinations have been identified (38, 39, 41, 145).

holds that proteasome inhibitors affect protein turnover, which is ultimately toxic to the cells. Bortezomib-mediated cell death has been shown to result from elevated apoptosis via multiple pathways, including the intrinsic apoptotic mitochondrial pathway, the extrinsic death-receptor pathway, and the endoplasmic reticulum stress response pathway (14–23). Many studies have found evidence for reduced activation of nuclear factor (NF)-κB due to bortezomib-mediated stabilization of the inhibitor IκB (24–29). In addition, bortezomib inhibits cyclin turnover, and this affects cyclin-dependent kinase (Cdk) activity (30, 31). Bortezomib also affects kinases such as JNK, tumor suppressors such as p53, and the Bcl-2 family of proteins (5, 17, 32, 33). However, most of the studies on cellular proteins have involved concentrations of drugs and/or treatment times that are orders of magnitude higher than those encountered upon clinical exposure, thereby raising the question as to whether these protein changes are the primary mechanisms by which the proteasome inhibitors contribute to cell death. Alternative theories are discussed below after a brief review of proteasomes.

INTRACELLULAR PROTEIN DEGRADATION

Protein degradation is an essential cellular function that has received considerable attention in the past few decades. Many years ago, lysosomes were considered to be the major waste disposal system of the cell. While lysosomes are important for the degradation of some cytoplasmic proteins, especially in autophagy-mediated degradation of organelles, it is now recognized that the ubiquitin/proteasome pathway is responsible for the degradation of approximately 80% of intracellular proteins, and this process is highly regulated (34).

Evidence for a specific protein degradation pathway came in the late 1970s when it was found that the degradation of many cellular proteins required ATP (35). The development of an ATP-dependent cell-free assay led to the discovery of the ubiquitin/proteasome system in which proteins are tagged for degradation by the addition of ubiquitin, a 76-residue cellular protein that serves many functions in addition to protein degradation (34, 36). Ubiquitin is attached to proteins by ubiquitin-ligases. The site of attachment on the targeted protein is nearly always a lysine residue, but on occasion other residues are used (37). Often, ubiquitin is itself ubiquitinated on one of seven lysine residues and/or its N terminus, resulting in polyubiquitin chains on the target protein. The specific lysine residue within ubiquitin used for polyubiquitination has functional consequences, and only some of these forms are targeted for degradation (34). Ubiquitinated proteins are recognized by regulatory subunits on the 26S proteasome complex, the ubiquitin chains

are removed and recycled, and the protein is unfolded and translocated into the interior by the proteasome complex where it is cleaved into peptide products by the active protease subunits (34). The 2004 Nobel Prize in Chemistry was given to Aaron Ciechanover, Avram Hershko, and Irwin Rose for the discovery of ubiquitin-mediated protein degradation, reflecting the importance of this process in basic cell biology.

PROTEASOME DIVERSITY

The proteasome complex is not a single entity but exists in many different forms due to variations in the composition of catalytic subunits, structural subunits, regulatory subunits, and posttranslational modifications (38–41). Multiple proteasome forms are typically present in a single cell type, and this can change in a dynamically regulated process. There is variation between cell types and tissues, suggesting that the proteasome is optimized for each cell's function. Various proteasome forms have dramatic differences in their substrate preferences and generate distinct sets of peptides (38–40, 42). In some studies, different proteasome forms have been found to show altered sensitivity to proteasome inhibitors (42, 43).

All proteasomes contain a common core, referred to as the 20S proteasome (based on its Svedberg sedimentation coefficient). This 20S core particle is about 700 kDa and contains 14 α and 14 β subunits arranged into four rings with a hollow center (**Figure 1*a***). The two outer rings are composed of seven α subunits (α1–7), and the two inner rings are composed of seven β subunits (β1–7). The α subunits are structural, with their N termini forming barriers that obstruct the movement of proteins into and out of the inner core. The protease activity of the complex is due to three β subunits present in each of the inner rings: β1, β2, and β5 (or the homologs described below). All of these catalytic subunits are threonine proteases that cleave substrates via a two-step process. First, the active site threonine residue attacks the substrate to form a covalent ester bond with the N-terminal portion of the substrate. Second, this intermediate is hydrolyzed to release the product and regenerate the active form of the protease. This mechanism is analogous to that of the well-studied serine enzyme trypsin. The active site threonine in the proteasome subunits is located on the N terminus of the protein following autocatalytic removal of a 20–60 residue propeptide (44). Thr1 is part of a catalytic triad together with Asp17 and Lys33 (45). The other four β subunits (β3, β4, β6, and β7) are homologs of the active subunits but are inactive as proteases. Three of these inactive subunits (β3, β4, and β6) do not have a threonine residue in the appropriate position and do not undergo propeptide removal. The β7 subunit undergoes removal of its propeptide and has a threonine on the N terminus. In addition, an acidic residue (Asp17 or Glu17) is conserved in β7 orthologs throughout the animal kingdom. However, the β7 subunit is not thought to participate in the cleavage of protein substrates due to the lack of Lys33. Active site-directed inhibitors that bind to the β1, β2, and β5 subunits do not bind to the β7 subunit, supporting the idea that this subunit is inactive (43). Interestingly, the β7 subunit (gene *PSMB4*) has been identified as a potential cancer driver gene that is upregulated in a variety of cancers (46–48).

The three catalytically active proteins have distinct cleavage specificities. The β2 subunit cleaves substrates with a basic residue (Lys, Arg) in the P1 site and is referred to as trypsin-like. The β5 is referred to as chymotrypsin-like because of its preference for hydrophobic residues in the P1 site. However, there is a preference for some hydrophobic substrates over others, with Leu, Phe, and Tyr being the most favorable and Trp, Ile, and Val being less favorable in the P1 position (49). The β1 subunit is often referred to as caspase-like or as having peptidylglutamyl hydrolyzing activity, but neither is technically correct because they imply specificity for only one amino acid

(Asp or Glu, respectively), whereas the β1 subunit cleaves substrates with either of these acidic amino acids. None of the three catalytic subunits is very efficient at cleaving substrates with Gly, Pro, Asn, Gln, or Cys in the P1 position, based on studies testing model protein substrates with purified 20S and 26S proteasomes (49) as well as studies examining the major proteasome products in cell extracts (50, 51).

In addition to the regular β1, β2, and β5 subunits, there are additional catalytic subunits named β1i, β2i, and β5i. The "i" designation refers to the immunoproteasome, which is the major proteasome form in antigen-presenting cells in the immune system (52). In addition, βi subunits are present in many cell types throughout the body and are induced under conditions of oxidative or inflammatory stress (38). Proteasomes have been identified with mixtures of β and βi subunits, which are referred to as intermediate or mosaic proteasomes. A distinct β5 subunit (β5t) is present in cortical thymic epithelial cell proteasomes. In these cells, the β1i and β2i forms are also expressed, producing a unique thymoproteasome. The seven α subunits are present in nearly all proteasomes with the exception of the spermatoproteasome that contains a unique α4s subunit (also known as α8).

Proteasomes with different β subunits in the 20S core produce distinct sets of peptide products (38, 53). One major difference is the β1i subunit, which has a Leu45 in place of Arg45 in β1, an important substrate-binding residue that confers selectivity to acidic residues. As a result, the β1i subunit cleaves substrates with branched-chain amino acids in the P1 position (52). β5 and β5i subunits have subtle differences in their substrate binding residues, and although both cleave at hydrophobic P1 residues, the β5i subunit is better at cleaving bulky hydrophobic residues (42). The thymoproteasome-specific β5t subunit also has differences in substrate-binding residues compared to the β5 and β5i subunits (54). As a result of these differences, the thymoproteasome and immunoproteasome produce different sets of peptides than the standard proteasome (54).

In most cell types, the 20S core particle exists together with regulatory complexes that also function as proteasome activators (**Figure 1*b***). The 19S proteasome activator, also known as PA700, is composed of 19 or more protein subunits and is responsible for the recognition of ubiquitinated proteins, removal of the ubiquitin chains, and the unfolding and translocation of the protein into the 20S core. The 19S complex is also responsible for the recognition of some nonubiquitinated substrates such as ornithine decarboxylase (55, 56). The 11S proteasome activator, also known as PA28, PA26, and REG, is composed of seven subunits (**Figure 1*b***). PA28αβ is a mix of α and β subunits (distinct from the α and β subunits of the 20S core), while PA28γ contains seven copies of the γ subunit. The 11S proteasome complex does not recognize ubiquitin or use ATP and is not able to unfold proteins. The third regulatory subunit is PA200, a monomeric protein of approximately 200 kDa (**Figure 1*b***), which also does not bind ubiquitin or use ATP (57). The regulatory subunits can occupy one or both sides of the 20S core (**Figure 1*c***). Two distinct regulatory subunits can bind to either end of a common 20S core, leading to hybrid forms with distinct properties (39). A common form is the 20S core with two 19S regulatory domains—this is termed the 26S proteasome and is ATP and ubiquitin dependent. In contrast, the 20S proteasome alone or in complex with 11S and/or PA200 primarily cleaves peptides and small unfolded/misfolded proteins (58, 59).

A large number of posttranslational modifications have been reported for the various proteasome subunit proteins, including phosphorylation, *N*-acetylation, glycosylation, myristylation, poly-ADP ribosylation, ubiquitination, and more (38, 40, 60). Some of these modifications have been found to affect proteasome assembly, stability, activity, and sensitivity to proteasome inhibitors (34, 38, 40, 60, 61).

FUNCTIONAL ROLES OF PROTEASOMES

Most of the literature on proteasomes refers to their degradative role, and this is clearly a major function of proteasomes. The various forms of the proteasome contribute to over 80% of protein turnover (34). Of the numerous proteins that are degraded by proteasomes, the following have been implicated in the therapeutic effect of proteasome inhibitors.

NF-κB Signaling

The NF-κB family of proteins includes NF-κB1, NF-κB2, and three class II proteins: RelA, RelB, and c-Rel (62). All of the NF-κB family proteins are transcription factors, and these proteins are constitutively inhibited within most cells. The canonical pathway of NF-κB activation requires proteasome-mediated degradation of IκB proteins, a family of constitutive inhibitors of NF-κB that bind and retain the complex in the cytoplasm. Phosphorylation of IκB leads to subsequent ubiquitination and degradation by the proteasome, thereby allowing NF-κB to enter the nucleus and activate the transcription of genes that promote cell survival. An alternative pathway, referred to as the noncanonical pathway, involves the phosphorylation of the NF-κB2 precursor protein p100 followed by proteasome-mediated cleavage of only the C-terminal inhibitory domain. This is distinct from the typical role of the proteasome in which entire proteins are cleaved. In the case of p100, the C terminus of the protein enters into the proteasome's inner chamber, but sequences in the middle of the protein prevent translocation of the entire protein, thereby preventing complete degradation. The active N-terminal fragment (p52) is subsequently released and dimerizes with class II family proteins (such as RelB), which in turn activates a different class of genes than those activated by the canonical NF-κB pathway (62).

Cellular Tumor Antigen p53

The protein p53 is a transcription factor that functions as a powerful tumor suppressor (63). It is often described as a master switch that activates DNA repair proteins, arrests cell growth, and initiates apoptosis if DNA damage cannot be repaired. The p53 protein is mutated or deleted in approximately half of all human cancers. The p53 protein is polyubiquitinated and degraded by the 26S proteasome. Under normal conditions, the p53 protein has a short half-life. Stress (e.g., DNA damage) greatly reduces the degradation of p53 and leads to rapid increases in the levels of this protein. Proteasome inhibitors that block p53 degradation could contribute to tumor suppression.

Cyclin and Cyclin-Dependent Kinases

Cyclins are a family of proteins that play an essential role in the progression of the cell cycle throughout the animal kingdom (64). Cyclins exert their effects by stimulating Cdks, which in turn regulate gene transcription and mRNA processing. Cellular levels of cyclins are controlled during the cell cycle by the balance between gene expression/protein translation and degradation by the proteasome. In addition, there are Cdk inhibitory proteins that are normally degraded by the proteasome. Some cyclins, such as cyclin D1, are frequently deregulated in cancer and are markers of disease progression (65). Inhibition of proteasomal degradation of the cyclins, Cdks, and/or Cdk inhibitors prevents cells from dividing and leads to cell death. Cyclins may be especially relevant to the action of bortezomib for treating mantle cell lymphoma, which is often caused by a gene translocation that causes the overexpression of cyclin D1 (66).

Other Protein Targets

Many other proteins are known to be degraded by the proteasome and have been proposed as candidates for the cytotoxic effect of proteasome inhibitors (67). The growth factor interleukin-6 plays an important role in multiple myeloma, and levels of interleukin-6 receptors may be impacted by proteasome inhibitors. It is also possible that the collective buildup of unfolded/misfolded proteins caused by proteasome inhibitors leads to endoplasmic reticulum stress, which in turn triggers the unfolded protein response and apoptosis.

Biosynthetic Roles of the Proteasome

In addition to the critical role that proteasomes play in protein degradation, there are also biosynthetic roles. Proteolysis can be considered biosynthetic if it produces an active molecule, analogous to neuropeptide biosynthesis in which an inactive precursor is selectively cleaved by proprotein convertases and carboxypeptidases to produce bioactive neuropeptides (68). Before discussing the biosynthetic roles of the eukaryotic proteasome, it is worth a brief digression to consider the bacterial proteasome. Archaebacteria express a proteasome that resembles the eukaryotic proteasome in many ways (69). Most importantly, the archaebacterial proteasome consists of a 20S core composed of α and β subunits, and the β subunit is a threonine protease that uses Asp17 and Lys33 for the catalytic triad, just like eukaryotic proteasomes. However, a major difference is that the archaebacterial proteasome contains 7 identical β subunits in each ring, for a total of 14 active subunits per 20S core particle (69). This makes sense for a system whose sole function is protein degradation. If eukaryotic proteasomes also functioned exclusively in protein degradation, it would be expected that they would also contain 7 active β subunits per ring and 14 per 20S core. However, eukaryotic proteasomes only contain 3 catalytically active subunits in each 7-subunit ring for a total of 6 active and 8 inactive β subunits per 20S core. This feature is highly conserved from yeast to humans, suggesting that the function of the eukaryotic proteasome is the conversion of proteins into peptides rather than only protein degradation.

One well-known biosynthetic function of the proteasome is the production of peptides that are expressed on the cell surface and are bound to class I major histocompatibility complex (MHC) proteins, a key part of the immune system's ability to differentiate self from nonself. However, only a small subset of peptides produced by proteasomal cleavages can bind to class I MHC proteins, as there are restrictions on peptide length and the requirement of residues in specific sites for MHC binding.

Most peptides produced by the proteasome were originally thought to be rapidly degraded by cellular peptidases, although this was primarily based on a theoretical concern: "rapid clearance of peptides released by proteasomes appears to be essential for cell viability, because…accumulation of undegraded peptide fragments of cell proteins in the cytosol could interfere with important protein-protein interactions, and such peptides could by themselves aggregate or be toxic" (70, p. 46,723). But what if a subset of the proteasome-generated peptides escaped rapid degradation by binding to proteins? If some peptides lasted for minutes, rather than seconds, these long-lasting peptides would potentially be bioactive, especially if their longevity was a result of binding to proteins that protected them from peptidases. This is conceptually similar to microRNA, in which small oligomers bind to larger oligomers, thus altering their function (71).

Studies examining the stability of cytosolic peptides do not support the dogma that all peptides are rapidly degraded. Two studies tested a small number of fluorescently labeled peptides and, while most were degraded within 10 s, some peptides survived for many minutes (72, 73). Other studies identified peptides that are protected from cellular degradation through binding to heat

shock proteins such as Hsp70 (74) and Hsp90 (75). Over the last decade, several laboratories have used mass spectrometry–based peptidomics techniques to identify peptides in mouse brain and other biological samples. These techniques, originally designed to detect neuropeptides, identified hundreds of peptides that arose from proteins present in cytosol, mitochondria, and/or nuclei (50, 51, 76–90). The peptides derived from these proteins are collectively called intracellular peptides to distinguish them from neuropeptides produced within the secretory pathway (71). Many of the same intracellular peptides found in human cell lines were also found in mouse, zebrafish, and other organisms (81, 91). Yeast (*Saccharomyces cerevisiae*) also produce intracellular peptides, and there are similarities between the peptides found in yeast, zebrafish, mouse, and human cells (91, 92). Short-term treatment of mammalian cells with proteasome inhibitors (described below) dramatically changed the levels of most intracellular peptides, indicating that these peptides are the products of proteasomal cleavage of cellular proteins (50, 51, 86, 90).

In addition to a role in producing peptides in the cytosol and nucleus, proteasomes may also generate peptides secreted from cells (93). In a recent study, about 40% of the 20S proteasomes in brain neurons were found to be associated with the plasma membrane. These membrane-bound proteasomes cleave intracellular proteins and release the peptides into the extracellular space (93). Further studies found that the protein substrates of the neuronal membrane proteasome were mainly nascent polypeptides, not full-length proteins, and the cleavage was ubiquitination independent (94). The neuronal plasma membrane-bound proteasome was sensitive to a nonpermeable inhibitor that altered the electrophysiological activity of the cultured cells within 10–30 s (93). These findings led to the proposal that proteasomes are involved in the generation of peptides that function in cell–cell communication, much like the neuropeptides produced within vesicles in the classic secretory pathway but without the need for storage in vesicles.

Another conceptually distinct way in which the proteasome can be considered biosynthetic is by the degradation of inhibitory domains, thereby leading to the activation of proteins. One well-studied example is the conversion of NF-κB precursor p105 into the active p50 domain, which is mediated by the proteasome (95). In this example, the proteasome binds to the C-terminal region of p105 and translocates this domain into the proteasome but does not degrade the entire protein. There are other examples of incomplete proteasomal processing of proteins, although the functional consequences of some of these events are unknown (38).

Taken together, proteasomes play many important cellular roles, including an essential role in the degradation of cellular proteins. An emerging view is that proteasomes are also involved in the production of bioactive proteins and peptides, and this should be taken into account when considering the mechanism by which proteasome inhibitors produce their therapeutic and side effects.

PROTEASOME INHIBITORS

The first synthetic proteasome inhibitor contained a peptide backbone with an aldehyde on its C terminus, which formed a reversible complex with the active site threonine (96). A modified version with a different peptide backbone, named ZLLLal, was more potent and cell permeable (97). ZLLLal was renamed MG-132 and is still used in laboratories to inhibit the proteasome. Other investigators replaced the aldehyde group with a vinyl sulfone, which bound irreversibly (98). However, both types of compounds are not very specific and also inhibit lysosomal and calcium-activated cellular proteases (99). The first highly selective proteasome inhibitor, lactacystin, was serendipitously identified in extracts of *Streptomyces* in a screen for the induction of neurites in a neuroblastoma cell line (100). Subsequently, a metabolite of lactacystin (clasto-beta-lactone) was found to be a highly selective irreversible inhibitor of the proteasome (101).

Epoxomicin was discovered in a screen of *Streptomyces* extracts that looked for antitumor activity (102). Further studies identified the proteasome as the main target of epoxomicin and also found that epoxomicin had anti-inflammatory properties (99). Epoxomicin's chemical structure consists of a peptide backbone with an epoxyketone on the C terminus—this forms a covalent bond with the active site threonine and leads to irreversible inhibition.

Soon after the discovery of the proteasome, the company MyoGenetics was founded to develop proteasome inhibitors, with the initial idea that such inhibitors would be useful to treat muscle-wasting conditions (103). Compounds developed by MyoGenetics included MG-262 and MG-341 (104). While the proteasome inhibitors were too toxic for treating muscle-wasting conditions, they showed cytotoxicity toward a broad range of human cancer cells, with an excellent correlation between their potency towards the chymotryptic-like proteasome activity and their ability to inhibit cell growth. One of these compounds, MG-341, was further developed as a drug. Its name was changed to PS-341 when MyoGenetics became ProScript and was later given the generic name bortezomib. Bortezomib is a reversible inhibitor of the proteasome, containing a peptide-like backbone and boronate group (**Figure 2**). Compared to similar peptide backbones

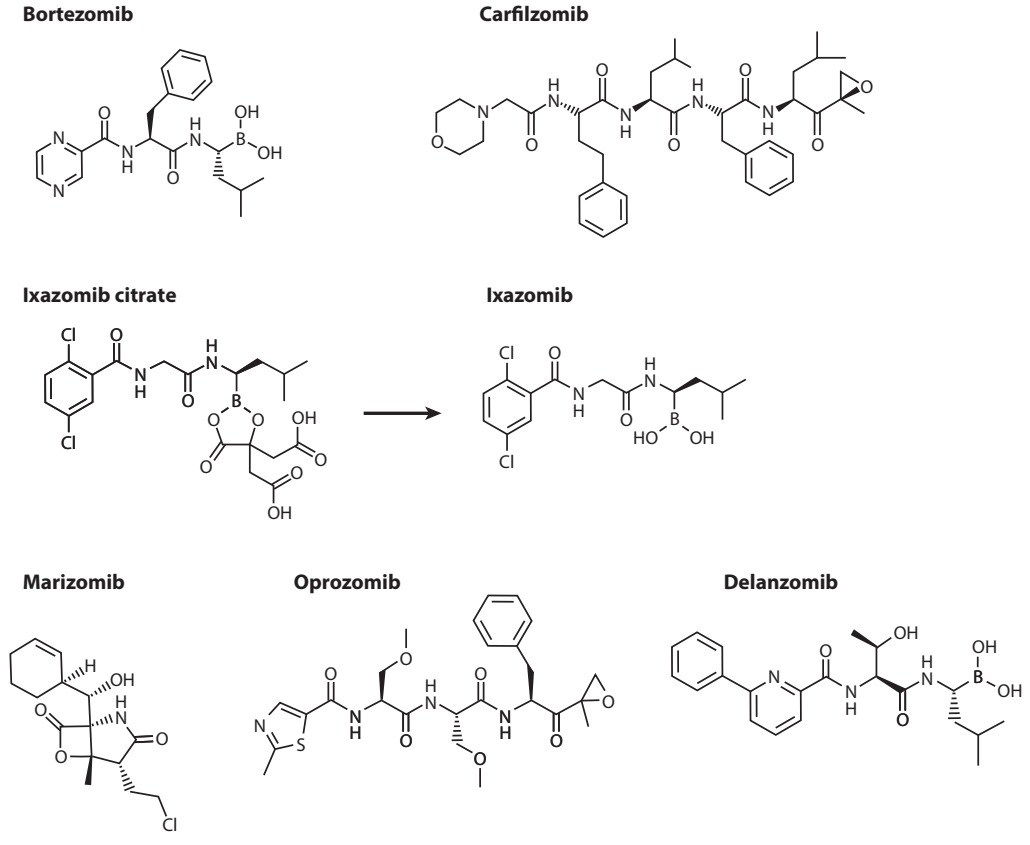


Figure 2

The proteasome inhibitors bortezomib, carfilzomib, and ixazomib citrate (which is converted in plasma into the active form, ixazomib) have been approved by the US Food and Drug Administration for treating multiple myeloma and are currently in clinical trials for additional uses. Marizomib, oprozomib, and delanzomib are in clinical trials for multiple myeloma as well as other applications.

containing an aldehyde group, the boronate binds more tightly to the active site threonine and thereby provides greater potency and selectivity toward the proteasome.

A phase I clinical trial of bortezomib in patients with refractory hematologic malignancies reported efficacy in some patients with multiple myeloma, mantle cell lymphoma, or non-Hodgkin lymphoma (103). Phase II studies focused on relapsed, refractory myeloma, and approximately one third of the patients responded, leading to fast-track FDA approval of bortezomib in 2003 (103). Subsequently, bortezomib was approved as a first-line treatment for new cases of multiple myeloma. Therapy combining bortezomib with a steroid such as dexamethasone and an immunomodulating agent such as lenalidomide have been found to be more effective than monotherapy (67). Bortezomib has also been approved for mantle cell lymphoma (67). Currently, there are over 200 open or planned clinical trials of bortezomib that are focused on the efficacy of bortezomib in other tumors, in combination with other drugs, and/or for noncancer applications such as graft-versus-host disease and other immune system–related applications (**https://clinicaltrials.gov**).

Carfilzomib (**Figure 2**) was approved by the FDA in 2012. Unlike bortezomib, carfilzomib is an irreversible proteasome inhibitor that contains an epoxyketone as the warhead, which forms a covalent bond with the threonine in the active site of the proteasome (13). The concept of the epoxyketone pharmacophore in carfilzomib came from epoxomicin.

Ixazomib citrate (**Figure 2**) was FDA-approved in 2015. It is orally active, whereas both bortezomib and carfilzomib require intravenous or subcutaneous administration (67). Ixazomib citrate is a prodrug that is rapidly converted into the active form (ixazomib) upon exposure to plasma (105). The active form contains a boronate moiety and is therefore a reversible inhibitor (5, 105). Patients resistant to bortezomib often show responses to carfilzomib or ixazomib.

Other compounds are currently in clinical trials, including marizomib, oprozomib, and delanzomib (1, 5, 13, 67). In addition to studies of these drugs for multiple myeloma, all are in clinical trials for patients with other types of tumors (**https://clinicaltrials.gov**). For example, several studies are testing marizomib for glioblastomas based on the ability of this drug to cross the blood–brain barrier (unlike bortezomib) and on animal studies showing that marizomib is effective in treating glioblastoma (106, 107). Marizomib is a natural product, named salinosporamide A, isolated from *Salinispora* bacteria found in ocean sediment. Marizomib is an irreversible inhibitor that contains a bicyclic β-lactone γ-lactam (**Figure 2**), as does the active form of lactacystin (clasto-lactacystin β-lactone). Oprozomib is structurally related to carfilzomib, with a peptide-like backbone and an epoxyketone warhead, and delanzomib is related to bortezomib, with a peptide-like backbone and a boronate warhead (**Figure 2**).

HOW DO PROTEASOME INHIBITORS KILL CANCER CELLS?

There is no question that the proteasome is the main target of bortezomib and the other drugs shown in **Figure 2**. More specifically, the target appears to be the chymotrypsin-like β5 catalytic subunit, based on the correlation between the inhibition of this subunit and its potency as an anticancer drug, although it cannot be ruled out that the inhibition of the β5i or the other subunits partially contributes to the drug's efficacy. For example, bortezomib inhibits the β5 and β5i subunits with comparable potency, and carfilzomib is approximately fivefold more potent toward the β5 than the β5i subunit (42, 43). There is little information on whether specific proteasome forms with different regulatory caps are more sensitive than others, as most in vitro biochemical studies have been done with purified 20S or 26S proteasomes. Furthermore, most biochemical studies on purified proteasomes measure activity using small synthetic substrates, typically four amino acids long, and these do not resemble typical substrates in the cell (e.g., ubiquitinated proteins,

misfolded proteins). Assays that measure the in vitro degradation of polyubiquitinated proteins (108) and the in situ degradation of fluorescent proteins (109) have been developed and recently reviewed (110).

Downstream Pathways Affected by Proteasome Inhibitors

Beyond the immediate target of the chymotrypsin-like β5 catalytic subunit of the proteasome, the downstream pathway that leads to cell death is less certain. A variety of pathways have been proposed, and it is possible that multiple pathways contribute to the mechanism. Several of the key proteins proposed to cause cell death are discussed above (e.g., NF-κB, p53, cyclin, and Cdks). Proteasome inhibitors also cause the accumulation of unfolded and misfolded proteins, which trigger the unfolded protein response and apoptosis. The extrinsic caspase-8 pathway and intrinsic caspase-9 pathways have also been proposed to be involved (67).

Although many studies have shown that the turnover of critical cellular proteins is altered by the treatment of cells with proteasome inhibitors, the doses of bortezomib required to alter protein levels are generally much higher than the doses required for cytotoxicity. For example, a highly cited study tested 5 μM of bortezomib for 1 h and found reduced degradation of IκBα in response to tumor necrosis factor (24). Many other studies used micromolar levels of bortezomib and/or long treatment times (6 h or more) to demonstrate altered levels of proteins (25–29). The effect of bortezomib on the rate of synthesis of cellular proteins in MCF-7 cells was examined using dynamic stable isotopic labeling and mass spectrometry–based proteomics (111). Despite the high concentration of bortezomib (2 μM), there was no major change in the biosynthetic rate of most proteins for the first 4 h of the study (111). A study by Kisselev and colleagues (112, p. 8,589) found that bortezomib and other proteasome inhibitors produced relatively small decreases in protein turnover, leading to their comment, "An obvious important question is how [therapeutic] concentrations of the inhibitors can block growth and cause apoptosis of myeloma cells."

Studies that incubate cells in the continued presence of high nanomolar or micromolar concentrations of bortezomib for 24–48 h do not reflect the concentration and time course of bortezomib in clinical scenarios. After subcutaneous administration, peak plasma levels are ~25–50 nM and this peak is sustained for 1–2 h (113–116). After intravenous injection, peak plasma levels are about tenfold higher than for subcutaneous administration but only for ~5 min—levels rapidly drop as bortezomib distributes to tissues (the volume of distribution is ~500 L). Both routes provide equal drug exposures and generally comparable therapeutic efficacy (115, 116). Proteasome activity in blood cells is inhibited by as much as 75% immediately after a single dose (113–116).

To mimic the exposure of cells in a clinical setting, a recent study treated myeloma cells with various concentrations of bortezomib for 1 h and further incubated the cells in the absence of drug for additional lengths of time (116). This treatment produced inhibition of the proteasome comparable to that observed in patients undergoing bortezomib therapy. All seven myeloma cell lines tested showed cytotoxicity with this treatment, but only one of these seven was sensitive to clinically relevant concentrations (116). In this study, robust changes in the levels of proteins such as poly ADP-ribose polymerase (PARP) were only detectable with bortezomib concentrations higher than what is achieved clinically (116).

One potential explanation of this apparent paradox is that the protein or proteins affected by bortezomib that cause cell death require only small, undetectable changes in their turnover to cause toxicity. Alternatively, the proteins that are the focus of most biochemical studies may not be the mediators of cell death, and instead other, short-lived proteins may be the major downstream mediators of cell death. For example, a recent study found that treatment of several different mantle cell lymphoma cell lines with relatively low concentrations of bortezomib (8–10 nM)

for 8 h produced dramatic increases in levels of NOXA, a short-lived proapoptotic Bcl-2 family protein (117). Treatment times shorter than 8 h were not reported in this study.

A related problem is that some effects of proteasome inhibitors occur very rapidly, long before changes in protein levels are detectable. For example, RPMI-8226 myeloma cells show a transient increase in intracellular Ca^{2+} levels within 5 min of exposure to 50 nM bortezomib (118). Another study found that 50 nM bortezomib caused a large reduction in the level of monoubiquitinated histone 2B (H2Bub1) at the earliest timepoint examined (2 h), leading the authors to state, "This 'nonproteolytic' effect of proteasome inhibition challenges the general assumption that proteasome inhibition functions primarily by preventing the degradation of specific proteins required for essential cellular processes" (119, p. 5,749). The level of H2Bub1 was also significantly decreased by the proteasome inhibitor MG132 after only 30 min of treatment (120). How can proteasome inhibitors cause rapid decreases in H2Bub1 or rapid increases in Ca^{2+}? Perhaps the effects of proteasome inhibitors are not mediated entirely through changes in protein levels.

Proteasome Inhibitors Lead to Dramatic Changes in Levels of Intracellular Peptides

It is well known that the proteasome cleaves proteins into peptides, but these peptides were generally ignored and thought to be rapidly degraded by cytosolic peptidases, with the exception of peptides that bound to MHC class I proteins (72, 73, 121, 122). As described above, many recent studies have detected a subset of proteasome-produced peptides in extracts of tissues and cultured cells. To identify the source of these peptides, cells were treated with various compounds, and quantitative peptidomics was used to compare levels in treated and untreated cells. Treatments that blocked lysosomal enzymes were ineffective at changing the levels of intracellular peptides. Similarly, increasing intracellular calcium to activate the calpains (a family of cytosolic endopeptidases) was also ineffective. In contrast, nearly every intracellular peptide was affected by short-term treatment with proteasome inhibitors (50, 51, 86). These effects were rapid, occurring within 30 min of exposure to the proteasome inhibitor (the shortest time point examined). The magnitude of the changes was large, with most peptides altered twofold or more. Most importantly, the effects were seen with clinically relevant concentrations of proteasome inhibitors—50 nM bortezomib caused major changes in levels of most peptides (50, 51, 86). Interestingly, while levels of most peptides were reduced by treatment of HEK293 cells with epoxomicin, treatment of these cells (and other cell lines) with bortezomib, carfilzomib, or ixazomib resulted in decreased levels of some peptides and elevated levels of other peptides (50, 51, 86, 88).

Could the dramatic changes in peptide levels contribute to either the therapeutic effect or the side effects of proteasome inhibitors? If these peptides are biologically active, it is likely that large changes in their concentrations would have physiological effects. Intracellular peptides have been proposed to be active based on two considerations. First, numerous studies have used synthetic peptides of 10–20 residues to perturb cellular function by disrupting protein–protein interactions (123–129). Although most of these studies have not considered that this ability of peptides to alter protein function is a natural process, the fact remains that small peptides can affect many cellular processes. The second theoretical consideration is that only a small subset of proteasome-cleaved peptides are detected in peptidomics studies, typically 1–3 peptides per protein and not the dozens of fragments that would be expected after proteasomal digestion (50, 51, 86, 88, 92). Because the observed peptides are not enriched in amino acids that confer stability (e.g., proline), their stability is most likely a result of binding to cellular proteins.

In 2004, soon after the first identification of intracellular peptides, Ferro and colleagues (130) proposed that these peptides were functional. In the time since this proposal, several studies have

investigated potential roles for endogenous intracellular peptides. One study found that peptides produced by the cleavage of mitochondrial proteins induced the cellular unfolded protein response in *Caenorhabditis elegans* (131). Another study found that endogenous peptides produced in *Drosophila* affected gene expression, although some of these peptides were thought to be produced from short coding sequences and not from proteasome-mediated cleavages (132). Recently, many studies have found biological activities for a number of peptides that were detected in cells/tissues and which are likely to be proteasome-mediated cleavage products (133–139). Thus, while still speculative, there is emerging evidence that peptides produced from the proteasome are functional within the cell. And, because the levels of these peptides are dramatically affected by short-term treatments with relatively low concentrations of proteasome inhibitors, these peptides may contribute to the physiological effects of proteasome inhibitors.

CONCLUSIONS AND PERSPECTIVES

In the 20 years since the first publication on bortezomib (PS-341), there has been remarkable progress, with three FDA-approved drugs and more in development. Bortezomib has gone from a third-line treatment for relapsed multiple myeloma to a front-line treatment for new cases. Drug combinations of proteasome inhibitors together with conventional anticancer drugs and new immunotherapy approaches are showing great promise. Other FDA-approved proteasome inhibitors are moving forward not only as treatments for bortezomib-resistant tumors but also for additional applications. Many ongoing clinical studies are testing the effectiveness of proteasome inhibitors in various types of cancer, including metastatic prostate cancer, neuroendocrine tumors, cutaneous T-cell lymphoma, non-Hodgkin lymphoma, and Hodgkin lymphoma (**https://clinicaltrials.gov**). New drugs in development that penetrate the blood–brain barrier may be useful for treating glioblastomas. Outside of oncology, proteasome inhibitors have shown efficacy as immunosuppressants in animal models, and numerous clinical trials are exploring these possibilities (140). An exciting direction of recent studies is the development of proteasome inhibitors that are more potent toward the immunoproteasome, specifically the β5i subunit. Such inhibitors may be especially effective as immunosuppressants with fewer side effects (141). Another direction is the development of inhibitors that are selective for the β2 trypsin-like activity, with the idea that combinations of these agents together with drugs like bortezomib (which targets β5 and also β1) can lead to greater proteasome inhibition (142, 143).

Over the past 20 years, much has been learned about the ubiquitin-proteasome system and the diversity of the catalytic and regulatory subunits that make up the various forms of the proteasome that are found in cells. An important question remains regarding the specific downstream effectors that mediate the therapeutic and side effects of the proteasome inhibitors. The conventional dogma is that the proteasome inhibitors block the activation of NF-κB and the degradation of other proteins, thereby causing cytotoxicity in cell types that are especially sensitive (e.g., multiple myeloma cells). While this has been shown in cell culture studies, the dose of drug and length of treatment required to show changes in NF-κB or other protein levels are not consistent with clinically relevant concentrations. This has caused several workers in the field to question whether the turnover of these proteins is the primary mechanism by which proteasome inhibitors are clinically effective.

The proteasome converts proteins into peptides, which were assumed to be rapidly degraded. While this appears to be true for the vast majority of the proteasome-generated peptides, a subset can be detected in cells and tissues, and levels of these peptides are dramatically altered by short-term treatment with relatively low concentrations of proteasome inhibitors. Whether this also occurs in patients treated with proteasome inhibitors has not yet been determined. Most

importantly, the functional consequences of altered peptide levels need to be identified. From a theoretical point of view, it is likely that endogenous peptides play a role in cellular function much like the complex role of small RNAs in cellular function (144).

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