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REVIEW ARTICLE

Proteasome inhibitors mechanism; source for design of newer therapeutic agents

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The proteasome was first identified as a high MW protease complex that gets resolved into a series of low MW protein species upon denaturation. As the dominant protease dedicated to protein turnover, the proteasome shapes the cellular protein repertoire. Our knowledge of proteasome regulation and activity has improved considerably over the past decade. Novel inhibitors, in particular, have helped to advance our understanding of proteasome biology. They range from small peptide-based structures that can be modified to vary target specificity to large macromolecular inhibitors that include proteins. Although these reagents have an important role in establishing our current knowledge of the proteasome's catalytic mechanism, many questions remain. The future lies in designing compounds that can function as drugs to target processes involved in disease progression. Our focus in this chapter is to highlight the use of various classes of inhibitors to probe the mechanism of the proteasome and to identify its physiological significance in the cell, so that the mechanism of inhibition of proteasome will work as a definite source for design of protocols for newer therapeutic agents for the treatment of inflammation and in cancer therapy.
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INTRODUCTION

Characterization of the catalytic mechanisms of the proteasome

Proteasome is found to catalyze the hydrolysis of amide bonds adjacent to a variety of amino acids. Chymotrypsin-like, trypsin-like and post-glutamyl peptide hydrolyzing activity[1,2] are the three categories of the substrates for the proteasome, based on the nature of the amino acid found in the P1 position adjacent to the scissile amide bond. Soon after the initial characterization of its biochemical properties, attention shifted to understanding the enzymatic mechanism of the proteasome. There is a need to identify a class of reagents capable of potent inhibition of a target protease. On the basis of catalytic mechanism, proteases were classified into four main groups and virtually all could be placed into aspartic, metallo, cysteine or serine protease families. However, initial biochemical studies of the proteasome quickly indicated that it did not fit into any of these classifications and thus, a new family must be established. Biochemical *anal* of the proteasome by variety of classical protease inhibitors can be performed taking advantage of the proteasome's large size. Benzyloxycarbonyl-glycyl-glycyl-leucinal, a peptide aldehyde transition-state analog of the substrate used to measure the chymotrypsin-like activity, exclusively inhibits that activity ($Ki=2.5\times10^{-4}$ M), while markedly activating the trypsin-like activity. The trypsin-like activity is inhibited by leupeptin ($Ki=1.2$ μM) and by sulfhydryl blocking agents, and activated by thiols, suggesting that this activity is due to a thiol protease.

The results indicate that cation-sensitive neutral endopeptidase is a multicatalytic protease complex, in which distinct proteolytic activities are associated with separate components of this high-MW protein.[3] Subsequently, a multitude of studies were performed using diverse sets of easily accessible, class-specific inhibitors.[4] Chelating agents indicated that the proteasome did not belong to the metallo-protease family and pepstatin ruled out its aspartic protease mechanism.[5]

Considering the general mechanisms for the proteolysis by other Ntn hydrolases and genetic, structural and inhibitor studies of the 20S proteasome, the following mechanism was proposed for the proteolysis catalyzed by the 20S proteasome (Figure 1a). In step 1, as the hydroxyl group of the amino terminal Thr-1 attacks the peptide carbonyl group of the scissile bond, a proton is transferred from the hydroxyl group of the nucleophile to the uncharged amine group of the same Thr-1, most likely through an intermediate water molecule. Evidence of the involvement of a water molecule comes from its proximity to the amino terminal Thr-1 in the active site of the catalytic subunit, as observed in the crystal structure of both penicillin acylase and the 20S proteasome.

The tetrahedral addition intermediate later breaks down to result in the formation of the covalent acyl-enzyme intermediate, with a proton transfer to the leaving group (step 2). Hydrolysis of the acyl enzyme intermediate is proposed to occur by a similar mechanism as described above for its formation. The free α-amine group of the amino terminal Thr-1 removes a proton from the attacking water

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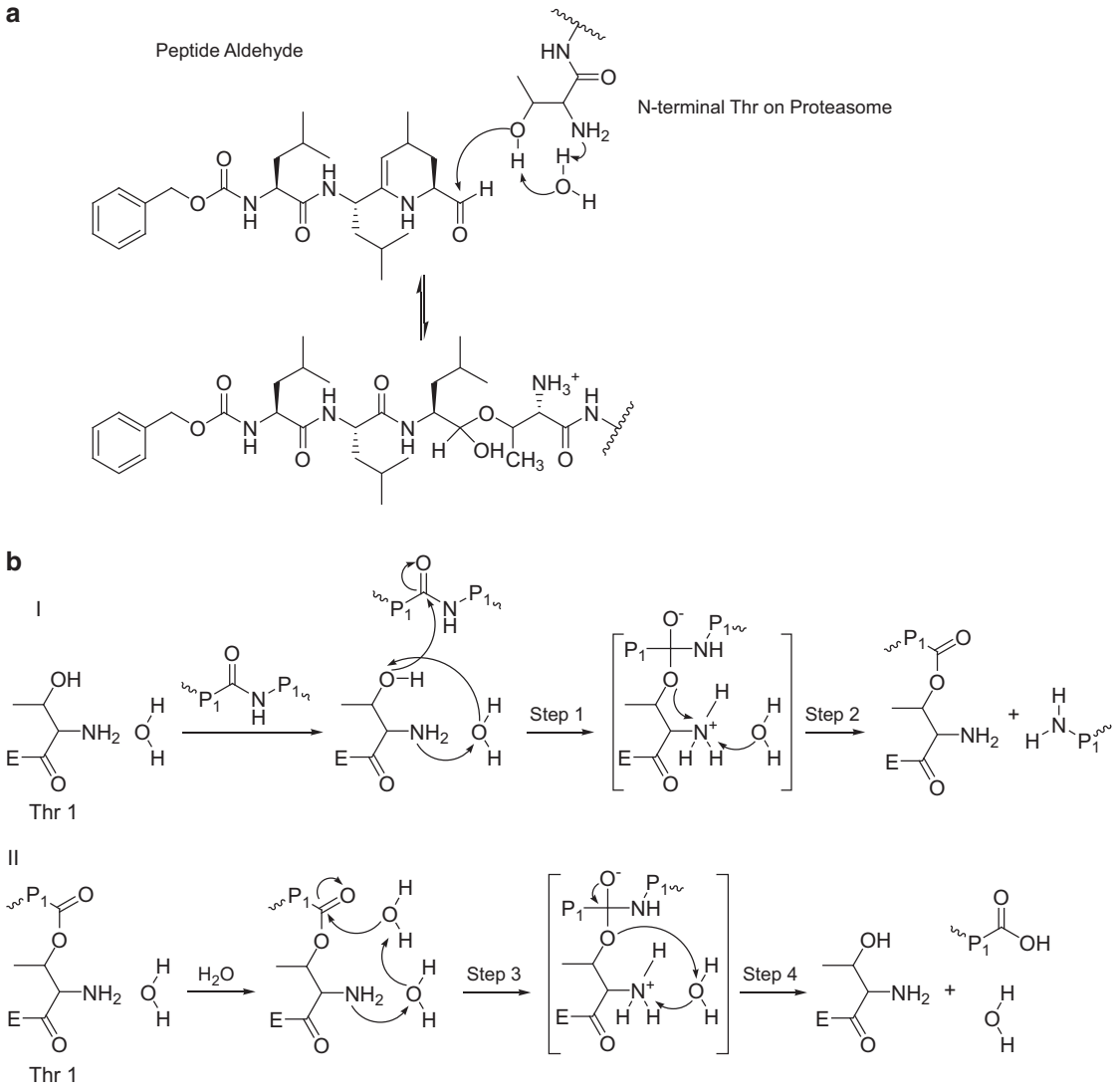


Figure 1 (**a**) The proposed mechanism for the inhibition of the 20S proteasome by the peptide aldehyde. (**b**) The proposed mechanism for the proteolysis catalyzed by the 20S proteasome.

molecule again through an intermediate water molecule (step 3). A proton is transferred to the oxygen atom of Thr-1 as the product is expelled from the tetrahedral addition intermediate[6] (step 4). Peptide aldehydes have long been used as inhibitors for both serine and cysteine proteases, given that the aldehyde functional group is readily subject to a nucleophilic attack by hydroxyl or thiol groups and that the proteasome uses the hydroxyl group of the amino terminal threonine as a nucleophile[6] (Figure 1b). The proteasome was also found to be resistant to inactivation by classical serine protease inhibitors such as di-isopropyl fluoro-phosphate and phenylmethylsulfonyl fluoride. However, it was determined that organic mercurials, known to be highly reactive toward thiol groups, profoundly inhibited the proteasome.[7] Moreover, additional thiol reactive compounds, such as N-methyl maleimide and iodo-acetic acid could partially block multiple proteasomal activities, with preferential inhibition of the trypsin-like activity. Thus, the proteasome was first classified as a thiol protease and the name 'macropain' was suggested to propose a link to the papain family of cysteine proteases.[1,7] Controversy over the classification of the proteasome's catalytic mechanism arose when several groups reported that prolonged exposure of the proteasome to high concentrations of di-isopropyl fluoro-phosphate resulted in inhibition of its chymotrypsin-like activity.

Proteasome inhibitors

Synthetic reversible inhibitors. Potency and specificity are two features often considered to be most critical when designing protease inhibitors. Failure to achieve either of these traits in inhibitor design can adversely influence our understanding of a given protease, as illustrated by the initial synthetic compounds used to block the proteasome. All these compounds lacked specificity, as they were originally designed to block non-proteasomal proteases. However, with the proteasome's catalytic mechanism established and its three-dimensional structure determined, synthetic chemistry could be used to further refine inhibitory compounds leading to greater potency and enhanced selectivity for the proteasome.

The first synthetic inhibitors designed to target the proteasome were peptide aldehydes as they were relatively easy to synthesize and previous studies indicated that small peptidic substrates could serve as a template for inhibitor design. As a result, numerous peptide sequences have been synthesized as aldehydes[9,10] and several have

proved widely useful. Peptide aldehydes such as leupeptin and calpain inhibitors I and II, as well as several closely related compounds, such as MG-132 (Cbz-Leu-Leu-leucinal) and MG-115 (Cbz-Leu-Leu-norvalinal; developed by Proscript; formerly Myogenics), are frequently used to block proteasome activity both *in vitro* and *in vivo*.[9,14] In addition, the tetra peptide aldehyde Z ± IE(OtBu)AL ± H developed by Wilk *et al*.[15,16] is among the commercially available proteasome inhibitors. Most of these compounds primarily inhibit the chymotrypsin-like activity of the proteasome, but are capable of modifying all three primary catalytic β-subunits at high concentrations. One of the drawbacks to these compounds is their reactivity towards both serine and cysteine proteases through formation of hemi-acetals or hemi thioacetals, with either hydroxyl or thiol nucleophiles present in the proteasome (Figure 2a). Specificity for the proteasome can only be achieved by design of peptide sequences optimized for proteasome binding. Owing to broad specificity to nucleophiles, very few examples of selective peptide aldehydes have ever been documented. Another drawback to the use of the aldehyde electrophile is its reactivity with free thiols and its instability in aqueous solution. Regardless of these shortcomings, the initial contributions of peptide aldehyde inhibitors laid the foundation for subsequent generations of proteasome inhibitors. By taking advantage of electrophiles that react specifically with a hydroxyl nucleophile, the first step toward designing compounds with increased selectivity for the proteasome will be taken.[16,17]

Peptide boron esters and acids are potent inhibitors of serine proteases that form reversible covalent interactions with the active hydroxyl site (Figure 2b). Partial list of compounds are found to inhibit the proteasome in a reversible manner. Schematic representations of inhibition mechanism shows site of initial attack by the active site *N*-terminal threonine found on multiple proteasomal β-subunits (arrows) that leads to form transient covalent adduct. Poor overlap between orbitals of the nonbonding electrons on sulfur with those of the vacant d-orbitals on boron produces lack of reactivity of these compounds towards cysteine proteases that results in a weak sulfur-boron bond. Furthermore, these derivatives are less reactive toward circulating nucleophiles in aqueous solutions than their aldehyde counterparts. Chemists and biochemists at Proscript (now Millennium Pharmaceuticals) and Cephalon found that changing the electrophile from an aldehyde to a boron acid or ester created compounds with reduced cross-reactivity toward cysteine proteases and significantly increased the potency of the proteasome.[18] Rivett *et al*.[16] also explored the potency and selectivity of a number of peptide boron acids and esters, including a boron ester derivative of the MG-132 aldehyde, described above. Further refinement of these lead compounds has resulted in the generation of dipeptide boron acids that are capable of inhibiting the proteasome at picomolar concentrations.[18,19] This high degree of potency for the proteasome essentially results in selective inhibitors of them. For example, the dipeptide boron ester, PS 341 requires 20 000 times higher concentrations to inhibit other abundant serine proteases.[18] These compounds are also highly bioavailable and are currently being pursued in clinical studies as potential anti-inflammatory agents.[19]

Several numbers of peptide-based electrophiles that reversibly target the proteasome's active site threonine have been explored. Most of these compounds contain a short di- or tri-peptide recognition element fused to a C-terminal modified amino acid (often an aliphatic residue such as leucine). Examples include the peptide β-keto carbonyls[17] (Figure 2c), β-keto amides[20] (Figure 2d) and β-keto aldehydes[21] (Figure 2e). All contain a highly electrophilic carbonyl carbon that forms a stable acetal or ketal linkage to the threonine hydroxyl when bound in the active sites

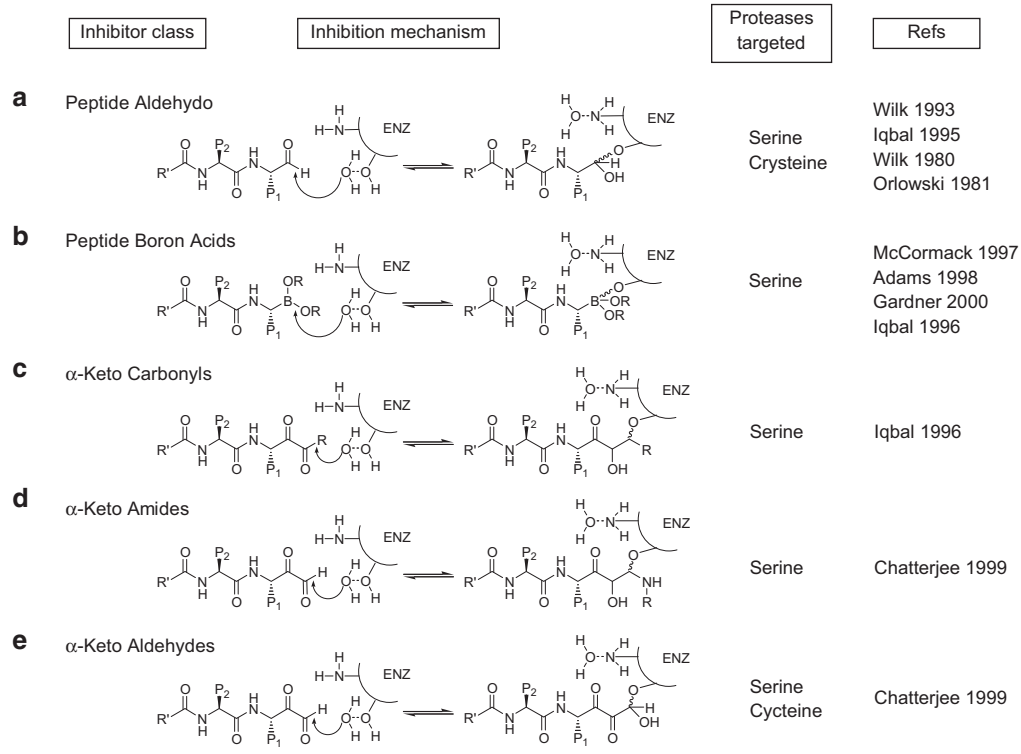
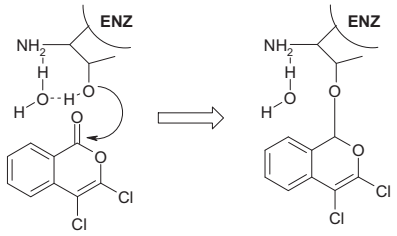
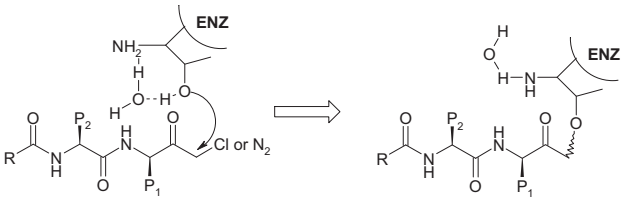
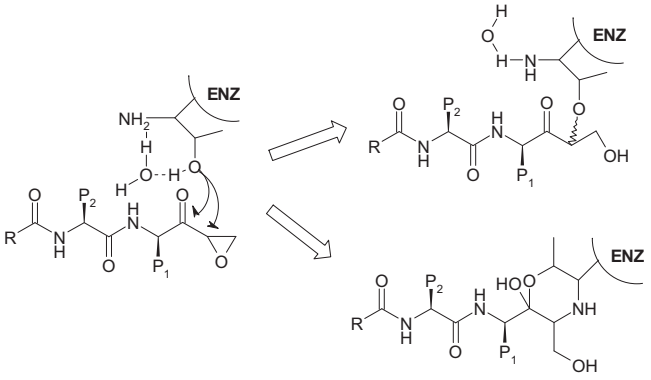
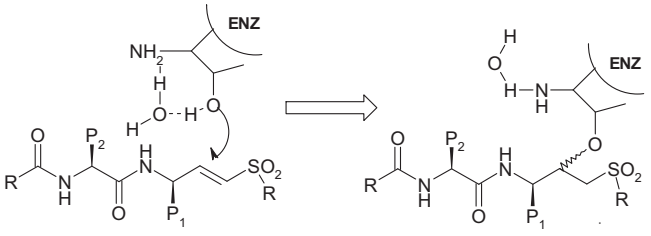


Figure 2 (**a–e**) General structures and mode of action of reversible synthetic proteasome inhibitors. Partial list of compounds found to inhibit the proteasome in reversible manner. Schematic representations of inhibition mechanism show site of initial attack by the active site *N*-terminal-threonine found on multiple proteasomal β-subunits (arrows), that leads to form transient covalent adduct.

Table 1 Inhibition mechanism for synthetic covalent inhibitors. (Suicide substrates)

Sr. No.	*Inhibitor class*	*Inhibition mechanism*	*Proteases targeted*
a	3,4-dichloroisocoumarin		Serine
b	Peptide chloromethyl/diazomethyl ketones		Serine cysteine
c	α′,β′-Epoxy ketones		Serine
d	Peptide vinyl sulphones		Serine cysteine

of the proteasome (Figures 2c–e). Moreover, compounds containing a β-keto amide at their *C*-terminus have the potential for extension of inhibitor structures into the S0 region of the target protease, located directly *C*-terminal to the site of amide bond hydrolysis (Figure 2d). Development of peptides containing binding elements with the hope to gain additional specificity and potency toward the proteasome's multiple active sites.[21] Unfortunately, all of these reversible inhibitors suffer from the same limitations associated with the peptide aldehydes, including broad specificity and instability in aqueous solution.

Synthetic covalent inhibitors (Suicide substrates). It is a major class of synthetic proteasome inhibitors having the compounds that inactivate the catalytic threonine nucleophile by irreversible covalent adduct formation (Table 1). These inhibitors are often referred to as suicide substrates, having wide spread use in biochemical studies of the proteasome. Furthermore, the covalent nature of these compounds allows protease activity to be traced using suitably labeled inhibitors. The general serine protease inhibitor 3, 4-dichloro isocoumarin (3, 4-DCI) was one of the first compounds found to function as a potent irreversible inhibitor of the proteasome.[3,8,23] Furthermore, Powers *et al.*[24,25] found that several structurally related isocoumarin derivatives, known to function as class-specific inhibitors of serine proteases, potently inhibited the proteasome.[24,25] These findings prompted classification of the proteasome as a serine protease.

Isocoumarins are potent mechanism-based heterocyclic irreversible inhibitors for a variety of serine proteases. Most serine proteases are inhibited by the general serine protease inhibitor 3,4-DCI, whereas isocoumarins containing hydrophobic 7-acylamino groups are potent inhibitors for human leukocyte elastase and those containing 7-alkylureido groups are inhibitors for procine pancreatic elastase. Isocoumarins containing basic side chains that resemble arginine are potent inhibitors for trypsin-like enzymes. A number of 3-alkoxy-4-chloro-7-guanidinoisocoumarins are potent inhibitors of bovine thrombin, human factor Xa, human factor XIa, human factor XIIa, human plasma kallikrein, porcine pancreatic kallikrein and bovine trypsin. Another cathionic derivative, 4-chloro-3-(2-isothioureidoethoxy) isocoumarin, is less reactive toward many of these enzymes, but is an extremely potent inhibitor of human plasma kallikrein.[24,25] Although many isocoumarin analogs possessing a variety of hydrophobic appendages have been synthesized, only 3,4-DCI shows appreciable activity against the proteasome.[8,22] This inhibitor functions as a masked acid chloride that binds to the active site near the base-activated hydroxyl side chain of threonine to form a covalent ester linkage (Table 1a). Inhibition is achieved by modification of one or more of the active sites of the proteasome. Although 3, 4-DCI initially found widespread use as a proteasome inhibitor, complications arose when it was discovered that in contrast to its potent inactivation of the chymotrypsin-like activity of the proteasome it simultaneously resulted in the inactivation of other activities.[4,23] Furthermore, 3,4-DCI treatment of proteasomes lead to the accelerated processing of selected protein substrates.[4,23] Carfilzomib (formerly PR-171; Proteolix, Inc., South San Francisco, CA, USA), is a tetrapeptide epoxyketone related to epoxomicin. There are two components of this agent, a peptide portion that binds to the substrate binding pocket(s) of the proteasome with high affinity and epoxyketone pharmacophore that interacts with the catalytic amino terminal threonine residue and irreversibly inhibits proteasome activity.[12] It binds the proteasome irreversibly, and its activity and mechanism of action in preclinical models of multiple myeloma are evaluated. Unlike other classes of proteasome inhibitors that show non-target specifcity, epoxomicin is highly specific for the 20S proteasome.[13]

PR-171 potently bound and inhibited the chymotrypsin-like subunit of the proteasome *in vitro*, in cellulo and *in vivo* at low concentrations. However, at higher concentrations, unlike bortezomib, which targeted the chymotrypsin-like and peptidyl-glutamyl peptide hydrolyzing activities *in vivo*, PR-171 also displayed significant inhibition of the trypsin-like and the peptidyl-glutamyl peptide hydrolyzing activities. PR-171-induced proteasome inhibition was associated with accumulation of polyubiquitinated substrates and pro-apoptotic Bax. Brief pulse PR-171 exposure, which simulates the *in vivo* pharmacokinetics of bortezomib, led to PR-171-mediated inhibition of cellular proliferation linked to induction of caspase-3-dependent apoptosis through both intrinsic (caspase-9) and extrinsic (caspase-8-dependent) pathways.[26] Lactacystin, an irreversible proteasome inhibitor, had no effect, but the β-lactone derivative of lactacystin, which directly reacts with proteasomes, inhibited the degradation of short-lived proteins. These inhibitors also blocked the rapid ubiquitin-dependent breakdown of a β-galactosidase fusion protein and caused accumulation of enzymatically active molecules in cells. The degradation of the bulk of cell proteins, which are long-lived molecules, was not blocked by proteasome inhibitors, but could be blocked by phenylmethylsulfonyl fluoride. A distinct type of proteasome inhibitor is lactacystin, a *Streptomyces* metabolite, which selectively inhibits multiple peptidase activities of mammalian proteasomes by covalently modifying the active site threonine residues of the β-subunits. The active chemical species that reacts with the proteasome is not lactacystin, but its spontaneous hydrolysis product *clasto*-lactacystin β-lactone (hereafter called β-lactone). In many different mammalian cells, treatment with lactacystin inhibits overall protein degradation, including the breakdown of short-lived and long-lived components.

Lactacystin was also capable of blocking, almost completely, peptide hydrolysis and degradation of ubiquitin-conjugated lysozyme by 26S proteasomes purified from wild-type yeast cells.[26] Chloromethyl ketones comprise a distinct class of commonly used covalent irreversible serine protease inhibitors. The diazomethyl ketones were initially thought to be reactive only toward cysteine proteases, but were later found to react with serine proteases as well and are closely related to chloromethyl ketones.[25] Mechanistic function of both classes of peptide electrophiles is similar to that of peptide aldehydes and boron acids. The chloride or diazo groups adjacent to the ketone moiety create a highly electrophilic site that is capable of reacting with activated nucleophiles of the proteasome (Table 1b). Peptide-based inhibitors lead to the development of this class of electrophiles as covalent inhibitors of the proteasome.[25] However, the low potency of these compounds toward the proteasome necessitated high concentrations of inhibitor, to elicit appreciable inhibition, limiting their use as proteasome inhibitors. The utility of this class of reagents as proteasome inhibitors is limited by their broad reactivity with many serine type proteases and their instability in solution.[24] Initially designed inhibitors to target serine proteases have been successfully converted into proteasome inhibitors. α′,β′-epoxyketone electrophiles have been incorporated into peptides sequences optimized for binding to the proteasome 26. The potent tri-peptide aldehyde inhibitor of the chymotrypsin-like activity of the proteasome Cbz-Ile-Ile-Phe-H, converted to the corresponding α′,β′-epoxyketone, produced a covalent inhibitor with a 40-fold improved potency.[27] The α′,β′-epoxyketones, similar to the β-keto amides and aldehydes, have two electron deficient carbon atoms that are susceptible to attack by the proteasome's threonine hydroxyl. This feature creates the potential for either reversible ketal formation with the carbonyl or irreversible ether formation through ring opening of the strained epoxide moiety (Table 1c).

Attack at the carbonyl carbon places the proteasome's terminal amino group in close proximity to the highly electrophilic epoxide ring, subsequent ring opening by the amino group would result in the formation of a stable six-membered ring (Table 1c). Evidence for this unusual 'double attack' as the primary mechanism for inhibition of the proteasome by α′,β′-epoxyketones was supported by recent studies of the natural product epoxomicin, which relies on the same electrophilic group for inhibition of the proteasome. Peptide vinyl sulfones are electrophiles, initially designed as cysteine protease inhibitors,[27,28]that function by formation of a covalent linkage with an active site nucleophile via a Michael addition (Table 1d). The same electrophile was reported to be resistant to attack by virtually all serine proteases.[27,28] The strong preference for a thiol nucleophile was believed to result from the 'soft' basic property of the thiol group that favors attack at the unsaturated carbon-carbon double bond.[27,28] However, several peptide vinyl sulfones were found to inhibit the proteasome through covalent bond formation, with the active site threonine hydroxyl (Table 1d). Replacement of the carboxylbenzoyl (cbz)-*N*-terminal capping group with a nitrophenol moiety produced a compound with increased potency that was easily modified by radioactive iodine. This class of electrophilic peptide proved to be valuable in affinity labeling and mechanistic studies of the proteasome.[29,30]

Bi-functional synthetic inhibitors—rational design based on structure. The complex biochemical mechanism of the proteasome was

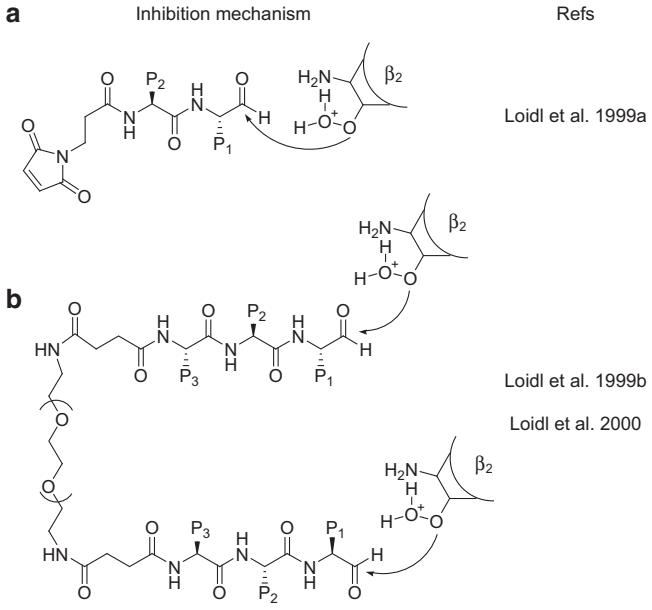


Figure 3 (**a, b**) Synthetic bivalent inhibitors of the proteasome. Structures of two classes of inhibitors that target multiple nucleophiles on multiple proteasomal β-subunits. In the case of class (**a**) selective inhibition of the β-2 proteasomal subunit is achieved by adduct formation b etween the active site threonine (β-2) and a side-chain cysteine of an adjacent non-catalytic β-subunit (β-3). Compounds of class (**b**) target two active site threonines on different catalytic β-sub units in the core 20S particle.

understood by the determination of its three-dimensional structure. The first structure was obtained for the archaebacterial form of the proteasome[10,11] and revealed a complex, comprising a single α- and β-type subunit each repeated 14 times, to create a highly symmetrical core complex. In contrast, the core of the yeast proteasome is made up of seven distinct α- and β-type subunits each repeated twice in the complex.[15] These structural studies provide valuable information for understanding the mechanism and topology of the complex. Initial structure-aided studies were aimed at creating inhibitors that could specifically target a single active site of the proteasome.[31]

The β-2 subunit of the yeast proteasome was shown to be responsible for the trypsin-like activity of the yeast proteasome.[32] It also possesses a unique feature in which a portion of its substrate-binding pocket lies in close proximity to a cysteine residue of the neighboring β-3 subunit.[15] Thus, the β-2 active site depends on contacts created by multiple subunits, and could potentially be targeted by reagents with two reactive electrophiles. Such β-2-specific reagents would represent a new class of inhibitors specific for the trypsin-like activity of the yeast proteasome. Using the cysteine-reactive maleimide group, Moroder *et al.*[31] synthesized a series of bi-functional peptide aldehydes (Figure 3a). The structure of the β-2 active site provided a guide for design of peptide scaffolds that placed the maleimide group in the S3 pocket of the β-2 subunit, proximal to the free thiol of the β-3 subunit. Replacement of the P3 acetyl-leucine residue with amaleoyl-β-alanine residue of the peptide aldehyde, Ac- LLnL-H, converted this compound from a potent, reversible inhibitor of the chymotrypsin-like activity into a specific, covalent inhibitor of the trypsin-like activity. This significant change in specificity was the direct result of a double covalent attack by threonine and cysteine in the active site of the β-2 subunit (Figure 3a). Further refinement of the P1 and P2 positions, to incorporate residues optimal for the trypsin-like activity, produced compounds with significant increases in potency. Thus, structural studies proved essential to the development of this novel, highly selective and highly potent class of proteasome inhibitors. The topology of the active sites of the proteasome was also used to generate inhibitors that could span multiple active sites.[32,33] The proteasome core structure contains two stacks of β-subunits, each active site is repeated twice and thus, can be targeted twice by a single compound that possesses reactive groups separated by the appropriate distance. An ethylene glycol polymer was selected as a scaffold for inhibitor design because it is composed of monomers that can be linked to create spacers of variable lengths, it contains no hydrolysable peptide bonds, and it is highly soluble in water. Compounds were synthesized by fusing potent peptide-aldehyde sequences end-to-end between a series of ethylene glycol monomers (Figure 3b). The distance between two active site threonine residues was calculated and was used to determine the number of monomers required.

The resulting compound, containing two identical peptide aldehydes specific for the chymotrypsin-like activity of the proteasome, was found to have a 100-fold increased potency toward the chymotrypsin-like activity as compared with the activity of the monomeric peptide aldehyde. Similarly, combining peptide sequences intended to target the trypsin-like activity of the proteasome, resulted in enhanced potency toward the trypsin-like activity. The versatility of the technique was also demonstrated by the production of hetero-bi-functional compounds that contain one chymotrypsin-like specific aldehyde and one trypsin-like specific aldehyde. These compounds are potent inhibitors of both activities.[32] Collectively, these studies highlight the importance of detailed structural information for inhibitor design. They also demonstrate how information from structural studies of complex, multi-component enzymes, such as the proteasome, can help to define mechanisms for controlling potency and selectivity of synthetic inhibitors. Structures of two classes of inhibitors that target multiple nucleophiles on multiple proteasomal β-subunits. In class (a) selective inhibition of β-2 subunit, achieved by adduct formation between the active site threonine (β-2) and a side chain cysteine of an adjacent non-catalytic β-subunit (β-3) class (b), targets two active site threonine on different catalytic β-subunits in the core 20 s particle.

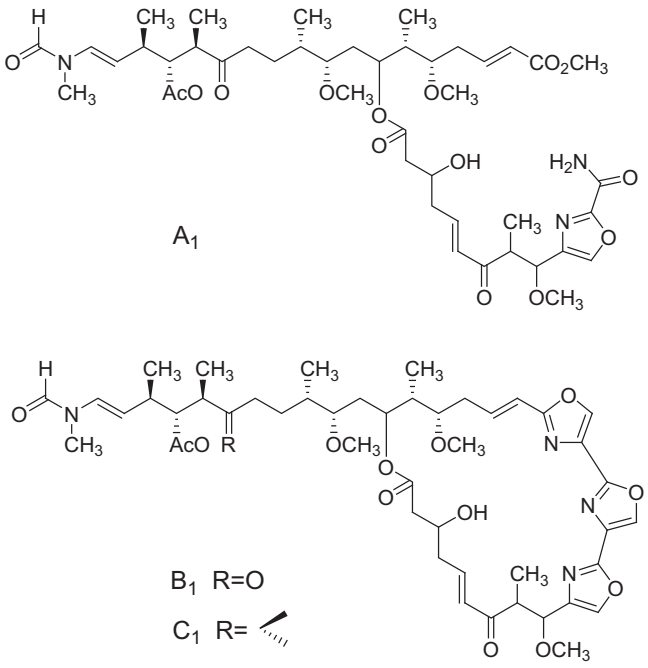


Figure 4 (A1, B1, CI). A new mycalolide derivative secomycalolide A (A1, B1, C1) from a marine sponge of the genus *Mycale*.

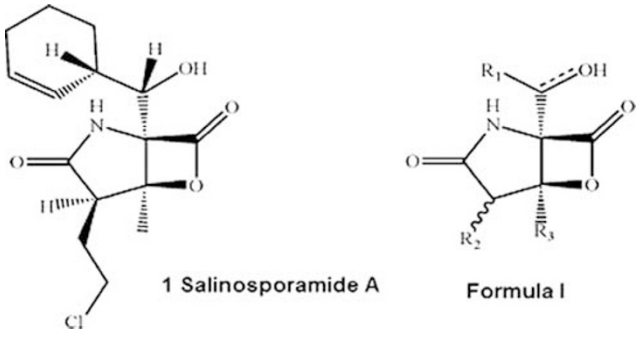


Figure 5 (1, NPI-0052) A novel analog of marine-derived potent proteasome inhibitor Salinosporamide A.

Natural proteasome inhibitors. A new mycalolide derivative secomycalolide A, (Figure 4A1) from a marine sponge of the genus *Mycale*, is isolated together with known mycalolide A (Figure 4B1) and 30 hydroxymycalolide A (Figure 4C1). They showed moderate inhibitory activities against chymotrypsin-like activity of the proteasome. Among three mycalolides, seco-oxyazole derivative Figure 4A1 showed the most potent inhibitory activity. So far, cytotoxicity and potent actin depolymerizing activity of mycalolides have been reported; however, this is the first report of proteasome inhibitory activity of mycalolides.[43] Although studying structural activity relationship of salinosporamide A, (1, NPI-0052) a novel marine-derived potent proteasome inhibitor (Figure 5) in development for treating cancer, a series of analogs were assayed for cytotoxicity, proteasome inhibition and inhibition of NF-κB activation.[45] Marked reductions in potency in cell-based assays accompanied replacement of the chloroethyl group with unhalogenated substituents. Halogen exchange and cyclohexene ring epoxidation were well-tolerated, whereas some stereochemical modifications significantly attenuated the activity.[44] Tyropeptins A and B, new proteasome inhibitors, were isolated from the culture broth of *Itasatospora* species MK993-dF2. They were purified using ethyl acetate extraction, silica gel column chromatography, Sephadex LH-20 column chromatography and high performance liquid chromatography. Tyropeptin A inhibited the chymotrypsin-like and trypsin-like activities of 20S proteasome with IC50 values of 0.1 μg ml^{-1} and 1.5 μg ml^{-1}, respectively, but did not inhibit the peptidylglutamyl-peptide hydrolyzing activity of 20S proteasome at a concentration of 100 μg ml^{-1}. The inhibitory activities of Tyropeptin A were about two times stronger than that of Tyropeptin B.[46]

Proteasome inhibitors as affinity labels

Affinity labeling techniques use the small molecule as a probe rather than an inhibitor. Upon modification, a target protein bound to a labeled probe can be resolved and labeling intensity can be used to determine the activity of that target protease. Small molecule inhibitors that are capable of covalently attaching themselves to their targets, in an activity-dependent manner, can be used as affinity labeling reagents. These small molecules are often designed to target a single enzyme thereby allowing *anal* of its function through inhibitor studies. Multiple targets can be assessed simultaneously by this approach, including species that may not have been identified previously. Thus, affinity labeling provides a direct indication of the global reactivity of the inhibitor in total cellular extracts.

Both lactacystin and epoxomicin are covalent, specific inhibitors of the proteasome that have been chemically synthesized, and therefore can be easily converted into a labeled form. For lactacystin, attachment of a tritium atom in place of hydrogen yielded an affinity label that could identify its cellular target.[35] Similarly, epoxomicin was chemically converted into an affinity label by attachment of a biotin moiety.[34] Both of these labeled compounds

were crucial for the identification of the proteasome as their primary protein target.[31,35] In addition to the obvious utility of these reagents for target identification, both classes of affinity probes can also be used to rapidly monitor proteasome activity under different physiological conditions. However, tritium-labeled lactacystin is difficult and expensive to synthesize, so its full potential as a probe will probably never be realized. On the other hand, biotin-epoxomicin is relatively easily produced and is likely to find increased use as a reagent for specific monitoring of proteasome activity. Consequently, careful consideration must be made when choosing compounds and conditions for affinity labeling studies of the proteasome. Peptide vinyl sulfones have also been developed as affinity labeling probes of the proteasome.[25,29]

Kinetic studies of the proteasome using inhibitors

This section will discuss some of the uses of proteasome inhibitors in kinetic studies of peptide hydrolysis. This makes conventional strategies of the kinetic *anal* study become ineffective because of the complexity resulting from the proteasome's multiple active sites. Most biochemical studies of the proteasome therefore rely heavily on inhibitors to help decipher these multiple proteolytic events. The use of the isocoumarin compound 3,4-DCI as a proteasome inhibitor led to the identification of additional proteasomal proteolytic activities distinct from the chymotrypsin-like, trypsin-like and post-glutamyl peptide hydrolyzing activities.[4,23,35]

The hydrolysis of certain protein substrates was accelerated in the presence of this isocoumarin inhibitor,[35] and products generated by DCI-treated proteasomes resulted in a majority of cleavages after branched aliphatic amino acids. The name branched amino-acid preferring (BrAAP) activity[23] was given for this distinct DCI-resistant activity. Several small fluorogenic substrates designed to mimic the polypeptides produced from DCI-treated proteasomes, include Cbz-Gly-Pro-Ala-Leu-Gly-p-amino benzoate and Cbz-Gly-Pro-Ala-Leu- Ala-p-amino benzoate. These substrates are cleaved by the BrAAP activity (after the leucine residue), and therefore can be used effectively to monitor this activity.[23] A fifth activity known as small neutral amino-acid preferring (SNAAP), which cleaves the same BrAAP-like substrates but has cleavage preference for Gly-Gly and Ala-Gly bonds was also identified. In contrast to the BrAAP activity, SNAAP is sensitive to DCI and a variety of other thiol reagents, but like BrAAP activity it is insensitive to treatment with the peptide aldehyde Z16- LLF-H.[23] Only three catalytic subunits have been found to exist in the proteasome core.[15] As five proteolytic activities have now been observed and no evidence exists for additional catalytically active proteasomal subunits, these newly defined BrAAP and SNAAP activities may result from hydrolysis by combined activity of the three active subunits. A series of elegant kinetic experiments analyzed BrAAP activity in the presence of peptide aldehydes that contained a P1 branched aliphatic or aromatic residue. The branched aliphatic P1 aldehydes exhibited simple inhibition kinetics with respect to the BrAAP substrate, whereas the aromatic substrates revealed a bi-phasic or partial inhibition of the BrAAP activity. Simple kinetic inhibition of the BrAAP activity correlated directly with compounds that show similar activity against both the chymotrypsin-like and post-glutamyl peptide hydrolyzing activities, and the bi-phasic or partial inhibitors of BrAAP inhibited specifically the chymotrypsin-like activity. These findings combined with mutational studies in yeast indicate that the BrAAP and SNAAP activities are not distinct activities, but rather a combination of the chymotrypsin-like and post-glutamyl peptide hydrolyzing activities of the proteasome.[32]

Analysis of substrate specificity for kinetic study

The amino-acid residue found at the site of hydrolysis of the proteasome defines its proteolytic activity. The ability of peptide aldehyde inhibitors containing hydrophobic P1 residues to block all three of the major proteolytic activities of the proteasome suggests that substrate specificity is regulated by multiple factors. Examination of the catalytic mechanism for the proteasome was accomplished by kinetic *anal* using several peptide reporters. A model based on these studies proposed that the 20S proteasome is a dynamic structure with multiple conformers having least two cooperative sites for hydrolysis of the chymotrypsin-like substrate. This study also suggested a model for substrate specificity in which active sites can bind substrates with diverse P1 residues leading to hydrolysis of a single substrate by more than one proteasomal active site. Modified peptides represent valuable tools for determining inhibitor specificity. Relatively large numbers of sequences can be synthesized to incorporate a desired electrophile creating substrates that can then be used to directly monitor binding to the proteasome's active sites or to indirectly monitor inhibition of proteolysis. These results suggest that positions distal to the site of amide bond hydrolysis represent a second critical binding determinant. Thus, information can be obtained regarding primary-sequence specificity of each catalytic subunit. Similar findings using tetra-peptide α', β'-epoxyketones further established that regulation of substrate specificity requires positions distal to the P1 site.[32] Although these studies represent a step toward the characterization of proteasomal substrate processing, a more systematic approach is needed to better define absolute substrate specificity. Extending this affinity labeling approach to include inhibitors that address the contributions of each of the possible 20 amino acids to binding to each of the three proteasomal active sites has recently been accomplished.[36] These studies will help lead to a better understanding of how the proteasome is able to perform the highly controlled process of protein breakdown. Furthermore, information from this study has led to the design of inhibitors that target a single subunit of the proteasome.

Proteasome inhibitors as therapeutic agents

Without mentioning of the possible uses of proteasome inhibitors as therapeutic agents, a review chapter of proteasome inhibitors is incomplete. Among the drugs used for cancer therapy, proteasome inhibitors have shown promise in disrupting regulatory proteins, thereby reducing and/or eliminating various neoplastic cancers. The theory behind the use of proteasomal inhibitors is that, of all the cellular proteins involved in maintenance, differentiation, immunity, growth, division and death, approximately 70–90% is eventually degraded by multi-catalytic proteasomes. By interfering with the normal function of proteasomes, cellular stress is induced leading to cell-cycle arrest and/or cell death. Many compounds that either interfere or block proteasomal function have been identified. These include β-lactones such as clasto-Lactacystin, peptidyl aldehydes such as MG132 (carbobenzoxy l-Leu-l-Leu-l-Leu) and peptidyl boronates such as Velcade (bortezomib/PS-341). Lactacystin (AG Scientific Inc., San Diego, CA, USA) binds covalently, whereas MG132 (Biomol International, Plymouth Meeting, PA, USA) and Velcade bind reversibly to the *N*-terminal Thr-residue of the β-1 subunit within the 26S proteasome. Aside from the anti-proteasomal activity of lactacystin and MG132, these compounds are known inhibitors of other proteases such as cathepsin A, cathepsin B and calpain 1, respectively. Regardless of the latter's protease activity, the primary effect of the inhibitors is directed on the proteasome. Proteasomal inhibitors increase the life-span of short-lived proteins and block the modification of proteins

involved in signaling pathways, resulting in changes that inhibit cell survival and proliferation. In early studies, Velcade was reported to induce DNA damage, arrest cellular cycling at the G1/S or G2/M phase and reduce NF-κB activity. When used to treat cancer cells, Velcade induced a dose-dependent cleavage of polyadenosine-5′-diphosphate-ribose polymerase, significant apoptosis, expression of p21WAF1 and G2/M cell-cycle arrest.

On the basis of these beneficial effects, Velcade is being tested in phase I to phase III clinical trials for a variety of solid tumors. Unfortunately, several side effects, including incomplete tumor regression, have been noted at the doses required for the induction of apoptosis, thus, necessitating the identification of other drugs that are effective at lower doses. Although many studies focused on the use of one proteasomal inhibitor for therapy, we hypothesized that the combination of proteasome inhibitors Lactacystin (AG Scientific Inc.,) and MG132 (Biomol International) may be more effective in inducing apoptosis. In addition, this regimen would enable the use of sublethal doses of individual drugs, thus reducing adverse effects. Results indicate a significant increase in apoptosis when LNCaP prostate cancer cells were treated with increasing levels of Lactacystin, MG132, or a combination of sublethal doses of these two inhibitors. Furthermore, induction in apoptosis coincided with a significant loss of IKKα, IKKβ and IKKγ proteins and NF-κB activity. In addition to describing effective therapeutic agents, we provide a model system to facilitate the investigation of the mechanism of action of these drugs and their effects on the IKK-NF-κB axis.[39] Bortezomib (INN, originally code-named PS-341; marketed as Velcade by Millennium Pharmaceuticals now Takeda Pharmaceutical Company Limited, Osaka, Japan) is the first therapeutic proteasome inhibitor to be tested on humans. It is approved in the United States for treating relapsed multiple myeloma and mantle cell lymphoma. The boron atom in bortezomib binds the catalytic site of the 26S proteasome with high affinity and specificity. In normal cells, the proteasome regulates protein expression and function by degradation of ubiquitinylated proteins, and also cleanses the cell of abnormal or misfolded proteins. Clinical and preclinical data support a role in maintaining the immortal phenotype of myeloma cells, and cell-culture and xenograft data support a similar function in solid tumor cancers. Although multiple mechanisms are likely to be involved, proteasome inhibition may prevent degradation of pro-apoptotic factors, permitting activation of programmed cell death in neoplastic cells dependent upon suppression of pro-apoptotic pathways.[40–42]

The proteasome is an enticing target for chemical intervention especially in view of its essential role in cellular physiology. Processing and activation of the transcription factor NFjB, a proteasome substrate that has implications in inflammation, makes it an ideal system to assess the role of proteasome inhibitors as anti-inflammatory agents.[36] Proteasome-mediated cyclin degradation is required for the initiation of mitosis, and this poses yet another role for inhibitors as anti-cancer agents.[37] It is not surprising that the biotech industry has taken a keen interest in inhibitor efficacy toward inflammation and cancer. Proscript (now Millennium Pharmaceuticals Inc., The Takeda Oncology Company, Cambridge, MA, USA) is applying peptide boron acid proteasome inhibitors to models of arthritis and delayed type hypersensitivity. Preliminary experiments using oral delivery of inhibitors have yielded promising results. However, questions still remain about the benefit of targeting an enzyme that is central to so many processes required for cell survival. Continued studies should uncover any benefits to proteasome inhibition as a means for therapeutic intervention.

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