Proteasome Inhibitors: Complex Tools for a Complex Enzyme

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1 Introduction

Over the last two decades it has become abundantly clear that the proteasome is the pivotal component in cytosolic catabolism. Since its initial purification and biochemical characterization, this multi-component enzyme has been found to be essential for the regulation of fundamentally important processes such as cell division, cell death, signal transduction, and immune surveillance (for reviews see Coux et al. 1996; Goldberg and Rock 1992; Rivett 1993). Central to understanding any enzyme is the need to perturb its function in a highly controlled manner. This has been achieved through recent advances in the development and

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use of both natural and synthetic inhibitors that are capable of blocking the proteolytic activity of the proteasome. These advances are the focus of this chapter that highlights the use of diverse classes of inhibitors to probe the mechanism of the proteasome as well as to identify its physiological significance in the cell.

2 Initial Inhibitor Studies – Characterizing the Catalytic Mechanism of the Proteasome

The proteasome was first identified as a high molecular weight protease complex that resolved into a series of low molecular weight protein species upon denaturation (Dahlmann et al. 1985; McGuire and DeMartino 1986; Orlowski and Wilk 1981; Tanaka et al. 1986; Wilk and Orlowski 1980). It was subsequently purified and found to catalyze the hydrolysis of amide bonds adjacent to a variety of amino acids (Orlowski and Wilk 1981; Wilk and Orlowski 1980). Classification of its broad substrate specificity into three categories based on the nature of the amino acid found in the P1 position adjacent to the scissile amide bond soon followed. These three activities: chymotrypsin-like, trypsin-like and post-glutamyl peptide hydrolyzing (PGPH) activity, were established based on their similarity to well characterized proteolytic enzymes (Orlowski and Wilk 1981; Wilk and Orlowski 1980). Soon after the initial characterization of its biochemical properties, attention shifted to understanding the proteasome's enzymatic mechanism.

A common means of analyzing a novel proteolytic enzyme makes use of small molecule inhibitors whose reactivity towards an attacking nucleophile of a protease is well defined. Thus, the identification of a class of reagents capable of potent inhibition of a target protease can aid in the characterization of its underlying mechanism. At the time of initial identification of the proteasome, proteases were classified into four main groups based on their catalytic mechanism, 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.

Rapid purification schemes that took advantage of the proteasome's large size made it possible to perform biochemical analysis of the proteasome using a variety of classical protease inhibitors. Initial studies by Orlowski and Wilk in the early 1980s established the utility of peptide aldehydes, identifying the natural product leupeptin as an inhibitor of the proteasome's trypsin-like activity (Orlowski and Wilk 1981; Wilk and Orlowski 1980, 1983a). Not surprisingly, this compound, with a basic arginine in the P1 position, showed little activity against the remaining two activities of the proteasome that preferred cleavage after acidic and hydrophobic P1 amino acids. The synthesis of a tri-peptide aldehyde (Cbz–Gly–Gly–

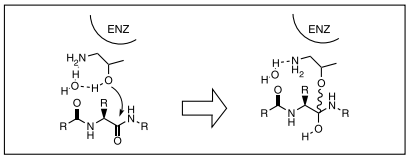
Leucinal) based on the sequence of a known peptide fluorogenic substrate resulted in a potent inhibitor of the chymotrypsin-like activity of the proteasome (Wilk and Orlowski 1983a). While these initial inhibitor studies provided useful new reagents for use in biochemical studies, the reactivity of peptide aldehydes towards both hydroxyl and thiol nucleophiles provided little information about the proteasome's catalytic mechanism.

Subsequently, a multitude of studies were performed using diverse sets of easily accessible, class-specific inhibitors (Cardozo et al. 1992; Dahlmann et al. 1985; McGuire and DeMartino 1986; Wilk and Orlowski 1980, 1983a). Chelating agents indicated that the proteasome did not belong to the metalloprotease family and pepstatin ruled out an aspartic protease mechanism (Dahlmann et al. 1985; McGuire and DeMartino 1986; Rivett 1985; Wagner et al. 1986). However, it was determined that organic mercurials, known to be highly reactive towards thiol groups, profoundly inhibited the proteasome (Dahlmann et al. 1985; McGuire and DeMartino 1986; Rivett 1985; Wagner et al. 1986; Wilk and Orlowski 1980; Zolfaghari et al. 1987). 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 (Dahlmann et al. 1985; McGuire and DeMartino 1986; Rivett 1985; Wagner et al. 1986; Wilk and Orlowski 1980; Zolfaghari et al. 1987). The proteasome was also found to be resistant to inactivation by classical serine protease inhibitors such as di-isopropyl fluorophosphate (DFP) and phenylmethanesulfonyl fluoride. 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 (Dahlmann et al. 1985; Rivett 1985; Wagner et al. 1986).

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 DFP resulted in inhibition of its chymotrypsin-like activity (Ishiura et al. 1986; Nojima et al. 1986; Wagner et al. 1986). Furthermore, Powers and Orlowski found that several structurally related isocoumarin derivatives, known to act as class-specific inhibitors of serine proteases, potently inhibited the proteasome (Harper et al. 1985; Kam et al. 1988; Orlowski and Michaud 1989). These findings prompted classification of the proteasome as a serine protease.

The cloning of several proteasome subunits from mammals and yeast (Emori et al. 1991; Fujiwara et al. 1989; Sorimachi et al. 1990) unfortunately provided little information to assist in the classification of the proteasome's catalytic mechanism as none of the subunits showed homology to any known proteases. It was not until the proteasome was crystallized in a complex with a peptide aldehyde inhibitor that the true active site nucleophile was revealed to be the N-terminal threonine residue found on the catalytic β-type subunits (Fig. 1; Lowe et al. 1995; Seemuller et al. 1995; Stock et al. 1995). Thus, the proteasome constitutes a new family of proteases that requires a free N-terminal threonine for activity.

Proteasome



Catalytic mechanism

3 Synthetic Inhibitors of the Proteasome

3.1 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. These compounds all 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 (Harding et al. 1995; Iqbal et al. 1995; Vinitsky et al. 1994; Wilk and Figueiredo-Pereira 1993) 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 (Harding et al. 1995; Lee and Goldberg 1996; Palombella et al. 1994; Rock et al. 1994). Additionally, the tetra peptide aldehyde Z–IE(OtBu)AL–H developed by Wilk and co-workers is among the commercially available proteasome inhibitors (Wilk and Figueiredo-Pereira 1993). 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 (Groll et al. 1997). 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 (Fig. 2a). Specificity for the proteasome can only be achieved by design of peptide sequences optimized for proteasome binding. In fact, very few examples of selective peptide aldehydes have ever been documented (Wilk and Figueiredo-Pereira 1993; Wilk and Orlowski 1983b) as they usually exhibit broad specificity. 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.

The first step towards designing compounds with increased selectivity for the proteasome was to take advantage of electrophiles that react specifically with a hydroxyl nucleophile (Adams and Stein 1996; Gardner et al. 2000; Iqbal et al. 1996; McCormack et al. 1997). Peptide boron esters and acids are potent inhibitors of serine proteases that form reversible covalent interactions with the active site hydroxyl (Fig. 2b). These compounds lack reactivity towards cysteine proteases as a consequence of poor overlap between orbitals of the non-bonding electrons on sulfur with those of the vacant d-orbitals on boron, resulting in a weak sulfur–boron bond. Furthermore, these derivatives are less reactive to circulating

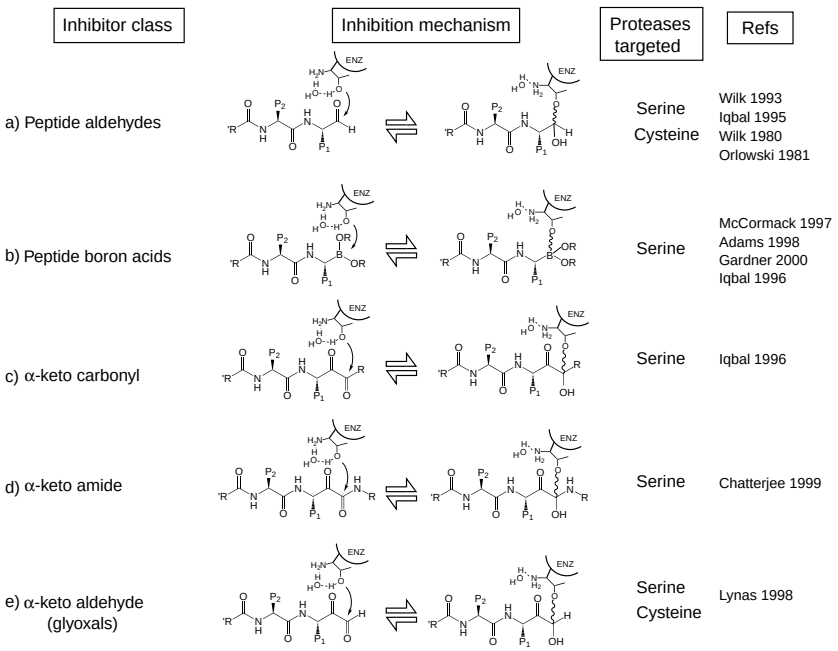


Fig. 2a–e. General structures and mode of action of reversible synthetic proteasome inhibitors. Partial list of compounds found to inhibit the proteasome in a reversible manner. Schematic representations of inhibition mechanism shows site of initial attack by the active site N-terminal threonine found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

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 towards cysteine proteases and dramatically increased potency for the proteasome (Adams and Stein 1996; Iqbal et al. 1996; McCormack et al. 1997). Rivett and co-workers 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 (Gardner et al. 2000). Further refinement of these lead compounds has resulted in the generation of di-peptide boron acids that are capable of inhibiting the proteasome at picomolar concentrations (Adams et al. 1998; Adams and Stein 1996; McCormack et al. 1997). This high degree of potency for the proteasome essentially results in selective inhibitors. For example, the di-peptide boron ester PS-341 requires 20,000 times higher concentrations to inhibit other abundant serine proteases (Adams et al. 1998). These compounds are also highly bio-available and are currently being pursued in clinical studies as potential anti-inflammatory agents (see below; Adams and Stein 1996).

Additional classes 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-terminally modified amino acid (often an aliphatic residue such a leucine). Examples include the peptide α-keto carbonyls (Iqbal et al. 1996; Fig. 2c), α-keto amides (Chatterjee et al. 1999; Fig. 2d), and α-keto aldehydes (Lynas et al. 1998; Fig. 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 of the proteasome (Fig. 2c–e). Moreover, compounds containing an α-keto amide at their C terminus have the potential for extension of inhibitor structures into the S′ region of the target protease, located directly C-terminal to the site of amide bond hydrolysis (Fig. 2d). Peptides containing these extended binding elements were developed with the hope of gaining additional specificity and potency towards the proteasome's multiple active sites (Lynas et al. 1998). Unfortunately, all of these reversible inhibitors suffer from the same limitations associated with the peptide aldehydes including broad specificity and instability in solution. To overcome these problems compounds must be generated that are potent enough to require a low dosage regime thus eliminating cross-reactivity and other toxic effects.

3.2 Synthetic Covalent Inhibitors

Another major class of synthetic proteasome inhibitors are compounds that inactivate the catalytic threonine nucleophile by irreversible covalent adduct formation (Fig. 3). Often referred to as suicide substrates, these inhibitors have found widespread use in biochemical studies of the proteasome. Furthermore, the covalent nature of these compounds allows protease activity to be traced using suitably labeled inhibitors (see Sect. 6).

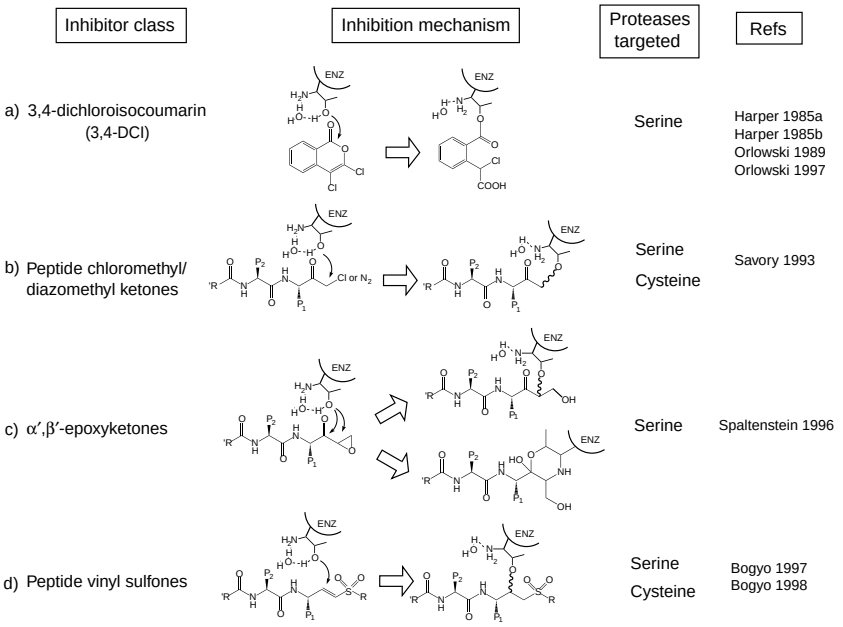


Fig. 3a–d. General structures and mode of action of irreversible synthetic proteasome inhibitors. Partial list of compounds that act as suicide substrates for the proteasome. Schematic diagrams show site of initial attack by the threonine hydroxyl and the resulting covalent adduct. In the case of the α′,β′ epoxyketones, multiple inhibition mechanisms are proposed

The general serine protease inhibitor 3,4 dichloroisocoumarin (3,4-DCI) was one of the first compounds found to act as a potent irreversible inhibitor of the proteasome (Harper and Powers 1985; Kam et al. 1988; Orlowski and Michaud 1989). While many isocoumarin analogs possessing a variety of hydrophobic appendages have been synthesized, only 3,4-DCI shows appreciable activity against the proteasome (Harper et al. 1985; Harper and Powers 1985; Kam et al. 1988; Orlowski and Michaud 1989). 3,4-DCI functions as a masked acid chloride that binds in the active site near the base-activated hydroxyl side chain of threonine to form a covalent ester linkage (Fig. 3a). Inhibition is achieved by modification of one or more of the active sites of the proteasome. While 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 activation of other activities (Cardozo et al. 1992; Orlowski et al. 1993). Furthermore, 3,4-DCI treatment of proteasomes lead to the accelerated processing of select protein substrates (Cardozo et al. 1992; Orlowski et al. 1993; Pereira et al. 1992). Many studies have attempted to ascertain the reason for the proteasome's varied response to 3,4-DCI, yet a detailed biochemical understanding of this phenomenon is lacking. Furthermore, the utility of this class of reagents as proteasome inhibitors is limited

by their broad reactivity with many serine-type protease as well as their instability in solution (Powers and Kam 1994). These reasons prompted many research groups to turn their attention to other classes of covalent proteasome inhibitors.

Chloromethyl ketones comprise a distinct class of commonly used covalent irreversible serine protease inhibitors. The diazomethyl ketones – close relatives of the chloromethyl ketones – were initially thought to be reactive only towards cysteine proteases, but were later found to react with serine proteases as well (Savory et al. 1993). The function of both classes of peptide electrophiles is mechanistically 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 (Fig. 3b). The ease with which these peptide-based inhibitors can be synthesized prompted the development of this class of electrophiles as covalent inhibitors of the proteasome (Savory et al. 1993). However, the low potency of these compounds towards the proteasome necessitated high concentrations of inhibitor to elicit appreciable inhibition, thus, limiting their use as proteasome inhibitors.

Many other classes of inhibitors initially designed to target serine proteases have been successfully converted into proteasome inhibitors. α′,β′ epoxyketone electrophiles have been incorporated into peptides sequences that have been optimized for binding to the proteasome (Spaltenstein et al. 1996). Conversion of the potent, tri-peptide aldehyde inhibitor of the chymotrypsin-like activity of the proteasome, Cbz–Ile–Ile–Phe–H, to the corresponding α′,β′ epoxyketone produced a covalent inhibitor with a 40-fold improved potency (Spaltenstein et al. 1996). The α′,β′-epoxyketones, like the α-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 irreversibly ether formation through ring opening of the strained epoxide moiety (Fig. 3c). 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 (Fig. 3c). 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 (see Sect. 5).

Peptide vinyl sulfones are electrophiles initially designed as cysteine protease inhibitors (Bromme et al. 1996; Palmer et al. 1995) that act by formation of a covalent linkage with an active site nucleophile via a Michael addition (Fig. 3d). The vinyl sulfone electrophile was reported to be resistant to attack by virtually all serine proteases (Bromme et al. 1996; Palmer et al. 1995). 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 (Bromme et al. 1996; Palmer et al. 1995). However, several peptide vinyl sulfones were found to inhibit the proteasome through covalent bond formation with the active site threonine hydroxyl (Fig. 3d; Bogyo et al. 1997, 1998). Remarkably, an

analog of the potent tri-peptide aldehyde MG-132, when converted to a vinyl sulfone, covalently inhibited all three activities of the proteasome. 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 (Bogyo et al. 1997, 1998). These aspects will be discussed in greater detail in Sect. 6.

4 Bi-Functional Synthetic Inhibitors – Rational Design Based on Structure

Determination of the three-dimensional structure of the proteasome provided the single greatest breakthrough in our understanding of its complex biochemical mechanism. The first structure was obtained for the archaebacterial form of the proteasome (Seemuller et al. 1995, Lowe et al. 1995) and revealed a complex comprised of 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 (Fig. 1; Groll et al. 1997). While the information from these structural studies was valuable for understanding mechanism and topology of the complex, they also paved the way for development of new classes of proteasome inhibitors the design of which is based on the detailed maps of the proteasome's inner cavity.

Initial structure-aided studies were aimed at creating inhibitors that could specifically target a single active site of the proteasome (Loidl et al. 1999a). The β2 subunit of the yeast proteasome was shown to be responsible for the trypsin-like activity of the yeast proteasome (Dick et al. 1998). It also possesses a unique feature in that a portion of its substrate-binding pocket lies in close proximity to a cysteine residue of the neighboring β3 subunit (Groll et al. 1997). Thus, the β2 active site depends on contacts created by multiple subunits and could potentially be targeted by reagents with two reactive electrophiles. Such β2-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 and co-workers synthesized a series of bi-functional peptide aldehydes (Loidl et al. 1999a; Fig. 4a). The structure of the β2 active site provided a guide for design of peptide scaffolds that placed the maleimide group in the S3 pocket of the β2 subunit, proximal to the free thiol of the β3 subunit. Replacement of the P3 acetyl-leucine residue with a maleoyl-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 dramatic change in specificity was the direct result of a double covalent attack by threonine and cysteine in the active site of the β2 subunit (Fig. 4a). Further refinement of the P1 and P2 positions, to incorporate residues optimal for the

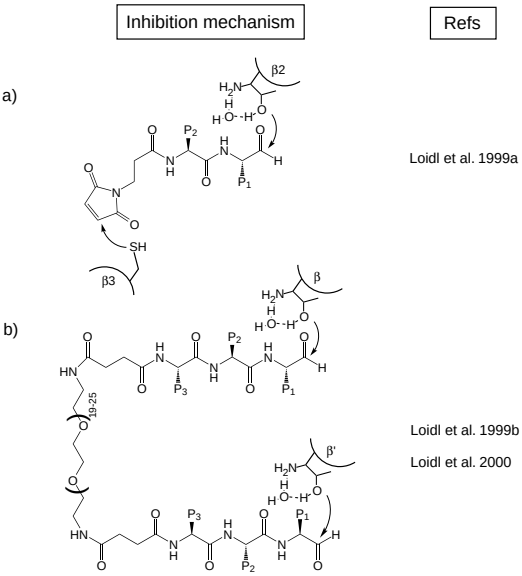


Fig. 4a,b. Synthetic bivalent inhibitors of the proteasome. Structures of two classes of inhibitors that target multiple nucleophiles on multiple proteasomal β-subunits. In the case of class (**a**) selective inhibition of the β2 proteasomal subunit is achieved by adduct formation between the active site threonine (β2) and a side-chain cysteine of an adjacent non-catalytic β-subunit (β3). Compounds of class (**b**) target two active site threonines on different catalytic β-subunits in the core 20S particle

trypsin-like activity, produced compounds with dramatic 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 (Loidl et al. 1999b, 2000). 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 hydrolyzable 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 (Fig. 4b). 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 towards the chymotrypsin-like activity as compared to 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 towards the trypsin-like activity. The versatility of the technique was also demonstrated by 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 (Loidl et al. 1999b).

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.

5 Natural Product Inhibitors of the Proteasome

5.1 Small Molecule Inhibitors

While chemists have expended a great deal of energy developing compounds that target enzymes such as the proteasome, nature often creates molecules that are far more specific and potent than anything made by the hands of a chemist. Unfortunately, it is often difficult or impossible to identify which natural products target a desired enzyme simply by inspection of structure or analysis of biochemical activity. In most cases compounds are singled out based on their biological effects in a pre-defined assay. Only after isolation of an active compound coupled with detailed biochemical analysis can the mode of action of the natural product be determined. Several new classes of proteasome inhibitors were identified through the synthesis of chemically modified versions of natural products that were then used to identify their biological targets (Fenteany et al. 1995; Meng et al. 1999a, 1999b). Another class of natural product inhibitors of the proteasome was uncovered by direct screening of microbacterial extracts for inhibition of proteasome activity (Koguchi et al. 1999, 2000a,b; Kohno et al. 2000).

The *Streptomyces* metabolite lactacystin was initially identified by its ability to inhibit cell cycle progression and induce neurite outgrowth in neuronal cell lines (Omura et al. 1991a,b). Chemists have produced synthetic versions of it, but its five chiral centers made its synthesis far from trivial (Fig. 5a). Initial structure/activity studies using synthetic analogs proved critical for determining lactacystin's mode of action (Fenteany et al. 1994). These studies showed that modification by deletion or inversion of steriochemistry of the hydroxyl or methyl groups attached to the lactam ring at carbons C6 and C7 resulted in complete loss of activity (Fenteany et al. 1994). Hydrolysis of the thioester at carbon C4 also abolished activity, while replacement of the N-acetyl cysteine residue with other thiol-containing groups that leave the thioester moiety intact had no effect (Fenteany et al. 1994). Similarly, the β-lactone derivative of lactacystin formed by an intra-molecular lactonization retained full activity, suggesting the importance of the thioester as an electrophilic site for attack by its target enzyme.

Derivatives of lactacystin in which a single hydrogen was replaced with its radioactive counterpart, tritium (Fenteany et al. 1995), showed that lactacystin modified predominantly one polypeptide in crude extracts, identified as the X (β5) subunit of the proteasome. Thus, lactacystin targets the proteasome by covalent modification of the active site threonine hydroxyl (Fig. 5a). Subsequent studies found that lactacystin blocks multiple activities of the proteasome through modification of all of the major catalytically active β subunits (Bogyo et al. 1997; Craiu

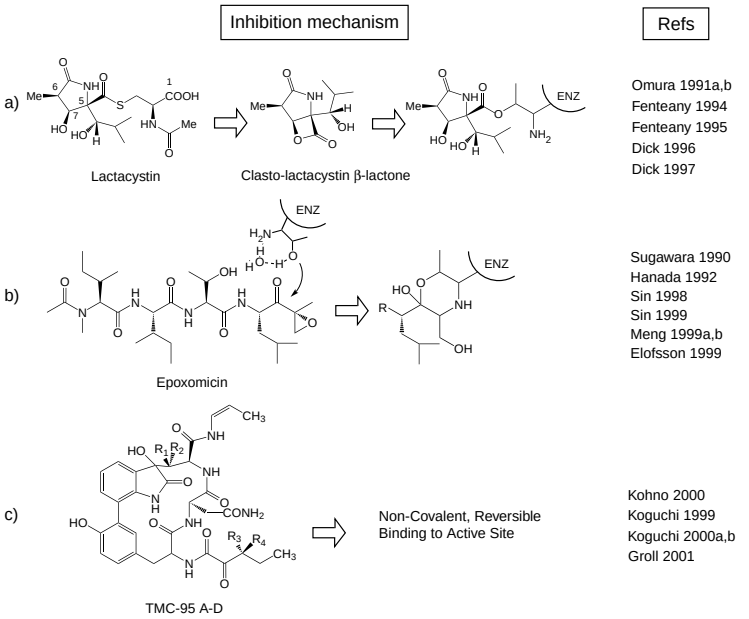


Fig. 5a–c. Natural product inhibitors of the proteasome. **a** The natural product lactacystin spontaneously reacts in basic aqueous solution to form the intra-molecular lactone (clasto-lactacystin β-lactone) which then reacts with the active site threonine of the proteasome. **b** Epoxomicin initially forms a covalent bond between the proteasome's N-terminal threonine hydroxyl at its C-terminal ketone carbonyl (not shown). This primary adduct formation is followed by a second attack by the terminal free amino group resulting in the formation of the stable six-membered ring adduct shown. **c** Cyclic peptide natural products TMC-95 A–D, from re-arrangement of the variable R groups (R1–R4)

et al. 1997). Other studies focused on kinetic analysis of the inhibition of the proteasome by lactacystin (Dick et al. 1996, 1997). These studies found that lactacystin acts by spontaneous lactonization between its thioester and the hydroxyl group at carbon C6 to form the highly reactive lactone, clasto-lactacystin β-lactone (Dick et al. 1996). This lactone species is the primary agent responsible for alkylation of the proteasome's active site. Moreover, lactonization is necessary for inhibition of the proteasome in intact cells, as lactacystin itself is not membrane permeable (Dick et al. 1997). Consequently, clasto-lactacystin β-lactone is now widely used and is commercially available.

The lack of structural resemblance of lactacystin to synthetic peptide-based compounds currently being used as protease inhibitors implied that it was highly specific for the proteasome. Several studies confirmed the selective nature of lactacystin, leading many to consider it as the gold standard by which to judge specificity of proteasome inhibitors (Craiu et al. 1997; Dick et al. 1997; Ostrowska et al. 1997). Since these initial studies of lactacystin's specificity, it has been tested against a wide range of protease targets and was eventually found to be cross-reactive with

cathepsin A. This lysosomal serine protease was effectively inhibited by low micromolar concentrations of lactacystin (Ostrowska et al. 1997) and may have escaped detection as a target owing to its lack of enzymatic activity in non-acidic assay conditions. Although it remains one of the most useful inhibitors currently available, the high cost of obtaining this compound from commercial sources financially limits its frequent use.

A second class of natural product proteasome inhibitors was isolated in screens of actinomycete fermentation broths for agents that blocked tumor growth (Hanada et al. 1992; Sugawara et al. 1990). Epoxomicin and eponemycin are modified peptides that contain an α′,β′-epoxyketone group at their C terminus attached to an aliphatic P1 amino acid (Fig. 5b). Eponemycin was also identified in assays of blood vessel formation as a potent inhibitor of angiogenesis (Oikawa et al. 1991), instigating a search for its biological targets.

At the core of both epoxomicin and eponemycin is an easily synthesized peptide backbone. This characteristic allowed the rapid facile synthesis of derivatives of epoxomicin in which the N-terminal acetate group was replaced with an aliphatic biotin linker (Meng et al. 1999a,b; Sin et al. 1998, 1999). This biotin-labeled epoxomicin derivative has identical activity to the parent natural product and was used to identify target proteins by affinity blotting. Moreover, the biotin molecule permitted simple affinity purification of targets using immobilized avidin. These studies identified four catalytic β-subunits of the proteasome as the primary targets of epoxomicin (X, LMP-7, Z, and MECL-1; Meng et al. 1999b). β-subunit modification correlated directly with inactivation of all three proteolytic activities of the proteasome and with anti-inflammatory activity in vivo (Meng et al. 1999b). Additional derivatives of epoxomicin were synthesized by optimizing the peptide backbone sequence to increase potency and selectivity for the chymotrypsin-like activity of the proteasome. Several of these derivatives showed as much as fivefold increased activity when compared to epoxomicin. Therefore simple chemical manipulation of the core peptide backbone sequence can be used to alter the selectivity and potency of this new class of proteasome inhibitors (Elofsson et al. 1999).

Epoxomicin contains an α′,β′-epoxyketone group at its C terminus such that when bound in the active site, two electrophilic carbon atoms are found in close proximity to the proteasome's nucleophilic threonine. The crystal structure of the epoxomicin-inhibited yeast proteasome was used to establish its mode of inhibition (Groll et al. 2000). Unlike other electrophilic peptide proteasome inhibitors that specifically target the hydroxyl nucleophile, epoxomicin reacts covalently with both the hydroxyl and the free amino groups of the N-terminal threonine, to produce a highly stable six-member ring (Fig. 5b). This unusual mode of inhibition provides an explanation for the extreme potency and selectivity of epoxomicin, as other inhibitors rarely possess an N-terminal nucleophile that can form this type of double adduct. To date, no other cellular targets of epoxomicin are known, reinforcing its utility as a new tool for studies of proteasome function.

A third class of compounds very recently identified in the fermentation broth of *Apiospora monagnei* was selected for its ability to directly inhibit the proteasome (Koguchi et al. 1999, 2000a,b; Kohno et al. 2000). Four compounds (TMC-95

A–D; Fig. 5c) were isolated that have potent activity against the proteasome and have nearly identical structures as determined by a combination of nuclear magnetic resonance, infra-red, and mass spectrometry (Kohno et al. 2000). The active species were modified cyclic peptides formed by a covalent link between a highly oxidized tryptophan side chain and the meta-carbon of a nearby tyrosine. The C terminus of the peptides contain a highly electrophilic α′-keto-carbonyl group, similar to that observed for several other synthetic inhibitors (Iqbal et al. 1996). While this class of natural products contains the electron-deficient α-keto-carbonyl group found on other previously characterized proteasome inhibitors, structural analysis of the yeast 20S proteasome bound to TMC-95A shows that it is not involved in direct covalent interaction with the active site hydroxyl (Groll et al. 2001). TMC-95A and its derivatives therefore represent the first class of truly non-covalent, selective inhibitors of the proteasome. These molecules will probably be the focus of much attention in the future as they provide a scaffold for the design of new classes of potentially therapeutically important proteasome inhibitors.

5.2 Protein Inhibitors of the Proteasome

In addition to the multitude of small molecule and peptide-based compounds, inhibitors can also take the form of large macromolecular protein structures. Most protein inhibitors of the proteasome were identified through screens of fractionated crude cellular extracts applied to purified proteasomes, which are then assayed for hydrolysis activity. In addition to endogenous proteins that modulate proteasome activity, several examples of macromolecular and exogenous protein inhibitors have been identified.

The paucity of endogenous inhibitors can be attributed to the limited number of large protease complexes that exist in the cell. The proteasome is one example of a large, complex protease that is comprised of a core particle, containing catalytically active β-subunits, that is able to form complexes with different multi-component cap complexes to facilitate protein breakdown. Some regulatory complexes facilitate activity of the 20S core and by analogy, similar mechanisms may exist that negatively modulate proteasome function (Coux et al. 1996). Furthermore, there are examples of endogenously synthesized protein inhibitors of nearly all classes of proteolytic enzymes (Barrett et al. 1998). Studies aimed at finding mediators for negative regulation of proteasomal proteolysis identified two high molecular weight protein complexes that bound to the proteasome and inhibited proteolysis of both protein and small fluorogenic substrates (Li et al. 1991; Li and Etlinger 1992). Denaturation of these 240- and 200-kDa complexes revealed that each consists of a single monomeric species with molecular weights of 40 and 50kDa, respectively. Biochemical studies established that the 40-kDa monomer was conjugated to ubiquitin and formed an ATP-stabilized complex with the 26S proteasome. The 40-kDa monomer was later determined to be δ-aminolevulinic acid dehydratase, the second enzyme in the pathway of heme synthesis (Guo et al. 1994). This intriguing discovery suggests that the inhibitory component of the proteasome was the result

of gene sharing. Moreover, the 240-kDa purified proteasome complex has dehydratase activity that when blocked has no effect on the ability of the complex to inhibit proteasomal proteolysis (Guo et al. 1994). The exact role of these large protein complexes is not clear but further studies will define their significance in bulk protein turnover.

A single monomeric protein of 31kDa was also identified as having an inhibitory influence on hydrolysis of both protein and small peptide substrates by the proteasome (Chu-Ping et al. 1992). This protein, isolated from red blood cells and given the name PI31, forms multimers under non-denaturing conditions and associates directly with the central core of the 20S proteasome. Recently PI31 was cloned and recombinantly expressed (McCutchen-Maloney et al. 2000; Zaiss et al. 1999). When applied in vitro to purified 20S proteasomes recombinant PI31 effectively blocks hydrolysis of several small peptide substrates. The sequence of this protein has no significant homology to any known protein family. It contains a proline-rich region at its C terminus and mutational studies suggest that this region contains the active domain (McCutchen-Maloney et al. 2000). Furthermore, truncation of the proline-rich domain results in C-terminal fragments with extended secondary structure that may form direct contacts with the proteasome core to modulate its activity. Studies performed in the presence of activator protein complexes such as the PA28 and PA700 caps indicate that PI31 competes for complex formation with these activators (Zaiss et al. 1999) suggesting an important regulatory role in the ubiquitin–proteasome pathway for protein breakdown.

Infectious pathogens provide an ideal opportunity to view natural mechanisms designed to block proteasome function. Viruses for example, may benefit from down-regulation of proteasome function, allowing endogenously synthesized viral proteins to evade processing into peptide fragments, a necessary process to elicit an immune response. The human immunodeficiency virus (HIV) Tat protein can function as a transcription factor and was shown to be stimulated by two of the ATPase subunits of the 26S proteasome. This observation prompted further biochemical studies on the interaction of Tat with the proteasome (Tsubuki et al. 1994). These studies lead to the finding that Tat was able to bind and inhibit the 20S proteasome core, thereby preventing the formation of complexes with the PA28 subunits, a requirement for antigen presentation (Seeger et al. 1997). Tat also exerts a small activation effect on the assembled 26S proteasome necessary for recognition and clearance of ubiquitinated protein substrates. Therefore inhibition by Tat leads to a specific blockade of the antigen presentation pathway without perturbing other critical functions of the proteasome.

6 Proteasome Inhibitors as Affinity Labels

Small molecules are often designed to target a single enzyme thereby allowing analysis of its function through inhibitor studies. A lack of specificity usually

plagues such designs, where absolute specificity of the reagent is often difficult to determine and inhibitors may effectively block unidentified enzymes. 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 to circumvent these difficulties. Affinity labeling techniques utilize 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 used to determine the activity of that target protease. Multiple targets can be assessed simultaneously by this approach, including species that may not have been identified previously. Thus, labeling provides a direct indication of the global reactivity of the inhibitor in total cellular extracts. Furthermore, indirect visualization of binding by unlabeled compounds can be accomplished through competition with labeled probes for the active site of a given target of interest. Several groups have taken advantage of chemically tagged suicide inhibitors to create several classes of useful new affinity probes for studying proteasome function.

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 (Fenteany et al. 1995). Similarly, epoxomicin was chemically converted into an affinity label by attachment of a biotin moiety (Sin et al. 1999). Both of these labeled compounds were crucial for identification of the proteasome as their primary protein target (Fenteany et al. 1995; Meng et al. 1999b). 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. Tritium-labeled lactacystin however, 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.

In addition to the natural product affinity labels, several other groups have generated labeled versions of known synthetic inhibitors of the proteasome. A ^{14}C-labeled analog of the coumarin inhibitor 3,4-DCI was synthesized (Orlowski et al. 1997) and used to identify the proteasome subunits that it modifies. Pre-treating proteasomes with peptide aldehyde inhibitors prior to labeling with probe allowed assignment of subunits targeted by these reagents. Similar studies made use of labeled peptide diazo-methylketones and chloro-methylketones (Reidlinger et al. 1997), but unlike the results from earlier studies (Bogyo et al. 1997), subunits that do not posses the catalytic N-terminal threonine residue were found to be labeled. This unexpected result is probably due to non-specific alkylations resulting from long incubations with high concentrations of purified enzymes using highly reactive labeled electrophiles. 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 (Bogyo et al. 1997, 1998; Nazif et al. 2000; Kessler et al. 2001). These compounds are simple peptide structures in which the C-terminal carboxylic

acid is replaced by the electrophilic vinyl sulfone electrophile. Simple attachment of a nitro-phenol or phenol moiety to a number of possible sites on the peptide backbone creates compounds that could be labeled with radioactive iodine. A variety of peptide sequences were used to produce probes that covalently label each of the six catalytically active β-subunits of the proteasome. Resolution of labeled proteins using either one- or two-dimensional gel electrophoresis permitted visualization of both overall levels of proteasomal activity as well as activity of individual subunits. This method therefore represents a considerable advantage over commonly used fluorogenic peptide substrates that provide information regarding only overall levels of proteasome activity.

These affinity labeling techniques highlight an often overlooked feature of covalent protease inhibitors, namely that specificity and potency are not always essential factors to be considered when designing new reagents. A compound having modest activity for a desired target that can be easily synthesized and modified for label attachment, for some applications, can be more effective than more potent counterparts that lack these features.

7 Kinetic Studies of the Proteasome Using Inhibitors

7.1 Studies of Catalytic Mechanism

The complexity resulting from the proteasome's multiple active sites makes conventional strategies of kinetic analysis ineffective. Most biochemical studies of the proteasome therefore rely heavily on inhibitors to help decipher these multiple proteolytic events. This section will discuss some of the uses for proteasome inhibitors in kinetic studies of peptide hydrolysis.

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 PGPH activities (Cardozo et al. 1992; Orlowski et al. 1993; Pereira et al. 1992). The hydrolysis of certain protein substrates was accelerated in the presence of this isocoumarin inhibitor (Pereira et al. 1992), and products generated by DCI-treated proteasomes resulted in a majority of cleavages after branched aliphatic amino acids. This distinct DCI-resistant activity was given the name branched amino acid preferring (BrAAP) activity (Orlowski et al. 1993). Several small fluorogenic substrates designed to mimic the polypeptides produced from DCI-treated proteasomes include Cbz–Gly–Pro–Ala–Leu–Gly–*p*-aminobenzoate and Cbz–Gly–Pro–Ala–Leu–Ala–*p*-aminobenzoate. These substrates are cleaved by the BrAAP activity (after the leucine residue), and therefore can be used to effectively monitor this activity (Orlowski et al. 1993). 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 is insensitive to treatment with the peptide aldehyde Z–LLF–H (Orlowski et al. 1993).

Only three catalytic subunits have been found to exist in the proteasome core (Groll et al. 1997). 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 (McCormack et al. 1998). 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 PGPH activities, and the bi-phasic or partial inhibitors of BrAAP inhibited specifically the chymotrypsin-like activity. These findings combined with mutational studies in yeast (Dick et al. 1998) indicate that the BrAAP and SNAAP activities are not distinct activities but rather a combination of the chymotrypsin-like and PGPH activities of the proteasome.

7.2 Analysis of Substrate Specificity

Traditionally, the amino acid reside found at the site of hydrolysis defines each proteolytic activity of the proteasome. However, this classification appears to be oversimplified. 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. A series of detailed kinetic and biochemical studies using inhibitors of varied peptide sequences are beginning to define the specificity elements used by the proteasome to determine how protein substrates are processed.

Examination of the catalytic mechanism for the proteasome was accomplished by kinetic analysis 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 (Stein et al. 1996). More extensive analysis revealed a non-linear dependence between steady-state velocity and inhibitor concentration, as the result of the peptide aldehyde inhibitor, Ac-LLnL-H, binding at multiple active sites in the complex. 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. This model also proposed that the P1 residue of a substrate has a relatively minor role in defining cleavage sites on a substrate.

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. Studies of extended peptide aldehydes showed that alteration in the P4 and P5 positions of inhibitors had, in some cases, a more profound effect on inhibitor potency than changes to the P1 position. These results suggest that positions distal to the site of amide bond hydrolysis represent a second critical binding determinant (T. Akopian, B. Gilbert, R.R. Rando, and A.L Goldberg, unpublished results). The importance of this distal P4 binding site was confirmed by direct labeling analysis using peptide vinyl sulfones (Bogyo et al. 1998). Addition of an aromatic P4 residue to the tri-leucine-containing core peptide vinyl sulfone dramatically changed its subunit labeling profile indicating the importance of this position for recognition by the proteasome's multiple active sites (Bogyo et al. 1998). Furthermore, these studies performed with peptide vinyl sulfones showed that changes in modification of individual catalytic β-subunits can be correlated with changes in inhibitory peptide sequences. 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 (Elofsson et al. 1999).

While these studies represent a step towards 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 of each of the three proteasomal active sites has recently been accomplished (Nazif et al. 2000). 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. These reagents are likely to be of particular importance for biochemical studies of proteasome function.

8 Proteasome Inhibitors as Therapeutic Agents

A review of proteasome inhibitors is incomplete without mention of the possible uses of proteasome inhibitors as therapeutic agents. 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 NF-κB, a proteasome substrate that has implications in inflammation, makes it an ideal system to assess the role of proteasome inhibitors as anti-inflammatory agents (Palombella et al. 1994).

Proteasome-mediated cyclin degradation is required for initiation of mitosis, and this poses yet another role for inhibitors as anti-cancer agents (Glotzer et al. 1991). It is not surprising that the biotech industry has taken a keen interest in inhibitor efficacy towards inflammation and cancer. Proscript (now Millennium

Pharmaceuticals) is applying peptide boron acid proteasome inhibitors to models for arthritis and delayed type hypersensitivity (Adams and Stein 1996). Preliminary experiments using oral delivery of inhibitors has 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.

9 Summary

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. While these reagents have played an important role in establishing our current knowledge of the proteasome's catalytic mechanism, many questions remain. Rapid advances in the synthesis and identification of new classes of proteasome inhibitors over the last 10 years serve as a positive indicator that many of these questions will soon be resolved. The future lies in designing compounds that can function as drugs to target processes involved in disease progression. It may be only a short while before the products of such research have safe application in a practical setting as the first inklings of possibilities are visible. Structural and combinatorial chemistry approaches are powerful techniques that will bring us closer to these goals.

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