Review

Inhibitors of the eukaryotic 20S proteasome core particle: a structural approach

Michael Groll a, *, Robert Huber b

a Abteilung für Physiologische Chemie, Ludwig-Maximilians-Universität München, Butenandtstr. 5, 81377 München, Germany
b Max Planck Institut für Biochemie, Abteilung Strukturforschung, Am Klopferspitz 18a, D-82152 Planegg Martinsried, Germany

Available online 7 October 2004

Abstract

The ubiquitin–proteasome pathway is particularly important for the regulated degradation of various proteins which control a vast array of biological processes. Therefore, proteasome inhibitors are promising candidates for anti-tumoral or anti-inflammatory drugs. N-Acetyl-Leu-Leu-Norleucinal (Ac-LLN-al, also termed calpain inhibitor I) was one of the first proteasome inhibitors discovered and has been widely used to study the 20S proteasome core particle (CP) function in vivo, despite its lack of specificity. Vinyl sulfones, like Ac-PRLN-vs, show covalent binding of the β-carbon atom of the vinyl sulfone group to the Thr1Oγ only of subunit β2. However, vinyl sulfones have similar limitations as peptide aldehydes as they have been reported also to bind and block intracellular cysteine proteases. A more specific proteasome inhibitor is the natural product lactacystin, which can be isolated from Streptomyces. It was found that this compound forms an ester bond only to the Thr1Oγ of the chymotrypsin-like active subunit β5 due to specific P1 interactions. In contrast to most other proteasome inhibitors, the natural α',β'-epoxyketone peptide epoxomicin binds specifically to the small class of N-terminal nucleophilic (Ntn) hydrolases (CPs belong to this protease family) with the formation of a morpholino adduct.

All previously described proteasome inhibitors bind covalently to the proteolytic active sites. However, as the proteasome is involved in a variety of biological important functions, it is of particular interest to block the CP only for limited time in order to reduce cytotoxic effects. Recently, the binding mode of the natural specific proteasome inhibitor TMC-95 obtained from Apiospora montagnei was investigated. The crystal structure revealed that the TMC-95 blocks the active sites of the CP noncovalently in the low nanomolar range.

This review summarizes the current structural knowledge of inhibitory compounds bound to the CP, showing the proteasome as a potential target for drug development in medical research.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Proteasome; Ubiquitin–pathway; Multifunctional protease complex; Ntn-hydrolase; Inhibitor

1. Introduction

Intracellular proteolysis is an essential cellular process. In eukaryotes the non-lysosomal protein degradation is performed by the strictly controlled complex enzymatic machinery of the ubiquitin–proteasome pathway. This degradation pathway is particularly important for the turnover of many critical proteins participating in a vast number of essential biological processes, such as cell proliferation, cell differentiation and inflammation [1–3]. The mechanism of degradation is strictly successive: protein substrates selected for destruction are marked by covalent addition of poly-ubiquitin chains (E1–E3-ubiquitin conjugation pathway), which are recognised and digested by the multifunctional 26S proteasome complex, a 2,500,000 Da proteolytic molecular machine. 26S proteasomes are formed from a cylinder-shaped multimeric protein complex referred to as the 20S proteasome core particle (CP), capped at each end by a regulatory component termed the 19S complex (regulatory particle, RP or PA700). Ubiquitinated substrates are processed at the active sites located within the inner cavity of the CP,
whereas the RP is responsible for recognition, unfolding and translocation of the selected substrates into the lumen of the CP. X-ray analysis of the proteasome crystals from the archaeon *Thermoplasma acidophilum* [4] revealed the architecture of the molecule at atomic resolution. The data showed that CPs are composed of four stacked rings, with each ring consisting of seven α- and β-type subunits, following an \( \alpha_1 \beta_1 \gamma \) stoichiometry. Proteolysis occurs within the central chamber at the active centres located at the \( \beta \)-subunits, with Thr1O\( \gamma \) acting as the nucleophile. Eukaryotic CPs are more complex in their architecture than prokaryotic CPs. In yeast, the α and β subunits have diverged during evolution into seven different subunits each, which occupy unique locations in two copies per CP [5]. The overall assembly of the CP is formed by contacts between characteristic and specific sequence insertions and extensions of the various α- and β-type subunits. In contrast to prokaryotic proteasomes which contain 14 identical proteolytic active sites, in eukaryotic CPs only subunits \( \beta_1 \), \( \beta_2 \) and \( \beta_5 \) harbour the nucleophilic Thr1O\( \gamma \), whereas the remaining \( \beta \)-subunits are inactive.

### 2. Proteolytic active site residues

The elucidation of the crystal structure of the CP from *T. acidophilum* in complex with the competitive inhibitor Ac-Leu-Leu-Norleu-al (calpain inhibitor I) showed binding of the inhibitor molecules only to the \( \beta \)-type subunits [4]. Thereby the functional aldehyde group of calpain inhibitor I forms a covalent hemiacetal bond with the N-terminal Thr1O\( \gamma \). This structure showed that 20S proteasomes belong to a new class of proteolytically active enzymes named threonine proteases. Thr1, Glu17 and Lys33 were identified by structural and mutational studies as the major catalytic residues. Additionally, Ser129, Asp166 and Ser169 located close to the active site threonine seem to be required for the structural integrity of the proteolytic centre and are probably involved in catalysis [4,8]. Crystallographic analysis of the wild type and mutants of the yeast 20S proteasome helped to understand its catalytic mechanism in more detail [5,9,10]. The proteolytically active Thr1O\( \gamma \) was shown to react either with electrophilic functional groups of inhibitors or peptide bonds of substrates, while Thr1N represents the proton acceptor. Ser129O\( \gamma \) and Ser169O\( \gamma \) form hydrogen bonds with the N-terminus of the nucleophilic threonine, which are coordinated by Asp168O, and the Thr1O\( \gamma \) is hydrogen bonded to Lys33N\( \gamma \). In yeast, subunit \( \beta_5 \) can be inactivated by replacement of Lys33 [10,11]. However, Lys33 is not acting as the proton acceptor, but contributes to the \( pK_a \) and status of protonation of the ionisable groups, since its pattern of hydrogen bonds indicates a positive charge. Even the conserved substitution of Lys33 with arginine results in proteolytic inactivation caused by the structural rearrangement of the guanidine group of Arg33 [10]. This rearrangement is possibly associated with the change of the intrinsic \( pK_a \) of Thr1O\( \gamma \). These observations indicate that exact layout of the residues responsible for catalytic activity of the \( \beta \)-subunit is absolutely essential. Generally, a nucleophilic water molecule is required for proteolysis. This water molecule, termed NUK, has been identified in the crystal structure of the yeast CP at high resolution as a bound solvent molecule in proximity to Thr1O\( \gamma \), Thr1N, Ser129O\( \gamma \) and Gly47N [5]. In 20S proteasomes the water molecule is supposed to function in two proteolytically important processes: first, to shuttle the proton between Thr1O\( \gamma \) and Thr1N, and second, to participate in the cleavage of the acyl-ester-intermediate, completing the proteolysis of the bound substrate.

### 3. 20S proteasomes belong to the Ntn-hydrolases

A set of hydrolases, including the 20S proteasome, which show no recognisable sequence similarity to each other, demonstrate similar topology. There are already 19 documented crystal structures of Ntn-hydrolases deposited in the RCSB protein data bank. The term “N-terminal nucleophile (Ntn)-hydrolases” reflects the fact that their N-terminal residue serves as a nucleophile [12]. It could be shown for penicillin acylase [13], a member of the Ntn-hydrolase family, that the nucleophilic water molecule is present in proximity to the N-terminal catalytic residue, similar to that which has been found in CPs. However, the mentioned above active site residues in Ntn-hydrolases are not conserved, which supports the postulated proton acceptor mechanism of 20S proteasomes. A processing step, common to all Ntn-hydrolases, results in the exposure of the N-terminal amino group acting as the nucleophile. Thus, 20S proteasomes undergo a defined maturation process, leading to formation of the active Ntn-protease complex. During maturation, the pro-segments of the \( \beta \)-subunit precursor complexes are removed by intramolecular proteolysis [9,14,15].

### 4. Catalytic roles and specificities of proteasomal subunits

The eukaryotic 20S proteasome shows different proteolytic activities. It could be shown in vivo that eukaryotic CPs are able to cleave after almost each amino acid [6,16,17]. However, in vitro assays with chromogenic substrates demonstrated that proteasomal activities are restricted to only five distinct cleavage preferences: chymotrypsin-like (CL), trypsin-like (TL), peptidyl-glutamyl-peptide-hydrolysing (PGPH), branched chain amino acid-preferring (BrAAP) and small neutral amino acid-preferring (SNAAP) activity. Structural and mutational studies of the yeast CP could identify the subunits responsible for these different activities [5,10,11]. Generally, the major residue responsible for the formation of each S1-specificity pocket is located in

---

**References:**


---
the position 45. Additionally, adjacent subunits in the β-rings contribute to the S1 pockets and significantly contribute to their selectivity (see Fig. 1). Structural analysis revealed that subunit β1 preferentially interacts with glutamate P1 residues of chromogenic substrates, due to the location of Arg45 in subunit β1. This observation and mutational analysis [18,19] restricted the PGPH-activity of the CP to this subunit. However, degradation experiments of yeast enolase have identified that subunit β1 has, besides its PGPH-specificity, limited BrAAP activity [20]. The crystal structure of the yeast CP:calpain inhibitor I complex shows the hydrophobic norleucine side chain of the inhibitor projecting towards the Arg45, providing an insight for the mechanism of this dual cleavage preference. Additional electron density in the vicinity of Arg45 was interpreted as a bicarbonate anion, compensating the unbalanced positive charge [9]. In the subunit β2 glycine is situated in the position 45. Consequently, the S1 pocket of subunit β2 is very spacious and therefore is suitable for very large P1 residues (see Fig. 1). Glu53 at the bottom of the S1 pocket is responsible for the trypsin-like activity of this subunit as it shows a high preference for basic P1 residues. These findings were verified by mutational analysis [10,11,21]. The chymotrypsin-like activity can be attributed to subunit β5 shaped in particular by Met45 (see Fig. 1). However, mutational analysis shows that subunit β5 has also the tendency to cleave after small neutral and branched side chains assigning additionally the BrAAP and SNAAP activity to this subunit. The crystal structures of the CP:lactacystin and CP:calpain inhibitor I complexes provide a possible structural explanation for the flexibility of the S1 pocket of this subunit. In case of the CP:lactacystin complex, the side chain of Met45 is oriented towards the isopropyl side chain of the inhibitor, thus minimizing the size of the S1 pocket. In contrast, in the crystal structure of the CP:calpain inhibitor I complex the Met45 side chain is displaced by the space-demanding norleucine side chain of the inhibitor, making the S1 pocket more spacious [5].

In mammalian CPs, γ-interferon induces replacement of the constitutive proteolytic subunits against immuno-subunits. This substitution has crucial influence on the quantity and distribution of MHC-class I molecules on the cell surface [22,23], since it enhances the production of oligopeptides, termed epitopes, which have specific C-terminal anchor residues showing higher affinity for MHC-class I molecules. So far, there exist no structural data for immuno-proteasomes. However, the superposition of the primary sequence of constitutive and related immuno-subunits reveals surprisingly high conservation of the substrate binding pocket residues. The only exception is found in subunit β1i, which contains two conspicuous differences in the S1 pocket, as compared to β1 [5,24]. In subunit β1i, Thr21 is replaced by Phe, and Arg45 by Leu. These substitutions, on one hand, minimize the S1 pocket of the immuno-subunit, and, on the other hand, change the overall charge of the pocket from positive to neutral. In accordance with this hypothesis, immuno-proteasomes have a reduced activity towards acidic residues, whereas the CL and BrAAP activity is drastically increased [5]. Furthermore, MHC-class I epitope presentation was reduced in mice knock-out mutants of subunit β1i [25–28]. Different from β1, the alignment of the primary sequences of β2 and β5 with β2i and β5i subunits in eukaryotes exhibits identity of important S1-pocket residues. Surprisingly, a severe
defect in MHC-class I presentation was found in vivo in mice mutants lacking these immuno-subunits [22], an observation not readily explained by the structures. It cannot be excluded that the flexibility and size of the specificity pockets during substrate binding can be altered by an induced fit, thereby changing the cleavage preference, as was already described for subunit β5 in complex with the calpain inhibitor I and lactacystin. Functional consequences of the subunit exchange in mammalian CPs need further investigation. It has been found that protein substrates can be cleaved by the proteasomes after almost every amino acid and residue, located in substrate binding pockets other than P1, and have a decisive influence on the cleavage preference (see below). Therefore, substrate specificity of the proteasomal subunits may reflect physical constraints due to the slightly altered local structure around each active site, rather than a strict selectivity for the P1-residues of the substrate.

5. Inhibitors of the 20S proteasome

5.1. Covalently bound inhibitors

A vast array of biological pathways is controlled by proteasomes, which is responsible for the regulated degradation of many critical proteins. Thus, proteasome inhibitors are promising candidates as anti-tumoral or anti-inflammatory drugs. The mode of action of the 20S proteasome has been first analyzed with nonspecific protease inhibitors. Several inhibitors showing greater specificity against the CP have been discovered, and they greatly facilitated in vivo as well as in vitro investigations of a proteasome role in biological processes.

N-Acetyl-Leu-Leu-Norleucinal (Ac-LLnL-al, also called calpain inhibitor I) was one of the first discovered proteasome inhibitors. Despite its lack of specificity, it has been widely used for analysing proteasome functions in vivo [29]. Reversible binding of this inhibitor abolishes the chymotrypsin-like and, to a lesser extent, the trypsin-like and post-acids activities of the eukaryotic CP. Ac-LLN-al binds with the formation of covalent hemiacetal bond to the Thr1O of all active subunits, as has been elucidated from the crystal structure of the yeast CP in complex with calpain inhibitor I (see Fig. 2a). The tripeptide aldehyde adopts a β-conformation and fills a gap between β-strands, forming hydrogen bonds with the residues 20, 21 and 47, with generation of an anti-parallel β-sheet structure. The norleucine side chain projects into the S1 pocket, whereas the leucine side chain at P2 is not in contact with the protein. The leucine side chain at P3 closely interacts with amino acids of the adjacent β-type subunit. Thus, both S1 and S3 specificity pockets play a prominent role in inhibitor binding [4,5]. However, despite the binding of the inhibitor to all proteolytic active centres, when used at high concentrations, in vitro and in vivo functional analysis demonstrated that calpain inhibitor I inactivates primarily the chymotrypsin-like active site of the yeast 20S proteasome.

Lactacystin, produced in Streptomyces sp., was the first identified natural proteasome inhibitor. It was discovered as an inducer of neurite outgrowth in a murine neuroblastoma cell line [30]. Radioactive lactacystin was shown to bind mainly to the proteasomal subunit β5 [31], thus effectively and irreversibly inhibiting the chymotrypsin-like activity. The trypsin-like and the post-acidic activities are also blocked, however, to a lower extent. In aqueous solutions at pH 8 the reactive compound of lactocystin is spontaneously hydrolyzed into clasto-lactacystin β-lactone, which inhibits the CP [32]. The crystal structure of the yeast CP:lactacystin complex confirmed that the inhibitor molecule covalently binds only to subunit β5 [5], which is in line with the observed chemical modification of subunit β5/β5i of the mammalian CP [31]. As can be seen from the crystal structure, lactacystin is bound to the 20S proteasome main chain atoms by a large number of hydrogen bonds (see Fig. 3a). The covalent binding of lactacystin to the N-terminal Thr1O, with the formation of an ester bond in the active site of subunit β5, causes the irreversible inhibition. The inhibitor selectively blocks proteolysis at subunit β5 due to the apolar nature of its S1 pocket. Lactacystin contains a less reactive head group and, therefore, needs longer time to react with the Thr1O than calpain inhibitor I. The neutral charge pattern of the S1 pocket of subunit β5 prolongs the mean residence time of lactacystin by characteristic ligand–protein interactions: Met45 mainly contributes to its neutral character, so that only this subunit can fix the inhibitor by hydrophobic interactions with its dimethyl side chain. This step is essential for the completion of the ester bond formation between the clasto-lactacystin β-lactone with Thr1O. In contrast to lactacystin, the aldehyde group of calpain inhibitor I binds to all proteolytic active sites, since this functional group is more reactive than the lactone ring. These observations indicate that in 20S proteasomes the functional head group of the inhibitors plays a significant role for selective and specific binding to single proteolytically active subunits.

The preference of lactacystin binding to single proteasomal subunits gave an impulse for the creation of synthetic compounds inhibiting individual active sites in eukaryotic CPs [33]. It became possible to design and synthesise specific inhibitors for single proteasomal subunits. Additionally, the structural data of CPs provided insights for the development of bifunctional inhibitors, which have several docking sites for covalent binding to the protein. For example, the trypsin-like activity of eukaryotic CPs is performed at the S1 pocket of subunit β2, which additionally harbours the N-terminal threonine. The S3 pocket of this proteolytically active site contains Cys118, which belongs to subunit β3. Maleoyl-[β]Ala-Val-Arginine-al (Mal-[β]AVR-al) has been designed to specifically block the trypsin-like activity of 20S proteasome in a bifunctional
The inhibitor was supposed to bind covalently with its maleimide-group in the P3-site to the S3-thiol-group of Cys118, and with its carboxy-terminal aldehyde group for hemiacetal formation with the Thr1-Og of subunit h2.

Structure-based modelling was required to measure the characteristic distance between the maleimide-side chain of the inhibitor and the thiol group of the P3 pocket. The calculations show that Ala-maleimide would perfectly meet these expectations. These structurally based predictions were confirmed by analysis of the crystal structure of the yeast CP:Mal-Ala-Val-Arg-al complex (see Fig. 2b). It was seen that the inhibitor molecule binds only to subunit β2 by hemiacetal formation. The presence of the covalent bond between the maleimide and the Cys118 residue of subunit β3 has been confirmed [33]. Remarkably, the IC50 value of Mal-βAVR-al for subunit β2 is 0.5 μM, which is 400 times less as compared to the IC50 value of calpain-inhibitor I (200 μM). Thus, Mal-βAVR-al represents a new type of inhibitor that is highly selective for the trypsin-like activity. Since Cys118 is conserved among all known primary structures of eukaryotic CPs, the new inhibitor promises to be an efficient tool for investigation of substrate degradation mechanisms. However, the reactivity of the maleimide group towards thiols limits the use of the inhibitor only to in vitro assays.

Previously described data stimulated further development of non-peptide specific inhibitors for single proteolytically active subunits of 20S proteasomes. The unique topography of the six proteolytic active subunits in the central chamber of eukaryotic CPs defines the distances between the active site Thr1-residues. With the usage of the known proteasomal layout, new bi- or multivalent proteasome inhibitors were designed. These inhibitors contain an oligomeric spacer of appropriate length, linking two monovalent binding head groups to each other, with the formation of homo- or heterobivalent compounds [34]. The spacer must be unable to form any secondary structure, in
order to reach the proteasomal central cavity. Unfolded peptides like gastrin (17mer) or secretin (27mer) were found to be rapidly degraded by the CP, and were therefore unsuitable for the role of spacer elements. Therefore, polyethylene-glycol (PEG) was selected as a possible spacer, since it is a linear, flexible, solvated and protease resistant polymer, which mimics unfolded peptide chains. Furthermore, the PEG spacer is hydrophilic and therefore prevents formation of hydrophobic cores, which would disturb the molecule entering the 20S proteasome. Spacer length for the various bifunctional inhibitors was chosen based on the proteasomal inter- and intra-ring distances. Finally, the N-termini of two tripeptide-aldehydes were linked to the related PEG-spacer, resulting in a bivalent protease-resistant CP-inhibitor. As expected, due to the flexibility of the PEG linker region, the crystal structure analysis of the CP:inhibitor complexes do not reveal a conformationally restricted PEG-moiety in any part of the density. Kinetic measurements of the proteolytic activity of the yeast CP showed that the bivalent inhibitors have IC₅₀ values in the low nanomolar range. In contrast, their monovalent analogues have much higher IC₅₀ values, thus the bivalent inhibitors increase the inhibition potency by two orders of magnitude. Interestingly, improved inhibition was achieved by using a heterogeneous population of polymeric spacers with a length distribution from 19 to 25 monomers to bridge various active sites. Furthermore, the general principle of bivalency is not only restricted to the use of peptide aldehydes as binding head groups. It could, in combination with more potent and selective inhibitor head groups (see below), result in the design of specific inhibitors of the CP acting in the picomolar range. The question

Fig. 3. Stereoview of yeast 20S proteasome subunits (coloured in white and grey) in complex with natural inhibitors (coloured in green): (a) lactacystin, (b) epoxomicin and (c) TMC95A. The layout of this figure is similar to that of Fig. 2. (a) Lactacystin covalently bound to subunit β5. The S1 pockets of subunit β1 and β2 differ from that of subunit β5 and do not interact with lactacystin. Met45 of subunit β5 (coloured in brown) specifically interacts with the branched side chain of lactacystin. In contrast to the crystal structure of the CP-calpain inhibitor I complex (see Fig. 3a), Met45 minimizes the size of the S1 pocket, thereby allowing optimal protein–ligand interactions, which account for the selectivity of this compound. (b) Epoxomicin, a natural Streptomyces metabolite, covalently bound to subunit β5. The electron density reveals the presence of a unique six-membered ring-system. This morpholino derivative results from adduct formation between epoxomicin and the proteasomal N-terminal Thr1Oγ and N (both bonds are coloured in magenta). Met45 of subunit β5 is arranged similarly as described in Fig. 2a, thus making the S1 pocket more spacious for the Leu-side chain of the inhibitor. (c) TMC-95A noncovalently bound to subunit β2. The natural specific 20S proteasome inhibitor from A. montagnei binds near the proteolytic active site, occupying the specificity pockets of the CP. TMC-95A adopts an extended conformation not modifying the nucleophilic Thr1 and is found in all proteolytically active sites. Optimal binding to the 20S proteasome is due to the strained conformation of TMC-95A, caused by the presence of the cross-link between the tyrosine and the oxindol side chain. The IC₅₀ values for stereoisomers in position C7 of TMC-95s (indicated by a black arrow) vary by two orders of magnitude. This observation is explained by the fact that for effective binding the hydroxyl group must be in its S-isomeric state, in order to avoid a steric clash with the carbonyl oxygen of residue 21 (coloured in brown).
whether PEG-linked bivalent inhibitors retain membrane permeability to be able to enter into the intracellular space has not been answered. However, PEG is known to be nontoxic, has low immunogenicity and low clearance rates, increases water solubility and may help in membrane transfer. These features make PEG-based bivalent inhibitors promising compounds for further investigations.

It was shown that the catalytic activity of the CP is efficiently and irreversibly inhibited by the α', β'-epoxyketone peptide natural product epoxomicin [35]. Unlike most other proteasome inhibitors, epoxomicin possesses high specificity for the CPs, and does not inhibit other proteases like calpain, trypsin, chymotrypsin, papain or cathepsins. The crystal structure of the yeast CP:epoxomicin complex revealed the molecular basis for selectivity of this class of inhibitors [36] (see Fig. 3b). The unexpected morpholino formation between the amino terminal threonine and epoxomicin was seen in the crystal structure of the complex, providing first insights into the unique specificity of epoxomicin for 20S proteasomes. The morpholino derivative formation is most likely a two-step process. First, activation of the Thr1O\textsuperscript{γ} is believed to occur by its N-terminal amino group acting as a base either directly or by a neighboring water molecule. A hemiacetal, representing the first step in the formation of the morpholino adduct, is produced by subsequent nucleophilic attack of the Thr1O\textsuperscript{γ} on the carbonyl carbon atom of the epoxyketone pharmacophore, as it has been observed in the CP:Ac-LLnL-al complex. The completion of the morpholino ring is performed by the N-terminus of Thr1, which opens the epoxide ring by an intramolecular displacement with consequent inversion of the C2 carbon atom. The requirement of both the free N-terminal amino group and the side chain nucleophile for adduct formation with the epoxyketone pharmacophore explains the observed specificity of epoxomicin for CPs. Thus, epoxomicin only interacts with the small class of Ntn-hydrolases and therefore is highly specific. However, since this cell-permeable natural product irreversibly blocks all proteolytically active subunits of the CP, its high cytotoxicity may limit the usage of this inhibitor as possible target for drug development.

Peptides possessing a vinyl sulfone moiety represent another class of proteasome inhibitors [37]. These compounds bind to CPs irreversibly, but are less reactive than aldehydes. Vinyl sulfones do not show high specificity for proteasomes, similar to peptide aldehydes, since they inhibit intracellular cysteine proteases such as cathepsins. The crystal structure of the CP:Ac-YLLN-vs complex shows covalent binding of the Thr1O\textsuperscript{γ} of all active subunits to the β-carbon atom of the vinyl sulfone group [38] (see Fig. 2c). Several elements that can be used to control the selectivity of synthetic inhibitors have been identified during scanning of libraries of peptide-based covalent CP inhibitors [5,33,34]. Additionally, a random positional search for selectivity of vinyl sulfones for proteasomes was performed. In this scan, the amino acid located directly at the site of hydrolysis was held constant, and sequences distal to that residue were varied across all natural amino acids [39]. Ac-PRLN-vs could be identified as a proteasomal inhibitor, specific for subunit β2. Remarkably, in comparison to Ac-YLLN-vs, which inhibits all proteolytically active sites in 20S proteasomes, Ac-PRLN-vs is highly selective, although these two compounds vary only in their P3 and P4 positions. The crystal structures of the yeast CP in complex with these two vinyl sulfones revealed that the inhibitors have identical P1 residues, which adopt the same conformation in the S1 pocket. However, it is the favourable interaction between the P3 residue and the S3 pocket, generated at the interface of neighbouring β-subunits, which explains their selectivity [38] (see Fig. 2c). These results demonstrate the possibility to design inhibitors, in which specificity can be controlled predominantly by interactions in the S3 pocket. Additionally, strong interactions at P1 may overcome the need for a favourable P3 residue. Lactacystin has been reported to specifically bind to subunit β5 due to its characteristic P1 site. This information, combined with findings of the importance of the P3 pocket, may facilitate the development of proteasomal inhibitors with tuneable selectivity for each of the active sites.

5.2. Noncovalently bound inhibitors

A common feature of all of the previously mentioned proteasome inhibitors is the formation of a covalent bond with the active site Thr1O\textsuperscript{γ} of the β-subunits. This usually induces apoptosis and causes cell death [3]. It can be expected that the cytotoxic effects of proteasomal inhibitors may be reduced by making their binding to single subunits of the CP reversible and time-limited. Recently, it was shown that the natural products from Apiospora montagnei, TMC-95s (TMC-95A, B, C and D), block the proteolytic activity of the CP selectively and competitively in the low nanomolar range [40,41]. These compounds, which are not related to any previously mentioned proteasome inhibitors, consist of a heterocyclic ring-system made of modified amino acids. The inhibitor binds to all three proteolytically active sites, as was elucidated from the crystal structure of the yeast CP:TMC-95A complex [42] (see Fig. 3c). TMC-95A is linked noncovalently to the active sites of the β-subunits, not modifying their N-terminal threonines. A tight network of hydrogen bonds connects TMC-95A with the protein, thus stabilizing its position. All these interactions are performed with main chain atoms and strictly conserved residues of the CP, revealing a common mode of proteasome inhibition among different species. The arrangement of TMC-95A in the CP is similar to the already reported aldehyde and epoxyketone inhibitors [36]. The n-propylene group of TMC-95A protrudes into the S1 pocket, making weak hydrophobic contacts with Lys33, whereas the S2 subsite is shallow and does not contribute to stabilization of TMC-95A, as has been already observed in the proteasome:Ac-LLnL-al adduct [5]. A major role in the different
IC₅₀ values among the different activities is ascribed to the side chain of the asparagine, which is inserted deeply into the S3 pocket. This observation is in line with the previously described results, obtained for the specific vinyl sulfone inhibitors. Additionally, the IC₅₀ values for the stereoisomers of TMC-95s vary by two orders of magnitude. The crystal structure of the CP:TMC95A complex gives explanations for this occurrence: for effective binding, the hydroxyl group present in R1/R2 of the TMC-95s must be in its S-isomeric state in order to avoid a steric clash with the carbonyl oxygen of residue 21; whereas the methyl group in R3/R4 is not sterically restricted (see Fig. 3c and Fig. 4b). Thus, the crystal structure revealed that the topology of proteasomal active sites is the prerequisite for selective and efficient inhibitor binding, and not the proteolytically active residues.

TMC-95 compounds do not inhibit other proteases like m-calpain, cathepsin L and trypsin [40]. The NMR-structure of unbound TMC-95A in solution [41], superimposed with the crystal structure of the CP:TMC-95A complex shows no conformational rearrangements of the inhibitor [42]. Optimal binding to the 20S proteasome is due to the strained conformation of TMC-95s, caused by the presence of the cross-link between the tyrosine and the oxindol side chain. Binding of TMC-95s does not require major rearrangements of ligand and protein and therefore is entropically favoured, in contrast to more flexible ligands. The structural superposition of proteasomal subunits in complex with TMC-95A and Ac-PRLN-vs shows a remarkable overlap of the backbone amides and the P1 and P3 residues, although TMC-95A binds noncovalently and Ac-PRLN-vs covalently to the active Thr1Og (see Fig. 4a). This demonstrates that if specific ligands of the inhibitor are presented in an optimal manner, covalent attachment to the catalytic nucleophile is no longer required. Combination of the crystal structures of CP:inhibitor complexes suggests the possibility to generate a basic inhibitor structure with the geometry of TMC-95s to create a variety of structures specific for each of the active sites of the CP. In particular, the P3 and P1 positions represent the sites for fine-tuning of the inhibitor selectivity for individual β-subunits (see Fig 4a).

Because of the promising therapeutic potential of proteasome inhibition, much attention has been paid to the development of synthetic and the discovery of natural ligands. The skeletal structure of the derivatised cyclic tripeptide TMC-95A is constrained into a 17-membered ring...
structure by an endocyclic biaryl system, which is the major component binding to the active-site clefts of the CP. The remaining part of the inhibitor is not involved in direct interactions with the protein, except the oxoindol group of the tryptophane, which forms a hydrogen bond with the main chain nitrogen of residue 23 (see Fig. 4b). Thus, the structural factors responsible for the differing activities of TMC-95A for different proteolytically active sites are the conformationally restricted P1 (C-terminal (Z)-prop-1-enyl moiety) and the central asparagine in P3. The total synthesis of the natural TMC-95A compound is highly complex [43,44], but the lead structure simplifies and facilitates the creation of TMC-95A analogues [45,46]. To verify the significance of the minimal core structure, it was decorated C-terminally with an n-propyl group (Nle-side chain) as P1 residue and N-terminally with a benzoyloxy carbonyl as N-protecting group, while the central Asn residue of TMC-95A was retained as P3 residue [47]. However, this analog showed a 10 times lower inhibition rate for subunit β5, compared to the natural compound. This observation has been a surprise, as the designed analog only differed from the natural inhibitor by lacking the two hydroxy groups at the tryptophan moiety. According to the X-ray structure of the CP:TMC-95A complex, these two modifications should not account for the substantially lower inhibition of the activity. Therefore, this effect may be due to the replacement of the conformationally restricted and bent (Z)-prop-1-enyl group with the more flexible n-propyl group as P1 residue. 

6. Biological effects of proteasome inhibitors

Since specific and efficient proteasome inhibitors have become available, a large collection of data documenting the critical roles of the proteasome and of the Ub pathway in many biological processes has been generated. Usage of these inhibitors enabled the identification of numerous proteasomal substrates. These results clearly show that, besides the necessary housekeeping functions of intracellular proteolysis, proteasome performs precise regulated degradation of key proteins to switch off specific pathways. The biological effects elicited by treatment of the cells with proteasomal inhibitors can be extremely diverse, depending on the cell type, cell proliferation status, nature and amount of the inhibitor, and time of exposure (see for example Ref. [50]). It must be taken into account, however, that most proteasome inhibitors are not exclusively specific, and their biological effect(s) may be partly explained by inhibition of other proteases. For example, peptide aldehydes and peptide vinyl sulfones at certain concentrations also inhibit intracellular cysteine proteases, such as cathepsins and calpains. Likewise, although the natural product lactacystin was initially thought to be highly specific for the proteasome, it was shown later to additionally inhibit cathepsin A [51].

As can be expected from the Ub–proteasome-dependent degradation of numerous proteins regulating cell-cycle (G1 and mitotic cyclins, CDK inhibitors, p53), cell proliferation is significantly affected by proteasome inhibitors. In fact, several of these compounds have been identified via their effect on cell growth. Proteasome inhibition can cause cell-cycle arrest at various stages: there are reports of arrest of the G1/S and the G2/M cell division stages [52]. Partly this effect can be due to protection of both positive and negative regulators of cell growth with following activation of conflicting signalling pathways, often triggering apoptosis [3,53]. In addition, proteasome inhibitors affect other biological processes such as cell differentiation, inflammatory and immune responses. For example, MG132 and lactacystin have been characterized as compounds that promote neurite
outgrowth in neuroblastoma cell lines [54], and several other proteasome inhibitors can block myoblasts to fuse with myotubes during muscle differentiation [55]. Proteasome inhibitors strongly stabilize the protein IκBα, which is selectively inhibiting the transcription factor NF-κB, being involved in the inflammatory response [56,57]. Proteasome inhibitors also represent interesting immuno-suppressive or immuno-modulating properties, since the proteasome is the main producer of antigenic peptides [58] and is directly involved in the control of immune response [59].

Proteasomal inhibitors have been proven to be a very useful tool in investigation of the contribution of proteasomes to the intracellular proteolysis. For example, they helped to identify that the Ub–proteasome pathway is involved in muscle atrophy in a variety of pathologies [60]. Interestingly, undegraded proteins seem to aggregate in a perinuclear region that appears to contain proteasomes, ubiquitin and chaperone proteins. As this region also contains γ-tubulin and corresponds to the centrosome and its periphery [61], it has been suggested that centrosome could be the centre, regulating proteasome function [62].

7. Perspectives

Proteasome inhibitors have a promising future for the treatment of diseases involving excessive proteolysis, like muscle atrophy (cachexia). However, due to the pleiotropic role of the Ub–proteasome system, the toxic side effects of these compounds may strongly limit their potential. Structural and functional information on proteasomal inhibitors allowed the Millennium Pharmaceuticals company to develop a proteasome inhibitor named Valcade (bortezomib), which passed in 2004 clinical phase III study, and now represents a newly approved prescription drug against multiple myeloma [63–65].

A future goal must be targeting proteasome inhibitors to the appropriate cells. Up to now, it has been difficult to use these inhibitors as drugs for correction of abnormal degradation of specific proteins. A possible solution could be to design inhibitors that block or retard degradation of only a specific set of substrates for a certain time and, thus, decrease the toxicity of the inhibitors. This goal also can be achieved by targeting the 19S complex, which is responsible for regulatory gating of the CP, and possesses ATPase and isopeptidase activity. Another field that remains to be explored is the family of endogenous protein complexes which modulate proteasomal activity. The discovery of new compounds influencing these regulators and inhibitors is a challenge for the future.

8. Uncited reference

[7]


