Review

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The persisting challenge of selective and specific proteasome inhibition‡

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Since the discovery of the proteasome and its structure elucidation intensive research programs in academic institutions and pharmaceutical industries led to identification of a wide spectrum of synthetic and natural small proteasomal inhibitors. Activity studies with these small molecules helped to deeply understand the complex biochemical organization and functioning of the proteasome. The new structural and biochemical insights placed the proteasome as an important anti-cancer drug target, as revealed by the dipeptide boronate proteasome inhibitor, bortezomib, which is currently used for treatment of multiple myeloma. Serious side effects and partial cell resistance against bortezomib demand creation and discovery of new improved generations of more specific and potent proteasomal inhibitors.

Keywords: proteasome; inhibitors; synthetic and natural products; crystallographic analysis; polyvalency

Background

In eukaryotic cells, most cytosolic and nuclear proteins are degraded by the ubiquitin–proteasome pathway, which is responsible for protein quality control, antigen processing, signal transduction, cell-cycle control, cell differentiation, and apoptosis [1–3]. As protein homeostasis is critically involved in cancer cell survival, targeting the balance between production and destruction of proteins that mediate proliferation and other key factors of malignancy has advanced to a major focus of cancer research [4–7]. Accordingly, the proteasome has emerged as a promising target for cancer therapy, and this therapeutic approach has recently been validated with the peptide boronate, bortezomib (VELCADE®) [8,9]. Indeed, for patients with multiple myeloma (MM), the peptide boronate exhibits unmatched anti-tumor activity, though not free of side effects [10]. Thus, the development of new generations of proteasome inhibitors as anti-cancer drugs still remains a challenge.

The executioner of the ubiquitin–proteasome pathway is the 26S proteasome, which is built up from the 700 000 Da proteolytic core particle (20S proteasome) and two 900 000 Da regulatory particles (19S regulatory complex). The 20S proteasome has a cylindrical shape showing two-fold symmetry and containing multiple catalytic centres located within the inner cavity of a molecular cage. It comprises 28 subunits, which are arranged in four seven-membered rings that stack upon each other, yielding an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ complex [11]. Compared to archaebacterial proteasomes, which have 14 identical, and thus, 14 proteolytically active sites [12], eukaryotic proteasomes contain only three proteolytically active β subunits per β ring (subunits β1, β2, and β3), whereas the other β subunits are inactive [13]. For a review see Ref. 14.

The first class of proteasome inhibitors were the tripeptide aldehydes calpain inhibitor I (Ac-Leu-Leu-Nle-H) and leupeptin from actinomycete (Ac-Leu-Leu-Arg-H). Structural analysis of these inhibitors revealed the mechanism of protein degradation catalysed by the nucleophilic *N*-terminal threonine hydroxy group (Thr1O$^{\gamma}$) and led to structural characterization of the substrate-binding pockets of the caspase-like (β1 subunit), trypsin-like (β2 subunit), and chymotrypsin-like (β5 subunit) catalytic sites, as illustrated in Figure 1 [12,13]. Peptide aldehydes were shown to form reversible covalent hemiacetals with the Thr1O$^{\gamma}$. Analysis of diverse other functional electrophiles such as boronates, vinyl sulfones, and natural product-based α', β'-epoxyketones provided additional insights into their various binding modes to the proteasomal active sites as well as into their ability to inhibit the different proteolytic activities of the proteasome; for a review, see Ref. 14. The peptide boronates were found to be much more potent inhibitors than aldehydes and vinyl sulfones. Low concentrations of boronate compounds are sufficient for significant inhibition of proteasome activity, and due to their high selectivity and low dissociation rates, this class of compounds has been used in various medical research programs and clinical experiments. The successful dipeptide boronate candidate bortezomib, which inhibits prevalently the β5 subunit, has now been approved as a prescriptive drug for treatment of relapsed and/or refracted multiple myeloma [8,15].

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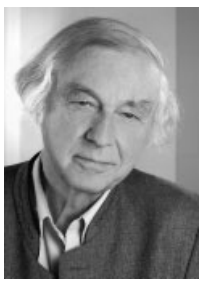
Biography

Luis Moroder was born at Urtijei in South Tyrol, Italy, in 1940. He had his early training in peptide chemistry with Professor E. Scoffone at the University of Padua and later spent a postdoctoral stage with Professor K. Hofmann in Pittsburgh. Since 1991, with distinguished visiting appointments elsewhere, he has been Head of the Laboratory of Bioorganic Chemistry at the Max Planck Institute of Biochemistry in Martinsried. He has been awarded the Max Bergmann Medal 1995, was Josef Rudinger Lecturer of the European Peptide Society in 2004 and Dimitrios Theodoropoulos Memorial Lecturer in 2008. He is author or coauthor of over 500 publications, and coeditor of the five-volume Houben-Weyl treatise 'Synthesis of Peptides and Peptidomimetics', and of the monograph 'Oxidative Folding of Peptides and Proteins'. His scientific interests include collagen model peptides, proteinase inhibitors, bioengineering of proteins, as well as photomodulation of conformation and bioactivity of peptides. Presently, he is Editor-in-Chief of the Journal of Peptide Science.



Robert Huber was born in 1937 in Munich, Germany. He studied chemistry at the Technische Universität München (TUM), where he also completed his Ph.D. and habilitation. Since 1972, he has been a member of the Max-Planck-Gesellschaft and Director at the Max Planck Institute of Biochemistry in Martinsried until his retirement in 2005. Since 1976, he also served at the TUM as a Professor. With distinguished appointments at various universities he serves as a member on the Boards and/or Scientific Advisory Boards of a number of academic institutions as well as of pharmaceutical and crop science companies, and is cofounder of two companies, Proteros and Suppremol, located at Martinsried. Robert Huber has made decisive contributions to the understanding of the structure and function of biological macromolecules, and in addition to the development of instruments for data collection, and to methods in protein crystallography, particularly Patterson methods, graphic methods, and refinement, to the use of electron-rich metal clusters, and most recently to the methods and instruments for crystal improvement. He is author or co-author of more than 730 publications, and his outstanding contributions to advancement of science have been recognized by numerous honorary doctorates, professorships, memberships in learned societies and awards, including the Otto-Warburg Medal, the Emil von Behring Medal, the Sir Hans Krebs Medal, the Linus Pauling Medal, Max Tishler Prize, and in 1988, the Nobel Prize for Chemistry, together with H. Michel and J. Deisenhofer.

Biography

Michael Groll was born in 1971 in Donauwörth, Germany. He studied Chemistry in Munich and then joined the group of Prof. Dr Robert Huber for postgraduate studies. In 1998, he received his Ph.D. for crystallographic and biochemical studies on the 20S proteasome from yeast. He continued to work as a postdoctoral fellow at the Max-Planck-Institute in Martinsried and in 2002 he joined the group of Prof. Finley at Harvard Medical School, being involved in the elucidation of structural and functional mechanisms of proteasomal regulation. From 2003 to 2006, he was running an independent research group at the Adolf-Butenandt-Institut for Physiological Chemistry at the Ludwig-Maximilians-Universität München, working particularly on protein translocation. In 2004, Michael Groll received his Habilitation from the Charité in Berlin for his work on structural and functional relationships between archaebacterial and eukaryotic 20S proteasomes. From 2006 to 2007, he accepted an appointment by the Charité, Medical School of the Humboldt-University of Berlin as a professor for Biochemistry. Since 2007, he is Chair of Biochemistry at the Department Chemie of the Technische Universität München and a member of the excellence cluster CIPSM (Center for Integrated Protein Science). His current main research interests are focused on functional and structural characterisation of multifunctional protein complexes; structural characterisation of protein-protein and protein-ligand interactions; development and practical application of synthetic and natural protein ligands as well as molecular flexibility and its biological significance.

Proteasome Inhibition by Pseudocovalent and Covalent Adduct Formation

As previously discussed, synthetic peptide aldehydes deactivate with a low degree of specificity all three proteasomal activities via hemiacetal bond formation. Interestingly, the fast reverse reaction of hemiacetal bonds is slowed down in the protease complex by formation of a hydrogen-bonding network between the generated hydroxy group of the ligand and the oxyanion-hole of the protein [12,13,16–18]. However, besides the functional reactive group, it is the dipeptide group which forms an antiparallel β-sheet at the active-site cleft and the characteristic ligand side-chain residues which finally determine the affinity of the compound to the distinct proteasomal active sites [19,20]. Amino acid aldehydes do not act as proteasome inhibitors, since the mean residence time of these compounds at the proteasomal active site is not sufficient to complete the chemical reaction cycle [21]. It is known that a functional aldehyde group is highly reactive; thus, peptide aldehydes are rather unspecific to the proteasome and block a broad range of different serine and cysteine proteases [22]. Surprisingly, it has been recently shown that the natural peptide aldehyde, fellutamide B (Figure 2), isolated from *Penicillium fellutanum*, potently induces the release of nerve growth factor (NGF) from fibroblasts and glial-derived cells by selectively inhibiting the 20S proteasome catalytic activity [23]. Furthermore, the most popular proteasome inhibitor which is used

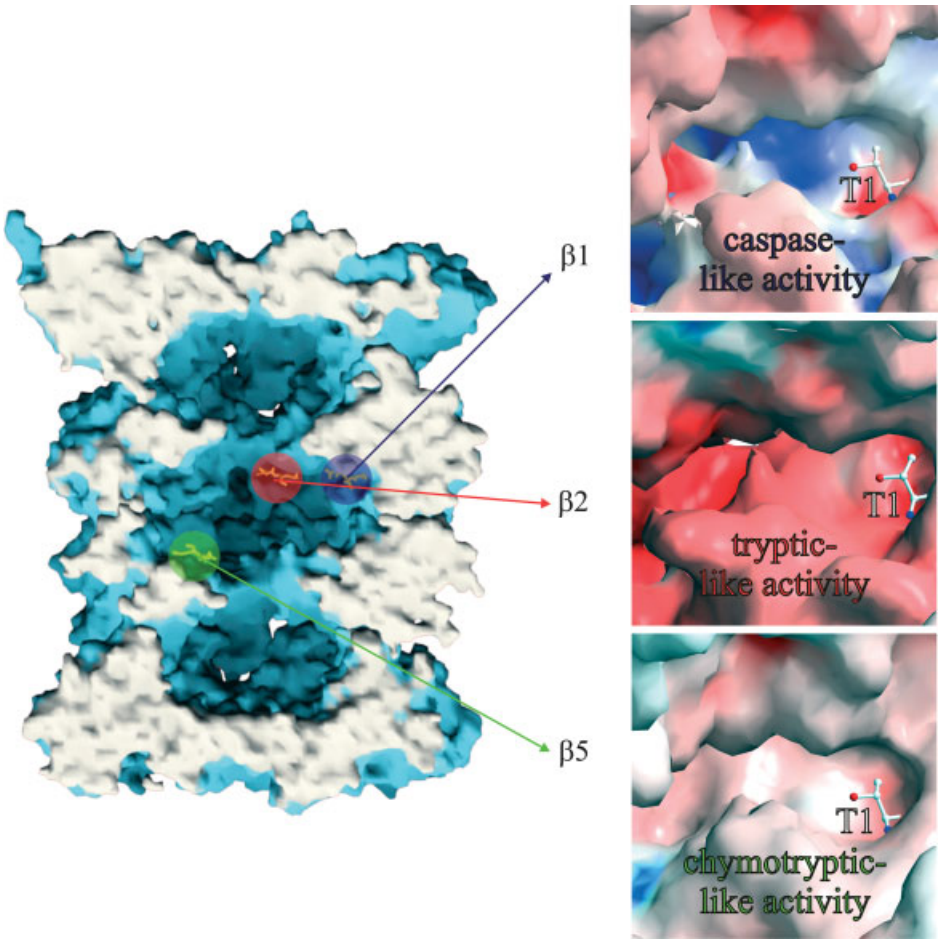


Figure 1. Surface representation of the inner chamber of eukaryotic proteasome and of the three active-site clefts of the β1, β2, and β3 subunits as derived from the crystal structure of yeast proteasome/calpain I inhibitor complex [13].

for *in vivo* and *in vitro* studies is the peptide aldehyde inhibitor, MG132 (Ac-Leu-Leu-Leu-H) [24]. Although peptide aldehydes are the best characterized proteasomal inhibitors, compounds possessing a vinyl sulfone moiety are also widely distributed and represent another class of proteasome inhibitors [25]. These compounds bind to proteasomes irreversibly but are less reactive than aldehydes. Vinyl sulfones act as Michael acceptors for soft nucleophiles such as thiols, leading to the formation of a covalent bond [26]. Although these inhibitors do not inhibit serine proteases, they show high specificity for intracellular cysteine proteases [27]. Compared to aldehydes and vinyl sulfones, peptide boronates such as bortezomib (Figure 2) turned out to be more specific for the proteasome [28], which was a surprise, since boronic acid derivatives were firstly described to inhibit most serine proteases. The high affinity of bortezomib for the proteasome can be explained by the threonine-boronate tetrahedral transition state complex, which is further stabilized by the amine-group of the *N*-terminal threonine [29]. The smallest class of compounds acting as selective and specific monovalent proteasome inhibitors are β-lactones (Figure 2) [30]. Proteasomal inhibition of these compounds is caused by hydrolysis of the β-lactone ring via the active-site Thr1O$^{\gamma}$, resulting in an acyl-enzyme complex [29]. Since formation of an acyl-enzyme complex also occurs during peptide bond hydrolysis of substrates, the β-lactone-binding of the inhibitor has to prevent its hydrolysis by the nucleophilic water molecule. Indeed, defined side-chains of β-lactones occupy the position of the nucleophilic water molecule at the catalytic center, thus interrupting the preferred trajectory of nucleophilic addition along the path approximately perpendicular to the plane of the ester group [31,32]. Compared to proteasomal peptide inhibitors, β-lactones do neither require a defined structural arrangement at the proteasomal active binding site nor a conserved structural motive for displacing the nucleophilic water molecule, indicating that β-lactones did not share a common ancestor and evolved separately during evolution. A new class of irreversible bivalent proteasome inhibitors are the natural product-based $\alpha'\beta'$-epoxyketones (Figure 2) [33]. Although epoxomicin binds similarly to the proteasomal active site as peptide aldehydes completing the anti-parallel β-sheet, there is a major difference: whereas peptide aldehydes form a reversible hemiacetal bond with the *N*-terminal nucleophilic threonine, $\alpha'\beta'$-epoxyketones form an irreversible morpholino bond formation by a two-step process [34]; for a review, see Ref. 14. First, nucleophilic attack of Thr1O$^{\gamma}$ on the carbonyl carbon atom of the epoxyketone pharmacophore produces a hemiacetal, as observed in the structure of proteasome–aldehyde inhibitor complexes. This hemiacetal bond facilitates opening of the epoxide ring via the Thr1-*N*-terminus, thereby forming the irreversible morpholino adduct. Therefore, compared to previously described proteasome inhibitors, epoximicin turned out to be highly specific for the small class of *N*-terminal nucleophilic hydrolases. Most recently, a new natural inhibitor of the proteasome, syringolin A (Figure 2), could

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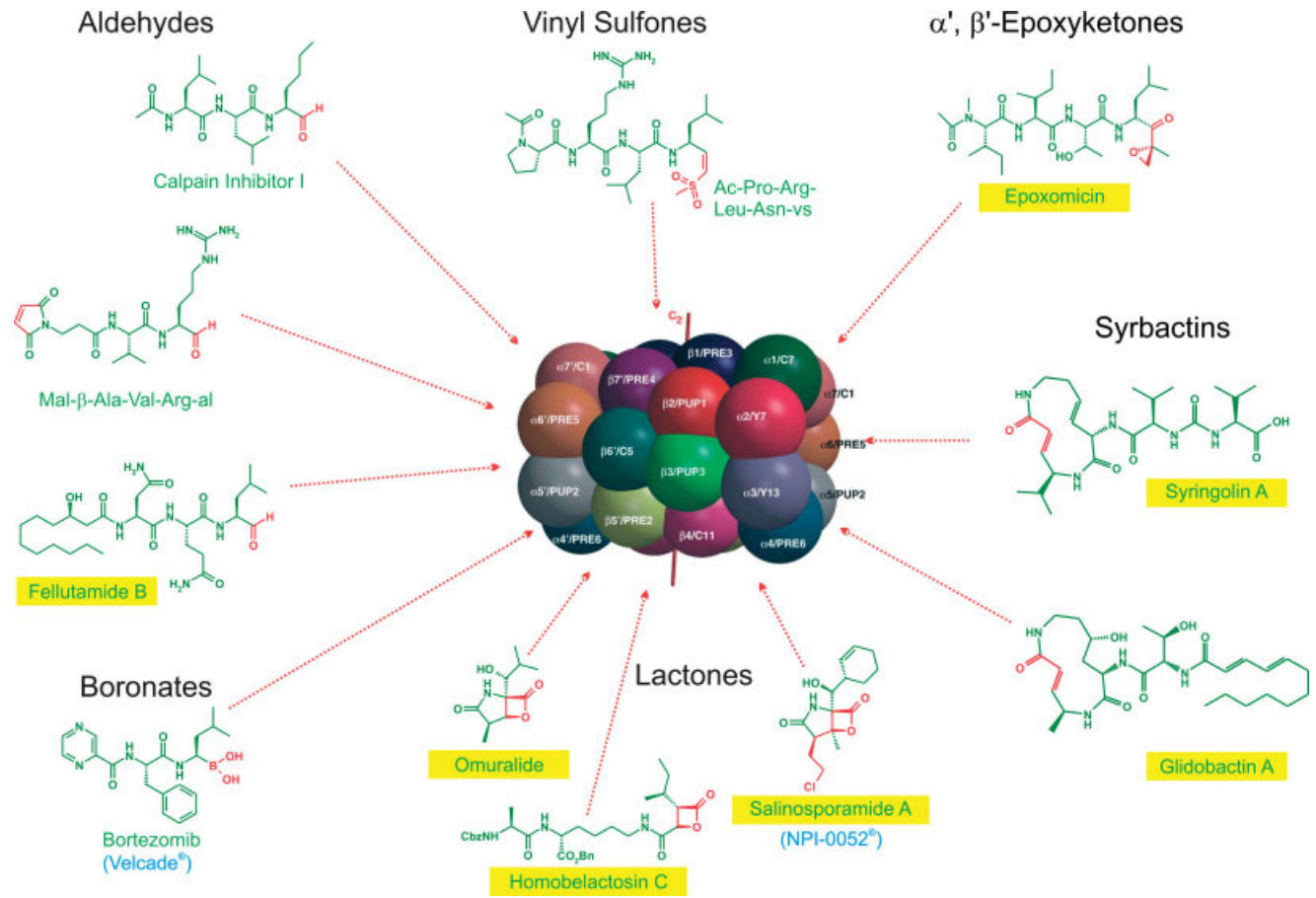

Figure 2. Overview of various different synthetic and natural (yellow background) compounds. The specific mode of actions of these small molecules to the active-site clefts of the proteasome have been determined by crystallographic analysis in complex with the yeast 20S proteasome; for review on X-ray structures see Ref. 14.

be identified, which acts as a plant pathogen virulence factor by proteasomal inhibition [35]. The crystal structure analysis revealed that syringolin A covalently binds to the hydroxy group of the active-site *N*-terminal threonine following a novel mechanism: Thr1O$^{\gamma}$ of the proteasome performs a Michael-type 1,4-addition to the double bond located in a 12-membered ring system of the inhibitor. The resulting covalent ether bond causes the irreversible inhibition. Interestingly, a unique model completely explaining the synthesis of the peptide part of syringolin A, including the functionally important reactive group, has been proposed on the basis of the architecture of non-ribosomal genes in syringolin A synthesis [35]. The model allowed identification of glidobactin A (Figure 2) which blocks proteasomal activity in the low nanomolar range. An NCBI database search of all sequenced bacterial genomes revealed homologues of these genes in the insect pathogen *Photorhabdus luminescens* and the human pathogen *Burkholderia pseudomallei*, the causing agent of melioidosis. Thus, it is most likely that these organisms are capable of synthesizing proteasome inhibitors of this new class of proteasome inhibitors termed syrbactins [35]. If this prediction is confirmed, it will be interesting to determine whether the respective compounds are involved in virulence.

Structures of the proteasome in complex with various synthetic and natural inhibitors reported in Figure 2 provided valuable insights into the architecture and organisation of substrate-binding pockets located near the proteasomal active centres [14,36]. The structural information on the binding mode of different chemical compounds stimulated the development of more potent inhibitors that block the individual proteolytic activities of this complex enzyme by changing structural and chemical aspects such as binding mode, specificity, and selectivity [37]. Among the members of this new generation of small proteasomal inhibitors, the analogue of epoxomicin PR-171 [38] with its high specificity for the chymotrypsin-like activity, and salinosporamide A (NPI-0052) [39], have emerged as promising candidates for anti-cancer therapy, and are presently in phase I human clinical trials for alternative treatments of patients intolerant or refractory to bortezomib [40,41].

Reversible Proteasome Inhibitors

All the previously described synthetic and natural proteasome inhibitors form pseudocovalent or irreversible covalent bonds with the active-site Thr1O$^{\gamma}$ of the β subunits. Application of these inhibitors *in vivo* was often found to induce apoptosis and, thus, cell death [42–44]. It may be reasonable to tune cytotoxic effects of proteasome inhibitors by increasing their specificity for the various active sites and, more importantly, by making their binding to the enzyme reversible and time-limited.

Among the diverse secondary metabolites of microorganisms, the TMC-95 family of cyclic tripeptides from *Apiospora montagnei* represents the only one that selectively and competitively blocks

the proteolytic activities of the proteasome in the low nanomolar range [45,46]. Furthermore, the TMC-95 compounds were inactive against other proteases such as calpain, cathepsins, caspases, elastase, thrombin, and trypsin [45]. The TMC-95 tripeptide derivatives, which are not related to any previously mentioned proteasome inhibitor, consist of a heterocyclic ring system deriving from carbon–carbon cross-bridging of a tyrosine side-chain with a highly hydroxylated tryptophan side-chain (Figure 3). The inhibitor binds to all three proteasomal proteolytically active centres, as determined by crystallographic analysis of the yeast proteasome in complex with TMC-95A, without covalent interaction with the active β subunits [47]. Instead, a tight network of hydrogen bonds is established between the ligand and the strictly conserved main-chain residues of the proteasome, supporting a common mode of proteasome inhibition among different species. Superposition of the crystal structure of TMC-95A with the NMR-structure of unbound TMC-95A in solution [46] showed no conformational rearrangements of the inhibitor upon binding to the proteasome [47]. Thus, optimal interaction of this inhibitor with the proteasome results from the constrained conformation of TMC-95A, which is imparted by the phenyl-oxindole side-chain clamp, and which pre-organizes the peptide backbone in an extended conformation to optimally fit the active-site clefts by formation of an anti-parallel β-sheet structure.

Using the structural information gained from crystallographic analysis of the TMC-95A/proteasome complex [47], in first instance, a minimal cyclic core with an oxindole-phenyl clamp was designed (Figure 3) [49]. As proof of principle, superposition of crystal structures of these structurally simplified TMC-95A analogs in complex with the proteasome to that of the original TMC-95A demonstrated an almost identical peptide backbone configuration. Secondly, the still-synthetically demanding oxindole-phenyl clamp was replaced by the more flexible endocyclic biphenyl-ether clamp of the isodityrosine type (Figure 3), which is known to pre-organize the peptide backbone in a rigid extended conformation [50–52]. The biphenyl-ether compound BIA-1a was decorated with an *N*-propyl group at P1 and an Asn residue at P3 in order to allow for structural and functional comparison of the endocyclic oxindole-phenyl and biphenyl-ether-bridged tripeptides with identical P1 and P3 residues [53]. Surprisingly, the crystal structure analysis of BIA-1a in complex with the proteasome showed that the ligand was exclusively bound to the trypsin-like active site, in proximity of the Thr1 hydroxy group of subunit β2 (Figure 3) [48], a fact that was attributed to the unique architecture of the specificity pockets of this active-site cleft. As BIA-1a and TMC-95A were exhibiting a high degree of consensus in their constrained conformation and binding mode, the strong interaction of BIA-1a with residues exclusively found in the trypsin-like active site, formed by subunits β2 and β3, is crucial for the high selective binding. These results provided the basis for the design of an advanced β2-selective compound for inhibition of the trypsin-like proteasome activity. Taking into account the selective inhibition of subunit β2 by the synthetic peptide vinyl sulfone, Ac-PRLN-vs [27,28], a modified endocyclic biphenyl-ether compound, BIA-2a with arginine side-chains in both P1 and P3 positions, was designed (Figure 3). Furthermore, the C-terminus of BIA-2a was extended by an amide group to achieve close contact with Thr1O$^{\gamma}$ [53]. Crystallographic analysis of BIA-2a in complex with the proteasome revealed formation of a covalent ester bond between the BIA-2a C-terminus and the Thr1O$^{\gamma}$, i.e. of the acyl-enzyme intermediate as the first step in amide hydrolysis (Figure 3) [48]. Amide hydrolysis by the nucleophilic water molecule was confirmed by mass-spectrometric analysis of yeast proteasome incubated with BIA-2a. This fact confirms that the proteolytically active sites of the proteasome maintain their functional efficiency while binding of the inhibitor BIA-2a in a reversible substrate-like manner, which causes no allosteric changes of the active-site residues [48]. The transient covalent derivatization of the active-site Thr1O$^{\gamma}$ by the clearly detectable ester-bond formation as an intermediate step of amide hydrolysis of BIA-2a supports the hypothesis that

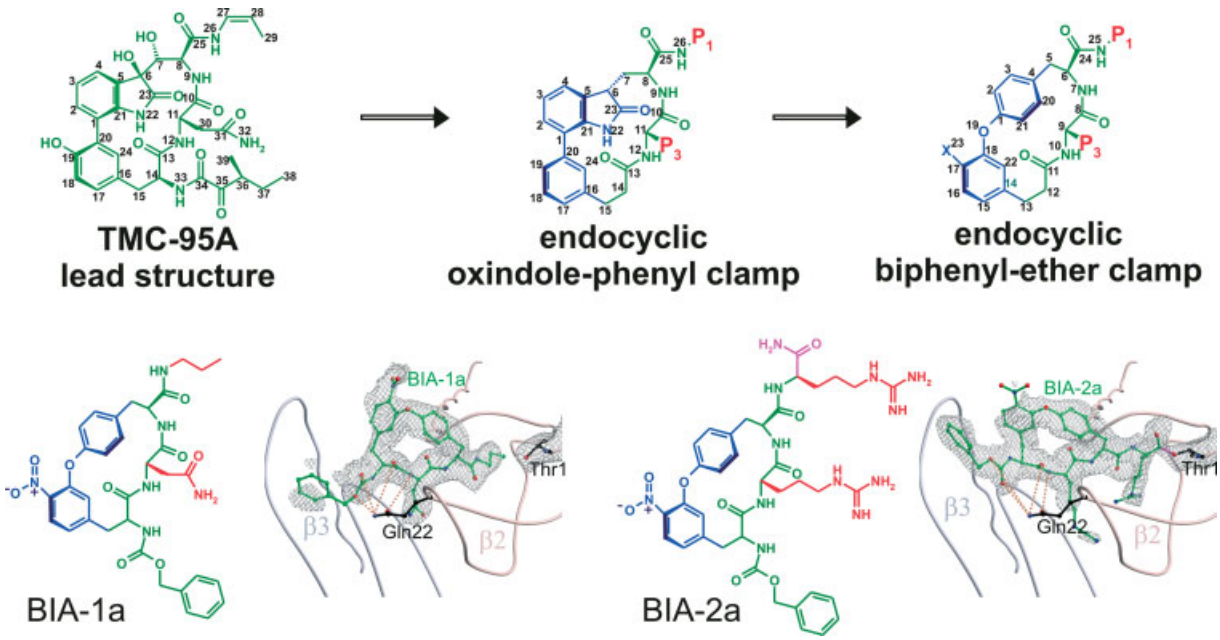

Figure 3. TMC-95A was identified as a new lead compound and allowed design and synthesis of structurally simplified endocyclic oxindole-phenyl compounds (BIA-1a and BIA-2a), which exhibit a novel structural biphenyl-ether clamp motive. Crystal structure analysis of BIA-1a (side-chains were similar as in the natural product TMC-95A) and BIA-2a (side-chains were designed to specifically bind to the trypsin-like active centre of the proteasome) in complex with the yeast 20S proteasome [48].

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the half-life of intermediate complexes of the proteasome with peptide substrates tightly bound to the primed sites of the active centres could well suffice for rates of aminolysis that compete with hydrolysis. This would conform to the reported peptide splicing activity of proteasome [54]. For additional information see Ref. 37.

The discussed structure-based evolution of the biphenyl-ether clamped BIA-2b allowed to convert the natural peptide TMC-95A with its two orders of magnitude stronger inhibition of the chymotrypsin-like, compared to the trypsin-like proteasome activity, into an inhibitor showing about two orders of magnitude stronger binding to the trypsin-like than to the chymotrypsin-like active centre.

Most recently, an additional natural cyclic octapeptide., i.e. argyrin A, derived from the myxobacterium *Archangium gephyra* [55], was identified as a potent proteasome inhibitor with promising anti-tumor activity [56]. Its tumor inhibition shown for a variety of human malignancies is attributed to inhibition of the $p27^{kip1}$ cyclin-dependent kinase inhibitor degradation by the proteasome. As shown in Figure 4, argyrin is a backbone-cyclized octapeptide. It inhibits the human proteasome caspase-, chymotrypsin-, and trypsin-like activities at potencies comparable to those of bortezomib, but the mode of action still has to be elucidated. Previous studies on degradation of linear, disulfide-bridged, and backbone-cyclized model peptides such as secretin, VIP, and gastrin, somatostatin-14 and *c*[D-Phe-His-Trp-Ala-Val-Gly-His-Leu-Leu], clearly confirmed fast proteolysis of the linear polypeptides by the proteasome from *T. acidophilum* [57]. However, no degradation of the cyclic nonapeptide as well as of the disulfide-bridged somatostatin was detected [58], supporting the notion that ubiquitinylated proteins are presented by the regulatory 19S complex for degradation by the 20S proteasome core particle. The very similar sizes of the cyclic 9-mer model peptide and of argyrin raises the question whether this natural product can enter the digestion chamber of the proteasome to block its active sites, or alternatively, interfere with the interaction with the 20S particle and the 19S complex required for presentation of the unfolded $p27^{kip1}$ cyclin kinase inhibitor for degradation. However, in the latter case, the differentiated inhibition potencies towards the three proteasomal activities can hardly be explained. Moreover, previous attempts to block the entrance of 20S proteasome with a phenylalanine amide derivatized β-cyclodextrin (Figure 4) have so far failed, although symmetry and size are well suited for such a capping (unpublished results).

Heterobifunctional Proteasome Inhibitors

From the X-ray structure of yeast proteasome, it was known that the conserved Cys118 residue of the β3 subunit protrudes into the S3 subsite of the β2 active site [13]. This reactive function allows for covalent grafting of a maleinimide-functionalized peptide aldehyde in juxtaposition of the active centre for hemiacetal formation with the Thr1O$^{\gamma}$. Based on modelling experiments, an ethylene spacer was selected to join the dipeptide aldehyde with the thiol-reactive maleinimide group, and the side-chains of Val and Arg were used to occupy the R1 and R2 residues, respectively, to address more specifically the β2 substrate-binding cleft (see Figure 5). Indeed, selective inhibition of the trypsin-like proteasomal activity with sub-micromolar affinity was achieved. X-ray structure of yeast proteasome-inhibitor adduct confirmed the covalent attachment to the Cys118 thiol group and exclusive hemiacetal formation with the β2 subunit active-site Thr1O$^{\gamma}$ [16]. Although this synthetic small molecule represents a successful example of the superb opportunities offered by crystal structures for the design of selective inhibitors, it remains an academic exercise as it cannot be applied to *in vivo* studies because of the high reactivity of the maleinimide function towards glutathione present at high concentration in cells.

Homo- and Heterobivalent Proteasome Inhibitors

By mimicking nature that ubiquitously exploits the principle of polyvalency to increase binding affinity and selectivity, multivalent binding has become an emerging strategy in drug design [59]. The advantages of such strategy result mainly from the entropic benefit, since the penalty for the loss of overall rotational and translational entropy is paid only once for multivalent ligands, rather than for each of the monovalent binding events.

Based on the assumption that the principle of polyvalency could well represent an alternative approach suited for increasing active-site selectivity of proteasome inhibitors, simple tripeptide aldehydes were used as model inhibitors [57]. For a review, see Ref. 60. From crystallographic analysis of the yeast 20S proteasome, a clear picture of the spatial display of the active centres on the two β rings was obtained with the tripeptide aldehyde Ac-Leu-Leu-Nle-H covalently linked as hemiacetals to the Thr1O$^{\gamma}$ of all six active sites (Figure 1). The X-ray coordinates allow to extract distances between the *N*-terminal Thr1 residues of the various active sites

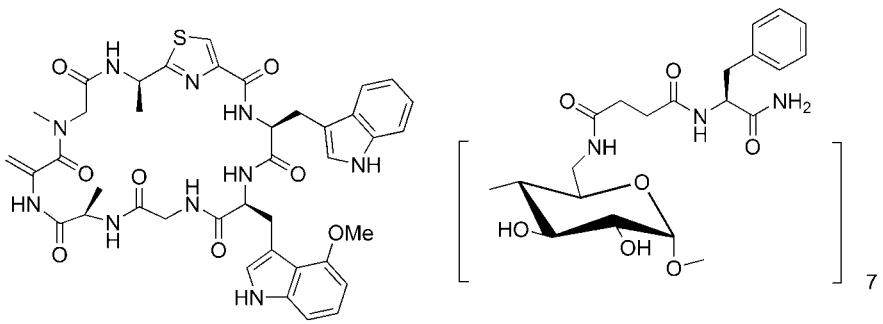

Figure 4. Structure of (**A**) the cyclic octapeptide argyrin A from the myxobacterium *Archangium gephyra* [55], and (B) the synthetic heptakis-[6-(succinylphenylalanine-amide)amido-6-deoxy]β-cyclodextrin.

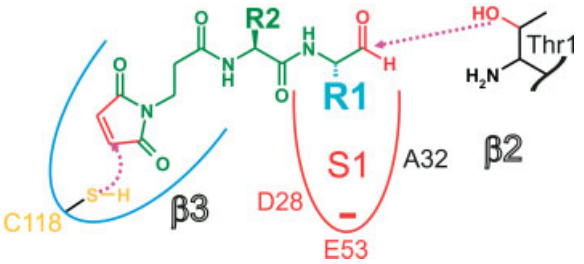

Figure 5. Schematic representation of the S subsites of the $\beta 2$ active site of the yeast 20S proteasome as structural model for the design of maleoyl-β-alanyl-dipeptide aldehydes as a new type of selective heterobifunctional inhibitors.

located on one ring or on the two staggered β rings (Figure 6) for the design of potential bivalent inhibitors that address adjacent active sites on a β ring (transannular) or on the two associated β rings (interannular).

First attempts to bridge the transannular 28 Å distance between the $\beta 1$-$\beta 2$ and $\beta 1'$-$\beta 2'$ active sites and to present, as required, the interacting head groups from both the primed and non-primed substrate-binding site have failed [57]. Thus, the main efforts were focused on interannular bivalent inhibitors which foresee a bridging of about 50 Å distances to allow for an access of two anchor groups to two identical or different active sites from the non-primed enzyme subsites. As proteasomes accept for digestion only fully unfolded linear polypeptides coming through the restricted bottle-neck of the α ring from the outside, linear polyoxyethylene (PEG) chains were selected as mimic of random-coiled polypeptide chains [57,61]. The pegylated tripeptide aldehydes $(PEG)_{19-25}$-Leu-Leu-Nle-H (**2**) and $(PEG)_{19-25}$-Arg-Val-Arg-H (**5**) were found to inhibit the proteasome with almost identical potency as the acetylated tripeptide aldehydes **1** and **4**, respectively (Table 1). Based on this observation, two interannular homobivalent inhibitors containing the tripeptide aldehydes -Leu-Leu-Nle-H (**3**) and -Arg-Val-Arg-H (**6**) as head groups for the $\beta 5/\beta 5'$ and $\beta 2/\beta 2'$ active-site pairs, respectively, were synthesized using a PEG spacer with a statistical distribution of 19–25 monomers, and thus, averaging a length of about 50 Å (Figure 6). The two homobivalent inhibitors inhibit in highly selective manner the chymotrypsin- ($\beta 5$) and trypsin-like ($\beta 2$) activities, respectively (Table 1). To examine the possibility of inhibiting simultaneously both the chymotrypsin- and trypsin-like activities, a heterobivalent inhibitor was synthesized containing the tripeptide aldehydes -Leu-Leu-Nle-H and -Arg-Val-Arg-H (**7**) as head groups. As expected, both proteasomal activities were inhibited with very similar potencies as those of the homobivalent inhibitors if the stoichiometry of this type of inhibitor is taken into account.

For bivalent ligands that in terms of entropy are optimally designed, exponentially enhanced binding affinities are expected. In the present case, however, an increase in potency by two orders of magnitude compared to the monovalent inhibitors was achieved. The relatively small but still significant gain in free energy of binding by the bivalent inhibitors has to be attributed to the high degree of flexibility of the spacer, and thus, to the loss of conformational entropy associated with the bidentated interaction [62,63].

As expected from the crystallographic analysis of proteasome/calpain I inhibitor complex [13], in the crystal structure of the bivalent inhibitor **3**/proteasome complex, the tripeptide moieties were found to occupy all six proteasomal active centres despite the very low inhibition of the trypsin- and caspase-like activities ($IC_{50} < 100$ µM) [57]. In contrast, the bivalent inhibitor **6** containing the tripeptide aldehyde -Arg-Val-Arg-H was detected only in the two trypsin-like $\beta 2$ and $\beta 2'$ centres, confirming the high degree of selectivity achieved with this ligand. As PEG components of various sizes are commonly used in transfection reagents [64,65] the bioavailability of such PEG-linked bivalent inhibitors should be sufficiently retained for *in vivo* applications.

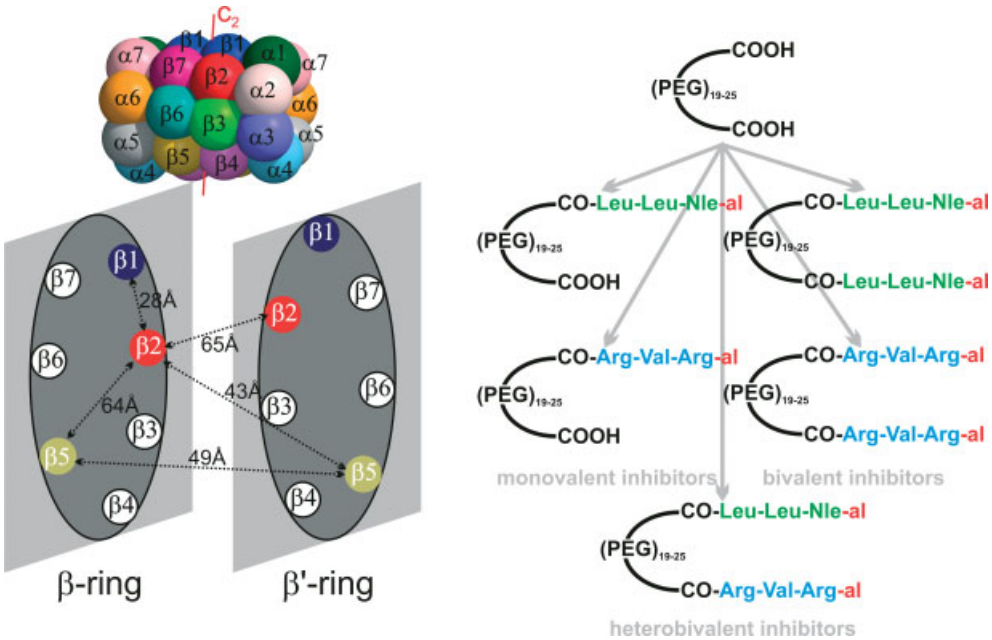

Figure 6. Schematic representation of the two central β rings of yeast 20S proteasome with the trans- and interannular distances between the Thr1 residues of the active sites.

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Table 1. Inhibition of 20S proteasome by mono- and bivalent inhibitors (IC_{50}, μM)

Inhibitor		Caspase-like	Trypsin-like	Chymotrypsin-like
Ac-Leu-Leu-Nle-H	(**1**)	<100	<100	2.1
CO-Leu-Leu-Nle-H | PEG-COOH	(**2**)	<100	<100	1.8
CO-Leu-Leu-Nle-H | PEG-CO-Leu-Leu-Nle-H	(**3**)	<100	<100	0.017
Ac-Arg-Val-Arg-H	(**4**)	<100	6.4	<100
CO-Arg-Val-Arg-H | PEG-COOH	(**5**)	<100	8.2	<100
CO-Arg-Val-Arg-H | PEG-CO-Arg-Val-Arg-H	(**6**)	<100	0.071	<100
CO-Leu-Leu-Nle-H | PEG-CO-Arg-Val-Arg-H	(**7**)	<100	0.097	0.031

Perspectives

A wide spectrum of different natural and synthetic proteasome inhibitors have been identified in the past years. Their exact modes of action as well as their binding characteristics to the proteasomal active sites are still to be clarified. For this purpose, the development of more appropriate fluorogenic substrates than those presently available and that bind to both the primed and non-primed sites to better mimic the endoproteolytic activities of proteasome are urgently required for a better assessment of active-site specificities of newly developed inhibitors. Crystal structure analysis of proteasome-ligand complexes significantly contributed to the recent progress in understanding the docking of proteasome ligands to their target molecule and mechanism of inhibition. It has been discovered that particularly natural compounds follow unique mechanisms of covalent or non-covalent binding to the proteasomal active centres, which can now be exploited for the design of more specific and selective proteasome inhibitors. The development of BIA-1a and BIA-2a inhibitors on the basis of the natural compound, TMC-95A, represents a first example of such inhibitor design. Combining the elegance of natural optimized inhibitors with the principle of polyvalency could well open new perspectives and opportunities in this challenging and therapeutically promising field of biomedical research.

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