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Advances in and applications of proteasome inhibitors

Bradley S Moore[1,2], Alessandra S Eustáquio[1] and Ryan P McGlinchey[1]

With the recent US Food and Drug Administration approval of bortezomib (Velcade®) for the treatment of relapsed multiple myeloma, the proteasome has emerged as a new therapeutic target with diverse pathology. Drug discovery programs in academia and the pharmaceutical industry have developed a range of low nanomolar synthetic and natural inhibitors of the 20S proteasome core particle that have entered human clinical trials as significant anti-cancer and anti-inflammatory leads. Moreover, proteasome inhibitors continue to serve as valuable research tools in cellular biology through the elucidation of important biological processes associated with the ubiquitin–proteasome pathway of protein degradation. This review will highlight recent advances in the development and application of proteasome inhibitors.

Addresses
[1] Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093, United States
[2] Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, CA 92093, United States

Corresponding author: Moore, Bradley S (bsmoore@ucsd.edu)

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Introduction

The ubiquitin–proteasome pathway in eukaryotes regulates many normal cellular processes including signal transduction, cell cycle control, transcriptional regulation, inflammation, and apoptosis through protein degradation and the maintenance of protein homeostasis [1,2,3•]. This primary route of regulated proteolysis of bulk and misfolded protein in mammalian cells is strictly controlled by the 26S proteasome complex, which recognizes polyubiquitinated proteins marked for elimination by the E1, E2, and E3 ubiquitinating enzymes (Figure 1). Upon recognition, unfolding and transfer of the de-ubiquitinated target protein by the 19S regulatory cap into the interior of the cylindrical 20S proteasome core particle, protein degradation is facilitated by catalytic β-subunits having nucleophilic N-terminal threonine (Thr1) residues. Although eukaryotic 20S proteasomes harbor seven different β-subunits in their twofold symmetrical $\alpha_7\beta_7\beta_7\alpha_7$ stacked complexes, only three β-subunits per β-ring [subunits β1 (caspase-like), β2 (trypsin-like), and β5 (chymotrypsin-like)] are proteolytically active (Figure 1). The disruption of this degradative process with small molecule inhibitors against one or more catalytic β-subunit has implications in a number of human diseases such as cancer, inflammation, and ischemic stroke and has exposed the proteasome as an important therapeutic target [4–7].

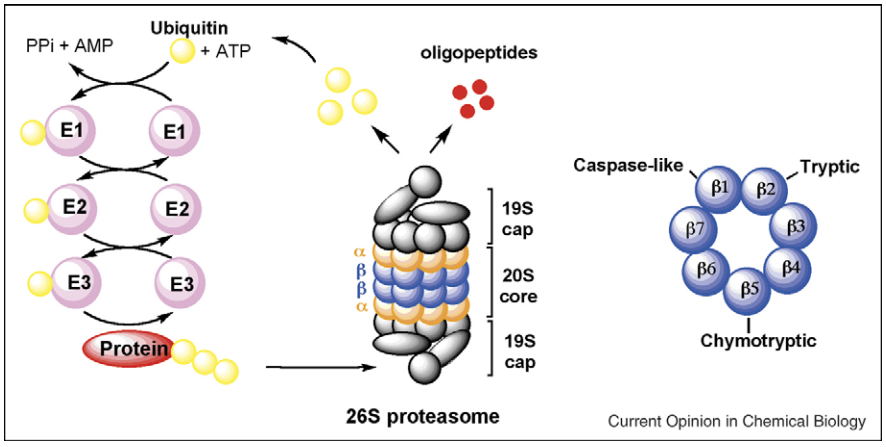
Chemical classes of proteasome inhibitors

The nucleophilic character of the proteasome is governed by the active site Thr1 residue of each catalytic β-subunit in which the side chain hydroxyl group reacts with peptide bonds of substrates as well as with electrophilic functional groups of inhibitors. Selectivity is dictated by the composition of the substrate binding pockets (termed S1, S2, S*n* and S1′, S2′, S*n*′ depending on proximity to the active site), which differs in the three catalytic β-subunits. A wide range of specific inhibitors has been developed as mechanism-based synthetic peptidyl electrophiles and natural products with IC_{50} values in the low nanomolar range [8••].

Tripeptide aldehydes such as the calpain inhibitor I (Ac-Leu-Leu-nLeu-al) and actinomycete natural product leupeptin (Ac-Leu-Leu-Arg-al) were the first class of inhibitors to probe the biochemistry of the proteasome active sites [9] and reveal that the proteasome belongs to a novel class of N-terminal threonine proteases [10]. While the peptide aldehydes form reversible covalent hemiacetal intermediates with Thr1O^{γ} primarily of the β5-subunit, their moderate reactivity (low micromolar) and lack of *in vivo* specificity (also inhibit serine and cysteine proteases) led to the exploitation of other binding head groups with greater potency and selectivity. Diverse functional groups such as vinyl sulfones [11], boronates [12] and natural product-based α′,β′-epoxyketones [13] were explored and provided a number of important leads.

Peptide boronates, which are aldehyde surrogates, are much more reactive with subnanomolar potency and are selective towards the proteasome over common proteases [12]. Owing to their high selectivity, potency and low dissociation rates, the peptide boronates are ideal candidates for drug development, and many analogs have been prepared and evaluated. The dipeptide boronic acid bortezomib (Velcade®, PS-341) (Figure 2), a reversible inhibitor of the β5-subunit, is the first in class proteasome inhibitor approved by the US Food and Drug Administration for the treatment of relapsed multiple myeloma

Figure 1



The ubiquitin–proteasome pathway. Intracellular proteins (e.g. phosphorylated through signaling pathways or misfolded) are targeted for degradation by a covalently bound ubiquitin tag [2]. Ubiquitylation is catalyzed by a multi-enzymatic system consisting of E1 (ubiquitin-activating enzyme), E2 (conjugating) and E3 (ligases). The 26S proteasome is an ATP-dependent protease complex comprised of a 20S catalytic core (organized into four stacked rings of seven subunits each, i.e. $\alpha_7\beta_7\beta_7\alpha_7$) and two 19S regulatory caps. 19S caps are responsible for recognition of ubiquitinylated proteins (lid) and also contain ATPase activity (base) required for linearization of large proteins facilitating their entry in the catalytic core. Three β-subunits (β1, β2, β5) are responsible for the different enzymatic activities of the proteasome and are differentially inhibited by small molecule inhibitors such as bortezomib and salinosporamide A. Figure adapted from ref. [42].

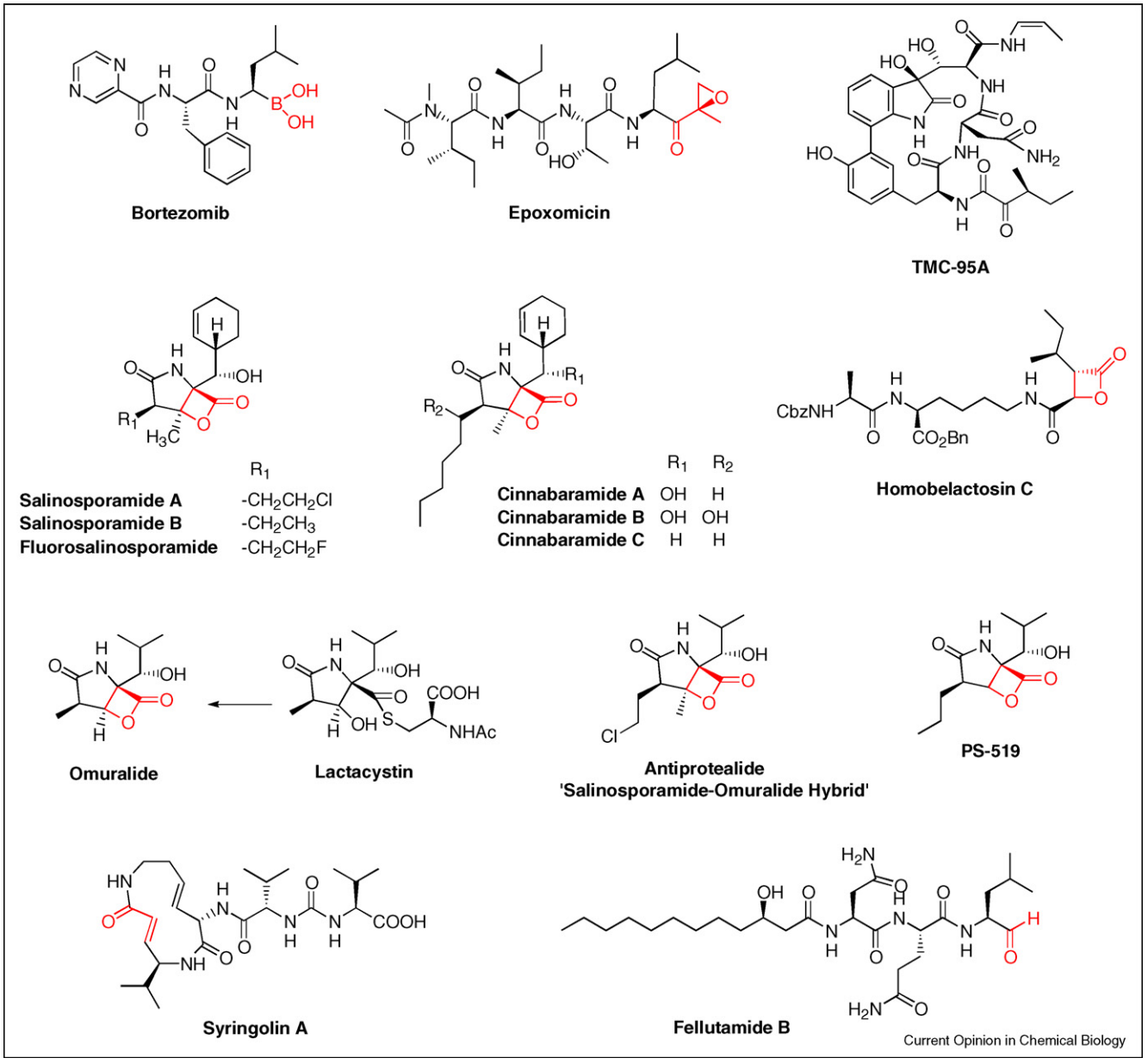
and mantle cell lymphoma [14]. Recently, the boronate derivative CEP-18770 harboring a threonine residue was advanced to preclinical development owing to its oral bioavailability and bortezomib-like pharmacology [15,16].

Irreversible non-aldehydic peptide inhibitors include the vinyl sulfones and the α′,β′-epoxyketones. While synthetic vinyl sulfones suffer from a lack of specificity, natural epoxyketone peptides such as epoxomicin (Figure 2) [13] are highly selective, potent and irreversible inhibitors of the proteasome. Numerous peptidyl epoxyketones bearing various chain lengths of acylated di- to tetra-peptides have been characterized from actinomycetes that primarily interact with the β5-subunit. Epoxomicin has a distinct mechanism of action in which the inhibitor forms a unique morpholino ring system between the epoxyketone functional group and Thr1 [17]. The significance of this mechanism provides epoxomicin's unique specificity for the proteasome, since other proteases do not have an N-terminal nucleophilic residue as part of their active sites. Hence, epoxomycin is unable to form the same stabilized morpholino adduct with proteases as it does with the proteasome. A synthetic analog of epoxomicin, PR-171 [18], which irreversibly inhibits the β5-subunit of the proteasome, is currently in phase I human clinical trials for the treatment of multiple myeloma and non-Hodgkin's lymphoma.

Since the discovery of bortezomib resistance in multiple myeloma cells, the natural product β-lactones have gained widespread attention as second-generation drug candidates. The streptomycete metabolite lactacystin was the first natural non-peptidic proteasome inhibitor [19]. Its low nanomolar reactivity toward the proteasomal β5-subunit is dependent on its transformation to *clasto*-lactacystin-β-lactone (omuralide) with concomitant loss of *N*-acetylcysteine (Figure 2) [20]. Nucleophilic attack of $Thr1O^{\gamma}$ on the β-lactone functional group generates a stable covalent adduct. Crystallization studies revealed that the side chain residues of omuralide play a significant role in the selectivity of the inhibitor to the proteasome and that they were important for prolonging its non-covalent binding in the active site to allow for covalent capture owing to its less reactive β-lactone functional group. The most clinically advanced lactacystin analog is PS-519 [21], a variant that features an *n*-propyl substitution at C7 (Figure 2). PS-519 is more potent than the natural product and is currently in clinical trials for acute stroke.

Recently new natural products related to omuralide that share its γ-lactam-β-lactone core yet have distinct substitution patterns have been discovered from actinomycetes with enhanced potency and selectivity (Figure 2). Salinosporamide A (NPI-0052) from the marine actinomycete *Salinispora tropica* [22,23] is currently in phase I human clinical trials for the treatment of multiple myeloma and other cancers. Studies of this natural product have shown enhanced potency over omuralide against the chymotrypsin-like proteasome activity (IC_{50} values of 1.3 nM versus 49 nM, respectively [22]) as well as extended activity against the other catalytic subunits. The deschloro analog salinosporamide B [24], which is

Figure 2

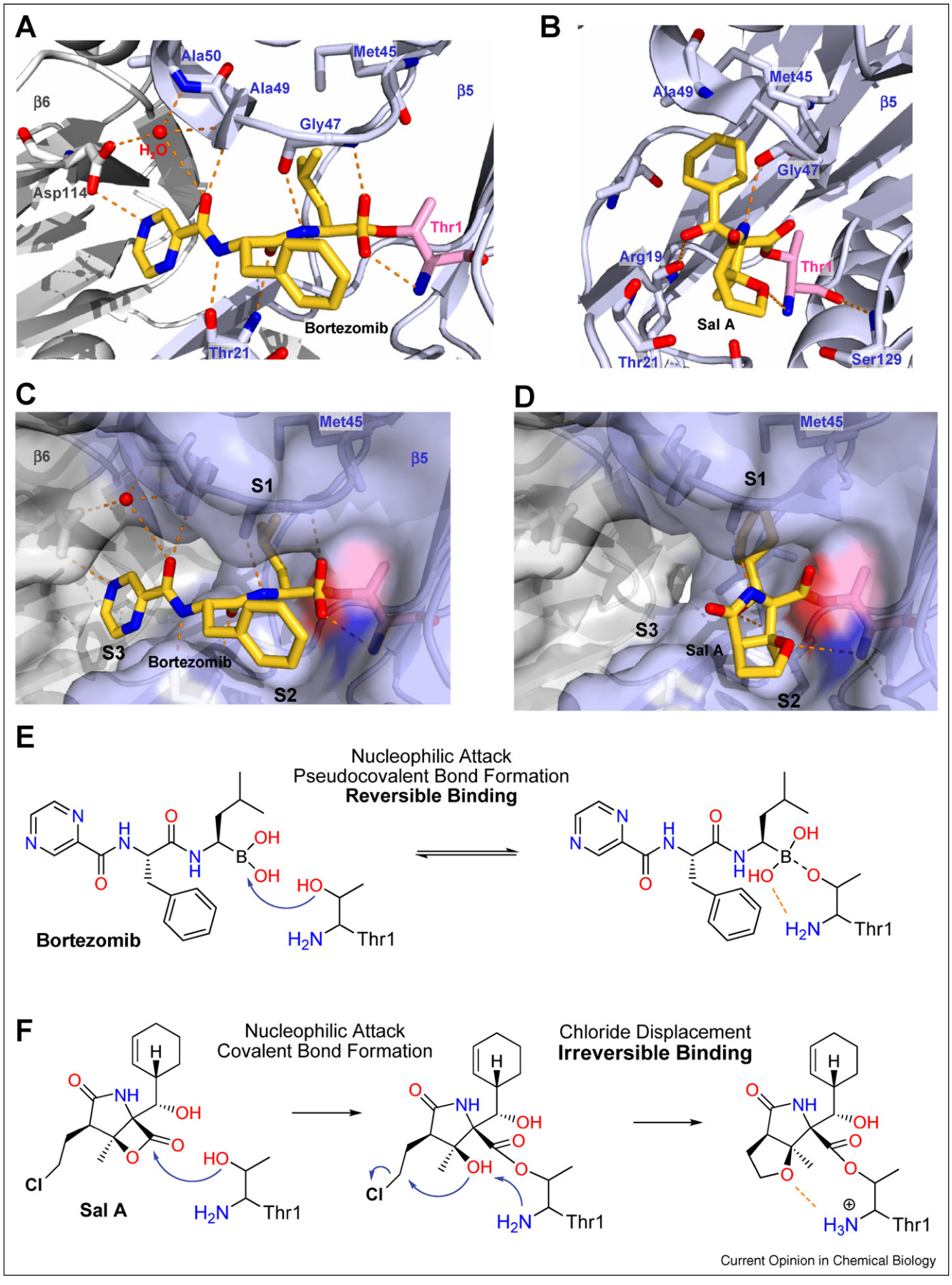


Representative structures of proteasome inhibitors. Electrophilic functional groups are depicted in red. See text for further discussion.

ten times less potent against the proteasome *in vitro*, first suggested that the chloro substituent in salinosporamide A is mechanistically important as later demonstrated structurally in complex with the yeast 20S proteasome (see next section). Other potent analogs of salinosporamide A include the synthetic salinosporamide-omuralide hybrid 'antiprotealide' [25,26•] and the bioengineered product fluorosalinosporamide [27], which is the most potent salinoporamide analog showing reversible binding activity. The most recent additions to this structural class

(Figure 3 Legend) Inhibitory mechanism of synthetic dipeptide boronic acid bortezomib and β-lactone natural product salinosporamide. Close-up view of bortezomib [33••] (**a**) and salinosporamide A [34••] (**b**) bound to Thr1 in the β5 active site of yeast 20S proteasome (PDB codes 2F16 and 2FAK, respectively). Hydrogen bonds are depicted as orange dashes and waters as red dots. (**c, d**) Surface representation showing the same view for each inhibitor as well as occupancy in specificity pockets S1, S2 and S3. (**e**) Reversible inhibitory mechanism of bortezomib is dictated by the boronic acid group [43]. (**f**) Chlorine is key for irreversible binding of salinosporamide A [34••]. Panels (**a**–**d**) were generated with PyMol [44].

Figure 3


A
Ala50
Met45
Ala49
β6
β5
Gly47
H2O
Asp114
Thr1
Bortezomib
Thr21
B
Ala49
Met45
β5
Gly47
Arg19
Thr1
Sal A
Thr21
Ser129
C
Met45
β6
β5
S1
S3
Bortezomib
S2
D
Met45
S1
S3
Sal A
S2
E
Nucleophilic Attack
Pseudocovalent Bond Formation
Reversible Binding
Bortezomib
Thr1
F
Nucleophilic Attack
Covalent Bond Formation
Chloride Displacement
Irreversible Binding
Sal A
Thr1
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are the cinnabaramides, which were isolated from a terrestrial streptomycete [28]. These structural analogs, which only differ from the salinosporamides in the C2 alkyl side chain, have comparable potency *in vitro* with IC_{50} values in the low nanomolar range. It remains, however, to be shown if the cinnabaramides have the same anticancer properties as salinosporamide A.

Further proteasome inhibitors of the β-lactone family include belactosines A and C (Figure 2) from *Streptomyces* sp. UCK14 that selectively inhibit the β5-subunit of the proteasome, with the modified homobelactosin C derivative (Figure 2) having an IC_{50} in the low nanomolar level [29]. Other natural proteasome inhibitors include the TMC-95 family of cyclic peptides from the fungus *Apiospora montagnei* [30], with TMC-95A being the only natural product inhibitor to non-covalently block all active sites of the proteasome selectively and competitively in the low nanomolar range [31].

The majority of the most potent natural proteasome inhibitors are derived from actinobacteria, which are uncommon amongst prokaryotes to synthesize a 20S proteasome complex. The simplified actinobacterial proteasome is composed of identical α and β subunits with an $\alpha_7\beta_7\beta_7\alpha_7$-stoichiometry and no regulatory caps reflective of the absence of ubiquitin in bacteria. While the mechanism for self-resistance in these bacteria that produce proteasome toxins has not yet been clarified, the recent first biosynthetic gene cluster analysis of the natural proteasome inhibitor salinosporamide A revealed an associated β-subunit that may be involved with resistance [32]. It will be intriguing to learn if other biosynthetic gene clusters associated with actinomycete proteasome inhibitors also harbor proteasome β-subunits, and if so, whether this genetic signature may enable the discovery of new inhibitor classes.

Molecular mechanism of action

High-resolution crystal structures of the 20S proteasome (mainly from yeast) in complex with all of the major inhibitors have been solved by Groll and coworker [8••]. These analyses illuminated their binding mode and mechanism of action at the molecular level and have been instrumental in the structure-based design of new inhibitors. Most proteasome inhibitors bind covalently to the catalytic Thr1 residue in the β5-subunit with the exception of the cyclic peptide TMC-95, which shows noncovalent binding in each catalytic subunit. Recent crystal structures of the yeast 20S proteasome with bound bortezomib [33••] and salinosporamide A [34••] have been reported and illustrate some of the guiding principles in proteasome inhibition (Figure 3).

As opposed to the reversible binding mode of bortezomib, binding of salinosporamide A to the proteasome has been shown to be irreversible [35,36]. Moreover, bortezomib and salinosporamide A differentially affect proteasome activities, that is at low concentrations salinosporamide A preferentially targets the chymotryptic (β5) and tryptic (β2) while bortezomib affects chymotryptic and caspase-like (β1) subunits [35].

The boronic acid moiety of bortezomib forms a (pseudo)-covalent bond to the nucleophilic hydroxyl side chain of Thr1. Further important interactions are summarized in Figure 3a. The inhibitor occupies specificity pockets S1, S2 and S3 (Figure 3c), which differ in charge and overall architecture depending on the subunit in question. Selectivity for the various proteasome active sites is controlled by P1 (leucine boronic acid moiety) and P3 (pyrazine-2-carboxyl group), while P2 (phenylalanine group) makes no contacts with the protein so that S2 pockets in all active sites can accept larger substituents. The leucine side chain induces a fit to Met45 of β5 involved in key proteasome–substrate interactions and the concerted movements generated upon binding allow additional hydrophobic contacts between P1 and S1. By contrast, P1 does not interact with the larger S1 pocket in β2. Furthermore, the S3 pocket of β2 fundamentally differs from β5 explaining bortezomib's lack of tryptic-like inhibitory activity. In case of β1, Asp114 in S3 (Figure 3a) is replaced by a histidine preventing interaction with P3 and vindicating the lower affinity for the caspase-like subunit [33••]. Figure 3e depicts bortezomib's binding mechanism.

As reported for omuralide, salinosporamide A is linked to the Thr1-hydroxyl of proteasome active sites by an ester bond with the carbonyl carbon of the β-lactone [34••] (Figure 3b). However, while omuralide occupies only β5 subunits, salinosporamide A interacts with all catalytic sites. The flexibility of Met45 (β5) affords accommodation of larger P1 sites (isopropyl in omuralide, and cyclohexenyl ring in salinosporamide A). Furthermore, the bulkier P1 group in salinosporamide A allows for additional hydrophobic interactions, helping explain at least in part the enhanced potency of salinosporamide A over omuralide [22,34••], and also the affinity to β2 which presents a larger S1 pocket, consistent to salinosporamide A's inhibition of tryptic activity as opposed to bortezomib [33••]. As shown in Figure 3d, the rather small β-lactone inhibitor occupies only specificity pockets S1 and S2. Yet, it represents an equipotent antitumor agent compared to bortezomib [36].

As mentioned for bortezomib, the P2 group projects into empty space. Therefore there is sufficient space to accommodate larger side chains as exemplified by the cinnabaramides [28]. Most important, P2 of β-lactone inhibitors appears to be fundamental in determining if binding is reversible or irreversible. Although omuralide has been reported to bind to the proteasome irreversibly

[20], based on a synthetic analog, binding of omuralide and of the deschloro analog salinosporamide B should be slowly reversible [34••]. After salinosporamide A becomes covalently tethered to Thr1, the resulting C3 hydroxyl displaces the C13 chlorine to yield an irreversibly bound adduct, since the newly formed tetrahydrofuran ring (i) blocks water attack on the ester bond preventing hydrolysis, (ii) engages C3O and circumvents reformation of the β-lactone, and (iii) the resulting protonated state of Thr1NH_2 results in inactivation of its catalytic activity (Figure 3b,f).

Therapeutic outlook

Proteasome inhibitors have been instrumental to our fundamental understanding and appreciation of the ubiquitin–proteasome system and are now rapidly emerging as important new treatment options in cancer. A new generation of proteasome inhibitors headed by salinosporamide A and PR-171 are presently being evaluated clinically and may offer alternative treatment to patients intolerant or whose disease is refractory to bortezomib. Comparative preclinical studies of these irreversible inhibitors as single agents suggest reduced toxicity and improved pathology [37,38], while combination therapy of salinosporamide A and bortezomib affords synergistic anti-multiple myeloma activity at reduced doses without the toxicity and resistance attributed to bortezomib alone [39]. The landscape of proteasome inhibitor-based therapeutics is quickly evolving with promise in other diseases beyond clinical oncology and represents an exciting example of translational medicine.

Primary resistance, as exemplified by bortezomid's ineffectiveness against some solid tumors, as well as acquired resistance may represent future hurdles for the wider applicability of proteasome inhibitors [5]. Therefore, further studies aimed to understand underlying mechanisms as well as the development of second-generation drugs are imperative. In this context, new proteasome inhibitors were reported during the production of this article. The plant pathogen virulence factor syringolin A from *Pseudomonas syringae* pv. *syringae* shows a novel mechanism of covalent binding to the proteasome representing a new class of inhibitors containing a reactive α,β-unsaturated carbonyl group that also includes glidobactin A (Figure 2) [40••]. Moreover, the fungal peptide aldehyde fellutamide B, a known inducer of nerve growth factor (NGF), was reported to inhibit the proteasome [41••]. The authors also show that other proteasome inhibitors induce production and secretion of NFG, suggesting that targeting the proteasome may aid in the treatment of neurodegenerative diseases. Together, these recent additions provide further examples of proteasome inhibition in nature as well as emphasize the vast therapeutic potential of small molecule proteasome inhibitors.

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