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Chemical Tools To Study the Proteasome

Martijn Verdoes,[a] Bogdan I. Florea,[a] Gijsbert A. van der Marel,[a] and Herman S. Overkleeft*[a]

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Proteolysis, or the processing and degradation of proteins, has emerged as one of the most widely studied processes in biology today. Long viewed as a dead end process, of importance only for the removal of obsolete peptides and proteins, proteolytic events are now associated with numerous biological events. The main proteolytic pathway in the eukaryotic cytoplasm and nucleus, responsible for the degradation of 80–90 % of all cellular proteins is known as the Ubiquitin Proteasome System (UPS). Proteasomes are the central proteases in this tightly controlled ATP- and ubiquitin-dependent proteolytic pathway. Proteasomes are multicatalytic, compartmentalized proteinase complexes. Their substrates include abnormal and damaged proteins, cell-cycle regulators, oncogens and tumor suppressors. Furthermore, proteasomal degradation is imperative for the generation of MHC class I antigenic peptides. The recent approval of a proteasome inhibitor as a cancer drug has boosted proteasome research. This microreview highlights the recent advances in the development of chemical tools to study proteasome activity.

1 Introduction

1.1 The Ubiquitin Proteasome System

The turnover of the majority of cellular proteins is controlled by the Ubiquitin Proteasome System (UPS, Figure 1). Being involved in the degradation of key regulatory proteins as well as clearance of misfolded and damaged proteins, the UPS plays a role in many cellular processes, such as cell-cycle control, differentiation, apoptosis, transcription processes and immune response. Poly-ubiquitination is a substrate's "molecular kiss of death", marking the protein for proteasomal degradation.[1] Ubiquitin, a 9 kDa, 76-residue protein is attached via its glycine-glycine *C*-terminus to a substrate. A cascade of enzymes is responsible for this process. First, ubiquitin is activated in an ATP-dependent manner by the ubiquitin-activating enzyme E1 through adenylation of the *C*-terminus. Subsequent nucleophilic attack of the E1 active site cysteine on the activated ubiquitin results in a thioester linkage and liberates adenosine monophosphate (AMP). Before transthioesterification of ubiquitin to an ubiquitin-conjugating enzyme E2, a second ubiquitin is bound in the adenylation site of E1, thereby increasing the affinity for E2.[2] The E3 ubiquitin ligase enzymes facilitate the transfer of ubiquitin to a lysine residue in the substrate to form an isopeptide linkage. Two independent mechanisms are discerned. In the first, the E3 binds a substrate and an ubiquitin carrying E2 simultaneously, after which the E2 transfers the ubiquitin to the substrate. In the second mechanism, the E3 binds a charged E2 alone and ubiquitin is first transferred to the E3 via a transthioesterification. The ubiquitin carrying E3 then binds a substrate to transfer the ubiquitin. This highly controlled multistep mechanism involves only two E1 enzymes,[3] a large family of E2 enzymes and an even larger set of E3 enzymes. Of these, it appears that the E3 ligases confer selectivity in ubiquitin-mediated protein degradation processes.[1] A substrate can be rescued from degradation after cleavage of the ubiquitin by so-called deubiquitinating enzymes (DUBs),[4] introducing an even higher degree of control. Poly-ubiquitin chains linked via the *C*-terminus to the side chain of lysine 48 are recognized by the 26S proteasome. During degradation of the substrate, ubiquitin is recycled by deubiquitinating enzymes. A fraction of the generated peptide fragments (between 3 and 25 amino acids, with an average of 8 to 12 amino acids) is trimmed by specific aminopeptidases, translocated into the ER by the Transporter associated with Antigen Presentation (TAP) and loaded on Major Histocompatibility Complex class I (MHC I) molecules.[5,6] The trimeric MHC I epitope complex is then transported to the cell surface to be exposed to the immune surveillance system. The majority of the proteolysis products are further degraded to single amino acids by peptidases to maintain protein homeostasis.

[a] Department of Bio-organic Synthesis, Leiden Institute for Chemistry, Leiden University,
POBox 9502, 2300 RA Leiden, The Netherlands
Fax: +31-71-527-4307
E-mail: h.s.overkleeft@chem.leidenuniv.nl

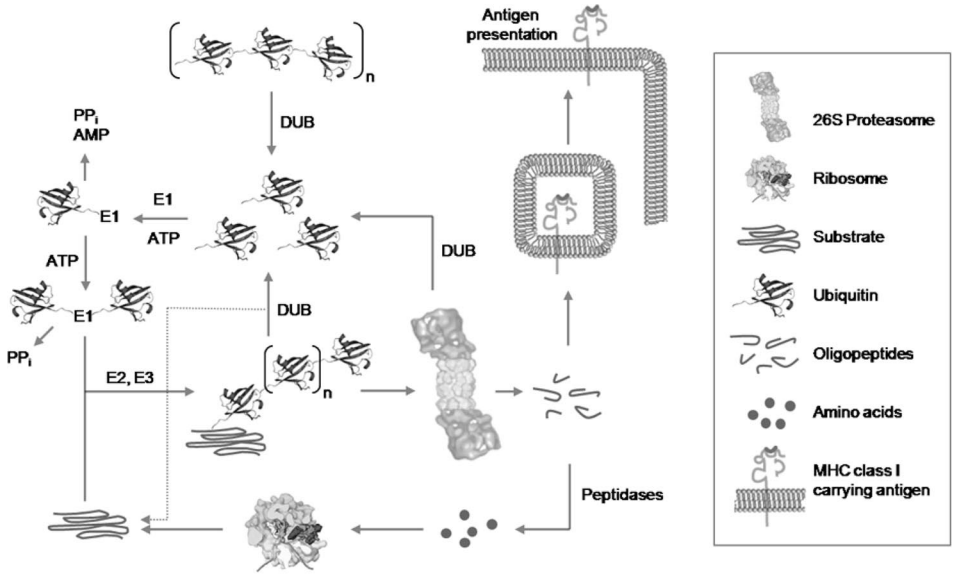

Figure 1. Schematic representation of the Ubiquitin Proteasome System.

1.2 Proteasomes

Proteasomes are highly conserved compartmentalized protease complexes belonging to the family of N-terminal nucleophilic (Ntn) hydrolases.[5,7,8] The 20S proteasome, a proteolytic nanotube, is a 720 kDa cylindrical protein complex composed of four stacked rings. In prokaryotes, the two outer rings consist of seven identical α subunits, sand-

Martijn Verdoes (March 6, 1980) began his education at Leiden University in the group of Jacques van Boom, continued at Harvard Medical School with Hidde Ploegh and received his MSc in chemistry in 2004 at the Leiden University. His Ph.D. research, within the group of Herman Overkleeft, was focused on synthesis of chemical probes to study proteasome activity. He received his Ph.D. degree in December 2008 and recently joined the group of Matthew Bogyo at Stanford University as a postdoctoral fellow.

Bogdan Florea (October 14, 1973) received his Ph.D. degree in 2002 for research on cationic polymers for gene delivery, under guidance of Hans Junginger. He then joined the group of Dik van Gent and Jan Hoeijmakers for a 2 year postdoctoral research on the biochemistry of DNA repair by end joining at the Erasmus Medical Center in Rotterdam. Since November 2004 he works as postdoctoral fellow in the group of Herman Overkleeft and Gijs van der Marel. He runs the proteomics facility of the department and is interested in characterizing interactions between chemical and biological entities at the molecular level.

Gijs van der Marel (April 3, 1952) received his training at Leiden University, where he graduated in 1977. He did his Ph.D. studies on the subject of DNA oligonucleotide synthesis together with Jacques van Boom and received his Ph.D. degree in 1981. He continued his career at Leiden University, first as assistant professor, then as associate professor and, since January 2005, as full Professor in organic synthesis. His research is focused on synthetic aspects of biopolymers, primarily nucleic acids, peptides, and carbohydrates, their hybrid structures, and their synthetic analogues.

Herman Overkleeft (April 12, 1969) received his Ph.D. education at the University of Amsterdam under the guidance of Upendra Pandit. After receiving his Ph.D. degree on the subject of the synthesis and application of iminosugar glycosidase inhibitors (1997), he moved to Leiden University for a two-year postdoctoral research stay in the group of Gijs van der Marel and Jacques van Boom. From 1999 to 2001 he was a postdoctoral fellow at Harvard Medical School, Department of Pathology, where he worked with Hidde Ploegh in the emerging area of chemical biology. In July 2001 he was appointed to the chair in bioorganic chemistry at Leiden University, where he currently is. His research interests include chemical biology, bioorganic chemistry and organic synthesis.

wiching the two inner rings that are build up of seven identical, proteolytically active β subunits. During evolution, these subunits diverged into seven distinct α subunits and seven distinct β subunits, four of which have lost their proteolytic character. Eukaryotic 20S core particles are build up of two rings consisting of seven different α subunits (α1-α7), capping the two inner rings which are build up of seven β subunits (β1-β7) (Figure 2, A). The three remaining proteolytically active β subunits are the β1 (Y/δ), β2 (Z) and β5 (X) subunits (Figure 2, B). The N-terminal threonine (Thr1) represents the proteolytically active residue and acts by nucleophilic attack of the γ-hydroxy on the carbonyl of the peptide bond destined to be cleaved (Figure 2, C). The α-amine of the threonine acts as the base in the catalytic cycle. Site-directed mutagenesis and kinetic studies using fluorogenic substrates revealed subtle differences in substrate specificities.[9] The active site located at the β5 subunit preferably cuts *C*-terminal of hydrophobic residues and is termed "chymotrypsin-like". Having a preference for cleaving after basic amino acids the β2 site is named "trypsin-like", whereas the β1 site cuts preferentially after acidic residues and is called "caspase-like". Despite the subunit preferences suggested by these designations, the proteasome subunits are rather more promiscuous. The proteolytic active sites reside inside the hollow proteasome microenvironment and can only be reached via the pores in the α rings, which are too narrow (10 to 15 Å) for folded proteins to enter.[10]

The 2.5 MDa 26S proteasome is formed when the 20S core particle is capped at either side by the 19S regulatory complex (PA700). The 19S regulatory complex is composed of two functionally different units, the base and the lid. The lid is responsible for substrate recognition and cleavage of the poly-ubiquitin chains. The base is composed of eight subunits, six of which are ATPases, and interacts directly with the α rings, facilitating the energy dependent unfolding of the substrate, opening of the pores in the α rings and translocation of the substrate into the proteolytic chamber of the 20S core particle.

MHC class I antigen presentation is in constant competition with aminopeptidases that degrade the available pool of potential antigenic peptides. To enhance MHC I antigen presentation upon infection, proteasome activity is altered to increase the pool of antigenic peptides.[5,11] Besides being constitutively expressed in immune-competent tissue, interferon-γ (IFN-γ) stimulates the expression of three additional catalytically active β subunits, β1i (Low Molecular weight Protein 2, LMP2), β2i (multicatalytic endopeptidase complex-like-1, MECL1) and β5i (LMP7). These subunits are incorporated in newly formed proteasome particles replacing their constitutive counterparts to form the so-called immunoproteasomes, which coexist next to the constitutive proteasomes. IFN-γ also upregulates the synthesis of the proteasome activator PA28 (11S cap).[12] PA28 binds to the α rings of the proteasome core particles to cause a conformational change in the N-terminal tails of the α subunits, resulting in the opening of the α-annulus, the gateway to the proteolytic chamber.

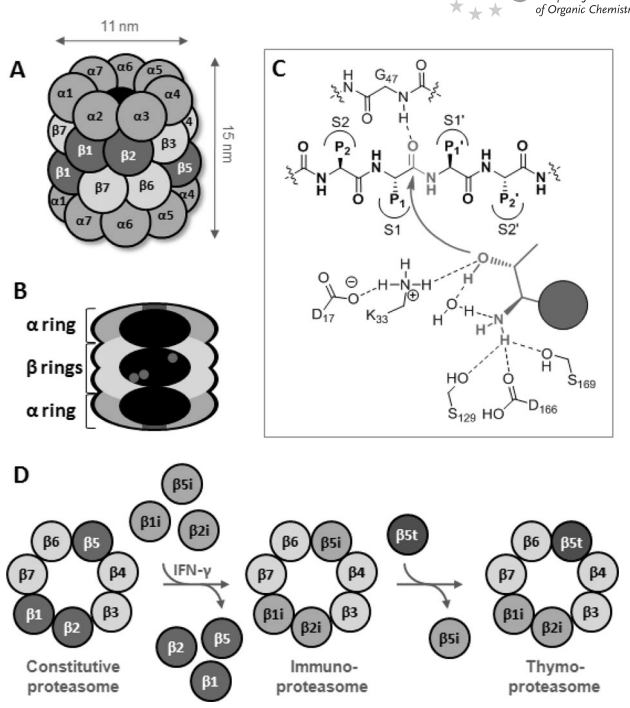

Figure 2. Schematic representation of the composition of the 20S proteasome. (**A**) Subunit composition of the 20S proteasome core particle. (**B**) Cross-section of the 20S proteasome showing the position of the catalytic residues. (**C**) Representation of the catalytic active site. (**D**) Top-view of the β ring of the constitutive proteasome, the immunoproteasome and the thymoproteasome.

Recently, an additional, seventh proteolytically active proteasome β subunit was identified in cortical thymic epithelial cells, which are responsible for the positive selection of developing T cells.[13] This new catalytic β subunit proved to be most closely related to the β5 and β5i subunits and was therefore named thymus specific β5, abbreviated as β5t. In approximately 20% of the 20S proteasome population in the thymus, β5t replaces the β5i subunit in immunoproteasomes to form the thymoproteasome. The chymotrypsin-like activity of thymoproteasomes is reduced by 60 to 70% as compared to the constitutive- and immunoproteasome, without an effect on the trypsin- and caspase-like activities. Although the exact role of β5t remains to be unraveled, it is plausible that compared to the constitutive- and immunoproteasomes, the thymoproteasomes produce low-affinity MHC I ligands rather than high-affinity ligands to support positive T-cell selection.[13]

2 Proteasome Inhibitors

2.1 Natural Product Proteasome Inhibitors

The importance of the proteasome for viability was capitalized upon by several microorganisms, demonstrated by the fact that these organisms produce natural products that are capable of blocking the proteasome. For example, the *Streptomyces* metabolite lactacystin (**1**)[14] is the precursor of the active proteasome inhibitor *clasto*-lactacystin β-lac-

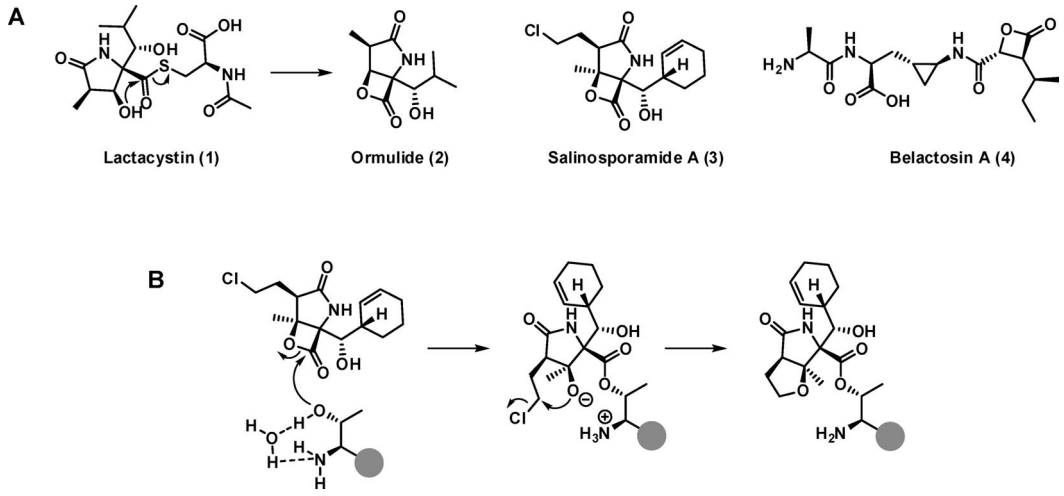

Figure 3. β-Lactone-containing natural products. (**A**) Structures and (**B**) mechanism of inhibition of β-lactone-containing natural products.

tone (**2**),[15,14f] also known as ormulide, which is formed under neutral conditions by lactonization (Figure 3). The structurally related proteasome inhibitor salinosporamide A (**3**, NPI-0052) is produced by *Salinispora tropica*, a marine bacterium which is found in ocean sediment.[16,14f] The mechanism by which these related compounds inhibit the proteasome is through nucleophilic attack of the N-terminal active site threonine of the proteasome on the β-lactone. This results in opening of the lactone and ester bond formation of the inhibitor to the active site threonine γ-hydroxyl.[17] In the case of salinosporamide A (**3**), the resulting oxanion ring-closes to an oxacycle by S_N2 displacement of the chlorine (Figure 3, B).[17,18] The β-lactone containing belactosin A (**4**),[19] isolated from a fermentation broth of *Streptomyces* sp. UCK14 inhibits the proteasome via a mechanism related to lactacystin.[17] Acylation of the proteasome active site threonine by β-lactone inhibitors is reversible, with a half-life of approximately 20 hours.[20]

A search for substances capable of reversing multi-drug resistance in tumor cells resulted in the isolation of the non-covalent proteasome inhibitor agosterol A (**5**, Figure 4) from a marine sponge of *Spongia* sp. collected in Mie Prefecture, Japan.[21] The highly selective, potent and non-covalent proteasome inhibitors TMC-95A-D[22a] {TMC-95A[22b] (**6**) is shown in Figure 4} have been discovered in the fermentation broth of *Apiospora montagnei* sacc. TC 1093. The polyphenol component of green and black tea, (–)-epigallocatechin-3-gallate (**7**) was shown to inhibit the chymotrypsin- and caspase-like activities of the proteasome.[23] Compound **7** reversibly deactivates the proteasome by acylation of the threonine γ-hydroxy as a result of nucleophilic attack on the ester carbonyl and subsequent transesterification.

Eponemycin (**8**, Figure 5, A) was isolated from *Streptomyces hygroscopicus* based on its in-vivo antitumor activity against murine B16 melanoma tumors.[24] The structurally related epoxomicin (**9**) was isolated from an *Actinomycetes* strain and was found to have antineoplastic activity.[25,26] The α′,β′-epoxy ketone "warhead" (the electrophilic moiety that reacts with the active-site nucleophile) containing natural products are highly specific inhibitors of the proteasome due to their unique inhibition mechanism (Figure 5, B).[17,27] First, the ketone is attacked by the N-terminal threonine γ-hydroxy group, just like it would attack the carbonyl of the peptide bond destined for cleavage, to give a reversible hemiacetal linkage. Next, the α-amine attacks the epoxide resulting in the irreversible formation of a very stable morpholine ring.

Strains of the plant pathogen *Pseudomonas syringae* pv. *syringae* secrete syringolin A (**10**, Figure 6, A), causing, for example, brown spot disease on beans.[28] The target of this virulence factor was found to be the proteasome, which is

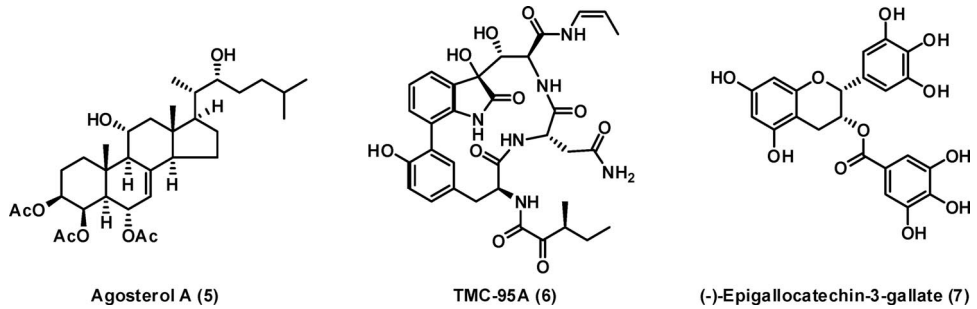

Figure 4. Structures of agosterol A (**5**), TMC-95A (**6**) and (–)-epigallocatechin-3-gallate (**7**).

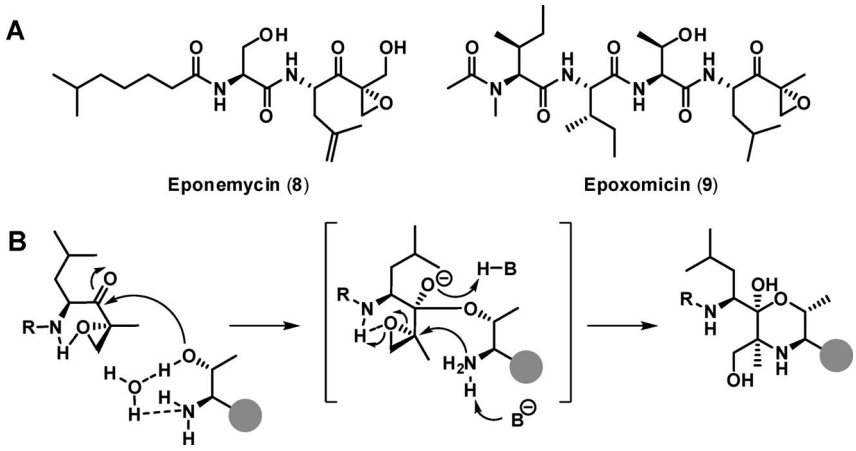

Figure 5. Epoxy ketone-containing natural products. (**A**) Structures and (**B**) mechanism of inhibition of epoxy ketone-containing inhibitors.

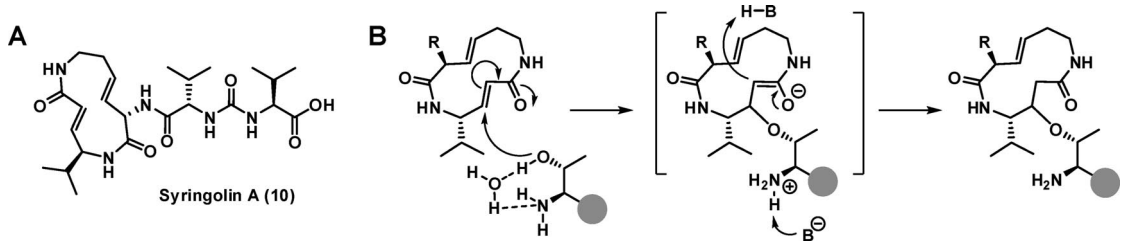

Figure 6. Syringolin A (**10**). (**A**) Structure of syringolin A (**10**) and (**B**) its mechanism of proteasome inhibition.

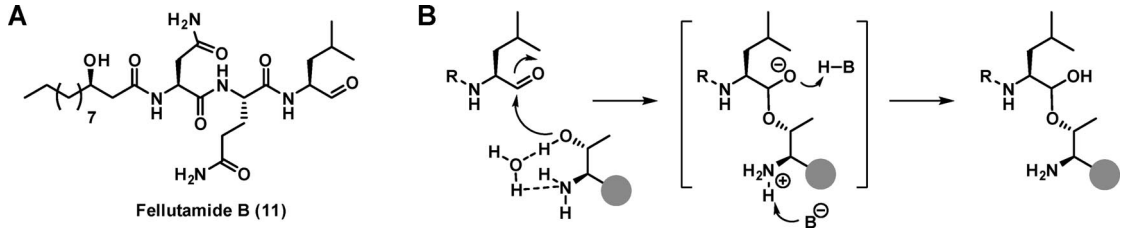

Figure 7. Fellutamide B (**11**). (**A**) Structure of fellutamide B (**11**) and (**B**) mechanism of inhibition of aldehyde-containing proteasome inhibitors.

blocked after Michael addition of the threonine γ-hydroxy on the macrocyclic α,β-unsaturated amide resulting in an irreversible ether bond formation.

Fellutamide B (**11**, Figure 7, A) is a cytotoxic peptide isolated from a marine fish-possessing fungus *Penicillium fellutanum*.[29] Peptide aldehydes such as fellutamide B inhibit the proteasome reversibly by the formation of a hemiacetal bond (Figure 7, B).[17]

2.2 Synthetic Peptide Based Proteasome Inhibitors

The observation that leupeptin (**12**, Ac-LLR-al, Figure 8), an inhibitor of the calcium-dependent, non-lysosomal cysteine protease calpain, inhibited the trypsin-like activity of the proteasome was the starting point of the development of synthetic peptide-based proteasome inhibitors.[30] In a study performed by Vinitsky et al., calpain inhibitor I (**13**, Ac-LLnL-al), calpain inhibitor II (**14**, Ac-LLM-al), Ac-LLF-al (**15**) and the α-keto ester Z-LLF-COOEt (**16**) were shown to inhibit the chymotrypsin-like activity of the proteasome.[31] Compared to its predecessors, Z-LLL-al (**17**, MG132) proved to be a more potent and more selective inhibitor of the proteasome as opposed to calpains and cathepsins,[32] and represents one of the most frequently used proteasome inhibitors to date. Since the development of these initial synthetic proteasome inhibitors, many aldehyde-based peptide inhibitors have been described.[33]

The challenging synthesis of lactacystin (**1**), combined with the fact that less synthetically demanding peptide aldehyde based inhibitors target calpains and cathepsins in addition to the proteasome, led to the development of the first

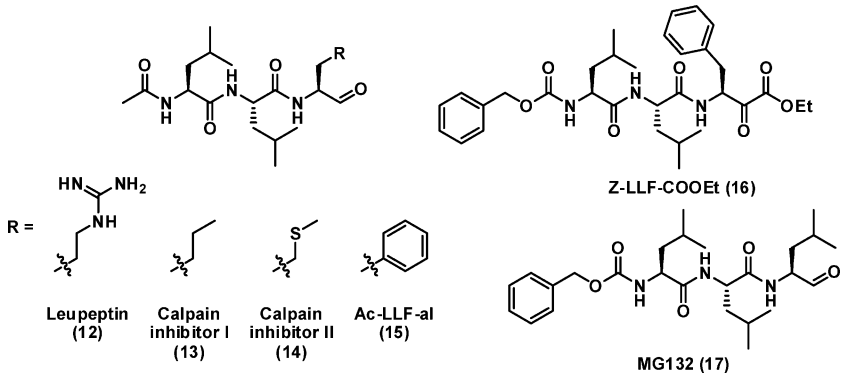

Figure 8. Synthetic peptide-based proteasome inhibitors.

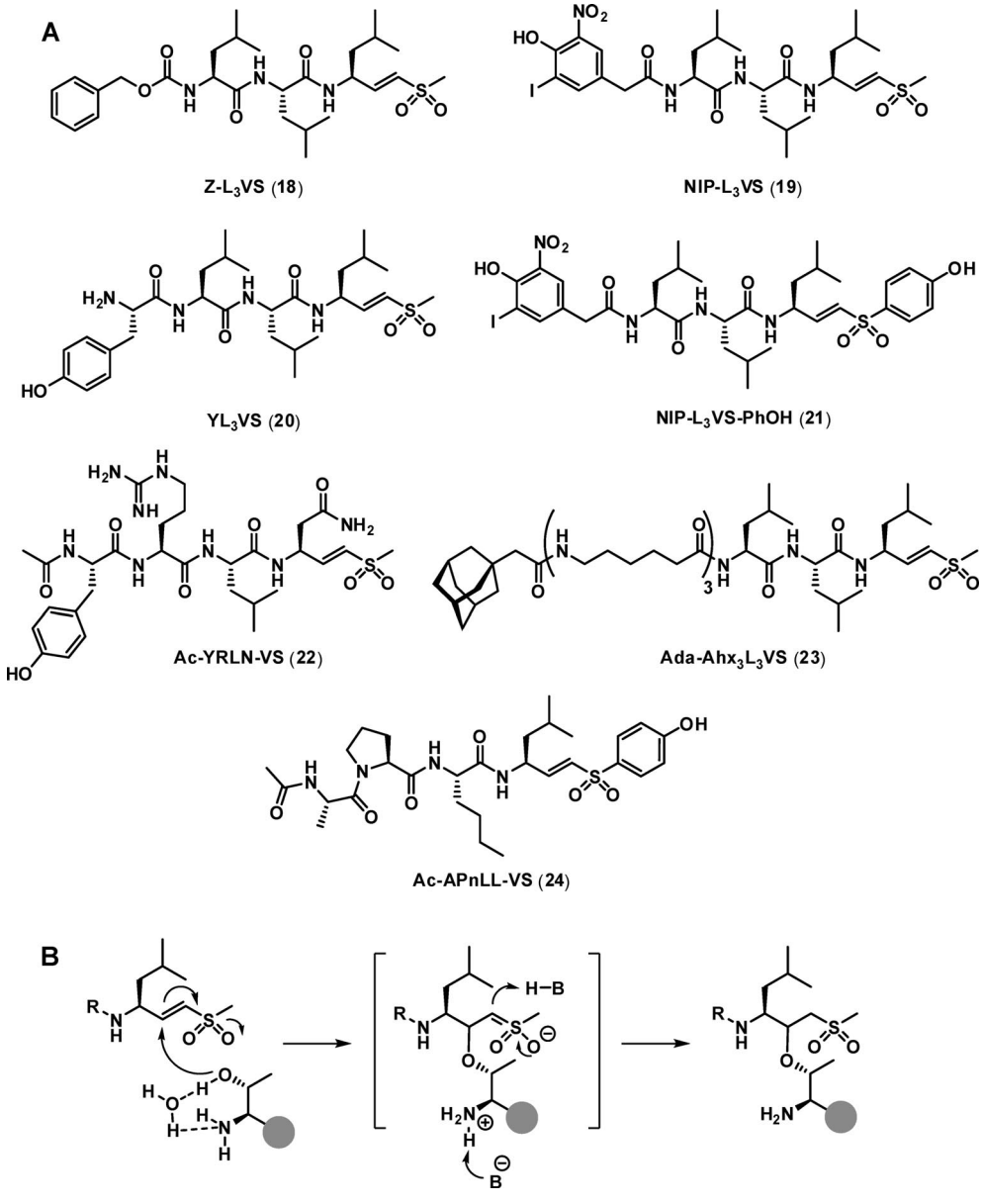

Figure 9. Vinyl sulfone-based proteasome inhibitors. (**A**) Structures and (**B**) mechanism of inhibition of vinyl sulfone-based proteasome inhibitors.

synthetic peptide vinyl sulfone-based proteasome inhibitors (Figure 9, A).[34] Although still targeting cathepsin S to some extend, Z-L_3VS. (**18**) and NIP-L_3VS. (**19**) covalently and irreversibly modified the γ-hydroxy of the N-terminal threonine by ether-bond formation via Michael addition (Figure 9, B).[17] In a later study, Bogyo et al. showed that removal of the N-terminal benzyloxycarbonyl in Z-L_3VS. (**18**) resulted in the loss of inhibitory potency.[35] After installation of a fourth amino acid with an aromatic or aliphatic side chain however, as in YL_3VS. (**20**), the inhibitory potency could be restored to even surpass that of Z-L_3VS. (**18**), with the most pronounced increase of potency observed for the trypsin-like activity.[35] Substitution of the methyl for a phenol in the vinyl sulfone warhead, as in NIP-L_3VS-PhOH (**21**), increased the potency for the caspase-like activity and to a lesser extent for the trypsin-like activity.[35] A P2 to P4 side chain residue positional scanning library of asparagine-derived vinyl sulfone-based proteasome inhibitors resulted in the β2 selective inhibitor Ac-YRLN-VS. (**22**).[36] The hypothesis that extended versions of the peptide-based vinyl sulfone inhibitors would be better mimics of natural proteasome substrates led to the synthesis of AdaAhx$_3$$L_3$VS. (**23**).[37] Indeed, this inhibitor proved to be more potent and pan-reactive towards the proteolytically active β subunits of the constitutive proteasome in living cells in comparison with, for example, Z-L_3VS. (**18**). Efforts to develop subunit specific proteasome inhibitors resulted in the β1 and β1i selective phenolic vinyl sulfone proteasome inhibitor **24**.[38]

Iqbal et al. described the derivatization of their previously developed peptide aldehyde proteasome inhibitor **25** into the corresponding α-ketoamide **26** and boronic ester **27** (Figure 10, A).[39] The latter proved to be a very potent proteasome inhibitor and represents the first of a new generation of inhibitors armed with a boronate-derived warhead. Soon thereafter, the boronic acid analogue of Z-LLL-al (**17**), MG262 (**28**) proved to be more than a 100-fold more potent then the parent compound MG132 (**17**).[40] In an effort to reduce the molecular weight and to simplify the synthesis, the sub-nanomolar dipeptidyl boronic acid proteasome inhibitor **29** (PS-341) was developed.[40] Despite the fact that peptidyl boronic acids have been described as inhibitors of serine proteases,[41] PS-341 (**29**) exhibits a very high selectivity over common serine proteases for the proteasome. Interestingly, PS-341 (**29**) was shown to bind β1, β5, β1i and β5i exclusively at therapeutic concentrations, leaving β2 and β2i untouched.[42] The mechanism of reversible inhibition entails the formation of a tetrahedral adduct with the γ-hydroxy of the active site threonine (Figure 10, B).[17,43]

The structure of the peptide-based proteasome inhibitors described above can be divided into three elements, being 1) the warhead, the electrophilic trap that reacts with the active-site nucleophilic residue, 2) the peptidic recognition element that serves as the homing sequence and 3) the N-terminal extension. Interchanging these structural elements of four commonly used proteasome inhibitors, Z-L_3VS. (**18**), AdaAhx$_3$$L_3$VS. (**23**), epoxomicin (**9**) and PS-341 (**29**), afforded a 15 member structural hybrid library. The epoxomicin-derived boronate **30** (Figure 10) proved to be the most potent pan-reactive proteasome inhibitor of this hybrid library and represents one of the most potent peptide-based proteasome inhibitor reported to date.[44]

Several synthetic analogues of the highly selective proteasome inhibitors eponemycin (**8**) and epoxomicin (**9**) have been synthesized (Figure 11). A structural hybrid library of

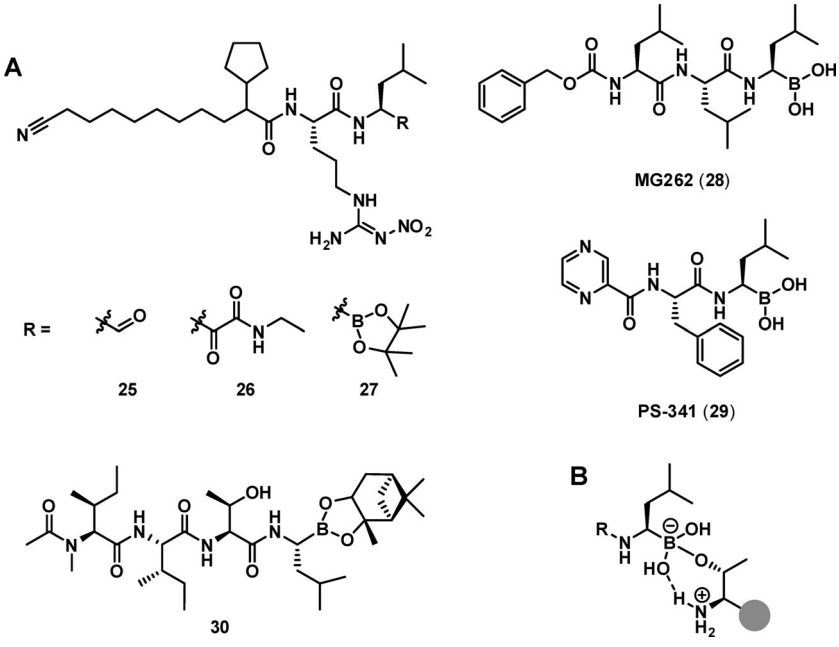

Figure 10. Boronate-based proteasome inhibitors. (**A**) Structures and (**B**) mechanism of inhibition of boronate-based proteasome inhibitors.

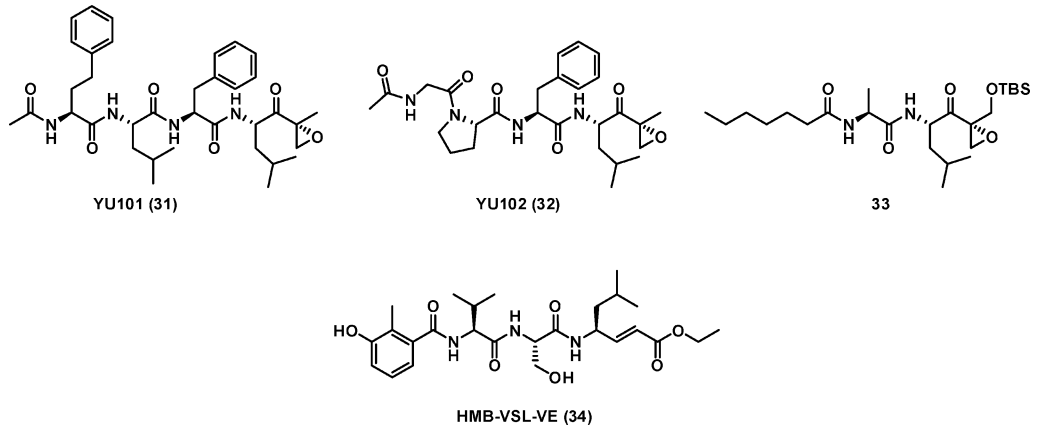

Figure 11. Synthetic epoxy ketone and vinyl ethyl ester based proteasome inhibitors.

the two inhibitors revealed that replacing the peptidic recognition element of epoxomicin (**9**) for the fatty acid N-terminal extension of eponemycin (**8**) resulted in a shift from a predilection for β2 and β5 towards β1 and β5.[45] The synthesis of a library of α′,β′-epoxy ketone warhead armed peptide inhibitors, varying in length and amino acid sequence resulted in Ac-hFLFL epoxy ketone (**31**, YU101), a selective inhibitor of the chymotrypsin-like activity.[46] In a subsequent study, the same group developed Ac-GPFL-epoxy ketone (**32**,YU102) as a selective inhibitor of the caspase-like activity of the proteasome.[47] The dihydroeponemycin analogue **33** was found to selectively inhibit the β1i subunit, over the other constitutive and immune-induced proteolytically active proteasome subunits.[48]

Marastoni et al. reported a new class of synthetic peptide-based proteasome inhibitors possessing a leucine-derived vinyl ethyl ester warhead. The authors report that HMB-VSL-VE (**34**, Figure 11) is a very potent and highly selective inhibitor of the trypsin-like activity of the proteasome.[49]

3 Probing the Proteasome

Activity-based probes[50] are very useful tools in proteasome research that allow for direct visualization of the proteolytically active proteasome subunits, rather than looking at activity using fluorogenic substrates.[51] A tritium labeled lactacystin molecule was synthesized to determine its cellular target.[14c] One of the first peptide-based activity-based proteasome probes was the radio-labeled ^{125}I-NIP-L_3VS. (**35**), developed by Bogyo et al. (Figure 12).[34] The biotinylated epoxomicin analogue **36** proved to be an important tool in the establishment of the proteasome being the target of epoxomicin.[26b] The biodistribution of PS-341 (**29**) after intraveneus dosing of rat was determined by the use of the radio-labeled probe [^{14}C]PS-341.[52] Potent proteasome probes were derived from the extended vinyl sulfone inhibitor AdaAhx$_3$L$_3$VS. (**23**) by the introduction of either a lysine ε-biotinamide to give AdaK(Bio)Ahx$_3$L$_3$VS. (**37**), or a radioiodinated tyrosine to result in AdaY(^{125}I)Ahx$_3$L$_3$VS. (**38**).[37]

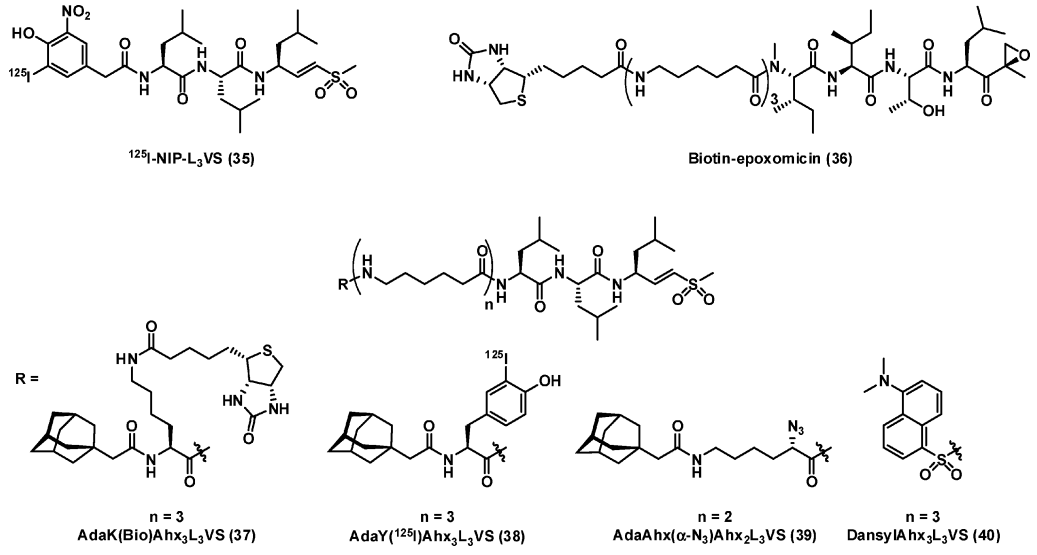

Figure 12. Epoxomicin-biotin and vinyl sulfone-based proteasome probes.

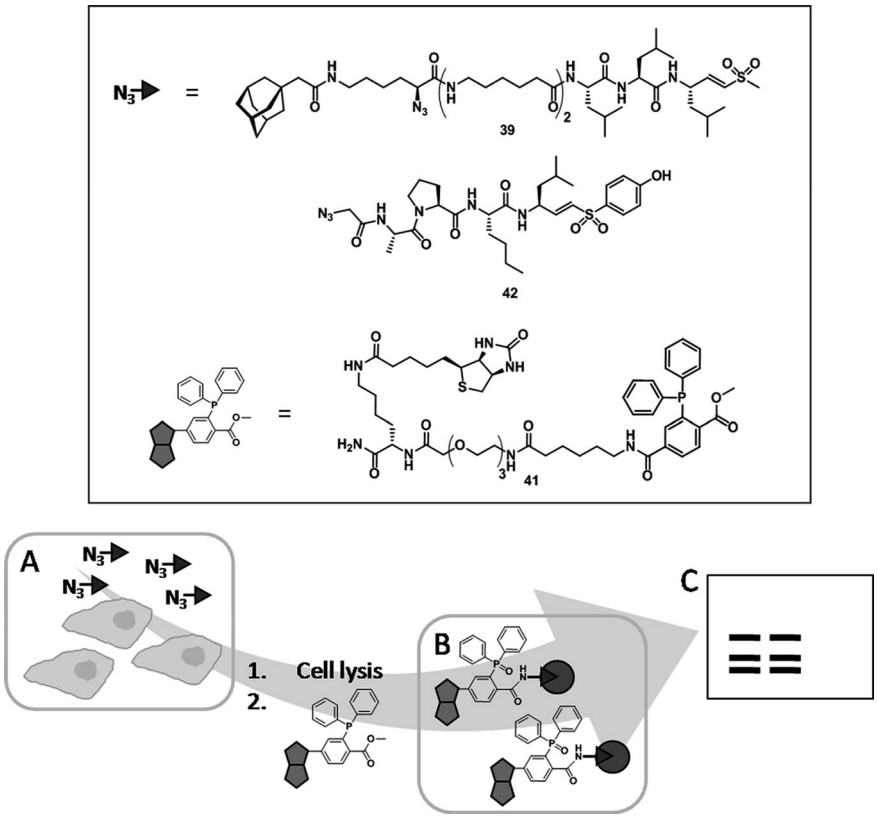

Figure 13. Schematic representation of a two-step proteasome labeling experiment. (**A**) Living cells are being exposed to the cell-permeable azide-containing proteasome probe (**38** or **42**), after which the cells are washed. (**B**) After cell lysis, the azide-modified proteins are treated with the Staudinger–Bertozzi reagent **41** to introduce a biotin moiety. (**C**) After denaturation of the proteins and separation on SDS-PAGE, the biotinylated proteins are visualized by streptavidin blotting.

The latter two probes are powerful tools for experiments in cell lysates or tissue homogenates, but the introduction of the reporter groups rendered the proteasome probes cell-impermeable. To alleviate this problem, the two-step labeling proteasome probe AdaAhx(α-N_3)Ahx$_2$L$_3$VS. (**39**) was synthesized.[53] Indeed, the introduction of the small biocompatible azido functionality in AdaAhx$_3$L$_3$VS. (**23**) did not affect the cell permeability. In a typical activity-based two-step proteasome labeling experiment living cells are exposed to AdaAhx(α-N_3)Ahx$_2$L$_3$VS. (**39**) (Figure 13). After cell lysis, the azido functionalized proteasome subunits are reacted with a ligation reagent equipped with a reporter group, allowing for the visualization of the labeled proteins. Ovaa et al. employed the biotinylated Staudinger–Bertozzi[54] reagent **41** (Figure 13) and proved that AdaAhx(α-N_3)Ahx$_2$L$_3$VS. (**39**) is a cell permeable pan-reactive two-step proteasome probe, representing the first example of a two-step labeling approach in activity-based protein profiling.[55] Employing a similar strategy, the β1 and β1i selective phenolic vinyl sulfone proteasome inhibitor **24** was transformed into the selective two-step labeling probe **42**.[38]

The subunit specificity of PS-341 (**29**) in living cells was determined with the use of the cell-permeable broad-spectrum proteasome probe dansylAhx$_3$L$_3$VS. (**40**, Figure 12).[42a] The weak fluorescent dansyl hapten enables the fast detection of proteolytically active proteasome subunits with anti-dansyl antibodies by SDS-PAGE and Western-blot analysis.

Replacing the dansyl for the strong fluorophore BODIPY TMR, resulted in MV151 (**43**, Figure 14) as a fluorescent, cell-permeable, and activity-based proteasome probe.[56] This probe enables fast and sensitive direct in-gel fluorescence readout of proteasome activity in vitro, in cells, and in mice, is compatible with live-cell imaging techniques and facilitates screening and determination of the subunit specificity of novel proteasome inhibitors. A related study that appeared shortly thereafter described the development of BODIPY FL equipped analogue **44** (Figure 14).[57]

Being highly selective for the proteasome, analogs of epoxomicin are attractive tools to label the proteasome. The development of easily accessible alkyne functionalized BODIPY dyes resulted in the synthesis of the green fluorescent epoxomicin-derived proteasome probe **45** (Figure 14), which was shown to label the proteolytically active proteasome subunits in vitro and in living cells.[58]

With the use of Azido-BODIPY-Ahx$_3$L$_3$VS. (**46**) and biotin-BODIPY-Ahx$_3$L$_3$VS. (**47**, Figure 14) we established that, in principle, the two-step labeling of azido modified proteolytically active proteasome subunits employing the Staudinger–Bertozzi[54] ligation proceeds in a quantitative man-

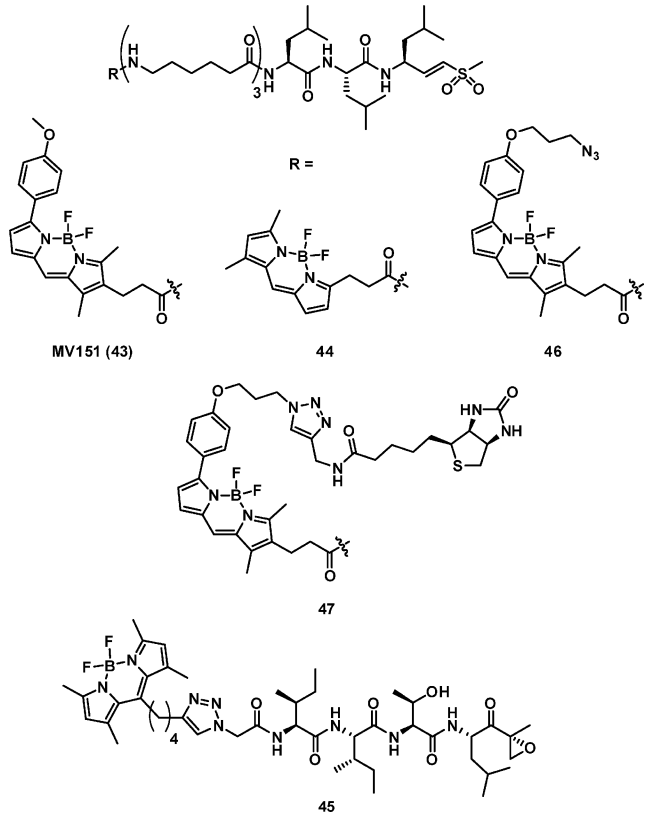

Figure 14. Fluorescent activity-based proteasome probes.

ner.[59] This essentially means that two-step activity-based protein profiling may proceed with equal efficiency with respect to protein tagging as contemporary one-step approaches.

4 The Proteasome as a Therapeutic Target

The ubiquitin proteasome system plays an essential role in the regulation of proteins engaged in cell-cycle progression (e.g. cyclins, activators of the cyclin-dependent kinase family which effect cell cycle progression), oncogens (e.g. growth factors), tumor suppressors (e.g. the transcription factor p53 and IκB, the inhibitor of NF-κB, the transcription factor nuclear factor-κB), and proteins involved in apoptosis (e.g. Bax, the pro-apoptotic Bcl-2-associated X protein).[20] Dividing cells are particularly sensitive to proteasome inhibition due to the tight control of cell-cycle regulators. The stabilization of both promoters and repressors results in cell-cycle arrest and induction of apoptosis, making cancer cells in particular susceptible for proteasome inhibitors. In several malignancies, NF-κB is overexpressed and stimulates cell proliferation and protects the cancer cells from apoptosis. NF-κB is expressed in an inactive form and requires activation by proteasome-mediated processing. Blocking the proteasome prevents activation and furthermore results in the stabilization of IκB and consequently in the inhibition of the anti-apoptotic function of NF-κB. Therefore, besides the direct effect of proteasome inhibition, it also displays a chemosensitizing effect in making cancer cells more susceptible to conventional chemotherapeutics.[60] Multidrug resistance in cancer therapy is partly caused by the overexpression of the ATP-dependent efflux pump P-glycoprotein. Inhibition of the proteasome disrupts the maturation of P-glycoprotein and circumvents it reaching the cell membrane.[61] This is another means for proteasome inhibitors to sensitize cancer cells for chemotherapeutics.

The proteasome inhibitors PS-341 (**29**, bortezomib), salinosporamide A (**3**, NPI-0052) and the epoxy ketone carfilzomib (**48**, Figure 15) have been studied extensively for their antineoplastic activity (Figure 15, A).[62,63] On May 13, 2003 the FDA granted accelerated marketing approval to Millennium Pharmaceuticals for bortezomib (**29**) as a single agent against multiple myeloma (plasma-cell cancer) in patients who have received at least two prior therapies and have demonstrated disease progression on the last therapy.[64] Later, on December 8, 2006 bortezomib (**29**) received marketing approval for the treatment of patients who have had at least one prior therapy for mantle cell lymphoma (a subtype of B-cell lymphoma and one of the rarer of the non-Hodgkin's lymphomas).[65] Promising results have been demonstrated for bortezomib (**29**) in combination with conventional drugs, like dexamethasone (member of the prednisone class of anti-inflammatory drugs), doxorubicin (a DNA intercalator, causing disruption of transcription and replication), melphalan and cyclophosphamide (nitrogen mustard alkylating agents with antiproliferative activity by the formation of non-replicating interstranded DNA) and as induction therapy prior to autologous stem cell transplantation.[62,63] Bortezomib (**29**) was shown to inhibit osteoclasts (bone tissue removing cells) and to enhance osteoblast (bone forming cells) activity, resulting in a positive effect on cancer-induced bone disease.[62] The most common adverse effects associated with bortezomib (**29**) are peripheral sensory neuropathy (effects the nerves that serve the hand and feet, causing pain, prickling, numbness and tingling of hands and feet), thrombocytopenia (decrease in the amount of platelets in blood), asthenia (symptoms of physical weakness and loss of strength), fatigue and gastrointestinal events. These side effects however are normally manageable. In contrast to the positive results with bortezomib (**29**) in hematologic malignancies, disappointing results were obtained in solid tumors. Resistance towards bortezomib (**29**) was shown to be correlated with the overexpression of proteasome subunits and a significant change in the proteasome activity profile.[66]

Two other proteasome inhibitors, NPI-0052 (**3**) and carfilzomib (**48**) have entered Phase I clinical trials.[62,63] NPI-0052 (**3**) has a preference for the chymotrypsin-like and the trypsin-like activity as opposed to the reversible inhibition of the chymotrypsin-like and caspase-like activity by bortezomib (Figure 15, B). NPI-0052 (**3**) was shown to initiate cancer cell-death via a different mechanism as compared to bortezomib and primarily relies on pro-apoptotic caspase-8 signaling pathways.[67] Furthermore, NPI-0052 (**3**) was proven to be orally bioactive, omitting intravenous administration of the drug and was shown to be less cytotoxic

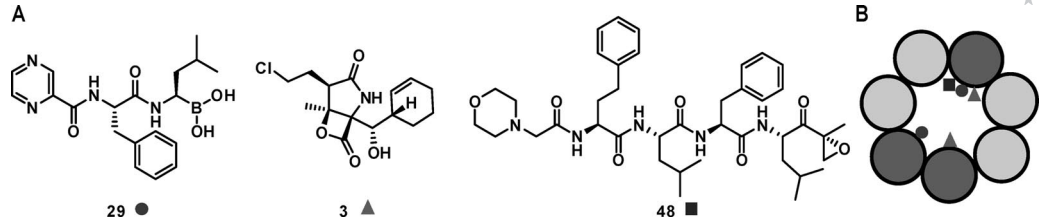

Figure 15. Proteasome inhibitor anticancer drugs. (**A**) Structures of the anticancer drugs bortezomib (**29**), NPI-0052 (**3**) and carfilzomib (**46**). (**B**) Schematic representation of catalytic proteasome subunits targeted by bortezomib (circle), NPI-0052 (triangle) and carfilzomib (square).

against healthy lymphocytes.[67] Carfilzomib (**48**) is a highly specific irreversible proteasome inhibitor showing preference for the chymotrypsin-like activity. It was shown to have increased efficacy in inhibiting proliferation and activation of apoptosis in patient-derived multiple myeloma cells compared to bortezomib (**29**).[68] Both carfilzomib (**48**) and NPI-0052 (**3**) have shown enhanced potency compared to bortezomib (**29**) and were proven to overcome resistance both to conventional drugs and bortezomib (**29**).[62,63,67,68]

5 Summary and Outlook

Although subject of extensive studies in the last decades, much remains to be discovered in the field of proteasome research. The interdisciplinary efforts on the interface of chemistry and biology have boosted the understanding of the role of proteasomes in a variety of processes. The individual role of the different proteolytically active proteasome subunits remains one of the big questions in proteasome research and pharmaceutical sciences. Bortezomib,[40] approved in the U.S. for treating relapsed multiple myeloma[64] and mantle cell lymphoma,[65] only targets β1 and β5 of the constitutive proteasome and β1i and β5i of the immunoproteasome.[42] An interesting research question is what subunit or which combination of subunits should be targeted to get the optimal anticancer therapeutic. Being involved in the generation of antigenic peptides loaded in MHC class I complexes, the contribution of each separate active proteasome subunit to the epitope repertoire is yet another question to be answered. To unravel the evolutionary advantage and the individual roles of the different proteolytically active subunits in cellular processess, antigen presentation and pharmacology, inhibitors that specifically target one of the seven proteolytic subunit would be highly valuable research tools.

Recently, it was found that the level of circulating 20S proteasomes in the plasma of multiple myeloma patients is of clinical significance as a parameter reflecting disease activity.[69] Moreover, the proteasome levels were shown to correlate with the response to chemotherapy. In patients (partially) responding to chemotherapy the circulating proteasome levels decreased significantly, whereas in non-responders no decrease was observed. Circulating proteasome levels were measured in serum samples by enzyme-linked immunoabsorbent assay (ELISA) techniques, after enrichment for 20S proteasomes. Because not only the abundance, but also proteasome activity is elevated in serum of various cancer patients,[70] the use of fluorescent activity-based proteasome probes in a diagnostic clinical setting would be a more straightforward and less expensive alternative for the quantification of circulating proteasome activity in plasma samples. Furthermore, differences in expression levels of proteolytically active proteasome subunits were shown to influence the sensitivity towards the proteasome inhibitor anticancer drug bortezomib.[71] In less sensitive cancer cells, the immunoproteasome levels as well as the constitutive β2 subunit levels are below the expression levels of that observed in sensitive cancer cells. Assessment of the proteasome labeling profile with fluorescent activity-based proteasome probes could predict the chance of success of a proteasome inhibitor-based antineoplastic therapy.

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