Chemical tools to probe the proteasome

Verdoes, M.

1 General Introduction

1.1 Introduction

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 Thesis is devoted to the development of chemical tools to study proteasome activity.

1.2 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 substrates "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 a 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

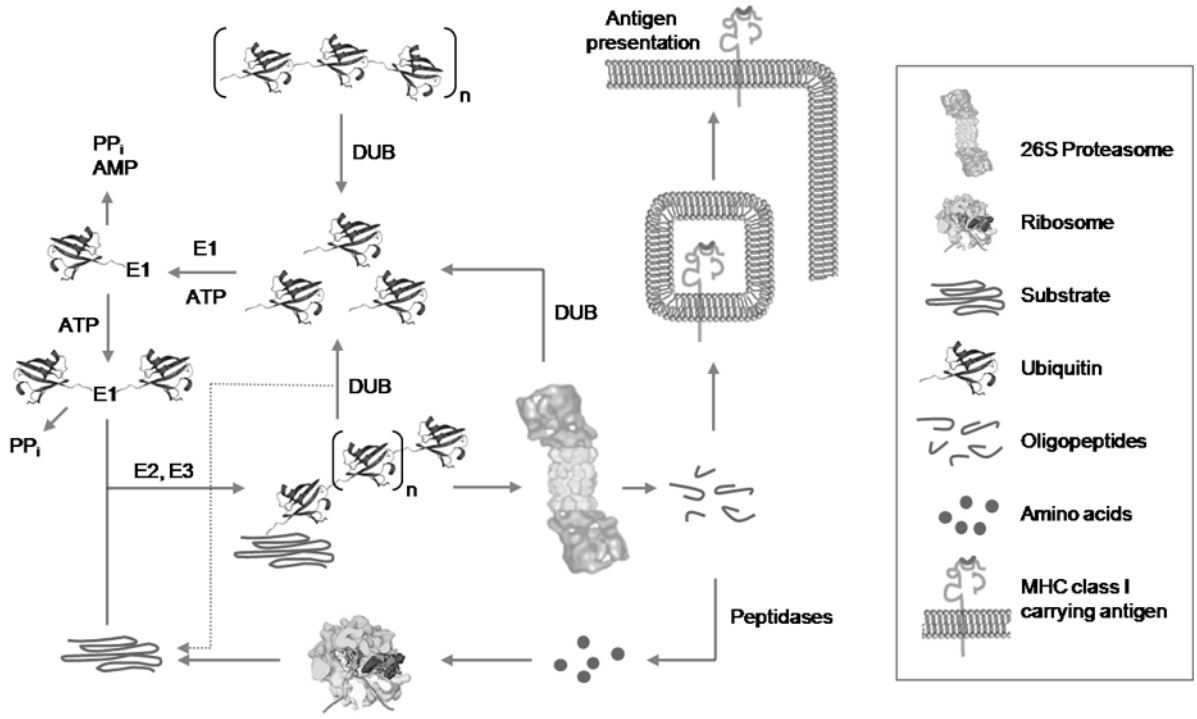


Figure 1. Schematic representation of the Ubiquitin Proteasome System.

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 by 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.

1.3 The proteasome

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,

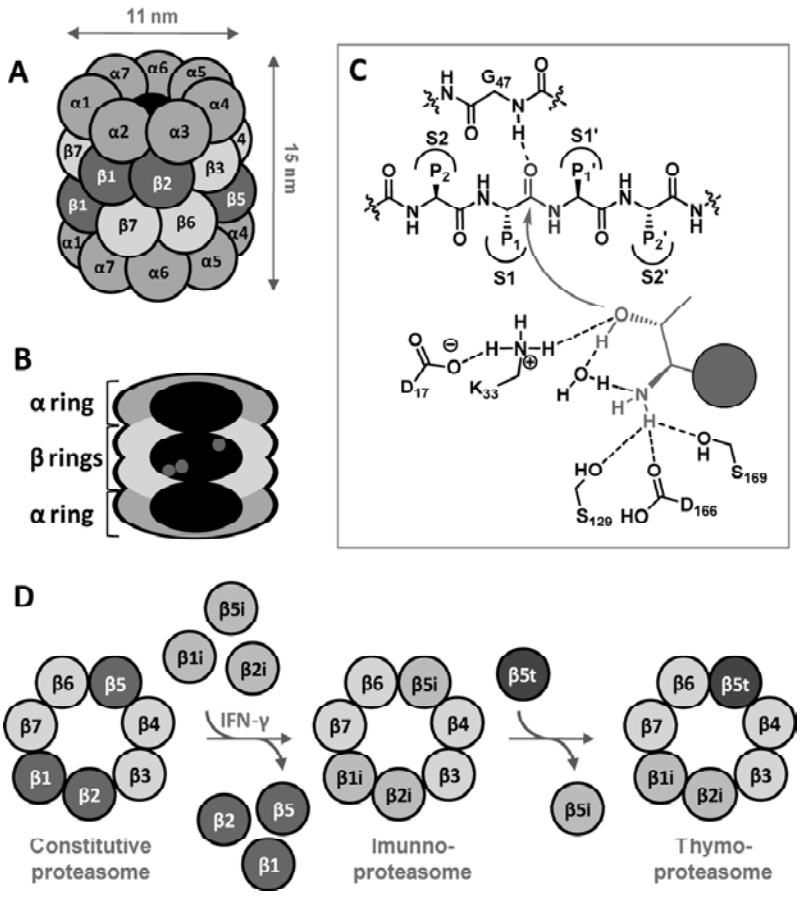


Figure 2. Schematic representation of the composition of the 20S proteasome.

(**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.

sandwiching the two inner rings which 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 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 2A). The three remaining proteolytically active β subunits are the β1 (Y/δ), β2 (Z) and β5 (X) subunits (Figure 2B). The *N*-terminal threonine (Thr1) represents the proteolytically active residue and acts by nucleophilic attack of the γ-hydroxyl on the carbonyl of the peptide bond destined to be cleaved (Figure 2C). 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 after 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-relevant 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.

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 selection.

1.4 Inhibitors of the proteasome

The importance of the proteasome for viability was capitalized upon by several micro-organisms, 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-lactone (**2**),[15] also known as ormulide, which is formed under neutral conditions by lactonization (Figure 3). A structurally related proteasome inhibitor salinosporamide A (**3**, NPI-0052) is produced by *Salinispora tropica*, a marine bacterium which is found in ocean sediment.[16] 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

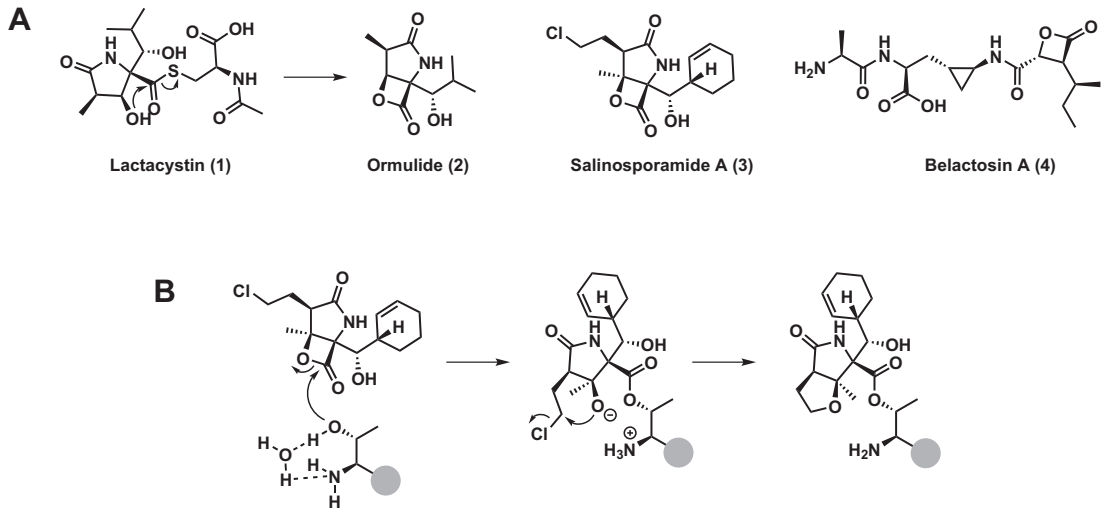


Figure 3. β-lactone containing natural products.

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

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 3B).[17,18] The β-lactone containing belactosin A (**4**),[19] isolated from a fermentation broth of *Streptomyces* sp. UCK14 inhibits the proteasome via the same mechanism.[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[22] (TMC-95A (**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] The latter reversibly deactivates the proteasome by acylation of the threonine γ-hydroxyl as a result of nucleophilic attack on the ester carbonyl and subsequent transesterification.

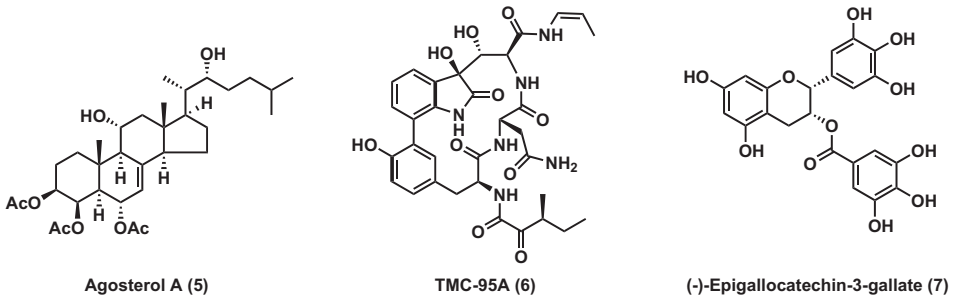


Figure 4. Structures of agosterol A (**5**), TMC-95A (**6**) and (-)-epigallocatechin-3-gallate (**7**).

Eponemycin (**8**, Figure 5A) was isolated from *Streptomyces hygroscopicus* based on its *in vivo* antitumor activity against murine B16 melanoma tumors.[24] The structurally homologues epoxomicin (**9**) was isolated from an *Actinomycetes* strain and was found to have antineoplastic activity.[25,26] The α′,β′-epoxyketone "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 5B).[17,27] First, the ketone is attacked by the *N*-terminal threonine γ-hydroxyl, 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.

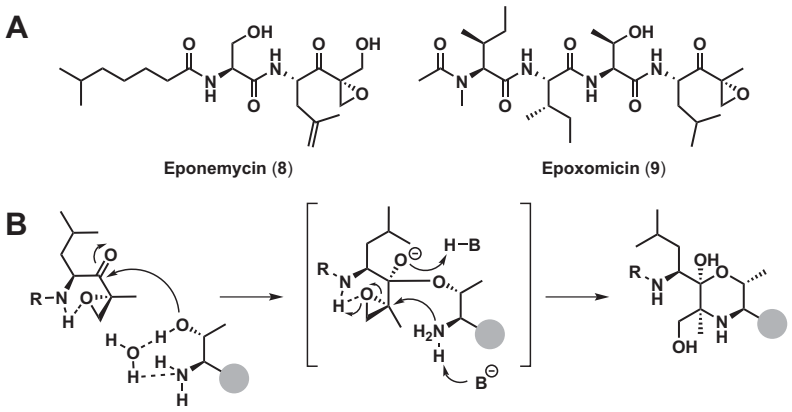


Figure 5. Epoxyketone containing natural products.

(**A**) Structures and (**B**) mechanism of inhibition of epoxyketone containing inhibitors.

Strains of the plant pathogen *Pseudomonas syringae* pv. *syringae* secrete syringolin A (**10,** Figure 6A), causing, for example, brown spot disease on bean.[28] The target of this virulence factor was found to be the proteasome, which is blocked after Michael addition of the threonine γ-hydroxyl on the macrocyclic α,β-unsaturated amide resulting in an irreversible ether bond formation.

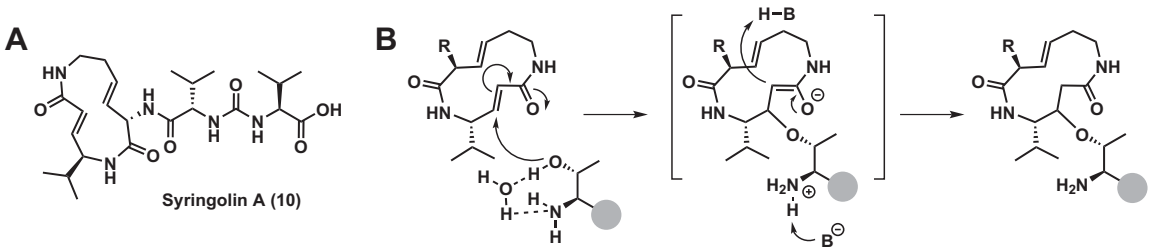


Figure 6. Syringolin A (**10**).

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

Fellutamide B (**11,** Figure 7A) 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 7B).[17]

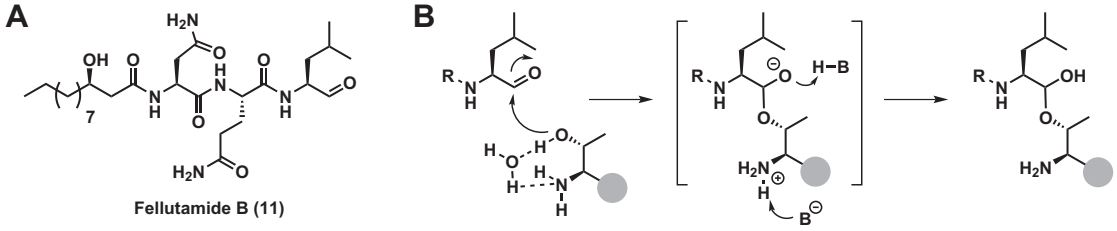


Figure 7. Fellutamide B (**11**).

(**A**) Structure of fellutamide B (**11**) and (**B**) mechanism of inhibition of aldehyde containing 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 meant 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-ketoester 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]

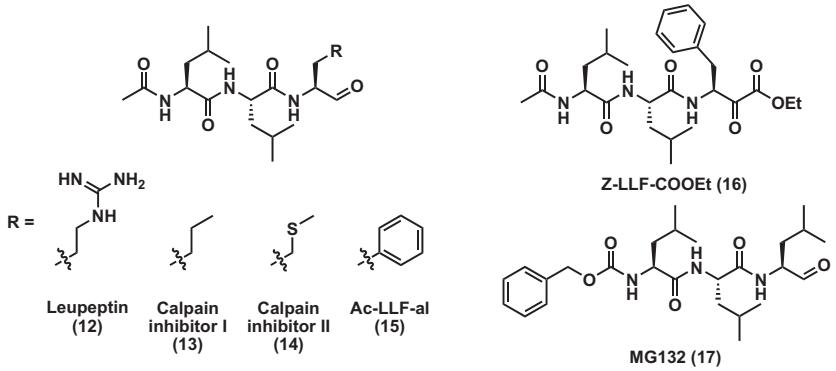


Figure 8. Synthetic peptide based proteasome inhibitors.

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 synthetic peptide vinyl sulfone based proteasome inhibitors (Figure 9A).[34] Although still marginally targeting cathepsin S, Z-L_3VS (**18**) and NIP-L_3VS (**19**) covalently and irreversibly modified the γ-hydroxyl of the *N*-terminal threonine by ether bond formation via Michael addition (Figure 9B).[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] By the installation of a fourth amino acid with an aromatic or aliphatic side chain however, like for example in YL_3VS (**20**), the inhibitory potency could be restored to even surpass that of Z-L_3VS (**18**), with the most dramatic increase of potency observed for the trypsin-like activity.[35] Substitution of the methyl for a phenol in the vinyl sulfone warhead, like in NIP-L_3VS-PhOH (**21**), dramatically increased the potency for the caspase-like activity and to a lesser extent for the trypsin-like activity.[35] A P2 to P_4 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 proteasome in living cells in comparison with, for example, Z-L$_3$VS (**18**). Efforts to develop subunit specific proteasome inhibitors resulted in the β1 and β1i selective phenolic vinyl sulfone proteasome inhibitor **24**.[38]

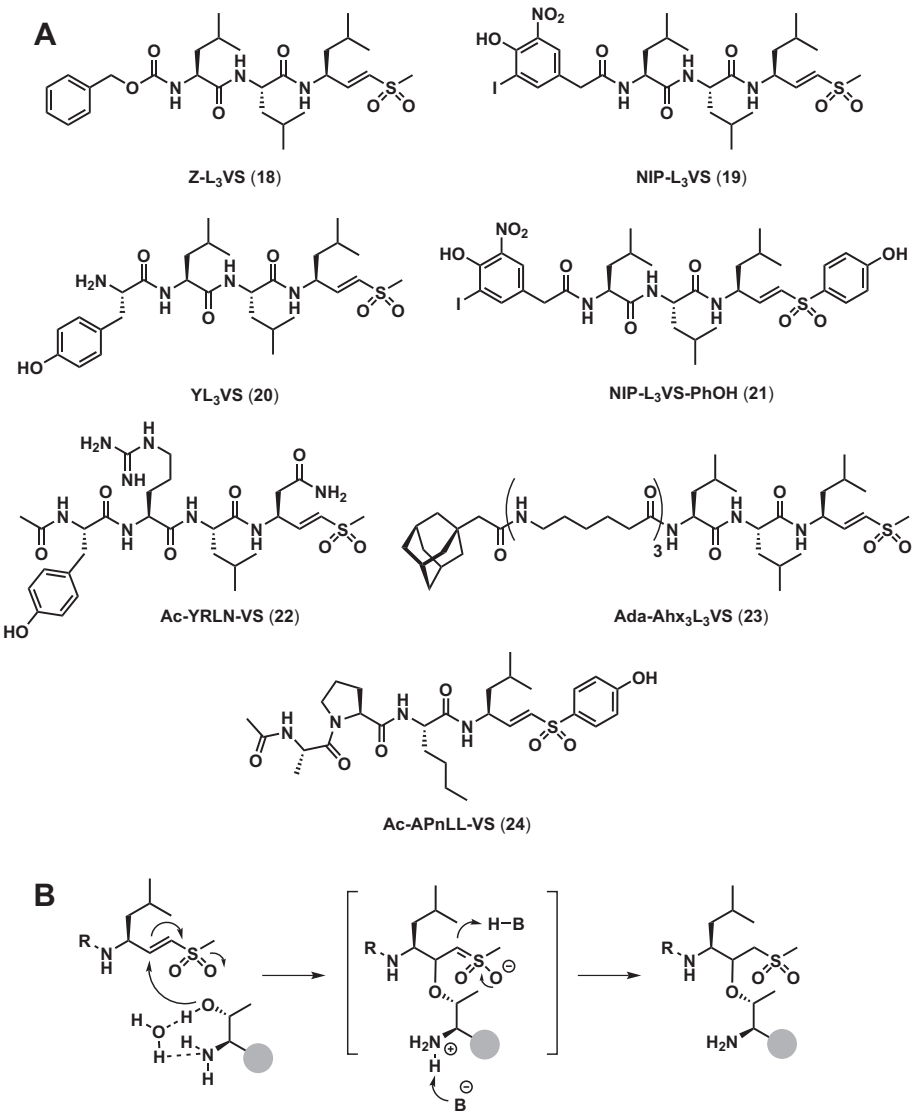


Figure 9. Vinyl sulfone based proteasome inhibitors.

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

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 10A).[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-hydroxyl of the active site threonine (Figure 10B).[17,43]

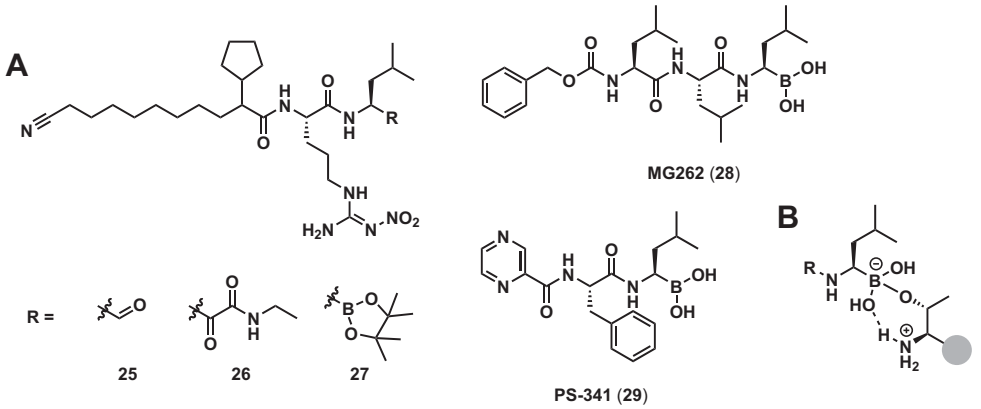


Figure 10. Boronate based proteasome inhibitors.

(**A**) Structures and (**B**) mechanism of inhibition of boronate based proteasome inhibitors.

Several synthetic analogues of the highly selective proteasome inhibitors eponemycin (**8**) and epoxomicin (**9**) have been synthesized (Figure 11). A structural hybrid library of 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.[44] The synthesis of a library of α′,β′-

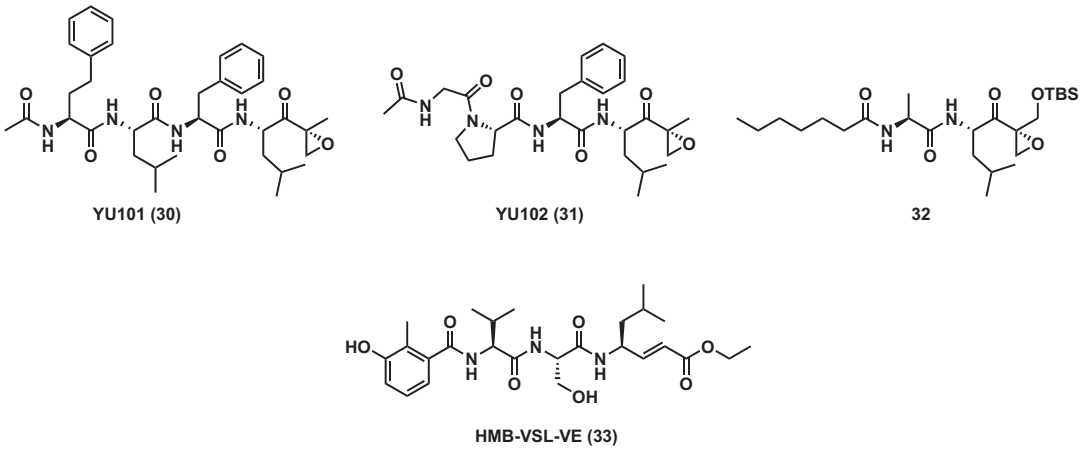


Figure 11. Synthetic epoxyketone and vinyl ethyl ester based proteasome inhibitors.

epoxyketone warhead armed peptide inhibitors, varying in length and amino acid sequence resulted in Ac-hFLFL-epoxyketone (**30**, YU101), a selective inhibitor of the chymotrypsin-like activity.[45] In a subsequent study, the same group developed Ac-GPFL-epoxyketone (**31**,YU102) as a selective inhibitor of the caspase-like activity of the proteasome.[46] The dihydroeponemycin analogue **32** was found to selectively inhibit the β1i subunit, over the other constitutive and immune-induced proteolytically active proteasome subunits.[47]

Marastoni *et al*. reported a new class of synthetic peptide based proteasome inhibitors possessing a leucine derived vinyl ethyl ester warhead. HMB-VSL-VE (**33**, Figure 11) was shown to be a very potent and highly selective inhibitor of the trypsin-like activity of the proteasome.[48]

1.5 Probing the proteasome

Activity-based probes 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.[49] One of the first activity-based proteasome probes was the radio-labeled ^{125}I-NIP-L_3VS (**34**), developed by Bogyo *et al*. (Figure 12).[34] The biotinylated epoxomicin analogue **35** 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 i.v. dosing of rat was determined by the use of the radiolabeled probe [^{14}C]PS-341.[50] 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 (**36**), or a radioiodinated tyrosine to result in AdaY(^{125}I)Ahx$_3$L$_3$VS (**37**).[37]

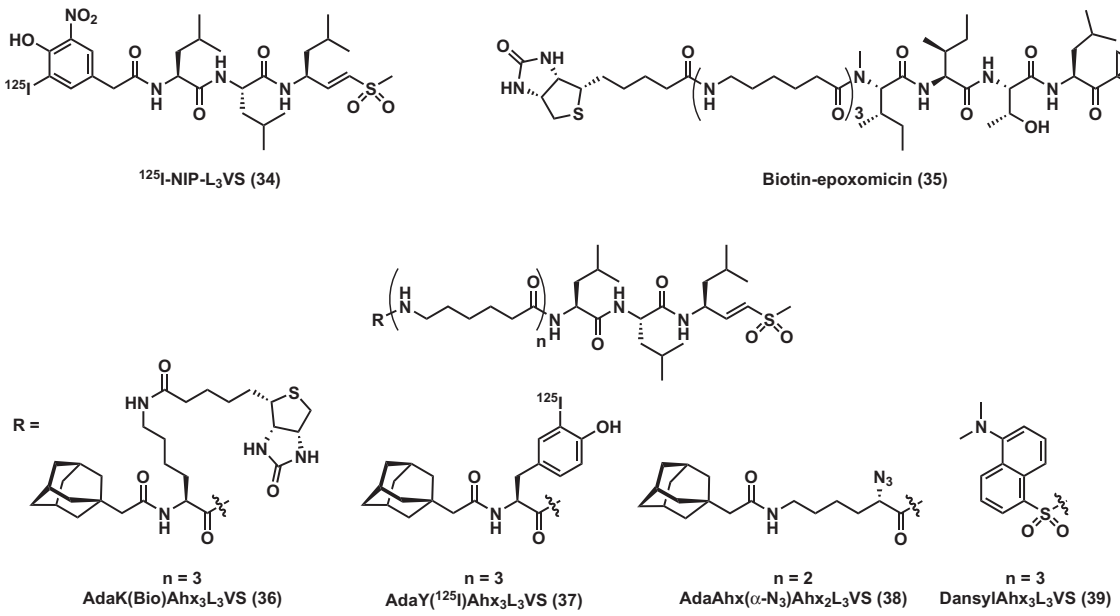


Figure 12. Epoxomicin-biotin and vinyl sulfone based proteasome probes.

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 (**38**) was synthesized.[51] 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 (**38**) (Figure 13). After cell lysis, the azido functionalized proteasome subunits can be 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[52] reagent **40** (Figure 13) and proved that AdaAhx(α-N_3)Ahx$_2$L$_3$VS (**38**) 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. Simultaneously, Speers *et al.* reported a two-step protease labeling approach for the activity-based profiling of serine hydrolases.[53] In their study, an azide-derivatized phenyl sulfonate ester probe was used to tag serine hydrolases with an azide moiety *in vivo*. After tissue homogenation, the azide labeled proteins were reacted with a rhodamine functionalized alkyne reagent (**41**) employing the copper(I)-catalyzed Huisgen [2+3]-cycloaddition ("click reaction") (Figure 14A).[54] Since then, several bioorthogonal

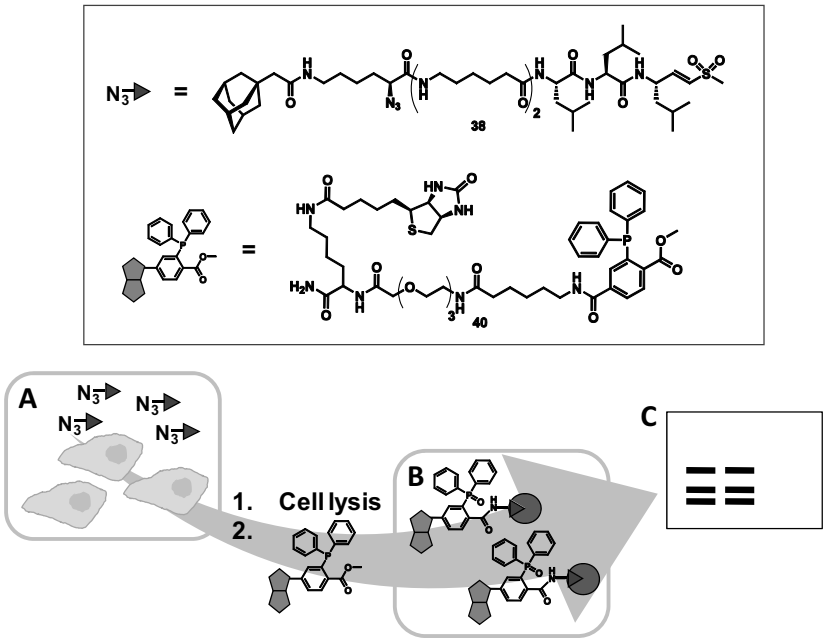


Figure 13. Schematic representation of a two-step proteasome labeling experiment.
(**A**) Living cells are being exposed to the cell-permeable azide containing proteasome probe (**38**), after which the cells are washed. (**B**) After cell lysis, the azide modified proteins are reacted with the Staudinger-Bertozzi reagent **40** to introduce a biotin moiety. (**C**) After denaturation of the proteins and separation on SDS-PAGE, the biotinylated proteins are visualized by streptavidin blotting.

chemoselective reagents have been developed for the ligation of a reporter group onto an azide functionalized biomolecule in cell-surface labeling experiments or activity-based protein profiling. To overcome the cytotoxicity of copper ions, the Bertozzi lab reported a series of strain promoted cyclooctyne copper-free click reagents.[55] The synthetically demanding DIFO (difluorinated cyclooctyne) **42** (Figure 14B) was applied for *in vivo* non-invasive imaging of metabolically azide labeled membrane-associated glycans in developing zebrafish.[56] Soon after, the easier accessible second-generation DIFO reagent **43** was reported.[57] In the same period, Boons and co-workers reported 4-dibenzocyclooctynol **44** for the visualization of metabolically labeled glycoconjugates on the surface of living cells.[58] In an effort to reduce aspecific labeling in *in vivo* imaging experiments, the quenched Staudinger-Bertozzi reagent **45** was synthesized.[59] Upon Staudinger reduction of the biomolecule bound azide, the quencher moiety is displaced and a fluorescent conjugate is formed (Figure 14C). Since the fluorophore on the unreacted ligation reagent is quenched, fluorescence will only appear after ligation with an azido modified biomolecule, making this tool ideal for imaging applications.

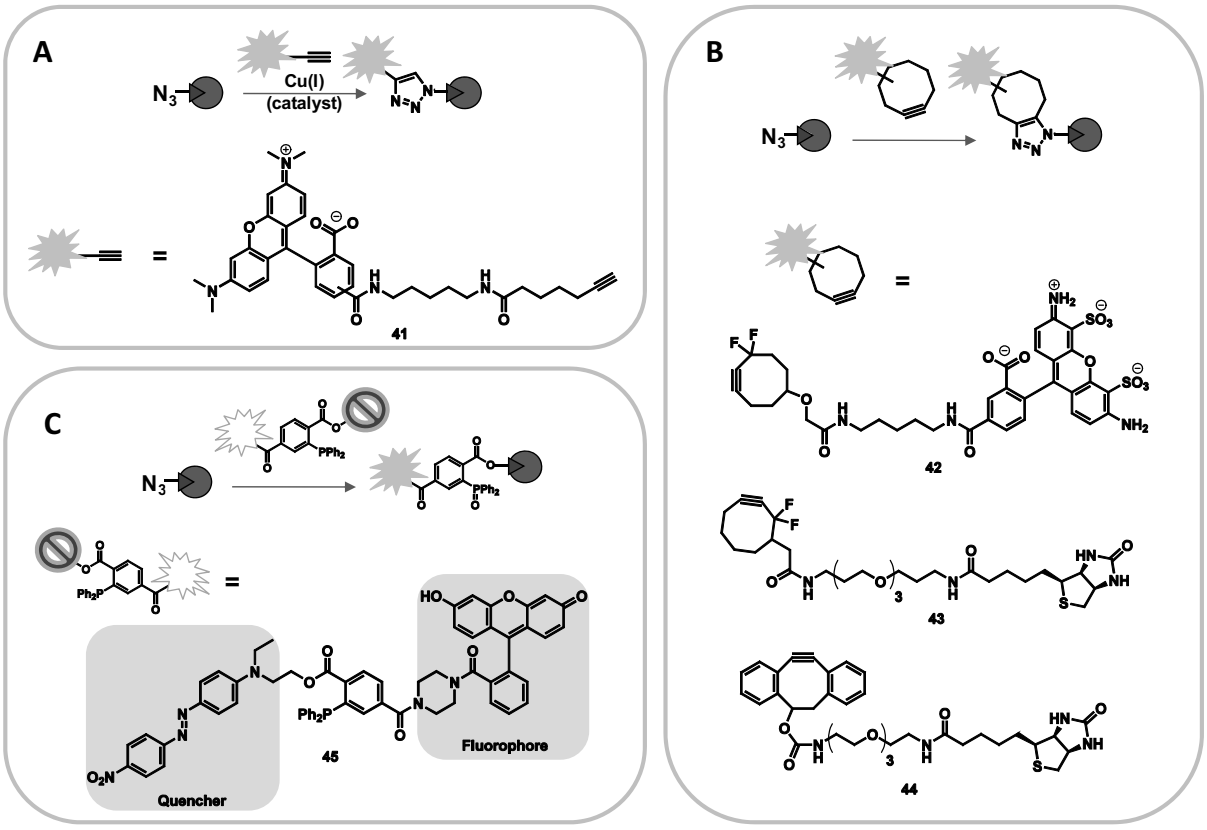


Figure 14. Schematic representation of two-step labeling approaches.

(**A**) The copper (I) catalyzed [2+3]-Huisgen cycloaddition. (**B**) Strain promoted [2+3]-cycloaddition. (**C**) Quenched Staudinger-Bertozzi reagent.

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 (**39**, 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.

1.6 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.

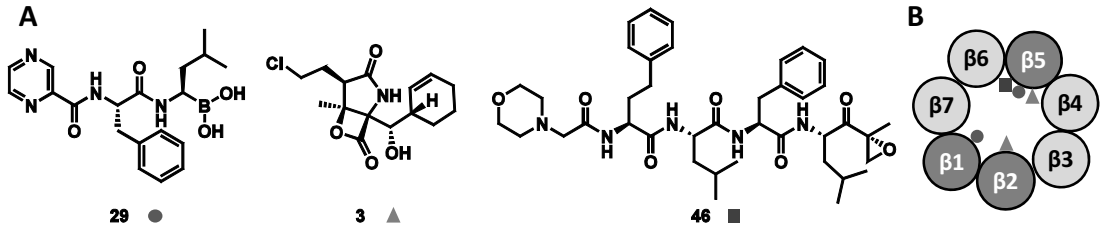


Figure 15. Proteasome inhibitor anti-cancer drugs.

(**A**) Structures of the anti-cancer 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).

The proteasome inhibitors PS-341 (**29**, bortezomib), salinosporamide A (**3**, NPI-0052) and the epoxyketone carfilzomib (**46**) have been studied extensively for their antineoplastic activity (Figure 15A).[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 (**46**) 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 15B). 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 against healthy lymphocytes.[67] Carfilzomib (**46**) 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 (**46**) 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]

1.7 Aim and outline of this Thesis

The research described in this Thesis aims at the development of novel chemical biology tools to study proteasome activity. **Chapter 2** describes the synthesis and characterization of the fluorescent, cell-permeable, and activity-based proteasome probe BODIPY TMR-Ahx$_3$L$_3$VS (**47**, MV151, Figure 16). 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.

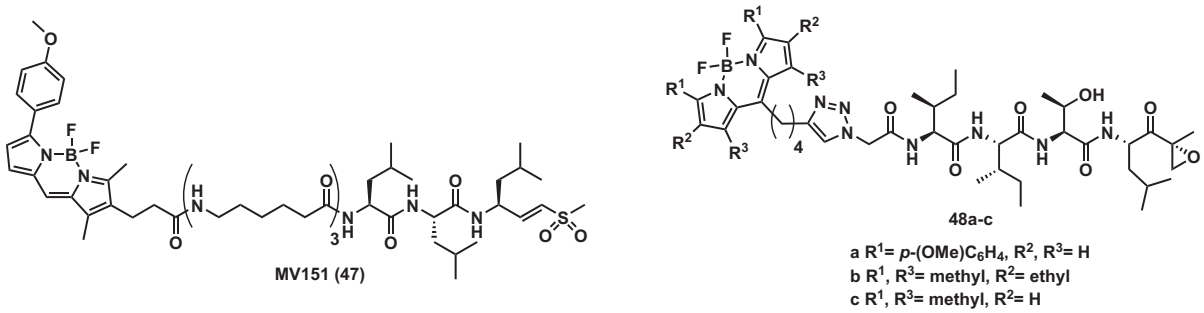


Figure 16. Structure of MV151 (**47**) and fluorescent epoxomicin derived proteasome probes **48a-c**.

The development of three easily accessible alkyne functionalized BODIPY dyes is discussed in **Chapter 3**. Using these fluorescent tags, three epoxomicin derived proteasome probes **48a-c** (Figure 16) were synthesized and shown to label the proteolytically active proteasome subunits *in vitro* and in living cells.

In **Chapter 4,** the synthesis of the bifunctional azido-BODIPY succinimidyl ester **49** (Figure 17) is described. Two sets of one- and two-step labeling proteasome probes were synthesized and were used to determine the efficiency of the Staudinger-Bertozzi ligation.

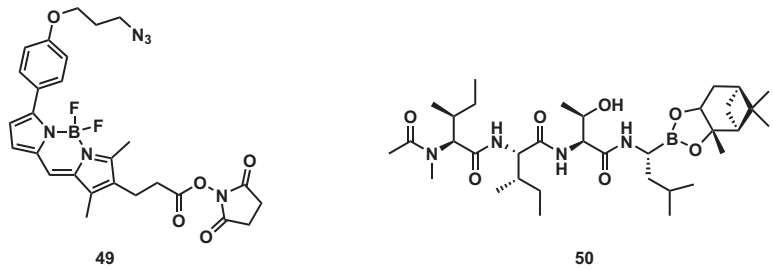


Figure 17. Azido-BODIPY-OSu (**49**) and epoxomicin derived boronic ester **50**.

Peptide based proteasome inhibitors can be divided in three structural entities, 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. In **Chapter 5** these three structural entities of five commonly used proteasome inhibitors are scrambled to afford a library of 15 hybrid proteasome inhibitors. Employing MV151, the inhibitory profile of the library was determined. One of the outcomes of this study is the discovery of the epoxomicin derived boronic ester **50** (Figure 17) as being one of the most potent peptide-based proteasome inhibitor reported to date.

A similar strategy was employed in **Chapter 6**, where warheads and peptidic recognition elements of previously reported subunit specific proteasome inhibitors were scrambled. A more potent β1 specific proteasome inhibitor **51** was developed, next to the β5 specific vinyl sulfone **52** (Figure 18). These hits were transformed into subunit specific two-step labeling probes **53** and **54** by the introduction of an azide moiety. Substitution of the naphthylacetamide in **52** for an azido-BODIPY moiety resulted in the fluorescent β5 specific two-step labeling proteasome probe **55**.

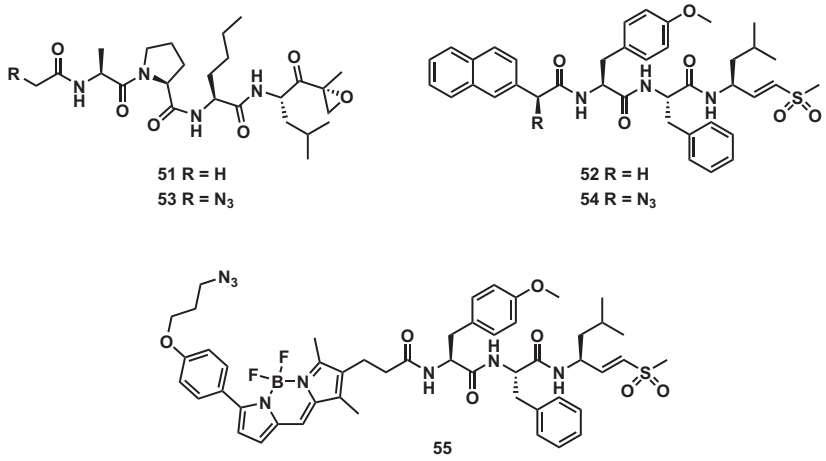
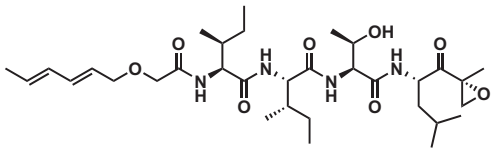


Figure 18. Subunit specific proteasome inhibitors and probes.

As discussed in the Chapter 1.5, two approaches for the two-step labeling of proteins have been developed, both relying on the azido moiety. In **Chapter 7** the applicability of the Diels-Alder reaction as a complementary two-step labeling approach of proteolytically active proteasome subunits is investigated. A panel of diene equipped epoxomicin analogues (like **56**, Figure 19) were synthesized and were shown to be able to inhibit the proteasome with potencies in the same order of magnitude compared to epoxomicin (**9**).



56

Figure 19. Diene functionalized epoxomicin analogue **56**.

In the final Chapter, the work described in this Thesis is summarized and future applications of the developed tools are proposed, as well as some suggestions for further research in line with that described in this Thesis.

References and notes

1. Hershko, A.; Ciechanover, A. *Annu. Rev. Biochem.* **1998**, *67*, 425-479.
2. (a) Pickart, C.M.; Eddins, M.J. *Biochim. Biophys. Acta.* **2004**, *1695*, 55-72. (b) Huang, D.T.; Hunt, H.W.; Zhuang, M.; Ohi, M.D.; Holton, J.M.; Schulman, B.A. *Nature* **2007**, *445*, 394-398.
3. Jin, J.; Li, X.; Gygi, S.P.; Harper, J.W. *Nature* **2007**, *447*, 1135-1138.
4. Wilkinson, K.D. *Semin. Cell. Dev. Biol.* **2000**, *11*, 141-148.
5. Rock, K.L.; Goldberg, A.L. *Annu. Rev. Immunol.* **1999**, *17*, 739-779.
6. Loureiro, J.; Ploegh, H.L. *Adv. Immunol.* **2006**, *92*, 225-305.
7. Baumeister, W.; Walz, J.; Zühl, F.; Seemüller, E. *Cell* **1998**, *92*, 367-80.
8. Voges, D.; Zwickl, P.; Baumeister, W. *Annu. Rev. Biochem.* **1999**, *68*, 1015-1068.
9. Dick, T.P.; Nussbaum, A.K.; Deeg, M.; Heinemeyer, W.; Groll, M.; Schirle, M.; Keilholz, W.; Stevanović, S.; Wolf, D.H.; Huber, R.; Rammensee, H.G.; Schild, H. *J. Biol. Chem.* **1998**, *273*, 25637-25646.
10. Wenzel, T.; Baumeister, W. *Nat. Struct. Biol.* **1995**, *2*, 199-204.
11. Strehl, B.; Seifert, U.; Krüger, E.; Heink, S.; Kuckelkorn, U.; Kloetzel, P.M. *Immunol. Rev.* **2005**, *207*, 19-30.
12. Whitby, F.G.; Masters, E.I.; Kramer, L.; Knowlton, J.R.; Yao, Y.; Wang, C.C.; Hill, C.P. *Nature* **2000**, *408*, 115-120.
13. (a) Murata, S.; Sasaki, K.; Kishimoto, T.; Niwa, S.; Hayashi, H.; Takahama, Y.; Tanaka, K. *Science* **2007**, *316*, 1349-1353. (b) Murata, S.; Takahama, Y.; Tanaka, K. *Curr. Opin. Immunol.* **2008**, *20*, 192-196.
14. (a) Omura, S.; Fujimoto, T.; Otoguro, K.; Matsuzaki, K.; Moriguchi, R.; Tanaka, H.; Sasaki, Y. *J. Antibiot.* **1991**, *44*, 113-116. (b) Fenteany, G.; Standaert, R.F.; Lane, W.S.; Choi, S.; Corey, E.J.; Schreiber, S.L. *Science* **1995**, *268*, 726-731.
15. (a) Dick, L.R.; Cruikshank, A.A.; Grenier, L.; Melandri, F.D.; Nunes, S.L.; Stein, R.L. *J. Biol. Chem.* **1996**, *271*, 7273-7276. (b) Corey, E.J.; Li, W.D. *Chem. Pharm. Bull.* **1999**, *47*, 1-10.
16. Feling, R.H.; Buchanan, G.O.; Mincer, T.J.; Kauffman, C.A.; Jensen, P.R.; Fenical, W. *Angew. Chem. Int. Ed.* **2003**, *42*, 355-357.
17. Borissenko, L.; Groll, M. *Chem. Rev.* **2007**, *107*, 687-717.
18. Groll, M.; Huber, R.; Potts, B.C. *J. Am. Chem. Soc.* **2006**, *128*, 5136-5141.
19. (a) Asai, A.; Hasegawa, A.; Ochiai, K.; Yamashita, Y.; Mizukami, T. *J. Antibiot.* **2000**, *53*, 81-83. (b) Asai, A.; Tsujita, T.; Sharma, S.V.; Yamashita, Y.; Akinaga, S. Funakoshi, M.; Kobayashi, H.; Mizukami, T. *Biochem. Pharmacol.* **2004**, *67*, 227-234.

20. Kisselev, A.F., Goldberg, A.L. *Chem. Biol.* **2001**, *8*, 739-758.
21. Aoki, S.; Yoshioka, Y.; Miyamoto, Y.; Higuchi, K.; Setiawan, A.; Murakami, N.; Chen, Z.S.; Sumizawa, T.; Akiyama, S.; Kobayashi, M. *Tetrahedron Lett.* **1998**, *39*, 6303-6306. (b) Tsukamoto, S.; Tatsuno, M.; van Soest, R.W.; Yokosawa, H.; Ohta, T. *J. Nat. Prod.* **2003**, *66*, 1181-1185.
22. Kohno, J.; Koguchi, Y.; Nishio, M.; Nakao, K.; Kuroda, M.; Shimizu, R.; Ohnuki, T.; Komatsubara, S. *J. Org. Chem*. **2000**, *65*, 990-995.
23. Nam, S.; Smith, D.M.; Dou, Q.P. *J. Biol. Chem.* **2001**, *276*, 13322-13330.
24. Sugawara, K.; Hatori, M.; Nishiyama, Y.; Tomita, K.; Kamei, H.; Konishi, M.; Oki, T. *J. Antibiot.* **1990**, 43, 8-18.
25. Hanada, M.; Sugawara, K.; Kaneta, K.; Toda, S.; Nishiyama, Y.; Tomita, K.; Yamamoto, H.; Konishi, M.; Oki, T. *J. Antibiot.* **1992**, *45*, 1746-1752.
26. (a) Meng, L.; Kwok, B.H.; Sin, N.; Crews, C.M. *Cancer Res.* **1999**, *59*, 2798-2801. (b) Sin, N.; Kim, K. B.; Elofsson, M.; Meng, L.; Auth, H.; Kwok, B. H.; Crews, C. M. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2283-2288. (c) Meng, L.; Mohan, R.; Kwok, B. H.; Elofsson, M.; Sin, N.; Crews, C. M. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 10403-10408.
27. Groll, M.; Kim, K.B.; Kairies, N.; Huber, R.; Crews, C.M. *J. Am. Chem. Soc.* **2000**, *122*, 1237-1238.
28. Groll, M.; Schellenberg, B.; Bachmann, A.S.; Archer, C.R.; Huber, R.; Powell, T.K.; Lindow, S.; Kaiser, M.; Dudler, R. *Nature* **2008**, *452*, 755-758.
29. (a) Shigemori, H.; Wakuri, S.; Yazawa, K.; Nakamura, T.; Sasaki, T.; Kobayashi, J.I. *Tetrahedron* **1991**, *47*, 8529–8534. (b) Hines, J.; Groll, M.; Fahnestock, M.; Crews, C.M. *Chem. Biol.* **2008**, *15*, 501-12.
30. Wilk, S.; Orlowski, M. *J. Neurochem.* **1980**, *35*, 1172-1182.
31. Vinitsky, A.; Michaud, C.; Powers, J.C.; Orlowski, M. *Biochemistry* **1992**, *31*, 9421-9428.
32. (a) Tsubuki, S.; Kawasaki, H.; Saito, Y.; Miyashita, N.; Inomata, M.; Kawashima, S. *Biochem. Biophys. Res. Commun.* **1993**, *196*, 1195-1201. (b) Tsubuki, S.; Saito, Y.; Tomioka, M.; Ito, H.; Kawashima, S. *J. Biochem.* **1996**, *119*, 572-576.
33. (a) Adams, J.; Behnke, M.; Chen, S.; Cruickshank, A. A.; Dick, L. R.; Grenier, L.; Klunder, J. M.; Ma, Y.-T.; Plamondon, L.; Stein, R. L. Bioorg. *Med. Chem. Lett.* **1998**, *8*, 333-338. (b) Zhu, Y.-Q.; Pei, J.-F.; Liu, Z.-M.; Lai, L.-H.; Cui, J.-R.; Li, R.-T. *Bioorg. Med. Chem.* **2006**, *14*, 1483-1496. (c) Kisselev, A. F.; Garcia-Calvo, M.; Overkleeft, H. S.; Peterson, E.; Pennington, M. W.; Ploegh, H. L.; Thornberry, N. A.; Goldberg, A. L. *J. Biol. Chem.* **2003**, *278*, 35869-35877. (d) Iqbal, M.; Chatterjee, S.; Kauer, J. C.; Das, M.; Messina, P.; Freed, B.; Biazzo, W.; Siman, R. *J. Med. Chem.* **1995**, *38*, 2276-2277. (e) Harding, C. V.; France, J.; Song, R.; Farah, J. M.; Chatterjee, S.; Iqbal, M.; Siman, R. *J. Immunol.* **1995**, *155*, 1767-1775. (f) Vivier, M.; Jarrousse, A.-S.; Bouchon, B.; Galmier, M.-J.; Auzeloux, P.; Sauzieres, J.; Madelmont, J.-C. *J. Med. Chem.* **2005**, *48*, 6731-6740. (g) Momose, I.; Umezawa, Y.; Hirosawa, S.; Iinuma, H.; Ikeda, D. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1867-1871. (h) Loidl, G.; Groll, M.; Musiol, H.-J.; Huber, R.; Moroder, L. *Proc. Natl. Acad. Sci. USA.* **1999**, *96*, 5418-5422. (i) Loidl, G.; Musiol, H.-J.; Groll, M.; Huber, R.; Moroder, L. *J. Peptide Sci*. **2000**, *6*, 36-46.
34. Bogyo, M.; McMaster, J.S.; Gaczynska, M.; Tortorella, D.; Goldberg, A.L.; Ploegh, H.L. *Proc. Natl. Acad. Sci. USA.* **1997**, *94*, 6629-6634.
35. Bogyo, M.; Shin, S.; McMaster, J.S.; Ploegh, H.L. *Chem. Biol.* **1998**, *5*, 307-320.
36. Nazif, T.; Bogyo, M. *Proc. Natl. Acad. Sci. USA.* **2001**, *98*, 2967-2972.
37. Kessler, B.M.; Tortorella, D.; Altun, M.; Kisselev, A.F.; Fiebiger, E.; Hekking, B.G.; Ploegh, H.L.; Overkleeft, H.S. *Chem. Biol.* **2001**, *8*, 913-929.
38. Van Swieten, P. F.; Samuel, E.; Orient Hernandez, R.; van den Nieuwendijk, A. M. C. H.; Leeuwenburgh, M. A.; van der Marel, G. A.; Kessler, B. M.; Overkleeft, H. S.; Kisselev, A. F. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3402-3405.

39. Iqbal, M.; Chatterjee, S.; Kauer, J. C.; Mallamo, J. P.; Messina, P. A.; Reiboldt, A.; Siman, R. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 287-290.
40. Adams, J.; Behnke, M.; Chen, S.; Cruickshank, A.A.; Dick, L.R.; Grenier, L.; Klunder, J.M.; Ma, Y.-T.; Plamondon, L.; Stein, R.L. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 333-338.
41. Walker, B.; Lynas, J.F. *Cell. Mol. Life Sci.* **2001**, *58*, 596-624.
42. (a) Berkers, C.R.; Verdoes, M.; Lichtman, E.; Fiebiger, E.; Kessler, B.M.; Anderson, K.C.; Ploegh, H.L.; Ovaa, H.; Galardy, P.J. *Nat. Methods* **2005**, *2*, 357-362. (b) Altun, M.; Galardy, P.J.; Shringarpure, R.; Hideshima, T.; LeBlanc, R.; Anderson, K.C.; Ploegh, H.L.; Kessler, B.M. *Cancer Res.* **2005**, *65*, 7896-7901.
43. Groll, M.; Berkers, C.R.; Ploegh, H.L.; Ovaa, H. *Structure* **2006**, *14*, 451-456.
44. Kim, K.B.; Myung, J.; Sin, N.; Crews, C.M. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3335-3340.
45. Elofsson, M.; Splittgerber, U.; Myung, J.; Mohan, R.; Crews, C.M. *Chem. Biol.* **1999**, *6*, 811-22.
46. Myung, J.; Kim, K.B.; Lindsten, K.; Dantuma, N.P.; Crews, C.M. *Mol. Cell* **2001**, *7*, 411-420.
47. Ho, Y.K.; Bargagna-Mohan, P.; Wehenkel, M.; Mohan, R.; Kim, K.B. *Chem. Biol.* **2007**, *14*, 419-430.
48. Marastoni, M.; Baldisserotto, A.; Cellini, S.; Gavioli, R.; Tomatis, R. *J. Med. Chem.* **2005**, *48*, 5038-5042.
49. Kisselev, A.F.; Goldberg, A.L. *Methods Enzymol.* **2005**, *398*, 364-378.
50. (a) no structural or synthetic data on [^{14}C]PS-341 available in literature. (b) Adams, J.; Palombella, V.J.; Sausville, E.A.; Johnson, J.; Destree, A.; Lazarus, D.D.; Maas, J.; Pien, C.S.; Prakash, S.; Elliott, P.J. *Cancer Res.* **1999**, *59*, 2615-2622.
51. Ovaa, H.; van Swieten, P.F.; Kessler, B.M.; Leeuwenburgh, M.A.; Fiebiger, E.; van den Nieuwendijk, A.M.C.H.; Galardy, P.J.; van der Marel, G.A.; Ploegh, H.L.; Overkleeft, H.S. *Angew. Chem. Int. Ed.* **2003**, *42*, 3626-2629.
52. Saxon, E.; Bertozzi, C.R. *Science* **2000**, *287*, 2007-2010.
53. A. E. Speers, G. C. Adam, B. F. Cravatt, *J. Am. Chem. Soc*. **2003**, *125*, 4686-4687.
54. (a) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem*. **2002**, *67*, 3057-3064. (b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem*. **2002**, *114*, 2708-2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596-2599.
55. (a) Agard, N.J.; Prescher, J.A.; Bertozzi, C.R. *J. Am. Chem. Soc.* **2004**, *126*, 15046-15047. (b) Agard, N.J.; Baskin, J.M.; Prescher, J.A.; Lo, A.; Bertozzi, C.R. *ACS Chem. Biol.* **2006**, *1*, 644-648 (c) Baskin, J.M.; Prescher, J.A.; Laughlin, S.T.; Agard, N.J.; Chang, P.V.; Miller, I.A.; Lo, A.; Codelli, J.A.; Bertozzi, C.R. *Proc. Natl. Acad. Sci. USA.* **2007**, *104*, 16793-16797. (d) Sletten, E.M.; Bertozzi, C.R. *Org. Lett.* **2008**, *10*, 3097-3099.
56. Laughlin, S.T.; Baskin, J.M.; Amacher, S.L.; Bertozzi, C.R. *Science* **2008**, *320*, 664-667.
57. Codelli, J.A.; Baskin, J.M.; Agard, N.J.; Bertozzi, C.R. *J. Am. Chem. Soc.* **2008**, *130*, 11486-11493.
58. Ning, X.; Guo, J.; Wolfert, M.A.; Boons, G.J. *Angew. Chem. Int. Ed.* **2008**, *47*, 2253-2255.
59. Hangauer, M.J.; Bertozzi, C.R. *Angew. Chem. Int. Ed.* **2008**, *47*, 2394-2397.
60. (a) Sterz, J.; von Metzler, I.; Hahne, J.C.; Lamottke, B.; Rademacher, J.; Heider, U.; Terpos, E.; Sezer, O. *Expert Opin. Investig. Drugs* **2008**, *17*, 879-895. (b) Orlowski, R.Z.; Kuhn, D.J. *Clin. Cancer Res.* **2008**, *14*, 1649-1657.
61. Loo, T.W.; Clarke, D.M. *FASEB. J.* **1999**, *13*, 1724-1732.
62. Sterz, J.; von Metzler, I.; Hahne, J.C.; Lamottke, B.; Rademacher, J.; Heider, U.; Terpos, E.; Sezer, O. *Expert Opin. Investig. Drugs* **2008**, *17*, 879-895.
63. Orlowski, R.Z.; Kuhn, D.J. *Clin. Cancer Res.* **2008**, *14* ,1649-1657.
64. (a) Kane, R.C.; Bross, P.F.; Farrell, A.T.; Pazdur, R. *Oncologist* **2003**, *8*, 508-513. (b) Bross, P.F.; Kane, R.; Farrell, A.T.; Abraham, S.; Benson, K.; Brower, M.E.; Bradley, S.; Gobburu, J.V.; Goheer, A.; Lee,

S.L.; Leighton, J.; Liang, C.Y.; Lostritto, R.T.; McGuinn, W.D.; Morse, D.E.; Rahman, A.; Rosario, L.A.; Verbois, S.L.; Williams, G.; Wang, Y.C.; Pazdur, R. *Clin. Cancer Res.* **2004**, *10*, 3954-3964.

65. Kane, R.C.; Dagher, R.; Farrell, A.; Ko, C.-W.; Sridhara, R.; Justice, R.; Pazdur, R. *Clin. Cancer Res.* **2007**, *13*, 5291-5294.
66. Kraus, M.; Rückrich, T.; Reich, M.; Gogel, J.; Beck, A.; Kammer, W.; Berkers, C.R.; Burg, D.; Overkleeft, H.; Ovaa, H.; Driessen, C. *Leukemia* **2007**, *21*, 84-92.
67. Chauhan, D.; Catley, L.; Li, G.; Podar, K.; Hideshima, T.; Velankar, M.; Mitsiades, C.; Mitsiades, N.; Yasui, H.; Letai, A.; Ovaa, H.; Berkers, C.; Nicholson, B.; Chao, T.H.; Neuteboom, S.T.; Richardson, P.; Palladino, M.A.; Anderson, K.C. *Cancer Cell* **2005**, *8*, 407-419.
68. Kuhn, D.J.; Chen, Q.; Voorhees, P.M.; Strader, J.S.; Shenk, K.D.; Sun, C.M.; Demo, S.D.; Bennett, M.K.; van Leeuwen, F.W.; Chanan-Khan, A.A.; Orlowski, R.Z. *Blood* **2007**, *110*, 3281-3290.