Towards subunit specific proteasome inhibitors

Linden, W.A. van der

1 General Introduction

1.1 Introduction

Protein quality and half life are essential for the function of a cell and are controlled in a tightly regulated fashion. Most of this work is carried out by the ubiquitin-proteasome system (UPS), which degrades 60-90% of all proteins in the nucleus and cytoplasm of the eukaryotic cell.[1,2] Central function in this process is proteolysis of substrates by the 26S proteasome, a large multicatalytic complex, crucial for cell viability. The proteasome degrades abnormal, damaged or misfolded proteins and is responsible for degrading short-lived regulatory proteins involved in cell differentiation, cell cycle regulation, transcriptional regulation, inflammation and apoptosis. Proteasomal degradation of substrates generates peptides, a fraction of which are used for MHC class I presentation. Hence, the proteasome is involved in almost all cellular processes and its deregulation is associated with many diseases. The proteasome contains catalytically active subunits with different substrate specificities and the role of these individual proteolytic activities of the proteasome in normal or diseased state is a question that is largely unanswered. This thesis aims at developing subunit specific inhibitors of the proteasome to study the individual function of proteasome active subunits *in vivo*.

1.2 Ubiquitin Proteasome System

The ubiquitin proteasome system is the major proteolytic machinery in the eukaryotic cell. Its central task is the degradation of proteins in an ATP and ubiquitin (Ub) dependent fashion. Ubiquitin, a 9 kDa highly conserved 76 residue protein, is attached to a target protein by an ATP-dependent cascade of E1, E2 and E3 enzymes.[3] Ubiquitin is activated by one of the two E1 enzymes,[4] which transfers ubiquitin to E2, a family of several dozen ubiquitin-conjugating enzymes. A member of the very large family of E3 enzymes then mediates the transfer of activated ubiquitin *via* its C-terminus to a lysine ε-amine of a pro-

tein substrate or an already linked ubiquitin.[5,6] Ubiquitination is further regulated by the action of one of the many deubiquitinating enzymes (DUB).[7] If a protein contains a chain of four or more Lys48-linked ubiquitin residues, it is recognised by the 26S proteasome as target for destruction (Figure 1.1A).[8] Ubiquitin is recycled as the ubiquitinated substrate is transported into the catalytic chamber of the proteasome, where the peptide is cut into small fragments. The average length of peptide products is 3-22 residues, with an average length of 8-12 amino acids.[9] Most peptides are degraded further into single amino acids downstream of the proteasome. About 1% escapes this fate and is trimmed N-terminally by cytosolic aminopeptidases, transported into the endoplasmatic reticulum (ER) to be loaded on a MHC class-I complex and presented at the cell surface. The proteasome is thus responsible for generating the C-terminus of most antigenic peptides.[10]

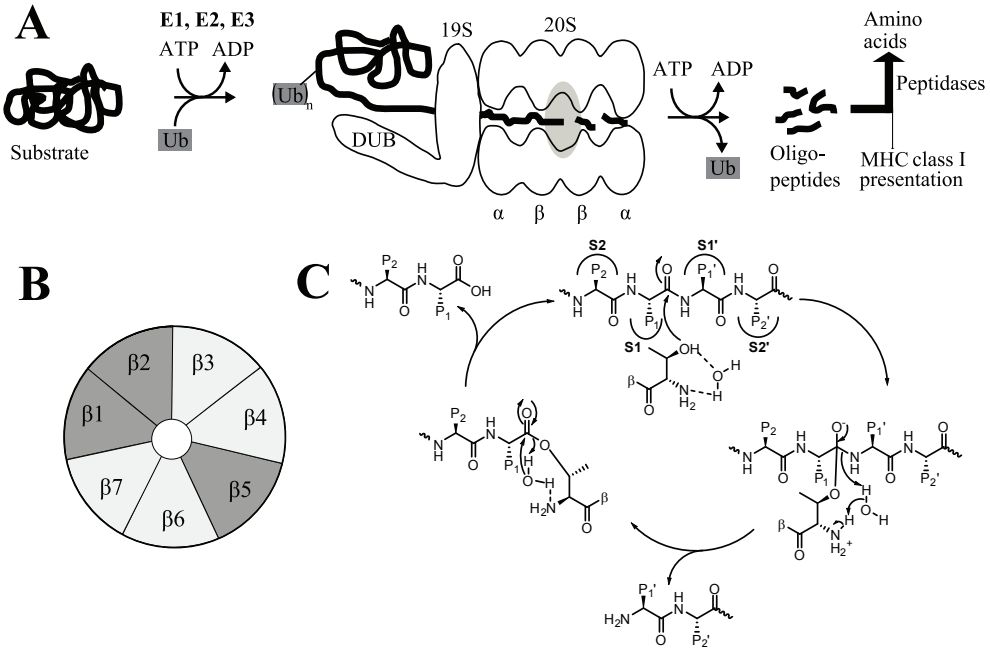

Figure 1.1: (**A**) Schematic presentation of protein catabolism by the proteasome. (**B**) Schematic top view of β ring of the constitutive proteasome. Proteolytic active subunits are depicted in dark gray (**C**) Proteolytic cycle of proteasome β subunit.

1.3 Structure and function of the proteasome

The term proteasome refers to a collection of different forms of proteasome, which all contain the catalytic 20S core particle. The eukaryotic 20S core particle is a 700 kDa complex and is built up from four heptameric rings, two rings of seven distinct α subunits and two rings of β_{1-7} subunits, stacked in the order αββα to form a hollow cylindrical structure 160Å by 120Å in dimension. The inside of this cylinder is partitioned into three cavities

of which the central cavity, lined by β subunits, contains the proteolytic active sites. Only β1, β2 and β5 contain proteolytic activity performed by the N-terminal threonine of these subunits (Figure 1.1B). Via a water molecule, the N-terminal amine group activates the γ hydroxyl of Thr1, which performs a nucleophilic attack on the target amide bond.[11] A tetrahedral intermediate is formed, which then degrades into an ester-bound peptide, releasing the amine product of the substrate cleavage. The ester bond is hydrolysed by a water molecule, regenerating the proteasome Thr1 residue and releasing the carboxylic acid product of the substrate (Figure 1.1C).

The catalytically active β subunits differ in their substrate preference, which is induced by differences in their substrate binding pockets. The β1 subunit (Y/δ) prefers cleaving after acidic residues (caspase-like activity), β2 (Z) prefers cleavage after basic residues (trypsin-like activity) and the β5 (X) prefers to cleave after hydrophobic residues (chymotrypsin-like activity). Substrate preference of the active β has been determined with fluorogenic peptides and their activities are not as strictly defined as their names suggest and partly overlap.[12] For example, next to its preference for acidic residues, β1 is able to cleave after branched amino acids, residues also processed by β5.[13]

Entry of protein substrate into the channel of the 20S proteasome is hindered by α N-termini, preventing unregulated entry of substrates in the catalytic chamber.[14] To open this gate to the proteasome, binding of a regulatory particle is required. Two 19S regulatory particle cap the 20S core particle to yield the 26S proteasome.[15] The 19S regulatory particle recognises and deubiquitinates ubiquitinated substrates and promotes their unfolding, while the base of the particle that binds to the α-ring of 20S contains Rpt-subunits that regulate the opening of the channel into the 20S core particle in an ATP-dependent fashion.[3,6,16,17]

In mammals, the cytokine interferon-γ induces the synthesis of three catalytic subunits with different substrate preference. These subunits, called β1i (LMP2), β2i (MECl1) and β5i (LMP7) replace their constitutive counterparts in newly formed immunoproteasomes.[18] Furthermore, interferon-γ induces the expression of a different regulatory complex, the 11S or PA28 regulator, able to doubly cap the 20S core particle or form a hybrid proteasome (11S-20S-19S).[19–21] The immunoproteasome displays substrate preferences slightly different from the constitutive proteasome.[22,23] Peptides with an average length of 8-10 amino acids are generated, with more basic and hydrophobic residues at their C-terminus. These peptides are more optimal for binding to the MHC class I complex for presentation on the cell surface.[10] For a long time, the function of the immunoproteasome was assumed to be only to increase the pool of antigenic peptides. However, the immunoproteasome displays increased efficiency in the degradation of oxidized proteins and its main function appears to preserve protein homeostasis after inflammation.[24,25] In cortical thymic epithelial cells a seventh active β subunit is expressed, which is called β5t. Compared to β5 or β5i, the β5t substrate pocket is more hydrophilic. Subunit β5t is found together with mainly β1i and β2i in so called thymoproteasomes. The chymotryptic activity of thymoproteasomes is decreased with respect to constitutive or immunoproteasomes and thymoproteasomes generate low-affinity MHC class I antigens. This altered pool of MHC class I ligands is important for the positive selection of developing thymocytes, although the exact role of β5t in this process is not yet clarified.[26,27]

1.4 Proteasome inhibitors

The UPS has a central role in protein degradation, which ensures its involvement in many processes. Much knowledge about the proteolytic role of the proteasome in a cellular pathway has been gathered with the aid of proteasome inhibitors. Over the last decades, an increasingly large number of proteasome inhibitors has been identified, showing a broad range of structural features and cellular activities. Nature provides a large spectrum of complicated molecules able to block proteasomal function and the apparent medicinal benefits of many of these molecules inspired organic chemists to expand the versatility of proteasome inhibitors even more. Often, a proteasome inhibitor contains an electrophile that is able to covalently capture the Thr1 Oγ of the proteasome and thereby blocking its catalytic activity. However, many inhibitors inhibit the proteasome in a non-covalent way. This Chapter gives an overview of classes of proteasome inhibitors.

1.5 Natural occurring proteasome inhibitors

In the search for new leads for medicine development, natural products are very often a source of inspiration for the development of new agents directed towards therapeutic targets. Natural products are commonly extracted from plant tissues, marine organisms or microorganism fermentation broths and screened for beneficial effects on various disease models. The cellular target of a biologically active molecule identified in such a screen is then sought for. In cases where natural products inhibit cell cycle progression, inflammation or microorganism growth, not rarely the proteasome is found to be inhibited. The variety of natural occurring proteasome inhibitors is very large and this diversity provides many lead structures for proteasome inhibitors.

1.5.1 Beta-lactones

The metabolite lactacystin (**1**, Figure 1.2A), produced by a *Streptomyces* strain, was identified as an inducer of differentiation of a mouse neuroblastoma cell line and inhibitor of cell cycle progression due to proteasome inhibition. Lactacystin itself is inactive against the proteasome, but upon lactonisation, the beta-lactone omuralide is formed (**2**, Figure 1.2A), a potent proteasome inhibitor.[28–30] Three other families of beta-lactone natural products, structurally related to omuralide, have been identified to date. In extracts of the marine actinomycete *Salinospora tropica*, salinosporamides were identified as agents cytotoxic to cancer cell lines, with salinosporamide A (**3**) as most potent member.[31] Highly related to salinosporamides are cinnabaramides, metabolites from terrestrial streptomycete *S. cinnabarinus* with cinnabaramide A (**4**) depicted in Figure 1.2A.[32] Belactosin A (**5**), a metabolite from *Streptomyces* sp. UCK14, displayes antitumour and antimicrobial activity though cell cycle inhibition, which was later ascribed to proteasome inhibition.[33,34] Beta-lactones inactivate the proteasome by nucleophilic attack of the proteasome Thr1 Oγ on the carbonyl of the lactone, thereby forming an ester adduct with the Thr1 Oγ of the active subunits (Figure 1.2B). In the case of salinosporamide A, as a result of beta-lactone opening, a second reaction occurs, forming a tetrahydrofuran ring by displacement of the chloride atom. The ester bond mediated active site inactivation by beta-lactones is reversible, although this is a slow process because the orientation of the inhibitor-proteasome adduct is such that

the entry of water into the active site is prevented, thereby preventing deacylation of the proteasome's catalytically active moiety.[35]

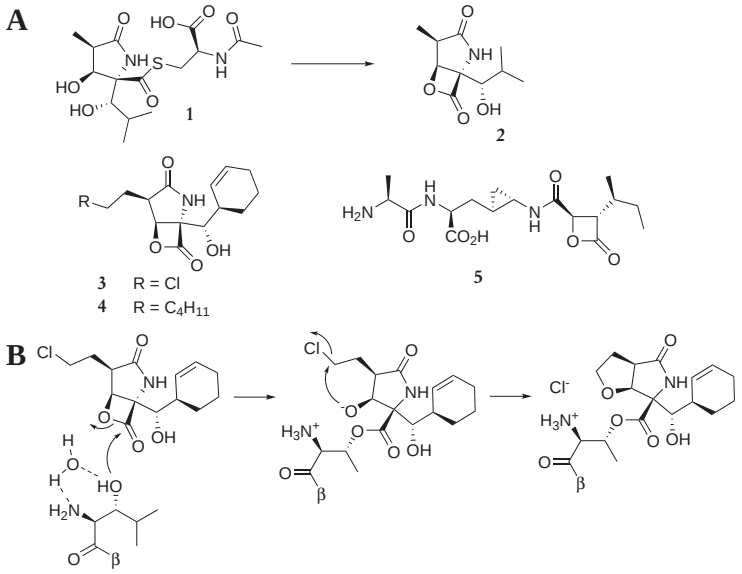

Figure 1.2: Structure (**A**) of beta-lactones found in nature and (**B**) proteasome inhibition mechanism of salinosporamide A.

1.5.2 Aldehydes

Leupeptin (**6**, Figure 1.3A) is a protease inhibitor with a broad range of enzyme families as target due to its reactive aldehyde electrophile. This compound, isolated from various strains of *Actinomycete*, inhibits several serine and cysteine proteases and was found to in-

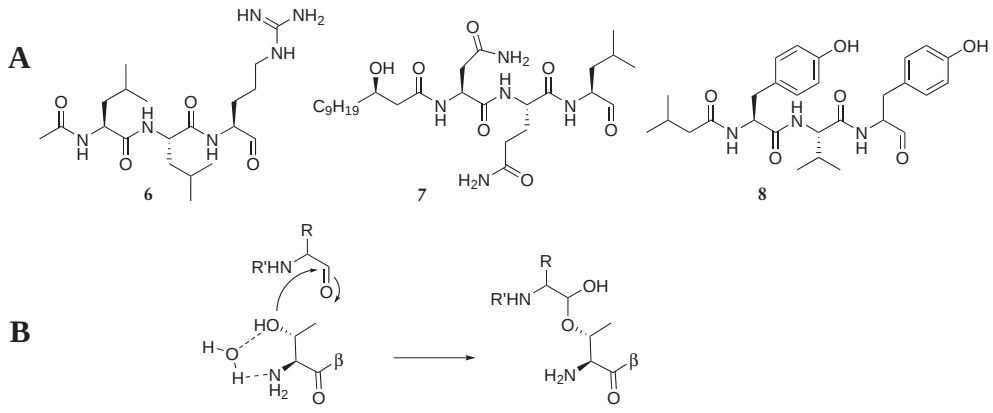

Figure 1.3: Structure (**A**) of aldehyde containing inhibitors found in nature and (**B**) their proteasome inhibition mechanism.

hibit the β2 activity of the proteasome as well.[36,37] Fellutamides are a family of cytotoxic peptides from marine fungus *Penicillium fellutanum*, and fellutamide A (**7**, Figure 1.3A) is a potent proteasome inhibitor with some preference for the β5 subunit.[38,39] Another natural occurring β5 preferring proteasome inhibitor is tyropeptin A (**8**, Figure 1.3A) isolated from *Kitasatospora* bacteria in a search for new proteasome inhibitors.[40,41] Proteasome inactivation by aldehydes occurs by attack of the proteasomal Thr1 Oγ on the aldehyde, forming a hemiacetal bond (Figure 1.3B). The hemiacetal bond with the proteasome is reversible and aldehydes are rapidly inactivated by oxidation to the carboxylic acid *in vivo* and therefore the effect of aldehydes is not sustained.

1.5.3 α',β'-epoxyketones

In a screen for agents with antitumor activity, natural occurring α',β'-epoxyketone containing peptides epoxomicin (**9**) and eponemycin (**10**, Figure 1.4A) were discovered in an *Actinomycete* and *Streptomycete* strain, respectively.[42,43] Closer analysis of fermentation broths of species of *Streptomyces* identified more linear α',β'-epoxyketone containing peptides analogous to epoxomicin or eponemycin, named TMC-86, TMC-89 and TMC-96.[44,45] This marked the discovery of truly selective proteasome inhibitors.[46,47] The selectivity of these compounds comes forth from the unique interaction of this electrophile with the proteasome Thr1 Oγ (Figure 1.4B). First, the Thr1 Oγ performs an attack on the carbonyl of the ketone, forming a reversible hemiacetal linkage. Next, the free amine from Thr1 opens the epoxide ring, irreversibly forming a morpholino ring.[48] Because many other common off-targets for proteasome inhibitors, such as cysteine or serine proteases, do not possess this N-terminal threonine functionality, these enzymes can not form the morpholino adduct as the proteasome does. Consequently, the proteasome is the only cellular target found for epoxomicin so far.[46,47]

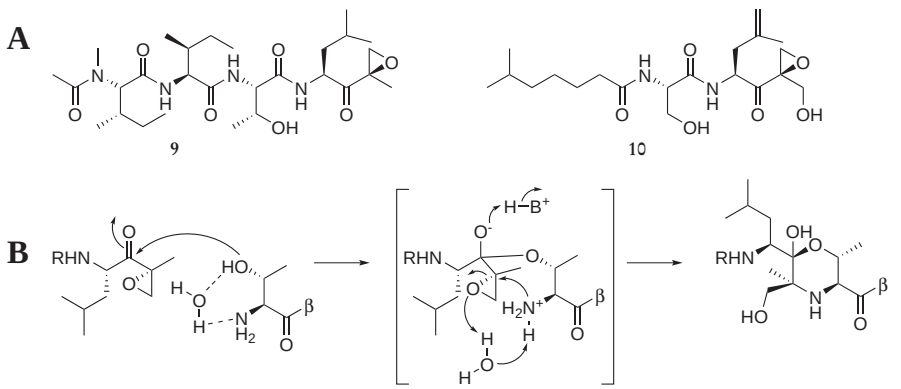

Figure 1.4: Structure (**A**) of α'-β'-epoxyketone containing inhibitors found in nature and (**B**) their proteasome inhibition mechanism.

1.5.4 Syrbactins

Syrbactins are a class of structurally related proteasome inhibitors, containing syringolins, glidobactins and the closely related cepafungins[49] as subfamilies.[50] Glidobactins were isolated from a *Burkholderia* species, in a search for antitumour and antimicrobial agents, but

its cellular target was then not yet determined.[51] Later, syringolin A (**11**, Figure 1.5A), secreted by plant pathogen *Pseudomonas syringae* pv. *syringae* as a virulence factor when invading a plant and the earlier found structurally related glidobactin A were both identified as potent inhibitors of the proteasome.[52–54] Syrbactins share a twelve-membered unsaturated lactam core structure. The α,β-unsaturated amide in this lactam structure undergoes Michael addition of the Thr1 Oγ of the proteasome catalytically active subunit to yield an irreversible ether band (Figure 1.5B).

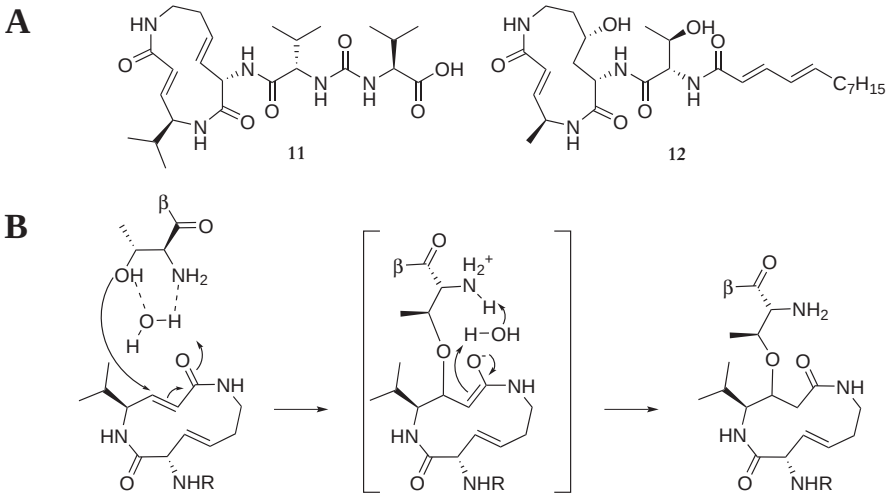

Figure 1.5: Structure (**A**) of syrbactins and (**B**) their proteasome inhibition mechanism.

1.5.5 Non-covalent natural peptoid proteasome inhibitors

The fungal plant pathogen *Apiospora montagnei* was found to produce complex cyclic peptoid molecules able to inhibit the proteasome with high selectivity.[45,55] TMC95A (**13**, Figure 1.6), the most potent of this family, inhibits the proteasome in a non-covalent way, as was deduced from crystallographic studies. A network of hydrogen bridges accomplishes the formation of an anti-parallel β-sheet in the active β-sites of the proteasome, causing potent competitive binding of this peptide-like structure.[56]Another , are isolated from myxobacteria.[57] Argyrin A (**14**, Figure 1.6), member of a naturally occurring cyclic peptide family called argyrins, was found to stabilize a tumour suppressor protein through inhibition of the proteasome.[58] The exact binding mechanism remains to be determined, but an analogue of argyrins lacking the dehydroalanine moiety lacks proteasome inhibitory capacities.[59] Scytonemide A (**15**), a cyclic peptide-like molecule isolated from cyanobacterium *Scytonema hofmanii*, potently inhibits the proteasome. Its proteasome binding mechanism has not yet been elucidated, although covalent inactivation by the imine functionality is not excluded.[60] PR39 is an arginine and proline rich antibacterial peptide derived from porcine bone marrow that was found to inhibit the proteasome by perturbing the 20S core particle of the proteasome in an allosteric fashion.[61]

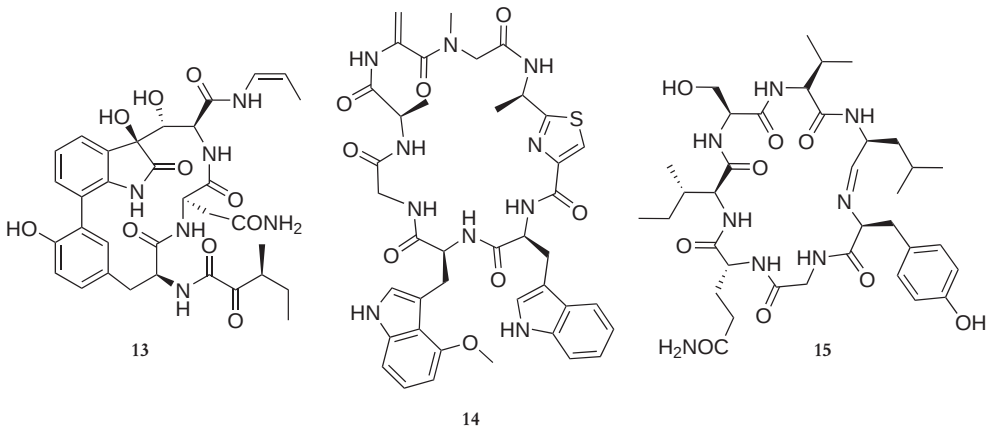

Figure 1.6: Structure of naturally occurring cyclic peptide proteasome inhibitors

1.5.6 Triterpenoids and flavonoid proteasome inhibitors

Triterpenoids form a diverse family of compounds, synthesised in plants from squalene, a precursor or steroids. A very large variety of triterpenoids have been isolated from plants used in traditional medicine. Subsequently, extracts of these plants were used in screens against various disease models.[62–64] A large number of triterpenoids were identified as 20S proteasome inhibitors, however, these molecules often have multiple cellular targets. Agosterol C (**16**, Figure 1.7), extracted from marine sponge *Acanthodendrilla* reverses multidrug resistance in tumour cell lines and has been found to inhibit the chymotryptic activity of the proteasome, although the mechanism of inhibition is unclear.[65,66] Withaferin A (**17**) and celastrol (**18**), terpenoids isolated from medicinal plants 'Indian Winter

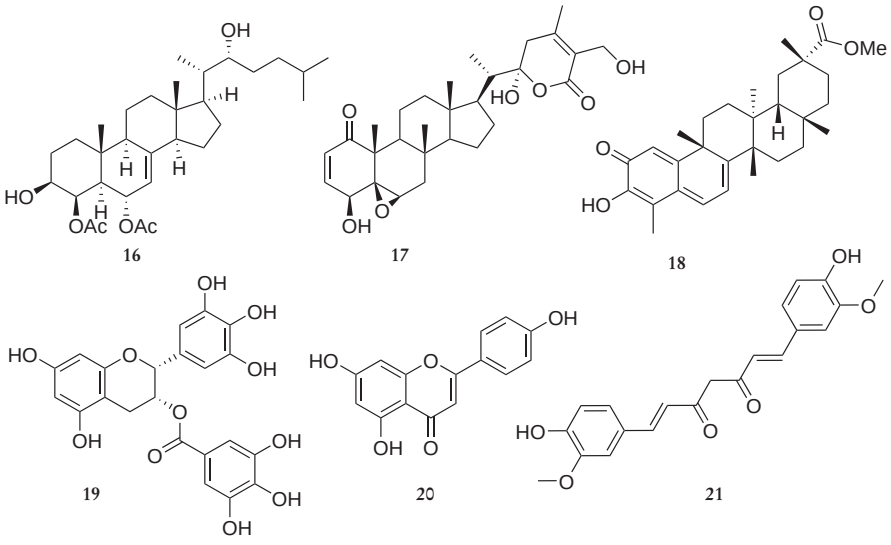

Figure 1.7: Structure of naturally occurring triterpenoid and flavonoid proteasome inhibitors.

Cherry' and 'Thunder of God vine', respectively, have been investigated for their anticancer properties. Withaferin A and celastrol inhibit β5 of the proteasome, and hypothetically, these two compounds are able to form a covalent adduct with cellular targets *via* unsaturated carbonyl moieties present in these triterpenoids.[67–69] Another large family of natural occurring compounds from which many show proteasome inhibition are the flavonoids. Green tea polyphenol (-)-epigallocatechin-3-gallate (**19**, Figure 1.7) potently inhibits the chymotryptic activity of the proteasome.[70] Docking studies suggest that the mechanism of proteasome inhibition could be acylation of the Thr1 hydroxyl.[71] Many other flavonoids commonly found in vegetable and fruit inhibit the chymotryptic activity of the proteasome.[72–74] Apigenin (**20**, Figure 1.7), found in celery seeds, is one of the most potent proteasome inhibitors and was found to have anticancer effects[75,76] Curcumin (**21**), found in turmeric[77] is also a proteasome inhibitor. Although docking studies suggest that the unsaturated ketone in flavonoids could be susceptible to nucleophilic attack, covalent proteasome inhibition by flavonoids has not been proven. The binding mode of these compounds, however, is not clearly competitive.[73] It remains unclear whether the anticancer effects of triterpenoids and flavonoids are caused solely through proteasome inhibition.

1.6 Synthetic proteasome inhibitors

With a very large variety of natural product proteasome inhibitors as inspiration, chemists have been synthesising proteasome inhibitors for about two decades. Natural products inspired scaffolds were optimised for selectivity for the proteasome, increasing potency and pharmacological benefits, and several new pharmacophores able to bind the proteasome have been found. An overview of synthetic proteasome inhibitors follows.

1.6.1 Reversible covalent proteasome inhibitors

Simple peptide aldehydes, such as the natural occurring leupeptin and the synthetic calpain inhibitor I and II (**22**, **23**, Figure 1.8), were found to inhibit the proteasome, but suffered from many off-targets.[1,37,78] This lack of selectivity sparked a large effort to develop more

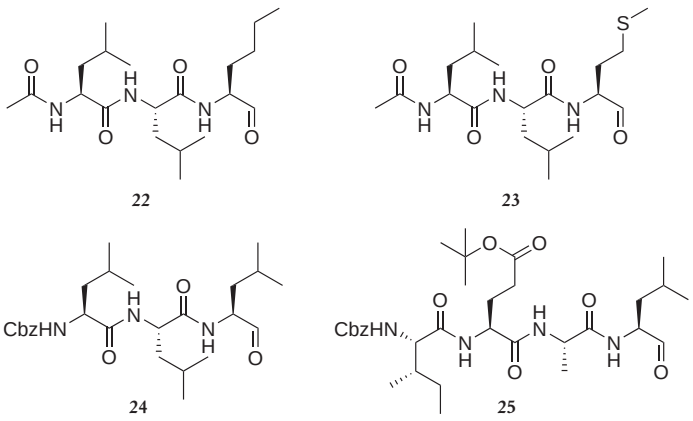

Figure 1.8: Structure of some synthetic aldehyde containing proteasome inhibitors.

proteasome specific peptide aldehydes. MG132 (**24**) displays more selectivity for the proteasome over other enzymes such as calpains or cathepsins and is still widely used as a model inhibitor of the proteasome.[78–80] Compound **25** was developed later as an even more specific proteasome inhibitor.[81,82] However, the reactivity of the aldehyde electrophile enables inhibition of targets other than the proteasome, limiting their use *in vivo*.

Since the discovery of peptide aldehydes, many other electrophiles have been synthesised and evaluated for their capacity to selectively inhibit the proteasome in a covalent reversible way. The boronic acid moiety yields inhibitors more potent than aldehyde counterparts and suffers less from cross-reactivity with cysteine proteases, although serine proteases can be modified by the boronic acid. Optimisation of the peptide sequence of a boronic acid inhibitor yielded bortezomib, which shows selectivity for the proteasome over most serine proteases (**26**, Figure 1.9A).[80] The empty orbital of boron acts as an electrophile, capturing the proteasomes active site hydroxyl in a reversible way (Figure 1.9B). Bortezomib has been approved by the FDA for the treatment of multiple myeloma. The success of this inhibitor in the treatment of cancer resulted in a surge of second generation boronic acid based inhibitors. For example, CEP18770 (**27**, Figure 1.9A) is an orally

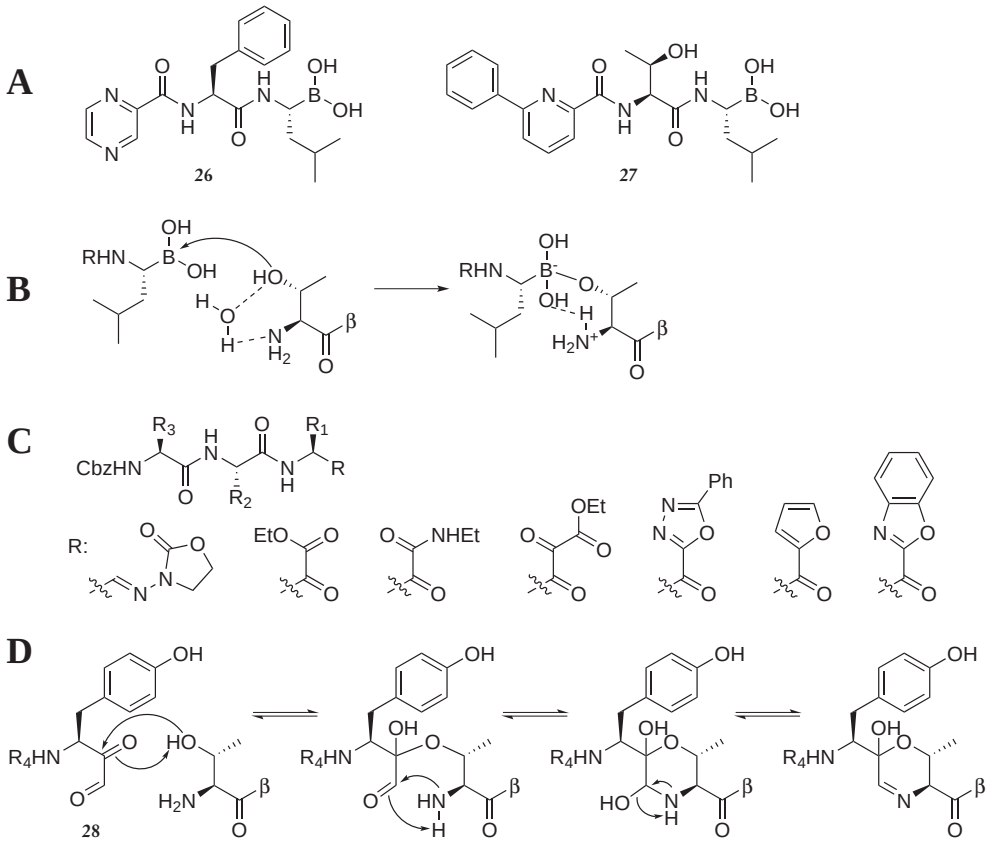

Figure 1.9: (**A**) Structure of boronic acid containing proteasome inhibitors with medicinal benefits. (**B**) Proteasome inhibition mechanism of boronic acid electrophile. (**C**) Structure of reversible covalent electrophiles (**D**) Proteasome inhibition mechanism of α-ketoaldehyde electrophile.

bioavailable selective proteasome inhibitor that is currently in clinical trials against multiple myeloma.[83]

An overview of other electrophiles able to bind the proteasome in a covalent and reversible way is provided in Figure 1.9C.[78,80,84–89] The mechanism of proteasome inhibition by the α-ketoaldehyde electrophile, present in the inhibitor Cbz-Leu-Leu-Tyr-COCHO (**28**), shows similarities with the epoxyketone electrophile. The catalytic Thr Oγ first forms a reversible hemiacetal linkage with the ketone of the α-ketoaldehyde. Then, the N-terminal amine of the proteasome reacts with the aldehyde group and reversibly forms a 5,6-dihydro-2*H*-1,4-oxazine ring (Figure 1.9D).[90] This bivalent inhibition mechanism of N-terminal threonine proteases explains the high selectivity of Cbz-Leu-Leu-Tyr-COCHO for the proteasome over proteases with different catalytic mechanisms.

1.6.2 Non-covalent synthetic proteasome inhibitors

The reactivity of many pharmacophores to bind the proteasome in many cases leads to side reactions of these covalent inhibitors with targets other than the proteasome. Non-covalent proteasome inhibitors lacking a reactive electrophile were developed. A library of 5-methoxy-1-indanones was screened for 20S proteasome inhibition, resulting in several linear peptides able to inhibit the chymotryptic activity of the proteasome non-covalently, with compound **29** (Figure 1.10) able to inhibit proteasome activity in cells.[91–93] The demanding synthesis of natural product TMC95A (**13**, Figure 1.6) led to the development of simplified cyclic analogues. These compounds, such as biaryl ether **30** (Figure 1.10) are less potent then TMC95A.[94,95] Optimisation of simplified TMC95A analogues led to

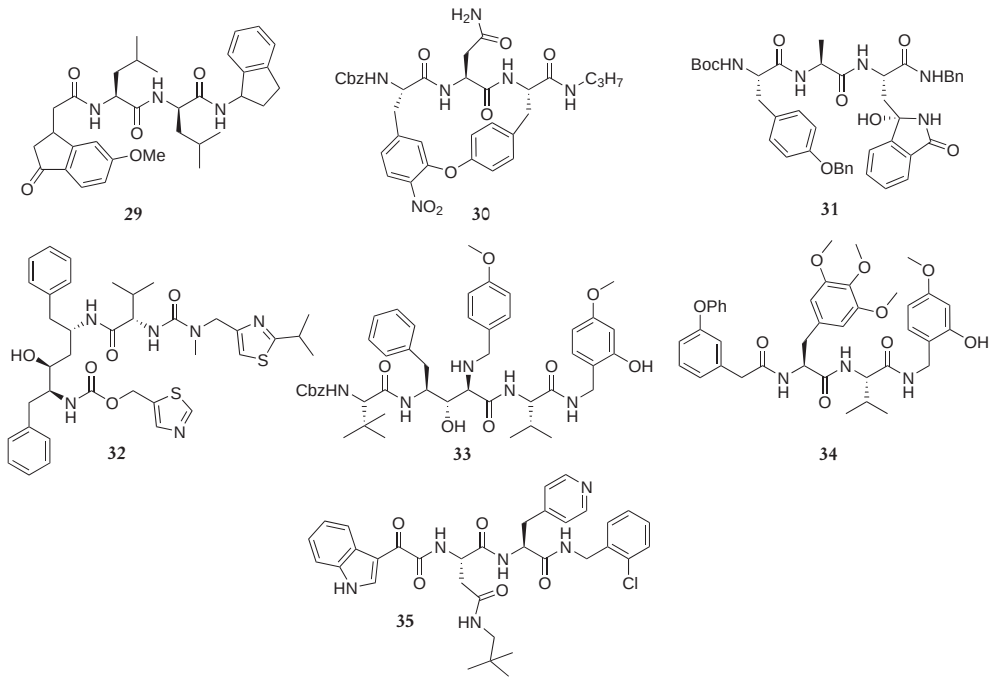

Figure 1.10: Structure of some noncovalent peptide based proteasome inhibitors.

linear analogue **31**, inhibiting the chymotryptic activity of the 20S proteasome.[96,97] Another source of inspiration for peptoid non-covalent proteasome inhibitors was found in HIV protease inhibitor ritonavir (**32**, Figure 1.10), which was found to inhibit the proteasome.[98] The related benzylstatine peptide **33** was synthesised in the course of a HIV protease project and optimisation of this motif yields a wide range of β5 selective proteasome inhibitors.[99–101] Further optimisation of linear peptide proteasome inhibitors led to **34** (Figure 1.10), which displays better cell-permeability than the benzylstatine peptides.[102] Independently, **35** was found to be a potent and cell permeable noncovalent inhibitor of proteasome chymotryptic activity.[93,103,104]

1.6.3 Peptide vinyl sulfones

Peptide vinyl sulfones, originally designed as inhibitors of cysteine proteases,[105] were discovered to irreversibly inhibit the proteasome when the aldehyde group in MG132 was replaced by a vinyl sulfone to give **36** (Figure 1.11A).[106] The proteasome's active site Thr1 Oγ attacks the vinyl sulfone in a Michael addition way, forming a stable ether linkage with the inhibitor (Figure 1.11B). The irreversibility of proteasome inhibition and ease of synthesis of these vinyl sulfone compounds enables the use of these scaffolds for activity-based probes (ABP). The first vinyl sulfone ABP for the proteasome is compound **37**, which is

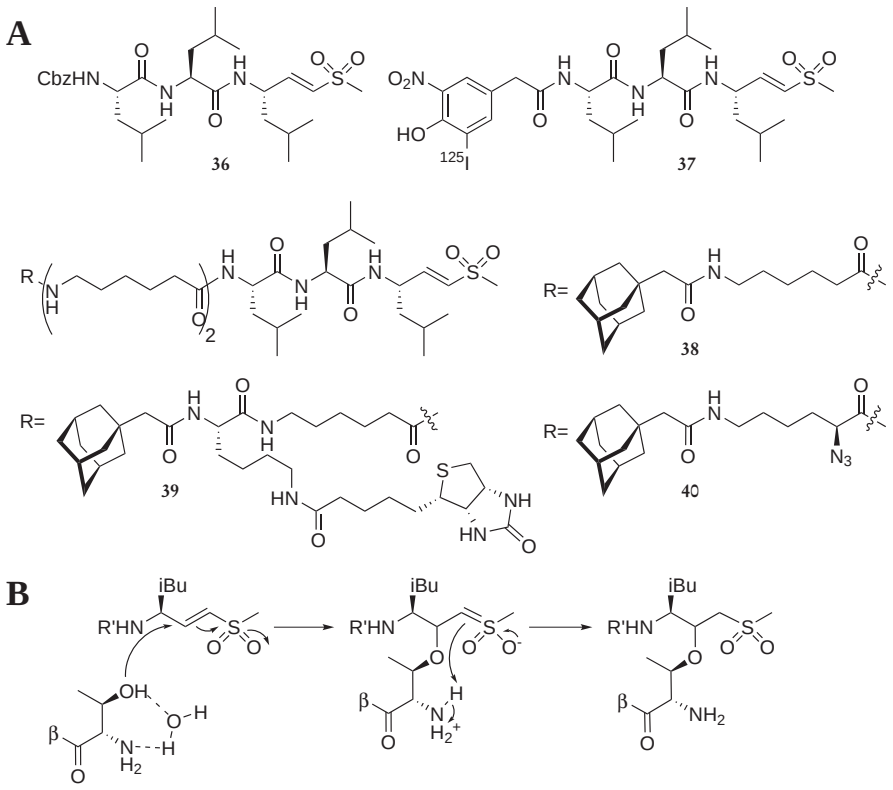

Figure 1.11: (**A**) Structure of vinyl sulfone inhibitors and ABPs. (**B**) Proteasome inhibition mechanism of vinyl sulfone electrophile.

labelled with ^{125}I and can be detected by autoradiography.[106] This probe has most affinity for the chymotryptic activity of the proteasome. N-terminal extension of the Leu_3-VS core, such as the adamantyl acetic acid-triaminohexanoic acid spacer in compound **38** yields an inhibitor with increased potency, good cell permeability and about equipotent activity for the three proteasomal subunits.[107] This finding was exploited by addition of a biotin as in compound **39** to yield an ABP that can be detected by immunoblotting. The presence of a biotin moiety, however, hampered cell permeability of this compound. To overcome this limitation, an azide group was introduced in the elongated inhibitor design to get a two-step labelling ABP, **40**. The azide moiety can be used to introduce a biotin after the probe bound its target, for example introduction of a biotin by the Staudinger-Bertozzi ligation.[108] This two-step labelling strategy is used often in cases where introduction of a large tag, such as biotin, negatively influences the biological characteristics of a probe. To be able to directly visualise labelled proteasomes in living cells, the Ahx_3-Leu_3-VS scaffold was decorated with a dansyl group to arrive at fluorescent ABP **41** (Figure 1.12).[109] This fluorescent ABP was used to study *in vivo* subunit specificity of anti-myeloma agent bortezomib. Substitution of the dansyl group by the BODIPY-TMR fluorophore yielded a probe (MV151, **42**) that allows detection of labelled proteasome subunits in vivo as well as directly in SDS-PAGE gels, greatly simplifying proteasome activity readout assays.[110] Azido-BODIPY probe **43** combines direct readout by fluorescence with the presence of an azide moiety, opening up the possibility to directly study two-step labelling.[111]

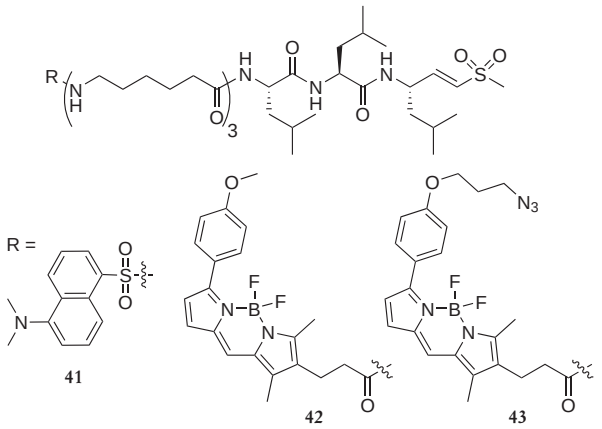

Figure 1.12: Structure of vinyl sulfone based fluorescent activity-based probes for the proteasome.

1.6.4 Synthetic peptide α',β'-epoxyketones

Synthetic epoxyketones were evaluated as proteasome inhibitors first by Spaltenstein et al. in 1996 (**44**, Figure 1.13).[112] Later, the cellular target of potent antitumour agents epoxomicin and eponemycin was determined to be the proteasome and the exclusive selectivity of epoxomicin for the proteasome was illustrated by biotinylated probe **45**.[46,47,113] Attachment of BODIPY fluorophores to the epoxomicin scaffold yielded three epoxomicin analogues with different fluorescent properties. BODIPY-TMR-epoxomicin (MVB003, **49**) and azido-BODIPY-epoxomicin **50** show excellent subunit labelling in cells and have been used

to study the β5t subunit.[114,115] The superior proteasome selectivity inherent to this electrophile lead to its widespread use in proteasome inhibitors and numerous epoxyketone peptides with remarkable potencies and selectivities have been synthesised. Several of these inhibitors will be discussed later in this Chapter.

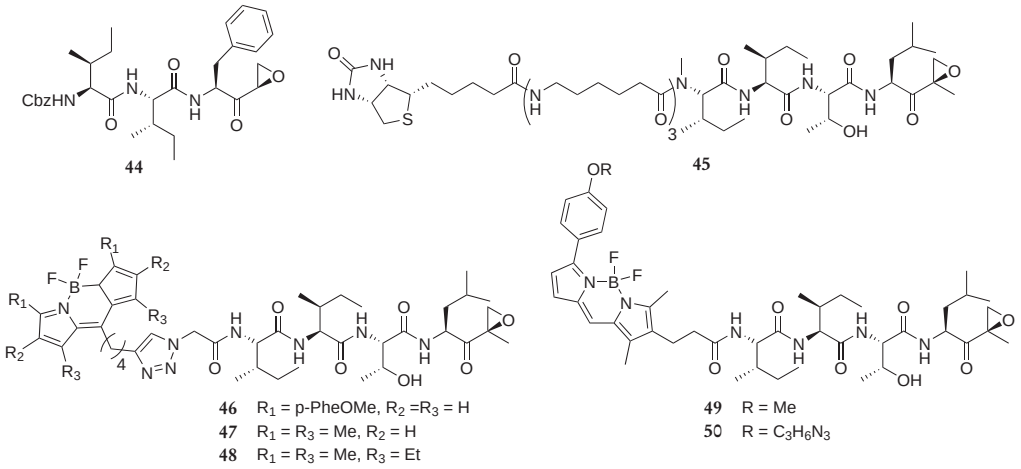

Figure 1.13: Structure of synthetic epoxyketone inhibitors and activity based proteasome probes.

1.7 Therapeutic implications of proteasome inhibition

Controlled protein degradation by the 26S proteasome is crucial for survival of eukaryotic cells. Among its targets are cell cycle regulators, tumour suppressors, oncogens and regulators of apoptosis. Tumour cells are more sensitive to proteasome inhibition than normal cells and this provides a therapeutic window for proteasome inhibitors to be used against cancers. Cytotoxicity of proteasome inhibitors in cancer cells is the result of several mechanisms.[116] Interference with timed destruction of cell cycle regulators induces cell cycle arrest in malignant cells. $P27^{Kip1}$ is a tumour suppressor and various malignancies display enhanced ubiquitination of this protein, lowering its concentration due to degradation by the proteasome. Proteasome inhibition rescues this protein, causing cell cycle arrest and thus inhibits tumour cell proliferation.[58] The endoplasmatic reticulum (ER) is responsible for the folding and maturation of proteins, and misfolded proteins are degraded by the proteasome. Exposure to proteasome inhibitors leads to prolonged accumulation of misfolded proteins which cause ER stress, a pro-apoptotic signal. Malignant cells are dividing quickly and have a high protein synthesis rate and are therefore more prone to pro-apoptotic ER stress. Apoptosis is tightly regulated by opposing activities of pro-apoptotic and anti-apoptotic proteins, whose levels are modulated by the proteasome. Inhibition of the proteasome leads to higher levels of pro-apoptotic proteins such as p53, Bax and Noxa, which are often inactivated in tumour cells.[117] The proteasome is responsible for degradation of IκB, an inhibitor of NF-κB. NF-κB is a transcription factor that regulates various immune and inflammatory responses, stimulates proliferation and suppresses apoptosis and is upregulated in many cancer types. Bortezomib and other proteasome inhibitors prevent

the degradation of IκB and thereby NF-κB is inactivated, reducing proliferation and sensitizing the cells to apoptosis. Proteasome inhibitors show synergistic enhancement of cytotoxicity of other anticancer agents, such as melphalan and cyclophosphamide (inducers of DNA damage), dexamethasone (anti-inflammatory agent), doxorubicin (DNA intercalator that disrupts transcription and replication), and thalidomide.[118]

In 2003, bortezomib received FDA approval to be used against multiple myeloma and later against mantle cell lymphoma. Treatment of multiple myeloma with bortezomib is associated with many side effects, among which are peripheral neuropathy and thrombocytopenia, and this limits the dosage and therapeutic window of this drug.[119] Upon prolonged exposure to bortezomib, cancers can become resistant to the action of bortezomib. This is thought to occur by a changed proteasome level and altered β subunit activity pattern.[120,121] In cultured cells resistant to bortezomib, a mutation in the S1 of β5 was found which reduces binding of bortezomib to this subunit. Whether this mutation plays a role in clinical resistance to bortezomib remains to be determined. Bortezomib displays no efficacy against solid tumours. These limitations of bortezomib sparked the generation of second generation proteasome inhibitors of which five are now in clinical trials (Figure 1.14).

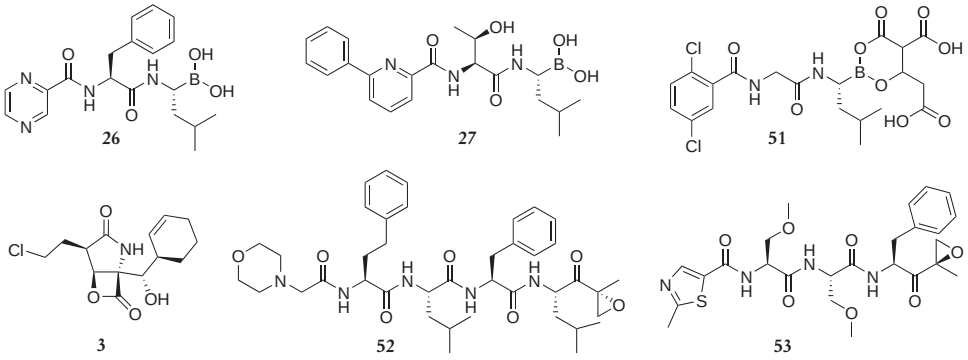

Figure 1.14: Structure proteasome inhibitor bortezomib (**26**) used clinically against multiple myeloma and structures of proteasome inhibitors in clinical trials against various types of cancer, CEP-18770 (**27**), MLN-9708 (**51**), salinosporamide A (marizomib, **3**), carfilzomib (**52**), ONX-0912 (**53**).

Reversible covalent inhibitors **27** and **51** both can be administered orally and are in clinical trials against multiple myeloma, and **51** shows better activity against solid tumours than bortezomib. Irreversible binding epoxyketone based inhibitor carfilzomib (**52**) inhibits the chymotryptic-like proteasome activity and induces cancer cell death more potently than bortezomib, and was found to overcome resistance of tumour cells to bortezomib. Epoxyketone **53** displays similar subunit preference to carfilzomib and is optimised for oral bioavailability. In cultures of malignant cells, beta-lactone salinosporamide A (clinical name currently marizomib, **3**), an inhibitor of chymotryptic and tryptic activity of the proteasome has been shown to overcome resistance to bortezomib, which inhibits chymotryptic and caspase-like proteasomal activity. Furthermore, salinosporamide A displays synergistic interaction with bortezomib, and is dependent on the stabilisation of pro-apoptotic proteasome substrates other than bortezomib for its anticancer efficacy.[122] Compounds salinosporamide A (**3**), carfilzomib (**52**) and ONX-0912 (**53**) are in clinical trials against haematological tumours and in a number of solid tumours.[117]

Bortezomib displays a suppressive effect on activated cells of the immune system and its activity against proliferating cells is most likely at the basis of this effect. Bortezomib prolongs the function of transplants in mouse models of transplantation, most likely due to ER-stress triggered induction of apoptosis in cells producing antibodies against the transplant.[123] Proteasome inhibition also shows promising results in models of autoimmune diseases.[124]

1.8 Subunit selective proteasome inhibitors

The proteasome is a very important part of cellular homeostasis in eukaryotic cells and modulators of proteasome activity have demonstrated a number of medicinal benefits. In mammals, seven distinct active β subunits are expressed and the individual role of each of these subunits in biological processes is not well understood. The function of proteolytic subunits of the proteasome cannot be studied individually as recombinantly expressed proteins since these proteolytic subunits only work in a fully assembled proteasome. Knockout of active β subunits is associated with defects in proteasome assembly and thereby obscures experimental outcome.[125] Subunit selective cell permeable inhibitors are useful tools to study individual subunit activities. Inhibition of individual proteasome subunits in vitro displayed reduction of degradation of model substrates depending on the subunit inactivated. Overall protein degradation in cells was halted only if two β sites were substantially inhibited, and cytotoxicity in most cancer cell lines could only be induced efficiently when, next to the important β5 subunit, either β1 or β2 is inhibited as well.[126–128] Whether the different mechanisms by which bortezomib and salinosporamide A induce apoptosis in malignant cells depends solely on their different proteasome subunit preference remains a question to be answered.

The proteasome and immunoproteasome are essential for the generation of MHC class I antigens but the role of each individual proteasome catalytic subunit on the generation of certain antigens is still subject of extensive study. However, mice lacking one or more immunoproteasome active β subunits show decreased efficiency of MHC class I presentation of pathogen epitopes and a lower T-cell response to these epitopes.[10,129] These knockout studies suggest that antigen presentation may be changed by altering the activity of a single subunit. Very subunit selective and cell permeable proteasome inhibitors can be a great aid to verify results from knockout studies and determine therapeutic potential of proteasome inhibitor mediated shaping of the antigenic pool.

To date, an increasing number of subunit selective proteasome inhibitors has been reported. Subunit selectivity for a large part has been realized by optimisation of the P1 and P3 amino acids in oligopeptoid inhibitors, interacting with the S1 and S3 proteasome substrate binding pockets, which differ the most among subunits β1, β2 and β5. This Chapter provides a summary of subunit selective inhibitors.

1.8.1 β1 subunit

The proteasomal β1 S1 selectivity pocket is characterised by a basic side chain of Arg45 of this subunit.[11] Consequently, peptides with an Asp or Glu at P1 show preference for β1. However, the β1 subunit also has the capacity to cleave after branched amino acids

and this promiscuity is demonstrated by the finding that Cbz-Gly-Pro-Phe-Leu aldehyde (**54**, Figure 1.15) shows preference for β1.[13] The proline at P3 is important in this motif since it seems to prevent binding to tryptic and chymotryptic sites. Optimisation of this motif led to YU102 (**55**) which is a less efficient β5 inhibitor due to lack of aromaticity at its N-terminus.[130] A scanning positional library of P1 Asp-aldehyde yielded optimised P2-P4 sequence Ac-Ala-Pro-nLe.[131] This P2-P4 motif was equipped with leucine p-phenol vinyl sulfone to give cell permeable inhibitor **56** with some selectivity for β1i over β1, and introduction of an N-terminal azide gave probe **57** (Figure 1.15).[132] Ultimately, the vinyl sulfone was changed for the more proteasome selective epoxyketone to arrive at NC001 (**58**) and azide analogue **59** as β1 selective standard inhibitors to date.[127,133] Attachment of a BODIPY fluorophore did not significantly decrease its β1 selectivity and resulted in β1 selective probe **60**.[133]

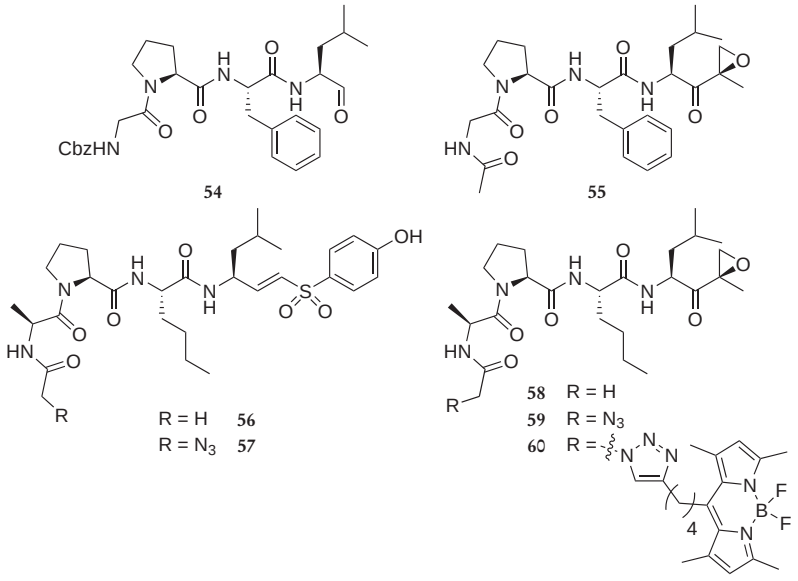

Figure 1.15: Structure of β1 selective inhibitors and activity-based probes.

1.8.2 β1i subunit

The immunoproteasome more efficiently generates peptides with basic and hydrophobic amino acids at their C-termini while its caspase-like activity is reduced and this has been ascribed to an altered activity of β1i with respect to β1. Subunit β1i of the immunoproteasome has changed significantly with respect to its constitutive counterpart.[11] Arg45 of β1 is replaced for leucine which abrogates caspase-like activity and rather results in a more chymotryptic-like activity of this subunit.[134] The inhibitors presented in Figure 1.15 do not efficiently discriminate between β1 and β1i. Among analogues of eponemycin (**10**) in which bulk was introduced at the P1' position of the inhibitor, in the form of silyl ethers, *tert*-butyldimethylsilyl ether containing compound **61** (Figure 1.16) was found selective for β1i over β1.[135] In this motif, P2 substitution for Lys and attachment of a BODIPY-650 or a fluorescein fluorophore resulted in β1i ABPs **62** and **63**.[136] Dipeptide aldehyde **64** has

selectivity for the immunoproteasome over constitutive proteasome and appears to target β1i.[137]

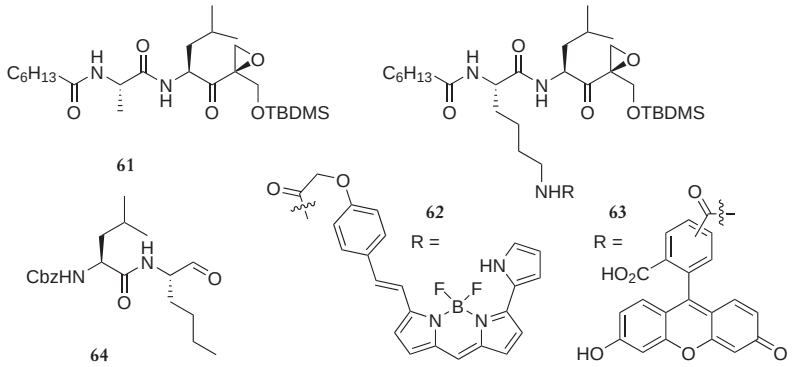

Figure 1.16: Structure of β1i selective inhibitors and activity-based probes.

1.8.3 β2 subunit

The β2 S1 pocket of the proteasome is spacious and characterised by Asp28 and Glu53 residues, explaining its preference for basic amino acid side chains.[11] Leupeptin is an inhibitor of β2 due to its arginine aldehyde electrophile. The S3 pocket of β2 contains a highly conserved cysteine side chain, Cys118, belonging to β3. The reactivity of this moiety has been exploited for binding by the bifunctional aldehyde **65** (Figure 1.17). The aldehyde captures Thr1 Oγ of β2, while the N-terminal maleimide is positioned to capture Cys118 in the S3 binding pocket, resulting in potent and selective β2 inhibition.[138]

The barrel shaped structure of the proteasome, in which the β subunits are in a defined distance from each other, was exploited by bivalent aldehyde inhibitors in which the two electrophiles are separated by a long PEG spacer. The presence of a second aldehyde at a defined distance from the first greatly increased potency of the inhibitor with respect to its monovalent counterpart. When the sequence Arg-Val-Arg-aldehyde was used, such as in **66**, a profoundly β2 selective inhibitor results.[139]

The S3 pocket of β2 has an acidic character due to the presence of two aspartic acids. A scanning positional library with asparagine vinyl sulfone at P1 stressed the importance of the P3 residue in a proteasome inhibitor for β2 selectivity. Introduction of basicity at P3 yielded compound AcPRLN-VS (**67**) as selective β2 inhibitor.[140] The requirement for basic amino acid residues in peptide based inhibitors decreases the ability of these compound to cross the cell membrane due to charged residues at physiologically pH. Ac-Arg-Leu-Arg-EK **68** is a recently discovered very specific inhibitor for β2 in cell lysate, however, this compound is only active in cells at high concentration. Monobasic peptides such as HMB-Val-Ser-Arg-EK **69** and **70** display more efficient β2 inhibition in cells.[128,141] Compounds with selectivity for the β2i subunit over constitutive β2, or vice versa, have not been reported to date.

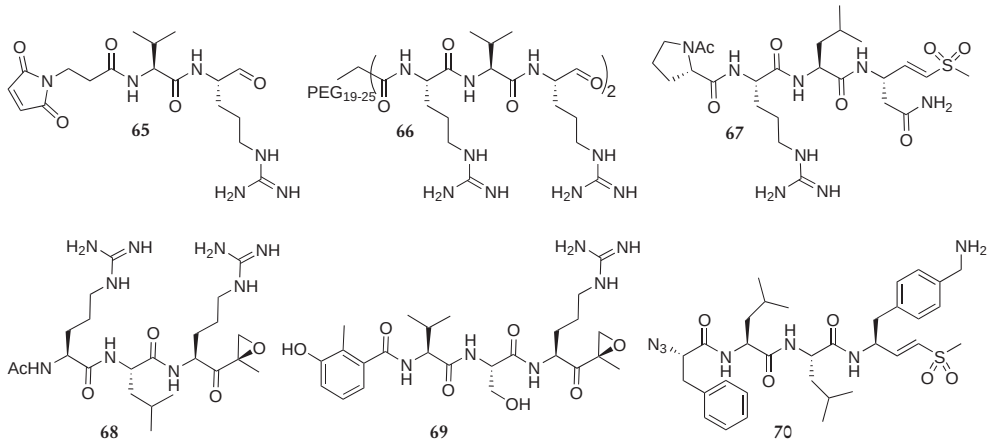

Figure 1.17: Structure of β2 selective inhibitors.

1.8.4 β5 subunit

The β5 subunit prefers hydrophobic substrates, which is illustrated by a great number of hydrophobic noncovalent proteasome inhibitors that target the β5 subunit, with examples **33**, **34**, **35** depicted in Figure 1.10. Epoxyketone YU101 (**71**, Figure 1.18), a very potent and selective inhibitor of chymotryptic activity of constitutive and immunoproteasome, was found by scanning the effect of introduction of large hydrophobic residues in the P2-P4 positions of a tetrapeptide epoxyketone.[142] Modification of the N-terminus of this compound to improve its pharmacological profile led to carfilzomib (**52**), currently in clinical trials against various cancers.[143,144]

In a medicinal chemistry effort that led to bioavailable epoxyketone ONX-0912 (**53**), multiple inhibitors have been discovered that inhibit chymotryptic activity of constitutive and immunoproteasome with about equal potency, for example **72** (Figure 1.18). However,

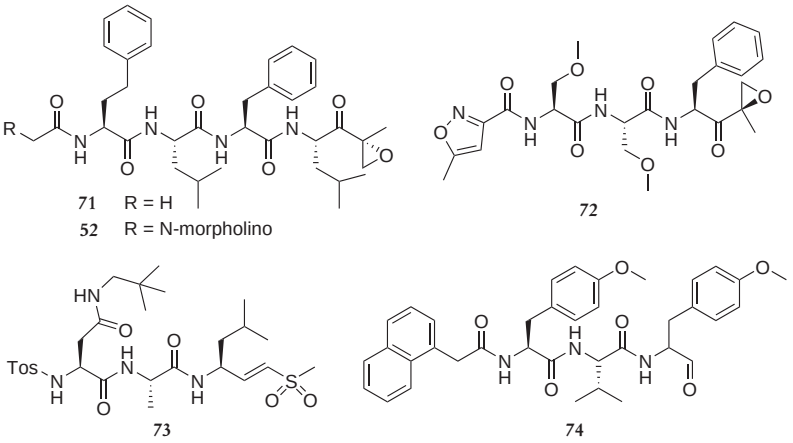

Figure 1.18: Structure of inhibitors selective for β5/β5i.

this study does not report on the effects of this compound on β1 or β2.[145] Hydrophobic bulky residues at the P3 position and aromaticity at the N-terminus of a proteasome inhibitor are factors beneficial for targeting of the chymotryptic activity of the proteasome. This is demonstrated by P3 neopentylasparagine containing potent and selective vinyl sulfone **73** (Figure 1.18), discovered when optimising a motif analogous to PSI (**25**) by screening different P3 residues and N-terminal caps.[87]

Optimisation of inhibition of chymotryptic activity of tyropeptin A (**8**) afforded reversible inhibitor **74** (Figure 1.18).[146] Structural elements from compound **74** inspired the synthesis of azide containing ABP **75** (Figure 1.19), a very selective irreversible inhibitor of β5.[127,133] Change of the naphthyl group in **75** for a BODIPY dye preserved β5 selectivity and afforded fluorescent and azide containing ABP **76**.[133] Incorporation of fluorinated amino acids at P2 and P3 of a tetrapeptide epoxyketone led to the discovery of compound **77**, which was found to be very selective for β5.[147] This inhibitor could be transformed into β5 selective fluorescent ABP by attachment of BODIPY fluorophore.[148]

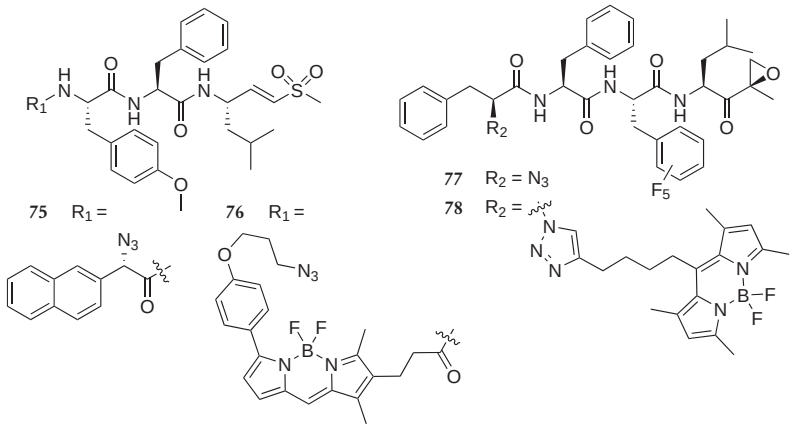

Figure 1.19: Structure of β5/β5i selective activity-based probes.

1.8.5 Discrimination between β5 and β5i subunit

The medicinal chemistry efforts that led to the discovery of ONX-0912 (**53**, Figure 1.14) identified compound **79** (Figure 1.20) as an inhibitor selective for β5 over β5i.[145] Interestingly, β5 inhibitor **79** differs from the equipotent β5/β5i inhibitor **72** (Figure 1.18) only in the amino acid at P1. Apparently, Phe at P1 is favourable for β5i preference while a β5 inhibitor seems more selective carrying Leu at P1.[145] Compound **80** targets β5i before β5 and this molecule indeed contains Phe at P1 and interestingly, it bears a D-alanine at P3.[145,149,150] PR957 is another β5i selective inhibitor, carrying Phe at P1 and like **80** possesses an aromatic P2 group, which could be favourable for β5i inhibition. PR957 causes decreased MHC class I presentation of a β5i dependent epitope.[151] This finding stresses the importance of the development of specific, cell permeable inhibitors of each of the active subunits of the proteasome to determine their ability to block presentation of certain epitopes.

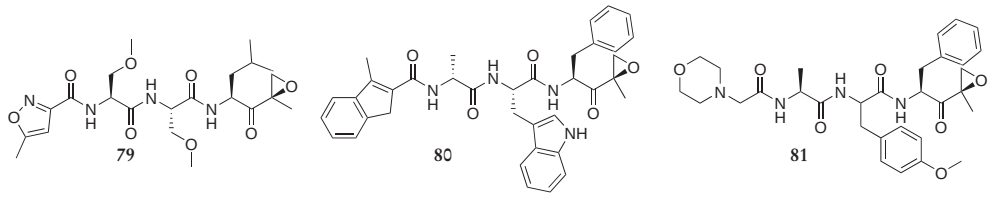

Figure 1.20: Structure of inhibitors able to discriminate between β5 and β5i.

1.9 Aim and outline of this Thesis

The research described in this Thesis aims at the development of novel subunit selective inhibitors of the proteasome. In Chapter 2 three vinyl sulfone analogues of three epoxyketone containing inhibitors described in literature are synthesised and characterised. Vinyl sulfones **82** and **83** (Figure 1.21) are more selective for β5 activity than their epoxyketone analogues.

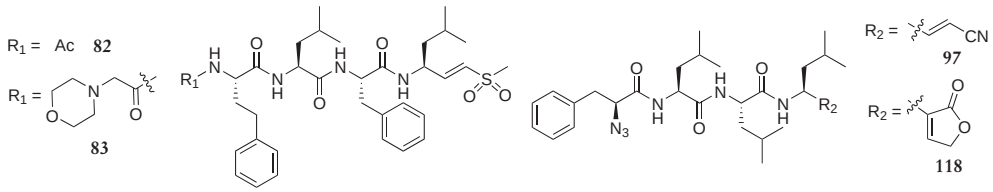

Figure 1.21: Structure of β5/β5i selective inhibitors **82** and **83**. Structure of Michael acceptors **97** and **118**.

In Chapter 3, ten Michael acceptors are coupled to three peptoid tails yielding thirty peptide-like compounds. These compounds are assessed on their capacity to inhibit the proteasome in cell lysate but none of the peptoid Michael acceptors are efficient proteasome inhibitors. Using the Staudinger-Bertozzi ligation followed by affinity purification, tryptic digestion of the isolated proteins and LC/MS^2 identification, the targets of **97** and **118** (Figure 1.21) in HEK293T and RAW264.7 cells are determined.

Syringolins are potent proteasome inhibitors found in nature and contain an unusual valine-urea-valine motif. In Chapter 4 this motif was built in peptoid epoxyketones and vinyl sulfones yielding a 16-membered library of proteasome inhibitors. The distance between the urea and electrophile dictates subunit selectivity. Tripeptide **182** (Figure 1.22) is the most potent and selective inhibitor of β1 in HEK cells reported to date. The longer tetrapeptides **190** and **192** preferentially inhibit β5 and are more potent than the parent compound, syringolin A.

In Chapter 5, three cyclooctynes are compared to the Staudinger-Bertozzi two-step labelling strategy using azido-BODIPY labelled proteasome as test system. Although cyclooctynes quantitatively convert azide labelled proteasomes at a lower concentration than the Staudinger-Bertozzi phosphane, cyclooctyne two-step labelling is associated with tremendous background labelling.

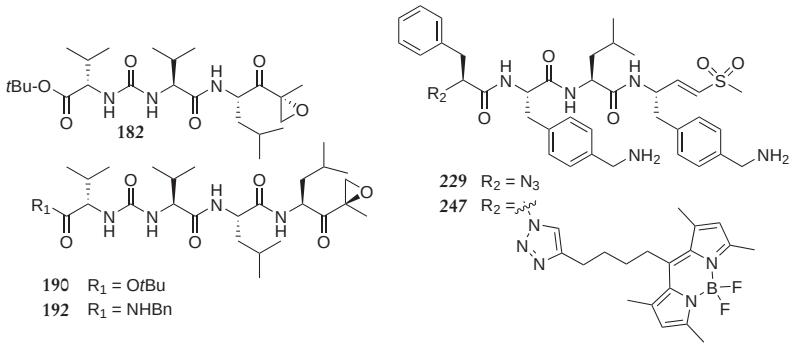

Figure 1.22: Structure of β1 selective inhibitor **182**, β5 selective inhibitors **190** and **192**, β2 selective inhibitor **229** and fluorescent β2 probe **247**.

Chapter 6 describes the synthesis and characterisation of a library of peptoid vinyl sulfones with basic amino acid side chains on P1 and P3. Compound **229**, with a benzylamine residue at both P1 and P3, is a potent and β2 selective proteasome inhibitor. Attachment of a BODIPY fluorophore yielded β2 selective probe **247** (Figure 1.22). The presence of two basic residues, however, reduced their entry into cells.