Design of selective inhibitors for human immunoproteasomes

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Chapter 1:

General introduction

Proteasomes are the major cytosolic and nuclear proteases and are responsible for the turnover of approximately 80% of proteins that are at the end of their lifetime.[1] As endoproteases, proteasomes degrade proteins into oligopeptides, the majority of which are further digested by cytosolic aminopeptidases, ultimately yielding single amino acids. In mammals, certain oligopeptides escape further degradation and serve as precursors for antigenic peptides displayed on MHC class I complexes. MHC class I complexes are expressed at the cell surface for immune surveillance and in this way, proteasomes play a vital role in our immune system by reporting on, for instance, viral infections.[2] In humans, two major proteasome isoforms are expressed and can co-exist within the same cell: constitutive proteasomes and immunoproteasomes. Constitutive proteasomes are expressed in all tissues whereas immunoproteasomes are constitutively expressed in immune-competent tissues and can be induced by inflammatory cytokines (for instance, IFN-γ). The existence of two proteasome particles, whose expression levels and functioning in (patho)physiological processes provide a selectivity window for clinical research and development. As of now there has not yet been capitalized upon this and that represents the overall aim of the research described in this Thesis: is it feasible to design a proteasome inhibitor that exclusively inhibits all immunoproteasome active sites while leaving constitutive ones untouched. Nowadays, three peptide-based proteasome inhibitors, bortezomib, carfilzomib and ixazomib, are in clinical use for the treatment of the haematological cancers, multiple myeloma, and mantle cell lymphoma.[3–5] All these compounds impartially target both proteasome isoforms, and thus target the constitutive proteasomes present in healthy tissues, causing adverse side effects. This, while in recent years it has become evident that certain haematological cancers predominantly (and in individual examples even exclusively) express immunoproteasomes.[6] For these cancers, immunoproteasome inhibition would be sufficient to reach a beneficial clinical effect.[7,8]

Both constitutive proteasomes and immunoproteasomes contain three distinct catalytic activities, the majority of which are targeted by the three clinical proteasome inhibitors, bortezomib, carfilzomib and ixazomib. Inhibition of multiple active sites simultaneously is a prerequisite to reach sufficient cytotoxicity.[6,9] For those haematological cancers that predominantly or exclusively express immunoproteasomes, compounds are required that selectively target the respective three catalytic activities.[6] For such compounds to be less toxic than the current clinical drugs they should not inhibit constitutive proteasomes. At the onset of the work described in this Thesis such compounds have not been discovered, but a plethora of peptide-based inhibitors that target individual activities, as well as compounds that are cross-reactive for the constitutive proteasome active sites and immunoproteasome active sites, were known. This knowledge provided the starting point of a rational inhibitor design campaign which led to the discovery of compounds with selective immunoproteasome inhibitory activity, targeting all three immunoproteasome active sites, and leaving the constitutive proteasome activities largely uninhibited. This introduction chapter provides the background of the conducted research. Proteasomes are introduced, with a focus on the inner core catalytic particles and where immunoproteasomes are distinct from constitutive ones. Then a short overview is given on existing proteasome inhibitors (including the clinically used ones), their mode of action and how based on their structure and activity new compounds can be designed in a rational fashion. Activity-based protein profiling of proteasome activities is a recurring theme throughout the Thesis as a rapid means to determine compound potency and selectivity and this technique is therefore also briefly introduced, after which the chapter ends with an overview of the experimental chapters and the specific research questions they address.

The ubiquitin-proteasome system

Proteasomal protein degradation is a tightly controlled process. Unlike lysosomal protein turnover, where proteases are confined within discrete subcellular compartments to which substrates are shuttled by endocytosis or autophacocytosis where they are the degraded by the concerted action of numerous proteases and other hydrolases, cytosolic and nuclear proteins exist side by side with proteasomes within the same cellular compartments. Proteins destined for degradation therefore need to be distinguished from functional proteins, which is done by tagging proteasome substrates with a poly-ubiquitin tag attached to the amine of a lysine residue.[10] Ubiquitin is a 76 amino acid protein that is expressed as a linear polymer composed of multiple ubiquitin monomers. These polymers are then processed to yield ubiquitin monomers, which are then transferred to specific lysine residues within cytosolic or nuclear proteins, and then extended through the attachment of additional ubiquitin moieties through lysine residues within the ubiquitin sequence. Depending on the site of modification and also the size and nature of the appended ubiquitin chain, ubiquitylated proteins can undergo various subcellular fates, of which targeting to proteasomal degradation is a major one.[1,11] Protein ubiquitylation is realized by a series of ligase enzymes termed E1, E2 and E3. E1 enzymes activate ubiquitin by creating a mixed anhydride composed of the ubiquitin C-terminus and adenosine monophosphate, which then reacts with an E1 cysteine thiol to create a thioester.[12] In a transesterification reaction, ubiquitin is then transferred to an E2 enzyme which then selects an E3 enzyme for another transesterification to yield a thioester-linked E3-ubiquitin construct. This then binds a substrate protein after which ubiquitin is transferred from the E3 cysteine to a substrate lysine side chain amine. A large variety of E2 and especially E3 enzymes ensure the creation of a wide variety of ubiquitylated proteins which may undergo a plethora of events.[1,10,12,13] One of these is proteasomal degradation, for which purpose a number of proteasome complexes have evolved that recognize certain ubiquitylated proteins, unfold these in an ATP-driven fashion, and degrade them into oligopeptides. The major mammalian proteasome degradation machinery comprises the 26S (or 30S) proteasome. This protein is constituted of two distinguishable polyprotein particles: the 20S core particle and the 19S cap particle (26S particles comprise of one of these each whereas in 30S particles one 20S core particle is capped by two 19S particles). The 19S particles contain ubiquitin recognition elements as well as deubiquitinating enzymes that remove ubiquitin chains from the proteasome substrates. The 20S particles in turn contain the proteolytic machinery, which can only be accessed by substrate proteins when unfolded. Protein unfolding and shuttling into the 20S proteolysis chamber is also affected by the 19S caps in an ATPase-driven fashion (**Figure 1**). Besides 26S (30S) particles a number of alternative particles may exist, with variation in the caps (PA700, PA200, PA28 $\alpha\,\beta$, PA28γ) and – of most relevance for the research described in this Thesis – the nature of the core particles.[1,14]

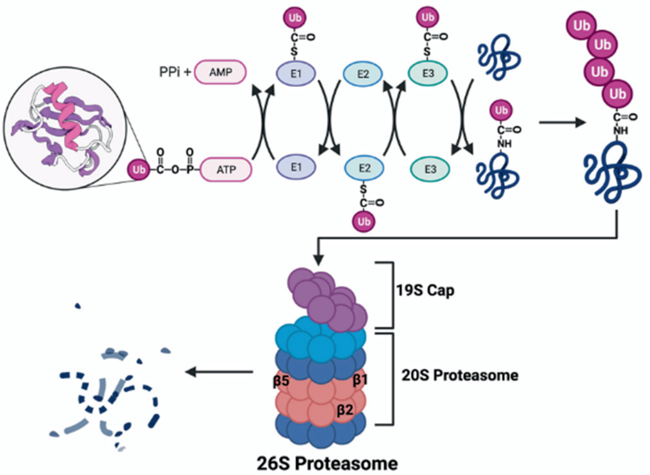


Figure 1. A schematic representation of the ubiquitin-proteasome system (UPS).[1,13]

20S core particles: Constitutive proteasomes and immunoproteasomes

The proteolytic activity of proteasomes resides within the 20S core particles, of which in mammals several isoforms exist and that may co-exist side by side. All particles are composed of four heptameric protein rings stacked on top of each other. The most common proteasome isoforms are the constitutive proteasome 20S core particles, which are C2-symmetrical species composed of two α-rings, each containing seven unique α-subunits (α1-α7) at the top and bottom, and two inner β-rings. The β-rings are composed of seven unique subunits (β1-β7), with proteolytic activity residing in the β1, β2 and β5 subunits. To distinguish between constitutive proteasome core particles and other ones, the constitutive proteasome 20S core particle is referred to as cCP and the catalytic activities residing therein as β1c, β2c and β5c (**Figure 1**).[1,2,15]

Each of the catalytically active subunits have an N-terminal threonine residue, which coordinates with a water molecule. Upon substrate binding the water molecule present mediates proton transfer between O^{γ} and NH_2 of the Thr1 residue, so that the O^{γ} can react with the scissile peptide bond, moving through a tetrahedral intermediate and resulting in peptide bond cleavage. This results in the formation of a substrate-proteasome adduct connected through an ester bond. This ester bond is then hydrolysed through nucleophilic attack of an activated water molecule, resulting in the regeneration of the N-terminal nucleophilic threonine residue and the release of the carboxylic acid containing part (**Figure 2**).[16,17]

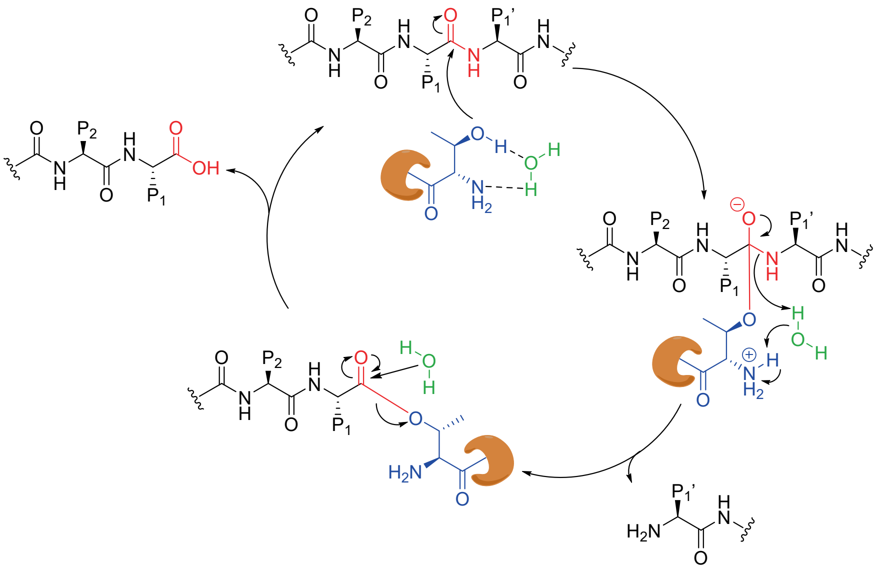
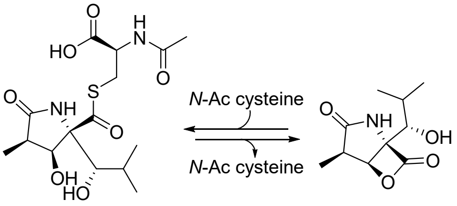


Figure 2. The catalytic mechanism of peptide bond hydrolysis by the proteasome. P indicates the position of the residue in respect to the scissile peptide bond (red: scissile peptide bond, blue: N-terminal active site threonine and green: water molecule).

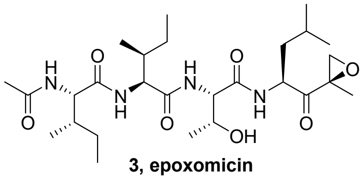
Compared to prokaryotic proteasomes, which have a conserved overall geometry but in which all α-subunits, as well as all β-subunits are identical (with all β-subunits catalytically active), constitutive proteasome cCPs harbour three distinct substrate preferences, with β1c cleaving predominantly after acidic residues, β2c after basic ones and β5c after hydrophobic ones. Besides the constitutive proteasome cCPs proteolytic activities, β1c, β2c and β5c, mammalian cells can express (constitutively for certain cell types and cytokine-induced expression for others) three alternative proteolytic activities. These are termed β1i (LMP2), β2i (MECL-1) and β5i (LMP7) and assemble, together with the remaining α- and β-subunits that also make up cCPs, into immunoproteasome core particles, or iCPs.[2,18] Compared to β1c, β2c and β5c, the iCP proteolytic activities have a diverged substrate specificity so that combined both proteasome species are able to produce a larger variety of product oligopeptides, which is thought to benefit MHC I-mediated antigen presentation.[2,19] In contrast to β1c, which favours acidic residues N-terminal to the scissile amide bind, β1i preferentially cleaves after hydrophobic residues. Differences between β2c and β2i (both preferentially cleaving after basic residues) and β5c and β5i (both preferentially cleaving after hydrophobic residues) is less drastic, but also here differences exist. For instance, the size and nature of the basic/hydrophobic residues that can be accommodated at the cleavage sites.[18,20,21] These differences can be capitalized upon in the development of chemical tools to study proteasome activities: substrates and in particular inhibitors. Besides cCP and iCP particles, also mixed proteasome core particles composed of cCP and iCP proteolytic activities can exist. To add to proteasome complexity, thymoproteasome core particles (tCPs) feature a third type of β5 catalytic activity termed β5t, which assemble jointly with β1i and β2i.[22] Finally, sperm proteasomes contain a unique α-subunit (α4) and are otherwise the same as constitutive proteasome cCPs.[23]

Proteasome inhibitors

Historically, insights in proteasome function and mode of action and the discovery and development of proteasome inhibitors have gone hand in hand. With some notable exceptions, most proteasome inhibitors (including the current clinically used inhibitors) are peptide-based, which makes sense when considering proteasome substrates, which are polypeptides. Peptide-based proteasome inhibitors are also the subject of the research described in this Thesis and for this reason will be considered exclusively here. The first effective proteasome inhibitors described in the literature comprise compounds derived from nature, and indeed many microorganisms have evolved the biosynthesis of proteasome inhibitors as part of their arsenal to combat competing species for living space. One of the first effective inhibitors discovered is lactacystin **1** (**Figure 3**), which in solution forms under expulsion of N-acetylcysteine, beta-lactone **2** as the actual active species.[24,25] This inhibitor proved to feature a slow-release mode of action and it was shown by biochemical, and later also structural biology studies that the beta-lactone, which is intrinsically reactive because of ring strain, reacts with the catalytic site N-terminal threonine to form a covalent (ester) enzyme-inhibitor adduct.[25] Mechanism-based inactivation is also the mode of action of what is arguably the most renowned of the natural product proteasome inhibitors: epoxomicin **3**.[26]



1, lactacystin **2, ß-lactone**



3, epoxomicin

Figure 3. Chemical structures of lactacystine **1** and its active species ß-lactone **2** (upper), and epoxomicin **3** (lower).[25,26]

Epoxomicin comprises a tetrapeptidyl compound, the C-terminus of which is an epoxyketone. This moiety presents two adjacent electrophiles to match the dual nucleophilicity of the 1,2-amino-alcohol that characterizes proteasome active sites (the N-terminal threonine moiety). Initially, the Thr1O$^\gamma$ nucleophile reacts with the electrophilic carbonyl moiety of the epoxyketone forming a hemiketal intermediate. Subsequently, in what was originally thought to yield a 1,4-morpholine, the Thr1N nucleophile reacts with the tertiary carbon of the epoxide to form a 1,4-morpholine ring. It was recently shown that the actual adduct formed comprises an 1,4-oxazepane (and not a 1,4-morpholine), which is formed after reaction of the Thr1N nucleophile with the secondary carbon of the epoxide instead of the tertiary one (**Figure 4**).[16,17] The epoxyketone moiety of epoxomicin, with its two adjacent electrophilic sites, takes advantage of the rather unique active site lay-out of proteasome catalytic subunits (the N-terminal amino-alcohol) and by this virtue, epoxyketone electrophiles are often found to be highly selective for proteasomes over other proteases that employ nucleophilic active site residues (cysteine proteases, serine hydrolases). This makes peptide epoxyketones, attractive starting points for drug discovery targeting proteasomes.

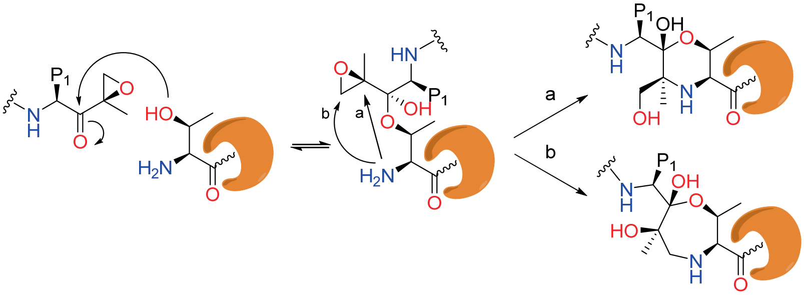


Figure 4. Covalent and irreversible inhibition mechanism of peptide epoxyketones as proteasome inhibitors. (**a**) 1,4-Morpholine ring formation as originally thought to be the mode of action, and (**b**) formation of an 1,4-oxazepane ring is now considered to be the most likely mode of action, based on structural studies.[16,17]

Lactacystin **1** and epoxomicin **3** as the two classical natural product proteasome inhibitors are matched, by studies from the same era, and by three classical synthetic designs: peptide aldehydes, peptide vinyl sulfones and peptide boronates. The archetypal peptide aldehyde, MG132 **4** comprises an N-terminally capped tripeptide with at the C-terminus an aldehyde. Proteasome active site threonine-OHs react with this aldehyde to form a transient covalent adduct: a hemi-acetal.[27] Peptide aldehydes are, in contrast to peptide epoxyketones not selective for threonine hydrolases over serine hydrolases or cysteine hydrolases and are therefore used mostly as research tools. Substitution of the aldehyde in **4** for a vinyl sulfone yields ZL$_3$VS **5**, which is an archetypal peptide vinyl sulfone proteasome inhibitor. Rather counter-intuitively, given that Michael acceptors appear more effective cysteine protease inactivators (and are often used as such), the proteasome active site N-terminal threonine have proven to react very efficiently to form a covalent, stable, ether bond.[27,28] The cross reactivity of Michael acceptor-type peptide-based inhibitors also make this class of compounds less suitable for drug discovery and, like with peptide aldehydes, peptide vinyl sulfones are largely used as research tools. The third class of synthetic, peptide-based proteasome inhibitors are the peptide boronates, with as one of the first examples described MG262 **6**. The mode of action of the peptide boronic acids deviates from the former examples in that, upon binding, no reaction takes place. Rather, a Lewis base (the threonine-OH) interaction with a Lewis acid (the boronic acid) follows, leading to a rather stable complex (**Figure 5**).[27,29]

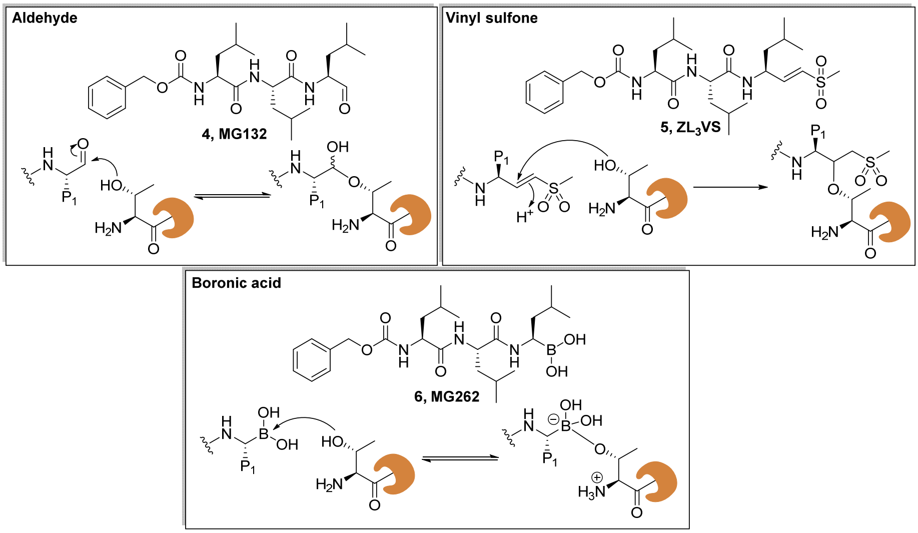


Figure 5. The chemical structures of MG132 **4**, ZL$_3$VS **5** and MG262 **6** and their associated inhibition mechanisms with the 20S proteasome catalytically active sites.[9,27–29]

In the early days of proteasome inhibitor discovery, attention was largely focused on the design and discovery of active site-reactive groups. Following these days, and as the composition of the various proteasome particles (cCP, iCP, tCP) and the substrate preference (as assessed by fluorogenic substrate hydrolysis, substrate polypeptide digestion and mutagenesis studies) became apparent, and based on the discovery that most of the first-generation inhibitors display little catalytic subunit selectivity, attention became focused on the design of proteasome active site selective inhibitors. The driving rationale behind these studies was the idea that, with such compounds, the individual contribution of the various cCP/iCP activities could be mapped by chemical knockout studies (selective inactivation of a single activity). This would then provide insight in, for instance, the production of pools of MHC I-antigenic peptides, and would also validate, or disprove, individual proteasome active subunits as clinical targets. By and large, these studies followed one of two complementary strategies. In the first, a C-terminal electrophile was selected to which a library of oligopeptides (normally from P2 up to P4, all or not N-terminally capped – P2, P3, P4 being the amino acid residues binding to the active site S2-S3-S4 sites, with S1 occupied by the P1 amino acid bearing the electrophile) was attached.[21] Canonical and non-canonical amino acids can be selected as P1 – P4 residues, giving rise to a large possible variety, necessitating pre-synthesis selection. This is done based on natural substrates and products formed and (as also done in this Thesis) also based on structural studies of (human) proteasomes complexed with existing inhibitors. These libraries are then screened on proteasome inhibition potency and selectivity using fluorogenic substrates and, as is also done in this Thesis, by competitive activity-based protein profiling. In the second strategy, libraries of fluorogenic substrates are assembled and subjected to proteasome digestion.[30] Deconvolution of the data (which proteasome active subunit is responsible for fluorescence emergence) yields oligopeptide sequences that are then transformed into candidate-inhibitors through instalment of a C-terminal aldehyde, vinyl sulfone, epoxyketone or boronic acid.[27]

In this vein, numerous compounds have been discovered in recent years that, to a more or lesser extent, display cCP/iCP catalytic subunit inhibitory activity. Here it should be noted that many of these studies did not have human proteasomes (the targets of the work described in this Thesis) as a primary target, both for practical reasons (human proteasomes are as not easily available as ones from other species for specific experiments, including structural biology studies) and for intrinsic reasons: pathogenic proteasomes are, besides human ones, also seen as drugs targets. The following examples do not cover all developments but have been selected based on their properties as human proteasome inhibitors, and because they form in part the basis of the rational, structure-based designs of immunoproteasome inhibitors as described in the experimental parts of this Thesis.

A representative set of cCP/iCP subunit-selective inhibitors is depicted in **Figure 6** In the first instance and because they, though evolutionary diverged, resemble each other closely, compounds able to selectively inhibit either β1c/β1i, β2c/β2i, or β5c/β5i were discovered. NC-001 **7** features a proline at P3 which induces a turn which is accepted by both β1c and β1i, but not by the other cCP/iCP active sites.[31] Even though the P1 residue (leucine) is hydrophobic (preferred by β1i) and not acidic (preferred by β1c) it blocks both active sites with almost equal potency, while leaving the other four intact. This, besides providing a research tool with which β1c and β1c can be inhibited simultaneously, also reveals the promiscuity of proteasome active sites for amino acid side chains, which is much more pronounced than for other proteases, like trypsin. LU-112 **8** with large, basic residues at P1 and P3 inhibits potently and selectively both β2c and β2i, whereas NC-005 **9**, characterized by an overall hydrophobic nature, targets selectively β5c/β5i.[32,33]

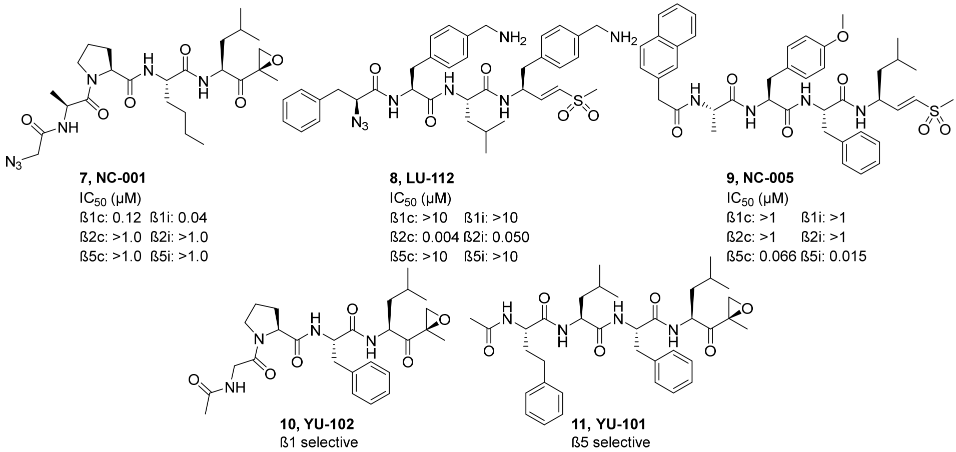
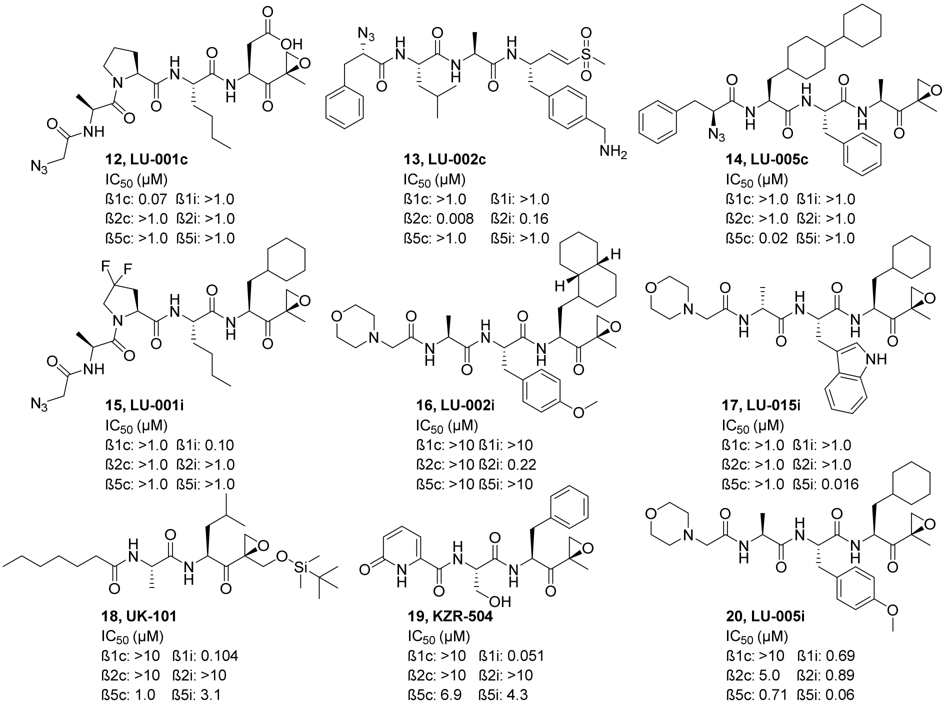


Figure 6. Chemical structures and inhibition profiles of the following subunit selective proteasome inhibitors: NC-001 **7**, LU-112 **8**, NC-005 **9**. The apparent inhibitory concentration values (IC_{50}) as determined by competitive activity-based protein profiling in µM are found underneath each chemical structure for each of the catalytically active subunits of human constitutive proteasomes and immunoproteasomes. In addition, two additional proteasome inhibitors, YU-102 **10** and YU-101 **11**, are displayed who also selectively target one of the human proteasomal catalytically active subunits.[31–35]

Ensuing studies were conducted with the aim to arrive at 'true' cCP/iCP subunit-selective inhibitors. For this purpose, human, or humanized proteasomes were co-crystalized with selected inhibitors, analysed and room for potential modification was sought for. Based on these structure-activity relationship analyses, and with the same strategy as for the β1c/β1i inhibitor (**7**) design by building in structural elements that are acceptable for one active subunit but not for the other ones, so building in structural penalties, a panel of next-generation inhibitors **12**-**20** was developed (**Figure 7**).[31] Comparison of structural elements in these with those at the same position (P1-P4) in the β1c/β1i, β2c/β2i, and β5c/β5i inhibitors reveals the validity of this approach. For instance, a large hydrophobic substituent at P1 is well tolerated by β5i, but not β5c, whereas the opposite holds true for the hydrophobic residue at P3, leading to two compounds that either selectively inhibit β5c (**14**) or β5i (**17**).[36–38] Placing an aspartic acid at P1 and a proline at P3 (**12**) yields a β1c-selective inhibitor, whereas a proline at P3 combined with a large hydrophobic residue at P1 yields a β1i-selective inhibitor (**15**).[21,38] Less clear from a structural perspective but with the same desired result comprises compounds **13** and **16**, with which β2c (**13**, basic residue at P1) and β2i (**16**), very large hydrophobic residue at P1) can be inhibited selectively, again with selectivity also over the four cCP/iCP active subunits.[20,39] It should be noted again that these selectivity profiles were not always exclusively established in human proteasomes. A case in point is LU-005i **20**, which was shown to be selective for β5i active sites in murine proteasomes.[36,38] Later research revealed that the selectivity for human proteasomes is less pronounced and that the activity of LU-005i **20** also extended towards the other two iCP active sites. This finding was an important driver for the research presented in this Thesis and will be further discussed in the final sections of this chapter.



12, LU-001c
IC_{50} (µM)
ß1c: 0.07 ß1i: >1.0
ß2c: >1.0 ß2i: >1.0
ß5c: >1.0 ß5i: >1.0

13, LU-002c
IC_{50} (µM)
ß1c: >1.0 ß1i: >1.0
ß2c: 0.008 ß2i: 0.16
ß5c: >1.0 ß5i: >1.0

14, LU-005c
IC_{50} (µM)
ß1c: >1.0 ß1i: >1.0
ß2c: >1.0 ß2i: >1.0
ß5c: 0.02 ß5i: >1.0

15, LU-001i
IC_{50} (µM)
ß1c: >1.0 ß1i: 0.10
ß2c: >1.0 ß2i: >1.0
ß5c: >1.0 ß5i: >1.0

16, LU-002i
IC_{50} (µM)
ß1c: >10 ß1i: >10
ß2c: >10 ß2i: 0.22
ß5c: >10 ß5i: >10

17, LU-015i
IC_{50} (µM)
ß1c: >1.0 ß1i: >1.0
ß2c: >1.0 ß2i: >1.0
ß5c: >1.0 ß5i: 0.016

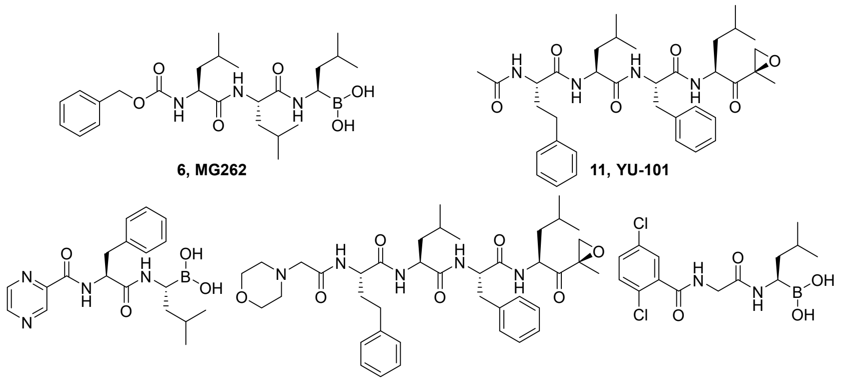
18, UK-101
IC_{50} (µM)
ß1c: >10 ß1i: 0.104
ß2c: >10 ß2i: >10
ß5c: 1.0 ß5i: 3.1

19, KZR-504
IC_{50} (µM)
ß1c: >10 ß1i: 0.051
ß2c: >10 ß2i: >10
ß5c: 6.9 ß5i: 4.3

20, LU-005i
IC_{50} (µM)
ß1c: >10 ß1i: 0.69
ß2c: 5.0 ß2i: 0.89
ß5c: 0.71 ß5i: 0.06

Figure 7. Chemical structures of selected subunit selective proteasome inhibitors: LU-001c **12**, LU-002c **13**, LU-005c **14**, LU-001i **15**, LU-002i **16**, LU-015i **17**, UK-101 **18**, KZR-504 **19** and LU-005i **20**, and their reported inhibition profiles. The apparent inhibitory concentration values (IC_{50}) as determined by competitive activity-based protein profiling in µM are found underneath each chemical structure for each of the catalytically active proteasome subunit.[7,9,21,37–41]

In around the same years that academic research on subunit-selective proteasome inhibitors took off, more applied research was done to establish whether derivatives of the first-generation broad-spectrum proteasome inhibitors (in particular, epoxomicin **3**, MG262 **6**) could be further developed into clinical drugs. Proteasomes have long been considered as drug targets in oncology, first because proteasome inhibition was seen as a way to interfere with the expression of (onco)genes and in later years because proteasome inhibition will induce an unfolded protein response from the ER, a well-characterized apoptotic trigger.[3,8] The latter mode of action is seen as the most beneficial one, since haematological cancers appear to be most sensitive to proteasome inhibition, especially malign plasma cells that feature a high turnover of misfolded or unfolded ER proteins. Three proteasome inhibitors have in the past decades made it to the clinic for the treatment of multiple myeloma and mantle cell lymphoma. Two of these, bortezomib **22** and ixazomib **24**, are peptide boronic acids, based on the structure of MG262 **6** while carfilzomib **23** comprises a close structural analogue of YU-101 **11** and epoxomicin **3** (**Figure 8**).[27,42,43]



6, MG262

11, YU-101

22, Bortezomib
IC_{50} (μM)
ß1c: 0.019 ß1i: 0.0043
ß2c: 0.452 ß2i: 0.636
ß5c: 0.041 ß5i: 0.0032

23, Carfilzomib
IC_{50} (μM)
ß1c: 0.130 ß1i: 0.0049
ß2c: 0.034 ß2i: 0.015
ß5c: 0.0016 ß5i: 0.0032

24, Ixazomib
IC_{50} (μM)
ß1c: 0.035 ß1i: 0.01
ß2c: ≥10.0 ß2i: 5.6
ß5c: 0.031 ß5i: 0.018

Figure 8. Chemical structures and inhibition profiles of clinical proteasome inhibitors, bortezomib **22**, ixazomib **23** and carfilzomib **24**. The apparent inhibitory concentration values (IC_{50}) as determined by competitive activity-based protein profiling in μM are found underneath the chemical structures of **22**-**24** for each of the catalytically active subunits of constitutive proteasomes and immunoproteasomes. In addition, the chemical structures of parent inhibitors MG262 **6** and YU-101 **11** are also shown.[6,9,27,34,42]

All three are efficacious, yet all three target all six active subunits of cCP and iCP proteasomes equally (the inhibition profiles in apparent IC_{50} values as depicted in **Figure 8** are determined by competitive activity-based protein profiling assays the working of which is explained in the next section). Arguably, the side effects associated with these three inhibitors, which are serious (patient compliance to the first-in-line drug, carfilzomib, drops severely after three months treatment) relate to their cCP cross-reactivity, which stimulated the research described in this Thesis towards true iCP-selective inhibitors: compounds that block β1i, β2i and β5i, but not β1c, β2c and β5c.[6] Before outlining the approach taken to discover such compounds, in the next section the mainstay biochemical assay, competitive activity-based protein profiling, that is used to assess all compounds developed, is briefly introduced.[44]

Comparative and competitive activity-based protein profiling of cCP and iCP proteasome particles

Activity-based protein profiling (ABPP) has been used for the past three decades to detect and identify enzymes from complex biological samples. In ABPP, activity-based probes (ABPs), which contain substrate-like elements connected to (in most cases) an electrophile and endowed with a reporter moiety (biotin, a fluorophore or a bioorthogonal tag), react within the active site of the target enzyme, or enzyme family to form a covalent and irreversible bond. Several proteasome inhibitors (peptide vinyl sulfones, peptide epoxyketones) react in a mechanism-based fashion, making them good starting points for the design of proteasome-targeting ABPs.[30,45] In fact, proteasomes were identified as the primary target of the natural product, epoxomicin, by Sin *et al.*, who synthesized an epoxomicin derivative **25** featuring a biotin moiety as the reporter group. Treatment of EL4 cell lysates (a murine lymphoma induced T lymphoblast, that expresses both cCPs and iCPs) with biotin-epoxomicin **25**, followed by streptavidin pull-down and mass spectrometry analysis yielded several ABP-reacted proteins, which later turned out to contain all six cCP/iCP active subunits.[42,46]

Following these initial studies, which were complemented by studies using radioactive lactacystin, many proteasome ABPs have been developed based on peptide epoxyketones and peptide vinyl sulfones. These studies culminated in a set of three ABPs, based on the three inhibitors shown in **Figure 9**, that when combined are able to resolve all six human cCP and iCP active subunits, after running ABP-treated cell extracts on an SDS-PAGE gel followed by in-gel fluorescence detection.[44] With this ABPP assay cell lysates can be rapidly analysed for their cCP and iCP catalytic activity content composition. For instance, treatment of lysates of HeLa cells, a cervical cancer cell line that exclusively expresses cCP active sites, with the three ABPS followed by SDS PAGE, and in-gel fluorescent detection yields three bands, a green band corresponding to β2c, a blue band (actually near-infra far-red fluorescence) corresponding to β1c and a red band corresponding to β5c (**Figure 9B**). Upon treatment of HeLa cells with IFN-γ they start to co-express iCP catalytic activities in a time dependent fashion, which can also be readily visualized using the above described ABPP assay. Raji cells, (a non-Hodgkin B-cell lymphoma cell line), constitutively express both proteasome isoforms, which after visualization with an ABPP assay gives 6 separate bands corresponding to all six iCP and cCP active sites. When applying this ABPP workflow to primary cancer cells from patients suffering from a haematological malignancy, the observation was made that iCP particles are often expressed predominantly to almost exclusively. This while most of these patients were treated with proteasome inhibitors that indiscriminately inhibit iCPs as well as cCPs.[6,44] Thus, the comparative ABPP assay as depicted in **Figure 9C** provided an important finding driving the research described in this Thesis: to discover and develop iCP-selective inhibitors that exclusively target all three of the iCP catalytic activities.

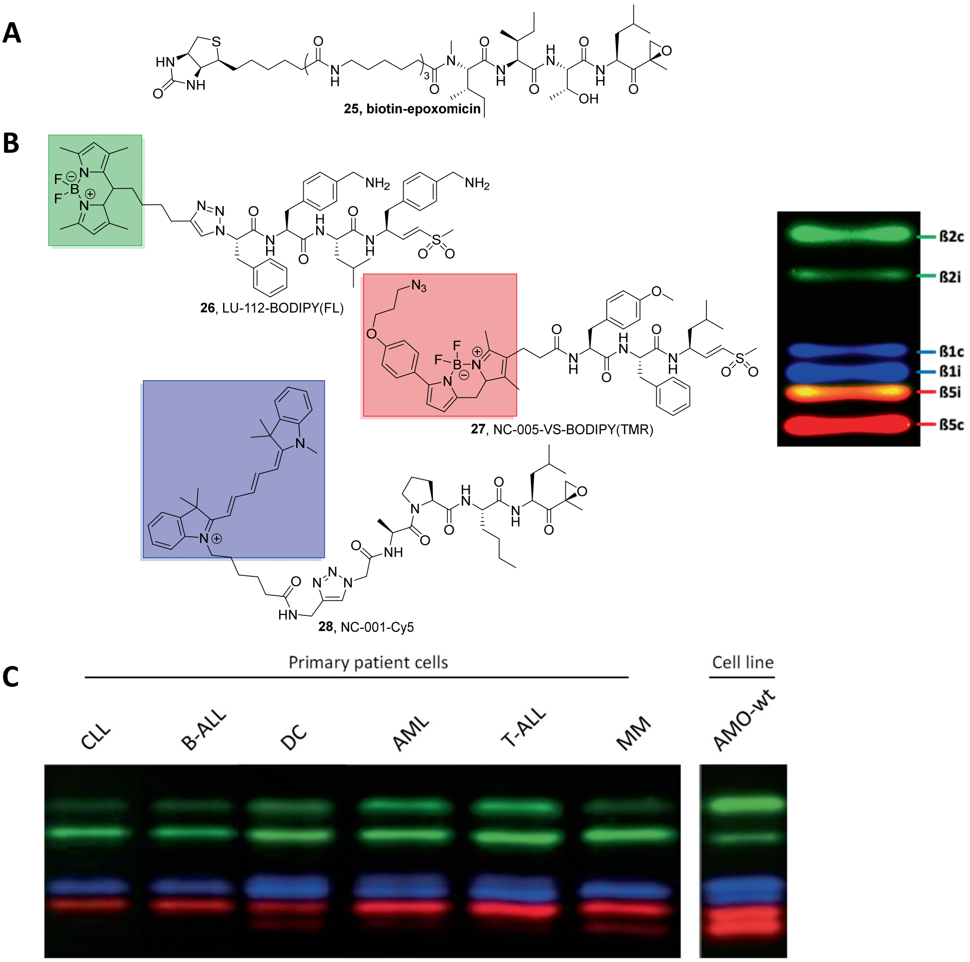

A
25, biotin-epoxomicin
B
26, LU-112-BODIPY(FL)
27, NC-005-VS-BODIPY(TMR)
28, NC-001-Cy5
ß2c
ß2i
ß1c
ß1i
ß5i
ß5c
C
Primary patient cells
Cell line
CLL
B-ALL
DC
AML
T-ALL
MM
AMO-wt

Figure 9. Multiplex activity-based protein profiling for assessment of proteasomal active sites. (**A**) Chemical structure of biotin-epoxomicin **25**. (**B**) Chemical structures of three activity-based probes used for labelling proteasomal active subunits in cell lysates. LU-112-BODIPY(FL) **26** (Ex: 505 nm, Em: 511 nm), labels the β2c and β2i subunits and is indicated as green on SDS-PAGE gels. NC-005-VS-BODIPY(TMR) **27** (Ex: 530 nm, Em: 560 nm) labels the β5c and β5i subunits and is indicated as red on SDS-PAGE gels. NC-001-Cy5 **28** (Ex: 600 nm, Em: 690 nm) labels β1c and β1i subunits and is indicated as blue on SDS-PAGE gels (left). In addition, an SDS-PAGE gel of all iCP and cCP subunits labelled with said ABPs shown (right). (**C**) An SDS-PAGE gel of primary patient cells derived from haematological cancers in comparison to the multiple myeloma derived cell line AMO-1. CLL: chronic lymphocytic leukaemia, B-ALL: B-cell acute lymphoblastic leukaemia, DC: acute leukaemia of plasmacytoid dendritic cells, AML: acute myeloid leukaemia, T-ALL: T-cell acute lymphoblastic leukaemia, MM: multiple myeloma.[44,46]

Competitive ABPP is complementary to comparative ABPP and allows the identification of active compounds, as well as their selectivity and potency, of libraries of putative proteasome inhibitors. Since most proteasome inhibitors designed in the literature, and also this Thesis, are peptide-based electrophiles that react within proteasome active sites to form a covalent and irreversible bond or form a long-lasting complex. In competitive ABPP, individual inhibitors are added at various concentrations and for varying time to a cell lysate (or a cell in case cell permeability is investigated) prior to addition of the three ABPs, SDS PAGE and in-gel fluorescence scanning.[44] In this way the selectivity and activity of the panel of subunit-selective inhibitors depicted in **Figures 6** and **7** was determined. **Figure 10** provides an image of the respective bands corresponding to the subunits that are inhibited by these compounds at the indicated concentration.

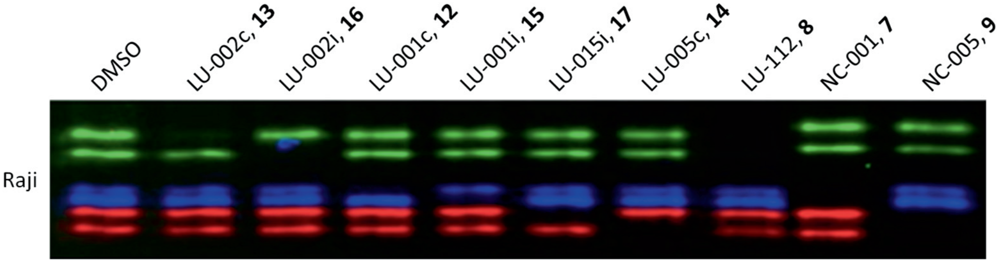
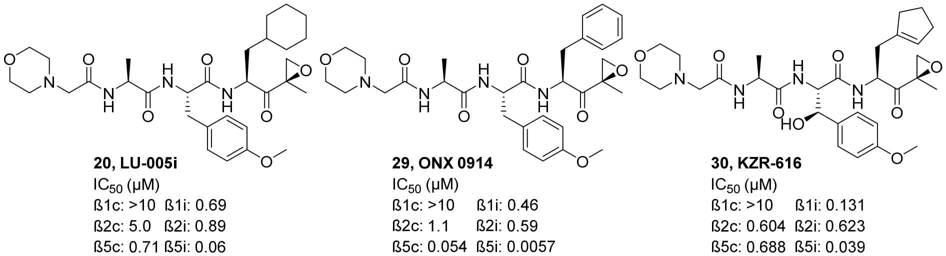


Figure 10. Proteasome subunit selective inhibition of the catalytically active ß-subunits in Raji lysates by subunit selective proteasome inhibitors at different concentrations (depicted above the gel) as assessed by competitive ABPP.[9,44]

LU-005i, ONX-0914 and KZR-616 as lead compounds

The work described in this Thesis has taken LU-005i **20**, originally described as a β5i-selective inhibitor and later found to display considerable activity against β1i and β2i as well, as a starting point. LU-005i **20** is a close structural analogue of ONX-0914 **29** and KZR-616 **30**, both of which also have considerable selectively inhibit all three iCP active subunits (**Figure 11**).[47–49] All three compounds also feature considerable cross-reactivity towards at least one cCP catalytic subunit, and further structure-guided designs were aimed to weed out these undesirable cross-reactivities. Of the three lead structures, ONX-0914 **29** has advanced most in terms of clinical development, targeting auto-immune diseases, next to haematological cancers. Auto-immune diseases comprise the second indication for which proteasome inhibitors are considered, but for which no proteasome inhibitors are in the clinic as of today. ONX-0914 **29** inhibits both β5c and β5i at low nanomolar concentrations and β1i and β2i at micromolar concentrations. ONX-0914 **29** mediated proteasome inhibition showed reductions in inflammation, cytokine gene expression and serum autoantibody reduction in rheumatoid arthritis mouse models.[2,50–53] Additionally, the compound induces apoptosis at pharmacologically relevant concentrations in an acute lymphoblastic leukaemia (ALL) cell line.[50] Moreover, Jenkins *et al*. showed that treatment of ONX-0914 **29** in conjunction with co-inhibition of the β2 active sites enhanced the observed anti-neoplastic activity.[50] LU-005i **30** closely resembles ONX-0914 **29**, with only the P1 position differentiating the two compounds. While ONX-0914 **29** proved to be the more potent inhibitor for each of the iCP subunits, LU-005i **20** proved to be the more selective one by greatly improving on the β5i over β5c selectivity. Just like ONX-0914 **29**, LU-005i **20** proved efficacious in the treatment of *in vitro* auto-immune disease models, showing that multi-immunoproteasome subunit inhibition effectively ameliorates autoimmunity through reduced cytokine secretion and impaired differentiation of naive T-helper cells.[54] KZR-616 **30** is a dual iCP inhibitor targeting β1i and β5i and is in clinical trials for both lupus nephritis and auto-immune hepatitis. In addition, similar to parent compound ONX-0914 **29**, KZR-616 **30** reduces cytokine secretion and proved to be efficacious in mouse arthritis models.[48]



20, LU-005i
IC_{50} (μM)
ß1c: >10 ß1i: 0.69
ß2c: 5.0 ß2i: 0.89
ß5c: 0.71 ß5i: 0.06

29, ONX 0914
IC_{50} (μM)
ß1c: >10 ß1i: 0.46
ß2c: 1.1 ß2i: 0.59
ß5c: 0.054 ß5i: 0.0057

30, KZR-616
IC_{50} (μM)
ß1c: >10 ß1i: 0.131
ß2c: 0.604 ß2i: 0.623
ß5c: 0.688 ß5i: 0.039

Figure 11. Chemical structures and inhibition profiles of some relevant multi-selective immunoproteasome inhibitors: LU-005i **20**, ONX 0914 **29**, KZR-616 **30**. The apparent inhibitory concentration values (IC_{50}) in Raji lysates as determined by competitive activity-based protein profiling in μM are found underneath each chemical structure for each of the catalytically active proteasome subunits.[38,48,49]

The research described in the doctoral thesis of Dr. E. Maurits re-expanded upon the structural features of LU-005i **20** and reported on several LU-005i analogues where P3 and P4 were systematically varied.[47] A subset of each peptide epoxyketone tested in these studies is depicted in **Figure 12**. The study compared LU-005i **20** with a set of P3 and P4 analogues, and showed that peptide epoxyketone featuring the N-terminal Morph-Ser(OBn) **33** motif did not inhibit any of the iCP or cCP active sites, while its P4 analogue BocPip-Ser(OBn) **38** proved to be a potent inhibitor for β1i, β2i, β5c and β5i. Additionally, BocPip-Ser(OBn) **38** proved as potent for β1i and β2i, but more potent for β5c when compared to LU-005i **20**. Inhibitors featuring a glycine residue at P3 (**31** and **36**) also proved to be weak inhibitors for each of the proteasomal active sites, independent of which P4 cap was featured. Similarly, inhibitors featuring an aminoisobutyric acid (Aib) residue at P3 (**35** and **40**) proved to be equally weak inhibitors compared to their P3 alanine analogues **32** and **37**. In addition, Morph-Ser derivative **34** proved to be more potent for β1i, β2i, β5c and β5i compared to its *O*-benzyl analogue **33**, with increases in potency of at least five-fold. Similarly, BocPip-Ser derivative **39** showed increased potency for β1i, β2c and β2i compared to its *O*-benzyl analogue **38**.[47]

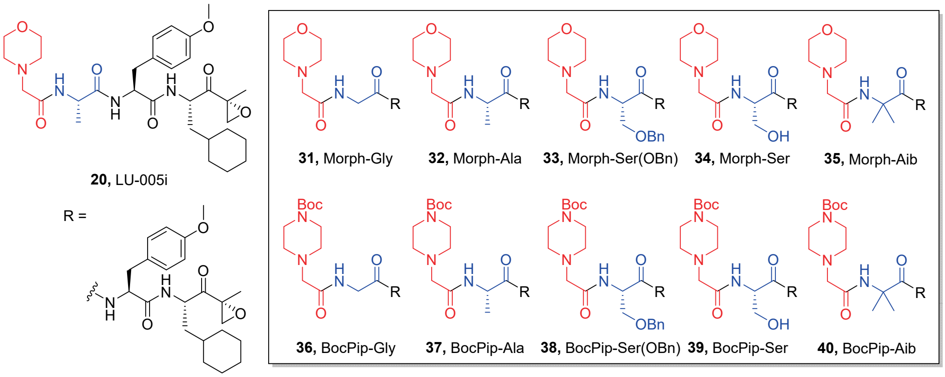


Figure 12. Chemical structures of P3 and P4 modified LU-005i based peptide epoxyketones that form the starting point of the studies described in **chapter two**. Compounds are named based on the abbreviation of the P4 cap followed by the amino acid abbreviation of P3. **A**) Chemical structure of LU-005i with alanine at P3 (in blue) and the N-terminal morpholine cap at P4 (in red). **B**) Chemical structures of ten P3 and P4 motifs found in the doctoral work of Dr. E. Maurits.[47]

Aim and outline of this thesis

The research described in this thesis aimed at the development of immunoselective proteasome inhibitors, either through varying the conventional recognition sequence of the inhibitors, or through introducing substitution of the electrophilic trap. Expanding on the doctoral thesis of Dr. E. Maurits, **chapter two** focuses on the synthesis of proteasome inhibitors that have serine analogues at P3 and feature a 2-morpholinoacetyl cap or *N*-Boc piperazinyl cap at P4. Each of the inhibitors in the small P3/P4 library was evaluated for its potency and selectivity as proteasome inhibitors, in addition to an analysis of two inhibitor-bound crystal structures of the β2 and β5 yeast proteasome subunits. **Chapter three** describes the synthesis of hydroxymethyl substituted epoxyketones, and esterified analogues thereof to probe the S' pocket with P1' substituted inhibitors. Biochemical evaluation of these substituted epoxyketones proved to greatly influence the inhibition profile when compared to the parent inhibitor LU-005i. Specifically, a set of *N*-Boc amino acid ester analogues proved to be immunoproteasome selective. Their de-N-Bocylated derivatives proved in some cases to further enhance the selectivity and potency for the immunoproteasome active sites. **Chapter four** expands on substituted epoxyketones, introducing the synthesis of three additional variations, as well as their effectivity and selectivity in inhibiting the 20S proteasome. Unfortunately, none of the novel epoxyketone substituted compounds were potent toward the 20S proteasome. **Chapter five** combines inhibitor design featured in chapters two and three, by synthesizing P3 and P1' modified peptide epoxyketones. Specifically, the epoxyketones of a set of P3 serine LU-005i analogues featured in chapter two, were substituted to feature a hydroxymethyl group and esterification thereof. Each of the successfully synthesized inhibitors were evaluated for their potency and selectivity for the human proteasome in two different haematological cancer cell lines. **Chapter six** summarizes the research described in this thesis and provides possible future directions.

References

1. G. A. Collins, A.L. Goldberg. The logic of the 26S proteasome. *Cell* **2017**, *169*, 792-806.
2. M. Groettrup, C. J. Kirk, M. Basler. Proteasomes in immune cells: more than peptide producers? *Nat. Rev. Immunol.* **2010**, *10*, 73-78.
3. E. E. Manasanch, R.Z. Orlowski. Proteasome inhibitors in cancer therapy. *Nat. Rev. Clin. Oncol.* **2017**, *14*, 417-433.
4. P. Moreau, P. Richardson, M. Cavo, R. Orlowski, J. San Miguel, A. Palumbo, J.L. Harousseau. Proteasome inhibitors in multiple myeloma: 10 years later. *Blood* **2012**, *120*, 947-959.
5. E. Scalzulli, S. Grammatico, F. Vozella, M.T. Petrucci. Proteasome inhibitors for the treatment of multiple myeloma. *Exp. Opin. Pharmacol.* **2018**, *19*, 375-386.
6. A. Besse, L. Besse, M. Kraus, M. Mendez-Lopez, J. Bader, B. Xin, G. de Bruin, E. Maurits, H.S. Overkleeft, C. Driessen. Proteasome inhibition in multiple myeloma: head-to-head comparison of currently available proteasome inhibitors. *Cell Chem. Biol.* **2019**, *26*, 340-351.
7. E. M. Huber, M.A. Groll. A nut for every bolt: subunit-selective inhibitors of the immunoproteasome and their therapeutic potential. *Cells* **2021**, *10*, 1929.
8. D. J. Sherman, J. Li. Proteasome inhibitors: harnessing proteostasis to combat disease. *Molecules* **2020**, *25*, 679.
9. G. de Bruin, Chemical tools to monitor and control human proteasome activities. Thesis, **2016** *Leiden University*.
10. J. Myung, K. B. Kim, C.M. Crews. The ubiquitin-proteasome pathway and proteasome inhibitors. *Med. Res. Rev.* **2001**, *21*, 245-273.
11. A. L. Schwartz, A. Ciechanover. Targeting proteins for destruction by the ubiquitin system: Implications for human pathobiology. *Annu. Rev. Pharmacol.* **2009**, *49*, 73-96.
12. L. Cappadocia, C.D. Lima. Ubiquitin-like protein conjugation: structures, chemistry, and mechanism. *Chem. Rev.* **2018**, *118*, 889-918.
13. S. M. Mali, Singh, S. K. Singh, Eid, E. Eid, A. Brik. Ubiquitin signaling: chemistry comes to the rescue. *J. Am. Chem. Soc.* **2017**, *139*, 4971-4986.
14. M. Groll, L. Ditzel, J. Lowe, D. Stock, M. Bochtler, H. Bartunikt, R. Huber. Structure of a 20S proteasome from yeast at 2.4Å resolution. *Nature* **1997**, *386*, 463-471.
15. L. Borissenko, M. Groll. 20S proteasome and its inhibitors: crystallographic knowledge for drug development. *Chem. Rev.* **2007**, *107*, 687-717.
16. M. Groll, K. B. Kim, N. Kairies, R. Huber, C.M. Crews. Crystal structure of epoxomicin:20S proteasome reveals a molecular basis for selectivity of α',β'-epoxyketone proteasome inhibitors. *J. Am. Chem. Soc.* **2000**, *122*, 1237-1238.
17. J. Schrader, F. Henneberg, R.A. Mata, K. Tittmann, T.R. Schneider, H. Stark, G. Bourenkov, A. Chari. The inhibition mechanism of human 20S proteasomes enables next-generation inhibitor design. *Science* **2016**, *353*, 594-598.
18. E. M. Huber, M. Basler, R. Schwab, W. Heinemeyer, C. Kirk, M. Groettrup, M. Groll. Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. *Cell* **2012**, *148*, 727-738.
19. K. L. Rock, I. A. York, T. Saric, A.L. Goldberg. Protein degradation and the generation of MHC class I-presented peptides. *Adv. Immunol.* **2002**, *80*, 1-70.
20. E. M. Huber, M. Groll. Inhibitors for the immuno- and constitutive proteasome: current and future trends in drug development. *Angew. Chem. Int. Ed.* **2012**, *51*, 8708-8720.
21. E. M. Huber, G. de Bruin, W. Heinemeyer, G. Soriano, H.S. Overkleeft, M. Groll. Systematic analyses of substrate preferences of 20S proteasomes using peptidic epoxyketone inhibitors. *J. Am. Chem. Soc.* **2015**, *137*, 7835-7842.

22. S. Murata, Y. Takahama, M. Kasahara, K. Tanaka. The immunoproteasome and thymoproteasome: functions, evolution and human disease. *Nat. Immunol.* **2018**, *19*, 923-931.
23. H. Uechi, J. Hamazaki, S. Murata. Characterization of the testis-specific proteasome subunit α4 in mammals. *J. Biol. Chem.* **2014**, *289*, 12365-12374.
24. R. W. Robertson, L. Zhang, D. Pasco, J. Fagan, D. Hilbert, K. Holmes, S. Rudikoff, G. Fenteany, R. Standeart, W. Lane, S. Choi, E. Corey, S. Schreiber. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Annu. Rev. Pharmacol. Toxicol.* **1994**, *14*, 268.
25. S. Omura, A. Crump. Lactacystin: first-in-class proteasome inhibitor still excelling and an exemplar for future antibiotic research. *J. Antibiot.* **2019**, *72*, 189-201.
26. M. Hanada, K. Suguwara, K. Kaneta, S. Toda, Y. Nishiyama, K. Tomita, H. Yamamoto, M. Konishi, T. Epoxomicin, a new antitumor agent of microbial origin. *J. Antibiot.* **1992**, *45*, 1746-1752.
27. A. Rentsch, D. Landsberg, T. Brodmann, L. Bulow, A. Girbig, M. Kalesse. Synthesis and pharmacology of proteasome inhibitors. *Angew. Chem. Int. Ed.* **2013**, *52*, 5450-5488.
28. M. Bogyo, J. McMaster, M. Gaczynska, D. Tortrella, A. Goldberg, H. Ploegh. Covalent modification of the active site threonine of proteasomal subunits and the Escherichia coli homolog HslV by a new class of inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6629-6634.
29. M. Groll, C. R. Berkers, H. L. Ploegh, H. Ovaa. Crystal structure of the boronic acid-based proteasome inhibitor bortezomib in complex with the yeast 20S proteasome. *Structure* **2006**, *14*, 451-456.
30. L. E. Sanman, M. Bogyo. Activity-based profiling of proteases. *Annu. Rev. Biochem.* **2014**, *83*, 249-273.
31. M. Britton, M. Lucas, S. Downey, M. Screen, A. Pletnev, M. Verdoes, R. Tokhunts, O. Amir, A. Goddard, P. Pelphrey, D. Wright, H. Overkleeft, A.F. Kisselev. Selective inhibitor of proteasome’s caspase-like sites sensitizes cells to specific inhibition of chymotrypsin-like sites. *Chem. Biol.* **2009**, *16*, 1278-1289.
32. P. P. Geurink, W. van der Linden, A. Mirabella, N. Gallastegui, G. de Bruin, A. Blom, M. Voges, E. Mock, B.I. Florea, G. van der Marel, C. Driessen, M. van der Stelt, M. Groll, H.S. Overkleeft, A. Kisselev. Incorporation of non-natural amino acids improves cell permeability and potency of specific inhibitors of proteasome trypsin-like sites. *J. Med. Chem.* **2013**, *56*, 1262-1275.
33. M. Screen, M. Britton, S. Downy, M. Verdoes, M. Voges, A. Blom, P. Geurink, M. Risseeuw, B. Florea, W. van der Linden, A. Pletnev, H. Overkleeft, A. Kisselev. Nature of pharmacophore influences active site specificity of proteasome inhibitors. *J. Biol. Chem.* **2010**, *285*, 40125-40134.
34. M. Elofsson, U. Splittgerber, J. Myung, R. Mohan, C.M. Crews. Towards subunit-specific proteasome inhibitors: synthesis and evaluation of peptide α’,β’-epoxyketones. *Chem. Biol.* **1999**, *6*, 811-822.
35. A. F. Kisselev. Site-specific proteasome inhibitors. *Biomolecules* **2022**, *12*, 54.
36. E. M. Huber, W. Heinemeyer, G. de Bruin, H. S. Overkleeft, M. Groll. A humanized yeast proteasome identifies unique binding modes of inhibitors for the immunosubunit β5i. *EMBO J.* **2016**, *35*, 2602-2613.
37. B. T. Xin, G. de Bruin, E. Huber, A. Besse, B.I. Florea, D. Filippov, G. van der Marel, A. Kisselev, M. van der Stelt, C. Driessen, M. Groll, H.S. Overkleeft. Structure-based design of β5c selective inhibitors of human constitutive proteasomes. *J. Med. Chem.* **2016**, *59*, 7177-7187.

38. G. de Bruin, E. Huber, B. Xin, E. van Rooden, K. Al-Ayed, K. Kim, A. Kisselev, C. Driessen, M. van der Stelt, G. van der Marel, M. Groll, H.S. Overkleeft. Structure-based design of β1i or β5i specific inhibitors of human immunoproteasomes. *J. Med. Chem.* **2014**, *57*, 6197-6209.
39. B. T. Xin, G. de Bruin, E. Huber, A. Besse, B.I. Florea, D. Filippov, G. van der Marel, A. Kisselev, M. van der Stelt, C. Driessen, M. Groll, H.S. Overkleeft. Structure-based design of inhibitors selective for human proteasome β2c or β2i subunits. *J. Med. Chem.* **2019**, *62*, 1626-1642.
40. M. J. Lee, D. Bhattarai, J. Yoo, Z. Miller, J. Park, S. Lee, W. Lee, J. Driscoll, K. Kim. Development of novel epoxyketone-based proteasome inhibitors as a strategy to overcome cancer resistance to carfilzomib and bortezomib. *J. Med. Chem.* **2019**, *62*, 4444-4455.
41. H. W. B. Johnson, J. Anderl, E. Bradley, J. Bui, J. Jones, S. Arastu-Kapur, L. Kelly, E. Lowe, D. Moebius, T. Muchamuel, C. Kirk, Z. Wang, D. McMinn. Discovery of highly selective inhibitors of the immunoproteasome low molecular mass polypeptide 2 (LMP2) subunit. *ACS Med. Chem. Lett.* **2017**, *8*, 413-417.
42. K. B. Kim, C.M. Crews. From epoxomicin to carfilzomib: chemistry, biology, and medical outcomes. *Nat. Prod. Rep.* **2013**, *30*, 600-604.
43. W. Harshbarger, C. Miller, C. Diedrich, J. Sacchettini. Crystal structure of the human 20S proteasome in complex with carfilzomib. *Structure* **2015**, *23*, 418-424.
44. G. de Bruin, B. Xin, M. Kraus, M. van der Stelt, G. van der Marel, A. Kisselev, C. Driessen, B. Florea, H.S. Overkleeft. A set of activity-based probes to visualize human (immuno)proteasome activities. *Angew. Chem. Int. Ed.* **2016**, *55*, 4199-4203.
45. S. Serim, U. Haedke, S.H.L. Verhelst. Activity-based probes for the study of proteases: recent advances and developments. *Chem. Med. Chem.* **2012**, *7*, 1146-1159.
46. N. Sin, B. Kyung, M. Elofsson, L. Meng, H. Auth, B. Kwok, C.M. Crews. Total synthesis of the potent proteasome inhibitor epoxomicin: a useful tool for understanding proteasome biology. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2283-2288.
47. C. Maurits. Targeting human proteasomes: Substrates, inhibitors and prodrugs. Thesis, Leiden university, **2020**.
48. H. W. B. Johnson, E. Lowe, J. Anderl, A. Fan, T. Muchamuel, S. Bowers, D. Moebius, C. Kirk, D. McMinn. Required immunoproteasome subunit inhibition profile for anti-inflammatory efficacy and clinical candidate KZR-616 ((2 S,3 R)- N-((S)-3-(Cyclopent-1-en-1-yl)-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)-3-hydroxy-3-(4-methoxyphenyl)-2-((S)-2-(2-morpholinacetamide)propanamido)propenamide). *J. Med. Chem.* **2018**, *61*, 11127-11143.
49. E. M. Huber, Heinemeyer, W. Heinemeyer, M. Groll. Bortezomib-resistant mutant proteasomes: structural and biochemical evaluation with carfilzomib and ONX-0914. *Structure* **2015**, *23*, 407-417.
50. T. W. Jenkins, S. Downey-Kopyscinski, J. Fields, G. Rahme, W. Colley, M. Israel, A. Maksimenko, S. Fiering, A. Kisselev. Activity of immunoproteasome inhibitor ONX-0914 in acute lymphoblastic leukemia expressing MLL–AF4 fusion protein. *Sci. Rep.* **2021**, *11*, 10883.
51. M. Basler, M. Lindstrom, J. LaStant, J. Bradshaw, T. Owens, C. Schmidt, E. Maurits, C. Tsu, H. Overkleeft, C. Kirk, C. Langrish, M. Groettrup. Co-inhibition of immunoproteasome subunits LMP2 and LMP7 is required to block autoimmunity. *EMBO Rep.* **2018**, *19*, e46512.
52. F. Oliveri, M. Basler, T. N. Rao, H. J. Fehling, M. Groettrup. Immunoproteasome inhibition reduces the T helper 2 response in mouse models of allergic airway inflammation. *Front. Immunol.* **2022**, *13*, 870720.

53. T. Muchamuel, M. Basler, M. Aujay, E. Suzuki, K. Kalim, C. Lauer, C. Sylvain, E. Ring, J. Shields, J. Jiang, P. Shwonek, F. Parlati, S. Demo, S. Bennet, C. Kirk, M. Groettrup. A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat. Med.* **2009**, *15*, 781-787.
54. M. Basler, E. Maurits, G. de Bruin, J. Koerner, H. Overkleeft, M. Groettrup. Amelioration of autoimmunity with an inhibitor selectively targeting all active centres of the immunoproteasome. *Br. J. Pharmacol.* **2018**, *175*, 38-52.