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Chemical tools to monitor and control human proteasome activities

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CHAPTER 12

Summary and future prospects

In this thesis the development of new tools to monitor and control proteasome activities are described. Proteasomes are multi-protein, multi-catalytic complexes responsible for the degradation of 80-90% of the proteins inside eukaryotic cells. Proteasomes contain a cylindrical 20S core particle (CP) and one or two 19S regulatory particles (RP). Proteins destined for degradation are tagged with a poly-ubiquitin chain, which is recognized by the 19S RP. Following ubiquitin removal and protein unfolding, the protein is translocated to the CP where the actual protein degradation takes place. The CP consists of four heptameric rings, two outer α -rings and two inner β -rings, with the catalytic subunits residing in the latter. The constitutive proteasome core particle (cCP), which is expressed in all mammalian tissues, contains three catalytically active subunits, namely β 1c (caspase-like, cleaving preferentially after acidic amino acids), β 2c (trypsin-like, cleaving preferentially after basic amino acids) and β 5c (chymotrypsin-like, cleaving preferentially after hydrophobic amino acids). Lymphoid cells, as well as cells that have been exposed to inflammatory cytokines express another proteasome core particle known as the immunoproteasome (iCP). In iCPs, β 1c, β 2c and β 5c are replaced by β 1i, β 2i and β 5i, respectively. The iCP catalytic activities have altered substrates preferences and iCP generated peptides have an averaged higher affinity for major histocompatibility complex class I (MHC-I), thus the iCP plays a major role in the immune system. In addition to cCPs and iCPs, mixed proteasomes (mCPs) may exist, in which both constitutive proteasome and immunoproteasome catalytic activities are found. Finally, thymic epithelial cells express thymoproteasomes (tCP), which play an important role in positive T-cell selection. In tCPs β 5i is replaced by β 5t in otherwise unchanged iCPs.

Proteasome inhibition is cytotoxic for certain types of cancer and is thought to lead to suppression of (auto)-immune diseases. Therefore, the proteasome is an important drug target in the field of oncology and immunology. Currently, several proteasome inhibitors are in use or in development for treatment of multiple myeloma and mantle cell lymphoma. Moreover, some iCP selective inhibitors are currently in clinical trials for the treatment of

auto-immune diseases. While in their development, $\beta 5$ subunits were considered as the most important target, all clinically used proteasome inhibitors appear to inhibit both $\beta 5c$ and $\beta 5i$, along with several other cCP/iCP catalytic activities. Indeed, in the last decade it has become clear that co-inhibition of additional proteasome catalytic subunits sensitizes cells to $\beta 5$ inhibition and is able to overcome resistance. For this reason, but also with the aim to unravel the contribution of the various catalytic subunits to protein turnover and MHC-I mediated antigen presentation, much research has been focussed on the discovery of proteasome catalytic subunit selective inhibitors. Associated with this, there is an increased interest in methods that allow simultaneous measurement of multiple proteasome catalytic activities. Such assays could aid the development of subunit selective inhibitors and determination of the proteasome composition in cell lines and (diseased) tissues. Related to this, methods to determine the presence and composition of mCPs have been developed. Briefly, the research described in this thesis reports on the development of new subunit-selective inhibitors and activity-based probes (ABPs), on the development of an assay to simultaneously monitor all cCP and iCP catalytic activities and on the development of a method that reports on CP catalytic active subunit composition. The tools that stem from the work described in this thesis can now be used to unravel the role of each individual catalytic subunit in a chemical genetics setting (selective and (near) complete inhibition of each individual subunit), and to clarify the role of mCPs, in, for instance, antigen presentation and cancer. Furthermore, these tools could possibly serve as leads in the discovery of agents for future treatment of cancer and autoimmune diseases.

Chapter 1 discusses the catalytic mechanism employed by proteasome active sites and the rationale behind proteasome inhibitors. **Chapter 2** presents an overview of the methods available to measure proteasome activity that have been developed to date. All methods rely either on the hydrolysis of substrates or on activity-based protein profiling (ABPP) and all methods have proven their worth in the search for new proteasome inhibitors, in determining the (relative) activity of the proteasome and in providing insight in the proteasome composition of a given biological sample.

Chapter 3 describes the development of a set of tools that enables monitoring and controlling the six catalytic activities of both human constitutive proteasomes and human immunoproteasomes. A cocktail of three ABPs, targeting either $\beta 1c/\beta 1i$ (Cy5-NC-001), $\beta 2c/\beta 2i$ (BODIPY(FL)-LU112) or $\beta 5c/\beta 5i$ (BODIPY(TMR)-NC-005), and each equipped with an orthogonal (in terms of excitation/emission) fluorophore was assembled.

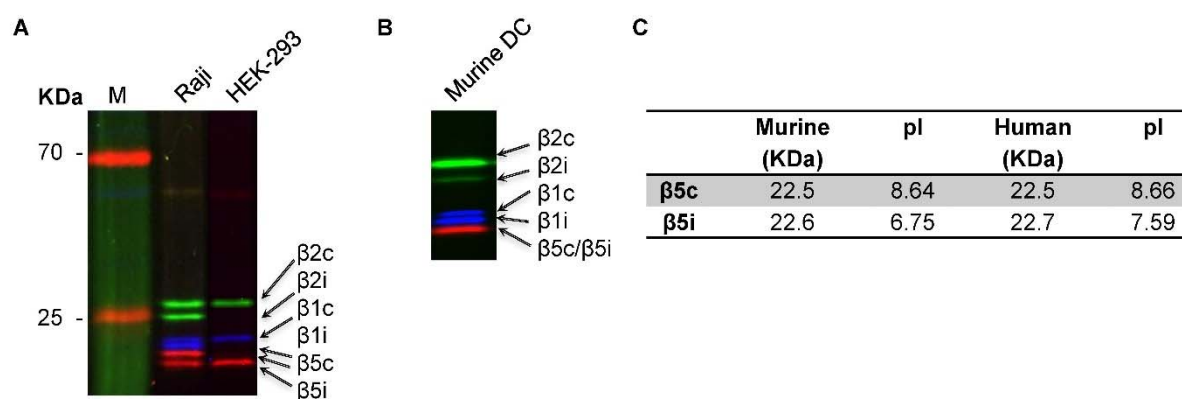


Figure 1. Proteasome ABP cocktail provides full separation of human cCP and iCP subunits on SDS-PAGE. Raji- or HEK-293 cell lysates (A) or murine dendritic cell (DC) lysates (B) were treated with the ABP cocktail, followed by SDS-PAGE and fluorescence imaging of the wet gel slabs. C) Molecular weight and pI values of human and murine β5c and β5i.

On SDS-PAGE, this cocktail provided full resolution of all six catalytic subunits of the human (immuno)proteasome modified by these probes (Figure 1A). This assay enables straightforward screening of putative proteasome inhibitors and rapid assessment of the proteasome composition of cell lines and patient samples, with respect to the relative abundance of each subunit. Using this cocktail, proteasome inhibitors selective for, either β1c, β2c, β2i or β5c were identified. Moreover, primary malignant haematological cells were revealed to express predominately iCP subunits; this in contrast to for instance multiple myeloma cell lines that express about equal levels of cCP and iCP subunits. Based on this observation, acute lymphoid leukemia (ALL) patient cells were treated with a combination of β1i and β5i inhibitors, which proved to be highly cytotoxic. Thus, selective iCP inhibition may be a valuable strategy to limit side effects associated with proteasome inhibitors currently used in the clinic in treating haematological cancers, especially since most cells have no or low expression of iCP subunits.

Since the β5c/β5i selective ABP BODIPY(TMR)-NC-005 does not separate murine β5c and β5i on SDS-PAGE, this probe cannot be used to determine the individual activity of these subunits in murine cell lines or tissues (Figure 1B). Murine β5c and β5i are slightly smaller compared to their human counterparts, and differ slightly in pI values and either of these differences, or a combination thereof, may be at the basis of the subtly different behaviour on SDS-PAGE (Figure 1C). The use of the ABPP assay described in Chapter 2 is thus limited to human cell lines or patient samples when full assessment of all cCP and iCP activities is desired. This is disadvantageous since often mouse models are used for *in vivo* studies. Interestingly, other β5c/β5i targeting ABPs, such as BODIPY-epoxomicin^{1, 2} and the probes described in chapter 6, are also not able to separate human β5c and β5i on SDS-PAGE, indicating that the nature of the fluorophore or inhibitor influences the resolution of the

subunits modified by these on SDS-PAGE. It would therefore be interesting to evaluate $\beta 5c/\beta 5i$ selective inhibitors equipped with different fluorophores, such as Cy3, Cy5, TAMRA and rhodamine in murine samples and compare these with human samples. Given the difference in pI values between human and murine $\beta 5i$, another solution to this problem might be to change the pH of the resolving gel. Furthermore, the percentage acrylamide and cross-links, size of the gel and the voltage at which the gel is resolved could be varied in order to find conditions which enable separation of murine $\beta 5c$ and $\beta 5i$.

While this assay enables rapid screening of putative proteasome inhibitors, high-throughput screening (HTS) of large compound libraries is not possible due to the gel-based format. To overcome this shortcoming, a fluorescence polarization competitive ABPP assay (FluoPol-ABPP) assay may be considered. Fluorescence polarization is used to study molecular interactions and is a measure of the apparent size of a fluorophore. The underlying principle is the slow tumbling rate of large molecules and high tumbling rate of small molecules. Small fluorophore containing molecules, such as ABPs, excited with plane-polarized light rotate in the excited state, resulting in the emission of depolarized light (low fluorescence polarization). Larger molecules, such as an ABP bound to a protein, rotate much slower, thereby emitting highly polarized light (high fluorescence polarization).³ Cravatt *et al.* have pioneered the application of FluoPol-ABPP in HTS.⁴ They developed an assay to screen for inhibitors of retinoblastoma-binding protein-9 (RBBP9) which was found to covalently interact with fluorophosphonate (FP)-rhodamine, a broad-spectrum serine hydrolase ABP. Based on these results, it might be possible to develop a proteasome FluoPol-ABPP, as depicted in Figure 2. However, several hurdles have to be taken. In FluoPol-ABPP assays, often μM concentrations of purified enzyme and nM concentration of ABP are needed in order ensure full binding of ABPs, thereby providing low background.⁴⁻⁶ However, given the size of the proteasome, this would require large amounts of purified 20S proteasome, which is rather expensive. Therefore, it would be desirable to perform proteasome FluoPol-ABPP in crude cell lysates with preferably also lower ABP concentrations. The probes described in chapter 3 are equipped with different fluorophores, and therefore it should be possible to monitor the activity of $\beta 1$, $\beta 2$ and $\beta 5$ simultaneously by FluoPol-ABPP. Fluorescence quenching will however likely occur (see chapter 10) in native samples, and a denaturation step is likely necessary following a competitive ABPP with the three probes and before fluorescence polarization is measured.

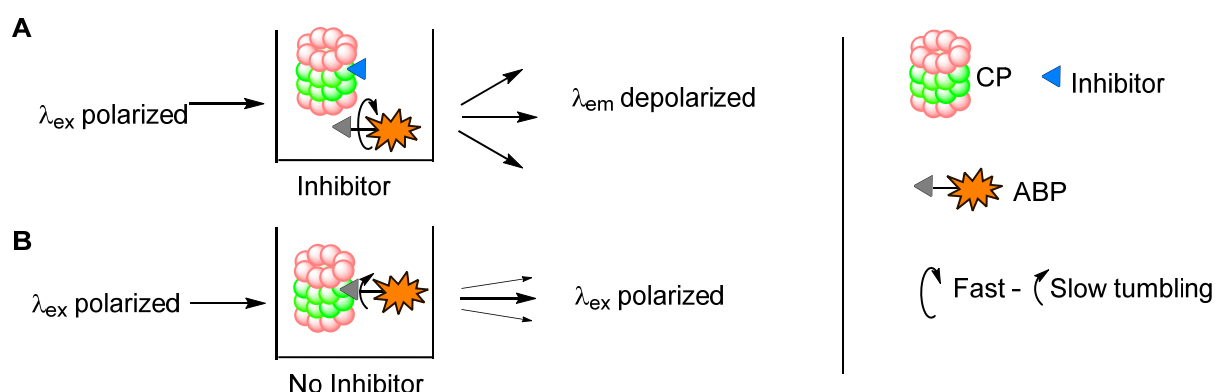


Figure 2. Proteasome FluoPol-ABPP. A) In presence of an inhibitor the ABP cannot bind, and the fast tumbling rate causes depolarization of the emitted light. B) In absence of an inhibitor, the ABP binds to the proteasome, resulting in slow rotation of the fluorophore, hence emission of highly polarized light.

Chapter 4 reports on a systematic analysis of substrate specificities of human cCPs and iCPs and yeast proteasome (yCP). For this, 18 oligopeptide epoxyketones (EK) were synthesized. The focused peptide library was assembled from Ala, Leu, Asp, Glu, Phe, Tyr, Ile or Val at P1; Ala or Leu at P2 and Leu or Pro at P3, and an acetyl N-cap. yCP crystals were soaked with the epoxyketones and X-ray structures were determined. This study provided detailed insight in substrate specificities of human proteasome subunits and yielded design parameters for subunit selective inhibitors. Interestingly, Val and Ile at P1 are highly disfavoured by all active sites of yeast and human proteasomes. Both yeast $\beta 1$ and human $\beta 1c$ prefer Asp, and not so much Glu, at P1, confirming that ‘caspase-like’ describes the specificity of $\beta 1(c)$ more accurately than the traditional ‘peptidyl-glutamyl peptide hydrolysing’. Implementation of the obtained knowledge led to the development of the $\beta 1c$ selective inhibitor LU-001c, as described in chapter 8. As expected, due to mutations in its S1 pocket, $\beta 1i$ prefers hydrophobic residues (Leu, Phe) at P1. The study also yielded a structural basis for the $\beta 1c/\beta 1i$ preference of inhibitors bearing Pro at P3. Compounds bearing Ala at P1 and Leu at P3 proved highly $\beta 5c$ selective (hardly any $\beta 5i$ inhibition observed), a result that was capitalized on in the development of $\beta 5c$ selective inhibitors (chapter 7). Interestingly, Glu at P1 in Ac-LAE-EK showed 30-fold selectivity for $\beta 2c$ over $\beta 2i$, although with moderate potency (IC_{50} : 3.6 μM). It may well be that the Asp53Glu mutation in $\beta 2i$, which prevents hydrogen bonding to the Glu side chain of Ac-LAE-EK, causes this observed $\beta 2c$ selectivity. These results may be exploited to further improve the moderate $\beta 2c$ selective inhibitor LU-002c described in chapter 3 (LU-002c, IC_{50} : 0.08 μM , selectivity: 20x). Although both compounds bear an Ala residue at P2, it is unlikely that in case of Ac-LAE-EK this causes $\beta 2c$ selectivity, this because none of the other epoxyketone with Ala at P2 proved to be $\beta 2c$ selective.

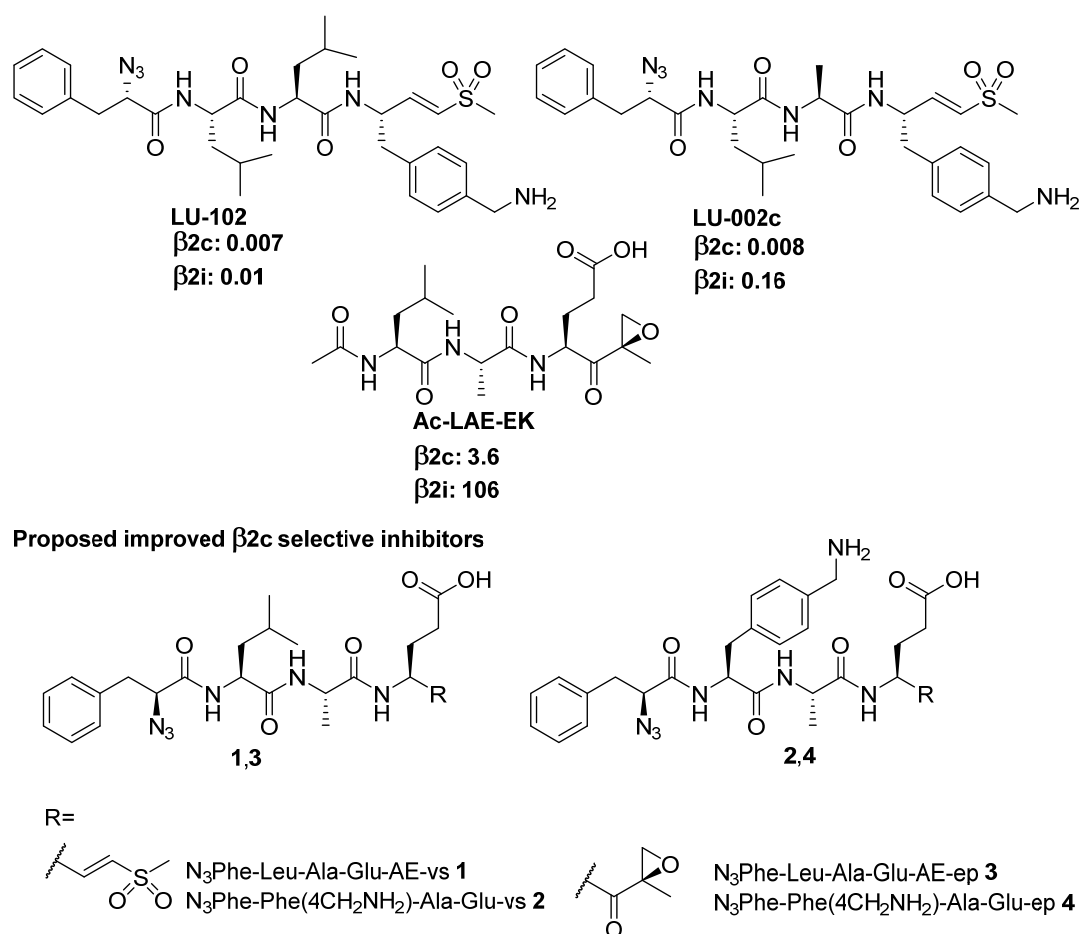


Figure 3. Structures of β_2c/β_2i selective inhibitor LU-102, β_2c selective inhibitors LU-002c and Ac-LAE-EK and proposed improved β_2c selective inhibitors 1-4.

However, since in LU-002c, the P2 Ala does cause β_2c selectivity, selectivity of an inhibitor is likely in part guided by the combination of amino acids present in this inhibitor. Considering this, several compounds can be proposed which may turn out to have improved β_2c selectivity compared to LU-002c and Ac-LAE-EK (Figure 3). Addition of a P4 residue could increase the potency of P1 Glu containing compounds **1** and **3**. Since LU-002c is equipped with a vinyl sulfone warhead and Ac-LAE-EK contains an epoxyketone, both warheads should be evaluated to determine their influence on selectivity. As has been shown previously⁷, a basic residue at P3 does increase β_2c/β_2i selectivity and potency and could thus further increase the potency of P1 Glu containing compounds. These considerations suggest that compounds **2** and **4** would be interesting targets to prepare and evaluate.

Chapter 5 describes the design and synthesis of improved β_1i and β_5i selective inhibitors. Incorporation of larger groups at P1 resulted in improved selectivity of existing β_5i selective inhibitors, which was the expected result given the structural data on murine cCP and iCP in complex with the literature β_5i -selective inhibitor, PR-957. Guided by these proteasome-

inhibitor structures, a focussed compound library was assembled featuring a number of bulky, hydrophobic residues at P1, specifically 1-naphthyl, 2-naphthyl, biphenyl, cyclohexyl and adamantyl. Taking the PR-957 structure and substituting the P1 phenylalanine for cyclohexylalanine (Cha) (LU-005i) yielded a compound featuring a five-fold improved $\beta 5i$ selectivity compared to the parent compound. Substituting the P1 Phe residue in PR-924 (another $\beta 5i$ selective inhibitor) for Cha resulted in the most selective $\beta 5i$ inhibitor known to date (LU-015i, 500-fold selective for $\beta 5i$ over $\beta 5c$, Figure 4). In a related study, analogues of the $\beta 1$ selective inhibitor NC-001, bearing different Pro residues at P3, were synthesized. Of these, in particular 4,4-F₂-Pro was found to induce $\beta 1i$ selectivity. Moreover, $\beta 5i$ selective inhibitors with either Phe or Cha at P1 were found to be selective for $\beta 1i$ over $\beta 1c$. Combining these two findings led to the highly potent and selective $\beta 1i$ inhibitor LU-001i (Figure 4).

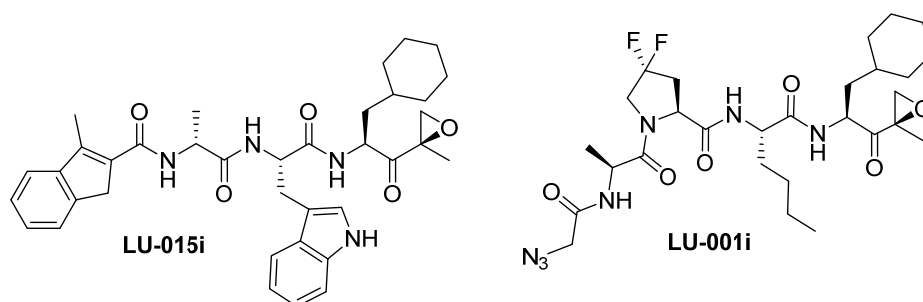


Figure 4. Structures of LU-015i ($\beta 5i$ -selective) and LU-001i ($\beta 1i$ selective).

The selective inhibitors described in chapter 5 were used to develop ABPs selective for $\beta 5i$ or $\beta 1i$, as is described in **chapter 6**. LU-001i was converted to Cy5-LU-001i (Figure 5) through copper catalysed azide-alkyne cycloaddition (click) with Cy5-alkyne. In order to obtain a $\beta 5i$ selective ABP, first the 3-methylindene cap of LU-015i was substituted for azido phenylalanine. However, attachment of a fluorophore at the azide provided ABPs with poor selectivity.

The S2 binding pocket of $\beta 5i$ (and all other active subunits) is spacious and solvent exposed. This allows the introduction of reporter moieties at the P2 position of subunit-selective inhibitors. Substituting the methyltyrosine at P2 position in the $\beta 5i$ -selective inhibitor LU-035i for a fluorescently labelled tyrosine residue gave Cy5-LU-035i (Figure 5), which proved to be potent and selective for $\beta 5i$. Both Cy5-LU-001i and Cy5-LU-035i can be used to completely block and tag their targeting subunits, with the other subunits remaining uncompromised.

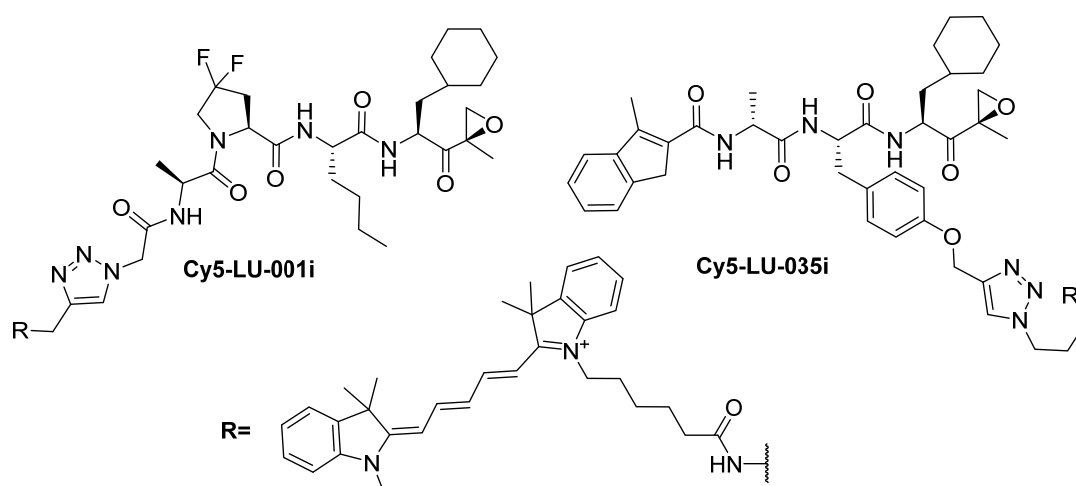


Figure 5. Structures of iCP subunit selective probes Cy5-LU-001i (β 1i selective) and Cy5-LU-035i (β 5i selective).

Chapter 7 details the development of highly selective β 5c targeting inhibitors. Now, crystal structures of murine cCP and iCP in complex with PR-957 guided the design of β 5c selective inhibitors. In this study, a focussed library of compounds, containing different bulky amino acids at the P3 position of the otherwise unchanged N₃Phe-xxx-Phe-Leu-EK sequence was prepared and evaluated. The compound featuring a Cha residue at P3 proved to be a low nanomolar β 5c inhibitor with some ten-fold selectivity for β 5c over β 5i. Various other compounds also proved to be β 5c selective, but were considerably less potent. Since it was previously established that P1 Ala is well tolerated by β 5c but not by β 5i (see chapter 4), a series of compounds was synthesized bearing an Ala at P1 and bulky amino acids at P3. The compound with Cha at P3 and Ala at P1 showed impeccable β 5c over β 5i selectivity, although β 2c/ β 2i are also targeted. At the same time, it was noted that compounds bearing a P3 biphenylalanine (BiPhe) are highly disfavoured by β 2c/ β 2i. Therefore, bicyclohexylalanine (BiCha, synthesized by hydrogenation of BiPhe, thus yielding a mixture of *cis/trans*-isomers – something to resolve as well in the future) was incorporated at P3 together with Ala at P1. The resulting compound (LU-005c) proved to be highly β 5c selective (over β 5i), did not target β 2c/ β 2i and can thus be considered as the most β 5c selective inhibitor known to date (Figure 6). Unfortunately, while LU-005c displayed high potency and β 5c selectivity in lysates, its *in situ* potency is drastically lower. Arguably, the compound is too lipophilic to cross the cell membrane. Substitution of the P4 azidophenylalanine residue by Leu, which was then N-capped with a 2-morpholino acetate moiety, resulted in compound LU-015c. Although this compound showed reduced selectivity compared to LU-005c, it showed increased cell permeability and allows full blockage of β 5c without inhibition of other subunits. Introduction of a BODIPY(FL) fluorophore at the P2 position of LU-015c yielded the β 5c-selective ABP, (BODIPY(FL)-LU-015i), with good selectivity for β 5c over β 5i, however, accompanied by significant β 2c/ β 2i labelling.

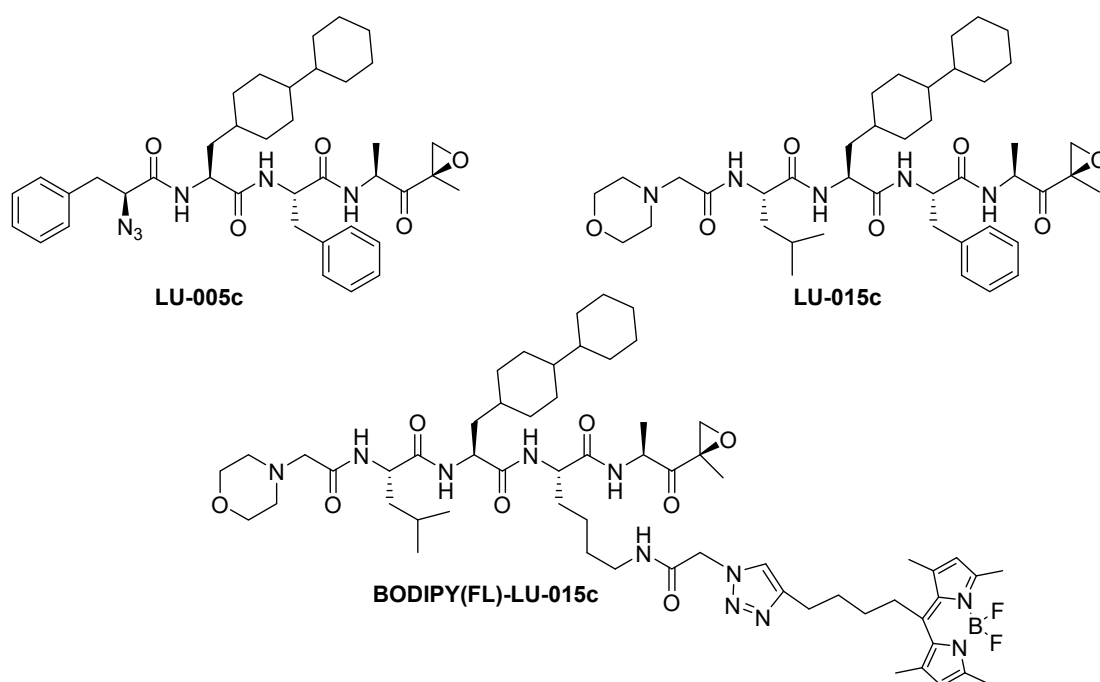


Figure 6. Structures of $\beta 5c$ selective inhibitors LU-005c and LU-015c and $\beta 5c$ selective ABP BODIPY(FL)-LU-015c

Chapter 8 describes the development of a potent, highly selective $\beta 1c$ inhibitor. From the systematic study described in chapter 4 it became apparent that $\beta 1c$ can be selectively targeted by the P1 Asp bearing compound Ac-PAD-EK (Figure 7). However, the apparent IC_{50} value in Raji lysate was rather high (1.4 μM for $\beta 1c$, >100 μM for all other subunits). Therefore, Asp epoxyketone was incorporated at P1 in the highly optimized $\beta 1$ targeting sequence of NC-001, resulting in compound LU-001c (Figure 7). The P4 residues (Ala) and extended P2 residues (Nle) may provide additional interactions and stabilization of the inhibitor, thereby enhancing the potency of LU-001c compared to Ac-PAD-EK. Indeed, the apparent IC_{50} value of LU-001c was found to be 20-fold lower (0.07 μM), while all other subunits remained unmodified up to 100 μM concentrations. LU-001c is equipped with a N-terminal azide moiety, which enabled the attachment of BODIPY-FL using click chemistry. In this way the highly potent and $\beta 1c$ targeting ABP BODIPY(FL)-LU-001c (Figure 7) was obtained, which can be used to completely block $\beta 1c$, without modifying other active proteasome subunits.

At neutral pH, the carboxylic acid moiety of LU-001c is negatively charged, rendering this compound cell impermeable thus precluding its use in intact cells. To overcome this limitation, a prodrug approach could be employed in which the carboxylic acid is protected as an ester. After cell membrane penetration, the ester protecting group is expected to be hydrolysed by esterases thereby liberating the active inhibitors.⁸

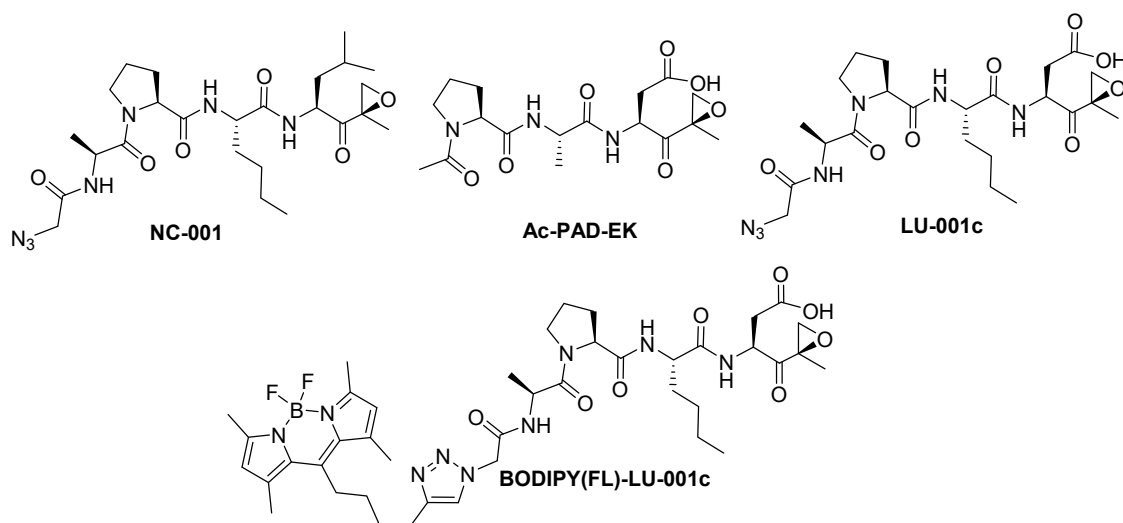
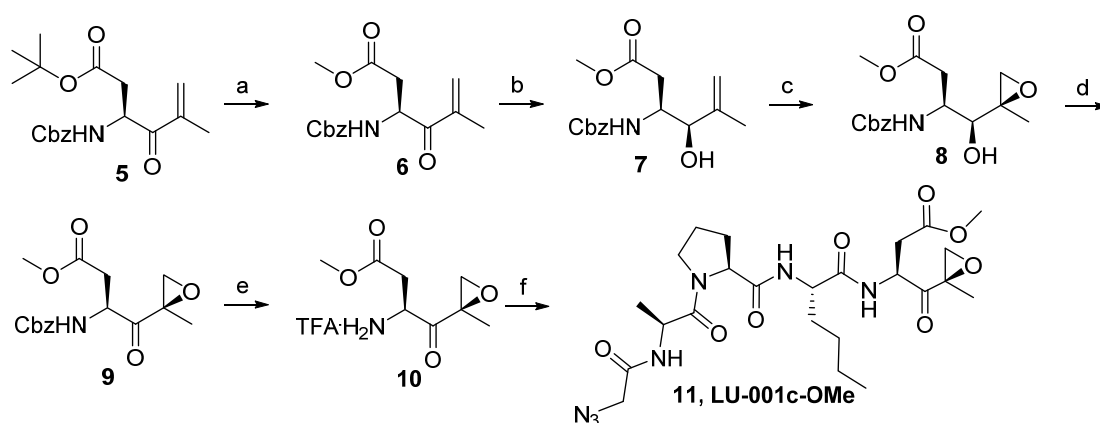


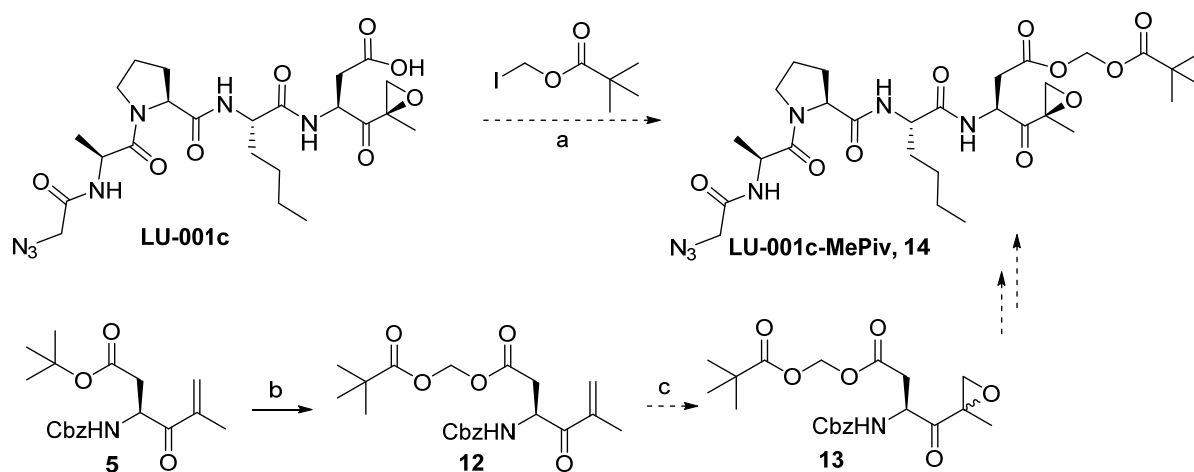
Figure 7. Structures of NC-001, Ac-PAD-EK, LU-001c and BODIPY(FL)-LU-001c

To explore this strategy, a methyl ester analogue of LU-001c was synthesized. For this, *tert*-butyl ester protected building block **5** was deprotected using TFA and the resulting carboxylic acid was converted to methyl ester **6** (Scheme 1). Luche reduction provided alcohol **7**, which was epoxidized to yield compound **8** as a mixture of two diastereomers, which could be separated using column chromatography. The major isomer was oxidized to provide epoxyketone **9**, which was hydrogenated to remove the Cbz protecting group. Finally, azide coupling provided compound **11**. The minor diastereomer of compound **8** was also taken further in the synthesis, however, biological evaluation of the resulting final compound showed impaired activity, indicating wrong epoxyketone stereochemistry.



Scheme 1. Synthesis of LU-001c-OMe (**11**). Reagents and conditions: a. 1. TFA; 2. DMAP, EDC, MeOH, 43%; b. $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$, NaBH_4 , MeOH, 0°C , 79%; c. $\text{VO}(\text{acac})_2$, tBuOOH , DCM, 0°C , 22%; d. DMP, DCM, 0°C , 70%; e. TFA, Pd/C, MeOH, H_2 ; f. $\text{N}_3\text{Gly-Ala-Pro-Nle-NHNH}_2$, tBuONO , HCl, DiPEA, DMF, -30°C , 18%.

Evaluation of LU-001c-OMe **11** in Raji lysates showed loss of selectivity and reduced potency for β 1c (IC_{50} : β 1c 0.46 μ M; β 1i: 0.95 μ M) indicating that hydrolysis of the methyl ester does not occur in lysates. Unfortunately, also in living RPMI-8226 cells the methyl ester is not hydrolysed, even not after a three hour treatment as is indicated by the high IC_{50} value for both β 1c/ β 1i (both 10 μ M). Nevertheless, LU-001c was found to be completely cell impermeable, indicating that protecting the carboxylic acid as ester does improve cell permeability. Possibly, the methyl ester is too hindered for esterase-mediated hydrolysis. In literature studies, (acyloxy)alkyl esters have been used to overcome the slow cleavage rate of alkyl ester β -lactam antibiotics.⁹ Cleavage of (acyloxy)alkyl esters is initiated at the distal ester moiety, followed by spontaneous elimination of the 'central' aldehyde providing the free carboxylic acid. To investigate (acyloxy)alkyl esters as prodrugs in β 1c inhibitors, the methyl pivalate ester (MePiv) can be taken as starting point (Scheme 2). The *tert*-butyl moiety provides additional hydrophobicity, which may increase cell permeability. The additional steric bulk may also prevent proteasome inhibition by the unprocessed compound. It has been attempted to perform a Luche reduction of compound **12**, however this proved to be unsuccessful. Therefore, it is proposed to directly epoxidize **12** after which the resulting mixture of diastereomers may be separated during final HPLC purification.¹⁰ Alternatively, LU-001c-MePiv **14** may be synthesized by alkylation of LU-001c with iodomethyl pivalate (Scheme 2).



Scheme 2. Proposed synthesis of LU-001c-MePiv. Reagents and conditions: a. DiPEA, DCM or DMF; b. 1. TFA; 2. Iodomethylpivalate, DiPEA, DCM; c. H_2O_2 , H_2O , benzonitrile, DiPEA, MeOH, 0-4 $^{\circ}C$.

The presence of a basic residue at P1, which may be accompanied by another basic residue at P3, in proteasome inhibitors induces $\beta 2c/\beta 2i$ selectivity. LU-102 (P1: Phe(4CH₂NH₂)) is the most potent and cell permeable $\beta 2c/\beta 2i$ selective inhibitor known to date, but shows a 30-fold increased apparent IC₅₀ value in living cells compared to cell lysates. This is likely caused by the positive charge on the benzylamine moiety, which impairs cell permeability. Introduction of an amine moiety with a pK_a value closer to physiological pH will lead to a less charged inhibitor, possibly resulting in an improved cell permeability. **Chapter 9** describes the synthesis and evaluation of compounds with various lysine analogues with reduced pK_a values at P1 and/or P3. In addition, histidine was explored as basic residue at P1. Allylic (pK_a 9.7) and propargylic (pK_a 8.9) ϵ -amine lysine analogues were synthesized via chiral phase transfer catalyzed alkylation of a glycine-based template. However, while most compounds proved to be $\beta 2c/\beta 2i$ selective, all displayed a severe loss of potency compared to LU-102 (Figure 8A). Interestingly, both P1 allylic and propargylic amine containing compounds showed similar potency and $\beta 2c/\beta 2i$ selectivity, indicating that lower pK_a values are tolerated. It seems therefore that the loss of potency can be explained by the shorter distance between the amine and the peptide backbone in lysine (analogues) compared to arginine in the first generation $\beta 2c/\beta 2i$ selective inhibitors¹¹ and to the benzylamine moiety in LU-102.⁷ It would therefore be desirable to explore more bulky, basic amino acids with a similar distance between the α -carbon and the amino-group as 4-aminomethyl-Phe of LU-102 to ensure optimal positioning of the amine group of the inhibitor and Asp53 of $\beta 2c$ and Glu53 of $\beta 2i$. Examples of such amino acids are homo-lysine analogues containing an allylic- or propargylic amine (Figure 8B).

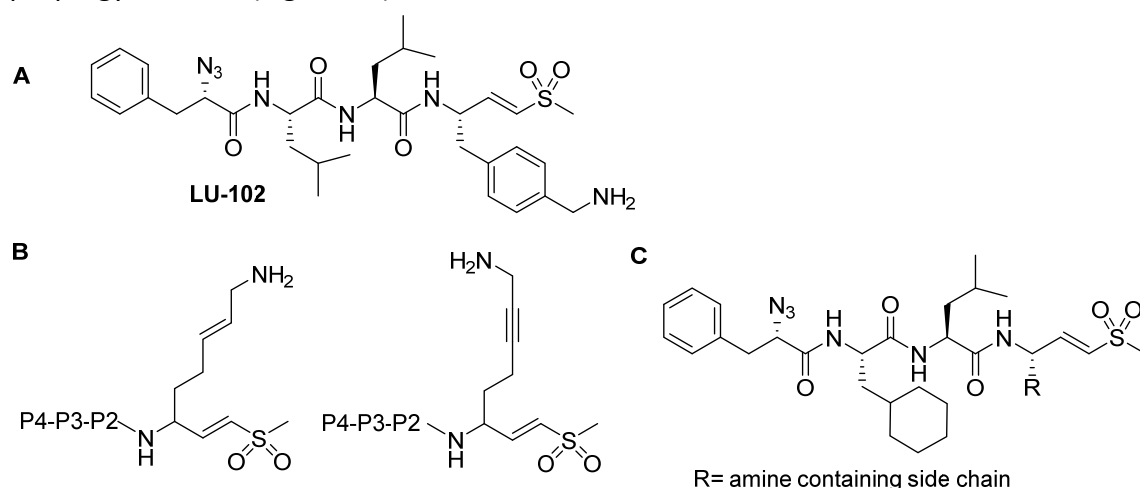


Figure 8. Structures of (proposed) $\beta 2c/\beta 2i$ selective inhibitors. A) Structure of LU-102. B) Structures of homo-lysine analogues with reduced pK_a values compared to lysine. C) Incorporation of cyclohexylalanine at P3 in $\beta 2c/\beta 2i$ selective inhibitors may increase potency and cell permeability.

Since cyclohexylalanine at P3 is well tolerated by $\beta 2c/\beta 2i$ (see chapter 6), its incorporation at the P3 position might improve the potency of $\beta 2$ selective inhibitors. Moreover, the lipophilic nature of the cyclohexyl moiety may increase cell permeability (Figure 8c).

From the panel of proteasome inhibitors with large residues at P3, as described in chapter 7, various compounds appeared to possess some $\beta 2i$ selectivity. This appeared in particular to hold for compounds featuring a *tert*-butyl serine residue at P3. The $\beta 2i$ selective inhibitor LU-002i (see chapter 3) shows good selectivity, however, is only moderately potent. Since LU-002i bears an alanine at P3, and the S3 pocket of $\beta 2c$ and $\beta 2i$ are rather spacious, *tert*-butyl serine at P3 may provide additional interactions, which may enhance the potency for $\beta 2i$ but to a lesser extent for $\beta 2c$ (Figure 9).

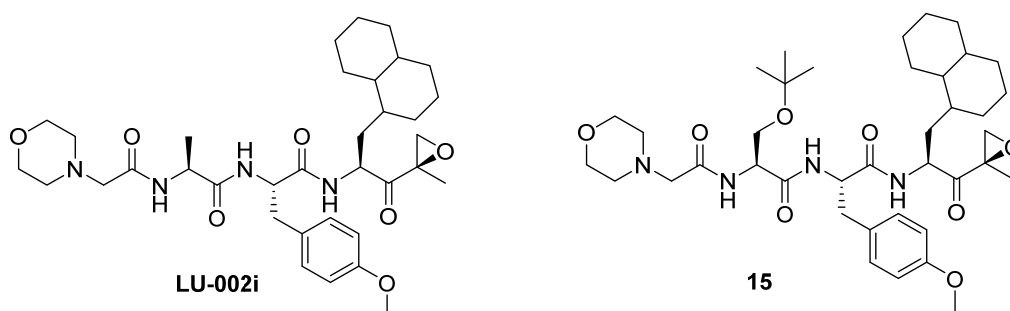


Figure 9. Structure of $\beta 2i$ selective inhibitor LU-002i and proposed inhibitor 15. Incorporation of a P3 *tert*-butyl serine may result in enhanced potency and/or selectivity for $\beta 2i$

During proteasome assembly the formation of pure cCP and iCP is favoured when all six catalytic subunits are expressed. However, also mixed proteasomes (mCPs) have been shown to exist in various tissues. In **chapter 10** a new assay is described, which provides insight in the proteasome composition of a given cell lysate. Since proteasome active sites are all in close proximity to each other, fluorescence resonance energy transfer (FRET) can take place between FRET donor and acceptor ABPs that are bound to separate active sites. When a cell lysate is separated on native PAGE, CPs stay intact and FRET signals emerge after fluorescent imaging of the wet gel slab. The optimal FRET donor-acceptor pair for this purpose was found to be the BODIPY(FL) (donor) and Cy5 (acceptor) fluorophores, which displayed close to 100% high FRET efficiency with minimal background. A set of ten ABPs equipped with either BODIPY(FL) or Cy5 and selective for either $\beta 1c/\beta 1i$, $\beta 2c/\beta 2i$, $\beta 5c/\beta 5i$, $\beta 1c$, $\beta 1i$, $\beta 5c$ or $\beta 5i$ was synthesised (as described in previous chapters). This set of ABPs was complemented by five proteasome inhibitors, each selective for a single catalytic subunit ($\beta 1c$, $\beta 1i$, $\beta 2i$, $\beta 5c$ or $\beta 5i$) and each with sufficient selectivity windows to allow full blockage of their target subunit without inhibition of the other five active subunits. When two of these inhibitors are used together, eight different subunit pairs can be blocked. By selecting the appropriate FRET ABP pair and selective inhibitor pair, FRET signals derived from eight

distinct subunit combinations were obtained. For instance, following inhibition of $\beta 1c$ and $\beta 5i$ using selective inhibitors, $\beta 1c/\beta 1i$ and $\beta 5c/\beta 5i$ targeting ABPs can only label $\beta 1i$ and $\beta 5c$. In case a FRET signal is observed, this indicates the presence of $\beta 1i/\beta 5c$ containing mCPs. CPs contain two β -rings, which do not have to be the same in subunits composition. Proteasome CPs containing different β -rings are termed asymmetric mixed proteasomes (m_a CPs). Using $\beta 1c$, $\beta 1i$, $\beta 5c$ and $\beta 5i$ selective ABPs, proteasomes asymmetric with respect to their $\beta 1$ and $\beta 5$ subunit composition could be visualized. For instance, when a sample is treated with a $\beta 1c$ targeting donor ABP and a $\beta 1i$ targeting acceptor ABP, an observed FRET signal reveals the presence of CPs asymmetric in their $\beta 1$ subunit composition.

The native PAGE FRET assay was used to analyse the proteasome composition of lysates from Raji cells, which constitutively express all six catalytic subunits. Furthermore, lysates of HeLa cells, which express mainly constitutive proteasome subunits were studied in comparison with lysates of HeLa cells in which immunoproteasome subunit expression was induced by a 24 h exposure to IFN- γ . Interestingly, although Raji cells and IFN- γ treated HeLa cells express similar ratios of constitutive proteasome subunit and immunoproteasome subunits, the relative FRET signals derived from mCPs were much higher in Raji cell lysates. This observation indicates that after immunoproteasome induction predominantly iCPs are formed, whereas in cells continuously expressing all six catalytic subunits the levels of mCPs are substantially higher.

The native PAGE FRET assay is not suitable for rapid screening of multiple samples, because running a native gel is rather time consuming. To overcome this limitation, a proteasome-capture-FRET assay can be envisioned, in which the total proteasome content of a cell lysate is captured on an anti- $\alpha 2$ coated 96-well plate (Figure 10). After removal of unbound proteins by washing of the wells, proteasome inhibitors are to be added, followed by the addition of FRET probes. Following removal of the unbound FRET probes by washing steps, the FRET signal is to be measured using a plate reader. Alternatively, anti- β subunit antibodies coated to a 96 well plate can be used to determine the composition of a subpopulation of proteasomes.

Compared to bortezomib, the recently approved (by the FDA) drug ixazomib exhibits a high off-rate for in particular $\beta 5c$ and $\beta 5i$ (see chapter 3). This feature may be at the basis of the reputedly improved pharmacodynamic and pharmacokinetic properties of ixazomib. Ixazomib bears a glycine at P2, thus no amino acid side chains for interaction with the proteasome subunit, which may be the reason behind the comparable higher instability of the ixazomib-proteasome complex. All proteasome subunit have spacious, solvent exposed S2 pockets. This invites the question whether large, sterically demanding moieties at P2 would affect the binding properties of boronic acid proteasome inhibitors.

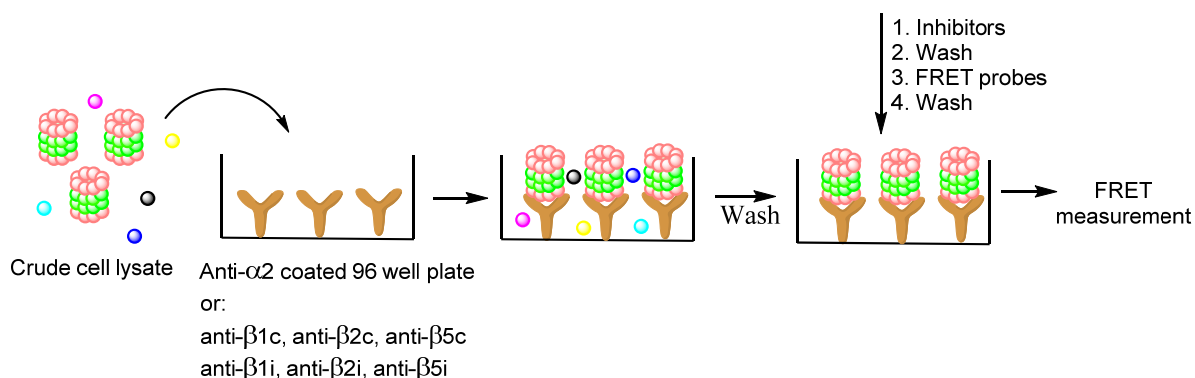


Figure 10. Proposed proteasome-capture-FRET assay

In **Chapter 11** the incorporation of adamantylalanine and carboranylalanine at the P2 position of bortezomib is described. Carboranes are considered to be ‘super-aromatic’, are highly hydrophobic and have been used as phenyl isosters. Besides this, carboranes are currently explored in boron-neutron capture therapy (BNCT), a potential anti-cancer therapy. A new enantioselective synthesis of both amino acids was developed, making use of Ellman’s *N*-tert-butylsulfinamide as the chiral auxillary and as key step an asymmetric Strecker reaction. Both amino acids could be synthesized as Fmoc building blocks in good yields and with high enantiomeric excess. The incorporation of these amino acids at the P2 position of bortezomib provided adamantezomib and carbortezomib. Both inhibitors displayed similar inhibition profiles and potencies compared to bortezomib, indicating that large residues are well tolerated at P2. However, both adamantezomib and carbortezomib showed a slight preference for $\beta 1i$ and $\beta 5i$ compared to $\beta 1c$ and $\beta 5c$. In addition, both inhibitors show higher off-rates for $\beta 5c/\beta 5i$ compared to bortezomib, indicating that large P2 substituents cause destabilization of the boronic acid- $\beta 5$ complex. While the off-rate of carbortezomib was found to be lower than ixazomib, adamantezomib displayed a higher off-rate. Moreover, the off-rate of both compounds appeared to be higher for $\beta 5c$ compared to $\beta 5i$, which increases their $\beta 5i$ selectivity. Therefore, adamantylalanine or carboranylalanine at P2 could be an important design parameter for the development of immunoproteasome selective inhibitors that have similar pharmacodynamic and pharmacokinetic properties as ixazomib.

All proteasome inhibitors described in this thesis target the non-primed site of active β -subunits. However, some inhibitors are bound by both the primed and non-primed sites, such as homobelactosin C¹², UK-101¹³ and α -keto phenylamides¹⁴ (Figure 11). These inhibitors have only a small moiety binding to the primed site (UK-101 and α -keto phenylamides) or to the non-primed site (homobelactosin C). In order to study the substrate preferences of the primed pockets, extended vinyl sulfones are proposed (Scheme 3).

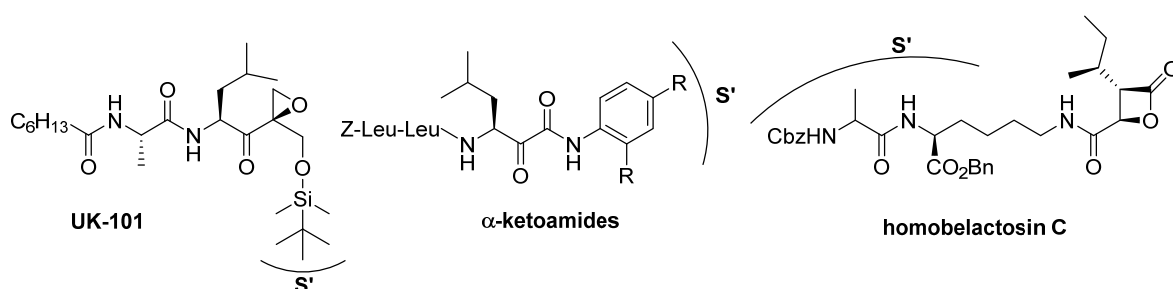
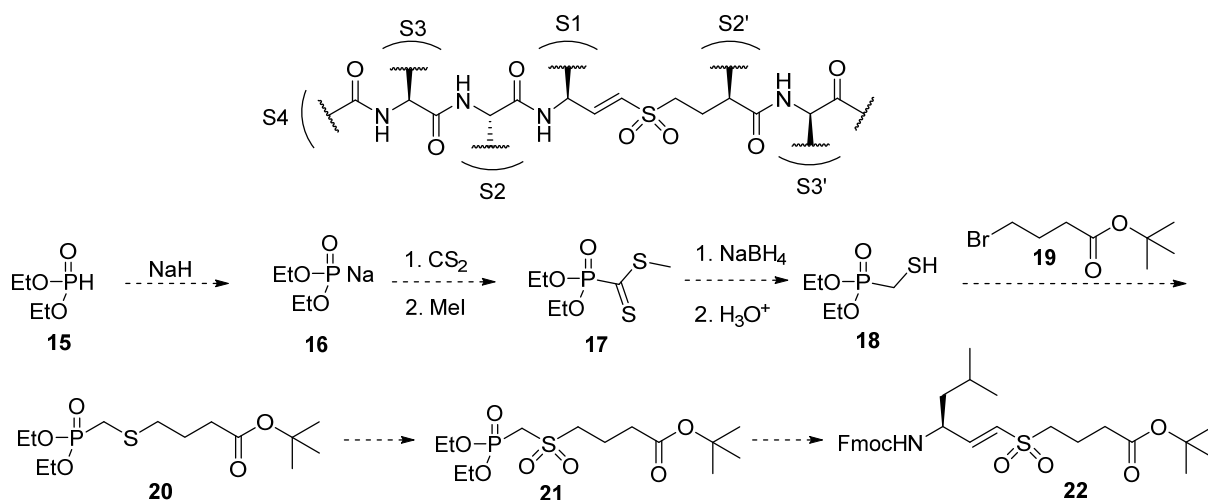


Figure 11. Structures of primed site targeting proteasome inhibitors. S' = primed site.

Epoxyketones are considered to be unsuited for this purpose, since the methyl group does not point into the direction of the primed site.¹⁵ Scheme 3 shows a proposed structure of an extended vinyl sulfone. The amino acids at $S1$, $S2$ and $S3$ can be chosen to tune subunit selectivity. Initially, the $S2'$ residue can be omitted to simplify the synthesis and in a later stage the $S2'$ and $S3'$ can be varied in order to explore the subunit preferences. For initial studies, key building block **22** is required, which bears orthogonally protected amine and carboxylic acids residues for sequential N- and C- terminal functionalization. A putative synthesis of **22** is shown in scheme 3. *In situ* generated sodium diethylphosphonate **16** can be reacted with carbondisulfide followed by the addition of methyl iodide to give compound **17**.¹⁶ Subsequent reduction and acidic hydrolysis¹⁷ will provide free thiol **18**, which can be reacted with bromide **19**. Phosphonate **20** can be oxidized to sulfone **21**, which can be reacted in a Horner-Wadsworth-Emmons reaction with Fmoc-leucinal. Initially, a focussed library with fixed $S1$ - $S4$ (for instance Ac-LLL-) and varying $S2'$ - $S3'$ substituents could be synthesized and evaluated for their inhibitory potency and subunit selectivity on Raji lysates. In case certain amino acids at $S2'$ - $S3'$ induce subunit selectivity, these may be combined with known subunit-selectivity inducing amino acids at $S1$ - $S4$. This may result in subunit selective inhibitors with improved potency and selectivity windows compared to existing ones.



Scheme 3. Structure of proposed extended vinyl sulfones and proposed synthesis of key intermediate **22**.

Experimental

Synthetic procedures

General procedures

Acetonitrile (ACN), dichloromethane (DCM), N,N-dimethylformamide (DMF), methanol (MeOH), diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma, Iris Biotech) were used as received. Column chromatography was performed on Screening Devices b.v. Silica Gel, with a particle size of 40–63 μm and pore diameter of 60 Å. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F254). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ (25 g/L) and $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4\cdot 2\text{H}_2\text{O}$ (10 g/L) in 10% sulphuric acid, a solution of KMnO_4 (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ^1H and ^{13}C NMR spectra were recorded on an AV-400 (400 MHz) or on a AV-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to CD_3OD as internal standard. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile 50/50 (v/v) and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution $R = 60,000$ at m/z 400 (mass range $m/z = 150$ –2,000) and dioctylphthalate ($m/z = 391.28428$) as a “lock mass”. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C_{18} 50 $\times$ 4.60 mm column (detection at 200–600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. The applied buffers were H_2O , ACN and 1.0% aq. TFA. Method: xx $\rightarrow$ xx% MeCN, 13.0 min (0 $\rightarrow$ 0.5 min: 10% MeCN; 0.5 $\rightarrow$ 8.5 min: gradient time; 8.5 $\rightarrow$ 10.5 min: 90% MeCN; 10.5 $\rightarrow$ 13.0 min: 10% MeCN). HPLC purification was performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μm 250 $\times$ 10 mm column and a GX281 fraction collector.

Methyl (S)-3-(((benzyloxy)carbonyl)amino)-5-methyl-4-oxohex-5-enoate (6)

Tert-butyl ester **5** (1.0 mmol, 347 mg) was dissolved in TFA. After 30 minutes the reaction mixture was concentrated and co-evaporated with toluene (3x). The crude product was dissolved in MeOH (10 mL) followed by the addition of DMAP (0.1 mmol, 12 mg) and EDC-HCl (2.0 mmol, 383 mg). After one hour the crude product was concentrated, redissolved in EtOAc, washed with 1M HCl, NaHCO_3 (2x), dried over Na_2SO_4 , filtered and concentrated. The product was purified by column chromatography (5 $\rightarrow$ 20% EtOAc/Pent) and concentrated yielding the title compound (133 mg, 43%). ^1H NMR (400 MHz, Chloroform-*d*) δ 7.33 (d, $J = 3.9$ Hz, 5H), 6.09 (s, 1H), 5.98 – 5.83 (m, 2H), 5.31 (dt, $J = 8.4, 5.7$ Hz, 1H), 5.09 (s, 2H), 3.64 (s, 3H), 2.86 (dd, $J = 15.9, 5.5$ Hz, 1H), 2.70 (dd, $J = 15.9, 5.7$ Hz, 1H), 1.90 (s, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 198.45, 170.94, 155.64, 141.89, 136.18, 128.53, 128.21, 128.09, 126.29, 67.09, 52.02, 51.47, 37.49, 17.95.

Methyl (3S,4R)-3-(((benzyloxy)carbonyl)amino)-4-hydroxy-5-methylhex-5-enoate (7)

Methyl ester **6** (0.43 mmol, 133 mg) was dissolved in MeOH (4.3 mL) and $\text{CeCl}_3\cdot 7\text{H}_2\text{O}$ (0.69 mmol, 257 mg) was added. When the reaction mixture became clear, it was cooled to 0 °C. NaBH_4 (0.56 mmol, 21 mg) was added portion wise and after 15 minutes the reaction mixture was quenched by the addition of AcOH. The mixture was co-evaporated with toluene and redissolved in EtOAc/ H_2O . The two layers were separated and the water layer was extracted with EtOAc (2x). The combined organic layers were washed with brine, dried over Na_2SO_4 , filtered and concentrated. Further purification was performed by column chromatography (10 $\rightarrow$ 40% EtOAc/Pent) yielding the title compound in a 6:4 mixture of diastereoisomers (105 mg, 79%). ^1H NMR (400

MHz, CDCl₃) δ 7.33 – 7.28 (m, 5H), 5.65 (d, J = 8.3 Hz, 0.6 H), 5.45 (d, J = 8.2 Hz, 0.4 H), 5.08 – 4.95 (m, 4H), 4.20 – 4.15 (m, 2H), 3.65 – 3.63 (m, 3H), 2.72 – 2.53 (m, 3H), 1.76 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 172.98, 156.07, 144.64, 144.40, 136.44, 128.56, 128.53, 128.20, 128.15, 128.12, 128.04, 112.64, 112.33, 76.52, 75.75, 66.87, 51.97, 51.93, 50.14, 36.94, 33.57, 18.88.

Methyl (3S,4S)-3-(((benzyloxy)carbonyl)amino)-4-hydroxy-4-((R)-2-methyloxiran-2-yl)butanoate (8).

To a solution of alkene **7** (1.0 mmol, 307 mg) in DCM (10 mL) at 0°C, VO(acac)₂ (0.1 mmol, 27 mg) and tBuOOH (5.5 M in decane, 3.0 mmol, 545 μ L) were added. After two hours the reaction mixture was concentrated and redissolved in EtOAc, washed with NaHCO₃ (2x), H₂O and brine, dried over Na₂SO₄, filtered and concentrated followed by column chromatography (10→40% EtOAc/Pent) yielding the desired compound (70 mg, 22%). ¹H NMR (400 MHz, CDCl₃) δ 7.35 – 7.28 (m, 5H), 5.74 (d, J = 9.3 Hz, 1H), 5.10 (s, 2H), 4.30 – 4.23 (m, 1H), 3.84 (d, J = 3.5 Hz, 1H), 3.64 (s, 3H), 2.91 (d, J = 4.5 Hz, 1H), 2.75 (s, 1H), 2.57 (dd, J = 13.8, 5.3 Hz, 3H), 1.39 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 172.08, 155.79, 136.40, 128.57, 128.22, 128.16, 73.20, 66.93, 57.37, 51.95, 50.06, 49.84, 34.39, 18.38.

Methyl (S)-3-(((benzyloxy)carbonyl)amino)-4-((R)-2-methyloxiran-2-yl)-4-oxobutanoate (9)

Epoxide **8** (0.2 mmol, 70 mg) was dissolved in DCM (2 mL) and cooled to 0°C. Dess-Martin periodane (0.8 mmol, 339 mg) was added as a suspension in DCM. After four hours the reaction mixture was quenched by the addition of NaHCO₃. The two layers were separated and the water layer extracted with DCM. The combined organic layers were washed with NaHCO₃ and brine, dried over Na₂SO₄, filtered and concentrated. The product was further purified by column chromatography (5→20% EtOAc/Pent) obtaining the title compound (44 mg, 70%). ¹H NMR (400 MHz, CDCl₃) δ 7.38 – 7.30 (m, 5H), 5.73 (d, J = 7.6 Hz, 1H), 5.11 – 5.04 (m, 2H), 4.61 – 4.56 (m, 1H), 3.67 (s, 3H), 3.15 (d, J = 4.6 Hz, 1H), 2.95 – 2.83 (m, 3H), 1.55 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 205.99, 170.57, 155.81, 136.13, 128.65, 128.33, 128.17, 67.18, 59.50, 52.55, 52.26, 51.07, 36.59, 16.99. $[\alpha]_D^{20}$ = -133° (c = 0.6, CHCl₃).

(S)-4-methoxy-1-((R)-2-methyloxiran-2-yl)-1,4-dioxobutan-2-aminium 2,2,2-trifluoroacetate (10)

To a solution of epoxyketone **9** (70 μ mol, 23 mg) in MeOH (5 mL) were added TFA (84 μ mol, 6.4 μ L) and Pd/C (10 mg). After two hours of stirring under a H₂ atmosphere the reaction mixture was filtered, concentrated and co-evaporated with toluene yielding the title compound (16 mg, 76%), which was directly used in the next step.

N₃Gly-Ala-Pro-Nle-Asp(OMe)-EK (11)

To a solution of N₃Gly-Ala-Pro-Nle-NHNH₂ (48 μ mol, 19 mg) in DMF (2 mL) at -30°C were added HCl (4M in dioxane, 270 μ mol, 68 μ L) and tBuONO (110 μ mol, 13 μ L). After stirring for three hours, deprotected epoxyketone **10** (53 μ mol, 16 mg) in DMF and DiPEA (480 μ mol, 84 μ L) were added. The mixture was allowed to react overnight while warming up to room temperature. The mixture was diluted in DCM, washed with water (2x) and brine, dried over MgSO₄, filtered and concentrated. Column chromatography (1→4% MeOH/DCM) and HPLC (32→36% MeCN/H₂O, 12 min.) yielded the desired compound as a white solid after lyophilisation (4.7 mg, 18%). ¹H NMR (600 MHz, MeOD) δ 4.78 – 4.75 (m, 1H), 4.61 – 4.58 (m, 1H), 4.51 – 4.41 (m, 1H), 4.21 – 4.12 (m, 1H), 3.89 – 3.83 (m, 1H), 3.79 – 3.73 (m, 1H), 3.69 – 3.56 (m, 1H), 3.32 (s, 3H), 3.21 – 3.15 (m, 2H), 2.92 – 2.88 (m, 2H), 2.81 – 2.65 (m, 2H), 2.30 – 2.15 (m, 1H), 2.08 – 1.94 (m, 3H), 1.79 – 1.72 (m, 1H), 1.63 – 1.56 (m, 1H), 1.47 – 1.44 (m, 3H), 1.41 – 1.37 (m, 1H), 1.37 – 1.10 (m, 8H), 0.94 – 0.83 (m, 3H). ¹³C NMR (151 MHz, MeOD) δ 207.08, 174.26, 174.15, 173.24, 171.91, 169.89, 61.77, 61.47, 60.32, 56.29, 54.68, 53.23, 52.58, 52.47, 50.28, 48.65, 36.25, 32.79, 30.44, 28.92, 28.24, 26.02, 23.44, 17.04, 16.80, 14.26. HRMS: calculated C₂₄H₃₈N₇O₈ 552.27819 [M+H]⁺; found 552.27762.

(pivaloyloxy)Methyl (S)-3-(((benzyloxy)carbonyl)amino)-5-methyl-4-oxohex-5-enoate (12).

Tert-butyl ester **5** (2.4 mmol, 820 mg) was dissolved in TFA. After 30 minutes the reaction mixture was concentrated and co-evaporated with toluene (2x). The product was redissolved in DMF (24 mL) and DiPEA (7.2 mmol, 1.23 mL, 3 equiv.) was slowly added. Iodomethyl pivalate (1.16 g, 4.8 mmol, 2 equiv.) was added and the mixture was allowed to react overnight. The reaction mixture was dissolved in EtOAc, washed with 1M HCl (2x), NaHCO₃ (2x), dried over Na₂SO₄, filtered and concentrated. Column chromatography (5→20% EtOAc/Pent) yielded the title compound (854 mg, 89%). ¹H NMR (400 MHz, CDCl₃) δ 7.46 – 7.20 (m, 5H), 6.09 (s, 1H), 5.92 (d, *J* = 8.3 Hz, 1H), 5.88 (s, 1H), 5.70 (s, 2H), 5.35 – 5.28 (m, 1H), 5.09 (s, 2H), 2.92 (dd, *J* = 16.2, 5.7 Hz, 1H), 2.73 (dd, *J* = 16.2, 5.3 Hz, 1H), 1.89 (s, 3H), 1.20 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 197.92, 177.00, 169.33, 155.56, 141.71, 136.08, 128.49, 128.18, 128.04, 126.39, 79.55, 67.09, 51.19, 38.69, 37.14, 26.79, 17.90.

Biochemical methods

General

Lysates of cells were prepared by treating cell pellets with 4 volumes of lysis buffer containing 50 mM Tris pH 7.5, 2 mM DTT, 5 mM MgCl₂, 10% glycerol, 2 mM ATP, and 0.05% digitonin for 15–60 min. Protein concentration was determined using Qubit® protein assay kit (Thermo Fisher). All cell lysate labelling experiments were performed in assay buffer containing 50 mM Tris pH 7.5, 2 mM DTT, 5 mM MgCl₂, 10% glycerol, 2 mM ATP. Cell lysate labelling and competition experiments were performed at 37°C. Prior to fractionation on 12.5% SDS-PAGE (TRIS/glycine), samples were boiled for 3 min in a reducing gel loading buffer. The 7.5x10 cm (L x W) gels were run for 15 min at 80V followed by 120 min at 130V. In-gel detection of (residual) proteasome activity was performed in the wet gel slabs directly on a ChemiDoc™ MP System using Cy2 setting to detect BODIPY(FL)-LU-112, BODIPY(FL)-epoxomicin and BODIPY(FL)-NC-001, Cy3 settings to detect BODIPY(TMR)-NC-005-VS and BODIPY(TMR)-epoxomicin and Cy5 settings to detect Cy5-NC-001. When the probes were used as a mixture the following concentrations were used: 100 nM Cy5-NC-001, 30 nM BODIPY(FL)-LU-112, 100 nM BODIPY(TMR)-NC-005-VS, as premixed 10x concentrated cocktail in DMSO which was incubated with cell lysate for 60 min, unless stated otherwise.

Competition experiments in cell lysate

Cell lysates (diluted to 10–15 µg total protein in 9 µL buffer) were exposed to the inhibitors (10x stock in DMSO) at indicated concentrations for 1 h at 37 °C, followed by addition of probe cocktail (10x stock, 1.1 µL) and SDS-PAGE as described in general methods.

Competition experiments in living RPMI-8226 cells

RPMI-8226 were cultured in RPMI-1640 media supplemented with 10% fetal calf serum, GlutaMAX™, penicillin, streptomycin in a 5% CO₂ humidified incubator. 5–8 × 10⁵ cells/mL were exposed to inhibitors for 1 h at 37 °C. Cells were harvested and washed twice with PBS. Cell pellets were treated with lysis buffer on ice for 15 min, followed by centrifugation at 14000 rpm for 5 min. Proteasome inhibition in the obtained cell lysates was determined using the method described above. Intensities of bands were measured by fluorescent densitometry and divided by the intensity of bands in mock-treated extracts. Average values of three independent experiments were plotted against inhibitor concentrations. IC₅₀ (inhibitor concentrations giving 50% inhibition) values were calculated using GraphPad Prism software.

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