Discovery and development of inhibitors selective for human constitutive proteasome and immunoproteasome active sites

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Summary and future prospects

9.1 Summary

This thesis describes the design and development of subunit-selective inhibitors of particular catalytically active subunits of human constitutive proteasomes and immunoproteasomes. Most existing proteasome inhibitors are oligopeptides composed of 2-4 amino acid residues, N-terminally capped and with the C-terminus adapted to give an electrophilic trap. Such compounds are also at the basis of the research described in this Thesis. Attention was directed to substitute specific amino acid residues by either synthetic, non-canonical amino acid derivatives (with a review on the synthesis of such items given in Chapter 2) or dipeptide isosteres. Alternatively, modifications of the electrophilic trap, specifically, the epoxyketone moiety, were investigated. In this way, and by the synthesis of focused libraries, in which in each case a number of structural analogues, rather than a single one, inhibitors selective for β5c, β2c and β2i were discovered, and a number of two-step activity-based probes for probing these activities *in vitro* and *in situ* were identified.

Chapter 1 shortly describes what (human) proteasomes are, and introduces the important proteasome inhibitors described to date and that serve as starting point for the research

described in this Thesis. **Chapter 2** provides an overview on literature procedures for the asymmetric synthesis of aliphatic, non-canonical α-amino acids, compounds that are important structural elements in several of the subunit-selective inhibitors identified through research described in the experimental chapters of this Thesis.

Epoxomicin was discovered as a selective proteasome inhibitor in 1992. It is a natural product characterized by the terminal epoxyketone as electrophilic trap. Based on the inhibition mechanism by which epoxyketone is thought to react with N-terminal threonine residues that make up the proteasome active site nucleophiles, a set of structural and functional analogues were made and evaluated as described in **Chapter 3**. These studies revealed that the 1,2-dicarbonyl moiety is a useful (known) dual electrophile, but that compounds with two adjacent epoxides are not and also that switching the epoxide and carbonyl from position is detrimental for proteasome inhibition. **Chapter 4** focuses on another under-investigated issue: the introduction of dipeptide isosteres as (physiologically potentially more stable) replacements of internal dipeptides in known proteasome inhibitors. Introduction of the 5-methylpyridin-2-one (a dipeptide isoster that can act as a hydrogen bond acceptor) let to a loss in proteasome inhibitory activity. **Chapter 5** reports on the development of new, potent and selective β5c inhibitors. The research described elaborates on known design principles that a large hydrophobic amino acid as P3 residue and a small hydrophobic one as P1 residue in oligopeptide epoxyketones yield inhibitors selective for β5c. Structure–activity relationships detail how β1c, β1i, β2c, β2i and β5i activities became resistant to inhibition as compounds were diversified stepwise. The most effective compounds were obtained as a mixture of cis- and trans-biscyclohexyl isomers, and an enantioselective synthesis campaign resolved this issue. Studies on yeast proteasome structures complexed with some of the compounds provide a rationale for the potency and specificity. Substitution of the N-terminus in the most potent compound for a more soluble equivalent led to a cell-permeable molecule that selectively and efficiently blocks β5c in cells expressing both constitutive proteasomes and immunoproteasomes. **Chapter 6** describes the development and evaluation of a set of potent and selective inhibitors of human proteasome β2c catalytic activities. Compounds were identified having small amino acids at P2 as distinguishing feature and that are selective for β2c over β2i (with all other cCP/iCP catalytic subunits remaining largely unmodified). Transformation of one of the most selective inhibitors into an activity-based probe gave loss of β2c selectivity and instead led to a compound that modifies preferentially β5c and β5i. **Chapter 7** describes how screening of a focused compound library led to the discovery of a β2i subunit-selective inhibitor. An activity-based probe is designed based on this inhibitor and that proves to be potent and selective for labeling β2i. Because the decalin system that characterizes this compound, termed LU-002i, was introduced as a mixture of diastereoisomers, efforts were taken to establish which of these stereoisomers carries most, or all, proteasome inhibition activity. These studies resulted in the identification of an enantiopure all-*cis* decalin containing compound that in all likelihood is the active ingredient of

the compound mixture that makes up LU-002i. **Chapter 8** deals with the synthesis of a set of norbornene modified proteasome inhibitors was synthesized for six proteasome subunits (β1c/i, β2c/i, β5c/i). Compounds for β1i, β2i and β5c/i proved to be useful two-step ABPs that effectively label their designed proteasome subunits in both Raji cell extracts and living Raji cells through IEDDA ligation. The compound designed for β1c subunit proved incapable of penetrating the cell membrane, but effectively labels β1c *in vitro*. The compound designed for β2c proved to be not selective, but its azide-containing LU-002c was used to supplement the labeling of β2c via azide-alkyne click ligation chemistry *in vitro* and *in situ*.

9.2 Future prospects

Screening for β5t selective inhibitors

Several years ago, a seventh catalytically active mammalian proteasome β-subunit was identified which is expressed exclusively in cortical epithelial cells of the thymus.[1] Accordingly, this activity was termed β5t and together with β1i and β2i, β5t forms the catalytically active species of thymoproteasomes. The exact function of β5t has yet to be established, however, mice lacking the β5t gene show problems with positive T-cell selection, indicating that β5t plays an important role in this process.[2] Structural studies comparing β5c and β5i with β5t revealed that in the β5t active site, nonpolar amino acids, such as alanine, valine and methionine (as present in β5c/β5i) are substituted for polar amino acids (serine and threonine). Likely, these substitutions are causative of the altered (compared to β5c/β5i) substrate preference: β5t has a 60% to 70% lower chymotrypsin activity, compared to β5c and β5i. A potential β5t-selective inhibitor arguably would be more polar than the commonly used chymotryptic-specific inhibitors. It should be noted that no effective β5t-selective inhibitor has been reported to date.

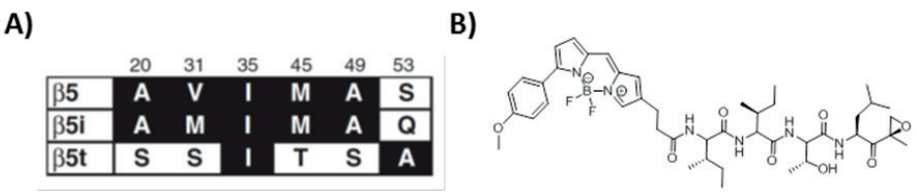

Figure 1. A) Alignment of amino acids forming the S1 pockets of β5, β5i and β5t. B) Structure of MVB003.

In the past decade, and discussed in part in this Thesis, a large and diverse library of proteasome inhibitors has become available. As well, activity-based protein profiling assays have been developed by means of which many proteasome activities, including murine β5t, can be visualized on SDS PAGE.[3] With the aim to establish whether the available proteasome inhibitor library contains compounds that could serve as starting point for the development of inhibitors selective for β5t, they were screened in a competitive activity-based protein profiling assay. Some of these proteasome inhibitors have been reported in the literatures [4-7]

and the rest is described in **Chapter 6** and **Chapter 7**. In this way, compounds were evaluated on their proteasome inhibition profile in whole tissue thymus homogenate from 3-week-old mice. In the initial screening, compounds were tested at 0.1 μM, 1.0 μM and 10 μM final concentrations and more than 200 in-house compounds were screened. Interesting compounds were selected and tested at a wider range of final concentrations (0.5 μM, 1.0 μM, 3.0 μM, 5.0 μM, 7.0 μM and 10 μM) to obtain the insight into inhibition of the other proteasome activities.

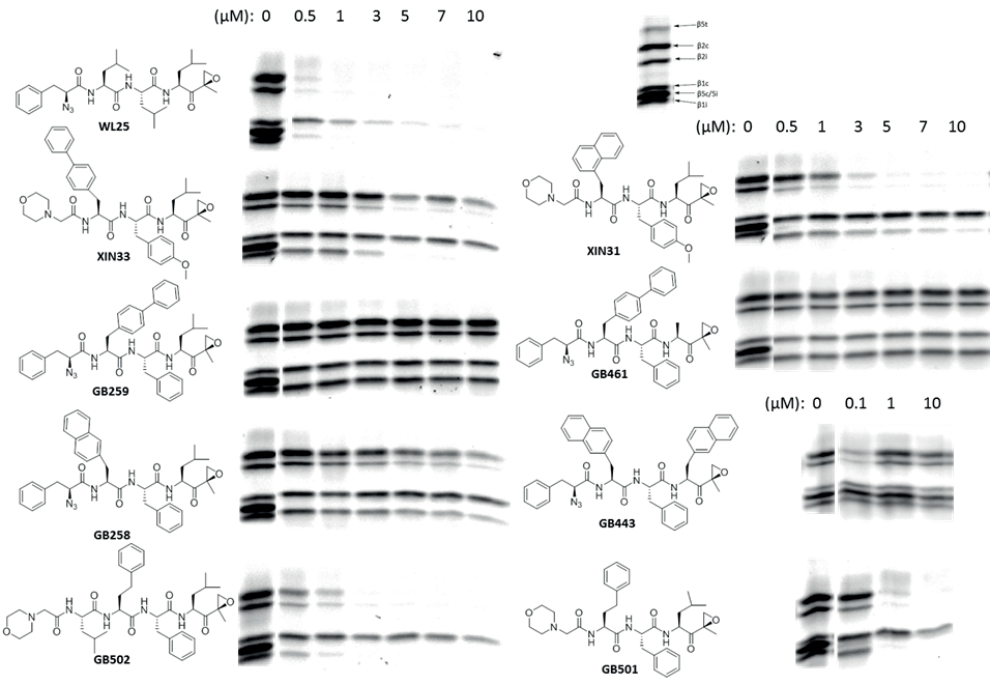

Figure 2. The structures and inhibition profiles of selected compounds.

From these studies, several compounds were identified that inhibit β5t rather potently. The potency and selectivity profiles of these compounds, which are termed WL25, XIN31, XIN33, GB258 and GB502, were established in a competitive activity-based protein profiling assay and the results are depicted in Figure 2. All compounds identified in this manner feature a leucine epoxyketone at P1. The compounds vary in the nature of their amino acid composition at P2-P3/4. As can be seen from Figure 2, XIN33 appears to be an effective β5t inhibitor, and almost complete inhibition can be observed and at a final concentration of 0.5 μM. XIN31, differing from XIN33 at P3 (napththyl versus biphenyl) also proved to be a potent β5t inhibitor, however neither of the compounds is selective and the same holds true for the other compounds identified from these studies.

It should be realized that the assay conditions used are not optimal in reporting on putative β5t inhibitors: whole thymus extracts are used instead of only extracts from cortical epithelial

cells (which are hard to harvest). As a consequence, β5t is underrepresented compared to the other constitutive proteasome and immunoproteasomes activities. As well, MVB003 may not have high affinity for β5t and these two issues combined make that the data as presented in Figure 2 are hard to interpret. Yet, based on the results depicted in Figure 2 and taking these shortcomings into account, peptide epoxyketones can be designed on paper that may turn out to be effective and selective β5t inhibitors. Most of the inhibitors tested feature leucine at P1, but substitution of Lue for Ala leads to improved β5t inhibition (Figure2, compare GB259 and GB461, it should be noted that residues at P2 and P3 are however also modified). A bulky side chain at this position is furthermore not desired (Figure 2, compare GB258 and GB443. It may well be that small, polar, non-charged residues (Ser or Thr) at P1 turn out to be optimal, as the thymoproteasome structure suggests that β5t would accommodate such groups rather well in the active site. Looking at position P2, 2 different moieties were observed to have favorable effects on β5t inhibition: Tyr(Me) and Phe (Figure 2, WL25 however being an exception). Either of these residues is recommended, but a Tyr could also be tried, because the size of Tyr residue is very similar to both of these side chains, and is more polar. Another tentative conclusion made based on the preliminary screen is that a bulky substituent at P3 (as in XIN33, GB259, GB461, XIN31, 2-Nal inGB258) is preferable, and one can imagine that bulky, polar, non-charged residues at this position would lead to more potent β5t inhibitors. Finally, residues at P4 are relatively unexplored and a series of residues – either amino acids or capping groups, or both, may be studied (the nature of the capping group potentially also contributing to solubility and bioavailability of the compounds). Based on the above-mentioned details, a set of potential selective β5t inhibitors is proposed (Figure 3).

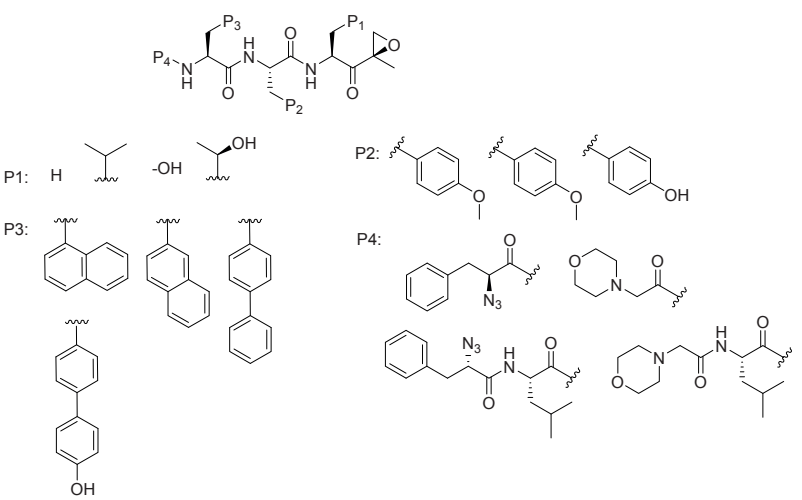

Figure 3. Proposed β5t selective inhibitors..

Putative new selective inhibitors and new warheads for proteasomes

In **Chapter 6**, the development of the β2c-specific inhibitor, LU-002c was described. Although it is the most selective β2c inhibitor described so far, the selectivity window is not very large.[4] As was observed in the discovery of β5c (chapter 5) and β2i (chapter 6) selective inhibitors, substituting the aromatic moiety of specific amino acids for its aliphatic counterpart resulted in much improvement in terms of subunit selectivity.[5] Based on the idea that a similar trend holds true for β2c selective inhibitor design, compound **1** is proposed, featuring a Cha(4-CH_2NH_2) residue at P1 (Figure 4). Alternatively, bicyclic systems **2** and **3** are proposed as rigid, cyclic analogues of the Cha(4-CH_2NH_2) moiety. The basic aliphatic moiety on P1 and the small moiety on P2 could lead to potential potent and selective inhibitors for β2c subunit as well.

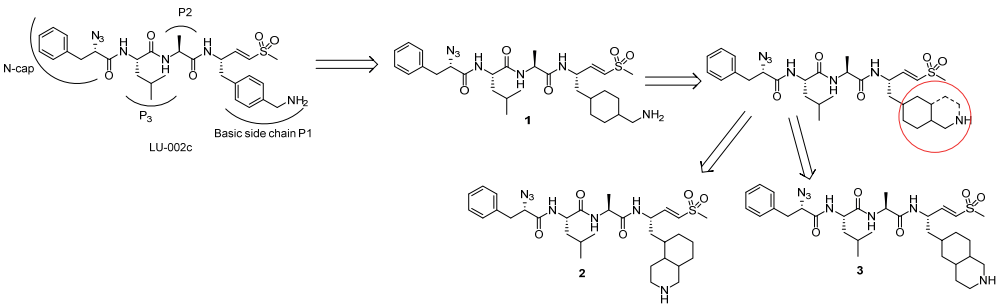

Figure 4. Proposed β2i selective inhibitors.

Although LU-002i is a selective β2i inhibitor,[4] it is not very potent (IC_{50}: 0.22 μM) and also co-inhibits β2c (IC_{50}: 2.5 μM). Based on the knowledge that β2c and β2i subunits favor basic residues on P1,[8] compounds **4** and **5** are designed, featuring amines while keeping the bicyclic system of the parent compound in place (Figure 5). According to previous work,[7] basic residues at P3 are also favored, both by β2c and β2i. Preliminary experiments revealed that compound **6** has no improved potency and selectivity towards β2i. This may be due to the nature of the large and aromatic side chain on P3. Because LU-002i features a small side chain (Ala) on P3, it may be wise to incorporate small and basic side chain on P3 position (**7** and **8**). Compound **9** is a potent dual β2i/ β5i inhibitor. Arguably, selectivity for β2i may be increased by disfavoring β5i inhibition, which may be accomplished by introducing a basic amine in the aliphatic ring at P1, as in compound **10**.

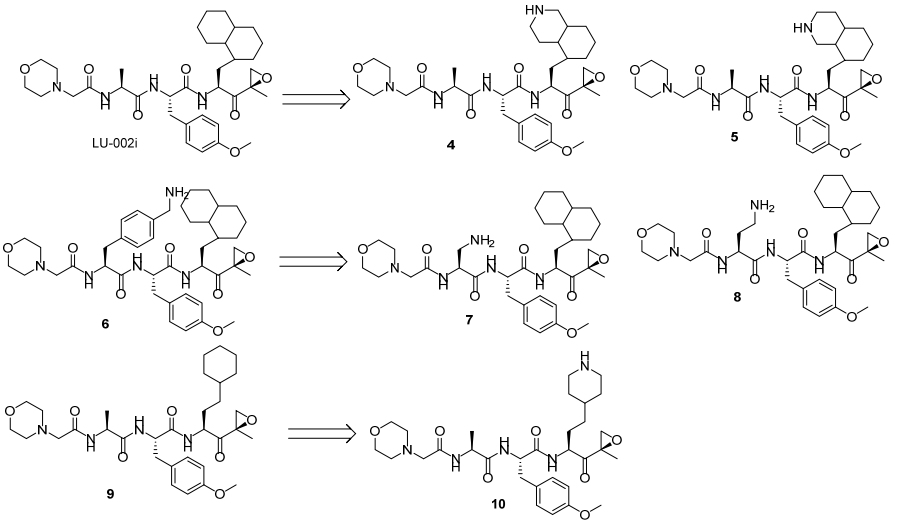

Figure 5. Proposed β2i inhibitors with improved potency compared to the existing compounds.

The epoxyketone warhead was originally thought to form a covalent morpholine ring adduct with the active site threonine.[9] However, recent structural studies of proteasomes complexed to epoxyketones strongly suggest that a seven-membered oxazepane ring is formed instead (Figure 6A).[10] With this information in mind, newly designed electrophiles can be imagined. For instance, compound **12** is proposed, which is an analog of **11** (epoxomicin) and may react within proteasome active sites to form an oxazepane adduct. Alternative structures are compounds **13** and **14**, compounds that also feature two electrophiles that may react with the proteasome active site threonine amino alcohols to form oxazepanes (Figure 6B).

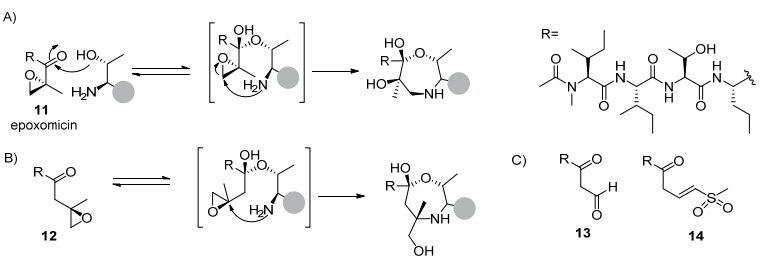

Figure 6. A) Mechanism of epoxyketone electrophile to form seven-member ring adduct. B) Structure of epoxyketone analog **12** and proposed mechanism of inhibition. C) Structures of compound **13** and **14**.

9.3 Experimental section

Material

Thymus organs were isolated from mice aged 1 to 3 months, homogenized in 3 volumes of ice-cold lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.025% digitonin, 0.2% NP40) with a tissue homogenizer and further disrupted by 2 x 3 s sonication. Lysates were cleared by cold centrifugation at 13,000 g. Protein concentration was determined by Bradford assay and the lysates were kept at -80 ^{o}C until use.

Competitive activity-based protein profiling assay

Equal amount of proteins were first exposed to inhibitors for 1h at 37 °C prior to incubation with probe MVB003 (0.5 µM) for another 1h, followed by 3 min of boiling with a reducing gel-loading buffer and fractionation on 12.5% SDS-PAGE. In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on a ChemiDoc MP system using Cy3 settings to detect probe MVB003.

9.4 References

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