# Chemical tools to monitor and control human proteasome activities

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# CHAPTER 1

# General introduction

## The proteasome

Proteasomes are multi-catalytic, multi-protein complexes that are found in all eukaryotic cells and are responsible for the majority of intracellular protein degradation. Proteins destined for degradation are tagged with a polyubiquitin chain, which is recognized by the 19S caps of 26S proteasomes (Figure 1A).[1] Following ubiquitin-chain removal and unfolding, the protein is translocated to the 20S core particle (CP), in which the proteolytic activity resides. 20S CPs consist of four heptameric rings, two outer α-rings and two inner β-rings, of which β1, β2, and β5 are catalytically active (Figure 1A).[2] The peptide backbone of the protein substrate is aligned in the β-subunit binding channel and the amino acid side chains (indicated by P) interact with the substrate binding pockets (indicated by S), which define the substrate specificity of the β-subunits (Figure 1A). The active β-subunits have an active site N-terminal threonine residue, which coordinates to a water molecule (Figure 1B).[3] The threonine hydroxyl group attacks the scissile peptide bond, resulting in cleavage of the peptide bond under formation of a proteasome substrate ester bond which is cleaved through attack of an activated water molecule.

In vertebrates, all cells express the constitutive proteasomes (cCP) of which the active subunits are termed β1c, β2c, and β5c. The substrate-binding channel C-terminal of the scissile peptide bond of a substrate is termed the non-primed site and the N-terminal part is termed the primed site. The substrate binding pockets of the active subunits differ in size, hydrophobicity and charge, which result in varying substrate specificities. The non-primed substrate binding pockets are considered to be determinative for the substrate preferences of the active β-subunits. β1c preferentially cleaves after C-terminal acidic amino acids and is therefore termed caspase-like, β2c prefers basic amino acids at P1 and is therefore termed trypsin-like and β5c is chymotrypsin-like, since it cleaves preferentially after hydrophobic amino acids. Part of the peptides generated by proteasomes are loaded onto major histocompatibility complex class I (MCH-I) and presented on the cell surface to cytotoxic T-cells. Lymphoid tissues

and cells that are exposed to inflammatory cytokines express an additional proteasome type, namely the 20S immunoproteasome (iCP) in which β1c, β2c, and β5c are replaced by β1i, β2i, and β5i.[4] These immunoproteasome subunits exhibit slightly changed substrate specificities and produces peptides with higher affinity for MHC-I. Cortical thymic epithelial cells express a third proteasome type, namely the thymoproteasome (tCP) which play an important role in positive T-cell selection.[5] tCPs contain the same catalytic active subunits as iCPs, with the exception that β5i is replaced by β5t.

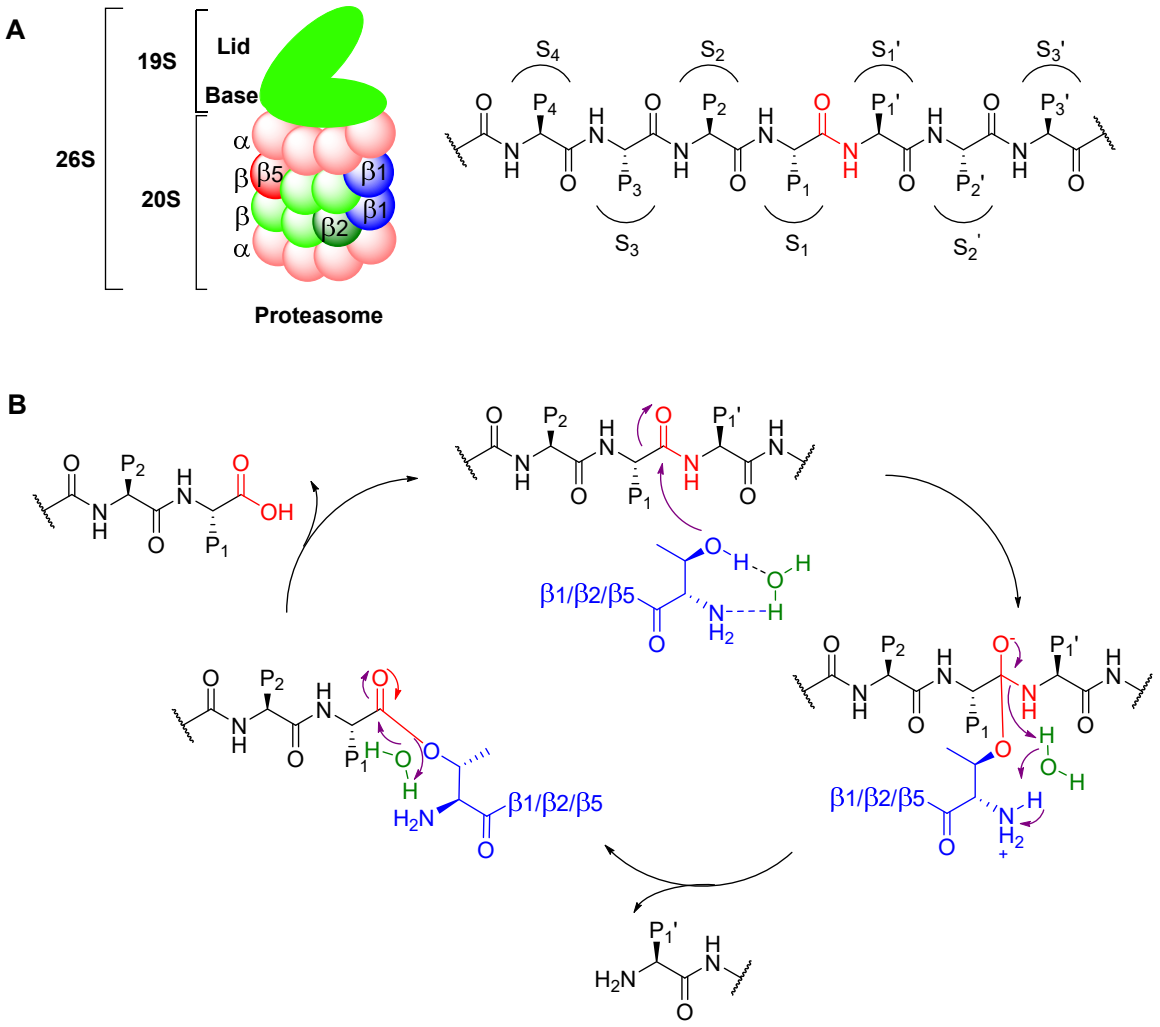


**Figure 1. The proteasome.** A) Schematic representation of the 26S proteasome and the substrate binding channel. B) Catalytic mechanism of peptide bond hydrolysis by the proteasome. 'P' indicates position with respect to the scissile peptide bond, 'S' indicates position of substrate binding pocket. Prime indicates primed sites. Red: scissile peptide bond, blue: active site threonine.

## Proteasome inhibitors

Proteasomes are important drug targets in the field of oncology and immunology. Proteasome inhibitors are currently used or in development for the treatment of various cancers (such as multiple myeloma and mantle cell lymphoma) and several auto-immune diseases.[6-8] The last decades, several proteasome inhibitors (PIs) have been discovered from nature and numerous have been synthesized for drug discovery purposes. PIs are often N-terminally capped di-, tri- or tetrapeptides with a C-terminal electrophilic trap, also termed warhead.[9] The peptide part of the inhibitor interacts with the non-primed part of the substrate binding channel, thereby positioning the warhead close to the catalytic active threonine residue. When the inhibitor is sufficiently stabilized by the substrate binding channel, the warhead may react to the active site threonine residue, in a reversible or irreversible fashion depending on the nature of the warhead. The mechanisms of four widely used warheads are depicted in Figure 2, namely aldehydes, boronic acids (both reversible), epoxyketones and vinyl sulfones (both irreversible).

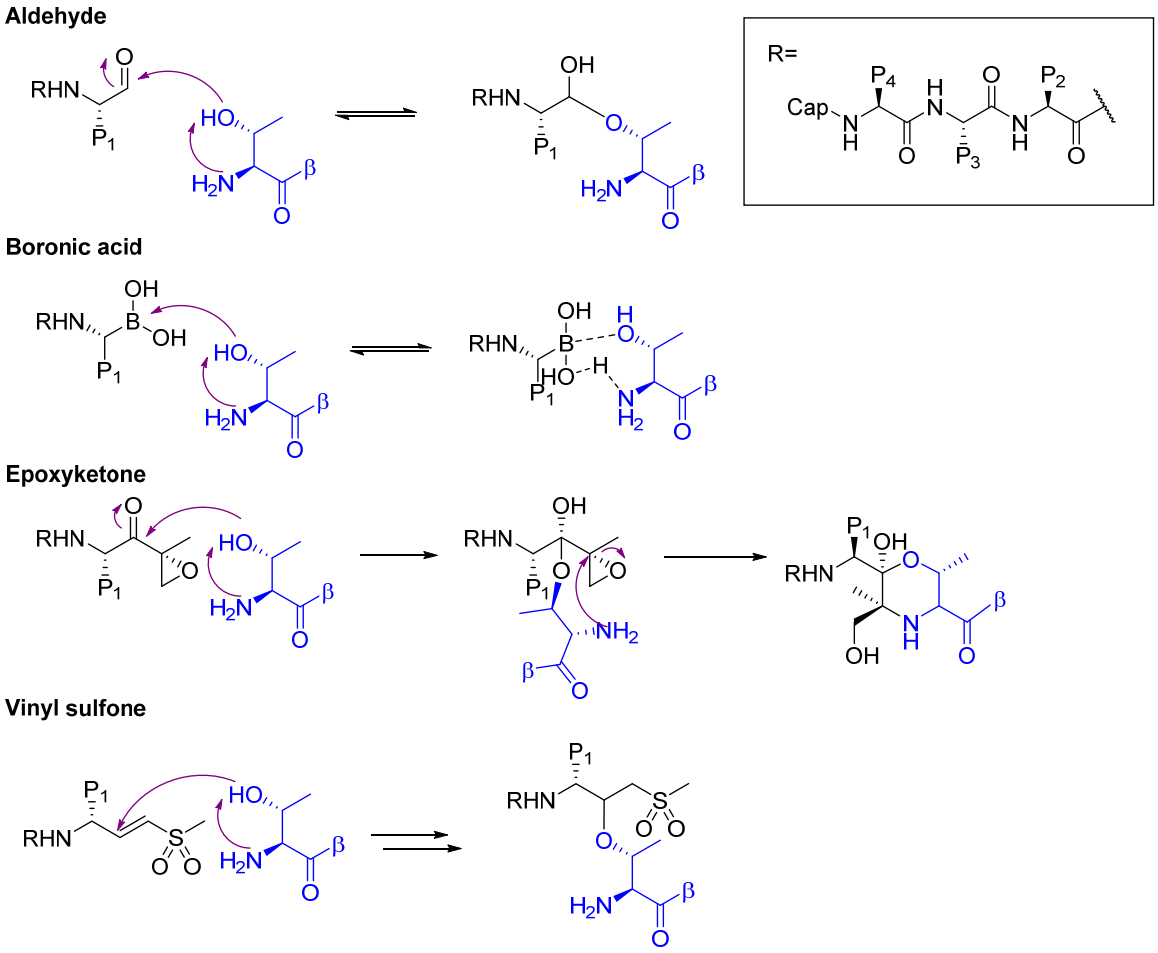


**Figure 2. Mechanisms of aldehyde, boronic acid, epoxyketone and vinyl sulfone electrophilic traps.**

Aldehydes form a hemiacetal with the N-terminal threonine residue of active proteasome subunits. However, the main disadvantage of aldehydes is their cross-reactivity towards cysteine proteases.[10] Boronic acids are found in various clinical drugs and candidates, and form a tetrahedral adduct with the active site threonine.[11] The clinical drug carfilzomib is equipped with an epoxyketone, which react irreversibly with the active site threonine residue by forming a morpholine adduct.[12] Epoxyketones are highly specific for proteasomes and no off-targets have been found to date. Vinyl sulfones have initially been used as cysteine protease inhibitors, but were found to also inhibit the proteasome. The hydroxyl moiety of the active site threonine reacts irreversibly with the vinyl sulfone via a conjugate addition.[13]

In the last decades, many proteasome inhibitors have been synthesized with the aim to obtain PIs that can be used as drugs or research tools. In particular, much research has been directed to the discovery of subunit selective inhibitors. Proteasome inhibitors can also be equipped with a reporter group, such as biotin or a fluorescent moiety. The resulting activity-based probes (ABPs) can report on the proteasome activity. Proteasome targeting ABPs can be used in competitive activity-based protein profiling (ABPP) to screen for new proteasome inhibitors, to quantify the relative amount of proteasomes in a given samples and to provide insight in proteasome composition.

## Aim and outline of this thesis

This thesis describes the development of multiple subunit-selective proteasome inhibitors and ABPs that can be used to determine the role of the individual proteasome subunits in biological processes such as antigen presentation and cancer. Moreover, an ABPP assay is described that enables rapid assessment of the activity of all catalytic active subunits of the cCP and iCP. Furthermore, an ABPP-fluorescence resonance energy transfer (FRET)-based assay is described that provides insight in proteasome composition.

**Chapter 2** provides a comprehensive overview of the ubiquitin-proteasome system and tools to monitor and quantify proteasome activity. Two main strategies that are employed to assay proteasome activity are substrate hydrolysis and ABPP. Both approaches are explained in detail and methods based on these strategies are discussed.

**Chapter 3** describes the development of an ABP cocktail that enables full resolution of all catalytic active subunits of human cCPs and iCPs on SDS-PAGE. This ABP cocktail can be used in competitive ABPP to screen for new proteasome inhibitors and to quantify the relative amounts of individual proteasome subunits in for instance primary patient cells. This chapter also describes a complete set of subunit selective inhibitors. The development of β5i, β1i, β5c and β1i selective inhibitors are described in the following chapters.

**Chapter 4** provides a systematic analysis of the substrate preference of yeast proteasomes (yCP) and human cCPs and iCPs. For this study, only proteinogenic amino acids were used in order to determine the natural substrate preferences of proteasome subunits. A series of tri- and tetrapeptide epoxyketones bearing different amino acids at P1, P2 and P3 were synthesized. Crystal structures of these inhibitors bound to yCPs were determined, revealing important insights which were further exploited to develop subunit selective inhibitors, as is described in the following chapters.

**Chapter 5** describes the design and synthesis of β5i and β1i selective inhibitors. Based on murine proteasome crystal structures it was reasoned increasing the steric bulk at P1 would result improved β5i selectivity. Various non-proteinogenic bulky amino acids were incorporated, of which only cyclohexylalanine provided increased β5i selectivity. Using a similar strategy, a highly selective β1i inhibitor was discovered. The β5i and β1i selective inhibitors could be used to completely inhibit β5i or β1i in cell lysates and living cells, without co-inhibition of the other subunits. Both β5i and β1i selective inhibitors were equipped with a fluorescent group and the resulting ABPs were highly selective for the corresponding subunits, as is described in **chapter 6**.

In contrast to β5i, β5c prefers large residues at P3 and small residues at P1, as was hypothesized form murine proteasome crystal structures. **Chapter 7** describes the synthesis of a small library of inhibitors with large, non-proteinogenic amino acids at P3 and leucine or alanine at P1. It was found that the combination of bicyclohexylalanine at P3 and alanine at P1 provided the highest β5c selectivity. Eventually, a β5 selective inhibitor was developed that can be used to completely inhibit β5c in cell lysates and living cells, without co-inhibition of the other subunits. In addition, an ABP that is selective for β5c over β5i was synthesized.

Based on the finding that aspartic acid at P1 provides β1c selectivity, as described in chapter 4, in **chapter 8** the development of a potent and selective β1c inhibitor and ABP is described. This inhibitor can be used to inhibit β1c in cell lysate, however, not in intact cells since the compound proved to be cell impermeable.

Basic residues at P1 and P3 induce β2c/β2i selectivity. LU-102, the most selective and potent β2c/β2i inhibitor known to date, showed impaired activity in living cells compared to cell lysates. This reduction in activity is likely caused by loss of cell permeability as result of its net positive charge at physiological pH. **Chapter 9** describes the incorporation of basic amino acids with reduced basicity in peptide vinyl sulfone proteasome inhibitors in order to identify a β2c/β2i selective inhibitor with improved cell permeability. The enantioselective synthesis of several lysine analogues and their incorporation into proteasome inhibitors is described. All synthesized inhibitors showed severe loss of activity compared to LU-102.

**Chapter 10** describes a gel-based FRET assay to provide insight in proteasome composition. In addition to cCPs and iCPs, mixed proteasomes (mCPs) exist, in which constitutive- and immunoproteasome subunits are incorporated. With the aid of the subunit selective inhibitors and ABPs described in the previous chapter specific FRET signals between different subunit pairs could be measured, which indicate the presence of mCPs. This method provides rapid insight in the types of mCPs that are present in any crude cell lysate.

**Chapter 11** describes the enantioselective synthesis of adamantylalanine and carboranylalanine and their incorporation at the P2 position of bortezomib. The resulting compounds proved to be potent proteasome inhibitors and displayed high off-rates and slight β5i selectivity.

**Chapter 12** provides a summary of this thesis and points at directions for further research.

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