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Chemical tools to monitor and control human proteasome activities

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CHAPTER 2

Tools and strategies to monitor and quantify proteasome activities

Introduction

The ubiquitin-proteasome system (UPS) degrades 80 to 90% of all proteins inside eukaryotic cells.¹ Proteins destined for degradation are tagged with a polyubiquitin chain.² In the first step of events leading to protein ubiquitination, ubiquitin (Ub), a small (8 KDa) protein, reacts with ATP to form the C-terminal mixed anhydride, ubiquitin-AMP (Figure 1). This process is catalysed by E1 Ub-activating enzymes that subsequently react with ubiquitin-AMP to form a ubiquitin C-terminal thioester involving an E1 active site cysteine thiol. Next and through trans-esterification, Ub is transferred to E2 Ub-conjugating enzymes. E3 ubiquitin ligases bind simultaneously to a specific combination of a substrate and an E2-Ub and catalyse the formation of an isopeptide bond between the ϵ -amine of a substrate lysine and the C-terminus of Ub (Figure 1). The human genome encodes for two E1-activating enzymes, dozens of E2-conjugating enzymes and hundreds of E3 ligases, which together ensure tight regulation of ubiquitin signalling. Poly-Ub chains can be formed by sequential ligations of Ub to either Lys6, 11, 27, 29, 33, 48 or 63 of an Ub residue attached to a substrate. Of these assemblies, Lys48 poly-Ub chains serve as a signal for degradation by proteasomes, which are multi-catalytic, multi-subunit protease complexes responsible for protein degradation.³ Alternative poly-Ub chains serve to guide a number of physiological processes unrelated to protein degradation. Proteasomes are composed of a 20S core particle (CP) that is capped by one or two 19S regulatory particles (RPs). RPs recognise poly-Ub chains, remove these and promote unfolding and translocation of the substrate into the hollow-cylindrical shaped CP, where protein degradation takes place (Figure 1). Proteins are processed into 3-12 amino acid peptides, which can be further degraded by aminopeptidases into single amino acids.⁴ About 1% of the proteasome-generated peptide pool is further trimmed to 8-9 amino acid peptides by downstream aminopeptidases, followed by transport to the endoplasmic reticulum (ER), where the peptides are loaded on major histocompatibility complex class I (MHC-I) molecules

and presented on the cell surface for immune surveillance. As such, proteasomes are key players in adaptive immunity and proteasome products presented by MHC-I report on, for instance, the presence of infecting viruses.⁵ CPs are C2-symmetrical protein complexes composed of 28 individual subunits assembled in four heptameric rings. The two outer α -rings interact with the regulatory particles and two inner β -rings harbour the proteolytic activity, which in eukaryotic proteasomes reside in three catalytically active β -subunits. Each active β -subunit possesses an N-terminal, catalytically active threonine residue, the hydroxyl moiety of which acts as the nucleophile in hydrolysis of the scissile peptide bond.⁶ In constitutive proteasomes, which are present in all tissues, the catalytic subunits are termed β 1c (caspase-like, cleaving preferentially after acidic residues), β 2c (trypsin-like, cleaving preferentially after basic residues) and β 5c (chymotrypsin-like, cleaving preferentially after hydrophobic residues). Next to cCPs, lymphoid tissues and cells that are exposed to the inflammatory cytokines interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) express immunoproteasomes (iCPs), in which β 1c, β 2c and β 5c are replaced by β 1i (branched amino acid preferring, BrAAP), β 2i (trypsin-like) and β 5i (chymotrypsin-like), respectively.⁷

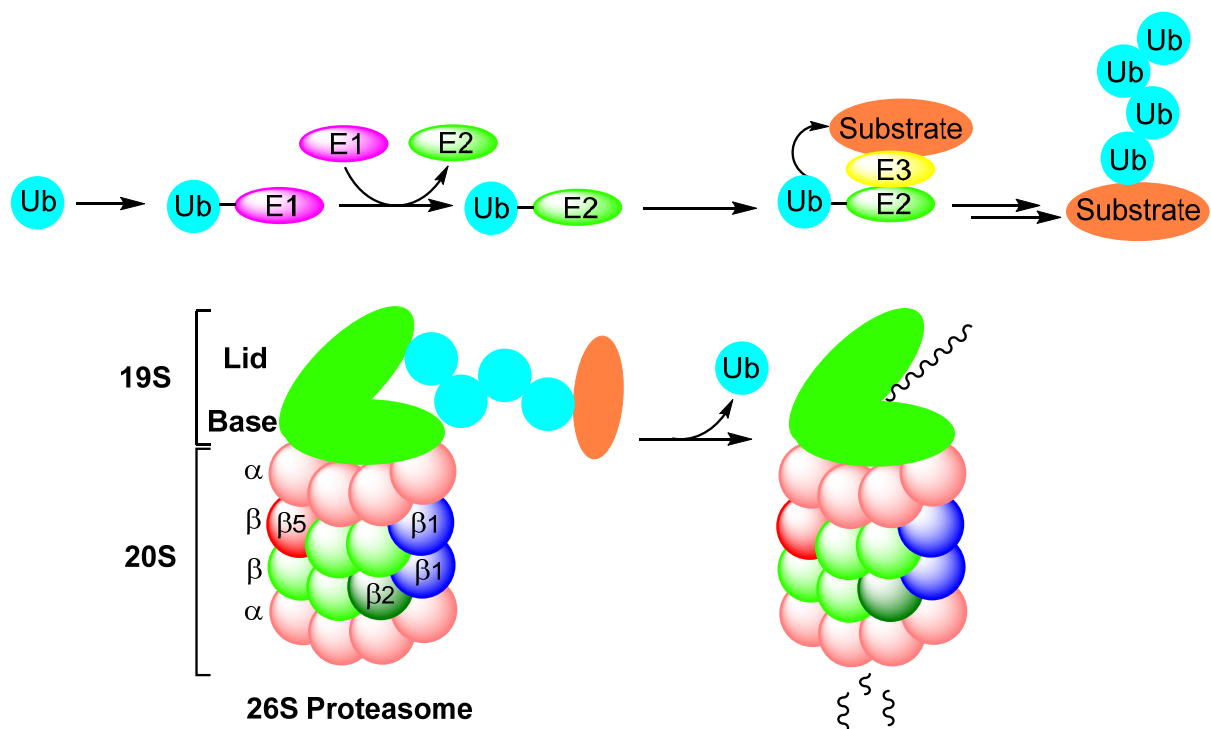


Figure 1. Schematic representation of ubiquitination of a substrate and subsequent recognition, deubiquitination and degradation by the 26S proteasome.

Whereas the substrate specificities of $\beta 2i$ and $\beta 5i$ largely resemble those of their constitutive proteasome counterparts, $\beta 1i$ prefers hydrophobic residues, as opposed to the acidic residues favoured by $\beta 1c$. As a result, compared to cCPs, iCPs predominantly produce peptides with basic and hydrophobic C-termini. Since MHC-I preferentially binds peptides with a hydrophobic residue at P1, iCPs may be superior to cCPs in supporting MHC-I-mediated antigen presentation.^{5, 8, 9}

In cortical thymic epithelial cells a third proteasome type is expressed, namely the thymoproteasome (tCP). tCPs are identical to iCP particles, with the exception that $\beta 5i$ is replaced by $\beta 5t$.¹⁰ Compared to $\beta 5i$ and $\beta 5c$, the substrate pocket of $\beta 5t$ is more hydrophilic and thus the chymotryptic activity of the proteasome is reduced.^{11, 12} tCPs generate peptide pools that have low affinity for MHC-I, and in this way are thought to promote positive selection of T-cells, one of the physiological functions of the thymus.¹³

Proteasomes are important drug targets for the treatment of cancer and autoimmune diseases.¹⁴ The peptide boronic acids, bortezomib (Velcade)¹⁵ and ixazomib (Ninlaro)¹⁶ as well as the peptide epoxyketone, carfilzomib (Kyprolis)¹⁷ are used clinically for the treatment of multiple myeloma and in some cases also mantle cell lymphoma. Myeloma cells excrete large amounts of immunoglobulins, and the numerous and continuously occurring errors in immunoglobulin synthesis and folding put a high burden on the UPS. For this reason, myeloma cells are particularly sensitive to proteasome inhibitors.¹⁸

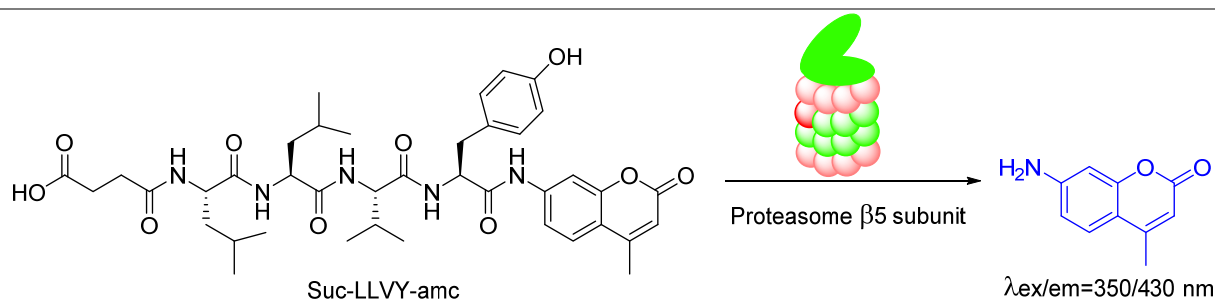
Elucidation of the physiological roles of proteasomes, as well as the development of proteasome inhibitors, necessitates tools and techniques that allow measurement of the activity of the individual catalytic β -subunits. In the past two decades, several methods have been developed to study proteasome activity. These methods rely either on hydrolysis of (fluorogenic) substrates or on covalent modification of the active site threonine by activity-based probes. This chapter provides an overview of these methods with a focus on recent developments.

Substrate hydrolysis assays

Substrate based probes have been widely used to monitor the activity of proteolytic enzymes. Such probes consist of a recognition sequence (usually a tri- or tetrapeptide) and a C-terminal reporter group that produces a measurable signal upon cleavage by the enzyme.¹⁹ In the past decades numerous fluorogenic, luminogenic and quenched substrate oligopeptides have been developed. In the following section relevant examples of these proteasome probes are given.

Fluorogenic substrates

Fluorogenic proteasome substrates generally are tri- or tetrapeptides, the N-terminus of which is capped and the C-terminus of which contain a fluorogenic reporter group. Upon enzymatic hydrolysis, the reporter group becomes fluorescent. Various fluorophores have been used in proteasome substrates, such as 7-amino-4-methylcoumarin (AMC or amc) and 2-naphthylamine (NA or na). Of these, AMC has the highest fluorescence quantum yield and is thus most widely applied. Proteasomes were discovered some three decades ago, and at that time it was not known that proteasomes harbour three different catalytic subunits. To obtain insight in the cleavage preferences of proteasomes, its proteolytic activities were determined using fluorogenic substrate peptides. Based on these studies, the activities of the proteasome were designated as chymotrypsin-like (Z-GGL-na hydrolysis), trypsin-like (Z-D-ALR-na hydrolysis) and peptidylglutamyl-peptide-hydrolyzing (PGPH, Z-LLE-na hydrolysis), which were later termed as $\beta 5c/\beta 5i$, $\beta 2c/\beta 2i$ and $\beta 1c$ respectively.^{20, 21} Another catalytic activity, which did not process these substrates, was found to hydrolyse after Leu or Ala of PAL or PAA containing substrates and was therefore designated as branched- or small neutral amino acid preferring (BrAAP or SNAAP) and later termed $\beta 1i$.²⁸ Since then, substrates for each catalytic activity have been optimized and Table 1 shows the fluorogenic substrates currently used to monitor the activity of individual proteasome subunits. Suc-LLVY-amc is the substrate of choice to monitor the activity of $\beta 5c$ and $\beta 5i$.²² Z-GGL-amc and Suc-AAF-amc can also be used for this purpose, however, those are hydrolysed much slower.²³



Subunit	Preferred	Other
$\beta 1c$	Ac-nLPnLD-amc	Ac-GPLD-amc; Z-LLE-na; Z-LLE-amc*
$\beta 1i$	Ac-PAL-amc*	
$\beta 2c/\beta 2i$	Ac-RLR-amc	Boc-LRR-amc; Z-ARR-amc; Bz-FVR-amc; Bz-VGR-amc Boc-LSTR-amc, Ac-KQL-amc*
$\beta 5c/i$	Suc-LLVY-amc*	Z-GGL-amc, Suc-AAF-amc
$\beta 5c$	Ac-WLA-amc*	
$\beta 5i$	Ac-ANW-amc*	

Table 1. Substrates to monitor proteasome subunit activities. The hydrolysis of Suc-LLVY-amc is shown as an example. *These substrates are also available as (peptide)₂-R110 substrates.

Suc-LLVY-amc is also hydrolysed by other chymotrypsin-like enzymes such as calpain, however, in cytosolic HeLa extract, non-proteasomal hydrolysis contributed to only 5% of total hydrolysis of this substrate.²³ Recently, substrates have been described that are hydrolysed selectively by $\beta 5c$ (Ac-WLA-amc) or $\beta 5i$ (Ac-ANW-amc).²⁴⁻²⁶ These substrates have been used to identify selective $\beta 5c$ inhibitors²⁵ and to quantify the relative amount of $\beta 5c$ and $\beta 5i$.²⁴

The catalytic activity of $\beta 1c$ was initially monitored by Z-LLE-na.²⁰ Cleavage of this substrate releases 2-naphtylamine, which is less fluorescent than AMC and fluoresces at a different wavelength, requiring different filter sets. Furthermore, Z-LLE-na is also hydrolysed at the Leu-Glu bond. To overcome some of these problems, NA was replaced by AMC, however the resulting substrate Z-LLE-amc was cleaved about 600 times slower than the original substrate.²⁷ Kisselev and co-workers found that the standard caspase substrate Ac-YVAD-amc is hydrolysed 50 times faster than Z-LLE-amc, indicating that $\beta 1c$ is caspase-like rather than PGPH.³⁷ However, this substrate was cleaved much slower than standard substrates for the chymotrypsin- and trypsin-like sites and, in addition, Ac-YVAD-amc is also hydrolysed N-terminally of the Asp-amc bond.²⁷ Using positional scanning libraries with Asp at P1, two substrates were identified that exhibited fast Asp-amc hydrolysis, without competing cleavage of other peptide bonds: Ac-nLPnLD-amc and Ac-GPLD-amc (with the former being the most rapidly processed). Ac-nLPnLD-amc is therefore the most optimal substrate currently available to monitor $\beta 1c$ activity. The S1 pocket of $\beta 1i$ is more hydrophobic compared to $\beta 1c$ due to two point mutations: R45L and T20V.¹² Therefore, substrates used to monitor the caspase-like activities of the proteasome are not hydrolysed by $\beta 1i$. To monitor $\beta 1i$ activity, Ac-PAL-amc was developed.²⁶ This substrate showed negligible hydrolysis by $\beta 1c$ and can therefore be used to selectively monitor $\beta 1i$ activity in presence of $\beta 1c$.^{25, 29}

Several substrates with R-amc bonds (Boc-LRR-amc; Z-ARR-amc; Bz-FVR-amc; Bz-VGR-amc; Boc-LSTR-amc) can be used to monitor the trypsin-like activities ($\beta 2c/\beta 2i$). However, K_m values for these substrates are rather high (>500 mM), and the substrates furthermore suffer from low specific activity.²³ Positional scanning of fluorogenic peptide libraries led to the development of Ac-RLR-amc, which specific activity is much higher, and for which the 26S proteasome has a much lower K_m value.^{18, 30} Ac-KQL-amc, with a basic substituent at P3, represents an alternative substrate to monitor $\beta 2c$ and $\beta 2i$ activity.²⁵

In recent years, rhodamine 110 (R110) substrates have become commercially available and are a useful alternative to AMC substrates. R110 fluoresces at a higher wavelength ($\lambda_{ex/em}=490/520$) and R110 substrates are more sensitive and suffer less from background fluorescence caused by additives present in the sample. The R110 fluorophore features two amines, each of which is modified to contain an oligopeptide. Relevant R110-based fluorogenic proteasome substrates are (Suc-LLVY)₂-R110 (reporting on $\beta 5c$ and $\beta 5i$), (Ac-KQL)₂-R110 (reporting on $\beta 2c$ and $\beta 2i$), (Z-LLE)₂-R110 (reporting on $\beta 1c$), (Ac-PAL)₂-R110 (reporting on $\beta 1i$), (Ac-ANW)₂-R110 (reporting on $\beta 5i$), and (Ac-WLA)₂-R110 (reporting on $\beta 5c$).

Fluorogenic substrates are often used on purified proteasomes. Their use in crude cell extracts is more complicated due to partial hydrolysis of the substrates by other enzymes.²³ This especially holds true for those substrates used to monitor the trypsin-like activities of the proteasome. In whole cell extracts, these substrates are processed in large parts by proteases (trypsin) other than proteasomes. Non-proteasomal hydrolysis is much less pronounced in cytosolic preparations (prepared using digitonin as detergent), but still accounts for up to 25% of total hydrolysis. Non-specific hydrolysis can be further reduced by partial proteasome purification via ultracentrifugation. Altogether, proper controls to account for non-proteasomal substrate hydrolysis have to be applied when fluorogenic substrates are used on cell extracts.^{23, 24, 31, 32}

Luminogenic substrates: Proteasome-Glo assay

As an alternative to fluorogenic substrates, luminogenic peptides can be used to detect and quantify proteasome activities.³³ As with fluorogenic peptides, luminogenic peptides can be used both to report on the activity of purified proteasomes and on crude tissue extracts containing proteasome activities, and in fact luminogenic peptides can often be applied in situations where fluorogenic peptides fall short. In what has become known as the 'proteasome-Glo assay', peptide-luciferin conjugates (specifically, Suc-LLVY-aminoluciferin, Z-LRR-aminoluciferin and Z-nLPnLD-aminoluciferin), which upon hydrolysis release the luciferase substrate aminoluciferin, are applied (Figure 2).³³ A single buffer containing digitonin, a peptide-luciferin conjugate, and luciferase is added to intact cells. Digitonin permeabilizes the cell membrane and after a short incubation time, luminescence can be measured directly. The Proteasome-Glo assay represents a major advance compared to fluorescent assays since it is not only faster and more convenient, but also significantly more sensitive, allowing a 20-fold reduction of the amount of cells.³³ Notwithstanding this, serine protease inhibitors need sometimes to be included in the assay, for instance when proteasome trypsin-like activities are subject of study. Moreover, background luminescence cannot be avoided in specific cell lines when probing for either chymotrypsin-like or trypsin-like activities. Inhibition of chymotrypsin-like or trypsin-like proteasome activities prior to conducting a proteasome-Glo assay lowers the amount of background labeling, but also makes the assay somewhat cumbersome.³⁴

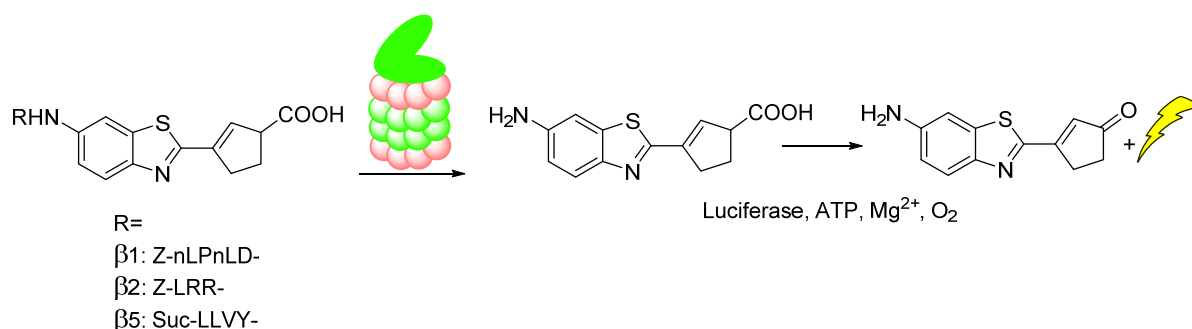


Figure 2. Proteasome-Glo assay. Peptide-aminoluciferin substrates are hydrolysed by the respective proteasome subunits, releasing aminoluciferin, which is consumed by luciferase to generate light.

Capture proteasome assay

As is evident from the above sections, measuring proteasome activities, especially trypsin-like activities, in crude cell extracts is complicated by the presence of other proteases. One way to overcome this problem is the capture-proteasome-assay (CAPA), in which the proteasome content of a cell lysate is captured on an anti-α2 antibody (MCP21) coated plate (Figure 3).³⁵ After a washing step, proteasome activities are readily measured using standard fluorogenic substrates (specifically, Suc-LLVY-amc, Z-LLE-amc and Boc-LRR-amc). Constitutive proteasomes, immunoproteasomes and mixed proteasomes (β5i or β5i-β1i)³⁶ could be captured from HEK-293 cells and various proteasome inhibitors were evaluated for their potency and selectivity for these proteasome subtypes. The capture assay can be conducted in a 96-well format, which allows screening of compound libraries on proteasome inhibitor content in a high-throughput format. Moreover, the capture assay eliminates the need for pre-purified proteasomes and, since no other proteases should bind the immobilized antibody, provides a more reliable measurement of especially the trypsin-like activities.

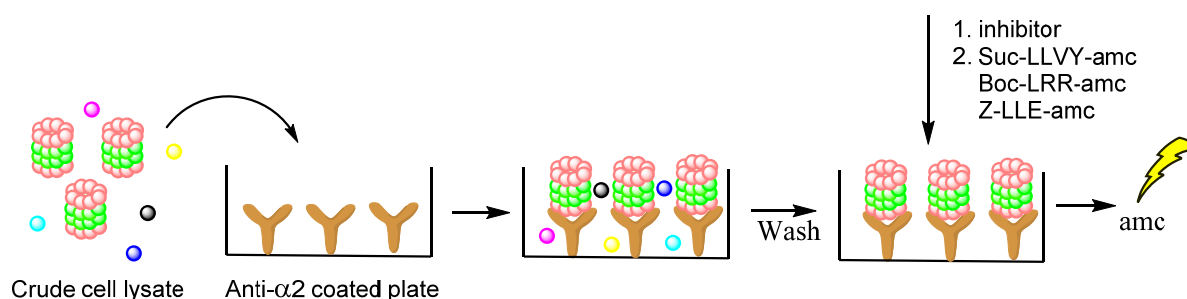


Figure 3. Capture proteasome assay. A crude cell lysate is added to an anti-α2 antibody coated plate. Unbound proteins are removed by washing steps and proteasome activity can be measured using fluorogenic substrates.

Simultaneous, multicolour monitoring of proteasome activities

Since β5 subunits determine the rate of protein degradation³⁷, for years, often only proteasomal β5 activities were monitored as a measure for total proteasome activity. As well, all proteasome inhibitors that are clinically approved or are in clinical trials have been

designed to primarily inhibit the $\beta 5$ activities.³⁸ However, in the last years it became clear that inhibition of $\beta 1$ or $\beta 2$ does sensitize cancer cells to selective $\beta 5$ inhibition, indicating the importance of these subunits as drug targets.^{39, 40} Furthermore, allosteric cross-talk between the different catalytic subunits, including allosteric up-regulation of one site by another subunit whose active site is occupied by an inhibitor, underscores current thought that the activity of all active subunits should be taken into account.³⁷ For these reasons, there is an increasing interest in the development of methods that allow simultaneous measurement of multiple proteasome activity sites. In a study aimed to deliver such methodology, Lawrence *et al.* developed a substrate for $\beta 5$, equipped with a high wavelength oxazine-based fluorophore ($\lambda_{\text{ex/em}}=663/678$), that is quenched internally by a tryptophan residue at P2 (Ac-HWSL-Lys(Fluorophore)).⁴¹ Upon hydrolysis a >20 fold increase in fluorescence was observed, and due to the distinct properties of the oxazine-fluorophore compared to AMC and NA, it can be used simultaneously with Z-LLE-na and Boc-LRR-amc. Simultaneous measurement of various combinations of two of the three activities (chymotrypsin-like activity, the caspase-like activity and the trypsin-like activities) with the appropriate compounds revealed that the rate of hydrolysis of each activity changes when a second substrate is present. This result indicates that the active subunits are indeed allosterically regulated.

In order to simultaneously monitor all three proteasome catalytic activities, Lawrence *et al.* extended their strategy and developed three quenched substrates, each equipped with a different fluorophore (Figure 4).⁴² The Acid-Blue 40 (AB40) fluorescent quencher was coupled to the ϵ -amine of lysine which was installed next to scissile bond of known peptide substrates (the HHSL sequence, which is cleaved at the S-L amide bond, was identified by positional scanning of libraries of tetrapeptide AMC substrates⁴³). The three complementary fluorophores were coupled to the N-terminus of the respective oligopeptides, yielding the conjugates as depicted in Figure 4. Upon proteasome-mediated removal of the quencher moiety in these compounds, a >30-fold increase in fluorescence intensity was observed in each case.

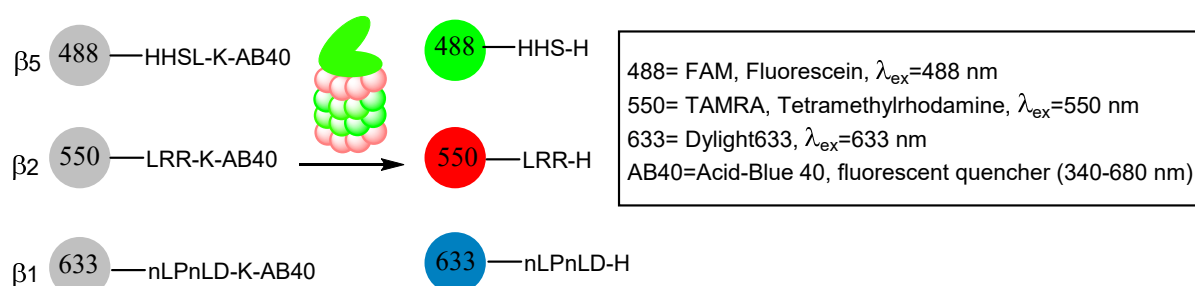


Figure 4. Simultaneous multicolour monitoring of proteasome activity by quenched substrates. Upon hydrolysis of the quenched substrates, the AB40 quencher is cleaved resulting in increased fluorescence. Due to the distinct properties of the fluorophores, the three substrates can be used to simultaneously monitor all three proteasome catalytic activities.

As result of allosteric regulation, the activity of each subunit was substantially lowered in presence of the three substrates. Therefore, low concentrations of substrates are required, which is a caveat because the resulting low hydrolysis rates cause limited sensitivity and long reaction times. Despite these drawbacks, this method was successfully applied to simultaneously measure the three proteasome activities in various samples.

Activity-based proteasome probes

Activity-based protein profiling (ABPP) has been used for decades to detect, identify and/or relatively quantify enzymes in complex samples, including intact cells and cell extracts.¹⁹ In ABPP, activity-based probes (ABPs) are employed to selectively, covalently and irreversibly bind to the active site of an enzyme in order to gain information regarding its activity and (relative) abundance. ABPs generally consist of three components, namely 1) a reactive group, often designated as ‘warhead’, which binds covalently to the active site residue of a protease; 2) a recognition sequence, providing affinity and selectivity for the target enzyme, and 3) a reporter group, such a fluorescent group or affinity tag (biotin), allowing detection of the enzyme by mass spectrometry, SDS-PAGE, fluorescence microscopy or *in situ* imaging (Figure 5). ABPP is complementary to the use of substrate probes. Whereas substrate probes report on (changes in) enzyme activity and turnover rate, ABPs report on the quantity of active enzymes, and not so much on their activity. In the proteasome field, the first ABPs were developed to identify the proteasome as target of natural product electrophiles with, amongst others, cytotoxic activity. More recently, proteasome (subunit-selective) targeting ABPs have been developed as tools to monitor proteasome activity and to identify new proteasome inhibitors.

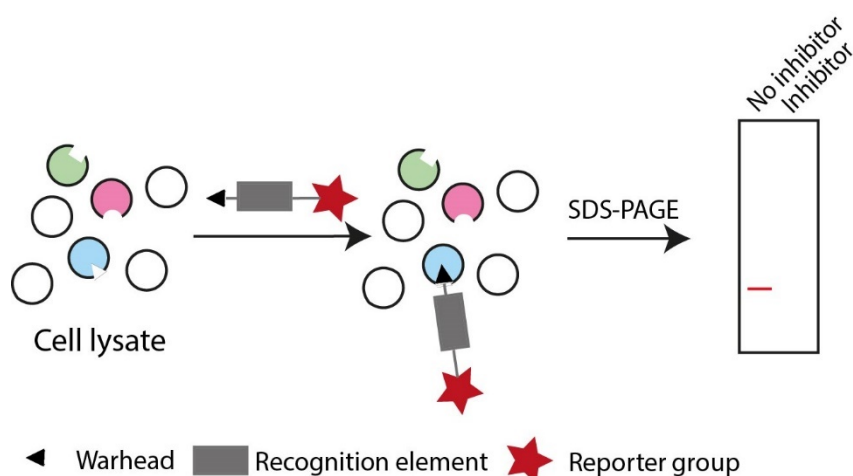


Figure 5. SDS-PAGE based ABPP. A crude cell lysate is treated with an ABP and analysed by SDS-PAGE, showing a band for the ABP-reactive enzyme or enzyme family (comparative ABPP). In the presence of a competitive inhibitor (competitive ABPP), the band disappears.

ABPs for target identification

The first proteasome inhibitors were discovered after grafting a reporting group onto bioactive natural products, suspected (by virtue of electrophilic moieties embedded in these structures) to inactivate their target through covalent and irreversible modification. Lactacystin (Figure 6) was isolated from *Streptomyces* and found to induce neurite outgrowth in Neuro-2a-cells and to inhibit cell cycle progression in an osteosarcoma cell line.^{44, 45} Lactacystin in time reacts intramolecularly to form *clasto*-lactacystin β -lactone, which was identified as the bioactive species. In order to identify its target, tritiated lactacystin was synthesized (Figure 6). On the basis of its labelling by [³H]-lactacystin, a 700 kDa complex was purified from bovine brain, which was identified to be the 20S proteasome. Lactacystin proved active against all three cCP activities with a preference for $\beta 5c$.⁴⁶ 2D-PAGE analysis of proteasome fractions derived from lymphoblasts that were treated with [³H]-lactacystin showed distinct spots for all catalytically active cCP and iCP proteasome subunits, indicating covalent binding of lactacystin to all six catalytic proteasome subunits.⁴⁷ Looking back, [³H]-lactacystin can be considered as the first proteasome targeting ABP.

Originally designed as mechanism-based cysteine protease inhibitors, peptide vinyl sulfones were found to be remarkably effective proteasome inhibitors some two decades ago. Peptide vinyl sulfones act as mechanism-based proteasome inhibitors due to conjugate addition of the N-terminal threonine alcohol to the Michael acceptor. Covalent proteasome modification appears irreversible and peptide vinyl sulfones can therefore serve as a basis for ABP development. Indeed, ¹²⁵I-NIP-L₃VS (Figure 6) was found to irreversibly react with all proteasome subunits, as revealed by SDS-PAGE.⁴⁸ The related ¹²⁵I-NIP-LLN-VS comprises the first ABP employed in screening of compound libraries for the identification of new proteasome inhibitors. Cellular extracts were incubated with a putative inhibitor, followed by labelling of residual proteasome activity by ¹²⁵I-NIP-LLN-VS, SDS-PAGE separation and autoradiographic analysis of the gel slabs. In this competitive ABPP setting, decreased band intensities correlate to inhibition of proteasome activity.⁴⁹ AdaY(¹²⁵I)-Ahx₃-L₃-VS emerged as a pan-reactive proteasome ABP from a study that aimed to demonstrate that N-terminally extended proteasome inhibitors are both more potent and less selective (with respect to proteasome catalytic activities modified) proteasome inhibitors than their tri- or tetrapeptide counterparts.⁵⁰ ABPP was also employed to identify the target of the natural product epoxomicin, for which anti-tumour and anti-inflammatory activities were observed following its discovery.⁵¹ For this purpose, epoxomicin was equipped with a biotin moiety (Figure 6), which can be detected following treatment with with avidin-horseradish peroxidase (HRP).⁵²

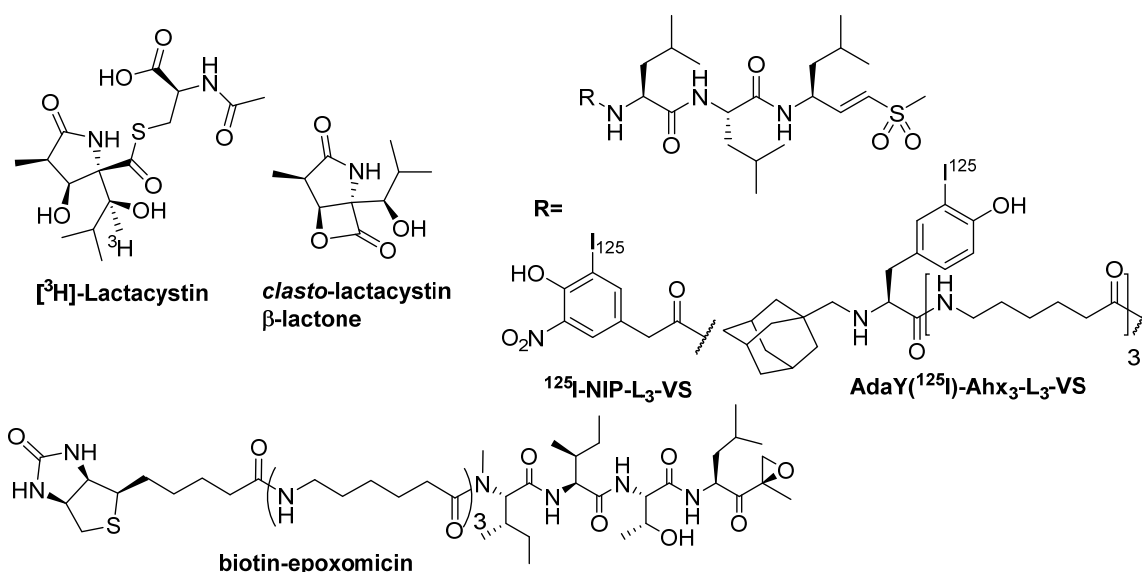


Figure 6. Structures of [³H]-lactacystin, active β-lactone *clasto*-lactacystin, ¹²⁵I-NIP-L₃-VS, AdaY(¹²⁵I)-Ahx₃-L₃-VS and biotin-epoxomicin.

Murine EL-4 cells were treated with biotin-epoxomicin, lysed and separated by SDS-PAGE after which biotin detection using avidine-horseradish peroxidase enhanced chemiluminescence revealed multiple bands. Upon trypsin digestion and MALDI-MS analysis of the corresponding bands, β5c, β5i, β2c and β2i were identified as targets of biotin-epoxomicin.⁵²

Pro-CISE assay

In order to quantify the amount of cCP and iCP subunits in cells of different origin, and to determine the inhibitory potency of a putative proteasome inhibitor against all six cCP and iCP active subunits, an enzyme-linked immunosorbent assay (ELISA) was developed (Figure 7).¹⁷ This assay, termed Pro-CISE, makes use of biotin-L₃-epoxyketone (EK), which covalently modifies the active site threonine residues of all six subunits when applied at high concentrations. After incubation of a cell lysate with biotin-L₃-EK, all biotin-containing molecules are captured upon incubation with streptavidin-sepharose beads under denaturing conditions. Subsequently, primary antibodies against each of the six catalytically active subunits are added in individual samples, followed by a secondary horseradish peroxidase (HRP) conjugated antibody. Finally, the wells are developed and luminescence is detected. Using purified iCP and cCP samples, standard curves for each of the six subunits can be obtained, enabling quantification of all six proteasome subunits.

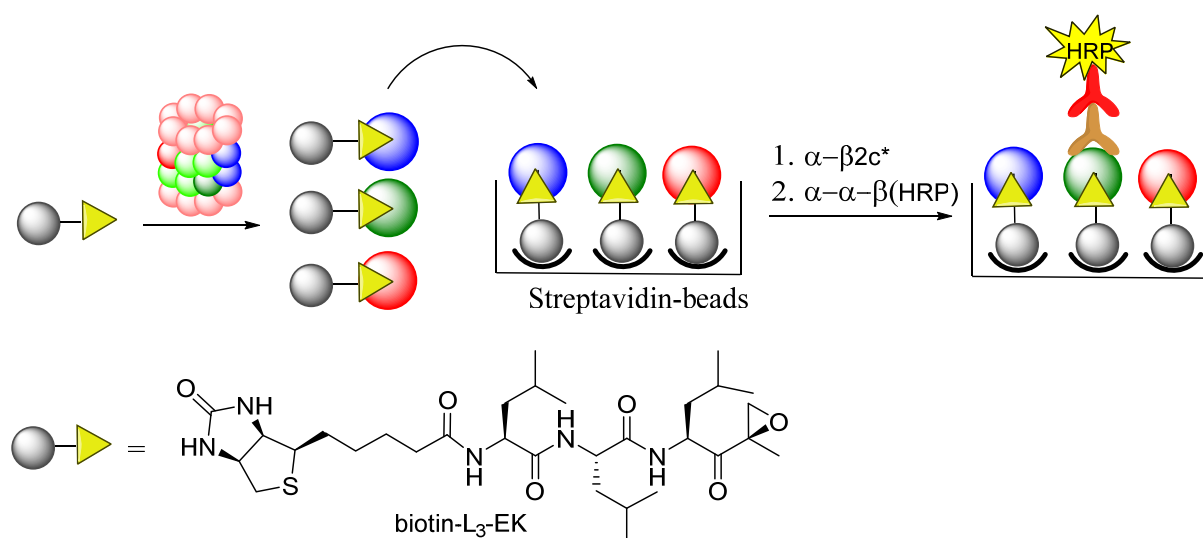


Figure 7. Pro-CISE assay. All active sites are covalently modified with biotin-L₃-EK, which is captured on streptavidin-beads in a 96-well filter-plate. Each of the active subunits can be detected by treatment with subunit-specific antibodies followed by treatment with a secondary HRP-conjugated antibody.

Fluorescent ABPs

In recent years, a number of fluorescent proteasome selective probes were developed that target all active subunits. With these, radioactive probes can be avoided and rapid screening of proteasome activity in multiple samples is feasible. Dansyl-Ahx₃-L₃-VS (Figure 8) comprises the first-in-class fluorescent proteasome ABP and the dansyl moiety can be detected, next to fluorescence read-out of gel slabs (which is a bit hampered by the low quantum yields of the fluorophore), also by dansyl-specific antibodies.⁵³ Using this probe, the subunit binding preferences of bortezomib and MG132 (Z-L₃-aldehyde) were determined. To overcome the weak fluorescent properties of dansyl-Ahx₃-L₃-VS, the BODIPY(TMR) analogue MV151 as well as a number of BODIPY-epoxomicin derivatives were developed (Figure 8).⁵⁴⁻⁵⁷ These ABPs and the vinyl sulfone-based ABP BODIPY(FL)-Ahx₃-L₃-VS⁵⁸ are used in competitive ABPP assays to determine the potency and specificity of putative proteasome inhibitors (Figure 5), in fluorescence microscopy as well as in flow cytometry experiments.

Triple colour ABPP-assay

Due to similar molecular weights of $\beta 1c$, $\beta 1i$, $\beta 5c$ and $\beta 5i$, the broad-spectrum vinyl sulfone- and epoxyketone-based ABPs are not able to provide full resolution of these subunits on 1D SDS-PAGE. Therefore, these probes cannot be used to determine the potency and subunit selectivity of putative proteasome inhibitors for each individual subunit. To overcome this limitation, ABPs that selectively target a subunit-pair were developed. These APBs were based on the previously developed $\beta 1c/\beta 1i$ -selective and $\beta 5c/\beta 5i$ -selective inhibitors, NC001 and NC005, respectively.³⁹

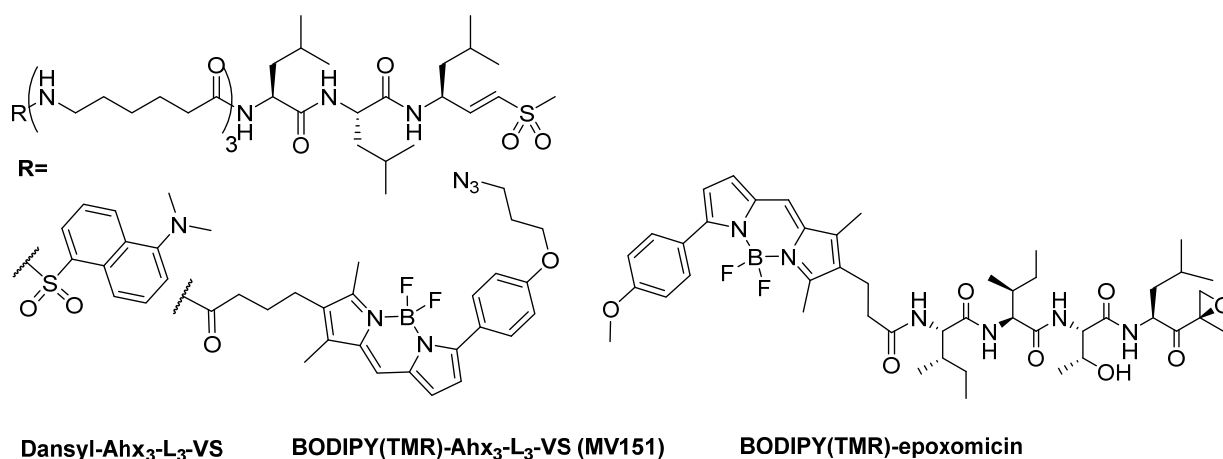


Figure 8. Examples of fluorescent, broad spectrum proteasome ABPs.

β 1c/ β 1i are selectively tagged by BODIPY(FL)-NC001 and β 5c/ β 5i by BODIPY(TMR)-NC005 (Figure 9), resulting in clear resolution of the respective subunits on SDS-PAGE.⁵⁵ In addition, since these ABPs are equipped with distinct fluorophores, they can be used to simultaneously resolve β 1c/ β 1i and β 5c/ β 5i on SDS-PAGE.⁵⁹⁻⁶¹ However, a separate analysis using BODIPY-epoxomicin or MV-151 has to be performed to determine β 2c/ β 2i activity. In order to further optimize and simplify the screening of inhibitors using competitive ABPP, a triple colour strategy was developed, making use of three subunit-pair selective ABPs, each equipped with a distinct fluorophore. BODIPY(FL)-LU112 was developed to label β 2c/ β 2i, but was found to also partially modify β 5c/ β 5i.⁶²

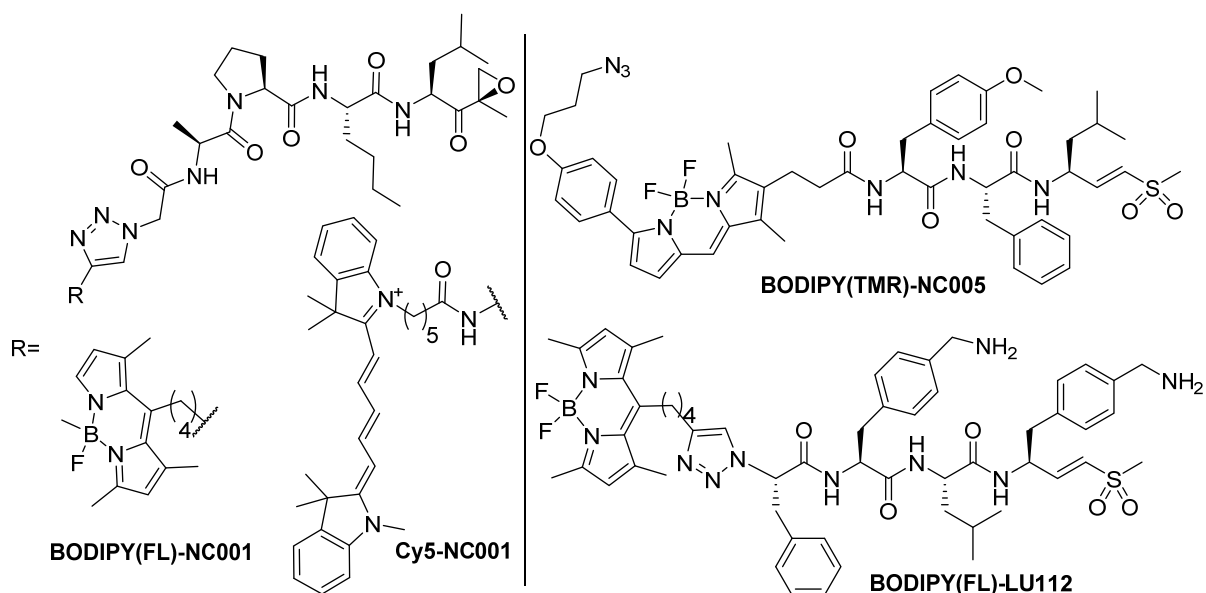


Figure 9. Structures of subunit-pair selective ABPs. The probes selectively target either β 1c/ β 1i (BODIPY(FL)-NC001 and Cy5-NC001), β 5c/ β 5i (BODIPY(TMR)-NC005) or β 2c/ β 2i (BODIPY(FL)-LU112).

However, when applied at optimal concentration, only minimal labelling of $\beta 5c/\beta 5i$ was found. In the end, a cocktail of probes consisting of optimized amounts of BODIPY(FL)-LU112, BODIPY(TMR)-NC005 and Cy5-NC001 was compiled (Figure 9). This probe cocktail labels all six active sites from human cCPs and iCPs simultaneously giving full resolution of the modified subunits on SDS-PAGE (Figure 10).⁶³ The cocktail can be used for rapid and straightforward screening of putative proteasome inhibitors, and was instrumental in the identification of new $\beta 2c$, $\beta 1c$ and $\beta 5c$ -selective inhibitors.

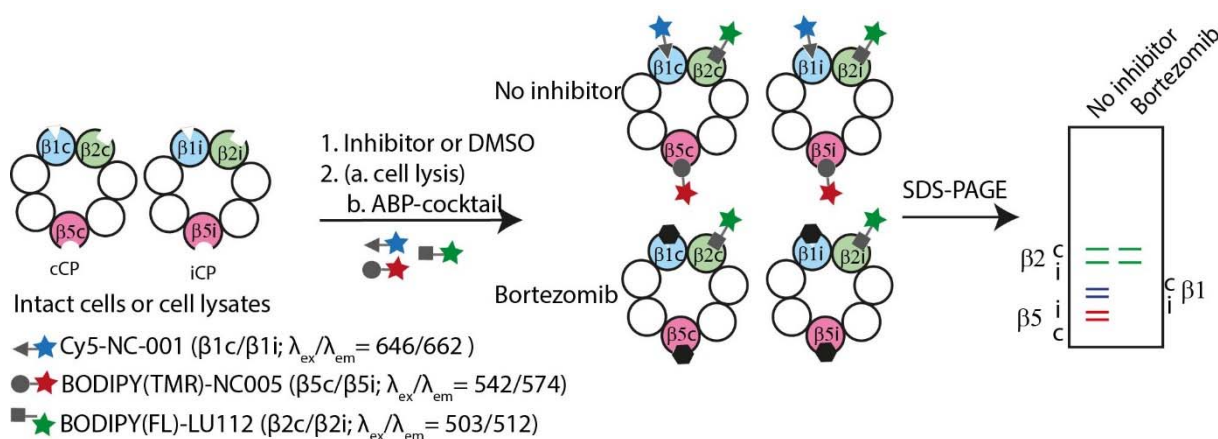
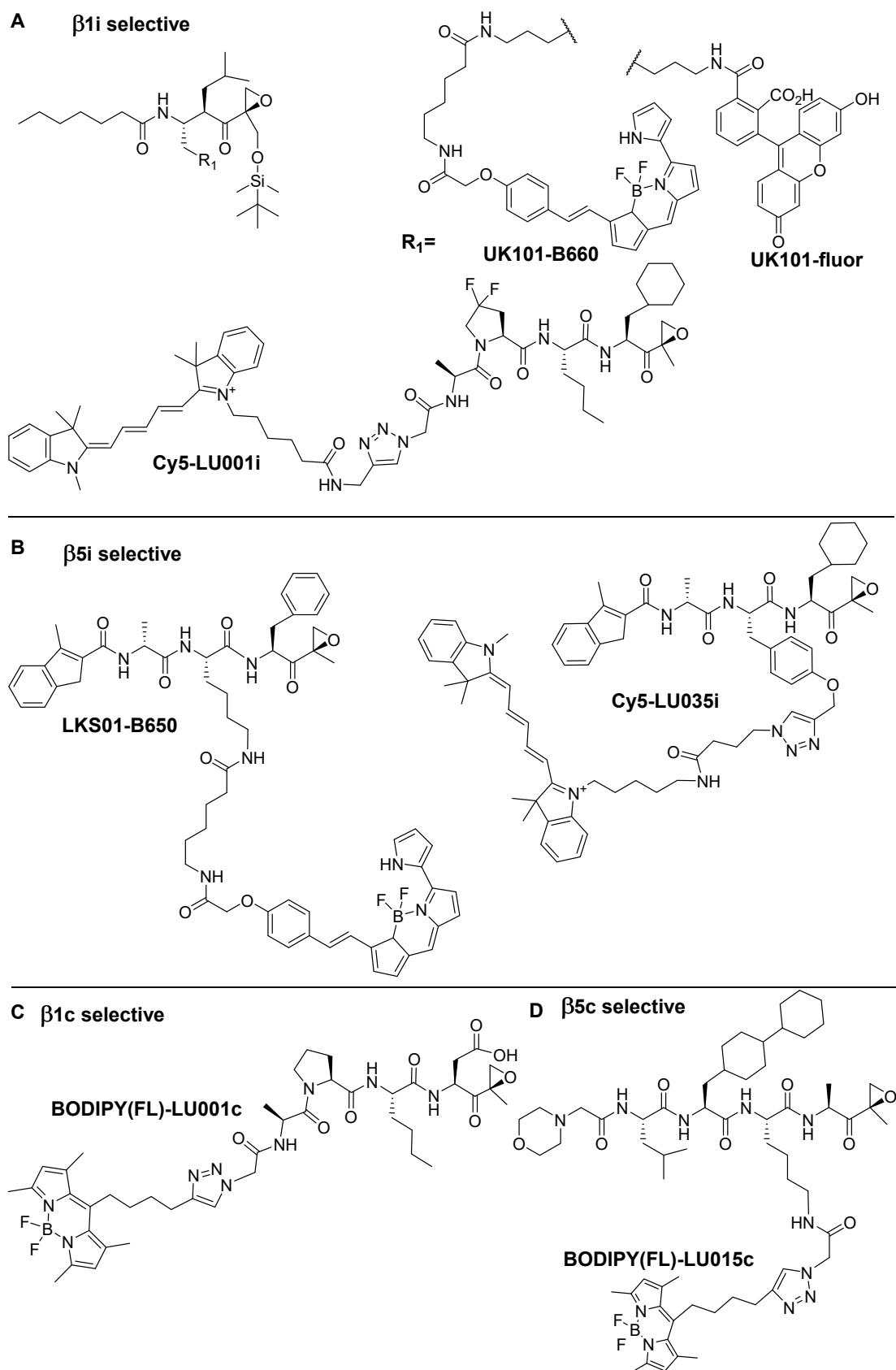


Figure 10. Schematic representation of multi-colour competitive ABPP. Intact cells or cell lysates are treated with inhibitor or DMSO, followed by cell lysis in case of intact cells, and addition of an ABP-cocktail consisting of Cy5-NC001, BODIPY(TMR)-NC005 and BODIPY(FL)-LU112. Subsequent SDS-PAGE analysis reveals full resolution of all six active subunits. Bortezomib inhibits the $\beta 1$ and $\beta 5$ subunits, indicated by disappearance of the corresponding bands.

iCP and cCP subunit selective ABPs

In the past decade, much research has been directed to the discovery of iCP selective inhibitors. In particular $\beta 5i$ -selective inhibitors were sought after, as they have potential as therapeutics against several auto-immune diseases.¹⁴ Based on the $\beta 1i$ selective inhibitor UK101⁶⁴ and the $\beta 5i$ selective inhibitor PR924,⁶⁵ Kim and co-workers developed ABPs selective for either $\beta 1i$ (UK101-Fluor and UK101-B660)⁶⁶ or $\beta 5i$ (LKS01-B650)⁶⁷ (Figure 11). Gel-based competition experiments and Western-blotting revealed that these probes maintain the selectivity of the parent inhibitors. These probes enable the assessment of cellular localization of $\beta 1i$ and $\beta 5i$ using fluorescence microscopy and show substantial co-localization indicating that these probes target fully assembled immunoproteasome complexes. Since $\beta 1i$ might serve as potential tumour biomarker and given the near-infrared properties of UK101-B660 and LKS01-B650, these ABPs might find their application in *in vivo* imaging for cancer screening, disease progression or subcellular localization of the immunoproteasome.⁶⁸ Recently, a $\beta 1i$ selective inhibitor (LU001i) as well as a $\beta 5i$ selective inhibitor (LU035i) with improved selectivity profiles compared to UK101 and PR924 have been developed.⁶⁰

Figure 11. Structures of iCP- (A. $\beta 1i$; B. $\beta 5i$) and cCP (C. $\beta 1c$; D. $\beta 5c$) subunit selective ABPs.

Based on these inhibitors, ABPs Cy5-LU001i and Cy5-LU035i were developed (Figure 11), which exhibit excellent selectivity for their target subunits (see chapter 6). Given the improved selectivity of the parent compounds, it may well be that these ABPs outperform UK101-B660, UK101-Fluor and LKS01-B650 on selective subunit tagging, though a direct comparison has not been made. Using related strategies, the recently discovered β 1c selective and β 5c-selective inhibitors⁶³ were transformed to fluorescent ABPs. BODIPY(FL)-LU001c is completely selective for β 1c, while BODIPY(FL)-LU005c has β 2c/ β 2i as off-target. However, when β 2c/ β 2i are selectively inhibited by a β 2c/ β 2i selective inhibitor, this probe can be used to modify β 5c selectively in presence of β 5i. These four cCP/iCP subunit selective ABPs have been successfully used in the development of a native-PAGE fluorescence resonance energy transfer (FRET) assays for the detection of mixed asymmetric proteasomes (see chapter 10).

Discussion/conclusion

Substrate hydrolysis-based assays and activity-based protein profiling (ABPP) are complementary methods to monitor proteasome activity. Both have been highly optimized and are currently used to screen for proteasome inhibitors and to assess the abundance of individual proteasome subunits and their relative activities. Substrate hydrolysis assays report predominantly on proteasome activity (turn-over rate) and less on abundance of each subunit, while the opposite holds true for ABPP. The applicability of substrate assays to measure proteasome activity has been given a major improvement by the development of cCP and iCP subunit selective substrates, allowing the independent measurement of β 1c, β 1i, β 5c and β 5i activities. In addition, the extensively optimized proteasome-Glo assay allows high throughput analysis of proteasome activity in cell lysates obtained from intact cells, without the need for (partial) proteasome purification. The drawback of substrate hydrolysis assays is the possibility of non-proteasomal hydrolysis, for which extensive controls are required. Another drawback is the lack of β 2c and β 2i selective substrates, making it impossible to measure β 2c and β 2i independently in cells expressing both subunits. Moreover, simultaneous monitoring of multiple proteasome activities using substrates is rather complicated and limited to the simultaneous measurement of caspase (β 1c), trypsin (β 2c/ β 2i) and chymotrypsin activities (β 5c/ β 5i). ABPP was initially used to identify the proteasome as target of certain inhibitors. In the last decade, this technique has evolved and now is often used to assess the activity of individual subunits. The Pro-CISE assay makes use of six subunit selective antibodies, which report on the presence of each active subunit after capture by a broad-spectrum biotinylated ABP. The main disadvantage is the length of the assay (several multiple hour incubations), the use of expensive antibodies, and the requirement of six individual assays to measure each catalytically active subunit. The availability of subunit selective ABPs resulted in the development of an SDS-PAGE based tri-colour ABPP assay that reports on the activity of all

active proteasome subunits in one experiment. This assay requires low amount of cells, is time efficient and does not require extensive controls. Therefore, the tri-colour ABPP assay can be used for screening of putative proteasome inhibitors and rapid assessment of relative abundances of each active proteasome subunit in cell extracts.

References

1. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425-79 (1998).
2. Komander, D. & Rape, M. The ubiquitin code. *Annu. Rev. Biochem.* **81**, 203-229 (2012).
3. Thrower, J.S., Hoffman, L., Rechsteiner, M. & Pickart, C.M. Recognition of the polyubiquitin proteolytic signal. *EMBO J.* **19**, 94-102 (2000).
4. Kisselev, A.F., Akopian, T.N., Woo, K.M. & Goldberg, A.L. The sizes of peptides generated from protein by mammalian 26 and 20S proteasomes: implication for understanding the degradative mechanism and antigen presentation. *J. Biol. Chem.* **274**, 3363-3371 (1999).
5. Sijts, E.J.A.M. & Kloetzel, P.-M. The role of the proteasome in the generation of MHC class I ligands and immune responses. *Cell. Mol. Life. Sci.* **68**, 1491-1502 (2011).
6. Groll, M. et al. Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* **386**, 463-71 (1997).
7. Ferrington, D.A. & Gregerson, D.S. Immunoproteasomes: structure, function, and antigen presentation. *Prog. Mol. Biol. Transl. Sci.* **109**, 75-112 (2012).
8. Romero, P., Corradin, G., Luescher, I.F. & Maryanski, J.L. H-2Kd-restricted antigenic peptides share a simple binding motif. *J. Exp. Med.* **174**, 603-12 (1991).
9. Kloetzel, P.M. & Ossendorp, F. Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. *Curr. Opin. Immunol.* **16**, 76-81 (2004).
10. Murata, S. et al. Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science* **316**, 1349-1353 (2007).
11. Florea, B.I. et al. Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit $\beta 5t$. *Chem. Biol.* **17**, 795-801 (2010).
12. Huber, Eva M. et al. Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. *Cell* **148**, 727-738 (2012).
13. Tomaru, U. et al. Exclusive expression of proteasome subunit $\beta 5t$ in the human thymic cortex. *Blood* **113**, 5186-5191 (2009).
14. Kisselev, A.F. & Groettrup, M. Subunit specific inhibitors of proteasomes and their potential for immunomodulation. *Curr. Opin. Chem. Biol.* **23**, 16-22 (2014).
15. Adams, J. et al. Potent and selective inhibitors of the proteasome: Dipeptidyl boronic acids. *Bioorg. Med. Chem. Lett.* **8**, 333-338 (1998).
16. Gentile, M. et al. Ixazomib for the treatment of multiple myeloma. *Expert Opin. Investig. Drugs* **24**, 1287-98 (2015).
17. Parlati, F. et al. Carfilzomib can induce tumor cell death through selective inhibition of the chymotrypsin-like activity of the proteasome. *Blood* **114**, 3439-3447 (2009).
18. Cenci, S. et al. Pivotal Advance: Protein synthesis modulates responsiveness of differentiating and malignant plasma cells to proteasome inhibitors. *J. Leukoc. Biol.* **92**, 921-931 (2012).
19. Sanman, L.E. & Bogoy, M. Activity-based profiling of proteases. *Annu. Rev. Biochem.* **83**, 249-273 (2014).
20. Wilk, S. & Orlowski, M. Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex. *J. Neurochem.* **40**, 842-9 (1983).
21. Orlowski, M. The multicatalytic proteinase complex, a major extralysosomal proteolytic system. *Biochemistry* **29**, 10289-97 (1990).
22. Stein, R.L., Melandri, F. & Dick, L. Kinetic characterization of the chymotryptic activity of the 20S proteasome. *Biochemistry* **35**, 3899-3908 (1996).

23. Kisselev, A.F. & Goldberg, A.L. Monitoring activity and inhibition of 26S proteasomes with fluorogenic peptide substrates. *Methods Enzymol.* **398**, 364-78 (2005).
24. Niewerth, D. et al. Interferon-gamma-induced upregulation of immunoproteasome subunit assembly overcomes bortezomib resistance in human hematological cell lines. *J. Hematol. Oncol.* **7**, 7 (2014).
25. Blackburn, C. et al. Characterization of a new series of non-covalent proteasome inhibitors with exquisite potency and selectivity for the 20S beta5-subunit. *Biochem. J.* **430**, 461-76 (2010).
26. Lin, G., Tsu, C., Dick, L., Zhou, X.K. & Nathan, C. Distinct specificities of mycobacterium tuberculosis and mammalian proteasomes for N-acetyl tripeptide substrates. *J. Biol. Chem.* **283**, 34423-34431 (2008).
27. Kisselev, A.F. et al. The caspase-like sites of proteasomes, their substrate specificity, new inhibitors and substrates, and allosteric interactions with the trypsin-like Sites. *J. Biol. Chem.* **278**, 35869-35877 (2003).
28. Orłowski, M., Cardozo, C. & Michaud, C. Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex. Properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral amino acids. *Biochemistry* **32**, 1563-1572 (1993).
29. Basler, M. et al. Why the structure but not the activity of the immunoproteasome subunit low molecular mass polypeptide 2 rescues antigen presentation. *J. Immunol.* **189**, 1868-1877 (2012).
30. Nazif, T. & Bogoy, M. Global analysis of proteasomal substrate specificity using positional-scanning libraries of covalent inhibitors. *Proc. Natl. Acad. Sci.* **98**, 2967-72 (2001).
31. Lightcap, E.S. et al. Proteasome inhibition measurements: clinical application. *Clin. Chem.* **46**, 673-683 (2000).
32. Crawford, L.J.A. et al. Comparative selectivity and specificity of the proteasome inhibitors BzLLCCHO, PS-341, and MG-132. *Cancer Res.* **66**, 6379-6386 (2006).
33. Moravec, R.A. et al. Cell-based bioluminescent assays for all three proteasome activities in a homogeneous format. *Anal. Biochem.* **387**, 294-302 (2009).
34. Wilkins, O.M. et al. Cell-line-specific high background in the Proteasome-Glo assay of proteasome trypsin-like activity. *Anal. Biochem* **451**, 1-3 (2014).
35. Vigneron, N., Abi Habib, J. & Van den Eynde, B.J. The capture proteasome assay: A method to measure proteasome activity in vitro. *Anal. Biochem* **482**, 7-15 (2015).
36. Guillaume, B. et al. Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules. *Proc. Natl. Acad. Sci.* **107**, 18599-18604 (2010).
37. Kisselev, A.F., Akopian, T.N., Castillo, V. & Goldberg, A.L. Proteasome active sites allosterically regulate each other, suggesting a cyclical bite-chew mechanism for protein breakdown. *Mol. Cell* **4**, 395-402 (1999).
38. Ping Dou, Q. & A. Zonder, J. Overview of proteasome inhibitor-based anti-cancer therapies: perspective on bortezomib and second generation proteasome inhibitors versus future generation inhibitors of ubiquitin-proteasome System. *Curr. Cancer Drug Targets* **14**, 517-536 (2014).
39. Britton, M. et al. Selective inhibitor of proteasome's caspase-like sites sensitizes cells to specific inhibition of chymotrypsin-like sites. *Chem. Biol.* **16**, 1278-1289 (2009).
40. Kraus, M. et al. The novel β 2-selective proteasome inhibitor LU-102 synergizes with bortezomib and carfilzomib to overcome proteasome inhibitor resistance of myeloma cells. *Haematologica* **100**, 1350-1360 (2015).
41. Wakata, A. et al. Simultaneous fluorescent monitoring of oroteasomal subunit catalysis. *J. Am. Chem. Soc.* **132**, 1578-1582 (2010).
42. Priestman, M.A. et al. Multicolor monitoring of the proteasome's catalytic signature. *ACS Chem. Biol.* **10**, 433-440 (2015).
43. Harris, J.L., Alper, P.B., Li, J., Rechsteiner, M. & Backes, B.J. Substrate specificity of the human proteasome. *Chem. Biol.* **8**, 1131-1141 (2001).
44. Omura, S. et al. Lactacystin, a novel microbial metabolite, induces neuritogenesis of neuroblastoma cells. *J. Antibiot. (Tokyo)* **44**, 113-6 (1991).

45. Fenteany, G., Standaert, R.F., Reichard, G.A., Corey, E.J. & Schreiber, S.L. A beta-lactone related to lactacystin induces neurite outgrowth in a neuroblastoma cell line and inhibits cell cycle progression in an osteosarcoma cell line. *Proc. Natl. Acad. Sci.* **91**, 3358-62 (1994).
46. Fenteany, G. et al. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268**, 726-731 (1995).
47. Craiu, A. et al. Lactacystin and clasto-lactacystin β -lactone modify multiple proteasome β -subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. *J. Biol. Chem.* **272**, 13437-13445 (1997).
48. Bogoy, M. et al. Covalent modification of the active site threonine of proteasomal β subunits and the Escherichia coli homolog HslV by a new class of inhibitors. *Proc. Natl. Acad. Sci.* **94**, 6629-6634 (1997).
49. Bogoy, M. in *Methods in Enzymology* (ed. Raymond, J.D.) 609-622 (Academic Press, 2005).
50. Kessler, B.M. et al. Extended peptide-based inhibitors efficiently target the proteasome and reveal overlapping specificities of the catalytic β -subunits. *Chem. Biol* **8**, 913-929 (2001).
51. Meng, L. et al. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. *Proc. Natl. Acad. Sci.* **96**, 10403-10408 (1999).
52. Sin, N. et al. Total synthesis of the-potent proteasome inhibitor epoxomicin: a useful tool for understanding proteasome biology. *Bioorg. Med. Chem. Lett.* **9**, 2283-2288 (1999).
53. Berkers, C.R. et al. Activity probe for in vivo profiling of the specificity of proteasome inhibitor bortezomib. *Nat. Methods* **2**, 357-362 (2005).
54. Verdoes, M. et al. Acetylene functionalized BODIPY dyes and their application in the synthesis of activity based proteasome probes. *Bioorg. Med. Chem. Lett.* **17**, 6169-6171 (2007).
55. Verdoes, M. et al. A panel of subunit-selective activity-based proteasome probes. *Org. Biomol. Chem.* **8**, 2719-2727 (2010).
56. Verdoes, M. et al. Chemical proteomics profiling of proteasome activity. *Methods Mol. Biol.* **328**, 51-69 (2006).
57. Verdoes, M. et al. A fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo. *Chem. Biol* **13**, 1217-1226 (2006).
58. Berkers, C.R. et al. Profiling proteasome activity in tissue with fluorescent probes. *Mol. Pharm.* **4**, 739-748 (2007).
59. Li, N. et al. Relative quantification of proteasome activity by activity-based protein profiling and LC-MS/MS. *Nat. Protocols* **8**, 1155-1168 (2013).
60. de Bruin, G. et al. Structure-based design of β 1i or β 5i specific inhibitors of human immunoproteasomes. *J. Med. Chem.* **57**, 6197-6209 (2014).
61. Huber, E.M. et al. Systematic analyses of substrate preferences of 20S proteasomes using peptidic epoxyketone inhibitors. *J. Am. Chem. Soc.* **137**, 7835-7842 (2015).
62. Geurink, P.P. et al. Incorporation of non-natural amino acids improves cell permeability and potency of specific inhibitors of proteasome trypsin-like sites. *J. Med. Chem.* **56**, 1262-1275 (2013).
63. de Bruin, G. et al. A set of activity-based probes to visualize human (immuno)proteasome activities. *Angew. Chem. Int. Ed.* **13**, 4199-4203 (2015).
64. Ho, Y.K., Bargagna-Mohan, P., Wehenkel, M., Mohan, R. & Kim, K.-B. LMP2-specific inhibitors: chemical genetic tools for proteasome biology. *Chem. Biol.* **14**, 419-430 (2007).
65. Singh, A.V. et al. PR-924, a selective inhibitor of the immunoproteasome subunit LMP-7, blocks multiple myeloma cell growth both in vitro and in vivo. *Br. J. of Haematol.* **152**, 155-163 (2011).
66. Carmony, K.C. et al. A bright approach to the immunoproteasome: Development of LMP2/ β 1i-specific imaging probes. *Bioorg. Med. Chem.* **20**, 607-613 (2012).
67. Sharma, L.K. et al. Activity-based near-infrared fluorescent probe for LMP7: A chemical proteomics tool for the immunoproteasome in living cells. *ChemBioChem* **13**, 1899-1903 (2012).
68. Carmony, K.C. & Kim, K.B. Activity-based imaging probes of the proteasome. *Cell Biochem. Biophys.* **67**, 91-101 (2013).

