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REVIEW ARTICLE

Activity-based probes for the multicatalytic proteasome

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Proteasomes are multisubunit protease complexes responsible for degrading most intracellular proteins. In addition to removing damaged proteins, they regulate many important cellular processes through the controlled degradation of transcription factors, cell cycle regulators, and enzymes. Eukaryotic proteasomes have three catalytic subunits, β1, β2, and β5, that each has different substrate specificities. Additionally, although we know that diverse cell types express proteasome variants with distinct activity and specificity profiles, the functions of these different pools of proteasomes are not fully understood. Covalent inhibitors of the protease activity of the proteasome have been developed as drugs for hematological malignancies and are currently under investigation for other diseases. Therefore, there is a need for tools that allow direct monitoring of proteasome activity in live cells and tissues. Activity-based probes have proven valuable for biochemical and cell biological studies of the role of individual proteasome subunits, and for evaluating the efficacy and selectivity of proteasome inhibitors. These probes react covalently with the protease active sites, and contain a reporter tag to identify the probe-labeled proteasome subunits. This review will describe the development of broad-spectrum and subunit-specific proteasome activity-based probes, and discuss how these probes have contributed to our understanding of proteasome biology, and to the development of proteasome inhibitors.

Introduction

Proteins are in a dynamic state of synthesis and degradation. Two main mechanisms exist to degrade proteins inside eukaryotic cells: the lysosome and the proteasome. In general, lysosomes digest extracellular or cell surface proteins that are taken up by the cell, and some intracellular proteins that have been engulfed in autophagosomes. However, most targeted degradation of intracellular proteins is carried out by the proteasome. These include damaged or misfolded proteins, transcription factors, cell cycle regulators, and proteins from intracellular pathogens. Proteins are typically targeted to the proteasome by tagging with K48-linked polyubiquitin chains, which are attached to proteins though the action of E1

Abbreviations
ABP, activity-based probe; ABPP, activity-based protein profiling; Ahx, 6-aminohexanoyl; CL, caspase-like; CTL, chymotrypsin-like; DUB, deubiquitinase; EK, epoxyketone; EMT, epithelial–mesenchymal transition; FRET, Förster resonance energy transfer; MS, mass spectrometry; nL, norleucine; TL, trypsin-like; Ub, ubiquitin; UPR, unfolded protein response; UPS, ubiquitin–proteasome system; VS, vinyl sulfone.

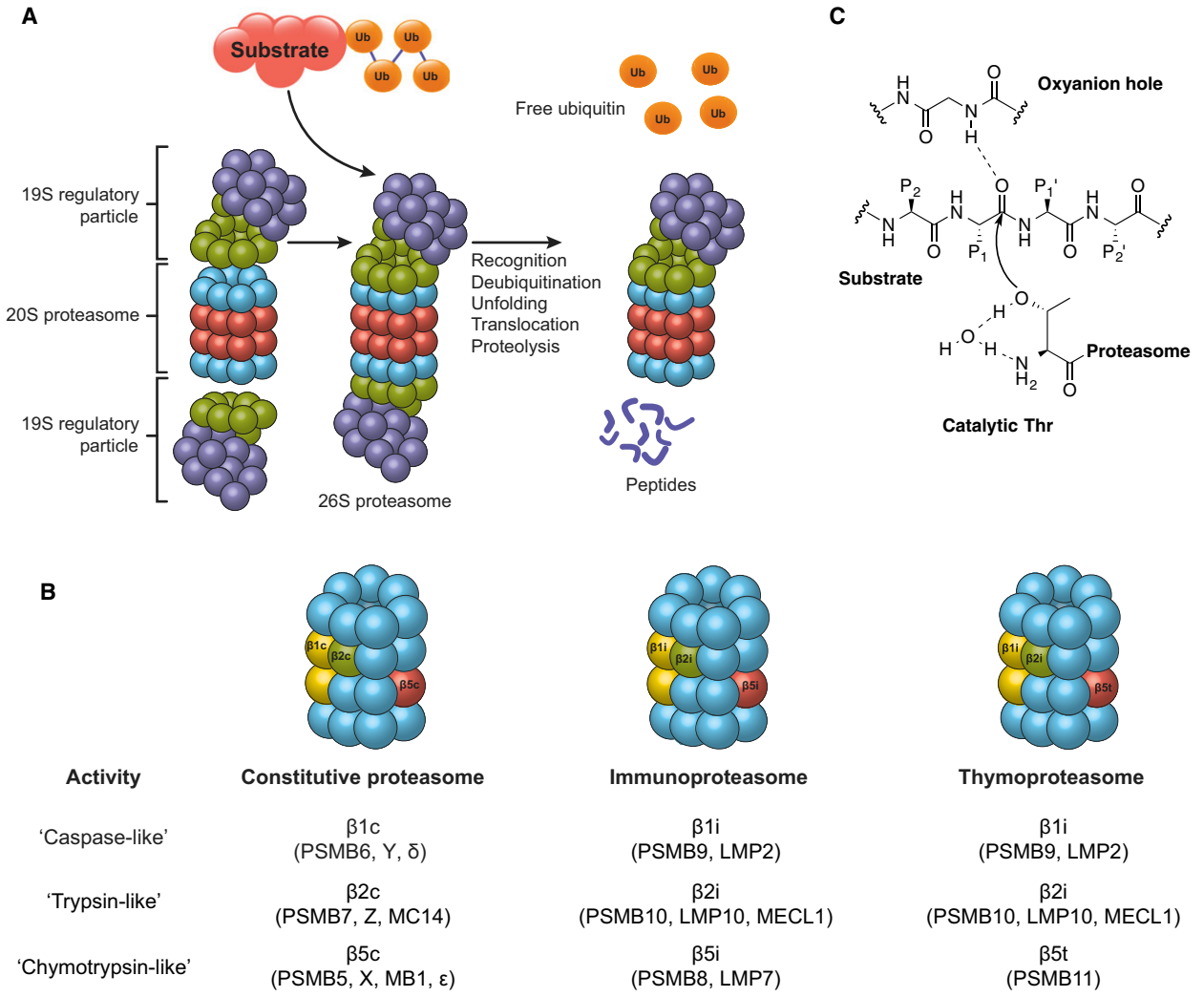
(ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin ligase) enzymes. Proteins can be rescued from proteasome-mediated degradation by deubiquitinases (DUBs). The proteasome, together with the Ub conjugation and deconjugation machinery, is referred to as the ubiquitin–proteasome system (UPS). The UPS not only recycles nonfunctional proteins but also controls a broad range of cellular processes though regulated proteolysis, including transcription, cell cycle progression, antigen presentation, apoptosis, and receptor-mediated endocytosis [1].

The eukaryotic proteasome (26S proteasome) is a multisubunit ATP-dependent protease. It consists of the 20S core particle, which contains multiple protease active sites, and one or two proteasome activators, of which the 19S regulatory particle is the dominant form [2] (Fig. 1A). The 19S cap contains subunits that recognize ubiquitinated proteins, which are then unfolded and deubiquitinated. The unfolded polypeptides are translocated into the 20S core, where proteolysis occurs to produce short peptides. The core particle consists of four rings of seven subunits: two outer rings of α subunits, and two inner rings of β subunits. The α subunits appear to have a regulatory or structural function. In archaea, all α subunits and all β subunits are identical, but higher organisms, including yeast, plants, and animals, have seven distinct α and seven distinct β subunits. Only the β1, β2, and β5 subunits are catalytically active (Fig. 1B). All share an unusual protease mechanism in which the N-terminal threonine hydroxyl group attacks the scissile peptide bond (Fig. 1C). The adjacent primary amine of the same residue acts as a general base, polarizing a nearby water molecule to deprotonate the hydroxyl during catalysis [3]. The β1, β2, and β5 subunits have distinct but overlapping selectivity. Based on experiments using fluorogenic substrates, β1 is referred to as 'caspase-like' (CL) or 'postglutamyl peptide hydrolyzing' (PGPH), β2 as 'trypsin-like' (TL), and β5 as 'chymotrypsin-like' (CTL), although these descriptions are undoubtedly an oversimplification [4].

Specific patterns of catalytic subunits may be expressed and assembled into the core 20S complex in different tissues or after certain stimuli. This enables modulation of proteasome activity as well as changes in the types of peptides produced by the complex. There are also tissue-specific variations of the proteasome [5] (Fig. 1B). Specifically, many cells of the immune system predominantly contain 'immunoproteasomes' in which the constitutively expressed catalytic subunits (β1c, β2c, and β5c) are replaced by interferon-induced immunoproteasome subunits (β1i, β2i, and β5i). In this review, reference to β1, β2, or β5, without the 'c' or 'i' suffix, implies both subunit types. Immunoproteasome expression can also be induced in nonimmune cells by proinflammatory cytokines. The immunoproteasome is thought to produce peptides that are optimized for binding to MHC class I molecules to increase the number of potential epitopes presented on the cell surface [6]. In particular, while β1c accepts peptides with an acidic P1 residue, β1i preferentially processes peptides with hydrophobic P1 residues which are required for efficient binding to MHC class I molecules [7]. The 'thymoproteasome' is a modification of the immunoproteasome, expressed only in the thymus, in which β5i is replaced with β5t. In addition to these three proteasome types, 'mixed' proteasomes can also form, containing both constitutive and immunoproteasome subunits. The formation and role of mixed proteasomes remains poorly understood [8].

Application of probes to functional studies of proteasome activity

Assessing the activity of individual proteasome subunits is important in order to understand how the activity of the complex is regulated, to distinguish the roles of individual subunits, and to assess the efficacy and selectivity of proteasome inhibitors. Direct measurement of the overall abundance of individual proteasome subunits is a poor surrogate for activity because each of the catalytic subunits is expressed in an inactive form and a propeptide must be removed to generate the N-terminal catalytic Thr residue after assembly of the complex [9]. Additionally, proteasome activity is regulated by multiple proteins and cofactors [2]. Therefore, the activity of the proteasome is often assessed using peptide substrates or substrate mimics. Fluorogenic peptides are most commonly used for this purpose. These tri- or tetrapeptides contain a fluorophore (typically 7-amino-4-methylcoumarin, AMC) at the C terminus, which is nonfluorescent when linked to the peptide but becomes fluorescent when released by proteolytic cleavage [10]. Fluorogenic peptides with specificity for trypsin-like, chymotrypsin-like, or caspase-like activity are commercially available and can be used for kinetic studies of three main types of activities of the proteasome. However, the potential for 'selective' substrates to be processed by multiple subunits limits their use for studying the activity of individual subunits. Furthermore, substrates are not sufficiently selective to distinguish constitutive from immunoproteasome activity due to the highly similar specificity patterns for these subunits. Finally, extended fluorogenic peptide substrates are usually not cell permeable and their use in lysates (rather than



Activity	Constitutive proteasome	Immunoproteasome	Thymoproteasome
'Caspase-like'	β1c (PSMB6, Y, δ)	β1i (PSMB9, LMP2)	β1i (PSMB9, LMP2)
'Trypsin-like'	β2c (PSMB7, Z, MC14)	β2i (PSMB10, LMP10, MECL1)	β2i (PSMB10, LMP10, MECL1)
'Chymotrypsin-like'	β5c (PSMB5, X, MB1, ε)	β5i (PSMB8, LMP7)	β5t (PSMB11)

Fig. 1. The proteasome—structure and catalysis. (A) The proteasome consists of the 20S core, which possesses protease activity, and one or more regulatory subunits, such as the 19S regulatory particle. Ubiquitinated proteins are recognized by the 19S cap where they are deubiquitinated, unfolded, and translocated into 20S, where proteolysis occurs; (B) Tissue-specific forms of the 20S core, with catalytic subunits shown. Multiple names are used for the same subunits (synonyms are shown in brackets). The principal catalytic activity type (based on P1 residue preferences) is also indicated; (C) Catalytic mechanism of the catalytic β-subunits of the proteasome.

with purified proteasomes) is often complicated by their propensity to be processed by other cellular proteases.

Activity-based probes (APBs) overcome many of the limitations of fluorogenic peptide substrates by reacting covalently and irreversibly with the proteasome active sites through an enzyme-catalyzed reaction. ABPs consist of 'three Rs': a reactive group, a recognition element, and a reporter tag [11]. For probes targeting Thr proteases, and other enzymes with active-site nucleophiles, the reactive group is an electrophile that forms a covalent bond to the active-site nucleophile. The recognition element typically mimics a substrate, and therefore can be made to selectively target an enzyme of interest, while the reporter tag is used to detect the labeled product of the reaction. ABPs therefore provide a significant advantage over substrate-based probes (such as fluorogenic peptides) as they allow direct identification of targets as well as direct monitoring of activity of individual subunits in the proteasome complex. As described below, ABPs have been developed that can distinguish all six types of constitutive and immunoproteasome subunits. They also show excellent selectivity for the proteasome over other proteases and in some instances can be used in live cells.

In this review, we will highlight advances in the development of broad-spectrum and subunit-selective ABPs that target the proteasome. We will focus on the aspects of probe design that confer selectivity, and make them particularly suited for certain applications (e.g., in-cell detection or affinity purification). We will also discuss the applications of these probes in basic science and clinical research. This is not meant to be an exhaustive list of all studies using proteasome ABPs; rather, we wish to illustrate a range of highly valuable applications of these probes.

Proteasome ABPs

Proteasome ABPs are mechanism-dependent inhibitors that are modified to enable detection of the labeled catalytic subunits. With a few exceptions, all proteasome ABPs share a similar design (Fig. 2A), consisting of:

(a) a reactive group ('warhead'), typically an epoxyketone (EK) or vinyl sulfone (VS), at the C terminus (Fig. 2B);
(b) a tri- or tetrapeptide recognition element;
(c) a reporter tag for detection (often a fluorophore), typically appended at the N terminus via a linker.

Consequently, the probes are frequently notated in the form label–linker–recognition element–warhead (e.g., BODIPY–Ahx_3–L_3–VS), or label–inhibitor (e.g., BODIPY–epoxomicin). Some laboratories apply their own systematic names: compounds from the Norris Cotton Cancer Center are NCxxy, where xx is a two-digit number, and y is the subunit inhibited (e.g., 1 for the β1 subunit), while compounds from Leiden University are LUxxy. However, nonsystematic names are also used (e.g., MV127), and probes may be referred to by different names in different publications, sometimes without reference to structures, making the literature somewhat confusing. For reference, chemical structures, probe names, and references are shown in Figs 3–6.

It is helpful to divide proteasome ABPs (and proteasome inhibitors) into two categories: 'broad-spectrum' (Fig. 3), which are reactive toward most proteasome subunits, and 'subunit-selective' (Figs 4–6), which show a strong preference for a single subunit type. This should not be regarded as a hard distinction, as broad-spectrum probes will not react at an identical rate with all subunits, and 'subunit-selective' probes often inhibit other subunits when used at higher concentrations. Subunit-selective probes which can differentiate between constitutive and immunoproteasome subunits of a given type have only been reported recently (see below). Selectivity is often dependent on probe concentration and reaction time, which need to be optimized for the probe and system of interest.

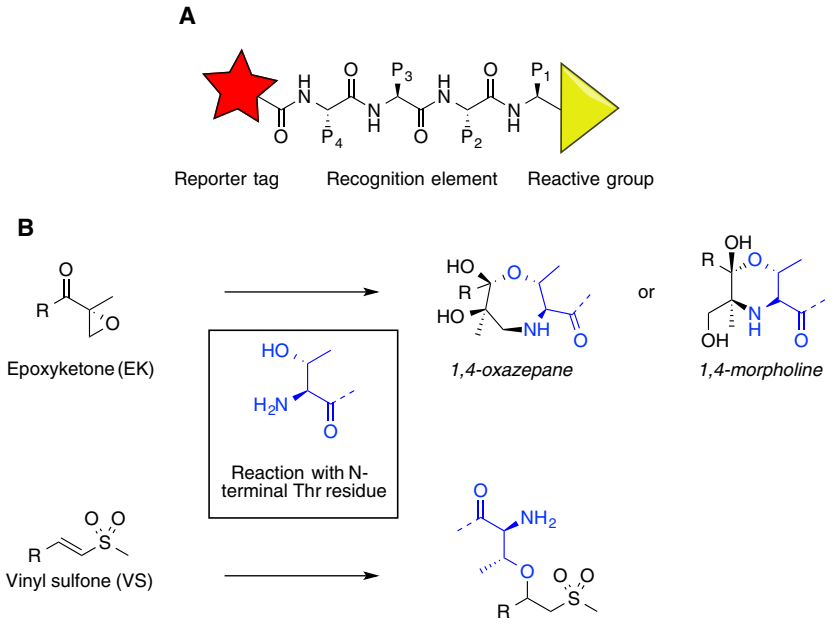
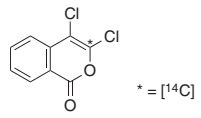
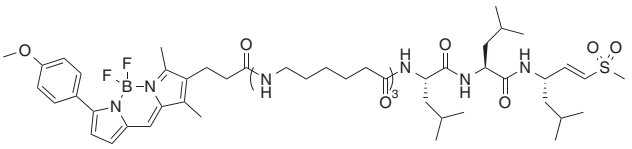


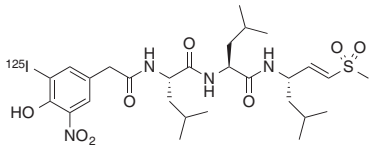
Fig. 2. (A) General structure of an activity-based probe targeting the proteasome; (B) Reaction of epoxyketone (EK) and vinyl sulfone (VS) reactive groups with catalytic Thr residues of proteasome. Epoxyketones were initially believed to form cyclic morpholine adducts with the N-terminal Thr [71], but recent high-resolution crystal structures suggest that the 1,4-oxazepane may be the preferred adduct [72].



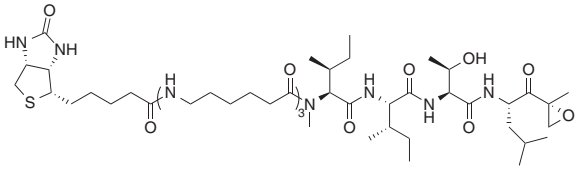
[^{14}C]-3,4-dichloroisocoumarin [12]



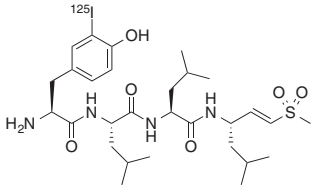
BODIPY(TMR)-Ahx$_3$-L$_3$-VS (MV151) [22]



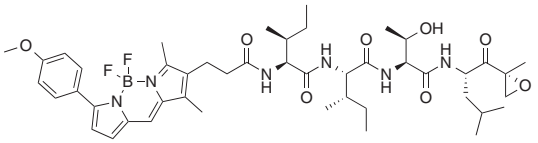
[^{125}I]-NIP-L$_3$-VS ([^{125}I]-NLVS) [14]



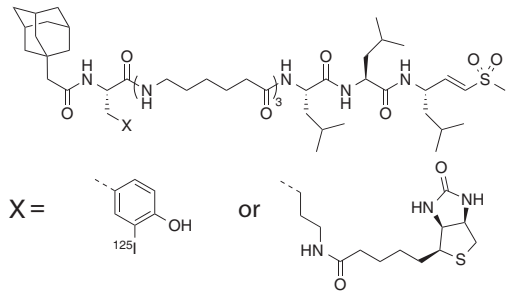
Biotin-Ahx$_3$-epoxomicin [28]



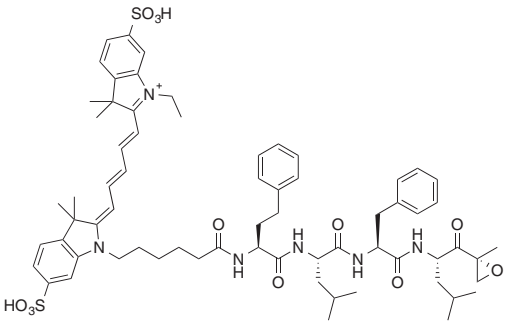
[^{125}I]YL$_3$-VS [15]



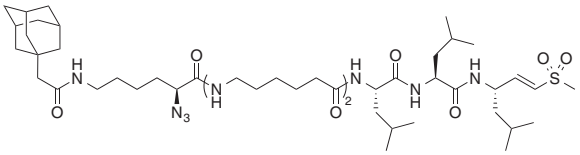
BODIPY(TMR)-epoxomicin (MBV003) [26]



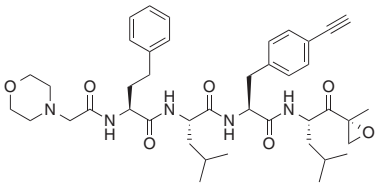
Ada-[^{125}I]Y-Ahx$_3$-L$_3$-VS
and
Ada-K(biotin)-Ahx$_3$-L$_3$-VS [17]



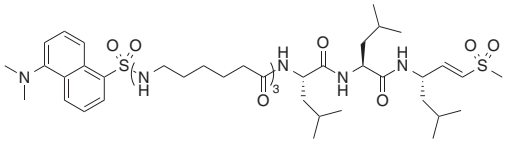
BMV037 [31]



Ada-Ahx(α-N$_3$)-Ahx$_2$-L$_3$-VS [18]

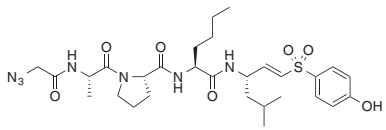


Alkynyl-carfilzomib (OP-829) [32]

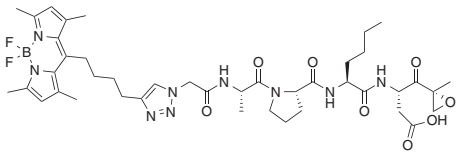


Dansyl-Ahx$_3$-L$_3$-VS (DALVS) [21]

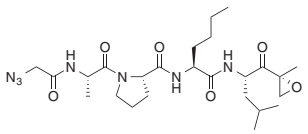
Fig. 3. Structures of broad-spectrum proteasome ABPs.



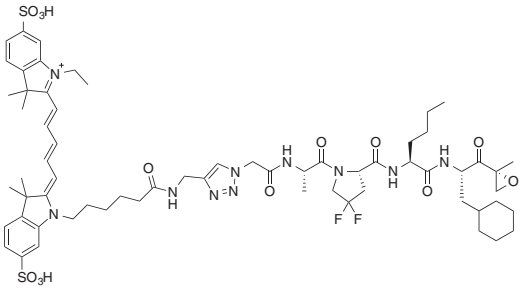
N_3-APnLL-VS-PhOH [37]



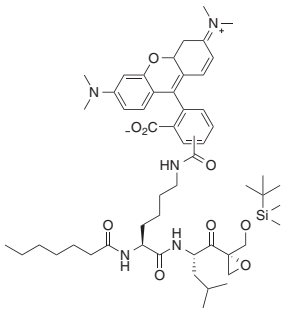
BODIPY(FL)-LU001c (β1c-selective) [40]



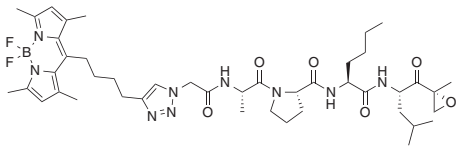
az-NC001 [38]



Cy5-LU001i (β1i-selective) [40]

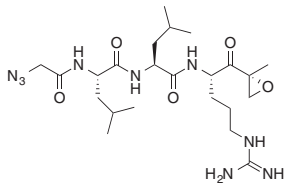


UK101-Fluor (β1i-selective) [41]

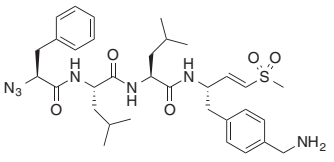


LW124 (BODIPY-NC001) [39]
(Cy5 analog also prepared [35])

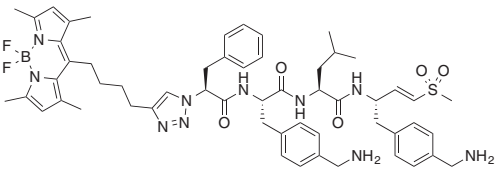
Fig. 4. Structures of β1-selective probes.



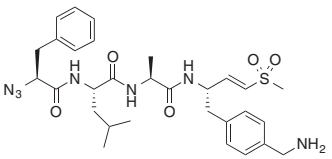
az-NC002 [42]



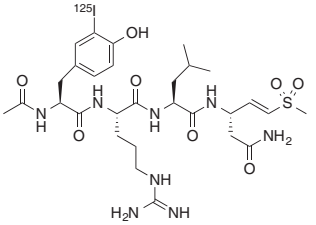
LU102 [43]



BODIPY(FL)-LU112 [35, 43]



LU002c (modestly β2c-selective) [35]

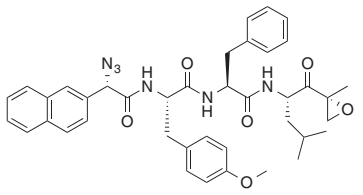


Ac-[^{125}I]YRLN-VS [16]

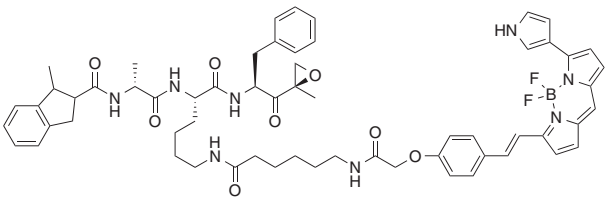
Fig. 5. Structures of β2-selective probes.

Broad-spectrum ABPs

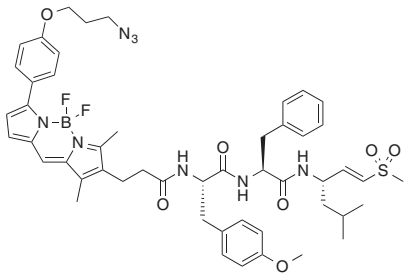
The earliest reported ABPs for the proteasome used a radiolabel for detection. The first reported probe, [^{14}C]-3,4-dichloroisocoumarin, was shown to label proteasome subunits. However, it also reacted broadly with serine and threonine proteases and so can only be effectively used with purified proteasomes [12,13]. The



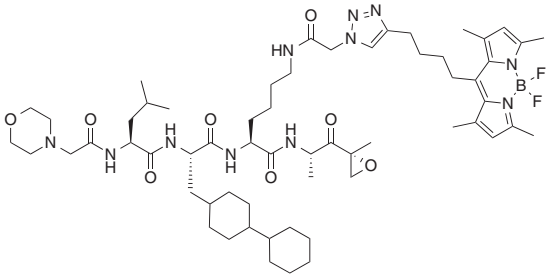
az-NC005 [38]



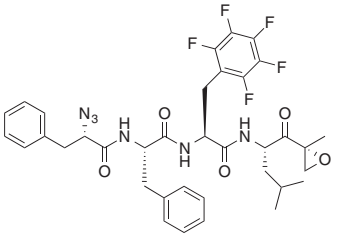
LKS01-B650 (β5i-selective) [73]



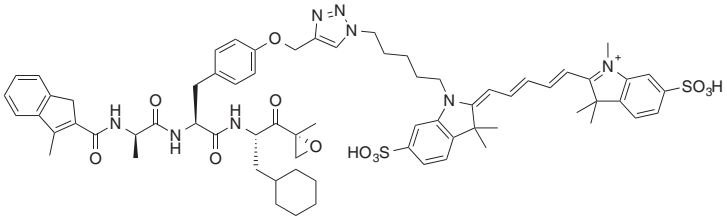
BODIPY-NC005-VS (MVB127) [39]



BODIPY(FL)-LU015c (β5c-selective) [40]



LU005 [74]
BODIPY analog also prepared (PR592) [74]



Cy5-LU035i (β5i-selective) [40]

Fig. 6. Structures of β5-selective probes.

first proteasome-selective probe, [^{125}I]-NIP-L_3-VS (NLVS), was reported by Bogyo *et al.* [14]. This cell-permeable compound showed good selectivity for the proteasome over other proteins. Remarkably, prior to this report, the VS warhead was thought to be highly selective for Cys proteases. Resolution of the ABP-labeled proteasome β-subunits by 1D SDS/PAGE failed to distinguish all the catalytic subunits, but use of 2D-SDS/PAGE enabled resolution of the subunits, including β1i and β5i. Replacing the nitrophenyl N-terminal cap with radioiodinated Tyr gave a more reactive probe ([^{125}I]-YL_3-VS) which labeled all six constitutive and immunoproteasome catalytic subunits [15]. More generally, a hydrophobic P4 residue was found to be required for activity, and changes in the distal portion of the probe could substantially affect selectivity [16]. Building on these observations, Kesser *et al.* designed an 'extended' probe, Ada-[^{125}I]Y-Ahx_3-L_3-VS. The large N-terminal extension increased potency and resulted in a probe that could label all catalytic subunits of both the constitutive and immunoproteasome. A biotin label was also introduced, but neither biotinylated nor radiolabeled probes were cell-permeable [17], in contrast to the parent inhibitor Ada-Ahx_3-L_3-VS.

To overcome the problem of cell permeability, Ovaa *et al.* [18] turned to a two-step labeling procedure. An azide-containing analog of the inhibitor Ada-Ahx_3-L_3-VS labeled proteasome subunits in live cells. After lysis, a biotin label was attached by Staudinger–Bertozzi ligation [19], and labeled proteasome subunits were detected by anti-biotin western blot. Alternatively, biotin-streptavidin affinity purification followed by tandem MS could be used to detect and identify labeled proteins [20]. Ovaa and coworkers subsequently reported a cell-permeable probe, dansyl-Ahx_3-L_3-VS (DALVS), that could be detected directly by immunoblot for the dansyl hapten [21]. However, the

dansyl group is excited by near-UV light and fluoresces with a low quantum yield. These properties make it unsuitable for sensitive detection of proteasome activity in cells by fluorescence microscopy, or by in-gel fluorescence detection (which could improve resolution and sensitivity by eliminating transfer and immunoblot steps). MV151 overcomes these shortcomings by appending the BODIPY(TMR) fluorophore to the same Ahx_3-L_3-VS inhibitory scaffold [22,23]. BODIPY-containing probes are much more sensitive than the dansyl-containing probe, and can be used to label proteasome subunits in lysate, whole cells or even *in vivo*. They are also suitable for fluorescence microscopy and flow cytometry experiments [24]. Verdoes *et al.* [25] also developed an azide-containing analog of MV151, to which biotin can be attached after labeling. This allows both visualization of proteasome reactivity and enrichment of probe-labeled proteins.

Broad-spectrum ABPs containing recognition elements other than L_3-VS have also been reported. Both fluorophore- [26,27] and biotin-tagged [28,29] analogs of the epoxyketone natural product epoxomicin have been prepared. Another proteasome-inhibiting natural product, syringolin A, has been prepared as a fluorophore conjugate [30]. For both compounds, the tagged analogs allowed identification of the proteasome as the biological target. These probes were also used to determine which subunits in the proteasome complex are targeted by each compound. These studies demonstrated that epoxomicin largely inhibits the β1 and β5 activity of the proteasome, while the preferred target of syringolin A is the β5 subunit, with somewhat weaker activity toward β2 and little effect on β1. Fluorophore- [31] and alkyne-tagged [32] analogs of the clinically approved proteasome inhibitor carfilzomib have also been reported, and the latter has been used to identify potential off-targets of the drug [32].

The profile of probe reactivity is often dependent on the type of samples being analyzed as well as the conditions use for probe labeling. For example, experiments using multiple different classes of cell-permeable probes have shown that there is a substantial difference in the labeling profile between live cells and lysates, perhaps due to partial dissociation of the proteasome during lysis [18,21,22]. These observations highlight the importance of measuring activity in live cells where possible. Additionally, probe reactivity can vary between organisms. In mammalian cells, MV151 is proteasome-selective and pan-subunit reactive, whereas in *Arabidopsis*, it labels multiple cysteine proteases in addition to the proteasome [33], and in *Plasmodium falciparum*, MV151 shows selective labeling of the β2 and β5 subunits [31]. It is therefore important to understand the specificity profiles of probes in the system of interest before using them to address proteasome function.

Subunit-selective ABPs

One of the major drawbacks in using broad-spectrum proteasome ABPs is the difficulty in distinguishing individual proteasome subunits. Notably, the β1c, β1i, β5c, and β5i subunits usually overlap on standard SDS/PAGE gels. 2D-SDS/PAGE provides better resolution, but due to the tedious nature of the analysis, is not suitable for analyzing multiple samples simultaneously. Subunit-selective probes can provide information about a specific subunit of interest, or alternatively, can be multiplexed using probes with different reporters to analyze multiple subunits simultaneously [34,35].

β1-selective probes (Fig. 4) typically employ recognition elements based on the Ala-Pro-norLeu-Leu sequence, which was reported to be a β1 substrate by Kisselev *et al.* [36]. The first β1-selective probe (N_3-APnLL-VS-PhOH) [37] used a phenolic vinyl sulfone warhead, previously shown to preferentially inhibit β1 sites [15]. Subsequently, an epoxyketone (NC-001) with the same peptide sequence was found to be more potent, without substantially affecting selectivity [38]. Both azide-tagged [37] and fluorophore-tagged [35,39] analogs of NC-001 have been reported. Modifications of the recognition element allow generation of either β1c- or β1i-selective probes (BODIPY(FL)-LU001c and Cy5-LU001i) [35,40]. A slightly different scaffold was used in the β1i-selective fluorescein-labeled probe UK101-Fluor, which uses an acylated lysine in P2, and a large hydrophobic moiety attached to the epoxide to mimic the P1′ position. UK101-Fluor and its BODIPY analog were shown to be suitable for imaging β1i activity in live cells by fluorescence microscopy [41].

Most β2-selective probes (Fig. 5) employ a basic amino acid in the P1 position, as expected from their trypsin-like substrate selectivity. Interestingly, a basic P3 residue is also favored by this subunit. The azide-tagged epoxyketone probe az-NC-002 (az-LLR-EK) selectively labels the β2 subunits in cells, as confirmed by gel-based assay, and by tandem MS after biotinylation and affinity purification [42]. The RLR recognition element was more active against purified proteasomes, but less active in cells, presumably due to poor cell permeability. Geurink *et al.* subsequently reported that vinyl sulfones are more potent and selective toward the β2 subunits than their epoxyketone analogs [43]. Furthermore, replacement of arginine with the non-natural amino acid 4-(aminomethyl) phenylalanine in either the P1 site (LU012) [43] or P1

and P3 sites (LU112) further improved affinity and chemical stability. These compounds have an azide handle that can be used for attachment of biotin or a fluorophore after labeling. However, introduction of the fluorophore prior to labeling reduced selectivity over the β5 subunit [43]. Despite the trypsin-like selectivity of β5, a basic residue in the P1 site is not required. In the first example of a subunit-selective probe, Nazif and Bogyo showed that Ac-[^{125}I]YRLN-VS selectively labeled β2, highlighting the importance of the P3 residue [16]. Recently, a β2c inhibitor bearing an azide group has been reported (LU-002c), but selectivity over the β2i subunit is modest [35].

β5-selective probes (Fig. 6) have a preference for hydrophobic and aromatic residues in most positions, consistent with the chymotrypsin-like activity of this subunit. Both VS and EK warheads have been evaluated, with EKs typically showing greater reactivity but reduced selectivity over β1 and β2 subunits [39,44]. Modification in the P1 and P3 positions can confer selectivity for either the β5c or β5i subunit: β5c favors a small hydrophobic group in P1 and a large hydrophobic group in P3, while β5i favors the reverse arrangement [35,40,45].

It is notable that for all three subunit types, incorporation of non-natural amino acids has been essential to obtain highly subunit-selective inhibitors and probes. This is consistent with the observation that the individual proteasome subunits, in the context of the 20S core complex, have overlapping substrate specificities [17]. Selective probes must therefore explore chemical space outside of that provided by naturally occurring amino acids.

Uses of proteasome ABPs

Activity-based probes have played an important role during the development of proteasome inhibitors for cancer therapy. As described below, they were used to identify the epoxyketone moiety (now used in the drug carflizomib) as a selective proteasome-targeting electrophile, to study the selectivity and pharmacodynamics of proteasome inhibitors, and to examine acquired resistance to proteasome inhibitor treatment. Other examples described below highlight how ABPs have been used to identify the proteasome as a potential target in noncancer indications, and to study basic proteasome biology.

Target identification

Several natural products were identified as proteasome inhibitors through their derivatization into ABPs. Lactacystin, the first natural product proteasome inhibitor identified, was prepared in tritiated form and used to track labeled proteins by fractionation, followed by Edman degradation [46]. This showed that the compound labeled the β5c and β5i subunits on their N-terminal threonine residues, helping to confirm that the N-terminal threonine is indeed the catalytic residue. Lactacystin is believed to cyclize to the β-lactone (Ormulide), which is then highly reactive toward the proteasome [47]. Subsequently, more potent β-lactone inhibitors have been identified, including salinosporamide A (marizomib), which is currently in phase I clinical trials.

The epoxyketone warhead, widely used in inhibitors, probes, and the drug carfilzomib, was first identified as a potent proteasome-targeting electrophile in the natural products epoxomicin and eponemycin [28,29,48,49]. Biotinylated variants of these compounds were prepared in order to isolate their biological targets, revealing that epoxomicin targets the β2 and β5 proteasome subunits. More recent studies, using more sensitive MS techniques, showed that biotin-epoxomicin labels all six catalytic β subunits [26,50]. Using biotinylated probes (for affinity purification followed by tandem MS to identify proteins) avoids the hazards of radioactivity and tedious fractionation used to identify targets.

Syringolin A (SylA) is a virulence factor produced by a strain of the bacterium *Pseudomonas syringae* that infects plants. It was initially shown to inhibit the eukaryotic constitutive proteasome based on experiments with fluorogenic substrates [51], but a fluorescently tagged analog, Rh-SylA, was used to examine the its selectivity. These experiments revealed that SyrA targets all proteasome subunits, with a preference for the β5 and β2 subunits over β1 [52]. In mammalian cells, the compound also reacts with immunoproteasome subunits [30].

Labeled versions of synthetic proteasome inhibitors have also been used to investigate proteasome selectivity. This has the advantage over competitive activity-based protein profiling (competitive ABPP, described below) of identifying nonproteasomal targets of the inhibitors. For example, azide-modified analogs of the subunit-specific inhibitors NC-001, NC-002, and NC-005 were used to demonstrate that the compounds were highly specific for the proteasome [38,42]. The small azide label had minimal effect on activity, unlike larger fluorophore labels that can adversely affect activity or selectivity [43]. However, labeling was inferred from an anti-biotin blot, which lacks sensitivity. A more recent study employed an alkyne-modified analog of carfilzomib, combined with streptavidin-biotin

affinity purification and tandem MS, to identify off-target activity in cells. Only two nonproteasomal proteins (CYP27A1 and GSTO1) were confidently identified as carfilzomib targets. These experiments confirm the impressive selectivity of carfilzomib for its therapeutic targets, despite its reactive, electrophilic warhead [32].

Competitive ABPP (compound screening and assessing selectivity)

Labeled ABPs for the proteasome are valuable tools for determining the selectivity of nonlabeled inhibitors, and for screening to identify novel subunit-selective inhibitors. In a typical competitive ABPP experiment, cells or lysate are first treated with a compound of interest, and then treated with a probe to label residual proteasome subunit activities. A reduction in probe labeling relative to an untreated control indicates that a compound targets that subunit.

In an important early application of competitive ABPP, Berkers *et al.* [21] used the probe DALVS to assess the activity of bortezomib toward different proteasome subunits in live cells. Bortezomib was initially designed as an inhibitor of β5 (chymotrypsin-like) activity [53,54]. However, 20 nM bortezomib completely inhibited labeling of β5 and β1 subunits in MM.1S (multiple myeloma) cells after 2 h, with little effect on β2 activity, thus identifying the β1 subunit as an additional target of bortezomib. Several other reports using DALVS [55,56] or Ada-[^{125}I]-Ahx$_3$-L$_3$-VS [57] confirmed the selectivity of bortezomib toward the β1 and β5 subunits. DALVS was also used to monitor proteasome activity in PBMCs from a multiple myeloma patient receiving bortezomib treatment. β1 and β5 labeling was largely abolished 1 h post-treatment, but β2 labeling was also somewhat reduced. By 72 h, full proteasome activity was restored [58]. It should be noted that although bortezomib is a reversible inhibitor of the proteasome, its off-rate is very slow, and therefore it can compete for proteasome active sites with irreversible covalent probes. Competitive ABPP is unlikely to be suitable for evaluating the selectivity of reversible proteasome inhibitors with fast off-rates, such as the recently approved ixazomib (MLN9708) [59].

Competitive ABPP has also been used to screen for novel proteasome inhibitors. Using broad-spectrum and substrate-specific ABPs, Li *et al.* demonstrated that *Plasmodium* and human proteasomes show differences in selectivity, and exploited these differences to identify compounds that selectively inhibit *Plasmodium* over human proteasome. While β5 inhibition is able to effectively block parasite replication, β2 inhibitors are less effective as a single agent but synergize with the antimalarial drug dihydroartemisinin. A combined β2/β5 inhibitor showed single-agent efficacy in a rodent model of malaria [31,60].

As noted above, a combination of subunit-specific ABPs with different labels can increase the resolution of individual subunits in gel-based detection methods. This can be valuable for characterizing compound selectivity in competitive ABPP assays. Li *et al.* report a detailed protocol for quantifying the proteasome activity of all constitutive and immunoproteasome subunits using three cell-permeable fluorescently labeled probes: LW124 (β1), MVB127 (β5), and MVB003 (nonspecific). LW124 and MVB127 allow good resolution of β1c, β1i, β5c, and β5i activity, while the two β2 subunits are easily resolved with MVB003. Competitive ABPP demonstrated that bortezomib is a more potent inhibitor of β1i than β1c [50]. This selectivity could not have been detected with nonsubunit-specific probes, or with fluorogenic substrates (which cannot distinguish between constitutive and immunoproteasome activity). In a more recent publication from the same laboratory, the authors applied a cocktail of three subunit-selective probes, Cy5-NC-001 (β1), BODIPY(FL)-LU-112 (β2), and MVB127 (β5), to resolve all six subunits simultaneously in a gel-based assay. This assay was then used to screen for new subunit-selective inhibitors, leading to the identification of several compounds with selectivity for individual constitutive or immunoproteasome subunits [35,45].

Profiling proteasome activity across different conditions or stimuli

Proteasome ABPs have been particularly valuable in assessing how proteasome activity responds to changes in cellular conditions. Several studies have examined how proteasome activity changes after prolonged treatment with bortezomib, in an effort to understand mechanisms of resistance to proteasome inhibitors. Typically, cells are cultured with gradually increasing concentrations of drug until they are able to tolerate concentrations that kill the parental line, and then an ABP is used to assess proteasome activity. Using the DALVS probe, Kraus *et al.* observed that overall proteasome activity was increased in bortezomib-adapted HL-60 (AML) cells. This included the β2 subunit, which is not a target of bortezomib. Furthermore, bortezomib showed little inhibitory effect on proteasome activity in adapted cells, while NIP-L$_3$-VS retained activity [58]. Other studies employing MV151 [61] or Rh-SylA [30] observed similar changes across adapted cell lines from different cancer types, including

myeloma (the primary indication for bortezomib). Increased proteasome activity was coupled with an overall decrease in protein synthesis [61]. Bortezomib typically increases the amount of misfolded proteins, triggering the unfolded protein response (UPR) leading to apoptosis [62]. These results therefore suggest that cancer cells can become resistant to bortezomib by reducing protein synthesis and increasing protein degradation, to avoid triggering the UPR [61]. Interestingly, a β2-selective inhibitor in combination with β1/β5-targeting bortezomib or carfilzomib could restore sensitivity in resistant cells, suggesting that β2 inhibitors may overcome resistance to current proteasome inhibitors [63].

Aside from studying drug resistance mechanisms, ABPs have been used to characterize changes in proteasome activity during other disease-relevant processes. For example, using both nonselective and subunit-selective ABPs, Kammerl *et al.* observed a reduction in both immunoproteasome activity and MHC class I antigen presentation in response to cigarette smoke. As immunoproteasome activity is a rate-limiting step for MHC class I antigen presentation, the authors speculated that cigarette smoke-impaired immunoproteasome function reduces MHC I-driven immune responses, making smokers more susceptible to viral lung infections [64]. ABP experiments have also suggested a role for the proteasome in epithelial–mesenchymal transition (EMT), an important developmental process that is also associated with metastasis [65]. A panel of fluorescent ABPs showed that β2c and β5c activity, but not β1c activity, were reduced during EMT. The authors subsequently showed that selective inhibition of β2 and β5 subunits promoted EMT and self-renewal. These findings suggest that proteasome inhibitors may, paradoxically, promote survival and metastasis in some cancer types. This contrasts with earlier studies using bortezomib, which found that proteasome inhibition suppressed metastasis [66]. Further study is required to clarify this potentially important effect of proteasome inhibitors.

Taken together, these experiments illustrate the power of ABPs to identify roles for the proteasome in the onset and progression of disease as well as in the response to treatment. The combination of ABPs with subunit-selective inhibitors is particularly valuable, as hypotheses about the involvement of particular proteasome subunits in a process can readily be tested with inhibitors.

Analyzing proteasome composition

How the makeup and activity of proteasome subunits affects proteasome function is not fully understood. Techniques to identify the active subunits present in specific proteasome populations in a cell, or within an individual proteasome complex, will help to define the relationship between proteasome composition and activity. Biotin- and BODIPY-epoxomicin probes were used to demonstrate that while the β5t subunit is catalytically active, it has distinct substrate specify from the β5c or β5i subunits. Active β5t could be detected in the thymus from young and adult mice, but in no other tissues. ABPs were uniquely suited for these studies, as the β5t subunit activity could not be analyzed with fluorogenic substrates (β5c/β5i-selective substrates are not cleaved by β5t, and pan-reactive substrates do not distinguish between subunits) [26].

Several groups have developed methods aimed at profiling proteasome composition. Western blotting, shotgun proteomics, or the ABPs described above can provide information about which subunits are present or active in a cell of interest, but cannot reveal the subunit composition of intact proteasomes. ABPs modified with a photocrosslinking group were reported by Geurnik, which can be used to isolate proteins found in proximity to active subunits. However, very few proteins were identified by this method [67]. Park *et al.* reported a FRET-based approach, where pairs of β1, β2, or β5-selective probes were tagged with FRET donor–acceptor pairs. A FRET signal between, for example, a Cy3-labeled β1 probe and a Cy5-labeled β5 probe would indicate that the β1 and β5 subunits are present in the same proteasome complex [68]. However, this method was hampered by the lack of probes that were able to distinguish constitutive from immunoproteasome subunits. Using a more extensive panel of selective probes, de Bruin *et al.* [40] identified populations of mixed proteasomes containing, for example, both β1i and β5c subunits.

Perspective

Far from simply being a 'trash can' for damaged proteins, the proteasome is a tightly regulated, heterogeneous protease complex with roles in controlling many cellular processes. However, how proteasome composition affects its activity is incompletely understood. The past two decades have produced a veritable 'toolbox' of ABPs to study the activity of all constitutive and immunoproteasome subunits, and also to identify which subunits are present simultaneously in the same complex. These tools will be useful to understand how proteasome composition and activity responds to stimuli, such as cytokines or infection. In particular, the FRET assay reported by de Bruin *et al.* is likely to be valuable for future studies of mixed proteasomes,

whose function in protein homeostasis and antigenic peptide production is currently unclear [40].

The proteasome is now a clinically validated target for several hematological cancers, and ABPs have played an important role in the development of these drugs, as described above. Recent work has identified parasite proteasomes as novel targets in infectious disease, including malaria, leishmaniasis, Chagas disease, and trypanosomiasis [60,69]. The mycobacterial proteasome has also been investigated as a therapeutic target, and inhibitors selective for the *Mycobacterium tuberculosis* proteasome over the human proteasome have been developed [70]. In their work on the *Plasmodium* proteasome as an antimalarial target, Li *et al*. made extensive use of ABPs to identify subunit-selective inhibitors of the *Plasmodium* proteasome that showed selectivity over human proteasomes. ABPs have the potential to identify differences between pathogen and host proteasomes, and can thus be used to investigate the therapeutic potential of proteasome inhibitors in infectious disease. More generally, ABPs are likely to prove to be useful tools to investigate the proteasome as a target for other nononcology indications.

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Author contributions

DSH, JAF, IEW, and MB discussed and developed the concept of the review and edited the manuscript. DSH wrote the review.

References

1 Ciechanover A (2005) Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat Rev Mol Cell Biol* **6**, 79–87.

2 Schmidt M & Finley D (2014) Regulation of proteasome activity in health and disease. *Biochim Biophys Acta* **1843**, 13–25.

3 Löwe J, Stock D, Jap B, Zwickl P, Baumeister W & Huber R (1995) Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution. *Science* **268**, 533–539.

4 Bensinger D, Neumann T, Scholz C, Voss C, Knorr S, Kuckelkorn U, Hamacher K, Kloetzel P-M & Schmidt B (2016) Elastase-like activity is dominant to chymotrypsin-like activity in 20s proteasome's β5 catalytic subunit. *ACS Chem Biol* **11**, 1800–1804.

5 Kniepert A & Groettrup M (2014) The unique functions of tissue-specific proteasomes. *Trends Biochem Sci* **39**, 17–24.

6 Groettrup M, Kirk CJ & Basler M (2010) Proteasomes in immune cells: more than peptide producers? *Nat Rev Immunol* **10**, 73–78.

7 Huber EM, Basler M, Schwab R, Heinemeyer W, Kirk CJ, Groettrup M & Groll M (2012) Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. *Cell* **148**, 727–738.

8 Dahlmann B, Ruppert T, Kuehn L, Merforth S & Kloetzel PM (2000) Different proteasome subtypes in a single tissue exhibit different enzymatic properties. *J Mol Biol* **303**, 643–653.

9 Schmidt M & Kloetzel PM (1997) Biogenesis of eukaryotic 20S proteasomes: the complex maturation pathway of a complex enzyme. *FASEB J* **11**, 1235–1243.

10 Kisselev AF & Goldberg AL (2005) Monitoring activity and inhibition of 26S proteasomes with fluorogenic peptide substrates. In *Methods in Enzymology* (Deshaies RJ, ed.), pp. 364–378. Academic Press, Cambridge, MA.

11 Sanman LE & Bogyo M (2014) Activity-based profiling of proteases. *Annu Rev Biochem* **83**, 249–273.

12 Orlowski M, Cardozo C, Eleuteri AM, Kohanski R, Kam CM & Powers JC (1997) Reactions of [^{14}C]-3,4-dichloroisocoumarin with subunits of pituitary and spleen multicatalytic proteinase complexes (proteasomes). *Biochemistry* **36**, 13946–13953.

13 Kuhn DJ, Hunsucker SA, Chen Q, Voorhees PM, Orlowski M & Orlowski RZ (2009) Targeted inhibition of the immunoproteasome is a potent strategy against models of multiple myeloma that overcomes resistance to conventional drugs and nonspecific proteasome inhibitors. *Blood* **113**, 4667–4676.

14 Bogyo M, McMaster JS, Gaczynska M, Tortorella D, Goldberg AL & Ploegh H (1997) Covalent modification of the active site threonine of proteasomal beta subunits and the Escherichia coli homolog HslV by a new class of inhibitors. *Proc Nat Acad Sci USA* **94**, 6629–6634.

15 Bogyo M, Shin S, McMaster JS & Ploegh HL (1998) Substrate binding and sequence preference of the proteasome revealed by active-site-directed affinity probes. *Chem Biol* **5**, 307–320.

16 Nazif T & Bogyo M (2001) Global analysis of proteasomal substrate specificity using positional-scanning libraries of covalent inhibitors. *Proc Nat Acad Sci USA* **98**, 2967–2972.

17 Kessler BM, Tortorella D, Altun M, Kisselev AF, Fiebiger E, Hekking BG, Ploegh HL & Overkleeft HS (2001) Extended peptide-based inhibitors efficiently target the proteasome and reveal overlapping

specificities of the catalytic β-subunits. *Chem Biol* **8**, 913–929.
18 Ovaa H, van Swieten PF, Kessler BM, Leeuwenburgh MA, Fiebiger E, van den Nieuwendijk AMCH, Galardy PJ, van der Marel GA, Ploegh HL & Overkleeft HS (2003) Chemistry in living cells: detection of active proteasomes by a two-step labeling strategy. *Angew Chem Int Ed Engl* **42**, 3626–3629.
19 Saxon E & Bertozzi CR (2000) Cell surface engineering by a modified Staudinger reaction. *Science* **287**, 2007–2010.
20 van der Linden WA, Geurink PP, Oskam C, van der Marel GA, Florea BI & Overkleeft HS (2010) Proteasome selectivity towards Michael acceptor containing oligopeptide-based inhibitors. *Org Biomol Chem* **8**, 1885–1893.
21 Berkers CR, Verdoes M, Lichtman E, Fiebiger E, Kessler BM, Anderson KC, Ploegh HL, Ovaa H & Galardy PJ (2005) Activity probe for in vivo profiling of the specificity of proteasome inhibitor bortezomib. *Nat Meth* **2**, 357–362.
22 Verdoes M, Florea BI, Menendez-Benito V, Maynard CJ, Witte MD, van der Linden WA, van den Nieuwendijk AMCH, Hofmann T, Berkers CR, van Leeuwen FWB *et al.* (2006) A fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo. *Chem Biol* **13**, 1217–1226.
23 de Jong A, Schuurman KG, Rodenko B, Ovaa H & Berkers CR (2011) Fluorescence-Based Proteasome Activity Profiling. In *Methods in Molecular Biology* (Drewes G & Bantscheff M, eds), pp. 183–204. Humana Press, Totowa, NJ.
24 Berkers CR, Leestemaker Y, Schuurman KG, Ruggeri B, Jones-Bolin S, Williams M & Ovaa H (2012) Probing the specificity and activity profiles of the proteasome inhibitors bortezomib and delanzomib. *Mol Pharmaceutics* **9**, 1126–1135.
25 Verdoes M, Florea BI, Hillaert U, Willems LI, van der Linden WA, Sae Heng M, Filippov DV, Kisselev AF, van der Marel GA & Overkleeft HS (2008) Azido-BODIPY acid reveals quantitative Staudinger-Bertozzi ligation in two-step activity-based proteasome profiling. *ChemBioChem* **9**, 1735–1738.
26 Florea BI, Verdoes M, Li N, van der Linden WA, Geurink PP, van den Elst H, Hofmann T, de Ru A, van Veelen PA, Tanaka K *et al.* (2010) Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit β5t. *Chem Biol* **17**, 795–801.
27 Verdoes M, Hillaert U, Florea BI, Sae Heng M, Risseeuw MDP, Filippov DV, van der Marel GA & Overkleeft HS (2007) Acetylene functionalized BODIPY dyes and their application in the synthesis of activity based proteasome probes. *Bioorg Med Chem Lett* **17**, 6169–6171.
28 Sin N, Kim KB, Elofsson M, Meng L, Auth H, Kwok BH & Crews CM (1999) Total synthesis of the potent proteasome inhibitor epoxomicin: a useful tool for understanding proteasome biology. *Bioorg Med Chem Lett* **9**, 2283–2288.
29 Meng L, Mohan R, Kwok BH, Elofsson M, Sin N & Crews CM (1999) Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. *Proc Nat Acad Sci USA* **96**, 10403–10408.
30 Clerc J, Florea BI, Kraus M, Groll M, Huber R, Bachmann AS, Dudler R, Driessen C, Overkleeft HS & Kaiser M (2009) Syringolin A selectively labels the 20 S proteasome in murine EL4 and wild-type and bortezomib-adapted leukaemic cell lines. *ChemBioChem* **10**, 2638–2643.
31 Li H, van der Linden WA, Verdoes M, Florea BI, McAllister FE, Govindaswamy K, Elias JE, Bhanot P, Overkleeft HS & Bogyo M (2014) Assessing subunit dependency of the Plasmodium proteasome using small molecule inhibitors and active site probes. *ACS Chem Biol* **9**, 1869–1876.
32 Federspiel JD, Codreanu SG, Goyal S, Albertolle ME, Lowe E, Teague J, Wong H, Guengerich FP & Liebler DC (2016) Specificity of protein covalent modification by the electrophilic proteasome inhibitor carfilzomib in human cells. *Mol Cell Proteomics* **15**, 3233–3242.
33 Gu C, Kolodziejek I, Misas Villamil J, Shindo T, Colby T, Verdoes M, Richau KH, Schmidt J, Overkleeft HS & van der Hoorn RAL (2010) Proteasome activity profiling: a simple, robust and versatile method revealing subunit-selective inhibitors and cytoplasmic, defense-induced proteasome activities. *Plant J* **62**, 160–170.
34 Willems LI, Li N, Florea BI, Ruben M, van der Marel GA & Overkleeft HS (2012) Triple bioorthogonal ligation strategy for simultaneous labeling of multiple enzymatic activities. *Angew Chem Int Ed Engl* **51**, 4431–4434.
35 de Bruin G, Xin BT, Kraus M, van der Stelt M, van der Marel GA, Kisselev AF, Driessen C, Florea BI & Overkleeft HS (2016) A set of activity-based probes to visualize human (immuno)proteasome activities. *Angew Chem Int Ed Engl* **55**, 4199–4203.
36 Kisselev AF, Garcia-Calvo M, Overkleeft HS, Peterson E, Pennington MW, Ploegh HL, Thornberry NA & Goldberg AL (2003) 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.
37 van Swieten PF, Samuel E, Hernández RO, van den Nieuwendijk AMCH, Leeuwenburgh MA, van der Marel GA, Kessler BM, Overkleeft HS & Kisselev AF (2007) A cell-permeable inhibitor and activity-based probe for the caspase-like activity of the proteasome. *Bioorg Med Chem Lett* **17**, 3402–3405.

38 Britton M, Lucas MM, Downey SL, Screen M, Pletnev AA, Verdoes M, Tokhunts RA, Amir O, Goddard AL, Pelphrey PM *et al.* (2009) Selective inhibitor of proteasome's caspase-like sites sensitizes cells to specific inhibition of chymotrypsin-like sites. *Chem Biol* **16**, 1278–1289.
39 Verdoes M, Willems LI, van der Linden WA, Duivenvoorden BA, van der Marel GA, Florea BI, Kisselev AF & Overkleeft HS (2010) A panel of subunit-selective activity-based proteasome probes. *Org Biomol Chem* **8**, 2719–2727.
40 de Bruin G, Xin BT, Florea BI & Overkleeft HS (2016) Proteasome subunit selective activity-based probes report on proteasome core particle composition in a native polyacrylamide gel electrophoresis fluorescence-resonance energy transfer assay. *J Am Chem Soc* **138**, 9874–9880.
41 Carmony KC, Lee D-M, Wu Y, Lee NR, Wehenkel M, Lee J, Lei B, Zhan CG & Kim KB (2012) A bright approach to the immunoproteasome: development of LMP2/β1i-specific imaging probes. *Bioorg Med Chem* **20**, 607–613.
42 Mirabella AC, Pletnev AA, Downey SL, Florea BI, Shabaneh TB, Britton M, Verdoes M, Filippov DV, Overkleeft HS & Kisselev AF (2011) Specific cell-permeable inhibitor of proteasome trypsin-like sites selectively sensitizes myeloma cells to bortezomib and carfilzomib. *Chem Biol* **18**, 608–618.
43 Geurink PP, van der Linden WA, Mirabella AC, Gallastegui N, de Bruin G, Blom AEM, Voges MJ, Mock ED, Florea BI, van der Marel GA *et al.* (2013) 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.
44 Screen M, Britton M, Downey SL, Verdoes M, Voges MJ, Blom AEM, Geurink PP, Risseeuw MDP, Florea BI, van der Linden WA *et al.* (2010) Nature of pharmacophore influences active site specificity of proteasome inhibitors. *J Biol Chem* **285**, 40125–40134.
45 Xin BT, de Bruin G, Huber EM, Besse A, Florea BI, Filippov DV, van der Marel GA, Kisselev AF, van der Stelt M, Driessen C *et al.* (2016) Structure-based design of β5c selective inhibitors of human constitutive proteasomes. *J Med Chem* **59**, 7177–7187.
46 Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ & Schreiber SL (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268**, 726–731.
47 Dick LR, Cruikshank AA, Grenier L, Melandri FD, Nunes SL & Stein RL (1996) Mechanistic studies on the inactivation of the proteasome by lactacystin. *J Biol Chem* **271**, 7273–7276.
48 Sin N, Meng L, Auth H & Crews CM (1998) Eponemycin analogues: syntheses and use as probes of angiogenesis. *Bioorg Med Chem* **6**, 1209–1217.
49 Meng L, Kwok BH, Sin N & Crews CM (1999) Eponemycin exerts its antitumor effect through the inhibition of proteasome function. *Cancer Res* **59**, 2798–2801.
50 Li N, Kuo CL, Paniagua G, van den Elst H, Verdoes M, Willems LI, van der Linden WA, Ruben M, van Genderen E, Gubbens J *et al.* (2013) Relative quantification of proteasome activity by activity-based protein profiling and LC-MS/MS. *Nat Protoc* **8**, 1155–1168.
51 Groll M, Schellenberg B, Bachmann AS, Archer CR, Huber R, Powell TK, Lindow S, Kaiser M & Dudler R (2008) A plant pathogen virulence factor inhibits the eukaryotic proteasome by a novel mechanism. *Nature* **452**, 755–758.
52 Kolodziejek I, Misas-Villamil JC, Kaschani F, Clerc J, Gu C, Krahn D, Niessen S, Verdoes M, Willems LI, Overkleeft HS *et al.* (2011) Proteasome activity imaging and profiling characterizes bacterial effector syringolin A. *Plant Physiol* **155**, 477–489.
53 Adams J, Behnke M, Chen S, Cruickshank AA, Dick LR, Grenier L, Klunder JM, Ma Y-T, Plamondon L & Stein RL (1998) Potent and selective inhibitors of the proteasome: dipeptidyl boronic acids. *Bioorg Med Chem Lett* **8**, 333–338.
54 Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, Maas J, Pien CS, Prakash S & Elliott PJ (1999) Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res* **59**, 2615–2622.
55 Chauhan D, Catley L, Li G, Podar K, Hideshima T, Velankar M, Mitsiades C, Mitsiades N, Yasui H, Letai A *et al.* (2005) A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from Bortezomib. *Cancer Cell* **8**, 407–419.
56 Crawford LJA, Walker B, Ovaa H, Chauhan D, Anderson KC, Morris TCM & Irvine AE (2006) Comparative selectivity and specificity of the proteasome inhibitors BzLLLCOCHO, PS-341, and MG-132. *Cancer Res* **66**, 6379–6386.
57 Altun M, Galardy PJ, Shringarpure R, Hideshima T, LeBlanc R, Anderson KC, Ploegh HL & Kessler BM (2005) Effects of PS-341 on the activity and composition of proteasomes in multiple myeloma cells. *Cancer Res* **65**, 7896–7901.
58 Kraus M, Rückrich T, Reich M, Gogel J, Beck A, Kammer W, Berkers CR, Burg D, Overkleeft H, Ovaa H *et al.* (2006) Activity patterns of proteasome subunits reflect bortezomib sensitivity of hematologic malignancies and are variable in primary human leukemia cells. *Leukemia* **21**, 84–92.
59 Kupperman E, Lee EC, Cao Y, Bannerman B, Fitzgerald M, Berger A, Yu J, Yang Y, Hales P, Bruzzese F *et al.* (2010) Evaluation of the proteasome

inhibitor MLN9708 in preclinical models of human cancer. *Cancer Res* **70**, 1970–1980.
60 Li H, O'Donoghue AJ, van der Linden WA, Xie SC, Yoo E, Foe IT, Tilley L, Craik CS, da Fonseca PCA & Bogyo M (2016) Structure- and function-based design of Plasmodium-selective proteasome inhibitors. *Nature* **530**, 233–236.
61 Rückrich T, Kraus M, Gogel J, Beck A, Ovaa H, Verdoes M, Overkleeft HS, Kalbacher H & Driessen C (2009) Characterization of the ubiquitin–proteasome system in bortezomib-adapted cells. *Leukemia* **23**, 1098–1105.
62 Deshaies RJ (2014) Proteotoxic crisis, the ubiquitin-proteasome system, and cancer therapy. *BMC Biol* **12**, 94.
63 Kraus M, Bader J, Geurink PP, Weyburne ES, Mirabella AC, Silzle T, Shabaneh TB, van der Linden WA, de Bruin G, Haile SR *et al.* (2015) The novel β2-selective proteasome inhibitor LU-102 synergizes with bortezomib and carfilzomib to overcome proteasome inhibitor resistance of myeloma cells. *Haematologica* **100**, 1350–1360.
64 Kammerl IE, Dann A, Mossina A, Brech D, Lukas C, Vosyka O, Nathan P, Conlon TM, Wagner DE, Overkleeft HS *et al.* (2016) Impairment of immunoproteasome function by cigarette smoke and in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **193**, 1230–1241.
65 Banno A, Garcia DA, van Baarsel ED, Metz PJ, Fisch K, Widjaja CE, Kim SH, Lopez J, Chang AN, Geurink PP *et al.* (2016) Downregulation of 26S proteasome catalytic activity promotes epithelial-mesenchymal transition. *Oncotarget* **7**, 21527–21541.
66 Teicher BA, Ara G, Herbst R, Palombella VJ & Adams J (1999) The proteasome inhibitor PS-341 in cancer therapy. *Clin Cancer Res* **5**, 2638–2645.
67 Geurink PP, Florea BI, van der Marel GA, Kessler BM & Overkleeft HS (2010) Probing the proteasome cavity in three steps: bio-orthogonal photo-reactive suicide substrates. *Chem Commun* **46**, 9052–9054.
68 Park JE, Wu Y, Carmony KC, Miller Z, Sharma LK, Lee D-M, Kim D-Y, Lee W & Kim KB (2014) A FRET-based approach for identification of proteasome catalytic subunit composition. *Mol BioSyst* **10**, 196–200.
69 Khare S, Nagle AS, Biggart A, Lai YH, Liang F, Davis LC, Barnes SW, Mathison CJN, Myburgh E, Gao M-Y *et al.* (2016) Proteasome inhibition for treatment of leishmaniasis, Chagas disease and sleeping sickness. *Nature* **537**, 229–233.
70 Lin G, Li D, de Carvalho LPS, Deng H, Tao H, Vogt G, Wu K, Schneider J, Chidawanyika T, Warren JD *et al.* (2009) Inhibitors selective for mycobacterial versus human proteasomes. *Nature* **461**, 621–626.
71 Groll M, Kim KB, Kairies N, Huber R & Crews CM (2000) Crystal structure of Epoxomicin:20S proteasome reveals a molecular basis for selectivity of α', β'-epoxyketone proteasome inhibitors. *J Am Chem Soc* **122**, 1237–1238.
72 Schrader J, Henneberg F, Mata RA, Tittmann K, Schneider TR, Stark H, Bourenkov G & Chari A (2016) The inhibition mechanism of human 20S proteasomes enables next-generation inhibitor design. *Science* **353**, 594–598.
73 Sharma LK, Lee NR, Jang ER, Lei B, Zhan CG, Lee W & Kim KB (2012) Activity-based near-infrared fluorescent probe for LMP7: a chemical proteomics tool for the immunoproteasome in living cells. *ChemBioChem* **13**, 1899–1903.
74 Geurink PP, Liu N, Spaans MP, Downey SL, van den Nieuwendijk AMCH, van der Marel GA, Kisselev AF, Florea BI & Overkleeft HS (2010) Incorporation of fluorinated phenylalanine generates highly specific inhibitor of proteasome's chymotrypsin-like sites. *J Med Chem* **53**, 2319–2323.