Review

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Future Medicinal Chemistry

Methods for the discovery of small molecules to monitor and perturb the activity of the human proteasome

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Regulating protein production and degradation is critical to maintaining cellular homeostasis. The proteasome is a key player in keeping proteins at the proper levels. However, proteasome activity can be altered in certain disease states, such as blood cancers and neurodegenerative diseases. Cancers often exhibit enhanced proteasomal activity, as protein synthesis is increased in these cells compared with normal cells. Conversely, neurodegenerative diseases are characterized by protein accumulation, leading to reduced proteasome activity. As a result, the proteasome has emerged as a target for therapeutic intervention. The potential of the proteasome as a therapeutic target has come from studies involving chemical stimulators and inhibitors, and the development of a suite of assays and probes that can be used to monitor proteasome activity with purified enzyme and in live cells.

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Regulation of protein synthesis and degradation is critical for the health of a cell. A careful balance between protein synthesis and degradation is strictly regulated by several cellular mechanisms, including the ubiquitin proteasome system [1], which is responsible for degradation of the majority of proteins that are misfolded or no longer needed by the cell. At the center of this system is the proteasome, a multicatalytic enzymatic complex that is responsible for recognizing, unfolding and hydrolyzing proteins that have been labeled for degradation [1,2]. The proteasome is supported by a series of ubiquitin-conjugating enzymes that facilitate linkage of multiple ubiquitin proteins to a target protein, marking it for degradation by the proteasome [2]. The 26S proteasome is composed of two parts: a 19S regulatory particle (RP) and the 20S core particle (sCP) (Figure 1) [3]. The RP binds labeled proteins, strips the ubiquitin, unfolds the protein and shuttles it into the sCP for hydrolysis [4,5]. The sCP is composed of four heptameric rings that assemble in an α-β-β-α fashion. The β rings house three catalytic subunits – β1, β2 and β5 – which each have unique hydrolysis activities (caspase-like, trypsin-like and chymotrypsin-like, respectively) and are responsible for cutting the linear protein sequence into short peptides [5,6]. These peptides are then recycled by the cell to aid in the synthesis of new proteins or can be loaded into MHC-I complexes to help immune cells recognize self cells from nonself cells [3,7]. The α heptameric rings of the sCP that flank either end of the β rings form a gate that controls entry of protein substrates into the core for hydrolysis [8,9]. Well-folded proteins are typically too bulky to enter the sCP without first being stripped of ubiquitin and unfolded by the RP, which offers protection against degradation of proteins that are still needed by the cell [4]. The sCP also exists in the cell in the uncapped form and is critical to the degradation of small disordered proteins that are able to pass through the gates formed by the α rings [10]. The uncapped sCP plays an important role in clearing proteins that are prone to aggregation, such as intrinsically disordered proteins [11–13].

Other forms of the proteasome exist, such as the thymoproteasome [14] and immunoproteasome. The immunoproteasome is expressed in cells derived from bone marrow and its production can be triggered in other cells upon stimulation by inflammatory signals [15]. When exposed to cytokines such as IFN-γ, the catalytic subunits of the sCP are replaced with three new subunits – the β1i, β2i and β5i – resulting in the production of the immunoproteasome

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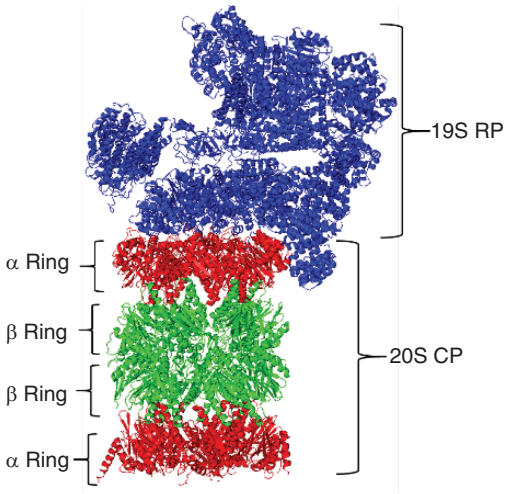

Figure 1. Structure of the 26S proteasome. The 26S proteasome is composed of two parts, the 19S regulatory particle and the 20S catalytic core particle (sCP). The 19S particle is responsible for recognizing ubiquitinated substrates and shuttling them into the sCP. The sCP then hydrolyzes the protein into short peptide sequences that can be recycled by the cell. The sCP also exists in the uncapped form. In this state, it degrades small, disordered proteins.

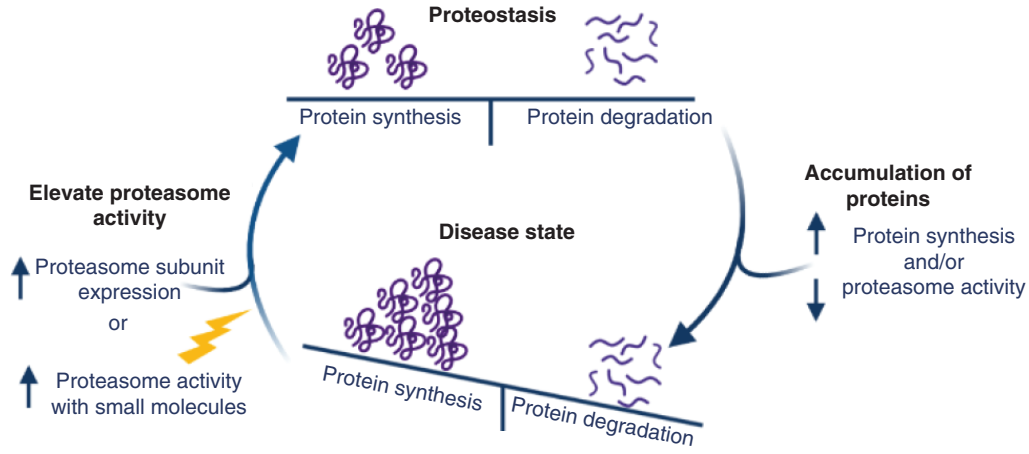

Figure 2. Maintaining proteostasis is critical to cell viability. The rate of protein synthesis must be matched by protein degradation to maintain proteostasis in the cell. If protein synthesis is too rapid, as is the case in several cancers, proteasomes are unable to degrade excess proteins efficiently, leading to protein accumulation and cell death. Similarly, if proteasome activity is suppressed (as is seen in some neurodegenerative diseases), proteins accumulate. To avoid death, cancer cells enhance proteasome activity by overexpressing genes encoding the proteasome subunits. Attention has also been turned to chemical stimulation of the proteasome as a mechanism to treat protein accumulation diseases (e.g., Parkinson disease) and pathologies related to aging.

(iCP). This happens in response to viral infections or inflammatory signals released near the cell. The iCP is more effective at generating peptide fragments that can be loaded into an MHC-I complex, which allows immune cells to recognize and eliminate virally infected cells [3]. Because the iCP plays a crucial role in immune cell recognition, it is also endogenously expressed in antigen-presenting cells such as dendritic cells [16,17]. Interestingly, the iCP has been shown to be produced in certain types of cancer cells such as breast cancer [18]. The iCP is also known to be important in autoimmune disorders such as multiple sclerosis [19] and rheumatoid arthritis [20].

Because tight regulation of protein degradation is required to maintain homeostasis, disruption of proteasome activity can have devastating impacts on a cell. Accumulation of unwanted proteins leads to activation of several stress pathways [12,21]. If proteasomes are unable to clear the accumulated proteins, cells die (Figure 2). Several pathologies result from protein accumulation, including neurodegenerative diseases such as Parkinson disease [22,23] and Huntington disease [24,25], as well as pathologies associated with aging [26–28]. Similarly, many types of cancer cells synthesize proteins at a faster rate than their normal counterparts and rely more heavily on the proteasome to clear unwanted proteins [29–31]. As such, the proteasome has emerged as an important target in treating a variety of diseases. Proteasome activity slows during aging, leaving older populations more susceptible to the pathologies

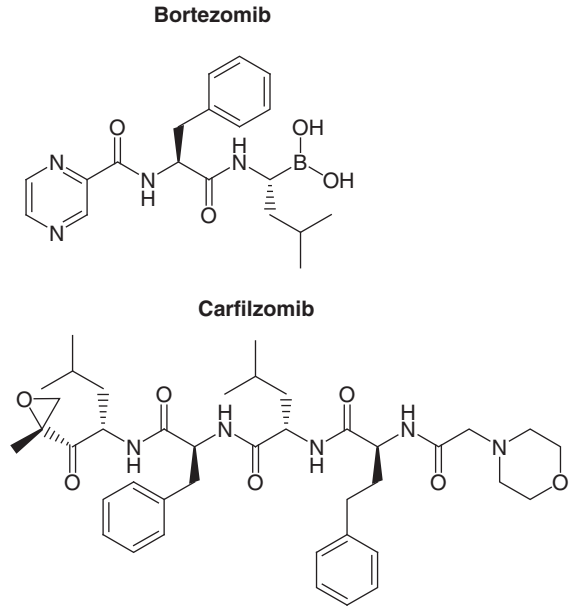

Figure 3. Structures of bortezomib and carfilzomib. Bortezomib and carfilzomib have both been approved by the US FDA for treatment of multiple myeloma. Bortezomib reversibly binds the β5 subunit via the boronic acid moiety. Carfilzomib irreversibly binds catalytic subunits via the epoxyketone moiety.

associated with protein accumulation [27]. On the contrary, centenarians and other long-lived mammals have been shown to have higher rates of proteasome activity, further providing evidence that maintaining proteasome function is important for preventing side effects of aging [32,33].

Because proteasome activity is implicated in several disease states, attention has been turned to chemical modulation of this activity through the use of small molecules. In the case of blood cancers that rely more heavily on proteasome activity to maintain homeostasis, blocking the activity with a small molecule inhibitor can trigger apoptosis [34,35]. The success of using proteasome inhibitors to treat blood cancers is reflected by the US FDA-approved drug bortezomib and its second-generation counterpart, carfilzomib (Figure 3) [36,37]. Bortezomib binds the β5 subunit of the sCP via an interaction between its boronic acid moiety and the catalytic threonine in the subunit's active site. Binding of bortezomib to the active site prevents the subunit from cleaving incoming polypeptide substrates, thereby reducing proteasome activity. Bortezomib has been extensively studied in multiple myeloma (MM), a type of B-cell blood cancer [38]. Because MM cells rely more heavily on proteasome activity than normal cells, reducing proteasome activity through the use of small molecules such as bortezomib represents a way to discriminate between healthy and cancer cells [39]. Although great success has been observed in treating MM patients with bortezomib, it is not uncommon for patients to display resistance to this drug. Proteasomes in healthy cells are also still impacted by bortezomib, and off-target side effects (e.g., neuropathy) are common [36,40].

Recently, our lab and others have demonstrated that in cells overexpressing α-synuclein, a protein shown to form aggregates in Parkinson disease, stimulating the proteasome with small molecules is effective in reducing aggregates [28,41]. Similarly, studies have also revealed that chemical stimulation of the proteasome can prolong the lifespan of yeast, a system commonly used to study aging [28]. Although it is established that modulating proteasome activity through the use of small molecules is a viable strategy to treat certain cancers and pathologies associated with aging, methods to discover small molecules that can interact with the non-catalytically active proteasome subunits are limited. Methods to monitor proteasome activity have been extensively reviewed elsewhere [42], so here we will focus on describing methods used to identify small molecule binders of proteasome subunits and ways in which these binders can be fully exploited to provide new insight into the proteasome as a therapeutic target.

Probes to monitor binding of small molecules to the catalytic subunits of the proteasome

The proteasome has emerged as an interesting target for chemical stimulation or inhibition to treat a variety of diseases. In order to rapidly and effectively screen small molecules for their ability to modulate proteasome activity, attention has been turned to the development of probes that bind catalytic proteasome subunits. Many of these probes are proteasome inhibitors that either bind all the catalytic subunits or show selectivity toward one subunit. Use of these probes is instrumental in determining to which subunits novel ligands bind and in monitoring proteasome activity in response to new ligands. Until recently, progress lagged in the creation of proteasome probes

that could be used to monitor proteasome activity in live cells, as they are often bulky and cannot penetrate the cell membrane [43]. However, our research group and others have established probes that are cell permeable and selective to either the standard proteasome or immunoproteasome [44,45]. With these probes, screening of small molecules for modulation of proteasome activity can be expedited and thoroughly characterized. In this section we will review commonly available proteasome inhibitors that have been developed into probes.

Covalent inhibitors of the proteasome

Over the last decade, significant progress has been made in the creation of probes to detect proteasomal subunits in lysate and, more recently, live cells. Both broad-spectrum and subunit-specific probes are now available to detect proteasome activity in cell lysates. Techniques for monitoring proteasome activity with these probes generally involve labeling the probe with a fluorophore or tag that can be detected by immunoblotting and incubating them with cell lysate [46]. Lysates are then subjected to SDS-PAGE. In the case of probes labeled with fluorophores, gels can be directly scanned for fluorescence. Because the probes form a covalent bond with the catalytic subunits of the proteasome, it can withstand this analysis. Probe-labeled proteasome subunits fluoresce in the gel and the binding patterns can be analyzed to determine the composition of proteasomes present in the sample. Similarly, probes may also be labeled with tags recognized by antibodies. In this case, after the probe is incubated with cell lysate, western blot analysis is used to study binding patterns of the probes to specific proteasome subunits. A suite of proteasome inhibitors has been developed into probes to study proteasome composition in cells, and current research is focused on utilizing these probes to monitor binding of novel small molecules to proteasome subunits and elucidate their impact on proteasome activity.

Broad-spectrum proteasome inhibitors

Broad-spectrum proteasome inhibitors were among the first types of small molecules to be developed into probes to monitor proteasome activity in cell lysate and live cells. Molecules in this class of inhibitors generally bind all catalytic subunits with equal affinity. A major hurdle in the design of probes to monitor proteasome activity in live cells is cell permeability. Although several proteasome inhibitors have been discovered and offer excellent specificity, they are often not cell permeable, greatly restricting their use to *in vitro* applications. One such inhibitor is AdaY(^{125}I)Ahx$_3$L$_3$VS, a radiolabeled substrate known to bind all catalytic subunits of relative equal affinity, which has been extensively studied and modified to better fit applications in cells (Figure 4) [46]. This inhibitor possesses a vinyl sulfone moiety which covalently binds catalytic subunits via a Michael reaction with the threonine residue located in the catalytic subunit. The N-terminal region houses an adamantanylacetate (Ada) moiety that was originally thought to make the probe more cell permeable. Coupled to the Ada moiety is a tyrosine residue with an I^{125} at the meta position, which facilitates radiolabeling of the probe to detect probe-bound proteasome subunits after SDS-PAGE analysis. The vinyl sulfone and Ada portions of the probe are separated by a linker structure which is resistant to proteolytic cleavage because it is not composed of natural amino acids that are readily degraded by proteases in the cell. Although this probe has been demonstrated to equally and selectively bind all subunits of the sCP, it is unable to cross the cell membrane [46].

In an effort to make this probe more cell permeable, a few research groups have studied the impact of exchanging the N-terminal Ada moiety for other chemical moieties [47]. Of note, the MV151 probe was generated by exchanging the Ada group of the AdaY(I^{125})Ahx$_3$L$_3$VS for boron-dipyrromethene (BODIPY) and tetramethylrhodamine moieties (Figure 4) [46]. Excitingly, not only has MV151 been shown to be more cell permeable than its parent structure, but it also contains fluorophores for effective readout in in-gel assays. MV151 has been shown to inhibit all three catalytic subunits of the sCP with similar affinity when probe concentrations are above 1 μM in cell lysate; below this concentration, MV151 shows more specificity toward the β2 and β5 subunits. Moreover, this probe has also been demonstrated to bind the immunoproteasome subunits β1i, β2i and β5i. The fluorescent tag also facilitates monitoring entry of the probe into cells using a fluorescent microscope. Similarly, the probe was also successfully used with cells engineered to express ubiquitin fused to green fluorescent protein (GFP) to study proteasome activity [46]. While the GFP–ubiquitin is normally quickly degraded by proteasomes in the cell, exposure to MV151 was successful in causing accumulation of the fusion protein, as evidenced by confocal microscopy images. MV151 offers several advantages over its parent structure because of the ease of detection both in-gel and in live cells with the BODIPY fluorophore and the improved cell permeability over the former. It is also feasible to change the fluorophore on this inhibitor to better suit cell assay needs and experiments that require multiple fluorophores to monitor proteasome activity, although each new fluorophore must be characterized

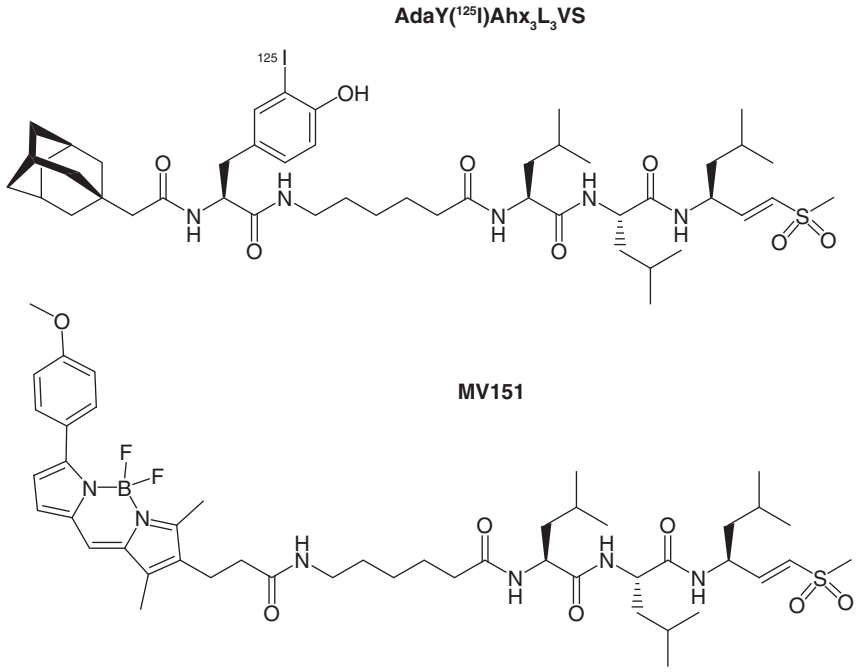

Figure 4. Broad spectrum proteasome inhibitor probes. (A) AdaY(^{125}I)Ahx$_3$L$_3$VS, a proteasome inhibitor that binds all catalytic subunits at concentrations greater than 1 μM. The vinyl sulfone moiety covalently binds catalytic threonine residues, and the radiolabeled iodine facilitates visualization of bound subunits after SDS-PAGE analysis. **(B)** MV151 was created using the same scaffold, but exchanging the radiolabel tag for BODIPY, a fluorescent tag. This new inhibitor has been demonstrated to retain the ability to covalently bind proteasome subunits while being easier to detect with fluorescent imaging after SDS-PAGE. It is also cell permeable.
BODIPY: Boron-dipyrromethene; TMR: Tetramethylrhodamine.

carefully to ensure the probe still nondiscriminately binds the catalytic proteasome subunits with similar affinity and remains cell permeable.

Broad-spectrum proteasome inhibitors have proven useful as a tool to study small molecules that modulate proteasome activity. Because these types of inhibitors bind nonspecifically to all catalytic subunits, they can be used in pulse-chase and competitive binding experiments in which a ligand of interest is allowed to incubate with pure proteasome or cell lysate, followed by the labeled inhibitor [34,46,48]. The binding patterns of the probe can be analyzed either through in-gel fluorescence or immunoblotting. Changes in the intensity of bands corresponding to a specific proteasome subunit reflect binding of the small molecule to a particular subunit. This approach has been used to study the binding affinity of bortezomib and MG132 in cell lysates. A modified version of AdaY(^{125}I)Ahx$_3$L$_3$VS that was conjugated to the dansyl fluorophore in place of the radiolabeled iodine moiety was successfully used to monitor proteasome activity in cells after treatment with either bortezomib or MG132 [47]. While bortezomib predominantly binds the β5 subunit, MG132 is known to bind both the β5 and β1 subunits [49]. After dosing cells with either bortezomib or MG132, the dansyl-conjugated proteasome inhibitor probe was then added to cell lysates. In bortezomib-treated lysates, the probe was effective at labeling both the β1 and β2 subunits but not the β5 subunit, indicating that bortezomib was bound and prevented linkage of the probe to this subunit. Likewise, the probe was able to label the β5 subunit of MG132-treated cell lysates, but not the β1 or β2, indicating the MG132 had interacted with these subunits.

Although broad-spectrum proteasome inhibitors are useful tools for studying the binding of new ligands to proteasome subunits, their use comes with drawbacks. First, the concentration at which broad-spectrum probes are used is crucial to their unbiased labeling of the catalytic subunits. As previously mentioned, use of MV151 at concentrations lower than 1 μM in cell lysate results in preferential binding of the probe to the β2 and β5 subunits. Similarly, conjugating new fluorophores to broad-spectrum proteasome inhibitors must be followed up with careful study of the binding affinities of the probe to all catalytic subunits. Bias toward one subunit of the proteasome is easy to introduce and requires careful attention to detail. Analyzing binding patterns that result from

competitive binding experiments between a novel ligand and a broad-spectrum probe can also be cumbersome, as this may differ depending on the probe used. Because the proteasome and immunoproteasome subunits have similar molecular weights, analyzing gels from cell lines dosed with a broad-spectrum inhibitor that are known to express both the standard and immunoproteasome (such as EL-4 lysate) [50] is challenging. Careful design of a broad-spectrum probe is also required, as the probes must be very specific to only catalytic proteasomal subunits; nonspecific labeling of other proteases has been reported with broad-spectrum probes and these can be mistaken for labeled proteasomes [51]. When designing new fluorescent broad-spectrum probes, it is important to conduct pulse-chase experiments in which cell lysate dosed with the probe is chased with known proteasome inhibitors. Chasing with known proteasome inhibitors should not alter the fluorescent labeling of the proteasome by the probe, so if a decrease in fluorescence is observed, this indicates that the probe is binding other proteases in the cell. These shortcomings and the desire to create proteasome inhibitors specific to a certain subunit have led to the development of several classes of subunit-specific probes that can be used to monitor the activity of individual proteasome subunits and discriminate between them.

Subunit-specific inhibitors of the proteasome

Subunit-specific proteasome inhibitors have greatly contributed to our understanding of proteasome composition and the activities of the catalytic subunits in cells. There is a suite of subunit-specific inhibitors that have been used for experiments not only with purified proteasome, but also with cell lysates. As with broad-spectrum inhibitors, subunit-specific probes are often conjugated to fluorophores so that specific subunits binding to a ligand can be identified. However, the use of fluorophores with subunit-specific probes must be carefully evaluated, as many groups have reported that adding a fluorescent tag can alter the specificity of the probe [51]. As a result, probes must be thoroughly tested to ensure their specificity remains the same. Extensive research has been conducted on the impact of the warhead on the specificity of probes to a particular subunit, as many – including epoxyketones, vinyl sulfones [52] and boronic acids [53] – are known to inhibit the proteasome. Originally, it was thought that the warhead does not impact the specificity of the ligand, as all the catalytic subunits use a similar mechanism to hydrolyze amide bonds. Rather, there are three binding pockets in each catalytic subunit that facilitate binding of an incoming substrate for cleavage by a specific active site. Differences in the interaction of the substrate/inhibitor with these binding pockets determine the type of cleavage that can occur: caspase-like, trypsin-like or chymotrypsin-like. The design of substrates that fit into these pockets is believed to be the best way to confer specificity of a probe for a subunit [6]. For example, the β1 subunit facilitates caspase-like cleavage, meaning peptides are normally cleaved after acidic amino acid residues; therefore, incorporation of an acidic residue in an inhibitor should confer specificity to the β1 subunit over the other catalytic subunits. Similarly, the β2 subunit preferentially cleaves with trypsin-like activity, meaning after basic residues. The β5 subunit cleaves with chymotrypsin specificity and cleaves after hydrophobic residues. The remaining two binding pockets also have subtle differences in binding preferences, and exploiting all three pockets has proven to be a successful strategy in designing inhibitors specific to each subunit. However, it has been demonstrated that altering the warhead does indeed change the specificity of the inhibitor to a subunit. This finding is discussed next.

Some of the most popular proteasome subunit-specific probes are NC-001 (β1- and β1i-specific), LU-112 (β2- and β2i-specific) and NC-005-VS (β5-specific) (Figure 5). NC-001 contains an epoxyketone warhead [54], while LU-112 and NC-005-VS possess a vinyl sulfone warhead [55,56]. NC-001 is cell permeable, and in one study almost 100% inhibition of the β1 subunit in B lymphocytes was achieved after 5 h of dosing at 2 μM [57]. Most importantly, NC-001 was shown not to significantly inhibit any of the other subunits at concentrations of up to 4 μM, suggesting it is very specific toward the β1 subunit. Similarly, longer doses of 24 h at high concentrations of NC-001 in cells did not result in inhibition of other proteasome subunits. However, NC-001 is known to interact with the β1i subunit, meaning its use must be carefully tailored to cells known only to express the sCP or iCP, as this probe cannot readily discriminate between the β1 subunits of both proteasome isoforms.

The LU-112 β2 subunit inhibitor was discovered by structure–activity relationship studies in which both epoxyketones and vinyl sulfone warheads were explored, as well as several chemical groups meant to bind the P1 and P3 pocket of the β2 subunit [56]. Given that the binding pockets of the β2 subunit preferentially interact with basic residues, several basic natural and unnatural amino acids were tested for their ability to confer specificity to this subunit. Interestingly, this study revealed that vinyl sulfones are more specific to the β2 subunit than epoxyketones, disproving the concept that the warhead does not contribute to probe specificity. Next, several basic chemical groups were placed in positions that would interact with the P1 and P3 binding pockets. Conjugating

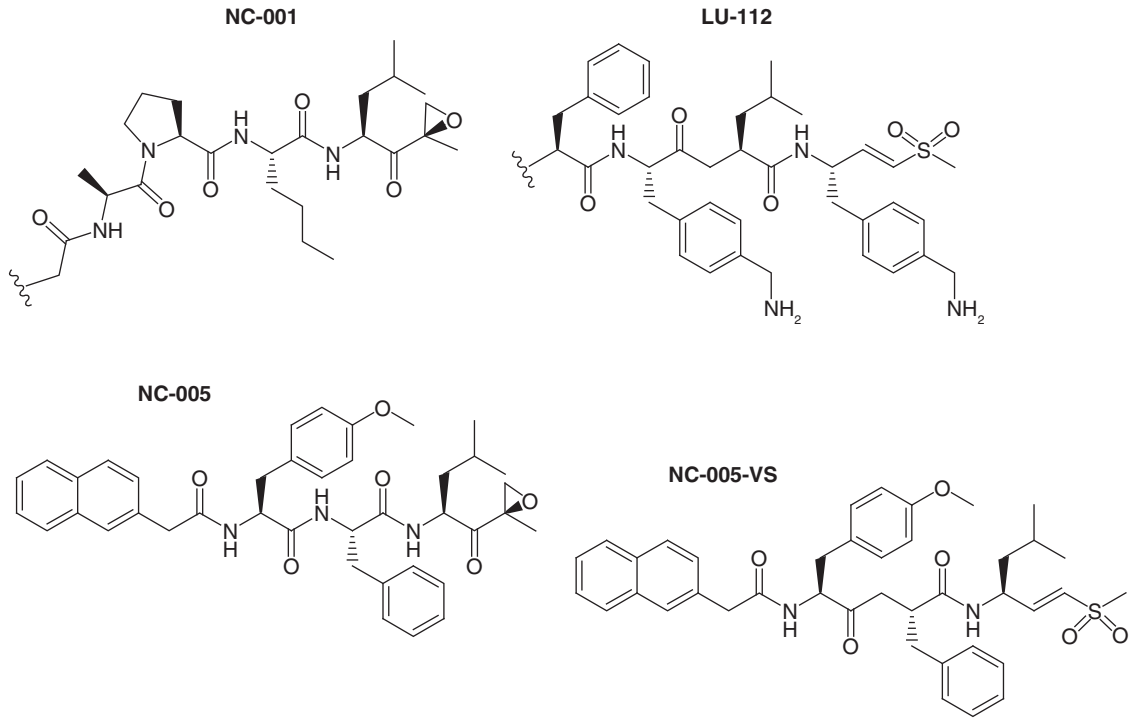

Figure 5. Structures of subunit-specific probes. Subunit-specific probes have enhanced the understanding of the proteasome and its activity in disease states such as cancer. NC-001 is specific to the β1/β1i subunits. LU-112 is specific to the β2/β2i subunit. NC-005 is the parent compound of NC-005-VS. Both of these inhibitors target the β5 subunit, but exchanging the epoxyketone of NC-005 for the vinyl sulfone shown in NC-005-VS provides more specificity to the β5 subunit. These probes have been conjugated to a variety of fluorophores and used to study the composition and activities of proteasomes in disease states and a multitude of cell types. Additionally, they have been used to monitor binding of small molecules to proteasome subunits.

benzylamines in both of these positions resulted in the most potent and specific β2 subunit inhibitor of all the chemical groups tested. Experiments to determine potency and specificity of all analogs were conducted in HEK293 cell lysate. Interestingly, LU-112 was less effective at inhibiting the β2 subunit of purified 26S proteasomes than proteasomes in HEK293 cell lysate, highlighting the importance of thoroughly characterizing the binding profiles of each subunit-specific probe. These data serves as a caution that results obtained from testing inhibitors with purified proteasomes do not always translate to cell lysate or intact cells.

Finally, NC-005-VS was initially developed to contain an epoxyketone warhead to facilitate binding the β5 subunit. This structure was based on a previously discovered inhibitor, the peptide aldehyde1-naptylacetyl(Nac)-4-methyl-tyrosine(mTyr)-phenylalanine-4-methyl-tyrosinal [57,58]. This inhibitor showed remarkable specificity to β5, and an epoxyketone derivative with a leucine residue was synthesized to produce NC-005. Excitingly, NC-005 has been shown to be cell permeable while retaining its specificity to the β5 subunit. Decrease in activity of the β1 and β2 subunit was not observed until almost complete inhibition of the β5 subunit. However, one drawback of this probe is that it also binds the β5i subunit. As is the case with NC-001, careful consideration of sCP and iCP expression must be utilized before using this probe to monitor proteasome activity. Although this probe is specific, further research in which the warhead was exchanged for a vinyl sulfone (NC-005-VS) revealed it was more specific to the β5 subunit than the epoxyketone derivative, further solidifying that the warhead does indeed contribute to subunit specificity [59].

Each of these subunit-specific probes has been developed into an activity probe by attaching a fluorophore to function as a reporter. Because the binding between the probes and the proteasome subunit is covalent, the resulting complexes can be run on SDS-PAGE and the gel can be directly imaged for fluorescence. Bands corresponding to each proteasome subunit can be visualized on the gels and the intensity can be quantified, providing a basis to determine the amount of each subunit present in a sample. These inhibitors have also been developed into probes that monitor proteasome activity through fluorescence resonance energy transfer (FRET), in which each probe

is conjugated to a fluorophore that, when excited, transfers energy to a second fluorophore in close proximity, which in turn emits a signal [60]. Because the catalytic proteasome subunits are close enough to facilitate FRET, sets of fluorophores conjugated to these subunit-specific probes have been used in cell lysates to monitor proteasome activity and composition [54].

Techniques to screen noncatalytic subunits of the proteasome for small molecule binders

Screening proteins for small molecule binders whose functions are unknown or that have no catalytic activity presents a unique set of challenges. Noncatalytic subunits compose the majority of the proteasome, but with no measurable enzymatic activity, screening them for small molecule binders can be difficult [29]. Although they do not carry out cleavage of peptides, these noncatalytic subunits play key roles in protein degradation, such as recognition of ubiquitinated proteins, opening and closing of the gates of the sCP to allow entry of substrates into the catalytic chamber and facilitating linkage of the RP and sCP [4]. Changes in expression of certain noncatalytic subunits are implicated in cancer [6]. Therefore, modulating the binding of these subunits through small molecules could enhance our understanding of the proteasome and its implications in disease. In recent years, several new techniques have emerged for screening nonenzymatic proteins and have enhanced the ability to discover new binders of proteasome subunits. Many of these screening methods utilize one-bead-one-compound libraries (OBOCs). The creation of OBOCs has made the process of synthesizing thousands of compounds streamlined and cost-effective [61]. Screening OBOCs against proteasome subunits has resulted in the discovery of several small molecule binders. Full characterization of these ligands and their interactions with the proteasome has enhanced our knowledge of the proteasome and revealed new subunits as potential drug targets for cancer and other diseases. In this section we will review common techniques to screen proteasome subunits for small molecule binders and discuss newly developed screening methods.

Screening OBOCs against proteasome subunits with magnetic beads

Magnetic beads are among the most commonly used tools to screen OBOCs against a biological target of interest [62]. This method entails incubating a protein of interest with beads of an OBOC. Next, excess unbound protein is washed away and the beads are incubated with an antibody specific to the protein. Only beads that are bound to the protein will be coated in the antibodies. Then the beads are exposed to magnetic beads decorated with a secondary antibody that binds the beads conjugated to the protein and primary antibody. 'Hit' beads that successfully bind the protein and are subsequently recognized by the primary and secondary antibody linked to a magnetic bead can be physically separated from non-hits using a strong magnet [63]. The identity of the small molecule on the library bead can then be established by a variety of mass spectrometry techniques or, if it is a DNA-encoded library, by sequencing the DNA (Figure 6) [64]. Although this method relies on several binding events to identify a hit ligand to a protein of interest, it has been successfully used to discover ligands for several oncoproteins and even proteasome subunits.

Recently, Rpn-13, a ubiquitin receptor found in the 19S RP, was screened against an OBOC consisting of 100,000 compounds using magnetic beads [65]. Six initial hits were discovered from this screening process. As a result, a library of derivatives from the top two hits was synthesized and screened against Rpn-13 using a fluorescence polarization assay in which each ligand was tagged with fluorescein and allowed to incubate with varying concentrations of Rpn-13. Binding of Rpn-13 to a ligand alters the ligand's ability to polarize light, and this change can be quantified [65,66]. This follow-up screen to the magnetic bead screen resulted in KDT-11, a peptoid composed of six amines (Figure 7). Excitingly, KDT-11 was shown to be toxic to multiple myeloma cells, which are known to rely heavily on proteasome activity for survival. It was not as toxic to HEK293T cells, a cell line that does not require enhanced proteasome activity for proliferation. KDT-11's affinity to Rpn-13 was determined to be ~2 μM, making it a moderately strong binder [65].

Although magnetic bead screening has been used successfully to identify ligands to proteins of interest, this method has several drawbacks, one of which is the likelihood of false positives. These are very common as a result of the multiple binding steps required to identify a hit, and it is not uncommon for library beads and magnetic beads to interact nonspecifically, resulting in a false hit [67]. To circumvent this issue, some groups have turned to using redundant OBOC libraries [68]. Because numerous binding and washing steps are required for this method, a strong interaction is required between ligand and protein. As a result, lower-affinity ligands are left out of this screening method, although they might be desirable depending on the application of hits after the screen. The final limitation of this screening method is the inability to prioritize hits. As a result, it is not possible to determine

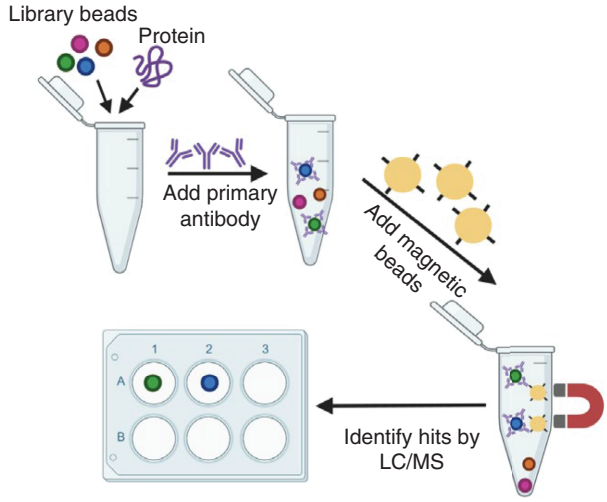

Figure 6. Screening one-bead-one-compound libraries with magnetic beads. The use of magnetic beads has enhanced the process of discovering new ligands to proteins of interest. Briefly, a protein incubates with beads of a one-bead-one-compound library, then a primary antibody against the protein is added and will only interact with beads bound to the protein. Magnetic beads coated with a secondary antibody are then added and bind only beads bound to the protein/primary antibody. A strong magnet is used to separate hits from non-hits and techniques such as LC/MS are used to identify the small molecule.
LC/MS: Liquid chromatography mass spectrometry.

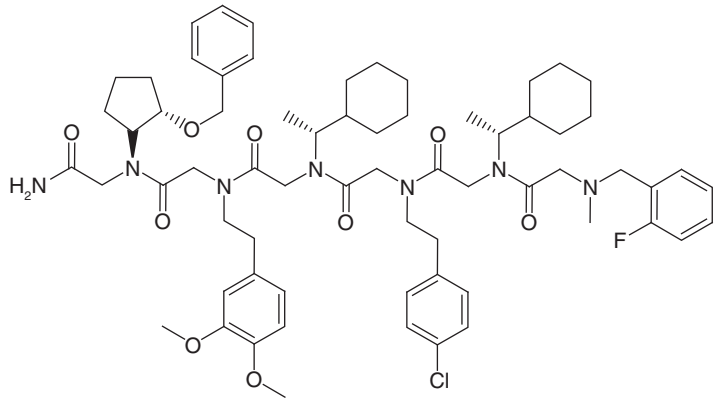

Figure 7. Structure of KDT-11. KDT-11 was identified as part of a screen against a one-bead-one-compound library using magnetic beads. It binds Rpn-13 with a K_D of ~2 μM and is toxic to multiple myeloma cells.

which hit provided the strongest interaction with the protein and which hits have lower affinities. Knowing this information from the initial screen would be useful in determining which hits are worth validating.

Newly developed techniques to screen OBOCs against proteasome subunits

In an effort to improve upon this screening method and reduce the limitations presented by this technique, our lab has recently designed a method to screen OBOCs in which hits can be prioritized [69]. By expanding and improving upon the techniques used to screen OBOCs, the process of identifying small molecules that bind proteasome subunits will become more streamlined. Our method relies on labeling a biological target of interest with a near-infrared range fluorophore [69]. The labeled protein is incubated with beads of an OBOC that have been individually split into the wells of a 96-well plate. The plate is then imaged for near-infrared fluorescence. The fluorescence intensities that result from the labeled protein binding a small molecule on bead can be quantified and provide a basis to rank hits (Figure 8). As a proof of concept, we allowed fluorescently labeled carbonic anhydrase to interact with several ligands of varying affinity synthesized on-bead [70]. Excitingly, clear differences were observable between

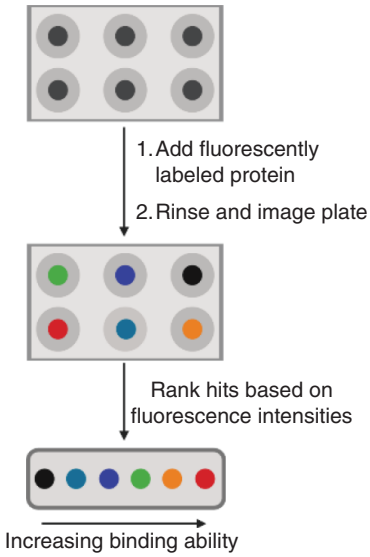

Figure 8. Screening scheme for one-bead-one-compound libraries using fluorescently labeled Proteins. Our new method to screen one-bead-one-compound libraries involves labeling a protein of interest with a near-infrared fluorophore. The protein is then incubated with beads from a one-bead-one-compound library that have been individually separated into the wells of a 96-well plate. Beads are then rinsed and the plate is imaged for fluorescence. The fluorescence intensities that result from the labeled protein binding the ligand on-bead can be quantified and provide a basis to rank hits.

low-, moderate- and high-affinity ligands in their binding affinities to carbonic anhydrase. Screening for a better Rpn-13 binder is currently ongoing. Because hits can be prioritized based on the initial screen, this method has potential to expedite the protein screening process. Similarly, the function of the protein does not need to be known, making this method an attractive way to screen noncatalytic subunits of the proteasome.

Another method that has recently been used to screen proteasome subunits against an OBOC is the thermal shift assay. This technique relies on the use of fluorescent dyes that bind hydrophobic regions of a protein [71]. Small molecules are cleaved from beads of an OBOC and a fixed concentration is added to the wells of a 96- or 384-well plate. Next, a fixed amount of a target protein is added, along with the fluorescent dye, and the protein/small molecule mixture is heated slowly. Over time, as the temperature rises, the protein begins to unfold, resulting in the exposure of hydrophobic regions that are normally not exposed to the environment. The fluorescent dye binds these regions and the fluorescence intensity can be quantitated [71]. The temperature at which the fluorescence intensity is maximal is the melting temperature of the protein (Figure 9A). The melting temperature changes if the protein binds a small molecule. For example, a small molecule that binds and stabilizes a protein could prevent it from denaturing, resulting in a melting temperature higher than that of the protein alone. Changes in melting temperature are used to determine whether a small molecule has bound a protein. Our lab has recently used this technique to identify a peptoid, TXS-8, that binds Rpn-6, a subunit of the 19S RP that facilitates linkage of the RP to the sCP (Figure 9B) [72]. Excitingly, TXS-8 is toxic to Burkitt lymphoma cells, which are known to rely more heavily on proteasome activity than noncancerous cells.

Techniques to discover & validate the activity of small molecule proteasome stimulators

While inhibitory drugs of the proteasome system have shown clinical applicability since the discovery of the first generation of inhibitors in 1994 [74], research in the proteasome stimulation field has lagged due to several challenges. Among them are a compensatory mechanism in the protein homeostasis network sustained by the autophagy pathway, the complexity of this macromolecular system, and a lack of tools to monitor proteasomal stimulation [74]. However, efforts have been made to overcome these challenges, and different approaches have been developed to expand our repertoire of tools and strategies to stimulate the proteasomal system. These approaches include genetic manipulation or utilizing small molecules that can interact with the sCP to increase its rate of degrading a substrate.

Discovery of proteasome stimulators: high-throughput screening assays

Considering that high-throughput screening is pivotal in the drug discovery process, efforts in the proteasome stimulation discovery field have aimed to develop tools that allow screening of large libraries of compounds. Since the discovery that aromatic lactones such as coumarins can act as short-wavelength fluorescent dyes, these compounds have been utilized as biochemical tools in several fields of health science [75]. Several fluorogenic peptide substrates have been developed to monitor proteasomal activity, and different fluorophores such as 2-naphthylamine and

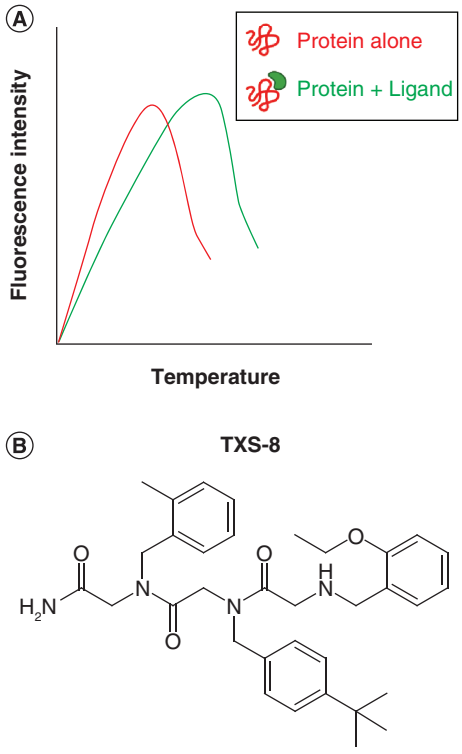

Figure 9. Identification of TXS-8 through a thermal shift assay. (A) Thermal shift assays rely on changes in the melting temperature of a protein in response to binding a small molecule. Fluorescent dyes that bind hydrophobic regions of proteins are used to monitor denaturation as the protein is heated. When a small molecule binds and stabilizes a protein, the melting temperature is increased and this shift can be quantified by measuring fluorescence intensity. **(B)** The structure of TXS-8, a peptoid binder of Rpn-6 identified from a thermal shift assay.

4-methoxy-2-naphthylamine have been utilized for such purposes; however, 7-amino-4-methylcoumarin (AMC) has the highest fluorescence strength (quantum yield) of these fluorophores and thus the most effective readout [76]. Peptide substrates containing AMC were originally developed in 1976 as an attempt to develop more sensitive fluorogenic tools with which to study the amidase activity of chymotrypsin [77]. Since then, AMC peptides have been extensively used to study the activity of several proteases [78–80]. In fact, since the discovery of the 'multicatalytic proteinase complex' in 1983 [81] – known today as the 20S isoform of the proteasome – AMC substrates have been employed to understand its mechanism, as well as in drug discovery campaigns. The Suc-LLVY-AMC substrate (Figure 10), first introduced in 1984 in a kinetic study of fluorogenic substrates on porcine calpain I and calpain II [82], is perhaps one of the most widely used fluorogenic substrates for the study of proteases with chymotrypsin-like activity, including the proteasome. In this substrate, the amidation of the peptide sequence to the coumarin scaffold quenches its fluorescence; upon cleavage of the tyrosine–AMC amide bond, the fluorescence is restored and can be monitored.

Proteasome activity assays using these substrates are performed using purified enzyme, and the change in fluorescence over time, mediated by the release of AMC, is measured using a spectrofluorometer or a plate reader. The rate of hydrolysis can be obtained and a comparison between the rate of cleavage of the reporter after an incubation period with different molecules of interest, and a control sample, can be used to assess the ability of molecules to modulate proteasome activity [76].

Despite the widespread use of AMC substrates in the proteasome field, their use in monitoring stimulation can be difficult. The AMC–peptide substrates are cleaved by the proteasome at a high rate in the absence of stimulatory molecules. This is partially because the probes are only a few amino acids long and readily enter the core of the sCP [83]. For this reason, our lab developed a FRET peptide (Figure 10) using DABCYL and EDANS as the FRET acceptor and donor, respectively [83]. This larger substrate has a relatively lower hydrolysis rate and a wider sensitivity window compared with the Suc-LLVY-AMC reporter, overcoming limitations such as false-negative results. As a result of its wider sensitivity window, this probe also facilitates discovery of weak stimulatory molecules.

While these assays with fluorogenic substrates are convenient and amenable for high-throughput screening, they do not mimic the endogenous substrates of the proteasome; therefore, potential molecules that increase the rate of hydrolysis of these reporters may not lead to enhanced cleavage of more physiologically relevant and longer substrates. For this reason, a more stringent and nonfluorogenic method using a quantitative mass spectrometry-based approach was developed [84]. In this assay, a 9-mer peptide with an N-terminal fluorenylmethoxycarbonyl

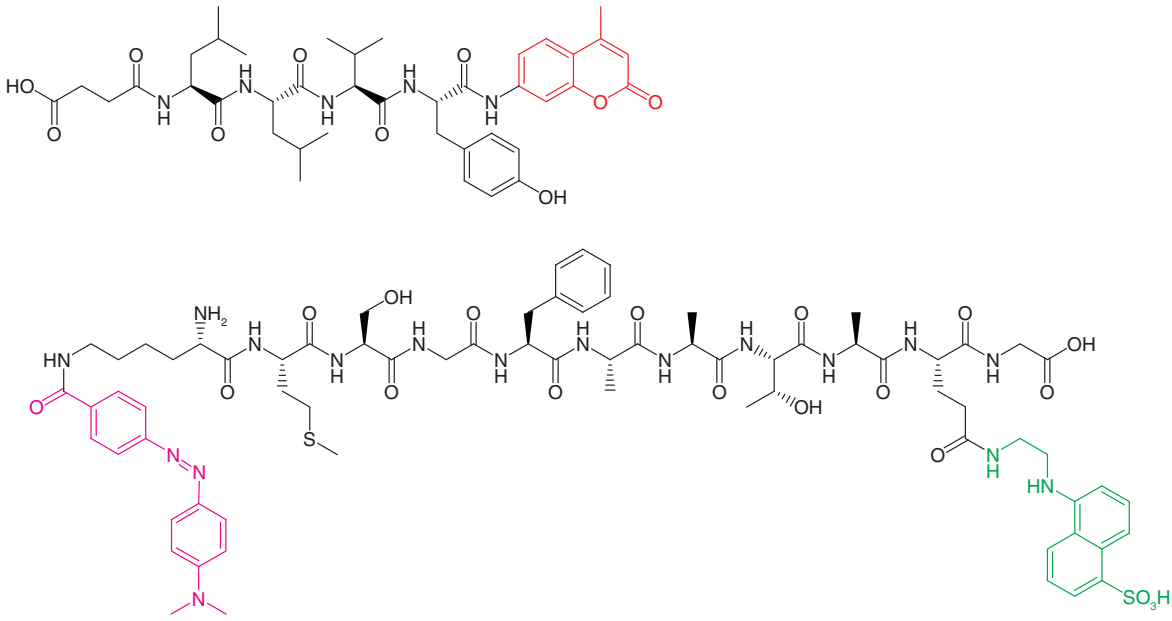

Figure 10. Structure of fluorogenic peptides used in high-throughput screening assays. (A) Structure of the Suc-LLVY-AMC substrate. In red is highlighted the 7-amino-4-methylcoumarin scaffold. **(B)** Structure of the FRET peptide using EDANS (green) and DABCYL (magenta) as fluorescence pair. FRET: Fluorescence resonance energy transfer.

group incorporated can be used to assess the ability of different molecules to increase the proteasome's catalytic activity. This method is not as high-throughput as a fluorescence assay, but can be employed for the analysis of large peptide substrates.

Discovery of proteasome stimulators: validation & dynamic structural studies

Following the identification of compounds with the ability to increase the turnover of peptide substrates from large libraries, cellular and gel-based assays are performed to determine efficacy of the molecules in more realistic environments; additionally, drug properties such as cell permeability and selectivity are required in a hit-to-lead process.

Gel-based assays constitute a simple and reproducible method to measure protein turnover using purified proteasome and substrate [83–86]. The protein substrate is incubated in Tris or HEPES buffer in the presence or absence of purified sCP. After running the samples on SDS-PAGE, they can be Coomassie-stained or immunoblotted. The amount of protein remaining is then quantified based on the intensity of the desired gel band. Next it is compared with the DMSO-treated sample in order to assess the small molecule's ability to increase the endogenous protein's cleavage as mediated by the proteasome. This method is commonly used to test proteasome stimulators for their ability to degrade a protein substrate [87]. Kinetic analysis can be performed by incubation at different time points, and the nature of protein substrate can be changed; α-synuclein and tau, two proteins known to be overexpressed in Alzheimer disease that result in oligomers that slow proteasome activity [88], have been used in these assays.

In a similar fashion to the AMC-based and FRET reporter assays, fluorescence can be used to assess a compound's ability to enhance proteasome activity in cells. However, considering the poor fluorescence signal obtained from AMC reporters and the high concentrations required to achieve satisfactory results, using these probes in live cells can lead to confusing results. For such a reason, our laboratory has developed a set of activity-based peptide–peptoid hybrid probes (TAS1–3) that incorporate variants of the LLVY recognition sequence of the chymotrypsin-like catalytic subunit of the sCP, a rhodamine-110 (Rh110) scaffold as fluorophore and a peptoid sequence to improve cell permeability and solubility (Figure 11A) [45]. Inspiration for these sCP probes came from the development of an activity probe to monitor the immunoproteasome in live cells [44]. These TAS probes are sensitive to both inhibitory and stimulatory molecules and can be used in live cells. Interestingly, it was demonstrated from the development of these probes that unnatural amino acids can be incorporated into activity-based probes and be recognized by the proteasome; additionally, these probes showed higher sensitivity compared with the 4 Suc-LLVY-AMC probe. A

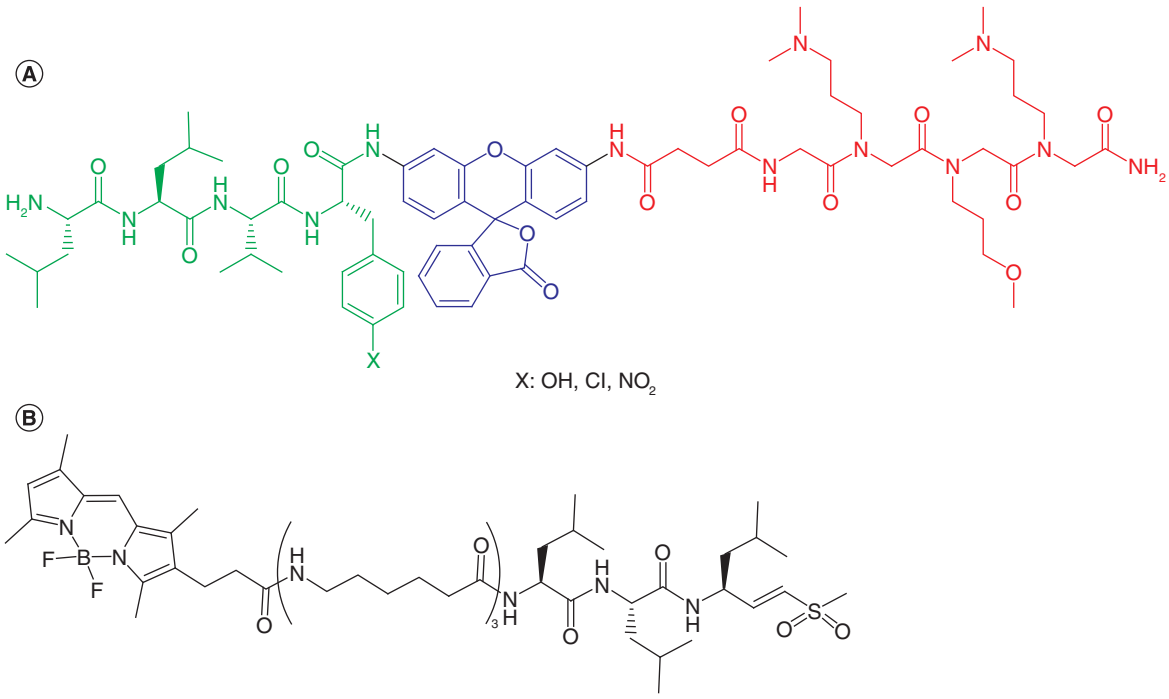

Figure 11. Newly developed probes to monitor proteasome activity. (A) Structure of the TAS probes, highlighting the 20S isoform recognition sequence in green, where TAS1 contains a tyrosine residue (X: OH), TAS2 a 4-Cl phenylalanine residue (X: Cl) and TAS3 a 4-NO2 phenylalanine residue. **(B)** Structure of covalent proteasome activity probe that can react with all three active sites.

kinetic analysis can be performed by comparing the rate of hydrolysis of the small molecule-treated sample versus the DMSO-treated control.

A number of covalent activity probes that can also monitor proteasome stimulation have been developed by the Ovaa group. Their probes are based on the covalent inhibitors MG132 and epoxomicin and include a fluorophore such as BODIPY (Figure 11B) [89]. They are effective in live cells or tissue. The sample is treated with the probe and then the cell lysate or tissue sample is subjected to analysis by SDS-PAGE. The amount of fluorescent signal observed in the gel at the molecular weight for any of the three active sites of the proteasome can then be quantitated. The group demonstrated the utility of these probes to identify a number of small molecule proteasome stimulators [90].

Since the discovery of GFP, fluorescent fusion proteins have been widely used to monitor proteins of interest in cells [91–93]. In fact, this strategy has also been employed to study the sCP when intrinsically disordered proteins such as α-synuclein or tau are fused to GFP [84]. Given the size and structured nature of GFP, the sCP cannot degrade it without help from the 19S RP to unfold it; however, the unstructured portion of the fused system can be cleaved by the sCP alone, meaning that the ratio of free GFP to the GFP fusion protein can be used to quantify the relative increase in sCP activity. The Tepe group has also used this system to monitor the degradation of ornithine decarboxylase, an endogenous protein substrate of the sCP [94]. It is noteworthy that this method measures the ability of the proteasome to degrade protein substrates in a ubiquitin-independent manner if the ratio of free GFP to fused GFP is quantitated, rather than just the decrease of the fusion protein.

A biophysical method that can be used to determine whether a small molecule is a stimulator of the sCP by affecting its gate opening is atomic force microscopy (AFM). This technique was developed in 1986 and has gained popularity as a tool because it uses liquid samples rather than crystals during the evaluation process [95,96]. Considering the complex multi-subunit nature of all proteasomal assemblies, it is to be expected that they display a high level of structural dynamics to comply with their biological processes [96]. In fact, the open and closed conformational states of the sCP correspond to the most prominent examples of conformational diversity. Conveniently, this high conformational degree, along with its colossal size, makes the proteasome suitable for AFM imaging because the different shapes can be recognized. The technique involves three steps: proteasome preparation, AFM

imaging and analysis of proteasome particles. The proteasomes are first immobilized for imaging, either electrostatically using a mica surface or using a His-tag and nickel-coated support system, and then the sample is placed into the AFM chamber. After tuning the microscope and engaging the probe for imaging, and once the quality of the image is acceptable, the scanning session is initiated. To assess the small molecule-mediated conformational effect on the proteasomal system, the ligand is gently injected into the chamber and the scanning is continued for several fields. The topography of the proteasomal complex is then analyzed by observing the α-rings section and the two states are determined by the observation of a concave surface on the edge (open state) or a central cavity (closed state) [96,97]. The ability of a small molecule to modulate the proteasome's gate dynamics is determined by the percentage increase in open-state conformations compared with a DMSO-treated control sample. This method was recently demonstrated by the Tepe group to classify the amount of sCPs that are in an opened conformation when dosed with a small molecule stimulator [86].

Future perspective

While inhibition of the active sites of the proteasome has been thoroughly studied and therapeutics developed, a number of other methods can be used to perturb proteasome activity to elicit a desired therapeutic response. Several interesting studies targeting the deubiquitinase activity of Rpn-11 (a subunit of the 19S RP) with small molecule inhibitors showed they could elicit cell death in a variety of cancers that require high proteasome activity to survive [98,99]. There are currently only a handful of small molecule binders to any of the 19S RP protein subunits.

New methods and small molecules are needed that bind the other subunits of the 26S/20S proteasome. However, in order to screen for new molecules that bind certain subunits, new methods that allow for the production and correct folding of the proteasome are required. Many subunits do not fold properly or may form different structures when they are not interacting with other proteasome subunits. This means that it may be better to develop cell-based systems with reporters for binding interactions. Methods such as microscale thermophoresis could also be used to evaluate the binding of a molecule to purified 26S/20S proteasome.

In addition to affecting proteasome assembly, the application of proteasome stimulators needs to be further evaluated. As described here, many tools have been developed to discover and quantify the amount of proteasome stimulation that can be induced by a small molecule. More in-depth studies on the cellular ramifications of increasing the ubiquitin-independent degradation of proteins are needed. These would include examining whether the potential protein substrates of the sCP can change in the presence of a stimulator, or if just more of the endogenous substrates are degraded. Additionally, are the peptide products from the degradation of a protein in the presence of a sCP stimulator different from the natural peptide degradation products? These questions must be addressed before the therapeutic relevance of a sCP stimulator can be determined.

Overall, the targeting of the proteasome with a small molecule remains an interesting field and one that is rapidly changing. The traditional inhibition of the active site subunits has been very fruitful, but there are many other mechanisms to change the natural activity of the proteasome and these should be investigated.

Executive summary

Background

- The proteasome can degrade proteins through both ubiquitin-dependent and ubiquitin-independent mechanisms.
- Small molecules have been discovered that can inhibit one or more of the catalytic subunits of the core particle.

Proteasome inhibitors

- Traditionally, the proteasome is inhibited using a covalent molecule that binds with one of the three catalytic active sites the proteasome uses to hydrolyze proteins into peptides.
- These scaffolds have also been used to generate activity-based probes.

Methods for discovering binders to proteasome subunits

- For proteasome subunits that can be expressed and fold correctly from bacteria or mammalian cell culture, several methods for screening for binders have been developed.
- These methods include thermal shift and fluorescence-based on-bead screening assays.

Proteasome stimulators

- A number of activity probes have been developed that allow for the detection of an increase in proteasome activity, either using purified enzyme or in live cells.
- These probes can also be used to evaluate the effect of small molecule stimulators and quantitate the amount of stimulation.

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•• **Improved probe for monitoring proteasome activity in live cells.**

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