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20S Proteasome Hydrolysis of LLVY Substrates to Determine Preferences for Moieties in its Primed Substrate Channel

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Abstract

The proteasome is an essential multi-catalytic enzyme in cells that is responsible for degrading proteins with a ubiquitin-dependent or -independent mechanism. Many activity-based probes, inhibitors, and stimulators have been developed to study or modulate the activity of the proteasome. The development of these proteasome probes or inhibitors have been based on their interaction with the amino acids of the β5 substrate channel proceeding the catalytically active threonine residue. There is potential for positive interactions with a substrate to increase selectivity or cleavage rate with the β5 substrate channel after the catalytic threonine as evidenced by the proteasome inhibitor belactosin. To study what moieties the proteasome could accept in its primed substrate channel, we developed a liquid chromatography-mass spectrometry (LC-MS) method to quantitate the cleavage of substrates by purified human proteasome. This method allowed us to rapidly evaluate proteasome substrates that contain a moiety that could interact with the S1' site of the β5 proteasome channel. We were able to determine a preference for a polar moiety at the S1' substrate position. We believe this information can be used in the design of future inhibitors or activity-based probes for the proteasome.

Graphical Abstract

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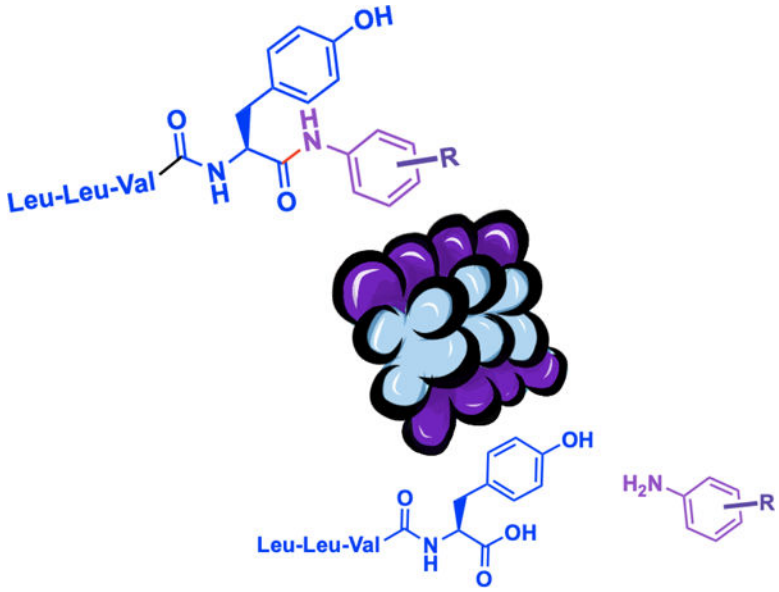
Associated Content

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2023.129233.

Conflict of interest

Prof. Trader is a shareholder and consultant for Booster Therapeutics, GmbH. Other author declares no conflict of interest.



The proteasome is a multiprotein, multicatalytic enzyme responsible for approximately 80% of protein degradation in the cell. The proteasome's 20S core particle (CP) is a barrel-like structure that houses three catalytic subunits with different cleavage specificities: where its β1 (caspase-like) subunit hydrolyzes after acidic amino acids, its β2 (trypsin-like) subunit cleaves after basic residues, and finally, its β5 (chymotrypsin-like) subunit after large and hydrophobic amino acids.[1–4] Many peptide-like inhibitors for the 20S, such as bortezomib and carfilzomib, have been designed to fit into the β5 unprimed specificity pockets (S1 through S4), Figure 1A, and contain an electrophilic warhead that covalently links to the catalytic threonine (Thr1).[5,6] Attention to the specificity pockets in the unprimed substrate channel have also allowed for selective inhibitors for the constitutive 20S proteasome (sCP) over its other major isoform, the immunoproteasome (iCP), that is expressed under inflammatory conditions.[7,8] In addition to sCP or iCP-selective inhibitors, many activity-based, luminescent, and fluorescent probes of various β-subunits have been developed to monitor the 20S proteasome's activity biochemically or in cells,[9–13] with β5 fluorogenic substrates shown in Figure 1B. These β5-specific probes have the common peptide recognition sequence of Leu-Leu-Val-Tyr (LLVY) that interact with the specificity pockets S1 through S4.

Despite the availability of many probes, little is known in regard to what the 20S proteasome can accept in the *primed* substrate channel, which is on the other side of the Thr1. One 20S inhibitor, belactosin A, and its analogs have been demonstrated to interact and inhibit the proteasome on the unprimed *and* primed substrate channel, and extensive structural-activity relationship (SAR) studies show that binding can be enhanced in the primed substrate channel, Figure 1C.[14–17] While this is an example that this inhibitor can benefit from contacts with the primed substrate channel, it is unclear if this is also true for activity probes or what other molecular groups that proteasome can accept in that S1' position. Varying hydrolysis or reactivity rates of probes by the 20S could be beneficial depending on the goal, such as monitoring how proteasome modulators (stimulators or inhibitors) perturb activity.

Here, we describe the evaluation of known LLVY-conjugated fluorogenic probes and new LLVY-conjugated unnatural substrates for the β5 catalytic subunit through a quantitative liquid chromatography - mass spectrometry (LC-MS) assay. We observed that the 20S proteasome accepted and cleaved the 7-amino-4-methylcoumarin (AMC) moiety significantly faster than rhodamine 110, indicating that rhodamine's bulky structure slows its ability to enter into the latent 20S for hydrolysis. While investigating nonfluorescent LLVY-R substrates, we determined that the 20S proteasome has a preference for substrates with slightly polar groups at the S1' position. Our results should be beneficial for guiding future development of 20S proteasome β5 inhibitors and probes.

Mass Spectrometry Analysis of LLVY Standard Curve Demonstrates Low Limit of Detection

Mass spectrometry (MS)-based enzymatic assays have been a rapid, label-free, and sensitive method to quantitate efficiency of substrate cleavage activity,[18,19] and we wanted to apply its versatility to analyze different Leu-Leu-Val-Tyr-conjugated substrate analogs' ability to be hydrolyzed by the sCP, Figure 2A.

Typically, small activity-based substrates of the proteasome have utilized fluorescence to monitor rate of hydrolysis. Since the different LLVY-conjugated substrates we explore in this study are non-fluorescent, we chose to use a single quadrupole LC-MS because they are accessible yet sensitive instruments. Before testing different substrates with the sCP, we determined the limit of detection (LOD) and limit of quantitation (LOQ) of the peptide recognition amino acid sequence that remains constant in the fluorogenic substrates in this study, or LLVY, Figure 2B. We determined the quantitation range to be between 20 to 1250 pmol. The low limit of quantitation of 20 pmol allows for us to detect small changes in hydrolysis activity between different LLVY-conjugated substrates.

The 20S Proteasome Cleaves Aminomethylcoumarin-conjugated Substrate Significantly Faster by MS.

To begin our studies, we first decided to characterize known β5 fluorogenic substrates. We evaluated if there were differences in sCP hydrolysis of AMC and rhodamine 110, which are the fluorophores for Suc-LLVY-AMC and TAS-1 or $(Suc\text{-}LLVY)_2Rh110$, respectively, and the binding of the fluorophore should near the S1' binding pocket. Since Suc-LLVY-AMC contains a succinate group on the *N*-terminus, it would not be able to ionize in positive mode MS and could not be directly compared to TAS-1, which has a free *N*-terminal LLVY conjugated to its fluorophore. We synthesized LLVY-AMC without the succinate group and compared this probe with commercially available Suc-LLVY-AMC by fluorescence to ensure cleavage rate was similar (Supporting Figure S1). While comparing AMC and Rh110 probes at 10 μM with 5 nM sCP over 1 hour, the relative fluorescent signal of TAS-1 was significantly higher than Suc-LLVY-AMC and our succinate-free fluorogenic probe, LLVY-AMC. $(Suc\text{-}LLVY)_2Rh110$ fluorescent signal was half of TAS-1, which we suspect is due to quenching from the neighboring Tyr after hydrolysis of one LLVY (Supporting Figure S1).

Though TAS-1's free rhodamine had a higher fluorescent signal, we anticipated actual cleavage of TAS-1 to be slower than LLVY-AMC due to the steric hindrance of rhodamine and therefore its potential difficulties of entering into the substrate channel of the sCP. By time-dependent MS analysis, LLVY-AMC was hydrolyzed by the sCP faster than TAS-1, Figure 2C. It is likely the LLVY-AMC substrate is cleaved faster due to its small size and ease at which it can enter the catalytic core as compared to TAS-1. Even though LLVY-AMC is hydrolyzed faster by the proteasome, AMC's weaker fluorescence properties require higher concentrations of probe and sCP to study sensitive and small changes in proteasome activity modulators, especially in live-cell cultures.

Smaller Aromatic Groups Conjugated to LLVY are Hydrolyzed Slower than TAS-1

To understand more how the proteasome accepts and cleaves unnatural substrates, we wanted to evaluate how other groups could be cleaved by the sCP, Table 1 (Supporting Information Scheme 1). Our first set of molecules included a variety of alkyl or benzyl groups to take an initial assessment of the tolerance of the sCP to accept these types of molecules as substrates. Surprisingly, they were cleaved very poorly, yielding little to no LLVY detected in the LC/MS assay (Figure 3, molecules **1**-**6** and Supporting Information Figure S2). After this result, we decided to focus on molecules that mimicked the structure of the fluorogenic probes, *i.e.* incorporating aniline derivatives with varying substitutions on the aromatic ring.

First, we analyzed how unsubstituted aniline, naphthalene, and anthracene would be hydrolyzed by the sCP. These substrates (molecules **7**-**10**) act as hydrocarbon and nonpolar versions of AMC, rhodamine 110, and other aromatic scaffolds that could be used for fluorescent or luminescent probe development. Again, we were surprised that molecules 7–10 were hydrolyzed significantly slower than sterically hindered TAS-1 (Figure 3). Despite being similar sizes to the overall AMC and rhodamine's carbon skeleton, these substrates could not be as efficiently degraded by the sCP (Supporting Information Figure S3). Compound **8** remained completely intact after sCP incubation, likely due to orientation and steric bulk of the naphthyl group. Taking a closer look at the trend of these conjugated substrates, it appeared that the more extended conjugated R groups are cleaved faster by the sCP, suggesting that space-filling groups in the primed substrate channel could help stabilize the substrate long enough for hydrolysis by Thr1. Additionally, molecules **9** and **10** are unsubstituted carbon versions of LLVY-AMC's coumarin and TAS-1's xanthene moiety, respectively, but the fluorogenic probes are cleaved at a significantly higher rate. Coumarin and xanthene may be more acceptable heterocycles in the substrate channel due to their added polarity, but more studies are needed to confirm this hypothesis.

For further investigations of what unnatural substrates the proteasome is able to cleave via the β5 channel, we measured hydrolysis with various direct and indirect electron-donating and electron-withdrawing groups in the *para* and *meta* position of aniline, molecules **11** through **23**. We had expected that the inductive electron-withdrawing groups could enhance

hydrolysis due to the increased electrophilicity of the amide bond between LLVY and the R group. However, molecules **11–14** showed no significant difference to aniline, molecule **7**.

From this set of substrates we observed that oxygen electron-donating groups (specifically when in the *meta* position) were able to be cleaved and obtain more free LLVY as compared to the previously tested molecules. For example, molecules **17** and **23** both have oxygens in the *meta* and *para* position, molecule **23** was cleaved the most out of the variety of aromatic R groups. This result indicates that polar R groups with less flexibility could engage the primed substrate channel leading to faster amide bond cleavage.

The catalytic threonine's reactivity of β5 does not appear dependent on the amide bond's electrophilicity (or lack thereof) being perturbed by the electron-donating or -withdrawing groups. We reason that the S1' and S2' in the substrate channel may prefer polar heteroatoms that could engage in dipole-dipole interactions or hydrogen bonding, mimicking a peptide or peptidomimetic scaffold. While this does not explain why molecule **18** (*para*-methyl) was able to cleave to the same extent as **16** (*para*-methoxy), this shows that more is to be explored regarding substrate preference in the primed substrate channel. Molecules **21** and **22** are cleaved at significantly different amounts by the sCP ($p \leq 0.001$), even though their major difference is a hydroxyl group in the *para* or the *meta* position, respectively. Since molecule **22** cleaves more than **21,** this once again suggests that direct electron donating effects do not impact hydrolysis rate and that rate could be more dependent on positioning of polar groups in the primed substrate channel.

To apply what we learned in our SAR studies of molecules **1** through **23**, we synthesized a different version of the LLVY-coumarin probe, Figure 4A, which uses 6-aminocoumarin instead of 7-amino-4-methylcoumarin as the R group. This molecule has the oxygen in the *para* position, as opposed to the LLVY-AMC, that has the oxygen in the *meta* position. Time-dependent MS analysis shows the sCP cleaved LLVY-AMC at a faster rate than the molecule with 6-aminocoumarin, Figure 4B. Despite the 6-aminocoumarin molecule's lower cleavage rate, it was still able to be cleaved by the sCP more efficiently than all other aromatic derivatives explored in this study, including TAS-1. This result again highlights the preference to have a polar and/or hydrogen bonding moiety at the *meta* position relative to the amide that is cleaved.

In the work presented here, we demonstrated the use of a single quadrupole LC-MS assay to evaluate how the sCP hydrolyzes a variety of LLVY-conjugated substrates. Differing from previous SAR studies that look into the sCP's unprimed substrate channel and its specificity, we investigated what types of substrates the sCP is willing to accept by changing what interacts with the *primed* substrate channel. After observing a low limit of detection in our assay, we studied known fluorogenic substrates that utilize aminocoumarin and rhodamine fluorophores. Through single time-point and also time-dependent MS studies, we found that R groups that had polar and/or hydrogen bonding groups in the *meta* position of an aromatic moiety at the S1' position was favored by the sCP. Interestingly, we found that sCP hydrolysis did not quickly hydrolyze substrates which had a more electrophilic amide bond (such as **11** through **14**), showing the sCP's hydrolysis rate may be more dependent on substrate engagement and orientation more than reactivity of the electrophilic moiety.

We then applied the knowledge we gained from this result by comparing two coumarin derived substrates, LLVY-AMC and LLVY-6-aminocoumarin, which followed the trend of *meta* directed oxygen groups being hydrolyzed at a faster rate. Additionally, with the sCP favoring unnatural polar substrates in the primed substrate channel, it would be interesting to explore if this preference also applies to polar natural amino acids, such as serine, threonine, glutamic acid, or aspartic acid. We anticipate that the results described here could contribute to future probe and inhibitor development to gain more selectivity for the β5 subunit and proteasome activity over other cellular proteases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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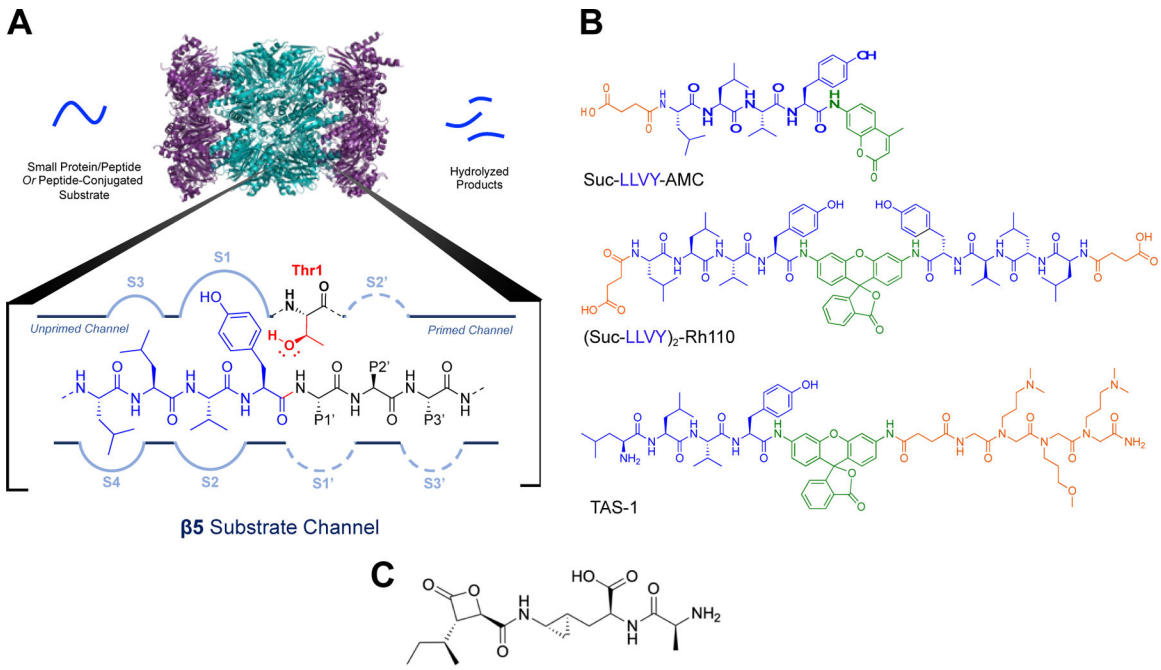

Figure 1.

(A) Once a substrate enters the sCP (PDB: 5LE5), it can be hydrolyzed by the β1, β2, and/or β5 catalytic subunit. Neutral and hydrophobic residues engage with the β5 unprimed substrate channel. (B) Several probes to monitor the activity of the β5 subunit have been developed. LLVY (blue) interacts with the unprimed substrate channel, and after being cleaved by Thr1, the aminocoumarin or rhodamine 110 moieties (green) produce a fluorescent signal. Regions of the probe that are added for non-specific protease stability are indicated in orange. (C) Structure of belactosin A, a 20S proteasome inhibitor that predominantly interacts with the primed substrate channel. The only aspects that do not interact with the unprimed pockets are the inhibitor's leucine group (which interacts with the S1 pocket) and the lactone (which reacts with the catalytic Thr1 for covalent inhibition).

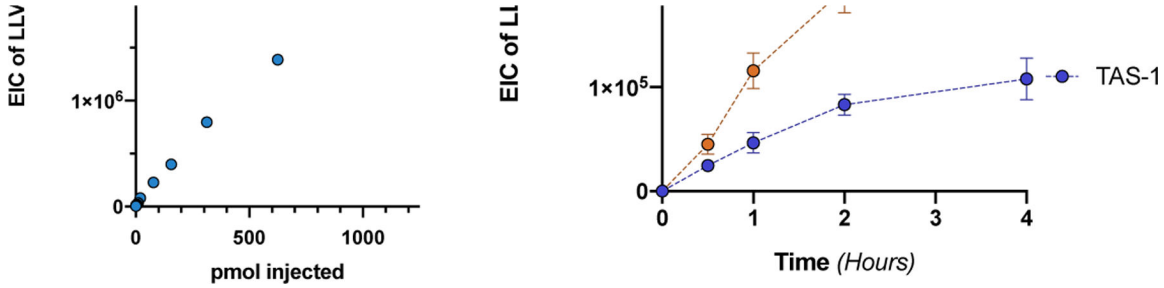

Figure 2.

(A) After incubation of an LLVY-conjugated substrate, such as TAS-1 or LLVY-AMC, with the 20S proteasome, a measurable amount of free LLVY will be present in the assay. By LC-MS, the EIC peak corresponding to free LLVY's [M+H] can be quantified and compared to other LLVY-conjugated substrates. (B) Standard curve of purified LLVY's pmol injected versus EIC of LLVY integrated. The limit of quantitation is 20 pmol. (C) LLVY-AMC was cleaved significantly faster by the 20S proteasome than TAS-1. Fluorogenic probes were approximately 21% and 7% cleaved after 4 hours, respectively.

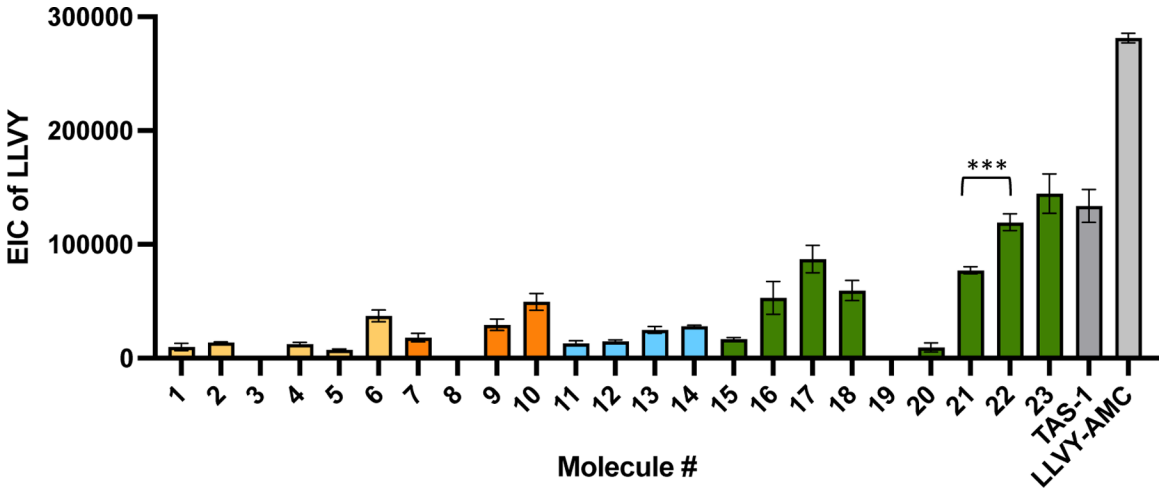

Figure 3.

The sensitivity of our MS assay allows us to compare these weakly cleaved substrates and gain insight into preferences of moieties in the unprimed substrate channel. These results highlight that the sCP prefers to aromatic groups with oxygens in the meta and/or para position relative to the amide bond to be cleaved. LLVY-AMC and TAS-1 were included as controls for each replicate. All substrates were tested in triplicate and one-way ANOVA analysis was applied to determine statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

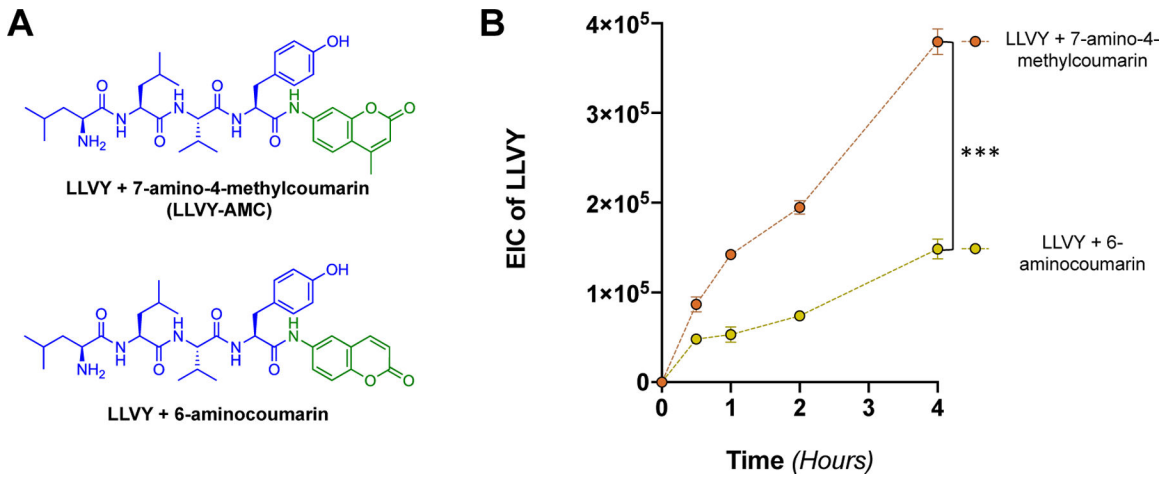

Figure 4.

(**A**) Structures of LLVY conjugated to two different aminocourmarin derivatives. (**B**) By time-dependent MS at 10 μM substrate and 5 nM sCP, the AMC derivative was cleaved more than the 6-aminocoumarin moiety. They were 32% and 11% cleaved after 4 hours, respectively. All substrates were tested in triplicate and one-way ANOVA analysis was applied to determine statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 1.

Summary of LLVY-R substrates that were incubated with 5 nM of 20S proteasome for 4 hours at 37 °C. We initially did not observe a dynamic range of hydrolysis when substrates were tested at 10 μM, indicating these LLVY-conjugated substrates are not as readily accepted as TAS-1 and LLVY-AMC, so substrates were tested at a higher concentration (25 μM) to gain insight on structural-activity relationships (SAR) and preference in the primed substrate pockets. All samples were run with positive control, 25 μM of TAS-1 and 5 nM sCP. Since our unnatural substrates did not get cleaved to the magnitude of LLVY-AMC, TAS-1 was used as a positive control for all studies for a more comparable scale.