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Fluorescent Probes with Unnatural Amino Acids to Monitor Proteasome Activity in Real-Time

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Abstract

The proteasome is an essential protein complex that, when dysregulated, can result in various diseases in eukaryotic cells. As such, understanding the enzymatic activity of the proteasome and what can alter it is crucial to elucidating its roles in these diseases. This can be done effectively by using activity-based fluorescent substrate probes, of which there are many commercially available that target the individual protease-like subunits in the 20S CP of the proteasome. Unfortunately, these probes have not displayed appropriate characteristics for their use in live cell-based assays. In the work presented here, we have developed a set of probes which have shown improved fluorescence properties and selectivity toward the proteasome compared to other cellular proteases. By including unnatural amino acids, we have found probes which can be utilized in various applications, including monitoring the effects of small molecule stimulators of the proteasome in live cells and comparing the relative proteasome activity across different cancer cell types. In future studies, we expect the fluorescent probes presented here will serve as tools to support the discovery and characterization of small molecule modulators of proteasome activity.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchembio.0c00634>.

Methods, Figures S1–S26, Tables S1–S3, and Appendices A–D (PDF)

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INTRODUCTION

Eukaryotic cells rely on the proteasome, a large enzyme complex with various catalytic activities, to maintain healthy protein levels and control a wide range of cell cycle pathways.^{1,2} Specifically, the 26S proteasome, comprised of the 19S regulatory particle (19S RP) and the 20S core particle (20S CP), degrades proteins that are damaged or otherwise no longer needed.^{2,3} Such proteins are tagged for degradation with a chain of ubiquitin (Ub) monomers, which is recognized and removed by the 19S RP before the protein is shuttled into the 20S CP to be hydrolyzed into small peptide units. The resulting peptide products can then be recycled for the synthesis of new proteins or other purposes as the cell needs.

The improper regulation of the hydrolysis activity of the proteasome has been implicated in several different disease types; this includes numerous cancers,⁴⁻⁹ which require a higher level of protein degradation to keep up with their increased protein load, and protein aggregation disorders.¹⁰⁻¹² As such, the proteasome has been recognized as a critical therapeutic target.¹³ For the past several decades, proteasome inhibitors have been studied and verified as a useful approach for a variety of hematological cancers, with the approval of bortezomib by the FDA for the treatment of multiple myeloma in 2003.¹⁴ More recently, small molecules which can stimulate the activity of the proteasome have also been discovered,¹⁵⁻¹⁸ providing a potential approach for the treatment of protein aggregation disorders, such as Parkinson's disease.¹⁹

Shortly after the discovery of the proteasome, a fluorescent substrate probe was described which can be used to monitor the protease-like activity of the 20S CP, specifically the $\beta 5c$ subunit.²⁰ This probe contains a modified four-mer peptide, with the sequence succinyl-Leu-Leu-Val-Tyr (Suc-LLVY), conjugated to a 4-amino-7-methylcoumarin (AMC) molecule (Figure 1A). In this structure, the amidation of the AMC molecule quenches its fluorescence, which can be restored upon the recognition of the peptide and cleaving of the Tyr-AMC amide bond. Additional peptide-AMC structures have since been designed which are selective for the specific protease-like subunits of the 20S CP. Together, these AMC-

based substrate probes have been vital tools for understanding a range of characteristics of the proteasome²¹⁻²⁴ and have been especially important in studying proteasome inhibitors.²⁵⁻²⁷

Although widely used, these AMC-based probes suffer from several limitations. For example, the poor fluorescence properties of AMC require the use of high concentrations of the given probe in order to obtain suitable signals for cell assays. At these concentrations, it is highly likely they are being cleaved by other cellular proteases and proteases in the cell environment. To circumvent this issue, assays can be performed using cell lysate and generic protease inhibitors, but this makes the evaluation of the effects of small molecule modulators of the proteasome confusing to assess.²⁸ Additionally, these substrate-based probes have since been developed as luminogenic substrates (known commercially as Proteasome-Glo from Promega) that can measure proteasome activity when incubated with a luciferase enzyme; however, this also requires the lysis of cells for measuring end-point activity. Previously reported substrate probes with altered fluorophores, although an improvement from AMC, are also primarily used in cell lysate.^{29,30} To overcome some these concerns, Urru and co-workers developed a Förster resonance energy transfer (FRET)-based probe with improved selectivity.³¹ To obtain suitable cell permeability, a peptide sequence corresponding to residues 48–57 of TAT was included, creating a probe which is 20 residues long and cumbersome to synthesize, unfortunately making its application for a high throughput screen challenging.

Beyond the substrate-based fluorescent probes for the proteasome, traditional activity-based probes based on proteasome inhibitors have also been reported.³²⁻³⁷ This includes several selective probes for the proteasome and immunoproteasome subunits developed by the Overkleeft group. Using fluorophore-labeled derivatives of their subunit-selective inhibitors, Overkleeft and co-workers have been able to determine the relative expression levels of the proteasome β -subunits in various cell lines³⁶ and have also been able to demonstrate the use of these probes in both live cells and mice with confocal microscopy.³² Others have developed similar activity-based probes, although the majority of these require SDS-PAGE analysis. Overall, these probes are best suited for comparing proteasome activity based on expression levels of each subunit or for the discovery and characterization of new proteasome inhibitors.

We sought to develop a fluorescent activity-based substrate probe to monitor proteasome activity, with the goal of using it for various applications in live cells for which the four-mer AMC probes are ineffective, including the evaluation of stimulators which do not alter the expression levels of the β -subunits. We also expected that a substrate-based activity probe would provide us with a method to monitor the proteasome's real-time activity instead of expression levels of the subunits. Additionally, we wanted a probe which can be sensitive to both inhibitors and stimulators, such that it would be useful in evaluating both types of molecules simultaneously.

Recently, we reported a new design for a fluorescent probe which is selective for the immunoproteasome.^{38,39} In this work, we demonstrated that using rhodamine 110 (Rh110) conjugated to an immunoproteasome-selective peptide on one side and a short peptoid on the

other provided an activity-based probe, named TBZ1, which could be used at low micromolar concentrations in live cells. By exchanging the peptide recognition sequence in TBZ1 for LLVY, we synthesized TAS1 (Figure 1B), which showed significant improvement in sensitivity compared to Suc-LLVY-AMC in live cells (Figure 1C) and cell lysate (Supporting Information Figure S1).

Here, we describe our evaluation of how incorporating unnatural amino acids improves the properties of TAS1. By using our Rh-probe scaffold, these probes show resistance to cleavage by proteases in human serum at sites which would lead to a fluorescent signal, demonstrating improved selectivity. Additionally, we demonstrated the versatility of these new probes, as they can be used to monitor the various levels of proteasome activity in different cell lines and can also be used to evaluate both small molecule inhibitors and stimulators of the proteasome. Compared to other methods to evaluate small molecule modulators of proteasome activity, these fluorescent substrate probes are more sensitive at lower concentrations and can be used for kinetic-based assays unlike the covalent-inhibitor-based activity probes.

RESULTS AND DISCUSSION

In our previous work, we demonstrated that our designed probe scaffold was able to improve upon several of the characteristics of its corresponding AMC probe, including sensitivity and assay compatibility.^{38,39} Upon exchanging the immunoproteasome-specific peptide for Leu-Leu-Val-Tyr, to generate TAS1 (Figure 1B), we created a similar substrate probe for the standard proteasome. To determine if TAS1 had any enhanced sensitivity compared to its AMC counterpart, the two probes were compared under the same conditions with purified 20S CP. Briefly, the probes were incubated in triplicate at 10 and 30 μ M with 9 nM 20S CP at 37 °C. The change in fluorescence was monitored for 2 h, and the end point values were averaged and graphed for comparison (Figure 1C). This data show that TAS1 has a signal greater than Suc-LLVY-AMC by more than an order of magnitude when used at either concentration. Since, unlike Suc-LLVY-AMC, TAS1 has the potential to have more than one cleavage site which could result in an increase in fluorescence (on either side of Rh110), we confirmed that the increase in signal is only from the recognition and cleavage of the peptide at the Tyr moiety by analyzing the 30 μ M TAS1 samples by LC/MS (Figure 2). These data showed that the only new peaks formed after incubating with purified 20S CP were the expected cleavage products, indicating that the fluorescence signal is not a result from cleavage of the bond between the Rh110 and the peptoid segment of TAS1.

One of the limitations to using activity-based substrate probes for the proteasome in cells is the ability of other proteases to also recognize and cleave at the desired amino acid, leading to a fluorescent signal. This nonproteasomal cleavage yields a falsely high fluorescence, requiring additional experiments to be able to properly interpret the data. For this reason, we were interested in determining if the addition of unnatural amino acids would provide improved selectivity for the proteasome compared to other proteases in cells. Although unnatural amino acids are abundant in proteasome inhibitors,^{13,40-43} suggesting they can be recognized for binding by the β -subunits, it is not well understood what types of noncanonical structures can be recognized and then subsequently cleaved. We chose to

synthesize and evaluate a small library of peptides for their ability to act as 20S CP substrates using LC/MS analysis, Figure 3. We varied the amino acid in the S1 position, replacing Tyr, as this is the amino acid at our desired cleavage site; the remainder of the 20S CP binding sequence was kept the same as TAS1. For the purpose of screening, we exchanged the Rh110-peptoid fragment with an Ala-Ala dipeptide, as it simplified the synthesis, and a fluorescent signal was not required for LC-MS-based quantitation. Since the peptide in TAS1 is recognized by the $\beta 5c$ active site, which has chymotrypsin-like activity, we focused our library on amino acids with hydrophobic side chains, including various phenylalanine (Phe) derivatives. In addition to noncanonical side chains, we were interested in whether the proteasome could recognize unnatural backbone structures, such as *N*-methylation and D-isomers, as these had not been previously reported in any proteasome activity-based probes. The peptides were synthesized in parallel fashion using standard Fmoc-based solid phase synthesis (Supporting Information Appendix A). Overall, the library included 15 unique sequences, along with Tyr and Ala derivatives as positive and negative controls, respectively.

To evaluate this set of peptides as potential 20S CP substrates, we applied a similar LC/MS-based assay to that used to discover TBZ1. Briefly, the peptides were dissolved in DMSO at a stock concentration of 600 μM . The peptides were then diluted in 50 mM Tris buffer, at pH 7.4, in a 96-well plate for a final assay concentration of 12 μM in two sets of triplicates. One set of triplicates served as a control, incubated in Tris buffer alone. For analysis, this would correspond to the ionization intensity of completely intact samples, which can then be compared to the amount of intact peptide remaining in 20S CP-treated samples. To the other triplicate was added 20S CP to a final concentration of 9 nM. All samples were incubated at 37 °C for 1 h before an equal volume of acetonitrile was added to denature the 20S CP. Samples were then analyzed by LC/MS (example spectra can be found in Supporting Information Appendix B).

For each peptide, the mass of the full (uncleaved) structure was extracted (extracted ion chromatogram, EIC) from each sample, and this peak was integrated to obtain the peak area. The peak areas for the control samples of individual structures were averaged and set equal to 1. Peak areas for 20S CP-treated samples were then scaled to the average for the untreated sample and the triplicates averaged to obtain a value for the fraction of full peptide remaining (Figure 4). To confirm that the peptide was being cleaved at the desired position, the mass of the corresponding four-mer cleavage product was found in each 20S CP-treated sample with significant decrease in full peptide observed.

Of the structures tested, 10 had a significant decrease in the amount of full peptide remaining compared to their respective untreated samples, while three peptides were cleaved better than the Tyr positive control. The library members containing unnatural backbone linkages showed mixed results: the sarcosine (Sar) peptide was determined to be cleaved significantly, while the 20S CP did not accept the peptide with the D-Phe moiety. For residues containing unnatural side chain functional groups, we observed that the aromatic structures, including all Phe analogues as well as thienylalanine (Thi), had the highest level of cleavage. By comparing Phe to cyclohexylalanine (Cha), we hypothesized the favorable cleavage of these aromatic derivatives could be contributed to by their planar configurations.

A comparison can also be made between the nonbranched alkyl side chains: norvaline (Nva), norleucine (Nle), and homoserine (Hse) all have close to 50% cleavage, while Ala is observed to have less than 10% of the peptide cleaved. This suggests that, while not cleaved as well as the aromatic functional groups, alkyl groups of a certain length can also be recognized by the 20S CP and hydrolyzed. Considering these results, we selected the five peptides cleaved more than 50%, as well as Sar since it was the only structure with an unnatural backbone to have significant cleavage observed, to test for human serum stability.

The hypothesis we considered was that unnatural amino acids, although capable of being cleaved by the proteasome, could be less prone to hydrolysis by other proteases in human serum or in cells than those with only natural amino acids. To test this hypothesis, we first optimized conditions for treating the peptides with human serum using the Tyr-containing peptide. We found that using 10% human serum v/v in Tris-buffer and incubating with the peptide for 10 min led to about 50% degradation of the peptide (Figure 5 and Supporting Information Appendix C).

When we tested each of the other six peptides from the library screen with the same procedure, we found that four of the peptides were cleaved significantly more by the 20S CP compared to when they are incubated in the human serum. Specifically, these were the four Phe derivatives tested. Unfortunately, there was no significant difference in cleavage of the Sar peptide, suggesting the *N*-methylation did not provide any special preference for the 20S CP. Overall, the three residues that performed the best were Phe, Phe(4-Cl), and Phe(4-NO₂). Because we were interested in the effects of unnatural amino acids in increasing the specificity of our 20S CP recognition sequence, we moved forward with Phe(4-Cl) and Phe(4-NO₂) as Tyr substitutes in our probe structure to create TAS2 and TAS3, respectively (Figure 1B and S2-S4).

With these new peptide sequences in hand, we returned to incorporating the Rh110-peptoid moiety and to compare the cleavage of TAS1–3 with the 20S CP. We incubated all three probes at various concentrations with 9 nM 20S CP and monitored the change in fluorescence using a microplate reader (Figures S5-S8). From this, we observed that a dose-dependent change in fluorescence was observed for all three probes in triplicate. In order to compare the three probes, we fit a linear regression for the points from 10 to 60 min for 30 μ M of each probe to obtain a rate for the change in fluorescence. Of the three, TAS1 was hydrolyzed the quickest (83.2 ± 0.5 RFU/min), although the slope for TAS2 was similar (63.8 ± 2.0 RFU/min). Unfortunately, TAS3 had a considerably higher level of background, which is a result of the properties of the Phe(4-NO₂), which resulted in a slower slope (28.1 ± 0.1 RFU/min). To confirm that the change in fluorescence was due to amide bond cleavage at the expected site on the peptide, we analyzed the samples at a concentration of 30 μ M by LC/MS (Figures S9-S11). As had been observed with TAS1, both TAS2 and TAS3 produced only the desired cleavage products.

We expected that the incorporation of the unnatural amino acids would provide protection for the fluorescent probes from serum proteases compared to the natural Tyr analogue, based on our previous serum stability results. In order to investigate this, we treated the probes under the same conditions, incubating for 10 min and then analyzing by LC/MS how much

of the whole probe remained intact. When initially incubating for only 10 min with TAS1, we saw no formation of new peaks in the TIC trace, Figure S12. For this reason, we chose to look at how the degradation changed with increased time. Each probe was incubated with 10% v/v human serum in Tris buffer for 0, 30, and 60 min and then analyzed by LC/MS (Supporting Information Appendix D). After 30 min, a new peak formed for each of the probes with a second new peak appearing after 60 min. Interestingly, neither of these peaks corresponded to the fluorogenic cleavage products, such that the serum proteases were not cleaving between the Phe derivatives and the Rh110. Instead, the first new peak that appeared corresponded to a removal of the *N*-terminal Leu while the second peak corresponded to the removal of the *N*-terminal Leu–Leu dipeptide. This would suggest that cleavage caused by the serum proteases would not lead to increased fluorescence and that, instead of the unnatural residues protecting the probes from nonspecific cleavage, it is the overall scaffold of the probes that provides this stability.

We next sought to determine if the TAS probes would be suitable for cell-based assays. To accomplish this, A549 cells were plated at a density of 5000 cells/well in a black 96-well plate with a black bottom. After allowing the cells to adhere to the plate, the samples were washed with PBS three times, then dosed with 10 μ M of each probe in modified KRBH buffer. The change in fluorescence was recorded on a microplate reader for 90 min. After allowing the signal to equilibrate for 25 min, each sample was set to zero, and the future time points were scaled accordingly. As was done with the biochemical data, a linear regression was fit to each set of data. From the slope, it is observed that the rate for TAS2 was the highest (Figure S13 and Table S1). To determine the in-cell selectivity, sets of samples with MG-132 (10 μ M) and epoxomicin (250 nM), proteasome inhibitors, were included for each probe. While epoxomicin is more selective, it is also more potent and therefore needed to be used at a lower concentration to avoid cell toxicity. These samples were preincubated with the inhibitors in cell culture media before washing with PBS and dosing with each probe in modified KRBH buffer. After plotting the average value over time and performing a linear regression, it was observed that the slope for each probe was decreased by about 50% or greater when cells were incubated with MG-132 (Table S1) and greater than 25% with epoxomicin (Table S2 and Figure S14). Plotting the average of each sample at their end point showed all probes had a significantly decreased signal when pretreated with either inhibitor (Figure 6A and B), supporting the expected selectivity of the probes for the 20S CP over other proteases. To ensure this decrease was not a consequence of cell death, cells were treated in the same way and analyzed using Cell-Titer Glo (Figures S15 and S16). Using the same protocol, cells were visualized using confocal microscopy, and the fluorescent signal was distributed inside the cell (Supporting Information Figure S17).

In addition to determining if our probes are capable of detecting a decrease in proteasome activity, we were also interested in monitoring molecules which have shown to increase proteasome activity, also known as proteasome stimulators. Various stimulators (Figure 7A) have been shown to be effective in cells by analyzing changes in GFP-fusion protein degradation.^{16,17} However, this method requires transfection of the GFP-fusion protein into cells and analysis was by Western blot. Given the promising results with inhibitors, we expected our probes to be sensitive enough to detect proteasome stimulation in a similar

manner. Using three molecules reported to have various degrees of stimulation,^{16,44} cells were dosed for 1 h before washing and adding in each probe. The samples were then monitored for changes in fluorescence and analyzed in the same way as inhibitor experiments (Figures 7B, S18, and S19).

With using all three probes, the stimulator molecules showed an increase in rate of cleavage compared to DMSO treated samples. We found TAS3 to have the highest change in activity level, which would provide the largest range in activities with various stimulators. This result is exciting because previous proteasome stimulators had been discovered using a biochemical assay which requires purified proteasome. As with all enzyme purifications, the batch to batch activity of purified proteasomes can vary greatly, leading to inconsistent results. The potential of using a TAS probe in cell-based screening would also expedite hit characterization, as they will already be known to have effective cell permeability. Additionally, our probes can be used in a variety of cell lines and in combination with other activity-based probes or cell markers.

Proteasome inhibitors approved by the FDA, such as bortezomib and carfilzomib, are effective in hematological cancers, due to these cells' strong dependency on proteasome activity to survive.^{43,45,46} Although a common way to determine a cell's dependency on proteasome activity is by treating cells with a proteasome inhibitor and observing its toxicity, many of these inhibitors have off-target effects which yield a falsely potent result. We sought to instead use TAS2 as a way to examine the relative proteasome activity levels in different cell lines as it is a more direct measure of the enzyme. TAS2 was chosen as it was shown to produce the highest signal in the A549 cell lines, Figure 6.

To compare cells lines, each was plated at the same density of 5000 cells/well in a black 96-well plate—for adherent cells lines A498, SK-MEL-2, MEL-92.1, SH-SY5Y, and HEK293T, the cells were allowed to adhere overnight before use in the assay; MM.1R cells were plated and used directly. Samples were incubated with either DMSO or TAS2 (10 μ M) for 15 min and washed with PBS three times before putting the plate in a microplate reader to record the fluorescence intensity over 90 min (Figures S20-S25). The experiment was performed in technical triplicate and experimental duplicate for each cell line to determine the rate of hydrolysis of TAS2, Table 1.

We were excited to see that a wide range of slopes is observed for the various cells lines. This suggests that there are different levels of proteasome activity associated with the cell lines tested. The values observed may suggest a trend between the slope for TAS2 and sensitivity of each cell line to proteasome inhibitors. For example, with the greatest slope, MM.1R is known to be strongly dependent on proteasome activity, and proteasome inhibition has been found to be a very effective treatment strategy for multiple myeloma.⁴³ We believe the variability of the MM.1R cell line is because some cells are lost during the wash protocol since they are nonadherent. However, they still possess the highest amount of proteasome activity, which was expected. On the other hand, HEK293T, a model noncancerous cell line, is not expected to have a high degree of proteasome activity and has one of the lowest slopes with TAS2.

We also sought to correlate this proteasome activity trend with susceptibility to a proteasome inhibitor by determining the IC_{50} values of MG-132 with these six cell lines. Cells were dosed for 48 h with increasing concentrations of MG-132 then analyzed using Cell-Titer Glo to determine cell viability (Figures S26 and Table S3). As expected, MG-132 was most toxic to MM.1R, with an IC_{50} of 264 nM. However, while the IC_{50} 's for the remaining cell lines were found to be higher than that of MM.1R, they did not follow the same trend as proteasome activity observed using TAS2. Despite this, we still believe comparing cell lines for proteasome activity based on fluorescence with TAS2 would still provide insight into the relative dependency of various cancer cell lines on proteasome activity and could be used to determine the threshold required for a cancer cell type to be highly susceptible to proteasome inhibitors. Also, TAS2 can be used to validate new proteasome inhibitors, to determine if their toxicity correlates with a decrease in proteasome activity or if there are potential off-target effects.

CONCLUSIONS

In the work presented here, we demonstrate the ability of the proteasome to recognize unnatural amino acids when incorporated into an activity-based probe. These probes, named TAS1–3 (Figure 1B), have demonstrated stability against nonproteasomal cleavage, an important characteristic for effective use in live cells. Additionally, all three probes have demonstrated versatility in various live cell applications, which will be beneficial to understanding the role of the proteasome in cancer as well as for examining potential therapeutics for both cancer and protein aggregation disorders. Overall, we expect the use of the probes to enhance the tools and assays already available for monitoring proteasome activity, leading to a better understanding of this critical protein complex and its effects on disease.

EXPERIMENTAL SECTION

For experimental details, including the synthesis of our probes and the procedure for utilizing them in live cells, see the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structures of (A) commercially available Suc-LLVY-AMC and (B) Rh-based peptide/peptoid hybrid probe. The dashed red line indicates where cleavage by the proteasome needs to occur to generate a fluorescent signal. (C) When comparing Suc-LLVY-AMC to our TAS1 with purified 20S CP, we can see the fluorescence signal for TAS1 is more than an order of magnitude higher at both concentrations tested. (D) A549 cells were dosed with 10 μM of TAS1 or Suc-LLVY-AMC, and the increase in fluorescence (RFU) was monitored over 60 min. The signal from the Suc-LLVY-AMC is significantly lower than the TAS1 signal, indicating a greater degree of sensitivity of TAS1 at this concentration.

Figure 2.

After incubating for 2 h with 20S CP, samples of 30 μ M TAS1 were analyzed by LC/MS to determine if the expected cleavage products were produced. Shown are example TIC traces for untreated (black) and 20S CP-treated (red). In the treated samples, we only observed two new peaks, corresponding to the 4-mer peptide and fluorescent Rh-peptoid fragments, along with a decrease in the full length TAS1. Corresponding structures are shown for each peak.

Figure 3.

Our library of unnatural amino acids is designed based on the LLVY 20S CP recognition sequence. The residue in position R (red) interacts with the S1 pocket of the $\beta 5$ -subunit then cleavage occurs at the C-terminal side (blue dashed line). The C-terminal Ala–Ala sequence was used as it is not expected to interfere with either 20S CP or general protease cleavage.

Figure 4.

The mass of each uncleaved peptide was extracted from all corresponding samples. The average amount of peptide in untreated samples was scaled to 1, and the treated samples were scaled accordingly from this. All samples are shown as an average of triplicates.

Figure 5.

Select peptides were incubated with 10% human serum, and the amount remaining after incubation was detected by an LC/MS assay. Several of those tested showed a preference for cleavage by the 20S CP compared to the serum. However, Thi and Sar showed near equal cleavage by both.

Figure 6.

A-549 cells were preincubated with either MG-132 (A) or epoxomicin (B) for 15 min. The signal from all three TAS probes was significantly reduced, indicating probe cleavage is through interacting with the proteasome.

Figure 7.

(A) Three small molecule stimulators previous validated using other proteasome stimulation assays. (B) TAS3 was able to detect stimulation, as determined by an increase in the % activity over 60 min as compared to the DMSO control (blue lines). Specifically, a difference can be observed between strong stimulators (e.g., MK-886) and weaker stimulators (TRC1 and TCM1).

Table 1.Six Different Cancer Cell Lines Were Incubated with TAS2 for 60 min^a

cell line	rate of hydrolysis (RFU/min)
MM.1R	82 ± 26
A498	40 ± 5
MEL-92.1	16 ± 3
SK-MEL-2	12 ± 1
HEK-293T	7 ± 3
SH-SY5Y	6 ± 1

^aThe amount of proteasome activity varied greatly indicating how much proteasome each of these cancer cell lines requires to survive.