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Monitoring the Immunoproteasome in Live Cells Using an Activity-Based Peptide–Peptoid Hybrid Probe

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Abstract

Activity-based probes have greatly improved our understanding of the intrinsic roles and expression levels of various proteins within cells. To be useful in live cells, probes must be cell permeable and provide a read-out that can be measured without disrupting the cells or the activity of the target. Unfortunately, probes for the various forms of the proteasome that can be utilized in intact cells are limited; commercially available probes are most effectively used with purified protein or cell lysate. The proteasome, both the 26S and various isoforms of the 20S CP, is an important target with reported roles in cancer, autoimmune disorders, and neurodegenerative diseases. Here, we present the development of a selective probe for the immunoproteasome, a specialized isoform of the 20S proteasome, that becomes expressed in cells that encounter an inflammatory signal. Using a one-bead, one-compound library of small peptides, we discovered a trimer sequence efficiently cleaved by the immunoproteasome with significant selectivity over the standard proteasome. Upon conjugating this sequence to rhodamine 110 and a peptoid, we generated a probe with a considerable improvement in sensitivity compared to that of current aminomethylcoumarin-based proteasome probes. Importantly, our probe was capable of labeling immunoproteasome-expressing cells while maintaining its selectivity over other cellular proteases in live cell cultures. We anticipate this probe to find wide utility for those that wish to study the immunoproteasome's activity in a variety of cell lines and to be used as a reporter to discover small molecules that can perturb the activity of this proteasome isoform.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/jacs.8b12873](https://doi.org/10.1021/jacs.8b12873).

Synthesis of probes, LC/MS methods, cell culture methods, and screening protocol; figures showing mass spectral characterization of molecules, hit validation data, biochemical characterization of probes and supplemental cell data; and tables of hit amino acid composition and hit peptide characterization (PDF)

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INTRODUCTION

The proteasome is a multiprotein complex critical to maintaining healthy protein homeostasis in eukaryotic cells because it is responsible for up to 90% of the protein degradation needs of a cell. In the ubiquitin-dependent pathway, the 26S proteasome, consisting of the 19S regulatory particle (19S RP) and the 20S catalytic particle (20S CP), degrades proteins that have been covalently modified with polyubiquitin chains.¹ For this process, the 19S RP first binds to and removes ubiquitin on tagged proteins and then unfolds and unwinds proteins to feed into the 20S CP for hydrolysis.² The 20S CP is a barrel-shaped complex containing four heptameric rings. The middle two β rings each contain three subunits with individual protease-like activities, and the outer α rings serve as gates in and out of the complex.

Without the RP, the CP is still capable of degrading small, disordered proteins in a ubiquitin-independent manner, and an average of about 60% of the CP in a cell exists as this uncapped form.³⁻⁵ Various isoforms of the 20S CP have been observed, depending on the cell type and environment. These include the standard CP (sCP), found in most healthy cells, and the immunoproteasome (iCP), present in immune cells and cells that have encountered various pro-inflammatory signals.⁶⁻⁹ These two isoforms differ in the primary structure of their catalytically active subunits: β_1 , β_2 , and β_5 . Under inflammatory conditions, such as exposure to interferon gamma (IFN- γ), the expression of β_{1i} , β_{2i} , and β_{5i} is turned on and these proteins are incorporated as new CPs are assembled. Overall, these structural changes result in a difference in the substrate preferences of the iCP compared to the sCP.¹⁰ As such, the same protein can be degraded by both isoforms but yield different peptide products. This is particularly useful because one of the major roles of the iCP is to produce MHC-I-compatible peptides. These different peptides can then be used to alert the immune system to the cause of inflammation with a smaller chance of wrongfully affecting healthy cells.

Both the sCP and iCP have been found to be overexpressed in various diseases.¹¹⁻²¹ However, because of their structural similarity, designing selective molecules is not trivial. Small-molecule-activity-based probes have been developed to monitor the activity of the sCP or iCP, with the most common being aminomethyl coumarin (AMC)-based peptides²² or peptides with fluorescence resonance energy transfer (FRET) pairs^{23,24} (Figure 2A). Several AMC-peptides are commercially available and include structures that are selective

for the different β subunits, including those of the iCP (Figure 2B). Unfortunately, these structures suffer from poor selectivity to other cellular proteases and cannot efficiently cross the cell membrane. As such, they are most often used for *in vitro* assays with either purified proteasomes or cell lysate.

Additionally, there have been iCP activity probes developed by the Overkleeft group and others.^{25,26} These probes can be used to decipher the 15 various proteasome subtypes. By treating with a set of FRET probes and then analyzing native-PAGE, they can determine what isoform of the proteasome is present in a cell line of interest. This is an elegant use of activity-based probes to evaluate proteasome composition but is limited in throughput because its analysis is performed by native-PAGE. These probes and others are also based on proteasome inhibitors, which limits their ability to be used to monitor proteasome activity because the probe then prevents any further proteasome activity.²⁷

We sought to improve upon the qualities of current proteasome probes to increase their versatility. Initially, we focused on selectivity toward the iCP. The iCP is a target of great interest for various cancers as well as autoimmune diseases, but few tools exist to determine the relative expression levels in live cells. We present here a rhodamine-based peptide-peptoid hybrid probe which has demonstrated improved selectivity and sensitivity in cells compared to commercially available structures. This probe can be easily used to evaluate the iCP activity in live cells in a plate reader or confocal microscope.

RESULTS AND DISCUSSION

With the solving of crystal structures for both the sCP and iCP,¹⁰ it is possible to rationally design selective molecules based on differences in substrate binding pockets. The crystal structures have also provided evidence for the selectivity of previously published inhibitors, such as bortezomib and carfilzomib.^{21,28,29} Using this crystallographic data and the features of known inhibitors, we hypothesized that a trimer peptide with residues favoring binding to its substrate binding pockets would allow for selective cleavage by the iCP. We chose to approach this by screening a one-bead, one-compound library of trimers attached to a common peptide. To select which amino acids to incorporate at each position of our library, we first focused on the binding pockets surrounding the $\beta 5$ subunits, which are responsible for the chymotrypsin-like activity of the CP (Figure 3A). For S1, the iCP has a larger hydrophobic pocket, so we selected residues with bulkier side chains for P1. The S2 pocket has a more conservative change from glycine in the sCP to cysteine in the iCP; therefore, amino acids for P2 are more random in structure. Finally, the S3 pocket in the iCP gains a hydroxyl group compared to the sCP. To take advantage of the change in hydrogen bond properties, amino acids selected for P3 are a mixture of hydrogen bond donors and acceptors. The side-chain structures for each position can be seen in Figure 3C. With 7 amino acids at each position, the full library contained 343 unique sequences attached to a common peptide. For the universal peptide sequence in the library, we chose to use OVA. Derived from chicken ovalbumin, OVA is a well-known model for MHC-I-compatible peptides³⁰⁻³⁶ and has favorable structural features for LC/MS analysis because it can be retained on a traditional C-18 column and ionizes well. Our library was synthesized using a modified version of OVA, exchanging an alanine for the phenylalanine in the sequence. This

was done to limit off-target cleavage because phenylalanine is a cleavage signal recognized by the $\beta 5$ subunit. The general structure of the library can be found in Figure 3B.

The library was synthesized using standard Fmoc-based solid-phase peptide synthesis with a split-and-pool method. In brief, the OVA sequence was first loaded onto Fmoc-Leu Wang resin. After Fmoc removal from the serine, the resin was split evenly into seven fractions, each receiving the necessary reagents for coupling one amino acid. When all couplings were complete, the resin was recombined for Fmoc removal, and the process was repeated for P2 and P3. Finally, an alanine was conjugated as the N-terminal amino acid, and its Fmoc was removed. Single beads were separated into wells of several 96-well plates, subjected to TFA cleavage and dried for storage. Twenty-four beads were used to check the quality of the library using MALDI. Of these, 90% were confidently matched to library sequences (Figure S1).

Looking for the peptides to be cleaved by the iCP, we planned to screen the library off resin. Cleaving a single bead in each well of a 96-well plate ensured that a single compound was contained in each sample. The next consideration we needed was how to screen for hits, which involved the destruction of the library compounds, while also being able to sequence the positive hits. To accomplish this, we settled on analyzing the screening samples by LC/MS. Using LC/MS provided us with the unique capability to separate the different components of our samples, including the full library peptide, cleavage products, and the iCP while simultaneously identifying them. Because hits will be considered to be sequences that produce the desired OVA peptide, we can also use LC/MS to quantify the relative amounts of OVA in each sample by comparing their extracted ion counts (EICs). Once samples have been recognized as hits, we can return to the LC/MS data for the sequencing of the library peptide. We primarily concentrated on sequencing the full peptide sequence, but the corresponding tetramer cleavage product was also expected to be observed in each sample, offering a second possible point for sequencing.

Optimizing the screening conditions (i.e., the concentration of iCP and incubation time) was essential because a balance between cleaving enough sample to observe an increase in OVA and leaving sufficient amounts of the full peptide for sequencing was necessary for a successful screen. We initially synthesized a control sequence, containing a phenylalanine in P1 and alanines in P2 and P3, for use in optimizing these conditions. This sequence was not expected to have any significant preference for the iCP or the sCP and thus also served as a baseline for later hit validation. Individual beads were separated into wells of a 96-well plate, cleaved, and dissolved in the proteasome assay buffer at an average concentration of 5.2 μ M, based on the average amount of peptide per bead. Samples were incubated at 37 °C with iCP at various concentrations and times in triplicate and then analyzed by LC/MS. Focusing on the amount of OVA produced as well as being able to confidently identify the full peptide, we found that incubating each well in the presence of 4 nM iCP for 6 h yielded the best balance of these two characteristics (Figure S2).

Library beads were screened in two separate rounds for a total of 403 beads, covering an extra 17% of library sequences. Example spectra can be seen in Figures 4A,B and S3. For each sample, the mass of OVA was extracted from the TIC trace and the peak was integrated

(Figure S4). Samples were then ranked within the 2 screening groups based on the area of the OVA peak, and the top 60 hits were reanalyzed to determine their corresponding library sequences. To further prioritize sequences for validation, the percent occurrence of each amino acid was calculated on the basis of the sequences of these hits (Table S2); nine sequences which contained the amino acids observed the most often were selected for validation. Additionally, we also determined the sequences of several low-ranking hits, selecting three to synthesize as negative controls.

Validation assays were performed under conditions similar to those for the screen. Briefly, each hit peptide (at 5 μ M) was incubated at 37 °C with 4 nM iCP for 6 h in triplicate and then analyzed by LC/MS. All peptides were also analyzed under identical conditions using 4 nM sCP. This was done to confirm that our library design yielded the desired selectivity and not simply sequences that could be efficiently cleaved by either isoform. The mass of OVA was extracted and integrated for each sample, averaging the triplicates. These values were compared to that of the control sequence with iCP, and those which had an increased amount of OVA were considered to be valid hits. Of the nine total hits, six were validated successfully. Additionally, all validated hits had at least 1.4-fold selectivity for the iCP (Figures 4C and S5). We chose to move forward with the Ala-Thr-Met-Trp sequence because this not only had highly efficient cleavage by the iCP but also was close to 5-fold selective for the iCP compared to the sCP.

Rhodamine 110 (Rh110) has several improved qualities over AMC that lead us to select it as the fluorophore in our probe. First, Rh110 has higher stability in biologically relevant buffers and media, making it more ideal for cell-based assays. Additionally, its greater fluorescence sensitivity compared to that of AMC allows for the use of a lower concentration of probes to achieve the same signal. Finally, current AMC-based probes suffer greatly from poor cell permeability and require significant quantities (high micromolar range), limiting their effectiveness as real-time tools.²² With its diamine structure, Rh110 contains two handles for modification, allowing us to further optimize the properties of our probes for improved cellular uptake.

Our probe design includes the sequence of our top hit conjugated to Rh110 with a peptoid fragment for cell permeability on the opposite side (Figure 5). This probe, referred to here as TBZ1, was synthesized on a Rink amide resin using standard peptoid and peptide synthesis (Figure 5A). Following the peptoid, Fmoc-Gly and succinic acid were subsequently coupled, providing the terminal acid group necessary for conjugating Rh110. The terminal acid was first activated with 1 equiv of COMU and then incubated at 60 °C for 3 h with 10 equiv of Rh110 chloride, 20 equiv of DIPEA, and 20 equiv of a proton sponge, which was added to provide improved deprotonation of the aromatic amine.³⁷ This reaction was followed by coupling Fmoc-Trp(boc)-OH to the second aromatic amine, using 10 equiv of amino acid, 10 equiv of COMU, 20 equiv of DIPEA, and 20 equiv of the proton sponge for 3 h at 60 °C. The succeeding amino acids were coupled using 6 equiv with 6 equiv of COMU and 12 equiv of DIPEA for 1 h at 37 °C. Each coupling was repeated a second time under the same conditions to ensure efficient coupling. The final probe was subjected to TFA cleavage and purified using RP-HPLC to at least 95% purity (Figure S6). In addition to our top hit sequence, we synthesized several controls in the same manner (Figure S7-S9). This includes

a negative hit from the screen as well as structures replacing either the peptide or peptoid segment. These structures can be found in Figure 5B.

Initially, we treated various concentrations of TBZ1 from 0 to 250 μM with both the iCP and sCP (Figure S10). All concentrations were incubated at 37 °C with the respective proteasomes (9 nM), and the increase in fluorescence was monitored for 60 min at 1 min intervals. We found that 31 μM TBZ1 gave a favorable profile, maintaining about 3:1 selectivity compared to that of the sCP (Figure 6).

The remaining probes were studied at 31 μM and monitored for an increase in fluorescence over 30 min. A summary of this data is provided in Figure 6. TBZ3, with the peptide sequence replaced with an acetyl group, does not result in any significant change in fluorescence. This suggests that the increase in fluorescence observed for TBZ1 is a result only of cleavage at the peptide structure and not the peptoid. Additionally, very minimal fluorescence is observed with TBZ2, which contains the peptide sequence of a negative hit, confirming that changes in fluorescence are dependent on peptide sequence. Finally, we also sought to compare TBZ1 to commercially available, iCP-selective substrate probe Ac-ANW-AMC. Under the same assay conditions, we found Ac-ANW-AMC to have very little change in fluorescence compared to TBZ1. Increasing the concentration of Ac-ANW-AMC still did not result in intensity similar to that of TBZ1 (Figure S12), confirming our expected increase in sensitivity. As a way to then compare the relative CP selectivities of TBZ1 and Ac-ANW-AMC, the fluorescence value of each probe at 30 min was set to 100% probe cleavage, and the earlier time points were scaled accordingly (Figure S13). This showed TBZ1 to have improved selectivity compared to that of Ac-ANW-AMC, most notably at shorter time points. Additionally, we sought to compare the subunit selectivity of each probe. Ac-ANW-AMC is referred to as being selective for the $\beta 5\text{i}$ subunit, which was also the designed target of our peptide library screen. As such, we performed the same biochemical assay with purified iCP described above in the presence of various concentrations of ONX-0914, a $\beta 5\text{i}$ -selective inhibitor with an IC_{50} of 65 nM for mouse $\beta 5\text{i}$ (Figure S17).^{10,44} When ONX-0914 is added to purified iCP directly before either probe, a decrease in the fluorescence signal is observed with increasing concentrations of inhibitor. This showed that TBZ1 is no less selective for $\beta 5\text{i}$ than Ac-ANW-AMC because the probe cleavage was significantly different between the two probes only at the highest concentration of ONX-0914 used (2X IC_{50}).

To further confirm that these results are due to cleavage specifically between our peptide and Rh and is selective for iCP cleavage, we analyzed TBZ1 samples by LC/MS (Figure 7, full spectra in Figure S15 and S16) with purified enzymes. We first analyzed TBZ1 under the same conditions as for the biochemical assay without any iCP (Figure S14) to confirm there was not any nonspecific degradation occurring during the assay or LC/MS run. In this, we observed only the two isomers of our probe. After treatment with the iCP, we observed two new peaks in the TIC spectrum. These corresponded to the expected Rh-peptoid and peptide fragments. The full mass of TBZ1 was extracted from each triplicate and compared between iCP and sCP samples. From this, we found that only 31% of uncleaved TBZ1 remained in iCP samples. When this analysis was performed using sCP, we observed that 77% remained uncleaved.

It has been well documented that the treatment of cultured cells with IFN- γ induces the expression of the three catalytic subunits ($\beta 1i$, $\beta 2i$, and $\beta 5i$) necessary to form the iCP, along with various other proteins involved in communication with the immune system.^{6,8,38-40} We sought to use IFN- γ treatment as a way to establish cell culture conditions for an iCP-expressing cell line. Initially, we tested A549 cells, a lung carcinoma. A549 has been demonstrated to express the immunoproteasome, though in varying degrees under different conditions.^{41,42} Cells were treated with 5 ng/mL IFN- γ each day for 4 days and then analyzed by Western blot, staining separately for $\beta 5$ and $\beta 5i$ (Figure 8A). In addition to being interested in the protein levels of $\beta 5i$ because it was the designed target of TBZ1, the incorporation of $\beta 5i$ during the assembly of the iCP occurs before that of $\beta 1i$ or $\beta 2i$, suggesting that it is one of the necessary triggers for the formation of iCPs.^{25,43} Therefore, $\beta 5i$ is considered to be a good indicator of the presence of the iCP. Untreated A549 cells showed little to no $\beta 5i$, offering a good negative control for later studies. After IFN- γ treatment, decreased expression of $\beta 5$ was observed with a corresponding increase in $\beta 5i$. These conditions are reproducible (Figure S18) and representative of those used for subsequent live cell experiments.

To determine the selectivity of TBZ1 in cells, both normal cells and cells treated with IFN- γ were plated at the same cell density into a 96-well plate for fluorescence readouts. After allowing sufficient time for the cells to adhere to the wells, TBZ1 was added and allowed to incubate for various time points. Following the initial incubation, wells were washed with PBS to remove any probe that had not been taken up by the cells, and the plate was incubated for an additional hour before the fluorescence was recorded. This data showed a significantly increased signal for IFN- γ -treated cells at 15 and 60 min compared to that of normal A549 cells. To confirm that this difference was not due to a difference in either the cell density or toxicity of TBZ1, we used CellTiter-Glo (Promega) to measure cell viability, which showed no significant difference between normal and IFN- γ -treated A549 cells plated in parallel with the fluorescence experiment (Figure S19). We initially intended to use TBZ4 as a way to confirm that the addition of a peptoid improved the cell penetration and protease stability of TBZ1. However, TBZ4 was not as efficiently cleaved at TBZ1 when used with purified iCP (Figure S11). Therefore, TBZ4 was not included in cell-based studies because it would be difficult to determine if a decrease in cell labeling was due to poor cell permeability or less efficient cleaving when inside the cell. Instead, to measure the cell permeability of TBZ1, we compared the fluorescence intensity for intact cells to that of an equal number of cells that had been lysed with a nondenaturing buffer (Figure S22). This showed TBZ1 to be about 35% cell-permeable. This is noteworthy because the peptoid sequence has not been investigated for optimal cell permeability.

We wanted to further demonstrate the selectivity of TBZ1 over other cellular proteases in cells and highlight its ability to effectively enter cells. To achieve this, we dosed IFN- γ -treated cells with a nonlethal concentration of ONX-0914 for 30 min before performing TBZ1 dosing. This data showed a significant decrease in fluorescence at all time points tested (Figure 8C). Using CellTiter-Glo showed that this decrease in signal was not due to cell death caused by iCP inhibition (Figure S20). This experiment was also repeated using normal A549 cells (Figure S21), showing no significant change in the fluorescence signal or cell viability between untreated cells and cells treated with ONX-0914. Because these cells

do not contain any iCP, this data provides further confirmation that the decrease in fluorescence observed in Figure 8C is due to the inhibition of the iCP.

As an additional way to visualize the cells treated with TBZ1, we analyzed IFN- γ cells using confocal microscopy. Following a procedure similar to that of the plate reader assay, IFN- γ -treated cells were plated on glass coverslip chambers coated with poly-D-lysine and were dosed with TBZ1 (31 μ M) or DMSO for 30 min. After being washed, the cells were returned to the incubator for 30 min before adding Hoechst and LysoTracker Red to label the nuclei and endosomes, respectively. Compared to cells treated with DMSO (Figure 8D-H), those treated with TBZ1 (Figure 8I-M) show increased staining due to probe cleavage within the cell. Background fluorescence is observed in the untreated cells (Figure 8G), as was also seen for cells alone in the plate reader assay. To confirm that cell fluorescence is increasing due to probe cleavage, we also performed a kinetic assay by treating cells with TBZ1 for either 0, 15, or 30 min before washing and returning samples to the incubator before imaging (Figure S23). By quantifying the mean cell fluorescence across each sample, this showed that the fluorescence increases over time, with both time points having average fluorescence values that are significantly higher than the background (Figure S24). Furthermore, very little background fluorescence is observed outside of the cell, suggesting that the fluorescence change observed is due to intracellular cleavage by the iCP rather than outside the cell by other proteases.

The use of LysoTracker Red shows that Rh110 fluorescence appears to be localized in acidic organelles, as represented by the yellow coloring in overlaid images (Figure 8M). To confirm that the release of TBZ1 fluorescence was not due to nonspecific cleavage in endosomes, we treated samples for confocal microscopy with ONX-0914 (Figure 8N-R), in the same way as was done in the plate reader assay. This showed a decrease in the fluorescence of both Rh110 (Figure 8Q compared to Figure 8L) and LysoTracker Red (Figure 8P compared to Figure 8K), with images appearing close to the background observed from untreated samples, suggesting that the fluorescence is dependent on TBZ1 being cleaved by the iCP.

CONCLUSIONS

In this work, we have discovered several short peptide sequences which, when incorporated into a larger structure, allow for specific cleavage by the immunoproteasome. This was accomplished by screening a library thoughtfully designed to exploit the differences between the iCP and sCP binding pockets. These sequences not only are efficiently cleaved by the iCP but also were observed to be modestly selective over the sCP without any further optimization. Of these, we chose to move forward with our top hit (Thr-Met-Trp) for further development as a fluorogenic probe.

When designing our probe, we kept in mind the pitfalls of current commercially available proteasome activity probes (Figure 2A,B). Namely, these are the lack of sensitivity of the fluorophore (aminomethylcoumarin) requiring the use of high concentrations in assays and the probes' poor cell permeability limiting live cell studies. To improve upon the former, we incorporated rhodamine 110 (Rh110) into our probe structure. In the published literature,

Rh110 has been used in protease substrates, typically including the same recognition peptide attached to both amines.^{45,46} However, because our sequence was observed to be efficiently cleaved, we chose to use one amine as a handle for incorporating a structure to improve cell permeability. Including a peptoid structure is known to increase cell permeability while also being resistant to proteolytic cleavage. This design strategy resulted in our final probe structure, named TBZ1 (Figure 5).

When used in biochemical assays, we found TBZ1 to maintain the selectivity observed for the original peptide used in hit validation (Figure 6, blue and red bars). Additionally, as expected, when used under the same assay conditions, the fluorescence signal of the commercially available iCP probe is barely above the baseline (Figure 6, purple bars). Even at the highest tested concentration of Ac-ANW-AMC (250 μ M), the signal is still 2 orders of magnitude lower than TBZ1 (Figures S10-S12). With the synthesis of various controls (TBZ2 and 3), we were able to demonstrate that the cleavage observed with TBZ1 is peptide-sequence-dependent and that no cleavage occurs between the rhodamine and the peptoid. This is further supported by LC/MS analysis of TBZ1 incubated with purified proteasomes, showing only the production of the expected products (Figures 7 and S14-S16).

We moved forward to demonstrate the ability of TBZ1 to be used with live cells while maintaining the selectivity observed in the biochemical assays. Furthermore, the observed signal can be significantly decreased when cells are dosed with ONX-0914, an iCP-selective inhibitor,⁴⁴ at a nonlethal dose. This reveals that TBZ1 is selective for the iCP not only over the sCP but also over other cellular proteases. Furthermore, with no change observed for normal A549 cells when treated with ONX-0914 (Figure S21), we confirmed that the change in fluorescence for A549 cells expressing the iCP is in fact due to the inhibition of iCP activity. Importantly, this data along with confocal microscopy analysis (Figure 8D-R) demonstrates the utility of TBZ1 with live cells, a critical characteristic for monitoring the activity of the iCP in real time. This will also be beneficial for the use of TBZ1 in more high-throughput applications as it simplifies sample preparation because cells do not need to be fixed and permeabilized.

When imaging cells treated with TBZ1, we observed some Rh110 fluorescence localized in acidic organelles, which are stained by LysoTracker Red (Figure 8M). By treating a separate sample with ONX-0914 and incubating for 30 min before adding TBZ1, we observed a decrease in Rh110 fluorescence as well as in LysoTracker Red staining to levels more similar to those observed for cells not treated with TBZ1. This data, along with the plate reader data from samples under the same conditions (Figure 8C), supports the fact that TBZ1 is not being cleaved in the endocytosis pathway and must first interact with the iCP to produce a fluorescence signal. Instead, we hypothesize that after being cleaved by the iCP, the fluorescent TBZ1 fragment is being packaged in exosomes, which can also be stained with LysoTracker Red, for excretion by the cells. Although further experiments will need to be performed to support this hypothesis, structurally similar molecules have been reported by Asanuma et al. for use in monitoring exocytosis in live cells,⁴⁷ suggesting that the Rh110-peptoid fragment of TBZ1 could be eliminated by the cell through a similar pathway.

TBZ1 has great potential in several different applications. First, with its ability to quickly label iCP-expressing cells, it can serve as a useful diagnostic tool. The expression of the iCP is observed in several autoimmune diseases and cancers, and the effective staining of these types of cells using TBZ1 would allow for their identification in a mixed population. To this end, we have preliminary flow cytometry data (Figure S25) which suggests that cell populations can be separated on the basis of increasing numbers of iCP-expressing cells. Along this same line, TBZ1 could be used in a cell culture to improve our understanding of the expression of the iCP, including how quickly it begins to be active after exposure to inflammatory cytokines and how distant from the point of $\text{INF-}\gamma$ exposure cells with iCP can be observed.

Another potential application for TBZ1 is as a tool for molecule discovery. Having a probe to monitor the iCP activity in cells allows for real-time observation of the effects that various molecules may have. However, the use of TBZ1 would not be limited to the investigation of inhibitors. With its great sensitivity, lower concentrations of TBZ1 could be used to monitor increases in iCP activity as well.

Overall, we have discovered a peptide sequence which can be selectively cleaved by the iCP and demonstrated its use in a fluorogenic substrate probe. This probe has shown usability in live cells, offering a broad scope of potential applications in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The proteasome exists in several different isoforms. The 26S form consists of a 20S catalytic particle (purple and blue) and a 19S regulatory particle (green = ATPase ring; orange = non-ATPase subunits). The 20S form can hydrolyze small, disordered proteins independent of the 19S RP. Upon encountering inflammatory signals, such as IFN- γ , three new β subunits are expressed and the iCP is formed. Immuno subunits are shown in navy blue. PDB ID 5GJQ.

Figure 2.

Several substrate probes for the proteasome have been reported, including several commercially available aminomethyl coumarin probes selective for the sCP ($\beta 5$, **A**) and the iCP ($\beta 5i$, **B**).

Figure 3.

(A) Overlaying the crystal structures from the human sCP and iCP at the active site shows the differences in substrate binding pockets of the two isoforms. (B) General design for our library, with the OVA (model antigen) in black and the three variable positions in red, blue, and green. (C) Seven amino acids were chosen for each position on the basis of structures hypothesized to favor substrate binding to the iCP.

Figure 4.

Our library was screened on the single-bead level and analyzed by LC/MS. Example LC/MS spectra show a positive (A) and a negative (B) hit, with peaks labeled for the corresponding structures. (C) To quantitatively rank hits, the mass of OVA was extracted from each sample. All synthesized hits were in the top 25% of samples based on the amount of OVA extracted. Shown in the table are hits synthesized for validation, compared to the control sequence (bolded). The bold line separates the hits that were found to be negative from our screen.

Figure 5.

(A) Scheme for the synthesis of Rh-based probes. Synthesis was achieved with standard Fmoc-based solid-phase synthesis. (Full synthesis details can be found in the Supporting Information.) (B) Structures of synthesized probes.

Figure 6.

Probes (31 μM) were mixed with iCP (9 nM) or sCP (9 nM) in tris HCl buffer (50 mM, pH 7.6, 50 μL). Samples were incubated at 37 $^{\circ}\text{C}$ while monitoring the change in fluorescence (ex –485(20) nm, em –535(20) nm). Each plotted point is an average of triplicates at each time point.

Figure 7.

TBZ1 (31 μ M) was incubated with 9 nM iCP of sCP for 55 min at 37 $^{\circ}$ C and then quenched with acetonitrile. The samples were analyzed by LC/MS. The sample treated with iCP (TIC blue trace) showed significant cleavage of TBZ1 at the desired location between the rhodamine moiety and the peptide. The sCP sample (TIC red trace) had more uncleaved probe as indicated by the larger peak at 6.3 min.

Figure 8.

(A) Western blot analysis of A549 cells after treatment with 5 ng/mL human IFN- γ . Compared to untreated cells, samples treated with IFN- γ showed an increase in $\beta 5i$, demonstrating the increase in expression of the iCP. Note that though both Western blots were from identical samples, the intensity of the GAPDH band is higher in the $\beta 5i$ blot because the required secondary antibody for $\beta 5i$ is antigoat, the host animal of the secondary antibody for GAPDH. (B) Untreated and IFN- γ -treated A549 cells were dosed with 31 μ M TBZ1 and incubated for various times. Fluorescence measurements showed a significant increase in the cleavage of TBZ1 in IFN- γ -treated cells compared to that of untreated cells. (C) When IFN- γ -treated A549 cells are dosed with ONX-0914, a significant decrease in TBZ1 cleavage is observed, demonstrating its selectivity for iCP cleavage in cells. (D–R) Confocal microscopy was used to demonstrate the intracellular localization of the fluorescence and the change in Rh110 fluorescence which occurs after ONX-0914 treatment. IFN- γ -treated cells were dosed with TBZ1 at 31 μ M (I–R) or DMSO (D–H) for

30 min before washing to remove any extracellular TBZ1. Nuclei were stained with Hoechst 33342 (blue) and LysoTracker Red DND-99 was used to label acidic organelles (red). (D, I, N) Bright field. (E, J, O) Hoechst 33342 (blue channel). (F, K, P) LysoTracker Red (red channel). (G, L, Q) Rh110 (green channel). (H, M, R) Overlay of blue, red, and green channels. These images demonstrate that the green fluorescence observed in TBZ1- treated cells is localized within acidic organelles inside the cell, as demonstrated by the yellow coloring in overlaid images. Treatment with ONX-0914 (20 μ M) for 30 min before adding TBZ1 reduces the LysoTracker Red (P) and Rh110 (Q) fluorescence to levels similar to those for the untreated cells (F and G).