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Establishment of A Suite of Assays That Support the Discovery of Proteasome Stimulators

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Abstract

Background—The proteasome catalyzes the degradation of many mis-folded proteins, which are otherwise cytotoxic. There is interest in the discovery of proteasome agonists, but previous efforts to do so have been disappointing.

Methods—The cleavage of small fluorogenic peptides is used routinely as an assay to screen for proteasome modulators. We have developed follow-on assays that employ more physiologically relevant substrates.

Results—To demonstrate the efficacy of this workflow, the NIH Clinical Collection (NCC) was screened. While many compounds stimulated proteasome-mediated proteolysis of the pro-fluorogenic peptide substrates, most failed to evince activity in assays with larger peptide or protein substrates. We also show that two molecules claimed previously to be proteasome agonists, oleuropein and betulinic acid, indeed accelerate hydrolysis of the fluorogenic substrate, but have no effect on the turnover of a mis-folded protein in vitro or in cellulo. However, two small molecules from the NCC, MK-866 and AM-404, stimulate the proteasome-mediated turnover of a mis-folded protein in living cells by 3- to 4-fold.

Conclusion—Assays that monitor the proteasome-mediated degradation of larger peptides and proteins can distinguish bona fide agonists from compounds only able to stimulate the cleavage of short, non-physiologically relevant peptides.

General significance—A suite of assays has been established that allows the discovery of bona fide proteasome agonists. AM-404 and MK-866 can be useful tools for cell culture experiments, and can serve as scaffolds to generate more potent 20S stimulators.

Graphical abstract

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Supporting Information

Additional data and detailed methods.

The authors declare no competing interests

Keywords

Proteasome; α -synuclein; Parkinson's disease; mass spectrometry; high-throughput screening; agonist

Introduction

The synthesis, folding, trafficking and degradation of proteins are all carefully coordinated to maintain proteostasis in healthy cells (1,2). Defective proteostasis, due to chemical damage or mutation, leads to the accumulation of mis-folded proteins, which can form highly cytotoxic amyloids. These species are central to the development of many diseases, including neurodegenerative conditions such as Alzheimer's and Parkinson's diseases (AD and PD). An important component in maintaining proteostasis is the rapid digestion of mis-folded species. While autophagy plays a role in this process, particularly under certain pathological conditions such as heart failure (3), the bulk of this "janitorial function" in healthy cells is mediated by the proteasome (4). Low levels of proteasome activity exacerbate protein-folding diseases, while higher proteasome activity ameliorate them (5–14). Furthermore, high levels of proteasome activity are correlated with long life (15).

The simplest form of the proteasome is the 20S core particle (CP), which is comprised of two copies each of 14 unique proteins (seven α sub-units and seven β sub-units) arranged as four stacked heptameric rings (Figure 1) (16). The three catalytic sites of the proteasome are located in the interior of the barrel-like 20S CP. Substrate entry must occur through a channel that is so narrow only unstructured peptide chains can pass (17). The 20S CP can associate with capping complexes on one or both ends of the barrel. For example, the 26S proteasome is comprised of the 20S CP and one or two copies of the 19S regulatory particle (RP). The 19S RP binds poly-ubiquitylated proteins, unfolds them in an ATP-dependent fashion, and recruits deubiquitinases to remove the ubiquitin chains so that they can be recycled (18). An alternative cap, the 11S complex is present in specialized immunoproteasome complexes (19) that process proteins into peptides suitable for transport to the cell surface and display in the form of MHC complexes.

Mis-folded proteins are turned over by both ubiquitin/ATP-dependent and ubiquitin/ATP-independent pathways. The former is mediated by the full 26S proteasome and depends

upon ubiquitin E3 ligases that target mis-folded proteins(4). Some of these E3s are associated with chaperones. However, about 30% of proteasomes in cells appear to lack a 19S RP cap, suggesting that the 20S CP plays a significant role in this process(20). Indeed, under oxidative stress eukaryotic cells induce the expression of a protein called ECM29 that promotes the dissociation of the 20S CP and 19S RP (21), consistent with 20S CP being the major catalyst for digestion of mis-folded protein and the cell wanting to increase its concentration when under stress. On the other hand, the 20S CP is a sluggish catalyst. This is due to the fact that the narrow substrate entry channel is gated by the N termini of the α -subunits that reach across the opening to interact with other α subunits and hinder substrates from diffusing through the channel (Figure 1). Indeed, one of the functions of both the 19S RP and the 11S cap is to open this gated channel, as demonstrated by their ability to stimulate peptidolysis *in vitro*. Another 20S CP cap, called PA28, also appears to perform this function and is important for maximal resistance to oxidative stress (22). Despite these capping complexes, many investigators suspect that there remains “naked” 20S CP in the cell that contributes to mis-folded protein turnover turnover (20). There is enormous interest in exploring this hypothesis but a critical reagent to do so is a potent agonist of the 20S CP.

The best-characterized 20S CP agonists are peptides. Goldberg and colleagues found that the seven C-terminal amino acids of the 19S RP ATPases Rpt2 and Rpt5 make direct contacts with α -subunits in the 20S CP and that these contacts are critical for stimulating peptidase activity *in vitro* (23–25). Seven residue synthetic peptides recapitulated this activity when mixed with purified 20S CP(25). Unfortunately, simple, linear peptides are neither cell permeable nor serum-stable and thus are not useful as probe molecules. Two papers appeared in 2007 that claimed more drug-like small molecules, oleuropein (26) and betulinic acid (27), stimulate 20S CP activity modestly. Like virtually all other studies of putative proteasome agonists, these employed a fluorogenic assay that uses a very small peptide substrate with a C-terminal coumarin functionality. When cleaved, a sharp increase in fluorescence results. Three peptide-coumarin substrates are available that allow the measurement of the chymotrypsin-, trypsin-, and caspase-like activities of the 20S CP. This assay is convenient and supports high-throughput analyses, but the substrate is decidedly non-physiological. Indeed, to our knowledge, none of the reports to date describing putative small molecule proteasome agonists have demonstrated unequivocal stimulation of the turnover of mis-folded proteins in living cells. Thus, it is fair to ask whether using this assay makes things “too easy”. Keeping the gated channel issue in mind, we wondered if some compounds might indeed tweak the gate just enough to allow a tiny substrate with the reporter group at its terminus to “poke its head” into the interior of the 20S, whereas a larger, more physiologically relevant substrate might still be unable to access the active sites.

To probe this, we sought to develop follow-on assays that would more stringently assess the activity of putative 20S CP agonists. We describe here several such protocols, shown schematically in Figure 2, including a new quantitative LC-MS assay with a peptide with a single cleavage site, and one that monitors the turnover of α -synuclein quantitatively in cells (Figure 2C). We show that betulinic acid and oleuropein are not, in fact, agonists of the 20S CP-catalyzed turnover of mis-folded proteins, though they indeed stimulate hydrolysis of the fluorogenic peptides. To attempt to identify *bona fide* agonists, we screened the 726 compounds in the NIH Clinical Collection (NCC) and discovered two molecules using the

fluorogenic assay, then vetted the screening hits in the more demanding assays. From this effort has come the discovery of two *bona fide* 20S CP small molecule agonists, MK-866 and AM-404.

2. Materials and Methods

2.1 Screening protocol for the NIH NCC Library

The 726 compounds in the NIH clinical collection (NIH NCC) were diluted to a final concentration of 200 μ M in phosphate buffered saline (PBS) in a 384 well plate format. From these “mother plates” aliquots were added to new 384 plates to provide a final concentration of 10 μ M of each compound. Also included in each well was 45 μ L of 200 μ M Succ-LVY-AMC dissolved in tris(hydroxymethyl)aminomethane-HCl (50 mM Tris-HCl, pH = 7.8). Before addition of the 20S CP the plate was read to detect any auto-fluorescent compounds. Any signal detected here was subtracted from the observed reading after adding the proteasome. Finally, human 20S CP was added to yield a final concentration of 3.5 nM to start the reaction. The plate was covered and allowed to incubate for 30 min at 37°C. After this time, the wells were scanned for a fluorescent signal produced by the release of the AMC after cleavage. Readings were then taken every 10 min for 2 hrs. Several wells lacked any small molecule to allow the basal rate of peptide turnover to be determined. The data were plotted in Prism as fluorescent signal versus time, generating a line whose slope corresponds to the rate of hydrolysis of the peptide (linear regression analysis). All experiments were done in triplicate, which revealed that the rates determined were accurate to $\pm$ 10%. A representative sample of the acquired data is shown in Supporting Information Figure S1.

2.2 LC-MS Agonist Assay

A solution of 200 μ M of the 20S peptide substrate was prepared in 50 mM Tris-HCl, pH = 7.8. 45 μ L of this solution was added to each well of a 96-well plate followed by 2.5 μ L of a Tris-HCl solution that contained 3.5 nM of human 20S CP and 2.5 μ L of a 200 μ M solution of the hit compound from the fluorescent screen. The plate was sealed and placed in an incubator for 2 hrs at 37°C with shaking at 60 rpm. The reaction was quenched with 5 μ L of trifluoroacetic acid (TFA). The reaction solutions were then subjected to ZipTip® pipette tips (100 μ L size) to collect the peptide and remove the Tris salt before LC-MS to reduce any ion suppression. This solution was then placed in an LC-MS vial and 30 μ L of the 50 μ L of the sample was injected. The chromatographic run employed the following conditions: 0–2 min 95% A, 5% B; 2–12 min linear gradient to 100% B; 12–15 min 100% B. A 4.6 $\times$ 100 mm, 3.5 μ m Zorbax SB-C18 column on an Agilent 1100 LC connected to an Agilent LC/MSD SL. LC-MS A solvent was 95% water, 5% acetonitrile, and 0.1% formic acid. LC-MS B solvent was 95% acetonitrile, 5% water, and 0.1% formic acid. ZipTip® pipette tips were purchased from EMD Millipore and used according to the manufacturer’s directions. The extracted ion chromatogram (EIC) of the peptide starting material and the cleaved product containing the Fmoc group were utilized for quantitation. The ratio of cleaved peptide to whole peptide was calculated for each individual run and all compounds were tested in triplicate to obtain an average. The amount cleaved was compared to the DMSO-

treated control to determine the percentage of peptide cleaved when treated with a small molecule (see Supporting Information Figure S3).

2.3 SDS-PAGE analysis of purified α -synuclein degraded by purified 20S CP

200 ng of purified α -synuclein (purchased from Novus Biologics) was incubated with 3.5 nM of purified 20S proteasome (purchased from Enzo Lifesciences) in 40 μ L of 50 mM Tris-HCl (pH = 7.8). The agonist or DMSO was then added to the mixture and allowed to incubate for two hours at 37°C. The reaction was then quenched with SDS loading buffer (4 $\times$). All of the sample was loaded into a 4–20% SDS-PAGE gel. Quantitation was done by also loading 200 ng, 100 ng, 50 ng, and 25 ng of pure α -synuclein in the same gel to ensure no difference in quantitation was due to image contrast differences. After SDS-PAGE electrophoresis, the proteins were transferred to a nitrocellulose blot. The blot was then blocked with 5% skim milk at room temperature for one hour. The blot was then drained and 1:1000 of anti- α -synuclein antibody was added in 5% milk at 4°C overnight. Next the blot was rinsed with PBS-T (0.01% tween 20) 3 $\times$ 10 minutes. The secondary LI-COR antibody (anti-rabbit) was added at a dilution of 1:10,000 in LI-COR block for one hour at room temperature. After rinsing again 3 $\times$ with PBS-T the blot was scanned with a LI-COR Odyssey. All quantitation of gel bands was done with ImageJ.

2.4 Analysis Of Protein Levels In Neurons Or Transfected Cells

12 hours prior to compound addition, each transfected T150 flask was split evenly between 10cm dishes (7–9 per flask). Each compound was added at the appropriate concentration maintaining the same DMSO concentration for each condition and all experiments performed in triplicate. Generally, 10 mM stocks of each compound were made in DMSO and 10 mL of media was added to each plate – for the highest concentration used (32 μ M), 32 μ L of a 10 mM stock dissolved in DMSO was added, providing a final DMSO concentration of 0.32%. Cells were grown for 16 hours and collected by cell scraper, centrifuged at 300 $\times$ g for 10 minutes, washed in PBS and snap frozen until all pellets had been collected. Pellets were lysed in 1mL of M-per cell lysis buffer (Thermo) containing Halt protease inhibitor cocktail (Thermo) following the manufacturers protocol. Protein concentrations were determined by OD₂₈₀ and all normalized prior to Western Blotting. Western blotting was performed for all proteins of interest by first running a SDS-PAGE electrophoresis and then transferring to a nitrocellulose blot. The blot was then blocked with 5% skim milk at room temperature for one hour. The blot was then drained and 1:1000 of the primary antibody of interest was added in 5% skim milk at 4°C overnight. Next the blot was rinsed with PBS-T (0.01% tween 20) 3 $\times$ 10 minutes. The secondary LI-COR antibody was added at a dilution of 1:10,000 in LI-COR block for one hour at room temperature. After rinsing again 3 $\times$ with PBS-T the blot was scanned with a LI-COR Odyssey. All quantitation of gel bands was done with ImageJ.

3. Results

3.1 Identification of proteasome inhibitors in vitro

We began our discovery efforts by screening the NCC of 726 compounds. These are clinically tested, highly drug-like, and their safety profiles are known (28). We first screened

all of the molecules at a concentration of 10 μ M, in triplicate, for their effect on cleavage of the chymotrypsin-coumarin (Succ-LLVY-AMC) probe by the purified 20S proteasome. A 384-well plate with 3.5 nM of purified 20S proteasome in each well was incubated for two hours at 37°C and a fluorescent reading was taken every 10 min. Oleuropein and betulinic acid were included as a positive control. For all 726 compounds and the 2 positive controls, the slope of the line generated by graphing the fluorescent signal over time was determined (Supporting Information Figure S1 and Table S1).

Somewhat surprisingly, the vast majority of compounds in the NCC inhibited the 20S CP-mediated peptidolysis reaction by more than 10%. While we do not understand the basis of this effect, it is possible that these small molecules are able to access the interior of the 20S CP barrel and hinder entry of the peptide substrate. As shown in Figure 3, only four compounds in the NCC provided a modest stimulation, whereas another eight at least did not inhibit the reaction by more than 10%. Therefore, we chose to treat these 12 compounds as primary screening hits.

This is hardly a stringent cut-off, but further evaluation of only 12 compounds was reasonable so we chose not to eliminate any possible candidate agonists at this stage, which proved to be a good decision (*vide infra*).

The 12 compounds were assessed further using an assay that employed the peptide substrate Fmoc-AMSGFAATG-CONH₂, which contains a single chymotryptic cleavage site in the middle of the sequence. Thus, the entire peptide must move into the interior of the 20S core particle in order to be digested. The same concentration of 20S proteasome and peptide substrate were utilized as in the fluorogenic assay so that the only deviation from the primary assay is the substrate size. After an incubation of two hours the sample was quenched with 1% TFA, desalted using a Zip Tip, and analyzed using liquid chromatography-ESI mass spectrometry (LC-MS). As shown in Figure 4, the peak representing the starting peptide (Figure 4A; retention time $\approx$ 9 minutes) was converted cleanly to a single product (Figure 4B–D) in the presence of the 20S proteasome. Note that the hydrophobic Fmoc group facilitates retention of the peptide on the reverse-phase column, so only the starting material and the Fmoc-containing fragment are seen in the chromatogram (Figure 4), allowing a ratiometric measurement of peptidolysis. Of the 12 compounds evaluated, only two, AM-404 and MK-886 increased the rate of hydrolysis of this larger peptide, as did oleuropein and betulinic acid (Figure 4 and Supporting Information Figures S3, S4 and Table S2). We thus chose to focus solely on these four compounds for further analysis.

3.2 Are AM-404 and MK-866 acting as detergents?

It has long been known that sodium dodecylsulfate (SDS) can stimulate 20S CP-catalyzed peptidolysis *in vitro*,⁽²⁹⁾ presumably by loosening the contacts that occlude the gate (Figure 1). Thus, a concern is that AM-404 and MK-866 are merely acting as detergents. A simple way to distinguish the action of a detergent from a true agonist is to examine the dose-dependence. Detergents such as SDS should exhibit a bell-shaped curve. This reflects an optimal concentration at which the gating interactions are melted, but also the general denaturation of the proteasome at higher SDS concentrations.

As shown in Figure 5, this is precisely the result observed in the 20S CP-catalyzed cleavage of the pro-fluorogenic peptide. Maximal hydrolysis was observed at 1 mM SDS, but higher detergent concentrations resulted in a steep reduction in activity. In sharp contrast, the dose-response curves of MK-886 and AM-404 showed a classic saturation curve with an EC₅₀ of 32 μ M (Supporting Information Figure S5). No drop –off in activity was observed at higher concentrations. This argues that these molecules are acting as true agonists and not as detergents.

3.3 Stimulation of α -synuclein turnover in cellulo

Next, AM-404 and MK-886 were tested in mammalian cells. HEK-293T cells were transfected with a plasmid that encodes a GFP- α -synuclein fusion protein (31). It was reported previously that the 20S proteasome has the ability to degrade only the α -synuclein portion of this protein hybrid because of the unstructured nature of α -synuclein (32,33). The highly stable, barrel-like structure of GFP is resistant to degradation by the 20S only. Thus, when cell lysates are analyzed by SDS-PAGE and Western blotting with an anti-GFP antibody, one can compare the intensities of the bands corresponding to the GFP- α -synuclein fusion protein and GFP alone to determine the extent of proteolysis. This ratiometric measurement is more straightforward and reproducible than measuring the disappearance of a protein band.

The cells were incubated for 16 hours with AM-404 or MK-886 (8–128 μ M) or DMSO as a control. The cells were then lysed and the lysates analyzed by SDS-PAGE and Western blotting with an anti-GFP antibody. Figure 6A shows the results from dosing the cells with 32 μ M of the two LC-MS validated hits, MK-886 and AM-404, and two hits not validated in the LC-MS assay, anagrelide and cefuroxime. (For the full dose response analysis see Supporting Information S6). The DMSO-treated cells contain only a small amount of free GFP, while MK-886- and AM-404-treated cells show a much more pronounced GFP band (also see Supporting Information Figure S7). Quantitation showed that AM-404 and MK-886 increased degradation of α -synuclein three-fold and four-fold, respectively (Supporting Information Table 3). In contrast, these compounds did not have any effect on the levels of several folded proteins or other well folded proteins fused to GFP (Figure 6B and Supporting Information Figure S8). Anagrelide and cefuroxime did not stimulate the degradation of α -synuclein in these cells (Figure 6A). When this experiment was repeated, but the cells were treated with a sub-lethal dose of Bortezomib, neither MK-886 nor AM-404 stimulated partial degradation of the fusion protein (Figure 6C). This confirms that this degradation is proteasome-mediated.

An alternative mechanism of action might be that AM-404 or MK-886 reduce the level of the 19S RP, providing a larger pool of 20S CP. To address this, N2A cells were treated with the compounds or vehicle (DMSO). Lysates prepared from these cells were analyzed by SDS-PAGE and Western blotting for Rpt2, a component of the 19S RP, and α 1, a component of the 20S proteasome. The ratio of 19S RP to 20S proteasome was then calculated based on band density (Figure 7) (also see supporting Information Figure S9 and Table S4). All four samples that were treated with a small molecule have less 20S proteasome relative to the 19S RP as compared to the DMSO control.

To ensure that these results were not peculiar to the GFP- α -synuclein fusion protein, HEK-293T cells were transfected with a plasmid that leads to overexpression of α -synuclein alone. After stopping protein synthesis with cycloheximide, the cells were treated with DMSO, AM-404, or MK-886 for 16 hours, lysed, and the lysates were analyzed by SDS-PAGE and Western blotting using an α -synuclein antibody. Figure 8 shows that AM-404 and MK-886 increase the rate of α -synuclein turnover by about two-fold and three-fold, respectively. The reduction in protein levels upon treatment of the cells with these small molecules was not due to general cytotoxicity resulting in fewer cells. MK-886 showed no cellular toxicity, while AM-404 showed only slight toxicity (Supporting Information Figure S11).

Two neuroblastoma cell lines were also tested to see if either small molecule had an effect on α -synuclein produced endogenously in these cells. Both SH-SY5Y and N2A cells were treated with 32 μ M of MK-886 or AM-404. After 16 hours, the cells were collected and lysed and the amount of α -synuclein analyzed by Western blotting. As shown in Figure 9, treatment with either MK-886 or AM-404 led to a decrease in α -synuclein levels by three- and two-fold, respectively, in both cell types.

3.4 MK-886 and AM-404 do not affect 26S proteasome-mediated proteolysis *in vitro*

As mentioned above, some mis-folded proteins are turned over by the 26S proteasome via a ubiquitin-dependent pathway. We carried out biochemical and cell biological experiments to determine if this pathway might contribute to the degradation of the substrates studied here and if MK-886 and AM-404 might affect this process. First a simple *in vitro* experiment was done in which the effect of the two small molecules on hydrolysis of the fluorogenic substrate was analyzed in the presence of purified 26S proteasome. As shown in Figure 10, no effect was observed, in contrast to the stimulation observed with the 20S CP (Figure 3).

However, as mentioned above, the pro-fluorogenic peptide is not a physiologically relevant substrate, so it was of interest to probe this issue in the context of living cells. To distinguish reactions mediated by the 20S CP or 26S proteasome *in cellulo*, one would need to completely inhibit 19S RP activity, thus blocking 26S activity without affecting 20S activity. Unfortunately, while a peptoid inhibitor of the yeast AAA ATPase Rpt4 has been reported, (34) this compound has poor activity against human 19S RP (H. Lim and T.K., unpublished data), and to the best of our knowledge, no other compounds capable of shutting down the 19S RP exist, so this is not possible. A reasonable alternative however, is presented by the peptoid KDT-11 (35), a recently reported inhibitor of one of the ubiquitin receptors in the proteasome, Rpn13. (36) While the 26S proteasome can process substrates without Rpn13, it does so at a reduced rate, as indicated by the buildup of poly-ubiquitylated proteins in Rpn13-inhibited cells (35,37). Thus, we asked if the stimulation of partial degradation of the GFP- α -synuclein fusion is reduced in cells treated with KDT-11.

As shown in Figure 11A, MK-866 and AM-404 stimulate the production of the GFP fragment to approximately the same extent in the presence or absence of KDT-11 (see also Figure 6). As anticipated, treatment of the cells with KDT-11 does result in a build-up of poly-ubiquitylated proteins (Figure 11B). The same higher levels of ubiquitylated proteins are also observed when cells are treated with KDT-11 and either MK-866 or AM-404. Taken

together, these data suggest that MK-866 and AM-404 have no effect on the 26S proteasome and that the digestion of the unstructured α -synuclein domain of the fusion protein is carried out by the 20S CP and not the 26S proteasome.

Discussion

Mis-folded proteins can aggregate into toxic oligomers, which are thought to be major contributing factors to several “protein folding diseases”. Studies in animal models and human cells have shown that stimulating proteasome activity through overexpression of limiting subunits has a beneficial impact in slowing the rate of some of these diseases, whereas proteasome inhibition exacerbates them (11,14). Thus, there is interest in exploring the hypothesis that pharmacological stimulation of proteasome activity might be an effective therapeutic strategy for suppressing protein folding diseases and perhaps aging itself.

Unfortunately, progress towards the development of proteasome agonists has been slow. The best work in the field involves a strategy to stimulate the proteasome-dependent turnover of ubiquitylated proteins indirectly. Finley and co-workers reported a small molecule that inhibits Usp14 (38), a deubiquitylase (Dub) that opposes ubiquitin (Ub)-dependent proteasome-mediated turnover by removing the poly-Ub tag from substrate proteins. Thus, the compound results in increased flux of polyubiquitylated substrates through the proteasome. Whether this approach would improve proteostasis in neurodegenerative or similar diseases is not yet clear. As mentioned in the introduction, there is evidence that at least some mis-folded proteins are turned over in a ubiquitin-independent fashion by the 20S CP (21). Thus, a direct agonist of the 20S CP would be invaluable.

Only a few reports of such compounds have appeared. (26,29,39–44) Most of these molecules are thought to mediate an allosteric conformational change that opens the gated entrance to the barrel-like core particle, which is almost completely closed in the resting state (Figure 1) (25). For example, Goldberg and colleagues have shown that this channel opening can be driven *in vitro* by 7–8 residue peptides from the extreme C-terminus of Rpt2 or Rpt5. This appears to recapitulate gate opening mediated by the 19S RP (25). Unfortunately, peptides are poor probe molecules given their low cell permeability and modest stability *in vivo*.

The natural product oleuropein, a major constituent of olive oil, and betulinic acid have been reported to stimulate 20S activity (27,45), which is interesting since these are more drug-like molecules. However, the only assay with which these molecules have been evaluated for agonist activity is the cleavage of the very small fluorogenic peptide substrates *in vitro* (Figure 2A). Neither molecule has been shown definitively to accelerate the ubiquitin- and ATP-independent digestion of mis-folded protein substrates *in cellulo* or even *in vitro*.

To facilitate a more rigorous analysis of putative proteasome agonists, we developed a suite of downstream assays that allow *bona fide* agonists to be distinguished from compounds that only have an effect in the fluorogenic assay. These include the LC-MS-monitored digestion of a nine amino acid substrate with a single cleavage site in the middle of the molecule, the

digestion of α -synuclein *in vitro* and the partial digestion of a GFP- α -synuclein fusion protein in living cells (Figure 2).

To identify new 20S proteasome agonists, we initially employed the fluorogenic assay to screen the NIH NCC. Twelve molecules were identified that either stimulated this reaction modestly or at least did not inhibit it significantly. However, when they were examined in the LC-MS-monitored digestion of the nine-residue peptide, only two of the hits showed substantial activity, MK-886 and AM-404. Both of these molecules also stimulated the turnover of α -synuclein *in vitro* by the 20S proteasome. Most importantly, MK-886 and AM-404 also stimulated the digestion of α -synuclein in living cells. This is most conveniently monitored by using the α -synuclein-GFP fusion protein as the substrate since the ratio of the bands corresponding to the fusion protein and the protease-resistant GFP can be measured easily in a Western blot using anti-GFP antibody (Figures 2C, and 7). However, we also demonstrated that these compounds accelerated the proteasome-mediated degradation of both recombinant and native α -synuclein in both neuronal and non-neuronal cell lines.

From a biological perspective, the data reported in this paper provide support for the point of view that a significant amount of the turnover of mis-folded proteins (as exemplified here by α -synuclein) occurs via a 20S CP-catalyzed, ubiquitin-independent pathway (20). Neither MK-866 nor AM-404 had any effect on the activity of the 26S proteasome *in vitro* (Figure 10) and slowing the processing of poly-ubiquitylated substrates by the 26S proteasome with the Rpn13 inhibitor KDT-11 had no effect on the ability of the agonists to stimulate α -synuclein degradation in cells (Figure 11). However, it is important to point out that a definitive approach to this question would require an inhibitor that completely shuts down 19S RP activity and none is available presently. Moreover, it will be important to test the generality of this conclusion by monitoring the turnover rate of a variety of mis-folded proteins.

While neither MK-886 nor AM-404 are particularly potent molecules, with EC_{50} s in the mid- μ M range, they display little or no cellular toxicity at these concentrations and thus may serve as useful probe molecules for cell culture assays, though not for animal experiments. To obtain more potent 20S CP agonists, medicinal chemistry could be employed to explore the activity of derivatives of MK-866 or AM-404. Obviously, since MK-886 and AM-404 are part of the NIH NCC, they have activity against other targets. MK-886 was initially developed as a FLAP inhibitor ($EC_{50} \approx 1 \mu$ M) and AM-404 was reported to be an inhibitor of anandamide transport with a K_i of 1.8μ M (46,47). Other FLAP or anandamide inhibitors do not affect the hydrolysis rate of the 20S CP (Supporting Information Figure S13), thus optimization efforts would hopefully “dial out” the undesired inhibitory activity of MK-886 and AM-404.

Perhaps more importantly though, this work sets the stage for new screening campaigns using larger libraries now that biochemical assays are in place to vet hits obtained from much higher throughput, but physiologically questionable, assays such as the fluorogenic peptide hydrolysis experiment. The fact that two *bona fide* 20S CP agonists were identified from a set of only 726 molecules (the NIH NCC) suggests that screening campaigns

involving hundreds of thousands of compounds have a good chance of turning up more potent 20S CP agonists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A workflow is described that allow bona fide proteasome agonists to be identified.
- Two compounds from the NIH Clinical collection, AM-404 and MK-866 are shown to be weak, but bona fide proteasome agonists.
- Two small molecules previously reported to be proteasome agonists stimulate the turnover of small, fluorogenic peptides, but not mis-folded proteins.

Figure 1. Cartoon depicting the opening of the gated channel to allow an unfolded substrate (black) access to interior of the 20S CP. Note that this cartoon is simplified in that the seven N-terminal peptides of the α -subunits do not play an equal role in gating the channel.

Figure 2.

A three-step validation process to facilitate the discovery of *bona fide* proteasome agonists. (A) In a 384-well plate the 20S proteasome is tested for its ability to cleave the AMC group following the tyrosine residue for any effect on the cleavage of its chymotrypsin-like ability. Using a plate reader, a reading can be taken every ten minutes to generate a rate of hydrolysis. (B) In our discovery method, we wished to employ a second validation step by application of a quantitative LC-MS assay. (C) Molecules that are validated in the LC-MS assay are then tested for their ability to stimulate the 20S in HEK-293T cells by monitoring the appearance of GFP. The amount of free GFP can be directly correlated to the amount of α -synuclein that was degraded from the fusion protein.

Figure 3.

(A) The rates of peptidolysis in the presence of all 726 compounds were determined in triplicate and then averaged by analyzing the fluorescent signal accumulated over time. Using this analysis method, 12 primary hits were found. Any molecule that generated a rate of fluorescence accumulation over time within 10% of the rate of the 20S alone was considered a primary hit, because in the next assay where the 20S proteasome is challenged with a larger substrate, a small molecule stimulator may have a more appreciable stimulatory effect. Oleuropein, a previously claimed to be a 20S agonist was included in the screen as a positive control. The Z' for this high throughput screen was 0.78 utilizing SDS as the highest signal control and DMSO as the lowest signal control. (B) Structures of four of the 12 hits discovered in this fluorescent activity screen, also see Supporting Information Figure S2.

Figure 4. Cleavage of a nine-residue, Fmoc-capped peptide containing a central chymotryptic cleavage site. (A) No proteasome. This represents uncleaved peptide. (B) Peptide + 20S. (C) Peptide + 20S + MK-866 (20 μ M). (D) Peptide + 20S + Anagrelide (20 μ M). All spectra are the total ion chromatograms. Note that only the starting peptide and the Fmoc-containing product are retained on the column.

Figure 5. Dose response curves for the effect of small molecules on the proteasome-mediated hydrolysis of the pro-fluorogenic peptide. (A) MK-886 and AM-404. (B) SDS. The rate of unstimulated 20S CP-mediated hydrolysis is shown by the dotted black line.

Figure 6.

Effect of select compounds on the partial proteolysis of a GFP-α-synuclein conjugate in HEK293T cells. (A) Western blot analysis for GFP cleaved from the GFP-α-synuclein conjugate and the effect of the 20S small molecule agonists. Quantitation of the free GFP band demonstrates that MK-886 increases the cleavage of the conjugate protein 4 fold; AM-404 increases cleavage by 3 fold, as compared to the negative control (cells that are treated with vehicle (DMSO)). Anagrelide and cefuroxime had no effect. (B) Effect of the compounds on the degradation of the proteins indicated. (C) Treatment of the cells with bortezomib, a potent proteasome inhibitor, (100 nM) abrogates the stimulation of α-synuclein digestion by MK-886 and AM-404.

Figure 7.

Effect of the small molecules indicated on the ratio of Rpt2 (19S RP) to $\alpha 1$ (20S CP), as determined by Western blotting. N2A cells were treated with AM-404, MK-886, or Oleuropein at 32 μ M, Bortezomib was used at 100 nM. Treated cells did not produce more 20S proteasome, thus we can attribute the degradation of the fusion protein in Figure 7 to more 20S proteasome activity rather than the cells expressing more proteasome.

Figure 8.

Effect of MK-866 and AM-404 on the turnover of α -synuclein. HEK-293T cells expressing α -synuclein were dosed with cycloheximide followed by DMSO, MK-866 or AM-404 (32 μ M). After 16 hrs, the cells were lysed, and the amount of α -synuclein remaining was quantitated by Western blotting, Supporting Information Figure S10.

Figure 9. Effect of MK-886 and AM-404 on the turnover of native α -synuclein in neurons. (A) SH-SY5Y and (B) N2A cells were treated with either MK-886 or AM-404. Cells were lysed and blotted for the presence of α -synuclein. Treated cells were compared to the DMSO-control cells to calculate the fold change (also see Supporting Information Figure S12 and Table S5).

Figure 10.
Effect of AM-404 and MK-886 on the rate of hydrolysis of the fluorogenic peptide by the 26S proteasome.

Figure 11.

HEKT-293T cells were again transfected to produce the α -synuclein-GFP fusion, but this time treated with KDT-11, a peptoid that decreases the ability of the 26S proteasome to degrade ubiquitinated proteins and does not affect the ability of the 20S to hydrolyze substrates. (A) When these cells were treated with either agonist the amount of free GFP was again increased as compared to the DMSO control, and similar to the results observed in Figure 6. (B) Ubiquitin accumulation is only observed in the presence of KDT-11 and the two agonists appear to have no effect on ubiquitin-dependent degradation, i.e. 26S proteasome activity.