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Small Molecule Enhancement of 20S Proteasome Activity Targets Intrinsically Disordered Proteins

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Abstract

The 20S proteasome is the main protease for the degradation of oxidatively damaged and intrinsically disordered proteins. When accumulation of disordered or oxidatively damaged proteins exceeds proper clearance in neurons, imbalanced pathway signaling or aggregation occurs, which have been implicated in the pathogenesis of several neurological disorders. Screening of the NIH Clinical Collection and Prestwick libraries identified the neuroleptic agent chlorpromazine as a lead agent capable of enhancing 20S proteasome activity. Chemical manipulation of chlorpromazine abrogated its D2R receptor binding affinity while retaining its ability to enhance 20S mediated proteolysis at low micromolar concentrations. The resulting small molecule enhancers of 20S proteasome activity induced the degradation of intrinsically disordered proteins, α -synuclein, and tau but not structured proteins. These small molecule 20S agonists can serve as leads to explore the therapeutic potential of 20S activation or as new tools to provide insight into the yet unclear mechanics of 20S-gate regulation.

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/acscchembio.7b00489. Synthesis, characterization, ¹H and ¹³C NMR spectra, and concentration–response curves of all compounds (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All synthetic work and docking experiments were performed by C.L.J. All biological assays were performed by E.N. with the exception of the D2R binding assay, which was performed by B.S. T.S.D. aided in the design and execution of the HTS assay.

Notes

The authors declare no competing financial interest.

Proteins undergo constant proteolytic degradation to regulate intracellular processes and maintain biological homeostasis.^{1,2} One of the main intracellular proteolytic pathways involves the proteasome, which is responsible for the degradation of misfolded, oxidatively damaged, and redundant proteins.^{3–6} In contrast to the 26S proteasome, which is primarily involved in ubiquitin-dependent protein degradation, intrinsically disordered proteins (IDPs), such as α -synuclein, and oxidatively damaged proteins are mainly targeted by the 20S proteasome for degradation.^{5,7–10} Disordered proteins are naturally short-lived; however basal levels are secured by forming proteolytically stable structured complexes with “nannies,” chaperones, or other protein complexes.^{4,11,12} However, mutations, overexpression, or proteasome dysfunction leads to accumulation of these nonsoluble species.¹³ When accumulation of intrinsically disordered proteins exceeds proper clearance in neurons, imbalanced pathway signaling¹⁴ or aggregation occurs,^{15,16} which have been implicated in the pathogenesis of several neurological disorders, including Parkinson’s disease (PD), Alzheimer’s disease (AD), Huntington’s disease (HD), and amyotrophic lateral sclerosis (ALS, Lou Gehrig’s disease).^{15–18} Proteasome enhancers may therefore offer a potential strategy to reduce the buildup of toxic proteins implicated in neurodegenerative diseases.

Several studies have illustrated the enormous potential of proteasome activation as a novel treatment strategy, but unfortunately very few molecules have been identified as direct or indirect enhancers of the proteasome-degradation pathway.^{19–23} Several of the few direct proteasome enhancers identified in the literature, including detergents such as sodium dodecyl sulfate (SDS), oleuropein,²⁴ and betulinic acid,²⁵ are questionable as true 20S agonists, since their activity does not translate under more physiologically relevant conditions.²⁶ However, Kodadek *et al.* recently reported a series of elegant assays to identify *bone fide* 20S agonists, and the authors identified two compounds, MK-866 and AM-404 (EC_{50} ’s $\sim 32 \mu\text{M}$ each), capable of enhancing 20S mediated protein degradation.²⁶ We present herein the discovery of a new class of molecules capable of enhancing 20S proteolytic activity *via* a ligand–20S proteasome interaction, which induces the selective degradation of disordered proteins, α -synuclein and tau, over structured proteins *in vitro* and in cell culture.

The human proteasome is comprised of a barrel-shaped 20S core particle (CP) capped by two 19S regulatory particles (RPs). The 20S CP is a threonine protease that consists of four stacked rings: the two inner β -rings contain three catalytic subunits ($\beta 5$, $\beta 2$, and $\beta 1$) that exhibit chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (Casp-L) proteolytic activity, respectively. The outer α -rings do not exhibit proteolytic activity but control access

to the proteolytic core chamber *via* an allosterically controlled gate-opening/closing mechanism²⁷ and thus guard the entrance to the interior catalytic core.²⁸ A number of reversibly associated regulatory particles (such as the 19S caps) dock onto the α -rings to form the 26S proteasome (Figure 1). Six ATPases (Rpt-1–6) on the base of the 19S lid insert a short C-terminal hydrophobic peptide chain (containing a Hb-Y-X motif) into the intersubunit pockets on the α -ring, inducing the open-gate active conformation (Figure 1).^{29–32} Recent cryo-EM structures have identified several distinct conformational forms of the proteasome, but the detailed mechanics of gate-regulation remain unclear.^{33,34}

The search for small molecules capable of enhancing 20S CP-mediated proteolysis was initiated by screening the NIH Clinical Collection and Prestwick libraries. Purified 20S proteasome was exposed to the compounds (10 μ M) in the presence of the fluorogenic chymotrypsin-like (CT-L) peptide substrate (Suc-LLVY-AMC). In this activity assay, proteolysis of the peptide substrate can only occur if a compound induces an open-gate conformation of the α -ring to allow proteolytic cleavage inside the core particle, as indicated by the release of aminomethylcoumarin from the peptide substrate.³⁵ The compounds were deemed inactive if the CT-L activity was <2 fold (at 10 μ M) over the vehicle. The neuroleptic agent chlorpromazine and its related analogues enhanced 20S proteolytic activity and indicated excellent reproducibility in a concentration–response assay with fresh stocks of the compounds (Figure 2D and Figure S1). The EC₅₀ values were determined and are shown in (Figure 2A). No fluorescence was detected in the absence of the AMC substrate (control) indicating no intrinsic fluorescent properties of the drugs in this assay.

To determine a possible mechanism of action of chlorpromazine mediated 20S activation, unbiased docking studies were undertaken utilizing Autodock Vina mated with PyRx for workflow management.^{36,37} Blind docking was done with the entirety of the 20S proteasome to remove selection bias and allow conformational preference to prevail. Remarkably, high predilection was suggested for binding into the α/α -intersubunit pockets of the α -rings, as seen with the endogenous Hb-Y-X-tails of the regulatory particles (RPs or caps). Chlorpromazine (CPZ) showed a preference for the $\alpha 1/\alpha 2$ -intersubunit cavity (Figure 2B, see also Figure S5 for a more detailed discussion of binding site preference of CPZ), which is the binding pocket for the Rpt3 tail of the 19S regulatory particle.³⁸ Search space was reduced and docking resubmitted. After several iterations, major binding sites of high incidence were isolated and docked in sequence to generate a small selection of highly probable docked poses (Figure 2C and Figure S5B).

Two proof-of-principle analogues, **1** and **2** (Figure 3B), were prepared by deprotonation of the phenothiazine with NaH and subsequent addition of the alkyl halide (1-bromo-4-methylpentane) or 1,4 butane sultone (1,2-oxathiane 2,2-dioxide) to render analogues **1** and **2**, respectively (Figure 3A). Both compounds were tested to validate our 20S α -ring docking-site hypothesis. According to Autodock Vina, the alkyl chain of **1** had very poor binding affinity for this pocket, whereas the sulfonic acid **2** has a possible binding interaction with Arg83 (Figure 2C). Consistent with this, we found that only compound **2** enhanced 20S proteolytic activity, whereas compound **1** did not (Figure 2E).

Next, we tested compounds **1** and **2** for the induction of proteolytic activity of each of the three catalytic sites ($\beta 5$, $\beta 2$, and $\beta 1$) of the 20S proteasome using the standard AMC-labeled peptide substrates.^{35,39} We found that only compound **2** induced the activation of the chymotrypsin-like (CT-L, EC₅₀ 4.9 μ M), tryptic-like (T-L, EC₅₀ 10.0 μ M), and caspase-like (Casp-L, EC₅₀ 4.8 μ M) activity (Figure S2). The parallel effects of all proteolytic sites is consistent with the induction of an open-gate conformation that allows all substrates to enter the chamber.²¹ The fold enhancement of 20S activity by compound **2** (~4 fold at 10 μ M) was equal to the activation of the 20S proteasome by the 19S caps (3 molar excess to ensure full 26S assembly; Figure S3).

We investigated whether compound **2** was capable of selectively enhancing 20S-mediated proteolysis over 26S-mediated proteolysis. The 26S proteasome is also capable of degrading the CT-L peptide probe, but in the 26S proteasome the α -rings are blocked by the two 19S caps (i.e., 19S-20S-19S, Figure 1). Treatment of the 26S proteasome with compound **2** did not affect 26S-mediated proteolysis of the CT-L peptide probe (Figure 2F). The inability of compound **2** to enhance 26S-mediated proteolysis is consistent with our proposed binding of **2** to the α -ring of the 20S proteasome, because this site is blocked by the 19S caps in the 26S proteasome.³⁸ Thus, we postulate that the enhanced 20S proteolytic activity (over 20S background activity) by compound **2** is due to its regulation of the 20S gate, rather than enhanced activity of the catalytic subunits.

Next, we evaluated whether the 20S agonist **2** preferentially induced 20S-mediated degradation of the intrinsically disordered protein, α -synuclein^{40,41} (associated with the pathogenesis of Parkinson's disease) in the presence of the structured protein, GAPDH. In this study, we mixed purified 20S proteasome with α -synuclein (α -syn) and GAPDH, in the presence of vehicle (positive control), compound **2** (3, 10, and 30 μ M), bortezomib (proteasome inhibitor, negative control), chlorpromazine (20S agonist), and SDS (common detergent for 20S activation *in vitro*). Western blot analysis shows that compound **2** significantly enhanced the rate of 20S-mediated degradation of α -syn compared to 20S/vehicle control (Figure 2G, lane 2 versus lane 3–5), in a concentration-dependent manner. Importantly, compound **2** does not enhance 20S-mediated degradation of the structured protein, GAPDH (Figure 2G), at any of the concentrations tested. α -Syn levels were unaffected by compound **2** in the absence of 20S or by pretreatment of 20S with bortezomib (BTZ, 3 μ M), which verifies that the α -syn clearance is due to induction of 20S proteolysis (Figure S7).

Following the success of the *in vitro* digestion of α -syn, we turned our attention to reducing the neuroleptic properties of the phenothiazines to limit their undesirable side effects. The neuroleptic properties of phenothiazines are mainly due to their ability to antagonize the dopamine D2 receptor (D2R)⁴² (and others)^{43,44} as its structure superimposes well with that of dopamine.^{45,46} The protonated alkylamine side chain⁴⁴ of these drugs binds to most/all of these receptors with different affinities, and multiple SAR studies have indicated that the amines are required for neuroleptic properties.^{47–49} We therefore aimed to reduce the D2R (dopamine receptor) antagonism of chlorpromazine by altering the N-chain functionality, while maintaining 20S activity. Guided by our docking model, several phenothiazine analogues were prepared (Figure 3A) with the goal of reducing D2R binding and validating

our proposed binding model. Treatment of 2-chloro-10*H*-phenothiazine with 1,2-oxathiolane 2,2-dioxide provided compound **3**. Alkylation of 2-chloro-10*H*-phenothiazine with 1-bromo-3-chloropentane provided the alkyl chloride **9a**, which was treated with NaI and sodium sulfite to render the 5-carbon sulfonic acid derivative **4**. Examination of the proposed docking poses of compound **2** indicated a possible binding interaction with Arg83 (Figure 2C). Consistent with this model, reduction of the chain length (compound **3**) or extension of the chain length (compound **4**) fails to interact efficiently with Arg83 and subsequently resulted in no significant enhancement of 20S-mediated CT-L proteolysis (Figure 3B). Even though compound **2** enhanced 20S proteolysis, its non-drug-like characteristics (negatively charged at physiological pH) and drop in activity at higher concentrations (Figure 3C, >35 μ M, i.e., possible detergent-like behavior at higher concentrations)²⁶ prompted us to extend our efforts to pursue more physiologically relevant derivatives. Therefore, alkyl chloride **9b** was treated with NaI and morpholine to render compound **5**. Similarly, addition of the propargyl or benzyl functionality to 2-chloro-10*H*-phenothiazine provided the products **10** and **8**, respectively. Carbonylation of **10** with CO₂ and ^{*n*}BuLi rendered compound **6**, and hydrolysis of the methylester **8** with NaOH provided the benzoic acid derivative **7** (Figure 3A).

The new phenothiazine derivatives were tested for their ability to displace the D2R antagonist [³H]-Spiperone using a radio ligand binding assay in intact HEK-293 (Figure S4). As reported in the phenothiazine literature,^{46,47,50} extension of the chain (analogue **5**) reduces the D2R binding but only modestly enhanced 20S activity (Figure 3B, 4-fold max, with EC₅₀ = 8.9 μ M). Elimination of the nitrogen in the chain (compound **1–4**, **6–8**) or chain length extension (compound **5**) effectively reduced the compounds' affinity for the dopamine receptor to physiologically insignificant potency (Figure 3B, *K_i* > 250 μ M). Docking of the propargylic acid **6** places the carboxylate in the same pocket but >5 Å away from Arg83 and was found to be unable to significantly enhance 20S activity. Docking of the benzoic acid derivative **7** places the carboxylate in a similar position as sulfonate **2**. Compound **7** did enhance 20S activity at relatively low concentrations (EC₅₀ = 6.4 μ M) but displayed partial agonist behavior, with only a 2–3-fold maximum increase in activity over the vehicle control. The methyl ester **8** also placed the ester near Arg83 (Figure S5C, within 2.5 Å) and gratifyingly enhanced 20S activity with much better efficacy (~10-fold maximum activity over vehicle control, Figure 3C). Unlike compound **2**, the methyl ester **8** displayed a classic saturation curve with an EC₅₀ = 15.6 μ M. Concentration response curves of all compounds are found in Figure S6. These derivatives are consistent with our docking model and may provide unique tools to gain further insight into the complex mechanics of 20S gate regulation. For example, induction of 20S gate opening using SDS (0.02%) followed by the addition of CPZ, or compounds **1**, **2**, **7**, and **8**, results in a reverse effect (i.e., a dose–response decrease in proteolysis of the fluorogenic CT-L peptide probe), highlighting the significance of the different 20S conformations in gate-regulatory mechanics (Figure S8).

We evaluated the ability of the phenothiazine derivatives to enhance the degradation of disordered proteins in cell culture by using green fluorescent protein (GFP)-labeled ornithine decarboxylase (ODC) as a model system. ODC is a classic example of an intrinsically disordered protein and a known 20S substrate.^{4,51} HEK 293 cells stably expressing ODC-

GFPSpark were treated with either vehicle; CPZ; compounds **1**, **7**, and **8**; proteasome inhibitor bortezomib (BTZ); or compound **2** for 24 h. ODC-GFPSpark degradation was monitored by immunoblot of cell lysates with a GFP antibody. Cells were treated with cycloheximide to ensure reduction of protein levels was due to a post-translational event. Only the active phenothiazines CPZ, **2**, **7**, and **8** reduced GFP-ODC levels, compared to the controls (vehicle and BTZ), thus validating the ability of these 20S agonists to degrade intrinsically disordered proteins in cell culture (Figure 3D). Importantly, the concentrations of structured protein GAPDH was unaffected by any of the 20S agonist (Figure 3D, lower panel).

Encouraged by these results, we evaluated the 20S agonists with the intrinsically disordered protein tau (associated with the pathogenesis of Alzheimer's disease)⁵² *in vitro* and in cell culture. We mixed purified 20S proteasome with disordered protein tau and structured proteins GAPDH, in the presence of vehicle (positive control), chlorpromazine (20S agonist), compounds **7** and **8** (30 μ M each), bortezomib (2 μ M BTZ, proteasome inhibitor, negative control), compound **2**, and the detergent SDS (0.02%). Western blot analysis shows that only CPZ and the active derivatives **2** and **8** significantly enhance the rate of 20S-mediated degradation of tau compared to 20S/vehicle control (Figure 4A, lane 2 versus lanes 3, 5, and 7). It is important to note that the detergent SDS (0.02%) prevents the 20S-mediated degradation of tau441 compared to 20S vehicle control (Figure 4A, lane 2 versus lane 8), as earlier reported.⁵³

Next, we evaluated the efficacy of the 20S agonists in glioblastoma (U87-MG) cells for the clearance of tau over various structured proteins. U87-MG cells were treated with 20 μ g/mL of cycloheximide to ensure changes in protein levels are at the post-translational level. Cells were treated with either vehicle; chlorpromazine (CPZ, 50 μ M); compounds **1**, **7**, and **8** (50 μ M each); proteasome inhibitor bortezomib (BTZ, 3 μ M); or compound **2** (50 μ M) for 24 h. Relative protein concentrations were evaluated by immunoblot of the cell lysates. As shown in Figure 4B, the 20S agonists CPZ and compounds **8** and **2** were all capable of significantly reducing tau concentrations compared to the vehicle control, consistent with our results in the purified protein assay (Figure 4A). Compounds **1** and **7** only enhanced modest clearance of tau (Figure 4B), which is also consistent with their lower *in vitro* activity compared to CPZ and compounds **2** and **8** (Figure 3B). We also probed for possible effects on various structured proteins (GAPDH, β -actin, and Hsp70) and proteasome subunits β 5 (subunit of the 20S) and Rpt1 (subunit of the 19S cap) by using their corresponding antibodies. The relative concentrations of the structured proteins GAPDH and β -actin were unchanged compared to the vehicle control. In addition, the level of Hsp70, which is actively involved in the 20S $\leftrightarrow$ 26S dynamic equilibrium⁵⁴ and itself a substrate for the 26S proteasome,^{55,56} was also unchanged upon treatment with the 20S agonists. Importantly, the reduction of tau did not appear to be due to changes in proteasome concentrations, as judged by the lack of changes in β 5 or Rpt1 concentrations (Figure 4B, lower two panels).

In conclusion, screening of the NIH Clinical Collection and Prestwick libraries for possible enhancers of the 20S proteasome identified the neuroleptic agent chlorpromazine as a lead agent. Chemical manipulation of chlorpromazine reduced its D2R receptor binding affinity while retaining its ability to enhance 20S mediated proteolysis *in vitro* and in cell culture.

Several 20S agonists were capable of reducing intrinsically disordered proteins, such as tau and α -synuclein, without affecting structure proteins. All data are consistent with a possible binding of these agents in the $\alpha 1/\alpha 2$ intersubunit pockets of the α -ring of the 20S proteasome, which induces an open-gate 20S conformation. These small molecule enhancers of 20S proteasome activity can serve as leads to explore the therapeutic potential of 20S activation and/or as new tools to provide insight into the yet unclear mechanics of 20S gate regulation.^{33,57}

METHODS

Materials/Reagents

Human proteasomes (20S, 26S) and fluorogenic substrates; N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC), carboxyl benzyl-Leu-Leu-Glu-7-amido-4-methylcoumarin (Z-LLE-AMC), *tert*-butyloxycarbonyl-Leu-Arg-Arg-7-amido-4-methylcoumarin (Boc-LRR-AMC), acetyl-Pro-Ala-Leu-7-amido-4-methylcoumarin (Ac-PAL-AMC), and bortezomib were obtained from Boston Biochem (Cambridge, MA). The nitrocellulose membrane, Clarity western ECL reagent, blocking grade milk, and precast SDS gels were from BioRad (Hercules, CA). Recombinant wild type α -synuclein, tau441, and GAPDH were obtained from Abcam (Cambridge, MA). Rabbit polyclonal anti- α -synuclein (C-20), antitau, goat antirabbit-HRP, rabbit polyclonal GAPDH-HRP, and GFP-HRP were purchased from Santa Cruz Biotechnologies. Compounds used for HTS were obtained from the NIH Clinical Collection and Prestwick libraries, through the MSU Assay Development and Drug Repurposing Core (ADDRC). Fresh phenothiazines (Figure 2A) were obtained from Sigma-Aldrich (St. Louis, MO). Human ornithine decarboxylase/ODC1 Gene ORF cDNA clone expression plasmid, N-GFPSpark, and the sinofection transfection reagent were bought from Sino Biological Inc. (China). Embryonic kidney cells (HEK293T) were a gift from Dr. Benita Sjögren, Department of Pharmacology and Toxicology, Michigan State University, while the glioblastoma astrocytoma (U-87 MG) cell line was obtained from ATCC.

Cells

Human embryonic kidney cells (HEK293T) or glioblastoma cells (U87-MG) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum, and 100 U/mL penicillin/streptomycin.

Proteasome Activity Assay

Activity assays were carried out in a 200 μ L reaction volume. Different concentrations of test compounds were added to a black flat/clear bottom 96-well plate containing 1 nM of either human constitutive 20S proteasome or 26S proteasome, in 50 mM Tris-HCl at pH 7.5 and allowed to sit for 10 min at RT. Fluorogenic substrates were then added and the enzymatic activity measured at 37°C on a SpectraMax M5e spectrometer by measuring the change in fluorescence unit per minute for 1 h at 380–460 nm. The fluorescence units for the vehicle control were set at a 100%, and the ratio of drug-treated sample set to that of vehicle control was used to calculate the fold change in enzymatic activity. Fold activity was plotted as a function of drug concentration, using graphpad prism 5. The fluorogenic substrates used

were Suc-LLVY-AMC (CT-L activity, 10 μ M), Z-LLE-AMC (Casp-L activity, 10 μ M), and Boc-LRR-AMC (T-L activity, 20 μ M). Magnesium chloride (5 mM) and ATP (2.5 mM) were included in assays containing the 26S proteasome. HTS was carried out in 384-well plates as described above with the exceptions that each compound was tested at a single concentration (10 μ M), and dispensing of assay reagents and compounds was automated.

Docking Studies

The crystal structure of the closed gate human proteasome was obtained from the PDB database (PDB ID: 4R3O). Molecules were generated in Chem-Bio3D, minimized using the MM2 force field, and converted to PDB. Docking was conducted in three stages utilizing AutoDock Vina mated to PyRx. **Stage I:** Each identified hit compound was docked against the entirety of the h20S proteasome (grid box $153.2 \times 138.0 \times 189.4$ Å) three times per compound with exhaustiveness set to 60. Active compounds displayed a preference for the α -rings (Figure S5A). **Stage II:** Following the results from stage I, docking was resubmitted with a new center at (136.2, -40.6, 60.5) and grid box dimensions reduced to $125.0 \times 138.0 \times 71.6$ Å and exhaustiveness left at 60 (Figure S5A). Stage II revealed numerous bound poses of each compound within the α -ring intersubunit pocket. **Stage III:** The binding pocket was isolated with a center at (159.7, -63.9, 69.8) and dimensions $30.98 \times 28.6 \times 30.1$ Å (Figure S5A). Exhaustiveness was raised to 80. Individual poses were manually inspected with higher energy binding poses preferred. Potential compounds to synthesize were docked in the same manner and those with similar binding preferences were synthesized and checked for 20S mediated CT-L activity. The rationale for the interpretation of our docking studies that led us to favor the $\alpha 1/2$ intersubunit binding pocket is provided in Supporting Information Figure S5.

In Vitro Degradation of α -Synuclein

Digestion of α -synuclein was carried out in a 50 μ L reaction volume made of 20 mM HEPES at pH 7.4, 2 mM EDTA, 1 mM EGTA, 0.5 μ M purified α -synuclein, 0.5 μ M GAPDH, and 15 nM purified human 20S proteasome. Briefly, 20S proteasome was diluted to 17 nM in the reaction buffer. Test compounds or vehicle (1 μ L of 50 $\times$ stock) was added to 44 μ L of 17 nM 20S and incubated at RT for 20 min. The substrate (5 μ L of 5 μ M GAPDH/synuclein mixture) was then added to the reaction mixture and incubated at 37°C for 1 h. The reactions were quenched with concentrated SDS loading buffer. After boiling for 5 min, samples were resolved on a 4–16% Tris-tricine SDS-PAGE and immunoblotted with rabbit polyclonal anti α -synuclein IgG (1:4000) and goat antirabbit HRP (1:5000)/anti-GAPDH-HRP. Blots were developed with ECL western reagent and imaged with X-ray film.

In Vitro Degradation of tau441

Tau degradation was carried out in the same way as α -synuclein with the exceptions that tau and GAPDH were used at a final concentration of 0.1 μ M, and samples were resolved on a 4–20% tris-glycine gel.

D2R Binding Assay

Human embryonic kidney (HEK-293) cells were plated in 100 mm plates and transfected with pcDNA3.1-D2R using Lipofectamine 2000 (Invitrogen; Waltham, MA) according to the manufacturer's instructions. Radioligand binding assays were performed 24 h after transfection essentially as described.⁵⁸ Cells were harvested in PBS and centrifuged for 5 min at 2000*g*. Cells were resuspended in Optimem media (Invitrogen) to 10⁶ cells/mL. A 50 μ L cell suspension (50 000 cells/well), 50 μ L of 1.5 nM [³H]-Spiperone (D2R antagonist), and 50 μ L of vehicle or displacing agents were added to wells in a 96-well microtiter plate. Binding reactions were allowed to reach equilibrium for 90 min at RT. Assays were terminated by rapid filtration over glass fiber filters using a Brandel cell harvester (Brandel; Gaithersburg, MD). Filters were thereafter washed with 5 mL of ice-cold PBS before being transferred to scintillation vials and incubated with 3 mL of scintillation fluid (Ultima Gold; PerkinElmer; Akron OH), at RT overnight. Vials were counted in a β -counter (Wallac 1209 Rackbeta; PerkinElmer) for 2 min/vial. All experiments were performed with duplicate samples. Data were analyzed with nonlinear regression using GraphPad Prism.

Generation of HEK 293 Cells Stably Expressing Human Ornithine Decarboxylase with N-terminal Spark Green Fluorescent Protein (ODC-GFPSpark)

HEK 293T cells were seeded at a density of 1 $\times$ 10⁵ cells/mL in a 24-well plate overnight. DNA (1 μ g of ODC-GFPSpark plasmid) was mixed with 250 μ L of serum free DMEM medium. Sinofection transfection reagent (5 μ L) was also mixed with 250 μ L of serum free medium in a separate vial. The separate mixtures were combined and allowed to sit at RT for 15 min. The mixture was then added to HEK 293 cells in a 24-well plate and allowed to incubate for 4 h at 37°C, with 5% CO₂, in a tissue culture incubator. The transfection medium was replaced with fresh complete culture medium (with 10% FBS). Three days later, cells were trypsinized and resuspended in hygromycin (100 μ g/mL) selection medium. Surviving clones were picked and expanded in hygromycin selection medium for 6 weeks. After three passages, stable expression was confirmed by confocal fluorescent imaging, using standard filters for GFP.

ODC-GFPSpark Degradation in HEK 293T Cells

HEK 293T cells stably expressing ODC-GFPSpark were seeded in a T-75 flask, in hygromycin selection medium 2 days prior to treatment, such that cells were about 70% confluent at the time of treatment. Cells were incubated with fresh medium (no hygromycin) containing 20 μ g/mL of cycloheximide, in combination with either vehicle, bortezomib (3 μ M), or 50 μ M of the indicated compounds for 24 h. ODC-GFPSpark degradation was monitored by immunoblot of cell lysates with GFP antibody.

Tau Degradation in U87MG Cells

Glioblastoma (U87MG) cells at about 70% confluency were incubated with medium containing 20 μ g/mL of cycloheximide, in combination with either vehicle, bortezomib (3 μ M), or 50 μ M of the indicated compounds for 24 h. Tau degradation was monitored by immunoblot of cell lysates with antibody against full length tau.

Statistical Analyses

Data are presented as mean $\pm$ standard deviation of at least three independent experiments. Western blots were quantified with imageJ and statistical analysis done with GraphPad Prism 5 software. An unpaired Student's *t* test was used for two samples, while one-way analysis of variance with a *post hoc* Bonferroni test was used for multiple comparisons of means

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fully assembled (active) 26S proteasome in a dynamic equilibrium with the 20S CP and activating complexes (“caps”).

Figure 2.

(A) EC₅₀ values of phenothiazines for enhancement of 20S proteasome chymotrypsin-like (CT-L) proteolytic activity. (B) Preferred docking site utilizing Autodock Vina of chlorpromazine (CPZ) in the α1/α2 intersubunit binding pocket of the α-ring of the 20S proteasome. (C) Overlay model of chlorpromazine and compound 2, docked in the α1/α2 binding pocket, and their interaction with Arg83. (D) Concentration response curves of phenothiazines (A) for enhancement of 20S proteasome CT-L activity. (E) 20S proteasome CT-L activity with varying concentrations of 1 and 2. (F) Measurement (RFU/h) of 20S CT-L-mediated proteolysis of fluorogenic CT-L peptide substrate versus 26S activity by compound 2 over vehicle control. (G) Western blot of a mixture of α-synuclein (α-syn) and GAPDH in the presence of purified 20S proteasome with vehicle, various concentrations of compound 2, proteasome inhibitor bortezomib (BTZ, 2 μM), chlorpromazine (CPZ, 10 μM), and SDS (0.02%).

Figure 3.

(A) Synthesis of compounds **1–8**: (a) NaH, THF, reflux 2 h, then addition of sultone reflux 12 h, cool to rt. (b) NaH, THF, alkyl halide, 1 h. (c, i) **9a** with NaI, acetone, reflux 3 days, then NaSO₃H, rt, 12 h for **4**, (ii) **9b** with NaI, acetone, reflux 3 days, then morpholine neat, reflux 2 h for **5**. (d) **10**, *n*BuLi, THF, -78°C , 20 min then CO₂ 2 h, warm to 10°C . (e) KOH, ethanol/water (1:1), reflux 2 h. (B) EC₅₀ values of 20S proteasome CT-L activity and maximum fold increase over vehicle control, % inhibition of [³H]-spiperone binding at the D2R and D2R K_i of chlorpromazine (CPZ) and analogues **1–8**. (C) Concentration response curve of compounds **2** and **8** (up to 70 μM) on 20S proteasome-mediated hydrolysis of the pro-fluorogenic CT-L substrate (Suc-LLVY-AMC). (D) HEK 293 cells stably expressing ODC-GFPspark treated with 20 $\mu\text{g}/\text{mL}$ of cycloheximide, in combination with either vehicle; CPZ (50 μM); compounds **1**, **7**, and **8** (50 μM each); proteasome inhibitor bortezomib (BTZ, 3 μM); or compound **2** (50 μM), for 24 h. ODC-GFPspark degradation was monitored by immunoblot of cell lysates with GFP antibody.

Figure 4.

(A) Western blot of a mixture of tau441 and GAPDH in the presence of 20S proteasome with vehicle, chlorpromazine (CPZ, 30 μ M), compounds **7** and **8** (30 μ M each), proteasome inhibitor bortezomib (BTZ, 3 μ M), **2** (30 μ M), and detergent SDS (0.02%). Relative tau density was determined using densitometry compared to untreated tau control. (B) Glioblastoma (U87-MG) cells were treated with 20 μ g/mL of cycloheximide, in combination with either vehicle, chlorpromazine (CPZ, 50 μ M), compounds **7** and **8** (50 μ M each), or proteasome inhibitor bortezomib (BTZ, 3 μ M) for 24 h. Tau degradation was monitored by immunoblot of cell lysates. Structured proteins (GAPDH, β -actin and Hsp70) and proteasome subunits (β 5 and Rpt1) were probed with corresponding antibodies.