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Supporting Information for:

Protein Degradation Profile Reveals Dynamic Nature of 20S Proteasome Small Molecule Stimulation

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Supporting Information Figure S2. Results of the protein degradation assay for TRC-1 and ursolic acid (UA) are shown in (**A**) for proteins smaller than 20 kDa and (**B**) protein larger than 20 kDa. For each graph, the proteins are shown in order of increasing disorder.

Supporting Information Figure S3. Comparison of the fold-change in degradation between the biochemical assay and the cellular GFP-fusion assay for actin (**A**), p53 (**B**), and α-synuclein (**C**). *$p < 0.05$; **$p < 0.01$

Supporting Information Figure S4. Global proteomic analysis of HEK cells treated with AM, MO and Btz. **A)** Heatmap representation of Z-scored LFQ values for all quantified proteins in each AM, MO and Btz with its respective DMSO control. Color scale represents the relative Z-score. Missing values are represented by blank spaces. **B)** Volcano plot representation of treatment compared to its corresponding DMSO control. In figure legend: Grey correspond to no significance; Green correspond to proteins with $Log_2(FC) \geq 0.5$; Blue correspond to proteins with $p \leq 0.05$, and Red correspond to proteins with both $p \leq 0.05$ and $Log_2(FC) \geq 0.5$. **C)** Venn diagram of overlapping proteins in BTZ (red), AM (green) and MO (blue).

Supporting Information Figure S5. Treatment induced regulation of proteasome subunits. Heatmap represents all quantified proteasome subunits identified in the Btz treatment condition compared to its DMSO control. Color scale represents the relative Z-score.

Supporting Information Tables:

Supporting Information Table S1. Results of the protein degradation assay. Shown are the percent degradation values calculated from the gel band quantitation analysis. Red = values that are significantly ($p < 0.05$) different from the "No 20S CP" samples. Blue = values that are significantly different from "No 20S CP" and "Basal Level" samples. Green = values that are significantly different from "Basal Level" samples but not "No 20S CP" samples.

Supporting Information Table S2. Statistical analysis of the protein degradation assay was performed using the t-test function in GraphPad Prism 8. The p-values from this analysis are shown in the table below.

Supporting Information Table S3. Uniprot ID and log_2(fold-change) of proteins shown to change due to proteasome activity in the MO-treated samples. *Separate Excel File

Supporting Information Table S4. Uniprot ID and log_2(fold-change) of proteins shown to change due to proteasome activity in the AM-treated samples. *Separate Excel File

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Appendix I: Gels from biochemical protein degradation assay

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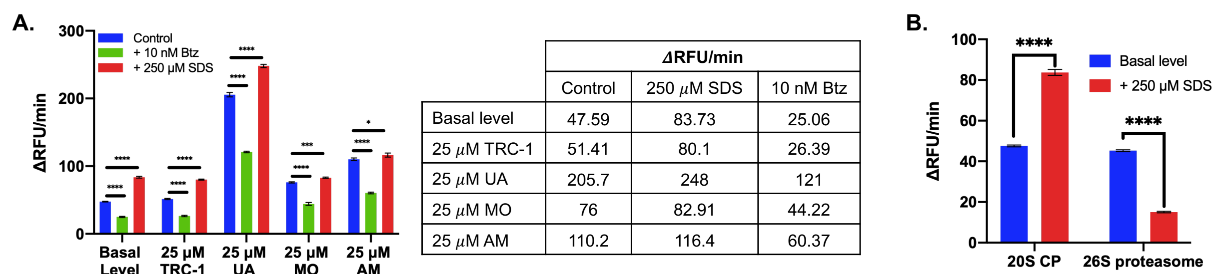
Methods

Biochemical Proteasome Activity Assays

TRC-1, ursolic acid, miconazole, and AM-404 were tested in a dose-response manner using our previously reported FRET reporter to determine the ability of each molecule to stimulate the 20S CP.[1] Briefly, each black 384-well plate contained the following controls in triplicate: No Proteasome and Basal Level. Each well contained 20 µM FRET reporter in Tris-HCl (50 mM, pH 7.6) and 2% DMSO in a total volume of 25 µL. Once the FRET solution was added in triplicate for the "No Proteasome" wells, the respective proteasome isoform was added to the remainder of the FRET solution at a final concentration of 5 nM. From this solution, 24.5 µL was added to the remaining wells. DMSO/compound was added by the addition of 0.5 µL DMSO or 50x µM compound in DMSO to the wells in triplicate, such that the final concentration of compound ranged from 0.39 to 200 µM. The plate was briefly centrifuged on a microplate centrifuge and was placed into a Tecan Infinite plate reader, which was set to heat the plate at 37°C. The excitation and emission wavelengths were set to 335 nm and 493 nm, respectively. The fluorescence was recorded every 2 min over a 60 min period, and the resulting fluorescence data was plotted against time using GraphPad Prism 8. A linear regression analysis was performed for the last 40 min of recorded fluorescence, as this is the most linear region. The slope of this line is described as the rate of hydrolysis of the FRET peptide reporter by the 20S CP. This procedure was repeated to test the effect of each molecule at 25 µM on the activity of the 26S proteasome. However, for the assay using the 26S proteasome, 1 mM ATP and 10 mM $MgCl_2$ were included in the Tris-HCl (50 mM, pH 7.6)

A similar assay was performed to examine the effect of each stimulator on the chymotrypsin-like activity of the 20S CP in the absence and presence of 250 µM sodium dodecyl sulfate (SDS) or 10 nM bortezomib (Btz). This assay was performed as described above with the following modifications: 20 µM Suc-LLVY-AMC was used instead of the FRET reporter. SDS or Btz were added to the solution of 20S CP prior to the addition of the small molecule stimulators. With the exception of these modifications, the assay was performed as described above. The ΔRFU/min were plotted in GraphPad Prism 8 and compared between samples, **Supporting Information Figure S1A**. For a comparison of how SDS impacts the activity of the 26S proteasome, a similar assay was performed using the 26S proteasome in Tris-HCl (50 mM, pH 7.6, 1 mM ATP, 10 mM $MgCl_2$), **Supporting Information Figure S1B**.



	ΔRFU/min		
	Control	250 µM SDS	10 nM Btz
Basal level	47.59	83.73	25.06
25 µM TRC-1	51.41	80.1	26.39
25 µM UA	205.7	248	121
25 µM MO	76	82.91	44.22
25 µM AM	110.2	116.4	60.37

Supporting Information Figure S1. A) The chymotrypsin-like activity of the 20S CP was monitored using 20 µM Suc-LLVY-AMC in the presence of DMSO (control), 25 µM TRC-1, 25 µM UA, 25 µM MO, and 25 µM AM all with and without 250 µM SDS or 10 nM bortezomib (Btz). The change in RFU/min is plotted and shown in the table for each treatment. **B)** The effect of 250 µM SDS on the activities of the 20S CP and 26S proteasome was also demonstrated using the Suc-LLVY-AMC reporter.

Protein Degradation Assay

Each protein was diluted in Tris-HCl (50 mM, pH 7.6) to a final concentration of 50 ng/μL. The small molecule 20S CP stimulators were dissolved in DMSO and diluted to a final concentration of 75 μM in Tris-HCl, containing 6% DMSO. A solution of 15 nM 20S CP was made by diluting the 2 μM purchased stock solution of human 20S CP (Boston Biochem Cat. #E-360) in Tris-HCl. The following samples were prepared for each compound and each protein in triplicate: No 20S CP, Basal Level, and 25 μM stimulator. The No 20S CP samples were prepared by mixing 4 μL of the 50 ng/μL protein solution and 8 μL of 3% DMSO in Tris-HCl. The Basal Level samples were prepared by mixing 4 μL of the 50 ng/μL protein solution, 4 μL of 6% DMSO in Tris-HCl, and 4 μL of 15 nM 20S CP. The 25 μM stimulator samples were prepared by mixing 4 μL of the 50 ng/μL protein solution, 4 μL of the 75 μM stimulator solution, and 4 μL of 15 nM 20S CP.

Each of the samples contained a total volume of 12 μL, a final DMSO concentration of 2%, and 200 ng of protein. The samples containing the 20S CP had a final concentration of 5 nM 20S CP, and the samples containing the small molecule had a final concentration of 25 μM stimulator.

Following preparation, the samples were incubated for 2 hr at 37°C. After this period, 4 μL of 4x SDS gel loading buffer was added to each sample, which were then heated for 5 min at 95°C. The samples were then loaded onto a 15-well gradient gel for SDS-PAGE. The gels were stained with Coomassie, imaged with a LICOR Odyssey, and quantitated using ImageJ. For each protein, the calculated band intensities were normalized. This allowed for the No 20S CP samples and the Basal Level samples to be compared for each protein across all samples for that protein (4 gels = 12 replicates for No 20S CP and Basal Level samples). The triplicate of stimulator treated samples were then compared to the total No 20S CP and Basal Level replicates from all gels. The results of this analysis are shown in **Supporting Information Table S1**, **Supporting Information Table S2**, **Figures 2C** and **2D**, **Supporting Information Figures S2A** and **S2B**, and **Appendix I**.

Supporting Information Table S1. Results of the protein degradation assay. Shown are the percent degradation values calculated from the gel band quantitation analysis. Red = values that are significantly ($p < 0.05$) different from the "No 20S CP" samples. Blue = values that are significantly different from "No 20S CP" and "Basal Level" samples. Green = values that are significantly different from "Basal Level" samples but not "No 20S CP" samples.

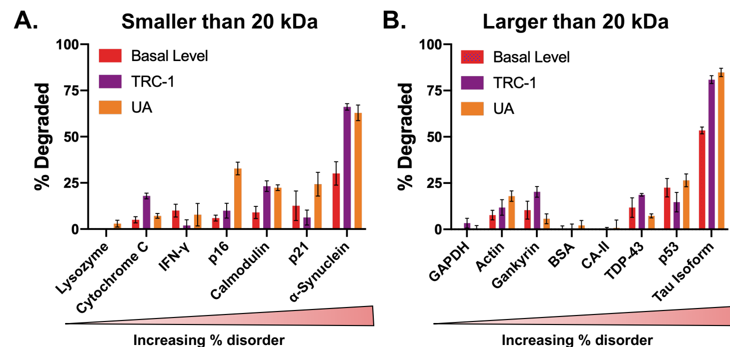
Protein	Degradation (%)				
	Basal Level	25 μM TRC-1	25 μM Ursolic Acid	25 μM Miconazole	25 μM AM-404
Lysozyme	(2)	(4)	3	14	3
GAPDH	(1)	3	(1)	(4)	2
Actin	8	12	18	22	24
Gankyrin	10	20	6	29	31
BSA	0	(1)	2	18	7
Carbonic Anhydrase 2	(5)	(2)	1	9	(2)
Cytochrome C	5	18	7	51	15
Interferon Gamma	10	2	8	31	28
p16	6	10	33	45	9
TDP-43	25	18	5	24	57
p53	23	15	23	34	49
Calmodulin	17	23	20	43	99
p21	13	6	24	43	23
α-Synuclein	30	66	63	94	90
Tau (1-383) Isoform D	53	81	85	96	97

The following proteins were tested: lysozyme (MP Biomedicals Cat. #02100834), GAPDH (Fisher Scientific Cat. #PILFP0008), actin (Novus Biologicals Cat. #NBP2-22670), gankyrin (AddGene Cat.

#31332), bovine serum albumin (BSA; Millipore Sigma Cat. #A8531), carbonic anhydrase 2 (Millipore Sigma Cat. #C7025), cytochrome C (Millipore Sigma Cat. #C7150), interferon gamma (BioLegend Cat. #713906), p16 (Novus Biologicals Cat. #NBP2-35199), TDP-43 (Novus Biologicals Cat. #AP-190-100), p53 (Novus Biologicals Cat. #SP-454-020), calmodulin (gift from Prof. Val Watts of Purdue University), p21 (Novus Biologicals Cat. #NBP2-22976), α-synuclein (Novus Biologicals Cat. #NBP2-54787), and tau isoform D (Novus Biologicals Cat. #NBP1-50881).

Supporting Information Table S2. Statistical analysis of the protein degradation assay was performed using the t-test function in GraphPad Prism 8. The p-values from this analysis are shown in the table below.

Protein	P-values				
	Basal Level	25 μM TRC-1	25 μM Ursolic Acid	25 μM Miconazole	25 μM AM-404
Lysozyme	No 20S CP: 0.397231	No 20S CP: 0.240080 Basal Level: 0.303763	No 20S CP: 0.400768 Basal Level: 0.084619	No 20S CP: 0.001311** Basal Level: 0.000021***	No 20S CP: 0.408289 Basal Level: 0.090490
GAPDH	No 20S CP: 0.211028	No 20S CP: 0.103094 Basal Level: 0.028158*	No 20S CP: 0.559181 Basal Level: 0.884347	No 20S CP: 0.060264 Basal Level: 0.254649	No 20S CP: 0.216438 Basal Level: 0.062564
Actin	No 20S CP: 0.000475***	No 20S CP: 0.000232*** Basal Level: 0.301277	No 20S CP: 0.000001*** Basal Level: 0.016051*	No 20S CP: <0.000001*** Basal Level: 0.002002**	No 20S CP: <0.000001*** Basal Level: 0.000807***
Gankyrin	No 20S CP: 0.000217***	No 20S CP: <0.000001*** Basal Level: 0.071137	No 20S CP: 0.031827* Basal Level: 0.374493	No 20S CP: <0.000001*** Basal Level: 0.002744**	No 20S CP: <0.000001*** Basal Level: 0.001058**
BSA	No 20S CP: 0.915222	No 20S CP: 0.817950 Basal Level: 0.806082	No 20S CP: 0.644264 Basal Level: 0.465765	No 20S CP: 0.001982** Basal Level: 0.000113***	No 20S CP: 0.126668 Basal Level: 0.031796*
Carbonic Anhydrase 2	No 20S CP: 0.002434**	No 20S CP: 0.517224 Basal Level: 0.171621	No 20S CP: 0.776868 Basal Level: 0.022479*	No 20S CP: 0.006342** Basal Level: 0.000019***	No 20S CP: 0.532365 Basal Level: 0.141248
Cytochrome C	No 20S CP: 0.001222**	No 20S CP: <0.000001*** Basal Level: 0.000227***	No 20S CP: 0.000840*** Basal Level: 0.437880	No 20S CP: <0.000001*** Basal Level: <0.000001***	No 20S CP: <0.000001*** Basal Level: 0.002205**
Interferon Gamma	No 20S CP: 0.000284***	No 20S CP: 0.520070 Basal Level: 0.654476	No 20S CP: 0.026286* Basal Level: 0.654476	No 20S CP: <0.000001*** Basal Level: 0.000392***	No 20S CP: <0.000001*** Basal Level: 0.001628**
p16	No 20S CP: 0.000548***	No 20S CP: 0.000359*** Basal Level: 0.203280	No 20S CP: <0.000001*** Basal Level: <0.000001***	No 20S CP: <0.000001*** Basal Level: <0.000001***	No 20S CP: 0.000630*** Basal Level: 0.318808
TDP-43	No 20S CP: 0.000058***	No 20S CP: <0.000001*** Basal Level: 0.205046	No 20S CP: 0.005458** Basal Level: 0.406684	No 20S CP: 0.000006*** Basal Level: 0.259367	No 20S CP: <0.000001*** Basal Level: <0.000001***
p53	No 20S CP: <0.000001***	No 20S CP: 0.000017*** Basal Level: 0.124828	No 20S CP: <0.000001*** Basal Level: 0.418167	No 20S CP: <0.000001*** Basal Level: 0.133102	No 20S CP: <0.000001*** Basal Level: 0.000016***
Calmodulin	No 20S CP: 0.000128***	No 20S CP: <0.000001*** Basal Level: 0.003457**	No 20S CP: <0.000001*** Basal Level: 0.004839**	No 20S CP: <0.000001*** Basal Level: <0.000001***	No 20S CP: <0.000001*** Basal Level: <0.000001***
p21	No 20S CP: 0.000347***	No 20S CP: 0.015451* Basal Level: 0.367027	No 20S CP: <0.000001*** Basal Level: 0.111140	No 20S CP: <0.000001*** Basal Level: 0.000421***	No 20S CP: <0.000001*** Basal Level: 0.132610
α-Synuclein	No 20S CP: <0.000001***	No 20S CP: <0.000001*** Basal Level: 0.000007***	No 20S CP: <0.000001*** Basal Level: 0.000022***	No 20S CP: <0.000001*** Basal Level: <0.000001***	No 20S CP: <0.000001*** Basal Level: <0.000001***
Tau (1-383) Isoform D	No 20S CP: <0.000001***	No 20S CP: <0.000001*** Basal Level: <0.000001***	No 20S CP: <0.000001*** Basal Level: <0.000001***	No 20S CP: <0.000001*** Basal Level: <0.000001***	No 20S CP: <0.000001*** Basal Level: <0.000001***



Supporting Information Figure S2. Results of the protein degradation assay for TRC-1 and ursolic acid (UA) are shown in (**A**) for proteins smaller than 20 kDa and (**B**) protein larger than 20 kDa. For each graph, the proteins are shown in order of increasing disorder.

Cell culture

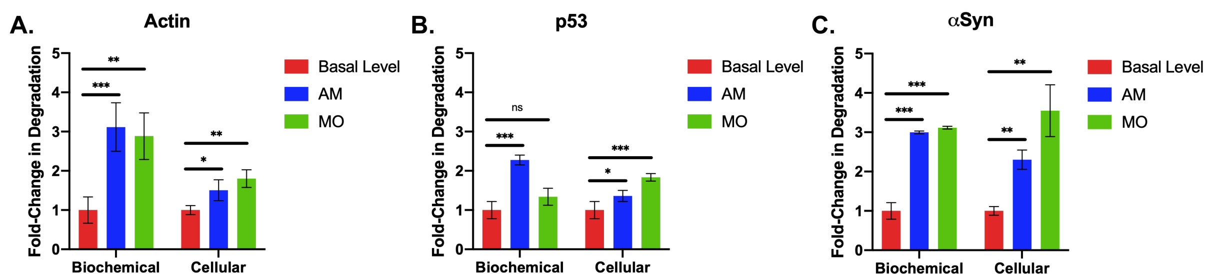
HEK-293T cells purchased from ATCC were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. Cultures were maintained at 37°C with 5% CO_2.

TAS3 probe analysis of 20S CP stimulators in HEK-293T cells

HEK-293T cells were plated on a black 96-well plate at 5000 cells/well. After 24 hr, the cells were treated with the following in three replicates: DMSO (0.001%, control) or 10 μM bortezomib. The plate was placed in the CO_2 incubator for 30 min. After this time period, the plate was removed, and the cells were treated with 25 μM TRC-1, 25 μM ursolic acid, 25 μM miconazole, or 25 μM AM-404 in triplicate. Treatment was performed by adding 10 μL of an 11x solution of compound diluted in medium, ensuring a final DMSO concentration of 0.001%. The plate was incubated in the CO_2 incubator for an additional 30 min. After this time period, the medium was removed. To each well was added 50 μL of 10 μM TAS3 in Krebs-Ringer Bicarbonate (KRBH) buffer, containing the respective control or compound at the aforementioned concentrations. The plate was immediately placed on the Tecan Infinite plate reader, heated at 37°C. Fluorescence intensity was recorded every 2 min over a 90 min period. A linear regression analysis was performed for the last 60 min of this period, as this is the most linear region. The resulting slope of this analysis is compared between samples to determine the increase or decrease in activity compared to the DMSO control.

Degradation of GFP-fusion proteins in HEK-293T cells

HEK-293T cells on a T-75 flask were transiently transfected to express a GFP-fusion protein (GFP-α-synuclein, GFP-p53, or Actin-GFP) using Lipofectamine 2000, 20 μg DNA, and Opti-MEM. After 24 hr, the transfected cells were plated on 6-well plates at 300,000 cells/well. The cells were treated 24 hr after plating with the following in triplicate: DMSO (0.001%, control), 25 μM AM-404, or 25 μM miconazole. Following 24 hr of treatment, the cells were lysed with a solution of M-PER containing Halt protease inhibitor cocktail. The cell lysates were normalized to total protein concentration and analyzed by immunoblot for GFP (**Appendix II**). Quantitative analysis was performed using ImageJ. For each sample, the ratio of free GFP to the GFP-fusion protein was calculated. This calculated ratio of all treated cell lysates was compared to the control (DMSO) to determine a change in 20S CP-mediated degradation. These changes were then compared to the changes observed in the biochemical degradation assay (**Supporting Information Figure S3**).



Supporting Information Figure S3. Comparison of the fold-change in degradation between the biochemical assay and the cellular GFP-fusion assay for actin (**A**), p53 (**B**), and α-synuclein (**C**). *p < 0.05; **p < 0.01; ***p<0.001

Statistical Analyses of Biochemical and Cellular Assays

For the fluorescent activity assays, a linear regression analysis was performed, and the slopes of the resulting lines were compared between the treated and basal level (DMSO control) samples. To determine if the changes were significant, a student's t-test analysis was performed using GraphPad Prism 8 to obtain p-values. A p-value less than 0.05 is considered significant.

Similarly, for the densitometry analyses of Coomassie-stained gels and Western blots, the calculated values were put into GraphPad Prism 8 to determine the statistical significance by performing the student's t-test function to obtain p-values.

Proteomics analysis of treated HEK-293T cells

HEK-293T cell treatment

HEK-293T cells were plated on T-25 flasks. The following day, the cells were treated with the following in four replicates: DMSO1 (0.001%, control 1), 25 μM miconazole (MO), DMSO2 (0.001%, control 2), 25 μM AM-404 (AM), 10 nM bortezomib (Btz), 25 μM MO + 10 nM Btz, or 25 μM AM + 10 nM Btz. DMSO1 and 25 μM MO treatment were performed at the same time, such that DMSO1 is the reference control for the MO treatment. Similarly, DMSO2 and the other cell treatments were performed at the same time, such that DMSO2 is the reference control for these treatments. Following 24 hr of treatment, the cells were collected and pelleted by centrifugation at 1000 xg for 5 min. The cell pellets were washed four times with PBS buffer and transported to the Purdue Proteomics Core Facility in PBS on ice.

Protein extraction

Cells were centrifuged at 6000 rpm for 5 min at 4°C. Pellets were resuspended in 200 μL of 4M urea buffer and homogenized using Precellys ® 24 Bead Mill Homogenizer (Berti) at 6500 rpm for 2 min at 30 sec/cycle. Freshly prepared serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to each tube at final concentration of 1 mM prior to homogenization. After homogenization, the lysates were sonicated on ice for 15 min and precipitated overnight at -20°C using 4 volume of cold (-20°C) 100% acetone. Samples were centrifuged at 14,000 rpm for 15 min at 4°C; the resulting pellets were washed twice using cold (-20°C) 80% acetone and dried in Vacuum Centrifuge for 5 min at 50°C before solubilizing in 50 μL of 8M urea. Samples were incubated at room temperature for 1 hour with continuous vortexing. The solution was centrifuged at 14,000 rpm for 15 min at 4°C to remove any undissolved pellets and cell debris. Protein concentration was measured by bicinchoninic acid (BCA) assay with BSA as a standard.

Fifty (50) μg protein from each sample was reduced, alkylated and digested using the previously published protocol.[2,3] Briefly, samples were reduced by incubating in 10 mM dithiothreitol (DTT) at 37°C for 45 min, and cysteine alkylated by incubating in 20 mM iodoacetamide (IAA) in the dark for 45 min at room temperature. Proteins were digested overnight at 37°C using mass spec grade trypsin and Lys-C mix (Promega Corporation, Madison, WI, USA) at 1:25 (w/w) enzyme-to-protein. The digested peptides were cleaned using C18 silica micro spin columns (The Nest Group Inc.) using the manufacturer's protocol. Peptides were eluted using 80% acetonitrile containing 0.1% formic acid (FA). The samples were vacuum dried and re-suspended in 3% acetonitrile and 0.1% formic acid. Peptide concentration was determined by BCA assay using BSA as standard. The concentration of peptides was adjusted to 0.2 μg/μL and 5 μL (1 μg total peptide) was used for LC-MS/MS analysis in Orbitrap Fusion Lumos Mass Spectrometer.

LC/MS-MS data acquisition

Samples were analyzed by reverse-phase LC-ESI-MS/MS system using the Dionex UltiMate 3000 RSLC nano System coupled to the Orbitrap Fusion Lumos Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were loaded onto a trap column (300 μm ID × 5 mm) packed with 5 μm 100 Å Acclaim PepMap C18 medium, and then separated on a reverse phase column (50-cm long × 75 μm ID) packed with 2 μm 100 Å Acclaim PepMap C18 silica (Thermo Fisher Scientific, Waltham, MA). The column temperature was maintained at 50°C. All the MS measurements were performed in the positive ion mode, using 160 min LC gradient and standard data-dependent mode.

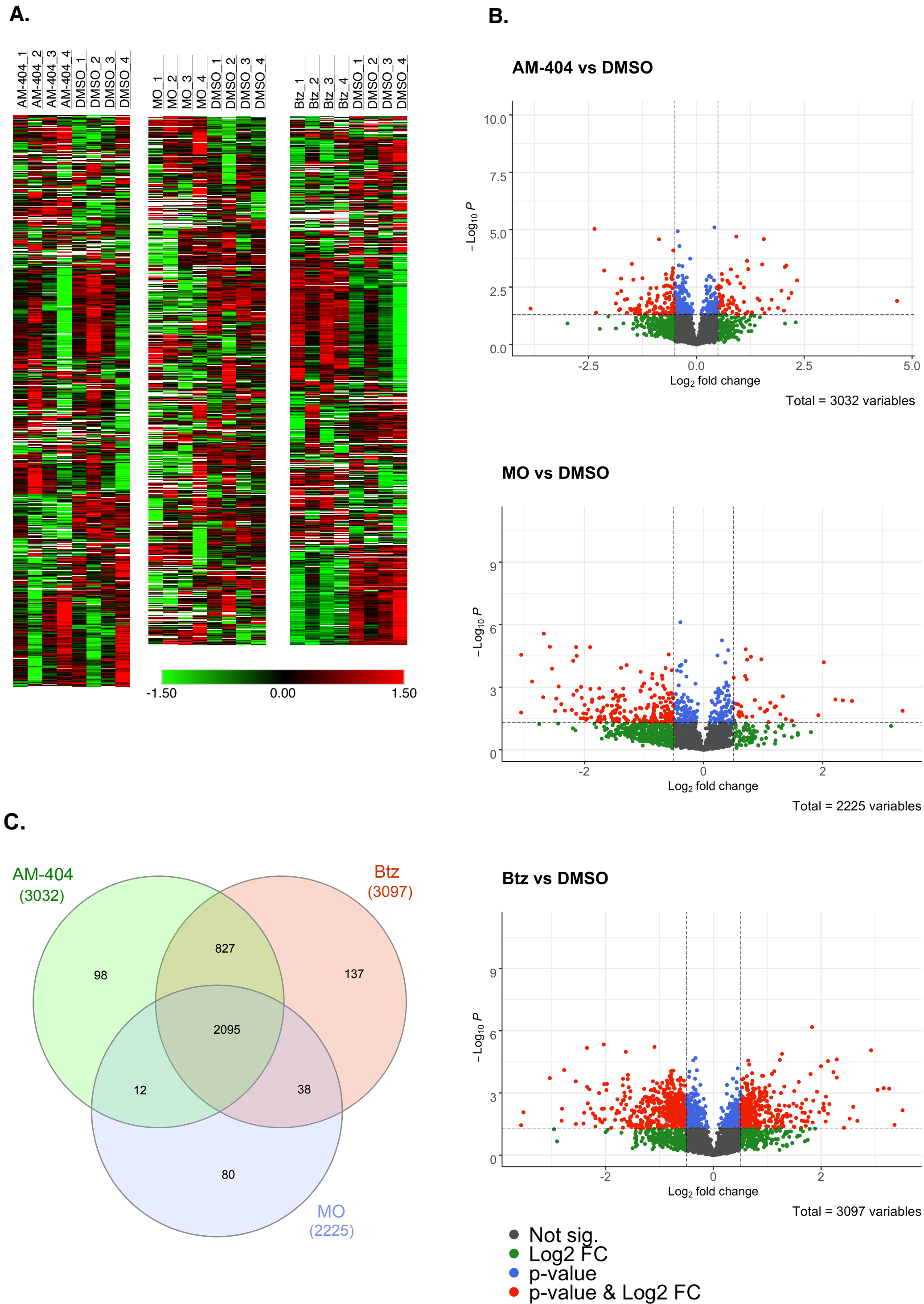
Mobile phase solvent A was 0.1% formic acid (FA) in water and solvent B was 0.1% FA in 80% acetonitrile (I). Loading buffer was 98% water/2% I/0.1% FA. Peptides were loaded to the trap column using a loading buffer for 5-min at 5 μL/min flow rate and eluted from the analytical column with a linear 110-min gradient of 3-27% of buffer B, then changing to 40% of B at 125 min, 100% of B at 135-min at which point the gradient was held for 10 min before reverting to 2% of B at 145-min. Peptides were separated from the analytical column at a flow rate of 300 nL/min. The column temperature was maintained at 50°C. The mass spectrometer was operated in positive ion and standard data-dependent acquisition mode with Advanced Peak Detection function activated. The fragmentation of precursor ion was accomplished by higher energy collision dissociation at a normalized collision energy setting of 30%. The resolution of Orbitrap mass analyzer was set to 120,000 and 15,000 for MS1 and MS2, with maximum injection time of 50 ms for both MS1 and MS2. The dynamic exclusion was set at 60 s to avoid repeated scanning of identical peptides; charge state was set at 2-7 with 2 as a default charge and mass tolerance of 10 ppm for both high and low. The full scan MS1 spectra were collected in the mass range of 375-1,500 m/z and MS2 in 300-1250 m/z. The spray voltage was set at 2 and AGC target of $4e^5$ for MS1 and $5e^4$ for MS2, respectively. MS1 data types were in profile mode and MS2 date types were in centroid mode. Three biological samples were analyzed for each organ. The instrument was calibrated at the start of each batch run and then every 72 hours using a calibration mix solution (Thermo Fisher Scientific). The performance of the instrument was monitored routinely using Hela cell digest from Thermo Scientific.

LC-MS/MS data analysis

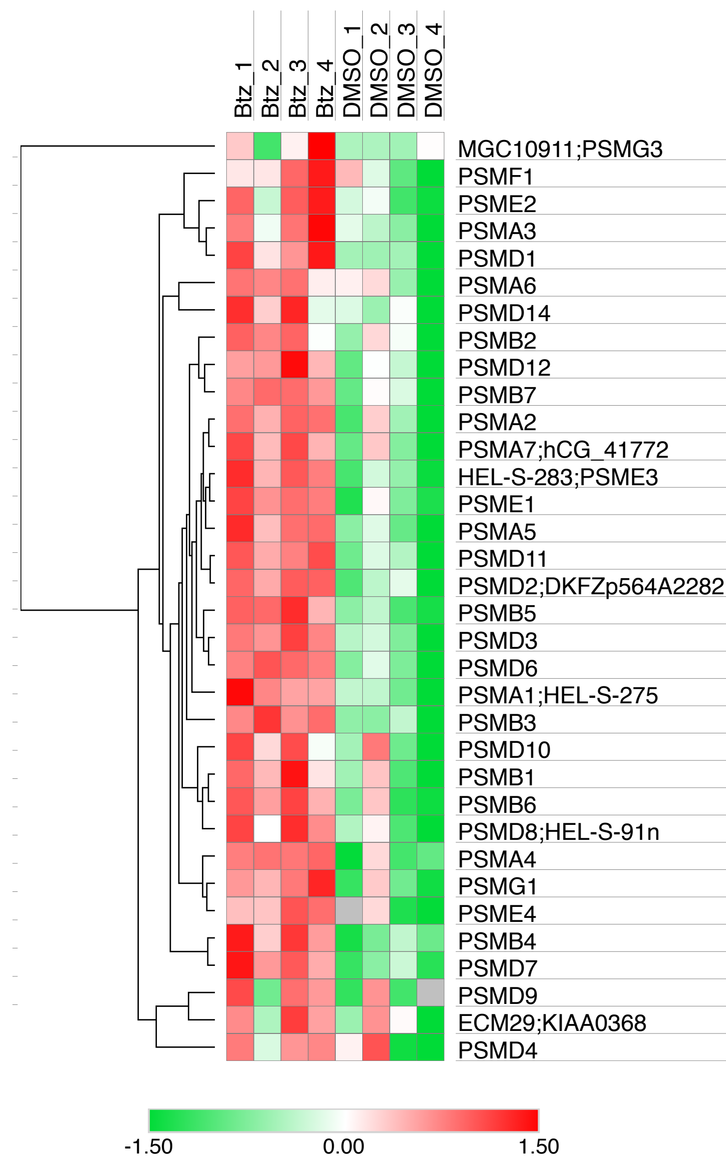
LC-MS/MS data were analyzed using MaxQuant software (version 1.6.3.3) against the *Homo sapiens* protein database (26,446 sequences as of 08/03/2019, www.unitprot.org) concatenated with a common contaminants and a reverse-decoy database.[4,5] Enzymes were setup as Trypsin and LysC, allowing up to 2 missed cleavages. Mass error was set to 10 ppm and 20 ppm for MS1 and MS2, respectively. Alkylation of cysteine and oxidation of methionine was set to fixed and variable modifications, respectively. The false discovery rate (FDR) threshold was set to 0.01 for both the peptides and the proteins. The peptide quantitation was conducted using 'unique plus razor peptides.' The razor peptides are the non-unique peptides assigned to protein/protein group with most other peptides.

The raw MaxQuant output file was then processed with Perseus (version 1.6.10.50). Proteins identified as "contaminants", "reverse" and "only identified by site" were removed, and LFQ values were Log_2 transformed. Proteins with Log_2(LFQ) values in at least 2 biological replicates were retained, and missing values were then imputed based on the column's normal distribution. T-test were performed with each treatment group against its corresponding DMSO control. Significantly regulated proteins were defined as proteins with a $p \leq 0.05$ and Log_2(fold change) ≥ 0.5. Gene ontology was performed using the Metascape software.

Those proteins that significantly changed in the MO-treated samples were examined in the Btz-MO-treated samples to determined which protein were changing due to proteasome activity, **Supporting Information Table S3**. For this analysis, a difference in Log_2(fold-change) of 0.5 or a 50% decrease in the Log_2(fold-change) observed in the MO-treated samples were considered significant. A similar analysis was performed for the proteins that significantly changed in the AM-treated samples, **Supporting Information Table S4**.



Supporting Information Figure S4. Global proteomic analysis of HEK cells treated with AM, MO and Btz. **A)** Heatmap representation of Z-scored LFQ values for all quantified proteins in each AM, MO and Btz with its respective DMSO control. Color scale represents the relative Z-score. Missing values are represented by blank spaces. **B)** Volcano plot representation of treatment compared to its corresponding DMSO control. In figure legend: Grey correspond to no significance; Green correspond to proteins with $Log_2(FC) \geq 0.5$; Blue correspond to proteins with $p \leq 0.05$, and Red correspond to proteins with both $p \leq 0.05$ and $Log_2(FC) \geq 0.5$. **C)** Venn diagram of overlapping proteins in BTZ (red), AM-404 (green) and MO (blue).



Supporting Information Figure S5. Treatment induced regulation of proteasome subunits. Heatmap represents all quantified proteasome subunits identified in the Btz treatment condition compared to its DMSO control. Color scale represents the relative Z-score.

References:

(1) Coleman, R. A.; Trader, D. J. A Sensitive High-Throughput Screening Method for Identifying Small Molecule Stimulators of the Core Particle of the Proteasome. *Curr. Protoc. Chem. Biol.* **2018**, *10* (4), e52. https://doi.org/10.1002/cpch.52.

(2) Harlow, K.; Suarez-Trujillo, A.; Hedrick, V.; Sobreira, T.; Aryal, U. K.; Stewart, K.; Casey, T. Temporal Analysis of Vaginal Proteome Reveals Developmental Changes in Lower Reproductive Tract of Gilts across the First Two Weeks Postnatal. *Sci. Rep.* **2019**, *9* (1), 1–8. https://doi.org/10.1038/s41598-019-49597-w.

(3) Barabas, A. J.; Aryal, U. K.; Gaskill, B. N. Proteome Characterization of Used Nesting Material and Potential Protein Sources from Group Housed Male Mice, Mus Musculus. *Sci. Rep.* **2019**, *9* (1), 1–10. https://doi.org/10.1038/s41598-019-53903-x.

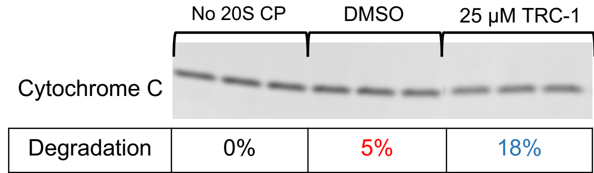
(4) Cox, J.; Mann, M. MaxQuant Enables High Peptide Identification Rates, Individualized p.p.b.-Range Mass Accuracies and Proteome-Wide Protein Quantification. *Nat. Biotechnol.* **2008**, *26* (12), 1367–1372. https://doi.org/10.1038/nbt.1511.
(5) Cox, J.; Neuhauser, N.; Michalski, A.; Scheltema, R. A.; Olsen, J. V.; Mann, M. Andromeda: A Peptide Search Engine Integrated into the MaxQuant Environment. *J. Proteome Res.* **2011**, *10* (4), 1794–1805. https://doi.org/10.1021/pr101065j.

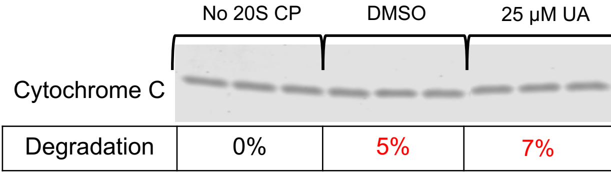
Appendix I: Gels from biochemical protein degradation assay

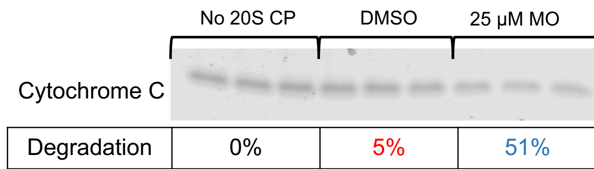
Red: Significantly different from No 20S CP

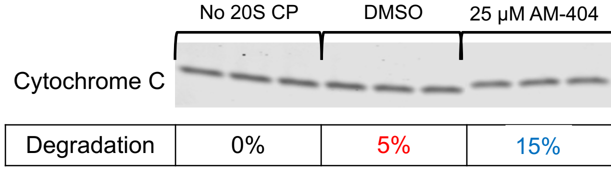
Green: Significantly different from DMSO

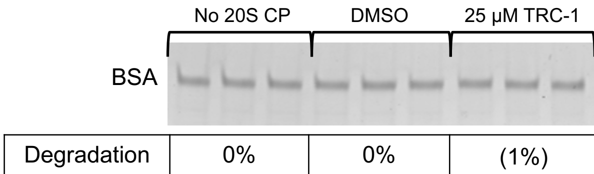
Blue: Significantly different from No 20S CP and DMSO

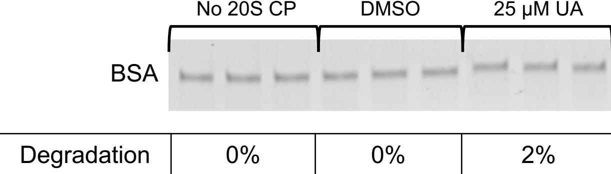


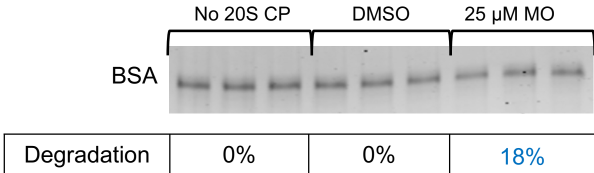


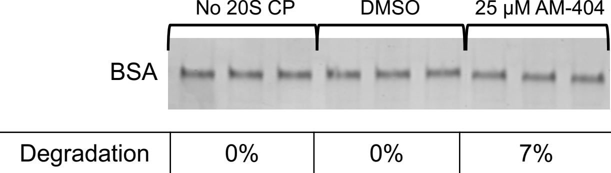


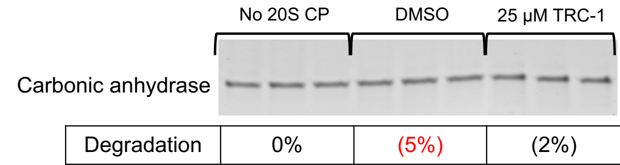


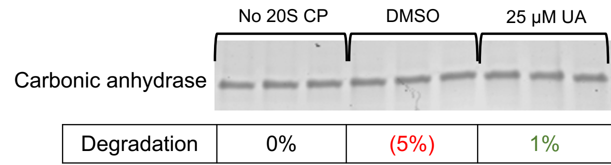


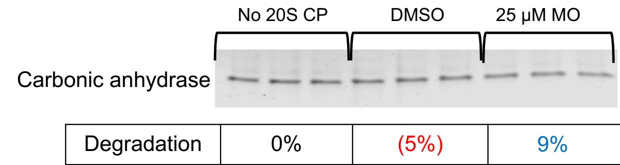


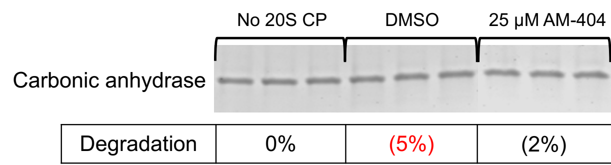


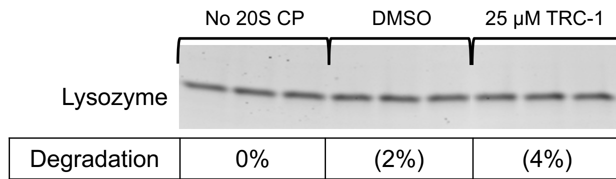


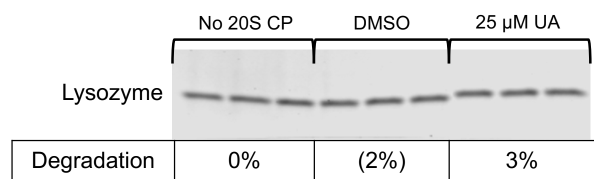


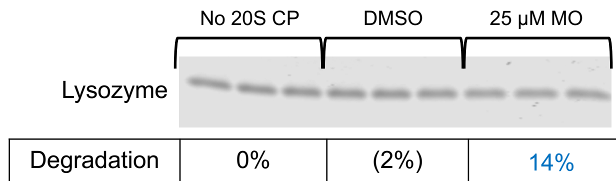


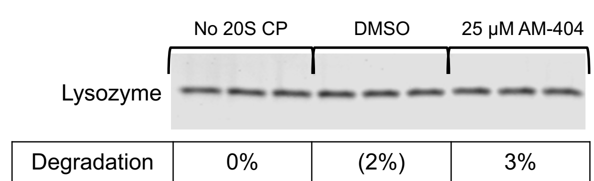


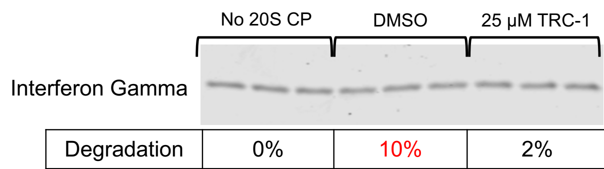


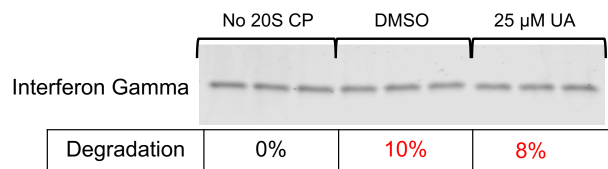

No 20S CP
DMSO
25 μM TRC-1
Lysozyme
Degradation
0%
(2%)
(4%)

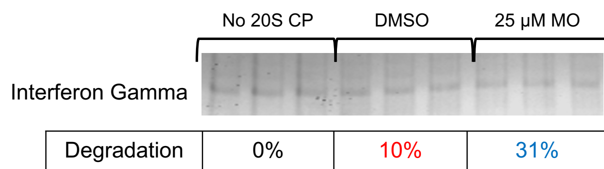

No 20S CP
DMSO
25 μM UA
Lysozyme
Degradation
0%
(2%)
3%

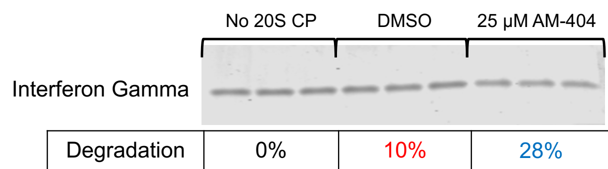

No 20S CP
DMSO
25 μM MO
Lysozyme
Degradation
0%
(2%)
14%

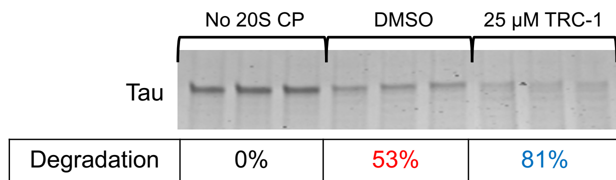

No 20S CP
DMSO
25 μM AM-404
Lysozyme
Degradation
0%
(2%)
3%

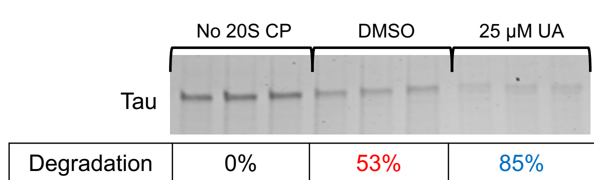

No 20S CP
DMSO
25 μM TRC-1
Interferon Gamma
Degradation
0%
10%
2%

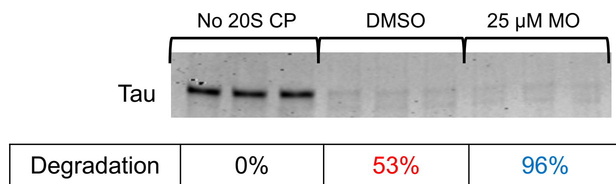

No 20S CP
DMSO
25 μM UA
Interferon Gamma
Degradation
0%
10%
8%

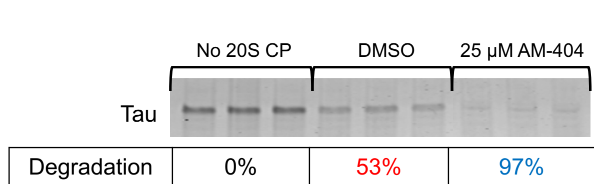

No 20S CP
DMSO
25 μM MO
Interferon Gamma
Degradation
0%
10%
31%

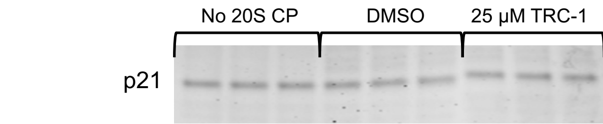

No 20S CP
DMSO
25 μM AM-404
Interferon Gamma
Degradation
0%
10%
28%


No 20S CP
DMSO
25 μM TRC-1
Tau
Degradation
0%
53%
81%

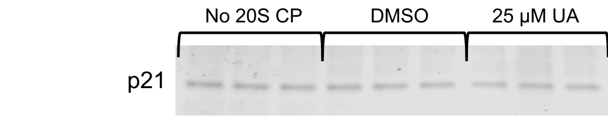

No 20S CP
DMSO
25 μM UA
Tau
Degradation
0%
53%
85%


No 20S CP
DMSO
25 μM MO
Tau
Degradation
0%
53%
96%

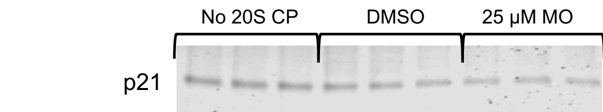

No 20S CP
DMSO
25 μM AM-404
Tau
Degradation
0%
53%
97%



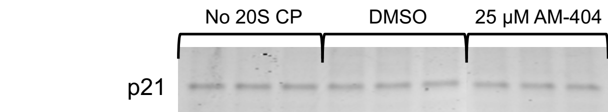
Degradation	0%	13%	6%



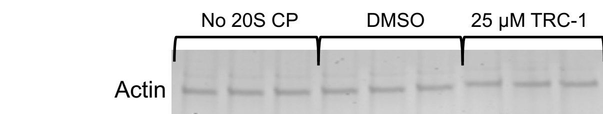
Degradation	0%	13%	24%



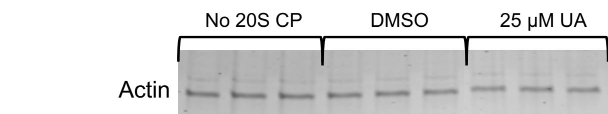
Degradation	0%	13%	43%



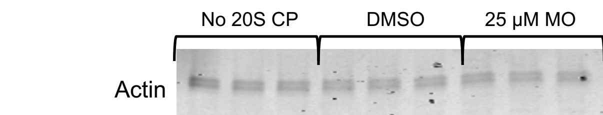
Degradation	0%	13%	23%



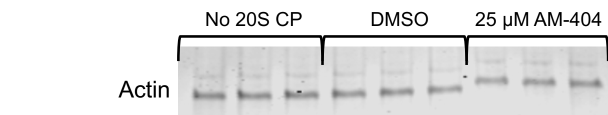
Degradation	0%	8%	12%



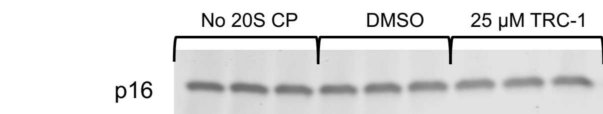
Degradation	0%	8%	18%



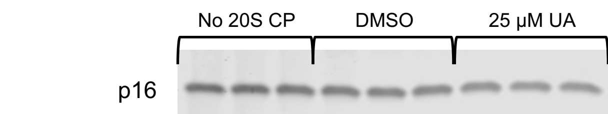
Degradation	0%	8%	22%



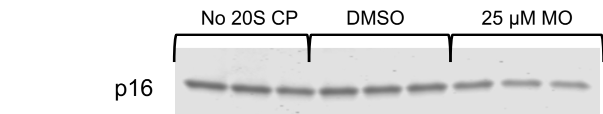
Degradation	0%	8%	24%



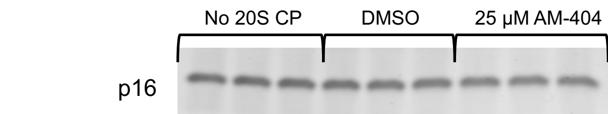
Degradation	0%	6%	10%



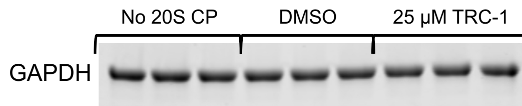
Degradation	0%	6%	33%



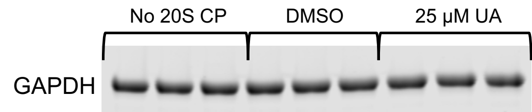
Degradation	0%	6%	45%



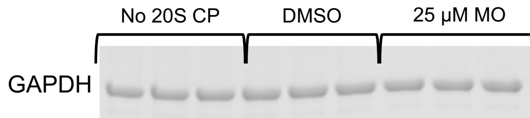
Degradation	0%	6%	9%



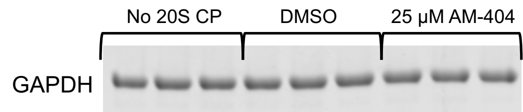
Degradation	0%	(1%)	3%



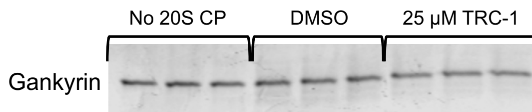
Degradation	0%	(1%)	(1%)



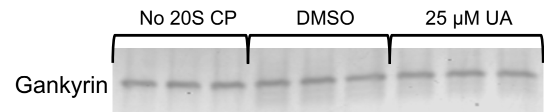
Degradation	0%	(1%)	(4%)



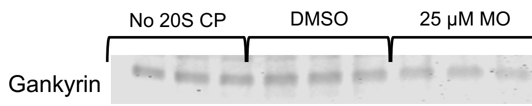
Degradation	0%	(1%)	2%



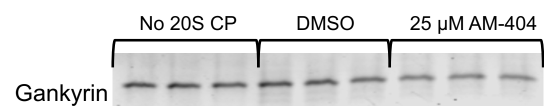
Degradation	0%	10%	20%



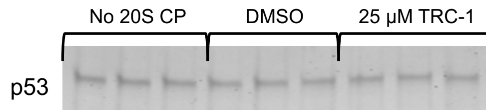
Degradation	0%	10%	6%



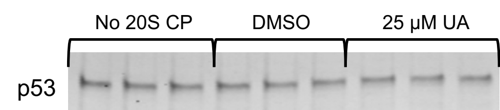
Degradation	0%	10%	29%



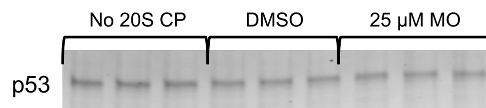
Degradation	0%	10%	31%



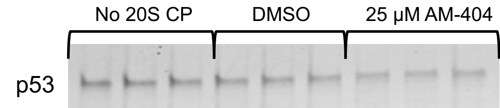
Degradation	0%	23%	15%



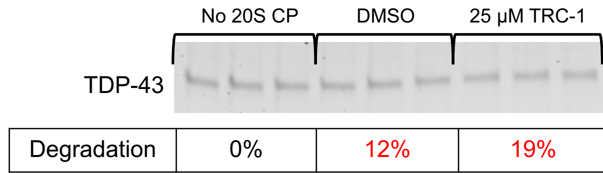
Degradation	0%	23%	26%

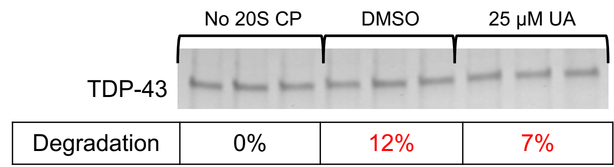


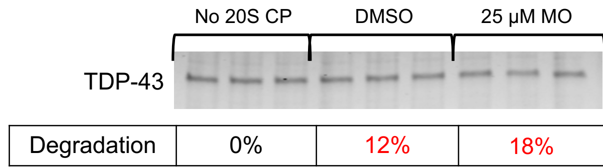
Degradation	0%	23%	30%

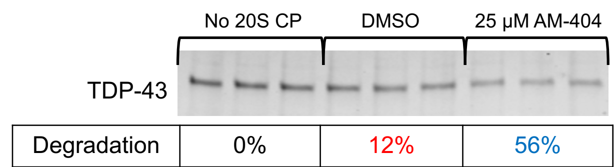


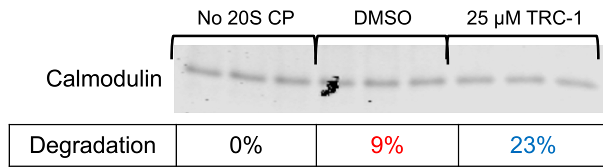
Degradation	0%	23%	51%

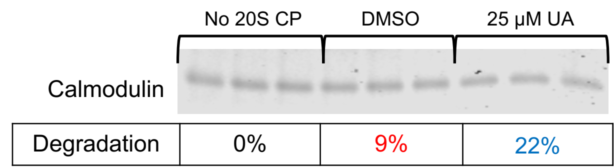

No 20S CP
DMSO
25 μM TRC-1
TDP-43
Degradation
0%
12%
19%

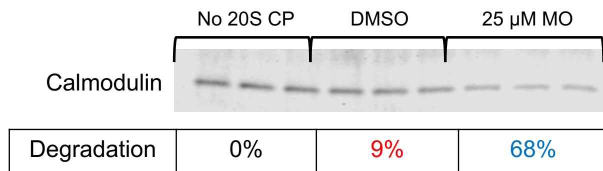

No 20S CP
DMSO
25 μM UA
TDP-43
Degradation
0%
12%
7%

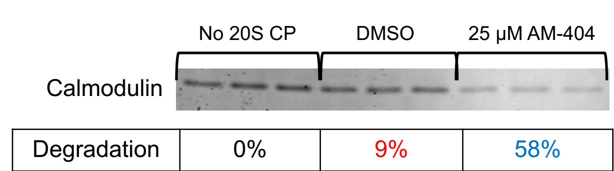

No 20S CP
DMSO
25 μM MO
TDP-43
Degradation
0%
12%
18%

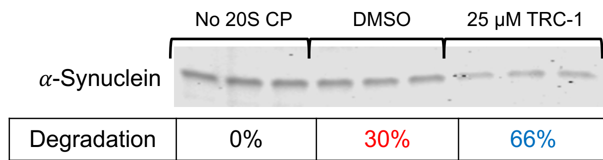

No 20S CP
DMSO
25 μM AM-404
TDP-43
Degradation
0%
12%
56%

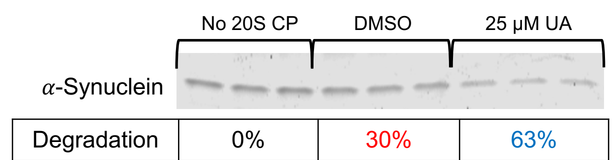

No 20S CP
DMSO
25 μM TRC-1
Calmodulin
Degradation
0%
9%
23%

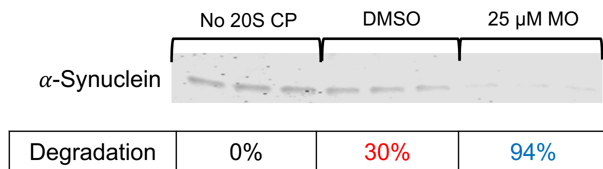

No 20S CP
DMSO
25 μM UA
Calmodulin
Degradation
0%
9%
22%

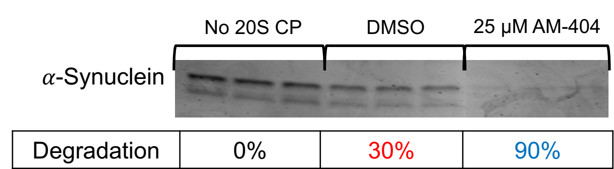

No 20S CP
DMSO
25 μM MO
Calmodulin
Degradation
0%
9%
68%


No 20S CP
DMSO
25 μM AM-404
Calmodulin
Degradation
0%
9%
58%


No 20S CP
DMSO
25 μM TRC-1
α-Synuclein
Degradation
0%
30%
66%


No 20S CP
DMSO
25 μM UA
α-Synuclein
Degradation
0%
30%
63%


No 20S CP
DMSO
25 μM MO
α-Synuclein
Degradation
0%
30%
94%


No 20S CP
DMSO
25 μM AM-404
α-Synuclein
Degradation
0%
30%
90%

Appendix II: Western blots of GFP-fusion proteins