Title: Proteasomal stimulation by MK886 and its derivatives can rescue tau-induced neurite pathology

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Materials and Methods

Cell Culture

HEK293T cells (ATCC) were cultured in phenol red-free Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS HI, Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), and 2 mM Glutamax (Invitrogen). SHSY5Y cell (ATCC) were cultured in phenol red-free DMEM:F12 (Gibco) with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), and non-essential amino acids (Gibco).

Proteasome Assay

HEK293T were transiently transfected with either empty vector (EV) or tau-GFP construct using Lipofectamine 3000 (Invitrogen) according to manufacturer's instructions. Briefly, 293T were plated onto 10 cm plates the day prior to transfection. 10ug plasmid DNA encoding empty vector or Tau-GFP was used for transfection. Forty-eight hours after Lipofectamine addition, the cells were passaged and plated into 6 well plates for the proteasome assay. Six hours after plating, the compounds (**1** – **8**) were added to the plates at 20 μM concentration. After a 20 hour incubation period, cells were washed with PBS and harvested in lysis buffer (50 mM Tris-HCL, pH 8.0, 150 mM NaCl, 1% sodium dodecyl sulfate, 0.5% Nonidet P-40, 0.5% sodium deoxycholate) with 1% protease (ProteoGuard EDTA-free cocktail, Takara) and 1% phosphatase inhibitors (Inhibitor cocktail, Sigma). After sonification, protein concentration of the cell lysates were determined using a bicinchoninic acid (BCA) Assay (Pierce).

Immunoblot protocol

Equal amounts of protein (5 – 20ug) were loaded with 4x reducing sample buffer (RPI Research Products) onto 12% Criterion TGX gels (Bio-Rad). Proteins were transferred to supported nitrocellulose membranes and probed using antibodies for GFP (ab290, abcam), tau5 (AHB0042, Thermo Fisher), α-tubulin-HRP (HRP-66031, Proteintech), beta(III)-tubulin (clone 2G10-TB3, Stemcell Technologies), NeuN (clone A60 MAB377, Millipore), tyrosine hydroxylase (clone TH-16 SAB 4200-697, Millipore). Blots were imaged on using a ChemiDoc MP imager and analyzed using Image Lab (Bio-Rad, Hercules, CA).

SHNS differentiation

SHSY5Y were passaged and transiently transfected with either EV, WT tau unlabeled, or tau-GFP using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The day after transfection, cells were passaged and plated in 6 well plates in differentiation media 1 (complete DMEM:F12 media supplemented with 10 μM retinoic acid (RA)). Cells were cultured in suspension using an orbital shaker for 4 days. One the fifth day of differentiation, the media was changed to differentiation media 2 (serum-free DMEM:F12 supplemented with 50 ng/mL BDNF) and cultured for an additional 5 days [2, 7]. At the end of the differentiation, SHNS were pooled and plated onto 0.1 mg/mL poly-L-lysine/50 μg/mL laminin coated glass bottom plates (MatTek) with MK analogs (2 μM concentration) or DMSO control.

SHNS Imaging and Neurite Analysis

Neurite outgrowth was quantified 36 hours after plating. SHNS were fixed in 4% paraformaldehyde with 4% sucrose for 30 min at RT. Fixed SHNS were washed three times with PBS and then blocked (10% donkey serum in PBS) for 1h at RT. Cells were then incubated in primary antibodies diluted in blocking buffer for 1h at RT. Primary antibodies used in this study were: β(III)-tubulin (Stemcell Technologies clone 2G10-TB3, 1:500); synaptophysin (Novus NB300-653, 1:100). This was followed by washing with PBS three times and incubating for 1h at RT in blocking buffer containing Alexa-Fluor488 or AlexaFluor568-conjugated isotype-specific secondary antibodies (Thermo Fisher). SHNS

were washed three times with PBS and then mounted and coverslipped using VECTASHIELD HardSet antifade with DAPI (Vector Laboratories). Stained SHNS were then imaged using an ImageXpress Pico (Molecular Devices) with a 4x and 10x objective using the DAPI and Cy5 filters. Neurite outgrowth analysis and quantification was performed using a custom MetaXpress module (Molecular Devices).

FLT-FRET Assay

FLT-FRET experiments were performed following the method established by Lo et al.[4]. Briefly, low passage HEK293T cells (ATCC) were plated in 10-cm tissue culture plates (Eppendorf) at 5e6 cells per plate in phenol red-free Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 2 mM Glutamax (Invitrogen), heat-inactivated 10% fetal bovine serum (FBS HI, Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Both donor only and donor and acceptor Tau FLT-FRET biosensor constructs (e.g., tau-mEGFP and tau-TagRFP; donor and acceptor, respectively) were transiently transfected for 24 hours at a 1:6, donor: acceptor ratio using Lipofectamine 3000 (Invitrogen).

The cells were harvested from the 10 cm plates by incubating with TrypLE (Invitrogen) for 2 min, washed three times in DPBS by centrifugation at 200 x g and filtered using 70 μm cell strainers (BD Falcon). Cell viability prior to screen was assessed using a trypan blue assay and confirmed to be >80%. Cells were diluted to 1.0E6 cells per mL. Expression of Tau-mEGFP and the Tau-mEGFP/Tau-TagRFP biosensors were confirmed by fluorescence microscopy prior to each FLT-FRET experiment (ImageXpress Pico, Molecular Devices, USA). During screening, cells (50 μl/well) were dispensed by a Multidrop Combi liquid dispenser (Thermo, Pittsburg, PA, USA) into 384-well black-bottom polypropylene assay plates containing the compounds and allowed to incubate at room temperature for 90 minutes before recording with the FLT plate reader (instrument manufactured by Fluorescence Innovations Inc, both owned and provided by Photonic Pharma LLC), as described previously[1, 4-6].

As described previously[1, 4-6], time-resolved fluorescence waveforms for each well were fit with single-exponential decays using least-squares minimization global analysis software to give donor-acceptor lifetime (τ_{DA}) and donor-only lifetime (τ_D).

MK and analog dose response plates were prepared by dissolving compounds to 10mM in DMSO, then serially diluted in 96-well mother plates. Dose response was performed across eight concentrations (1nM to 50μM). Compounds (0.5μl) were transferred from mother plates into assay plates using a Mosquito HV liquid handler (TTP Labtech Ltd, UK). Dose response plates were sealed and stored at −20 °C until use.

Phenotypic Autophagy LC3 Biosensor Assay

HEK293T were transfected with the pMRX-IP-GFP-LC3-RFP-LC3ΔG construct (Addgene plasmid #84572; http://n2t.net/addgene:84572; RRID:Addgene_84572). [3] Cells were placed under selection using puromycin and then sorted using a FACSAria II Cell Sorter (BD Biosciences). Sorted, stable cells expressing the LC3 biosensor were then transiently transfected with WT tau (unlabeled) construct using the protocol for Lipofectamine 3000, as described previously. Characterization of the biosensor response was conducted using known stimulators (serum free starvation, 100nM rapamycin) and inhibitors (100nM bafilomycin A1) of autophagy for 24 hours (Supplemental Figure S5) to establish the experimental range for the GFP/RFP ratio.

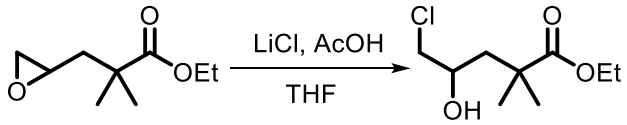
LC3 biosensor cells expressing tau were treated with MK compounds at 5 μM concentration for 24 hours. Cells were imaged using the FITC and TRITC filters on the ImageXpress Pico prior to treatment and also at 24 hours using the 10X and 40X objectives. A custom module on MetaXpress imaging software was used to calculate the GFP/RFP ratio.

Statistical Analyses.

All data are presented as the mean ± SEM of triplicate experiments. Statistical analyses were performed using GraphPad Prism 9.0 (Graph Pad Software) using ANOVA with Dunnett's test (for comparing multiple groups).

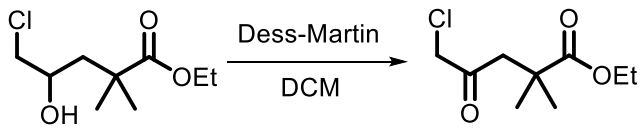
MK and Analog Synthesis

General materials and equipment: bulk solvents and chemicals were purchased commercially from Sigma-Aldrich, Fisher Chemicals, TCI America, Acros, and Oakwood chemicals. Anhydrous THF was obtained from a MBraun MB-SPS-800 solvent dispensing system. Diisopropylamine was dried in potassium hydroxide pellets, freshly distilled, and stored with 4A molecular sieves shortly before use. Other chemicals were used directly from the bottle without purification. Moisture- or air-sensitive reactions were conducted under an atmosphere of N_2 or Ar in oven-dried glassware. Reaction progress was monitored by thin layer chromatography (TLC) carried out using Macherey-Nagel Alugram SIL G/UV254 pre-coated sheets. A hand-held UV lamp was used to visualize the plates. A rotary evaporator was used to remove solvents under reduced pressure. Flash column chromatography was performed using a SiliaFlash P60 silica gel (40-60 μm) purchased from Silicycle. ^{1}H NMR spectra were obtained on a Varian 400 MHz or Varian 600 MHz spectrometer in the specified solvent and ^{13}C NMRs were collected solely on a Varian 600 MHz spectrometer. ^{1}H NMR peaks are reported as: chemical shift (multiplicity, J coupling in Hz, integration). Mass spectrometry data was obtained on an Agilent ESI-TOF instrument. Purity of final compounds **1 - 8** was determined on Agilent 1260 HPLC system. Separations were accomplished with a reversed phase C18 column (Gemini-NX 5μm, 150 × 4.60 mm, Phenomenex, Torrance, United States) at 25 °C and the following solvent system: solvent A–Optima water/10 mM ammonium acetate buffer pH=3.5; solvent B–HPLC grade acetonitrile. The mobile phase was maintained at a flow rate of 0.3 mL/min with a gradient of 95% A/5% B to 5% A/95% B over 5 min and a 5 min hold at 5% A/95% B (for all HPLC chromatograms). Each run was followed by a 2 min post-run to equilibrate to 95% A/5% B before the next run. HPLC traces were recorded with a diode array detector at 280 nm ultraviolet wavelength. Percent purity was assessed based on peak areas. All final compounds used in biological assays had over 95% purity.



S1 **S2**

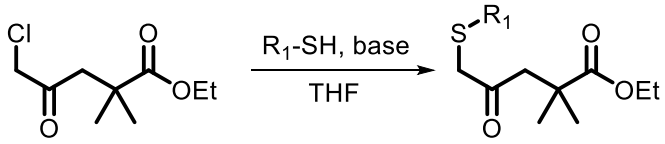
Synthesis of **S2**: **S1** was synthesized following reported procedures by Schimdt et. al. 0.636 g (15 mmol, 1.5 eq) anhydrous LiCl was dissolved in a mixture of 1.8 mL (30 mmol, 3 eq) glacial acetic acid and 8 mL anhydrous THF. To this solution was added 1.72 g (10 mmol, 1 eq) of the epoxide intermediate **S1**. The reaction was mixed well and allowed to sit at room temperature overnight. The progress of the reaction was accompanied by the formation of large amount of LiOAc crystals. The reaction was diluted with ether and washed with saturated sodium bicarbonate and brine. Removing the solvent in vaccum gave 1.86 g chlorohydrin **S2** as colorless oil (8.9 mmol, 89% yield). The product was used in the next step without further purification. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.12 (q, *J* = 7.1 Hz, 2H), 3.90 (s, 1H), 3.52 (dd, *J* = 10.9, 4.5 Hz, 1H), 3.46 (dd, *J* = 10.9, 6.3 Hz, 1H), 1.96 (dd, *J* = 14.6, 9.5 Hz, 1H), 1.60 (dd, *J* = 14.6, 2.3 Hz, 1H), 1.32 – 1.18 (m, 9H).



S2 **S3**

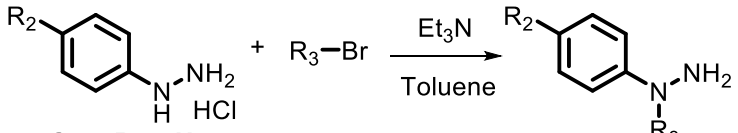
Synthesis of **S3**: A solution of 1.67 g (8 mmol, 1 eq) **S2** in 40 mL DCM was added 4 g (9.6 mmol, 1.2 eq) Dess-Martin periodinane. After the suspension was stirred at room temperature for 3 hours, the

reaction was sequentially washed with sat. sodium bisulfite solution, sat. sodium bicarbonate solution, and brine. The organic layer was concentrated to give 1.4 g **S3** as colorless oil (6.88 mmol, 86% yield), which was used without further purification. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.11 (q, *J* = 7.1 Hz, 2H), 4.04 (s, 2H), 2.83 (s, 2H), 1.33 – 1.15 (m, 9H).



S3 **S4a-c**

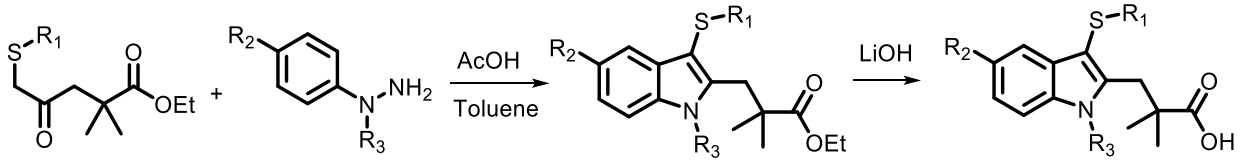
General procedure for the synthesis of S4a-c: To a solution of 2.2 mmol (1 eq) thiol in 10 mL anhydrous THF was added base (1.2 eq NaH with 1 eq tert-butyl mercaptan, 2 eq triethylamine with 1 eq of benzyl mercaptan, 1 eq of sodium ethane thiolate were used for the synthesis of **S4a**, **S4b** and **S4c** respectively). 0.413 g (2 mmol, 0.9 eq) **S3** was added subsequently, and the reaction carried out at room temperature for 4 hours. Ethyl acetate was added, and the organic layer was washed with water and brine. Rotavaping the solvent gave the corresponding thioether. **S4a**: 0.224 g (0.86 mmol), 43% yield. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.10 (q, *J* = 7.1 Hz, 2H), 3.24 (s, 2H), 2.91 (s, 2H), 1.42 – 1.02 (m, 18H). **S4b**: 0.542 g (1.84 mmol) product, 92% yield ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.35 – 7.28 (m, 5H), 4.14 (q, *J* = 7.1 Hz, 2H), 3.66 (s, 2H), 3.05 (s, 2H), 2.87 (s, 2H), 1.27 – 1.20 (m, 9H). **S4c**: 0.423 g (1.82 mmol) product**,** 91% yield**.** ^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.10 (q, *J* = 7.1 Hz, 2H), 3.15 (s, 2H), 2.92 (s, 2H), 2.48 (q, *J* = 7.4 Hz, 2H), 1.29 – 1.15 (m, 12H).



S5a: R_2 = H
S5b: R_2 = OPh
S5c: R_2 = iPr

S5d-g

General procedure for the synthesis of **S5d-g**: 2 mmol (1 eq) of phenylhydrazine hydrochloride (**S5a**, **S5b, S5c)**, 2.2 mmol (1.1 eq) of 4-substituted benzyl bromide (4-chlorobenzyl bromide, 4-phenylbenzyl bromide), and 6 mmol (3 eq) of triethylamine were dissolved in toluene and refluxed for 4 hours. The reaction was washed with water and brine. The organic layer was concentrated. For **S5d** and **S5e**, the crude mixture was acidified with HCl in dioxane and recrystallized from ethanol and ether in the form of hydrochloride salt. For **S5f** and **S5g**, the crude mixture was purified via flash silica chromatography. **S5d HCl**: 0.219 g (0.8 mmol) product from 0.373 g (2 mmol) **S5c**, 40% yield; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.32 (s, 2H), 10.14 (s, 1H), 7.37 (d, *J* = 8.3 Hz, 2H), 7.32 (d, *J* = 8.3 Hz, 2H), 7.19 (d, *J* = 8.5 Hz, 2H), 7.12 (d, *J* = 8.4 Hz, 2H), 4.59 (s, 2H), 2.82 (sep, *J* = 6.9 Hz, 1H), 1.13 (d, *J* = 6.8 Hz, 6H). **S5e HCl**: 0.172 g (0.74 mmol) product from 0.289 g (2 mmol) **S5a**, 37% yield; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.44 (s, 3H), 7.40 – 7.25 (m, 6H), 7.14 (d, *J* = 7.9 Hz, 2H), 7.06 (t, *J* = 7.3 Hz, 1H), 4.66 (s, 2H). **S5f**: 0.31 g (0.98 mmol) product from 0.373 g (2 mmol) **S5c**, 49% yield; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.57 (t, *J* = 6.6 Hz, 4H), 7.47 – 7.29 (m, 5H), 7.15 (d, *J* = 7.5 Hz, 2H), 7.05 (d, *J* = 7.2 Hz, 2H), 4.57 (s, 2H), 3.56 (s, 2H), 2.85 (sep, *J* = 7.1 Hz, 1H), 1.23 (d, *J* = 6.9 Hz, 6H). **S5g**: 0.45 g (1.39 mmol) product from 0.473 g (2 mmol) **S5b**, 70% yield; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.37 – 7.20 (m, 6H), 7.10 – 6.89 (m, 7H), 4.49 (s, 2H), 3.52 (s, 2H).



S4a-c **S5c-g** **S6a-h** **Compounds 1-5, 7, 8**

General procedure for the synthesis of final compounds **1-5, 7, 8**: To a solution of 0.5 mmol (1 eq) phenylhydrazine (**S5c-g**) (1.1 eq of triethylamine for HCl salts) and 0.524 mmol (1.05 eq) thioketone (**S4a-c**) in 2 mL toluene was added 0.7 mL AcOH and allowed to react at room temperature overnight. DCM was added and the organic layer was neutralized with saturated $NaHCO_3$, concentrated and briefly purified via flash silica chromatography. The combined fractions were concentrated, dissolved in a mixture of 2 mL THF, 2 mL MeOH, and 2 mL H_2O, and added 63 mg (1.5 mmol, 3 eq) of LiOH hydrate. The reaction was heated to 60 °C for 1 hour. 30 mL 0.1 M hydrochloric acid was added, and the product was extracted with ethyl acetate. The organic layer was concentrated. The crude products were recrystallized in DCM and hexanes to give pure compounds as white crystalline solid.

Compound **1** (MK886): 99 mg, 42% yield over two steps. ^{1}H NMR (400 MHz, dmso) δ 12.41 (s, 1H), 7.44 (s, 1H), 7.33 – 7.20 (m, 3H), 6.96 (d, *J* = 8.4 Hz, 1H), 6.83 (d, *J* = 4.8 Hz, 2H), 5.44 (s, 2H), 3.17 (s, 2H), 2.92 (t, *J* = 6.9 Hz, 1H), 1.23 – 1.09 (m, 15H), 1.06 (s, 6H). ^{13}C NMR (151 MHz, dmso) δ 178.98, 143.62, 140.77, 137.59, 135.68, 132.21, 131.56, 129.03, 128.10, 121.51, 116.96, 110.89, 104.41, 48.19, 46.93, 43.59, 33.83, 33.65, 31.37, 25.52, 24.95. HRMS (ESI-): m/z calc for $[M-H]^-$ = 470.1920; found 470.1900

Compound **2** (MK1H): 78 mg, 45% yield over two steps. ^{1}H NMR (400 MHz, dmso) δ 12.29 (s, 1H), 10.84 (s, 1H), 7.36 (s, 1H), 7.24 (d, *J* = 8.4 Hz, 1H), 6.93 (d, *J* = 8.4 Hz, 1H), 3.09 (s, 2H), 2.91 (sep, *J* = 6.9 Hz, 1H), 1.20 (d, *J* = 5.6 Hz, 6H), 1.15 (s, 9H), 1.05 (s, 6H). ^{13}C NMR (151 MHz, dmso) δ 179.14, 142.60, 139.87, 134.53, 131.53, 120.71, 116.39, 111.54, 101.86, 47.75, 43.01, 35.52, 33.97, 31.32, 25.31, 25.09. HRMS (ESI-): m/z calc for $[M-H]^-$ = 346.1841; found 346.1856

Compound **3** (MK5H): 120 mg, 56% yield over two steps. ^{1}H NMR (400 MHz, dmso) δ 12.44 (s, 1H), 7.68 – 7.54 (m, 1H), 7.43-7.32 (m, 1H), 7.29 (d, *J* = 6.0 Hz, 2H), 7.15-7.02 (m, 2H), 6.81 (d, *J* = 6.2 Hz, 2H), 5.49 (s, 2H), 3.19 (s, 2H), 1.16 (s, 9H), 1.07 (s, 6H). ^{13}C NMR (151 MHz, dmso) δ 178.97, 143.74, 137.49, 137.09, 132.22, 131.57, 129.02, 128.05, 122.30, 120.70, 120.15, 111.21, 104.68, 48.18, 46.91, 43.59, 33.64, 31.39, 25.57. HRMS (ESI-): m/z calc for $[M-H]^-$ = 428.1451; found 428.1457

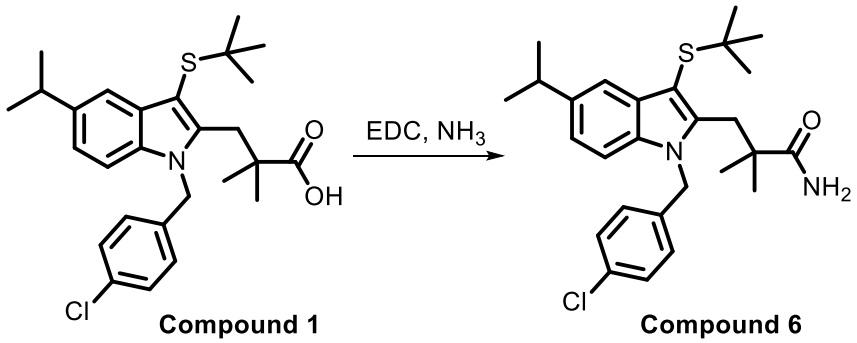
Compound **4** (OPh): 165 mg, 63% yield over two steps. ^{1}H NMR (400 MHz, dmso) δ 12.40 (s, 1H), 7.41 (d, *J* = 8.6 Hz, 1H), 7.38 – 7.27 (m, 4H), 7.15 (s, 1H), 7.05 (t, *J* = 7.3 Hz, 1H), 6.93 (d, *J* = 8.7 Hz, 2H), 6.86 (d, *J* = 8.7 Hz, 3H), 5.51 (s, 2H), 3.20 (s, 2H), 1.13 (s, 9H), 1.09 (s, 6H). ^{13}C NMR (151 MHz, dmso) δ 178.87, 158.55, 151.14, 145.08, 137.39, 133.76, 132.44, 132.30, 130.28, 129.09, 128.08, 123.03, 118.11, 115.24, 112.57, 109.51, 104.55, 48.26, 47.13, 43.60, 33.77, 31.31, 25.57. HRMS (ESI-): m/z calc for $[M-H]^-$ = 520.1713066; found 520.1723

Compound **5** (bPh): 146 mg, 57% yield over two steps. ^{1}H NMR (400 MHz, dmso) δ 12.45 (s, 1H), 7.58 (d, *J* = 7.8 Hz, 2H), 7.54 (d, *J* = 7.9 Hz, 2H), 7.47 (s, 1H), 7.39 (t, *J* = 7.6 Hz, 2H), 7.34 – 7.27 (m, 2H), 6.98 (d, *J* = 8.4 Hz, 1H), 6.91 (d, *J* = 7.9 Hz, 2H), 5.51 (s, 2H), 3.24 (s, 2H), 2.94 (sep, *J* = 6.9 Hz, 1H), 1.22 (s, 6H), 1.20 (s, 9H), 1.11 (s, 6H). ^{13}C NMR (151 MHz, dmso) δ 179.01, 143.78, 140.69, 139.99, 139.53, 137.79, 135.80, 131.59, 129.32, 127.87, 127.32, 127.01, 126.76, 121.44, 116.95, 110.97, 104.20, 48.24, 47.25, 43.62, 33.85, 33.75, 31.39, 25.59, 24.97. HRMS (ESI-): m/z calc for $[M-H]^-$ = 512.2623108; found 512.2643

Compound **7** (SBn): 129 mg, 51% yield over two steps. ^{1}H NMR (400 MHz, dmso) δ 12.38 (s, 1H), 7.39 – 7.30 (m, 3H), 7.20 (d, *J* = 8.4 Hz, 1H), 7.17 – 7.06 (m, 3H), 7.01 – 6.91 (m, 3H), 6.74 (d, *J* = 8.5 Hz, 2H), 5.33 (s, 2H), 3.78 (s, 2H), 2.94 (sep, *J* = 6.9 Hz, 1H), 2.66 (s, 2H), 1.23 (d, *J* = 6.8 Hz, 6H), 1.03 (s, 6H). ^{13}C NMR (151 MHz, dmso) δ 178.80, 142.14, 140.86, 139.41, 137.46, 135.49, 132.16, 129.75, 129.14, 128.99, 128.38, 128.19, 126.94, 121.45, 115.78, 111.06, 103.91, 46.69, 43.25, 39.76, 33.90, 33.23, 25.47, 24.98. HRMS (ESI-): m/z calc for $[M-H]^-$ = 504.1763916; found 504.1777

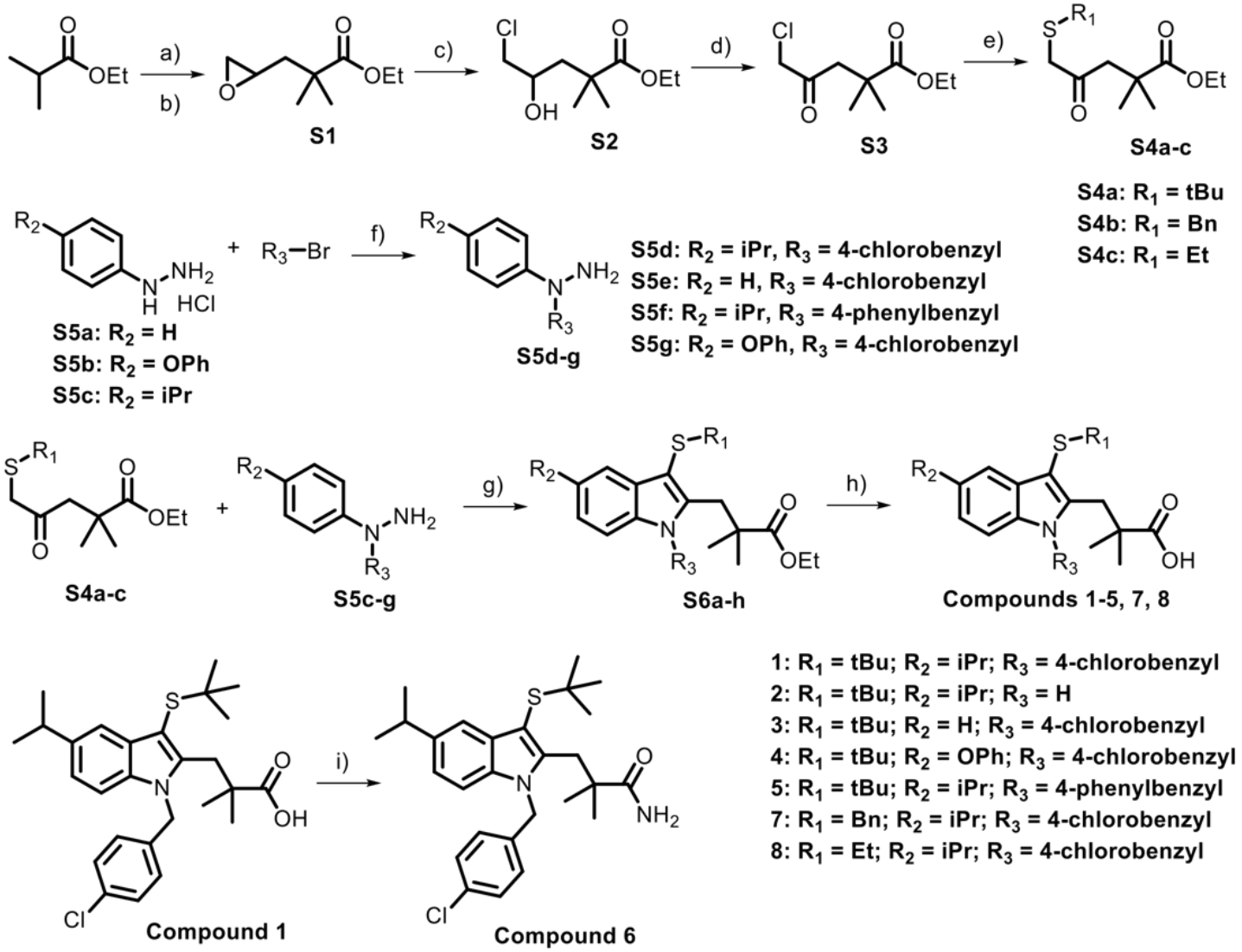
Compound **8** (SEt): 106mg, 48% yield over two steps. ^{1}H NMR (400 MHz, dmso) δ 12.44 (s, 1H), 7.41 (s, 1H), 7.31 (d, *J* = 7.2 Hz, 2H), 7.25 (d, *J* = 8.4 Hz, 1H), 6.99 (d, *J* = 8.3 Hz, 1H), 6.83 (d, *J* = 8.1 Hz, 2H), 5.43 (s, 2H), 3.15 (s, 2H), 2.95 (sep, *J* = 6.9 Hz, 1H), 2.62 (q, *J* = 7.3 Hz, 2H), 1.22 (d, *J* = 6.9 Hz, 6H), 1.11 (s, 6H), 1.02 (t, *J* = 7.9 Hz, 3H). ^{13}C NMR (151 MHz, dmso) δ 178.91, 141.77, 140.84,

137.57, 135.75, 132.15, 129.82, 129.02, 128.14, 121.45, 115.85, 111.19, 104.77, 46.81, 43.46, 33.89, 33.72, 29.47, 25.47, 24.97, 15.12. HRMS (ESI-): m/z calc for $[M-H]^-$ = 442.1607424; found 442.1606

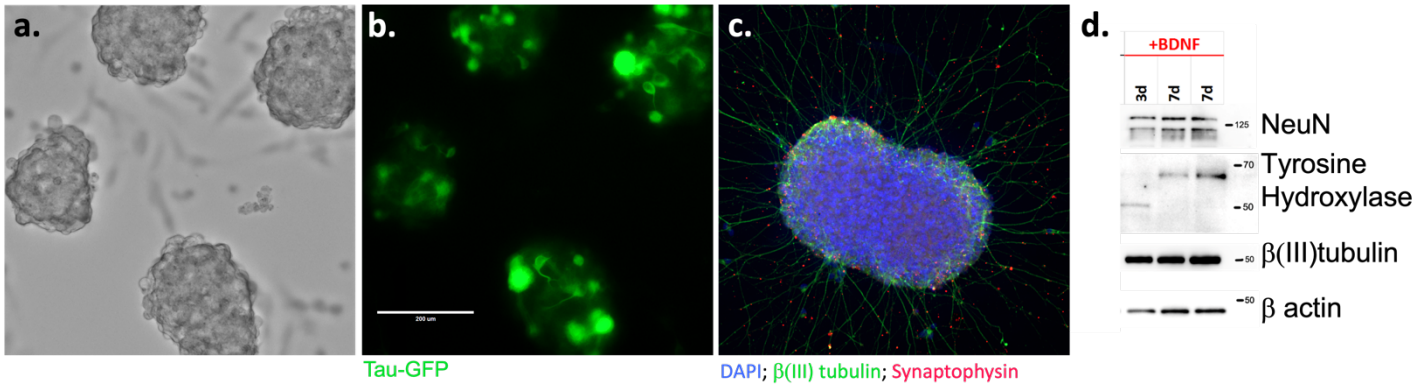


Compound 1 **Compound 6**

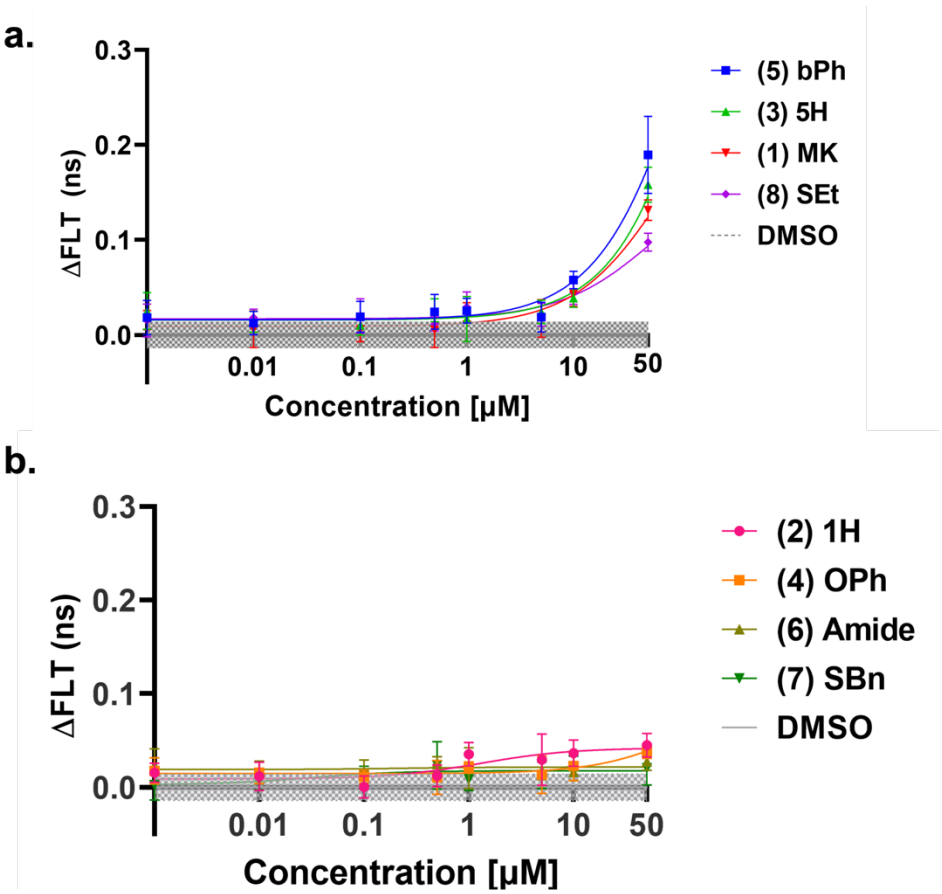
Synthesis of compound **6** (amide): To a solution of 94 mg (0.2 mmol, 1 eq) compound **1** in 5 mL DCM was added 42 mg (0.22 mmol, 1.1 eq) EDC and stirred in room temperature for 10 min. 57 uL (0.4 mmol, 2 eq) 7M ammonia in MeOH was added to the mixture and stirred for an additional 1 hour. The reaction was washed with water and brine. The solvent was removed and crude product recrystallized from DCM and hexanes to give pure compound **6** as a while solid (56 mg, 60% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.63 (s, 1H), 7.18 (d, *J* = 8.5 Hz, 2H), 7.10 – 7.00 (m, 2H), 6.75 (d, *J* = 8.4 Hz, 2H), 5.45 (s, 1H), 5.40 (s, 2H), 5.18 (s, 1H), 3.23 (s, 2H), 3.01 (sept, *J* = 6.9 Hz, 1H), 1.30 (d, *J* = 6.9 Hz, 7H), 1.26 (m, 15H). ^{13}C NMR (151 MHz, dmso) δ 179.49, 144.28, 140.66, 137.87, 135.62, 132.12, 131.63, 128.99, 128.11, 121.35, 116.92, 110.84, 104.12, 48.17, 46.79, 43.66, 34.21, 33.84, 31.37, 25.88, 24.97. HRMS (ESI+): m/z calc for $[M+Na]^+$ = 493.2056; found 493.1259



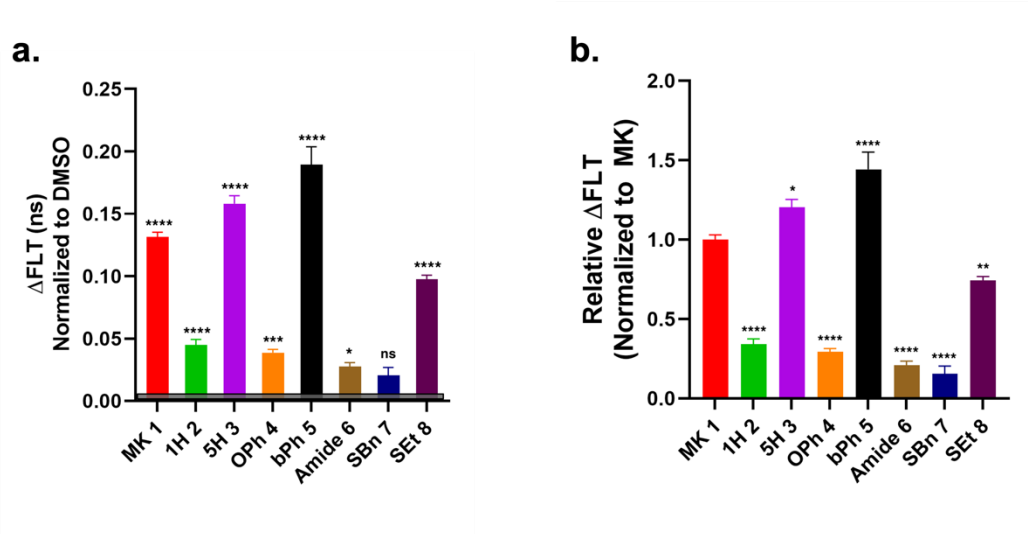
Supplemental Scheme 1. Synthetic routes for MK886 and analogs. Reagents and conditions: a) nBuLi, iPr_2NH, THF, -78 °C, 1h; b) epibromohydrin; c) AcOH, LiCl, THF, rt, overnight; d) Dess-Martin periodinane, THF, rt, 1h; e) NaH for tBuSH, Et_3N for other thiols, R_1-SH, THF, rt, 2h; f) Et_3N, toluene, reflux, 4h; g) toluene, AcOH, rt, overnight; h) LiOH, MeOH, H_2O, THF, 50 °C, 1h; i) EDC, 7M NH_3 in MeOH, THF, rt, 1h.



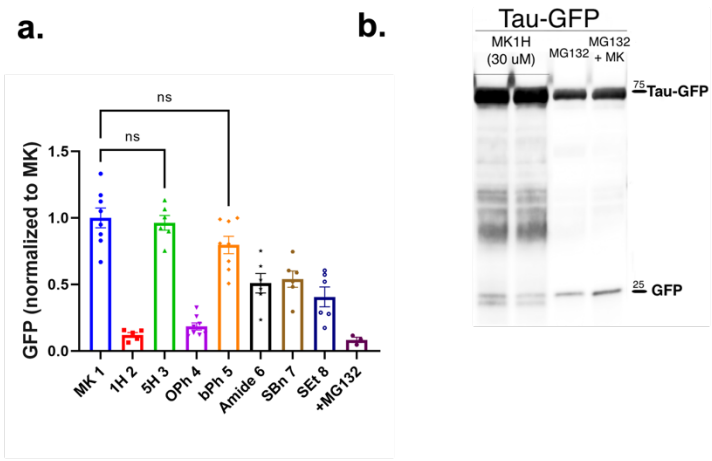
Supplemental Figure 1. SHSY5Y Neurosphere Culture. a. SHSY5Y cells form neurospheres (SHNS) in suspension culture on an orbital shaker. **b.** Premature SHNS expressing tau-GFP to highlight the neuronal-like cells with short processes within the neurosphere bodies. **c.** Mature SHNS, when plated, extend neuronal projections which are stained with markers for neuron specific marker - β(III)-tubulin (green), pre-synaptic marker – synaptophysin (red), and DAPI (blue) labels the neurosphere body. **d.** Biochemical analysis of SHNS at early and late differentiation timepoints, further validates the presence of mature neurons within the SHNS. Markers for mature neurons, NeuN and β(III)-tubulin, are present, as well as increased expression of tyrosine hydroxylase, confirming the presence of dopaminergic neurons.



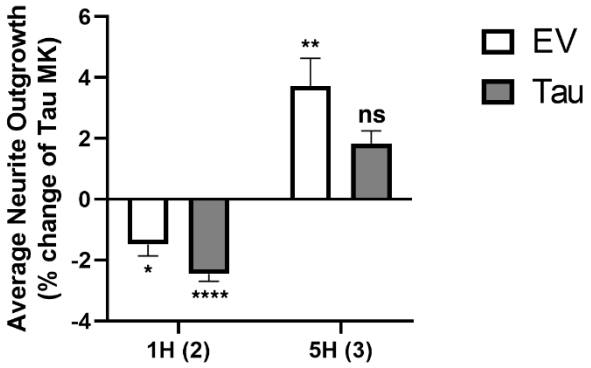
Supplemental Figure 2. MK and analog dose response in tau FLT-FRET biosensor. All analogs, except SBn (**7**), displayed a significant ΔFLT relative to DMSO with MK (**1**), 5H (**3**) , bPh (**5**), and SEt (**8**) showing an increased response (3 SD) at 50μM (**a.**) relative to the other 4 derivatives (**b.**).



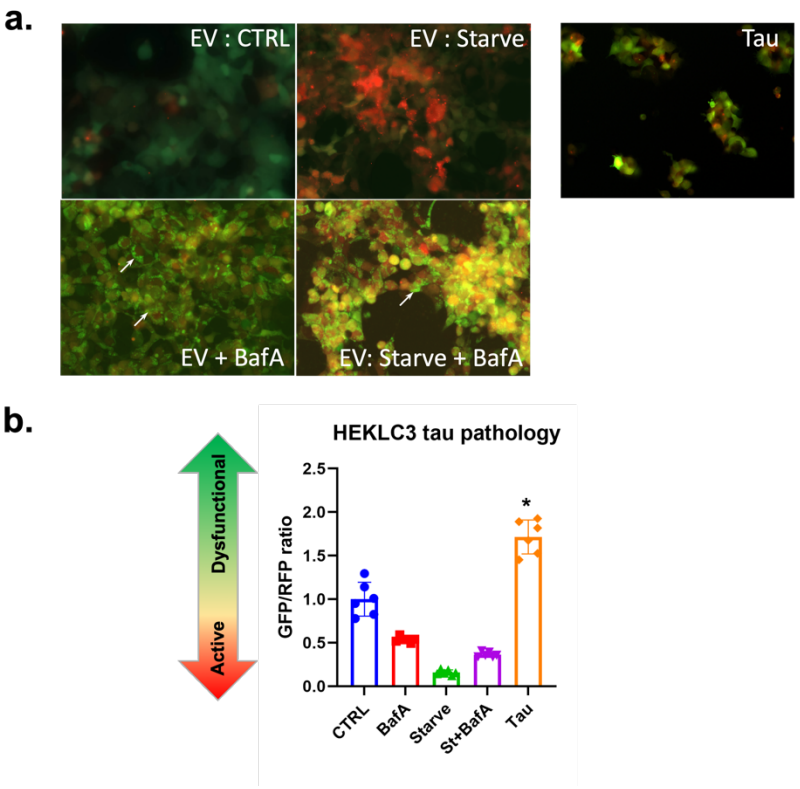
Supplemental Figure 3. Cellular FLT-FRET response of MK analogs compared to DMSO control at 50 µM concentration. **a**. MK and all analogs, except for SBn, produce a significant increase in FLT relative to DMSO treatment (shown as gray horizontal bar), suggesting that most compounds can modulate tau-tau interaction to some extent. Derivatives 5H and bPh show increased change in FLT even greater than MK. Experimental values presented are from n = 8, **** = $p < 0.0001$, *** = $p < 0.001$, * = $p < 0.05$, and ns is not significant. **b**. Replot of 3a with statistical comparison to MK treatment is shown and a full dose response for each compound is presented in Supplemental Figure S2.



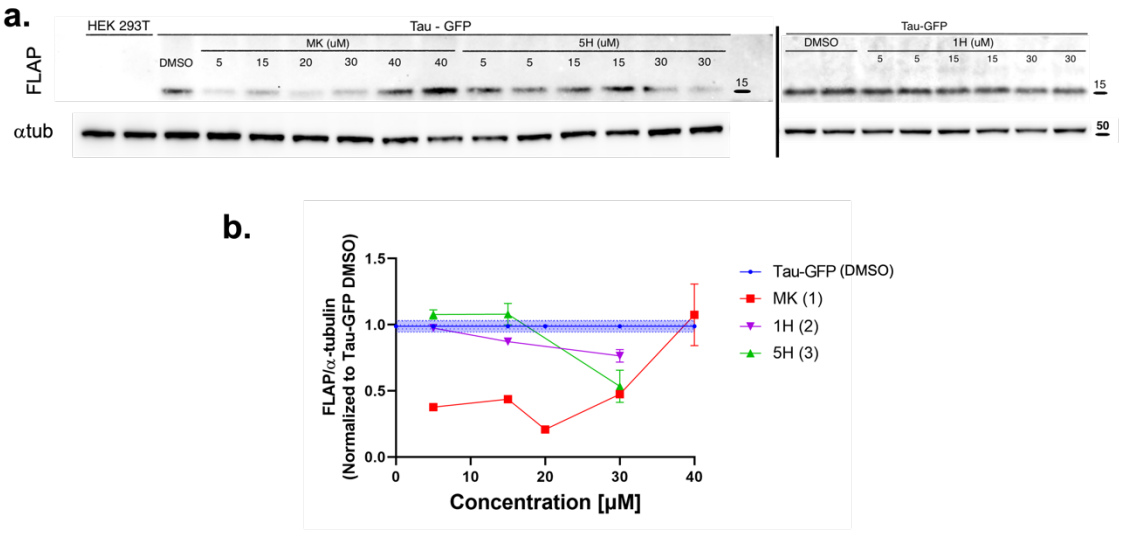
Supplemental Figure 4. Proteasome Assay for all MK analogs (1 - 8). a. Quantification of cleaved GFP immunoblot from tau-GFP expressing HEK293T cells treated with MK analogs (**1** – **8**). Replot of GFP cleavage (Figure 3b) with normalization and statistical comparison to MK treated cells. Compounds 5H and bPh are not significantly different from MK treatment, while all remaining compounds are significantly lower than MK.**b.** Comparison of 30 μM MK1H (inhibitor) to known proteasomal inhibitor MG-132 (negative control).



Supplemental Figure 5. Replot of neurite outgrowth with 1H and 5H treatment, relative to MK. Neurite outgrowth from SHNS treated with 1H and 5H were normalized to tau SHNS treated with MK. 1H significantly decreased neurites for both EV and tau SHNS ($p < 0.05$) and ($p < 0.0001$), respectively. Treatment of tau SHNS with 5H was not significantly different then tau SHNS treated with MK.

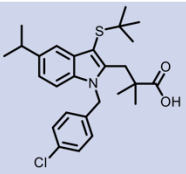
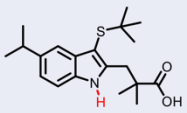
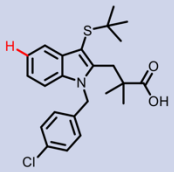
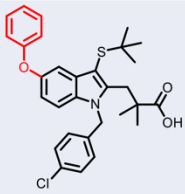
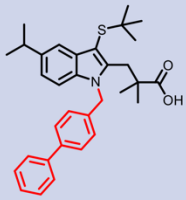
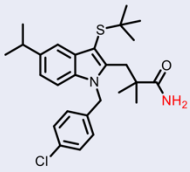
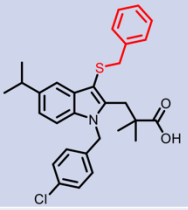
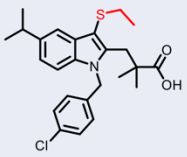


Supplemental Figure 6. Characterization of phenotypic autophagy LC3 biosensor. a. Live cell images of HEK293T cells expressing the (GFP-LC3-RFP-LC3ΔG) construct. Under basal conditions, the cells are a muted green with some red, indicating some basal level of autophagy is present. Under (serum free) starvation conditions, the cells become more red in appearance because activation of autophagy leads to a decrease in the GFP signal as the sequestered material within the autolysosomes are degraded, while RFP-LC3ΔG remains in the cytosol. Treatment with 100 nM Bafilomycin A1 (BafA), a vacuolar H+ ATPase, inhibits fusion of autophagosomes with the lysosomes and subsequent accumulation of autophagosomes (green punta, arrows) is accompanied by a brighter green signal. A combination of starvation and BafA treatment leads to cells that are yellow (mix of green and red) as the accumulation of autophagosomes is heightened by the increased induction of autophagy by starvation and increased cleavage of GFP-LC3-RFPLC3ΔG. The LC3 biosensor was transiently transfected to express unlabeled wildtype 2N4R tau. Tau expression under basal conditions caused an increase in the amount of GFP. **b.** Quantification of images in **a.** show the change in GFP/RFP ratio under positive and negative control conditions. Under basal conditions, expression of tau increased the GFP/RFP ratio significantly over EV controls ($p < 0.05$).



Supplemental Figure 7. Effect of MK, 1H and 5H on FLAP expression (inflammatory chaperone). a. Immunoblot probing for FLAP expression in HEK293T cells. FLAP expression increases in cells that are transfected with tau-GFP (72-hour timepoint after transfection). Treatment with MK between 5 – 30 μM lowers the amount of FLAP present, while at 40 μM MK concentration, FLAP is comparable to DMSO treated cells. FLAP expression in cells treated with 1H were slightly lower than controls and were reduced by only 20% at a 30 μM concentration. 5H also did not decrease FLAP expression until the 30 μM concentration, but it was able to reduce FLAP expression to the same levels as 30 μM MK. Untransfected cells are also shown for comparison and do not express an appreciable amount of FLAP. **b.** Quantification of FLAP expression shows increased FLAP expression for both 1H and 5H compared to MK at the 20 μM concentration used for proteasome experiments.

Supplemental Table 1. Summary of MK and analog response.

Compound #	Name	Structure	20S Proteasome	FRET (ΔFLT)	Neurite Outgrowth	Autophagic Flux	FLAP Expression
1	MK886		↑↑↑	↑↑	↑↑	↑↑	↓↓↓
2	1H		↓↓↓	–	↓↓	↓↓	↓
3	5H		↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↓
4	OPh		↓↓	–			
5	bPh		↑↑	↑↑↑			
6	Amide		–	–			
7	SBn		–	–			
8	SEt		–	↑			

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