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Supporting Information

for

Modulation of the Aβ peptide aggregation pathway by KP1019 limits Aβ-associated neurotoxicity

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Experimental

Materials and methods

All reagents were purchased from commercial suppliers and used without further purification unless otherwise specified. KP1019 was synthesized using a previously reported procedure.[1] $A\beta_{1-28}$ and $A\beta_{1-42}$ were purchased from 21st Century Biochemicals (Marlborough, MA). $A\beta_{1-42}$ was monomerized by first dissolving in *ca.* 2 mL of 1, 1, 1, 3, 3, 3-hexafluoroisopropanol with subsequent sonication to ensure complete dissolution. The solution was incubated overnight at 4 ^{o}C and then aliquoted into *ca.* 0.25 mg quantities and evaporated for later use. Fluorescence measurements were obtained using a Synergy 4 Fluorometer plate reader from BioTek. TEM images were obtained using a Tecnai G2 F20 scanning TEM (STEM) operating at 200 kV. Peptide aggregates were separated on a 10-20% Mini-PROTEAN® Tris-Tricine Precast Gel from Bio-Rad followed by Western blotting using an anti-Aβ antibody 6E10 (Covance) and a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Cayman Chemicals). Peptide bands were visualized using the Thermo Scientific Supersignal West Pico Chemiluminsecent Substrate kit.

Thioflavin-T Assay

In a 96-well plate, the inhibitory effect of KP1019 (0.25 – 2 eq.) on $A\beta_{1-42}$ aggregation was investigated. $A\beta_{1-42}$ was prepared in 1:1 DMSO/H_2O to a final volume of 200 μL. Peptide concentration was measured using a Thermo Nicolet UV nanodrop and an $\varepsilon = 1450\ M^{-1}$ for $A\beta_{1-42}$.[2] Final Aβ concentration in the assay was 5 μM. KP1019 was prepared as a 50 μM stock solution in 1:1 DMSO/H_2O and diluted with PBS pH 7.4 buffer to the desired concentration. ThT was prepared as a 50 μM stock solution in 10% DMSO/H_2O. Final concentration of ThT was 5 μM in all wells with a final DMSO concentration of 1%. CR was used as positive control since this compound is a potent inhibitor of Aβ peptide aggregation.[3] CR was prepared as a 50 μM stock solution in 1:1 ethanol/PBS pH 7.4 and had a final concentration of 10 μM. All wells were brought to a final volume of 200 μL using PBS pH 7.4. ThT fluorescence was measured by excitation at 404 nm and monitoring of the emission at 477 nm. Measurements were performed in quadruplicate at 24 and 48 hours.

Transmission Electron Microscopy (TEM)

Samples were prepared following a previously reported method.[4] Briefly, glow-discharged grids (Formvar/Carbon 300-mesh, Electron Microscopy Sciences) were incubated with a 10 μL sample for 5 minutes following the completion of either the ThT fluorescence assay or native gel electrophoresis incubation. Excess sample was removed via Kim-wipe. 3 x 10 μL drops of syringe-filtered 5% uranyl acetate solution were placed onto a sheet of parafilm and grids placed onto the drop, removed immediately, repeated for the second drop, and then placed on the last drop for 1 minute incubation. Excess uranyl acetate was removed between drops. The grid was then allowed to dry for at least 15 minutes at room temperature. Images were captured at 200 kV and 25,000X magnification.

EPR Measurements and Simulations

X-band (9.4 GHz) EPR spectra were collected using a Bruker EMXplus spectrometer equipped with a PremiumX microwave bridge and HS resonator. All measurements were conducted at 20 K, using a Bruker ER 4112HV helium temperature-control system and continuous-flow cryostat. Final concentrations of KP1019 and $A\beta_{1-28}$ were 1.5 mM and 0.5 mM, respectively. Peptide and KP1019 were incubated at 37 ^{o}C under constant agitation. Samples (250 μL) were obtained after 2 and 24 hour incubation periods, mixed with 50 μL glycerol and frozen in liquid N_2 in 4 mm outer-diameter quartz tubes. A weak background signal was subtracted from each spectrum. Bruker WinEPR Simfonia[5] simulations were performed to deconvolute the spectra into separate spectral components of KP1019 alone, and protein-coordinated KP1019 (KP1019-$A\beta_{1-28}$).

Dot Blot Assay

To further evaluate the specific region of the Aβ peptide KP1019 interacts with, a dot blot assay was performed at varying KP1019:$A\beta_{1-42}$ ratios and time periods. A 96-well plate was incubated at 37 ^{o}C for a total of 72 hours. $A\beta_{1-42}$ was reconstituted in 1:1 DMSO:H_2O in a total volume of 200 μL. The final $A\beta_{1-42}$ concentration was 10 μM in the 96-well plate. KP1019 was prepared as a 250 μM stock solution in 1:1 DMSO:H_2O. Final KP1019 concentrations ranged from 10 – 100 μM in the 96-well plate. PBS pH 7.4 was then added to the wells to a final volume of 100 μL. 10 μL aliquots were obtained immediately following mixing (time 0), and at each 24 hour timepoint thereafter up to 72 hours. From these aliquots, a 2 μL drop was absorbed onto a sheet of nitrocellulose. The nitrocellulose membrane was immediately blocked for 1 hour at room temperature using 3% BSA in TBS-T under constant agitation, followed by incubation with a primary antibody (6E10) overnight at room temperature. The membrane was washed 4 x 15 minutes with TBS buffer then incubated with a horseradish peroxidase conjugated goat anti-mouse secondary antibody in 2% BSA solution for 2-3 hours at room temperature. The membrane was washed with TBS buffer for 4 x 15 minutes and then incubated at room temperature with the Thermo Scientific Supersignal West Pico Chemiluminsecent Substrate kit for 5 minutes and visualized with a Fujifilm Luminescent imager.

Native Gel Electrophoresis and Western Blotting

The ability of KP1019 to regulate the aggregation of $A\beta_{1-42}$ was further evaluated by molecular weight separation on a 10 – 20% gradient tris-tricine gel and visualized using western blotting techniques. For the inhibition experiment, each sample was incubated for 24 hours under constant agitation at 37 ^{o}C in a 96-well plate, covered with a lid and sealed with parafilm. Final concentrations, diluted in PBS pH 7.4, were as follows: 25 μM $A\beta_{1-42}$, 0 – 2 eq. KP1019. Disaggregation experiments commenced by incubating a 96-well plate with 25 μM $A\beta_{1-42}$ for 24 hours, followed by incubation for 24 hours with 0.25 – 2 eq. KP1019. Samples were loaded onto a 10-20 % gradient tris-tricine gel (Bio-Rad) and run at 100 V for 100 minutes in a tricine running buffer, followed by transferring to a nitrocellulose membrane for 3 hours at 40 V in a 4 ^{o}C cold room. The membrane was immediately blocked in 3 % BSA solution in tris-buffered saline containing 0.1 % Tween-20 (TBS-T) for 1 hour at room temperature, followed by incubation with a primary anti-Aβ antibody (6E10) overnight at room temperature under constant

agitation. The membrane was washed 4 x 15 minutes with TBS buffer and then incubated at room temperature with a horseradish peroxidase conjugated goat anti-mouse secondary antibody in 2 % BSA solution. The membrane was washed with TBS buffer for 4 x 15 minutes, incubated with the Thermo Scientific Supersignal West Pico Chemiluminsecent Substrate kit for 5 minutes and visualized with a Fujifilm Luminescent imager.

Alamar Blue Cell Viability Assay

SH-SY5Y cells were a gift from Prof. Frank Lee in the Department of Health Sciences at Simon Fraser University. Cells were grown in T-75 tissue culture flasks (Greiner) until 80-90% confluency. Media consisted of 1:1 DMEM/Ham's F-12 nutrient mixture (Sigma-Aldrich), 1% Penicillin/Streptomycin solution (Life Technologies), 1% MEM non-essential amino acid solution (Gibco), and 10% Fetal Bovine Serum (ATCC). Media was replaced every 3-4 days. Trypsinized cells were pelleted, re-suspended in DMEM/Ham's F-12 media, and seeded into a 96-well plate at a density of *ca.* 20,000 cells/well using a haemocytometer. Cells were placed in differentiating media containing DMEM/Ham's F-12, 1% Penicillin/Streptomycin solution, 1% MEM Non-essential amino acid solution, 1% fetal bovine serum, and 10 μM retinoic acid. Media was changed every 3-4 days. Cells were allowed to differentiate for 5-7 days before incubation experiments were initiated. Cells were treated with varying KP1019 concentrations to determine if the concentration range used in the incubation studies (20 – 100 μM) demonstrated any toxicity (Figure S2). A 5 mM KP1019 stock solution was prepared in 20% DMSO/PBS buffer at pH 7.4. The toxicity of KP1019 was evaluated using concentrations ranging from 500 nM – 1 mM (Fig. S2). The experimental concentration range used for KP1019 was outside of its toxicity range (>300 μM). $A\beta_{1\text{-}42}$ was also evaluated for toxicity using concentrations ranging from 10 – 40 μM (Figure S3). Significant Aβ toxicity at 20 μM was observed and used as the concentration for all cell studies. In all toxicity experiments, differentiated cells were incubated at 37 ^{o}C for 24 hours at a final volume of 100 μL with pre-mixed KP1019:$A\beta_{1\text{-}42}$ species incubated for 5 minutes, 2 hours, and 24 hours. To measure the cell viability, Alamar Blue reagent (10 μL, Life Technologies) was added to each well and allowed to incubate for 2 hours at 37 ^{o}C. Fluorescence measurements were performed by monitoring the emission at 600 nm after excitation at 570 nm. % Cell Viability was determined by comparing all measurements to wells containing cells only. All experiments were completed in quadruplicate.

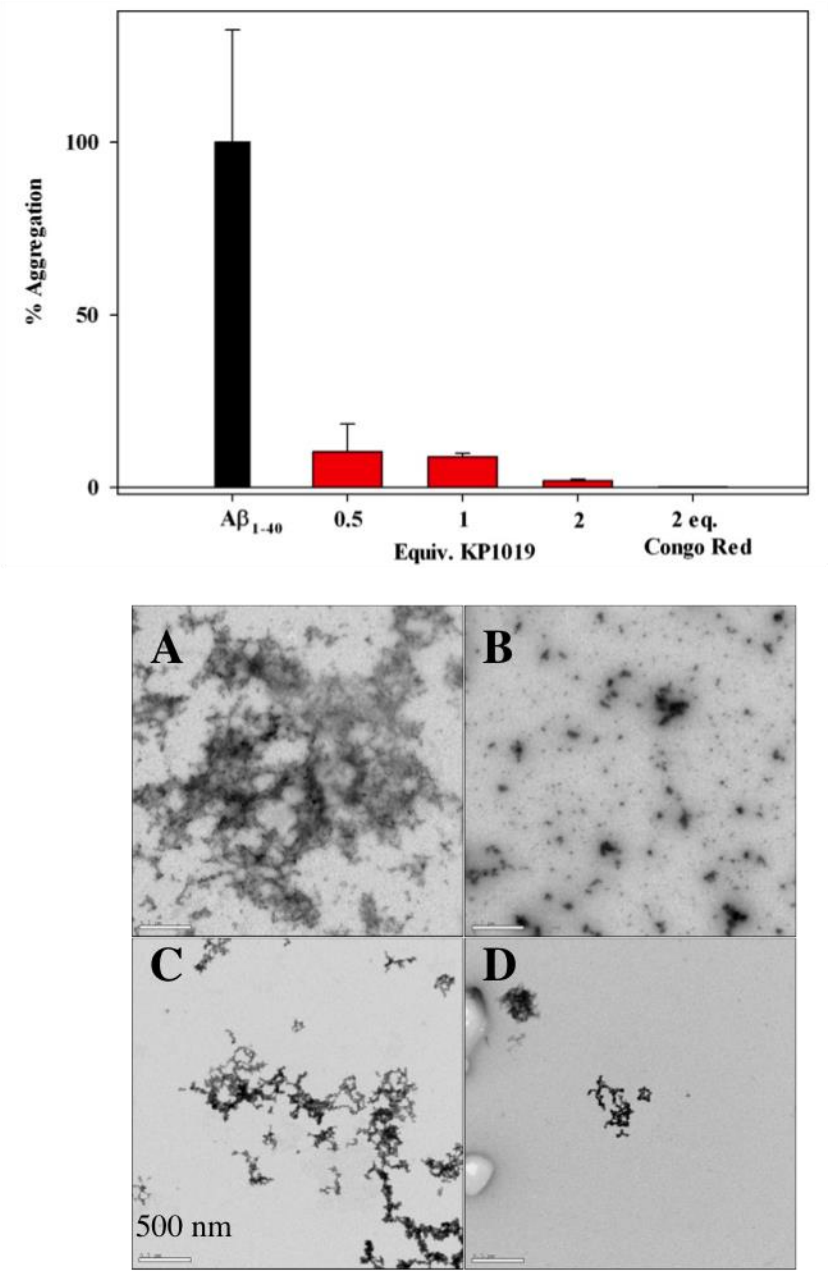

Figure S1: $A\beta_{1\text{-}40}$ (5 μM) was incubated for 48 hours in the absence and presence of varying concentrations of KP1019 (0.5 – 2 eq.) and the known aggregation inhibitor Congo Red. Even at 0.5 eq. KP1019/$A\beta_{1\text{-}40}$, KP1019 is still able to limit the fibrillization process (top). TEM images were obtained after 48 hours of incubation (bottom). When $A\beta_{1\text{-}40}$ was incubated in the absence of KP1019, significant fibrillar aggregates are observed (bottom, A). Upon addition of 2 eq. Congo Red, an apparent decrease in aggregate size is clear (bottom, B). When 0.5 and 1 eq. KP1019 are incubated with $A\beta_{1\text{-}40}$, a concentration-dependent decrease in aggregate size was characterized (bottom, C and D).

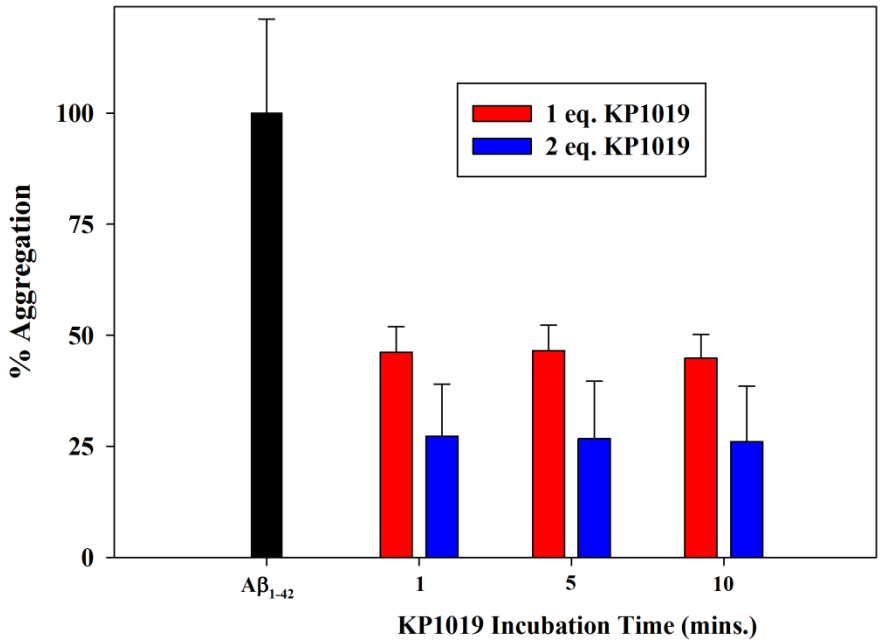

Figure S2: $A\beta_{1-42}$ (5 μM) was incubated for 48 hours in the presence of ThT (5 μM) causing a significant increase in fluorescence indicative of peptide aggregation. Upon addition of either 1 or 2 eq. KP1019, an immediate decrease in fluorescence was observed and did not change even after 10 mins.

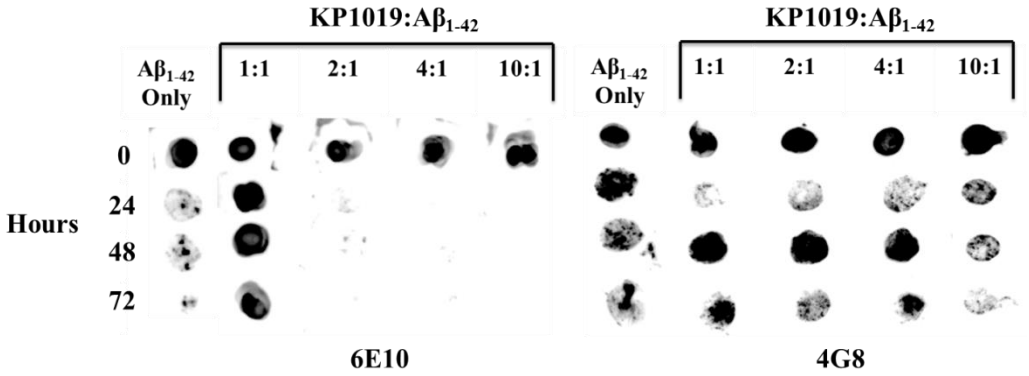

Figure S3: 24, 48, and 72 hour time course dot blot assay using 6E10 (left) and 4G8 (right) primary anti-Aβ antibodies. KP1019 (10 – 100 μM) binding to $A\beta_{1-42}$ (10 μM) reduced the immunoreactivity of the N-terminus primary antibody 6E10, but did not inhibit the immunoreactivity of the 4G8 primary antibody for the hydrophobic region of the $A\beta_{1-42}$ peptide. Interestingly, when the peptide is incubated in the absence of KP1019, 6E10 immunoreactivity decreased over time, likely due to slow aggregation and precipitation of the peptide.

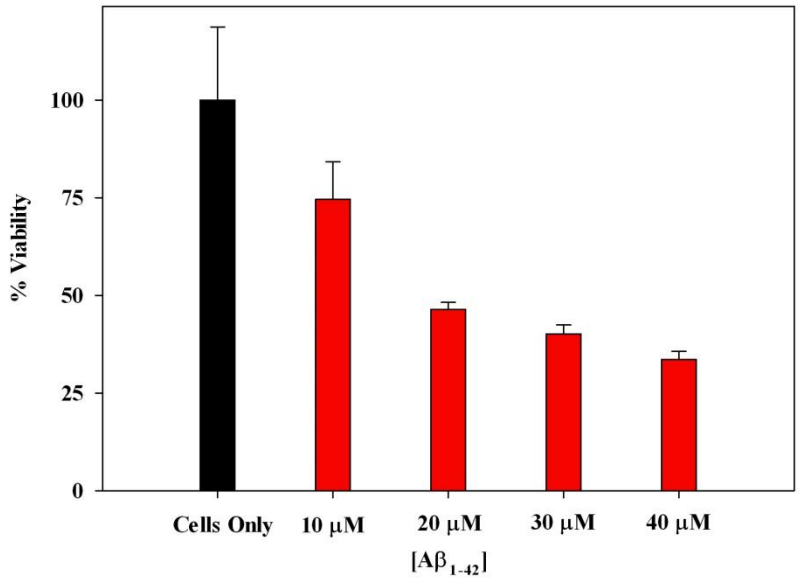

Figure S4: Differentiated SH-SY5Y cells were treated with variable [$A\beta_{1-42}$] in quadruplicate measurements for 24 hours. To assess cell viability, an Alamar Blue assay was used to evaluate $A\beta_{1-42}$-associated neurotoxicity. 20 μM $A\beta_{1-42}$ was used in all other measurements as this was determined to be an optimal concentration to evaluate the rescuing of cell viability with KP1019.

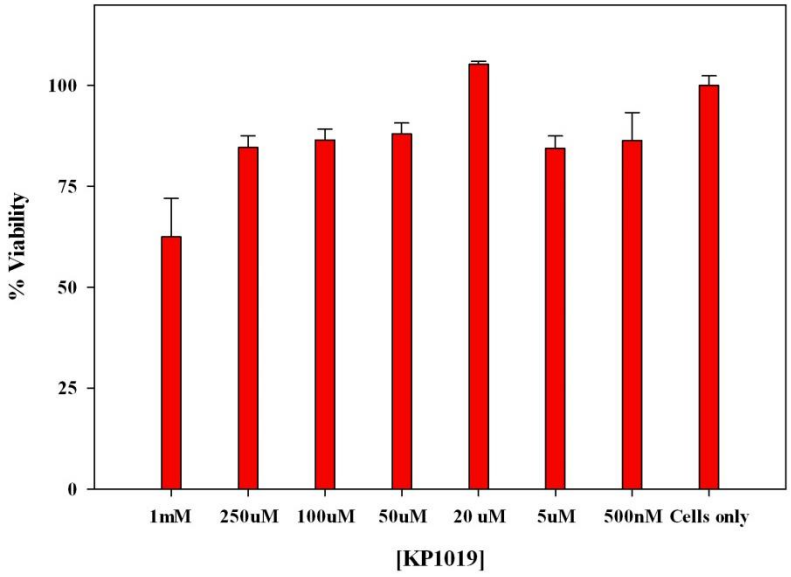

Figure S5: Differentiated SH-SY5Y cells were treated with variable [KP1019] in quadruplicate measurements and evaluated for toxicity using an Alamar Blue cell viability assay. KP1019 is relatively non-toxic up to 250 μM under these conditions.

References

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5. WINEPR SimFonia, Bruker Analytik GmbH, Copyright 1994–1997.