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SUPPORTING INFORMATION

Inhibition of tau-derived hexapeptide aggregation and toxicity by a self-assembled cyclic D,L-α-peptide conformational inhibitor

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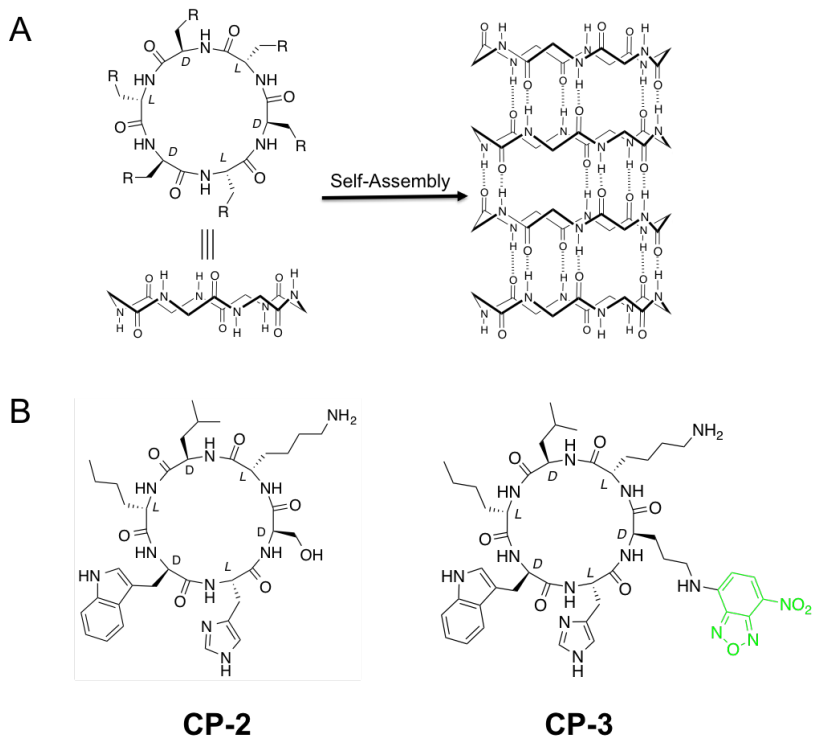

Scheme S1 a) Schematic representation of nanotube assembly from cyclic D,L-α-peptide hexamers. For clarity, all of the side-chains have been omitted. b) Chemical structures of the two cyclic D,L-α-peptide analogues investigated in this study.

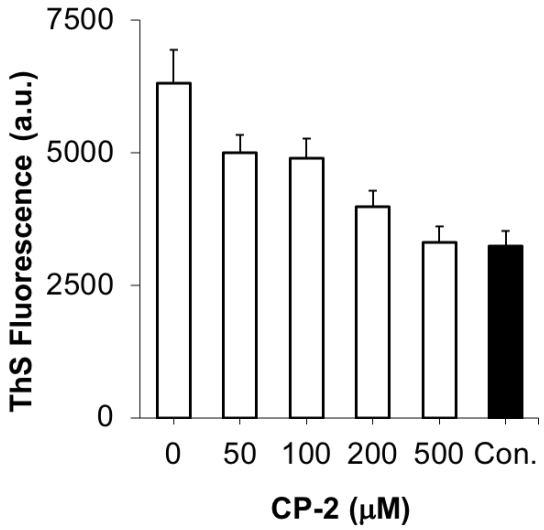

Fig. S1 Dose dependent anti-amyloidogenic activity of the cyclic D,L-α-peptide **CP-2**. Monomeric AcPHF6 (100 μM) was treated for 30 min without or with increasing amounts of **CP-2** in MOPS buffer (20 mM, pH 7.2) at 25 °C, and aggregation was then induced by 1% heparin. The ThS fluorescence assay was used to determine the degree of aggregation (Ex. 436 nm and Em. 470 nm). The ThS fluorescence obtained from a MOPS solution was used as a control.

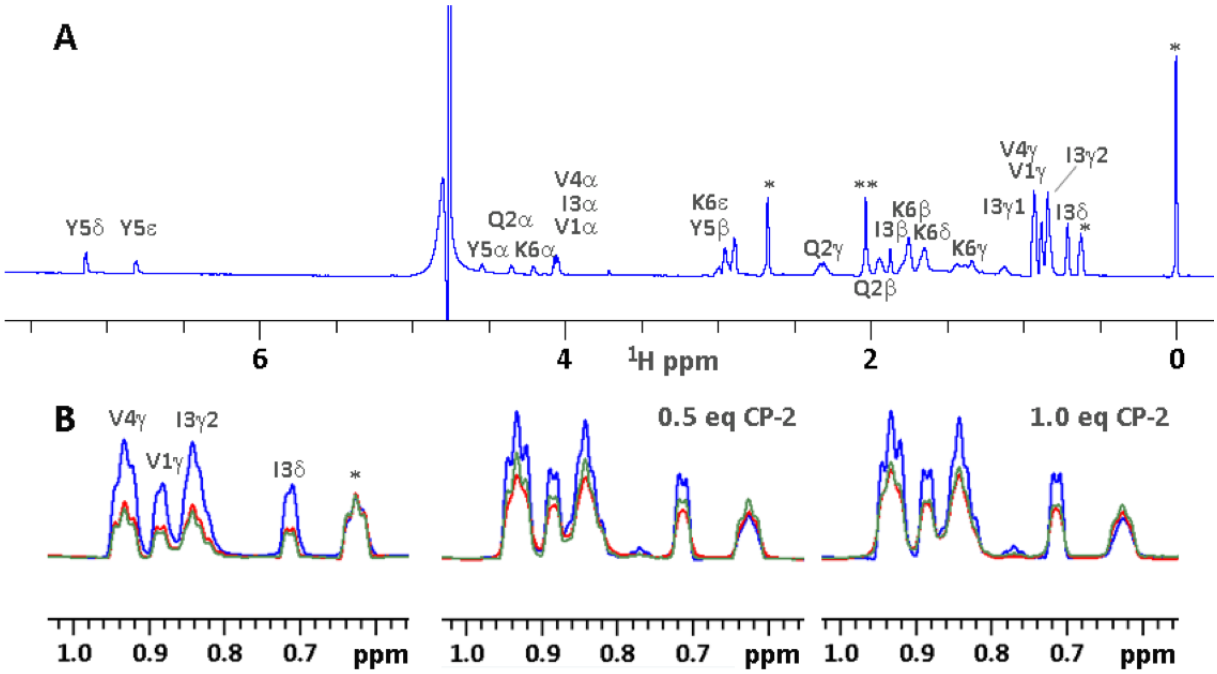

Fig. S2 NMR analysis of **CP-2** antiamyloidogenic activity. (a) ^{1}H spectrum of AcPHF6 with assignments obtained from combination of TOCSY and NOESY spectra. NMR measurements were conducted on a sample of AcPHF6 (100 µM) in 50 mM d_3-NaAc buffer in 95% $^{2}H_2O$/5% d_6-DMSO, at 16.4 T and 298 K. 1D experiments were acquired with HDO suppression by presaturation. * represents the NMR signals derived from 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) used as a reference for chemical shifts correction. ** represents the residual protonated DMSO present in the sample. (b) Methyl regions of ^{1}H spectra of AcPHF6 in the absence **CP-2** (left) or presence of 0.5 eq. (50 µM, middle) and 1 eq. (100 µM, right) of **CP-2**. Shown are comparisons between the spectrum before (blue) and immediately upon (red) addition of 1 µM heparin. The spectrum 1 h later is shown in green. The decrease in NMR signals is indicative of a reduction in soluble (monomeric) AcPHF6, which is much more pronounced in the absence of **CP-2** (~55%) as opposed to samples containing the peptide (~30%). All measurements were performed on a DRX700 Bruker spectrometer using a cryogenic triple-resonance TCI probehead equipped with z-axis pulsed field gradients.

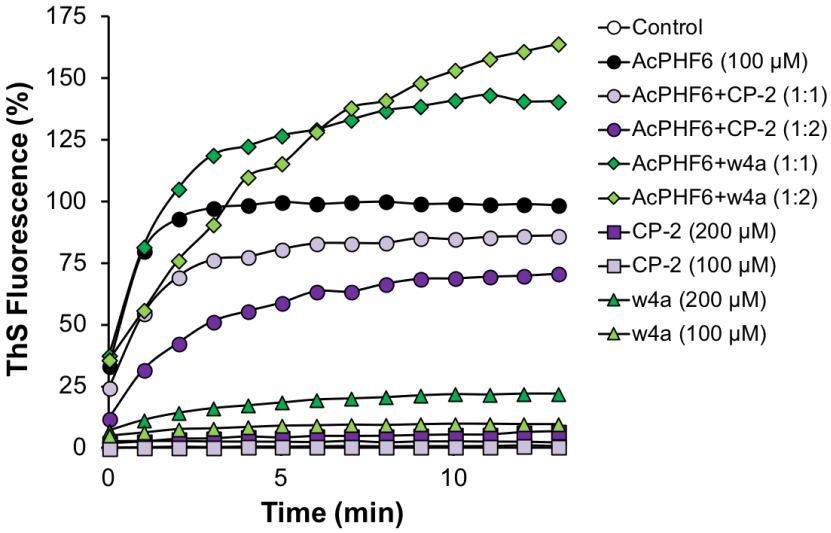

Fig. S3 Effect of increasing concentrations of [w4a]-**CP-2** mutant [lJaHsK] on the aggregation kinetics of AcPHF6 in presence of 1% heparin. Monomeric AcPHF6 (100 µM) was treated without or with increasing amounts of [w4a]-**CP-2** or **CP-2** in MOPS buffer (20 mM, pH 7.2) at 25 °C, and aggregation was then induced by 1% heparin and followed over 20 min. The ThS fluorescence assay was used to determine the degree of aggregation (Ex. 436 nm and Em. 470 nm). The ThS fluorescence obtained from a MOPS solution was used as a control.

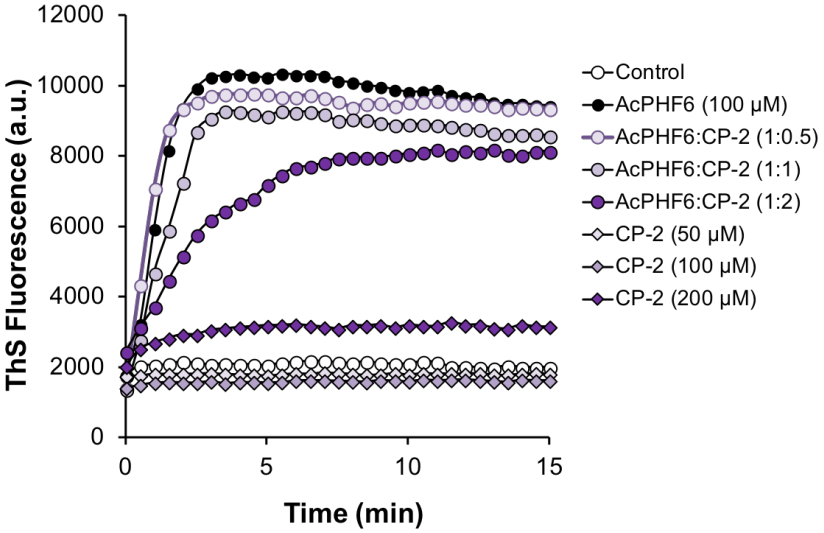

Fig. S4 Effect of 10% heparin on antiamyloidogenic activity of **CP-2**. Monomeric AcPHF6 (100 µM) was treated with increasing amounts of **CP-2** in MOPS buffer (20 mM, pH 7.2) at 25 °C, and aggregation was then induced by 10% heparin and followed over 20 min using the ThS fluorescence assay. The ThS fluorescence obtained from a MOPS solution was used as a control. Data are representative of three independent experiments carried out in triplicate.

Materials and Methods

All chemicals and reagents were of analytical grade. 2-Chlorotrityl-chloride, 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acid derivatives, and all other reagents for solid-phase peptide synthesis were purchased from Shanghai Hanhong Scientific (Shanghai, China) and used as received. Unless otherwise stated, all other chemicals were obtained from Sigma-Aldrich (Rehovot, Israel). Peptide analogs were synthesized using solid-phase peptide synthesis, employing the common Fmoc strategy.[1] Cyclic D,L-α-peptides were synthesized on 2-chlorotrityl chloride resin, as described.[2] AcPHF6 (Ac-VQIVYK-NH_2) was synthesized on Rink amide resin. Coupling reactions were carried out for 45 min using O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and diisopropylethylamine (DIEA) (5:15 eq.) in N-methyl-2-pyrrolidone (NMP). The peptides were cleaved from the resin by a solution of 95:2.5:2.5 trifluoroacetic acid:triisopropylsilane:water for 2–3 h and purified by preparative RP-HPLC using a C18 column. The purified peptide (>95%) was then analyzed by analytical HPLC and MS. HRMS m/z: calculated for $C_{38}H_{63}N_9NaO_9$ 813.4725, found 813.4480.

Synthesis of CP-3. The linear peptide Leu-D-Nle-Trp-D-His(Trt)-Orn(Dde)-D-Lys(allyl) was synthesized on 2-chlorotrityl chloride resin, as described.[2a] Following assembly of the linear sequence, the allyl protecting group was removed under N_2 atmosphere by treating the resin with $Pd(PPh_3)_4$ and $PhSiH_3$ (0.25:10 eq.) in DCM for 4 h. The resin was washed with a solution of 1% sodium dimethylthiocarbamic acid in DMF followed by 1% DIEA in DMF. The peptide was then cyclized while still on resin, using HOAt and DIC (5:5 eq.) in DMF for 4 h followed by PyBOP and DIEA (5:15 eq.) overnight. Following cyclization, the Dde protecting group was removed by 2% hydrazine hydrate in DMF. The removal of the Dde group was monitored spectroscopically at 290 nm. The resin was then washed thoroughly with DMF, and a solution of 4-chloro-7-nitro-1,2,3-benzoxadiazole (NBD) and DIEA (2:2 eq.) in DMF was added to the resin for overnight. Finally, the crude peptide was cleaved from the resin and purified by preparative HPLC (>95%), as described. HRMS m/z: calculated for $C_{46}H_{62}N_{14}NaO_9$ 977.4722, found 977.4741.

Synthesis of NBD-conjugated PHF6. PHF6 was synthesized on Rink amide resin as described. Following the assembly of the peptide on the resin, the N-terminus of the peptide was conjugated to NBD, using NBD-Cl and DIPEA (2:2 eq.) in DMF for 16 h. The peptide was then

cleaved from the resin and purified by preparative HPLC (>95%), as described. HRMS m/z: calculated for $C_{42}H_{62}N_{12}NaO_{11}$ 933.4559, found 933.4553.

Thioflavin S (ThS) aggregation assay. Lyophilized AcPHF6 powder was first monomerized by dissolving it in hexafluoroisopropanol (HFIP) at a concentration of 1 mM. The solvent was then evaporated by a stream of N_2 and the residual HFIP was removed by placing the sample in high vacuum for 1 h. The inhibitory activity of the cyclic D,L-α-peptides on AcPHF6 aggregation was then determined as described.[3] In brief, to each well of a black 96-well flat-bottom plate containing MOPS buffer (35 μL, 20 mM, pH 7.2) were added freshly monomerized AcPHF6 (10 μL, 1 mM in DDW), increasing amounts of the cyclic D,L-α-peptides (5 μL, dissolved in 5% DMSO in DDW), and a solution of ThS (40 μL, 200 μM) in MOPS buffer. Aggregation was initiated by addition of heparin (10 μL, 10 or 100 μM) in MOPS buffer, and the fluorescence of amyloid-bound ThS was followed using a plate reader (Infinite M200, Tecan, Switzerland) at excitation and emission wavelengths of 436 and 470 nm, respectively.

Thioflavin T (ThT) aggregation assay. The inhibitory activity of **CP-2** on AcPHF6 aggregation was also determined by ThT assay, as described by Nagaraj *et al*.[4] In brief, Lyophilized AcPHF6 was dissolved in HFIP at a concentration of 0.75 mM. The monomerized HFIP solution of AcPHF6 (0.75 mM, 7 μL) was then added to a black 96-well flat-bottomed plates containing ThT (100 μM, 10 μL) in PBS (50 mM, pH 7.2), different concentration of **CP-2** in DMSO (100%, 10 μL) and PBS (73 μL) to yield final concentrations of 50 μM AcPHF6, 10 μM of ThT and increasing concentrations of **CP-2** in 10% DMSO. The unsealed plates were then placed in a plate reader (Infinite M200, Tecan, Switzerland) thermostated at 25 ºC, and the fluorescence of amyloid-bound ThT was monitored with 2 min intervals for 4 h using excitation and emission wavelengths of 440 and 480 nm, respectively. The plates were shaken for 10 sec before each reading with an orbital shaking amplitude of 3 mm.

Transmission electron microscopy (TEM) analysis. Samples were prepared for TEM studies in a similar manner to the ThS aggregation assay, except for the ThS addition. Each sample contained AcPHF6 (5 μL, 200 μM in DDW) and increasing amounts of **CP-2** or vehicle (5 μL in 25% DMSO/DDW) diluted in MOPS buffer (35 μL, 20 mM, pH 7.2). Aggregation was initiated

by addition of heparin (2 μM, 5 μL) in the MOPS buffer. Samples (5 μL) were then loaded onto glow-discharged, carbon-coated Formvar/copper grids (SPI supplies, West Chester, PA), blotted with a filter paper, and dried for 1 min. The samples were negatively stained with 2% uranyl acetate in water (5 μL) for 1 min, blotted with filter paper, and dried for an additional 1 min. Samples were then analyzed by a Tecnai G2 TEM (FEI TecnaiTM G2, Hillsboro, OR) operated at 120 kV.

Circular dichroism (CD) spectroscopy. CD measurements were carried out using a Chirascan spectrometer (Applied Photophysics, UK). To a solution of AcPHF6 (50 μL, 200 μM in DDW) and **CP-2** (50 μL, 100 and 400 μM in 20% acetonitrile in DDW) or vehicle (50 μL, 20% acetonitrile in DDW) in MOPS buffer (380 μL, 5 mM, pH 7.2), a solution of heparin (20 μL, 20 μM) in MOPS buffer was added. Measurements were then performed at room temperature without dilution in a cell with 1 mm optical path length, and the spectra were recorded from 260 to 190 nm with a step size and a bandwidth of 1 nm. The spectra are the average of three measurements after background subtraction. The experiments were repeated thrice.

AcPHF6-induced toxicity. Rat pheochromocytoma cells (PC-12) were maintained in low-glucose DMEM supplemented with horse serum (10%) and fetal bovine serum (FBS; 5%), L-glutamine, penicillin, and streptomycin in a 5% CO_2 atmosphere at 37 °C. To evaluate the toxic effect of AcPHF6, cells were grown in 0.1 mL of medium for overnight in a 96-well plate (7,000 cells per well). Increasing amounts of AcPHF6 in DDW (10 μL) were then added to each well, and the cells were incubated for another 24 h. Control wells received DDW (10 μL). Cell viability was then determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[5]

Protecting effect of CP-2 on AcPHF6-induced toxicity. To evaluate the protecting effect of **CP-2** on AcPHF6-induced toxicity, AcPHF6 (5 μL, 100 μM in DDW) was incubated with increasing concentrations of **CP-2** (5 μL, in 2.5% DMSO in DDW) or with vehicle (5 μL, 2.5% DMSO in DDW) in sterile MOPS buffer (40 μL, 20 mM, pH 7.2) for 1 h at room temperature. PC-12 cells (7,000 cells per well) in 90 μL of medium were then treated with the samples (10 μL), which were diluted in the growth medium by a factor of 10. Control wells received vehicle (10 μL)

containing 2.5% DMSO (100% cell viability). After 24 h incubation, cell viability was determined by the MTT assay.

Preparation of liposomes and calcein release assay. Large unilamellar vesicles (LUVs) of 1,2-dioleoyl-sn-3-phosphatidyl-glycerol (DOPG) were prepared and encapsulated with calcein as described before.[6] In brief, DOPG (Avanti, Alabama) was dissolved in chloroform and dried overnight under vacuum. The resulting thin film was hydrated in a calcein solution (0.5 mL, 70 mM in 25 mM MOPS, pH 7.4), giving a final lipid concentration of ~ 14 mM. The solution was hydrated by vortexing it every 15 min for 1 h. The suspension was then exposed to at least 7 cycles of freezing in liquid nitrogen and thawing in a 50 ºC water bath. The suspension was extruded through a 100 nm pore filter at least 12 times using the LiposoFast instrument (Avestin, Canada). Vesicles were chromatographically separated from the calcein solution using a pre-equilibrated Pharmacia PD-10 column. Fractions were then chosen based on their maximal dye content by treating the small aliquots of the collected fractions (10 μL of sample diluted in 100 μL MOPS buffer (20 mM, pH 7.2)) with Triton X-100 (0.1% w/v) and monitoring the fluorescence of the released dye (excitation 485 nm, emission 520 nm). Dynamic light scattering (DLS) measurement of the diluted samples in PBS demonstrated a monodispersed size of approximately 125 nm.

For dye release experiments, the calcein-encapsulated LUVs (10 μL) were incubated with either increasing concentrations of AcPHF6 (10 μL, 250 – 1000 μM in DDW) or with a solution of AcPHF6 (10 μL, 1 mM in DDW) in the absence or presence of **CP-2** (10 μL, 1.5 mM in 5% DMSO in DDW) in MOPS buffer (70 μL, 20 mM, pH 7.2), and the fluorescent intensity was monitored in a plate reader (Infinite M200, Tecan, Switzerland) for 4 h.

Cellular uptake of NBD-PHF6 by PC-12 cells. PC-12 cells (50,000 cells) were grown overnight on 35 mm disposable glass-bottomed tissue culture plates (MatTek). The medium was then replaced with fresh DMEM medium containing 1% FBS and NBD-PHF6 (10 μM), and the cells were incubated at 37 ºC for 3.5 h. Cells were then washed with fresh DMEM containing 10% FBS medium and stained with Hoechst (1 μg mL^{-1}, Sigma-Aldrich, USA) and Alexa Fluor® 680 wheat germ agglutinin (WGA, 10 μg mL^{-1}, Invitrogen, USA) for nuclei and membranes, respectively. Live cell imaging was performed on an Olympus FV-1000 confocal microscope operated at 37 ºC. For intracellular localization of NBD-PHF6, cells were grown and treated

with NBD-PHF6 (10 μM) as described above and stained with LysoTracker® Red DND-99 (60 μM) for 30 min and Hoechst and visualized at 37 ºC.

Uptake of CP-2-NBD (CP-3) by PC-12 cells. PC-12 cells were grown on 35 mm disposable glass-bottomed tissue culture plates as described. The medium was replaced with a fresh DMEM containing 1% FBS, and cells were treated with **CP-3** (8 μM) for 3.5 h. The cells were then washed with fresh DMEM containing 10% FBS medium and stained with Hoechst, Alexa Fluor® 680 WGA, and LysoTracker® Red DND-99 as described and visualized.

References:

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