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Supporting Information

Potent Tau Aggregation Inhibitor D-Peptides Selected against Tau-Repeat 2 Using Mirror Image Phage Display

Marwa Malhis[+], Senthilvelrajan Kaniyappan[+], Isabelle Aillaud, Ram Reddy Chandupatla, Lisa Marie Ramirez, Markus Zweckstetter, Anselm H. C. Horn, Eckhard Mandelkow, Heinrich Sticht, and Susanne Aileen Funke*

Experimental section

Preparation of D-PHF6* fibrils for mirror image phage display selection

PHF6* stock solution was prepared by dissolving the lyophilized acetylated D-enantiomeric PHF6* in hexafluoro-2-propanol (HFIP) to a molarity of 1.5 mM. PHF6* fibrillizes spontaneously during incubation at room temperature. The fibrillization was started by incubating 100 μM PHF6* in 50 mM NaPi buffer (Sodium Phosphate buffer), pH 7.0 with 10 μM ThT at room temperature for 42 h. NaPi and 10 μM ThT without addition of peptide was used as negative control. Fibril formation of PHF6* was monitored using the ThioflavinT (ThT) assay. For ThT fluorescence measurements, 70 μL of the sample were pipetted into a black 96-well half area clear flat-bottom plate, three replicates per sample. The fluorescence measurement was performed using a photometer POLARstar optima (BMG-Labtechnologies, Ortenberg, Germany), excitation/emission wavelengths were set at 440/490 nm. Mean and standard deviations of results were calculated using Microsoft Excel 2013 (Microsoft Corp.).

Mirror image phage display selection

First, the fibrillized D-enantiomeric PHF6* peptide, prepared as described above, was immobilized on polystyrene 96-well microtiter plate (Greiner Bio-One International GmbH, Frinckenhausen). The plate was covered with gas permeable sealing film and incubated at 4°C overnight with 300 rpm agitation. The next day, the coating solution was poured off, and the phage display selection was performed using the Ph.D.-12 Phage Display Peptide Library Kit (New England Biolabs, Frankfurt a.M., Germany) according to the instructions of the manufacturer.

Single clone ELISA (Enzyme Linked Immunosorbent Assay)

Single phage ELISA was performed to test the binding properties of a selected phage clone to PHF6* fibrils. Wells of polystyrene 96-well microtiter plate (Greiner Bio-One International GmbH, Frinckenhausen) were coated with PHF6* fibrils diluted in coating buffer 0.1 M $NaHCO_3$, pH 8.6 at a concentration of 100 µg/ml. As a negative control, a coating buffer without the target protein was added to the control wells. The plate was covered with gas permeable sealing film and incubated at 4°C, 300 rpm overnight. Next morning, after discarding the coating solution, the wells were completely filled with the blocking buffer (5 mg/ml BSA in 0.1 M $NaHCO_3$, pH 8.6) and incubated at 4°C, 300 rpm for 2h. In a separate plate, to exclude possible plastic binding phages, 2 x 80 µl of each purified individual phage clone solution were pipetted into two wells of the 96-well plate, mixed with 80 µl of the blocking buffer and incubated on a shaker at RT for 20 minutes. After discarding the blocking buffer from the first plate, the plate was washed 6 times with TBST (0.5% Tween 20 in Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.6)). The incubated diluted phage eluates were transferred to PHF6* fibril coated wells and to the control wells, followed by incubation at RT, 300 rpm for 1h. After washing the plates six times, 200 µl of HRP-conjugated anti-M13 antibody (diluted in blocking buffer 1:5000) were added to the adequate wells and incubated at RT, 300 rpm for 1h. Subsequently, the plate was washed six times and 100 µl of TMB substrate solution were added to each well and incubated for 15 minutes at RT with gentle agitation. The reaction was stopped by adding 100 µl of 20% H_2SO_4. The absorption was measured at 450 nm with the plate reader Multiscan Go (Thermo Fisher Scientific, Darmstadt, Germany).

Peptides

All of the peptides were synthesized commercially (JPT Peptide Technologies, Berlin, Germany) with minimum purities of 95 %. For 5-carboxyfluorescein (FAM) labeled peptides, an additional lysine residue was attached to the C-terminus of the peptides. For Alexa647

labeled peptides, an additional cysteine residue was attached to the C-terminus of the peptides. The hexapeptides PHF6 and PHF6* were purchased with N-terminus capped by acetylation to allow the self- aggregation and filaments formation. The acetylated hexapeptides mimic the aggregation propensity of these segments within Tau protein.

Full-length Tau protein expression and purification

The gene of the large isoform of human Tau40 contains 1323 base pairs, which encodes a full-length Tau protein with 441 amino acids (Tau441 or Tau 2N4R). This gene was commercially synthesized and cloned into a pET28A(+) vector (Genentech, San Francisco, USA). Full-length Tau protein expression and purification were carried out according to Margittai et al. [1] and KrishnaKumar et al. [2].

Thioflavin T (ThT) fluorescence assays for monitoring PHF6 and PHF6* fibrillization inhibition

The fibrillization conditions of the hexapeptide segments PHF6 and PHF6* were established as described above, the only difference was the use of 50 µM of PHF6 solution to prepare PHF6 fibrils. Also, 100 µM PHF6* or 50 µM PHF6 were incubated with the respective peptide at a concentration of 1:10 (PHF6 or PHF6*: peptide) in NaPi buffer. No heparin was used in the assays. ThT was added to the sample at a concentration of 10 µM. As a positive control, PHF6 or PHF6* was incubated without peptides in NaPi buffer with 10 µM ThT. As a negative control, NaPi buffer was incubated with ThT. The samples were pipetted into a 96-well black µclear flat-bottom plate, the plate was covered with sealing film and incubated at RT for 36 h. The relative fluorescence intensity was measured at 440/490 nm at a POLARstar optima microtiter plate reader. The mean of three absorption values was calculated using Microsoft Excel 2013 (Microsoft Corp.), as well as the standard deviation.

Inhibition of full-length Tau protein aggregation determined by ThT assay

Purified recombinant Tau protein at a concentration of 24 µM was incubated with 6 µM heparin and 10 µM ThT in an HEPES buffer. The peptides (MMPD2, MMPD6, MMD3 and MMD3rev) were added in molar ratio 1:10 (Tau:peptide). As a positive control, Tau was incubated with heparin and ThT. As negative controls, the fluorescence of Tau alone with ThT was measured as well as the fluorescence of the buffer HEPES in the presence of ThT. The samples were pipetted into a black 96-well µclear flat-bottom plate, the plate was covered with sealing film and incubated at 37°C for 72 h. The relative fluorescence intensity was read out at 440/490 nm on a POLARstar optima microtiter plate reader. The relative fluorescence of the samples with peptides were normalized to the positive controls, which were each set to be 100%. The mean of three absorption values was calculated using Microsoft Excel 2013 (Microsoft Corp.), as well as the standard deviation.

Inhibition of Tau$^{RD\Delta K}$ aggregation determined by ThS (Thioflavin S) assay

The peptides (TLKIVW, APT, MMD3, MMD3rev and W-MINK) were tested for their ability to inhibit the aggregation of the 4-repeat domain of Tau with ΔK280 mutation (Tau$^{RD\Delta K}$) *in vitro*, monitored by ThS fluorescence assay. Tau aggregation assays were performed under reducing conditions. Before the addition of heparin and peptides, final concentration of 1mM DTT was added to the Tau protein solution and heated at 95 degree Celsius for 15 min. 10 µM of Tau$^{RD\Delta K}$ was incubated with different concentrations (1 nM to 200 µM) of different peptides in the presence of heparin 16,000 daltons (H16K) (2.5 µM) in BES buffer pH 7.0 and incubated at 37°C for 24 h. Aggregation of Tau was monitored by using 40 µM of the amyloid-dye ThioflavinS (ThS) (excitation-440 nm; emission 521 nm). ThS intensity was monitored for 24 h with readout every 15 min using a Tecan spectrofluorometer (Lab System, Frankfurt).

Characterization of the binding properties of the selected peptides to PHF6* fibrils and Tau fibrils by ELISA

Polystyrene 96-well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated with Tau fibrils at a concentration of 10 µg/ml in $NaHCO_3$ pH 8.3, or with PHF6* fibrils at a concentration of 50 µg/ml in $NaHCO_3$. As a negative control, only $NaHCO_3$ buffer without the target protein was incubated in the wells. The plates were covered with sealing film and incubated at RT with 300 rpm agitation for 1 h. After 3 times of washing with PBST 1% (phosphate-buffered saline (pH 7.4) with 0.1 % Tween 20), the wells were blocked with 1% BSA (IgG free) (Roth, Karlsruhe, Germany) in PBS for 1h at RT and 300 rpm followed by 3 times washing with PBST. The FAM-labelled peptide MMD3-Lys(FAM)-NH2 was dissolved in PBST and added in increasing concentrations 1µg/ml, 5 µg/ml, 10 µg/ml and 20 µg/ml, in the case of Tau fibrils; and 10 µg/ml, 20 µg/ml and 50 µg/ml in the case of PHF6* fibrils, respectively, the peptide was incubated for 1h at RT and 300 rpm. The plates were washed 3 times with PBST and horseradish peroxidase-conjugated sheep anti-FITC secondary antibodies (Bio-Rad, München, Germany) diluted 1:5000 in PBST were used to detect the bound peptide. Subsequently, the TMB substrate solution was prepared from TMB Peroxidase EIA Substrate Kit (Bio-Rad Laboratories, Muenchen) and transferred to the relevant wells. Finally, the reaction was stopped with 20% H_2SO_4 and the plates were read at 450 nm (Multiskan GO, Thermo scientific, Germany). The mean of three absorption values was calculated using Microsoft Excel 2013 (Microsoft Corp.), as well as the standard deviation.

NMR titration experiments with hTau40

The 10 mM stock solution of MMD3 (*D*-DPLKARHTSVWY) peptide was prepared in Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.6). ^{15}N-labeled Tau protein (hTau40, Uniprot ID 10636-8, 441 residues) was prepared as previously described [3]. To examine the effect of *D*-enantiomeric peptide MMD3 on ^{1}H-^{15}N backbone resonances of hTau40, NMR

titrations were performed in which 2D ^{1}H-^{15}N SOFAST-Heteronuclear Multiple Quantum Coherence (HMQC) [4] spectra of ^{15}N-labeled hTau40 were monitored upon addition of MMD3. Spectra were recorded at 5°C on a Bruker 800 MHz spectrometer equipped with a triple-resonance cryoprobe. NMR samples of 18 µM ^{15}N-labeled hTau40 were prepared in 50 mM sodium phosphate buffer at pH 6.8, 0.01% NaN_3, 90% H_2O/10% D_2O, with unlabeled peptide at concentrations of 54 µM, 180 µM, and 540 µM, which corresponded to hTau40:peptide mole ratios of 1:3, 1:10, and 1:30, respectively. The samples were incubated overnight (~16h) at 37°C prior to spectral acquisition. In control experiments, reference samples containing hTau40 in the abovementioned phosphate buffer were titrated with TBS. NMR signal intensity ratios, I/I_0 (where I represents the peak intensity of hTau40 amide backbone resonances in the presence of the peptides, and I_0 is the peak intensity in the absence of the peptides), were calculated for each titration point. All spectra were processed using TopSpin version 3.6.2 (Bruker) and analyzed using Sparky [5]. Averaged, normalized chemical shift perturbations (CSP) were calculated as $CSP = \sqrt{[0.5\ [(\Delta\delta\ H)^2 + (\Delta\delta\ N)^2/25]}$.

Inhibition of Tau aggregation determined by dynamic light scattering

DLS measurements were performed after 24h of Tau aggregation. 20 µl of the sample was placed in quartz batch cuvette (ZEN2112) and thermally equilibrated at 25°C for 2 min in Zetasizer Nano S (Malvern, Herrenberg) instrument before the measurement was performed. Particle size distribution obtained from the sample is based on the intensity of the scattered light. Intensity values were obtained as an average of three measurements with 15 runs each. The results are expressed as volume graph.

Pelleting assay

After 24 h of Tau aggregation, 60 µl of the samples (with and without the inhibitor peptides) were centrifuged in a Beckmann Coulter (AvantiRCentrifuge J-26 XP) at 61,000 rpm for 60

min at 4°C using TLA 100.3 rotor. After centrifugation, the supernatant was separated from the pellet. The pellet was dissolved in BES buffer (equal volume to that of supernatant) and western blot was performed. 13 µl of each sample was diluted with 5X SDS sample buffer (2 µl) was heated at 95°C for 5 min and resolved on 4-20% SDS gels (BIO-RAD #4561096). Tau protein was detected by chemi-luminescence based western blot method using Pan-tau K9JA antibody as primary antibody and anti-rabbit HRP labelled secondary antibody. Image quantification was performed using ImageJ.

Atomic force microscopy (AFM)

For AFM imaging, 2 µM of protein sample diluted in adsorption buffer (PBS, pH 7.4) was placed on a freshly cleaved mica disc and incubated for 10 min. The unbound protein was removed by washing with PBS, pH 7.4 (3 times). AFM imaging was done in oscillation mode for all Tau samples in liquid. Using cantilever (MSNL10) the surface was approached in an oscillation mode with an amplitude set point of 70% (0.7V) of the target amplitude (1V). Once the surface was reached, the minimal contact between the sample and cantilever was maintained by altering the amplitude set point manually. The images were acquired at a scan rate of 1Hz with the resolution of 512 by 512 pixels. The images were acquired using the JPK Nano Wizard ultra-speed AFM microscope facility at CAESAR. The acquired images were processed by the JPK data processing software**.**

***In silico* analysis of binding mode of MMD3 and MMD3rev to the PHF6* fibril**

Modelling of inhibitor binding was based on the known experimental structures of the PHF6* aggregation site (PDB codes 5V5B, 5V5C; [6]). The overall binding mode of D-peptides to Tau-fibrils was adapted from the previous modelling studies of Sievers et al. [7] and Dammers et al. [8]. The register of the interaction was guided by a previous model the Tau-bound W-MINK peptide [7], which exhibits a similar pattern of aromatic and basic residues. Modelling was

performed using USCF Chimera [9] and Sybyl 7.3 (Tripos Inc., St. Louis, MO, USA); VMD [10] was used for visualization.

Fluorescence activated cell sorting (FACS)

Inducible (doxycycline) N2a cells expressing $Tau^{RD\Delta K}$ (induced for 48 h) were incubated with different concentrations (25, 50 and 100 µm) of A647 fluorophore-labelled peptides for 24 h. Cells were trypsinized and centrifuged and the cell pellet was diluted in 1 ml of warm PBS. Red laser was used to excite A647 (excitation-650 nm; emission 665 nm). 20,000 cells per sample were counted for A647 fluorescence using Gallios-Beckmann Coulter flow cytometry.

Immunocytochemistry (IC)

Inducible N2a-$Tau^{RD\Delta K}$ cells were incubated for 48 h with doxycycline (DOX). 100 µM of A647-labelled peptides MMD3 and MMD3rev were incubated with N2a cells for 24 h. After 24 h, the N2a cells were fixed (3.7% formaldehyde, 4% sucrose in PBS for 30 min at 37°C) and permeabilized (0.5% tritonX-100, 5% BSA in PBS for 6 min at RT). Cells were imaged using a cell voyager confocal microscope.

S1

Supplementary Table 1: Selected peptides from mirror image phage display selection against D-PHF6* fibrils. The peptides' sequences were determined after DNA sequencing of the positive phages. Each sequence was given a number in the list. D-peptides of sequences 4, 9, and 17 were investigated in more detail, see below.

No.	sequence	No.	sequence
1	YPVRAVPNQSGQ	16	NLWKGLDGSGRT
2	YVTHYNANYSNL	17 (MMP6)	HSDLWRRSFELM
3	YSLRLTSVTAPT	18	TPSYLMPLAPHT
4 (MM3)	DPLKARHTSVWY	19	SLLHPNAIMPRT
5	MPHLHPSSANWS	20	DEDQQVHYQIWR
6	GRDMPMSALMRH	21	HPAPHRYHSNLH
7	NHNHGYPITHRT	22	TRTATLADNSWL
8	GIALSEPVPNHH	23	HLTATELANSYH
9 (MMP2)	WPHDTKRYLFPA	24	MKAHHSQLYPRH
10	YPMHPGYGTKLG	25	SYPSNALSLHKY
11	YSGVSRGHSHGP	26	NHSDKQMSSAFL
12	YVPANNYHLHSP	27	SLSPAGYTRLSL
13	SLSPIFIQNGTN	28	DHMPPYHWRPWD
14	NLPPERGHLSWI	29	KIHHSLTIRTAA
15	SDASMQNKLPLW		

S2

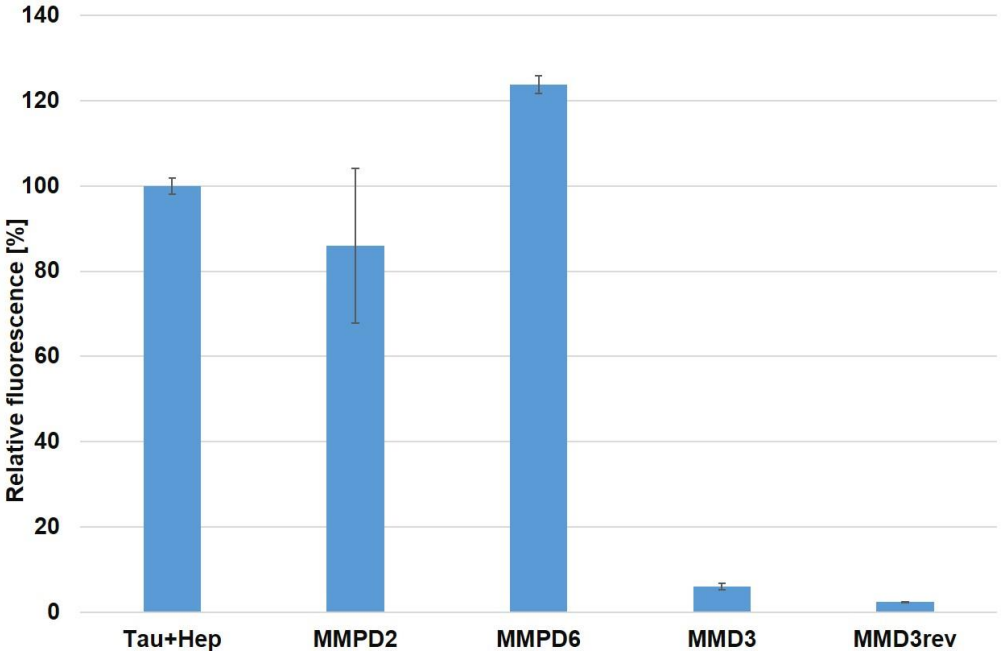

Figure S2: The ability of the selected peptides MMPD2, MMPD6, MMD3 and MMD3rev to reduce or inhibit full-length Tau fibril formation. 24 µM Tau, 6 µM heparin and 10 µM ThT were incubated at 37°C with and without the peptides at a molar ratio of 1:10 (Tau:peptide). ThT fluorescence was measured for 72h at 490 nm in relative units (mean +/- standard deviations of results, three replicates per run). The relative fluorescence for the Tau and heparin control without peptides and after 72 hours incubation (saturation level) was set at 100%.

S3

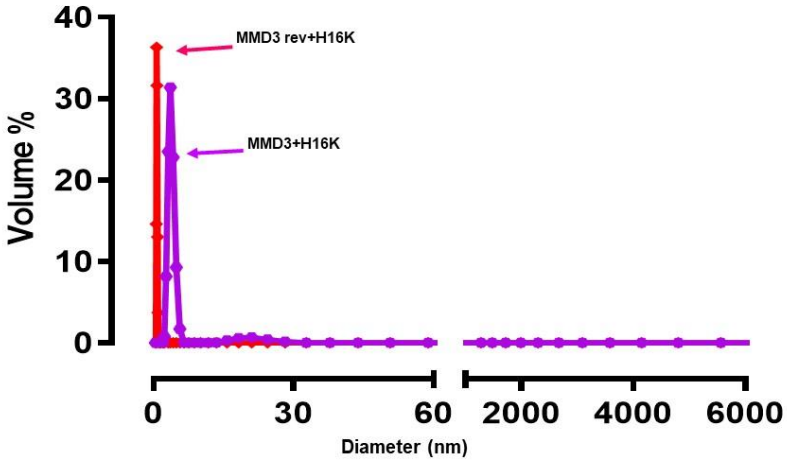

Figure S3: Peptides alone do not form larger aggregates

2.5 µM of heparin 16000 (H16K) and 100 µM of peptides were incubated together for 24h and the hydrodynamic radius was measured. Hydrodynamic sizes of MMD3 peptide (violet curve) and MMD3 reverso (red curve) are <5 nm in diameter which confirm that peptides alone in the presence of heparin do not form larger aggregates

S4

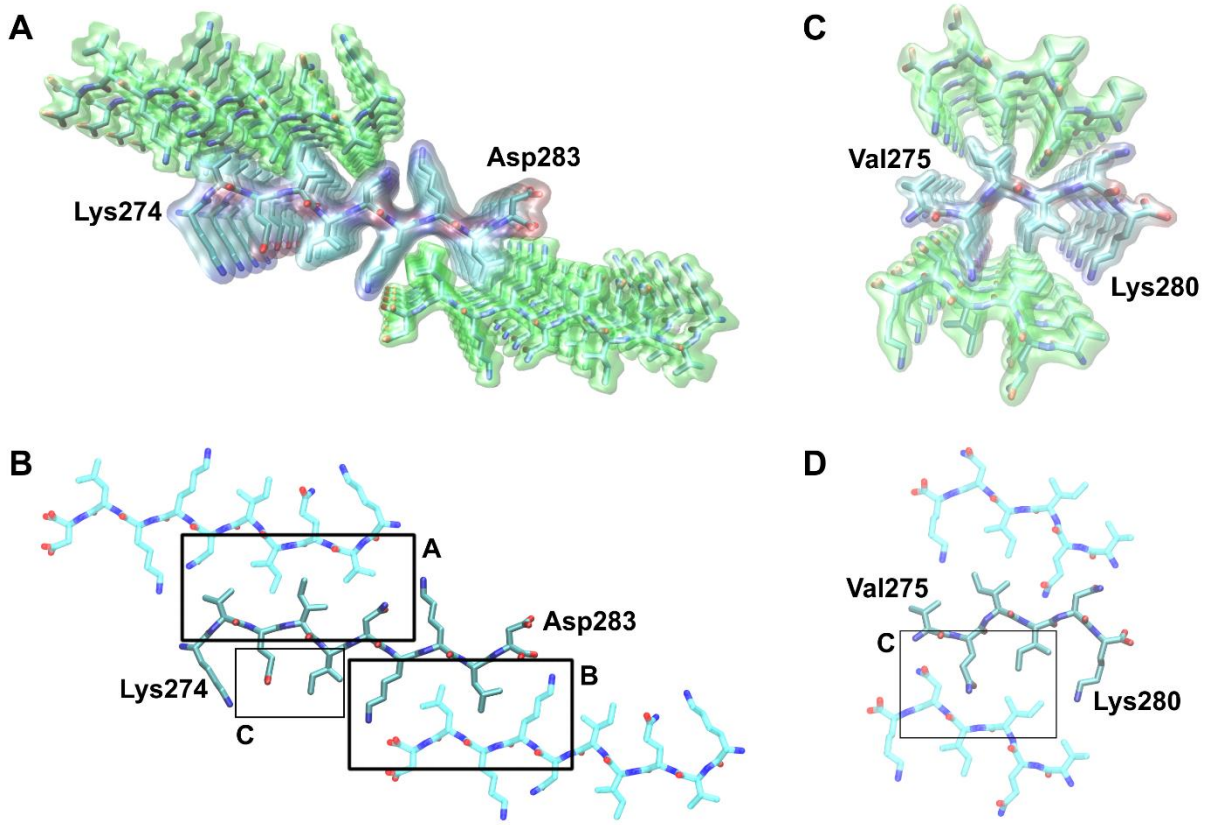

Figure S4: Structures of amyloid fibrils formed by the PHF6* fragment of tau

(A) View along the fibril axis of the polymorph 1 aggregate formed by tau residues Lys274-Asp283 (PDB code: 5V5B). (B) Detailed view of one fibril layer of polymorph 1 indicating the two interfaces (A, B) involved in aggregate formation. The thin box highlights the residues involved in the formation of interface C in polymorph 2. (C) View along the fibril axis of the polymorph 2 aggregate formed by tau residues Val275-Lys280 (PDB code: 5V5C). (D) Detailed view of one fibril layer of polymorph 2 indicating interface C involved in aggregate formation.

S5

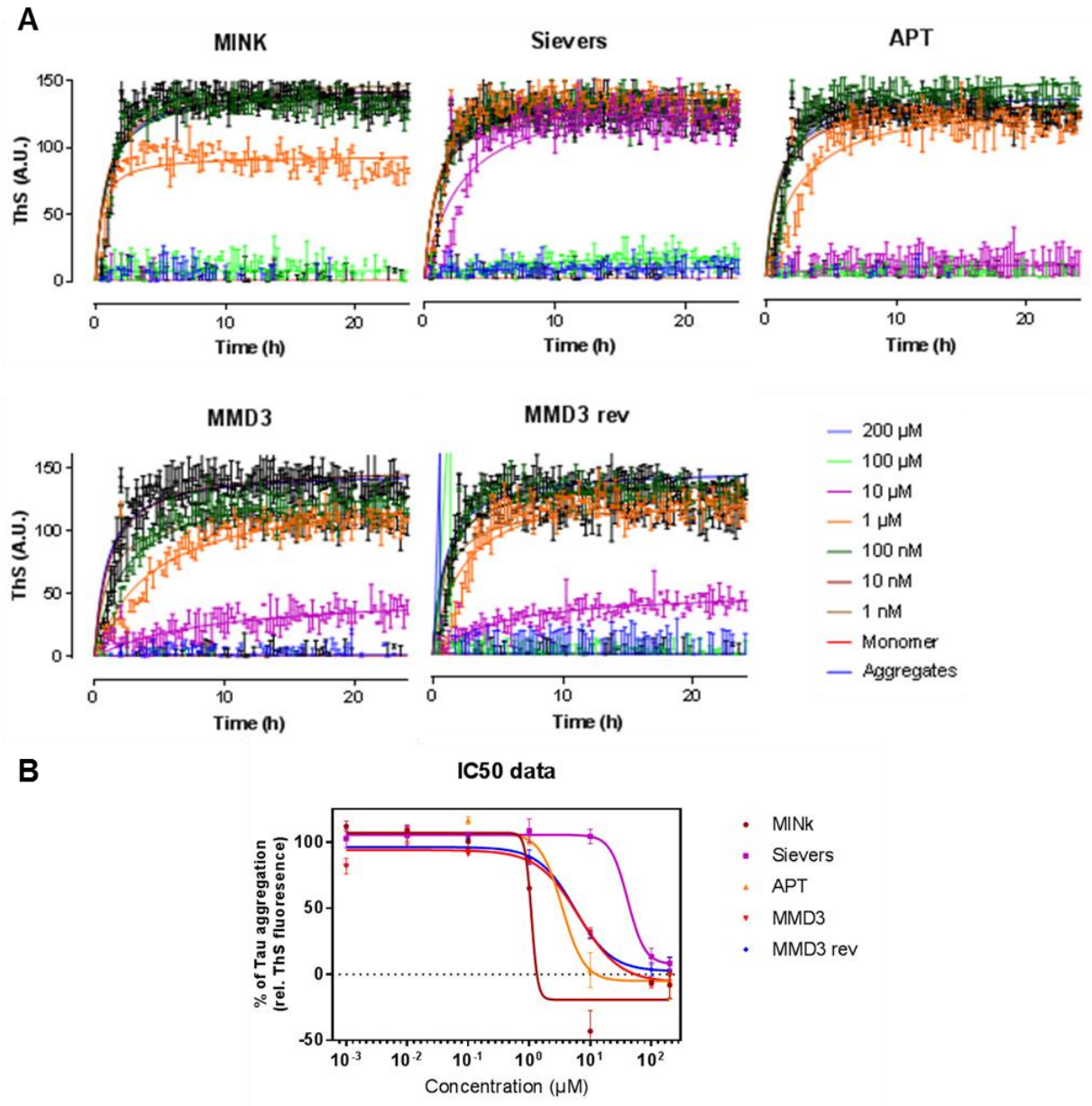

Figure S5: Inhibition of the aggregation of Tau$^{RD\Delta K}$ determined by ThS assay

(A) Aggregation of Tau$^{RD\Delta K}$ in the presence of different peptides over the period of 24 hours. Different peptides inhibit the aggregation at different rates. The representative plots are shown in A for the respective peptides. Except for the peptide described by Sievers and colleagues [7], all other peptides were able to reduce the aggregation from ~1 µM concentrations. **(B)** The ThS-fluorescence (as indicator for the extent of Tau aggregation) after 24h incubation in the presence of peptides was plotted as percentage of the untreated control to find out the IC50 values.

References

[1] L. R. Margittai M, *Proc Natl Acad Sci USA* **2004**, *101*, 10278–10283.

[2] V. G. KrishnaKumar, S. Gupta, *Preparative biochemistry & biotechnology* **2017**, *47*, 530–538.

[3] T. Ukmar-Godec, P. Fang, A. Ibáñez de Opakua, F. Henneberg, A. Godec, K.-T. Pan, M.-S. Cima-Omori, A. Chari, E. Mandelkow, H. Urlaub et al., *Science advances* **2020**, *6*, eaba3916.

[4] P. Schanda, E. Kupce, B. Brutscher, *Journal of biomolecular NMR* **2005**, *33*, 199–211.

[5] W. Lee, M. Tonelli, J. L. Markley, *Bioinformatics* **2014**, *31*, 1325–1327.

[6] P. M. Seidler, D. R. Boyer, J. A. Rodriguez, M. R. Sawaya, D. Cascio, K. Murray, T. Gonen, D. S. Eisenberg, *Nature chemistry* **2018**, *10*, 170–176.

[7] S. A. Sievers, J. Karanicolas, H. W. Chang, A. Zhao, L. Jiang, O. Zirafi, J. T. Stevens, J. Münch, D. Baker, D. Eisenberg, *Nature* **2011**, *475*, 96–100.

[8] C. Dammers, D. Yolcu, L. Kukuk, D. Willbold, M. Pickhardt, E. Mandelkow, A. H. C. Horn, H. Sticht, M. N. Malhis, N. Will et al., *PloS one* **2016**, *11*, e0167432.

[9] Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera., *J Comput Chem* **2004**, *25*, 1605–1612.

[10] Humphrey W, Dalke A, Schulten K, *J Mol Graph.* **1996**, *14*, 33–38.