Supporting Information

Residue-based propensity of aggregation in Tau amyloidogenic hexapeptides AcPHF6* and AcPHF6

Abha Dangi¹,²,³ Abhishek Ankur Balmik³,⁴, Archana Ghorpade¹,², Nalini Vijay Gorantla³,⁴, Shweta Kishor Sonawane³,⁴, Subashchandrabose Chinnathambi³,⁴*, Udaya Kiran Marelli¹,²,³*

¹Central NMR Facility and ²Division of Organic Chemistry, CSIR-National Chemical Laboratory, Dr.Homi Bhabha Road, 411008 Pune, India
³Academy of Scientific and Innovative Research (AcSIR), 110025 New Delhi, India
⁴Neurobiology Group, Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Dr.Homi Bhabha Road, 411008 Pune, India

Keywords: Alzheimer's, Tau peptides, Tau aggregation, Ac-PHF6*, AcPHF6, TEM

*Corresponding authors:

Udaya Kiran Marelli, Ph.D.; Tel. +91-20-25902563; Email: m.udayakiran@ncl.res.in

Subashchandrabose Chinnathambi, Ph.D.; Tel. +91-20-25902232, Fax. +91-20-25902648; Email: s.chinnathambi@ncl.res.in
Experiments and Methods

Chemicals and Reagents

All the amino acids required for solid phase peptide synthesis, peptide coupling reagents (HCTU, HOAt) and rink amide resin were purchased from GL Biochem (Shanghai) Ltd. DIPEA, diethyl ether and TIPS were obtained from Spectrochem. Piperidine and NMP were procured from Avra Laboratories. Dry DCM was purchased from Acros Organics. Pyridine was obtained from Finar. HPLC gradient grade acetonitrile was obtained from Thomas Baker. BES, ThS and ANS were purchased from Sigma. Heparin, NaCl and NaN3 were purchased from MP Biomedicals. PIC was purchased from Calbiochem. Carbon coated copper grids of 400 mesh size were purchased from Ted Pella Inc. Cell culture media and reagents like DMEM, FBS, PBS and Penstrep were purchased from Invitrogen.

Synthesis of peptides by solid phase peptide synthesis (SPPS):

All the peptides were synthesized by Fmoc based solid phase peptide synthesis using the rink amide resin. For the synthesis of each peptide, 150 mg resin was swollen in 5 ml of dry DCM for 1 hour, followed by the Fmoc deprotection of the resin using 20% piperidine in DMF. After Fmoc deprotection, the first amino acid was loaded onto the resin using 2 eq. of HCTU, 4 eq. of DIPEA in 5 ml of NMP for 2 hrs. Fully loaded resin was then subjected for capping using acetic anhydride and pyridine in 3:2 ratio. From this stage, elongation of the peptide chain was carried out by repeated Fmoc deprotection (using 20 % piperidine in DMF) and coupling of the amino acids (using 2 eq. of Fmoc protected amino acid, 2 eq. of HCTU, 4 eq. of DIPEA in 5 ml of NMP for 2 hrs) up to the desired length of the peptide. After the coupling of the final amino acid and deprotection of its Fmoc group, acetylation of the terminal free amine was carried out using acetic anhydride and pyridine. The final linear peptide was cleaved from the resin in three cycles using 5 ml of cleaving cocktail TFA: TIPS: H2O (9 ml: 0.5 ml: 0.5 ml) each time for 1 hr. The cleaved contents were solvent dried by solvent evaporation on rotary evaporator and crude peptide was precipitated in cold diethylether, which was taken forward for reverse phase HPLC purification.

Far-UV CD Spectroscopy

CD spectra were acquired for all peptides in a cuvette with a path length of 1 mm under nitrogen atmosphere at 25 °C on Jasco J-815 CD spectrometer. For CD, peptides were dissolved in 5 mM MOPS buffer at a concentration of 1 mg/ml. The scans were carried out at a scan speed 100 nm/min and bandwidth of 1 nm with a scan range 190 to 250 nm. The final spectra were taken as an average of 5 acquisitions. The buffer baseline was set with 5 mM MOPS buffer at pH 7.2 and subtracted from the measured spectra for each sample. The final subtracted values were plotted using SigmaPlot 10.0 (Systat software).

NMR Spectroscopy

For NMR experiments, a solution of 5 mM concentration of each compound dissolved in DMSO-\textit{d}_6 was used. \textit{^1}H NMR experiments for all the compounds were recorded at 298 K on
Bruker AvanceIII HD 700 MHz NMR spectrometer equipped with a BBO probe. Variable temperature experiments for calculating the amide NH temperature co-efficients were carried out on Bruker Avance II 400 MHz NMR spectrometer equipped with BBFO probe. A temperature range of 295 K to 325 K with 5 K increments was used.

TEM Analysis

2 μM of peptides were applied on 400 mesh carbon coated copper grids for 1 minute followed by washing twice with filtered miliQ water for 30 seconds each and negative staining with 2% uranyl acetate for 1.5 minutes. The grids were dried at least 24 hours before scanning by TECNAI T20 Transmission Electron Microscope at 120 KV.

ThS Fluorescence Assay

100 μM peptides were resuspended in the assembly buffer composed of 20 mM BES at pH 7.4, 25 μM heparin, 25 mMNaCl, 0.01% NaN₃ and 20 μL PIC for aggregation. This preparation was incubated at 37°C and the fluorescence was measured at every 24 hours. The peptides were diluted to 5 μM with 20 μM of ThS and incubated in the dark for 10 minutes. ThS fluorescence of 20 mM BES was taken as blank. The ThS fluorescence was measured in duplicate for all samples and blank at 521 nm by exciting the fluorophore at 440 nm in Tecan Infinite Series Pro200 spectrofluorimeter.

ANS Fluorescence Assay

5 μM of peptides set-up for aggregation were incubated with 100 μM of ANS in the dark for 10 minutes. The ANS fluorescence was measured with excitation and emission wavelength of 390 and 475 nm respectively in Tecan Infinite Series Pro200 spectrofluorimeter. All readings were taken in duplicate and the fluorescence of 20 mM BES buffer was subtracted from each sample.

Cell viability assay

The cell viability assay was performed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Neuro2a cells were seeded in 96 well plate at the density of 1X10⁴ cells/well and incubated for 24 hours. The aggregated peptides from the 168 hours incubated reaction mixture were added to the seeded cells in the serum starved media for 24 hours. Further, the cells were incubated with 0.5 mg/ml MTT reagent for 4 hours. The formazan crystals formed with dissolved in DMSO, which gave a purple colour to the solution. This absorbance was read in Tecan Infinite Series Pro200 spectrofluorimeter at 570 nm in triplicates and the blank subtraction was made from absorbance by media incubated with MTT reagent in the similar way. The background subtraction was made with the readings at 660 nm.

Statistical Analysis

Experimental analysis of the studies carried out in duplicates (n=2) were tested for significance by two tailed student unpaired t-test in SigmaPlot 10.0 (Systatsoftwares).
Statistical significance was represented as ns - non-significant; * - P ≤ 0.05; ** - P ≤ 0.01; *** - P ≤ 0.001.

**HPLC Purification of peptides (1-6 and 1’-6’):**

All the peptides were purified by reverse phase HPLC on Waters 2545 Quaternary Gradient Module with 2489 UV/Visible Detector using YMC Pack ODS-A-HG C18 column of pore size S-10µm, 12nm, 250 mm * 20 mmI.D. A flow rate of 8ml/min was used for the purification.

| S. No. | Peptide         | HPLC Method                        | Retention Time |
|--------|-----------------|------------------------------------|----------------|
| 1      | VQINK(1)        | 40%ACN :60% water (run time: 10min)| 2.7min         |
| 2      | AQIINK(2)       | 40%ACN :60% water (run time: 15min)| 3.0min         |
| 3      | VAIINK(3)       | 40%ACN :60% water (run time: 15min)| 3.1min         |
| 4      | VQAINK(4)       | 50%ACN :50% water (run time: 15min)| 2.5min         |
| 5      | VQIANK(5)       | 50%ACN :50% water (run time: 12min)| 2.6min         |
| 6      | VQIIAK(6)       | 30%ACN :70% water (run time: 15min)| 2.3 min        |
| 7      | VQIVYK(1’)      | 30%ACN :70% water (run time: 15min)| 6.4min         |
| 8      | AQIVYK(2’)      | 30%ACN :70% water (run time: 15min)| 7.6min         |
| 9      | VAIYK(3’)       | 40%ACN :60% water (run time: 15min)| 6.3min         |
| 10     | VQAVYK(4’)      | 40%ACN :60% water (run time: 15min)| 8.3min         |
| 11     | VQIAYK(5’)      | 40%ACN :60% water (run time: 15min)| 7.2min         |
| 12     | VQIVAK(6’)      | 45%ACN :55% water (run time: 10min)| 5.3min         |
Analytical HPLC of peptides (1-6 and 1'-6'):

Purity by analytical HPLC of all the peptides was determined using YMC Pack ODS-A-HG C18 column of pore size S-10µm, 12nm, 250 mm * 4.6 mm I.D. on Waters 2545 Quaternary Gradient Module with 2489 UV/Visible Detector. A flow rate of 1ml/min was used for the analytical run.

| Sr. No | Peptide | HPLC Gradient | Retention Time |
|--------|---------|---------------|----------------|
| 1      | VQIINK (1) | 10 To 90% in 15 min | 3.4 min |
| 2      | AQIINK (2) | 10 To 90% in 15 min | 3.69 min |
| 3      | VAIINK (3) | 10 To 90% in 15 min | 2.59 min |
| 4      | VQAINK (4) | 10 To 90% in 15 min | 3.1 min |
| 5      | VQIANK (5) | 10 To 90% in 15 min | 2.69 min |
| 6      | VQIIAK (6) | 10 To 90% in 15 min | 3.6 min |
| 7      | VQIVYK (1') | 10 To 90% in 15 min | 2.19 min |
| 8      | AQIVYK (2') | 10 To 90% in 15 min | 2.45 min |
| 9      | VAIVYK (3') | 10 To 90% in 15 min | 2.36 min |
| 10     | VQAVYK (4') | 10 To 90% in 15 min | 3.2 min |
| 11     | VQIAKYK (5') | 10 To 90% in 15 min | 2.78 min |
| 12     | VQIVAK (6') | 10 To 90% in 15 min | 2.60 min |
Supporting Table 3: HPLC chromatograms of the peptides (1-6 and 1'-6')

| S. No. | Peptide  | HPLC Chromatogram          |
|--------|----------|----------------------------|
| 1      | VQIINK   | ![HPLC Chromatogram](image1) |
|        | (1)      |                            |
| 2      | AQIINK   | ![HPLC Chromatogram](image2) |
|        | (2)      |                            |
| 3      | VAIINK   | ![HPLC Chromatogram](image3) |
|        | (3)      |                            |
| 4      | VQAINK   | ![HPLC Chromatogram](image4) |
|        | (4)      |                            |
| 5      | VQIANK   | ![HPLC Chromatogram](image5) |
|        | (5)      |                            |
| 6      | VQIIAK   | ![HPLC Chromatogram](image6) |
|        | (6)      |                            |
| 7 | VQIVYK (1') |
|---|--------------|
| 8 | AQIVYK (2') |
| 9 | VAIYVK (3') |
| 10 | VQAVYK (4') |
| 11 | VQIAYK (5') |
| 12 | VQIVAK (6') |
**Supporting Table 4: HRMS data of peptides (1-6 and 1’-6’)**

| S. No. | Peptide  | Exact Mass | HRMS (M+1) | HRMS Plot |
|--------|----------|------------|------------|-----------|
| 1      | VQIINK   | 754        | 755        | ![HRMS Plot 1](image1) |
|        | (1)      |            |            |           |
| 2      | AQIINK   | 726        | 727        | ![HRMS Plot 2](image2) |
|        | (2)      |            |            |           |
| 3      | VAIINK   | 697        | 698        | ![HRMS Plot 3](image3) |
|        | (3)      |            |            |           |
| 4 | VQIAK (4) | 712 | 713 |
|---|-----------|-----|-----|

| 5 | VQIAK (5) | 712 | 713 |
|---|-----------|-----|-----|

| 6 | VQIAK (6) | 711 | 712 |
|---|-----------|-----|-----|
**Supporting Table 5**: Concentration of peptides 1-6 and 1'-6' used for CD spectroscopic studies.

| Peptide     | Concentration (in mg/ml) | Concentration (in mM) |
|-------------|--------------------------|-----------------------|
| VQIINK (1)  | 1 mg/ml                  | 0.755                 |
| AQIINK (2)  | 1 mg/ml                  | 0.726                 |
| VAIINK (3)  | 1 mg/ml                  | 0.697                 |
| VQAINK (4)  | 1 mg/ml                  | 0.712                 |
| VQIANK (5)  | 1 mg/ml                  | 0.712                 |
| VQIIAK (6)  | 1 mg/ml                  | 0.711                 |
| VQIVYK (1’) | 1 mg/ml                  | 0.790                 |
| AQIVYK (2’) | 1 mg/ml                  | 0.761                 |
| VAIYK (3’)  | 1 mg/ml                  | 0.732                 |
| VQAVYK (4’) | 1 mg/ml                  | 0.747                 |
| VQIAYK (5’) | 1 mg/ml                  | 0.761                 |
| VQIVAK (6’) | 1 mg/ml                  | 0.698                 |
Supporting Table 6: Temperature Co-efficients of amide protons in peptides 1-6 and 1’-6’

The temperature dependence of amide proton resonances was calculated from 1D 1H-NMR spectra acquired in DMSO-$d_6$ from 300K to 325 K at 5K intervals on Bruker Avance 400 MHz NMR spectrometer.

Temperature co-efficient = \((\Delta \delta / \Delta T) \times 1000\)

| S. No | Peptide       | NH1    | NH2    | NH3    | NH4    | NH5    | NH6    |
|-------|---------------|--------|--------|--------|--------|--------|--------|
| 1     | VQIINK (1)    | 8.105  | 8.096  | 7.955  | 7.883  | 7.803  | 7.772  |
|       |               | (-4.0) | (-5.6) | (-6.4) | (-4.8) | (-4.4) | (-4.4) |
| 2     | AQIINK(2)     | 8.115  | 8.105  | 8.093  | 7.939  | 7.666  | 7.664  |
|       |               | (-3.6) | (-5.2) | (-4.8) | (-6.4) | (-3.2) | (-3.2) |
| 3     | VAIINK(3)     | 8.107  | 8.107  | 7.989  | 7.906  | 7.813  | 7.770  |
|       |               | (-0.6) | (-6.4) | (-7.6) | (-6.0) | (-5.6) | (-5.2) |
| 4     | VQAINK(4)     | 8.116  | 8.086  | 7.92   | 7.913  | 7.897  | 7.788  |
|       |               | (-5.2) | (-4.0) | (-4.0) | (-5.2) | (-5.2) | (-4.4) |
| 5     | VQIANK(5)     | 8.113  | 8.033  | 8.033  | 7.896  | 7.896  | 7.719  |
|       |               | (-4.0) | (-4.8) | (-5.2) | (-4.8) | (-5.6) | (-4.0) |
| 6     | VQIAK(6)      | 8.135  | 8.010  | 7.924  | 7.790  | 7.770  | 7.750  |
|       |               | (-4.0) | (-6.0) | (-4.8) | (-4.8) | (-4.4) | (-5.2) |
| 7     | VQIVYK (1’)   | 8.109  | 7.944  | 7.902  | 7.891  | 7.785  | 7.733  |
|       |               | (-3.6) | (-5.2) | (-4.8) | (-6.0) | (-4.4) | (-4.8) |
| 8     | AQIVYK (2’)   | 8.105  | 8.078  | 7.918  | 7.865  | 7.722  | 7.697  |
|       |               | (-3.2) | (-4.0) | (-5.2) | (-6.0) | (-4.4) | (-4.0) |
| 9     | VAIVYK(3’)    | 8.068  | 7.894  | 7.883  | 7.865  | 7.767  | 7.676  |
|       |               | (-4.8) | (-4.8) | (-5.6) | (-4.8) | (-4.4) | (-4.4) |
| 10    | VQAVYK(4’)    | 8.074  | 7.893  | 7.893  | 7.880  | 7.861  | 7.706  |
|       |               | (-3.6) | (-3.6) | (-4.4) | (-4.4) | (-5.6) | (-4.0) |
| 11    | VQIAYK(5’)    | 8.121  | 7.991  | 7.908  | 7.830  | 7.830  | 7.689  |
|       |               | (-3.6) | (-4.8) | (-4.4) | (-4.8) | (-5.2) | (-4.2) |
| 12    | VQIVAK(6’)    | 8.112  | 8.000  | 7.892  | 7.799  | 7.764  | 7.742  |
|       |               | (-4.0) | (-4.8) | (-4.4) | (-4.4) | (-5.2) | (-4.4) |
**NMR spectroscopic data:** NMR spectroscopic data for all the peptides was obtained either on Bruker Avance 500 MHz spectrometer equipped with a BBO probe or Bruker Avance 400 MHz spectrometer equipped with a BBFO probe.

The $^1$H NMR data for all the peptides was obtained both in DMSO-$d_6$ and phosphate buffer at 298 K. The data in phosphate buffer was recorded with NMR water suppression method.

A general assignment of the region wise $^1$H NMR chemical shifts for these peptides in all the given NMR spectra is

- 6.5 – 8.5 ppm: NH and aromatic proton chemical shifts
- 3.5 – 5.0 ppm: $H\alpha$ protons chemical shifts
- 0.5 – 3.0 ppm: $H\beta$, $H\gamma$, $H\delta$, $H\epsilon$ and other protons

Akin to that in DMSO-$d_6$, none of the peptides showed any dispersed amide or $H\alpha$ chemical shifts implying an absence of a well ordered secondary structure.
Supporting Figure 1: $^1$H NMR of 1 (Ac-VQIINK-NH$_2$) on 500 MHz at 298 K in DMSO-$d_6$.

Supporting Figure 2: $^1$H NMR of 2 (Ac-AQIINK-NH$_2$) on 500 MHz at 298 K in DMSO-$d_6$. 
Supporting Figure 3: $^1$H NMR of 3 (Ac-VAIINK-NH$_2$) on 500 MHz at 298 K in DMSO-$d_6$.

Supporting Figure 4: $^1$H NMR of 4 (Ac-VQAINK-NH$_2$) on 500 MHz at 298 K in DMSO-$d_6$. 
Supporting Figure 5: $^1$H NMR of 5(Ac-VQIANK-NH$_2$) on 500 MHz at 298 K in DMSO-$d_6$.

Supporting Figure 6: $^1$H NMR of 6(Ac-VQIIAK-NH$_2$) on 500 MHz at 298 K in DMSO-$d_6$. 
Supporting Figure 7: $^1$H NMR of $1'(\text{Ac-VQIVYK-NH}_2)$ on 500 MHz at 298 K in DMSO-$d_6$.

Supporting Figure 8: $^1$H NMR of $2'(\text{Ac-AQIVYK-NH}_2)$ on 500 MHz at 298 K in DMSO-$d_6$. 
Supporting Figure 9: $^1$H NMR of 3′(Ac-VAIVYK-NH$_2$) on 500 MHz at 298 K in DMSO-$d_6$.

Supporting Figure 10: $^1$H NMR of 4′(Ac-VQAVYK-NH$_2$) on 500 MHz at 298 K in DMSO-$d_6$. 
Supporting Figure 11: $^1$H NMR of 5'(Ac-VQIAYK-NH$_2$) on 500 MHz at 298 K in DMSO-$d_6$.

Supporting Figure 12: $^1$H NMR of 6'(Ac-VQIVAK-NH$_2$) on 500 MHz at 298 K in DMSO-$d_6$. 
Supporting Figure 13: $^1$H NMR of 1 (Ac-VQIINK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.

Supporting Figure 14: $^1$H NMR of 2 (Ac-AQIINK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.
Supporting Figure 15: $^1$H NMR of 3 (Ac-VAINK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.

Supporting Figure 16: $^1$H NMR of 4(Ac-VQAINK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.
Supporting Figure 17: $^1$H NMR of 5(Ac-VQIAK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.

Supporting Figure 18: $^1$H NMR of 6 (Ac-VQIAK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.
Supporting Figure 19: $^1$H NMR of 1'(Ac-VQIVYK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.

Supporting Figure 20: $^1$H NMR of 2'(Ac-AQIVYK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.
Supporting Figure 21: $^1$H NMR of 3'(Ac-VAIVYK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.

Supporting Figure 22: $^1$H NMR of 4'(Ac-VQAVYK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.
**Supporting Figure 23:** $^1$H NMR of 5'(Ac-VQAYK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.

**Supporting Figure 24:** $^1$H NMR of 6'(Ac-VQIVAK-NH$_2$) on 500 MHz at 298 K in Phosphate Buffer.
ThS and ANS assay results

Supporting Figure 25: A) Aggregation of peptides 1-6 monitored by using ThS fluorescence shows the effect of alanine incorporated at various positions in VQIINK hexapeptide. VQIINK aggregation was found to be affected by substitution of Glu or Asn residues where these substitutions enhanced the aggregation propensity. B) Peptides 1'-6' showed similar trend in their aggregation propensity as peptide 3' with glutamine to alanine substitution showed increased aggregation as compared to the other peptides in the group. C) Maximum fluorescence at the end-point (168 hours) of assay showed increased aggregation of peptides 3 and 6 while peptides 2, 4 and 5 showed no aggregates formation. Aggregate formation was observed for peptide 1 even though the ThS fluorescence suggested otherwise as lower end-point ThS value of 30 a.u. was obtained for peptide 1. D) Absolute fluorescence values at the end-point for peptide 1' and 3' were obtained as 48 and 100 a.u., respectively while minimal or no aggregation was observed for other variants. E, F) ANS fluorescence assay was carried out to monitor the change in hydrophobicity of peptides 1-6 and 1'-6'. There was minimal
hydrophobic change in synthetic peptides as compared to peptides 1-6. ANS fluorescence assay for peptides 1'-6' showed that hydrophobicity of 1' and 3' increases upon aggregation as compared to other variants. (Statistical significance determined by comparison with parent peptides 1 and 1’ by unpaired student t-test and represented as: ns - non-significant; * - P \leq 0.05; ** - P \leq 0.01; *** - P \leq 0.001).

Relative toxicity of 1-6 and 1'-6' hexapeptides on neuro2a cells: Formation of Tau aggregates and its deposition in neuronal cells in the form of NFTs is known to be toxic and cause neurodegeneration. Thus, we carried out cell viability assay on neuro2a cells using the end product of peptide aggregation assay to explore whether alanine substitution imparts toxicity to the peptides. The aggregated 1-6 and 1'-6' peptides at 168 hours were studied for their toxicity in neuro2a cells (Supporting Fig. 13). The cells were treated with 1-10 μM of the aggregated peptides for 24 hours. The aggregated 1-6 peptides did not show toxicity to neuro2a cells up to 5 μM concentration. The highest concentration of 10 μM for all aggregated 1-6 peptides also showed 60% viability as compared to untreated control (Supporting Fig. 13A). The cell viability assay suggested that the concentration of 0.1 and 1 μM for all 1'-6'peptides were not toxic to neuro2a cells. The concentrations of 2-10 μM of peptides 1'-6' showed reduced viability as compared to untreated control. A relatively better tolerance by cells is observed for 6' aggregated peptide that resulted in 80% viability at 10 μM concentration whereas the other peptides showed less than 50% viability at the same concentration (Supporting Fig. 13B).

Supporting Figure 26: Cell viability assays for aggregated peptides 1-6 and 1'-6'. The aggregation assay end-point samples were tested for its effect on cell viability of neuro2a cells. A) The viability assay for aggregated peptides 1-6 does not show toxicity to neuro2a cells. B) The viability assay for aggregated peptides 1'-6' showed reduction in viability with increasing concentration in comparison to peptides 1-6. Cell viability was found to be sustained for 6' peptide.