Structure-based design of non-natural amino-acid inhibitors of amyloid fibril formation

Stuart A. Sievers1*, John Karanicolas2,3*, Howard W. Chang1*, Anni Zhao1*, Lin Jiang1*, Onofrio Zirafi4, Jason T. Stevens3, Jan Münch5, David Baker2 & David Eisenberg1

Many globular and natively disordered proteins can convert into amyloid fibrils. These fibrils are associated with numerous pathologies as well as with normal cellular functions, and frequently form during protein denaturation. Inhibitors of pathological amyloid fibril formation could be useful in the development of therapeutics, provided that the inhibitors were specific enough to avoid interfering with normal processes. Here we show that computer-aided, structure-based design can yield highly specific peptide inhibitors of amyloid formation. Using known atomic structures of segments of amyloid fibrils as templates, we have designed and characterized an all-D-amino-acid inhibitor of the fibril formation of the tau protein associated with Alzheimer’s disease, and a non-natural L-amino-acid inhibitor of an amyloid fibril that enhances sexual transmission of human immunodeficiency virus. Our results indicate that peptides from structure-based designs can disrupt the fibril formation of full-length proteins, including those, such as tau protein, that lack fully ordered native structures. Because the inhibiting peptides have been designed on structures of dual-β-sheet ‘steric zippers’, the successful inhibition of amyloid fibril formation strengthens the hypothesis that amyloid spines contain steric zippers.

The finding that dozens of pathologies, including Alzheimer’s disease, are associated with amyloid fibrils has stimulated research on fibril inhibition. One approach uses the self-associating property of proteins, including those, such as tau protein, that lack fully ordered native structures. Because the inhibiting peptides have been designed on structures of dual-β-sheet ‘steric zippers’, the successful inhibition of amyloid fibril formation strengthens the hypothesis that amyloid spines contain steric zippers.

These structures reveal a common motif called a steric zipper, in which a pair of β-sheets is held together by the interdigitation of their side chains. Using as templates the steric-zipper structures formed by segments of two pathological proteins, we have designed inhibitors that cap fibril ends. As we show, the inhibitors greatly slow the fibril formation of the parent proteins of the segments, offering a route to designed chemical interventions and supporting the hypothesis that steric zippers are the principal structural elements of these fibrils.

One of the two fibril-like steric zippers that we have chosen as a target for inhibitor design is the hexapeptide VQIVYK, residues 306–311 of the tau protein, which forms intracellular amyloid fibrils in Alzheimer’s disease. This segment has been shown to be important for fibril formation of the full-length protein and itself forms fibrils with biophysical properties similar to full-length tau fibrils. Our second template for inhibitor design, identified by the ‘3D profile’ algorithm, is the steric-zipper structure of the peptide segment GGVLVN from the amyloid fibril formed by 248PAP286, a proteolytic fragment containing residues 248–286 of prostatic acid phosphatase, a protein abundant in semen. 248PAP286 fibrils, also known as semen-derived enhancer of virus infection (SEVI), enhance human immunodeficiency virus (HIV) infection by orders of magnitude in cell culture studies, whereas the monomeric peptide is inactive.

Our computational approach to designing non-natural peptides that inhibit fibril formation is summarized in Fig. 1 for the VQIVYK segment of tau protein; the same general strategy is used for the GGVLVN segment of 248PAP286. In both systems, we design a tight interface between the inhibiting peptide and the end of the steric zipper to block additional segments from joining the fibril. By sampling 1 or 14 amino acids, or commercially available non-natural amino acids, we can design candidate inhibitors with side chains that maximize hydrogen bonding and hydrophobic interactions across the interface.

We propose that the steric-zipper structures of the VQIVYK and GGVLVN segments represent the spines of the fibrils formed by the parent proteins containing these segments. Supporting our hypothesis are our results that D-amino-acid inhibitors designed on the VQIVYK steric-zipper template inhibit fibril formation not only of the VQIVYK segment, but also of two tau constructs, K12 and K19–24 (Fig. 2a). Similarly, the peptide composed of non-natural amino acids designed on the GGVLVN template inhibits the fibril formation of 248PAP286 and greatly inhibits the HIV infectivity of human cells in culture.

To design a D-amino-acid hexapeptide sequence that interacts favourably with the VQIVYK steric zipper, and prevents further addition of tau molecules to the fibril, we used the Rosetta software. This led to the identification of four D-amino-acid peptides: D-TLKIVW, D-TWKLVL, D-DYYFEF and D-YVIIER, in which the prefix signifies that all α-carbon atoms are in the D configuration (Fig. 2b, c, Supplementary Figs 1 and 2 and Supplementary Table 1). In the D-TLKVW design model (Fig. 2b, c and Supplementary Fig. 1), the inhibitor packs tightly across the top of the VQIVYK steric-zipper structure, maintaining all main-chain hydrogen bonds. The side-chain hydrogen bonding between layers of stacked Gln 307 residues is replaced in the designed interface by an interaction with D-Lys 3. Several hydrophobic interactions between D-TLKVW and the two VQIVYK β-strands contribute to the favourable binding energy (Supplementary Table 1). In the design, the D-peptide blocks the addition of another layer of VQIVYK, both above the D-peptide and across on the mating β-sheet (Supplementary Fig. 3). D-Leu 2 of the designed inhibitor prevents the addition of a VQIVYK molecule above it through a steric clash with Ile 308 of VQIVYK and on the mating sheet through a clash with Val 306 and Ile 308 (Supplementary Fig. 3). These steric clashes involving D-Leu 2 are intended to block fibril growth.
We used fluorescence spectroscopy and electron microscopy to assess whether the designed D-peptides inhibit the fibril formation of the tau segment VQIVYK and of the tau constructs K12 and K19. Of our designed inhibitors, D-TLKVW is the most effective (Supplementary Fig. 4). Electron microscopy, performed after three days, verified that incubation with equimolar D-TLKVW prevents K19 fibril formation, which would otherwise have occurred within the elapsed time (Fig. 1, upper right). D-TLKVW delays fibril formation of VQIVYK, K12 and K19 even when present in sub-equimolar concentration (Supplementary Fig. 5). A fivefold molar excess of D-TLKVW delays K12 fibril formation for more than two weeks in some experimental replicates (Supplementary Fig. 5c, d). In tenfold molar excess, D-TLKVW prevents the fibril formation of K12 for more than 60 hours in the presence of preformed K12 fibril seeds, suggesting that the peptide interacts with fibrils (Fig. 2d). Also, kinetic analysis shows that the fibril elongation rate decreases in the presence of increasing concentrations of inhibitor peptide (Supplementary Fig. 6). The large increase in lag time in unseeded reactions may be due to interactions with small aggregates formed during the process of fibril formation.

To investigate the specificity of the designed inhibitor, we tested scrambled sequence variants of D-TLKVW that have poor (that is, with small aggregates formed during the process of fibril formation.)

Figure 1 | Design and characterization of peptide inhibitors of amyloid fibril formation. Tau constructs form fibrils in vitro (top left; scale bar, 200 nm). The VQIVYK segment in isolation forms fibrils and microcrystals (bottom left; fibril scale bar, 200 nm; microcrystal scale bar, 100 μm). The atomic structure of the fibril-like VQIVYK segment reveals a characteristic steric zipper motif comprising a pair of interacting β-sheets (purple and grey) running along the fibril axis (grey arrow) (bottom right). We designed a D-amino-acid peptide to bind to the end of the steric zipper template and prevent fibril elongation (middle right). The D-peptide (red) is designed to satisfy hydrogen bonds and make favourable non-polar interactions with the molecule below, while preventing the addition of other molecules above and on the opposite β-sheet. As shown in vitro, the designed D-peptide prevents the formation of fibrils when incubated with tau K19 (upper right; scale bar, 200 nm).

Figure 2 | Designed D-peptide delays tau K12 fibril formation in a sequence-specific manner. a, Tau construct composition. The longest human tau isoform found in the central nervous system, hTau40 (Uniprot ID, P10636-8), contains four microtubule-binding repeats, R1 to R4, whereas K12 and K19 lack R2. The black bars at the amino termini of R2 and R3 represent the fibrillogenic segments VQIINK and VQIVYK, respectively. b, The inhibitor D-TLKV (red) is designed to interact with atoms on both β-strands of the VQIVYK steric zipper (grey) primarily through hydrophobic packing and hydrogen-bonding interactions. c, The inhibitor interacts with the VQIVYK β-strand below. The transparent spheres show where the two molecules interact favourably. Black and red dashes indicate main-chain and side-chain hydrogen bonds, respectively. Stereo views of b and c are shown in Supplementary Fig. 1. d, The seeded fibril formation of 50 μM K12 in the presence and absence of a tenfold molar excess of peptide was monitored by Thioflavin S fluorescence. In the presence of the scrambled peptide D-TIWKVL (dark green) and alone (black), seeded K12 fibril formation occurs with almost no lag time. However, D-TLKVW prevents fibril formation for days (maroon). e, At equimolar concentrations, D-TLKVW (red) inhibits the fibril formation of 50 μM K12, D-TIKWVL (blue), with only three residues scrambled, shows weak inhibition. However, no inhibition is observed for either D-TIWKVL (green) or D-LKTWIV (cyan). f, The replacement of D-Leu 2, designed to clash with VQIVYK on the opposite sheet, with D-Ala eliminates the inhibition of fibril formation.
both to the inhibitor and, separately, to a scrambled hexapeptide, D-LKTWIV. We used a blind counting assay and found that, relative to Nanogold alone, D-TLKIVW shows a significant binding preference for the end of fibrils, in contrast to the scrambled control peptide, D-LKTWIV (Fig. 3a and Supplementary Fig. 10).

As a further test of the model, we used NMR to characterize the binding affinity of D-TLKIVW for tau fibrils. The 1H NMR spectra for D-TLKIVW were collected in the presence of increasing concentrations of VQIVYK or K19 fibrils. Because neither K19 nor VQIVK contains tryptophan, we were able to monitor the 1H resonance of the indole proton of the tryptophan in our inhibitor. When bound to a fibril, the inhibitor, D-TLKIVW, is removed from the soluble phase and the 1H resonance is diminished (Fig. 3b and Supplementary Fig. 11). As a control, we measured spectra for the non-inhibiting peptide D-LKTWIV present with D-TLKIVW in the same reaction mixture. As shown in Fig. 3b, the presence of VQIVYK fibrils at a given concentration reduces the D-TLKIVW indole resonance much more than it does the D-LKTWIV indole resonance. Spectra of the two peptides are shown in Supplementary Fig. 12. By monitoring the D-TLKIVW indole resonance over a range of VQIVYK fibril concentrations, we estimate the apparent dissociation constant of the interaction between D-TLKIVW and VQIVYK fibrils to be $\approx 2 \mu M$ (Supplementary Fig. 11a and Methods). This value corresponds to a standard free binding energy of $\approx -7.4 \text{ kcal mol}^{-1}$, with $\approx -2.5 \text{ kcal mol}^{-1}$ from non-polar interactions and $\approx -4.9 \text{ kcal mol}^{-1}$ from six hydrogen bonds (Methods). Repeating the NMR binding experiment with K19 fibrils yields a similar trend (Supplementary Fig. 11b). To determine whether D-TLKIVW has affinity for soluble VQIVYK, we measured 1H NMR spectra of D-TLKIVW and D-LKTWIV in the presence of increasing amounts of soluble VQIVYK. Only a slight change in the respective chemical shifts of the indole proton peaks of D-TLKIVW and D-LKTWIV is observed, even at a 70-fold molar excess of VQIVYK (Supplementary Fig. 13). This, together with the ability of the peptide to prevent seeded fibril formation, suggests that D-TLKIVW does not interact with monomers but rather with a structured, fibril-like species.

As another test of our design model, we replaced the D-Leu residue with D-Ala in D-TLKIVW. Our structural model suggests that D-Leu 2 of D-TLKIVW is important for preventing tau fibril formation because of its favourable interaction with the Ile residue of the VQIVYK molecule below and with Ile and the first Val of VQIVYK across the steric zipper (Fig. 2b, c and Supplementary Fig. 1). The D-Ala replacement eliminates these interactions and, furthermore, removes a steric clash that would occur were another VQIVYK molecule placed across from the inhibitor (Supplementary Fig. 3 and Supplementary Table 1). When the D-Ala variant is incubated with VQIVYK and the tau constructs, it has no inhibitory effect on fibril formation (Fig. 2f and Supplementary Fig. 14). This confirms that D-Leu 2 is critical for the efficacy of D-TLKIVW, consistent with our model.

In summary, although our electron microscopy, NMR and D-Ala replacement results support a model in which the designed peptide D-TLKIVW binds to the ends of tau fibrils, they do not constitute proof that the inhibitors bind exactly as anticipated in the designs (Supplementary Fig. 15).

To expand on our design methodology, we computationally designed an inhibitor of 248PAP286 fibril formation containing non-natural t-amino acids (Fig. 4b and Supplementary Fig. 16), using the GGVLVN structure as a template (Fig. 4a and Supplementary Table 2). This peptide, Trp-His-Lys-chAla-Trp-hydroxyTic (WW61), contains an Ala derivative, $\beta$-cyclohexyl-$\alpha$-alanine (chAla) and a Tyr/Pro derivative, 7-hydroxy-(S)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (hydroxyTic), both of which increase contact area with the GGVLVN template. The non-natural chAla forms hydrophobic interactions with the Leu residue in the steric-zipper interface, and hydroxyTic supports the favourable placement of chAla through hydrophobic packing (Fig. 4b and Supplementary Fig. 16b).
Moreover, we propose that the bulky side chains and steric constraints of hydroxyTic provide hindrance to further fibril growth.

This designed peptide, WW61, effectively delays both seeded and unseeded fibril formation of 248PAP286 in vitro (Fig. 4c and Supplementary Figs 17 and 18). In the presence of a twofold molar excess of this inhibitor, seeded fibril formation is efficiently blocked for more than two days (Fig. 4c). Furthermore, we see that increasing the concentration of this inhibitor extends the fibril formation lag time (Supplementary Fig. 19). These inhibition assay results were further confirmed by electron microscopy (Supplementary Fig. 20). As a control for specificity, we tested the effect of GIHKQK, from the amino terminus of 248PAP286 and PYKLN, a peptide with the same charge as WW61. Neither peptide affected fibril formation kinetics, indicating that the inhibitory activity of the designed peptide is sequence specific (Supplementary Fig. 21).

Because 248PAP286 fibrils (SEVI) have been shown to enhance HIV infection22, using a functional assay we investigated whether WW61 is able to prevent this enhancement. In this experiment, we treated HIV particles with 248PAP286 solutions that had been agitated for 20 hours (to allow fibril formation) in the presence or absence of WW61, and infected TZM-bl indicator cells. As has been previously observed, SEVI efficiently enhanced HIV infection22. However, 248PAP286 incubated with the designed inhibitor prevented HIV infection (Fig. 4d).

We performed several control experiments to verify that the lack of infectivity observed in the assay is indeed due to the inhibition of SEVI formation. First we confirmed that in the absence of SEVI the designed inhibitor WW61 does not affect HIV infectivity (Supplementary Fig. 22a). We also found that the control peptides GIHKQK and PYKLN, which do not inhibit 248PAP286 fibril formation, fail to decrease HIV infectivity (Supplementary Fig. 22b). Additionally, we observed that WW61 has no inhibitory effect on polylysine-mediated HIV infectivity27, further ruling out a non-specific electrostatic interaction mechanism (Supplementary Fig. 22a). Together, these results demonstrate that a peptide capable of preventing 248PAP286 fibril formation also inhibits the generation of virus-enhancing material.

Structure-based design of inhibitors of amyloid fibril formation has been challenging in the absence of detailed information about the atomic-level interactions that form the fibril spine. So far, one of the most successful structure-based approaches to preventing fibril formation has been to stabilize the native tetrameric structure of transthyretin28. That approach is well suited to the prevention of fibril formation of proteins with known native structures, but other proteins involved in amyloid-related diseases, such as tau protein, amyloid-β and 248PAP286, lack fully ordered native structures29. Our structure-based approach makes it possible to design inhibitors independent of native structure. Instead, the templates are atomic-level structures of short, fibril-forming segments14,15. By using these fibril-like templates, and adopting computational methods successful in designing novel proteins and protein–protein interfaces23,24, we have created specific inhibitors of proteins that normally form fibrils. These results support the hypothesis that the steric zipper is a principal feature of tau-related and SEVI fibrils, and suggest that, with current computational methods and steric-zipper structures, we have the tools to design specific inhibitors to prevent the formation of other amyloid fibrils.

METHODS SUMMARY

We used crystal structures of hexapeptide segments of VQIVYK and GGVLNV as templates to design peptide inhibitors using the Rosetta software25. Briefly, this algorithm searches possible side-chain conformations (called rotamers) of all amino acids in a peptide β-strand backbone stacked onto the fibril end of both segment structures. The Rosetta software is extended to sample the approximate 3D profile method for identifying fibril-forming protein interfaces25,30. We have created specific inhibitors of proteins related to diseases, such as tau protein, amyloid-β and 248PAP286, in vitro. J. Cell Biol. 271, 8545–8548 (1996).

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Author Contributions S.A.S., J.K., D.B., J.M. and D.E. designed the project. J.K. and S.A.S. created the design protocol. J.K. designed the D-peptides. L.J. expanded the design methodology and designed the non-natural amino-acid peptides. S.A.S., H.W.C. and A.Z. performed the fluorescence experiments and electron microscopy, and analysed kinetic data. A.Z. determined the structure of GGVLVN. O.Z. performed the HIV infectivity experiments. J.T.S. determined the tau fibril elongation rates. S.A.S. performed the NMR experiments. S.A.S., J.K. and D.E. wrote the manuscript and coordinated contributions by other authors.

Author Information Atomic coordinates and structure factors for the reported GGVLVN structure have been deposited in the Protein Data Bank with accession code 3PPD. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to D.E. (david@mbi.ucla.edu).
METHODS

Computational design. Computational designs were carried out using the Rosetta software37 (http://www.rosettaconsortium.org). This algorithm involves building side-chain rotamers of all amino acids onto a fixed protein backbone. The lowest energy set of side-chain rotamers is then identified as those which minimize an energy function containing a Lennard–Jones potential, an orientation-dependent hydrogen-bond potential, a solvation term, amino-acid-dependent reference energies and a statistical torsional potential that depends on the backbone and side-chain dihedral angles.

t-aminooic-acid tau inhibitors. The crystal structure of VQIVYK (ref. 15; Protein Data Bank ID, 2ON9) was used as a starting scaffold for computational design. To take full advantage of the statistical nature of the rotamer library and some terms in the Rosetta energy function, the stereochemistry of the fibril scaffold was inverted so that design would take place using t- amino acids. An extended t-peptide was aligned with the N, C and O backbone atoms of the t-fibril scaffold. This t-peptide was subsequently redesigned, keeping all atoms of the t-fibril fixed. The stereochemistry of the final design model was then inverted, yielding a t-peptide designed to cap an t-fibril. We inspected the finished models to confirm that inversion of the stereochemistry at the Thr and Ile Cβ atoms did not make the designs energetically unfavourable. Energetic consequences of incorporating a t inhibitor peptide in the middle of an t fibril were subsequently evaluated to ensure that fibril propagation could not continue after association of an inhibitor. Calculations of the area buried and shape complementarity were performed with AREAIMOL31 and SC32, respectively.

t-peptide 248PAP256 inhibitors. The crystal structure of GGVLVN (PDB ID, 3PDD) was used as a template for the following design procedure. An extended t-peptide was aligned according to crystal symmetry. Small, random perturbations of the t-peptide were performed to optimize the rigid-body arrangement between the fibril template and the peptide inhibitor. Full sequence optimization of the inhibitor was performed using the Rosetta software package, allowing residues directly contacting the inhibitor to repack; other scaffold residues remained fixed. Because the design calculations use a discrete rotamer representation of the side chains, we next performed simultaneous quasi-Newtonian optimization of the inhibitor rigid-body orientation, the side-chain torsion angles and, in some cases, the backbone torsion angles using the full-atom Rosetta energy function. This optimization was essential to the subsequent assessment of the inhibition of the design. Several iterative runs of small perturbations in inhibitor placement, interface design and refinement were performed to improve hydrogen-bonding and packing interactions. The designs that ranked highest on the basis of the total binding energy between the inhibitor and the fibril scaffold and the interfacial shape complementarity were subsequently synthesized and tested.

For each initial active t-peptide design, the non-natural t amino acids were incorporated using a growth strategy. Non-natural amino acids, structurally similar to those of initial active designs, were selected on the basis of their solubility, side-chain shape and commercial availability. Side-chain conformations were approximately sampled by adopting side-chain torsion angles from those in their natural counterparts. Sequence optimization of the inhibitor was performed and the optimal set of rotamers identified using Monte Carlo simulated annealing with the full-atom energy function described above. The resulting designs were ranked on the basis of the total binding energy between the inhibitor and the fibril scaffold.

Tau construct expression and purification. PNG2 expression vectors (derived from PET-3b39) containing either the K12 or K19 gene were provided by E. Mandelkow34. Expression in BL21(DE3) Escherichia coli was induced with 1 mM isopropyl thiogalactoside when the absorbance 0.600 nm was between 0.8 and 1.0, and cells were collected after 3–4 h. K12 and K19 were purified on the basis of previously described methods35. Cells were pelleted for 20 min at 4,700g and resuspended in 20 mM MES, pH 6.8, 1 mM EDTA, 0.2 mM MgCl2, 5 mM DTT, 1 mM PMSF and a protease inhibitor cocktail. The cells were sonicated for approximately 1 min of 25 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM EDTA, 1 mM MgCl2, 2 mM DTT and 0.1 mM PMSF at 4°C to remove large aggregates (final concentration, −1 mM). Filtered VQIVYK (20 μL) was added to each reaction well. ThS fluorescence was monitored at room temperature every 2 min using a SpectraMax M5 fluorometer with 2 s of mixing before each reading.

Amyloid-b fibril formation assay. Lyophilized amyloid-b(1–42) was diluted to 0.2 mg/ml in 50 mM NH4OH and filtered with a 0.2-μm filter. The reaction mixture contained a final concentration of 11.5 μM amyloid-b(1–42), 10 μM Thioflavin T (ThT), 23 mM NH4OH in 100 mM bicine, pH 9.1, and 11.5 μM t-TKIVW in reactions with peptide present. Reactions were split into four replicates and the ThT fluorescence signal was measured every minute (excitation wavelength, 440 nm; emission wavelength, 510 nm), at 37°C, with continuous shaking at 960 r.p.m. with a 1-mm diameter in a Varioskan fluorometer. Electron microscopy. Sample (5 μL) was applied to glow-discharged, 400-mesh carbon-coated, formvar films on copper grids (Ted Pella) for 3 min. Grids were rinsed twice with distilled water and stained with 1% uranyl acetate for 90 s. Grids were examined in a Hitachi H-7000 transmission electron microscope at 75 keV or a JEOL JEM1200-EX operating at 80 keV.

Tau fibril formation kinetic analysis. The nucleation (kn) and propagation (kp) rates were determined by fitting the form of the Fingl mechanism40. Plateau values were determined and the remaining parameters were fitted using the ‘leasqr’ nonlinear least-squares regression function (http://fly.isti.cnr.it/pub/software/octave/leasqr/) through the OCTAVE software package (http://www.gnu.org/software/octave/).

Preparation of peptide–gold conjugates. Peptide–Nanogold conjugates were prepared as described earlier for similarly sized peptides35. Briefly, 60 nmol of the peptides CGGG-(D)-TLKIVW and CGGG-(D)-LKTWIV (CS Bio) were dissolved in 110 mM NaOH and filtered with a 0.2-μm filter. The reaction mixture contained a final concentration of 11.5 μM amyloid-b(1–42), 10 μM Thioflavin T (ThT), 23 mM NH4OH in 100 mM bicine, pH 9.1, and 11.5 μM t-TKIVW in reactions with peptide present. Reactions were split into four replicates and the ThT fluorescence signal was measured every minute (excitation wavelength, 440 nm; emission wavelength, 510 nm), at 37°C, with continuous shaking at 960 r.p.m. with a 1-mm diameter in a Varioskan fluorometer.

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Preparation of samples for Nanogold binding experiments. Nanogold conjugated inhibitor (or control) (10 mM) was incubated with 1.67 μM K9 fibrils (by monomer) in MOPS buffer (25 mM, pH 7.2) for 1 h. We applied 5 μl of it to a glow-discharged, 400-mesh carbon-stabilized copper grid (Ted Pella) for 3 min. The grids were washed twice with H2O and 10 μl of the Goldenhance reagent was applied for 10 s. The grids were washed five times with H2O and negatively stained with 2% uranyl acetate.

Quantification and localization of Nanogold binding. For each sample, 75 Nanogold particles <15 nm in diameter were counted and classified as bound or unbound. The 15-mm cut-off was chosen to exclude unbound, but adjacent, particles enlarged by Goldenhance that only apparently bind fibrils. To establish the localization of the binding observed, individual Nanogold particles bound to fibrils were categorized as bound to the fibril end or side. In both of these experiments, sample identities were concealed from the microscopist to ensure unbiased counting. Grids were examined with a JEOL JEM1200-EX and images were recorded using DIGITALMICROGRAPH (Gatan).

Statistical analysis of Nanogold binding. We compared counts of Nanogold-conjugated peptides and unconjugated Nanogold bound to fibrils or localizing to fibril ends. Twenty-one unconjugated Nanogold particles out of 75 counted bound to fibrils. We modelled Nanogold particles bound to fibrils using a binomial distribution with parameters n = 75 (sample size: number of observations) and P = 0.28 (probability of success). In a separate experiment, 22 unconjugated Nanogold particles bound to fibrils that localized to fibril ends, following a binomial distribution with n = 105 and P = 0.21. Because the number of counts is fairly large, we assumed a normal distribution and used a standard Z-test to compare the number of bound Nanogold–peptide conjugates with the expected distribution based on the number of bound, unconjugated Nanogold particles. We used an analogous analysis to determine the significance of localization to fibril ends.

The numbers of Nanogold–α-TLKIVW conjugates bound to fibrils (Xbound = 43, n = 75) and bound Nanogold–α-TLKIVW conjugates localizing to the end of fibrils (Xend = 49, n = 86) were significantly different from the corresponding numbers for Nanogold alone, whereas the number of Nanogold–α-LKTWIV conjugates bound (Xbound = 15, n = 75) or the number localized to fibril ends (Xend = 17, n = 100) did not differ significantly from the corresponding numbers for Nanogold alone.

VQIVVK preparation for binding studies. Acetylated and amidated VQIVVK peptide (Genscript) was dissolved to 1 mM in 25 mM MOPS, pH 7.2, and incubated at room temperature for at least 24 h. Fibrils were washed with H2O, concentrated using an Amicon ultrafiltration filter with a 3.3-kDa molecular mass cut-off and resuspended in H2O to a final concentration (by monomer) of 4 mM. Soluble VQIVVK was prepared by dissolving VQIVVK peptide (CS Bio) with free amino and carboxyl termini in H2O.

1H NMR sample preparation and measurements. NMR samples were prepared with 5% D2O and 10 mM NaOAc, pH 5.0. n-peptides were added from 1 mM stocks in H2O to a final concentration of 100 μM. Soluble and fibrillar VQIVVK and tau protein were added at indicated concentrations to make a final volume of 550 μl. 1H NMR spectra measured at 500 MHz were collected on a Bruker DRX500 at 283 K. n-peptide resonance was suppressed through presaturation. Spectra were processed with XWINNMR 3.6.

Binding constant estimations. NMR data were analysed to estimate a binding constant for the interaction between α-TLKIVW and VQIVVK fibrils. At about 1,000 μM VQIVVK (concentration as monomer), 50% of α-TLKIVW is bound (Supplementary Fig. 11). The steric-zipper model suggests that there are two non-polar and polar areas buried by the interaction between D-TLKIVW with the VQIVVK steric-zipper template, and preparation of Alzheimer-paired helical filaments in vitro. J. Mol. Biol. 299, 35–51 (2005).


Palonen, M., Valpuesta, J. M., Medina, M., Montejo de Garcini, E. & Avila, J. Polymerization of tau into filaments in the presence of heparin: the minimal antigens for 5 min at room temperature. Peptide concentrations were 150, 30, 6, and 0.75 μg ml−1 during pre-incubation with virus stocks. Thereafter, 20 μl of each mixture was added to 180 μl of TZM-bl cells seeded the day before (104 well). Two days later, infection rates were determined by quantifying β-galactosidase activities in cellular lysates using the Gal-Scan assay (Applied Biosystems, T1027). Luminescence was recorded on an Orion microplate luminometer as relative light units per second.

Effect of WW61 on fibril-mediated enhancement of HIV-1 infection. The CCR5 tropic molecular HIV-1 clone NL4_3/92TH014-2 was generated by transient transfection of 293T cells with proviral DNA. Supernatants were collected 48 h later and p24 concentrations determined by ELISA. TZM-bl reporter cells encoding a luc gene under the control of the viral LTR promoter were obtained through the NIH AIDS Research and Reference Reagent Program and provided by Dr John C. Kappes, Dr Xiaoyun Wu and Tranzyme. HIV-1 (40 μl) containing 0.1 ng of p24 antigen was incubated with 40 μl of mixtures of 248PAP and inhibitory peptide, WW61, that was either freshly prepared or had been agitated for 24 h. Peptide concentrations and experimental conditions during agitination were similar to those described above. Thereafter, 20 μl of the mixtures were used to infect 180 μl of TZM-bl cells seeded the day before (106 well). Two days later, infection rates were determined by quantifying β-galactosidase activities in cellular lysates using the Gal-Scan assay (Applied Biosystems, T1027). Luminescence was recorded on an Orion microplate luminometer as relative light units per second.

Effect of WW61 on polylysine-mediated enhancement of HIV-1 infection. Polylysine (Sigma Aldrich) (50 μl) was mixed with an equal volume of WW61. Thereafter, 35-μl fivefold dilutions of the polylysine–WW61 mixture or polylysine alone were incubated with the same volume of virus and incubated for 5 min at room temperature. Polylysine–WW61 concentrations were 100, 20, 4, 0.8, 0.16, 0.032, 0.0064, and 0.00128 μg ml−1 during pre-incubation with virus stocks. Thereafter, 20 μl of each mixture was added separately to 180 μl of TZM-bl cells (tenfold dilution) and the infection rate was determined two days later as described above.

Effect of WW61, GIHKQK and PYKWLK on HIV-1 infection. Each peptide (40 μl) was incubated with an equal volume of virus containing 1 ng of p24 antigens for 5 min at room temperature. Peptide concentrations were 150, 30, 6, 1.2 and 0.24 μg ml−1 during pre-incubation with virus stocks. Thereafter, 20 μl of each mixture was added to 180 μl of TZM-bl cells (tenfold dilution) and the infection rate was determined as above.

37. Palonen, M., Valpuesta, J. M., Medina, M., Montejo de Garcini, E. & Avila, J. Polymerization of tau into filaments in the presence of heparin: the minimal antigens for 5 min at room temperature. Peptide concentrations were 150, 30, 6, and 0.75 μg ml−1 during pre-incubation with virus stocks. Thereafter, 20 μl of each mixture was added to 180 μl of TZM-bl cells (tenfold dilution) and the infection rate was determined two days later as described above.

Effect of WW61, GIHKQK and PYKWLK on HIV-1 infection. Each peptide (40 μl) was incubated with an equal volume of virus containing 1 ng of p24 antigens for 5 min at room temperature. Peptide concentrations were 150, 30, 6, and 0.75 μg ml−1 during pre-incubation with virus stocks. Thereafter, 20 μl of each mixture was added to 180 μl of TZM-bl cells (tenfold dilution) and the infection rate was determined as above.