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A Peptide Hairpin Inhibitor of Amyloid β-Protein Oligomerization and Fibrillogenesis†

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ABSTRACT: Amyloid β-protein (Aβ) self-assembly is linked strongly to Alzheimer's disease. We found that PP-Leu, a tridecapeptide analogue of broad-spectrum antiviral peptides termed θ-defensins, potently inhibits Aβ oligomer and fibril formation. This effect appeared to be mediated through sequestration of the amyloidogenic Aβ peptide in colloid-like assemblies. PP-Leu comprises a turn formed by a D-Pro−L-Pro amino acid dyad and stabilized by a disulfide bond, a motif that was exceptionally resistant to endoproteinase K digestion. This combination of assembly inhibitory activity and protease resistance suggests that PP-Leu may have potential therapeutic value.

Alzheimer's disease (AD) affects an estimated 5.2 million Americans and is the fifth-leading cause of death among those over the age of 65 (*1*−*3*). AD is characterized by cerebral extracellular amyloid fibril formation by the amyloid β-protein (Aβ) and by intraneuronal paired helical filament formation by the protein tau (*3*). Aβ exists predominately as a 40- or 42-amino acid protein. Oligomerization and fibrillogenesis of Aβ are thought to cause AD (*4*, *5*). Effective inhibitors of Aβ assembly thus have been sought (*6*−*8*).

Several groups have investigated the use of peptide and peptidomimetic inhibitors (*6*−*8*). Soto et al. used small "β-sheet breaker" peptides that bind to Aβ and prevent its assembly into toxic structures (*7*). Assembly inhibitors also have been produced using short *N*-methyl peptides homologous to the central hydrophobic cluster region of Aβ, Leu_{17}−Ala_{21}, which is important in Aβ fibril formation (*8*). Recently, Fradinger et al. (*9*) reported the synthesis of peptide inhibitors designed from hydrophobic C-terminal segments of Aβ. Common features of these inhibitors are their hydrophobicity and their propensity to incorporate into β-sheets. These characteristics also are displayed by potent antiviral peptides, termed θ-defensins, and by peptidic analogues of the toxin invariant domain of cholesterol-dependent cytolysins (*10*, *11*).

To determine whether θ-defensins or cytolysins might also be active in inhibiting Aβ assembly, we used thioflavin T (ThT) fluorescence to monitor the development of β-sheet structure in mixtures of Aβ42 and each of 10 of these potential inhibitors (data not shown). Nine of the inhibitors formed extended β-sheet structures themselves, precluding their further use. However, one compound, the θ-defensin analogue PP-Leu, was active and did not display significant ThT binding (data not shown). PP-Leu (Figure 1) is a 13-amino acid peptide hairpin stabilized by a Cys_2−Cys_{13} disulfide bond and a type II′ β-turn formed by a D-Pro_7−L-Pro_8 moiety (*12*). Such cyclic peptides can possess exceptional structural stability and protease resistance, important properties for maximizing biological activity and half-life in vivo (*13*).

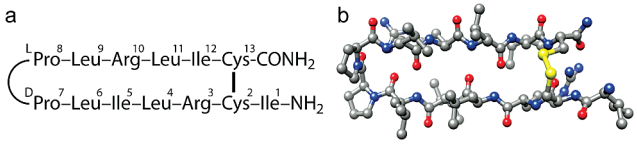

FIGURE 1: (a) Primary structure and (b) ball-and-stick model of PP-Leu. Atoms are color-coded: C, gray; O, red; N, blue; S, yellow.

To explore the inhibitory effects of PP-Leu systematically, we determined the concentration dependence of PP-Leu inhibition of Aβ assembly. To do so, ThT fluorescence assays were performed using freshly prepared, aggregate-free Aβ42 incubated at 37 °C (Figure 2). Aβ42, the longer of the two predominant Aβ isoforms in humans, is thought to be the key pathologic agent in AD (*3*, *4*). Aβ42 incubated alone exhibited a rapid increase in ThT fluorescence that reached a plateau after ≈24 h and then declined thereafter. This behavior is characteristic of amyloid assembly reactions (*14*). PP-Leu diminished the rate of Aβ42 assembly, and the final level of ThT bound, in a concentration-dependent manner. At 24 h, samples of PP-Leu and Aβ42 at molar ratios of 1:5 and 1:1 yielded ThT signals that were ≈$^1/_2$ and ≈$^1/_4$ of that of Aβ42 alone, respectively. At 5:1 and 10:1 molar ratios, no significant increase in ThT binding was observed over time. No ThT signal increase was observed in these latter samples even if the incubation was extended to 300 h (data not shown).

To determine whether PP-Leu blocked fibril formation, transmission electron microscopy (TEM) was performed on aliquots removed during ThT assays performed with PP-Leu and Aβ42 at a 5:1 molar ratio (Aβ concentration of 55 μM). The ThT signal remained constant (< 25 fluorescence units, which is very low) throughout the assay, demonstrating that insignificant β-sheet formation occurred. Aβ42 incubated alone exhibited an ≈50% increase in the magnitude of the ThT signal during the assay and produced abundant amyloid fibrils (Figure 3a). These fibrils display a twisted morphology, widths of 10 ± 1.8 nm, and lengths approaching 1 μm. PP-Leu samples incubated alone produced a mesh comprising both straight and curved assemblies with widths of 4 ± 0.5 nm and lengths of 29 ± 6 nm (Figure 3b). Aβ42 incubated with PP-Leu produced a PP-Leu-like mesh (Figure 3c), not the uniform surface of fibrils seen in the absence of

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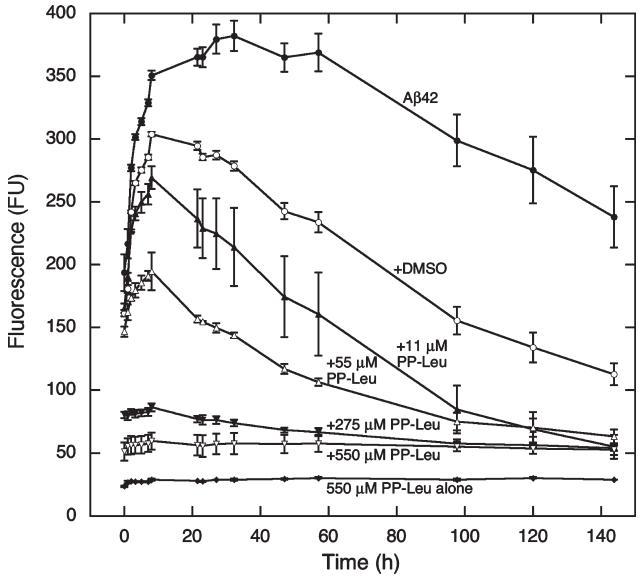

FIGURE 2: Thioflavin T (ThT) binding. Aβ42, at a concentration of 55 μM, was incubated at 37 °C alone, with vehicle (DMSO), or with PP-Leu. β-Sheet structure then was monitored using ThT. Fluorescence units (FU) are arbitrary.

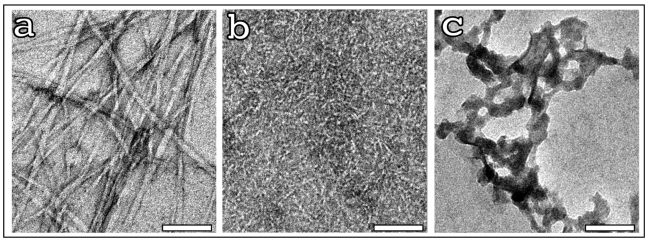

FIGURE 3: Electron microscopy of (a) Aβ42, (b) PP-Leu, and (c) Aβ42 with PP-Leu. The scale bar is 100 nm.

PP-Leu (Figure 3a). The widths of the structures in this mesh were ≈20 nm.

Monitoring the kinetics of increasing ThT fluorescence demonstrated that the midpoint of the conformational transition of the disordered Aβ monomer to the assembled β-sheet-rich fibril occurred at ≈11 h (Figure S1). To determine if PP-Leu blocked fibril elongation, the compound was added to a fibril formation system after 11 h. As seen in Figure S1, addition of PP-Leu at an Aβ42:PP-Leu molar ratio of 1:5 resulted in an immediate and significant diminution in fluorescence to levels indistinguishable from those observed at the beginning of the experiment. This decline occurred over a 3 h time period, after which fluorescence remained relatively constant for ≈48 h before slowly declining during the remaining ≈100 h of the incubation. In comparison, addition of PP-Leu to Aβ42 at the same Aβ42:PP-Leu molar ratio, but at the start of the experiment, produced a 50% lower initial fluorescence, a smaller absolute increase in the magnitude of the ThT signal during the first 24 h, and a declining signal therafter. The final level of ThT fluorescence in samples in which PP-Leu was added after initiation of fibril formation was higher than that in samples in which PP-Leu was added before fibril formation began. In the vehicle alone [5% (v/v) DMSO] control, a small initial decrease in the magnitude of the ThT signal and a smaller maximal ThT signal were observed relative to those of the untreated control.

We next determined the effect of PP-Leu on Aβ42 oligomerization by using photo-induced cross-linking of unmodified proteins (PICUP) to rapidly and efficiently "freeze" metastable Aβ oligomers in a state amenable for study by SDS−PAGE (*15*). In the absence of cross-linking, Aβ42 displayed monomers and trimers (Figure S2, lane 2) whereas PP-Leu was monomeric (Figure S2, lane 3). The Aβ42 trimer band is an SDS-induced artifact (*15*). Un-cross-linked mixtures of PP-Leu and Aβ42 at molar ratios of 1:1 and 5:1 produced oligomer patterns indistinguishable from that of un-cross-linked Aβ42 alone (Figure S2, lanes 4 and 5). After cross-linking, Aβ42 produced an oligomer distribution comprising monomers through octamers (Figure S2, lane 6), with nodes at monomer and pentamer/hexamer (paranuclei). Addition of PP-Leu to Aβ42 at a 1:1 molar ratio inhibited paranucleus formation almost completely and caused a very significant shift of the oligomer frequency distribution to lower orders (≤4) (Figure S2, lane 8). A 5-fold molar excess of PP-Leu had a greater effect on Aβ42 oligomerization, blocking oligomerization entirely and producing an oligomer distribution similar to that of un-cross-linked Aβ42, but with even less trimer (Figure S2, lane 9).

In theory, the inhibition of Aβ42 oligomerization could have resulted from effects of PP-Leu on the PICUP chemistry itself. To test this hypothesis, we assessed the cross-linking potential of the irradiated cross-linking agents APS and Ru(bpy) incubated with or without PP-Leu prior to their addition to Aβ-containing tubes (Figure S3a). Preirradiation of APS and Ru(bpy) produced an Aβ42 oligomer distribution ranging from monomer through hexamer, with an increased population distribution toward trimer and tetramer (Figure S3b, lane 1). Preirradiation of the reagents in the presence of a 5-fold molar excess of PP-Leu produced an oligomer size distribution that was indistinguishable from that produced in the absence of PP-Leu (cf., Figure S3b, lanes 1 and 2). Preirradiation of the cross-linking reagents, followed by their addition to a PP-Leu/Aβ42 mixture [5:1 molar ratio (Figure S3b, lane 3)], produced an oligomer size distribution qualitatively similar to that produced in an equivalent cross-linking reaction in which all reactants were irradiated together (Figure S2, lane 9). These results thus do not support the hypothesis but instead support the conclusion that PP-Leu does indeed inhibit Aβ42 oligomerization.

Taken together, the data discussed thus far suggest that PP-Leu may disrupt structures existent in growing fibrils, preventing further assembly, or have a direct effect on incoming Aβ monomer units that otherwise would bind to fibril ends. Several amyloid inhibitors are known to form aggregates that inhibit protein aggregation through sequestration within colloidal phases (*16*). PP-Leu also forms non-amyloid aggregates (Figure 3b), a property that may explain its mechanism of action. A sequestration mechanism has been reported in studies of the Parkinson's disease-associated protein α-synuclein (*17*). These studies showed that the protein β-synuclein, like PP-Leu, formed nonfibrillar assemblies that inhibited α-synuclein fibril formation. Importantly, β-synuclein coexpression in α-synuclein transgenic mice alleviated motor deficits, neurodegeneration, and α-synuclein accumulation (*18*). PP-Leu, like β-synuclein, thus may have therapeutic potential.

Cyclic peptides have enhanced structural stability relative to linear peptides, an important property with respect to biological activity and stability in vivo. To determine formally whether PP-Leu also exhibited enhanced stability, we compared the protease sensitivity of PP-Leu in its native cyclic (disulfide) and linear (disulfide reduced) forms. To do so, proteinase K (PK), a potent nonspecific protease, was used to digest oxidized and reduced forms of PP-Leu at 37 °C at high (1:1) enzyme:substrate (E:S)

Table 1: Proteinase K Digestion of Oxidized and Reduced PP-Leu

peptide fragment	peptide sequence	observed mass (oxidized)[a]	mass error (oxidized)[b]	observed mass (reduced)[a]	mass error (reduced)[b]
3−11	RLILPPLRL	n.o.[c]	n.o.[c]	1112.6	0.2
5−11	ILPPLRL	844.1	0.0	843.3	−0.8
6−12	LPPLRLI	844.1	0.0	843.3	−0.8
6−11	LPPLRL	730.7	−0.2	730.7	−0.2
7−12	PPLRLI	730.7	−0.2	730.7	−0.2
7−11	PPLRL	596.6*	0.8	596.3*	0.5
5−9	ILPPL	574.5	−0.2	574.3	−0.4

[a]Mass is for the singly sodiated $[M + Na^+]^+$ peptide fragment, except where indicated by an asterisk, in which case the ion is the singly protonated $[M + H^+]^+$ peptide fragment. [b]Observed average mass minus calculated average mass. All experiments were performed in triplicate. [c]n.o. = not observed.

ratios. Digestion of oxidized and reduced PP-Leu yielded peptide fragments that were expected on the basis of the peptide cleavage specificity of PK (Table 1), with the exception that an additional peptide fragment, PP-Leu(3−11), was observed in the cleavage of reduced PP-Leu. Protease digestion progress curves (Figure S7) show that oxidized PP-Leu was digested rapidly and that reduced PP-Leu was digested even more rapidly (digestion of the latter peptide essentially was complete by 1 h). The normalized initial digestion rate of oxidized PP-Leu was ≈10%/h, whereas that of reduced PP-Leu was ≈88%/h, ≈9-fold higher (Figure S7, inset). Aβ40 was used to assess the kinetics of proteolysis of a statistical coil conformer, a conformer that should be substantially less protease resistant than the structured PP-Leu peptide. We observed almost complete Aβ40 digestion within 1 min (Figure S7), a kinetics far more rapid than that of either reduced or oxidized PP-Leu. These results show that native, cyclic PP-Leu displays the increased protease resistance predicted for such peptides.

In conclusion, a 13-residue peptide hairpin, PP-Leu, blocks the formation of the extended β-sheets necessary for Aβ fibril growth, disrupts the structure of preformed fibrils, and potently inhibits Aβ oligomerization. The mechanism of inhibition appears to be Aβ sequestration, as observed with β-synuclein (*17*, *18*) and novel C-terminal Aβ inhibitors (*9*). Importantly, PP-Leu shows substantial protease resistance. Taken together, these results suggest that PP-Leu may be of value as an AD therapeutic.

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures, materials and methods, and Figures S1−S7. This material is available free of charge via the Internet at http://pubs.acs.org.

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