

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

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ABSTRACT: Amyloid β -protein ($A\beta$) 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 $A\beta$ oligomer and fibril formation. This effect appeared to be mediated through sequestration of the amyloidogenic $A\beta$ 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 endoprotease 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 ($A\beta$) and by intraneuronal paired helical filament formation by the protein tau (3). $A\beta$ exists predominately as a 40- or 42-amino acid protein. Oligomerization and fibrillogenesis of $A\beta$ are thought to cause AD (4, 5). Effective inhibitors of $A\beta$ 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 $A\beta$ 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 $A\beta$, Leu₁₇–Ala₂₁, which is important in $A\beta$ fibril formation (8). Recently, Fradinger et al. (9) reported the synthesis of peptide inhibitors designed from hydrophobic C-terminal segments of $A\beta$. 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 $A\beta$ assembly, we used thioflavin T (ThT) fluorescence to monitor the development of β -sheet structure in mixtures of $A\beta$ 42 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 Cys₂–Cys₁₃ disulfide bond and a type II' β -turn formed by a D-Pro₇–L-Pro₈ 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).

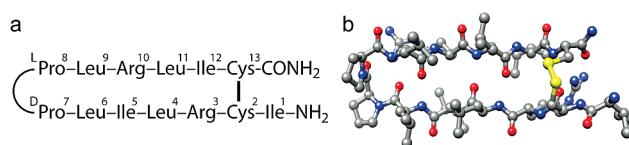


FIGURE 1: (a) Primary structure and (b) ball-and-stick model of PP-Leu. Atoms are color-coded: C, gray; O, red; N, blue; S, yellow.

To explore the inhibitory effects of PP-Leu systematically, we determined the concentration dependence of PP-Leu inhibition of $A\beta$ assembly. To do so, ThT fluorescence assays were performed using freshly prepared, aggregate-free $A\beta$ 42 incubated at 37 °C (Figure 2). $A\beta$ 42, the longer of the two predominant $A\beta$ isoforms in humans, is thought to be the key pathologic agent in AD (3, 4). $A\beta$ 42 incubated alone exhibited a rapid increase in ThT fluorescence that reached a plateau after \approx 24 h and then declined thereafter. This behavior is characteristic of amyloid assembly reactions (14). PP-Leu diminished the rate of $A\beta$ 42 assembly, and the final level of ThT bound, in a concentration-dependent manner. At 24 h, samples of PP-Leu and $A\beta$ 42 at molar ratios of 1:5 and 1:1 yielded ThT signals that were \approx 1/2 and \approx 1/4 of that of $A\beta$ 42 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 $A\beta$ 42 at a 5:1 molar ratio ($A\beta$ concentration of 55 μ M). The ThT signal remained constant ($<$ 25 fluorescence units, which is very low) throughout the assay, demonstrating that insignificant β -sheet formation occurred. $A\beta$ 42 incubated alone exhibited an \approx 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 μ m. 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). $A\beta$ 42 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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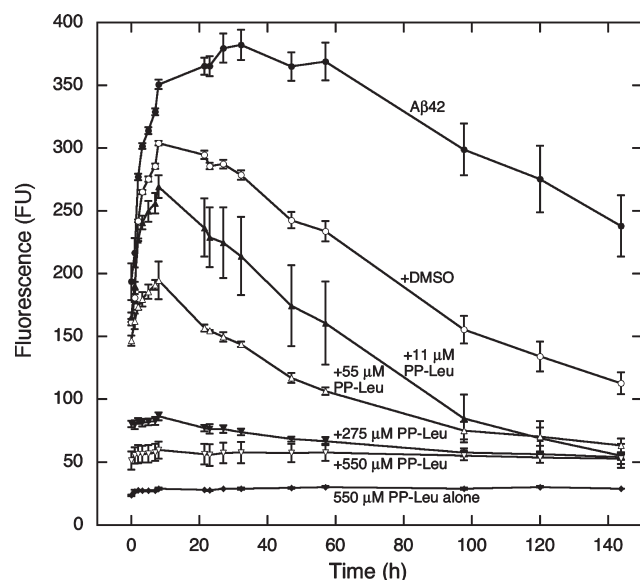


FIGURE 2: Thioflavin T (ThT) binding. A β 42, at a concentration of 55 μ M, was incubated at 37 $^{\circ}$ C alone, with vehicle (DMSO), or with PP-Leu. β -Sheet structure then was monitored using ThT. Fluorescence units (FU) are arbitrary.

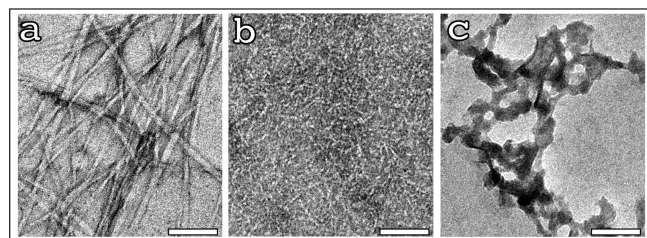


FIGURE 3: Electron microscopy of (a) A β 42, (b) PP-Leu, and (c) A β 42 with PP-Leu. The scale bar is 100 nm.

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

Monitoring the kinetics of increasing ThT fluorescence demonstrated that the midpoint of the conformational transition of the disordered A β monomer to the assembled β -sheet-rich fibril occurred at \approx 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 A β 42: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 \approx 48 h before slowly declining during the remaining \approx 100 h of the incubation. In comparison, addition of PP-Leu to A β 42 at the same A β 42: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 thereafter. 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 A β 42 oligomerization by using photo-induced cross-linking of unmodified proteins (PICUP) to rapidly and efficiently “freeze” metastable

A β oligomers in a state amenable for study by SDS-PAGE (15). In the absence of cross-linking, A β 42 displayed monomers and trimers (Figure S2, lane 2) whereas PP-Leu was monomeric (Figure S2, lane 3). The A β 42 trimer band is an SDS-induced artifact (15). Un-cross-linked mixtures of PP-Leu and A β 42 at molar ratios of 1:1 and 5:1 produced oligomer patterns indistinguishable from that of un-cross-linked A β 42 alone (Figure S2, lanes 4 and 5). After cross-linking, A β 42 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 A β 42 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 (\leq 4) (Figure S2, lane 8). A 5-fold molar excess of PP-Leu had a greater effect on A β 42 oligomerization, blocking oligomerization entirely and producing an oligomer distribution similar to that of un-cross-linked A β 42, but with even less trimer (Figure S2, lane 9).

In theory, the inhibition of A β 42 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 A β -containing tubes (Figure S3a). Preirradiation of APS and Ru(bpy) produced an A β 42 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/A β 42 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 A β 42 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 A β 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 $^{\circ}$ 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

^aMass 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. ^bObserved average mass minus calculated average mass. All experiments were performed in triplicate. ^cn.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 $\approx 10\%/h$, whereas that of reduced PP-Leu was $\approx 88\%/h$, ≈ 9 -fold higher (Figure S7, inset). A β 40 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 A β 40 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 A β fibril growth, disrupts the structure of preformed fibrils, and potently inhibits A β oligomerization. The mechanism of inhibition appears to be A β sequestration, as observed with β -synuclein (17, 18) and novel C-terminal A β 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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