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Stoichiometric Inhibition of Amyloid β-Protein Aggregation with Peptides Containing Alternating α,α-Disubstituted Amino Acids

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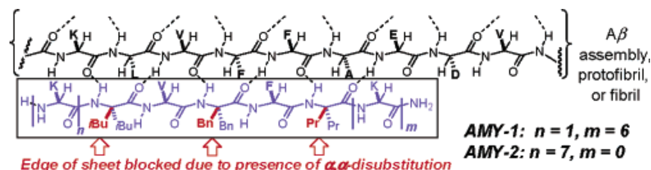
A large body of evidence suggests that amyloid precursor protein (APP) overexpression and faulty processing—which cause overproduction of the amyloid β-protein (Aβ) and increased production of its longer isoforms—are strong risk factors for familial and sporadic forms of Alzheimer's Disease (AD).[1,2] The predominant hypothesis in the field is that the neuronal toxicity and cell death observed in AD are caused by the aggregation products (oligomeric assemblies)[1−3] of Aβ that can continue to self-assemble into protofibrillar or fibrillar structures. Thus a general strategy to prevent or treat AD may be to interrupt or inhibit the assembly of Aβ into these toxic forms.

Herein, we present the use of novel peptide analogues containing α,α-disubstituted amino acids (ααAA) in the hydrophobic core of Aβ (KLVFF); these materials interact with Aβ to yield nonfibrillar assemblies that do not progress to fibrils. We discovered that certain ααAA-containing peptides greatly alter the rate of $A\beta_{1-40}$ aggregation and the resulting aggregate morphology. Through microscopy studies of these inhibitor compounds in the presence of Aβ, we found that fibril formation was inhibited and globular assemblies resulted for both short- and long-term incubation periods. Circular dichroism spectroscopy (CD) indicated that these novel peptides interacted with Aβ by forming β-sheet secondary structures, which further assembled to form the globular aggregates we observe.

A number of research groups have investigated peptides related to the central hydrophobic core (residues 17−20) of the Aβ as potential "blockers" of Aβ aggregation and/or fibrillogenesis.[4] Nordstedt and co-workers found that short peptide congeners and analogues of the KLVFF sequence were effective at halting Aβ fibril formation,[5] while Soto[4b] and Meredith[6] discovered that analogues of this sequence serve as fibril dissolution agents. Researchers at Praecis Pharmaceuticals also evaluated peptides conjugated to a variety of groups as modulators of Aβ polymerization.[7] Murphy, Kiessling, and co-workers used an alternative approach—attaching a disrupting group to the termini of the hydrophobic core (KLVFF)—which altered aggregation rates and reduced toxicity.[8]

Our design of inhibitors of Aβ fibril formation builds on the hypothesis that peptides, which contain the hydrophobic core of Aβ, can interact with the corresponding residues of Aβ via self-recognition and disrupt the self-assembly of Aβ into fibrils. In particular, we have utilized an alternating ααAA/L-amino acid design to give a peptide that interacts with Aβ by hydrogen bonding as well as by side-chain interactions, but has one hydrogen bonding edge blocked. The ααAAs that have side-chain groups larger than methyl have been shown to stabilize extended peptide conformations (e.g., homooligomers of diethylglycine).[9] Thus we hypothesized that peptides with this design (Scheme 1, AMY-1 and AMY-2) could have strong affinity for β-sheet assemblies of Aβ and also prevent further aggregation by blocking one face of the assembly. The synthesis of the AMY-1 and AMY-2 peptides used methods previously reported by our laboratory.[10]

Scheme 1. Design of Peptides with ααAAs as Blockers of Aβ Assembly



We find that AMY-1 (oligolysine unit on the C-terminus) dramatically alters the progression of the β-sheet secondary structure associated with the $A\beta_{1-40}$ protein (Figure 1). After several days in aqueous buffer, the structure of $A\beta_{1-40}$ protein alone changes from random coil (minima at 197 nm and maxima at 220 nm) to β-sheet (maxima at 197 nm and minima at 217 nm) (Figure 1A).

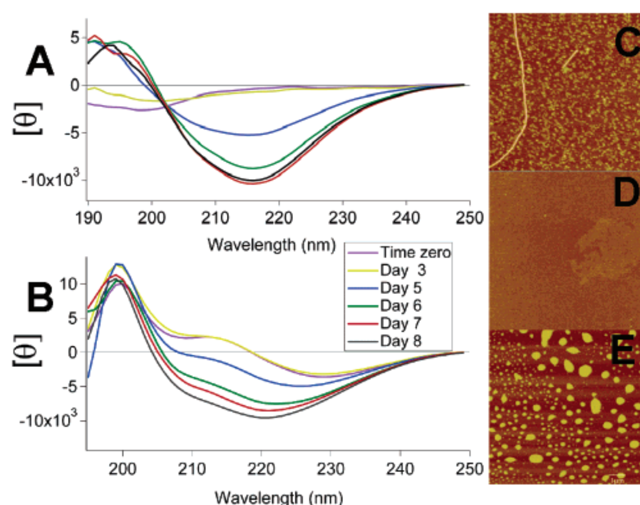


Figure 1. Aggregation of $A\beta_{1-40}$ mitigated by ααAA-based inhibitors. All incubations were performed in PBS (0.050 M, 0.150 M NaCl, pH 7.4) at 37 °C. (A) CD of Aβ (50 μM) for $t = 0-8$ days; (B) CD of Aβ (50 μM) co-incubated with AMY-1 (50 μM) for $t = 0-8$ days; 10 μm × 10 μm ex situ tapping mode SFM images acquired on mica of (C) Aβ (50 μM) after 8 days, (D) Aβ (50 μM) and AMY-1 (50 μM) after 8 days, and (E) Aβ (50 μM) and AMY-2 (50 μM) for 1.5 h at 37 °C. [θ] units: deg cm² dmol⁻¹.

This is followed by concomitant formation of mainly protofibrils, which are approximately 4−7 nm high,[11] as observed by scanning force microscopy (SFM; Figure 1C). This is typical of the pattern that we and others have observed where Aβ protein forms small oligomeric aggregates, then protofibrils that progress to fibrils (in Figure 1C is shown one larger fibril among a sea of protofibrils). Contrastingly, equimolar mixtures of AMY-1 and Aβ lead to an unusual CD signature, which over time appears more β-sheet-like (maxima at 202 nm and minima at 220 nm; Figure 1B).[12] Samples containing equimolar concentrations of AMY-1 and Aβ do not exhibit any protofibrillar aggregates when examined with ex situ

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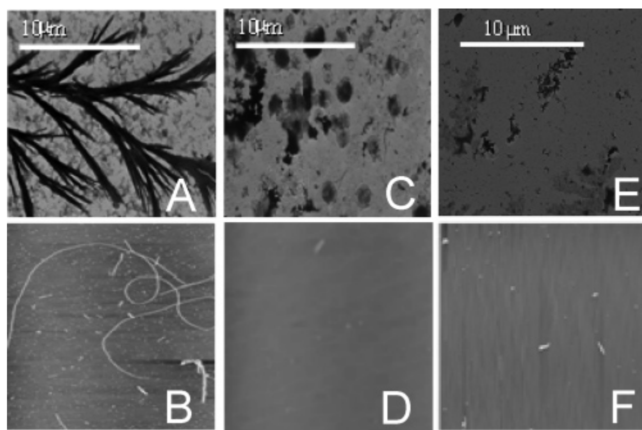

Figure 2. Effect of AMY-1 on Aβ_{1-40} aggregation at varying inhibitor concentrations. A, C, and E are TEM images; B, D, and F are SFM images. Aβ (50 μM) incubated for 4.5 months at 25 °C in PBS; (A) and (B) Aβ alone; (C) and (D) with 50 μM AMY-1; (E) and (F) with 5 μM AMY-1. SFM images are 10 μm × 10 μm scans.

SFM after 8 days. All that was observed was a layer of protein adsorbed onto the mica substrate (Figure 1D). We also determined that AMY-2 (oligolysine on the N-terminus) behaves very differently than AMY-1. Mixing Aβ and AMY-2 solutions results in rapid (<10 min) formation of turbid solutions, thereby precluding CD analysis (sample opaqueness). Microscopy studies of Aβ with AMY-2 (1:1) display large nonfibrillar aggregates on the order of ~1 μm (Figure 1E).

Most interestingly, we have found that AMY-1 significantly alters the pathway of Aβ assembly, as AMY-1/Aβ mixtures exhibit no fibrillization or gelation even after months at room temperature (Figure 2). Transmission electron microscopy (TEM) images of Aβ_{1-40} stored at room temperature for 4.5 months display large branched fibrillar structures (≥10 μm; Figure 2A). SFM of this same sample[13] showed that the remaining material was composed of small fibrillar structures (Figure 2B). Importantly, equimolar mixtures of AMY-1 and Aβ stored at room temperature for 4.5 months showed no sign of precipitate; TEM (Figure 2C) and SFM images (Figure 2D) displayed no signs of fibril formation but rather only globular, nonfibrillar protein aggregates. Even at substoichiometric concentrations of AMY-1 (5 μM AMY-1:50 μM Aβ), very little fibrillization of Aβ was found (Figure 2; E and F) for the same time period.

The results presented here suggest a very different mechanism for the inhibition of Aβ fibrillization by AMY-1 than by other peptide-based inhibitors. AMY-1 and AMY-2 are very stable and do not aggregate in solution. In the presence of Aβ, the AMY peptides may act much like cosurfactants.[14] We have performed surface activity analyses of the inhibitors and found that they do not show micellar-like activity up to millimolar concentrations (Supporting Information), and they do not have significant surface activity at the concentrations at which they were studied (5−50 μM). The very different behavior of AMY-1 and AMY-2, which have the hydrophilic Lys tail on the C- and N-termini, respectively, suggests some directionality to their interaction with Aβ. One way to rationalize this is to suggest that AMY-1 disrupts the intermolecular assembly of the hydrophobic C-terminal portion of Aβ, thus producing smaller particles.[15] AMY-2 disrupts only the hydrophilic N-terminal assembly of Aβ, which has less of an impact on Aβ aggregation.[16] Thus, AMY-2 interaction with Aβ leaves the C-terminus of Aβ accessible for nucleation, which potentially leads to larger particle formation.

In conclusion, we have developed a novel ααAA-containing peptide-based approach to inhibit the aggregation of Aβ protein in vitro. Our studies show that peptides with alternating L-amino acids and ααAAs incorporated into the hydrophobic core of Aβ dramatically alter Aβ protein aggregation behavior. Spectroscopic studies suggest that these novel peptides interact through the formation of β-sheet assemblies, but the mechanism and duration of inhibition is unique relative to that of other peptide and non-peptide-based inhibitors. A plausible mechanistic pathway explaining inhibition of fibril formation is that the inhibitor peptides interact by intercalation into the Aβ globular aggregate, disrupting the formation of fibrils by taking the assembly off-pathway. Further characterization of the AMY/Aβ aggregate size, structure, and stability as well as in vitro and in vivo toxicity assays are underway.

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Supporting Information Available: Experimental details; surface tension results for AMY-1 and AMY-2; CD spectra of AMY-1. This material is available free of charge via the Internet at http://pubs.acs.org.

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