

Design of β -Amyloid Aggregation Inhibitors from a Predicted Structural Motif

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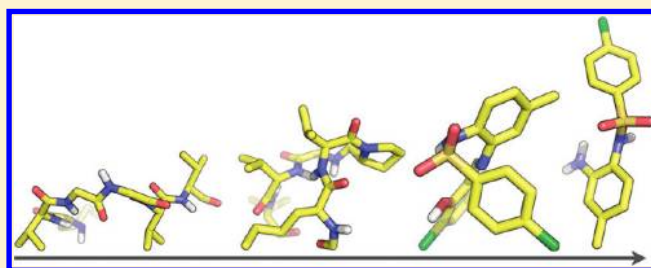
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S Supporting Information

ABSTRACT: Drug design studies targeting one of the primary toxic agents in Alzheimer's disease, soluble oligomers of amyloid β -protein ($A\beta$), have been complicated by the rapid, heterogeneous aggregation of $A\beta$ and the resulting difficulty to structurally characterize the peptide. To address this, we have developed [Nle³⁵, D-Pro³⁷] $A\beta_{42}$, a substituted peptide inspired from molecular dynamics simulations which forms structures stable enough to be analyzed by NMR. We report herein that [Nle³⁵, D-Pro³⁷] $A\beta_{42}$ stabilizes the trimer and prevents mature fibril and β -sheet formation. Further, [Nle³⁵, D-Pro³⁷] $A\beta_{42}$ interacts with WT $A\beta_{42}$ and reduces aggregation levels and fibril formation in mixtures. Using ligand-based drug design based on [Nle³⁵, D-Pro³⁷] $A\beta_{42}$, a lead compound was identified with effects on inhibition similar to the peptide. The ability of [Nle³⁵, D-Pro³⁷] $A\beta_{42}$ and the compound to inhibit the aggregation of $A\beta_{42}$ provides a novel tool to study the structure of $A\beta$ oligomers. More broadly, our data demonstrate how molecular dynamics simulation can guide experiment for further research into AD.



■ INTRODUCTION

Alzheimer's disease (AD) is a degenerative brain disorder and a major cause of dementia for which no cure is known. Currently, >35 million people worldwide are believed to suffer from the disease and with the aging of the population this number is expected to increase by a factor of 3–4 over the next 40 years.¹ AD is characterized by two pathological hallmarks, extracellular deposits composed primarily of amyloid β -protein ($A\beta$) fibrils and intracellular, hyperphosphorylated tau fibrils. While the identity of the molecular species responsible for the initiation of AD pathology has been debated historically, genetic evidence from early onset familial AD,^{2–5} and experimentally observed neurotoxicity of $A\beta$,^{6–11} have indicated that $A\beta$ assembly into β -sheet rich aggregates is a crucial, early stage, neurotoxic element in AD.

Although $A\beta$ is an important neurotoxin in the etiology of AD, the heterogeneous nature of $A\beta$ assemblies has made it difficult to study particular toxic $A\beta$ oligomers individually. An early hypothesis of $A\beta$ toxicity stated that the energetically stable, insoluble $A\beta$ fibrils were the primary pathologic species.¹² However, a large body of evidence demonstrated poor correlation between fibril content and disease severity, thus potentially contradicting the original amyloid fibril hypothesis.^{13–16} Instead, evidence connecting synaptotoxicity and

early cognitive deficits to soluble $A\beta$ oligomers has emerged.^{9,17,18} Still, because of the rapid aggregation kinetics of $A\beta$ and the existence of $A\beta$ oligomers in dynamically changing mixtures, it has been difficult to elucidate the exact mechanism of assembly and the relationship between specific oligomer structures and their toxic activity. Bitan et al. have used photo-induced cross-linking of unmodified proteins (PICUP) to demonstrate the rapid equilibration of monomers and oligomers in freshly prepared $A\beta$, with distinct oligomer size distributions for $A\beta_{40}$ and $A\beta_{42}$.¹⁹ Recently, the relationship between oligomer order and toxicity was elucidated for $A\beta_{40}$ monomer through tetramer using PICUP-stabilized oligomers.²⁰ Nonetheless, testing the biological activity of single oligomeric $A\beta$ species has been highly challenging.

An additional consequence of the heterogeneous composition of $A\beta$ assemblies is the difficulty determining the structure of soluble $A\beta$ oligomers under physiological conditions. Traditional high-resolution methods, such as X-ray crystallography and solution-state NMR spectroscopy, have been unsuccessful at determining the structure of the pathologically and physiologically relevant soluble $A\beta$ oligomers. Efforts have been

Received: September 2, 2011

Published: March 15, 2012

made to stabilize low-molecular-weight oligomers, but to date, high-resolution structures of $A\beta$ oligomers are not available. To gain insight into the structural features of $A\beta$ oligomers and the mechanisms by which they cause neurotoxicity, more information is needed.

Toward this end, we have previously reported the results of a Markov state model molecular dynamics (MSM-MD) simulation of four C-terminal fragments of $A\beta_{42}$ at physiological concentrations.²¹ Because of the strong dependence of aggregation kinetics on the exact amino acid sequence of the C-terminus, C-terminal fragments of $A\beta_{21-43}$ were used in the simulation. The study revealed that $A\beta$ trimers were a semi-stable state in the aggregation pathway, in which monomers contained a β -turn in the C-terminus. Similar structural motifs containing a C-terminal β -turn had been previously predicted in coarse-grained simulations.²² On the basis of these results, a Gly³⁷→D-Pro substitution was predicted to stabilize the β -turn. Given that this turn is not seen in any proposed structure of $A\beta$ fibrils, it was hypothesized that the Gly³⁷→D-Pro substitution would stabilize soluble forms of $A\beta_{42}$.

Recently, we have reported experimental studies of the structure and behavior of an $A\beta$ analogue containing both Gly³⁷→D-Pro and Met³⁵→Nle (norleucine) substitutions ([Nle³⁵,D-Pro³⁷] $A\beta_{42}$).²³ The Nle substitution was included to preclude oxidation of Met³⁵, which can impact assembly kinetics and toxicity.²⁴ Solution-state NMR studies of [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ showed that this analogue indeed formed a C-terminus β -turn centered around residues 37–39. Additional evidence from atomic force microscopy (AFM) and circular dichroism (CD) spectroscopy suggested that [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ did not form fibrils and instead was stable in soluble oligomeric forms comprising 3–7 monomers. Here, we report further characterization of the behavior of [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ and its effect on the aggregation of WT $A\beta_{42}$. In addition, intrigued by the results of these studies, we used ligand-based drug design to identify a small molecule capable of recapitulating the behavior of [Nle³⁵,D-Pro³⁷] $A\beta_{42}$. In doing so, we have identified and characterized novel modulators of the $A\beta_{42}$ aggregation pathway capable of stabilizing $A\beta_{42}$ oligomers that may be used for detailed structural and mechanistic investigations of their interactions with their cellular targets. Our studies demonstrate the utilization of molecular simulations to guide experiment in developing small-molecule inhibitors targeting toxic amyloidogenic protein oligomers.

RESULTS

[Nle³⁵,D-Pro³⁷] $A\beta_{42}$ Forms Stable Oligomers, Not Fibrils, In Solution. In a previous computational study, Kelley et al. hypothesized that a turn-promoting substitution such as Gly³⁷→D-Pro would stabilize trimeric $A\beta$ oligomers.²¹ To test this hypothesis, an $A\beta_{42}$ analogue with the Gly³⁷→D-Pro substitution was synthesized. An additional Met³⁵→Nle substitution was introduced to avoid oxidation of the native methionine at position 35 during assay. Thioflavin T experiments, in which fluorescence of the dye is proportional to the extent of β -sheet character of the protein aggregates, were performed to assess the extent of $A\beta$ aggregation (Figure 1). Following four days of incubation, the [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ samples showed negligible fluorescence whereas substantial fluorescence was measured for WT $A\beta_{42}$, indicating negligible β -sheet character in the substituted peptide. This result is consistent with our predictions that a Gly³⁷→D-Pro substitution stabilizes oligomers.

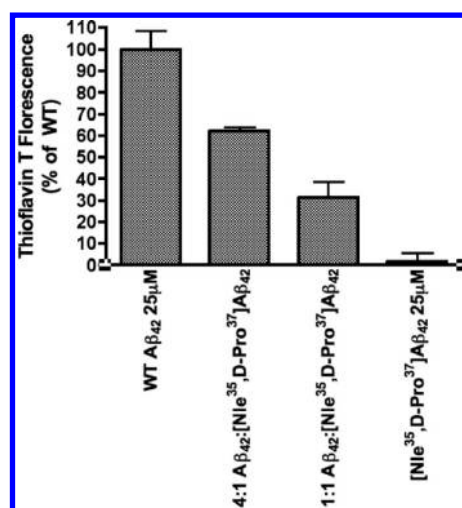


Figure 1. ThT fluorescence experiments of mixtures incubated for four days containing [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ and $A\beta_{42}$. Mixtures contained 25 μ M $A\beta_{42}$ with [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ at 6.25 μ M in the 4:1 mixture and 25 μ M in the 1:1 mixture. Samples were incubated at 37 °C for the duration of the experiment.

To further evaluate the effect of the Gly³⁷→D-Pro substitution on fibril formation, peptide preparations were imaged using transmission electron microscopy (TEM) at the same time point as the ThT fluorescence measurement. $A\beta_{42}$ showed extensive fibril formation with codeposition of amorphous aggregates (Figure 2A,B). In contrast, in identically prepared solutions of [Nle³⁵,D-Pro³⁷] $A\beta_{42}$, we observed only amorphous aggregates and no fibrils (Figure 2C,D). Additionally, [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ samples aggregated for up to 6 months showed no fibril formation (data not shown).

Our previous simulations had indicated that, apart from the C-terminal turn, [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ is largely disordered in solution and should not form stable secondary structure elements. To assess this, [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ was analyzed by circular dichroism (CD). At time zero, both WT $A\beta_{42}$ and [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ exhibited spectra characteristic of a statistical coil, with a minimum at 197 nm (Figure 3). The spectrum of WT $A\beta_{42}$ changed gradually over the course of the five-day experiment to develop a minimum at 217 nm and a maximum at 196 nm, indicative of β -sheet. In contrast, the spectrum of [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ exhibited almost no change over five days.

Finally, to investigate the effect of [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ on equilibrium mixtures of freshly prepared $A\beta_{42}$, we conducted PICUP experiments, fractionated the cross-linked oligomers using SDS-PAGE, and visualized them by silver staining as previously reported (Figure 4A).²⁵ Quantitative analysis of the gels with ImageJ indicated a 70% increase in the relative abundance of the trimer state for [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ compared with WT $A\beta_{42}$ (Figure 4B). Concomitant with the increase in trimer abundance, we observed a decrease in monomer and tetramer in [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ relative to WT $A\beta_{42}$, consistent the prediction that the Gly³⁷→D-Pro substitution stabilizes $A\beta_{42}$ trimer.

As a control, we examined an analogue with an identical amino acid composition to [Nle³⁵,D-Pro³⁷] $A\beta_{42}$ but with sequence positions randomly assigned ($A\beta_{\text{scramble}}$). In both turbidity and Thioflavin T experiments, $A\beta_{\text{scramble}}$ aggregated substantially more rapidly than WT $A\beta_{42}$ (Supporting Information Figure 3). This result demonstrates that any D-proline

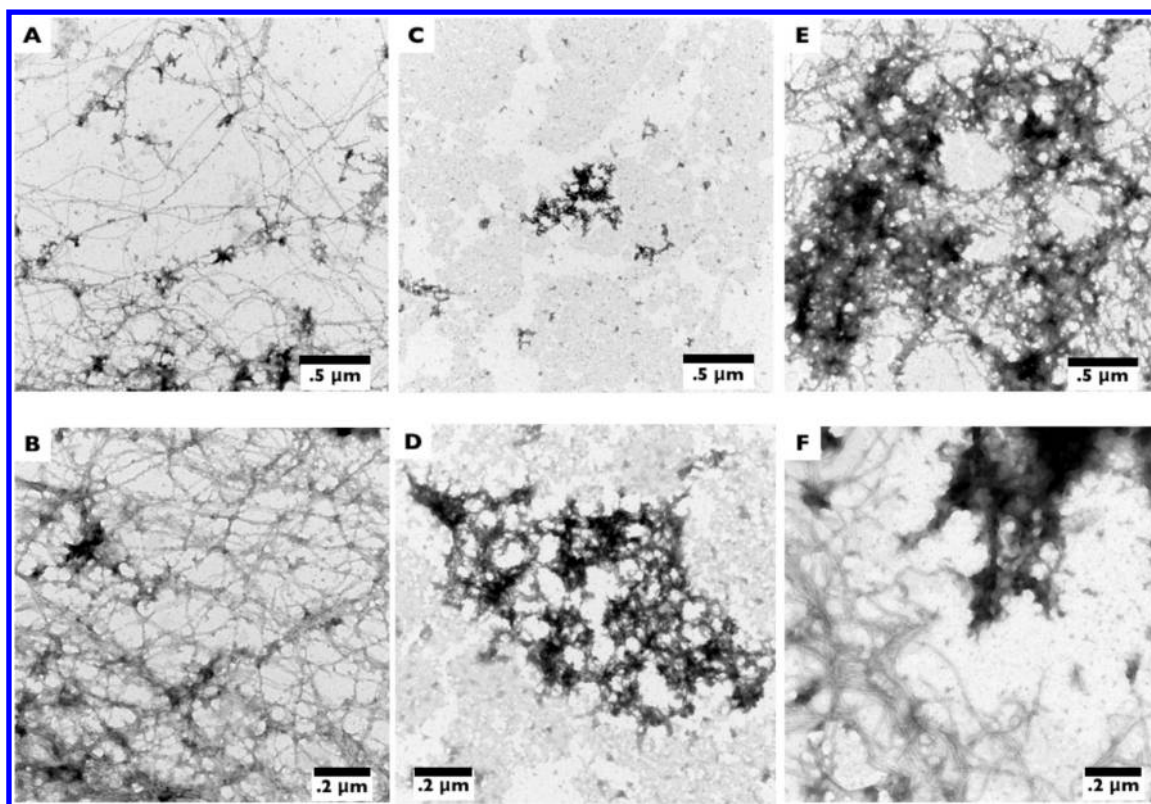


Figure 2. Morphological analysis of mixtures of $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ and $\text{A}\beta_{42}$ after a four-day incubation by TEM. Solutions containing $25 \mu\text{M}$ $\text{A}\beta_{42}$, (A) and (B), $25 \mu\text{M}$ $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$, (C) and (D), or a 1:1 mixture of $25 \mu\text{M}$ $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ and $25 \mu\text{M}$ $\text{A}\beta_{42}$, (E) and (F), were incubated at 37°C until immediately prior to imaging. After incubation, samples were transferred to carbon-coated Formvar grids and stained with uranyl acetate.

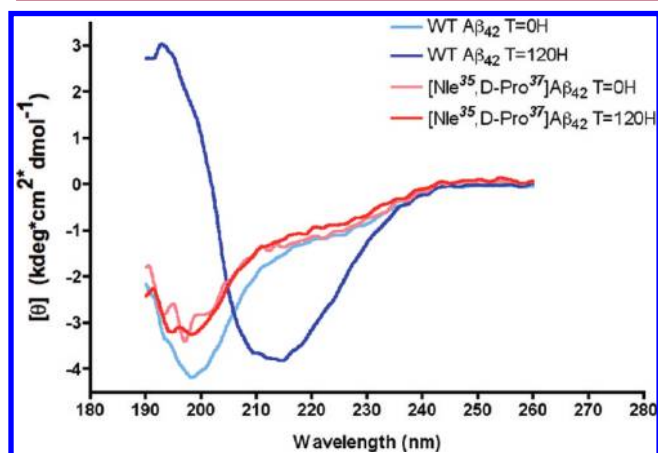


Figure 3. Secondary structure analysis of $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ and $\text{A}\beta_{42}$ by CD spectroscopy. CD spectra of $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ and $\text{A}\beta_{42}$ taken immediately after dissolution of the monomer (light-blue and pink, respectively), and after five-day incubation (dark-blue and red, respectively). Samples of either $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ or $\text{A}\beta_{42}$ ($25 \mu\text{M}$) were incubated at 37°C with continuous shaking for the duration of the experiment.

substitution alone is not sufficient to replicate the effects of the $\text{Gly}^{37} \rightarrow \text{D-Pro}$ substitution on aggregation. Additionally, a selenomethionine substituted peptide $[\text{Sem}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ was created for attempted X-ray studies. This peptide gave similar ThT and PICUP results as $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$, thus demonstrating that the $\text{Met}^{35} \rightarrow \text{Nle}$ substitution is not required to reproduce the effects of $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ (Supporting

Information Figure 4). These results agree with other PICUP studies which demonstrate that a $\text{Met}^{35} \rightarrow \text{Nle}$ substitution slightly destabilizes the trimer state and is otherwise dissimilar from the distribution of $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$.²⁶

$[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ Reduces Aggregation in Mixtures with WT $\text{A}\beta_{42}$ in a Dose-Dependent Manner. Next, we asked whether $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ could interact with and affect the aggregation of WT $\text{A}\beta_{42}$. To answer this question, we measured ThT fluorescence in solutions of $25 \mu\text{M}$ WT $\text{A}\beta_{42}$ mixed with 25 or $6.25 \mu\text{M}$ $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ (1:1 or 4:1 $\text{A}\beta_{42}/[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ concentration ratio, respectively). Following four-day incubation experiments, the fluorescence of the mixtures was intermediate between the values observed for each peptide alone and inversely proportional to $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ concentration (Figure 1). The fluorescence signal of the 1:1 and 4:1 mixtures were $31.4 \pm 12.5\%$ and $62.2 \pm 3.3\%$ that of WT $\text{A}\beta_{42}$ alone, respectively.

Morphological examination by TEM of the $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}:\text{A}\beta_{42}$ mixtures supported the ThT data (Figure 2E,F). At a 1:1 molar ratio, the extent of mature fibril formation was reduced and an increase in amorphous aggregated was observed. Taken together with ThT data, the results suggest that $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ interacts with WT $\text{A}\beta_{42}$, decreases formation of β -sheet-rich $\text{A}\beta_{42}$ fibrils, and increases the relative abundance of nonfibrillar assemblies.

Identification of a Small-Molecule $\text{A}\beta_{42}$ Aggregation Inhibitor. Intrigued by the effects of $[\text{Nle}^{35}, \text{D-Pro}^{37}]\text{A}\beta_{42}$ on aggregation, we conducted a ligand-based screen to identify small molecules capable of recapitulating structural and chemical motifs of the substituted peptide. The previously reported

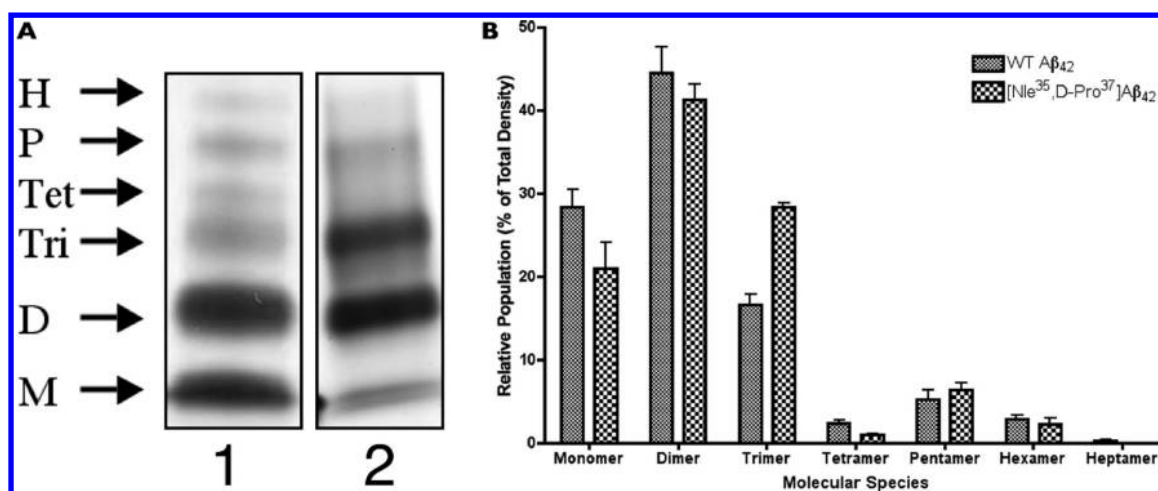


Figure 4. PICUP reveals distinct oligomer size distributions for [Nle³⁵,D-Pro³⁷]Aβ₄₂ and Aβ₄₂ following cross-linking immediately after preparation of fresh monomer. (A) SDS-PAGE of cross-linked samples of 25 μM Aβ₄₂ and [Nle³⁵,D-Pro³⁷]Aβ₄₂ (1 and 2, respectively), following silver staining. Arrows to the left of the gel images indicate the identity of oligomer bands, as deduced from the molecular weight marker. (B) Densitometric analysis of [Nle³⁵,D-Pro³⁷]Aβ₄₂ and Aβ₄₂ created using ImageJ. The data are the result of three independent experiments, each of which is well represented by the images in (A).

NMR structure of [Nle³⁵,D-Pro³⁷]Aβ₄₂ was stripped of side chain atoms, except for the D-Pro, and used as the query for the screen (Figure 5A). In doing so, molecules mimicking the β-turn structure and recapitulating hydrogen-bonding interactions were selected. The top 100 scoring hits from the Maybridge Screening Database were purchased and investigated for their ability to reduce Aβ₄₂ aggregation.

The compounds were screened in a single-dose ThT aggregation assay (data not shown). Compounds identified as potential hits were further evaluated in a dose–response ThT aggregation assay. Of these compounds, 4-chloro-*N*-[2-[(*E*)-(3,5-dichloro-2-hydroxyphenyl)methylideneamino]-4-methylphenyl] benzenesulfonamide (**1**) demonstrated reproducible, dose-dependent decreases in fluorescence (Figure 5B,C). **1** was found to inhibit WT Aβ₄₂ aggregation as read by ThT fluorescence with an IC₅₀ of 13 μM.

Characterization of the Effects of **1 on WT Aβ₄₂ Aggregation.** We then evaluated the effect of **1** on the secondary structure of aggregated Aβ₄₂ by CD. In initial scans, identically prepared solutions of WT Aβ₄₂ and a 1:1 ratio of drug/Aβ₄₂ showed a minimum absorbance around 197 nm and were otherwise characteristic of a statistical coil conformation (Figure 6A). Over the course of the five-day aggregation, the WT Aβ₄₂ spectra shifted to have an absorbance maximum around 196 nm and minimum at 217 nm, indicating the transition of the WT peptide from a statistical coil to β-sheet rich conformation. In comparison, the CD spectrum of the 1:1 solution did not change over the course of the experiment and remained consistent with a random coil structure.

To supplement our data on the effect of **1** on Aβ₄₂ fibril formation, we investigated the formation of fibrils by TEM in solutions of the compound at a 1:1 ratio to Aβ₄₂. As reported above, WT Aβ₄₂ forms mature fibrils over the course of a four-day aggregation experiment (Figure 4A,B). However, in the 1:1 mixture, no fibrils were observed and only amorphous aggregates were seen (Figure 6B). In comparison with [Nle³⁵,D-Pro³⁷]Aβ₄₂, which did not completely block fibril formation at this concentration ratio, **1** was more effective than [Nle³⁵,D-Pro³⁷]Aβ₄₂ at inhibiting fibril growth.

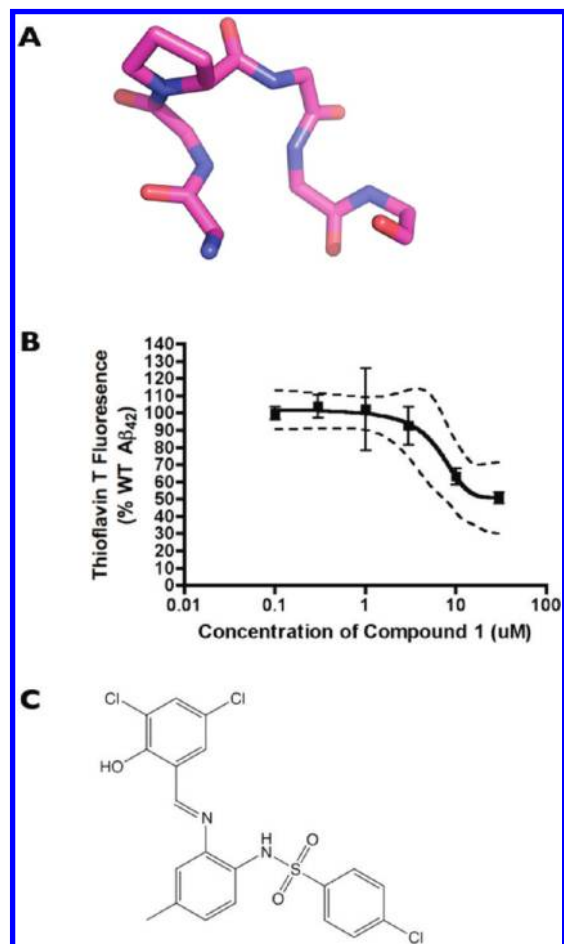


Figure 5. (A) Query used in the ligand-based drug design screen to identify **1**, as visualized in PyMol. Residues 35–40 of the NMR structure of [Nle³⁵,D-Pro³⁷]Aβ₄₂ were stripped of side chain atoms except for the D-Pro residue. Carbons are shown in purple, oxygen in red, and nitrogen in blue. (B) Dose–response effect of **1** on Aβ₄₂ aggregation as measured by ThT fluorescence. Aβ₄₂ was incubated at a concentration of 25 μM in the presence of the indicated concentrations of **1**. (C) Structure of **1**.

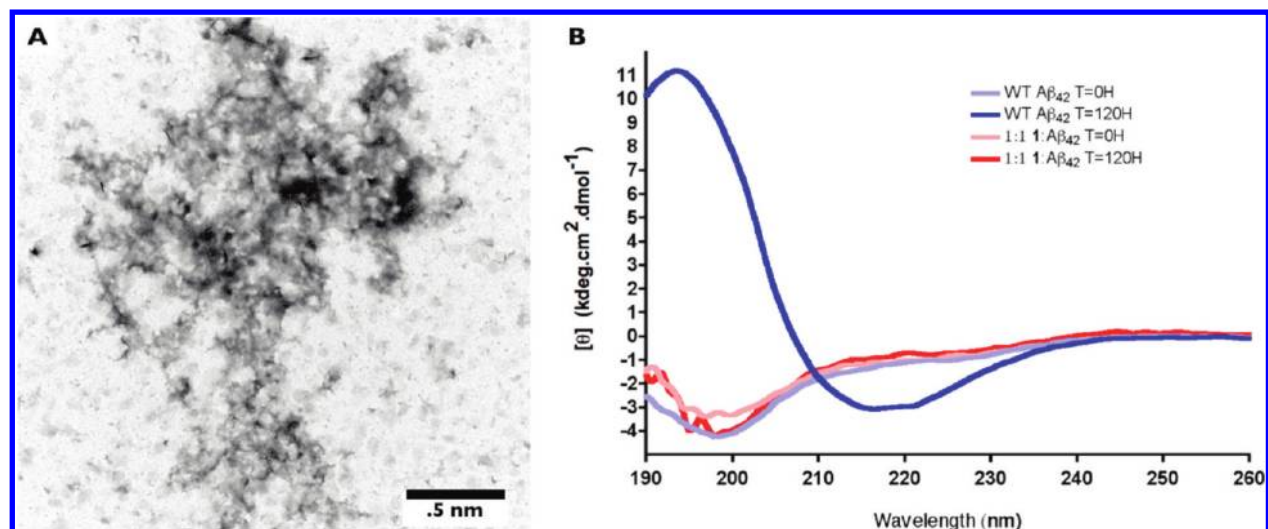


Figure 6. Analysis of the effect of **1** on the fibril and secondary structure formation of $A\beta_{42}$. (A) TEM image of a 1:1 mixture of 25 μM **1** and 25 μM $A\beta_{42}$. Samples were incubated for four days at 37 $^{\circ}\text{C}$. After incubation, samples were transferred to carbon-coated Formvar grids and stained with uranyl acetate. (B) CD spectra of 25 μM $A\beta_{42}$ and a 1:1 mixture of 25 μM **1** and 25 μM $A\beta_{42}$ incubated for five days at 37 $^{\circ}\text{C}$. Spectra for $A\beta_{42}$ alone and the 1:1 mixture were obtained at $t = 0\text{d}$ (light-blue and pink, respectively), and after a five-day incubation (dark-blue and red, respectively).

Neurotoxicity of $[\text{Nle}^{35}, \text{D-Pro}^{37}]A\beta_{42}$ and **1.** We then examined the effect of $[\text{Nle}^{35}, \text{D-Pro}^{37}]A\beta_{42}$ and **1** on cell viability using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay in differentiated PC-12 cells. Cells exposed to 5, 10, or 20 μM $[\text{Nle}^{35}, \text{D-Pro}^{37}]A\beta_{42}$ demonstrated a dose-dependent decrease in cell viability (Figure 7). The toxicity was weaker than that of $A\beta_{42}$, which led

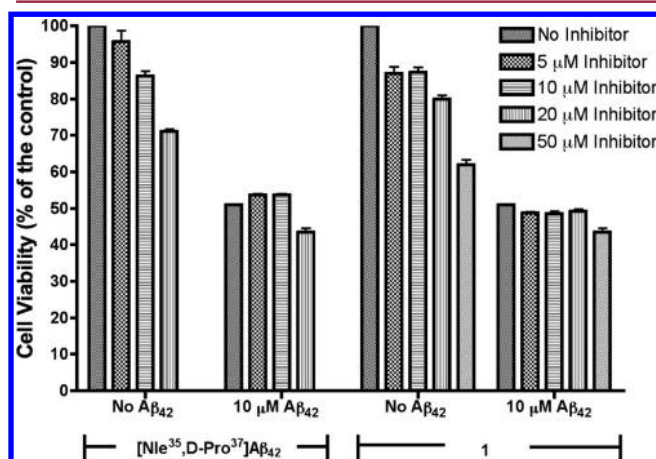


Figure 7. Results of MTT reduction assay to assess the effect of $[\text{Nle}^{35}, \text{D-Pro}^{37}]A\beta_{42}$ or **1** on the viability of PC-12 cells in the presence and absence of 10 μM $A\beta_{42}$. Assay was performed after maintaining cells with $A\beta_{42}$ mixtures for 48 h.

to a 45% decrease in viability at 10 μM , while $[\text{Nle}^{35}, \text{D-Pro}^{37}]A\beta_{42}$ showed only 14% decrease in viability at this concentration. **1** also showed a dose-dependent increase in toxicity, reducing cell viability by 40% at 50 μM . (Figure 6)

Stability Assessment of **1 and Identification of Active Fragment.** Given the intriguing effects of **1** on $A\beta_{42}$, we sought to verify its stability under experimental conditions by LC-MS. We found that upon entering an aqueous environment **1** reached an equilibrium with several degradation products (Figure 8). Dechlorination of **1** at the para chlorine, followed by epoxidation, yields a product with a mass 20 g/mol lighter than **1**,

identified as **1–20** (mass spectra and NMR available in the Supporting Information). Either **1** or **1–20** undergoes hydrolysis of the imine to yield the degradation products **2** or **2–20**, respectively, and **3**. Unexpectedly, mass chromatograms and UV spectra of samples incubated up to 24 h were identical within error, indicating that the equilibrium does not trend toward any product on time scales relevant to these experiments. When compound **2** was analyzed alone with the same protocol, the product **2–20** was not identified, suggesting that the dechlorination/epoxidation occurs only in the parent compound, **1**.

To assess the activity of the fragments relative to compound **1**, we obtained **2** and **3** to evaluate in ThT assays. While compound **3** was found to be inactive (data not shown), compound **2** exhibited a dose-dependent inhibition of $A\beta_{42}$ aggregation (Figure 9). Interestingly, the inhibition of $A\beta_{42}$ by **2** exhibited notably different kinetics in comparison to **1**. At $t = 0\text{d}$ (Figure 9A), the IC_{50} of **1** as measured by percent of fluorescence of an untreated control was 62 μM and the IC_{50} of **2** was 30 μM . Over the course of the four-day aggregation, **1** increased in efficacy in comparison to control with an IC_{50} of 13 μM at $t = 4\text{d}$ (Figure 9B). Conversely, compound **2** showed similar efficacy after the aggregation period, with an IC_{50} of 23 μM at $t = 4\text{d}$.

DISCUSSION

In our effort to design a stable oligomer of $A\beta_{42}$, we have predicted²¹ that the substitution $\text{Gly}^{37} \rightarrow \text{D-Pro}$ would result in enhanced β -hairpin formation and increased stability of low-order oligomers, particularly trimers. Here, our PICUP results confirmed these computational predictions, demonstrating a $70.7 \pm 5.5\%$ increase in trimer abundance of $[\text{Nle}^{35}, \text{D-Pro}^{37}]A\beta_{42}$ compared to $A\beta_{42}$ (Figure 4). The CD, Thioflavin T, and TEM experiments demonstrated that the $\text{Gly}^{37} \rightarrow \text{D-Pro}$ substitution prevented formation of β -sheet-rich fibrils and promoted formation of amorphous assemblies devoid of regular secondary structure elements (Figures 1–3). The data suggest that the β -turn stabilized by the $\text{Gly}^{37} \rightarrow \text{D-Pro}$ mutation either (a) stabilizes the small, soluble oligomers to such a degree as to prohibit further aggregation into fibrils and/or (b) the

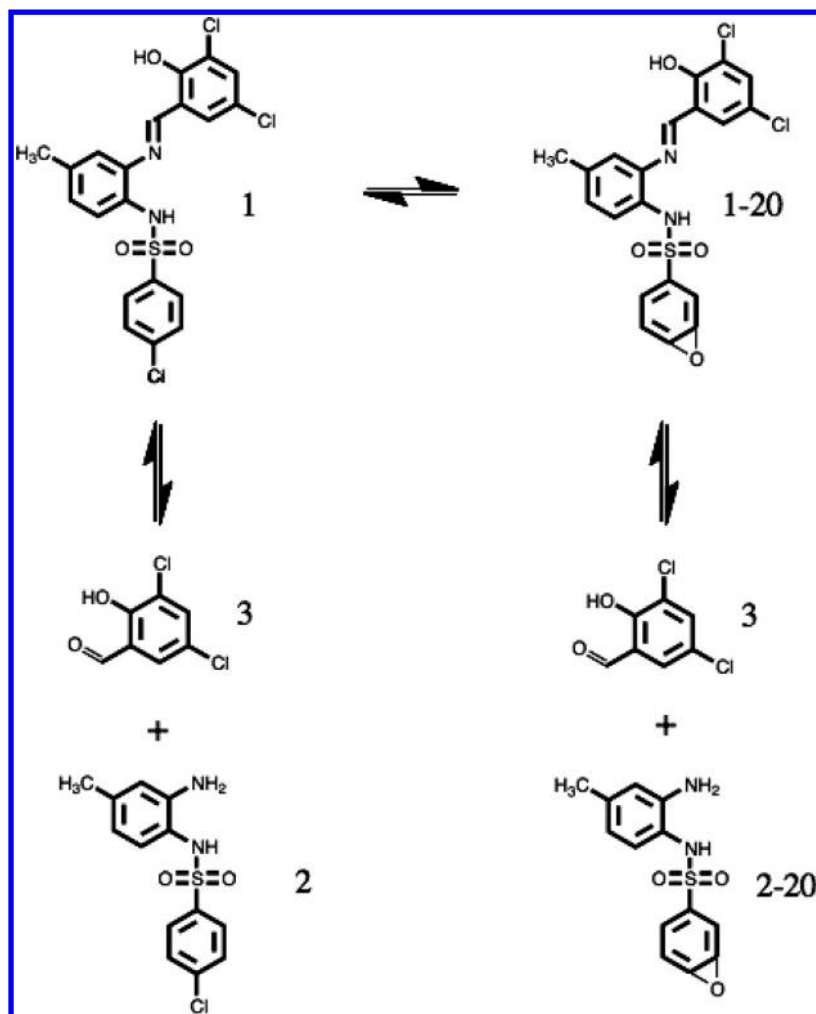


Figure 8. Equilibrium reaction diagram of the products created upon dissolution of **1** in aqueous buffer, pH = 7.4. Dechlorination of **1** at the para position yields product **1–20**. Hydrolysis of the imine bond of **1** yields **2**, and analogous hydrolysis of **1–20** yields **2–20**, in addition to the aldehyde **3**.

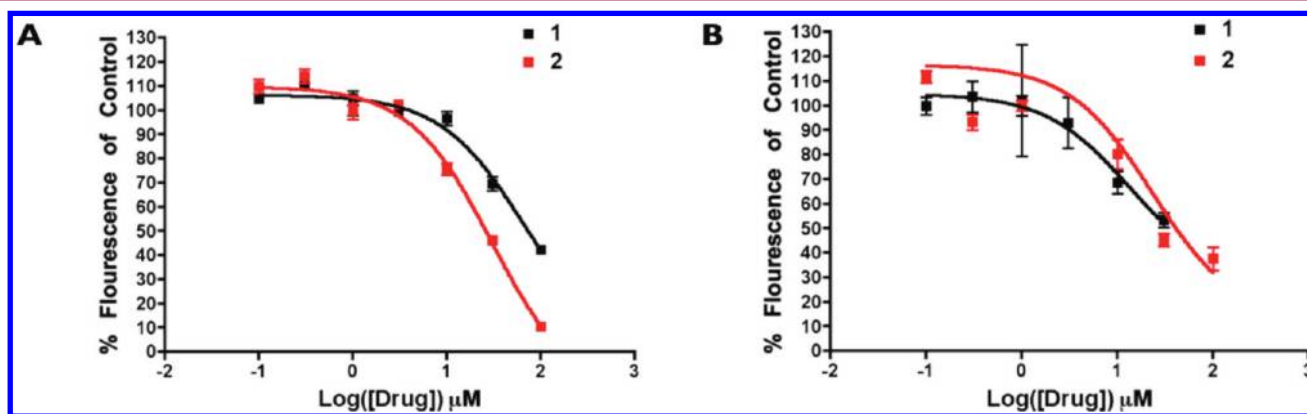


Figure 9. Results from ThT assays measuring the dose dependence of ThT fluorescence in solutions of $A\beta_{42}$ aggregated with the indicated concentrations of **1** and **2**. (A) Fluorescence measured immediately after resuspending $A\beta_{42}$ and combining with either **1** or **2**. (B) Fluorescence of identical samples, measured after a four-day aggregation period.

conformation assumed by $[Nle^{35},D-Pro^{37}]A\beta_{42}$ represents a conformation that is off pathway of fibril formation all together. Interestingly, although other parts of $A\beta$ are unaltered, most notably, the central hydrophobic cluster (residues 17–21), which is known to be important for β -sheet formation, the Gly³⁷→D-Pro substitution is sufficient for preventing formation of β -sheets.

Importantly, for the development of our research on $[Nle^{35},D-Pro^{37}]A\beta_{42}$ toward a lead compound for Alzheimer's disease, the change in aggregation induced by the Gly³⁷→D-Pro substitution is not limited to solutions containing only $[Nle^{35},D-Pro^{37}]A\beta_{42}$ but is observed in mixtures of $[Nle^{35},D-Pro^{37}]A\beta_{42}$ and WT $A\beta_{42}$ as well. The results from ThT and TEM analysis of mixtures of $[Nle^{35},D-Pro^{37}]A\beta_{42}$ and WT $A\beta_{42}$ clearly

indicate that [Nle³⁵,D-Pro³⁷]A β ₄₂ interacts with WT A β ₄₂ to reduce the extent of aggregation and fibril formation when compared to A β ₄₂ alone (Figures 1,3). At a 1:1 ratio, aggregation as read by ThT was reduced by 60% when compared to WT peptide alone. In TEM images, fibrils were reduced in the solution containing a mixture of [Nle³⁵,D-Pro³⁷]A β ₄₂ and A β ₄₂.

We further attempted to recapitulate the effects of [Nle³⁵,D-Pro³⁷]A β ₄₂ on A β ₄₂ aggregation with a small molecule. Structural and chemical motifs from the NMR structure of [Nle³⁵,D-Pro³⁷]A β ₄₂ were used in a ligand-based drug discovery effort. Using this approach, we discovered **1** and found that it inhibited A β ₄₂ β -sheet formation with an IC₅₀ of 13 μ M over a four-day aggregation (Figure 5). Results from CD and TEM experiments were consistent with the ThT data and indicated that **1** completely inhibited A β ₄₂ fibril and β -sheet formation at a 1:1 molar ratio (Figure 6). Considering the results of the LC-MS stability assessment in combination with the ThT studies with compound **2**, the substructure represented by **2** clearly is the primary fragment of **1** responsible for inhibition of A β ₄₂. Although **3** does not inhibit aggregation on its own, the addition of fragment **3** to fragment **2** (yielding **1**) significantly alters the kinetics of the aggregation in comparison to **2** alone. The data suggest that **2** has a faster rate of association with A β ₄₂ in comparison to **1**.

Both [Nle³⁵,D-Pro³⁷]A β ₄₂ and **1** were found to cause a dose-dependent decrease in cell viability. Notably, [Nle³⁵,D-Pro³⁷]A β ₄₂ is significantly less toxic than WT A β ₄₂ (Figure 7). Recently, O'Nuallain et al. found that freshly prepared dimers of [Cys²⁶]A β ₄₂ did not block long-term potentiation (LTP) but that protofibrils built from these dimers did reduce LTP.²⁷ Similarly, our results suggest that the trimer stabilized by [Nle³⁵,D-Pro³⁷]A β ₄₂ is a minimally toxic species, in comparison to WT A β ₄₂. Given the results, it is hypothesized that [Nle³⁵,D-Pro³⁷]A β ₄₂ and **1** reduce A β ₄₂ aggregation by either stabilizing soluble oligomers to such an extent that further aggregation is prohibited and/or by stabilizing a conformation of A β ₄₂ that is off pathway altogether of protofibril and fibril formation.

CONCLUSIONS

We designed [Nle³⁵,D-Pro³⁷]A β ₄₂ based on a structural motif observed in the structure of the A β ₄₂ trimer predicted from molecular dynamics simulations. Recently, we have reported solution NMR structures confirming the existence of the predicted β -hairpin, and the data herein demonstrate a 70% increase in the relative population of the trimer in freshly prepared solutions. The use of computation to predict the structure of a stable species in a heterogeneous aggregation pathway and guide the development of an experimental investigation is notable and suggests a methodology for developing aggregation inhibitors for other amyloid-related diseases, which are complicated due to a lack of structural information. Both [Nle³⁵,D-Pro³⁷]A β ₄₂ and the compounds identified based on its structure, **1** and **2**, are able to interact in solution with A β ₄₂ to stabilize soluble oligomers and reduce fibril formation. The shift in oligomer distribution induced by [Nle³⁵,D-Pro³⁷]A β ₄₂ correlated to a reduction in toxicity compared to A β ₄₂. Given the evidence herein that [Nle³⁵,D-Pro³⁷]A β ₄₂, **1**, and **2** inhibit A β ₄₂ aggregation and β -sheet formation, we hope to further exploit their structural motifs to develop more efficacious and less toxic lead compounds.

MATERIALS AND METHODS

Sample Preparation. WT A β ₄₂ was purchased from a single production lot from Bachem (Bachem H-6466 lot 1013710). Synthetic peptide [Nle³⁵,D-Pro³⁷]A β ₄₂ (DAEFRHDSGY¹⁰EVHHQKLVFF²⁰-AEDVGSNKG³⁰IIGL³⁵NLV⁴²PGVV⁴⁰IA) was synthesized by Anaspec (San Jose, CA). Because the work was carried out in two different laboratories, with slightly different protocols, two sample preparations exist. Peptides were prepared for all experiments in Eppendorf Protein Lo-Bind tubes (Eppendorf no. 022431081). For Thioflavin T studies, the entire contents of the 1 mg A β ₄₂ peptide vial was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma no. 325244) to a concentration of 1 mg/mL and incubated for 2 h at room temperature. After aliquoting the solution, samples were covered with Parafilm in which holes were poked. The solvent was evaporated using a SpeedVac for 1 h and the dry peptide films stored at -80 °C. Immediately before experiments, samples were dissolved in DMSO (Sigma no. D2438), Biotechnology Performance grade) at 2 mM and sonicated for 5 min. The peptide solutions were diluted with PBS, pH 7.4 (GIBCO no. 10010) to the desired concentration. Final DMSO concentration was kept below 3%. For all other experiments, peptides were prepared as described previously.²⁸ Dried peptide films were dissolved by adding sequentially 10% of the final volume 60 mM NaOH, 45% water, and 45% 20 mM NaH₂PO₄ buffer. After dissolution in 60 mM NaOH, peptides were sonicated for 1 min in a bath sonicator. Peptide concentration was measured by Bradford protein assay (Bio-Rad) or quantitative amino acid analysis and was generally 70–80% of the nominal concentration. Peptide mixtures were prepared immediately prior to the beginning of experiments.

Compounds were purchased from the Maybridge Screening Collection (Maybridge Chemical Company, Cornwall, UK) and Sigma. Compounds were dissolved in DMSO to 10 mM and then diluted with PBS to the desired concentration. Purity of the compounds was assessed by NMR, and the degradation of the parent compound was investigated by LC-MS. For MTT and CD experiments, which are sensitive to the presence of DMSO, **1** was dissolved to 10 mM in NaOH and then diluted further with PBS.

PICUP/SDS-PAGE. Photo-induced cross-linking of unmodified proteins (PICUP) was performed as described previously.²⁵ Briefly, 1 μ L of 40 mM ammonium persulfate (APS, Aldrich) and 1 μ L of 2 mM tris(2,2'-bipyridyl)dichlororuthenium(II) (Ru(Bpy)) (Aldrich) were added to 18 μ L of 25 μ M WT A β ₄₂ sample in a clear PCR tube. Irradiation was carried out for 1 s. The cross-linking reaction is quenched immediately by adding 1 μ L of 1 M DTT and 10 μ L of Tricine sample buffer (Invitrogen). Samples are then analyzed using 1 mm thick, 10–20% Tris-Tricine gradient gels (Invitrogen) and silver stained (SilverXpress, Invitrogen). Following air drying, gels were analyzed using ImageJ. Reported values are from averaging of three separate gels.

Circular Dichroism. Samples of 25 μ M WT peptide, in the absence or presence of [Nle³⁵,D-Pro³⁷]A β ₄₂ or **1** were incubated at 37 °C with continuous agitation using an orbital shaker at 200 rpm. Spectra were recorded every 24 h during 5 days using a J-810 spectropolarimeter (Jasco, Easton, MD) equipped with a thermostable sample cell at 37 °C using 1 mm path-length cuvettes. Spectra were collected from 190 to 260 nm with 1 s response time, 100 nm/min scan speed, 0.2 nm resolution, and 1 nm bandwidth. Spectra were averaged after background subtraction.

Thioflavin T Fluorescence. Samples were incubated at 37 °C for four days in triplicates with shaking. Then 10 μ L samples were added in black, flat-, and clear-bottom 96-well plates (Corning no. 3631) to 190 μ L of 25 μ M Thioflavin T (Sigma no. T3516) in 50 mM glycine, pH 8 (Fisher no. G48–212). After a 5 min incubation with the dye, the fluorescence of ThT was measured using a Spectromax M5 fluorometer (Molecular Devices) at an excitation wavelength of 446 nm and emission at 490 nm.

Transmission Electron Microscopy. Samples of A β ₄₂ were incubated in the absence or presence of [Nle³⁵,D-Pro³⁷]A β ₄₂ or **1** for four days at 37 °C. Aliquots of 7 μ L were spotted onto 400 mesh, glow-discharged, carbon-coated Formvar grids (Electron Microscopy

Science, Hatfield, PA) for 2 min and stained with 7 μ L of 1% uranyl acetate. The samples were analyzed using a transmission electron microscope JEM1200-EX (JEOL).

MTT Assay. MTT assays were conducted as previously reported.²⁶ Rat pheochromocytoma (PC-12) cells were maintained in F-12 nutrient mixture with Kaighn's modification (F-12K) with 15% heat-inactivated horse serum and 2.5% FBS at 37 °C in an atmosphere of 5% CO₂. For cell viability assays, cells were plated in 96-well plates at a density of 30000 cells per well in differentiation media (F-12K, 0.5% FBS, 100 μ M nerve growth factor) and maintained for 48 h.

To assess the biological activity of A β ₄₂, [Nle³⁵,D-Pro³⁷]A β ₄₂, and **1**, solutions were prepared as indicated above and diluted into RPMI media to yield concentrations 10 times higher than experimental concentrations. Aliquots of 10 μ L were added to PC-12 cells to yield final concentrations of 10 μ M WT A β ₄₂ and incubated for 15 h. Cell viability was assessed qualitatively by visual observation and quantitatively by the CellTiter 96 nonradioactive cell proliferation assay (Promega). Briefly, 15 μ L of dye solution was incubated with the cells for 3 h. Then 100 μ L of solubilization/stop solution was added and the plates were incubated overnight in the dark to ensure complete solubilization. Plates were read by using a Synergy HT microplate reader (BioTek), and the absorbance at 570 nm (formazan product) minus the absorbance at 630 nm (background) was recorded. At least three independent experiments with six replicates ($n = 18$) were carried out, and the results were averaged.

Virtual Screening. A representative structure from the previously reported NMR studies into the [Nle³⁵,D-Pro³⁷]A β ₄₂ peptide was used as a basis for the ligand-based screen. Residues 35–40 were extracted from the full structure, and side chain atoms were deleted except for the proline atoms. The Maybridge Screening Collection was used as a screening database. Molecules were stripped of salts, and conformers of the remaining molecules were created using Omega (version 2.3, OpenEye Scientific Software). The screening database was then compared to the modified peptide structure using the program ROCS (version 2.3, OpenEye Scientific Software). The Explicit Mills Dean force field was used, with the exception that ring interactions were turned off. The top 100 compounds ranked by combo Tanimoto after optimization by shape and color and were selected for evaluation by ThT. Compound **1** was ranked 48th out of 56897 compounds screened (details available as Supporting Information).

■ ASSOCIATED CONTENT

■ Supporting Information

Results from experiments conducted with A β _{scramble} and [Sem³⁵,D-Pro³⁷]A β ₄₂. LC-MS and ¹H NMR of **1**, **2**, and **3**. Rankings and structures of top scoring compounds from the virtual screen. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ ACKNOWLEDGMENTS

We acknowledge the UCLA Jim Easton Consortium Alzheimer's Drug Discovery and Biomarker Development, the NIH Nanomedicine Center (PN1 EY016525), and the Stanford Center for Molecular Analysis and Design (CMAD) for funding. We also acknowledge NSF award CNS-0619926 and no. 0960306 (MRI-R2: Acquisition of a Hybrid CPU/GPU and Visualization Cluster for Multidisciplinary Studies in

Transport Physics with Uncertainty Quantification) for computing resources.

■ ABBREVIATIONS USED

A β , amyloid β -protein; AD, Alzheimer's disease; Nle, norleucine; D-Pro, D-proline; ThT, Thioflavin T; PICUP, photo-induced cross-linking of unmodified peptides

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