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Transthyretin-Derived Peptides as β-Amyloid Inhibitors

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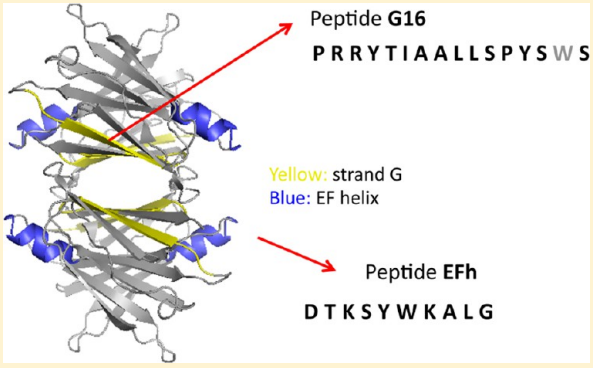
ABSTRACT: Self-association of β-amyloid (Aβ) into soluble oligomers and fibrillar aggregates is associated with Alzheimer's disease pathology, motivating the search for compounds that selectively bind to and inhibit Aβ oligomerization and/or neurotoxicity. Numerous small-molecule inhibitors of Aβ aggregation or toxicity have been reported in the literature. However, because of their greater size and complexity, peptides and peptidomimetics may afford improved specificity and affinity as Aβ aggregation modulators compared to small molecules. Two divergent strategies have been employed in the search for peptides that bind Aβ: (i) using recognition domains corresponding to sequences in Aβ itself (such as KLVFF) and (ii) screening random peptide-based libraries. In this study, we propose a third strategy, specifically, designing peptides that mimic binding domains of Aβ-binding proteins. Transthyretin, a plasma transport protein that is also relatively abundant in cerebrospinal fluid, has been shown to bind to Aβ, inhibit aggregation, and reduce its toxicity. Previously, we identified strand G of transthyretin as a specific Aβ binding domain. In this work we further explore and define the necessary features of this binding domain. We demonstrate that peptides derived from transthyretin bind Aβ and inhibit its toxicity. We also show that, although both transthyretin and transthyretin-derived peptides bind Aβ and inhibit toxicity, they differ significantly in their effect on Aβ aggregation.



KEYWORDS: *Alzheimer's, amyloid, aggregation, beta-amyloid, peptide-based drugs, transthyretin, intrinsically disordered*

Alzheimer's disease (AD) is the most common age-associated neurodegenerative disorder, affecting more than 35 million people worldwide. Two of the primary pathological characteristics of AD, observed primarily in the hippocampus and cerebral cortex, are the deposition of β-amyloid (Aβ) in extracellular senile plaques and the formation of intracellular neurofibrillary tangles derived from hyperphosphorylated tau protein. Aβ, a proteolytic product of amyloid precursor protein (APP), spontaneously self-assembles through a multistep process into soluble oligomers and insoluble fibrils. Although the precise mechanism of AD pathogenesis is unknown, several studies have suggested that the aggregation of Aβ plays a key causal role.[1,2]

Compounds that can alter Aβ production and/or aggregation have been extensively explored as therapeutic agents against AD, although to date none have been clinically effective.[3] Numerous small-molecule inhibitors of Aβ aggregation have been demonstrated to interact with Aβ, alter Aβ aggregation, and/or prevent Aβ-induced toxicity.[4] Many of these compounds contain multiple aromatic or substituted aromatic rings; a few examples include curcumin,[5] epigallocatechin gallate,[6] resveratrol,[7] and Brilliant Blue G.[8] Sugars such as inositol[9] and charged compounds such as 3-amino-1-propanesulfonic acid[10] are also reportedly effective modulators of Aβ aggregation and/or toxicity.

An alternative approach is to exploit the use of peptides and peptidomimetics that bind to Aβ, modulate aggregation, and/or inhibit toxicity. Peptides have potential advantages over small molecules in terms of better target affinity and specificity, and lower toxicity, but have been less explored as therapeutics because of their short-lifetime in vivo as well as limitations in delivery method.[11,12] As strategies are developed to address these issues, peptides are increasingly considered as attractive compounds for drug development.[11,12]

In general, two approaches have been used to design and discover peptides and peptidomimetics that bind to Aβ.[13,14] One method is based on self-complementation.[15] Because Aβ self-assembles, peptides derived from Aβ sequences are argued to act as "recognition elements". The most commonly used recognition element is the central domain of Aβ (KLVFF, Aβ(16−20)), which, in pioneering work by Tjernberg and co-workers, was shown to bind full length Aβ and prevent its aggregation.[16] To improve activity, the KLVFF base has been modified by adding disrupting domains of hydrophilic residues,[17,18] incorporating proline,[19] or grafting onto scaffold proteins such as GFP[20] or antibodies.[21] Promising results were obtained in a few in vivo studies; for example, a retro-inverso peptide based on KLVFF reduced amyloid deposits in transgenic mice.[22] The second approach is to screen libraries to select for peptides that bind to Aβ species with high affinity.

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Using this approach, several groups have identified peptides or peptidomimetics that interact with Aβ monomers or fibrils, and alter Aβ aggregation and/or toxicity.[23−26]

Alternatively, one could design peptides that bind to Aβ based on knowledge of complementary binding proteins. This strategy is based on the idea of mimicking naturally occurring heterologous interactions between Aβ and any of a number of natural binding partners. This is a promising strategy to modulate disease-associated protein−protein interaction due to the potential for high specificity, but this approach has not yet been extensively used for developing Aβ inhibitors.

A few natively folded proteins have been identified as putative binders of Aβ. One of these is transthyretin (TTR), a stably folded homotetrameric transport protein that circulates in blood and cerebrospinal fluid. Upregulation of TTR synthesis was identified in a gene expression screen of transgenic mice that produce Aβ deposits but do not show signs of neurodegeneration.[27] Additional studies provided further support for the hypothesis that TTR protects against Aβ toxicity in vivo.[28,29] We and others have shown that TTR binds to Aβ and prevents its toxicity in vitro, and that inhibition of toxicity requires TTR-Aβ binding.[30−33] TTR binds preferentially to Aβ oligomers compared to fibrils, but binds only weakly to monomers;[34] this binding pattern is potentially advantageous if Aβ oligomeric intermediates are the most toxic species, as is widely postulated.[35] TTR completely abrogated in vitro Aβ toxicity at substoichiometric (1:100 TTR:Aβ) molar ratio.[33] To the best of our knowledge, this result indicates that TTR possesses greater efficacy as an Aβ toxicity inhibitor than other reported materials. Substoichiometric efficacy can be explained if inhibition of toxicity requires binding preferentially to Aβ oligomers.

Recently, we presented evidence that Aβ binds to TTR through two different binding domains: strand G in the inner β-sheet, and the EF helix/loop.[33,36] Leu110 on strand G and Leu82 on the EF loop were identified as two critical residues that mediate the interaction between Aβ and TTR (Figure 1). TTR mutants L110A and L82A showed significantly reduced Aβ binding and did not protect neuronal cells against Aβ-induced toxicity, thus directly linking binding to inhibition of toxicity.[33] Identification of this region on TTR as the Aβ binding "pocket" has been confirmed and extended recently.[37]

Based on these results, we speculated that peptides that mimic the Aβ binding domain on TTR might reproduce the protective effect of TTR against Aβ. Given the demonstration of TTR-mediated neuroprotection in vivo, along with TTR's strong preference for binding Aβ oligomers rather than monomers, we hypothesized that TTR-derived peptides may have superior efficacy compared to peptides designed using the self-complementation strategy. In this work, we explored whether peptides of the same linear sequence as these binding domains can serve as replacements for TTR, and we determined minimum requirements for binding. We examined whether these designed peptides affect Aβ aggregation, and/or inhibit Aβ toxicity in primary neuronal cultures. These studies are the first step in designing peptidomimetics based on TTR that could be highly efficacious against Aβ.

RESULTS AND DISCUSSION

SPOT Analysis to Identify Minimum Binding Requirements. In our prior peptide array work, we identified the 12-mer TIAALLSPYSYS (residues 106−117 of TTR) as the strongest Aβ binder of all contiguous sequences tested.[33]

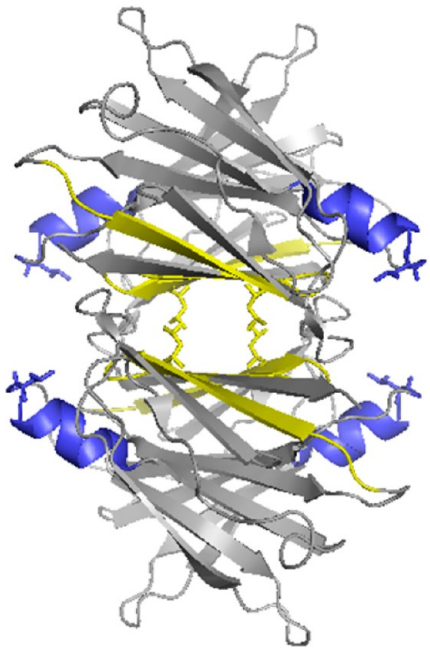

Figure 1. Ribbon structure of transthyretin (PDB entry 1DVQ) tetramer, showing residues corresponding to peptide G16 (residues 102−117, yellow) and peptide EFh (residues 74−83, blue). Leucine side chains for L82 (blue) and L110 (yellow) are shown explicitly. Each monomer contains two four-stranded β-sheets and a single helix.

Sequences displaced by 6 residues either N-terminally (SGPRRYTIAALL) or C-terminally (SPYSYSTTAVVT) did not bind Aβ. In the current study, we tested several variants of TIAALLSPYSYS (Figure 2). First we tested 12-residue fragments at higher resolution, where the register was shifted *N*-terminally by 2 or 4 amino acids (spots **5** − **6**, compared to the base case at spot **7**). Compared to **7**, binding was weak for **6**, and nonexistent for **5**. We tested shorter (6−10-residue) fragments of the base sequence (**1**−**4**, **8**−**9**, and **20**−**23**). Of the 10-mers, strong binding was detected only for **3** (TIAALLSPYS, Figure 2). Of the 6-residue and 8-residue fragments, none bound Aβ except possibly weak binding for spot **9** (LLSPYSYS). These data indicate that a minimum length of 10 residues is needed (although caution must be used in interpreting the minimum length, as membrane effects may interfere with binding to shorter peptides). In addition, we conclude that the *N*-terminal PRRY is not required for Aβ binding, and both the hydrophobic hexamer TIAALL as well as *C*-terminal residues, SPYS or SPYSYS, are needed. Interestingly, several studies have demonstrated amyloid fibril formation from the related peptide, TTR(105−115) (YTIAALLSPYS).[38−40]

We next tested whether individual amino acids were required for binding. With TIAALLSPYSYS as the base case, we mutated each residue to alanine (**10**−**19**). Strong binding of Aβ was detected for **10**, **14**, and **15**, indicating that T106, S112, and P113 are not required features of the binding domain. Two mutations at serine (S115A and S117A, **17** and **19**) reduced but did not eliminate binding. Aβ binding was completely eliminated with mutations in I107, L110, L111, Y114, or Y116 (**11**, **12**, **13**, **16**, and **18**). We previously identified L110 as a critical binding residue,[33] so we also mutated this residue to Met, Ser, or Lys (**24**−**26** in Figure 2); all three mutations led to loss of Aβ binding, confirming the importance of this Leu

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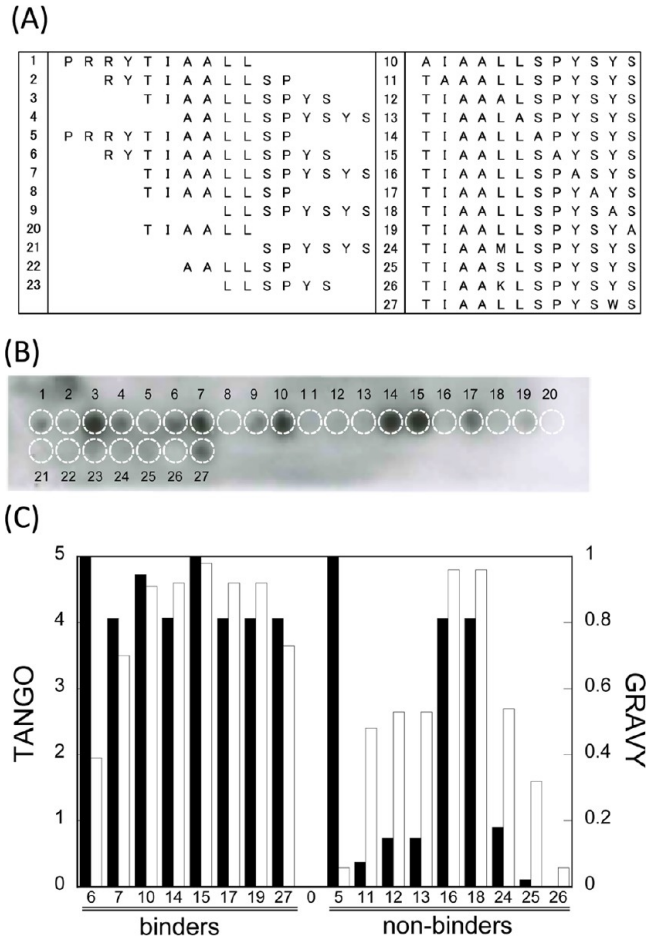

Figure 2. Aβ binding to G-derived peptides. (A) Peptide sequences corresponding to each spot on the membrane. (B) Aβ was bound to the peptide array; then bound Aβ was transferred to a PVDF membrane and imaged by Western blot analysis. (C) Comparison of amyloidogenicity (TANGO, solid bars) and hydrophobicity (GRAVY, open bars) scores for binders and nonbinders. The TANGO Score was calculated by analyzing each sequence for amyloidogenicity in TANGO, summing up the total score for each position, and then dividing by the number of residues. The GRAVY score is the grand average of hydropathy and was calculated using an online calculator www.gravy-calculator.de/.

residue. Finally, we observed that substitution of Y116 with W116 was tolerated (**27**), although there was some reduction in signal.

We categorized all 12-mers as either binders or nonbinders based on the SPOT data (**5**–**7**, **10**–**19**, and **24**–**26**). To determine whether there were specific attributes that distinguished binders from nonbinders, we analyzed these sequences for amyloidogenicity (as calculated by TANGO[41]) and hydrophobicity (as calculated by GRAVY[42]) (Figure 2C). Amyloidogenicity is influenced by hydrophobicity so there is some correlation between the two scores, but additional factors such as propensity to form β-sheet secondary structural elements or charge effects are incorporated into the TANGO score. For the most part, loss of hydrophobicity (**5**, **26**) or loss of amyloidogenicity (**11**, **12**, **13**, **24**, **25**) led to loss of binding. The exception to this rule was mutation of tyrosine to alanine (**16**, **18**). This mutation increased hydrophobicity relative to the base case, and retained amyloidogenicity, but binding was lost. Mutation of tyrosine to tryptophan (**27**) did not result in a loss of binding. Taken together, these results point to two types of requirements for binding of TTR-mimetic peptides to Aβ: (a) hydrophobic residues isoleucine and leucine in the N-terminal domain TIAALL, and (b) aromatic groups in the C-terminal domain SPYSYS. The latter domain is striking in its tyrosine and serine content. It has been demonstrated that complementary binding domains on synthetic antibodies, composed of only serine and tyrosine in various arrangements, can retain high antigenic diversity and affinity.[43,44] Sequences rich in tyrosine and serine comprise a set of universal binding domains:[45] tyrosines are large and amphipathic, participating in cation−π, hydrophobic, and hydrogen bonding interactions, while serines add flexibility.

Solution-Phase Binding of TTR-Derived Peptides to Aβ. Based on these results, we synthesized two peptides corresponding to residues 106−117 (denoted G12) and 102−117 (G16) of TTR. Y116 was mutated to W in G16 in order to use tryptophan for concentration determination. We also synthesized two controls: a scrambled sequence peptide (Gsc), and a peptide (GLA) in which one leucine was mutated to alanine. GLA was designed based on our previous results in which we showed that an L110A TTR mutant bound less Aβ than wt, and was unable to prevent Aβ toxicity.[33] Finally, we synthesized a peptide corresponding to the EF helix/loop, residues 74−83 of TTR (EFh). G12 was insoluble in water but was soluble in DMSO; all other peptides were soluble in water. The sequences of all peptides are listed in Table 1.

Two methods were used to test for interaction between Aβ and the TTR-derived peptides: cross-linking followed by gel electrophoresis, and protease protection. G16, Gsc, GLA, G12, or EFh was mixed with Aβ (10:1 molar excess of Aβ), cross-linked using the PICUP method, separated by gel electrophoresis, and analyzed for Aβ by Western blot (Figure 3a). Aβ alone produced a ladder of monomer, dimer, trimer, tetramer, and a few larger oligomers, consistent with other reports in the literature.[46] We observed a smear of oligomers (∼30 to ∼100 kDa) in G16−Aβ mixtures that was absent with Aβ alone, indicating that G16 interacts with Aβ and increases the average size of Aβ aggregates. The effect of GLA and especially Gsc on Aβ size distribution was much less pronounced, suggesting that the interaction between Aβ and G16 is specific. Neither G12 nor EFh had any effect on Aβ aggregate size; we conclude that

Table 1. Sequences of TTR-Derived Peptides

peptide name	sequence	TTR residue numbers	molecular mass (expected/measured)	modification from native TTR sequence
G16	PRRYTIAALLSPYSWS	102−117	1922.2/1922.03	Y117W
Gsc	LPRATYSSIYLPSRWA	102−117	1922.2/1922.04	scrambled sequence
GLA	PRRYTIAAALSPYSWS	102−117	1880.1/1880.06	Y117W, L110A
G12	TIAALLSPYSYS	106−117	1326.5/1348.61[a]	none
EFh	DTKSYWKALG	74−83	1209.3/1209.66	none

[a]The sodium adduct accounts for the higher measured mass than expected.

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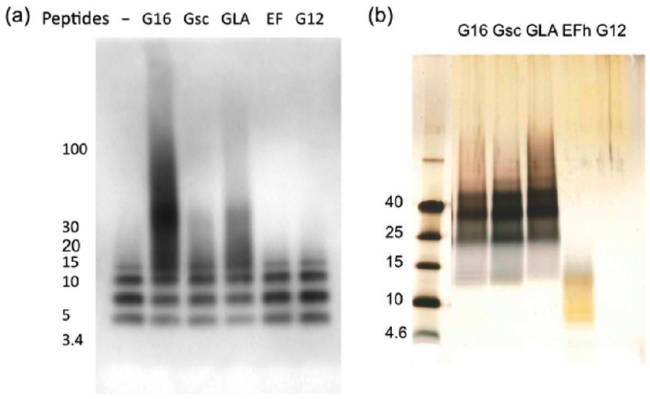

Figure 3. (A) Photoinduced cross-linking of Aβ incubated without (−) or with peptides G16, Gsc, GLA, EFh, and G12. Position of molecular weight markers (in kDa) is shown on left. Aβ concentration was 24 μM, and peptide concentration was 2.4 μM. Aβ was detected by Western blot with anti-Aβ antibody 4G8. (B) Photoinduced cross-linking of G16, Gsc, GLA, EFh, and G12. Peptide concentration was 24 μM, and peptides were visualized by silver staining.

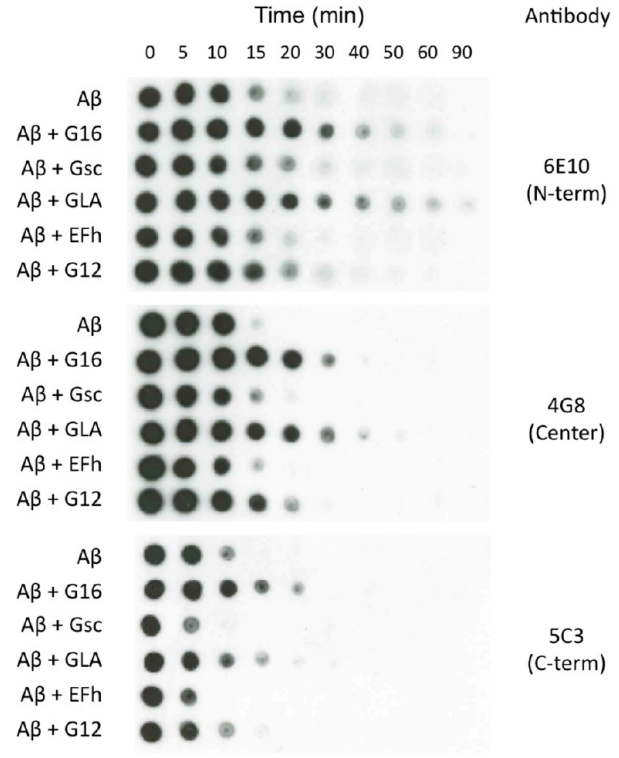

Figure 4. Proteolytic fragmentation assay of Aβ incubated with or without TTR-derived peptides G16, Gsc, GLA, G12, and EFh. Aβ concentration was 20 μM, and peptide concentration was 40 μM.

neither interacts strongly with Aβ. It is likely that G12's insolubility precludes interaction with Aβ, and that EFh's lack of α-helical structure (data not shown) prevents its binding to Aβ.

For comparison, solutions of the TTR-derived peptides alone were cross-linked and analyzed by gel electrophoresis. As shown in Figure 3b, EFh stained poorly, and G12 was insoluble and did not enter the gel. For all other peptides, discrete oligomers were observed at ~24−25 kDa (corresponding to ~12-mers), ~35 kDa (~18-mers), and ~45 kDa (24-mers). In the absence of cross-linker, all peptides ran true to molecular weight (data not shown).

As another test of binding between the TTR-derived peptides and Aβ, we incubated Aβ alone or with one of the peptides, and measured Aβ proteolysis rates. The rationale for the experiment is that binding of the TTR-derived peptide should inhibit access of proteases to Aβ and delay proteolytic degradation.[47] Three different monoclonal antibodies were used to probe for Aβ degradation, as they probe different regions of Aβ. These experiments were carried out at 2-fold peptide molar excess. As shown in Figure 4, Aβ degradation was detected within 10 min by 5C3 (C-terminal specific antibody) and within 15 min by 6E10 (N-terminal specific) and 4G8 (central domain-specific). Neither Gsc, EFh, or G12 had any significant effect on the time course of Aβ degradation. These results are consistent with the cross-linking studies, indicating little to no interaction of G12, Gsc, or EFh with Aβ.

With both G16 and GLA, all three epitopes were protected from proteolysis for significantly longer times than Aβ alone. These results are partially consistent with the cross-linking study. Both assays demonstrate an interaction between G16 and Aβ. For GLA, there was much less interaction detected in the PICUP assay compared to the proteolysis assay. We suspect that the difference derives from the Aβ:peptide ratio and the peptide concentration: specifically, the cross-linking experiments were run under Aβ excess (test peptide concentration = 2.4 μM) while the protease-resistance experiments were conducted with peptide excess (test peptide concentration = 40 μM). Low-affinity interaction between GLA and Aβ becomes apparent at the higher GLA concentration. We suspect that this weak interaction is due to its similar hydrophobicity but reduced amyloidogenicity compared to G16.

Effect of G16 on Aβ Aggregation Kinetics. We next probed for evidence of interaction between Aβ and TTR-derived peptides by measuring the effect on Aβ aggregation kinetics using light scattering (Figure 5). Under the conditions of the experiment, Aβ alone aggregated slowly, as measured by both the mean aggregate size (Figure 5a) and the intensity of scattered light (Figure 5b). Samples containing Aβ and G16 or Gsc were tested next. At the low peptide concentration of these experiments, there was no detectable scattering above noise from the peptides in the absence of Aβ (data not shown). G16 dramatically increased the average size and the rate of growth of Aβ aggregates (Figure 5). The control peptide Gsc had no effect on Aβ aggregation.

To identify reasons for the large increase in aggregation caused by G16, we examined samples by TEM. Aβ incubated for 20 h at 37 °C contained a mix of short flexible "protofibrils" and a few long fibrillar aggregates (Figure 6a). Addition of G16 at a 12-fold Aβ excess resulted in a marked shift in morphology: numerous spherical globules appeared, along with a network of short and thin entangled "protofibrils" (Figure 6b). G16 alone contained a few flattened micellar-like structures (Figure 6c). After 9 days of incubation, precipitates were visible to the naked eye in the Aβ sample but none were observed in the Aβ + G16 sample. Approximately ~80% of Aβ, but only ~25% of the Aβ +G16 mixture, could be pelleted by centrifugation after 9 days of incubation.

ThT fluorescence is generally indicative of aggregates with cross-β sheet structure.[48−50] ThT fluorescence intensity of all samples was low immediately after sample preparation, and increased after 20 h. ThT fluorescence in mixtures of Aβ + G16

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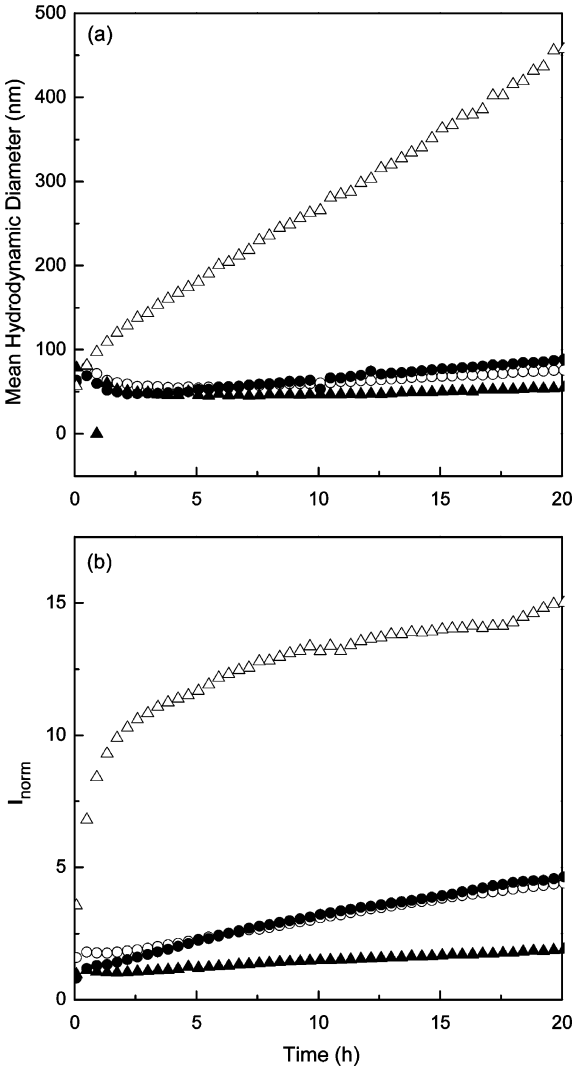

Figure 5. Effect of TTR-derived peptides and mTTR on Aβ aggregate growth kinetics. The mean hydrodynamic diameter (a) and average scattering intensity (b) were measured by dynamic light scattering. Samples contained 140 μM Aβ alone (○) or Aβ with peptide G16 (△), with peptide Gsc (●), and with mTTR (▲). Concentration for G16, Gsc, and mTTR was 12 μM. At the conditions of these experiments, concentrations of G16, Gsc, and mTTR were too low to contribute to the scattering signal.

was modestly reduced (~20% compared to Aβ alone) whereas Gsc had no effect (Figure 7).

Taken together, we hypothesize that G16 oligomers (observed both in PICUP and TEM) scavenge Aβ monomers and/or small Aβ oligomers, creating larger soluble globular oligomeric assemblies. G16 reduces or eliminates further growth of Aβ fibrils, while having little or no effect on pre-existing Aβ fibrils. This explanation is consistent with the increase in the molecular weight of cross-linked Aβ aggregates in the presence of G16, the large increase in aggregate size and scattering intensity detected by light scattering, the shift in morphology observed by TEM, the decrease in the formation of precipitable aggregates, and the small decrease in thioflavin T fluorescence.

Comparison to TTR. mTTR is an engineered transthyretin mutant that is stable as a monomer;[51] solvent exposure of strand G is much higher in monomeric than tetrameric TTR

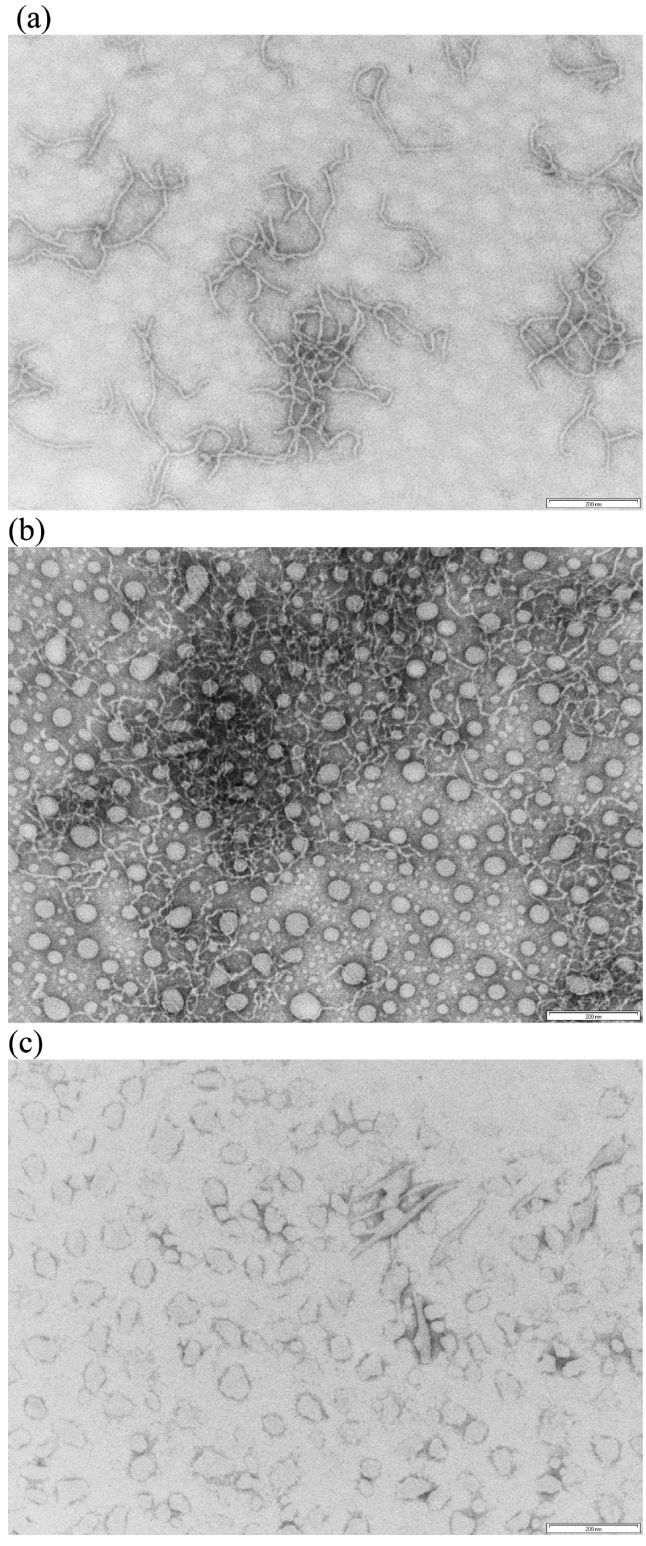

Figure 6. TEM images, taken after 20 h incubation at 37 °C: (a) 140 μM Aβ, (b) 140 μM Aβ + 12 μM G16, and (c) 12 μM G16. Scale bar is 200 nm in all images.

(Figure 1) . Like G16, mTTR reduced ThT fluorescence of Aβ (Figure 7). In sharp contrast to G16's effect, mTTR inhibited rather than enhanced Aβ aggregation (Figure 5). This result is consistent with our previous report that mTTR decreased Aβ aggregation, as measured by both arrest of growth of aggregate size as well as inhibition of formation of new aggregates.[33]

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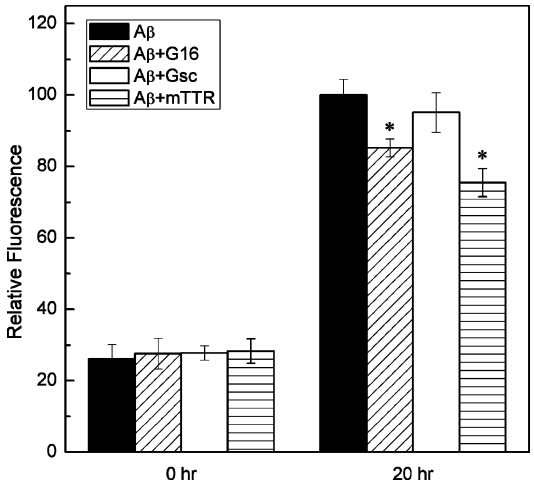

Figure 7. Thioflavin T fluorescence intensity. Samples were prepared with Aβ alone (30 μM) or with the indicated peptide or protein (2.5 μM), and then analyzed immediately or incubated for 20 h prior to analysis. Samples were diluted 6.5-fold into ThT-containing solution, and fluorescence emission intensity was measured immediately. Background intensity was measured with ThT-containing solution and subtracted. *Differs from Aβ alone ($p < 0.05$).

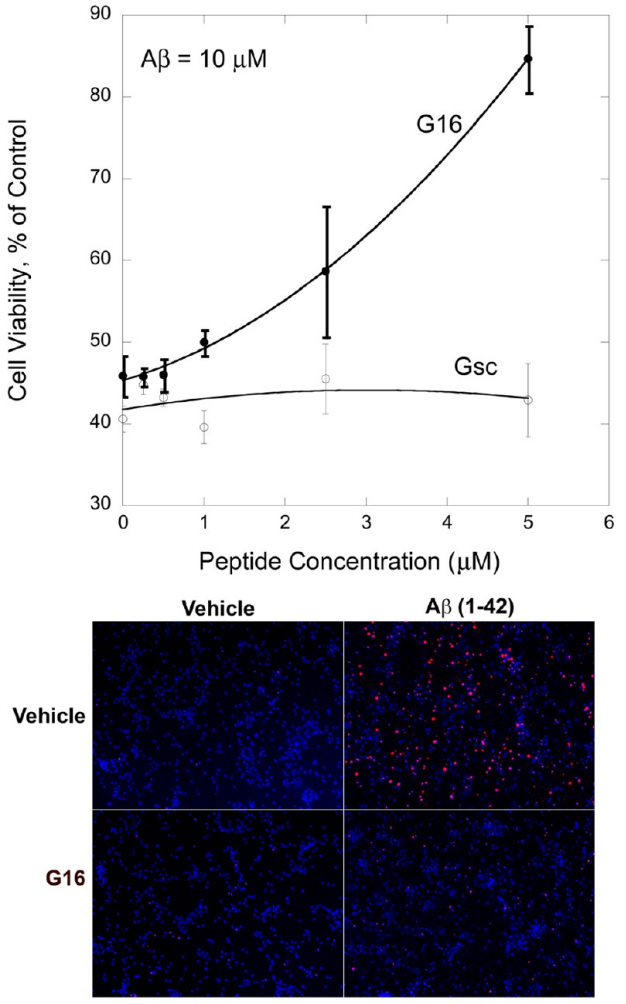

Figure 8. (top) Toxicity was measured in primary neuronal cultures using the MTS assay. G16 or Gsc was added at the indicated concentration and incubated with Aβ (10 μM) for 1 h before addition to neuronal cultures. (bottom) TUNEL staining was used to confirm the results of the MTS assay. Aβ alone (10 μM) was toxic; 5 μM G16 prevented Aβ toxicity.

Previously we showed by TEM that Aβ fibrils were shorter in the presence of mTTR, but there was no change in the morphology.[34] Thus, although both mTTR and G16 bind to Aβ, presumably via similar binding domains, the outcome of that binding interaction is quite different. mTTR binds to Aβ aggregates and prevents their continued growth, but does not cause significant conformational changes. In contrast, remodeling of Aβ to large globular aggregates is a consequence of G16 binding to Aβ. There are several possible explanations for differences between G16 and mTTR in their effect on Aβ aggregation. One possibility is that the oligomeric nature of G16 facilitates multivalent binding to Aβ and subsequent formation of clusters of oligomers. Since mTTR does not self-associate under our experimental conditions, it also does not coalesce Aβ oligomers into larger aggregates. Another possibility is that the greater conformational flexibility of the G16 binding surface may facilitate its adaptation to and remodeling of Aβ, while steric restrictions from the nonbinding scaffold of mTTR prevent remodeling.

Effect of TTR-Derived Peptides on Aβ Toxicity. Given that G16 bound to Aβ but displayed different effects on Aβ aggregation than did TTR and mTTR, we tested whether G16 was effective at inhibiting Aβ toxicity. Since Aβ oligomers are widely believed to be more toxic than fibrils,[35] and since our data indicated that G16 greatly increased the appearance of soluble globules in Aβ, we were concerned that G16 might actually enhance toxicity. Using an MTS assay, we observed that 10 μM Aβ was toxic to primary neuronal cultures and that G16 inhibited Aβ toxicity in a dose-dependent manner (Figure 8, top). No inhibition of toxicity was observed for Gsc (Figure 8, top). Neither G16 nor Gsc alone was toxic (data not shown.) The results from MTS assay were confirmed by TUNEL staining (Figure 8, bottom). We conclude that G16 inhibits Aβ toxicity at substoichiometric ratio, as a consequence of its binding. The fact that both G16 and TTR inhibit toxicity, although they have very different effects on Aβ aggregation, suggest that it is the binding interaction per se that is the relevant measure for impact on toxicity rather than the Aβ aggregation state. It has been hypothesized that Aβ toxicity is not linked to the *presence* of prefibrillar aggregate(s), but rather to the *process* of their growth into fibrils.[52] This might help to explain why both TTR and G16 prevent Aβ induced toxicity even though they have different effects on Aβ aggregation.

However, G16 is not as effective as mTTR. Complete protection against 10 μM Aβ was afforded by mTTR at 0.2 μM (2.8 μg/mL) and by G16 at 5 μM (10 μg/mL). (mTTR alone was tested and caused no loss of cell viability compared to control, data not shown.) It is possible that G16 affinity for Aβ is lower than that of mTTR, because the flexible linear peptide is not in the best conformation to support binding, or because there are other noncontiguous parts of the inner sheet of mTTR that are necessary for highest affinity. Alternatively, self-assembly of G16 could reduce its effective concentration. We do not know if G16 self-assembly is helpful (by increasing opportunity for multivalent association) or harmful (by reducing effective concentration). A third possibility is that the difference in efficacy of G16 versus mTTR indicates that enhancement of aggregation and remodeling of aggregates is not as effective as suppression of aggregation. Future steps include the synthesis of peptides that are conformationally

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constrained or that do not self-associate, and grafting of TTR-derived sequences onto minimal protein scaffolds, in an attempt to bridge the gap in efficacy between native TTR and TTR mimics.

Our results demonstrate that G16, a TTR-derived peptide that was chosen based on previous identification of relevant binding domains on TTR, can specifically interact with Aβ, affect Aβ aggregation, and inhibit Aβ toxicity. The amyloidogenic nature of G16 appears to be a critical property of the peptide (Figure 2c). In fact, after 9 days incubation at 37 °C, G16 itself forms short stiff fibrils (Figure 9). Our data seem to

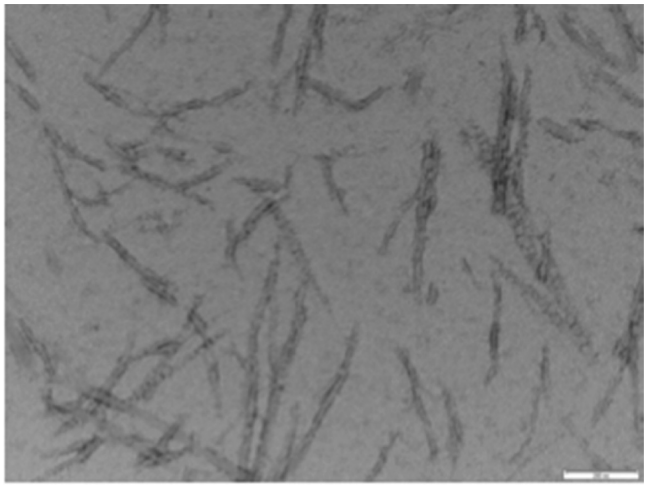

Figure 9. EM of G16 taken after 9 day incubation at 37 °C and 12 μM.

suggest that when two disordered peptides, Aβ and G16, both of which by themselves can assemble into fibrillar aggregates, are mixed, they interact but form nonfibrillar globules.

TTR is not the only native protein that is known to bind Aβ. Other proteins, such as cystatin C[53] or clusterin,[54] also bind Aβ oligomers. TTR, cystatin C, and clusterin are all present in cerebrospinal fluid, with the former at somewhat higher concentrations (~50 nM for clusterin, ~250 nM for TTR, and ~500 nM for cystatin C[54,55]). Thus, there are proteins other than TTR that are abundant in CSF and that could serve as natural protective agents against Aβ toxicity. We chose TTR as the template for this study because of our prior work with TTR and Aβ,[28,33,36] but similar approaches could be used with clusterin, cystatin C, or other proteins.

TTR may act as a general scavenger of amyloidogenic peptides beyond Aβ, possibly using the same binding domain. In a recent study, Cascella et al. demonstrated that TTR binds to oligomers of the amyloidogenic protein HypF-N and suppresses its toxicity.[56] These results suggest that TTR serves as a general scavenger of amyloid-prone peptides and proteins, possibly mediated by the same binding domain.

METHODS

Peptide Synthesis and Purification. All materials were from Fisher Scientific (Waltham, MA) unless otherwise stated. Peptides were synthesized using standard Fmoc solid-phase methods on the Symphony peptide synthesizer (Protein Technologies, Inc., Tucson, AZ). The resin used was Fmoc-PAL-PEG-PS from Applied Biosystems (Foster City, CA). Extended cycles and double couplings were used to improve yield. All peptides were modified with N-terminal acetylation and C-terminal amidation. Peptides were cleaved manually from the resin by suspending the resin in a cleavage cocktail of 81.5% trifluoroacetic acid (TFA), 5% thioanisole, 5% phenol, 5% water, 2.5% ethanedithiol (Fluka, Buchs, Switzerland), and 1% triisopropylsilane for 4 h at room temperature with occasional shaking. For peptides which do not contain Arg residue (EFh and G12), a cleavage cocktail of 95% TFA, 2.5% water, and 2.5% triisopropylsilane was used instead. Cleaved peptides were dripped into cold *t*-butyl methyl ether on ice, and precipitated by centrifugation. Precipitated peptides were then dried in vacuum, dissolved in 40% acetonitrile/water and lyophilized for purification.

Crude peptides were purified by reverse-phase HPLC on a Vydac C18 column (Grace Davison Discovery Science, Bannockburn, IL). Peptides were eluted from the column with a linear gradient of acetonitrile and water with 0.1% TFA. Purified peptide was lyophilized, and identity was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Purified peptides were dissolved in 0.22 μm filtered water, aliquoted, snap-frozen, and stored at −80 °C. G12 was dissolved in DMSO since it was not soluble in water. The concentration of peptide was determined by comparison to intrinsic fluorescence of tryptophan standards in 8 M urea. The concentration of G12 was determined using BCA protein assay (Pierce, Rockford, IL).

Aβ Sample Preparation. Aβ(1−40) was purchased from Anaspec, Inc. (Fremont, CA) as lyophilized powder and used without further purification. For dynamic light scattering and SPOT peptide array analysis, Aβ stock in 8 M urea was prepared as described,[57] snap-frozen in ethanol with dry ice, and stored at −80 °C. For PICUP and proteolytic fragmentation analysis, lyophilized Aβ was dissolved in prechilled hexafluoroisopropanol (HFIP, Acros Organics, Geel, Belgium) to a concentration of 0.5 mM. After 30 min at room temperature, Aβ was divided into aliquots of 50 μg and HFIP was evaporated overnight. Dried peptides were stored at −20 °C. Aβ was reconstituted by dissolving in 50 mM NaOH to 1 mg/mL, sonicating for 5 min and diluting into phosphate-buffered saline (PBS) [10 mM Na_2HPO_4/NaH_2PO_4 and 150 mM NaCL (pH 7.4)] to the desired concentration (25 or 30 μM). To neutralize the NaOH and adjust the pH, HCl was added. Reconstituted Aβ was centrifuged at 16,000 × g for 10 min and top 95% was collected and sonicated briefly before use. Aβ samples were used immediately or after incubation for 24 h.

SPOT Peptide Array. Peptides (sequences shown in Table 1) were synthesized onto a cellulose membrane (Sigma-Genosys, St. Louis, MO). The SPOT membrane was incubated with preaggregated Aβ, and bound Aβ was transferred to PVDF membrane and detected as previously described.[36]

Photoinduced Cross-Linking of Aβ and TTR-Peptides. The photoinduced cross-linking of the unmodified protein (PICUP) experiment was performed according to the protocol of Fancy and Kodadek[58] with some modifications.[59] Aβ prepared with HFIP/NaOH treatment at 30 μM as described was incubated at 37 °C for 24 h and mixed with TTR-derived peptides to a final concentration of 24 μM Aβ and 2.4 μM peptides. Samples of Aβ with and without peptides were incubated at 37 °C for 1 h prior to cross-linking. Cross-linking was done as previously described.[33] Cross-linked samples were heated at 95 °C for 5 min and separated on a 10−20% Tris-Tricine gradient gel (Invitrogen, Carlsbad, CA). Peptides were transferred onto a 0.2 μm poly(vinylidene difluoride) (PVDF) membrane at 25 V for 90 min. The membrane was blocked with 5% nonfat dry milk in T-TBS [Tris-buffered saline (TBS); 20 mM Tris and 150 mM NaCl (pH 7.4) with 0.05% (v/v) Tween 20] overnight at 4 °C, and reacted with monoclonal mouse anti-Aβ antibody 4G8 (Covance, Princeton, NJ) at a 1:6000 dilution in T-TBS with 5% nonfat dry milk for 2 h. After washing, the membrane was incubated with the antimouse immunoglobulin/HRP at a 1:6000 dilution in T-TBS with 5% nonfat dry milk for 1 h, washed, and visualized by means of the ECL Western Blotting Analysis System (GE Healthcare, Buckinghamshire, U.K.).

TTR-derived peptides were cross-linked and analyzed for self-association. Twenty microliters of peptides at 24 μM in PBS was incubated at 37 °C for 1 h and cross-linked. Cross-linked samples were then heated at 95 °C for 5 min and separated on a 10−20% Tris-Tricine gradient gel. Peptides were visualized by silver staining (SilverStain Kit, Pierce).

Proteolytic Fragmentation Assay. The proteolytic fragmentation assay of Aβ with TTR-derived peptides was performed based on the protocol by Ladiwala et al.[47] with some modification. Aβ was prepared at 25 μM with HFIP/NaOH treatment and incubated for 24 h at room temperature. Aβ alone (20 μM) or with peptide (40 μM) was incubated for 30 min at room temperature. Proteinase K (Promega, Madison, WI) was added to final concentration of 0.5 μg/mL and 1.5 μL of Aβ sample was dotted onto a 0.45 μm nitrocellulose membrane (Pierce) after incubation for 0, 5, 10, 15, 20, 30, 40, 50, 60, and 90 min. The proteolytic reaction was quenched quickly as the membrane was dried after deposition. The dried membranes were blocked with 5% nonfat dry milk in T-TBS overnight at 4 °C and reacted with monoclonal antibodies against Aβ; 6E10 (Covance) against Aβ(3−8), 4G8 against Aβ(18−22), and 5C3 (EMD Millipore) against C-terminus of Aβ(1−40) diluted to 0.2 μg/mL in T-TBS with 5% nonfat dry milk. After washing, the membranes were incubated with antimouse immunoglobulin/HRP at a 1:4000 dilution in T-TBS with 5% nonfat dry milk. The membranes were then washed and visualized with the ECL Western Blotting Analysis System.

Dynamic Light Scattering (DLS). Aβ stock in 8 M urea prepared as stated above was thawed and diluted into filtered PBSA [PBS with 0.02% w/v NaN_3] or PBSA containing TTR-derived peptides or mTTR. Aβ alone (140 μM) or mixed with peptides (12 μM) or with mTTR (0.16 mg/mL) was filtered through 0.45 μm filter directly into a light scattering cuvette and placed into a bath of the index-matching solvent decahydronaphthalene with temperature controlled to 37 °C. Light scattering data were collected using a Brookhaven BI-200SM system (Brookhaven Instruments Corp., Holtsville, NY) and an Innova 90C-5 argon laser (Coherent, Santa Clara, CA) operating at 488 nm and 150 mW. The average scattering intensity at 90° was measured over a 20 h interval and data were normalized to the total mass concentration. The z-averaged hydrodynamic diameter was determined from the autocorrelation function using the method of cumulants.

Thioflavin T (ThT) Fluorescence Assay. ThT stock solutions was prepared in water and filtered through 0.22 μm filters followed by measurement of the concentration using an extinction coefficient of 26 620 M^{-1} cm^{-1}. ThT stock solution was then diluted to 20 μM in PBS prior to the measurement. Aβ alone (30 μM) and Aβ with 2.5 μM G16, Gsc, or mTTR were prepared in PBSA and analyzed immediately or incubated for 20 h at 37 °C. Twenty microliter of protein samples were then mixed with 130 μL of 20 μM ThT. ThT fluorescence emission was measured using a QuantaMaster spectrofluorometer (PTI, Birmingham, NJ), with excitation at 440 nm and emission spectra recorded from 450 to 550 nm. Three serial spectra were averaged for each sample and the background signal of ThT in PBS was subtracted from the averaged data. Fluorescence intensity at a wavelength of 480 nm of each samples were compared.

Expression and Purification of mTTR. Recombinant human transthyretin mutant F87M/L110 M (mTTR) was produced and purified as previously described in detail.[34]

Transmission Electron Microscopy (TEM). Aβ alone (140 μM) or with G16 (12 μM), and G16 alone (12 μM) were prepared in PBSA and incubated for 20 h at 37 °C. A drop of sample was placed on a pioloform-coated grid and stained with methylamine tungstate stain. Images were then taken with a Philips CM120 scanning transmission electron microscope (FEI Corp., Eindhoven, The Netherlands).

In Vitro Cellular Toxicity. Primary cortical neuronal cultures were generated as described previously.[33] One mg/mL Aβ(1−42) (American Peptide, Sunnyvale, CA) was prepared in phosphate buffered saline (PBS) and then diluted to a final concentration of 10 μM with peptide (G16 or Gsc) for 1 h in NBM supplemented with B27 minus antioxidant, L-glutamine, and PS. Aβ alone or with peptide was then added to cells at 6 days in vitro (DIV), and viability was assessed at 8 DIV. The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2*H*-tetrazolium salt] assay (Promega) was used according to the manufacturer's instructions to assess cell viability as described previously.[33] Triplicate measurements were taken at each condition; data are reported relative to medium-treated cells. For TUNEL staining, cells were fixed for 1 h with 4% paraformaldehyde followed by brief treatment with permeabilization solution (0.1% Triton-X-100 in 0.1% Sodium Citrate) on ice. Permeabilized cells were labeled with the Roche (Indianapolis, IN) In Situ Cell Death Detection kit according to manufacturer's instructions. Apoptotic cells are identified by fluorescein labeling of DNA strand breaks with the TdT enzyme. Cells were counter stained with DAPI and imaged using a Zeiss microscope.

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P.Y.C. and R.M.M. designed and planned the overall study. P.Y.C. synthesized peptides and performed biochemical characterization studies. G.J. performed cellular toxicity studies. All authors contributed to data analysis and interpretation, figure preparation, and manuscript writing.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

Aβ, beta-amyloid; AD, Alzheimer's disease; APP, amyloid precursor protein; CD, circular dichroism; CSF, cerebrospinal fluid; DLS, dynamic light scattering; DIV, days in vitro; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2*H*-tetrazolium salt; NBM, neurobasal medium; NTA, nanoparticle tracking analysis; PMS, phenazine methosulfate; PBS, phosphate-buffered saline; PICUP, photoinduced cross-linking of unmodified proteins; PS, penicillin/streptomycin; RBP, retinol-binding protein; TBS, Tris-buffered saline; TTR, transthyretin; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; wt, wild-type

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