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Transthyretin Mimetics as Anti- β -Amyloid Agents: A Comparison of Peptide and Protein Approaches

Dr. Kayla M. Pate, Dr. Brandon J. Kim, Prof. Eric V. Shusta, and Prof. Regina M. Murphy

Department of Chemical and Biological Engineering, University of Wisconsin – Madison, 1415 Engineering Drive, Madison WI 53706 (USA)

Abstract

β -Amyloid ($A\beta$) aggregation is causally linked to neuronal pathology in Alzheimer's disease; therefore, several small molecules, antibodies, and peptides have been tested as anti- $A\beta$ agents. We developed two compounds based on the $A\beta$ -binding domain of transthyretin (TTR): a cyclic peptide cG8 and an engineered protein mTTR, and compared them for therapeutically relevant properties. Both mTTR and cG8 inhibit fibrillogenesis of $A\beta$, with mTTR inhibiting at a lower concentration than cG8. Both inhibit aggregation of amylin but not of α -synuclein. They both bind more $A\beta$ aggregates than monomer, and neither disaggregates preformed fibrils. cG8 retained more of its activity in the presence of biological materials and was more resistant to proteolysis than mTTR. We examined the effect of mTTR or cG8 on $A\beta$ binding to human neurons. When mTTR was co-incubated with $A\beta$ under oligomer-forming conditions, $A\beta$ morphology was drastically changed and $A\beta$ -cell deposition significantly decreased. In contrast, cG8 did not affect morphology but decreased the amount of $A\beta$ deposited. These results provide guidance for further evolution of TTR-mimetic anti-amyloid agents.

Keywords

aggregation; β -amyloid peptides; cyclic peptides; pluripotent stem cells; transthyretin

Introduction

Alzheimer's disease (AD) is the most common form of dementia, affecting approximately 5.4 million people in the US alone.^[1] With this number expected to triple by 2050,^[1] there is a desperate need for AD therapeutics that target the disease pathology; unfortunately, currently available FDA-approved drugs provide only symptomatic relief. β -Amyloid ($A\beta$) is a key component in AD pathology and therefore has been a main therapeutic target. $A\beta$ is the proteolytic cleavage product of the amyloid precursor protein APP. Variable cleavage releases several different $A\beta$ isoforms, of which the most common are $A\beta_{1-40}$ and $A\beta_{1-42}$. Although monomeric $A\beta$ is natively unfolded and nontoxic, the peptide spontaneously self-

Correspondence to: Regina M. Murphy.

Conflict of interest

The corresponding author holds a US patent on the cyclic peptide described in the manuscript.

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associates into soluble oligomers and insoluble aggregates with high β -sheet content and fibrillar morphology. The soluble oligomers are believed by many researchers to be the most neurotoxic species, while the insoluble fibrils eventually deposit as the amyloid plaques that are one of the defining features of AD brain pathology. $A\beta$ is one of many amyloidogenic proteins associated with degenerative diseases; other well-known examples including amylin (islet amyloid polypeptide or IAPP) in type II diabetes and α -synuclein in Parkinson's disease.^[2]

Because $A\beta$ aggregation is intrinsically linked to $A\beta$ toxicity, numerous small molecules, such as the polyphenols epigallocatechin-3-gallate and curcumin, 3-amino-1-propanesulfonic acid and scyllo-inositol, have been developed, with the goal of binding $A\beta$, inhibiting $A\beta$ aggregation, and decreasing toxicity.^[3] However, most of these $A\beta$ aggregation inhibitors have modest affinity and require stoichiometric or greater quantities; furthermore, selectivity and specificity are often unreported, and none have proved to be clinically effective.^[3, 4]

$A\beta$ - $A\beta$ interactions leading to aggregate formation are stabilized by a large contact area between adjacent $A\beta$ monomers. Inhibition of protein-protein interactions with small molecules is challenging because the protein-protein surface area is typically large, relatively featureless, and relatively flexible.^[5] Thus, small-molecule approaches to inhibiting $A\beta$ aggregation may suffer from poor selectivity and unwanted side effects.^[6] Monoclonal antibodies offer higher affinity and excellent specificity; several anti- $A\beta$ antibodies (such as bapineuzumab and solanezumab) have been developed that showed promise in animal studies but have been largely unsuccessful in clinical trials.^[3e, 7] Antibodies that specifically target $A\beta$ oligomers and aggregates, rather than monomers, may be an essential feature for clinical success.^[7d] Drawbacks of monoclonal (or other protein therapeutics) include poor oral bioavailability, high cost, and susceptibility to proteolysis.^[6] A third approach is to generate peptides and peptidomimetics that bind to $A\beta$. Peptides and their derivatives occupy a space midway between small-molecule and protein therapeutics. Their larger size affords improved selectivity and affinity over small-molecule drugs, while chemical synthesis provides opportunities to incorporate features that enhance bioavailability and stability while lowering cost of manufacture relative to protein therapeutics.^[5, 6]

A common strategy for targeting $A\beta$ is to incorporate a self-recognition domain derived from an $A\beta$ sequence.^[3a,d,f-h, 5, 8] Alternatively, $A\beta$ -binding peptides have been identified by screening phage-display libraries.^[3f, 9] Our strategy for designing anti- $A\beta$ agents is distinct from these other approaches (Figure 1). We start with evidence, from animal studies, that transthyretin (TTR) overexpression protects against $A\beta$ deposition and toxicity.^[10] TTR is a homotetrameric protein that functions as a carrier for thyroxine and forms a complex with retinol binding protein (RBP) for the transport of retinol. TTR has been shown to decrease $A\beta$ toxicity in neuronal cell cultures and in transgenic animal models^[11] and is co-localized with amyloid deposits in human AD brain.^[11c] This motivated our group and others to investigate TTR- $A\beta$ interactions, leading to the discovery that the main $A\beta$ -binding site of TTR resides in the "inner" β -sheet, including specific residues in strands G and H.^[10a,b, 11b,d, 12] Relying on these discoveries, we synthesized peptides that mimicked the $A\beta$ -

binding site of TTR. Our first iteration, a linear peptide, was moderately successful at inhibiting A β fibrillogenesis and decreasing A β toxicity.^[13] To increase peptide stability, we then created a cyclic peptide which showed greater inhibitory capability.^[14] Further evolution produced cG8, which increased efficacy by increasing the adoption of an antiparallel β -strand structure and decreasing the tendency to self-associate.^[15]

As an alternative to synthetic peptides, we also tested an engineered TTR mutant, F87M/L110M (mTTR). Unlike its wild-type tetrameric parent, mTTR is stable as a monomer and does not aggregate.^[11d, 16] Our previous studies have shown that, relative to wild-type (tetrameric) TTR, mTTR is more effective at binding to A β and decreasing A β toxicity, probably because of greater access of A β to its putative binding site.^[10b, 11d, 12b, 13] mTTR is also unable to bind thyroxine and RBP, so it does not interfere with the normal functions of wild-type TTR. Furthermore binding of RBP to wild-type TTR decreases its ability to inhibit A β aggregation (manuscript in preparation).^[12a] mTTR does not suffer from that concern.

In the study presented here, cyclic peptide cG8 and engineered protein mTTR were directly compared for their potential as anti-A β agents. Specifically, cG8 and mTTR were compared for 1) efficacy at inhibiting A β fibrillogenesis at low dose, 2) activity against other amyloidogenic proteins, 3) relative binding of A β monomers, oligomers or fibrils, 4) ability to disaggregate preformed A β fibrils, 5) interference from other proteins and lipids, and 6) stability against acidic conditions or proteolytic cleavage. Additionally, the association of A β to induced pluripotent stem cell (iPSC)-derived neurons as a function of A β aggregation state was imaged by confocal fluorescence microscopy, and the impact of cG8 or mTTR on cell association was evaluated. Combined, these findings highlight the relative advantages and disadvantages of using a protein versus peptide as a therapeutic anti-A β agent and provide guidance for future development of a therapeutic that combines the advantages of both mTTR and cG8.

Results

cG8 and mTTR are effective inhibitors of A β ₁₋₄₀ and A β ₁₋₄₂ fibril formation

A β fibril formation was measured using a thioflavin T (ThT) assay, a dye that fluoresces in proportion to the mass of amyloid fibril. Freshly prepared A β was ThT-negative, as expected. The fluorescence signal increased at 24h and again at 48 h after preparation, indicating aggregation of A β into ThT-positive fibrils (Figure 2, white bars). Inhibition of A β aggregation by TTR, cG8 or mTTR was compared at varying A β /inhibitor molar ratios of 20:1, 50:1, and 75:1. After 24 or 48 h co-incubation, TTR decreased the fibril mass compared with A β alone, even at 75-fold excess A β (Figure 2A). cG8 was also able to decrease A β aggregation at ratios of 20:1 (24 h and 48 h) and 50:1 (48 h, Figure 2B), and was overall similarly effective as TTR. mTTR not only proved effective at decreasing A β aggregation at all molar ratios (Figure 2C) but outperformed TTR at 20:1 and 50:1 ratios ($p < 0.05$). The enhanced performance of mTTR vis-à-vis TTR is consistent with previously obtained results, and can be attributed to lowered steric hindrance of A β to its binding site.^[11d]

To ensure aggregation inhibition was not A β isoform-dependent, ThT aggregation assays were repeated with A β ₁₋₄₂ at A β ₁₋₄₂/inhibitor molar ratios of 10:1 and 20:1. Results were similar to that of A β ₁₋₄₀: both cG8 and mTTR successfully decreased A β ₁₋₄₂ fibril formation (Figure 3A).

cG8 and mTTR inhibit some but not all amyloidogenic proteins

A cross- β conformation, a fibrillar morphology, and binding of ThT are features that A β shares with other amyloidogenic proteins. Several anti-A β agents have been reported to interact with multiple amyloidogenic proteins,^[23] likely due to the structural properties shared by amyloid aggregates.^[23f, 24] For example, EGCG inhibits fibrillogenesis not only for A β , but also for amylin, α -synuclein, huntingtin, and prion.^[25] Antibodies such as A11 show broad cross-reactivity with oligomeric amyloid-related intermediates.^[23f, 24] While having a therapeutic capable of targeting multiple amyloidogenic proteins has its benefits, it also brings into question the specificity of these compounds. We reasoned that, if cG8 and/or mTTR were recognizing an amyloid-related structural feature on A β , they may also recognize other amyloidogenic proteins and inhibit their aggregation. ThT fluorescence intensity was used to determine if cG8 or mTTR could inhibit the aggregation of amylin or α -synuclein. Both cG8 and mTTR significantly decreased amylin aggregation even at 20-fold excess amylin (Figure 3B) but neither had any impact on α -synuclein aggregation (Figure 3C). Thus, cG8 and mTTR are not broad-spectrum anti-amyloid agents. This result suggests that cG8 and mTTR recognize similar epitopes that A β and amylin share but that α -synuclein does not possess.

cG8 and mTTR bind more oligomeric and fibrillar than monomeric A β but do not disaggregate preformed fibrils

cG8b or mTTRb were immobilized on NeutraAvidin-coated plates, and binding of A β monomer (fresh), oligomers (RT, 24 h), and fibrils (37 °C, 24 h) was determined by ELISA, with detection by the anti-A β antibody 6E10. A direct quantitative comparison between cG8b and mTTRb should not be made due to potential differences in the amount of peptide versus protein immobilized in each well. However, both cG8b and mTTRb bind substantially more A β in oligomer-rich and fibril-rich environments than when there is only A β monomer (Figure 4A,B).

Because cG8 and mTTR bound to both A β oligomers and fibrils, we asked whether either could disaggregate preformed fibrils. cG8 and mTTR were incubated with preformed A β fibrils at 20:1, 10:1, 2:1 and 1:1 A β /inhibitor molar ratio for 48 h, after which ThT fluorescence was measured. No significant decrease in ThT fluorescence was observed (Figure 4C,D, data not shown). Thus, despite the fact that coincubation at A β /inhibitor ratios as low as 50:1 or 75:1 inhibited formation of A β fibrils, even at equimolar concentrations, cG8 and mTTR do not disaggregate fibrils. This feature has also been reported for TTR^[26] and is advantageous as it avoids the possibility that fibrils might be inadvertently disaggregated into the more toxic oligomeric form.

cG8 retains A β binding in the presence of other biological macromolecules better than mTTR

Anti-amyloid agents will need to target A β within a biological environment containing proteins, lipids, carbohydrates and other macromolecules. Therefore, we next checked whether cG8 and mTTR retain A β binding in the presence of proteins and lipids. cG8b and mTTRb were immobilized on NeutrAvidin-coated plates. Mouse brain tissue lysate or lysate from human iPSC-derived neurons was spiked with oligomeric A β and then added to each well. Final samples contained 5 $\mu\text{g mL}^{-1}$ A β and 0, 1, 5, 15, or 30 mg dL^{-1} lysate protein. Binding of A β decreased in the presence of both tissue and cell lysate. However, A β binding to cG8b was significantly less affected than binding to mTTRb (Figure 5A). At 30 mg dL^{-1} tissue lysate, A β binding to cG8b was $52 \pm 6\%$ of control (no lysate) while binding to mTTR was only $24 \pm 5\%$ of control.

To isolate whether lipids or proteins were responsible for the decrease in A β binding, porcine brain total lipid extract or FBS was spiked with A β and then added to immobilized cG8b and mTTRb. At typical CSF concentrations (2–4 mg dL^{-1}),^[27] lipids had no effect on A β binding; however, at high lipid concentrations (600 and 1800 mg dL^{-1}) binding of A β to both cG8b and mTTRb was decreased (Figure 5B). As with tissue lysate, lipids affected A β binding to cG8b significantly less than binding to mTTRb. Proteins proved to have a greater effect on A β binding than lipids, decreasing binding at concentrations typical of CSF (15 and 30 mg dL^{-1}) (Figure 5C). At high levels of FBS (5000 and 15000 mg dL^{-1}), A β binding to cG8b reached a plateau corresponding to approximately 60% of control, while A β -mTTRb binding was virtually eliminated. Combined, these results demonstrate that most of the loss in A β binding is due to interference from protein components in cell homogenates, and that cG8 is less susceptible than mTTR to interference.

cG8 and mTTR differ in stability against protease

cG8 and mTTR were incubated in SGF (pH 1.2) or SIF (pH 6.8) without enzymes for 24h at 37°C. Neither cG8 nor mTTR showed any indication of degradation after exposure to either buffer (mass spectrometry data not shown), nor was there any loss in their ability to inhibit fibril formation (Figure 6A,B). Pepsin was used to evaluate the resistance of cG8 and mTTR to enzyme degradation. mTTR degraded so quickly that even the initial sample contained protein fragments instead of intact protein, and no fragments of 5 kDa or larger were detected after 2 h (Figure 6D). cG8 was intact at $t=0$ and was only partially degraded after 2 h incubation with pepsin at 37°C (Figure 6C). Besides intact cG8, two fragments were detected of $m/z=1244.8$ and 1345.8. We used Peptide Cutter (https://web.expasy.org/peptide_cutter/) to predict that pepsin would cleave cG8 between Lys/Leu or between Leu/Ser (between residues 8/9 or 9/10 if ^DPro is residue 1). Cleavage at Leu/Ser and secondary cleavage at Val/Val or Val/Thr (residues 20/21 or 21/22, SSPYSYSQTKV or SSPYSYSQTKVV) would produce fragments of 1246.3 or 1345.5 Da. The latter cleavage site is not a preferred site for pepsin but could indicate unusual strain induced by the ^DPro-Pro template. Other possible fragments include VVTdPPRYTIK (1244.5) and VTdPPRYTIKLS (1345.6), again suggesting an unusual cleavage site. MS/MS would be required to positively identify fragmentation sites.

mTTR changes the morphology and quantity of cell-associated A β

The inhibition of A β toxicity by mTTR and TTR-inspired peptides in mouse neuronal cultures has previously been reported, with mTTR showing much greater efficacy.^[14] In this work we used human iPSC-derived neurons to detect cellular A β binding as a function of aggregation state, and to determine if cG8 and/or mTTR altered the cellular binding and internalization of A β . To do this, cells were treated with A β monomer, oligomer, or fibrils prepared alone or with cG8 or mTTR. Control treatments tested the effect of cG8 and mTTR added to preformed oligomers and fibrils. The experimental strategy is illustrated in Figure 7. Images were collected after staining for A β with both permeabilized and nonpermeabilized cells, to compare total (internalized plus cell surface-associated) versus extracellular (cell surface-associated) A β .

A β monomer, oligomers, or fibrils were prepared as illustrated in Figure 7A and added to iPSC-derived human neuronal cultures. Cells were exposed to A β (5 μ M final concentration) for 24 h before cells were washed, stained and imaged. With all three treatments, some A β became cell associated (Figure 8, Supporting Information Figure 3). Treatment with A β monomer produced a few small, punctate deposits (Figure 8A). This result suggests that little to no additional aggregation of A β monomer occurs during the 24 h cell treatment time, probably because of the decreased A β concentration. Noticeably greater amounts of deposited A β were observed with oligomer or fibril treatments, and the extensive deposits had a web-like appearance (Figure 8D,G). The fraction of each image containing A β , normalized to account for differences in cell density, was estimated as \approx 5% for A β monomer, \approx 63% for A β oligomers, and \approx 53% for A β fibrils (Supporting Information Table 1). The visual appearance of deposited A β aggregates for both oligomer and fibril preparations was similar, suggesting that oligomers progressed to fibrils during the 24 h cell exposure (total aggregation time of 48 h). Together with the monomer result (Figure 8A), this is consistent with a distinction between initiation of new aggregates versus growth and maturation of preexisting aggregates. A β deposits were clearly cell-associated, as there was no fluorescence in areas of the wells that were cell-free or in control wells that received A β treatment but contained no cells (data not shown).

Next, cG8 or mTTR was incubated with A β monomer (20:1 A β /inhibitor) for 1 h and then the neurons were exposed to the A β /inhibitor mixture for 24 h. After washing, cells were stained for A β (green) and cG8 or mTTR (red). Neither cG8 nor mTTR caused a measurable change in the amount of A β deposition or the morphology of the deposited A β , relative to A β alone (Figures 8B,C). mTTR co-localized with deposited A β , as indicated by yellow, whereas there was no observable co-localization of cG8 with A β . There was little to no nonspecific association of cG8b or mTTRb to the cells (Supporting Information Figure 4).

Next, cG8 or mTTR was added to A β and the mixtures were incubated under conditions that would normally lead to A β oligomers (RT, 24 h, Figure 7B). Neurons were exposed to these mixtures for 24 h, after which the cells were washed and stained. cG8 caused a modest decrease in the amount of cell-associated A β , from \approx 63% in absence of cG8 to \approx 47% in presence of cG8 (Figure 8E). There was no evidence that cG8 co-localized with A β deposits. In contrast, mTTR significantly decreased the amount of A β deposition, to \approx 15%. More

strikingly, there was a distinct morphological change into smaller, globular aggregates (Figure 8F). Furthermore, co-localization of mTTR with A β is clearly evident.

cG8 or mTTR was added to A β and the mixtures were incubated under conditions that would normally lead to A β fibrils (37 °C, 24 h, Figure 7B), followed by a 24 h exposure of the neurons to the solutions. The physical morphology of the A β deposits was not changed by addition of either cG8 or mTTR (Figure 8H,I). The quantity of deposited A β was partially decreased by cG8 (from \approx 53% to \approx 33%) but not by mTTR. mTTR strongly co-localized with cell-associated A β (Figure 8I) while there was little to no co-localization of cG8 (Figure 8H). Control experiments in which cG8 or mTTR was added to preformed oligomers or fibrils just prior to cell exposure (Figure 7C) showed no distinct changes in the morphology, and no change in the quantity of cell-associated A β , except for a small (\approx 10%) decrease when cG8 was added to pre-formed fibrils (Supporting Information Figures 5 and 6). Additionally, there was little to no co-localization of either inhibitor to cell-associated A β fibrils (Supporting Information Figure 6), suggesting that mTTR incorporates into the growing aggregates rather than binding to fibrils after they are formed. This result is consistent with our finding that mTTR does not break up preformed A β fibrillar aggregates (Figure 4).

A comparison of total (internalized plus cell surface-associated) A β and extracellular (cell surface-associated) A β provides additional insight (Figure 9, Supporting Information Table 1). The comparison is between two separately prepared wells, so the results should be interpreted cautiously. When A β monomer or fibrils was added to cells, there was no difference between the morphology or the quantity of extracellular and total cell-associated deposits (Figure 9A,C). With A β oligomers, there were also no observable morphological differences between extracellular and total cell-associated deposits (Figure 9B). However, there was a modest decrease in the quantity of extracellular versus total cell-associated deposits (63% versus 48%). Taken together, these results suggest that A β oligomers, but not monomers or fibrils, are partially internalized.

Because our analysis suggested that A β oligomers, but not monomers or fibrils, were internalized, we tested whether the inhibitors altered internalization. When A β was co-incubated with cG8 for 24 h at room temperature (Figure 7B) prior to cell exposure, the total cell-associated deposited A β was greater than extracellular A β (\approx 47% total versus \approx 23% extracellular, Figure 10B), suggesting that although cG8 decreases the quantity of both extracellular and internalized A β , it does not prevent A β oligomer internalization. The notable morphological change induced by mTTR under these conditions, previously described for total cell-associated A β (Figure 8F), was also observed for extracellular A β (Figure 10C). Additionally, total and extracellular A β were similar (\approx 14% total versus \approx 11% extracellular, Figure 10C). Co-incubation of cG8 or mTTR with A β as monomer or under fibril-forming conditions prior to cell treatment changed neither the amount nor the morphology of extracellular A β relative to total A β (Supporting Information Figure 7 and 8).

Discussion

Previously, we demonstrated that mTTR and TTR-inspired peptides inhibit A β fibrillogenesis and decrease A β toxicity in mouse neuronal cultures.^[13, 15] In this study, we evaluated mTTR and cG8 for their efficacy, stability, selectivity and mode of action. Our aim was to compare critical parameters that influence the choice of protein versus peptide-based compounds for anti-amyloid therapeutic strategies.

mTTR and cG8 were both effective at inhibiting amyloid fibril formation by either A β isoform, A β ₁₋₄₀ and A β ₁₋₄₂ (Figures 2 and 3A). Both were also effective inhibitors of amylin but neither was effective against α -synuclein aggregation (Figure 3B,C). This demonstrates that mTTR and cG8 have a similar degree of selectivity with respect to their interaction with amyloidogenic proteins. Their efficacy against amylin might arise from the fact that amylin is of similar length and shares 24% sequence similarity to A β ^[20] with the greatest degree of similarity in the domains crucial for self-association.^[28] α -synuclein monomers are natively disordered, like A β , but the protein is much larger (\approx 14 kDa) and has only 12% sequence similarity.^[20] Kellock et al.^[23g] recently reported on the design of a peptide that inhibits aggregation of A β , amylin and TTR in support of the notion of cross-interaction of these three amyloidogenic proteins.

mTTR and cG8 both bind greater amounts of A β oligomers and fibrils than monomers (Figure 4A,B). A β oligomers are distinct from monomers in the presence of hydrophobic patches or cavities (as detected by ANS fluorescence) on the former. As oligomers rearrange and assemble into fibrils, ANS fluorescence partially decreases and ThT fluorescence (indicative of extended cross- β conformation) increases. We hypothesize that selectivity toward oligomers and fibrils occurs because of binding of mTTR or cG8 to hydrophobic patches. As described previously, our data are consistent with the hypothesis that binding of mTTR to oligomers completely arrests both the adoption of extended cross- β conformation and further self-assembly.^[11d] The mechanism of action of cyclic peptides is similar, except that cyclic peptides also re-directs the self-association of a fraction of A β into large non-fibrillar amorphous clusters.^[14] Although both mTTR and cG8 potently inhibit fibrillogenesis of A β , neither disaggregates preformed A β fibrils (Figure 4C,D). This is a favorable feature of an anti-A β compound if intermediate oligomeric aggregates are indeed the most toxic species.

While cG8 and mTTR share many features in common, important differences were also observed. Although cG8 successfully inhibited A β fibrillogenesis at substoichiometric ratios, with overall similar dose-dependence as wild-type TTR, mTTR was more effective than cG8, suppressing fibrillogenesis even at a 75-fold excess A β (Figure 2). We hypothesize that the greater efficacy of mTTR is attributable to its attaining a stable anti-parallel two- β -strand conformation^[15] that fully mimics TTR's A β -binding site, while cG8 displays conformational heterogeneity and its structure is not as constrained. On the other hand, mTTR is more sensitive to the presence of biological materials (cell lysate, lipids or proteins), suffering a greater decrease in A β binding in their presence than does cG8 (Figure 5). We hypothesize that the decrease in efficacy in the presence of proteins is attributable to nonspecific binding of the proteins to cG8 or mTTR, and that there is less nonspecific

binding to cG8 than to mTTR because of the smaller size of the former. A much larger fraction of the residues in cG8 are part of A β -binding domain (the functional part) compared with that in mTTR. An improved therapeutic might therefore be achieved by trimming down the non-essential residues in mTTR while retaining a sufficient scaffold to stabilize the conformation of the A β -binding domain.

When exposed to the lower pH of either simulated intestinal or simulated gastric fluids, both inhibitors resisted degradation and retained their ability to inhibit A β aggregation (Figure 6). However, mTTR was extremely susceptible to enzymatic degradation while cG8 proved more stable, likely due primarily to its cyclic structure. This reflects a significant stability advantage of synthetic peptides, where protease-resistant features such as D-amino acids, other non-natural amino acids, or cyclization can be readily incorporated into the design.

We next examined association of A β with human iPSC-derived neurons as a function of A β aggregation state. To our knowledge this is the first such study reported in the literature. With pre-aggregated A β (oligomeric or fibrillar), but not with monomeric A β , at least half of the cell surface was covered with deposited A β . Oligomers, but not monomers or fibrils, appeared to be partially internalized after 24 h cell exposure. This observation, though preliminary, could explain why oligomers are more toxic than fibrils.

When A β was co-incubated with mTTR or cG8 prior to contact with neurons, mTTR was tightly integrated into the cell-associated A β aggregates whereas there was little to no colocalization of cG8 with A β . Under conditions where A β alone forms oligomers (presumably the most toxic A β species), mTTR substantially decreased the amount of A β deposited on cells and drastically altered the physical morphology of the deposits (Figure 8). In contrast, cG8 had no discernable effect on A β aggregate morphology, but decreased the quantity of A β that deposited on the cells. cG8 did not prevent A β internalization, but the amount of A β internalized was lower, presumably because the total cell-associated A β was reduced.

Under conditions where A β alone forms fibrils, neither mTTR nor cG8 affected the morphology of cell-associated A β deposits. This was an unexpected result given that both compounds strongly suppressed fibrillogenesis at similar conditions per the ThT assay (Figure 2). There are several possible explanations. First, even if the total A β fibril concentration is reduced with cG8 or mTTR, only a small quantity of A β fibrils might be needed to coat the cells. Second, contact with cells could catalyze A β fibril formation even with inhibitors present. This explanation seems unlikely, because there was no evidence of A β fibril formation during the 24 h cell exposure period from the monomer preparation. A third possibility is that cross- β structure (and hence ThT binding) of the aggregates is lost even though there is no observable change in the aggregate morphology. This explanation is unlikely, especially for mTTR, because light scattering and particle tracking experiments have shown that mTTR not only inhibits ThT signal but also prevents aggregates from growing in size.^[11d]

Taken together, these data suggest that there are different ways in which mTTR and cG8 interfere with A β toxicity. As shown in prior work, mTTR arrests A β aggregate growth

entirely whereas cG8 redirects aggregation down a non-amyloid pathway.^[13, 15] We speculate that mTTR, by retaining A β in a smaller and less aggregated state, decreases the quantity of A β deposited on cell membranes and inhibits internalization, thereby reducing A β -induced cellular toxicity. The striking effect of mTTR on A β -cell interactions was observed only when co-incubation occurred under oligomer-forming conditions. mTTR had no influence on morphology or quantity of cell-associated A β deposition when co-incubated under fibril-forming conditions even though mTTR was clearly integrated into the deposits. This suggests that mTTR may be the most effective at arresting A β growth and preventing A β toxicity when the rate of A β aggregation is moderate. On the other hand, whether co-incubated under oligomer- or fibril-forming conditions, cG8 decreased A β deposition but did not change the morphology nor prevent internalization. Because cG8 binds A β (Figure 4) and decreases the quantity of cell-associated A β deposits, but is not observed to be integrated into these deposits, we speculate that cG8 acts by preventing binding of a fraction of A β aggregates to cells. Experiments are currently underway to explore in greater depth the cellular response to A β , in the human iPSC-derived neuron model, and to elucidate the mechanisms by which inhibitors of fibrillogenesis may decrease A β toxicity.

Conclusions

This study provides a direct comparison of protein versus peptide, each designed as a mimic of the A β -binding domain on wild-type TTR, and each designed to associate with A β oligomers and inhibit amyloid fibril formation. By many measures, mTTR and cG8 were similar. Each however has its advantages and disadvantages. Specifically, mTTR is effective to lower concentrations, and has a strong impact on both the morphology and the quantity of A β deposited on cells. On the other hand, cG8's cyclic structure and smaller size results in better stability against proteolysis and less interference from nonspecific biological materials. Studies with human iPSC-derived neurons provide insight into the mechanism by which each inhibitor decreases toxicity. Combined, these results provide guidance for future work to design a molecule that combines the advantages of each.

Experimental Section

Chemicals and reagents

A β ₁₋₄₀ and A β ₁₋₄₂ were purchased from Bachem (Torrance, CA). Amylin and α -synuclein were purchased from Anaspec (Fremont, CA). Thioflavin T (ThT) and an Amersham ECL Western Blotting Analysis System were purchased from Sigma-Aldrich (St. Louis, MO) and Sulfo-NHS-LC-Biotin was purchased from VWR (Radnor, PA). Anti-A β 6E10 antibody was purchased from Covance (Princeton, NJ) and superclonal anti-mouse HRP antibody was purchased from Pierce Chemical (Rockford, IL). All other materials except where noted were purchased from Fisher Scientific (Hampton, NH).

A β sample preparation

A β ₁₋₄₀ was used in most experiments and will be denoted as A β whereas subscripts will be used to indicate when A β ₁₋₄₂ was used. A β was dissolved to 12 mgmL⁻¹ in urea (8M), glycine (10 mM) buffer (pH 10.0) to monomerize. After 1 h incubation at RT, A β stock was

aliquoted, snap-frozen, and stored at -80°C until use. Prior to experimentation, NaOH (1M) was added to A β at a 1:8 volume ratio and incubated for 20 min at RT to dissolve any A β aggregates formed during the freeze-thaw cycle. A β was then diluted into phosphate buffered saline with azide (PBSA, 10 mM Na $_2$ HPO $_4$ /NaH $_2$ PO $_4$, 150 mM NaCl, 0.02% NaN $_3$, pH 7.4). For testing of A β monomer, A β was used immediately after dilution into PBSA. A β oligomers were formed by incubating A β at RT for 24 h while A β fibrils were formed by incubation at 37°C for 24 h. ANS and ThT fluorescence confirmed monomer (no ANS, no ThT), oligomers (high ANS, low ThT), and fibrils (low ANS, high ThT) (Supporting Information Figure 1).^[17] A β_{1-42} was prepared and stored in the same fashion except that a 1:2 NaOH to A β_{1-42} volume ratio was required to remove preformed aggregates.

TTR and mTTR preparation

As previously described in detail,^[12b] recombinant human transthyretin (TTR) and transthyretin mutant F87M/L110M (mTTR) was produced and purified. Identity was confirmed by mass spectrometry and purity by gel electrophoresis. Biotinylated mTTR (mTTRb) was produced by combining mTTR with Sulfo-NHS-LC-Biotin (10 mM in H $_2$ O) at a 1:5 ratio and incubating for 30 min at RT. Free biotin was removed by centrifugation through 3 kDa MWCO filters (14 000 rpm, 4°C , 10 min). ThT analysis confirmed that mTTRb retained the ability to inhibit A β aggregation (data not shown).

cG8 Preparation cG8 was prepared using Fmoc solid-phase methods and benzotriazol-1-yl-oxytrityrrolidinophosphonium hexafluorophosphate (PyBOP) for head-to-tail cyclization as previously described in detail.^[15, 18] Biotinylated cG8 (cG8b) was synthesized by New England Peptide (Gardner, MA) by replacing Val19 with Fmoc-Glu(biotinyl-PEG)-OH. cG8b was received as a linear peptide still on the resin. Peptide was cleaved from resin by diluting in dichloromethane containing hexafluoroisopropanol (HFIP, 20%). The solution was placed on a shaker for 1 h then centrifuged (2000 rpm, 2 min) and the peptide containing supernatant was removed. Additional HFIP/CH $_2$ Cl $_2$ was added to the resin and the process was repeated. The combined supernatant was filtered and the HFIP/CH $_2$ Cl $_2$ was evaporated under nitrogen. For head-to-tail cyclization, the peptide was dissolved in CH $_2$ Cl $_2$ containing PyBoP (25 mg) and diisopropylethylamine (DIPEA, 1.25%) and shaken under nitrogen for 18 h. The solution was evaporated off under nitrogen and cyclization was repeated. After cyclization, the remaining protecting groups were removed by incubating the peptide in Reagent K for 2 h. The peptide was then treated with cold tert-butyl methyl ether (t-BME), pelleted by centrifugation (2000 rpm, 4°C , 10 min), and air dried. cG8b was purified by RP-HPLC on a Vydac C18 column as previously described.^[15] ThT analysis confirmed that cG8b retained the ability to inhibit A β aggregation (data not shown).

Thioflavin T (ThT) fluorescence assay

ThT is a fluorescent probe commonly used to monitor A β aggregation due to its binding to the β -sheet structure that is a hallmark of amyloidogenic proteins.^[17b, 19] To compare the inhibitory capability of cG8 and mTTR to that of TTR, A β monomer (28 μM) in PBSA was combined with each inhibitor at an A β to inhibitor molar ratio of 20:1, 50:1, or 75:1. A β aggregated alone was used as a control. To determine the ability of cG8 and mTTR to

disaggregate A β fibrils, A β monomer was incubated in PBSA for 24h at 37°C to allow fibrils to form. A β fibrils were then combined with cG8 or mTTR for a final A β concentration of 28 μ M and at either a 2:1 or 1:1 A β to inhibitor molar ratio. A β fibrils were used as a control. All samples were incubated for 48 h at 37°C.

Stock ThT solutions were prepared by dissolving ThT into filtered PBS. At times 0, 24, and 48 h, A β samples were diluted in ThT stock for final concentrations of 2 μ M A β and 10 μ M ThT. Fluorescence ($\lambda_{\text{excitation}}=440$ nm, $\lambda_{\text{emission}}=460-500$ nm) was measured using a QuantaMaster spectrometer (PTI, Birmingham, NJ). For each sample, three spectra were collected and the fluorescent intensity at 480 nm was averaged, baseline (PBSA, ThT) subtracted, and normalized to the respective control. TTR, cG8, and mTTR alone were ThT negative. Results are reported as the mean \pm SEM of 3–4 independent experiments.

A β_{1-42} , amylin, and α -synuclein studies

ThT fluorescence was used to determine the ability of cG8 and mTTR to inhibit the aggregation of A β_{1-42} , amylin, and α -synuclein. A β_{1-42} was prepared as described above. Amylin was diluted in cold HFIP to 500 μ M and incubated for 1 h at RT. Dissolved protein was then aliquoted and HFIP was evaporated off overnight. Dried protein aliquots were stored at -80°C until use. To initiate aggregation, amylin was diluted to 1 mM in NaOH (5 mM) and incubated at RT for 10 min, then combined with cG8 or mTTR in PBS (40 mM Na₂HPO₄/NaH₂PO₄, 150 mM NaCl, pH 7.4).

α -Synuclein was prepared per the manufacturer's instructions. Briefly, the protein was dissolved in 10 mM PBS and, after 10 min incubation at RT, centrifuged (12000 rpm, 4°C, 10 min) to remove any undissolved material. The protein was then aliquoted, snap-frozen, and stored at -80°C until use. α -synuclein was combined with cG8 or mTTR in 10 mM PBS and agitated (600 rpm) to promote aggregation.

For all three proteins, experiments were conducted at 20 μ M protein and at molar ratios of 10:1 and 20:1 protein to inhibitor. All samples were incubated for 48h at 37 °C and ThT fluorescence was measured as described above. Results are reported as the mean \pm SEM of 2–4 independent experiments.

Sequence similarity comparing A β , amylin and α -synuclein was calculated using EMBOSS Needle.^[20]

Enzyme-linked immunoassay (ELISA)

Pre-blocked NeutrAvidin protein-coated ELISA plates were used as previously described^[15] to determine A β binding to cG8b and mTTRb under various conditions. The plates were coated with either cG8b or mTTRb (5 $\mu\text{g mL}^{-1}$) for 1 h then washed three times with PBST (0.05% Tween20). To assess binding of different A β species, A β monomer, oligomers, and fibrils were diluted in PBS to 1, 3, and 5 $\mu\text{g mL}^{-1}$ and added to the plate for 1 h at 37 °C. To assess binding under physiologically relevant conditions, A β oligomers were diluted to a final concentration of 5 $\mu\text{g mL}^{-1}$ A β in PBS containing wild-type mouse cortex tissue lysate (0–30 mgdL⁻¹). This experiment was repeated with cell lysate from induced pluripotent stem cell (iPSC)-derived human neurons. To evaluate lipid interference, lipids extracted

from porcine brain tissue (Avanti Polar Lipids, Alabaster, AL) were combined with A β oligomers. Final samples contained either lipid concentrations similar to the concentration in cerebrospinal fluid (CSF) (2 and 4 mgdL⁻¹) or lipids in excess (600 and 1800 mgdL⁻¹). Similarly, the effect of proteins was tested at biological relevant levels of fetal bovine serum (FBS, 15 and 30 mgdL⁻¹) and at excess FBS concentrations (5000 and 15000 mgdL⁻¹).

After incubation with A β , the plate was again washed and anti-A β 6E10 antibody (1:3000) was added to the plate for 1 h with gentle shaking. After washing, superclonal anti-mouse HRP antibody (1:3000) was added to the plate for 1 h with gentle shaking. The plate was again washed before addition of Ultra-TMB solution. After full color development, the reaction was stopped by adding equal volume of sulfuric acid (2M). The absorbance (450 nm) was measured using an EL800 Universal Microplate Reader (Biotek Instruments Inc., Winooski, VT). For each sample, four wells were prepared and the absorbance intensity averaged. Control experiments showed that cG8b and mTTRb-coated wells receiving buffer, tissue lysate, FBS, or lipid without A β showed negligible signal (<3%) as did non-coated wells that received A β (data not shown). For A β samples containing biological materials, the absorbance was normalized to the absorbance for A β in buffer (PBST or tissue lysis buffer). Results are reported as the mean \pm SEM of 3 independent experiments.

Tissue lysate preparation

Cortex tissue for use in the ELISA assay was harvested from a 4 month, male, wild-type mouse and was a generous gift from Dr. Jeffery Johnson (School of Pharmacy, UW-Madison, WI). Lysis buffer containing TritonX-100 (1% v/v), dithiothreitol (DTT, 1mM), and protease and phosphatase inhibitors was added at 10 μ L of lysis buffer per mg of tissue. Tissue was sonicated until homogenized then centrifuged (16 000 rpm, 4 °C, 30 min) to remove cell debris. Total protein concentration of the supernatant was determined via BCA assay. Lysate aliquots were stored at -80°C until use.

Stability assay

The stability of cG8 and mTTR from pepsin-induced degradation was determined by combining 20-fold excess cG8 or mTTR to pepsin (dissolved in 10 mM HCl) and incubating for 2 h at 37°C. At 0 and 2 h, pepsin was deactivated by adding NaOH (1M) to neutralize the pH.

The stability of cG8 and mTTR was also assessed using simulated gastric fluid (SGF, pH 1.2, without enzyme) and simulated intestinal fluid (SIF, pH 6.8, without enzyme) as previously described.^[15] cG8 and mTTR were each diluted into either SGF or SIF to 0.1 mgmL⁻¹ and incubated at 37 °C. At 0 and 24 h, the samples were diluted in equal volume cold H₂O and NaOH (1M) was added to SGF samples to neutralize the pH. All samples were prepared for mass spectrometry by being spotted directly onto the plate, dried, and quickly washed with water. Samples were analyzed using MALDI-ToF mass spectrometry.

To test the activity of cG8 and mTTR after incubation in SGF and SIF, inhibitor samples were diluted 35-fold in PBSA to neutralize the pH and combined with A β (28 μ M) at an A β to inhibitor molar ratio of 20:1. To account for any effect the residual biological fluids may have on A β aggregation, the same final amount of SGF or SIF present in the A β -inhibitor

samples (1.4%) was added to A β alone. Samples were incubated for 48h at 37°C and ThT fluorescence was measured as described above. Results are reported as the mean \pm SEM of 3 independent experiments.

Cell culture maintenance and differentiation

CS03iCTRn2 derived EZ spheres^[21] were cultured as previously described.^[22] EZ spheres were maintained in DMEM/F12 (70:30) media supplemented with epidermal growth factor (100 ngmL⁻¹; Pepro-tech, Rocky Hill, NJ), basic fibroblast growth factor (100 ngmL⁻¹; WiCell Research Institute, Madison, WI), heparin (2 μ gmL⁻¹; Sigma–Aldrich), B27 minus Vitamin A (2%), and penicillin/streptomycin (1 %). Cells were grown in an ultra-low attachment flask and approximately two thirds of the media was replaced with fresh media every 2 days. Once a week, cells were passaged using a mechanical dissociation technique as described previously.^[22] For differentiation to EZ sphere derived neurons, EZ spheres were dissociated into singular cells using Accutase (Innovative Cell Technologies, San Diego, CA) and seeded onto Matrigel-coated plates (WiCell Research Institute, Madison, WI). Cells were seeded onto a 24-well, black-sided, glass bottom plate at 20000 cells per well for immunostaining. Cells were differentiated into neurons using neuron differentiation media containing DMEM/F12 (70:30) media, heparin (2 μ gmL⁻¹), B27 minus Vitamin A (2%) and penicillin/streptomycin (1 %). Neuron differentiation media was replaced every 2 days for 14 days. Successful differentiation was confirmed by staining with β -III tubulin and nestin (Supporting Information Figure 2).

Immunostaining

Immunostaining with confocal microscopy was used to image A β bound to iPSC-derived neurons, and to determine whether cG8 and/or mTTR alter the total (internalized plus cell surface associated) A β or the ratio of extracellular (cell surface associated) A β to total A β . A β monomer (50 μ M) in PBS was incubated alone (control) or at a 20:1 molar ratio with either cG8b or mTTRb under conditions that normally create either fibrils (37 °C, 24 h) or oligomers (RT, 24 h). Biotinylated cG8 and mTTR were used so that they could be visualized using Neutravidin Protein, DyLight 550. Samples were diluted in media for final concentrations of 5 μ M A β , 0 (controls) or 0.25 μ M of either cG8 or mTTR, and <15% PBS. Samples were added to 14 day differentiated neurons for 24 h.

All chemicals and antibodies were diluted in PBS for immunostaining. Following 24 h treatment, cells were washed with PBS, fixed using paraformaldehyde (4%, 15 min, 37°C), and permeabilized using Triton X-100 (0.1 %), glycine (10 mM) buffer (10 min). Cells were blocked (30 min) using goat normal serum (5%) before being probed with DyLight 550 (1:500, 1 h). After additional PBS washes, cells were probed with anti-A β 6E10 antibody (1:500, 1 h), washed, and probed with a goat anti-mouse Alexa Fluor 488 antibody (10 μ gmL⁻¹, 1 h) to allow visualization of cell associated A β . DAPI (1:1000, 10 min) was used for nuclei visualization. For visualization of only extracellular A β , cell permeabilization was omitted. After staining, cells were kept in PBS for imaging. Images were obtained with a Nikon A1R-Si+ Confocal Microscope using a three-channel, 1024V1024 scanning format. For each sample, a single mid-slice image was obtained using a 20V objective as well as a 5

slice, *z*-stack image obtained using a 20V objective with the scan area set to a zoom factor of 3.

ImageJ software (NIH, Bethesda, MD) was used to export the ND2 files into TIFF images and to convert the *z*-stack images into max intensity *z*-projections. To provide semi-quantitative analysis, ImageJ was used to determine the fraction of pixels in each image that contained green (AlexaFluor 488, indicating deposited A β) or blue (DAPI, indicating cell nuclei). The relative amount of deposited A β was normalized by the amount of surface occupied by cells in each image by calculating the ratio (fraction green/fraction blue).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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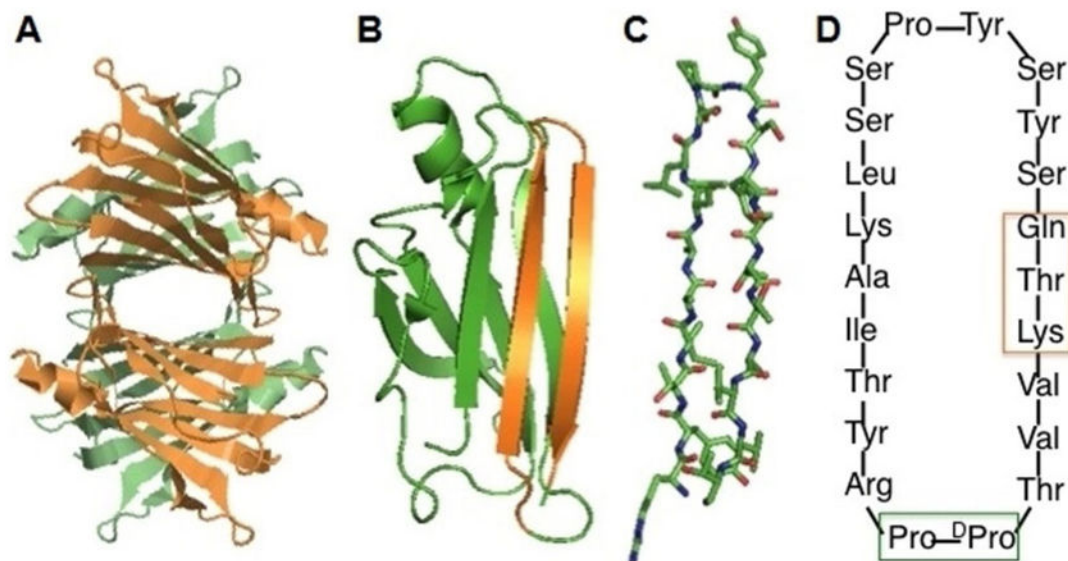


Figure 1.

Transthyretin structure and TTR-derived mTTR and cG8. (A) Ribbon structure of TTR tetramer. Created from PDB ID: 1DVG in the RCSB Protein Data Bank. (B) TTR monomer, with strands G and H highlighted in orange. (C) Stick representation of residues in strands G and H. Sequence shown is from wild-type human TTR: RYTIAALLSPYSYSTTAVVT. (D) Sequence of cG8. N-to C-linkage is clockwise. Green box shows template to enforce β -turn to link the N-terminus of strand G to C-terminus of strand H. Orange box shows substitutions to allow cyclization. The linear peptide is synthesized with the trifunctional glutamate attached to resin, which converts into glutamine upon cyclization and release from resin.

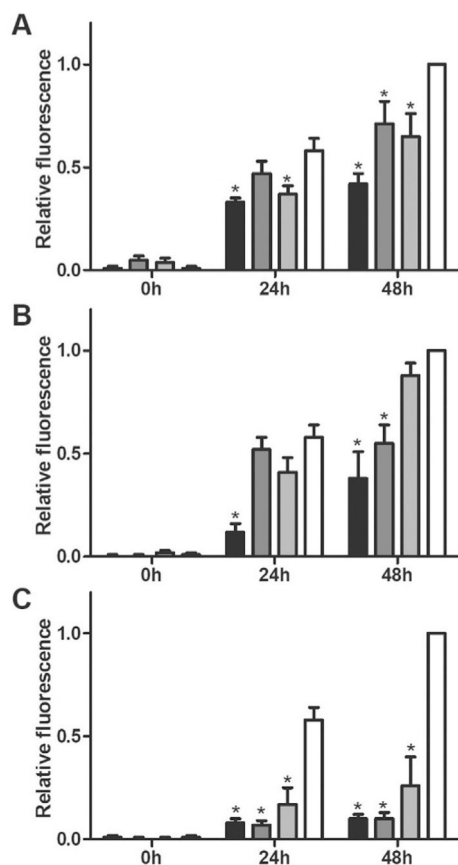


Figure 2. cG8 and mTTR retain TTR's ability to inhibit A β aggregation. A β monomer was combined with either TTR (A), cG8 (B), or mTTR (C) and aggregated at 37°C for 48 h. All samples contained 28 μ M A β with inhibitor/A β molar ratio of 1:20 (black), 1:50 (dark grey), or 1:75 (light grey) and are compared to A β alone (white). Aggregation was monitored via ThT fluorescence and data was blank (ThT in PBS) subtracted then normalized to the fluorescence of the control at 48 h. Error bars indicate SEM, $n=3-4$ for samples, $n=8$ for control. * $p<0.05$ vs. control.

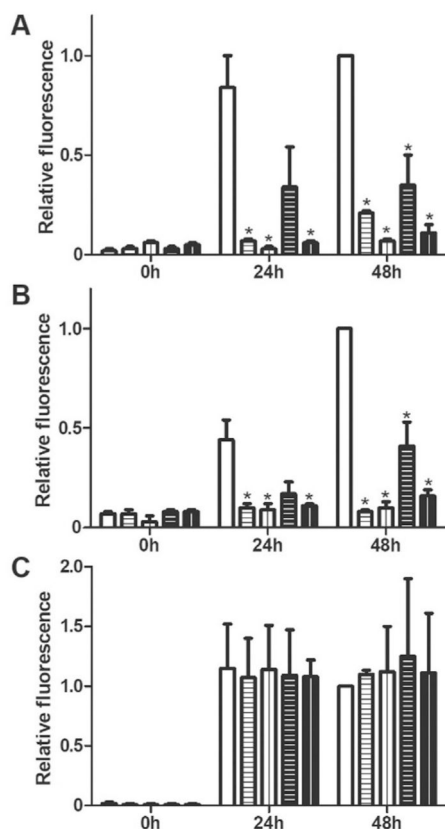


Figure 3. cG8 and mTTR inhibit the aggregation of $A\beta_{1-42}$ and amylin but not α -synuclein. $A\beta_{1-42}$ (A), amylin (B), and α -synuclein (C), all at $20 \mu\text{M}$ final concentration, were combined with cG8 (horizontal lines) or mTTR (vertical lines) at inhibitor/amyloid protein ratios of 1:10 (white bars, black lines), or 1:20 (black bars, white lines) and compared to control (white bars, amyloid protein with no added inhibitor). Aggregation was monitored via ThT fluorescence and data was blank (ThT in PBS) subtracted then normalized to the respective control at 48 h. Error bars indicate SEM, $n=2-4$. * $p < 0.05$ vs. control.

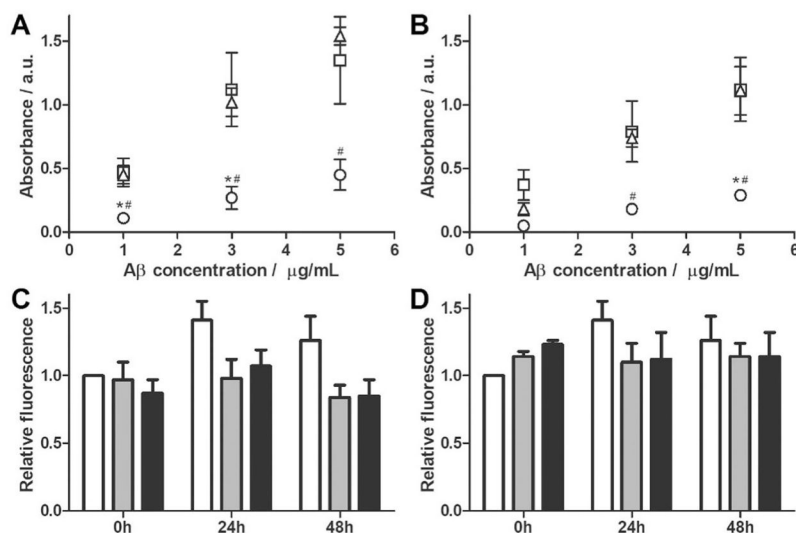


Figure 4. cG8 and mTTR bind to multiple A β species but do not disaggregate A β fibrils. (A–B) NeutrAvidin plates were coated with 5 $\mu\text{g mL}^{-1}$ of either cG8b (A) or mTTRb (B) and the relative binding of A β monomer (fresh, ○), oligomer (24 h, RT, □) or fibril (24 h, 37°C, Δ) was measured. Data was blank (PBS, 0 mg mL^{-1} A β) subtracted. Error bars indicate SEM, $n=3$. * $p<0.05$ vs. oligomer. # $p<0.05$ vs. fibril. (C–D) Preformed A β fibrils were combined with cG8 (C) or mTTR (D). Final samples contained 28 μM A β alone (white bars), or at A β /inhibitor molar ratios of 2:1 (grey bars), or 1:1 (black bars). Disaggregation was monitored via ThT fluorescence and data was normalized to the fluorescence of the control at 0 h. Error bars indicate SEM, $n=3$ for samples, $n=6$ for control.

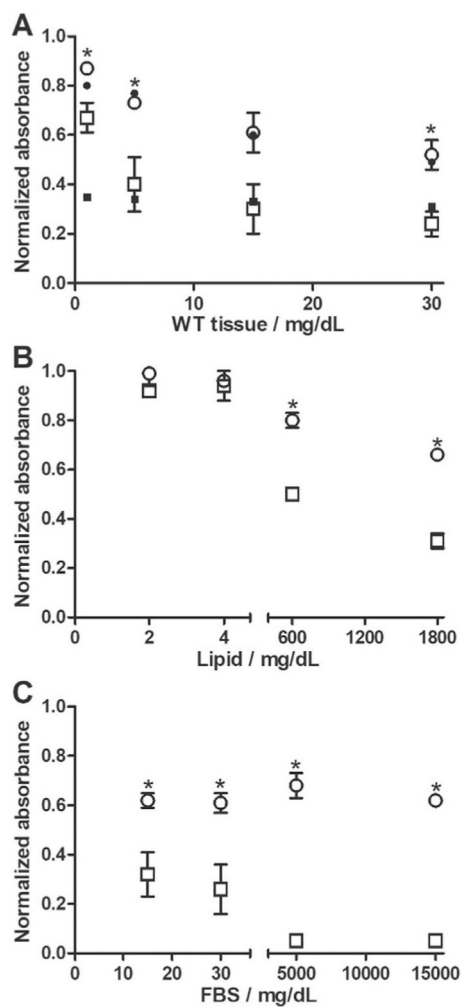


Figure 5. cG8 and mTTR bind A β aggregates in the presence of biological materials. NeutrAvidin plates were coated with 5 $\mu\text{g mL}^{-1}$ of either cG8b (O) or mTTRb (\square) and the relative binding of 5 $\mu\text{g mL}^{-1}$ of A β in the presence of wild-type mouse cortex tissue lysate (A), human neurons (A, closed symbols, $n=1$), lipids (B), or FBS (C) was measured. Data normalized to binding of A β in buffer alone. Error bars indicate SEM, $n=3$. * $p<0.05$ vs. mTTRb.

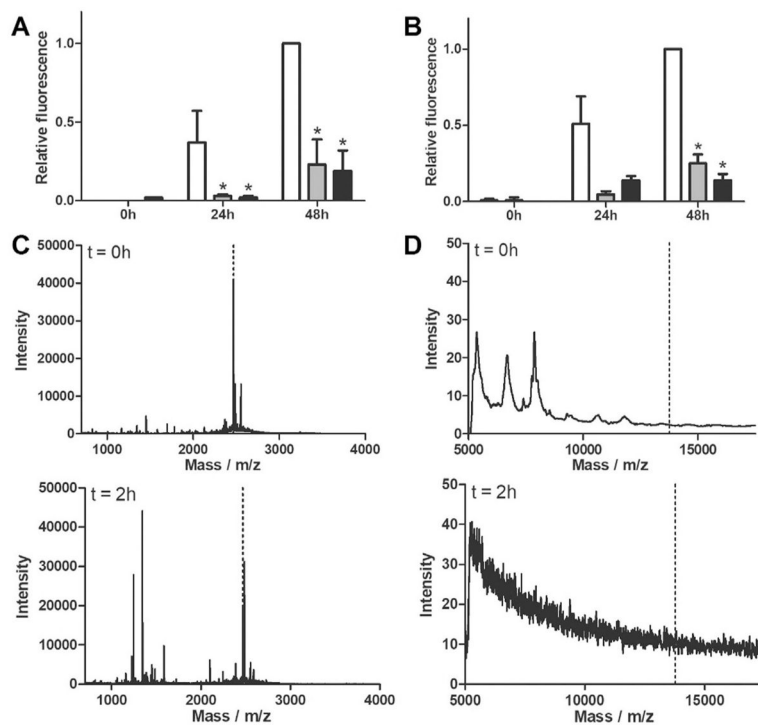
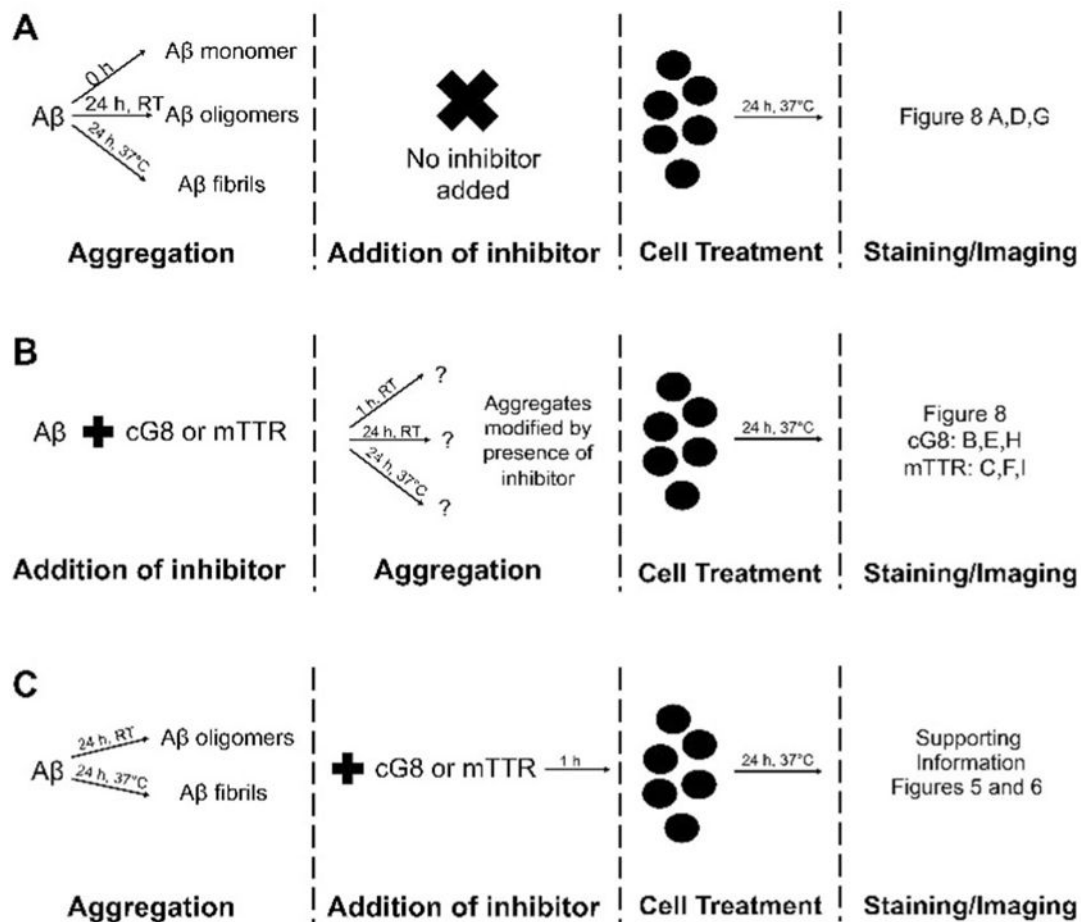


Figure 6. Activity of cG8 and mTTR in biological fluids and stability from enzyme degradation. A–B) cG8 (grey bars) and mTTR (black bars) were incubated in either SGF (A) or SIF (B) for 24 h at 37°C and then combined with Aβ monomer. Final samples contained 28 μM Aβ alone (control, white bars) or at 20:1 Aβ to inhibitor molar ratio. Aggregation was monitored via ThT fluorescence and data was blank (ThT in PBS) subtracted. Error bars indicate SEM, $n=3$. * $p<0.05$ vs. control. C–D) cG8 (C) and mTTR (D) were incubated at a 20:1 ratio with pepsin for 0 and 2 h at 37°C. Degradation was assessed via mass spectrometry. Dashed lines indicate correct molecular weight of intact protein or peptide.

**Figure 7.**

Treatment of human iPSC-derived neurons with Aβ. Aβ monomer, oligomers, and fibrils were formed in the absence (A) or presence (B) of cG8 or mTTR. Additionally, cG8 and mTTR were incubated with preformed oligomers or fibrils prior to cell treatment (C). Cell treatment lasted 24 h before washing, staining, and confocal imaging.

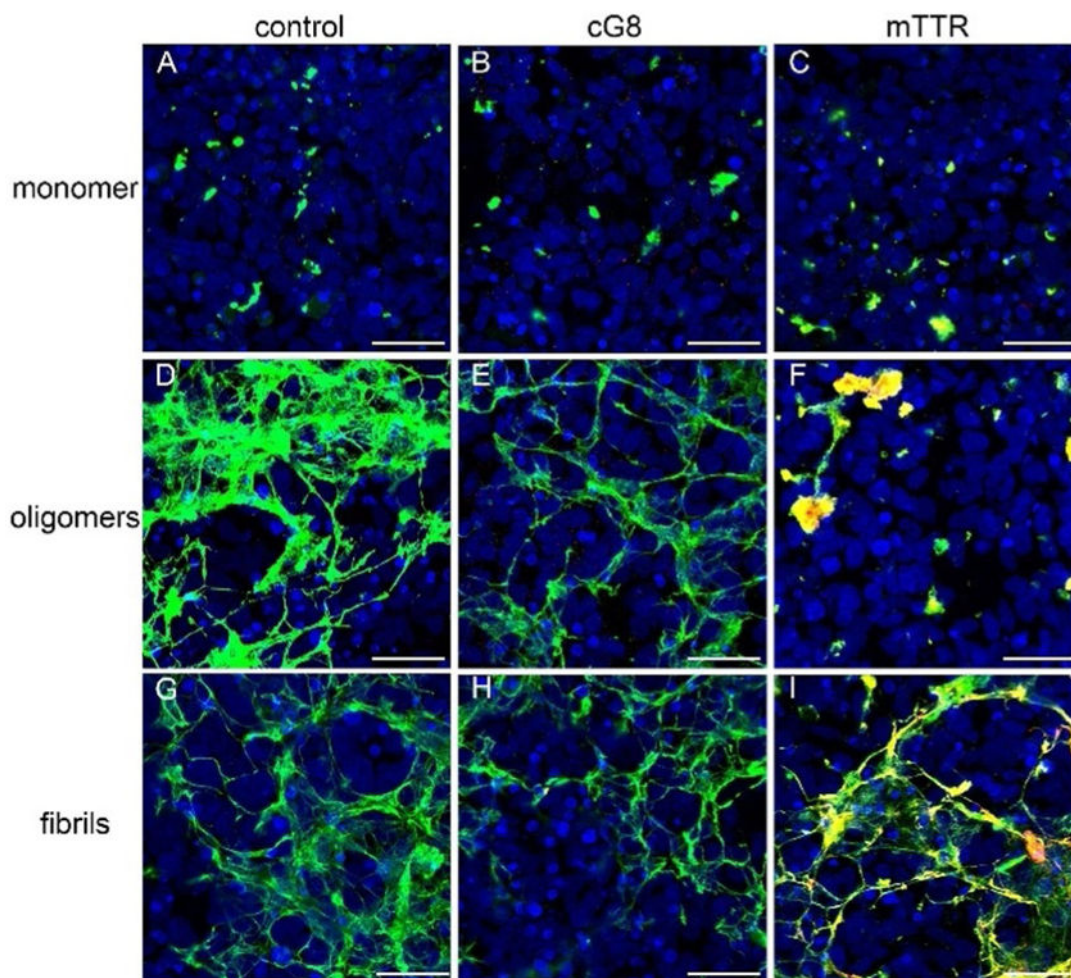


Figure 8. mTTR alters A β deposition on human neurons. A β monomer (top row), oligomers (middle row), and fibrils (bottom row) were prepared alone (control, left column) or in the presence of cG8 (middle column) or mTTR (right column). After 24 h cell treatment, cells were immunostained to visualize cell nuclei (DAPI, blue), cell associated A β (6E10, green), and cell associated cG8b or mTTRb (Neutravidin, red). Co-localization of A β and cG8b or mTTRb is shown in yellow. Cells were imaged using a Nikon A1R-Si+ confocal microscope and 20V objective with a zoom factor of 3. Representative images are a max intensity z-projection of 5 slice, z-stack images. Scale bars=50 μ m.

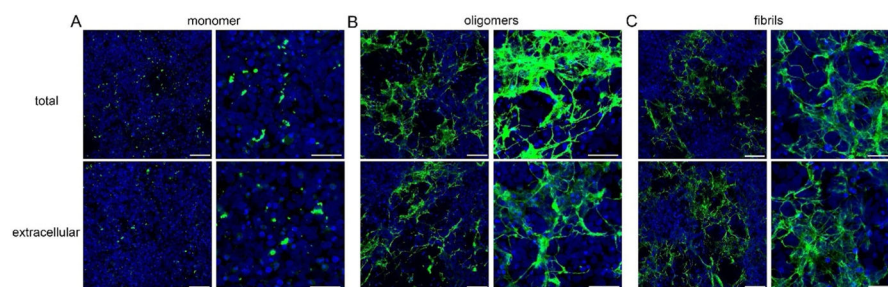


Figure 9.

A β oligomers are internalized more than A β monomer or fibrils. Cells were treated with A β monomer (A), oligomers (B), and fibrils (C) for 24 h then stained and images as previously described (Figure 8). Each treatment was done in duplicate to allow for staining of the total amount of A β (top row) and just the extracellular A β (bottom row). Both a single slice image (left side of panels, scale bars=100 μ m) and a z-stack image (right side of panels, scale bars=50 μ m) were obtained.

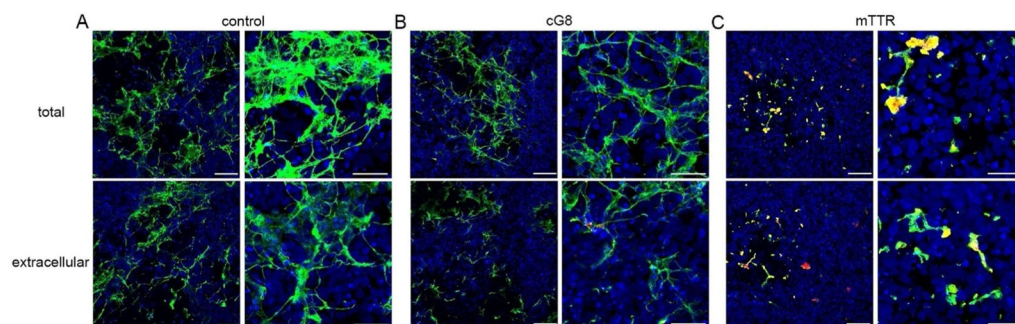


Figure 10. cG8 does not prevent internalization of A β when present during oligomer formation. A β was co-incubated with cG8 (B) or mTTR (C) under oligomer forming conditions. Cells were then treated for 24 h with control oligomers (A) or those formed in the presence of cG8 or mTTR. Cells were stained and imaged as previously described (Figure 8). Each treatment was done in duplicate to allow for staining of the total amount of A β (top row) and just the extracellular A β (bottom row). Both a single slice image (left side of panels, scale bars=100 μ m) and a z-stack image (right side of panels, scale bars=50 μ m) were obtained.