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Article

Calorimetric Studies of Binary and Ternary Molecular Interactions between Transthyretin, Aβ Peptides, and Small-Molecule Chaperones toward an Alternative Strategy for Alzheimer's Disease Drug Discovery

Ellen Y. Cotrina, Ana Gimeno, Jordi Llop, Jesús Jiménez-Barbero, Jordi Quintana, Gregorio Valencia, Isabel Cardoso, Rafel Prohens, and Gemma Arsequell*

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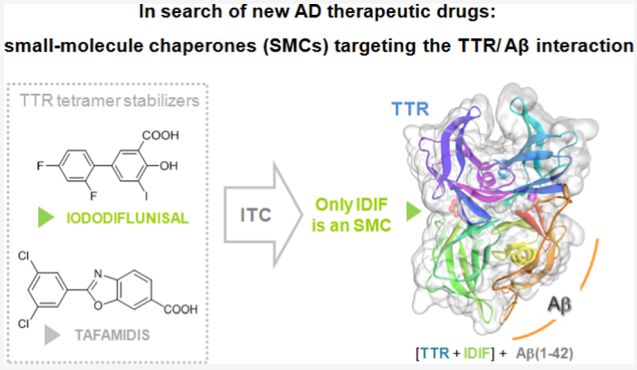
ABSTRACT: Transthyretin (TTR) modulates the deposition, processing, and toxicity of Abeta (Aβ) peptides. We have shown that this effect is enhanced in mice by treatment with small molecules such as iododiflunisal (IDIF, **4**), a good TTR stabilizer. Here, we describe the thermodynamics of the formation of binary and ternary complexes among TTR, Aβ(1−42) peptide, and TTR stabilizers using isothermal titration calorimetry (ITC). A TTR/Aβ(1−42) (1:1) complex with a dissociation constant of K_d = 0.94 μM is formed; with IDIF (**4**), this constant improves up to K_d = 0.32 μM, indicating the presence of a ternary complex TTR/IDIF/Aβ(1−42). However, with the drugs diflunisal (**1**) or Tafamidis (**2**), an analogous chaperoning effect could not be observed. Similar phenomena could be recorded with the shorter peptide Aβ(12−28) (**7**). We propose the design of a simple assay system for the search of other chaperones that behave like IDIF and may become potential candidate drugs for Alzheimer's disease (AD).



INTRODUCTION

Alzheimer's disease (AD) is a complex neurodegenerative brain disease characterized by extracellular amyloid plaques, intracellular neurofibrillary tangles, and neuronal death.[1] The amyloid hypothesis of AD has guided a huge effort in drug discovery and development, leading to many small-molecule and biological drug candidates.[2,3] Regrettably, only five treatment options are currently approved to treat this disease,[4] but none is a truly disease-modifying intervention. In spite of this sad situation, a number of novel therapeutic approaches are currently being investigated. One of them is targeting protein−protein interactions (PPi) between Aβ and other amyloid-binding proteins such as gelsolin,[5] ApoJ (clusterin),[6,7] ApoE,[8,9] human serum albumin (HSA),[10,11] humanin,[12] the neuronal Tau protein,[13] and transthyretin (TTR).[14−16]

The present investigation relates to TTR, which is a 55 kDa homotetramer multifaceted protein responsible for the transport of thyroid hormones (thyroxine, T4) and retinol in plasma and cerebrospinal fluid (CSF).[17] Several physiological and epidemiological clues point to a possible direct involvement of TTR in AD. One of the most significant observations is the decreased TTR levels in CSF in AD patients that parallels similar variations in CSF-Aβ levels[18−20] and suggests that TTR is a biomarker of AD.[21] TTR is the main Aβ-binding protein in the CSF.[14,22,23] This binding is believed to naturally prevent Aβ aggregation and toxicity in this media. This putative neuroprotective effect of TTR is also supported by a number of biochemical and animal studies, some of them, conducted in one of our consortiated laboratories.[24−26]

TTR tetrameric stability appears as a relevant factor in its interaction with the Aβ peptide. Supporting this hypothesis, in vitro studies showed that amyloidogenic TTR variants bind with lower affinity to Aβ peptide than does the wild-type (wt) or nonamyloidogenic TTR,[27] also affecting the ability to avoid Aβ aggregation and toxicity.[28] Recently, some researchers have suggested that TTR interferes with Aβ amyloid formation by redirecting oligomeric nuclei into nonamyloid aggregates.[29]

Since TTR binds T4 in its central hydrophobic channel, we have suggested that, in AD, TTR is destabilized and its clearance accelerated, thus explaining its lower levels.[30] TTR is

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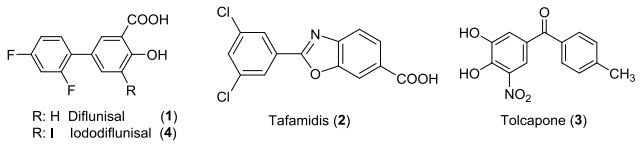
also an amyloidogenic protein. Thus, TTR stability is also a key factor in familial amyloid polyneuropathy (FAP),[31] a TTR-related hereditary amyloidosis. TTR tetrameric stabilization has been defined as the basis for one of the possible therapeutic strategies in FAP.[32−35] Some of the TTR tetramer stabilizers are drugs, such as the NSAID diflunisal (**1**),[36] the orphan drug Tafamidis (**2**),[37−40] and Tolcapone (**3**),[41,42] a drug for the treatment of Parkinson's disease recently repositioned for FAP (Scheme 1).

Scheme 1. Chemical Structures of TTR Tetramer Kinetic Stabilizers[a]



[a]Diflunisal (**1**), Tafamidis (**2**), and Tolcapone (**3**) are registered drugs.

By using in vitro studies, we have earlier demonstrated that TTR/Aβ interactions can be enhanced by a small set of tetramer-stabilizing compounds,[28] one of them being iododiflunisal (IDIF, **4**), a small-molecule iodinated derivative of the NSAID diflunisal (**1**) (Scheme 1).[43−45] Remarkably, in vivo administration of IDIF (**4**) to a mice model of AD resulted in the binding and stabilization of the TTR tetramer, decrease in brain Aβ levels and deposition, and improvement in the cognitive functions that are impaired in this AD-like neuropathology.[46]

In this study, we have used isothermal titration calorimetry (ITC),[47−49] a powerful biophysical technique for the quantitative analysis of PPi.[50−53] ITC provides the complete thermodynamic profile in terms of free energy (ΔG), enthalpy (ΔH), entropy (ΔS), binding constant (K_d), and stoichiometry (n) of the interaction from a single experiment. Since ITC is extremely sensitive to the energetics of conformational changes and intermolecular interactions, this technique is one of the gold standard biophysical methods that can be used to interrogate ternary molecular systems,[54−58] such as the one formed by TTR, Aβ peptides, and IDIF (**4**). Thus, the goal of the present study was to determine the thermodynamic parameters of the intermolecular interaction in solution between TTR and Aβ(1−42). We also wanted to elucidate the structural bases for the enhancement of the TTR/Aβ interaction driven by our chaperone compound IDIF (**4**). With these aims and for comparative reasons, we have also assayed if other known TTR tetramer stabilizer drugs, such as the drugs diflunisal (**1**) and Tafamidis (**2**), behave as chaperones of the TTR/Aβ interaction. In addition and following the clues revealed by previous structural information gathered by STD-NMR experiments, we have also investigated if shorter Aβ peptide sequences perform similarly in stabilizing these systems.[59]

RESULTS AND DISCUSSION

ITC Analysis of Binary and Ternary Complex Formation between TTR, Aβ(1−42), and TTR Tetramer Stabilizers. To characterize the binding process of the full-length Aβ(1−42) to TTR, we have used a depsipeptide precursor of Aβ(1−42). This depsipeptide precursor is converted into the corresponding native Aβ(1−42) peptide, in situ, by a change in pH.[60,61] This is a guarantee that Aβ(1−42) is in a monomeric state, free of aggregates, at the beginning of each experiment. Thus, the binary complex TTR/Aβ(1−42) was prepared by the titration of a solution of TTR (20 μM) by a solution of Aβ(1−42) (200 μM) yielding the diagrams and calorimetric constants reported in Figure 1.

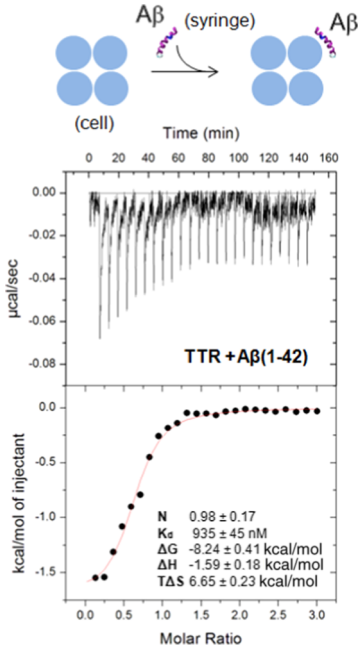


Figure 1. Isothermal titration calorimetry (ITC) studies. The binary complex [TTR + Aβ(1−42)] at pH 7.4 in 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer, 10 mM glycine, and 5% dimethyl sulfoxide (DMSO) (final concentration) at 25 °C.

The calculated binding constant for the formation for this (1:1) TTR/Aβ(1−42) complex is K_d = 0.94 μM. A comparison of this figure with other literature data of K_d constants of TTR binding with other Aβ peptides can only be done with a TTR/Aβ(1−40) complex, which stands at K_d = 24 μM.[62] Although both Aβ sequences are very closely related, their amyloid properties are rather different, Aβ(1−42) being more amyloidogenic.[63] This property may likely be the cause of this difference. In any event, we have also repeated this experiment with Aβ(1−40), which in our conditions yields K_d = 7.1 μM (Figure S19).

Furthermore, to study the effect of the TTR tetramer stabilizers on the TTR/Aβ(1−42) complex, binary complexes of TTR/stabilizers were first prepared and analyzed (Figure S17). In a second set of experiments, the binary complexes were subsequently titrated with Aβ(1−42) solutions. In Figure 2, this procedure is expressed by the equation: (TTR + stabilizer) + Aβ(1−42). IDIF (**4**) (Figure 2A) and Tafamidis (**2**) (Figure 2B) were used as stabilizers.

The calorimetric constants for the stabilizers' interactions are reported in Table 1. The thermodynamic profile showed that IDIF (**4**) has a cooperative effect, the binding of Aβ(1−42) + [IDIF (**4**) + TTR] with K_d = 0.32 μM is approximately threefold stronger than that of [Aβ(1−42) + TTR] with K_d = 0.94 μM, and again a strong enthalpy/entropy compensation is observed in this system when IDIF (**4**) is the ligand. These results confirm the chaperoning effect exerted by

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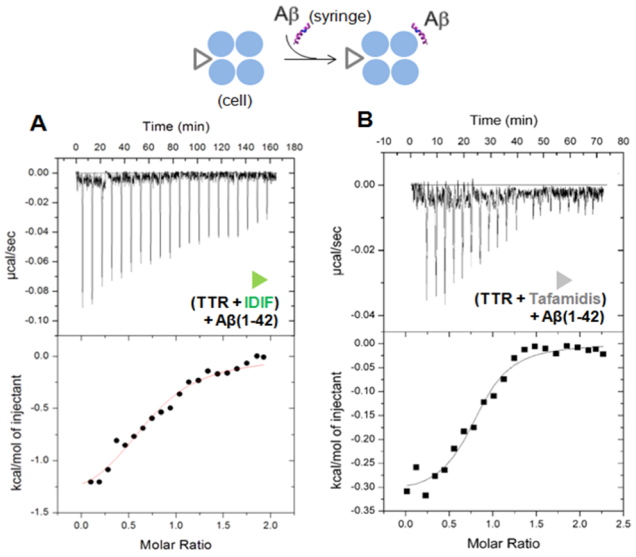

Figure 2. ITC studies. (A) Titration of [TTR + IDIF (**4**)] complex with Aβ(1−42). (B) Titration of [TTR + Tafamidis (**2**)] complex with Aβ(1−42). All of them at pH 7.4 in 25 mM HEPES buffer, 10 mM glycine, and 5% DMSO (final concentration) at 25 °C.

IDIF (**4**) at enhancing the TTR/Aβ interaction. Interestingly, an analogous stabilizing effect of IDIF (**4**) is observed when tested on TTR/Aβ(1−40) complexes (Figure S20 and Table S2). On the other hand, Tafamidis (**2**) falls rather behind IDIF (**4**), with a binding constant of K_d = 1.05 μM that is very close to the original TTR/Aβ(1−42) complex (0.94 μM) indicating that Tafamidis (**2**) has a negligible effect.

Thioflavin T (ThT) Analysis of the Aggregation Properties of the Binary and Ternary Complexes of TTR, Aβ(1−42) and TTR Tetramer Stabilizers. The possible chaperoning effect of the TTR stabilizers in preventing TTR/Aβ(1−42) complex aggregation has been studied by ThT fluorescence assays, which monitor the increase of fluorescence during the aggregation process.[64] The ThT assays were performed to study the aggregation of Aβ(1−42) alone or in the presence of TTR or when TTR had been preincubated with the TTR tetramer stabilizer drugs IDIF (**4**) or Tafamidis (**2**). The results from ThT assays (Figure 3 and Table 2) corroborated our ITC results. The aggregation of Aβ(1−42) was reduced in the presence of TTR, and even more when TTR was complexed with IDIF (**4**), but not when TTR was complexed with Tafamidis (**2**). An almost negligible ThT signal was detected when analyzing the [TTR + IDIF (**4**)] complex, indicating that only the small-molecule IDIF (**4**) has a chaperone effect further enhancing the TTR/Aβ interaction. These results obtained by ThT fluorescence assays have also been corroborated by turbidity assays (Figure S6).

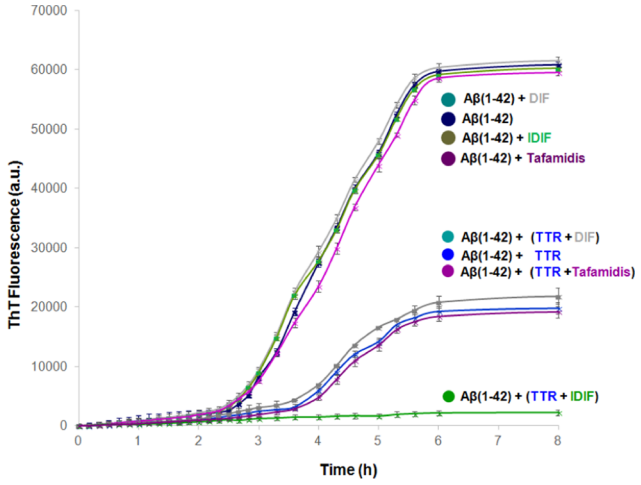

Figure 3. ThT assays of the aggregation of Aβ(1−42) alone (50 μM), in complex with TTR (25 μM), or in complex with TTR stabilized with different small compounds (50 μM), [TTR/IDIF (**4**), TTR/DIF (**1**), and TTR/Tafamidis (**2**)]. ThT fluorescence was measured at 37 °C each 10 min for 3 h, then each 20 min from 3 to 6 h, and then at 8 h.

Table 2. Percentage of Fibril Formation Obtained from ThT Assays[a]

	ThT (au)	% fibril formation
Aβ(1−42)	60 810 ± 566	99 ± 1
Aβ(1−42) + TTR	19 836 ± 913	25 ± 1
Aβ(1−42) + [TTR + IDIF (**4**)]	2224 ± 439	6 ± 1
Aβ(1−42) + [TTR + DIF (**1**)]	21 852 ± 946	26 ± 2
Aβ(1−42) + [TTR + Tafamidis (**2**)]	19 188 ± 923	24 ± 2

[a]The concentrations used: Aβ(1−42) (50 μM) and TTR (25 μM) for the different small compounds (50 μM).

ITC Studies of the Interaction between TTR and Short Aβ Sequences. In our previous STD-NMR spectroscopy studies in solution,[59] we have identified structural elements implicated in the TTR/Aβ interaction that indicate the close proximity of the V18, F19, and F20 fragment of the Aβ(12−28) sequence to V94, F95, and T96 of TTR, highlighting V18 to F20 as the main structural motif for the recognition process. This Aβ(12−28) peptide is reported in the literature to essentially exhibit the same neurotoxic behavior and fibril formation properties as the full-length Aβ(1−42) peptide.[65−67] To confirm that these are the key structural elements involved in the TTR/Aβ(1−42) complex, we have prepared the following short sequences of Aβ(1−42), namely, Aβ(1−11) (**5**), Aβ(10−20) (**6**), Aβ(12−28) (**7**), and Aβ(25−35) (**8**) and subsequently characterized their interaction with TTR (Table 3) by ITC.

Table 1. Thermodynamic Parameters for the Titration of (A) Aβ(1−42) and (TTR), (B) Ternary Complex of Aβ(1−42) and [TTR + IDIF (4)], and (C) Ternary Complex of Aβ(1−42) and [TTR + Tafamidis (2)] at 25 °C

	Assay	n	K_d (μM)	ΔG (Kcal/mol)	ΔH (Kcal/mol)	TΔS (Kcal/mol)
A	TTR+Aβ(1-42)	0,98 ± 0,17	0,94 ± 0,04	-8,24 ± 0,41	-1,59 ± 0,18	6,65 ± 0,23
B	[TTR+IDIF (**4**)] + Aβ(1-42)	0,89 ± 0,15	0,32 ± 0,03	-8,87 ± 0,35	-1,47 ± 0,19	7,39 ± 0,17
C	[TTR+Tafamidis (**2**)] + Aβ(1-42)	0,80 ± 0,08	1,05 ± 0,05	-8,11 ± 0,02	-0,34 ± 0,02	7,77 ± 0,05

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Table 3. Sequences of Amyloid Peptides Used in This Study: Aβ(1−42) and Other Short Amyloid β Sequences, Including Three Aβ(12−28) Ala Mutants (9, 10, and 11)

1 42	Abeta peptide	#
DAEFR HDSGY EVHHQ KLVFF AEDVG SNKGA IIGLM VGGVV IA	Aβ (1-42)	
DAEFR HDSGY E	Aβ (1-11)	(**5**)
Y EVHHQ KLVFF	Aβ (10-20)	(**6**)
VHHQ KLVFF AEDVG SNK	Aβ (12-28)	(**7**)
EDVG SNKGA IIGLM	Aβ (25-35)	(**8**)
VHHQ KLAFF AEDVG SNK	V18A Aβ (12-28)	(**9**)
VHHQ KLVAF AEDVG SNK	F19A Aβ (12-28)	(**10**)
VHHQ KLVFA AEDVG SNK	F20A Aβ (12-28)	(**11**)

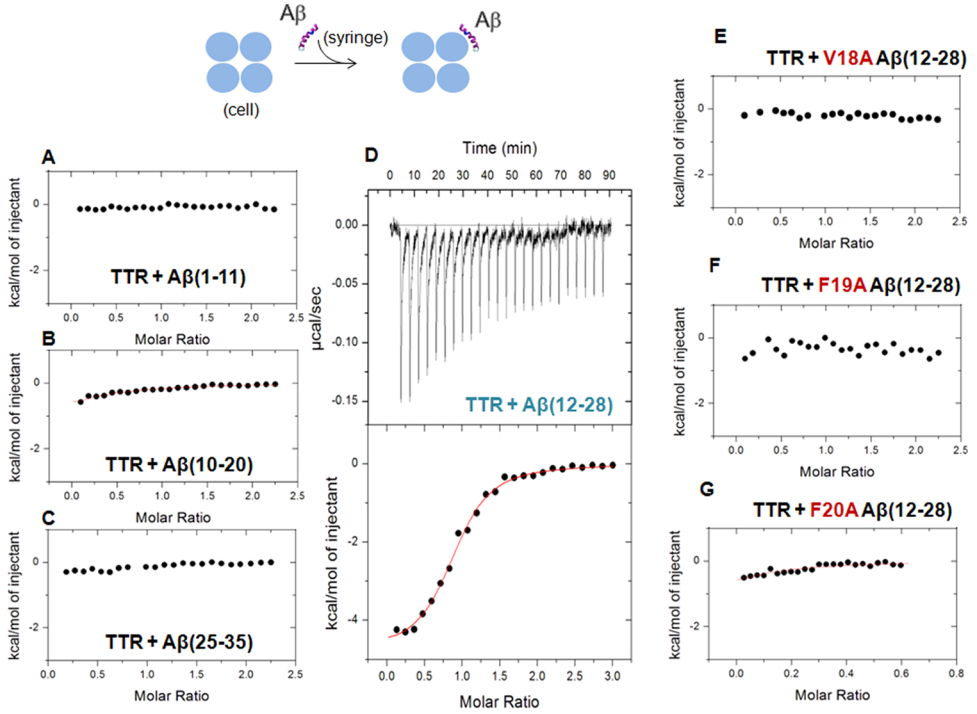

Figure 4. ITC analysis of different short sequences of amyloid β Aβ(1−42) binding to TTR at pH 7.4 in 25 mM HEPES buffer, 10 mM glycine, and 5% DMSO at 25 °C. The binary systems are: (A) TTR + Aβ(1−11) (**5**), (B) TTR + Aβ(10−20) (**6**), (C) TTR + Aβ(25−35) (**8**), (D) TTR + Aβ(12,28) (**7**), (E) TTR + V18A Aβ(12−28) (**9**), (F) TTR + F19A Aβ(12−28) (**10**), and (G) TTR + F20A Aβ(12−28) (**11**).

Table 4. Thermodynamic Parameters for the Complex Formation between Different Short Sequences of Aβ and TTR at 25 °C

Assay	n	K_d (µM)	ΔG (Kcal/mol)	ΔH (Kcal/mol)	TΔS (Kcal/mol)
TTR + Aβ(1-11) (**5**)	-	-	-	-	-
TTR + Aβ(10-20) (**6**)	0,50 ± 0,10	14,90 ± 0,50	-6,58 ± 0,38	-0,84 ± 0,05	5,74 ± 0,33
TTR + Aβ(25-35) (**8**)	-	-	-	-	-
TTR + Aβ(12-28) (**7**)	1,00 ± 0,15	3,00 ± 0,20	-7,76 ± 0,41	-4,52 ± 0,30	3,23 ± 0,12
TTR+ V18A Aβ(12-28) (**9**)	-	-			
TTR + F19A Aβ(12-28) (**10**)	-	-			
TTR + F20A Aβ(12-28) (**11**)	-	-			

ITC studies for the binary complexes between different short sequences of Aβ and TTR are summarized in Figure 4. Only the binding isotherm of the binary complex between Aβ(12−28) (**7**) and TTR showed a typical thermodynamic profile (Figure 4D). Accordingly, a full thermodynamic characterization was performed (Table 4). The thermograms for the binary complexes between TTR and Aβ(1−11) (**5**) (Figure 4A) and TTR and Aβ(25−35) (**8**) (Figure 4C) show negligible enthalpy changes, confirming that there was no significant interaction between each of these sequences and TTR. In the case of the binding of TTR to Aβ(10−20) (**6**) (Figure 4B), a very low enthalpy change was observed. Thus, these results are in agreement with those from our previous STD-NMR spectroscopy studies.[59]

To provide further insights into the interaction between the specific sequence Aβ(12−28) and TTR, three Ala mutants in the residues V18 to F20 of the Aβ(12−28) were prepared (Table 4) and ITC experiments were performed. The binding

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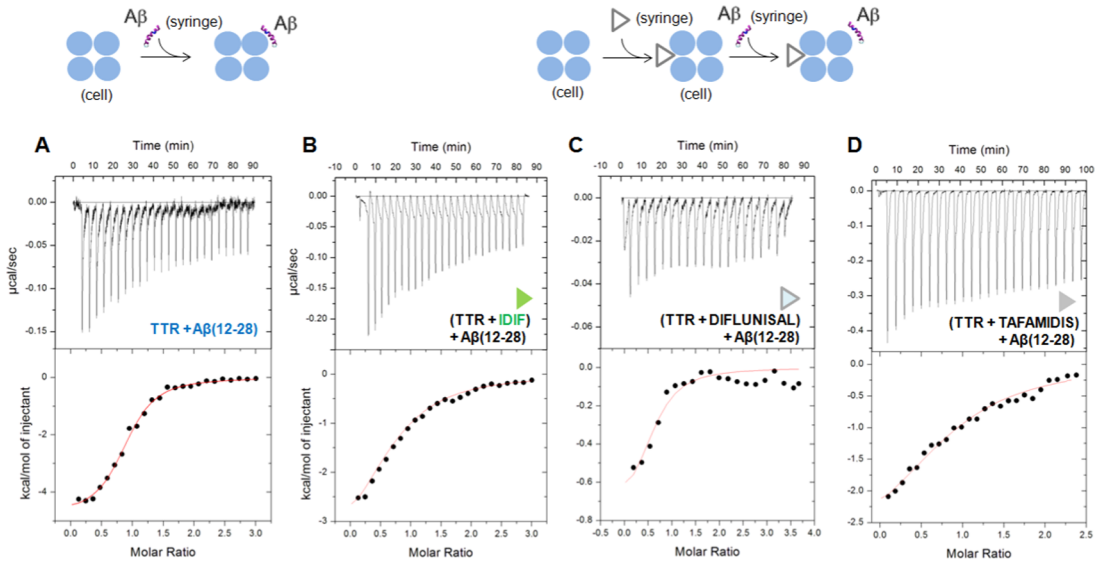

Figure 5. ITC analysis of (A) binary complex TTR + Aβ(12−28), (B) ternary complex [TTR + IDIF (**4**)] and Aβ(12−28), (C) ternary complex [TTR + Diflunisal (**1**)] and Aβ(12−28), and (D) ternary complex [TTR + Tafamidis (**4**)] and Aβ(12−28). All of these ITC studies were performed at pH 7.4 in 25 mM HEPES buffer, 10 mM glycine, and 5% DMSO (final concentration) at 25 °C.

Table 5. Thermodynamic Parameters for the Titration of (A) Binary Complex Aβ(12−28) and TTR, Ternary Complexes (B) Aβ(12−28) and [TTR + IDIF (4)] and (C) Aβ(12−28) and [TTR + Diflunisal (1)], and (D) Ternary Complex of Aβ(12−28) and [TTR + Tafamidis (2)] at 25 °C

	Assay	n	K_d (μM)	ΔG (Kcal/mol)	ΔH (Kcal/mol)	TΔS (Kcal/mol)
A	TTR+Aβ(12-28)	1.00 ± 0.15	3.00 ± 0.20	-7.76 ± 0.25	-4.52 ± 0.12	3.23 ± 0.14
B	(TTR+ IDIF) + Aβ(12-28)	1.00 ± 0.12	0.81 ± 0.03	-8.31 ± 0.32	-2.48 ± 0.11	5.83 ± 0.21
C	(TTR+ Diflunisal) + Aβ(12-28)	0.87 ± 0.15	2.70 ± 0.14	-7.85 ± 0.44	-0.78 ± 0.18	7.07 ± 0.26
D	(TTR + Tafamidis) + Aβ(12-28)	0.90 ± 0.10	2.80 ± 0.12	-7.22 ± 0.24	-3.13 ± 0.10	4.08 ± 0.15

isotherms obtained between Ala mutants of Aβ(12−28) (**9**, **10**, and **11**) and TTR are also shown in Figure 4.

As it can be deduced from these ITC results, replacement of any residue from V18 to F20 for Ala has a detrimental effect in the binding of Aβ(12−28) to TTR, indicating that these residues are essential for the interaction with TTR.[59]

ITC Studies of the Binary and Ternary Complexes between TTR, Aβ(12−28) and IDIF (4), Diflunisal (1), and Tafamidis (2). To investigate if IDIF shows the same chaperoning character as in the previous TTR/Aβ(1−42) complexes against this shorter, Aβ(12−28) model peptide, we have performed ITC studies and compared the interaction between Aβ(12−28) with TTR alone or with TTR preincubated with the TTR tetramer stabilizers IDIF, diflunisal, and Tafamidis. Results are shown in Figure 5 and the full thermodynamic characterizations are displayed in Table 5.

The interactions with Aβ(12−28), both the one with TTR and the one with TTR complexed with IDIF, are enthalpy-driven with a favorable entropic contribution. The titration of the binary complex (TTR + IDIF) with Aβ(12−28) has a notable improvement of binding (K_d = 0,81 μM) compared to the [TTR + Aβ(12−28)] binary complex (K_d = 3,00 μM) (Table 5). When TTR is stabilized with IDIF (**4**), the complex with Aβ(12−28) is almost three times stronger than with TTR alone.

These results highlight that although diflunisal (**1**) and Tafamidis (**2**) are good TTR tetrameric stabilizers, these compounds do not enhance the TTR/Aβ interaction, and therefore not all TTR tetramer stabilizers are chaperones of the TTR/Aβ interaction, and they need to be assayed for this specific purpose.

Transmission Electron Microscopy (TEM) Study of the Aggregation of Complexes Formed by TTR, Aβ(12−28) and Either IDIF (4) or Tafamidis (2). To further confirm the chaperone effect of IDIF on the TTR/Aβ(12−28) interaction, as suggested by the ITC experiments, we studied the morphology of the species of Aβ(12−28) by transmission electron microscopy (TEM). After 48 h of incubation at 37 °C, the Aβ(12−28) peptide alone (Figure 6A−C) formed abundant, long and complex fibrils, higher-ordered structured fibrils, constituted by several protofilaments, which presented as more rigid (Figure 6A,B) and with twisting of the fibrils (Figure 6B, arrows) or as more relaxed fibrils with the protofilaments laterally assembled (Figure 6C).

This ultrastructural analysis showed that the presence of TTR clearly prevented Aβ(12−28) fibrillization, resulting in the presence of fewer, less complex fibrils and small aggregates

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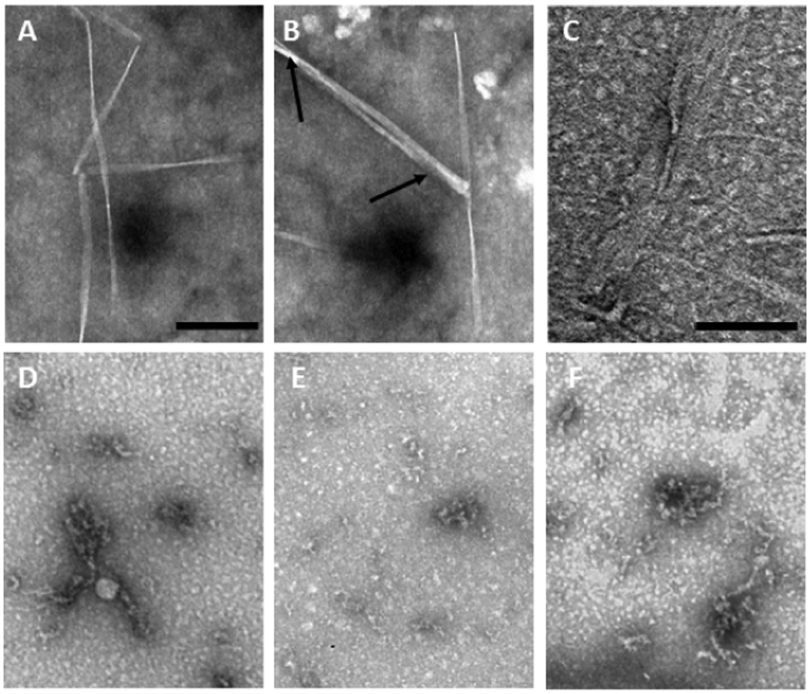

Figure 6. Morphologic assessment by TEM of the influence of TTR on Aβ(12−28) aggregation after 48 h of incubation at 37 °C. (A−C) Aβ(12−28) peptide alone, (D) Aβ(12−28) in the presence of TTR, (E) Aβ(12−28) in the presence of TTR preincubated with IDIF, and (F) Aβ(12−28) in the presence of TTR preincubated with Tafamidis. Scale bar (A, B, D, E, and F) = 200 nm; C = 100 nm.

(Figure 6D), compared to the control, the Aβ(12−28) alone (Figure 6A−C), which presented long and complex fibrils.

Importantly, here we showed that preincubation of TTR with IDIF completely abolished the presence of Aβ(12−28) fibrils and only round particles and prefibrillar species were visualized (Figure 6E). However, when TTR was preincubated with Tafamidis (Figure 6F), there was no significant effect beyond the effect of TTR itself, since small fibers were detected.

CONCLUSIONS

These calorimetric studies demonstrate that TTR forms (1:1) complexes with Aβ(1−42) with K_d = 0.93 μM. In the presence of the TTR tetramer stabilizer IDIF, these complexes are chaperoned showing K_d = 0.31 μM. This effect was not detected when using the drug Tafamidis (**2**) instead of IDIF (**4**). In addition, it was observed that the shorter Aβ sequence, Aβ(12−28) in complex with TTR imitates almost exactly the calorimetric behavior of the full Aβ(1−42) in complex with TTR. The effect of the TTR tetramer stabilizers IDIF, diflunisal, and Tafamidis upon these later complexes is analogous to the ones formed by full Aβ(1−42). The magnitude of this effect is stabilizing for IDIF but negligible for diflunisal (**1**) and Tafamidis (**2**). We hope that using this simpler and easy-handling Aβ(12−28) peptide, screening strategies for the identification of compounds chaperoning the TTR−Aβ peptides complexes could be realized. In turn, these strategies could aid in the search for potential drug candidates in AD drug discovery.

EXPERIMENTAL SECTION

Chemical Compounds. Amyloid β peptides Aβ(1−11) (**5**), Aβ(10−20) (**6**), Aβ(12−28) (**7**), and Aβ(25−35) (**8**) as trifluoroacetate salts were purchased from Bachem AG (Switzerland) (ref.: H-2956, H-1388, H-7910, and H-1192, respectively). Depsi-Aβ(1−42) peptide, a chemically modified β-amyloid (1−42) precursor containing an isoacyl dipeptide at residues Gly-Ser, was available from GenScript (ref.: RP10017-1, purity by HPLC > 96%). Aβ(1−40) peptide was available from rPeptide (β-amyloid (1−40), Ultra Pure, HFIP, ref: A-1153-2, purity > 97%, Lot#05271640H, www.rpeptide.com). *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid) (HEPES), glycine, *Tris*(hydroxymethyl)-aminomethane (Tris), dimethyl sulfoxide (DMSO), and the NSAID drug diflunisal (DIF, **1**) were purchased from Sigma-Aldrich (D3281, purity > 98%) and used without further purification. The small-molecule compound iododiflunisal (IDIF, **4**) was synthesized in our lab IQAC-CSIC by iodination of the NSAID diflunisal (**1**) following our procedures.[44] The drug Tafamidis (**2**) was prepared in our lab following the procedures described in the literature.[68] Purity of all final compounds was proved to be ≥95% by means of HPLC, HR-MS, and NMR techniques.

Solid-Phase Peptide Synthesis of Aβ(12−28) Peptide and Mutants of Aβ(12−28) Peptide. Amyloid peptide sequences Aβ(1−11) (**5**) and Aβ(12−28) (**7**) were purchased from Bachem AG (Switzerland) as trifluoroacetate salts (H-2956 and H-7910, respectively). H-2956 showed purity by HPLC > 96%, and H-7910 showed purity by HPLC > 96%. The Aβ(12−28) peptide and its corresponding mutants [V18A Aβ(12−28) (**9**); F19A Aβ(12−28) (**10**); and F20A Aβ(12−28) (**11**)] were synthesized by manual Solid-Phase Peptide Synthesis (SPPS) using Fmoc chemistry with the corresponding Fmoc-protected amino acids. Cleavage from resin was performed using TFA/H_2O/TIS (95:2.5:2.5) (v/v/v), and the peptides were precipitated with *tert*-butyl methyl ether. The peptides were purified by reversed-phase−high-performance liquid chromatography (RP−HPLC) using a VersaFlash system and characterized by analytical RP−HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS) (purity by HPLC > 96%). The characterization of the Aβ(12−28) peptide prepared in our lab was compared to a commercial sample acquired from Bachem (H-7910).

Preparation of Aβ(1−42). The native Aβ(1−42) peptide was obtained from depsi-Aβ(1−42) peptide (Genscript, RP10017-1, purity by HPLC > 96%), a chemically modified β-amyloid (1−42)

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precursor, by a switching procedure involving a change in pH and immediate use.

Recombinant Wild-Type Human TTR (wt rhTTR) Production and Purification. Human wild-type rhTTR gene was cloned into a pET expression system and transformed into *Escherichia coli* BL21(DE3) Star. The phTTRwt-I/pET-38b(+) plasmid was provided by Prof. Antoni Planas (IQS, URL). The production of recombinant protein was performed at the Erlenmeyer scale, and the production and purification of protein were done as described previously following an optimized version of our protocol.[69] Recombinant wild-type hTTR was produced using a pET expression system. The expressed protein only contains an additional methionine on the *N*-terminus compared to the mature natural human protein sequence. wt rhTTR protein was expressed in *E. coli* BL21−(DE3) cells harboring the corresponding plasmid. Expression cultures in 2xYT-rich medium containing 100 μg/mL kanamycin were grown at 37 °C to an optical density (at 600 nm) of 4 (OD600 ≈4); then induced by addition of IPTG (1 mM final concentration); grown at 37 °C for 20 h; harvested by centrifugation at 4 °C, 10,000 rpm for 10 min; and resuspended in a cell lysis buffer (0.5 M Tris−HCl, pH 7.6). Cell disruption and lysis were performed by French press followed by a sonication step at 4 °C. Cell debris was discarded after centrifugation at 4 °C, 11,000 rpm for 30 min. Intracellular proteins were fractionated by ammonium sulfate precipitation in three steps. Each precipitation was followed by centrifugation at 12 °C, 12,500 rpm for 30 min. The pellets were analyzed by SDS-PAGE (14% acrylamide). The TTR-containing fractions were resuspended in 20 mM Tris−HCl, 0.1 M NaCl, pH 7.6 (buffer A) and dialyzed against the same buffer. It was purified by ion-exchange chromatography using a Q-Sepharose High-Performance (Amersham Biosciences) anion-exchange column and eluting with a NaCl linear gradient using 0.1 M NaCl in 20 mM Tris−HCl pH 7.6 (buffer A) to 0.5 M NaCl 20 mM Tris−HCl pH 7.6 (buffer B). All TTR-enriched fractions were dialyzed against deionized water in three steps and were lyophilized. The protein was further purified by gel filtration chromatography using a Superdex 75 prep grade resin (GE Healthcare Bio-Sciences AB) and eluting with 20 mM Tris pH 7.6 and 0.1 M NaCl. Purest fractions were combined and dialyzed against deionized water and lyophilized. The purity of the protein preparations was > 95% as judged by SDS-PAGE. Average production yields were 150−200 mg of purified protein per liter of culture. Protein concentration was determined spectrophotometrically at 280 nm using a calculated extinction coefficient value of 17 780 M^{-1} cm^{-1} for wtTTR. The protein was stored at −20 °C.

Isothermal Titration Calorimetry (ITC) Assay. Experiments were carried out in a VP-ITC (MicroCal, LLC, Northampton, MA). In a titration experiment, the ligand in the syringe is added in small aliquots to the macromolecule, in our case the TTR protein in the calorimeter cell, which is filled with an effective volume that is sensed calorimetrically. The TTR solution of 20 μM and Aβ or ligand solutions of 200 μM were prepared in the same buffer. The titrant was injected over 20 or 30 times at a constant interval of 300 s with a 450 rpm rotating stirrer syringe into the sample cell containing its binding partner. All of the solutions were prepared with a 25 mM HEPES buffer, 10 mM glycine, pH 7.4, and 5% DMSO (final concentration), and it was corroborated that in these conditions, TTR and Aβ(1−42) are stable. The Aβ(1−42) working solution was prepared at 200 μM and used immediately to avoid premature aggregate formation. The TTR stock solution was prepared at 40 μM. Ligand stock solution was prepared at 10 mM in DMSO. All solutions were prepared in the same buffer and filtered prior to use. In the control experiments, the titrant (ligand or Aβ) was injected into the buffer in the sample cell to measure the heat of dilution. This value of the heat of dilution was subtracted from the titration data. The experiments were performed at 25 °C. Titration data were analyzed by evaluation software MicroCal Origin, Version 7.0. The binding curves were fitted by the nonlinear regression method to one set of sites binding model. This leads to the calculation of K, n, ΔH, ΔS, and ΔG. Each experiment was conducted three times, and the mean value with standard deviations is provided.

Transmission Electron Microscopy (TEM). Aβ(12−28) peptide (100 μM), alone or with TTR (20 μM) (alone or preincubated with a stabilizer for 1 h at 37 °C), was incubated at 37 °C for 48 h. For visualization by TEM, 5 μL sample aliquots were absorbed to a carbon-coated collodion film supported on 200-mesh copper grids, for 5 min, and negatively stained with 1% uranyl acetate. Grids were exhaustively examined with a JEOL JEM-1400 transmission electron microscope equipped with an Orious Sc1000 digital camera.

ThT Fluorescence Assay. Samples were prepared in 25 mM HEPES buffer, 10 mM glycine, pH 7.4, and 5% DMSO (final concentration) containing 20 μM ThT. The Aβ(1−42) peptide was adjusted to 50 μM, TTR to 25 μM, and the small-molecule compound to 50 μM as final concentrations. Briefly, samples of Aβ(1−42) alone or with TTR or with TTR preincubated with a small molecule were mixed with ThT. Fluorescence spectra were acquired in cells thermostated at 37 °C, with 15 s of shaking at 500 rpm every 30 min. ThT fluorescence assays were acquired in each cell of a 96-well plate containing 200 μL of sample. Excitation was at 430 nm, and emission spectra were recorded at 485 nm using a Beckman Coulter DTX 880 Multimode Detector plate reader. The results are the mean values of four replicates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.9b01970.

Recombinant wild-type human (wt rhTTR) production and purification and MALDI-TOF MS; turbidity assays of the binary and ternary assay complex formation using Aβ(1−42); synthesis of Aβ(12−28) (**7**) and three Ala mutants (**8**, **9**, and **10**); additional isothermal titration calorimetry (ITC) studies (binary and ternary interactions and control experiments) (PDF)

Molecular formula strings of key compounds (CSV)

■ AUTHOR INFORMATION

Corresponding Author

Gemma Arsequell − *Institut de Química Avançada de Catalunya (I.Q.A.C.-C.S.I.C.), 08034 Barcelona, Spain;* orcid.org/0000-0002-7009-3738; Email: gemma.arsequell@iqac.csic.es

Authors

Ellen Y. Cotrina − *Institut de Química Avançada de Catalunya (I.Q.A.C.-C.S.I.C.), 08034 Barcelona, Spain*

Ana Gimeno − *CIC bioGUNE, Basque Research and Technology Alliance (BRTA), 48160 Derio, Spain;* orcid.org/0000-0001-9668-2605

Jordi Llop − *CIC biomaGUNE, Basque Research and Technology Alliance (BRTA), 20014 San Sebastian, Spain;* orcid.org/0000-0002-0821-9838

Jesús Jiménez-Barbero − *CIC bioGUNE, Basque Research and Technology Alliance (BRTA), 48160 Derio, Spain; Ikerbasque, Basque Foundation for Science, 48009 Bilbao, Spain; Department of Organic Chemistry II, Faculty of Science & Technology, University of the Basque Country, 48940 Leioa, Bizkaia, Spain;* orcid.org/0000-0001-5421-8513

Jordi Quintana − *Research Programme on Biomedical Informatics, Universitat Pompeu Fabra (UPF-IMIM), 08003 Barcelona, Spain;* orcid.org/0000-0002-7059-7418

Gregorio Valencia − *Institut de Química Avançada de Catalunya (I.Q.A.C.-C.S.I.C.), 08034 Barcelona, Spain*

Isabel Cardoso − *IBMC—Instituto de Biologia Molecular e Celular, 4200-135 Porto, Portugal; i3S—Instituto de*

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Investigação e Inovação em Saúde, Universidade do Porto, 4200-135 Porto, Portugal

Rafel Prohens − *Unitat de Polimorfisme i Calorimetria, Centres Científics i Tecnològics, Universitat de Barcelona, 08028 Barcelona, Spain;* orcid.org/0000-0003-0294-1720

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jmedchem.9b01970

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

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■ ABBREVIATIONS

AD, Alzheimer's disease; TEM, transmission electron microscopy; ThT, thioflavin T; TTR, transthyretin; CSF, cerebrospinal fluid; IDIF, iododiflunisal; on, overnight; PPi, protein−protein interactions; SPPS, solid-phase peptide synthesis

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