A pair of peptides inhibits seeding of the hormone transporter transthyretin into amyloid fibrils

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Abstract

The tetrameric protein transthyretin is a transporter of retinol and thyroxine in blood, cerebrospinal fluid, and the eye, and is secreted by the liver, choroid plexus, and retinal epithelium, respectively. Systemic amyloid deposition of aggregated transthyretin causes hereditary and sporadic amyloidoses. A common treatment of patients with hereditary transthyretin amyloidosis is liver transplantation. However, this procedure, which replaces the patient’s variant transthyretin with the wild-type protein, can fail to stop subsequent cardiac deposition, ultimately requiring heart transplantation. We recently showed that preformed amyloid fibrils present in the heart at the time of surgery can template or seed further amyloid aggregation of native transthyretin. Here we assess possible interventions to halt this seeding, using biochemical and electron microscopy assays. We found that chemical or mutational stabilization of the transthyretin tetramer does not hinder amyloid seeding. In contrast, binding of the peptide inhibitor TabFH2 to ex vivo fibrils efficiently inhibits amyloid seeding by impeding self-association of the amyloid-driving strands F and H in a tissue-independent manner. Our findings point to inhibition of amyloid seeding by peptide inhibitors as a potential therapeutic approach.

Transthyretin amyloidosis (ATTR) is a fatal disease caused by the abnormal aggregation of the protein transthyretin (TTR). TTR, a transporter of retinol and thyroxine in blood, cerebrospinal fluid, and the eye, is secreted by the liver, choroid plexus, and retinal epithelium, respectively. TTR amyloid deposits are found in virtually every organ of ATTR patients, but the heart and nerves are often the first to fail. Although more than 140 known hereditary mutations in the ttr gene result in an early onset of the disease, wild-type TTR is found not only co-depositing with mutant TTR in hereditary ATTR cases but also in sporadic cases in which only wild-type TTR is present. Wild-type ATTR, or senile systemic amyloidosis, manifests as an age-related disease, and is often overlooked and underdiagnosed (1, 2).

The current standard of care for hereditary cases is liver transplantation, which does not always cure the condition. Through this procedure, most of the circulating mutant TTR is replaced with the wild-type form that is secreted by the implanted liver. However, this surgery is not sufficient to stop amyloid cardiac deposition in many patients who require heart transplantation a few years later. Our recent studies suggest the reason for such continued cardiomyopathy: preformed TTR fibrils present in cardiac tissues of ATTR patients at the time of surgery have the capacity to catalyze or seed fibril aggregation of
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wild-type TTR that is secreted by the implanted liver (3).

The stabilization of the functional non-amyloidogenic form of transthyretin is currently under clinical assessment. The functional and most abundant form of TTR is tetrameric, with a hydrophobic central tunnel that binds thyroxine. Kelly and colleagues have established that conversion of native transthyretin to amyloid fibrils is preceded by dissociation of tetrameric TTR to monomers, which then undergo a conformational change and form fibrils (4). Based on this premise, extensive biochemical studies have led to the discovery of compounds such as tafamidis and diflunisal that bind within the hydrophobic central tunnel of TTR and stabilize this binding within the hydrophobic central tunnel of TTR and stabilize the native structure, inhibiting its aggregation in vitro (5–7). These two ligands stabilize tetrameric transthyretin in vivo and delay the progression of disease in many patients. However, the efficacy of these ligands is reduced when administered at late stages of the disease (8, 9).

In our recent studies, we have developed and optimized peptide inhibitors that are designed to cap the tip of TTR fibrils and block further amyloid aggregation (3, 10). This structure-based drug design strategy started with the identification of two amyloid-driving segments of transthyretin: β-strands F and H (10). We then determined the structures of the two segments in their amyloid state and designed peptide inhibitors that block self-association and protein aggregation in vitro. This first generation of inhibitors was further optimized in a second study (3).

Amyloid seeding offers a potential therapeutic target to be explored. Clinical observations indicate that amyloid seeding greatly contributes to ATTR pathogenesis (11). In our previous study, we found that tetramer stabilizers do not inhibit amyloid seeding catalyzed by amyloid fibrils extracted from an ATTR-D38A patient (3). In contrast, our peptide inhibitors halted this process. Here we expand our studies by evaluating the efficacy of both tetramer stabilization and peptide inhibitors in blocking amyloid seeding of ATTR fibrils extracted from affected tissue of several types.

Results

Tetramer stabilization by ligands does not inhibit TTR amyloid formation induced by ATTR-D38A ex-vivo seeds.

We previously found that the presence of tafamidis or diflunisal at 180 μM is not sufficient to prevent amyloid seeding of wild-type TTR catalyzed by ATTR fibrils extracted from the explanted heart of an ATTR-D38A patient (3). Although ATTR-D38A patients manifest slow progressive polyneuropathy and autonomic symptoms similar to the most common form of hereditary ATTR, ATTR-V30M, they also develop cardiac dysfunction (12). Here we expand our previous study by evaluating the effect of these ligands at various concentrations.

We first confirmed the ability of tafamidis and diflunisal to inhibit protein aggregation in the absence of seeds. Consistent with previous studies, we found that both tafamidis and diflunisal efficiently inhibit wild-type TTR aggregation in vitro under acidic conditions in the absence of seeds (Fig. 1a,b) (5–7). For these aggregation assays, we incubated 1 mg/ml recombinant wild-type TTR with increasing amounts of stabilizers at pH 4.3 and monitored protein aggregation both by absorbance at 400 nm and immuno-dot blot of the insoluble fraction, as we previously described (10). After 4 days in the absence of inhibitor, virtually the total amount of TTR present was converted to aggregates. In the presence of either tafamidis or diflunisal, TTR aggregation was diminished in a dose-dependent manner (Fig. 1a,b).

We next studied the effect of various concentrations of tafamidis and diflunisal on protein aggregation in the presence of ATTR-D38A ex-vivo seeds. In our previous study, we observed that the addition of fibril seeds extracted from ATTR cardiac tissue accelerates aggregation but only of wild-type TTR at pH 4.3 but also monomeric TTR under physiological conditions (3). Additionally, we tested the effect of tafamidis and diflunisal at 180 μM on amyloid seeding and found that this concentration was not sufficient to hinder the process. Here we evaluate the effect of these ligands at various concentrations (Fig. 1c-f). For this assay, we incubated 0.5 mg/ml recombinant wild-type TTR with 30 ng/μl ATTR-D38A ex-vivo seeds and increasing amounts of ligands. We monitored fibril formation for 24 hours by Thioflavin T fluorescence (ThT), by immuno-dot blot of the insoluble fraction (Fig. 1c-f), and by protein quantification of insoluble fractions (Fig. 1g), as we previously described (10). We found that these ligands did not reduce or delay seeded fibril formation even at ligand concentrations that resulted in full inhibition of unseeded TTR aggregation (Fig. 1).

Tetramer stabilization does not inhibit TTR amyloid formation induced by any of the ATTR ex-vivo seeds studied

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In our previous study, we report that our specimen obtained from an ATTR-D38A patient contains type B ATTR amyloid fibrils made of full-length TTR (3, 13). In order to rule out the possibility of pathology-based specificity, we analyzed seeding inhibition by ligands with seven additional ATTR samples (Fig 2). For this assay, we incubated 0.5 mg/ml recombinant wild-type TTR with 30 ng/µl ATTR ex-vivo seeds in the presence or the absence of 180 µM stabilizers. We monitored fibril formation by quantifying the protein content in the insoluble fraction after 24 hours of incubation at 37 °C. As in our ATTR-D38A experiments, we found that the addition of tafamidis or diflunisal did not reduce the accumulation of insoluble material in the presence of seeds extracted from any of the other seven ATTR cardiac specimens. These findings suggest that tetramer stabilization by ligands may not be an effective strategy to halt amyloid seeding under the studied conditions.

T119M-derived stabilization fails to inhibit amyloid seeding

We then evaluated tetramer stabilization by mutagenesis, analyzing the effect of the T119M TTR variant on amyloid seeding. This variant exhibits high tetrameric stability resulting in a significant delay of the onset of hereditary neuropathic ATTR in patients who carry both ttr-V30M and ttr-T119M genes (5, 14, 15). Although a sample containing 1 mg/ml recombinant T119M variant remained soluble after days of incubation at a low pH that causes native TTR to dissociate (10) (Fig. 3a), the addition of 30 ng/µl ATTR-D38A ex-vivo seeds to 0.5 mg/ml recombinant T119M did result in seeded fibril formation (Fig. 3b). Notably, unlike T119M, the TTR variant T119W, which blocks self-association of strand H (10), did show a significant decrease in protein fibril formation upon seeding (Fig. 3b). We also found that although ex-vivo seeds caused the aggregation of a TTR variant that blocks self-association of the strand F, S85P/E92P, the lag phase was significantly longer (Fig. 3b-c). Consistent with our previous work (3), these findings suggest that blocking self-association of amyloidogenic TTR segments may be an effective approach to stop fibril formation when ATTR amyloid seeds are present.

TabFH2 halts amyloid seeding caused by ex-vivo seeds extracted from all ATTR samples evaluated

In a previous study, we developed peptide inhibitors that were designed to cap the tip of amyloidogenic segments of TTR in their amyloid state (10). Recently, we have shown that the optimized peptide inhibitor TabFH2 blocks amyloid seeding driven by fibrils extracted from the explanted heart of a patient who carries the hereditary mutation ATTR-D38A (3). Note that TabFH2 is a cocktail of two peptides, TabF2 and TabH2, that target the two amyloid-driving segments of transthyretin (3, 10). To better compare the efficacy of the peptide inhibitor TabFH2 with tafamidis and diflunisal, we evaluated TabFH2 with the same set of assays. We first found that TabFH2 inhibits TTR aggregation in the absence of ATTR seeds (Fig. 4a). For this assay, we incubated 1 mg/ml recombinant wild-type TTR with increasing amounts of TabFH2 and monitored protein aggregation for 4 days by absorbance at 400 nm, and visualized the insoluble fraction by immuno-dot blot, as described above. In addition, we analyzed the inhibitory effect of TabFH2 in our amyloid seeding assays, using the same conditions as in the preceding experiment (Fig. 1c-f): we incubated 0.5 mg/ml of recombinant wild-type TTR with 30 ng/µl ATTR-D38A seeds and TabFH2 at various concentrations, for 24 hours (Fig. 4b-d). We found that TabFH2 completely inhibits TTR amyloid seeding at concentrations higher than 180 µM with intermediate effect at lower doses. Longer incubation resulted in similar results (Fig. 4e). Altogether, our results indicate that TabFH2 inhibits TTR aggregation when seeded by ex-vivo patient amyloid fibrils, in a dose-dependent manner (Fig. 4).

We next examined the effectiveness of TabFH2 inhibitors in other pathological cases, by evaluating its inhibitory activity on our additional ATTR ex-vivo samples (Fig. 5). For this assay, we incubated 0.5 mg/ml recombinant wild-type TTR with 30 ng/µl ATTR ex-vivo seeds extracted from a total of 9 patients, in the presence or the absence of 180 or 360 µM TabFH2. We monitored fibril formation by quantifying the protein content in the insoluble fraction after 24 hours of incubation at 37 °C (Fig. 5a) and immuno-dot blot of the insoluble fraction (Fig. 5b). The presence or the absence of protein aggregates was confirmed by electron microscopy (Fig. 5c). A scrambled version of our peptide inhibitor, H1, was included as a negative control. We found that the efficacy of TabFH2 was dose-dependent, and although its efficiency differs from sample to sample, for every sample full inhibition was observed at the highest concentration analyzed (Fig. 5a-c). This variability did not correlate with mutational background of the ex-vivo seeds. In contrast, we found an inverse relationship between TabFH2...
effectiveness and the amount of TTR C-term fragments in the *ex-vivo* resuspension (Fig. 5d). The content of fragmented TTR, present in type A amyloidosis, was quantified from two independent western blots using an antibody that specifically recognizes TTR C-terminal fragments, as we previously described (3). Pearson's correlation coefficient between amyloid seeding in the presence of 180 µM TabFH2 and TTR fragment content was of 0.90 (R-square=0.80) indicating a positive correlation. In addition, we evaluated the effect of inhibitors on the seeds extracted from an ATTR-V30M patient (Fig. 5e-f). As mentioned above, ATTR-V30M is the most common form of hereditary ATTR that manifests progressive polyneuropathy and autonomic symptoms (12). We observed a similar pattern: TabFH2 offers an inhibitory effect whereas tafamidis and diflunisal show limited inhibition of seeding (Fig. 5e-f).

**TabFH2 inhibitory activity is tissue independent**

We extracted *ex-vivo* seeds from small quantities of two additional tissue types to evaluate tissue specificity of TabFH2. We extracted ATTR seeds from five samples of adipose tissue collected in fat pad biopsies, including one case of wild-type ATTR and four cases of hereditary ATTR, and two samples of labial salivary glands from two ATTR-V30M patients (Table 1). Since biopsy specimens were considerably smaller than the cardiac tissue samples (30-150 mg vs. 1-5 g), we downsized the amyloid extraction protocol accordingly. Otherwise, the procedure remained as previously described (3). The extraction from fat pad biopsies produced a very limited amount of insoluble material. We were therefore forced to reduce the amount of *ex-vivo* seeds used in amyloid seeding assays to 5 ng/µl. Due to the formation of structurally different species or perhaps a result of the small amount of seeds that were added to the assay, the ThT signal was insufficient to draw any conclusion. For this reason, we opted to follow amyloid seeding by image-based computational quantification of protein aggregates after 24 hours of incubation (Fig. 6). We collected images of the bottom of 96 well plates by optical microscopy using the Celigo S Imaging system. The images showed the formation of UV-positive aggregates in those samples that contained *ex-vivo* seeds and no apparent aggregation in the control sample (Fig. 6a). The inhibitory effect of TabFH2 was confirmed by a significant reduction of amyloid conversion at 180 µM (Fig 6b). Together, our results indicate that TabFH2 efficiently inhibits amyloid seeding caused by wild-type and mutant *ex-vivo* seeds in a tissue-independent manner, for the range of tissues we studied.

**TabFH2 inhibits amyloid seeding by binding to ATTR fibrils**

Our peptide inhibitors were originally designed and optimized to cap the tip of TTR fibrils by binding β-strands F and H (3, 10). To validate our design, we next assessed binding of TabFH2 to ATTR seeds (Fig. 7). We first immobilized 5 µg of ATTR-D38A seeds on each of eight anti-TTR pre-coated wells. Binding of ATTR seeds to the bottom of the wells was confirmed later by BCA protein assay of unbound material. We then added increasing amounts of TabFH2 (0-500 µM) to pretreated wells. After 2 hours of incubation, we analyzed the remaining amount of TabFH2 in the sample by HPLC. Anti-TTR pre-coated wells treated with buffer, but not seeds, were used as negative control to detect unspecific binding of TabFH2 to the well. While the majority of TabFH2 remained in the sample after incubation in negative control wells, most of TabFH2 was absent when wells were pretreated with ATTR seeds (Fig. 7d). Our results indicate that the mechanism of action of our peptide inhibitors involves binding to ATTR fibrils.

**Discussion**

Extensive studies by others have established that ligands such as tafamidis and diflunisal bind within TTR and stabilize its tetrameric structure, diminishing its rate of dissociation and hence its conversion to amyloid fibrils (5–7). The data of Fig. 1a and 1b confirm the stabilization of TTR by tafamidis and diflunisal. Indeed tafamidis has been prescribed for the treatment of neuropathic ATTR-V30M and ameliorates disease progression when administered at disease stage I (16–19). Diflunisal has shown positive neurological effects in ATTR patients at different stages (9, 20). Despite the stabilization, our results show that disease-related seeds convert wild-type TTR into amyloid fibrils in the presence of these ligands at concentrations that fully inhibit aggregation of recombinant transthyretin in the absence of seeds (Fig. 1 and 2) (21, 22). Thus our results offer a plausible explanation for the reported limited efficacy of tafamidis over the long term, when administered at late stages, or in patients with severe cardiac involvement (8, 23, 24). That is, stabilization of tetrameric TTR may be insufficient in situations in which seeded polymerization dominates rather than *de-novo* nucleation of TTR seeds.
Genetically stabilized TTR fails to inhibit seeded fibril formation. The genetic variant T119M of TTR and its capacity to delay fibril formation was originally found in an ATTR-V30M family because of its protective effects; this variant remains soluble at pH 4.3 for weeks, if not months (Fig. 3a) (10, 14, 25). However, in our experiments, this stabilized variant does not halt conversion to amyloid in the presence of ex-vivo seeds (Fig. 3b, c). These findings may explain why heterozygous individuals carrying both the hereditary amyloidogenic trt-V30M allele and the stabilizing trt-T119M allele in time develop ATTR (14, 15). In ATTR-V30M/T119M patients, we hypothesize that the mutation T119M may delay the progression of ATTR by reducing de-novo nucleation.

The limited effect of TTR tetramer stabilization in our experiments does not contradict the well-established mechanism of de-novo formation of TTR amyloid (26). In our previous study, we showed that the conversion of TTR to amyloid fibrils requires the dissociation of tetramers and partial unfolding of monomers, which takes place as the pH is lowered to 4.3, exposing the adhesive amyloidogenic segments. The monomeric variant MTTR, which exposes these segments, can be seeded at physiological pH (3). TTR dissociation and subsequent seeded or unseeded polymerization may be triggered by a variety of factors, such as protease cleavage or local pH disturbances. In our assays, amyloid seeding is also performed at low pH to weaken the quaternary structure and to lead to tetramer dissociation. Under acidic conditions, the addition of seeds to recombinant TTR causes acceleration of fibril formation through seeded polymerization. We reason that this seeded polymerization results in fibrils that are resilient to disassembly, thereby making the pathway irreversible. Because the interaction of TTR with the tetramer stabilizers is not irreversible, the presence of seeds will eventually reduce both monomeric and tetrameric pools while generating irreversible amyloid fibrils.

Capping of TTR amyloid fibrils by designed peptides is an effective approach to inhibit TTR fibril seeding. Our results show that in cases for which stabilization of tetrameric TTR by ligands may not be fully effective in halting fibril formation (Fig. 1 and 2), capping of TTR fibrils by designed blockers of fibril elongation is effective (Fig. 4-6). This may be of special importance for cardiac ATTR patients, who are often diagnosed when manifesting advanced TTR deposition and have limited treatment options. In the conditions of our experiments, TabFH2 blockers are effective in halting fibril formation caused by wild-type ATTR seeds and also by seeds of at least six disease-related variants extracted from ex-vivo cardiac specimens (Fig. 5). Overall, we find that the inhibition of amyloid seeding by peptide inhibitors is an effective strategy independent of pathological variant or tissue (Fig. 5 and 6).

Structure-based inhibition of amyloid aggregation by peptides is an emergent strategy that has shown promising results. Our structure-based strategy has generated peptide inhibitors of tau fibril formation that is associated with Alzheimer's disease (27, 28), SEVI amyloid aggregation that enhances HIV transmission (28), and p53 aggregation associated with certain types of ovarian cancer (29). In our previous work on inhibition of TTR, we generated TTR peptide inhibitors that block both protein aggregation and amyloid seeding catalyzed by ex-vivo ATTR fibrils (3, 10). Moreover, diseased flies showed motor improvement and a reduction of TTR deposition after treatment with our peptide inhibitors (30). In this study, we expand the evaluation of peptide inhibitors to question their inhibitory capacity in comparison to two tetramer stabilizers and mutagenesis-based stabilization. Figure 8 depicts how we envision the mechanism by which TabFH2 and tetramer stabilizers inhibit amyloid seeding and aggregation, respectively. The peptide inhibitor TabFH2 was previously designed to bind strands F and H, the two amyloid driving segments of TTR, thereby halting self-association and further polymerization (3, 10). Other groups have expanded on our work on the inhibition of TTR aggregation by impeding self-association of the amyloid-driving strands F and H of TTR using monoclonal antibodies (31, 32). Our results indicate that the inhibition of amyloid seeding by peptide inhibitors may represent a potential therapeutic strategy for ATTR when tetramer stabilization is not sufficient to halt disease progression.

**Experimental procedures**

**Antibodies:**

The antibodies used in this study include rabbit anti-human transthyretin polyclonal antibody (DAKO, Agilent Technologies; immuno-dot blots and western blots 1:10,000) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (ThermoFisher Scientific, immuno-dot blots and western blots 1:5,000). Anti-truncated TTR was generously provided by
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Gunilla Westermark (labeled as 1898, western blots 1:5,000).

Patients and Tissue Materials:
Twenty-one ATTR patients carrying wild-type (n=4) or TTR mutations (n=17) were included in this study (refer to Table 1 for full details). Cardiac tissue specimens were obtained from several laboratories from explanted hearts or by autopsy. Adipose tissues were obtained from needle biopsy procedures performed at the University Medical Centre Groningen. Salivary gland tissues were obtained from surgical biopsy procedures performed at the Hospital Santo António in Porto. The UCLA Office of the Human Research Protection Program granted exemption from IRB review because all specimens were anonymized. These studies abide by the Declaration of Helsinki principles.

Extraction of amyloid fibrils from tissue samples
Amyloid fibrils were extracted from fresh-frozen human tissue following a previously described protocol (3). In short, thawed tissue sample, resuspended in 10 mL of 0.15 M NaCl, was minced with a motorized homogenizer and pelleted by centrifugation at 15,000 rpm for 30 minutes. The pellet was subject to further cycles of resuspension, homogenization, and centrifugation first with 0.15 M NaCl solution 7 times, followed by distilled water 3 times. Since there was less starting material for both adipose tissue and salivary gland specimens, the extraction protocol was downsized in volume accordingly. The final pellet was lyophilized, and amyloid content of the extracts was confirmed by electron microscopy. TTR content of the samples was analyzed by anti-TTR western blot.

Purification of Recombinant TTR
Recombinant transthyretin was prepared as described previously (10). To summarize, E. coli cells (Millipore Rosetta DE3 pLysS Competent Cells) were transformed with a pET24(+) vector carrying the sequence for either wild-type or a mutant of transthyretin. The expressed recombinant protein was harvested and purified by nickel affinity chromatography with a HisTrap column (GE Healthcare). The appropriate fractions were pooled and further purified by size exclusion on a Superdex 75 gel filtration column (GE Healthcare). Recombinant transthyretin was stored in 10 mM sodium phosphate pH 7.5, 100 mM KCl, and 1 mM EDTA at -20 °C.

Western Blot of Tissue Extracts
TTR content of tissue extracts was confirmed by western blotting as described previously (3). To summarize, equal amounts of total protein were loaded on a 4-12% NuPAGE BisTris Gel (ThermoFisher Scientific) and separated by gel electrophoresis in denaturing conditions. TTR was detected after transfer to a nitrocellulose membrane with polyclonal anti-human transthyretin antibody or anti-truncated TTR antibody and horseradish peroxidase conjugated secondary goat anti-rabbit IgG. SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFisher Scientific) was used according to manufacturer’s instructions to visualize TTR content. Truncated TTR content of cardiac seeds was quantified by ImageJ using two independent western blots.

Congo red staining and TTR content quantification of adipose tissue
Abdominal fat smears were made as previously described (33). Slides were stained with alkaline Congo red (34) and apple-green birefringence under polarized light were semiquantitatively scored as follows: 0 (negative), 1 (minute, <1% surface area), 2 (little, between 1 and 10%), 3 (moderate, between 10 and 60%), and 4 (abundant, >60%). The remaining abdominal fat tissue was weighed and washed. Proteins were resuspended with a Tris-guanidinium hydrochloride solution, and TTR content was measured by ELISA. Briefly, microtiter plates were coated overnight with the extracts and human native TTR protein (Abcam, Cambridge, UK), which served as control, in several dilutions. Detection was done by using rabbit anti-human TTR polyclonal antibodies (DAKO, Agilent Technologies) followed by horseradish peroxidase- conjugated goat anti-rabbit IgG antibody (DAKO, Agilent Technologies) and visualized by a color reaction with TMB ELISA substrate. Plates were scanned at 450 nm after stopping the reaction with sulfuric acid.

Peptides
Peptides were synthesized at > 97% purity by GL Biochem (Shanghai) Ltd. (Shanghai, China). Purity and molecular weight were confirmed by MALDI-TOF and reversed phase HPLC. TabFH2 is an equimolar cocktail of RRRRHAHPFV(N-methyl)EFT and RRRSAYTNPTS(N-methyl)AVT, as previously described (3). Peptides were dissolved in 0.22 µm filtered water to 5 mM stock solution. These working solutions were further diluted prior to use to the final concentration.
Non-seeded TTR Aggregation

TTR aggregation assays were done as previously described (10). Briefly, 1 mg/mL TTR sample in 100 mM sodium acetate pH 4.3, 100 mM KCl, 1 mM EDTA was incubated in the presence or absence of inhibitor - diflunisal, tafamidis or TabFH2 – at 37°C for 4 days. TTR aggregation was followed by measuring sample turbidity at 400 nm and by anti-TTR immuno-dot blot of the insoluble fraction.

TTR Amyloid Seeding Assay

Extracted tissue samples were used to seed the formation of transthyretin amyloid fibrils following a protocol described previously (3). In short, extracts were washed twice in 1% sodium dodecyl sulfate and twice in 10 mM sodium phosphate pH 7.5, 100 mM KCl, 1 mM EDTA. Next, the extracts were sonicated at minimum intensity with 5 second pulses for a total of 10 minutes. Protein concentration of the samples was determined by Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). Seeding reactions contained 0.5 mg/mL of recombinant protein, 30 ng/µL of cardiac extract or in the case of adipose and gland extracts 5 ng/µL, 5 µM thioflavin T, 100 mM sodium acetate pH 4.3, 100 mM KCl, and 1 mM EDTA. The inhibitors mentioned, diflunisal, tafamidis, and TabFH2 were added at concentrations described in the figures. Thioflavin T fluorescence emission was measured at 482 nm with excitation at 440 nm in a FLUOstar Omega (BMG LabTech) plate reader. Plates were incubated at 37°C for 24 hours with orbital shaking at 700 rpm between measurement points. In all assays, measurements were normalized by subtracting the initial ThT measurement and considering the maximum signal as 100%. Protein aggregates were visualized by both bright field and UV and TTR aggregation was quantified using a Celigo S Imaging system. The insoluble fraction was obtained by centrifuging the sample and resuspending the pellet in guanidinium hydrochloride. Protein content of the pellet was determined by measuring absorbance at 280 nm.

Anti-TTR immuno-dot blot

TTR aggregation was confirmed by immuno-dot blot as described previously (10). After obtaining the insoluble fraction by centrifugation and resuspension in guanidinium hydrochloride, 15 µL of sample was dotted onto a nitrocellulose membrane (0.2 µm, Bio-Rad). TTR content was visualized using polyclonal rabbit anti-human transthyretin (DAKO), horseradish peroxidase conjugated goat anti-rabbit IgG antibody (ThermoFisher Scientific), and SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFisher Scientific).

Transmission Electron Microscopy

Amyloid content of tissue extracts and inhibition of fibril formation was confirmed by transmission electron microscopy. 5 µL of sample was applied to a glow discharged carbon coated electron microscopy grid (CF150-Cu, Electron Microscopy Sciences) for 4 minutes. After three quick rinses in distilled water, grids were stained with 2% uranyl acetate for 2 minutes. Samples were visualized using a T12 Quick CryoEM and CryoET (FEI) transmission electron microscope using an acceleration voltage of 120 kV equipped with a Gatan 2,048 x 2,048 CCD camera.

Detection of TabFH2 binding to ATTR seeds

TabFH2 binding to ATTR seeds was analyzed by HPLC. First, ATTR-D38A fibrils were immobilized on anti-TTR pre-coated well plates (Prealbumin ELISA kit, Abcam) as follows. 50 ml samples containing 0.1 mg/ml ATTR seeds were added on each well. Control wells were equally treated with buffer (10 mM sodium acetate pH 7.5, 100 mM KCl, 1 mM EDTA). After 1-hour incubation at room temperature, samples were then removed and wells were washed twice with buffer. Immobilization of the total amount of fibrils was confirmed by BCA protein assay of the remaining sample. 50 µL samples that contained increasing concentrations of TabFH2 (0-500 mM) were added to independent wells pretreated with ATTR seeds or buffer. After an incubation of 2 hours at room temperature, samples were transferred to new tubes and snap frozen until further analysis. Unbound TabFH2 was detected by chromatography after 0.10 nm filtration, on a Waters 1525 HPLC System (SpectraLab), with a Proto 300 C18 5 mm 250 x 4.6 mm analytical reverse phase column (Higgins Analytical). Flow rate = 1.0 ml/min; solvents: A = 0.1% trifluoroacetic acid in water, and B = 0.1% trifluoroacetic acid in acetonitrile. The column was equilibrated with 10% B for 5 minutes, followed by a gradient from 10% to 60% B in 30 minutes, and a 2 minute wash at 95% B. TabFH2 eluted in two peaks after approximately 17 and 20 min from the start. Peaks were integrated by Breeze2 software and Prism was used for graphing.
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Conflict of interest statement: The authors and UCLA have filed an international patent application for the TTR inhibitors (No. PCT/US17/40103). D.S.E. is an advisor and equity holder of ADRx, Inc. L.S. is a consultant of ADRx, Inc.

Author contributions: LS and DSE were responsible for project conception. LS directed all experiments. LS, BAN, KC, YW, AO, and JHL performed experimental procedures. TC, JB, and MDB contributed new reagents and analytic tools; LS, BAN, KC, and DSE analyzed data. LS wrote the paper.
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FOOTNOTES

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The abbreviations used are: TTR, transthyretin; ATTR, transthyretin amyloidosis; ThT, thioflavin T.
### Tables

#### Cardiac Tissue Specimens

| Mutation     | Origin       | Sex | Age | Neuropathy signs                      | Weight (g) |
|--------------|--------------|-----|-----|---------------------------------------|------------|
| ATTR-WT\(^1\) | Post mortem  | m   | 84  | No                                    | 1.6        |
| ATTR-WT\(^2\) | Post mortem  | m   | 70  | No, but amyloid found at autopsy      | 2.8        |
| ATTR-WT\(^3\) | Transplant   | m   | 78  | No                                    | 3.4        |
| ATTR-P24S    | Transplant   | m   | 65  | No                                    | 2.3        |
| ATTR-V30M    | Post mortem  | m   | 60  | Yes                                   | 2.5        |
| ATTR-D38A    | Transplant   | f   | 59  | Unknown                               | 4.5        |
| ATTR-T60A    | Post mortem  | m   | 57  | Moderate                              | 2.2        |
| ATTR-I84S\(^1\) | Post mortem | f   | 56  | No, but amyloid found at autopsy      | 2.7        |
| ATTR-I84S\(^2\) | Post mortem | m   | 53  | Mild                                  | 1.1        |

#### Fat biopsy specimens

| Mutation       | Congo Red (0-4) | Sex | Age | Wet fat (mg) | ng TTR/ mg fat |
|----------------|-----------------|-----|-----|--------------|----------------|
| ATTR-WT        | 3               | m   | 73  | 58.9         | 51.8           |
| ATTR-V30M      | 4               | f   | 47  | 78.5         | 618.7          |
| ATTR-A45G      | 4               | m   | 77  | 91.8         | 657.7          |
| ATTR-G47E      | 4               | m   | 35  | 31.3         | 433.3          |
| ATTR-V122del   | 3               | m   | 59  | 17.7         | 84.2           |

#### Labial salivary gland biopsy specimens

| Mutation       | Sex  | Age   |
|----------------|------|-------|
| ATTR-V30M\(^1\) | Unknown | Unknown |
| ATTR-V30M\(^2\) | Unknown | Unknown |

**Table 1. Tissue specimens used for amyloid extraction.** A total of 8 cardiac, 5 adipose, and 2 glandular specimens were included in this study. Patients presenting the same TTR variant are indicated by superscripts.
Figure 1. Tetramer stabilizers inhibit TTR aggregation in the absence of seeds and fail to inhibit amyloid seeding caused by ATTR-D38A ex-vivo seeds. (a, b) Inhibition assay of TTR aggregation in the absence of seeds, measured by absorbance at 400 nm. Increasing amounts of diflunisal (a) or tafamidis (b) were added to 1 mg/ml recombinant wild-type TTR and the sample was incubated for 4 days at pH 4.3. Absorbance measured after 4 days of incubation in the absence of ligand was considered 100% aggregation because no soluble TTR was detected. n=3, Error bars, SD. Right insets, full inhibition of TTR aggregation by diflunisal (a) or tafamidis (b) was confirmed by anti-TTR dot blot of insoluble fractions (IF) collected by centrifugation. (c, d) Inhibition assay of amyloid seeding at pH 4.3, monitored by ThT fluorescence. Increasing amounts of diflunisal (c) or tafamidis (d) were added to 0.5 mg/ml recombinant wild-type TTR and ATTR-D38A seeds. All replicates are shown, n=3. a.u., arbitrary units. Insets, anti-TTR dot blot of insoluble fractions. All samples were spotted onto the same nitrocellulose membrane and subjected to the same procedure; splicing was needed for presentation purposes. (e, f) Short-time view of the lag phase of c and d, respectively. n=3, Error bars, S.D. (g) Protein content quantification of the insoluble fractions collected from c and d, measured by 280 nm absorbance. AU, absorbance units. Notice that tafamidis at only its highest concentration tested (360 µM) results in a significant reduction of ThT signal (d). However, this reduction does not correlate with a reduction of protein content in the insoluble fraction (g). Diflunisal treatment did not inhibit amyloid seeding of TTR under tested conditions (c, g).
Figure 2. Tetramer stabilizers do not inhibit amyloid seeding caused by any of the cardiac samples analyzed. (a) Inhibition assay of amyloid seeding of ex-vivo seeds incubated with recombinant wild-type transthyretin in the absence or presence of 180 µM tafamidis or 180 µM diflunisal. After 24 h of incubation at pH 4.3, the insoluble fraction was collected, and protein content was quantified by absorbance at 280 nm. Consistent with the results observed in Fig. 1, the addition of diflunisal or tafamidis does not result in an apparent reduction of amyloid seeding. AU, absorbance units. (b) Anti-TTR dot blot of the insoluble fractions collected from a, shows that tetramer stabilizers do not reduce the formation of insoluble TTR-derived material when seeded with ATTR ex-vivo fibrils.
Figure 3. Mutational tetramer stabilization does not halt amyloid seeding. (a) Aggregation assay of 1 mg/ml non-aggregating recombinant TTR mutants in the absence of seeds at pH 4.3, followed by absorbance at 400 nm. The assay shows that the T119M and other non-aggregating variants do not aggregate at pH 4.3 if unseeded. Aggregation of wild-type transthyretin was considered as 100%. Right inset, anti-TTR dot blot of insoluble fractions collected by centrifugation after 4 days of incubation. n=3. Error bars, S.D. (b) Amyloid seeding assay of non-aggregating mutants in the presence of ATTR-D38A ex-vivo seeds, monitored by ThT fluorescence at pH 4.3. Notice that T119M does aggregate when seeded. Right inset, anti-TTR dot blot of insoluble fractions collected after 24 hours of incubation. All replicates are shown, n=3. a.u. arbitrary units. (c) Short-time view of the lag phase of the amyloid seeding assay shown in b. n=3. Error bars, S.D. *p ≤ 0.05 and **p ≤ 0.005.
Figure 4. The anti-amyloid peptide inhibitor TabFH2 inhibits TTR aggregation and amyloid seeding caused by ATTR ex-vivo seeds extracted from ATTR-D38A cardiac tissue. (a) Inhibition of TTR aggregation by TabFH2 in the absence of seeds, measured by absorbance at 400 nm. Increasing amounts of TabFH2 were added to 1 mg/ml recombinant wild-type TTR and the sample was incubated for 4 days at pH 4.3. Absorbance measured after 4 days of incubation in the absence of TabFH2 was considered 100% aggregation because no soluble TTR was detected. n=3, Error bars, SD. Right inset, anti-TTR dot blot of insoluble fractions (IF) collected by centrifugation after 4 days of incubation. (b) Inhibition of amyloid seeding by TabFH2 at pH 4.3, monitored by ThT fluorescence. Increasing amounts of TabFH2 were added to 0.5 mg/ml recombinant wild-type TTR and 30 ng/µl ATTR-D38A seeds. All replicates are shown, n=4. a.u., arbitrary units. Inset, anti-TTR dot blot of insoluble fractions collected by centrifugation after 24 hours of incubation. All samples were spotted onto the same nitrocellulose membrane and subjected to the same procedure; splicing was needed for presentation purposes. (c) Short-time view of the lag phase of the assay shown in b. n=4, Error bars, S.D. (d) Protein content quantification of the insoluble fractions collected from b, measured by 280 nm absorbance. AU, absorbance units. Notice that the reduction of ThT fluorescence observed in b correlates with the decrease of total protein and TTR content in the insoluble fractions, shown in c and the right inset of b, respectively. (e) Comparison of inhibition of amyloid seeding by tafamidis, diflunisal, and TabFH2 when incubated for 4 days, measured by ThT fluorescence. 360 µM inhibitor was added to 0.5 mg/ml recombinant wild-type TTR and 30 ng/µl ATTR-D38A seeds. n=3, Error bars, SD. Inset, anti-TTR dot blot of insoluble fractions collected by centrifugation after 4 days of incubation.
Inhibition of amyloid seeding

Figure 5. TabFH2 inhibits amyloid seeding caused by ex-vivo ATTR seeds extracted from all cardiac extracts studied. (a) Inhibition of amyloid seeding by TabFH2 in the presence of cardiac ex-vivo seeds, measured by protein content quantification of insoluble fractions. 30 ng/µl ex-vivo seeds extracted from the hearts of eight ATTR patients were added to 0.5 mg/ml recombinant wild-type transthyretin in the presence of 0, 180 or 360 µM TabFH2. The insoluble fractions were collected by centrifugation after 24 hours of incubation. The insoluble protein content was measured by absorbance at 280 nm. The results show that full inhibition of amyloid seeding by TabFH2 is achieved at the highest concentration for every ATTR ex-vivo sample. n=3. Error bars, S.D. *p ≤ 0.05 and **p ≤ 0.005, ***p ≤ 0.0005. (b) TTR immuno-dot blot of insoluble fractions collected by centrifugation from the assay shown in a, with consistent results. The experiment shown in Fig. 5a and 5b was performed in combination with the assay shown in Fig. 2; therefore the control samples without seeds are the same. All samples, including those from Fig. 2, were spotted onto the same nitrocellulose membrane and subjected to the same procedure; splicing was needed for presentation purposes. (c) Electron micrographs of the samples containing 360 µM TabFH2 collected from a. These are uncorrected images generated directly from a Gatan 2kX2k CCD camera. Scale bar, 200 nm. (d) Correlation between amyloid seeding capacity in the presence of 180 µM TabFH2, and relative quantity of truncated TTR of ex-vivo ATTR seeds. Notice that the presence of truncated TTR facilitates seeding. Truncated TTR content was quantified by ImageJ from two independent western-bLOTS. Linear regression and Pearson r were obtained by OriginLab. (e) Comparison of inhibition of ATTR-V30M amyloid seeding by tafamidis, diflunisal, and TabFH2, measured by ThT fluorescence. 180 µM or 360 µM inhibitor was added to 0.5 mg/ml recombinant wild-type TTR and 30 ng/µl ATTR-V30M seeds. All replicates are shown, n=4. a.u., arbitrary units. Inset, anti-TTR dot blot of insoluble fractions collected by centrifugation after 24 hours of incubation. (f) Electron micrographs of the samples collected from e.
Inhibition of amyloid seeding

Figure 6. Inhibition of amyloid seeding caused by ex-vivo ATTR seeds extracted from human fat pad and salivary gland biopsies. (a) Representative optical micrographs of aggregates of recombinant wild-type TTR formed after 24 hours of incubation with 5 ng/µl fat-extracted ATTR-WT or gland-extracted ATTR-V30M1 ex-vivo seeds, as visualized on a Celigo S Imaging system under bright field and UV channels. Images are composites generated by the Celigo S Imaging system. Scale bar, 1 mm; all images have the same scale. Note that the addition of TabFH2 results in the reduction of UV-positive aggregates. Three control samples are included: samples containing only seeds, recombinant wild-type TTR aggregates formed after incubation with 30 ng/µl cardiac ATTR-D38A seeds, and recombinant wild-type TTR in the absence of seeds (rec.WT). (b) UV intensity-based quantification of protein aggregates after 24 hours of incubation of recombinant wild-type TTR with ATTR ex-vivo seeds extracted from various adipose tissue and salivary gland samples (respectively labeled with italic a and g). The presence of 180 µM TabFH2 resulted in the inhibition of amyloid seeding for all studied samples. Cyan line and rectangle represent the UV intensity mean and S.D. range in the absence of seeds, respectively. n=3. Error bars, S.D. *p ≤ 0.05 and **p ≤ 0.005, ***p ≤ 0.0005, for the comparison between samples with and without TabFH2.
Inhibition of amyloid seeding

Figure 7. TabFH2 binds ATTR ex-vivo seeds. Increasing amounts of TabFH2 were added to independent wells pretreated with ATTR seeds (treated with seeds) or buffer (treated with buffer), and unbound TabFH2 was detected by reverse-phase chromatography. TabFH2 samples before treatment were included in the analysis (untreated). (a) Representative HPLC elution profile for a TabFH2 concentration of 500 µM showing two peaks: TabH2 eluting at ~17 ml and TabF2 eluting at ~21 ml. (b) Analysis of TabFH2 recovery at all concentrations, measured as peak area integrated from HPLC elution profiles. The graph shows that the recovery of both TabF2 and TabH2 when wells were pretreated with ATTR seeds was significantly lower than when pretreated with buffer.
Figure 8. Model of TTR amyloid seeding and its inhibition. TTR dissociation provides monomers that are susceptible to nucleation and fibril formation through self-association of amyloid-driving segments. TTR ligands such as tafamidis or diflunisal stabilize the tetrameric form, decelerating tetramer dissociation and unseeded polymerization. After fragmentation of fibrils, small fragments may serve as seeds that template fast polymerization. The peptide inhibitor TabFH2 does not affect tetramer stability. Instead, TabFH2 binds to seeds, hindering self-recognition and seeding. Both strategies seem synergistic and could potentially be used in combination.
A pair of peptides inhibits seeding of the hormone transporter transthyretin into amyloid fibrils
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