Tafamidis, a potent and selective transthyretin kinetic stabilizer that inhibits the amyloid cascade


The transthyretin amyloidoses (ATTR) are invariably fatal diseases characterized by progressive neuropathy and/or cardiomyopathy. ATTR are caused by aggregation of transthyretin (TTR), a natively tetrameric protein involved in the transport of thyroxine and the vitamin A–retinol-binding protein complex. Mutations within TTR that cause autosomal dominant forms of disease facilitate tetramer dissociation, monomer misfolding, and aggregation, although wild-type TTR can also form amyloid fibrils in elderly patients. Because tetramer dissociation is the rate-limiting step in TTR amyloidogenesis, targeted therapies have focused on small molecules that kinetically stabilize the tetramer, inhibiting TTR amyloid fibril formation. One such compound, tafamidis meglumine (Fx-1006A), has recently completed Phase II/III trials for the treatment of Transthyretin Type Familial Amyloid Polyneuropathy (TTR-FAP) and demonstrated a slowing of disease progression in patients heterozygous for the V30M TTR mutation. Herein we describe the molecular and structural basis of TTR tetramer stabilization by tafamidis. Tafamidis binds selectively and with negative cooperativity (Kds ≈ 2 nM and < 200 nM) to the two normally unoccupied thyroxine-binding sites of the tetramer, and kinetically stabilizes TTR. Patient-derived amyloidogenic variants of TTR, including kinetically and thermodynamically less stable mutants, are also stabilized by tafamidis binding. The crystal structure of tafamidis-bound TTR suggests that binding stabilizes the weaker dimer–dimer interface against dissociation, the rate-limiting step of amyloidogenesis.

drug | aggregation inhibition

Amyloid diseases appear to be caused by the extracellular accumulation of protein aggregates, including cross-β-sheet amyloid fibrils for which these maladies are named (1–3). Wild-type (WT) and/or mutant transthyretin (TTR) amyloidogenesis leads to the TTR amyloidoes (ATTR), with phenotypes including peripheral neuropathy (4, 5) and/or cardiomyopathy (6, 7). ATTR are progressive and fatal within 10 y of onset. The most common mutations are V30M, which causes familial amyloid polyneuropathy (TTR-FAP) (8), and V122I, which causes familial amyloid cardiomyopathy (TTR-CM) (9). In the elderly, WT-TTR amyloidogenesis leads to the cardiomyopathy called senile systemic amyloidosis (SSA) (6, 7).

Homotetrameric WT-TTR comprises 127-amino-acid, β-sheet-rich subunits and is made primarily by the liver, which secretes it into the blood, where it binds to and carries holo retinol-binding protein (10, 11). The native state of TTR features two largely unoccupied thyroxine (T4)-binding sites (Fig. 1) that are generated by the weaker dimer–dimer interface of TTR (12–15). Rate-limiting tetramer dissociation about this interface generates dimers that rapidly dissociate into monomers (14, 16, 17). Partial monomer unfolding, likely involving β-strand dissociation (18), promotes misassembly into soluble oligomers and amyloid fibrils through a thermodynamically favorable downhill process (15, 19). Misfolded monomers and oligomers have been implicated as neurotoxic species (20–23). Disease-associated mutations destabilize the TTR tetramer and some increase the velocity of rate-limiting tetramer dissociation (17, 24–26).

In populations of Portuguese descent, inheritance of one copy of V30M TTR typically leads to TTR-FAP with high clinical penetrance; however, a few V30M carriers develop mild pathology or no disease. These carriers have a different mutation, T119M, on their second TTR allele (27), resulting in the formation of T119M/V30M heterotetramers, which are more stable than heterotetramers comprising V30M and WT subunits (17, 28, 29). Tetramer dissociation is slowed proportional to the number of T119M subunits in the heterotetramer, due to destabilization of the dissociative transition state (17). Therefore, increasing the kinetic barrier for tetramer dissociation, which is the initial and rate-limiting step of TTR amyloidogenesis, is the likely mechanism by which T119M prevents TTR-FAP in V30M/T119M compound heterozygotes (17, 28).

Because lowering the native state energy through small-molecule binding to the TTR tetramer also increases the activation energy associated with tetramer dissociation (15, 17, 30–33), a mechanism known to ameliorate TTR-FAP based on the T119M interallelic trans suppression data, we predicted that small-molecule TTR kinetic stabilizers (amyloid inhibitors) would be disease-modifying therapies for ATTR (15, 17).

Of the multiple TTR kinetic stabilizer structures reported (15, 30–32, 34–45), the benzoxazoles (32) were pursued to identify an orally bioavailable candidate exhibiting potent and selective TTR binding in blood, while lacking nonsteroidal antiinflammatory (NSAID) activity, which is contraindicated in patients with cardiomyopathy. Tafamidis, or 2-(3,5-dichloro-phenyl)-benzoxazole-6-carboxylic acid, meets all of these criteria and was selected for clinical development. Herein we show that tafamidis selectively binds TTR with negative cooperativity and kinetically


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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3TCT).

1To whom correspondence should be addressed. E-mail: Christine.Bulawa@pfizer.com.

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stabilizes WT-TTR and mutant tetramers under denaturing and physiologic conditions, inhibiting amyloidogenesis. A high-resolution crystal structure reveals the details of how tafamidis binds to the weaker dimer–dimer interface of TTR.

**Results**

**Tafamidis Stabilizes TTR Under Fibril-Promoting Conditions.** Incubation of WT or mutant TTR homotetramers (3.6 μM) at a pH of 4.4–4.5 over 72 h results in tetramer dissociation, partial monomer denaturation, and misassembly into amyloid fibrils and other aggregates (Fig. 1) (19, 46). Purified WT, V30M, and V122I homotetramers were incubated with tafamidis at concentrations ranging from 0 to 7.2 μM. The pH was adjusted to either 4.4 or 4.5 to promote an identical rate of amyloidogenesis among these sequences (without tafamidis). After 72 h (aggregation is complete without tafamidis), amyloidogenesis was quantified by measuring turbidity at 350 and 400 nm (16). Consistent with previous studies (32), tafamidis dose-dependently inhibited WT-TTR amyloidogenesis (Fig. 2). Importantly, this study shows that tafamidis stabilized the two most clinically significant amyloidogenic mutant homotetramers, V30M-TTR and V122I-TTR, with potency and efficacy comparable to that of WT-TTR (Fig. 2). Tafamidis reached its half-maximal effective concentration (EC$_{50}$) at a tafamidis:TTR tetramer molar ratio of <1 (EC$_{50}$ of 2.7–3.2 μM, corresponding to a tafamidis:TTR molar ratio of 0.75–0.90). Under these conditions, the occupancy of less than one binding site of TTR by tafamidis reduces TTR fibril formation by 50%, consistent with reports that binding to only one T$_{4}$-binding site is sufficient to kinetically stabilize tetrameric TTR (33).

**Tafamidis Kinetically Stabilizes TTR Under Denaturing Conditions.** The TTR tetramer must dissociate before urea can unfold its β-sheet–rich tertiary structure (47). Therefore, at high urea concentrations, the rate of tetramer dissociation is linked irreversibly to fast monomer unfolding, which is easily followed by far UV circular dichroism spectroscopy (14, 24, 25, 33). TTR (1.8 μM) was incubated together with tafamidis (tafamidis:TTR tetramer molar ratios of 0, 1, and 2) and urea (6.5 M final concentration) to denature any monomer that is produced, and circular dichroism spectra were collected at 0 and 72 h (the time required for maximum denaturation without tafamidis). As shown in Fig. 3, only 33% of the TTR tetramer dissociates after a 72-h period at a tafamidis:TTR tetramer molar ratio of 1 and less than 3% of tetramers dissociate when tafamidis is used at twice the concentration of TTR, indicating dose-dependent kinetic stabilization of the tetramer.

**Tafamidis Kinetically Stabilizes TTR Under Physiologic Conditions.** It is difficult to detect folded monomeric or misfolded monomeric TTR upon dissociation from the tetramer under physiologic conditions. However, it is possible to indirectly detect monomer formation by using subunit exchange experiments (28, 48, 49). It was previously demonstrated that tetramer dissociation is rate limiting for subunit exchange, that the tetramer dissociates to monomers through transient dimers on a biologically relevant
timescale, and that folded monomers reassociate to form mixed tetramers under nondenaturing conditions (14, 48, 49). To study subunit exchange, two homotetramers were used. One was composed of WT-TTR subunits that were labeled at the N terminus with a negatively charged FLAG-tag (Fig. 4A, 4 FLAG). The other was made up of untagged WT subunits (Fig. 4A, 0 FLAG). After these tetramers were mixed, time-dependent subunit exchange between them could be quantified in buffers at physiologic pH using ion-exchange chromatography (48). A TTR kinetic stabilizer such as tafamidis is expected to decrease the rate of tetramer dissociation, which would slow subunit exchange between the WT-TTR homotetramer and FLAG-tagged WT-TTR homotetramer. When subunit exchange is complete, the monomers are statistically distributed, with tetramers 0 FLAG, 1 FLAG, 2 FLAG, 3 FLAG, and 4 FLAG being present in a ratio of 1:4:6:4:1. Because each FLAG tag adds approximately six negative charges to each TTR subunit, the more FLAG-tagged TTR subunits in the tetramer, the longer the retention time on an anion exchange column, allowing separation of all five tetramers and quantification of the extent of exchange as a function of time (48).

Solutions of homotetrameric WT-TTR (0 FLAG, 1.8 μM) and homotetrameric WT-TTR bearing an N-terminal acidic FLAG tag (4 FLAG WT-TTR, 1.8 μM) were each individually preincubated with tafamidis as a function of concentration (tafamidis:TTR tetramer molar ratios of 0, 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50). The subunit exchange reaction was initiated by mixing the homotetramers (25 °C; pH 7), and exchange was quantified by anion-exchange chromatography over the course of 8 d (except for the tafamidis:TTR tetramer molar ratio of 1.50, which was studied for 11 d). The exchange was complete in the vehicle control (Fig. 4A, gray line) after 96 h of incubation as shown by the statistical distribution of subunits. In the presence of tafamidis (tafamidis:TTR tetramer molar ratio of 1.50), negligible tetramer dissociation was observed after 96 h (Fig. 4A, black line), with a calculated fraction exchange of <5% (Fig. 4B, orange line), and after 11 d the fraction of exchange was still <10%. At a molar ratio of 1.25, <20% subunit exchange was observed after 96 h. Even at a molar ratio of 1, the fraction of exchange was only 15% after 24 h (70% was exchanged in the absence of tafamidis). Stabilization over this period is highly relevant because the TTR half-life in plasma is 24 h. Collectively, these data demonstrate that tafamidis dose-dependently decreases the rate of tetramer dissociation at physiologic pH.

**Tafamidis Binds with High Affinity to TTR at Its T4-Binding Sites.** We used two methods to determine the binding constants of tafamidis to WT-TTR. The first employs a modeling analysis of the subunit exchange time courses (Fig. 4B) at neutral pH as a function of tafamidis concentration (49). The appearance of the various mixed tetramers and the disappearance of the homotetramers appear to be simple unimolecular processes with the same apparent rate constant, $k_{app}$, which is equal to the tetramer dissociation rate constant, $k_{diss}$. In the absence of ligands (49). As TTR cannot exchange subunits when the tetramer is bound to a ligand (33, 49), $k_{app} = f_{unbound} \times k_{diss}$ in the presence of ligands, where $f_{unbound}$ is the fraction of TTR tetramers that do not have bound ligands.

Fitting the data in Fig. 4B to a single exponential and dividing the resulting $k_{app}$ values by $k_{diss}$ gave $f_{unbound}$ at a series of tafamidis concentrations. These $f_{unbound}$ vs. ligand concentration data were then fit with a second-order binding polynomial (50) to determine $K_{d1} = 2 \text{nM}$ and $K_{d2} = 154 \text{nM}$, the dissociation constants for the first and second binding sites of TTR (49).

Isothermal titration calorimetry (ITC) was also used to determine the binding constants of tafamidis to TTR (45). A solution of tafamidis was titrated into an ITC cell containing WT-TTR (17 μM) in identical buffers. The initial 2.5-μL injection of tafamidis (500 μM) was followed by forty-nine 5-μL injections (25 °C), and the heat evolved was measured as a function of time after each injection. After subtracting the blank, the area under each injection peak was integrated, corresponding to

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**Fig. 4.** Subunit exchange between WT-TTR and FLAG-tagged WT-TTR under physiologic conditions, monitored by anion exchange chromatography. Black square, WT-TTR; white square, FLAG-tagged WT-TTR. Homotetramers of WT-TTR (0 FLAG; 1.8 μM) and WT-TTR tagged with an N-terminal acidic FLAG tag (4 FLAG; 1.8 μM) were mixed with tafamidis at different concentrations (tafamidis:TTR tetramer molar ratios: 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50) and incubated at 25 °C at a pH of 7. Samples were analyzed by anion exchange chromatography at the indicated times. (A) FPLC trace after 96-h incubation of 0 Flag TTR with 4 Flag TTR in the absence (gray) or presence (black) of tafamidis at a tafamidis:TTR tetramer molar ratio of 1.5. (B) Dose-dependent stabilization of TTR by tafamidis. At each time point, the extent of exchange was calculated by dividing the peak area of each tetramer by the sum of the peak areas for all of the tetramers. The fraction exchange was calculated by dividing the extent of exchange of 2 FLAG at each data point by 0.375 multiplied by 100. The predicted complete extent of exchange for 2 FLAG is 0.375, based on the statistical distribution of 1:4:6:4:1 for tetramer 0 FLAG–4 FLAG, respectively. Values for samples of tafamidis:TTR tetramer molar ratio = 1.5 at 220 and 264 h (not shown) were 9.5% and 9.8%, respectively.
this orientation, the meta ligand and Glu54/54 residues of TTR represented by dotted lines (Lys15′ and Glu54/54′ residues of TTR. Because these low B-value waters are not conserved in other TTR apo structures or ligands, TTR structures and because these H-bonds are solvated, we do not anticipate that they are a driving force for tafamidis binding to TTR, but rather a consequence of binding. This combination of hydrophobic and ionic interactions appears to bridge adjacent dimers to kinetically stabilize the TTR tetramer (14, 15, 34).

Tafamidis Stabilizes Selectively to TTR in Human Plasma. We next examined the relative binding selectivity of tafamidis for TTR compared with the ~4,000 additional proteins in plasma. Tafamidis (7.2 μM) was incubated with human plasma (3.6 μM TTR) overnight; TTR and any bound tafamidis were then immunocaptured using a resin-bound polyclonal antibody to TTR (51). After washing the immobilized TTR, we liberated the TTR–tafamidis complex from the resin with triethylamine and determined the stoichiometry of tafamidis relative to TTR by reverse-phase HPLC analysis (51). Under these conditions, tafamidis exhibits a binding stoichiometry of 0.81 ± 0.02 (SI Appendix, Fig. S2), confirming previous results (32). Because several wash steps were used, likely resulting in tafamidis loss (52), the stoichiometry determined should be considered a lower limit.

Tafamidis Stabilizes WT, V30M, and V122I TTR in Human Plasma. WT, V30M, and V122I TTR were evaluated because these sequences are linked to SSA and the majority of TTR-FAP and TTR-CM cases, respectively. To assess kinetic stabilization, tafamidis was preincubated with plasma samples, and urea (4.8 M) was added as a denaturation stress. After 1, 2, 3, and 4 d, aliquots were withdrawn and treated with glutaraldehyde to cross-link the TTR tetramer remaining (53). Samples were then analyzed by SDS/PAGE, followed by Western blotting using anti-TTR antiserum to measure the remaining amount of TTR tetramer. When the TTR monomer is unfolded in 4.8 M urea, it is heavily modified by glutaraldehyde, such that it is no longer recognized by the TTR antibody (53). A human plasma sample from a healthy volunteer exhibited nearly complete WT-TTR denaturation by day 3 of incubation in the absence of tafamidis, and complete denaturation after 4 d (Fig. 6A). In the presence of 3.6, 5.4, and 7.2 μM tafamidis (corresponding to tafamidis:TTR tetramer molar ratios of ~1.0, 1.5, and 2.0, respectively) dose-dependent stabilization is observed even after 4 d (Fig. 6A). Tafamidis (7.2 μM) also strongly stabilized TTR heterotetramers containing WT and mutant subunits in plasma samples from patients carrying V30M or V122I mutations (Fig. 6B and C). These ex vivo results demonstrate the efficacy of a 7.2-μM tafamidis dose to stabilize a variety of TTR tetramers in the presence of urea (strongly denaturing), even though urea likely lowers the binding affinity of tafamidis to TTR.

TTR stabilization in plasma was also observed by using a modification of the method described above, in which the Western blotting was replaced by an immunoturbidity measurement (54), a readout that is amenable to automation used in clinical laboratories. The large antibody–TTR tetramer complexes that self-assemble precipitate out of solution; thus, the amount of turbidity can be used to quantify the TTR tetramer as the heavily glutaraldehyde-modified TTR monomer is not detected by the antibody (53). Preincubation with tafamidis stabilized TTR in a dose-dependent manner, similar to that observed with Western blotting (SI Appendix, Fig. S3 A and B).

Fig. 5. Crystal structure of tafamidis bound to TTR. The coordinates are available in the Protein Data Bank under accession code 3TCT. (A) 3D ribbon diagram depiction of the TTR tetramer with tafamidis bound. The four TTR monomers are individually colored. (B) Magnified image of tafamidis bound in one of the T4-binding sites. Connolly analytical surface representation (translucent gray, hydrophobic; translucent purple, polar) depicts the hydrophobicity of the binding site. The 3,5-chloro groups are placed in the HBPs and 3′ making hydrophobic interactions, whereas the carbamate of tafamidis engages in water-mediated H-bonds with the Lys15′ and Glu54/54′ residues of TTR represented by dotted lines (Lys15′–water H-bond not shown owing to tafamidis orientation). Fig. 5 was generated using the software Molecular Operating Environment, MOE (Chemical Computing Group).
Tafamidis Stabilizes a Broad Range of Pathogenic TTR Variants. Plasma samples from patients harboring mutations exhibiting a wide range of kinetic and/or thermodynamic stabilities (24–26), including V30M, Y69H, F64S, I84S (lower thermodynamic stability), V122I (lower kinetic stability), and L111M (lower thermodynamic and kinetic stability), were subjected to urea denaturation, and stabilization was analyzed by immunoturbidity (except for L111M, which was analyzed by Western blot). In a broad spectrum of TTR variants, tafamidis significantly reduced the disappearance of TTR tetramer (SI Appendix, Figs. S4 A and B) by kinetic stabilization.

Discussion

Although fibrils are the common observable pathology in many amyloid diseases, there is a growing realization that it may be the process of amyloid fibril formation, featuring nonfibril intermediates, that actually causes proteotoxicity (55). Thus, for treatment, it is optimal to arrest the amyloid cascade (15), which is how tafamidis functions (Fig. 1). Like interallelic transpression, which prevents FAP onset, we showed that tafamidis, by binding to TTR, raises the energy barrier for tetramer dissociation and prevents fibril formation under both denaturing and physiologic conditions and slows TTR-FAP progression by kinetic stabilization after FAP onset (59). Tafamidis-mediated kinetic stabilization of TTR was demonstrated by slowed subunit exchange under physiologic conditions (Fig. 4) and slowed tetramer dissociation under denaturing conditions (Fig. 3).

That tafamidis occupies the T4-binding sites in TTR raises the energy barrier for tetramer dissociation under denaturing or urea-mediated denaturation stress. Tafamidis-mediated kinetic stabilization of TTR tetramer in a dose-dependent manner. Human plasma from patients with V30M TTR-FAP (β, n = 4) and V122I TTR-CM (C, n = 4) was obtained and pooled by genotype. Stabilization by tafamidis (7.2 μM) was compared with vehicle (DMSO). Tafamidis stabilized both V30M WT and V122I WT heterotetramers.

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