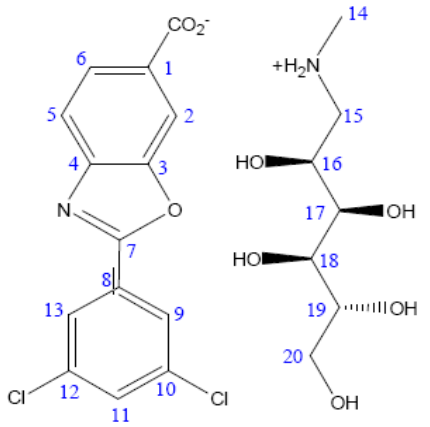
Supporting Information

Tafamidis, a Potent and Selective Transthyretin Kinetic Stabilizer That Inhibits the Amyloid Cascade

Bulawa *et al*.

METHODS

Synthesis of tafamidis meglumine.



4-Amino-3-hydroxybenzoic acid (AHBA) is reacted with HCl (3 to 6 M equivalents) in methanol (8 to 9 L/kg). Methyl t-butyl ether (TBME) (9 to 11 L/kg) is then added to the reaction mixture. The product, methyl 4-amino-3-hydroxybenzoate hydrochloride salt, is isolated by filtration and then reacted with 3,5-dichlorobenzoyl chloride (0.95 to 1.05 M equivalents) in the presence of pyridine (2.0 to 2.5 M equivalents) in dichloromethane (DCM), (8 to 9 L/kg) as a solvent. After the distillation of DCM, acetone and water are added to the reaction mixture, producing methyl 4-(3,5-dichlorobenzoylamino)-3-hydroxy-benzoate. This is recovered by filtration and reacted with p-toluenesulfonic acid monohydrate (0.149 to 0.151 M equivalents) in toluene (12 to 18 L/kg) at reflux with water trap. Treatment with charcoal is then performed. After the distillation of toluene, acetone (4-6 L/kg) is added. The product, methyl 2-(3,5-dichlorophenyl)-benzoxazole-6-carboxylate, is isolated by filtration and then reacted with LiOH (1.25 to 1.29 M equivalents) in the presence of tetrahydrofuran (THF) (7.8 to 8.2 L/kg) and water (7.8 to 8.2 L/kg) at between 40 and 45 °C. The pH of the reaction mixture is adjusted with aqueous HCl to yield 2-(3,5-dichloro-phenyl)-benzoxazole-6-carboxylic acid, the free acid of tafamidis. This is converted to the meglumine salt by reacting with N-methyl-D-glucamine (0.95 to 1.05 M equivalents) in a mixture of water (4.95 to 5.05 L/kg)/isopropyl alcohol (19.75 to 20.25 L/kg) at 65-70 °C. Tafamidis meglumine (d-glucitol, 1-deoxy-1-(methylamino)-,2-(3,5-dichlorophenyl)-6-benzoxazole carboxylate) is then isolated by filtration.

The following fragments were identified from electrospray ionization mass spectra acquired in positive-ion mode: meglumine M+ ($C_7H_{18}NO_5^+$, m/z = 196.13), M (carboxylate form) +2H ($C_{14}H_6Cl_2NO_3$, m/z = 308.13), M (salt) + H ($C_{21}H_{24}Cl_2N_2O_8$, m/z = 504.26).

^{1}H-nuclear magnetic resonance spectra were acquired on a 700 MHz Bruker AVANCE II spectrometer in acetone:D2O (~8:2). Data were reported as chemical shift in ppm (δ), multiplicity (s = singlet, dd = double of doublets, m = multiplet), coupling constant (*J* Hz), relative integral and assignment: δ = 8.14 (m, $J_{H2\text{-}H5}$ = 0.6 and $J_{H2\text{-}H6}$ = 1.5, 1H, H_2), 8.02 (dd, $J_{H9\text{-}H11}$ = 1.9 and $J_{H13\text{-}H11}$ = 1.9, 2H, H_9 and H_{13}), 7.97 (dd, $J_{H6\text{-}H5}$ = 8.25, 1H, H_6), 7.67 (dd, $J_{H5\text{-}H2}$ = 0.6 and $J_{H5\text{-}H6}$ = 8.25, 1H, H_5), 7.58 (m, $J_{H11\text{-}H9}$ = 1.9 and $J_{H11\text{-}H13}$ = 1.9, 1H, H_{11}), 4.08 (m, $J_{H16\text{-}H17}$ = 4.9, 1H, H_{16}), 3.79 (dd, $J_{H17\text{-}H18}$ = 2.2, 1H, H_{17}), 3.73 (dd, $J_{H19\text{-}H20}$ = 3.2, 1H, H_{20}), 3.69 (m, $J_{H19\text{-}H20}$ = 3.2, 1H, H_{19}), 3.61 (m, $J_{H18\text{-}H19}$ = 12.25, 1H, H_{18}), 3.58 (m, $J_{H19\text{-}H20'}$ = 5.8 and $J_{H20\text{-}H20'}$ = 11.7, 1H, $H_{20'}$), 3.19 (m, $J_{H15\text{-}H15'}$ = 12.9 and $J_{H15'\text{-}H16}$ = 9.25 and $J_{H15\text{-}H16}$ = 3.5, 2H, H_{15}).

Immunoturbidity Assay for Stabilization of TTR Tetramer in Human Plasma. Urea denaturation of TTR in human plasma and chemical crosslinking was performed as described (see text and Fig. 6.) with minor modifications, except that TTR was quantified by immunoturbidity. Human plasma samples were thawed on ice and insoluble material was removed by centrifugation. For each, 4 μL was removed, and the initial TTR concentrations were determined by immunoturbidity. For each stabilization determination, 80 μL aliquots of each plasma sample were retained and 1.6 μL of either 5% dimethyl sulfoxide (DMSO) or 360 μM tafamidis in 5% DMSO was added. After incubation at room temperature for 15 minutes, 120 μL of urea buffer (8 M urea, 40 mM sodium phosphate, 80 mM KCl, pH 7.4) was added and samples were mixed and incubated at room temperature for the indicated time (typically 48 h). All samples were cross-linked with 3.2 μL of 25% glutaraldehyde. After 4 minutes, the reaction was quenched with 5.6 μL of 1.85 M $NaBH_4$ (freshly prepared in 0.1 N NaOH) and incubated for 5 minutes. Postdenaturation TTR concentrations (4 μL) were determined by immunoturbidity. Olympus OSR6175 reagent and Prealbumin Calibrator ODR3029 were used according to the manufacturers' instructions.

To assess the correlation between the two detection methods, we analyzed plasma samples after urea treatment and glutaraldehyde crosslinking in parallel by Western blot and immunoturbidity. In the control samples, the amount of TTR detected by immunoturbidity decreased from an initial value of 22 mg/dL to 3 mg/dL after 3 d in urea. In the presence of tafamidis, 13 mg/dL of TTR remained; a level that was in good agreement with results from the Western blot assay (Fig. S3A).

Crystallographic Analysis of Tafamidis Bound to TTR. WT-TTR was purified from an *Escherichia coli* expression system, as described previously (2). The WT-TTR was concentrated to 6 mg/mL in 10 mM NaP_i, 100 mM KCl, at a pH of 7.6, and cocrystallized at room temperature with tafamidis at a 5-fold molar excess using the vapor-diffusion sitting drop method. Crystals were grown from 1.395 M sodium citrate, 3.5% v/v glycerol at a pH of 5.5. The crystals were cryoprotected with 10% v/v glycerol. Data

were collected at beam line 11-1 at the Stanford Synchrotron Radiation Lightsource (SSRL) at a wavelength of 0.9795 Å. The data set was integrated and scaled using HKL2000 (3). The diffraction data were indexed in space group $P2_12_12$ with two subunits per asymmetric unit with unit cell dimensions a = 42.75 Å, b = 85.00 Å, and c = 64.34 Å. The crystal structure was determined by molecular replacement using the model coordinates of 2FBR (4) in the program Phaser (5) to 1.3 Å resolution. Further model building and refinement were completed using Refmac (6). Hydrogens were added during refinement, and anisotropic *B*-values were calculated. Final models were validated using the JCSG quality control server incorporating Molprobity (7), ADIT (http://rcsb-deposit.rutgers.edu/validate) WHATIF (8), Resolve (9) and Procheck (10). Data collection and refinement statistics are presented in Table S1.

References

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A

Molar ratio Tafamidis:TTR	Heat kcal/mol	Molar ratio Tafamidis:TTR	Heat kcal/mol
0.165	-5.299	2.906	-0.269
0.274	-5.491	3.015	0.064
0.384	-5.617	3.125	-0.125
0.494	-5.417	3.234	-0.202
0.603	-5.546	3.344	0.048
0.713	-5.385	3.454	0.087
0.823	-5.464	3.563	-0.064
0.932	-5.633	3.673	-0.211
1.042	-5.604	3.783	-0.046
1.151	-5.355	3.892	0.143
1.261	-5.405	4.002	0.024
1.371	-5.315	4.112	-0.340
1.480	-5.124	4.221	-0.186
1.590	-5.086	4.331	-0.130
1.700	-4.737	4.440	0.138
1.809	-4.958	4.550	0.060
1.919	-3.518	4.660	0.121
2.029	-3.131	4.769	-0.140
2.138	-1.962	4.879	-0.109
2.248	-1.099	4.989	0.030
2.357	-0.574	5.098	-0.093
2.467	-0.457	5.208	0.044
2.577	-0.144	5.317	-0.010
2.686	-0.072	5.427	0.115
2.796	0.019		

B

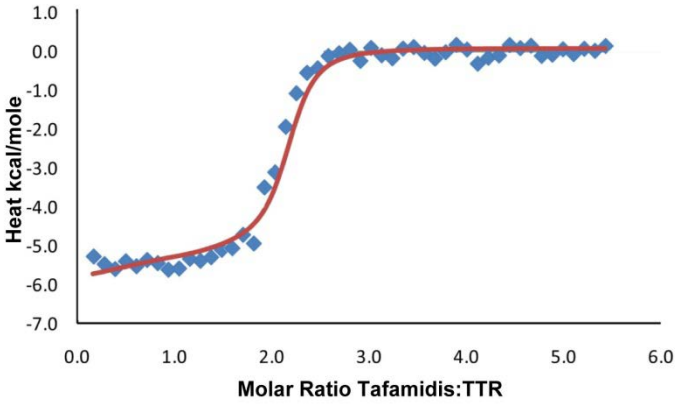

Fig. S1. Determination of the dissociation constants for tafamidis binding to WT transthyretin using isothermal titration calorimetry (ITC). Dissociation constants for tafamidis:WT-TTR were determined using a Microcal MCS isothermal titration calorimeter (Microcal Inc., Northhampton, MD). 75 µL of 20 mM tafamidis in DMSO were mixed into 3 mL of ITC buffer (100 mM phosphate with 100 mM KCl, 1 mM EDTA, pH 7.6). This solution, containing 500 µM tafamidis, was titrated into an ITC cell containing 17 µM WT-TTR in the same buffer. The initial injection of 2.5 µL of tafamidis solution was followed by 49 injections of 5 µL each (25 °C). Integration of the thermogram after the subtraction of blanks yielded a binding isotherm that fit best to a model of two interacting sites exhibiting negative cooperativity. The data were fit by a nonlinear least squares approach with four adjustable parameters: K_{d1}, $\Delta H1$, K_{d2}, and $\Delta H2$ using the ITC data analysis module in ORIGIN version 2.9 provided by Microcal.

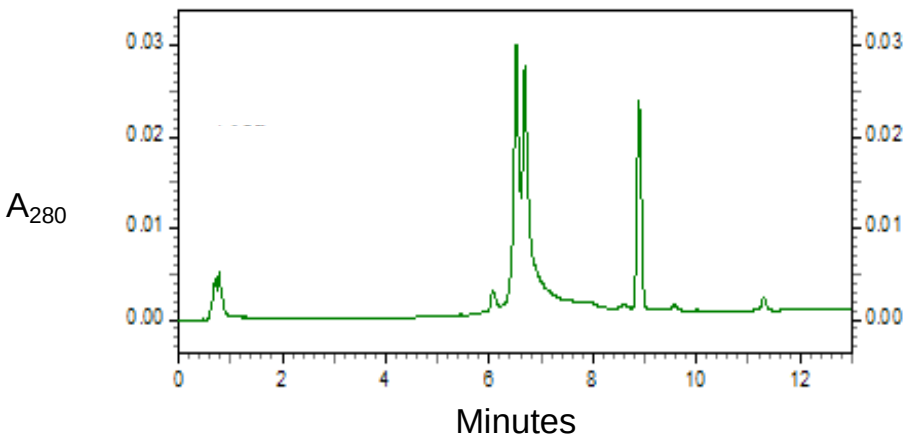

Fig. S2. Tafamidis selectively binds TTR in human plasma. Human plasma sample (pool, n = 11, 3.6 μM) was incubated with 7.2 μM tafamidis. TTR with bound tafamidis was captured using anti-TTR resin (polyclonal rabbit antibody to native human TTR coupled to seharose beads). After washing, the TTR:tafamidis complex was dissociated with triethylamine and the amount of TTR and free tafamidis was quantified by reverse-phase HPLC (see text). TTR eluted as a doublet with retention times of 6.3 and 6.5 minutes; the peak at 6.3 minutes corresponds to cysteine-modified TTR (1). Tafamidis eluted as a single peak at 8.8 minutes. Four independent determinations were performed; a representative chromatogram is shown. Quantification of the peak areas yielded a TTR:tafamidis stoichiometry of 0.81 ± 0.02.

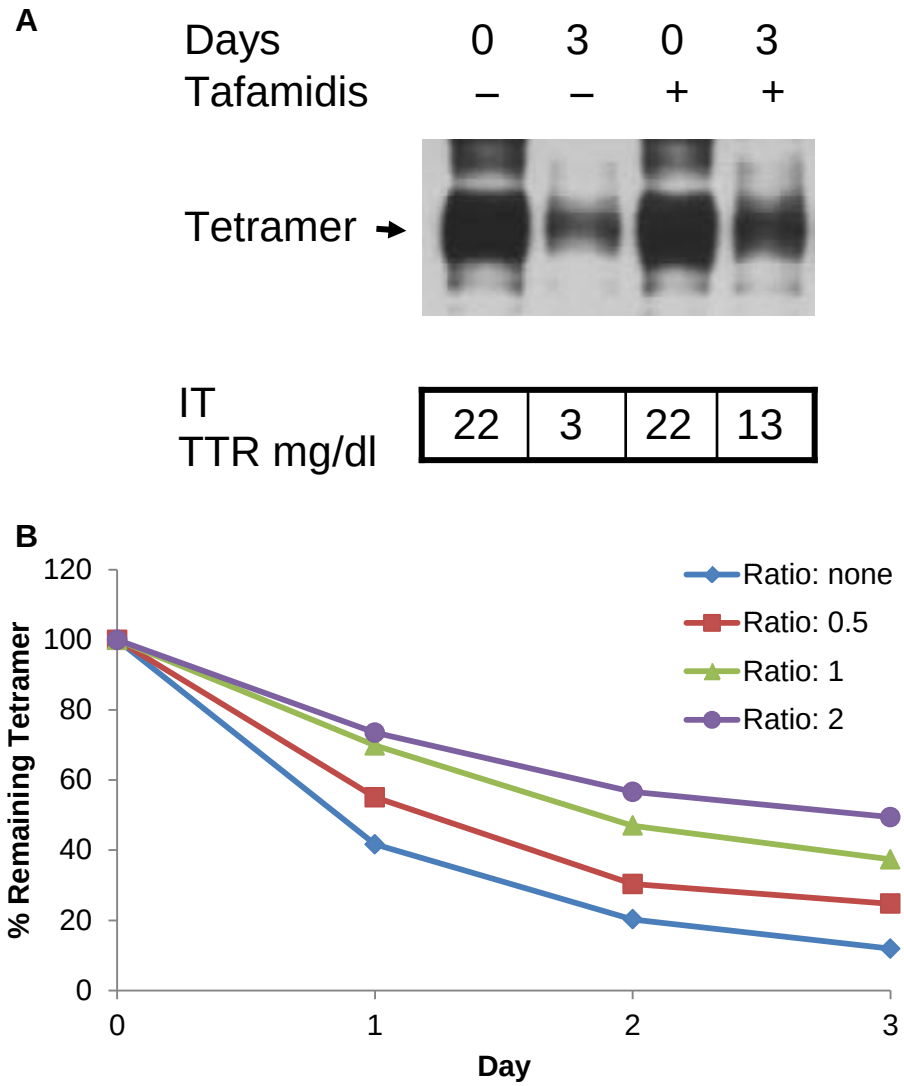

Fig. S3. Novel TTR stabilization assay using immunoturbidity. Human plasma (pool, n = 16, normal individuals, Golden West Biologicals Inc. Temecula, CA). The premeasured TTR level is 4.3 µM. (*A*) Urea denaturation (DMSO vs. 7.2 µM of tafamidis) was performed for 3 d as described for Western blot assay. The remaining TTR tetramer was analyzed by both immunoblotting (photo) and immunoturbidity. A similar degree of stabilization was detected by both methods. (*B*) Samples were denatured at the indicated molar ratios of tafamidis to TTR, and the TTR tetramer was analyzed by immunoturbidity.

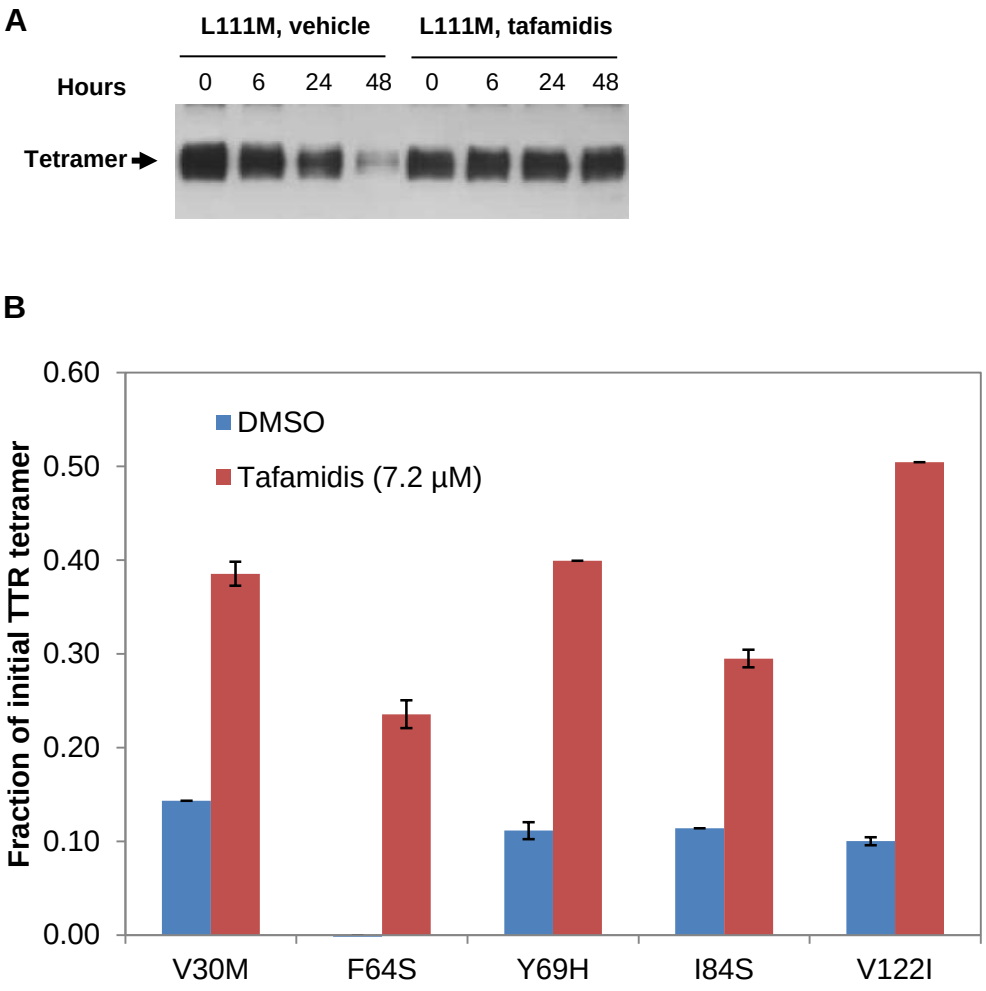

Fig. S4. Stabilization of kinetically and/or thermodynamically destabilized TTR variants with tafamidis. A spectrum of amyloidogenic TTR variants was tested for their ability to be stabilized by tafamidis in the presence of urea. (*A*) Human plasma from patients with L111M (n = 4) was tested using the Western blot method (see text and Fig. 6) with 10 µM tafamidis. (*B*) Plasma samples from patients with the indicated mutation were treated with urea in the presence of DMSO or 7.2 µM tafamidis and the amount of TTR tetramer present before and after denaturation (48 h) was determined by immunoturbidity. The results are presented as fraction of initial TTR tetramer = post denaturation level (mg/dL)/predenaturation level (mg/dL). Error bars represent the standard deviation of replicate stabilization determinations (n = 10 for V30M, n = 3 for V122I, n = 2 for others). Plasma samples are from single individuals with the exception of V122I and V30M (pools of 3 and 12 individuals, respectively).

Table S1. **Data collection and refinement statistics**.

	WT-TTR • (tafamidis)$_2$
Data Collection	
Beamline	SSRL 11-1
Wavelength (Å)	0.9795
Resolution (Å)	1.30 (1.30-1.35)[a]
Space group	$P2_12_12$
a, *b*, *c* (Å)	42.75, 85.00, 64.34
No. molecules in the a.u.	2
No. observations	408,156 (32,300)
No. unique reflections	58,308 (5,569)
Completeness (%)	99.4 (96.5)
R_{sym} (%)[b]	3.5 (63.0)
Average I/σ	51.5 (2.4)
Redundancy	7.0 (5.8)
Refinement statistics	
Resolution (Å)	85.1-1.30
No. reflections (working set)	55,182 (3,915)
No. reflections (test set)	2,940 (183)
R_{cryst} (%)[c]	16.1 (26.4)
R_{free} (%)[d]	19.2 (29.8)
Average B-values (Å^2)	
TTR	16.1
Ligand	19.2
Wilson B-value	16.9
Ramachandran plot	
Most favored (%)	93.0
Additionally allowed (%)	7.0
Generously allowed (%)	0
Disallowed (%)	0
R.M.S deviations	
Bond lengths (Å)	0.019
Angles (°)	1.56

[a] Values in parentheses are for the outer shell.
[b] $R_{sym} = \sum_{hkl} \left| I-\langle I\rangle \right| \sum_{hkl} I$.
[c] $R_{cryst} = \sum_{hkl} \left| F_o-F_c \right| \sum_{hkl} F_o$.
[d] R_{free} is the same as R_{cryst}, but for 5% of data excluded from the refinement.