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Chemoselective Small Molecules that Covalently Modify One Lys in a Non-enzyme Protein in Plasma

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Abstract

A small molecule that could bind selectively to and then react chemoselectively with a non-enzyme protein in a complex biological fluid, such as blood, could have numerous practical applications. Herein, we report a family of designed stilbenes that selectively and covalently modify the prominent plasma protein transthyretin in preference to more than 4000 other human plasma proteins. They react chemoselectively with only one of eight Lys ϵ -amino groups within transthyretin. The crystal structure confirms the expected binding orientation of the stilbene substructure and the anticipated conjugating amide bond. These covalent transthyretin kinetic stabilizers exhibit superior amyloid inhibition potency, compared to their non-covalent counterparts, and prevent cytotoxicity associated with amyloidogenesis. While there are a few prodrugs that, upon metabolic activation, react with a Cys residue inactivating a specific non-enzyme, we are unaware of designed small molecules that react with one Lys ϵ -amine within a specific non-enzyme in a complex biological fluid.

Small molecules that react chemoselectively with a unique non-enzyme protein in a complex biological environment are rare. Such molecules are envisioned to have imaging, pharmacology and protein engineering applications. Pharmacologic examples include Plavix and related thienopyridines, which are oxidized in the liver yielding a thiol that forms a disulfide with a Cys residue in the P2Y₁₂ receptor, inactivating it¹. There are β -amino ketones identified by high throughput screening that β -eliminate to form an α,β -unsaturated ketone and then react with a proximal Cys residue in the thyroid hormone receptor, inhibiting it². There are also small molecules that are known to bind to and react with one

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Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

AUTHOR CONTRIBUTIONS

S. Choi performed the chemical syntheses, all biochemical assays and wrote the majority of the paper, S. Connelly performed the crystallization, crystallographic structure determination and the structural analyses and wrote the crystallography section of the paper, N.R. carried out all the cell-based assays and wrote that section of the manuscript, I.A.W. supervised the crystallographic work and edited the manuscript, J.W.K. supervised the chemical biology and edited the paper.

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JWK is a founder, shareholder and paid consultant for Foldrx Pharmaceuticals, Inc., a biotechnology company that specializes in the discovery and development of drug therapies for transthyretin amyloidoses.

Lys ϵ -amino group within a purified antibody in buffer³. These conjugates, after purification, are envisioned to become injectable drugs (www.covx.com). However, we are unaware of compounds that react with a specific Lys ϵ -amino group in a non-enzyme protein in the context of a complex biological sample.

For pharmacologic applications, it would be advantageous if an orally bioavailable, appropriately reactive small molecule formed a conjugate with an endogenous non-enzyme protein. This strategy could be used to conjugate potent drug candidate substructures, whose individual pharmacologic properties are undesirable, to an endogenous protein such that the conjugate exhibited activity and a favorable half-life and distribution. Lys ϵ -amine covalent modifiers could also be used to modulate non-enzyme function. We focus on a third application in this paper, the creation of Lys ϵ -amine covalent modifiers that form a conjugate with an endogenous protein preventing it from aggregating and leading to a gain-of-toxic-function amyloid disease.

Human amyloid diseases, like the transthyretin (TTR) amyloidoses, are named after the characteristic extracellular cross- β -sheet amyloid fibril deposits that result from the misassembly of a specific protein^{4,5}. The amyloidogenesis-associated cytotoxicity that appears to be central to amyloid disease etiology is linked to an aging-associated decline in cellular protein homeostasis, or proteostasis, capacity^{4,6}.

Senile systemic amyloidosis (SSA) affecting > 10% of the aged population, results from wild type (WT) TTR amyloidogenesis leading to cardiomyopathy^{7,8}. Deposition of a destabilized TTR mutant causes earlier onset familial amyloid polyneuropathy (FAP) and/or cardiomyopathy (FAC)⁹⁻¹¹. No effective treatment is available for SSA or FAC. The only strategy to ameliorate FAP is gene therapy mediated by liver transplantation, wherein the WT-TTR / mutant-TTR genes in the liver (which synthesize plasma-bound TTR) are replaced by WT-TTR / WT-TTR genes¹², resulting in a dramatic reduction in amyloidogenic TTR concentration in the blood. However, continued WT-TTR deposition in the heart post-transplantation results in cardiomyopathy, limiting effectiveness. Thus, general chemotherapeutic approaches for the TTR amyloidoses are sought⁷.

Transthyretin is a 55 kDa homotetrameric protein composed of 127-residue β -sheet- rich subunits¹³. TTR transports thyroxine (T_4) and holo-retinol binding protein (RBP) in the blood and in the cerebrospinal fluid (CSF), using non-overlapping binding sites^{14,15}. The more labile dimer-dimer interface of the TTR tetramer creates two funnel-shaped T_4 binding sites that are 99% unoccupied in the blood, because thyroid binding globulin and albumin transport the vast majority of T_4 in humans^{7,13}.

TTR tetramer dissociation is rate limiting for the generation of partially folded monomers that spontaneously self-assemble into TTR amyloid fibrils^{7,16-22}. Reversible occupancy of one of the two T_4 binding sites by a high affinity small molecule is known to be sufficient to kinetically stabilize the entire TTR tetramer through differential stabilization of the ground state over the dissociative transition state, thus preventing amyloidogenesis^{7,19,20,23-31}. Below, we report on a family of designed stilbenes that very selectively bind to TTR in human plasma and, when bound, chemoselectively react with the ϵ -amino group of Lys-15 of TTR, affording an amide bond. These covalent TTR kinetic stabilizers prevent 10-20% more TTR amyloid fibril formation than their non-covalent counterparts, which are exceptional TTR kinetic stabilizers in their own right. The cytotoxicity linked to TTR amyloidogenesis is also prevented by these covalent TTR kinetic stabilizers.

RESULTS

Design of chemoselective covalent TTR kinetic stabilizers

Previous non-covalent TTR kinetic stabilizer structure-based design and/or structure-activity relationship studies demonstrate the efficacy of non-covalent TTR kinetic stabilizers^{13,23,24,27,28,32-37}. Ester and thioester derivatives of previously optimized *trans*-stilbene-based non-covalent TTR kinetic stabilizers²⁴ (Table 1) were prepared utilizing Horner-Wadsworth-Emmons couplings between a benzaldehyde and benzyl halide (Supplementary Scheme 1). Placement of the ester or thioester group at the 3-position on the aromatic ring expected to occupy the outer T₄ binding cavity²⁴ enables the TTR lysine 15 (K15) ε-amine to approach the carbonyl at the Burgi-Dunitz angle, facilitating amide bond conjugation (Table 1)³⁸. The K15 and K15' side chains are in proximity in each T₄ binding site in the tetramer, thus putative pK_a perturbation leads to one K15 ε-amine group and one K15' ε-ammonium group at pH 7³⁹.

The chemoselectivity of the conjugation reaction of compounds 1-4

The ability of candidate covalent kinetic stabilizers **1-4** to form a chemoselective amide bond with the K15 residue of WT-TTR was assessed by incubating the recombinant WT-TTR tetramer (3.6 μM) with compounds **1-4** (7.2 μM, the minimum concentration required to covalently modify both T₄ binding sites) for 18 h at 25 °C. The stilbene-TTR conjugate was quantified by reverse-phase (RP)-HPLC using a denaturing acetonitrile gradient. The resulting chromatograms exhibit two peaks of nearly equal intensity in the case of highly chemoselective compounds (Fig. 1a, top chromatogram, and Supplementary Fig. 1a-c, top chromatograms), as it is only possible to benzoylate two of the four subunits at K15 (Table 1), affording conjugation yields of 90-100% (the molar absorptivity changes associated with benzoylation were accounted for). Liquid chromatography-mass spectrometry (LC-MS) analysis confirmed that the first peak is the unmodified WT-TTR subunit (13893 m/z [M + 2H]⁺ calculated, 13893 m/z observed), whereas the second peak is the stilbene-WT-TTR conjugate (14271 m/z M⁺ for benzoylation by **1** and 14143 m/z [M + 2H]⁺ for benzoylation by **2-4** (shown in red, see Table 1, top right)).

Analysis of the kinetics of the TTR conjugation reaction demonstrates that the rate of conjugation can vary dramatically, even when the leaving group is identical (Fig. 1b, cf. **1** and **2**). This is not surprising since the reaction rate is dependent on the binding constants, the leaving group potential, and the exact binding geometry; the latter two are envisioned to be the major contributors to the differences in reaction rates observed. Covalent kinetic stabilizers **2** and **3** exhibit a conjugation half-life of 5–15 min (Fig. 1b), whereas compounds **1** and **4** exhibit t_{50s} of 143 and 247 min, respectively. Importantly, all the TTR covalent kinetic stabilizers react completely with TTR within 24 h, the half-life of TTR in plasma.

A strictly analogous experiment performed with the K15A homotetrameric TTR mutant confirmed that K15 was essential for the observed chemoselective reaction between TTR and **1-4** (Fig. 1a, bottom trace and Supplementary Fig. 1a-c, bottom traces). Compound **4** was the most chemoselective covalent kinetic stabilizer identified *in vitro*, as no reaction could be detected between it and K15A-TTR mutant (Fig. 1a, bottom trace). However, it is important to note that compounds **1-3** were only slightly less chemoselective (< 5% reaction with K15A-TTR) (Supplementary Fig. 1a-c, bottom traces).

To demonstrate that the covalent kinetic stabilizers can bind to K15A-TTR like they do to WT-TTR, compound **4** was pre-incubated with K15A-TTR. K15A-TTR and any compound bound to it was immunocaptured using a Sepharose-resin-conjugated anti-TTR antibody⁴⁰. After high-pH-mediated dissociation, HPLC analysis showed that 1.4 out of a maximum of 2 eq. of **4** was non-covalently bound to the K15A-TTR homotetramer (Supplementary Fig.

2), demonstrating that covalent kinetic stabilizer binding to K15A-TTR is not impaired. Therefore, compounds **1-4** do not react with K15A-TTR simply because the Lys-15 ϵ -amino group is absent, not because they cannot bind.

Placement of the ester or thioester at the 4-position of the stilbene aryl ring putatively occupying the outer T₄ binding pocket (e.g., compounds **5-7**, Supplementary Fig. 3) either resulted in more non-chemoselective conjugation, as exemplified by the reactivity of **6** with the K15A mutant subunits (Supplementary Fig. 4b, lower panel), or lower reactivity, as illustrated by the lower modification yield of the WT-TTR subunits by **7** (Supplementary Fig. 4c, upper panel). However, compound **5** exhibits promising data, suggesting that it may be possible to place a conjugating functional group at the 4-position in another context. There are numerous examples of transforming non-covalent enzyme inhibitors into covalent inhibitors, and it is likely that additional functional groups at the 3- or 4-position will find utility in covalent kinetic stabilization of TTR.

The potency of **1-4** as amyloid inhibitors relative to their non-covalent counterparts **8** and **9**

The utility of the chemoselective covalent kinetic stabilizers **1-4** to inhibit WT-TTR amyloidogenesis was evaluated next utilizing the previously validated acid-mediated fibril formation assay⁴¹. Compounds **1-4** (3.6 or 7.2 μ M) were pre-incubated with WT-TTR (3.6 μ M) for 18 h (the half-life of TTR in plasma is 24 h) before initiating TTR amyloidogenesis (pH jump to 4.4). The extent of amyloidogenesis was quantified at a fixed time point (72 h), as done previously, by measuring sample turbidity (400 nm), shown to be equivalent to thioflavinT monitoring of amyloidogenesis²¹. Potent non-covalent kinetic stabilizers, many exhibiting subnanomolar dissociation constants, allow ~10% WT-TTR fibril formation at a concentration of 7.2 μ M and ~40% WT-TTR fibril formation at a concentration equal to that of the TTR tetramer (3.6 μ M)^{23-28,32,33,36,42}. TTR incubated with compounds **1-4** exhibits < 4% (7.2 μ M) and 12-21% (3.6 μ M) of the fibril formation exhibited by WT-TTR alone after 72 h (Table 1), less than half of the TTR amyloidogenesis allowed by the best non-covalent kinetic stabilizers after 72 h^{23-28,32,33,36,42}.

To further scrutinize the potency of covalent kinetic stabilizers **2** and **4**, not against a spectrum of non-covalent kinetic stabilizers as above, but relative to their strictly structurally analogous highly potent and selective non-covalent counterparts **8** and **9**²⁴ (Fig. 2), the extent of inhibition of acid-mediated WT-TTR (3.6 μ M) fibril formation by lower concentrations (2.7 μ M and 1.8 μ M) of the kinetic stabilizers was compared over a 120 h time course instead of at a fixed time (72 h). The covalent kinetic stabilizers inhibited 10-20% more fibril formation than their non-covalent counterparts when applied at 2.7 μ M (Fig. 2a) or 1.8 μ M (Supplementary Fig. 5). This is highly significant, given that the non-covalent inhibitors have been previously optimized for potency and selectivity²⁴.

The inhibition of WT-TTR (3.6 μ M) amyloidogenesis by **1-4** is dose dependent, with apparent IC₅₀ values in the range of 1.96 – 2.26 μ M, while the previously optimized²⁴ non-covalent kinetic stabilizers **8** and **9** exhibited higher IC₅₀ values around 3 μ M (Fig. 2b). The exquisitely high binding affinity of **8** and **9** to TTR necessarily renders the apparent IC₅₀ of **8** and **9** similar to that of stabilizers **1-4** *in vitro*. In order for the incorporation of covalency into the TTR kinetic stabilization mechanism to translate into potency *in vivo*, the compound must bind with very high selectivity to TTR in plasma (demonstrated below), react with TTR rapidly relative to its 24 h half-life (demonstrated *in vitro*), and exhibit a plasma distribution, concentration and half-life facilitating a near quantitative conjugation yield (to be demonstrated).

Compounds 1-4 inhibit V30M TTR amyloidogenesis

Covalent kinetic stabilizers **1-4** (3.6 and 7.2 μM) also efficiently inhibit V30M-TTR (3.6 μM) amyloidogenesis associated with FAP (Table 1, values in blue font). As expected, **1-4** exhibited decreased potency relative to WT-TTR amyloidogenesis, owing to the decreased thermodynamic stability of the V30M-TTR tetramer.

Benzoylation of K15 and occupancy of the T₄ site together contribute to the superiority of the covalent kinetic stabilizers

Previous studies demonstrate that electrostatic repulsions between the proximal pairs of K15 and K15' residues destabilize the TTR tetramer structure³⁹. In an attempt to understand whether the kinetic stabilization of TTR by conjugation to **1-4** results solely from benzoylation-induced reduction of the K15-mediated electrostatic repulsions or whether bridging hydrophobic interactions between proximal TTR subunits mediated by the stilbene component of the kinetic stabilizers also contribute, WT-TTR or K15A-TTR (3.6 μM) was incubated with the non-covalent kinetic stabilizers **8** or **9** (7.2 μM) for 72 h at pH 4.4. Although K15A-TTR is much less amyloidogenic than WT-TTR (demonstrating that reduction of the charge-charge repulsions by benzoylation is likely stabilizing), additional stabilization of K15A-TTR was observed in the absence of benzoylation (**8** and **9** cannot covalently modify TTR; Supplementary Fig. 6). This experiment demonstrates that both diminishing the K15-K15' electrostatic repulsions through benzoylation and subunit bridging through non-covalent hydrophobic interactions with neighboring subunits comprising the weaker dimer interface of TTR together contribute to the kinetic stabilization of TTR.

Assessing the selectivity of 1-4 for binding to TTR over other plasma proteins

The ability of **1-4** to bind to and then react with TTR over the > 4000 other human proteins in blood plasma was assessed next. Untreated recombinant TTR in buffer is homogeneous and, thus, its subunits exhibit a single RP-HPLC peak under denaturing conditions (Supplementary Fig. 7). RP-HPLC analysis of TTR captured from human plasma, using a Sepharose-resin-conjugated anti-TTR antibody⁴⁰, and dissociated with a high pH treatment revealed two RP-HPLC peaks of nearly equal intensity (Fig. 1c, bottom chromatogram), because about half of the TTR subunits form a disulfide bond with the amino acid Cys, and a very small amount of the Cys in TTR gets S-sulfonated, $-\text{C}_\alpha\text{H}-\text{C}_\beta\text{H}_2-\text{S}-\text{SO}_3(\text{H})_4$. Compounds **1-4** (10.8 μM) were incubated for 24 h at 37 °C with human plasma, wherein the TTR concentration is 3.6–5.4 μM . Immunocapture/HPLC analysis of TTR⁴⁰ revealed the ratio of the TTR monomer to the TTR–stilbene-conjugate (Fig. 1c, Supplementary Fig. 8, and Table 1, rightmost column), demonstrating selective binding to and reaction with plasma TTR. Covalent kinetic stabilizers **2** (Table 1 and Supplementary Fig. 8) and **3** (Table 1 and Fig. 1c, middle chromatogram) exhibited the highest modification yield in plasma, very close to the maximum of 50% (the molar absorptivity changes associated with benzoylation were accounted for). It is not surprising that the two TTR kinetic stabilizers exhibiting rapid (Fig. 1b) and selective (Fig. 1c and Supplementary Fig. 1) TTR conjugation reactions perform best in human plasma.

Mass spectrometry is an especially gratifying method for the assessment of covalent kinetic stabilizer modification of TTR. LC-MS analysis (Supplementary Fig. 9) following treatment by **3** reveals that the additional peaks in the chromatogram (Fig. 1c; middle chromatogram) correspond to the TTR-benzoyl component of **3** = 14012 m/z, and under the rightmost broad peak is the Cys-SS-TTR benzoyl component of **3** = 14131 m/z and the S-sulfonated-TTR benzoyl component of **3** = 14092 m/z. LC-MS analysis of the benzoylation of TTR by **1** reveals strictly analogous species (Supplementary Fig. 10).

Dose-dependent kinetic stabilization of the TTR tetramer by **4**

Since TTR tetramer dissociation is rate limiting for amyloidogenesis¹⁹, the extent of TTR kinetic stabilization is revealed by measuring tetramer dissociation rates and the degree of dissociation as a function of the concentration of **4**. Tetramer dissociation kinetics are measured by linking the slow dissociation step to the rapid monomer unfolding step—assessed by far-UV circular dichroism (CD) spectroscopy^{7,19,31}. Recombinant TTR (1.8 μ M) was pre-incubated for 18 h as a function of the concentration of **4** (0.45–3.6 μ M). Dissociation (accelerated by the addition of 6 M urea) and denaturation of TTR, monitored over 144 h by far-UV CD, was diminished proportionally to the concentration of **4** added (Fig. 2c). Only slightly less TTR kinetic stabilization was observed at 1.8 vs 3.6 μ M, indicating that covalent attachment of **4** to one T₄ site in the TTR tetramer is sufficient to impose kinetic stability on the entire tetramer, as demonstrated previously²⁹.

Covalent kinetic stabilizers prevent TTR-amyloidogenesis-associated cytotoxicity

WT-TTR purified at 4 °C is cytotoxic to IMR-32 human neuroblastoma cells, possibly because of an altered tetramer structure that facilitates amyloidogenesis^{44,45}. Non-covalent TTR kinetic stabilizers (e.g., resveratrol) are known to prevent amyloid-formation-associated cytotoxicity, whereas structurally related compounds with poor TTR binding capacity do not inhibit cytotoxicity^{44,45}. Compounds **2** and **4** or the corresponding non-covalent kinetic stabilizers **8** and **9** (2, 4, 6 and 8 μ M) were pre-incubated with WT-TTR (8 μ M) for 18 h at 37 °C and added to IMR-32 cells. Cell viability was measured after 24 h by a resazurin reduction assay. Metabolically active cells reduce the resazurin redox dye to resorufin, a soluble fluorescent compound⁴⁶. The percentage of viable cells was calculated relative to cells treated with vehicle only (cell culture media and DMSO = 100% viable). Pre-incubation of WT-TTR with compounds **2**, **4**, **8**, or **9** clearly and dose-dependently prevents IMR-32 cytotoxicity (Fig. 3). However, this assay is not capable of uncovering the differences between the covalent (**2** and **4**) and the non-covalent (**8** and **9**) kinetic stabilizers, which is not surprising given the high selectivity and the potency of the non-covalent kinetic stabilizers **8** and **9**. None of these compounds were cytotoxic to IMR-32 cells in the absence of WT-TTR (Supplementary Fig. 11).

Crystal structure of the WT-TTR–(benzoyl substructure of **4**)₂ complex

The crystal structure of the WT-TTR–(benzoyl substructure of **4**)₂ covalent conjugate was determined to 1.35 Å resolution (Table S1, Fig. 4a). Since the conjugated ligand resides on the crystallographic 2-fold axis, the observed electron density represents an average of the two possible symmetry-related conformations⁴⁷. Electron density for the benzoyl substructure of **4** and the K15 (K15') side chain up to the δ methylene are clear and unambiguous. While the electron density for the amide bond is clearly visible, symmetry averaging makes it difficult to be absolutely certain whether the amide bond is in a *cis* or *trans* conformation. Modeling with a *trans* amide bond did not agree as well as modeling the amide in a *cis* conformation. Moreover, the *trans* bond angles and lengths were atypical and the crystallographic R-values were higher, suggesting that the *cis* amide affords a lower overall energy structure in the crystal given the constraints on the orientation of the bound stilbene and the K15 (K15') side chain. Future NMR studies are warranted to explore this subject in more detail.

The 3,5-dimethyl-4-hydroxyphenyl substructure of the TTR conjugate occupies the inner T₄ binding cavity (Fig. 4b), consistent with the preferred binding orientation of non-covalent stilbene-based high pK_a phenols^{24,25}. The methyl substituents extend into the two symmetry-related halogen binding pockets 3 and 3'. The 4-OH substituent on the aryl makes bridging hydrogen bonds with the Ser117 and 117' side chains of adjacent TTR subunits (Fig. 4b).

The conjugating amide bond orders the ordinarily flexible K15 side chain and the substructure of **4** such that their B-values ($18.4 \text{ \AA}^2$) approach those of the protein ($13 \text{ \AA}^2$), suggesting that the stilbene substructure is rigidly held in the pocket. As the *p*-fluorophenol leaving group of **4** is not observed in the density, all bound **4** must have reacted covalently with the lysine ϵ -amine nucleophile. Even though a 5-fold excess of **4** was used in formation of the TTR–(benzoyl substructure of **4**) conjugate crystals, the electron density indicated that the other 7 lysines, and all other residues, were unmodified, consistent with the high chemoselectivity of **4**, as demonstrated above.

A structurally homologous, non-covalent TTR kinetic stabilizer recently completed a successful phase II/III placebo-controlled clinical trial for the amelioration of FAP, demonstrating that it is unlikely that T₄ or holo-retinol binding protein or retinol homeostasis will be perturbed in any significant fashion upon TTR conjugate formation with **1-4** (www.foldrx.com). Comparisons of the structures of WT-TTR–(benzoyl substructure of **4**)₂ (PDB accession code 3HJ0) and WT-TTR with two non-covalently bound resveratrol molecules (**10**; PDB accession code 1DVS) ($0.389 \text{ \AA}$ RMSD; Supplementary Figs. 12 and 13) and WT-TTR in complex with RBP (PDB accession code 1QAB) (Supplementary Fig. 14) further support this hypothesis.

DISCUSSION

Small molecules that bind selectively to and then react with a specific Lys ϵ -amino group composing a specific non-enzyme protein in a complex biological sample are rare. We report a designed small family of such compounds herein that should be useful for a range of applications, beyond the amelioration of TTR amyloidosis. Another application of small molecule TTR conjugating structures includes their covalent attachment to low MW drugs that exhibit unfavorable pharmacokinetic (PK) or pharmacodynamic (PD) properties by themselves⁴⁸. Ideally, the small molecule TTR covalent modifier–drug fusion would be orally bioavailable. Upon formation of the TTR–(covalent modifier–drug)_{n<2} conjugate in plasma, the covalently linked drug should protrude from the surface of TTR and take on the favorable PK and PD properties of TTR (conjugated drug concentration up to $10 \text{ }\mu\text{M}$ with a half-life of 24 h). Such TTR–small molecule covalent modifier substructure–drug conjugates could significantly expand the chemical diversity of pharmacologic agents for the treatment of disease.

METHODS

Reverse phase HPLC analysis

Recombinant WT-TTR was expressed as described previously⁴¹. For analysis of test compounds with TTR, each compound ($1.5 \text{ }\mu\text{L}$ in DMSO, 1.44 mM) was added to $300 \text{ }\mu\text{L}$ of WT-TTR or K15A-TTR ($3.6 \text{ }\mu\text{M}$ in phosphate buffer [10 mM sodium phosphate, 100 mM KCl, 1 mM EDTA, pH 7.0]). The samples were vortexed, and incubated for 18 h at $25 \text{ }^\circ\text{C}$. Reverse phase high performance liquid chromatography (RP-HPLC) was performed on a Water 600 E multi-solvent delivery system, using a Waters 486 tunable absorbance detector, a 717 autosampler, and a ThermoHypersil Keystone Betabasic-18 column ($150 \text{ \AA}$ pore size, $3 \text{ }\mu\text{m}$ particle size, and mobile phase A; 0.1% TFA in 95% H_2O + 5% CH_3CN and mobile phase B, 0.1% TFA in 95% CH_3CN + 5% H_2O). Linear gradients were run from 100:0 to 0:100 A:B over 27 min.

Fibril formation of Transthyretin

Compounds ($5 \text{ }\mu\text{L}$ of 1.44 mM solution in DMSO) were added to $495 \text{ }\mu\text{L}$ of TTR ($7.2 \text{ }\mu\text{M}$, in phosphate buffer). The mixtures were vortexed and incubated for 18 h at $25 \text{ }^\circ\text{C}$. To obtain

a final pH of 4.4, 500 μ L of 100 mM acetate, 100 mM KCl, 1 mM EDTA, pH 4.2 was added, and the mixtures were incubated at 37 °C for 72 h without agitation. The solutions were vortexed to evenly distribute any precipitate, and turbidity was measured at 400 nm using a spectrophotometer.

Modification of WT-TTR and K15A-TTR by test compounds in human plasma

The modification ratio of TTR by kinetic stabilizers in human plasma was evaluated as described⁴⁰ with minor modifications. Briefly, 7.5 μ L of compounds (1.44 mM in DMSO) were added to 1 mL of human plasma and incubated at 37 °C for 24 h on a rocker (30 rpm). A 1:1 (v/v) slurry of unfunctionalized sepharose resin in 10 mM Tris, 140 mM NaCl, pH 8.0 (TSA) was added and the solutions were incubated for 1 h at 4 °C on a rocker plate, followed by brief centrifugation. Two 400 μ L aliquots of the supernatants were added to 300 μ L of 1:1 (v:v) slurry of anti-TTR antibody-conjugated sepharose resin in TSA and gently rocked at 4 °C for 20 min (30 rpm). After centrifugation, the resin was washed by shaking for 1 min in 1 mL of TSA containing 0.05% saponin (three times) and then 1 mL of TSA (twice). The TTR and bound test compound were dissociated from the resin by adding 155 μ L triethylamine (100 mM, pH 11.5) and vortexing for 1 min. After centrifugation, 135 μ L of solution containing test compound and TTR was analyzed by RP-HPLC as described above using linear gradients from 100:0 to 0:100 A:B over 45 min.

Urea-induced dissociation kinetics study

Compounds (1.25, 2.5, 5, 7.5, and 10 mM in DMSO) were diluted 10 fold in EtOH. Such stocks (7.2 μ L) were added to 200 μ L of TTR (18 μ M in phosphate buffer) to afford final concentrations of 4.5, 9, 18, 27, and 36 μ M. These mixtures were vortexed briefly and incubated for 18 h at 25 °C, before adding 100 μ L to 900 μ L of 6.67 M urea in phosphate buffer). The mixtures were vortexed and incubated in the dark at 25 °C without agitation. CD spectra were measured at 215-218 nm (0.5 nm steps, 5 times, 3s averages) at the indicated times.

Cell-based assay

The human neuroblastoma IMR-32 cell line (CCL-127 ATCC) was maintained in Opti-MEM (Invitrogen), supplemented with 5% FBS, 1 mM Hepes buffer, 2 mM L-glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin and 0.05 mg/mL CaCl_2 (complete cell medium). Cells (6,000 cells/well) were seeded in black wall, clear bottom, tissue culture treated 96-well plates (Costar) in complete cell medium and incubated overnight at 37 °C.

Recombinant WT-TTR purified in the cold was used as the cytotoxic insult. The protein was buffer exchanged in Hanks' Balanced Salt Solution (HBSS) at 10 °C using a Centriprep device (10 kDa MWCO, Millipore). Compounds (16 mM in DMSO) were diluted 1:1000 with WT-TTR (16 μ M in HBSS, filter sterilized) or with HBSS only, vortexed and incubated for 18 h at 37 °C. WT-TTR (16 μ M in HBSS) containing the same amount of DMSO was prepared in parallel and incubated under the same conditions.

The medium was removed from the cells and replaced immediately by TTR or TTR +compound or HBSS previously diluted 1:1 in Opti-MEM supplemented with 0.8 mg/mL BSA, 2 mM Hepes buffer, 4 mM L-glutamine, 200 units/mL penicillin, 200 μ g/mL streptomycin and 0.1 mg/mL CaCl_2 . The cells were incubated 24 h at 37 °C. Cell viability was evaluated by a resazurin reduction assay. Briefly, 10 μ L of resazurin (500 μ M, PBS) was added to each well and fluorescence quantitated in a plate reader (Ex/Em 530/590nm, Tecan Safire2, Austria) after 1 h incubation at 37 °C. Cell viability was calculated as percentage of fluorescence relative to cells treated with vehicle only (100% viability) after subtraction of blank fluorescence (wells without cells). Averages and standard errors from 2

independently performed experiments, each performed at least in triplicate, were calculated using GraphPrism (San Diego).

Crystallization and structure determination of the WT-TTR–(benzoyl substructure of **4**)₂ complex

WT-TTR concentrated to 4 mg/mL in 10 mM sodium phosphate buffer, 100 mM KCl (pH 7.6) was incubated at room temperature with a 5-fold molar excess of compound **4**. The vapor-diffusion sitting drop method was employed for crystallization. Crystals from TTR with compound **4** grew from 1.395 M sodium citrate, 3.5% v/v glycerol at pH 5.5. The crystals were cryo-protected with 10% v/v glycerol. Data were collected at beamline GM/CA-CAT 23-IDB at the APS (Advanced Photon Source) at a wavelength of 1.0333 Å. All data sets were integrated and scaled using HKL2000⁴⁹. The diffraction data from the crystal were indexed in orthorhombic space group P2₁2₁2 with two subunits (one dimer) per asymmetric unit. The crystal structure was determined by molecular replacement using the model coordinates of 2FBR³⁶ with the program Phaser⁵⁰. The structure possessed excellent stereochemical properties with 92.5% of residues in the most favored regions and 7.5% in the additionally allowed regions, further model building and refinement is discussed in the supplementary material.

Supplementary Material

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References

1. Savi P, et al. Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost.* 2000; 84:891–896. [PubMed: 11127873]
2. Estebanez-Perpina E, et al. Structural insight into the mode of action of a direct inhibitor of coregulator binding to the thyroid hormone receptor. *Mol Endocrinol.* 2007; 21:2919–2928. [PubMed: 17823305]
3. Guo F, et al. Breaking the one antibody-one target axiom. *Proc Natl Acad Sci USA.* 2006; 103:11009–11014. [PubMed: 16822849]
4. Cohen E, et al. Opposing activities protect against age-onset proteotoxicity. *Science.* 2006; 313:1604–1610. [PubMed: 16902091]
5. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science.* 2002; 297:353–356. [PubMed: 12130773]
6. Balch WE, Morimoto RI, Dillin A, Kelly JW. Adapting proteostasis for disease intervention. *Science.* 2008; 319:916–919. [PubMed: 18276881]
7. Johnson SM, et al. Native state kinetic stabilization as a strategy to ameliorate protein misfolding diseases: a focus on the transthyretin amyloidoses. *Acc Chem Res.* 2005; 38:911–921. [PubMed: 16359163]
8. Westermarck P, Sletten K, Johansson B, Cornwell GG. Fibril in senile systemic amyloidosis is derived from normal transthyretin. *Proc Natl Acad Sci USA.* 1990; 87:2843–2845. [PubMed: 2320592]

9. Coelho T. Familial amyloid polyneuropathy: new developments in genetics and treatment. *Curr Opin Neurol.* 1996; 9:355–359. [PubMed: 8894411]
10. Jacobson DR, et al. Variant-sequence transthyretin (isoleucine 122) in late-onset cardiac amyloidosis in black Americans. *N Engl J Med.* 1997; 336:466–473. [PubMed: 9017939]
11. Sekijima Y, et al. The Biological and Chemical Basis for Tissue Selective Amyloid Disease. *Cell.* 2005; 121:73–85. [PubMed: 15820680]
12. Holmgren G, et al. Clinical improvement and amyloid regression after liver transplantation in hereditary transthyretin amyloidosis. *Lancet.* 1993; 341:1113–1116. [PubMed: 8097803]
13. Klabunde T, et al. Rational design of potent human transthyretin amyloid disease inhibitors. *Nat Struct Biol.* 2000; 7:312–321. [PubMed: 10742177]
14. Monaco HL, Rizzi M, Coda A. Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein. *Science.* 1995; 268:1039–1041. [PubMed: 7754382]
15. Wojtczak A, Luft J, Cody V. Mechanism of molecular recognition. Structural aspects of 3,3'-diiodo-L-thyronine binding to human serum transthyretin. *J Biol Chem.* 1992; 267:353–357. [PubMed: 1730601]
16. Colon W, Kelly JW. Partial denaturation of transthyretin is sufficient for amyloid fibril formation in vitro. *Biochemistry.* 1992; 31:8654–8660. [PubMed: 1390650]
17. Liu K, et al. A glimpse of a possible amyloidogenic intermediate of transthyretin. *Nat Struct Biol.* 2000; 7:754–757. [PubMed: 10966644]
18. Jiang X, et al. An engineered transthyretin monomer that is nonamyloidogenic, unless it is partially denatured. *Biochemistry.* 2001; 40:11442–11452. [PubMed: 11560492]
19. Hammarstrom P, Wiseman RL, Powers ET, Kelly JW. Prevention of transthyretin amyloid disease by changing protein misfolding energetics. *Science.* 2003; 299:713–716. [PubMed: 12560553]
20. Hammarstrom P, Schneider F, Kelly JW. Trans-suppression of misfolding in an amyloid disease. *Science.* 2001; 293:2459–2462. [PubMed: 11577236]
21. Hurshman AR, White JT, Powers ET, Kelly JW. Transthyretin aggregation under partially denaturing conditions is a downhill polymerization. *Biochemistry.* 2004; 43:7365–7381. [PubMed: 15182180]
22. Babbes ARH, Powers ET, Kelly JW. Quantification of the thermodynamically linked quaternary and tertiary structural stabilities of transthyretin and its disease-associated variants: The relationship between stability and amyloidosis. *Biochemistry.* 2008; 47:6969–6984. [PubMed: 18537267]
23. Adamski-Werner SL, Palaninathan SK, Sacchettini JC, Kelly JW. Diflunisal analogues stabilize the native state of transthyretin. Potent inhibition of amyloidogenesis. *J Med Chem.* 2004; 47:355–374. [PubMed: 14711308]
24. Johnson SM, Connelly S, Wilson IA, Kelly JW. Toward optimization of the linker substructure common to transthyretin amyloidogenesis inhibitors using biochemical and structural studies. *J Med Chem.* 2008; 51:6348–6358. [PubMed: 18811132]
25. Johnson SM, et al. Bisaryloxime Ethers as Potent Inhibitors of Transthyretin Amyloid Fibril Formation. *J Med Chem.* 2005; 48:1576–1587. [PubMed: 15743199]
26. Miroy GJ, et al. Inhibiting transthyretin amyloid fibril formation via protein stabilization. *Proc Natl Acad Sci USA.* 1996; 93:15051–15056. [PubMed: 8986762]
27. Oza VB, et al. Synthesis, structure, and activity of diclofenac analogues as transthyretin amyloid fibril formation inhibitors. *J Med Chem.* 2002; 45:321–332. [PubMed: 11784137]
28. Razavi H, et al. Benzoxazoles as transthyretin amyloid fibril inhibitors: synthesis, evaluation, and mechanism of action. *Angew Chem Int Ed Engl.* 2003; 42:2758–2761. [PubMed: 12820260]
29. Wiseman RL, et al. Kinetic Stabilization of an Oligomeric Protein by a Single Ligand Binding Event. *J Am Chem Soc.* 2005; 127:5540–5551. [PubMed: 15826192]
30. Foss TR, et al. Kinetic stabilization of the native state by protein engineering: Implications for inhibition of transthyretin amyloidogenesis. *J Mol Biol.* 2005; 347:841–854. [PubMed: 15769474]
31. Foss TR, Wiseman RL, Kelly JW. The pathway by which the tetrameric protein transthyretin dissociates. *Biochemistry.* 2005; 44:15525–15533. [PubMed: 16300401]

32. Johnson SM, Connelly S, Wilson IA, Kelly JW. Biochemical and structural evaluation of highly selective 2-arylbenzoxazole-based transthyretin amyloidogenesis inhibitors. *J Med Chem.* 2008; 51:260–270. [PubMed: 18095641]
33. Petrassi HM, Klabunde T, Sacchettini JC, Kelly JW. Structure-Based Design of N-Phenyl Phenoxazine Transthyretin Amyloid Fibril Inhibitors. *J Am Chem Soc.* 2000; 122:2178–2192.
34. Purkey HE, et al. Hydroxylated polychlorinated biphenyls selectively bind transthyretin in blood and inhibit amyloidogenesis: rationalizing rodent PCB toxicity. *Chem Biol.* 2004; 11:1719–1728. [PubMed: 15610856]
35. Baures PW, Oza VB, Peterson SA, Kelly JW. Synthesis and evaluation of inhibitors of transthyretin amyloid formation based on the non-steroidal anti-inflammatory drug, flufenamic acid. *Bioorg Med Chem.* 1999; 7:1339–1347. [PubMed: 10465408]
36. Green NS, Palaninathan SK, Sacchettini JC, Kelly JW. Synthesis and characterization of potent bivalent amyloidosis inhibitors that bind prior to transthyretin tetramerization. *J Am Chem Soc.* 2003; 125:13404–13414. [PubMed: 14583036]
37. Miller SR, Sekijima Y, Kelly JW. Native state stabilization by NSAIDs inhibits transthyretin amyloidogenesis from the most common familial disease variants. *Lab Invest.* 2004; 84:545–552. [PubMed: 14968122]
38. Burgi HB, Dunitz JD, Lehn JM, Wipff G. Stereochemistry of Reaction Paths at Carbonyl Centers. *Tetrahedron.* 1974; 30:1563–1572.
39. Hammarstrom P, Jiang X, Deechongkit S, Kelly JW. Anion shielding of electrostatic repulsions in transthyretin modulates stability and amyloidosis: insight into the chaotrope unfolding dichotomy. *Biochemistry.* 2001; 40:11453–11459. [PubMed: 11560493]
40. Purkey HE, Dorrell MI, Kelly JW. Evaluating the binding selectivity of transthyretin amyloid fibril inhibitors in blood plasma. *Proc Natl Acad Sci USA.* 2001; 98:5566–5571. [PubMed: 11344299]
41. Lashuel HA, Wurth C, Woo L, Kelly JW. The most pathogenic transthyretin variant, L55P, forms amyloid fibrils under acidic conditions and protofilaments under physiological conditions. *Biochemistry.* 1999; 38:13560–13573. [PubMed: 10521263]
42. Baures PW, Peterson SA, Kelly JW. Discovering transthyretin amyloid fibril inhibitors by limited screening. *Bioorg Med Chem.* 1998; 6:1389–1401. [PubMed: 9784876]
43. Zhang Q, Kelly JW. Cys10 mixed disulfides make transthyretin more amyloidogenic under mildly acidic conditions. *Biochemistry.* 2003; 42:8756–8761. [PubMed: 12873136]
44. Reixach N, et al. Tissue damage in the amyloidoses: Transthyretin monomers and nonnative oligomers are the major cytotoxic species in tissue culture. *Proc Natl Acad Sci USA.* 2004; 101:2817–2822. [PubMed: 14981241]
45. Reixach N, et al. Cell based screening of inhibitors of transthyretin aggregation. *Biochem Biophys Res Commun.* 2006; 348:889–897. [PubMed: 16904635]
46. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem.* 2002; 267:5421–5426.
47. Wojtczak A, Cody V, Luft JR, Pangborn W. Structure of rat transthyretin (rTTR) complex with thyroxine at 2.5 angstrom resolution: first non-biased insight into thyroxine binding reveals different hormone orientation in two binding sites. *Acta Crystallogr D Biol Crystallogr.* 2001; 57:1061–1070. [PubMed: 11468389]
48. Marinec PS, et al. FK506-binding protein (FKBP) partitions a modified HIV protease inhibitor into blood cells and prolongs its lifetime in vivo. *Proc Natl Acad Sci U S A.* 2009; 106:1336–1341. [PubMed: 19164520]
49. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* 1997; 276:307–326.
50. Storoni LC, McCoy AJ, Read RJ. Likelihood-enhanced fast rotation functions. *Acta Crystallogr D Biol Crystallogr.* 2004; 60:432–438. [PubMed: 14993666]

Figure 1.

Reverse phase (RP)-HPLC analysis of the chemoselectivity of compounds **1-4** in recombinant WT-TTR vs K15A-TTR homotetramer solutions and human plasma. (a) C₁₈-RP-HPLC analysis of WT-TTR (top) or K15A-TTR (bottom) pre-incubated (18 h) with candidate covalent kinetic stabilizer **4** (analogous data for **1-3** are described in Supplementary Fig. 1). (b) Rate of WT-TTR-(stilbene)_n conjugate formation analyzed by C₁₈-RP-HPLC. Samples were analyzed in triplicate and the error bars represent standard deviations. (c) C₁₈-RP-HPLC assessment of the modification of TTR in human blood plasma by compounds **3** and **4** (analogous data for **1** and **2** are shown in Supplementary Fig. 8).

Figure 2.

A comparison of the potency of covalent kinetic stabilizers and their non-covalent counterparts and an assessment of WT-TTR tetramer dissociation kinetics in the presence of a covalent kinetic stabilizer. (a) Comparison of the potency of non-covalent kinetic stabilizers **8** and **9** (2.7 μ M) with that of covalent kinetic stabilizers **2** and **4** (2.7 μ M) assessed by their capacity to inhibit recombinant WT-TTR (3.6 μ M) amyloidogenesis over a 120 h time course. (b) Concentration-dependent kinetic stabilization of recombinant WT-TTR by **1-4** and their non-covalent counterparts **8** and **9** assessed by % fibril formation after 72 h. (c) Kinetic stabilizer **4** prevents WT-TTR dissociation in a concentration-dependent fashion. Urea-mediated WT-TTR (1.8 μ M) dissociation time course in the absence and presence of compound **4** as a function of the indicated concentrations, evaluated by linking slow tetramer dissociation to rapid and irreversible monomer denaturation in 6 M urea measured by far-UV circular dichroism at 215–218 nm¹⁹. Samples were analyzed in triplicate and the error bars represent standard deviations.

Figure 3.

Inhibition of WT-TTR cytotoxicity in human IMR-32 neuroblastoma cells as a function of the dose of covalent and non-covalent TTR kinetic stabilizers. WT-TTR was pre-incubated at 37 °C in the absence (black bars) or presence of compounds for 18 h and then added to the cell culture media. The final concentration of WT-TTR was 8 μM and the final concentrations of compounds were 8, 6, 4 and 2 μM, as indicated. Cell viability was assessed using the resazurin reduction assay after 24 h. Cell viability results are reported relative to cells treated with vehicle only (100% cell viability)⁴⁴. Columns represent the means of 2 independently performed experiments (n = 6) and the error bars represent standard errors.

Figure 4.

Crystal structure of the WT-TTR-(benzoyl substructure of **4**)₂ conjugate, showing the amide bond linkage to the Lys-15 ε-amino group (PDB 3HJ0). Individual TTR monomers are uniquely colored. (a) Three-dimensional ribbon diagram depiction. (b) Magnified image of the benzoyl substructure derived from **4** covalently bound in one of the T₄ binding sites. A Connolly analytical surface representation (green = hydrophobic, purple = polar, and blue = exposed) depicts the hydrophobicity of the binding site. The 3,5-methyl groups are placed in the halogen binding pockets 3 and 3' and bridging hydrogen bonds are formed between the 4-OH of the benzoyl substructure of **4** and the Ser117 and 117' hydroxyls from adjacent TTR monomers. Figure generated using MOE (2006.08), Chemical Computing Group, Montreal, Canada).

Table 1

Inhibition of purified recombinant WT-TTR and V30M-TTR amyloidogenesis *in vitro* by covalent kinetic stabilizers **1-4** and quantification of the chemoselective covalent modification of TTR in blood plasma by the candidate covalent kinetic stabilizers **1-4** *ex vivo*. Shown schematically below is one of the two T₄ binding sites comprising two subunits of TTR.

Compound	R	XR'	% Fibril Formation ^a		IC ₅₀ (μM) ^b	% Modification of TTR subunits in Human Blood Plasma ^c
			7.2 μM	3.6 μM		
1	Br		2% (±1%)	12% (±1%)	2.00	36% (±0.4%)
			3% (±0.3%)	17% (±0.2%)		
2	CH ₃		2% (±1%)	16% (±2%)	2.00	48% (±1.3%)
			3% (±0.2%)	34% (±0.5%)		
3	CH ₃		4% (±1%)	21% (±2%)	2.26	49% (±0.7%)
			3% (±0.2%)	33% (±2%)		
4	CH ₃		1% (±1%)	16% (±1%)	1.96	32% (±3.3%)
			7% (±0.2%)	36% (±3%)		

^a Percent fibril formation of WT-TTR (3.6 μM, black font) and V30M-TTR (3.6 μM, blue font) in the presence of candidate covalent kinetic stabilizers (7.2 and 3.6 μM) at pH 4.4, 72 h, 37 °C.

^b IC₅₀ reported is apparent since these compounds bind and then react with WT-TTR.

^c Maximum modification percent by candidate covalent kinetic stabilizers is 50% because only 2/4 TTR subunits can be modified in each tetramer after incubation (18 h).

The covalently attached benzoyl fragment from **1-4** is shown in red.