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A competition assay to identify amyloidogenesis inhibitors by monitoring the fluorescence emitted by the covalent attachment of a stilbene derivative to transthyretin

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ABSTRACT

Herein we demonstrate that competition between candidate kinetic stabilizer binding to transthyretin (TTR) and stilbene binding to and reaction with the same thyroxine sites within TTR can be utilized to discover potent and highly selective non-covalent TTR amyloidogenesis inhibitors. We report two stilbenes, **S1** and **S2**, for use in distinct competition assays. Each bind selectively to TTR and then chemoselectively react to form an amide bond with the Lys-15 residue of TTR, creating a fluorescent conjugate. We used 28 TTR kinetic stabilizers exhibiting a known spectrum of plasma TTR binding selectivities and TTR amyloid fibril inhibition efficacies to validate the 'TTR fluorescence conjugate competition assay'. The kinetic stabilizers competed with **S1** for binding to recombinant TTR in buffer and with **S2** for binding to endogenous levels of TTR in human blood serum. In both assay scenarios, we demonstrate that the lower the TTR–stilbene conjugate fluorescence after a 3 h competition, the greater the binding selectivity and potency of the candidate TTR kinetic stabilizer. These assays, particularly the assay utilizing **S2** in human serum, replace two assays previously utilized to gather the same information. While not the focus of this manuscript, it is clear that the 'TTR fluorescence conjugate competition assay' could be adapted for high throughput screening applications.

1. Introduction

Protein aggregation, including amyloid fibril formation or amyloidogenesis, is genetically and pathologically linked to the proteotoxicity thought to cause the post-mitotic cell degeneration characteristic of human amyloid diseases.[1–4] Transthyretin (TTR) is one of more than 30 non-homologous human proteins that misassemble into amyloid fibrils, a cross-β-sheet quaternary structure, and other aggregate morphologies leading to the TTR amyloid diseases.[5–15] Amyloidogenesis appears to be exacerbated by an aging-associated decline in cellular protein homeostasis or proteostasis capacity.[16–21]

TTR amyloidogenesis is associated with cardiomyocyte degradation[8] and/or peripheral and autonomic nervous system degeneration in humans.[5,15] In senile systemic amyloidosis, wild type (WT) TTR amyloidogenesis in the heart leads to a cardiomyopathy, affecting as much as 20% of the population over the age of 70.[7,9,11,22,23] Familial amyloid polyneuropathy (FAP) results from aggregation and deposition of one of over 100 destabilized TTR variants in the peripheral nervous system, affecting about 10,000 individuals worldwide.[10,15,24–26] Familial amyloid cardiomyopathy (FAC) is caused by the deposition of one of a few TTR variants in the heart (e.g., V122I-TTR, affecting 3–4% of African Americans).[8,27–29] The central nervous system selective amyloidoses (CNSA) are rare and are associated with amyloid fibril formation from the most destabilized TTR variants (e.g., D18G-TTR and A25T-TTR).[12,30–32]

Transthyretin is a tetrameric protein composed of 127-amino-acid β-sheet-rich subunits (Fig. 1A). TTR is synthesized by the liver for secretion into the blood, by the choroid plexus for secretion into the cerebrospinal fluid (CSF), and by the eye for utilization there.[33–36] While TTR transports thyroid hormone (T_4) and the *holo*-retinol binding protein in the blood and CSF in humans, the vast majority (>99%) of the T_4 sites in the blood are unoccupied because T_4 in blood is largely carried by thyroid binding globulin and albumin.[37–39]

The structure of the TTR tetramer creates two T_4 binding sites at the weaker dimer–dimer interface that is bisected by the crystallographic C_2 axis (Fig. 1A, binding of thyroxine shown along the C_2 axis).[39] The two T_4 binding sites are interconverted by C_2-symmetry axes perpendicular to the crystallographic two-fold axis.[11,40–45] There are subtle conformational changes that occur upon ligand binding to the first T_4 binding site in TTR that alter the binding constant of the same small molecule for the second T_4 binding site.[46]

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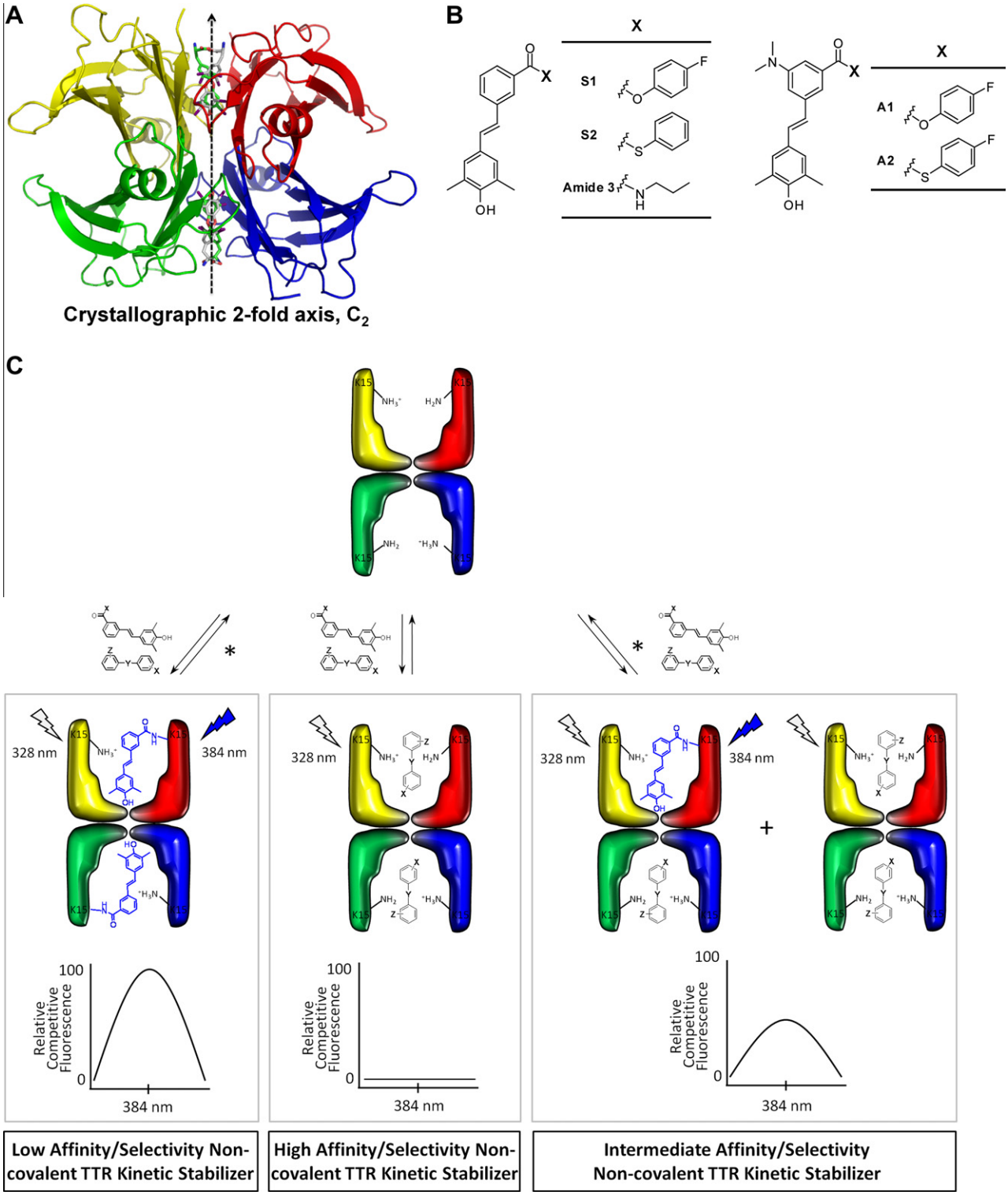

Figure 1. (A) Crystal structure (PDB 1ROX) of the TTR tetramer with thyroxine (T_4) bound at the weaker dimer–dimer interface, the interface bisected by the crystallographic C_2 axis. (B) Chemical structures of fluorogenic probes **S1** and **S2** and the fluorescent non-covalent propylamide stilbene analogue **Amide 3**, and **A1** and **A2**, previously published auxochromic analogs of stilbenes **S1** and **S2**.[81] (C) General principle of the fluorescence-based competition assay. Candidate non-covalent kinetic stabilizers compete with **S1** or **S2** for the T_4 binding pocket, reducing the fluorescence generated by amide bond formation with TTR (* indicates that only binding of **S1** or **S2** is reversible).

Generally, ligands bind to TTR with negative cooperativity, although there are rare exceptions of non- and positively-cooperative ligand binding to the T_4 binding sites.[11,47–58] Previous studies demonstrate that high affinity ligand binding to one T_4 site is sufficient to kinetically stabilize the entire TTR tetramer,[59] by selectively stabilizing the native quaternary structure to a greater extent than the dissociative transition state.[43,47–58,60–70]

Since transthyretin amyloidogenesis causes degeneration of tissues that do not synthesize TTR, including the heart, it appears that extracellular TTR tetramer dissociation, partial monomer

unfolding, and spontaneous misfolded monomer misassembly into amyloid fibrils and other aggregate morphologies in affected tissues is the most likely pathway by which proteotoxicity occurs.[71–77] Since dissociation of the tetramer is rate limiting for TTR amyloidogenesis, compounds that bind to tetrameric TTR and prevent dissociation by making the dissociation barrier insurmountable are highly sought after,[11,65] especially since Tafamidis, a kinetic stabilizer of TTR, has recently been demonstrated to successfully halt FAP-associated neurodegeneration in a placebo-controlled clinical trial (www.foldRx.com).

Historically, clinically promising TTR kinetic stabilizers have been identified using a combination of two assays: an in vitro acid-mediated TTR fibril formation assay and a plasma TTR binding selectivity assay, carried out in that order.[60,61,66,78] In the in vitro acid-mediated TTR fibril formation assay, the candidate small molecule (3.6 and 7.2 μM) is pre-incubated with recombinant TTR (3.6 μM) before initiating fibril formation by lowering the pH to 4.4 (37 °C). TTR fibril formation is assessed over a three-day time course or at the end of a 72 h incubation period either by measuring turbidity, or less often by following thioflavin T fluorescence.[77,79] Adapting this approach for a high-throughput screening (HTS) format is difficult because of the large quantities of recombinant TTR required (0.6 mg to test one compound in triplicate) and the long incubation period required (72 h). Because denaturing conditions are employed to accelerate the rate of amyloidogenesis to make the assay convenient on a laboratory time scale,[71] there is always the concern that the dissociation constants of selected kinetic stabilizers could be altered by the acidic conditions.

Because the in vitro fibril formation assay is performed with recombinant TTR in buffer, it does not account for the possibility that a putative TTR amyloidogenesis inhibitor could bind with higher affinity to another protein in a complex biological fluid like human blood, reducing the compound's effectiveness against TTR amyloidogenesis. Therefore, the plasma TTR binding selectivity assay has been historically employed next to further assess the ability of a putative kinetic stabilizer to bind to TTR over the approximately 4000 other proteins in human blood plasma, including thyroid binding globulin and albumin.[78] The candidate kinetic stabilizer (10 μM) is incubated with human plasma for 24 h and then TTR (at an endogenous concentration of about 5 μM), along with any bound candidate kinetic stabilizer, is immunoprecipitated with an anti-TTR antibody conjugated to Sepharose resin. After washing the immunocaptured TTR with buffer, the complex is dissociated at high pH and the binding stoichiometry of the small molecule relative to TTR is determined by reverse-phase high performance liquid chromatography (RP-HPLC).[78] Although this method sensitively assesses plasma TTR binding stoichiometry and thus selectivity, it would be costly to automate this assay for a HTS format. Moreover, this assay generally under-determines TTR kinetic stabilizer stoichiometry owing to the pre-HPLC washing steps.

This paper demonstrates that the previously reported stilbene covalent TTR modifiers **S1** and **S2**[80] (Fig. 1B), that bind highly selectively to the T_4 binding sites within TTR and then react with the pK_a perturbed Lys-15 ε-amino group of TTR, create amide linked conjugates that are fluorescent. Herein we demonstrate that a competition between these stilbenes (**S1** or **S2**) and a non-covalent candidate kinetic stabilizer that binds to the same thyroxine sites within TTR (Fig. 1C) serves as an assay to discover potent and selective non-covalent TTR kinetic stabilizers that prevent amyloidogenesis. The lower the TTR–stilbene conjugate fluorescence in the competition assay, the greater the TTR blood serum binding selectivity and potency of the candidate kinetic stabilizer (Fig. 1C). Importantly, we demonstrate that human blood serum and **S2** can be employed to discover potent TTR kinetic stabilizers that also bind highly selectively to TTR over the 4000+ other proteins present in serum. Thus, the 'TTR fluorescent conjugate competition assay', especially the assay carried out with **S2** in serum, can be used to discover potent and selective TTR amyloidogenesis inhibitors—replacing the two assays previously required.[60,78] The 28 TTR kinetic stabilizers exhibiting known plasma TTR binding selectivity and amyloid fibril inhibition efficacy were used to benchmark the competition assay. These data suggest that the assay utilizing **S2** in human serum is sufficient to generate highly potent and selective TTR amyloidogenesis inhibitors.

2. Results and discussion

2.1. Fluorescence generated through a reaction between S1 or S2 and TTR

Previously, we reported a family of related stilbenes, including **S1** and **S2** (Fig. 1B), that bind to TTR highly selectively and then covalently modify TTR in preference to more than 4000 other proteins that make up human plasma.[80] These covalent modifiers react with only one of eight Lys ε-amino groups (that of Lys-15) present in TTR, affording a TTR–stilbene conjugate.[80]

Evidence is presented below that the ester substructure of **S1**, and particularly the thioester substructure of **S2**, quenches the stilbene fluorophore when bound to the T_4 binding sites of TTR prior to reaction, strictly analogous to the quenching derived from the same functional groups on **A1** and **A2** (Fig. 1B), auxochromic analogs of stilbenes **S1** and **S2** previously employed for fluorescent imaging of TTR in the secretory pathway of mammalian cells.[81] This study on the photophysics of stilbene **A2** reveals that the thioester functionality is very effective at internally quenching the fluorescence, thus only when the conjugate forms with TTR is fluorescence observed.[81] Ester **S1** and thioester **S2** react with WT-TTR in buffer to afford fluorescent TTR–$(\text{stilbene})_{n\leqslant 2}$ conjugates analogous to those reported for analogs **A1** and **A2**.[81] Incubation of recombinant WT-TTR (3.6 μM) with **S1** or **S2** (7.2 μM) for 24 h in phosphate buffer (pH 7) produced fluorescence resulting from amide bond conjugation (Fig. 2A and B, respectively). The excitation (Ex.) spectra are shown as dotted black traces (λ_{max} = 328 nm), while emission (Em.) spectra are depicted by blue traces (λ_{max} = 384 nm). The basal level of fluorescence from the 328 nm excitation of stilbene **S2** in buffer was undetectable (Fig. 2B, red trace), whereas **S1** was barely detectable (Fig. 2A, red trace), consistent with internal thioester and ester singlet state quenching mechanisms, respectively.[81] Strictly analogous incubation of recombinant V30M-TTR tetramer (amyloid disease-associated variant) with **S1** and **S2** for 24 h afforded similar fluorescence spectra (Supplementary Fig. 1A and B). Importantly, no significant fluorescence was detected upon **S1** or **S2** binding to K15A-TTR, which lacks the Lys-15 ε-amino group and, therefore, cannot form the amide bond with **S1** or **S2** that is required to create the TTR–stilbene fluorescent conjugate (Fig. 2A and B, solid black traces near the *x*-axis).[80,81] A blue shift (relative to buffer alone) in the very weak fluorescence of **S1** was observed with K15A-TTR, providing evidence that **S1** is binding to the T_4 binding sites within the K15A-TTR homotetramer (additional evidence is provided below). Demonstrated binding in the absence of fluorescence, when considered in the context of previously published photophysical studies on **A2**, suggest that the intense 384 nm fluorescence from the stilbene–WT-TTR conjugate does not simply result from binding or placement of the stilbene in the unique hydrophobic environment of the T_4 binding site. Instead, amide bond conjugation is required to observe fluorescence. With most stilbenes, environment-sensitive fluorescence is observed when the trans–cis photoisomerization relaxation mechanism is inhibited by protein binding;[82–87] however with **A2**, trans–cis photoisomerization and fluorescence in hydrophobic environments is not

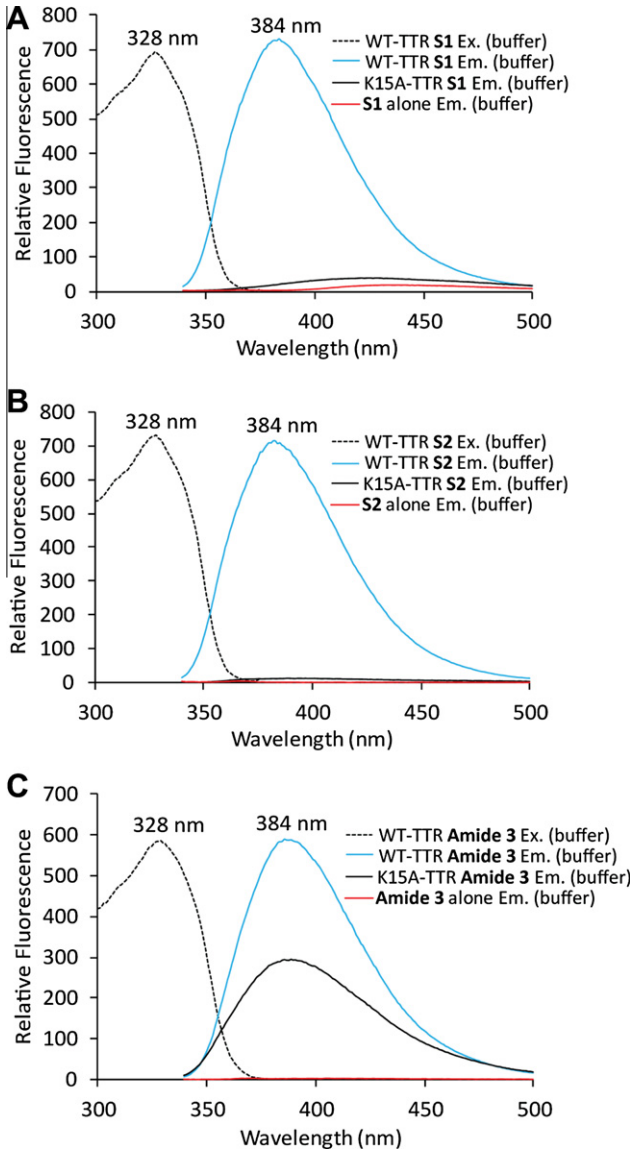

Figure 2. Fluorescence observed after a 24 h incubation of the reactive stilbene-based TTR modifiers **S1** (A) and **S2** (B) with recombinant WT-TTR (blue traces). (C) Fluorescence of the non-reactive stilbene **Amide 3** upon incubation with recombinant WT-TTR (blue trace). Each stilbene (7.2 μM) was also incubated with recombinant K15A-TTR (3.6 μM) for 24 h (solid black traces). The fluorescence spectra of the stilbenes (7.2 μM) alone in aqueous buffer are shown (red traces), and the excitation spectra are presented as dotted black traces. See text for excitation (Ex.) and emission (Em.) details.

observed. Thus, it is clear that the thioester of **A2** is quenching its inherent fluorescence.[81] All indications are that **S1** and **S2** behave analogously, however additional photophysical experiments are desirable to better understand these molecules.

To provide evidence that chemoselective Lys-15 amide bond conjugation to the stilbene structure creates the blue-shifted, ~560-fold (**S1**) and ~1100-fold (**S2**) increase in fluorescence intensity displayed in Figure 2A and B (compared to **S1** and **S2** alone in buffer), recombinant WT-TTR and K15A-TTR homotetramers were incubated with a stilbene analog already possessing a propyl amide bond (**Amide 3**, Fig. 1B). Indeed, the presence of a *meta*-carboxamide appended to the stilbene renders the chromophore fluorescent upon binding to TTR (Fig. 2C), in contrast to its quenched ester or thioester analogs.[81] Incubation of **Amide 3** (7.2 μM) with recombinant WT-TTR (3.6 μM) for 24 h reveals a ~400-fold increase in fluorescence intensity (Fig. 2C, blue trace), compared to **Amide 3** alone in buffer (red trace), supporting the hypothesis that the ester and thioester *meta* substituents quench **S1** and **S2** fluorescence resulting from TTR binding. In further support of this hypothesis, **S1** and **S2** remain non-fluorescent upon binding to K15A-TTR, whereas **Amide 3** exhibited a ~190-fold increase in fluorescence intensity upon binding to K15A-TTR (Fig. 2C, solid black trace, 24 h incubation). The fluorescence intensity is diminished relative to **Amide 3** binding to WT-TTR binding, presumably due to changes in the T_4 binding site environment of mutant K15A-TTR. **Amide 3** fluorescence in buffer is undetectable (Fig. 2C, red trace), likely because of the trans–cis photoisomerization-based quenching mechanism[81] and due to absence of a hydrophobic environment.

The requirements for stilbene–TTR conjugate fluorescence were further probed by adding **S1**, **S2**, or **Amide 3** to recombinant WT-TTR and recording time-dependent emission spectra. Addition of **Amide 3** to WT-TTR confirmed that these *meta*-substituted stilbenes bind rapidly to TTR (within 1 min), resulting in an ~360-fold increase in fluorescence intensity (Fig. 3A) relative to **Amide 3** alone

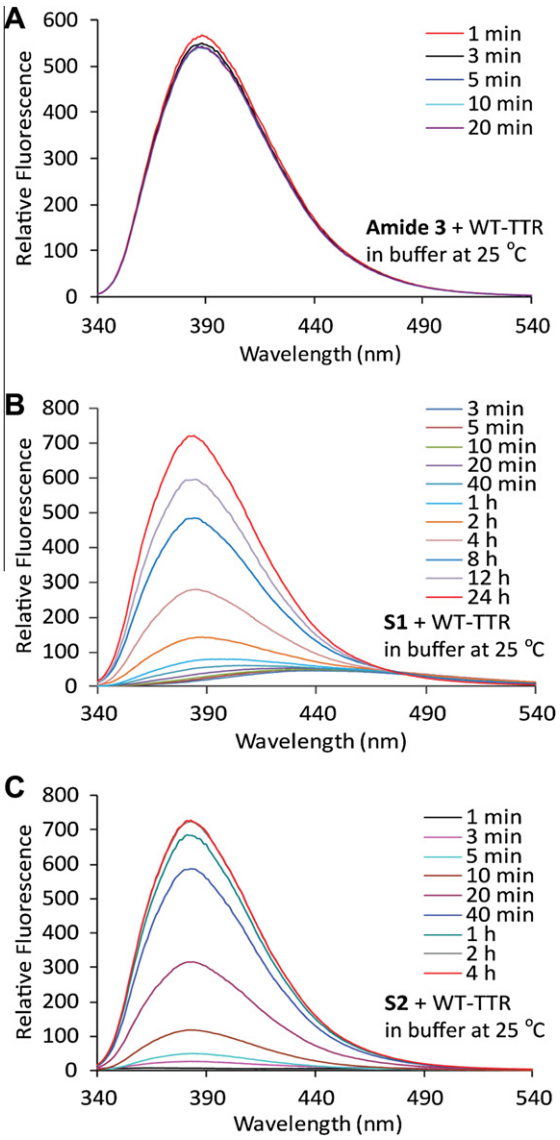

Figure 3. Time-dependent stilbene–TTR conjugate fluorescence spectra from recombinant WT-TTR (3.6 μM) treated with **Amide 3** (7.2 μM) (A), **S1** (7.2 μM) (B), or **S2** (7.2 μM) (C).

in buffer (Fig. 2C, red trace). The 360-fold change is within standard deviation of the 400-fold increase observed after a 24 h incubation of **Amide 3** with WT-TTR (Fig. 2C, blue trace). The deviation is likely caused by changes in room temperature, as fluorescence is temperature dependent. The slight time-dependent decrease in intensity is likely due to photobleaching of **Amide 3**. Addition of **S1** to WT-TTR reveals that binding is not sufficient to afford fluorescence, as fluorescence was not observed within 3 min. Instead, amide-bond-mediated conjugate formation is required, which proceeded with a 24 h time course (Fig. 3B), as discerned by the progressive increase in TTR–(stilbene)$_{n\leqslant 2}$ conjugate fluorescence. The addition of **S2** to WT-TTR further demonstrated that binding alone (within 1 min) was insufficient for the acquisition of fluorescence, instead conjugation was required, which occurred over a much faster time course (t_{50} = 22 min) owing to the more reactive thioester in **S2** (thiophenol pK_a = 6.6) relative to the ester in **S1** (*para*-fluorophenol pK_a = 9.8) (Fig. 3C). The rate of **S1** or **S2** amide bond conjugation to recombinant WT-TTR, monitored by RP-HPLC, correlates nicely with the time-dependent increase in TTR–(stilbene)$_{n\leqslant 2}$ conjugate fluorescence (Supplementary Fig. 2). Collectively, these data suggest that binding to TTR and amide bond conjugation to TTR are both required to observe conjugate fluorescence from **S1** and **S2**. In the absence of amide bond conjugation, ester/thioester quenching appears to dominate.

2.2. Kinetic stabilizer competition assay utilizing latent fluorophores S1 and S2

To assess whether the TTR fluorescent conjugate competition assay depicted in Figure 1C is sufficient to replace both the in vitro acid-mediated TTR fibril formation assay and the plasma TTR binding selectivity assay for the discovery of new potent and selective TTR kinetic stabilizers, we utilized a collection of kinetic stabilizers (**1**–**28**; Fig. 4) that had already been analyzed by the in vitro acid-mediated TTR fibril formation assay (compound at 7.2 μM, WT-TTR at 3.6 μM) and the plasma TTR binding selectivity assay (candidate inhibitor plasma concentration 10.8 μM).[43,47,62,67,69,78] The collection of TTR kinetic stabilizers chosen exhibit a range of in vitro TTR fibril inhibition potencies at pH 4.4 and a range of binding stoichiometries to TTR in human plasma, as shown in Table 1.

2.2.1. A linear correlation exists between the extent of TTR–(stilbene)$_{n\leqslant 2}$ conjugate fluorescence and the individual efficacy scores of test kinetic stabilizers using recombinant TTR in buffer

Reversible binding and irreversible covalent TTR modifying stilbene **S1** (7.2 μM) was placed in competition (Fig. 1C) with the non-covalent TTR kinetic stabilizers (7.2 μM) listed in Table 1 and

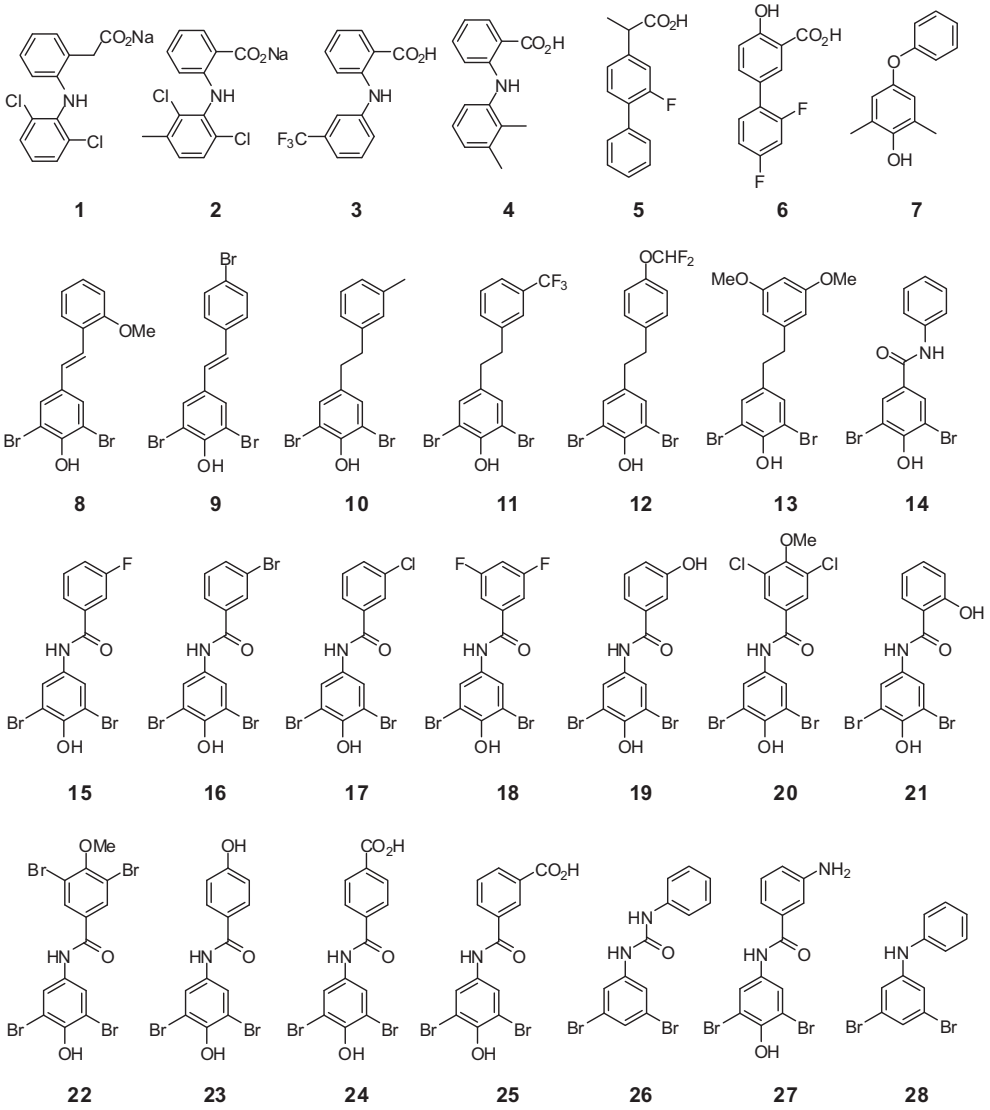

Figure 4. Chemical structures of test compounds **1**–**28** (non-covalent kinetic stabilizers of TTR) evaluated.

Table 1
In vitro acid-mediated TTR amyloid fibril inhibitor potency, plasma TTR binding stoichiometry, and the individual efficacy scores of non-covalent TTR kinetic stabilizers **1–28**

Compound	Fibril formation[a] (%)	Stoichiometry binding[b]	Efficacy score[c]	% Fluorescence from **S1** in recombinant TTR (37 °C)[d]	% Fluorescence from **S2** in serum (37 °C)[d]
1	17	0.04	0.29	85 (21)	99 (22)
2	3	0.14	0.37	62 (15)	85 (18)
3	2	0.2	0.39	44 (13)	97 (21)
4	7	0.2	0.37	65 (16)	96 (20)
5	29	0	0.24	93 (22)	96 (20)
6	2	0.13	0.37	75 (17)	86 (19)
7	8	0.31	0.40	76 (18)	81 (17)
8	0	1.67	0.89	−6 (2)	0 (1)
9	2	1.22	0.73	−6 (2)	2 (2)
10	3	1.5	0.81	−7 (1)	2 (2)
11	1	1.4	0.79	−6 (2)	3 (3)
12	1	1.08	0.69	−3 (3)	42 (12)
13	1	1.3	0.76	3 (5)	30 (10)
14	2	0.58	0.52	42 (12)	61 (16)
15	13	0.68	0.49	18 (9)	54 (15)
16	8	0.95	0.60	13 (8)	28 (8)
17	8	1.09	0.64	13 (8)	28 (8)
18	10	1.02	0.61	12 (7)	35 (11)
19	9	1.4	0.73	26 (9)	18 (5)
20	9	1.48	0.75	4 (6)	26 (7)
21	2	1.14	0.70	2 (4)	29 (9)
22	10	1.13	0.64	4 (6)	44 (13)
23	21	1.33	0.61	32 (10)	16 (4)
24	11	0.49	0.44	57 (14)	45 (14)
25	11	0.16	0.34	77 (19)	85 (18)
26	9	0.02	0.31	82 (20)	100 (23)
27	25	1.21	0.55	35 (11)	24 (6)
28	8	0.13	0.35	76 (18)	100 (23)

[a] Kinetic stabilizer at 7.2 μM, TTR 3.6 μM, pH 4.4 after 72 h. Values previously published.[43,47,62,69,78]
[b] Kinetic stabilizer added to human plasma at a final concentration of 10.8 μM. Values previously published.[43,47,62,69,78]
[c] See Eq. 1 in text.
[d] % Competition fluorescence after 3 h incubation and ranked order of test compounds in parenthesis.

shown in Figure 4, first employing recombinant WT-TTR (3.6 μM) in phosphate buffer (pH 7) at 25 °C and monitoring the time-dependent fluorescence changes over a time course of 8 h, after increasing the temperature to 37 °C (Fig. 5A). Maximum fluorescence was observed with recombinant WT-TTR (3.6 μM) in the absence of a test compound after a 3 h incubation period (Fig. 5A). Increasing the temperature to 37 °C (Fig. 5A) notably increased the kinetics relative to the conjugate formation kinetics at 25 °C (Fig. 3B). The extent of fluorescence was diminished in the presence of known TTR kinetic stabilizers in every case, as expected due to competitive binding to the same site that **S1** binds to in order to create the fluorescent conjugate. Low **S1**-derived WT TTR–$(stilbene)_{n\leqslant 2}$ conjugate fluorescence after a 3 h competition with a non-covalent kinetic stabilizer (Fig. 5A) indicates that the candidate is a promising TTR kinetic stabilizer/inhibitor of TTR amyloidogenesis. Non-covalent TTR kinetic stabilizers that bind to TTR with high affinity and create fluorescence at 384 nm after excitation at 328 nm will be scored as false negatives, which is a limitation of transforming this assay into a screen for the discovery of new kinetic stabilizers.

Data from the competition assay at 3 h (Fig. 5A) revealed that there was a poor linear correlation between the extent of recombinant TTR–$(stilbene)_{n\leqslant 2}$ conjugate fluorescence and the previously reported amyloid fibril inhibitor potency obtained using the acid-mediated fibril formation assay (R^2 = 0.18; Fig. 5B). The competition assay's fluorescence intensity at 3 h (Fig. 5A) correlates much better with the previously determined plasma TTR binding stoichiometry data (R^2 = 0.83; Fig. 5C). The **S1** TTR fluorescent conjugate competition assay's fluorescence data evaluated at 3 h (Fig. 5A) correlated best with the individual efficacy scores of the candidate kinetic stabilizers (R^2 = 0.89; Fig. 5D). The individual efficacy score of a candidate kinetic stabilizer results from the product of data derived from two assays: specifically, data from the in vitro acid-mediated fibril formation assay (100% − % of fibril formation (% F.F.)) and data from the plasma TTR binding stoichiometry assay reflecting the binding selectivity of the TTR kinetic stabilizer for TTR in human blood plasma (1 + TTR binding stoichiometry in human blood plasma (S.B.)) (Eq. 1 and Table 1). Candidate kinetic stabilizer individual efficacy scores exhibit a maximum of 1 for the most potent and selective TTR kinetic stabilizers and a minimum of 0.

$$\text{Individual efficacy score} = \frac{(100\% - \%\ \text{F.F.}) \times (1 + \text{S.B.})}{300\%} \qquad (1)$$

When the fluorescence of the TTR–$(stilbene)_{n\leqslant 2}$ conjugate derived from **S1** in the competition assay at 3 h is less than 50% of the maximal value attained when no TTR kinetic stabilizer was added, promising TTR amyloidogenesis inhibitors are identified (the kinetic stabilizers shown in the red portion of Fig. 5D). Kinetic stabilizers in this zone typically exhibit less than 10% fibril formation in vitro (72 h incubation of 3.6 μM TTR with 7.2 μM kinetic stabilizer at 37 °C, pH 4.4) (cf. compounds **3** and **14**, Table 1) or display a plasma TTR binding stoichiometry exceeding 1 out of a maximum of 2 (e.g., compounds **23** and **27**), or both (e.g., compounds **8**–**13**). Importantly, candidate TTR amyloidogenesis inhibitors exhibiting poor plasma TTR binding selectivity but good in vitro fibril inhibition potency (e.g., compounds **2** and **4**, Table 1) were identified as being poor candidates (the gray area of Fig. 5D). Based on the excellent linear correlation between the data derived from the competition assay and the individual efficacy scores exhibited by 28 established TTR kinetic stabilizers (Fig. 5D), we expect this single step TTR fluorescent conjugate competition assay in buffer to accurately predict the efficacy score, integrating amyloid inhibition potency and plasma TTR binding stoichiometry of candidate

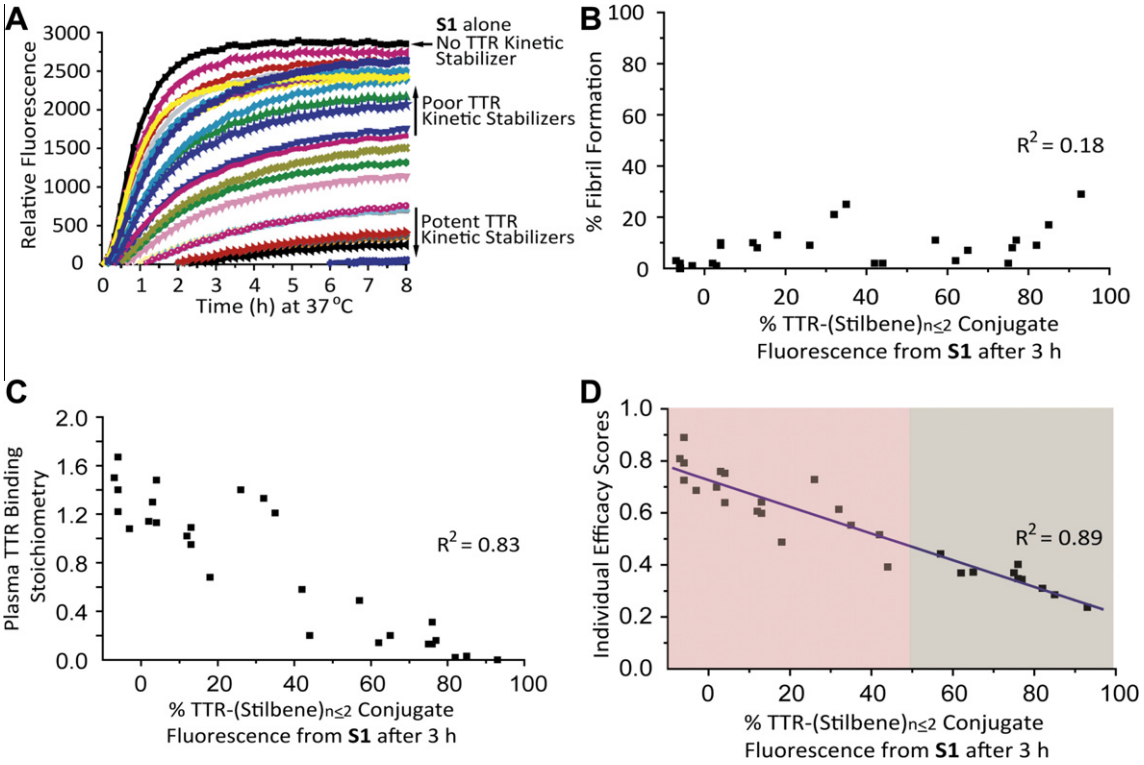

Figure 5. (A) Fluorescence change monitored for 8 h in the presence of candidate non-covalent TTR kinetic stabilizers and latent fluorogenic probe **S1** in recombinant WT-TTR solutions. (B) A poor linear correlation between the extent of TTR–(stilbene)$_{n\leq 2}$ conjugate fluorescence derived from **S1** after a 3 h competition and the previously reported amyloid fibril inhibitor potencies. (C) Linear correlation between the extent of TTR–(stilbene)$_{n\leq 2}$ conjugate fluorescence derived from **S1** after a 3 h competition and the previously reported plasma TTR binding stoichiometry data. (D) Linear correlation between the extent of TTR–(stilbene)$_{n\leq 2}$ conjugate fluorescence after a 3 h competition with **S1** in recombinant WT-TTR solutions and the individual efficacy scores of kinetic stabilizers **1**–**28**. Data points in the area shaded in red represent the compounds that allow less than 10% fibril formation in vitro or exhibit a plasma TTR binding stoichiometry exceeding 1, or both.

TTR kinetic stabilizers. Thus, it appears that the competition assay can be used to replace the two aforementioned assays, at least in buffer.

Initial attempts at an **S2**-based competition assay employing recombinant WT-TTR (3.6 μM) in buffer at 25 °C were unsuccessful because the reaction rate was too fast to discern differences between similar candidate kinetic stabilizers. However, it is likely that a successful **S2**-based assay could result from altering the concentration of **S2** and the temperature at which the assay is conducted.

2.2.2. A linear correlation exists between the extent of TTR–(stilbene)$_{n\leq 2}$ conjugate fluorescence derived from S2 in human serum and the individual efficacy score

To assess whether the fluorescent conjugate competition assay can also be performed in human serum (serum lacks cells and the proteins used in blood clotting), we first determined the approximate concentration of TTR in human serum (7.5 ± 1 μM) (Supplementary Fig. 3). Maximum conjugate fluorescence derived from **S2** was observed at a concentration of **S2** at 15 μM (25 °C) in the absence of a TTR kinetic stabilizer, 6–7 h into the time course (Supplementary Fig. 3). For the competition assay, we utilized candidate kinetic stabilizers at a concentration of 15 μM and **S2** at a concentration of 5 μM because of the faster reaction rate of **S2** with the ε-amine group of Lys15 and, more importantly, because **S2** exhibits no environment-sensitive fluorescence due to serum protein binding—as a consequence of the internal thioester quenching of its singlet excited state discussed above. The extent of TTR–(stilbene)$_{n\leq 2}$ conjugate fluorescence was diminished in the presence of candidate kinetic stabilizers that also bind competitively to the thyroxine sites, as expected.

There is a very poor correlation between the extent of TTR–(stilbene)$_{n\leq 2}$ conjugate fluorescence from **S2** in the serum competition assay and the previously reported amyloid fibril inhibitor data (R^2 = 0.04; Supplementary Fig. 4A). In stark contrast, there is an excellent linear correlation between the extent of TTR–(stilbene)$_{n\leq 2}$ conjugate fluorescence derived from **S2** (5 μM) measured after a 3 h competition with the established kinetic stabilizers (15 μM) in serum and the previously reported plasma TTR binding stoichiometry data (R^2 = 0.93; Fig. 6B). However, since the **S1** correlation using recombinant TTR in phosphate buffer (R^2 = 0.83; Fig. 5C) is also very good, it is arguable whether employing serum is worth the additional trouble from this perspective alone. Low **S2**-derived TTR–(stilbene)$_{n\leq 2}$ conjugate fluorescence nicely predicts candidate TTR amyloidogenesis inhibitors that exhibit high binding selectivity for serum TTR (stoichiometric binding exceeding 1). In fact, all but one kinetic stabilizer allowing less than 50% maximal fluorescence (the area of Fig. 6B shaded in red) exhibits a binding stoichiometry to plasma TTR exceeding 1. There is also a very good correlation between conjugate fluorescence in the competition assay employing **S2** in serum and the individual efficacy scores of the kinetic stabilizers employed (R^2 = 0.87; Fig. 6C), comparable to the use of **S1** in buffer (R^2 = 0.89; Fig. 5D). That the **S2**-derived fluorescence is very low in the case of the best kinetic stabilizers demonstrates that even if **S2** binds to other serum proteins, such as albumin, the fluorescence of **S2** does not increase because of the internal thioester quenching mechanism discussed above.

While the fluorescent conjugate competition assays employing serum TTR and **S2** or recombinant TTR and **S1** are largely in agreement, there were differences. For example, compounds **3**, **12**, **15**, **22**, and **23**, apparently bind to other serum proteins, reducing their

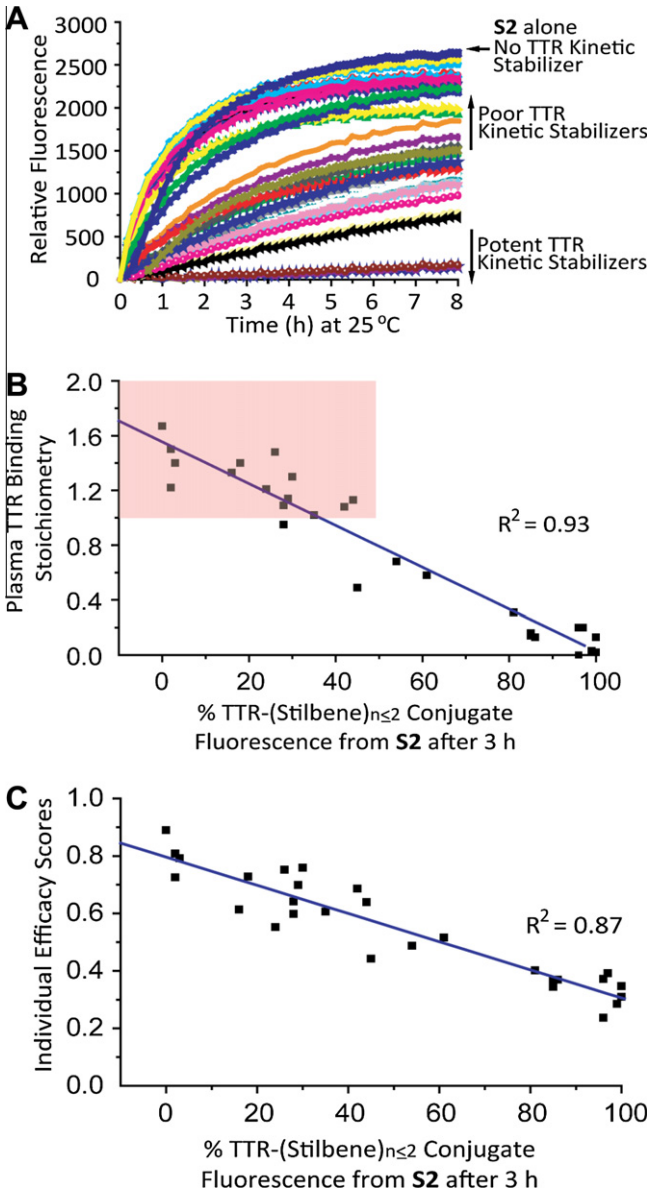

Figure 6. (A) Fluorescence change monitored for 8 h in the presence of candidate non-covalent TTR inhibitors and latent fluorogenic probe **S2** in human serum. (B) Linear correlation between the extent of TTR–$(stilbene)_{n\leqslant 2}$ conjugate fluorescence after a 3 h competition with latent fluorogenic probe **S2** and the previously reported plasma TTR binding stoichiometry data. Data points in the area shaded in red represent the compounds that exhibit a plasma TTR binding stoichiometry exceeding 1. (C) Linear correlation between the extent of TTR–$(stilbene)_{n\leqslant 2}$ conjugate fluorescence derived from **S2** after a 3 h competition and the previously reported individual efficacy scores of kinetic stabilizers **1**–**28**.

competitiveness with the covalent TTR modifier **S2** (Table 1, cf. last two columns, rank ordering of candidate kinetic stabilizers in the distinct experimental paradigms in parentheses). If a single screen were to be done to search for novel TTR kinetic stabilizers, we would suggest using the serum competition assay with **S2,** as this eliminates compounds that bind to other serum proteins.

3. Conclusion

We have developed easily executed TTR fluorescent conjugate competition assays to discover potent and selective non-covalent inhibitors of TTR amyloidogenesis. These plate reader-based assays utilize covalent modifiers of TTR that are non-fluorescent when bound to TTR, but become fluorescent when they chemoselectively react with the Lys-15 residue of TTR. Non-covalent candidate TTR kinetic stabilizers allowing less than 50% of maximum TTR–$(stilbene)_{n\leqslant 2}$ conjugate fluorescence after a 3 h competition with TTR modifiers **S1** in buffer or **S2** in serum are promising kinetic stabilizers. The very good correlation between individual efficacy scores and TTR–$(stilbene)_{n\leqslant 2}$ conjugate fluorescence in these competition assays strongly suggests that the competition assays reported herein will be useful for discovering new kinetic stabilizers and for carrying out structure–activity relationship studies, particularly in the context of human serum. These assays appear to be poised to identify TTR kinetic stabilizers either in buffer or in serum without having to resort to the acid-mediated fibril formation assay and the plasma TTR binding stoichiometry measurements used previously—which were historically integrated to create individual efficacy scores for each compound. With regard to reducing these assays to a high throughput screen to discover TTR kinetic stabilizers, the correlation shown in Figure 6C between individual efficacy scores and TTR–$(stilbene)_{n\leqslant 2}$ conjugate fluorescence suggests that using **S2** in serum seems ideal for this purpose. Utilizing **S2** in serum eliminates kinetic stabilizers that appear promising in the recombinant TTR assay in buffer, but prove to bind to other serum proteins.

4. Experimental section

4.1. Synthesis of compound Amide 3

4.1.1. General synthetic methods

^1H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz spectrometer. Chemical shifts are reported relative to internal $CDCl_3$ (Me_4Si, δ 0.0) and DMSO-d_6 (δ 2.50 for ^1H and δ 39.52 for ^{13}C). Reverse phase high performance liquid chromatography (RP-HPLC) was performed on a Waters 600E multi-solvent delivery system, using a Waters 486 tunable absorbance detector, a 717 autosampler, employing a ThermoHypersil Keystone Betabasic-18 column (150 Å pore size, 3 μm particle size). The 'A' mobile phase comprises 0.1% TFA in 94.9% H_2O + 5% CH_3CN and the 'B' mobile phase is made up of 0.1% TFA in 94.9% CH_3CN + 5% H_2O. Final compound purities were determined by analytical RP-HPLC and were >95% in purity. Mass spectrometry data was collected at The Scripps Research Institute Center for Mass Spectrometry.

4.1.2. (*E*)-3-(4-Hydroxy-3,5-dimethylstyryl)-*N*-propylbenzamide (Amide 3)

To a solution of (*E*)-3-(4-(methoxymethoxy)-3,5-dimethylstyryl)benzoic acid (0.1 g, 0.32 mmol),[80] EDC (0.125 g, 0.64 mmol), HOAT (87.1 mg, 0.64 mmol), and DMAP (7.8 mg, 0.064 mmol) in 2 mL of DMF was added propylamine (54 μL, 0.64 mmol) at 25 °C. The reaction mixture was stirred overnight and diluted with EtOAc. The solution was washed with brine and dried with Na_2SO_4. The solution was filtered and concentrated to get crude (*E*)-3-(4-(methoxymethoxy)-3,5-dimethylstyryl)-*N*-propylbenzamide.

4.1.2.1. MOM deprotection. To a solution of crude (*E*)-3-(4-(methoxymethoxy)-3,5-dimethylstyryl)-*N*-propylbenzamide in 2 mL of THF and 1 mL of MeOH was added 0.5 mL of concentrated HCl. The reaction mixture was refluxed overnight and was diluted in EtOAc. The organic layer was washed with brine and dried with Na_2SO_4. The organic layer was filtered and concentrated. The residue was subjected to chromatography over silica gel (hexanes/EtOAc = 2.5/1) to **Amide 3** (97 mg, 98% in two steps). ^1H NMR (500 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.57 (dd, J = 7.8, 7.8 Hz, 2H), 7.38 (dd, J = 7.7, 7.7 Hz, 1H), 7.16 (s, 2H), 7.06 (d, J = 16.3 Hz, 1H), 6.95 (d, J = 16.3 Hz, 1H), 6.16 (s, 1H), 4.84 (s, 1H), 3.44 (m, 2H), 2.28 (s, 6H), 1.66 (m, 2H), 1.01 (t, J = 7.4 Hz, 3H); ^{13}C NMR (125 MHz,

$CDCl_3$) δ 167.68, 152.46, 138.35, 135.24, 129.74, 129.10, 128.89, 128.76, 127.05, 125.03, 124.97, 124.69, 123.40, 41.81, 22.93, 15.99, 11.45; ESI-MS: *m/z* (MH^+): 310.1801 (calcd), 310.1806 (found).

4.2. Fluorescent conjugate formation

WT-TTR was expressed and purified from an *E. coli* expression system as described previously.[79] The covalent modifiers (**S1** or **S2**) or the non-covalent TTR ligand **Amide 3** (5 μL of a 1.44 mM stock solution in DMSO, final concentration: 7.2 μM) were added to 1 mL of a solution of WT-TTR (0.2 mg/mL, final concentration: 3.6 μM) in 10 mM phosphate, 100 mM KCl and 1 mM EDTA (pH 7.0) in a 2 mL Eppendorf tube. The samples were vortexed, and incubated for 24 h at 25 °C. The fluorescence changes were monitored using a Varian Cary 50 spectrofluorometer at 20 °C in a 1 cm path length quartz cell. The excitation slits was set at 5 nm and the emission slits was set at 10 nm. The samples were excited at 328 nm and the emission spectra were collected from 330 to 550 nm (Fig. 2). The time-dependent fluorescence changes were monitored in similar manner (Fig. 3).

4.3. TTR fluorescent conjugate

One microliter of a candidate kinetic stabilizer/amyloidogenesis inhibitor (0.72 mM stock solution in DMSO, final concentration: 7.2 μM) was added to 100 μL of recombinant WT-TTR (3.6 μM) in 10 mM sodium phosphate, 100 mM KCl and 1 mM EDTA (pH 7.0) in a 96-well plate (Costar black, clear bottom). The plate was sealed and vortexed slowly for 4 h at 25 °C and then, 1 μL of covalent TTR modifier **S1** (0.72 mM stock solution in DMSO, final concentration: 7.2 μM) was added to each well. The fluorescence changes were monitored every 10 min using a microplate spectrophotometer reader (Gemini SpectraMax®, Molecular Devices, Sunnyvale, CA) for 8 h at 37 °C. The fluorescence (λ_{ex} = 328 nm and λ_{em} = 384 nm) was measured from the bottom of the plate without shaking.

For the competition assay with human serum, 100 μL of human serum (Sigma–Aldrich), 1 μL of test compounds (1.5 mM stock solution in DMSO, final concentration: 15 μM), and 1 μL of covalent fluorogenic modifier **S2** (0.5 mM stock solution in DMSO: final concentration: 5 μM) were employed. The fluorescence changes every 10 min were monitored using a microplate spectrophotometer reader (Gemini SpectraMax®, Molecular Devices, Sunnyvale, CA) for 8 h at 25 °C.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.12.050.

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