

Toward Optimization of the Second Aryl Substructure Common to Transthyretin Amyloidogenesis Inhibitors Using Biochemical and Structural Studies[†]

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Transthyretin (TTR) amyloidogenesis inhibitors are typically composed of two aromatic rings and a linker. We have previously established optimal structures for one aromatic ring and the linker. Herein, we employ a suboptimal linker and an optimal aryl-*X* substructure to rank order the desirability of aryl-*Z* substructures—using a library of 56 *N*-(3,5-dibromo-4-hydroxyphenyl)benzamides. Coconsideration of amyloid inhibition potency and ex vivo plasma TTR binding selectivity data reveal that 2,6, 2,5, 2, 3,4,5, and 3,5 substituted aryls bearing small substituents generate the most potent and selective inhibitors, in descending order. These benzamides generally lack undesirable thyroid hormone receptor binding and COX-1 inhibition activity. Three high-resolution TTR•inhibitor crystal structures (1.31–1.35 Å) provide insight into why these inhibitors are potent and selective, enabling future structure-based design of TTR kinetic stabilizers.

Introduction

An aging-associated decline in proteostasis capacity can lead to aggregation-linked gain-of-toxic-function protein misfolding diseases such as the amyloidoses, especially when proteome maintenance is further challenged by the inheritance of mutant misfolding-prone proteins or by environmental factors.^{1–6} Transthyretin (TTR^a) is one of more than 30 human amyloidogenic proteins whose misfolding and/or misassembly into a variety of aggregate structures, including cross- β -sheet amyloid fibrils, appears to cause proteotoxicity.^{7–11} What the TTR toxic structures are and how toxicity arises are key unanswered questions.

To become amyloidogenic outside the cell, tetrameric TTR must first undergo rate-limiting dissociation, allowing the resulting monomers to partially unfold and misassemble.⁹ Another possibility is that TTR amyloidogenesis competes with folding and TTR tetramerization within the cellular secretory pathway, leading to intracellular proteotoxicity. Thus, proteotoxicity could have its origins both within and outside the cell and this issue remains to be resolved. Aggregation of wild-type transthyretin (WT-TTR), and the resulting proteotoxicity appears to cause senile systemic amyloidosis (SSA), a cardiac disease affecting up to $\approx 15\%$ of the population over age 65.^{9,12–14} Deposition of the V122I-TTR variant leads to familial amyloid cardiomyopathy (FAC) in up to 4% of African Americans carrying at least one V122I-TTR allele, while amyloid-associated cardiomyopathy linked to the proteotoxicity arising from other TTR variant aggregates has a lower penetrance.^{15,16} Amyloidogenesis of V30M-TTR, or the aggregation of one of

nearly 100 other rarer TTR mutations leads to familial amyloid polyneuropathy (FAP), usually presenting with peripheral neuropathy and sometimes autonomic and organ system involvement.¹⁷ The much rarer central nervous system selective amyloidoses (CNSA) result from deposition of highly destabilized TTR variants (e.g., D18G and A25T) in the brain, but not in the periphery. This is because the liver, which secretes TTR into the blood, detects these variants as misfolding prone and degrades them, unlike the choroid plexus, which is a more permissive secretor of misfolding-prone variants into the brain.^{18–24} Without treatment, the TTR amyloidoses are fatal.

The only currently accepted therapeutic strategy to ameliorate FAP is gene therapy mediated by liver transplantation, wherein an FAP-associated mutant TTR/WT-TTR liver is replaced by a WT-TTR/WT-TTR secreting liver, eliminating the presence of amyloidogenic mutant TTR in the blood.^{25–27} Unfortunately, WT-TTR deposition often continues post-transplantation in the heart, leading to cardiomyopathy, consistent with the hypothesis that an age-dependent decline in proteostasis contributes to the etiology of the TTR amyloidoses.^{1,28} Because liver transplantation must be performed early in the course of the disease to be effective, and owing to the shortage of livers, the expense associated with transplantation, and the requirement for life-long immune suppression, a generally applicable, oral small molecule therapeutic strategy for all the TTR-based amyloid diseases is highly desirable.^{9,29,30}

Transthyretin transports the *holo*-retinol binding protein complex and thyroxine (T₄) in the blood.^{9,31–34} The four 127-amino acid β -sheet rich subunits of transthyretin create two distinct dimer–dimer interfaces.^{9,35,36} The energetically weaker interface forms two concave thyroxine binding pockets. Small molecule binding to only one or both of the T₄ sites differentially stabilizes the tetrameric ground state over the dissociative transition state, imposing kinetic stability by precluding dissociation required for amyloidogenesis.^{9,35–38}

Optimism that such small molecule kinetic stabilizers of TTR^{24,39–47} will be effective against the TTR amyloidoses is warranted based on human genetic evidence. T119M subunit inclusion into tetramers otherwise composed of V30M disease-associated subunits in compound heterozygotes kinetically

[†] Atomic coordinates have been deposited in the RCSB Protein Data Bank (www.pdb.org) and are available under accession codes 3ESN (WT-TTR in complex with **2d**), 3ESO (WT-TTR in complex with **3b**), and 3ESP (WT-TTR in complex with **5d**).

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^a Abbreviations: TTR, transthyretin; COX-1, cyclooxygenase-1; WT, wild type; HBP, halogen binding pocket; PGE₂, prostaglandin E₂; T₄, thyroxine; T₃, triiodothyronine; NSAID, nonsteroidal anti-inflammatory drug.

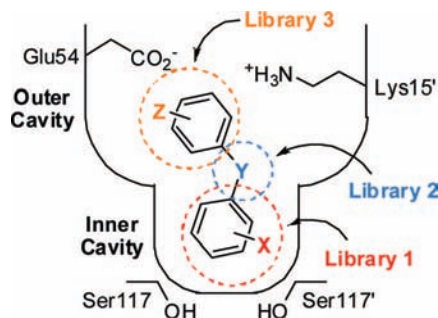


Figure 1. Schematic depiction of one T₄ binding pocket occupied by a typical small molecule TTR aggregation inhibitor. Y represents a linker of variable chemical structure (e.g., NH, O, CH=CH, C(O)NH, etc.) joining the two aryl rings, which typically bear a combination of alkyl, carboxyl, halide, trifluoromethyl, or hydroxyl substituents (X and Z). Library 3, the focus of this manuscript, explores the SAR of the aryl-Z substructure. While the aryl-X substructure was initially envisioned to bind within the inner cavity of the thyroxine binding site, previous crystallographic data reveal that inhibitors can bind opposite to the expected orientation.^{34,51,52,59–61} Thus, it may not be the case that the aryl-X substructure of an inhibitor will occupy the inner binding cavity as illustrated. For conceptual simplicity in terms of integrating the data from the three inhibitor substructure optimization libraries, this initial schematic has been retained.

stabilizes the tetrameric structure of TTR, preventing amyloidogenesis and ameliorating FAP.^{9,37,48–50}

To increase the probability of identifying TTR kinetic stabilizers with desirable pharmacological properties, three small molecule libraries have recently been designed and synthesized to optimize the three substructures comprising a typical TTR amyloidogenesis inhibitor (Figure 1): the two aromatic rings and the linker joining them.^{51,52} In each library, one fixed substructure was purposefully chosen to be nonoptimal to afford a wider range of TTR amyloidogenesis inhibitor potencies and plasma TTR binding selectivities that are together used to rank order the numerous substructures of interest. Data from the first library, focused on optimization of the aryl-X ring, revealed that thyroid hormone-like aryl substitution patterns (3,5-X₂-4-OH, where X = CH₃, F, Cl, Br, I) lead to potent and highly selective TTR amyloidogenesis inhibitors lacking undesirable thyroid hormone receptor and cyclooxygenase-1 (COX-1) binding activities.⁵¹ The second library, designed to reveal optimal linker substructures, demonstrates that directly linking two aryls, or tethering them through nonpolar *E*-olefin or -CH₂CH₂-substructures, generates the most potent and selective TTR amyloidogenesis inhibitors that also lack the undesirable thyroid hormone and COX-1 activities outlined above.⁵² In this study, we utilize a nonoptimal amide linker and an optimal aryl-X substructure in all library members to rank order candidate aryl-Z substructures. The intention of this three-part series of papers is to generate optimal substructure information that can ultimately be used to predict the structures of potent and selective TTR kinetic stabilizers that lack the thyroid hormone receptor binding capacity and COX-1 inhibition activity contraindicated for cardiomyopathy applications. These activities should increase the probability of finding a TTR kinetic stabilizer with desirable pharmacologic properties going forward.

Herein, we report a 56-member library wherein all members are composed of an optimal 3,5-dibromo-4-hydroxyphenyl aryl-X ring attached to the NH of a nonoptimal secondary amide linker connected to variable aryl-Z rings, which are the subject of this optimization study (Figure 2). Numerous aryl-Z substituents and substitution patterns dramatically increase inhibitor potency and binding selectivity in comparison to the parent

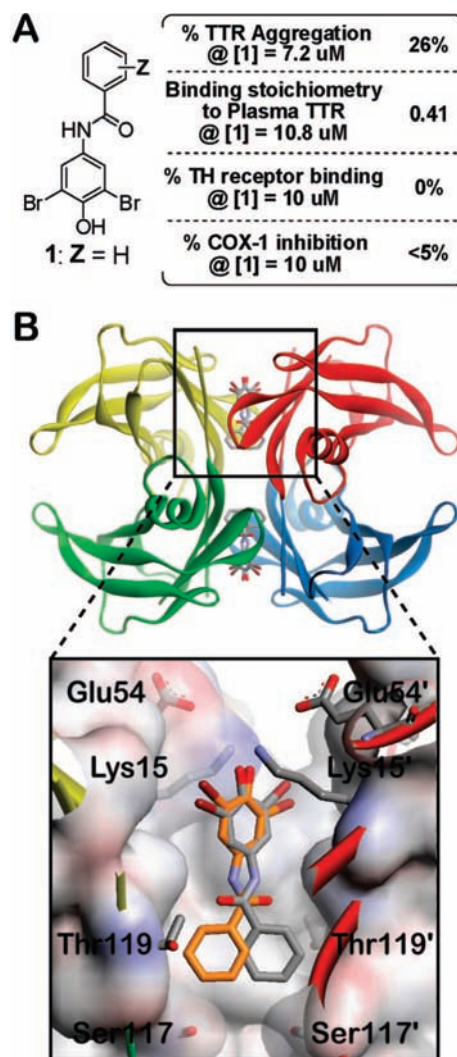


Figure 2. Selection of an appropriate molecular scaffold for structure-guided drug design to identify favorable aryl-Z substituents and substitution patterns. (A) *N*-(3,5-Dibromo-4-hydroxyphenyl)benzamide (**1**) was previously found to be a moderately potent and selective inhibitor of TTR amyloidogenesis, allowing 26% aggregation of WT-TTR in vitro (7.2 μ M inhibitor, 3.6 μ M TTR, pH 4.4, 37 $^{\circ}$ C, 72 h) and displaying a stoichiometry of 0.41 equiv bound to TTR in human blood plasma ex vivo.⁵² Importantly, compound **1** does not target the thyroid hormone (TH) receptor or inhibit COX-1 activity. (B) Molecular modeling was performed using the previously determined TTR•(I)₂ crystal structure (PDB accession code 3CN4), in which compound **1** binds in the reverse orientation with its 3,5-dibromo-4-hydroxyphenyl substructure occupying the outer binding site cavity, projecting the aryl-Z ring under evaluation into the halogen binding pockets of the inner cavity.⁵² Individual TTR monomers in the ribbon diagram representation have been color coded red, yellow, green, and blue for differentiation. The zoomed image of one site shows **1** bound in its two symmetry-related binding modes (orange and gray). The protein surface is shown colored by electrostatic potential, with the front portion cut away to allow easier visualization into the pocket.

N-(3,5-dibromo-4-hydroxyphenyl)benzamide inhibitor (**1**), while generally avoiding undesirable binding to the thyroid hormone receptor and COX-1 inhibition. High-resolution X-ray structures of three TTR•inhibitor complexes (1.31–1.35 \AA) rationalizes their potency and selectivity.

Results

Design and Synthesis of the Aryl-Z Optimization Library. In the recently published linker optimization component (part 2) of this three-part study, *N*-(3,5-dibromo-4-

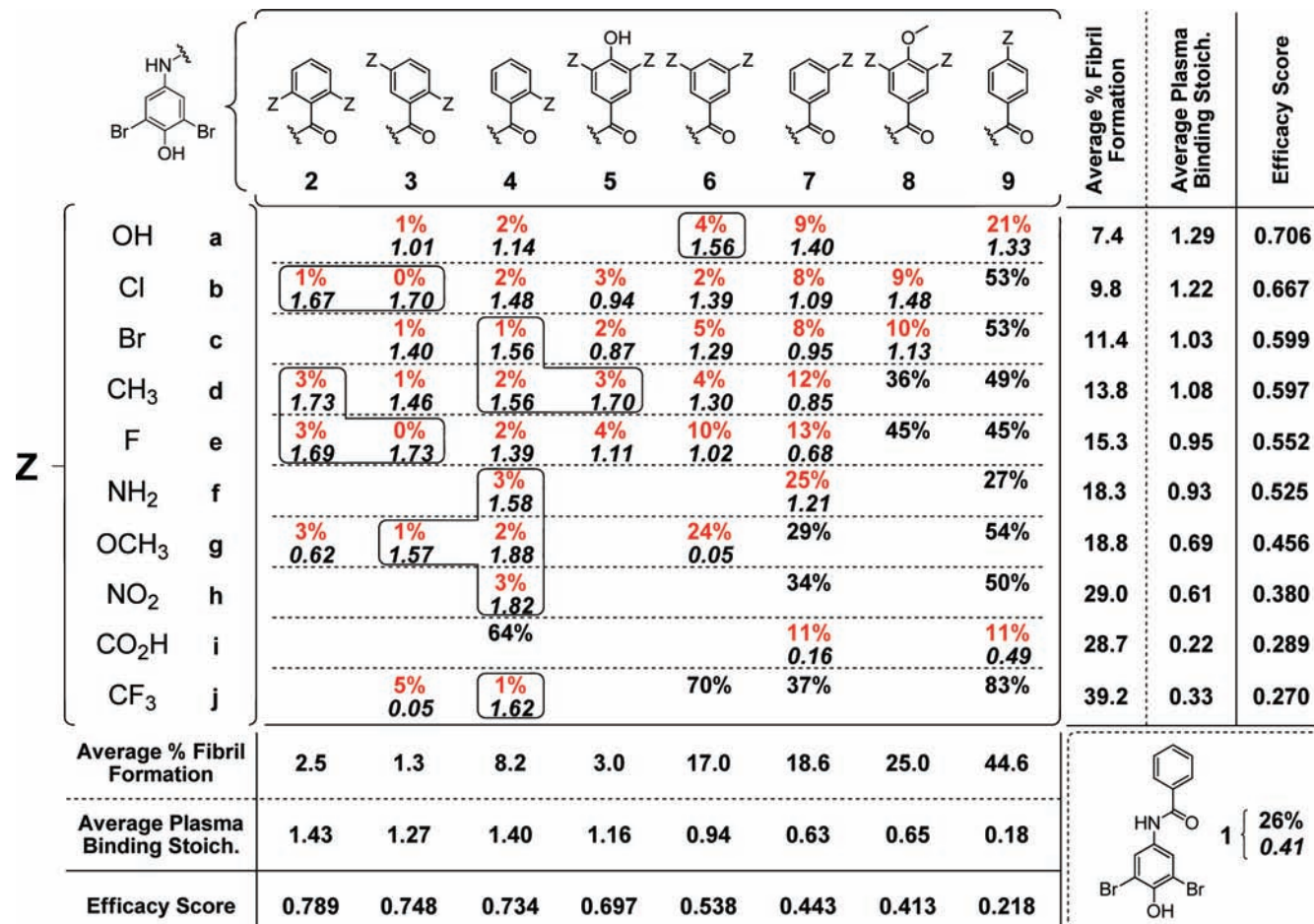


Figure 3. Extent of inhibition of TTR aggregation and stoichiometry of inhibitor bound to TTR in human blood plasma. Percent (%) values represent the extent of in vitro WT-TTR fibril formation in the presence of inhibitor (7.2 μ M inhibitor, 3.6 μ M TTR, pH 4.4, 37 $^{\circ}$ C, 72 h) relative to aggregation in the absence of inhibitor (100%), with the best values shown in red (<25% aggregation; errors are typically less than ± 5 percentage points). The average stoichiometries of the most potent aggregation inhibitors bound to TTR in human blood plasma ex vivo are shown in italics (10.8 μ M inhibitor, 1.8–5.4 μ M TTR; theoretical maximum binding stoichiometry = 2). Those exhibiting exceptional binding selectivity to TTR are boxed (errors are typically less than ± 0.1).

hydroxyphenyl)benzamide (**1**) was established to be a moderately potent and selective transthyretin amyloidogenesis inhibitor (Figure 2A),⁵² lacking contraindicated thyroid hormone receptor binding or COX-1 inhibition properties. The amide linker is isostructural with the most potent 2-arylbenzoxazole and E-olefin linkers, but is nonoptimal because its polarity is poorly matched with the hydrophobic properties of the TTR subsite that surrounds the linker. Thus the bisarylamide library described within to optimize the aryl-Z ring should prove to be highly relevant, especially considering that all library members are made up of an optimized aryl-X ring identified in the first paper in this series.⁵¹

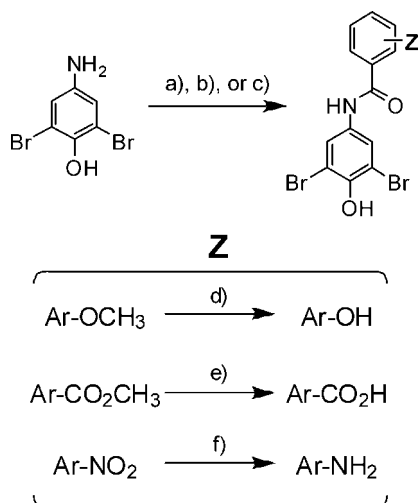
The TTR·(**1**)₂ crystal structure (PDB accession code 3CN4) solved previously⁵² enables structure-based design principles to be used to identify favorable aryl-Z substituents and substitution patterns, thus enhancing the probability for success. Compound **1** binds such that the 3,5-Br₂-4-hydroxyphenyl substructure (having a calculated pK_a of 6.5) occupies the outer thyroxine binding site (true of nearly all low pK_a phenols structurally characterized to date) where the phenolate can interact with the Lys-15 and 15' side chain ϵ -NH₃⁺ groups (Figure 2B).^{51,52} This orientation projects the aryl-Z ring into the inner cavity, where its substituents can occupy the symmetric hydrophobic depressions referred to as halogen binding pockets 3 and 3' (HBP-3 and 3'). Upon slight repositioning of the aryl-Z ring, it appears that ortho and meta substituents could interact

favorably with HBP-3 and 3'. In addition, polar meta or para substituents (e.g., amino groups or high pK_a phenols) could enhance binding affinity through hydrogen bonding with the Ser-117/117' hydroxyls.

Certain aryl-Z substructures, such as low pK_a phenols, could change the binding orientation, such that the 3,5-Br₂-4-hydroxyphenyl substructure common to all library members now occupies the inner binding cavity. If this were to occur, modeling suggests that the same aryl-Z substructures bearing ortho and meta alkyl and halide substituents could interact favorably with the hydrophobic HBP-1 and 1', while meta or para carboxyl, amino, or phenolic substituents could make electrostatic interactions with the Lys-15 and 15' ϵ -NH₃⁺ groups or the Glu-54 and 54' carboxylate groups.

Using structure-based principles as a rough guideline, a library of 56 bisarylamides was synthesized to evaluate 10 different aryl-Z substituents (**a–j**) in eight distinct substitution patterns (**2–9**) (Figure 3). Coconsideration of amyloid inhibition and ex vivo plasma TTR binding selectivity data using a simple equation allows us to rank order the aryl-Z substructures from most desirable (highest score) to least desirable using an efficacy score (Figure 3; see below).

Synthesis of these bisarylamides was accomplished primarily via coupling of substituted benzoyl chlorides (phthalic anhydride in the case of **4i**) with 4-amino-2,6-dibromophenol (Scheme 1) to form the amide bond. The amide bond coupling reactions

Scheme 1. General Synthesis of the Bisarylamide Inhibitors under Evaluation^a

^a (a) Ar-COCl, THF (20–95%); (b) Ar-CO₂H, SOCl₂, heat, concentrate, then 4-amino-2,6-dibromophenol, THF (9–76%); (c) phthalic anhydride, THF (**4i**, 66%); (d) BBr₃, CH₂Cl₂ (19–87%); (e) LiOH·H₂O, THF, MeOH, H₂O (**7h**, 70%; **9h**, 64%); (f) Sn, HCl/AcOH (**4f**, 93%; **7f**, 41%; **9f**, 86%).

occurred rapidly in THF and, after 1 h of mixing, the resultant bisarylamides were precipitated by dilution with water. In many cases, simple filtration and rinsing of the solid with saturated sodium bicarbonate (no basic rinse in the case of **4i**) resulted in compounds of acceptable purity for biophysical evaluation (>95% purity as determined by RP-HPLC and ¹H NMR). Compounds not meeting this criterion were further purified by column chromatography over silica. Several compounds required further substituent conversions (i.e., anisole or ester deprotection or NO₂ reduction to NH₂), accomplished using standard procedures (refer to Scheme 1 and the Experimental Section and Supporting Information). In some cases, synthesis of the appropriate benzoyl chloride building block was required, accomplished by reacting the commercially available benzoic acids with thionyl chloride at elevated temperatures, followed by concentration in vacuo.

Small Molecule-Mediated Inhibition of WT-TTR Amyloidogenesis. The ability of the bisarylamide library members to inhibit WT-TTR aggregation was evaluated using the acid-mediated aggregation assay used previously to identify optimal aryl-X and linker-Y substructures.^{51,52} Briefly, physiologically relevant concentrations of WT-TTR (3.6 μM) were incubated with a candidate inhibitor (7.2 μM) prior to subjecting the solution to partial denaturation conditions that enable ≈90% of WT-TTR to aggregate in the absence of an inhibitor after 72 h (pH 4.4, 37 °C). The extent of TTR aggregation (Figure 3) was monitored by measuring sample turbidity in the presence of the test compound, expressed as a percentage of TTR aggregation observed in inhibitor-free samples (assigned to be 100% fibril formation). Of the 56 new compounds evaluated, 41 display increased inhibitor potencies relative to the parent compound **1** (<25% fibril formation, values in red). Twenty-eight of the benzamides completely inhibit TTR aggregation (<5% fibril formation) within the 72 h time frame of the assay.

Binding Selectivity of Amyloidogenesis Inhibitors to TTR in Human Blood Plasma. The 41 compounds displaying increased inhibitor potencies relative to **1** (i.e., <25% fibril formation) were further evaluated for their ability to bind selectively to TTR in blood plasma using an ex vivo TTR plasma binding selectivity assay.^{51–53} Briefly, the candidate

inhibitor (10.8 μM) was incubated in human blood plasma in the dark at 37 °C for 24 h. Transthyretin, with any bound inhibitor, was then captured by a resin-conjugated anti-TTR antibody and any unbound material was washed away (including weakly or nonspecifically bound inhibitors). The captured TTR·(inhibitor)_n complex was then dissociated from the antibody under alkaline conditions, and TTR and inhibitor stoichiometry was quantified by RP-HPLC. Results represent the average stoichiometry of inhibitor bound to TTR in blood plasma (Figure 3, lower black italicized values), the maximum value being 2 owing to the presence of the two thyroxine binding sites in each tetramer. Of the 41 inhibitors evaluated, 31 display average binding stoichiometries exceeding 1 equiv bound per TTR tetramer, 14 of which are exceptionally selective, displaying >1.5 equiv bound (boxed values). Six bisarylamides display modest yet likely sufficient binding stoichiometries (0.5–1.0 equiv bound), while the remaining four exhibit minimal binding selectivity to TTR (<0.5 equiv).

Evaluating the Potent TTR Amyloidogenesis Inhibitors for COX-1 Enzymatic Inhibition and Binding to the Thyroid Hormone Nuclear Receptor.

The 41 most potent TTR aggregation inhibitors were further evaluated for their ability to inhibit COX-1 enzymatic activity and for their binding to the thyroid hormone nuclear receptor. These analyses were contracted out to the Cerep laboratories in Redmond, WA (refer to the Experimental Section for a detailed description of the assay protocols).^{51,52,54,55} For the COX-1 inhibition analyses, results represent the % inhibition of arachidonic acid conversion to PGE₂ due to competitive binding of test compound (10 μM) to COX-1 (Figure 4, lower, black values). Of the 41 compounds evaluated, all but four display <5% inhibition of COX-1 activity: compounds **4e**, **4h**, **7i**, and **8c** display slight to modest (10–33%) COX-1 inhibition. For the thyroid hormone receptor binding analyses, the % displacement of [¹²⁵I]-labeled triiodothyronine (T₃, the primary thyroid hormone) was determined from competitive binding of test compound (10 μM) to the thyroid hormone receptor (Figure 4, red, italicized values). Of the 41 compounds evaluated, five display >10% displacement of T₃ from the thyroid hormone nuclear receptor (**3a**, **4a**, **5e**, **6e**, and **7a**), the maximum activity observed being 28% for compound **6e**.

X-ray Crystallographic Analysis of WT-TTR Bound to Inhibitors 2d, 3b, and 5d. Crystal structures of compounds **2d**, **3b**, and **5d** in complex with WT-TTR were determined to 1.35, 1.31, and 1.35 Å resolution, respectively (Table 1). In all three structures, the observed electron density allowed unambiguous placement of the kinetic stabilizer. The 3,5-Br₂-4-hydroxyphenyl substructure in **2d**, **3b**, and **5d** bound in the outer thyroid hormone binding pocket, as was the case for the parent TTR·(**1**)₂ structure (Figure 5).⁵² As anticipated, the bromine substituents extend into HBP-1 and 1', while the putative phenolate makes electrostatic interactions with the Lys-15 and 15' ε-NH₃⁺ groups. In all cases, at least one of the aryl-Z substituents of **2d**, **3b**, and **5d** positioned in the inner binding cavity projected their methyl or chloro substituents into HBP-3 and 3'.

In the **2d** and **3b** structures, an ordered water molecule is located between the Ser-117 hydroxyls of each T₄ binding site, which is not observed in the TTR·(**1**)₂ structure (Figure 5, red dot(s)). In the TTR·(**5d**)₂ structure, this ordered water molecule is absent and instead the 4-phenolic substituent creates a hydrogen bonding network that bridges the Ser-117 and 117' hydroxyls on adjacent TTR subunits. While additional ordered

Figure 4 shows the chemical structures of compounds 2 through 9, which are variations of a parent ligand. The parent ligand is a 3,5-dibromo-4-hydroxyphenyl ring with an amide group. The variations are defined by the substituent Z on the aryl ring. The table below provides data on the displacement of T₃ from the thyroid hormone receptor and the inhibition of COX-1 by these compounds.

Z	Substituent	2	3	4	5	6	7	8	9
OH	a	13% <5%	11% <5%			0% <5%	13% <5%		8% <5%
Cl	b	3% <5%	5% <5%	3% <5%	0% <5%	3% <5%	5% <5%	10% <5%	
Br	c		0% <5%	8% <5%	0% <5%	6% <5%	6% <5%	0% 23%	
CH ₃	d	6% <5%	1% <5%	1% <5%	5% <5%	4% <5%	2% <5%		
F	e	7% <5%	0% <5%	0% 33%	21% <5%	28% <5%	3% <5%		
NH ₂	f			6% <5%			0% <5%		
OCH ₃	g	1% <5%	1% <5%	8% <5%		0% <5%			
NO ₂	h			2% 10%					
CO ₂ H	i						0% 17%		2% <5%
CF ₃	j		2% <5%	7% <5%					

Figure 4. Thyroid hormone receptor T₃ displacement and COX-1 inhibition for the most potent aggregation inhibitors (those displaying <25% aggregation). The extent that the test compounds (10 μ M) competitively bind to the thyroid hormone receptor and displace T₃ is shown in red italics (errors are typically less than ± 2 percentage points). COX-1 inhibition results are shown below in black, with values representing the % inhibition by the test compounds (10 μ M) of COX-1-mediated conversion of arachidonic acid to prostaglandin-E₂ (errors are typically less than ± 6 percentage points).

water molecules have been modeled elsewhere, these do not make significant interactions with any of the bound inhibitors.

Taking the positioning of the parent ligand in the TTR•(1)₂ cocrystal structure as a reference, compound **2d** appears to bind ~ 0.4 Å deeper within the pocket, while compounds **3b** and **5d** bind ~ 0.3 Å and ~ 1.4 Å shallower, respectively. The noncoplanarity of the aryl-X and aryl-Z rings observed in the TTR•(1)₂ cocrystal structure was also observed for these new kinetic stabilizers (46–51° for **2d**, 41–44° for **3b**, and 43–46° for **5d**, compared to 42–45° for **1**).

Discussion

Optimal Aryl-Z Substructures for Creating Potent TTR Amyloidogenesis Inhibitors that also Exhibit High TTR Binding Selectivity in Blood. To use the previously employed substructure ranking calculation⁵² that coconsiders inhibition efficacy and plasma TTR binding selectivity, we first averaged the percent fibril formation (% FF_{ave}) and plasma TTR binding stoichiometry (PS_{ave}) values across the columns, reflecting different substitution patterns, and across rows, reflecting the influence of a given substituent in several contexts. Compounds that were not tested in the plasma selectivity assay because they allowed >25% aggregation of TTR were assigned plasma stoichiometry values of 0 for averaging purposes, as experience demonstrates that poor aggregation inhibitors typically display little, if any, binding to TTR in blood plasma. To obtain an “efficacy score” for each aryl-Z substituent and substitution pattern, the % FF_{ave} and PS_{ave} values were then input into eq 1.

$$\text{efficacy score} = \frac{(100\% - \%FF_{ave}) \times (1 + PS_{ave})}{300\%} \quad (1)$$

Subtraction of the % FF_{ave} values from 100% converts the data

into % inhibition. The PS_{ave} values are adjusted by adding 1 in order to be able to differentiate inhibitors with plasma binding stoichiometries of 0 from each other based on % inhibition data. Division by 300% restricts the efficacy scores to a range between 0 and 1, the latter being the best score. Coconsideration of efficacy and binding selectivity using eq 1 affords clear rank-orderings of the different aryl-Z substituents and substitution patterns (Figure 3). Nearly all of the 2,6, 2,5, 2,3,4,5, and 3,5 substitution patterns afforded excellent TTR amyloidogenesis inhibitors that exhibited selective binding to TTR in plasma ex vivo, in descending order. The hydroxy, Cl, Br, CH₃, F, and NH₂ substituents generally afforded excellent TTR amyloidogenesis inhibitors that also exhibited selective TTR binding in plasma, in descending order. Strictly analogous ranking of aryl-X substituents and substitution patterns as well as optimal linkers now allows highly efficacious and selective inhibitor structures to be predicted.

Generally the Most Potent and Selective TTR Amyloidogenesis Inhibitors Interact Minimally with the COX-1 Enzyme and the Thyroid Hormone Receptor. Because many of the potent TTR amyloidogenesis inhibitors in this aryl-Z optimization library bear resemblance to some common nonsteroidal anti-inflammatory drugs (NSAIDs), their ability to inhibit the COX-1 enzyme was evaluated. COX-1 binding is undesirable, especially for familial amyloid cardiomyopathy patients, as NSAID activity can further compromise renal blood flow.⁵⁶ Of the 41 compounds evaluated, four display some COX-1 inhibition (**4e**, **4h**, **7i**, and **8c**).

Because these compounds all contain the thyroid hormone-like 3,5-Br₂-4-OH aryl-X ring, we evaluated the 41 most potent kinetic stabilizers for their ability to bind to the thyroid hormone nuclear receptor. Five compounds, the majority bearing OH or F substituents in distinct substitution patterns, displace >10% of T₃ from the thyroid hormone receptor (**3a**, **4a**, **5e**, **6e**, and

Table 1. Data Collection and Refinement Statistics for the Crystal Structures of WT-TTR in Complex with Inhibitors **2d**, **3b**, and **5d**

	WT-TTR/ 2d	WT-TTR/ 3b	WT-TTR/ 5d
data collection			
beamline	SSRL 9–2	SSRL 9–2	SSRL 9–2
wavelength (Å)	0.9795	0.9795	0.9795
resolution (Å)	1.35 (1.35–1.40) ^a	1.31 (1.31–1.36)	1.35 (1.35–1.36)
space group	<i>P</i> 2 ₁ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	42.65, 84.62, 64.94	42.50, 85.00, 64.74	42.58, 85.34, 64.28
no. of molecules in a.u.	2	2	2
no. of observations	346443 (22334) ^a	353372 (16464)	358657 (16884)
no. of unique reflections	51708 (4558) ^a	54365 (3829)	55178 (4221)
redundancy	6.7 (64.9) ^a	6.5 (4.3)	6.5 (4.0)
completeness (%)	98.4 (88.1) ^a	95.1 (68.0)	96.4 (75.1)
<i>R</i> _{sym} (%) ^b	3.7 (50.1) ^a	3.4 (45.2)	4.0 (33.1)
average <i>I</i> /σ	38.3 (2.3) ^a	39.1 (2.3)	36.6 (3.6)
refinement statistics			
resolution (Å)	64.96–1.35	64.68–1.31	64.28–1.31
no. of reflections (working set)	49039 (3100) ^a	51574 (2573)	52337 (2930)
no. of reflections (test set)	2629 (173) ^a	2744 (142)	2794 (156)
<i>R</i> _{cryst} (%) ^{a, c}	17.3 ^{a, c}	17.7	17.7
<i>R</i> _{free} (%) ^{a, d}	18.8 ^{a, d}	20.4	20.0
no. of protein/ligand/water atoms	891884/38/215	891884/38/208	891884/44/209
average <i>B</i> values			
protein	16.6	15.2	14.9
ligand	19.0	19.3	19.4
Wilson <i>B</i> value	20.8	18.4	17.5
Ramachandran plot			
most favored (%)	92.1	92.1	92.1
additionally allowed (%)	7.9	7.9	7.9
generously allowed (%)	0	0	0
disallowed (%)	0	0	0
rms deviations			
bond lengths (Å)	0.018	0.018	0.019
angles (deg)	1.73	1.78	1.84

^a Numbers in parentheses are for highest resolution shell of data. ^b $R_{\text{sym}} = \sum_{\text{hkl}} \|I - \langle I \rangle\| / \sum_{\text{hkl}} I$. ^c $R_{\text{cryst}} = \sum_{\text{hkl}} \|F_o - F_c\| / \sum_{\text{hkl}} F_o$. ^d R_{free} is the same as R_{cryst} but for 5% of data excluded from the refinement.

7a). The random distribution of substitution patterns suggests that this undesirable characteristic could be prevented by employing different linkers and by minimizing the use of the 3,5-Br₂-4-OH aryl-*X* ring.⁵² It might be prudent to pursue future inhibitors bearing hydroxyl and fluoro substituents in *meta* positions with caution.

Structural Characterization of Potent and Selective TTR Amyloidogenesis Inhibitors with Optimal Aryl-Z Substitution Patterns. Structures of kinetic stabilizers **2d**, **3b**, and **5d** in complex with WT-TTR (Figure 5) support the hypothesis that low *pK_a* phenols, including 3,5-Br₂-4-hydroxyphenyl substructures, bind in the outer cavity of the thyroxine binding site where their phenolates interact with the Lys-15 and 15' side chain ε-NH₃⁺ groups, whereas high *pK_a* phenols like 3,5-(CH₃)₂-4-hydroxyphenyl prefer to occupy the inner binding cavity, H-bonding with the Ser-117 and 117' hydroxyl groups.^{51,52} That **5d** is one of the most potent and selective TTR amyloidogenesis inhibitors discovered to date is not surprising, as it incorporates both preferred subsite binding aryls. On the basis of previous structural data^{51,52} and that obtained herein, it is likely that the remaining compounds bind with their 3,5-Br₂-4-OH aryl ring occupying the outer binding cavity.

Even though the aryl-Z substituents under evaluation are fairly diverse, every one is capable of increasing inhibitor potency and selectivity if displayed in the proper substitution pattern. Bisarylamides bearing ortho aryl-Z substituents are generally more potent than their counterparts bearing meta and especially para substituents, almost independent of the nature of the substituent. Consistent with previously published TTR•(inhibitor)₂ X-ray cocrystal structures, the three structures determined

in this study reveal that the aryl-*X* and aryl-*Z* rings are oriented out-of-plane with respect to one another.^{9,34,38,57–62} With ortho substituents, unbound inhibitors may have a steric predisposition to the nonplanar bound conformations, thereby displaying more favorable binding energetics.

Para-substitution, reflected in the **8** and **9** series, generally exhibits decreased potency and selectivity, likely due to the inability of these aryl-*Z* rings to engage the HBP and because of steric clashing with the Ser-117/117' side chains when positioned in the inner binding cavity. The inability of many of these substituents to hydrogen bond with the Ser-117/117' hydroxyls in the inner binding cavity or the Lys-15/15' ε-NH₃⁺ and/or Glu-54/54' carboxyl groups when positioned in the outer binding cavity could also reduce ligand binding affinity and thus inhibitor potency. This hypothesis is consistent with the observation that hydrogen bonding capable substituents such as phenols (**9a** and **5b–e**) and carboxyls (**9i**) are well tolerated in this position.

It is clear that alkyl and halide substituents on the aryl-*Z* ring produce the most potent and selective inhibitors, likely owing to their hydrophobic and charge transfer interactions with the halogen binding pockets, as described in more detail in the first study and elsewhere.^{51,63,64} The hydroxyl substituent on the aryl-*Z* ring also contributes to high potency and selectivity over a range of substitution patterns, likely owing in large part to its ability to hydrogen bond.

Concluding Remarks

The systematic optimization of the three substructures comprising a typical transthyretin kinetic stabilizer (i.e., the two

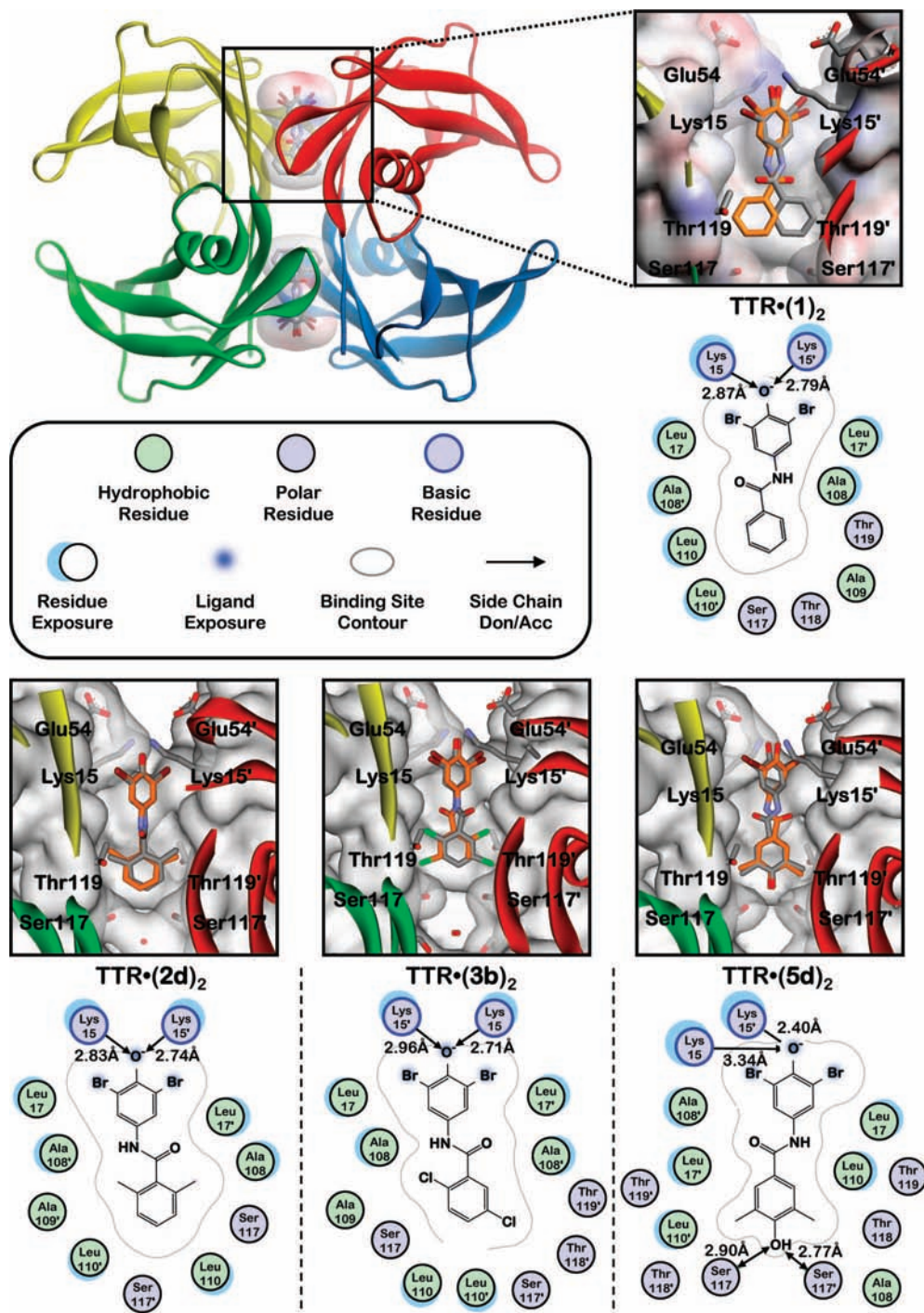


Figure 5. X-ray crystallography structures and schematic representations of key features of homotetrameric WT-TTR cocrystallized with inhibitors **1**, **2d**, **3b**, and **5d** (PDB accession codes 3CN4,⁵² 3ESN, 3ESO, and 3ESP, respectively). The zoomed images show the respective inhibitors bound in one binding site in their two symmetry-related binding modes (orange and gray), with the protein surface shown colored by electrostatic potential (nb: the front portion has been cut away to allow easier visualization into the pocket). Schematic representations of each bound inhibitor are presented as two-dimensional topology diagrams (generated using MOE (2006.08), Chemical Computing Group, Montreal, Canada). Inhibitors are shown in only one of their two symmetry-related binding modes for clarity. Important hydrogen bonding and electrostatic interactions between protein side chains and the ligands are indicated by arrows, with distances shown in Å. A graphical legend for interpretation of key binding site characteristics is displayed below the TTR tetramer ribbon diagram. Ligand exposure indicates specific portions of the ligand structures that are solvent accessible (i.e., not completely buried within the binding pocket). Residue exposure indicates those amino acids whose side chains and/or peptide backbones are partially solvent accessible.

aromatic rings and the linker joining them) has enabled the rank ordering of substructures that can be combined to produce potent and selective inhibitors. Because most of these TTR kinetic stabilizers are devoid of thyroid hormone receptor binding and COX-1 inhibition activity, it is likely that kinetic stabilizers derived from combinations of the best aryl and linker substructures

will be as well, mitigating toxicity in the envisioned long-term use of these drugs.^{51,52}

The high-resolution X-ray crystallographic data obtained from this three-part series supports the hypothesis that thyroid-hormone-like, 3,5-*X*-4-hydroxyphenyl aryl substructures (*X* = halide) prefer to bind in the outer binding site when combined

with most other substituted aromatics, including phenols exhibiting high pK_a s.^{51,52} These structural insights enable us to move beyond simply screening collections of compounds to now being able to confidently employ structure-based drug design for the development of diverse, potent, and highly selective transthyretin amyloidogenesis inhibitors. Use of the structural data, along with the rankings of the three substructural elements enabled by this three-part series, is currently motivating the synthesis of inhibitors that should be exceptionally potent and selective and thus potential clinical candidates. Upon the identification of clinical candidates, it will be highly desirable to evaluate their cellular and organismal toxicity.

Experimental Section

General Synthetic Methods. Unless otherwise stated, all chemicals were purchased from commercial suppliers and used without further purification. Reaction progress was monitored by thin-layer chromatography on silica gel 60 F254 coated glass plates (EM Sciences). Flash chromatography was performed using 230–400 mesh silica gel 60 (EM Sciences). NMR spectra were recorded on a Bruker 500 MHz spectrometer. ¹H NMR spectra were calibrated to the DMSO-*d*₆ solvent peak at 2.49 ppm. Reverse phase high performance liquid chromatography (RP-HPLC) was performed using a Waters 600 E multisolvent delivery system employing a Waters 486 tunable absorbance detector and 717 autosampler. Samples were chromatographically separated using a ThermoHypersil-Keystone Betabasic-18 column (model 71503-034630, 150 Å pore size, 3 µm particle size), eluting with a H₂O:CH₃CN gradient solvent system. Linear gradients were run from either 100:0, 80:20, or 60:40 A:B to 0:100 A:B (A = 95:5 H₂O:CH₃CN, 0.25% trifluoroacetic acid (TFA); B = 5:95 H₂O:CH₃CN, 0.25% TFA). Final compound purities were additionally evaluated under distinct RP-HPLC conditions by chromatographically separating samples using a Vydac-C₄ column (model 214TP5415, 300 Å pore size, 5 µm particle size), eluting with a H₂O:MeOH gradient solvent system. Linear gradients were run from 100:0 to 0:100 C:D (C = 99.75% H₂O, 0.25% TFA; D = 100% MeOH). Representative synthetic procedures are presented below, while experimental details for compounds synthesized analogously are presented in the Supporting Information.

Representative Amide Coupling Procedures: Method A. 4-Amino-2,6-dibromophenol (~0.3–0.7 mmol, 1.0 equiv) was mixed with the respective substituted benzoyl chloride (~0.3–0.7 mmol, ~1–1.2 equiv) in THF (~0.5–2.0 mL) at ambient temperature. After ~1 h, the reaction mixtures were diluted with H₂O (~20 mL), sonicated, and the resulting precipitates were filtered, rinsed with H₂O, collected, sonicated with sat. NaHCO₃ (~10 mL), filtered, rinsed with H₂O, and collected. When necessary, compounds were further purified by flash chromatography over silica, employing a hexanes:EtOAc elution system. All compounds were characterized by ¹H NMR and RP-HPLC and were >95% in purity. See the Supporting Information for specific synthetic procedures and characterization data.

Representative Amide Coupling Procedures: Method B. Substituted benzoic acids (~0.3–0.7 mmol, ~1–1.2 equiv) were stirred in SOCl₂ (~2.0 mL) at ~70–80 °C. After ~1 h, the reactions were concentrated and then the resulting acid chlorides were mixed with 4-amino-2,6-dibromophenol (~0.3–0.7 mmol, 1.0 equiv) in THF (~0.5–2.0 mL) at ambient temperature. After ~1 h, the reaction mixtures were diluted with H₂O (~20 mL), sonicated, and the resulting precipitates were filtered, rinsed with H₂O, collected, sonicated with sat. NaHCO₃ (~10 mL), filtered, rinsed with H₂O, and collected. When necessary, compounds were further purified by flash chromatography over silica, employing a hexanes:EtOAc elution system. All compounds were characterized by ¹H NMR and RP-HPLC and were >95% in purity. See the Supporting Information for specific synthetic procedures and characterization data.

Representative Anisole Deprotection Procedures. Boron tribromide (0.5–3.5 mmol of 1 M BBr₃ in hexanes, 5–10 equiv)

was added to a stirring mixture of the respective anisole (0.1–0.35 mmol, 1 equiv) in anhydrous CH₂Cl₂ (5–10 mL), and the reaction was stirred at room temperature under an argon atmosphere. After 18 h, the reaction was quenched with MeOH (5 mL), extracted into EtOAc (50 mL), and washed with 1 N HCl (25 mL), sat. NaHCO₃ (25 mL), and brine (25 mL). The organics were then dried over Na₂SO₄, filtered, and concentrated. Compounds were purified by flash chromatography over silica, employing a hexanes:EtOAc elution system. All compounds were characterized by ¹H NMR and RP-HPLC and were >95% in purity. See the Supporting Information for specific synthetic procedures and characterization data.

Representative Methyl Ester Hydrolysis Procedures. LiOH·H₂O (0.5–0.85 mmol, ~4 equiv) was added to a stirring mixture of the respective methyl ester (0.13–0.2 mmol, 1 equiv) in H₂O/MeOH/THF (0.5/0.5/1.5 mL), and the reaction was stirred at room temperature. After 18 h, the reaction was acidified with 1 N HCl and extracted into EtOAc (50 mL), washed with H₂O (2 × 25 mL) and brine (25 mL), dried over Na₂SO₄, filtered, and concentrated. Compounds were characterized by ¹H NMR and RP-HPLC and were >95% in purity. See the Supporting Information for specific synthetic procedures and characterization data.

Representative NO₂ to NH₂ Reduction Procedures. Tin powder (0.6–2.2 mmol, 4.0–4.7 equiv) was added to a stirring mixture of the respective nitro compound (0.13–0.55 mmol, 1 equiv) in an HCl/AcOH mixture (0.2/2.0 mL). After 18 h, the reaction was diluted with H₂O (25 mL), neutralized with NaHCO₃, extracted into EtOAc (50 mL), and the organics were washed with H₂O (25 mL) and brine (25 mL), dried over Na₂SO₄, filtered, and concentrated. Compounds were purified by flash chromatography over silica, employing a hexanes:EtOAc elution system. All compounds were characterized by ¹H NMR and RP-HPLC and were >95% in purity. See the Supporting Information for specific synthetic procedures and characterization data.

Evaluating Small Molecule-Mediated Inhibition of WT-TTR Amyloidogenesis. WT-TTR was purified from an *Escherichia coli* expression system as described previously.⁶⁵ To a 495 µL aliquot of 0.4 mg/mL WT-TTR (7.2 µM, 10 mM phosphate, pH 7.0, 100 mM KCl, 1 mM EDTA) in a disposable cuvette was added 5.0 µL of the desired small molecule in DMSO (1.44 mM) and the sample was vortexed briefly. After incubating for 30 min at ambient temperature, the pH of the sample was lowered to 4.4 by addition of 500 µL of acidic buffer (100 mM acetate, pH 4.2, 100 mM KCl, 1 mM EDTA), and the solution was briefly vortexed and incubated in the dark for 72 h at 37 °C (final protein and inhibitor concentrations were 3.6 and 7.2 µM, respectively). The sample was then vortexed to evenly distribute any precipitate, and the turbidity was measured at 400 nm in a Hewlett-Packard model 8453 UV–vis spectrophotometer. The extent of WT-TTR aggregation (% fibril formation, *FF*; % inhibition = 100 – %*FF*) was determined by comparing the sample turbidity in the presence of small molecule as a percentage of control WT-TTR sample incubated with 5.0 µL of pure DMSO (representing 100% aggregation, 0% inhibition). All samples were performed in at least triplicate, with average values obtained presented in Figure 3 (errors are typically less than ±5 percentage points).

Evaluating the Binding Selectivity of Amyloidogenesis Inhibitors to TTR in Human Blood Plasma. The procedure for the antibody capture method to evaluate the stoichiometry of inhibitor bound to TTR in human blood plasma has been described in detail elsewhere.⁵³ Briefly, 7.5 µL of a 1.44 mM DMSO solution of test compound was incubated with human blood plasma (1.0 mL) in a 2 mL microfuge tube at 37 °C for 24 h on a rocker (30 rpm). Then, 125 µL of a 1:1 v/v slurry of unfunctionalized sepharose resin in TSA (10 mM Tris, pH 8.0, 140 mM NaCl) was added and the mixture was incubated for another hour at 4 °C on a rocker (18 rpm). The sample was then centrifuged and the supernatant divided into 2 aliquots of 400 µL each, which were each added to 200 µL of a 1:1 v/v slurry of sepharose resin conjugated to an anti-TTR antibody in TSA, and the sample was incubated again at 4 °C for 20 min on a rocker (18 rpm). The sample was then centrifuged, the supernatant removed, and the TTR bound resin was washed

three times (10 min each wash) with 1 mL of TSA/0.05% saponin, then twice (10 min each wash) with 1 mL of TSA at 4 °C on a rocker (18 rpm). After centrifugation and removal of the final supernatant, dissociation of the TTR and bound test compound from the resin-bound antibody was achieved through addition of 155 μ L of aqueous triethylamine (100 mM, pH \sim 11.5) and rocking (18 rpm) at 4 °C for 30 min. The suspension was then centrifuged and 145 μ L of the supernatant containing both TTR and test compound was removed, neutralized with 0.84 mL of glacial acetic acid (to prevent peak bleeding during HPLC analysis), and then analyzed by RP-HPLC to determine the stoichiometry of small molecule bound to TTR (the test compound-TTR complex dissociates and the small molecule and protein are chromatographically separable under the HPLC buffer conditions). Quantification of test compound and TTR is achieved by comparing the integrated peak areas to standard curves: the ratio of the amount of test compound to TTR yields the binding stoichiometry, of which a theoretical maximum value of 2 is possible owing to the two T_4 binding sites per TTR tetramer. Analyses were performed in duplicate from at least two different blood plasma samples (i.e., at least four analyses), with average values obtained presented in Figure 3 (errors are typically less than ± 0.1).

Evaluating the Inhibition of COX-1 Enzymatic Activity by the Potent TTR Amyloidosis Inhibitors. The evaluation of the most potent TTR aggregation inhibitors (displaying <25% fibril formation) at inhibiting COX-1 activity was contracted out to the Cerep laboratories in Redmond, WA. Compound analyses were performed using assay catalog reference no. 777-1hr, which uses procedures developed by Glaser et al.⁵⁴ A brief experimental protocol as provided by Cerep is outlined below. The enzyme (~ 2 μ g) was preincubated in the absence (water control) or presence of test compound (10.0 μ M) for 20 min at 22 °C in 250 μ L of buffer (100 mM Tris-HCl, pH 8, 2 mM phenol, 1 μ M hematine). Arachidonic acid (4 μ M) was then added to initiate the reaction (no arachidonic acid added for basal control measurements). After incubation at 22 °C for 10 min, the reaction was quenched by addition of 2 M HCl and 1 M Tris-HCl (pH 7.8) and cooling at 4 °C. Prostaglandin- E_2 (PGE $_2$) quantification was performed using an EIA detection kit with measurements made using a microplate reader. Values of analyses performed in singleton are presented in Figure 4 (black values), which represent the % inhibition of arachidonic acid conversion to prostaglandin- E_2 due to competitive binding of test compound to COX-1 (previous studies typically show representative errors in the data of less than ± 6 percentage points).^{51,52} Control analyses were performed analogously, with the standard inhibitory reference compound diclofenac tested at several concentrations to obtain an inhibition curve from which its IC $_{50}$ is calculated (12 nM).

Evaluating the Binding of the Potent TTR Amyloidosis Inhibitors to the Thyroid Hormone Nuclear Receptor. The evaluation of the most potent TTR aggregation inhibitors (displaying <25% fibril formation) at binding to the thyroid hormone receptor was contracted out to the Cerep laboratories in Redmond, WA. Compound analyses were performed using assay catalog reference no. 855, which uses procedures developed by Inoue et al.⁵⁵ The experimental protocol as provided by Cerep is briefly outlined below. Liver membrane homogenates (100 μ g protein) were incubated for 18 h at 4 °C with 0.1 nM [125 I]-labeled triiodothyronine ([125 I]T $_3$, the primary thyroid hormone) in the absence or presence of test compound (10.0 μ M) in 500 μ L of buffer (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 2 mM EDTA, 10% glycerol, and 5 mM β -mercaptoethanol). The samples were then vacuum filtered through glass fiber filters (GF/B, Packard), rinsed several times with ice-cold buffer (50 mM Tris-HCl and 150 mM NaCl), and the filters were dried and counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). Nonspecific binding, determined in the presence of 1 μ M T $_3$, was subtracted from the [125 I]T $_3$ binding results. Values of analyses performed in singleton are presented in Figure 4 (red italicized values), which represent the % displacement of [125 I]T $_3$ due to competitive binding of test compound to the thyroid hormone

receptor (previous studies typically show representative errors in the data of less than ± 2 percentage points).^{51,52} Control analyses were performed analogously with T $_3$ tested at several concentrations to obtain a competition curve from which its IC $_{50}$ is calculated (0.38 nM).

X-ray Crystallographic Analysis of WT-TTR Bound to Inhibitors 2d, 3b, and 5d. WT-TTR was purified from an *E. coli* expression system as described previously.⁶⁵ The protein was concentrated to 4 mg/mL in 10 mM NaP $_i$, 100 mM KCl, at pH 7.6 and cocrystallized at room temperature with various inhibitors at a 5 molar excess using the vapor-diffusion sitting drop method. Crystals were grown from 1.395 M sodium citrate, 3.5% v/v glycerol at pH 5.5. The crystals were cryoprotected with inhibitor-free 1.395 M sodium citrate, 10% v/v glycerol at pH 5.5. Data were collected on beam line 9–2 at the Stanford Synchrotron Radiation Laboratory (SSRL) at a wavelength of 0.9795 Å. Data sets were integrated and scaled using HKL2000.⁶⁶ The crystals were indexed in orthorhombic space group $P2_12_12$ with two subunits per asymmetric unit (see Table 1 for full data collection and refinement statistics). The three crystal structures were determined by molecular replacement using the 2FBR⁶⁷ coordinates in the program Phaser.⁶⁸ Further model building and refinement were completed using Coot⁶⁹ and Refmac.⁷⁰ For all structures, hydrogens were added during refinement and anisotropic B values calculated. Final models were validated using the JCSG quality control server (<http://jcsgrsv2/QC>) incorporating Molprobity,⁷¹ ADIT (<http://rcsb-deposit.rutgers.edu/validate>) WHATIF,⁷² Resolve,⁷³ and Procheck.⁷⁴ Full data collection and refinement statistics are presented in Table 1.

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Supporting Information Available: Detailed synthesis and characterization data on additional compounds prepared analogously to the general synthetic procedures presented in the Experimental Section, tabulation of compound purity as determined by C $_4$ and C $_{18}$ RP-HPLC, and calculated phenolic pK $_a$ values. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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