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Discovering Transthyretin Amyloid Fibril Inhibitors by Limited Screening

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Abstract—Insoluble protein fibrils, resulting from the self-assembly of a conformational intermediate are implicated to be the causative agent in several human amyloid diseases including familial amyloid polyneuropathy (FAP) and senile systemic amyloidosis (SSA). These diseases are associated with transthyretin (TTR) amyloid fibrils, which appear to form in the acidic partial denaturing environment of a lysosome or endosome. Here we identify several structural classes of small molecules that are capable of inhibiting the TTR conformational changes facilitating amyloid fibril formation. A small molecule inhibitor that stabilizes the normal conformation of a protein is desirable as a promising approach to treat amyloid diseases and to rigorously test the amyloid hypothesis, the apparent causative role of amyloid fibrils in amyloid disease.

Introduction

Transthyretin (TTR) is one of seventeen different proteins that are known to form amyloid fibrils in vivo.[1–5] Wild type TTR fibrils are thought to be the disease causing agent in senile systemic amyloidosis in patients typically >80 years of age, whereas one of 54 TTR mutations are the main component of the fibrils in a group of diseases generally called familial amyloid polyneuropathy, where the age of onset is typically much earlier.[6,7] Several amyloid diseases are thought to be caused by the conversion of a normally soluble and functional human protein into an alternatively folded state that renders the protein capable of self assembly into putatively neurotoxic amyloid fibrils.[1–3,5,8–13] Transthyretin amyloid diseases were among the first fibril formation processes demonstrated to involve protein conformational changes.[1,14] Our laboratory has been using reduced pH (5.0 ± 0.5) to induce transthyretin amyloid formation in vitro, as acid is the in vivo denaturant.[1,2]

Transthyretin is found in plasma (3.6 μM) and cerebral spinal fluid (CSF) and is composed of four identical 127 amino acid β-sheet rich subunits. The tetrameric structure binds and transports thyroxine (T4) and the retinol binding protein (RBP). X-Ray studies of TTR show two funnel-shaped binding sites for T4, each defined by a dimer–dimer interface.[15,16] In plasma, only 10–15% of TTR has T4 bound (K_a's of 10^8 and 10^6, negative cooperativity), because thyroxine binding globulin is the major thyroxine carrier ($K_a = 6 \times 10^9$). However in CSF, TTR is the main thyroid carrier. At lower pH, such as the pH encountered in a lysosome or endosome, tetrameric transthyretin dissociates into a conformationally altered monomeric structure that self-associates into amyloid fibrils.[1–4,11] Recently, we have demonstrated that the normal tetrameric β-sheet rich fold of TTR can be stabilized under amyloid forming (acidic) conditions by small ligands that bind and prevent amyloid fibril formation in vitro.[17,18] Thyroxine (**1**, T_4) and the non-steroidal antiinflammatory drug Flufenamic acid (**2**) were among the first compounds shown to be capable of preventing TTR amyloid fibril formation. These ligands function by preventing the amyloidogenic conformational changes that convert the normally soluble protein into a conformational intermediate that assembles into amyloid fibrils.[17,18] The fact that TTR fibril formation is almost never observed in the brain is likely explained by the two T4 ligands bound to TTR in the CSF, which we now know stabilize the protein against amyloid fibril

Key words: Transthyretin; amyloid fibril; inhibition; screening.
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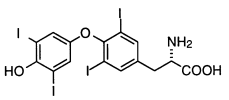
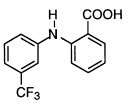
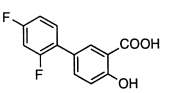
formation.[17] Recently, a cocrystal structure of Flufenamic acid and TTR was determined which provides the basis for a combination structure-based drug design/screening effort.[18] The screening effort described here will help identify important binding features of transthyretin ligands as well as identifying new structural platforms on which important pharmacophoric groups could be presented in the TTR binding cavity.[18] Additional studies show that Diflunisal (**3**) is similar to Flufenamic acid (**2**) in terms of inhibiting TTR fibril formation.[19]



1, Thyroxine (T_4)



2, Flufenamic Acid



3, Diflunisal

In this paper we extend our previous knowledge regarding TTR fibril formation inhibitors by screening a number of compounds available from commercial sources that appear to be complimentary to the TTR binding site.[18] We have utilized information obtained from published studies on transthyretin binding ligands to select compounds which contain either all or portions of the previously described pharmacophores.[15,16,20–28] In addition, we screened structural classes of molecules which are currently found in a variety of pharmaceuticals including flavones, tetrahydroquinolines, dihydropyridines, and benzodiazepines. The ability of this group of compounds to bind to transthyretin was not previously tested. In addition, several conformationally constrained ring systems were evaluated to help delineate the bound conformation of the active molecules. Here we identify lead structures which aid our current efforts to develop improved TTR amyloid inhibitors for evaluation in animal models and ultimately in the clinic.[29,30]

Inhibiting protein conformational changes with small molecules has associated with it special challenges not typically encountered with enzyme inhibition. Enzymes retain their tertiary structure over the course of several hours, allowing the inhibitors to dissociate and rebind many times. Unfortunately, an unliganded amyloidogenic protein under amyloid forming conditions changes conformation and associates, denying the alternatively folded protein another chance to bind the inhibitor of the conformational change. Hence, the conformational change commits the protein to amyloid formation unless the activation barrier(s) for refolding are low enough to allow the associated alternatively folded protein to be in equilibrium with the folded protein, which is possible, but not likely. This scenario is highly unlikely for transthyretin, based on the high activation barriers that are known to intervene between the folded tetramer and the amyloidogenic monomeric intermediate.[31] Our current knowledge suggests that both of the ligand binding sites in transthyretin must be occupied to suppress TTR amyloid formation over a 72 h period.[17,18] Hence, an inhibitor must be present at 7.2 μM concentration in order to completely shut down fibril formation, assuming that the K_d is an order of magnitude or more below the physiological TTR concentration (3.6 μM). Inhibitors that have very slow off rates are highly desirable for this application, as dissociation of the ligand can lead to largely irreversible protein conformational changes. Flufenamic acid has many of the desirable features of a conformation change inhibitor, including an apparently slow off rate.[18]

Results

The compounds assayed in this study were purchased in the highest grade available from either Aldrich Chemical Co., Fluka, Lancaster Synthesis, Research Biochemicals Inc., or Sigma Chemical Co. and were used without further purification.

The compounds were assessed for their ability to inhibit amyloid fibril formation in a 72 h stagnant fibril formation assay described previously by our laboratory.[2,17] This screen subjects wild type transthyretin to acid mediated fibril formation in acetate buffer at pH 4.4 in the absence or presence of a potential amyloid fibril inhibitor. The extent of fibril inhibition is then determined by the change observed in the optical density (light scattering) measured on a UV spectrometer at either 330 nm or 400 nm relative to a triplicate TTR standard lacking inhibitor (this averaged optical density is assigned to be 100% TTR amyloid fibril formation). The inhibitor flufenamic acid (**2**) is always included as a control in each run to be sure the expected inhibition of TTR fibril formation is observed (i.e., only 4 ± 3% conversion of soluble TTR to amyloid fibrils is observed in the presence of 10.8 μM flufenamic acid). Potential inhibitors were prepared as 5.4 mM solutions in pure DMSO, hence only 2 μL of the DMSO solution was required to reach the highest desired inhibitor concentration (36 μM, 10× the TTR concentration) for screening utilizing a 300 μL assay volume. In the case of the lower inhibitor concentrations (3×TTR, 1×TTR, and 0.5×TTR), serial dilutions were made of a 10×TTR solution such that 2 μL of the DMSO solution provided the desired concentration of compound to be tested. Potential inhibitor solutions were prepared by weighing a known amount of the compound and making the appropriate addition of DMSO. The remainder of the assay was performed as described in the experimental section. All the compounds were tested in triplicate for each inhibitor concentration. Testing was initially carried out at 36 μM, 10× the plasma TTR concentration,

to determine whether there was sufficient activity to warrant further assessment at lower inhibitor concentrations. We divided the compounds in this screen into seven classes based on either the presence or the absence of certain structural homologies. Compounds **4**–**18**, Figure 1 are either available therapeutics or have some known biological potency and these, like compounds **19**–**30**, Figure 2, were included to either challenge or support the pharmacophoric hypothesis as initially understood. Screening diverse structures serves to identify additional structural platforms for inhibitor design purposes. The remaining compounds, **31**–**80**, were selected based on particular structural features anticipated to be important for the binding interaction with the TTR tetramer, Figures 3–7. Most of these compounds are aromatics and several have a biaryl structure with an acidic functionality on at least one of the rings. When comparing inhibitors it is important to remember that we report the percent conversion of soluble transthyretin into fibrils over a 72 h period relative to transthyretin in the absence of inhibitor, hence 100% conversion (fibril formation) reflects no inhibition,

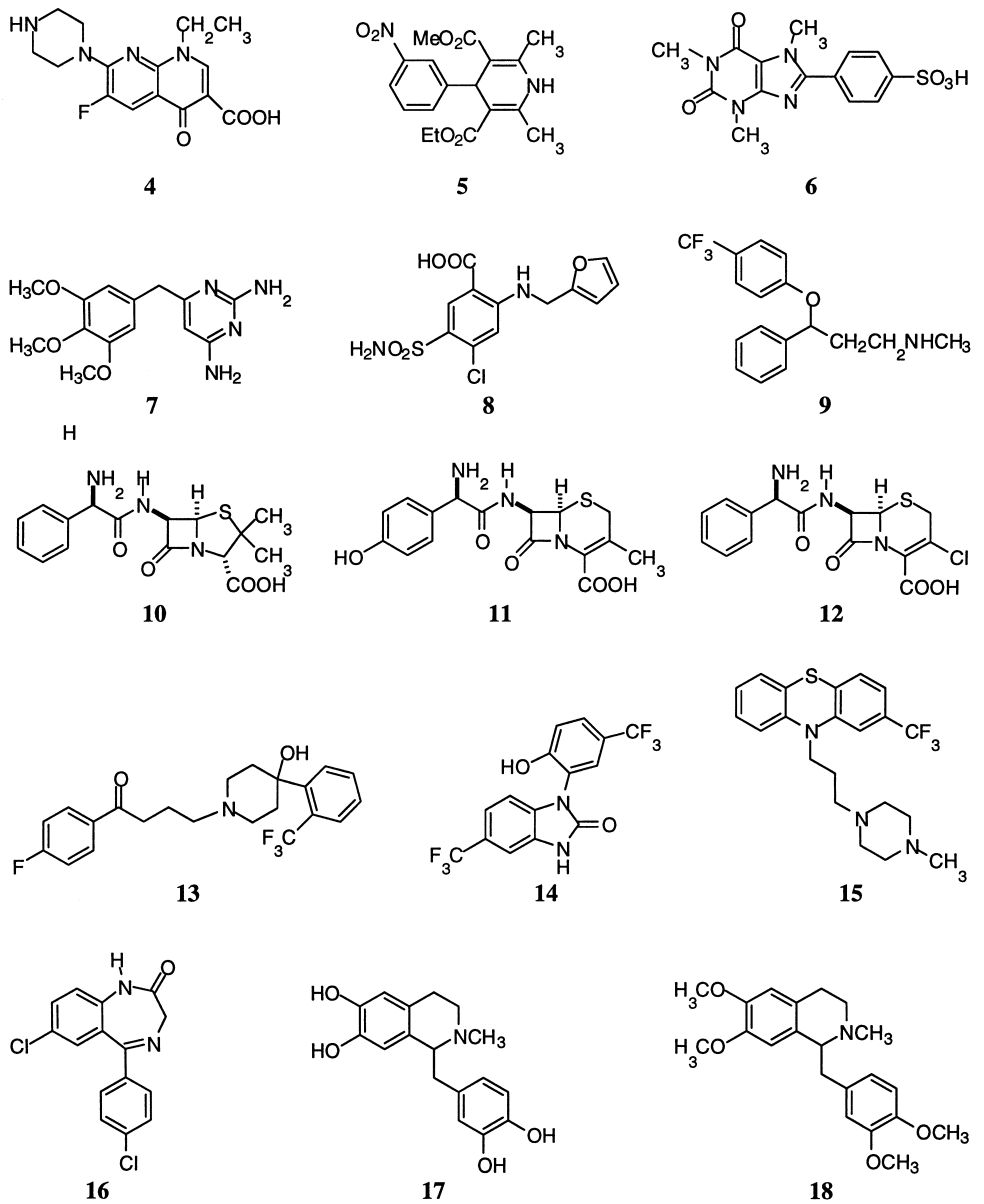


Figure 1. Structures of known pharmacologically active compounds assayed for their ability to inhibit TTR amyloid fibril formation.

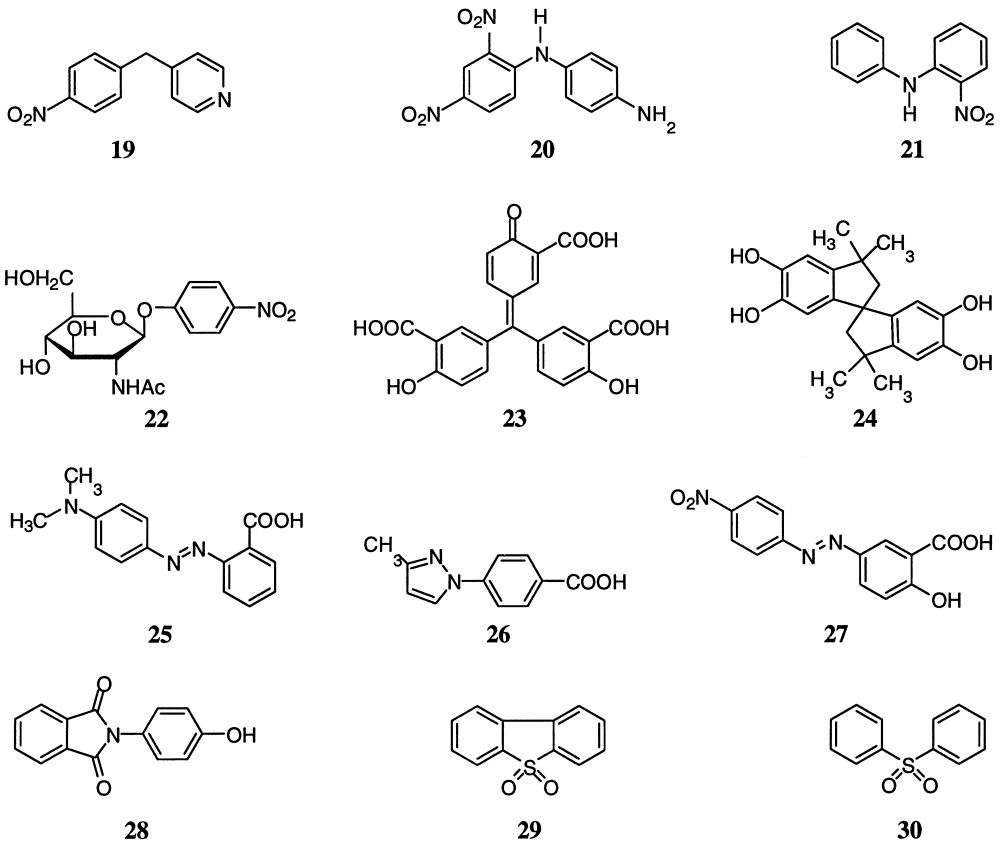


Figure 2. Small molecule structures evaluated as transthyretin amyloid fibril inhibitors to challenge the previously identified pharmacophoric hypothesis including the need for a carboxylic acid and a biaryl system.

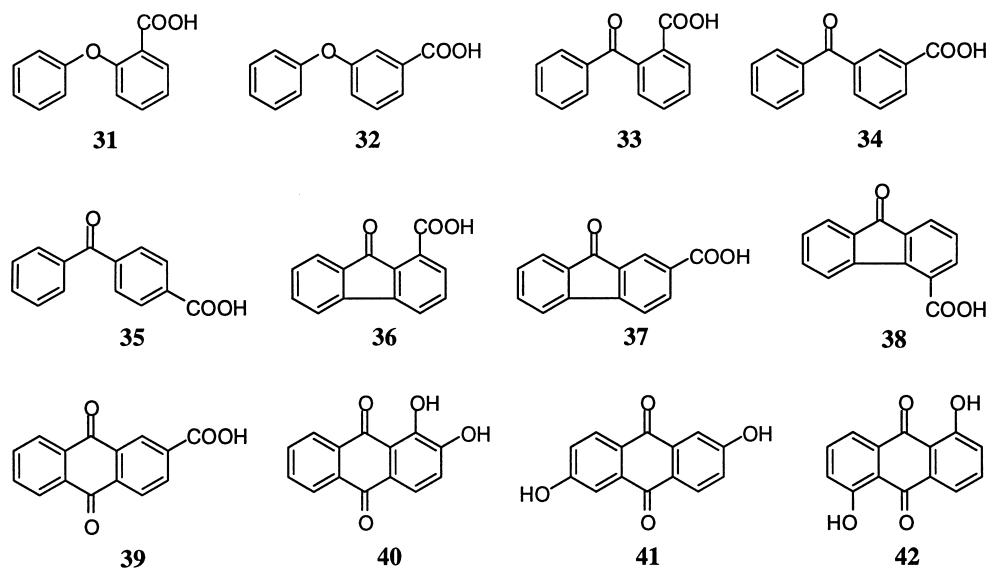


Figure 3. Biaryl ethers, benzophenones, fluorenones and anthraquinones with varying homology to Flufenamic acid which were evaluated as transthyretin amyloid fibril inhibitors.

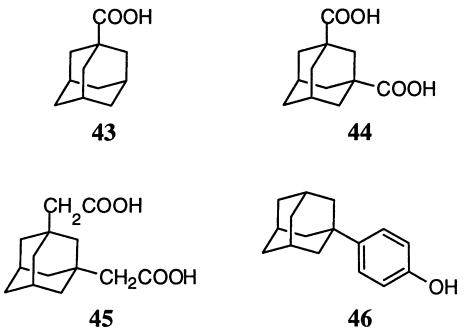


Figure 4. Adamantane based small molecule structures screened as possible TTR amyloid fibril inhibitors.

whereas 0% conversion is complete inhibition of TTR fibril formation.

The efficacy of compounds **4**–**30**, Figures 1 and 2, as TTR fibril formation inhibitors is given in Table 1. These compounds in general do not show good TTR fibril inhibitory activity (>50% fibril formation at 36 mM, 10× the TTR concentration). Several of these compounds (**4**, **10**–**13**, **15**, **17**–**19**, and **22**) produced OD readings greater than the TTR samples undergoing fibril formation in the absence of inhibitor (the positive controls), indicating a greater extent of fibril formation in the presence of the small molecules. This may be due to either fibril promotion by these compounds or to the fact that the small molecules themselves are precipitating in the presence of the protein and not in the control solutions, or a combination of these effects. Compound **20** (Fig. 2) is interesting because only 32% of TTR forms fibrils in its presence, yet it does not contain a carboxylic acid or phenolic hydroxyl group thought to be important for inhibition via an interaction with the ammonium groups of Lys-15 from each TTR subunit. Similarly, compound **21** has no acidic functional group appended, yet is capable of some inhibition. This compound can be considered isosteric to *N*-phenylanthranilic acid, which was previously reported to inhibit fibril formation nearly completely at relatively high concentration (only 2–3% conversion at 36 μM). The conformationally constrained compounds **23** and **24** resulted in 31% and 28% conversion to fibrils, respectively, whereas Methyl Red (**25**) was a slightly poorer inhibitor, allowing conversion of 44% of TTR into fibrils. Mordant Orange I (**27**) was a much better inhibitor allowing only 15% fibril conversion at 10.8 μM, its solubility limit in the buffer. This compound was listed as being 80% pure, the remaining 20% was not characterized.

Fibril inhibition data for compounds **31**–**56** at 36 μM (10× the physiological TTR concentration) is given in Table 2. Compounds **31**–**42**, Figure 3, are close structural homologues to Flufenamic acid in that the biaryl system composing these compounds is separated by a

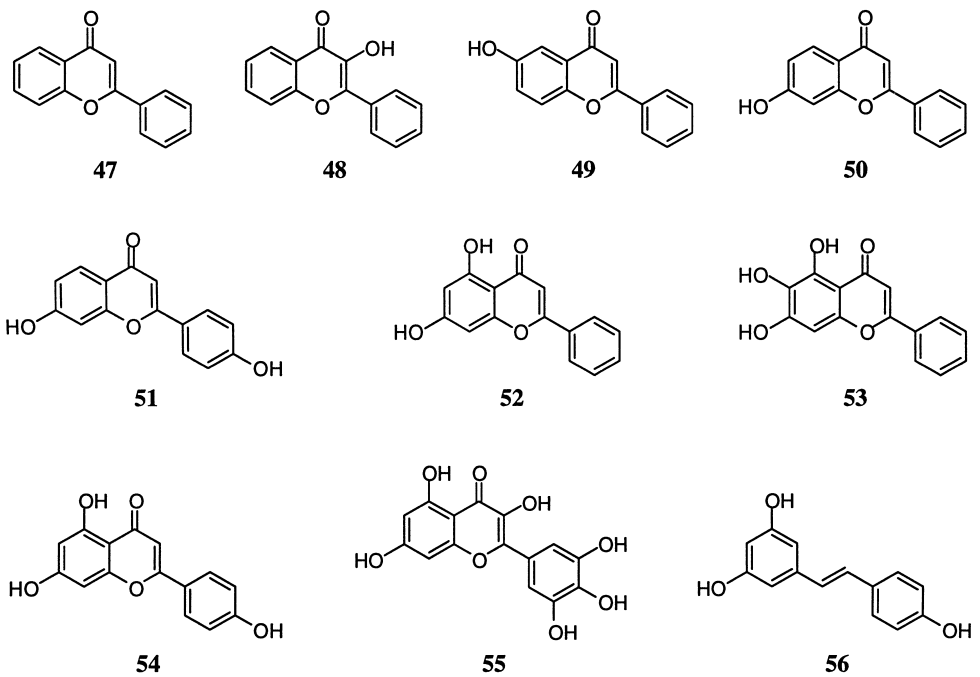


Figure 5. Structures of plant-derived flavones and stilbene derivatives assayed for their efficacy as transthyretin fibril inhibitors.

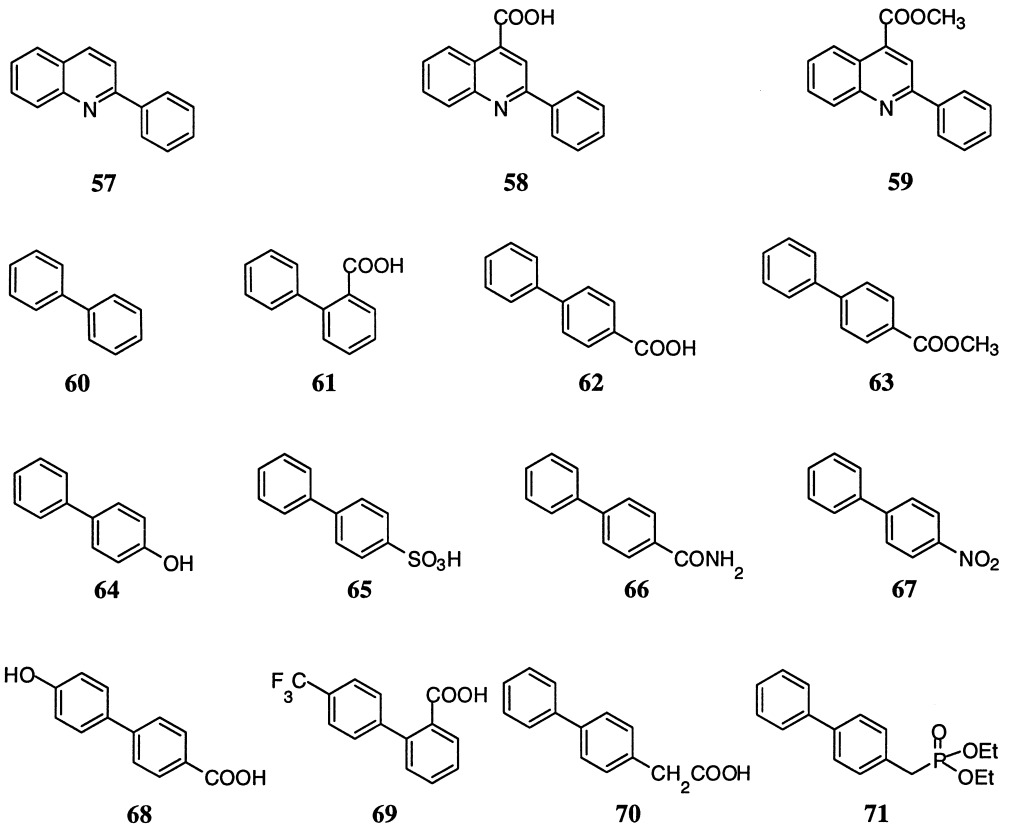


Figure 6. Biphenyl-related small-molecule structures screened as transthyretin fibril formation inhibitors in this study.

single atom. Furthermore, all of these compounds contain an acidic functional group in the form of either a carboxylic acid or phenolic hydroxyl group. The non-conformationally constrained *meta*- and *para*-carboxylic acid containing analogues (**32**, **34**, and **35**) were the best inhibitors of the group exhibiting only 5–6% amyloid fibril formation at 36 μM. Neither **31** nor **33**, having an *ortho*-carboxylic acid functionality, could inhibit amyloid fibril conversion below 50% of the control. The fluorenone and anthraquinone derivatives (**40-42**), Figure 3, not bearing a carboxylic acid were ineffective as TTR fibril formation inhibitors. The compounds which did contain a carboxylic acid (**36**–**39**) were modestly effective with compound **39** in this group being the best with only 4% amyloid fibril formation observed. The four adamantane analogues, **43**–**46**, Figure 4, included in this study were not effective at inhibiting transthyretin amyloid fibril formation in the μM concentration range.

We also included a series of flavone compounds, **47**–**55**, Figure 5, with varying numbers of phenolic hydroxyl groups. In addition, structurally related resveratrol, **56**, was included in this group. Resveratrol has been isolated from skin of red grapes and is believed to be one of the therapeutically useful compounds present in red wine and grape juice. In general, this class of compounds is effective at inhibiting transthyretin amyloid fibril formation. Only compounds **47** and **48** gave more than a 33% conversion to amyloid when tested at 36 μM. The more highly substituted flavones, such as **51**–**55**, kept the conversion from normally folded TTR to amyloid fibrils to less than 10%. Apigenin (**54**) was the best of all of the flavones, completely inhibiting fibril formation at a concentration of 36 μM. The trans-stilbene Resveratrol (**56**) was an equally good inhibitor at this concentration.

Compounds **32**, **38**, **39**, **54**, and **56** were evaluated as inhibitors at 10.8 μM concentration, three times the TTR concentration, and the results are presented in Table 3. All were capable of keeping the fibril formation conversion below 50%. Compounds **32** and **54** were very good inhibitors, exhibiting 5 and 6% conversion, respectively. Resveratrol was an excellent inhibitor in that it did not allow the conversion of soluble TTR into amyloid fibrils when present at 10.8 μM concentration as determined by the OD measurement, indicating stilbenes of this type are exciting lead compounds.

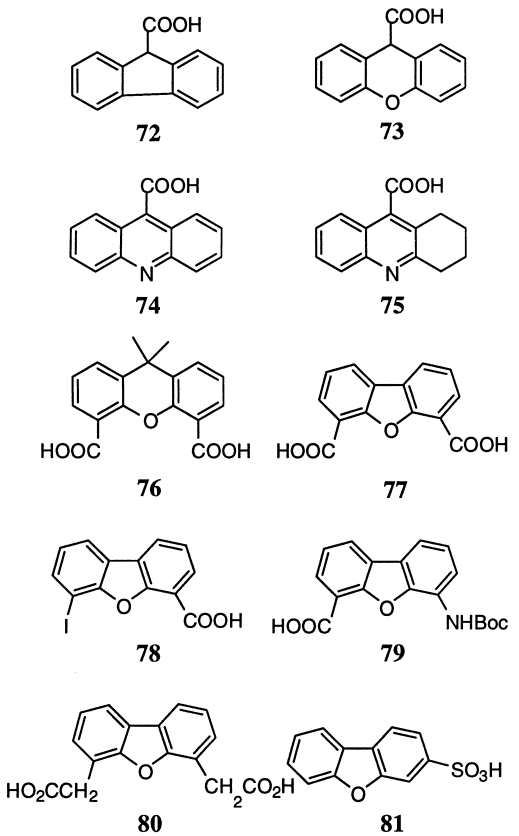


Figure 7. Xanthene, acridine, and dibenzofuran derived compounds evaluated as transthyretin fibril formation inhibitors in this study.

Table 1. Amyloid fibril inhibition by the compounds shown in Figures 1 and 2[a]

Compd	Percent fibril formation[b]	Compd	Percent fibril formation[b]
4	107	**18**	109
5	87	**19**	101
6	92	**20**	32
7	62	**21**	54
8	36	**22**	119
9	88	**23**	31
10	111	**24**	28
11	108	**25**	44
12	119	**26**	68
13	118	**27**	15
14	91	**28**	79
15	117	**29**	53
16	92	**30**	85
17	111		

[a]The TTR concentration was 3.6 μM and the compounds were evaluated at either 36 μM (**4**–**26**) or 10.8 μM (**27**–**30**).
[b]Control fibril formation was evaluated in each assay in the absence of drug and was considered to be 100%.

A collection of biphenyl compounds (**57**–**71**), Figure 6, were tested at 36 μM and the results are outlined in Table 4. The three isoquinoline containing compounds, **57**, **58**, and **59**, ranged in inhibitor efficacy from poor to modest with amyloid conversion yields of 95%, 34%, and 47%, respectively. The biphenyl series, **60**–**71**, contain both ineffective and effective inhibitors. The most effective biphenyls as inhibitors were **62**, **64**, and **65**, all containing an acidic functional group. These compounds inhibited the conversion nearly completely with only 4 to 5% yield of amyloid being produced at 36 μM inhibitor concentration. Compound **68**, having a phenol and a carboxylic acid, was a less effective inhibitor allowing a 20% conversion to fibrils. The biphenyl compound **66** with a primary amide substituent was only modestly effective as an inhibitor with 39% TTR amyloid fibril formation observed.

Selected compounds from the biphenyl series, Figure 6, were further tested at 10.8 μM and 3.6 μM, with the results presented in Table 5. Results from the inhibitor Flufenamic acid were also included to put everything in perspective. Compounds **60**, **63**, **66**, **67**, and **69** were ineffective inhibitors at these lower concentrations. Compound **62** was the best fibril inhibitor with only 2.3% fibril formation observed at 10.8 μM. The other two analogues, **64** and **65**, drop off in activity significantly, affording 63% and 52% yields of fibril conversion, respectively, at 3.6 μM inhibitor concentration.

The final series of compounds can be considered to be conformationally constrained Flufenamic acid analogues.

Table 2. Amyloid fibril inhibition by the compounds shown in Figures 3–5[a]

Compd	Percent fibril formation[b]	Compd	Percent fibril formation[b]
31	82	**44**	100
32	5	**45**	96
33	53	**46**	76
34	5	**47**	64
35	6	**48**	80
36	18	**49**	21
37	44	**50**	33
38	9	**51**	9
39	4	**52**	6
40	78	**53**	5
41	59	**54**	–[c]
42	109	**55**	10
43	101	**56**	–[c]

[a]The TTR concentration was 3.6 μM and the compounds were evaluated at 36 μM.
[b]Control fibril formation was evaluated in each assay in the absence of drug and was considered to be 100%.
[c]No fibril formation was observed at 36 μM inhibitor.

Table 3. Amyloid fibril inhibition with selected compounds at 10.8 μM concentration[a]

Compd	Percent fibril formation[b]
32	5
38	43
39	32
54	6
56	–[c]

[a]The TTR concentration was 3.6 μM and the compounds were evaluated at 10.8 μM.
[b]Control fibril formation was determined in each assay in the absence of drug and was considered to be 100%.
[c]No fibril formation was observed at 10.8 μM inhibitor concentration.

Table 4. Amyloid fibril inhibition by the compounds shown in Figure 6[a]

Compd	Percent fibril formation[b]	Compd	Percent fibril formation[b]
57	95	**65**	4
58	34	**66**	39
59	47	**67**	91
60	96	**68**	20
61	85	**69**	37
62	4	**70**	79
63	10	**71**	69
64	5		

[a]The TTR concentration was 3.6 μM and the compounds were evaluated at 36 μM (**57**–**70**) or 10.8 μM (**71**).
[b]Control fibril formation was determined in each assay in the absence of small molecules and was considered to be 100%.

Compounds **72**–**78** and **81**, Figure 7, were tested directly at 10.8 μM concentration, 3× the TTR concentration. The fluorene derivative **72** was the most effective of the non-dibenzofuran compounds with 43% amyloid fibril conversion at this concentration, Table 6. The dibenzofuran analogues, **77**, **78**, and **81** tested at 10.8 μM proved to be very good amyloid fibril inhibitors. In particular, **77** and **78** were excellent inhibitors at this concentration with less than 5% fibril formation observed. Derivatives **79** and **80** were modestly effective at 36 μM (Table 6).

The most promising inhibitors **2**, **32**, **39**, **54**, **56**, **62**, and **77** were selected from the above assays and tested at 10.8 μM, 3.6 μM, and 1.8 μM concentrations (i.e., 3×, 1×, and 0.5× the physiological TTR concentration), Figure 8. The TTR for this assay was freshly purified and greater than 95% pure as indicated by SDS–PAGE. The results for this transthyretin fibril formation screen are given in Table 7. Compound **39** is not very effective at low concentration, and rapidly becomes ineffective at inhibiting TTR fibril formation as the concentration is lowered. In contrast, the remaining lead compounds are excellent transthyretin amyloid fibril inhibitors, allowing only 2–7% of soluble TTR to be converted to amyloid at 10.8 μM and 24–53% of TTR converted to amyloid at 3.6 μM inhibitor, under conditions where only one of the two binding sites are occupied. At 1.8 μM, the compounds no longer can keep TTR fibril formation to below 50% conversion, as expected owing to the 3.6 μM TTR concentration and the requirements discussed in the last paragraph of the introduction. Flufenamic acid (**2**), the analogous analogue **32**, resveratrol (**56**), and the biphenyl-*p*-carboxylate **62** were approximately as effective as one another despite structural differences, although it is likely that more detailed studies could reveal distinct differences. The quantitative congo red fibril analysis as a function of added inhibitor agree very nicely with the light scattering data (Figure 8) further supporting the efficacy of these inhibitors.[2]

Discussion

We have previously reported the transthyretin amyloid fibril inhibitor Flufenamic acid,[18] an antiinflammatory drug, and have recently completed another study showing

Table 5. Amyloid fibril inhibition by selected compounds from Figure 6[a]

	Concn (μM)			Concn (μM)	
	10.8	3.6		10.8	3.6
Compd	Percent fibril formation[b]		Compd	Percent fibril formation[b]	
2	4	11	**65**	13	52
60	98	—[c]	**66**	79	95
62	2	14	**67**	96	—[c]
63	82	91	**68**	37	68
64	25	63	**69**	89	—[c]

[a]The TTR concentration was 3.6 μM and the compounds were evaluated at either 10.8 μM, 3.6 μM or both.
[b]Control fibril formation was determined in each assay in the absence of drug and was considered to be 100%.
[c]Not determined.

Table 6. Amyloid fibril inhibition by the compounds shown in Figure 7[a]

Compd	Percent fibril formation[b]	Compd	Percent fibril formation[b]
72	43	**77**	3
73	57	**78**	5
74	84	**79**	33
75	89	**80**	53
76	86	**81**	31

[a]The TTR concentration was 3.6 μM and the compounds were evaluated at 10.8 μM (**72**–**78** and **81**) or 36 μM (**79**–**80**). Flufenamic inhibition control values were the same as in Table 5.
[b]Control fibril formation was determined in each assay in the absence of inhibitor and was considered to be 100%.

the amyloid inhibitory efficacy of the related compounds, Diflunisal and *N*-benzyl-*p*-aminobenzoic acid.[19] There are significant structural differences amongst the molecules that are known to inhibit TTR fibril formation, that is, those molecules mentioned in the previous sentence and thyroxine, as well as the new inhibitors indentified herein. Previous structures of native TTR[32,33] as well as cocrystal structures of TTR bound with thyroxine ,[16] 3,3′-diiodo-L-thyronine,[15] 3′,5′-dibromo-2′,4,4′,6-tetrahydroxyaurone,[26] Milrinone,[27] and Flufenamic acid[18] have been reported. These structures illustrate the different binding modes compounds can adopt when binding to transthyretin. The two equivalent binding sites in TTR are shaped like a silhouette of the human head and shoulders. The inner binding site

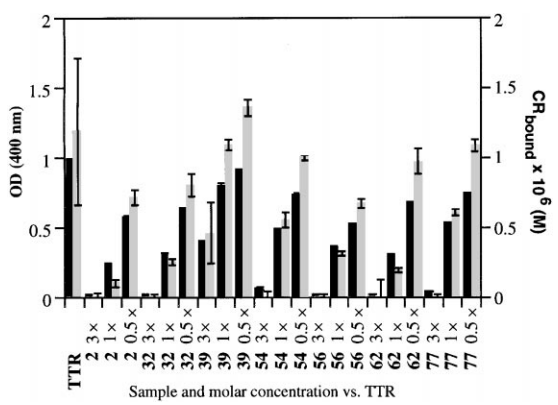


Figure 8. A bar graph representing the extent of TTR amyloid fibril formation with selected inhibitors at 3×, 1×, and 0.5× the TTR concentration (3.5 μM). Dark bars represent fibril formation as evaluated by the optical density (OD) reading of the solution in a UV spectrometer at 400 nm. Lighter bars represent the extent of TTR fibril formation as evaluated by Congo Red binding. Each assay was done in triplicate and the error bars indicate the standard deviation.

Table 7. Amyloid fibril inhibition with selected compounds at varying concentrations[a]

	Concn (μM)		
	10.8	3.6	1.8
Compd	Percent fibril formation[b]		
2	2	24	59
32	2	33	64
39	40	82	93
54	6	49	75
56	2	33	64
62	2	30	69
77	4	53	76

[a]The TTR concentration was 3.6 μM and the selected compounds were evaluated at 10.8, 3.6, and 1.8 μM (3×, 1×, and 0.5× the TTR concentration respectively).
[b]Control fibril formation was evaluated in each assay in the absence of inhibitor and was considered to be 100%.

(the head) and the outer binding site (the shoulders) are identified in Figure 9. Preliminary results suggest that not all inhibitors utilize the inner binding site. In this paper we screened 78 known compounds of diverse structure and compounds similar to known inhibitors such as Flufenamic acid or Diflunisal to identify new inhibitors. Five structurally diverse transthyretin amyloid fibril inhibitors were indentified in this study, the biphenylether **32**, flavone **54**, stilbene **56**, biphenyl **62** and the dibenzofuran **77**.

Of the known pharmacologically active compounds (**4**–**15**), Figure 1, only furosamide (**8**) showed modest activity as a TTR fibril inhibitor. The cocrystal structure of Milrinone with TTR provided the rationale for testing **5**. The inability of **5** to inhibit fibril formation is likely due to a difference in the steric requirements resulting from the additional substituents which prevent binding to TTR. Interestingly, Enoxacin (**4**), Ampicillin (**10**), Cefadroxil (**11**), Cefaclor (**12**), Trifluperidol (**13**), and Trifluoperazine (**15**) do not inhibit fibril formation, in fact they may increase fibril formation. However, this may result from precipitation of the compound in the presence of the protein, but not in TTR's absence, as we always make sure that the potential inhibitor does not precipitate and scatter light on its own.

Compounds **19**–**30**, Figure 2, were tested to critically evaluate the perceived requirement for an acidic functional group on an aromatic substructure to interact with the Lys-15 residues in TTR. The inactivity of bisarylamine **21** demonstrates that a nitro group is not nearly as effective as the carboxylic acid found in *N*-phenylanthranilic acid, the latter exhibiting only 3% fibril formation at 36 μM inhibitor concentration.[19] Compounds **23** and **24** are highly constrained due to

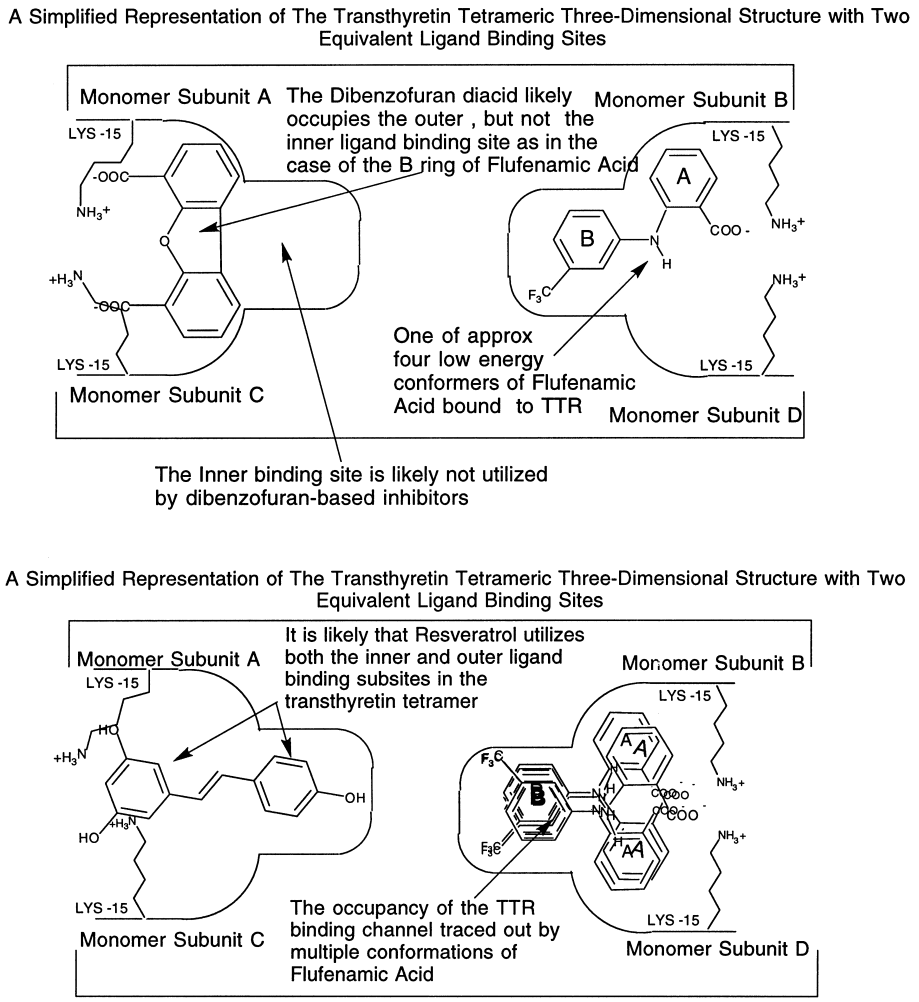


Figure 9. A simplified representation of the two ligand binding sites in the transthyretin tetramer. Note that each binding site has a smaller inner and larger outer binding pocket. The inner binding pocket is not expected to be occupied by the dibenzofuran ligand shown in the upper left, whereas the B ring of Flufenamic acid occupies the inner binding pocket while the A ring occupies the outer binding pocket.

delocalization and the spiro ring system, respectively. Both are modestly effective amyloid fibril inhibitors. In the case of **24**, the phenolic hydroxyl group serves as the acidic functionality, the ring system providing the shape/size complementarity to the TTR binding pocket. We are seeking more structural information on the inhibitors because of the very different conformations (planar versus perpendicular) expected for the biaryl ring systems of **23** and **24**. The two dyes, Methyl Red (**25**) and Mordant Orange I (**27**) were also tested. Of the two, **27** (80% pure) was the most effective with good fibril inhibition observed at 10.8 μM. Compound **26** was not very active despite having a pseudo biaryl system functionalized with a carboxylic acid. Even though compounds **29** and **30** were not very effective inhibitors at 10.8 μM, the improved activity of **29** in comparison to **30** suggests that the bound conformation prefers coplanarity, although this logic could prove to be flawed if **29** and **30** bind differently.

Insight on the preferred conformation of a particular series was sought by examining compounds **31**–**42**, Figure 3. The most active inhibitors in this series includes compounds **32**, **34**, **35**, **38** and **39**, the common thread being two aromatic rings linked by a one atom spacer, with a *m*- or *p*-positioned carboxylic acid. We strongly suspect that the tricyclic systems bind in the outer binding pocket only (Figure 9), differently than the bicyclic systems, but this remains to be verified by structural methods. It is not clear whether the inactivity

of **31** is due to intramolecular hydrogen bonding or the inability to attain the desired conformation owing to steric reasons. The inability of compounds **40**–**42** to inhibit fibril formation to the same extent as **39** indicates the carboxylic acid group generally improves the inhibitory activity.

The adamantane series **43**–**46** tested the ability to replace one or both of the aromatic rings in a biaryl system with another large hydrophobic structure, adamantane, which appears to be able to fit in the TTR binding site. None of these compounds were effective at inhibiting amyloid fibril formation.

TTR was reported to bind to plant flavanoids.[34] Therefore we selected a series of these analogues to determine which, if any, were effective at inhibiting amyloid fibril formation, Figure 5. Flavone (**47**) itself is not very effective at inhibiting fibril formation, nor is 2-hydroxyflavone (**48**). Incorporation of an acidic phenolic OH onto the aryl ring as in **49** and **50** greatly increase their inhibitor efficacy. Inhibitory power increases with the further addition of phenolic functionality (**51**, **52**, **53** and **54**). Out of these compounds, apigenin (**54**) is the most potent with only 5% fibril formation observed at 10.8 μM. The additional phenolic OHs present in myricetin (**55**) reduced activity. This may be due to the presence of the 2-hydroxy group since this addition in **48** has reduced activity when compared to **47**.

In addition to the flavanoids, we also tested the stilbene analogue resveratrol (**56**). This compound shares structural homology with apigenin and was even more effective than apigenin at inhibiting TTR amyloid formation in vitro. It is possible that resveratrol and apigenin bind differently, we won't know until the crystal structure for each is solved bound to TTR, Figure 9.

The biphenyl analgesic Diflunisal was previously shown to be an effective TTR amyloid fibril inhibitor in vitro.[19] A series of biphenyl compounds and biphenyl analogues were screened to explore the versatility of this skeleton, Figure 6. The isoquinoline series **57**–**59** demonstrates the importance of the carboxylic acid substituent. The activity of the monosubstituted biphenyls, **62**–**66** and **68**, Figure 6, demonstrate the utility of the biphenyl substructure as the basis for transthyretin amyloid fibril inhibitors. The *para*-substituted acid **62** was the most effective inhibitor. The *ortho*-substituted carboxylic acid **61** was not very effective, likely due to the inability to present the acid properly at the binding site in transthyretin. Replacing the acid with a phenolic OH (**64**) or a sulfonic acid (**65**) affords inhibitors that are very similar to the best compound **61**. Inhibition is not observed when the acid is replaced with either an amide (**66**) or a nitro group (**67**).

The symmetry equivalent binding of Flufenamic acid to TTR suggests that a molecule which presents a carboxylic acid functionality central to two positions of the carboxyl-bearing aromatic ring may have improved binding and inhibition activity, Figure 9.[18] We assayed **72**–**75**, Figure 7, anticipating improved binding and fibril inhibition from these compounds. The isoquinoline **58** can also be considered to bind in such a fashion and we expected to improve on the observed inhibition by fusing on the third ring. Only the fluorene derivative **72** had modest inhibitory activity when tested at 10.8 μM.

The xanthene and dibenzofuran analogues, **76**–**81**, Figure 7, combine several of the necessary structural features for binding into a single group of compounds. These compounds can be considered to be conformationally constrained biphenyls and are structurally related to compounds **36**, **37**, and **62**. Compound **76** was not an effective inhibitor although the similar dibenzofuran (**77**) was very effective at fibril inhibition. The xanthene tricyclic rings are not coplanar, perhaps explaining the inability of **76** to inhibit. The spacing and the orientation of the carboxyl groups is also different in **76** and **77**. This may also be related to the steric demand of the interior methyl groups if this compound binds across the pocket, as expected, Figure 9. The presence of a single carboxylic acid in dibenzofuran **78** did not significantly alter the inhibitory potential at 10.8 μM as compared to the dicarboxylic acid **77**. Addition of a sterically demanding Boc group on an amine (**79**) or the creation of a one methylene homologated acid (**80**) resulted in a decrease in efficacy. The sulfonic acid (**81**) was a modestly effective inhibitor at 10.8 μM.

The most promising structurally diverse inhibitors from the screen (**32**, **39**, **54**, **56**, **62**, and **77**) were tested at 10.8 μM, 3.6 μM, and 1.8 μM concentrations (3×, 1×, and 0.5× the 3.6 μM TTR concentration), Figure 8. Compounds **32**, **56**, and **62** were determined to be equivalent to Flufenamic acid (**2**) in inhibiting transthyretin amyloid fibril formation, despite the different structural basis of each of these compounds. Compound **32** is not radically different than Flufenamic acid and is likely to bind in a similar conformation, placing the unsubstituted phenyl ring deep in the pocket, Figure 9. It is expected that **56** and **62** will bind similarly with the less-substituted phenyl ring reaching deep into the aromatic binding pocket. Biphenyl **62** can be considered conformationally homologous to a biaryl ether as both will be expected to prefer some degree of perpendicularity between the planes of the two rings. Whether resveratrol (**56**) binds with a coplanar orientation of the two rings or with an altered conformation is unknown at this time. Dibenzofuran **77** is likely to bind by bridging the entrance of the binding site, that is, to occupy

the space occupied by both of the orientations of the carboxylated A ring of Flufenamic acid in the Flufenamic acid-TTR cocrystal structure, but with better contacts for the two carboxylic acids than in compounds **72**–**75**, Figure 9.

Conclusion

We have utilized a screen for TTR amyloid fibril formation to test 78 small molecules of varying structure in an effort to challenge and better formulate a pharmacophoric hypothese for TTR inhibitor efficacy. The results of these experiments confirm that a carboxylic acid functional group optimizes the interaction of the inhibitor with the TTR binding site, most likely via an electrostatic interaction of Lys-15 from one or more TTR subunits. The results also suggest that molecules which either bind deep in the binding pocket or which can take advantage of the dual binding modes observed in the Flufenamic acid–TTR cocrystal structure will be the most effective at fibril inhibition, Figure 9. The most important contribution from this study is the identification of the biphenyl, dibenzofuran, biaryl ether, stilbene and flavone skeletons as lead substructures that can be functionalized in a systematic fashion to design better transthyretin amyloid fibril inhibitors. Cocrystallization studies with transthyretin are underway to determine the binding conformation(s) of lead compounds identified from this screen.

Experimental

Fibril formation assay. Wild-type TTR was purified from an *E. coli* expression system described previously. Potential inhibitors were prepared as 5.4 mM solutions in pure DMSO such that only 2 μL of solution was required to reach the desired concentration (36 μM, 10× TTR) in a 300 μL assay. In the case of the lower concentrations (3× TTR, 1× TTR, and 0.5× TTR), serial dilutions were made of the 10×TTR solution such that 2 μL provided the desired amount of compound to be tested. All of the compounds were prepared by weighing a known amount of material and making the appropriate addition of DMSO. The amyloid fibril inhibition assays were described previously.[2,17,18] Briefly, a series of eppendorf tubes filled with 200 mM sodium acetate buffered 100 mM KCl solutions (0.3 mL) containing 2 μL of different concentrations of potential inhibitor were prepared at pH 4.4. The TTR stock solution in 10 mM phosphate, pH 7.6, 100 mM KCl, 1 mM EDTA was diluted appropriately to obtain a final TTR concentration of 0.2 mg/mL (3.6 μM). In addition, two control samples containing either TTR and no inhibitor or potential inhibitors with no TTR were also evaluated. All solutions were incubated at 37 °C for 72 h and the extent of fibril formation was measured by OD at 330 nm (or 400 nm for compounds that significantly absorb at 330 nm) and by a quantitative Congo red binding assay, both assays were described in detail previously.[2,17,18] The control experiment with Flufenamic acid (10.8 μM Flufenamic acid; 3×TTR) was carried out each time providing information about the reliability of static light scattering as a screen. The 5–10% variance in fibril formation conversion yield amongst different TTR preparations does not significantly affect the screening process because the differences between the most promising inhibitors and weak inhibitors is much greater than the typical errors involved.

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