Small-Molecule Inhibitors

Benzoxazoles as Transthyretin Amyloid Fibril Inhibitors: Synthesis, Evaluation, and Mechanism of Action**

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Transthyretin (TTR) is one of twenty nonhomologous proteins, the misfolding, aggregation, and deposition (amyloidogenesis) of which is linked to amyloid disease.[1,2] TTR is a homotetramer composed of 127 amino acid subunits[3] that carries thyroxine[4] and holo-retinol binding protein[5] in the blood. The TTR tetramer is non-amyloidogenic, but undergoes dissociation, monomer misfolding, and misassembly into numerous aggregated structures including amyloid under partially denaturing conditions (for example, at low pH values).[6] Amyloidogenesis by wild type (wt) TTR appears to cause senile systemic amyloidosis,[7] whereas amyloidogenesis by one of over 80 TTR mutants results in either familial amyloid polyneuropathy[8] or cardiomyopathy.[9] Incorporation of T119M *trans*-suppressor subunits into tetramers otherwise composed of disease-associated subunits (such as V30M or L55P) inhibits amyloidogenesis in vitro and ameloriates disease in V30M carriers by increasing the dissociative activation barrier and thereby kinetically stabilizing the tetramer.[10,11] TTR has two largely unoccupied thyroxine

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binding sites that are created by its quaternary structural interface.[12] The tetramer can be stabilized by small-molecule-binding to these sites, potentially providing a means to treat TTR amyloid disease with small-molecule drugs.[13] Many families of compounds have been discovered, the binding of which stabilizes the tetrameric ground state to a degree proportional to the small-molecule dissociation constants K_{d1} and K_{d2}.[11] This binding also effectively increases the dissociative activation barrier and inhibits amyloidosis by kinetic stabilization.[11] Such inhibitors are typically composed of two aromatic rings, with one ring bearing halogen substituents and the other bearing hydrophilic substituents.[13–20] Benzoxazoles substituted with a carboxylic acid at C4–C7 and a halogenated phenyl ring at C2 also appeared to complement the TTR thyroxine binding site. A small library of these compounds was therefore prepared by dehydrocyclization of *N*-acyl amino hydroxybenzoic acids (Scheme 1).[21,22]

The benzoxazoles were evaluated by using a series of analyses of increasing stringency. A previously developed fibril-formation assay was used as the first screen.[13,15] wt TTR (3.6 μM) was incubated for 30 min (pH 7, 37 °C) with a test compound (7.2 μM). Since at least one molecule

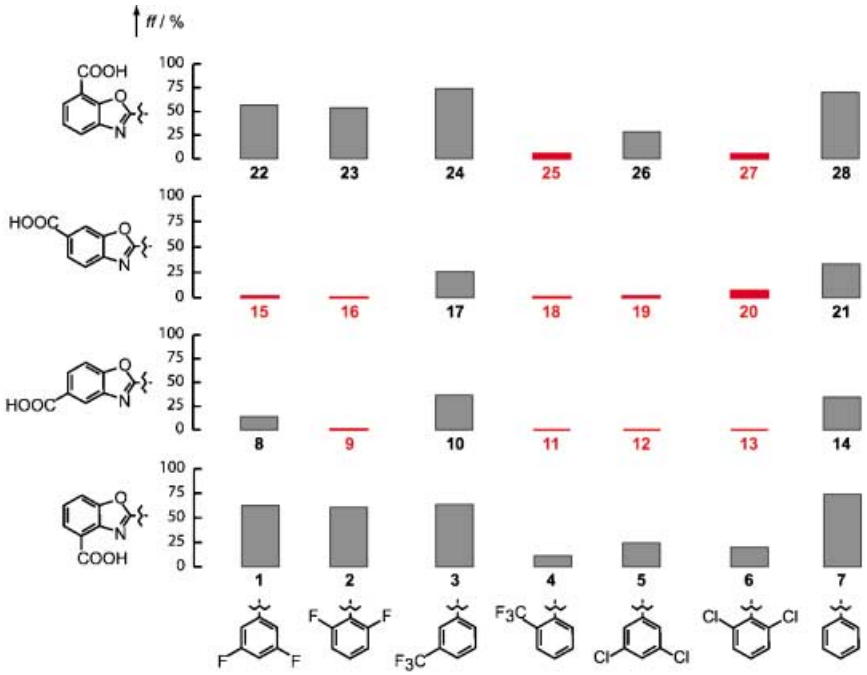
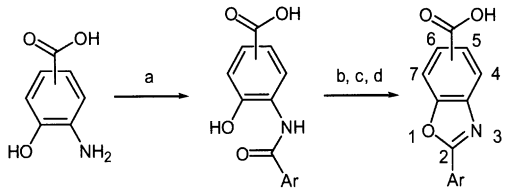


Figure 1. Suppression of TTR fibril formation by compounds **1**–**28**. The position of the carboxy group on the benzoxazole is shown on the left-hand side, while the C2 phenyl ring is shown at the bottom. The bars indicate the percent fibril formation (*ff*), that is, the amount of fibrils formed from TTR in the presence of **1**–**28** relative to the amount formed by TTR in the absence of inhibitor (which is defined as 100%). The most effective compounds (lowest *ff*) are indicated in red.



Scheme 1. General synthesis of benzoxazoles: a) ArCOCl, THF, pyridine (Ar = phenyl, 3,5-difluorophenyl, 2,6-difluorophenyl, 3,5-dichlorophenyl, 2,6-dichlorophenyl, 2-(trifluoromethyl)phenyl, and 3-(trifluoromethyl)phenyl); b) $TsOH \cdot H_2O$ (Ts = tosyl), refluxing xylenes; c) $TMSCHN_2$, benzene, MeOH; d) LiOH, THF, MeOH, H_2O (8–27% yield over four steps).

of the test compound must bind to each molecule of TTR tetramer to be able to stabilize it, a test compound concentration of 7.2 μM is only twice the minimum effective concentration. The pH value was then adjusted to 4.4, the optimal pH for fibrilization. The amount of amyloid formed after 72 h (37 °C) in the presence of the test compound was determined by turbidity at 400 nm and is expressed as % fibril formation (*ff*), 100% being the amount formed by TTR alone. Of the 28 compounds tested, 11 reduced fibril formation to negligible levels (*ff* < 10%; Figure 1, red bars).

The 11 most-active compounds were then evaluated for their ability to bind selectively to TTR over all other proteins in the blood.[23] Human blood plasma (TTR conc. 3.6–5.4 μM) was incubated for 24 h with the test compound (10.8 μM) at 37 °C. The TTR and any bound inhibitor were immunoprecipitated using a sepharose-bound polyclonal TTR antibody. The TTR with or without bound inhibitor was liberated from the resin at high pH value, and the inhibitor:TTR stoichiometry was ascertained by HPLC analysis (Figure 2).[23] Benzoxazoles with carboxylic acids in the 5- or 6-position, and 2,6-dichlorophenyl (**13**, **20**) or 2-trifluoromethylphenyl (**11**, **18**) substituents at the 2-position displayed the highest binding stoichiometries. In particular, **20** exhibited excellent inhibitory activity and binding selectivity. Hence, its mechanism of action was characterized further.

To confirm that **20** inhibits TTR fibril formation by binding strongly to the tetramer, isothermal titration calo-

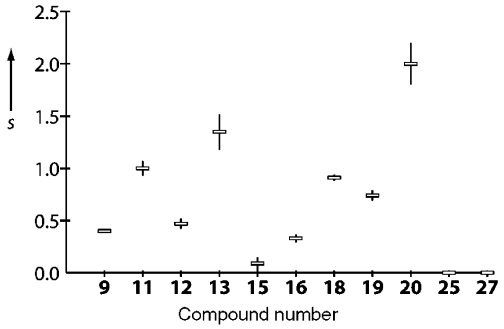


Figure 2. Stoichiometry (*s*) of benzoxazoles bound to TTR after incubation in human blood plasma; the maximum possible value of *s* is 2. The thin vertical lines indicate the measurement error. The stoichiometries shown are not corrected for wash-associated loss of small molecules and therefore are lower limits for the true stoichiometries.

rimetry (ITC) and sedimentation velocity experiments were conducted with wt TTR. ITC showed that two equivalents of **20** bind with average dissociation constants of $K_{d1} = K_{d2} = 55$ (± 10) nM under physiological conditions. These values are comparable to the dissociation constants of many other highly efficacious TTR amyloidogenesis inhibitors. For the sedimentation velocity experiments, TTR (3.6 μM) was incubated with **20** (3.6 μM, 7.2 μM, 36 μM) under optimal fibrilization conditions (72 h, pH 4.4, 37 °C). The tetramer (55 kDa) was the only detectable species in solution with **20** at 7.2 or 36 μM. Some large aggregates formed with **20** at 3.6 μM, but the TTR remaining in solution was tetrameric.

T119M subunit inclusion and small-molecule binding both prevent TTR amyloid formation by raising the activation barrier for tetramer dissociation.[11] The ability of an inhibitor to do this is most rigorously tested by measuring its efficacy at slowing tetramer dissociation in 6 M urea, a severe denaturation stress.[11] Thus, the rates of TTR tetramer dissociation in 6 M urea in the presence and absence of **20**, **21**, or **27** were compared (Figure 3). TTR (1.8 μM) was completely denatured after 168 h in 6 M urea. In contrast, **20** at 3.6 μM prevented tetramer dissociation for at least 168 h (> 3× the half-life of TTR in human plasma). With an equimolar amount of **20**, only 27% of TTR denatured in 168 h. Compound **27** (3.6 μM) was much less able to prevent tetramer dissociation (90% unfolding after 168 h), even though it was active in the fibril formation assay. Compound **21** did not hinder the dissociation of TTR at all. These results show that inhibitor binding to TTR is necessary but not sufficient to kinetically stabilize the TTR tetramer under strongly denaturing conditions; it is also important that the dissociation constants are very low (or that the off rates are very slow). Also, the arrangement of functional groups on **20** is apparently optimal for stabilizing the TTR tetramer; moving the carboxylic acid from C6 to C7, as in **27**, or removing the chlorines, as in **21**, severely diminishes its activity.

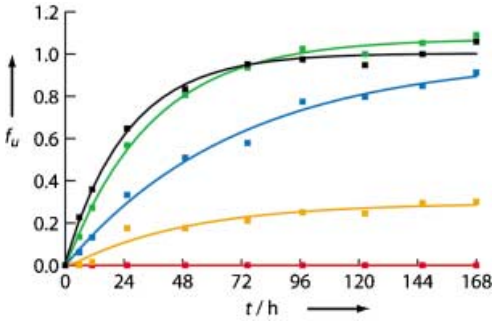


Figure 3. Dissociation as a function of time (t) for wt TTR (1.8 μM) in 6 M urea without inhibitor (——), or in the presence of 3.6 μM of **20** (——), **21** (——), or **27** (——), or 1.8 μM **20** (——). The dimensionless extent of tetramer dissociation (f_u, or fraction unfolded) was determined from the intensity of the circular dichroism spectrum at 215–218 nm. Although this is a measure of secondary structure, it corresponds indirectly to tetramer dissociation because dissociation must precede denaturation, and monomer denaturation is virtually irreversible and instantaneous in 6 M urea (the half time for denaturation is approximately 70 ms).

The role of the substituents in **20** is evident from its cocrystal structure with TTR (Figure 4). Compound **20** orients its two chlorine atoms near halogen binding pockets 2 and 2′ (so-called because they are occupied by iodine atoms when thyroxine binds to TTR). The 2,6 substitution pattern

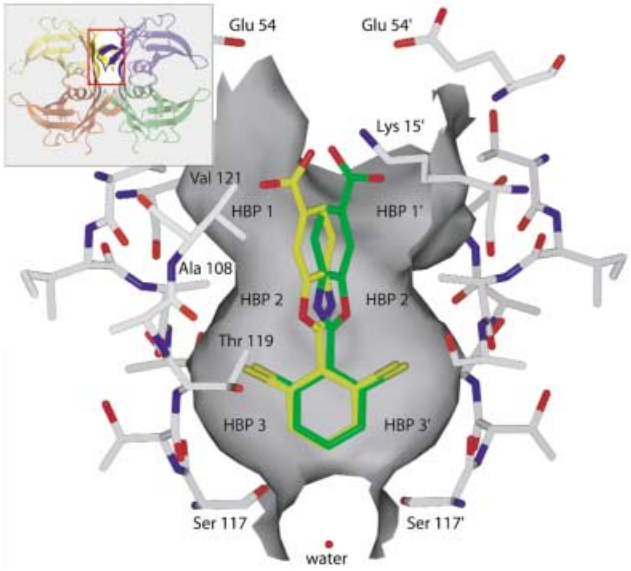


Figure 4. X-ray cocrystal structure of **20** bound to TTR. The green and yellow structures represent its two symmetry equivalent binding modes. The surface of the binding site is shown in gray. Equivalent residues in different subunits are distinguished with primed and unprimed residue numbers, as are the pairs of halogen binding pockets (HBPs). The inset shows an entire TTR tetramer (each subunit colored differently) with the binding site highlighted. Details of the crystal structure are given in the Supporting Information.

on the phenyl ring forces the benzoxazole and phenyl rings out of planarity, optimally positioning the carboxylic acid on the benzoxazole unit to hydrogen bond with the ε-NH_3^+ groups of Lys 15/15′. Hydrophobic and van der Waals interactions between the aromatic rings of **20** and the side chains of Leu 17, Leu 110, Ser 117, and Val 121 contribute additional binding energy (the side chains of Leu 17 and leu 110 obscure the view of **20** in Figure 4 and were therefore omitted for clarity).

In summary, 28 benzoxazole compounds were each prepared in four steps. Many of these compounds effectively inhibited TTR amyloid formation in vitro and bound selectively to TTR in blood plasma. Urea denaturation experiments demonstrated that **20** was particularly effective at kinetic stabilization of the TTR tetramer. The X-ray crystal structure of the TTR–**20** complex provides a structural basis for understanding its ability to stabilize the native state. The benzoxazole compounds presented here represent a new class of TTR inhibitors that may have better pharmacological properties than those described previously.[13–20] Their efficacy in vivo will be determined in animal models for TTR amyloidosis.[24]

Experimental Section

The general procedure for benzoxazole synthesis and complete characterization of the products (^{1}H and ^{13}C NMR spectroscopy and high-resolution mass spectra) are given in the Supporting Information. The analytical ultracentrifugation and X-ray crystallography experiments are also detailed in the Supporting Information. The procedures for the fibril formation assay,[13,15] the immunoprecipitation assay,[23] ITC[14,20] and urea-induced dissociation kinetics[11] have been described elsewhere.

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