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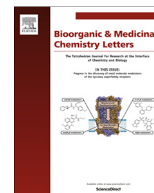

Semi-quantitative models for identifying potent and selective transthyretin amyloidogenesis inhibitors

Stephen Connelly [a], David E. Mortenson [b,c], Sungwook Choi [e], Ian A. Wilson [a,d], Evan T. Powers [a], Jeffery W. Kelly [b,c,d,*], Steven M. Johnson [f,*]

[a] Department of Integrative Structural and Computational Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, United States
[b] Department of Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, United States
[c] Department of Molecular Medicine, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, United States
[d] The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, United States
[e] Department of New Drug Discovery and Development, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejon 305-764, Republic of Korea
[f] Department of Biochemistry & Molecular Biology, Indiana University, School of Medicine, Van Nuys Medical Sciences Building, MS 0013D, 635 Barnhill Dr, Indianapolis, IN 46202, United States

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ABSTRACT

Rate-limiting dissociation of the tetrameric protein transthyretin (TTR), followed by monomer misfolding and misassembly, appears to cause degenerative diseases in humans known as the transthyretin amyloidoses, based on human genetic, biochemical and pharmacologic evidence. Small molecules that bind to the generally unoccupied thyroxine binding pockets in the native TTR tetramer kinetically stabilize the tetramer, slowing subunit dissociation proportional to the extent that the molecules stabilize the native state over the dissociative transition state—thereby inhibiting amyloidogenesis. Herein, we use previously reported structure-activity relationship data to develop two semi-quantitative algorithms for identifying the structures of potent and selective transthyretin kinetic stabilizers/amyloidogenesis inhibitors. The viability of these prediction algorithms, in particular the more robust *in silico* docking model, is perhaps best validated by the clinical success of tafamidis, the first-in-class drug approved in Europe, Japan, South America, and elsewhere for treating transthyretin aggregation-associated familial amyloid polyneuropathy. Tafamidis is also being evaluated in a fully-enrolled placebo-controlled clinical trial for its efficacy against TTR cardiomyopathy. These prediction algorithms will be useful for identifying second generation TTR kinetic stabilizers, should these be needed to ameliorate the central nervous system or ophthalmologic pathology caused by TTR aggregation in organs not accessed by oral tafamidis administration.

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Human genetic, biochemical and pharmacologic evidence implicates rate-limiting transthyretin (TTR) tetramer dissociation, followed by rapid monomer misfolding and misassembly, as the cause of several degenerative diseases exhibiting overlapping phenotypes, collectively referred to as the transthyretin amyloidoses.[1–16] The amyloidogenic TTR monomer misassembles into a variety of aggregate structures during amyloidogenesis, including cross-β-sheet amyloid fibrils, for which these diseases are named.[17–19] Amyloidogenesis of wild-type (WT) TTR or aggregation of certain mutants along with WT-TTR in heterozygotes leads to cardiomyopathies, affecting up to 500,000 individuals (disorders historically called senile systemic amyloidosis (SSA) and familial amyloid cardiomyopathy (FAC), respectively).[14,20] Amyloidogenesis of distinct TTR mutants along with WT-TTR in heterozygotes results in a primary autonomic and peripheral neuropathy, often called familial amyloid polyneuropathy (FAP). The latter disease has historically been treated by liver transplant-mediated gene therapy, wherein the mutant-TTR/WT-TTR liver (which secretes destabilized TTR heterotetramers) is replaced by a WT-TTR/WT-TTR liver (which secretes a more stable WT-TTR homotetramer). Interestingly, slowing the course of peripheral disease progression by liver transplantation has led to the appearance of TTR aggregation in the central nervous system (CNS) and eyes, which manifests as a consequence of treatment-associated lifespan extension.[21–26]

* Corresponding authors at: Department of Biochemistry & Molecular Biology, Indiana University, School of Medicine, Van Nuys Medical Sciences Building, 635 Barnhill Dr, Indianapolis, IN 46202, United States (S.M. Johnson). Department of Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, United States (J.W. Kelly).

E-mail addresses: jkelly@scripps.edu (J.W. Kelly), johnstm@iu.edu (S.M. Johnson).

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Another strategy to prevent TTR amyloidogenesis is to fashion small molecules that bind selectively in human blood to one or both of the thyroxine (T_4) binding sites comprising the tetramer made up of WT or mutant and WT subunits. Selective binding to the native tetrameric ground state of TTR over the dissociative transition state raises the kinetic barrier for subunit dissociation, substantially slowing TTR aggregation. The extent of kinetic stabilization of tetrameric TTR determines the extent to which amyloidogenesis is inhibited.[27–31] A placebo-controlled clinical trial in V30M FAP patients (a prominent mutation causing tetramer destabilization), along with a 12-month extension study, demonstrates the efficacy of this strategy in slowing the progression of autonomic and peripheral neuropathy.[32,33]

Our studies carried out over the last two decades to develop small molecule TTR amyloidogenesis inhibitors have revealed that optimal TTR kinetic stabilizers are typically composed of two aryl rings joined by linkers of variable chemical composition.[28,29,34–55] Fig. S1 and Table S1 in the Supporting Information contain compilations of the structures and experimental results for the majority of the inhibitors procured or synthesized by the Kelly laboratory during this period. Binding of these small molecules to one or both of the generally unoccupied, funnel-shaped, T_4 binding pockets strengthens the weaker dimer-dimer interface of TTR by non-covalently bridging adjacent monomeric subunits through specific hydrophobic and electrostatic interactions, as exemplified in the TTR·(**201**)$_2$ crystal structure (Fig. 1). To gauge the efficacy of candidate molecules to bind to the T_4 pockets and kinetically stabilize the TTR tetramer from dissociating and aggregating in complex biological environments, we rely on two primary assays: 1) an *in vitro* acid-mediated TTR aggregation assay carried out with recombinant TTR in buffer; and 2) an *ex vivo* TTR immunoprecipitation/HPLC assay to quantify the stoichiometry of a candidate kinetic stabilizer bound to TTR in blood plasma. These two assays are briefly explained below, with complete experimental details presented in the Supporting Information.[56,57]

The acid-mediated TTR aggregation assay (pH 4.4; 100 mM acetate buffer) probes the ability of a candidate small molecule kinetic stabilizer to bind tetrameric TTR *in vitro* and prevent amyloidogenesis under denaturing conditions that destabilize the tetramer, and largely convert the dissociated TTR monomers to aggregates within 3 days in the absence of a kinetic stabilizer. The best inhibitors prevent >90% of TTR aggregation over a 72 h time course when incubated at a concentration twice that of tetrameric TTR (i.e., 7.2 μM candidate inhibitor versus 3.6 μM tetrameric TTR). We have traditionally presented results as % fibril formation (% FF), with complete arrest of TTR aggregation (i.e., 100% inhibition) corresponding to 0% FF.

Since plasma TTR is the form that undergoes amyloidogenesis and causes degenerative phenotypes in the periphery, a kinetic stabilizer candidate must bind selectively to TTR over all the other proteins in blood plasma to be efficacious. The *ex vivo* TTR immunoprecipitation/HPLC quantification assay probes the ability of candidate kinetic stabilizers to bind selectively to TTR over the 4000+ other proteins that are present in human blood plasma. Selectively binding to TTR over albumin, which is the primary T_4 transport protein in the blood, can be particularly challenging: TTR represents <0.5% by mass of the total plasma proteome, whereas albumin comprises ~57%.[58] Potent TTR kinetic stabilizers can display average plasma TTR binding stoichiometry values, also referred to as the Plasma Binding Selectivity (PS) values, between zero (i.e., the candidate small molecule binds predominantly to plasma proteins other than TTR) and the theoretical maximum of two (i.e., the candidate kinetic stabilizer binds very selectively to TTR in blood plasma and exhibits a slow dissociation rate), owing to the presence of the two T_4 binding sites per TTR tetramer. A PS value of 0 renders a compound useless as a TTR amyloidogenesis inhibitor *in vivo*, whereas a PS value of 1 or higher is acceptable as our previous studies show that only one inhibitor bound per TTR tetramer is sufficient to kinetically stabilize the WT-TTR tetramer against amyloidogenesis.[29] In addition to mitigating off-target toxicity during the envisioned life-long use of a kinetic stabilizer to ameliorate a TTR amyloid disease, achieving a high PS value may also allow administration of lower amounts of drug while still maintaining efficacy.

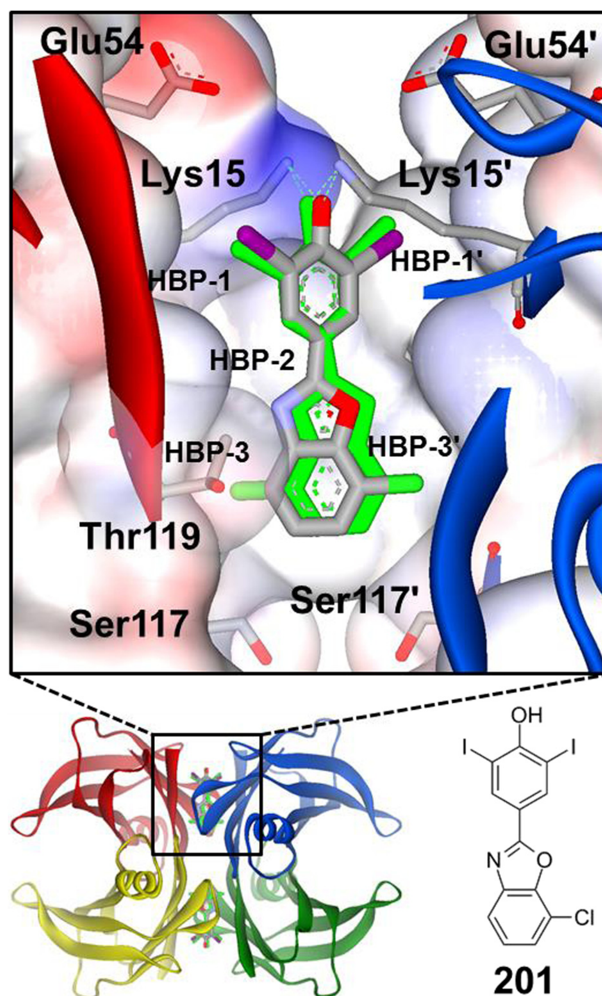

Fig. 1. X-ray structure of the TTR·(**201**)$_2$ complex (PDB ID 5TZL) highlights the interactions known to be important for tight binding to TTR. Compound **201** is bound in its equivalent symmetry-related binding modes (grey and green, respectively), which results from ligand binding along the crystallographic 2-fold axis. The omit F_O-F_C density (contoured at ±3.5σ) for **201** is shown in Fig. S3 of the Supporting Information. The binding pocket is characterized by a smaller inner cavity and a larger outer cavity, throughout which are distributed three pairs of symmetric hydrophobic depressions, referred to as the halogen binding pockets (HBPs). The iodine and chlorine atoms of **201** reside within HBPs 1 and 3, respectively. Primed amino acids or HBPs refer to symmetry-related monomers of TTR comprising each T_4 binding pocket. The phenolate of **201** makes charged interactions with the Lys 15 and 15′ residues in the outer cavity; however, it is known that other kinetic stabilizers composed of phenols exhibiting a higher pK_a preferentially bind in the opposite orientation so that the phenols can hydrogen bond with the Ser-117 and 117′ residues in the inner binding cavity (details of this phenomenon have been previously reported).[46–50]

Our early studies looked at the potential of repurposing approved non-steroidal anti-inflammatory drugs (NSAIDs) to serve as TTR kinetic stabilizers, and thus inhibit the process of TTR amyloidogenesis.[38–41] During these investigations, we discovered that diflunisal (compound **389**) is a viable TTR kinetic stabilizer and inhibitor of TTR aggregation *in vitro*. Diflunisal is an effective TTR

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