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Native State Kinetic Stabilization as a Strategy To Ameliorate Protein Misfolding Diseases: A Focus on the Transthyretin Amyloidoses

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ABSTRACT

Small molecule-mediated protein stabilization inside or outside of the cell is a promising strategy to treat protein misfolding/misassembly diseases. Herein we focus on the transthyretin (TTR) amyloidoses and demonstrate that preferential ligand binding to and stabilization of the native state over the dissociative transition state raises the kinetic barrier of dissociation (rate-limiting for amyloidogenesis), slowing and in many cases preventing TTR amyloid fibril formation. Since T119M-TTR subunit incorporation into tetramers otherwise composed of disease-associated subunits also imparts kinetic stability on the tetramer and ameliorates amyloidosis in humans, it is likely that small molecule-mediated native state kinetic stabilization will also alleviate TTR amyloidoses.

Introduction to the Transthyretin Amyloidoses

There are 20 secreted human proteins whose misfolding or misassembly outside the cell is linked to the gain of toxic function diseases known as the amyloidoses.[1−3] Each specific disease typically arises from the misfolding of one protein into cross-β-sheet structures. There is substantial genetic evidence supporting the amyloid hypothesis: the idea that the multistep process of protein misassembly, ultimately leading to amyloid fibrils, causes neurodegenerative diseases such as the transthyretin amyloidoses. Amyloid formation from an initially folded protein like transthyretin requires partial denaturation; hence, maintenance of the native state can prevent the process of amyloidogenesis from commencing.[4−12] The most reliable strategy to achieve this goal is to make the denaturation activation barrier insurmountable under physiological conditions. We refer to this therapeutic strategy as kinetic native state stabilization, which is demonstrated herein in the context of the transthyretin (TTR) amyloidoses.[13−15]

Table 1. Prominent TTR Variants Associated with Transthyretin Amyloidoses

TTR sequence	age of onset	disease
WT	>80	senile systemic amyloidosis (SSA)
V122I	60s	familial amyloid cardiomyopathy (FAC)
V30M	30−60	familial amyloid polyneuropathy (FAP)[a]
D18G	40s	central nervous system amyloidosis (CNSA)[b]

[a] Associated with >80 TTR variants in addition to V30M. [b] Associated with ∼10 TTR variants in addition to D18G.

Significant evidence links the process of TTR dissociation, misfolding, and misassembly to the tissue degeneration characterizing the amyloid diseases senile systemic amyloidosis (SSA), familial amyloid cardiomyopathy (FAC), familial amyloid polyneuropathy (FAP), and the recently discovered central nervous system selective amyloidosis (CNSA, Table 1).[16−28] SSA is a late onset sporadic disease associated with wild-type transthyretin (WT-TTR) deposition in the heart,[16,17] whereas the familial diseases (FAP, FAC, and CNSA) result from tissue selective deposition of one of >80 TTR variants.[16,18−28] The familial diseases are typically more severe and can appear as early as the second decade.[22] If left unchecked, these diseases often lead to organ dysfunction and ultimately death within 10 years.

Transthyretin is a 55-kDa homotetrameric protein composed of 127-amino acid β-sheet-rich subunits (Figure 1A).[29] Transthyretin tetramers are composed of a near-statistical distribution of mutant and wild-type subunits in heterozygotes, yet the tetrameric structure depicted in

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Steven M. Johnson was a Research Associate at Boehringer-Ingelheim Ltd. (Quebec) and is currently finishing his Ph.D. studies at The Scripps Research Institute in the Kelly Laboratory. His research interests include the design and synthesis of small molecules that inhibit the amyloidogenesis of transthyretin by a kinetic stabilization mechanism.

Yoshiki Sekijima received his M.D. in 1991 and his Ph.D. in 1998 at Shinshu University School of Medicine (Japan). He performed postdoctoral research at The Scripps Research Institute in the Kelly Laboratory. He recently demonstrated that diflunisal kinetically stabilizes transthyretin in humans after oral dosing and revealed that intracellular quality control influences extracellular misfolding pathology.

Nora S. Green received her Ph.D. from the University of California, Los Angeles (2001), and performed postdoctoral research in the Kelly Laboratory at The Scripps Research Institute focusing on bivalent ligands that impose kinetic stabilization on transthyretin. She is now an Assistant Professor at the Randolph-Macon College in Ashland, VA.

R. Luke Wiseman received his Ph.D. (2005) from The Scripps Research Institute doing research in the Kelly Laboratory. His interests are mainly focused on the kinetic stabilization of transthyretin by trans-suppression and small molecule binding and on the relationship between the transthyretin folding energetics and secretion efficiency. He is currently a postdoctoral fellow in David Ron's Laboratory at New York University.

Sara L. Adamski-Werner received her Ph.D. (2001) from the University of Illinois at Urbana-Champaign and performed postdoctoral research in the Kelly Laboratory focusing on the synthesis of small molecule inhibitors that stabilize the native state of transthyretin. She left The Scripps Research Institute in 2003 and is now a Research Associate at Senomix Inc. (La Jolla, CA).

Jeffery W. Kelly received his Ph.D. degree from the University of North Carolina at Chapel Hill (1986) and performed postdoctoral research at The Rockefeller University. In 1989, he became Assistant Professor at Texas A&M University where he was subsequently promoted to the rank of Professor. He joined the Department of Chemistry at The Scripps Research Institute in 1997 as the Lita Annenberg Hazen Professor of Chemistry. Three years later, he became Dean of Graduate Studies and Vice President of Academic Affairs. His research focuses on the chemical basis of protein folding and misfolding and the influence of small molecules on these processes in vivo.

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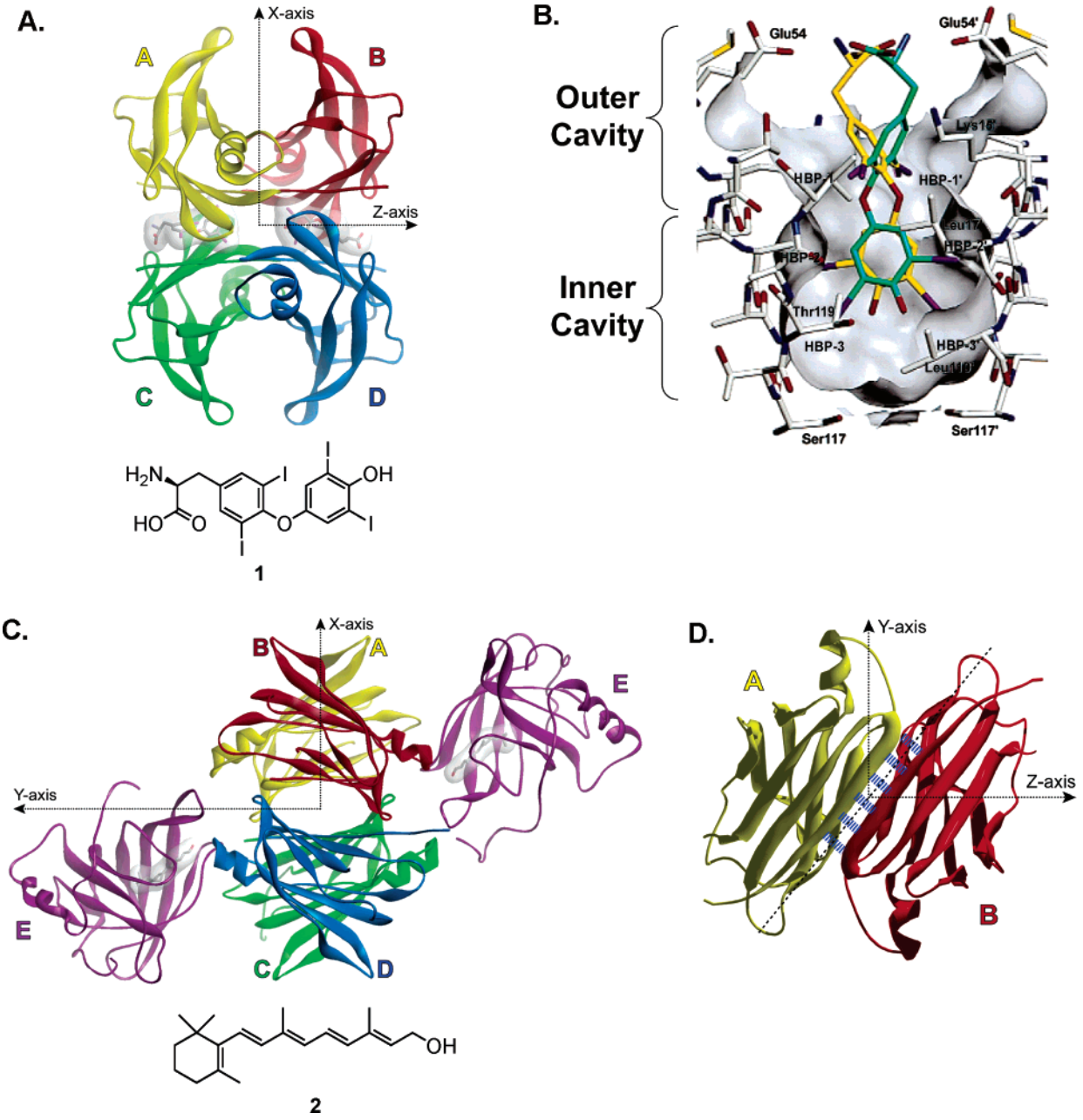


FIGURE 1. Panel A presents a ribbon diagram depiction of the human WT-TTR·$(T_4)_2$ cocrystal structure (1ICT) viewed perpendicular to the T_4 binding channel (*Z*-axis). Thyroxine (**1**) is shown in stick representation with its CPK surface depicted (transparent). Panel B presents an expanded view of one T_4 binding pocket with T_4 shown in its two symmetry-related binding modes (green and yellow) with the binding site surface shown in gray.[33] Primed and unprimed residues or HBPs (halogen binding pockets) refer to symmetry-related monomers of TTR. Panel C presents a ribbon diagram depiction of the WT-TTR·(*holo*-RBP)$_2$ cocrystal structure (1QAB) viewed down the *Z*-axis or the T_4 binding channel (view in Figure 1A rotated by 90° about the *X*-axis). All-*trans*-retinol (**2**) is shown in stick representation with its CPK surface depicted (transparent). Panel D presents a ribbon diagram depiction of the AB dimer interface (dashed line), shown with H-bonding (blue dashed lines) that propagates the extended intersubunit β-sheet (the CD dimer is identical).

Figure 1A remains invariant for all but the most destabilized mutants associated with CNSA.[24,25,28,30] Since TTR does not cross the blood−brain barrier, its concentration is maintained independently in the cerebrospinal fluid (CSF, 0.04−0.4 μM tetramer) and blood (1.8−5.4 μM tetramer) by secretion primarily from the choroid plexus and liver, respectively.[31,32] Although TTR is the primary carrier of thyroxine (T_4, **1**, Figure 1A,B[33]) in the CSF and a tertiary carrier in blood plasma, the vast majority (>99.5%) of the two T_4 binding sites within TTR in both fluids are unoccupied.[31,32] Transthyretin also carries substoichiometric levels of *holo*-retinol binding protein in the blood (Figure 1C), utilizing binding sites that are orthogonal to the T_4 sites.

While it is not yet clear how and where TTR forms amyloid in humans, biophysical studies reveal that tetramer dissociation is the rate-limiting step for amyloidogenesis and that the natively folded monomer must first undergo partial denaturation to become competent for

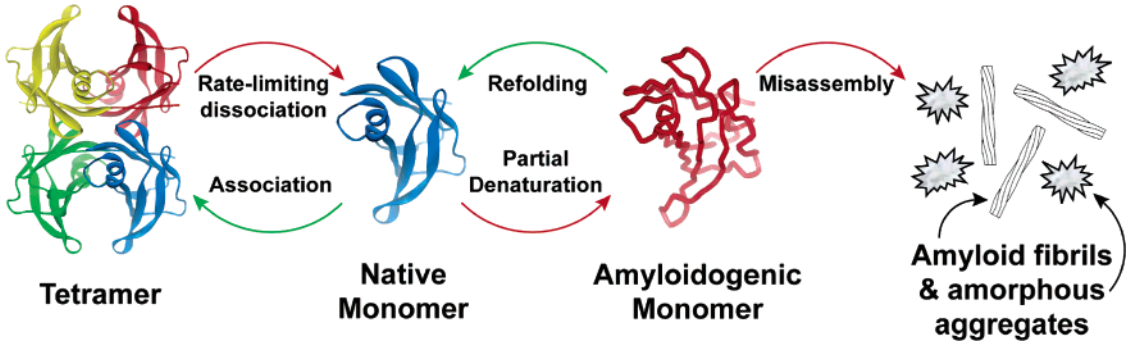


FIGURE 2. Schematic of the TTR amyloidogenesis pathway based on biophysical studies with insight from pathological studies. Rate-limiting tetramer dissociation affords natively folded monomers that can either reassociate or partially unfold. At sufficient concentration, the misfolded monomers can assemble via a downhill polymerization to afford a variety of aggregate morphologies including amorphous aggregates and amyloid fibrils. The small soluble TTR oligomers formed early in this pathway are reported to be cytotoxic.[27,65]

misassembly through a nonnucleated thermodynamically favorable process (Figure 2).[4−8,13,14,34,35] This occurs very inefficiently under physiological conditions but is accelerated under acidic conditions because the tetramer to natively folded monomer to partially denatured monomer equilibria are shifted toward the latter, facilitating amyloidogenesis.[4−8,34]

The only treatment currently available for FAP is gene therapy mediated by liver transplantation, wherein the FAP variant/WT-TTR secreting liver is surgically replaced by a WT/WT liver. This decreases plasma variant TTR levels to less than 5% of pretransplant levels, halting disease progression in most patients, at least initially.[36−38] Unfortunately, cardiac amyloidosis often progresses in familial patients even after transplantation due to the continued deposition of WT-TTR.[39] Despite the inability of liver transplantation to prevent WT-TTR deposition, it has provided invaluable insight in terms of demonstrating that TTR amyloidosis can be treated by lowering the concentration of the more amyloidogenic sequence in heterozygotes.

Interallelic Trans-Suppression Prevents TTR Amyloidosis in Humans by Kinetic Stabilization

In 1993, Coelho et al. reported a compound heterozygous Portuguese family that expresses the highly penetrant V30M FAP variant, yet these individuals do not develop disease.[40,41] The V30M-TTR plasma concentration of these compound heterozygotes was the same as that found in V30M/WT Portuguese heterozygotes who develop FAP. The resistant family is distinct in that they express a T119M variant instead of WT-TTR from their second allele, resulting in the production of seven different TTR tetramers of varying V30M and T119M subunit stoichiometry (Figure 3A).[13,14] The inclusion of T119M subunits into tetramers also composed of disease-associated subunits raises the kinetic barrier of tetramer dissociation (as demonstrated below), protecting these individuals from disease by a process we refer to as interallelic trans-suppression.[13,14,40,41]

By adding an acidic tandem flag-tag sequence to the N-terminus of the T119M subunits, one can separate the five tetramers of defined stoichiometry by anion exchange chromatography and evaluate them individually, Figure 3A.[14,42] Increasing the T119M subunit stoichiometry relative to V30M in the tetramer dramatically reduces the amyloidogenesis rate under acidic conditions (Figure 3B) and the rate of tetramer dissociation in urea (Figure 3C).[14] The dissociation rate in 6 M urea varies linearly with T119M subunit stoichiometry (data shown in ref 14). Thermodynamic cycle analysis of T119M and WT homotetramers reveals that these have very similar thermodynamic stabilities (Figure 4A).[14] In contrast, analysis of tetramer dissociation and association kinetics demonstrates that the T119M homotetramer has a substantially higher dissociation barrier than that of the WT homotetramer.[13,14] Therefore, the protective influence of the T119M suppressor subunit inclusion arises from destabilization of the tetramer dissociation transition state, thereby kinetically stabilizing the native tetramer, as opposed to thermodynamic stabilization of the native state. The M119 side chains project into the thyroxine binding pockets (Figure 4B)[43] and impart kinetic stability; hence alterations in this region conferred by small molecule binding may do the same, a hypothesis supported by perturbation of this quaternary structural interface by mutagenesis and protein engineering.[44−48]

Discovering Small Molecules that Bind to the Thyroxine Binding Sites of TTR

TTR has two unique dimer−dimer interfaces (Figure 1A). The more robust AC/BD interface bisected by the *X*-axis is stabilized by interstrand hydrogen bonding (Figure 1A,D).[29,32,44] The energetically weaker AB/CD interface creates the two funnel-shaped hydrophobic T_4 binding cavities and encompasses the *Z*-axis (crystallographic 2-fold axis, Figure 1A,B). Each of the two thyroxine binding sites is characterized by a small inner cavity and a larger outer cavity with three pairs of symmetric depressions distributed throughout that are referred to as the halogen binding pockets (HBPs), wherein the iodine atoms of T_4 reside (Figure 1B). Although the HBPs are primarily hydrophobic, TTR conformational changes can reveal hydrogen bond donor and acceptor sites.[33] Thyroxine

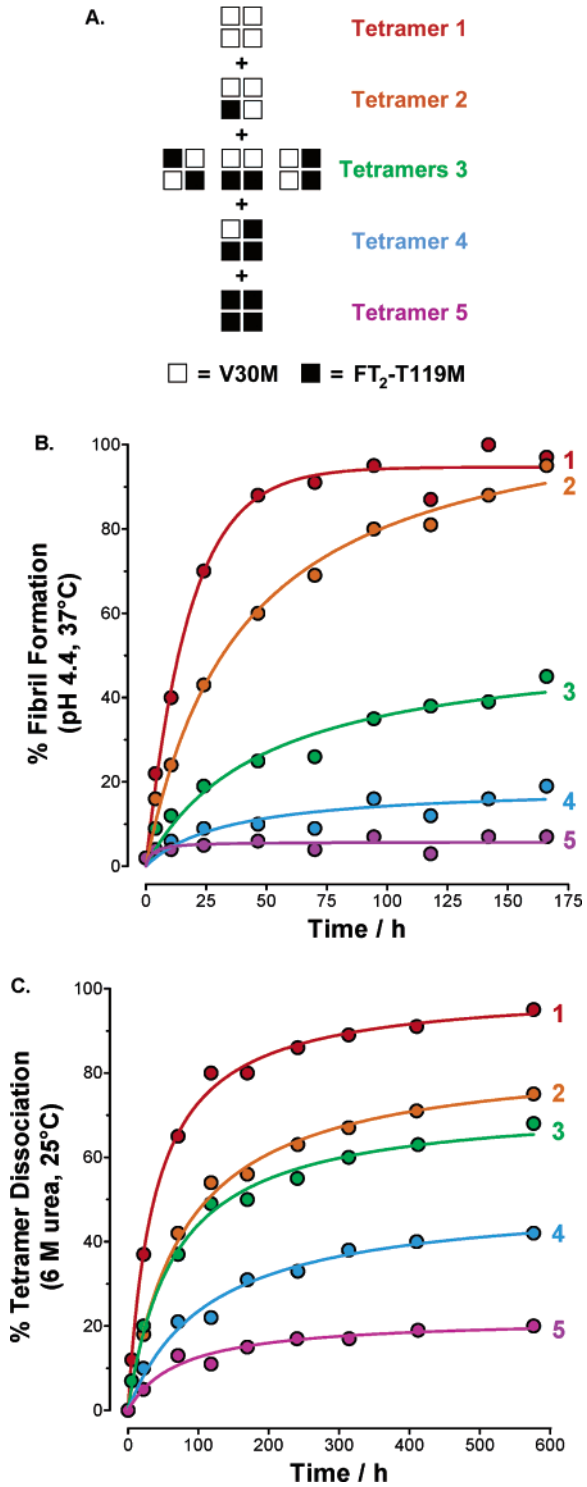


FIGURE 3. Panel A shows a schematic representation of the seven different T119M, V30M-TTR, or mixed tetramers produced during coexpression of the alleles. T119M subunits were appended with a tandem flag-tag (FT) sequence ({DYKDDDDK}$_2$) attached to the N-terminus, which enables separation and isolation of each subunit stoichiometry via anion exchange chromatography (tetramers 3 are chromatographically inseparable).[14,42] Panel B shows acid-mediated aggregation time courses of tetramers **1**−**5** followed by turbidity (3.6 μM tetrameric TTR, pH 4.4, 37 °C).[14] Panel C shows urea-mediated tetramer dissociation time courses of tetramers **1**−**5** (1.8 μM tetrameric TTR, 25 °C, 6.0 M urea) evaluated by linking tetramer dissociation to the rapid, irreversible monomer unfolding as detected by tryptophan fluorescence.[14]

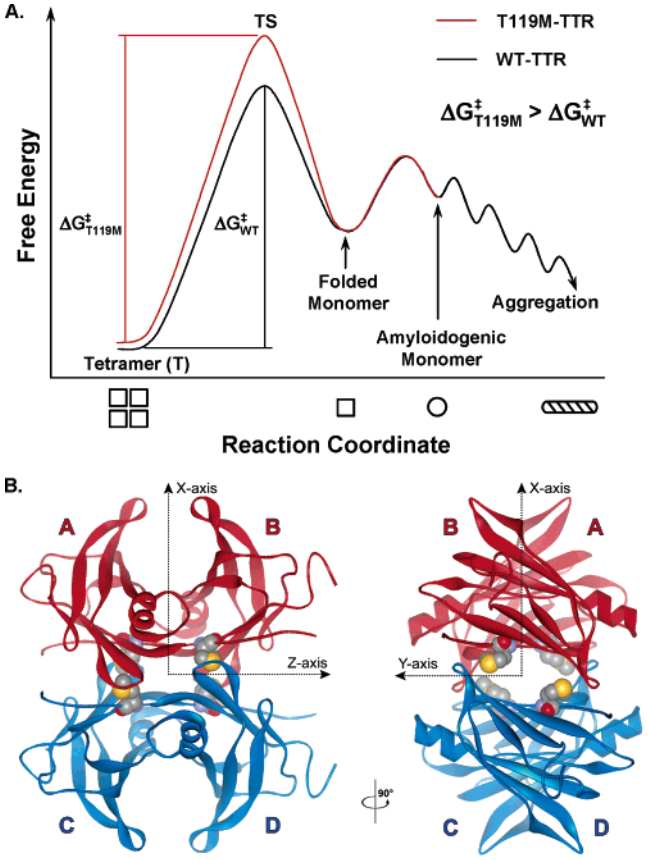


FIGURE 4. Panel A presents an energy landscape diagram depicting the mechanism by which T119M subunit inclusion into the tetramer functions to inhibit amyloid formation. T119M homotetramers have a destabilized transition state (TS), thereby increasing the activation barrier associated with tetramer dissociation ($\Delta G^{\ddagger}$) in relation to WT-TTR ($\Delta G^{\ddagger}_{T119M} > \Delta G^{\ddagger}_{WT}$). The increased activation barrier decreases the concentration of folded monomers (□) that can misfold (○) and aggregate to form amyloid.[14] Panel B presents ribbon diagram views of the T119M-TTR crystal structure (1BZE) with the T119M side chains shown in CPK representation. The weaker dimer−dimer interface, AB (red)/CD (blue), is perturbed by this mutation.

binds to these two sites with negative cooperativity, which appears to arise because T_4 binding to the first site causes TTR conformational changes, rendering the second site less suitable for binding.[31,49,50]

Over the past decade, hundreds of small molecules that bind with high affinity to the thyroxine binding sites within TTR have been identified.[26,33,50−63] Most, like thyroxine, bind with negative cooperativity; however, a minority of small molecules display non- or positively cooperative binding, although the structural basis for this is not yet clear.[14,57,64] Some of these small molecule ligands were discovered through focused screening, whereas the majority were created based on TTR·(small molecule)$_2$ co-crystal structures utilizing structure-based design principles.[33] Small molecule TTR ligands are typically composed of two aromatic rings, either linked directly as a biaryl or separated by linkers of variable chemical structure (Figure 5). Typically, one aromatic ring is substituted with a polar substituent while the other ring displays

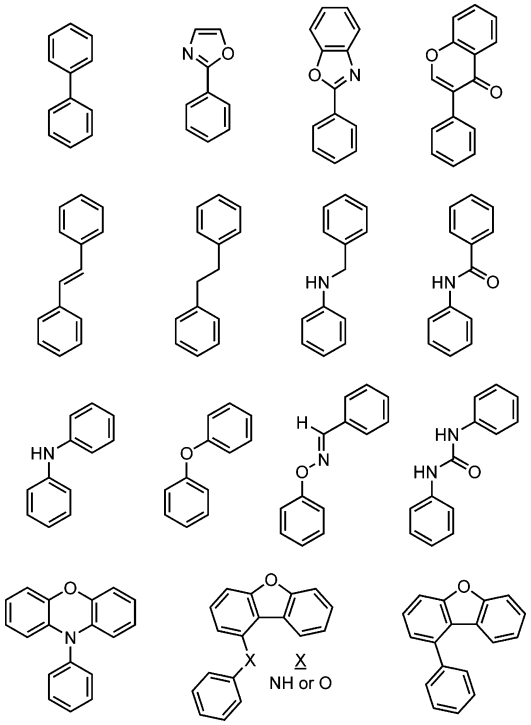


FIGURE 5. Line drawings of the structural cores of the better TTR amyloidogenesis inhibitors. One aromatic ring typically bears polar substituents (CO_2H, OH), whereas the other bears halides, trifluoromethyl, or alkyl substituents.[26,33,50−62]

halogenated substituents, alkyl groups, or a combination thereof (substituents not shown in Figure 5). Polar substituents (i.e., carboxylates or phenolates) can make important electrostatic interactions with the Lys-15 ϵ-NH_3^+ group and to some extent with the Glu-54 carboxylic acid when these rings are positioned in the outer binding site (Figure 1B). The halogenated or alkylated aryl rings complement the hydrophobicity of the inner binding pocket by occupying a subset of the HBPs. As a consequence of these interactions, small molecules typically bind to TTR in the "forward mode" wherein the aromatic ring bearing an anionic substituent prefers the outer binding pocket owing to electrostatic interactions with the Lys-15 ϵ-NH_3^+ group.[33,52,54−56,59,62] Occasionally structure-based design leads to unpredicted results such as ligands that bind in the "reverse mode" (i.e., with the carboxylic acid substituted ring oriented into the inner binding pocket), some of which display high binding affinity.[33,52,56]

Small Molecule-Mediated Kinetic Stabilization of the Native State of Transthyretin

Since T119M TTR subunit inclusion prevents FAP by kinetic stabilization of the tetramer, we have focused on small molecules that act analogously by preferentially binding to and stabilizing the native state of TTR over the dissociative transition state.[14,63] This is the most conservative therapeutic strategy because it prevents the process of amyloidogenesis from beginning. Moreover, this approach should be effective even if the misfolded monomer or small soluble TTR oligomers mediate cytotoxicity, which has recently been reported.[27,65] While numerous thyroid hormone analogues bind to the largely unoccupied[31,32] thyroxine binding sites and impose kinetic stabilization on tetrameric TTR, we desire high-affinity ligands that are neither thyroid agonists nor antagonists to mitigate toxicity in long term or prophylactic use.

Biophysical experiments demonstrate that inhibitor (I) binding to the first (T·I) and second ($T{\cdot}I_2$) thyroxine binding sites additively increase the activation barrier associated with tetramer dissociation ($\Delta G^{\ddagger}$), accomplished by preferential stabilization of the tetrameric ground states relative to the dissociative transition state ($\Delta G^{\ddagger}_{T\cdot I_2} > \Delta G^{\ddagger}_{T\cdot I} > \Delta G^{\ddagger}_{T}$, Figure 6A), imposing native state kinetic stabilization.[14] Kinetic stabilization is demonstrated by evaluating tetramer dissociation rates as a function of inhibitor concentration under a variety of conditions. Urea-mediated denaturing conditions enable the rate of tetramer dissociation to be monitored by linking dissociation to rapid monomer unfolding, detected by far-ultraviolet circular dichroism spectroscopy.[14,56,59,61] Tetramer dissociation kinetics under physiological conditions are evaluated by monitoring subunit exchange between WT-TTR and flag-tag WT-TTR homotetramers, possible because monomer reassembly into tetramers is fast relative to rate-limiting tetramer dissociation.[42,66] In both cases, the rate of tetramer dissociation decreases as the stoichiometry of bound small molecule increases (Figure 6B, analogous subunit exchange data not shown).[14,56,59,61,66]

Since tetramer dissociation is rate-limiting for fibril formation, it is also useful to evaluate the rate of TTR amyloidogenesis as a function of inhibitor concentration to demonstrate kinetic stabilization. The rate of acid-mediated amyloidogenesis decreases as the stoichiometry of bound small molecule increases (Figure 6C). Many inhibitors dramatically slow acid-mediated amyloidogenesis, whereas the best completely inhibit aggregation at a concentration (7.2 μM) twice that of the physiological concentration of TTR (3.6 μM tetramer).[26,33,50−62] Because both acid- and urea-mediated denaturation alter the small molecule binding constants, the subunit exchange method appears to be best suited to rank order compound efficacy under physiological conditions.[66]

That small molecule binding stabilizes and preserves the native quaternary structure of TTR is best demonstrated by equilibrium analytical ultracentrifugation utilizing acidic conditions (pH 4.4, 37 °C), where TTR (3.6 μM tetramer) efficiently dissociates and self-assembles into high molecular weight aggregates in the absence of inhibitor. TTR samples (3.6 μM tetramer) preincubated with inhibitor (7.2−10.8 μM) are evaluated after several days of acid incubation (aggregation is nearly complete after 72 h in the absence of inhibitor), revealing the extent of tetrameric TTR retained. The best inhibitors maintain the tetrameric structure of TTR.[26,33,52−56,58−62]

Inspection of numerous TTR·$(\text{inhibitor})_2$ X-ray cocrystal structures suggested that it was possible to design a single molecule that occupies both thyroxine binding sites

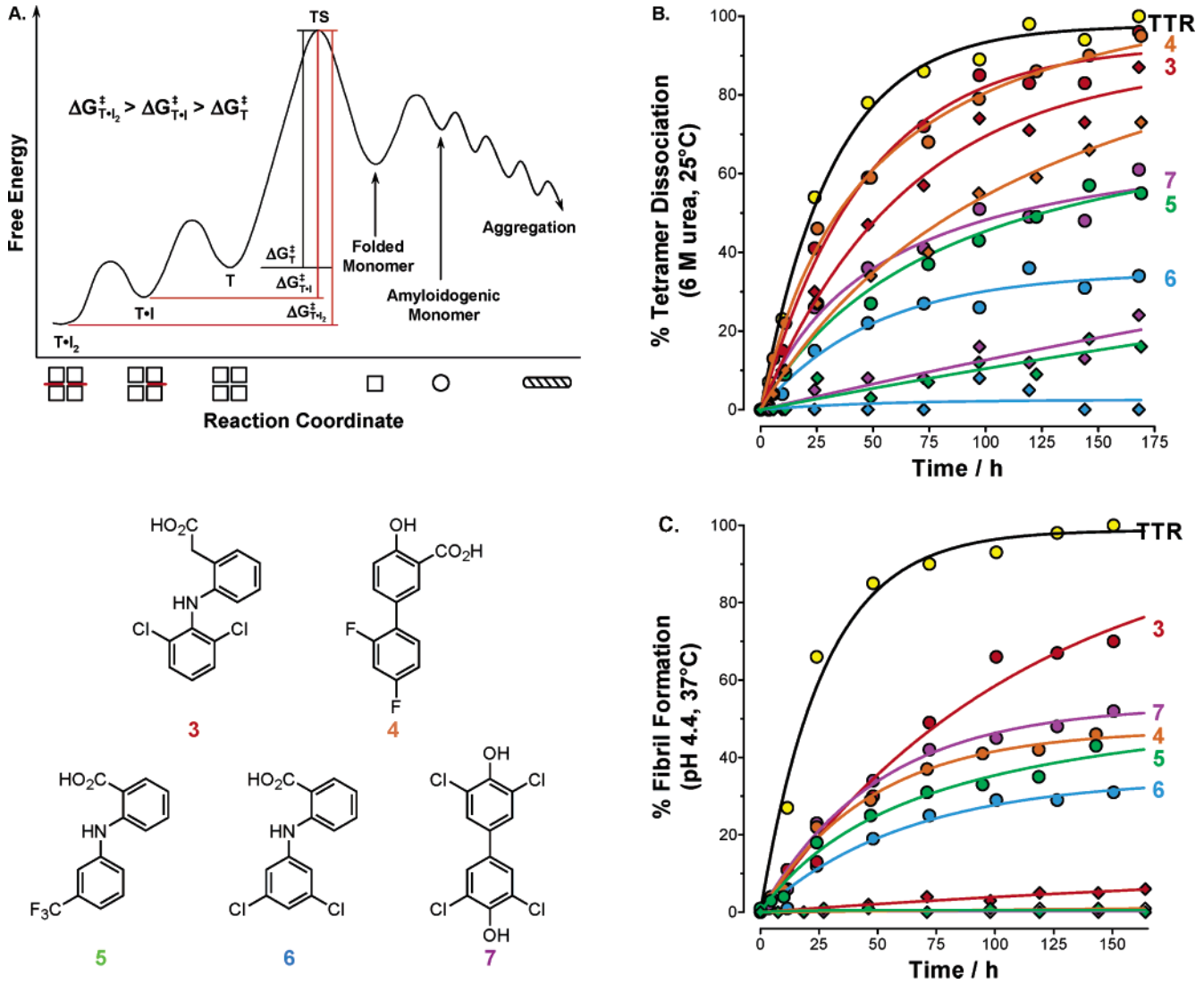


FIGURE 6. Panel A presents an energy landscape diagram depicting the mechanism by which selective small molecule binding to the native state inhibits amyloid formation. Inhibitor binding to one (T·I) or both ($T\cdot I_2$) thyroxine binding sites increases the activation barrier associated with tetramer dissociation ($\Delta G^{\ddagger}$) through stabilization of the tetrameric ground state relative to the dissociative transition state ($\Delta G^{\ddagger}_{T\cdot I_2} > \Delta G^{\ddagger}_{T\cdot I} > \Delta G^{\ddagger}_{T}$), thereby decreasing the concentration of folded monomers (□) that can misfold (○) and aggregate.[14] Panel B shows urea-mediated TTR tetramer dissociation time courses for inhibitors **3**−**7**, evaluated by far-ultraviolet circular dichroism (25 °C, 6.0 M urea, 1.8 μM tetrameric TTR); inhibitors were evaluated at 1.8 (○) and 3.6 μM (◇) concentrations. Panel C shows acid-mediated TTR aggregation time courses for inhibitors **3**−**7** followed by turbidity (pH 4.4, 37 °C, 3.6 μM tetrameric TTR); inhibitors were evaluated at 3.6 (○) and 7.2 μM (◇) concentrations.

simultaneously (Figure 7A). These bivalent inhibitors were expected to have a higher binding affinity to TTR than their monovalent counterparts. Symmetric and asymmetric bivalent inhibitors were synthesized, the latter featuring components having a strong binding orientation preference.[67] Although these inhibitors do not bind to preassembled tetrameric TTR, they are incorporated into the tetramer during its folding and assembly as revealed by biophysical and X-ray cocrystallographic analyses (Figure 7B). The TTR·bivalent inhibitor complexes do not dissociate or form amyloid on any reasonable biological time scale, demonstrating their high kinetic stability. Mammalian cell-based studies reveal that these inhibitors are endoplasmic reticulum (ER) permeable and template the folding and assembly of TTR in this organelle, a requirement for their function in humans.[28]

Although inhibitor binding to both thyroxine binding sites clearly imposes kinetic stability upon the entire tetramer, we have recently established that occupancy of only one thyroxine binding site within TTR is sufficient to impart kinetic stabilization on the entire tetramer.[48] The sufficiency of occupying only one binding site to prevent tetramer dissociation has been demonstrated by various methods including covalently tethering one inhibitor to a TTR tetramer, using subunit exchange analysis as a function of inhibitor concentration, and indirectly by covalently tethering the A and C subunits.[44,48,66] These results suggest that an inhibitor exhibiting strong nega-

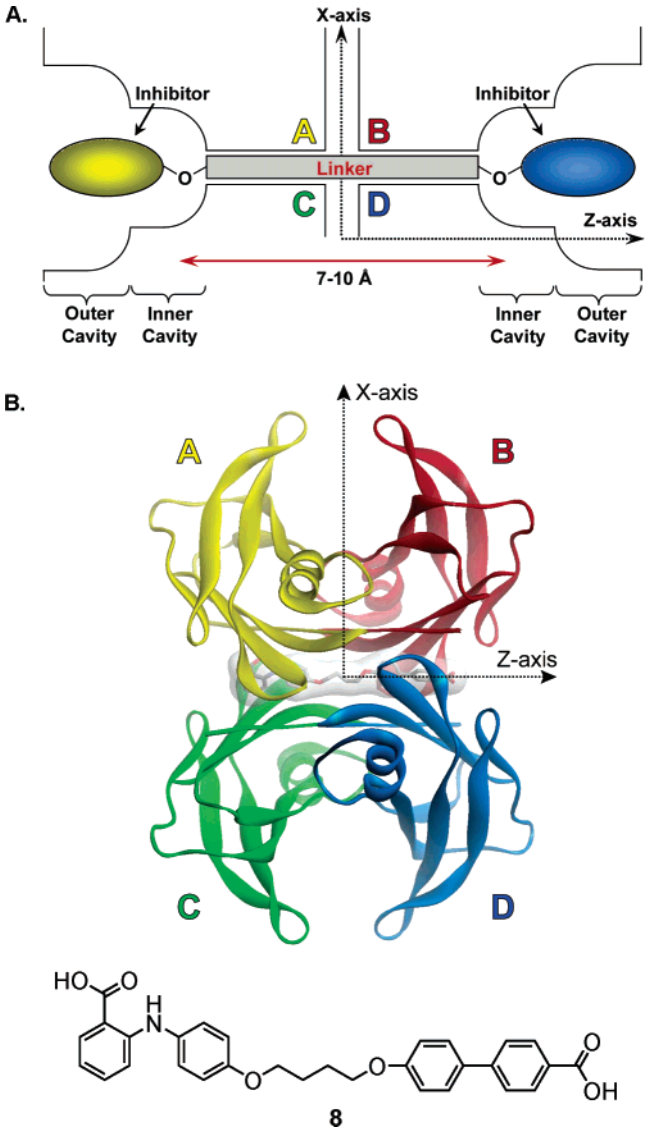


FIGURE 7. Panel A shows a schematic representation of a bivalent amyloidogenesis inhibitor bound to tetrameric TTR. Small molecule inhibitor substructures (yellow and blue) bound in each of the two thyroxine binding sites are connected by a linker that fills the channel.[67] Panel B shows the crystal structure of bivalent inhibitor **8** simultaneously occupying both thyroxine binding pockets.[67] The inhibitor, in only one of its two symmetry-equivalent binding modes, is shown in stick representation with a transparent CPK surface.

tively cooperative binding would be ideal for pharmaceutical applications, although this remains to be proven in vivo.

Effective Amyloidogenesis Inhibitors Must Bind Selectively to TTR in Vivo

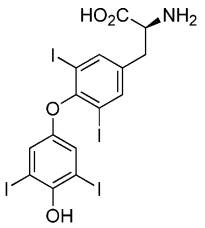
Transthyretin amyloidogenesis inhibitors must be able to bind selectively to TTR over the plethora of other proteins in blood to impose kinetic stabilization on TTR and minimize interference in other biological processes. Therefore, we have and are developing methods to measure the binding stoichiometry of inhibitors to TTR in biological fluids.[68] Incubation of a potential inhibitor (10.8 μM) with blood plasma (~5 μM TTR), followed by the capture of TTR, TTR·I, and TTR·I_2 by a resin-conjugated antibody enables HPLC analysis of the average inhibitor binding stoichiometry. Because of required wash steps, this approach can lead to underestimation of the true binding stoichiometry; however, inhibitors exhibiting high binding stoichiometry (>1.5, 2 being maximal) necessarily have low dissociation constants from TTR. The most potent inhibitors also exhibiting high TTR plasma binding selectivity (**6**, **7**, and **9**−**14**) are presented in Figure 8.[56−59,61,62,68] Thyroxine (**1**) and three FDA approved nonsteroidal antiinflammatory drugs (NSAIDS), diclofenac (**3**), diflunisal (**4**), and flufenamic acid (**5**), that have proven to be good TTR aggregation inhibitors are also displayed for comparison.

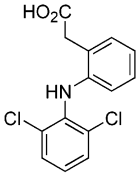
Emerging evidence suggests that thyroid hormone-like substitution patterns confer excellent TTR plasma binding selectivity (e.g., 3,5-dihalide-4-hydroxy substructures).[56,57,59,61,62,68] Preliminary unpublished data demonstrates that many of these compounds are not thyroid hormone receptor agonists or antagonists. As a consequence, in the future we will be more inclined to utilize thyroid hormone-like substructures to seek inhibitors with optimal pharmacological profiles.

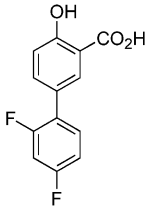
Small Molecule Kinetic Stabilization of TTR; Proof of Principle in Human Clinical Trials

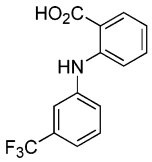
Given that the drug development process can be arduous, we were fortunate to discover three NSAIDs (diclofenac **3**, diflunisal **4**, and flufenamic acid **5**) that inhibit TTR amyloidosis owing to their chemical structure, not because of their NSAID activity. These provide the opportunity to immediately test whether small molecule-mediated kinetic stabilization of TTR will ameliorate amyloidosis in humans, an outcome we are optimistic about because interallelic trans-suppression prevents FAP by an analogous mechanism. Diflunisal and flufenamic acid exhibit notable inhibition of both acid-mediated aggregation and urea-mediated tetramer dissociation of the most common disease-associated variants.[26] The excellent oral bioavailability of diflunisal (>200 μM plasma concentration at its typical 250 mg dosage) and its ability to stabilize TTR in human plasma (Yoshiki Sekijima, unpublished results), in combination with its demonstrated safety record (albeit in a different patient population), makes diflunisal a good candidate for a placebo-controlled, multicenter FAP clinical trial, which has already begun (directed by Dr. Martha Skinner and Dr. John Berk at Boston University).

Because diflunisal has liabilities associated with its NSAID activity (especially in familial patients who typically have renal amyloid deposition and compromised function of the kidneys and gastro-intestinal tract), there is incentive for the continued development of new, selective TTR kinetic stabilizers. One such compound discovered at the Scripps Research Institute lacking NSAID activity has thus far exhibited favorable pharmacology and toxicology profiles, providing hope that it will be a suitable candidate for clinical trials soon.

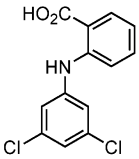


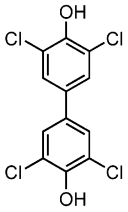


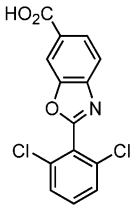


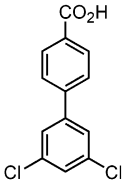


		1	**3**	**4**	**5**
% *f.f.*	**7.2 μM I**	5%	10%	1%	1%
	3.6 μM I	43%	42%	24%	27%
Binding Stoichiometry		0.86	0.04	0.14	0.20
K_{d_1}, K_{d_2}/nM		44, 2080	60, 1200	75, 1110	30, 250

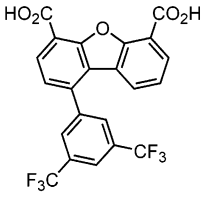


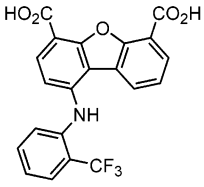


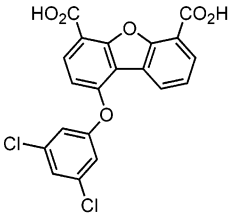


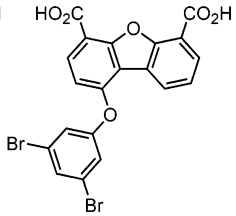


		6	**7**	**9**	**10**
% *f.f.*	**7.2 μM I**	0%	0%	7%	5%
	3.6 μM I	32%	17%	N/A	41%
Binding Stoichiometry		1.43	1.36	2.00	1.56
K_{d_1}, K_{d_2}/nM		5, 5	3, 3	N/A	N/A









		11	**12**	**13**	**14**
% *f.f.*	**7.2 μM I**	0%	0%	2%	1%
	3.6 μM I	30%	25%	36%	35%
Binding Stoichiometry		1.54	1.69	1.98	1.68
K_{d_1}, K_{d_2}/nM		N/A	N/A	N/A	N/A

FIGURE 8. Structures, dissociation constants, fibril formation inhibition potency (100% *ff* observed in the absence of inhibitor, 3.6 μM tetrameric TTR), and plasma binding stoichiometry (maximum of 2 at an inhibitor concentration of 10.8 μM) data for the most promising TTR amyloidogenesis inhibitors discovered to date (**6**, **7**, and **9**−**14**). Thyroxine (**1**) and three FDA-approved NSAIDs found to be inhibitors of TTR aggregation are shown for comparison: diclofenac (**3**); diflunisal (**4**); and flufenamic acid (**5**).[26,33,50−62] TTR (3.6 μM) % aggregation is shown for both 7.2 and 3.6 μM inhibitor (I) at pH 4.4, 72 h (37 °C).

Implications for Other Protein Misfolding/ Misassembly Diseases

The studies discussed herein demonstrate that kinetic stabilization of the native state of a misfolding competent protein is an effective strategy to prevent the sampling of unfolded or partially denatured states leading to amyloidogenesis. Therapeutic strategies utilizing kinetic stabilization mechanisms are currently being developed for a number of protein aggregation diseases, including amyotrophic lateral sclerosis (ALS), where superoxide dismutase (SOD) aggregation is associated with some

forms of the disease,[69] and familial lysozyme amyloidosis, where variants of lysozyme render the protein amyloidogenic.[12] A recent publication demonstrates that small molecules can stabilize the native SOD dimer, preventing dissociation and subsequent aggregation associated with ALS.[69] A camelid monoclonal antibody that binds to the native state of lysozyme also has been demonstrated to prevent misfolding and subsequent aggregation.[12] These results highlight the potential for treating protein aggregation diseases with therapeutic strategies that take advantage of native state kinetic stabilization.

Small molecule-mediated kinetic stabilization of the native state of a protein may also be an effective therapeutic strategy to prevent loss of function protein misfolding diseases (e.g., lysosomal storage diseases and cystic fibrosis), where pathology is associated with the inability of a protein to efficiently fold in the ER, precluding its trafficking to its destination environment (e.g., the lysosome or plasma membrane, respectively).[70,71] Recent publications focusing on small molecule-assisted folding of mutation-compromised enzymes (e.g., β-glucocerebrosidase and α-galactosidase variants associated with Gaucher's and Fabry's disease, respectively) demonstrate that the addition of small molecules (pharmacological chaperones) can stabilize the variant protein in the endoplasmic reticulum, allowing for its proper trafficking to the lysosome.[71,72] While it is likely that many pharmacological chaperones operate through a kinetic stabilization mechanism, this remains to be proven. These examples demonstrate that small molecule-mediated stabilization of the native state can be used to ameliorate loss of function protein misfolding diseases in addition to alleviating gain of toxic function diseases associated with aggregation.

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