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The Transthyretin Amyloidoses: From Delineating the Molecular Mechanism of Aggregation Linked to Pathology to a Regulatory Agency Approved Drug

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Abstract

Transthyretin (TTR) is one of the many proteins that are known to misfold and aggregate (i.e., undergo amyloidogenesis) *in vivo*. The process of TTR amyloidogenesis causes nervous system and/or heart pathology. While several of these maladies are associated with mutations that destabilize the TTR native quaternary and/or tertiary structure, wild type TTR amyloidogenesis also leads to the degeneration of post-mitotic tissue. Over the past twenty years, much has been learned about the factors that influence the propensity of TTR to aggregate. This biophysical information led to the development of a therapeutic strategy, termed “kinetic stabilization”, to prevent TTR amyloidogenesis. This strategy afforded the drug, tafamidis (trade name: Vyndaqel®), which was recently approved by the European Medicines Agency for the treatment of Transthyretin Familial Amyloid Polyneuropathy (TTR-FAP), a common familial TTR amyloid disease. Tafamidis is the first, and currently the only, medication approved to treat TTR-FAP. Here we review the biophysical basis for the kinetic stabilization strategy and the structure-based drug design effort that led to this first-in-class pharmacologic agent.

Introduction

Transthyretin (TTR) is a tetrameric protein found in the bloodstream at a concentration of ≈ 5 μM, comprising identical 127-amino-acid β-sheet-rich subunitsin homozygotes (Figure 1A).[1–3]In heterozygotes, the TTR tetramers are made up of variant and/or wild type subunits, combined in a statistical fashion.[4] The established function of TTR in the blood is to transport *holo*-retinol binding protein.[5]While TTR could transport up to 2 equivalents of retinol binding protein bound to retinolper TTR tetramer, the average stoichiometry of *holo*-retinol binding protein bound to TTR in blood is ~0.5 equivalents.[5–8]While TTR is the major carrier of thyroxine (T_4) in the blood of rodents, utilizing binding sites that are orthogonal to those used for *holo*-retinol binding protein, the T_4 binding sites are effectively

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unoccupied in humans.[9]There are three T_4 binding proteins in human blood: TTR, albumin, and thyroid binding globulin (TBG). Of these three, albumin has the highest concentration ([albumin] = 620 μM; [TTR] = 5 μM; [TBG] = 0.3 μM) and thyroid binding globulin has the highest affinity for T_4 ($K_{d,TBG}$ = 0.1nM; $K_{d,TTR}$ = 15 nM; $K_{d,albumin}$ = 1.5 μM).[10,11]These facts, combined with the low T_4 concentration in blood (0.1 μM), mean that very little (< 10%) of the T_4 in human blood is bound to TTR, and virtually all (>99 %) of the T_4 binding sites in TTR are unoccupied and available for small molecule binding. This circumstance will be taken advantage of as described below in our kinetic stabilization strategy to prevent TTR amyloidogenesis.[9,12] While TTR is the primary carrier of T_4 in cerebrospinal fluid (CSF), the low concentration of T_4 in CSF leads to the situation being similar to that in the blood, in that the vast majority of the T_4 binding sites are unoccupied.[13,14]

The TTR tetramer has two distinct dimer-dimer interfaces, the less stable of which is bisected by the crystallographic 2-fold axis,[15] or the Z-axis. It is this interface that creates the two identical funnel-shaped T_4 binding sites (Figure 1A, B).[1,12,16]The largely hydrophobic T_4 binding sites each have a small inner binding subsite and a larger outer subsite (Figure 1B).[12,17,18] The T_4 binding sites display positively (Lys 15) and negatively (Glu 54) charged side chains at their periphery, which complement the zwitterionic structure of T_4 (Figure 1B).[2,12,18] The three pairs of symmetric hydrophobic depressions that line the T_4 binding site are referred to as the halogen binding pockets (HBPs), so named because they are occupied by the iodine atoms of T_4 (Figure 1B, dark red balls).[19,20]

TTR is one of at least thirty different human proteins whose extracellular misfolding and/or misassembly (amyloidogenesis) into a spectrum of aggregate structures is thought to cause degenerative diseases referred to as amyloid diseases, named after the characteristic cross-β-sheet assemblies called amyloid fibrils which are one of the structures formed from the process of aggregationor amyloidogenesis.[21–23]Genetic, pharmacologic, pathologic and biochemical evidence all suggest that human amyloid diseases result from the process of amyloidogenesis.[24–27]Amyloid can form from intrinsically disordered proteins that have no defined tertiary structure (e.g., the Huntington's-disease-related exon 1 of the Huntingtin protein, or the Alzheimer'sdisease-related amyloid-β peptide).[28] In such cases, pathogenesis is often triggered by gene duplication or aberrant post-translational processing or modifications.[29–31] Amyloid can also result from the partial unfolding of proteins that normally adopt a well-defined tertiary and/or quaternary structure.[32–35] Substantial evidence supporting the "conformation change hypothesis" was published in the 1990s, revealing that this category of proteins must partially unfold in order to misassemble into aggregates, including amyloid fibrils.[23,32–38]Transthyretin is an example of a protein that has to undergo conformational changes in order to become amyloidogenic.[32,33]Partial unfolding exposes stretches of largely uncharged hydrophobic residues in an extended conformation that efficiently misassemble into largely unstructured spherical aggregates that ultimately undergo conformational conversion into cross-β sheet amyloid structures.[39–43]

Amyloid Diseases in General

Aging is the most significant risk factor for the development of both sporadic and inherited amyloidoses.[44,45]Aging-associated deficiencies in stress-responsive signaling probably causes or exacerbates compromised protein homeostasis (or proteostasis), leading to the demise of tissue that does not easily regenerate.[46–49]Mutations within the amyloidogenic proteins, while not absolutely required for human amyloid diseases, often predispose the proteins toward amyloidogenesis by destabilizing the non-amyloidogenic native state, and sometimes by making the amino acid sequences exposed in partially folded states more amyloidogenic, accelerating the onset of post-mitotic tissue degeneration.[50–53] While much remains to be learned about the molecular underpinnings of why aging is the most important

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risk factor for the onset of amyloid diseases, it is clear that activation of the heat shock response stress-responsive signaling pathway is markedly protective in worm and mouse models of Alzheimer's disease,[46,47] and worm models of Huntington's disease.[54–56]Investigations are underway to sort out the mechanism by which stress-responsive signaling pathways protect against the pathology of the amyloidoses.

Sporadic and Inherited TTR amyloidoses

In humans, both wild type TTR tetramers and mixed tetramers compromised of mutant and wild type subunits can dissociate, misfold and aggregate–with the process of amyloidogenesis leading to the degeneration of post-mitotic tissue. Deposition of wild type TTR outside and apparently, ultimately within the cardiomyocytes of the heart appears to cause senile systemic amyloidosis (SSA), a late onset sporadic cardiomyopathy, affecting as much as 15% of males>80 years of age and significant numbers as young as 60 years of age.[57,58]The TTR amyloidoses associated with point mutations in the TTR gene include familial amyloid polyneuropathy (FAP), familial amyloid cardiomyopathy (FAC), and the rare central nervous system selective amyloidoses (CNSA).[59–65] These patients are almost always heterozygotes, meaning that their tetramers are composed of mutant and/or wild type TTR subunits, generally statistically distributed.[4] These autosomal dominant, inherited (or familial) TTR amyloidoses are typically earlier onset than the sporadic disease SSA. For example, L55P FAP has an onset at around 20 years of age,[67] where as V30M FAP symptoms surface around age 30 in the Portuguese population.[59,65,68] More than one hundred mutations have been associated with the familial TTR amyloidosis.[69,70]Generally, the more destabilizing the mutant subunits are to the TTR tetramer structure, the earlier the onset of amyloid disease,[50,71] although surprisingly the most destabilized TTR mutants, D18G and A25T, are not the most pathogenic.[72,73] The pathogenic potential of a TTR variant is determined by a combination of its instability and its cellular secretion efficiency.[71] Highly destabilized TTR mutants are subject to cellular quality control by the proteostasis network, and are thus degraded intracellularly by endoplasmic reticulum-associated degradation mediated by the proteasome.[71]As suggested by their names, FAP first manifests with autonomic and peripheral nervous system symptoms, while FAC presents with cardiacabnormalities—however, many patients have both nervous system and cardiac symptoms, especially late in the disease course, suggesting that these patients may have more general post-mitotic tissue dysfunction than previously thought.[66] It is not currently understood why a particular mutant prefers a particular site of deposition, with the exception of the CNSA-associated TTR mutants, although tissue selective amyloid deposition probably is influenced by the extracellular matrix unique to that tissue. The CNSA-associated mutants are generally the most destabilizing and lead to central nervous system pathology because the choroid plexus, which synthesizes TTR for secretion into the CSF, is more permissive than the liver, the primary source of TTR in the bloodstream, in terms of secreting highly destabilized and extremely amyloidogenic TTR tetramers.[71] This may be due to the relatively high concentration of T_4 in the choroid plexus, which appears to bind to and stabilize the mutant TTR tetramer in the endoplasmic reticulum by a pharmacologic chaperoning mechanism, affording sufficient stabilization to evade endoplasmic reticulum-associated degradation.[71] Once secreted, the lower extracellular T_4 concentration in the CSF would then favor ligand dissociation, leaving the destabilized TTR tetramer to undergo dissociation, misfolding and amyloidogenesis.

As mentioned above, the initial pathology caused by some TTR variants comes from their selective destruction of cardiac tissue, while that from other TTR variants comes from compromising the peripheral and autonomic nervous system; the basis for this initial selectivity in clinical presentation remains unclear.[59,60,65,74,75]The tissue damage caused by TTR amyloidogenesis appears to stem largely from the toxicity of small, diffusible TTR

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aggregates,[61,76]although accumulation of extracellular amyloid may contribute and almost certainly compromises organ structure in the late stages of the TTR amyloidoses.[77] In either case, however, we hypothesize that these post mitotic tissues are especially vulnerable to proteotoxicity because these tissues do not readily regenerate. The vulnerability of specific post-mitotic tissues to a given TTR mutation may have to do with the ineffectiveness of the extracellular matrix and the glycosaminoglycans in particular in protecting against cytotoxicity.[78,79]If left unchecked, amyloidogenesis of most TTR sequences leads to compromised function of both the heart (and probably other muscles) and the autonomic and peripheral nervous systems.[59,60,65,74,75]Ultimately, death occurs within approximately a decade after the onset of symptoms.[59,74,75,80–83]

Therapeutic Strategies under Development for the TTR and Other Amyloidoses

Emerging strategies to treat human amyloid diseases center on reducing the concentration of the amyloidogenic protein or peptide. Since the rate and extent of amyloidogenesis is highly dependent on the concentration of the amyloidogenic peptide and/or the population of the amyloidogenic protein conformation(s)[84–86], lowering their concentration offers a potential therapeutic strategy. For example, one strategy to treat Alzheimer's disease is to reduce the production of amyloid β (Aβ), the intrinsically disordered amyloidogenic peptide whose misassembly leads to Alzheimer's disease, by inhibiting the β- or γ-secretases that generate the Aβ from the trans-membrane amyloid precursor protein.[87–89] Another strategy is to decrease the concentration of Aβ monomers and oligomers, clearing them by any one of several antibody-mediated mechanisms; several clinical trials using monoclonal anti-Aβ antibodies are ongoing (http://www.clinicaltrials.gov).[90–92] In light chain amyloidosis, the clonal plasma cells in the bone marrow are eliminated with chemotherapy agents to dramatically reduce the concentration of the amyloidogenic light chain protein in the blood.[93–97]Light chain amyloid disease has been ameliorated in numerous individuals by eliminating the amyloidogenic light chain.[93–97]

The currently practiced strategy to ameliorate FAP associated with mutant TTR aggregation is liver transplantation.[98,99]In this surgical procedure, a patient that is heterozygous for a disease-associated TTR mutation has their liver replaced with one from a donor that is homozygous for wild type TTR.[98]Since TTR is mainly synthesized by the liver, this amounts to a surgical form of gene therapy. While initially effective for the ~90% of patients that survive the surgery (liver transplantation of FAP patients reduces the serum concentration of the V30M mutant to <5% of pre-transplant levels),[103,104] progression of WT TTR amyloidosis after about a decadeultimately leads to cardiomyopathy.[98,105,106]This surgical gene therapy strategy appears to be less effective for FAP-associated with TTR variants other than V30M, probably because these non-V30M patients are generally older when they are transplanted.[105,107]Combined heart and liver transplantation has been employed for FAC patients, where as heart transplants have been used to ameliorate SSA.[100–102]It is important to realize that liver transplantation does not prevent the development of the life threatening arrhythmias in familial amyloid polyneuropathy,[105] and of course liver and/or heart transplant patients require life-long immunosuppression, which creates its own challenges.

Emerging additional approaches to lower the concentration of amyloidogenic TTR include antisense oligonucleotide (Isis Pharmaceuticals) and RNA interference (Alnylam Pharmaceuticals) strategies to lower the TTR mRNA levels.[108,109] Antisense oligonucleotides specific for human TTR mRNA have been shown to inhibit hepatic synthesis of TTR in mice transgenic for a human amyloid-associated TTR sequence.[108]Parenteral administration of a TTR-specific antisense oligonucleotide, however,

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had no effect on the expression of TTR by the choroid plexus, which is the source of TTR in the brain.[109] This is likely desirable because there are numerous reports that normal TTR levels in the brain appear to be protective against other amyloid diseases, such as Alzheimer's disease.[110,111] There is a risk that continued amyloidogenic TTR synthesis in the brain, albeit at lower levels, could put FAP patients at risk for late onset central nervous system selective TTR amyloidosis. Antisense oligonucleotides are now being tested clinically as well by Isis Pharmaceuticals (http://www.clinicaltrials.gov). Alnylam Pharmaceuticals is using double-stranded RNA (RNAi) directed against transthyretin mRNA to lower its levels,[112] which in turn lower the TTR plasma protein concentrations of TTR FAP-associated variants without affecting the TTR level in the CSF. The TTR-targeting RNAi, when formulated in the appropriate lipid, is quite effective at degrading hepatic TTR mRNA. This approach against the TTR amyloidosis is now being explored in human clinical trials (http://www.clinicaltrials.gov). This strategy is expected to be effective for FAC and SSA, as lowering mutant and/or wild type TTR should decrease TTR aggregation efficiency thought to cause cardiomyopathy.

The Kinetic Stabilizer Strategy to Ameliorate the TTR Amyloidoses: Leveraging an Understanding of the Molecular Mechanism of Aggregation Linked to Pathology

Another strategy for ameliorating the amyloidoses caused by the misfolding and misassembly of a protein like TTR or lysozyme, which normally adopt folded, non-amyloidogenic 3-D structures, focuses on preventing the conformational excursions from the native state or partial denaturation that renders them amyloidogenic (Figure 2).[12,23,113–116] Stabilizing the properly folded, non-amyloidogenic conformations of these proteins is considered to be the most conservative approach for treating these maladies, because it is still unclear which misfolded or misassembled TTR, light chain or lysozyme conformations / quaternary structures lead to proteotoxicity.[61,76,117–120] There is mounting evidence that stopping the process of amyloidogenesis without necessarily clearing the deposited amyloid fibrils[121] is sufficient to stop the degeneration of post-mitotic tissue and disease progression.[27]

In the case of TTR amyloidogenesis, the tetramer must first dissociate and then the natively folded monomer must undergo partial denaturation in order for the TTR subunits to become aggregation competent.[12,24,32,33,51,114,122–124] Several mechanisms have been proposed to explain the aggregation of amyloidogenic proteins.[85,86] In the most widely accepted mechanism, multiple chains of the amyloidogenic protein assemble into an oligomeric nucleus in the rate-limiting step, before the rate of amyloid fibril formation becomes substantial. This scenario is referred to as a nucleated polymerization (Figure 3, top).[86] For other proteins, e.g., Aβ whose aggregation appears to cause Alzheimer's disease, rapid oligomerization of the amyloidogenic protein into spherical or amorphous micelle-like assemblies is observed and these undergo slow conversion into amyloid fibrils in a process associated with a high activation barrier. The latter mechanism is referred to as a nucleated conformational conversion and appears to govern Aβ aggregation, at least in vitro (Figure 3, middle panel).[125] TTR aggregation proceeds by yet a third mechanism, referred to as a downhill polymerization reaction.[84] After rate-limiting tetramer dissociation, and monomer misfolding, the partially denatured TTR monomers aggregate very efficiently because the misassembled dimer is more stable than the dimer and the misassembled trimer is more stable than the dimer, etc. TTR aggregation does not require nucleus formation, is not amenable to seeding, and is limited only by the relatively low activation barriers of the bimolecular association of misfolded TTR monomers and oligomers, thus the downhill polymerization designation (Figure 3, bottom panel).[84] After monomeric TTR undergoes

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partial denaturation, it spontaneously misassembles into a variety of aggregate morphologies, including amyloid fibrils and more structurally diverse aggregates exhibiting varying extents of cross-β-sheet structure (Figure 4).[84,126,127]Because TTR aggregation is very efficient once the misfolded monomer state is reached, it seems unwise to try to block TTR aggregation after rate-limiting tetramer dissociation.

The tetramer–monomer–unfolded monomer equilibria (Figure 4) are strongly thermodynamically linked,[128] thus, destabilization of either the tetramer or the monomer (and perhaps even the dimer in some mutants) can enhance TTR amyloidogenicity.[50,51,53,72,73,122,127–131] Generally, the disease-associated TTR mutations characterized to date either decrease the tetrameric quaternary structural stability or the monomer'stertiary structure stability, or both.[50,51,53,71–73,122,127,129–131]The efficiency of TTR amyloidogenesis is dominated by the extent of thermodynamic destabilization, which determines the concentration of TTR adopting an amyloidogenic conformation. TTR tetramer dissociation kinetics, which control the maximal rate of amyloidogenesis, appear to play a less significant role–as some disease-associated mutant homotetramers dissociate more quickly and others more slowly than wild type TTR homotetramers.[50,71]The V122I TTR FAC variant is amyloidogenic because it forms a relatively unstable tetramer and it dissociates rapidly, but its monomers are as stable as those of wild type TTR.[130] In contrast, the L55P TTR variant forms a stable tetramer, but its monomers are unstable leading to its efficient amyloidogenesis.[128]

It is not known what triggers TTR tetramer dissociation and monomer misfolding *in vivo*. We hypothesize that tetramer dissociation and monomer misfolding could occur in the acidic vesicles that transport TTR to the cell surface or in the acidified endocytic vesicles that take TTR into the cell, either by a receptor-mediated process or by macropinocytosis, or the like.[32,33]Tetramer dissociation does occur at physiological pH, albeit slowly, as evidenced by TTR subunit exchange occurring under physiological conditions with a $t_{1/2}$ of ~ 1 day.[4,114,126,127,132,133]TTR tetramer dissociation and monomer misfolding *in vitro*is notably enhanced through the use of acidic denaturing conditions, supporting our hypothesis that an acidified vesicle including endosomes and lysosomes could be responsible for triggering amyloidogenesis *in vivo*.[32,33,134–137]

Just before the Kelly laboratory began developing small molecules that bind to the unoccupied T_4 binding sites in plasma TTR to slow or prevent tetramer dissociation (Figure 4), (the rate limiting step of TTR amyloidogenesis), Coelho and colleagues-reported a Portuguese family that appeared to exhibit suppression of TTR amyloid disease phenotypes.[138,139]This compound heterozygous family expresses the V30M mutation associated with highly penetrant FAP on one allele, yet they do not develop polyneuropathy. Instead of expressing wild type TTR from their second allele, they express a T119M TTR variant, resulting in the formation of mixed TTR tetramers that exhibit a statistical distribution of V30M and T119M subunits.[4,24]

Subsequent biophysical studies by our laboratory revealed that T119M subunit incorporation into tetramers otherwise composed of V30M subunits proportionately reduces the amyloidogenesis rate under acidic conditions and the rate of tetramer dissociation at neutral pH in urea (Figure 5A).[24,114]Wild type and T119M TTR homotetramers have very similar thermodynamic stabilities, but differ dramatically in their dissociation kinetics (Figure 5A, B).[24,50,114] The dissociation rate of the T119M TTR homotetrameris~ 25-foldslower than the dissociation rate of the wild type TTR homotetramer, demonstrating that the T119M TTR homotetramer has a much higher dissociation barrier relative to that of the wild type TTR homotetramer (Figure 5B). Hence, T119M subunit inclusion into a tetramer otherwise composed of disease-associated subunits raises the kinetic barrier of tetramer dissociation by

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destabilizing the dissociative transition state (Figure 5B), protecting these individuals from amyloidogenesis and disease by a process referred to as interallelic trans-suppression.[24,114,138,139]

The M119 side chains may impart kinetic stability to the TTR tetramer by increasing the surface area of the contacts between the weaker of the two dimer-dimer interfaces in the dissociative transition state: the AB/CD interface bisected by the crystallographic two-fold or Z axis (Figure 5C).[15,16,140,141] This interface creates the two symmetrical T_4 binding sites.[19,20] Furthermore, perturbation of this quaternary structural interface by mutagenesis or protein engineering also kinetically stabilizes the tetramer.[15,16]

Collectively, these observations suggest that TTR amyloidogenesis could be suppressed and amyloid pathology ameliorated by kinetically stabilizing the weaker dimer-dimer interface of TTR. We envisioned that small molecule binding to one or both of the T_4 binding sites should stabilize the AB/CD dimer-dimer interface in the dissociative transition state by simultaneously interacting with the A and C and/or B and D subunits across the weaker dimer–dimer interface of the tetramer, analogous to the hydrophobic bridging interactions enabled the T119M mutation (Figure 5C).[12,114,116,132,142] The human genetic data mentioned above along with the corresponding biochemistry strengthened our resolve to discover small molecules that could bind to the normally unoccupied TTR T_4 binding sites in blood and prevent amyloidogenesis through kinetic stabilization of the TTR tetramer (Figures 4 and 5).[12,114,116,143] This pharmacologic principle was first demonstrated with T_4, a natural TTR ligand, and 2,4,6-triiodophenol, when it was found that they inhibited TTR amyloidogenesis.[116] This proof-of-principle experiment justified a robust screening[144–148] and structure-based drug design[17,18] program to find small molecule TTR ligands that bind tightly and selectively to TTR, kinetically stabilizing the native, non-amyloidogenic quaternary structure.[12,114] It is important that TTR kinetic stabilizers lack thyroid hormone receptor agonism or antagonism, while also exhibiting minimal to no binding to the other 4000 or more proteins found in the blood.[12] Poor binding selectivity to TTR would increase the concentration of the kinetic stabilizer required to inhibit TTR aggregation and possibly lead to off-target binding-associated toxicity that would derail a clinical development program.

The Discovery and Design of Kinetic Stabilizers of TTR

Over one thousand aromatic small molecules exhibiting structural complementarity to the T_4 binding sites within TTR have been designed and synthesized by taking advantage of $ligand_2$•TTR structural information to optimize kinetic stabilizer design.[12,17,18,114,116,142,143,149–164] Numerous structurally distinct TTR kinetic stabilizers were identified early in this program using screening approaches, including naturally derived flavonoid, xanthone derivatives, as well as biaryls and some of these facilitated the generation of the early $ligand_2$•TTR structural information.[144–146,148] Structure-based drug design and screening hits guided our synthetic chemistry, affording compounds in multiple structural families including: bisaryloxime ethers, biphenyls, 1-aryl-4,6-biscarboxydibenzofurans, 2-phenylbenzoxazole and biphenylamines (Figure 6A).[12,17,18,114,116,142,143,149–164] Potent compounds have also been identified through halogenation of nonsteroidal anti-inflammatory drugs (NSAIDs), such as salicylic acid, diflunisal, and flufenamic acid.[153,165,166] We also recently employed a substructure combination strategy[163] to arrive at potent and selective TTR kinetic stabilizers, where in we systematically ranked the candidate substructures composing a typical TTR kinetic stabilizer, the two aromatic substructures and the linker, using fibril inhibition potency and plasma TTR binding selectivity data.[159–161] Of the 92 stilbene and dihydrostilbene candidate kinetic stabilizers predicted to be potent and selective by the substructure

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combination strategy, nearly all potently inhibit TTR fibril formation and 17 of these exhibited a binding stoichiometry of >1.5 (out of a maximum of 2) to plasma TTR, while displaying minimal binding to the thyroid hormone receptor (<20% hormone displacement). These potent and selective TTR kinetic stabilizers also rescue cells from the cytotoxic effects of TTR amyloidogenesis.[163] Others have also identified TTR kinetic stabilizers using a variety of the approaches mentioned above.[167–177]

TTR kinetic stabilizers are typically composed of three substructures: two differentially substituted aryl rings connected by a linker (Figure 6B).[159–161] Each ring occupies one of the two subsites of the T_4 binding site in TTR.[12,17,18,163] Substituted aryl rings occupying the outer binding subsite form salt bridges with the Lys-15 and/or 15'ε-ammonium groups and / or engage in complementary hydrophobic interactions with halogen binding sites 1 /1' and/or 2/2', where as the functionalized aromatic rings occupying the inner binding subsite can engage in hydrogen bonds with Ser-117 and/or 117' and/or occupy the hydrophobic halogen binding pockets 2/2' and/or 3/3'.[12,17,18] These rings can either be linked directly, as in the case of the biphenyls,[143,154] or can be connected through short hydrophobic linkers,[12,160] as in the case of the stilbenes (Figure 6A). The linker typically interacts with the hydrophobic side chains of Leu-17 and/or 17', Ala-108 and/or 108', Leu-110 and/or 110', and Val-121 and/or 121'.[160] These complementary interactions between the kinetic stabilizer and TTR combine to differentially stabilize the ground state over the dissociative transition state, making tetramer dissociation extremely slow under physiological conditions (Figures 4 and 5).[12,114]

Most TTR kinetic stabilizers bind to the T_4 binding sites with negative cooperativity, apparently resulting from conformational changes within the tetramer upon binding to the first T_4 site.[12,142,178] Strikingly, occupancy of only one T_4 binding site is sufficient to impart kinetic stabilization on the entire TTR tetramer.[142] This was demonstrated unequivocally by tethering a kinetic stabilizer via a linker to Cys10 in a single monomer of a TTR tetramer.[142] Such a chemically modified TTR tetramer was found to be highly resistant to denaturation and aggregation. This data is enabling in that it allows patients to be treated with lower doses of kinetic stabilizers that are sufficient to occupy one of the two T_4 binding sites.[124]

TTR kinetic stabilizers must exhibit high binding affinity and high binding selectivity to plasma TTR over the blood plasma proteome in order to be useful pharmacologic agents.[9,12,159–161,163] There are >4000 proteins in blood plasma, including albumin. Albumin binds promiscuously to many small molecules and, by doing so, could prevent TTR kinetic stabilizers from binding to TTR. In a recent review, and in the original papers, we provide a comprehensive list of recently synthesized TTR kinetic stabilizers along with their so-called efficacy scores, which integrate TTR amyloidogenesis inhibitor potency and TTR binding selectivity in plasma.[18,159,160,163] Furthermore, as mentioned above, candidate kinetic stabilizers must not interact with the thyroid hormone receptor, a major concern given the structural similarity of some kinetic stabilizers with triiodothyronine (T_3, the primary thyroid hormone) and T_4 (the prohormone). NSAID activity is also undesirable in candidate TTR kinetic stabilizers, as cyclooxygenase inhibition is contraindicated for treating TTR cardiomyopathy patients, who often have impaired renal blood flow.[159–161,179]

Testing the Kinetic Stabilizer Strategy in an FAP Clinical Trial

The pharmacokinetic and pharmacodynamic properties of most of the small molecules that are potent TTR kinetic stabilizers *in vitro* and exhibit excellent plasma TTR binding selectivity *ex vivo* have not been evaluated. To date, only two small molecules have been assessed in animal safety studies and in human clinical trials.[27,159–161,180] One of these, diflunisal, is an FDA-approved NSAID, which binds to TTR with negative cooperativity

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(K_{d1}= 75 nM, K_{d2}= 1100 nM).[153,166,180,181] Diflunisal exhibits only modest binding selectivity to TTR over all the other plasma proteins and displays modest binding affinity to TTR *in vitro*. Nevertheless, recent Phase I clinical trials showed that diflunisal kinetically stabilizes TTR tetramers in human plasma because of its excellent oral bioavailability and high plasma concentrations after oral dosing (250 mg BID).[180,181] A placebo-controlled, multicenter phase III clinical trial to test the efficacy of diflunisal for the treatment of FAP, FAC and SSA is currently fully enrolled, with the results expected within the next two years (http://www.clinicaltrials.gov/). The enhanced sensitivity of some patients to the gastrointestinal, cardiac, and renal side effects of taking 0.5 g of diflunisal a day may limit its applicability to those who can tolerate this dose.

Another small molecule discovered by the Kelly laboratory[143] and developed by FoldRx Pharmaceuticals (acquired by Pfizer in September 2010) was recently shown to slow the progression of FAP in a placebo controlled, double-blind Phase II/III clinical trial (http://www.clinicaltrials.gov/).[27] Tafamidis, or 2-(3,5-dichloro-phenyl)-benzoxazole-6-carboxylic acid (Figure 7A; trade name: Vyndaqel®), was shown to bind highly selectively to TTR in human plasma and with negative cooperativity (K_ds ~ 2 nM and ~200 nM) to TTR (manuscript in preparation). Tafamidis reaches its EC_{50} for preventing TTR fibril formation at a tafamidis: TTR tetramer ratio of <1, consistent with tafamidis effectively stabilizing TTR when it occupies only one of TTR's two T_4 binding sites. Tafamidis dose-dependently kinetically stabilizes TTR under denaturing conditions (in the presence of 6.5 M urea) and under physiologic conditions. According to structural modeling, tafamidis is envisioned to bind to TTR such that it stabilizes the weaker dimer-dimer interface through a combination of specific hydrophobic and electrostatic interactions (Figure 7B). Importantly, tafamidis kinetically stabilizes a broad spectrum of TTR variants, suggesting a broadly applicable therapeutic for all the TTR amyloidosis (manuscript in preparation).

The 18-month duration phase II/III clinical trial for tafamidis enrolled > 100 patients heterozygous for V30M TTR, the clinically most important FAP-associated mutation (≈ 10,000 FAP cases world-wide).[27] Treatment with tafamidis (20 mg, once a day) slowed progression of peripheral and autonomic neuropathy, as measured by neurologic examination of the lower limbs, nerve conduction studies, among other measures of neuropathy; see the European Medicines Agency website (www.ema.europa.eu) for more details.[27] Tafamidis treatment also improved autonomic nervous system dysfunction and cachexiaas reflected by an increase in the modified body mass index (mBMI). Finally, tafamidis slowed the decline in the total quality of life, as measured by the Norfolk quality of life questionnaire used for diabetic neuropathy (QOL-DN).[27] Tafamidis was approved by the European Medicines Agency in November 2011 for the treatment of FAP (www.ema.europa.eu). Approval by the United States Food and Drug Administration is expected in 2012. It seems unlikely that the amyloid fibrils in the tafamidis treated V30M FAP patients were cleared over the course of this trial based on amyloid P component imaging data revealing that amyloid is rarely cleared post liver transplantation.[121] What seems almost certainis that the process of TTR amyloidogenesis is stopped in the patients taking tafamidis, although further studies will be required to demonstrate this assertion. The lack of misassembly intermediates in FAP patients with slowed disease progression suggests that it is the process of TTR amyloidogenesis, and not the amyloid fibrils themselves, that cause the TTR amyloidoses.

New Directions in the Kinetic Stabilizer Approach

In addition to the above-mentioned monovalent TTR kinetic stabilizers, bivalent kinetic stabilizers that simultaneously bind to both T_4 sites have also been developed (Figure 8A). Initial work by Green et al. showed that bivalent kinetic stabilizers bind with 1:1

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stoichiometry to TTR during tetramer formation within the cell, but do not bind to already-formed tetramers.[152] More recently, the Pepys group has developed bivalent palindromic ligands that bind with high affinity to preformed TTR tetramers under physiological conditions.[167] These bivalent ligands stabilize the tetramer more potently than monovalent ligands. In addition, they preferentially bind mutant TTR over wild type TTR, perhaps because the inherent instability of mutant TTR allows for easier access to T_4 binding sites in the tetramer. It is not yet clear whether these molecules have suitable TTR plasma binding selectivity, solubility, and appropriate pharmacokinetic and pharmacodynamics properties to serve as clinical candidates.

Covalent kinetic stabilizers of TTR that are more potent inhibitors of amyloidogenesis than their non-covalent counterparts have also recently been reported.[162]Stilbenes, conceived of by structure-based design, that selectively bind to TTR in preference to the more than 4000 other human plasma proteins and then react chemo-selectively with only one of eight lysine ε-amino groups (Lys15) within transthyretin have been reported. The crystal structure confirms the expected binding orientation of the stilbene substructure and the conjugating amide bond (Figure 8B). While these covalent transthyretin kinetic stabilizers exhibit superior amyloid inhibition potency compared to their non-covalent counterparts*in vitro*, and prevent cytotoxicity associated with the process of amyloidogenesis, their safety, pharmacokinetics and pharmacodynamicsremain to be evaluated.

Perspective and Conclusions

Structure-based drug design principles[12,17,18]enabled the efficient design of tafamidis,[143] a high affinity, highly selective TTR kinetic stabilizer that slows the progression of TTR amyloid disease in FAP patients.[27]This kinetic stabilizer approach for halting aggregation and preventing the degeneration of post-mitotic tissue is also being explored in other amyloid diseases as well. For example, small molecules have been used to stabilize the mutant superoxide dismutase-1 associated with familial amyotrophic lateral sclerosis,[182]and β_2-microglobulin associated with dialysis-related amyloidosis.[183] In addition, camelid antibodies have been employed to stabilize lysozyme to prevent lysozymeamyloidosis.[115] Thus, the kinetic stabilizer strategy, now clinically validated for TTR amyloidosis, has the potential to ameliorate additional degenerative diseases. A modification of this approach, the discovery of small molecules that bind to monomeric intrinsically disordered protein conformational ensembles, altering their aggregation propensity, is also being explored for Alzheimer's disease. We envision a pharmacologic future, where in synergy in ameliorating the human amyloid diseases is achieved by using small molecule kinetic stabilizers (or the equivalent),[12] in combination with drugs that lower the concentration of the amyloidogenic protein of interest,[108,109]and also in combination with drugs that enhance the capacity of the proteostasis network to achieve proteome maintenance.[46,48]

Highlights

Wild type and mutant transthyretin (TTR) causes amyloid diseases in humans

Amyloid formation requires tetramer dissociation (rate limiting) and monomer misfolding

Transthyretin tetramers can be kinetically stabilized by binding small molecules

Tafamidis, a kinetic stabilizer of TTR, is the first drug approved to treat a TTR amyloidosis

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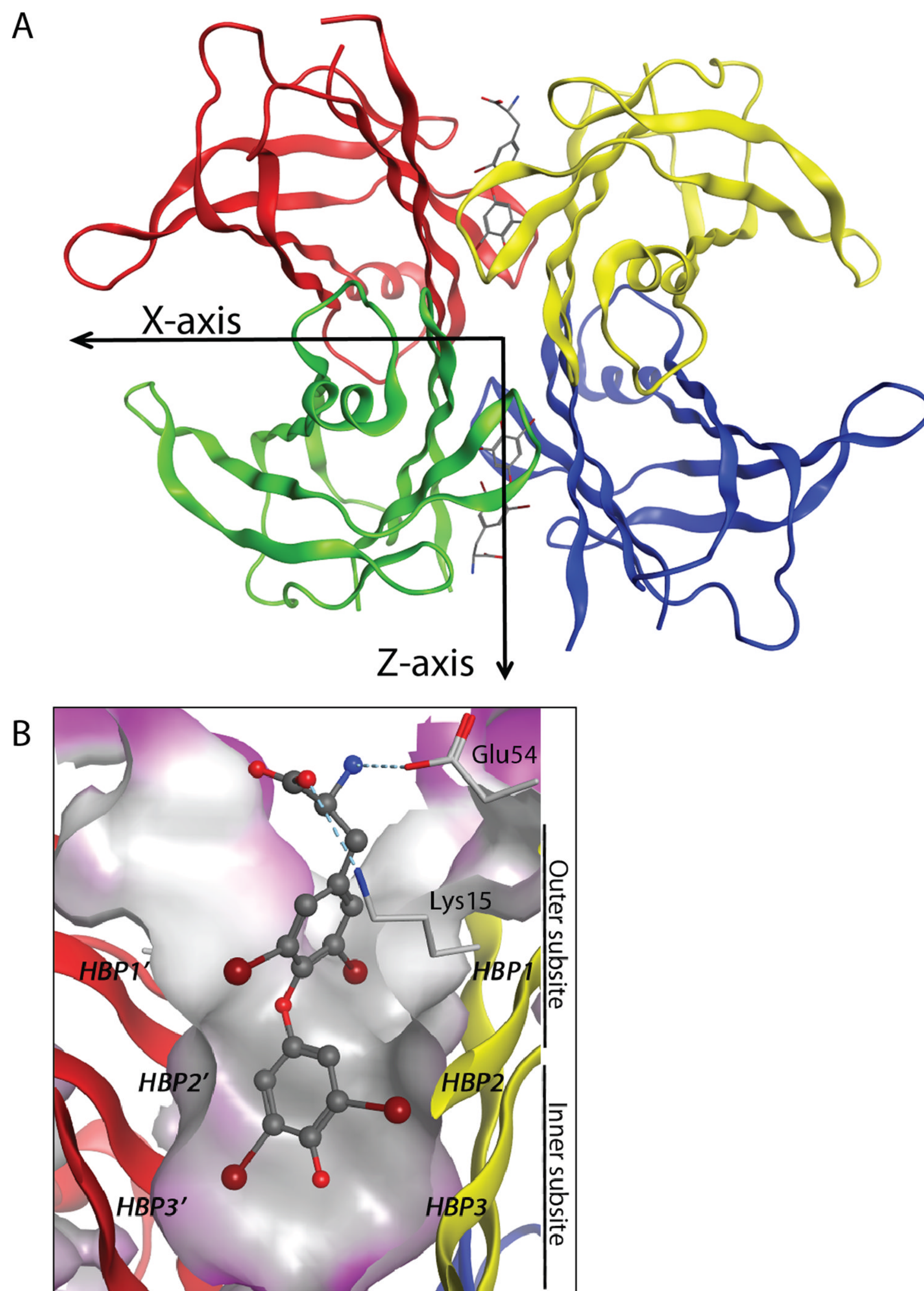

Figure 1.

Structure of transthyretin (TTR; PDB code: 2ROX). (A) Ribbon diagram depiction of TTR with the crystallographic two-fold axis (Z-axis) bisecting the T_4 binding channel comprising the weaker of TTR's two dimer-dimer interfaces(B)B. Close-up view of onethyroid hormone binding site with T_4(shown as a ball-and-stick representation) bound, showing the iodide substituents occupying symmetry-related halogen binding pockets. Primed amino acids refer to those comprising symmetry-related subunits. Hydrogen bonds are shown as light blue, dashed lines. Figure adapted from Connelly et al.[18]

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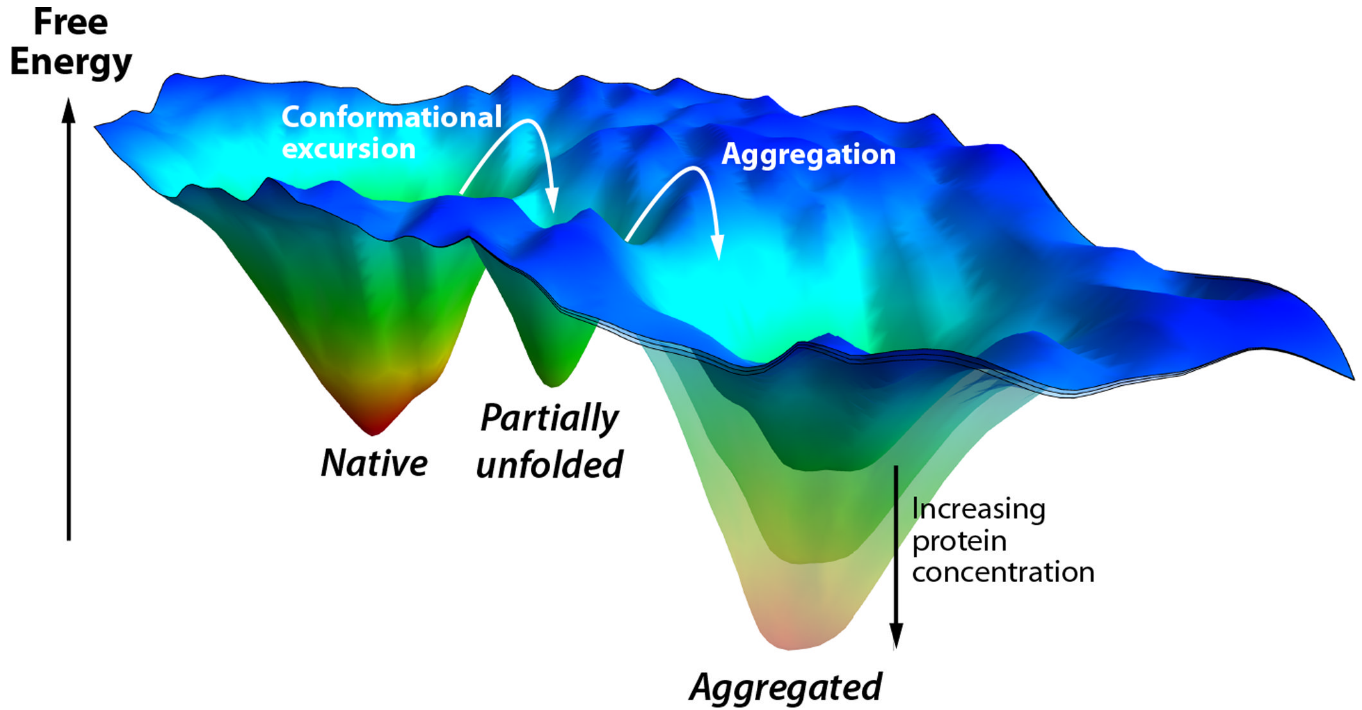

Figure 2.
Folding free energy landscape of an amyloidogenic protein that normally forms a well folded 3D structure, but can also aggregate as a consequence of a conformational change, e.g., TTR or lysozyme. Three energy wells are shown: the native state, a partially unfolded amyloidogenic intermediate, and an aggregated state. Conformational excursions convert the native state to the partially unfolded state, which can then aggregate. The stability of the aggregated state depends on the protein concentration. At low protein concentrations, it would be less stable than the native state, and therefore not substantially populated. As the protein concentration increases, it becomes increasingly stable, and will eventually become the most stable state.

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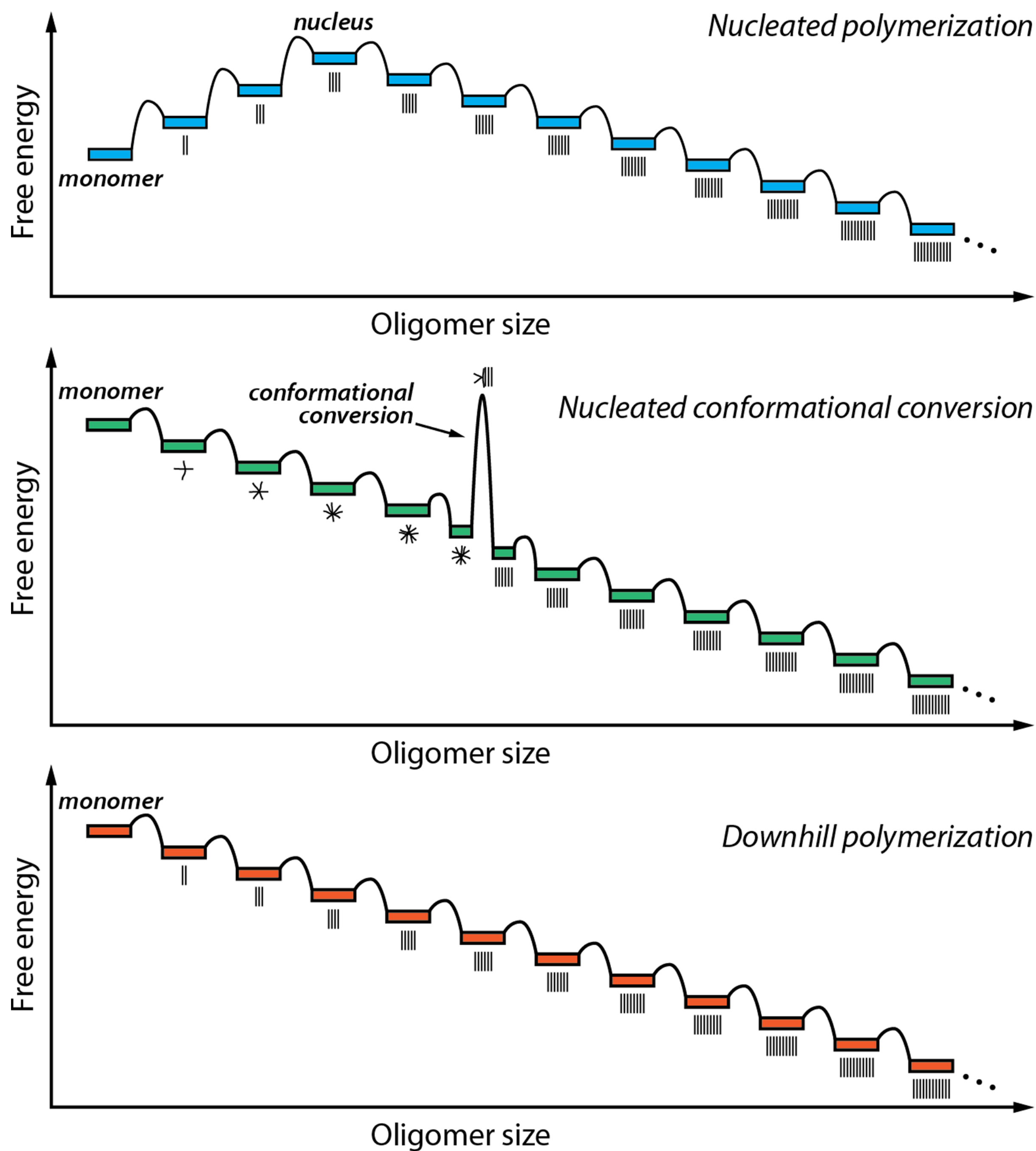

Figure 3.

Energy diagrams associated with three distinct mechanisms of protein aggregation. In a nucleated polymerization (top), the initial association events are unfavorable until a critical sizeis reached. The oligomer of this size is referred to as the nucleus. Subsequent steps are favorable, making further growth favorable for oligomers larger than the nucleus. In a nucleated conformational conversion (middle), facile initial association steps form amorphous oligomers. Oligomers of a certain size can undergo a rate-limiting conversion step, in which they change from an amorphous structure to a cross-β-sheet fibrillar state. Subsequent steps are favorable, as in the nucleated polymerization. In a downhill polymerization (bottom), the mechanism by which TTR aggregates, all of the association

steps are favorable after formation of the amyloidogenic intermediate, and there is no kinetic barrier to oligomerization. The aggregates shown are ordered, but they need not be; TTR forms a collection of aggregate structures.

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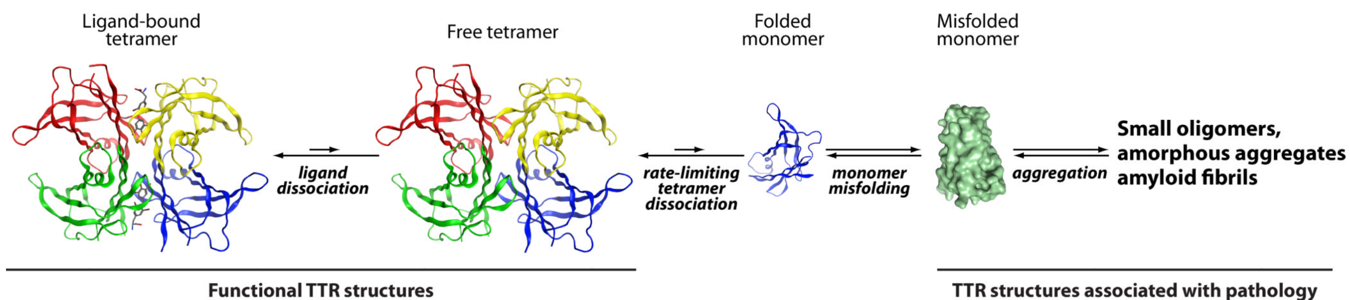

Figure 4.

TTR amyloid cascade. In order for TTR to form amyloid, the tetramer must first dissociate (the rate-limiting step) and then the natively folded monomer must undergo partial denaturation to become competent to misassemble into a variety of aggregate morphologies, including oligomers and amyloid fibrils. Ligands (such as thyroxine, shown in gray and red) stabilize the tetramer and thus prevent amyloidogenesis.

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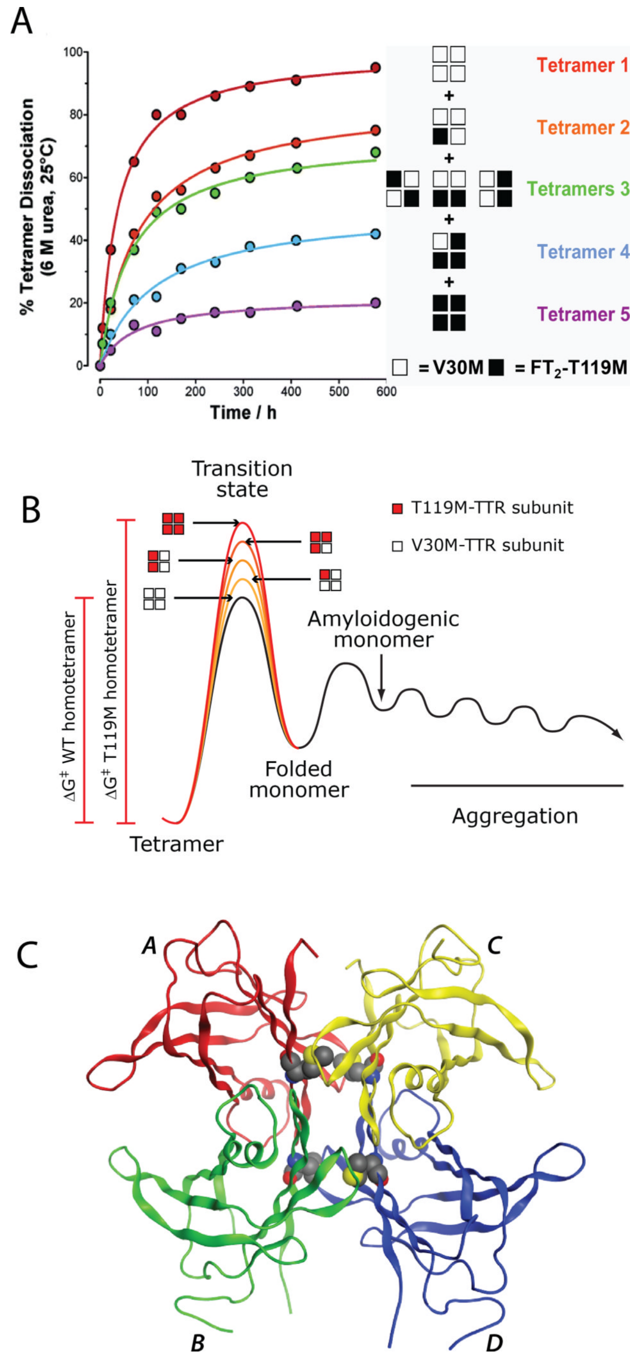

Figure 5.

Kinetic stabilization through T119M TTR subunit incorporation into TTR tetramers.(A) Urea-mediated tetramer dissociation time courses of the T119M TTR homotetramer, wild type TTR homotetramer, or mixed tetramers produced by co-expression of the two different subunits, the stoichiometry being indicated on the right. (B) Free energy diagram illustrating that the increase in activation energy required for tetramer dissociation is proportional to the number of T119M subunits comprising the tetramer. (C) Ribbon diagram depiction of T119M TTR, where in the 119M side chains shown in CPK representation stabilize the weaker of TTR's two dimer-dimer interfaces (PDB code: 1BZE). Figure adapted from Hammarstrom et al. [24].

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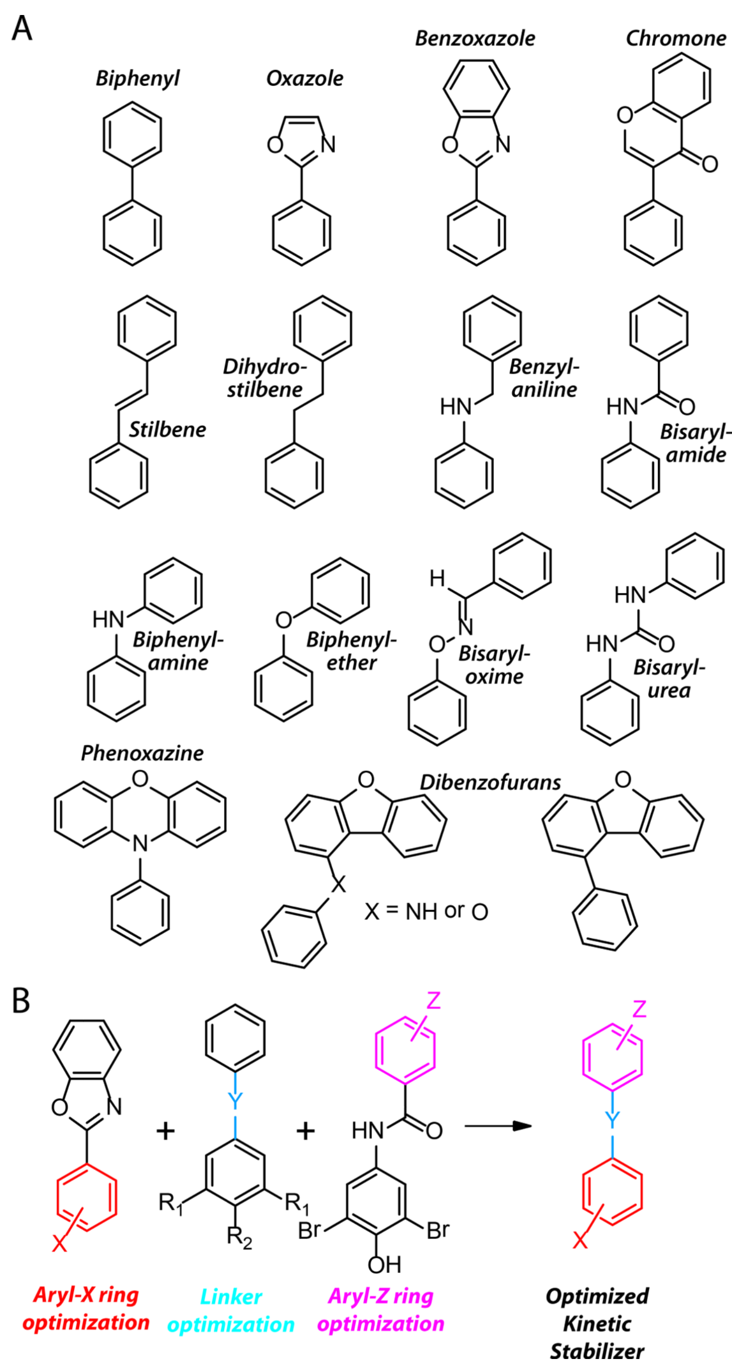

Figure 6.

The structural diversity of TTR kinetic stabilizer core structures. (A) Line drawings of the structural cores underpinning the 1000+ TTR kinetic stabilizers synthesized to date. Adapted from Johnson et al.[12] (B) Schematic depiction of the substructure combination strategy to create potent and highly selective TTR kinetic stabilizers. Individual elements of candidate TTR kinetic stabilizers are varied and the most potent and selective substructures of the candidates are combined to create potent, highly selective TTR kinetic stabilizers. Adapted from Choi et al.[163]

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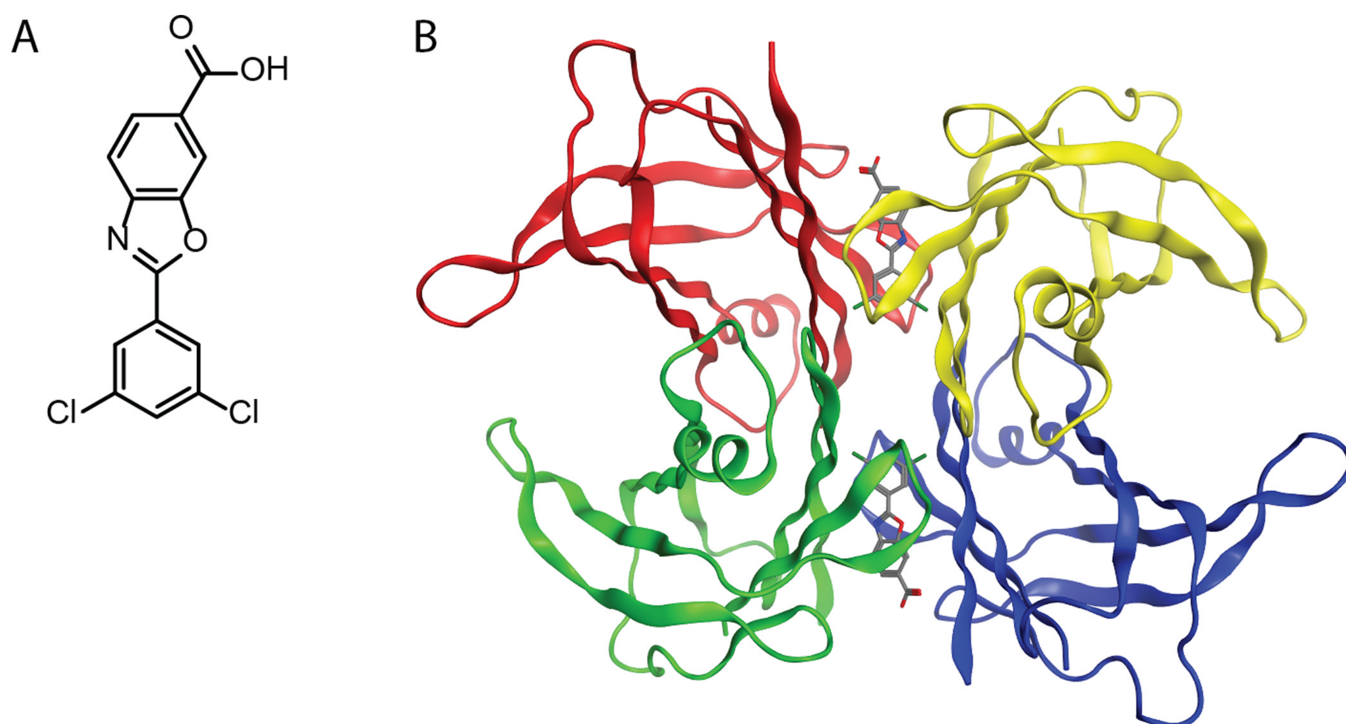

Figure 7.
(A) Line drawing of tafamidis. (B) Structural model of how tafamidis is envisioned to bind to and kinetically stabilize TTR.

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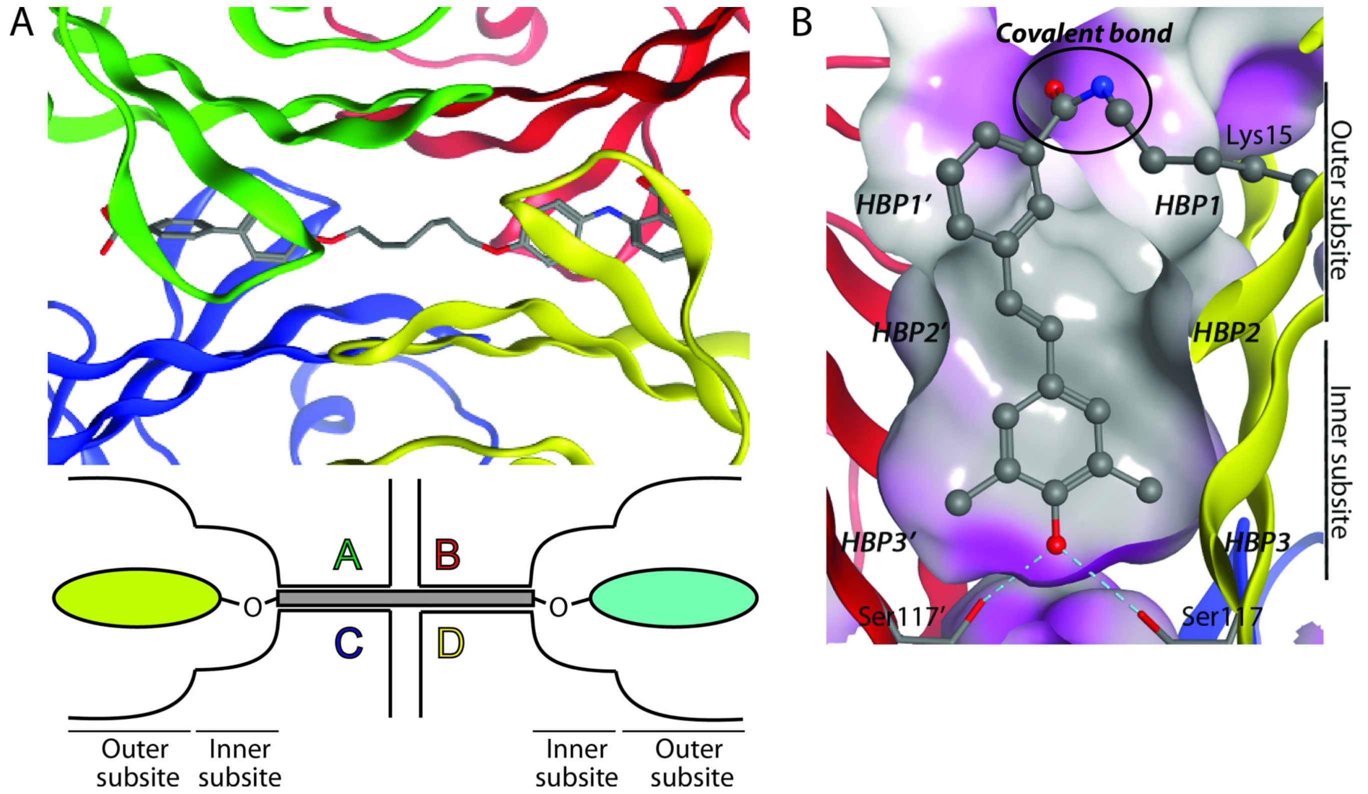

Figure 8.

(A) Top: Close-up view of a bivalent TTR kinetic stabilizer bound to the thyroid hormone binding sites (PDB code:2FLM). Bottom: Schematic representation of a bivalent TTR kinetic stabilizer bound simultaneously to both T_4 binding sites of tetrameric TTR. Figure adapted from Green et al.[152] (B) Close-up view of a covalent kinetic stabilizer attached via an amide bond to Lys 15 in one thyroid hormone binding site of TTR (PDB code: 3HJ0).