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A Current Pharmacologic Agent *versus* The Promise of Next Generation Therapeutics to Ameliorate Protein Misfolding and/or Aggregation Diseases

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Abstract

The list of protein aggregation-associated degenerative diseases is long and growing, while the portfolio of disease-modifying strategies is very small. In this review and perspective, we assess what has worked to slow the progression of an aggregation-associated degenerative disease, covering the underlying mechanism of pharmacologic action and what we have learned about the etiology of the transthyretin amyloid diseases and likely amyloidoses in general. Finally, we introduce emerging therapies that should apply more generally to protein misfolding and/or aggregation diseases that rely on adapting the protein homeostasis or proteostasis network for disease amelioration.

Introduction

Amyloidogenesis refers to the concentration-dependent, multi-step process of protein aggregation–resulting either from the alteration of the tertiary structure of a natively folded protein or the linked conformational changes and misassembly of an intrinsically disordered protein [1,2]. Compelling evidence suggests that the process of aggregation from one or several of more than 30 amyloidogenic proteins causes unique degenerative diseases [3]. These include Alzheimer's and Parkinson's diseases, the transthyretin amyloidoses, and light chain amyloidosis that result from the abnormal proliferation of cancer cells that secrete amyloidogenic light chains. Amyloidogenesis can occur intra- and/or extracellularly, affording numerous distinct aggregate structures including insoluble, fibrous cross-β-sheet assemblies, known as amyloid fibrils, on one end of the structural continuum [4] and at the other end, soluble oligomers exhibiting a range of secondary structures, including β-sheet-rich structures [2,5]. The process of amyloidogenesis is known to compromise the function of post-mitotic tissues, such as the heart or the peripheral, autonomic or central nervous

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systems by mechanisms under intense investigation [2,6]. While amyloid fibrils are a distinctive feature of amyloid diseases, there is no consensus on what drives the pathology of these maladies. Some investigators hypothesize that soluble, non-native, β-sheet-rich oligomers may be the major proteotoxic structure, more problematic than the amyloid fibrils themselves [7,8].

The precise factors that trigger amyloid pathology in humans remain incompletely characterized [9,10]. While inherited mutations generally make proteins prone to misfolding and aggregation [11,12], it is not clear why the process of amyloidogenesis only results in pathology later in life. One explanation may be aging-associated deficiencies in stress-responsive signaling pathways that regulate cellular proteostasis network capacity [13,14]. It has been hypothesized that the attenuated ability to activate stress-responsive signaling pathways upon aging results in increased protein aggregation that becomes pathogenic, triggering the onset of many age-onset sporadic and inherited amyloid diseases [14,15].

Current therapeutic approaches for the treatment of human amyloid diseases target various aspects of the amyloidogenesis cascade. A proven approach for ameliorating light chain amyloidosis is to use chemotherapy agents to kill the plasma cells secreting the light chains that aggregate. Antisense and RNAi approaches under development reduce the amyloidogenic protein concentration by mRNA degradation, whereas another approach removes aggregates and amyloid fibrils by antibody-mediated clearance [2,16]. Herein, we cover a related strategy—blocking the protein misfolding that commences the amyloidogenic cascade. The amyloidogenic protein that is the focus of the first part of this review is transthyretin which is associated with the transthyretin amyloidoses [12,17–19].

Transthyretin as an amyloidogenic protein

Transthyretin (TTR) is a protein produced in and secreted by the liver, by the choroid plexus and by the retinal pigment epithelial cells [20–22]. TTR is a 55 kDa tetramer made up of identical subunits (Figure 1A), each adopting a β-sheet-rich secondary structure comprising 127 amino acids [23]. The quaternary structure of transthyretin is characterized by two distinct dimer-dimer interfaces, the least stable of which, bisected by the X-axis, forms two C_2-symmetric funnel-like binding sites for thyroxine (T_4) [24,25]. In blood, TTR transports $\approx$ 0.5 eq. of *holo*-retinol binding protein and a very small amount of T_4, due to the presence of other T_4 carrier proteins.

In order for TTR to form amyloid fibrils, its tetramer must undergo rate-limiting dissociation to natively folded monomers, but this is not sufficient (Figure 1B) [18,26]. Partial monomer denaturation enables misassembly of TTR into structurally diverse aggregates, including amyloid fibrils—provided the concentration is high enough [27,28]. This process is best described as a downhill polymerization, wherein the misfolded monomer misassembles spontaneously, affording progressively more stable aggregate structures (Figure 1C) [29]. Three sequence-dependent factors determine the rate of TTR aggregation: 1) the rate of TTR homo-or heterotetramer (mutation carrier) dissociation, which is almost always rate-limiting [12,18,26,30]; 2) the rate of monomer misfolding, the extent of which is influenced by the thermodynamic stability of the natively folded monomers [26,29,31]; and 3) the total extracellular TTR concentration, which is controlled by the cells' proteostasis network

capacity that determines the partitioning of TTR between degradation or folding and secretion by sensing the stability of TTR [12].

In the autosomal dominantly inherited or mutation-associated TTR amyloidoses—including familial amyloid polyneuropathy (FAP) [32], and familial amyloid cardiomyopathy (FAC) [33,34]—aggregation of both mutant and wild type (WT) TTR leads to the degenerative phenotype. FAP compromises the function of the peripheral and autonomic nervous systems and initially manifests as a loss of temperature and pain sensation in the feet [35]. The progression of FAP leads to cachexia and often death within 10 years, if untreated. The onset of the familial forms of TTR amyloidosis can be as early as age 20 [36]. Aggregation of exclusively WT-TTR is the TTR amyloid disease affecting the most patients, typically men over age 60 [37,38]. This disease, called senile systemic amyloidosis (SSA), is a cardiomyopathy that is fatal approximately 5 years after initial diagnosis, if untreated. Hereditary amyloid FAP and/or FAC can also be caused by the aggregation of other proteins, such as apolipoprotein A1 [39,40].

Milestone discoveries

Liver transplantation–mediated TTR gene therapy was the first therapeutic approach for the treatment of V30M FAP [41,42]. In this strategy, a liver secreting heterotetrameric TTR, comprising mutated and/or wild-type monomers, was replaced with a donor liver secreting the more stable homotetrameric WT-TTR tetramers (Figure 2A, and 3). Although profoundly innovative, liver transplantation as a treatment strategy involves risks, including 10% surgery-related mortality and the requirement of life-long immunosuppression (increasing the risk of infection). Moreover, it was not anticipated that WT-TTR would continue to deposit after liver transplantation, leading to cardiomyopathy [43].

In 1992, Coelho and coworkers described a Portuguese family carrying the V30M mutation that developed a rather benign type of FAP, if they developed the disease at all [44,45]. These individuals produced the V30M-TTR mutation from allele 1 and the T119M-TTR from allele 2, affording tetramers comprising a statistical distribution of V30M and T119M subunits (in contrast to the normal heterozygous FAP patient whose tetramers are a statistical mix of V30M and WT subunits) [44,45]. T119M-TTR subunit incorporation into tetramers composed of disease-associated TTR sequences kinetically stabilized the resulting tetramers by increasing the activation energy required for tetramer dissociation. The activation barrier increases proportional to the number of T119M subunits making up the tetramer [19,46]. These findings suggested that strategies which increase the tetramer dissociation barrier have the potential to slow disease progression.

Building on the observation that TTR aggregation can be accelerated under acidic conditions (pH=4–5) [47,48], leading to aggregation on a convenient laboratory time scale (72 h), our laboratory evaluated the influence of T_4 binding to TTR with regard to selectively stabilizing the native tetramer over the dissociation transition state to slow tetramer dissociation—the rate limiting step of amyloidogenesis. We found that formation of a TTR•T_4 complex prevented TTR aggregation by kinetically stabilizing TTR, i.e., by dramatically slowing tetramer dissociation [17]. Furthermore, we demonstrated that numerous ligands can bind to the tetramer and kinetically stabilize TTR, proportional to their binding constants [49–52].

Structure-based design of TTR kinetic stabilizers

This logic motivated us to design and synthesize > 1000 candidate small molecule kinetic stabilizers of TTR [53,54]. The exact process of structure-based design has been explained previously [51–53]. Briefly, small molecules that inhibited acid-mediated TTR aggregation were co-crystalized with TTR, leading to crystal structures of TTR bound to kinetic stabilizers. These structures guided small molecule modifications that have the potential to enhance binding affinity and selectivity. Promising candidates were defined as those which reduced TTR aggregation to less than 10% of vehicle control in the acid-mediated TTR aggregation assay [55–58] and did not exhibit nonsteroidal anti-inflammatory (NSAID) activity (a contraindicated activity for cardiomyopathy as NSAIDs further restrict renal blood flow) [55–57]. A candidate kinetic stabilizer's ability to bind to TTR over the 4000+ additional proteins in plasma, including albumin, was assessed and over the years a few different methods have been utilized, each exhibiting some limitations [59–61]. The TTR subunit exchange assay, carried out in human plasma, is currently the best method to assess binding selectivity [61]. This method also quantifies kinetic stabilization under physiological conditions.

In 2003, our laboratory described the synthesis and evaluation of a library of benzoxazoles as candidate TTR kinetic stabilizers [58]. Of the 28 compounds envisioned by structure-based design, 11 prevented TTR aggregation in the acid-accelerated fibril formation assay, and a further six were found to selectively bind to TTR in blood plasma. These TTR kinetic stabilizers were among those considered for development by FoldRx Pharmaceuticals into an orally available therapeutic for the treatment of TTR-related amyloidoses. 2-(3,5-dichloro-phenyl)-benzoxazole-6-carboxylic acid, later named tafamidis, was one of those compounds (Figure 2B) [62]. Tafamidis proved to be orally bioavailable and it prevented TTR tetramer dissociation of wild-type and pathogenic TTR variants under fibril-promoting and physiological conditions [62]. Tafamidis was found to bind to TTR with high affinity (K_{d1}= 3 nM, K_{d2}= 278 nM), which undoubtedly contributed to its high binding selectivity towards TTR in complicated biological environments like blood [62]. Importantly, tafamidis lacked NSAID activity.

The X-ray crystal structure revealed that tafamidis occupied the T_4-binding sitesas designed, and its affinity for TTR was driven by a combination of hydrophobic and electrostatic interactions (Figure 2C) [62]. Tafamidis binding strengthened the weaker dimer-dimer interface via multi-subunit hydrophobic and electrostatic interactions, resulting in the kinetic stabilization of TTR tetramer (Figure 2D). Binding of only one tafamidis molecule per TTR tetramer is sufficient to achieve enough TTR kinetic stabilization to arrest aggregation [63].

In 2011, the results of a phase II/III 18-month randomized double-blind placebo-controlled clinical trial of tafamidis for the treatment of FAP were reported, revealing that the predetermined endpoints were met in the efficacy evaluable population [64]. Tafamidis delayed neuropathic progression in these heterozygous patients harboring the V30M mutation. In comparison to placebo controls, these individuals exhibited 52% less neurologic deterioration demonstrated by 53% and 80% preservation of large- and small-nerve fiber function, respectively. Additionally, tafamdis treatment significantly prevented weight loss, a factor used in the assessment of a change in the quality of life of patients

undergoing therapy. A subsequent 12-month, open-label extension study further demonstrated the long-term safety of tafamidis, its tolerability and efficacy in slowing disease progression [65]. The European Medicines Agency, later joined by the Japanese Pharmaceuticals and Medical Devices Agency as well as other regulatory agencies, approved tafamidis for the treatment of FAP. A multicenter, placebo-controlled, double-blinded clinical trial is well underway to determine the efficacy of tafamidis for the treatment of patients diagnosed with transthyretin-related cardiomyopathy (clinicaltrials.gov) [66].

Therapeutics of the Future

Despite immense effort from the biotechnology and pharmaceutical industries, as well as academia, the list of regulatory agency approved therapies that modify the course of protein aggregation-associated degenerative diseases remains very short [2]. It seems likely that the design of clinical trials focusing on patients already exhibiting substantial neuronal loss is contributing to the high failure rate of drug candidates [2]. Moreover, it is now becoming clear that the pathology of some degenerative diseases may be due to aggregation of more than one protein. For example, multiple proteins are aggregating in Alzheimer's disease, not just Aβ and Tau. Thus repairing the ability to maintain the proteome [67–69] and performing more sophisticated trials might together contribute to increasing the list of efficacious drugs.

Towards this end, one promising future therapeutic strategy involves manipulation of the cellular protein homeostasis (proteostasis) network to minimize the accumulation of harmful aggregated proteins [67–69]. The proteostasis network comprises a variety of competing and integrated biological pathways, including chaperone folding pathways, as well as the ubiquitin proteasome and autophagy degradation pathways. The basic function of the cellular proteostasis network is to make a decision to either facilitate the folding of a given protein or to degrade it (Figure 4). Various stressors—a fever, a viral infection, the presence of ingested oxidants or oxidants produced by dysfunctional mitochondria, etc.—can cause protein misfolding and/or aggregation. We hypothesize that when we are young, protein aggregation and misfolding caused by such stresses are countered by stress-responsive signaling pathways, which increase proteostasis network capacity to meet demand and remedy misfolding and/or aggregation, required for normal organismal function [70–73]. In the cytosol and nucleus of cells, the heat shock response (HSR) stress-responsive signaling pathway, mediated by the transcription factor HSF1, induces ~500 and represses ~1000 genes to ensure the maintenance of proteostasis in those cellular compartments [74]. The unfolded protein response (UPR) stress-responsive signaling pathway regulates proteostasis within the secretory pathway of eukaryotic cells and also contributes to extracellular proteostasis maintenance [75,76]. A growing body of evidence suggests that the inability to activate stress-responsive signaling pathways as humans age leads to exacerbated improper folding and aggregation [13–15]. Thus, a therapeutic strategy that restores normal regulation of the cellular proteostasis network(s) could potentially have a broad, positive impact on cellular and organismal function, and hopefully could halt the progression of aggregation and protein misfolding diseases [77–79].

Recent publications demonstrate the potential benefits of proteostasis network manipulation as a therapeutic strategy to ameliorate diseases of protein conformation. Small-molecule mediated manipulation of Hsp90–co-chaperone machinery or the Hsp70–co-chaperone pathway in the cytosol has been shown to impede the effects of misfolding and aggregation of various proteins associated with the onset of a number of neurodegenerative diseases including Parkinson's, Huntington's and Alzheimer's diseases [80–85].

While many uncertainties about the role of stress-responsive signaling remain to be addressed [86–88], multiple preclinical studies indicate that selective modulation of stress-responsive signaling pathways reduces the damaging effects of protein misfolding and aggregation [14]. Selective enhancement of either the HSR or the UPR has been shown to extend life span in various misfolding disease animal models [79,89,90]. In a recent report, Das and coworkers demonstrated that inhibition of stress-induced phosphatase PPP1R15A, a component of the PERK arm of the UPR, ameliorated degeneration in both cell and animal models of Charcot-Marie-Tooth 1B disease and amyotrophic lateral sclerosis [91]. Interestingly, the small molecule Sephin1 identified in this study did not bind to related and constitutive phosphatase PPP1R15B, sparing undesired pro-apoptotic stress response activation [91]. Cellular studies demonstrate that activation of a specific arm(s) of the UPR stress-responsive signaling pathway increases secretion of functional misfolding-prone proteins associated with loss-of-function diseases [92,93] while preventing secretion of dysfunctional proteins [94]. Moreover, arm-selective UPR activation has been demonstrated to achieve intracellular degradation of the mutant aggregation-prone variants while simultaneously allowing for the proper folding and secretion of functional wild type amyloidogenic proteins in heterozygotes [78,95,96]. In the extracellular space, UPR activation reduces the concentration of misfolding-prone proteins, which should reduce concentration-dependent amyloidogenesis [95,97]. A reduction in aggregation is also likely achieved owing to the secretion of the Hsp40 co-chaperone ERdj3, as a consequence of activation of the ATF6 arm of the UPR [98]. Small molecule ligands that modulate the activity of specific arms of stress-responsive signaling pathways are envisioned to be valuable [75,79,88,99].

Removal of dysfunctional and aggregated proteins through direct activation of cellular degradation pathways is being explored intensively as a therapeutic strategy for the amelioration of protein misfolding/aggregation diseases [100–104]. Animals appear to lack the dedicated disaggregase that yeast have. Instead, proteins can be degraded by the ubiquitin proteasome system or aggregates can be cleared by acid denaturation and proteolysis in the lysosome, which can be accessed through multiple pathways, including autophagy routes [102,105,106]. In some organelles, especially the mitochondria, specialized proteases exist to degrade the proteome [107].

Activating the proteasome is an attractive approach for enhancing the degradation capacity of the cytosolic proteostasis network, an approach that is currently being investigated preclinically in aggregation-associated degenerative diseases [108,109]. The proteasome-associated deubiquitination enzymes, or the DUBs, negatively regulate the proteasome by removing ubiquitin from a subset of proteasome clients, reducing the efficiency of degradation. Thus, DUB inhibitors are being developed by several biotechnology and

pharmaceutical companies to enhance the degradation of client proteins associated with neurodegenerative disorders, including α-synuclein and tau, to treat Parkinson's disease and the tauopathies including Alzheimer's disease [108,109].

Degradation of soluble and aggregated proteins in the lysosome is a very attractive strategy to remedy aging-linked degenerative diseases that appear to be caused by the aggregation of one or more proteins. Numerous research groups are focused on discovering autophagy activating small molecules—this field is vast and cannot be adequately reviewed here [105,110–124]. However, there is enough preliminary data to motivate extensive studies to determine whether enhancing proteostasis by activating aggregate clearance mechanisms will be useful for the treatment of human degenerative diseases.

Conclusion and Perspectives

The list of disease-modifying strategies for human amyloid diseases is very short. Besides directly stabilizing an amyloidogenic protein, as we have done in the case of the transthyretin amyloidoses, we envision that therapies that rely on adapting the proteostasis network will also be very useful for ameliorating amyloid diseases. We envision that mechanistically distinct drugs will be used in combination with a plethora of additional potential strategies, including activation of the immune system to clear distinct aggregate types [2].

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Highlights

- Protein misfolding and misassembly leads to amyloid diseases and tissue degeneration.
- Genetic and aggregation insights led to a drug for the transthyretin amyloidoses.
- Tafamidis prevents the progression of the otherwise fatal transthyretin amyloidoses.
- An understanding of the cellular proteostasis networks is emerging.
- Proteostasis regulators should be useful for treating protein misfolding diseases.

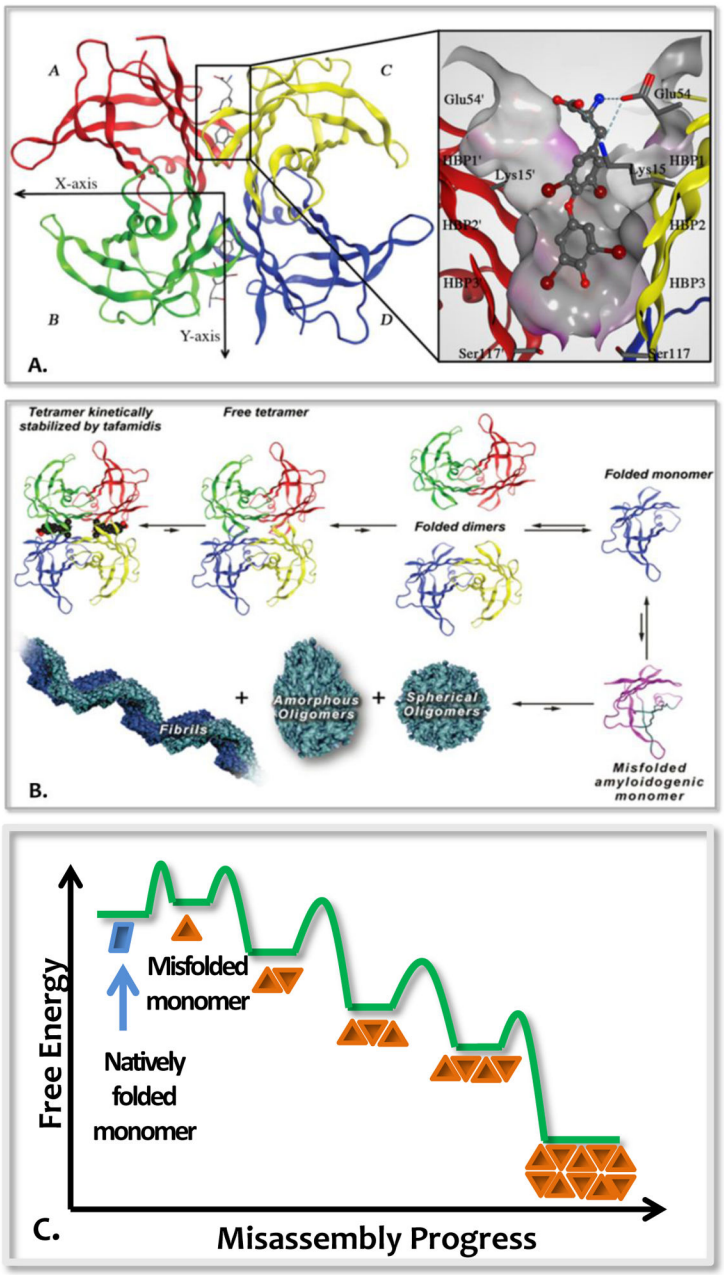

Figure 1.

Transthyretin structure and amyloidogenesis cascade. (A) Ribbon diagram depiction of the structure of homo-tetrameric TTR with T_4 occupying the ligand-binding site. (B) TTR amyloidogenic cascade. (C) Free energy diagram consistent with TTR aggregation.

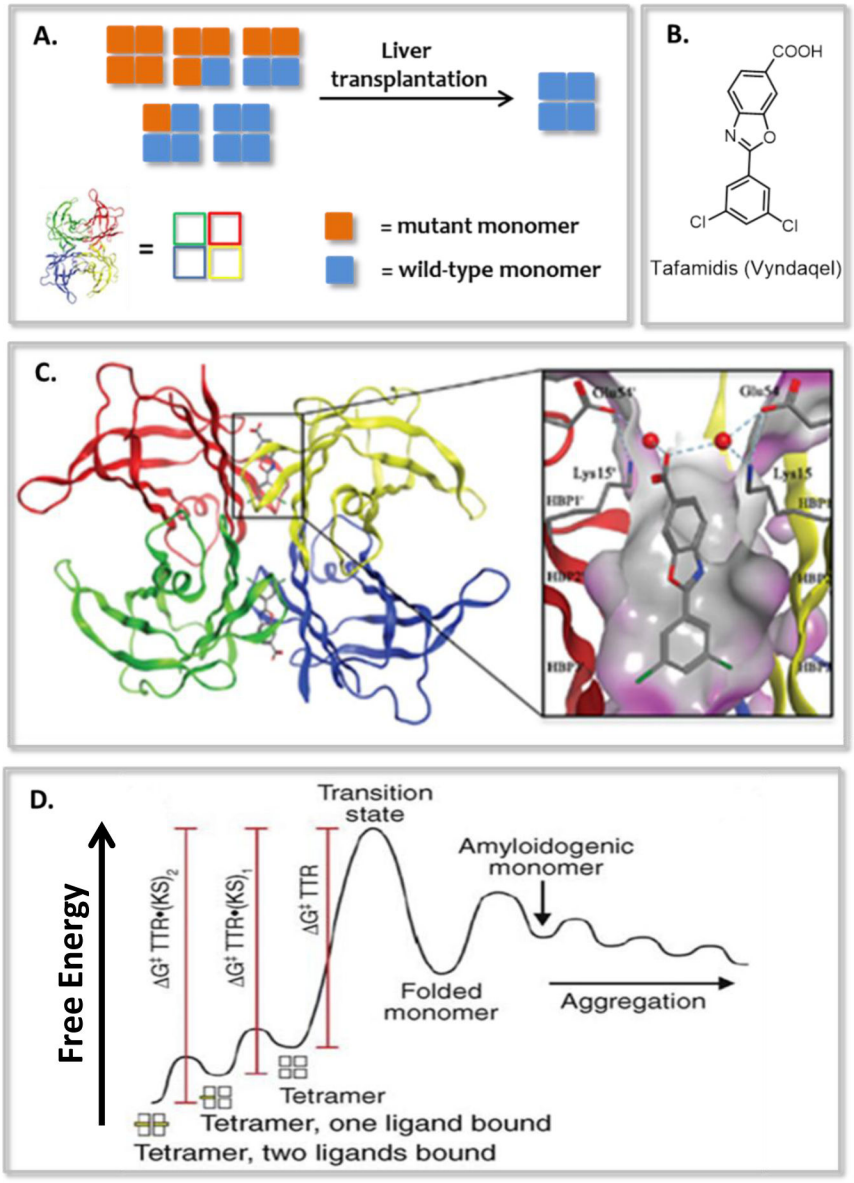

Figure 2.

Summary of key findings that led to a tafamidis-based kinetic stabilization strategy for the treatment and/or prevention of transthyretin-related amyloidoses. (A) Original liver transplantation-based strategy for replacing kinetically less stable heterotetrameric TTR with kinetically more stable wild type homotetrameric TTR for slowing the progression of familial amyloid polyneuropathy. (B) Line drawing of the tafamidis structure. (C) Structure of the $(tafamidis)_2$•TTR complex that dissociates slowly. (D) Mechanism of TTR stabilization by tafamidis.

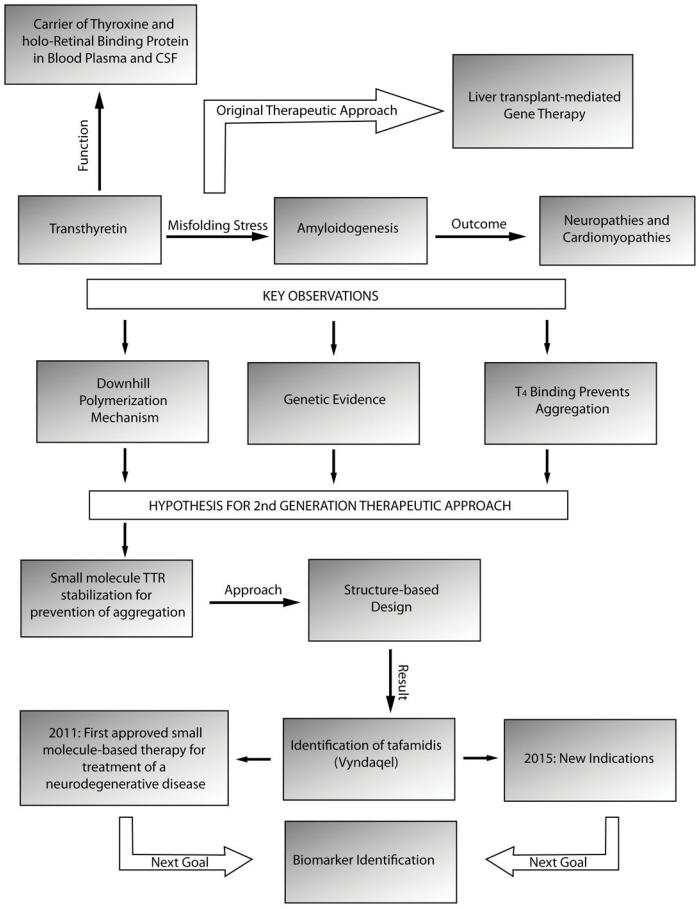

Figure 3.

Discovery path to tafamidis. Numerous observations summarized on this flow chart led to the discovery of tafamidis, its regulatory agency approval, and continuing biomarker discovery efforts.

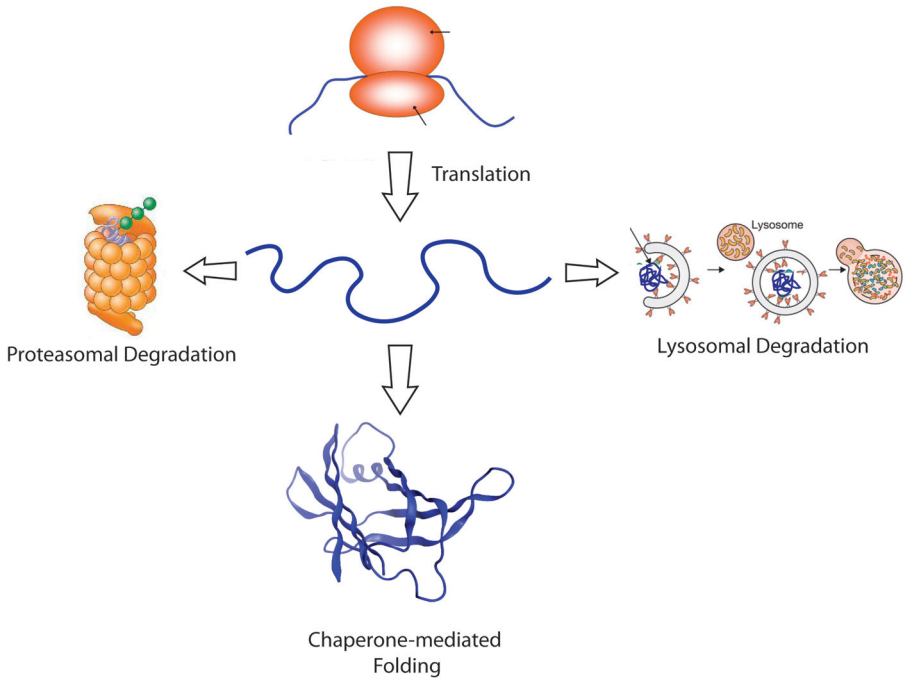

Figure 4.

The main decision to be made by the protein homeostasis, or proteostasis, network is to fold or refold a protein or degrade it by the ubiquitin proteasome system or by one of several lysosomal degradation pathways (autophagy is shown). While it is clear that intrinsically disordered proteins are degraded both by the proteasome and by the lysosome, it is less clear which proteostasis network components engage this class of proteins to keep them soluble and functional.