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# Small molecule protein binding to correct cellular folding or stabilize the native state against misfolding and aggregation

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**Abstract**

Protein misfolding diseases are caused by the difficulty of a protein to attain or stably maintain its native three-dimensional structure. In 2011, the first small molecule that specifically binds to the folded state of a protein was approved by a regulatory agency to treat a protein misfolding disease (tafamidis, transthyretin amyloidosis). Subsequently, folded state binders for three additional pathologies were approved. All of these molecules bind specifically to and stabilize the native state of a misfolding-prone protein and either correct cellular folding or stabilize the native state against misfolding and aggregation. We will use these four case studies to explain how protein folding coupled to small molecule binding is a promising approach to treat a variety of human maladies.

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## Introduction

Protein misfolding diseases are a group of heterogeneous disorders wherein the difficulty of a protein to attain or stably maintain its native three-dimensional structure leads to pathology [1]. Loss-of-function maladies typically arise due to the inability of a protein to efficiently fold and traffic within the cell. In contrast, gain-of-toxic-function diseases result from the aggregation of the misfolded protein inside or outside of a cell, often coupled to aging-associated deficiencies in the proteostasis network (PN).

Until 2011, disease-modifying small molecule drugs targeting the conformational aspects of protein misfolding diseases were not available in the pharmacy [2]. However, over the previous 40 years, a large body of experimental data had been generated using purified proteins or in cells demonstrating that it was possible to enable protein folding or prevent protein misfolding and aggregation using small molecules that have an ability to bind specifically and with high affinity to the natively folded state.

Pioneering studies published in the 1970s showed that it was possible to stabilize the $O_2$-bound state of a mutated form of hemoglobin (HbS) by binding and reacting cyanate, aspirin, or generic aldehydes with the N-termini of its subunits [3–5]. When unbound to $O_2$, aberrant polymerization of HbS occurs, causing cell sickling linked to vaso-occlusive crises that are a feature of sickle cell disease (SCD). Thus, these covalent modifiers and later renditions in the form of substituted benzaldehydes to stabilize the non-aggregation-prone $O_2$-bound state [6,7] foreshadowed drugs to appear 50 years later for treating SCD (discussed below). Starting from the 1980s, small molecules with binding affinity for the native state of enzymes started to be used to show the principle of correcting protein folding inside cells to treat misfolding diseases. For example, the addition of galactose to cells expressing mutant α-galactosidase A was shown to promote the folding and trafficking of galactosidase A variants associated with a lysosomal storage disease (LSD) [8], exemplifying drugs to appear 40 years later for treating Fabry disease (FD) (discussed below). In 1996, thyroxine and 2,4,6-triiodophenol were added to recombinant tetrameric transthyretin (TTR) in the buffer to prevent its dissociation, misfolding, and aggregation under acidic denaturing conditions, simulating the environment of the lysosome [9]. This report foreshadowed the feasibility of using the anti-aggregation drug tafamidis to treat TTR amyloidosis (ATTR). Tafamidis is the first disease-modifying, regulatory-agency-approved drug that targets a protein conformation to ameliorate a degenerative disease. Additional non-drug examples include using organic

phosphate derivatives to stabilize acylphosphatases [10,11] and patented small molecules to stabilize the dimeric folded state of superoxide dismutase-type 1 [12], demonstrating the general applicability of this concept. The rationale behind these articles was that a small molecule binding to the native state could increase the thermodynamic and/or kinetic stability of the native state of the protein, reducing the populations of non-native or misfolded states, as well as the frequency of structural fluctuations leading to aggregation, while increasing the population of the native state (Figure 1).

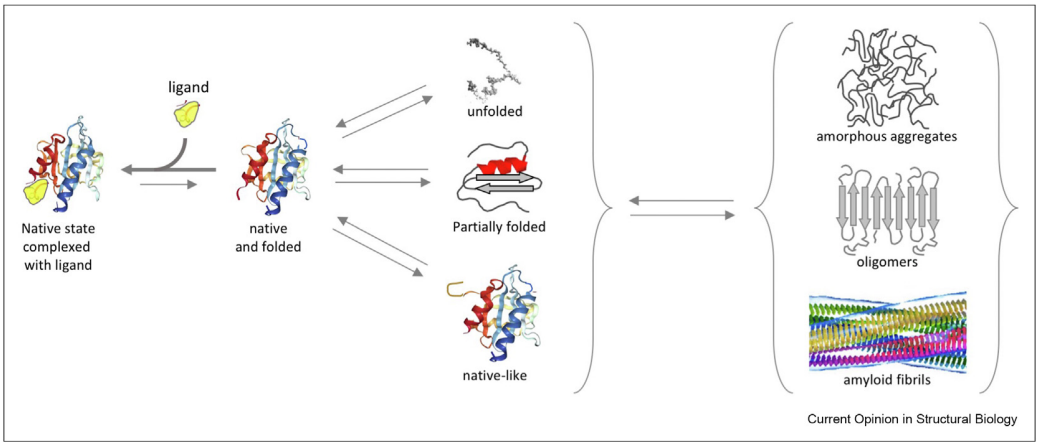
In this review, we will describe four proteins for which ligand binding has been shown not only to inhibit protein misfolding *in vitro* as a proof-of-principle, but has also led to the successful design of disease-modifying small molecules. These small molecules have provided satisfactory results in clinical trials leading to drugs that have been later approved by drug agencies in the United States, Europe, and other countries for slowing the relentless progression of protein misfolding diseases.

## Transthyretin and associated amyloidosis

The ATTRs are degenerative diseases resulting from rate-limiting dissociation of the native tetrameric TTR structure (wild-type homotetramer or mutant and wild-type heterotetramers), followed by monomer misfolding and low-energy barrier misassembly to afford a variety of TTR aggregate structures, including cross-β amyloid fibrils (Figure 2) [13,14]. The native state of TTR is a tetramer comprising β-sheet-rich 127-residue subunits and featuring two binding sites for thyroxine at the dimer-to-dimer interface (Figure 2). Over 130 TTR mutations have been described that cause familial ATTR with autosomal dominant inheritance [15]. Disease-associated mutations lead to kinetic and thermodynamic destabilization of the natively folded tetramer, resulting in TTR aggregation [16]. Depending on the sequence(s) of TTR undergoing aggregation, and likely additional genetic factors, degeneration of the peripheral nervous system and/or the autonomic nervous system, and/or the central nervous system, and/or the heart and other organ systems can occur. This tissue tropism may be based on the structural ensemble of aggregates afforded [13]. ATTRs are, therefore, a heterogeneous group of diseases, including sporadic TTR amyloid cardiomyopathy (ATTRwt-CM or SSA), familial TTR polyneuropathy (ATTRv-PN or FAP), familial TTR amyloid cardiomyopathy (ATTRv-CM or FAC), and more rarely, familial central nervous system TTR amyloidosis.

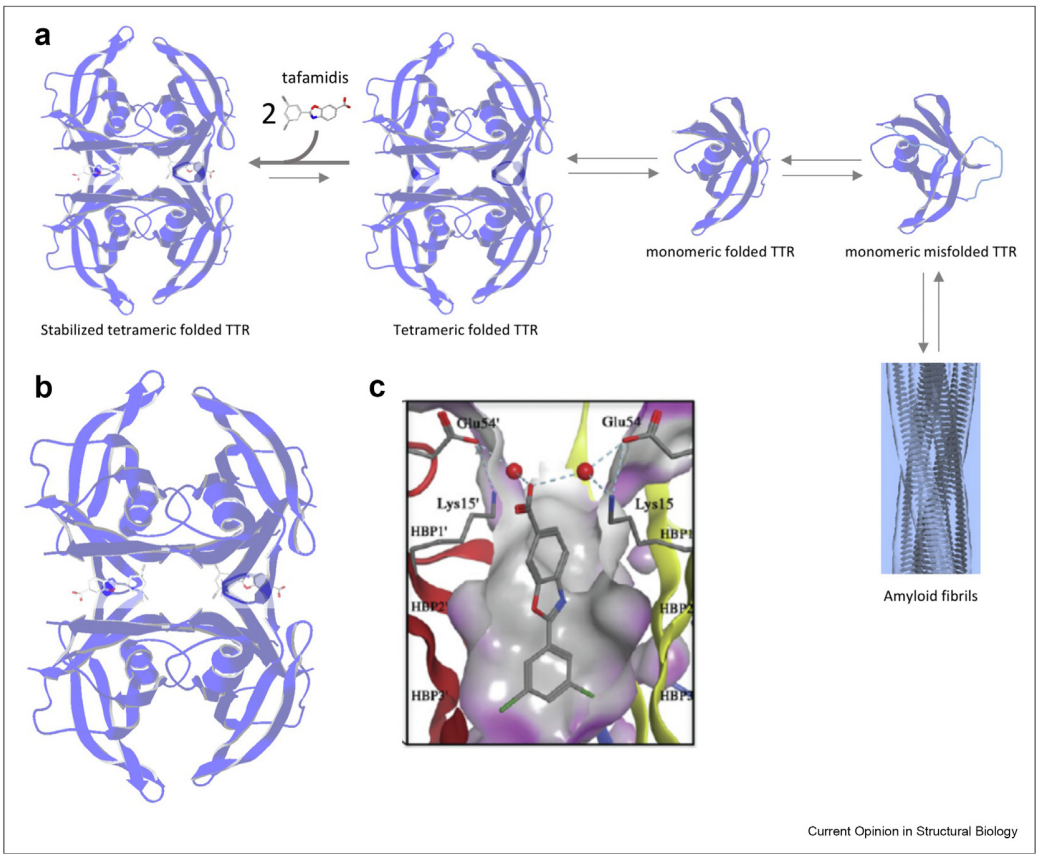
Because TTR tetramer dissociation is rate limiting for TTR aggregation and the TTR thyroxine-binding sites are largely unoccupied in blood, a kinetic stabilizer strategy for preventing TTR aggregation was conceived in 1996 based on stabilization of the native tetramer through small molecule binding [9]. In this strategy, small molecules, such as thyroxine or 2,4,6-triiodophenol, were envisioned to bind to native tetrameric TTR selectively over the dissociative transition state, increasing the activation free energy of tetramer dissociation to an extent inversely proportional to the dissociation constant of the TTR-small molecule complex, dose-dependently slowing down TTR tetramer dissociation (Figure 2) [9,17,18]. Structure-based drug discovery principles were then used to design more than 1000 candidate TTR kinetic stabilizers, of which 2-arylbenzoxazoles emerged as promising candidates owing to their preclinical potency and safety profiles

**Figure 1**




The native (folded) state can unfold completely, partially or undergo thermal fluctuations to generate the unfolded, partially folded or native-like ensembles, respectively (middle). All non-native states have the potential to be aggregation prone and can generate various types of aggregates, including amorphous aggregates, structured oligomers, and amyloid fibrils (right). The addition of a ligand to form a ligand-native state complex stabilizes the folded state (left) so that the alternative amyloidogenic species depicted in the center of the figure decrease in the population at equilibrium and/or form more slowly. Readapted with permission from Chiti and Dobson, 2017.

**Figure 2**




Stabilization of TTR by tafamidis. (**a**) TTR is a tetramer that needs to monomerize and then misfold before it can aggregate into an ensemble of misassembled non-native structures, including amyloid fibrils. Two molecules of tafamidis bind a tetramer at the two thyroxine binding sites with a 2:1 stoichiometry, resulting in thermodynamic and kinetic stabilization of the tetramer relative to monomers. Light gray, blue, red, and dark gray colours indicate C, N, O, and Cl atoms, respectively. (**b**) Tetrameric folded TTR complexed with two molecules of tafamidis. The structure was created with Protein Data Bank (PDB) entry 6E72. (**c**) Enlargement of one binding site for tafamidis, whose chemical structure is also reported in Table 1. Gray, blue, red, and green colours indicate C, N, O, and Cl atoms, respectively. Connolly analytical surface representation of TTR (translucent gray and purple indicate hydrophobic and hydrophilic portions, respectively). The Cl atoms are placed in a hydrophobic pocket, whereas the tafamidis carboxylate forms H-bonds with two Lys15 and two Glu54 residues from distinct subunits (dotted lines). Readapted with permission from Bulawa et al., 2012. TTR, tetrameric transthyretin.

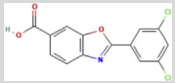
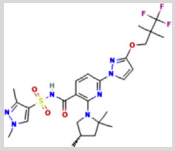
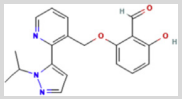
[19,20]. In 2011, one of these compounds, tafamidis, was approved by the EMA for the treatment of stage I ATTRv-PN (press release by EMA, URL) based on a placebo-controlled phase III clinical trial [21]. It was the first drug to slow down the progression of an amyloid disease, and more generally, the first disease-modifying drug that targets a native protein conformation. Tafamidis was also approved by the FDA and EMA in 2019 and 2020, respectively, for the treatment of ATTRv-CM and ATTRwt-CM at stages I and II (press release by FDA, URL; press release by EMA, URL) after a successful placebo-controlled phase III clinical trial [22].

Tafamidis orally dosed at 61 mg once daily achieves an average plasma concentration of 24 μM, sufficient to decrease the wild-type TTR tetramer dissociation rate by 95%, while exhibiting a safety profile that is indistinguishable from placebo controls [22,23]. Inhibiting the aggregation of newly synthesized TTR is sufficient to stop disease progression in most patients. That 30% of ATTRv-PN patients do not respond to tafamidis despite effective correction of the proteinopathy is consistent with the role that hyperactivated immune cells may be playing in the destruction of nervous systems, wherein aggregates are one of several activators of microglia in the brain and macrophages in the peripheral organ systems [24].

Owing to the clinical trial metrics established by the success of tafamidis and the earlier success of liver transplant-mediated TTR gene-replacement therapy, wherein the organ secreting mutant-destabilized TTR heterotetramers into the blood is replaced by a liver secreting only more stable wild-type TTR homotetramers [25], two pharmaceutical companies became energized to lower TTR levels as a therapeutic strategy.

**Table 1**

**Drugs approved by the FDA/EMA for the treatment of protein misfolding diseases.**

| Protein misfolding disease | Protein name | Drug name | Brand name | Chemical structure | Mechanism of action | Year of FDA approval | Year of EMA approval |
|---|---|---|---|---|---|---|---|
| Transthyretin amyloidosis (ATTR)[a] | Wild-type or Mutant TTR[a] | Tafamidis | Vyndaqel® or Vyndamax® |  | Binding to the two thyroxine binding sites of native tetrameric TTR endowing TTR with thermodynamic and kinetic stability | 2019[e] | 2011[f] and 2020[g] |
| Fabry disease (FD)[b] | Mutant α-GAL A[b] | Migalastat or DGJ | Galafold® |  | Binding to the active site of the fully folded enzyme (α-GAL A) with consequent stabilization | 2018 | 2016 |
| Cystic fibrosis (CF)[c] | F508del mutant CFTR[c] | Ivacaftor (VX-770) Tezacaftor (VX-661) elexacaftor (VX-445) | Trikafta® or Kaftrio® |  | Ivacaftor promotes channel gating of CFTR, whereas the correctors putatively bind to distinct domains of CFTR enabling their folding | 2019[c] | 2020[c] |
| Sickle cell disease (SCD)[d] | Hb with E6V in β-chain (HbS) | Voxelotor or GBT440 | Oxbryta® |  | Covalent/reversible binding of the aldehyde group with the α-chain N-terminus of oxy-HbS to prevent deoxy-HbS polymerization | 2019 | pending |

[a] Heterogeneous group of diseases including non-familial TTR amyloidosis with cardiomyopathy (ATTRwt-CM or SSA), familial TTR amyloidosis with polyneuropathy (ATTRv-PN or FAP), familial TTR amyloidosis with cardiomyopathy (ATTRv-CM or FAC), and rare forms of familial central nervous system TTR amyloidosis (ATTRv-CNS).

[b] Only patients with FD with 'amenable mutants', that is, α-GAL A variants recovering sufficient enzymatic activity upon folded state stabilization.

[c] Only patients with CF homozygous or heterozygous for the F508del mutation of CFTR. Chemical structure and years refer to the latest approved molecule (elexacaftor or VX-445).

[d] All patients with SCD.

[e] Approved by the FDA in 2019 to treat both ATTRwt-CM and ATTRv-CM at stages I and II.

[f] Approved by the EMA in 2011 to treat ATTRv-PN at stage I.

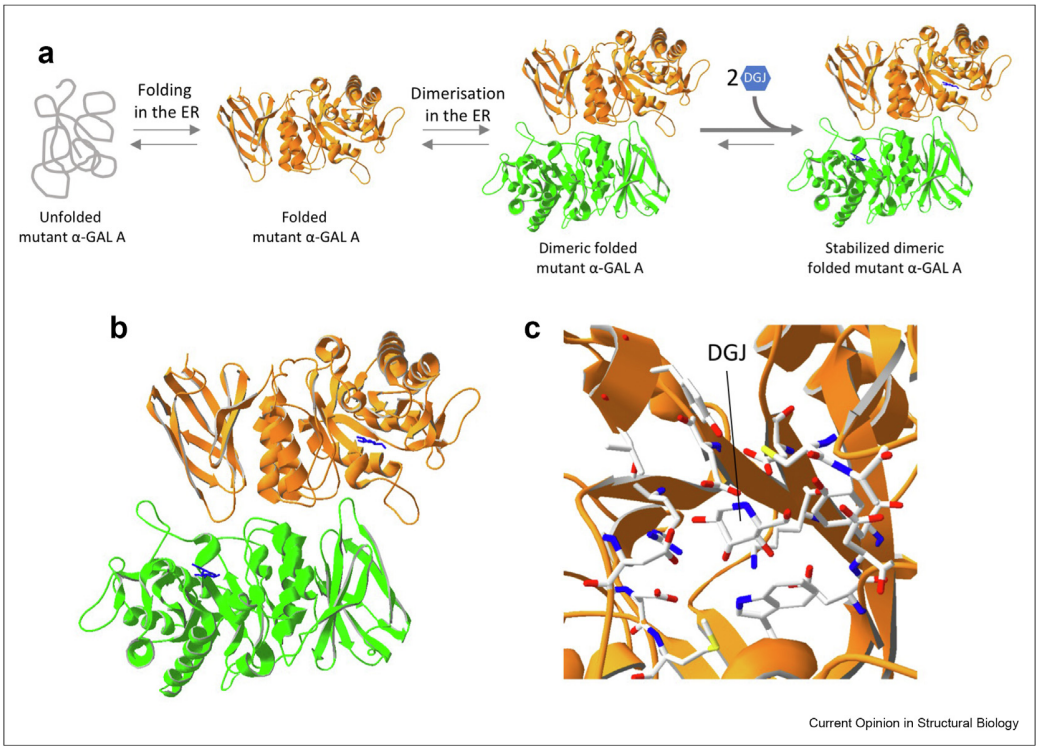
[g] Approved by the EMA in 2020 to treat both ATTRwt-CM and ATTRv-CM at stages I and II.

Thus, Ionis Pharmaceuticals and Alnylam Pharmaceuticals introduced the antisense oligonucleotide drug (Inotersen) and the RNAi drug (Patisiran), respectively, to lower TTR mRNA levels and inhibit mRNA translation into TTR [26,27]. These drugs lead to a >75% decrease in the TTR concentration in the blood, lowering TTR levels below the critical concentration required for aggregation [26,27]. Today, three drugs are sold to treat ATTR, tafamidis (Vyndaqel®, Vyndamax® by Pfizer), Patisiran (Onpattro® by Alnylam) and Inotersen (Tegsedi® by Ionis). All of them have been approved by numerous regulatory agencies, including the EMA and FDA (Table 1). A CRISPR-based TTR knockdown has recently been reported in human livers to ameliorate ATTR in a few polyneuropathy patients, although this is a phase I clinical trial result that merits a registration trial before regulatory agency approval is considered [28].

## α-galactosidase A and Fabry disease

Pathological conditions where attainment of the folded state of a mutant protein is challenging include the LSDs, exemplified by FD. Typically in LSDs, the folding-challenged enzyme fails to fold in the endoplasmic reticulum and traffic to the lysosome and is therefore degraded by the endoplasmic reticulum-associated degradation (ERAD) pathway. FD is generally caused by having a folding-deficient mutation on one allele coding for lysosomal α-galactosidase A (α-GAL A) [29,30] and an unfoldable α-GAL A truncation mutation encoded by the second allele. Lysosomal α-GAL A is a dimeric 398-residue α/β enzyme (Figure 3) that catalyzes the hydrolysis of α-1,4-glycosidic bonds formed by the terminal α-galactosyl groups of glycolipids and glycoproteins [29–31]. Rarely do the FD-associated missense mutations compromise the active site of the enzyme, leading to its complete inactivation [30]. Instead, many of the 1000 mutations so far described compromise α-GAL A folding in the neutral pH environment of the endoplasmic reticulum; for the small population of folded mutant α-GAL A attained in the endoplasmic reticulum, trafficking of this population to the acidic environment of the lysosome demonstrates that this folded mutant population is functional [29–

**Figure 3**




Stabilization of mutant α-GAL A by 1-deoxygalactonojirimycin (DGJ). (**a**) Folding, dimerization and ligand binding of mutant α-GAL A by two molecules of DGJ. Orange and green indicate the two α-GAL A subunits, respectively. Blue refers to DGJ. The binding of two molecules of DGJ to the α-GAL A dimer (one for each subunit) thermodynamically stabilizes the folded dimer of the mutant protein in the ER, preventing its degradation by ERAD. This pharmacological chaperoning promotes α-GAL A dimer trafficking to the lysosome. (**b**) Dimeric folded α-GAL A complexed with 2 molecules of DGJ. Colours as in A. (**c**) Enlargement of the binding site of one α-GAL A subunit, showing the complex with one DGJ molecule, whose chemical structure is also reported in Table 1. White, blue, red, and yellow colours indicate C, N, O, and S atoms, respectively. Readapted with permission from Lieberman et al., 2009. ERAD, endoplasmic reticulum-associated degradation; ER, endoplasmic reticulum; α-GAL A, α-galactosidase A.

31]. Because of the low functional levels of mutant α-GAL A in the lysosomes of these patients, the lysosomes of many cell types accumulate globotriaosylceramide (Gb3), which is the main substrate of the enzyme, resulting in a well-defined clinical phenotype.

In 1995, D-galactose was shown to stabilize the folded state of representative α-GAL A mutants, leading to their corrected folding in the endoplasmic reticulum and trafficking to the lysosome, therefore enhancing lysosomal enzymatic activity in COS-1 and lymphoblast cells [8]. Four years later, a galactose analog named 1-deoxygalactonojirimycin (DGJ) was established to bind reversibly and with a higher affinity to α-GAL A mutants than galactose [32]. This small molecule became the drug named migalastat, which was granted orphan drug status by the FDA and EMA in 2004 and 2006, respectively (Figure 3).

Amicus Therapeutics demonstrated the efficacy of DGJ in two placebo-controlled phase III clinical trials (NCT00925301 and NCT01458119) and called the drug Galafold®. Galafold was authorized by the the EMA and FDA in 2016 and 2018, respectively, to be marketed as the first oral drug for the treatment of FD with 'amenable mutants' (press release by EMA, URL; press release by FDA, URL). Today, Galafold is generally referred to as a pharmacological chaperone therapy (Table 1) and is one of the treatments of choice besides enzyme replacement therapy [33].

## Cystic fibrosis transmembrane conductance regulator and cystic fibrosis

Another successful application of folding-promoting drugs can be found in the treatment of cystic fibrosis (CF). More than 2000 cystic fibrosis transmembrane conductance regulator (CFTR) mutations have been described to cause CF, although the deletion of Phe508 (F508del) is by far the most common disease-associated variant, found in 80% of patients with CF [34]. CFTR is a 1480-residue transmembrane protein consisting of a small α-helical Lasso motif, two hexa-helix transmembrane domains (TMD1/TMD2), two α/β nucleotide-binding domains (NBD1/NBD2), and a largely intrinsically disordered regulatory domain (RD) connecting the NBD1 and TMD2 (Figure 4) [35]. The protein forms a channel that allows the flow of anions (mainly $Cl^-$ ions) across the cell membranes of epithelia, including lung epithelial cells [36]. F508del and many other CFTR mutants do not fold correctly in the endoplasmic reticulum and are degraded through the ERAD process [37,38]. Consequently, a very small pool of properly folded CFTR reaches the cell surface, and this small population is largely inactive because ion channel gating is dysregulated [37,38].

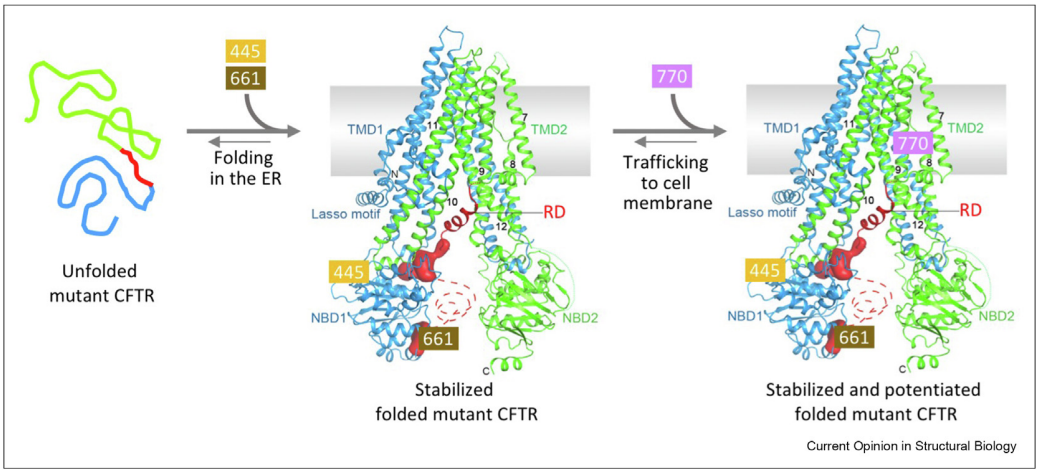
Until 2015, patients with CF were typically treated with ivacaftor (also known as VX-770), which is a potentiator of defective ion channel gating for a subset of CFTR variants, after successful completion of a placebo-controlled phase III clinical trial [39]. This molecule was first coupled to lumacaftor (VX-809) in 2015 and then to tezacaftor (VX-661) in 2018, two pharmacological chaperones approved by the FDA after successful clinical trials [40–43]. These molecules increase the fraction of CFTR that can fold and traffic to the apical cell membrane and were hypothesized to bind to residues 370–380 of CFTR, spanning the C-terminal end of NBD1 and the N-terminal residues of the following linker (Figure 4) [44,45]. These molecules are generally referred to as 'correctors' in the CF field [38], and unlike the situation with the other three case studies, the exact details of how these molecules function remains unclear. More significant clinical improvement was observed by adding a third small molecule, named elexacaftor (VX-445), in two separate placebo-controlled phase III clinical trials concluded in 2019 on patients with CF who harbor one mutated CFTR allele [46] or who were homozygous for F508del [47], respectively. Similar to tezacaftor and lumacaftor, this new molecule was found to act as a corrector (Figure 4) [38]. However, unlike tezacaftor and lumacaftor, this new molecule was hypothesized to bind to the NBD1 of CFTR and have a synergistic effect with tezacaftor (Figure 4), as a consequence of its ability to bind to a distinct site in the CFTR protein [38]. More recently, elexacaftor was also found to act as a potentiator of CFTR by promoting channel opening, again acting synergistically with ivacaftor [48,49].

Triple combination therapy based on ivacaftor/tezacaftor/elexacaftor for the treatment of CF caused by the F508del mutation was approved by the FDA and EMA in 2019 and 2020, respectively (press release by FDA, URL; press release by EMA, URL). This combination therapy is currently marketed by Vertex Pharmaceuticals under the brand names Trikafta® and Kaftrio® in the US and Europe, respectively (Table 1).

## Hemoglobin and sickle cell disease

The latest medication targeting a protein conformational state to be approved by regulatory agencies works by ligand-binding and reaction-based stabilization of an allosteric state of hemoglobin bearing the Glu6Val mutation in its β subunits (HbS). It has been clear for decades that HbS lacks solubility and polymerizes into fibrils when deoxygenated in distant capillary vessels, leading to red cell sickling, vaso-occlusive crises, hemolysis, and anemia, processes that shorten the lifespan and healthspan of patients with SCD. The deoxygenated conformation of HbS is amenable to nucleated polymerization, a process described by Bill Eaton in 1974 [50] (Figure 5a). The deoxygenated state, called the T state, can misassemble because the Glu6Val mutation in its β subunits enables misassembly of a native

**Figure 4**




Stabilization of mutant CFTR by elexacaftor (VX-445) and tezacaftor (VX-661) and ion channel potentiation by ivacaftor (VX-770). Mutant CFTR folding occurs in the endoplasmic reticulum (ER) to a very low yield, but the yield is enhanced by 'correctors' VX-445 and VX-661. Mutant CFTR reaches the cell membrane and its gating activity is improved by 'potentiator' VX-770. Blue, green, and red indicate Lasso/TMD1/NBD1, TMD2/NBD2, and RD, respectively. Light orange, dark brown, and light purple indicate elexacaftor (VX-445), tezacaftor (VX-661) and ivacaftor (VX-770), respectively. The chemical structure of the elexacaftor is also reported in Table 1. Readapted with permission from Liu et al., 2017. CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nucleotide binding domain; RD, regulatory domain; TMD, transmembrane domain.

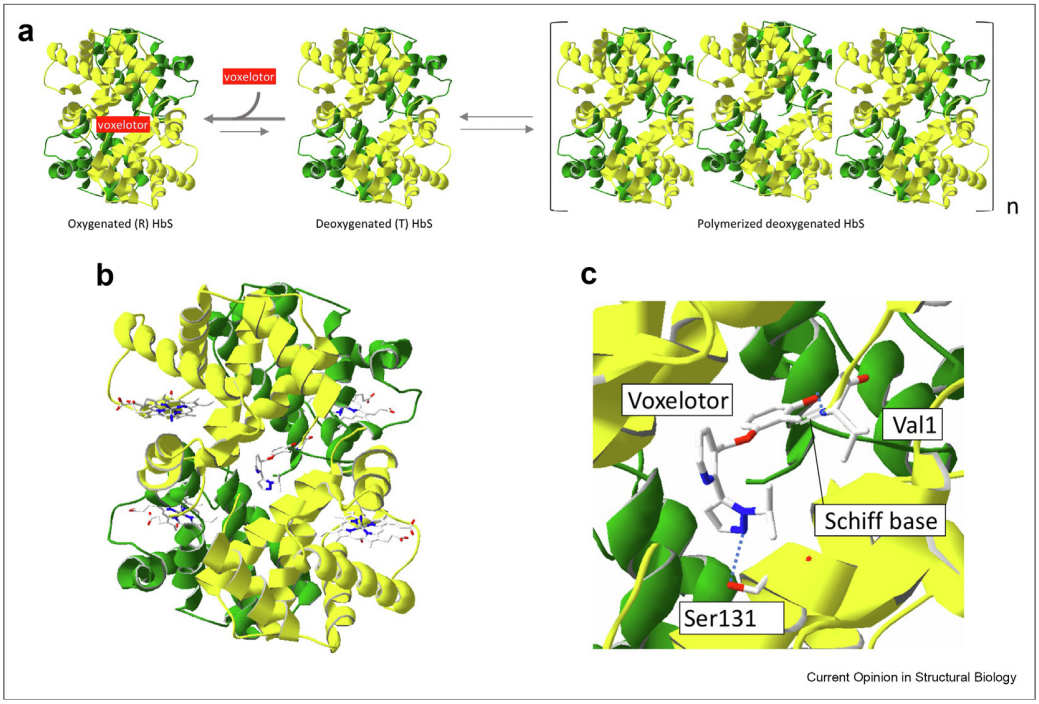
conformation, whereas the oxygenated state or R state of HbS is stable and soluble (Figure 5a). Hence, preventing deoxygenation under hypoxic conditions prevents HbS polymerization.

Wild-type hemoglobin (HbA) is an archetypical protein in structural biology, currently described in most biochemistry textbooks to introduce protein structure, function, and allosteric regulation. Its structure was solved at 5.5 Å by Perutz et al. [51] and later refined at progressively increasing resolutions from many organism sources in both the T and R states. Tetrameric HbA (or HbS) is an all-α protein, comprises two α and two β chains of 141 and 146 residues, respectively, and is associated with 4 hemes for its function of binding $O_2$ in red cells and its delivery of $O_2$ to all tissues (Figure 5a,b). The early elucidation of the HbA structure in both the R and T states enabled the rational design of proof-of-principle small molecules against SCD. These proof-of-principle small-molecule stabilizers of the HbS R state were described earlier than the small molecules that target the other conformational diseases featured in this review, although a disease-modifying drug was approved by a regulatory agency only recently.

Empirical observations first showed that the N-termini of the α and β chains could bind covalently and reversibly to generic aldehydes, increasing the affinity of HbA and HbS for $O_2$, stabilizing the non-aggregation-prone R-state [5]. Structural investigation of the R and T states of HbA revealed differences in the distance between the two N-termini of the α chains and distribution of surrounding residues [6]. Three benzaldehydes substituted with a hydrophobic substructure terminating in an acidic group at the ideal distance from the aldehyde were therefore designed to bind covalently and reversibly to one N-terminus and form a salt bridge with the other only in the R or oxygenated state; thus this resulted in an increased affinity of HbA and HbS for $O_2$, preventing HbS misassembly and red cell sickling. The compound named BW12C was notably active in this regard [6]. Other substituted benzaldehydes were later designed to improve oral bioavailability and pharmacokinetics, leading to tucaresol [7], 5-HMF [52], INN-312 [53], as well as others. None of them were evaluated in human SCD clinical trials.

A major breakthrough was achieved with the synthesis and analysis of voxelotor, also called GBT440 [54]. Global Blood Therapeutics discovered this potent covalent allosteric effector of HbS, harboring a substituted benzaldehyde functionality that reacts with only one of the two α-chain N-termini because the second is shielded by an H-bond between the pyrazole of voxelotor and Ser131 of the second α chain (Figure 5b,c). This was a critical outcome towards treating SCD because it resulted in a 1:1 stoichiometry of voxelotor to HbS (Figure 5b,c). Indeed, there is a high concentration of HbS in human blood and this result was probably critical for making investors more confident that a lower, but still high dose of voxelotor, could be used to safely treat SCD. The resulting Schiff-base between the N-terminus and the aldehyde group is stabilized via an

**Figure 5**




Stabilization of tetrameric oxygenated HbS by voxelotor (GBT440). (**a**) HbS exists in two native conformations, oxygenated (R) and deoxygenated (T) states. Polymerization proceeds from the T state. Voxelotor binds to and reacts in a 1:1 stoichiometry with the R state, stabilizing it. This shifts the equilibrium to the R state and lowers the population of the T state, preventing its polymerization. Yellow and green indicate the α and β chains, respectively. The red square represents voxelotor. (**b**) Covalent conjugate between tetrameric oxygenated HbS and voxelotor (in the center). The four heme groups are also indicated (one per subunit). (**c**) Enlargement of the structure of the conjugate formed between HbS and voxelotor, whose chemical structure is also reported in Table 1. Voxelotor reacts with HbS through Schiff base formation with the N-terminus of Val1 within the first α chain comprising tetrameric HbS. The Schiff base is stabilized by the indicated H-bond (dotted line). The voxelotor pyrazole forms an H-bond with Ser131 of the second α chain (dotted line). Yellow and green indicate the α and β chains, respectively. White, blue and red colours indicate C, N, and O atoms, respectively. Figure created with PDB entry 5E83 (Oxenberg et al., 2016).

intramolecular H-bond with the hydroxyl group at the ortho-position of voxelotor (Figure 5c). The chemistry of the molecule was also designed to complement the hydrophobic and hydrogen bonding attributes of a nearby pocket of the oxygenated state of HbS, resulting in stabilization of the oxygenated conformation of HbS (Figure 5c). Compared with previously evaluated molecules, voxelotor was shown to exhibit a higher affinity for purified and cellular oxygenated HbS, it displays a higher partitioning between red cells and plasma and exhibits suitable pharmacokinetics [54]. Voxelotor was also shown to inhibit HbS polymerization *in vitro*, impede sickling of HbS-containing red cells, and in a murine model of SCD it reduced cell sickling and prolonged red cell half-life [54].

Voxelotor was found to increase HbS levels significantly while reducing anemia and hemolysis in a human placebo-controlled phase III clinical trial [55]. Thus, the FDA approved GBT440 as a first-in-class medicine for the treatment of SCD in 2019, sold under the brand name Oxbryta® by Global Blood Therapeutics (press release by FDA, URL). This clinical success encouraged others to design related compounds that may offer additional benefits, including non-covalent stabilizers of the R state of HbS [56,57]. At present, four drugs are available to treat SCD, hydroxyurea (Hydrea®, Droxia®, Siklos®, Mylocel®), voxelotor or GBT440 (Oxbryta®), L-glutamine (Endari®), and crizanlizumab or SEG101 (Adakveo®). Hydroxyurea received FDA approval in 1998 and the other three in 2019, with approval by the EMA still pending. Among them, voxelotor is the only one that targets HbS (Table 1).

## Encouraging results on other misfolding-prone proteins with stabilizing ligands

The clinical success with tafamidis, as a stabilizer of the native tetrameric state of TTR, has encouraged the identification of small molecules that bind at the native monomer–monomer interface of dimeric immunoglobulin light chains (LCs) and function as stabilizers. In a pioneering study by Eisenberg and coworkers, a pool of 27 hydrophobic or aromatic compounds was used to identify small molecules that bind to the monomer–monomer interface of dimeric LC variable domains [58]. A 650,000-molecule library was then used to

discover small molecules that bind to the monomer–monomer interface of full-length LCs secreted by clonal plasma cells using a protease sensitivity assay reporting on the folded state stability [59]. Because light chain amyloidosis (AL) is both a plasma cell cancer and an LC misfolding and aggregation disease, it is currently treated with cytotoxic drugs to kill the cancer cells secreting full-length LCs. It is challenging to kill all of the cancer cells, and because resistant plasma cells secrete full-length LCs that can misfold and aggregate into non-native structures giving rise to organ toxicities, this strategy is only partially effective for ameliorating AL. Structure-based drug design has led to full-length LC stabilizers exhibiting 3000-fold potency increases relative to the small molecules initially crystallized, and the hope is that this approach will afford stabilizers that are complementary to the cytotoxic drugs for treating AL [60].

Pharmacological hope is also being offered for the reversal of cataract disease in aged human eyes. Cataracts are caused by the aggregation of crystallin proteins in the eye lens and cataract formation is the cause of ca. 90% of blindness in humans in developed countries [61]. Two independent studies identified 5-cholesten-3b,25-diol (also known as compound 29 or VP1-001) and lanosterol as promising small molecules to reverse aggregation of crystallins *in vitro* and in transfected cells, while also reducing cataract formation in transgenic mouse models and aged animals, such as mice, rabbits, dogs, and even humans [62,63]. In the first case, it was found that the small molecule binds specifically to the native state of an αB-crystallin dimer leading to its stabilization and increase in population at the expense of crystallin aggregates [64]. These compounds are currently being optimized for pet and human use.

An approach to stabilize the native state of misfolding-prone proteins, that represents an alternative to the use of small molecules as native state binders exemplified herein, is based on the use of single-domain camelid antibodies (nanobodies) that bind strongly to protein native states. Nanobodies have been shown to prevent aggregation of a number of amyloidogenic proteins associated with amyloid diseases, including lysozyme [65], the folded cellular prion protein [66], β2-microglobulin [67], and gelsolin [68], generally inhibiting conformational excursions into conformational ensembles that can misassemble into amyloid fibrils. All these approaches await clinical trials in humans.

## Conclusions

The examples presented herein on FD and CF demonstrate that small molecule binding to the native state of misfolding-prone proteins, that is, α-GAL A and CFTR, respectively, can enhance the ability of a protein to attain and maintain its native folded structure in cells in the context of loss-of-function diseases, in which the pathological condition typically originates from the inability of a protein to efficiently fold and traffic within the cell. The other two cases described herein focus on ATTR and SCD, demonstrating that native state binders can also enhance the ability of TTR and oxygenated HbS, respectively, to maintain a native three-dimensional structure after it is attained, inhibiting its misfolding and/or aggregation thought to cause gain-of-toxic-function phenotypes. The disease amelioration in all four cases is based on a common hypothesis that pharmacological stabilization of the native state of proteins susceptible to misfolding helps attain and maintain non-pathological protein conformations, supporting protein function. Numerous small molecules that bind specifically to and stabilize the native/folded state of a protein are under development by biotechnological and pharmaceutical companies, as well as by scientists in academia and other institutes of basic and applied research. That regulatory agencies have approved 4 native state stabilizer drugs over the past 10 years for general use by medical practitioners in numerous countries indicates the modernity of the rationale and its generic applicability in the future for treating other challenging maladies associated with abnormal protein conformations. The recently demonstrated ability to develop small molecule binders of specific conformations of the native state ensemble of enzymes to activate them represents a substantial pharmacological opportunity for disease amelioration as well [69].

## Credit author statement

**Fabrizio Chiti:** Conceptualization, Methodology, Writing - Original Draft, Visualisation, Supervision; **Jeffery W Kelly**: Conceptualization, Methodology, Writing - Review & Editing, Supervision.

## Conflict of interest statement

JWK discovered tafamidis at the Scripps Research Institute, is a shareholder of FoldRx Pharmaceuticals, acquired by Pfizer Inc in October 2010 (the companies that developed tafamidis into a drug), and receives royalty payments from tafamidis patents and sales.

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