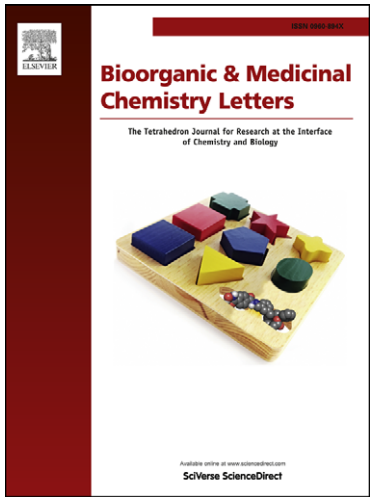

BMCL Digest

Recent Developments in Targeting Protein Misfolding Diseases

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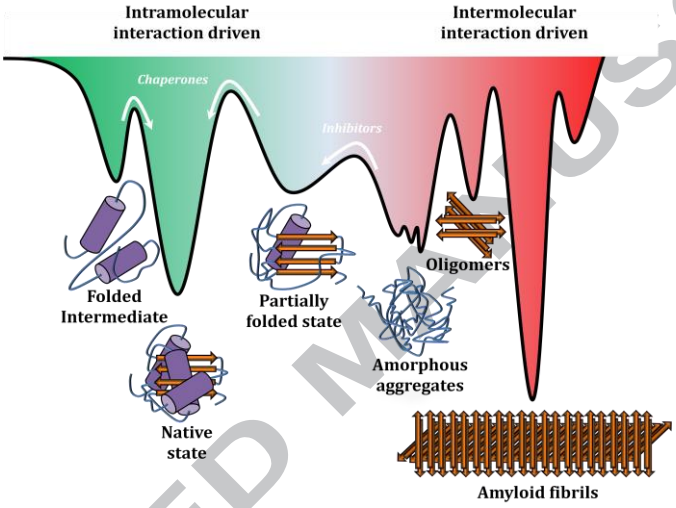

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ABSTRACT

Protein misfolding is an emerging field that crosses multiple therapeutic areas and causes many serious diseases. As the biological pathways of protein misfolding become more clearly elucidated, small molecule approaches in this arena are gaining increased attention. This manuscript will survey current small molecules from the literature that are known to modulate misfolding, stabilization or proteostasis. Specifically, the following targets and approaches will be discussed: CFTR, glucocerebrosidase, modulation of toxic oligomers, serum amyloid P (SAP) sections and HSF1 activators.

Protein folding is the process by which a protein attains a well defined three-dimensional structure known as its native state. This tertiary structure is reached through the folding of the polypeptide chain starting from a disordered state referred to as the unfolded state. The unfolded protein has both high entropy and high free energy (see Figure 1). The high entropy is due to the large number of possible conformational states while the high free energy reflects the protein instability. As the protein starts to fold, the free energy decreases and the number of accessible conformational states decrease as well. The folded native conformation is reached when the free energy is at a minimum, however local minima can trap the protein in an intermediate state slowing the folding process. Aggregation occurs when folding intermediates or partially folded states expose hydrophobic amino acid residues or regions that are largely buried in the native state. Aggregation is mainly driven by hydrophobic forces and results in the formation of amorphous aggregates, instable oligomers and ultimately amyloid fibrils (Figure 2). These thermodynamically stable structures are accessible to proteins under denaturing conditions and are also driven by the protein sequence.

The cellular machinery has evolved a stringent quality control system as part of the proteostasis network to ensure proper protein folding, trafficking and degradation.[1] However, despite the exquisite proteostasis network control, a number of proteins still fail to reach or maintain their native conformation leading to protein misfolding. When unfolded or misfolded proteins cannot be refolded by protein chaperones, they are targeted to the proteasome or the lysosome for degradation. Several factors contribute to misfolding: somatic or genetic mutations, aging, changes in the intracellular environment such as pH, temperature, oxidative stress and metal ions. Protein misfolding is linked to a large number of diseases, including cystic fibrosis, Alzheimer's disease, Parkinson's disease, and ALS (Lou Gehrig's disease) and is becoming increasingly more common as the population ages. These diseases can be defined and classified as "loss" or "gain" of protein function.[2] Loss of function (LOF) occurs if the improper folding results in the protein failing to achieve its functional conformation or reach its required location in the cell. Examples of diseases and targets associated with loss of function are shown in Table 1.

Table 1. Diseases and targets associated with loss of function

LOF Disease	LOF Protein
Cystic Fibrosis	CFTR
Gaucher's disease	Glucocerebrosidase
Hypogonadotropic hypogonadism	GNRH
Nephrogenic diabetes insipidus	V2R
COPD, emphesyma	A1AT
Fabry disease	Alpha-galactosidase

Retinitis pigmentosa	Rhodopsin

Gain of function (GOF) occurs when a protein accumulates, leading to toxic oligomers or aggregates that can adversely affect cell function. Examples of diseases and targets associated with gain of function are shown in Table 2.

Table 2. Diseases and targets associated with gain of function

GOF Disease	GOF Protein
Alzheimer's Disease	Amyloid Beta, Tau
Type II Diabetes	Amylin
Parkinson's disease	α-Synuclein
ALS (Lou Gehrig's Disease)	SOD1
Huntington's Disease	Huntingtin protein
Creutzfeldt-Jakob Disease (mad cow)	Prion protein
Familial amyloid polyneuropathy	TTR

Drug discovery strategies that restore protein folding and function can be grouped into three different categories:

1. Stabilization of a specific misfolding-prone or mutant protein using pharmacological chaperones. A small molecule pharmacological chaperone increases the population of the folded state by direct binding and stabilization, thus pulling the protein towards a lower free energy minimum (Figure 1). Examples of mutated proteins that can be stabilized by correctors or chaperones include CFTR, glucocerebrosidase (activation or inhibition) and rhodopsin mutations.

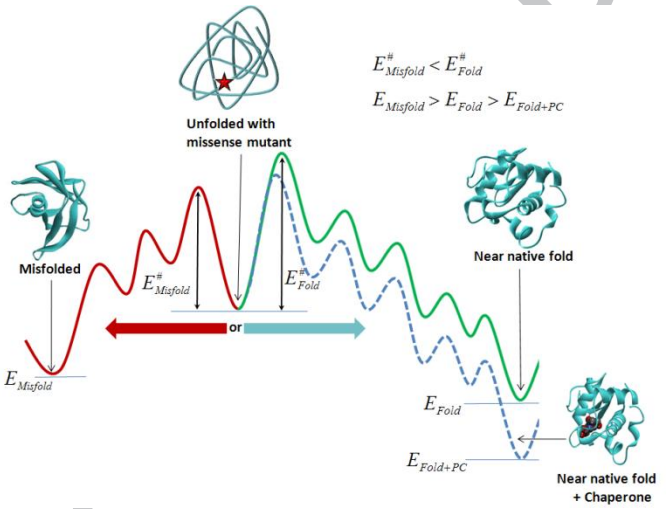


Figure 1: Schematic representation of protein folding energetics and how a pharmacological chaperone increases protein stability. For clarity purposes, the various protein states (misfolded, unfolded, folded and folded in presence of a chaperone) are shown on the same energy landscape. The reader should note that some proteins fold co-translationally while others are natively unfolded.

2. Inhibition of aggregation of a specific amyloid-prone protein that stabilizes the native state or the partially folded state and prevents the formation of oligomers or amyloids (see Figure 2). Examples of aggregated proteins that could be inhibited at the partially folded state include SOD1, prion proteins, and lysozyme.

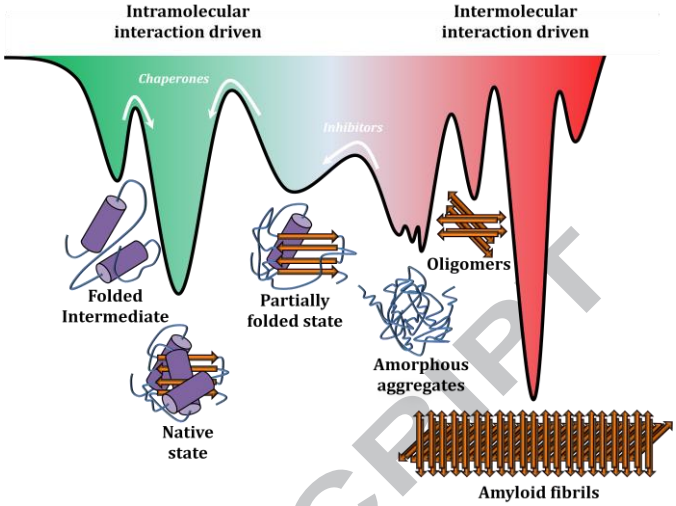


Figure 2: Folding intermediates leading to oligomer and amyloid formation.

3. Enhancing proteostasis by modulating the biological capacity of the cell's quality control protein network. Small molecule proteostasis regulators induce the unfolded protein response. This leads to the coordinated up-regulation of natural chaperones or the endoplasmic reticulum quality control capacity. The chaperones, co-chaperones and folding enzymes can reconstruct the folding free energy of mutant enzymes, "pushing" more protein toward the native state by lowering the energy of intermediate and transition states and thus minimizing misfolding. Examples of proteostasis targets include HSF1 (heat shock transcription factor 1), HSPs (heat shock proteins), ubiquitin targeted proteins, and proteases. Examples of diseases and targets associated with proteostasis are shown in Table 3.

Table 3. Diseases and targets associated with proteostasis.

Potential Indication	Proteostasis Target
Huntington disease	HSF1
Parkinson's disease	Hsp90
Neurodegenerative diseases	Usp14
Lysosomal storage diseases	Ryanodine receptor

Several reviews were published recently covering various topics related to protein misfolding.[3-7] In the present digest, we will focus on recent developments within the 3 aforementioned strategies of protein function restoration. Specifically, within strategy 1, correctors of CFTR for the treatment of cystic fibrosis and glucocerebrosidase chaperones for the treatment of Gaucher's disease will be highlighted. Novel approaches towards ameliorating the aggregation of misolded proteins will also be discussed in the toxic oligomer and serum amyloid P (SAP) sections. Although, HSF1 is not the only proteostasis target being pursued in this space, the large number of recent publications on the identification of HSF1 activators aimed at enhancing the proteostasis network makes it a suitable topic of discussion in the present digest. Within the scope defined above, protein truncation and nonsense stop codons are considered out of the mandate of this digest and are not discussed herein.

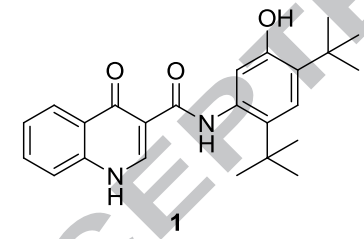
Cystic Fibrosis: CFTR Correctors

Cystic Fibrosis (CF) is the most common lethal genetic disease affecting Caucasians. Worldwide, approximately 70,000 people suffer from this disease.[8] CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel that regulates epithelial ion and water

transport in multiple organs, including the lung, pancreas, liver, and intestinal tract. In the lung, the loss of CFTR mediated chloride and bicarbonate secretion is believed to cause airway surface dehydration that leads to thick, sticky mucus accumulation, infection, inflammation and destruction of tissue. Lung disease is the primary cause of morbidity and mortality for those with CF. There is a huge unmet medical need to develop a curative treatment for CF patients since it is a fatal disease with a current median survival age of 37 years.

More than 1900 mutations in the CFTR gene have been identified and described, though many are extremely rare.[9] CFTR mutations have been grouped into 5 classes, based on the molecular mechanisms leading to the CFTR protein malfunction. Class I mutations contribute to the formation of proteins with incomplete length and provide protein with complete loss of activity (i.e., mutation: G542X). Class II mutations are those that lead to abnormal maturation of the CFTR protein in the ER and Golgi apparatus. The effect of these mutations is premature degradation, leading to a loss of function, since CFTR does not reach the cell membrane. Examples of class II LOF mutations are F508del, I507del, and S549R. The gene product having mutations of class III is properly synthesized, transported, and incorporated into the cell membrane, but has decreased functional activity in its gating properties, i.e., mutation: G551D/S. Mutations of class IV cause abnormalities in the structure of the transmembrane protein and therefore reduce the conduction of the chloride channel (i.e., mutations R117H, R334W). Lastly, mutations altering the stability of mRNA represent class V CFTR gene mutations.[9]

Kalydeco™ (Ivacaftor or VX-770, **1**, Figure 3), was approved in January, 2012 as the first disease modifying drug for patients with a class III gating mutation, G551D/S.[10] Although Kalydeco is not correcting the folding or trafficking, it rectifies the gating defect of cell surface (mutant) CFTR. Kalydeco is currently approved for ~5% of all CF patients.



1

(VX-770, Ivacaftor, Kalydeco™)

Figure 3: Structure of Kalydeco™, **1**.

The most prevalent mutation present in CFTR, accounting for more than 90% of CF patients, is when at least one allele contains a deletion of the phenylalanine at position 508.[11] This is an example of a class II mutation, where the protein does not make it to the cell surface. F508del CFTR also has a gating defect in the small percentage of protein that does make it to the cell surface. Therefore, any therapy correcting the trafficking of F508del will likely need to be combined with a potentiator.

CFTR is comprised of 1480 amino acids and is classified as an ABC transporter. CFTR consists of five domains, two nucleotide binding domains (NBD1 and NBD2), a regulatory domain (R), and two membrane spanning domains (MSD1 and MSD2), See Figure 4. Protein activity is regulated by cAMP-dependent protein kinase A (PKA)[12] and also by binding of two ATP molecules at the NBD1 and NBD2 dimer interface.

Designing a pharmacological chaperone for F508del CFTR would be the most direct approach to modulate misfolding. Unfortunately, structure based drug design (SBDD) for CFTR is challenging due to lack of an X-ray crystal structure. The published CFTR homology models are based on ATPases with low sequence similarity and with no R domain.[13-17] The R domain in CFTR does not have resemblance to any known crystal structure.[13]

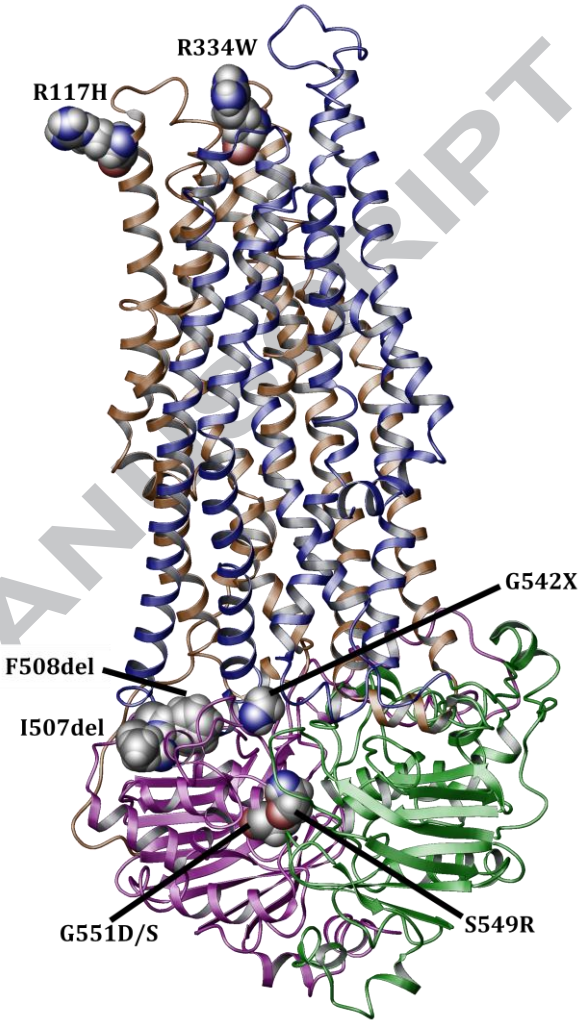


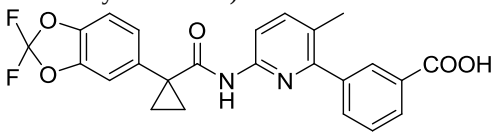
Figure 4: Homology model developed by Dalton et al.[18] of CFTR with 7 prominent mutations (described in the text) shown in CPK representation. MSD1, MSD2, NBD1 and NBD2 are highlighted in orange, blue, purple and green, respectively. The F508del mutation is in NBD1. Figure was generated using Schrodinger's Maestro v9.3.[19]

Although the F508del mutation is in NBD1, the crystal structure of F508del NBD1 and wild type (WT) NBD1 show very little structural difference.[20] However, it should be noted that the construct of the crystallized human NBD1 contains two solubilizing mutations, while the construct of the crystallized human F508del NBD1 contains several mutations (in addition to F508), in order to increase both solubility and stability.[20] Additionally, the crystal structures were obtained at 4°C, at which temperature there may be cold correction of protein. Further studies will be necessary to assess whether these mutations could affect the structure of F508del NBD1. Nevertheless, the structural similarity between WT NBD1 and F508del NBD1 hints at the kinetic nature of CFTR folding. Any mutation that disrupts the folding mechanism by delaying the process of folding gets degraded by the endoplasmic reticulum associated degradation (ERAD) pathways. Serohijos and team[21] performed molecular dynamic simulations to identify meta-stable

intermediate states appearing on WT and mutant NBD1 folding pathways. They observed that the NBD1 WT and mutant folding pathways are populated differently and their kinetic accessibilities of transitional intermediates are distinct, indicating the direct effect of the Phe508 deletion on NBD1 folding.[21]

For patients who have F508del CFTR, the only therapy currently available is supportive care. However, it has been shown that F508del CFTR is poised for repair and can be rescued by small molecules. There are several recent reviews on molecules that modulate CFTR and there is also a collection of CFTR modulators available in the public domain.[22-24] The mechanism of action of these F508del "correctors" is unknown, as most have been identified through phenotypic screening.

There are currently two small molecule correctors in the clinic for evaluation in CF patients with the F508 deletion. These compounds rescue F508del CFTR from degradation and help transit it to the cell surface. VX-809[25] (**2**, Lumacaftor, Figure 5), in combination with Kalydeco, has been tested in homozygous and heterozygous F508del CF patients. The complete findings from this trial are expected in 2013, but initial Phase IIa study results in homozygous F508del patients have recently been published.[26] In addition to VX-809, Vertex Pharmaceuticals has also initiated a Phase II clinical trial with a second corrector, VX-661 (structure not yet available).[27]



2
(VX-809, Lumacaftor)

Figure 5: Structure of VX-809, **2**

Gaucher Disease: Glucocerebrosidase Pharmacological Chaperones

Lysosomal storage diseases (LSDs) encompass over 40 inherited metabolic diseases that result from lysosomal dysfunction. The dysfunction is a consequence of the deficiency of a single enzyme required for the metabolism and of substrates such as glycolipids, glycans, polysaccharides and cholesterol. LSDs are classified based on the kind of substrate that accumulates.[28] Each LSD disorder results from different enzyme deficiencies. Gaucher disease (GD) is caused by the accumulation of glycolipids called glucosylceramides. The function of glucocerebrosidase is to hydrolyze the β-glycosidic linkage of glycosylceramide. Depending on the accumulating organ, a broad spectrum of clinical manifestations can be observed, including anemia, thrombocytopenia, bone disease and massive liver and spleen enlargement. Moreover, in the neuronopathic forms, brain deterioration is observed.

There are three GD clinical types, based upon the presence and progression of neurological disease manifestations. Type 1 disease (GD1), also referred to as non-neuronopathic GD, lacks primary central nervous system (CNS) involvement. Neuronopathic or CNS forms of the disease include types 2 and 3, where type 2 (acute neuronopathic) is characterized by infantile onset and rapid progression of neurological symptoms, and type 3 (chronic neuronopathic) is characterized by onset later in childhood and slower progression.

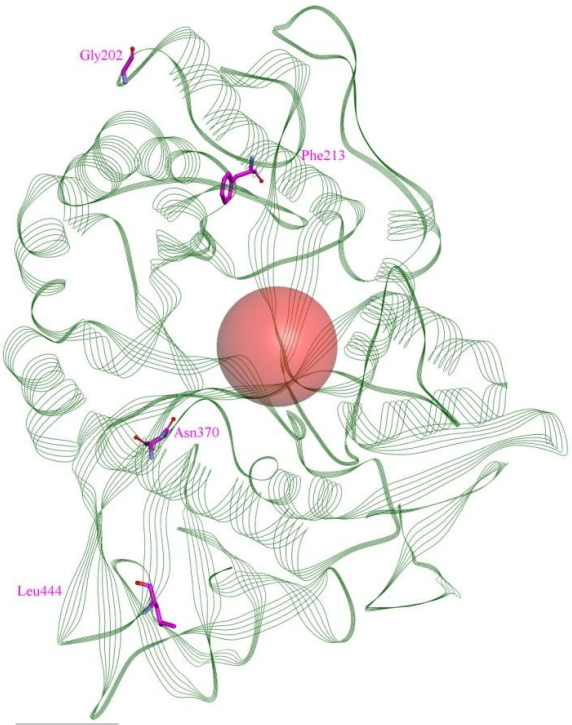
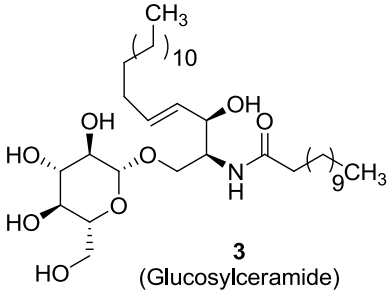


Figure 6: Crystal structure of GCase; line ribbon representation with missense mutations Gly202, Phe213, Asn370 and Leu444 represented in magenta stick. Red sphere identifies the catalytic binding pocket available in GCase. Figure 6 was generated with 2V3D coordinates using Discovery Studio 3.5.[29]

GCase, Figure 6, is a 516 residue enzyme with more than 300 known mutations.[30] Many of these mutations are shown to be benign, allowing GCase to fold to its native state and function normally.[31] A few missense GCase mutations either partially or completely abolish catalytic activity.[32] This occurs through the reduction of GCase stability resulting in premature proteosomal degradation.[33] These missense mutations are N370S, L444P, F213I, and G202R, as highlighted in Figure 6.[34-36] Among these, N370S is by far the most common mutation and is primarily associated with GD1. The L444P allele is most frequently associated with the neuronopathic variants.[37]

The most common treatment for GD1 is enzyme replacement therapy (ERT), by infusion of exogenous enzymes to process accumulated glycolipid substrates. It has been shown that when GCase is infused intravenously at regular intervals, it can lead to successful treatment of many of the systemic manifestations of the disease, and has greatly improved the quality of life for patients with GD1. However, the inability of recombinant enzymes to cross the blood–brain barrier prevents amelioration of the CNS associated symptoms in the neuronopathic forms (2 and 3) of GD.



3
(Glucosylceramide)

Figure 7: Structure of glucosylceramide, **3**

The second treatment option for GD is substrate reduction therapy (SRT). The concept of this therapy is to reduce the amount of the natural substrate that GCase should be metabolizing in the lysosome. The structure of the natural substrate, glucosylceramide, **3**, is shown in Figure 7. Despite ERT and SRT successes in patients with GD1, these therapies are ineffective against type 2 and 3 neuronal forms of the disease.

A benefit of applying the pharmacological chaperone approach to GCase, is that chaperones have the potential to cross the blood-brain barrier, providing an opportunity to treat additional symptoms of the disease. Isofagomine, **4**, Figure 8, a competitive small molecule inhibitor of GCase, at sub-inhibitory concentrations, has been shown to act as a pharmacological chaperone leading to increased catalytic activity.[38] The major effect of isofagomine is to facilitate the folding of newly synthesized GCase in the ER, thereby increasing the lysosomal concentration of the enzyme.

The first generation of GCase pharmacological chaperones including isofagomine (**4**), NB-DNJ (**5**), NN-DNJ (**6**) and ND-DNJ (7) are natural and alkylated iminosugars, which are inhibitors of GCase, Figure 8.[37] These iminosugars resemble the structure of the natural glycoside substrate for GCase. Therefore, it is not surprising that these compounds have high affinity for the catalytic pocket of glucosidases and poor isoenzyme selectivity. Although these compounds promoted GCase trafficking, they prevented its enzymatic activity in the lysosome due to the fact that they are also potent GCase inhibitors.[37] It was found that there is a narrow therapeutic window between improving translocation and inhibiting enzyme activity.[39] In fact, isofagomine was discontinued in phase II clinical trials for failing to meet efficacy expectations, even though almost all of the patients enrolled experienced an increased level of GCase in white blood cells.[40]

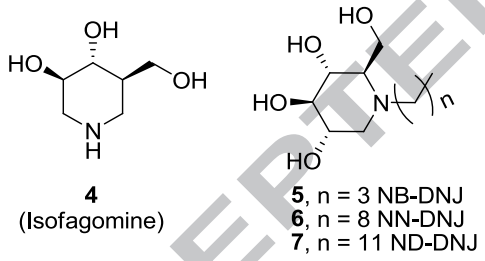


Figure 8: First generation of pharmacological chaperones, **4-7**. (NB-DNJ = N-butyldeoxynojirimycin; NN-DNJ = N-nonyl deoxynojirimycin; ND-DNJ = N-dodecyl deoxynojirimycin).

The lessons from first generation chaperones suggest that an ideal chaperone should have weak binding to mutant GCase in the endoplasmic reticulum (where it is formed) and no inhibitory effects in the lysosome (where it functions). This demands that chaperoning activity at the ER should be a balance between folding enhancement and GCase activity. Reports on imino-sugar derivatives with a more varying chaperone-inhibitor balance have recently been reported in the literature.[41-52]

Alternatively, it is possible that pharmacological chaperones binding to an allosteric pocket of GCase may provide a therapeutic advantage by increasing translocation to the lysosome without hindering its function. From the structural perspective, non-glycomimetic small molecules also have the potential advantage of increasing selectivity over the other glycosidases. The first non-glycomimetic chaperones were identified through the use of a quantitative high throughput screen on a structurally diverse library consisting of 59,815 compounds.[53] The primary screen was aiming to identify binders to WT GCase using an enzymatic assay. A subsequent follow up assay in patient cell-based fibroblasts expressing mutant GCase was used to distinguish those compounds with chaperone activity. Three structurally distinct classes of compounds, namely aminoquinolines, sulfonamides and triazines (**8-10** in Figure 9), showed good potency and efficacy. More importantly, these compounds exhibited selectivity against α-glucosidase, α-galactosidase and β-hexosaminidase. Mutant cells treated with 40 uM of **8** or **9** showed an increase in N370S mutant GCase activity and enhanced lysosomal co-localization, indicating chaperone activity.

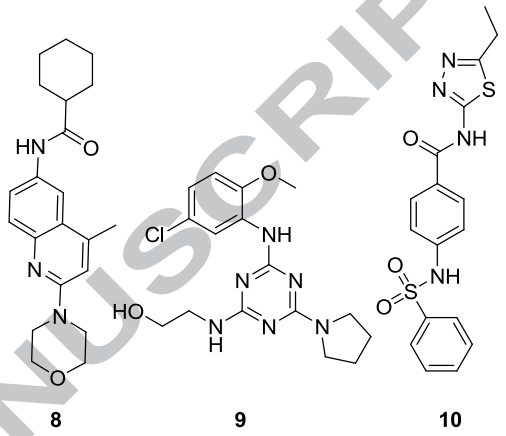


Figure 9: First non-iminosugars, **8-10**, identified as pharmacological chaperones for GCase

Recently, an HTS screen was reported using enzyme homogenates from the spleen of a patient with Gaucher disease with the N370S/N370S genotype.[54-58] Under these conditions, GCase is believed to be present in a "normal" physiological environment bound to the native activator saposin C and other co-factors. Using this assay, a library of 250,000 compounds was screened and novel activators of mutant GCase, **11-13**, were identified, Figure 10.

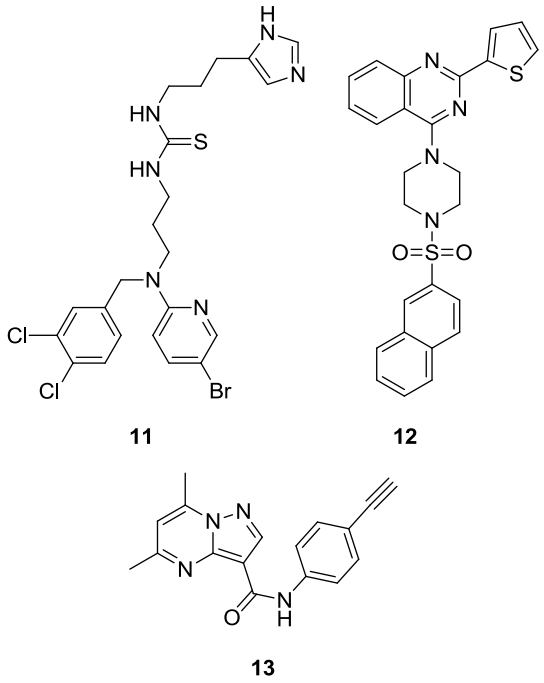


Figure 10: GCase pharmacological chaperones, **11-13**.

The activity of the primary hits was confirmed in subsequent cell-based assays using patient-derived fibroblasts. Translocation

experiments using Gaucher fibroblasts showed that these compounds facilitated translocation of GCase to the lysosome and thus act as chaperones. These compounds were shown to bind directly to GCase using microscale thermophoresis (fluorescence using an IR laser), however the exact binding site of these compounds is not known.

Amyloidoses: Targeting Toxic Oligomers

Until recently, it was believed that the gain of function (GOF) toxicity in protein misfolding diseases was associated with the accumulation of the amyloids or fibrils formed by the misfolded protein. The latest research however suggests that the oligomers, and not the fibrillar forms, are the most toxic species, both *in vitro* and *in vivo*.[59-63] Metastable oligomeric structures have been observed in the preparations of amyloid-forming peptides such as α-synuclein, tau, prion, TTR, SOD1, Aβ, and many others. When produced intracellularly, oligomers expose flexible hydrophobic surfaces that might contribute to trapping vital proteins. When produced extracellularly, oligomers can cause potentially toxic alterations of cell membranes.[64] The binding of these oligomers to a variety of cell-surface receptor signaling molecules has been hypothesized to be the cause of the toxicity in protein misfolding diseases.[65] Furthermore, it has been proposed that these pre-fibrillar aggregates may initiate a number of cellular signals and responses that can become self-perpetuating, independent of the initial protein misfolding event.[66]

As the field moves toward the common consensus that it is the oligomers (and not the aggregates) which cause the toxicity, the approaches to tackling these disorders must adapt accordingly. The methods and molecules that will be designed to remove aggregates versus oligomers will inherently be very different.

One strategy is to look for molecules that bind to the soluble oligomers in order to preclude them from interacting with cell surface receptors. In other words, the target in a screen would be the oligomeric species itself, as shown below in Figure 11.

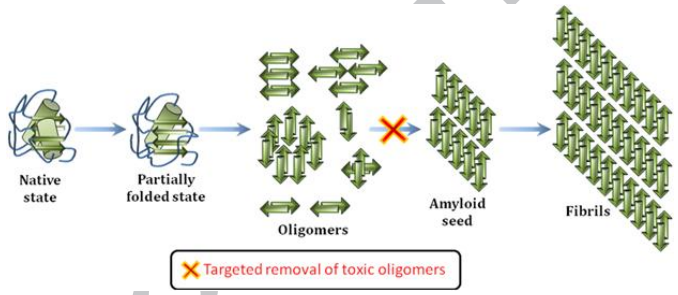


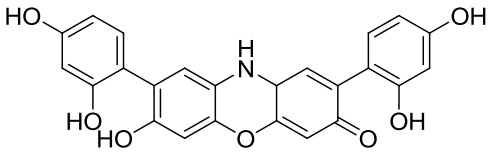
Figure 11. Schematic representing the idea of toxic oligomer sequestration

Key to this strategy is to develop a method to generate stable and soluble oligomers *in vitro*, though their preparation is not an easy task. Despite the enormous importance of these molecules, detailed knowledge regarding the structure of Aβ oligomers and the biological mechanism behind their toxicity have remained unclear. This knowledge gap can be partly ascribed to the difficulty of preparing stable and well-defined oligomeric Aβ species. Oligomers made using standard methods are heterogeneous and contain a mixture of different Aβ species (monomers, multimers, various oligomers, protofibrils, fibrils etc), which makes them difficult to study.[67, 68] Additionally, the oligomers that are prepared are quite unstable. Typically, oligomers prepared and purified using standard methods stay in solution for only a few hours.[69] The preparation of stable and soluble oligomers is not an easy task. However, scientists at Crossbeta Biosciences[70] reported a method for synthesizing 1-42 peptide (Aβ) type oligomer, implicated in Alzheimer's disease (AD). The reported Aβ oligomers were claimed to be stable for weeks.[71] The identification of small molecules that bind to such an oligomer and prevent their aggregation could be of considerable therapeutic value.

Another method that is being investigated is to remove the toxic oligomer via its conversion into a non-toxic aggregate. Incorporation of toxic oligomers into protective amyloid-like protein inclusions has been observed to reduce toxicity in mammalian cells and mouse models.[72] Scientists at the Medical College of Georgia have recently published work that HspB1 (also known as heat shock protein 25) has an important role in protein aggregation diseases. Using biochemical methods, light scattering, and microscopy methods, they have shown that HspB1 sequesters toxic Aβ oligomers and accelerates their conversion into non-toxic aggregates.[73]

Narayan *et al.*[74] recently described how the extracellular chaperone clusterin sequesters all of the different oligomeric forms of Aβ peptide to form stable, non-toxic Aβ-clusterin aggregates. This work has shown that the sequestration of disease-associated proteins into insoluble protein inclusions reduces their cytotoxicity and alleviates cellular dysfunction.

Similarly, the small molecule O4, **14**, Figure 12, has been shown to bind directly to the hydrophobic amino acid residues in Aβ peptides and stabilizes the self-assembly of the seeding-competent, β-sheet-rich protofibrils and fibrils.[75] Notably, the O4-mediated acceleration of amyloid fibril formation efficiently decreases the concentration of small, toxic Aβ oligomers in complex, heterogeneous aggregation reactions. This result supports the hypothesis that toxic oligomers can be fibrillized efficiently with the use of a small molecule.



14 (O_4)

Figure 12. Structure of O4, **14**.

One of the key challenges is to be able to accurately describe the structure and dynamics of the heterogeneous population of species formed during amyloid aggregation. This step is essential in order to determine which oligomeric species is responsible for causing the disease. If the structure of the toxic molecule is elucidated, it should be more straightforward to identify ways to prevent their formation, enhance their removal or block their effects.[76]

To further complicate the oligomer hypothesis, it is possible that oligomers do not exist as a single molecule, but instead are only present as dynamic and heterogeneous oligomers.[77] Further research in this field is required to fully elucidate the structures of the toxic species in GOF misfolding diseases.

Amyloidoses: Serum Amyloid P (SAP) Inhibition

Serum amyloid P (SAP) is a plasma glycoprotein that is a member of the pentraxin family of proteins. SAP is synthesized in the liver and consists of five non-covalently associated identical subunits that form a donut-like structure. SAP succumbed to X-ray crystallography which elucidated that the symmetric pentameric disc is approximately 100 angstroms in diameter, 35 angstroms in depth, and contains a 20 angstrom pore in the center, as shown in Figure 13.[78] Each SAP subunit consists

of 204 amino acids in a single polypeptide chain with a metal-binding site containing two calcium ions.

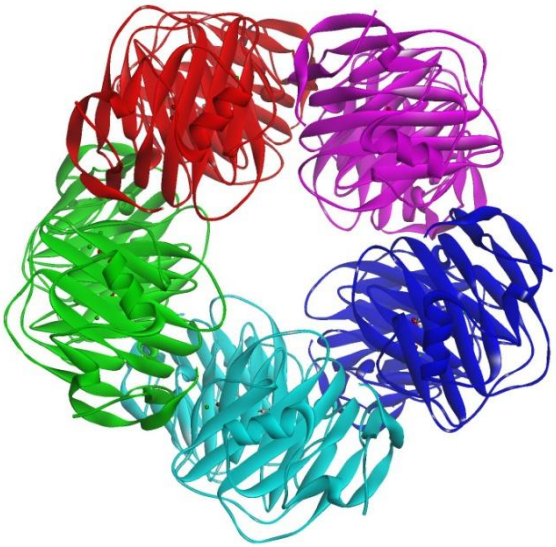


Figure 13: Top view of decameric SAP X-ray crystal structure, shown using 2A3W coordinates. Figure is generated using Discovery Studio 3.5.[29]

Although SAP is evolutionary-conserved in all vertebrates, the function of circulating SAP is not well understood. SAP is best known as a universal constituent of fibril deposits that are found in all amyloid diseases. SAP binds to apoptotic cells, double-stranded DNA and chromatin in a calcium-dependent manner.[79] Therefore, it has been hypothesized that SAP functions as a scavenging protein which is able to recognize nuclear cell debris released during apoptotic and necrotic cell death, masking them from the immune system. More recent work suggests that SAP facilitates phagocytosis of apoptotic bodies by macrophages, indicating that it plays a role in innate immunity.[80] However, SAP knockout mice developed normally and have an average lifespan, suggesting that SAP blockade may not be detrimental.[81]

Since there are many different proteins responsible for the clinically significant amyloidoses, a specific treatment for each GOF disease would require a specific drug to target the pre-fibrillar protein precursor for each different amyloidosis. On the other hand, all amyloid deposits contain a SAP component. Therefore, SAP has become a target for drug discovery and development. SAP can comprise up to 14% of the dry mass of any type of an amyloid.[82] In fact, researchers have taken advantage of SAP's affinity to amyloids by developing a clinical diagnostic technique called SAP scintigraphy where radiolabelled SAP protein is injected into patients to locate areas of amyloid deposition.[83, 84]

Although the specific interactions of SAP binding to amyloids are not known, SAP is believed to encase fibrils thus stabilizing them by inhibiting their removal via normal scavenging mechanisms. Since SAP alone is highly resistant to proteolytic cleavage, when bound to amyloid fibrils, it in turn protects the amyloid from degradation.[85] Actually, layers of SAP have been observed on the surface of amyloid fibrils.[86] SAP's critical role in the prevention of amyloid removal makes it a key contributor to the pathogenesis of all amyloid diseases.

In order to disrupt the binding of SAP to amyloids, Roche, in collaboration with the Imperial College of Medicine in London, performed an HTS searching for competitors of SAP binding to the amyloid formed from Aβ oligomers. From this work, Ro 63-8695, **15**, a palindromic bis-D-proline compound was discovered, Figure 14.

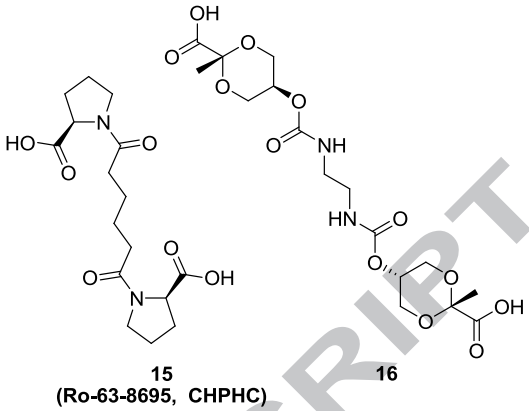


Figure 14: Structure of Ro 63-8695, **15** and 2A3W crystal structure ligand, **16**.

Ro 63-8695, also known as CPHPC (abbreviation of (R)-1-[6-[(R)-2-**c**arboxy-**p**yrrolidin-1-yl]-6-oxo-**h**exanoyl]**p**yrrolidine-2-**c**arboxylic acid), was found to prevent SAP from binding to amyloid fibrils both *in vitro* and *in vivo*.[87] A crystal structure of the SAP-CPHPC complex revealed a decamer in which 5 CPHPC molecules crosslinked and dimerized 2 SAP pentamers. The coordinates of the SAP-CPHPC complex are not available in the public domain. However, a co-crystal structure with a different multivalent SAP inhibitor, **16**, has been published, Figure 15.[88]

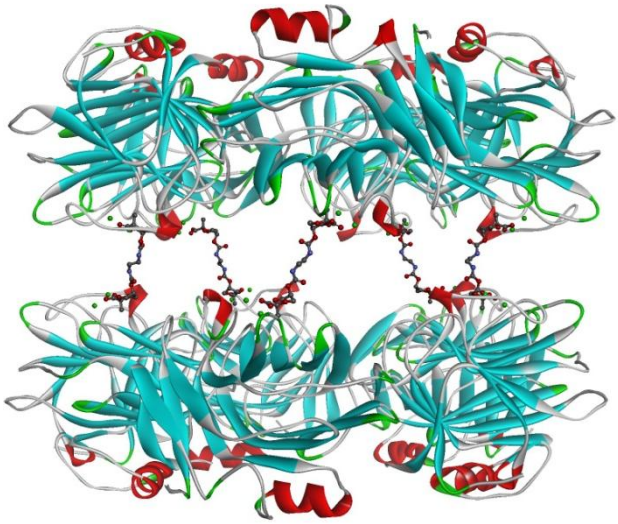


Figure 15: Five molecules of **16** crosslinking 2 SAP pentamers, pdb 2A3W, generated using Discovery Studio 3.5.[29]

As anticipated, the SAP-CPHPC complex is perceived as abnormal by the liver and is instantly cleared, leading to a profound depletion of SAP from circulation, continuing for as long as the drug is administered.[89] In mouse models of systemic amyloidosis, CPHPC was found to dramatically decrease circulating levels of SAP. Perhaps even more important, CPHPC was found to bind to SAP that had already bound to amyloids, dissociating the SAP from the fibrils, and causing amyloid regression.[87] For these reasons, this compound was advanced to clinical trials to 'knock out' human SAP. This was the first example of a clinical trial aimed at using a small molecule to deplete a specific plasma protein from circulation and tissues.

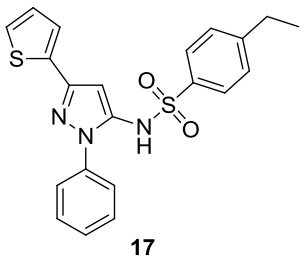
CPHPC has been investigated in the clinic for several GOF diseases including AA amyloidosis, Amyloid Light Chain (AL) amyloidosis, hereditary TTR amyloidosis, apolipoprotein AI amyloidosis, fibrinogen A amyloidosis, gelsolin amyloidosis, lysozyme amyloidosis and Alzheimers disease.[87, 89] CPHPC depleted circulating SAP by more than 90% while the drug was administered, however most of the SAP remained bound to amyloid fibrils, even after months of treatment.[90] The reason for the lack of SAP dissolution could be due to the fact that human SAP binds more avidly than mouse SAP. Moreover, there is a continuous production of 50-100 mg of new hSAP per day.[91] CPHPC was found to be well tolerated by patients and has been administered to more than 60 subjects for a total of more than 50 patient years without any adverse effects. However, since there was no evidence of amyloid regression, CPHPC has not been approved as a single agent drug. Currently, CPHPC is being evaluated in a clinical trial in combination with a fully humanized mouse monoclonal anti-human SAP antibody.[92, 93]

Proteostasis Modulation: Heat Shock Transcription Factor 1 (HSF1)

HSF1 is the master transcriptional regulator that controls the heat shock response (HSR). It maintains proteostasis and resistance to cellular stress through the production of heat shock proteins (HSPs). Selective activation of HSF1 with a small molecule induces the entire protein chaperone network. Chaperone proteins work synergistically in cells to combat protein aggregation, enabling the body's natural response to effectively reduce protein misfolding.[94, 95]

Pharmacological activation of HSF1 and transcriptional activation of genes encoding protein chaperones can be achieved by different mechanisms. HSF1 can be activated by molecules that cause cell stress or alternatively, by molecules that inhibit the protein chaperones. Small molecules that activate HSF1 by promoting protein misfolding or cellular stress are unlikely to be useful in the chronic treatment of diseases, as these molecules ultimately promote cellular dysfunction and lead to cell death. Therefore, pharmacological agents that specifically activate HSF1 without triggering a cell death pathway will be required.

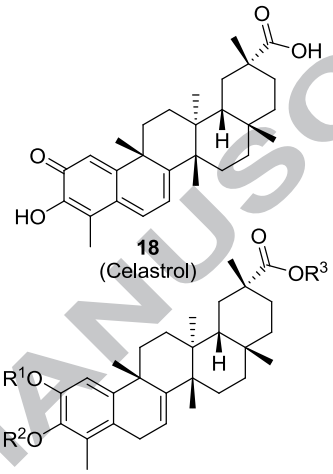
Neef *et. al.*[96] performed a high throughput screen using a humanized yeast based assay and reported a pyrazole sulfonamide compound **17**, Figure 16. Compound **17** was shown to activate HSF1 in mammalian and fly cells. It was also found to elevate protein chaperone expression and ameliorate protein misfolding and cell death in polyQ-expressing neuronal precursor cells. Moreover, **17** was able to protect against cytotoxicity in a fly model.[96] Though the specific mechanisms by which compound **17** promotes HSF1 expression remain unclear, it has been proposed that it interacts with the T complex protein 1 ring complex (TRIC) – a cytosolic chaperonin complex.



17

Figure 16: Structure of HSF1 activator, **17**.

Morimoto and coworkers[97] performed a screen of bioactive small molecules that activates human heat shock response and identified celastrol, **18**, and its derivatives, **19-21**, as shown in Figure 17. While many HSR activators show delayed kinetics of induction, triterpenoids **18-21** induce HSF1 rapidly, with kinetics similar to those observed for heat shock in normal cells. This rapid induction of the heat shock response represents a new class of HSF1 inducers. HSF1 activation by celastrol was shown to promote molecular chaperone expression and increases viability upon lethal heat shock in yeast and mammalian cells.[98] Although the ability of Celastrol to promote heat shock and antioxidant pathways has proven to be efficacious in reducing protein aggregation and cytotoxicity in models of ALS,[99, 100] Alzheimer's disease,[101] Huntington's disease[102] and Parkinson's disease[103] the therapeutic potential of celastrol is limited due to its inherent cytotoxicity.[104-106]



18
(Celastrol)

19; R^1 = H, R^2 = H, R^3 = H
20; R^1 =C(O)Me, R^2 = C(O)Me, R^3 = H

Figure 17: Structures of Celastrol, **18**, and derivatives, **19-21**.

Zhang and coworkers[107] from CytRx also identified several small molecule amplifiers of the heat shock response pathway. They developed a high content target based primary screen,[108] and validated their assay by confirming a previously identified 3,4-dichloroisocoumain, serine protease inhibitor[109] as an enhancer of HSF1. The screening campaign included ~4,000 compounds, including known bioactive libraries such as LOPAC and the NIH clinical collection. Three potent gedunin derivatives, **22-24**, and a sapanone A derivative, **25**, were discovered as HSF1 activators, Figure 18. They showed these compounds rescued cells from cell death caused by the potent and selective 26S proteasome inhibitor MG-132. Furthermore, RNAi knockdown of HSF1 significantly reversed the cytoprotective effects, confirming that these compounds have an HSF1-dependent mechanism of action. HSF1 amplifiers **22-25** were also tested in two mammalian cell based models of Huntington's disease (HD), and were found to improve survival.[107]

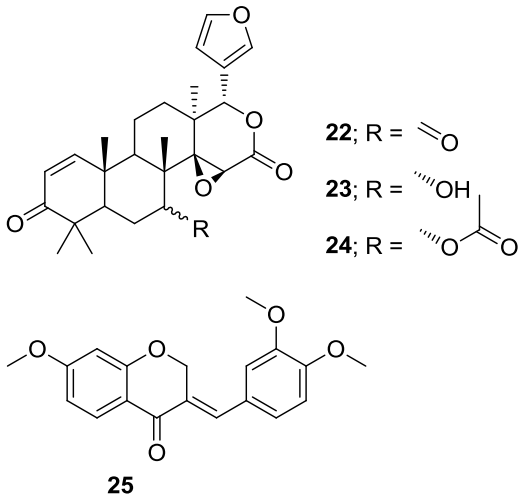


Figure 18: Structures of HSF1 activators, **22-25**.

The promising space of protein misfolding presents exciting drug discovery opportunities in a wide range of therapeutic areas. Innovative screening technologies and improved understanding of protein misfolding kinetics and thermodynamics are already providing novel ways of correcting misfolded proteins in LOF diseases, as shown in the case of CFTR and GCase. Significant progress is being made to better understand the toxicity of misfolded oligomeric intermediates and amyloid formation. This knowledge will likely pave the way to the development of novel therapeutics to treat the many devastating diseases caused by GOF amyloidoses. Finally, modulating protein misfolding by targeting the proteostasis network, such as activating HSF1, and shifting misfolded proteins towards their native state holds promising therapeutic potential. Although drug discovery applications in the field of proteostasis are still emerging, our growing understanding of these targets may provide intriguing opportunities to tackle a large number of diseases caused by protein misfolding.

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