Chemical Chaperones—A New Concept in Drug Research

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Low-molecular-weight compounds are able to stabilize the conformation of proteins that are defective in patients of inherited diseases. Unspecifically acting chemical chaperones, including osmolytes, can increase the fraction of the correctly folded variant protein encoded by the mutated gene. More recently, the concept of specifically acting chemical chaperones has been applied to two sphingolipid storage diseases, Fabry's disease[1] (Figure 1) and Gaucher's disease[2] (Figure 2). Studies in cultured cells and, in the case of Fabry's disease also in the animal model, revealed that administration of inhibitors led to a significant increase in the activity of the variant enzymes and to a substantial improvement of therapeutic parameters.

Molecular Chaperones

The function of proteins depends on their correct three-dimensional structure. The loss of their native conformation by denaturation leads to functional deficiency. Whereas many proteins can autonomously fold into their native structure, that is, in the absence of other cellular components,[3] the folding of other proteins requires the assistance of additional factors, like protein-disulfide-isomerases, peptidyl-prolyl-*cis*–*trans*-isomerases, and molecular chaperones.[4–6] A molecular chaperone is a protein that transiently associates with folding intermediates, prevents aggregation of intermediately exposed hydrophobic protein surfaces, and facilitates the adoption of the native structure. Many of these molecular chaperones act rather unspecifically and promote the correct folding, assembly, and targeting of a whole series of unrelated proteins. Recent work has revealed that the isomerization of secondary amide bonds might also contribute to chaperone function.[7]

Of particular importance for the folding of glycoproteins are lectin chaperones in the endoplasmic reticulum (ER). They ensure that only correctly folded proteins are transported along the exocytic pathway to their destination, for example, the Golgi apparatus, the cell surface, the extracellular space, or the lysosomal compartment.[8] This quality control system involves the membrane-bound chaperone calnexin and the soluble chaperone calreticulin. Both proteins bind to α-glycosidically linked terminal glucose residues on monoglucosylated *N*-glycans on glycoproteins and, together with a protein-disulfide-isomerase, promote correct folding.[9] Hydrolytic cleavage of the glucose residue from the glycan chain by glucosidase II terminates the association of glycoprotein substrate and the lectin chaperone. If the glycoprotein has not adopted its correct structure, a glucose residue is re-added to the *N*-glycan by a glucosyltransferase that acts as a folding sensor. This process of de-glucosidation and re-glucosidation is repeated until the native conformation of the glycoprotein is acquired. Persistently misfolded proteins are retranslocated into the cytosol, where they are ubiquitinylated and degraded by the proteasome.[10]

In inherited diseases such as cystic fibrosis, mutations in the gene of a protein can lead to folding-defective variants that do not successfully pass the quality control system of the ER, so that only an insufficient fraction of the variant gene product reaches its destination.

Chemical Chaperones

A series of low-molecular-weight compounds like dimethylsulfoxide, methyl-β-cyclodextrin, and the cellular osmolytes glycerol and trimethylamine-*N*-oxide, as well as some ions, can compensate for defects in protein folding and stabilize proteins in their native conformation.[11–13] For these substances, the term 'chemical chaperone' was coined.[14] For example, calcium ions promote the correct folding of LDL-receptor-related protein (LRP; LDL = low-density lipoprotein).[15] Substances like glycine betain, choline, proline, or trehalose are able to restore the activity of the mutated protein chaperone DnaK in *Escherichia coli*.[16]

The majority of these chemical chaperones requires relatively high (millimolar) concentrations to be efficient. Like many molecular chaperones, they show limited specificity and facilitate the folding of a range of different proteins. Among the possible mechanisms discussed for their effects are the stabilization of misfolded proteins, the prevention of aggregation, and the activation of alternative chaperone systems.[17]

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Specific Chemical Chaperones

In addition to the rather unspecifically acting chemical substances described above, other low-molecular-weight compounds were found to *specifically* stabilize certain proteins by binding to their native conformation. For example, 6-aminohexanoic acid serves as a specific chemical chaperone for apolipoprotein A by occupying lysine binding sites on the protein.[18] Hapten ligands like *p*-nitrophenylphosphocholine stabilize the native conformation of immunoglobulins.[19] Methotrexate, an inhibitor of dihydrofolate reductase, binds to the three-dimensional structure of this enzyme and stabilizes its conformation.[20] Similarly, opioid derivates stabilize the delta opioid receptor in the ER.[21] Although these compounds exert their effect by a mechanism different from the rather unspecifically acting chemical chaperones, in both cases the fraction of correctly folded protein is increased.

Evidence is now accumulating that chemical chaperones hold the promise to correct a range of inherited human diseases that involve protein folding deficiencies. This will be described in more detail for specifically acting chaperones applied to two lysosomal storage disorders, Fabry's and Gaucher's diseases.

Fabry's Disease

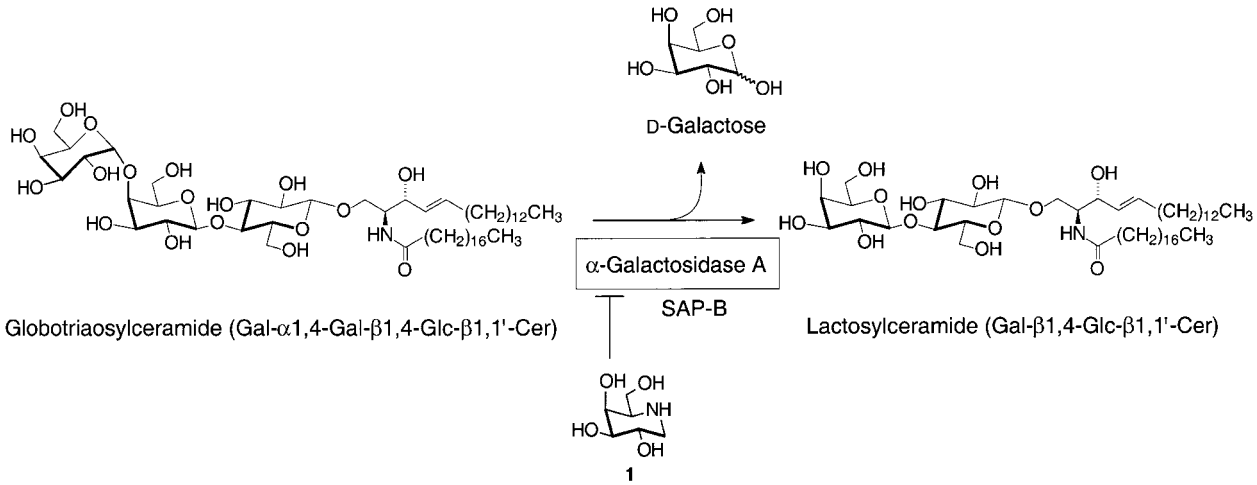
The first patients of Fabry's disease were described in 1898 by two dermatologists independently, the Briton Anderson and the German Fabry. Fabry's disease is a rare, X-chromosomal-linked metabolic disorder, which results from a defect of a lysosomal glycosidase, α-galactosidase A. This deficiency leads to the accumulation of enzyme substrates with terminally α-glycosidically bound galactose residues. In particular, the deposition of globotriaosylceramide (Scheme 1) within vascular epithelial cells accounts for the symptoms of the disease. The enzyme is a homodimer of 50 kDa subunits; its complementary DNA (cDNA) and gene have been cloned and numerous mutations (>160) have been characterized.[22]

Fabry's disease is characterized by lipid depositions within the skin leading to painful lesions and pain in the extremities. Through secondary effects like myocardial infarction, stroke, or renal failure, the average life expectancy of the patients is reduced to about 42 years. A variant form of the disease, which shows a milder progression and a primary impairment of the heart muscle, has been attributed to enhanced residual activity of the defective enzyme of more than 5% of normal values. In patients with normal progression of the disease, the residual enzyme activity is often barely detectable. As for most lysosomal storage disorders, the activity of the defective enzyme is not completely lost, but reduced to small but finite values. According to the so called threshold theory,[23] which has been confirmed for the course of several of these diseases, the severity and progression of the disease correlate with the residual degrading capacity in the lysosomes of the patients' cells.[24] The application of chemical chaperones has now allowed the residual activity of mutant α-galactosidase A to be increased to a significant extent.

D-Galactose as a Specific Chemical Chaperone

Various mutations in the gene of α-galactosidase A lead to variant forms of the protein that are functionally active but unstable and that normally do not pass the quality control system in the ER. Experiments with COS-1 cells and lymphoblasts expressing α-galactosidase A with such mutations show a significant increase in enzyme activity when the cells are cultured in the presence of D-galactose.[25, 26] Upon binding of D-galactose, the conformation of the mutant enzyme is stabilized. While the variant enzyme is especially labile in conditions of neutral pH, it remains stable over a longer period of time after reaching the acidic surrounding within the lysosomal compartment.

This observation prompted a clinical trial with a male patient of 55 years of age who suffered from the cardiac variant of Fabry's disease and showed the α-galactosidase A mutation



Scheme 1. *Enzymatic reaction catalyzed by α-galactosidase A, which is deficient in patients with Fabry's disease. In the presence of the sphingolipid-activator protein SAP-B (saposin-B), the enzyme cleaves glycolipids like globotriaosylceramide. 1-Deoxygalactonojirimycin (**1**) is a competitive inhibitor of the enzyme but also acts as a chemical chaperone in the ER.*

G328R. An infusion of D-galactose (1 g kg^{-1} body weight) for four hours per day over three days increased the residual activity of the enzyme, for example, in the lymphocytes of the patient from 7 to 10% of normal values. This modest increase in enzyme activity was sufficient to significantly ameliorate the patient's condition. After two years of treatment with three daily infusions, the conditions of the patient were improved to such an extent that the heart transplantation initially required was not necessary any more.[27]

1-Deoxygalactonojirimycin

Since D-galactose binds only weakly to α-galactosidase A and, in addition, is metabolically unstable, experiments with 1-deoxygalactonojirimycin **1** (Scheme 1), a potent competitive inhibitor of α-galactosidase A, were performed.[1] In protonated form, nojirimycin and its derivatives like **1** mimic the charge distribution of the transition state of a glycosidase reaction.[28] Upon binding of the competitive inhibitor, the conformation of mutant α-galactosidase A was expected to be stabilized. Indeed, it was found that the addition of **1** at a concentration of 20 μM to the culture medium of lymphoblasts expressing various defective forms of α-galactosidase A (R301Q, Q279E) enhanced the enzyme activity about 8-fold, up to 45% of normal values. The elevation of enzyme activity persisted five days after removal of the inhibitor. Similar results were obtained in COS cells and in fibroblasts of transgenic mice overexpressing the α-galactosidase A mutation R301Q. The oral administration of **1** to these mice (30 mg kg^{-1} body weight) also led to a significant increase in enzyme activity in some organs, for example, 18-fold in the heart muscle. Data concerning the life span of the treated animals were not included in the publication; however, it was reported that daily doses of 3 mg of **1** per kg of body weight were well tolerated by the animals over a period of 140 days. In a further study, a series of imino sugar derivatives were evaluated for their ability to enhance α-galactosidase A activity in lymphoblasts expressing mutant enzyme forms.[29] The stabilizing effect of these compounds was found to correlate with their respective inhibitory potential. The finding that the enzyme activity was elevated in the presence of the inhibitor was only observed at low inhibitor concentrations of up to 20 μM; higher doses reduced the enzyme activity.

Inhibitors as Stabilizers

It has been demonstrated previously that binding of an analogous gluco-configurated nojirimycin derivative to a glucosidase is strongly pH dependent, with an 80-fold higher affinity at pH 6.5 than at pH 4.5.[30] This might explain the two opposing effects of nojirimycin compounds as both inhibitors and stabilizers of glycosidases: In the neutral pH conditions of the ER, the inhibitors bind strongly to the enzyme. This stabilizes the native conformation and allows even mutant, less stable variants of a protein to pass the quality control system of the ER and reach the correct destination in the cell. In the case of α-galactosidase A, the protein is transported to the lysosome. In this acidic environment, the inhibitor is released and the protein remains stable. Only at higher inhibitor concentrations would the onset of enzyme inhibition be observed. Thus, in the case of mutant enzymes that retain catalytic activity but are predisposed to misfolding and premature degradation, enzyme inhibitors can stabilize the native protein conformation. This might enable correct transport and maturation of the protein, without necessarily being accompanied by reduction of enzyme activity. In contrast, enzyme activity of the variant protein might be enhanced, as in the case of α-galactosidase A.[1]

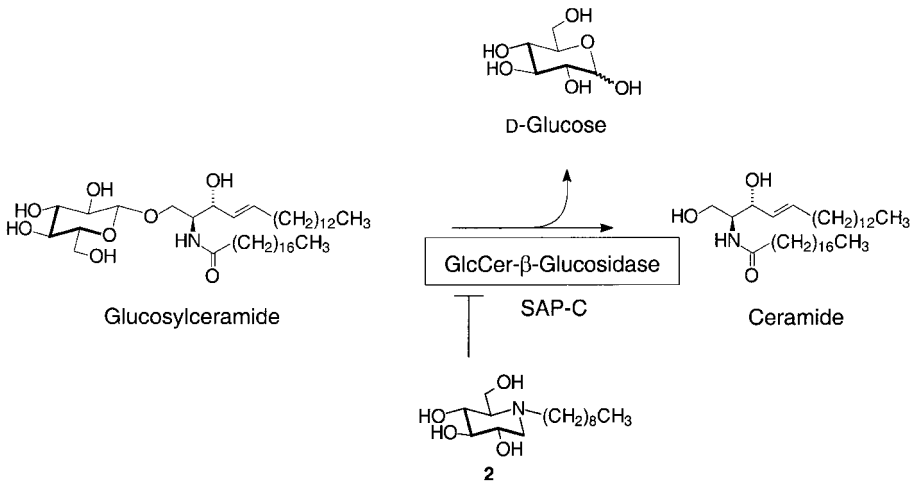
Gaucher's Disease

Gaucher's disease, initially described in 1882, is the most prevalent of the lysosomal storage disorders.[31] The deficiency of the enzyme glucosylceramide-β-glucosidase leads to the accumulation of its nondegradable substrate glucosylceramide. In the most common form of the disease, type I, the reticuloendothelial system of the patients is predominantly affected. The morphology of macrophages, which have especially large amounts of glucosylceramide to degrade, is characteristically changed due to the lipid storage. The occurrence of these so-called Gaucher cells in the liver, lymph nodes, and spleen causes an enlargement of these organs, and infiltration of the bone marrow results in painful skeletal lesions and in a reduction of hematopoietic cells. In the less frequent forms of the disease, types II and III, degeneration of the central nervous system is also observed. Whereas Gaucher's disease is rare in relation to the entire population with an incidence of 1 in 40 000 – 100 000 births, it has a significantly higher frequency among the Ashkenazi Jewish population with an incidence of 1 in 800. With the exception of Fabry's disease,[32] type I Gaucher's disease is currently the only inherited sphingolipid storage disorder for which a causal treatment is possible.[33] In both cases, the defective enzyme of the patients is replaced by intravenous infusions of the recombinant protein. This therapy normalizes the blood parameters and reverses the hepatosplenomegaly. However, this treatment shows the typical shortcomings of enzyme replacement therapies: The enzyme has to be administered by an implanted catheter or through weekly infusions lasting for several hours. Since the exogenously supplied protein cannot pass the blood – brain barrier, it is not effective for the treatment of neurological damage. Furthermore, the yearly costs of this therapy amount to $ 100 000 – 750 000 per patient.

It has recently been reported that the addition of *N*-(*n*-nonyl)deoxynojirimycin (**2**, Scheme 2) to cultured cells stabilizes glucosylceramide-β-glucosidase, the enzyme that is deficient in Gaucher's disease patients. Activity of the variant enzyme is enhanced to such an extent that a significant improvement of therapeutic values could be achieved.[2] This represents an additional example for the concept of specifically acting chemical chaperones, which now promises to broaden the therapeutic spectrum for inherited diseases.

Chemical Chaperones and Gaucher's Disease

As hypothesized for several other inherited disorders, many gene mutations underlying Gaucher's disease apparently result



Scheme 2. *Enzymatic reaction of β-glucosylceramide-β-glucosidase, which is deficient in patients with Gaucher's disease. In the presence of the sphingolipid-activator protein SAP-C, the enzyme cleaves glucose from glucosylceramide in the lysosome.* N-(n-*Nonyl)deoxynojirimycin (**2**) acts as chemical chaperone in the ER.*

in enzyme variants that remain functionally active but are prone to misfolding and instability and presumably do not pass the quality control system in the ER. It was assumed that the most common mutation causing Gaucher's disease (N370S) falls into this category. A series of potential chemical chaperones has been evaluated for their effect on cell lines that are homozygous for this mutation and show only drastically reduced glucosylceramide-β-glucosidase activity. Among the heterocycles tested were several deoxynojirimycin analogues, which had already proven their applicability as chemical chaperones in the study on Fabry's disease. This work revealed that the addition of *N*-(*n*-nonyl)deoxynojirimycin (**2**) at subinhibitory concentrations of 10 μM increases the activity of mutant glucosylceramide-β-glucosidase by up to 2-fold. This increase persisted for six days after withdrawal of the drug. In analogy to the effect of **1** on the variant α-galactosidase A underlying Fabry's disease, the authors propose that **2** stabilizes the correct folding of defective glucosylceramide-β-glucosidase and thereby ensures its proper transport to the lysosome. Clinical data suggest that the concomitant increase in enzyme activity in patients' cells is sufficient to accomplish therapeutic effects and to treat the metabolic disorder.

Iminosugars and Alternative Therapeutic Approaches

Current therapeutic strategies for lysosomal storage diseases aim to restore the defective degrading capacity in the lysosome through organ transplantation, gene therapy, stem cells, or enzyme replacement therapy.[34] A further approach is to reduce the biosynthesis of the accumulating nondegradable substrate with low-molecular-weight inhibitors like *N*-butyldeoxynojirimycin in the so-called 'substrate deprivation' strategy.[35] The studies summarized in this Minireview underline the importance of iminosugar derivatives, which so far are employed as inhibitors of glycosidases[36] and of ceramide-β-glucosyltransferase,[35] as potential specifically acting chemical chaperones for the treatment of these and similar metabolic disorders. As a further example, the use of deoxygalactonojirimycin derivatives for the possible treatment of GM1-gangliosidosis, a β-galactosidase deficiency, has to be mentioned.[37]

Future Perspectives

Numerous inherited diseases are caused by genetic defects in molecular chaperones,[38–39] for which partial functional compensation is conceivable through the development of low-molecular-weight compounds that can act as chemical chaperones.[40]

Furthermore, as summarized here for two lysosomal storage disorders, many mutations underlying metabolic disorders lead to variant enzymes that remain functionally active, but are predisposed to misfolding or instability. Evidence is now accumulating that these mutations, which normally lead to premature protein degradation in the ER, are amenable to chemical chaperoning. This can either be achieved by specific ligands or inhibitors, as reported for Gaucher's and Fabry's diseases, or by nonspecifically acting chemical chaperones. For example, the deficiency in alpha-1-antitrypsin Z in transgenic animals expressing only a mutated, misfolded variant could be corrected by the administration of 4-phenylbutyric acid and other chemical chaperones.[41] Clinical studies suggest that the administration of 4-phenylbutyrate is also a viable therapeutic approach in the treatment of cystic fibrosis.[42] The natural osmolyte trimethylamine *N*-oxide was found to correct assembly defects of the mitochondrial branched-chain alpha-ketoacid-decarboxylase, which is deficient in maple syrup disease.[43]

Many of these substances acting as chemical chaperones can be orally administrated, they might cross the blood-brain barrier, and they are far less expensive to produce than recombinant enzymes. Potential specifically acting chemical chaperones are new targets for drug design and chemical synthesis. The reported studies demonstrate the potential of this concept for the treatment of genetic metabolic disorders.

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