Misfolded Proteins

International Edition: DOI: 10.1002/anie.201505147
German Edition: DOI: 10.1002/ange.201505147

# pH-Responsive Pharmacological Chaperones for Rescuing Mutant Glycosidases

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***Abstract:*** *A general approach is reported for the design of small-molecule competitive inhibitors of lysosomal glycosidases programmed to 1) promote correct folding of mutant enzymes at the endoplasmic reticulum, 2) facilitate trafficking, and 3) undergo dissociation and self-inactivation at the lysosome. The strategy is based on the incorporation of an orthoester segment into iminosugar conjugates to switch the nature of the aglycone moiety from hydrophobic to hydrophilic in the pH 7 to pH 5 window, which has a dramatic effect on the enzyme binding affinity. As a proof of concept, new highly pH-responsive glycomimetics targeting human glucocerebrosidase or α-galactosidase with strong potential as pharmacological chaperones for Gaucher or Fabry disease, respectively, were developed.*

Lysosomal storage disorders (LSDs) are a heterogeneous group of rare inherited metabolic disorders that most commonly arise from mutations in lysosomal glycosidases.[1] Under normal conditions, newly synthesized lysosomal proteins correctly fold in the endoplasmic reticulum (ER) and are then translocated to the lysosomes. Aberrant mutant enzymes that fail to fold correctly, however, are arrested in the ER and redirected to undergo ER-associated degradation. While enzyme replacement therapies exist for some LSDs, such as Gaucher disease, Pompe disease, and Fabry disease, and substrate reduction therapy is also available for Gaucher disease, the use of pharmacological chaperones (PCs) to rescue the endogenous mutant enzyme by stabilizing the folding conformation and restoring trafficking appears to be a promising and more general therapeutic option.[2] Moreover, PCs also have the potential to attenuate the unfolded protein response,[3] which leads to ER stress, contributes to the neuropathogenesis of LSDs,[4] and is suspected to be responsible for the observed link between mutations in lysosomal glycosidases, especially glucocerebrosidase (GCase, the defective enzyme in Gaucher disease) but also others such as α-galactosidase A (α-Galase, the defective enzyme in Fabry disease), and Parkinson disease.[5]

Most of the reported PCs are reversible inhibitors[6] or transient covalent inactivators[7] of the corresponding LSD-associated glycosidase. Generally, strong binding is achieved through the interplay of glycone and non-glycone type interactions involving the catalytic site and allosteric regions of the enzyme, thereby stabilizing the folded protein state in the ER.[8] Dissociation of the PC:glycosidase complex once in the lysosome is a prerequisite to allow substrate processing and turnover. The high initial substrate concentration in patient cells favors this exchange, but further equilibration can unveil the inhibitory character of the drug, cancelling the chaperone benefit and thwarting a medically relevant result, which is presumably the reason behind several frustrating clinical trial failures.[9] Herein, we report a new class of highly pH-responsive glycosidase ligands that have been tailored to undergo fast self-inactivation in the lysosome, thereby maximizing chaperone versus inhibitor behavior. We demonstrate the validity of such an approach for the design of pharmacological chaperones for GCase and α-Galase.

Previous studies have shown that $sp^2$-iminosugar representatives with a configurational pattern matching that of D-glucose behaved as strong competitive inhibitors of GCase and as active site-directed PCs of Gaucher disease associated mutants when bearing hydrophobic groups, but the inhibitory capacity is virtually abolished when such substituents were hydrophilic.[10,11] We envisioned that equipping the non-

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201505147.

glycone segment in this type of conjugates with an appropriate acid-sensitive functionality could be exploited to program a sharp switch in the hydrophobic–hydrophilic balance of the molecule on going from the neutral ER to the acidic lysosome, thereby leading to irreversible dissociation of the PC:enzyme complex after correct processing and trafficking of the mutant enzyme. Among the battery of functional groups reported in the literature to impart pH sensititvity,[12–14] the orthoester group was selected for our purpose since it represents one of the most acid-labile motifs, remaining stable under neutral conditions and undergoing fast cleavage in a physiologically useful pH window (Figure 1).[15,16]

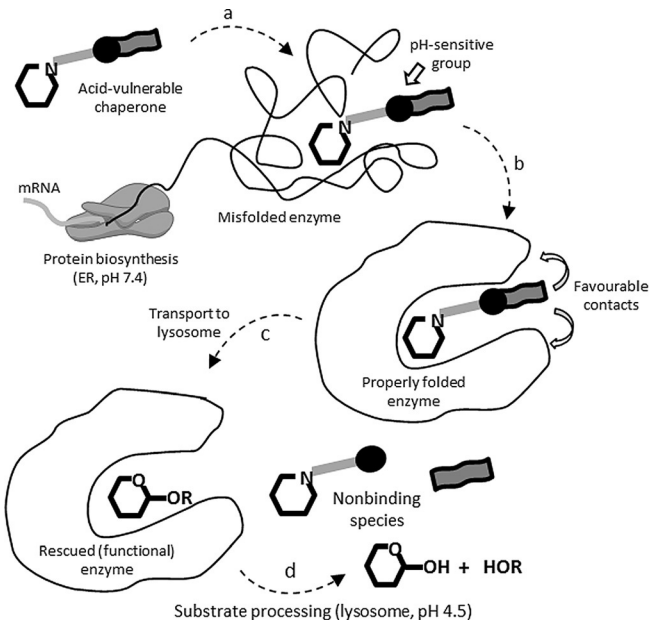
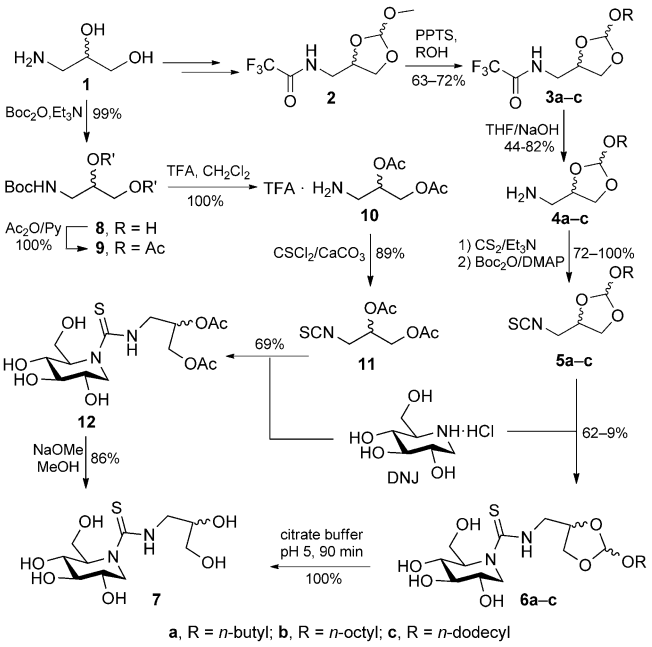



***Figure 1.*** Schematic representation of the proposed pH-responsive pharmacological chaperone prototype and the expected mutant enzyme enhancement mechanism: a) chaperone-assisted folding of the mutant enzyme, b) formation of an enzyme:chaperone complex stabilized by interactions involving the aglycone, c) restoration of trafficking to the lysosome, d) acid-promoted chaperone inactivation and substrate processing.

Coupling of isothiocyanates **5a–c** (Scheme 1)[17] with 1-deoxynojirimycin (DNJ), a classical iminosugar glucopyranose mimic,[18] afforded the corresponding $sp^2$-iminosugar adducts **6a–c** with total chemoselectivity and high yields. The incorporation of a thiourea linker in the conjugation step was an integral part of our approach. First, it favors hydrogen bonding over electrostatic interactions with amino acid residues at the catalytic site,[19] thus preventing enhanced enzyme binding in the lysosome as a consequence of protonation. Second, it has been shown to impart a high glycosidase discrimination capacity.[20] Accordingly, compounds **6a–c** selectively inhibited GCase among lysosomal glycosidases (α-glucosidase, β-galactosidase, α-galactosidase, β-hexosaminidase and α-galactosaminidase) at pH 7.0, with half maximal inhibitory concentration ($IC_{50}$) values in the μM to nM range (Figure 2).[21]




***Scheme 1.*** Synthesis of the orthoester-armed pH-sensitive pharmacological chaperones **6a–c** from DNJ and the corresponding product of acid hydrolysis (**7**). PPTS = pyridinium *p*-toluenesulfonate, THF = tetrahydrofuran, Boc = *tert*-butoxycarbonyl, DMAP = 4-dimethylaminopyridine, Py = pyridine, TFA = trifluoroacetic acid.

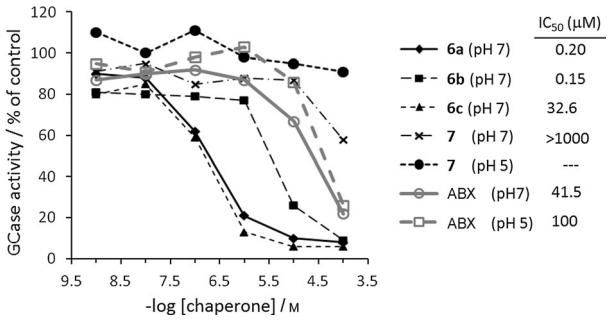



***Figure 2.*** GCase inhibition curves and $IC_{50}$ values for the orthoester-armed DNJ derivatives **6a–c** (pH 7) and the hydrolysis product **7** (pH 7 and pH 5). For comparative purposes, the inhibition curves (grey lines) and $IC_{50}$ values for ambroxol (ABX; pH 7 and 5) are also shown.

All three orthoester-armed DNJ–thiourea compounds (**6a–c**) were stable in neutral aqueous solutions at room temperature (less than 10% degradation after 3 d). However, they readily underwent hydrolysis at pH 5 to give **7** (the structure of which was confirmed by synthesis; Scheme 1), formic acid, and the corresponding *n*-butyl, *n*-octyl or *n*-dodecyl alcohols, with 50% and 100% of the starting material been consumed after 25 and 90 min, respectively (HPLC monitoring; Figure 3 and Figure S1 in the Supporting information). We were delighted to see that the dramatic shift in the hydrophobic–hydrophilic balance promoted by the increase in acidity virtually cancelled the ability of the compound to inhibit GCase ($IC_{50}$ value for the hydrolysis

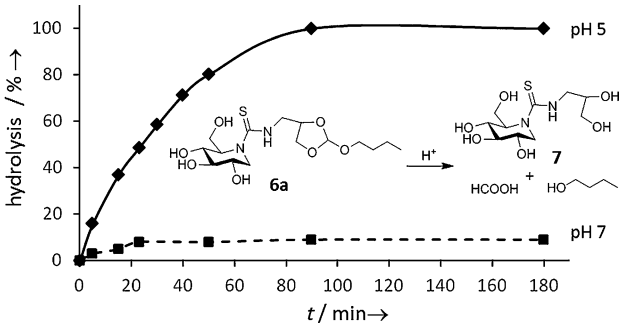



*Figure 3.* Plots of the rate of hydrolysis of orthoester **6a** at pH 5 and pH 7 as determined by HPLC. Similar plots were obtained for **6b** and **6c**.

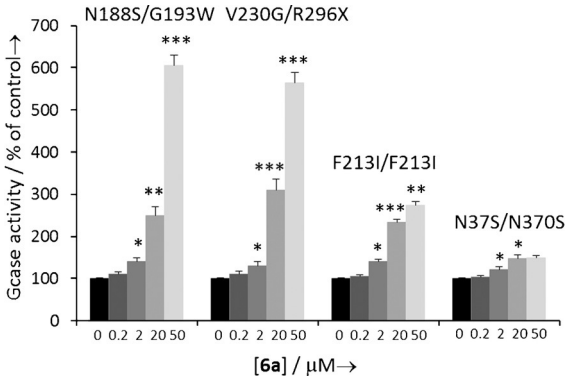



*Figure 4.* Chaperone activity of **6a** in cultured human fibroblasts of type 3 (N188S/G193W), type 2 (V230G/R296X and F213I/F213I), and type 1 (N370S/N370S) Gaucher patients (in situ cell enzyme assay). No significant chaperone activity was observed in fibroblasts harbouring the L444P/L444P GCase mutation. Each bar represents the mean ± standard error of the mean (SEM) of 3 determinations each done in triplicate. $*p<0.05$, $**p<0.01$, $***p<0.001$ (*t*-test).

product **7** is greater than 1 mM at pH 7 and essentially no inhibition was observed at pH 5; Figure 2). In comparison to ambroxol (ABX), a nonglycomimetic pharmacological chaperone in preclinical trials for the treatment of Gaucher disease,[22] the new orthoesters **6a–c** were found to be 1.3 to 200-fold better GCase ligands at the neutral pH of the ER, whereas at the acidic pH values of the lysosomes, the hydrolysis product **7** was at least a 3-fold weaker ligand than ABX (91 vs 26% GCase inhibition at 0.1 mM).

The capacity of the pH-responsive inhibitors **6a–c** to enhance the activity of GCase was next assessed in Gaucher patient fibroblasts with the N188S/G193W mutation, which is associated with the type 3 (neuropathic) phenotype of the disease. The butyloxy derivative **6a** ($IC_{50}$ 0.20 μM; $K_i$ 0.42 μM for GCase inhibition at pH 7) proved much more efficient than the octyloxy and dodecyloxy counterparts **6b** and **6c** in this assay, with remarkable 2.5- and 6-fold activity increases at 20 and 50 μM, respectively (to be compared with a maximum enhancement of 1.8 and 1.5-fold at 20 μM for **6b** and **6c**, respectively). Compound **6a** additionally proved efficient in enhancing GCase activity in V230G/R296X or F213I/F213I type 2 (acute neuropathic) Gaucher disease fibroblasts by 3-fold or 2-fold at 20 μM and 5.5-fold or 2.5-fold at 50 μM, respectively (Figure 4 A). Activity enhancement was more modest (1.5-fold) in N370S/N370S type 1 (non-neuropathic) Gaucher fibroblasts, whereas the L444P/L444P mutation (type 3), which is located at a non-catalytic domain of the enzyme, was refractory to treatment with **6a**. This trend is in agreement with that already encountered for other nojirimycin-derived $sp^2$-iminosugar chaperones.[11,23] In the same experimental set up, compound **7** was essentially inactive in wild-type or Gaucher fibroblasts with the above mutations, as either a chaperone or inhibitor, at concentrations above 1 mM (Figure S2 in the Supporting Information).

To further challenge our mechanistic hypothesis, N188S/G193W fibroblast cultures were incubated with 50 μM solutions of **6a**, **6b**, or **7** and then washed and lysed. The lysates were then screened by mass spectrometry. The presence of the glycomimetics could be readily assessed in the negative mode by their pseudomolecular peaks $[M-H]^-$ at *m/z* 375, 435, and 295, respectively. Control experiments confirmed the integrity of the compounds in the culture medium in the absence of the cells (Figure S5 in the Supporting Information). Lysates from fibroblasts treated with **6a** showed exclusively the presence of **7**, meaning that full hydrolysis occurred in the cellular environment. Fluorescent immunolabelling further evidenced the increase in the amount of GCase in the fibroblasts after treatment with **6a**, thus demonstrating that the PC:enzyme complex escaped the ER and reached the lysosomes (Figure 5 and Figures S3 and S4 in the Supporting Information). Since

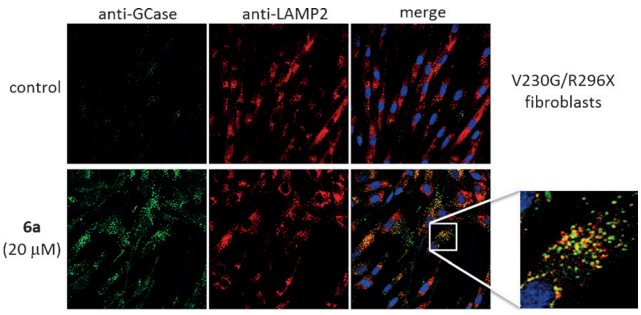



*Figure 5.* Confocal microscopy images showing the intracellular distribution of GCase (green) in the absence (control) or presence of the pharmacological chaperone **6a** (20 μM). Inmmunolabelling of the lysosome-associated membrane protein LAMP-2 (red) was used for co-localization experiments. Fluorescence intensity is normalized to the intensity of nuclear staining with 4',6-diamidino-2-phenylindol (DAPI; blue).

the hydrolysis product **7** is not a ligand of GCase, we can reasonably assume that the GCase activity measured in lysates indeed corresponds to active GCase that is available for substrate processing in the lysosome.[24]

In contrast to the behavior observed for **6a**, mass spectra of lysates from fibroblasts treated with **6b** under identical conditions indicated only partial hydrolysis (Figure S6 in the Supporting Information), which provides a rational for its much poorer chaperone behavior compared with **6a**. This result also indicates that the nature of the hydrophobic substituent may have a strong effect in the pH responsiveness

under physiological conditions, probably by altering the kinetics of the PC:enzyme dissociation process.

The above strategy was purposely conceived to allow modifications at both the glycone and non-glycone moieties of the glycomimetic conjugates in order to target lysosomal glycosidases other than GCase. As a practical demonstration, the concept has been further validated by preparing the 1-deoxygalactonojirimycin (DGJ) derivative **13**, the C4-epimer of **6a** (Figure 6A and the Supporting information). Compound **13** selectively inhibited lysosomal α-Galase at pH 7

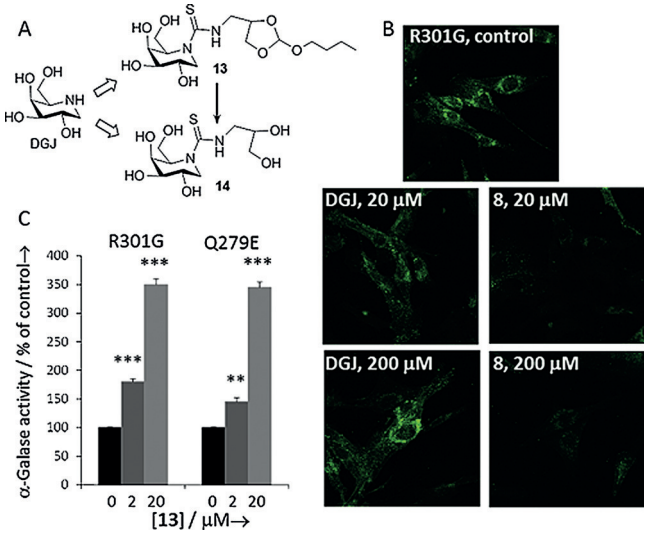



***Figure 6.*** A) Structures of 1-deoxygalactonojirimycin (DGJ), the orthoester conjugate **13**, and the hydrolysis product **14**. B) Chaperone activity of **13** in cultured human fibroblasts from Fabry patients with the R301G and Q279E α-Galase mutations (each bar represents the mean ± SEM. of 3 determinations each done in triplicate; $^{**}p < 0.01$, $^{***}p < 0.001$ (*t*-test). C) Confocal microscopy images showing the intracellular distribution of globotriaosylceramide (green) in the absence (control) or presence DGJ or **13** (20 and 200 μM).

($IC_{50}$ 0.9 μM) and was readily transformed into the inactive species **14** at pH 5. Moreover, **13** behaved as an efficient chaperone in fibroblasts from Fabry patients with the R301G or Q279E mutations, leading to activity increases up to 3.5-fold at 20 μM (Figure 6B). Immunolabelling of globotriaosylceramide, the main substrate accumulated in Fabry lysosomes, almost totally disappeared after treatment with chaperone **13** under these conditions (Figure 6C), thus indicating restoration of α-Galase activity. Interestingly, no reversion from chaperone to inhibitor behavior was observed, even at 200 μM, which is consistent with total inactivation of **13** in the lysosome. In a control experiment, the parent iminosugar DGJ, which behaves as a chaperone at 20 μM (although less efficiently than **13**), turned into a potent α-Galase inhibitor at 200 μM, resulting instead in substrate accumulation (Figure 6B, bottom). Implementing this strategy based on orthoester conjugation to access highly pH-responsive chaperones in a broader range of lysosomal storage disorders is currently in progress in our laboratories.

## Acknowledgements

The Spanish Ministerio de Economía y Competitividad (contract numbers SAF2013-44021-R and CTQ2010-15848), the Ministry of Education, Culture, Science, Sports and Technology of Japan (KAKENHI) and the Ministry of Health, Labour and Welfare of Japan (H17-Kokoro-019, H20-Kokoro-022) are acknowledged. Cofinancing from the European Regional Development Funds (FEDER and FSE), the Fundación Mehuer, and the Junta de Andalucía (contract number FQM-1467) is also acknowledged. K.H. was supported by Takeda Science Foundation. G. Tiscornia was supported by Genzyme Corporation, USA. T.M.-B. thanks the Junta de Andalucía for a postdoctoral fellowship. Technical assistance from the research support services of the University of Seville (CITIUS) is also gratefully acknowledged.

**Keywords:** chaperones · glycosidases · inhibitors · pH sensitivity · protein folding

**How to cite:** *Angew. Chem. Int. Ed.* **2015**, *54*, 11696–11700
*Angew. Chem.* **2015**, *127*, 11862–11866

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Received: June 5, 2015
Published online: August 7, 2015

  