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Tuning glycosidase inhibition through aglycone interactions: pharmacological chaperones for Fabry disease and GM_1 gangliosidosis†

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Competitive inhibitors of either α-galactosidase (α-Gal) or β-galactosidase (β-Gal) with high affinity and selectivity have been accessed by exploiting aglycone interactions with conformationally locked sp^2-iminosugars. Selected compounds were profiled as potent pharmacological chaperones for mutant lysosomal α- and β-Gal associated with Fabry disease and GM_1 gangliosidosis.

Inappropriate performance of any of the multiple hydrolytic enzymes involved in lysosomal catabolic pathways is at the origin of a range of pathologies known as lysosomal storage disorders (LSDs).[1] Many of the mutations associated with these diseases translate into enzymes that retain partial catalytic activity *in vitro* but exhibit impaired cellular trafficking as a consequence of aberrant folding. Among the therapeutic approaches under investigation are the parenteral infusion of exogenous enzymes (*enzyme replacement therapy*, ERT)[2] and the reduction in the influx of the accumulated substrate (*substrate reduction therapy*, SRT) by administration of an inhibitor of the substrate-producing enzyme.[3] Pharmacological chaperones (PCs) constitute a promising new treatment paradigm and several compounds are currently being evaluated for LSDs.[4] In this approach, a ligand of the target glycosidase promotes those conformational changes that are required for efficient folding, restoring trafficking.[5] Although somewhat counterintuitive, competitive inhibitors can increase steady-state lysosomal levels of active enzymes through this rescue mechanism. At the massive lysosomal substrate concentration, the inhibitor would be replaced from the active site of the enzyme and the metabolic activity recovered.

With few exceptions,[6] PCs under study mimic the glycone moiety of the putative substrate. This is the case of the iminosugars 1-deoxynojirimycin (**1**, DNJ) or 1-deoxygalactonojirimycin (**2**, DGJ).[7] The fact that the same glycone moiety is shared for enzymes acting on anomeric substrates frequently leads to an insufficient selectivity of these types of inhibitors for clinical application, however.[8] Incorporation of alkyl substituents into iminosugar frameworks has been shown to improve the affinity towards certain glycosidases and their drugability as PCs, but fails to provide a tool for switching between enzymes with reverse anomeric selectivity.[9] Recently, we demonstrated that replacing the sp^3 amine-type nitrogen typical of classical iminosugars by a pseudoamide-type (urea, thiourea, isourea, isothiourea) nitrogen atom (sp^2-iminosugars) provided excellent opportunities to shape the glycosidase inhibitory properties.[10] For instance, 5-*N*,6-*S*-[*N*′-(*n*-octyliminomethylidene)]-6-thionojirimycin (**3**, 6S-NOI-NJ) and the D-*galacto* configured analogue 6S-NOI-GNJ (**4**) behaved as potent, competitive and selective inhibitors of lysosomal β-glucosidase (β-glucocerebrosidase, GCase).[11] Both epimers **3** and **4** were able to increase the GCase activity in GCase-deficient Gaucher disease (GD, the LSD with the highest prevalence) fibroblasts, being particularly efficient in the case of neuronopathic-associated mutations (Fig. 1).[11]

The outstanding selectivity of the sp^2-iminosugars **3** and **4** for β-glucosidase relies on a subtle configurational–conformational switch mechanism controlled by aglycone interactions. In the unbound state, the anomeric hydroxyl adopts the axial orientation (α-configuration) dictated by the generalized anomeric effect, with the piperidine ring in the $^4C_1$ chair conformation and the imine group in the *E*-configuration. Crystallographic evidence indicated that **3** and **4** are accommodated in the active

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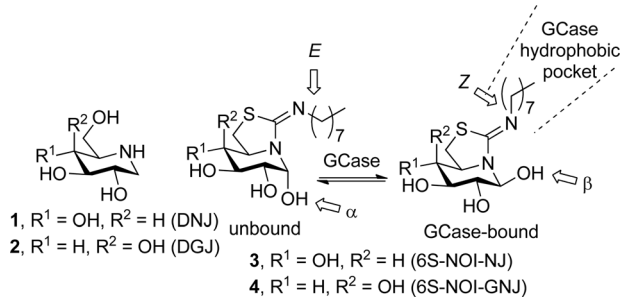
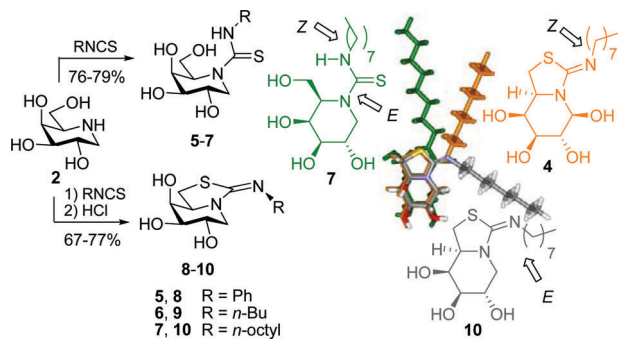


Fig. 1 Structures of the iminosugars DNJ and DGJ and of the sp^2-iminosugars 6S-NOI-NJ and 6S-NOI-GNJ. The configurational–conformational switch process experienced by the latter upon binding to β-glucocerebrosidase (GCase) is schematically depicted.

site of the enzyme in the β-configuration, however, with the *N*′-alkyl chain in the *Z*-orientation (Fig. 1).[12] Actually, GCase possesses a hydrophobic pocket at the entrance of the active site that is perfectly matched by the *N*′-substituent in this particular conformation. In contrast, overlapping of the active sites of α-glycosidase or α- and β-galactosidase revealed steric clashes that hampered binding.

The above results strongly suggest that fine tuning of aglycone interactions has the potential to become a powerful strategy for elaborating selective active-site directed PCs for LSDs. To test this hypothesis, we have focused on the lysosomal α- and β-galactosidases (α-Gal and β-Gal), whose deficiency results in the LSDs known as Fabry disease (FD) and GM_1 gangliosidosis (GM1), respectively. Two series of conformationally locked sp^2-iminosugar galactomimetic probes, namely the monocyclic and bicyclic DGJ derivatives **5–7** and **8–10** (Scheme 1, left), were synthesized and shown to have remarkable glycosidase selectivities, which translated into potent pharmacological chaperoning activities in mutant proteins associated with FD and GM1, respectively.

The above-mentioned X-ray and docking data revealed that the anomeric hydroxyl in **4** was not directly involved in strong contacts with amino acid residues at the active site of glycosidases. Its removal should broaden the available conformational space for *N*-substituents. On the other hand, the dissymmetry in the substitution pattern at C-1 and C-5 was expected to promote preferred *E*,*Z*- and *E*-configurations at the thiourea segment and the cyclic isothiourea group, in **5–7** and **8–10**, respectively.



Scheme 1 Synthesis of the conformationally locked mono (**5–7**) and bicyclic DGJ derivatives (**8–10**). The 3D molecular models of the new octyl thiourea (**7**, in green) and isothiourea (**10**, in grey) derivatives, overlapped with that of the GCase-bound reducing derivative 6S-NOI-DGJ (**4**, in orange), illustrate the sharp differences in the relative orientations of the *N*′-substituent in their ground state conformations.

This situation will force distinct orientations of the aglycone moiety that will be anticlock- and clockwise displaced with respect to the scenario found for GCase-bound **4**, thus enabling galactosidase binding (Scheme 1, right).

DGJ thioureas **5–7** were accessed in one step, with total chemoselectivity and good yield, by direct coupling of the parent iminosugar **2** with isothiocyanates. Attempts to promote further cyclization by treatment with triflic anhydride or mesyl chloride in dry *N*,*N*′-dimethylformamide[13] led to extensive hydrolysis of the sulfonylating reagents. Only traces of the target isothioureas **8–10** were detected. To our delight, the transformation proceeded smoothly in methanol under acid catalysis without the need for any additional additive. The reaction can be effected in one pot from iminosugar **2** by sequential isothiocyanate coupling–intramolecular acid-promoted cyclisation, which makes this general strategy very well-suited for library generation and optimization schemes. Dynamic NMR experiments and NOE data fully supported the predicted conformational assignment indicated in Scheme 1.

Compounds **5–10** were first evaluated for their inhibitory properties against a panel of commercial glycosidases including α-Gal from coffee, belonging to the same CAZy family (GH27) as that of human α-Gal A, and β-Gal from *Escherichia coli* (*E. c.*) and bovine liver, both classified in the same clan GH A as human lysosomal β-Gal (Table 1).[14] Thioureas **5** and **7** and isothioureas **9** and **10**, showing the strongest inhibition potency for α-Gal and β-Gal, respectively, were selected for further evaluation against human lysosomal glycosidases. They exhibited high affinity and total specificity for human α-Gal and β-Gal, respectively, among several lysosomal enzymes. Inhibition data are collected in Table 1 in comparison with data for DGJ (**2**) and *N*-octyl-4-*epi*-β-valienamine (NOEV), the best performing PCs for FD and GM1 gangliosidosis reported to date.[15] Most interestingly, the inhibition potency was about ten-fold higher at neutral than at acidic pH, a favourable feature considering that mutant enzyme rescue by pharmacological chaperones requires strong binding at the ER but dissociation of the complex once in the lysosome.

Table 1 Inhibitory activity of compounds **5–10** against commercial (K_i, μM) and human (*H. s.*, lysosomal) glycosidases (IC_{50}, μM).[a] For comparative purposes, values of the reference compounds DGJ (**2**) and NOEV are also included

	Commercial enzymes (K_i, μM)			Human enzymes (pH 5.0/7.0; IC_{50}, μM)	
	α-Gal (coffee)	β-Gal (*E. c.*)	β-Gal (bov.)	α-Gal *H. s.*	β-Gal *H. s.*
2[7]	0.016	12.5	616	0.06/0.01	25/n.d.[b]
NOEV[15]	n.d.	n.d.	0.87	n.i.	0.13/0.02
5	0.0019	5.2	526	4.5/0.2	n.i.
6	0.044	189	98	n.d.	n.d.
7	0.029	18	25	37/5.0	n.i.
8	18	13	6.9	n.d.	n.d.
9	134	7.3	2.9	n.i.	32/3.1
10	81	0.65	0.2	n.i.	6.0/0.5

[a] No inhibition (n.i.) was observed for **5–10** on α-glucosidase (baker yeast), amyloglucosidase (*Aspergillus niger*), isomaltase (yeast), α-mannosidase (Jack bean) and β-mannosidase (*Helix pomatia*) at concentrations up to 2 mM, or on human lysosomal α-Glc, β-Glc (GCase) or *N*-acetyl-β-D-glucosaminidase at concentrations up to 0.1 mM. [b] Not determined.

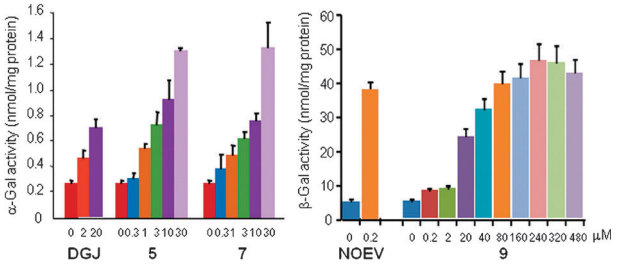


Fig. 2 Effect of thioureas **5** and **7** on α-Gal activity in mutant R301G FD fibroblasts (left) and of isothiourea **9** on β-Gal activity in mutant R201C GM1 fibroblasts (right). Each bar represents the mean ± SEM of three determinations each done in triplicate.

The effects of compounds **5** and **7** on α-Gal were further evaluated in human skin fibroblasts derived from Fabry patients homozygous for the R301G mutation, known to lead to folding defects in the enzyme but not compromising the catalytic site. The parent iminosugar **2** (DGJ) was used as a control. About 3-fold increases in enzyme activity were reached after 4 days of incubation with thioureas **5** and **7** at 3 μM, equalling the results obtained with DGJ at its optimal concentration of 20 μM. By increasing the concentrations of the PCs to 30 μM, a maximal enhancement of 5-fold was achieved (Fig. 2). Neither **5** nor **7** exhibited cell toxicity at concentrations up to 500 μM.

The *N*′-octylisothiourea **10** turned to be toxic to human fibroblasts at concentrations over 100 μM and was discarded at this stage. The *N*′-butyl analogue **9** was nontoxic at concentrations up to 640 μM. When used at concentrations higher than 100 μM, it led to a 6-fold β-Gal activity enhancement in fibroblasts from patients homozygous for the juvenile GM1 mutation R201C, comparable to that achieved with the reference compound NOEV.[15] Although a biologically useful chaperoning activity was only observed from 20 μM concentration, the chaperon:inhibitor balance was very favourable, since the mutant enzyme activity continued to increase with the concentration of **9** up to 240 μM (Fig. 2).

In summary, we have succeeded in the design and synthesis of sp^2-iminosugar ligands that can discriminate between lysosomal α- and β-galactosidases from which pharmacological chaperones active against trafficking-incompetent FD and GM1 mutants have been identified. Since the general approach allows modifications of the hydroxylation profile and the nature of the substituents, this strategy may facilitate the discovery of PCs for other LSDs through targeting the corresponding glycosidases.

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