A Systematic Investigation of Iminosugar Click Clusters as Pharmacological Chaperones for the Treatment of Gaucher Disease

Antoine Joosten, Camille Decroocq, Julien de Sousa, Jérémie P. Schneider, Emile Etamé, Anne Bodlenner, Terry D. Butters, and Philippe Compain

A series of 18 mono- to 14-valent iminosugars with different ligands, scaffolds, and alkyl spacer lengths have been synthesized and evaluated as inhibitors and pharmacological chaperones of β-glucocerebrosidase (GCase). Small but significant multivalent effects in GCase inhibition have been observed for two iminosugar clusters. Our study provides strong confirmation that compounds that display the best affinity for GCase are not necessarily the best chaperones. The best chaperoning effect observed for a deprotected iminosugar cluster has been obtained with a tetravalent 1-deoxynojirimycin (DNJ) analogue (3.3-fold increase at 10 μM). In addition, our study provides the first evidence of the high potential of prodrugs for the development of potent pharmacological chaperones. Acetylation of a trivalent DNJ derivative, to give the corresponding acetate prodrug, leads to a pharmacological chaperone that produces higher enzyme activity increases (3.0-fold instead of 2.4-fold) at a cellular concentration (1 μM) reduced by one order of magnitude.

Introduction

Since its recent discovery, the pharmacological chaperone concept has experienced spectacular growth in the field of inherited diseases caused by improperly folded proteins.[1] In particular, promising success has recently been achieved in the treatment of glycosphingolipid lysosomal storage disorders (GLSDs)[2, 3] with iminosugar-based chaperones reaching phase II and phase III clinical trials. GLSDs are a small group of rare diseases characterized by deficiency of glycosidases involved in the catabolism of glycosphingolipids in the lysosome.[4] These inherited enzyme deficiencies result in pathological lysosomal accumulation of glycolipids, with widespread clinical consequences.

Pharmacological chaperone therapy (PCT) is based on the use of reversible inhibitors of these deficient enzymes capable of enhancing their residual hydrolytic activity at subinhibitory concentrations.[2, 3] Since the seminal work of Asano and Fan in 1999, which showed that the residual α-galactosidase A activity in lymphoblasts of patients with Fabry disease could be significantly enhanced by treatment with 1-deoxygalactonojirimycin (1a, Amigal, Scheme 1),[5] hundreds of small molecules, mainly iminosugars, have been evaluated as pharmacological chaperones (PCs) for the treatment of GLSDs.[5] The scientific interest in pharmacological chaperone therapy is also highlighted by the exponential growth in citations of articles dealing with this concept (≈ 200 citations in 2004, more than 2000 in 2012). Indeed, use of PCT for the treatment of GLSDs offers many advantages over the other established therapies,[6] based either on substitutive enzymotherapy (enzyme replacement therapy, ERT)[7] or on inhibitors of glycolipid substrate biosynthesis (substrate reduction therapy, SRT).[8, 9] PCT targets the cause of GLSDs by restoring the hydrolytic activity of the mutated enzymes, whereas the other two therapies reduce the accumulation of un-degraded glycosphingolipids either by the administration of a recombinant protein as an exogenous substrate (ERT) or by the dysregulation of the glycosphingolipid metabolic pathway (SRT).[10] PCT combines the benefits of

Scheme 1. Iminosugar-based pharmacological chaperones.

[a] Dr. A. Joosten, Dr. C. Decroocq, J. de Sousa, J. P. Schneider, E. Etamé, Dr. A. Bodlenner, Prof. P. Compain
Laboratoire de Synthèse Organique et Molécules Bioactives (SYBIO)
Université de Strasbourg/CNRS (UMR 7509)
Ecole Européenne de Chimie, Polymères et Matériaux
25 rue Becquerel, 67087 Strasbourg (France)
E-mail: philippe.compain@unistra.fr

[b] J. de Sousa, Dr. T. D. Butters
Glycobiology Institute, Oxford University
South Parks Road, Oxford OX1 3QU (UK)

[c] Prof. P. Compain
Institut Universitaire de France
103 Bd Saint-Michel, 75005 Paris (France)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201300442.
a small-molecule approach, including oral bioavailability and the potential to cross the blood–brain barrier, with the specificity of an enzyme-directed approach.

Despite all this promise, however, a number of scientific challenges might impede the development of drugs based on PCT. The first relates to the mechanism by which pharmacological chaperones increase the residual hydrolytic activity of the mutant enzymes involved in GLSDs. There are currently two opposite views concerning the molecular basis of pharmacological chaperoning activity. According to the more accepted theory, the pharmacological chaperone acts at neutral pH in the endoplasmic reticulum (ER) by inducing or stabilizing the correct conformation of the misfolded but catalytically active enzyme, preventing its degradation by a “quality-control” mechanism (endoplasmic-reticulum-associated degradation, ERAD). In sharp contrast, Wei et al. have very recently proposed that the main role of pharmacological chaperones might be to increase the resistance of a correctly folded but catalytically deficient enzyme to proteases in the acidic lysosomes. Since the report of this theory, which is based in part on crystallographic evidence, few studies have been performed to resolve these conflicting views. However, the debate is still open, and more research is needed. Beyond its fundamental interest, a clear understanding of the chaperoning effect would constitute a decisive leap forward towards the rational drug design of potent pharmacological chaperones.

A second obstacle to therapeutic application of pharmacological chaperones might be achievement of insufficient levels of residual cellular activity enhancement. For example, almost all the PCs reported to date for type I Gaucher disease, the most prevalent GLSD, double to triple the residual cellular activity of N3T7S β-glucocerebrosidase (GCase) whatever their affinity for the mutant enzyme. These enhancement values are comparable to that obtained for N-nonyl-1-deoxyxojirimycin (NN-DNJ, Scheme 1), the first example of a PC for Gaucher disease, reported in the literature ten years ago. Treatment with isofagomine (2, Plicera), the leading clinical candidate for chaperone therapy for Gaucher disease, led to an increase in enzymatic activity for all patients enrolled in a phase II trial without serious side effects. However, Plicera might not move forward to phase III development because significant clinical improvements were observed for only one patient out of eighteen. This result could indicate that the doubling of the residual GCase activity reported in the case of Plicera is not sufficient for a therapeutic application.

In this context, one current question is how to get closer to a second generation of pharmacological chaperones that would display improved enhanced activity. Because only very few rational criteria for the design of such compounds are available, new approaches and concepts have to be explored.

Mechanism-based inactivators or iminosugars bearing carbonyl groups on their pseudo-anomeric carbon atoms, for example, have been found to be promising new class of PCs. In connection with our studies on multivalent effect in glycocidase inhibition, we have recently explored the potential of multivalency for the design of efficient PCs containing four DNJ–cyclodextrin conjugates. The heptavalent iminosugar 16 (Scheme 2) was found to be a nm inhibitor of recombinant GCase (ceredase), whereas the corresponding monovalent analogue displayed inhibition in the μM range. Compound 16 increased residual GCase activity in fibroblasts from Gaucher patients by 53% at 10 μM, a chaperoning effect comparable to that observed for the corresponding monovalent iminosugar (60% increase at 10 μM). The ability of 16 to gain access to the ER and/or the lysosomes was thus unambiguously demonstrated by the significant enhancement of GCase activity in Gaucher fibroblasts. However, in view of the high value of inhibition in vitro observed for 16, the cellular concentration of 10 μM required to achieve a chaperoning effect is relatively high. We hypothesized that this result could be explained by having a lower cellular permeability than the corresponding monovalent analogue.

On the basis of this proof-of-concept preliminary study, we have performed a systematic investigation of structure–activity relationships by synthesizing and evaluating a panel of tri- to 14-valent systems with different alky spacer lengths (C₆ or C₉). In particular, we have investigated the effects of size, valency, ligand topology, and scaffold structure on GCase binding affinity and chaperoning activity (Scheme 2). The two cyclodextrin-based 14-valent systems 19 and 21, for example, were synthesized to explore the impact of ligand spatial orientation. Smaller systems of lower valency, as well as acetylated analogues designed as potential prodrugs (10 and 17), were prepared with the aim of facilitating cellular uptake. Iminosugars 4–7 were also synthesized and evaluated as monovalent controls to assess any possible multivalent effect in GCase inhibition and/or chaperoning activity. Here we describe the full details of our study, from synthesis to cellular assays.

Results and Discussion

Synthesis of multivalent iminosugars

Our synthetic strategy was based on experience gained in our previous work on multivalent iminosugars and involved attachment of the iminosugar moieties onto the scaffolds by Cu-catalyzed azide–alkyne cycloaddition (CuAAC). N-Alkyl derivatives of DNJ, the first family of PCs disclosed for Gaucher disease, were chosen as the peripheral ligands. Multivalent systems containing analogues of α-1-C-alkyl-iminoxylitol (α-1-C-alkyl-DIXs, 3, Scheme 1), which are highly potent nanomolar inhibitors of human GCase, were also synthesized and evaluated.

We started with the multivalent iminosugars in the iminooxyitol series. The syntheses of tri- and tetravalent iminosugars 11 and 14 were performed in five and three steps, respectively, from pentaerythritol (22, Schemes 3 and 4). Trivalent iminooxyitol derivative 11 was synthesized by direct microwave Cu-catalyzed 1,3-cycloaddition between triproargyl ether 23, prepared in three step from 22, and the corresponding azido iminosugar precursor 24. Subsequent clean O-deacetylation of adduct 25 by use of Amberlite IRA 400 (OH-) anion-exchange resin afforded the expected trivalent iminosugar in a very good yield (80% for the two steps).
Compound 14, the tetravalent analogue of 11, was synthesized according to the same convergent strategy (Scheme 4). The tetrapropargyl ether 26, prepared in one step from pentaerythritol (22), reacted with the azide precursor 24. Subsequent deprotection provided the desired tetravalent C₆ iminosugar 14.

The same two key reactions—CuAAC reaction followed by O-deacetylation—were again applied for the synthesis of iminosugar 20, containing 14 DIX-based ligands (Scheme 5). The two cycloaddition partners were 29, the N-Boc-protected analogue of azido iminosugar 24, and per-(2,6-di-O-propargyl)-β-cyclodextrin (28), obtained in only one step from β-cyclodextrin (β-CD). The protection and deactivation of the endocyclic nitrogen in 29 was nevertheless found to reduce the yield of the CuAAC reaction and ultimately the efficiency of the whole process.

For the synthesis of 18, the heptavalent analogue of iminosugar 20, the alkyne substrate for the CuAAC was heptakis(2,3-di-O-methyl-6-O-propargyl)cyclomaltoheptaose (31), obtained in four steps from β-CD (Scheme 5). To complete the series, hepta- and 14-valent CD-based iminosugar clusters 15–17, 19, and 21 were efficiently synthesized through CuAAC reactions by our reported strategy, together with tri- and tetravalent DNJ derivatives 8–10 and 12–13, respectively.

Monovalent controls were also prepared to allow assessment of the multivalent effects. Iminosugars 4–6 were synthesized as described previously. Iminosugar 33 was treated with pent-1-yne in the presence of CuSO₄·5H₂O and sodium ascorbate to afford 25 (80%) and 11 (100%).
ascorbate to yield the corresponding fully deprotected adduct 7 after treatment with TFA (Scheme 6).

Scheme 4. Reagents and conditions: a) CuSO₄·5H₂O cat., sodium ascorbate, DMF/H₂O (4:1), MW, 80 °C, to afford 27 (83%); b) Amberlite IRA 400 (OH⁻), MeOH/H₂O (1:1), to afford 14 (100%).

Biological results

Having a library of tri- to 14-valent systems and the corresponding monovalent controls to hand, we first evaluated their inhibitory activities toward human placental GCase (Table 1). With the exceptions of 14-valent DNJ clusters 15, 19, and 20, which behaved as reversible mixed (competitive/non-competitive) inhibitors, and tetravalent DIX cluster 14, the complex inhibition mode of which could not be determined, all the other iminosugars were found to be competitive inhibitors of GCase. Less-potent inhibition was observed for multivalent systems with the shorter linker (C₆) in the DNJ series. Multivalent presentation of the DNJ motif in these compounds was either detrimental to or irrelevant for inhibition of GCase.

Replacement of the DNJ ligand by the much more potent DIX ligand[32] led to dramatic improvements in the affinities for GCase, as reflected in Kᵢ increases of three orders of magnitude. In this series (compounds 7, 11, 14, 18, and 20), a small but significant[33] multivalent effect was observed for the tetravalent DIX derivative 14, with inhibition potency increased approximately eightfold (≈2 on a molar basis) over the corresponding monovalent analogue 7.

The influence of linker length on GCase inhibition was evaluated by comparison of DNJ click clusters possessing two different alkyl spacer lengths. Extension of the spacer length by three carbons (from C₆ to C₉) in the DNJ series led to improvement in inhibition, with Kᵢ decreases of one to three orders of magnitude. Again, a small but significant multivalent effect was observed only for one compound: the heptavalent DNJ derivative 16, with inhibition potency increased approximately 14-fold (≈2 on a molar basis) over the corresponding monovalent analogue 5. It is noteworthy that the multivalent effect observed for 16 is one order of magnitude lower than that of 14.

Scheme 5. Reagents and conditions: a) CuSO₄·5H₂O cat., sodium ascorbate, DMF/H₂O (4:1), MW, 80 °C, to afford 30 (44%); b) TFA, CH₂Cl₂, then Amberlite IRA 400 (OH⁻), MeOH/H₂O (1:1), to afford 20 (66%); c) 24, CuSO₄·5H₂O cat., sodium ascorbate, DMF/H₂O (4:1), MW, 80 °C, to afford 32 (89%); d) Amberlite IRA 400 (OH⁻), MeOH/H₂O (1:1), to afford 18 (82%).

Scheme 6. Reagents and conditions: a) pent-1-yne, CuSO₄·5H₂O cat., sodium ascorbate, THF/H₂O (1:1), to afford 34 (89%); b) TFA, H₂O then IR 120 (H⁺), to afford 7 (64%).
Table 1. Biological evaluation of iminosugars.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ligand</th>
<th>Valency</th>
<th>Linker length</th>
<th>$\text{CC}_{50}^{\text{a}}$ [µM]</th>
<th>$K_i^{\text{b}}$ [nm]</th>
<th>Fold increase$^{\text{c}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>DNJ</td>
<td>1</td>
<td>6</td>
<td>20</td>
<td>1660 ± 210</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>DNJ</td>
<td>3</td>
<td>6</td>
<td>100</td>
<td>23 500 ± 1500</td>
<td>2.5 ± 0.1 at 50 µM</td>
</tr>
<tr>
<td>12</td>
<td>DNJ</td>
<td>4</td>
<td>6</td>
<td>100</td>
<td>7630 ± 620</td>
<td>_R</td>
</tr>
<tr>
<td>15</td>
<td>DNJ</td>
<td>7</td>
<td>6</td>
<td>20</td>
<td>12 000 ± 2000</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>19</td>
<td>DNJ</td>
<td>14</td>
<td>6</td>
<td>50</td>
<td>14 000 ± 34000</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>DIX</td>
<td>1</td>
<td>6</td>
<td>20</td>
<td>35 ± 2</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>11</td>
<td>DIX</td>
<td>3</td>
<td>6</td>
<td>20</td>
<td>29 ± 1</td>
<td>1.7 ± 0.1 at 0.5 µM</td>
</tr>
<tr>
<td>14</td>
<td>DIX</td>
<td>4</td>
<td>6</td>
<td>20</td>
<td>14 ± 5</td>
<td>1.2 ± 0.2 at 0.1 µM</td>
</tr>
<tr>
<td>18</td>
<td>DIX</td>
<td>7</td>
<td>6</td>
<td>100</td>
<td>50 ± 2</td>
<td>2.1 ± 0.2 at 1 µM</td>
</tr>
<tr>
<td>20</td>
<td>DIX</td>
<td>14</td>
<td>6</td>
<td>50</td>
<td>22 ± 11</td>
<td>_R</td>
</tr>
<tr>
<td>3a</td>
<td>DIX</td>
<td>1</td>
<td>9</td>
<td>n.d.$^{\text{d}}$</td>
<td>26 ± 3</td>
<td>1.9 ± 0.1 at 10 nm</td>
</tr>
<tr>
<td>5</td>
<td>DNJ</td>
<td>1</td>
<td>9</td>
<td>100$^{(22)}$</td>
<td>758 ± 65</td>
<td>1.2 ± 0.1 at 1 µM</td>
</tr>
<tr>
<td>1b</td>
<td>DNJ</td>
<td>1</td>
<td>9</td>
<td>n.d.$^{(22)}$</td>
<td>300$^{(27a)}$</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>DNJ (OAc)</td>
<td>1</td>
<td>9</td>
<td>50</td>
<td>n.i.$^{(19)}$</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>9</td>
<td>DNJ</td>
<td>3</td>
<td>9</td>
<td>&gt; 100$^{(22)}$</td>
<td>1070 ± 70</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>DNJ (OAc)</td>
<td>3</td>
<td>9</td>
<td>&gt; 100</td>
<td>n.i.$^{(19)}$</td>
<td>3.0 ± 0.1 at 1 µM</td>
</tr>
<tr>
<td>13</td>
<td>DNJ</td>
<td>4</td>
<td>9</td>
<td>50$^{(22)}$</td>
<td>284 ± 19</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>16</td>
<td>DNJ</td>
<td>7</td>
<td>9</td>
<td>50</td>
<td>55 ± 3</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>17</td>
<td>DNJ (OAc)</td>
<td>7</td>
<td>9</td>
<td>50</td>
<td>n.i.$^{(19)}$</td>
<td>_R</td>
</tr>
<tr>
<td>21</td>
<td>DNJ</td>
<td>14</td>
<td>9</td>
<td>200</td>
<td>61 ± 6</td>
<td>1.6 ± 0.2 at 0.1 µM</td>
</tr>
<tr>
<td>control$^{(23)}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

[a] HL60 cells were treated with various concentrations (1–500 µM) of compounds for three days, and the compounds’ cytotoxicities were evaluated as described in the Experimental Section. [b] Not determined. [c] Not cytotoxic. [d] The $K_i$ values were determined for human placental GCase with 4-methylumbelliferyl-$\beta$-d-glucoside. Inhibition, when detected, was competitive unless specified otherwise. [e] Mixed inhibition: the $K_i$ value corresponding to the competitive term is given, and the noncompetitive contribution had an apparent inhibition constant $K_i' = (77 600 ± 40 300) \text{ nm}$. [f] Mixed inhibition: the $K_i$ value corresponding to the competitive term is given, and the noncompetitive contribution had an apparent inhibition constant $K_i' = (22 900 ± 12 300) \text{ nm}$. [g] $K_i$ value determined with human placental GCase with 0.5 µM 4-methylumbelliferyl-$\beta$-d-glucoside. [h] Mixed inhibition: the $K_i$ value corresponding to the competitive term is given, and the noncompetitive contribution had an apparent inhibition constant $K_i' = (11 ± 10) \text{ nm}$. [i] No inhibition detected at 5 µM. [j] Gaucher fibroblasts (N370S) were cultured in the presence of compounds in various concentrations (1–200 µM) for three days, after which GCase activity was measured. The fold increase in enzyme activity is relative to untreated cells (i.e., normalized value = 1). The maximum fold increase as shown in the table appeared at 10 µM unless specified otherwise (see the Supporting Information). [k] Control cells (no compound addition) were analyzed after addition of water or 0.01% DMSO. [l] No significant chaperoning activity observed.

Initially measured in our first preliminary study based on IC$_{50}^{\text{a}}$ values.$^{(23)}$

The peracetylated mono- or multivalent DNJ analogues 6, 10, and 17, designed as potential prodrugs, were found to display no inhibition of GCase. This result might be explained in part by the fact that these compounds are not deprotected under the conditions of the in vitro assays. Cytotoxicity assays were performed before the investigation of the influence of the iminosugar clusters and their monovalent analogues on human GCase activity in N370S fibroblasts (Table 1). All the compounds had CC$_{50}^{\text{a}}$ values (the concentration at which the number of cells or cell proliferation is reduced by 50%) greater than 20 µM. With the exceptions of compounds 13 and 16, the multivalent deprotected iminosugars are less toxic than or equally toxic as their corresponding monovalent derivatives. These two iminosugar clusters, which displayed higher toxicity values, had the longer spacer length.

The assay of N370S GCase activation was then performed (Table 1). For comparison, α-C-nonyl-DIX (3a) and NN-DNJ (1b) were also evaluated, and their chaperoning activities were found to be comparable to that already reported.$^{(17,27a)}$ Of the 14 multivalent iminosugars evaluated, five were found to display weak to no significant chaperoning activity (Table 1). The best results were obtained with two DNJ clusters with the C$_9$ spacer, compounds 10 and 13, which demonstrated threefold and 3.3-fold increases at 1 and 10 µM, respectively, in GCase activity.

In the DNJ series with the C$_6$ linker, no improvement in the chaperoning activity was observed, and only compounds 15 (heptavalent) and 19 (14-valent) demonstrated effects comparable to that of the corresponding monovalent derivative 4 at a cellular concentration of 10 µM (Figure 1).

Interesting results were obtained when the spacer length was increased by three carbon atoms (Figure 2). The presence of the N-alkyltriazolyl substituent in monovalent control 5

![Figure 1](image-url) The influence of iminosugars 4 (●), 15 (×), and 19 (○) on β-glucocerebrosidase activities in N370S Gaucher fibroblasts. The fold increases in enzyme activity are relative to untreated cells (i.e., normalized value = 1) and shown as relative enzyme activities. Means ± SD obtained from experiments performed in triplicate are shown.

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
The most promising result came from peracetylated trivalent DNJ analogue 10 (Table 1, Figure 4). This molecule, small in comparison with the corresponding heptavalent and 14-valent analogues,[35] was designed as a produg with the aim of facilitating cellular uptake and permeability. Compound 10 (3.0-fold increase) displayed enhancement comparable to that seen with 1b but at a cellular concentration ten times lower (1 μM). The trivalent cluster 10, which was not an inhibitor of GCase in vitro, showed better enhancement than its monovalent analogue 6 (1.2-fold increase). The chaperoning effect was lost with systems of higher valency, as is shown in the case of the corresponding heptavalent analogue 17.

The positive impact of acylation of the OH group on chaperone activity was clearly demonstrated by comparison of the enzyme activity enhancement by 10 (3.0-fold increase at 1 μM) and that by the corresponding deprotected analogue 9 (2.4-fold increase at 10 μM). Taken together, these data support the idea that compound 10 acts as an acetate produg of 9 with improved cellular uptake and permeability. Once inside the cell, endogenous esterases presumably convert 10 into the corresponding active chaperone 9.[36] However, additional unknown mechanisms of action in addition to this produg effect may not be discounted. To the best of our knowledge, the results obtained with 10 constitute the first demonstration that the produg concept might be a promising option for the design of pharmaceutical chaperones for GLSDs. It is also noteworthy that trivalent cluster 10 is among the less toxic iminosugars evaluated in this study (CC_{50} > 100 μM).

Conclusions

In summary, we report a systematic structure–activity relationship study in which we have synthesized 14 iminosugar clusters and evaluated their inhibition and chaperoning activities toward GCase. Small but significant multivalent effects in GCase inhibition were observed for two iminosugar clusters. Our study provides strong confirmation that compounds that display the best affinities for GCase, as judged by their in vitro inhibition potencies, are not necessarily the best chaperones of the mutant enzyme. This observation, recently also made for a series of amphiphilic DNJ derivatives,[36] is strongly accentuated in the case of multivalent iminosugars such as 20 and 13. The lack of correlation between inhibition and chaperoning activity is an additional issue for the rational design of pharmaceutical chaperones because no prediction based on structure–inhibition relationship studies can be made. The best chaperoning effect for a deprotected iminosugar cluster (3.3-fold increase at 10 μM) was observed in the DNJ series with tetravalent compound 13, a sub-micromolar GCase inhibitor.

Our study also provides the first evidence of the great potential of prodrugs for the development of potent pharmacological chaperones. Acetylation of the trivalent iminosugar 9,
giving the corresponding peracetate prodrug 10, led to a pharma-
cological chaperone that displayed higher enzyme activity
increases (3.0-fold instead of 2.4-fold) at cellular concentrations
reduced by one order of magnitude. Future studies combining
prodrug and multivalent strategies might provide a new gen-
eration of potent pharmacological chaperones for the treat-
mant of lysosomal diseases.

Experimental Section

General methods: CHCl₃ was distilled over CaH₂ under Ar. All re-
actions were performed in standard glassware or microwave reac-
tior vials purchased from Biotage. Microwave reactions were carried
out with a Biotage Initiator microwave synthesizer. Column chro-
matography: silica gel 60 (230–400 mesh, 0.040–0.063 mm) was
purchased from E. Merck. Flash chromatography was carried out
with Grace Reveleris flash system equipped with UV/Vis and
ELSD detectors. TLC was performed on aluminum sheets coated
with a Grace Reveleris flash system equipped with UV/Vis and
solvent peaks as references. Carbon multiplicities were assigned by
distortionless enhancement by polarization transfer (DEPT) experi-
ments. NMR spectra were recorded on a Bruker AC 300 or AC 400 with
a Bruker MicroTOF spectrometer. Specific rotations were determined at room temper-
ature (20 °C) with a PerkinElmer 241 polarimeter and the use of
sodium light (l = 589 nm).

General procedure A — CuAAC reaction: Alkyne, azide,
CuSO₄·5H₂O (0.1 equiv per alkyne) and sodium ascorbate
(0.2 equiv per alkyne) in DMF/H₂O (4:1, 2.5 to 5 mL) were placed
successively in a 5 mL microwave reaction vial. The resulting sus-
ension was heated under microwave irradiation at 80 °C for
30 min, water was added (10 mL), and the aqueous phase was
extracted with AcOEt (3 × 12 mL). The organic phases were combined,
dried over Na₂SO₄, and concentrated in vacuo. Traces of copper
salts were removed by filtration over a short pad of silica gel with
eution with CH₃CN/H₂O/NH₄OH (15:0.5:0.5), and the residue was
salts were removed by filtration over a short pad of silica gel with
saturated NaHCO₃ solution (1 mL). After 15 min of stirring, the resin was filtered and
concentration to 0.2 mL and then purified by flash chromatography (SiO₂, CH₂Cl₂/MeOH 100:0
to 98:2) to give the desired iminosugar.

General procedure B — deacetylation reaction: Amberlite resin
IRA 400 (OH -) (6 g per mmol Ac) was added to a solution of acety-
ated iminosugar in a mixture of MeOH and H₂O (1:1), and the solu-
tion was stirred for 4 h with the aid of a rotary evaporator (at
atmospheric pressure). The resin was then filtered off and washed
with methanol and water. The solvents were then removed under reduced
pressure to give the desired deprotected iminosugar.

α-1-C-(6-Azidoxyethyl)-2,3,4-tri-O-acetyl-1,5-dideoxy-1,5-imino-
xylitol (24): Trifluoroacetic acid (1.2 mL) was added to a solution of
39 (136 mg, 0.280 mmol) in CH₂Cl₂ (4.5 mL), and the mixture was
stirred for 2 h at RT. It was then concentrated to dryness, the resi-
due was dissolved in CH₂Cl₂ (10 mL), and the organic phase was
washed with saturated NaHCO₃ solution, (2 × 10 mL), dried over
Na₂SO₄, filtered, and concentrated. The residue was purified by flash
column chromatography (CH₂Cl₂/MeOH 100:0 to 95:5) to afford
24 (83 mg, 77%) as a pale yellow oil. [α]D = +3.0 (c = 1, CHCl₃); ³H NMR (300 MHz, CDCl₃): δ = 1.11–1.39 (m, 6 H; H-7, H-8, H-9), 1.39–1.46 (m, 9 H; H-3), 1.48–
1.66 (m, 4 H; H-4, H-10), 1.94–2.06 (m, 9 H; CH₂(CH₂)₃CH₂), 2.69 (br. J =
11.3 Hz, 1 H; H-5b), 2.52 (t, J = 6.4 Hz, 2 H; H-12), 4.09–4.56 (m, 2 H;
H-5a, H-1), 4.68–5.01 (m, 2 H; H-4, H-2), 5.25 ppm (t, J = 9.8 Hz, 1 H;
H-3); ¹C NMR (75 MHz, CDCl₃): δ = 20.8, 20.9 (C-CH₃), 24.7, 25.2,
25.4, 26.5, 26.7, 28.3 (C-3), 28.9, 39.0, and 40.1 (C-5), 51.4 (C-11),
52.5 and 53.5 (C-6), 69.3, 69.8, 70.4, 71.0, 71.06 (C-2, C-3, C-4), 81.1
(C-2), 153.3 and 153.4 (C-1), 169.4, 169.6, 169.76, 169.9, 170.4 ppm (C-3
(COCH₃)); IR (neat): ν = 2098 (N=O); HRMS (ESI): m/z calc'd for C₂₇H₂₉N₄NaO₇: 575.2473 [M+Na]⁺; found: 570.239.

α-1-C-(6-Azidoxyethyl)-2,3,4-tri-O-acetyl-1,5-dideoxy-1,5-imino-
xylitol (24): Trifluoroacetic acid (1.2 mL) was added to a solution of
29 (136 mg, 0.280 mmol) in CH₂Cl₂ (4.5 mL), and the mixture was
stirred for 2 h at RT. It was then concentrated to dryness, the resi-
due was dissolved in CH₂Cl₂ (10 mL), and the organic phase was
washed with saturated NaHCO₃ solution, (2 × 10 mL), dried over
Na₂SO₄, filtered, and concentrated. The residue was purified by flash
column chromatography (CH₂Cl₂/MeOH 100:0 to 95:5) to afford
24 (83 mg, 77%) as a pale yellow oil. [α]D = +3.0 (c = 1, CHCl₃); ³H NMR (300 MHz, CDCl₃): δ = 1.11–1.39 (m, 6 H; H-7, H-8, H-9), 1.39–1.46 (m, 9 H; H-3), 1.48–
1.66 (m, 4 H; H-4, H-10), 1.94–2.06 (m, 9 H; CH₂(CH₂)₃CH₂), 2.69 (br. J =
11.3 Hz, 1 H; H-5b), 2.52 (t, J = 6.4 Hz, 2 H; H-12), 4.09–4.56 (m, 2 H;
H-5a, H-1), 4.68–5.01 (m, 2 H; H-4, H-2), 5.25 ppm (t, J = 9.8 Hz, 1 H;
H-3); ¹C NMR (75 MHz, CDCl₃): δ = 20.8, 20.9 (C-CH₃), 24.7, 25.2,
25.4, 26.5, 26.7, 28.3 (C-3), 28.9, 39.0, and 40.1 (C-5), 51.4 (C-11),
52.5 and 53.5 (C-6), 69.3, 69.8, 70.4, 71.0, 71.06 (C-2, C-3, C-4), 81.1
(C-2), 153.3 and 153.4 (C-1), 169.4, 169.6, 169.76, 169.9, 170.4 ppm (C-3
(COCH₃)); IR (neat): ν = 2098 (N=O); HRMS (ESI): m/z calc'd for C₂₇H₂₉N₄NaO₇: 575.2473 [M+Na]⁺; found: 570.239.
Preparation of 14: 27.5 mg (80%) was prepared according to General Procedure A, starting from 2,2-bis(prop-2-ynyl)methyldiisopropylpropan-1-ol (32.4 mg, 0.023 mmol) and iminosugar 24 (36.1 mg, 0.094 mmol, 3.3 equiv). \( \text{C}_{29} \text{H}_{34} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). \( \text{C} \) for 25 min. Water (a few mL) was added, and the aqueous phase was extracted with AcOEt (3 x 10 mL). The combined organic layers were washed with water (10 mL) and then were dried (NaSO4) and concentrated. The residue was purified by flash chromatography (CH2Cl2/MeOH 98:2 to 90:10). In order to remove copper from the product, the residue was dissolved in CHCl3 (1 mL). NH4OH (10% solution, 3 mL) was added, and the mixture was stirred for 15 min. The phases were separated, and the aqueous phase was extracted with CHCl3 (2 x 1 mL). The combined organic layers were dried over NaSO4 and concentrated. The residue was again purified by flash chromatography (CH2Cl2/MeOH 98:2 to 90:10) to afford 30 (44 mg, 44%) as a yellow solid and as a mixture of rotamers according to NMR data. \( \text{C}_{29} \text{H}_{33} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). 

Preparation of 16: 27.5 mg (80%) was prepared according to General procedure A, starting from triakos(prop-2-ynyl)methylmethylene (26,28) 63 mg, 0.022 mmol and iminosugar 24 (37.2 mg, 0.097 mmol, 4.4 equiv). \( \text{C}_{29} \text{H}_{38} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). 

Preparation of 17: 43.5 mg (83%) was prepared according to General procedure A, starting from tetraakos(prop-2-ynyl)methylmethylene (26,28) 63 mg, 0.022 mmol and iminosugar 24 (37.2 mg, 0.097 mmol, 4.4 equiv). \( \text{C}_{29} \text{H}_{38} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). 

Preparation of 18: 27.5 mg (80%) was prepared according to General procedure A, starting from triakos(prop-2-ynyl)methylmethylene (26,28) 63 mg, 0.022 mmol and iminosugar 24 (37.2 mg, 0.097 mmol, 4.4 equiv). \( \text{C}_{29} \text{H}_{38} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). 

Preparation of 19: 27.5 mg (80%) was prepared according to General procedure A, starting from triakos(prop-2-ynyl)methylmethylene (26,28) 63 mg, 0.022 mmol and iminosugar 24 (37.2 mg, 0.097 mmol, 4.4 equiv). \( \text{C}_{29} \text{H}_{38} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). 

Preparation of 20: 27.5 mg (80%) was prepared according to General procedure A, starting from triakos(prop-2-ynyl)methylmethylene (26,28) 63 mg, 0.022 mmol and iminosugar 24 (37.2 mg, 0.097 mmol, 4.4 equiv). \( \text{C}_{29} \text{H}_{38} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). 

Preparation of 21: 27 mg (80%) was prepared according to General procedure A, starting from triakos(prop-2-ynyl)methylmethylene (26,28) 63 mg, 0.022 mmol and iminosugar 24 (37.2 mg, 0.097 mmol, 4.4 equiv). \( \text{C}_{29} \text{H}_{38} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). 

Preparation of 22: 27 mg (80%) was prepared according to General procedure A, starting from triakos(prop-2-ynyl)methylmethylene (26,28) 63 mg, 0.022 mmol and iminosugar 24 (37.2 mg, 0.097 mmol, 4.4 equiv). \( \text{C}_{29} \text{H}_{38} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). 

Preparation of 23: 27 mg (80%) was prepared according to General procedure A, starting from triakos(prop-2-ynyl)methylmethylene (26,28) 63 mg, 0.022 mmol and iminosugar 24 (37.2 mg, 0.097 mmol, 4.4 equiv). \( \text{C}_{29} \text{H}_{38} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). 

Preparation of 24: 27 mg (80%) was prepared according to General procedure A, starting from triakos(prop-2-ynyl)methylmethylene (26,28) 63 mg, 0.022 mmol and iminosugar 24 (37.2 mg, 0.097 mmol, 4.4 equiv). \( \text{C}_{29} \text{H}_{38} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544). 

Preparation of 25: 27 mg (80%) was prepared according to General procedure A, starting from triakos(prop-2-ynyl)methylmethylene (26,28) 63 mg, 0.022 mmol and iminosugar 24 (37.2 mg, 0.097 mmol, 4.4 equiv). \( \text{C}_{29} \text{H}_{38} \text{N}_{4} \text{O}_{3} \) (Found: 440.2547, calcd: 440.2544).
(OH−), (1.4 meq·mL−1, 0.81 g) was added. The mixture was stirred for 4.5 h at 20 °C with the aid of a rotary evaporator (at atmospheric pressure). After concentration, the residue was dissolved again in water and washed with ether to remove minor impurities. The phases were separated, and the solvent was evaporated to give 20 (18 mg, 66% over two steps) as a white solid.  

**1H NMR (400 MHz, MeOD):** δ = 1.30–1.48 (8 mg, 98%; H-7′ to H-9′, H-6′a, 1.48–1.64 (m, 14H, H-6′b), 1.81–1.99 (m, 28H, H-10′), 2.76 (bd, J = 13.0 Hz, 14H, H-5′a), 2.81–2.90 (m, 14H, H-1′), 3.01 (d, J = 13.4 Hz, 14H, H-5′b), 3.40 (t, J = 8.9 Hz, H-7′), 3.47–3.54 (m, 35H, H-2′, H-7′, H-10′), 3.69–3.78 (m, 42H, H-6′, H-5′, H-3′, H-3), 4.33 (t, J = 6.2 Hz, 14H, H-11′), 4.42 (t, J = 6.9 Hz, 14H, H-11′), 4.48–4.64 (m, 14H, H-7′ or H-10′), 4.80–4.91 (m, 14H, H-5′a or H-10′a, 1H, H-1), 5.00 (d, J = 12.3 Hz, 7H, H-7b or H-10b), 7.71 (s, 7H, H-9′ or H-12′), 8.07 ppm (s, 7H, H-9′ or H-12′); 13C NMR (100 MHz, MeOD): δ = 26.9, 27.4, 30.2, 30.7 (C-6′ to C-9′), 31.3 (C-10′), 47.5 (C-5′), 51.3 (C-11), 55.6 (C-1′), 64.4 (C-11′), 65.2 and 66.1 (C-7′, C-10), 70.5 (C-6′, 70.7 (C-2′ or C-4′), 71.4 (C-5′), 71.9 (C-2′ or C-4′), 73.9 (C-3′), 74.6 (C-3′), 81.2 (C-2′), 84.2 (C-4′), 102.5 (C-1′), 125.1 and 125.7 (C-9′, C-12), 145.3 ppm (C-8′, C-11′), 135.3 ppm (C-9′, C-11′), 91.2 (OH); MS (MALDI-TOF): m/z calcd for C_{48}H_{50}O_{19}Na: 825.51 [M+H]^+; average found: 825.62.

**Preparation of 32:** Compound 32 (108.5 mg, 94%) was prepared according to General procedure A, starting from heptakis(2,3,6-tri-O-methyl-2-proparyl)cyclomaltoheptaose (31, 43 mg, 0.027 mmol) and inosimannos 24 (80 mg, 0.208 mmol, 1.1 x equiv) (δ = 42.0 (C-1′, CHCl)); δ = 1.06 (m, 35H, H-6′, H-7′, H-8′). The culture medium was replaced every two to three days, and all cells used in this study were between the fifth and the 15th passages.

**HL60 culture:** HL60 cells were cultured in RPMI1640 medium supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U·mL−1), and streptomycin (100 mg·mL−1). Compound 32 (10 pg, 0.27 mmol) was added to the culture medium 24 h prior to the addition of the inhibitors. The cells were incubated for 36 h at 37 °C and exposed to 5 μM of the inhibitor before use. The cells were harvested by centrifugation and washed with PBS (pH 7.4) and counted using a haemocytometer.

**β-Glucocerebrosidase inhibition assay:** Human placental β-glucocerebrosidase was purified from tissue, and activity against glucosylceramide was determined as described previously[22] Enzyme activity was measured in the well of a flat-bottomed 96-well (300 μL) plate: inhibitor solution (1 μL) enzyme solution (2 μL) and 47 μL substrate solution of 4-methylumbelliferyl-β-D-glucoside in citrate/phosphate buffer (pH 5.2, 0.1 M) containing 0.25% sodium taurocholate, 0.10% TX100) at 37 °C for 30 min of incubation. The reaction was stopped by addition of a solution of sodium carbonate (0.5 M, 200 μL), and fluorescence was measured by use of an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Inhibition constants were generated for placental β-glucocerebrosidase (K_{i} for 4-MU-β-D-glucoside (1.9 ± 0.3 μM) with use of 0.25 to 2 mm substrate concentrations for K determinations and 0.5 mm substrate concentration for IC_{50} determinations. All experiments were performed in triplicate, and nonlinear regression was performed with Prism software. Standard deviations were obtained directly from Prism software for K values.

**Determination of IC_{50} values:** Percentage inhibition was plotted against the log of the inhibitor concentration, and a nonlinear regression was fitted with the aid of Prism software. The IC_{50} value for each compound was calculated from the value of the log of the inhibitor concentration at 50% inhibition of enzyme activity for each three series of data, and the mean value of IC_{50±SD} was determined with Microsoft Excel.

**Determination of K_{i} values:** K_{i} values were determined by use of a suitable range of substrate and inhibitor concentrations. Velocity values were plotted against [S] and subjected to nonlinear regression by use of Prism software. The different inhibition type models were tested on the substrate/velocity curves, and the inhibition mode fitting best with experimental data was selected, providing K_{D±SD}. Lineweaver–Burk plots (1/[V] against 1/[S]) were also drawn with the aid of Prism software reflecting K_{M} and V_{max} values found from the nonlinear regression to show the inhibition mode type.

**Cytotoxicity assays:** A CellTiter 96 aqueous non-radioactive cell proliferation assay kit (Promega) was used to measure the effect of each compound on cell proliferation by the manufacturer’s protocol. HL60 cells were seeded in 96-well plates at 5 × 10^{3} cells per well and were incubated for three days in the presence of various concentrations of inhibitor. An aliquot (40 μL) of MTS-phosphine methosulfate mixture (20:1) 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium) was added to each well, and the samples were incubated for 3–4 h. The absorbance was read at 490 nm. Experiments were performed in triplicate, and the cytotoxicity concentrations (CC_{50}) at which absorbance

These are not the final page numbers!
at 490 nm was 50% of that of an untreated control, were determined.

β-Glucocerebrosidase activation assays: Having been distributed over 96-well plates, the cells were incubated for three days with various inhibitor concentrations up to 50 μM. Cells were washed twice in phosphate-buffered saline and incubated for three extra hours with fresh medium. All enzyme activation measurements were performed by adding homogenate substrate solution (50 μl per well, 4-methylumbelliferyl-β-D-glucoside (5 mM) in citrate phosphate buffer (pH 5.2, 0.1 M) containing 0.25% sodium taurocholate, 0.1% TX100) at 37°C for 3 h incubation. The reaction was stopped by addition of glycine (1 M, 50 μl) in sodium hydroxide solution (1 M) before measurement of the β-glucocerebrosidase activity. The fluorescence was measured with use of an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Enzyme activation is defined as the fold increase in enzyme activity in treated cells relative to untreated cells. Results were plotted with Prism software. All experiments were performed in triplicate, and the mean values ± SDs were determined with the aid of Prism software.

Acknowledgements

This work was supported by the Institut Universitaire de France (IUF), the CNRS (UMR 7509), the University of Strasbourg, the Agence Nationale de la Recherche (ANR, grant numbers 08JC-0094-01 and 11-B507-003-02), and doctoral fellowships from the French Department of Research to C.D. The authors express their gratitude to Michel Schmitt for NMR measurements.

Keywords: Gaucher disease - inosimogars - multivalency - pharmacological chaperones - produgs - structure-activity relationships


All attempts to prepare a pure sample of the tetravalent DIX analogue of 14 with a C8 linker proved unsuccessful. Because of isomerization side-reactions of C=C double bonds in the reactants during the key cross-metathesis step, an inseparable mixture of the expected azido iminosugar precursor along with the two corresponding analogues incorporating a C9 or a C10 alkyl spacer was obtained, as judged by NMR and MS analysis. To have an idea of the impact of longer alkyl spacers on GCase binding affinity and chaperoning activity, we nevertheless synthesized the corresponding hetero-tetravalent analogues. Interestingly, this mixture of tetravalent DIX clusters with a distribution of C8 to C10 alkyl spacers was found to display strong inhibition toward GCase but no significant chaperoning activity.

A Systematic Investigation of Iminosugar Click Clusters as Pharmacological Chaperones for the Treatment of Gaucher Disease

The right combination: Combining prodrug and multivalent strategies has led to an unprecedented pharmacological chaperone. A trivalent acetylated iminosugar is able to increase mutant N370S β-glucocerebrosidase (GCase) activity levels by therapeutically relevant amounts, by as much as threefold in cells, and at lower concentrations than required with the corresponding deprotected analogue.