ChemPubSoc Europe

DOI: 10.1002/cmdc.201402023

Glucocerebrosidase Enhancers for Selected Gaucher Disease Genotypes by Modification of α-1-*C*-Substituted Imino-D-xylitols (DIXs) by Click Chemistry

Jenny Serra-Vinardell,[a] Lucía Díaz,[b] Josefina Casas,[b] Daniel Grinberg,[a] Lluïsa Vilageliu,[a] Helen Michelakakis,[c] Irene Mavridou,[c] Johannes M. F. G. Aerts,[d] Camille Decroocq,[e] Philippe Compain,*[e, f] and Antonio Delgado*[b, g]

A series of hybrid analogues was designed by combination of the iminoxylitol scaffold of parent 1C9-DIX with triazolylalkyl side chains. The resulting compounds were considered potential pharmacological chaperones in Gaucher disease. The DIX analogues reported here were synthesized by CuAAC click chemistry from scaffold 1 (α-1-*C*-propargyl-1,5-dideoxy-1,5-imino-D-xylitol) and screened as imiglucerase inhibitors. A set of selected compounds were tested as β-glucocerebrosidase (GBA1) enhancers in fibroblasts from Gaucher patients bearing different genotypes. A number of these DIX compounds were revealed as potent GBA1 enhancers in genotypes containing the G202R mutation, particularly compound DIX-28 (α-1-*C*-[(1-(3-trimethylsilyl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl]-1,5-dideoxy-1,5-imino-D-xylitol), bearing the 3-trimethylsilylpropyl group as a new surrogate of a long alkyl chain, with approximately threefold activity enhancement at 10 nM. Despite their structural similarities with isofagomine and with our previously reported aminocyclitols, the present DIX compounds behaved as non-competitive inhibitors, with the exception of the mixed-type inhibitor DIX-28.

Introduction

Lysosomal storage disorders constitute a group of genetic diseases characterized by the accumulation of non-degraded substrates within the lysosomes.[1] These types of disorders are usually classified according to the nature of the substrate accumulated. In particular, sphingolipidoses are the result of the abnormal lysosomal metabolism of glycosphingolipids. Among the different sphingolipidoses, Gaucher disease (GD) is particularly relevant, due to its prevalence. It is characterized by the accumulation of glucosylceramide (GlcCer), as a result of the deficiency of β-glucocerebrosidase (GCase or GBA1), a lysosomal β-glucosidase that hydrolyses GlcCer into glucose and ceramide.[2] The disease has classically been divided into three types, based on neurological involvement: type 1 (non-neuronopathic), type 2 (acute neuronopathic), and type 3 (subacute neuronopathic).

Despite current therapeutic approaches for the treatment of GD, such as enzyme replacement and substrate reduction therapies, alternative strategies are desirable in order to avoid the drawbacks associated with the above classical approaches.[3,4] The deficiency of GBA1, in most instances, results from the presence of one or several enzyme mutations that give rise to misfolded forms of the enzyme that are prematurely removed in the endoplasmic reticulum (ER) by the ER-associated degradation (ERAD) system before reaching the lysosome.[5] In this context, pharmacological chaperones have become an active field of research.[6]

[a] *J. Serra-Vinardell,[+] Prof. Dr. D. Grinberg, Prof. Dr. L. Vilageliu*
Departament de Genètica, Universitat de Barcelona (UB)
IBUB; CIBER de Enfermedades Raras (CIBERER)
Av. Diagonal 643, 08028, Barcelona (Spain)

[b] *Dr. L. Díaz,[+] Dr. J. Casas, Prof. Dr. A. Delgado*
Research Unit on BioActive Molecules (RUBAM)
Departament de Química Biomèdica
Institut de Química Avançada de Catalunya (IQAC-CSIC)
Jordi Girona 18, 08034 Barcelona (Spain)

[c] *Prof. Dr. H. Michelakakis, Dr. I. Mavridou*
Department of Enzymology & Cellular Function
Institute of Child Health, Athens 11527 (Greece)

[d] *J. M. F. G. Aerts*
Department of Medicinal Biochemistry, Academic Medical Center
Meibergdreef 15, 1105 AZ, Amsterdam (The Netherlands)

[e] *Dr. C. Decroocq, Prof. Dr. P. Compain*
Laboratoire de Synthèse Organique et Molécules Bioactives
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

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

[g] *Prof. Dr. A. Delgado*
Facultat de Farmàcia, Unitat de Química Farmacèutica (Unitat Associada al CSIC), Universitat de Barcelona (UB)
Avda. Joan XXIII, s/n, 08028 Barcelona (Spain)
E-mail: delgado@rubam.net

[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201402023.

Application of this concept to the development of alternative strategies for GD has focused on the discovery of small molecules able to target the mutated enzyme, thus preventing its premature degradation, giving rise to an enzyme activity enhancement. Although classical approaches for the development of pharmacological chaperones have relied on the use of ligand-related reversible enzyme inhibitors, other approaches based on allosteric ligands have recently been disclosed.[7,8]

Since Sawkar et al.[9] demonstrated that N-nonyldeoxynojirimicin (NN-DNJ) produced a 1.5- to 2-fold increase in the activity of the N370S mutation, several molecules have been evaluated, primarily for mutations N370S and L444P,[10,11] the two most frequent GD mutant alleles. In particular, isofagomine (IFG), which was shown to produce one of the highest increases in the N370S mutant GBA1,[12,13] entered clinical trials. However, after the results of Phase I and II, the trials were discontinued.[10,14]

In recent years, our laboratories have been actively working on the discovery of new small GBA1 inhibitors as potential pharmacological chaperones of diverse mutant forms of this enzyme. Thus, a series of potent iminocyclitol derivatives[15,16] culminated in the second generation iminoxylitol derivative 1C9-DIX (Figure 1), whose efficiency as pharmacological chaperone at low nanomolar concentration in N370S fibroblasts from GD patients was reported.[17] Interestingly, this compound was used by Overkleeft as a chemical tool to understand GC metabolism and the basis of GD.[18,19]

Similarly, with a series of aliphatic *N*-alkyl aminocyclitols,[20,21] interesting GBA1 enhancement in patient fibroblasts with different enzyme mutations were obtained.[22] Subsequent modifications at the nitrogen side chain using Cu-promoted alkyne–azide cycloaddition (CuAAC) between a parent aminocyclitol and a set of azides, carefully chosen to ensure a high degree of diversity in the resulting library, led to aminocyclitols of the general structure AC-x (see Figure 1). These were reported as

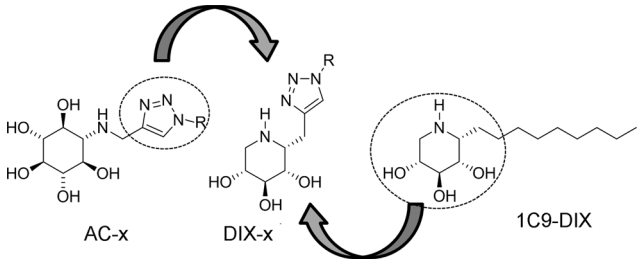


Figure 1. Conceptual design of DIX-x compounds by molecular hybridization of the iminoxylitol scaffold present in 1C9-DIX with the N-substituted triazolyl side chain of AC-x compounds.

potent GBA1 inhibitors[23,24] and also exhibited interesting in vitro and in cellular enzyme enhancement activities toward several GBA1 mutations (unpublished results).

Results and Discussion

Collection design

The remarkable effects elicited by the *N*-substituted triazolylalkyl side chain in aminocyclitols AC-x prompted us to use this structural motif to explore the chemical diversity around the iminoxylitol scaffold present in compound 1C9-DIX (Figure 1). In conceptually related approaches, the use of click chemistry to explore the chemical diversity in N-substituted 1-deoxynojirimycin[25] and the thiol-ene click reaction in α-1-*C*-substituted imino-D-xylitols[26] have also been reported. In this way, a small library of iminoxylitols, DIX-x (Figure 1), arising from the substitution of the parent 1,5-dideoxy-1,5-imino-D-xylitol scaffold (DIX) with some of the most interesting triazolylalkyl side chains used in compounds AC-x,[23] was assembled by means of CuAAC chemistry from iminoxylitol **1** and the azides shown in Figure 2 and Scheme 1. The required azides were selected

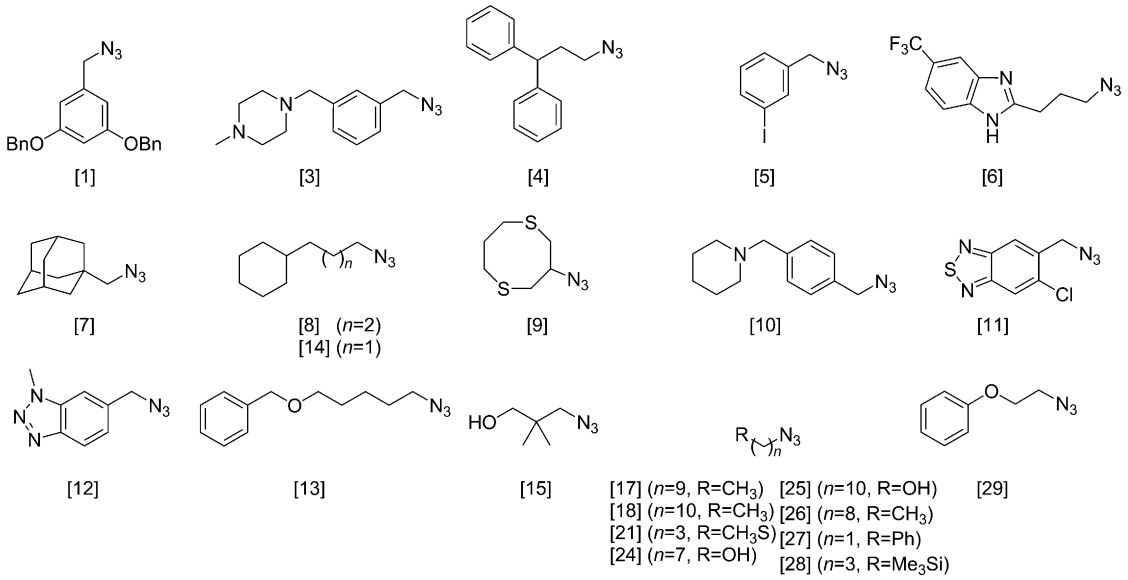
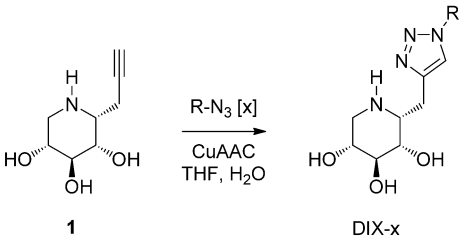
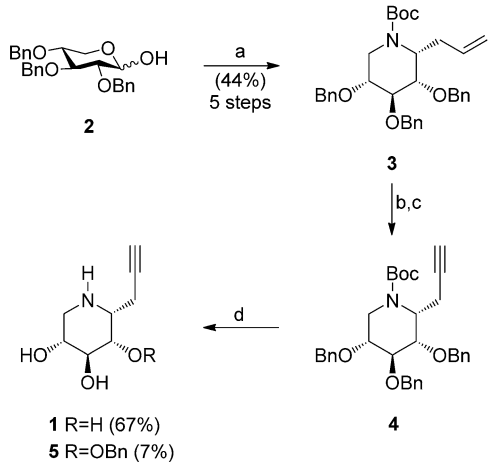


Figure 2. Azides used in the click chemistry reaction of iminoxylitol **1** under conventional CuAAC conditions. Azide numbering has been maintained for comparison with data reported in ref. [23].



Scheme 1. General approach to DIX-x compounds. The azide partner is denoted by [x] (for azide structures, see Figure 2).



Scheme 2. *Reagents and conditions*: a) for details, see Ref. [27]; b) OsO_4, $NaIO_4$, THF/H_2O (1:1), RT, 3 h; c) $(MeO)_2P(O)C(N_2)C(O)Me$, K_2CO_3, MeOH, RT, 5 h, 68% over two steps; d) BCl_3, pentamethylbenzene, CH_2Cl_2, −78 °C, 4 h.

from a collection of commercial precursors (mainly alcohols and bromides), which were filtered as previously described.[23] In addition, a new silyl-containing side chain was found to confer interesting properties to the resulting DIX adduct (see below).

Synthesis of iminoxylitol scaffold 1

Our approach was based on the protected advanced intermediate **3**,[27] recently synthesized in one of our groups in five steps and 44% overall yield from 2,3,4-tri-*O*-benzyl-D-xylopyranose (**2**)[28] (Scheme 2). Conversion of alkene **3** into the corresponding alkyne **4** was performed in two steps (68% combined yield) by means of Lemieux-Johnson oxidative cleavage and reaction of the intermediate aldehyde with an excess of the Bestmann–Ohira reagent.[29] At this stage, the chemoselective deprotection of the O-benzyl groups in **4** proved difficult, and our first attempts using boron trichloride[30] or TMSI[31,32] led to a complex mixture of products. In this context, the deprotection of O-benzyl groups using boron trichloride in the presence of an alkyne functionality were described as troublesome,[23] due to the reactivity of the C–C triple bond. Fortunately, a combination of boron trichloride and pentamethylbenzene was found to be optimal, leading to the concomitant deprotection of the O-benzyl and N-*tert*-butoxycarbonyl groups. The interest in pentamethylbenzene as a cation scavenger for related boron trichloride-mediated debenzylation of aryl benzyl ethers was previously reported.[33] The main advantage of this scavenger is that it does not decrease the Lewis acidity of boron trichloride. This one-pot process afforded the desired iminoxylitol **1** in 67% yield after chromatographic purification. It is noteworthy that the corresponding monobenzylated analogue **5**, resulting from a partial debenzylation reaction, was also isolated in 7% yield. ^1H and ^{13}C-HMBC NMR experiments showed that the benzyl group was attached to the C2 position of the iminoxylitol system.

Library synthesis and preliminary screening

The iminoxylitols used in this study were obtained by the CuAAC of scaffold **1** with the azides shown in Figure 2, following our previously reported protocol (see Experimental Section).[23] Reactions were carried out on a small scale (~8 mg of scaffold and a 1.2 molar ratio of the required azide; Figure 2) in 1 mL of an H_2O/THF (1:1) mixture for 24 h at room temperature to ensure total consumption of the starting scaffold. The identity of the resulting DIX adducts was confirmed by UPLC-MS analysis of the crude reaction mixtures. As the components of the click reaction (copper salts and ascorbic acid) do not interfere with the enzymatic assay,[23] the crude mixtures were individually screened as GBA1 inhibitors in recombinant imiglucerase to determine the IC_{50} value at the optimal pH for enzyme activity (5.2) and at the neutral pH of the ER (7.0) (Table 1).

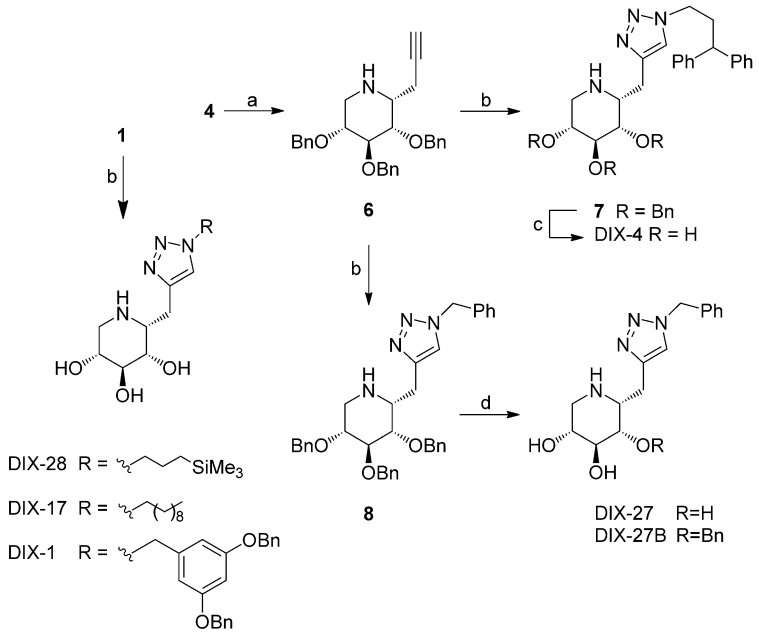
On the basis of the results obtained from in situ screening of the crude CuAAC reaction mixtures (see below), the five most promising iminoxylitol derivatives were selected for further biological evaluation. Compounds DIX-1, DIX-4, DIX-17,

Table 1. IC_{50} values for the library of iminoxylitols DIX toward imiglucerase at pH 5.2 and pH 7.0.[a]

Iminoxylitol	IC_{50} [nM]		Iminoxylitol[b]	IC_{50} [nM]	
	pH 5.2	pH 7.0		pH 5.2	pH 7.0
DIX-1	7.9 ± 0.2	6.1 ± 0.31	DIX-14	12.5 ± 0.5	6.8 ± 0.3
DIX-3	166.0 ± 8.3	116.0 ± 5.7	DIX-15	355.0 ± 16.5	314.8 ± 15.1
DIX-4	8.7 ± 0.3	4.6 ± 0.2	DIX-17	7.6 ± 0.5	5.5 ± 0.2
DIX-5	17.2 ± 0.6	7.5 ± 0.4	DIX-18	7.6 ± 0.4	10.0 ± 0.4
DIX-6	160.0 ± 7.5	57.5 ± 3.1	DIX-21	112.0 ± 5.1	50.5 ± 2.3
DIX-7	11.6 ± 0.5	5.9 ± 0.3	DIX-24	83.0 ± 3.9	44.5 ± 2.1
DIX-8	8.1 ± 0.3	5.1 ± 0.2	DIX-25	18.9 ± 0.7	12.1 ± 0.5
DIX-9	29.4 ± 1.2	10.4 ± 0.4	DIX-26	5.5 ± 0.3	5.4 ± 0.3
DIX-10	223.0 ± 10.5	115.9 ± 4.7	DIX-27	7.7 ± 0.4	7.2 ± 0.3
DIX-11	33.0 ± 1.4	20.9 ± 1.1	DIX-28	7.8 ± 0.3	6.4 ± 0.2
DIX-12	223.0 ± 10.8	136.4 ± 6.5	DIX-29	94.0 ± 4.5	27.3 ± 1.2
DIX-13	8.2 ± 0.5	5.8 ± 0.3	1C9-DIX	6.8 ± 0.3[b]	3.9 ± 0.2

[a] All compounds, except 1C9-DIX, were tested as crude mixtures from the CuAAC reaction between **1** and the appropriate azide (Figure 2). Inhibitors were tested at five different concentrations from a 47 mM click reaction mixture, assuming a quantitative conversion of the starting iminoxylitol (for details, see Experimental Section). [b] Data taken from Ref. [16].

DIX-27, and DIX-28 were thus resynthesized and fully characterized (Scheme 3). With the aim of facilitating purification of the iminosugars obtained by CuAAC, iminoxylitols were prepared in two steps from the corresponding azides and protected alkyne **6**, which was obtained, in turn, by treatment of **4** with trifluoroacetic acid (TFA). This strategy, which was first performed to synthesize DIX-4, DIX-27, and DIX-27B gave unsatisfactory results for DIX-1, DIX-17, and DIX-28, which were finally obtained in acceptable yields by click reactions from fully deprotected **1**. The boron trichloride-mediated removal of the O-benzyl groups from **7** and **8** required longer reaction times and higher temperatures to avoid partial debenzylation reactions, as exemplified by the formation of DIX-27B. In fact, this iminoxylitol was obtained as the major compound from debenzylation of **8** and, due to its structural singularity, was included in the GBA1 enhancement assays described below.



Scheme 3. *Reagents and conditions*: a) TFA/CH_2Cl_2 (1:4), RT, 1 h, 93%; b) $CuSO_4{\cdot}5H_2O$, Na ascorbate, azide, H_2O/THF (1:1), RT, 15 h, 66% (DIX-28); 60% (DIX-17); 78% (DIX-1); 79% (**7**); 86% (**8**); c) BCl_3, CH_2Cl_2, −60 °C→RT, 16 h, 80%; d) BCl_3, CH_2Cl_2, −60 °C→0 °C, 4 h, 40% (DIX-27); 59% (DIX-27B).

Imiglucerase is the recombinant DNA-produced analogue of human β-glucocerebrosidase used in enzyme replacement therapy of GD. The results of the imiglucerase inhibitory assay are shown in Table 1. In general, all compounds behaved as potent imiglucerase inhibitors, with IC_{50} values in the nanomolar range. It is worth mentioning the higher inhibitory potency elicited by iminoxylitol derivatives in comparison with the corresponding aminocyclitol counterparts. Thus, for identical side chain substitution, even in crude reaction mixtures, compounds with the iminoxylitol scaffold (see Figure 1) gave rise to significantly more potent inhibitors than the corresponding aminocyclitols counterparts, which were active in the micromolar range.[23,24]

As with the aminocyclitol analogues,[23,24] compounds with an aliphatic chain (DIX-17, DIX-18, and DIX-26) were among the most potent members of the series with IC_{50} values in the low nanomolar range, similar to that of 1C9-DIX. In general, the affinity of alkylated iminosugar[15–17] or aminocyclitol[24] derivatives toward GBA1 increases with the length of the alkyl chain. However, long alkyl chain derivatives may also be cytotoxic, mainly due to membrane insertion and pore formation.[34] To shorten the alkyl chain length while maintaining an optimal level of lipophilicity, a 3-trimethylsilylpropylgroup was introduced as a substituent by means of azide [28] (see Figure 2). Interestingly, the resulting iminoxylitol, DIX-28, showed an inhibitory potency similar to that of the above longer alkyl chain analogues. In agreement with this observation, the short chain and polar DIX-15 was a less potent member of the library, while other short chain analogues bearing strongly lipophilic aryl substituents, such as DIX-1, DIX-4, and DIX-27 were among the most potent inhibitors. The negative effect of a terminal polar substituent is again evidenced in DIX-3, DIX-6, DIX-10, and DIX-21, which were less potent members of the series, albeit with IC_{50} values still below the micromolar range.

Biochemical studies with purified compounds

From the above preliminary screening with crude CuAAC reaction mixtures, iminoxylitols DIX-1, DIX-4, DIX-17, DIX-27, DIX-27B, and DIX-28 were selected for further biochemical studies and resynthesized for complete chemical characterization (Scheme 3). The IC_{50} values (see Table 2) were within the range of those obtained with the crude reaction mixtures. The new DIX compounds behaved as non-competitive imiglucerase inhibitors, with the exception of DIX-28, which showed mixed-type inhibition kinetics (Figure 3). In all cases, the K_i values were in the nanomolar range, with the exception of the weak inhibitor DIX-27B.

Table 2. Inhibition data for selected compounds toward imiglucerase.

Compd	IC_{50} [nM]		K_i [nM][a]	
	pH 5.2	pH 7.0	pH 5.2	pH 7.0
DIX-1	4.7 ± 0.2	4.3 ± 0.2	1.1 ± 0.1	1.9 ± 0.1
DIX-4	10.2 ± 0.4	7.7 ± 0.3	3.7 ± 0.2	3.7 ± 0.2
DIX-17	5.1 ± 0.3	4.7 ± 0.3	4.4 ± 0.2	3.5 ± 0.2
DIX-27	26.7 ± 1.2	22.2 ± 1.1	16.5 ± 0.7	10.1 ± 0.4
DIX-27B	83.8 ± 4.7	71.4 ± 3.8	> 100	> 100
DIX-28	6.5 ± 0.2	3.8 ± 0.2	2.6 ± 0.1[b]	1.7 ± 0.1[c]
1C9-DIX	6.8 ± 0.3[e]	3.9 ± 0.2	2.2 ± 0.1[d]	2.2 ± 0.1[d,e]

[a] Noncompetitive inhibitors (unless otherwise noted). [b] Mixed-type inhibitor ($\alpha = 3.6$). [c] Mixed-type inhibitor ($\alpha = 2.3$). [d] Data taken from Ref. [16]. [e] Competitive inhibitor.

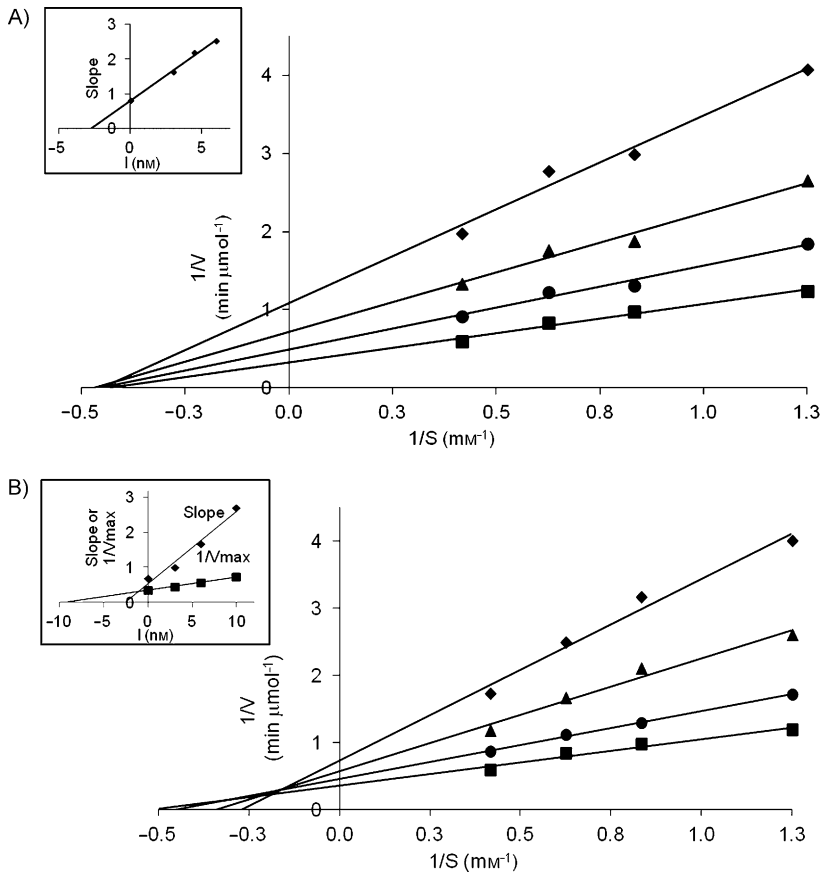


Figure 3. Kinetics for the inhibition of imiglucerase by DIX-17 and DIX-28. Double reciprocal plot of imiglucerase incubated at different concentrations of substrate and compounds. A) DIX-17: 0 μM (■); 3 μM (●); 4.5 μM (▲); 6 μM (◆). B) DIX-28: 0 μM (■); 3 μM (●); 6 μM (▲); 10 μM (◆). Regression lines arise from data obtained in two different experiments performed in triplicate.

Biological studies with purified compounds

The selected compounds were nontoxic in a wild-type (WT) fibroblasts MTT assay at concentrations up to 300 nM after 6 days incubation (Figure S1). The above compounds were next evaluated for their ability to enhance GBA1 residual activity in several GD genotypes (Figures 4 and 5, and Table 1; see also, Figures S2 and S3 in the Supporting Information). In particular, six different genotypes, together with WT fibroblasts, were used in this study (see Experimental Section).

1C9-DIX,[17] with a reported enhancement of N370S enzyme activity (1.6-fold at 10 nM), was used as a reference. In order to determine whether this compound was active against other mutant GBAs, we tested it on the fibroblasts used in this study. The best results were found for those bearing the genotypes G202R/[L444P; E326K] (genotype E) and G202R/G202R (F) (2–3-fold increase, at 10 nM). For N370S/N370S (C), an enhancement similar to that previously reported was observed (Figure 4).

DIX-28 exhibited the best activity enhancement, reaching a 2.5–3-fold increase for genotypes E and F at 10 nM (Figure 4) and up to 4–5-fold increase at 100 nM (Table S1 and Figure S2). This compound behaved similarly to 1C9-DIX at low concentrations (10 nM) but showed a wider enhancement window, as it was significantly more efficient than 1C9-DIX at higher concentrations (Figure 5). Considering that a relatively small amount of enzyme activity may be enough to avoid substrate accumulation,[35] the observed increase in activity could be clinically significant. Compound DIX-28 also showed a remarkable effect on the N370S/N370S genotype, close to that of 1C9-DIX at 10 nM (Figure 4) and even greater than that of 1C9-DIX at 100 nM (Table S1). This is relevant, considering the high prevalence of the N370S mutation, which is related to the non-neuronopathic GD type I.

In all cases, the selected DIX compounds showed preferential GBA1 enhancement towards genotypes E and F (Figure 4; see also Table S1 in the Supporting Information). Compound DIX-1 was the most potent member of the series toward the latter, with a twofold increase in activity at concentrations as low as 0.05 nM (Figure S2). This trend was found even for the weak inhibitor DIX-27B, which showed a modest activity enhancement at 100 nM concentration (Table S1 in the Supporting Information). As the concentration that gave a maximal activity enhancement differed for different compounds, a wide range of concentrations was analyzed for each product and genotype (Figure 5; see also Figures S2 and S3 in the Supporting Information).

It is worth noting that genotypes E and F contain the G202R mutation, which affects trafficking of the enzyme, precludes its transport to the lysosome,[36] and is associated with the neuronopathic phenotype of the disease. It has been reported that both the N370S and G202R mutations are located in the catalytic domain,[37] but the latter is located much farther from the active site than N370S. We found that, in most cases, compounds that increase the activity of N370S/N370S (genotype C) also increase the activity of genotypes containing the G202R mutation, as reported by others.[38] Moreover, the difference is greater in genotypes containing the G202R mutation than in the N370S/N370S genotype. Some authors suggest that, while the N370S mutation affects substrate binding and catalytic activity, the G202R mutation destabilizes GBA but does not disrupt the catalytic activity of the folded protein.[38] This could be the reason for the good results of several pharmacological chaperones toward the G202R mutation. In this regard, several compounds have been evaluated against this mutation with successful results, such as a DNJ analogue described by Sawkar

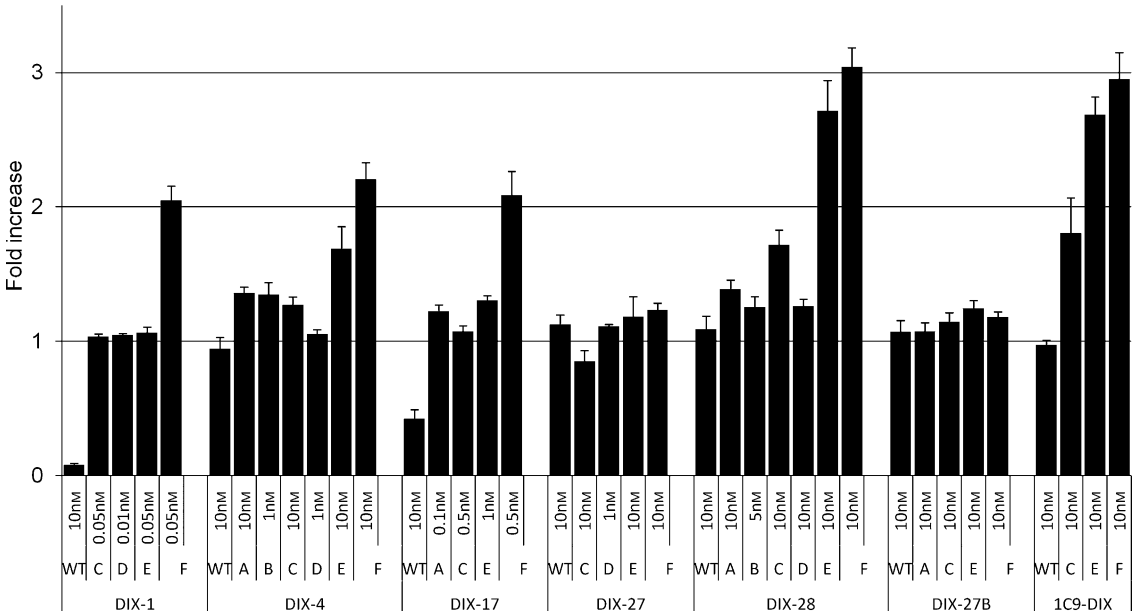


Figure 4. Enhancement of residual GBA1 activity of selected compounds in fibroblasts from GD patients. Fold increase: Increase of the residual GBA1 activity in GD fibroblasts in comparison with untreated cells at different DIX concentrations (for complete data, see Table S1 in the Supporting Information). Genotypes: WT: wild-type; A: [D409H;H255Q]/[D409H;H255Q]; B: [D409H;H255Q]/L444P; C: N370S/N370S; D: L444P/L444P; E: G202R/[L444P;E326K]; F: G202R/G202R. Values are the median ± confidence interval (CI) of 3–6 separate experiments performed in triplicate.

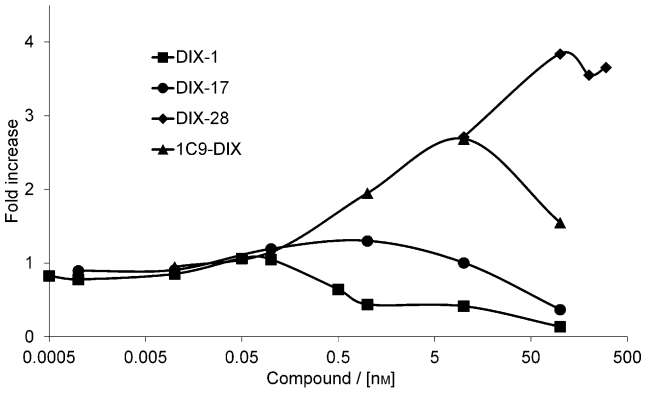


Figure 5. Activity enhancements (fold increase relative to untreated cells) for selected DIX compounds at different concentrations toward fibroblasts containing the G202R/[L444P;E326K] genotype.

et al.[38] with a 270% increase in activity, bicyclic nojirimycin analogues with sp^2 iminosugar structure[39] and approximately a 250% increase in activity, and an azepine analogue[40]]with a modest 20% increase in activity. The best results were obtained for isofagomine derivatives,[41] with a 7.2-fold increase in activity. However, it must be mentioned that this result was obtained at a concentration of 150 μM, which is above the threshold for clinical use.

Finally, none of the DIX compounds proved efficient against genotype D (Figure 4; see also Table S1 in the Supporting Information), and only a negligible effect was observed for some of the compounds against genotypes A and B. In the case of genotypes containing the L444P mutation, it is important to note that this mutation is not located at the catalytic domain of GBA and may be the reason why none of our compounds produced a significant enhancement in activity. In fact, examples of pharmacological chaperones able to target this mutation are scarce in the literature. Calystegine B_2 was the first to be reported on fibroblasts with the L444P/L444P genotype (230% activity increase at 10 μM)[11] and, more recently, some bicyclic iminocyclitols have shown promise as enzyme enhancers in induced pluripotent stem cell (iPSc) lines with the L444P/G202R genotype[42] and in monkey kidney fibroblast COS-7 cells expressing the L444P mutation.[43]

On the other hand, none of the DIX compounds showed appreciable GBA2 inhibition (in mouse testes homogenates) at concentrations up to 1 μM, which indicates an irrelevant effect on this enzyme. With regard to other lysosomal enzymes, compounds were inactive against the two human α-glycosidases tested, namely α-glucosidase and α-galactosidase. On the other hand, human β-galactosidase was slightly inhibited by compound DIX-17 (25% inhibition) at 10 μM, a concentration four orders of magnitude higher than required for the observed GBA1 activity enhancements (Table S2 in the Supporting Information). Finally, despite the fact that activity of human β-hexosaminidase was somehow affected by several of the tested compounds, no good concentration–inhibition correlation was observed. In summary, these compounds exhibited an excellent selectivity towards GBA1. With regard to other carbohydrate-processing enzymes, 1C9-DIX was recently described as an inhibitor of the cytosolic β-glucosidase GBA3.[18] However, as this enzyme does not appear to modify GD manifestations, no efforts along this line were carried out.

Immunofluorescence staining and confocal microscopy imaging were used to determine whether DIX-28 increased trafficking of the G202R mutant enzyme to the lysosome

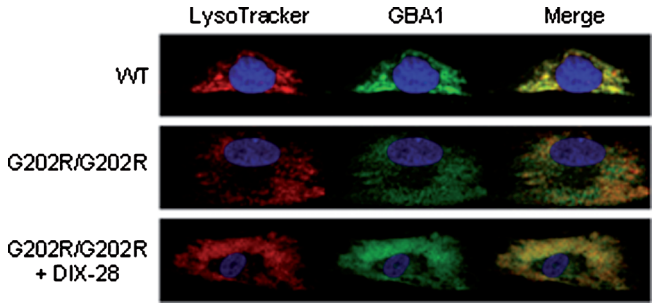


Figure 6. Confocal laser microscope images of immunofluorescence staining for lysosomes (red) and GBA1 (green) in cultured fibroblasts derived from a healthy individual (WT) and a GD patient with the G202R/G202R genotype untreated (middle) and after treatment with DIX-28 (bottom). Nuclei were stained with DAPI (blue). Immunofluorescent labeling of GBA1 was performed with the 8E4 monoclonal antibody, and LysoTracker was used to detect lysosomes.

(Figure 6). Fibroblasts from a Gaucher patient homozygous for the G202R mutation were incubated for 6 days with or without 100 nM of DIX-28. In treated cells, GBA colocalization of the mutant enzyme with a lysosomal marker (LysoTracker) clearly increased, as shown by higher stain intensity compared to untreated cells (Figure 6). In fact, the degree of colocalization following treatment was similar to that observed in wild-type fibroblasts (Figure 6). These results are in agreement with the idea that the G202R mutation does not disrupt the catalytic site. Thus, compound DIX-28 induced the correct folding and trafficking of the enzyme into the lysosome, as shown in Figure 6 and, once there, the enzyme could perform its role, as indicated by the activity results shown here (Figures 4 and 5; see also Table S1 in the Supporting Information).

Conclusions

A new series of α-1-C-substituted DIXs were designed by combination of the iminoxylitol scaffold present in parent 1C9-DIX with the triazolylalkyl side chains present in a series of aminocyclitols previously reported by our groups as GBA1 enhancers with application in GD. The resulting hybrid structures were synthesized using standard CuAAC click chemistry from scaffold **1** and were initially screened as imiglucerase inhibitors. In general, for identical side chain substitution, the DIX scaffold gives rise to appreciably more potent inhibitors than the corresponding aminocyclitol counterparts. The most potent members of the series were resynthesized and tested as GBA1 activity enhancers in fibroblasts from GD patients bearing different genotypes. In general, the DIX compounds reported here were shown to be potent GBA1 enhancers in genotypes containing the G202R mutation, which is responsible for a neuronopathic phenotype of the disease. In particular, 1C9-DIX and the silyl derivative DIX-28 showed approximately threefold activity enhancements at 10 nM, and four- to fivefold increases in activity at 100 nM (Table S1 in the Supporting Information). Moreover, these two compounds are also among the most potent members of the series toward the highly prevalent N370S mutation. Combining click chemistry and an in situ screening approach allowed identification of the 3-trimethylsilylpropyl group as a promising surrogate of long alkyl chains that are known to induce cytotoxicity.[34] Interestingly, incorporation of this group into DIX-28 gives rise to a compound with low cytotoxicity exhibiting a remarkable GBA1 enhancement. Regardless of their structural similarities with IFG and with our previously reported aminocyclitol analogues,[23] the present DIX compounds behaved as non-competitive inhibitors, with the exception of the mixed-type inhibitor DIX-28. However, even though our compounds were inspired by the common "active site-directed ligand strategy" that has classically guided the design of pharmacological chaperones,[6] our results represent an interesting breakthrough that deserves further attention.

Experimental Section

Chemistry

General: CH_2Cl_2 was distilled over CaH_2 under argon. MeOH was distilled over Mg/I_2 under argon. Tetrahydrofuran (THF) was dried by passage through an activated alumina column under argon. Flash column chromatography was carried out using silica gel 60 (230-400 mesh, 0.040–0.063 mm) purchased from Merck. Automatic flash chromatography was carried out in a Grace Reveleris flash system equipped with UV/Vis and ELSD detectors. Thin-layer chromatography (TLC) was performed on aluminum sheets coated with silica gel 60 F_{254} purchased from Merck. IR spectra (cm^{-1}) were recorded on a Perkin–Elmer SpectrumOne spectrophotometer. NMR spectra were recorded on a Bruker AC 300 or Bruker 1C 400 spectrometer, with residual solvent peaks used as reference. Carbon multiplicities were assigned by distortionless enhancement by polarization transfer (DEPT) experiments. ^1H and ^{13}C signals were assigned by COSY (correlation spectroscopy), HSQC (heteronuclear single quantum correlation spectroscopy), and HMBC (heteronuclear multiple-bond correlation spectroscopy) experiments. Coupling constants (*J*) are in Hertz (Hz), and multiplicity is described as (s) singlet, (d) doublet, (t) triplet, (q) quadruplet, and (br) broad. Electrospray ionization (ESI)-high-resolution mass spectrometry (HRMS) mass spectrometry was carried out on a Bruker MicroTOF spectrometer. Specific rotations were determined at room temperature (20 °C) in a PerkinElmer 241 polarimeter for sodium ($\lambda =$ 589 nm). Purity of compounds used for enzyme assays was $\geq$ 95%, as judged by HPLC analysis (Gemini C18 column, 4.6 mm × 250 mm, 5 μm) under the following chromatographic conditions: mobile phase A, water containing 0.1% *v/v* trifluoroacetic acid (TFA); mobile phase B, CH_3CN containing 0.1% *v/v* TFA; flow rate of 1.0 mL min^{-1}; detection, SATIN-ELS (evaporative light scattering) ($\lambda =$ 254 nm); gradient elution, 0 min, from 80% A/20% B to 0% A/100% B over 20 min. Each run was followed by a 3 min wash with 80% CH_3CN and 20% water.

α-1-*C*-Propargyl-2,3,4-tri-*O*-benzyl-1,5-dideoxy-1,5-*tert*-butoxycarboxylimino-D-xylitol (4): OsO_4 (2.5% *w/w* in *t*BuOH, 0.058 mmol, 590 μL, 10% mol) was added to a solution of **3** (313 mg, 0.576 mmol) in THF/water (1:1, 7.4 mL), followed by addition of $NaIO_4$ (246 mg, 1.151 mmol, 2 equiv). The mixture was stirred for 3 h at RT, then water (3 mL) was added to the reaction. The aqueous phase was extracted with CH_2Cl_2 (3 × 10 mL). The extracts were combined and dried over Na_2SO_4. The solution was filtered, and the solvent was removed in vacuo. The crude aldehyde (320 mg) was used in the next step without purification. This residue was dissolved in MeOH (9 mL), and K_2CO_3 (159 mg,

1.152 mmol, 2 equiv) was added. Then, Bestmann–Ohira reagent (104 μL, 0.691 mmol, 1.2 equiv) was added, and the solution was stirred for 5 h at RT. Et_2O (20 mL) and a solution of $NaHCO_3$ (5%, 15 mL) were added. The aqueous phase was extracted with Et_2O (3×15 mL), and the extracts were combined and dried over Na_2SO_4. The solution was filtered and concentrated in vacuo. The residue was purified by automatic flash column chromatography (EtOAc/petroleum ether, 0:1 to 1:4) to afford a **4** as a colorless oil as a mixture of rotamers, according to NMR data (212 mg, 68% over two steps): $R_f = 0.52$ (EtOAc/petroleum ether, 1:3); $[\alpha]_D^{20} = -14.0$ ($c = 1$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.45$ (s, 9H), 1.94 (br s, 1H), 2.50 (ddd, $J = 17.5$, 11.0 and 2.4 Hz, 1H), 2.61–2.85 (m, 2H), 3.34–3.70 (m, 3H, H-2), 4.13 (br dd, $J = 13.5$ and 5.3 Hz, 0.5H), 4.42–4.58 (m, 1H), 4.60–4.78 (m, 4H), 4.78–4.98 (m, 2.5H), 7.27–7.40 ppm (m, 15H); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 16.1$, 28.4, 40.0; 41.5, 50.8, 52.4, 70.3, 72.9, 73.2, 73.3, 75.7, 75.8, 77.4, 78.3, 78.4, 79.0, 79.4, 80.5, 80.7, 81.9, 82.0, 127.7, 127.88, 128.97, 128.02, 128.4, 128.6, 138.1, 138.3, 138.9, 154.8 ppm; IR (neat): $\tilde{\nu} = 1694$ cm^{-1} (C=O); HRMS (ESI): m/z [M+Na$^+$] calcd for $C_{34}H_{39}NNaO_5$: 564.272, found: 564.271.

α-1-*C*-Propargyl-1,5-dideoxy-1,5-imino-D-xylitol (1) and α-1-*C*-propargyl-2-*O*-benzyl-1,5-dideoxy-1,5-imino-D-xylitol (5): Pentamethylbenzene (1.790 g, 12.07 mmol, 15 equiv) was added to a solution of **4** (436 mg, 0.805 mmol) in CH_2Cl_2 (16 mL). Then, BCl_3 (7.24 mL, 7.24 mmol, 9 equiv) was added dropwise at −78 °C, and the mixture was stirred for 4 h. MeOH/H_2O (1:20, 2 mL) was added at −78 °C, and the mixture was evaporated to dryness. This step was repeated twice, and the residue was purified by column chromatography (CH_3CN/H_2O/NH_4OH, 15:0.5:0.5 to 10:0.5:0.5). Monobenzylated compound **5** eluted first and was obtained as a colorless oil (14.5 mg, 7%): $R_f = 0.45$ (CH_3CN/H_2O/NH_4OH, 10:0.5:0.5); $[\alpha]_D^{20}$ −22.0 ($c = 0.33$, MeOH); ^{1}H NMR (400 MHz, MeOD): $\delta = 2.36$ (t, $J = 2.6$ Hz, 1H), 2.42 (ddd, $J = 16.8$ Hz, 7.8 and 2.6 Hz, 1H), 2.48 (ddd, $J = 16.8$ Hz, 7.4 and 2.5 Hz, 1H), 2.78 (dd, $J = 13.2$ and 5.1 Hz, 1H), 3.03 (dd, $J = 13.1$ and 3.3 Hz, 1H), 3.22 (td, $J = 7.6$ and 3.3 Hz, 1H), 3.52 (br q, $J = 5.0$ Hz, 1H), 3.63 (dd, $J = 5.0$ and 3.4 Hz, 1H), 3.85 (t, $J = 5.2$ Hz, 1H), 4.62 (d, $J = 11.3$ Hz, 1H), 4.74 (d, $J = 11.3$ Hz, 1H), 7.25–7.38 (m, 3H), 7.38–7.45 ppm (m, 2H); ^{13}C (100 MHz, MeOD): $\delta = 19.8$, 47.1, 54.8, 69.8, 70.7, 71.8, 74.1, 78.8, 81.7, 128.9, 129.39, 129.44, 139.4 ppm; IR (neat): $\tilde{\nu} = 3291$ cm^{-1} (O–H); HRMS (ESI): m/z [M+H$^+$] calcd for $C_{15}H_{20}NO_3$: 262.144, found: 262.145. The second fraction was constituted of compound **1** obtained as a white solid (92.5 mg, 67%): $R_f = 0.29$ (CH_3CN/H_2O/NH_4OH, 10:0.5:0.5); $[\alpha]_D^{20} = -9.0$ ($c = 1$, MeOH); ^{1}H NMR (400 MHz, MeOD): $\delta = 2.31$ (t, $J = 2.6$ Hz, 1H), 2.36 (ddd, $J = 16.6$ Hz, 7.4 and 2.5 Hz, 1H), 2.46 (ddd, $J = 16.6$ Hz, 7.8 and 2.5 Hz, 1H), 2.80 (dd, $J = 13.3$ and 4.1 Hz, 1H), 3.05 (dd, $J = 13.3$ and 2.7 Hz, 1H), 3.09 (td, $J = 7.5$ and 2.6 Hz, 1H), 3.55 (br qd, $J = 4.1$ and 1.0 Hz, 1H), 3.69-3.73 (m, 1H), 3.76 ppm (t, $J = 4.5$ Hz, 1H); ^{13}C NMR (100 MHz, MeOD): $\delta = 20.5$, 47.5, 55.4, 70.4, 71.2, 71.3, 71.3, 82.0 ppm; IR (neat): $\tilde{\nu} = 3282$ cm^{-1} (O–H); HRMS (ESI): m/z [M+H$^+$] calcd for $C_8H_{14}NO_3$: 172.097, found: 172.096.

α-1-*C*-Propargyl-2,3,4-tri-*O*-benzyl-1,5-dideoxy-1,5-imino-D-xylitol (6): TFA (1 mL) was added to a solution of **4** (113 mg, 0.21 mmol) in CH_2Cl_2 (4 mL). After stirring for 1 h at RT, the reaction mixture was evaporated to dryness. The residue was dissolved in CH_2Cl_2 (10 mL) and washed with a 5% aq solution of $NaHCO_3$ (10 mL). The aqueous phase was extracted with CH_2Cl_2 (3×10 mL), and the extracts were combined and dried over Na_2SO_4. The solution was filtered and evaporated to afford **6** (86 mg, 93%) of sufficient purity, as judged by ^{1}H NMR, to be used directly in the next CuAAC step: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.01$ (t, $J = 2.7$ Hz, 1H), 2.20 (br s, 1H), 2.40–2.58 (m, 2H), 2.90 (dd, $J = 13.1$ and 6.2 Hz, 1H), 3.05 (dd, $J = 13.2$ and 4.0 Hz, 1H), 3.27 (td, $J = 7.7$ and 3.4 Hz, 1H), 3.45 (br q, $J = 5.1$ Hz, 1H), 3.61–3.72 (m, 2H), 4.57 (d, $J = 11.7$ Hz, 1H), 4.59 (s, 2H), 4.65 (d, $J = 11.5$ Hz, 1H), 4.66 (s, 2H), 7.22–7.39 ppm (m, 15H).

α-1-*C*-((1-(3,3-Diphenylpropyl)-1*H*-1,2,3-triazol-4-yl)-methyl)-2,3,4-tri-*O*-benzyl-1,5-dideoxy-1,5-imino-D-xylitol (7): $CuSO_4{\cdot}5H_2O$ (2.0 mg, 0.008 mmol, 0.1 equiv) and sodium ascorbate (3.2 mg, 0.016 mmol, 0.2 equiv), dissolved in water (1 mL), was added to a solution of **6** (35.5 mg, 0.080 mmol) and 1,1-diphenyl-3-azidopropane (26.7 mg, 0.113 mmol, 1.4 equiv) in THF (1 mL). The mixture was stirred overnight at RT. The mixture was diluted with EtOAc (5 mL), and the phases were separated. The organic phase was washed with a 10% aq solution of NH_4OH (5 mL) and then dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 1:0 to 95:5) to afford **7** as a pale yellow oil (43 mg, 79%): $R_f = 0.44$ (CH_2Cl_2/MeOH, 95:5); $[\alpha]_D^{20} = -5.0$ ($c = 1$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.12$ (br s, 1H), 2.78 (q, $J = 7.4$ Hz, 2H), 2.98-3.20 (m, 4H), 3.48–3.55 (m, 1H), 3.55-3.64 (m, 1H), 3.68 (dd, $J = 6.3$ and 3.8 Hz, 1H), 3.89 (t, $J = 6.0$ Hz, 1H), 4.02 (t, $J = 8$ Hz, 1H), 4.36 (t, $J = 7.2$ Hz, 2H), 4.68 (d, $J = 11.5$ Hz, 1H), 4.74 (s, 2H), 4.77–4.87 (m, 3H), 7.28–7.57 ppm (m, 26H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 24.7$, 36.0, 44.5, 48.3, 48.7, 55.2, 72.1, 72.5, 74.1, 76.8, 77.4, 78.1, 122.0, 126.8, 127.68, 127.70, 127.73, 127.8, 127.86, 127.87, 127.93, 128.1, 128.4, 128.5, 128.9, 138.6, 138.7, 143.3, 145.8 ppm; IR (neat): $\tilde{\nu} = 3260$ cm^{-1} (N–H); HRMS (ESI): m/z [M+H$^+$] calcd for $C_{44}H_{47}N_4O_3$: 679.364, found: 679.365.

α-1-*C*-(1-Benzyl-1*H*-1,2,3-triazol-4-yl)-methyl-2,3,4-tri-*O*-benzyl-1,5-dideoxy-1,5-imino-D-xylitol (8): $CuSO_4{\cdot}5H_2O$ (2.3 mg, 0.010 mmol, 0.1 equiv) and sodium ascorbate (3.7 mg, 0.020 mmol, 0.2 equiv), dissolved in water (1 mL), were added to a solution of **6** (42 mg, 0.095 mmol) and benzylazide (16.5 mg, 0.124 mmol, 1.3 equiv) in THF (1 mL). The mixture was stirred overnight at RT. The mixture was diluted with EtOAc (5 mL), and the phases were separated. The organic phase was washed with a 10% aq solution of NH_4OH (5 mL) and then dried over Na_2SO_4, filtered and evaporated. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 1:0 to 95:5) to afford **8** as a pale yellow oil (47 mg, 86%): $R_f = 0.39$ (CH_2Cl_2/MeOH, 95:5); $[\alpha]_D^{20} = -3.0$ ($c = 1$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.07$ (br s, 1H), 2.96–3.15 (m, 4H), 3.43–3.59 (m, 2H), 3.62 (dd, $J = 6.3$ and 4.0 Hz, 1H), 3.83 (t, $J = 6.2$ Hz, 1H), 4.60 (d, $J = 11.7$ Hz, 1H), 4.69 (s, 2H,), 4.71–4.79 (m, 3H), 5.52 (d, $J = 15.0$ Hz, 1H), 5.58 (d, $J = 14.8$ Hz, 1H), 7.28–7.54 ppm (m, 21H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 24.7$, 44.4, 54.1, 55.2, 72.1, 72.4, 74.0, 76.8, 77.4, 78.1, 121.7, 127.6, 127.70, 127.73, 127.86, 127.92, 128.0, 128.10, 128.15, 128.39, 128.44, 128.7, 129.1, 135.0, 138.6, 138.7, 146.4 ppm; IR (neat): $\tilde{\nu} = 3295$ cm^{-1} (N–H); HRMS (ESI): m/z [M+H$^+$] calcd for $C_{36}H_{39}N_4O_3$: 575.302, found: 575.301.

α-1-*C*-((1-(3,3-Diphenylpropyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1,5-dideoxy-1,5-imino-D-xylitol (DIX-4): BCl_3 (1 M in CH_2Cl_2, 0.4 mL, 0.4 mmol, 6 equiv) was added dropwise to a solution of **7** (44 mg, 0.065 mmol) in CH_2Cl_2 (2.5 mL) at −60 °C. The solution was allowed to warm to RT and was stirred overnight. Then, MeOH/H_2O (20:1, 3 mL) was added, and the solution was evaporated to dryness. This step was repeated, and the residue was purified by column chromatography (CH_3CN/NH_4OH/H_20, 15:0.5:0.5) to afford DIX-4 as a white solid (21 mg, 80%): $R_f = 0.29$ (CH_3CN/NH_4OH/H_2O, 10:1:1); $[\alpha]_D^{20} = -6.5$ ($c = 1$, MeOH); ^{1}H NMR (300 MHz, MeOD): $\delta = 2.69$ (q, $J = 7.4$ Hz, 2H), 3.11 (dd, $J = 14.8$ and 7 Hz, 1H), 3.16–3.30 (m, 2H), 3.44 (dd, $J = 13.2$ and 2.0 Hz, 1H), 3.70–3.82 (m, 2H), 3.88–4.01 (m, 3H), 4.33 (t, $J = 7.2$ Hz, 2H), 7.09–7.23 (m, 3H), 7.23–7.37 (m, 7H),

7.82 ppm (s, 1H); ^{13}C NMR (75 MHz, MeOD): δ = 25.9, 36.8, 47.5, 49.6, 50.1, 56.3, 67.8, 68.0, 69.6, 125.0, 127.6, 128.8, 129.7, 142.9, 145.0 ppm; IR (neat): $\tilde{\nu}$ = 3330, 3025 cm^{-1} (O–H and N–H); HRMS (ESI): *m/z* [*M*+H$^+$] calcd for $C_{23}H_{29}N_4O_3$: 409.225, found: 409.223.

α-1-*C*-((1-Benzyl-1*H*-1,2,3-triazol-4-yl)-methyl)-1,5-dideoxy-1,5-imino-D-xylitol (DIX-27) and α-1-*C*-((1-benzyl-1*H*-1,2,3-triazol-4-yl)-methyl)-2-*O*-benzyl-1,5-dideoxy-1,5-imino-D-xylitol (DIX-27B): BCl_3 (1 M in CH_2Cl_2, 0.5 mL, 0.50 mmol, 6 equiv) was added dropwise to a solution of **8** (47 mg, 0.082 mmol) in CH_2Cl_2 (0.5 mL) at −60 °C. The mixture was allowed to warm to 0 °C over 3 h. Then, MeOH/H_2O (20:1, 3 mL) was added and, after 10 min of stirring at 0 °C, the solvent was evaporated. This step was repeated, and the residue was purified by column chromatography (CH_3CN/NH_4OH/H_2O, 15:0.5:0.5 to 10:0.5:0.5). Monobenzylated compound DIX-27B was eluted first (19 mg, 59%): $[\alpha]_D^{20}$ = −2.0 (*c* = 1, MeOH); ^{1}H NMR (300 MHz, MeOD): δ = 2.80-3.03 (m, 3H), 3.10 (dd, *J* = 13.6 and 2.2 Hz, 1H), 3.26-3.37 (m, 1H), 3.55-3.60 (m, 1H), 3.64 (br t, *J* = 3.4 Hz, 1H), 3.74–3.79 (m, 1H), 4.52 (d, *J* = 11.9 Hz, 1H), 4.57 (d, *J* = 12.2 Hz, 1H), 5.56 (s, 2H), 7.18–7.41 ppm (m, 10H), 7.76 (s, 1H); ^{13}C NMR (75 MHz, MeOD): δ = 27.6, 48.3, 54.9, 56.2, 67.7, 68.6, 73.3, 77.2, 124.4, 128.8, 128.9, 129.1, 129.4, 129.5, 130.0, 136.8, 139.5, 145.8 ppm; IR (neat): $\tilde{\nu}$ = 3276, 3032, 2918 cm^{-1} (O–H, N–H); HRMS (ESI): *m/z* [*M*+H$^+$] calcd for $C_{22}H_{27}N_4O_3$: 395.208, found: 395.207. The second fraction was constituted of DIX-27, obtained as a pale yellow oil (10 mg, 40%): $[\alpha]_D^{20}$ = −4.0 (*c* = 0.55, MeOH); ^{1}H NMR (300 MHz, MeOD): δ = 3.11 (dd, *J* = 15.1 and 6.9 Hz, 1H), 3.17–3.28 (m, 2H), 3.43 (dd, *J* = 13.1 and 1.8 Hz, 1H), 3.70–3.74 (m, 1H), 3.78 (br td, *J* = 8.4 and 1.8 Hz, 1H), 3.90–3.99 (m, 2H), 5.59 (s, 2H), 7.28–7.43 (m, 5H), 7.92 ppm (s, 1H); ^{13}C (75 MHz, MeOD): δ = 25.9, 47.5, 55.0, 56.3, 67.8, 68.0, 69.6, 124.9, 129.3, 129.6, 130.0, 136.7, 143.3 ppm (C-7); IR (neat): $\tilde{\nu}$ = 3240, 3124, 3001 cm^{-1} (O–H, N–H); HRMS (ESI): *m/z* [*M*+H$^+$] calcd for $C_{15}H_{21}N_4O_3$: 305.161, found: 305.160.

General method for CuAAC from scaffold 1

Iminoxylitol **1** and the corresponding azide (1.3 equiv) were solubilized in THF (1 mL). Next, $CuSO_4$·5H_2O (2.5 mg, 0.010 mmol, 0.1 equiv) and sodium ascorbate (0.2 equiv) in water (1 mL) were successively added. The mixture was stirred overnight at RT, then the solvents were evaporated to dryness. The crude product was filtered through a plug of Celite and purified by flash chromatography on silica gel (CH_3CN/NH_4OH/H_2O, 15:0.5:0.5).

α-1-*C*-((1-(3,5-bis(Benzyloxy)benzyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1,5-dideoxy-1,5-imino-D-xylitol (DIX-1): Following the general method, iminoxylitol **1** (16 mg, 0.093 mmol) was combined with 1-azidomethyl-3,5-bis(benzyloxy)benzene[23] (42 mg, 0.122 mmol) to afford DIX-1 as a white solid (37.5 mg, 78%): R_f = 0.12 (CH_3CN/NH_4OH/H_2O, 10:0.5:0.5): $[\alpha]_D^{20}$ = −3.0 (*c* = 0.17, MeOH); ^{1}H NMR (300 MHz, MeOD): δ = 2.99 (dd, *J* = 14.7 and 7.2 Hz, 1H), 3.03–3.15 (m, 2H), 3.22–3.30 (m, 1H), 3.57 (td, *J* = 7.4 and 2.2 Hz, 1H), 3.61–3.65 (m, 1H), 3.73–4.81 (m, 1H), 3.87 (t, *J* = 3.8 Hz, 1H), 5.03 (s, 4H), 5.47 (s, 2H), 6.53–6.58 (m, 2H), 6.59–6.62 (m, 1H), 7.23–7.44 (m, 10H), 7.78 ppm (s, 1H); ^{13}C NMR (75 MHz, MeOD): δ = 26.4, 47.5, 54.9, 56.2, 69.0, 69.3, 70.4, 71.2, 103.1, 108.4, 124.7, 128.6, 128.9, 129.5, 138.4, 138.8, 144.6, 161.8 ppm; IR (neat): $\tilde{\nu}$ = 3253 cm^{-1} (O–H and N–H); HRMS (ESI): *m/z* [*M*+H$^+$] calcd for $C_{29}H_{33}N_4O_3$: 517.245, found: 517.244.

α-1-*C*-((1-Decyl-1*H*-1,2,3-triazol-4-yl)methyl)-1,5-dideoxy-1,5-imino-D-xylitol (DIX-17): Following the general method, iminoxylitol **1** (18 mg, 0.105 mmol) was combined with 1-azidodecane[23] (25 mg, 0.137 mmol) to afford DIX-17 as a white solid (22.2 mg, 60%): R_f = 0.22 (CH_3CN/NH_4OH/H_2O, 10:1:1); $[\alpha]_D^{20}$ = −8.0 (*c* = 1, MeOH); ^{1}H NMR (300 MHz, MeOD): δ = 0.90 (t, *J* = 6.8 Hz, 2H), 1.17–1.46 (m, 14H), 1.80–197 (m, 2H), 3.12 (dd, *J* = 14.8 and 6.6 Hz, 1H), 3.17–3.29 (m, 2H), 3.44 (dd, *J* = 13.1 and 2.0 Hz, 1H), 3.71–3.74 (m, 1H), 3.78 (td, *J* = 7.3 and 1.7 Hz, 1H), 3.90–4.01 (m, 2H), 4.39 (t, *J* = 7.1 Hz, 2H), 7.91 ppm (s, 1H); ^{13}C NMR (75 MHz, MeOD): δ = 14.4, 23.7, 25.9, 27.5, 30.1, 30.4, 30.5, 30.6, 31.3, 33.0, 47.5, 51.4, 56.3, 67.9, 68.0, 69.6, 124.8, 142.9 ppm; IR (neat): $\tilde{\nu}$ = 3348 cm^{-1} (O–H and N–H); HRMS (ESI): *m/z* [*M*+H$^+$] calcd for $C_{18}H_{35}N_4O_3$: 355.271, found: 355.270.

α-1-*C*-((1-(3-(Trimethylsilyl)propyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1,5-dideoxy-1,5-imino-D-xylitol (DIX-28): Following the general method, iminoxylitol **1** (17 mg, 0.099 mmol) was combined with 3-azidopropyl)trimethylsilane[44] (20.3 mg, 0.129 mmol) to afford DIX-28 as a white solid (21.6 mg, 66%): R_f = 0.43 (CH_3CN/NH_4OH/H_2O, 10:1:1); $[\alpha]_D^{20}$ = −7.5 (*c* = 1, MeOH); ^{1}H NMR (300 MHz, MeOD): δ = 0.0 (s, 9H), 0.44–0.56 (m, 2H), 1.83–1.98 (m, 2H), 3.12 (dd, *J* = 14.9 and 6.7 Hz, 1H), 3.18–3.39 (m, 2H), 3.45 (dd, *J* = 13.1 and 1.9 Hz, 1H), 3.71–3.76 (m, 1H), 3.79 (td, *J* = 7.5 and 1.5 Hz, 1H), 3.90–4.01 (m, 2H), 4.37 (t, *J* = 7.1 Hz, 2H), 7.92 ppm (s, 1H); ^{13}C NMR (75 MHz, MeOD): δ = −1.9, 14.3, 25.9, 26.3, 47.5, 54.4, 56.4, 67.8, 68.0, 69.6, 124.9, 142.8 ppm; IR (neat): $\tilde{\nu}$ = 3336, 3022 (O–H and N–H) cm^{-1}; HRMS (ESI): *m/z* [*M*+H$^+$] calcd for $C_{14}H_{29}N_4O_3Si$: 329.201, found: 329.200.

General method for parallel click chemistry and direct screening: A solution of iminoxylitol **1** (8 mg, 0.047 mmol) and the corresponding azides [1]–[29] (0.055 mmol) in a mixture of H_2O/THF (1:1, 1 mL) in a 5 mL screw cap vial was treated with a catalytic amount of $CuSO_4$·5H_2O (around 250 μg), followed by sodium ascorbate (5 μmol, around 1 mg). After stirring for 24 h at RT, an aliquot was analyzed (UPLC-HRMS) to confirm click adduct formation. The crude reaction mixtures containing compounds DIX were used directly as mother solutions (47 mM) for in vitro IC_{50} calculation of imiglucerase inhibition (Table 1).

In vitro activity was determined as previously reported.[24] IC_{50} values were determined by plotting percent activity versus log [*I*], using at least five different inhibitor concentrations. Type of inhibition and K_i values for the most active inhibitors were determined by Lineweaver–Burk and Dixon plots of assays performed with different concentrations of inhibitor and substrate. IC_{50} data from crude click chemistry mixtures are reported in Table 1. Data from individually synthesized compounds are reported in Table 2.

Biological evaluation

Fibroblast culture assay: Skin fibroblasts were obtained from non-neurological (type 1) and neurological (types 2 and 3) GD patients with distinct genotypes, which were diagnosed, in most cases (genotypes: N370S/N370S, L444P/L444P, [D409H;H255Q]/[D409H;H255Q], G202R/G202R, [D409H;H255Q]/L444P), at the Institute of Child Health, Athens. In one case (G202R/[L444P;E326K]), diagnosis was performed at the Institut de Bioquímica Clínica, Barcelona. Fibroblasts from healthy individuals were used as controls. Fibroblast cultures were established following routine procedures in Dulbecco's modified Eagle's medium (DMEM) with 10% inactivated fetal bovine serum (FBS).

Lysosomal glucocerebrosidase assay (GBA1): For the assay of GBA1 in intact cells, 10000 cells were plated into 24-well assay plates over 6 days in DMEM with 10% FBS serum at 37 °C under 5% CO_2, either with or without DIX compounds at different concentrations. Culture media was replaced at day 3 with fresh media supplement-

ed with the corresponding compound dissolved in DMSO. Cells were washed, and the enzyme assay was performed as follows: substrate (100 μL, 5 mM 4MU-β-glucopyranoside) in 0.1 M acetate buffer (pH 5.2) was added to each well, up to a total volume of 260 μL, then the plates were incubated for 1 h at 37 °C. The enzyme reaction was stopped with 2 mL of 100 mM glycine/NaOH, pH 10.7, and the released fluorescence was measured (λ_{ex}: 355 nm; λ_{em}:460 nm). For each experiment, untreated (no compound added) and treated cells were plated in quadruplicate. Nonspecific GBA1 activity was evaluated by addition of CBE (2 h 0.5 mM) to control wells and was shown to account for about 1% of the activity in control fibroblasts.

Non-lysosomal glucocerebrosidase assay (GBA2): The effect of DIX compounds on GBA2 activity was determined in mouse testes homogenates, following a procedure described by Walden et al.[45] with modifications. Homogenates were prepared in 50 mM potassium phosphate buffer, pH 5.8 (1:3; *w/v*) using a manual glass homogenizer and were centrifuged for 15 min at 13500 rpm at 4 °C (Beckman J2-21). The pellets were washed three times in phosphate buffer and resuspended in the same buffer (1:1; *w/v*). The concentration was adjusted to 1 mg mL^{-1} of protein, and the aliquots were stored at −80 °C. For the GBA2 assay, aliquots of mouse testes homogenates were pre-incubated at room temperature with conduritol β-epoxide (CBE) (Toronto Research Chemicals, Downsview, ON, Canada) at a final concentration of 2.5 mM for 30 min. Then, 20 μL of homogenates and 5 μL of 50 mM potassium phosphate buffer (pH 5.8), supplemented with products to the desired concentration, were incubated at 37 °C for 15 min. Then, 15 μL of 4-metylumbelliferyl-β-D-glucoside (Sigma) was added to a final concentration of 3 mM, and the mixture was incubated at 37 °C for 120 min. The reaction was stopped by adding 100 μL of 100 mM glycine/NaOH, pH 10.7, and the released fluorescence was measured (λ_{ex}: 355 nm; λ_{em}:460 nm).

Cytotoxicity assay: The cytotoxicity of the selected compounds and the cell viability over a period of 6 days were tested by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay[23] (see Supporting Information).

Immunofluorescence staining and confocal microscopy imaging: For immunofluorescence, 5000 cells per well were seeded overnight on 10 mm glass coverslips (Marienfeld) in 24-well plates. The cells were then incubated for 6 days in fresh medium (DMEM, 10% FBS) at 37 °C under 5% CO_2 in the absence or presence of 100 nM of DIX-28. Culture media was replaced every 3 days with fresh media supplemented with 100 nM of DIX-28. The untreated cells were incubated with 0.1% DMSO. On day 6 of treatment, the cells were incubated for 1 h with 75 nM LysoTracker Red DND-99 (L7528; Invitrogen) at 37 °C. Then, the cells were washed twice with phosphate-buffered saline (PBS) and fixed for 10 min with 4% paraformaldehyde at room temperature. The cover slips were then washed 4 times for 5 min each with 0.3 M PBS, and the cells were permeabilized with 0.1% Tween in PBS. The cells were then incubated with primary antibody (mouse monoclonal anti-GBA 8E4, diluted 1:100)[46] in a solution of PBS with 10% NDS (normal donkey serum, Millipore) and 0.1% Tween at 4 °C overnight. The coverslips were then washed three times with 0.3 M PBS and incubated for 1 h with secondary antibody anti-mouse (Cy2-conjugated AffinPure donkey anti-mouse IgG (H+L), diluted 1:100, Jackson Immuno Research Laboratories, Inc.) followed by DAPI staining (1:10000 dilution in a solution of PBS with 10% NDS and 0.1% Tween for 10 min; Invitrogen). Staining was viewed with a Leica TCS-SP2, and the images were analyzed using Fiji-Image J software.

Statistical analysis

For all measures, the hypothesis of normality was rejected, and the nonparametric Mann-Whitney U test was used. Normal distribution was assessed by Kolmogorov-Smirnov test. The SPSS statistical program was used for statistical analysis.

Supporting Information

Toxicity of DIX compounds (MTT assay) and GBA1 enhancements by DIX compounds on G202R/G202R, N370S/N370S, and GBA1 enhancements by DIX compounds on different genotypes. Copies of ^{13}C and ^{1}H NMR spectra for DIX compounds (19 pages).

Abbreviations

CuAAC: Cu-promoted alkyne-azide cycloaddition; DAPI: 4′,6-diamidino-2-phenylindole; DIX: 1,5-dideoxy-1,5-imino-D-xylitol; ER: endoplasmic reticulum; GBA1: β-glucocerebrosidase; GD: Gaucher disease; GCase: β-glucocerebrosidase.

Acknowledgements

This work was supported by the Institut Universitaire de France (IUF), the French National Center for Scientific Research (CNRS), the University of Strasbourg (France), the French National Agency for Research (ANR) (grant no. 11-BS07-003-02), the Spanish Ministry of Science and Innovation (MICINN) (grant SAF 2011-25431), and the Generalitat de Catalunya, Spain (grants 2009SGR-1072 and 2009SGR-971). The following fellowships are also gratefully acknowledged: a doctoral fellowship from the French Department of Research to C.D, a FI predoctoral fellowship from the Generalitat de Catalunya to J.S.-V., and a JAE-predoctoral fellowship from the Consejo Superior de Investigaciones Científicas (CSIC), Spain to L.D. The "CIBER de Enfermedades Raras" is an initiative of the Instituto de Salud Carlos III (Spain). The authors thank the Institut de Bioquímica Clínica (Barcelona, Spain) for collaboration, and Genzyme Corporation (Cambridge, MA, USA) for a generous supply of imiglucerase (Cerezyme).

Keywords: activity enhancement · chaperones · click chemistry · Gaucher disease · glucosyl ceramide · iminoxylitol

[1] F. M. Platt, B. Boland, A. C. van der Spoel, *J. Cell Biol.* **2012**, *199*, 723 – 734.
[2] E. Beutler, G. A. Grabowski in *The Metabolic and Molecular Bases of Inherited Diseases* (Eds.: C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle), McGraw-Hill, New York, **2001**, pp. 3635 – 3668.
[3] F. M. Platt, R. H. Lachmann, *Biochim. Biophys. Acta - Mol. Cell Res.* **2009**, *1793*, 737 – 745.
[4] M. Beck, *IUBMB Life* **2010**, *62*, 33 – 40.
[5] S. S. Vembar, J. L. Brodsky, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 944 – 957.
[6] D. Ringe, G. A. Petsko, *J. Biol.* **2009**, *8*, 80.
[7] R. A. Denny, L. K. Gavrin, E. Saiah, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 1935 – 1944.
[8] I. Bendikov-Bar, G. Maor, M. Filocamo, M. Horowitz, *Blood Cells Mol. Dis.* **2013**, *50*, 141 – 145.
[9] A. R. Sawkar, W. C. Cheng, E. Beutler, C. H. Wong, W. E. Balch, J. W. Kelly, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15428 – 15433.
[10] R. E. Boyd, G. Lee, P. Rybczynski, E. R. Benjamin, R. Khanna, B. A. Wustman, K. J. Valenzano, *J. Med. Chem.* **2013**, *56*, 2705 – 2725.

[11] J. M. Benito, F. J. M. García, O. C. Mellet, *Expert Opin. Ther. Pat.* **2011**, *21*, 885 – 903.
[12] H. H. Chang, N. Asano, S. Ishii, Y. Ichikawa, J. Q. Fan, *FEBS J.* **2006**, *273*, 4082 – 4092.
[13] R. Steet, S. Chung, W. S. Lee, C. W. Pine, H. Do, S. Kornfeld, *Biochem. Pharmacol.* **2007**, *73*, 1376 – 1383.
[14] K. J. Valenzano, R. Khanna, A. C. Powe, R. Boyd, G. Lee, J. J. Flanagan, E. R. Benjamin, *Assay Drug Dev. Technol.* **2011**, *9*, 213 – 235.
[15] L. Yu, K. Ikeda, A. Kato, I. Adachi, G. Godin, P. Compain, O. Martin, N. Asano, *Bioorg. Med. Chem.* **2006**, *14*, 7736 – 7744.
[16] P. Compain, O. R. Martin, C. Boucheron, G. Godin, L. Yu, K. Ikeda, N. Asano, *ChemBioChem* **2006**, *7*, 1356 – 1359.
[17] F. Oulaidi, S. Front-Deschamps, E. Gallienne, E. Lesellier, K. Ikeda, N. Asano, P. Compain, O. R. Martin, *ChemMedChem* **2011**, *6*, 353 – 361.
[18] N. Dekker, T. Voorn-Brouwer, M. Verhoek, T. Wennekes, R. S. Narayan, D. Speijer, C. E. Hollak, H. S. Overkleeft, R. G. Boot, J. M. Aerts, *Blood Cells Mol. Dis.* **2011**, *46*, 19 – 26.
[19] T. Wennekes, R. J. B. H. N. van den Berg, T. J. Boltje, W. E. Donker-Koopman, B. Kuijper, G. A. van der Marel, A. Strijland, C. P. Verhagen, J. Aerts, H. S. Overkleeft, *Eur. J. Org. Chem.* **2010**, 1258 – 1283.
[20] P. Serrano, J. Casas, M. Zucco, G. Emeric, M. Egido-Gabas, A. Llebaria, A. Delgado, *J. Comb. Chem.* **2007**, *9*, 43 – 52.
[21] M. Egido-Gabás, D. Canals, J. Casas, A. Llebaria, A. Delgado, *ChemMedChem* **2007**, *2*, 992 – 994.
[22] G. Sánchez-Ollé, J. Duque, M. Egido-Gabás, J. Casas, M. Lluch, A. Chabás, D. Grinberg, L. Vilageliu, *Blood Cells Mol. Dis.* **2009**, *42*, 159 – 166.
[23] L. Díaz, J. Casas, J. Bujons, A. Llebaria, A. Delgado, L. Díaz, *J. Med. Chem.* **2011**, *54*, 2069 – 2079.
[24] L. Díaz, J. Bujons, J. Casas, A. Llebaria, A. Delgado, *J. Med. Chem.* **2010**, *53*, 5248 – 5255.
[25] J. D. Diot, I. G. Moreno, G. Twigg, C. O. Mellet, K. Haupt, T. D. Butters, J. Kovensky, S. G. Gouin, *J. Org. Chem.* **2011**, *76*, 7757 – 7768.
[26] E. D. Goddard-Borger, M. B. Tropak, S. Yonekawa, C. Tysoe, D. J. Mahuran, S. G. Withers, *J. Med. Chem.* **2012**, *55*, 2737 – 2745.
[27] C. Decroocq, L. M. Laparra, D. Rodriguez-Lucena, P. Compain, *J. Carbohydr. Chem.* **2011**, *30*, 559 – 574.
[28] O. N. Nadein, A. Kornienko, *Org. Lett.* **2004**, *6*, 831 – 834.
[29] S. Müller, B. Liepold, G. J. Roth, H. J. Bestmann, *Synlett* **1996**, 521 – 522.
[30] S. Desvergnes, Y. Vallee, S. Py, *Org Lett.* **2008**, *10*, 2967 – 2970.
[31] O. R. Martin, O. M. Saavedra, F. Xie, L. Liu, S. Picasso, P. Vogel, H. Kizu, N. Asano, *Bioorg. Med. Chem.* **2001**, *9*, 1269 – 1278.
[32] O. Gaurat, J. Xie, J. Valery, *J. Carbohydr. Chem.* **2003**, *22*, 645 – 656.
[33] K. Okano, H. Tokuyama, T. Fukuyama, *Chem. Asian J.* **2008**, *3*, 296 – 309.
[34] H. R. Mellor, F. M. Platt, R. A. Dwek, T. D. Butters, *Biochem. J.* **2003**, *374*, 307 – 314.
[35] U. H. Schueler, T. Kolter, C. R. Kaneski, G. C. Zirzow, K. Sandhoff, R. O. Brady, *J. Inherited Metab. Dis.* **2004**, *27*, 649 – 658.
[36] M. Schmitz, M. Alfalah, J. M. Aerts, H. Y. Naim, K. P. Zimmer, *Int. J. Biochem. Cell Biol.* **2005**, *37*, 2310 – 2320.
[37] H. Dvir, M. Harel, A. A. McCarthy, L. Toker, I. Silman, A. H. Futerman, J. L. Sussman, *EMBO Rep.* **2003**, *4*, 704 – 709.
[38] A. R. Sawkar, S. L. Adamski-Werner, W. C. Cheng, C. H. Wong, E. Beutler, K. P. Zimmer, J. W. Kelly, *Chem. Biol.* **2005**, *12*, 1235 – 1244.
[39] Z. Luan, K. Higaki, M. Aguilar-Moncayo, H. Ninomiya, K. Ohno, M. I. Garcia-Moreno, C. O. Mellet, J. M. G. Fernandez, Y. Suzuki, *ChemBioChem* **2009**, *10*, 2780 – 2792.
[40] S. D. Orwig, Y. L. Tan, N. P. Grimster, Z. Yu, E. T. Powers, J. W. Kelly, R. L. Lieberman, *Biochemistry* **2011**, *50*, 10647 – 10657.
[41] Z. Yu, A. R. Sawkar, L. J. Whalen, C. H. Wong, J. W. Kelly, *J. Med. Chem.* **2007**, *50*, 94 – 100.
[42] G. Tiscornia, E. L. Vivas, L. Matalonga, I. Berniakovich, M. B. Monasterio, C. Eguizábal, L. Gort, F. González, C. O. Mellet, J. M. G. Fernández, A. Ribes, A. Veiga, I. J. C. Belmonte, *Hum. Mol. Genet.* **2013**, *22*, 633 – 645.
[43] P. Alfonso, V. Andreu, A. Pino-Angeles, A. A. Moya-Garcia, M. I. Garcia-Moreno, J. C. Rodriguez-Rey, F. Sanchez-Jimenez, M. Pocovi, C. O. Mellet, J. M. G. Fernandez, P. Giraldo, *ChemBioChem* **2013**, *14*, 943 – 949.
[44] A. Amore, R. van Heerbeek, N. Zeep, J. van Esch, J. N. Reek, H. Hiemstra, J. H. van Maarseveen, *J. Org. Chem.* **2006**, *71*, 1851 – 1860.
[45] C. M. Walden, R. Sandhoff, C. C. Chuang, Y. Yildiz, T. D. Butters, R. A. Dwek, F. M. Platt, A. C. van der Spoel, *J. Biol. Chem.* **2007**, *282*, 32655 – 32664.
[46] J. M. Aerts, W. E. Donker-Koopman, G. J. Murray, J. A. Barranger, J. M. Tager, A. W. Schram, *Anal. Biochem.* **1986**, *154*, 655 – 663.

Received: February 11, 2014
Published online on June 27, 2014