Enzyme Inhibition

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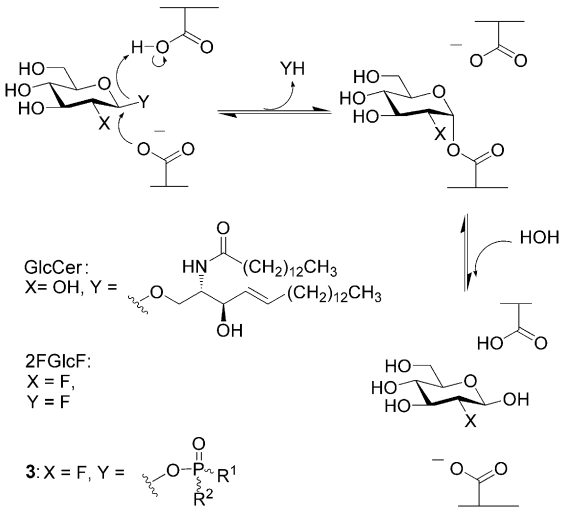
Tailoring the Specificity and Reactivity of a Mechanism-Based Inactivator of Glucocerebrosidase for Potential Therapeutic Applications**

*Brian P. Rempel, Michael B. Tropak, Don J. Mahuran, and Stephen G. Withers**

Gaucher's disease[1,2] is a lysosomal storage disorder that most commonly arises from mutations in lysosomal β glucocerebrosidase (GCase). Mutant GCase is not trafficked to the lysosome correctly, thus leading to an accumulation of unprocessed substrate. While enzyme replacement therapies exist, a potentially less expensive and systemic treatment for Gaucher's disease lies in pharmacological chaperones.[1–3] These small molecules stabilize the folded conformation of the mutant GCase,[4,5] thus allowing successful trafficking to the lysosome.[6] Some recent examples of pharmacological chaperones of GCase are reversible competitive inhibitors.[7–12] An alternative, but untested strategy would employ transient covalent inactivators, such as activated fluorosugars, to stabilize the enzyme. GCase hydrolyzes glucosylceramide (GlcCer, Scheme 1)[13] through a double-displacement catalytic mechanism,[14,15] which involves a covalent glucosyl–enzyme intermediate. These activated fluorosugars function as mechanism-based glycosidase inhibitors through the formation of a long-lived, but catalytically competent covalent glycosyl–enzyme intermediate (Scheme 1).[16–18] Indeed, 2-deoxy-2-fluoro-β-D-glucosyl fluoride (2FGlcF) is a known inactivator of GCase, and forms a glucosyl–enzyme intermediate with a hydrolytic half-life of 1300 min.[19] Importantly, 2FGlcF is fully bioavailable and functional in rats, in which it inactivates GCase in all organs, including the brain.[20] 2FGlcF is a slow inactivator of GCase however ($k_i/K_i = 0.023\ \text{min}^{-1}\,\text{mM}^{-1}$),[19] and thus an improved fluorosugar with a more selective leaving group was sought. An ideal leaving group (aglycone) would be inherently reactive, and equipped with a pair of hydrophobic substituents that mimic the ceramide of the natural substrate GlcCer. Herein, we report the development of a new class of fluorosugar glycosidase inactivators bearing tunable phosphorus-based leaving groups that react with GCase over 4000 times faster than 2FGlcF. We demonstrate the function of one such compound as a pharmacological chaperone for GCase.



Scheme 1. Chemical reaction mechanism of GCase and mode of inactivation.

Previous studies showed that effective fluorosugar inactivators incorporate leaving groups with $pK_a \leq 4.0$.[16,21] Leaving-group candidates that also allow incorporation of lipidlike substituents would include carboxylate, sulfate, and phosphate groups. Acyl ester hydrolysis limits the usage of carboxylate groups, while sulfate groups are likely too reactive and permit incorporation of only one alkyl group. Dialkyl phosphate ($pK_a \approx 1.5$) and phosphonate groups ($pK_a \approx 3$)[22] of general formula **3**, however, are attractive candidates with anticipated reasonable stability toward spontaneous decomposition in aqueous solution. Furthermore, these groups offer flexibility in the choice of alkyl groups in order to optimize binding or pharmacokinetic behavior. This approach was first evaluated with three different phosphate groups, bearing O-methyl, O-octyl, and O-benzyl substituents.

Scheme 2 shows the synthetic route used to produce fluorosugars **3a–d**. The dialkyl phosphate and phosphonate precursors, which are commercially available or readily accessible,[23–25] were coupled with the known[26] bromide **1** under silver-mediated glycosylation conditions to furnish acetate-protected precursors **2a–d**. Yields for the glycosylation step reached up to 87%, while yields for the methoxide-catalyzed deprotection steps were in the range of 65–86%.

[*] B. P. Rempel, Prof. S. G. Withers
Department of Chemistry, University of British Columbia
Vancouver, B.C. V6T 1Z1 (Canada)
E-mail: withers@chem.ubc.ca
Dr. M. B. Tropak, Dr. D. J. Mahuran
Division of Genetics and Genome Biology
The Hospital for Sick Children
Toronto, ON. M5G 1X8 (Canada)

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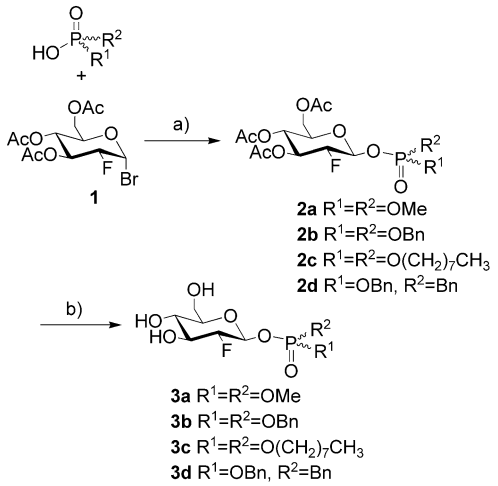
Supporting Information for this article (including full details of the synthesis and characterization of compounds **3a–d**, in vitro enzymatic assays, and cell-based studies) is available on the WWW under http://dx.doi.org/10.1002/anie.201103924.

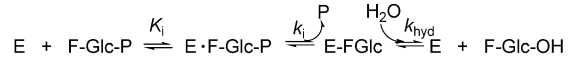


Scheme 2. Synthesis of fluorosugars bearing phosphorus-based aglycones: a) Ag_2CO_3, MeCN, **2a**: 87%, **2b**: 53%, **2c**: 15%, **2d**: 63%; b) NaOMe, MeOH, **3a**: 71%, **3b**: 65%, **3c**: 86%, **3d**: 83%.

Full synthetic details and product characterization are provided in the Supporting Information. Compounds **2d** and **3d** were both prepared and enzymatically evaluated as a 1:1 mixture of diastereomers at the phosphorus stereocenter.

In order to test whether these leaving groups provide lipid specificity, compounds **3a**–**d** were evaluated as inactivators of not only GCase, but also of the well-studied and broadly specific model retaining β-glucosidase from *Agrobacterium sp.* (Abg)[27,28] in order to test whether these leaving groups provide lipid specificity. Kinetic data were evaluated according to the kinetic scheme seen in Scheme 3.

Rapid, time-dependent inactivation was seen in all cases. Furthermore, inactivation was so rapid that it was generally not possible to measure individual values of k_i and K_i, but only values of the second-order rate constant k_i/K_i. These values for both enzymes are shown in Table 1. The complete set of kinetic data are available in the Supporting Information, and are summarized in Table 1.



Scheme 3. Kinetic mechanism for covalent inactivation of a retaining glycosidase by an activated fluorosugar.

Table 1: Selected kinetic data for inactivators **3a**–**3d**, with data for 2FGlcF included as reference.[19,21,27]

Inactivator	k_i/K_i [min^{-1} mM^{-1}] Abg	k_i/K_i [min^{-1} mM^{-1}] GCase
3a $R^1=R^2=OMe$	1.23	0.052
3b $R^1=R^2=OBn$	8.0	61
3c $R^1=R^2=O(CH_2)_7CH_3$	17	98
3d $R^1=OBn$, $R^2=Bn$	11	29
2FGlcF	14.8	0.023

Compounds **3a**–**d** inactivate Abg at rates similar to those of 2FGlcF; this result is consistent with the broadly similar leaving-group pK_a values of the reagents. This behavior suggests that Abg does not interact significantly with these unnatural aglycones. Thus, although they are useful inactivators, no specific benefit accrues in this case through use of this aglycone. The 'parent' inactivator of this class (**3a**) likewise functions as a time-dependent inactivator of GCase, with an efficiency comparable to that of 2FGlcF. However, consistent with our hypothesis, the incorporation of more lipidlike, hydrophobic groups dramatically increases inactivation efficiency, with the di-O-octyl reagent **3c** inactivating GCase about 4300 times faster than the parent di-O-methyl phosphate **3a**. This greatly increased enzymatic reactivity, despite its equivalent inherent chemical reactivity, arises from the recruitment of transition-state-stabilizing interactions that have evolved within the enzyme to enable efficient hydrolysis of glucosyl ceramide. The similar reactivity of the dibenzyl derivative **3b** further suggests that it is the general hydrophobicity that is important rather than specific binding interactions with the alkyl groups.

As suspected, these dialkyl phosphate derivatives are somewhat labile under the assay conditions, with the dibenzyl derivative **3b** undergoing hydrolysis with a half-life of approximately 15 min. In an effort to improve this stability while retaining enzymatic reactivity, compound **3d**, which bears the dibenzyl phosphonate aglycone, was synthesized. Consistent with the higher pK_a value of its aglycone, **3d** is considerably more stable and undergoes spontaneous hydrolysis with a half-life of 630 min. Importantly, however, **3d** remained a potent inactivator of GCase. Based on its ease of synthesis, high efficiency as a GCase inactivator, and relative stability toward spontaneous hydrolysis, **3d** was selected for further studies with GCase to probe enzyme stabilization and chaperoning efficiency.

A fluorescence-based heat denaturation assay was used to test the ability of **3d** to stabilize the folded conformation of wild-type GCase[29,30] at both the neutral pH value found in the endoplasmic reticulum and the low pH value of the lysosome (see the Supporting Information). The known[7,9,11] pharmacological chaperone isofagomine was also tested as a positive control. Remarkably, at neutral pH value, GCase with bound **3d** has a melting temperature (T_m) of 69 °C, which is some 22 °C higher than that of the free enzyme ($T_m = 47$ °C; see Figure S4 in the Supporting Information). By contrast, isofagomine stabilizes GCase by only 14 °C under similar conditions (pH 7.0), reported here (Figure S4) and previously.[3,31]

In order to test its ability as a pharmacological chaperone, fibroblast cells from a patient homozygous for the N370S point mutation (the most common mutation causing Gaucher's disease[2]) were continuously treated with **3d** over five days with regular changes of media supplemented with a fresh dose of compound every other day. Lysates from treated fibroblasts were analyzed by Western blot (Figure 1). A dose-dependent increase in GCase levels was seen in cells treated with **3d** relative to cells treated with vehicle (DMSO) only. When cells were treated with only a single dose of compound **3d**, noticeable increases in GCase were seen only at higher

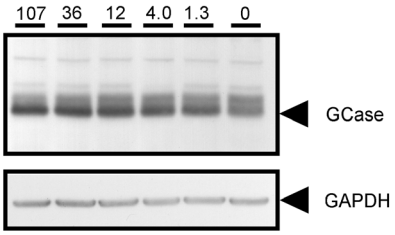


Figure 1. Western blot analysis of GCase from patient fibroblast cells that bear an Asn370Ser point mutation. Cells were grown for five days in the presence of media that contained **3d** (107 μM, 36 μM, 12 μM, 4.0 μM, 1.3 μM, or DMSO only). The **3d**-containing medium was added to the cells and changed every second day with fresh **3d**-containing medium. The two bands in the upper box correspond to different glycosylation states of GCase, while the band in the lower box corresponds to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as a loading control. DMSO = dimethylsulfoxide.

concentrations of **3d** (36–107 μM), a result that is likely caused by the slow decomposition of **3d** in aqueous buffer (see Figure S3). Daily treatment with **3d** led to an increase in GCase levels similar to those observed in treatment every other day. In conjunction with the previous demonstration that the inactivated enzyme slowly recovers activity on a timescale compatible with treatment,[20] this result augurs well for a new class of pharmacological chaperones for GCase. Radiolabeled versions may also prove valuable as selective positron emission tomography (PET) probes for imaging GCase in vivo.[32]

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