Activity-based probes for retaining -glucosidases: Novel tools for research and diagnostics

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Chapter 3

Tuning the leaving group in 2-deoxy-2-fluoro-\(D\)-glucoside results in improved activity-based retaining \(\beta\)-glucosidase probes

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**Graphical abstract** – The inhibitory potency of 2-deoxy-2-fluoro-\(D\)-glucosides towards GBA is drastically improved by implementation of \(N\)-phenyl trifluoroacetimidate as leaving group, while the C2-fluor results in temporary trapping of the nucleophile.
Abstract

Retaining β-glucosidases, like the lysosomal glucocerebrosidase (GBA), are known to be inhibited by 2-deoxy-2-fluoro-D-glucosides. This inhibition is based on stabilization of the glycosyl-nucleophile intermediate by its 2-fluorine keeping the active-site occupied. In principle, 2-deoxy-2-fluoro-D-glucosides can be employed as activity-based probes (ABPs) for labeling of β-glucosidases. We here report that the potency with which 2-deoxy-2-fluoro-D-glucoside type ABPs label GBA is drastically improved by introducing an N-phenyl trifluoroactimidate-leaving group at its anomeric center. Protonation by the general acid–base catalyst in the active-site turned out to be a prerequisite, making the imidate probe a genuine mechanism-based β-glucosidase inactivator.

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The design of tailored carbohydrates to function as enzyme inhibitors has been paramount to the general understanding of the reaction mechanism employed by glycosyl hydrolases. This is well exemplified by the fluorine-substituted glycosides, designed by Withers and co-workers in the 1980s (Fig 1a). Introduction of an electron-withdrawing fluorine substituent close to the anomeric C1 carbon, they were able to trap a covalent glycosyl-enzyme intermediate (Fig 1b). This adduct accumulated because the deglycosylation step (II – III) was sufficiently slowed down by the enhanced stability of the ester bond, induced by the fluorine at the C2 position of the glycoside, where the reactive anomeric leaving group enabled the initial glycosylation step (I – II, Fig 1a). Thanks to the use of such glycosides, the nucleophilic residues in the active site of many glycosidases have been identified. Structural biology has also benefitted greatly from these fluoroglycosides, and X-ray crystallography studies using these probes have resulted in snapshots of the Michaelis complex (I), the covalent adduct (II), and the product complex (III). While they are indispensable tools for structural biology studies, the fluoroglycosides, such as 1 and 2 (Fig 1b), are less suited for activity-based protein profiling (ABPP). The ABPP technology relies on mechanism-based inactivation of the enzyme, followed by visualization of the bound enzyme using a reporter group (often BODIPY, biotin or FLAG-
peptide). In the case of lysosomal acid β-glucosidase (GBA), a retaining exo-glucosidase associated with Gaucher’s storage disorder, cyclophellitol β-epoxide based probes as ABPs and β-aziridines including ABP 11 (Fig 1c) were found to be much more potent activity-based probes (ABPs) than the corresponding 2-deoxy-2-fluoro-D-glucosides 1 and 2.

The reason for the lower potency of the 2-deoxy-2-fluoro-D-glucosides as ABPs may be two-fold, namely that (1) the deactivating fluorine atom at C2 deactivates the ABP as a whole, making it less reactive for the glycosylation step, and (2) with the implementation of the fluorine atom at the C2 position, an important recognition element for GBA, namely the C2-hydroxyl, has been sacrificed. In addition, the leaving group may influence the potency of a compound as ABP. Of interest, the anomeric fluoride or 2,4-dinitrophenol moieties in 1 and 2 are less dependent on protonation by the general acid–base residue in the active site in order to be expelled.

We therefore reasoned that tuning of the leaving group could result in more reactive 2-deoxy-2-fluoro-D-glucosides. Introducing an anomeric leaving group that invites protonation by the catalytic acid/base residue might display a higher potency for GBA. Based on anomeric leaving groups commonly employed in synthetic carbohydrate chemistry, S-tolyl compound 3, anomeric sulfoxides 4/5, N-phenyl trifluoroacetimidate probe 6 and diphenylphosphate probe 7 were selected. Because the target enzyme GBA allows (and actually prefers) bulky hydrophobic substituents at the C6 position, all probes are equipped with a green-fluorescent BODIPY dye for direct visualization. Probes 3–7 were readily synthesized starting from D-glucose, and the sulfoxide probes 4/5 have been obtained as single, yet unidentified sulphur stereoisomers.

Results

Comparison to irreversible mechanism-based inhibitors – First, the apparent IC_{50} values of 2-deoxy-2-fluoro-D-glucoside probes 1–7 were established (Table 1, inhibition curves are displayed in Fig 2a, see next page). As revealed in Table 1, the ‘classic’ inhibitors 1 and 2, having an anomeric fluorine or dinitrophenol substituent, are relatively poor inhibitors of GBA, as is phosphate probe 7. Thioacetal probe 3 and sulfoxide probes 4 and 5 did not inhibit GBA. In contrast, imidate probe 6 inhibited GBA completely, and an IC_{50} value of ~5.5 μM was determined, which is in fact somewhat lower than the IC_{50} of known inhibitor conduritol β-epoxide 8 (CBE, ~9.49 μM).

To prove that the abolished enzyme activity was a result of inhibition of active enzyme via a covalent inhibitor-enzyme intermediate, 2 picomoles of GBA was incubated with different concentrations of probes 1, 2, 6 and 7 for 30 min, with β-epoxide ABP 9 and β-aziridine ABP 11 were included in this experiment for comparison, followed by
visualization of the enzyme mixture after gel electrophoresis and fluorescent scanning of the slab gels. As shown in Fig 2b, fluorescently labeled enzyme could be observed for probes 1, 6 and 7, while 2,4-dinitrophenyl probe 2 showed no labeling even at 50 μM. To clearly visualize GBA, 50 μM of fluoride probe 1 and phosphate probe 7 were minimally needed. GBA could be visualized using as little as 500 nM of fluorescent imidate probe 6. Although the gel depicted in Fig 2b reveals that imidate probe 6 is not as potent as cyclophellitol β-epoxide based ABP 9 or β-aziridine based ABP 11 (both label GBA in the picomolar range), the minimal concentration for labeling is 100-fold lower than the concentration required for fluoride probe 1.

Covalent attachment of cyclophellitol β-epoxide based probe ABP 9 requires access to the nucleophile of GBA11. In Fig 2c is illustrated that labeling of GBA by probe 1, 6 and 7 also occurs via the active-site. Labeling is successfully abrogated by pre-incubation with active-site inhibitors such as conduritol β-epoxide 8, cyclophellitol β-epoxide, β-epoxide 10 or AMP-DNM, and denaturation by boiling also results in absence of labeling.

Figure 2 | In vitro labeling of recombinant GBA. (a) Relative concentration-dependent inhibition of recombinant GBA (imiglucerase) β-glucosidase activity in vitro, with 2-deoxy-2-fluoro-β-glucoside 1 (closed circle, ● – ●), 2 (open circle, ○ – ○), 3 (open square, □ – □), 6 (closed square, ■ – ■) and 7 (open triangle, △ – △) compared to β-epoxide 9 (green square, □ – □), β-epoxide 10 (red square, ■ – ■) and β-aziridine 11 (green circle, ○ – ○). Data are mean ± SD. (b) Detection limit after incubation of 2 pmol GBA with indicated concentrations of probe 1–7 compared to β-epoxide 9 and β-aziridine 11. (c) Labeling of recombinant GBA (imiglucerase, 2 pmol) was blocked by incubating with irreversible inhibitors CBE 8, cyclophellitol, red β-epoxide 10, reversible inhibitor AMP-DNM or after denaturing conditions (1% (w/v) SDS for 4 min at 100 °C) prior to labeling with green ABPs β-epoxide 9, β-aziridine 11, probe 1, 6 or 7. Inter-gel comparisons via 9/10-labeled imiglucerase (asterisk; ●).

Table 1 | Inhibitory properties. Apparent in vitro IC₅₀ values of 2-deoxy-2-fluoro-β-glucosides 1–7 compared to conduritol β-epoxide 8, β-epoxide ABPs 9 and 10, and β-aziridine ABP 11.

<table>
<thead>
<tr>
<th>Compound</th>
<th>in vitro GBA IC₅₀ (nM)</th>
</tr>
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<tbody>
<tr>
<td>Difluor 1</td>
<td>&gt; 10⁴</td>
</tr>
<tr>
<td>2,4-dinitrophenyl 2</td>
<td>&gt; 10³</td>
</tr>
<tr>
<td>S-tolyl 3</td>
<td>&gt; 10³</td>
</tr>
<tr>
<td>Anomeric sulfoxide 4/5</td>
<td>&gt; 10³</td>
</tr>
<tr>
<td>N-phenyl trifluoracetimidate 6</td>
<td>5,458 ± 130</td>
</tr>
<tr>
<td>Diphenylphosphate 7</td>
<td>&gt; 10³</td>
</tr>
<tr>
<td>CBE 8</td>
<td>9,497 ± 42.8</td>
</tr>
<tr>
<td>β-epoxide 9</td>
<td>1.24 ± 0.04</td>
</tr>
<tr>
<td>β-epoxide 10</td>
<td>1.94 ± 0.08</td>
</tr>
<tr>
<td>β-aziridine 11</td>
<td>0.98 ± 0.06</td>
</tr>
</tbody>
</table>
**Binding requires active GBA** – Since the optimal pH for GBA activity is 5.2, and cyclophellitol β-epoxide based ABP 9 and 10 both label GBA accordingly, the pH-dependent labeling by probes 1, 6 and 7 was assessed. As shown in Fig 3a, fluorescently labeled enzyme could be observed for each probe, with a maximum intensity at pH 5.0–5.5, overlapping with the optimum pH of 5.2 and labeling by β-epoxide ABP 9 (Fig 3a). The acid stability of probes 1, 2, 6 and 7 was evaluated by incubating at 37 °C overnight in McIlvaine buffers of pH 4.0, 5.2 and 7.0, and subsequent LC-MS analysis of the probe mixtures. This revealed that probes 1 and 2 were not significantly hydrolyzed. Imidate probe 6 showed >50% hydrolysis at pH 4.0, but only trace amounts of the hydrolyzed probe were detected at pH 5.2 and 7.0. Hydrolysis of phosphate 7 started immediately, leading to full conversion to the hydrolyzed probe within 24 h at the three pH values analyzed.

**Acid/base (in)dependent binding mechanisms** – The requirement of probes 1, 6 and 7 for catalysis by the acid/base residue was evaluated using mutant GBA enzyme, in which the glutamic acid residue (E235) was substituted for a glycine (E235G) or a glutamine (E235Q). Homogenates of cells over-expressing wild-type or mutant mycHis-tagged GBA were incubated with probes 1, 6, 7, cyclophellitol β-epoxide ABP 9 or β-aziridine ABP 11 for 2 h and 24 h, followed by pull-down of the (labeled) acid/base-substituted GBA mutants with nickel-agarose beads (see Fig 3b for the input/pull-down results, next page). As

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**Figure 3 | Prerequisites for labeling to GBA.** (a) Effect of pH on labeling of GBA (imiglucerase) with β-epoxide 9, 2-deoxy-2-fluoro-α-glucosides 1, 6, or 7. (b) Labeling of GBA active-site mutants. Over-expressed mycHis-tagged GBA, either as wild-type or lacking the acid/base (E235G or E235Q), was labeled for 2 h (top) or 24 h (bottom) with β-aziridine 11, β-epoxide 9, probe 1, 6 or 7 in total lysate (left), or isolated from labeling in total lysate after pull-down (right), with α-myc as loading control (bottom). Inter-gel comparisons facilitated by imiglucerase (asterisk; †) as positive control.
displayed in Fig 3b, labeling of the wild-type enzyme was observed with all four probes upon incubation for 2 h. Fluoride probe 1 (used at 50 μM) did not label mutant GBA within 2 hours, similar as imidate 6 (used at 500 nM) and phosphate 7 (used at 5 μM). In contrast, after 24 hours incubation with probes, mutant GBA was labeled with fluoride probe 1, apparently independent of the presence of the acid/base residue as described previously for glycosylfluorides and other glycosidases. Cyclophellitol β-epoxide based ABP 9, used at 5 nM, labeled gradually the glutamine-mutant E235Q as detected after 24 h incubation, but not the glycine-mutant E235G. Potent labeling of E235G and E235Q GBA by β-aziridine ABP 11, similar to wild-type GBA, was observed after 2 and 24 h and in line with previous reports, as β-aziridine type cyclophellitols bind the nucleophile independently of the acid/base status (Fig 3b). It follows from these results that fluoride probe 1 does not require acid–base catalyzed protonation to bind covalently in the active site of GBA. In contrast, the labeling experiment with probes 6 and 7 confirmed that the presence of the acid–base catalyst was a prerequisite for their active binding, analogous to the synthetic activation of imidate and phosphate moieties under acidic conditions. The labeling of the glutamine-mutant E235Q by the cyclophellitol β-epoxide based probe can be accounted for by the activation of the β-epoxide in ABP 9 through the formation of a hydrogen bond with the installed glutamine residue. This type of activation is obviously less effective than protonation by the natural glutamic acid residue, as revealed by the slower labeling of the E235Q mutant with respect to the wild-type GBA, and apparently is not sufficient to activate the imidate and phosphate leaving groups in 6 and 7.

**In situ labeling of GBA in fibroblasts** – The ability of probes 1, 6, and 7 to label GBA in living cells was also investigated, including β-epoxide ABP 9 and β-aziridine ABP 11 for comparison. To this end, confluent human skin fibroblasts were grown in the presence of 1 or 10 μM of the 2-deoxy-2-fluoro-D-glucoside probes 1, 6 and 7 for 2 hours (Fig 4, left panel) and 24 hours (Fig 4, right panel). β-Epoxide ABP 9 and β-aziridine ABP 11 were used at 1 or 10 nM to allow time-dependent labeling in a similar time-frame as the 2-deoxy-
2-fluoro-$D$-glucoside probes. After lysis of the cells, the lysates were treated with red fluorescent cyclophellitol $\beta$-epoxide ABP 10 to label any free GBA enzyme. Imidate 6-labeled GBA at a concentration of 1 $\mu$M after 2 h (Fig 4, left panel, green trace), allowing 49% of residual labeling by red $\beta$-epoxide ABP 10 (Fig 4, left panel, red trace). After labeling for 24 h, the residual labeling decreased to 15%. Incubating with 10 $\mu$M of probe 6 for 2 h resulted in complete covalent blocking of the enzyme (Fig 4, right panel, green trace), with only minimal residual labeling (6%), which did not decrease further after 24 h. Fluoride probe 1 only showed labeling at 10 $\mu$M, resulting in 70% residual labeling after 2 h, and 26% after 24 h. Phosphate probe 7 gave a significant amount of residual labeling (71%) after 24 h at the highest concentration (10 $\mu$M). Green-fluorescent $\beta$-epoxide ABP 9 slowly labeled GBA at the lowest concentration (88% and 81% residual labeling after 2 h and 24 h, respectively, with 1 nM). Faster labeling was observed at 10 nM, leading to a decrease in residual labeling from 82% (2 h) to 41% (24 h).

**Discussion**

The results described here show that the usefulness of 2-deoxy-2-fluoro-$D$-glucosides as ABPs can be improved by tuning the anomeric aglycone. Both imidate probe 6 and phosphate probe 7 are more potent inhibitors of GBA than contemporary fluoride and 2,4DNP probe 1 and 2, and label the enzyme more efficiently. Probes 6 and 7 both require protonation by the general acid/base residue, indicating that they are genuine mechanism-based inhibitors, in comparison to the commonly used anomeric fluoride probes which only require the action of a nucleophilic residue. Imidate probe 6 showed a higher hydrolytic stability than phosphate probe 7, highlighting the opportunities for the imidate probe when used in biological experiments, for example as a mechanism-based enzyme chaperone to promote proper folding of mutant GBA, following a strategy recently outlined by Withers and co-workers. The lower activity of the imidate with respect to the cyclophellitol-based probe can be caused by the absence of the C2 hydroxyl, which is implicated to have a decisive role in inducing an optimal fit in the active site of GBA.

In conclusion, tuning of the reactive aglycone in a 2-deoxy-2-fluoro $\beta$-glucosidase probe has resulted in a potent and useful inhibitor of GBA. Installation of an $N$-phenyl trifluoroacetimidate leaving group, originally developed by Yu and co-workers for glycosylation chemistry, led to a hydrolytically stable probe, which holds great promise in applications such as ABPP and chaperone technology. Its ease of synthesis, regardless of carbohydrate configurations, renders this probe highly suitable in the design of ABPs targeting other retaining glycosidases. In addition, both the amino and carbon substituents of the imidate functionality can be varied to further tune the reactivity of the probes and open up possibilities to develop $endo$-glycosidase probes.
Experimental Section

**General methods** – Synthesis of 2-deoxy-2-fluoro-D-glucoside probes 1–7 as described, synthesis of cyclophellitol β-epoxide ABPs 9 and 10 were synthesized as described and cyclophellitol β-aziridine as published. Chemicals were obtained from Sigma-Aldrich if not otherwise indicated. Recombinant GBA was obtained from Genzyme. Fibroblasts were obtained with consent from donors. Cell lines were cultured in HAMF12-DMEM medium (Invitrogen) supplied with 10% (v/v) FBS.

**Enzyme activity assays** – The activity of GBA was assayed at 37 °C by incubating with 3.75 mM 4-methylumbelliferyl-β-D-glucopyranoside (4MU-β-D-Glc) as substrate in 150 mM McIlvaine buffer, pH 5.2, supplemented with 0.1% (w/v) BSA, 0.2% (w/v) sodium taurocholate, and 0.1% (v/v) Triton X-100. To determine the apparent IC₅₀ value, GBA was pre-incubated with a range of inhibitor dilutions for 30 min at 37 °C. After stopping the substrate reaction with excess NaOH-glycine (pH 10.3), fluorescence was measured with a fluorimeter LS30 (Perkin Elmer) using λₑₓ 366 nm and λₑᵐ 445 nm.

**Detection limit** – Imiglucerase (10 μL, 100 nM) was prepared in 150 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100. The enzyme was incubated with a range of probe concentrations (10 μL, 50 μM to 10 nM final concentration, DMSO) for 60 min at 37 °C. The sample was denatured with 5x Laemmli buffer (50% (v/v) 1M Tris-HCl, pH 6.8, 50% (v/v) 100% glycerol, 10% (w/v) DTT, 10% (w/v) SDS, 0.01% (w/v) bromophenol blue), boiled for 4 min at 100 °C, and separated by electrophoresis on 7.5% (w/v) SDS-PAGE gel running continuously at 90 V.10, 16 Wet slab gels were scanned on fluorescence using the Typhoon Variable Mode Imager (Amersham Biosciences) using λₑₓ 488 nm and λₑᵐ 520 nm (band pass filter 40 nm) for green fluorescent probes 1–7, β-epoxide ABP 9 and β-aziridine ABP 11, and λₑₓ 532 nm and λₑᵐ 610 nm (band pass filter 30 nm) for red fluorescent β-epoxide ABP 10.

**Competition for the active site** – Imiglucerase (10 μL, 100 nM) was prepared in 150 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100. The enzyme was pre-incubated with CBE 8 (10 μL, 20 mM in H₂O), cyclophellitol (10 μL, 2 mM in H₂O), β-epoxide ABP 10 (10 μL, 2 μM in H₂O), or AMP-DNM (10 μL, 2 mM in H₂O) for 30 min at 37 °C, or with 10 μL 2% (w/v) SDS and boiled for 4 min at 100 °C. The pre-incubated mixtures were labeled with β-epoxide ABP 9 (10 μL, 30 mM in H₂O), probe 1 (10 μL, 150 μM in H₂O), probe 6 (10 μL, 1.5 μM in H₂O), or probe 7 (10 μL, 15 mM in H₂O) for 30 min at 37 °C. Finally, samples were analyzed by SDS-PAGE and fluorescence scanning.

**pH-dependent labeling** – Imiglucerase (10 μL, 10 nM) was prepared in 1.5 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100, and incubated with 150 mM McIlvaine buffer of pH 2–9 (25 μL), containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100, for 30 min at 37 °C. Pre-incubated enzyme was labeled with β-epoxide ABP 10 (5 μL, 8 nM in H₂O), probe 1 (5 μL, 400 μM), probe 6 (5 μL, 4 μM), or probe 7 (5 μL, 40 μM) for 30 min at 37 °C. Finally, samples were analyzed by SDS-PAGE and fluorescence scanning.

**Labeling of mutant GBA** – All probe solutions were prepared in 150 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate, 0.1% (v/v) Triton X-100, and protease inhibitor cocktail (Roche). Homogenate (20 μL) of COS-7 cells over-expressing wild-type and acid/base mutant (E235G and E235Q) GBA was incubated with β-aziridine ABP 11 (20 μL, 2 μM), β-epoxide ABP 9 (20 μL, 2 μM), probe 1 (20 μL, 200 μM), probe 6 (20 μL, 2 μM), or probe 7 (20 μL, 20 μM) for either 2 h or 24 h at 37 °C. The samples were split in two, and one half (20 μL) was directly denatured etcetera (vide infra). The labeled homogenate (20 μL) was incubated with Ni-agarose beads (5 μL) and native lysis buffer (100 μL, pH 8.0) containing NaCl (300 mM) and imidazole (10 mM) while rotating for 1 h at 4 °C. The samples were centrifuged for 3 min at 800 rpm, cleaned with wash buffer (200 μL, pH 8.0) containing NaCl (300 mM) and imidazole (20 mM) for 10 min at 4 °C, and repeated thrice. After centrifugation for 10 min at 800 rpm, pellet was resuspended in McIlvaine buffer (20 μL, pH 5.2) containing 0.2% (w/v) taurocholate, 0.1% (v/v) Triton X-100. Finally, samples were analyzed by SDS-PAGE and fluorescence scanning. Western blotting was accomplished by transfer of the protein for 1 h at 12 V, followed by blocking of the membrane with 2% (w/v) BSA in TBST buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20), overnight treatment with 1:2,000 diluted primary mouse α-myc mAb (Cell Signaling, b118, 2% (w/v) BSA in
TBST), washing with TBST for 20 min (repeated 6 times), followed by 1:10,000 diluted secondary rabbit α-mouse IRD680 (Cell Signaling, 2% (w/v) BSA in TBST), subsequent washing with TBST for 20 min (repeated 6 times), and read-out on Odyssey infrared scanner.

**Labeling in fibroblasts** – Wild-type human skin fibroblasts were grown to confluency (RPMI medium) for 3 days and cultured in the presence of β-epoxide ABP 9 (0/1/10 nM), β-aziridine ABP 11 (0/1/10 nM), probe 1 (0/1/10 μM), probe 6 (0/1/10 μM), or probe 7 (0/1/10 μM) (probe solutions in PBS buffer) for 2 or 24 h at 37 °C. The cells were lysed by scraping in potassium phosphate buffer (100 μL, 25 mM, pH 6.5) containing 0.1% (v/v) Triton X-100 and protease inhibitor cocktail (Roche). After determination of protein concentration (BCA kit, Pierce), 21 μg (2 h) or 27 μg (24 h) total protein was loaded per lane. The homogenates (35 μL) were incubated with β-epoxide ABP 10 (5 μL, 800 nM in McIlvaine buffer, pH 5.2, containing taurocholate, 0.1% (v/v) Triton X-100, and protease inhibitor cocktail) for 30 min at 37 °C. Finally, samples were analyzed by SDS-PAGE and fluorescence scanning.
References


