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A potent and selective activity-based probe for human retaining α-galactosidases

6.1 Introduction

Lysosomal degradation of glycosphingolipids is mediated by the consecutive action of several glycosidases, each displaying a unique substrate specificity. A deficiency in one of the lysosomal glycosidases can cause accumulation of the corresponding substrates in lysosomes and consequently lead to a lysosomal storage disorder. Although the primary defects underlying such disorders are similar, they generally show a completely different disease progress, phenotype and clinical manifestation. An attractive approach to study glycosidases and their involvement in disease is the use of activity-based probes (ABPs) that allow the visualization of active enzymes in their natural environment. This method is especially useful for the class of retaining glycosidases, since these form a covalent enzyme-substrate intermediate during hydrolysis and are thus amenable to targeting by ABPs that react covalently with the catalytic nucleophile in the active site.

The human enzyme α-galactosidase A (αGal A) is a retaining glycosidase that resides in lysosomes and cleaves terminal α-linked galactosyl moieties from polysaccharides, glycolipids and glycoproteins. It is one of only two human enzymes within the family of retaining α-galactosidases. The related lysosomal enzyme α-N-acetylgalactosaminidase, also termed α-galactosidase B (αGal B), hydrolyzes substrates with a terminal N-
acetylgalactosamine (NAGA) moiety. The two proteins share 46% amino acid sequence identity and their active site differs only in two amino acid residues, which are responsible for the interaction with the substrate’s C2-substituent. Consequently, αGal B can accommodate the larger C2 substituent in NAGA as compared to α-galactose while it also tolerates a C2-hydroxyl substituent and is thus capable of hydrolyzing α-galactosides, though with lower efficiency than NAGA substrates. By replacing the two unique active site residues in either αGal A or αGalB with the corresponding residues from the other enzyme, the specificities of the two enzymes can be interchanged. The hydrolysis of α-galactosides by both enzymes occurs through a double displacement mechanism resulting in cleavage of the glycosidic bond and the release of free galactose with overall retention of the α-anomeric configuration (Figure 6.1A). Two carboxylic acid residues are required in the active site to function as a nucleophile and a general acid/base. In human αGal A, these residues have been identified as the aspartic acid residues D170 and D231, respectively. Furthermore it was found that the enzyme forms a homodimer with each monomer containing at least three distinct N-linked glycosylation sites that are essential for the lysosomal transport of αGal A mediated by the mannose-6-phosphate receptor.

A deficiency in αGal A is at the basis of the lysosomal storage disorder Fabry disease, an X-linked recessive disorder characterized by the lysosomal accumulation of glycosphingolipids with terminal α-galactosyl moieties. More than 400 mutations in the gene encoding αGal A (GLA) are known that lead to malfunctioning or absence of the enzyme. Symptoms of the classic manifestation of Fabry disease include skin lesions, chronic pain, corneal opacity, intolerance to heat, inability to sweat and micro-albuminuria. Moreover, non-specific complications can develop later in life including progressive kidney disease, cardiac symptoms and cerebrovascular disease. The primary storage lipid globotriaosylceramide is further metabolized to globotriaosylsphingosine, which is highly

![Figure 6.1. A) Mechanism of substrate hydrolysis by αGal A. B) Proposed mechanism by which ABPs 1 and 2 bind to αGal A.](image-url)
A potent and selective activity-based probe for human retaining α-galactosidases elevated in plasma of Fabry patients and believed to be responsible for many of the symptoms. However, there appears to be no clear correlation between the GLA mutation, residual αGal A activity, the amount of lipid storage and the nature or onset of clinical symptoms. Diagnosis of Fabry disease is presently confirmed by demonstrating reduced αGal A activity using fluorogenic substrate assays. Additionally, elevated plasma levels of globotriaosylsphingosine can be used to confirm diagnosis of classic Fabry disease. In atypical patients that usually display uncharacteristic symptoms in combination with relatively high residual αGal A activity, diagnosis is generally less straightforward than in classic patients. The currently applied treatment for Fabry disease is enzyme replacement therapy, in which patients receive recombinant human αGal A to restore enzyme function. Two recombinant protein products are currently available, Replagal produced by Shire and Fabrazyme produced by Genzyme. These enzymes have an identical amino acid sequence but a different glycosylation pattern as a result of the different cell lines they are produced in, which causes a different tissue distribution and dose response. Unfortunately, the effectiveness of enzyme replacement therapy for the treatment of Fabry disease appears to be limited.

The development of specific ABPs that enable monitoring of endogenous levels of catalytically active retaining α-galactosidases will provide useful new research tools for the investigation of αGal A. The α-galactopyranose-configured probes synthesized as described in Chapter 5 might serve this purpose (1-5; Figure 6.2). These probes were designed to bind in the active site of retaining α-galactosidases by mimicking a galactopyranoside substrate.

Figure 6.2. Structures of novel retaining α-galactosidase ABPs 1-5 and inhibitor 6, known reversible inhibitors of αGal A (7) and αGal B (7, 8), and retaining β-glucosidase ABP 9.
and contain an α-configured electrophilic aziridine or epoxide moiety to allow attack by the catalytic nucleophile (Figure 6.1B). The ABPs are functionalized with a Bodipy fluorophore (2, 4), a biotin tag (5) or an azide (1, 3) as a ligation handle for two-step labeling. The azide-tagged ABPs may also be used as control probes and serve as α-galactosidase inhibitors. In addition, the non-tagged epoxide-based inhibitor 6 was synthesized as a galactopyranose-configured isomer of the known retaining β-glucosidase inhibitor cyclophellitol.15-17 This chapter describes the biological evaluation of compounds 1-6 as retaining galactosidase inhibitors and the use of fluorescently labeled ABP 2 for the imaging of recombinant αGal A as well as endogenous activity of human retaining α-galactosidases in cell extracts.

6.2 Results and discussion

The inhibitory potential of compounds 1-6 on recombinant αGal A (Fabrazyme) was first assessed by measuring residual enzyme activity using the fluorogenic substrate 4-methylumbelliferyl α-D-galactoside (4-mu α-gal) after 30 min of preincubation with varying concentrations of the probes. The residual activity was plotted against the inhibitor concentration (Figure 6.3A) and these curves were used to calculate apparent IC\(_{50}\) values (Table 6.1). Aziridines 1 and 2 proved to be very potent inhibitors of αGal A with apparent IC\(_{50}\) values of 3.2 and 2.0 nM, respectively. Epoxide 6 inhibited the enzyme with 10,000-fold lower potency, and only reached full inhibition after a prolonged incubation time. Modification of the hydroxyl group at C6 by substitution with an azide (3) or functionalization with a tag (4, 5) proved to be detrimental for inhibitory potency since no

Figure 6.3. Inhibition of recombinant αGal A activity by 1, 2 and 6. A) Fabrazyme was treated for 30 min with the inhibitors after which residual activity was determined from hydrolysis of 4-mu α-gal. B) Effect of 30 min preincubation (+) (dotted lines) as compared to simultaneous addition of 4-mu α-gal and probes (-) (solid lines) on inhibition. The displayed inhibitor concentrations in both cases represent those without addition of the fluorogenic substrate solution.
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Table 6.1. Inhibition of recombinant αGal A activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Apparent IC&lt;sub&gt;50&lt;/sub&gt; (30 min preincubation)</th>
<th>% inhibition 30 min</th>
<th>6 hrs</th>
<th>Apparent IC&lt;sub&gt;50&lt;/sub&gt; without preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aziridine 1</td>
<td>3.2 nM</td>
<td>100 (10 µM)</td>
<td>100 (10 µM)</td>
<td>44 nM</td>
</tr>
<tr>
<td>Bodipy-aziridine 2</td>
<td>2.0 nM</td>
<td>100 (10 µM)</td>
<td>100 (10 µM)</td>
<td>24 nM</td>
</tr>
<tr>
<td>Epoxide 6</td>
<td>30 µM</td>
<td>85 (100 µM)</td>
<td>100 (100 µM)</td>
<td>&gt;100 µM</td>
</tr>
<tr>
<td>Azido-epoxide 3</td>
<td>n.i.</td>
<td>0 (100 µM)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Bodipy-epoxide 4</td>
<td>n.i.</td>
<td>0 (100 µM)</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Biotin-epoxide 5</td>
<td>n.i.</td>
<td>0 (100 µM)</td>
<td>n.d</td>
<td>n.d</td>
</tr>
</tbody>
</table>

Inhibition of Fabrazyme as determined from hydrolysis of 4-mu α-gal after preincubation for 30 min or 6 hrs with the indicated concentrations of 1-6. Apparent IC<sub>50</sub> values were calculated from plots of residual αGal A activity as a function of probe concentration with a preincubation time of 30 min (Figure 6.3A) or without preincubation (Figure 6.3B). n.i.: no inhibition observed up to 100 µM. n.d.: not determined.

Inhibition was observed under these conditions using up to 100 µM of the probes. These findings are in contrast to previously reported data on the inhibition of retaining β-glucosidases using analogous C6-modified epoxide probes. In order to confirm the non-reversible mode of binding by probes 1, 2 and 6, inhibition of αGal A activity after preincubation with the probes was compared to that obtained by simultaneous addition of the fluorogenic substrate and the ABPs (Figure 6.3B). The evident shifts in the plots of residual activity against probe concentration and the associated 10-fold lower IC<sub>50</sub> values after preincubation as compared to simultaneous addition of inhibitors and fluorogenic substrate (Table 6.1) indicate that the binding of the probes is indeed irreversible.

In a next experiment the selectivity of compounds 1-6 was evaluated by measuring inhibition of recombinant galactocerebrosidase, a closely related retaining galactosidase that hydrolyzes β-linked instead of α-linked galactosyl moieties. Residual enzyme activity was measured using the fluorogenic substrate 4-methyl-umbelliferyl β-D-galactoside (4-mu β-gal) after 30 min of preincubation with the probes. The aziridines 1 and 2 were able to inhibit this enzyme but with 7,000-30,000 fold lower potency than αGal A (Figure 6.4A and Table 6.2). Interestingly, no evidence of irreversible binding could be obtained (Figure 6.4B). On the other hand, it was found that the non-tagged epoxide 6 inhibits galactocerebrosidase irreversibly and with similar potency to αGal A. The functionalized epoxides 3 and 4, which do not inhibit αGal A activity, appeared to be weak inhibitors of galactocerebrosidase. Together these data demonstrate that the aziridines 1 and 2 should enable selective targeting of αGal A without affecting galactocerebrosidase, while epoxide 6 inhibits both enzymes.
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Figure 6.4. Inhibition of recombinant galactocerebrosidase activity by 1, 2, 4 and 6. A) Recombinant galactocerebrosidase (βGal) was treated for 30 min with the inhibitors after which residual activity was determined from hydrolysis of 4-mu β-gal. B) Effect of 30 min preincubation (+) as compared to simultaneous addition of 4-mu β-gal and probes (-) on inhibition. The displayed inhibitor concentrations in both cases represent those without addition of the fluorogenic substrate solution.

Having established the inhibitory potential of the probes, aziridine ABPs 1 and 2 were next used to visualize recombinant αGal A on gel. Labeling of Fabrazyme with Bodipy-tagged ABP 2 for 1 hr resulted in fluorescent labeling of the enzyme in a concentration-dependent manner (Figure 6.5A). The smaller lower-running band most likely represents a non-glycosylated form of αGal A. The reaction appears to occur in a 1:1 ratio, since saturation is reached at 10 to 30 nM of probe when 20 nM of αGal A is used. Denaturation of the enzyme prior to labeling led to complete disappearance of the signal on gel, confirming that catalytically active enzyme is required for binding of the probe. Pretreatment of Fabrazyme with azide-tagged aziridine 1 resulted in concentration-dependent disappearance of the fluorescent labeling by ABP 2 (Figure 6.5B). In addition, it was shown that the enzyme can be

Table 6.2. Inhibition of recombinant galactocerebrosidase activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Apparent IC₅₀ (µM)</th>
<th>% inhibition 30 min</th>
<th>% inhibition 6 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aziridine 1</td>
<td>106</td>
<td>42 (100 µM)</td>
<td>56 (100 µM)</td>
</tr>
<tr>
<td>Bodipy-aziridine 2</td>
<td>14</td>
<td>63 (100 µM)</td>
<td>61 (100 µM)</td>
</tr>
<tr>
<td>Epoxide 6</td>
<td>5</td>
<td>97 (100 µM)</td>
<td>98 (100 µM)</td>
</tr>
<tr>
<td>Azido-epoxide 3</td>
<td>n.d.</td>
<td>20 (100 µM)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Bodipy-epoxide 4</td>
<td>40</td>
<td>56 (100 µM)</td>
<td>91 (100 µM)</td>
</tr>
<tr>
<td>Biotin-epoxide 5</td>
<td>n.i.</td>
<td>0 (100 µM)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Inhibition of recombinant galactocerebrosidase as determined from hydrolysis of 4-mu β-gal after preincubation for 30 min or 6 hrs with probes 1-6. Apparent IC₅₀ values were calculated from plots of residual galactocerebrosidase activity as a function of probe concentration with a preincubation time of 30 min (Figure 6.4A). n.i.: no inhibition observed up to 100 µM. n.d.: not determined.
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Figure 6.5. Labeling and inhibition of recombinant αGal A. A) Labeling of Fabrazyme (10 ng) with 1 nM - 1 µM of Bodipy-aziridine 2 for 1 hr; where indicated the enzyme was denatured prior to labeling. B) Fabrazyme (50 ng) labeled with 100 nM 2 after preincubation with 1 nM - 1 µM of aziridine 1 for 1 hr. C) Two-step labeling of Fabrazyme using 1 µM aziridine 1 followed by copper(I)-catalyzed click reaction with 0.5 - 50 µM of Bodipy-alkyne 10 or biotin-alkyne 11; control samples were treated with 0.3 µM 2 instead of click reagents. D) Fabrazyme or Replagal labeled with 100 nM 2 after preincubation with inhibitors 7 (10 µM), 8 (100 mM), 6 (100 µM) or probe 1 (100 nM) for 1 hr. All gels are 10% SDS-PAGE with fluorescent readout or visualization by streptavidin Western blotting (C, lower panel).

visualized after targeting by ABP 1 by performing a copper(I)-catalyzed click reaction with Bodipy- and biotin-functionalized alkyne reagents 10 and 11 (Figure 6.5C). Replagal, the second recombinant enzyme that can be used for enzyme replacement therapy in Fabry disease, was also labeled effectively with probe 2 (Figure 6.5D). Labeling of either of the two enzymes was completely abolished by preincubation with aziridine probe 1 or epoxide inhibitor 6. The labeling was also blocked by the competitive α-galactosidase inhibitor deoxygalactonojirimicin (7) but not by the selective αGal B inhibitor N-acetylgalactosamine (8) (see Figure 6.2).

The optimal pH for αGal A activity is around pH 4.6, consistent with the acidic pH of the lysosomal environment. By treating recombinant αGal A with ABP 2 in buffers of varying pH, it was revealed that the labeling is indeed pH-dependent with an optimum around pH 5 (Figure 6.6A). However, at high pH values (6 or 7) there was a discrepancy between the enzymatic activity as determined by fluorogenic substrate hydrolysis and the intensity of fluorescent labeling on gel (Figure 6.6C). This phenomenon is in agreement with previous findings on the labeling of retaining β-glucosidases by the corresponding aziridine probe 9 (Figure 6.2) and may be explained by the fact that the aziridine moiety forms a better leaving group than the aglycon of the (fluorogenic) substrate and therefore allows for a more efficient reaction with the active site nucleophile at high pH. In order to confirm the
mechanism-based inhibition of αGal A by ABP 2, mutants of αGal A lacking either the active site nucleophile (D170) or the general acid/base residue (D231) were generated. Reaction of the resulting recombinant enzymes with aziridine 2 demonstrated that absence of either of the aspartic acid residues leads to complete disappearance of the fluorescent signal on gel (Figure 6.6B). Hence, both residues are essential for labeling of the enzyme by the ABP which underscores the proposed binding mechanism as shown in Figure 6.1B.

Next, attention was focused on the labeling of endogenous α-galactosidases in cell extracts. Treatment of wild-type (WT) fibroblast extracts with Bodipy-aziridine 2 gave two fluorescently labeled proteins on gel (Figure 6.7A). In contrast, only one fluorescent signal, corresponding to the upper band in the WT fibroblasts, was visible in fibroblast extracts from αGal A deficient Fabry patients. This indicates that the lower signal, which is completely absent in Fabry cells, is αGal A. The identity of this protein can be further confirmed by the fact that the labeling in WT extracts was blocked by deoxygalactonojirimicin (7) but not by N-acetylgalactosamine (8). Likewise, competition of the upper signal by both inhibitors revealed that this protein is αGal B. The non-fluorescent aziridine ABP 1 was also able to bind both enzymes. Interestingly, epoxide inhibitor 6 selectively blocked labeling of αGal A without affecting αGal B. With the aim to determine whether Bodipy-aziridine 2 is more selective for αGal A or αGal B, the probe concentration and reaction time used for labeling in WT fibroblast lysates were reduced (Figure 6.7B). Although it proved impossible to obtain selective labeling of only one of the two enzymes, quantification of the fluorescent bands in Figure 6.7B revealed that the ratio of αGal A/αGal B labeling is higher after 2 min of labeling (1/0.9) than after 1 hr (1/2.1) which indicates that ABP 2 preferentially targets αGal A (Figure 6.7C).
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Figure 6.7. Labeling of endogenous α-galactosidases in cell extracts. A) Wild-type (WT) or Fabry (F) fibroblast extracts were treated with 100 nM 2 for 1 hr; where indicated extracts were preincubated for 1 hr with inhibitors 7 (10 μM), 8 (100 mM), 6 (100 μM) or probe 1 (1 μM). B) Labeling of WT fibroblast extracts with 10 or 100 nM 2 for 2 - 60 min. C) Ratio of αGal A/αGal B labeling as determined from quantification of gel bands in B. Gels are 10% SDS-PAGE with fluorescent readout followed by coomassie brilliant blue staining. ‘M’: protein marker.

In a final experiment the simultaneous labeling of retaining α-galactosidases and retaining β-glucosidases was examined by using α-galactosidase ABP 2 together with β-glucosidase ABP 9²⁰, which is equipped with a different Bodipy fluorophore than 2 and can therefore be visualized using different scanner settings for in-gel fluorescent readout. Labeling of recombinant glucocerebrosidase (GBA1), a lysosomal retaining β-glucosidase, and αGal A with the two probes at the same time gave selective labeling of the anticipated enzymes without any observed cross-reactivity (Figure 6.8). Similarly, simultaneous treatment of fibroblast extracts with both ABPs enabled the selective labeling of endogenous αGal A and αGal B activity by aziridine 2 and GBA1 activity by probe 9.
Figure 6.8. Simultaneous labeling of retaining α-galactosidases and β-glucosidases. Recombinant αGal A, recombinant GBA1 or WT fibroblast extracts were labeled with 100 nM 2 and 100 nM 9 and resolved by 10% SDS-PAGE with fluorescent readout in Cy3 (2) and Cy2 (9) channels followed by coomassie brilliant blue staining. ‘M’: protein marker.

6.3 Conclusion

A very potent aziridine-based fluorescent activity-based probe was identified (2) that enables profiling of endogenous αGal A and αGal B activity in cell extracts. The two enzymes can be separated on gel and are easily distinguished by competition with known inhibitors, which makes aziridine 2 a valuable probe to study the activity of human retaining α-galactosidases in vitro. Epoxide 6 is an irreversible inhibitor of αGal A that also inhibits galactocerebrosidase but not αGal B. Bodipy-aziridine 2 can be used in combination with the previously reported retaining β-glucosidase ABP 9 to study both enzyme classes simultaneously. A noteworthy difference between the two ABPs is the labeling of mutant enzymes lacking the active site nucleophile or general acid/base residue. While it was demonstrated that GBA1 labeling by aziridine 9 occurs in the absence of the acid/base residue,20 aziridine 2 does not label either of the αGal A mutants and may thus be considered to be a ‘true’ activity-based probe that can report accurately on active enzyme levels. Bodipy-aziridine 2 may provide a useful new tool for studying human retaining α-galactosidases and their role in the development of Fabry disease, for evaluating the various phenotypic variants of the disease, and for diagnostic purposes and the assessment of the effects of therapeutic intervention. In addition, labeling of αGal B activity may turn out to be useful to study a related lysosomal storage disorder that is caused by αGal B deficiency, Schindler disease.21
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Experimental procedures

General

Fibroblasts were obtained with consent from control donors and Fabry patients. Cell lines were cultured in DMEM/F-12 medium (Invitrogen) supplied with 10% (v/v) FBS in an incubator at 5% CO2 and 37 °C. Recombinant α-galactosidase A was obtained from Genzyme (Fabrazyme) and Shire (Replagal), recombinant β-glucocerebrosidase (GBA1) was obtained from Genzyme (Cerezyme). For all in vitro assays, a 100 ng/µL stock solution of Fabrazyme or Replagal was prepared in McIlvaine pH 4.6/H2O 1/1 (v/v), to which 100 ng/µL (0.1%) BSA was added for stabilization of the recombinant proteins. Cell lysates were prepared from cell pellets by resuspension in 50 mM potassium phosphate buffer (pH 6.0, 0.1% (v/v) Triton X-100) and sonication on ice for 3x 1 s with 1 min interval, after which the protein concentration was determined by BCA assay. SDS-PAGE analysis: in-gel fluorescence was measured on a Bio-Rad ChemiDoc MP scanner using Cy3 settings (Green Epi illumination, 605/50 Filter) or Cy2 settings (Blue Epi illumination, 530/28 Filter). As a loading control gels were stained with Coomassie Brilliant Blue. For Western blotting, the proteins were transferred onto a PVDF membrane (Bio-Rad Trans-Blot Turbo Transfer Pack) using a Bio-Rad Trans-Blot Turbo Transfer System. Membranes were blocked with 1% BSA in TBS-t(+) (0.1% Tween 20) for 1 hr at room temperature, hybridized with antibodies, washed with TBS-t(+) and TBS and then visualized using an ECL+ Western blotting detection kit (Amersham Biosciences). For detection of biotinylated proteins, membranes were hybridized with Streptavidin-HRP for 1 hr at room temperature (1:10,000 in blocking buffer) (Molecular Probes, Life Technologies). For detection of αGal A, membranes were hybridized with rabbit anti-GLA antibody at 4 °C overnight (1:500 in blocking buffer) followed by goat anti-rabbit-HRP antibody for 1 hr at room temperature (1:10,000 in blocking buffer) (Santa Cruz Biotechnology). The protein standard is a PageRuler Plus Prestained Protein Ladder (Thermo Scientific).

Fluorogenic substrate assay on recombinant αGal A

Fabrazyme (1 ng, 20 fmol per experiment) was diluted in McIlvaine buffer pH 4.6/H2O 1/1 (v/v) containing 0.1% BSA (10 µL) and exposed to the indicated concentrations of compounds 1-6 (10 µL 2x solution in H2O) for 30 min at 37 °C, before addition of 100 µL substrate mix (1.5 mg/mL 4-methylumbelliferyl-α-D-galactopyranoside in McIlvaine pH 4.6/H2O 1/1 (v/v) + 0.1% BSA). After incubation at 37 °C for 20 min, the reaction was quenched with 2.5 mL 0.3 M glycine, pH 10.6 and fluorescence was measured with a fluorimeter LS55 (Perkin Elmer) using λex 366 nm and λem 445 nm. For tests of irreversibility, the substrate mix was added simultaneously with the inhibitors to the enzyme (without any preincubation) and incubated for 20 min. All samples were corrected for background fluorescence (sample without enzyme) and residual enzyme activity was calculated as compared to a control sample incubated without inhibitors. Displayed values represent mean values from duplicate experiments and error bars indicate standard deviation (SD). Graphpad Prism 5 software was used to determine apparent IC50 values.

pH-dependent activity assay. Fabrazyme (1 ng, 20 fmol per experiment) in McIlvaine buffer pH 4.6/H2O 1/1 (v/v) containing 0.1% BSA (10 µL) was mixed with 100 µL substrate mix of various pH values (1.5 mg/mL 4-methylumbelliferyl-α-D-galactopyranoside in McIlvaine pH 3 - pH 8/H2O 1/1 (v/v) + 0.1% BSA). After incubation at 37 °C for 20 min, the reaction was quenched and fluorescence measured as described above. Displayed values represent mean values from 5 experiments and error bars indicate standard deviation (SD).

Fluorogenic substrate assay on recombinant galactocerebrosidase

Recombinant galactocerebrosidase was expressed in Cos-7 cells by transfection with cDNA encoding human galactocerebrosidase (gift from professor T. M. Cox, Cambridge). The culture medium containing the secreted recombinant protein was used directly for the fluorogenic substrate assay. Medium was diluted 2/1 (v/v) with
Mcllvaine buffer pH 4.3 (10 µL total volume) and exposed to the indicated concentrations of compounds 1-6 (10 µL 2x solution in H2O) for 30 min at 37 °C, before addition of 100 µL substrate mix (0.23 mg/mL 4-methylumbelliferyl-β-D-galactopyranoside in Mcllvaine pH 4.3/H2O 1/1 (v/v) with 0.2 M NaCl and 0.1% BSA). After incubation at 37 °C for 30 min, the reaction was quenched with 2.5 ml 0.3 M glycine, pH 10.6 and fluorescence was measured with a fluorimeter LS55 (Perkin Elmer) using λex 366 nm and λem 445 nm. For tests of irreversibility, the substrate mix was added simultaneously with the inhibitors to the enzyme (without any preincubation) and incubated for 30 min. All samples were corrected for background fluorescence (sample without enzyme) and residual enzyme activity was calculated as compared to a control sample incubated in the same manner but without inhibitors. Displayed values represent mean values from triplicate experiments and error bars indicate standard deviation (SD). Graphpad Prism 5 software was used to determine apparent IC50 values.

**In vitro labeling assays using recombinant αGal A**

In a typical experiment, Fabrazyme (10 ng, 0.20 pmol per experiment) was diluted in Mcllvaine buffer pH 4.6/H2O 1/1 (v/v) (9 µL total volume) and exposed to the indicated concentrations of ABP 2 (1 µL 10x solution in DMSO) for 1 hr at 37 °C. For denaturation of samples, Fabrazyme was heated to 100 °C for 5 min in Mcllvaine buffer pH 4.6/H2O 1/1 (v/v) containing 1% SDS (9 µL total volume) before addition of probe 2. After labeling, the reaction mixtures were boiled for 5 min at 100 °C with 4 µL 4x Laemmli’s sample buffer containing 2-mercaptoethanol and resolved on 10% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using Cy3 settings.

**Competition assays.** Fabrazyme or Replagal (10 ng, 0.20 pmol per experiment) were first exposed for 1 hr at 37 °C to either 10 µM of 7, 100 mM of 8, 1 µM of 1, or 100 µM of 6 (0.5 μL 20x solution in DMSO), before labeling with 100 nM 2 (0.5 µL 2 µM in DMSO) as described above.

**pH-dependent labeling assay.** Fabrazyme (50 ng, 1.0 pmol) was labeled with 50 nM of ABP 2 (1 µL 0.5 µM, 0.5 pmol, 0.5 eq.) as described above using Mcllvaine buffers of pH 3 - pH 8. Gel bands were quantified using Image Lab 4.1 (Bio-Rad) software. Displayed values represent mean values from 5 independent experiments.

**Copper(I)-catalyzed click reaction on recombinant αGal A**

Fabrazyme (100 ng, 2.0 pmol per experiment, 1 µL 100 ng/µL) was mixed with 50 mM potassium phosphate buffer pH 4.6 (3.5 µL) and 1 µM of ABP 1 (0.5 µL 10 µM in DMSO, 5.0 pmol, 2.5 eq.). The reaction was incubated for 1 hr at 37 °C, before being diluted with 50 mM NaOAc containing 0.1% SDS (10 µL) for copper(I)-catalyzed click reaction using a slightly modified literature procedure. After successive addition of 1 mM TBT (2 µL 10 mM in DMSO), 2.5 mM DTT (0.5 µL 100 mM in H2O) and 5 mM CuSO4 (1 µL 100 mM in H2O), the reaction mixtures were treated for 1 hr at 37 °C with 0.5, 5 or 50 µM of either Bodipy-alkyne 10 or biotin-alkyne 11 (1 µL 20x solution in DMSO; respectively 10, 100 and 1000 pmol; 5, 50 and 500 eq.). As a control, after exposure to ABP 1 the mixtures were diluted with 50 mM potassium phosphate buffer pH 4.6 (4 µL) and labeled with 0.3 µM ABP 2 (1 µL 3 µM in DMSO, 3.0 pmol, 1.5 eq.) for 1 hr at 37 °C instead of click reaction. The reaction mixtures were then boiled for 5 min at 100 °C with 7 µL 4x Laemmli’s sample buffer containing 2-mercaptoethanol and resolved on 10% SDS-PAGE. In-gel visualization of the fluorescent labeling by ABP 2 and click reaction with 10 was performed in the wet gel slabs directly using Cy3 settings. Proteins labeled by click reaction with 11 were detected by streptavidin Western blotting.

**Generation of αGal A mutants D170G and D231G**

**Molecular cloning and site-directed mutagenesis.** Design of cloning primers and primers for mutation introduction were based on NCBI Reference sequence for human αGal A NM_000169.2 (GLA). The full-length αGal A wild-type
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cDNA sequence was cloned from human mRNA (Hek293 cells) in pcDNA-DEST40 using the Gateway cloning system (Invitrogen). Full length cDNA was generated using the PfX50 DNA polymerase (Invitrogen) and cloned in pDONR-221 with the following primers:
- forward: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTACCACCATGCAGCTGAGGAACCCAGA-3'
- reverse: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAAGTAAGTACGCTTATGACATC-3'.

Site-directed mutagenesis was performed using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) in order to change the nucleophilic (D170) and acid/base (D231) aspartic acid to glycine residues, using the following primers:
- nucleophile D170G: 5'-GATCTGCTAAAATTTGGTGGTTGTTACTGTGACAG-3'
- acid/base D231G: 5'-GGCGAAATTTTGCTGGCATTGATGATTCCTGGAAAAG-3'.

Recombinant protein expression. Cos-7 cells were cultured in HAMF12-DMEM (Invitrogen) supplied with 10% (v/v) FCS. Confluent cells were transfected with FuGENE (Roche) and either wild-type αGal A, D170G αGal A mutant, D231G αGal A mutant or chitotriosidase as a mock transfection control. The cells were harvested after 72 hours by scraping in 25 mM potassium phosphate buffer (pH 6.5, 0.1% (v/v) Triton X-100, protease inhibitor cocktail (Roche)). Control cells were not transfected.

Labeling of αGal A mutants

Cos-7 lysates (10 μg total protein per experiment) from cells transfected with either WT αGalA, D170G or D231G mutants, chitotriosidase or control cells were diluted in McIlvaine buffer pH 4.6 (9 μL total volume) and exposed to 100 nM of ABP 2 (1 μL 1 μM in DMSO) for 1 hr at 37 °C. The reaction mixtures were then boiled for 5 min at 100 °C with 4 μL 4x Laemmli’s sample buffer containing 2-mercaptopethanol and resolved on 10% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using Cy3 settings. Next, αGal A levels were detected by performing anti-GLA Western blotting.

In vitro labeling assay using cell lysates

In a typical experiment, fibroblast lysates (20 μg total protein per experiment) were diluted in McIlvaine buffer pH 4.6 (9 μL total volume) and exposed to 100 nM of ABP 2 (1 μL 1 μM in DMSO) for 1 hr at 37 °C. Where indicated, a reduced reaction time or probe concentration was used. The reaction mixtures were then boiled for 5 min at 100 °C with 4 μL 4x Laemmli’s sample buffer containing 2-mercaptopethanol and resolved on 10% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using Cy3 settings. The gel bands in the fluorescence scan were quantified using Image Lab 4.1 (Bio-Rad) software to calculate the ratio of labeling between αGal A (lower signal) and αGal B (upper signal).

Competition assays. Fibroblast lysates (20 μg total protein per experiment) were first exposed for 1 hr at 37 °C to either 10 μM of 7, 100 mM of 8, 1 μM of 1, or 100 μM of 6 (0.5 μL 20x solution in DMSO), before labeling with 100 nM 2 (0.5 μL 2 μM in DMSO) as described above.

Simultaneous labeling of retaining α-galactosidases and β-glucosidases

Wild-type fibroblast lysates (20 μg total protein), recombinant αGal A (Fabrazyme, 10 ng, 0.20 pmol), recombinant GBA1 (Cerezyme, 12 ng, 0.20 pmol) or a mixture of both recombinant proteins were diluted in McIlvaine buffer pH 4.6/H2O 1/1 (v/v) (9 μL total volume) and labeled with 100 nM aziridine 2 plus 100 nM aziridine 9 (each 0.5 μL 2 μM in DMSO, 1 pmol, 5 eq.) for 1 hr at 37 °C. The reaction mixtures were then boiled for 5 min at 100 °C with 4 μL 4x Laemmli’s sample buffer containing 2-mercaptopethanol and resolved on 10% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly using Cy3 settings for probe 2 and Cy2 settings for probe 9.
References