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Potent and Selective Activity-Based Probes for GH27 Human Retaining α-Galactosidases

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Supporting Information

ABSTRACT: Lysosomal degradation of glycosphingolipids is mediated by the consecutive action of several glycosidases. Malfunctioning of one of these hydrolases can lead to a lysosomal storage disorder such as Fabry disease, which is caused by a deficiency in α-galactosidase A. Herein we describe the development of potent and selective activity-based probes that target retaining α-galactosidases. The fluorescently labeled aziridine-based probes **3** and **4** inhibit the two human retaining α-galactosidases αGal A and αGal B covalently and with high affinity. Moreover, they enable the visualization of the endogenous activity of both α-galactosidases in cell extracts, thereby providing a means to study the presence and location of active enzyme levels in different cell types, such as healthy cells versus those derived from Fabry patients.

Glycosidases are responsible for the hydrolysis of glycosidic bonds in (oligo)saccharides and glycoconjugates and are crucial for the breakdown of glyco(sphingo)lipids in lysosomes. A deficiency in a specific glycosidase may 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.[1] 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.[2,3] This method is especially useful for retaining glycosidases that form a covalent enzyme−substrate intermediate during hydrolysis.

Within the CAZY GH27 family of retaining α-galactosidases, two human enzymes are known, both of which reside in lysosomes. The enzyme α-galactosidase A (αGal A) cleaves terminal α-linked galactosyl moieties, while α-*N*-acetylgalactosaminidase, also termed α-galactosidase B (αGal B), hydrolyzes substrates with a terminal α-linked *N*-acetylgalactosamine (NAGA) moiety.[4,5] The active site of αGal B differs from that of αGal A in only two amino acid residues, which allow it to accommodate the larger C2 substituent in NAGA compared with α-galactose.[6] Consequently, αGal B is also capable of hydrolyzing α-galactosides.

A deficiency in αGal A causes the inherited glycosphingolipidosis called Fabry disease.[4,7] More than 400 mutations in the gene encoding αGal A that lead to malfunctioning or absence of αGal A are known. The primary storage lipid globotriaosylceramide is further metabolized to globotriaosylsphingosine, which is highly elevated in plasma of Fabry patients and believed to be responsible for many of the symptoms.[8,9] However, there appears to be no clear correlation between residual αGal A activity, 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 the diagnosis of classic Fabry disease.[9,10] The currently applied treatment for Fabry disease is enzyme replacement therapy, in which patients receive recombinant αGal A (either Replagal produced by Shire or Fabrazyme produced by Genzyme).[11,12] The effectiveness of this treatment appears to be limited.[9]

To obtain new research tools for the investigation of αGal A, we set out to develop specific ABPs that target retaining α-galactosidases and enable monitoring of endogenous levels of enzymatic activity. Recently we described several ABPs for a related class of enzymes, retaining β-exoglucosidases.[3,13] ABP **1** (Chart 1A) was designed to bind these enzymes by mimicking a β-linked glucoside substrate and reacting covalently with the active-site nucleophile. Herein we describe the development of two fluorescently labeled ABPs that target αGal A and αGal B (**3** and **4**; Chart 1B) and allow imaging of endogenous α-galactosidases in cell extracts.

Because the mechanism of substrate hydrolysis by retaining α-galactosidases is very similar to that of retaining β-glucosidases, we decided to use β-glucopyranose-configured ABP **1** as a basis for the design of our α-galactosidase ABPs. In 1988, Tong and Ganem[14] reported on an aziridine-based α-galactosidase inactivator. In their design, the aziridine nitrogen is part of both the piperidine and aziridine that make up the molecule and therefore is not available for functional modification. Despite this

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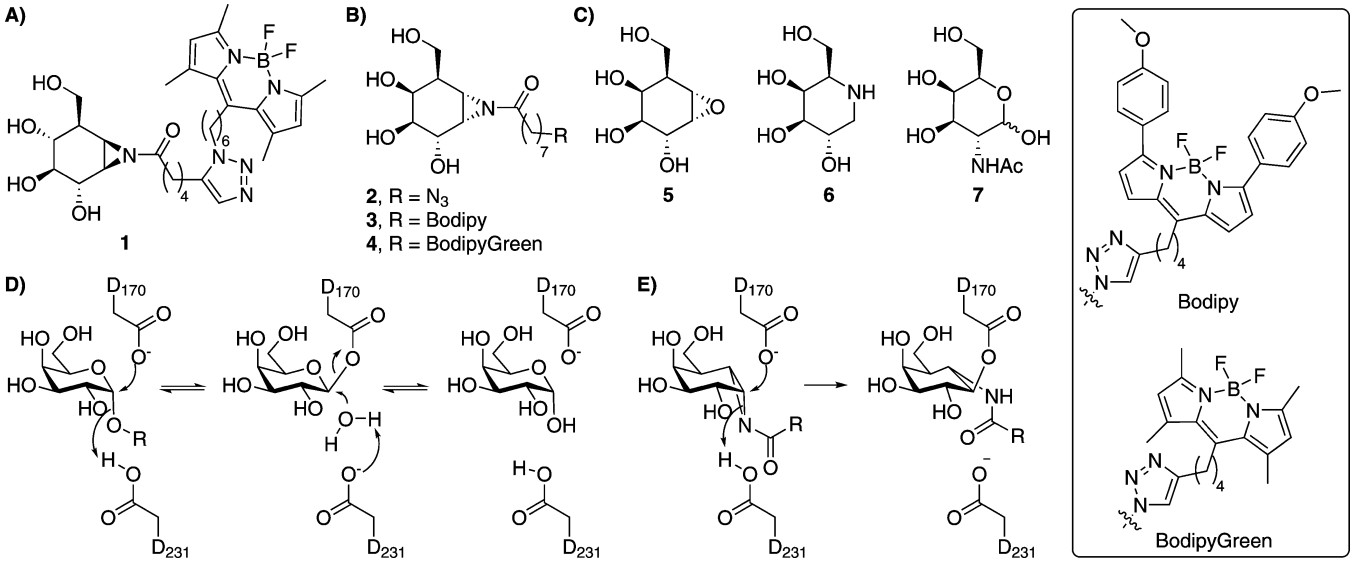
Chart 1. Design of ABPs for Retaining α-Galactosidases: (A) Known Retaining β-Exoglucosidase ABP 1; (B) Novel Fluorescent α-Galactosidase ABPs 3 and 4 and Azide-Derivatized Probe 2; (C) Novel Inhibitor (5) and Known Reversible Inhibitors of αGal A (6) and αGal B (6 and 7); (D) Mechanism of Substrate Hydrolysis by αGal A; (E) Proposed Mechanism of αGal A Binding by ABPs 3 and 4



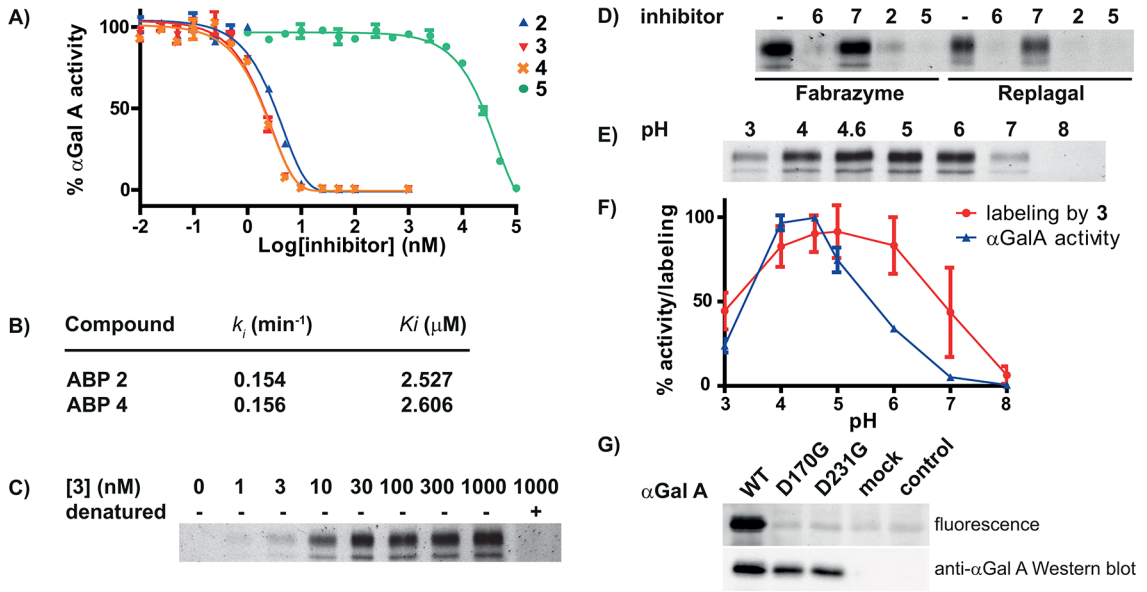


Figure 1. Labeling and inhibition of recombinant αGal A. (A) Residual activity of Fabrazyme as measured by hydrolysis of 4-mu α-gal after 30 min of preincubation with probes **2**–**5**. (B) Calculated k_i and K_i from ABPs **2** and **4**. (C) Fabrazyme (10 ng) labeled with 1 nM–1 μM of Bodipy-aziridine **3** for 1 h; the enzyme was denatured prior to labeling where indicated. (D) Fabrazyme or Replagal labeled with 100 nM **3** after preincubation with inhibitor **6** (10 μM), **7** (100 mM), **2** (100 nM), or **5** (100 μM) for 1 h. (E) Fabrazyme labeled with 50 nM **3** in buffers of varying pH. (F) Quantification of gel bands in D (circles) compared with Fabrazyme activity on 4-mu α-gal (triangles) at different pH values. (G) Labeling of recombinant wild-type αGal A (WT) and D170G and D231G mutants in lysates of transfected Cos-7 cells with 100 nM **3**; "mock", mock-transfected cells; "control", nontransfected cells. Gels are 10% SDS-PAGE with fluorescent readout (C–E, G) followed by anti-αGal A Western blotting (G).

caveat, that paper underscores the validity of our approach to label GH27 α-galactosidase through covalent modification. The same holds true for later reports by Brumer[15] and Withers[16] on the use of 2,2-difluorogalactosides and 5-fluorogalactosides, respectively, to identify the active-site nucleophile in α-galactosidases.

The catalytic mechanism follows a two-step process (Chart 1D). In the first step, an aspartic acid residue in the active site of αGal A (D170) attacks the anomeric position to cleave the glycosidic bond and create a covalent enzyme–substrate intermediate. At the same time, another aspartic acid residue that acts as a general acid (D231) protonates the leaving aglycon. Next, this residue, now acting as a general base, deprotonates a water molecule to hydrolyze the enzyme–substrate intermediate.[17] A change in the configuration of probe **1** so that it mimics an α-galactopyranoside should lead to specific targeting of catalytically active retaining α-galactosidases. Following this line, we synthesized galactopyranose-configured ABPs **3** and **4** (Chart 1B) in which the electrophilic aziridine moiety is in an α-configuration to allow attack by the catalytic nucleophile (Chart

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1E). The nonfluorescent aziridine **2** may be used as a control probe and serve as an α-galactosidase inhibitor. In addition, we synthesized epoxide-based inhibitor **5** (Chart 1C) as a galactose-configured isomer of the known retaining β-glucosidase inhibitor cyclophellitol.

The inhibitory potential of compounds **2**–**5** on recombinant αGal A was first assessed by measuring the 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 (Figure 1A). Aziridines **2**, **3**, and **4** proved to be very potent inhibitors of αGal A with apparent IC_{50} values of 2.0–3.2 nM [see the Supporting Information (SI)]. Epoxide **5** inhibits the enzyme with 10^4-fold lower potency and reaches full inhibition only after prolonged incubation time. In order to confirm the nonreversible mode of binding by probes **2**–**5**, the inhibition of αGal A activity after preincubation with the probes was compared with that obtained by simultaneous addition of the fluorogenic substrate and the ABPs (see the SI). Preincubation clearly leads to a higher percentage of inhibition, resulting in approximately 10-fold lower IC_{50} values compared with simultaneous addition of the inhibitors and fluorogenic substrate, indicating that the binding of the probes is indeed irreversible.

Next we evaluated the use of fluorescently tagged ABP **3** for visualization of recombinant αGal A on gel. As shown in Figure 1C, incubation with probe **3** for 1 h results in fluorescent labeling of the enzyme in a concentration-dependent manner. The reaction appears to occur in a 1:1 ratio, since saturation is reached at 10–30 nM probe when 20 nM αGal A is used. Denaturation of the enzyme prior to labeling leads to complete disappearance of the signal on gel, confirming that catalytically active enzyme is required for binding of the probe. The lower band in Figure 1C (and the following panels D and E) are likely differently glycosylated isoforms, and treatment of the samples with the endoglycosidase PNG F results in the formation of a single, lower-running band (see the SI).

The two different recombinant enzymes that are used for enzyme replacement therapy in Fabry disease, Fabrazyme and Replagal, are both labeled effectively with probe **3** (Figure 1D). Either labeling is completely abolished by preincubation with the nonfluorescently labeled aziridine probe **2** or epoxide inhibitor **5**. The labeling is also blocked by the competitive α-galactosidase inhibitor deoxygalactonojirimicin (**6**) but not by the selective αGal B inhibitor *N*-acetylgalactosamine (**7**) (Chart 1C).

The optimal pH for αGal A activity is around 4.6, consistent with the acidic pH of the lysosomal environment. When recombinant αGal A is treated with ABP **3** in buffers of varying pH, it becomes apparent that the labeling is indeed pH-dependent, with an optimum around pH 5 (Figure 1E).

In order to confirm the mechanism-based inhibition of αGal A by ABP **3**, we generated mutants of αGal A lacking either the active-site nucleophile (D170) or the general acid/base residue (D231). Reaction of the resulting recombinant enzymes with ABP **3** demonstrated that absence of either of the aspartic acid residues leads to disappearance of the fluorescent signal on gel (Figure 1G). Hence, both residues are essential for labeling, which underscores the proposed binding mechanism as shown in Chart 1E.

Next we turned our attention to the labeling of endogenous α-galactosidases in cell extracts. Treatment of wild-type (WT) fibroblast extracts with Bodipy-aziridine **3** gives two fluorescently labeled proteins on gel (Figure 2). In contrast, only one fluorescent signal, corresponding to the upper band in the WT

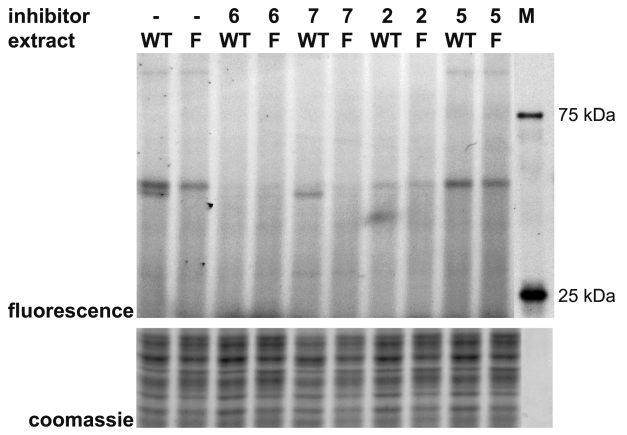

Figure 2. Labeling of endogenous α-galactosidases in cell extracts. Wild-type (WT) or Fabry (F) fibroblast extracts were treated with 100 nM **3** for 1 h and analyzed on 10% SDS-PAGE with fluorescent readout. Where indicated, extracts were preincubated with inhibitor **6** (10 μM), **7** (100 mM), **2** (1 μM), or **5** (100 μM) for 1 h. Coomassie brilliant blue staining was used as a loading control. "M" denotes protein marker.

fibroblasts, appears 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 was further confirmed by the fact that the labeling in WT extracts is blocked by **6** but not by **7**. Likewise, competition of the upper signal by both inhibitors reveals that this protein is αGal B. Nonfluorescent aziridine **2** is also able to bind both enzymes. Interestingly, epoxide inhibitor **5** selectively blocks labeling of αGal A without affecting αGal B. It is noteworthy that the same compound also irreversibly inhibits the recombinant human enzyme β-galactosylcerebrosidase (see the SI). In addition to ABP **3**, BodipyGreen-functionalized aziridine **4** enables similar fluorescent labeling of the activity of the two retaining α-galactosidases in fibroblast extracts (see the SI).

In final experiments, we examined the enzyme specificity of the synthesized probes. First, we simultaneously labeled retaining α-galactosidases and retaining β-glucosidases using probe **3** together with β-glucosidase ABP **1**, which is equipped with a different Bodipy dye and can be visualized using different scanner settings. Labeling of recombinant glucocerebrosidase (GBA), 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 3). Similarly, simultaneous treatment of fibroblast extracts with both ABPs enabled the selective labeling of endogenous αGal A

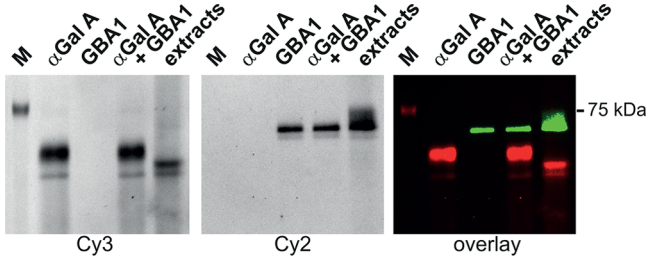

Figure 3. Simultaneous labeling of retaining α-galactosidases and retaining β-glucosidases. Recombinant αGal A, GBA, or WT fibroblast extracts were labeled with 100 nM **3** and 100 nM **1** and resolved by 10% SDS-PAGE with fluorescent readout in Cy3 (**3**) and Cy2 (**1**) channels. "M" denotes protein marker.

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and αGal B activity by aziridine **3** and GBA activity by probe **1**. Finally, in order to assess the enzyme specificity, we established the inhibition potency (IC_{50}) of compound **4** on retaining β-glucosidase, retaining α-glucosidase, and retaining β-galactosidase (see the SI). These enzymes are inhibited, but at much higher concentrations, which underscores the selective labeling as witnessed in Figures 2 and 3.

In conclusion, we have developed two potent aziridine-based fluorescent ABPs (**3** and **4**) that enable profiling of aGal 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 aziridines **3** and **4** valuable probes to study the endogenous activity of human retaining α-galactosidases in vitro. Epoxide **5** is an irreversible inhibitor of αGal A but not αGal B. However, it was found that modification of the hydroxyl group at C6 by substitution with an azide or a reporter entity is detrimental to inhibitory potency (ABPs **8**−**10**; see the SI). These findings are in contrast to those observed for the labeling of GBA by C6-modified cyclophellitol analogues. Another difference with the β-glucosidase ABPs is the labeling of mutant enzymes lacking the active-site nucleophile or general acid/base residue. While it was demonstrated previously that GBA labeling by aziridine **1** occurs in the absence of the acid/base residue, aziridine **3** 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. We consider probes **3** and **4** to be useful new tools for studying human α-galactosidases and their role in the development of Fabry disease, evaluating the various phenotypic variants of the disease, and assessing the effects of therapeutic intervention. In addition, labeling of αGal B may turn out to be useful for studying Schindler disease, a related lysosomal storage disorder that is caused by αGal B deficiency.[18] Finally, ABPs **3** and **4** may find use in screening for pharmacological chaperones aimed at increasing αGal A activity.[19]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, supporting figures and tables, and full-sized figures with Coomassie brilliant blue loading controls. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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