Enzyme Labeling

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Novel Activity-Based Probes for Broad-Spectrum Profiling of Retaining β-Exoglucosidases In Situ and In Vivo**

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Retaining β-exoglucosidases are a broad class of glycosidases widely found in nature. In humans, four members of this family are known to date. Of these, the lysosomal enzyme glucocerebrosidase (GBA1) and the non-lysosomal glucosylceramidase (GBA2) catalyze the hydrolysis of glucosylceramide.[1–6] Deficiency of GBA1 forms the basis for the most common inherited lysosomal storage disorder, Gaucher disease.[2] Recently, mutations of GBA1 have been reported to markedly increase the risk for Parkinsonism,[7–9] and excessive degradation of glucosylceramide by GBA2 may play a role in neuropathology.[4,10–12] The cytosolic broad-specificity β-glucosidase (GBA3) is thought to be involved in degrading xenobiotic β-glucosides.[13,14] The intestinal lactase/phlorizin hydrolase (LPH) is able to hydrolyze lactose as well as some hydrophobic β-glucosides including glucosylceramide.[15] Deficient LPH activity underlies lactose intolerance.[16]

In recent years, small molecules that interact with β-glucosidases, either as inhibitors or as molecular chaperones, have received growing interest in medicinal chemistry.[17,18] An important issue is the target specificity of such compounds, particularly given the limited knowledge of the physiological role of GBA2/3. Until recently, few tools were available to monitor active retaining β-exoglucosidases in living cells, and the effect of potential inhibitors on these cells.[19] Recently, fluorescent activity-based probes (ABPs) have been developed that allow labeling of active GBA1 molecules.[20] These cyclophellitol epoxide-based ABPs **1** and **2** covalently bind to the nucleophile (residue E340) of GBA1 and allow visualization of active enzyme in living cells and mice (Figure 1 a,b). The BODIPY extension at C6 markedly increases the affinity for binding to the GBA1 catalytic pocket.[20] At the same time, GBA2, GBA3, and LPH do not or only poorly bind ABPs **1** and **2**. All the β-exoglucosidases studied to date can, however, hydrolyze the artificial substrate 4-methylumbelliferyl-β-D-glucopyranoside, in which the aglycon is located at the C1 position. Based on this we decided to add a reporter group near this position in the ABP, and used the aziridine-analogue of cyclophellitol as a scaffold to develop broad-spectrum retaining β-exoglucosidase ABPs.

Cyclophellitol-aziridine has been found to be a potent mechanism-based retaining β-exoglucosidase inhibitor.[21–24] Herein, we reveal the development of aziridine ABPs **3** and **4** and demonstrate their merits as broad-spectrum retaining β-exoglucosidase probes capable of tagging the human and murine enzymes GBA, GBA2, GBA3, and LPH as well as a wide variety of non-mammalian β-exoglucosidases.

The synthesis of ABPs **3** and **4** is described in the Supporting Information (Scheme S1). Whereas epoxides **1** and **2** are selective nanomolar GBA1 inhibitors, aziridines **3** and **4** inhibit GBA1, GBA2, and GBA3 in the low nanomolar to high picomolar range (IC_{50} values are given in Figure S1) Recombinant human GBA1 (Imiglucerase, Genzyme) was used to further study the potency of **3**. GBA1 is a retaining β-exoglucosidase in which E340 acts as a nucleophile and E235 as an acid/base during catalysis.[25] Pre-incubation of GBA1 with the irreversible inhibitors conduritol β-epoxide (CBE), (azido-)cyclophellitol, and ABP **2** completely blocked covalent labeling by ABP **3** (Figure 2 a). As expected, pre-incubation with non-fluorescent ABP **8** (see Scheme S1 for the formula of **8**) also blocked labeling by **3**, as did high concentrations of the competitive inhibitor AMP-DNM.[4] These findings confirm that covalent labeling of GBA1 with **3** involves the active site, and in particular E340.

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Supporting information for this article (experimental details) is available on the WWW under http://dx.doi.org/10.1002/anie.201207771. Details of synthesis, purification and analysis of ABPs **3** and **4** and intermediates are provided, as are full details of enzyme activity, molecular cloning, site-directed mutagenesis, and fluorescence microscopy. Full gels are also provided in the Supporting Information.

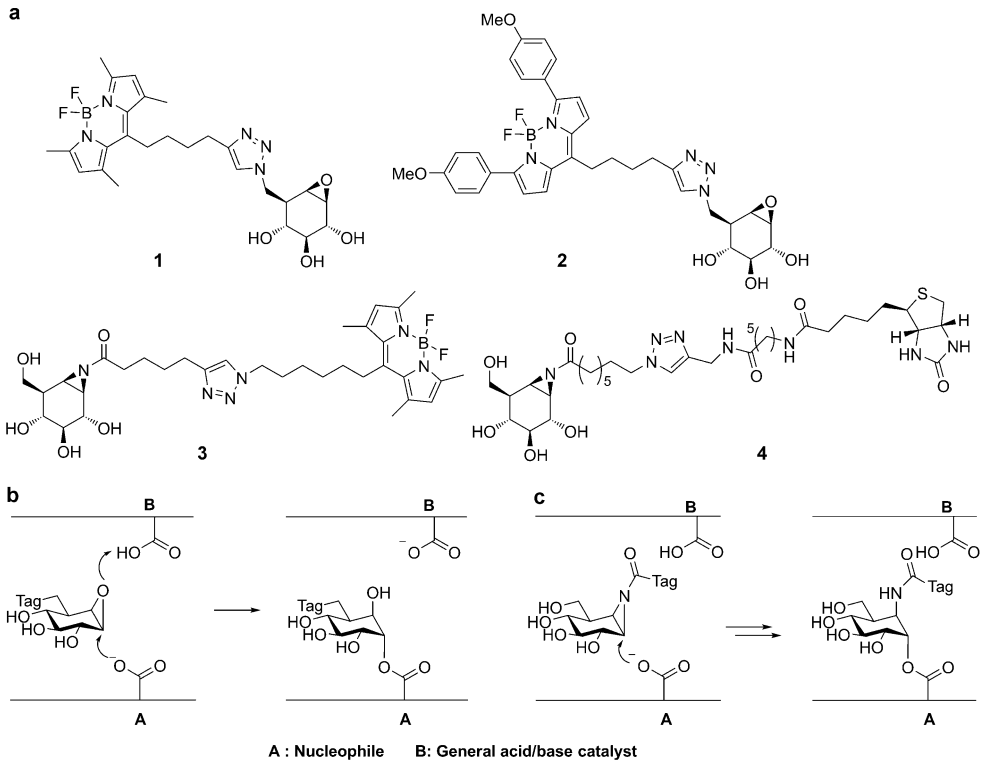

Figure 1. a) Cyclophellitol-epoxide type ABPs **1** and **2** and broad-spectrum cyclophellitol aziridine ABPs **3** and **4**. b,c) Mechanism of irreversible inhibition of retaining β-*exo*-glucosidases by b) ABP **1** or **2** and c) ABP **3** or **4**.

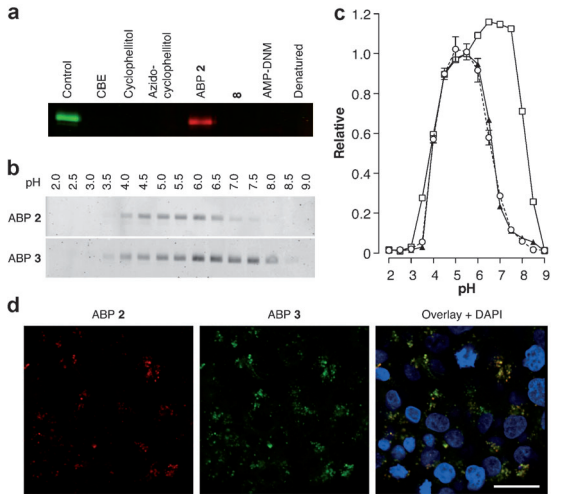

Figure 2. In vitro labeling of recombinant GBA1. a) Labeling with **3** was blocked in the presence of various inhibitors or by denaturing the protein (full gel shown in Figure S2). b) Effect of pH value on labeling of GBA1 using **2** or **3** (full gel shown in Figure S4). c) Plot showing the quantification of in-gel fluorescence from (b) of GBA1 labeled with ABP **2** (▲), **3** (□), and compared with GBA1 enzymatic activity profile towards 4-methylumbelliferyl-β-D-glucopyranoside (○). Values have been normalized to the conditions at pH 5, error bars indicate the standard deviation. d) In situ labeling of GBA1. Representative spectral imaging micrographs of cells labeled with ABPs **2** (red) and **3** (green), and nuclei stained with DAPI (blue). Regions with overlapping signal of **2** and **3** are yellow. Scale bar = 25 μm (complete micrograph analysis is shown in Figure S6).

Complete irreversible denaturation of GBA1 by exposure to high temperature and 1% (*w*/*v*) SDS abolishes labeling by ABP **3** to GBA1 (Figure 2a). Thus aziridine ABP **3** behaves like the epoxide ABPs **1**/**2** towards labeling and sensitivity of GBA1 inhibition (see Figure S3 for labeling sensitivity).

We had found previously that labeling with ABPs **1** and **2** coincides exactly with the pH profile of enzymatic activity towards 4-methylumbelliferyl-β-D-glucopyranoside (Figure 2c). In contrast, the cyclophellitol aziridine-based ABP **3** is able to label GBA1, also at high pH conditions where it is inactive towards the artificial substrate 4-methylumbelliferyl-β-D-glucopyranoside (Figure 2b,c). It is intriguing that ABP **3** is able to label GBA1 at high pH values under which the enzyme is inactive, this suggests that labeling by this ABP does not require enzyme-based protonation. The acylated nitrogen in the aziridine structure, a reasonably good electrophile, appears to not need protonation, thereby eliminating the need for acid/base catalysis. To test this, we substituted the acid/base E235 and nucleophile E340 in myc/His-tagged GBA1 separately to both glutamine and glycine by site-directed mutagenesis, giving recombinant GBA1 with E235G, E235Q, E340G, and E340Q mutations. As expected, neither ABP labeled GBA1 when the nucleophile was mutated to either a glycine or glutamine, but ABP **3** was able to label GBA1 when only the acid/base was mutated. Interestingly, significant labeling of E235Q by **3** was observed, indicating that the glutamine activates the acylated aziridine through hydrogen bonding (Figure S5). These findings offer an explanation for the different pH dependence of labeling of GBA1 by the cyclophellitol epoxide-type ABP **2** and cyclophellitol aziridine-type ABP **3**.

We next studied the ability of ABP **3** to label GBA1 in living cells. For this purpose, we incubated cultured HepG2 cells with a suboptimal amount (5 nM) of ABP **2** for two hours. After washing and incubation with 5 mM α-cyclodextrin to remove any free ABP **2**, cells were washed and then labeled with 10 nM of ABP **3** for 16 hours. Finally, fixed cells were examined by confocal-laser scanning microscopy. As shown in Figure 2d, incubation with both ABP **2** and **3** resulted in a similar staining of lysosomes.[20]

We next overexpressed the human β-exoglucosidases GBA2, GBA3, and LPH in COS-7 cells. Then, lysates of the

cells were incubated with 100 nM ABP **3** as well as **2** at different pH-values (Figure S7). Figure 3a shows the labeling at optimal pH values for each β-glucosidase. ABP **2** specifically labels GBA1 in the various cell lysates whereas

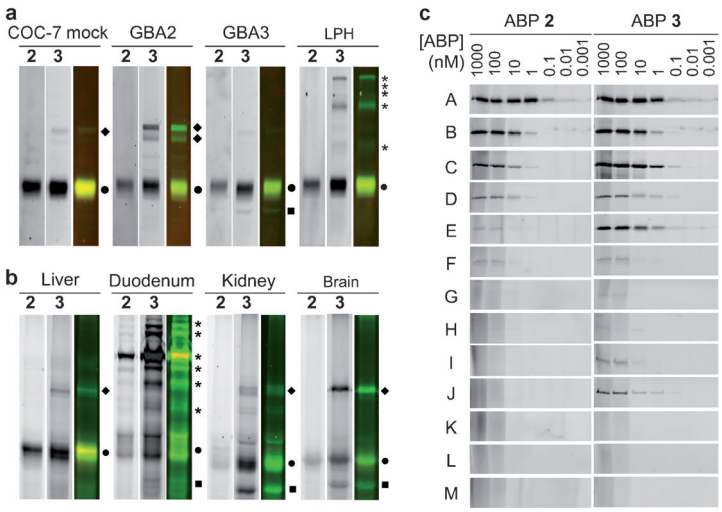

Figure 3. In vitro labeling of multiple retaining β-exoglucosidases. a) In vitro fluorescent labeling of endogenous GBA1 (●) and recombinant GBA2 (◆), GBA3 (■), and LPH (*) in homogenates of COS-7 cells using **2** and **3**. Overlaid image of each cut out gel is shown at right panel of each set. b) Labeling of endogenously expressed β-glucosidases in homogenates of murine liver, duodenum, kidney, and brain using **2** and **3**. Overlaid image of each cut out gel is shown at right panel of each set. Equal fluorescence in both channels is yellow in the overlaid image. c) Analysis of binding efficacy in vitro for non-mammalian glycosidases for different concentrations of ABP **2** and **3** at the corresponding optimal pH and buffer conditions A) *Homo sapiens* GBA1; B) *R. etli* CFN42 GBA; C) *B. fragilis* NCTC GBA; D) *Agrobacteria sp.* GBA; E) *C. thermocellum* GBA; F) *Th. Maritime* GH1; G) *A. niger* GBA; H) Sp1599; I) *P. dulcis* GBA; J) *A. fumigates* GBA; K) *A. niger* cellulase C1184; L) *T. viride* xylanase; M) *T. lanuginosus* xylanase). See also Figures S9–S12.

cyclophellitol aziridine-type ABP **3** is able to, in addition, label all other β-glucosidases: GBA2 MW = 102 kDa, GBA3 MW = 54 kDa, and LPH MW is a doublet of 210 kDa and 160 kDa.

To test the specificity of ABP **3**, we incubated homogenates of various mouse tissues with 100 nM probe **3** for 30 minutes. An identical labeling was performed with **2**. Figure 3b shows that **2** specifically labels GBA1 in the tissue homogenates, with the exception of faint labeling of LPH in the duodenum. In sharp contrast, ABP **3** not only labeled GBA1 in all tissue homogenates, but also GBA2 in the liver, kidney, and brain, GBA3 in kidney and brain, and LPH in the duodenum. No other proteins were modified by ABP **7**, illustrating the high degree of specificity. We confirmed labeling of GBA1 and GBA2 by cyclophellitol aziridine-type probes by labeling mouse spleen homogenate and HEK cell lysate with biotin aziridine ABP **4**, followed by streptavidin pull-down and mass-spectrometry based identification of the captured proteins (Figure S8).

We next extended our investigation to non-mammalian glucosidases that are commercially available. About one microgram of each individual enzyme was incubated with different concentrations of the ABPs **3** or **2** at the optimal pH determined for each enzyme and with the required additives (Figure S11, S12). Labeling was analyzed following gel electrophoresis (Figure 3c). ABP **2** labels extremely well human GBA1, but not the other glucosidases tested, with the exception of the β-glucosidases of *R. etli*, *B. fragilis*, and *Agrobacterium sp.* Less-intense labeling was observed for the β-glucosidase from *C. thermocellum* and *Thermotoga maritime* GH1. ABP **3** labeled the enzymes mentioned above more avidly, plus also β-glucosidase from *P. dul*cis and *A. fumigatis.* Diminished labeling was observed for SP1599 and β-glucosidases from *A. niger* and *A. fumigatus.* Neither **2** nor **3** reacted with the xylanases and cellulase. No clear correlation between labeling of β-glucosidases and their classification in the glycosidase protein families (CAZy) was observed (Table S2). At present, it is not clear how important the region of the binding pocket with the aglycon is for labeling by ABP **3**. A more systematic survey of the structural requirements for covalent labeling, including studies with crystals of various retaining β-exoglucosidases with and without bound **3**, may help in answering this question.

We next investigated the ability of ABP **3** to label retaining β-glucosidases in living mice. For this purpose, we injected intravenously mice with 10, 100, or 1000 pmol (about 0.2 $\mu g\,kg^{-1}$, 2 $\mu g\,kg^{-1}$, and 20 $\mu g\,kg^{-1}$, respectively) of ABP **1** or **3**. After two hours, animals were deeply anesthetized with FFM mix before perfusion with PBS, and then various tissues were isolated. Homogenates of tissues were prepared, aliquots (1 μg) were subjected to gel electrophoresis, and fluorescently labeled proteins were visualized (Figure 4a–d).

In brain (Figure 4a), no proteins were labeled by treatment of mice with ABP **3** or **1**. In contrast, in vitro incubation of a brain homogenate of a vehicle-treated mouse shows labeling of GBA1 with 1000 pmol ABP **1** or **3**. Also, GBA2 and GBA3 in brain homogenates are labeled with ABP **3**. In duodenum (Figure 4b), a prominent labeling of LPH had occurred in ABP **3** and **1** treated mice. It should, however, be noted that in animals treated with a low dose of **3** (but not with **1**), LPH was maximally labeled. This indicates a marked difference in affinity for in vivo labeling of LPH by ABP **3** versus **1**. The duodenum-derived image (Figure 4b) reveals a complex labeling pattern compared to the other tissues (also observed in the duodenum extracts, Figure 2b). Likely the added bands stem from LPH that has been partially processed by digestive proteases, although it cannot be excluded that α-glucosidases are targeted by our ABPs. In kidney (Figure 4c), ABP **1** treatment only resulted in a dose-dependent labeling of GBA1. ABP **3** treatment resulted, in addition, in dose-dependent labeling of GBA3. GBA2 was already maximally labeled in animals treated with the lowest dose of ABP **3**. Finally, in liver (Figure 4d), ABP **1** treated mice showed only dose-dependent labeling of GBA1, whilst in ABP **3** treated mice additional labeling of GBA2 occurred.

The ability of **3** to profile multiple retaining β-exoglucosidases in situ and in vivo and in their enzymatically active

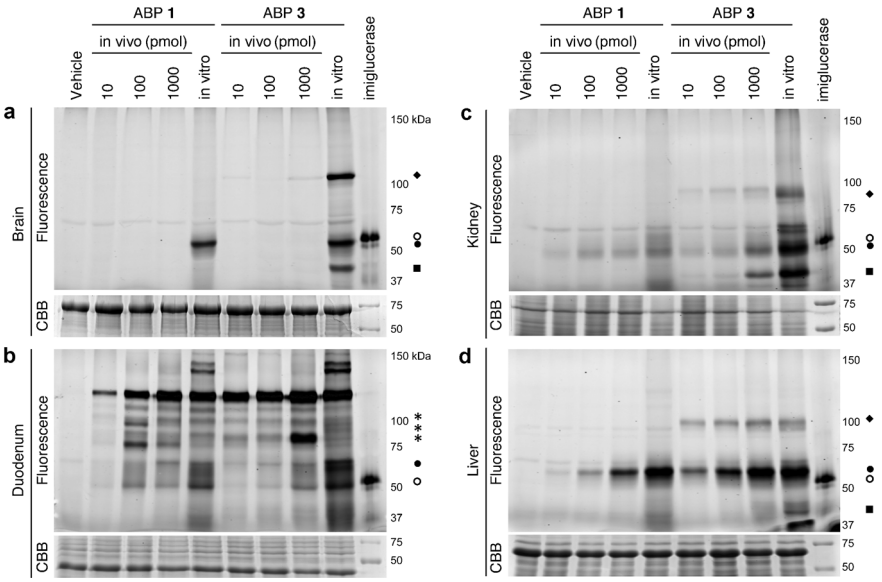

Figure 4. In vivo labeling of endogenous retaining β-exoglucosidases in mice using **1** and **3**. Fluorescently labeled proteins were analyzed in tissue homogenates of a) brain, b) duodenum, c) kidney, and d) liver. Marked are human recombinant GBA1 (○), endogenous GBA1 (●), GBA2 (◆), GBA3 (■), LPH and its degradation fragments (*). Full gels are shown in Figure S13. CBB = Coomassie Brilliant Blue staining.

form has great potential. It allows investigations on a variety of enzymes such as GBA2, GBA3, and LPH. Further investigations of GBA1 and GBA2 using ABPs are of particular interest because of the observed link between deficiency in GBA1 and the risk for the development of Parkinsonism.[7–9] It is conceivable that impaired degradation of glucosylceramide in GBA-deficient lysosomes is compensated by non-lysosomal hydrolysis by GBA2.[10] GBA2 may thus play an important role in pathophysiological processes associated with GBA1 deficiency. The new ABPs **3** and **4** are elegant tools to study GBA2 in this respect. ABPs **3** and **4**, and to a lesser extent **1** and **2**, also may be used to study LPH, whose deficiency causes lactose intolerance.

In conclusion, we have developed new potent broad-spectrum activity-based probes to monitor retaining β-glucosidases in vitro, in situ, and in vivo. Our new cyclophellitol aziridine-based ABPs, together with our previously reported cyclophellitol probes, are a highly useful pair of probes for the study of human β-glucosidases in relation to disease states. Modification of the acyl aziridine scaffold, for instance through a strategy by which broad-spectrum serine hydrolase inactivators were turned into selective inhibitors,[26] may lead to selective irreversible inhibitors of GBA2/3 and LPH for subsequent biological studies on these enzymes. Finally, the design of ABPs **3**/**4**, with the reporter pointing towards the area normally occupied by the substrate aglycon, suggests that configurational isomers of **3** could be designed that may result in effective activity-based probes that are able to label a variety of retaining glycosidases.

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