A Specific Activity-Based Probe to Monitor Family GH59 Galactosylceramidase, the Enzyme Deficient in Krabbe Disease


Galactosylceramidase (GALC) is the lysosomal β-galactosidase responsible for the hydrolysis of galactosylceramide. Inherited deficiency in GALC causes Krabbe disease, a devastating neurological disorder characterized by accumulation of galactosylceramide and its deacylated counterpart, the toxic sphingoid base galactosylsphingosine (psychosine). We report the design and application of a fluorescently tagged activity-based probe (ABP) for the sensitive and specific labeling of active GALC molecules from various species. The probe consists of a β-galactopyranose-configured cyclophellitol-epoxide core, conferring specificity for GALC, equipped with a BODIPY fluorophore at C6 that allows visualization of active enzyme in cells and tissues. Detection of residual GALC in patient fibroblasts holds great promise for laboratory diagnosis of Krabbe disease. We further describe a procedure for in situ imaging of active GALC in murine brain by intra-cerebroventricular infusion of the ABP. In conclusion, this GALC-specific ABP should find broad applications in diagnosis, drug development, and evaluation of therapy for Krabbe disease.

Introduction

Glycoside hydrolase family 59 (GH59) human galactosylceramidase (GALC, galactocerebrosidase) is an 80 kDa protein responsible for the lysosomal turnover of galactosylceramide and galactosylsphingosine. Newly synthesized GALC contains at least four N-linked glycosylation sites, which are responsible for lysosomal trafficking through the mannose-6-phosphate receptor.[1] After entering the lysosomes, the enzyme is cleaved into 30 and 50 kDa subunits without effect on enzymatic activity.[2] Crystal studies indicate that no dissociation of these subunits occurs.[3] Substrate hydrolysis by GALC occurs through a Koshland double displacement mechanism with overall retention of the α-anomeric configuration of the released galactopyranoside (Scheme 1A). The two carboxylic acid residues in the active site that function as a nucleophile and a general acid/base have been identified as the glutamic acid residues E258 and E182, respectively.[1, 2]

Deficiencies in GALC are at the basis of the autosomal recessive lysosomal storage disorder Krabbe disease, also termed globoid cell leukodystrophy. More than 70 mutations in the gene encoding GALC have been implicated in the development of this disease.[1] The main pathological consequences are found in the peripheral and central nervous systems. The
mechanism behind this neuropathology has not been fully elucidated. Reduced activity of GALC results in reduced catabolism of galactosphingolipids, including galactosylceramide and galactosylsphingosine (psychosine). Galactosylceramide is the main lipid component of myelin, the protective sheath around neuron axons that is essential for correct functioning of the nervous system. Accumulation of the toxic metabolite galactosylsphingosine eventually leads to apoptosis of myelin-forming cells and consequent demyelination and neurodegeneration. Infantile Krabbe disease is usually diagnosed before one year of age and lethal before the age of two years. Early symptoms include limb stiffness, developmental delay, and severe irritability. When diagnosed in adolescents or adults, other symptoms can be observed, such as seizures, feeding difficulties, slowing of mental and motor development, muscle weakness, spasticity, deafness, and blindness. The onset and severity of symptoms, as well as the course of the disease in adult Krabbe patients, is highly variable, even in patients carrying the same mutation. Diagnosis can be confirmed by measuring residual GALC activity in leukocytes or cultured skin fibroblasts, which is usually 0–5% of normal levels. However, the amount of residual activity is neither directly correlated to the clinical symptoms nor to the course of the disease. Carriers might have as little as 10–20% of normal GALC activity without being affected. The only available treatment for Krabbe disease involves symptomatic treatment and physical therapy. Clinical trials with hematopoietic stem cell transplantation, which aim to restore GALC activity in the central nervous system and thereby prevent further demyelination, hold promise in slowing the course of juvenile Krabbe disease when diagnosed at an early stage.

Further investigations of GALC and its involvement in Krabbe disease would benefit from the availability of an activity-based probe (ABP) that specifically targets this enzyme. Numerous fluorescent β-galactosidase probes have been reported in literature. However, most of these are reversible and therefore cannot be used in, for example, gel-based assays. Examples include substrates consisting of a β-galactose moiety with a luminescent or fluorogenic tag attached to the aglycon position that is released after cleavage by the enzyme, as well

**Scheme 1.** Galactocerebrosidase and structures of inhibitors and probes 1–7. A) Mechanism of substrate hydrolysis by GALC. B) Proposed mechanism of GALC binding by compounds 1–4. C) Structures of novel retaining β-galactosidase inhibitor 1 and ABPs 2–4. β-Glucopyranosyl-configured compound 5 targets glucocerebrosidase. ABB166 (6) and galactosylsphingosine (psychosine, 7) are competitive GALC inhibitors.
as fluorescently tagged competitive inhibitors. In addition, a few ABPs that enable irreversible mechanism-based labeling of retaining β-galactosidases have been reported. These include suicide substrates bearing a latent quinone methide precursor as the aglycon, to which a fluorescent or fluorogenic tag is attached, and 2-fluorogalactosidases in which the hydroxy group at C6 is substituted with an azide, which enables two-step labeling by Staudinger–Bertozzi ligation. To date, none of these probes has been used for the labeling of human retaining β-galactosidases.

Our work on activity-based retaining glycosidase probes used the natural product and retaining β-glucosidase inhibitor, cyclophellitol, as starting point. Substituting the epoxide for an azidiridine and grafting a reporter group onto the azidiridine nitrogen yielded ABPs that were broadly selective for various members within a given class of retaining glycosidases. Glycosidase family selectivity was dictated by the configuration of the cyclophellitol azidiridine derivative, an approach that was shown valid for GH1-retaining β-glucosidases, GH79-retaining α-galactosidases and, most recently, for GH29-retaining α-fluco-
sidases. In our first forays into activity-based glycosidase profiling, however, we studied cyclophellitol derivatives modified at C6 (glucopyranose numbering) with a fluorophore. These epoxide probes exhibited much higher selectivity than their azidiridine analogues. Because of their high selectivity and potency for human lysosomal glucosylceramidase (GBA)—the enzyme deficient in Gaucher patients—we now routinely use the fluorescent cyclophellitol derivatives to monitor GBA activity in vitro, in situ, and in vivo in healthy and Gaucher models.

We realized that this probe design might also hold potential for the development of a GALC ABP suitable for monitoring this enzyme in the context of Krabbe disease (Scheme 1B), the more so as GALC and GBA have related glucosylceramide substrates. Here we describe the evaluation of β-galactopyranose-configured epoxides 1–4 as inhibitors and ABPs for GALC (Scheme 1C). The cyclophellitol core of these probes, synthesized as reported, was designed to mimic the substrate’s terminal galactosyl moiety and bind covalently to the target enzyme through nucleophilic attack of the catalytic residue in the active site on the β-configured electrophilic epoxide moiety (Scheme 1B). The non-tagged inhibitor 1 was included in our studies as a galactose-configured isomer of the known retaining β-glucosidase inhibitor cyclophellitol. For potential use in two-step activity-based profiling studies, in ABP 2 the primary hydroxy group is substituted with an azide that can be used for two-step labeling by copper(I)-catalyzed or copper-free strain promoted alkyne-azide [2+3] cycloaddition chemistry or Staudinger–Bertozzi ligation. In addition, this probe could serve as a control probe and an inhibitor. ABPs 3 and 4 were obtained by functionalization of 2 with a BODIPY fluorophore and a biotin tag, respectively.

Results

Labeling and inhibition of recombinant galactocerebrosidase

First we evaluated the ability of compounds 1–4 to inhibit recombinant GALC by measuring residual enzyme activity using the fluorogenic substrate 4-methylumbelliferyl β-D-galactopyranoside (4-MU β-Gal) after 30 min of pre-incubation with varying concentrations of the probes. Plots of residual activity against inhibitor concentration reveal a clear dose-dependent inhibition of GALC by all probes (Figure 1A). The apparent IC_{50} values calculated from these curves are shown in Table 1. The non-tagged epoxide 1 proved to be a very potent inhibitor of GALC with an apparent IC_{50} value of 38 nM. Substitution of the hydroxy group at the C6 position with an azide (2), however, resulted in a dramatic loss of potency with an almost 2000-fold increase of the apparent IC_{50} value (70 μM). We found probe 2 to reach full inhibition after a prolonged incubation time. Interestingly, the inhibitory potency was partially restored by incorporation of a BODIPY dye (3), but not a biotin tag (4), at the same position. The second order rate constants for inhibition (k_{i}/K_{i}) of compounds 1 and 3 were in agreement with this observation (see Table 1 and Figure S1 in the Supporting Information). Hence, it appears that the hydrophobic fluorophore leads to enhanced binding of the probe to its target enzyme and might be better tolerated in the active site of the enzyme than a small polar azide moiety. A similar result has been found previously for the inhibition of retaining β-glucosi-
dases by analogous C6-modified probes, although the benefi-
cial effect of the BODIPY dye was much larger in that case.

On the contrary, previous findings suggest that neither of the α-configured isomers of the C6-functionalized epoxide probes 2–4 appears to inhibit retaining α-galactosidases, indicating that this phenomenon is dependent on the specific active site features and substrate tolerance of each individual glycosidase.

To demonstrate the irreversibility of GALC inhibition by ABP 3 we preincubated GALC with ABP 3 for different time periods. Next, residual enzymatic activity in the samples was determined and aliquots of the same samples were subjected to gel electrophoresis to quantify ABP-labeled GALC (Figure S2).
Enzyme activity was irreversibly lost during the pre-incubation in time- and concentration-dependent manner. Loss of activity of GALC by pre-incubation with ABP₃ correlated with its fluorescent labeling. To further demonstrate irreversibility of labeling, we incubated GALC with ABP₃ for one hour and separated enzyme and small compound by using a spin dialysis cartridge with 7k Da cutoff. No activity was found to be recovered over time after removal of free unbound ABP₃ from the enzyme (Figure S3). We also incubated enzyme and ABP₃ briefly (2 min) before performing the same separation: in this case part of the separated enzyme population was still active. Again, the amount of enzyme activity did not increase following the separation from free ABP₃. Analysis of labeled GALC revealed no loss of label following separation of pre-labeled enzyme from free ABP₃ (Figure S3). In order to assess the selectivity of inhibitors 1–4 for retaining β-galactosidases we determined inhibition of recombinant α-galactosidase A (Fabrazyme) by using a similar assay with the fluorogenic substrate 4-methylumbelliferyl α-D-galactoside. We did not detect any inhibition after 30 min of pre-incubation with up to 100 μM of the probes (data not shown).

Having shown that compounds 1–4 were able to irreversibly inhibit GALC, we next assessed the visualization of the recombinant enzyme on gel by using fluorescently labeled ABP₃. Exposure to BODIPY-epoxide 3 for 1 h resulted in concentration-dependent fluorescent labeling of a band around 80 kDa, corresponding to the molecular weight of non-dissociated GALC (Figure 1B). The labeling was completely abolished by denaturation of the enzyme prior to addition of the ABP, confirming the specific binding of the probe to catalytically active enzyme. In addition, a second fluorescently labeled band of approximately 60 kDa was visible at higher probe concentrations. This protein most likely represents serum albumin, a component of the cell culture medium. Unwanted labeling of albumin by BODIPY-functionalized probes can be caused by non-specific interactions of the hydrophobic dye with the protein. Evidence for the non-specific nature of these interactions is provided by the fact that denaturation of the protein by boiling in assay buffer prior to labeling with ABP₃ resulted in an even stronger fluorescent signal, which could only be eliminated by addition of the surfactant SDS.
Pre-treatment of samples with non-tagged inhibitor 1 led to complete disappearance of the fluorescent labeling of GALC by BODIPY-tagged epoxide 3, whereas the labeling of albumin was unaffected (Figure 1C). Pre-incubation with azide- and biotin-tagged probes 2 and 4 resulted in partial blocking of the fluorescent labeling of GALC. We also used biotinylated ABP 4 to visualize GALC activity directly by streptavidin western blotting (Figure 1D). In agreement with the higher IC₅₀ value of this probe as compared to its fluorescently labeled analog 3, a relatively large amount of probe is required to label the enzyme. At the highest concentration tested (10 μM), a single biotin-labeled band was clearly visible that corresponds to catalytically active GALC.

**pH dependence of galactocerebrosidase labeling**

As GALC is a lysosomal enzyme, its activity is highest in a slightly acidic environment with an optimum around pH 4.3.[1] We examined the pH dependence of GALC labeling by BODIPY-functionalized ABP 3 by exposing the recombinant enzyme to the probe in buffers of varying pH (Figure 1E). The intensity of fluorescent labeling of GALC is highest at pH 4–5, whereas it is almost completely abolished at pH 3 and lower or pH 7 and higher. Quantification of the fluorescent gel bands revealed that the amount of labeling by ABP 3 overlaps perfectly with the enzymatic activity, as determined by fluorogenic substrate hydrolysis (Figure 1F). These results indicate that binding of the probe occurs in an activity-based manner and support the proposed binding mechanism depicted in Scheme 1B.

**Selectivity of galactocerebrosidase labeling**

Next, we set out to validate our hypothesis that selective labeling of retaining β-galactosidases and retaining β-glucosidases could be achieved with differently configured epoxide-based ABPs. For this purpose, we used β-galactosidase ABP 3 and the previously reported β-glucosidase ABP 5[28] (Scheme 1C) to label either recombinant GALC or recombinant glucocerebrosidase, a lysosomal retaining β-glucosidase. The two ABPs were functionalized with different BODIPY fluorophores and could therefore be visualized by using different scanner settings for in-gel fluorescent readout. Although GALC was labeled exclusively by probe 3, glucocerebrosidase was labeled by ABP 5 but not by its stereoisomer, 3 (Figure 1G). The absence of cross-reactivity demonstrated the selective targeting of each of these enzymes by the appropriately configured ABPs.

To further demonstrate the specificity of probe 3 towards GALC and to show its application to label endogenous enzyme, we used the probe to label various tissue extracts of Twitcher (twi/twi) mice.[32] These animals are a naturally occurring model of Krabbe disease and lack GALC protein. We compared the labeling of tissues of wild-type (wt), Twitcher, and heterozygous mice with ABP 3 and ABP 5. As expected, labeling by β-glucosidase probe 5 occurred in all tissues of all the genotypes (Figure 2A). However, incubation of tissue lysates with GALC probe 3 resulted in fluorescent labeling of a single band of approximately 50 kDa in the kidney, brain, and sciatic nerve of wt and heterozygous animals but not of Twitcher mice (Figure 2A). This band corresponds to the 50 kDa subunit of GALC that is formed after proteolytic cleavage of the enzyme in the lysosome. The fact that no labeling by probe 3 was detected in wt liver is in line with the low expression levels of GALC in this organ.[33] To confirm the identity of the labeled proteins, we performed a streptavidin affinity purification after labeling of mouse kidney lysates with biotinylated ABP 4. Proteins were subjected to LC-MS/MS identification following tryptic digestion. Analysis of those proteins specifically enriched in samples treated with probe as compared to the no-probe control revealed the specific labeling of several peptides from the N-terminal part of the active 50 kDa GALC protein by ABP 4 (Table S1).

Next, we studied competition of ABP 3 labeling of GALC by two known competitive inhibitors of the enzyme, ABB116[31] (Scheme 1C, compound 6) and galactosylsphingosine or psychosine (Scheme 1C, compound 7). Labeling of GALC in mouse brain and kidney lysates by probe 3 was fully competed by both inhibitors at millimolar concentrations; this confirmed that the ABP binds to the active site of the enzyme (Figure 2B).

Besides GALC, lysosomes contain another acid β-galactosidase involved in degradation of various substrates like ganglioside GM1, lactosylceramide, glycoproteins, and keratan sulfate-derived oligosaccharides.[34–36] Malfunctioning of this β-galactosidase can cause GM1 gangliosidosis and Morquio B syndrome.[34, 37] To evaluate the enzyme specificity of probe 3 and epoxide inhibitor 1 within the family of retaining β-galactosidases, we investigated their effect on the enzymatic activity of lysosomal acid β-galactosidase and GALC. To distinguish the two enzyme activities, we followed the protocol described by Martino et al., which employs AgNO₃, as a selective competitive inhibitor of lysosomal acid β-galactosidase.[38] Pre-incubation of brain and kidney lysates with 10 μM of probe 3 resulted in a significant decrease in GALC activity (ca. 50% of untreated values) in these tissues, whereas β-galactosidase activity was unaltered (Figure 2C and D). In contrast, incubation of the lysates with 0.5 μM of inhibitor 1 completely abrogated both enzyme activities.

The human body contains one additional β-galactosidase in the small intestine: lactase, also known as lactase-phlorizin hydrolase (LPH).[39, 40] This enzyme, which also has β-glucosidase activity, cleaves lactose into galactose and glucose, and deficiency in its activity causes lactose intolerance. We transfected human embryonic kidney (HEK293) cells with LPH and studied labeling of the enzyme by ABP 3 and ABP 5 by using concentrations of these probes at which they are known to target LPH (Figure S4).[28] Whereas the β-glucosidase ABP 5 resulted in prominent labeling of LPH, this was negligible for ABP 3, again indicating specificity of ABP 3 for binding to GALC and not LPH.

**In situ and in vivo GALC labeling**

Finally, we examined labeling of GALC by ABP 3 in intact cells and tissues. We noted earlier that the stereoisomer ABP 5 was
Figure 2. Labeling of tissues of wild-type, GALC-deficient (Twitcher) and heterozygous mice. A) Homogenates of brain, kidney, sciatic nerve and liver of wt (Galc+/+), carrier (Galc+/−), and Twitcher (Galc−/−) mice were incubated with ABP 3 (1 μM) or ABP 5 (100 nM). Samples were analyzed by 7.5% SDS-PAGE with fluorescent readout, followed by CBB staining. M: protein marker. B) Competition of ABP 3 labeling of GALC in mouse kidney and brain homogenates by ABB166 (6) and psychosine (7). C) Kidney homogenates were pre-incubated with 10 μM ABP 3 and 0.5 μM compound 1 for 1 h, after which the residual acid β-galactosidase and GALC enzyme activity was determined. D) Brain homogenates were pre-incubated with 10 μM ABP 3 and 0.5 μM inhibitor 1 for 1 h, after which the residual acid β-galactosidase and GALC enzyme activity was determined. Data (n = 3 per group, mean ± SD) were analyzed by using one-way ANOVA followed by the Dunnett’s multiple comparison test: *p < 0.05; **p < 0.01; ***p < 0.001.
able to penetrate cells by diffusion and efficiently labels lysosomal glucocerebrosidase in situ.\textsuperscript{28} Cultured HEK293 cells, with and without overexpression of GALC, were exposed for 1 hour to 5 nM ABP 3, and confocal fluorescence microscopy was used to detect labeled GALC in the unfixed cells. The fluorescent labeling in control HEK293 cells was low (Figure 3A), in agreement with the virtual absence of endogenous GALC labeling in HEK cells extracts (Figure S4). Low expression levels of GALC were further confirmed by western blot (not shown). However, in cells overexpressing GALC, a perinuclear vesicular labeling, characteristic of lysosomes, was observed (Figure 3A). The perinuclear vesicular labeling pattern of lysosomes was confirmed by labeling of fixed cells with the lysosomal marker LAMP1 (Figure 3A, last panel).

Next, we examined ABP 3 labeling of endogenous GALC in the brain of living mice. For this purpose, we intra-cerebroventricularly infused mice for 2 h with 1 nM ABP 3. The animals were sacrificed, and brain slices were examined by confocal fluorescence microscopy. Pronounced perinuclear labeling was detected in cells of the cerebellar cortex, overlapping with immuno-histochemical detection of GALC and lysosomal marker LAMP1 (Figure 3B).

To investigate the potential of ABP 3 for the diagnosis of Krabbe disease, we labeled fibroblasts from Gaucher and Krabbe disease patients and healthy volunteers simultaneously with ABP 3 and ABP 5. Labeling was preceded by an affinity purification of glycosylated proteins with concanavalin A beads. A band of approximately 50 kDa, corresponding to human GALC, was labeled by ABP 3 in fibroblasts of the control subjects and Gaucher disease patients (Figure 4) but was virtually absent in samples from individuals diagnosed with Krabbe disease. As expected, active GBA labeled by ABP 5 was much less prominent in fibroblasts of Gaucher disease patients compared to the other cell types (Figure 4).

Discussion and Conclusion

Prompted by the successful design of β-glucopyranose-configured epoxide-based probes that are able to label lysosomal glucocerebrosidase (E.C. 3.2.1.45) in an activity-based manner with high selectivity and sensitivity,\textsuperscript{28} we examined a similar approach for the related enzyme galactocerebrosidase (E.C. 3.2.1.46). The β-galactopyranose-configured epoxide-based probes 1–4 were demonstrated to inhibit recombinant and endogenous rodent GALC covalently and irreversibly. Of these probes, non-tagged epoxide inhibitor 1 was the most potent inhibitor. Substitution of the hydroxy group at C6 with an azide (2) resulted in loss of potency, whereas installment of

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\caption{In situ labeling of GALC in intact cells in culture and in vivo in mouse brain. A) In situ labeling with ABP 3 of control HEK293 cells (top) and HEK293 cells overexpressing GALC (bottom). Left to right: nuclear DAPI staining, ABP 3 labeling, and overlay of DAPI staining and ABP 3 labeling (all in unfixed cells), and overlay of immuno-detection of lysosomal membrane marker LAMP1 and DAPI staining in fixed cells. Scale bars in third panels: 10 μm, scale bars in right panels: 20 μm. B) In vivo labeling with ABP 3 of wt mouse cerebellar cortex following i.c.v. administration. Left to right: DAPI staining, ABP 3 labeling, anti-GalC Ab (top) or anti-LAMP1 Ab (bottom), and overlay of ABP 3 and Ab staining. Scale bar: 10 μm.}
\end{figure}
Primers were designed based on NCBI reference sequence m and ABPs targets neither the related glycosidase nor any of the other retaining β-galactosidases known to be present in humans, lysosomal acid β-galactosidase (EC 3.2.1.24) and intestinal LPH (E.C. 3.2.1.106). We previously noted the same high degree of selectivity for BODIPY-functionalized β-galactopyranose-configured epoxide S. At low concentration, this probe selectively labeled lysosomal glucocerebrosidase but not the non-lysosomal glucosylceramidase GBA2, cytosolic β-glucosidase GBA3, and LPH, all other enzymes degrading glucosylceramide.

GALC is synthesized as a precursor of about 80 kDa that is processed into 30 and 50 kDa fragments after lysosomal uptake. The two fragments do not dissociate but remain linked to each other by disulfide bridges. The cleavage of the already active 80 kDa precursor does not affect the enzymatic activity. In line with this, we found that ABP 3 labels recombinantly produced 80 kDa precursor GALC well. In lysates of GALC-overexpressing HEK293 cells, we could detect both the 80 kDa precursor and the 50 kDa mature subunit with ABP 3 (data not shown). The 50 kDa GALC subunit, containing the catalytic nucleophile residue E258, was the major GALC form visualized with ABP 3 in kidney, brain, and sciatic nerve of wt and heterozygous mice and is absent in the same tissues of Twitcher animals.

Importantly, the reported β-galactopyranose-configured epoxide-based probes can be applied as diagnostic tools in monitoring Krabbe disease by visualizing the levels of residual GALC activity, as was demonstrated here by the labeling of active enzyme in control compared to Krabbe disease fibroblasts. The probes could also be used in activity-based protein profiling studies, aiding in the development of novel therapeutic strategies by facilitating the screening of potential chaperones interacting with the catalytic pocket of GALC. Last but not least, the probes will likely prove of great value to evaluate efficacy of experimental therapies in mouse models of Krabbe disease.

### Experimental Section

#### Synthesis of β-galactopyranose-configured epoxide-based probes

β-Galactopyranose-configured cyclophellitol epoxides 1–4 were synthesized as previously reported.

#### Enzymes

Recombinant murine GALC was expressed in HEK293 cells, as previously described. The mouse enzyme is 83% homologous to human GALC. Culture medium containing the secreted recombinant protein was used directly for fluorogenic substrate assays and labeling assays. Recombinant α-galactosidase A (Fabrazyme) and recombinant β-glucocerebrosidase (Cerezyme) were obtained from Genzyme.

#### Cells

HEK293 cells (ATCC CRL1573) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose (Gibco), supplemented with 10% FBS (Bodinco) and 100 units/mL penicillin/streptomycin (Gibco). HEK293 cells with and without stable overexpression of murine GALC were generated as previously described.

#### Animals

Twitcher mice (twi/twi), a natural model of Krabbe disease resulting from a mutation in the GALC gene, along with wild-type littermates, were generated by crossing heterozygous (+/−twi) mice in-house. Heterozygous C57BL/6J B6.CE-GalC−/− mice (stock number 000845) were obtained from the Jackson Laboratory (Bar Harbor, USA). Mouse pups were genotyped as previously described.

#### Fibroblasts

Fibroblasts were obtained from skin biopsies with informed and signed consent from the donors.

#### Expression and labeling of human lactase phlorizin hydrolase (LPH)

Primers were designed based on NCBI reference sequence NG_008104.2. The full-length cDNA sequence was cloned into pcDNA3.1 in frame with the myc/His vector (Invitrogen). Confluent HEK293 cells were transfected with empty pcDNA3.1 vector or the vector with the described insert, in conjunction with FuGENE (Roche), and harvested after 72 h by scraping into potassium phosphate (KPi) buffer (25 mM, pH 6.5) supplemented with 0.1% (v/v)
Triton X-100 and protease inhibitor cocktail (Roche). A volume equivalent to 50 μg of protein was labeled with 1 μM (final concentration) of ABP 3 or ABP 5 for 2 h at 37 °C.

**SDS-PAGE analysis and fluorescence scanning:** Protein samples (recombinant enzyme, cell and tissue homogenate) were denatured by adding 5× Laemmli sample buffer containing 2-mercaptoethanol (1% of sample volume) and boiling for 4 min at 100 °C. The samples were then run on a 7.5% or 10% SDS-PAGE gel, and wet slab gels were scanned for fluorescence by using a Typhoon Variable Mode Imager (Amersham Biosciences, Piscataway, NJ, USA) with λex = 488 nm and λem = 520 nm (band pass: 40) for green fluorescence, ABP 5 and λem = 532 nm and λem = 610 nm (band pass: 30) for red fluorescent ABP 3. As a loading control, gels were stained with Coomassie Brilliant Blue (CBB) and destained with milliQ water.

**Western blotting:** Proteins were transferred onto a PVDF membrane by using a Bio-Rad Trans-Blot Turbo Transfer System. Membranes were blocked with 1% BSA in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) for 1 h at room temperature, hybridized with streptavidin-horseradish peroxidase (HRP) for 1 h at room temperature (1:10000 in blocking buffer) (Molecular Probes, Life Technologies), washed with TBST and TBS, and then visualized by using an ECL + western blotting detection kit (Amersham Biosciences). Protein standards were PageRuler Plus Prestained Protein Ladder (Thermo Scientific) and biotinylated protein marker (Bio-Rad).

**Proteomics:** One kidney from a five week old wt mouse was homogenized in 150 mM McIlvaine buffer, pH 4.5 supplemented with protease inhibitors. The homogenate (100 μL, containing 3.5 mg protein) was incubated for 2 h at 37 °C with ABP 4 (100 μL, 0.18 mm) in McIlvaine buffer (150 mM, pH 4.5). Analysis was performed as previously reported.142 Peptides were desalted on stage tips48 and analyzed with a trap-elute system on a C18 reversed-phase nano LC with a 45 min 10–60% ACN/0.1% formic acid gradient and a Thermo LTQ-Orbitrap mass spectrometer by using a top 3 data-dependent protocol (60000 resolution, m/z range 300–2000, 1000 ms fill time in the Orbitrap, 35 units of CID energy for MS/MS fragmentation, 120 ms max fill time, AGC 50 e3, and 750-count threshold). Ions of z = 2+ and higher were selected to be fragmented twice within 10 s, prior to exclusion for 150 s. Peak lists were extracted and searched against the Uniprot mouse (decoy) database, with carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification, 20 ppm peptide tolerance, trypsin as protease, and with two missed cleavages allowed by using a Mascot (matrix science) search engine.

**Fluorogenic substrate assay for recombinant GALC:** Culture medium containing recombinant GALC was diluted 2:1 (v/v) with McIlvaine buffer (10 μL, pH 4.3) and exposed to the indicated concentrations of compounds 1–4 (10 μL 2× solution in H2O) for 30 min at 37 °C, before addition of substrate mix (100 μL, 0.23 mg mL−1 4-methylumbelliferyl-β-d-galactopyranoside in McIlvaine buffer (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 and fluorescence was measured as described above. Displayed values represent mean values from six experiments, and error bars indicate SD.

For tests of pH dependence, culture medium containing recombinant GALC was diluted 2:1 (v/v) with McIlvaine buffer (10 μL, pH 4.3) and mixed with substrate mix of various pH values (100 μL, 0.23 mg mL−1 4-methylumbelliferyl-β-d-galactopyranoside in McIlvaine buffer (pH 3–8)/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, and fluorescence was measured as described above. Displayed values represent mean values from six experiments, and error bars indicate SD.

**Fluorogenic substrate assay for recombinant a-galactosidase A:** Fabrazyme was diluted to a concentration of 0.1 ngl−1 in McIlvaine buffer (pH 4.6)/H2O (1:1, v/v) containing 0.1% BSA for stabilization of the recombinant protein. A solution of Fabrazyme (1 ng, 20 fmol per experiment, 10 μL, 0.1 ng mL−1) was exposed to the indicated concentrations of compounds 1–4 (10 μL 2× solution in H2O) for 30 min at 37 °C, before addition of substrate mix (100 μL, 1.5 mgmL−1 4-methylumbelliferyl-a-d-galactopyranoside in McIlvaine buffer (pH 4.6)/H2O (1:1, v/v) containing 0.1% BSA). After incubation at 37 °C for 20 min, the reaction was quenched glycine (0.3 M) adjusted with NaOH to pH 10.6, and fluorescence was measured by using an LS55 fluorometer (PerkinElmer) with λem = 366 nm and λem = 445 nm. 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.

**In vitro labeling assays with recombinant GALC:** In a typical experiment, culture medium containing recombinant GALC (5 μL) was diluted with McIlvaine buffer (pH 4.3)/H2O (2:1, v/v; 14 μL) and exposed to the indicated concentrations of ABPs 3 or 4 (1 μL of a 20× solution in DMSO) for 1 h at 37 °C. Labeling of denatured enzyme was performed by pre-heating the enzyme to 100 °C for 3 min in buffer with or without 1% SDS (9 μL total volume) before addition of the probes. One half of each sample was resolved on 10% SDS-PAGE. In-gel visualization of the fluorescent labeling by probe 3 was performed directly on the wet gel slabs. In the case of probe 4, biotinylated proteins were detected by performing streptavidin western blotting.

**Competition assays:** Culture medium containing recombinant GALC (5 μL) in McIlvaine buffer (pH 4.3)/H2O (2:1, v/v; 14 μL) was first exposed for 1 h at 37 °C to either 1 (0.2 μM), 2 (10 μM), or 4 (10 μM, all 1 μL of 20× solution in DMSO), before labeling with 3 (0.2 μM, 1 μL of 4 μM stock in DMSO) as described above.

**pH-dependent labeling assay:** Culture medium containing recombinant GALC (5 μL) was diluted with McIlvaine buffers (pH 3–8)/H2O (2:1, v/v; 14 μL) and labeled with ABP 3 (0.5 μM; 1 μL of 10 μM stock in DMSO) for 1 h at 37 °C. Gel bands were quantified by using Image Lab 4.1 (Bio-Rad) software. Displayed values represent mean values (± SD) from four independent experiments.

**In vitro labeling assays with recombinant GALC and glucocerebrodase:** Recombinant glucocerebroidase (Cerezyme) in McIlvaine buffer (pH 5.2)/H2O (1:1, v/v; 9 μL 0.22 μM, 2.0 pmol, 0.12 μg per experiment) or culture medium containing recombinant GALC (2.5 μL) in McIlvaine buffer (pH 4.3)/H2O (2:1, v/v; 6.5 μL) were exposed to either 1 μM of epoxide 3 or 1 μM of epoxide 4 (μL of 10 μM stock in DMSO) for 1 h at 37 °C. The reaction mixtures were then resolved on 7.5% SDS-PAGE, and in-gel visualization of the fluorescent labeling was performed directly on the wet gel slabs.

**In vitro labeling assay with mouse tissue homogenates:** Animals were first anesthetized with a dose of Hypnorm (0.315 mgmL−1 1
fluanisone) and Dormicum (5 mg mL⁻¹ midazolam). The given dose was 80 µL/10 g body weight. Animals were sacrificed by cervical dislocation. Tissues were collected, snap frozen in liquid N₂, and stored at −80 °C. Later, homogenates from the frozen material were made in KPi buffer (25 mM, pH 6.5), supplemented with 0.1% (v/v) Triton X-100 and protease inhibitors, and protein concentration was determined (BCA kit, Pierce). For labeling experiments, a volume of tissue homogenate equivalent to 50 µg (for kidney, brain and liver) or 25 µg of protein (for sciatic nerve) was completed to 10 µL with water and incubated for 30 min at 37 °C with 1 µM of ABP 3 (10 µL 2 µM in McIlvaine buffer 150 mM pH 4.3) or 100 nM of ABP 5 (10 µL 200 nM in McIlvaine buffer 150 mM pH 5.2, 0.2% (w/v) sodium taurocholate, 0.1% (v/v) Triton X-100). The samples were then resolved on 7.5% SDS-PAGE and analyzed as described above. For competition experiments the indicated concentrations of psychosine (7) and ABB166 (6) dissolved in water were pre-incubated with the tissue lysate for 30 min on ice before addition of ABP 3 (final concentration 1 µM) and subsequent incubation at 37 °C for 15 min.

Fluorescent substrate assay with mouse tissue homogenates: For measurement of β-galactosidase and β-galactocerebrosidase activity, we followed the protocol previously described by Martin et al. with slight alterations. Briefly, a volume of tissue lysate equivalent to 7.5 µg of protein was completed with water (6.25 µL total volume) and exposed to the indicated concentration of ABP 3 or inhibitor 1 (6.25 µL of a 2x solution in McIlvaine buffer, pH 4.3) for 60 min at 37 °C. Afterwards, AgNO₃ (12.5 µL of 110 µM solution in water; 11 µM) and substrate mix (100 µL, 0.23 mg mL⁻¹ 4-methylumbelliferyl-β-D-galactopyranoside in McIlvaine pH 4.3) were added, and the mixture was incubated for 45 min (for β-galactosidase) or 90 min (for GALC) at 37 °C. The reaction was then quenched with glycine (0.3 M) adjusted with NaOH to pH 10.6, and the fluorescence was measured with a fluorimeter as described above. Data (n = 3 per group) represent mean ± SD. Statistical analysis was performed by using one-way ANOVA, followed by Dunnett’s test.

ABP intra-cerebroventricular administration: For this purpose, we followed a previously described procedure. Briefly, intra-cerebroventricular stainless steel guide cannulas were implanted in the lateral ventricle by using the following stereotaxic coordinates: AP: 0.3, L 1.0, and V:−2.2. After a recovery period of seven days, a needle connected to a tube was introduced in the guide cannula and a solution of ABP 3 (1 mM in PBS) was administered at a rate of 0.1 µL min⁻¹ for 10 min. After 2 h, the animals were sacrificed by CO₂, euthanasia and transcardially perfused with 250 mL of 0.9% saline solution. Brains were isolated and immediately frozen for further biochemical and histological analysis.

Fluorescent and immuno-histochemical analysis of brain sections: Brains were cut in 30 µm slices with a cryostat and attached to SuperFrost slides (Thermo Scientific). All of the following steps were performed in the dark to protect the fluorescence of the ABPs. Slides were extensively washed in 0.01 M TBS to remove non-specific fluorescent and were covered in DAPI (Vector Lab, Burlingame, USA) mounting media.

For the immuno-histochemical analysis, brain slices were also extensively washed in TBS and then incubated overnight in TBS with 0.5% (v/v) Triton X-100, 0.025% (v/v) gelatin, and rabbit-raised primary antibodies against LAMP1 (Millipore) and GALC (12887-1-AP, Proteintech, Chicago, IL, USA) at a concentration of 1:1000. After primary antibody incubation, slides were incubated for 2 h with the appropriate secondary antibodies (1:10000) conjugated with fluorescent dyes: donkey anti-rabbit Alexa 488/Alexa 594 (Life Technologies). The slides were then rinsed three times with TBS, mounted, and covered with DAPI to be observed in a confocal laser scanning microscope (Leica SP5). Images were taken with λex = 488 nm for Alexa 488 and 561 nm for Alexa 594 and ABP 3. To capturing images, a 63x objective was used. In the case of single-cell images, a further threefold magnification was used.

Fluorescence microscopy: HEK293 cells with and without stable overexpression of murine GALC were cultured on glass slides precoated with poly-L-lysine. Cells were incubated with ABP 3 (5 nM) in the medium for 1 h. Next, the cells were washed twice with PBS, and new medium was added. The cells were placed in the incubator overnight (approximately 16 h) before being washed again and then mounting the glass slides without fixation. For LAMP1 staining, the cells were fixed with 3% paraformaldehyde (v/v) before being washed and blocked in donkey serum. Afterwards, the cells were stained with LAMP1 antibody for 1 h, washed and incubated with the secondary Alexa 594-conjugated antibody (see above). The cells were observed by using a confocal laser scanning microscope (Leica SP5) as described previously.

Fibroblast concanavalin A affinity purification and ABP labeling: Cell pellets (from T-75 cm² flasks) of fibroblasts from patients and healthy volunteer were lysed in KPi buffer (pH 6.5, 100 µL, 25 mM), supplemented with 0.1% (v/v) Triton X-100 and protease inhibitors. The lysate was incubated overnight at 4 °C with pre-washed concanavalin A beads (200 µL, Sigma–Aldrich). The beads were then washed with wash buffer according to the manufacturer’s instructions. Bead-bound glycoproteins were labeled with ABP 3 (1 µM) in McIlvaine buffer (pH 4.3) for 1 h at 37 °C, followed by labeling for 30 min with ABP 5 (0.1 µM) in McIlvaine buffer (150 mM, pH 5.5, 0.2% (w/v) sodium taurocholate, 0.1% (v/v) Triton X-100). The samples were boiled in the presence of 5× Laemmli sample buffer and resolved by 10% SDS-PAGE.

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