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A specific activity-based probe to monitor family GH59 galactosylceramidase – the enzyme deficient in Krabbe disease

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Abstract: 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 visualizing active enzyme in cells and tissues. The 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 intracerebroventricular infusion of the ABP. In conclusion, the GALC-specific ABP should find broad applications in diagnosis, drug development and evaluation of therapy of Krabbe disease.

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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 via 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 *β*-anomeric configuration of the released galactopyranoside (Figure 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.[1,3]

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 system. The mechanism behind this neuropathology has not yet been fully elucidated.[1,2] Reduced activity of GALC results in a reduced catabolism of galactosphingolipids including galactosylceramide and galactosylsphingosine (psychosine). Galactosylceramide is the main lipid component of myelin, the protective sheath around neuron axons and essential for correct functioning of the nervous system. Accumulation of the toxic metabolite galactosylsphingosine eventually leads to apoptosis of myelin-forming cells and consequently demyelination and neurodegeneration.[4,5] Infantile Krabbe disease is usually diagnosed before one year of age and lethal before the age of two. Early symptoms include limb stiffness, developmental delay, and severe irritability. When diagnosed in adolescents or adults other symptoms may 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

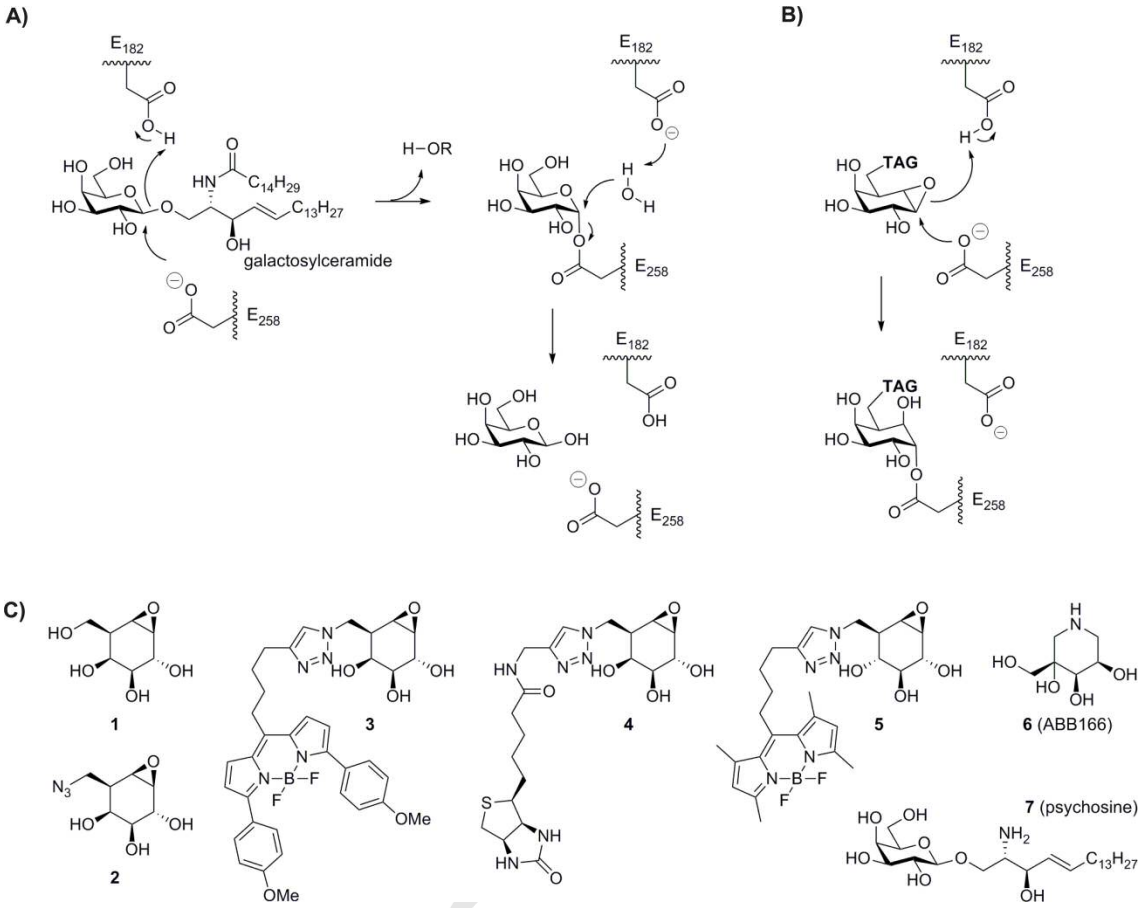

Figure 1. Galactocerebrosidase and structures of inhibitors and probes **1**-**7**. A) Mechanism of substrate hydrolysis by GALC. B) Proposed mechanism of GALC binding by compounds **1**-**4**. C) Structures of novel retaining *β*-galactosidase inhibitor **1** and ABPs **2**-**4**. *β*-Glucopyranosyl-configured compound **5** targets glucocerebrosidase. ABB166 (**6**)[31] and galactosylsphingosine (psychosine, **7**) are competitive GALC inhibitors.

the same mutation.[6,7] 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 may 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.[8,9]

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 can therefore not 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,[10-16] and fluorescently tagged competitive inhibitors.[17,18] In addition, a few ABPs that enable non-reversible 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[19–22] and 2-fluorogalactoside inhibitors in which the hydroxyl group at C6 is substituted with an azide, which enables two-step labeling via Staudinger-Bertozzi ligation.[23] 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 uses the natural product and retaining *β*-glucosidase inhibitor, cyclophellitol, as starting point.[24] Substituting the epoxide for an aziridine and grafting a reporter group onto the aziridine nitrogen yields ABPs broadly selective for various members within a given class of retaining glycosidases. Glycosidase family-selectivity is dictated by the configuration of the cyclophellitol aziridine derivative, an approach that was shown valid for GH1 retaining *β*-glucosidases, GH79 retaining *α*-galactosidases and, most recently, for GH29 retaining *α*-fucosidases.[25–27] In our first forays into activity-based glycosidase profiling, however, we studied cyclophellitol derivatives modified at C6 (glucopyranose

numbering) with a fluorophore.[28] These epoxide probes exhibit much higher selectivity than their aziridine 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.[28]

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 (Figure 1B), the more so since GALC and GBA have related glycosylceramide substrates. Here we describe the evaluation of *β*-galactopyranose-configured epoxides **1**-**4** as inhibitors and ABPs for GALC (Figure 1C). The cyclophellitol core of these probes, synthesized as reported,[24,29,30] was designed to mimic the substrate's terminal galactosyl moiety and bind covalently to the target enzyme via nucleophilic attack of the catalytic residue in the active site on the *β*-configured electrophilic epoxide moiety (Figure 1B). The non-tagged inhibitor **1** was included in our studies as a galactose-configured isomer of the known retaining β-glucosidase inhibitor cyclophellitol.[24,28] For potential use in two-step activity based profiling studies, in ABP **2** the primary hydroxyl group is substituted with an azide that can be used for two-step labeling via copper(I)-catalyzed or copper-free strain promoted alkyne-azide [2+3] cycloaddition chemistry or Staudinger-Bertozzi ligation. In addition, this probe may 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 galactocerebrosidase (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 2A). 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 hydroxyl 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 (*ki*/*K*i) of compounds **1** and **3** were in agreement with this observation (see Table 1 and Supporting Figure 1). 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*-glucosidases by analogous C6-modified probes, although the beneficial effect of the Bodipy dye was much larger in that case.[28] 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,[26] indicating that this phenomenon is dependent on the specific active site features and substrate tolerance of each individual glycosidase.

To demonstrate the non-reversibility of GALC inhibition by ABP **3** we pre-incubated 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 (Supporting Figure 2). Enzyme activity was irreversibly lost during the pre-incubation in time- and concentration-dependent manner. Loss of activity of GALC by pre-incubation with ABP **3** correlated with its fluorescent labeling. To further demonstrate irreversibility of labeling, we incubated GALC with ABP **3** for one hour and separated enzyme and small compound using a spin dialysis cartridge with 7 kDa cutoff. No activity was found to be recovered over time after removal of free unbound ABP **3** from the enzyme (Supporting Figure 3). We also incubated enzyme and ABP **3** 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 ABP **3**. Analysis of labeled GALC revealed no loss of label following separation of pre-labeled enzyme from free ABP **3** (Supporting Figure 3). In order to assess the selectivity of inhibitors **1**-**4** for retaining *β*-galactosidases over the related class of retaining *α*-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** are able to irreversibly inhibit GALC, we next assessed the visualization of the recombinant enzyme on gel using fluorescently labeled ABP **3**.

Table 1. Inhibition of recombinant galactocerebrosidase activity.

Compound	Apparent IC_{50} (μM)[a]	*ki*/*K*i ($\mu M^{-1}min^{-1}$)	Percentage inhibition (%) 30 min	6 hrs
Epoxide **1**	0.038	12.06	98 (1 μM)	100 (1 μM)
Azido-epoxide **2**	70	N.A.	62 (100 μM)	96 (100 μM)
Bodipy-epoxide **3**	2.8	0.014	97 (100 μM)	100 (100 μM)
Biotin-epoxide **4**	30	N.A.	81 (100 μM)	97 (100 μM)

[a] Inhibition of recombinant GALC as determined from hydrolysis of 4-MU *β*-gal after pre-incubation for 30 min or 6 h with probes **1**-**4**. Apparent IC_{50} values were calculated from residual GALC activity as a function of probe concentration (Figure 2A). Second order rate constants were calculated from progress curves over time (Supporting Figure 1).

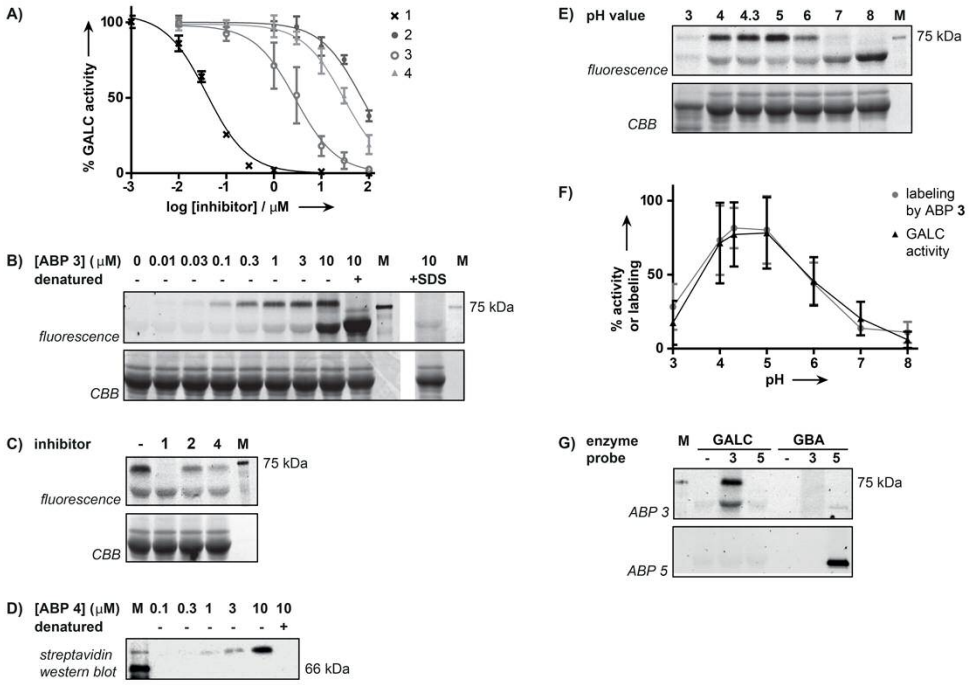

Figure 2. Labeling and inhibition of recombinant GALC. A) Inhibition of recombinant GALC activity. Recombinant GALC was treated for 30 min with inhibitors **1**-**4** after which residual activity was determined from hydrolysis of 4-MU *β*-gal. B) Labeling with 0.01 - 10 µM of Bodipy-epoxide **3** for 1 h; where indicated enzyme was denatured prior to labeling by boiling at 100°C for 3 min in assay buffer (+) or in buffer with 1% SDS (+SDS). C) Labeling with 0.2 µM **3** after pre-incubation with inhibitors **1** (0.2 µM), **2** (10 µM) or **4** (10 µM) for 1 hr. D) Labeling with 0.1 - 10 µM of biotin-epoxide **4** for 1 h; where indicated enzyme was denatured prior to labeling by boiling at 100°C for 3 min. E) Recombinant GALC was labeled with 0.5 µM **3** for 1 h in buffers of varying pH. Samples were analyzed by 10% SDS-PAGE with fluorescent readout followed by Coomassie brilliant blue (CBB) staining (B, C, E) or streptavidin western blotting (D). 'M': protein marker. F) Quantification of gel bands in A (circles) as compared to GALC activity on 4-Mu *β*-Gal (triangles) at different pH values. G) Selective labeling of retaining *β*-galactosidases and retaining *β*-glucosidases with differently configured epoxide ABPs. Recombinant GALC and glucocerebrosidase (GBA) were labeled with 1 µM of ABP **3** or ABP **5** for 1 h and labeled proteins were resolved by 7.5% SDS-PAGE with fluorescent readout.

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 2B). 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 ABP **3** 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**, while the labeling of albumin was unaffected (Figure 2C). 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 2D). In agreement with the higher IC_{50} value of this probe as compared to its fluorescently labeled analogue **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

Since GALC is a lysosomal enzyme, its activity is highest in a slightly acidic environment with an optimum around pH 4.3.[3] 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 2E). The intensity of fluorescent labeling of GALC is highest at pH 4-5 while 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 2F). These results indicate that binding of the probe occurs in an activity-based manner and support the proposed binding mechanism depicted in Figure 1B.

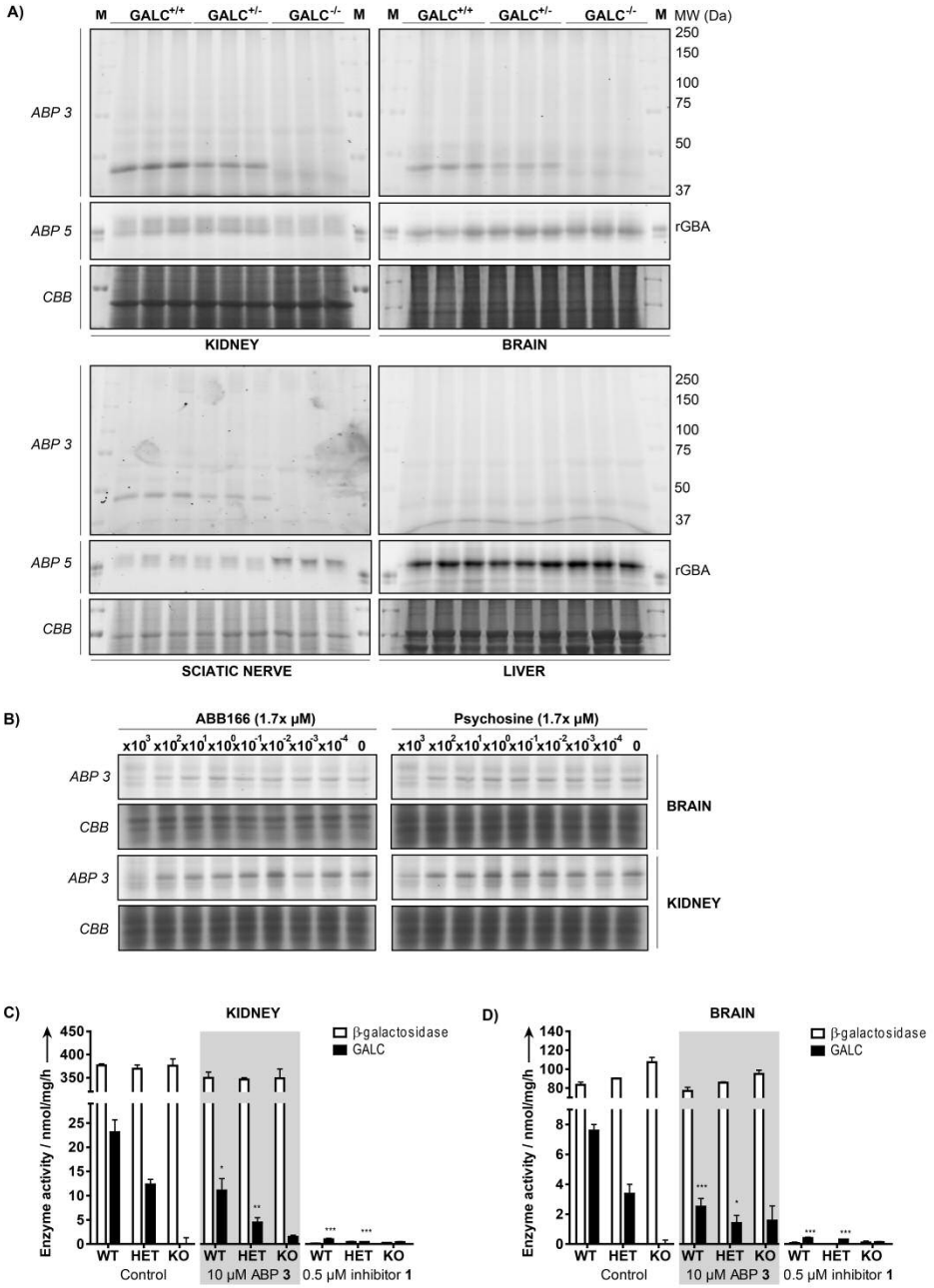

Figure 3. Labeling of tissues of wt, GALC-deficient (Twitcher) and heterozygous mice. A) Homogenates of brain, kidney, sciatic nerve and liver of wt (Galc+/+), carrier (Galc+/-), and Twitcher (Galc-/-) mice were incubated with ABP **3** (1 µM) or ABP **5** (100 nM). Samples were analyzed by 7.5% SDS-PAGE with fluorescent readout followed by Coomassie brilliant blue (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 using one-way ANOVA followed by the Dunnett's multiple comparison test: * P < 0.05; ** P < 0.01; *** P < 0.001.

Selectivity of galactocerebrosidase labeling

Next we set out to validate our hypothesis that selective labeling of retaining *β*-galactosidases and retaining *β*-glucosidases can 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] (Figure 1C) to label either recombinant GALC or recombinant glucocerebrosidase, a lysosomal retaining *β*-glucosidase. The two ABPs are functionalized with different Bodipy fluorophores and can therefore be visualized using different scanner settings for in-gel fluorescent readout. While GALC was labeled exclusively by probe **3**, glucocerebrosidase was labeled by ABP **5** but not by its

stereoisomer **3** (Figure 2G). The absence of cross-reactivity demonstrates 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 3A). 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 3A). 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 reveals the specific labeling of several peptides from the *N*-terminal part of the active 50 kDa GALC protein by ABP **4** (see Supporting Table 1).

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

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 the 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 $AgNO_3$ 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 significant decrease of GALC activity (approximately 50% of untreated values) in these tissues, while *β*-galactosidase activity was unaltered (Figure 3C 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 HEK293 cells with LPH and studied labeling of the enzyme by ABP **3** and ABP **5** using a concentration of this probe at which it is known to target LPH (Supporting Figure 4).[28] Whilst the *β*-glucosidase ABP **5** gave 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** is able to penetrate cells by diffusion and efficiently labels lysosomal glucocerebrosidase *in situ*.[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 non-fixed cells. The fluorescent labeling in control HEK293 cells was low (Figure 4A), in agreement with the virtual absence of endogenous GALC labeling in HEK cells extracts (see Supporting Figure 4). Low expression levels of GALC were further confirmed by western blot (not shown). However, in cells overexpressing GALC a perinuclear vesicular labeling, characteristic for lysosomes, was observed (Figure 4A). The perinuclear vesicular labeling pattern of lysosomes was confirmed by labeling of fixed cells with the lysosomal marker LAMP1 (Figure 4A, last panel).

Next, we examined ABP **3** labeling of endogenous GALC in the brain of living mice. For this purpose, we intracerebroventricularly infused mice for 2 hours 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 immunohistochemical detection of GALC and lysosomal marker LAMP1 (Figure 4B).

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 subject and Gaucher disease patient (Figure 5), being virtually absent in samples from the individual diagnosed with Krabbe disease. As expected, active GBA labeled by ABP **5** was much less prominent in fibroblasts of Gaucher disease patient compared to the other cell types (Figure 5).

Discussion and conclusions

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,[28] we examined a similar approach for the related enzyme galactocerebrosidase (GALC, 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

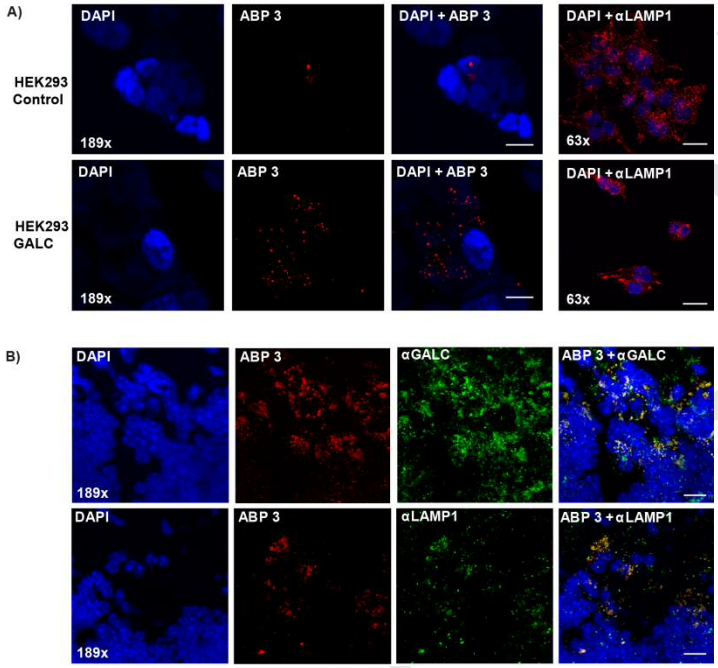

Figure 4. *In situ* labeling of GALC in intact cells in culture and *in vivo* in mouse brain. A) *In situ* labeling with ABP **3** of control HEK293 cells (top) and HEK293 cells overexpressing GALC (bottom). Left to right: nuclear DAPI staining, ABP **3** labeling, and overlay of DAPI staining and ABP **3** labeling (all in non-fixed cells), and overlay of immuno-detection of lysosomal membrane marker LAMP1 and DAPI staining in fixed cells. Scale bars in third panels are 10 µm, scale bars in right panels are 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.

epoxide inhibitor **1** is the most potent inhibitor. Substitution of the hydroxyl group at C6 with an azide (**2**) results in loss of potency, whereas installment of a Bodipy tag at the same position (**3**) partially restores the inhibitory potency. A high resolution crystal structure of murine GALC has recently been reported.[3] Soaking of crystalized enzyme with ABP **3** should yield an explanation for the noted differences in affinity of inhibitor **1** and ABPs **2**-**4**.

Bodipy-functionalized ABP **3** and biotinylated ABP **4** enable the visualization of catalytically active GALC on gel. The epoxide probes label the enzyme in an activity-based manner. The selectivity of the labeling by ABP **3** is remarkable. In particular, Bodipy-epoxide **3** differs only in the configuration of a single substituent from the previously reported glucopyranose configured stereoisomer **5**, yet the two probes enable selective targeting of GALC and glucocerebrosidase, respectively. At low concentrations, ABP **3** neither targets the related glycosidase *α*-galactosidase A, and importantly none of the other retaining *β*-galactosidases known to be present in humans, lysosomal acid *β*-galactosidase (EC 3.2.1.24) and intestinal lactase-phlorizin hydrolase (LPH) (E.C. 3.2.1.106). Of note, we earlier noted the same high degree of selectively for Bodipy-functionalized *β*-glucopyranose-configured epoxide **5**.[28] At low concentration, this probe selectively labels lysosomal glucocerebrosidase but not the non-lysosomal glucosylceramidase GBA2, cytosolic *β*-glucosidase GBA3 and LPH,[28] all other enzymes degrading glucosylceramide.

GALC is synthesized as 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 via disulfide bridges.[1] The cleavage of the already active 80 kDa precursor does not affect the enzymatic activity.[41] In line with this, we found that recombinantly produced 80 kDa precursor GALC labels well with ABP **3**. In lysates of GALC overexpressing HEK293 cells we could detect both 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, is 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 may 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.

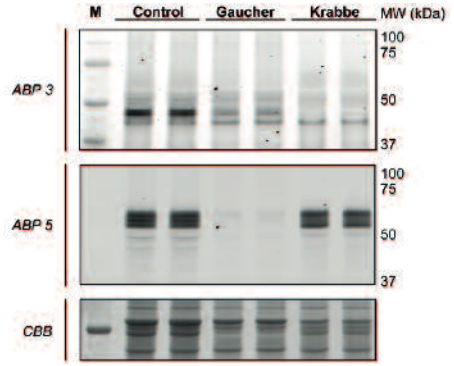

Figure 5. Labeling of Gaucher and Krabbe patient fibroblasts with ABPs **3** and **5**. Fibroblasts from Gaucher and Krabbe disease patients, and healthy volunteer were lysed and incubated overnight with Concanavalin A beads for enrichment of glycosylated proteins. Bead-bound GALC was labeled with ABP **3** (1 μM) followed by labeling of GBA with ABP **5** (0.1 μM). Samples were resolved by 10% SDS-PAGE with fluorescent readout followed by Coomassie brilliant blue (CBB) staining. 'M': protein marker.

Experimental Section

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

The synthesis of *β*-galactopyranose-configured cyclophellitol epoxides **1**-**4** has been reported.[24]

Enzymes

Recombinant murine galactocerebrosidase (GALC) was expressed in human embryonic kidney 293 (HEK293) cells as previously described.[1] The mouse enzyme is 83% homologous to human GALC. [1] The 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

Human embryonic kidney 293 (HEK293) cells (ATCC CRL 1573) 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 over-expression of murine GALC were generated as previously described.[1]

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. The heterozygous C57BL/6J B6.CE-*Galc*^{twi}/J mice (stock number 000845) were obtained from The Jackson Laboratory (Bar Harbor, USA). Mouse pups were genotyped as previously described.[33] Mice (± 3 weeks old) received the rodent AM-II diet (Arie Blok Diervoeders, Woerden, The Netherlands). Animals were housed and experiments were conducted according to approved protocol by the Institutional Animal Welfare Committee of the Academic Medical Centre Amsterdam in the Netherlands.

Twelve week-old wt C57Bl/6 mice were used for intracerebroventricular (i.c.v.) administration of ABP **3**. The animals were kept in individual cages at constant temperature (23°C+/- 2 °C) and a 12/12h light/dark cycle. They were exposed to ad libitum food and water before and after the experimental procedures.

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. 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 in 25 mM potassium phosphate (KPi) buffer (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 5x Laemmli sample buffer containing 2-mercaptoethanol (1/4th 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 using the Typhoon Variable Mode Imager (Amersham Biosciences, Piscataway, NJ, USA), using λ_{ex} 488 nm and λ_{em} 520 nm (band pass 40) for green fluorescent ABP **5** and λ_{ex} 532 nm and λ_{em} 610 nM (band pass 30) for red fluorescent ABP **3**. As a loading control, gels were stained with Coomassie Brilliant Blue (CBB) and de-stained with milliQ water.

Western blotting

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 Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) for 1 h at room temperature, hybridized with Streptavidin-HRP for 1 h at room temperature (1:10,000 in blocking buffer) (Molecular Probes, Life Technologies), washed with TBST and TBS and then visualized using an ECL+ western blotting detection kit (Amersham Biosciences). Protein standards are PageRuler Plus Prestained Protein Ladder (Thermo Scientific) and biotinylated protein marker (Bio-Rad).

Proteomics

One kidney from a 5 week old wt mouse was homogenized in 150 mM McIlvaine buffer, pH 4.5 supplemented with protease inhibitors. From the homogenate 100 μL (containing 3.5 mg protein) were incubated for 2 h at 37°C with 100 μL of 0.18 mM ABP **4** in 150 mM McIlvaine buffer pH 4.5. The analysis was performed as previously reported.[42] Peptides were desalted on stage tips[43] and analyzed with a trap-elute system on C18 reversed phase nano LC with a 45 min 10-60% ACN/0.1% formic acid gradient, hyphenated to a Thermo LTQ-orbitrap mass spectrometer using a top 3 data dependent protocol at 60.000 resolution, m/z range 300-2000, 1000 msec fill time in the Orbitrap, for MS/MS fragmentation 35 units of CID energy, 120 msec max fill time, AGC 50 e3 and a threshold of 750 counts. Ions of z = 2+ and higher were selected to be fragmented

twice within 10 sec prior to exclusion for 150 sec. Peak lists were extracted and searched against the Uniprot mouse (decoy) database, with carbamidomethylation of cysteine as fixed and oxidation of methionine as variable modifications, 20 ppm peptide tolerance, trypsin as protease and 2 missed cleavages allowed using a Mascot (matrix science) search engine.

Fluorogenic substrate assay of recombinant GALC

Culture medium containing recombinant GALC was diluted 2/1 (v/v) with McIlvaine buffer pH 4.3 (10 µL total volume) and exposed to the indicated concentrations of compounds **1**-**4** (10 µL 2x solution in H_2O) for 30 min at 37°C, before addition of 100 µL substrate mix (0.23 mg/mL 4-methylumbelliferyl-*β*-*D*-galactopyranoside in McIlvaine pH 4.3/H_2O 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 NaOH-glycine, pH 10.6 and fluorescence was measured with a fluorimeter LS55 (Perkin Elmer) using λ_{ex} 366 nm and λ_{em} 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. Displayed values represent mean values from triplicate experiments and error bars indicate standard deviation (SD). Graphpad Prism 5 software was used to determine apparent IC_{50} values.

For tests of pH-dependence, culture medium containing recombinant GALC was diluted 2/1 (v/v) with McIlvaine buffer pH 4.3 (10 µL total volume) and mixed with 100 µL substrate mix of various pH values (0.23 mg/mL 4-methylumbelliferyl-*β*-*D*-galactopyranoside in McIlvaine pH 3 - pH 8/H_2O 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 measured as described above. Displayed values represent mean values from 6 experiments and error bars indicate standard deviation (SD).

Fluorogenic substrate assay of recombinant *α*-galactosidase A

Fabrazyme was diluted to a concentration of 0.1 ng/µL in McIlvaine pH 4.6/H_2O 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/µL) was exposed to the indicated concentrations of compounds **1**-**4** (10 µL 2x solution in H_2O) 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/H_2O 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 NaOH-glycine, pH 10.6 and fluorescence was measured with a fluorimeter LS55 (Perkin Elmer) using λ_{ex} 366 nm and λ_{em} 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 using recombinant GALC**

In a typical experiment, culture medium containing recombinant GALC (5 µL) was diluted with McIlvaine buffer pH 4.3/H_2O 2/1 (v/v) (14 µL) and exposed to the indicated concentrations of ABPs **3** or **4** (1 µL 20x 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 in the wet gel slabs directly. In 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/H_2O 2/1 (v/v) (14 µL) was first exposed for 1 h at 37°C to either 0.2 µM of **1**, 10 µM of **2** or 10 µM of **4** (1 µL 20x solution in DMSO), before labeling with 0.2 µM **3** (1 µL 4 µM in DMSO) as described above.

pH-dependent labeling assay

Culture medium containing recombinant GALC (5 µL) was diluted with McIlvaine buffers of pH 3 - pH 8/H_2O 2/1 (v/v) (14 µL) and labeled with 0.5 µM of ABP **3** (1 µL 10 µM in DMSO) for 1 h at 37°C. Gel bands were quantified using Image Lab 4.1 (Bio-Rad) software. Displayed values represent mean values (± SD) from 4 independent experiments.

***In vitro* labeling assays using recombinant GALC and glucocerebrosidase**

Recombinant glucocerebrosidase (Cerezyme) in McIlvaine buffer pH 5.2/H_2O 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/H_2O 2/1 (v/v) (6.5 µL) were exposed to either 1 µM of epoxide **3** or 1 µM of epoxide **5**[28] (1 µL 10 µM 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 in the wet gel slabs directly.

***In vitro* labeling assay using mouse tissue homogenates**

Animals were first anesthetized with a dose of Hypnorm (0.315 mg/mL phenyl citrate and 10 mg/mL fluanisone) and Dormicum (5 mg/mL midazolam). The given dose was 80 µL/10 g body weight. Animals were sacrificed by cervical dislocation. Tissues were collected, snap frozen in liquid N_2 and stored at -80°C. Later, homogenates from the frozen material were made in 25 mM KPi buffer, 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.

Fluorogenic substrate assay using mouse tissue homogenates

For measurement of *β*-galactosidase and *β*-galactocerebrosidase activity we followed the protocol previously described by Martino et al.[38] 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 2x solution in McIlvaine buffer pH 4.3) for 60 min at 37°C. Afterwards 12.5 µL of 110 µM $AgNO_3$ in water (final concentration 11 µM) and 100 µL of substrate mix (0.23 mg/mL 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 2.5 mL 0.3 M NaOH-glycine 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 using one-way ANOVA followed by Dunnett´s test.

ABP intracerebroventricular administration

For this purpose, we followed the procedure previously described.[44] Briefly, intracerebroventricular stainless steel guide cannulas were implanted in the lateral ventricle using the following stereotaxic coordinates: AP 0.3, L +1.0 and V -2.2. After a recovery period of 7 days, a needle connected to a tube was introduced in the guide cannula and a solution of ABP **3** (1 nM in PBS) was administered with a rate of 0.1 µL/min for 10 minutes. After 2 hours, the animals were sacrificed by CO_2 euthanasia and transcardially perfused with 250 mL of 0.9% of saline solution. Brains were isolated and immediately frozen for further biochemical and histological analysis.

Fluorescent and immunohistochemical analysis of brain sections

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

For the immunohistological analysis, brain slices were also extensively washed in TBS, and next 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, MA, USA) and GALC (12887-1-AP, Proteintech, Chicago, IL, USA) in a concentration of 1:1000. After the primary antibody incubation, slides were incubated for 2 h with the appropriate secondary antibodies (1:1000) conjugated with fluorescent dyes; donkey anti rabbit Alexa 488/Alexa 594 (Life Technologies, Paisley, UK). 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 made using an excitation wavelength of 488 nM for Alexa 488, and 561 nM for Alexa 594 and ABP **3**. For capturing of pictures a 63x objective was used. In the case of single cell images a further zoom of 3 times was used.

Fluorescence microscopy

HEK293 cells with and without stable over-expression of murine GALC were cultured in glass slides pre-coated with poly-L-lysine. Cells were incubated with 5 nM of ABP **3** in the medium for 1 h. Next the cells were washed with PBS twice and new medium was added. The cells were placed in the incubator overnight (approximately 16 h) before being washed again and the glass slides mounted 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 using a confocal laser scanning microscope (Leica SP5) as described previously.[25]

Fibroblast Concanavalin A affinity purification and ABP labeling

Cell pellets (from T-75cm^2 flasks) of fibroblasts from patients and healthy volunteer were lysed in (100 µL) 25 mM KPi buffer, pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitors. The lysate was incubated overnight at 4°C with 200 µL of pre-washed Concanavalin A beads (Sigma-Aldrich). The beads were then washed with wash buffer according to manufacturer's instructions. Bead-bound glycoproteins were labeled with 1 µM of ABP **3** in McIlvaine buffer pH 4.3 for 1 h at 37°C followed by labeling for 30 min with 0.1 µM of ABP **5** 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 5x Laemmli sample buffer and resolved by 10% SDS-PAGE.

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Keywords: galactosylceramidase • hydrolase • Krabbe disease • inhibitor • fluorescent probes

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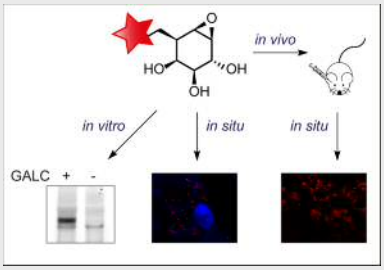
Entry for the Table of Contents (Please choose one layout)

FULL PAPER

We report the design and application of a fluorescently labeled *β*-galactopyranose-configured cyclophellitol-epoxide probe. The activity-based probe allows sensitive detection of active molecules of the lysosomal *β*-galactosidase galactosylceramidase (GALC) *in vitro*, *in situ* and *in vivo*.



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A specific activity-based probe to monitor family GH59 galactosylceramidase – the enzyme deficient in Krabbe disease