Activity-based probes for retaining -glucosidases: Novel tools for research and diagnostics
Kallemeijn, Wouter

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Ultra-sensitive in situ visualization of active glucocerebrosidase molecules

Martin D. Witte\textsuperscript{a,*}, Wouter W. Kallemeijn\textsuperscript{b,*}, Jan Aten\textsuperscript{c}, Kah-Yee Li\textsuperscript{a}, Anneke Strijland\textsuperscript{b}, Wilma E. Donker-Koopman\textsuperscript{b}, Adrianus M. C. H. van den Nieuwendijk\textsuperscript{a}, Boris Bleijlevens\textsuperscript{b}, Gertjan Kramer\textsuperscript{b, f}, Bogdan I. Florea\textsuperscript{a}, Berend Hooibrink\textsuperscript{d}, Carla E. M. Hollak\textsuperscript{c}, Roelof Ottenhoff\textsuperscript{b}, Rolf G. Boot\textsuperscript{b}, Gijsbert A. van der Marel\textsuperscript{a}, Herman S. Overkleeft\textsuperscript{a} and Johannes M. F. G. Aerts\textsuperscript{b}

* These authors contributed equally to this work.

Published in Nature Chemical Biology 6, 907–913 (2010)

Graphical abstract – Cyclophellitol β-epoxide type activity-based probes exploit the catalytic double-displacement mechanism of GBA to covalently, irreversibly modify its nucleophile, enabling ultra-sensitive fluorescence detection of the enzyme in situ. Figure partially modified from the publication-featuring ‘News & Views’-editorial ‘Getting lucky in the lysosome’ (Goddard-Borger ED, et al. (2010) Nat Chem Biol. 6, 881–883).
Abstract

Deficiency of glucocerebrosidase (GBA) underlies Gaucher disease, a common lysosomal storage disorder. Carriership for Gaucher disease has recently been identified as major risk for Parkinsonism. Presently, no method exists to visualize active GBA molecules \textit{in situ}. We here report the design, synthesis and application of two fluorescent activity-based probes (ABPs) allowing highly specific labeling of active GBA molecules \textit{in vitro}, in cultured cells and in mice \textit{in vivo}. Detection of \textit{in vitro} labeled recombinant GBA on slab gels after electrophoresis is in the low attomoles. Using cell or tissue lysates, we obtained exclusive labeling of GBA molecules. We present evidence from fluorescence-activated cell sorting analysis, fluorescence microscopy and pulse-chase experiments of highly efficient labeling of GBA molecules in intact cells as well as tissues of mice. In addition, we illustrate the use of the fluorescent probes to study inhibitors and tentative chaperones in living cells.

Author affiliations

\textsuperscript{a}Department of Bio-organic Synthesis, Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands. Department of \textsuperscript{b}Medical Biochemistry, \textsuperscript{c}Pathology, \textsuperscript{d}Cell Biology and Histology, \textsuperscript{e}Endocrinology and Metabolism, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. \textsuperscript{f}Department of Mass Spectrometry, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands.
The lysosomal hydrolase glucocerebrosidase (GBA) hydrolyzes glucosylceramide\(^1\)\(^,\)\(^2\). This ubiquitously expressed enzyme is initially synthesized as a 519-residue protein that co-translationally acquires four N-linked glycans. After the removal of its signal peptide, GBA undergoes no further post-translational proteolytic modification and does not acquire mannose-6-phosphate moieties in the Golgi apparatus. The expression of disease in individuals with a defective GBA is remarkably heterogeneous. Substantial deficiency results in Gaucher disease, and recently carriership has been recognized as major risk for Parkinsonism\(^2\)\(^,\)\(^3\). The manifestation of Gaucher disease is highly variable, ranging from the common non-neuronopathic (type I) variant to more severe manifestations with lethal neurological complications (type II and III variants) and extreme cases with abnormalities in skin permeability (so-called ‘collodion babies’)\(^3\). The marked phenotypic heterogeneity is only partly explained by differences in underlying mutations in the GBA gene. The hetero-allelic presence of N370S GBA, the most frequent mutation in Caucasian individuals, protects against a neuronopathic manifestation, whereas homozygosity for L444P GBA is associated with severe neurological symptoms\(^3\). Several studies have indicated that the relationship between GBA genotype and Gaucher phenotype is not very strict\(^4\). Even phenotypic heterogeneity among identical twins has been reported, suggesting that additional factors influence the \textit{in situ} residual activity of GBA\(^5\).

Two treatments for Gaucher disease presently exist: enzyme therapy and substrate reduction therapy. Enzyme therapy is based on chronic intravenous administration of recombinant GBA (imiglucerase; trade name: Cerezyme\textsuperscript{TM}\(^6\)\(^,\)\(^7\)). Substrate reduction therapy is based on chronic oral administration of \(N\)-butyldeoxynojirimycin, an inhibitor of the enzyme glucosylceramide synthase, which catalyzes the formation of glucosylceramide\(^8\)\(^,\)\(^9\). More recently, an alternative approach has received considerable attention, so-called chaperone therapy. Common in Gaucher patients are mutant forms of GBA that show impaired folding and retention in the endoplasmic reticulum, ultimately resulting in elimination \textit{via} the ubiquitin-proteasome system, a process known as ER-associated degradation (ERAD)\(^10\)\(^–\)\(^15\). Studies have investigated small compounds, designated ‘chemical chaperones’, that are able to increase the amount of GBA by stabilizing and/or promoting folding of the enzyme. One extensively studied example is isofagomine (IFG), which is a potent competitive inhibitor interacting with the catalytic pocket\(^16\)\(^–\)\(^20\). Beneficial effects on the amount and lysosomal localization of mutant GBA forms in cultured cells have been reported for IFG, but the assays used to demonstrate increased degradative capacity have been quite artificial: cells are exposed to high concentrations of fluorogenic substrate at acidic pH\(^17\). It is not likely that exposing cells to low pH and millimolar concentrations of artificial 4-methylumbelliferyl \(\beta\)-\(D\)-glucopyranoside (4MU-\(\beta\)-\(D\)-Glc) substrate for a prolonged period reflects faithfully the \textit{in situ} ability of the enzyme to degrade glucosylceramide. Pharmacologic chaperones like IFG will only exert a positive clinical effect at a particular dose range: their concentration should be sufficiently high to promote folding of the enzyme in the endoplasmic reticulum to increase transport to lysosomes,
whereas the concentration in lysosomes should also be sufficiently low to prevent marked inhibition of catalytic activity.

The present lack of a suitable method for specific visualization of active GBA molecules is a major limitation in research on Gaucher disease and Parkinsonism, as well as the development of new therapies. For this reason, we embarked on the development of such a method utilizing the retaining β-glucosidase activity of GBA for its activity-based probe (ABP) labeling. Its catalytic double-displacement mechanism of its retaining β-glucosidase activity has been elucidated in detail (Fig 1a). Briefly, the unprotonated nucleophile residue E340 in GBA performs the initial nucleophilic attack on the anomeric C1 carbon in the β-D-glucose moiety; in parallel the acid/base E235 protonates the glycosidic bond oxygen, resulting in hydrolysis of the aglycone and concomitant formation of a covalently bound enzyme-substrate intermediate (see Fig 1a).

Epoxides like conduritol β-epoxide (CBE 1) and cyclophellitol 2 (Fig 1b) form first a non-covalent inhibitor–enzyme complex that then reacts with the E340 carboxylate to form a covalent bond, thus acting as irreversible inhibitors (Fig 1c). Cyclophellitol 2 resembles more closely the structure of D-glucoside substrates and is the more potent irreversible inhibitor of the two. We capitalized on this by grafting boron dipyrromethene difluoride (BODIPY) fluorophores on to the cyclophellitol core through a spacer (Fig 1b). We here demonstrate highly efficient labeling of GBA in situ by these probes and reveal their use in monitoring GBA activity in Gaucher fibroblasts.

**Figure 1 | Background.** (a) Double-displacement mechanism of the retaining β-glucosidase GBA. (b) Acid/base-dependent nucleophile-binding mechanism of cyclophellitol β-epoxides. (c) Structures of conduritol β-epoxide (CBE 1), cyclophellitol β-epoxide 2, β-epoxide ABP 3 (β-deoxy-β-azidocyclophellitol β-epoxide), β-epoxide ABP 4 (MDW933, green fluorescent), β-epoxide ABP 5 (MDW941, red), non-reactive probes 6 and 7, and reversible, competitive GBA inhibitor isofagomine (IFG) 8.
Results

Design and synthesis of activity-based probes – Cyclophellitol 2 and 8-deoxy-8-azidocyclophellitol ABP 3 were synthesized and their inhibitory properties toward recombinant GBA (Imiglucerase, Genzyme) were tested (see Fig 1b). Cyclophellitol 2 and its azido analog β-epoxide ABP 3 were found to be far more potent inhibitors of GBA than CBE 1. Click ligating BODIPY moieties to β-epoxide ABP 3 gave fluorescent inhibitors β-epoxide ABP 4 and β-epoxide ABP 5 (Fig 1b). Examination of the inhibitory properties revealed that β-epoxide ABPs 4 and 5 were comparably potent as irreversible inhibitors, being markedly superior to CBE 1 and even surpassing cyclophellitol 2 and β-epoxide ABP 3 (Table 1). The apparent half-maximal inhibitory concentration (IC\textsubscript{50}) values of both fluorescent compounds (ABP 4: IC\textsubscript{50} = 1.24 ± 0.04 nM; ABP 5: IC\textsubscript{50} = 1.94 ± 0.08 nM) were very similar, being about 100– and 1,000-fold lower than those of cyclophellitol 2 and β-epoxide ABP 3 (IC\textsubscript{50} = 0.15 ± 0.009 μM and 0.12 ± 0.004 μM, respectively) and CBE 1 (IC\textsubscript{50} = 9.49 ± 0.042 μM). We determined next the inhibition constants – the Ki (the equilibrium constant for initial binding), the rate constant (k\textsubscript{i}) and the relative rate constant k\textsubscript{i}/Ki – for CBE 1, cyclophellitol 2 and β-epoxide ABPs 3 to 5 using a continuous substrate release assay (see Supplementary Figure S1a for progress curves; Fig S1b for k’ plots). A general trend that we observed for the equilibrium constant for initial binding was that increased hydrophobicity resulted in decreased Ki values (Table 1). Comparison of relative rate constants demonstrated that the fluorescent probes inhibited GBA 22–, 34– and 4,300-fold better than β-epoxide ABP 3, cyclophellitol 2 and CBE 1.

The affinity of β-epoxide ABPs 4 and 5 for GBA was unexpectedly high. For a better understanding of this finding, we performed molecular docking analysis using a crystal structure of GBA (PDB: 2V3E)\textsuperscript{25}. The docking model revealed that at minimum free energy of the simulated enzyme–ligand complex, both fluorescent probes efficiently bound to the GBA active site. The docking model showed that the BODIPY moiety could be positioned in two different hydrophobic pockets, each time with the β-epoxide of the glycan moiety positioned 3–4 Å away from E340 in the active-site, close enough to allow nucleophilic addition of the E340 carboxylate (Fig S2a, c). Both orientations gave similar minimal free energy values (Fig S2a, c).

The hydrophobic interactions of the BODIPY moieties contribute significantly to the high affinity interaction of β-epoxide ABPs 4 and 5 with GBA. Minimum free energy values

<table>
<thead>
<tr>
<th>Compound</th>
<th>GBA IC\textsubscript{50} (nM)</th>
<th>k\textsubscript{i} (min\textsuperscript{-1})</th>
<th>K (μM)</th>
<th>k\textsubscript{i} / K (μM\textsuperscript{-1}min\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBE 1</td>
<td>9.497 ± 42.8</td>
<td>0.217 ± 0.026</td>
<td>53 ± 10.8</td>
<td>0.004</td>
</tr>
<tr>
<td>Cyclophellitol 2</td>
<td>152 ± 9.32</td>
<td>0.078 ± 0.010</td>
<td>0.152 ± 0.026</td>
<td>0.514</td>
</tr>
<tr>
<td>β-epoxide ABP 3</td>
<td>121 ± 4.77</td>
<td>0.035 ± 0.003</td>
<td>0.044 ± 0.007</td>
<td>0.794</td>
</tr>
<tr>
<td>β-epoxide ABP 4</td>
<td>1.24 ± 0.04</td>
<td>0.127 ± 0.024</td>
<td>0.007 ± 0.002</td>
<td>17.76</td>
</tr>
<tr>
<td>β-epoxide ABP 5</td>
<td>1.94 ± 0.08</td>
<td>0.208 ± 0.063</td>
<td>0.008 ± 0.003</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Table 1 | Binding constants of presented probes. Parameters describing inhibitor–GBA affinity (K) and rate of covalent attachment to GBA nucleophile E340 (k) values were calculated from progress curves ± SEM.
for probes 4 and 5 (\(-8.1 \text{ kcal mol}^{-1}\) and \(-8.4 \text{ kcal mol}^{-1}\)) were significantly lower than for β-epoxide ABP 3 (\(-5.2 \text{ kcal mol}^{-1}\)). Notably, when we compared the crystal structure of CBE 1-bound GBA (2VT0) with the enzyme structure 2V3E in which was modeled β-epoxide ABP 4 or 5 (Fig S2b, d), the cyclitol moiety not completely overlap with CBE 1. This discrepancy is likely explained by differences in coordinates of the crystal structures, as well as the different positioning of β-epoxide ABPs 4 and 5 before the pre-nucleophilic attack compared to the already nucleophile-bound CBE 1. The binding of CBE 1 to E340 likely alters the local protein structure.

**In vitro labeling of GBA with the fluorescent ABPs** – To examine labeling of recombinant GBA by β-epoxide ABP 4 and 5, we incubated the enzyme for 30 min at 37 °C with mixtures of both probes at pH 5.2, with 0.2% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100, an optimal condition for enzymatic activity in vitro and activity-based labeling. After the incubation, we resolved the protein preparations with SDS-PAGE and analyzed the labeled proteins by fluorescence scanning of the slab-gel on a Typhoon Variable Mode Imager (Fig 2a, next page). Labeled recombinant GBA migrated at the expected mass of 59 kDa. At equimolar concentrations of β-epoxide ABPs 4 and 5, both probes bound the enzyme equally well. Thus, labeling of GBA with both probes was comparable, as expected given their similar inhibition constants. Boiling of the samples before electrophoresis had no impact on the detection of the fluorescently labeled protein on the slab gel, indicating that the probes were firmly attached. The presence of reducing agent also did not affect the covalent binding of the probes.

We determined the sensitivity of detection of labeled GBA by incubating 2 pmol GBA with an excess of β-epoxide ABP 4 (20 nmol at 1 mM concentration) for 1 h at 37 °C and subsequent titration of the amount applied on the gel (Fig 2b). We could detect as little as 20 attomoles, or \(20 \times 10^{-18} \text{ mol}\), GBA by fluorescence scanning.

Next, we incubated equal amounts of GBA (2 pmol) for 30 min with decreasing amounts of β-epoxide ABP 4 and applied all protein to a gel. Incubation with as little as 20 attomol of probe resulted in detectable GBA on the slab-gel (Fig 2c). Apparently, nearly the entire pool of probe had been covalently bound to recombinant GBA, consistent with its high affinity for binding. These experiments indicated that ultrasensitive detection of GBA was feasible on slab-gels following in vitro labeling with the fluorescent ABPs 4 and 5.

Additionally, we analyzed the site of binding of the probe on GBA using a competition assay. Prior to labeling with β-epoxide ABP 4, we incubated recombinant GBA with 2 mM CBE 1 for 30 min (Fig 2d). Pre-incubation with CBE 1, as shown via crystallography to bind to E340 to 26, blocked labeling completely. Similarly, we also noticed competition with labeling by the competitive inhibitor AMP-DNM (Fig 2d) 27. These results from competition experiments indicated that indeed the probe was bound in the catalytic center of GBA. Moreover, we unambiguously identified the site of binding of β-epoxide ABPs 3 and 4 by mass spectrometry. Using tryptic digestion and LC-MS/MS, we detected active-
site fragments of GBA that showed a shift in mass coinciding with binding of ABP 3 to E34028. A similar experiment with ABP 4 did not render detectable tryptic fragments of interest, most likely because the attachment of the hydrophobic moieties impaired ionization. This complication was circumvented by treating GBA labeled with β-epoxide ABP 4 with hydroxylamine before tryptic digestion. This released the probe from GBA and concomitantly converted the modified residue into a hydroxamic acid. The outcome of this experiment demonstrated that ABP 4 also bound covalently to E34028.

Enzymatically active GBA molecules, i.e. with a functional nucleophile and acid/base residue, are a prerequisite for labeling with the cyclophellitol β-epoxide probes, as demonstrated by the lack of labeling of GBA that had been denatured by boiling (Fig 2d). The same conclusion could be drawn from the pH dependence of irreversible inhibition of GBA by the fluorescent probes. It exactly coincided with the pH profile of enzymatic activity toward 4MU-β-D-Glc (Fig 2f).

**Labeling of GBA in cell and tissue extracts** – To determine the labeling specificity of both fluorescent probes, we incubated homogenates of cultured cells and mouse tissues with 100 nM green fluorescent β-epoxide ABP 4 for 30 min at 37 °C and analyzed the preparation with SDS-PAGE. In the case of homogenates of cultured RAW cells, fluorescence scanning showed exclusive labeling of GBA by ABP 4. The various GBA
forms, with molecular mass ranging 58–66 kDa owing to glycan differences, were visualized (Fig 3a). It was striking that incubation of cell lysates with the probes in vitro did not result in fluorescent labeling of other cellular proteins.

Furthermore, we observed very similar fluorescent labeling results – that is, highly specific labeling of GBA – using lysates of several mouse tissues (Fig 3b). Homogenates of mouse intestine were the only exception, most likely owing to labeling of high–molecular weight lactase and fragments thereof. Lactase/phlorizin hydrolase (LPH) shows both β-glucosidase and β-galactosidase activity, residing in two different catalytic pockets, and is known to covalently bind CBE 1. It therefore was no complete surprise that β-epoxide ABP 4 also labels LPH. Incubation for 30 min with 1 mM of ABP 4 resulted in ~90% inhibition of lactase (β-galactosidase pocket IV) activity and complete inhibition of β-glucosidase activity (pocket III) of LPH. Next, we demonstrated that a high concentration of lactose (250 mM, Fig 3c) reduced labeling of LPH (Fig 3d). Later on, see Chapter 4, we noted that β-epoxide ABP 4 labels with higher affinity the phlorizin hydrolase pocket (β-glucosidase pocket III) than the lactase pocket (β-galactosidase pocket IV) of LPH.

In situ labeling of GBA in cultured cells – We investigated whether labeling of GBA in intact cells was also feasible. For this purpose we added β-epoxide ABPs 4 or 5 to the culture medium at a concentration of 5 nM. At different time points, we harvested the cells and determined the GBA activity in the cell homogenates with artificial substrate (Fig 4a, next page). Even in intact cells, both probes inactivated GBA. Apparently, the more hydrophobic β-epoxide ABP 5 can more easily reach intracellular GBA. It is unlikely that uptake occurred only by endocytosis, given the fast speed of labeling and its occurrence at low temperature at which endocytosis is blocked (Fig 4b). Direct uptake of the probes, either by diffusion of the amphiphilic structures or by facilitation by transporters seems likely.

We studied in situ labeling of GBA in cells using fluorescence-activated cell sorting (FACS) analysis. We first pre-incubated cells in the absence or presence of CBE 1 and

---

**Figure 3** | In vitro ABP-labeling. (a) Fluorescent labeling of GBA in homogenates of RAW cells using β-epoxide ABP 4. Proteins were detected by fluorescence scanning of the slab-gel (left) and total proteins by Coomassie Brilliant Blue (CBB, right). (b) Fluorescent labeling of murine tissue lysates exposed to β-epoxide ABP 4. (c) Labeling of LPH by β-epoxide ABP 4 in murine duodenum in the absence and presence of 250 mM lactose. (d) Quantification of ABP-emitted fluorescence in c: in the absence (open; ▲) and presence (closed; ■) of lactose. Data represent mean values ± SD. Arrows indicate molecular weight of imiglucerase (~59 kDa).
subsequently incubated the cells with a sub-saturating or an excess amount of the green fluorescent β-epoxide ABP 4. FACS analysis revealed dose-dependent fluorescent labeling of cells and no labeling above background in cells pre-treated with CBE 1 (Fig 4c). To show the versatility of the probes, we performed pulse-chase experiments using cultured cells. For this purpose, we incubated fibroblasts overnight with 10 nM red fluorescent β-epoxide ABP 5 (Fig 4d). Subsequently, we treated the cells with 10 nM of green fluorescent ABP 4, harvested them at different time points (0–48 h) and subjected aliquots of cell homogenates to gel electrophoresis (see Fig 4d for the lifecycle of GBA as visualized in this manner). It should be noted that GBA chase labeled with the green fluorescent ABP 4 appeared gradually from the cells with an estimated half-life of about 30 h. The obtained half-life was roughly similar to the half-life determined previously using conventional pulse-chase labeling with radioactive methionine

These positive results prompted us to analyze labeling of the cells with fluorescence microscopy (Fig 4e). For this purpose, we cultured fibroblasts for 2 h with 5 nM β-epoxide

![Image of Figure 4](image-url)

**Figure 4 | In situ labeling of GBA.** (a) Time-dependent in situ inactivation of GBA in wild-type fibroblasts by 5 nM β-epoxide ABP 4 (open circles, ●-●) and ABP 5 (closed squares, □-□). Inactivation determined in vitro with artificial 4MU-β-α-Glc substrate. Data represent values ± SD. (b) FACS analysis of wild-type fibroblasts incubated for 300 min with 0 nM (red), 2 nM (blue) and 10 nM (green) β-epoxide ABP 4, or pre-treated with 300 μM CBE 1 prior to incubation with 2 nM (brown) and 10 nM (purple) ABP 4. (d) Pulse-chase of wild-type fibroblasts for 0–48 h. After overnight incubation with red β-epoxide ABP 5, cells were chased with green β-epoxide ABP 4. (e) Representative fluorescence micrographs of wild-type fibroblasts treated with β-epoxide ABP 5 and subsequent immunohistochemistry. After spectral imaging, channels were unmixed into autofluorescence (grey), AlexaFluor488 fluorescence of GBA visualized with monoclonal antibody 8E4 (green), β-epoxide ABP 5 BODIPY fluorescence of GBA (red), overlay of the latter two, and detailed focus (square), where equal green and red fluorescence yields a yellow overlay. In all pictures, nuclei are stained with DAPI (blue). Scale bar represents 20 μm.
ABP 5. We also visualized GBA protein by indirect immunofluorescence using the specific α-GBA monoclonal antibody 8E4\textsuperscript{30}. Using multispectral image analysis, we could specifically distinguish the respective fluorescent emission spectra from autofluorescent background (Fig 4e). The intracellular pattern of labeling with ABP 5 showed an almost complete overlap with the detection of GBA using monoclonal antibody 8E4 (Fig 4e).

As probes with a hydrophobic BODIPY moiety might nonspecifically be retained in membranes, in particular the plasma membrane, we studied this possibility more closely. First, we cultured fibroblasts obtained from a Gaucher patient homozygous for the RecNCI GBA mutation, a mutation resulting in premature degradation of GBA by the proteasome, with β-epoxide ABP 5. In contrast to control fibroblasts, the patient’s cells did not show labeling in the lysosomal compartment by ABP 5 (Fig 5). As expected, immunofluorescence using monoclonal 8E4 showed a lysosomal staining pattern for GBA in the control cells whereas RecNCI cells revealed staining for GBA in the cytosol, most likely because of high levels of endoplasmatic reticulum-associated degradation (ERAD) and proteasomal degradation in these cells. Control fibroblasts cultured with ABP 5 revealed labeling of the lysosomal compartment (Fig S3).

Next, fibroblasts were cultured for 16 h in presence of 3 mM CBE 1 to irreversibly inhibit GBA molecules to block subsequent labeling. When the same cells were cultured for 2 h with 5 nM β-epoxide ABP 5, again in presence of CBE 1, no clear lysosomal labeling was observed. To test further specificity, probe 6 and probe 7, non-reactive analogs of β-epoxide ABPs 4 and 5, lacking a β-epoxide, were synthesized. These control probes did competitively, but not irreversibly, inhibit GBA \textit{in vitro} with IC\textsubscript{50} values of 41 and 95 μM.

![Figure 5](image_url)  \textbf{Figure 5 | In situ labeling of GBA.} Representative fluorescence micrographs of wild-type (top row) and RecNCI Gaucher (bottom row) fibroblasts treated with β-epoxide ABP 5 and subsequent immunohistochemistry. After spectral imaging, channels were unmixed into autofluorescence (grey), AlexaFluor488 fluorescence of GBA visualized with monoclonal antibody 8E4 (green), ABP 5 BODIPY fluorescence of GBA (red), overlay of the latter two, and detailed focus (square), where equal green and red fluorescence yields a yellow overlay. In all pictures, nuclei are stained with DAPI (blue). Scale bar represents 20 μm.
Culturing of fibroblasts for 2 h with 5 nM probe 7 resulted in hardly any detectable labeling. In the presence of CBE 1, probe 7 also only weakly labeled the fibroblasts, in a pattern suggesting non-specific interaction with membranes (Fig S3).

We next studied the labeling of GBA by β-epoxide ABP 5 in intact fibroblasts using time-lapse microscopy. Fibroblasts treated with ABP 5 showed very rapid fluorescent labeling of lysosome-like structures. With 5 nM ABP 5, labeling reached a maximum within 15 min. Even after 100 h of exposure to the compound, cells did not show any signs of apoptosis or toxicity.

Finally, we examined the possibility of labeling GBA in mice by intravenously administering 100 pmol green fluorescent β-epoxide ABP 4 dissolved in phosphate-buffered saline to adult male wild-type mice. As a control, matched mice received the buffer solution intravenously. After 2 h, we sacrificed the animals, prepared tissue extracts, labeled the lysates with excess red fluorescent β-epoxide ABP 5 to visualize unlabeled GBA and subsequently, fluorescent proteins were visualized by SDS-PAGE and scanning of the slab-gel (Fig 6 shows the outcome of a typical experiment). In most tissues – shown are lung and liver – ABP 4 had already labeled in vivo a considerable proportion of GBA (Fig 6a). Consistently, in such tissues the probe also irreversibly inhibited a large proportion of GBA in the living mouse (Fig 6b).

An exception in this respect was the brain (Fig 6a), in which β-epoxide ABP 4 apparently labeled almost no GBA in vivo and GBA-associated β-glucosidase activity was not reduced. As observed earlier, intestinal fractions showed in vivo labeling of GBA and other proteins of multiple molecular masses probably reflecting LPH (Fig 6a).

![Figure 6](image_url)

**Figure 6 | Labeling of GBA in intact mice.** Adult mice received intravenously 100 pmol β-epoxide ABP 4 and were sacrificed after 2 h. (a) In vivo labeled GBA (left panels) compared to in vitro labeled residual GBA (right) in lungs, liver, brain and duodenum. Arrows indicate molecular weight of imiglucerase. (b) Residual GBA β-glucosidase activity in tissues of β-epoxide ABP 4-treated mice (closed; ■) versus matched control (open; □). Data represent mean values ± SD.

**Analysis of Gaucher materials** – We investigated labeling of mutant GBA in fibroblasts from a normal individual and from several Gaucher donors (a N370S GBA homozygote, a L444P homozygote and a RecNCI homozygote manifesting as collodion Gaucher, almost entirely lacking active GBA protein) by treating cell lysates with 10 nM β-epoxide ABP 4 for 1 h and subjecting these to SDS-PAGE. A comparison of cells from a normal subject and
from a Gaucher donor homozygous for L444P GBA revealed that the amount of L444P GBA was markedly lower in the Gaucher donor (Fig 7a).

It is indeed known that L444P GBA undergoes largely premature degradation by ERAD as a result of impaired folding\textsuperscript{11}. This phenomenon is far less striking in the case of N370S GBA\textsuperscript{10}. Cells from a Gaucher donor homozygous for N370S GBA also showed reduced labeling of GBA but to a lesser extent than cells from a L444P homozygote (Fig 7a). As expected, cells from the RecNCI collodion Gaucher did not show any labeled GBA (Fig 7a).

We obtained similar results by analysis of the fibroblast extracts using Western blotting and the α–human GBA antibody 8E4, although the detection limit of this method was far inferior (Fig 7a). It has been noted earlier that excess CBE\textsubscript{1} completely irreversibly inhibits N370S GBA like wild-type enzyme\textsuperscript{20, 31}. We noted no significant differences in inactivation by β-epoxide ABPs 4 or 5 of GBA activity in intact cells of a normal subject and a N370S GBA homozygote (Fig 7b).

Analysis of GBA in spleen from a Gaucher patient with solely N370S GBA showed the presence of a lower amount of β-epoxide ABP 4-labeled GBA protein as compared to control tissue (Fig 7c).

**Impact of isofagomine on N370S GBA in cultured fibroblasts** – It has been reported that for cells from N370S GBA homozygotes, prolonged incubation with isofagomine (IFG 8) yields an increase in GBA activity\textsuperscript{32}. The interaction of IFG 8 with the catalytic pocket has been intensely studied, including at the level of crystals\textsuperscript{21}. We therefore examined whether incubation of N370S GBA homozygous fibroblasts with IFG 8 increased the activity of GBA and the amount of enzyme that can be labeled with fluorescent probes.

We cultured wild-type cells for 7 d with different concentrations of IFG 8 (0, 10, 30 and 300 nM) and subsequently incubated them for 2 h with or without excess β-epoxide ABP 5 in the presence of the original concentration of IFG 8. Determination of the activity of GBA in homogenates of cells not treated with ABP 5 using 4MU-β-D-Glc as substrate revealed an
IFG 8 dose–dependent increase in enzyme activity (Fig 8a). The homogenates of cells labeled with ABP 5 showed a modest dose-dependent increase in labeled GBA (Fig 8b). Surprisingly, the increase in enzymatic activity in cells treated with high dose IFG 8 was relatively higher than the increase in ABP-labeled GBA protein.

To test whether high concentrations of IFG 8 interfered with the labeling of GBA with ABPs, we studied the competition by IFG 8 for fluorescent ABP labeling. For this purpose, we pre-incubated recombinant GBA (velaglucerase), attached to 8E4 immobilized to Sepharose beads, labeled with β-epoxide ABP 4, in the presence of 0–100 μM IFG 8 (left), or after wash-out of IFG 8 (right). (d) Quantification of β-epoxide ABP 4-emitted fluorescence in the presence (closed; ■) or absence (open; □) of IFG 8 in c. Data represent mean values ± SD. (e) In situ IC50 of IFG 8 using 40 μM FDG. Activity assessed by FACS as relative FDG hydrolysis per cell. Data represent mean values ± SD.

Additionally, to analyze if indeed the catalytic pocket of GBA, present in living cells exposed to high concentrations of IFG 8, is occupied by this compound, we determined the in situ enzymatic activity of GBA using fluorescein-di-β-D-glucopyranoside (FDG) as substrate. Fibroblasts were treated with various concentrations of IFG 8 for 20 min, then 40 μM FDG was added to the medium for 1 h and its hydrolysis was determined by FACS33. The IC50 of IFG 8 for hydrolysis of FDG by intact cells was about 1 μM (Fig 8e). At 300 nM IFG 8, the in situ activity of GBA was decreased approximately 25% in combination with the concentrations of FDG used.

It should be kept in mind that IFG 8 is a reversible inhibitor (see also Fig 8c, d). When making a lysate of cells exposed to IFG 8, the inhibitor is markedly diluted compared to the in situ situation. Consequently, in vitro assays may result in an overestimation of the true increase in enzymatic activity in cells exposed to IFG 8.
Discussion

The need for a method allowing visualization of active GBA molecules in situ in living cells is evident. It is of importance to understand better what the precise cell- and tissue-distribution of active GBA molecules is as this may improve understanding of Gaucher disease pathogenesis. Moreover, demonstration of a true increase in active GBA molecules by tentative chaperones is of interest. At present, the detection of GBA still relies on the use of antibodies that do not distinguish between active and inactive GBA molecules and cannot label enzyme in intact cells. Our search for a suitable probe for activity-based labeling of GBA in situ has yielded the desired result. As starting point for the development of such an ABP, we selected cyclophellitol 2, a known potent irreversible inhibitor of GBA that forms a covalent adduct. Next we linked, via a spacer, hydrophobic BODIPY moieties to β-epoxide ABP 3. Serendipitously, this led to even more potent irreversible inhibitors. Molecular docking analysis suggested that interaction of the hydrophobic BODIPY moiety in β-epoxide ABPs 4 and 5 with a hydrophobic pocket at the surface of GBA guides the β-epoxide to a position close to nucleophile residue E340. This effect may plausibly underlie the remarkable avidity of the two fluorescent compounds as activity-based probes. The labeling of GBA with the fluorescent probes 4 and 5 seemed to proceed exactly via the expected mechanism for cyclophellitol inhibition of a β-glucosidase. We could indeed demonstrate that labeling could be blocked with CBE 1 or β-epoxide ABP 3 and potent competitive inhibitors such as hydrophobic deoxynojirimycins could compete away labeling. Labeling also required the folded enzyme and occurred proportional to enzymatic activity at different pH.

The affinity of both ABPs for GBA is in fact quite notable: truly ultra-sensitive detection of GBA molecules was obtained. The high affinity of the fluorescent probes for GBA offered the opportunity to label the enzyme very specifically. With cell or tissue lysates, exclusive labeling of GBA molecules was observed following electrophoresis. In the case of homogenates of intestine alone we noticed labeling of other proteins, probably fragments of LPH known to covalently interact with CBE 1. The highly selective labeling of GBA is notable when taking into account the fact that GBA is a very low-abundance protein and constitutes < 10⁻⁵ of all cellular or tissue protein. Another favorable feature of these probes is their ability to enter various cellular compartments. It will be of interest to determine the precise mechanism(s) more closely, although it is already clear that cellular entry seems not to depend on endocytosis. The entry of the probes into living cells allows their use in FACS analysis, and one can perform pulse-chase experiments in intact cells as we have demonstrated. Time-lapse microscopy confirmed that GBA can be labeled very efficiently in intact fibroblasts. We obtained no indications that fluorescent labeling of GBA was toxic to the cells. Initial experiments also indicated that in mice cellular GBA can be labeled with fluorescent cyclophellitol β-epoxide type compounds. Notably, the brain showed a different picture. Almost no brain GBA was labeled in mice upon intravenous
administration of β-epoxide ABP 4. This may suggest that ABP 4 does not effectively pass the blood-brain barrier or is actively removed from the brain by some P-glycoprotein. The fluorescence features of the green– and red fluorescent probes are intrinsically suboptimal for in situ imaging of labeled GBA in tissues or whole animals.

The potential applications for activity-based fluorescent probes 4 and 5 are substantial. They offer an alternative to antibodies, which are species-specific and cannot reach compartmental GBA in intact cells. Moreover, in contrast to antibodies, our probes uniquely label active GBA molecules. One tentative area of application of the fluorescent probes may be diagnosis of Gaucher disease, in particular the demonstration of low amounts of active GBA molecules in fibroblasts, or blood cells of patients. This is helpful, as low amounts of active GBA molecules are usually associated with severe, neuronopathic Gaucher disease. Another area of application for these fluorescent probes may be found in the analysis of compounds for their possible inhibitory or chaperone effects. As we demonstrated, the beneficial effect of IFG 8 on N370S GBA in cultured fibroblasts could be confirmed with activity-based labeling. This finding is of importance as it implies that at an optimal concentration of IFG 8, occupation of the catalytic center by the competitive inhibitor is in situ sufficiently low to allow labeling by the fluorescent probe. In other words, at an appropriate concentration IFG 8 indeed increases GBA levels and intralysosomal enzymatic capacity. This finding for IFG 8 is not entirely unexpected, as it has been proposed that at the low intralysosomal pH IFG 8 interacts less well with β-glucosidases than at neutral pH in the endoplasmic reticulum35. Our observations render support for the approach of chaperone therapy, although the dosing of drugs in patients to reach optimal (steady-state) concentrations in various tissues may prove to be a major challenge.

Our approach of selective detection of GBA molecules using fluorescently labeled irreversible inhibitors allows unprecedented, ultra-sensitive in vivo monitoring of active enzyme molecules. It can be envisioned that the same approach is also feasible for other glycosidases, and another challenging perspective is the future use of the fluorescent probes in living animals. Two approaches can be envisioned. In the first approach, the fluorescent probes are used to report on local GBA activity. In the second strategy, recombinant GBA is labeled with the fluorescent probe so that after administration, trafficking of the construct can be monitored in a strategy that is related to another recently reported strategy that uses active site labeling of recombinant and purified GBA with a radio-tag36. In conclusion, the reported fluorescent activity-based probes offer very versatile research tools to visualize active GBA, ultra-sensitively and specifically. This accomplishment may be not only relevant for Gaucher disease but also for Parkinsonism.
Experimental Section

Activity-based probes – For a detailed description of synthesis of all ABPs described in this manuscript, the reader is referred to the Supplementary Information.28

General methods – Chemicals were obtained from Sigma-Aldrich if not otherwise indicated. Recombinant GBA was obtained from Genzyme. Monoclonal α-human GBA antibody 8E4 was produced from hybridoma cells as described earlier.37 Gaucher patients were diagnosed on the basis of reduced GBA β-glucosidase activity and demonstration of an abnormal genotype.38 Fibroblasts were obtained with consent from donors. Cell lines were cultured in HAMF12-DMEM medium (Invitrogen) supplied with 10% (v/v) FBS.

Enzyme activity assays – The β-glucosidase activity of GBA was assayed at 37 °C by incubating with 3.75 mM 4-methylumbelliferyl-β-D-glucopyranoside (4MU-β-D-Glc) as substrate in 150 mM McIlvaine buffer, pH 5.2, supplemented with 0.1% (w/v) BSA, 0.2% (w/v) sodium taurocholate, and 0.1% (v/v) Triton X-100. To determine the apparent IC50 value, GBA was incubated with a range of inhibitor dilutions for 30 min at 37 °C prior to the addition of artificial substrate. After stopping the substrate reaction with excess NaOH-glycine (pH 10.3), fluorescence was measured with a fluorimeter LS30 (Perkin Elmer) using λ EX 366 nm and λ EM 445 nm. Lactase of LPH was quantified by measuring liberated D-glucose from lactose.39 The β-glucosidase activity by LPH was measured using 4-methylumbelliferyl-β-D-glucopyranoside (4MU-β-D-Glc) as substrate. In situ activity of GBA in cells was measured the hydrolysis of fluorescein-di-β-D-glucopyranoside (FDG) as substrate and read-out by FACS.

Determination of inhibition constants – Previous discontinuous methods have been used to determine the IC50 and inhibition constants of glycosidase inhibitors. This method however proved to be unsuitable for the determination of the inhibition constants of β-epoxide ABPs 3 to 5 due to their high affinity/fast binding. Therefore the inhibition constants have been determined in a continuous substrate assay,40, 41 where substrate hydrolysis and (irreversible) enzyme inhibition proceed concurrently, making the situation more complex than the discontinuous as described in Scheme 1.

\[
\begin{align*}
[E] + [S] & \overset{K_m}{\underset{[I]}{\rightleftharpoons}} [ES] \overset{k_{out}}{\rightarrow} [E] + [P] \\
\end{align*}
\]

Time-dependent interactions of inhibitor (I) with free GBA (E) was considered to occur in separate stages, with a rapid reversible interaction (Kᵢ) followed by a slower, irreversible reaction that transforms the reversible enzyme-inhibitor complex (EI) into an irreversible enzyme-inhibitor complex (EI*). Kinetic experiments were performed in black Greiner 96-well plates (flat bottom) with 7.5 mM 4MU-β-D-Glc in 150 mM McIlvaine buffer (pH 5.2, supplemented with 0.2% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100 and 0.1% (w/v) BSA) was supplemented with different inhibitor concentrations and total was pre-warmed to 37 °C for 15 min. At t₀, 2 ng pre-warmed GBA was added per well. After 15 s horizontal shaking, released 4MU was measured each minute in a TECAN GENios plate-reader (λEX 340 nm, λEM 465 nm) for 1 h. Results were verified using a BioTEK Synergy plate-reader (λEX 360 nm, λEM 465 nm) for 90 min. Fluorophore bleaching was corrected by parallel control blanks and each measurements was performed in n = 9 wells/inhibitor concentration. The apparent rate constants k’ were obtained by fitting progress curves with one-phase association equation 1.

\[
[\text{EI}^*] = ([\text{EI}^*]_{\text{max}} - [\text{EI}^*]_0)(1 - e^{-kt})
\]
Plotting the obtained \( k' \) values versus [I] yielded rectangular hyperbole functions and estimates of \( K' \) and \( k_i \) were subsequently obtained from equation 2.

\[
    k' = \frac{k_i [I_i]}{K_i' + [I_i]}
\]

The \( K_i \) value in the absence of substrate was obtained by disseminating the \( K' \) with equation 3:

\[
    K_i = \frac{K_i'}{1 + [S]/K_m}, \text{ where } K_m = 1.2 \text{ mM}
\]

**Molecular docking** – CBE 1, cyclophellitol 2, \( \beta \)-epoxide ABPs 3 to 5 ligand structures were drawn, lowest energy conformers calculated and saved as .pdb structure files in MarvinDraw. Rotatable bonds in ligands were defined and structure saved as ligand .pdbqt files using AutoDockTools (ADT). The following RCSB PDB crystal structures were taken: 2V3E and 2VT0, and crystal water molecules were removed (PyMol). Structure 2VT0 were aligned to 2V3E for structure comparison. Autodock Vina input files were prepared from PDB structure files using ADT. Polar hydrogens were added and files were saved as receptor .pdbqt files. Docking experiments using flexible ligands were run using standard settings, with exhaustiveness adjusted to 12 (Autodock Vina), with search space boundaries spanning 90 Å around the active site of GBA. Each compound:structure match was docked repeatedly (\( n = 9 \)) and resulting .pdbqt files were analyzed for top lowest free energy associated with the complex (a), positioning near the active site (\( \beta \)) and correct coordination within the active site (\( \gamma \)) (PyMol).

**Labeling competition between \( \beta \)-epoxide ABPs 4 and 5** – Imiglucerase (2 pmol) was prepared in 150 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100. The enzyme was incubated with \( \beta \)-epoxide ABPs 4 and 5 at different concentration ratios (0.001–10 \( \mu \)M) for 30 min at 37 °C. The sample was denatured with 5x Laemmli buffer (50% (v/v) 1M Tris-HCl, pH 6.8, 50% (v/v) 100% glycerol, 10% (w/v) DTT, 10% (w/v) SDS, 0.01% (w/v) bromophenol blue), boiled for 4 min at 100 °C and separated by electrophoresis on 7.5% (w/v) SDS-PAGE gel running continuously at 90 V. Wet slab-gels were scanned for fluorescence using the Typhoon Variable Mode Imager (Amersham Biosciences) using \( \lambda_{EM} \) 488 nm and \( \lambda_{EM} \) 520 nm (band pass filter 40 nm) for green fluorescent \( \beta \)-epoxide ABP 4 and \( \lambda_{EM} \) 532 nm and \( \lambda_{EM} \) 610 nm (band pass filter 30 nm) for red fluorescent \( \beta \)-epoxide ABP 5.

**Detection limit of \( \beta \)-epoxide ABP 4** – Imiglucerase (2 pmol) was prepared in 150 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100. The enzyme was incubated with 20 nmol (1 mM) \( \beta \)-epoxide ABP 4 for 1 h at 37 °C. The sample was diluted sequentially, separated by SDS-PAGE and fluorescence scanned. Alternatively, Imiglucerase (2 pmol) was incubated with 2,000–0.0002 fmol \( \beta \)-epoxide ABP 4 for 1 h at 37 °C and analyzed.

**Competition for the active site** – Imiglucerase (2 pmol) was prepared in 150 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100. The enzyme was pre-incubated with 2 mM CBE 1, 1 mM AMP-DNM for 30 min at 37 °C or with 10 \( \mu \)L 2% (w/v) SDS and boiled for 4 min at 100 °C. The pre-incubated mixtures were labeled with 100 nM \( \beta \)-epoxide ABP 4 for 30 min at 37 °C. Finally, samples were analyzed by SDS-PAGE and fluorescence scanning.

**Influence of pH on inhibitory potential** – To determine the apparent IC\(_{50}\) value at pH 3–8, GBA was pre-incubated with a range of inhibitor dilutions for 30 min at 37 °C whilst solubilized in 150 mM McIlvaine buffer containing 0.1% (w/v) BSA, 0.2% (w/v) sodium taurocholate, and 0.1% (v/v) Triton X-100 and adjusted to pH 3–8. After 30 min, the activity of GBA was assayed by incubating with 3.75 mM 4MU-\( \beta \)-D-Glc as substrate in 150 mM McIlvaine buffer, pH 5.2, with the aforementioned constituents. After stopping the substrate reaction with excess NaOH-glycine (pH 10.3), fluorescence was measured with a fluorimeter LS30 (Perkin Elmer) using \( \lambda_{EM} \) 366 nm and \( \lambda_{EM} \) 445 nm.

**Labeling of GBA in cell lysates and tissue homogenates** – After determination of protein concentration (BCA kit, Pierce), 10 \( \mu \)g total protein was incubated with 100 nM \( \beta \)-epoxide ABP 4 or 5 in 150 mM McIlvaine buffer, pH 5.2, containing 0.2% (w/v) sodium taurocholate, 0.1% (v/v) Triton X-100 and protease inhibitor cocktail (Roche), for 30 min at 37 °C. Hereafter, labeled proteins were visualized by fluorescence scanning of the SDS-PAGE gels and total protein was stained by coomassie brilliant blue (Fermentas). \( \beta \)-Epoxide ABP 4-emitted fluorescence was quantified using ImageQuant 5.1.
**In situ labeling of GBA in cultured cells** – Confluent wild-type skin fibroblasts were incubated with 5 nM β-epoxide ABP 4 or 5 in the culture medium for 0–1,400 min. Hereafter, cells were washed extensively with PBS and lysed by scraping in 25 mM potassium phosphate buffer (pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitor cocktail (Roche)) and GBA β-glucosidase activity was measured as described.

**Effect of temperature on in situ labeling of GBA** – Confluent wild-type skin fibroblasts were incubated with 0–1,000 nM β-epoxide ABP 4 in the culture medium for 2 h at 18 or 37 °C. Hereafter, cells were washed extensively with PBS and lysed by scraping in 25 mM potassium phosphate buffer (pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitor cocktail) and GBA β-glucosidase activity was measured as described.

**Fluorescence-activated cell sorting** – Fibroblasts were cultured in the presence or absence of 300 μM CBE 1 overnight. Next, cells were incubated with 2 and 10 nM β-epoxide ABP 4 for 300 min, suspended by trypsinization and analyzed by FACS using FACS Vantage (B.D. Bioscience), λ EX 488 nm, λ EM 530 nm (band pass filter 30 nm).

**Pulse-chase experiments** – Fibroblasts were pulse-labeled overnight with 10 nM β-epoxide ABP 5, extensively washed with PBS and subsequently chase-labeled with 10 nM β-epoxide ABP 4 for 0–48 h. At each time-point, cells were washed extensively with PBS and lysed by scraping in 25 mM potassium phosphate buffer (pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitor cocktail (Roche)), and labeled GBA was visualized by fluorescence scanning following SDS-PAGE.

**Fluorescence microscopy and multispectral imaging** – Wild-type and RecNCI fibroblasts were cultured on glass slides. Cells were incubated with 5 nM β-epoxide ABP 5 or probe 7 for 2 h. Next, cells were washed, fixed with 3% (w/v) p-formaldehyde in PBS for 15 min, washed and incubated first with 0.05% (w/v) saponine for 15 min, next with 0.1 mM ammonium chloride in PBS for 10 min and then with 3% (w/v) BSA in PBS for 1 h. Slides were then incubated with 1:500 α-GA mAb 8E4, subsequently visualized with secondary goat α-mouse pAb-conjugated AlexaFluor488, and nuclei were stained with DAPI. Cells were examined by epi-fluorescence microscopy (Leica DM5000B) with an HCX PL APO ×63 1.40 oil immersion objective. Filter blocks used were A4 (λ EX 360/40 nm band pass, 400 nm dichromatic mirror, λ EM 470/40 nm band pass suppression) for DAPI, L5 (λ EX 480/40 nm band pass, 505 nm dichromatic mirror, λ EM 527/30 nm band pass) for AlexaFluor488 and N2.1 (λ EX 515–560 nm band pass, 580 nm dichromatic mirror, λ EM 590 nm long pass suppression) for β-epoxide ABP 5 and probe 7. Fluorescence analysis was performed by multispectral imaging using a Nuance N-MSI-420-20 camera with Nuance 2.10 software (Cambridge Research & Instrumentation). Data sets were acquired at λ EM 440–500 nm for A4, λ EM 500–580 nm for L5, and λ EM 580–720 nm for N2.1 filter blocks, each at 10 nm intervals. In each experiment, unlabeled control cells were imaged to define the auto-fluorescence spectral library. Separate spectral libraries for DAPI, AlexaFluor488 and β-epoxide ABP 5 or probe 7, each obtained from single-stained cells, were used to un-mix the triple staining patterns into the individual components and separation from auto-fluorescence. Nuance software was used to construct composite images.

**Time-lapse microscopy** – Fibroblasts were cultured in chamber slides (Lab-Tek II, Nunc, Roskilde, Denmark) and incubated with 5 nM β-epoxide ABP 4 or 5. Hereafter, mineral oil was immediately layered thinly on the medium and cells were cultured in the incubator enclosing the microscope, with a 10% (v/v) CO2 atmosphere at 37 °C. During 2 h, cells were imaged every 5 min using fluorescence microscopy (N2.1 filter) and phase-contrast bright-field microscopy (Leica IR-BE with Z-motor drive and a Plan APO 63×/1.40 oil immersion objective (Leica Microsystems, Rijswijk, The Netherlands), equipped with a KX85 camera, Apogee Instruments, Auburn, CA, USA). To minimize photo-toxicity, fluorescence imaging was limited to 2 h to monitor for possible toxicity due to the presence of β-epoxide ABP 4 or 5, after 2 h live-cell imaging was continued with bright field microscopy for another 98 h. An auto-focus routine was applied during acquisition. Images were analyzed using TimeLapseAVI 5.1.4 software.

**Mass spectrometric analysis** – GBA (5 μg) was labeled with 6 μM β-epoxide ABP 3 in standard McIlvaine buffer (pH 5.2) for 1 h at 37 °C, whereafter mixture was reduced with 10 mM dithiothreitol at pH 8.0 (adjusted with 200 mM NH4HCO3) for 30 min at 60 °C. After alkylation with 15 mM iodoacetamide for 30 min at room temperature (RT), the mixture was digested with 50 ng trypsin (Promega) overnight, at 37 °C, and then desalted with reversed phase C18-Ziptips (2 μg capacity, Millipore). After acetonitrile activation and sample loading, the Ziptips were washed with 0.1% (v/v) aqueous trifluoroacetic acid and eluted with 60% (v/v) acetonitrile.
acetonitrile, 0.1% (v/v) trifluoroacetic acid in water. Prior to LC-MS, samples were diluted 10-fold in 0.1% aqueous trifluoroacetic acid.

**Identification of the site of binding of β-epoxide ABP 4** – GBA (40 μg) was labeled with 20 μM β-epoxide ABP 4 in standard McIlvaine buffer (pH 5.2) for 1 h at 37 °C, then precipitated with chloroform/methanol (C/M)\(^5\). Pellet was rehydrated in 8 M urea with 100 mM NH\(_4\)HCO\(_3\), alkylated with 200 mM iodoacetamide for 30 min at RT in the dark and desalted by C/M. After dispersed in 8 M urea with 50 mM Na\(_2\)CO\(_3\) buffer (pH 9.4), protein incubated with 5 M hydroxylamine hydrochloride, 4 M urea and 25 mM Na\(_2\)CO\(_3\) (pH 9.2, equilibrated with NaOH), overnight at 37 °C, precipitated with C/M, re-dissolved in 8 M urea with 100 mM NH\(_4\)HCO\(_3\), and digested overnight with 500 ng trypsin in digest buffer (Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM CaCl\(_2\), 2% (v/v) acetonitril). Peptides were collected and desalted on StageTips\(^6\).

**LC-MS analysis** – Tryptic peptides were analyzed on a Surveyor nanoLC system (Thermo) hyphenated to a LTQ-Orbitrap mass spectrometer (Thermo). Gold and carbon coated emitters (OD/ID = 360/25 μm tip ID = 5 μm), trap column (OD/ID = 360/100 μm packed with 25 μm robust Poros 10R2/15 μm BioSphere C\(_18\) 5 μm 120 Å) and analytical columns (OD/ID = 360/75 μm packed with 20 cm BioSphere C\(_18\) 5 μm 120 Å) were from Nanoseparations (Nieuwkoop, The Netherlands). The mobile phases (A: 0.1% FA/H\(_2\)O, B: 0.1%FA/ACN) were made with ULC/MS grade solvents (Biosolve). The emitter tip was coupled end-to-end with the analytical column via a 15 mm long Teflon tubing sleeve (OD/ID 0.3x1.58 mm, Supelco, USA) and installed in a stainless steel holder mounted in a nano-source base (Upchurch scientific, Idex, USA).

**General mass spectrometric conditions** – Electrospray voltage of 1.8 kV was applied to the emitter, no sheath and auxiliary gas flow, ion transfer tube temperature 150 °C, capillary voltage 41V, tube lens voltage 150V. Internal mass calibration was performed with air-borne protonated polydimethylcyclosiloxane (m/z = 445.12002) and the plasticizer protonated diocyl phthalate ions (m/z = 391.2829) as lock mass\(^5\). Samples (10 μL) were pressure loaded on the trap column with a 10 μL min\(^{-1}\) flow for 5 min followed by peptide separation with a gradient of 35 min 5–30% B, 15 min 30–60% B, 5 min A at a flow of 300 μL min\(^{-1}\) split to 250 nL min\(^{-1}\) by the LTQ divert valve. For each data dependent cycle, one full MS scan (300–2000 m/z) acquired at high mass resolution (60,000 at 400 m/z, AGC target 10\(^6\), maximum injection time 1,000 ms) in the Orbitrap was followed by 3 MS/MS fragmentations in the LTQ linear ion trap (AGC target 5x10\(^5\), max injection time 120 ms) from the three most abundant ions. MS\(^2\) settings were: collision gas pressure 1.3 mT, normalized collision energy 35%, ion selection threshold of 500 counts, activation q = 0.25 and activation time of 30 ms. Fragmented precursor ions that were measured twice within 10 s were dynamically excluded for 60 s and ions with z < 2 or unassigned were not analyzed. Data from MS\(^2\) was validated manually.

**Labeling of GBA in live mice** – The appropriate ethics committee for animal experiments approved all experimental procedures. C57Bl/6J mice were obtained from Harlan and fed a commercially available lab diet (RMH-B; Hope Farms). Two Npc1 BALB/c wild-type (+/+) mice were injected intravenously with 100 μl PBS or 100 μl 100 nM β-epoxide ABP 4 dissolved in PBS. After 2 h of administration the mice were anesthetized with FFM mix (1 ml of fentanylcitrate, 1 ml of midazalam and 2 ml of distilled water), and blood, urine and organs were collected and directly frozen in liquid nitrogen. Homogenates were made in 25 mM potassium phosphate buffer, pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and labeled with 100 nM β-epoxide ABP 5. Homogenates were analyzed by fluorescence scanning after SDS-PAGE, and ABP-emitted fluorescence was quantified using ImageQuant (5.1), and verified in-gel by presence of 50 fmol equimolar ABP 4 and 5-labeled imiglucerase.

**Analysis of Gaucher materials** – Human wild-type and mutant GBA fibroblasts (N370S homozygote, L444P homozygote and RecNCI homozygote manifesting as collodion Gaucher) were grown to confluency and lysed by scraping in 25 mM potassium phosphate buffer (pH 6.5, supplemented with 0.1% (v/v) Triton X-100). Lysates were labeled with 10 nM β-epoxide ABP 4 for 1 h at 37 °C and labeled GBA was analyzed by fluorescence scanning of SDS-PAGE gel. Western blotting was accomplished by transfer of the protein for 1 h at 12 V, followed by blocking of the membrane with 2% (w/v) BSA in TBST buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20), overnight treatment with 1:1,000 diluted primary mouse α-human GBA mAb (8E4, 2% (w/v) BSA in TBST), washing with TBST for 20 min (repeated 6 times), followed by 1:10,000 diluted secondary rabbit α-mouse
IRD680 (Cell Signalling, 2% (w/v) BSA in TBST), subsequent washing with TBST for 20 min (repeated 6 times), and read-out on Odyssey infrared scanner.

**In situ labeling of N370S GBA** – Wild-type and N370S homozygote fibroblasts were incubated with 0–100 nM β-epoxide ABP 4 or 5 in the medium for 2 h at 37 °C. Hereafter, cells were washed extensively with PBS and lysed by scraping in 25 mM potassium phosphate buffer (pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitor cocktail (Roche)) and GBA β-glucosidase activity was measured as described.

**In vitro labeling of GBA in spleen homogenates** – Spleen samples from wild-type control and N370S homozygote were homogenized in 25 mM potassium phosphate buffer (pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitor cocktail (Roche)). After determination of protein concentration (BCA kit, Pierce), 100 μg total protein was labeled with 0–100 nM β-epoxide ABP 4 for 1 h at 37 °C. Finally, samples were denatured and labeling analyzed by fluorescence scanning.

**Effect of chaperone IFG 8 on GBA β-glucosidase activity** – Confluent N370S homozygote fibroblasts were cultured for one week with 0–300 nM IFG 8. After extensive washing with PBS, cells were lysed by scraping in 25 mM potassium phosphate buffer (pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitor cocktail (Roche)). After determination of protein concentration (BCA kit, Pierce), GBA β-glucosidase activity was measured as described before. The incubation mixture contained the same concentration of IFG 8 as to which cells were exposed, 3 mM 4MU-β-D-Glc, 0.2% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100 in 150 mM McIlvaine buffer, pH 5.2.

**Effect of chaperone IFG 8 on labeling with β-epoxide ABP 5** – Confluent N370S homozygote fibroblasts were cultured for one week with 0–300 nM IFG 8. Hereafter, cells were incubated with 10 nM β-epoxide ABP 5 for 2 h, lysed by scraping in 25 mM potassium phosphate buffer (pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitor cocktail). After determination of protein concentration (BCA kit, Pierce), 25 μg total protein was denatured and labeling was assessed by fluorescence scanning of SDS-PAGE gels. ABP-emitted fluorescence was quantified using ImageQuant (5.1), and verified in-gel by presence of 50 fmol equimolar ABP 4– and 5-labeled imiglucerase.

**In situ hydrolysis of FDG in presence of IFG 8** – Confluent GBA wild-type fibroblasts were incubated with 0–20 μM IFG 8 for 2 h at 37 °C and subsequently with 50 μM fluorescein-di-β-D-glucopyranoside (FDG) for 1 h at 37 °C. Next, cells were suspended by trypsinization and analyzed by FACS using FACS Vantage (B.D. Bioscience), λ<sub>EX</sub> 488 nm, λ<sub>EM</sub> 530 nm (band pass filter 30 nm).

**Reversibility of IFG 8 binding** – Velaglucerase (Shire PLC, 2 pmol) was incubated with 0–1 mM IFG 8 in 150 mM McIlvaine buffer (pH 5.2, supplemented with 0.2% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100) for 1 h at 37 °C, whilst capturing GBA with mouse α-human GBA 8E4 mAb, immobilized on Sepharose-Prot A beads. After centrifugation at 800 rpm, pellet was either washed with aforementioned McIlvaine buffer or not, and incubated with 10 nM β-epoxide ABP 4 for 15 min at 37 °C. Finally, samples were denatured, subjected for SDS-PAGE and labeled GBA analyzed by fluorescence scanning. ABP-emitted fluorescence was quantified using ImageQuant (5.1), and verified in-gel by presence of 50 fmol equimolar ABP 4– and 5-labeled imiglucerase.
References

Supplementary Information

Supplementary Figure S1 | Inhibition constants as determined for recombinant GBA with 4MU-β-α-Glc. (a) Progress curves of 4MU liberation (corrected for blank and 4MU-fluorophore photo-bleaching), adjusted to the relative maximal activity at 90 min (n = 3). Control (straight line) versus CBE 1 (50–500 μM: light to dark line); cyclophellitol 2 and β-epoxide ABP 3 (50–500 nM); β-epoxide ABP 4 and β-epoxide ABP 5 (2–16 nM). (b) Apparent k’ versus [I]₀ plots. Data-points represent mean of n = 9 individual k’ ± SEM. Solid lines represent best fit according to equation described in Experimental Section. Dashed lines represent 95% intervals calculated for the fitted line.
Supplementary Figure S2 | Molecular docking of β-epoxide ABPs 4 and 5 in GBA crystal structure 2V3E. (a) Lowest free energy conformers of β-epoxide ABPs 4 and 5 (black sticks with semi-transparent space-filling spheres) docked on GBA crystal structure 2V3E (grey semi-transparent space-filling model) with acid/base residue E235 (dark grey sticks) and nucleophile residue E340 (light grey sticks). Two common docked structures (Trench I, II) with identical lowest free energies calculated for β-epoxide ABPs 4 and 5 (−8.2, −8.4 kcal mol\(^{-1}\), respectively). (b) Detail of coordination of β-epoxide ABPs 4 and 5 in the active-site of GBA, with calculated distances to acid/base and nucleophile. Overlay with crystal structure 2VT0 containing CBE 1, covalently bound to E340 is shown (nucleophile in white and bound CBE in lighter grey).
Cyclophellitol β-epoxide ABPs

Supplementary Figure S3 | In situ localization of active GBA as visualized specifically by β-epoxide ABP 5. Spectral imaging micrographs of wild-type fibroblasts treated with red fluorescent β-epoxide ABP 5 (top row), with ABP 5 after CBE 1 treatment (second row). Cells treated with non-reactive, red fluorescent probe 7 in the absence (third row) or presence of CBE 1 (fourth row). Autofluorescence (grey) was unmixed from ABP 5 BODIPY fluorescence (red); nuclei were stained with DAPI (blue).