CHAPTER 1

Development of activity-based protein profiling methods for *in vitro* and *in situ* visualization of glucocerebrosidase

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ABSTRACT

Glycosidases mediate the fragmentation of glycoconjugates in the body, including the vital recycling of endogenous molecules. Several inherited diseases in men concern deficiencies in lysosomal glycosidases degrading glycosphingolipids. Prominent is Gaucher disease caused by an impaired lysosomal β-glucosidase (glucocerebrosidase; GBA) and resulting in pathological lysosomal storage of glucosylceramide (glucocerebroside) in tissue macrophages. GBA is a retaining glucosidase with a characteristic glycosyl–enzyme intermediate formed during catalysis. Using the natural suicide inhibitor cyclophellitol as lead, mechanism-based irreversible inhibitors of GBA equipped with a fluorescent reporter was developed. These covalently link to the catalytic nucleophile residue of GBA and permit specific and sensitive visualization of active enzyme molecules. The amphiphilic activity-based probes (ABPs) allow *in situ* detection of active glucocerebrosidase in cells and organisms. Furthermore, they may be used to biochemically confirm the diagnosis of Gaucher disease and they might assist screening for small compounds interacting with the catalytic pocket. While the focus of this chapter is ABPs for β-glucosidases and Gaucher disease, the described concept has meanwhile been extended to other retaining glycosidases and related disease conditions as well.

1.1 Introduction

The human body contains a huge number of glycoconjugates that are ongoingly synthesized and degraded by glycosidases. As first described by Daniel E. Koshland, the hydrolysis of the glycosidic bond by glycosidases results in inversion or retention of the anomeric stereochemistry in the glycon.[1] Inverting glycosidases employ two carboxylic acid residues, typically spaced 6–11 Å apart and positioned on opposing sides of the substrate glycoside, allowing joint entry of substrate and a water molecule.[2] The reaction proceeds through a single oxocarbenium ion-like transition state. In contrast, retaining glycosidases use a double-displacement mechanism that proceeds via a glycosyl–enzyme intermediate, flanked by two oxocarbenium ion-like transition states, preserving the initial configuration at the anomeric center. Two adjacent carboxylic acid residues, spaced 5.5 Å apart, function as the catalytic nucleophile and acid/base residue.[3] Variations on the classical Koshland-type mechanisms have been identified.[4]

Among the many cellular glycoconjugates are glycosphingolipids (GSLs), consisting of ceramide lipid backbones extended with oligosaccharides.[5] Their recycling involves fragmentation in lysosomes through sequential action of glycosidases and acid ceramidase. A genetic deficiency of a lysosomal glycosidase involved in GSL degradation causes accumulation of the corresponding lipid substrate.[6] Most prominent among these inherited glycosphingolipidoses are Gaucher disease (GD), Krabbe disease, Tay-Sachs disease and Fabry disease. Albeit monogenetic, the lysosomal disorders show remarkable variation in nature of symptoms, age of onset and progression of clinical presentations.

Over the years, GD has acted as frontrunner in fundamental research on lysosomal glycosidases and subsequent development of rational therapies for enzymopathies.[7] One and a half century ago Ernest Gaucher described a patient with a markedly enlarged spleen showing accumulation of lipid-laden phagocytes. It was soon recognized that this patient represented a distinct disease entity, subsequently referred to as Gaucher disease.[8] The accumulating lipid in the lysosomes of storage macrophages (Gaucher cells) of GD patients was identified as glucosylceramide (glucocerebroside). Next, Roscoe Brady and co-workers demonstrated that deficiency of glucocerebrosidase (GBA, acid β-glucosidase; EC. 3.2.1.45) is the molecular cause of GD.[9] Over 200 mutations in the GBA gene (locus 1q21) have meanwhile been linked to the disease.[10] The clinical manifestation of GD is remarkably heterogeneous, ranging from lethal

neonatal complications to an almost asymptomatic course.[8] The most prevalent manifestation, type 1 GD, does not involve pathology of the central nervous system. Major symptoms are enlargement of spleen and liver, infiltration of the bone marrow by storage cells, thrombocytopenia, anemia and bone disease. GD types 2 and 3 are accompanied by fatal neurological symptoms developing in the first years of life or at later age, respectively. The most severe manifestation is the so-called collodion baby, born with lethal skin permeability.[11] For several GBA genotypes considerable variability in disease severity has been documented, even among monozygotic twins.[12, 13] Deficiency of GBA has recently been recognized as a risk factor for multiple myeloma.[14] Intriguingly, carriers of a mutant GBA allele are also at markedly increased risk for developing Parkinsonism.[15]

The life cycle and structural features of GBA have been studied in great detail. The acquired fundamental knowledge has been translated to (experimental) therapeutic interventions. Given the success of bone marrow transplantation, presently gene therapy of GD is pursued, aiming at macrophage-specific expression of GBA in genetically modified hematopoietic stem cells.[16] Small compound glycomimetics (chemical chaperones) promoting folding and/or increasing the structural stability of GBA are searched and designed.[17] Substrate reduction therapy inhibiting biosynthesis of glucosylceramide through oral administration of inhibitors of glucosylceramide synthase has already been registered as treatment of GD (Miglustat, Actelion; Eliglustat, Sanofi-Genzyme).[18, 19] Moreover, intravenous supplementation of macrophage-targeted recombinant GBA results in major visceral improvements in type 1 GD patients, but fails to prevent neurological manifestations in type 2 and 3 GD patients.[20] Currently, several enzyme preparations have been registered for enzyme replacement therapy of type 1 GD.[7] The success of enzyme replacement therapy has led to similar approaches for other lysosomal enzymopathies in the lysosomal turnover of GSL, mucopolysaccharides and glycogen. Glycosidases, particularly those implicated in rare lysosomal storage diseases, receive consequently great attention. New tools to visualize active glycosidases in living cells and organisms are warranted.

GBA is a retaining β-glucosidase with E340 as nucleophile and E325 as acid/base residue (**Fig. 1.1**, upper panel).[21] Cyclophellitol **1** (**Fig. 1.1**, lower panel), isolated from the *Phellinus sp.* mushroom, is a mechanism-based inhibitor of GBA, reacting far more potently and specifically with GBA than cyclitol epoxide conduritol B-epoxide (CBE).[22, 23] Cyclophellitol has been

successfully used as a lead for the development of high affinity ABPs for retaining β-glucosidases, in particular for the human enzyme GBA.[24, 25] Grafting an azide group onto the C6 position of cyclophellitol **1** (glucopyranose numbering) yielded azido-cyclophellitol **2** (**Fig. 1.1**, lower panel). When **2** was covalently extended at C6 with BODIPY fluorophore moieties, the resulting compounds **3** and **4** were even 100-fold more potent inhibitors for GBA than parent cyclophellitol **1**.[24] The fluorescent ABPs, green-fluorescent β-epoxide **3** (MDW933) and red-fluorescent β-epoxide **4** (MDW941) allow immediate fluorescence scanning of SDS-PAGE gels with ABP-labeled proteins.[24] The attachment of the bulky BODIPY moiety at the C6-position in cyclophellitol enhanced the potency and specificity of the inhibitor for GBA.

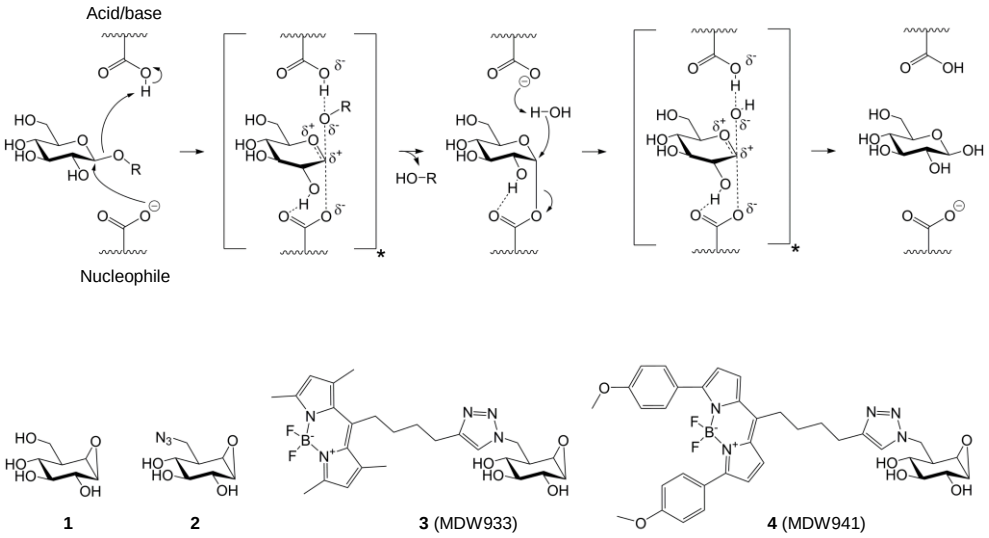

Figure 1.1. Catalytic mechanism of retaining glycosidases and ABPs. Upper panel: Catalytic mechanism of a retaining β-glycosidase, characterized by a covalent glycosyl–nucleophile adduct, flanked by two oxocarbenium ion-like transition states (*). The transition states are stabilized by hydrogen bonding (finely dotted line) between the C-2 hydroxyl group of the substrate and the nucleophile. Lower panel: Structures of cyclophellitol β-epoxide 1, azido-cyclophellitol **2** (KY170), fluorescent β-epoxide **3** (MDW933, green fluorescent) and β-epoxide **4** (MDW941, red fluorescent).

Active GBA can be labeled in lysates of cells with **3** or **4** and subsequently visualized by fluorescence scanning following SDS-PAGE electrophoresis. **Fig. 1.2** shows ABP-labeled GBA in lysates of human fibroblasts. Multiple molecular weight isoforms are detected, reflecting variations in the N-linked glycan composition of GBA.[26] Active GBA can also be labeled in living cells thanks to the cell permeability of the amphiphilic ABPs.[24] **Fig. 1.3** Shows the *in situ*

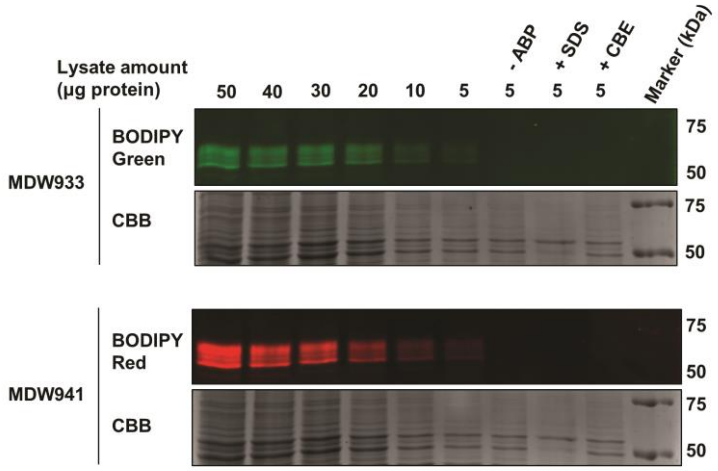

Figure 1.2. *In vitro* MDW933 (3) and MDW941 (4) labeling of GBA in human fibroblast lysates. Different amounts of samples were labeled with 100 nM MDW933 or MDW941 for 30 min at 37 °C, denatured, resolved using 7.5 % polyacrylamide SDS-PAGE, and scanned for BODIPY green or red fluorescence (upper and lower panel respectively). Coomassie Brilliant Blue G250 (CBB) staining was used to visualize total protein. No ABP was added to the negative control sample (- ABP). 2 % SDS was added to one sample (+SDS) and incubated for 5 min at 98°C prior to ABP addition. 1 mM CBE was added to one sample (+CBE) for 30 min at 37 °C prior to ABP addition.

labeling with **4** of GBA in human fibroblasts as visualized by confocal fluorescence microscopy.

The β-glucosyl configured cyclophellitols find broad applications as inspired by the seminal work by Cravatt and co-workers with ABPs.[27, 28] It has been earlier reported that they can be used in biochemical confirmation of the diagnosis GD through the demonstration of reduced ABP-labeled GBA in fibroblasts.[24, 29] The ABPs can be employed across species to visualize active GBA.[24, 30, 31] *In situ* ABP-labeling of active GBA in cultured cells and mice is feasible. In the case of cells, the ABP can be applied to the medium. In the case of mice, it needs to be intravenously infused.[32] Since BODIPY-tagged ABPs poorly penetrate the brain, *in situ* visualization of GBA in this tissue requires intrathecal administration of the ABP.[33] The use of the ABPs in correlative light and electron microscopy (CLEM) is presently pursued. Equipping the β-glucosyl

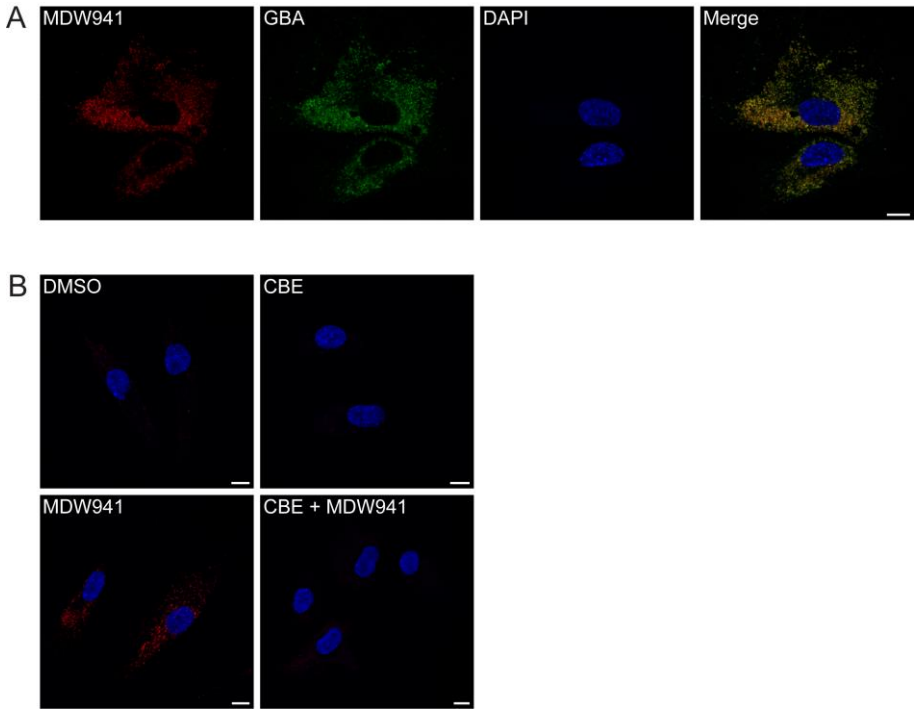

Figure 3. *In situ* labeling with MDW941 detects GBA in human fibroblasts by confocal fluorescence microscopy. A) In situ labeling with MDW941 for 2 h at 5 nM followed by immunostaining of GBA showing significant co-localization of active GBA (red) with total GBA (green). B) Active GBA is detected with MDW941, while pre-incubation with CBE followed by MDW941 results in loss of the fluorescent signal. Scale bars, 10 µm.

configured cyclophellitol with a biotin at C6 drastically decreased its activity against GBA[24] and was not suitable for streptavidin-bead pull down application, but novel biotin ABPs with longer linker are currently being developed for GBA (General Discussion and Future Prospects, this thesis). In principle, proteins strongly interacting with GBA could be enriched in this manner and identified by proteomics.

Another future application of the ABPs could be (ABPP) screening of compound libraries for structures showing affinity for the catalytic pocket of GBA. Such compounds may be of interest to be developed further as therapeutic chemical chaperones.[17] The β-glucosyl configured cyclophellitol ABP has been also used to study the interaction of isofagomine with GBA in intact cells.[24] Finally, the BODIPY-equipped ABPs, not penetrating the brain possibly due to active Pgp-mediated removal, may be used to pharmacologically generate a visceral model of

Gaucher disease without the fatal neuropathology in GBA-deficient mice.

Here, a detailed protocol for GBA detection using ABPs **3** and **4** is presented in this chapter. The first section lists the equipment and materials (**1.2**); the second section details the protocol for *in vitro* ABP labeling and SDS-PAGE-based fluorescence detection for GBA (**1.3**); the third section concerns the protocol for *in situ* ABP labeling of GBA in intact cells followed by SDS-PAGE-based fluorescence detection (**1.4**); and the fourth section reports on the protocol for *in situ* ABP labeling of GBA in intact cells followed by detection using fluorescent microscopy (**1.5**). Meanwhile, the concept of cyclophellitol-type ABPs has been extended to other retaining glycosidases (General introduction, this thesis). Thus, this protocol can also be used as a basis for activity-based protein profiling using other cyclophellitol-based or cyclophellitol-aziridine-based ABPs targeting different disease-relevant glycosidases.

1.2 List of equipment and materials

Equipment

Spectrophotometer
Heating block
Tabletop centrifuge
1.5-mL Microcentrifuge tubes
200-µL PCR tubes
15- and 50-mL Centrifuge tubes
Micropipettors
Micropipettor tips
SDS-PAGE equipment and glass plates
Fluorescence imager or scanner (with settings for BODIPY green or red fluorescence)
Sterile 6-well and 12-well plates
Coverslips
Incubator at 37 °C with 5 % CO2
Parafilm
Confocal fluorescence microscope
Microscope slides

Materials

a. 25 mM Potassium phosphate buffer (KPi) pH 6.5 (1 L)

Component	Final concentration	Stock concentration	Amount
K_2HPO_4	7.5 mM	1 M	7.5 mL
KH_2PO_4	17.5 mM	1 M	17.5 mL

*Add 950 mL of ultrapure-H_2O, adjust the pH to 6.5 and add H_2O to a total volume of 1 L

b. Cell lysis buffer (50 mL)

Component	Final concentration	Stock concentration	Amount
KPi buffer pH 6.5	25 mM	25 mM	49.5 mL
Protease inhibitor - cocktail tablet (Roche)	-		1 tablet
Triton X-100	0.1 % (v/v)	10 % (v/v)	0.5 mL

*Note: Store at -20 °C in 1 mL aliquots

c. 750 mM McIlvaine buffer pH 5.2 (40 mL)

Component	Final concentration	Stock concentration	Amount
Citric acid	0.27 M	0.5 M	21.4 mL
Na_2HPO_4	0.46 M	1 M	18.6 mL

*Note: 0.01% (w/v) NaN_3 can be added to both Na_2HPO_4 and citric acid stocks as bacteriostatic agent

d. 150 mM McIlvaine buffer pH 5.2 (40 mL)

Component	Final concentration	Stock concentration	Amount
Citric acid	0.054 M	0.1 M	21.4 mL
Na_2HPO_4	0.093 M	0.2 M	18.6 mL

*Note: 0.01% (w/v) NaN_3 can be added to both Na2HPO4 and citric acid stocks as bacteriostatic agent

e. 5x Laemmli sample buffer (10 mL)

Component	Final concentration	Stock concentration	Amount
Tris-HCl pH=6.8	0.3 M	1.25 M	2.5 mL
Glycerol	50% (v/v)	100% (v/v)	5 mL
Sodium dodecyl sulfate	10% (v/v)	-	1 g
Bromophenol blue	0.1% (v/v)	1% (w/v)	1 mL

*Add ultrapure-H_2O to a total volume of 10 mL

1.3 Protocol: Fluorescent detection of ABP-labeled GBA on wet slab gels

MDW933 (**3**) and MDW941 (**4**) are dissolved in DMSO and stored in small aliquots at -20 °C until use. The labeling can be performed on samples *in vitro* or directly in intact cells (*in situ*). For the *in vitro* labeling procedure, the samples and ABP are diluted separately in McIlvaine buffer pH 5.2 and combined for a 30 min incubation at 37 °C, and denatured for SDS-PAGE. For the *in situ* labeling procedure, MDW933 or MDW941 is diluted in culture medium and applied to the cell culture for a 2 h incubation at 37 °C. Subsequently, the cells are washed three times in PBS and lysed in lysis buffer. Proteins from both *in vitro* and *in situ* labeled samples are resolved by SDS-PAGE and wet slab gels are scanned for BODIPY green or red fluorescence using a fluorescence scanner.

Storage of ABPs

Aliquots of MDW933 and MDW941 are made in either 1 mM or 50 µM concentrations, in a volume of 1–2 µL.

1. To make the 1 mM stock, add 100 µL DMSO to the tube containing 100 nmol lyophilized MDW933 or MDW941, vortex to dissolve and transfer to a 1.5 mL Eppendorf tube.

2. Make 1 µL or 2 µL aliquots for the 1 mM stock in PCR tubes (200 µL volume).

3. To make the 50 µM stock, add 38 µL DMSO to one tube of the 2 µL 1 mM stock, vortex to dissolve and make aliquots of 1 µL in PCR tubes.

4. Place the PCR tubes into containers or 50 mL centrifuge tubes and store them at -20°C. MDW933 and MDW941 are stable at -20 °C for long-term storage (> 1 year).

*Tip

One tube of 1 µL 50 µM MDW933 or MDW941 is sufficient for the *in vitro* labeling of 66 samples at 100 nM labeling concentration (SDS-PAGE loading volume = 20 µL), or 1 x 6-well plate of culture cells at 5 nM labeling concentration (1 mL culture medium per well).

**Caution

1. Avoid freeze-thawing the ABP stocks (in DMSO) more than twice.

2. Do not store and re-use ABPs that have been diluted in H_2O or buffer.

3. Protect the ABPs from light.

In vitro labeling and in-gel detection

1. Prepare lysates and homogenates in lysis buffer and measure protein concentration by the BCA assay (Pierce, BCA protein assay kit).

2. On ice, prepare 2–5 µL samples in 1.5 mL tubes, containing 5–50 µg total protein, or 0.5–50 fmole GBA molecules of purified enzyme stock.

3. Add McIlvaine buffer (pH 5.2) to the samples, to a total volume of 10 µL, and incubate on ice for 5 min. Use 150 mM McIlvaine buffer for lysate volumes ≤ 2 uL, and 750 mM for lysate volumes of 2–5 µL.

4. Prepare 300 nM MDW933 or MDW941 diluted in McIlvaine buffer (pH 5.2, 150 mM or 750 mM) using the 50 µM MDW933 or MDW941 stock (e.g. 1 µL 50 µM stock + 166 µL McIlvaine buffer).

5. Add 5 µL 300 nM MDW933 or MDW941 to each sample (10 µL) and incubate at 37°C on a thermoshaker or in a waterbath for 30 min.

6. After the incubation, add 3.75 µL 5x Laemmli sample buffer to the sample, and incubate at 98 °C for 5 min.

7. Resolve the proteins by SDS-PAGE (7.5 % or 10 % polyacrylamide gels) and quantify the labeling on a Typhoon scanner (GE Healthcare) using the following fluorescence settings: green

BODIPY: λ_{ex} = 488 nm, λ_{em} = 500–550 nm; red BODIPY: λ_{ex} 594 nm, λ_{em} = 605–645 nm.

*Tip

1. For optimal labeling and fluorescence detection in lysates and homogenates, the protein concentration in the prepared cell lysates or tissue homogenates is best kept at 10-20 μg/μL.

2. When performing labeling on purified GBA, supplement the McIlvaine buffer with 0.1% (v/v) Triton X-100 and 0.2% (w/v) sodium taurocholate.

3. Optimal labeling visualization is achieved with 0.5–50 fmole GBA per sample.

4. Include one sample without MDW933 or MDW941 treatment as negative control for assessing autofluorescence from the samples.

1.4 Protocol: *In situ* labeling and in-gel detection

Cells are treated with 5 nM MDW933 or MDW941 at 37 °C for 2 h, washed three times with PBS, lysed in lysis buffer and detached by scraping. The homogenate is resolved by SDS-PAGE and the fluorescent signal on the gel is quantified by fluorescent scanning.

1. Maintain normal human dermal fibroblasts (Lonza) in DMEM containing 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Sigma, D6546), or F-12 Ham/DMEM (Sigma, D8062), both supplemented with 10% (v/v) FBS, 100,000 units/L penicillin, 100 mg/L streptomycin and 2 mM glutamax.

Transfer the fibroblasts to 6-well plates and let them grow until at least 80 % confluency.

2. In a 15 mL centrifuge tube, combine 10 mL pre-warmed culture medium (37 °C) with 1 μL of 50 μM MDW933 or MDW941.

3. Aspirate the culture medium from the cell culture plate and add 1 mL of the prepared 5 nM MDW933 or MDW941 diluted in the pre-warmed culture medium.

4. Incubate for 2 h in a 37 °C incubator.

5. After the incubation, aspirate the MDW933 or MDW941-containing medium and wash the cells three times with 1 mL PBS.

6. Aspirate the PBS, place the culture plate on ice and add 50 µL pre-chilled 25 mM KPi buffer pH 6.5 (+0.1% (v/v) Triton X-100 and protease inhibitor cocktail) to each well of the plate.

7. Use a cell scraper or the back of a P200 pipette tip to detach the fibroblasts from the plate and collect the cells in 1.5 mL Eppendorf tubes.

8. Vortex, store the samples at -80 °C for 1 h, and thaw the samples on ice.

9. Determine the protein concentrations using the BCA assay (Pierce BCA protein assay kit) and a spectrophotometer (absorption set at 562 nm). The typical protein concentration for *in situ* treated fibroblast lysates is between 1–2 µg/µL.

10. For SDS-PAGE, samples containing the same amount of protein (typically 5-30 µg protein) are diluted in 25 mM KPi buffer (+0.1% (v/v) Triton X-100 and protease inhibitor cocktail), in a total volume of 15 µL, denatured by incubation with 3.75 µL 5x Laemmli sample buffer at 98 °C for 5 min, and resolved by SDS-PAGE.

11. Quantify fluorescence on wet slab gel using a Typhoon scanner (GE Healthcare).

*Tip

For ABP labeling experiment involving different ABP concentrations, or treatment at different time points, an alternative labeling method can be followed. For this method, first pre-dilute the ABP stock (1 mM or 50 µM) in DMSO into 1000x of the desired labeling concentrations in sterile 1.5 mL tubes, then directly pipette 1 µL of the prepared ABP dilutions per well to the 6-well plate (containing 1 mL refreshed medium per well). It is important to ensure proper mixing of the ABP in the culture medium.

1.5 Protocol: *In situ* labeling of GBA for fluorescence microscopy

To localize active GBA, the cells are *in situ* labeled with MDW941 by applying the ABP to the culture medium. It is important to include controls for autofluorescence, i.e. DMSO treated cells, and for the specificity of the ABP signal. For the latter, the cells are incubated with CBE prior to the in situ labeling with ABP. This compound will bind to the active GBA molecules, thereby blocking all binding by the ABP. Any remaining signal will represent unbound ABP.

1. The day before the experiment, transfer normal human dermal fibroblasts to four wells of a 12-well plate that contain autoclaved coverslips (Menzel, 15 mm diameter). Allow the cells to adhere to the coverslips by incubating them overnight in a humidified incubator at 37 °C with 5 % CO_2.

2. The cells should have reached ~70 % confluency on the day of the in situ labeling. Remove the culture medium and place 1 mL of fresh medium in each well.

3. Two wells are treated with CBE as a control for the specificity of the signal. Dissolve CBE (Enzo Life Sciences Inc.) in ultrapure-H_2O at a concentration of 150 mM, e.g. by adding 200 µL ultrapure-H_2O to 5 mg CBE. Add 2 µL of CBE per well (final concentration will be 0.3 mM) and incubate the cells at 37°C for 16 h.

4. After the 16 h incubation, remove the culture medium from the wells and wash twice with PBS. Subsequently, place 1 mL of culture medium in each well.

5. Dilute MDW941 in DMSO to a concentration of 5 µM, e.g. by adding 1 µL of the 1 mM stock to 200 µL DMSO. Subsequently, add 1 µL of the 5 µM dilution to one well that was pre-incubated with CBE and to one untreated well. This final concentration of 5 nM will label ~50 % of total endogenous GBA in these cells, but optimal concentrations should be determined for each cell line. Add 1 µL of DMSO to the second untreated well as a control for autofluorescence. From this step onwards it is important to protect the cells from light.

6. Incubate the cells for 2 h at 37 °C.

7. At the end of the incubation, remove the culture medium from the wells and wash 3 times with 1 mL PBS.

8. Fix the cells by placing 1 mL of 4 % (w/v) formaldehyde/PBS in each well and incubate for 25 min at RT. A stock of 16 % formaldehyde/H_2O can be made as described in Slot & Geuze, 2007.[34] Dilute this stock 1:4 in PBS, e.g. by mixing 4 mL of 16 % formaldehyde with 1.6 mL 10x concentrated PBS and 10.4 mL distilled H_2O.

9. Remove the fixative and wash the coverslips with PBS by placing 1 mL in each well. Remove the PBS and repeat this wash two times. To stain all GBA in the cells (active and inactive) for immunofluorescence microscopy continue with step 10. To mount the coverslips directly, skip

steps 10-14 and proceed with step 15.

10. Prepare a piece of parafilm with 200 µL drops of 2 % (w/v) bovine serum albumin/ 0.1% (w/v) saponin in PBS (= permeabilization buffer). Incubate the coverslips onto these drops, with the cells facing the drop, for 10 min. Cover the coverslips with a petri dish wrapped in aluminum foil to protect from light and evaporation.

11. Incubate the coverslips for 1 h at RT onto 100 µL drops of 1:500 diluted mouse anti-GBA monoclonal antibody 8E4 (generated in the Aerts lab) in permeabilization buffer.

12. Transfer the coverslips to 200 µL drops of permeabilization buffer and incubate for 10 min. Repeat this washing step twice.

13. Incubate the coverslips for 1 h at RT onto 100 µL drops of 1:500 diluted Alexa Fluor 488-coupled donkey anti-mouse IgG (H+L) (Invitrogen).

14. Wash the coverslips three times on 200 µL drops of permeabilization buffer.

15. Wash the coverslips quickly by dipping them in distilled H_2O.

16. Drain off the H_2O from the coverslips and mount them on a microscope slide (VWR) with ProLong Diamond antifade reagent containing DAPI (Molecular Probes). Allow the coverslips to dry overnight, seal with nail polish and store at 4°C.

17. Image the cells under a Leica SP8 confocal microscope with a 63x/1.40 NA HC Plan Apo CS2 oil immersion objective and equipped with a hybrid detector (HyD). Image MDW941 with excitation at 552 nm, emission 590–650 nm, Alexa Fluor 488 with excitation at 488 nm, emission 500–540 nm and DAPI with excitation at 405 nm, emission 420–480 nm.

*Tip

The concentration of *in situ* applied MDW933 or MDW941 can be adjusted from 5 nM to 100 nM, according to cell types and confluency, and it is advisable to validate the percentage of GBA inhibition by *in vitro* 4-methylumbelliferyl substrate assay.[35]

1.6 Conclusion

Deficiency of GBA leads to Gaucher disease and constitutes a risk factor for multiple myeloma and Parkinsonism. The biochemical and clinical study on Gaucher disease has enabled tremendous therapeutic development in the past decades, which has encouraged the research and therapeutic development on other lysosomal storage diseases involving glycosidases. The availability of the here-described ABPs specifically labeling GBA in a mechanism-based manner offer new valuable tools for research on the enzyme, diagnosis and therapy development. Detailed protocols for *in vitro* and *in situ* detection of GBA using these ABPs were described in this chapter, which should also serve as a basis for ABP detection of other glycosidases relevant in lysosomal storage diseases.

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