Transfer of "goods" from plants to humans: Fundamental and applied biochemical investigations on retaining glycosidases

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Chapter 6

Cross species investigations with activity-based probes. Future prospects

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Abstract

The potential use of ABPs cross species was investigated. Studied were lysates derived from Bright Yellow tobacco cell lines (BY2) and the food supplement Beano containing a fungal (*A. niger*) α-galactosidase.

The investigations with BY2 cell lysates led to the discovery of a plant ß-glucosidase that is potently labeled by the ß-glucose configured epoxide cyclophellitol ABP and its identity was next established by proteomics. The enzyme, here named B56, is active towards NBD-GlcCer lipid, revealing its potential use in therapy of Gaucher disease.

ABPs could be also successfully applied to label the α-galactosidase from *A. niger* present in the food supplement Beano. The enzyme was found to be active towards artificial 4-methulumbelliferyl-α-galactoside but not towards NBD-Gb3.

As Fabry patients suffer from intestinal complications, possibly induced by accumulating lysoGb3 in the intestine, future application of other αGAL enzymes might be considered for diminishing such toxic agents.

Introduction

Retaining glycosidases are remarkably conserved among species in their catalytic mechanism based on double displacement. This reaction is essentially governed by appropriately placed nucleophile and acid/base residues in the catalytic pocket (Davies and Henrissat 1995). Due to the conserved structural prerequisites for catalytic activity, glycosidases across species are found to be targeted by activity-based probes (ABPs) consisting of a correctly configured cyclophellitol with a reporter group linked to it (Witte et al. 2010).

Retaining glycosidases fulfil important functions in human physiology. For example, in the lysosomes of cells specialized glycosidases are essential in turnover of glycoconjugates. Examples are the acid ß-glucosidase (glucocerebrosidase, GBA) hydrolyzing glucosylceramide (GlcCer) and α-galactosidase A (GLA) hydrolyzing globotriaosylceramide (Gb3) (Ferraz et al. 2014). Inherited deficiencies in these enzymes of glycosphingolipid metabolism cause the lysosomal storage disorders Gaucher disease and Fabry disease, respectively (Beutler and Grabowski 2001; Desnick et al. 2003).

Cyclophellitol and cyclophellitol-aziridine are known suicide inhibitors of ß-glucosidases (Kallemeijn et al. 2014b; Li et al. 2014; Kuo et al. 2018). Different sets of ABPs have been designed to label the retaining ß-glucosidases present in human cells and tissues. One set consists of cyclophellitol (-epoxide) with linked to C6 (C8 cyclophellitol numbering) a spacer with attached reporter (fluorophore or biotin) (Witte et al. 2011). The other set consists of cyclophellitol-aziridine with linked to the nitrogen a spacer with attached reporter. The first set of ABPs specifically labels GBA, whilst the second set labels all human ß-glucosidases (GBA, GBA2, GBA3 and LPH) (Kallemeijn et al. 2012, 2014a).

Differently configured cyclophellitol-aziridines have been subsequently designed to label various classes of retaining glycosidase (Witte et al. 2011; Kallemeijn et al. 2012; Willems et al. 2014a; Kallemeijn et al. 2014a; Jiang et al. 2015, 2016; Marques et al. 2017; Wu et al. 2017; Artola et al. 2018). For example, galactose-configured cyclophellitol-aziridine ABP labels the highly homologous α-galactosidase enzymes GLA and α-N-acetylgalactosaminidase (NAGA) (Kytidou et al. 2017). Earlier we labeled with the same ABP an ubiquitous retaining α-galactosidase in *Nicotiana benthamiana (N. benthamiana)* (Kytidou et al. 2018). The protein, named A1.1, was identified, cloned and characterized. Crystallography revealed a high similarity in 3D-structure with the human counterparts GLA and NAGA (Kytidou et al. 2018). The A1.1 enzyme shows activity towards Gb3, albeit this lipid does not occur in

plants. The A1.1. α-galactosidase differs from GLA and NAGA in complete lack of N-glycans and a broader pH optimum (Kytidou et al. 2018). Its potential value to treat patients suffering from Fabry disease with recombinant A1.1 is being investigated.

The metabolism of the glycosphingolipid GlcCer is of great interest in relation to various diseases. Impaired lysosomal degradation due to deficient GBA activity causes formation of lipid-laden macrophages (Gaucher cells) that are thought to contribute to the hepatomegaly, splenomegaly, and cytopenia in Gaucher patients (Ferraz et al. 2014). Accumulation of GlcCer is partly prevented by its conversion to glucosylsphingosine (GlcSph) by the lysosomal enzyme acid ceramidase (Dekker et al. 2011; Ferraz et al. 2016). Recent studies suggest that GlcSph is a toxic agent that promotes gammopathy in Gaucher patients and multiple myeloma as well as increases the risk for Parkinson disease in carriers of a mutant GBA allele (Siebert et al. 2014; Nair et al. 2016). GlcCer is also degraded by the cytosol-face GBA2, a membrane-bound ß-glucosidase (Ferraz et al. 2014). GBA2 not only acts as hydrolase but also as transglucosidase transferring glucose from GlcCer to cholesterol to yield glucosylcholesterol (GlcChol) (Marques et al. 2016). For a number of decades type Gaucher patients are treated by enzyme replacement therapy (ERT) involving chronic intravenous infusion with recombinant GBA (Barton et al. 1991; Brady 2003). ERT allows correction of Gaucher cells, but does not prevent all complications in Gaucher patients, for example skeletal disease and neurological impairment (Ferraz et al. 2014).

Plants contain also the glycosphingolipid GlcCer and therefore very likely also possess a glucosyl-ceramidase fragmenting the lipid to ceramide and glucose, similar in function to GBA1 (chapter 2). Plants might also contain a transglucosylating ß-glucosidase, similar in function to GBA2. Glucosylated sterols are also abundant plants and yet unidentified enzymes involved in their synthesis and degradation have to exist (Grille et al. 2010). In this investigation, cyclophellitol and cyclophellitol-aziridine ABPs (Figure 1) were used in *Nicotiana tabacum* Bright yellow-2 cells (BY-2) to study the retaining ß-glucosidases in plants.

Another example of cross species use of glycosidase ABPs is provided by experiments performed with Beano®, a dietary supplement used to reduce flatulence after consumption of beans and cabbage. The supplement degrades complex carbohydrates present in such foods and thus prevents intestinal bacteria producing excessively intestinal gas (Prestige Brands Holdings, Inc., 2016a). A key component of Beano is an α-galactosidase from the fungus *Aspergillus niger (A. niger)* according to the producer (Prestige Brands Holdings, Inc., 2016b).

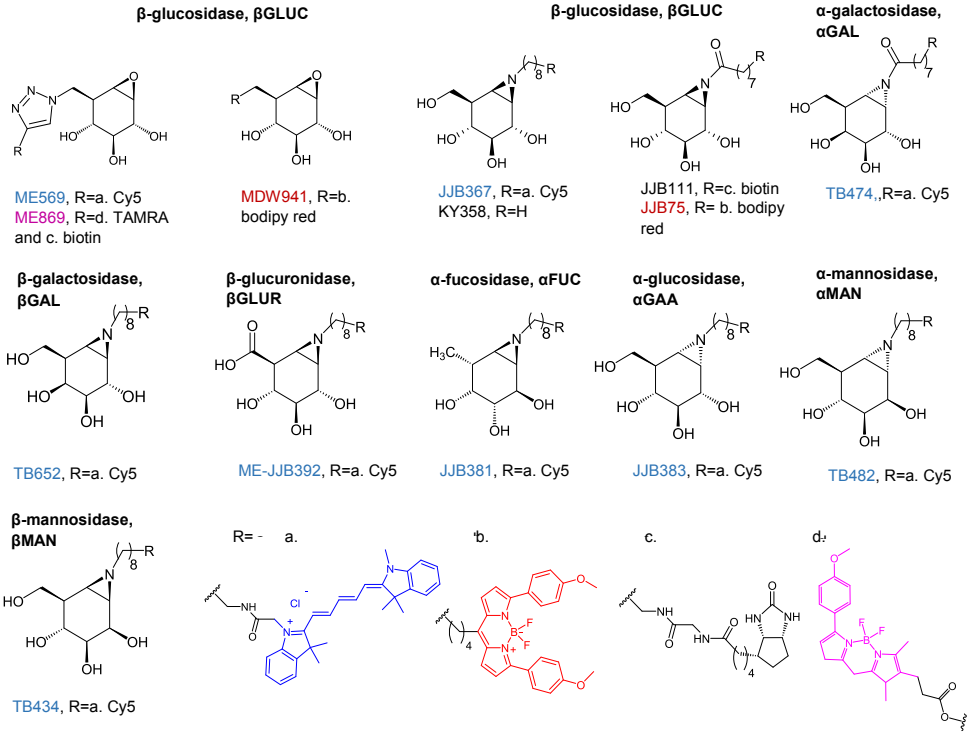

Figure 1. Chemical structures and codes of the ABPs used in this study and their targeting enzymes.

Results

Targeting *Nicotiana tabacum* BY-2 cells ß-glucosidases

An investigation regarding ß-glucosidases involved in glycolipid metabolism in *N. tabacum* BY-2 cells was conducted. In this study use was made of the cyclophellitol ABP and cyclophellitol-aziridine ABP and lysates of cultured BY-2 cells as source of enzymes (Figure 2). Cells were cultured in Linsmaier and Skoog medium (Linsmaier and Skoog 1965) supplemented with 0,09 M sucrose and 0.2 mg/lt 2,4-dichlorophenoxyacetic acid and were harvested at day 7 of sub-culturing. Cell lysates were prepared in ice cold lysis buffer (10 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 6, with 0,15 M NaCl and 0,1 % Triton X-100), using the FastPrep-24 machine (MP Biomedicals). Then, lysates were used directly for experiments or were further fractionated into soluble or insoluble fractions after centrifugation for 10 min at 16,000 rpm, 4 ^{o}C. B-glucosidase activity present in all fractions was first measured using 1.25 mg/mL of the 4-methylumbelliferyl (4MU) -ß-D-glucopyranoside substrate, at pH5 (Figure 2A). Activity present in soluble fraction was around 10 times lower than the one measured in full lysate. Insoluble fraction contained the highest activity, possibly due to high protein content of the preparation. Then, the fractions were incubated with 0.5 µM ME569, JJB367 or TB474 at pH5 conditions, for 30min, 37 ^{o}C. Figure 2B shows that a single protein with apparent MW at 57 kDa was labeled by the epoxide ABP, ME569. The protein was more prominent in the soluble fraction. Multiple proteins were labelled using the epoxide ABP, JJB367 at apparent MWs of 79, 73, 70, 65 kDa. Some of them were present in the soluble and others in the insoluble fraction. Use was made of the α-galactosidase configure ABP, TB474 to visualize α-galactosidases in the various fractions. Proteins with MWs of 45 and 39 kDa were labeled, as earlier observed with *N. benthamiana* leaves (Kytidou et al. 2018) (Chapter 3).

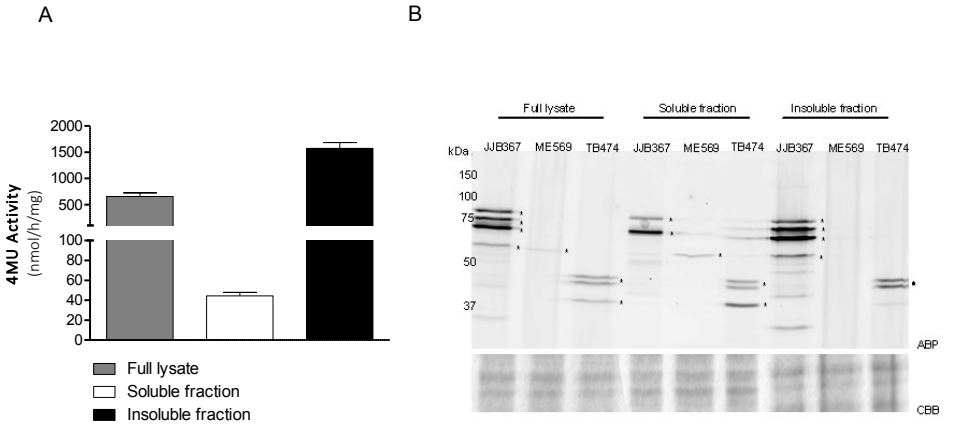

Figure 2. Targeting retaining glycosidases in BY2 cells. A. 4MU activities of BY2 lysates, as measured using the 4MU-β-D-glucopyranoside substrate. error bars indicate standard deviations, n=4 biological replicates. B. Full and fractionated BY2 cell lysates were incubated for 30min, 37ºC, pH5 with 0.5µM JJB367, ME569 or TB474 ABPs. The samples were then applied to 10 % SDS-PAGE gels and fluorescent scanning of the gels followed. Interestingly, a specific protein was labeled using the epoxide ME569, at 57 kDa. Multiple proteins were identified using JJB367 and the already characterized α-galactosidases using TB474.

N-glycosylation profile of the labeled BY2 ß-glucosidases as revealed by Concanavalin A binding.

Lysates were incubated with Concanavalin A (Con A) beads to separate proteins into a lectin binding fraction and non-binding fraction. Enzymatic activity was first measured, using 4-MU-ß-glucoside, in all fractions (Figure 3A). Results revealed that most enzymatic activity was present in the supernatant (non-binding fraction) fractions. Then, samples were incubated with 0.5 µM MDW941 at a pH of 5 or 0.5 µM JJB367 at a pH of 5 (conditions determined to be optimal for labeling). According to figure 3B the ME569-ABP-identified protein (MW 57 kDa) was present in the non-binding fraction upon Con A separation, suggesting that the protein might not contain N-linked glycans. Some of the proteins labeled with JJB367 were in the Con A binding fraction and others in the non-binding fraction.

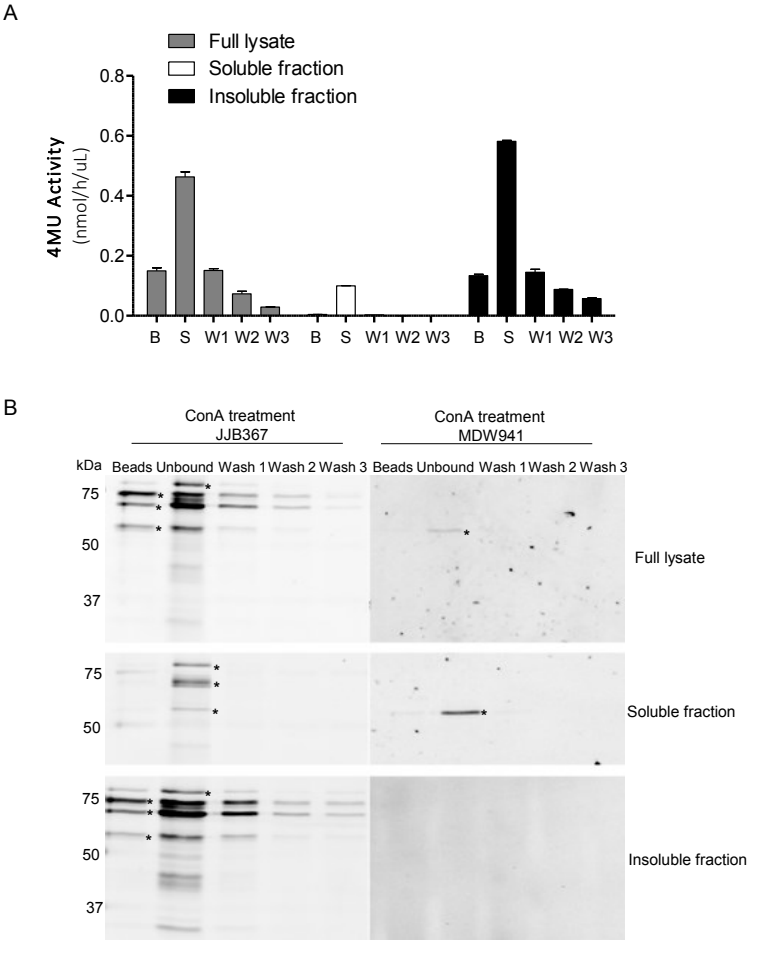

Figure 3. Studying the N-glycosylation profile of BY2 β -glucosidases. Concanavalin A (Con A) treatment of BY2 cell lysates following 4MU activity measurements (A) and ABP labeling (B). BY2 cell lysates were first incubated for 1hr, 4ºC while rolling with ConA beads. After incubation, centrifuge of the samples took place and the different fractions were obtained (beads (B), supernatant/ unbound (S), wash 1,2,3 (W1, W2, W3)). Then, the fractions were incubated for 30 min, 37 ºC, with 0.5 µM JJB367 or MDW941.

Next, biotinylated versions of the ABPs (JJB111 and ME869) (Figure 1) were used to target proteins followed by a pulldown with streptavidin-beads, as earlier described (Jiang et al. 2015, 2016). The nature of the enriched proteins was determined using LC-MS/MS based proteomics exactly as earlier described (Jiang et al. 2015, 2016). Measurement of the biotinylated targets was performed via on bead and in gel trypsin digestions, using the aziridine biotinylated ABP, JJB11 and also the newly synthesized cyclophellitol-epoxide-biotinylated ABP, ME869. Table 1 shows the outcome of on-bead digestions

using JJB111 in soluble fraction. The ME869, was found to label the protein A0A1S4CL56, named as B56, with predicted MW of 57 kDa, consistent with that observed for the labeled protein (Table 1). In the CAZy database, B56 is based on its amino acid composition classified as member of GH family 5. The GH5 family consists of glycoside hydrolases with various functions, including endo-ß-1,4-glucanase / cellulase, endo-ß-1,4-xylanase, ß-glucosidase, ß-mannosidase, glucan ß-1,3-glucosidase, exo-ß-1,4-glucanase / cellodextrinase, glucan endo-1,6-ß-glucosidase, mannan endo-ß-1,4-mannosidase, cellulose ß-1,4-cellobiosidase, chitosanase, ß-primeverosidase, xyloglucan-specific endo-ß-1,4-glucanase, endo-ß-1,6-galactanase, ß-1,3-mannanase, arabinoxylan-specific endo-ß-1,4-xylanase, mannan transglycosylase, lichenase / endo-ß-1,3-1,4-glucanase, ß-glycosidase, endo-ß-1,3-glucanase / laminarinase, ß-N-acetyl-hexosaminidase, chitosanase, ß-D-galactofuranosidase, ß-galactosylceramidase, endoglycoceramidase, ß-glucosylceramidase and steryl ß-glucosidase (www.cazy.org). More recently human GBA1 has been classified from GH5 to GH30 (Ben Bdira et al. 2018).

Table 1. List of on-bead identified JJB111 protein targets.

UniProt code	Protein name	MW (Da)	Score	SeqCov (%)	GH Family
A0A1S4CR57	beta-glucosidase BoGH3B-like	68860	685	30	3
A0A1S3ZME1	probable beta-D-xylosidase 6	88005	418	23	3
A0A1S4CL56	probable glucan 1,3-beta-glucosidase A isoform X2	57212	269	44	5
A0A1S4C677	beta-galactosidase	98264	163	24	35
A0A1S3XJ27	beta-glucosidase BoGH3B-like isoform X1	69022	136	17	3
A0A1S4AT57	glucan endo-1,3-beta-glucosidase, basic vacuolar	40474	100	25	17
A0A1S3ZFV9	lysosomal beta glucosidase-like	70183	81	6	3
A0A1S3ZJI2	beta-galactosidase 17-like	36223	72	24	35
A0A1S4C5S1	probable beta-D-xylosidase 5	89322	67	1	3
A0A1S3YQ73	beta-glucosidase 18-like	61221	44	7	1
A0A1S3XGA2	beta-xylosidase/alpha-L-arabinofuranosidase 2-like	86389	40	5	3

Activity of BY2 glycosidases towards NBD-GlcCer substrate.

It will be of great interest to study more closely A56 regarding various enzymatic and structural features. It has been observed that part of the enzymatic activity towards the fluorogenic substrate 4MU-ß-glucoside in the soluble fraction of BY2 lysate is inhibited by MDW941. Its pH optimum is about 5.0 and the activity seems not to be bound by Concanavalin A (Figure 3). Lysates of BY2 cell were found to degrade NBD-glucosylceramide to NBD-ceramide and this activity was inhibitable with ABP1 and ABP2 (Figure 4).

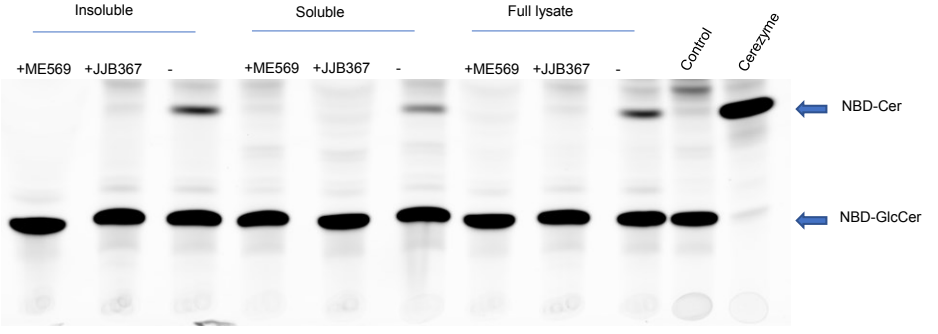

Figure 4. NBD-GlcCer lipid hydrolysis via BY2 cell lysates. Full and fractionated BY2 cell lysates were pre-incubated with or without 10uM ME569 or JJB367, for 2h, 37ºC. Then, the samples were incubated with 2uM of C6: NBD-GlcCer, for 2h 37ºC, pH5. Cerezyme was used as a positive control of the lipid hydrolysis reaction. BY2 cell lysates contain enzymes able to hydrolyse the human NBD-lipid, and they are inhibited by both epoxide and aziridine ABPs (ME569 and JJB367, respectively).

A straight-forward approach to characterize better B56 would be to overexpress the enzyme and purify it, along the same line as earlier performed for the A1.1. α-galactosidase (Kytidou et al. 2018). It should be in particular established whether the B56 protein shows activity towards natural GlcCer and GlcChol (chapter 2). In addition, its ability to act as transglucosidase should be tested (Marques et al. 2016). Moreover, its subcellular localization will be interest. Information on stability of the protein and resistance towards proteases is required to get an impression of its value to modulate glycolipid metabolism in humans. Presently available data suggest, but do not proof, that A56 lacks N-glycans given its poor binding to Concanavalin A.

Cyclophellitol-aziridine ABPs were found to label proteins A0A1S4CR57, A0A1S3ZME1 and A0A1S3ZFV9 with MWs of 69, 88 and 70 kDa, respectively. All three proteins are predicted to belong to GH3, a family of various ß-glucosidases including cell wall degrading enzymes [ß-glucosidase, xylan 1,4-ß-xylosidase, ß-glucosylceramidase, ß-N-acetylhexosaminidase, α-L-

arabinofuranosidase, glucan 1,3-ß-glucosidase, glucan 1,4-ß-glucosidase, isoprimeverose-producing oligoxyloglucan hydrolase, coniferin ß-glucosidase, exo-1,3-1,4-glucanase, ß-N-acetylglucosaminide phosphorylases, ß-1,2-glucosidase, ß-1,3-glucosidase, xyloglucan-specific exo-ß-1,4-glucanase / exo-xyloglucanase (http://www.cazy.org/GH3.html) .

Indeed, fluorescence microscopy revealed labeling of the cell wall by fluorescent cyclophellitol-aziridine ABP (Figure 5). The labeling was inhibited after overnight incubation with an ABP without the fluorophore (KY358), revealing that it was specific targeting enzyme molecules. Both JJB367 and JJB75 fluorescent ABPs were used for the same microscopy experiment, here presented only results using JJB75 (Figure 5). Since the BY2 cultures used during these experiments were harvested at day 3 of post culturing, the ABP-labelled proteins seem to be mainly the ones at MW of ~69 (Figure S1). Possibly targeting, the enzyme ß-glucosidase BoGH3B (A0A1S4CR57) of GH3 family, as also measured during in-gel proteomics analysis. In addition, to further support the previous, i*n situ* ABP labeling presented at figure S2, reveals that using 0.5 µM concentration of JJB75 probe (concentration used during microscopy experiments) mainly targets enzyme(s) present at MW of 69 kDa. One can further exploit the localization of targeted proteins after ABP labeling also via fractionating the samples and isolating the vacuoles or extracellular area of BY2 cells.

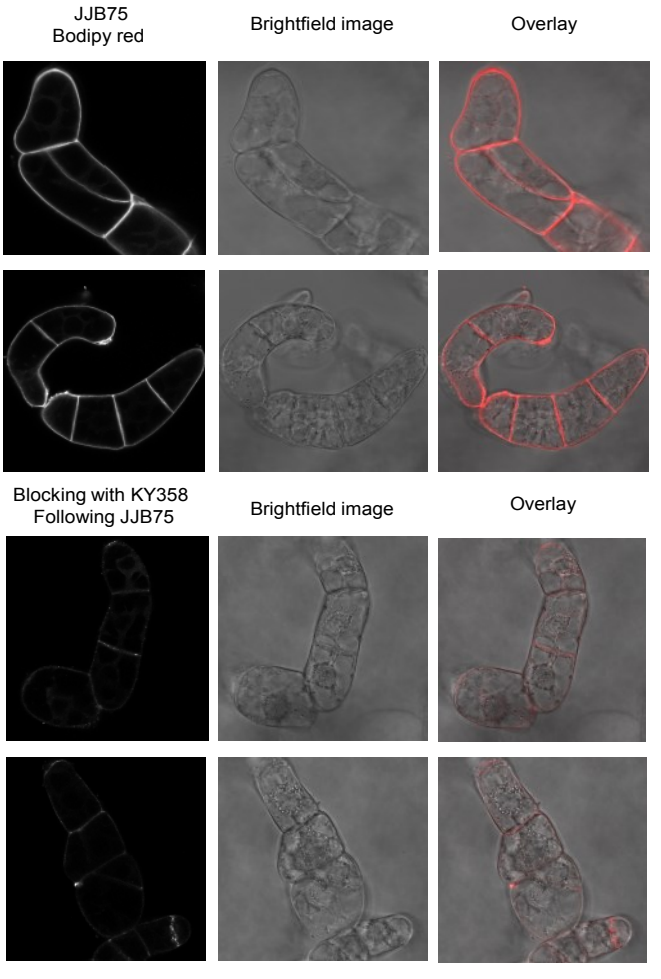

Figure 5. Live confocal microscopy of BY2 cells using ABPs. 3 days old BY2 cells (500 µL) were incubated in a 12-well plate with 0.5 uM of JJB75 for 2-3 h. To ensure that the labeling was specific, samples were also pre-incubated overnight with the non-fluorescent ABP, KY358 and then JJB75 labeling followed as before. The signal was lost after pre-incubation with the non-fluorescent ABP suggesting that the labeling was specific.

In silico model of B56 and its active site similarities with human GBA1 enzyme

There are no striking amino acid sequence similarities of B56 with the human GBA1 enzyme, and its homologues in mice *(Mus musculus)* and zebrafish *(Danio rerio).* The protein shows amino acid sequence similarities with bacterial and yeast enzymes. A hypothetical structural model of the ß-glucosidase B56 was made, in Swiss-model program, using as a structural model the Glucan 1,3-beta-glucosidase of the yeast *Candida albicans* (Figure 6).

The proteins have 36 % sequence similarity and both belong to GH family 5. The modeled 3D-structure was superimposed on the established 3D-structure of human GBA1 (PBD code: 2NT0). Overall the proteins were very different but their active site shared conserved moieties. Most importantly, two glutamic acid residues serving as the nucleophile and acid/base moieties of the retaining mechanism, were conserved among the two enzymes. In human GBA1 the catalytic residues are E235 and E340 and in B56 putatively E346 and E450. B56 is a predicted monomer, without N-linked glycans in contrast to the human enzyme which is a natural dimer, having 5 predicted N-linked glycosylation sites of which 4 are used (Ben Bdira et al. 2018).

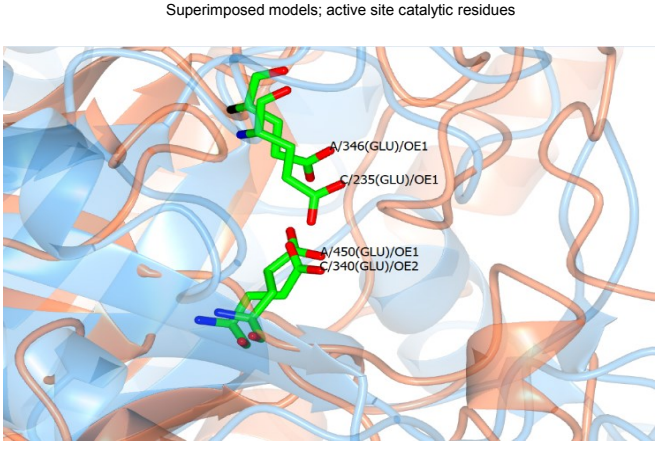

Figure 6. *In silico* structural models of B56 and the human GBA1 (PBD code: 2NT0). Superimposed active sites of human GBA1 (light blue) and B56 (light orange). The two catalytic residues (E235/E340 for human GBA1 and E346/E450 for B56) are conserved between the two proteins.

Screening for different retaining glycosidases in BY-2 cells using different configured ABPs

In a pilot investigation lysate of BY-2 cells were labeled with ABPs directed against different classes of retaining glycosidases (see figure 1; α-glucosidase, α-galactosidase, ß-mannosidase, α-fucosidase, ß-galactosidases, ß-glucuronidase, α-mannosidase). Discrete proteins were visualized with most probes (Figure 7), except for α-fucosidase. Thus, the same approach could be used to identify retaining glycosidases for other research questions of interest.

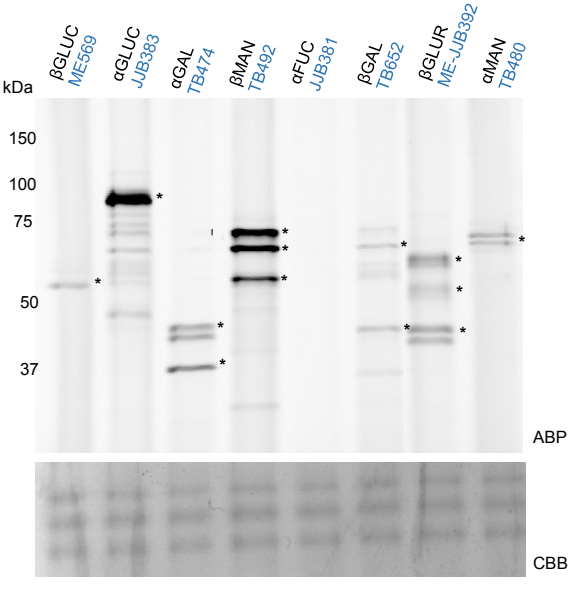

Figure 7. Screening for different retaining glycosidases in BY2 cell lysates, using different ABPs. 20ug of total protein were incubated with 0.5uM of different Cy5-labeled ABPs (ME569, JJB383, TB474, TB492,JJB381,TB652,ME-JJB392,TB480). Most of the ABP labeling revealed possible candidate enzymes, yet to be characterized and identified.

Fungal α-galactosidase: investigations with ABP (ABP labeling Beano® α-galactosidase)

Alpha-galactose configured TB474 was used to study the content on α-galactosidases of Beano® (Figure 1). For this purpose, Beano tablets were dissolved in water. The obtained solution was analyzed on protein content and incubated with TB474. A-galactosidases belong to family GH27 and GH36. They hydrolyze on galactose oligosaccharides, galactomannans and galactolipids, whose terminal 1,6-α-D-galactosyl moieties are release (https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.22). *A. niger* is

known to express 4 α-galactosidases (α-Gal I-IV) (Ademark et al. 2001a). The enzymes α-Gal II, III and IV are isoforms of AglB (GH27), whilst α-Gal I (GH36) is entirely distinct (Ademark et al. 2001b).

Using TB474 directed against α-galactosidases in the Beano solution, discrete proteins were labeled. Proteins with apparent MW of 60-65 kDa were labelled. Ademark and colleagues found that a protein closely related to A. niger α-galactosidase B consisted of two subunits with a molecular weight of 64 kDa (Ademark et al. 2001a, b). Beano contains also an invertase with sucrose activity (Prestige Brands Holdings, Inc., 2016b). This protein of approximately 135 kDa is unlikely to be the labelled protein with apparent MW of about 100 kDa reflects.

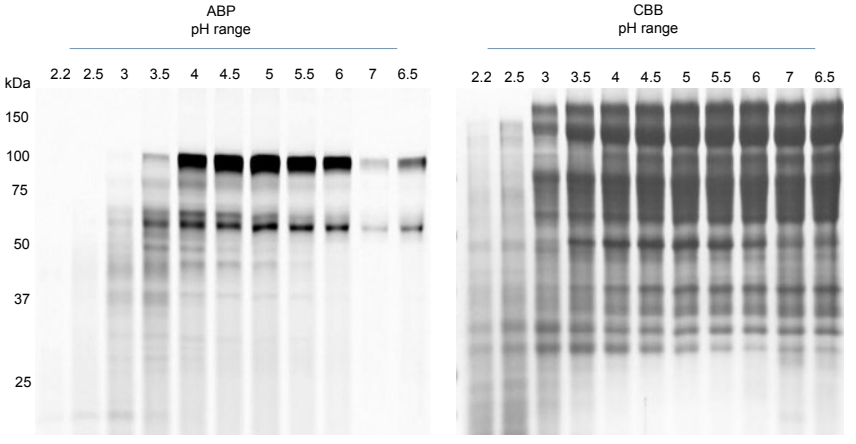

Figure 8. ABP labeling of alpha galactosidases present in Beano® supplement. The ABP labeling performed using 0.5uM of TB474 and the incubations performed in different pH buffer conditions (pH range 2.2- 6.5). The proteins are optimum labeled at pH 4.5-5. Coomassie staining of the gel followed to ensure equal loading and detect the general protein content of the supplement.

It was next examined whether incubation of Beano solution with TB474 led to inactivation of α-galactosidase activity. Most activity was lost following incubation with ABP and enzyme assay for 1 hour.

Next, apparent IC50 values of the potential natural substrate raffinose and D-galactose against human, fungal and plant α-galactosidases were determined (table 2). Competition of ABP labeling by the same substrates and with the additional tetrasaccharide stachyose was also tested (Figure 8). In this experiment Beano α-galactosidase, human α-galactosidase GLA, and recombinant plant α-galactosidase A1.1 were compared. It was noted that raffinose and stachyose strongly competed labeling of *A. niger* enzyme,

indicating that these are likely substrates. This was also the case for plant A 1.1., but not so for human GLA.

Table 2. Apparent IC50 values for *in vitro* inhibition of fungal, human and plant α-galactosidases by raffinose and D-galactose.

IC50s (mM)	Beano	Fabrazyme	A1.1
Raffinose	317	1779	187
D-galactose	327	83	10

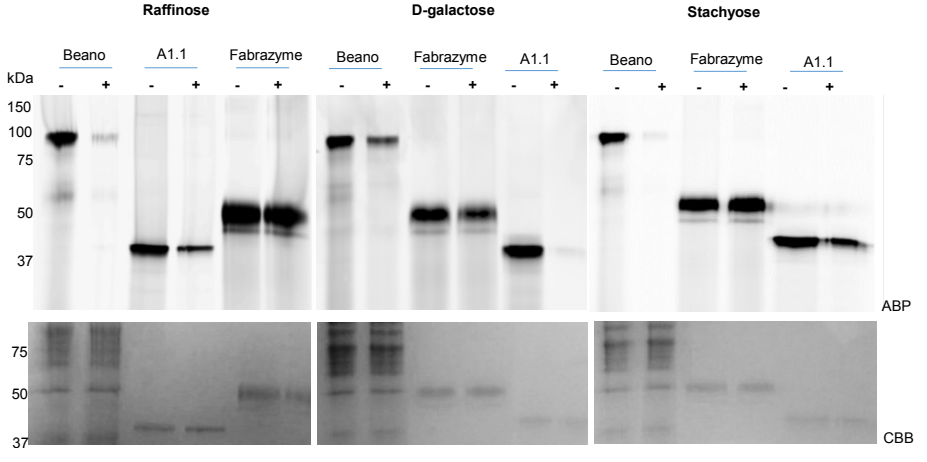

Figure 9. ABP signal competition with raffinose, D-galactose and stachyose. All enzymes, were pre-incubated for 1h, 37ºC, with and without 500mM of the trisaccharide raffinose, monosaccharide D-galactose or the tetrasaccharide stachyose. Then ABP labeling followed using 0.5 μM of TB474, for 30min, 37 ºC. The samples were then run on 10% SDS-PAGE gels and competition of the ABP signal revealed that Beano and A1.1 enzymes were highly inhibited by all saccharides, whereas human enzyme (Fabrazyme) was inhibited by D-galactose.

Finally, it was tested whether Beano solution was able to degrade NBD-GB3 (Figure 9). The enzyme despite its high activity towards the artificial fluorogenic 4MU-α-galactoside, which was comparable to that of Fabrazyme (recombinant human α-galactosidase A), it was not found active towards the NBD-GB3 lipid.

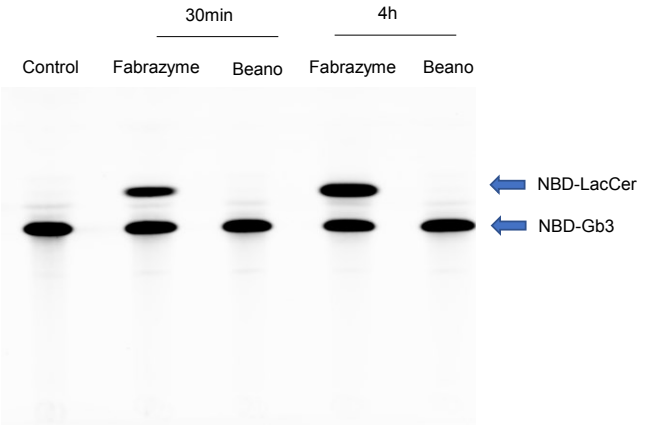

Figure 10. Beano and Fabrazyme activity towards NBD-Gb3. NBD-Gb3 lipid (2μM) was incubated with the same 4MU-aGAL activity input of Beano and Fabrazyme enzymes in different time points (30min and 4h). Beano enzyme cannot degrade the lipid substrate in contrast to Fabrazyme.

Investigations on intestinal α-galactosidases may be of great interest in relation to Fabry disease (Ferraz et al. 2014). As the result of deficiency of the lysosomal enzyme α-galactosidase A, Fabry disease patients accumulate Gb3 and the de-acylated form of the lipid called globotriaosylsphingosine or lysoGb3 (Aerts et al. 2008). LysoGb3 is formed from Gb3 by lysosomal acid ceramidase and part of the water-soluble lipid may leave the body via urine and bile (Ferraz et al. 2016). It has recently been reported that lysoGb3 impacts on microbiota and decreases production of the beneficial short-chain fatty acid butyrate (Aguilera-Correa et al. 2019). The investigation indicates that intestinal lysoGb3 has harmful effects and may contribute to the commonly reported intestinal complaints of Fabry patients (Hoffmann et al. 2007). Our investigations suggest that the Beano α-galactosidase may have no therapeutic potential regarding gastrointestinal complaints of Fabry patients since it seems unable to degrade (lyso)Gb3.

Discussion

The studies described in this chapter further illustrate how ABPs can conveniently be used in fundamental research. Firstly, ABPs can be used to identify likely substrates by testing competition of ABP labeling by compounds (Figure 7). The same principle is applied in ABPP (activity-based profiling) screening of drug laboratories as was demonstrate by Lahav *et al.* in their search for potent inhibitors of the retaining human ß-glucosidase GBA2 (Lahav et al. 2017). Secondly, ABPs can be used to identify the various classes of retaining glycosidase in different species. Examples for this are provided by the work of the van der Hoorn laboratory regarding plant glycosidase (Chandrasekar et al. 2014; Husaini et al. 2018). Along the same line, the panel of available ABPs labeling distinct glycosidases was used by Kuo et al. to determine target and off-target interactions of inhibitors such as conduritol B-epoxide and cyclophellitol, aiming to covalently inhibit the lysosomal ß-glucosidase GBA1 (Kuo et al. 2019). The outcome of this investigation allowed design of a very specific GBA1 suicide inhibitor allowing generation of genuine Gaucher models (Artola et al. 2019).

Materials and methods

BY2 cell culturing and lysis. BY-2 cells were cultured in Linsmaier and Skoog medium (Linsmaier and Skoog 1965) supplemented with 0,09 M sucrose and 2,4-dichlorophenoxyacetic acid (0.2 mg/l). The medium was autoclaved and kept at 4 °C until further use. Cells were sub-cultured every seven days (1-5 mL cells into 100 mL medium). BY2 cultures were kept in dark on a rotary shaker , 130 rpm at 27 °C (Nagata et al. 1992). Cell lysates were made using ice cold lysis buffer (10 mM Na_2HPO_4/NaH_2PO_4, pH 6, 0,15 M NaCl, 0,1 % Triton X-100 with additional protease inhibitor), using 500 µL of buffer per gram of cells. Then the cells were further lysed using the FastPrep-24 (MP Biomedicals). After lysis, part of the samples was collected (Full lysate) and the rest was fractionated, after centrifuge at 16,000rpm for 10 min, 4 °C. The obtained supernatant was collected (soluble fraction) and also the pellet (insoluble fraction). Protein concentration of the samples was measured using the Micro BCA Protein Assay Reagent Kit (PIERCE), according to manufacturer protocol. The samples were stored at -20 °C until further use.

Beano® lysates. 1 tablet of Beano® (Prestige Brands Holdings, Inc), was dissolved in 1 mL MQ. Protein content of the sample followed, using the Micro BCA Protein Assay Reagent Kit (PIERCE). The sample was aliquoted and stored at -20 °C until further use.

Fluorescent ABP labeling and SDS-PAGE analysis. *In vitro* ABP labeling assay was performed at 37 °C, for 30 min to 1h, using different ABPs (Figure 1) at final concentration 0.5 µM, volume 20 µL. The incubation was done in 150 mM McIlvaine buffer, pH5, using 20 µg of total protein concentration. SDS-PAGE loading buffer was then added to the samples, following boiling at 95 °C, for 5min. Then samples were applied on 10 % SDS-PAGE gels and fluorescent scanning of the gels tool place as previously described (Willems et al. 2014b).

Concanavalin A beads. Cell lysates were incubated for 1h, 4°C while rolling with Concanavalin-A (ConA) Sepharose beads (GE healthcare Bio-Sciences) and the protocol was followed exactly as previously described (Kytidou et al. 2018).

Proteomics analysis. *On bead proteomics analysis:* 450 µL of BY2 cell lysates (Full lysate, soluble, insoluble fraction), having a protein content between 2-5 mg/mL, were incubated with 5 µM of the biotinylated aziridine ABP, JJB111 or with DMSO (control) overnight at room temperature. After incubations,

proteins were linearized with 2 % SDS, precipitated using chloroform/ methanol precipitation, cystine bridges were reduced and alkylated following the exact protocol described at Jiang et al. 2016 (Jiang et al. 2016). Pull downs with avidin agarose beads (Pierce) 50 µL per sample were followed. The beads were washed with PBS and added to each sample in 1 mL PBS with beads per sample. The samples were incubated with the beads overnight at 4 °C while rolling. The beads were isolated through centrifugation (2500 g, 2 min) and a sample was taken of the supernatant: post-pulldown sample. This sample, together with the pre-pulldown sample, was used for SDS-PAGE analysis of the pulldown efficiency. The beads were washed with 0,5 % (w/v) SDS in PBS (1x) and PBS (3x). Then samples were prepared for proteomics analysis exactly as described at (Jiang et al. 2016; Kytidou et al. 2018).

In gel proteomics analysis :50 mL BY2 lysates were first incubated with 50 mL of diluted (x10) ConA beads overnight, 4 ^{o}C while rotating. Then, the sample was centrifuged max. speed, for 15min, 4 ^{o}C and supernatant was collected. The supernatant sample was then concentrated using the Amicon ultra-centrifugal tubes, according to the manufacture's protocol, up to 2 mL. Then, 200 µL of the sample were incubated with or without 2 µM of ME869 or JJB111 probes, overnight at room temperature. After ABP incubations the samples were treated exactly as described for the on-bead proteomics analysis, until the streptavidin bead incubations. In these experiments, the Dynabeads™ MyOne™ Streptavidin beads were used and the protocol was exactly as previously described (Kytidou et al. 2018). After binding to streptavidin beads, samples were applied on 10 % pre-cast SDS-PAGE gels (Bio rad). Excise of the bands followed, and the samples were further treated for proteomics analysis as previously described (Jiang et al. 2016; Wu et al. 2017). The Synapt G2Si mass spectrometer (Waters) operating with Masslynx for acquisition and ProteinLynx Global Server (PLGS) for peptide identification was used for analysis. Peak lists containing parent and daughter ions were compiled in .mgf format and searched against the Swiss-Prot (version 2017).

Enzyme activity assays. 4MU activity assays were performed exactly as previously described (Kallemeijn et al. 2012; Kytidou et al. 2017).

Microscopy. BY2 cultures at day 3 of sub-culturing were transferred in 12-well plates (500 µL). 0.5 µM of JJB75 (red bodipy) or JJB367 (Cy5) ABPs were applied directly to the cells and they were incubated for 2-3 h, at 130 rpm, 27 ^{o}C in the dark. Cells were also pre-incubated overnight with 60 µM of KY358, non-fluorescent ABP and then fluorescent ABP labeling followed. Then, the cells were washed 5x times with PBS to ensure that the there is no free probe

left and placed in champers for fluorescent detection at EVOS microscope (Thermo Fisher Scientific). For Cy5, λex= 638 nm, λem= 650–700 nm. For red bodipy, λex 594 nm, λem = 605-645 nm.

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Supplementary Material

ß-Glucosidase profile in BY2 cell cultures over time.

Activity based probe profiling was performed for BY2 cells cultured for different days. The results reveal that ß-glucosidases are differently expressed over time. In parallel, 4MU activities of the lysates were measured, reflecting changed ß-glucosidase expression.

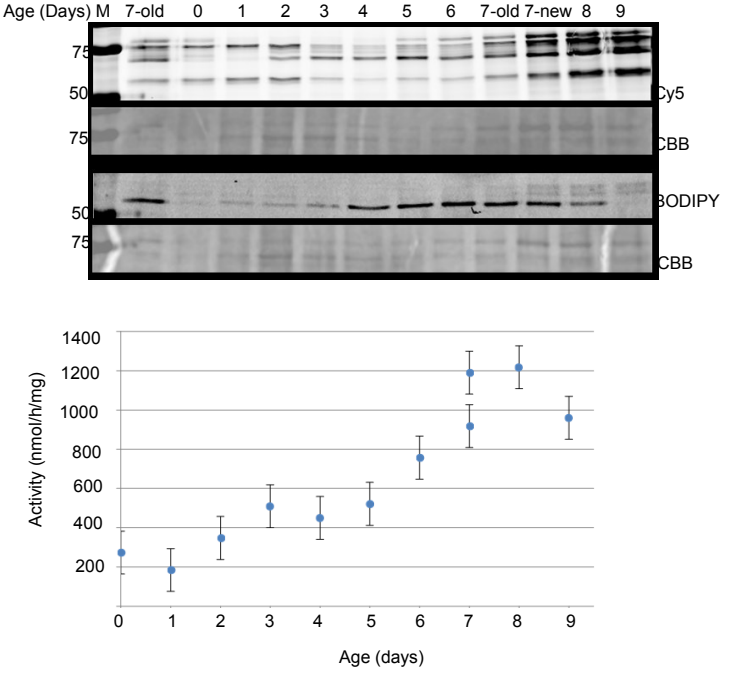

Figure S1. Beta-glucosidases of BY-2 cells with increasing of duration of cell culture. **A**: Detection by labeling with 0,5 µM β-glucosidase ABPs containing aziridine (JJB 367) and epoxide (MDW 941) warheads (0,5 % DMSO, 2 h, pH 5.0). **B**: Detection by measurement of activity towards 4-MU β-D-glucopyranoside at pH 5.

In situ Labelling of Cells with Activity Based Probes

The cell permeability of ABPs was examined by incubation of BY-2 cells. ABPs were added in the medium and cells were harvested after 120 minutes and lysed. Then, ABP labeling was analysed through SDS-PAGE and enzyme activity assays. ABP1 was used during *in situ* incubation; ABP2 is the one added in excess at lysis to ensure that the observed labelling with ABP1 did not happen during the preparation of lysates. As is clear from the gels in figure S2,

JJB75 (aziridine-Bodipy Red) labels enzymes at a lower concentration than JJB367 (aziridine-Cy5), suggesting that either the cell permeability of the more hydrophobic JJB75 is better or that JJB75 has a higher affinity for its counterpart enzymes. Interestingly, JJB367 labels a protein around 70 kDa that is not labelled by JJB75 at the same concentration. Therefore, this protein seems to have a higher affinity for JJB367. When incubating with high concentrations, JJB75 shows binding to a wider range of proteins than JJB 367. This is also reflected in the activity assay, where the *in situ* addition of JJB 75 leads to a larger inhibition of enzyme activity.

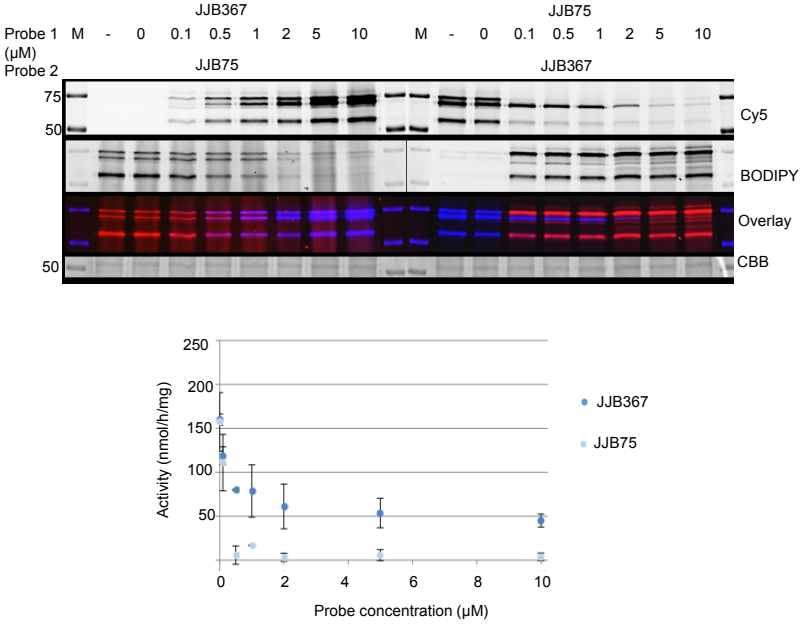

Figure S2. Incubation of BY-2 cells with the β-glucosidase aziridine ABPs JJB 367 (Cy5) and JJB 75 (Bodipy Red) in varying concentrations for 2 h. **A**: Lysis buffer supplemented with 5 μM of the contrasting colour to label residual protein. **B**: Activity in 4-MU β-D-glucopyranoside, without probe in the lysis buffer. M: Marker, band size shown in kDa.