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Chemical biology studies on retaining α -glucosidases

Su, Q.

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Chapter 7

Summary and future prospects

The research described in this Thesis centered on retaining exo- β -glucosidases implicated in health and disease, in particular the human inherited lysosomal storage disorder, Gaucher disease (GD). Acid lysosomal β -glucosidase (GBA1), non-lysosomal β -glucosidase (GBA2), and cytosolic broad-specificity β -glucosidase (GBA3) all employ a Koshland double replacement mechanism in the hydrolysis of β -D-glucosides. Activity-based probes (ABPs) labelling retaining β -glucosidases are used throughout this Thesis to visualize human GBA1, GBA2 and GBA3, as well as analogous enzymes from other species. **Chapter 1** comprises a brief introduction on the human enzymes, their connection to human disease (Gaucher disease, Parkinson's disease) as well as on reagents and tools to study their activity. The first part of this thesis (**Chapters 2-4**) focus on the identification of selective inhibitors and activity-based probes (ABPs) for each of the three human retaining β -glucosidases, GBA1, GBA2 and GBA3. **Chapter 2** describes studies on the potency and selectivity of β -D-xylose-configured cyclophellitol aziridine ABPs towards the three human retaining β -glucosidases. In these studies, β -D-xylose-configured cyclophellitol, thus the compound lacking the hydroxymethylene as present in cyclophellitol, was found to be a GBA1 selective inhibitor, this in contrast to cyclophellitol itself which inhibits GBA2 and GBA3 as well. **Chapter 3** reports on the finding that β -D-arabinofuranose configured (β -D-Araf) cyclitol aziridines can be used to design GBA2-selective ABPs – compounds that did not exist prior to the in this Chapter described studies. **Chapter 4** describes the discovery of some relatively GBA3-selective mechanism-based inhibitors and ABPs. Their cross-reactivity towards GLB1 and GALC can be ameliorated by the inclusion of non-fluorescent inhibitors selective for these retaining β -galactosidases, enabling for monitoring of GBA3 activity in the presence of GBA1 and GBA2. The second part of this thesis centers on the identification of retaining β -glucosidase homologues in other species using established β -glucosidase ABPs and inhibitors. **Chapter 5** details studies on *Caenorhabditis elegans* (*C. elegans*) retaining β -glucosidases while **Chapter 6** characterizes a retaining β -glucosidase (termed B56) in *Nicotiana tabacum* and similarities and differences between these enzymes and human GBA1 and GBA2 are discussed in these chapters.

New GBA2-selective inhibitors and probes. GBA2 has attracted considerable attentions in last decade because of its involvement in human health and disease (see Chapter 3).¹⁻³ The discovery that β -D-Araf cyclitol aziridines equipped with fluorescent aziridine N-substituents are GBA2-selective ABPs may open new directions for medicinal chemistry. Inhibition of GBA2 ameliorates the phenotypes in GBA1-deficient type 1 GD and Niemann-Pick disease type C (NPC) mouse models, which highlights GBA2 as a potential therapeutic target.⁴⁻⁶ Conversely, GBA2 deficiency, such as homozygous mutant of GBA2, has been linked to hereditary spastic paraplegia and autosomal-recessive cerebellar ataxia.³ Miglustat **4** (N-butyl-deoxynojirimycin, NB-DNJ), an iminosugar inhibitor of glucosylceramide synthase (GCS), has been approved for treatment of mild type 1 GD and NPC.⁷⁻⁹ Besides inhibiting GCS, Miglustat **4** also potently inhibits GBA2.¹⁰ The clinical benefits of Miglustat **4** towards NPC may be partially attributed to this competitive inhibition of GBA2, which is relatively abundant in the brain and CNS.⁴ Therefore, the development of GBA2-selective inhibitors presents an attractive avenue for studying the enigmatic role of GBA2 in human (patho)physiology, for instance in central nervous system (CNS) motor coordination. Building upon the β -D-Araf aziridine scaffold, mechanism-based, covalent, and irreversible GBA2-selective inhibitors are thus proposed, as potential drug candidates and also to create bona fide GBA2-deficient models, complementing *GBA2* gene knockout approaches.¹¹⁻¹³ Ideally, GBA2 inhibitors insert well into cell membranes (where GBA2 acts) and are able to pass the blood-brain barrier (BBB). Therefore, modifications to enhance hydrophobicity, such as installing hydrophobic substitutions like adamantyl or biphenyl groups on the nitrogen of β -D-Araf aziridine, are suggested, also based on the precedent of cyclophellitol-C8-modified GBA1-selective inhibitors **5** and **6** in the generation of a GBA1-deficient zebrafish larvae model.¹⁴ Notably, the nature of the β -D-Araf aziridine tag impacts GBA2 selectivity over GBA1 and GBA3. For instance, Cy5-tagged and biotin-tagged β -D-Araf aziridine ABPs **3** and **7**, are much less selective for GBA2 than BODIPY-tagged derivatives **1** and **2**. At present, there is no crystal structure of human GBA2. The bacterial GBA2 homologue TxGH116,¹⁵ with an available crystal structure and a catalytic pocket comparable to that of human GBA2, may help in revealing the binding model of proposed GBA2-selective β -D-Araf aziridine

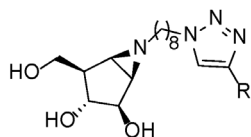
compounds (in Figure 1) and human GBA2. This, combined with structural studies on GBA1-complexed inhibitors may assist in the design of more active and more selective inhibitors.^{14,16,17}

In previous studies, overexpressed GBA2 constructs of different nature in cultured cells were found at different locations. GBA2-GFP was found at the plasma membrane¹⁸ while overexpressed, native GBA2 localized at the ER and Golgi.¹⁹ An earlier fractionation study of spleen identified endogenous GBA2 associated with an endosomal fraction.²⁰ The fluorescent β -D-Araf ABPs may provide a straightforward way to show the sub-cellular localization of endogenous GBA2 through directly binding to the catalytic active site of endogenous enzyme. The first attempt, as described in Chapter 3, to use β -D-Araf ABP **2** to reveal sub-cellular localization of native GBA2 in wild-type HEK293T cells did not present visible punctate labelling, possibly due to the dispersed distribution and low expression of GBA2 in these cells. To optimize the visualization of endogenous GBA2 by microscopy, several strategies could be employed, namely (1) the use of more advanced and sensitive fluorescence microscopy with higher quantum efficiency; (2) the use of other wild-type cell lines with more abundant GBA2; (3) substitution of the BODIPY group for a fluorescent group with higher quantum yield such as BODIPY-TMR to increase the fluorescence signal; or (4) the application of agents to amplify the labelled-GBA2 signal after initial labelling with tagged β -D-Araf ABP. With respect to the latter, one can envisage to first label GBA2 with BODIPY-tagged β -D-Araf ABP, then employ an anti-BODIPY primary antibody which is subsequently detected by a fluorescent secondary antibody for conjugating primary antibody to increase the fluorescence signal for microscope detection. The GBA2 selectivity window of β -D-Araf ABPs **1** and **2** proved already sufficient to selectively label GBA2 in different biological samples when used at appropriate concentrations, but also label GBA1 when high concentrations are used. Therefore, there is room for improvement in the GBA2 selectivity of these ABPs. In the future, new β -D-Araf ABPs with varying fluorescent substitutions at the aziridine might provide probes with even superior GBA2 selectivity.

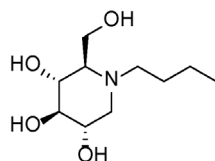
Recently, Shimokawa *et al.* identified a β -D-arabinofuranosidase in bacteria belonging to the same GH116 family as human GBA2.²¹ It may be speculated that β -D-Araf molecules are primary substrates for GH116 enzymes (including human GBA2) in nature, and that therefore β -D-Araf compounds fit the catalytic pocket of human GBA2 well. It is intriguing to explore whether the β -D-Araf cyclitol aziridine ABPs could react well with other GH116 enzymes, which may expand the utility of these β -D-Araf ABPs as the selective GH116 enzymes labelling probe. At present, a GBA2-selective fluorogenic substrate is not available, and it may be relevant to assess whether 4-methylumbelliferyl β -D-arabinofuranoside (4-MU- β -D-Araf) **8** (Figure 1) are hydrolyzed by human GBA2, and as well by other GH116 family glycoside hydrolases.

Compounds

β -D-Arabinofuranose configured
aziridine-based ABPs

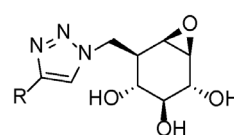


- 1 R = BODIPY green
- 2 R = BODIPY red
- 3 R = Cy5
- 7 R = biotin



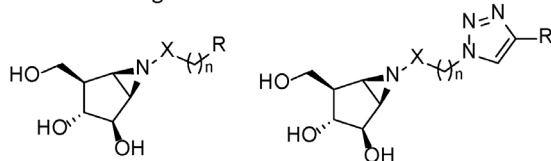
Miglustat 4
N-butyl-deoxynojirimycin
(NB-DNJ)

Cyclophellitol GBA1-
selective inhibitors



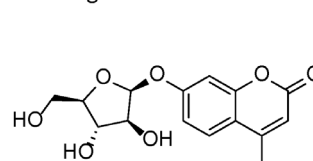
- 5 R = biphenyl
- 6 R = adamantyl

Proposed new β -D-arabinofuranose
configured aziridine-based inhibitors



X = CH₂ or CO R = biphenyl or adamantyl

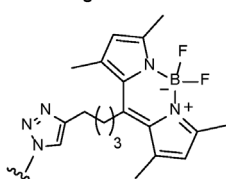
Proposed β -D-arabinofuranose
configured 4-MU substrate



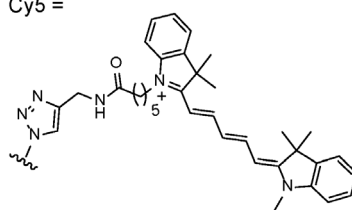
8

Tags

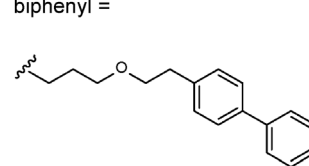
BODIPY green =



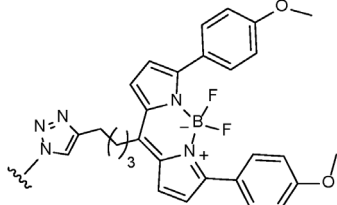
Cy5 =



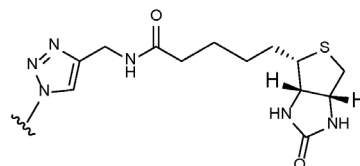
biphenyl =



BODIPY red =



biotin =



adamantyl =

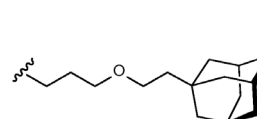


Figure 1. Compounds of (proposed) GBA2-selective inhibitors, ABPs, and fluorogenic substrates.

C. elegans as a model to study retaining β -glucosidases. The small round nematode *C. elegans*, the first multicellular organism whose genome was fully sequenced²², has been used as a model to study autophagy, neurobiology, and aging.²³⁻²⁷ As described in Chapter 5, enzymes identified by proteomic analysis are the hGBA1 resembling protein C-GBA1-3 (UniProt code: G5ECR8) encoded by *gba-3* gene and the hGBA2 resembling protein C-GBA2 (UniProt code: O01893) encoded by *R08F11.1* gene. The lack of detection of other putative β -glucosidases (encoded by *gba-1*, *2*, *4*, or *hpo-13*) may possibly be attributed to either low expression or lower reactivity towards the β -glucosidase ABPs used in Chapter 5. The *klo-1* and *klo-2* genes of *C. elegans* theoretically encode proteins that exhibit approximately 35% identity with human cytosolic broad-specificity β -glucosidase (hGBA3). These possible paralogues of hGBA3 in *C. elegans* merit characterization using existing β -glucosidase ABPs as well as new ones such as described in Chapter 4.

The pathways of carbohydrate and lipid metabolism in *C. elegans* are in general quite similar to those in mammals.²⁸ The occurrence of β -glucosidases in *C. elegans* implies that the nematode could be used as convenient organismal model to study β -glucosidases in relation to glycolipid metabolism. The glycosphingolipid d17iso-GlcCer of *C. elegans* is similar to mammalian GlcCer except for a slight difference in the sphingoid base (C17iso-sphingosine instead of C18-sphingosine). The lipid d17iso-GlcCer has been reported to play a vital role in growth of the nematode.^{29,30} The identified putative β -glucosidases C-GBA1-3 and/or C-GBA2 are hypothesized to be involved in metabolism of d17iso-GlcCer and future research, including lipid metabolism studies, may point out whether C-GBA1-3 degrades d17iso-GlcCer in *C. elegans*. One approach could involve using a *gba-3* knockout *C. elegans* strain, (available from the Caenorhabditis Genetics Center), to analyze changes in glycosphingolipid as compared to wild-type strains. Additional putative β -glucosidase KO nematode strains could be theoretically generated by genetic methods such as transgenesis, RNA interference (RNAi), and CRISPR-Cas9.³¹

Transglycosylation, another capacity of retaining β -glucosidases besides hydrolysis (see Chapter 1), presents an intriguing avenue for further study.³²⁻³⁵ GlcChol is a glycolipid formed in mammalian cells by transglycosylation, particularly by GBA2. The presence of GlcChol in *C. elegans* was observed (Chapter 5) and comprises the first report on the occurrence of the glycosylated sterol in the nematode. The physiological role of GlcChol in *C. elegans* is still unknown and this holds true also for the enzymes involved in GlcChol metabolism. Future studies should inform whether GlcChol in *C. elegans* is formed through transglycosylation and if so by which β -glucosidase.

In conclusion, the past decades have witnessed the design of inhibitors and ABPs designed for retaining exo- β -glucosidases have been successfully generated and established. The toolbox for glucosidase research provides powerful and diverse instruments to study aspects of particular glucosidases of interest. The work described in this thesis has further extended the toolbox, and those tools have been applied to shed light on retaining exo- β -glucosidases and their homologues in other species.

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