General Discussion and Future Prospects

Activity-based probes for retaining exo-glycosidases: novel research tools for lysosomal storage disorders

The central theme of this thesis are molecular probes labeling retaining exo-glycosidases implicated in health and diseases such as lysosomal storage disorders (LSDs). The structure of these so-called activity-based probes (ABPs) is based on configurational isomers of a cyclophellitol or cyclophellitol aziridine scaffold, to which a reporter tag (e.g. a fluorophore or a biotin) is attached. When incubated with sample, their substrate mimicry and the latent electrophilic trap (epoxide or aziridine) allow them to react upon proximity-driven protonation with their target glycosidase's catalytic nucleophile, resulting in a stable ester bond that irreversibly inactivates the enzyme. Through the grafted reporter tag on the probe, the covalent ABP-enzyme complex can be readily visualized or affinity-enriched, allowing quantitative and qualitative analysis on the spatiotemporal distribution of the labeled enzyme *in vitro*, *in situ*, and *in vivo* by the activity-based protein profiling (ABPP) platform (**General Introduction**, this thesis).

This thesis aims to explore novel applications of glycosidase ABPs to study these enzymes' role in health and disease, in particular in the inherited lysosomal storage diseases (LSDs). It further aims to expand the ABPP platform to other retaining exo-glycosidases such as α-glucosidase, β-glucuronidase, α-L-iduronidase, α/β-mannosidases, and β-galactosidases. Different goals for the thesis work were formulated and reached: (1) setting up ABP profiling protocols for glycosidases (**Chapter 1**), (2) developing two novel *in vivo* applications for ABPs in LSD research (**Chapter 2-3**), and (3) characterizing in detail the mechanism of action, potency, target specificity, and potential applications of six novel classes of ABPs (**Chapter 4-7**). The described work builds on past research utilizing cyclophellitol ABPs labeling the lysosomal glucocerebrosidase (GBA)[1] and galactocerebrosidase (GALC)[2], and cyclophellitol aziridine N-acyl or N-alkyl variants targeting either retaining exo-β-glucosidases (GBA, GBA2, GBA3, and LPH), retaining exo-α-galactosidases, or retaining exo-α-L-fucosidases[3–6]. These ABPs, via the afore-mentioned reaction mechanism, allow sensitive and specific visualization of target enzymes across species and sample types (recombinant enzyme, cell lysates, tissue homogenates, whole cells, and tissue sections) using detection methods such as SDS-PAGE-based fluorescence scanning, fluorescence-activated cell sorting (FACS), fluorescence microscopy, and mass spectrometry (LC-MS/MS). Demonstrated applications include the diagnostic confirmation of LSDs (Gaucher disease, Fabry disease, and Krabbe disease),[1–3] the assessment of bodily distribution of labeled enzymes,[7, 8] the investigation of active-site pocket occupancy of GBA by potential small molecule chaperons,[1] and the identification of the active-site nucleophile of the

labeled enzymes.[9]

The first part of the thesis concerns Gaucher disease. **Chapter 1** systematically describes ABP protocols for gel-based and microscopy-based detection for GBA; these can be adapted to detect other lysosomal glycosidases with ABPs. Next are presented two novel *in vivo* applications of ABPs, being: (1) assessing target specificity of existing pharmacological agents for inducing cell/animal models of Gaucher disease (**Chapter 2**), and (2) generating such cell/animal models using compounds modified from the cyclophellitol-based ABPs (**Chapter 3**). Both applications exploit the cell and tissue permeability of ABPs and their highly selective inactivation of GBA by binding to the catalytic nucleophile E340. The first study established the relevance and limitations of conduritol B epoxide (CBE) and cyclophellitol (CP) in generating Gaucher cell/animal models, while the second investigation led to the identification of superior GBA inhibitors (CPs with installed biphenyl or adamantly moiety at C8).

The second part of the thesis reports on the characterization of novel cyclophellitol aziridine ABPs with a differentially configured cyclophellitol core. For this use was made of enzymatic assays to establish potency of inhibition and kinetic parameters; gel-based ABPP to assess target specificity and labeling at various conditions (concentration of ABP, incubation time, pH, temperature, sample types; chemical proteomics to identify the labeled targets, and protein X-ray crystallography (performed at the University of York) to acquire structural insights regarding the ABP-enzyme complex. It is demonstrated that the examined ABPs allow mechanism-based labeling and activity-based protein profiling (ABPP) for the corresponding LSD-relevant glycosidases, including GH31 lysosomal α-glucosidase (GAA) (**Chapter 4**), GH2 β-glucuronidase (GUSB) (**Chapter 4**), GH39 α-L-iduronidase (IDUA) (**Chapter 5**), GH38 lysosomal α-mannosidase (MAN2B1) (**Chapter 6**), GH2 β-mannosidase (MANBA) (**Chapter 6**), and GH35/GH59 lysosomal β-galactosidase/galactocerebrosidase (GLB1/GALC) (**Chapter 7**). For all ABPs, Cy5- and biotin-tagged variants are available and both variants successfully label their target enzymes. Besides the α-L-iduronidase ABPs that are less potent than other ABP classes, all ABPs are able to visualize glycosidases in the context of complex biological samples. Additional in-class (or similar class) glycosidase targets were identified for several ABPs, consistent with previous findings that cyclophellitol aziridine ABPs are broad-spectrum probes: the in-class targets for the α-glucosidase ABPs were the other GH31 α-glucosidases ER α-glucosidase II (GANAB), maltase-glucoamylase (MGAM), and sucrose-

isomaltase (SI); GH79 heparanase (HPSE) for the β-glucuronidase ABPs; four other GH38 α-mannosidases (MAN2A1, MAN2A2, MAN2B2, and MAN2C1) for the α-mannosidase ABPs; two other GH35 proteins GLB1-like protein 1 and 2 (GLB1L and GLB1L2) for the β-galactosidase ABPs. Of note, all cyclophellitol aziridine ABPs at high concentrations tend to also label GBA, with the exception of the α-L-iduronidase and α-mannosidase ABPs. Specific visualization of the target enzyme class may be achieved by pre-blocking of GBA with selective inhibitors/ABPs for this enzyme.

I. Unique research possibilities and applications offered by the glycosidase ABPs

a. Superior spatial detection

One important feature of the studied ABPs is that they allow one to directly visualize active glycosidase molecules. As such ABP labeling fundamentally differs from detecting an enzyme through measurement of its activity towards fluorogenic substrates or natural substrates. When applied *in situ* to detect the spatial distribution of active glycosidase molecules, the ABP method provides better resolution than zymography based on enzyme activity assays. A recent study reported on detection of active GBA molecules by fluorescent ABPs in human skin sections with higher resolution compared to fluorogenic substrate-based zymography[10] This study established that active GBA molecules largely reside in the extracellular lipid matrix between the viable epidermis and the lower part of stratum corneum. The *in situ* ABP detection technique, conceivably combined with the gel-based detection procedure, may be used to elucidate the (patho)physiological roles of GBA in diseases such as atopic dermatitis,[11] multiple myeloma,[12] and neurological diseases such as amyotrophic lateral sclerosis,[13] Lewy-body Dementia,[14] and Parkinson disease.[15]

b. *In situ* and *in vivo* labeling

The amphiphilic ABPs readily cross cellular membranes, which allows *in situ* and *in vivo* labeling of lysosomal glycosidases that can be coupled to either gel-based, FACS, or fluorescence microscopy-based detection.[1, 16] This approach is superior to detection of enzymes using antibodies, which do not crosses cellular membranes and report glycosidase activity. Recently, cell permeable fluorescence-quenched substrates were developed by the group of Vocadlo and shown to reveal the *in situ* activities by GBA[17, 18] Similar probes are designed by the same researchers for other glycosidases (unpublished data). By virtue of their covalent mode of

inactivation/labeling, ABPs offer several unique applications. One example is to be found in studying the life cycle of glycosidases in cells by pulse-chase experiment,[19, 20] in which the formation, post-translational modification, and degradation can be monitored. Proof of principle in this connection has been obtained for GBA.[1] It can be envisioned that pulse-chase labeling with ABPs can be exploited to systematically examine the effect of different mutations, modifier genes, or other cellular factors on the life cycle of GBA. Such studies may shed further light on Gaucher disease and Parkinson disease. The approach could be extended to other lysosomal glycosidases for which ABPs are available.

c. Distinguishing between isozymes

When coupled to gel-based detection, distinction among different enzymes or isoforms labeled by the same ABP is feasible, obviously provided that these differ in molecular weights (**Chapter 1**). Examples are the β-glucosidases GBA and GBA2, where the former is about 50-65 kDa, and the latter is about 100 kDa.[3, 9, 21] Isoforms of GBA differ in glycan composition, i.e. there are distinct GBA glycoforms between 50-65 kDa),[8] Isoforms may reflect the glycosidase's cellular location (e.g. ER, Golgi, or lysosome). Isoforms may also stem from proteolytic processing, for example the 85 kDa vs. 64 kDa mature forms of GLB1[22, 23]. In general, combining the three approaches to detect enzymes (activity assay, ABP labeling, and antibody labeling) offers the most comprehensive assessment at both protein and activity level. The combination of these three methods also assists best the biochemical confirmation of diagnosis of lysosomal storage diseases caused by defects in glycosidases.

d. Sophisticated analysis of therapeutic enzymes

Another unique advantage offered by ABPs is their potential use to track the cellular uptake and bodily distribution of therapeutic enzymes, as in enzyme replacement therapy (ERT) for LSDs. The labeling of a therapeutic enzyme with ABP is elegant and targeted, taking place at a well-defined catalytic nucleophile in the catalytic pocket. One study has compared head-to-head two registered therapeutic enzymes for ERT of Gaucher disease, imiglucerase and velaglucerase for their receptor-binding and uptake in cultured dendritic cells and macrophage, and for their bodily distribution in treated mice.[8] The enzymes were labeled with similar ABPs with distinct fluorophores, next mixed equimolar after which the uptake and bodily distribution of each was assessed in parallel.[8] In this thesis, the α-L-iduronidase ABPs was successfully

employed to monitor the cellular uptake and lysosomal targeting of labeled therapeutic IDUA molecules in cultured fibroblasts from patients of Hurler disease, mucolipidosis type II, and type III disease, using both fluorescence microscopy and gel-based methods (**Chapter 5**).[24] Similarly, a recently discovered non-glycosylated α-galactosidase from plant was pre-labeled by α-galactose configured ABPs, and the uptake and delivery to lysosomes of the fluorescent enzyme by cultured fibroblasts from a Fabry patient—via a lectin-independent pathway—was visualized.[25] More recently, correlative light and electron microscopy (CLEM) has been utilized to visualize endocytosed ABP-labeled imiglucerase in treated human fibroblasts expressing mannose receptors, revealing efficient delivery in individual lysosomes that contained endogenous GBA molecules as visualized with ABPs with other fluorophores.[26] The newly available ABPs for different LSD-related glycosidases, described in this thesis, may facilitate the assessment of distribution, processing, and efficacy of therapeutic enzymes following their administration to cells or animal models. Future installation of detection tags compatible with infrared-red or radioisotopic detection may even allow real-time monitoring of bodily distribution of the labeled enzymes in animal models or in patients. Existing examples are cysteine protease ABPs containing a ^{64}Co tag (with or without an additional Cy5 tag),[27–29] a serine protease ABP coupled to an ^{111}In tag,[30] and a GBA ABP having a 2-deoxy-2-^{18}F group.[31]

e. Multiplexing

An advantage of ABPs is the possibility offered to design multiplex readouts. Careful selection of ABPs with distinct fluorophores allows simultaneous profiling of multiple glycosidases, even in living cells or animals. Elegant simultaneous ABP profiling of endocannabinoid hydrolases in the mouse brain has been demonstrated.[32] **Chapter 2** of this thesis provides examples of multiplex ABP detection for active retaining exo-β-glucosidases, α-glucosidases, and β-glucuronidase in cultured cells. Cells were incubated with ABPs having distinct fluorophores (BODIPY green, BODIPY red, or Cy5) that label the above mentioned enzymes. Upon cell lysis and subsequent gel-based fluorescence detection, different glycosidases labeled *in vivo* were visualized in parallel. In this manner, decrease in active enzyme molecules can be simultaneously assessed. By adding a subcellular fractionation step prior to gel-based detection, it is conceivable that one can also visualize the comparable/distinct subcellular localization of different glycosidases. Furthermore, the multiplex setup coupled to gel-based detection would also allow convenient assessment of post-translational modification status (e.g.

N-linked glycosylation, proteolytic processing) for multiple enzymes. One potential application for the multiplex ABPP setup is to elucidate the molecular mechanisms underlying cellular processes affecting the transport and function of lysosomal glycosidases. For example, it has been noted that the presence of HEPES, a common pH-buffering additive to cell culture medium, could cause in cultured cells lysosomal stress and abnormality in GBA's glycosylation status.[33] The multiplex ABP incubation, coupled to subcellular fractionation technique and gel-based readout, may thereby be applied to investigate whether upon HEPES treatment GBA is specifically affected in its glycosylation status, compared to other lysosomal glycosidases (for which ABPs are available). Furthermore, it can also be used to screen for the impact of other types of stressors on the lysosomal routing and lysosomal processing/stability of GBA, compared to other lysosomal glycosidases. Impact of different types of enzyme mutations may be similarly examined in the context of delineating genotype-phenotype correlation in LSDs. Furthermore, the multiplex readout may also be used in applied research. It is in principle compatible with different types of sample, such as cells, tissues, urine and plasma, thus assisting screening and diagnostic confirmation of LSDs.

f. Investigating *in vivo* target engagement of glycosidase inhibitors

One of the most powerful applications of the cell permeable glycosidase ABPs is their use in assessing *in vivo* target engagement of (covalent) inhibitors. The *in vivo* target engagement of a drug is crucial to elicit proper therapeutic responses. The same holds for enzyme inhibitors used to induce authentic pharmacological cell/animal models of human disease. Lack of awareness of off-targets of a given small molecule can be fatal,[34] and ABPs are an excellent tool to profile potential off-targets of covalent enzyme inhibitors.[35] As mentioned, the irreversible GBA inhibitor conduritol B epoxide (CBE) with conceived specificity for GBA is commonly used for generating Gaucher disease models. It was closely examined in this thesis in cell and animal models to which extent there is interaction *in vivo* of CBE with off-target glycosidases (other β-glucosidases, α-glucosidases and β-glucuronidase; enzymes for which ABPs were available) (**Chapter 2**). Observed competition by CBE of the ABP labeling of off-target glycosidases provides evidence for undesirable co-inhibition of these enzymes. The investigation identified GBA2 and GAA as major off-target glycosidases at higher CBE concentrations. A narrow window for selective GBA inactivation exists, but when using CBE to induce a Gaucher disease model the experimenter should better check for potential concomitant inhibition of

GBA2 and GAA. The same investigation revealed that cyclophellitol (CP), a closer mimic of glucose than CBE, inhibits GBA and GBA2 with equal affinity in all the studied models.[21]

The assessment of *in vivo* target engagement is not necessarily restricted to covalent, mechanism-based inhibitors. For example, highly potent (nanomolar) reversible iminosugar inhibitors of GBA2 based could be examined for their *in vivo* selectivity for GBA2, GBA and GBA3.[36] Apparently, active-site targeted inhibitors with high affinity stay associated with the enzyme upon lysis of cells and freeze-thawing of lysates, and their ongoing active-site pocket occupancy can be readily visualized by *in vitro* applied ABPs. Obviously, such assays require considerable fine-tuning based on the features of the inhibitor tested and ABP employed in the read-out.

g. Use as mechanism-based inhibitors to inactivate enzymes on demand

The cyclophellitol and cyclophellitol aziridine ABPs can be used to selectively inhibit certain glycosidase *in vitro* or *in vivo*. The installation of the flexible hydrophobic linker (with or without a fluorophore) in fact often enhances the inhibitory potency of the compound towards their targeted glycosidases compared to just the cyclophellitol or cyclophellitol aziridine core alone;[1, 3] sometimes the specificity profile also improves as in the case of ABPs for GBA (where a bulky, hydrophobic moiety at the C8 position of cyclophellitol prevents the labeling towards GBA2).[37] For example, the cyclophellitol ABPs can be used as a more specific GBA inhibitor compared to CBE; the cyclophellitol aziridine ABPs can inhibit besides GBA also GBA2 and GBA3, while not inhibiting glucosylceramide synthase (GCS), a common off-targets by iminosugar inhibitors aiming at GBA2.[38] When combined, these potent, specific, and cell-permeable ABPs would offer convenient tools for studying the role of GBA and GBA2 in sphingolipid metabolism in healthy and disease state.

In addition, the GBA-specific ABPs have been explored as a pharmacological agent in inducing Gaucher-like models (**Chapter 3**).[37] Past research has found that the BODIPY-containing cyclophellitol ABP does not penetrate the brain well of treated mice,[7] and this is consistent with the finding in adult zebrafish (**Chapter 3**). Thus, this ABP can only generates type 1 (non-neuropathic) Gaucher model at best. Due to GBA's association with other neurological disorders such as Parkinson's disease and Lewy-Body Dementia, and that current ERT and SRT therapies do not correct neurological symptoms in Gaucher patients,[39, 40] GBA

inactivation in the animal brain is desired. Although this has been achieved with genetic GBA knockout mice[41] and fish models[42, 43], pharmacological perturbation still offers several advantages, including the experimental simplicity, tunability of the extent of GBA inactivation, and that it allows one to simply start and stop enzyme inactivation and examine the response in the starting and recovering phase.[44] Currently, only the cyclophellitol ABPs/inhibitors labeling GBA are suitable for generating an LSD model, as all others label multiple enzyme targets. The galactose configured cyclophellitol aziridine ABPs[2] might be a potential lead in creating Krabbe disease animal model. Future studies might consider replace its BODIPY fluorophore with other hydrophobic moieties (such as biphenyl or adamantyl) to achieve better brain penetration. However, due to the much lower *in vitro* inhibitory potency, the *in vivo* efficacy and concomitant inhibition towards other enzymes in animals should be thoroughly examined.

h. Discovery of novel glycosidases, including cross species

The ability of gel-based ABPP to simultaneously visualize multiple labeled targets having similar reactivity but distinct molecular properties (molecular weight, pH profile, and glycosylation status) provides a convenient platform for discovering novel glycosidases in given samples. Labeled bands (with or without affinity-enrichment by biotin ABP) can be further excised, tryptic digested, and identified by proteomics using LC-MS/MS instruments. The same approach has been widely applied in the case of ABPs targeting proteases.[45, 46] The α-galactose configured cyclophellitol aziridine ABPs were also used to investigate reactive α-galactosidases in plants, which in *Nicotiana benthamiana* led to the identification of A1.1, a non-glycosylated enzyme stable across a broad pH range, and found to degrade the non-plant sphingolipids Gb3 and lysoGb3 elevated in Fabry patients.[25] Similarly, the broad substrate specificity of some glycosidase ABPs have been be used to "fish out" novel glycosidases in other organisms such as plants.[47, 48] In this thesis, the β-galactose configured cyclophellitol aziridine ABPs identified in the mouse kidney extracts two putative β-galactosidases in the GH35 family: the GLB1-like protein 1 and 2 (GLB1L and GLB1L2) (**Chapter 7**). This is substantiated by the occurrence of ABP-labeled bands in other mouse tissue extracts showing distinct molecular weights, glycosylation profile, and pH range than GLB1 and GALC. In the future, the biology and potential contribution of GLB1-like proteins to LSDs stemming from β-galactosidase deficiency (e.g. GM1 gangliosidosis, Morquio B syndrome, Krabbe disease) should be carefully examined.

i. Detecting interaction partners of glycosidases

For proteomics identification of glycosidases, biotin-tagged ABPs are commonly used to allow enrichment of labeled enzyme with streptavidin carriers. In most of the present biotinylated glycosidase ABPs, the biotin moiety is embedded in the catalytic pocket of the enzyme and binding to streptavidin is only feasible upon denaturation of the enzyme. An exception in this connection forms one GBA ABP that is a cyclophellitol-epoxide with an extended linker preceding the biotin moiety: likely when this ABP is bound to native enzyme the biotin is accessible to streptavidin, allowing pull-down.[49] This ABP additionally contains an internal BODIPY fluorophore within the linker, making detection of the affinity-enriched ABP-enzyme complex compatible with fluorescence scanning on wet slab-gels, and thus facilitates their subsequent in-gel tryptic digest and proteomic analysis. It is conceived that such extended, dual functional extensions may be installed on other configurational isomers of cyclophellitol and cyclophellitol aziridine cores. Such novel probes could be employed to pull down native enzymes and co-purify interacting protein partners. Combined with prior sub-cellular fractionation, this could provide valuable information on the protein interaction partners for each of the glycosidases at distinct cellular locations. Identification of interacting proteins is particularly warranted for the non-lysosomal glucosylceramidase (GBA2). The exact mode of its membrane association, its precise localization, and biological functions is still enigmatic. Poorly understood is the reported association between GBA2 mutation and with locomotor dysfunction whilst pharmacological inhibition of GBA2 activity in Miglustat-treated Gaucher disease and Niemann-Pick type C patients is well tolerated.[50, 51] In the case of the lysosomal GBA, protein interaction partners that should be identified with native ABP pull-downs as positive controls include LIMP2 and saposin C. Discovery of novel GBA interacting proteins might be of interest to generate new insights and clues in the pathophysiology of Gaucher and Parkinson disease.

j. Discovery of small molecule interactors of glycosidases

ABPs can in principle be also used to identify small molecules (including peptides) interacting with the catalytic pocket. Such interactors might be developed into pharmacological chaperones to stabilize (mutant) enzyme.[52] Recently, a high-throughput *in vitro* screening platform has been developed using the glycosidase ABPs coupled to fluorescence polarization (FluoPol) assay, in which the ratio of free ABP and bound ABP can be measured by reading out the extent of polarized fluorescence (where the bound ABP emits more polarized fluorescence).[36] Identified in this way have been several potent and specific inhibitors of GBA2,

and the same approach is presently extended to GBA, α-glucosidase, and α-mannosidase (unpublished work by Daniel Lahav, Dep. Bio-organic Synthesis, Leiden University). The screening can in principle also be performed in living cells, given the cell-permeability of some ABPs. Alternatively, the fluorescent quench glycosidase substrates developed by Vocadlo and co-workers provides another useful tools that is compatible with high-throughput microscopy-based screening in living cells.[17, 18] Nevertheless, no matter the type of initial screen, ABP can be used as a confirmation tool to assess the amount of active glycosidase molecules in different tissues of treated animals, where *in vivo* imaging techniques for glycosidase activity are not yet available. This can be performed with gel-based detection for tissue homogenates and microscopy-based detection in tissue sections, as earlier discussed. The existing FluoPol and gel-based ABPP platforms are presently used to identify selective inhibitors for various glycosidases (ongoing work Daniel Lahav, Dep. Bio-organic Synthesis, Leiden University). Finally, crystallography studies on the formed Michaelis complex and covalent complex of the ABP towards the target glycosidase may provide a structural-guided design of specific inhibitors towards a given glycosidase, for example to create a better inhibitor/ABP towards GH39 α-L-iduronidase (**Chapter 5**).[24]

k. Confirming LSD diagnosis

Diagnosis of LSDs is primary based on clinical symptoms. It is often combined with genotyping and demonstration of reduced enzyme activity in the case of enzymopathies.[53] Additionally used for confirmation of diagnosis is demonstration of accumulating metabolites or elevated biomarkers.[54] For several LSDs there are now (newborn) screening programs in some parts of the world,[55] largely based on genotyping followed by metabolite analysis and/or enzyme activity assays. The cyclophellitol and cyclophellitol aziridine ABPs can assist in some cases in the biochemical confirmation of the diagnosis of an LSD caused by deficiency of a corresponding glycosidase. Available ABPs include those labeling GBA (Gaucher disease),[1] GLA (Fabry disease),[4] FUCA1 (fucosidosis),[5] GALC (Krabbe disease),[2] GAA (Pompe disease) (**Chapter 4**),[56] GUSB (Sly syndrome) (**Chapter 4**),[57] MAN2B1 (α-mannosidosis) (**Chapter 6**), MANBA (β-mannosidosis)(**Chapter 6**), and GLB1 (GM1 gangliosidosis and Morquio B syndrome) (**Chapter 7**). Of note, ABP labeling can not only visualize lack of active enzyme molecules, but also abnormalities in post-translational processing (such as glycosylation and proteolytic cleavage). The latter abnormalities can also be detected with antibodies but these are

not always available. Proof of concept for the diagnostic potential of ABPs has been provided for Gaucher disease,[1] Fabry disease,[4] Krabbe disease,[2] and Pompe disease (**Chapter 4**)[56] with patient fibroblasts. Future application might exploit the use of ABPs in other patient materials, such as dried blood spots and urine, to assist in the confirmation of LSD diagnosis.

Finally, an attractive potential application for α-galactose configured ABPs to discuss warrants discussion, that is their use to identify female heterozygotes of Fabry disease that may develop an attenuated form of the disease. Fabry disease (α-galactosidase (GLA) deficiency) is an X-linked disorder, and as the result of random X-chromosome inactivation some cells of Fabry disease heterozygotes lack the normal enzyme whereas other cells are entirely normal depending on which copy of the X-chromosome was inactivated.[58] Reliable diagnosis of Fabry disease carriers by simple enzyme activity assays is virtually impossible[59]: only the measurement of isolated hair root cells, showing random X-chromosome inactivation) allows confirmation of the status of carriers.[60] The labeling of cellular GLA with appropriate ABP might offer a convenient solution for carrier detection. Labeled cells can be sorted by FACS: concomitant demonstration of cells with normal labeling intensity and cells lacking label would point to a heterozygote. A complication is that the presently available GLA ABP also labels the enzyme N-acetylgalactosaminidase (NAGA) that is not affected in Fabry disease. A more specific ABP for GLA should be ideally designed.

II. Current limitations and future prospects

The electrophilic trap (warhead)

The broad-specificity cyclophellitol aziridine ABPs offer mechanism-based labeling of in class glycosidases. This makes them excellent tools for simultaneous gel-based profiling and for discovery of novel glycosidases. However, the high reactivity of aziridine with nucleophiles, even beyond in-class targets, also makes them unattractive for microscopic imaging, as multiple enzymes may be labeled. Cyclophellitol epoxides allow highly specific labeling of GBA and GALC. Unfortunately, its α-galactose configurational isomers did not react with retaining exo-α-galacosidases.[61] The same was observed for exo-α-glucosidase (unpublished data, Dep. Bio-organic Synthesis, Leiden University). Future studies could investigate whether the C8-tagged cyclophellitol epoxide with other extensions would offer better target specificity. Also worth considering is to generate cyclophellitol ABPs containing other types of warhead that are less

reactive than aziridine but are still compatible with installing a linker/tag at the aglycon position.

The recognition element/linker

The β-glucosidases (GBA and GBA2) are prone to become labeled by higher concentrations of cyclophellitol aziridine ABPs with various configurations of the cyclophellitol core. This was observed for ABPs with α-glucose (**Chapter 4**),[56] β-glucuronic acid (**Chapter 4**),[57] β-mannose (**Chapter 6**), and β-galactose (**Chapter 7**) configurations. For the α-glucose configured ABPs, their labeling of β-glucosidases has been attributed to the half chair ($^4H_3$) conformational mimicry of cyclophellitol aziridine to β-glucosidases' substrates at the transition-state.[62] Another explanation for the aziridine ABPs' cross-reactivity with β-glucosidases might be the presence of the N-alkyl extension (with or without a fluorophore) at the aglycon side. The major substrate of both GBA and GBA2, glucosylceramide, contains such flexible alkyl chains at the aglycon side, and it has been noted that these are important for the hydrophobic interaction of the substrate glucosylceramide with GBA during catalysis.[63] Indeed, it was noted that the bare β-galactose configured cyclophellitol aziridine core is a much weaker β-glucosidase inhibitor when compared to its N-alkylated analogues. In the future, structural modification on the alkyl chain might prevent these ABP's concomitant labeling on β-glucosidases, such as increasing the hydrophilicity, or decreasing the structural flexibility.

Interestingly, a conceptually new compound, cyclophellitol with a cyclic sulfate electrophilic warhead, has recently been developed as a selective inhibitor of α-glucosidases over β-glucosidases by mimicking the $^4C_1$ chair conformation of the Michaelis complex observed in α-glucosidases.[62] In the future, other types of nucleophilic trap favoring the $^4C_1$ chair conformation and allowing the attachment of a reporter tag at the reducing-end aglycon side should be explored for the synthesis of selective ABPs.

The detection tag

Currently used fluorophores have their limitations. The BODIPY fluorophores, while providing excellent quantum yield,[64] are usually inferior to the Cy5 fluorophore when used in samples such as cell lysates or animal tissue homogenates, as these often contain background fluorescence at overlapping wavelength. They are also not always synthetically attainable, and sometimes they are unstable during purification and storage.[65] In addition, ABPs with the BODIPY fluorophores are less brain-penetrant, which may be due to the presence and action

of p-glycoprotein or multidrug resistance protein pumps that actively excrete BODIPY-containing molecules across the blood-brain barrier.[66, 67] The Cy5 fluorophore offers better signal-to-noise ratio, but on the other hand appears less soluble in aqueous solution, and is prone to label non-specifically, particularly in cells and zebrafish (observations Dep. Medical Biochemistry, Leiden University). Similarly, oligonucleotides modified with the positively-charged Cy3 or Cy5 dye, but not the non-charged Alexa fluorophores, have been found to aspecifically accumulate at the mitochondrial membrane.[68] All of these issues might underlie the poor labeling and inactivation of enzyme by Cy5 cyclophellitol ABP in zebrafish (**Chapter 3**). Taken together, a non-charged fluorophore with good solubility in aqueous solution would be ideal for visualizing purposes. Another way to improve sensitivity of ABP detection under fluorescence microscopy is to use a two-step labeling strategy. For example, cells can be firstly treated with ABPs containing a norbornene tag (**Chapter 7**), and after fixation these can be covalently attached with tetrazene-Cy5 fluorophores *via* inverse electron-demand Diels-Alder reaction (IEDDA, **Chapter 7**). In this manner, issues concerning Cy5's solubility and aspecificity in cells might be overcome. The tetrazine can in principle also be coupled to structures containing multiple fluorophores, thus allowing signal amplification. The ABPs can also be installed with a isotopic tag, allowing real-time imaging of labeled therapeutic glycosidases using techniques such as positron emission tomography (PET)[27–29, 31] or single-photon emission computed tomography (SPECT)[30] in living animals.

For affinity-enrichment using biotinylated ABPs, the described methods in this thesis only allow enrichment of denatured proteins. As mentioned above, this is due to the inaccessibility of the biotin tag to streptavidin resin, when the glycosidase is in its native conformation. Replacing the biotin moiety with the dual functional tag with an extended linker (described in an earlier section) might allow native pull-down of glycosidases in cell lysates or tissue homogenates. The conceived advantages of this setup include: (1) "cleaner" enrichment, as endogenous biotinylated proteins would not be enriched by the streptavidin resin when they are in the native conformation (unpublished data); (2) easier confirmation of the enrichment results. After pull-down, samples can be resolved by SDS-PAGE, and the labeled glycosidases can be easily detected by fluorescence scanning. Sample resolved by SDS-PAGE can be directly used for in-gel tryptic digestion for subsequent LC-MS/MS detection; (3) co-affinity enrichment of other associated proteins with the labeled glycosidase (discussed in an earlier section). The proposed ABPs should be obviously carefully examined regarding target specificity and cell-permeability.

III. Conclusion

Over the past years, carefully designed and characterized small molecules tailored for specific proteins, such as the herein described activity-based probes labeling retaining exo-glycosidases, have opened up new research possibilities in the traditional medical biochemistry field. In the future, further development of these chemical biology tools will no doubt continue to offer novel insights on the physiological and pathological roles of relevant enzymes, as well as facilitate the development of therapies and improvement of disease diagnosis, as exemplified in this thesis for the lysosomal storage disorders.

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