Cyclophellitol and its derivatives: synthesis and application as beta-glycosidase inhibitors

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2 Activity-Based Retaining β-Exoglucosidases Probes

Their Rational Design and Biological Applications

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2.1 Introduction

Biochemical and biological research on the structure and function of carbohydrates and glycoconjugates are complicated by their heterogeneity and transient occurrence in nature. Moreover, their biosynthesis, in contrast to proteins and nucleic acids, is not directly controlled by the genetic code, which means that the nature of the eventual carbohydrate structures in a given organism (termed glycome) cannot be extracted from the genetic material of the organism. Instead, the nature of the glycome is determined by the substrate levels in conjunction with the combined action of glycosyl transferases and glycosidases.[1-3]

Studies toward the glycome are further complicated by its structural complexity as the chemical space covered by carbohydrate-containing compounds is vast. Carbohydrates and glycoconjugates are constructed from a large variety of monosaccharide building blocks, while in contrast nucleic acids and peptide/proteins are synthesized from a relatively small set of building blocks. A limited set of monosaccharides (predominantly D-glucose, D-mannose, D-galactose, D-glucuronic acid, D-xylose, D-ribose, *N*-D-glucosamine, *N*-D-galactosamine, L-idose and D-neuraminic acid) is used to construct the glycome in human, but bacterial glycomes contain up to hundreds of monosaccharides differing in stereochemistry and functional group pattern. Monosaccharide building blocks can be, and are interconnected through glycosidic bonds to various positions of the core of other monosaccharides forming oligomeric structures, known as oligosaccharides and polysaccharides.[4-5] Hybrid molecules like glycolipids, glycopeptides as well as glyconjugates involving other biomolecules also exist. Nucleic acids and amino acids are linked through achiral linkages (phosphodiester bonds and amide bonds, respectively), whereas the glycosidic linkages in carbohydrate structures involve a chiral anomeric carbon centre, increasing the structural complexity even further.

The structural complexity in conjunction with the non-templated encoded properties of the glycome limits the use of biomolecular techniques, and therefore other means of studying the glycome need to be employed. One attractive and commonly used strategy is perturbation, which can be achieved by manipulating the corresponding glycoprocessing enzymes, glycosyl transferases and glycosidases. A variety of natural and synthetic (fluorogenic) substrates and inhibitors that predominantly act on glycosidases exist.[6-7] With this set of compounds, the activity of a given glycosidase can be monitored or inhibited, yet insight in the presence and/or nature of glycoprocessing enzymes in a biological sample cannot be established directly and unambiguously. Activity-based protein profiling (ABPP) does provide the means to do so, but requires that suitable activity-based probes (ABP) are available. This Chapter discusses how by rational design suitable ABPs for retaining β-exoglucosidases can be obtained.

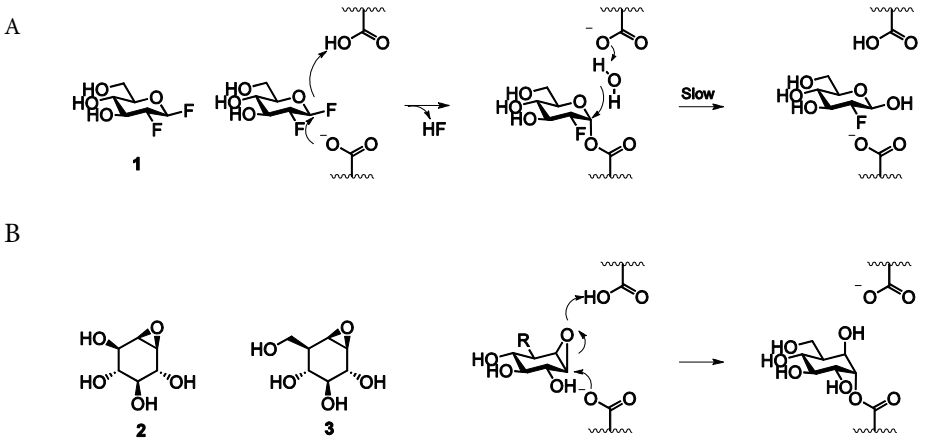
2.2 The Chemical Approach

Both the nature of the substrate and the mechanism employed by the given enzyme are taken into consideration when designing ABPs for a specific enzyme/class of enzymes. Ideally, the enzyme of interest forms a covalently bound enzyme-substrate intermediate at a certain stage of the catalytic cycle. Analysis of such a covalent intermediate allows

the design of a mechanism-based inhibitor, which in contrast to the natural substrate cannot proceed to the ensuing catalytic reactions as the formed covalent intermediate is (much) more stable than the corresponding enzyme-substrate adduct. This strategy has met with most success in the design of ABPs for hydrolytic enzymes, in particular serine hydrolases, cysteine proteases and threonine proteases.[8-11] Glycosidases are a large family of hydrolytic enzymes that catalyze the cleavage of acetal linkages, characteristic for oligosaccharides and glycoconjugates to form hemi-acetal linkages.[3] Mechanistic studies on glycosidases revealed that retaining glycosidases develop covalent intermediates during the hydrolysis, following the Koshland double displacement mechanism[12] as discussed in Chapter 1. In contrast to inverting glycosidases, the enzymes that do form a covalent adduct are amenable to active site labeling and therefore ABPP.

As stated, inhibitors that proceed through the catalytic process are good leads for ABP development if they form a long-lived covalent intermediate. With respect to retaining β-glucosidases, two compound classes that meet this requirement have been studied in detail in the past decades: 2-deoxy-2-fluoroglucosides and cyclitol epoxides.[13-14] Substitution of the 2-hydroxyl by an electron-withdrawing fluorine, as in compound **1**, results in the formation of an enzyme-glucoside adduct that is comparatively more stable than that formed from the natural substrate. The presence of the 2-fluorine destabilizes both oxocarbenium ion transitions states and thereby also the rate of formation and breakdown of the glucosyl-enzyme adduct. Incorporation of a good

Figure 1. Overview of mechanism-based retaining β-exoglucosidases inhibitors and their mode of action. (A) 2-deoxy-1,2-difluoroglucose **1** and (B) conduritol β-epoxide **2** and cyclophellitol **3**.



leaving group (here: fluorine) is thus a prerequisite to assure that the formation of the glucosyl-enzyme adduct proceeds uneventfully.[13] It should be noted that 2-deoxy-2-fluoroglycosides were employed by the Withers laboratory in a seminal paper demonstrating the involvement of the covalent enzyme-substrate adducts in the action of retaining glycosidases and thus proving the double displacement mechanism hypothesized by Koshland correct.[15]

In an alternative design, replacement of the glucoside by a cyclitol analogue equipped with a reactive epoxide generates a stable covalent ester adduct after enzymatic catalysis (protonation of the epoxide followed by nucleophilic substitution), thereby effectively inactivating the enzyme. As much as five decades ago, Legler and co-workers reported on the use of conduritol β-epoxide (CBE, **2**) for this purpose.[16,17] The most effective inhibitor of this class is the natural product, cyclophellitol **3**. This compound closely resembles β-glucopyranose in configuration and substitution pattern and as such appeared a highly potent mechanism-based inhibitor of retaining β-exoglucosidases from different origins.[18-19]

2.2.1 Development of a Human Glucocerebrosidase Activity-Based Probe

Human glucocerebrosidase, or GBA, catalyzes the hydrolysis of glucosylceramide to glucose and ceramide. This enzyme is responsible for the penultimate step in the turnover of glycosphinglipids, which, when defective results in the lysosomal storage disorder Gaucher disease. Malfunctioning in the GBA encoding gene can lead to partial malfunctioning of the enzyme and hence accumulation of its substrate glucosylceramide.[20,21] Two Gaucher therapies are practiced in the clinic. In enzyme replacement therapy, patients are treated with recombinant GBA, whereas in substrate reduction therapy glucosylceramide levels are downregulated through partial inhibition of the enzyme responsible for glucosylceramide biosynthesis: glucosylceramide synthase.[22-24] A third potential clinical strategy that received increasing attention in recent years is called chemical (or pharmacological) chaperone therapy.[25] This therapy aims to enhance the activity of mutant GBA through stabilizing molecules. Both for monitoring GBA levels in healthy and Gaucher patients, and for assessment of the effect of interference in glucosylceramide metabolism, it would be advantageous to have access to potent and selective activity-based GBA probes.

With the aim to develop those probes, a comparative study was performed on the merits of the two scaffolds described earlier - 2-deoxy-2-fluoroglucosides and cyclitol epoxides - as activity-based GBA probes. Figure 2 depicts the four probes that were designed for this purpose: two direct probes and two probes relying on two-step bio-

Figure 2. Mechanism-based inhibitors **4**-**7** and reversible inhibitor AMP-DNM **8** for comparative studies.

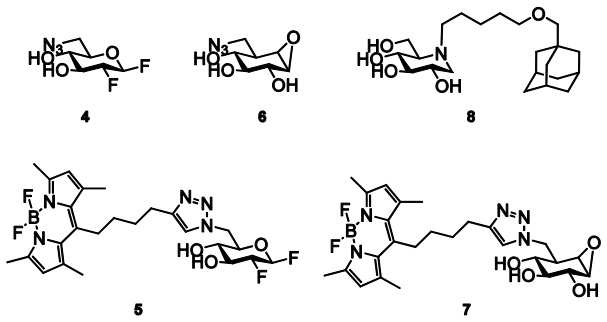


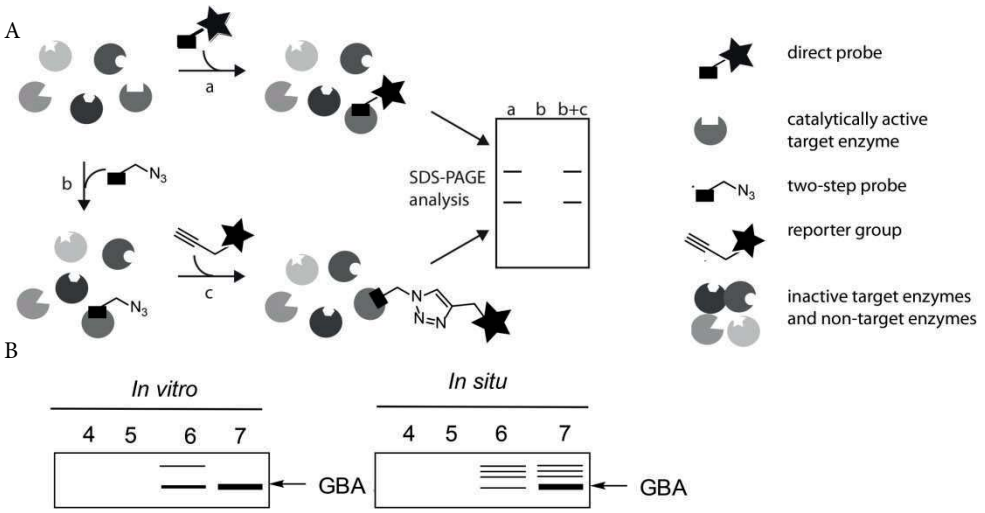
Table 1. Apparent IC_{50} of **2**-**8** for almond β-glucosidase and glucocerebrosidase.

Compound	Almond β-glucosidase	Glucocerebrosidase
	IC_{50} (μM)	IC_{50} (μM)
2	461	9.49
3	3	0.35
4	>10000	1665
5	>1000	785
6	27	0.120
7	56.5	0.0012
8	-	0.2

orthogonal ligation. GBA belongs to the family of exoglucosidases, an enzyme class that is rather specific to the nature of their substrates. It was therefore considered unlikely that the attachment of a bulky reporter group would be accepted within the enzyme active site and hence fluoroglucoside **4**, its click-conjugated fluorescent counterpart **5**, as well as 8-deoxy-8-azidocyclophellitol **6** and its congener BODIPY-cyclophellitol derivative **7** were designed.[26-27] Comparison of the inhibitory potency of these compounds relative to that of the known glucosidase inhibitors **2**, **3** and adamantane pentyloxydeoxynojirimycin (AMP-DNM, MZ21, **8**) for both almond retaining β-exoglucosidase (ABG) and GBA yielded a rather surprising result (Table 1). The ABG inhibitory potency of the small set of compounds was as expected. The cyclitol epoxides out perform the 2-deoxy-2-fluoroglycosides, with **3** as the most potent inhibitor. Partial to complete loss of inhibition of ABG and GBA was observed with the C6-modified fluoroglucosides. In contrast, cyclophellitol derivative bearing a bulky fluorescent group at C8 proved to be the far most potent GBA inhibitor. [26-,7]

This superior inhibition becomes also evident in a comparative direct and two-step bioorthogonal ABPP experiment on the four compounds. Figure 3A depicts a general strategy for direct and two-step labeling on cells and cell extracts, whereas Figure 3B gives a representative image of the potency and specificity of the various ABPs on GBA. Labeling of GBA with BODIPY-cyclophellitol derivative **7** proved to be very clean both *in vitro* and *in situ*, much more than when applying the indirect probe **6** in a two-step bioorthogonal approach under copper (I)-catalyzed click reactions conditions. No GBA-specific labeling was achieved with either the 2-deoxy-2-fluoroglucosides directly or with two-step probes **4** and **5**. The latter result is perhaps not so surprising as 2-deoxy-2-fluoroglucosides are rather poor glucosidase inhibitors, since the hydroxyl group at C2 of the corresponding substrates is an important structural feature in binding to the enzyme active site.

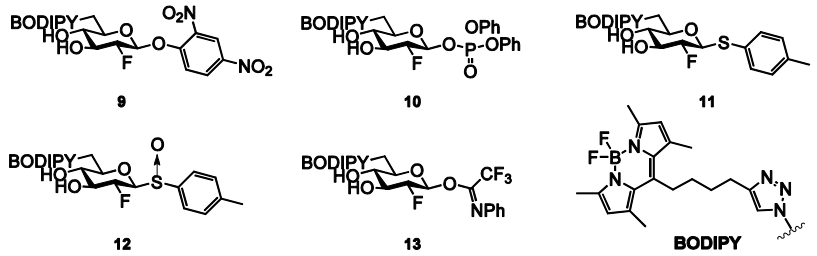
Figure 3. Direct and two-step bio-orthogonal labeling of GBA in cells and cell extracts.



(A) Schematic representation of the general workflow. In a direct labeling approach (a), a proteome is treated with a direct probe. The labeled enzymes are directly visualized with SDS-PAGE followed by fluorescence imaging. In a two-step labeling approach, a proteome is treated with a two-step probe (b) followed by ligation with a reporter group (c). Subsequently, the enzymes are visualized with SDS-PAGE followed by fluorescence imaging; (B) *In situ* and *in vitro* labeling of GBA with **4**-**7**. BODIPY-cyclophellitol derivative **7** is the most effective ABP *in vitro* and *in vivo*.

Another intrinsic feature of 2-deoxy-2-fluoroglucosides that sets these apart from cyclitol epoxides is their tempered reactivity as a result of the electron-withdrawing fluorine at C2. To offset this disadvantage, good anomeric leaving groups (fluoride, dinitrophenyl) are often employed. Figure 4 depicts a few structures that were employed to look further into the labeling activity of this class of compound.[28] Anomeric imidate **13** proved by far the most potent of these series. Moreover, 1,2-difluoroglucoside **5** labeled mutant GBA, in which the general acid/base residue was mutated (Glu235Gln) equally well, while imidate **9** proved inactive towards this mutant. Arguably, imidate **13** is therefore a more "true" ABP that actually recruits the catalytic residues. At the same time, BODIPY-cyclophellitol derivative **7** proved to be a better inhibitor than imidate **13** by several orders of magnitude and therefore, cyclophellitol **1** appears the superior scaffold for retaining glycosidase ABP design.

Figure 4. Tuning the leaving goup on 2-deoxy-2-fluoroglucosides yields comparatively more potent GBA ABPs.

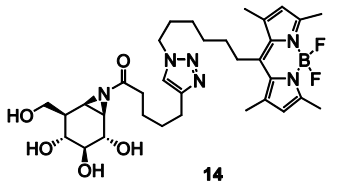


2.2.2 Cyclophellitol Aziridine is a Broad-Spectrum Activity-Based Retaining β-Exoglucosidase Probe

The human genome contains at least four retaining β-exoglucosidase genes. Next to GBA, these are the non-lysosomal retaining β-glucosidase (GBA2), cytosylic β-glucosidase (GBA3) with broad substrate specificity and lactase phlorizin hydrolase (LPH).[29] LPH is an intestinal, dual-activity glycosidase which can process both β-glucosides and β-galactosides as substrates. Among these, only GBA and to a lesser extent LPH, were labeled by BODIPY-cyclophellitol derivative **7** and the bulky reporter group is apparently not accepted by the other enzymes. Indeed, and as stated before, one would expect exoglycosidases to be rather particular to the substitution pattern and configuration of the glycomimetic emulating the corresponding natural substrate. In contrast to this assumption, however exoglycosidases are often less selective towards the aglycon as illustrated by a wide variety of natural - and artificial substrates

(including fluorogenic substrates commonly used for glycosidase kinetic studies). Figure 5 depicts broad-spectrum retaining β-exoglucosidase probe **14**. The design of this molecule is based on the assumption that by pointing the bulky reporter group towards the direction that is normally occupied by the substrate aglycon would result in a mechanism-based inhibitor accepted by all the enzymes mentioned.[29]

Figure 5. Broad-spectrum-based ABP **14**.



14

Table 2. Head to head comparison of **7** and **14**.

Glucosidase	Epoxide 7	Aziridine 14
GBA	+	+
GBA2	-	+
GBA3	-	+
LPH	-	+
Bacterial	+	+

A key aspect to ABPP studies is, next to the design of an ABP also its synthesis. This is often not an easy task and one complicating factor is that ABPs are intrinsically reactive. Their reactivity needs to be balanced in a way that they are stable under physiological conditions, yet react efficiently with their target enzymes. The synthesis and purification of ABP **14**, which will be discussed in detail in Chapter 4 and related aziridine ABPs have to be conducted with great care as the probes are both acid-and base labile.[30]

A head-to-head comparison of GBA-specific probe **7** and aziridine **14** is presented in Table 2. As expected, aziridine **14** labels all four human retaining β-exoglucosidases depending on their expression in various tissues. Similar to epoxide **7**, aziridine **14** is both cell- and tissue permeable, and therefore both probes are amenable for *in vivo* labelings experiments. The question why GBA, but not the other enzymes, tolerates and actually prefers a bulky substituent at the primary position remains unanswered. Both probes react with a considerable number of bacterial glucosidases and some of them appeared to be evolved from endoglycosidases. It is likely that GBA is evolutionary related to these bacterial enzymes.[29]

2.3 Biological Research and Evaluation

In general, ABPs find various applications in biology research. They can be used in comparative ABPP studies to determine the enzyme activity levels in different proteomes (for instance healthy versus diseased state) and in the evaluation of the

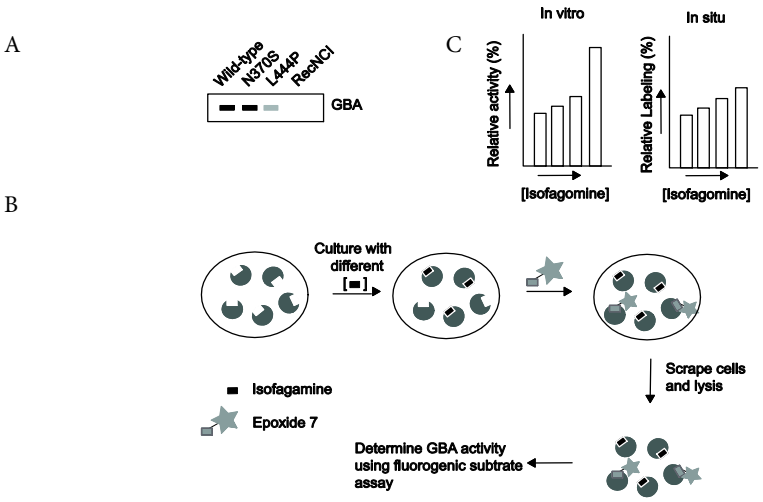
potency and selectivity of putative inhibitors of a given enzyme targeted by the ABP (competitive ABPP).[31, 32] Depending on the bio-availability of the probes, these studies can be conducted in an *in vitro*,- *in situ*,-and *in vivo* research settings. Such studies are common practice with serine hydrolase, cysteine protease, and threonine protease probes, yet only start to emerge in the field of glycobiology as suitable glycosidases probes were until recently not available. In the following two sections, two examples of biochemical and biological studies are described to highlight the potential of activity-based glycosidase probes in chemical biology research.

2.3.1 In situ Monitoring of Active Site Directed GBA Chemical/Pharmacological Chaperones

Chemical or pharmacological chaperones form a conceptually new approach to treat inherited diseases that are characterized by point mutations in a given hydrolytic enzyme, which causes its partial dysfunctioning. Gaucher disease is caused by point mutations in the GBA gene that lead to an overall lower enzyme activity. This lower enzyme activity is caused by a comparably lower number of GBA copies that reach the lysosome, rather than by a lower activity of an individual GBA protein. Indeed, probing tissues from different Gaucher type patients with epoxide **7** (Figure 6A) shows GBA labeling in varying intensities, corresponding to the severity of the disease. The intrinsic reactivity of the GBA mutants towards epoxide **7** is largely invariable and therefore the partial loss in lysosomal activity is thought to rely on the partial impairment of folding of the mutant enzymes in the endoplasmic reticulum (ER).[26] Chemical/pharmacological chaperone strategies aim at correcting this impaired folding through stabilization of the enzymes in its proper fold by inhibition of the enzyme active site. It should be kept in mind that in this way a large number of enzymes can travel to the lysosomal compartment, but will be accompanied by their active site inhibitor. In other words, an increase in the enzyme activity within lysosomes may not be the actual result. For the first time, BODIPY-cyclophellitol derivative **7** allows probing of the intracellular GBA activity with or without the presence of the chemical chaperone isofagamine as depicted in Figure 6B. After treatment of cell cultures with isofagomine and ensuing incubation with ABP **7**, the sample is lysed and the GBA activity is monitored using a fluorogenic substrate assay. For various reasons, including dilution of the chemical chaperone, such a research setting may not reflect the intracellular situation, which appears to be true as shown in Figure 6C. *In vitro* measurement reveals a marked increase in activity of mutant (N370S) GBA activity in the presence of isofagamine, while *in situ* measurement with ABP **7** shows

comparatively a less pronounced activity increase. It should be noted that these assays are rather complicated and that care has to be taken in the interpretation of the results. At the same time, this observation should serve as a warning to the field: for an enzyme active site directed chemical chaperone to be effective, it should bind within the ER to stabilize the enzyme in its proper fold and once it enters the lysosomal compartments the compound should dissociate to become an inactive bystander. It may not be easy to reach this feature using iminosugars as they are intrinsically basic by nature and therefore prone to be trapped in an acidic environment. Interestingly, the Withers laboratory recently proposed the use of 2-deoxy-2-fluoroglycosides as potentially useful alternative chemical chaperones based on their mechanism-based binding, followed by slow but sure release to produce 2-deoxy-2-fluoroglucose as such an inert molecule.[33]

Figure 6. *In situ* monitoring of GBA with ABP **7**.



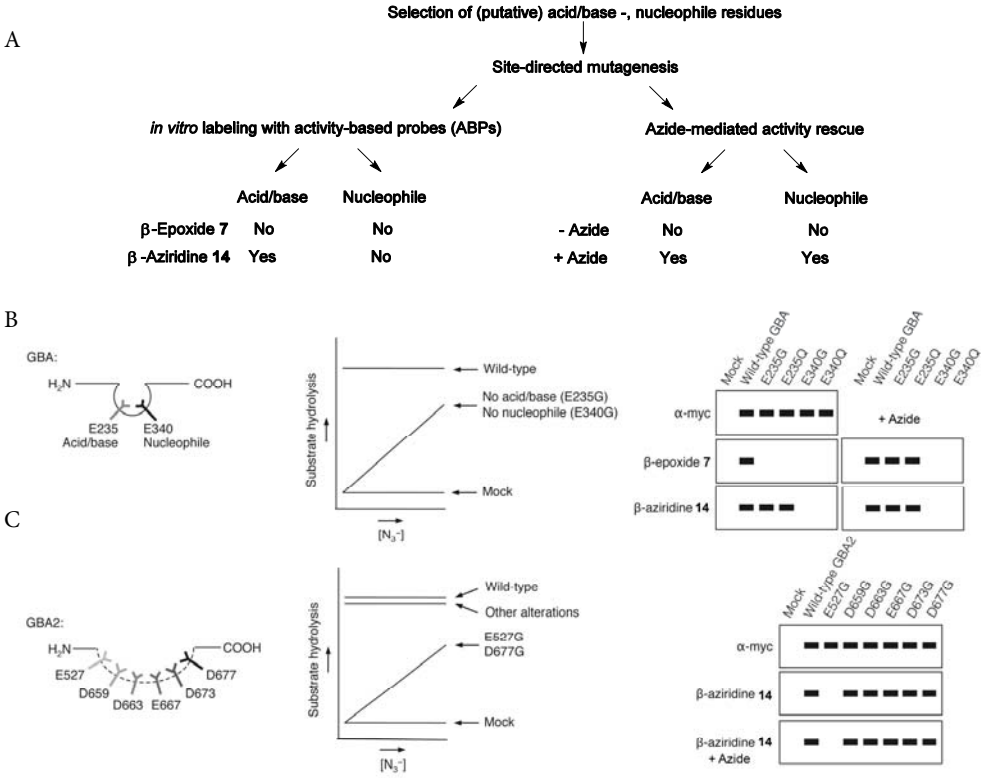
(A) Detection of GBA in Gaucher tissues. GBA in wild-type and homozygous N370S, L444P and collodion fibroblast were labeled with ABP **7**, (B) General workflow for *in vitro* and *in situ* GBA activity profiling in the presence of isofagamine, (C) *In vitro* and *in situ* effect of the pharmalogical chaperone, isofagamine, on GBA activity.

2.3.2 Mapping of human retaining β-glucosidase active site residues

An intrinsic nature of activity-based probes is their covalent attachment to enzyme active site nucleophiles. In case the nature of these is unknown they can in fact be assigned using activity-based probes as summarized in Figure 7A. This approach entails a combination of site-directed mutagenesis of putative catalytic carboxylic residues and

their identification by sodium azide-mediated rescue experiments in the presence of an universal substrate, for instance, 2,4-dinitrophenyl β-D-glucose (DNPGlc). Subsequently, discrimination between the general acid/base and nucleophilic residue can be accomplished by the combined experiments of ABP labeling and sodium azide rescue experiments. Such studies can be executed on recombinant purified enzymes and therefore both 2-deoxy-2-fluoroglucosides and cyclitol epoxides/aziridines can be used for this purpose. Figure 7B provides a representative example with ABP **7** and **14**. In contrast to GBA and GBA3, the active site acid/base and nucleophile of GBA2 were unknown. Moreover, at least six aspartate/glutamate residues appeared suitable candidate-nucleophiles. Following a similar strategy as for GBA (Figure 6B), the general acid/base (D677) and catalytic nucleophile (E527) were determined as outline in Figure 7C.[34]

Figure 7. Retaining β-exoglucosidase active site mapping using ABP **7** and **14**.

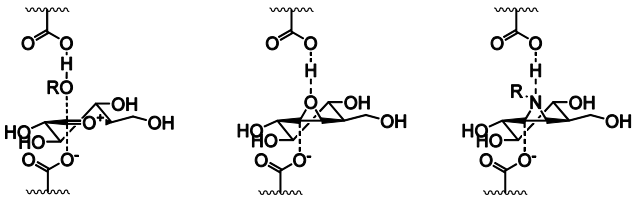


(A) Schematic representation of the general workflow, (B) GBA active site mapped using APB **7** and ABP **14**, (C) GBA2 active site mapped using ABP **14**.

2.4 Conclusion

In conclusion, rational design has resulted in the development of a panel of active and and selective ABPs for retaining β-exoglucosidases. Cyclophellitol **3** is a natural product and it is therefore fair to state that nature has paved the way for these studies. The first-generation probes, represented by epoxide **7**, provided a rather surprising result: an active and highly selective probe for GBA, key enzyme in Gaucher disease. Arguably, this design principle with a reporter group grafted at the C8, will not meet success when applied to other retaining glycosidases. In contrast, the cyclophellitol aziridine scaffold holds more promise. Numerous retaining exoglycosidases, presented in the CaZy database, follow the general Koshland mechanism, which means that they are in principle amenable to ABPP. Using cyclophellitol aziridine as scaffold, new glycosidase ABPs classes can be designed by emulating the configuration and substitution pattern of the corresponding substrate glycosidase.
Another intriguing feature of the cyclophellitol and cyclophellitol aziridine probes is their high potency. The putative half-chair conformation adopted by the cyclophellitol **3** might be a contributing factor, next to offering a suitable electrophile (epoxides, acylaziridine) to the general acid/base catalyst. This configuration presumably resembles that of the natural substrate oxocarbenium ion configuration upon aglycon protonation (Figure 8).

Figure 8. Cyclitol epoxides and cyclitol aziridines may feature ideal conformational behaviour for retaining β-glucosidase inhibition.



These probes are not only highly potent, but they also appear to react almost instantaneously, further suggesting they fit exceedingly well in the active site. All this bodes well for the future development of ABP aimed at other retaining glycosidases and perhaps - given that there are literature speculations on the covalent intermediacy of some glycosyl transferases- also for glycosyl transferases.

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