General Introduction

Lysosomal glycosidases: inherited deficiency, molecular aspects, and novel chemical probes

Carbohydrates are the most abundant biomolecules on earth and they are besides nucleic acids, proteins, and lipids one of the four major macromolecules essential for life.[1] They are structurally very diverse as the result of the occurrence of different monosaccharide building blocks, and different linkages among these. The most utilized monosaccharides are pentose and hexose (five- or six- carbon sugars), whose ketone or aldehyde can spontaneously react with one of its own hydroxyl, forming a stable five-membered ring (furanose, e.g. fructose and ribose) or a six-membered ring (pyranose, e.g. glucose). This is accompanied by two possible stereochemical outcomes (α- or a β-anomer, where the hemiacetal hydroxyl points to opposite directions from the ring plane). In addition, the monomers can have different hydroxyl configurations (e.g. glucose, galactose, and mannose), and their hydroxyl(s) may be substituted with other types of functional group (e.g. N-acetyl, carboxylic acid, sulfate). The ten commonly utilized monosaccharide building blocks in life are glucose, galactose, mannose, glucuronic acid, L-iduronic acid, neuraminic acid, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), L-fucose, and xylose (**Fig. 1A**).[2] When these are linked together through glycosidic linkages at different hydroxyl positions (on the reducing-end sugar), a large number of possible polymeric structures (oligosaccharides and polysaccharides) can be formed. For example, a simple hexasaccharide constituting of a single type of hexose can form 1.05 x 10^{12} different isomers.[3] Finally, carbohydrates can also linked to other biomolecules, such as protein and lipids, forming glycoconjugates that are also structurally diverse (**Fig 1B**). These entail specific cellular functions such as energy storage (i.e. glycogen and starch), structural integrity (i.e. cellulose and chitin), and a plethora of fundamental cellular processes in eukaryotes, ranging from glycoprotein folding and targeting, inter- and intra-cellular signaling, and cellular interaction/recognition events underlying infection, immune response, and cancer.[1] In vertebrates, glycan synthesis is carried out by ~200 glycosyltransferases, mostly are Golgi enzymes that transfer monosaccharides to the glycans' non-reducing end using high-energy nucleotide sugar donors.[2, 6]

In contrast, glycan degradation largely takes place in lysosomes. The lysosomes are ubiquitous cellular organelles with an acid interior; they contain a range of acid hydrolases fragmenting macromolecules that may enter lysosomes through endocytosis and autophagy. Degradation products are exported from lysosomes via specific transporters for re-use in the cytosol. Only ~30 glycosidases (glycoside hydrolases) orchestrate the step-wise degradation of

glycosidic bonds presented on the structurally diverse glycoconjugates. These lysosomal glycosidases are proficient catalytic machines that can accelerate the hydrolysis of glycosidic bonds by a factor of 10^{17}.[7] Most of them hydrolyze a specific type of reducing-end monosaccharide with either α- or β-anomeric linkage, from glycans presented on glycosphingolipids or liberated from other types of glycoconjugate. Paradoxically, the impressive substrate specificity and high catalytic efficiency of lysosomal glycosidases forms the basis of a number of inherited metabolic diseases in man (**Part I**). Deficiency of a single lysosomal glycosidase can cause major disturbances in metabolism, ultimately translating in clinical

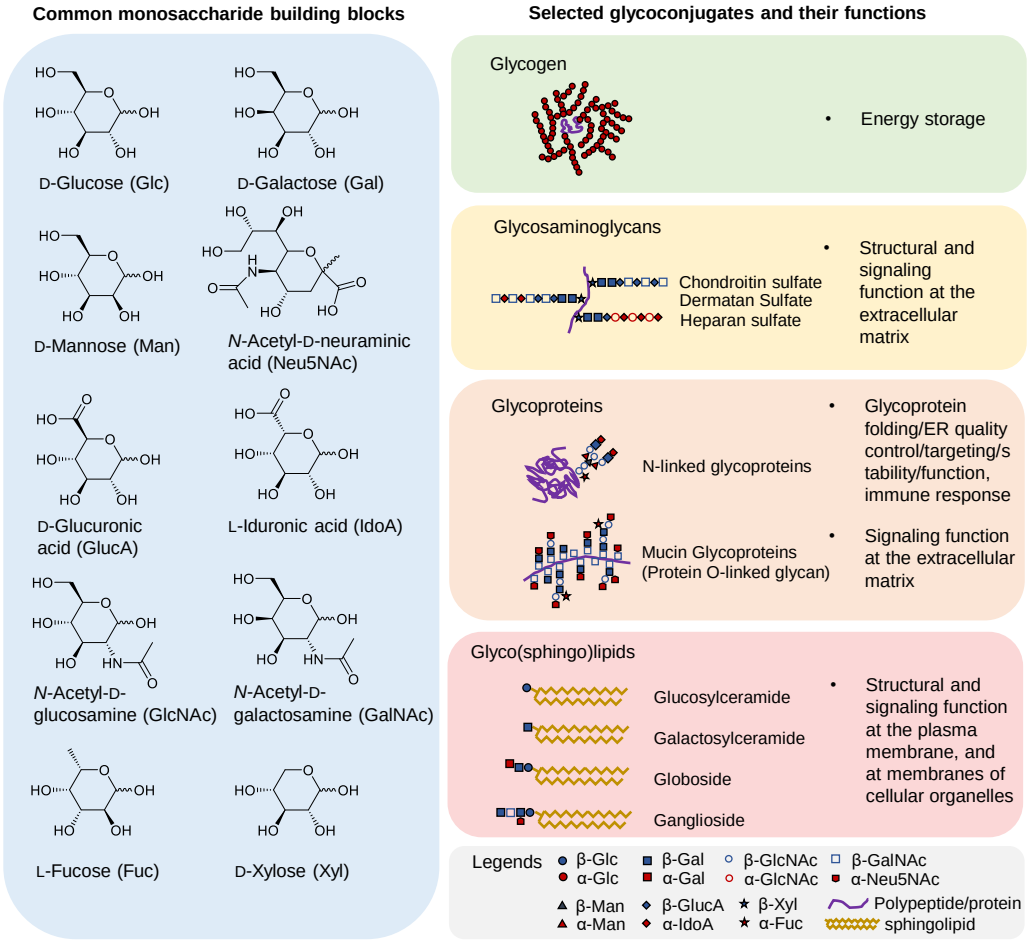

Figure 1. Structures of A) monosaccharide building blocks and B) a selected set of glycoconjugates with their main cellular functions.

symptoms. Research on lysosomal glycosidases and corresponding diseases have prompted the design of mechanism-based chemical probes for enzyme profiling to facilitate investigations on glycosidases in health and disease in an unprecedented manner (**Part II**).

Part I. Glycosidases and inherited metabolic diseases

Pioneering work in the 50's and 60's revealed that inborn deficiencies of particular lysosomal glycosidases are the molecular basis of a number of inherited metabolic disorders, collectively coined as lysosomal storage disorders (LSDs).[8–10] Individual LSDs are rare, and the combined incidence rate is about 1 in 5,000–8,000 newborns.[11, 12] The prevalence of some LSDs can be however high in specific populations: for example, the prevalence of Gaucher disease among Ashkenazi Jewish individuals is reported to be 1 in 450–850 newborns.[13, 14] To date, more than 50 discrete LSDs are recognized. Lysosome dysfunction may result from defects in formation of lysosomes, stability of the cell organelle, export of degradation products and failure of fragmentation processes. Most LSDs are caused by primary defects in enzymes: about one-third of the LSDs is caused by deficiencies of lysosomal glycosidases (**Table 1**).

The best studied LSD is Gaucher disease (GD), where deficiency of lysosomal β-glucosidase (glucocerebrosidase; GBA) leads to prominent accumulation of the substrate glucosylceramide in lysosomes of tissue macrophages that transform into lipid-laden Gaucher cells. These viable storage macrophages are thought to underlie characteristic symptoms of GD patients such as hepatosplenomegaly and pancytopenia (shortage of red blood cells and platelets).[15] In the case of GD, the progression and severity of disease, and occurrence of particular signs and symptoms varies greatly among patients, even homozygotic twins.[16, 17] The cause for this phenotypic variation of GD is presently largely unclear, hypothetically being attributed to modifier genes and other factors. [18–22] More severely affected GD patients (type 2 and type 3) develop marked skeletal disease, fatal neurological symptoms and impaired skin permeability. The pathophysiological mechanisms driving the latter symptoms have not been elucidated yet.[14] Alternative excessive metabolism of glucosylceramide by the cytosolic β-glucosidase GBA2 as well as the formation of toxic glucosylsphingosine from accumulating glucosylceramide in lysosomes is considered to contribute to particular symptoms of GD patients.[15, 21] Mutations in the *GBA* gene are at present also the highest known risk factor for developing Parkinson's disease[23] and Dementia of Lewy Bodies.[24, 25] The surprising link between partial lysosomal GBA impairment and risk for neurodegenerative disease remains enigmatic.

Table 1. Selected lysosomal glycosidases and underlying lysosomal storage diseases.

Enzyme	Gene	Major Substrates (Glycosidic Linkage)	Associated Diseases	Clinical Features
Glucocerebrosidase (GH45, E.C. 3.2.1.21)	*GBA*	Glucosylceramide (glucose β-1-ceramide))	Gaucher Disease (OMIM # 230800 (Type I), # 230900 (Type II), # 23100 (Type III))	Type I: Hepatosplenomegaly, anemia, thrombocytopenia, bone lesions Type II: Neurodegeneration and hepatosplenomegaly in infancy Type III: Intermediate between type I and II
Acid α-glucosidase (GH31, E.C. 3.2.1.20)	*GAA*	Glycogen (glucose α-1,4-glucose)	Pompe Disease (Glycogen Storage Disease Type II) (OMIM # 232300)	Infantile form: Cardiomegaly, hepatomegaly, hypotonia, severe muscle weakness, respiratory failure Late-onset form: Progressive muscle weakness
β-galactosidase (GH35, E.C. 3.2.1.23)	*GLB1*	Ganglioside GM1, Keratan sulfate (galactose β-1,4-saccharide)	GM1 Gangliosidosis (OMIM # 230500 (Type I), # 230600 (Type II), # 230650 (Type II)) Morquio Disease Type B (Mucopolysaccharidosis type IVB, MPS 4B) (OMIM # 253010)	Type I (infantile): Pyschomotor deterioration, central nervous system complication, hepatosplenomegaly, macular cherry-red spot, skeletal dysplasia, facial dysmorphism Type II (late infantile/juvenile): Pyschomotor deterioration, seizure, localized skeletal problem Type III (adult): Localized skeletal and central nervous system involvement Morquio Disease Type B: Bone abnormality, corneal clouding, hepatomegaly
Galactocerebrosidase (GH59, E.C. 3.2.1.46)	*GALC*	Galactosylceramide (galactose β-1-ceramide)	Krabbe Disease (OMIM # 245200)	Mental and motor degeneration, extreme irritability, spasticity
α-galactosidase A (GH27, E.C. 3.2.1.22)	*GLA*	Globotriaosylceramide, galabiosylceramide (galactose α-1,4-saccharide)	Fabry Disease (OMIM # 301500)	Extreme pain and paresthesia, renal failure, hypohydrosis, corneal opacity, angiokeratoma
β-mannosidase (GH2, E.C. 3.2.1.25)	*MANBA*	Oligosaccharides (mannose β-1,4-GlcNAc)	β-mannosidosis (OMIM # 248510)	Developmental delay, mental retardation, hearing loss, angiokeratoma
Lysosomal α-mannosidase (GH38, E.C. 3.2.1.24)	*MAN2B1*	Oligosaccharides (mannose α-1-2, -1,3, and 1,6-mannose)	α-mannosidosis (OMIM # 248500)	Facial dysmorphism, psychomotor impairment, hearing loss

Table 1. (Continued)

Enzyme	Gene	Major Substrates (Glycosidic Linkage)	Associated Diseases	Clinical Features
β-glucuronidase (GH2, E.C. 3.2.1.31)	*GUSB*	Heparan sulfate, Chondroitin sulfate (glucuronic acid β-1,4-saccharide)	Sly Syndrome (Mucopolysaccharidosis Type VII, MPS 7) (OMIM # 253220)	Hepatomegaly, skeletal abnormalities, facial dysmorphism, mental impairment, hydrops fetalis (in severely affected patients)
α-L-iduronidase (GH39, E.C. 3.2.1.76)	*IDUA*	Dermatan sulfate, heparin sulfate (iduronic acid α-1,4-saccharide)	Hurler Syndrome (Mucopolysaccharidosis Type I-H, MPS 1H) (OMIM # 607014) Scheie Syndrome (MPS 1S) (OMIM # 607016) Hurler-Scheie Syndrome (MPS 1H/S) (OMIM # 607015)	Hurler: Facial dysmorphism, corneal clouding, mental retardation, hernias, hepatosplenomegaly, dysostosis multiplex Scheie: Milder phenotype than Hurler Hurler-Scheie: Intermediate in phenotype
α-L-fucosidase (GH29, E.C. 3.2.1.51)	*FUCA1*	Protein N-linked glycan (fucose α-1,2-galactose, fucose α-1,3-, 1,4-, or 1,6-GlcNAc)	Fucosidosis (OMIM # 230000)	Angiokeratoma, facial dysmorphism, progressive psychomotor degeneration, dysostosis multiplex
Hexosaminidase A/B (heterodimer) (GH 20, E.C. 3.2.1.52)	*HEXA* *HEXB*	Ganglioside GM2, oligosaccharides	Tay-Sachs Disease (HEXA deficiency, GM2 Gangliosidosis Type I) (OMIM # 272800) Sandhoff Disease (HEXB deficiency, GM2 Gangliosidosis Type II) (OMIM # 268800)	Tay-Sachs: Mental retardation, paralysis, dementia, blindness, macular cherry-red spot Sandhoff: Clinically undistinguishable from Tay-Sachs Disease
α-galactosaminidase/ α-galactosidase B (GH27, E.C. 3.2.1.49 / 22)	*NAGA*	Mucin glycoprotein from human blood group A and AB (GalNac α-1,3-glycoprotein)	Schindler Disease (OMIM # 609241 (Type I & III), # 609242 (Type II, Kanzaki Disease)	Type I (infantile form): Neuroaxonal dystrophy, vision loss, hearing loss, seisure Type II (adult form): Mild cognitive impairment, hearing loss, angiokeratoma, Type III: Intermediate between Type I and II
α-glucosaminidase (GH89, E.C. 3.2.1.50)	*NAGLU*	Heparan sulfate (GlcNAC α-1,4-saccharide)	Sanfillipo Disease Type B (Mucopolysaccharidosis type IIIB, MPS 3B)	Progressive neurodegeneration, behavioral problems
Neuraminidase (GH 33, E.C. 3.2.1.18)	*NEU1*	Glycopeptide, oligosaccharide, glycoproteins (sialic acid α-2,3-, 2,6-, or 2,8-glycoconjugates)	Sialidosis (Mucolipidosi Type I, ML I) (OMIM # 256550)	Neurologic abnormalities, facial dysmorphism, spinal deformity, macular cherry-red spot

Pompe disease is another relatively common LSD caused by deficiency of a lysosomal glycosidase, in this case the acid α-glucosidase (GAA).[26] Affected patients are characterized by abnormal lysosomal accumulation of undegraded glycogen in muscle and bone cells, leading to progressive muscle weakness, respiratory failure, and in the severe cases death in early childhood (**Table 1**).[26] GM1 gangliosidosis is an LSD in the subgroup of (glyco)sphingolipidosis, in which the primary accumulating substrate is the (glyco)sphingolipid GM1 ganglioside. It is caused by deficiency in the lysosomal β-galactosidase (GLB1).[27] The lysosomal accumulation of GM1 ganglioside affects both the central and peripheral nervous system, resulting in neurodegeneration in affected patients (**Table 1**).[28] Hurler syndrome (Mucopolysaccharidosis Type I, MPS 1)[29] and Sly syndrome (MPS 7)[30] are mucopolysaccharidoses, in which enzyme deficiency (α- L-iduronidase and β-glucuronidase, respectively) causes accumulation of glycosaminoglycans (GAGs, or mucopolysaccharides). Patients typically exhibit abnormality in bone and connective tissues, as well as in the brain (**Table 1**), due to impaired GAG metabolism. Finally, the oligosaccharidoses α- and β-mannosidosis are caused by deficiency in lysosomal α- or β-mannosidases.[31, 32] Both diseases are characterized by the accumulation of undegraded oligosaccharides deriving from N-linked glycoproteins and lipid carriers of the N-linked glycan, which manifests in clinical features such as mental retardation and facial dysmorphism (**Table 1**).

Enzyme replacement therapy The realization that a single enzyme deficiency underlies specific LSD has prompted the design of therapeutic approaches that aim to either replace the defective enzyme with a normal one, to stabilize and activate the residual mutant enzyme, or to reduce the accumulating substrates for reducing their cytotoxic effects (**Fig. 2**).[12] Gaucher disease, in particular the non-neuropathic type 1 variant, has been the frontrunner in development of therapy approaches. Building on the finding that bone marrow transplantation of GD patients, replacing the white blood cell lineage with hematopoietic stem cells capable of producing GBA competent macrophages, renders major clinical improvements, correction of macrophages in type 1 GD patients by enzyme supplementation, so-called enzyme-replacement therapy (ERT) has been actively studied.[33] First conceived by Brady and co-workers in 1960s,[34] ERT with macrophage-targeted GBA results in remarkable reduction of hepatosplenomegaly, anemia, and thrombocytopenia in Gaucher patients.[35, 36] The originally approved therapeutic enzyme was isolated from human placenta, and its complex-type N-glycans were chemically modified into terminal high-mannose N-glycans,[35] resulting in efficient uptake by (affected) macrophages

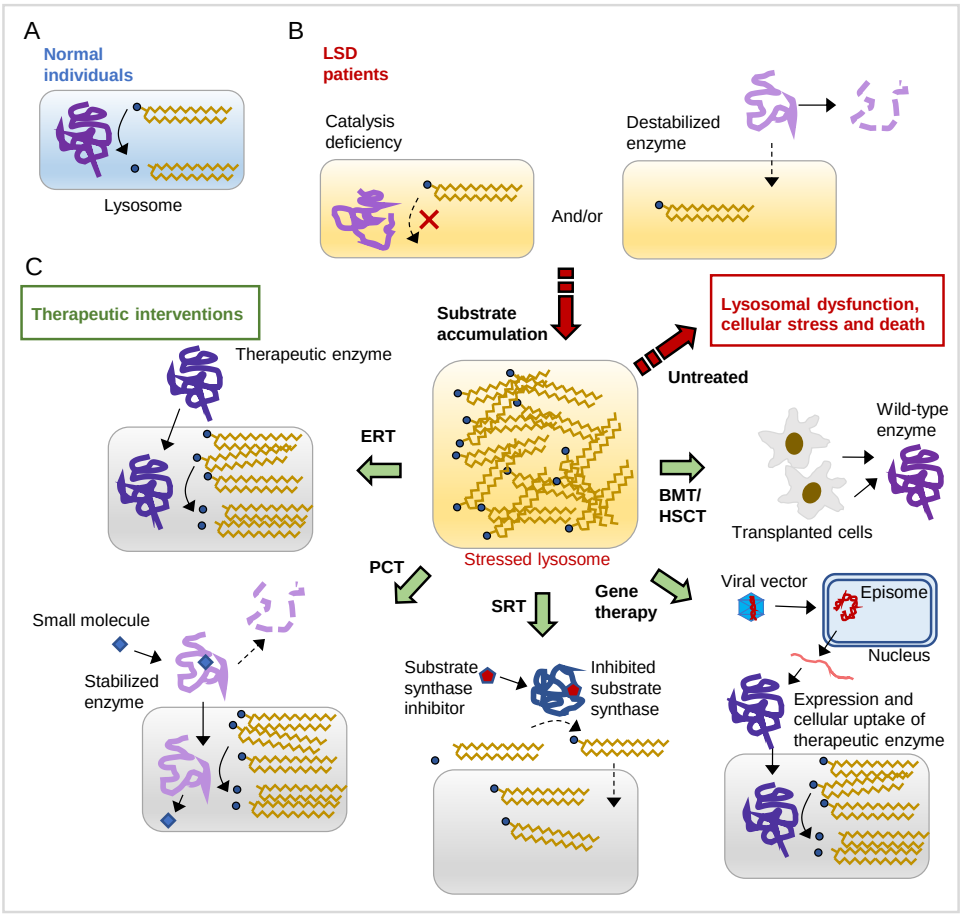

Figure 2. Lysosomal storage diseases and current therapeutic strategies. A) Substrate hydrolysis by glycosidases in normal individuals. B) Enzyme defects in lysosomal storage disease (LSD) patients lead to lysosomal dysfunction. C) Therapeutic strategies for LSDs. *ERT*, enzyme replacement therapy; *PCT*, pharmacological chaperon therapy; *SRT*, substrate reduction therapy; *BMT*, bone-marrow transplantation; *HSCT*, hematopoietic stem cell transplantation.

expressing the mannose receptors. Later therapeutic enzymes were produced in Chinese hamster ovary (CHO) cells (Imiglucerase, Cerezyme®). Now several therapeutic glucocerebrosidase preparations, produced in various cell types including carrot cells, are registered for therapeutic use in type 1 GD patients.[37, 38] The success of ERT for type 1 Gaucher disease, aided with the passing of Orphan Drug Act in the US in 1983, prompted the development of ERT for other diseases by the pharmacological industry. Approved by the Food and Drug Administration (FDA) in the USA or similar agencies in other parts of the world are therapeutic enzymes for Pompe disease (Aglucosidase alfa, Myozyme®/Lumizyme®), Fabry disease (Agalsidase alfa, Fabrazyme®

and Agalsidase beta, Replagal®), Hurler syndrome (Laronidase, Aldurazyme®),[32] and recently Sly syndrome (Vestronidase alfa, Mepsevii®)[39] and α-mannosidosis (Velmanase alfa, Lamzede®).[40]

Other therapeutic options A caveat of presently registered therapeutic enzymes is their poor penetration to certain types of tissue (including bone, muscle), but most particular the brain. At present, bone marrow transplantation or hematopoietic stem cells transplantation is investigated for Gaucher disease[41] and remain the only viable therapeutic options for some LSDs affecting the central nervous system, such as Krabbe disease and GM1 gangliosidosis.[27, 42, 43] Two other major therapeutic strategies for LSDs based on small molecules are now available on the market, namely pharmacological chaperon therapy (PCT) and substrate reduction therapy (SRT). The former makes use of small chemicals that interact with enzymes with folding defects, which stabilize the enzyme in during its maturation and promote their correct transportation to the lysosomes.[44] Such therapy is currently only available for Fabry disease,[45] while other types of chaperone therapy, such as induction of heat-shock proteins to stabilize glycosidase folding and function, are also under development.[46] For SRT, the aim is not to correct for the defective enzyme, but to reduce the amount of accumulating substrates by inhibiting the enzymes accounting for their synthesis.[47, 48] To date, Gaucher disease is the only LSD with lysosomal glycosidase deficiency with approved SRT options based on orally administered inhibitors of glucosylceramide synthase: the iminosugar Miglustat (N-butyl-deoxynojirimycin)[49] and the more potent and specific ceramide mimic Eliglustat.[50] Another actively pursued therapeutic approach for LSDs is gene therapy. Studied are several approaches, in modifying patients' hematopoietic stem cells *ex vivo* with lentiviral vectors encoding wild-type enzymes, or injecting the patient with engineered adeno-associated viral (AAV) vectors encoding the enzyme.[51, 52] The promotor and the site of injection for the later approach can be tailored according to the disease and affected tissue types, and the treatment would only require one-time injection—an obvious advantage compared to the life-long treatment regimen for ERT, PCT, and SRT. Safety remains the major concern, which has translated to strict manufacturing regulation and thus high pricing—the current major hurdle for developing such therapy.

Diagnosis Traditionally, the diagnosis of an LSD is made based on clinical symptoms, and later confirmed by genetic and biochemical analyses. However, due to the rarity and associated poor awareness of LSDs, as well as their considerable heterogeneity in clinical presentation, in most countries their correct diagnosis remains challenging.[22, 53] This prompts new-born screening

(NBS) programs in many parts of the world, aiming to detect patients before they show the first symptoms. Early treatment of many LSDs is increasingly considered to be essential to achieve maximum therapeutic outcome. The first-line screening sometimes builds on genetic testing. However, many LSDs show poor genotype-phenotype correlations, and in addition it remains challenging to interpret novel (mild) mutations as being a polymorphism or disease-causing trait.[54] Detection of biomarkers (surrogate biochemical markers of disease manifestation and/or progression) assist diagnosis, but for many LSD these are unavailable.[15, 55] In the case of LSDs caused by enzyme deficiencies, biochemical activity assays are viewed as the gold standard. In many cases these indeed allow reliable confirmation of diagnosis. Traditionally, enzyme activity assays involve measurement of residual activity in cultured patients' fibroblasts or collected white blood cells using artificial fluorogenic substrates, such as 4-methylumbelliferyl (4-MU) glycosides. Such assays may suffer from low dynamic range,[56] cross activity from other enzymes, and inability to multiplex with other enzyme assays. More recently, microfluidic-based 4-MU assay platform[57, 58] and an MS-based multiplex method using deuterium natural substrates[59, 60] have been developed and implemented in several NBS programs in the USA and other parts of the world, using dried-blood spots collected from patients as enzyme source. A major obstacle is the need for specialized laboratories employing validated measurements, still unavailable in most parts of the world. Furthermore, each method has its own limitations, as the microfluidic method does not solve the low-dynamic range issue intrinsic to the 4-MU assay, and the MS-based method requires laborious procedures and considerable technical know-how.[54]

Part II. Lysosomal glycosidases: from molecular understanding to novel chemical tools

Fundamental research in the last decades on lysosomal enzymes, including glycosidases, has revealed some commonalities among these enzymes. The hydrolases show optimal activity at acid pH, coinciding with the low lysosomal pH value. In general, lysosomal glycosidases are relatively resistant against proteolytic degradation and survive for more than one day in the lytic lysosome environment. For many of the lysosomal glycosidase the 3D-structure has been determined by means of crystallography and X-ray diffraction.

Life cycle of lysosomal glycosidases It is now well-established that lysosomal glycosidases are formed at the endoplasmic reticulum and are subsequently are routed to the lysosomes via the mannose-6-phosphate (M6P)-dependent pathway. Nascent lysosomal glycosidases are upon translation firstly targeted into the ER via their N-terminal signal peptide

(typically 20-25 amino acids), which is cleaved from the nascent preproproteins by the ER-resident signal peptidase.[61] The resulting proproteins are glycosylated at selective Asn residues (Asn-X-Ser/Thr, where X cannot be Pro or Asp) with the pre-synthesized $Glc_3Man_9GlcNac_2$ glycans transferred *en bloc* from the dolichol carrier (**Fig. 3**, step 1).[62] N-glycans prove to be crucial for the correct folding of many glycoproteins in the ER. Only after successful folding, successive trimming of terminal glucoses and of one terminal mannose in N-glycans takes place (by processing α-glucosidase I (MOGS), II (GANAB) and ER α-mannosidase (MAN1B1)). Next, the N-glycoproteins are transported to the Golgi apparatus for further modification and sorting (**Fig. 3**, step 2−4).[63] In the Golgi, the terminal α-1,2-linked mannoses in the N-glycans of lysosomal proteins are cleaved by the processing mannosidases (**Fig. 3**, step 5). This enables the terminal α-1,6-linked mannose to be modified at the 6 position with a N-acetylglucosamine (GlcNAc)-1-phosphate group, catalyzed by GlcNAc-1-phospotransferase (**Fig. 3**, step 6).[64] Next, the GlcNAc residue on the GlcNAc-1-phosphate group is released at the *trans*-Golgi network (TGN) by GlcNAc phosphodiesterase (i.e. uncovering enzyme), exposing the mannose-6-phosphate (M6P) group.[65] Two dedicated M6P receptor proteins (MPR300 in particular and MPR47 to far lesser extent) recognize the M6P groups presented on the glycosidases, and shuttle them via clathrin-coated vesicles to the early or late endosomes (**Fig. 3**, step 7−9).[61]

Some lysosomal glycosidases are co-transported to the lysosomes in a protein complex, such as the β-galactosidase (GLB1)-neuraminidase (Neu1)-cathepsin A complex.[66] Glucocerebrosidase (GBA) does not acquire M6P moieties in its glycans and contrary to other lysosomal glycosidases uses an M6P-independent lysosomal targeting pathway involving its hydrophobic association with the lysosomal integral protein 2 (LIMP2) (**Fig. 3**, step a−b).[67, 68, 69] As the early/late endosomes mature into lysosomes, gradual acidification of the lumen from pH 6.0 to below 5.0 facilitates (partial) dissociation of GBA from LIMP2 and dissociation of other lysosomal glycosidases from the MPRs (in particular MPR300) (**Fig. 3**, step c). LIMP2 stays in lysosomes, while MPRs are recycled back to the TGN via retrograde transport (**Fig. 3**, step 10).[61] An estimated 10 % of the total cellular MPRs reside at the plasma membrane, where they function in "fishing back" the secreted lysosomal glycosidases (both from the same cell or from other cells) to the lysosomes via endocytosis (**Fig. 3**, step 11−13).[61] In the lysosome, most of the glycosidases (e.g. α-galactosidase, α- L-fucosidase, α-glucosidase, β-glucuronidase, α- L-iduronidase, α-mannosidase, β-galactosidase, and galactocerebrosidase) undergo proteolytic

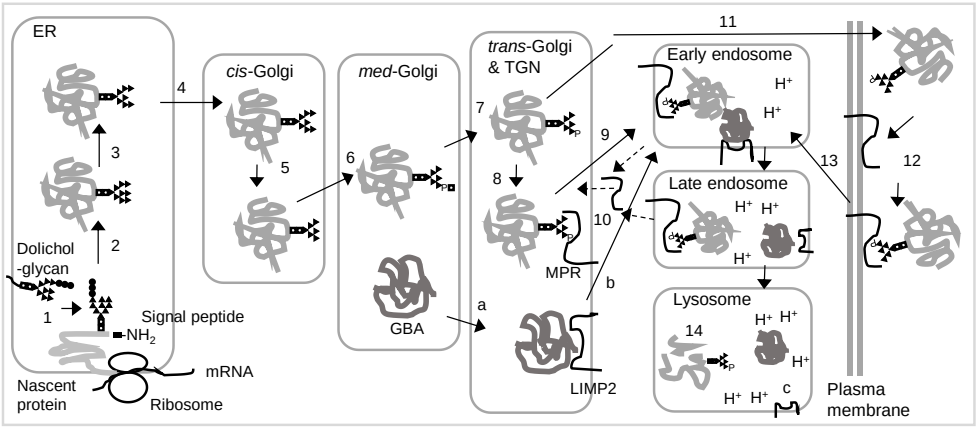

Figure 3. Lysosomal targeting of glycosidases. *TGN*, trans-Golgi network. *MPR*, mannose-6-phosphate receptor. *GBA*, glucocerebrosidase. *LIMP2*, lysosomal integral protein 2. *P*, phosphate group. Dashed lines, retrograde transport of MPR from early and late endosomes to TGN. Glycosylation on GBA is omitted for clarity.

cleavage (**Fig. 3**, step 14), producing the final mature enzymes.[70]

Catalytic mechanisms It has been observed in the first half of 20th century that most glycosidases can be categorized into two groups according to the stereochemistry outcome of their hydrolysis—either into inverting glycosidases or retaining glycosidases. These groups utilize distinct catalytic mechanisms, as firstly described by Daniel E. Koshland Jr. in 1953.[71] Inverting glycosidases typically possess a catalytic pocket 10.5 Å in width, which allow the accommodation of the glycoside substrate plus a water molecule.[72] When correctly orientated, the catalytic base deprotonate the water molecule, which attacks the electron-poor anomeric carbon by the assistance of the catalytic acid that attracts electrons from the glycosidic oxygen, distorting the glycosidic bond and disrupt the exo-anomeric effect that stabilizes the glycosidic linkage (**Fig. 4A**).[73] The reaction proceeds through a transient oxocarbenium-like transition state adopted by the substrate glycon (the sugar part), and completes in one-step where the hydroxyl replaces the aglycon (the rest of the reducing-end structures linked to the anomeric carbon) situated on the opposing side of the symmetric plane in a typical S_N2 mechanism, resulting a product with the inversion of stereochemistry (**Fig. 4A**). For retaining glycosidases, which include most lysosomal glycosidases, the S_N2 mechanism occurs twice (Koshland double-displacement mechanism), resulting in the net retention of stereochemistry on the product. The catalytic pocket is narrower (typically 5.5 Å apart), and the reaction occurs with protonation of the glycosidic oxygen by the

catalytic acid/base (typically aspartic acid/aspartate or glutamic acid/glutamate) and simultaneous nucleophilic attacks at the anomeric carbon by the catalytic nucleophile (aspartate or glutamate). This results in a transient oxocarbenium transition state, followed by a covalent substrate-enzyme intermediate (**Fig. 4B**).[72] Next, the catalytic acid/base deprotonates a water molecule, which displaces the nucleophile in a similar reaction sequence adopted by the inverting glycosidases, producing a product with retained stereochemistry (**Fig. 4B**).

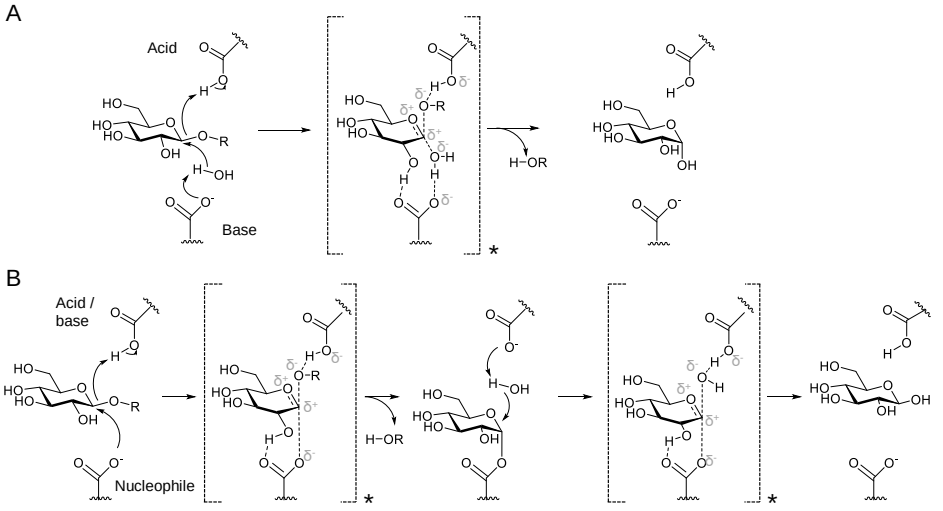

Figure 4. Reaction mechanism of exo-glycosidases towards a β-glucoside. A) Inverting mechanism. B) Retaining double-displacement mechanism. *, transition state. *R*, aglycon.

It is also known that glycosidases distort their sugar substrates during the course of their catalysis, and typical sets of conformational changes on the substrate's glycon during the reaction itinerary can be assigned for each glycosidase class (CAZy classification, discussed later).[74, 75] For example, the glucose on a β-glucoside, such as the β-glucose presented on a glucosylceramide, initially adopts a lowest-energy $^{1}C_{4}$ conformation, but as it accommodates in the active-site pocket of a retaining β-glucosidase, such as glucocerebrosidase, it is distorted to a skew-boat conformation ($^{1}S_{3}$) that facilitates both protonation by the acid/base and the nucleophilic attack by the nucleophile (**Fig. 5**). The glycon is further distorted to a half-chair conformation ($^{4}H_{3}$) during the transient transition state, and upon the formation of the new glycosidic bond with the nucleophile the glycon adopts a $^{4}C_{1}$ chair conformation, becoming an α-glucose (**Fig. 5**).[74, 75] The second half of the reaction distorts the glycon conformation in a reverse order, producing the product with identical conformation to the substrate (**Fig. 5**).

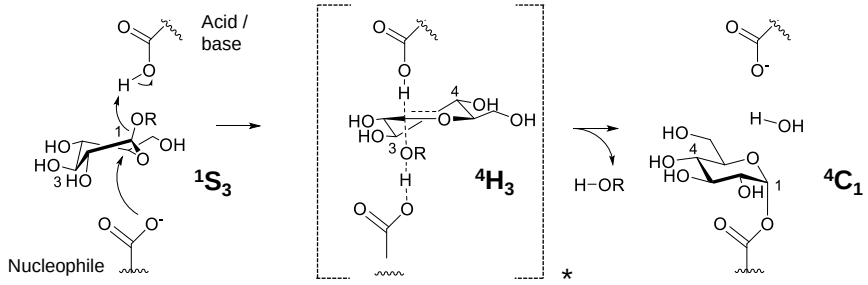

Figure 5. Distortion of substrate conformation by retaining β-glucosidase. Bold, conformation adopted by the glycon. *, transition state. *R*, aglycon.

Glycosidase can be additionally classified based on the site of hydrolysis in the substrate. Exo-glycosidase releases terminal monosaccharide from the reducing end of the substrate. Their catalytic sites is typically pocket-shaped, allowing interactions with only monosaccharide at the reducing end.[76] In contrast, endo-glycosidase have a wider cleft- or tunnel-shaped catalytic site, which can accommodate multiple sugar residues at the reducing end of the to-be-hydrolyzed glycosidic bond.[76]

Sequence-based classification In order to study glycosidases in a systematic approach, a framework named Carbohydrate-Active Enzyme (CAZy) database has been setup, which groups glycosidases into over 100 Glycoside Hydrolase (GH) families.[77] This classification system is based on amino acid sequence homology, instead of grouping glycosidases solely by their catalytic activity (the Enzyme Commission (E.C.) system). Using this system, it turns out that glycosidases classified into the same GH family usually possess similar structural features and reaction mechanisms. This in turn proves to be useful in predicting catalytic activities by proteins with known sequence, as well as the activity of a known active-site-interacting molecules towards enzymes in the same GH family. The second implication has greatly facilitated the design of glycosidase active-site interactors as well as inhibitors, and has assisted the development of activity-based probes (ABPs), as discussed in the next section.

Mechanism-based profiling Enzymes with known reaction mechanism invite the design of chemical probes that would react covalently with the enzyme's active site catalytic amino acids, allowing the detection of the enzymes through reporter moiety grafted on the probe. If the reaction depends on enzymatic activity rather than affinity alone, the probe is termed activity-

based probe (ABP). Crucial for an ABP is its ability to form covalent linkage to the target enzyme, for a period long enough to allow their detection. Key structural elements include a reactive "warhead"—typically an electrophile, a recognition element that confers specificity towards the target enzyme(s), and a reporter group for detection (or a chemical handle for subsequent installation of reporter group) (**Fig. 6A**).[78]

Known covalent inhibitors of GBA have been successfully used as scaffolds to design the desired ABPs for the enzyme. The suicide inhibitor conduritol B epoxide (CBE, **Fig. 6B**) irreversibly inactivates β-glucosidases and (with lower potency) other glycosidases in a mechanism-based manner.[79, 80] It has been exploited for the generation of a Gaucher-like mouse model,[81] and for the identification of active sites of both retaining β- and α-glucosidases.[80, 82] Cyclophellitol, a later discovered natural suicide inhibitor[83] and now synthetically available[84, 85], also reacts with β-glucosidases in a mechanism-based manner and with higher potency (**Fig. 6C**).[86, 87] Even superior potency is observed by the synthetic compound cyclophellitol aziridine, in which the epoxide on cyclophellitol is replaced by an aziridine group (**Fig. 6B**).[88]

Based on the cyclophellitol scaffold, the first true ABP for lysosomal glycosidase has been generated.[89] It was synthesized by installing an azide group at the methoxy carbon (C6 by glucopyranoside numbering; C8 by cyclophellitol numbering (**Fig. 6B**)) and the subsequent Cu(I)-catalyzed "click" reaction with a BODIPY-alkyne, which generates a BODIPY-substituted cyclophellitol (**Fig. 6D**, ABP **1**).[89] The ABPs (two BODIPY variants) label glucocerebrosidase (GBA) in a mechanism-based manner and with high potency and specificity, allowing detection of endogenous glucocerebrosidase by SDS-PAGE, fluorescence-activated cell sorting (FACS), and fluorescence microscopy, and were demonstrated to be useful in the study of GBA life cycle, inhibitor screening (for potential PCT), and diagnosis of Gaucher disease.[89] Later, a suite of cyclophellitol and cyclophellitol aziridine-based ABPs containing fluorophores and biotin have been developed for retaining β-glucosidases (GBA, non-lysosomal glucosylceramidase (GBA2), cytosolic β-glucosidase (GBA3), and lactase phlorizin hydrolase (LPH)) (**Fig. 6D**, ABP **2**, **3**),[90] GH27 α-galactosidases,[91] GH29 α- L-fucosidases,[92] and galactocerebrosidase (GALC).[93] The designing principle relies on tuning the cyclophellitol scaffold to match the configuration of the substrates from the target glycosidase, and this approach indeed has proved to be successful in the specific detection of enzymes in particular GH family.[94] Structural-activity-relationship (SAR)

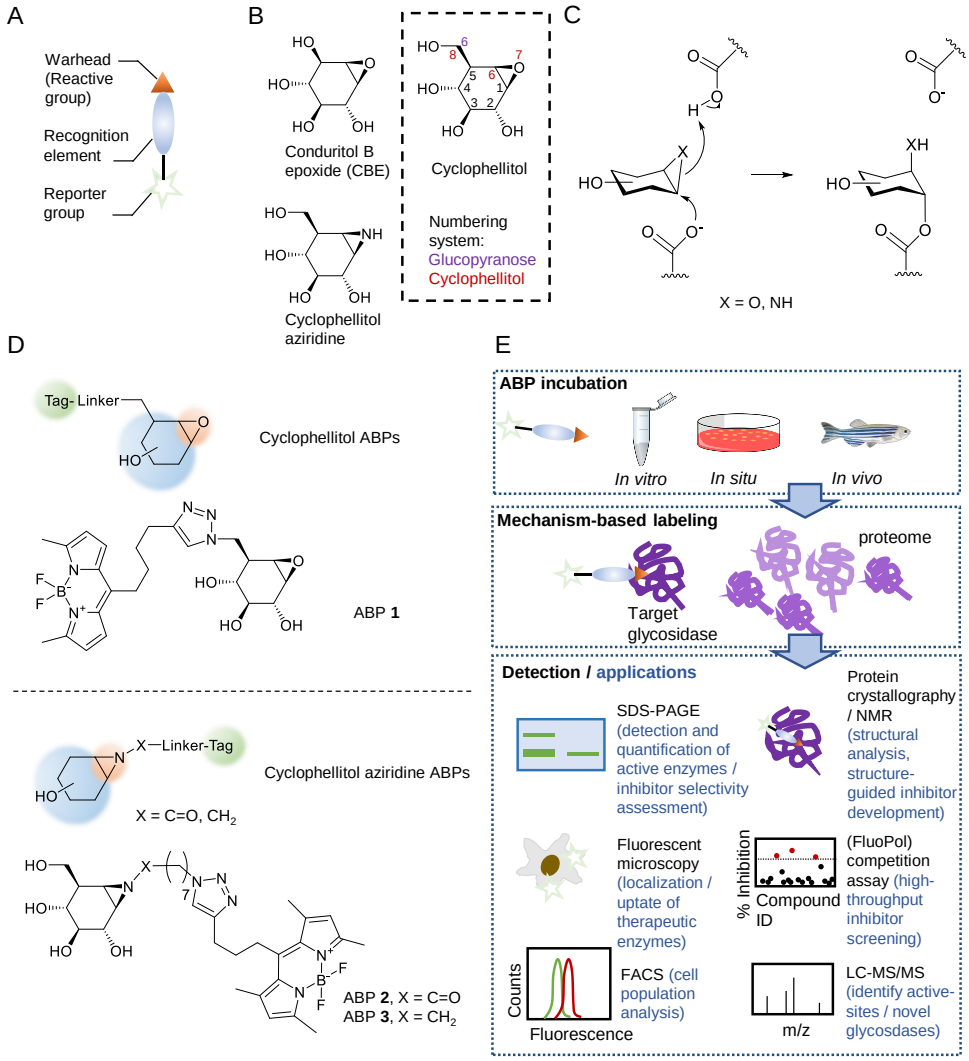

Figure 6. Mechanism-based activity profiling for retaining exo-glycosidases. A) Structural elements of activity-based probe (ABP). B) Structures of selected irreversible glycosidase inhibitors. C) Mechanism-based inactivation by cyclophellitol- and cyclophellitol aziridine-based structures. D) Structures of glycosidase activity-based probes (ABPs) based on cyclophellitol- (upper) and cyclophellitol aziridine (lower) scaffolds. ABP **1**, epoxide ABP for glucocerebrosidase[89]; ABP **2** and **3**, aziridine ABPs for β-glucosidases[90, 96]. Color scheme same as in A). E) Workflow for activity-based protein profiling (ABPP).

studies has confirmed this observation, as deleting the C2- or C4-hydroxyls on the aziridine ABP render the probes unselective towards specific glycosidase family.[95] SAR study also revealed that ABPs with the N-alkyl linker is equally potent than their N-acyl counterparts, while being more

synthetically tractable and more stable during their synthesis and purification[96] Meanwhile, this designing strategy has been extended for other (lysosomal) retaining glycosidases, for example GH31 α-glucosidases (Chapter 4, this thesis),[97] GH2 β-glucuronidases (Chapter 4, this thesis),[98] GH39 α-L-iduronidases (Chapter 5, this thesis),[99] GH38 α-mannosidases (Chapter 6, this thesis), GH2 β-mannosidases (Chapter 6, this thesis), and GH59 and GH35 β-galactosidases (Chapter 7, this thesis).

Applications of glycosidase activity-based probes Developed applications for the mechanism-based glycosidase inhibitors and ABPs include specific visualization of endogenous glycosidases in complex samples such as cell lysates and tissue homogenates based on their distinct pH range and molecular weight using SDS-PAGE and fluorescence scanning,[100] in intact cells using fluorescent microscopy[100] and fluorescence-activated cell sorting (FACS),[89] and in whole tissue sections by fluorescent microscopy (**Fig. 6E**).[93, 101, 102] These methods should prove to be useful in diagnostic conformation of LSDs, in investigations on outcome of lysosomal glycosidase mutations, and in the present development of LSD therapies such as PCT and gene therapy. Therapeutic enzymes for LSDs can also be pre-labeled by the ABPs, which allows their detection by these mentioned methods[99, 103]—crucial in monitoring ERT efficacy in pre-clinical and clinical settings. The ABPs can also be used to assess potency and specificity of known glycosidase inhibitors in intact cells or animals (Chapter 2, this thesis)[104] or in an *in vitro* high-throughput setup such as the fluorescence polarization (FluoPol) assay for discovering potential glycosidase interactors (inhibitors/stabilizers/activators) for PCT (**Fig. 6E**).[105] The covalent nature of labeling also allow structural analysis of (mutant) glycosidase (with or without ABP) by protein crystallography[93, 97-99] or NMR[106], which would offer structural insights crucial for the development of small-molecule chaperones for PCT (**Fig. 6E**). On the other hand, mechanism-based inhibitors, owing to their substrate specificity, can also be used to generate LSD cell and animal models (Chapter 3, this thesis).[107] Last but not least, ABPs appended with a biotin moiety would allow affinity-enrichment and identification of the labeled targets by LC-MS(/MS) based proteomics, which is useful in identification of glycosidase catalytic residues[108] or of unknown glycosidases (Chapter 7, this thesis) (**Fig. 6E**), with the later has already been exploited in plants.[109, 110] This thesis aims to illustrate a number of applications by the mechanism-based inhibitors and ABPs in the LSD field, as well as to present detailed biochemical characterization of novel ABPs targeting other (LSD-relevant) retaining exo-glycosidases (Chapter 4–7, this thesis).

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