Chemical biology of glucosylceramide metabolism fundamental studies and applications for Gaucher disease

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General Introduction

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The human body produces various classes of complex glycoconjugates with very diverse functions, including glycolipids, glycoproteins, glycosaminoglycans, mucopolysaccharides and glycogen (Figure 1). Like other macromolecules, complex glycoconjugates are subject to recycling. Through autophagy or endocytotic pathways, complex glycoconjugates ultimately end up in lysosomes, the perinuclear acid compartments of cells specialized in degradation of macromolecules[1,2]. Lysosomes equipped with a broad range of glycosidases efficiently fragment glycoconjugates to release individual monosaccharides. A set of specialized transporter proteins in the lysosomal membrane facilitates the export of the simple sugars to the cytosol where further metabolism proceeds[3,4]. The release of nutrients by lysosomes, in particular amino acids, is sensed by lysosome-associated mTORC1 and linked to TFEB-regulated expression of genes coding for proteins required for lysosome biogenesis and autophagy[5–7]. Genetic defects in the intralysosomal turnover of glycoconjugates lead to a number of inherited lysosomal storage diseases, collectively a significant proportion of the inborn errors of metabolism in man[8–10].

Proteins involved in lysosomal glycoconjugates turnover.

The turnover of glycoconjugates in lysosomes requires a machinery of specialized proteins[8–10]. Table 1 provides an overview of the presently known lysosomal glycosidases and monosaccharide transport proteins. A considerable number of inborn errors of metabolism are due to primary defects in one of these proteins (see Table 1). For example, one inherited metabolic disorder, Salla disease, is due to primary defects in the lysosomal membrane protein mediating export of sialic acid. Defects in the accessory GM2 activator protein cause GM2 gangliosidosis, a neurodegenerative disease. Mutations in the prosaposin gene, affecting one or several of its intralysosomally generated accessory proteins (saposins A, B, C and D) cause impairments in degradation of glycolipids and associated disease manifestations. The majority of the inborn errors in lysosomal glycoconjugate degradation are caused by genetic defects in specific lysosomal glycosidases. Deficiency of almost every known lysosomal glycosidase has meanwhile been linked to an inherited metabolic disease with characteristic clinical presentation. This observation illustrates the great substrate specificity of each of the glycosidases and the apparent absence of functional redundancy.

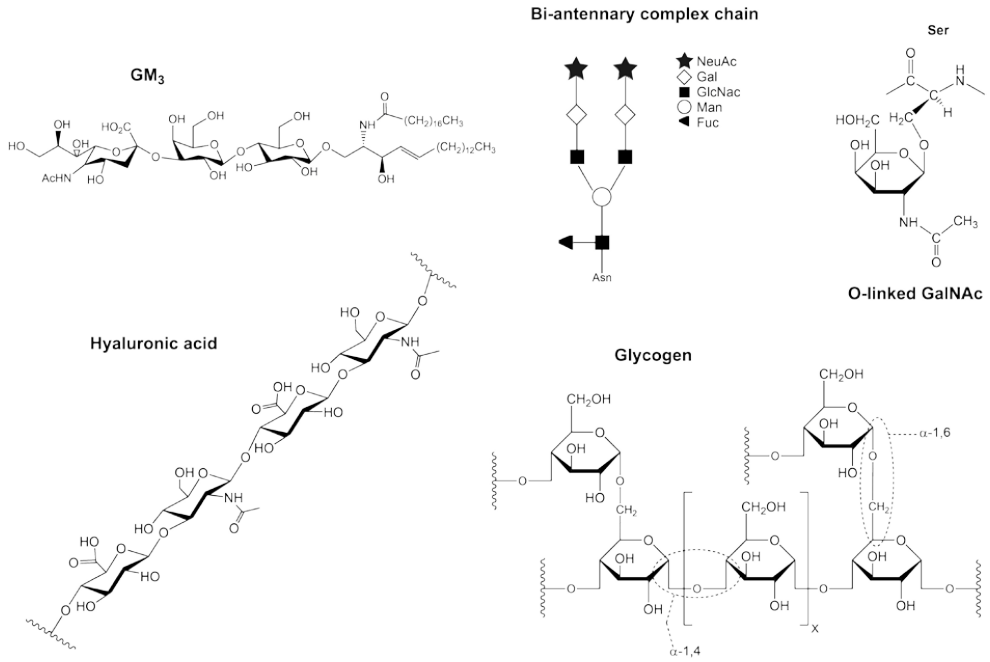


Figure 1. Examples of complex glycoconjugates in man. Upper row, left to right: glycolipid (example GM3), glycoprotein N-linked glycan (example bi-antennary complex chain), glycosaminoglycans (example O-linked saccharide). Lower row, left to right: mucopolysaccharides (example hyaluronic acid), glycogen (example branched structure with alpha-1,4 and alpha-1,6 linkages).

Classification of glycosidases.

Glycosidases (*a.k.a.* glycohydrolases), present in all forms of life, are often classified as either *retaining* or *inverting* enzymes according to the stereochemical outcome of the hydrolysis reaction[11]. Glycosidases are also grouped as *exo* or *endo* enzymes, dependent upon their ability to cleave at the, usually the non-reducing, end or in the middle of a saccharide chain. More recently sequence-based classifications of glycosidases have become popular[12]. The CAZy (CArbohydrate-Active EnZymes) classification (data base at CAZy Family Glycoside Hydrolase web site) distinguishes about 100 distinct (GH) protein families[13–15]. It offers predictions of retaining *versus* inverting mechanism, active site residues and possible substrates and it is supported by CAZypedia, an online encyclopedia of carbohydrate active enzymes. Based on three-dimensional structural similarities, sequence-based families have been classified into 'clans' of related structure and even an extended hierarchical classification of glycosidases has been proposed[16].

Table 1. Lysosomal proteins mediating glycoconjugate turnover and associated inherited diseases. Sources: OMIM, Expasy, Genetics home reference, Uniprot

Protein	Gene	Function	Substrate	Inherited disease
Acid β-Galactosidase	*GLB1*	Hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides	GM1 ganglioside	GM1 gangliosidosis
β-Hexosaminidase α subunit	*HEXA*	Hydrolysis of terminal non-reducing N-acetyl-D-hexosamine residues in N-acetyl-β-D-hexosaminides	GM2 ganglioside	GM2 gangliosidosis (Tay-Sachs)
β-Hexosaminidase β subunit	*HEXB*	Hydrolysis of terminal non-reducing N-acetyl-D-hexosamine residues in N-acetyl-β-D-hexosaminides	GM2 ganglioside	GM2 gangliosidosis (Sandhoff)
Sialidase/Neuraminidase	NEU1	Cleave terminal sialic acid residues	GM3 ganglioside	Sialidosis
α-Galactosidase A	*GLA*	Hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides	Globotriaosylceramide (Gb3)	Fabry
Glucocerebrosidase	*GBA*	Catalyzes the breakdown of the glycolipid glucosylceramide (GlcCer) to ceramide and glucose	Glucosylceramide (GlcCer)	Gaucher
Galactosylceramidase	*GALC*	Catalyzes the breakdown of the glycolipid galactosylceramide (GalCer) to ceramide and galactose	Galactosylceramide (GalCer)	Krabbe
Acid α-Glucosidase	GAA	Catalyzes the breakdown of glycogen into glucose	Glycogen	Glycogen Storage Disease type II (Pompe)
α-L-Iduronidase	IDUA	Catalyses the hydrolysis of unsulfated α-L-iduronosidic linkages in dermatan sulfate	Glycosaminoglycans/ mucopolysaccharides	Mucopolysaccharidosis MPS I (Hurler/Scheie)
α-Mannosidase	MAN2B1	Catabolism of N-linked carbohydrates released during glycoprotein turnover	Non-reducing α-D-mannose residues in α-D-mannosides	α-Mannosidosis
β-Mannosidase	MANBA	Cleaves the single β-linked mannose residue from the non-reducing end of all N-linked glycoprotein oligosaccharides	Terminal, non-reducing beta-D-mannose residues in β-D-mannosides	β-Mannosidosis

Catalytic mechanisms.

The catalytic reaction of inverting and retaining glycosidases is fundamentally different[17,18]. Inverting glycosidases utilize two catalytic amino acid residues, typically carboxylate residues, that act as acid and base respectively and no covalent glycosyl-enzyme intermediate is generated during the reaction (Figure 2). In contrast, retaining glycosidases utilize a two-step mechanism in which each step results in inversion, leading to net retention of stereochemistry. Again two residues are involved, usually carboxylates. One acts as nucleophile and the other as acid/base. In the first step the nucleophile attacks the anomeric center, resulting in the release of the aglycon and formation of a covalent glycosyl enzyme intermediate, assisted by the protonated carboxylate of the acid/base residue. Next, the now deprotonated carboxylate acts as a base and assists a nucleophilic water to hydrolyze the

glycosyl enzyme intermediate, releasing the saccharide (Figure 2)[19]. An exception is formed by classes of chitinases (families GH18 and GH20) where the enzymes employ so-called substrate-assisted catalysis (Figure 2). These retaining chitinases use a single glutamate as catalytic residue. This glutamate in their catalytic $(\beta\alpha)_8$-barrel domain acts in concert with the carbonyl oxygen atom of the substrate's C2 *N*-acetyl group that functions as the nucleophile[20]. This mechanism is used by the human chitinase CHIT1, named chitotriosidase[21–24], as well as by the homologous AMCase (acidic mammalian chitinase; CHIT2) arisen by gene duplication[25,26].

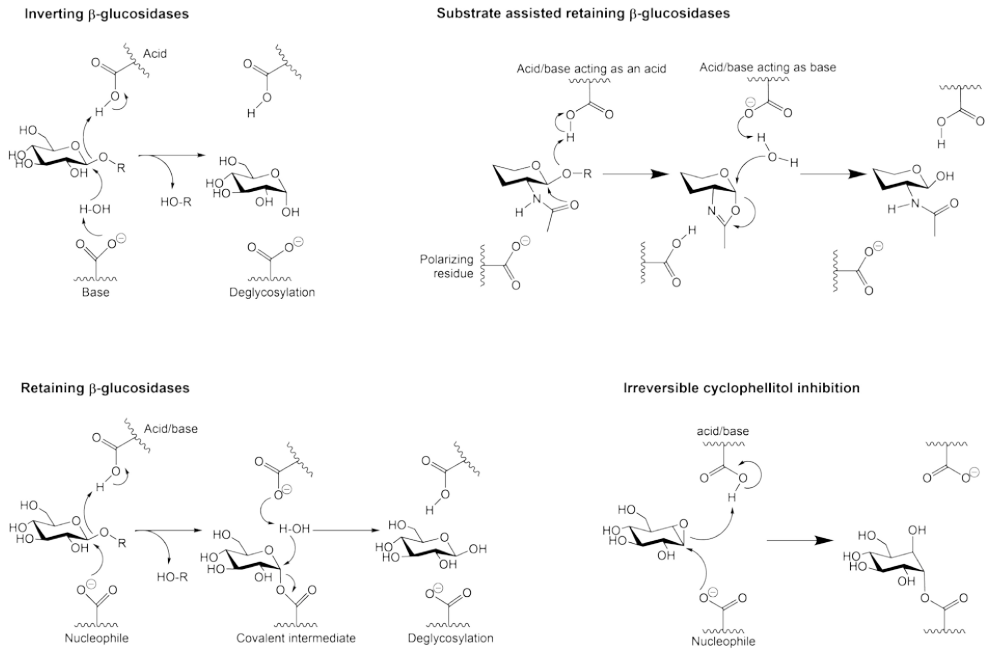


Figure 2. Catalytic mechanisms of glycosidases. Upper left: Inverting mechanism. Lower left: Retaining mechanism. Upper right: Substrate-assisted retaining mechanism. Lower right: Irreversible inhibition by cyclophellitol. Upper left, lower left and lower right figures adapted from WW Kallemeijn, upper right figure adapted from Gloster and Davies, 2009.

Glycosphingolipids: synthesis and lysosomal degradation.

Glycosphingolipids (GSLs), firstly identified by Johannes Thudichum at the end of the nineteenth century[27], are structural components of membranes. The outer leaflet of the plasma membrane bilayer is particularly rich in GSL. Their generic structure comprises a lipid moiety, being an N-acylated sphingosine named ceramide (Cer). Attached to the C1-hydroxyl of Cer is a monosaccharide (glucose or galactose) to which further sugars may be added[28]. Through van der Waals interactions GSLs form transient semi-ordered domains with

cholesterol molecules in membranes. Specific proteins preferentially reside in these so-called 'lipid rafts' and mediate signaling events there [29–31]. During their life cycle GSLs travel through various subcellular compartments (Figure 3). GSLs are synthesized at the endoplasmic reticulum (ER) as started by the enzyme serine palmitoyltransferase (SPT) generating a keto-sphinganine structure by the condensation of serine and fatty acyl-CoA, usually palmitoyl-CoA[32]. Next, keto-sphinganine is transformed to sphinganine by a reductase. A set of ceramide synthases (CerS 1-6)[33], each enzyme with acyl-CoA length preference, catalyze the N-acylation of sphinganine. Generated dihydroceramides are rapidly converted to ceramides by the action of dihydroceramide desaturase (DES)[34,35]. Subsequently, part of the newly formed Cer gets galactosylated inside the ER to galactosylceramide (GalCer), a reaction catalyzed by galactosylceramide synthase using UDP-galactose as sugar donor[36]. Alternatively, Cer molecules are transported by the protein CERT to the cytosolic leaflet of membranes of the cis-Golgi apparatus[37]. There, the enzyme glucosylceramide synthase (GCS) transfers glucose from UDP-glucose to Cer, generating β-glucosylceramide (glucocerebroside; GlcCer)[38]. Part of the formed GlcCer is immediately translocated to the luminal leaflet of the Golgi membrane via an unknown mechanism[39]. Inside the Golgi apparatus, GlcCer is next modified by stepwise addition of further sugars catalyzed by glycosyltransferases, yielding a broad spectrum of complex GSLs such as gangliosides and globosides[28,40]. Sulfation of specific lipids by sulfotransferases may also take place, adding to the structural diversity of GSLs[40,41]. After the modifications in the Golgi apparatus, GSLs reach the outer leaflet of the plasma membrane via membrane flow to play their various roles in interactions with the outside world.

Export of GSLs from cells may occur via nascent HDL particles, however most GSLs molecules remain in the plasma membrane. Ultimately, GSLs are internalized via endocytosis, ending up in multi-vesicular bodies within late endosomes destined for degradation inside lysosomes. Likewise, exogenous GSLs, for example components of endocytosed lipoproteins or phagocytosed cell debris and senescent cells, undergo lysosomal degradation. This is a coordinated process in which terminal sugar moieties are removed from GSLs in a stepwise manner by sequential action of glycosidases, assisted by specific accessory proteins (GM2 activator protein and saposins A-D)[42]. The degradation pathways of the most simple ganglioside GM3 and globoside Gb3 are depicted in Figure 4. The sphingolipid Cer is ultimately formed, either from GalCer by galactocerebrosidase (GALC) or GlcCer by glucocerebrosidase (GBA). Lysosomal acid ceramidase (AC) splits ceramide to free fatty acid and sphingosine that are subsequently exported. In the cytosol sphingosine can be re-used by CerS enzymes in the so-called salvage pathway to generate ceramide molecules[43]. Alternatively, sphingosine can be modified to sphingosine-1-phosphate (S1P) via sphingosine kinases (SK1 and SK2), whereafter S1P lysase (SPL) degrades it to phosphatidylethanolamine and 2-trans-hexadecenal[44,45].

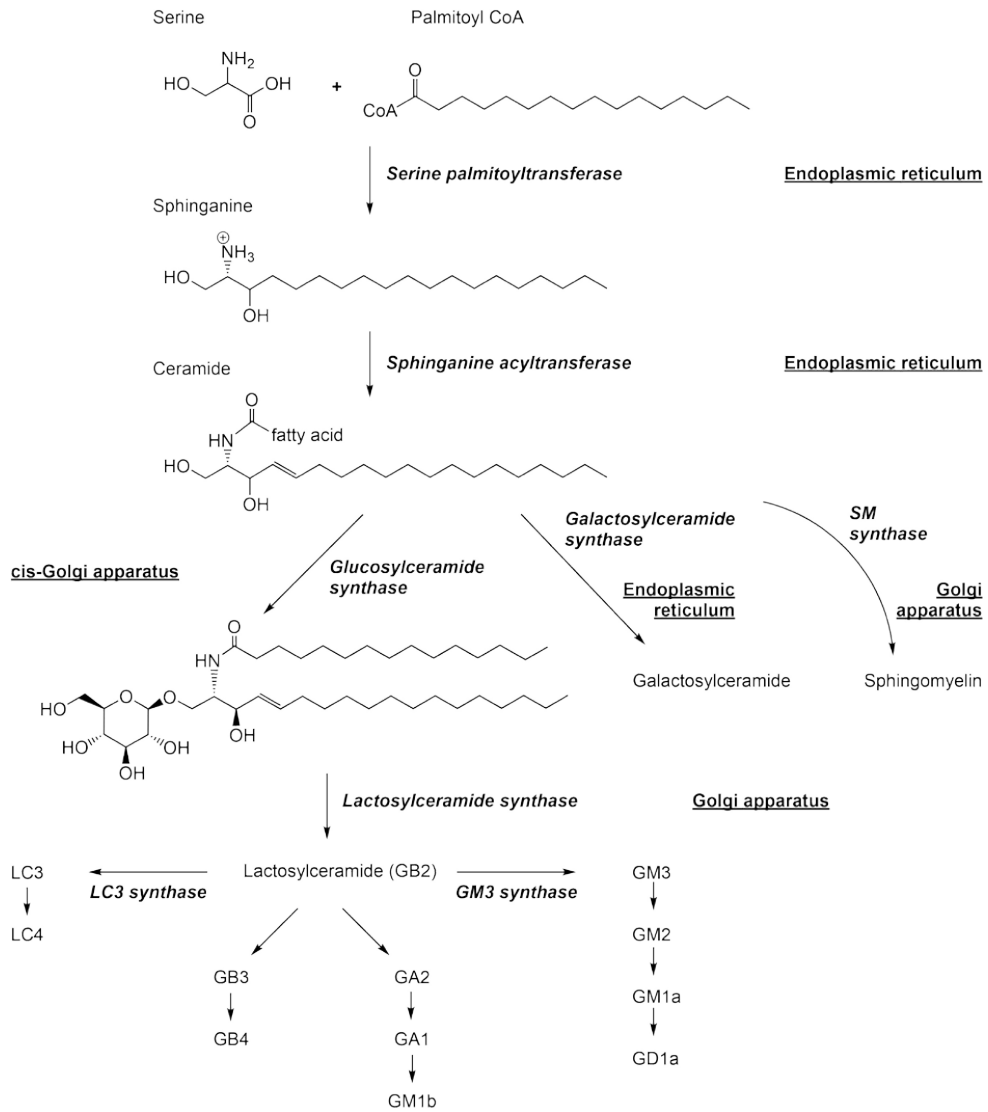


Figure 3. Schematic overview of GSL synthesis: enzymes and topology. Enzymes are indicated in bold and italic, topology is underlined and bold.

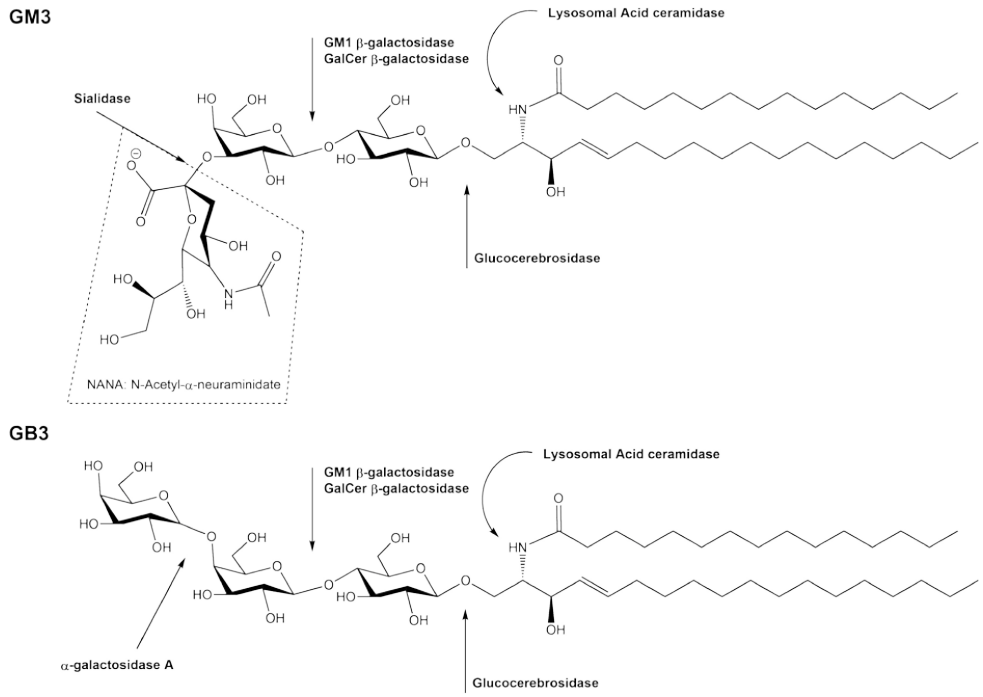


Figure 4. Overview of lysosomal degradation of ganglioside GM3 and globoside Gb3. Top: GM3 degradation in lysosomes. Bottom: Gb3 degradation in lysosomes. Involved enzymes are designated in **bold**.

Glucocerebrosidase*: *glucosylceramide hydrolase.

Glucocerebrosidase (GBA) is a retaining β-glucosidase, encoded by the *Gba1* gene at locus q21 of chromosome 1[46]. It is synthesized as 497 amino acid polypeptide at ER-associated ribosomes with a regular N-terminal signal sequence allowing co-translational translocation to the lumen of the ER[47]. Inside the ER, the signal peptide is removed and glycans are attached to the amino acids N19, N59, N146 and N270 of the nascent GBA, an essential modification for correct folding of the protein[48,49]. Next, within one hour of their synthesis, folded GBA molecules bind to the triple helical structure in the apical region of integral membrane protein LIMP-2 (lysosomes integral membrane protein 2, encoded by the *Scarb2* gene) containing trafficking information in its cytoplasmic tail[50,51]. Incorrectly folded GBA molecules failing to associate with LIMP-2 are removed from the ER and degraded in proteasomes[52]. Of note, GBA is not synthesized as inactive precursor but as active glycosidase, contrary to some other lysosomal hydrolases like α-glucosidase, acid ceramidase and cathepsins that require proteolytic processing to active enzyme[53]. Again in sharp contrast to most other lysosomal hydrolases, GBA does not acquire mannose-6-phosphate recognition moieties mediating mannose-6-phosphate receptor-mediated sorting to lysosomes[54]. Instead, the complex of GBA/LIMP-2 traverses the Golgi apparatus where most N-glycans in GBA are modified from high mannose-type to complex type-structures[53]. The precise manner in which the complex GBA/LIMP-2 is routed to lysosomes is unknown, but physically distinct vesicles from those containing mannose-6-phosphate receptors with their lysosomal hydrolase cargo are involved[55]. The travel of newly formed GBA from the ER to lysosomes is surprisingly slow in some cultured cells, taking several hours[53]. The survival of GBA in lysosomes is limited to 24-36 hours, at least as suggested by observations with cultured cells. Its proteolytic breakdown in cultured cells can be largely inhibited by leupeptin and E64, inhibitors of cysteine proteases[56,57]. Table 2 presents an overview of lysosomal proteases with corresponding inherited diseases in man.

Table 2. Lysosomal proteases and associated inherited diseases.
Sources: OMIM, Expasy, Genetics home reference, Uniprot

Protein	Gene	Type	Disease
Cathepsin A	CTSA	Serine	Galactosialidosis and Glycoproteinosis
Cathepsin C	CTSC	Cysteine	Papillon-Levèfre syndrome
Cathepsin D	CTSD	Aspartyl	Neuronal Ceroid Lipofuscinosis (CLN10)
Cathepsin F	CTSF	Cysteine	Kufs disease
Cathepsin K	CTSK	Cysteine	Pycnodysostosis (PKND)

After reaching acid late endosomes/lysosomes, GBA dissociates from LIMP-2, presumably due to the protonation of a specific histidine in LIMP-2's triple helical structure[58]. In lysosomes the enzyme GBA meets at acidic pH optimal for catalytic activity with saposin C, an 80 amino acid protein generated from 70 kDa prosaposin[59]. Saposin C stimulates enzymatic activity of GBA towards glucosylceramide, presumably by facilitating entry of lipophilic substrate in the catalytic pocket[42]. During catalysis GBA utilizes the double-displacement mechanism like most other retaining glucosidases. The key catalytic residues in GBA are the nucleophile glutamate 340 and acid/base glutamate 325[60]. As reaction intermediate the glucose of the GlcCer substrate becomes covalently linked to E340 and is released by subsequent attack of a nucleophilic water molecule assisted by E235. By the same mechanism, cyclophellitol and conduritol B-epoxide (CBE) irreversibly inhibit GBA. Cyclophellitol and CBE both form a permanent conjugate with the nucleophile E340 of GBA (Figure 2)[61]. Cyclophellitol scaffolds have been successfully used to design functionalized activity-based probes allowing *in situ* visualization of GBA[62,63] (see also section below). The 3D structure of GBA has been solved by X-ray diffraction crystal analysis, indicating a typical (β/α)8 TIM barrel catalytic core domain III, a three-strand antiparallel β-sheet flanked by a loop and a perpendicular strand (domain I) and an Ig-like fold formed by two β-sheets (domain II)[64,65]. A molecular dynamics model of

GBA has also been generated[66]. GBA has not yet been co-crystalized with saposin C and interaction of the two proteins has only been modelled *in silico*[67,68]. The part of saposin C essential for interacting with GBA has been determined with NMR and by site-directed mutagenesis[67,69,70]. How saposin C exactly stimulates enzymatic activity of GBA is still enigmatic. Saposin C is thought to perturb phospholipid-containing membranes at acid pH and thus offer better access for GBA to its lipid substrate[71]. However, saposin C also promotes activity of GBA towards water soluble artificial β-glucoside substrates[72]. It seems likely that binding of saposin C to GBA affects the enzyme's conformation. This is suggested by the observation that saposin C protects against inhibitory binding of α-synuclein to the enzyme[73]. Furthermore, saposin C influences resistance against degradation by lysosomal cysteine-proteases[74,75]. It has recently been shown that a LIMP-2 helix 5-derived peptide binds directly to GBA *in vitro*[51]. The helix 5 peptide fused to a cell-penetrating peptide was found to activate endogenous lysosomal GBA[51]. Beneficial transient interactions of LIMP-2 with GBA in lysosomes can at present not be excluded (see also chapter 3 of this thesis).

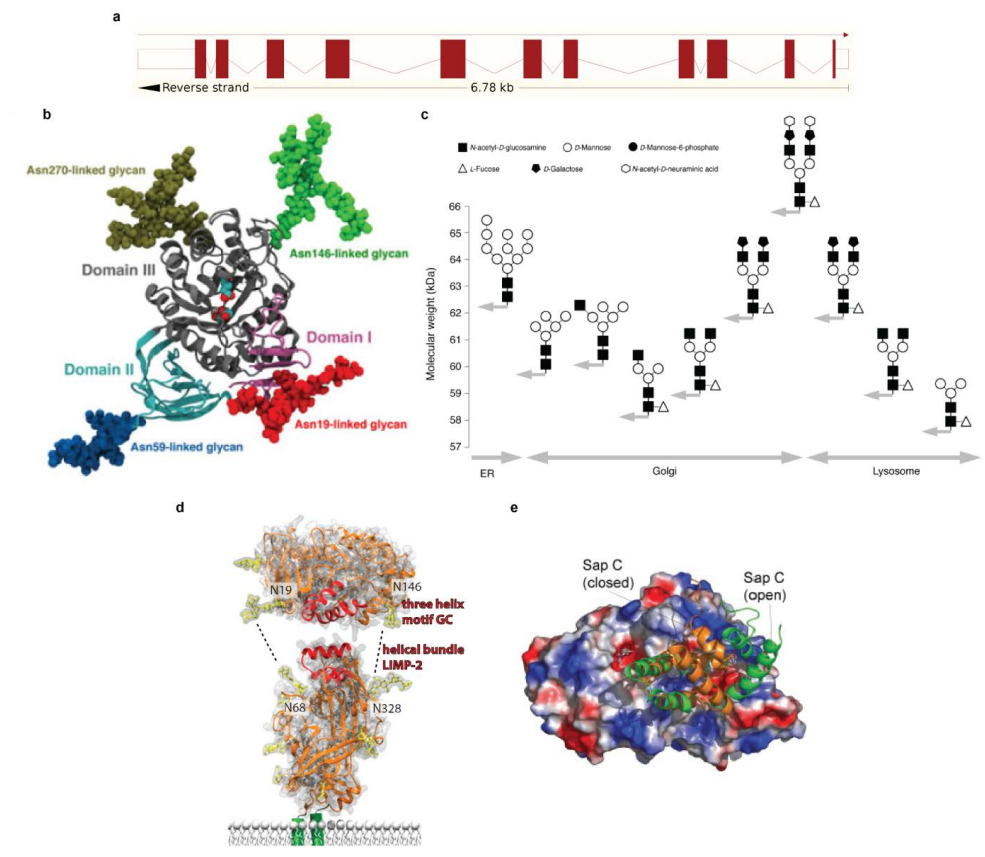


Figure 5. Glucocerebrosidase: a graphical narrative of composition, life cycle and interactors. a) Schematic overview of *Gba1* gene. Source: ENSEMBL; b) 3D structure of GBA showing various domains and 4 N-linked glycans, adopted from Pol-Fachin et al. 2016; c) Schematic life cycle of GBA from ER via Golgi apparatus to lysosome: molecular mass maturation by glycan modifications made by WW Kallemeijn; symbols: ■=*N*-acetyl-*D*-glycosamine, ○=*D*-mannose, ●=*D*-mannose-6-phosphate, Δ=*L*-Fucose, ⬣ =*D*-galactose, ⬡=*N*-acetyl-*D*-neuraminic acid. d) LIMP-2 as transporter of GBA, model adopted from Zunke et al. 2016; e) Lysosomal interaction of saposin C with GBA, model adopted from Atrian et al. 2008. SapC docked onto GBA with GlcCer molecule visible in catalytic pocket.

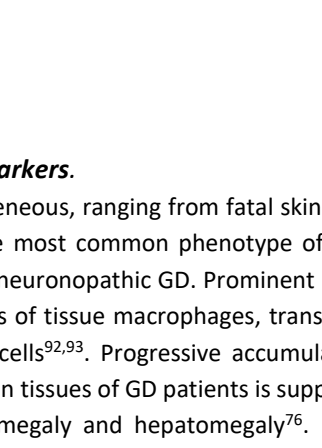
Impaired lysosomal GlcCer degradation: molecular causes of disease.

Defects in GBA cause intralysosomal accumulation of GlcCer in characteristic tubular structures[76]. Inherited mutations in the *Gba1* gene constitute the molecular basis for the relatively common lysosomal storage disorder named Gaucher disease (GD). A patient suffering from GD was firstly described in 1882 by the French dermatologist Philippe E.C. Gaucher[77]. His thesis at the University of Paris consisted of a case report describing a young woman with unexplained massive splenomegaly. Soon it was realized that this patient represented a distinct disease entity that was subsequently referred to as Gaucher's disease. The chemical nature of the accumulating lipid in GD tissues was first correctly elucidated by Aghion in 1934[78]. Only 50 years ago, deficiency of glucocerebrosidase (acid β-glucosidase) was identified as the cause of GD, independently by Patrick, Brady and co-workers [79,80]. At present more than 200 mutations in the *Gba1* gene have been linked with GD[81]. Next to truncations and splicing defects, several hundred amino acid substitutions in GBA have been shown to cause GD. The position of amino acid substitutions in the protein, in the catalytic or another domain, proves to poorly predict the clinical severity of GD patients[82]. Some substitutions in the folding domain that are positioned far away from the catalytic pocket have nevertheless major negative consequences. For instance, the substitution L444P in GBA causes faulty folding of most enzyme molecules in the ER and subsequent proteasome mediated degradation[53]. Homozygosity for L444P GBA nearly always leads to a severe neuronopathic course of GD, albeit with great individual variability in onset and progression[76]. Premature degradation may also occur with mutations in the catalytic domain. In fact, quite many of the documented mutations in GBA lead to defective folding and reduced transport to lysosomes[83]. An exception is the N370S GBA substitution, the prevalent *Gba1* mutation among Caucasians GD patients. The amino acid substitution is in a loop close to the catalytic pocket and it was found to affect pH optimum and kinetic parameters such as affinity for substrate[57,84,85]. There are controversial reports regarding the impact of the N370S substitution in GBA on initial folding of the enzyme in the ER, claimed to be impaired as well as normal[86,87]. Certainly, the intralysosomal stability of N370S GBA is markedly reduced[56,88]. Hetero- and homo-allelic presence of the *Gba1* gene coding for N370S GBA is associated with a non-neuronopathic type 1 course of disease[76,89]. Otherwise, *Gba1* genotype – GD phenotype correlations are relatively poor, illustrated most explicit by the occurrence of phenotypically discordant monozygotic twins[90,91].

Gaucher disease: clinical manifestation and biomarkers.

The clinical presentation of GD is remarkably heterogeneous, ranging from fatal skin defects to an almost asymptomatic course of disease[76]. The most common phenotype of among Caucasian GD patients is referred to as type 1 or non-neuronopathic GD. Prominent in these patients is the ongoing storage of GlcCer in lysosomes of tissue macrophages, transforming into the characteristic enlarged lipid-laden Gaucher cells[92,93]. Progressive accumulation of these alternatively activated lipid-laden macrophages in tissues of GD patients is supposed to underlie the development of symptoms like splenomegaly and hepatomegaly[76]. Massive accumulation of Gaucher cells in the bone marrow is thought to contribute to hematological abnormalities, such as anemia and the commonly encountered thrombocytopenia. Other, highly variable, signs and complications associated with GD are skeletal deterioration, neuropathology including oculomotor apraxia and peripheral neuropathy as well as polyclonal and monoclonal gammopathies[76]. GD patients developing lethal complications in the central nervous system are classified as type 2 GD (infantile onset) and type 3 GD (late infantile/juvenile onset). Complete deficiency of GBA activity results in pre-natal/neo-natal phenotypes characterized by lethally aberrant permeability of the skin (so-called collodion baby showing severe ichthyosis)[94–96].

Major symptoms	Classification
Enlarged liver	Type 1, 2 & 3
Enlarged spleen	Type 1, 2 & 3
Bone disease	Type 1, 2 & 3
Bone marrow infiltration	Type 1, 2 & 3
Anemia	Type 1, 2 & 3
Thrombocytopenia	Type 1, 2 & 3
Pulmonary disease	Type 1, 2 & 3
Gammopathies	Type 1, 2 & 3
Cancer risk	Type 1, 2 & 3
Cholesterol choletithiasis	Type 1, 2 & 3
Neurodegeneration	Type 2 & 3
Epilepsy	Type 2 & 3
Eye movement disturbances	Type 3
Skin permeability	Lethal



Protein Biomarkers
PARC/ CCL18
Chitotriosidase
S-gpNMB
MIP-1α and MIP-1β

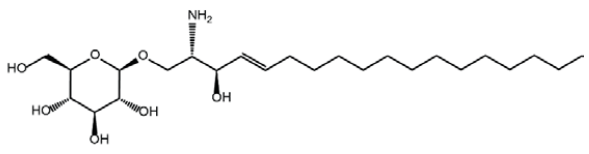


Figure 6. Gaucher disease; a graphical narrative of clinical heterogeneity, Gaucher cells and biomarkers. Top left: Major symptoms and phenotype classification. Top right: Gaucher cell picture adopted from Aerts et al. 2003. Bottom left: Protein biomarkers of Gaucher cells. Bottom right: Glucosylsphingosine.

Carriers of mutant *Gba1* alleles do not develop Gaucher cells and characteristic GD symptoms, intriguingly however they, like patients, are at significantly increased risk for α-synucleinopathies such as Parkinsonism and Lewy-body dementia[97–99]. Given the poor prognostic value of *Gba1* genotype, early demonstration of onset of disease in individuals with aberrant GBA is essential. Several sensitive plasma biomarkers for the presence of Gaucher cells in the body have been identified, being proteins produced and secreted by lipid-laden macrophages, such as the chitinase chitotriosidase and the chemokine CCL18[21,93,100]. Gaucher cells also release soluble fragments of the membrane-proteins CD163 and gpNMB into the circulation, leading to markedly elevated plasma concentrations[101,102]. In addition, glucosylsphingosine (GlcSph), deacylated GlcCer, is several hundred-fold increased in plasma of symptomatic GD patients and may serve as biomarker[103,104]. These surrogate markers of disease in plasma are increasingly used as guidance in clinical management, especially after the availability of therapies for type 1 GD (see section below)[105,106]. Defects in saposin C impairing GBA activity lead to clinical symptoms similar to those presented by GD patients[107]. Mutations in the *Scarb2* gene causing deficiency of LIMP-2 result in markedly reduced GBA levels in many cell types in man and mice[108–110]. However, the corresponding human disease, action myoclonus renal failure syndrome (AMRF), is clinically very different from GD[111,112]. In LIMP-2 deficient mice relatively little GlcCer accumulates in tissues, a phenomenon explained by the ability of most cells to efficiently convert accumulating GlcCer in lysosomes to GlcSph by the action of lysosomal acid ceramidase[113,114]. White blood cells of AMRF patients and LIMP-2 deficient mice have a surprisingly high residual GBA content, possibly due to re-uptake of faulty secreted GBA. This considerable residual enzyme activity in macrophages seems sufficient to prevent formation of Gaucher cells and associated symptoms as occurring in GD patients. In line with this, AMRF patients do not show elevated plasma levels of Gaucher cell markers such as chitotriosidase, CCL18 and gpNMB, but plasma glucosylsphingosine is increased although far less spectacularly than in GD patients[112]. Plasma glucosylsphingosine abnormalities in type 1 GD patients have been found to be corrected upon various therapeutic interventions similar to the validated Gaucher cell biomarker chitotriosidase, indicating that most of the excessive plasma glucosylsphingosine in type 1 GD patients stems from their Gaucher cells[114].

***Pathophysiology of Gaucher disease*.**

The most poorly understood aspects of GD are the pathophysiological mechanisms underlying the complex clinical picture of the disorder. A closer inspection of patients shows that numerous cell types and tissues can become affected. Ichtyotic skin disease is observed only in GD patients without virtual GBA activity. The ratio of glucosylceramide to ceramide in the

stratum corneum, determined by GBA, seems critical for correct barrier function of the skin[94]. Clinical symptoms related to the central nervous system such as epilepsy, apraxia and scoliosis are usually observed only in GD patients with markedly reduced GBA activity, but on the other hand even GD carriers show an increased risk for Parkinsonism[97]. Peripheral neuropathy also occurs in GD patients with an otherwise relative mild disease course and with significant residual GBA activity[115]. Growth retardation and signs of insulin resistance point to hormone disturbances, again also occurring in milder affected type 1 GD patients[116]. Some of the liver-related symptoms like hepatomegaly and gallstones occur frequently in GD patients; however, cirrhosis is more rare and associated with a more severe disease course in general[76]. Cardiac valve calcification is a symptom specifically occurring in GD patients with D409H mutated GBA, suggesting a very specific, but still enigmatic, mechanism[76,117–120]. Splenomegaly is again a very common sign of disease and associated with accumulation of Gaucher cells in the organ. Frequent in GD patients is polyclonal gammopathy, and quite often this evolves into monoclonal gammopathy[121]. Ultimately this can even lead to the development of multiple myeloma and amyloidosis[122–124]. Bone marrow filtration is another regular sign in GD patients and likely contributes besides splenomegaly to the common thrombocytopenia and anemia, albeit the latter generally develops only in more severely affected patients[76]. The skeletal disease and bone remodelling in GD patients is heterogeneous and focal of nature[76]. It seems not to correlate well with other disease manifestations and circulating biomarkers of Gaucher cells[125]. Osteoporosis is often encountered in GD patients, potentially linked to impaired osteoblasts rather than increased osteoclast activity[125,126]. It is unclear whether the presence of Gaucher cells in tissues explains the entire spectrum of symptoms and signs in GD patients. Indeed, the lipid-laden macrophages are viable cells able to secrete various proteins promoting the influx of further monocytes to disease loci and stimulating ongoing inflammation and tissue remodeling[92,93]. As discussed in more detail in chapter 6 of the thesis, it is conceivable that secondary abnormalities in GD patients stemming from adaptations to the primary lysosomal GlcCer accumulation contribute to specific symptoms. Briefly, compensatory increased metabolism of GlcCer by the cytosolic β-glucosidase GBA2[127–130] might promote loss of motor coordination by Purkinje cell loss[131]. It might also lead to excessive formation of potential toxic metabolites such as ceramide and glucosylated compounds, e.g. cholesterolglucoside[131,132]. Another adaptation in GBA deficient cells, the intralysosomal formation of GlcSph from accumulating GlcCer, may even be pathogenic as such. Excessive GlcSph has been linked to B-cell lymphoma[133–135]. It has very recently been reported that glucosylsphingosine in GD patients acts as auto-antigen driving B-cell proliferation and it is proposed to directly promote the development of multiple myeloma[133]. Of note, deacylation of accumulating storage GSLs to corresponding glycosphingoid bases is not unique for GD and AMRF, but also occurs in Fabry disease and Krabbe disease[136–138]. Again, toxicity of the generated glycosphingoid bases is considered: excessive galactosylsphingosine is thought to be neurotoxic in Krabbe disease patients[139,140] and excessive globotriaosylsphingosine (lysoGb3) is claimed to be toxic for nociceptive peripheral neurons and podocytes in Fabry disease patients[141,142].

Therapies for Gaucher disease.

Based on seminal studies by Brady and collaborators at the National Institutes of Health in Bethesda, U.S.A. half a century ago[143], the first effective treatment for GD was introduced, so-called enzyme replacement therapy (ERT)[144,145]. This treatment is based on two-weekly intravenous administration of glycan modified human recombinant GBA to supplement macrophages with enzyme. Initially GBA was isolated from human placenta and its N-glycans were enzymatically modified *in vitro* to expose terminal mannose residues favoring endocytotic uptake of enzyme via the mannose receptor, a lectin receptor present on tissue macrophages[146]. An effective ERT enzyme preparation was commercially developed by Genzyme (Boston, U.S.A.), involving large scale isolation of enzyme from placental extracts[143]. A few years later the placental enzyme preparation was replaced by a recombinant GBA produced in Chinese hamster ovary cells. Now several GBA preparations are in use for ERT of type 1 GD, all leading to reversal of organomegaly and hematological complications, stabilization of skeletal disease and markedly increased quality of life[147–151]. Clinical improvement is accompanied by corrections in plasma biomarkers of Gaucher cells and glucosylsphingosine. The quantification of plasma chitotriosidase is widely applied to monitor disease progression and response to therapy. Corrections in plasma chitotriosidase of GD patients have been found to correlate with corrections in organomegaly and to be associated with improvements in hematological abnormalities. Moreover, the extent of correction in chitotriosidase correlates with the incidence of long term complications such as pulmonary hypertension, multiple myeloma and Parkinsonism[106]. It soon became apparent that neurological manifestations in more severely affected GD patients are not prevented by ERT because the therapeutic enzyme fails to pass the blood-brain barrier[147]. An alternative treatment of type 1 GD is offered by so-called substrate reduction therapy (SRT)[152–155]. Here, three times daily GD patients take a small compound inhibitor of GCS orally, the key enzyme in glucosylceramide and subsequent glycosphingolipid biosynthesis. Two drugs (*Miglustat* and *Eliglustat,* Figure 7) are registered for treatment of type 1 GD patients. *Miglustat* (N-butyl-deoxynojirimycin), already registered in 2001, is a relative weak and non-specific inhibitor of GCS. Lately the far more potent and specific GCS inhibitor *Eligustat* (*N*-[(1*R*,2*R*)-1-(2,3-dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-(1-pyrrolidinyl)-2-propanyl] octanamide) has also been registered for SRT of type 1 GD patients[156–158]. The latter drug does not penetrate the brain well and is not considered suitable to treat neuronopathic variants of GD. The design of brain-permeable specific inhibitors of GCS is actively pursued by pharmaceutical industry and academic researchers. Brain-permeable N-(5'-adamantane-1'-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM)[159] has earlier been identified as high nanomolar GCS inhibitor. It was observed that ido-variants of AMP-DNM inhibit GCS with the same efficacy but with much less affinity for GBA. Based on these compounds, a new generation of deoxynojirimycin type GCS inhibitors with IC_{50} values in the very low nanomolar range has been recently developed[160].

Table 3. Therapy approaches for Gaucher disease. ERT: Enzyme Replacement Therapy; SRT: Substrate Reduction Therapy; CT: Chaperone Therapy; LST: Lysosomal Stabilization Therapy; GT: Gene Therapy.

Therapy	Agent	Mode of action	Target	Status	Limitation	References
ERT	Recombinant GBA1	Enzyme supplementation	Macrophages	Registered	Costly, only for GD1	Barton 1990 & 1991
SRT	GCS inhibitor	Substrate reduction	Viscera	Registered	Costly, only for GD1	Platt 2001, Aerts 2006, Cox 2000, Elstein 2004
CT	GBA1 chaperone	Folding promotion	Total body	Experimental	Also inhibits enzyme	Boyd 2013, Fan 2003, Benito 2011, Jung 2016, Chang 2006, Yu 2007, Steet 2007, Tropak 2008, Lieberman 2009, Khanna 2010, Babajani 2012, Zimran 2013, Bendikov-Bar 2013
LST	Arimoclomol, others	Enzyme stabilization	Total body	Experimental	No known adverse effects	Kirkegaard 2016
GT	DNA construct	cDNA/gene correction	Stem cells	Experimental	Safety, Immune response	Dahl 2015

There is active research on additional treatments of GD, particularly for the non-neuronopathic variants for which there remains an unmet clinical need. Chemical chaperones of GBA might offer a novel additional treatment avenue. With this approach, small compounds interacting with the catalytic site of the enzyme should chaperone folding of (mutant) GBA in the ER, resulting in increased transport of enzyme to the lysosome[161–164]. In essence, chemical chaperones promoting correct conformation of GBA might also stabilize the enzyme intralysosomally. Whether the latter could offer clinical benefit is debated: chemical chaperones interacting with the active site of lysosomal GBA intrinsically also inhibit its enzymatic activity. As many GD patients produce mutant forms of GBA that are impaired in folding and/or lysosomal stability, these might profit from chaperone-based therapies. There is an explosive increase in reports on the design and synthesis of potential chemical chaperones for GBA. Recent reviews elegantly cover some of the glycomimetics classes currently under investigation as chaperones[165,166]. Many are reversible competitive, or mixed-type, inhibitors of GBA. The most extensively studied small compound so far has been isofagomine (IFG, Figure 7), which was the subject of several pre-clinical studies as well as a clinical study that did not meet the full expectations[167–176]. IFG is a potent competitive GBA inhibitor with an IC_{50} of approximately 30 nM at pH 5.2 and 5 nM at pH 7.0[177]. Compounds like IFG will only exert a beneficial effect on GBA in a delicate concentration window that is likely difficult to reach concomitantly in various tissues of GD patients. The effects of oral

administration of *Ambroxol* (Figure 7), a weak mixed-type inhibitor of GBA have been investigated in studies with cells and small numbers of type 1 GD patients[177–180]. Impressive reductions in spleen and liver volumes of *Ambroxol*-treated type 1 GD patients have been documented, as well as reductions the GD biomarker chitotriosidase.

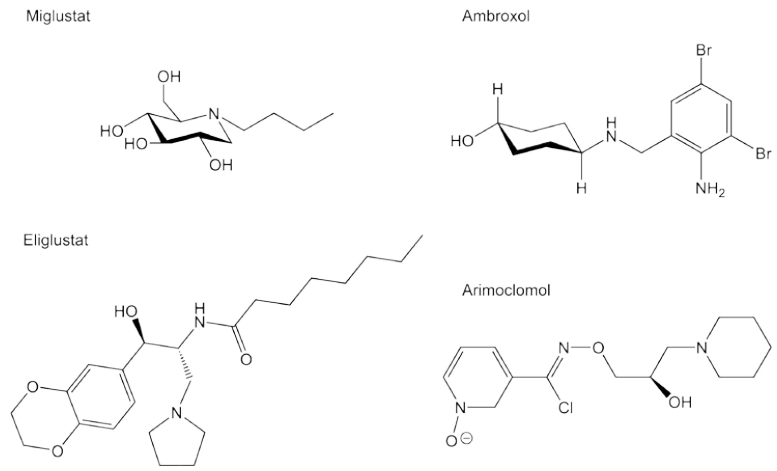


Figure 7. Small compound therapeutics for Gaucher disease. Top left: Miglustat. Lower left: Eliglustat. Top right: Ambroxol. Lower right: Arimoclomol.

N370S GBA is the prevalent mutant enzyme among Caucasian type 1 GD patients[76]. For example, almost every Dutch type 1 GD patient was found to possess at least one *Gba1* allele coding for N370S enzyme[89]. Significant amounts of N370S GBA molecules reach lysosomes[56,88], their intralysosomal stability is however reduced. Fibroblasts homozygous for N370S GBA show a marked increase in enzyme activity when cultured in the presence of leupeptin inhibiting lysosomal proteolysis[56]. Given this observation, a potential alternative treatment for such individuals could be lysosomal stabilization therapy (LST), in which agents promoting intralysosomal survival of GBA would be administered. Several approaches to be considered in this direction are the use of selected lysosomal protease inhibitors and glyco-mimetics acting as stabilizing chemical chaperone. Since GBA also exerts β-xylosidase activity (Chapter 7 of this thesis), xylo-mimetics might also be worthwhile to test as potential stabilizers. Little consideration as potential beneficial stabilizers has so far been given to agents binding to the aglycon site of GBA. An approach proposed for treatment of Niemann Pick disease types B and C is the administration of *Arimoclomol* (Figure 7)[181], a small compound boosting formation of endogenous Hsp70, a protein that assists (re)folding of unfolded mutant enzymes, even in the lysosome[182]. *Arimoclomol* is hoped to also exert positive effects in GD patients. Finally, targeting of (fragments of) saposin C or LIMP-2 to lysosomes might offer other ways to stabilize GBA *in situ* and augment GlcCer degradative capacity.

During a discussion of future GD treatments, gene therapy has to be mentioned[183]. Given the positive outcome of bone marrow transplantation in type 1 GD patients, genetic modification of hematopoietic stem cells has been, and still is, seriously considered as therapeutic avenue. Pioneering trials with retroviral vectors to introduce *Gba1* cDNA in hematopoietic stem cells of GD patients did not result in permanent correction of white blood cells[184], however in recent times encouraging data have been obtained with lentiviral gene therapy in type 1 GD mice[185]. Moreover, the exciting new possibilities for gene corrections using CRISPR-CAS technology may further promote a revival in research on gene therapy as therapeutic modality for GD[186].

***Chemical biology tools: activity-based probes, isotope encoded and clickable GSLs, fluorogenic caged substrates*.**

Diagnosis of GD and fundamental research on the disorder were accelerated with the availability of an antibody toolbox. Polyclonal and monoclonal antibodies directed to GBA quickly found valuable applications in fundamental studies on the life cycle and subcellular localization of GBA, the diagnosis of GD patients and even the purification of therapeutic enzyme[187–189]. In recent times novel chemical biology tools have been designed allowing investigations on GBA and *in vivo* GlcCer metabolism in an unprecedented manner.

One recent breakthrough is the development of activity-based probes (ABPs) for GBA. The irreversible inhibition of GBA by cyclophellitol (see section above and Figure 2) was exploited for the design of ABPs for the enzyme[62,63]. Initially, β-glucopyranosyl-configured cyclophellitol-epoxides modified at C6 (glucopyranose numbering) with a fluorescent BODIPY (Figure 8) were synthesized as mechanism-based probes for GBA[62]. These ABPs bind covalently and with high specificity in a mechanism-based manner, to the catalytic nucleophile residue E340 of GBA[62]. *In situ* labeling of active GBA with the ABPs in intact cells is feasible, visualizing the intralysosomal location of the enzyme. Intravenous infusion of mice with these ABPs results in specific labeling of GBA in various tissues, except brain and eye[63]. Intracerebroventricular administration of the ABPs to mice allows visualization of active GBA in brain with high spatial resolution[190]. Reductions in active GBA molecules can be detected after the exposure of cultured GD fibroblasts to ABP and subsequent analysis of ABP-labeled protein by gel electrophoresis and quantitative fluorescence scanning[62]. The successful approach for design of GBA directed ABPs was successfully reproduced for the lysosomal β-galactosylceramidase (galactocerebrosidase, GALC), deficient in Krabbe disease[191]. Next, a broad spectrum ABP was generated by the design of β-glucopyranosyl-configured cyclophellitol-aziridines with a fluorophore modification (Figure 8)[63]. In these probes the fluorophore is positioned more closely to the position of the aglycon in substrates of β-glucosidases. Therefore, these ABPs covalently label a broad class of human β-glucosidases, including GBA, GBA2, GBA3 and lactase-phloridzin hydrolase[57]. Meanwhile, cyclophellitol-type ABPs with distinct sugar configuration have been developed as ABPs for α-glucosidases, α-fucosidase, α-mannosidases, α-iduronidase, α-galactosidases, β-galactosidases, β-mannosidases and β-glucuronidases[192–195], (unpublished data: Artola, Jiang, Beenakker and Kuo).

The ABPs can be conceived to find application in the diagnosis of lysosomal storage diseases as well in fundamental research. Of note, the ABPs can be applied cross species given the conserved catalytic pockets of glycosidases. They can be also equipped with biotin instead of fluorophores, allowing convenient purification by means of streptavidin-based pull down and subsequent identification of proteins by proteomics[195,196]. This procedure should also allow identification of interacting proteins with the ABP-targeted enzyme. The GBA ABPs can be further optimized to monitor the precise localization of enzyme in life cells, by super-resolution microscopy as well as by correlative light and electron microscopy. For this cyclophellitol derivatives can be fitted out with a norbonene at C8 (cyclophellitol numbering) to generate a mechanism-based inhibitor projected to be (due to the bulk at this position) selective for GBA. Pulse labeling of tissue culture and washing away unbound probe can be followed by treatment with fluorogenic tetrazine. Only after inverse-electron demand Diels Alder the dye becomes fluorescent, thus limiting background labeling[197].

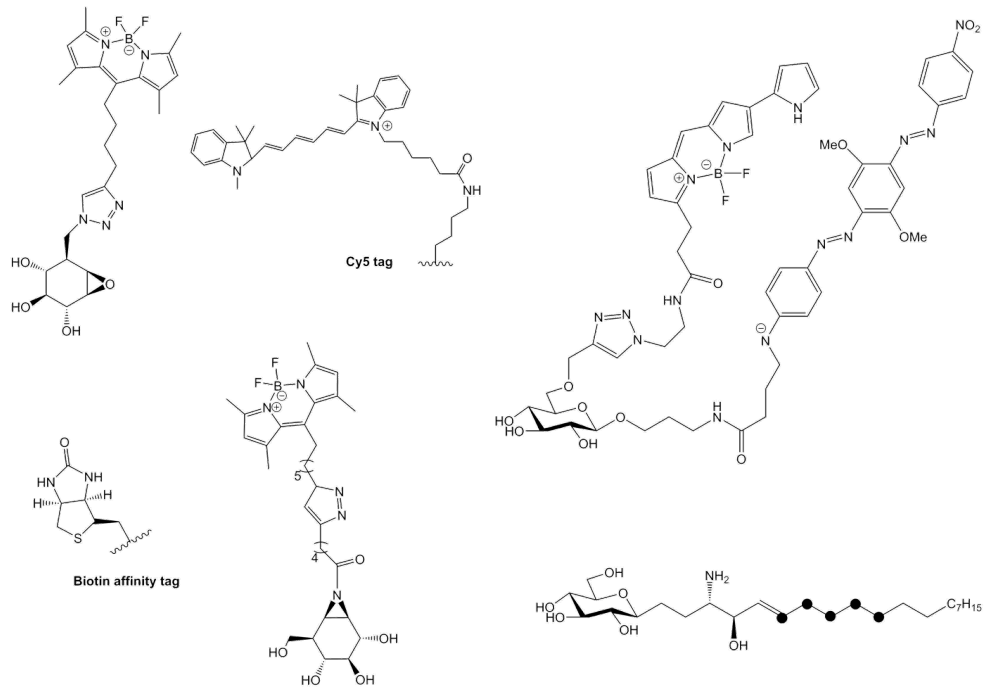


Figure 8. Chemical biology tools: ABPs, isotope and di-azirine-GSLs, and caged GBA substrate. Upper left panel: Cyclophellitol-epoxide with BODIPY and Cy5 fluorophore; Lower left panel: Cyclophellitol-aziridine with BODIPY and biotin; Upper right panel: Caged fluorogenic substrate for GBA[198]; Lower right panel: Isotope labeled glucosylsphingosine.

Other important chemical research tools developed in the last decades are reversible and irreversible inhibitors targeting various enzymes (GBA, GBA2 and GCS) involved in glucosylceramide metabolism[62,199]. Vocadlo and co-workers recently reported the design of a caged fluorogenic substrate specific for GBA (Figure 8)[198]. This substrate, suited for activity measurements in living cells, should find broad application. Ideally however, *in situ* activity of GBA towards natural substrates is detected. New tools for this are glycosphingolipids and glycosphingoid bases encoded with ^{13}C atoms. These isotope labeled lipids synthesized at the Leiden Institute of Chemistry can be used as internal standards in LC-MS/MS quantification of lipids[200,201]. Moreover, they can be used in the analysis of enzymatic activity towards natural substrates in cultured cells[113]. Particularly, feeding of cells with isotope (glyco)sphingoid bases offers a convenient way to monitor *in situ* lipid metabolism over time. A similar approach can be employed with mice to which isotope labeled lipids are intravenously administered. Recently photoactivatable lipid analogs have been developed that can be activated by UV light to form a covalent linkage to their protein-binding partners[202,203]. There now are commercial diazirine-functionalized and clickable lipids like pacFA, pacSph and pacGlcCer. As demonstrated in this thesis, pacGlcCer is very suitable to study *in vitro* features of the catalytic pocket of GBA.

Transglycosylation.

Several retaining glycosidases have been found to also efficiently transglycosylate, i.e. to transfer the released sugar from substrate to another acceptor than a water molecule. A thorough historical account of the realization that glycosidases may transglycosylate is provided by the review of Hehre on the topic[204]. Acceptors in transglycosylation reactions can be sugars, as is the case with chitinases such as chitotriosidase[205]. Other glycosidases like GBA may transglucosylate sugar to other structures such as for example retinol and sterol. Glew and co-workers were the first to demonstrate that GBA catalyzes the transfer of glucose from 4-methylumbelliferyl-β-glucoside to retinol and other alcohols[206]. Akiyama and colleagues more recently reported that *in vitro* GBA generates 25-NBD-cholesterol-glucoside through transglucosylation from GlcCer and 25-NBD-cholesterol[207]. This finding was recently recapitulated by Marques *et al.* showing GBA mediated transglucosylation of natural cholesterol with GlcCer as donor[132]. Importantly, it was demonstrated that also the cytosolic β-glucosidase GBA2 generates GlcChol through transglucosylation, again using GlcCer as donor. A sensitive quantitative detection of GlcChol by LC-MS/MS using $^{13}C_5$-isotope labeled GlcChol as internal standard enabled the detection of the glucosylated sterol in human plasma and cultured cells[132]. Analysis of mouse organs revealed that GlcChol is present in almost all tissues, with relative high amounts in the thymus, of interest in view of the noted abnormalities in NKT and B-cells in GD patients[208–210]. It has been speculated by Mistry and colleagues that elevated GlcCer or GlcSph via binding to CD1 may be causing this phenomenon[208]. GlcChol should also be considered a serious candidate in this respect.

Interestingly, GlcChol is not directly formed by GCS by transfer of glucose from UDP-glucose to ceramide[132]. GBA2 is found to be largely responsible for biosynthesis of GlcChol using GlcCer as glucose donor. Whilst GBA seems to normally degrade GlcChol, high intralysosomal cholesterol concentrations as in Niemann Pick type C disease favor formation of GlcChol by GBA. Induction of lysosomal cholesterol accumulation in cells with U18666A causes a rapid increase in GlcChol, which is abolished by selective inactivation of GBA[132]. Pharmacological inhibition of GBA2 leads to reduction of GlcChol in cultured cells, plasma of mice and plasma of GD patients. The same is observed upon lowering of GlcCer by inhibition of GCS, further indicating that the availability of GlcCer is an important driver in formation of GlcChol through transglucosylation[132].

The recent discovery of additional metabolites linked to GlcCer seems highly relevant for a better understanding of the complex clinical picture of GD. The finding suggests that GBA may be responsible for metabolism of multiple glucosylated metabolites and not only GlcCer. Hypothetically, other metabolites than GlcCer might more directly underlie some disease manifestations of GD patients. The realization that GlcCer acts as intermediary metabolite donating glucose to other metabolites may help to understand better in the future the complex and highly variable clinical outcome of inherited disturbances in GlcCer metabolism such as GD.

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