Lipophilic iminosugars : synthesis and evaluation as inhibitors of glucosylceramide metabolism

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1 General Introduction and Outline

Glycosphingolipids, Carbohydrate-processing Enzymes and Iminosugar Inhibitors

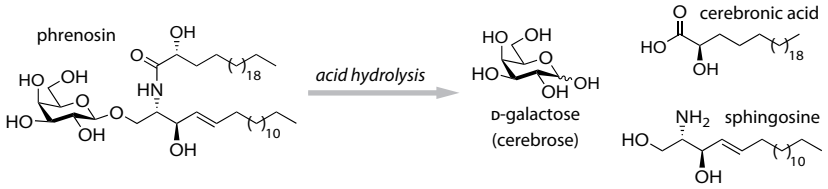
General Introduction

The study described in this thesis was conducted with the aim of developing lipophilic iminosugars as selective inhibitors for three enzymes involved in glucosylceramide metabolism. Glucosylceramide, a β-glycoside of the lipid ceramide and the carbohydrate D-glucose, is a key member of a class of biomolecules called the glycosphingolipids (GSLs). One enzyme, glucosylceramide synthase (GCS), is responsible for its synthesis and the two other enzymes, glucocerebrosidase (GBA1) and β-glucosidase 2 (GBA2), catalyze its degradation. Being able to influence glucosylceramide biosynthesis and degradation would greatly facilitate the study of GSL functioning in (patho)physiological processes. This chapter aims to provide background information and some history on the various subjects that were involved in this study. The chapter will start out with a brief overview of the discovery of GSLs and the evolving view of the biological role of GSLs and carbohydrate containing biomolecules in general during the last century. Next, the topology and dynamics of mammalian GSL biosynthesis and degradation will be described with special attention for the involved carbohydrate-processing enzymes. Following this, the known functions of GSLs in health and diseases will be discussed together with the therapeutic opportunities for inhibitors of glucosylceramide metabolism. The chapter ends with an introduction on iminosugars and a concise overview of the presently known small-molecule inhibitors of the three targeted enzymes.

1.1 About Thudichum's Discovery of (Glyco)sphingolipids and Glycobiology.

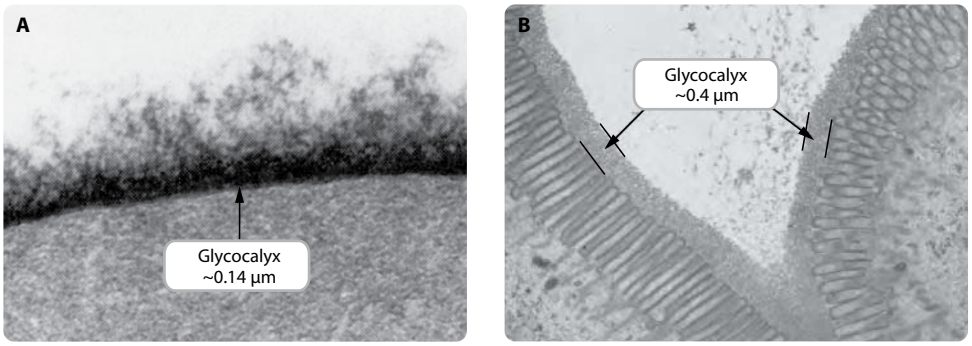
Johan L.W. Thudichum was born in 1829 and after attending the Medical School in Giessen – being taught among others by Justus von Liebig – he embarked on a prosperous scientific career. After having been active on subjects ranging from urology to vinology he embarked at the end of the 1870s on a study of the chemical composition of the brain. During these investigations he isolated several compounds from ethanolic brain extracts that he named cerebrosides. One of these, phrenosin, he subjected to acid hydrolysis and this produced three distinct components after fractional crystallization (Scheme 1). One he identified as a fatty acid and another proved to be an isomer of D-glucose that he coined cerebrose, now known as D-galactose. The third component with an 'alkaloidal nature' however presented 'many enigmas' to Thudichum and therefore he named it sphingosine, after the myth of the Sphinx's riddle.[1,2]

Scheme 1. Compounds isolated by Thudichum after the acid hydrolysis of phrenosin.



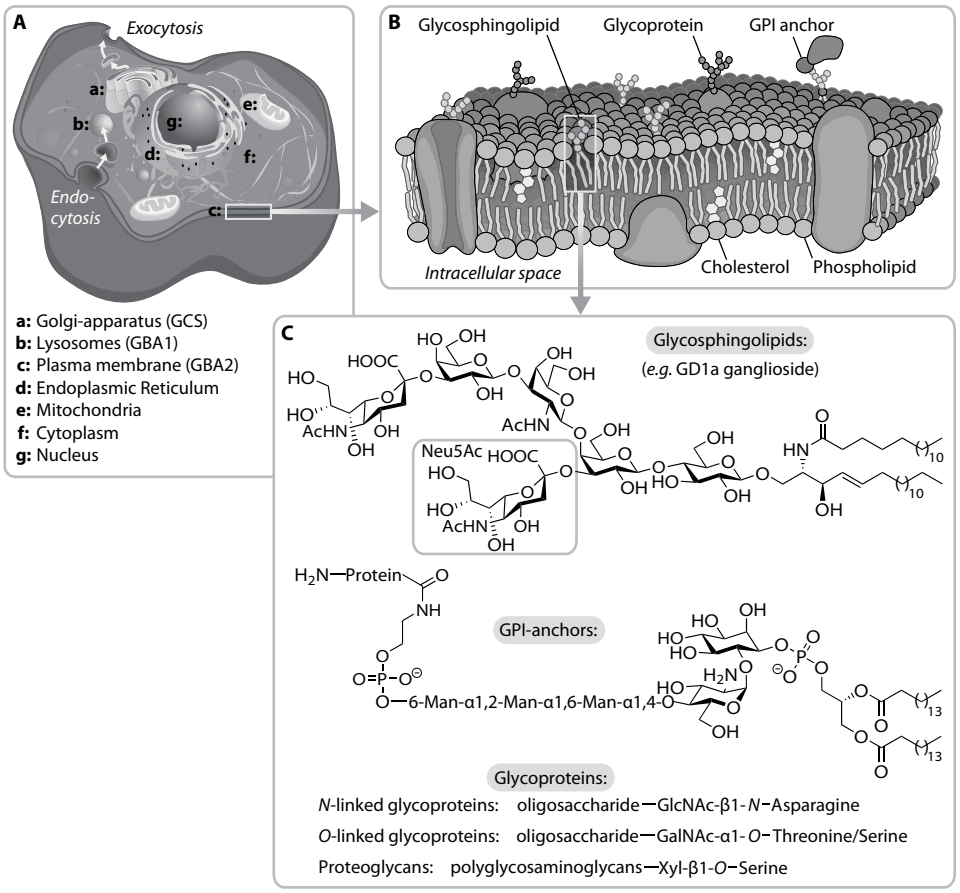
Thudichum's discovery did not receive due recognition during his lifetime (1829–1901), because up to about 1910 the authorities in this field fiercely defended the hypothesis that brain matter consisted of one giant molecule, the protagon, from which all simpler compounds were derived as breakdown products.[1] However, by the 1930s, Thudichum was fully vindicated and in 1947, Herbert E. Carter eventually published the molecular structure for sphingosine and proposed the term sphingolipids (SLs) for its derivatives.[3] Nowadays it is known that galactosylsphingolipids, like phrenosin, are among the most prevalent sphingolipids found in the brain, functioning as critical components in the myelin isolation of the axons of neuronal cells.

Figure 1. The glycocalyx covering an erythrocyte (**A**)[4] and the microvilli of intestinal absorptive cells (**B**).[6]



By the 1960s, numerous more complexly glycosylated sphingolipid derivatives had been discovered. In many of those the sialic acid, 5-*N*-acetylated neuraminic acid (Neu5Ac; Figure 2C), proved to cap the oligosaccharide.[4,5] These glycosphingolipids are generally named gangliosides. During this time, electron microscopy imaging of tissues and cells that were stained for carbohydrates also showed that most mammalian cells are covered with a dense and complex layer of carbohydrates, called the glycocalyx (Figure 1).[4,6]

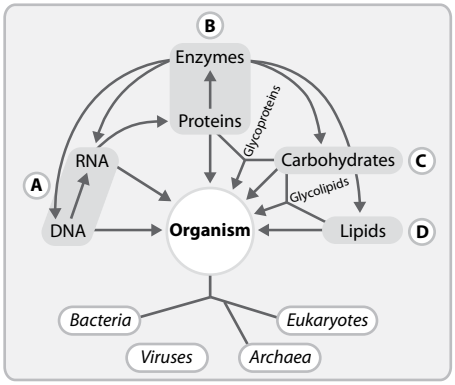
Figure 2 A: Cross-section of eukaryotic cell; **B**: Plasma membrane section; **C**: Structure of glycoconjugates.



The glycocalyx covers most mammalian cells and consists of a wide variety of oligosaccharides that are anchored to the plasma membrane as glycoconjugates with either a plasma membrane associated protein or lipid (Figure 2B). There are two types of lipid glycoconjugates. The first, GSLs, are anchored in the membrane via their ceramide lipid part. Ceramide consists of sphingosine that is *N*-acylated with a fatty acid. The *N*-acyl tail in ceramide is variable, but in mammalian glycoconjugates the most encountered is the *N*-palmitoylated (C_{16}) ceramide (Figure 2C). The glycosyl phosphatidylinositol (GPI) anchors are the second type of glycolipid.[7] These complex constructs consist of a

phosphatidylinisitol membrane anchor to which a (glycosylated) protein is attached via a tetrasaccharide linker. After biosynthesis, the lipid anchor of the GPIs is often remodeled and in yeast the diacylglycerol part is exchanged for a ceramide. Most oligosaccharides that are linked to a membrane protein do so via either the amino acid side chain amide of asparagins (*N*-linked glycoproteins) or the hydroxyl of serines or threonines (*O*-linked glycoproteins).[8] Until the 1980s it was mainly thought that the primary location of oligosaccharides was extracellular, on the cell surface or its intracellular topological equivalent, the endoplasmic reticulum (ER) and the Golgi apparatus. Also, besides their already known importance as a metabolic source of energy via glycolysis they were mainly thought to perform a structural role in cell biology and physiology. However, research by Hart and others during the eighties proved that proteins in the cytoplasm and nucleus of eukaryotic cells are also extensively glycosylated. Especially, cytosolic and nuclear serine and threonine residues are dynamically modified with an *O*-linked *N*-acetylglucosamine that seems to occur as abundant and often at the same sites as serine/threonine phosphorylation.[9,10]

Figure 3. Overview of interactions between the four molecular building blocks of life **A**: Nucleic acids, **B**: Proteins, **C**: Carbohydrates and **D**: Lipids.[14]



It is now known that the types and amounts of oligosaccharides linked to lipids and proteins on the outside and inside of the cell vary continuously depending on cell types and (patho)physiological conditions. Carbohydrates and their glycoconjugates play essential roles in cell to cell interaction and communication processes, regulation of protein activity and embryonal development amongst others. For instance, glycosphingolipids (GSLs) and glycoproteins on the surface of erythrocytes are at the root of the A/B/O blood antigen system. Dynamic glycosylation and deglycosylation of unfolded proteins secreted into the ER after translation regulates correct protein folding and quality control of protein synthesis. The research into these and other biological functions of carbohydrates and their conjugates, called glycobiology,[11-13] is expanding the understanding of how organisms function and the vital role of carbohydrates herein (Figure 3).[8,14]

1.2 Mammalian (Glyco)sphingolipid Metabolism.

Most of the enzyme catalyzed pathways of SL and GSL metabolism that take place in the ER, Golgi apparatus and lysosomes have been identified. Due to the lipophilic nature of the substrates in this metabolism most of the enzymes involved are integral membrane bound proteins. The following sections will describe SL and GSL metabolism from start to finish with an overview presented in Figure 7 on page 24.

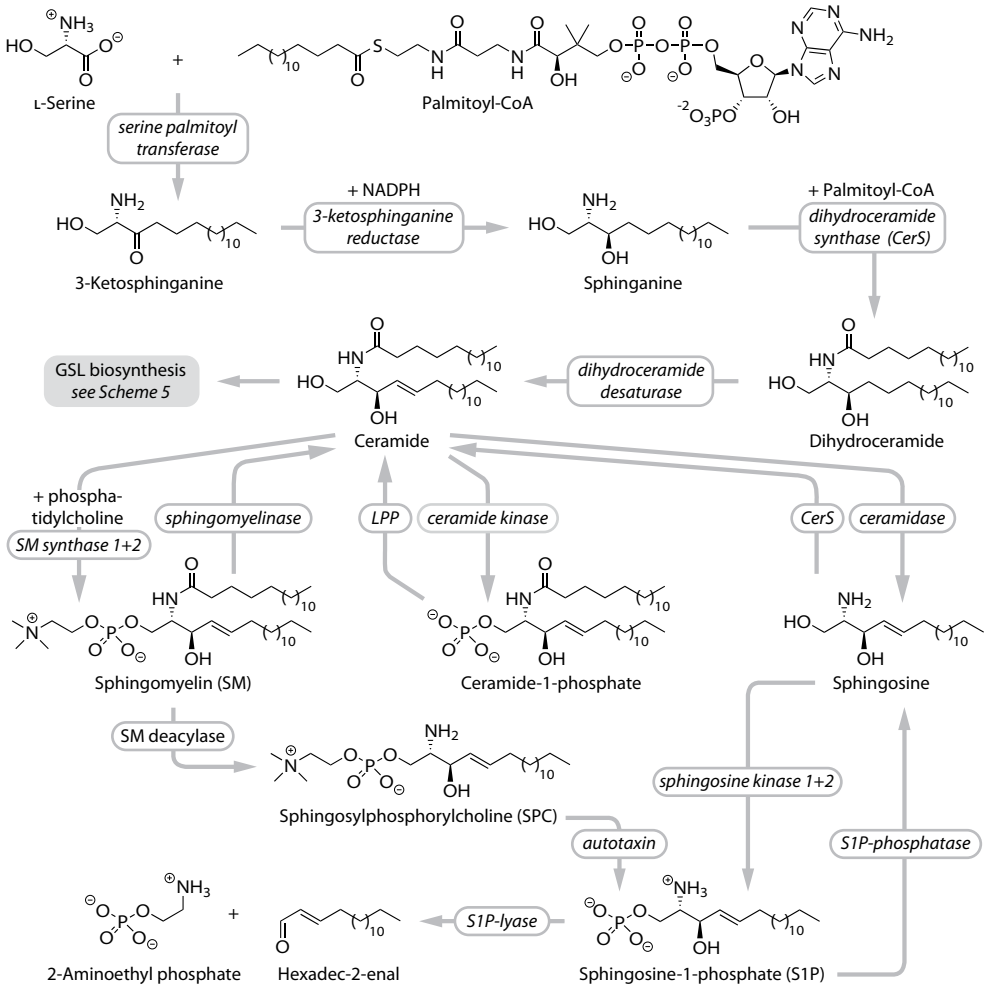
1.2.1 Sphingolipid Metabolism.[15-19] The *de novo* biosynthesis of SLs starts in the cytosolic leaflet of the ER membranes. Here ceramide is synthesized by a sequence of four enzyme catalyzed reactions from L-serine and two molecules of coenzyme A (CoA) activated fatty acid (see Scheme 2 on the next page). Palmitoyl-CoA is almost always used in the synthesis of 3-ketosphinganine. However, varying CoA-activated esters are used in the *N*-acylation of sphinganine. Depending on the tissue and function of SLs and GSLs, the length and saturation of the *N*-acyl tail of ceramide is highly variable in SLs and GSLs. Throughout this thesis however only the more common type of ceramide is depicted, which is made using two molecules of CoA-activated palmitic acid.

Next, the formed ceramide in the ER is a key precursor in the synthesis of five other sphingolipids and two distinct classes of GSLs. First, ceramide is transported by the recently discovered transport protein, CERT, to the cytosolic membrane of the *trans*-Golgi apparatus.[20,21] Here it randomly flip-flops and equilibrates between the cytosolic and luminal side of the *trans*-Golgi membrane. On the luminal inside sphingomyelin synthase 1 converts ceramide into sphingomyelin (SM) by transfer of a phosphorylcholine headgroup from phospholipids. The positively charged SM can no longer flip-flop unassisted. A second enzyme, SM2, is located at the plasma membrane and converts ceramide into sphingomyelin at this location. A neutral and acidic form of the enzyme sphingomyelinase is able to regenerate ceramide from SM. This enzyme is located predominantly in the lysosomes and at the plasma membrane, but is also excreted extracellularly. Alternatively, ceramide can also be phosphorylated by ceramide kinase (CERK). The kinase is transported from the cytosol to the plasma membrane upon specific signals. Its product, ceramide-1-phosphate, can be hydrolyzed back to ceramide by lipid phosphate phosphatase (LPP).[22] Acid, alkaline and neutral ceramidases, located respectively in the lysosome, plasma membrane and Golgi/ER, are capable of deacylating ceramide to generate pools of sphingosine at specific cellular locations. This sphingosine can be phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinase 1 that operates at the plasma membrane and can also be excreted extracellularly. A second sphingosine kinase 2 is located at the ER near the nucleus.[23] S1P levels can be downregulated in turn by S1P-phosphatase. Finally, there is the SL, sphingosylphosphorylcholine (SPC), of which the presence in mammalian cells and plasma has been known for a long time.[24,25] Only recently however has research started to tentatively reveal aspects of its metabolism and biological functions. The extracellularly excreted enzyme, SM deacylase, hydrolyzes the acyl tail from SM to generate SPC. A

dedicated catabolic enzyme for SPC has not been found yet. The plasma circulating enzyme autotaxin however, whose primary function is the formation of lysophosphatidic acid, is capable of converting SPC into S1P.

The cellular orchestration of these complex interconversions between SLs is called the sphingomyelin cycle and is a testament to the role of these SLs in extra- and intracellular signaling pathways. The only currently known exit pathway from this interconnected SL metabolism is degradation of S1P by S1P-lyase in the ER.

Scheme 2. Overview of the biosynthesis and catabolism of mammalian sphingolipids.



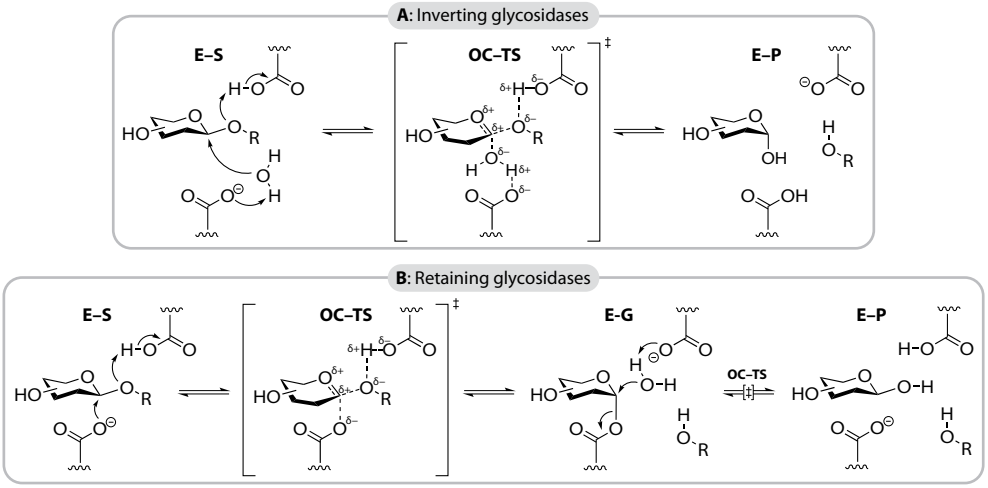
1.2.2 Glycosyltransferase and Glycosidase Mode of Action. Before discussing the biosynthesis and catabolism of GSLs this section will first describe how the two classes of carbohydrate-processing enzymes that regulate these processes work. Glycosyltransferases and glycosidases respectively catalyze the formation and hydrolysis of glycosidic bonds.

The regulation of their expression and activity is responsible for the enormous complexity of carbohydrate structures found in nature. Each of the two classes can be subdivided into families based on similarities in their amino acid sequence. Currently, 113 families of glycosidases and 91 families of glycosyltransferases are known.[26]

The glycosidases[27-29] are the most thoroughly studied of the two. The research until today has shown that there is much diversity in the 3D structure and folding among glycosidases as opposed to a highly conserved active site architecture. The active site of the various glycosidase families functions via one of two fundamental mechanisms that differ in the stereochemical outcome of the reaction and result in either inversion or retention at the anomeric center. The two enzymes, GBA1 and GBA2, responsible for degradation of glucosylceramide are both examples of glycosidases. GBA1 is known to operate as a retaining glycosidase for GBA2 it is not known yet.

Inverting glycosidases operate via a single-displacement mechanism in which water attacks the anomeric center and displaces the aglycone. This reaction is assisted in the enzyme's active site by two carboxylic acid residues from either aspartic or glutamic acid side chains. These side chains are separated by approximately 10 Å that allows simultaneous entry of both the substrate and water in the active site. One of the carboxylic acids acts as a general base for the attacking water molecule and the other as a general acid that protonates the glycosidic bond (Scheme 3A). Displacement of the aglycone by water produces the hemi acetal product via an oxo-carbenium-ion-like transition state (OC-TS).

Scheme 3. General mechanism for inverting (**A**) and retaining (**B**) glycosidases (a β-glycoside is shown here).



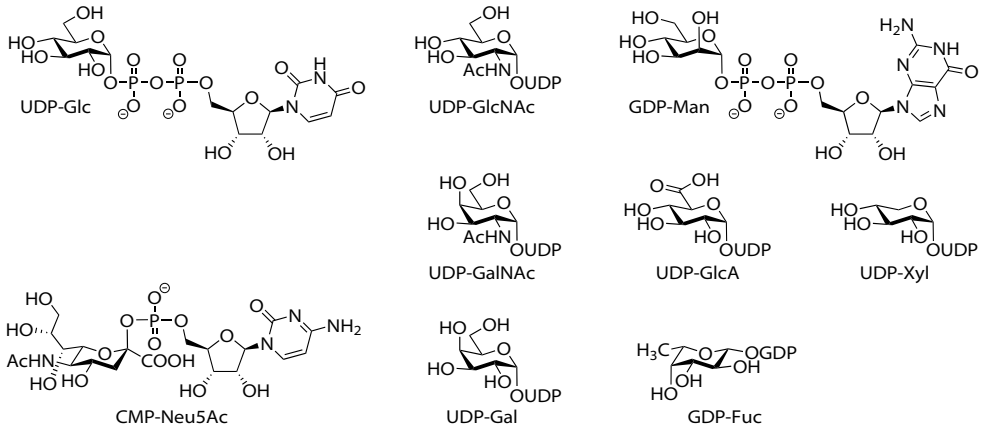
E–S: Enzyme–substrate complex; **OC–TS**: Oxocarbenium-ion-like transition state; **E–G**: Covalent enzyme–glycoside intermediate; **E–P**: Enzyme–product complex; R = substrate aglycon.

In 1953, Koshland proposed that retaining glycosidases operate via a double displacement mechanism that involves a covalent enzyme-glycosyl intermediate. However, Philips

proposed a stabilized oxo-carbenium-ion as intermediate. In 2001, Withers and co-workers were able to confirm the Koshland model by isolation and characterization of the covalent intermediate for the retaining glycosidase, hen egg-white lysozyme.[30] Extensive studies confirmed that this mechanism is used by almost all retaining glycosidases and that it is catalyzed by two carboxylic acid residues that are approximately 5 Å apart. The first step, much the same as in the inverting glycosidases, involves the protonation of the leaving group oxygen by one of the carboxylic acid residues. However, the narrower active site does not accommodate water at this stage and instead the closely positioned second carboxylate residue attacks at the anomeric center to produce a covalent enzyme-glycosyl intermediate. The aglycon diffuses out of the active site and in a second step a water molecule attacks the anomeric center of the intermediate under base catalysis of the remaining carboxylate to achieve hydrolysis (Scheme 3B).

Glycosyltransferases[31] use activated donor carbohydrates that contain a (substituted) phosphate as leaving group. Mammalian glycosyltransferases almost exclusively catalyze glycosidic bond formation using one of nine activated carbohydrate donors that contain an anomeric nucleoside (di)phosphate (Figure 4). Nucleotide carbohydrate-dependent glycosyltransferases are referred to as Leloir enzymes.

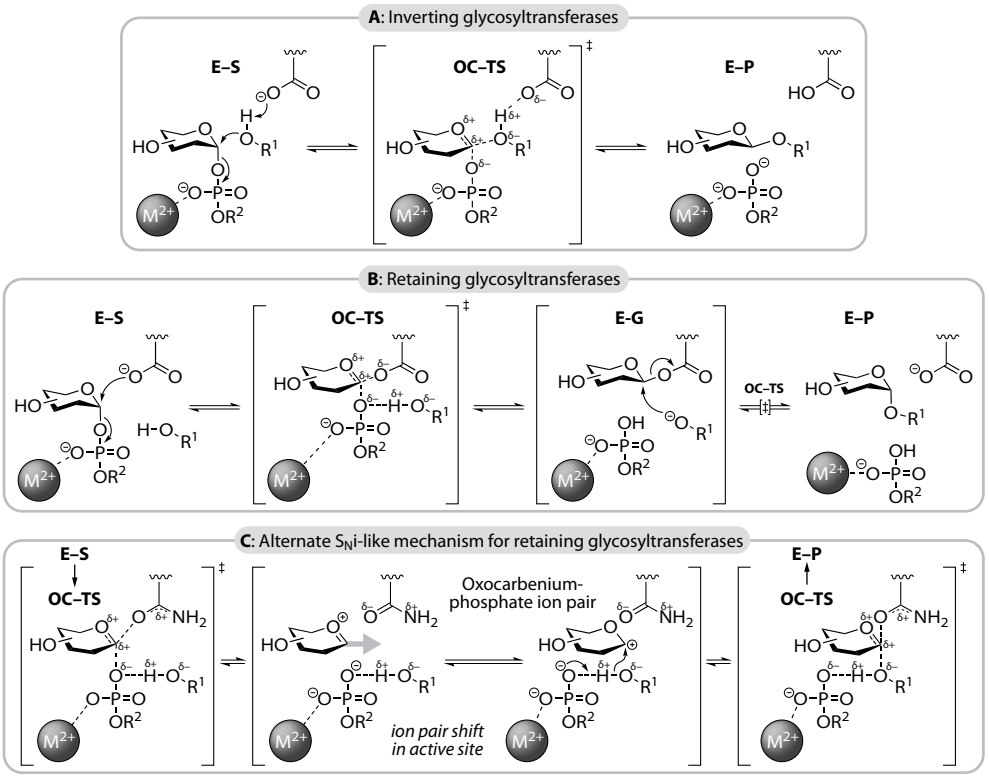
Figure 4. The nine nucleotide-carbohydrate donors used by mammalian glycosyltransferases.



Structural and mechanistic investigations of transferases have lagged behind those of glycosidases. However, the available X-ray crystal structures and amino acid sequences have already indicated that contrary to glycosidases only two common structural folds exist among transferases. The GT-A and GT-B fold differ in the topology of the so-called Rossmann fold, which is a common fold in proteins that bind nucleotide containing ligands. Just as for glycosidases, two mechanistic classes can be defined that are differentiated by the stereochemical outcome of either inversion or retention at the anomeric center of the reaction product. This mechanistic subdivision is unrelated to the presence of a GT-A or GT-B fold. Departure of the nucleotide-phosphate leaving group in GT-A fold transferases is typically facilitated by a divalent metal cation that

is coordinated in the active site by an aspartic-X-aspartic (DXD) motif. GT-B fold transferases use appropriately placed positively charged amino acid residues in the active site instead of a metal cation.

Scheme 4. General mechanism for inverting (**A**) and retaining (**B**) glycosyltransferases and an alternate S_Ni mechanism (**C**) for retaining transferases (an α-glycoside donor and GT-A transferase are shown here).



E–S: Enzyme–substrate complex; **OC–TS**: Oxocarbenium-ion-like transition state; **E–G**: Covalent enzyme–glycoside intermediate; **E–P**: Enzyme–product complex; M^{2+}: divalent magnesium or manganese cation. R^1 = acceptor hydroxyl; R^2 = donor nucleoside/nucleoside monophosphate.

The mechanism of inverting transferases involves a direct displacement S_N2-like mechanism and seems conserved among most GT-A/B transferases of this type (Scheme 4A).[31] The enzyme, GCS, responsible for glucosylceramide biosynthesis is an inverting glycosyltransferase. The mechanism of retaining transferases remains less clear. A double displacement mechanism, similar to inverting glycosidases has long been thought to occur, but trapping of the covalent enzyme-glycosyl intermediate that is required for proof of this mechanism has so far eluded researchers (Scheme 4B). There also seems to be a lack of conserved architecture in the active site where the nucleophilic amino acid side chain should be positioned. In the case of the LgtC retaining transferase from the *Neisseria meningitidis* bacteria the primary amide from glutamine was located at this position but its mutation to an alanine did not abolish all transferase activity.[32]

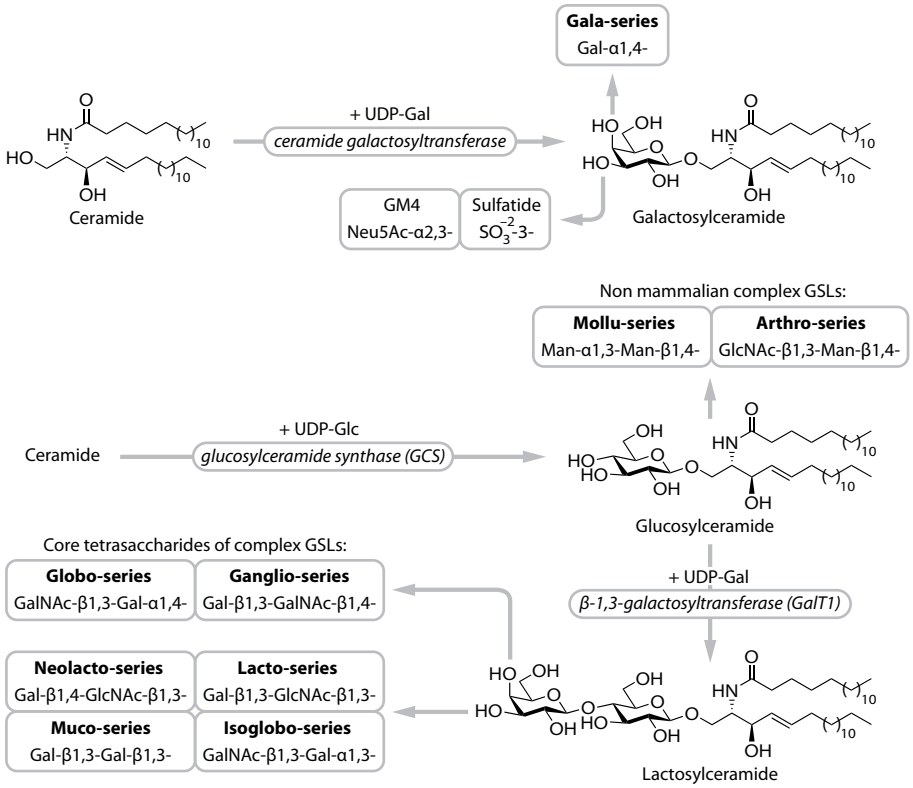
An alternate S_Ni-like (S_N1 internal return variation) mechanism for retaining transferases is gaining support among researchers and involves a discrete short-lived ion pair intermediate (Scheme 4C).[31] In this mechanism the nucleotide-diphosphate leaving group acts as a base for the acceptor and coordinates with it. The positively charged oxo-carbenium ion forms a discrete ion pair with the negatively charged phosphate-acceptor complex on the same face as where the phosphate is disconnected. A shift of this ion pair in the active site then allows attack of the acceptor from the same direction as the leaving group – resulting in retention. Due to the variation in the active site architecture of retaining glycosyltransferases it is likely that both of the discussed mechanisms (B and C) and hybrids of them occur depending on the transferase.

1.2.3 Mammalian Glycosphingolipid Biosynthesis. Besides the previously discussed SLs, ceramide is transformed into two distinct monosaccharide-containing GSLs. After its synthesis on the cytosolic side of the ER membrane, ceramide equilibrates to the luminal side via random flip-flopping. A recent study showed that when ceramide was inserted into the external leaflet of a phosphatidylcholine unilamellar vesicle it equilibrated to the inner leaflet with a half-time ($t_{½}$) below 1 min at 37 °C.[33]

Once ceramide arrives at the luminal side of the ER it is transformed into galactosylceramide by ceramide galactosyltransferase (CGalT).[34,35] This GSL is further diversified into the Gala-series of GSLs via either sulfation or glycosylation with Neu5Ac at its 3-*O*-position or further extension to oligosaccharides at its 4-*O*-position via a second α-linked D-galactose (Scheme 5).[17] ER localized ceramide is transported via vesicular transport to the cytosolic side of the *cis*-Golgi apparatus membrane. Here the membrane bound glycosyltransferase, glucosylceramide synthase (GCS), catalyzes the glycosylation of the primary hydroxyl in ceramide using UDP-glucose as donor glycoside. Interestingly, a recent study indicated that a region of the ER that is closely associated with mitochondria also shows an enzymatic activity capable of generating glucosylceramide.[36]

As mentioned, glucosylceramide synthase is an inverting transferase (Family 21; GT-A fold).[26] Its cDNA sequence was reported by Ichikawa in 1996 and encodes for a 45 kDa protein.[37] It possesses an *N*-terminal hydrophobic transmembrane stretch that anchors the enzyme to the cytosolic face of the Golgi membrane together with a hydrophobic loop near the *C*-terminal region.[38,223] A study by Pagano and co-workers has shown that GCS forms hetero dimers or oligomers with an unidentified 15 kDa protein.[39] Via site-directed mutagenesis and sequence comparisons with other transferases they also identified several active site amino acid residues and an amino acid near the *N*-terminus (His-193) that was important for substrate binding and inhibition of GCS by the inhibitor, PDMP (**7**; Figure 9 page 28).[40] GCS also possesses a DXD metal coordinating motif, but it appears to not require a divalent metal for catalysis.[41]

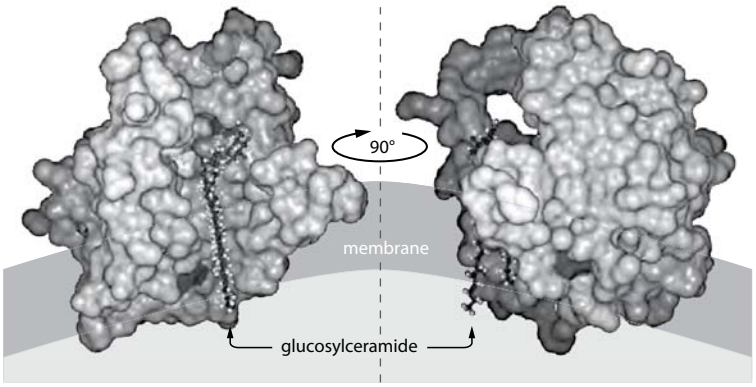
Scheme 5. Overview of the biosynthesis of (mammalian) glycosphingolipids.



Due to its tight membrane association no X-ray crystal structure of GCS has been determined yet. Based on the available data, Butters and co-workers did develop a computational model of GCS that is depicted in Figure 5 on the next page and shows a partly membrane immersed ceramide binding groove.[42] The product of GCS action, glucosylceramide, occupies a key position in the biosynthesis of GSLs because besides the Gala-series all more complex GSLs are derived from it. The fact that both GCS and glucosylceramide face the cytosolic side of the cellular membranes are distinguishing features in GSL biosynthesis: further synthesis of complex GSLs takes place exclusively on the inside (lumen) of the Golgi apparatus. Thus, glucosylceramide needs to traverse the Golgi lipid bilayer.

When glucosylceramide is introduced to the outer leaflet of a model membrane it only slowly flip-flops across unassisted ($t_{½}$ = 5 h at 20 °C). However, in the Golgi-apparatus membrane glucosylceramide undergoes rapid transbilayer movement ($t_{½}$ = 3 min at 20 °C). Studies indicate that an ATP-independent Golgi-localized 'flippase' protein is responsible that however has not been identified yet.[43] Other research has shown that the ATP-dependant P-glycoprotein multidrug transporter located throughout the cell is capable of acting as a rapid flippase for fluorescently labeled (NBD) glucosylceramide, galactosylceramide and sphingomyelin, but not lactosylceramide.[44]

Figure 5. Two views of a computational model of GCS with glucosylceramide bound in cleft.[42]



It has been determined that the majority of complex GSLs are synthesized at the *trans*-Golgi as opposed to glucosylceramide at the *cis*-Golgi.[45] It had been assumed that glucosylceramide was transported to the *trans*-Golgi by vesicular flow. However, De Matteis and co-workers recently reported that the protein FAPP2 is responsible and essential for this relocation.[46] Van Meer and co-workers reported that FAPP2 also transports glucosylceramide to the ER and that the closely related GLTP transport protein is capable of transporting it to the cell surface.[47]

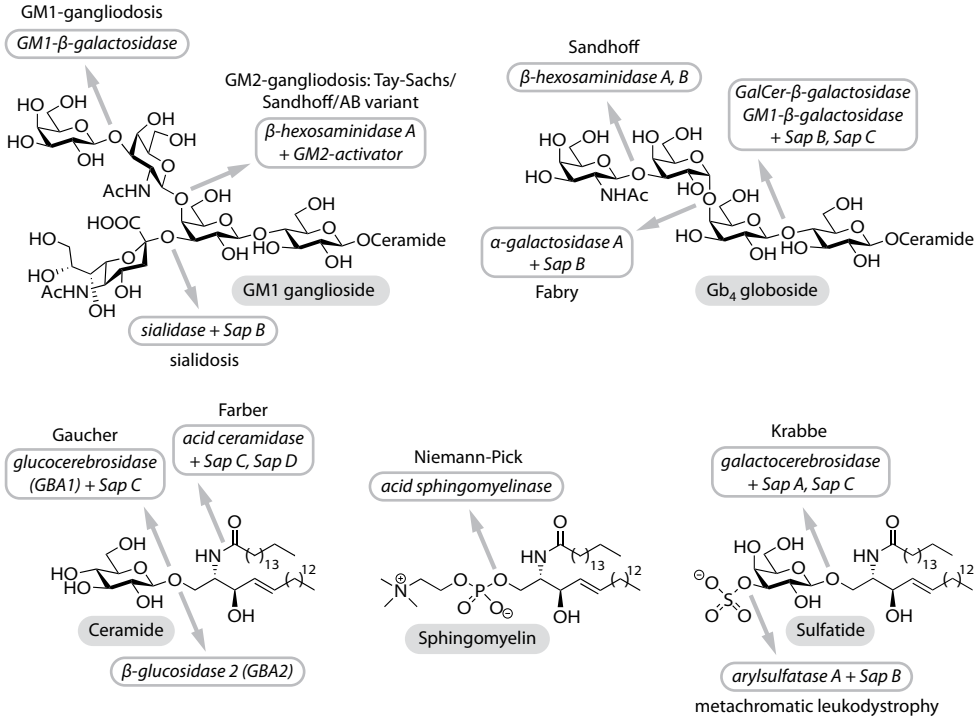
Having arrived at the *trans*-Golgi and flipped to the inside, the biosynthesis of GSLs continues with the synthesis of lactosylceramide by GalT1 (Scheme 5). Lactosylceramide is sequentially extended at either the 3-*O*-positon or the 4-*O*-position in a stepwise fashion by a panel of specialized transferases. This eventually results in the six distinct GSL-series that together form the cellular pallet of hundreds of unique GSLs in mammals – the core tetrasaccharides of these are depicted in Scheme 5.[48,49] Most of these GSLs consist of alternating and branched combinations of α- or β-linked glucose, galactose, *N*-acetylglucosamine and *N*-galactosamine. At their non-reducing end numerous of these complex GSLs are terminated with either L-fucose or acidic Neu5Ac. Two other non-mammalian series of complex GSLs also exist and originate from a β1,4-linked mannopyranoside to glucosylceramide. Complex GSLs in the Mollu-series[50] have been isolated from freshwater bivalves (*e.g.* molluscs) and GSLs from the Arthro-series[51] from several species of arthropods (*e.g. Drosophila* flies).

After their biosynthesis GSLs are transported to the cell surface by exocytosis where they perform their functions, which are described in sections 1.3.1 to 1.3.11. The maintenance and change of GSL patterns on the cell surface requires a delicate balance between GSL biosynthesis and their degradation (catabolism).

1.2.4 Mammalian (Glyco)sphingolipid Catabolism. The catabolism of GSLs starts by endocytosis of GSL containing regions of the plasma membrane. GSL containing regions of the membrane associate with the membrane protein caveolin-1.[52] Grouping of this

protein induces a flask-like invagination of the plasma membrane – called a caveolae – that is taken up by the cell and enters endocytotic vesicular flow.[53] The endosomes can be transported to the Golgi for alteration of their SL and GSL content or to the lysosomes. The GSL containing domains are thought to form smaller vesicles inside the endosomes (Figure 7). These so-called multi-vesicular bodies are targeted to the lysosome for degradation. After merger with a lysosome the catabolism of GSLs starts.[54]

Scheme 6. Overview of mammalian (glyco)sphingolipid catabolism. Responsible enzyme/glycosidase and activator protein are indicated at glycosidic linkage. The catabolism associated hereditary diseases are indicated above/below and discussed in section 1.3.3.

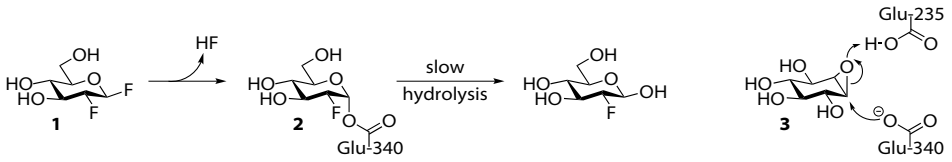


Catabolism takes place at the membrane surface of internal lysosomal membrane vesicles (Figure 7). The perimeter membrane of the lysosome is protected from degradation by a glycocalix composed of lysosome resistant glycoproteins.[54] Carbohydrate residues from the non-reducing end of the GSL oligosaccharides are sequentially cleaved off one carbohydrate residue at a time by the action of exo-glycosidases (Scheme 6). Contrary to the biosynthetic enzymes all catabolic glycosidases are non-membrane bound and dissolved in the lysosome. However, their GSL substrates are embedded in intralysosomal membranes. Therefore GSLs with less than four carbohydrate residues require the presence of specific (glyco)sphingolipid activator proteins (Sap) that assist the glycosidases in interacting with their target substrate. Five such proteins are currently known, saposin-A, -B, -C, -D and the GM2-activator protein. It is telling of their role in

catabolism that for *in vitro* assays of these glycosidases their action can be replaced by detergents. Scheme 6 provides an overview of the glycosidases and activator proteins associated with GSL degradation.[17,54]

The penultimate step in GSL catabolism is the hydrolysis of the β-glycosidic bond in glucosylceramide by glucocerebrosidase (GBA1) to yield D-glucose and ceramide. The retaining glycosidase GBA1 (family 30) is a ~65 kDa protein in its glycosylated form and the activator protein saposin C is essential for its *in vivo* functioning.[54] In 1994, Withers and co-workers identified the active site catalytic nucleophile as the side chain carboxylate of glutamic acid-340.[55] This was ascertained by feeding the enzyme mechanism-based inhibitor **1** that reacts with this nucleophile to provide a stable covalent enzyme-'substrate' intermediate (**2**) that could be analyzed by mass spectrometry (Scheme 7). Inhibitor **1** achieves this via the electron negative fluorine atom on the 2-position that drastically slows down the second hydrolysis step by increasing the required activation energy to the oxocarbenium-ion-like transition state. This also holds for the first step, but here the reactive anomeric fluoride leaving group compensates for this. In 2003, Futerman and co-workers published the first X-ray crystal structure of GBA1.[56] Later a crystal structure of GBA1 was reported with the irreversible covalent inhibitor, conduritol-B-epoxide (**3**; CBE), bound to Glu-340 (Scheme 7). This study also confirmed Glu-235 as the acid/base catalyst of GBA1.[57]

Scheme 7. Overview of the action of mechanism based glycosidase inhibitors **1** and CBE (**3**).



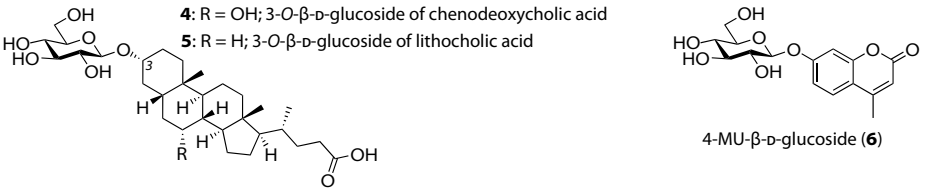
Interestingly, Futerman and co-workers also performed a structural comparison of GBA1 with closely related glycosidases and found a close correspondence with a bacterial xylanase.[58] Although mammals also produce D-xylose containing oligosaccharides (proteoglycans), a mammalian xylanase has so far not been found yet. With β-xylanase activity having been detected in rat lysosomes[59] and GBA1 being able to hydrolyze artificial β-xylosides,[60] GBA1 might represent a candidate for this missing xylanase.

Recently, Saenger, Maier and co-workers published the X-ray crystal structure of the GBA1 activator, saposin C.[61] This study and another report[62] propose that saposin C assists by a dual action. First, Sap C associates with the glucosylceramide carrying intralysosomal vesicles. When two Sap C bound vesicles encounter each other the two Sap C proteins dimerize via domain swapping that in turn induces the two vesicles to fuse together (clip-on model).[61] Additionally, in areas of these vesicles where Sap C proteins bind and congregate they decreases membrane thickness and cause perturbed membrane edges that facilitates interaction of GBA1 with glucosylceramide.[62] Sap C has also been shown to directly bind to GBA1 and thereby increase its enzymatic activity.[224]

Besides glucosylceramide based GSLs, the Gala-series GSLs are also degraded in the lysosome (Scheme 6). All these GSLs eventually yield a pool of monosaccharides and ceramide. Deacylation of ceramide to a fatty acid and sphingosine by acid ceramidase represents the final catabolic step of GSLs in the lysosome. Sphingosine, the fatty acids and the monosaccharides are all recycled by the cell.

Contrary to all other GSLs, glucosylceramide is also catabolized via a second non-lysosomal pathway. Aerts and co-workers reported this hydrolytic activity in 1993.[63] Recently, the activity was identified as β-glucosidase 2 (GBA2).[60,64] GBA2 was previously already known for its capacity to hydrolyze bile acid glucosides (*e.g.* **4** and **5**; Figure 6) and extensively investigated to this end by Matern and co-workers.[65-67] GBA2 is a 105 kDa protein with a transmembrane region and has not been assigned yet to a specific family of glycosidases. Contrary to GBA1, it is not sensitive to inhibition by CBE (**3**).[60] The enzyme has a neutral pH optimum opposed to the acidic optimum of GBA1. *N*- and *C*-terminal fusion proteins of GBA2 with green fluorescent protein show the highest fluorescence near the plasma membrane.[60] Addition of the fluorescent substrate, 4-methylumbelliferyl-β-D-glucoside (**6**; Figure 6), to the medium of cell cultures that express GBA2 shows almost instantaneous GBA2 activity that indicates it might be anchored to the outer plasma membrane.[60] GBA2 was also found to be enriched in the apical membrane of epithelial cells.[68] Additionally, experiments with fluorescently labeled glucosylceramide showed that the ceramide generated by GBA2 action was rapidly converted to sphingomyelin.[60] This might be explained if GBA2 is co-localized with the enzyme SMS2 on the outer plasma membrane. The function of GBA2 is currently not known. However, inhibition of GBA2 activity in certain strains of mice is associated with impaired spermatogenesis, a result that is confirmed in studies with a GBA2 knock out mouse model.[64,69,70]

Figure 6. Structure of 4-methylumbelliferyl-β-D-glucoside (**6**) and two bile acid glucosides (**4** and **5**).



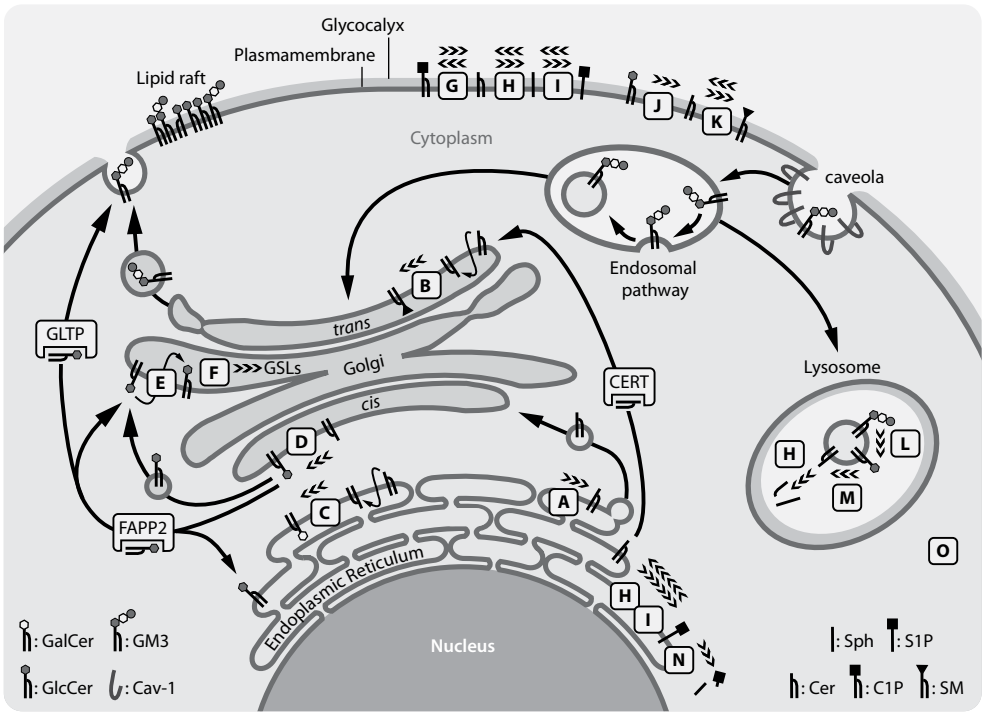
Two other distinct glycosidases have also been implicated in glucosylceramide catabolism, but their activity has not been fully substantiated yet**.** Two publications have reported that the β-glucosidase, LPH, is capable of hydrolyzing glucosylceramide. LPH is a ~300 kDa retaining glycosidase (family 1) that is sensitive to CBE (**3**). The enzyme is membrane bound at the outer plasma membrane and is exclusively expressed in the microvilli of intestinal epithelial cells. LPH is also able to hydrolyze galactosylceramide, lactosylceramide and glucosyl- and galactosylsphingosine, but not GM1 ganglioside (structure in Scheme 6).[71,72] LPH might therefore play a role in the intestinal digestion of food derived GSLs. Humans on an typical Western diet ingest roughly 300 mg of (glyco)

sphingolipids per day, mainly by consumption of egg, dairy and meat products.[73]

The β-glucosidase, GBA3, is a cytosolic retaining glycosidase (family 1) with a broad substrate specificity that includes artificial hydrophobic β-glucosides and β-galactosides. Recently determined X-ray structures of GBA3 revealed its two catalytic residues (nucleophile: Glu-371; acid/base: Glu 165). The ~60 kDa protein is CBE (**3**) insensitive and expressed in the cytosol and operates optimally at a neutral pH. Its reported ability to degrade glucosylceramide is still topic of debate.[74,75]

The facts contained in the previous sections about (glyco)sphingolipid biosynthesis and catabolism, with emphasis on the cellular topology and dynamics, are summarized in Figure 7. Most of the enzymes involved in (glyco)sphingolipd metabolism have been elucidated. The next challenge lies in understanding the complex dynamics and topology of this metabolism, which plays a crucial part in the functions of SLs and GSLs in both health and disease.[18]

Figure 7. Cellular topology and dynamics of mammalian SL/GSL biosynthesis and catabolism.



A: *De novo* synthesis of ceramide; **B**: Synthesis of sphingomylin (SM) by SMS1; **C**: Synthesis of GalCer by CGalT; **D**: Synthesis of GlcCer by GCS **E**: GlcCer flippase; **F**: Synthesis of lactosylceramide and complex GSLs (*e.g.* GM3); **G**: Synthesis of ceramide-1-phosphate (C1P) by CERK and hydrolysis by LPP; **H**: Deacylation of ceramide to sphingosine (Sph) by ceramidase; **I**: Synthesis of sphingosine-1-phosphate (S1P) by sphingosine kinase 1 and hydrolysis by S1P-phosphatase; **J**: Hydrolysis of GlcCer by GBA2 (by LPH in intestines?); **K**: Synthesis of SM by SMS2 and hydrolysis by sphingomyelinase; **L**: Stepwise hydrolysis of complex GSLs; **M**: Hydrolysis of GlcCer by GBA1; **N**: Degradation of S1P by S1P-lyase; **O**: GlcCer hydrolysis by GBA3 (?); Cav-1: Caveolin-1 (GSL endocytosis).

1.3 Functions of (Glyco)sphingolipids in Health and Disease.

GSLs and SLs were initially thought to be merely structural membrane components. However, the large heterogeneity in SL and GSL structures due to variation in the sphingosine base, *N*-acylation and glycosylation pattern suggests a high degree of functional complexity. Research over the past decades has proven that (glyco) sphingolipids are involved in many (patho)physiological process. As in many other areas of biology much has been learned about SLs and GSLs from the study of diseases where their metabolism and functioning has gone awry. The following sections describe some of the biological functions that are attributed to SLs and GSLs in health and disease with a focus on GSLs. For several of the GSL associated diseases, inhibitors of glucosylceramide metabolism are being used or investigated for therapeutic applications and these will also be summarized.

1.3.1 Sphingolipids and Cellular Signaling. The SLs have been implicated in numerous intra- and extracellular signaling processes. Sphingomyelin is the most common SL and comprises as much as 30% of the total membrane lipids in certain tissues.[76] In the plasma membrane, sphingomyelin predominantly associates with cholesterol and GSLs to form microdomains. Besides this role in membrane structure, sphingomyelin mainly seems to serve as a reservoir and precursor for the site specific generation of other bioactive SLs. This role as a precursor is facilitated by the fact that it can be synthesized at two different locations in the cell and converted to ceramide by the neutral, alkaline and acid sphingomyelinases distributed throughout the cell. As shown in Scheme 2, ceramide can be converted to ceramide-1-phophate and via sphingosine to sphingosine-1-phosphate. After exposure to heat shock, oxidative stress and other damaging conditions, cells produce elevated levels of ceramide among other responses. These elevated ceramide levels have been implicated as second messengers in signal transduction pathways that lead to cell death (apoptosis).[18] Less is known about the function of ceramide-1-phosphate, but it is implicated in inflammatory signaling pathways and as a promoter of cell survival.[22,77] Sphingosine is thought to serve as an intracellular regulator for the activity of several kinases. Sphingosine-1-phosphate is an extracellular ligand for several G-protein coupled receptors and is involved in many signaling pathways involving cell migration, cell growth and vascular maturation (angiogenesis). In general, sphingosine-1-phosphate has an opposite effect to ceramide in that it promotes cell growth and survival.[22,77] Finally, the less well studied sphingosylphosphorylcholine has been shown to stimulate cell division and has also been implicated in pro-inflammatory signaling pathways.[24,25] It appears that a delicate balance between the various levels of these bioactive SLs and where in the cell they are generated is essential for maintaining health.

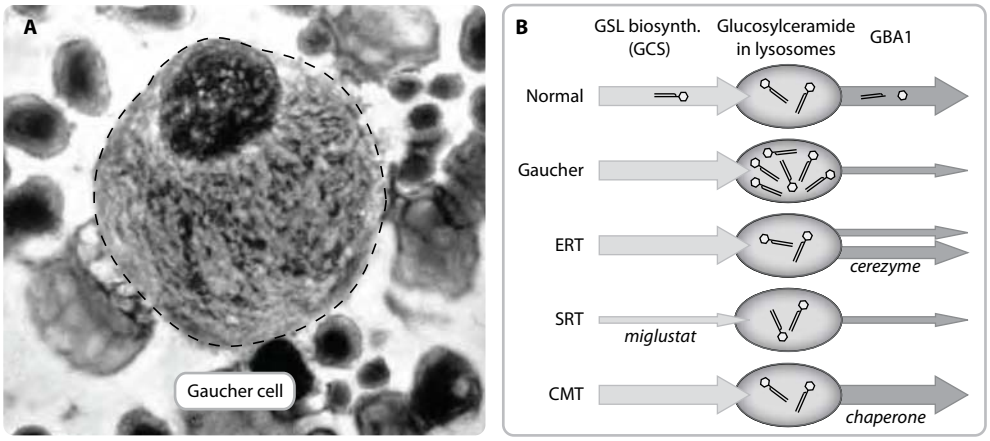
1.3.2 Glycosphingolipids and Lipid Rafts. The functions of GSLs on the cell surface can be roughly divided into two basic functions: involvement in cell adhesion/recognition processes by interactions with GSLs and lectins on other cells, and in modulation of

signal transduction by influencing receptor proteins on the cell surface. During the late eighties it was discovered that GSLs and SLs don't always distribute homogeneously in the outer plasma membrane. Instead they often form defined microdomains, also called lipid rafts, together with cholesterol.[78-81] Many proteins have been found to be associated with these microdomains including most GPI anchored proteins, flotillins, caveolins, certain G proteins coupled receptors, the epidermal growth factor receptor and the insulin receptor. The microdomains exist in a gel-like liquid ordered phase (l_o) that have a lower diffusion rate then the surrounding liquid disordered (l_d) phospholipid-rich plasma membrane. Initially, these lipid rafts were isolated by extraction of membranes at 4 °C in the presence of specific detergents and were therefore called detergent resistant microdomains (DRMDs). The existence of lipid rafts was questioned for a long time with the valid question being wether the composition and dynamics of detergent extracted membranes at 4 °C resembled their composition at 37 °C in live cells. However, since then evidence in favor of lipid rafts has increased and they have also been visualized in living cells by other less intrusive techniques like Förster/fluorescence resonance energy transfer (FRET) and single molecule fluoresence. With their existence confirmed the following current definition of lipid rafts illustrates the future challenges in their further characterization: membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Although most current research on rafts focuses on the outside of the cell, their original proposed role was in intracellular sorting. Using BODIPY labeled GSLs, Pagano and co-workers have shown that lipid rafts also occur intracellularly and that GSLs in these lipid rafts play an important role in caveolae mediated endocytosis and endosomal sorting of many cellular proteins.[82,83]

1.3.3 Lysosomal Sphingolipidoses. The importance of SLs and GSLs in mammalian physiology is underlined by the fact that virtually no hereditary diseases exist that impair their biosynthesis. Systemic deletion of the GCS gene in a mouse model resulted in lethality during the early stages of embryogenesis.[84] On the contrary, a hereditary disease exists for almost every catabolic step in SL and GSL degradation (see Scheme 6 on page 21). Collectively known as the sphingolipidoses, they are each caused by mutations in genes that encode the lysosomal SL or GSL catabolic enzymes.[17,54] The mutation causes lowered lysosomal levels of the enzyme or impairment of its catalytic activity, thus leading to deficient degradation and accumulation of the enzyme's substrate in the lysosomes. The most prevalent among the sphingolipidoses is Gaucher disease with an incidence of about 1:50000 among the general population.[85-88] Gaucher disease is an autosomal recessive disorder caused by mutations in the GBA1 gene. Impaired degradation of glucosylceramide occurs in all cells of the body, but pathologic accumulation in the lysosomes mainly occurs in the macrophages. These cells have a heightened influx of GSLs due to their phagocytosis of senescent blood cells and other necrotic cells in the body. The severe storage of glucosylceramide in the macrophages results in an increase

in size (~20 » ~100 μM) and a transformation into so-called Gaucher cells that cause the observed pathology (Figure 8A).

Figure 8 A: Transformed macrophage; **B**: Schematic overview of Gaucher disease and its potential therapies.



The clinical symptoms were first described by Philippe Gaucher in 1882 and currently over 200 distinct mutations in the GBA1 gene are known to cause Gaucher disease. The symptoms observed in patients with these mutations vary from mild to severe and correlate closely with residual lysosomal GBA1 activity. Mutations that cause complete GBA1 deficiency result in skin permeability issues and result in lethality either prenatally or shortly after birth.[88] Gaucher disease symptoms are categorized into three different phenotypes (type I, II and III). Type I Gaucher disease is the most common and its symptoms among others can involve enlargement of liver and spleen, weakened bones and anaemia. The more severe types II and III also involve neurological symptoms. Type II is the most severe form and symptoms set in shortly after birth and lead to death within the first year of life. Type III manifests itself during later childhood and shows a slower progression of neurological complications. Scheme 6 provides an overview of the other sphingolipidoses for which the details are not discussed here but are extensively reviewed in literature.[17,54] Contrary to Gaucher disease, in some of these sphingolidoses it is a deficiency in the activator protein that causes lysosomal accumulation. In Krabbe and Fabry disease an *N*-deacylated metabolite of the primary storage material is responsible for many of the clinical symptoms – globotriaosylsphingosine[89] in Fabry disease and psychosine[54] in Krabbe disease.

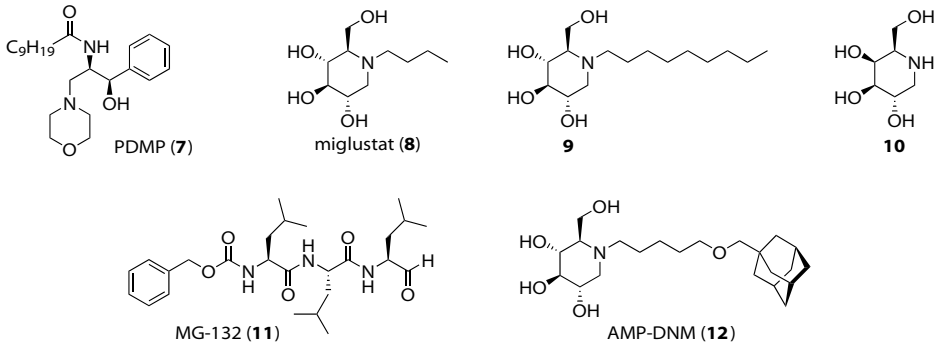
1.3.4 Therapeutic Strategies for Gaucher Disease. Many of the sphingolipidoses remain untreatable, but especially for type I Gaucher disease several therapies are available and also under development. The theoretical basis for all these therapeutic approaches is the 'threshold theory' which states that the rate of substrate influx into the lysosomes and the degradation capacity determine the onset and severity of the disease (Figure 8B). In

a cellular model the overt onset of glucocylceramide storage in macrophages was shown to occur only when overall GBA1 activity in the lysosomes drops below ~15% of normal activity.[90] Therefore even minor improvements of lysosomal GBA1 activity can result in major improvements for patients.

The first therapy to be developed for Gaucher disease was enzyme replacement therapy (ERT). In the 1970 and 80s, Brady and co-workers developed the intravenous administration of GBA1 that was purified from human placenta tissue to Gaucher patients.[91,92] A proportion of this functional GBA1 enzyme ends up in the lysosomes of Gaucher cells and thereby temporarily increases the degradation capacity (Figure 8B). During the 1990s, the placenta derived enzyme was replaced by a recombinant GBA1 derivative.[93] At present about 5000 type I Gaucher patients receive ERT with this form of GBA1, called cerezyme. Drawbacks of this therapy are its intravenous delivery, its high costs and the impossibility to treat Gaucher patients with neurological symptoms due to the inability of cerezyme to pass the blood-brain barrier.

Already in 1980, Radin and Vunnam proposed the concept that downregulation of glucosylceramide influx into the lysosomes by inhibiting GCS with inhibitor PDMP (**7**) could alleviate the symptoms of Gaucher disease (Figure 8B).[94] In 1994, Platt and co-workers reported the inhibition of GCS by the *N*-butylated 1-deoxynojirimycin derivative **8** and the use of this effect to lower glucosylceramide accumulation in an *in vitro* model of Gaucher disease.[95] The concept and results with **8** were further developed into what is now called substrate reduction therapy (SRT). After clinical trials in Gaucher patients, **8** (now called miglustat or zavesca) was approved as an orphan drug in SRT for Gaucher disease in 2002. The drug is administered orally to type I Gaucher patients for whom ERT is unsuitable. Close to a hundred patients are presently receiving SRT with **8**.[96-98]

Figure 9. Structures of compounds mentioned in sections 1.3.4 to 1.3.11.

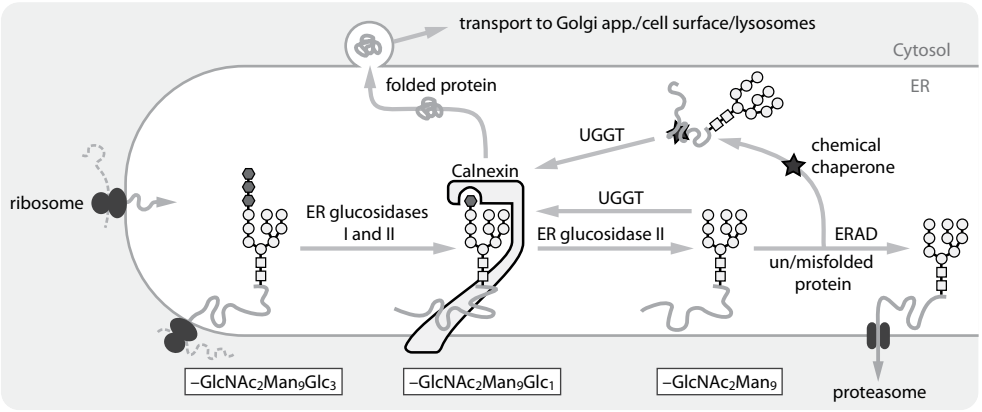


The clinical studies with miglustat and longer *N*-alkyl chain derivatives have shown that they are metabolically stable and excreted mostly via the kidney (plasma $t_{½}$ = 6.3 h). Oral administration of **8** (100 mg, 0.46 mmol), three times a day, results in a steady-state plasma concentration of 5 μM after ~5 weeks that is sufficient to partially inhibit GSL

biosynthesis.[97] The entry of these iminosugars into the cell (within 1 minute) appears to occur via passive diffusion or endocytosis and membrane flip-flopping. A longer *N*-alkyl chain (like in **9**) results in more protein and membrane binding and this phenomenon might be one of the reasons for their higher inhibition potency for GCS when compared to **8**.[99] Experiments with radiolabeled iminosugar derivatives showed that the compounds were able to pass the blood-brain barrier.[100] This represents a major advantage over ERT, because it enables the potential treatment of Gaucher disease with a neurological symptoms – currently 30 type III patients receive SRT with **8**. The radiolabel study also revealed that longer *N*-alkyl chain iminosugars displayed a slower penetration into the body from the intestine, but eventually did reach higher overall levels. The effect of lowering GSL levels with **8** is reversible and the levels show full recovery within 24 h after removal of **8**. The toxicity of **8** is low and the cellular LD_{50} lies in the high mM-range.[98] The side effects of **8** at therapeutic concentrations are predominantly caused due to inhibition of other carbohydrate-processing enzymes. The major side-effects associated with SRT for Gaucher disease are related to the ability of **8** to inhibit glycosidases in the intestinal microvilli that results in diarrhea, flatulence and abdominal bloating. Miglustat is also able to inhibit ER glucosidases I and II that play a major role in the quality control of protein synthesis and folding. However, this requires concentrations of **8** in 10000-fold excess compared to the required dose for inhibition of GCS. It is the quality control of protein synthesis and folding in the ER where a potential third therapeutic opportunity for treating Gaucher disease and related sphingolipidoses exists.

After transcription of the GBA1 gene in the nucleus the generated messenger RNA is translated by ribosomes that are bound to the cytosolic membrane of the ER. The synthesized protein is secreted into the luminal compartments of the ER. Here the largely unfolded protein is decorated with a distinct *N*-linked oligosaccharide (*N*-GlcNAc-$_2$Man$_9$Glc$_3$) after which it enters the folding/quality control machinery. The protein folds into its active conformation in which it is assisted and monitored by various chaperone proteins (see Figure 10 on the next page). Many of the Gaucher associated mutations of the GBA1 gene result in improper or retarded folding in the ER. Consequently, many GBA1 proteins never reach the lysosome and are instead rejected, secreted into the cytosol, and degraded by the proteasome. The third therapy that is currently in development for Gaucher disease is called chaperone mediated therapy (CMT).[85,101,102] It is based on the concept that an active-site directed inhibitor of an enzyme (a pharmacological chaperone) can already bind an enzyme during the folding process in the ER and thereby stabilize the proper protein conformation. This stabilization of the critical region of the enzyme during folding might result in a larger percentage of properly folded enzymes that reaches the lysosome (Figure 8B).

Figure 10. Overview of ER protein folding + quality control and the putative action of a chaperone in CMT.



Description: During protein folding, ER glucosidases I and II remove two of the three glucose residues after which the chaperones calnexin (membrane bound) or calreticulin (solubilized) recognize the trimmed oligosaccharide and assist the protein in folding. If ER glucosidase II cleaves the final glucose residue and if the protein is properly folded it is transported via vesicle flow for further processing in the Golgi (glycosylation). However, if after ER glucosidase II action it is not yet properly folded, UDP-glucose glycoprotein:glucosyltransferase (UGGT) recognizes the protein and probes its folding. The protein is now either reglucosylated by UGGT and reassisted in folding by calnexin/calreticulin or rejected and entered into the ER-associated degradation pathway (ERAD).

Application of this concept was first reported in 1999 by Fan and co-workers for the deficient α-galactosidase in the sphingolipidose, Fabry disease.[103] They observed that the iminosugar inhibitor, galactostatin (**10**; Figure 9) enhanced enzyme activity in cells of Fabry patients, when administrated at concentrations lower than those required for inhibition of the enzyme. Since this first report, the CMT concept has also been demonstrated to work for several common mutated forms of GBA1 in Gaucher disease – albeit in cellular assays. Many reports have since appeared that evaluate a multitude of different inhibitors, mainly iminosugar-based, for application in CMT for Gaucher disease (see Figures 19–22 on pages 45 to 47 for an overview of their structures with references). Most reports describe the incubation for 4–5 days of the inhibitor together with cells that express a specific Gaucher disease associated deficient GBA1 after which the artificial 4MU-glucoside substrate (**6**) is added and GBA1 activity is determined. The activity measurement is repeated in the presence of CBE (**3**) to correct for the activity of GBA2. Several studies have also shown with fluorescent markers of both GBA1 and the lysosome that increased amounts of GBA1 indeed reach the lysosome after treatment. However, it has yet to be shown that actual glucosylceramide degradation increases in the lysosomes of intact treated cells. A recent report showed that a combination of the iminosugar chaperone **9** with the proteasome inhibitor MG-132 (**11**) results in a synergistic effect and greater improvements of GBA1 activity in the lysosome.[104] However, because **9** also inhibits GCS, GBA2 and ER glucosidases I and II; and MG-132 also has secondary activities it is far from clear what actions of these compounds actually lead to the observed improvement in GBA1 activity.

1.3.5 Glycosphingolipids and the Brain. Starting with Tudichum's work, the importance of GSLs in brain tissue was noticed early on in GSL research. Although still not understood, especially Neu5Ac-terminal acidic gangliosides seem to play an important role in neurochemistry. They are found in high concentrations in brain tissue and can constitute up to 25% of the outer membrane lipid content. During embryogenesis and the post-natal period a small subset of acidic gangliosides are highly expressed in the developing brain. In the adult brain the levels of gangliosides are much lower but many more types of gangliosides are expressed.[18] Several knock out mouse models of the glycosyltransferases in GSL biosynthesis have shed some light on their functioning in the brain. Selective deletion of GCS in neural cells prevented the formation of the brain gangliosides and resulted in the birth of animals with severe neural defects that died within 3 weeks.[105] Knock out models of several transferases involved in the biosynthesis of more complex gangliosides have also been developed. These models indicate that a certain degree of functional redundancy exists among the brain gangliosides, because the mutant animals show only minor defects and other gangliosides seem to substitute for the functions of the missing ones.[18,106] Brain gangliosides have also been implicated in several neurological diseases. Parkinson like symptoms are one of the hallmarks of more severe forms of Gaucher disease.[107] Thus decreased functioning of GBA1 may predispose people for Parkinson disease. GSLs also seem to play a contradictory role in Alzheimer's disease. Activity of GCS was found to be lowered in brains affected with Alzheimer.[108] This caused an increase in ceramide and a decrease in levels of complex GSLs that in turn caused abnormal functioning of neural cells. Abnormal functioning could be prevented by infusions with ganglioside GM1. Contrary to this, GM1 enriched lipid rafts have also been shown to play a critical role in the pathology of Alzheimer disease by promoting the formation of amyloid deposits or plaques by aggregation of amyloid β-protein.[109]

1.3.6 Glycosphingolipids and the Skin. GSLs and SLs play vital roles in normal skin functioning. Ceramides and keratins are the essential components of the epidermal stratum corneum that makes the skin of all land dwelling animals impermeable to water and thereby prevents lethal dehydration.[110] The ceramides occupy the extracellular spaces of the stratum corneum and are characterized by *N*-acylation of the sphingosine backbone with long ω-hydroxy fatty acid chains (C_{30}–C_{36}).[17] The ceramides are thought to be excreted into the extracellular space by exocytosis of glucosylceramide and sphingomyelin intermediates. Skin cells simultaneously excrete vesicles that contain GBA1, Sap C and sphingomyelinase that hydrolyze these intermediates to generate skin ceramides at the required location. Indeed glucosylceramide constitutes ~4% of the total epidermal lipid mass. A knock out mouse model with a keratinocyte specific GCS deficiency recently proved the vital role of glucosylceramide as a intermediate in maintaining skin barrier functioning.[111] The mutant animals displayed a grossly abnormal stratum corneum and died of dehydration within 5 days after birth. Inhibition of GBA1 activity by topical exposure of skin to CBE (**3**) also caused impaired skin functioning.[110] Correspondingly,

skin abnormalities are also observed in patients with severe forms of Gaucher disease. Many skin diseases such as psoriasis also show abnormal SL and GSL metabolism.

Recently it was shown that one of the causes of the severe skin disorder, *Harlequin ichthyosis*, was a mutation that results in a deficient ABCA12 lipid transporter. This deficiency results in impaired extracellular delivery of glucosylceramide. The pathology could be remedied in a cellular model by corrective ABCA12 gene transfer.[112]

In patients with atopic dermatitis the enzyme SM deacylase was found over expressed together with 300% higher levels of sphingosylphosphorylcholine (SPC).[113] Extracellularly excreted SM deacylase in the epidermis was also found to be capable of deacylating glucosylceramide to glucosylsphingosine.[114] The increased activity of this enzyme has been proposed to contribute to dermatitis pathology by preventing sufficient generation of ceramides in the stratum corneum.

1.3.7 (Glyco)sphingolipids and Cystic Fibrosis. Cystic fibrosis is an autosomal recessive hereditary disorder caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) – a member of the family of ABC transporter proteins. The mutations cause lowered expression and activity of CFTR that affects the exocrine organs (*e.g.* lungs, intestines and pancreas). One of the clinical symptoms that play a major role in the morbidity of cystic fibrosis is a gradual destruction of lung tissue through chronic and recurrent bacterial infections by *Pseudomonas aeruginosa*. Recent research has revealed that a delicate balance of SLs and GSLs is required for normal CFTR functioning and that a SL imbalance is involved in respiratory system pathology of cystic fybrosis.

One facet of the respiratory system pathology is excessive angiogenesis in the infected lung tissue. This process is promoted by binding of sphingosine-1-phosphate to extracellular receptors. In healthy individuals, CFTR is involved in the active uptake of extracellular sphingosine-1-phosphate, which decreases the availability of this SL for promoting angiogenesis during inflammation.[115] Pier and co-workers have shown that CFTR localizes to GM1 ganglioside-containing lipid rafts upon contact of lung epithelial cells with *P. aeruginosa*.[116] They also proved that direct binding of *P. aeruginosa* to CFTR in lipid rafts is essential for recruitment of the major vault protein to rafts, internalization of the bacteria and proper host resistance to further infection.[117]

Ceramide also plays a vital role in both the normal and aberrant immune response against *P. aeruginosa* infection of lung tissue. First, ceramide that is produced from sphingomyelin via sphingomyelinase at the basolateral membrane of airway epithelial cells inhibits anion transport by CFTR at the apical membrane and thereby augments cystic fibrosis pathogenesis.[118] Gulbins and co-workers have shown that *P. aeruginosa* infection also activates acid sphingomyelinase that locally releases ceramide into lipid rafts.[119] These ceramide enriched lipid rafts reorganize into larger and more stable rafts that are required for internalization and clearance of *P. aeruginosa* infection.

Cystic fibrosis patients show an age-dependant accumulation of ceramide in the epithelial lung tissue. CFTR dysfunction impairs cellular influx of Cl^- counter anions for H^+ acidification of specific cellular compartments. This phenomenon induces a lysosomal increase in pH (4.5 » 5.9) that decreases the activity of acid ceramidase by > 90% and even reverses its activity to generate ceramide. On the other hand the activity of acid sphingomyelinase is only inhibited by 35%. This metabolic imbalance causes an increase in ceramide. The excessive ceramide levels cause cell death by apoptosis that in turn causes a hypersensitivity to *P. aeruginosa* infection creating a vicious cycle of tissue infection and destruction.[120]

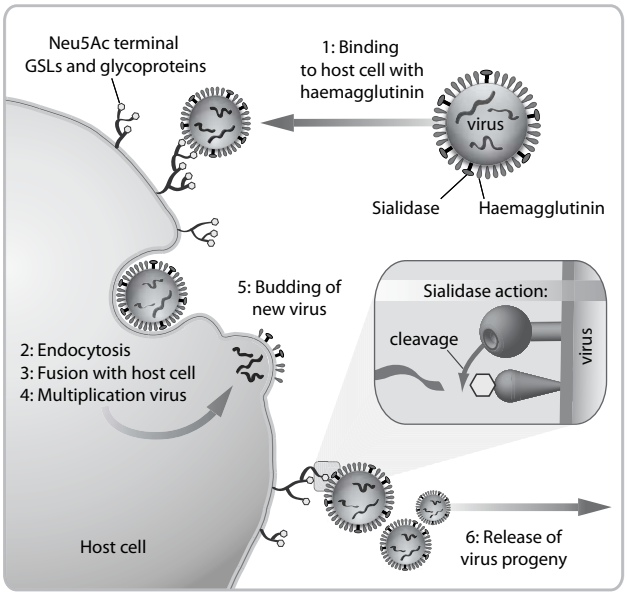
The most common CFTR mutation (F508del) that causes cystic fibrosis results in improper folding of most CFTR in the ER and degradation by the proteasome via ERAD. Becq and co-workers have reported that treatment with miglustat (**8**) is able induce a 12% increase in properly folded CFTR, which resulted in improved ion transport in cystic fibrosis epithelial cells.[121,122] They reasoned that the ability of **8** as an α-glucosidase inhibitor decreases F508del-CFTR degradation via ERAD during folding and *N*-glycan trimming in the ER. A recent patent by Aerts and Boot however demonstrates that treatment of F508del-epithelial cells with AMP-DNM (**12**), at concentrations that do not cause inhibition of α-glucosidase, also markedly improves the activity of ion transport by F508del-CFTR. In their experiment **12** inhibits GBA2 that shares the same cellular localization as CFTR.[68] The proposed mechanism of action is that inhibition of GBA2 lowers the level of ceramide in the microenvironment of F508del-CFTR and thereby improves its functioning.

1.3.8 Glycosphingolipids and Pathogens. GSLs are found at increased concentrations on the outer membranes of apical cells (*e.g.* cells that line the inside of stomach, intestines, respiratory track). Most types of these apical cells represent the initial barrier of the body with the external world and are therefore also the first to make contact with potential pathogens. Correspondingly, many pathogens have evolved mechanisms for exploiting these GSLs for infecting and invading their host. Especially, GSL-rich lipid rafts with terminal Neu5Ac carbohydrates are often hijacked by viruses, bacteria and protozoans for their propagation. The HIV-1, Ebola and Marburg viruses all use lipid rafts in binding to, entry in and budding from host cells.[123]

One of the most well studied examples is that of the influenza virus family that has only three different surface proteins of which the two most abundant are specifically aimed against the human host's terminal Neu5Ac GSLs and glycoproteins. The protein, called haemagglutinin, is a specific lectin for Neu5Ac and enables the virus to bind to the host cell after which it is endocytosed and multiplies itself (see Figure 11). The role of the second viral protein, a sialidase called neuraminidase, is to process progeny virus particles when they bud from the host cell, by hydrolyzing Neu5Ac from both the host cell and budding virus particle to achieve release from the host cell and prevent self-agglutination

of viruses. A major factor determining why humans are not yet readily infected by the H5N1 'bird-flu' is because it currently preferentially recognizes the terminal α2-3 linked Neu5Ac in bird respiratory tracks, where humans predominantly possess terminal α2-6 linked Neu5Ac in their respiratory tracks.[124]

Figure 11. Infuenza virus lifecycle and sialidase/haemagglutinin action.



1-Deoxynojirimycin based iminosugar derivatives such as miglustat (**8**; Figure 9) have been successfully applied to suppress the propagation of various viruses in cellular models.[125,126] Many viruses express glycoproteins on their surface and glucosidase inhibitors can disrupt proper processing of viral *N*-glycoproteins in the host ER by inhibiting trimming by ER glucosidase I+II and therefore calnexin/calreticulin binding. However, so far this has not resulted in a viable antiviral due to the excessive concentrations of inhibitor needed to achieve relevant levels in the ER when orally administered to patients.

An example of a bacterial pathogen that abuses host GSLs is *Helicobacter pylori*. It causes gastric ulcers and infects the gastric lining by lectin binding to the several host GSL among which the sialyl dimeric Lewis-X antigen (Neolacto series with one terminal Neu5Ac and two terminal L-fucose residues).[127] Depletion of surface GSLs by inhibition of GCS with miglustat (**8**) or PDMP (**7**) has been shown to successfully impair adherence of several bacteria species to host cells.[128,129] Another example is *Vibrio cholerae*. It expresses a sialidase that removes terminal Neu5Ac from complex GSLs (*e.g.* ganglioside GD1a) on the apical surface of the host's intestinal epithelial cells. This action exposes apical GM1 gangliosides to which the cholera toxin can bind that after internalization causes diarrhea.[130,131] As seen in the case of chronic respiratory track infection by *P. aeruginosa*

in cystic fibrosis, SLs and GSLs can also play an essential role in staging a proper immune response to infection by a pathogen.

1.3.9 (Glyco)sphingolipids in Immunology and Inflammation. A GSL derived from marine sponges and not found in humans, α-galactosylceramide, is a ligand for a specific subset of immune T cells, invariant natural killer T (iNKT) cells. Upon recognition of α-galactosylceramide, which is presented by CD1d molecules on antigen presenting cells, the iNKT cells rapidly secrete cytokines (IL-4 and interferon-γ) and downregulate cell surface T cell receptors. This results in the activation of various cells of the innate and adaptive immune system.[132,133]

One of the clinical symptoms in the sphingolipidosis, Sandhoff disease, is a reduced amount of iNKT cells. The deficient enzymes in Sandhoff disease are β-hexosaminidases A and B. Several studies have proposed that the inavailability of a specific degradation product of these enzymes, isoglobotrihexosylceramide (iGb3; Gal-α1,3-lactosyl-ceramide), almost not formed in Sandhoff disease patients, is responsible for this effect. They have labeled iGb3 as the endogenous CD1d lipid ligand by which newly generated iNKI cells are positively selected in the thymus for proper functioning.[134] This GSL has also been implicated in the activation of NKT cells. Dendritic cells, activated by lipopolysaccharide from *Salmonella typhimurium*, were found to activate NKT cells by presentation of iGb3 to them.[135] These results were contradicted by two recent studies that showed the absence of iGb3 in the thymus of humans and that knock out mice for iGb3 biosynthesis possess a normal immune response.[136,137] However, observations that intacellular CD1d antigen loading occurs in low pH endosomal/lysosomal compartments and is dependant on the presence of saposin activator proteins does suggest that another GSL may still be the ligand. This is further substantiated by a report that GCS deficient cells either by gene knock out or PDMP (**7**) inhibition are unable to activate iNKT cells.[138]

A recent study found that the presence of GSLs from the acidic ganglioside series in lipid rafts increases the cell surface expression of Toll-like recepeptor 2 (TLR2) in brain microglia cells. These GSLs also enhanced the interaction of TLR2 with its intracellular adaptor protein Myd88 that leads to an inflammatory condition in the brain.[139]

Treatment of a chemically induced inflammatory condition of the bowels in a mouse model with the GCS inhibitor **12** showed a considerable reduction in the inflammatory condition. This indicates that GSL biosynthesis is involved in the inflammatory cascade of inflammatory bowel diseases.[140]

1.3.10 Glycosphingolipids and Cancer. Most tumor cells exhibit altered GSL patterns on their surface, abnormal SL signaling and increased GSL biosynthesis that together play a major role in tumor growth, angiogenesis and metastasis.[141,142] The human sialidase, Neu3, is found in caveolae containing lipid rafts in the plasma membrane and cleaves

terminal Neu5Ac residues from GSLs. It is overexpressed in many types of cancer and plays an important role in their malignant character.[143] Tumor cells also actively shed specific gangliosides from the cell surface in order to cloak themselves from the body's immune system.[144]

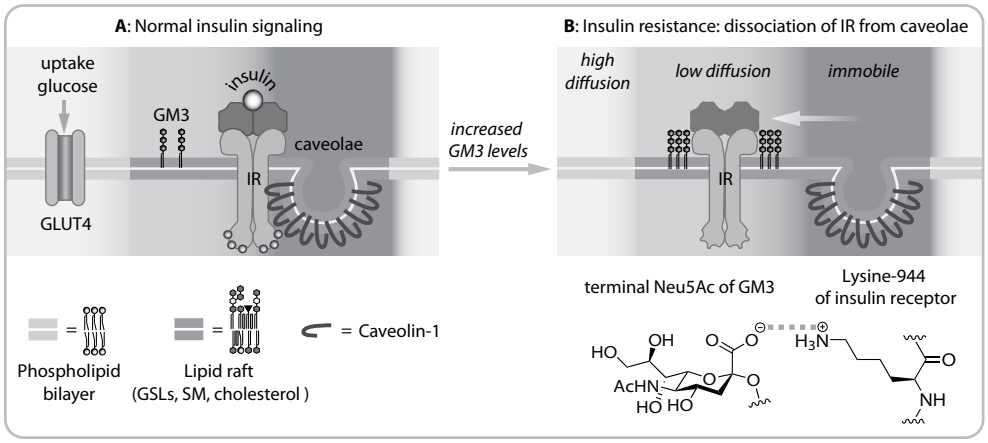
The effect of many chemotherapy agents and radiotherapies for treating cancers relies on their ability to increase levels of ceramide in tumor cells in order to activate ceramide-mediated apoptosis. Many tumors have increased expression levels and activity of GCS. This is thought in part to function as a detoxification method for the increased ceramide levels by conversion to glucosylceramide. Drug resistant cancer cell lines show up to threefold higher levels of glucosylceramide. Many tumors also achieve drug resistance by actively pumping out the drugs via the family of ABC transporter proteins. Overexpression of the most common of these efflux pumps, P-glycoprotein, coincides with abnormally high GCS activity in multidrug-resistant breast cancer, leukemia, melanoma and colon cancer. P-glycoprotein is a 170 kDa plasma membrane anchored protein that is situated in GSL containing lipid rafts. With expenditure of ATP it is capable of transporting a wide range of non-charged amphiphilic molecules – glucosylceramide among others – from the cytosol to the outer plasma membrane. Overexpression of the P-glycoprotein efflux pump is actually one of the most consistant hallmarks of drug resistance of many pathogens; antimalaria resistance of *Plasmodium falciparum*, chemotherapy resistance by the protozoan *Leishmania* and resistance to macrolide antibiotics by *S. pneumoniae*.[145,146]

Research has shown that inhibition of GCS by treatment of tumor cells with *N*-alkylated iminosugars (**8** and its derivatives) reduces tumor growth.[147,148] GCS inhibition with PDMP (**7**) and GCS silencing by iRNA's has been reported to reverse P-glycoprotein induced drug resistance in tumor cells.[149] However, other recent reports have also claimed there is no connection between GCS activity and drug resistance of cancers.[150]

1.3.11 (Glyco)sphingolipids and the Insulin Receptor. The insulin receptor is localized on the cell surface in GSL-containing lipid rafts that also house the membrane protein caveolin-1. This membrane protein creates stabile flask-like invaginations in the lipid raft called caveolae. The insulin receptor has a binding domain for caveolin-1 and their co-localization appears to be crucial for proper functioning of the insulin receptor (Figure 12A).[151] Many studies have also pointed to the role of SLs and GSLs in the pathology of insulin resistance in obesity linked type 2 diabetes.[152-154] Ceramide, sphingosine and sphingosine-1-phosphate are elevated in type 2 diabetes patients and obese insulin resistant mouse models. Ceramide has been demonstrated to inhibit insulin-stimulated glucose uptake by GLUT4 translocation and glycogen synthesis. The inhibitory effect of ceramide originates from its ability to block phosphorylation of Akt and protein kinase B in the downstream insulin signaling cascade.

The levels of GSLs such as glucosylceramide and ganglioside GM3 are also increased in a state of insulin resistance. Inokuchi and co-workers recently reported that the insulin receptor is also capable of binding GM3 in microdomains via interaction of the amine residue of lysine-944 with the acidic Neu5Ac of GM3 (Figure 12B). The study showed that the mobility of the insulin receptor is increased in GM3-enriched microdomains via this interaction that eventually causes insulin resistance by complete dissociation of the receptor from the caveolae.[151] Recent research also demonstrated that Gaucher disease, in which patients also have elevated GM3 levels, is associated with insulin resistance.[155] Several studies have shown that down regulation of GSL levels by inhibition of GCS by AMP-DNM (**12**)[156] or PDMP (**7**)[157] improves sensitivity of the insulin receptor in several type 2 diabetes animal models. Inhibition of GCS by **12** does not to influence the expression levels of components in the insulin signaling pathway, which is in line with its GSL level lowering mode of action.[158]

Figure 12. Lipid raft environment of the insulin receptor (IR) in normal conditions (**A**) and insulin resistance (**B**).



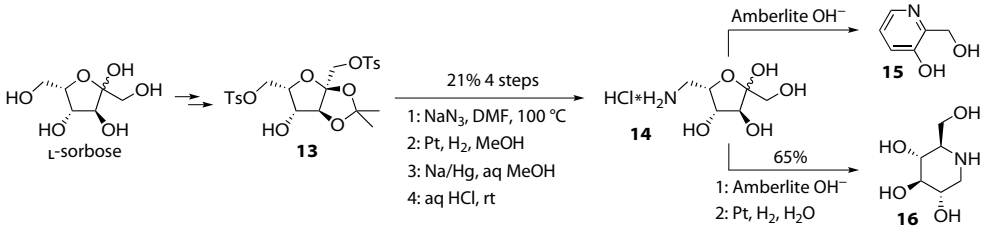
1.4 From Iminosugars to Inhibitors of Glucosylceramide Metabolism.

As can be judged from the previous sections, GSLs are involved in many biological processes both in health and disease of which most are still not fully understood. Small molecule inhibitors of the enzymes involved in the metabolism of glucosylceramide, GCS, GBA1 and GBA2, have been used as a handle to change the cellular levels of GSLs, for therapeutic purposes such as in SRT and CMT or to probe GSL functioning. Most of these inhibitors are based on the naturally occurring class of alkaloids called iminosugars. Iminosugars are carbohydrate derivatives in which the endocylic oxygen is replaced by a nitrogen atom. Contrary to many other naturally occurring compounds, iminosugars were first designed and synthesized in an organic chemistry laboratory before their isolation from a natural source.

1.4.1 The Discovery of Iminosugars. Ever since the ground breaking work on the chemistry and structure of carbohydrates by Emile Fisher during the 1880s and 90s,[159] organic chemists have used carbohydrates as starting materials in the synthesis of other either natural or non-natural derivatives. During the 1960 several groups investigated replacing the oxygen atom in carbohydrates with another heteroatom. The group of Paulsen chose the nitrogen atom and in 1966 they reported the conversion of known L-sorbose derivative (**13**) into a 6-amino-6-deoxy-L-sorbose derivative, which formed a furanose as its hydrochloric acid salt (**14**) and eliminated three water molecules to give a pyridine derivative (**15**) in neutral or alkaline solution (Scheme 8). However, when the free base of the amine was immediately hydrogenated it produced a 1-deoxy glucose analog with a ring nitrogen (**16**) accompanied by a minor amount of the equivalent C-5/L-idose epimer.[160,161]

Besides the quest to synthesize iminosugars, no real applications existed at the time for these compounds. In 1968, Inouye and co-workers characterized and synthesized an unstable antibiotic, nojirimycin (**17**), isolated from strains of *Streptomyces* (structure in Scheme 9).[162] When hydrogenated, **17** transformed into its stable 1-deoxynojirimycin analog which corresponded exactly to the glucose derivative (**16**) synthesized by Paulsen. In 1976, **16** was isolated from the mulberry tree and certain species of bacteria. Further research proved that **16** was a potent inhibitor of glycosidases and a whole field of research into the natural occurrence, synthetic preparation and inhibitory properties of **16** and its derivatives, nowadays called iminosugars, was initiated.[163,164]

Scheme 8. Synthesis of 1-deoxynojirimycin (**16**) as reported by Paulsen and co-workers.

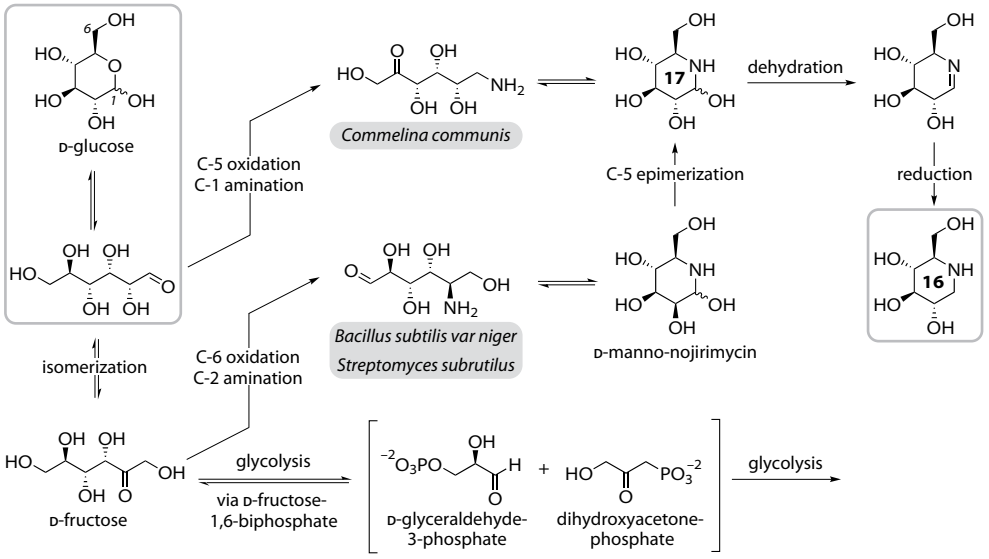


1.4.2 Biosynthesis of 1-Deoxynojirimycin. Compared to the large number of synthetic routes developed for the archetypical iminosugar **16**, for a relatively long time little was known about how **16** was synthesized in nature. Hardick and co-workers[165,166] researched its biosynthesis in two species of bacteria and Shibano and co-workers[167] in the plant *Commelina communis*. These studies proved that **16** is made from D-glucose in both the two species of bacteria and the plant. However, its biosynthesis from D-glucose follows two distinct paths. Both studies elucidated the biosynthesis of **16** through ^{13}C-1 labeled D-glucose feeding experiments and ^{13}C-NMR analysis of metabolites.

Hardick and co-workers showed that in both bacteria species D-glucose is first converted to D-fructose – via an enzyme catalyzed Lobry de Bruyn-Alberda van Ekenstein

isomerization (Scheme 9). Next, the amination and epimerization of C-2 and oxidation of C-6 in an undetermined order results in an intermediate that cyclizes to D-manno-nojirimycin. This intermediate is epimerized at C-2 to **17** and subsequently dehydrated at C-1 and reduced to provide **16**. Besides the expected ^{13}C labeling at C-6 of **16** they also found minor labeling at C-1. This result combined with the incorporation of ^{13}C at C-1 and C-6 in **16** after ^{13}C-1-D-glyceraldehyde feeding experiments proved that before entering the biosynthesis of **16**, D-fructose is also in equilibrium with the first steps of glycolysis.

Scheme 9. Proposed biosynthetic routes for **16** in a plant and two species of bacteria.



Shibano and co-workers found that in *Commelina communis* the C-1 position of **16** possessed the majority of the label. This indicated that here a more direct route of amination at C-1 and oxidation of C-5 was followed to produce **16**. Also here the label was divided over the C-1 and C-6 position that indicated prior equilibration of D-glucose to D-fructose and glycolysis (Scheme 9)

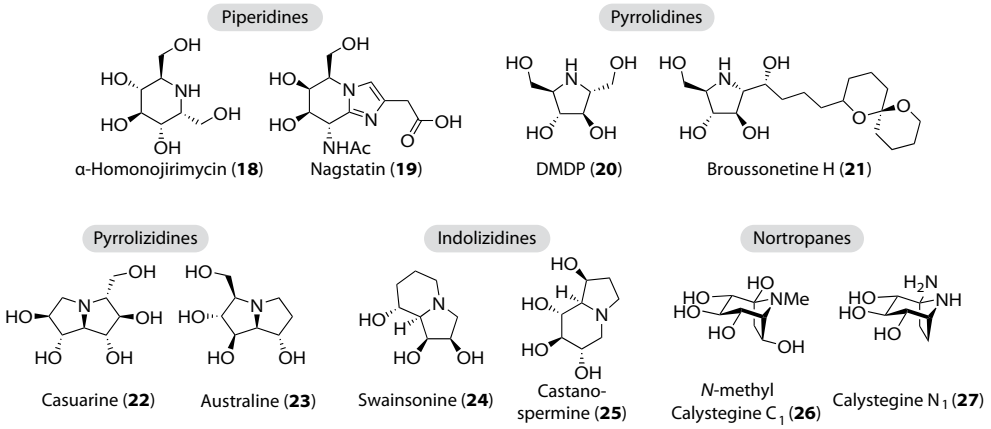
Besides **16**, countless other iminosugars have been isolated from natural sources such as plants, bacteria and fungi – as described in various comprehensive reviews[168,169] and books.[163,164] All these iminosugars are categorized in five classes based on their structure (Figure 13 on the next page). 1-Deoxynojirimycin (**16**) and nojirimycin (**17**) belong to the class called the piperidines. The most recent class discovered is that of the nortropanes during the 1990s. The calystegines from this class are even found at low concentrations in many common foods such as egg-plant, potatoes, tomatoes and chilli peppers.

One unifying property among these structurally diverse classes of iminosugars is their ability to inhibit glycosidases[168] and to a lesser extent glycosyltransferases.[170] Their structural diversity also results in a large degree of selectivity among iminosugars in the

inhibition of specific glycosidases and transferases. The known inhibitory activities of many natural and synthetic iminosugars as well as their therapeutic applications have been listed in two books on iminosugars.[163,164]

Promoted by therapeutic applications for this ability to inhibit carbohydrate-processing enzymes and difficulties in isolating large amounts from natural sources, much research since the 1970s has also focused on developing methodology for iminosugar synthesis.[163,164,171,172] During the late 1980s and early 90s, research into the application of iminosugars as antivirals led to the discovery of *N*-alkylated 1-deoxynojirimycins as potent ER glucosidase I and II inhibitors. One of these was miglustat (**8**) that in 1994 was also found to be an inhibitor of GCS. This discovery initiated the development of iminosugars as inhibitors of glucosylceramide metabolism. The next three sections will provide an overview of the different inhibitors of GCS, GBA1 and GBA2 that have so far been discovered – with a focus on iminosugar based inhibitors. Two reviews by Delgado and co-workers provide an overview of inhibitors of SL metabolic enzymes and also of GCS/GBA1.[173,174]

Figure 13. The five iminosugar classes with two examples of naturally occurring members.



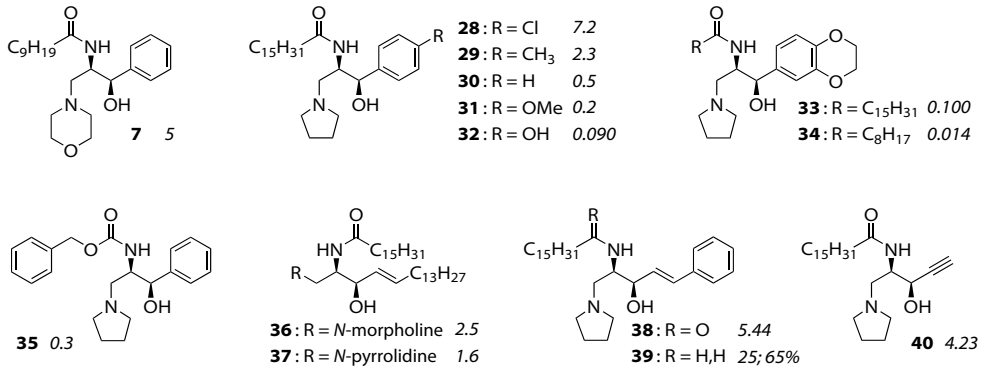
1.4.3 Inhibitors of Glucosylceramide Synthase. GCS is quite unique among glycosyl transferases in that an extended and structurally diverse range of potent inhibitors exist for it. In 1980 Vunnam and Radin reported the synthesis and activity of PDMP (**7**), the first example of a GCS inhibitor. It was part of a series of ceramide analogues in which the unsaturated alkyl chain of the sphingosine backbone was replaced by a phenyl group and the primary hydroxyl by several heterocycles (Figure 14).[94] Only analogues with D-*threo* stereochemistry – the opposite of ceramide – proved to inhibit GCS and kinetic analysis showed **7** to be uncompetitive for UDP-glucose and a mixed competitive inhibitor for ceramide.[175] Switching the heterocycle to a pyrrolidine (**30**) resulted in a tenfold increase in potency.[176] Subsequent investigation of *para*-substitions on the phenyl ring of **30** revealed a relationship between the IC_{50}, the hydrophilicity and the electron donating

capacity of the *para*-position (**28**–**32**; Figure 14).[177] An ethylenedioxy modification of the phenyl ring (**33**)[177] in combination with a shorter acyl C_9 chain has resulted in the most potent PDMP derivative (**34**) and GCS inhibitor to date.[178] Two other studies have shown that the acyl chain can be replaced by a benzyloxy carbonyl (**35**)[179] but not with an alkyl chain (**38** *vs.* **39**).[180] Derivatives **36** and **37** that more closely mimic ceramide also inhibited GCS comparably to PDMP.[181] Finally, van Calenbergh and co-workers recently reported that replacing the sphingosine mimicking aryl moiety of PDMP with a terminal alkyne (**40**) also results in a potent inhibitor of GCS.[180]

Evaluation of the selectivity of a structural homologue of **33** with an IC_{50} of 24 nM for GCS revealed that it did not inhibit the enzymes GBA1, GBA2, ER α-glucosidases I+II, debranching enzyme, sucrase and maltase.[182] Another study showed that **33** does not inhibit GCS in lower animals, plants, fungi or bacteria, but only inhibits human GCS.[183]

In general, the D-*threo* PDMP structure has proven a flexible and productive core for the design of GCS inhibitors. During the development of PDMP and its derivatives it was discovered that many of these compounds also inhibit the lysosomal phospholipase A2 that is capable of acylating ceramide to 1-*O*-acylceramide. Its inhibition results in increased cellular ceramide levels, which can lead to secondary effects of these inhibitors.[184]

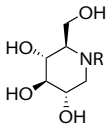
Figure 14. Overview of PDMP-based GCS inhibitors (in italic: IC_{50} values in μM or % inhibition at μM).



In 1994, Platt and co-workers reported that **8** (miglustat)[95] is able to inhibit GCS, which has since been used to develop substrate reduction therapy for Gaucher disease, as described in section 1.3.4. Due to this applications much attention has focused on the structure–activity relationship (SAR) of *N*-alkylated iminosugars.[42,185] These studies have shown that *N*-propyl (**44**) represents the minimum length for inhibition of GCS and that lengthening the *N*-alkyl chain further improves inhibition of GCS up to *N*-decyl alkylation (**47**; see Table 1 on the next page).[42] Linear aliphatic *N*-alkylation does not appear to be crucial as the IC_{50} of *N*-benzyl **58** is comparable to **8** (Figure 15).[185] Also *N*-5-(adamantan-1yl-methoxy)-pentyl (AMP) derivative **12**, published by Aerts *et al.* in 1998, proved a very potent inhibitor of GCS.[186] Lengthening the *N*-alkyl chain also increases

the cytotoxicity of these compounds (Table 1).[42] The exact nature of this toxicity is not fully understood but might be caused by membrane solubilisation due to their behavior as detergent amphiphiles. Van den Broek *et al.* have shown that introduction of an ether function into the *N*-alkyl chain decreases cytotoxicity (**47** compared to **51** in Table 1).[187]

Table 1. IC_{50} in µM or % inhibition at µM and cytotoxicity for *N*-alkylated 1-deoxynojirimycins.

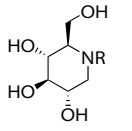


Compound	GCS *in vitro*	Cell proliferation CC_{50}	ER α-glucosidase I *in vitro*	GBA1 *in vitro*
16: R = H	2 mM (0%)[a]	> 5 mM[a]	1.44[a]	240[e]
42: R = Me	200 (31%)[b]	-	-	150[e]
43: R = Et	200 (52%)[b]	-	-	-
44: R = Propyl	200 (69%)[b]	-	-	700[e]
8: R = Butyl	34.4[c]	> 10 mM[a]	0.57[a]	270[e]
45: R = Hexyl	23.8[c]	> 1 mM[c]	-	13[e]
46: R = Octyl	16.6[c]	984.1[c]	-	0.82[e]
9: R = Nonyl	7.4[c]	118.9[c]	0.29[a]	0.66[e]
47: R = Decyl	3.1[c]	95.5[c]	0.48[d]	-
48: R = Dodecyl	5.2[c]	39.7[c]	-	0.05[f]
49: R = Hexadecyl	3.4[c]	25.1[c]	-	-
50: R = Octadecyl	4.0[c]	36.6[c]	-	-
51: R = 7-oxadecyl	3.2[a]	> 5 mM[a]	0.29[a]	-
52: R = 7,10,13-trioxa-tetradecyl	200 (93%)[d]	-	-	-

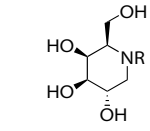
a[188]; b[189]; c[42]; d[185]; e[190]; f[191]

Miglustat (**8**) was first reported in 1988 by Fleet *et al.* as part of a panel of inhibitors of HIV virus replication.[192] Inhibition of ER α-glucosidase I and II causes this effect and in an attempt to dissociate this activity from GCS inhibition Platt and co-workers investigated modification of the iminosugar part of **8**. This study proved that *N*-butylated 2-acetamido-, D-*manno*- and L-*fuco*-1-deoxynojirimycin no longer inhibit GCS. However, D-*galacto* derivatives (**53** and **54**) do inhibit GCS and no longer inhibit ER glucosidase I or GBA1 (Figure 15).[189] Recently reported results have shown that L-*ido* derivatives (**55** and **56**[193]) and L-*altro* **57** also still inhibit GCS.[98,193]

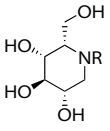
Figure 15. Overview of piperidine based GCS inhibitors (in italic: IC_{50} values in µM or % inhibition at µM).



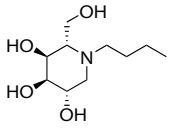
8 : R = Butyl *34.4*
9 : R = Nonyl *7.4*
12 : R = AMP *0.2*



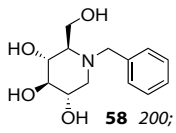
53 : R = Butyl *40*
54 : R = Nonyl *10.6*



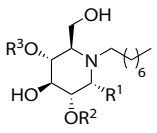
55 : R = Butyl *27*
56 : R = Pentyl *4.0*



57 *70*



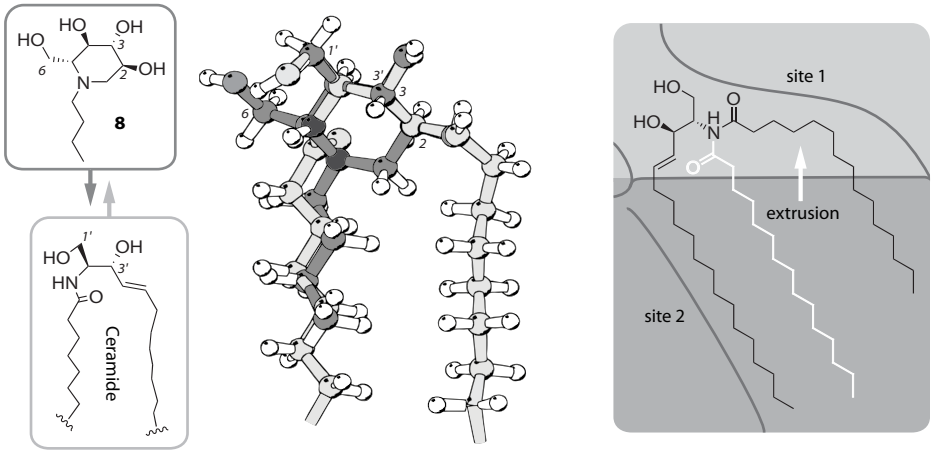
58 *200; 73%*



59 : R^1 = Octyl; R^2 = R^3 = H *174*
60 : R^1 = H; R^2 = Octyl, R^3 = H *164*
61 : R^1 = R^2 = H, R^3 = Octyl *134*
62 : R^1 = H; R^2 = R^3 = Octyl *1 mM; 26%*

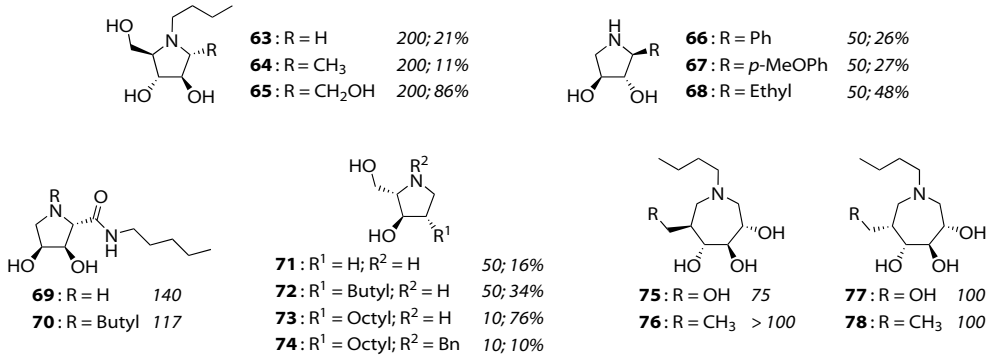
Due to the lack of a crystal structure for GCS it is not known how 1-deoxynojirimycin derived inhibitors bind its active site and what SAR governs this. A study by Butters and co-workers of the kinetics of GCS inhibition by **8** showed that it was non-competitive for UDP-glucose and competitive for ceramide.[185] On the other hand compound **9** proved non-competitive for both substrates.[42] These results combined with the close structural similarities of **8** and ceramide has led Butters to tentatively designate **8** as a ceramide mimic (Figure 16). A proposed model explains the difference between the binding kinetics of **8** and **9** by the presence of two hydrophobic sites for binding of ceramide in a predicted computational model of GCS. In this model a membrane embedded ceramide is required to be partially extracted for proper positioning of its primary hydroxyl in the active site. The sphingosine backbone binds with transmembrane site 2 and the acyl tail is partly extruded from the membrane to bind site 1. In this model the less lipophilic **8** is predicted to bind site 1 and the lipophilic **9** at site 2.[42] This model indicates that the attachment of a second lipophilic moiety at the 2-*O*-position hydroxyl of **8** or **9** could result in a better mimic of ceramide. However, a recent study by Compain, Martin and co-workers that reported the synthesis and evaluation of di- or trialkylated derivatives **59**–**62** showed this did not result in more potent GCS inhibitors (Figure 15).[194] Research described in Chapter 5 of this thesis corroborates this finding.

Figure 16. Model of miglustat (**8**) as a ceramide mimic as proposed by Butters and co-workers.[42,185]



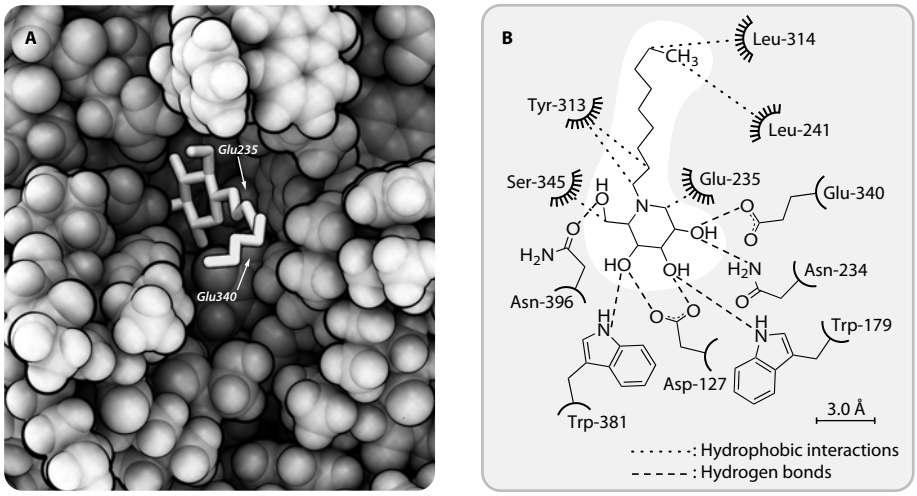
Several pyrrolidine iminosugars have also proven to be inhibitors of GCS. In 2000, Butters and co-workers revealed that DMDP derivative **65** was an inhibitor comparable in activity to **5** (Figure 17 on the next page).[185] Two studies by Davis and co-workers have also identified several pyrrolidine inhibitors (**66**–**70**).[195,196] Génisson *et al.* have developed pyrrolidines that mimic the sphingosine backbone (**71**-**74**) among which **73** proved to be a potent GCS inhibitor.[197] Finally, Blériot and co-workers recently reported moderately active azepane based inhibitors of GCS (**75**-**78**).[198]

Figure 17. Pyrrolidine and azepane based GCS inhibitors (in italic: IC_{50} values in μM or % inhibition at μM).



1.4.4 Inhibitors of Glucocerebrosidase. As mentioned in the previous section, miglustat (**8**) also inhibits GBA1 and increasing the length and hydrophobicity of the *N*-alkyl chain, similar as for GCS, also improves GBA1 inhibition (Table 1, **8**, **9** and **12** in Figure 19). Attachment of amantadine – itself an anti-influenza drug – to the *N*-alkyl tail via an amide linkage to produce AMP-DNM derivative **79** does not decrease GBA1 inhibition.[199] Inhibitor **79** has shown potential as a pharmacological chaperone of Gaucher disease associated GBA1.[199,200]

Figure 18. X-ray crystal structure of **9** in active site of GBA1 (**A**). Coordination of **9** by active site residues (**B**).[201]

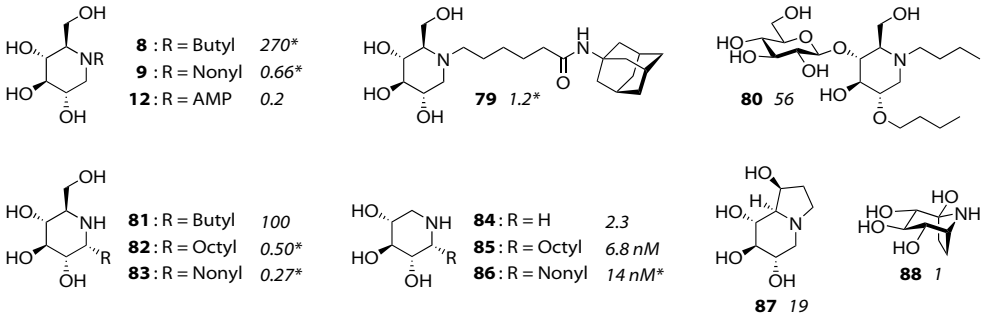


In 2007 Futerman, Sussman and co-workers published two crystal structure of GBA1 with **8** and **9** bound in its active site. Binding of **8** and **9** was very similar with the nonyl moiety of **9** making additional hydrophobic interactions with leucine residues near the entry to the active site (Figure 18). Remarkably, the nitrogen atom of both inhibitors did not coordinate with the two catalytic glutamic acid residues (Glu-340/235) or any

other active site residue.[201] These observations indicate that 1-deoxynojirimycin-based inhibitors of GBA1 appear to act as fortuitous binders of the active site. Extensive studies of the binding kinetics of numerous iminosugar-type inhibitors and their target glycosidases has shown that in general 1-deoxynojirimycin-based inhibitors are not true transition state analogs. To achieve true transition state mimicry for glycosidases and the associated increases in binding affinity requires sp^2 hybridization at the pseudo anomeric centre of the iminosugar (*e.g.* Nagstatin **19**[202] in Figure 13).[203]

With the ceramide mimicking model of **8** as a basis (Figure 16 left), Compain, Martin and co-workers reported the β-4-*O*-glucosylation of two *N*-alkylated 1-deoxynojirimycins. According to the model glycosylation at this position should result in mimics of glucosylceramide. Glycosylation of miglustat (**8**) resulted in loss of GBA1 inhibition. However, a 2-*O*, *N*-dibutylated-1-deoxynojirmycin – itself not a GBA1 inhibitor – did display inhibition of GBA1 upon β-4-*O*-glucosylation (**80**).[204]

Figure 19. Overview of structures and IC_{50} values in μM (italic) for GBA1 inhibitors.



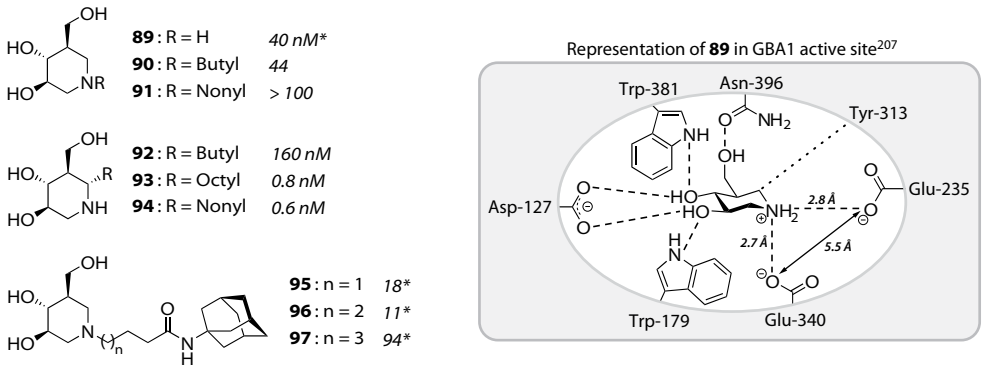
*: **8**[205]; **9**[104,206]; **79**[200,207]; **82**+**83**[190], **86**[208] show *in vivo* pharmacological chaperone activity for mutated GBA1.

Two other studies by the same group showed that D-*gluco*- (**81**–**83**)[190] and D-*xylo*-α-aza-*C*-glycosides (**84**–**86**)[208] are very potent GBA1 inhibitors (Figure 19). Evaluation of a series of naturally occurring iminosugars by Fan and co-workers identified castanospermine (**87**) and several calystegines (**88**: calystegine B2) as inhibitors of GBA1.[191] Isofagomine (**89**; see Figure 20 on the next page) proved to be the most potent GBA1 inhibitor in this screening (IC_{50} 40 nM) and was subsequently evaluated as a chemical chaperone in cells of Gaucher, Fabry and G_{M1}-gangliosidosis patients with promising results.

Petsko and co-workers reported the crystal structure of GBA1 with **89** in its active site in 2007 (Figure 20 right).[207] The secondary hydroxyls of **89** are coordinated by the same residues as reported for **8** and **9**, but it differs in that its nitrogen is coordinated by the two catalytic residues. Fan and co-workers have shown that a great difference in potency for GBA1 inhibition is observed between *N*-alkylation (**90** and **91**) of **89** or a alkyl tail on the C-6 position (**92**–**94**).[209] From this last series, isofagomine derivative **94** with an IC_{50} of 0.6 nM represents the most potent GBA1 inhibitor reported to date. Kelly and co-

workers have since shown that *N*-alkylation with an alkyl spaced amantadine-amide also provides GBA1 inhibitors (**95**–**97**) that are efficient pharmacological chaperones for two common mutated forms (N370S and G202R) of GBA1 in Gaucher disease.[210]

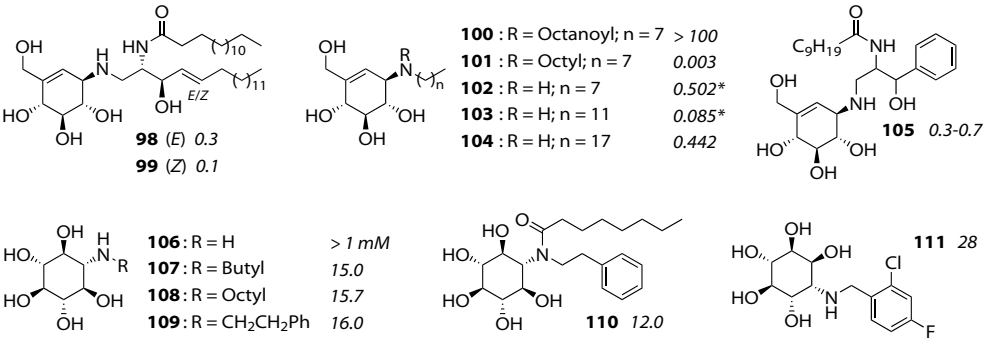
Figure 20. Overview of structures and IC_{50} values in μM (italic) for isofagomine-based GBA1 inhibitors.



*: Active as pharmacological chaperone for mutated GBA1 during *in vivo* evaluation.[191,210-212]

Besides the piperidine-based iminosugars, lipophilic aminocyclitols constitute a second important class of GBA1 inhibitors. In 1995, Ogawa and co-workers reported the *N*-alkylation of a naturally occurring aminocyclitol, β-valienamine, with either an *E* or *Z* ceramide moiety. This resulted in **98** and **99** that represented the first known selective inhibitors of GBA1 (Figure 21).[213] Replacing the complex ceramide moiety by one[214] or two[215] aliphatic alkyl chains also produced potent inhibitors (**100**–**104**) that were shown not to inhibit GCS. Derivatives of β-valienamine (**105**) inspired by PDMP also produced inhibitors of GBA1 regardless of the stereochemistry of the PDMP part. Using α-valienamine or a saturated β-valienamine analogue, β-validamine, in this design resulted in significantly decreased GBA1 inhibition.[216]

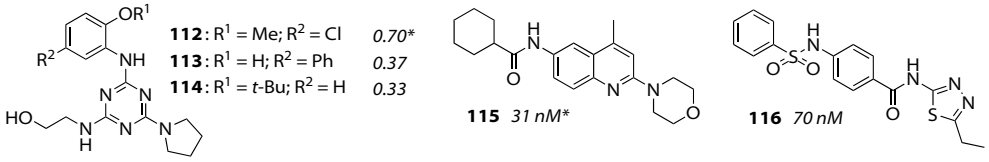
Figure 21. Overview of structures and IC_{50} values in μM (italic) for aminocyclitol-based GBA1 inhibitors.



*: Active as pharmacological chaperone for mutated GBA1 during *in vivo* evaluation.[217,218]

Delgado and co-workers have reported *N*-alkylated aminocyclitols **107**–**109** as GBA1 inhibitors (Figure 21). These were obtained by regio- and stereocontrolled opening of conduritol-B-epoxide (**3**). Aminocylitol **110** was the most potent GBA1 inhibitor and remarkably does not contain a basic amine function that is usually required for inhibition of GBA1.[219] These compounds were also evaluated as GCS inhibitors, but proved inactive. In a second combinatorial chemistry study by Delgado and co-workers, substitution of the nitrogen atom in two amincylitols cores was investigated that resulted in GBA1 inhibitors similar in structure and activity to **107**–**109**, as well as a new class of inhibitors, namely, **111**.[220]

Figure 22. Overview of structures and IC_{50} values in μM (italic) for aromatic-based GBA1 inhibitors.

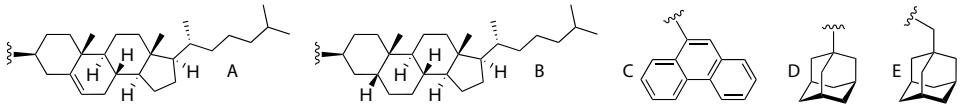


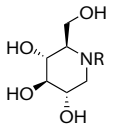
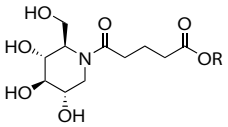
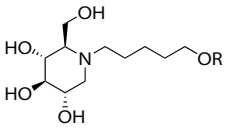
*: Active as pharmacological chaperone for mutated GBA1 during *in vivo* evaluation.[221]

Finally, Sidransky and co-workers recently identified three novel classes of aromatic, achiral GBA1 inhibitors (**112**, **115** and **116**; Figure 22) by high throughput screening of a library of 59815 compounds.[221] Compound **112** proved the most promising for use as a GBA1 chemical chaperone and was further developed. Modifications at the pyrrolidine nitrogen and primary hydroxyl of **112** did not improve its potency, but several modifications of the phenyl ring did (**113** and **114**).[222]

1.4.5 Inhibitors of β-Glucosidase 2. Before its identification as GBA2, Aerts and co-workers published a series of 1-deoxynojirimycin derivatives in 1999 that were evaluated in an enzyme assay for inhibition of the unidentified non-lysosomal glucosylceramidase activity (GBA2), GBA1 and GCS. As previously seen for GCS and GBA1 inhibition, longer *N*-alkyl chains here also resulted in more potent inhibitors of GBA2 (**8**, **44**, **117** and **118** in Table 2).[186] Several derivatives were also equipped with various C_5-spaced hydrophobic moieties and evaluated in an enzyme assay (see Table 2 on the next page). Hydrophobic moieties attached to the ring nitrogen via an amide linkage provided inactive to weak inhibitors of GBA2, GBA1 and GCS (**119**–**123**). However, potent inhibitors of GBA2 and also GBA1 and GCS were obtained when a hydrophobic 5-cholesteroloxy-pentyl (**124**) or 5-(adamantane-1yl-methoxy)-pentyl (**12**) moiety was attached to the ring nitrogen. Especially compound **12** with an IC_{50} of 1 nM proved a very potent inhibitor of GBA2.

Table 2. Apparent *in vitro* IC_{50} values in μM for 1-deoxynojirimcyin derivatives.[186]



Compound		GCS	GBA1	GBA2	Lysosomal α-glucosidase
	**44**: R = Propyl	> 100[a]	332	0.120	9.2
	8: R = Butyl	50	400	0.230	0.1
	117: R = Pentyl	40[a]	8.5	0.038	3.7
	118: R = Heptyl	30[a]	13.5	0.028	1.3
	**119**: R = A	> 100[a]	NI	NI	NI
	120: R = B	> 100[a]	NI	NI	NI
	121: R = C	> 100[a]	0.44	39	NI
	122: R = D	> 100[a]	4.1	306	NI
	123: R = E	> 100[a]	3.2	461	NI
	**124**: R = A	7[a]	0.77	0.097	7.2
	12: R = E	0.2	0.2	0.001	0.4

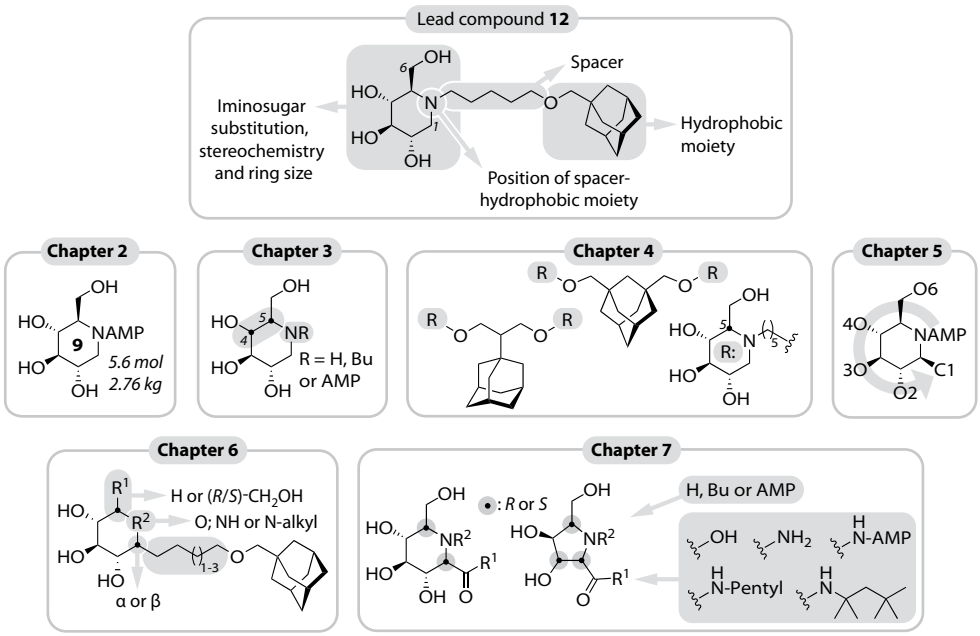
[a]unpublished data from Aerts *et al.*; NI = no inhibition at 100 μM .

Outline of Thesis

The library of lipophilic iminosugars published by Aerts and co-workers in 1999 initiated the research described in this thesis. Its most potent member, **12**, was a submicromolar inhibitor of GCS, GBA1 and GBA2. As can be judged from the inhibitory potencies as described in sections 1.4.3 to 1.4.5 many of the iminosugars, including **12**, currently in use or in development as therapeutics are not selective for their target enzyme. Being able to influence glucosylceramide biosynthesis and degradation in more selective fashion would facilitate the study of GSL functioning in (patho)physiological processes. More selective inhibitors would also help to elucidate their mode of action as therapeutics. Consequently, in the here presented study lipophilic iminosugar **12** was chosen as a lead compound in achieving this goal to develop selective inhibitors for GCS, GBA1 and GBA2.

Modifications of the structure of **12** can be achieved as follows (Figure 23): 1) variation of the length and nature of the spacer; 2) altering the hydrophobic adamantan-1-yl-methoxy group; 3) modification of the stereochemistry, substitution patterns and ring size of the iminosugar moiety and 4) alteration of the attachment site of the adamantan-1-yl-methoxy-spacer moiety on the iminosugar core.

Figure 23. Overview of potential modification sites in lead compound **12** and the chapters in this thesis.



For the study of the effects of inhibitor **12** in various biological applications a large supply of it was needed. Consequently, **Chapter 2** describes the development and implementation of a large scale synthetic route for **12** (Figure 23). The research described in **Chapter 3** investigates the mode of action by which **12** improves glycemic control in models of type 2 diabetes by varying the C-4/C-5 stereochemistry and *N*-alkylation of the iminosugar moiety. Dimeric iminosugar derivatives of **12** are develop and evaluated as potential bivalent inhibitors in **Chapter 4**. In **Chapter 5**, the synthesis and biological evaluation of derivatives of **12** is described in which AMP-moiety is moved to alternate positions on the 1-deoxynojirmycin ring. **Chapter 6** deals with the synthesis and evaluation of a small library of α/β-aza-*C*-glycoside derivatives based on **12**. **Chapter 7** presents the preparation of four diverse libraries of pyrrolidine and piperidine iminosugars in a combinatorial fashion via the tandem Staudinger/aza-Wittig/Ugi three-component reaction and their biological evaluation. **Chapter 8** provides a short summary of the research described in chapters 2 to 7 as well as an overview of results from work that is currently in progress for this study and some prospects for new research avenues.

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