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Glycobiology

Glycosphingolipids—Nature, Function, and Pharmacological Modulation

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The discovery of the glycosphingolipids is generally attributed to Johan L. W. Thudichum, who in 1884 published on the chemical composition of the brain. In his studies he isolated several compounds from ethanolic brain extracts which he coined cerebrosides. He subjected one of these, phrenosin (now known as galactosylceramide), to acid hydrolysis, and this produced three distinct components. One he identified as a fatty acid and another proved to be an isomer of D*-glucose, which is now known as* D*-galactose. The third component, with an "alkaloidal nature", presented "many enigmas" to Thudichum, and therefore he named it sphingosine, after the mythological riddle of the Sphinx. Today, sphingolipids and their glycosidated derivatives are the subjects of intense study aimed at elucidating their role in the structural integrity of the cell membrane, their participation in recognition and signaling events, and in particular their involvement in pathological processes that are at the basis of human disease (for example, sphingolipidoses and diabetes type 2). This Review details some of the recent findings on the biosynthesis, function, and degradation of glycosphingolipids in man, with a focus on the glycosphingolipid glucosylceramide. Special attention is paid to the clinical relevance of compounds directed at interfering with the factors responsible for glycosphingolipid metabolism.*

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1. Introduction

Sphingolipids (SLs) and glycosphingolipids (GSLs) are essential structural components of mammalian cell membranes and largely reside at the cell surface. Sphingolipids are composed of a ceramide moiety with an N-acylated sphingosine group.[1–3] Either glucose or galactose is linked to the primary hydroxy group of the sphingosine moiety through a β-glycosidic bond, thereby giving rise to the simplest glycosphingolipids: glucosylceramide and galactosylceramide. Linkage of a phosphorylcholine moiety results in sphingomyelin, a very abundant membrane lipid. Further additions of oligosaccharides and sulfate groups to glycosphingolipids give rise to a broad range of complex glycosphingolipids.[4,5] Those with a capping *N*-acetylneuraminic acid are known as gangliosides (Figure 1). The biosynthesis and degradation of glycosphingolipids involve numerous enzymes that act at various subcellular locations (Figure 2).

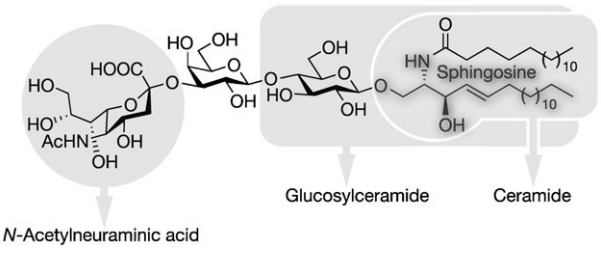

Figure 1. Structure of the glycosphingolipid ganglioside GM3.

1.1. Sphingolipid Metabolism

The biosynthesis of SLs[6–10] starts at the cytosolic leaflet of membranes of the endoplasmic reticulum (ER), where dihydroceramide is synthesized by a sequence of three enzyme-catalyzed reactions from L-serine and two molecules of coenzyme A (CoA) activated fatty acid (Scheme 1). The predominant mammalian sphingoid bases contain 18 carbon atoms, but small amounts of other (C_{12-26}) sphingoid bases also occur.[11] Depending on the tissue, the length and saturation of the N-acyl tail in SLs and GSLs can vary significantly.[12] In humans this is achieved by six distinct dihydroceramide synthase (*CerS*) genes (formerly known as *LASS* genes) that encode for a family of CerS enzymes with differing selectivity for the CoA-activated fatty acid.[13] In this Review only one common type of sphingolipid is depicted, which is generated from the most abundant fatty acid, palmitic acid. The majority of dihydroceramide is transformed into ceramide by dihydroceramide desaturase. However, in epithelial cells of the skin and small intesines, the SL phytoceramide also occurs at significant levels. Phytoceramide is formed by DES2-mediated[14] C-4 hydroxylation of dihydroceramide and can be converted into phytosphingosine by a specialized alkaline ceramidase.[15] It has recently become clear that ceramide can also be generated for biosynthetic purposes by acylation of sphingosine stemming

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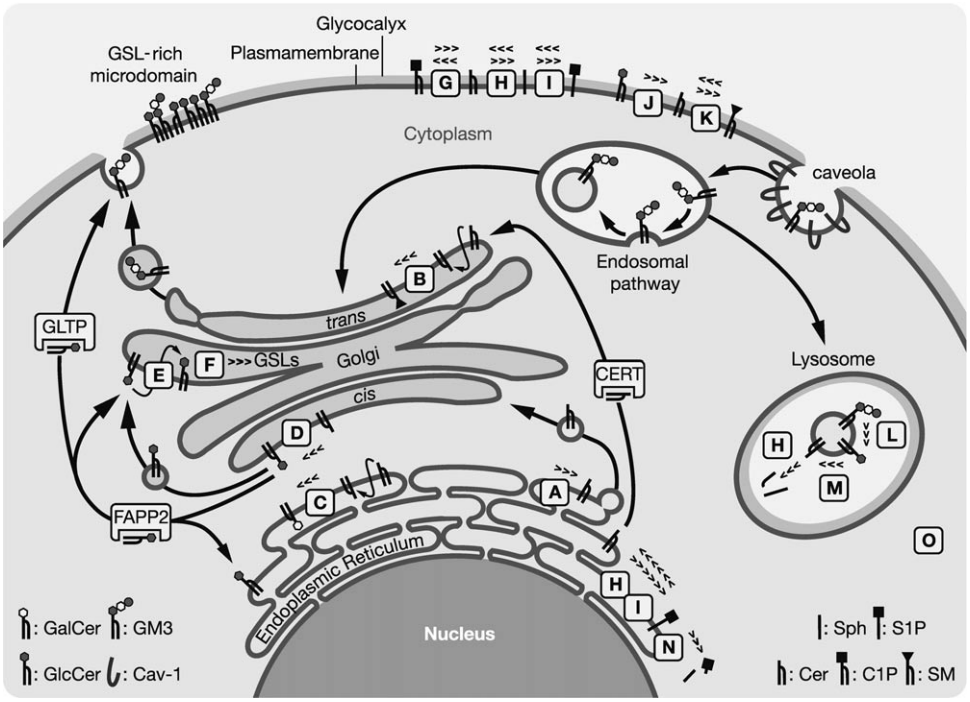

Figure 2. Cellular topology and dynamics of mammalian SL/GSL biosynthesis and catabolism. A) De novo synthesis of ceramide; B) synthesis of sphingomylin (SM) by SMS1; C) synthesis of GalCer by CGalT; D) synthesis of GlcCer by GCS; E) GlcCer flippase; F) synthesis of lactosylceramide and complex GSLs (for example 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 (and LPH in the 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) hydrolysis of GlcCer by GBA3 (?); Cav-1: Caveolin-1 (mediates endocytosis of GSLs).

from lysosomal degradation of sphingolipids. This pathway is generally referred to as the salvage pathway.[16]

Ceramide is the key precursor in the synthesis of various sphingolipids and glycosphingolipids. Ceramide is transported from the ER by the transport protein CERT to the cytosolic leaflet of the trans-Golgi apparatus membrane.[17–19] Here it equilibrates between the cytosolic and luminal side of the trans-Golgi membrane. On the luminal inside, sphingomyelin synthase 1 (SMS1) converts ceramide into sphingomyelin (SM) by transfer of a phosphorylcholine head group from phosphoglycerolipids. The zwitterionic SM no longer passes appreciably through the membrane. A second enzyme, SMS2, is located at the plasma membrane and converts ceramide there into sphingomyelin. A neutral and acidic form of the enzyme sphingomyelinase is able to regenerate ceramide from SM. The latter enzyme is located in the lysosomes and at

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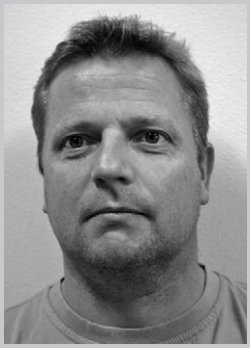
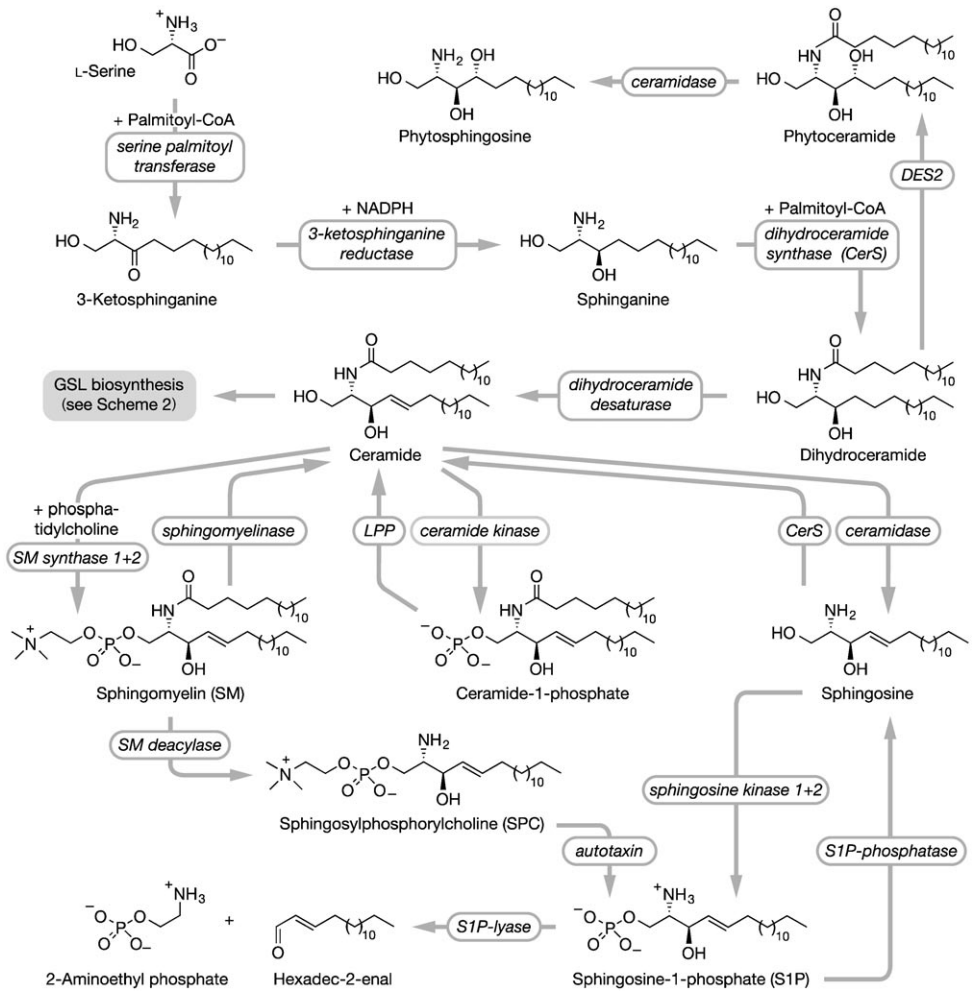


Rolf Boot completed his PhD thesis on Gaucher disease and chitotrisidase, a Gaucher cell derived human chitinase, and is currently assistant Professor in Medical Biochemistry at the Academic Medical Center of the University in Amsterdam. His research focuses on mammalian chitinases and the pathogenic mechanisms and therapeutic interventions of lysosomal storage disorders as well as identification and application of novel biomarkers for these disorders.

Richard van den Berg completed his PhD at Leiden University in 2001 under the mentorship of Jacques van Boom. He continued as a postdoctoral fellow at Leiden University and was appointed lecturer in 2008. His research concerns the development of lipophilic iminosugars and sphingolipid-related compounds.

Gijs van der Marel completed his PhD under the supervision of Jacques van Boom at Leiden University, where he is currently Professor in Synthetic Organic Chemistry. His research focuses on the development of synthetic methodology towards carbohydrates and nucleic acids, including hybrid structures and non-natural analogues.



Scheme 1. Overview of the biosynthesis and catabolism of mammalian sphingolipids.

the outer leaflet of the plasma membrane, but is also excreted. In an alternative pathway, ceramide is phosphorylated at the plasma membrane by ceramide kinase (CERK). Its product, ceramide-1-phosphate, can be hydrolyzed by lipid phosphate phosphatase (LPP) to give ceramide.[20] Acid, alkaline, and neutral ceramidases, which are 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 (SK1), which operates at the plasma membrane and in the extracellular matrix. A second sphingosine kinase, SK2, is located at the ER near the nucleus.[21] S1P levels can be downregulated in turn by S1P phosphatase. The presence of sphingosylphosphorylcholine (SPC) in mammalian cells and plasma has been known for a long time,[22,23] but only recently were some of its metabolism and biological functions revealed. The extracellularly excreted enzyme, SM deacylase, hydrolyzes the acyl tail from SM to generate SPC. A dedicated catabolic enzyme for SPC has not yet been found, but the plasma-circulating enzyme autotaxin 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 the degradation of S1P by S1P-lyase at the ER.

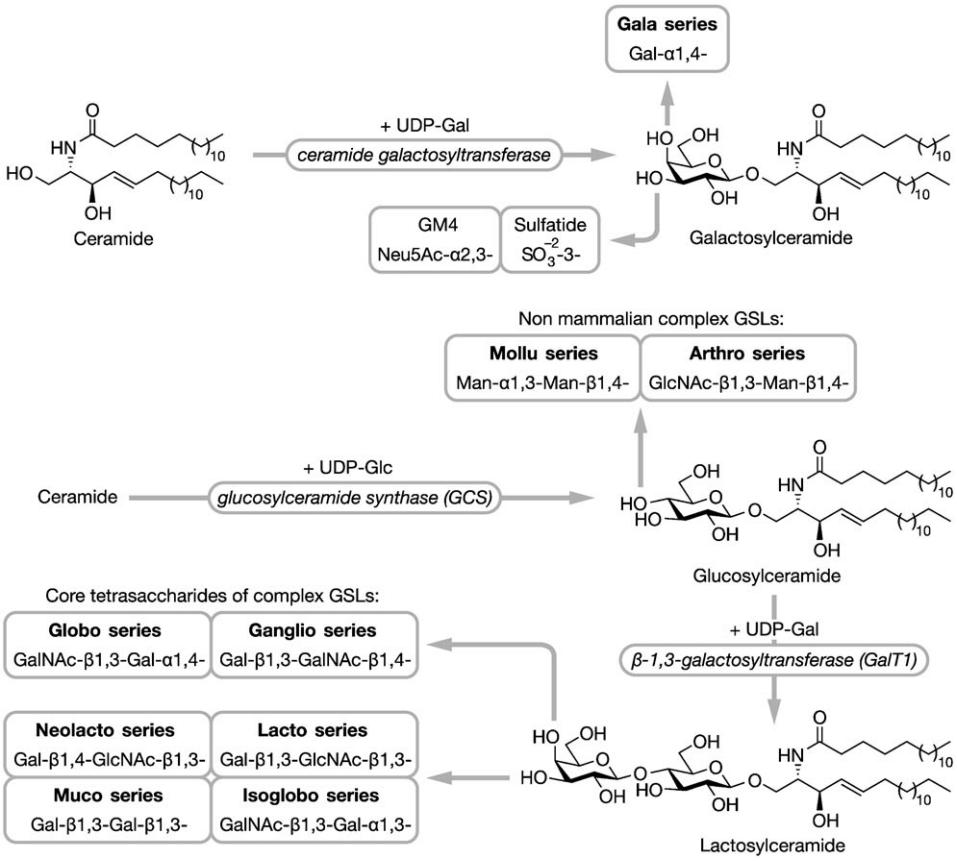
Herman Overkleeft is Professor in Bioorganic Chemistry at Leiden University. His current research interests concern the development of chemical biology strategies to study enzymes in their physiologically relevant environment, with a focus on the glucosylceramide metabolising enzymes, the ubiquitin-proteasome pathway, lysosomal proteases, kinases, and matrix metalloproteases.

Hans Aerts is Professor in Medical Biochemistry at the Academic Medical Center of the University of Amsterdam. His PhD concerned the characterization of glucocerebrosidase, the enzyme deficient in Gaucher disease. His subsequent and current research concerns the therapy and pathogenesis of lysosomal storage disorders, identification and application of biomarkers for these diseases, and the role of glycosphingolipids in manifestations of metabolic syndrome.

1.2. Mammalian Glycosphingolipid Biosynthesis

After its synthesis on the cytosolic side of the ER membrane, ceramide equilibrates to the luminal side. When it is inserted into the external leaflet of a phosphatidylcholine unilamellar vesicle it equilibrates to the inner leaflet with a half-life ($t_{1/2}$) below 1 minute at 37 °C.[24] Ceramide is transformed into galactosylceramide by ceramide galactosyltransferase (CGalT) in the lumen of the ER of some cell types.[25,26] This GSL is further diversified into the Gala series either by sulfation or glycosylation at its 3-O-position with Neu5Ac or

by further extension to oligosaccharides at its 4-O-position through a second α-linked D-galactose (Scheme 2).[8] Ceramide is also transported from the ER to the cytosolic side of the cis-Golgi apparatus membrane by a not yet understood CERT-independent mechanism.[19,27] Here the membrane-bound glycosyl transferase, glucosylceramide synthase (GCS), catalyzes the glycosylation of the primary hydroxy group in ceramide using UDP-glucose as a donor glycoside. A recent study indicated that a region of the ER that is closely associated with mitochondria also shows enzymatic activity that is capable of generating glucosylceramide.[28] Glucosylceramide synthase is an inverting transferase (family 21; GT-A fold).[29,30] 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.[31] One study found that GCS forms heterodimers or oligomers with an unidentified 15 kDa protein.[32] Site-directed mutagenesis and sequence comparisons with other transferases identified several active-site amino acid residues, including His193 located near the N terminus, that were important for binding to both substrate and the GCS inhibitor D-*threo*-(1*R*,2*R*)-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP; **1** in Figure 6).[33] GCS also possesses a metal-coordinating DXD motif, but it does not require a divalent metal for catalysis.[34]

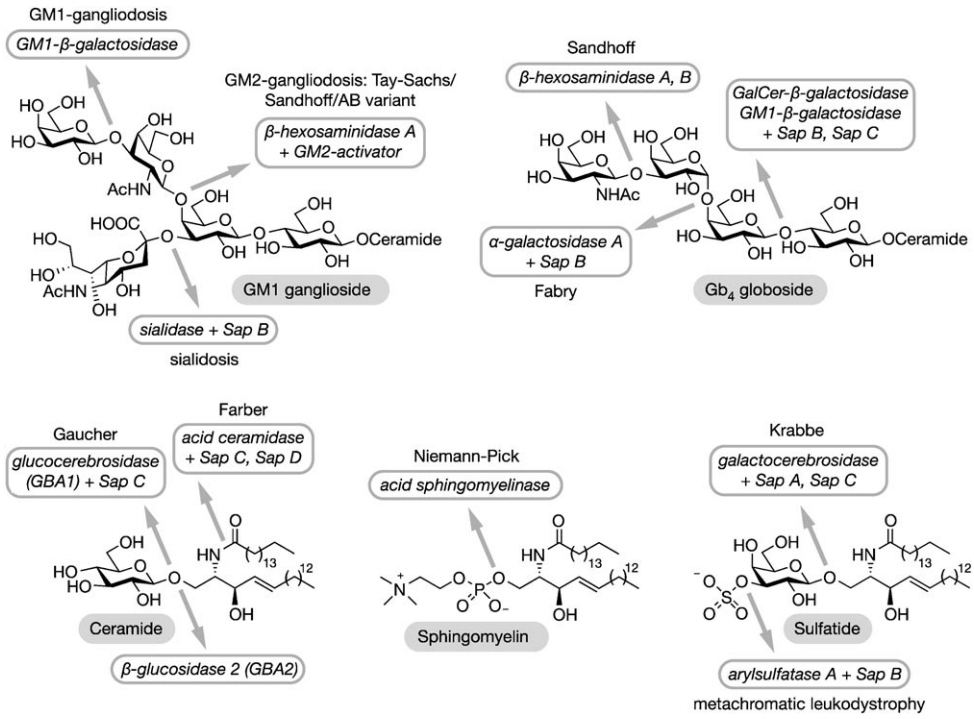


Scheme 2. Overview of the biosynthesis of (mammalian) glycosphingolipids.

Glucosylceramide occupies a key position in the biosynthesis of many GSLs. The fact that both GCS and glucosylceramide face the cytosolic side of the cellular membrane is a characteristic feature in GSL biosynthesis: further synthesis of complex GSLs takes place exclusively at the luminal inside the Golgi apparatus. When glucosylceramide is introduced to the outer leaflet of a model membrane it only slowly equilibrates to the luminal side when unassisted ($t_{1/2}$ = 5 h at 20 °C). However, glucosylceramide undergoes rapid transbilayer movement in the Golgi-apparatus membrane ($t_{1/2}$ = 3 min at 20 °C). Studies indicate that an ATP-independent Golgi-localized "flippase" protein is responsible.[35] The ATP-dependent multidrug transporter P-glycoprotein located throughout the cell acts as a rapid flippase for artificial, fluorescently labeled (NBD) glucosylceramide, galactosylceramide, and sphingomyelin, but not for lactosylceramide.[36] De Matteis and coworkers recently showed that FAPP2 is required for the synthesis of complex GSLs because it mediates the nonvesicular transport of glucocylceramide to distal Golgi compartments, and proposed that FAPP2 is also responsible for the relocation of glucosylceramide to the luminal leaflet of trans-Golgi membranes where further GSL synthesis takes place.[37,38] Van Meer and coworkers reported that FAPP2 may also transport glucosylceramide to the ER. In addition to this, the closely related GLTP transport protein is capable of transporting glucosylceramide to the cytosolic leaflet of the plasma membrane (Figure 2).[39,40]

Having arrived at the luminal leaflet of trans-Golgi membranes, the biosynthesis of GSLs continues with the synthesis of lactosylceramide by GalT1. Lactosylceramide is extended sequentially at either the 3-O-positon or the 4-O-position in a stepwise fashion. The core tetrasaccharides of the GSL series are depicted in Scheme 2. Most of these GSLs consist of alternating and branched combinations of α- or β-linked glucose, galactose, *N*-acetylglucosamine, and *N*-galactosamine. At their nonreducing end, many of these complex GSLs are terminated with either L-fucose or acidic Neu5Ac. Two other nonmammalian series of complex GSLs originating from a β-1,4-linked mannopyranoside to glucosylceramide also exist. Complex GSLs in the Mollu series[41] have been isolated from freshwater bivalves (for example, molluscs) and

GSLs from the Arthro series[42] from several species of arthropods (for example, *Drosophila* flies). Of particular interest are the gangliosides, that is, lactosylceramide-derived sphingolipids containing capping *N*-acetylneuraminic acid (sialic acid) units. A bewildering number of gangliosides exists and a convenient shorthand nomenclature has been developed by Svennerholm, a pioneer in ganglioside research.[43] In this nomenclature G stands for ganglioside, A for asialo-, M for monosialo-, D for disialo-, and T for trisialoganglioside.[44,45] Specific sialyl transferases convert lactosylceramide stepwise into GM3, GD3, and GT3. Lactosylceramide and each of its sialylated derivatives serve as precursors for complex gangliosides of the 0, a, b, and c series. These different series are characterized by the presence of no (0 series), one (a series), two (b series), or three sialic acid residues (c series) linked to the 3-position of the inner galactose moiety. Gangliosides from the 0 and c series are only found in trace amounts in adult human tissues.



Scheme 3. Overview of mammalian (glyco)sphingolipid catabolism. The enzyme/glycosidases and activator proteins responsible are indicated at the glycosidic linkage. The associated sphingolipidose (see Section 2) that is manifested by an enzyme defect is also shown.

1.3. Mammalian (Glyco)Sphingolipid Catabolism

Catabolism of complex glycosphingolipids is a stepwise process that predominantly takes place in endosomes and lysosomes. Glycosphingolipids reach the endosomal–lysosomal compartment in various ways. Receptor-mediated endocytosis of low-density lipoprotein (LDL) delivers glycosphingolipids to the lumen of lysosomes. Phagocytosis of larger structures, such as senescent cells containing glycosphingolipids, occurs by specialized phagocytes, such as macrophages. Another major pathway in most cells involves endocytosis of the plasma membrane.[46,47] Glycosphingolipid-rich membrane parts are internalized and fuse with early endosomes. Here, glycosphingolipids destined for degradation are sorted through formation of intraluminal vesicles (multivesicular bodies) which reach the lysosome.[48,49] The endolysosomal catabolism of glycosphingolipids takes place at the surface of either the internal membrane vesicles or endocytosed lipoproteins (Figure 2). The lysosomal membrane itself is protected from degradation by a glycocalix, which consists of heavily glycosidated membrane proteins.[50] Carbohydrate residues from the nonreducing end of the GSLs are sequentially released by the action of exoglycosidases. In contrast to the biosynthetic enzymes, none of the catabolic glycosidases are bound to the membrane. 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 (SAPs), which assist the glycosidases in their interaction with their target substrate. Five such proteins are currently known: saposin-A, -B, -C, -D, and the GM2-activator protein. Evidence of the role of these glycosidases in catabolism comes from in vitro assays, where their function can be replaced by detergents. Scheme 3 provides an overview of the glycosidases and activator proteins associated with GSL degradation.[8,48,50]

Glucosylceramide is degraded into ceramide and glucose by the enzyme glucocerebrosidase (GBA1; glucosylceramide-β-glucosidase).[51] GBA1 is a retaining glycosidase (family 30), and the activator protein saposin C is essential for its function in vivo.[50] In 1994, Withers and co-workers identified the catalytic nucleophile at the active site as the side-chain carboxylate group of glutamic acid 340.[52] In 2003, Futerman and co-workers published the first X-ray crystal structure of GBA1.[53] Recently, Saenger and co-workers published the X-ray crystal structure of the GBA1 activator saposin C.[54,55] Ceramide is cleaved into sphingosine and fatty acid by acid ceramidase. Ceramide degradation can also take place in other parts of the cell by neutral ceramidases. Sphingosine can be either reacylated to ceramide or used as a substrate for sphingosine-1-phosphate (S1P) synthesis.[56]

Metabolism of endocytosed glycosphingolipids is not restricted to lysosomes. A limited amount of glucosylceramide derived from the degradation of complex glycosphingolipids may escape further lysosomal degradation and

reenter the glycosphingolipid biosynthesis pathway.[57] In addition, direct metabolic remodeling of glycosphingolipids at the plasma membrane may result in local formation of simpler glycosphingolipids from complex ones.[58] The occurrence of nonlysosomal glucosylceramidase activity has long been known, and was recently identified as β-glucosidase 2 (GBA2).[59–61] GBA2, a 105 kDa protein with a transmembrane region, has not yet been assigned to a specific family of glycosidases. In contrast to GBA1, it is not sensitive to inhibition by conduritol B epoxide (**2**; CBE; Figure 3).[60] The enzyme has a pH optimum in the neutral region, as opposed

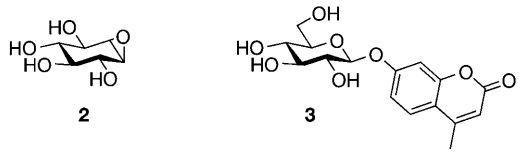


Figure 3. Structure of conduritol B epoxide (**2**) and 4-methylumbelliferyl-β-D-glucoside (**3**).

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. Addition of the fluorescent substrate 4-methylumbelliferyl-β-D-glucoside (**3**; Figure 3) to cell cultures that express GBA2 results in almost instantaneous GBA2 activity, which indicates it might be anchored to the outer plasma membrane. Furthermore, experiments with fluorescently labeled glucosylceramide showed that the ceramide generated by GBA2 action was rapidly converted into sphingomyelin, thus suggesting that GBA2 co-localizes with SMS2.[60] The function of GBA2 is currently not known, but its inhibition in mice is associated with impaired spermatogenesis, a result that is confirmed in studies with a GBA2 knock-out mouse model.[61–63]

Two other distinct glycosidases have also been implicated in glucosylceramide catabolism. The β-glucosidase lactase-phlorizin hydrolase (LPH) is capable of hydrolyzing glucosylceramide. LPH is an approximately 300 kDa retaining glycosidase (family 1) that is sensitive to CBE (**2**). It is bound to the outer plasma membrane and is expressed exclusively in the microvilli of intestinal epithelial cells. LPH is also able to hydrolyze galactosylceramide, lactosylceramide, as well as glucosyl- and galactosylsphingosine, but not GM1 ganglioside.[64,65] LPH might, therefore, play a role in the intestinal digestion of food-derived GSLs.[66] The human β-glucosidase GBA3 is an approximately 60 kDa retaining glycosidase (family 1) located in the cytosol. It is insensitive to CBE (**2**) and operates optimally at a neutral pH. In vitro studies with recombinant GBA3 indicate it has a broad substrate specificity that includes glucosylceramide and galactosylceramide. Recently determined X-ray structures of GBA3 revealed its two catalytic residues (nucleophile: Glu371; acid/base: Glu165).[67,68]

2. Inherited Sphingolipidoses

The importance of endolysosomal catabolism of sphingolipids is best illustrated by the existence of a group of inherited disorders in humans caused by deficiency in lysosomal catabolic pathways, the sphingolipidoses (Scheme 3).[50] Inherited deficiencies in a specific lysosomal enzyme or activator protein result in accumulation of the corresponding (glyco)sphingolipids.[69–71] The most common of the sphingolipidoses is Gaucher disease,[72] an autosomal recessive disorder caused by deficient glucocerebrosidase activity.[73,74] The manifestation of Gaucher disease is remarkably heterogeneous: its onset can occur from birth up to an almost asymptomatic course at old age. The underlying mutations in the *GBA1* gene show some correlation with the severity of disease manifestation and, in particular, the development of neurological symptoms. A low residual enzyme activity in leukocytes or fibroblasts is associated with a more severe progression of the disease.[75,76] The most common mutation in the *GBA1* gene, which encodes the amino acid substation N370S, is usually associated with a relatively benign course of the disease, with no neuropathology involved. N370S-GBA1 is normally synthesized and delivered to lysosomes, but shows catalytic abnormalities.[77,78] In sharp contrast, the other common L444P mutation results in a polypeptide that folds poorly in the ER.[77] Homozygotes for L444P-GBA1 develop a severe, neuropathic course of the disease. In contrast to other lysosomal glycosidases, GBA1 does not acquire mannose-6-phosphate moieties, but is sorted and transported to lysosomes by interaction with the integral membrane-protein LIMP-2.[79–81] Deficiency in LIMP-2 may, therefore, also result in reduced cellular GBA1 activity.[82] Since GBA1 requires the activator protein saposin C for efficient intralysosomal degradation of glucosylceramide, deficiency in this accessory protein also results in the accumulation of glucosylceramide in cells.[83] The majority of Gaucher patients have one N370S-GBA1 allele and develop a nonneuropathic, so-called type 1, disease. In these patients, accumulation of the substrate glucosylceramide is restricted to tissue macrophages. These heavily lipid-laden macrophages, named Gaucher cells, have a characteristic appearance (Figure 4a). Gaucher cells are viable and secrete characteristic proteins such as chitotriosidase and CCL-18.[84,85] Elevated levels of these proteins are found in Gaucher patients, and their measurement is currently used to monitor disease progression as well as efficacy of therapeutic interventions[86] (Figure 4b; discussed in Section 4.3). The presence of large numbers of Gaucher cells in various tissues results in characteristic clinical signs such as hepatosplenomegaly, pancytopenia, and skeletal deterioration. The constant release of hydrolases and cytokines by Gaucher cells and surrounding phagocytes is thought to underlie the pathological features of the disorder.[87–91]

Inherited deficiencies in acid sphingomyelinase (Niemann–Pick disease A/B), acid ceramidase (Farber disease), galactocerebrosidase (Krabbe disease), β-hexosaminidase (Sandhoff and Tay–Sachs disease), acid β-galactosidase (GM1-gangliosidosis), acid α-galactosidase A (Fabry disease), and in the saposin and GM2 activator genes also result in sphingolipidoses (Scheme 3). Interestingly, in the case of Krabbe disease and Fabry disease not only do the glycosphingolipid substrates accumulate, but also their *N*-deacylated derivatives (psychosine and globotriaosylsphingo-

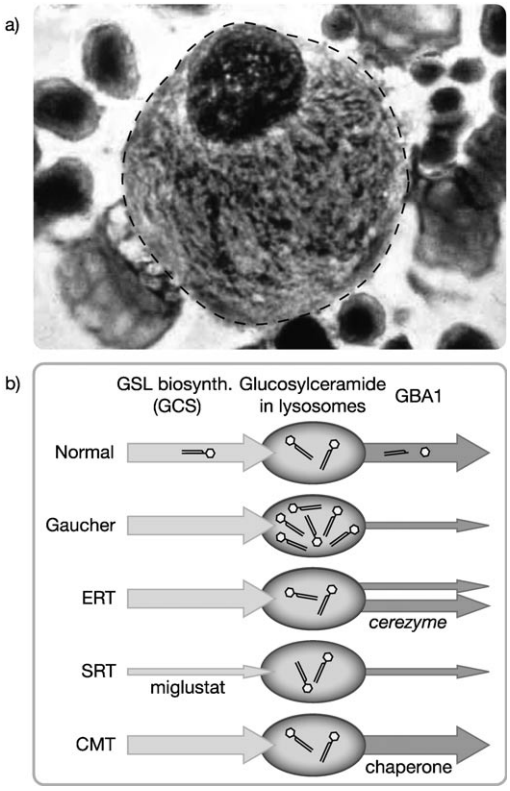


Figure 4. a) Gaucher cell; b) schematic overview of Gaucher disease and its potential therapies.

sine, respectively). Both play a crucial role in the pathophysiology of these disorders.[50,92] The importance of SLs and GSLs in mammalian physiology is evident from the fact that there are few hereditary diseases that impair their biosynthesis.[71] Exeptions are GM3 synthase deficiency,[93] hereditary sensory neuropathy I (deficiency in a subunit of serine palmitoyltransferase),[94] and deficiency in the α-1,4-galactosyltransferase Gb_3 synthase (resulting in the rare P blood group phenotype).[95] Systemic deletion of the *GCS* gene in a mouse model resulted in death during the early stages of embryogenesis.[96]

3. Glycosphingolipids 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 as a result of variation in the sphingoid 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 processes.[97,98] The following sections describe some of the biological functions that are attributed to SLs and GSLs, with a focus on GSLs.

3.1. Glycosphingolipids and Lipid Rafts

The function of GSLs on the cell surface can be roughly divided into two basic functions: 1) involvement in cell adhesion/recognition processes by interactions with GSLs and lectins on other cells, and 2) modulation of signal transduction by influencing receptor proteins on the cell surface. Unlike phosphoglycerolipids, GSLs and SLs do not distribute homogeneously in the outer plasma membrane. Instead it is postulated that they, together with cholesterol, form semiordered lipid microdomains, also called lipid rafts.[99–103] Certain proteins appear to associate with these microdomains, including GPI-anchored proteins, flotillins, caveolins, G-protein-coupled receptors, and certain receptor tyrosine kinases such as the epidermal growth factor receptor and the insulin receptor. The lipid microdomains exist in a gel-like liquid-ordered phase (l_o), which has a lower diffusion rate than the surrounding liquid-disordered (l_d) phosphoglycerolipid-rich plasma membrane.

Initially, proof of the existence of lipid microdomains was their isolation by extraction of membranes at 4 °C in the presence of specific detergents: the so-called detergent-resistant membrane domains (DRMDs). However, the physiological relevance of the data obtained by this method is still under debate, since the composition and dynamics of membranes extracted with detergent at 4 °C may be quite different from the situation in live cells at 37 °C.[104] The following current definition of lipid rafts illustrates the existing challenges for further study: membrane rafts are small (10–200 nm), heterogeneous, highly dynamic (ca. 0.1–1000 ms), sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. The existence and physiological role of lipid rafts is still very much under debate and less intrusive techniques are required to detect and study lipid rafts in living cells. One such technique, stimulated emission depletion (STED) microscopy, has recently made it possible to show in living cells that SLs and GPI-anchored proteins are indeed transiently trapped in cholesterol-containing domains (ca. 20 nm).[105] Several recent reviews document the current state of research into lipid rafts.[106,107] Although most current research into lipid rafts focuses on the outside of the cell, their original proposed role was in intracellular sorting. By using BODIPY-labeled GSLs, Pagano and co-workers demonstrated that GSL-containing microdomains also occur intracellularly, and that these play an important role in caveolae-mediated endocytosis and endosomal sorting of many cellular proteins and lipids.[108,109]

3.2. Sphingolipids and Cellular Signaling

SLs have been implicated in numerous intra- and extracellular signaling processes as both signaling molecules and secondary messengers.[110,111] Sphingomyelin is the most common SL and comprises as much as 30 % of the total membrane lipids in certain tissues.[112] In the plasma membrane, sphingomyelin is postulated to associate predominantly with cholesterol and GSLs to form microdomains. It is synthesized at two different locations in the cell and converted into ceramide by the neutral, alkaline, and acid sphingomyelinases distributed throughout the cell. Heat shock, oxidative stress, and other damaging conditions

induce cells to produce an elevated level of ceramide, which has been implicated in signal-transduction pathways that lead to apoptosis.[9] Ceramide can be converted into ceramide-1-phosphate and via sphingosine to sphingosine-1-phosphate (Scheme 1). Less is known about the function of ceramide-1-phosphate, but it has been implicated in inflammatory signaling pathways and as a promoter of cell survival.[20,113] Sphingosine is thought to serve as an intracellular regulator of 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 angiogenesis. In general, sphingosine-1-phosphate has the opposite effect of ceramide, in that it promotes cell growth and survival.[20,113] Finally, the less well studied sphingosylphosphorylcholine has been shown to stimulate cell division and has also been implicated in proinflammatory signaling pathways.[22,23]

3.3. Glycosphingolipids in Immunology

α-Galactosylceramide, a GSL derived from marine sponges and not found in humans, has become the model ligand for studying the binding and presentation of glycolipids by CD1d molecules to invariant natural killer T (iNKT) cells. Upon recognition of α-galactosylceramide by iNKT cells, a cascade of events results in the activation of various cells of the innate and adaptive immune system.[114]

The mammalian GSL isoglobotrihexosylceramide (iGb3; Gal-α-1,3-lactosylceramide) has been proposed to be the endogenous self-ligand, which mediates positive selection of developing iNKT cells for normal functioning.[115] These results were recently contradicted by two studies.[116,117] However, observations that intracellular CD1d antigen loading occurs in endosomal/lysosomal compartments with low pH values and that it is dependent on the presence of saposin activator proteins suggest that another GSL may still be the self-ligand. This is further substantiated by a report that GCS-deficient mice (either by gene knock-out or PDMP (**1**) inhibition) are unable to present antigen on CD1d and activate their iNKT cells.[118] A more extensive overview of the role of (glyco)sphingolipids in immunology is given in several recent reviews.[119,120]

3.4. Glycosphingolipids and the Brain

Starting with Thudichum's work, the importance of GSLs in brain tissue was noticed early on in GSL research. Although still not understood, Neu5Ac-terminal acidic gangliosides in particular seem to play an important role in neurochemistry. They are found in high concentrations in brain tissue and constitute up to 25% of the lipid content of the outer membrane. During embryogenesis and the postnatal period a small subset of acidic gangliosides is highly expressed in the developing brain. The levels of gangliosides are much lower in the adult brain, but many more different types of gangliosides are expressed.[121] Several knock-out mouse models of the glycosyltransferases in GSL biosynthesis have shed some light on their function 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 three weeks.[122] Knock-out models of several transferases involved in the biosynthesis of more complex gangliosides indicate that a certain degree of functional redundancy exists among the brain gangliosides.[9,123] Brain gangliosides have also been implicated in several neurological diseases. Intriguingly, a markedly increased incidence of carriers of mutant GBA1 alleles has been identified among individuals that developed Parkinson disease.[124] Thus, decreased functioning of GBA1 may predispose people for Parkinson disease. Furthermore, a lowered GCS activity in the brain tissue of Alzheimer patients[125] causes an increase in ceramide and a decrease in the levels of complex GSLs, which in turn may cause abnormal functioning of neural cells in Alzheimer patients. Abnormal functioning could be prevented by infusions with ganglioside GM1. In contrast to this, GM1-enriched membrane microdomains 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.[126]

3.5. Glycosphingolipids and the Skin

GSLs and SLs play vital roles in the normal functioning of the skin. Ceramides and keratins are the essential components of the epidermal stratum corneum, which makes the skin of all land-dwelling animals impermeable to water, thereby preventing lethal dehydration.[127] Ceramides occupy the extracellular space of the stratum corneum and are characterized by N-acylation of the sphingoid backbone with long ω-hydroxy fatty acid chains (C_{30}–C_{36}).[8] The ω-hydroxy group in these ceramides can be acylated with fatty acids or covalently bound to epidermal proteins.[128] Ceramide in keratinocytes of the epidermis often (ca. 40%)[129] contain phytosphingosine instead of sphingosine. In the epidermis, ceramides are excreted into the extracellular space by exocytosis of sphingomyelin intermediates and free or protein-bound[130] glucosylceramide. Keratinocytes simultaneously excrete vesicles that contain GBA1, saposin C, and sphingomyelinase which hydrolyze these intermediates to generate skin ceramides at the required location. Indeed, glucosylceramide constitutes about 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 an intermediate in maintaining the ability of the skin to act as a barrier.[131] The mutant animals displayed a grossly abnormal stratum corneum and died of dehydration within five days after birth. Inhibition of GBA1 activity by topical exposure of the skin to CBE (**2**) also caused impaired functioning of the skin.[127] In accordance, skin abnormalities are also observed in patients with severe forms of Gaucher disease. Many skin diseases such as psoriasis are also characterized by 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 transfer of a corrective *ABCA12* gene.[132]

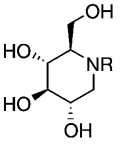
The enzyme SM deacylase was found to beoverexpressed and levels of sphingosylphosphorylcholine (SPC) 300% higher in patients with atopic dermatitis.[133] Extracellularly excreted SM deacylase in the epidermis is also capable of deacylating glucosylceramide to glucosylsphingosine.[134] The increased activity of this enzyme has been suggested to contribute to dermatitis pathology by preventing sufficient generation of ceramides in the stratum corneum.

3.6. Glycosphingolipids and Pathogens

GSLs are found at increased concentrations on the outer membranes of apical cells that line the inside of the stomach, intestines, and respiratory track. Apical cells represent the initial barrier of the body to the external world and are the first to make contact with potential pathogens. Many pathogens have evolved mechanisms that exploit apical cell surface GSLs to infect and invade their host. Membrane microdomains rich in GSLs with terminal Neu5Ac residues are often targeted by viruses, bacteria, and protozoans. The HIV-1, Ebola, and Marburg viruses all use these membrane microdomains in binding to, entry in, and budding from host cells.[135]

One of the best studied examples is that of the influenza virus (A/B/C) family, which is a very topical subject with the current pandemic of H1N1 flu ("swine flu"). The influenza virus has only three different surface proteins, of which the two most abundant are specifically aimed against the terminal Neu5Ac group on the GSLs and glycoproteins of the human host. The protein, called hemagglutinin (H), is a specific lectin for Neu5Ac and enables the virus to bind to the host cell, after which it is endocytosed. A major factor why humans are not yet readily infected by the H5N1 flu ("bird flu") is because its hemagglutinin currently preferentially recognizes the terminal α-2-3-linked Neu5Ac in bird respiratory tracts, whereas humans predominantly possess terminal α-2-6 linked Neu5Ac in their upper respiratory tracts[136] (a switch the H1N1 flu ("Spanish flu") of the 1918 pandemic was able to make).[137] The role of the second viral protein, a sialidase called neuraminidase (N), 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 result in release from the host cell and prevent self-agglutination of viruses.[136] Besides the currently used antiviral drugs—the neuraminidase inhibitors Tamiflu and Relenza—1-deoxynojirimycin-based iminosugars such as Miglustat (**4**; see Table 1) have also been applied successfully to suppress the propagation of various viruses in cellular models.[138,139] Many viruses express glycoproteins on their surface, and glucosidase inhibitors can disrupt the proper processing of viral *N*-glycoproteins in the host ER by inhibiting the trimming by ER glucosidase I + II and calnexin/calreticulin binding. Although this has not yet resulted in a viable antiviral drug, the successful and ongoing development of iminosugars as therapeutics for the treatment of GSL-related disease has benefited greatly from this pioneering work.

Table 1: Inhibitory profile (IC_{50} in vitro in μM) and cytotoxicity for *N*-alkylated 1-deoxynojirimycins.



	GCS	Cell proliferation CC_{50}	GBA1
7: R = H	2 mM (0%)[a][201]	>5 mM[201]	240[203]
20: R = Me	200 (31%)[a][202]	–	150[203]
21: R = Et	200 (52%)[a][202]	–	–
22: R = propyl	200 (69%)[a][202]	–	700[203]
4: R = butyl	34.4[196]	>10 mM[201]	270[203]
23: R = hexyl	23.8[196]	>1 mM[196]	13[203]
24: R = octyl	16.6[196]	984.1[196]	0.82[203]
25: R = nonyl	7.4[196]	118.9[196]	0.66[203]
26: R = decyl	3.1[196]	95.5[196]	–
27: R = dodecyl	5.2[196]	39.7[196]	0.05[204]
28: R = hexadecyl	3.4[196]	25.1[196]	–
29: R = octadecyl	4.0[196]	36.6[196]	–
30: R = 7-oxadecyl	3.2[201]	>5 mM[201]	–
31: R = 7,10,13-trioxatetradecyl	200 (93%)[a][197]	–	–

[a] Percent inhibition at stated concentration.

Another example of a bacterial pathogen that abuses host GSLs is *Helicobacter pylori*. It causes gastric ulcers and infects the gastric lining through the binding of lectins to several host GSLs, including the sialyl dimeric Lewis-X antigen (neolacto series with one terminal Neu5Ac and two terminal L-fucose residues).[140] Depletion of surface GSLs by inhibition of GCS with Miglustat (**4**) or PDMP (**1**) impairs the adherence of several bacteria species to host cells.[141,142] Finally, *Vibrio cholerae* expresses a sialidase that removes terminal Neu5Ac from complex GSLs (for example, ganglioside GD1a) on the apical surface of the intestinal epithelial cells of the host. This action exposes apical GM1 gangliosides to which the cholera toxin can bind, which eventually causes diarrhea after being internalized.[143,144]

3.7. Glycosphingolipids and Cancer

Most tumor cells show altered GSL patterns on their surface as well as abnormal SL signaling and increased GSL biosynthesis, which together play a major role in tumor growth, angiogenesis, and metastasis.[145,146] The human sialidase Neu3 is found on the plasma membrane in caveolae-containing microdomains and cleaves terminal Neu5Ac residues from GSLs.[147] It is overexpressed in many types of cancer and plays an important role in tumor growth and survival.[148] Tumor cells also actively shed specific gangliosides from the cell surface to cloak themselves from the body's immune system.[149]

The effectivness of many chemotherapy agents and radiotherapies in treating cancers has been found to rely on their ability to increase levels of ceramide in tumor cells so as to activate ceramide-mediated apoptosis. Many tumors have increased expression and activity of GCS. This is thought in

part to function as a detoxification method for the increased ceramide levels by conversion into 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 through 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 membrane microdomains. Through the consumption of ATP it is capable of transporting a wide range of noncharged amphiphilic molecules—including glucosylceramide—from the cytosol to the outer plasma membrane. Overexpression of the P-glycoprotien efflux pump is actually one of the most consistent hallmarks of drug resistance in many pathogens, including the antimalaria resistance of *Plasmodium falciparum*, chemotherapy resistance by the protozoan *Leishmania*, and resistance to macrolide antibiotics by *S. pneumoniae*.[150,151]

3.8. (Glyco)Sphingolipids and Insulin Resistance

The incidence of obesity is markedly increasing as a result of the increase in the amount and caloric content of food as well as diminishing physical activity. Obesity is associated with an increased risk for lowered insulin sensitivity. This so-called insulin resistance is characterized by reduced insulin-mediated glucose uptake and impaired suppression of lipolysis as well as hepatic glucose production. Another common feature of obese individuals is low-grade inflammation, mediated by cytokines secreted by macrophages in their adipose tissue, which further promotes insulin resistance.[152] In the last decade evidence has accumulated that points to a key role of excessive lipids in the etiology of obesity-induced insulin resistance.[153] Lipid excess in tissues is associated with impaired insulin signaling and action. A strong inverse correlation exists between the intramyocellular lipid content and the insulin-stimulated glucose uptake of the whole body.[154] It is thought that the surplus of triglycerides and free fatty acids delivered to insulin-responsive tissues results in local high concentrations of other lipids, including sphingolipids. Excessive amounts of sphingolipids hamper the insulin signaling and promote inflammation. For example, in cell models, increased concentrations of ceramide lead to inhibition of insulin signaling at the level of Akt/protein kinase B (PKB).[155] Since the role of ceramide and glycosphingolipids in insulin resistance has been extensively reviewed,[156–159] only the most recent findings that stress the importance of glycosphingolipids in regulating insulin responsiveness are discussed.

The insulin receptor is localized at the cell surface in GSL-containing lipid microdomains (Figure 5a). The interaction of gangliosides with the insulin receptor was originally described by Nojiri et al.,[160] who demonstrated the ganglioside-mediated inhibition of the insulin-dependent cell growth of leukemic cell lines. Tagami et al. were the first to demonstrate that the addition of GM3 ganglioside to cultured adipocytes

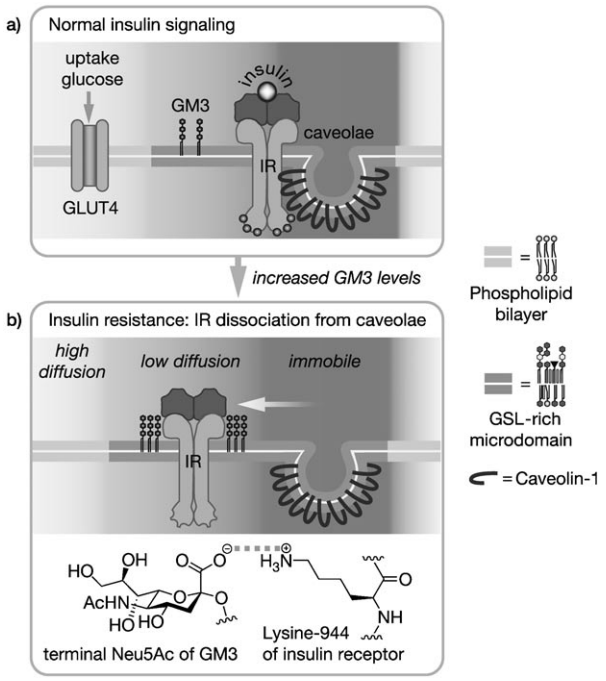


Figure 5. The insulin receptor (IR) and its membrane environment under normal conditions (a) and during insulin resistance (b).

suppresses phosphorylation of the insulin receptor and its downstream substrate IRS-1, thereby resulting in reduced glucose uptake.[161] Inokuchi and co-workers reported that exposure of cultured adipocytes to TNF-α increases GM3 and inhibits IR and IRS-1 phosphorylation. This was found to be counteracted by PDMP (**1**), an inhibitor of glycosphingolipid biosynthesis.[162] Mutant mice lacking GM3 have been reported to show an enhanced phosphorylation of the insulin receptor of skeletal muscle after ligand binding, and to be protected from high fat diet induced insulin resistance.[163] Consistent with this is the recent report on increased insulin sensitivity and glucose tolerance in mice with increased expression of the GM3-degrading sialidase Neu3.[164] In contrast, GM3 levels are elevated in the muscle of certain obese, insulin-resistant mouse and rat models.[161] Altered sphingolipid metabolism, as reflected by increased glycosphingolipid levels, has recently also been documented in relation to neuronal pathology in diabetic retinopathy.[165] More recently, Kabayami et al. provided evidence that the interaction of GM3 with the insulin receptor is mediated by a specific lysine residue located just above the transmembrane domain of the receptor, and that excess levels of GM3 promote dissociation of the insulin receptor from caveolae, a location which is essential for transduction of the insulin signal (Figure 5b).[166]

The value of pharmacologically lowering excessive glycosphingolipid levels to increase insulin sensitivity has recently been demonstrated. Holland et al. reported that inhibition of the synthesis of ceramide, the precursor of glycosphingolipids, markedly improves glucose tolerance and prevents the onset of overt diabetes in obese rodents.[167] Zhao et al. demonstrated that inhibition of the first step in the biosynthesis of

glycosphingolipids, which is catalyzed by GCS, exerts beneficial effects. The GCS inhibitor Genz-123346 (**5**; Figure 6) lowered blood glucose and HbA1C levels and improved

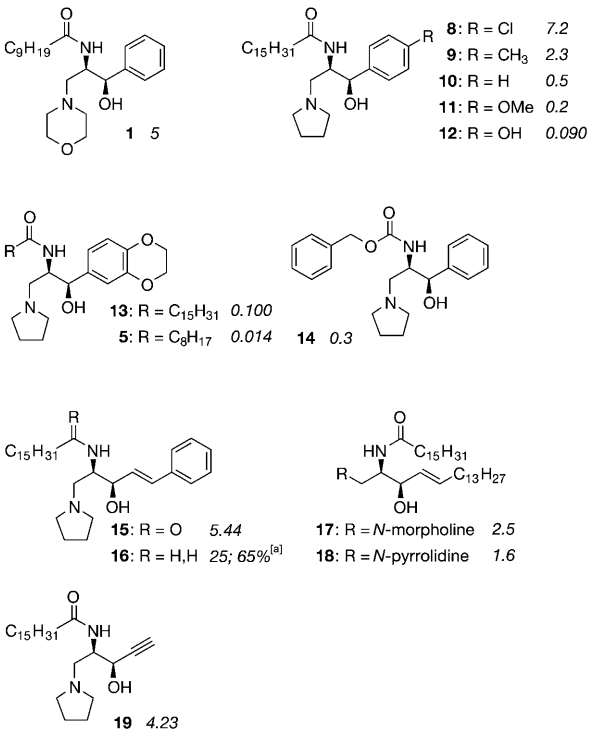


Figure 6. Overview of the structures and IC_{50} values (in μM, italic) of PDMP-based GCS inhibitors. [a] Percent inhibition at inhibitor concentration (in μM).

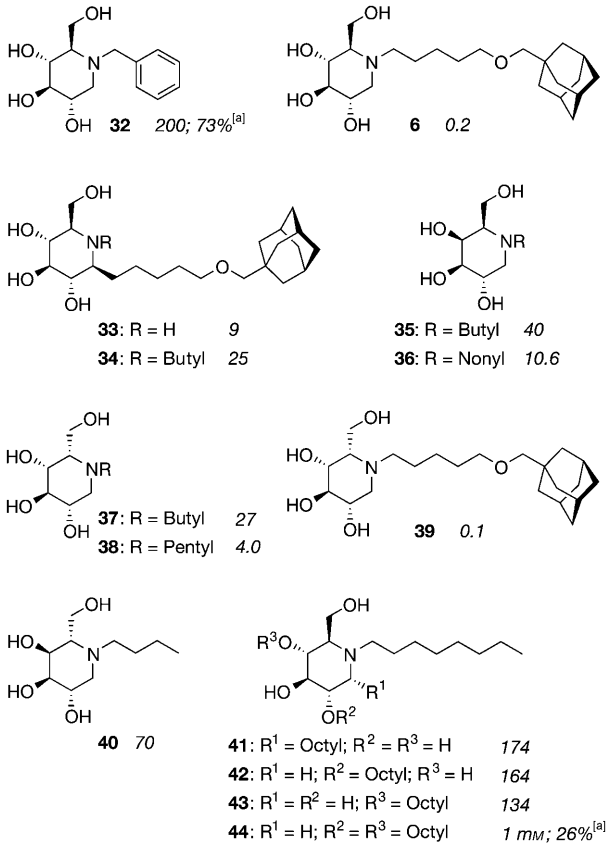


Figure 7. Overview of the structures and IC_{50} values (in μM, italic) of piperidine-based GCS inhibitors. [a] Percent inhibition at the specified inhibitor concentration (in μM).

glucose tolerance in insulin-resistant rodents.[168] Finally, we showed that treatment of various insulin-resistant rodent models with the hydrophobic iminosugar *N*-(5-adamantane-1-yl-methoxy)-pentyl-1-deoxynojirimycin (**6**; Figure 7), a well-tolerated and potent inhibitor of GCS, very markedly lowered circulating glucose levels, improved oral glucose tolerance, reduced HbA1C, and improved insulin sensitivity in muscle and liver.[169] An additional beneficial feature of **6** is its ability to improve the adipocyte function and reduce inflammation in adipose tissue of obese mice.[170] Reduction in the amount of glycosphingolipids by treatment with **6** was also found earlier to exert beneficial effects in mouse models of inflammatory bowel disease.[171]

4. Modulating GSL Metabolism: Inhibitors and Chaperones

Small-molecule inhibitors of the enzymes involved in the metabolism of glycosphingolipids have found widespread use as research tools, are the subject of therapeutic investigations, and in some examples have found clinical use in combating GSL-related disease. Of particular interest are compounds that interfere with glucosylceramide metabolism. Among the class of covalent glycosidase inhibitors, CBE (**2**) has mainly been used for the irreverisble inhibition of GBA1.[172] However, most of the inhibitors of GBA1 and GCS reported to date are reversible inhibitors based on the naturally occurring iminosugar 1-deoxynojirimycin (**7**; see Table 1) and its structural and stereochemical congeners. Iminosugars are carbohydrate derivatives in which the endocylic oxygen atom is replaced by a nitrogen atom, and by this virtue may inhibit glycosidases[173–175] and to a lesser extent glycosyltransferases.[176,177] The structural diversity of iminosugars results in a large degree of selectivity in their inhibition of specific glycosidases and transferases.[178,179]

Given the difficulties in isolating large quantities of iminosugars from natural sources, much research since the 1970s has focused on developing synthetic methods for the synthesis of iminosugars.[178–181] During the late 1980s and early 1990s, research into the application of iminosugars as antivirals led to the discovery of *N*-alkylated 1-deoxynojirimycins as potent inhibitors of ER glucosidase I and II. One of these is Miglustat (**4**), which was also found to be an inhibitor of GCS.[182] This discovery promoted research into the development of new iminosugars as inhibitors of glucosylceramide metabolism. The next sections provide an overview of the different inhibitors of GCS and GBA1 that have so far been discovered, with a focus on iminosugar-based inhibitors. Recent reviews by Delgado et al. provide an overview of inhibitors of SL metabolic enzymes.[183,184]

4.1. 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 (**1**), the first example of a GCS inhibitor. PDMP was part of a series of ceramide analogues in which the unsaturated alkyl chain of the sphingosine backbone is replaced by a phenyl group and the primary hydroxy group by a variety of heterocycles (Figure 6).[185] Only analogues with an L-*threo* configuration—the opposite of ceramide— inhibited GCS. Kinetic analysis showed **1** to be uncompetitive for UDP-glucose and a mixed competitive inhibitor for ceramide.[186] Switching the heterocycle to a pyrrolidine (**10**) resulted in a tenfold increase in potency.[187] Subsequent investigation of *para* substituents on the phenyl ring of **10** revealed a relationship between the IC_{50}, the hydrophilicity, and the electron-donating capacity of the *para* position (**8**–**12**; Figure 6).[188] An ethylenedioxy group on the phenyl ring (**13**)[188] in combination with a shorter acyl chain gave the most potent PDMP derivative (**5**) and GCS inhibitor to date.[168,189] Two other studies have shown that the acyl chain can be replaced by a benzyloxy carbonyl group (**14**)[190] but not by an alkyl chain (**15** versus **16**).[191] Derivatives **17** and **18**, which more closely mimic ceramide, were comparable to PDMP in inhibiting GCS.[192] Van Calenbergh and co-workers have shown that replacing the sphingosine-mimicking aryl moiety of PDMP with a terminal alkyne (**19**) also results in a potent inhibitor of GCS.[191]

Evaluation of the selectivity of a close structural homologue of **13** with an IC_{50} value of 24 nM for GCS revealed that it does not inhibit the enzymes GBA1, GBA2, ER α-glucosidases I + II, debranching enzyme, sucrase, and maltase.[189] Another study showed that **13** does not inhibit GCS in lower animals, plants, fungi, or bacteria, but only inhibits human GCS.[193] During the development of PDMP (**1**) and its derivatives it was discovered that many of these compounds also inhibit the lysosomal phospholipase A2. This enzyme, amongst others, is capable of acylating ceramide to 1-*O*-acylceramide. Its inhibition can result in phospholipidosis[194] and increased cellular ceramide levels.[195]

In 1994, Platt et al. reported that **4** (Miglustat)[182] is an effective GCS inhibitor. Since then, the same research group has devoted considerable attention to elucidating the mode of action by which alkylated deoxynojirmycin derivatives inhibit GCS.[196,197] These studies have shown that an *N*-propyl chain (**22**) is the minimum length for inhibition of GCS and that lengthening the *N*-alkyl chain further improves inhibition of GCS up to the *N*-decyl group (**26**, Table 1).[196] Linear aliphatic N-alkylation does not appear to be crucial, as the IC_{50} value of *N*-benzylated **32** is comparable to that of **4** (Figure 7).[197] Lengthening the *N*-alkyl chain also increases the cytotoxicity of these compounds (Table 1).[196] The exact nature of this toxicity is not fully understood, but might be caused by membrane solubilization as a consequence of them acting as detergent amphiphiles. Van den Broek et al. have shown that the introduction of an ether function into the *N*-alkyl chain decreases the cytotoxicity (**26** compared to **30** in Table 1).[198] The *N*-5-(adamantan-1yl-methoxy)-pentyl (AMP) functionalized 1-deoxynojirmycin **6** proved to be a very potent GCS inhibitor.[199] Translocation of the AMP moiety to other positions of 1-deoxynojirmycin abolished its ability to inhibit GCS, with the exception of β-aza-*C*-glycoside derivatives **33** and **34**.[200]

Miglustat (**4**) was first reported in 1988 by Fleet et al. as one of a series of inhibitors of HIV virus replication.[205] Inhibition of ER α-glucosidase I and II causes this effect and, in an attempt to dissociate this activity from GCS inhibition, Platt et al. investigated the modification of the iminosugar section in **4**. This study revealed that *N*-butylated 2-acetamido-, D-*manno*-, and L-*fuco*-1-deoxynojirimycin no longer inhibit GCS. However, D-*galacto* derivatives (**35** and **36**) do inhibit GCS and no longer inhibit ER glucosidase I or GBA1 (Figure 7).[202] Recent results have shown that L-*ido*-(**37**–**39**) and L-*altro*-configured (**40**) lipophilic iminosugars also represent a promising class of selective GCS inhibitors.[206–208] Compound **39** represents the most potent iminosugar-based GCS-selective inhibitor reported to date.[208]

The lack of a crystal structure for GCS has resulted in there being little structural information that explains the mode of binding of lipophilic iminosugars to the GCS active site. A study by Butters et al. of the kinetics of GCS inhibition by **4** suggested that it was noncompetitive for UDP-glucose and competitive for ceramide.[197] On the other hand, compound **25** proved to be noncompetitive for both substrates.[196] These results, combined with the structural similarities of **4** and ceramide, led Butters et al. to tentatively designate **4** as a ceramide mimic.[196] This hypothesis indicates that the attachment of a second lipophilic moiety at the 2-O-position of **4** or **25** could result in a better mimic of ceramide. However, a recent study by Compain, Martin, and co-workers on di- or trialkylated derivatives **41**–**44** showed this did not result in more potent GCS inhibitors (Figure 7).[209]

Several pyrrolidine iminosugars have also proven to be GCS inhibitors. In 2000, Butters et al. revealed DMDP derivative **47** as a GCS inhibitor of comparable activity as **4** (Figure 8).[197] Two studies by Davis and co-workers have also identified several pyrrolidine inhibitors (**48**–**52**).[210,211] Génisson and co-workers have developed pyrrolidines that mimic the sphingosine backbone (**53**–**56**), with **55** proving to be a potent GCS inhibitor.[212] Finally, Blériot and co-workers recently reported moderately active azepane-based inhibitors of GCS (**57**–**60**).[213]

4.2. Inhibitors of Glucocerebrosidase

As mentioned in the previous section, Miglustat (**4**) also inhibits GBA1. Research has shown that, similar to GCS inhibition, increasing the length of the *N*-alkyl chain in **4** also improves GBA1 inhibition (Table 1, **4**, **25**, and **6** in Figure 9). Attachment of amantadine to the *N*-alkyl tail by an amide linkage to produce AMP-DNM derivative **61** does not decrease GBA1 inhibition.[214] Attachment of an AMP moiety to the O2- or O6-hydroxy group of 1-deoxynoijirimycin also provided submicromolar GBA1 inhibitors.[200]

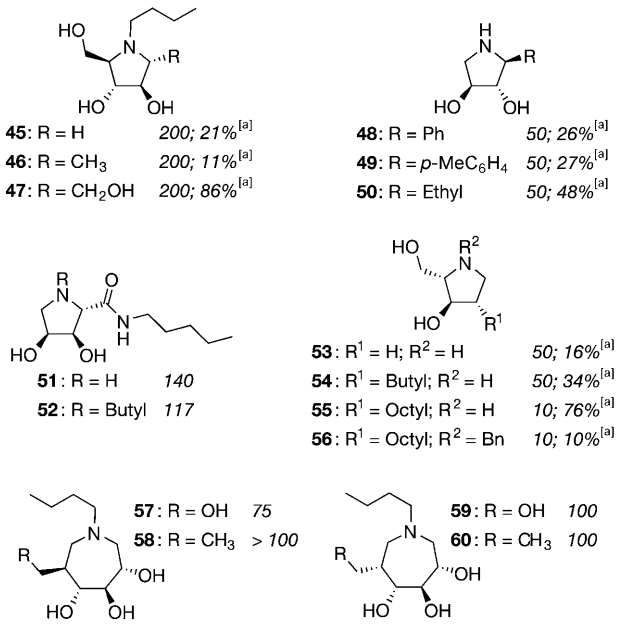


Figure 8. Overview of the structures and IC_{50} values (in μM, italic) of pyrrolidine- and azepane-based GCS inhibitors. [a] Percent inhibition at inhibitor concentration in μM.

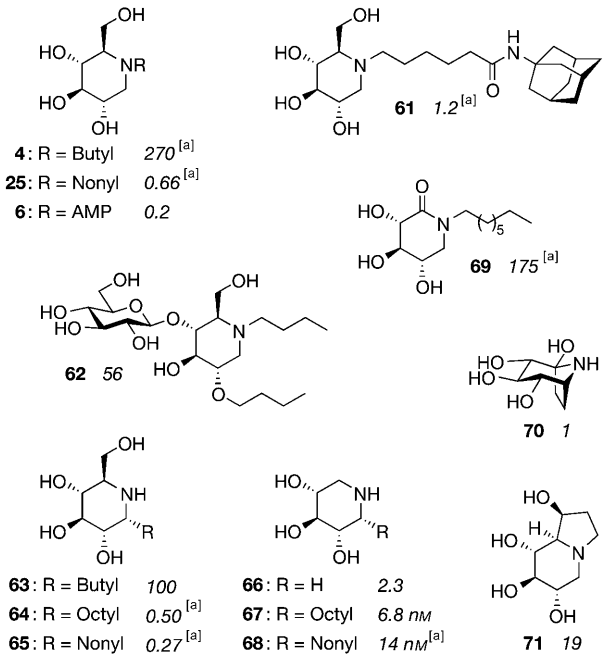


Figure 9. Overview of the structures and IC_{50} values (in μM, italic) of piperidine-based GBA1 inhibitors. [a] Reported as a pharmacological chaperone for variant GBA1: **4**,[215] **25**,[216,217] **61**,[214,218] **64**,[203] **65** and **68**,[219] **69**.[220]

In 2007 Futerman, Sussman, and co-workers published two crystal structures of GBA1 with either **4** or **25** bound in its active site. The binding of **4** and **25** was very similar, with the nonyl moiety of **25** making additional hydrophobic interactions with leucine residues near the entry to the active site (Figure 10). Remarkably, the nitrogen atom of both inhibitors

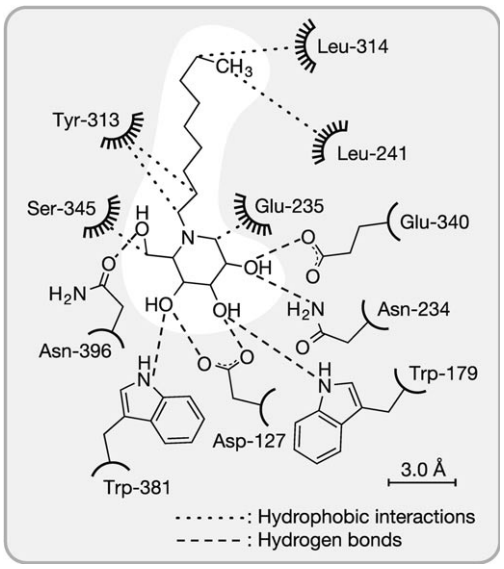


Figure 10. Coordination of **25** to active-site residues, based on the X-ray crystal structure of GBA1 complexed with **25**.[221]

did not appear to coordinate with the two catalytic glutamic acid residues (Glu340/235) or any other active-site residue.[221]

With the ceramide-mimicking model of **4** as a basis, 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 glucosylceramide mimics. The glycosylation of Miglustat (**4**) resulted in loss of GBA1 inhibition. However, *N*-butyl-2-*O*-butyl-1-deoxynojirmycin—itself not a GBA1 inhibitor—did display inhibition of GBA1 after β-4-*O*-glucosylation (**62**; Figure 9).[222]

Two other studies by the same research group showed that D-*gluco*- (**63–65**)[203] and D-*xylo*-α-aza-*C*-glycosides (**66–68**)[219] are very potent GBA1 inhibitors (Figure 9). Recently, the *N*-substituted D-*xylo*-δ-lactam **69** was shown to be a weak GBA1 inhibitor, but by acting as a pharmacological chaperone it was able to achieve a sixfold increase in the activity of variant (mutant) N370S-GBA1 (see Section 4.3).[220] An evaluation of naturally occurring iminosugars by Fan and co-workers identified several calystegines (**70**: calystegine B2) and castanospermine (**71**) as inhibitors of GBA1.[204] However, isofagomine (**72**; Figure 11) proved to be the most potent GBA1 inhibitor in this screening (IC_{50}: 40 nM) as well as a pharmacological chaperone of GBA1 variants.

Petsko and co-workers reported in 2007 the crystal structure of GBA1 with **72** in its active site (Figure 11 bottom).[223] The secondary hydroxy groups of **72** are coordinated by the same residues as reported for **4** and **25**, but it differs in that the nitrogen atom coordinates with the two catalytic residues. Fan and co-workers have shown that a large difference in the degree of GBA1 inhibition is observed between the N-alkylation (**73** and **74**) of **72** and alkylation at C-6 (**75–77**).[224] The isofagomine derivative **77**, with an IC_{50} value of 0.6 nM, represents the most potent GBA1 inhibitor reported to date. Kelly and co-workers have since shown that N-alkylation of **72** with alkyl-spaced amantadi-

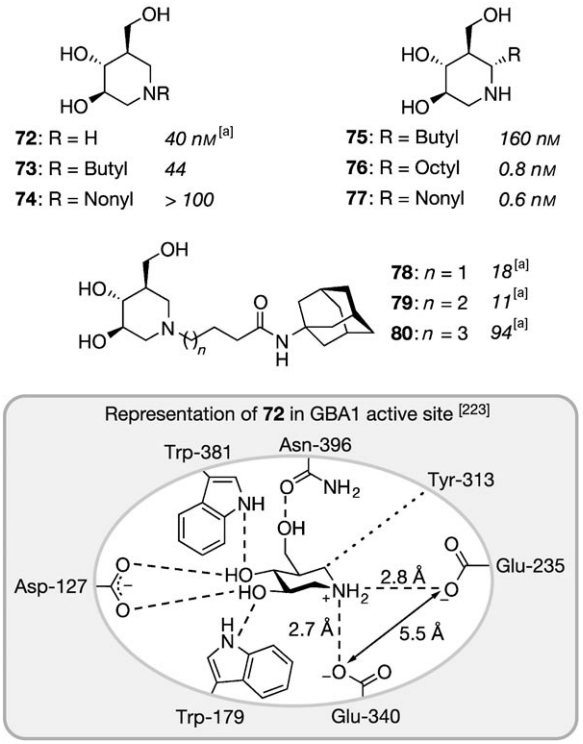


Figure 11. Overview of the structures and IC_{50} values (in μM, italic) of isofagomine-based GBA1 inhibitors. [a] Reported as a pharmacological chaperone for variant GBA1.[204, 225–227]

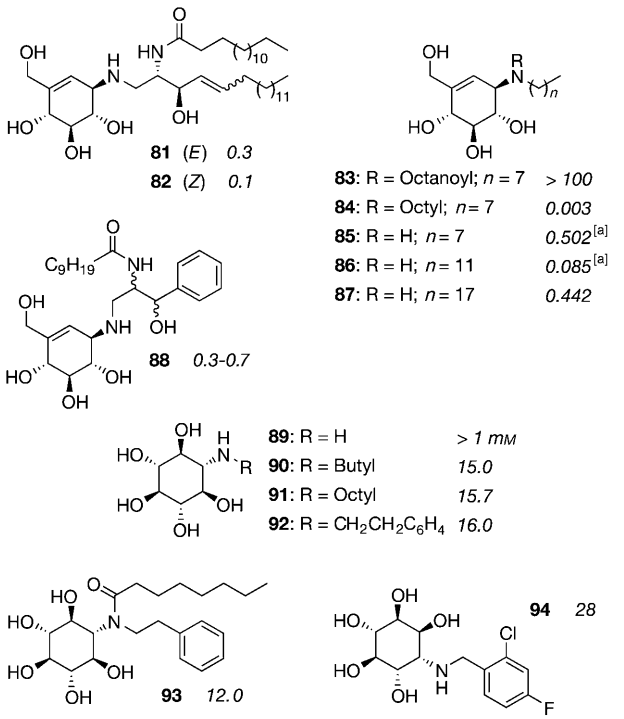


Figure 12. Overview of the structures and IC_{50} values (in μM, italic) of aminocyclitol-based GBA1 inhibitors. [a] Reported as a pharmacological chaperone for variant GBA1.[232, 233]

neamides provides moderate GBA1 inhibitors (**78–80**) with pharmacological chaperone activity.[225]

Lipophilic aminocyclitols constitute a second important class of GBA1 inhibitors. In 1995, Ogawa and co-workers reported the N-alkylation of the naturally occurring aminocyclitol β-valienamine to give **81** and **82**, which represented the first known selective inhibitors of GBA1 (Figure 12).[228] Replacing the complex ceramide moiety by one[229] or two[230] aliphatic alkyl chains also produced potent inhibitors (**83–87**), which were shown not to inhibit GCS. Derivatives of β-valienamine (**88**) inspired by PDMP were also inhibitors of GBA1, regardless of the configuration of the PDMP unit. The use of α-valienamine or the saturated β-valienamine analogue β-validamine resulted in significantly decreased GBA1 inhibition.[231]

Delgado and co-workers have reported N-alkylated aminocyclitols **89–92** as GBA1 inhibitors (Figure 12). These were obtained by regio- and stereocontrolled opening of CBE (**2**; see Figure 3). Aminocylitol **93** was the most potent GBA1 inhibitor and remarkably does not contain a basic amine function that is usually required for inhibition of GBA1.[234] These compounds were also evaluated as GCS inhibitors, but proved to be inactive. In a second combinatorial study by Delgado and co-workers, substitution of the nitrogen atom in two aminocyclitol cores was investigated, which resulted in GBA1 inhibitors similar in structure and activity to **90–92**, as well as a new class of inhibitors, namely, **94**.[235]

Finally, Sidransky and co-workers identified three novel classes of aromatic, achiral GBA1 inhibitors (**95**, **98**, and **99**; Figure 13) by high-throughput screening of a library of 59815

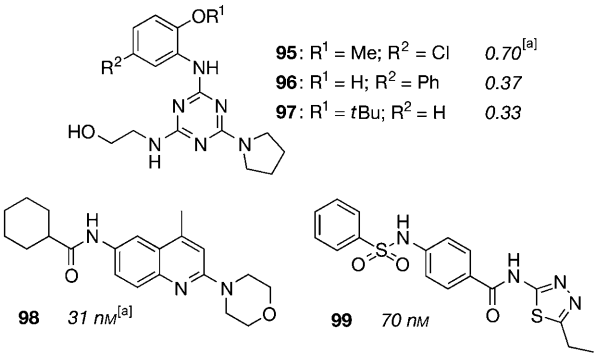


Figure 13. Overview of the structures and IC_{50} values (in μM, italic) of achiral aromatic GBA1 inhibitors. [a] Reported as a pharmacological chaperone for variant GBA1.[236]

compounds.[236] Modifications at the pyrrolidine nitrogen atom and primary hydroxy group of **95** did not improve its potency, but several modifications of the phenyl ring did (**96** and **97**).[237]

4.3. Therapeutic Applications

For the last few decades type I Gaucher disease has acted as a testing ground for the development of therapies for inherited lysosomal storage disorders. The first therapy that was developed for this disease was enzyme-replacement therapy (ERT; Figure 4b). Brady and co-workers demonstrated that intravenous administration of GBA1, purified

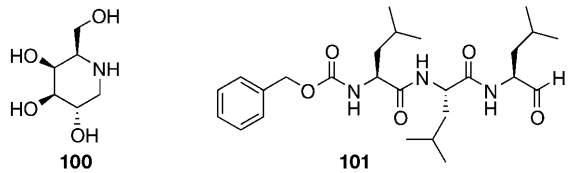
from human placental tissue, exerted beneficial effects in Gaucher patients.[238,239] During the 1990s, the placenta-derived enzyme was replaced by a recombinant GBA1 derivative.[240] At present, several thousand type I Gaucher patients worldwide receive ERT with this recombinant GBA1, named Cerezyme. Drawbacks of this therapy are its intravenous administration, the high costs, and the inability to treat or prevent neurological symptoms in more severely affected Gaucher patients (because of the lack of penetration of Cerezyme in the brain).

In 1980, Vunnam and Radin already proposed that downregulation of glucosylceramide influx into the lysosomes by inhibiting GCS with inhibitor PDMP (**1**) could alleviate the symptoms of Gaucher disease.[185] In 1994, Platt et al. reported the inhibition of GCS by the *N*-butylated 1-deoxynojirimycin derivative (**4**), and its ability to lower glucosylceramide accumulation in an in vitro model of Gaucher disease.[182] The concept was further developed into what is now called substrate-reduction therapy (SRT; Figure 4b). After clinical trials in Gaucher patients, **4** (now named Miglustat or Zavesca) was approved in 2002 as an orphan drug in SRT for Gaucher disease.[241,242] For more extensive discussion of SRT for Gaucher disease see the recent reviews.[243,244]

Studies with **4** and longer *N*-alkyl chain derivatives have shown that they are metabolically stable and excreted mostly through the kidneys (plasma $t_{1/2} = 6.3$ h). Oral administration of Miglustat (100 mg, 0.46 mmol) three times a day results in a steady-state plasma concentration of 5 μM after about 5 weeks that only partially inhibits GSL biosynthesis.[241] Experiments with radiolabeled iminosugar derivatives suggest that *N*-alkyl deoxynojirimycins are able to pass the blood–brain barrier.[245] The major side effects associated with SRT for Gaucher disease are related to the ability of **4** to inhibit glycosidases in the intestinal microvilli, which results in diarrhea, flatulence, and abdominal bloating. Miglustat also inhibits ER glucosidases I and II; however, this requires toxic concentrations in 10000-fold excess compared to the required dose for inhibition of GCS.

A third therapy that is currently in development for Gaucher disease is called chaperone-mediated therapy (CMT; Figure 4b).[246–248] It is based on the concept that a reversible active-site-directed inhibitor of an enzyme (a pharmacological chaperone) can already associate with a variant enzyme during the folding process in the ER (Figure 14). When the inhibitor is applied at subinhibitory concentrations, the chaperone can assist in proper folding and stabilization during trafficking. This may result in a larger percentage of the properly folded active enzyme reaching its cellular destination. Here, the chaperone either dissociates from the active site or is displaced by the enzyme's substrate. Several of the Gaucher-associated mutations in the *GBA1* gene result in improper or hindered folding of GBA1 in the ER. Consequently, many variant (mutant) GBA1 proteins never reach the lysosome and are instead degraded by the ERAD pathway (Figure 14).

The first example of a pharmacological chaperone stabilizing a lysosomal glycosidase was reported in 1999 by Fan et al. for variant forms of α-galactosidase A from patients with Fabry disease.[249] They observed that the iminosugar inhibitor galactostatin (**100**; Figure 15) enhanced the enzyme levels in cells of Fabry patients when administrated at concentrations lower than those required for inhibition of



100 **101**

Figure 15. Structure of compounds **100** and **101**.

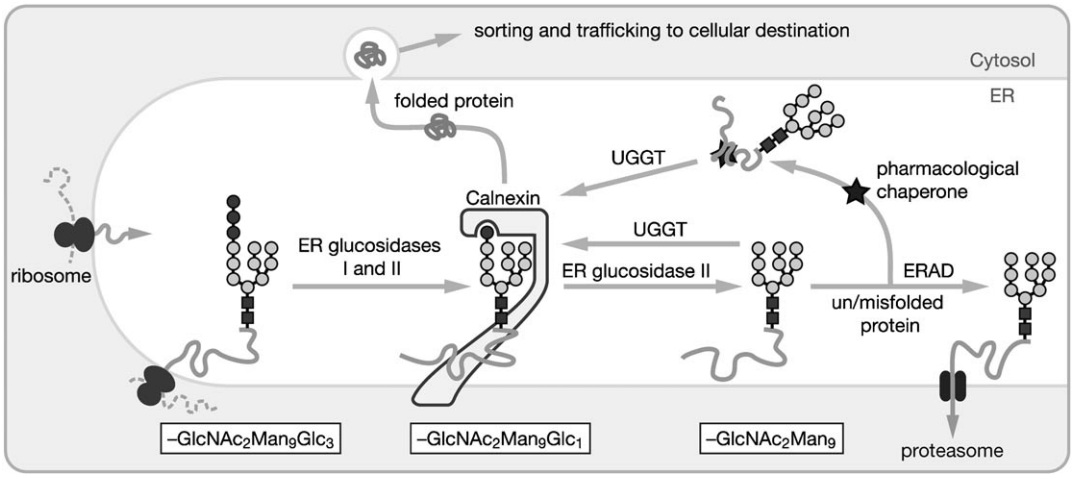


Figure 14. Overview of ER protein folding, quality control, and the putative action of a pharmacological chaperone in CMT. ER glucosidases I and II remove two of three glucose residues, after which the chaperones calnexin or calreticulin recognize the trimmed oligosaccharide and assist in protein folding. ER glucosidase II cleaves the final glucose residue. If the protein is properly folded, sorting and trafficking to the cellular destination occurs. If the protein is not yet properly folded, the UDP-glucose glycoprotein glucosyltransferase (UGGT) recognizes the protein and probes its folding. The protein is now either reglucosylated by UGGT and reassisted in folding or rejected and targeted to the ER-associated degradation pathway (ERAD).

the enzyme. The CMT concept has since also been demonstrated to be effective for several common variant forms of GBA1 in Gaucher disease—albeit in artificial 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 compounds labeled with an [a] in Figures 9 and 11–13). Most reports describe the incubation of the inhibitor together with cells that express a specific Gaucher disease associated deficient GBA1 for four to five days, after which the artificial 4MU-glucoside substrate (**3**; see Figure 3) is added and the GBA1 activity determined. The activity measurement is repeated in the presence of CBE (**2**; see Figure 3) to correct for the activity of GBA2. Besides an increase in enzyme activity, additional evidence for the improved stability of chaperone-bound variant GBA1 has been the observation that the protein dynamics at its active and secondary site decrease (by amide hydrogen/deuterium-exchange MS)[250] and its thermostability increases.[214,250] Several studies have also shown that increased amounts of GBA1 indeed reach the lysosome after treatment. However, it has yet to be shown that degradation of glucosylceramide actually increases in the lysosomes of intact treated cells. A recent report showed that a combination of the iminosugar chaperone **25** with the proteasome inhibitor MG-132 (**101**; Figure 15) results in a synergistic effect and greater increases in the GBA1 activity in the lysosome.[216] However, as **25** also inhibits GCS, GBA2, and ER glucosidases I and II, and since MG-132 also has secondary activity, it is far from clear which actions of these compounds actually lead to the observed improvement in the GBA1 acivity. It is of interest to mention recent findings regarding the most common CFTR mutation (F508del), which causes cystic fibrosis. The F508del mutation results in improper folding of the CFTR protein in the ER and degradation by the proteasome. Becq and co-workers have reported that treatment with Miglustat (**4**) was able to induce a 12% increase in properly folded CFTR, which resulted in improved ion transport in cystic fibrosis epithelial cells.[251,252] The authors proposed that **4** may inhibit ER α-glucosidases I + II, which are involved in the quality control of variant F508del-CFTR, and thus prevent its degradation via ERAD. Given the relative insensitivity of the ER α-glucosidases for inhibition by **4**, this explanation does not seem to be very attractive and some other effects of **4** can not be excluded.

5. Summary and Outlook

Sphingolipids and glycosphingolipids are involved in a wide range of physiological processes in health and disease. Tremendous progress in fundamental science and applied biomedical research, especially in the last few decades, has followed on from the initial discovery of this diverse class of compounds by Thudichum. At the same time, it should be equally clear that we are just at the beginning of unraveling and exploiting the role and therapeutic potential of SLs/GSLs and their metabolic pathways, and many years of exciting research at the interface of glycobiology and lipid biology lie ahead of us. Despite the large number of potential therapeutic targets, only compounds interfering with factors involved in the biosynthesis and degradation of glucosylceramide have evolved to become validated therapeutic agents (Miglustat (**4**) as a GCS inhibitor) or may become so (pharmacological chaperones for variant GBA1). Here, it must be stressed that both the mode of action and therapeutic value of iminosugar chaperones need to be established, and it is our expectation that this will come to pass in the next few years. The fact that glucosylceramide is central in many GSL metabolic pathways indicates that targeting its biosynthesis through GCS inhibition may also lead to therapies for lysosomal storage disorders caused by the accumulation of more complex GSLs. Alternatively, inhibitors of downstream glycosyl transferases involved in the synthesis of complex GSLs are much sought after. Future research on the poorly understood finding that GCS is susceptible to iminosugar inhibition, while most other glycosyl transferases are not, may guide this research. Despite the wealth of literature data on the development of iminosugars, this field is far from mature. For example, iminosugar compound libraries are small and scattered, and their activity towards glycoprocessing enzymes has rarely been investigated. The discovery of Miglustat (**4**), originally developed as a trimming ER α-glucosidase inhibitor, as a GCS inhibitor and its subsequent development into a clinical drug underscores our opinion that the assembly and broad screening of iminosugar libraries is a worthy research objective. In this fashion, insight into the specifics of still poorly understood glycoprocessing enzymes may be obtained. Here we note that, in doing such studies, one may find that so-called specific inhibitors turn out to possess more side activities than initially expected. As an intriguing example, we have noted from our work that all deoxynojirimycin-type lipophilic inhibitors with GCS inhibitory potency, including **4**, besides targeting GBA1, also target the nonlysosomal GBA2.[199,200,208] Neither its role nor the dangers associated with its permanent blockage are understood yet. Altogether, extensive iminosugar compound libraries combined with in vitro and in vivo assays to monitor their effect on a broad array of relevant glycoprocessing enzymes may well lead to the discovery of new therapeutic strategies, as was recently demonstrated by the identification of GCS as a potential clinical target for diabetes II.[168,169] Finally, and importantly, we believe that it is imperative to be able to carefully monitor (G)SL concentrations and their alteration upon interfering with their metabolic enzymes, and we foresee growing interest in the emerging field of directed lipidomics.

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