# Novel Intrinsic and Extrinsic Approaches to Selectively Regulate Glycosphingolipid Metabolism

by

Mustafa Ali Kamani

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# Abstract

Glycosphingolipid (GSL) metabolism is a complex process involving proteins and enzymes at distinct locations within the cell. Mammalian GSLs are typically based on glucose or galactose, forming glucosylceramide (GlcCer) and galactosylceramide (GalCer). Most GSLs are derived from GlcCer, which is synthesized on the cytosolic leaflet of the Golgi, while all subsequent GSLs are synthesized on the lumenal side. We have utilized both pharamacological and genetic manipulation approaches to selectively regulate GSL metabolism and better understand its mechanistic details. We have developed analogues of GlcCer and GalCer by substituting the fatty acid moiety with an adamanatane frame. The resulting adamantylGSLs are more water-soluble than their natural counterparts. These analogues selectively interfere with GSL metabolism at particular points within the metabolic pathway. At 40 μM, adaGlcCer prevents synthesis of all GSLs downstream of GlcCer, while also elevating GlcCer levels, by inhibiting lactosylceramide (LacCer) synthase and glucocerebrosidase, respectively. AdaGalCer specifically reduces synthesis of globotriaosylceramide ($Gb_3$) and downstream globo-series GSLs. AdaGalCer also increases Gaucher disease N370S glucocerebrosidase expression, lysosomal localization and activity. AdaGSLs, therefore, have potential as novel therapeutic

agents in diseases characterized by GSL anomalies and as tools to study the effects of GSL modulation.

Two predominant theories have been developed to explain how GlcCer accesses the Golgi lumen: one involving direct translocation from the cytosolic-to-lumenal leaflet of the Golgi by the ABC transporter P-glycoprotein (P-gp, ABCB1, MDR1), and the other involving retrograde transport of GlcCer by FAPP2 to the ER, followed by entry into the vesicular transport system for Golgi lumenal access. To examine the *in vivo* involvement of P-gp in GSL metabolism, we generated a knockout model by crossbreeding the Fabry disease mouse with the P-gp knockout mouse. HPLC analyses of tissue $Gb_3$ levels revealed a tissue-specific reduction in MDR1/Fabry mice. TLC analyses, however, did not show such reduction. In addition, we performed a gene knockdown study using siRNA against P-gp and FAPP2. Results show these siRNA to have distinct effects on GSL levels that are cell-type specific. These results give rise to the prospect of unique therapeutic approaches by targeting P-gp or FAPP2 for synthesis inhibition of particular GSL pathways.

*In the name of God, the most Kind, the most Merciful*

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# Table of Abbreviations

| | |
|---|---|
| ABC | ATP-Binding Cassette |
| Ada | Adamantyl |
| BFA | Brefeldin A |
| CBE | Conduritol β epoxide |
| Cer | Ceramide |
| CerS | Ceramide synthase |
| CsA | Cyclosporine A |
| DGJ | Deoxygalactonojirimycin |
| DNJ | Deoxynojirimycin |
| EET | Enzyme Enhancement Therapy |
| ERAD | Endoplasmic reticulum-associated degradation |
| ERT | Enzyme Replacement Therapy |
| FAPP | Phosphatidylinositol four-phosphate adaptor protein |
| FBS | Fetal Bovine Serum |
| GalCer | Galactosylceramide |
| GalT | Galactosyltransferase |
| $Gb_3$ | Globotriaosylceramide |
| $Gb_4$ | Globotetraosylceramide |
| GCC | Glucocerebrosidase |
| GCS | Glucosylceramide synthase |
| Gg3 | Gangliotriaosylceramide |
| Gg4 | Gangliotetraosylceramide |
| GLA | α Galactosidase A |

| | |
|---|---|
| GlcCer | Glucosylceramide |
| GM1 | α-N-acetylneuraminosylgangliotetraosylceramide |
| GM2 | α-N-acetylneuraminosylgangliotriaosylceramide |
| GM3 | α-N-acetylneuraminosyllactosylceramide |
| GSL | Glycosphingolipid |
| IFG | Isofagomine |
| LacCer | Lactosylceramide |
| Lc3 | Lactotriaosylceramide |
| Lc4 | Lactotetraosylceramide |
| LSD | Lysosomal Storage Disease |
| MCD | Methyl-β-cyclodextrin |
| MDR | Multidrug Resistant |
| MRP | Multidrug Resistance Associated Protein |
| nLC4 | Neolactotetraosylceramide |
| P4 | D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol |
| P-gp | P-glycoprotein |
| SAP | Sphingolipid activator protein |
| SM | Sphingomyelin |
| SRT | Substrate Reduction Therapy |
| SSEA | Stage-specific embryonic antigen |
| TGN | Trans Golgi Network |
| TLC | Thin-Layer Chromatography |
| VT | Verotoxin |

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# Chapter 1: Introduction

## *1.1 Glycosphingolipids: The Beginning*

The discovery and isolation of the first glycosphingolipid (GSL) by Johann Thudichum in 1874 established the foundation of sphingolipidomics—the detailed study of sphingolipids. This GSL was given the name cerebroside by its discoverer. Thudichum subjected it to acid hydrolysis and identified three products: an isomer of glucose, a fatty acid and a third component "...in commemoration of the many enigmas which it presented to the inquirer, I have given the name of sphingosin..." (Thudichum, 1962; Wennekes et al., 2009). This symbolic nomenclature is based on the Greek mythological creature, the Sphinx, who killed everyone who incorrectly answered her riddles. In the words of Sen-itiroh Hakomori, "...the romantic prefix *sphingo*-, introduced by Thudichum in his name for the compounds, remains appropriate...The function of many glycosphingolipids in neuronal structures continues to be a riddle." (Hakomori, 1986a). In the quarter-century since Hakomori's statement, much has been discovered regarding the structural, functional and regulatory roles of not only neuronal GSLs, but GSLs of all cell-types from all the body's tissues. Much, however, still remains to be elucidated in the continually expanding field of sphingolipidomics (Merrill, 2011).

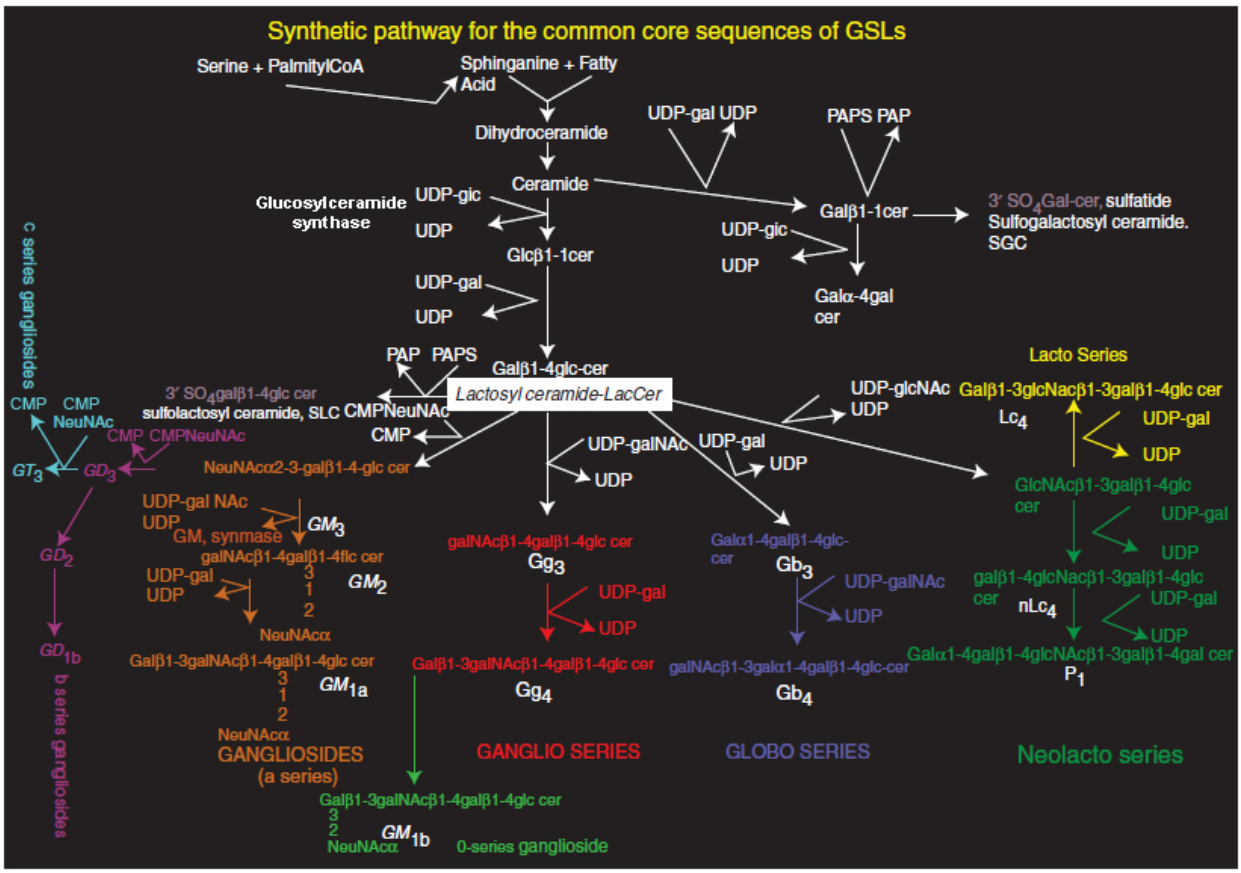
## *1.2 Glycosphingolipid Structure*

GSLs are composed of a hydrophobic sphingoid backbone and a hydrophilic head group, making them amphipathic. Structurally, GSLs are a very diverse class of molecules. This structural heterogeneity arises from differences in the sphingoid base, namely variations in the backbone alkyl chain length, saturation, hydroxylations and branching alkyl chains, as well as

variations in the number, type and linkages of the sugars attached to the sphingoid base, making sphingolipids a very diverse class of molecules (Vesper et al., 1999). Mammalian sphingolipids consist of an estimated 600 or greater different sphingolipid head group structures; combined with variations in the hydrophobic backbone, this leads to a potential collection of over 10,000 different structures (Merrill, 2011); collectively, these comprise the sphingolipidome.

The hydrophilic head group can be phosphate-based, forming the phosphosphingolipids, or it can be a sugar, forming the glycosphingolipids (Merrill, 2011). The first sugar linked to ceramide is either glucose, galactose or, rarely, fucose (Watanabe et al., 1976), generating glucosylceramide (GlcCer), galactosylceramide (GalCer) or fucosylceramide, respectively, and leading to the formation of distinct GSL sub-classes. Glucose-based GSLs comprise the vast majority of total GSLs, while galactose-based GSLs are few in number.

Addition of galactose to GlcCer via a β 1-4 linkage forms lactosylceramide (LacCer), the key precursor within the glucose-based GSL pathway, serving as a substrate for all GSL sub-types. Addition of galactose to LacCer in an α1-4 linkage forms globotriaosylceramide ($Gb_3$), the first amongst the globo-series GSLs; Lc3 synthase forms lactotriaosylceramide (Lc3) via a β1-3 N-acetylglucosamine (GlcNAc) linkage, generating the lacto- and neolacto-series; the addition of α2-3 linked sialic acid to LacCer synthesizes α-N-acetylneuraminosyllactosylceramide (monosialyl ganglioside, GM3); β1-4-linked N-acetylgalactosamine (GalNAc) to LacCer forms gangliotriaosylceramide (asialo-GM3) (Takematsu et al., 2011). The GSL synthesis pathway is depicted in Scheme 1.1. A more comprehensive collection of approximately 450 sphingolipid structures is available at http://sphingomap.org.




Scheme 1.1. **GSL biosynthesis pathway.** The pathway for glucose and galactose-based GSL synthesis is depicted. The various GSL sub-classes for the glucose-based pathway are indicated by colour differences. Image taken from Lingwood (2011).

The fatty acid linked to sphingosine in the GSL backbone is not a random association; rather, particular fatty acids are selected for the different GSLs (Stiban et al, 2010). For example, two lacto-series GSLs that are positional isomers contain different acyl chain lengths, with one containing predominantly C16 fatty acids, while the other is mainly composed of C20-24 fatty acids (Kannagi et al., 1982). Both GSLs are ultimately derived from the same precursor—GlcCer. How such selectivity is conferred is not precisely known, but may be functionally relevant (Lingwood, 2011). It appears that glycosyltransferases responsible for GSL biosynthesis may be selective for their substrate acyl chain. This, in turn, may link the glycosyltransferases to specific fatty acid-selective ceramide synthases.

GSLs are not essential for cell survival in culture, but they are necessary at the tissue differentiation level (Ichikawa et al., 1994; Liang et al., 2010; Yamashita et al., 1999b). The mouse B16 melanoma cell line (GM-95) is deficient in the simplest GSL, glucosylceramide (GlcCer); the cells have altered morphology and slower growth rates than the parental MEB-4 cells (Ichikawa et al., 1994). Knockout of the gene encoding the enzyme responsible for GlcCer synthesis is embryonic lethal in mice (Yamashita et al., 1999b).

## *1.3 Metabolism*

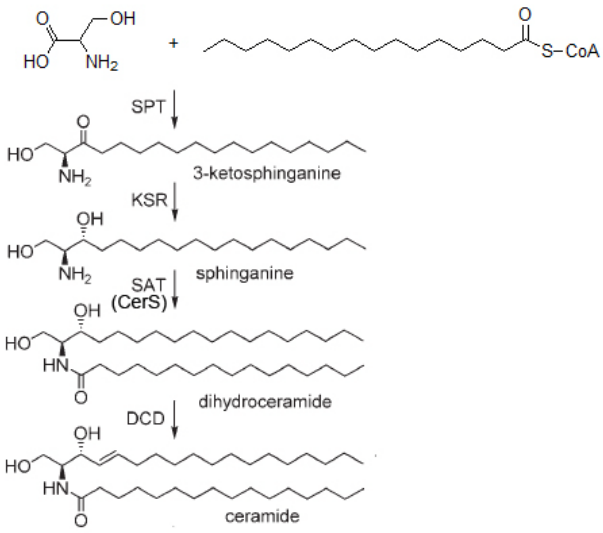
GSLs can be synthesized *de novo* or through a recycling pathway, the former being predominant in actively dividing cells (Gillard et al., 1998; Sillence, 2007). In addition, the relative contribution of each pathway to sphingolipid synthesis appears to be cell-type specific (Mullen et al., 2012). As part of the recycling pathway, the sugar moieties of GSLs are cleaved by appropriate exoglycosidases, forming ceramide, which is then deacylated to produce sphingosine. This sphingosine is then the substrate of a CerS to re-synthesize ceramide (Gillard et al., 1998).

Given the sequential, step-wise synthesis and processing pathway of glycoproteins from core glycosylation and processing in the ER to transit through the *cis*, *medial* and *trans* Golgi while undergoing trimming and addition of sugars (Rothman and Orci, 1990), it was not unreasonable to assume GSL synthesis behaved similarly, with GlcCer synthase located at the cis-Golgi and the enzymes for more complex GSLs sequentially located in more distal Golgi compartments. However, evidence to the contrary has accumulated: GlcCer synthase is found predominantly in the *cis*, *medial*, and *trans* Golgi, while other glycosyltransferases are also present in the *cis*

Golgi (D'Angelo et al., 2007; Halter et al., 2007); most of the GSL glycosyltransferases are found throughout the Golgi (Halter et al., 2007); GM3 synthase has been reported in proximal Golgi compartments (Iber et al., 1992; Trinchera and Ghidoni, 1989); GlcCer may be transported back to the ER before accessing downstream biosynthetic enzymes (Halter et al., 2007).

### 1.3.1 Ceramide Synthesis

Ceramide is synthesized on the cytosolic leaflet of the ER (Buton et al., 2002). *De novo* ceramide synthesis begins by a condensation reaction between serine and palmitoyl-CoA to generate 3-ketosphinganine, whose reduction forms dihydrosphingosine (Mullen et al., 2012) (Scheme 1.2). This species is acylated to produce dihydroceramide. This can occur through two mechanisms: an acyl-CoA-dependent ceramide synthase (CerS) reaction or an acyl-CoA-independent reaction called reverse ceramidase (Gatt, 1963; Gatt, 1966; Mullen et al., 2012). Six different mammalian ceramide synthases have been identified (CerS1 to CerS6) (Mullen et al., 2012). There is controversy as to the membrane topology and number of transmembrane segments in these proteins; different groups have suggested five or six transmembrane segments, with both N- and C-termini cytosolic or with a lumenal N-terminus and cytosolic C-terminus (Kageyama-Yahara and Riezman, 2006; Mizutani et al., 2005; Stiban et al., 2010). The enzymes are located at the ER and mitochondrial membranes, and reverse ceramidase activity also exists in mitochondria (Bionda et al., 2004; Hirschberg et al., 1993; Mullen et al., 2012). Ceramide synthases control sphingolipid synthesis for both the *de novo* and salvage pathways (Mullen et al., 2012).

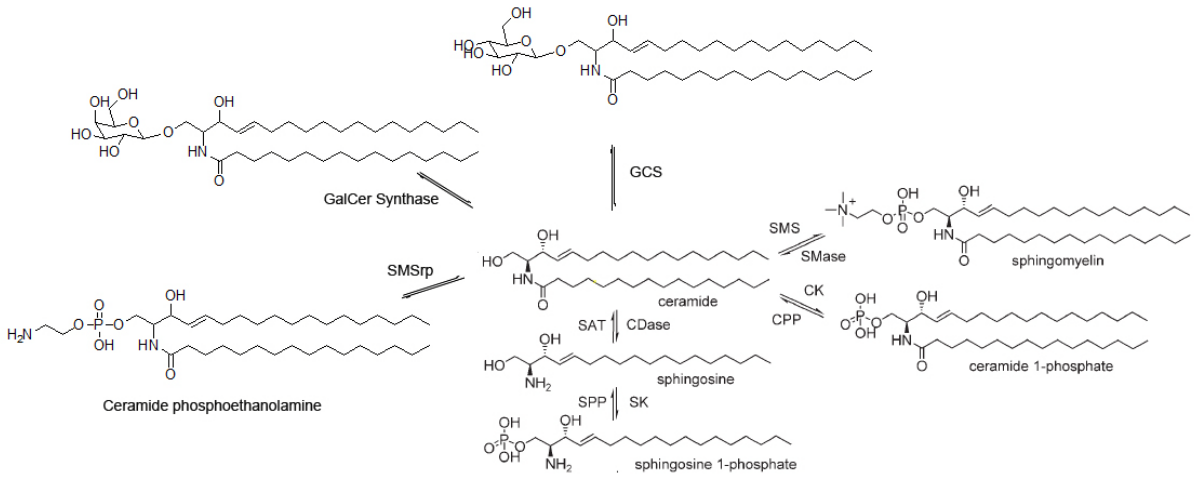



Scheme 1.2. ***De novo* ceramide synthesis.** SPT: serine palmitoyl transferase, KSR: 3-ketosphinganine reductase, SAT: sphinganin N-acyl transferase, CerS: ceramide synthase, DCD: dihydroceramide desaturase. Figure adapted from Nussbaumer (2008).

Importantly, the ceramide synthases selectively differ in their preference of acyl-chain lengths (Mullen et al., 2012). CerS1, for example, predominantly makes $C_{18:0}$ ceramides, while CerS2 is biased towards $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$ acyl-chains (Laviad et al., 2008; Mullen et al., 2012; Venkataraman et al., 2002). The ability to synthesize ceramides with diverse acyl-chains is functionally relevant. Aberrant expression of particular CerSs—either upregulation or downregulation—produces a different pool of ceramides, which in turn, can induce different signaling pathways to stimulate, for instance, apoptosis or ER stress (Mullen et al., 2012). In addition, the particular CerS expressed is tissue selective: CerS1, for instance, is expressed predominantly in the brain and skeletal muscle, while CerS2 is abundant in the liver and kidney (Jiang et al., 1998; Laviad et al., 2008; Mullen et al., 2012), further emphasizing the functional significance of the ceramide subtypes.

Ceramide synthases also acylate sphingosine produced from the salvage pathway, i.e. from breakdown of sphingomyelin or GSLs. From the *de novo* pathway, a 4,5-*trans* double bond is introduced into dihydroceramide by a desaturase to produce ceramide, which serves as the

backbone for the various sphingolipids (Mullen et al., 2012). Two different desaturases have been identified, with DES1 responsible for the *trans* double bond formation, while DES2 hydroxylates dihydroceramide to produce 4-hydroxysphinganine, commonly referred to as phytosphingosine, which is the sphingoid base found in skin ceramides (Merrill, 2011), as well as fungi and plants (Dickson, 1998). The ceramides that have been synthesized are then used as substrates for the addition of various polar head groups—phosphate, phosphocholine, galactose or glucose (Scheme 1.3).




Scheme 1.3. **Sphingolipid products from ceramide**. The various products synthesized from the ceramide precursor are shown. GCS: glucosylceramide synthase, SMS: sphingomyelin synthase, SMase: sphingomyelinase, CK: ceramide kinase, CPP: ceramide-1-phosphate phosphatase, SK: sphingosine kinase, SPP: sphingosine-1-phosphate phosphatase, CDase: ceramidase, SAT: sphingosine N-acyl transferase, SMSrp: SMS related protein. Figure adapted from Nussbaumer (2008)

## 1.3.2 Phosphosphingolipids

### 1.3.2.1 Ceramide phosphoethanolamine synthesis

Ceramide phosphoethanoamine is produced in very minor quantities in the ER lumen (Vacaru et al., 2009). Although not abundant, it appears to be involved in maintaining ceramide homeostasis, as inhibiting its synthesis significantly increases ceramide levels (Vacaru et al., 2009).

### 1.3.2.2 Ceramide-1-Phosphate and Sphingosine-1-Phosphate

Ceramide is transported from the ER to the Golgi by the protein ceramide transporter (CERT) (Hanada et al., 2003). This protein is biased towards long-chain (C16 fatty acid) ceramide rather than the very-long-chain ceramides (Merrill, 2011). This ceramide is then phosphorylated at the *trans* Golgi by ceramide kinase, yielding the bioactive species ceramide-1-phosphate (C1P) (Gault et al., 2010). Similarly, sphingosine generated by breakdown of ceramide can be phosphorylated by sphingosine kinases at the ER or the cell surface to produce sphingosine-1-phosphate (S1P) (Chalfant and Spiegel, 2005). Both C1P and S1P have been implicated in cell survival, cell proliferation and in the inflammatory response (Chalfant and Spiegel, 2005). Thus, the interconvertability between S1P, sphingosine, ceramide and C1P allows for control of the balance between pro-apoptotic and pro-survival signals (Chalfant and Spiegel, 2005).

### 1.3.2.3 Sphingomyelin synthesis

Ceramide that is transported to the Golgi by CERT can flop to the lumenal leaflet, where sphingomyelin synthase 1 (SMS1) transfers a phosphocholine group to ceramide to form sphingomyelin (SM) (Wennekes et al., 2009). SM is the most common and well-studied among the phosphosphingolipids (Merrill, 2011). At least two different SM synthases exist—SMS1 activity resides in the lumen of the *trans* Golgi (Huitema et al., 2004) or *cis* Golgi (Jeckel et al., 1990), while the catalytic activity of SMS2 exists predominantly on the extracellular leaflet of the PM but is also present to a lesser extent at the *trans* Golgi (Gault et al., 2010; Huitema et al., 2004). It is thought that SMS1 is involved in *de novo* SM synthesis, while SMS2 acts within the

salvage pathway, following conversion of SM to ceramide at the cell surface by sphingomyelinase (Hanada et al., 2007; Wennekes et al., 2009).

### 1.3.3 Glycosphingolipids

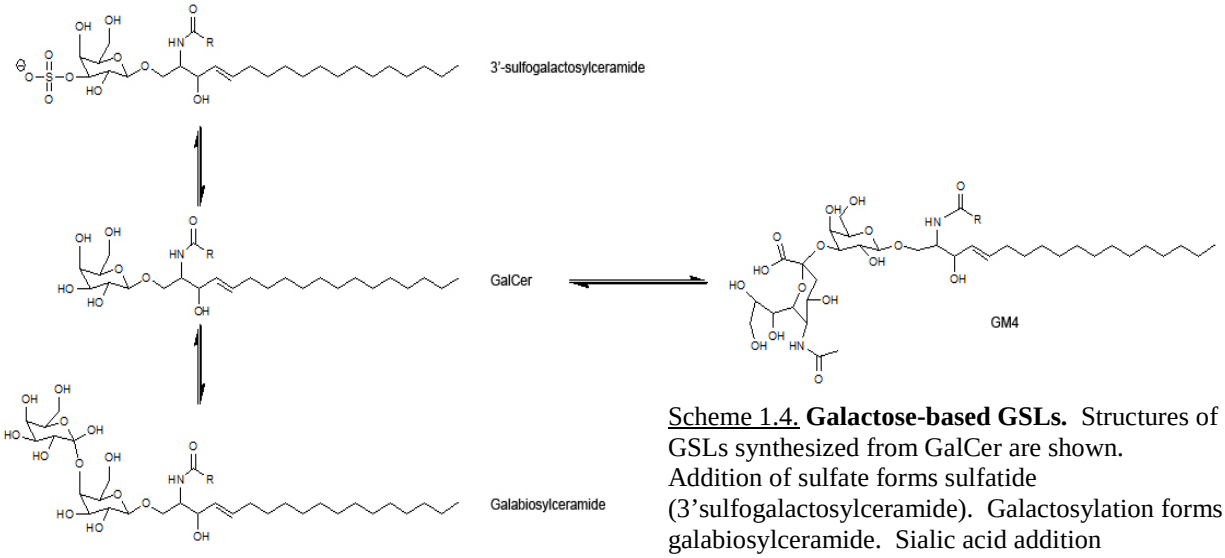
#### 1.3.3.1 Galactose-based GSLs

Ceramide synthesized in the ER can be galactosylated by UDP-galactose:ceramide galactosyltransferase (GalT1, GalCer synthase) to produce GalCer (Gault et al., 2010). GalCer-based GSLs usually have a 2-hydroxy fatty acid (HFA), but can also contain a non-hydroxy fatty acid (NFA), each synthesized in a distinct location of the cell (Burger et al., 1996). The former is made on the lumenal leaflet of the ER, while the latter is synthesized on the cytosolic leaflet of the Golgi (Burger et al., 1996). HFA-GalCer synthase has high homology to UDP-glucuronyltransferases (Burger et al., 1996).

HFA-GalCer synthase is a type I membrane protein (cytoplasmic C-terminus and lumenal N-terminus) (Maccioni et al., 2011a; Sprong et al., 1998). The lumenal N-terminal domain contains the catalytic site (Burger et al., 1996; Sprong et al., 1998). Tissue distribution of GalCer synthase is limited primarily to the kidney, testis, intestine, Schwann cells and oligodendrocytes (Gault et al., 2010; Stahl et al., 1994). Galactose-based GSLs are important components of myelin sheath and Schwann cells (Ichikawa and Hirabayashi, 1998). Cells expressing high levels of GalCer, such as Schwann cells, also express UDP-Gal transporter 2 (UGT2) at the ER, a splice variant of the Golgi UGT1 (Merrill, 2011; Sprong et al., 2003).

#### 1.3.3.2 Gala-series GSLs

Synthesis of galabiosylceramide and sulfatide (sulfogalactosylceramide) from β-GalCer (Scheme 1.4) occurs on the lumenal side of the Golgi (Burger et al., 1996; Buton et al., 2002) by α-1,4-galactosyltransferase (A4GalT) and 3'phosphoadenosine 5'-phosphosulfate:galactosylceramide sulfotransferase (GalCer sulfotransferase), respectively (Maccioni et al, 2011a; Kojima et al., 2000). The vast majority of endogenous galabiosylceramide and sulfatide in MDCKII cells contain an NFA-ceramide backbone; this necessitates translocation of NFA-GalCer from the cytosolic to the lumenal leaflet of the Golgi (Burger et al., 1996). The translocation mechanism has not yet been elucidated.




Scheme 1.4. **Galactose-based GSLs.** Structures of GSLs synthesized from GalCer are shown. Addition of sulfate forms sulfatide (3'sulfogalactosylceramide). Galactosylation forms galabiosylceramide. Sialic acid addition synthesizes GM4.

### 1.3.3.3 Glucose-based GSLs

For glucose-based GSLs, ceramide is trafficked from the ER to the cytosolic leaflet of the Golgi, either by CERT or a CERT-independent mechanism—likely vesicular traffic (Giussani et al., 2008; Halter et al., 2007; Hanada et al., 2003). Here, UDP-glucose:ceramide glucosyltransferase (glucosylceramide synthase, GCS, EC 2.4.1.80) catalyzes the transfer of a

glucose residue to ceramide to form GlcCer. Synthesis of GlcCer appears to occur at both the *cis* and *trans* Golgi (D'Angelo et al., 2007; Halter et al., 2007; Jeckel et al., 1992; Lannert et al., 1998). That the catalytic site of GCS is cytoplasmically oriented (Coste et al., 1986; Jeckel et al., 1992) is unlike that found for any other glycosyltransferase (with the exception of NFA-GalCer synthase).

GCS is a 394 amino acid type III membrane protein (a short N-terminal region projecting in the lumenal side, a single transmembrane segment and a longer cytoplasmic tail) with a predicted molecular weight of 44,853 (Ichikawa et al., 1996). It has been said to possess a single transmembrane segment and a long cytoplasmic tail (Ichikawa et al., 1996). The protein is highly hydrophobic at the N-terminus, where the signal anchor sequence is located, and near the C-terminus (Ichikawa et al., 1996). The cDNA of GCS has a large G+C-rich 5'-untranslated region that is thought to be part of a CpG island—these usually flank promoters of essential genes (Ichikawa et al., 1996). Accordingly, GCS is ubiquitously expressed in tissues (Ichikawa et al., 1996).

While GCS has a predicted size of approximately 45 kDa, it runs anomalously on SDS-PAGE as a 38 kDa polypeptide (Marks et al., 1999). This irregular migration is not due to a cleavage event, as the C-terminus has been shown to be intact and deletion of N-terminal residues leads to even faster migration on SDS-PAGE (Marks et al., 1999). Consequently, Marks et al suggested that the extreme hydrophobicity of the protein causes it to run faster than expected on SDS-PAGE (Marks et al., 1999). The cytoplasmic C-terminus of GCS is important for catalytic activity, as exposure to trypsin or deletion of the C-terminal 8 residues significantly impairs activity (Marks et al., 1999). Cross-linking studies have revealed GCS to exist as a heterodimer

or hetero-oligomer that migrates as a 50 kDa (or higher) species on SDS-PAGE (Marks et al., 1999). The identity of the small protein(s) with which GCS associates is unknown.

All subsequent GSLs are synthesized on the lumenal leaflet of the Golgi (Jeckel, 1994); thus, a mechanism must exist for GlcCer Golgi lumenal access. The precise mechanism remains to be solved; however, it appears multiple processes are involved (Manuscript in preparation). One theory involves direct translocation of GlcCer by a flippase, while the other involves transport by a carrier protein followed by vesicular transport (more below).

## *1.4 Lipid Flippase*

The ER is a self-synthesizing, or biogenic, membrane (Pomorski and Menon, 2006). Most of the glycerophospholipid biosynthetic enzymes are membrane proteins in the ER with catalytic sites cytoplasmically oriented (Pomorski and Menon, 2006). Thus, newly synthesized phospholipids must be translocated to the lumenal leaflet to generate a bilayer structure. Since phospholipids contain a polar head group, translocation through the hydrophobic membrane interior is energetically unfavourable; thus, spontaneous translocation is slow (Pomorski and Menon, 2006). Flip-flop in biogenic membranes, however, is rapid, indicative of a mechanism facilitating such an event. Flipping refers to the translocation of a molecule to the cytoplasmic leaflet of a membrane bilayer, while flopping corresponds to the reverse process (i.e. translocation to the non-cytoplasmic leaflet) (Daleke, 2007). Unfortunately, identification of the ER phospholipid flippase or flippases has remained elusive; however, the responsible protein(s) seems to lack stereospecificity and does not distinguish between the different phospholipids (Pomorski and Menon, 2006).

While transbilayer lipid translocation at the ER is energy-independent and does not discriminate between phospholipids, at the PM, late Golgi and endosomes, this is an ATP-dependent process responsible for maintaining lipid asymmetry across the bilayer, with aminophospholipids being primarily transported towards the cytoplasmic membrane leaflet (Pomorski and Menon, 2006). Both P-type ATPases and ABC transporters have been implicated in this process, with the former typically being involved in translocation toward the cytoplasmic leaflet and the latter toward the exoplasmic leaflet (Pomorski and Menon, 2006). In addition, the PM of some cells contain a phospholipid scramblase that disrupts the lipid asymmetry, thereby modulating function in the outer leaflet (Pomorski and Menon, 2006). Apoptotic signalling induces phospholipid asymmetry by inhibiting activity of the P-type ATPase or activating the phospholipid scramblase (Bevers and Williamson, 2010). Evidence also shows that ATP-dependent flippases may be required for the formation of intracellular transport vesicles (Pomorski and Menon, 2006): in Tangier disease, defective ABCA1, which normally translocates phosphatidylserine to the outer leaflet of the plasma membrane, increases endocytosis (Zha et al., 2001); in yeast, loss of two P-type ATPases, responsible for translocating phosphatidylserine and phosphatidylethanolamine from the exoplasmic to the cytosolic leaflet of the plasma membrane, impairs endocytosis (Pomorski et al., 2003).

### 1.4.1 ABC Transporters

The ATP-binding cassette (ABC) transporter superfamily corresponds to a group of transmembrane proteins that transport substances across the membrane by using the energy from ATP hydrolysis (Oldham et al., 2008). The proteins are involved in the transport of a wide range of substrates across the membrane against their concentration gradients (Klappe et al., 2009). Approximately 50 different proteins have been identified in humans within this

transporter superfamily, with each member sub-classified into one of seven groups (ABCA to ABCG) (Dean et al., 2001; Ueda, 2011). Evidence indicates various ABC transporters are localized within rafts, with alterations in raft composition affecting transporter function (Klappe et al., 2009).

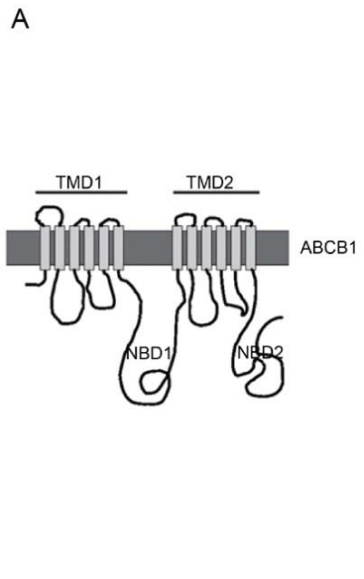
Structurally, ABC transporters are composed of two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) involved in ATP hydrolysis on the cytoslic side of the membrane (Oldham et al., 2008). However, some half-transporters exist, being comprised of a single TMD and NBD; these must dimerize for transport functionality (Dean et al., 2001).

### 1.4.2 P-glycoprotein (P-gp)

P-gp (ABCB1, MDR1) is a member of the ATP binding cassette (ABC) transporter superfamily that is encoded by the ABCB1 gene (Dean et al., 2001). It was the first ABC transporter to be cloned and characterized (Dean et al., 2001; Riordan et al., 1985). P-gp was identified by selecting Chinese hamster ovary cells for resistance to colchicine (Juliano and Ling, 1976). These drug-resistant cells express a 170 kDa glycoprotein at the cell surface. The name P-glycoprotein was assigned to this protein on the basis of its ability to alter the permeability of drugs (Juliano and Ling, 1976). P-gp has been commonly called multidrug resistance 1 (MDR1) due to its overexpression in tumour cells and function in resisting the cytotoxic effects of chemotherapeutic agents (Sharom, 2008). P-gp plays an important role in the body by pumping out toxic compounds, thereby preventing their accumulation in vital organs (Cascorbi, 2011; Fromm, 2004; Ueda, 2011). To fulfill this role, P-gp has a broad range of hydrophobic substrates—those that can potentially cross the membrane bilayer for intrusion into cells (Ueda,

2011). As such, Pgp is also responsible for resistance to many chemotherapeutic agents (Dean et al., 2001; Sharom, 2008).

P-gp is a 1280 amino acid protein with two TMDs, each containing six transmembrane segments, and two NBDs (Chen et al., 1986). Schematic and structural models are shown in Scheme 1.5. Substrate efflux or translocation is coupled to ATP hydrolysis in the NBD sites (Ueda, 2011). Two distinct models have been proposed for substrate translocation: the 'pump' model and the 'flippase' model. The flippase mechanism of ABCB1-mediated transport was proposed by Higgins and Gottesman on the basis that binding and transport of substrate from the cytosol directly to the extracellular medium—as suggested in the 'pump model'—is unable to explain the wide range of substrates recognized by the protein (Higgins and Gottesman, 1992). In the pump model, substrate that has entered into the cytoplasm interacts with ABCB1 and is directly transported into the extracellular medium without interaction with the lipid bilayer (Higgins and Gottesman, 1992). As part of the flippase mechanism, it is hypothesized that substrate initially interacts with the lipid bilayer, followed by interaction with ABCB1, which flips the substrate from the cytosolic to the exoplasmic leaflet (Higgins and Gottesman, 1992). Drugs that are efficiently transported, therefore, tend to be largely hydrophobic. Similar to the flippase model, the 'hydrophobic vacuum cleaner' model involves extraction of the substrate from the cytosolic membrane leaflet followed by direct extrusion to the exoplasmic medium (Hennessy and Spiers, 2007).




Scheme 1.5. **ABCB1 structural schematic and models.** (A) A schematic of ABCB1. Each transmembrane domain (TMD) consists of six transmembrane segments. Image adapated from Zhang (2007). (B) Structural representation of the human ABCB1. The image is a screenshot from a molecular dynamics simulation of human ABCB1 derived from homology models. The image represents the "fully open extracellularly" state of the protein. Image adapted from Wise (2012).

Recently, using molecular dynamics simulations and homology models of human ABCB1 based on crystal structures of the mouse and bacterial ABCB1 homologues, a mechanism was proposed suggesting that following substrate penetration into the membrane bilayer and subsequent drug binding to ABCB1, the protein undergoes a transition from having the intracellular domain completely "open" to partially open to partially open on the extracellular side to fully open extracellularly (Wise, 2012). According to this mechanism, the final opening of the drug binding domain to the extracellular side of the membrane can either lead to substrates dissociating into the extracellular matrix or into the exoplasmic membrane leaflet (Wise, 2012). This is consistent with the flippase model of substrate transport.

### 1.4.2.1 Tissue Distribution

ABCB1 is expressed on the apical surface of the entire intestine, serving a barrier function to prevent absorption of toxic molecules (Cascorbi, 2011). It is also highly expressed in hematopoietic stem cells (Licht et al., 1994), kidney and liver, although expression in liver is variable (Cascorbi, 2011). P-gp is also important in three blood-tissue barriers: the blood-brain-

barrier to protect from toxic molecule infiltration into the central nervous system, the blood-testis-barrier and the maternal fetal interface in the placenta to protect the fetus from potentially toxic compounds ingested by the mother during pregnancy (Cascorbi, 2011; Fromm, 2004).

### 1.4.3 ABC Transporters and Lipid Transport

It has been estimated that approximately half of the human ABC transporters are involved in lipid transport (van Meer et al., 2006). The first such example was identified in 1993 when knockout of mouse ABCB4 resulted in the absence of phosphatidylcholine (PC) from bile (Smit et al., 1993; van Meer et al., 2006). Since then, several other members of this superfamily have been shown or suggested to transport lipids: ABCA3 is expressed in alveolar cells, where it is thought to transport pulmonary surfactant lipids (Nagata et al., 2004; Ueda, 2011); ABCG5 and ABCG8 transport plant sterols to prevent their uptake into intestinal cells (Berge et al., 2000; Ueda, 2011); ABCD1 is involved in the transport of long-chain fatty acids (van Meer et al., 2006); ABCA1 is involved in translocation of PS to the extracellular leaflet of the plasma membrane (Zha et al., 2001) and in the generation of HDL and, therefore, cholesterol homeostasis (Kellner-Weibel and De La Llera-Moya, 2011; Ueda, 2011); ABCA2 appears to play a role in SM and GM1 homeostasis in the brain, as the brains of mice deficient in the protein show diminished SM levels and GM1 accumulation (Sakai et al., 2007a; Ueda, 2011); ABCA12 transports GlcCer in keratinocytes, with defects in the protein leading to loss of the lipid barrier in skin and the pathology known as harlequin ichthyosis (Sakai et al., 2007b). Interestingly, ABCA12 has also been localized to the Golgi (Sakai et al., 2007b), while ABCA2 is expressed in both the Golgi and lysosomal membranes, the primary biosynthetic and degradation compartments, respectively, in sphingolipid metabolism (Sakai et al., 2007a).

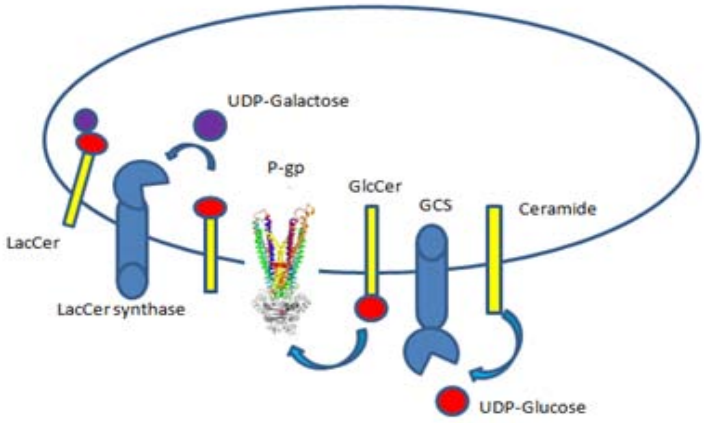
### 1.4.3.1 ABCB1-dependent translocation of GlcCer

In addition to the lipid translocation functions carried out by the various ABC transporters mentioned above, ABCB1 is also capable of lipid tranlocation. In order to study intracellular GlcCer transport, fluorescent analogues are often employed, the most common being $C_6$-NBD-GlcCer. This GlcCer analogue, in addition to $C_6$-GlcCer and $C_8C_8$-GlcCer, is translocated to the exoplasmic leaflet of the apical PM in epithelial cells transfected with a plasmid engineering expression of ABCB1 (Van Helvoort et al., 1996). Reconstitution of recombinant P-gp into liposomes similarly translocates GlcCer analogues in a cell-free system, suggesting a flippase function for P-gp (Eckford and Sharom, 2005).

Furthermore, ABCB1, like ABCA2 and ABCA12, has been found at the membrane of the Golgi (Molinari et al., 1994). Given ABCB1expression at the Golgi and transport activity towards GlcCer analogues, it was questioned whether natural GlcCer is also a substrate for the protein (Borst et al., 2000). This is particularly intriguing given the requirement for GlcCer translocation from the cytosolic to the lumenal leaflet of Golgi. For GlcCer Golgi lumenal access, one theory involves an energy-dependent translocation event carried out by ABCB1 (De Rosa et al., 2004; Lala et al., 2000) (Scheme 1.6). ABCB1 may be localized to the same membrane region as GCS or GlcCer may be trafficked to the membrane region containing ABCB1. GlcCer would then be directly flipped by ABCB1 from the cytosolic to the lumenal leaflet of the Golgi.

In support of this hypothesis, transfection of the human MDR1 cDNA encoding ABCB1 into MDCK cells causes a drastic increase in GSLs, particularly in $Gb_3$ containing shorter chain fatty acids, while wild-type MDCK cells have undetectable $Gb_3$ levels (Arab and Lingwood, 1998;

Lala et al., 2000). Correspondingly, MDR cells expressing P-gp are more sensitive to verotoxin (VT) (Farkas-Himsley et al., 1995) (Details of the VT-$Gb_3$ binding are presented in Section 1.16.3). In addition, inhibitors against ABCB1, such as cyclosporine A and ketoconazole, inhibit neutral GSL synthesis (De Rosa et al., 2004). This effect of ABCB1 inhibitors on GSL metabolism is not a general phenomenon of ABC transporter inhibition, as inhibition of the multidrug resistance-associated protein (MRP) with the MRP inhibitor probenecid does not alter GSL levels (Lala et al., 2000). Although ABCB1 appears to be a candidate flippase for GlcCer, some studies have reported the translocation event being ATP-independent (Buton et al., 2002), while others have demonstrated that only short-chain GlcCer analogues, and not natural GlcCer, are substrates (Halter et al., 2007).




Scheme 1.6. **P-gp-mediated flippase model for GlcCer.** GlcCer is synthesized on the cytosolic leaflet of the Golgi by GlcCer synthase (GCS). P-gp then translocates GlcCer to the lumenal leaflet, whereupon LacCer synthase can transfer galactose to form LacCer.

### 1.4.3.2 ABCB1 and GCS

An interesting line of work has linked ABCB1 to GCS such that overexpression of GCS is accompanied by elevated levels of ABCB1, and downregulation of GCS reduces ABCB1, sensitizing drug-resistant cells to doxorubicin (Gouazé et al., 2005; Liu et al., 2010). In drug-resistant cells, GCS may, therefore, be a route for removal of the pro-apoptosis molecule

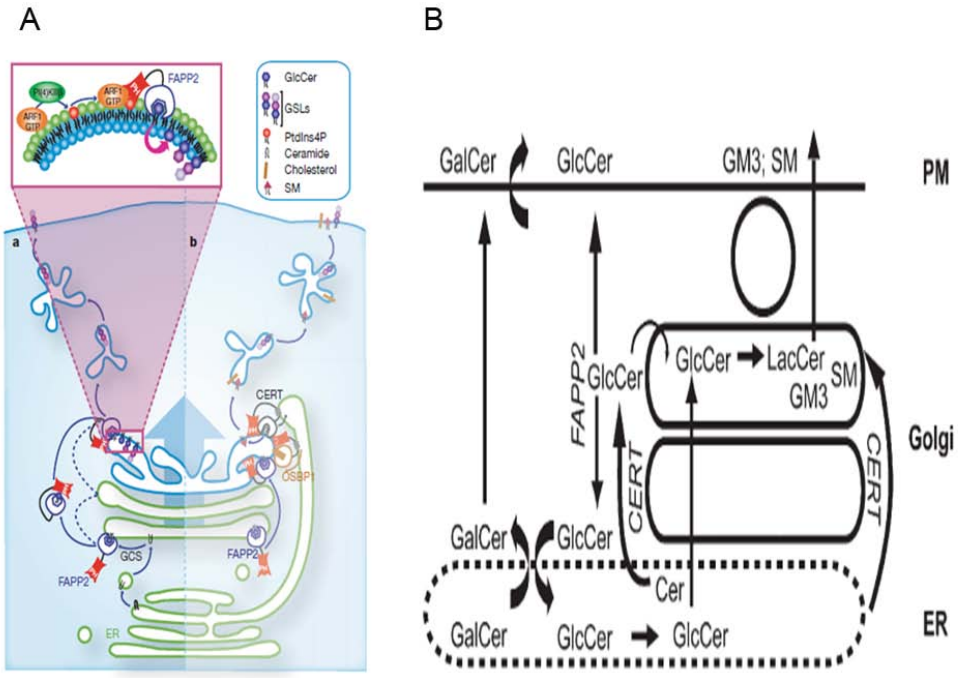
ceramide. Subsequent flipping of GlcCer by P-gp would then protect it from non-lysosomal glucosylceramidases. This has implications in cancer therapy in defining new approaches to overcome multidrug resistance that target GCS.

## *1.5 FAPP2*

The second theory for GlcCer access to the Golgi lumen involves transport of GlcCer by a specific glycolipid transfer protein. The phosphatidylinositol-four-phosphate-adaptor proteins 1 and 2 (FAPP1 and FAPP2, also known as pleckstrin homology domain-containing family A member 3 (PLEKHA3) and PLEKHA 8, respectively) are cytosolic proteins that are recruited to the *trans* Golgi by interaction of their pleckstrin homology (PH) domains with phosphatidylinositol-4-phosphate (PI4P) (Godi et al., 2004) and the small GTPase ADP-ribosylation factor (ARF1) (Dowler et al., 2000; Godi et al., 2004). The FAPPs have been shown to be involved in the transport of cargo from the TGN to the PM, with FAPP2 also being involved in the apical transport of proteins (Godi et al., 2004; Vieira et al., 2005). While human FAPP2 is 80% identical and 90% similar to human FAPP1 at the N-terminus, it differs at the C-terminus by the presence of a glycolipid transfer protein (GLTP) domain. GLTPs, first identified by Metz and Radin (Metz and Radin, 1980), are cytosolic proteins 22-24 kDa in size that are specific for glycolipids (Brown et al., 1985; Lin et al., 2000; Metz and Radin, 1980) and able to mediate their intermembrane transfer *in vitro* (Brown et al., 1985).

Two different models have been proposed as part of the FAPP2 theory of GlcCer transport. In one model, ceramide is trafficked to the *cis* Golgi, whereupon GCS catalyzes the transfer of glucose to ceramide, forming GlcCer (Scheme 1.7A). FAPP2 then carries GlcCer to a distal Golgi membrane region (non-vesicular transport) (D'Angelo et al., 2007), followed by

translocation to the lumenal leaflet of the Golgi by an unknown mechanism. Transport of GlcCer by FAPP2 is dependent on PI4P and ARF1 (D'Angelo et al., 2007). In the second model (Scheme 1.7B), ceramide is transported to the *trans* Golgi, where it is glucosylated to form GlcCer. FAPP2 then transports GlcCer back to the ER, where, it is able to passively translocate across the ER membrane, which is less ordered—due to the presence of very little cholesterol and sphingolipids—and follow the secretory pathway for access to the Golgi lumen (Halter et al., 2007; Papadopulos et al., 2007; Lippincott-Schwartz and Phair, 2010). It has been shown that a fluorescent GlcCer analogue is translocated across the ER membrane by an ATP-independent process (Chalat et al., 2012)




Scheme 1.7. **FAPP2-mediated GlcCer transport models.** (A) The D'Angelo et al. (2007) model shows GlcCer synthesis at the *cis* Golgi. FAPP2 then transports GlcCer to the *trans* Golgi, where a translocation event by an unknown mechanism delivers GlcCer to the lumenal leaflet of the Golgi. Image obtained from D'Angelo et al. (2007). (B) In the Halter et al. (2007) model, GlcCer is synthesized at the *trans* Golgi. FAPP2 can then deliver GlcCer to the cell surface or back to the ER, where it flips to the lumenal leaflet. GlcCer then follows the vesicular trafficking pathway for Golgi lumenal access. Image obtained from Halter et al. (2007).

Cao et al (Cao et al., 2009) have suggested the possibility that FAPP2 acts as a sensor to maintain GSL homeostasis. GlcCer on the cytosolic leaflet of the Golgi (i.e. GlcCer that has not been translocated to the lumenal leaflet) is a signal that complex GSLs have accumulated and their syntheses are no longer required. FAPP2 would bind GlcCer (as well as PI4P and Arf1) at the TGN, thereby tubulating the membrane and forming transport carriers. Such tubulation confounds GlcCer transport function.

While it is possible that FAPP2 shuttles GlcCer directly to the cytosolic leaflet of the PM, another mechanism for GlcCer transport to the cell surface may involve translocation of GlcCer into the TGN or a post-Golgi compartment (Lannert et al., 1998). Here, the absence of LacCer synthase means GlcCer cannot be converted to higher GSLs and would be transported to the cell surface by vesicular traffic (Lannert et al., 1998).

## *1.6 LacCer*

Regardless of the mechanism, the next GSL synthesized is LacCer, the key precursor for all glucose-based GSL sub-classes. LacCer synthase activity has been localized to the *trans* Golgi, but is also expressed at the *cis/medial* Golgi (Halter et al., 2007; Lannert et al., 1998).
Two different genes have been identified whose proteins synthesize LacCer, β4GalT5 and β4GalT6 (Chatterjee and Pandey, 2008). These proteins may be expressed differentially developmentally or in different cells. β4GalT5 is constitutively expressed in most tissues, while β4GalT6 is predominantly found in embryonic and adult brain cells (Chatterjee and Pandey, 2008). The enzymes catalyze the transfer of UDP-galactose to GlcCer, forming LacCer. Thus, these, and all other downstream GSLs, depend on particular sugar-nucleotide substrates in addition to the GSL susbtrate (Lannert et al., 1998). The sugar-nucleotide species must also be

translocated into the Golgi lumen (and the ER lumen for GalCer synthesis) (Burger et al., 1996; Lannert et al., 1998), which is achieved by organelle-specific nucleotide transporters (Hirschberg et al., 1998). In addition to the UDP-galactose transporter, transporters for CMP-sialic acid, UDP-GlcNAc, UDP-GalNAc and GDP-fucose are also found in the Golgi membrane (Hirschberg et al., 1998).

LacCer synthase (β4GalT5) is also involved in protein galactosylation; this, coupled with the presence of two different LacCer synthases, complicates the study of specific effects caused by gene knockout (Pontier and Schweisguth, 2012). In contrast, GCS is only involved in GSL synthesis; glycoproteins do not contain β-linked glucose (Ichikawa et al., 1996). LacCer appears to be a pro-survival signal, with reactions that lead to LacCer generation causing cell proliferation, migration and angiogenesis (Chatterjee and Pandey, 2008). In contrast, conversion of LacCer to other products, either by degradation or synthesis of downstream GSLs, often serves as a cell death signal (Chatterjee and Pandey, 2008).

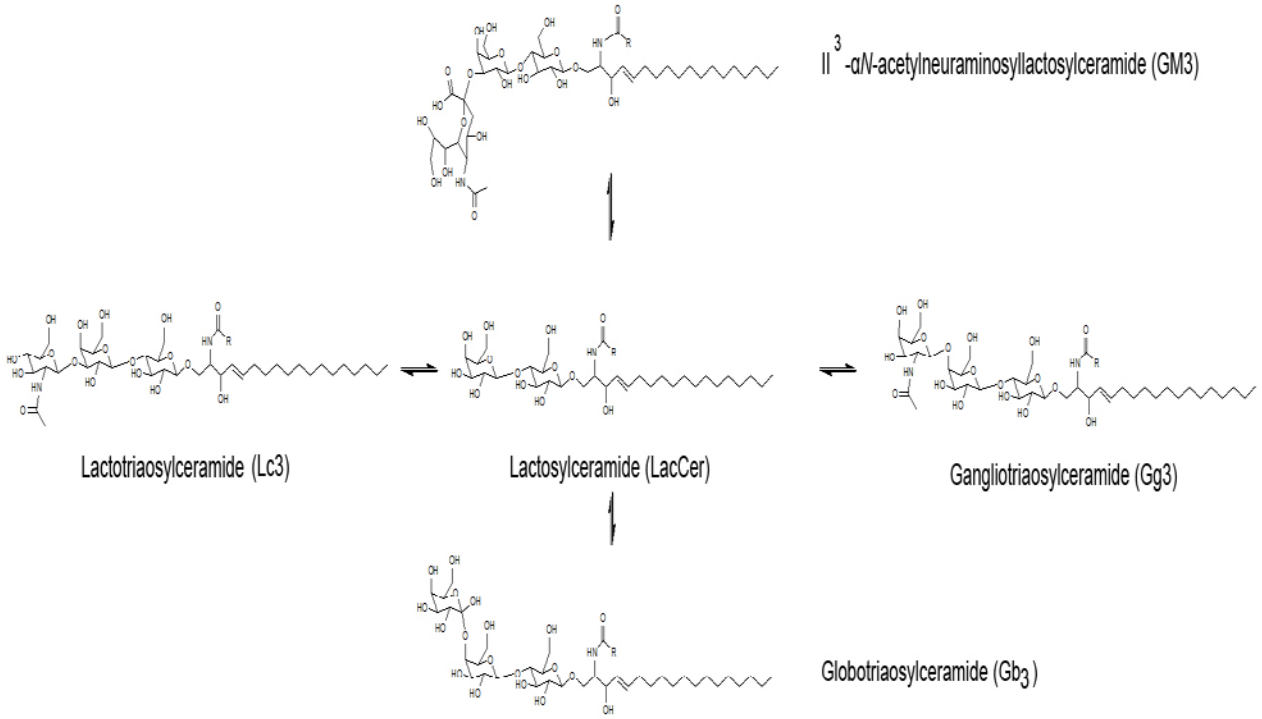
## *1.7 Glycosyltransferase knockout*

Various gene manipulation studies have been performed in mice on the glycosyltransferases to examine the phenotypic effect of loss of particular glycosyltransferase genes. The outcomes of some of these studies have been summarized by Pontier and Schweisguth (Pontier and Schweisguth, 2012). Knockout of GlcCer (Yamashita et al., 1999) and LacCer (Nishie et al., 2010) synthases are embryonic lethal; $Gb_3$ synthase KO mice appeared to be normal (Okuda et al, 2006), while GM3 synthase KO mice show enhanced insulin signalling (Yamashita et al., 2003) and neurological symptoms (Yoshikawa et al., 2009); GalCer synthase KO causes neurodegeneration (Coetzee et al., 1996) in addition to males being sterile (Pontier and

Schweisguth, 2012; Fujimoto et al., 2000); Lc3 synthase KO causes organ enlargement and reduces mouse lifespan (Kuan et al., 2010; Merrill, 2011); GalNAcT knockout mice express only the simple gangliosides GM3 and GD3 and develop behavioural neuropathies (Chiavegatto et al., 2000). A summary of other ganglioside synthase knockout mice is given by Yu et al (2012). A conditional GCS KO mouse was established deficient in enzyme expression in the nervous system (Yamashita et al., 2005). Mice showed abnormal neurological behaviour and loss of Purkinje cells.

## *1.8 Complex GSL Synthesis*

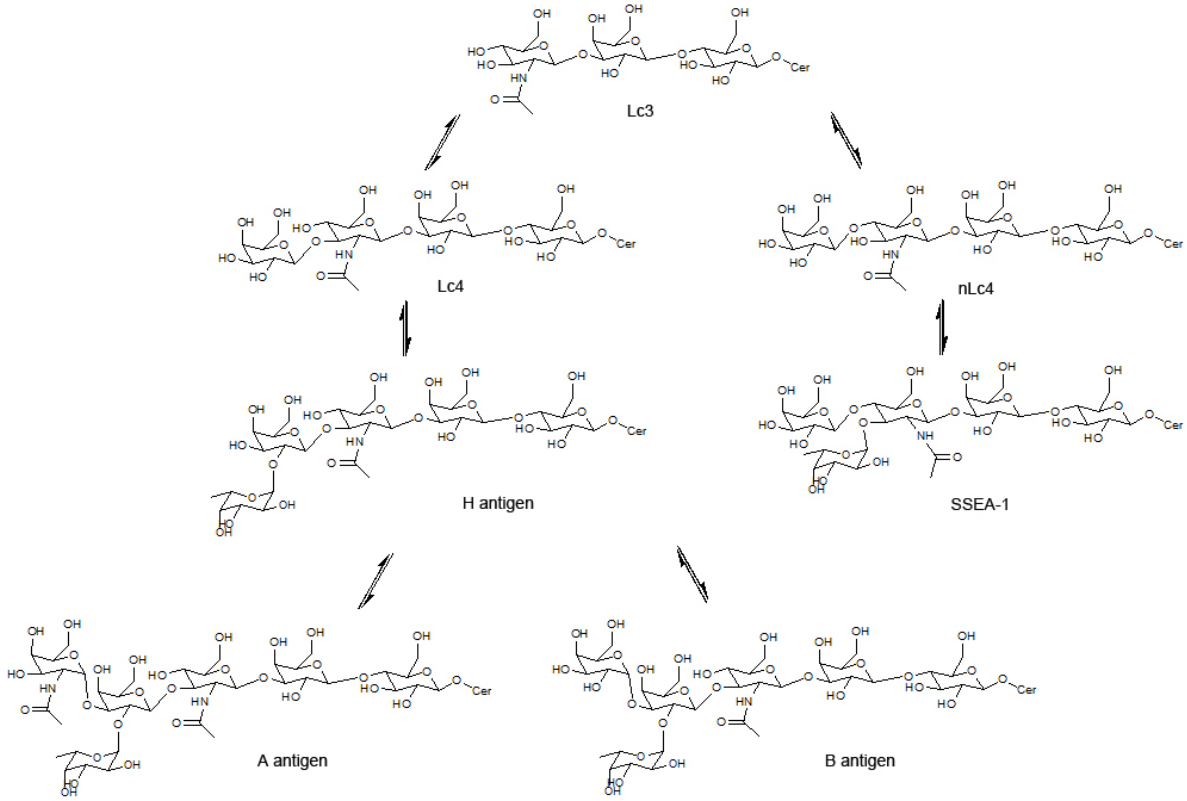
The glycosyltransferases within the Golgi are typically type II membrane proteins (cytoplasmic N-terminus and lumenal C-terminus) with catalytic activity lumenally-oriented (Maccioni et al., 2011b). As mentioned previously, LacCer is the precursor for all the sub-classes of glucose-based GSLs (Schemes 1.1 and 1.8). It remains unclear, however, as to what determines which biosynthetic pathway LacCer follows. It is possible that different pools of LacCer exist for the different pathways. This would likely require enzymes for the various sub-classes to be expressed at different locations in the Golgi. Studies to this effect have shown GM3 synthase to be predominantly localized in the *trans* Golgi, while $Gb_3$ synthase expression has been shown in both the *cis* Golgi and the *trans* Golgi network (TGN) (Halter et al., 2007; Yamaji et al., 2010).




Scheme 1.8. **GSL biosynthetic products from LacCer.** LacCer is the major precursor for the various glucose-based GSL subclasses. Addition of GlcNAc to LacCer forms Lc3 and the lacto-series. Galactosylation of LacCer forms $Gb_3$ and the globo-series. Addition of GalNAc to LacCer forms Gg3 and the ganglio-series. Sialic acid addition to LacCer forms GM3 and the first of the gangliosides.

### 1.8.1 Lacto-series

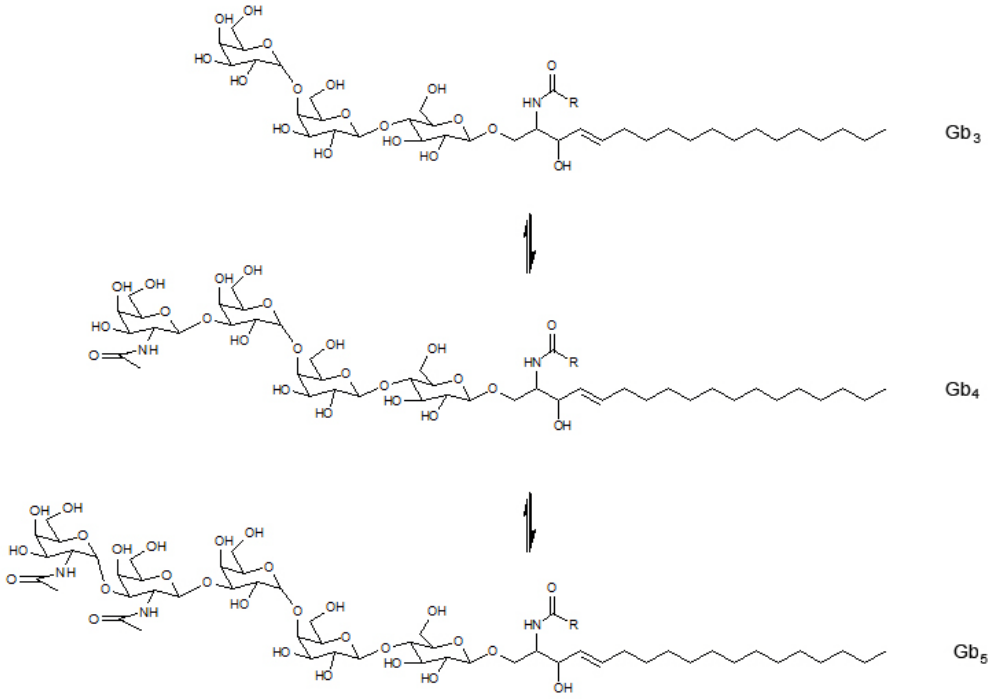
Lc3 (lactotriaosylceramide) is the first amongst the lacto-series GSLs, synthesized from LacCer by UDP-N-acetylglucosamine:β-galactose β1,3-N-acetylglucosaminyltransferase (Lc3 synthase, β3GlcNAcT) (Merrill, 2011) (Scheme 1.9). β1,3GalT can then synthesize Lc4, while β1,4GalT uses Lc3 to make nLc4, the first of the neolacto-series GSLs (Merrill, 2011).




Scheme 1.9. **Lacto-series GSL synthesis pathway.** Lc3 is the precursor for Lc4 and nLc4. Fucosylation of nLc4 produces SSEA-1. Fucosylation of Lc4 produces the H antigen, which is the precursor of the A and B antigens, synthesized by addition of GalNAc or galactose, respectively.

## 1.8.2 Globo-series

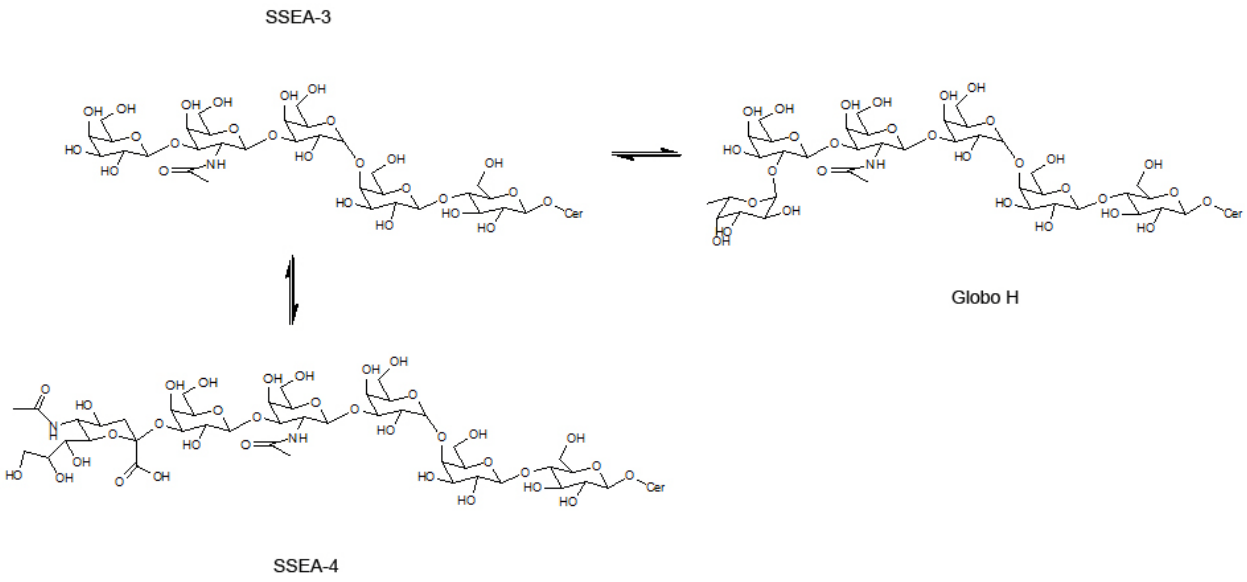
Biosynthesis of the globo series begins with formation of globotriaosylceramide ($Gb_3$) via addition of galactose to LacCer by A4GalT ($Gb_3$ synthase), the same enzyme that uses GalCer as a substrate to synthesize galabiosylceramide ($Gb_2$) (Kojima et al., 2000) (Scheme 1.10). The enzyme has been shown in both the *cis* and *trans* Golgi (Halter et al., 2007; Yamaji et al., 2010). Subsequent addition of GalNAc forms $Gb_4$. An α1-3 linked GalNAc to $Gb_4$ produces globopentosylceramide ($Gb_5$), also known as the Forssman antigen (Yoda et al., 1980).




Scheme 1.10. **Globo-series GSLs.** $Gb_3$ is the first among the globo-series GSLs. Addition of GalNAc forms $Gb_4$ and further GalNAc addition produces $Gb_5$.

### 1.8.3 Stage-specific embryonic antigens

Addition of galactose to $Gb_4$ by β3GalT5 produces a glycolipid that is also called $Gb_5$ but is more commonly known as stage-specific embryonic antigen-3 (SSEA-3); sialylation of this product forms SSEA-4 (Yanagisawa, 2011) (Scheme 1.11). Fucosylation of SSEA-3 forms Globo H. SSEA-3, SSEA-4 and Globo H are all classified as globo-series GSLs. SSEA-1 is synthesized by fucosylation of nLC4 (Scheme 1.9) and is, therefore, a member of the neolacto-series.




Scheme 1.11. **Stage-specific embryonic antigens**. Galactosylation of $Gb_4$ forms SSEA-3. Sialylation of SSEA-3 forms SSEA-4, while fucosylation of SSEA-3 produces Globo H.

### 1.8.4 Blood Group Determinant GSLS

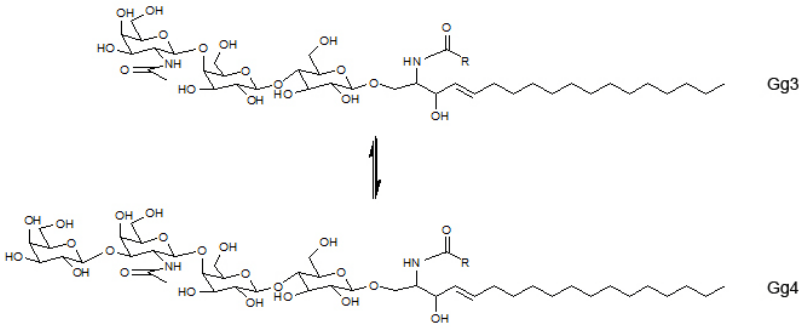
The ABO blood group designation is based on the presence of A and B carbohydrate antigens in the blood. These antigens are either part of erythrocyte glycoproteins or GSLs (Daniels, 2009). The *A* gene product catalyzes the transfer of GalNAc to the H antigen to form the A antigen, while the *B* gene product transfers a galactose to the H antigen to form the B antigen (Daniels, 2009) (Scheme 1.9). The H antigen is formed by fucosylation of Lc4. Due to the lack of active enzyme from the *O* allele, blood group O contains only the H antigen (Daniels, 2009).

Similar to the ABO blood group, the Lewis antigens are carbohydrate antigens linked to GSLs at the erythrocyte surface. Instead of fucosylation of the terminal galactose in Lc4, Lewis a ($Le^a$) is made by α1,4-fucosylation of the GlcNAc in Lc4 (Wu, 2011). Subsequent α1,2-fucosylation

of the terminal galactose of $Le^a$ forms $Le^b$. $Le^x$ and $Le^y$ are identical to $Le^a$ and $Le^b$, respectively, except that the fucose is connected to GlcNAc in $Le^x$ and $Le^y$ by an α1,3 linkage.

### 1.8.5 Ganglio-series

The Ganglio-series GSLs are related to gangliosides, but lack the sialic acid. β1,4-*N*-acetylgalactosaminyltransferase (β1,4GalNAc-T, GM2 synthase, EC 2.4.1.92) catalyzes the transfer of a GalNAc residue to LacCer to form gangliotriaosylceramide (Gg3, asialoGM2) (Giraudo and Maccioni, 2003) (Scheme 1.12). Galactosylation of Gg3 forms gangliotetraosylceramide (Gg4, asialoGM1), a GSL that appears to be involved in inhibiting the epithelial-to-mesenchymal transition (EMT) (Guan et al., 2009; Guan et al., 2010).




Scheme 1.12. **Ganglio-series GSLs.** Addition of GalNAc to LacCer forms Gg3 and subsequent galactosylation synthesizes Gg4.

### 1.8.6 Gangliosides

Gangliosides correspond to a large group consisting of acidic GSLs. They are further subdivided into three categories: *a*, *b*, *c*. Each class varies in the number and/or position of

sialic acid residues attached. The most common gangliosides are represented by the *a* series. These include the monosialyl gangliosides GM3, GM2 and GM1.

The first acidic ganglioside within the glucose-based GSL pathway, GM3, is generated from LacCer by the addition of a sialic acid by CMP-N-acetyl-neruraminate:lactosylceramide α2,3-sialyltransferase (GM3 synthase, SAT-I) (Giraudo and Maccioni, 2003). The simplest ganglioside, however, is GM4 (sialosylgalactosylceramide) (Scheme 1.4), a rare galactose-based GSL with two sugar residues (Ando et al., 1978; Ledeen et al., 1973). GD3, the first *b*-series ganglioside is synthesized from GM3 by SAT-II adding a second sialic acid in an α2-8 linkage and GT3, the first amongst the *c*- series, is made by SAT-III adding a third sialic acid to GD3 (Lloyd and Furukawa, 1998; Merrill, 2011). Synthesis of more complex gangliosides of each class involves the same enzyme-catalyzed reaction regardless of the substrate; for instance, addition of GalNAc to the terminal galactose of LacCer, GM3, GD3 and GT3 forms Gg3, GM2 (Scheme 1.13), GD2 and GT2, respectively, while galactosylation of these products forms Gg4, GM1, GD1b and GT1c, respectively (Giraudo and Maccioni, 2003; Lloyd and Furukawa, 1998). As it turns out, a single enzyme is responsible for the synthesis of Gg3, GM2, GD2 and GT2 from their respective precursors (Lutz et al., 1994; Pohlentz et al., 1988); one enzyme is responsible for the formation of Gg4, GM1a, GD1b and GT1c (Iber et al., 1989; Lloyd and Furukawa, 1998); a single enzyme synthesizes GM1b, GD1a, GT1b and GQ1c (Lloyd and Furukawa, 1998; Pohlentz et al., 1988).

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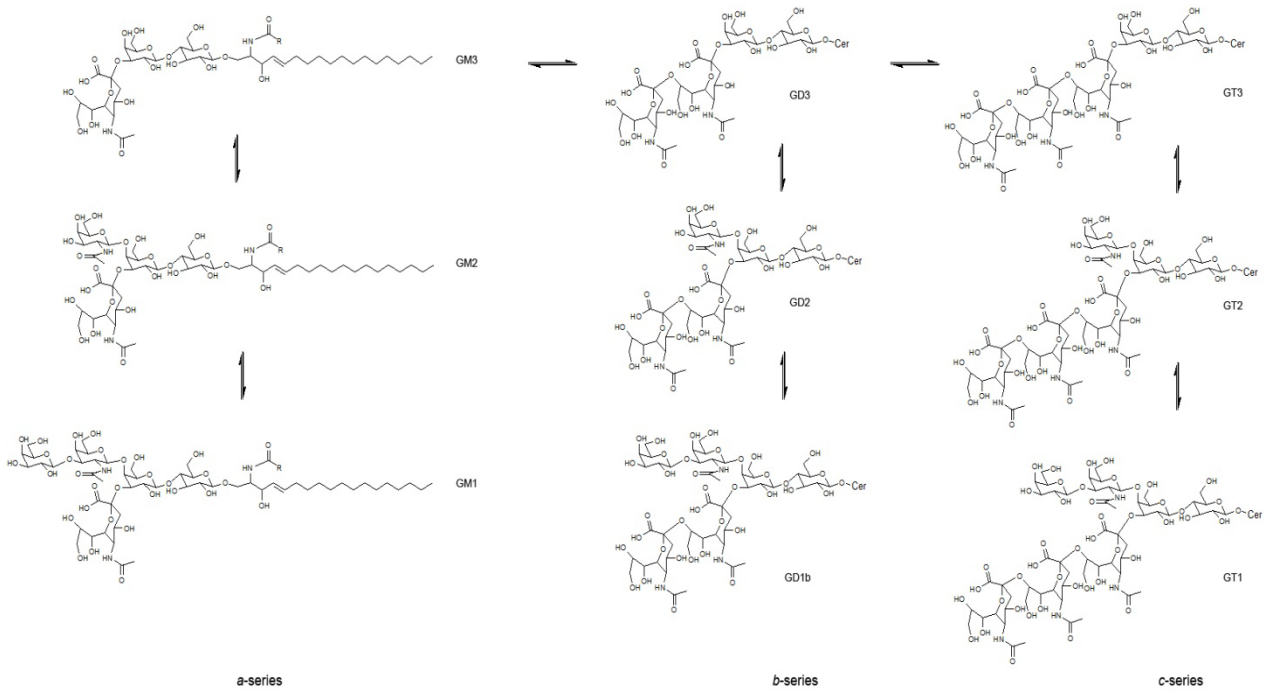



Scheme 1.13. G**anglioside structures.** The first *a*-series ganglioside is synthesized by sialylation of LacCer. Subsequent addition of GalNAc forms GM2 and galactosylation of GM2 produces GM1. *b*-series gangliosides are formed by addition of a second sialic acid residue (middle column). A third sialic acid is added in *c*-series gangliosides (right column).

### 1.8.7 Fucolipids

Fucolipids are found in human erythrocytes (McKibbin, 1978). They typically contain five or more sugars, although fucosylceramide has also been reported to accumulate in some human colon tumours; thus, the relatively large carbohydrate head groups make fucolipids water-soluble (McKibbin, 1978; Watanabe et al., 1976). Lc3 is the precursor of most fucolipids, with those based on Lc4 being classified as type 1, while those based on nLc4 are labelled type 2 (McKibbin, 1978). Fucolipids are mostly found in rapidly proliferating tissues such as the bone marrow and gastrointestinal epithelium, and are not common in brain or heart (McKibbin, 1978). Fucosyltransferase catalyzes the addition of fucose to gangliosides (Merrill, 2011);

fucosylGM1, for example, is seen in small cell lung cancer (Vangsted, 1994). Fucolipids also comprise many of the blood group antigens (as described in Section 1.8.4).

### 1.8.8 Glycosyltransferase Complexes

A similar localization of particular enzymes acting consecutively within the GSL biosynthetic pathway has given rise to the suggestion that such enzymes can exist within an actual complex. Using Golgi subfractions, it was shown that GM3 synthase activity peaks with LacCer synthase activity and that levels of LacCer itself were undetectable (Lannert et al., 1998). Not only does this suggest, according to the authors, that LacCer synthesis is the rate-limiting step in this GSL biosynthetic pathway (Lannert et al., 1998), but it also raised the possibility that the enzymes for these two reactions are localized near one another for the reaction to proceed so rapidly.

Indeed, evidence has revealed that sequential GSL biosynthetic enzymes organize into functional complexes (Bieberich et al., 2002; Giraudo and Maccioni, 2003). The GalNAc transferase (UDP-GalNAc:lactosylceramide/GM3/GD3 β-1,4-*N*-acetylgalactosaminyltransferase; GalNAcT) responsible for formation of Gg3, GM2 and GD2 from LacCer, GM3 and GD3 respectively exists in a complex with the downstream galactosyltransferase (UDP-Gal: Gg3/GM2/GD2 β-1,3-galactosyltransferase; GalT2) that forms Gg4, GM1 and GD1b (Giraudo et al., 2001). These enzymes are localized to the TGN (Giraudo et al., 1999). Co-immunoprecipitation and fluorescence analyses revealed this complex to be distinct both spatially and in its composition from that formed by LacCer synthase and the two sialyltransferases forming GM3 and GD3 (Giraudo and Maccioni, 2003). Importantly, LacCer synthase appears not to interact with the downstream sialyltransferase (SAT-II) alone; rather, both enzymes exist within the same complex by virtue of a mutual interaction with SAT-I

(Giraudo and Maccioni, 2003). Using a different cell system, however, it was shown that stable transfection of the gene encoding SAT-II into cells normally containing low endogenous activity of the protein increases the activity of GalNAcT and vice versa (Bieberich et al., 2002), suggesting cell-type dependence of complex composition and formation.

Interestingly, the enzymes forming a complex actually associate with one another within the ER and are transported to the Golgi already existing as a complex. This becomes relevant in the event of a mutation in one member of the complex that normally prevents its exit from the ER; assuming such a mutation is not within the regions involved in interaction, a pre-formed complex should allow normal traffic to the Golgi. This has been shown for ER exit mutants of GalT2 and GalNAcT, where mutant protein traffic is rescued by a wild-type interacting partner capable of associating with the ER export machinery and not by non-interacting proteins (Giraudo and Maccioni, 2003).

### 1.8.9 Pathway after LacCer

At branch points within GSL biosynthesis, the pathway followed generally depends upon expression of the relevant glycosyltransferases; however, many other factors are also involved, including the expression of particular nucleotide-sugar pyrophosphatases, which can act to limit the availability of sugar-nucleotide substrates for corresponding enzymes (Maccioni et al., 2011a). Given expression of each enzyme using LacCer as a substrate (LC3 synthase, $Gb_3$ synthase, GM3 synthase, Gg3 synthase), it is unclear what determines the metabolic fate of LacCer. Studies in this regard show globo-series GSLs to be dominant over the gangliosides (Takematsu et al., 2011). More specifically, it was shown that introduction of $Gb_3$ synthase into $Gb_3$-negative cells that express GM1 not only induced $Gb_3$ expression, but also decreased GM3

and GM1 levels (Takematsu et al., 2011). Furthermore, it turns out that LacCer conversion to $Gb_3$ by $Gb_3$ synthase is not required to cause this reduction in gangliosides, as a catalytically-inactive mutant $Gb_3$ synthase was sufficient to reduce GM1 levels (Takematsu et al., 2011).

The mechanism by which $Gb_3$ synthase negatively regulates ganglioside levels has not entirely been elucidated. Consistent with its dominant effect, both wild-type and inactive $Gb_3$ synthase interact with LacCer synthase (β4GalT6) to form a complex (Takematsu et al., 2011). However, as mentioned earlier, SAT-I (GM3 synthase) also forms a complex with LacCer synthase, which precludes complex formation as a plausible mechanism, although $Gb_3$ synthase appears to reduce the LacCer synthase-GM3 synthase interaction (Takematsu et al., 2011).

In one study, although the ceramide synthase inhibitor fumonisin B1 prevented formation of ceramide, long-term treatment showed a remarkable increase in certain GSLs (Meivar-Levy and Futerman, 1999). In particular, the activities of GCS, LacCer synthase and $Gb_3$ synthase were increased, in addition to there being elevated GCS transcript levels. Activities of SM synthase and GM3 synthase were unaltered. According to that study, when substrate is limited, GlcCer synthesis is favoured over SM and $Gb_3$ synthesis takes precedence over GM3 synthesis, while the converse is true under high substrate concentrations. These results suggest that maintenance of homeostatic levels of $Gb_3$ is important in certain cell types.

### 1.8.10 Multiple Locations of Glycosyltransferases

It was previously thought that the simple monohexosylceramides are synthesized at the *cis* Golgi, while more complex GSLs are made in distal Golgi compartments (Buton et al., 2002). This was rationalized based on glycoprotein maturation proceeding via a stepwise transit from

*cis* to *trans* Golgi. More recent investigations have discredited this rather simplistic view (Halter et al., 2007). Although the predominant Golgi region for GlcCer synthesis remains controversial, GlcCer synthase expression has been shown in the *cis, medial* and *trans* Golgi (D'Angelo et al., 2007; Halter et al., 2007). LacCer and GM3 synthases are primarily localized to the *trans* Golgi (Halter et al., 2007; Lannert et al., 1998). Other studies, however, have reported GM3 synthase to be localized to more proximal Golgi compartments (Iber et al., 1992; Trinchera and Ghidoni, 1989).

Varying assignments of enzyme localizations may relate to the different substrate analogues that are often used to measure enzyme activity, including fluorescent, short acyl chain and short ceramide backbone analogues. It is possible that enzymes in particular locations are specific for a particular aglycone. This would imply that glycosyltransferases are not confined to a single subsite within the Golgi, but rather, may be distributed throughout the Golgi, with greater densities in particular regions, as was shown by Halter et al (Halter et al., 2007).

### 1.8.11 Regulation of Glycosyltransferases

GSL levels can be controlled by regulating glycosyltransferase and glycosidase levels and activities responsible for their synthesis and degradation, respectively. This can be achieved at the level of transcription as well as by post-translational modification. The ganglioside expression profile is altered during development from simple to complex gangliosides (Yu et al., 1988), a process that has been shown to correlate with changes in expression of the corresponding glycosyltransferases (Ngamukote et al., 2007). For the developing mouse brain, levels of most glycosyltransferase transcript remain unchanged from embryonic day 12 to

adulthood; however, GalNAcT is an exception, with elevated transcript levels being detected from embryonic day 14 and being maintained into adulthood (Ngamukote et al., 2007). A corresponding change in GSL expression from simple gangliosides (GM3, GD3) to more complex gangliosides was observed. Gene levels of most glycosidases also remain unchanged, implying that glycosyltransferase, and not glycosidase, expression regulates the ganglioside profile during development (Ngamukote et al., 2007). Transcription itself can also be regulated through binding of activators or repressors in the upstream promoter region. Sp1 and AP2 are two such transcription factors that promote transcription of the mouse GM3 synthase gene (Xia et al., 2005).

With regards to post-translational regulation of glycosyltransferase activity, it has been shown that phosphorylation inhibits activity of SAT-IV, while it stimulates activity of GalNAcT (Bieberich et al., 1998). This was shown both by inhibition of phosphatases as well as activation of kinases. The distinct effects on two enzymes by the same modification is thought to be a mechanism for increasing cellular GM1 levels, as stimulation of GalNAcT facilitates conversion of GM3 to GM2, while inhibition of SAT-IV prevents conversion of GM1 to GD1a (Bieberich et al, 1998).

### 1.8.11.1 Epigenetic Regulation of Glycosyltransferases

Epigentic regulation of gene expression refers to modulation in gene expression by DNA modifications without altering the DNA sequence (Hsieh and Gage, 2004). This can occur by DNA methylation or histone modification via events such as acetylation, phosphorylation, methylation and ubiquitination (Hsieh and Gage, 2004). In light of the aforementioned

developmental regulation of ganglioside expression, it was recently shown that in the developing mouse brain, increased expression of GalNAcT is correlated to elevated histone acetylation (Suzuki et al., 2011). In particular, it was shown that histone acetylation was increased from embryonic day 12 through post-natal day 10 and into adulthood for GalNAcT, but not for SAT-II (Suzuki et al., 2011). In addition, inhibition of histone de-acetylation similarly increased GalNAcT and complex ganglioside expression. These results indicate that transcriptional activation during development is under epigenetic control (Suzuki et al., 2011).

## *1.9 GSL synthesis by the recycling pathway*

GSLs can also be synthesized by a salvage pathway by utilizing products from the degradation of existing GSLs. As part of the recycling pathway, in the late endosome and lysosome, the sugar moieties of GSLs are cleaved by appropriate glycosidases to eventually form ceramide (Kitatani et al., 2008). In addition, SM is degraded to ceramide by acid sphingomyelinase. Ceramide is then deacylated by acid ceramidase to produce sphingosine, which exits the lysosome (Kitatani et al., 2008). This sphingosine can then serve as a substrate for a CerS to re-synthesize ceramide for re-entry into the GSL synthesis pathway (Kitatani et al., 2008; Mullen et al., 2012). A second minor salvage pathway for GSL synthesis involves recycling of native or partially catabolized GSLs from late endosomes into the Golgi (Gillard et al., 1998). In this way, existing GSLs can be further glycosylated for synthesis of more complex GSLs. This pathway, however, makes very little contribution towards GSL synthesis. The relative contributions of the *de novo* and salvage pathways to GSL synthesis were examined using an inhibitor of sphinganine synthesis (β-chloroalanine) and an inhibitor of sphinganine/sphingosine acylation (fumonisin B1) (Gillard et al., 1998). It was observed GSL synthesis primarily occurs via the major recycling pathway, while *de novo* synthesis was prominent in actively dividing

cells (Gillard et al., 1998). It has been suggested that exploiting the salvage pathway is a means by which cells conserve energy (Gillard et al., 1998).

## *1.10 GSL Catabolism*

Like many macromolecules, GSLs are primarily degraded in the lysosome. GSLs reach the lysosome by a number of different mechanisms, including endocytosis of the plasma membrane, receptor-mediated endocytosis of low-density lipoprotein (LDL) and phagocytosis by macrophages (Wennekes et al., 2009). In the lysosome, sugars are enzymatically cleaved sequentially by exoglycosidases, eventually yielding free sugars and ceramide. Ceramide is then de-acylated by acid ceramidase back to sphingosine, which can be recycled and re-enter the biosynthetic pathway (as mentioned above) or can be broken down to simpler end products that are no longer classified as sphingolipids (Gillard et al., 1998; Merrill, 2011; Mullen et al., 2012).

Unlike the biosynthetic glycosyltransferases, which are membrane proteins (Maccioni et al., 2011b), the lysosomal hydrolases are soluble (Wennekes et al, 2009). In general, a particular GSL species is hydrolyzed by a single lysosomal enzyme. LacCer catabolism, however, is unique in that two different enzymes (along with corresponding cofactors) can degrade it: galactosylceramidase and GM1-β-galactosidase (Zschoche et al., 1994). While most GSL hydrolases are found in the lysosome and glycosyltransferases are found in the Golgi, there are some hydrolases that also exist in other locations of the cell, including at the PM (Sonnino et al., 2010). Their presence at the PM suggests a regulatory role of GSLs at the cell surface (Pontier and Schweisguth, 2012).

Defects in a GSL catabolic enzyme leads to accumulation of the corresponding GSL and the lysosomal storage disorder (LSD) pathology (Vellodi, 2005). Over 50 different LSDs have been identified to date (Grabowski, 2008). While the prevalence of each individual disorder is relatively low—Gaucher disease (GD), the most common of the diseases, affects 1 in 40,000 to 1 in 50,000 individuals (Cox and Cachón-González, 2012)—taken together as a class, they affect a significant number of individuals with a combined prevalence between 1 in 2500 to 1 in 7500 (Mechtler et al., 2012; Fletcher, 2006, Cox and Cachón-González, 2012). Table 1 lists some sphingolipid lysosomal hydrolases and the disease associated with a defective enzyme.

| Enzyme | Substrate | Disease Caused By Enzyme Defect |
|---|---|---|
| Glucocerebrosidase | Glucosylceramide | Gaucher |
| GalCer-β-galactosidase<br>GM1-β-galactosidase | LacCer | --- |
| α-Galactosidase A | $Gb_3$<br>Galabiosylceramide | Fabry |
| Sialidase | GM3 | Sialidosis |
| β-Hexosaminidase | GM2<br>Gg3<br>$Gb_4$ | Tay-Sachs<br>Sandhoff AB variant<br>Sandhoff |
| GM1-β-galactosidase | GM1<br>Gg4 | GM1 gangliosidosis |
| Arylsulfatase | Sulfatide | Metachromatic leukodystrophy |
| β-galactosylceramidase | GalCer | Krabbe |
| Acid ceramidase | Ceramide | Farber |
| Sphingomyelinase | Sphingomyelin | Niemann Pick |

Table 1.1. **Sphingolipid catabolic enzymes.** Some of the enzymes responsible for sphingolipid catabolism, their specific substrate(s) and the disease associate with defective enzyme are listed. No LSD has been assigned to defects in the enzyme responsible for LacCer breakdown.

### 1.10.1 Sphingolipid Activator Proteins

Many GSL catabolic enzymes require sphingolipid activator proteins (SAPs) for their function, and defects in these proteins can also cause storage disease (Kolter and Sandhoff, 2006). In

particular, the catabolic enzymes for GSLs with less than four carbohydrate residues require SAPs (Kolter and Sandhoff, 2005). SAPs function either by facilitating interaction of the membrane-bound GSL and the soluble lysosomal hydrolase or by activating the enzyme itself (Kishimoto et al., 1992; Kolter and Sandhoff, 2006). SAPs can be substituted *in vitro* by detergents. There are presently five known SAPs: GM2 activator protein and saposin A-D. GM2 activator protein is required for breakdown of GM2 by β-hexosaminidase A (Kolter and Sandhoff, 2006). This protein acts to present GM2 to the enzyme's active site. To accomplish this, GM2 activator inserts into the bilayer of lipid vesicles that are inside the lysosome. GM2 activator contains a lipid recognition site, allowing it to bind to the ceramide backbone of GM2, causing a conformational change and subsequent presentation of GM2 to β-hexosaminidase A (Wendeler et al., 2004).

SAP A-D are small glycoproteins that belong to the saposin-like protein family (Kolter and Sandhoff, 2006). All the saposins are part of the precursor protein, prosaposin; hence, their nomenclature is based on their position within the primary prosaposin sequence (Kishimoto et al., 1992). Although structurally similar, they are specific for different substrates and catabolic enzymes. All the saposins are able to bind GSLs, but they differ in their ability to stimulate enzyme activity (Kishimoto et al., 1992; Wendeler et al., 2004). Thus, defects in saposins lead to different sphingolipidoses. SAP-C was discovered in 1971 and is required for lysosomal degradation of GlcCer by glucocerebrosidase and is stimulated by negatively-charged lipids (Brown and Mattjus, 2007; Ho and O'brien, 1971). It appears, therefore, that activator proteins can also be independently regulated, adding to the complexity of GSL homeostasis.

### 1.10.2 GlcCer Catabolism

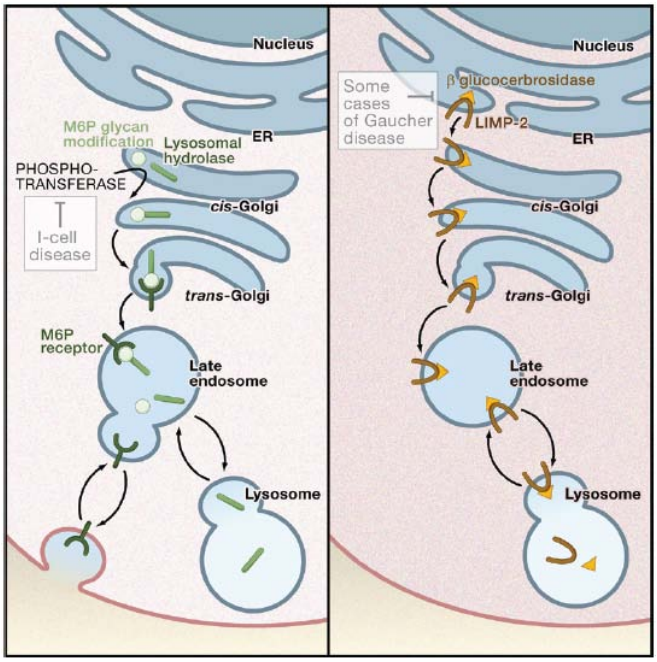
GlcCer is degraded to ceramide and glucose in the lysosome by glucocerebrosidase (acid-β-glucosidase, β-glucosylceramidase, GBA, EC 3.2.1.45). The enzyme requires the presence of SAP-C. Structurally, Glu340 serves as the catalytic nucleophile and Glu235 the general acid/base (Lieberman et al., 2007). Asn370 is located 13 Å from the active site; however, mutation of this residue to serine, the most common mutation found in Gaucher disease patients, significantly impairs enzyme activity (Lieberman et al., 2007). Asn370 apparently stabilizes a helical turn in loop 1 of the enzyme such that a mutation to serine destabilizes loop 1, reducing the efficiency of substrate binding (Lieberman et al., 2007).

#### 1.10.2.1 Glucocerebrosidase trafficking

Glucocerebrosidase is unique in terms of its transport mechanism to the lysosome. Whereas other lysosomal hydrolases are targeted to the lysosome by the mannose-6-phosphate (M6P) pathway, glucocerebrosidase reaches the lysosome in a M6P-independent fashion (Aerts et al., 1988), relying instead on lysosomal integral membrane protein 2 (LIMP-2) for this function (Reczek et al., 2007) (Scheme 1.14). The idea that proteins can be targeted to the lysosome independent of the M6P pathway came through observations in the LSD mucolipidosis II, in which patients suffer from deficiency of GlcNAc phosphotransferase (Ginsel and Fransen, 1991). Lack of this enzyme prevents phosphorylation of mannose on N-linked glycoproteins in the Golgi. However, patients still exhibited normal expression of some enzymes within the lysosome, suggesting a M6P-independent pathway (Ginsel and Fransen, 1991).

LIMP-2 is a type III transmembrane protein with short N- and C-terminal cytoplasmic domains, two TM domains and a long lumenal loop (Griffiths, 2007; Reczek et al., 2007). Mouse

embryonic fibroblasts deficient in M6P receptors did not have any effect on glucocerebrosidase trafficking, suggesting M6P-independent transport of the enzyme (Reczek et al., 2007). Furthermore, tissues from LIMP-2 knockout mice have elevated GlcCer levels (Reczek et al., 2007). In the absence of LIMP-2, glucocerebrosidase is secreted from cells (Reczek et al., 2007). Importantly, LIMP-2 is also capable of binding L444P glucocerebrosidase, which is normally retained in the ER and degraded, indicating that LIMP-2 is also present in the ER and is capable of binding the enzyme at the more neutral pH (Reczek et al., 2007). The model proposed for the LIMP-2-mediated delivery of glucocerebrosidase to the lysosome involves binding within the ER, traffic through the Golgi by vesicular transport, association of LIMP-2 to the adaptor protein AP-3 for incorporation into clathrin-coated vesicles, transit through an endosomal compartment and delivery to the lysosome (Reczek et al., 2007). Here, the acidic pH may facilitate dissociation. Whether or not LIMP-2 is recycled back to neutral compartments—similar to M6P receptors—is unknown. It has, therefore, been postulated that part of the variability in Gaucher disease patient phenotypes may be due to secondary mutations in LIMP-2 (Reczek et al., 2007).




Scheme 1.14. **Lysosomal hydrolase trafficking pathways.** GSL catabolic hydrolases are typically delivered to the lysosome via the mannose-6-phosphate (M6P) pathway (left panel). Transit of these enzymes through the Golgi during the maturation process involves phosphorylation of mannose residues. This is recognized by M6P receptors in the TGN. The receptor-enzyme complex dissociates in the late endosome, leading to recycling of the receptor and delivery of the enzyme to the lysosome. For glucocerebrosidase, LIMP-2 binds to the enzyme in the ER. The complex traffics through the Golgi and is only dissociated once at the lysosome. Image taken from Griffiths (2007).

### 1.10.2.2 Non-lysosomal degradation of GlcCer

The lysosome is not the exclusive centre for GSL degradation. GlcCer, for instance, can be catabolized outside the lysosome—perhaps at the PM—by non-lysosomal glucosylceramidases (GBA2 and GBA3) (Boot et al., 2007; Hayashi et al., 2007). In addition, glucocerebrosidase activity has also been reported in Golgi fractions, which may be attributed to protein en route to the lysosome (Lannert et al., 1998). GBA2 was first described as the bile acid β-glucosidase (EC 3.2.1.45) (Boot et al., 2007). GBA2 differs from glucocerebrosidase in several respects. First, while the latter is a soluble lysosomal enzyme, GBA2 is a non-lysosomal integral membrane protein that loses its activity upon exposure to detergents (Boot et al., 2007; Van Weely et al., 1993). In addition, glucocerebrosidase is stimulated by negatively charged phospholipids and bile salts, while GBA2 does not show such an effect (Boot et al., 2007). GBA2 is also insensitive to conduritol β epoxide inhibition and its pH optimum is more neutral than the lysosomal enzyme (Wennekes et al., 2009). Functionally, GBA2 appears to be important in male fertility, as GBA2 knockout mice accumulate GlcCer in the testis (in addition

to the liver and brain, where GBA2 is also expressed) and have abnormal sperm formation, leading to reduced fertility (Yildiz et al., 2006). Interestingly, GlcCer that accumulates in other tissues does not lead to increases in organ volumes as seen in Gaucher disease (Yildiz et al., 2006). This reinforces an idea proposed previously that glucocerebrosidase may also activate other signaling pathways that lead to the disease phenotype (Jmoudiak and Futerman, 2005).

The ceramide generated by GBA2-mediated breakdown of a fluorescent GlcCer analogue has been shown to be rapidly converted to SM, suggesting co-localization with SMS2 at the cell surface (Boot et al., 2007). Different studies, however, have reported GBA2 to be either at the PM or the ER, with Yildiz et al suggesting that GBA2 knockout leads to ER storage disorder (Boot et al., 2007; Yildiz et al., 2006).

Cytosolic-β-glucosidase (GBA3, klotho-related protein, EC 3.2.1.21) was also identified as an enzyme capable of breaking down GlcCer (Hayashi et al., 2007). GBA3 is a cytosolic protein that can hydrolyze various β-$_D$-glucosides. Another GlcCer degrading enzyme has also been reported—lactase phlorizin hydrolase (LPH, EC 3.2.1.62/108) is an intestinal disaccharidase important for lactose hydrolysis (Rings et al., 1994; Wennekes et al., 2009). Expression of LPH is restricted to the microvilli of intestinal epithelial cells (Wennekes et al., 2009). Its specificity is not limited to GlcCer, being capable of hydrolyzing lysoGlcCer, lysoGalCer, GalCer and LacCer as well (Buller et al., 1989; Kobayashi and Suzuki, 1981; Leese and Semenza, 1973). The specific localization of LPH may be indicative of a digestive and absorptive function for dietary sphingolipids (Vesper et al., 1999; Wennekes et al., 2009).

## *1.11 GSL Subcellular Location*

GSLs are predominantly found on the exoplasmic leaflet of the plasma membrane, where they serve important structural and functional roles (see section 1.12-1.14). While most GSLs are localized to the exoplasmic leaflet of the cell surface, GlcCer is enriched in intracellular membranes (Sillence, 2007). It has been shown that GlcCer accumulation in neurons can cause increased calcium release from the ER either directly—via interaction with ER calcium channels—or indirectly—via depletion of inositol trisphosphate, which would increase expression of the ryanodine receptor in the ER (Korkotian et al., 1999).

GSLs are also found on nuclear and mitochondrial membranes (Ledeen and Wu, 2006; Morales et al., 2003). Forssman antigen and GM1 have been shown at the nuclear envelope (Lucki and Sewer, 2012). GM1 is found on both the inner and outer nuclear membranes, being associated with a sodium/calcium exchanger responsible for maintaining nuclear calcium homeostasis (Lucki and Sewer, 2012). GSLs may have reached the nucleus through retrograde transport (Burger et al., 1996), although it is unclear why only a specific subset of GSLs are localized here. Likewise, it has been proposed that other GSLs may also be trafficked from the Golgi to the ER and flipped to the cytosolic leaflet by the non-specific phospholipid flippase to bind to the cytoskeleton (Burger et al., 1996). SM is also found in the nucleus and can be degraded by nuclear sphingomyelinase to ceramide, which plays a role in apoptosis induction (Ledeen and Wu, 2008).

The presence of GSLs in mitochondria is accompanied by the reported localization of several glycosyltransferases in a special subcompartment of the ER called the mitochondria-associated membrane (MAM), leading to synthesis of GlcCer, LacCer and gangliosides within this

compartment (Ardail et al., 2003). This is in contrast to the earlier evidence that mitochondria lack GSLs and cholesterol (Van Meer, 1989). In addition to their presence within cellular membranes, GSLs are also found in the plasma at low concentrations (Clarke, 1981). GSLs in the plasma, particularly the water-insoluble neutral GSLs, are typically associated with lipoproteins, primarily LDL (Clarke, 1981). In patients with familial hypercholesterolemia, GSL levels are significantly elevated; this excess over normal patients is associated with LDL (Clarke, 1981).

## *1.12 GSLs Organize into Membrane Microdomains*

In contrast to the conclusion from the fluid mosaic model of cell membranes that lipids can freely diffuse laterally within the membrane and act as the solvent for proteins (Simons and Ikonen, 1997; Singer and Nicolson, 1972), lipids can, in fact, form lateral microdomains. Lipids in membranes do not necessarily diffuse freely laterally (Karnovsky et al., 1982). Evidence that lipids can form microdomains in the membrane was shown by Karnovsky et al (Karnovsky et al., 1982; Pike, 2009). Later, it was shown that the lipid compositions of the two membranes in polarized epithelial cells, apical and basolateral, which are separated by tight junctions, are distinct in their exoplasmic leaflets, with the apical membrane being enriched in sphingolipids (van Meer and Simons, 1988). The delivery of sphingolipids to the exoplasmic leaflet of the cell membrane was thought to be due to accumulation of these molecules in the lumenal leaflet of the Golgi at a region where proteins destined for the cell surface are sorted; thus, vesicular transport delivers these proteins together with sphingolipids to the cell surface (Simons and Ikonen, 1997; van Meer and Simons, 1988).

The concept of lipid rafts was first introduced in 1997 (Simons and Ikonen, 1997). In this model, sphingolipids were proposed to associate laterally with one another. Any gaps between adjacent sphingolipids are filled with cholesterol. These rafts were shown to be insoluble in Triton X-100 at 4°C (Simons and Ikonen, 1997). Thus, lipid rafts were initially defined as cholesterol and GSL-enriched regions on the membrane that are resistant to detergent extraction (Pike, 2009). "This proposal for compartmentalization by lipid rafts suggested a non-random membrane architecture specifically geared to organize functionality within the bilayer" (Lingwood and Simons, 2010). Over the years, the understanding of lipid rafts has evolved, which has led to a corresponding evolution in the definition of rafts. The consensus definition of lipid rafts, established in 2006 at the Keystone Symposium of Lipid Rafts and Cell Function is as follows: "Membrane rafts are small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions." (Pike, 2006). Importantly, the term "membrane raft" replaced "lipid raft" due to the realization that raft formation depends on both lipids and proteins (Pike, 2006).

Membrane rafts do not simply refer to a single region on a membrane; rather, multiple rafts in different regions of the membrane may exist (Pike, 2004). This, in turn, results in heterogeneity in terms of the composition of rafts (Pike, 2004). Membrane rafts are dynamic assemblies that can exist transiently or be stable structures (Lingwood and Simons, 2010; Pike, 2006). The definition of a membrane raft is also no longer dependent on being resistant to detergent extraction on the grounds that this "does not provide physiologically relevant information" and the observation that some proteins that are not resistant to extraction by detergents have been shown by other methods to exist within rafts (Pike, 2006).

Rafts are important structurally and functionally. The initial observations by Karnovsky et al led them to question whether particular proteins may reside in these microdomains (Karnovsky et al., 1982). Brown and Rose (1992) first showed a protein with a glycosylphosphatidyl inositol (GPI) anchor can be recovered from detergent-resistant, GSL-enriched domains (Brown and Rose, 1992). As it turns out, many important proteins are localized in these microdomains, such as the epidermal growth factor receptor (EGFR) and insulin receptor (IR) (Balbis and Posner, 2010; Kabayama et al., 2007). Furthermore, exclusion from membrane rafts may impair function and contribute to disease pathology, such as is seen for the insulin receptor (Kabayama et al., 2007). Absence of this receptor from caveolae leads to insulin resistance (see section 1.14.6 for details) (Kabayama et al., 2007).

## *1.13 GSL/Cholesterol interaction*

GSLs and cholesterol have an intimate relationship in biological membranes. Indeed the Niemann-Pick C-causing mutation of NPC1, a transport protein responsible for the transport of LDL-derived cholesterol, leads not only to lysosomal accumulation of cholesterol, but also of GSLs (Wang and Song, 2012; Xu, 2010). Conversely, GSL storage in Gaucher, Fabry and Sandhoff diseases impairs cellular cholesterol efflux (Glaros et al., 2005). In addition, in LSDs, endocytic trafficking of fluorescent GSL analgoues is directed predominantly to the lysosomes rather than to the Golgi; sorting to the Golgi can be achieved by depleting cells of cholesterol (Puri et al., 1999).

GSLs and cholesterol are typically localized to the same membrane region, forming membrane raft microdomains. It had been proposed that the GSL aglycone and the microenvironment in

the membrane can modulate GSL conformation such that multiple presentations of the same GSL modulates receptor function (Lingwood, 1996). Recently, it has been shown that cholesterol regulates GSL conformation and, therefore, receptor function (Lingwood et al., 2011). Using liposomes containing either GM1 or $Gb_3$, binding of cholera toxin and verotoxin, respectively, was significantly reduced in the presence of cholesterol. In the presence of cholesterol, the GSL glycan headgroup is tilted towards the membrane ('membrane parallel'), while in the absence of cholesterol, the glycan is more membrane perpendicular (Lingwood et al., 2011; Yahi et al., 2010). The membrane parallel configuration makes the GSL receptor 'invisible' to ligands. This has been shown by immunohistochemistry analysis of GM1 and $Gb_3$ in human kidney sections, verotoxin binding of red blood cells and GSL exposure upon sperm capacitation (Lingwood et al., 2011). In each case, cholesterol extraction renders accessible a pool of GSLs that were previously undetectable.

## *1.14 Sphingolipid Function*

For many years, it was thought that GSL function in membranes was exclusively structural (Wennekes et al., 2009). According to Koscielak, "the primary evolutionary function of GSLs is to provide [the cell membrane] with an energetically inexpensive carbohydrate protective coat..." (Kościelak, 2012). Over the years, however, it has been proven that sphingolipids have a central role in a number of different functions including cell proliferation, development, protein trafficking, apoptosis and receptor function modulation among many others that have been described. These functions of GSLs are dependent not only on the glycan headgroups; rather, the hydrophobic aglycone is equally important (Lingwood, 1996; Wennekes et al., 2009). In virtually all these roles, genetic or pharmacological suppression of GSLs can disrupt normal function.

### 1.14.1 GSL Regulation of Endocytosis and Intracellular Protein Trafficking

GSL depletion alters the typical endocytic pathway by re-routing molecules to other compartments; for example, the fluorescent LacCer analogue, BODIPY-LacCer, was directed to the Golgi in control cells, while GSL depletion re-directed it to endosomes (Sillence et al., 2002). Interestingly, for the converse situation, with elevated GSL levels such as in GSL LSDs, trafficking is also impaired and BODIPY-LacCer is directed to the lysosome (Chen et al., 1999). GSLs, therefore, appear to be intricately involved in vesicular trafficking.

GSLs have also been shown to be required for the correct intracellular transport of tyrosinase from the Golgi to the melanosome (Sprong et al., 2001). Using GM95 cells, which lack all GSLs, it was shown that tyrosinase is expressed but cannot process tyrosine to L-DOPA, the rate-limiting step in melanin synthesis within the melanosome. Lack of melanin was shown to be due to mislocalization of tyrosinase to the Golgi rather than the melanosome. This defect is overcome by restoring GSL synthesis, either by transfection of GCS or GalCer synthase, or by exogenous addition of lysoGlcCer (Sprong et al., 2001). Surprisingly, exogenous GlcCer itself was unable to correct the trafficking defect. It was suggested that this may be due to a requirement for a cytosolic pool of GlcCer (Sprong et al., 2001).

A gradient of lipid expression exists within cellular membranes, such that the plasma membrane contains the highest concentration of sphingolipids, the ER contains the lowest concentration and the Golgi has intermediate levels between the two (Lippincott-Schwartz and Phair, 2010). This distribution is functionally relevant, as low ER sphingolipid levels results in a less ordered membrane allowing for protein entry for folding, while the high cell surface concentrations

creates a thicker membrane with higher permeability stringency (Lippincott-Schwartz and Phair, 2010).

It has been hypothesized that GSL accumulation in the lumenal leaflet of a distinct region of the Golgi serves as a sorting centre for proteins destined for delivery to the apical plasma membrane (Simons and Ikonen, 1997; van Meer and Simons, 1988). The transport of both sets of macromolecules, therefore, appears to be linked. Recenly, a model of the Golgi was developed that suggests that the partitioning of phospholipids and sphingolipids/cholesterol into unique domains creates membrane regions of varying thickness into which integral membrane proteins, which are sorted according to transmembrane thickness, selectively partition (Bretscher and Munro, 1993; Lippincott-Schwartz and Phair, 2010). Molecules in these domains are either selectively retained, trafficked to the ER or trafficked towards the cell surface.

### 1.14.2 LacCer regulates Cell Proliferation

Regulation of cell proliferation is of paramount importance, as evidenced by uncontrolled growth in cancer. LacCer has been assigned a role in cell proliferation by activation of a signalling cascade. A number of different signals can trigger LacCer synthesis by activating LacCer synthase, including oxidized LDL, EGF and platelet-development growth factor (PDGF) (Chatterjee and Pandey, 2008). LacCer then activates NADPH oxidase to produce reactive oxygen species (ROS), which initiates a signalling cascade involving the MAP-kinase pathway and culminates in cell proliferation (Bhunia et al., 1996). Contrary to the ability of LacCer to induce cell proliferation, simpler and more complex GSLs are thought to generally inhibit this process (Chatterjee and Pandey, 2008), although several GSLs have been associated with various tumours (Hakomori, 1986b).

GD3 has also been implicated in cell proliferation. In fact, GD3 is a tumour-associated ganglioside (Zeng et al., 2002) that has been reported to be overexpressed in breast cancer (Cazet et al., 2010). Breast cancer cells expressing GD3 synthase stimulated serum-free proliferation of these cells and showed increased phosphorylation of MAP-kinase (Cazet et al., 2010). Signalling through the MAP-kinase pathway for cell proliferation was shown to occur via increased activation of the receptor c-Met (Cazet et al., 2010). In another study, microarray analysis of cells stably knocked down in GD3 synthase expression revealed downregulation of genes known to be involved in cell proliferation (Zeng et al., 2002). Maintaining homeostatic levels of GD3, therefore, is important for regulation of cell growth.

### 1.14.3 GSLs Facilitate Cell Adhesion

OxLDL-induced synthesis of LacCer also activates a MAP-kinase-independent pathway for intercellular adhesion in neutrophils and endothelial cells (Chatterjee and Pandey, 2008). As part of this pathway, OxLDL, TNF-α or shear stress stimulates LacCer synthesis, which activates NADPH oxidase for production of ROS. In LacCer-rich neutrophils, LacCer signalling stimulates expression of CD11/CD18 to stimulate adhesion of neutrophils to endothelial cells (Arai et al., 1998). It was later revealed that C16 LacCer does not associate with particular kinases within microdomains, while C24:1 LacCer does (Sonnino et al., 2009). In endothelial cells, LacCer induces expression of intercellular adhesion molecule-1 (ICAM-1), allowing for adhesion of neutrophils expressing CD11/CD18 (Bhunia et al., 1998). Similarly, LacCer has also been implicated in angiogenesis (Rajesh et al., 2005). Vascular endothelial growth factor (VEGF) induces LacCer synthesis, initiating a signalling cascade that results in

the cell-surface expression of platelet endothelial cell adhesion molecule-1 (PECAM-1), a protein involved in angiogenesis (Rajesh et al., 2005).

Mouse melanoma B16 cells adhere to endothelial cells expressing LacCer and Gg3 as part of the metastasis process (Kojima et al., 1992; Otsuji et al., 1995). Using plates coated with LacCer or Gg3, B16 cells were shown to adhere to them, proliferate and become motile (Kojima and Hakomori, 1991). The adhesion of these cells is glycan dependent between the cellular GM3 and either LacCer or Gg3. In addition, signal transduction proteins are also enriched within the GSL-enriched microdomains containing GM3, leading to activation of signalling pathways upon GM3 adhesion (Iwabuchi et al., 1998).

### 1.14.4 Sphingolipids are involved in Apoptosis Signalling

Ceramide is a second messenger in stress responses; its production *de novo* or by SM catabolism is stimulated by stress inducers such as TNFα, UV light and chemotherapeutic agents (Chalfant and Spiegel, 2005). The effects exerted by ceramide are not usually achieved by dihydroceramide; this is a testament to the selectivity of the targets of ceramide signaling and to the importance of the desaturase responsible for ceramide formation *de novo* (Merrill, 2011). Ceramides, and therefore ceramide synthases, have been linked to apoptosis (Bose et al., 1995; Mullen et al., 2012). In particular, production of $C_{16:0}$ by CerS5 and CerS6 appears to be involved in the process (Mullen et al., 2011; Mullen et al., 2012). Elevations in ceramide can trigger apoptosis through a variety of signaling pathways, including via protein kinase C, serine/threonine protein phosphatases and cathepsin D (Pettus et al., 2002), though such responses are tissue/cell-dependent. Ceramide can also be phosphorylated at the *trans* Golgi and possibly the PM by ceramide kinase (CERK) to form the bioactive species ceramide-1-

phosphate (Gault et al., 2010). Both ceramide-1-phosphate and its deacyl analogue sphingosine-1-phosphate are pro-survival signals, stimulating cell growth and angiogenesis among other functions (Chalfant and Spiegel, 2005).

Apoptosis initiation by CD95 cross-linking at the cell surface increases ceramide levels, which is followed by elevations in GD3 (De Maria et al., 1997). GD3 disrupts the mitochondrial membrane by dissipating the membrane potential, leading to the release of cytochrome c from mitochondria (De Maria et al., 1997). GD3 also prevents translocation of the pro-survival signal NF-κB into the nucleus (Colell et al., 2001). In addition, GD3 stimulates formation of reactive oxygen species, which can activate caspases (Zhang, 2004). Since particular GSLs are involved in apoptotic signalling, cancer therapeutics have attempted to exploit this to interrupt the uncontrolled proliferation characteristic of cancer cells (Zhang, 2004). As an example, antibodies against GM2 have been shown to induce apoptosis in GM2-positive lung cancer cells (Nakamura et al., 1999).

### 1.14.4.1 Sphingolipids and Autophagy

Autophagy, a Greek term first coined by Christian de Duve literally meaning “self-eating”, is a regulatory process induced for the clearance of damaged proteins and organelles or to promote survival under conditions of nutrient deprivation (Glick et al., 2010). The process involves sequestration of cargo to be degraded within a double-membraned autophagosome, followed by fusion of the autophagosome with the lysosome to form the autolysosome and subsequent degradation of cargo within this structure (Glick et al., 2010). An intricate relationship exists between autophagy and apoptosis, with autophagy being a means for cell death or survival under different conditions (Young et al., 2013). In addition, both processes have several

regulators in common (Young et al., 2013). Similar to their involvement in apoptosis, sphingolipids are also involved in the process of auotphagy. Ceramide and gangliosides have been implicated in stimulating autophagy, with both being involved in autophagy induction and in the process of autophagosome formation (Young et al., 2013). Interestingly, LSDs have been implicated in preventing autophagy (Settembre et al., 2008). It is thought that accumulation of lysosomal substrates in LSDs causes dysfunction, preventing autophagosome fusion with the lysosome (Settembre et al., 2008). This leads to a block in autophagy, followed by accumulation of dysfunctional organelles and ultimately apoptosis.

### 1.14.5 GM3-Mediated Regulation of Epidermal Growth Factor Receptor (EGFR) Signalling

EGFR is a transmembrane protein that is important in cell proliferation and is overexpressed in various carcinomas (Kawashima et al., 2009). EGF binding to its receptor causes EGFR dimerization and autophosphorylation of tyrosine residues, initiating a signaling cascade (Jorissen et al., 2003). EGFR contains 12 N-linked oligosaccharides, loss of which reduces receptor activation (Kawashima et al., 2009). It was shown that GM3—and GM1 to a lesser extent—inhibit growth of A431 cells by inhibiting EGFR tyrosine phosphorylation (Bremer et al., 1986). EGFR exists at the cell surface within GSL and cholesterol-enriched membrane rafts (Jorissen et al., 2003). GSL depletion by PDMP inhibition of GSL biosynthesis stimulates EGFR phosphorylation (Meuillet et al., 2000). The inhibitory mechanism was proposed to be via carbohydrate-to-carbohydrate interaction; more specifically, the interaction is between the GlcNAc termini on the N-glycans of EGFR and the carbohydrate of GM3 (Yoon et al., 2006). This interaction prevents receptor dimerization and, therefore, activation (Kawashima et al., 2009). Interestingly, the aglycone appears to be capable of modulating inhibitory activity, as

lysoGM3 more effectively inhibits EGFR phosphorylation (Haga et al., 2008). From this proposed mechanism of interaction, it was inferred that GM3 regulates cell proliferation in normal cells, which contain some GlcNAc-terminated N-glycans on EGFR; in contrast, cancer cells like A431 have complex N-glycans containing sialic acid, which do not interact with GM3, and thereby proliferate uncontrollably (Kawashima et al., 2009).

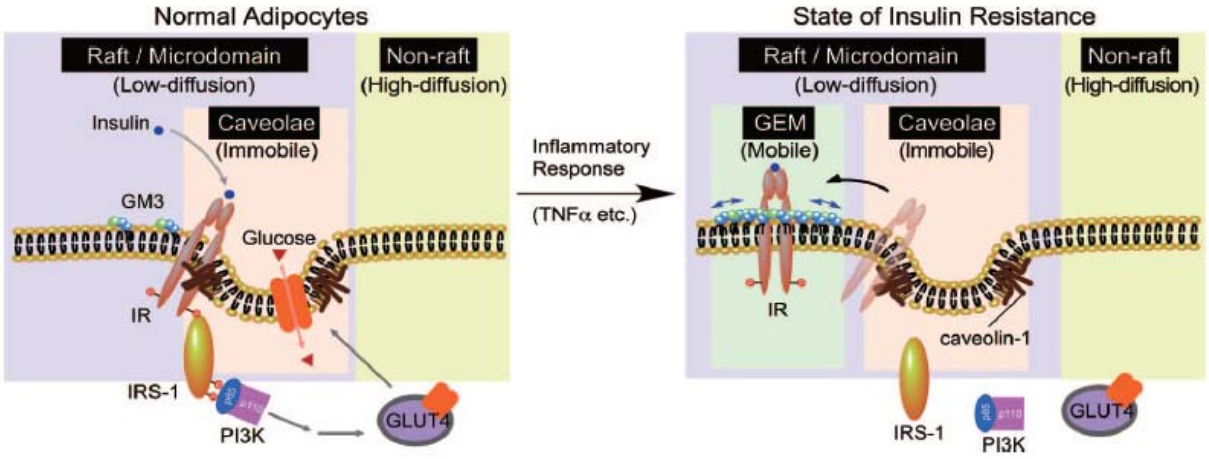
Recently, it has been shown that the juxtamembrane Lys642 residue in EGFR is important in the EGFR-GM3 interaction, as mutation to glycine prevents the inhibitory effects of GM3 on EGFR phosphorylation (Coskun et al., 2011). A proposed model suggests that monomeric EGFR interacts with GM3 and is raft-associated; EGF binding may result in dissociation from membrane rafts to facilitate dimerization and activation (Coskun et al., 2011; Jorissen et al., 2003).

### 1.14.6 GM3 Modulates Insulin Receptor (IR) Signalling

Signalling through the IR is initiated by insulin binding the IR, followed by receptor dimerization, tyrosine autophosphorylation of the IR and recruitment and phosphorylation of its downstream substrates IRS-1 and IRS-2 (Langeveld and Aerts, 2009). This leads to binding and activation of phosphoinositide-3-kinase (PI3K), which produces phosphatidylinositol (3,4,5)-triphosphate (PIP3) and activates protein kinase C (PKC). Proteins with PH domains, such as protein kinase B (PKB), bind to PIP3 resulting in their activation. Activation of PKB and PKC stimulates glucose transporter 4 (GLUT4) transport to the cell surface, leading to glucose uptake by the cell. (Langeveld and Aerts, 2009).

GM3 was identified as an inhibitor of IR tyrosine phosphorylation (Nojiri et al., 1991). TNF-α-induced insulin resistance leads to elevated levels of GM3, by increasing both expression of GM3 synthase and activity of the enzyme (Tagami et al., 2002). This inhibits insulin signalling through the IR by preventing tyrosine phosphorylation of both the IR and IRS-1, which ultimately suppresses insulin-dependent glucose uptake by cells. Inhibiting GSL biosynthesis overcomes this block in signalling, while exogenous supplementation of GM3 to normal cells inhibits phosphorylation, signifying the importance of GSLs and GM3, in particular, in this process (Tagami et al., 2002). The GM3-IR association is an electrostatic interaction based on a lysine residue (Lys944) in the IR that is exposed on the cell surface, very near the transmembrane domain, and the negatively charged sialic acid of GM3 (Kabayama et al., 2007). This is similar to the recent finding of a juxtamembrane lysine residue in the EGFR mentioned above.

Insulin signalling depends on IR localization within caveolae, while GM3 does not associate with these domains (Kabayama et al., 2007; Tagami et al., 2002). Based on these findings, a model was proposed in which the IR in normal cells is sequestered within the immobile caveolae where insulin-dependent signalling takes place (Kabayama et al., 2007) (Scheme 1.15). In the insulin-resistant state, TNF-α stimulates GM3 synthesis. Elevated GM3 interacts with the IR to dissociate it from caveolae and move it into a mobile GSL-enriched microdomain from which insulin signalling cannot occur.




Scheme 1.15. **Model of GM3-mediated inhibition of insulin signalling.** In normal adipocytes (left panel), the IR is localized within caveolae, where it can respond to insulin stimulation by initiating a signalling cascade that leads to GLUT4 expression at the cell's surface. In the insulin-resistant state (right panel), elevated levels of TNF α induces GM3synthesis. GM3 interacts with the IR, dissociating it from caveolae, thereby preventing insulin signalling from taking place. Image taken from Kabayama et al. (2007).

### 1.14.7 GSLs in Development

GSLs are essential for the development of organisms (Pontier and Schweisguth, 2012). Their importance in development is evidenced by defects in embryonic differentiation caused by knockdown of *Ugcg* (Liang et al., 2010; Yamashita et al., 1999). Furthermore, mutation or knockdown of *Ugcg* proves lethal in *C. elegans*, *D. melanogaster* and *M. musculus* (Pontier and Schweisguth, 2012).

GSLs have been shown to be essential in regulating the EMT process in embryonic development (Guan et al., 2009). In particular, EMT induction is associated with depletion of certain GSLs, namely Gg4 (Guan et al., 2009; Guan et al., 2010). In addition, the GSL expression profile changes during development and differentiation (Liang et al., 2010). SSEA-3 and SSEA-4 are highly expressed in human embryonic stem cells (hESCs) and are used as markers for isolation of these cells, while their expression is significantly diminished in differentiated embroid body (EB) outgrowth cells (Liang et al., 2010; Rao, 2007). In addition, other globo- and lacto-series

GSLs are also expressed in hESCs, but are significantly reduced in EB outgrowth cells. These include Globo H, $Gb_4$, $LC_4$, fucosyl (n)$Lc_4$ and disialyl $Gb_5$ (Liang et al., 2010). In contrast, expression of the gangliosides GM3, GM2, GM1, $GD1_a$, $GD1_b$, GD3 and $GT1_{a\text{-}c}$ increase following differentiation (Liang et al., 2010). In accordance with the observed changes in GSL expression between undifferentiated and differentiated stem cells, expression of the corresponding glycosyltransferases is also altered (Liang et al., 2010). Pontier and Schweisguth made an interesting observation that many stem cell markers used for purification of stem cells happen to be proteins localized to membrane microdomains (Pontier and Schweisguth, 2012). Thus, it was postulated that changes in expression of these markers during differentiation may correlate to changes in GSLs and expression of GSL biosynthetic enzymes (Pontier and Schweisguth, 2012).

### 1.14.8 Sphingolipids Prevent Water Loss from Skin

The outer epidermis layer—stratum corneum—of skin is responsible for maintaining a barrier against pathogens and dehydration (Holleran et al., 2006). In order to fulfill this function, cells in the stratum corneum secrete large quantities of lipid, primarily GlcCer (Feingold, 2007; Holleran et al., 2006). This lipid-rich extracellular matrix prevents loss of water from the skin (Feingold, 2007). The importance of ceramide is evidenced by their reduction being associated with various skin diseases including atopic dermatitis and lamellar ichthyosis (Choi and Maibach, 2005). Furthermore, lack of CerS3 results in the inability to synthesize ultra-long acyl chains (greater than C26), leading to loss of the skin's barrier function and death of mice shortly after birth from water loss (Jennemann et al., 2012).

GlcCer comprises the vast majority of the sphingolipid species in lamellar bodies, where lipids accumulate prior to secretion and subsequent processing into various ceramides (Holleran et al., 2006). It has been shown that GlcCer synthase activity increases during epidermal differentiation. Since GlcCer must be hydrolyzed for ceramide production, glucocerebrosidase activity and expression is also increased in the outer epidermal layers (Takagi et al., 1999) . Consistent with the requirement for glucocerebrosidase, Type II Gaucher disease patients show ultrastructural defects in the outer epidermal layer (Holleran et al., 1994). Similarly, defects in the protein responsible for transport of GlcCer into lamellar bodies, ABCA12, also cause skin disease (Holleran et al., 2006; Sakai et al., 2007b).

### 1.14.9 Dietary Sphingolipids

Sphingolipids are found in most foods, albeit in relatively small quantities (Vesper et al., 1999). GSLs and SM from food pass through the stomach intact. Some are broken down in the small intestine and colon in rats and mice (Schmelz et al., 1994). LPH is strategically located in this regard to fulfill part of this function. The sphingosine product of hydrolysis is taken up by intestinal cells for further degradation to release fatty acids or for re-conversion to higher sphingolipids (Schmelz et al., 1994). Interestingly, in mice induced to develop colon tumours, supplementing their diet with SM reduced the number of aberrant colonic crypt foci (early markers of colon cancer) (Dillehay et al., 1994). In addition, such mice had more benign adenomas as opposed to the adenocarcinomas in mice fed a standard diet (Schmelz et al., 1996). Supplementing GSLs in the diet has also been reported to be just as effective, or more effective, than SM addition at preventing aberrant colonic crypt formation (Vesper et al., 1999). Gangliosides in milk have been proposed to provide protection against pathogens (Vesper et al., 1999; Zopf and Roth, 1996). Healthy pre-term infants who were given a milk formula

supplemented with gangliosides had fewer *E. coli* in their feces and higher numbers of fecal bifidobacteria, bacteria with a symbiotic relationship with humans (Rueda et al., 1998) .

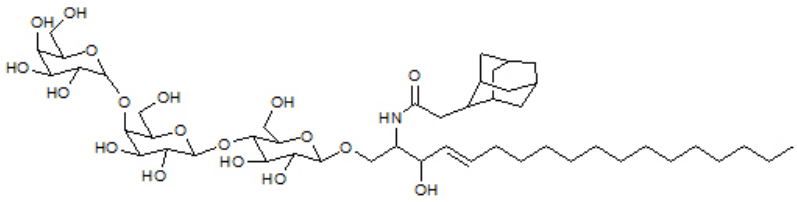
## *1.15 Functional Importance of the GSL Aglycone*

Evidence for the importance of the aglycone in GSL receptor function is provided by the inability of verotoxin (VT) to bind to the lipid-free trisaccharide of $Gb_3$ or to the terminal disaccharide galabiose, even though the primary recognition epitope lies within the carbohydrate (Boyd et al., 1994; Lindberg et al., 1987). Studies with different deoxy$Gb_3$ analogues have shown distinct hydroxyl groups to have specific roles in binding the different VT subtypes (Nyholm et al., 1996). Not only is the aglycone necessary for VT binding, modifications in the lipid backbone also affect binding (Kiarash et al., 1994). In addition, the $Gb_3$ acyl chain affects retrograde transport of VT, such that transport to the ER/nucleus corresponds to C16 or C18 $Gb_3$, while C22 and C24 $Gb_3$ tend to traffic VT only to the Golgi (Arab and Lingwood, 1998; Lingwood, 2003). Importantly, this means that incorporation of a fluorescent probe within the lipid backbone may alter the trafficking, and therefore, functional, properties of the GSL itself, complicating the study of GSLs.

Although differences within the aglycone appear to be minor and insignificant to be functionally relevant, the “biochemical effects due to minor structural variations of the lipids are amplified in multivalent GSL organizations” (Mylvaganam and Lingwood, 2003; 2005). As an example, the reactivity of an antibody against gangliotriaosylceramide is subtype dependent, with increased binding to the subtype containing 2-hydroxylated C16 acyl chain (Kannagi et al., 1983).

### 1.15.1 AdamantylGSLs

GSLs have limited solubility in water, with neutral GSLs being completely insoluble. Our lab has previously designed analogues of GSLs by replacing the fatty acid moiety with a rigid adamantane (or norbornane) frame (Lingwood, 2003; Mylvaganam and Lingwood, 1999). This has been done for $Gb_3$, GalCer, LacCer and SGC (Lingwood, 2003; Mylvaganam and Lingwood, 1999). $Gb_3$ has low solubility in water, partitioning more than 95% into the organic phase in a chloroform/water mixture. Surprisingly, ada$Gb_3$ (Scheme 1.16) was found almost entirely in the aqueous phase. Such a drastic shift in water-solubility was unexpected given the substitution of one hydrophobic moiety—fatty acid—with another more hydrophobic and less water-soluble group—adamantane. Similar observations were made for SGC and adaSGC, with the former partitioning predominantly in the organic phase and the latter in the aqueous phase (Whetstone and Lingwood, 2003). The adamantyl analogue of adaGalCer, the simplest GSL—and thereby containing the highest hydrocarbon to sugar ratio—partitions approximately 5% into the aqueous phase, while the natural counterpart is found entirely in the organic phase (Mylvaganam and Lingwood, 1999).




Scheme 1.16. **AdamantylGb$_3$ structure.** The fatty acid of $Gb_3$ is replaced by the adamantane frame.

The enhanced solubility in water was thought to be due to formation of low order aggregates at high dose, while at low concentrations, the proposed model involved the sphingosine backbone wrapping around the adamantane frame (Lingwood, 2003; Mylvaganam and Lingwood, 1999).

This would minimize exposure of the hydrophobic surface to the aqueous medium (Mylvaganam and Lingwood, 2003; 2005). The volume of such a species may approach the maximum limit for a hydrophobic molecule to exist as a monomer in aqueous solution (Mylvaganam and Lingwood, 2003; 2005).

The interaction of gp120 with $Gb_3$ is increased in the presence of cholesterol, likely due to assembly into a raft-like organization (Lingwood, 2003). Interestingly, interaction of gp120 with ada$Gb_3$ resembles this situation, suggesting that the $Gb_3$-cholesterol organization is inherent within the ada$Gb_3$ molecule (Mahfoud et al., 2002). Similarly, using a discontinuous sucrose gradient commonly employed for separation of detergent-resistant domains, $Gb_3$ was only bound by fluorescent VT B subunit at the light density interface characteristic of membrane rafts, in the presence of cholesterol (Lingwood, 2003). Ada$Gb_3$ was bound by $VT_B$ in the absence of cholesterol. Ada$Gb_3$, in fact, inhibits VT1 binding of $Gb_3$ with an $IC_{50}$ of 1 μM (Mylvaganam and Lingwood, 1999).

## *1.16 Sphingolipids and Disease*

### 1.16.1 Lysosomal Storage Disorders

Genetic defects causing functional or expression deficiencies in lysosomal hydrolases, in their associated cofactors, or in transport mechanisms leading to degradation result in accumulation of particular molecules and the clinical pathology known as lysosomal storage disease (LSD) (Vellodi, 2005). Defects causing sphingolipid accumulation are called sphingolipidoses, a subclass within the LSDs. Over 50 different LSDs have been identified (Cox and Cachón-González, 2012). While the frequency of each individual disease is relatively low—Gaucher disease, the LSD with the highest incidence, has a prevalence of 1 in 40,000-50,000

(Grabowski, 2008) –taken together, they affect approximately 1 in 2500-7500 individuals (Mechtler et al., 2012; Cox and Cachón-González, 2012). A specific subset of the sphingolipidoses in which GSLs accumulate represent the GSL LSDs.

#### 1.16.1.1 Gaucher Disease

Gaucher disease results from mutations in β-glucosylceramidase (glucocerebrosidase, EC 3.2.1.45) and is characterized by GlcCer accumulation (Brady et al., 1965). Over 300 different mutations have been identified in the glucocerebrosidase gene (Grabowski, 2008). The residual enzyme activity in Gaucher patients has been estimated to be 5-25% of normal activity (Jmoudiak and Futerman, 2005). The disease has been subdivided into three types varying on a spectrum of neuronopathic symptoms, with Type I displaying no neuronopathic symptoms and Type II suffering from the most severe symptoms. Type II Gaucher disease is the early onset acute neuronopathic form. Patients suffer from severe CNS symptoms and usually do not survive beyond the age of 3 years, with a median age of death at 9 months (Grabowski, 2008; Jmoudiak and Futerman, 2005). Type III is either childhood or adolescence-onset with mild-to-severe CNS manifestations (Grabowski, 2008).

Type I Gaucher disease is the most common and is considered to be a disease of macrophages as GlcCer accumulation is predominantly seen in visceral macrophages (Grabowski, 2008). Macrophages with accumulated GlcCer are called Gaucher cells (Bussink et al., 2006). Type I Gaucher disease onset varies from childhood to later in life. The most common mutation leading to type I Gaucher disease involves a substitution of asparagine for serine at position 370. This mutation is present in 70% of affected Ashkenazi Jews and 25% in the remaining patients. Patients typically suffer from hepatosplenomegaly, bone disease (osteopenia, osteoporosis, bone

pain, deformities), and thrombocytopenia (Grabowski, 2008). Many children also suffer from growth retardation (Kaplan et al., 2006). Although many patients suffer from considerable increases in tissue size, which for spleen can be 25 times that of normal, accumulation of GlcCer accounts for less than 2% of the total mass increase (Cox, 2001). Thus, either the enzyme deficiency or the accumulation of GlcCer substrate activate other pathways causing such a phenotype (Jmoudiak and Futerman, 2005).

There is debate as to whether the N370S mutant enzyme still traffics normally to the lysosome but manifests catalytic deficiencies (Wennekes et al., 2009), or whether the enzyme is unable to fold and is, therefore, retained in the ER for ER-associated degradation (Ron and Horowitz, 2005). On the one hand, patients homozygous for the N370S mutation exhibit variable disease severity, with a more severe phenotype associated with reduced Endo-H resistance of glucocerebrosidase (Ron and Horowitz, 2005). This reflects greater ER retention of the enzyme. Furthermore, approximately 60% of newly synthesized N370S glucocerebrosidase is degraded within 24 h, while isofagomine is able to facilitate transport of the vast majority of the mutant enzyme to its appropriate destination—the lysosome (Steet et al., 2006). On the other hand, N370S glucocerebrosidase has been shown to reach the lysosome, albeit with slower kinetics (Schmitz et al., 2005).

Gaucher disease is typically diagnosed by analysis of glucocerebrosidase activity. While DNA sequencing can identify different GBA mutants, among the many mutations identified in the gene, some have only been seen in single families; thus, evaluation of enzyme activity is important (Beutler et al., 2005; Grabowski, 2008). In general, patients with one mild mutation in GBA (such as N370S) combined with any other mutation in the second allele (mild, severe or

null) will suffer from mild disease severity (type I); two severe mutant alleles will typically lead to types II or III disease (Beutler et al., 2005). In addition, an allele harbouring the L444P mutation can also contain other mutations—these are called complex alleles (Grabowski, 2008). Two complex alleles, or one complex allele and one with L444P, often lead to type II disease (Grabowski, 2008).

One established marker of Gaucher disease is the presence of chitotriosidase in the blood (Malaguarnera, 2006). The enzyme is a member of the chitinase family and is capable of breaking down chitin (Malaguarnera, 2006). It is secreted by macrophages (Gaucher cells) into the blood circulation and is used clinically as a marker to monitor efficacy of Gaucher disease treatments. More recently, glucosylsphingosine (lysoGlcCer) has been reported to be a potential Gaucher disease marker (Dekker et al., 2011). LysoGlcCer was originally detected in the spleen and skin fibroblasts of Gaucher disease patients, and later in the brain as well (Nilsson and Svennerholm, 1982; Raghavan et al., 1973). In type I Gaucher disease patients, elevations of plasma lysoGlcCer relative to controls was considerably greater than GlcCer accumulation (Dekker et al., 2011).

Since GlcCer breakdown by glucocerebrosidase requires saposin C, mutations in the Sap-C domain of prosaposin leading to defects in saposin C also result in Gaucher disease. Surprisingly, lysoGlcCer also accumulates in this Gaucher disease subtype, even though the water-solubility properties of this species were expected to make its degradation by glucocerebrosidase sap-C independent (Dekker et al., 2011).

#### *1.16.1.1.1 Gaucher Disease Mouse Model*

A ‘Gaucher’ mouse was originally established by injecting mice with the irreversible glucocerebrosidase inhibitor conduritol β epoxide (CBE) to mimic the accumulation of GlcCer characteristic of the disease (Kanfer et al., 1975). This model, however, requires continual injections due to new enzyme synthesis (Stephens et al., 1981). Inactivation of the enzyme using a covalent inhibitor also circumvents utilization of the mouse model for the study of therapeutics, as potential pharmacological chaperones will be unable to induce activity and enzyme supplementation would simply be inactivated by circulating CBE. A subsequent model generated by introduction of the disrupted glucocerebrosidase gene into embryonic stem cells followed by injection into blastocysts resulted in death within 24 h of birth (Tybulewicz et al., 1992). Homozygous N370S mice were similarly lethal in the neonatal period (Xu et al., 2003).

Recently, a conditional glucocerebrosidase knockout mouse has been established by using lox-Cre recombination (Sinclair, 2007). This resulted in viable mice with glucocerebrosidase deficiency in liver, spleen and bone marrow, thereby mimicking the Gaucher disease phenotype. Fromt this model, transgenic mice were established, expressing either human wild-type, N370S or L444P glucocerebrosidase.

### 1.16.1.2 Fabry Disease

Fabry disease (also called Anderson-Fabry’s disease) is caused by defects in α-galactosidase A (GLA, EC 3.2.1.22), leading to accumulation of globotriasoylceramide ($Gb_3$) (Brady et al., 1967). It is the lone disease amongst the sphingolipidoses that is X-linked recessive; all other sphingolipidoses are inherited as autosomal recessive (Kolter and Sandhoff, 2006). Fabry disease has an estimated incidence of one in 40,000 to 117,000 (Beck et al., 2004). Over 400

different mutations have been identified in the GLA gene. Unlike Gaucher disease, where the N370S mutation is found in the majority of patients (Futerman et al., 2004), there is no single predominant Fabry disease mutant (Pastores and Lien, 2002).

GLA is a homodimer, with each monomer consisting of two domains (Garman and Garboczi, 2004). Domain 1 contains the active site. Mature GLA consists of 398 amino acids, with 13 residues predicted to directly interact with the α-galactose substrate (Garman and Garboczi, 2004). Mutation in any of these 13 residues leads to severe classic Fabry disease (Ishii et al., 2007). Mutations at a distance from the active site reduce the protein's stability by disrupting its hydrophobic core (Garman and Garboczi, 2004). Such mutations reduce the efficiency of folding and therefore, lysosomal transport.

Fabry disease is classified into two subtypes: early onset (or classic) Fabry disease, in which patients suffer from little or undetectable enzyme activity, and late-onset disease, in which patients do have some residual enzyme activity (Ishii et al., 2007). Fabry disease patients typically suffer from chronic pain, renal impairment, skin lesions, cardiomyophathy and cerebral vascular disease (Lidove et al., 2007; Motabar et al., 2010). Clinical manifestations and disease severity do not always correlate with $Gb_3$ and GLA levels. Heterozygous females generally have less severe symptoms than hemizygous males, but they may also exhibit similar symptoms to hemizygous males, rendering the female phenotype difficult to predict and one that varies considerably (Aerts, 2008; Kolter and Sandhoff, 2006). In addition, mouse models of Fabry disease have been developed that accumulate $Gb_3$ in various tissues, but do not exhibit clinical symptoms (Ohshima et al., 1997).

Similar to the observation of lysoGlcCer accumulation in Gaucher disease patients, lysoGb$_3$ significantly accumulates in the plasma of Fabry disease patients (Aerts, 2008). LysoGb$_3$ is a potent inhibitor of GLA; thus, it was proposed that the water-soluble lsyoGb$_3$ formed in one cell can be released into the bloodstream and be taken up by another cell to inhibit the otherwise functional GLA in this cell (Aerts, 2008). Alternatively, lysoGb$_3$ that is taken up may be acylated to Gb$_3$, resulting in a non-lysosomal Gb$_3$ pool that would not be susceptible to degradation by enzyme replacement therapy (Aerts, 2008). These explanations may account for the severity of disease observed in some female heterozygotes.

### 1.16.2 Glucocerebrosidase and GM3 in Parkinson disease (PD)

PD is the second most frequent neurodegenerative disorder (Campbell and Choy, 2012). Recently, a number of studies have reported a link between Gaucher disease and PD. A relationship between the two diseases was first noted by clinical reports of Gaucher disease patients expressing symptoms of PD and subsequent confirmation of GBA mutations in some PD patients (Machaczka et al., 1999; Manning-Boğ et al., 2009; Sidransky et al., 2009). It has been suggested that GBA deficiency itself—and not elevated GlcCer levels—may predispose Gaucher disease patients to PD, as patients harbouring the non-neuronopathic N370S mutation have been diagnosed with PD (Tayebi et al., 2003). In addition, it has been shown that the relatives of some Gaucher disease patients who are carriers of GBA mutations, but asymptomatic for Gaucher disease, also developed PD (Goker-Alpan et al., 2004). Furthermore, analysis of 57 PD subjects revealed GBA mutations in 14%, a frequency greater than that estimated within the Ashkenzai Jewish population (Goker-Alpan et al., 2004). In general, PD patients harbouring GBA mutations suffered from earlier onset of the disease (Sidransky et al., 2009).

While clinical case studies have shown a potential link between the two diseases, determining the biochemical link has been difficult. In an effort to study such a link, Manning-Bog et al. treated cells and mice with the GBA inhibitor, conduritol β epoxide, and detected elevated levels of α-synuclein, a characteristic of PD (Macphee and Stewart, 2012; Manning-Boğ et al., 2009). In fact, α-synuclein actually possesses a GSL binding domain that preferentially binds GM3 (Fantini and Yahi, 2011). The E46K mutation, representing a familial form of PD, has increased affinity for GM3. Wild-type monomeric α-synuclein is disordered and attains an α-helical conformation upon binding to negatively-charged lipids at the cell surface (Zakharov et al., 2007). The E46K mutation increases fibrillization (Greenbaum et al., 2005) resulting in oligomers of the proteins that form channel-like structures upon binding to the membrane (Zakharov et al., 2007). This leads to non-specific leaking of molecules (Zakharov et al., 2007). Although wild-type protein also forms channels in DOPC membranes, these are functionally distinct from the mutant, with the latter forming hyperactive channels (Di Pasquale et al., 2010). The presence of small amounts of GM3, a minor human brain ganglioside, in these membranes reverses channel properties to that of the wild-type (Di Pasquale et al., 2010). Since GBA inhibition—and mutation—facilitates α-synuclein aggregation, it was thought that GlcCer itself may be able to induce fibrillization. In lipid dispersions composed of 75% GlcCer, soluble oligomeric intermediate formation is stabilized by GlcCer, which eventually leads to fibril formation (Mazzulli et al., 2011).

### 1.16.3 Sphingolipids as receptors for bacteria and toxins

Several bacteria and toxins use GSLs as receptors for binding to and invading cells. Cholera toxin (CT) from *Vibrio cholerae* binds to GM1 and fucosyl-GM1 (Yanagisawa et al., 2006;

King and van Heyningen, 1973); verotoxin (VT) from enterohemorrhagic *Escherichia coli* binds to $Gb_3$ (Lingwood et al., 1987); *Hemophilus influenzae* binds to $Gg_3$, $Gg_4$ and SGC (Busse et al., 1997); *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Candida albicans*, *Helicobacter pylori*, *Chlamydia trachomatis* and *Chlamydia pneumoniae* bind to $Gg_3$ and $Gg_4$ (Krivan et al., 1988; Krivan et al., 1991; Lingwood et al., 1993; Yu et al., 1994); *H. Pylori* also binds to LacCer (Chatterjee and Pandey, 2008).

CT is composed of a catalytic A subunit and five receptor-binding B subunits (Lingwood, 2011). GM1 and fucosyl-GM1 serve as the receptor for the B-subunit (Masserini et al., 1992; Van Heyningen, 1974); however, other GSLs have also been reported to be bound by CT, although with lower affinity, including GD1b, GT1b, GM2 and Gg4 (Angstrom et al., 1994; Cumar et al., 1982; Lauer et al., 2002; Yanagisawa et al., 2006). CT binding to GM1 within membrane rafts facilitates internalization of the complex and retrograde transport through the endosomes, TGN, Golgi and into the ER (Lingwood, 2011). Here, the A subunit is separated from the B subunits and is retrotranslocated into the cytosol where it catalyzes the transfer of ADP-ribose to a GTP binding protein family member, leading to a signalling cascade that causes diarrhea (Basset et al., 2010). The trafficking pathway following internalization appears to be specific to GM1, as mutation of CT causing it to specifically bind the ganglioside GD1a does not follow this retrograde pathway (Wolf et al., 1998). In addition, the aglycone of GM1 is important; varying the acyl chain modulates the toxin's effects (Lingwood, 2011).

Similar to CT, VT also contains a single A subunit and five receptor-binding B subunits, which specifically bind the receptor GSL $Gb_3$ (Lingwood, 2011). Receptor binding again facilitates internalization of the VT-$Gb_3$ complex, following the same retrograde pathway as CT-GM1.

Once in the cytosol, the A subunit is able to exert its cytotoxic effects by inactivating protein synthesis. The $Gb_3$ acyl chain length appears to modulate intracellular trafficking of the VT-$Gb_3$ complex (Arab and Lingwood, 1998).

### 1.16.4 GM3 and Diabetes

The primary cause of type 2 diabetes is considered to be insulin resistance (Inokuchi, 2006). Adipose tissue is a major source of TNF-α (Hotamisligil et al., 1995), whose expression is increased in obese mice (Hotamisligil et al., 1993). This cytokine was, therefore, implicated in obesity-induced insulin resistance (Hotamisligil et al., 1993; Inokuchi, 2006). Subsequently, it was identified that TNF-α increases GM3 levels by inducing expression of GM3 synthase (Tagami et al., 2002). GM3 interacts with the insulin receptor, dissociating it from caveolae where it is required for insulin signalling to take place (Kabayama et al., 2007) (For details on the GM3-insulin receptor interaction, see GSL Functions in section 1.14.6). Pharmacological inhibition of GSL synthesis reduces GM3 levels and restores insulin sensitivity in animal models (Inokuchi, 2006; Zhao et al., 2007).

### 1.16.5 Sphingolipids and Cancer

Similar to the role of GM3 in diabetes, GM3 also plays a role in cancer. GM3 interacts with EGFR, thereby preventing EGF stimulation and the downstream signalling cascade that ultimately leads to proliferation and differentiation (Bremer et al., 1986; Kawashima et al., 2009) (For details on this interaction, see GSL Functions in section 1.14.5). Unlike the situation for diabetes, where elevated GM3 adversely affects insulin signalling, higher levels of this GSL

should theoretically prove beneficial in treating the uncontrolled growth characteristic of cancer cells.

Aberrant expression of other sphingolipids also contributes to cancer. Increase in CerS1 and CerS5 increases sensitivity to chemotherapeutic agents (Min et al., 2007). Furthermore, elevated $C_{16:0}$ ceramide and reduced $C_{18:0}$ ceramide have been observed in head and neck cancer, possibly due to reduced expression of CerS1 (Koybasi et al., 2004). Increased ceramide levels have also been detected in breast tumours together with increased CerS2 and CerS6 expression (Erez-Roman et al., 2010; Schiffmann et al., 2009).

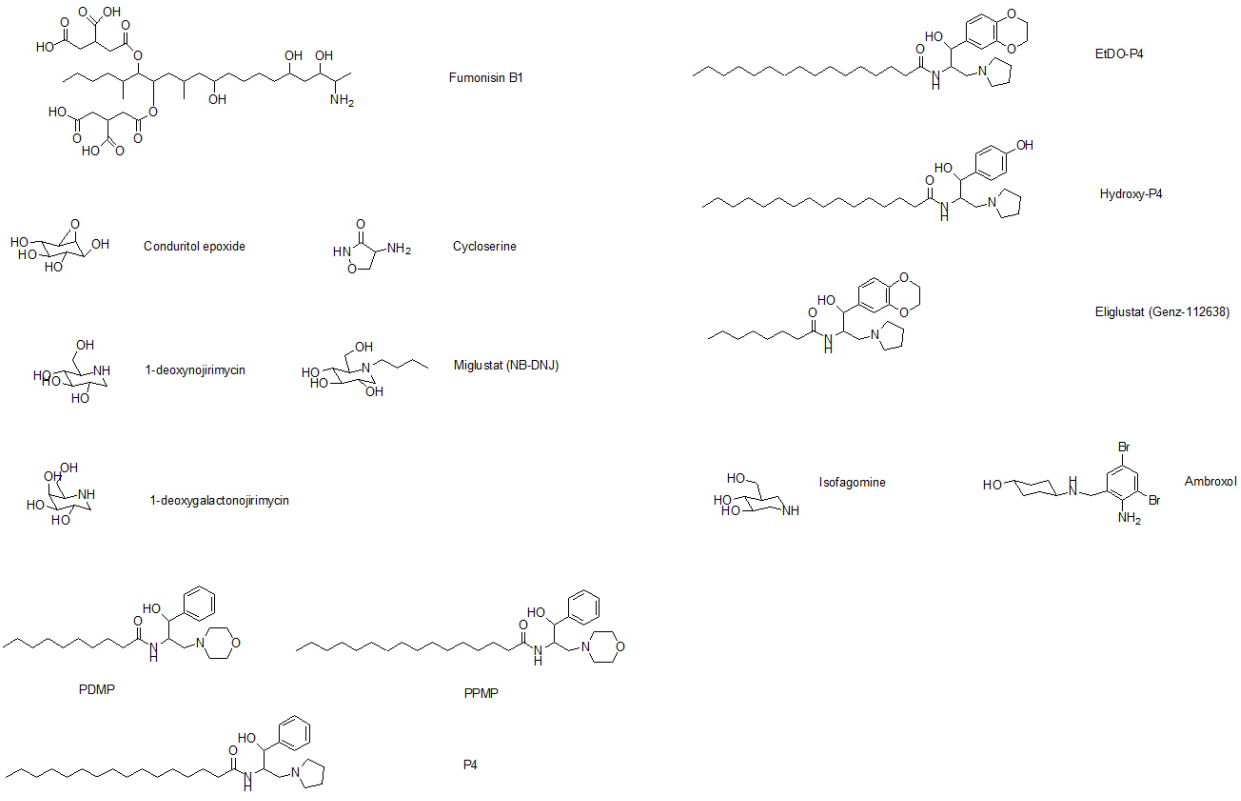
Several other GSLs have been reported to be elevated in different cancers, some of them being labelled as tumour-associated GSL antigens (Hakomori, 1986b); for example: Globo H is overexpressed in epithelial tumours of various cancers (Hakomori, 1986b; Liang et al., 2010); fucosylceramide has been isolated from colon and small cell lung carcinomas (Nilsson et al., 1984; Watanabe et al., 1976); $Gb_3$, fucosyl-GM1 and sulfatide are also found in several tumours (Hakomori, 1986b; Merrill, 2011; Nilsson et al., 1984). As has already been mentioned, GCS contributes to the multidrug resistance phenotype of cancer cells overexpressing P-gp. LacCer and β4GalT5 have also been implicated in a variety of cancers (Chatterjee and Alsaeedi, 2012). Given that β4GalT5 is also involved in N-glycosylation of glycoproteins, a role for LacCer, however, cannot directly be inferred simply by examining levels of its synthetic enzyme.

## *1.17 Inhibitors of GSL Metabolism*

### 1.17.1 General structure

The design of enzyme inhibitors is often based on structural features of the enzyme's natural substrate. Modifications are subsequently made to enhance or reduce the inhibitor's affinity for the enzyme. For metabolic enzymes, a given substrate is usually acted upon by two different enzymes—one anabolic that is involved in its conversion to a more complex molecule, and one catabolic that is responsible for its breakdown. This structure-based inhibitor design approach, therefore, often leads to compounds that inhibit the activities of both synthetic and degradative enzymes. In addition, off-pathway enzymes also have activity towards different molecules. *In vivo*, however, these molecules may not encounter such enzymes.

Active-site inhibitors usually compete with the natural substrate for access to the enzyme. Inhibitors may also covalently bind to the enzyme, rendering the inhibition irreversible. For clinical purposes, however, reversibility of enzyme inhibition is important so as to reduce a given enzyme's activity while still allowing for sufficient enzyme activity to fulfill necessary cellular functions. Modifications of substrate analogues at particular positions usually lead to defined effects on inhibition. For example, removing the C-2 hydroxyl group tends to weaken sugar binding to glycosidases, while removing the C-1 hydroxyl group enhances binding (Dong et al., 1996). Aza sugars—with the ring oxygen replaced by nitrogen—are potent glycosidase inhibitors (Dong et al., 1996). Several inhibitors of sphingolipid metabolism have been identified—those occurring naturally—or chemically synthesized. Some of the common ones are described below, with their chemical structures depicted in Scheme 1.17.




Scheme 1.17. **Inhibitors of GSL metabolism.** The structures of various inhibitors of GSL biosynthesis and catabolism are depicted.

## 1.17.2 Fumonisin B1

Fumonisin B1 (FB1) is a fungal toxin produced by *Fusarium* (Marasas, 2001) that inhibits ceramide synthesis (Wang et al., 1991). Structurally, it is similar to ceramide and it specifically inhibits CerS in a competitive manner (Merrill Jr. et al., 1993). Since FB1 specifically inhibits CerS, the compound is used to delineate between ceramide synthesized *de novo* and that generated by sphingomyelinase and glucocerebrosidase (Mullen et al., 2012; Stiban et al., 2010).

## 1.17.3 Conduritol epoxide (CBE)

CBE is a covalent inhibitor that is selective for glucocerebrosidase (Rempel and Withers, 2008). It does not inhibit activity of GBA2; thus, it can be used to delineate between lysosomal vs non-lysosomal catabolism of GlcCer. CBE-treated cells are also used as a model for Gaucher disease, owing to the accumulation of GlcCer. In fact, CBE treatment of human blood-derived macrophages results in morphological changes resembling Gaucher cells (Yatziv et al., 1988). Furthermore, CBE has been injected into mice to facilitate GlcCer storage and simulate a Gaucher disease model, although the degree of storage was not to the extent of Gaucher disease (Stephens et al., 1978). In this mouse model, the residual glucocerebrosidase activity required to eliminate symptoms was determined to be 12-16%. Knowledge of this threshold enzyme activity is important to design EET agents to achieve this target value.

### 1.17.4 Cycloserine

$_{L}$-cycloserine irreversibly inhibits the first enzyme in *de novo* sphingolipid synthesis, serine palmitoyltransferase (Ikushiro et al., 2004). By inhibiting the first step in GSL biosynthesis, not only is GSL synthesis prevented, but synthesis of ceramide is also blocked. The enantiomer of $_{L}$-cycloserine, $_{D}$-cycloserine, is a natural compound found in *Streptomyces* and is used to treat *Mycobacterium tuberculosis* infections (Lowther et al., 2010). $_{L}$-cycloserine is not found naturally and must be synthesized.

### 1.17.5 Deoxynojirimycin (DNJ)

1-DNJ (and N-butyl DNJ) is an imino sugar—containing a ring nitrogen as the heteroatom rather than oxygen—that was shown to inhibit activity of the ER α-glucosidase I and II responsible for N-linked oligosaccharide processing (Saunier et al., 1982). In examining other

effects of N-butyl DNJ, Platt et al. discovered the inhibitory effect of the compound on GSL biosynthesis by inhibiting GCS (Platt et al., 1994a). In addition, NB-DNJ proved to be a more potent inhibitor of GSL biosynthesis (50 μM) than the ER glucosidases (mM range) (Platt et al., 1994a). Interestingly, *N*-methyl-DNJ did not inhibit GSL synthesis, while *N*-hexyl-DNJ was also inhibitory, suggesting the importance of the alkyl chain length. NB-DNJ is currently being used clinically for Gaucher disease therapy under the name Zavesca ® (Actelion Pharmaceuticals).

In an effort to design similar compounds that retain GSL synthesis inhibition but without an effect on ER glucosidases, the galactose analogue of DNJ, *N*-butlydeoxygalactonojirimycin (NB-DGJ) was synthesized (Platt et al., 1994b). NB-DGJ inhibition of GSL synthesis was comparable to its glucose-based counterpart, while the fucose and N-acetylglucosamine analogues did not have an effect. Furthermore, NB-DGJ does not inhibit the ER glucosidases or glucocerebrosidase, with the latter being moderately inhibited by NB-DNJ (Platt et al., 1994b).

### 1.17.6 1-Deoxygalactonojirimycin (1-DGJ)

Interestingly 1-DGJ, which lacks the alkyl chain in NB-DGJ, is actually a potent inhibitor of α-galactosidase A (Fan et al., 1999). 1-DGJ markedly increases activity of mutant GLA from Fabry disease patients. Binding of the compound to mutant GLA allows the protein to overcome its trafficking defect—likely misfolding—by serving as a pharmacological chaperone, facilitating maturation of the enzyme and thereby allowing it to be trafficked to the lysosome (Fan et al., 1999).

### 1.17.7 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP)

PDMP was the first GCS inhibitor developed (Rao Vunnam and Radin, 1980; Wennekes et al., 2009). It was synthesized as a ceramide analogue following findings that substitution of the long alkene portion of sphingosine with a benzene ring, and shortening the acyl chain length are inhibitory towards GCS (Rao Vunnam and Radin, 1980). While PDMP strongly inhibits GlcCer synthesis, it also causes ceramide accumulation independent of GCS inhibition (Abe et al., 1995). Subsequent improvements on this compound were, therefore, developed by extending the acyl chain (PPMP) (Abe et al., 1995), switching the heterocyclic group to a pyrrolidino ring (P4) (Abe et al., 1995), adding an ethylenedioxy group to the benzene ring (EtDO-P4) (Lee et al., 1999), shortening the acyl chain (GENZ-112638) (McEachern et al., 2007) or replacing the ethylenedioxy group with a hydroxy functionality (Lee et al., 1999). The newer generation PDMP analogues do not raise ceramide levels (Lee et al., 1999). With these newer generation compounds being more potent and specific inhibitors of GCS, they may serve as effective agents to reduce accumulation of GlcCer (or downstream GSLs).

### 1.17.8 Isofagomine (IFG)

IFG is a glucose analogue in which the ring oxygen is replaced by a carbon and the anomeric carbon with a nitrogen (Jespersen et al., 1994). The compound was designed by Jespersen et al. as a transition state analogue, with the positive charge on the nitrogen resembling the carbocation transition state resulting from glycosidic bond cleavage (Jespersen et al., 1994). The compound strongly inhibits glucocerebrosidase, with an inhibition constant $K_i$ of approximately 0.1 μM (Dong et al., 1996). Given that lack of a C-2 hydroxyl is expected to weaken binding to the enzyme, the observed increase is likely due to the introduction of nitrogen at C-1 (Dong et al., 1996). Dong et al. suggest that the strong inhibition of

glucocerebrosidase is likely due to electrostatic interaction between the positive charge generated at the nitrogen and the carboxyl groups in the enzyme's active site that are situated close to the anomeric position of the substrate.

Treatment of N370S Gaucher disease fibroblasts with IFG increases mutant glucocerebrosidase expression and activity approximately 2.5- and 3-fold, respectively (Steet et al., 2006). IFG is capable of inhibiting both N370S and wild-type glucocerebrosidase at both neutral and acidic pH and the amount of lysosomal N370S is also elevated. Thus, IFG likely binds to the mutant enzyme within the ER, allowing it to achieve a more native conformation to exit the ER and reach its destination in the lysosome. Removing IFG from culture medium has shown the enzyme to be stable for up to 72 h (Steet et al., 2006). Further, since IFG is a reversible inhibitor of glucocerebrosidase, removal from the medium results in rapid recovery of enzyme activity (Steet et al., 2006). Based on these results, IFG reached phase II clinical trials under the name Plicera (Amicus Therapeutics).

### 1.17.9 Ambroxol (ABX)

ABX is a United States Food and Drug Administration (FDA) approved drug for the treatment of airway mucous hypersecretion that acts by breaking down the mucous (Li et al., 2011). ABX is also capable of stabilizing mutant glucocerebrosidase as well as increasing its activity (Maegawa et al., 2009). ABX has just entered clinical trials for the treatment of Type I Gaucher disease.

## *1.18 Therapeutic Options for Sphingolipidoses*

The sphingolipidoses result in accumulation of lipid molecules. Since only a slight increase in catabolic enzyme activity is required to alleviate clinical symptoms (Jmoudiak and Futerman, 2005), therapeutic approaches can be designed with two basic objectives: (1) increase the amount or residual activity of the target lysosomal enzyme. (2) decrease synthesis of the stored molecule. With these two goals in mind, a number of different therapeutic approaches have been designed, some of which are currently in clinical use.

### 1.18.1 Enzyme Replacement Therapy

Enzyme replacement therapy involves injecting patients with recombinant enzyme to compensate for defective enzyme. Cerezyme ® (Genzyme Corporation) is currently being used to treat Gaucher disease patients. While effective at reversing several disease symptoms, such as reducing the size of enlarged organs, relieving bone pain and increasing platelet counts (Grabowski, 2008; Grabowski et al., 2009), ERT suffers from the exorbitant costs to patients, which often exceed $100,000 annually. In addition, this approach suffers from the inability to cross the blood-brain-barrier, rendering it incapable to treat neurological symptoms. A short circulation half-life also limits its impact, necessitating continual infusions (Futerman et al., 2004; Kacher et al., 2008).

ERT for Gaucher disease was first proposed by Brady in 1966, and it was shown in 1974 that glucocerebrosidase purified from placenta and delivered intravenously could substantially reduce GlcCer levels (Barton et al., 1991). Over the years, ERT for Gaucher disease has seen three different enzyme preparations approved by the FDA: ceredase (alglucerase, Genzyme), cerezyme (imiglucerase, Genzyme) and Vpriv (velaglucerase alfa, Shire).

Ceredase, approved by the FDA in 1991, was originally developed by extracting glucocerebrosidase from placenta (Aerts et al., 2010). In order to target the enzyme to macrophages, which express large amounts of mannose receptors at the cell surface, the enzyme was processed to expose mannose chains (Aerts et al., 2010). Cerezyme was FDA-approved in 1994 (Aerts et al., 2010). It is a recombinant glucocerebrosidase analogue produced in Chinese hamster ovary (CHO) cells (Brumshtein et al., 2010). After purifying the enzyme, it is treated with exo-glycosidase to expose core mannose chains for macrophage targeting (Brumshtein et al., 2010). Vpriv was approved by the FDA in 2010 (Aerts et al., 2010). Vpriv is human glucocerebrosidase produced by Shire's proprietary Gene-Activation ® technology, which involves introduction of a promoter by recombination upstream of the glucocerebrosidase gene (Zimran et al., 2007). This promoter activates glucocerebrosidase expression in the target human fibroblast cell line (Aerts et al., 2010; Zimran et al., 2007). The mannosidase I inhibitor kifunensine is used during cell culture, resulting in secretion of an enzyme that mainly contains mannose residues (Brumshtein et al., 2010). Vpriv contains longer chain high-mannose glycans than cerezyme, leading to increased uptake (Brumshtein et al., 2010). Clinical trials have revealed the enzyme to be well-tolerated and to exert similar effects to cerezyme (Zimran et al., 2007). This technology has also been used to produce recombinant enzyme for Fabry disease and Mucopolysaccharidosis II (Zimran et al., 2007).

Replagal (agalsidase alfa, Shire) and Fabrazyme (agalsidase beta, Genzyme) are two recombinant GLA products used for ERT in Fabry disease patients. The two treatments differ in the cell line used for production, leading to variations in their N-linked glycans (Garman and Garboczi, 2004); Replagal is produced in a human cell line, while Fabrazyme is produced in CHO cells. Replagal is produced by Shire's proprietary gene activation technology in which a

regulatory DNA sequence is introduced into a human cell line upstream of the GLA gene, thereby activating the gene for protein production. One of the problems associated with these treatments is the development of neutralizing antibodies against the enzymes in patients receiving ERT, thereby preventing efficient $Gb_3$ catabolism (Linthorst et al., 2004).

### 1.18.2 Substrate Reduction Therapy (SRT)

Since cellular pathology and manifestation of clinical symptoms is caused by accumulation of sphingolipids, preventing their synthesis may also alleviate symptoms (van Gelder et al., 2012). As such, SRT makes use of small molecule inhibitors of particular enzymes within the GSL metabolic pathway to block GSL synthesis, while at the same time, having little or no affect on catabolic enzymes. This is often a difficult task to achieve, as inhibitors are typically structural analogues of natural enzyme substrates, rendering them accessible by both synthetic and degradative enzymes. Slight modifications of molecules are usually necessary to optimize the relative affinities for the opposing enzymes. Small molecule inhibitors also frequently have off-target effects by inhibiting proteins in different pathways (Belmatoug et al., 2011).

N-butyldeoxynojirimycin (NB-DNJ, miglustat, Zavesca ®) was the first SRT agent approved for use in Type I Gaucher disease in 2003 (Ficicioglu, 2008). In addition to decreases in GlcCer levels, treatment has shown reduction in liver and spleen volumes and increases in platelet counts (Elstein et al., 2004; Pastores et al., 2005). Side effects include diarrhea and weight loss, but these subsided in the second and third years of treatment (Elstein et al., 2004). In addition, Zavesca ® affects spermatogenesis (Futerman et al., 2004). Although still expensive, the advantages of SRT include oral administration and the ability of molecules to potentially cross the BBB (Futerman et al., 2004). Since NB-DNJ inhibits the first step in glucose-based GSL

synthesis, it also prevents synthesis of all glucose-based GSLs, which may have deleterious effects over the long-term.

### 1.18.3 Enzyme Enhancement Therapy

It has been shown that GSLs accumulate when catabolic enzyme activity falls below a threshold value (Butters et al., 2005). Thus, improving activity above this critical value may ameliorate the disease phenotype (Fan, 2008). As part of EET, small molecules (or substrate analogues) serve as pharmacological chaperones of a misfolded protein whose catalytic centre is intact, allowing for a more stable conformation that can pass the ER quality control mechanisms and continue along the secretory pathway to reach its appropriate destination in the lysosome (Fan et al., 1999). Thus, the pharmacological chaperone must have a strong enough affinity to the enzyme allowing for binding while at the same time an affinity that is not so strong to prevent subsequent binding of the target substrate. EET is attractive since only a small increase in residual enzyme activity is required to ameliorate the LSD phenotype (Fan et al., 1999). Since the EET agent must be able to bind to the enzyme, reversible inhibitors of the enzyme are often used.

The idea of using enzyme inhibitors in a counterintuitive manner as pharmacological chaperones to facilitate folding of mutant misfolded enzymes was first put into effect by Fan et al (Fan et al., 1999). The group used 1-deoxygalactonojirimycin (1-DGJ), known to be a potent inhibitor of α-galactosidase A, to enhance activity of the enzyme in Fabry lymphoblasts. At sub-inhibitory concentrations, the compound increased mutant enzyme expression in lysosomes, without altering mRNA levels (Fan et al., 1999). 1-DGJ (migalastat) is currently in phase III clinical trials for treatment of Fabry disease under the name Amigal (TM) (Amicus

Therapeutics, GlaxosmithKline). Similarly, IFG (Plicera, Amicus Therapeutics, Shire HGT) reached phase II clinical trials as a pharamacological chaperone for glucocerebrosidase in Gaucher disease (Zimran, 2011). Exr-202 (Ambroxol, ExSAR Corporation) is another EET agent that has recently entered clinical trials.

The large numbers of different mutations that can typically cause a protein to misfold render EET difficult to address each of these mutants. This is due to the small molecule chaperones being designed for particular mutants. In addition, this approach will be ineffective against null mutants. EET has shown promise in clinical trials for Gaucher disease, Fabry disease and GM2 gangliosidoses (Valenzano et al., 2011).

As an alternative approach that should be independent of the misfolding mutation, our lab has targeted the retrotranslocon as a means of reducing degradation of mutant enzymes (Manuscript in preparation). We have inactivated cholera toxin or verotoxin and inserted 9 to 18 leucines at the N-terminus. Following binding of the mutant toxin to its cell surface receptor—GM1 and $Gb_3$ respectively for cholera toxin and verotoxin—and retrograde transport, the added leucines will serve to slow down migration through the translocon (sec61, derlins). Thus, the exit site for mutant enzyme en route to degradation by ERAD is blocked, allowing for increased opportunity to achieve an acceptable conformation for transport to the Golgi. We have shown this to be effective in the case of glucocerebrosidase in homozygous N370S Gaucher disease fibroblasts and HeLa cells transfected with ΔF508 CFTR. Treatment by this method would have to be pulsed in such a way to prevent toxic accumulation of misfolded proteins, as many proteins are known to be misfolded and degraded by ERAD as a normal quality control mechanism (Vembar and Brodsky, 2008).

### 1.18.3.1 Endoplasmic Reticulum Associated Degradation (ERAD)

Protein folding is prone to errors, with proteins being able to populate partially folded intermediate states that may lead towards the native state or may trap the polypeptide and prevent acquisition of native conformation (Jahn and Radford, 2005; Vembar and Brodsky, 2008). In fact, it has been estimated that 30 % of wild-type proteins misfold and are degraded by the ER quality control system (Fan, 2008). To ensure a cell's proteins are synthesized in the appropriately folded form as required by the cell, proteins must pass the ER quality control system (Vembar and Brodsky, 2008). Failure to meet the conditions necessitated by the cell leads to degradation of the protein. One aspect of this quality control system is endoplasmic reticulum associated degradation (ERAD), which is the pathway for degradation of terminally misfolded proteins. ERAD is part of the cell's quality control mechanisms, preventing toxic accumulation of misfolded proteins.

When a protein is unable to achieve its correct folded state, hydrophobic regions that are normally buried in the core may become exposed (Vembar and Brodsky, 2008). Molecular chaperones bind to these regions to prevent polypeptide aggregation, giving the nascent polypeptide time to fold (Vembar and Brodsky, 2008). In the case of glycoproteins, translocation of the nascent polypeptide into the ER through the translocon coincides with addition of the N-linked oligosaccharide $GlcNAc_2$-$Man_9$-$Glc_3$ (GlcNAc- N-acetylglucosamine; Man- mannose; Glc; glucose) (Vembar and Brodsky, 2008). Glucosidase I and II then remove the two terminal glucose residues. The resulting monoglucosylated substrate is recognized by the ER lectin-like chaperones calreticulin and calnexin, allowing the protein to fold (Vembar and Brodsky, 2008). The remaining glucose is then cleaved by glucosidase II, facilitating

dissociation from the chaperones and vesicular transport to the Golgi (Vembar and Brodsky, 2008).

Polypeptides that do not acquire the appropriate folded state are re-glucosylated by UDP-glucose:glycoprotein glucosyltransferase (UGGT), facilitating re-entry into the calnexin cycle (Vembar and Brodsky, 2008). In the case of terminally misfolded polypeptides, ER α-mannosidase I cleaves the terminal mannose to form $GlcNAc_2$-$Man_8$, which may be recognized by other ERAD targeting proteins, such as ER degradation enhancing α-mannosidase-like lectin (EDEM) (Hoseki et al., 2010). Substrates are then targeted to the retrotranslocon. Substrates are retrotranslocated into the cytoplasm, ubiquitylated and targeted for degradation by the proteasome (Vembar and Brodsky, 2008).

### 1.18.4 Gene Therapy

Recently, with advances in molecular biology tools and techniques, gene therapy has garnered much attention. This approach involves supplying cells with a functional copy of the defective gene. Cells must take up the supplied vector and stably express the target protein. Since the lysosomal hydrolases are expressed ubiquitously, gene therapy of LSDs would allow all tissues to be treated simultaneously (Tomanin et al., 2012). Importantly, newly synthesized enzyme does not exclusively traffic to the lysosome; rather, some is also secreted into the circulation, thereby allowing other cells to take up enzyme via the cell-surface mannose-phosphate receptor (Tomanin et al., 2012). Retroviral and lentiviral vectors are typically used to transduce the target gene into cells. Gene therapy to deliver GBA DNA has successfully reversed disease abnormalities in a Gaucher disease mouse model (McEachern et al., 2006). It is thought that gene therapy will be effective for treatment of neuronopathic symptoms—direct injection into

the brain (not the preferred route) or CNS penetration using hematopoietic stem cells transduced with lentiviral vectors *ex vivo*—but efficient delivery to the brain systemically remains an obstacle (Biffi et al., 2004; Kolter and Sandhoff, 2006; Tomanin et al., 2012). In one clinical study, gene therapy was used to administer ceroid lipofuscinosis, neuronal 2 (CLN2) cDNA to ten children suffering from late infantile neuronal ceroid lipofuscinosis (LINCL) (Worgall et al., 2008). The study revealed significant reduction in the rate of neurological decline relative to the control group.

# Chapter 2: Rationale and Objectives

The ability to specifically regulate GSL metabolism is both of biological and clinical importance. By interfering with GSL metabolism at particular steps, thereby preventing synthesis of GSLs downstream of these steps, we can examine the functional importance of GSLs no longer being synthesized. In addition, we are able to assess for compensatory mechanisms that may be activated in response to accumulation or depletion of particular GSLs. Clinically, GSLs are relevant in various diseases, some of which have been described in Section 1.16. Thus, the development of strategies to exogenously manipulate GSL metabolism is of great importance.

Unfortunately, GSL biosynthesis is not as clear-cut as a single substrate encountering one enzyme for the formation of a single product; rather, the GSL repertoire is very diverse, and a given enzyme can use as substrates GSLs with differences in the aglycone and sometimes even in the carbohydrate (Kojima et al., 2000; Merrill, 2011). Moreover, LacCer is a substrate for several different biosynthetic enzymes, leading to the formation of the various GSL sub-classes (Pontier and Schweisguth, 2012). As described in the Introduction, it is not clear what determines the preferred metabolic path for LacCer.

The process by which complex GSL synthesis is initiated also poses a problem: the first glucose-based GSL, GlcCer, is synthesized on the cytosolic leaflet of the Golgi; however, LacCer synthase and downstream glycosyltransferases have their catalytic activities oriented on the lumenal side of the Golgi. Thus, a mechanism must exist to allow GlcCer access to the Golgi lumen. As outlined previously, two predominant theories exist: one involving direct translocation via the ABC transporter, P-gp, and the other involving GlcCer retrograde transport

to the ER, followed by entry into the secretory pathway. It is not known if one pathway functions at the expense of the other, if they are both involved within the same pathway, if they act within distinct pathways or if additional proteins are also involved.

### *Specific Aims*

To address some of these problems, the body of work described in this thesis is divided into three chapters with a central theme: identifying new approaches to selectively regulate GSL metabolism. We have synthesized analogues of GSLs by substituting the acyl chain with an adamantane frame. The resulting adaGSLs show increased water solubility, while retaining membrane solubility and receptor function (Mylvaganam and Lingwood, 1999). The characterization of two analogues of monohexosyl ceramides, adamantyl glucosylceramide (adaGlcCer) and adamantyl galactosylceramide (adaGalCer), is described in chapter 3. These compounds are evaluated for their utility as therapeutic reagents by monitoring their ability to correct defects in LSDs.

In order to clearly define the role of ABCB1 *in vivo*, we describe a new mouse model using the Fabry mouse, which accumulates $Gb_3$, and crossbreeding it with the ABCB1 knockout mouse. By analysing the tissue GSL, and particularly $Gb_3$, levels in Fabry/ABCB1 offspring, a role for ABCB1 is GSL biosynthesis can more definitively be assigned. Within the same context, in chapter 5, a gene silencing study is described in which expression of ABCB1 and FAPP2 in cells is transiently knocked down by siRNA treatment. The aim of this study is to evaluate the relative contributions of these two proteins to GSL biosynthesis. In addition, other candidate proteins are described that may be involved in GlcCer Golgi lumenal access.

# Chapter 3: Adamantyl glycosphingolipids provide a new approach to the selective regulation of cellular glycosphingolipid metabolism.

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Adamantyl glycosphingolipids provide a new approach to the selective regulation of cellular glycosphingolipid metabolism.

**Mustafa Kamani, Murugespillai Mylvaganam, Robert Tian, Brigitte Rigat, Beth Binnington and Clifford Lingwood**

## *3.1 Abstract:*

Mammalian glycosphingolipid (GSL) precursor monohexosyl ceramides are either glucosyl or galactosyl ceramide (GlcCer, GalCer). Most GSLs derive from GlcCer. Substitution of the GSL fatty acid with adamantane generates amphipathic mimics of increased water solubility, while retaining receptor function. We have synthesized adamantylGlcCer (adaGlcCer) and adamantylGalCer (adaGalCer). AdaGlcCer and adaGalCer partition into cells to alter GSL metabolism. At low dose, adaGlcCer increased cellular GSLs by inhibition of glucocerebrosidase (GCC). Recombinant GCC was inhibited at pH 7 but not 5. In contrast, adaGalCer stimulated GCC at pH 5 but not 7, and like adaGlcCer, corrected N370S mutant GCC traffic from the endoplasmic reticulum to lysosomes. AdaGalCer reduced GlcCer levels in normal and lysosomal storage disease (LSD) cells. At 40 µM adaGlcCer, lactosylceramide (LacCer) synthase inhibition depleted LacCer (and more complex GSLs), such that only GlcCer remained. In Vero cell microsomes, 40 µM adaGlcCer was converted to adaLacCer and LacCer synthesis was inhibited. AdaGlcCer is the first cell LacCer synthase inhibitor. At 40 µM adaGalCer, cell synthesis of only $Gb_3$ and $Gb_4$ was significantly reduced and a novel product, adamantyl digalactosylceramide ($adaGb_2$) was generated, indicating substrate competition for $Gb_3$ synthase. AdaGalCer also inhibited cell sulfatide synthesis and microsomal $Gb_3$ synthesis. Metabolic labeling of $Gb_3$ in Fabry LSD cells was selectively reduced by adaGalCer and $adaGb_2$ was produced. $AdaGb_2$ in cells was 10-fold more effectively shed into the extracellular medium than the more polar $Gb_3$, providing an easily eliminated “safety valve” alternative to $Gb_3$

accumulation. Adamantyl monohexosyl ceramides thus provide new tools to selectively manipulate normal cellular GSL metabolism and reduce GSL accumulation in cells from LSD patients.

## *3.2 Introduction:*

Glycosphingolipid (GSL) homeostasis is essential to ensure cellular integrity. GSLs have important functional roles in cell signaling pathways, membrane transport and cell recognition via intercellular interactions (Hakomori and Igarashi, 1995; Sillence, 2007). GSLs can modulate membrane receptor function (Nishio et al., 2004; Yoon et al., 2006), are altered in cancer (Bieberich, 2004; Kovbasnjuk et al., 2005) and cell growth (Chatterjee, 2008), and provide a primary target for host cell interaction with microbial pathogens (Lloyd et al., 2007). In addition, GSL accumulation has been identified to contribute to the pathology of many other diseases, including Parkinson disease (Manning-Boğ et al., 2009; Velayati et al., 2010), Alzheimer's disease (Yahi et al., 2010), type 2 diabetes (Tagami et al., 2002; Yew et al., 2010), growth factor dysregulation (Kawashima et al., 2009), polycystic kidney disease (Natoli et al., 2010), atherosclerosis (Bietrix et al., 2010) and cystic fibrosis (Norez et al., 2009). However, most significantly, defects in GSL catabolism lead to toxic accumulation of GSL substrates and the GSL lysosomal storage disease (LSD) pathologies, a subclass of disorders within the 40 known LSDs (Heese, 2008).

GSL metabolism comprises a complex, branched network of transferases, hydrolases, activators and trafficking, rendering the process difficult to manipulate selectively. Current therapeutic approaches for LSDs include enzyme replacement therapy (ERT), enzyme enhancement therapy (EET) and substrate reduction therapy (SRT) (Heese, 2008). ERT uses a recombinant enzyme

to compensate for the defective enzyme (Van Der Ploeg et al., 1991). Although effective in reversing some clinical symptoms, such as organomegaly (Barton et al., 1991), it is not economically sustainable, with an annual cost over $100,000 per patient (Burrow et al., 2007). The recombinant enzyme also cannot cross the blood-brain-barrier, rendering this approach ineffective against neurological symptoms (Heese, 2008; Hoffmann and Mayatepek, 2005).

EET uses small molecules to stabilize a more native conformation of partially misfolded mutant proteins, to avoid endoplasmic reticulum associated degradation (ERAD) and allow traffic to the lysosome (Fan, 2008). One well-characterized pharmacological chaperone that reached phase II clinical trials is isofagomine, an inhibitor of glucocerebrosidase (β-glucosylceramidase, GCC) that rescues enzyme activity in Gaucher disease (GD) cells, which suffer from glucosylceramide (GlcCer) accumulation due to defective catabolism (Steet et al., 2006; Zimran, 2011). The utility of this approach extends only to misfolding mutants, and given the many mutations that may compromise folding, many different agents may be required (Hruska et al., 2008; Pastores and Lien, 2002).

SRT involves inhibition of GlcCer synthase to reduce the total GSL content (Fan, 2008). Its utility extends to LSDs involving neurological symptoms, as the inhibitors are able to cross the blood-brain-barrier. However, SRT is subject to unwanted side effects, especially at the more effective higher doses, where additional inhibition of glucosidases may occur (Butters et al., 2005). N-butyl-deoxynojirimycin is an SRT agent approved for use by GD patients and has been shown to be effective in reversing organomegaly, but suffers from low efficacy (Cox et al., 2000; Futerman and Hannun, 2004). Other inhibitors of GlcCer synthase include 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (Radin et al., 1993) and subsequent

improvements with enhanced selectivity (Abe et al., 1992; Lee et al., 1999). These inhibitors are non-carbohydrate mimics of GlcCer and the most recent derivative shows much promise for the selective depletion of all GSLs (McEachern et al., 2007).

Inhibition of GlcCer synthase offers the opportunity to modulate the other disease processes in which GSLs play a role. However, such inhibition depletes cells of all GSLs, not just the offending accumulated species. Approaches which more selectively deplete a single, or subset, of GSLs may avoid side effects resulting from total GSL ablation. As a first step towards this goal, we have made GSL inhibitors to selectively target the two key precursor GSLs of GSL biosynthesis. We have previously made amphipathic analogues of GSLs by substituting the fatty acid moiety with an adamantane frame. These adamantyl GSLs (adaGSLs) show a major increase in water solubility, while retaining receptor function of the parent GSL in solution (Lingwood et al., 2006). Adamantyl$Gb_3$, a soluble mimic of globotriaosylceramide, $Gb_3$ (Mylvaganam and Lingwood, 1999), preferentially partitions into aqueous phase, as opposed to organic solvents, which completely sequester $Gb_3$. Unlike the water soluble, lipid-free $Gb_3$ oligosaccharide, ada$Gb_3$ retains high affinity verotoxin (VT) binding in aqueous solution (Mylvaganam and Lingwood, 1999) and indeed shares some properties of $Gb_3$-cholesterol complexes in solution (Mahfoud et al., 2002), which may relate to its several bioactivities (De Rosa et al., 2008; Lund et al., 2006). Exchange of the GSL fatty acid moiety for adamantane also proved effective to generate a water soluble, ligand binding, bioactive mimic of sulfogalactosylceramide (sulfatide) (Mamelak et al., 2001; Park et al., 2009; Whetstone and Lingwood, 2003), suggesting that this could represent a general method for the generation of soluble bioactive GSL mimics. AdaGSLs are amphipathic mimics which retain receptor

function (Lingwood et al., 2006) and are taken up by living cells (Park et al., 2009). We investigated whether adaGSLs can partition into cells and alter cellular GSL metabolism.

GSL biosynthesis is essentially based on the synthesis of two precursor GSLs, GlcCer, upon which 90% of GSLs are based, and galactosylceramide (GalCer) (Van Meer and Holthuis, 2000). In this study, we characterize two GSL analogues— adamantyl glucosylceramide (adaGlcCer) and adamantyl galactosylceramide (adaGalCer)—in terms of their effects on cellular GSL metabolism. These analogues serve as inhibitors and alternative substrates to redirect cellular GSL metabolism in a selective, targeted manner and are a new means to control aberrant GSL turnover. These, and similar, adaGSLs provide an approach to manipulate cellular GSL metabolism for the selective depletion of a single GSL, or GSL series.

## *3.3 Experimental Procedures*

### 3.3.1 Reagents

Sodium hydroxide, 1-adamantaneacetic acid, dimethylformamide (DMF), triethylamine (TEA), dichloromethane (DCM), coldwater fish gelatin, 4-chloro-1-naphthol, $MgCl_2$, dithiothreitol, sucrose, $MnCl_2$, 4-methylumbelliferyl β-D-glucopyranoside, resorcinol, DMSO, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), saponin were all obtained from Sigma-Aldrich. UDP-[$^3$H]-Galactose and $^{14}$C-Galactose were purchased from American Radiolabeled Chemicals, Inc. Horse-raddish peroxidase (HRP)-conjugated goat anti-mouse and goat-anti-rabbit antibodies were from Bio-Rad Laboratories. DAPI, Alexa fluor 488 chicken anti-rabbit IgG for GCC and Alexa fluor 594 goat anti-mouse IgG for LAMP-1 or PDI were obtained from Molecular Probes.

Methanol, $CHCl_3$, silica and Tris base were obtained from Caledon Laboratory Chemicals. All tissue culture reagents: HEPES, 1X RPMI, Eagle's minimal essential medium (EMEM), Dulbecco's MEM (DMEM)/F12, Alpha modification of EMEM (AMEM), fetal bovine serum (FBS), penicillin/streptomycin, D-PBS, normal goat serum were purchased from Wisent, Inc. Verotoxin (VT1) was purified as described previously (Lingwood et al., 2006; Petric et al., 1987). The LAMP-1 antibody was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

The following reagents were purchased from the source indicated in parentheses: HCl (Fisher Scientific), $C_{18}$ Sep-pak (Waters), Thin-layer chromatography (TLC) plates (Macherey-Nagel Inc.), 30% $H_2O_2$ (EM Science), bovine buttermilk GlcCer (Matreya, LLC), $NH_4OAc$ (BioShop Canada, Inc.), conduritol β epoxide (Toronto Research Chemicals), paraformaldehyde (EMS), mouse monoclonal anti-rat PDI (Stressgen Bioreagents).

### 3.3.2 Adamantyl glycosphingolipid synthesis

20 mg of bovine buttermilk glucosylceramide was dried and incubated in a $P_2O_5$ chamber overnight. GlcCer was deacylated in 10 mL 1.0 M NaOH (methanolic) at 72°C over four days to form lysoGlcCer. The reaction mixture was neutralized with 5-6 M $HCl_{(aq)}$. Solvent was removed on a rotary evaporator to a thick, syrupy liquid, which was dissolved in water and passed through a $C_{18}$ column to de-salt. LysoGlcCer was eluted with methanol and then applied to a silica column in $CHCl_3/CH_3OH$ (98:2, v/v). LysoGlcCer was eluted with $CHCl_3/CH_3OH/H_2O$ (90:10:0.5, v/v/v).

A 0.5 M solution of 1-adamantaneacetic acid and a 0.25 M solution of BOP were prepared in dimethylformamide/dichloromethane/triethylamine (5:5:1, v/v/v). LysoGlcCer, 20 mg (43.4 µmol; 2.17 µmol/mg) was re-dissolved in 0.5 mL 5:5:1. 130 µmol of 0.5 M 1-adamantaneacetic acid was added to 1.5 mL 5:5:1 and the tube was cooled to -70°C in a dry ice/ethanol bath. To this was added 108 µmol BOP. After incubating for 10 min at -70°C, lysoGlcCer was added to the reaction tube and the reaction was allowed to proceed at -70°C for 1.5 h. The reaction was stopped by raising to room temperature, followed by the addition of 10 mL $H_2O$. DCM was removed under a stream of nitrogen. Product mixture was de-salted by $C_{18}$ chromatography and purified by silica column. AdaGlcCer eluted with 90:10:0.5 ($CHCl_3$:$CH_3OH$:$H_2O$). The reaction scheme was identical for synthesis of adaGalCer.

### 3.3.3 Mass spectroscopic analysis

Mass spectra were recorded on a $QSTAR_{XL}$ spectrometer with an o-MALDI source (ABI/MDS Sciex, Concord, ON). Sample was mixed with an equal volume of DHB and was spotted on a MALDI-plate. Compounds were dissolved in $CH_3OH$-NaCl solution that was prepared by adding a saturated solution of $NaCl_{(aq)}$ to LC-MS Chromasolv ® methanol.

### 3.3.4 Cell culture

Normal, Fabry disease (FD) and GD patient lymphoblasts and skin fibroblasts (kindly provided by Dr D.Mahuran (Research Institute, The Hospital for Sick Children)) were maintained in 1X RPMI 1640 medium supplemented with 10 or 15% FBS and 1 µg/mL penicillin/streptomycin. For cell treatments, cells were grown in medium containing 10, 20 or 40 µM adaGSL or 2 µM D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4) for three or four days. For

untreated cells, ethanol was added to the medium, up to the highest volume of adamantyl analogue used, for a final concentration of 0.4% (v/v). Vero (African green monkey kidney epithelial) and Daoy (human medulloblastoma) cells were maintained in Eagle's mnimal essential medium (EMEM), supplemented with 5% FBS and antibiotics (as above). BHK (hamster kidney) cells were cultured in DMEM/F12, supplemented with 5% FBS. Normal and FD fibroblasts were cultured in α-modification of EMEM (AMEM), supplemented with 10% FBS and antibiotics (as above). For metabolic labeling studies, treatments were for three days, with the addition of 1.5 μCi [$^{14}$C]-galactose, fresh adaGSL and/or fresh medium on day 3 for 24 h. Alternatively, cells were pre-treated for 3 h, followed by culture with fresh analogue and [$^{14}$C]-galactose overnight.

### 3.3.5 GSL Extraction

Adherent cells were washed, trypsinized, counted, and then pelleted at 1200 x *g* for 9 min and washed in PBS. Total cellular lipids were extracted in $CHCl_3/CH_3OH$ (2:1, v/v) with shaking for 3 h or 24 h. For total GSL preparations, extracts were passed through glass wool to filter cellular debris, and dried under nitrogen. For neutral GSLs, a Folch partition ($CHCl_3/CH_3OH/H_2O$, 2:1:0.6) of the extracts was performed and the lower phase was dried under nitrogen. Phospholipids were saponified with 0.5 mL of 0.5 M methanolic NaOH for 1 h at 37°C or overnight at room temperature. Extracts were neutralized with 0.5 mL of 0.1 M methanolic $NH_4OAc$ and 0.5 mL of 0.5 M $HCl_{(aq)}$. For total GSL extraction, methanol was diluted to < 20% with water and the preparation was applied to a $C_{18}$ column. Salts were removed with water and GSLs eluted with 5 mL $CH_3OH$, followed by 3 mL $CH_3Cl/CH_3OH$ (2:1). Eluates were pooled, dried and re-suspended in $CH_3Cl/CH_3OH$ (2:1) equivalent to $10^5$ cells/μl.

### 3.3.6 TLC and VT1 overlay

The GSL extract from an equivalent of $10^6$ adherent cells or $2x10^6$ lymphoblasts was applied to a TLC plate and separated in $CH_3Cl/CH_3OH$ (98:2), followed by $CH_3Cl/CH_3OH/H_2O$ (65:25:4 or 140:60:11 v/v/v). Brief iodine staining of sphingomyelin was used as an internal loading control. Iodine stain was removed by brief heating. GSLs were then visualized by spraying TLC plates with orcinol and heating at 110°C until bands appeared.

For ganglioside isolation, acidic GSLs were separated by first passing the total GSL extract through a DEAE-Sephadex column. Neutral GSLs were eluted with methanol washes and acidic GSLs were eluted with 0.25 M methanolic $NH_4OAc$. Acidic GSLs were de-salted by $C_{18}$ column chromatography. An extract from an equivalent of $1.5x10^6$ cells was separated by TLC first in 98:2, then 55:45:10 ($CHCl_3/CH_3OH$/0.25% KCl). The plate was lightly sprayed with resorcinol/HCl and heated at 110°C out of air contact until ganglioside bands appeared.

For VT1 overlays (Nutikka et al., 2003), GSLs from an equivalent of $0.5\text{-}1x10^6$ cells were separated by TLC. Plates were dried and blocked with 1% fish gelatin in 50 mM Tris-buffered saline (TBS), pH 7.4, for 1 h at room temperature or overnight at 4°C. Plates were washed with TBS and then incubated with 0.35 μg/mL VT1 B subunit in TBS for 1 h at room temperature or overnight at 4°C. After washing in TBS, plates were incubated with polyclonal rabbit anti-$VT1_B$ antibody. Plates were washed in TBS and then incubated with HRP-conjugated goat anti-rabbit antibody. VT1-bound GSLs were visualized with a 3 mg/mL solution of 4-chloro-1-naphthol in methanol mixed with 5 volumes of TBS and 0.015% $H_2O_2$.

GSLs were quantified and compared by densitometry using ImageJ. GSL intensities were normalized with the intensity of the sphingomyelin internal control.

### 3.3.7 α-Galactosidase assay

Lactosylceramide (LacCer), $Gb_2$ and media GSL extracts were mixed with 50 µL 1% (w/v) taurodeoxycholate and dried under nitrogen. Samples were re-suspended in 200 µL 50 mM sodium citrate buffer (pH 6.0, 2 mM EDTA, 1% BSA) and sonicated. GSLs were digested with 0.5 units coffee bean α-galactosidase at 37$^{o}$C for 4 h. An additional 0.5 units was added to one medium extract at 4 h and $H_2O$ added to the other, and incubation of all reactions was continued overnight (Bailly et al., 1986).

### 3.3.8 Sulfatide Detection

TLC overlay immunoassay was performed as previously described (Colsch et al., 2008) with some modifications. Briefly, GSL extract dissolved in $CHCl_3/CH_3OH$ (2:1) was separated by TLC first in 98:2 ($CHCl_3/CH_3OH$) and then in 60:35:8 ($CHCl_3/CH_3OH/H_2O$). The plate was dried using a hair dryer, treated with a solution of polyisobutylmethacrylate (PIBM) for 5 min, dried and treated with PIBM for an additional 3 min. The plate was blocked with 1% BSA-TBS for 30 min and incubated with 1µg/mL Sulph1 antibody (Fredman et al., 1988) overnight at room temperature. Following washing, the plate was incubated with HRP-conjugated goat anti-mouse antibody for 1 h and then washed and visualized by electro chemiluminescence.

### 3.3.9 Cell-free glycosyltransferase assay

Vero cells were washed, scraped and counted. Cells were centrifuged at 1200 x *g* and the supernatant was discarded. Cells were re-suspended in homogenization buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 M sucrose) and were Dounce homogenized with 35 strokes. Homogenate was collected and centrifuged at 4°C and 800 x *g* for 10 min. The supernatant was centrifuged at 4°C and 100 000 x *g* for 80 min. The supernatant was discarded and the pellet was re-suspended in buffer (100 mM HEPES, 0.05% Triton X-100, 0.5 M sucrose) and centrifuged at 4°C and 800 x *g* for 10 min. The resulting supernatant (microsomal fraction) was kept at -80°C until required. Protein quantitation was performed using the BCA assay (Pierce).

A master mix composed of 100 mM Tris-HCl, pH 6.7, 10 mM $MnCl_2$, 0.3 µCi/reaction UDP-[$^3$H]-galactose, and $H_2O$ was prepared. GlcCer, LacCer and adaGSL were prepared by mixing with Triton (0.3% w/v final concentration per reaction) and drying under nitrogen, followed by re-dissolving in water. 30 µg GlcCer or LacCer and/or 40 µM adaGSL and/or water was added to microfuge tubes, followed by 70 µL of master mix and 30 µg enzyme (i.e. microsomal fraction). Tubes were incubated at 37°C with agitation for 3 h, after which contents were transferred to glass tubes containing $CHCl_3/CH_3OH$ (2:1). The neutral GSL extraction procedure was followed, as described above.

### 3.3.10 Inhibition of Glucocerebrosidase

Compounds (adaGlcCer, adaGalCer, conduritol β epoxide) were dissolved in DMSO to a concentration of 7.5 mM for adaGSLs and 500 mM for conduritol β epoxide (CBE). A three-fold serial dilution of the adamantyl GSLs from 7.5 mM to 10 µM was prepared. CBE concentrations of 500, 250, 100, 40, 16, 6.4, 2.5 mM were prepared.

An inhibition assay was performed as previously reported (Rigat and Mahuran, 2009). Briefly, 1 µl of GSL analogue was added in each well of one column in a 96-well plate, and DMSO was added to one well. 25 µl of 5mM 4-methylumbellilferyl glucopyranoside fluorescent substrate was added to each well, followed by 25 µl of either 1.24 µg/ml GCC (Cerezyme ®) in pH 5 citrate-phosphate (CP) buffer (McIlvaine buffer) or 0.62 µg/mL in pH 7 CP buffer. Wells with CP buffer only (blank substrate) were also included. Plates were incubated at 37°C for 20 min; thereafter, 200 µl 0.1 M 2-amino-2-methyl-1-propanol was added to stop the reaction. Fluorescence intensities were measured using a Spectramax Gemini EM MAX (Molecular Devices Corp.) fluorometer and detected at excitation and emission wavelengths set to 365 nm and 450 nm, respectively.

### 3.3.11 Glucocerebrosidase assay

Bovine buttermilk GlcCer and adaGlcCer were mixed with 1% taurodeoxycholate and dried under nitrogen. These were re-suspended in 80 µL of pH 5 or 7 CP buffer, followed by addition of either buffer or 0.24 µg of Cerezyme ®, and incubated overnight at 37°C. Reaction was stopped by Folch partition. The upper phase was removed and lower phase washed twice with $CH_3OH/H_2O$ (1:1). Lower phase was dried, re-suspended in 2:1 and separated by TLC in 90:10:0.5, followed by iodine and orcinol detection.

### 3.3.12 Indirect immunofluorescence and confocal microscopy imaging

Indirect immunolabeling was performed using a previously described protocol (Birmingham and Brumell, 2006) with small modifications. In brief, cells were seeded at low density onto 18 mm

diameter coverslips for about 16 h, then washed and fixed with 2.5% paraformaldehyde in PBS, pH 7.2, for 30 min at 37$^{o}$C. Blocking and permeabilization was performed for 1 h at room temperature with 0.2% saponin and 10% normal goat serum in phosphate buffered saline with calcium and magnesium (SS-PBS). Primary and secondary antibodies were diluted in SS-PBS and overlaid on the coverslips for 1 h at room temperature; secondary antibodies were similarly overlaid for 1 h in the dark at room temperature; extensive washes with PBS were performed after primary and secondary antibody incubations. Nuclear staining was done with DAPI at 1/50,000 in PBS. Coverslips were mounted onto glass slides using fluorescent anti-fading mounting medium (DakoCytomaton). Primary antibodies used were rabbit polyclonal IgG anti-human beta-glucocerebrosidase (a gift from Dr D. Mahuran), mouse monoclonal IgG1 anti-human LAMP-1 and mouse monoclonal anti-rat PDI. Secondary antibodies were Alexa fluor 488 chicken anti-rabbit IgG for GCC and Alexa fluor 594 goat anti-mouse IgG for LAMP-1 or PDI at a 1/200 dilution in SS-PBS. Samples were analyzed using a Zeiss Axiovert confocal laser microscope equipped with a 63 x 1.4 numerical aperture Apochromat objective (Zeiss) and LSM 510 software; DAPI-stained nuclei were detected on the same system with a Chameleon two-photon laser. To provide a qualitative estimate of GCC levels from the fluorescent signals, the same confocal microscope settings were maintained throughout all confocal sessions for the same pair of primary antibodies (GCC plus LAMP-1 or GCC plus PDI). Confocal images were imported and contrast/ brightness adjusted using Volocity 5 program (Improvision Inc.). A representative cell is shown for each set of staining.

### 3.3.13 GCC Western blot

Normal and GD fibroblasts were treated ± 40 µM adaGlcCer or adaGalCer for four days. Cells were washed and trypsinized, then pelleted at 1200 x *g* for 10 min and washed in PBS. Cells

were suspended in lysis buffer (PBS, 0.1% taurodeoxycholate) and lysed by five cycles of alternately freezing (in a bath of dry ice/ethanol) and thawing (37°C water bath). Suspension was centrifuged at 800 x *g* for 10 min at 4°C. Total cellular protein was quantitated by BCA assay. 30 µg of protein lysate was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane at 100 V for 70 min. Membrane was blocked with 5% TBS-tween (M-TBST) for 1 h, followed by incubation with 1 in 1000 (in M-TBST) rabbit anti-human GCC (kindly provided by Dr. D. Mahuran) overnight at 4°C. Membrane was incubated with 1 in 10,000 donkey anti-rabbit HRP for 1 h, followed by ECL detection.

### 3.3.14 Extracellular GSL content analysis

Following treatment of GD and normal patient lymphoblasts with adaGalCer for four days, the growth medium was lyophilized overnight. Contents were re-dissolved in water and a Folch partition was formed. The neutral GSL isolation procedure was then followed, as described above. The GSL extract from an equivalent of $2x10^6$ cells was separated by TLC and visualized by orcinol or VT binding.

## *3.4 Results*

### 3.4.1 AdaGSL synthesis

The reaction scheme for synthesis of adaGalCer is shown in Figure 3.1. Product yield was approximately 90%. Following synthesis and purification of the products, MALDI-TOF mass spectrometry was performed to verify identity of the product (Figure 3.2).

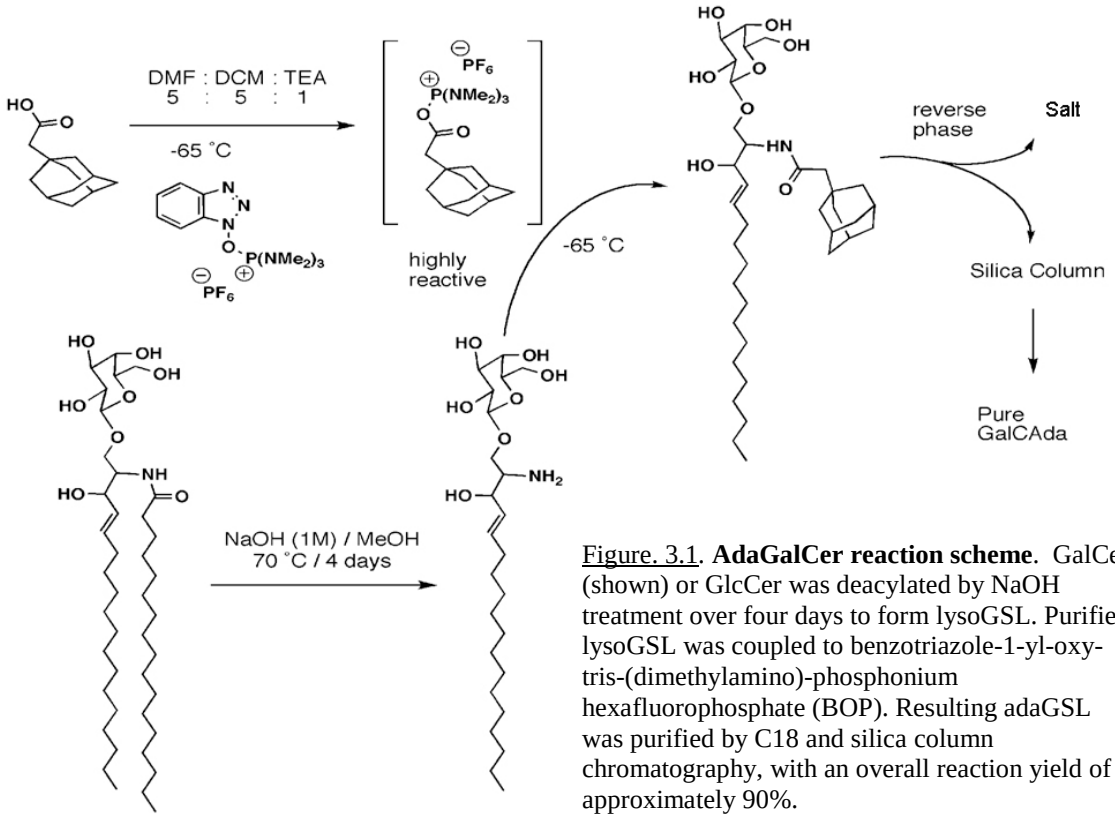



Figure. 3.1. **AdaGalCer reaction scheme**. GalCer (shown) or GlcCer was deacylated by NaOH treatment over four days to form lysoGSL. Purified lysoGSL was coupled to benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP). Resulting adaGSL was purified by C18 and silica column chromatography, with an overall reaction yield of approximately 90%.

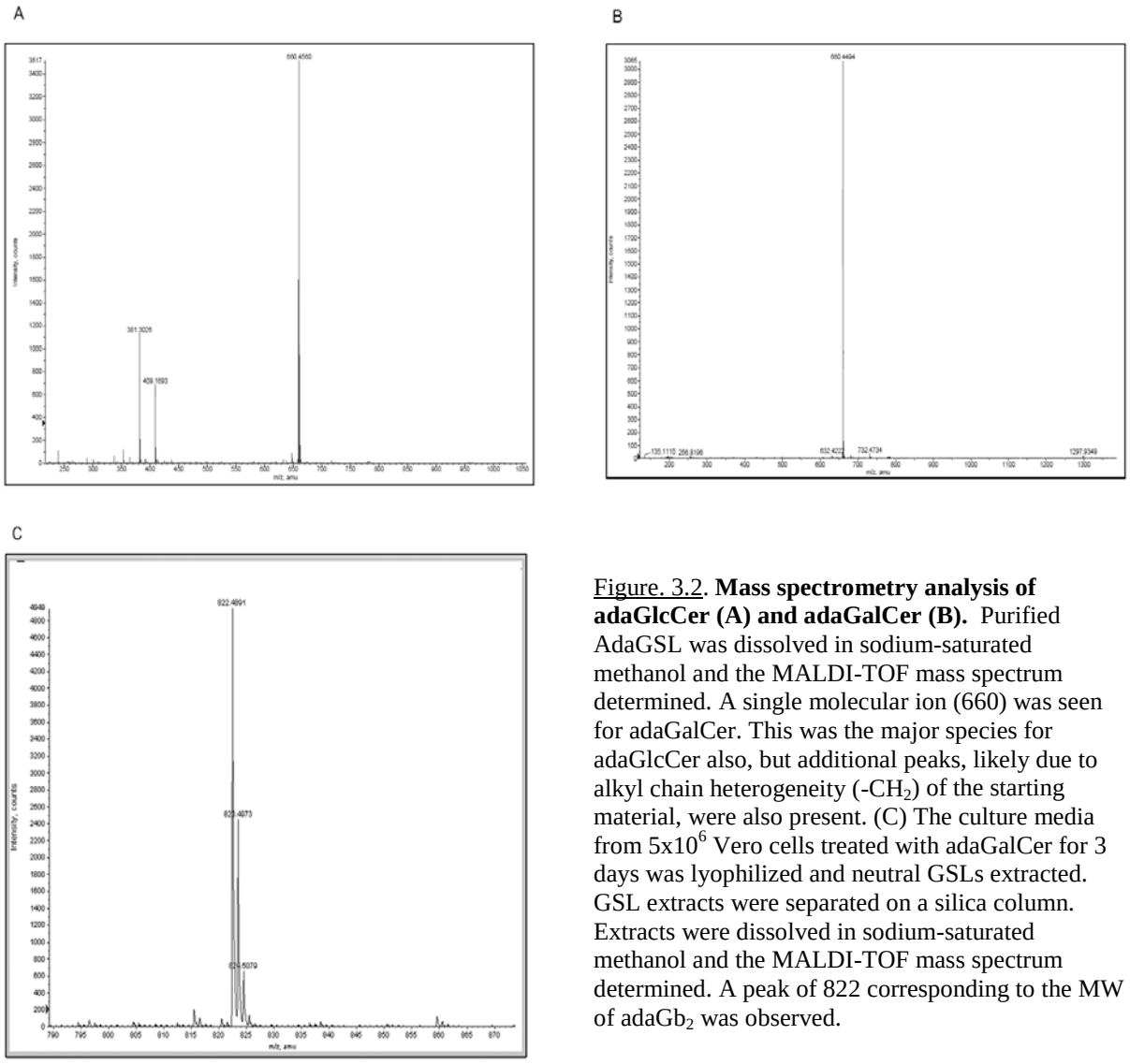



Figure. 3.2. **Mass spectrometry analysis of adaGlcCer (A) and adaGalCer (B).** Purified AdaGSL was dissolved in sodium-saturated methanol and the MALDI-TOF mass spectrum determined. A single molecular ion (660) was seen for adaGalCer. This was the major species for adaGlcCer also, but additional peaks, likely due to alkyl chain heterogeneity ($-CH_2$) of the starting material, were also present. (C) The culture media from $5x10^6$ Vero cells treated with adaGalCer for 3 days was lyophilized and neutral GSLs extracted. GSL extracts were separated on a silica column. Extracts were dissolved in sodium-saturated methanol and the MALDI-TOF mass spectrum determined. A peak of 822 corresponding to the MW of $adaGb_2$ was observed.

### 3.4.2 AdaGlcCer and adaGalCer treatment alter cellular GSL levels

Vero cells were treated with adaGSLs at three different doses for three days and the effect on GSL content was determined (Figure 3.3i, A-C). 10 and 20 µM adaGlcCer resulted in elevated levels of all neutral GSLs. In contrast, a major reduction in all GSLs downstream of GlcCer was observed at 40 µM. GlcCer levels were further elevated at the higher dose.

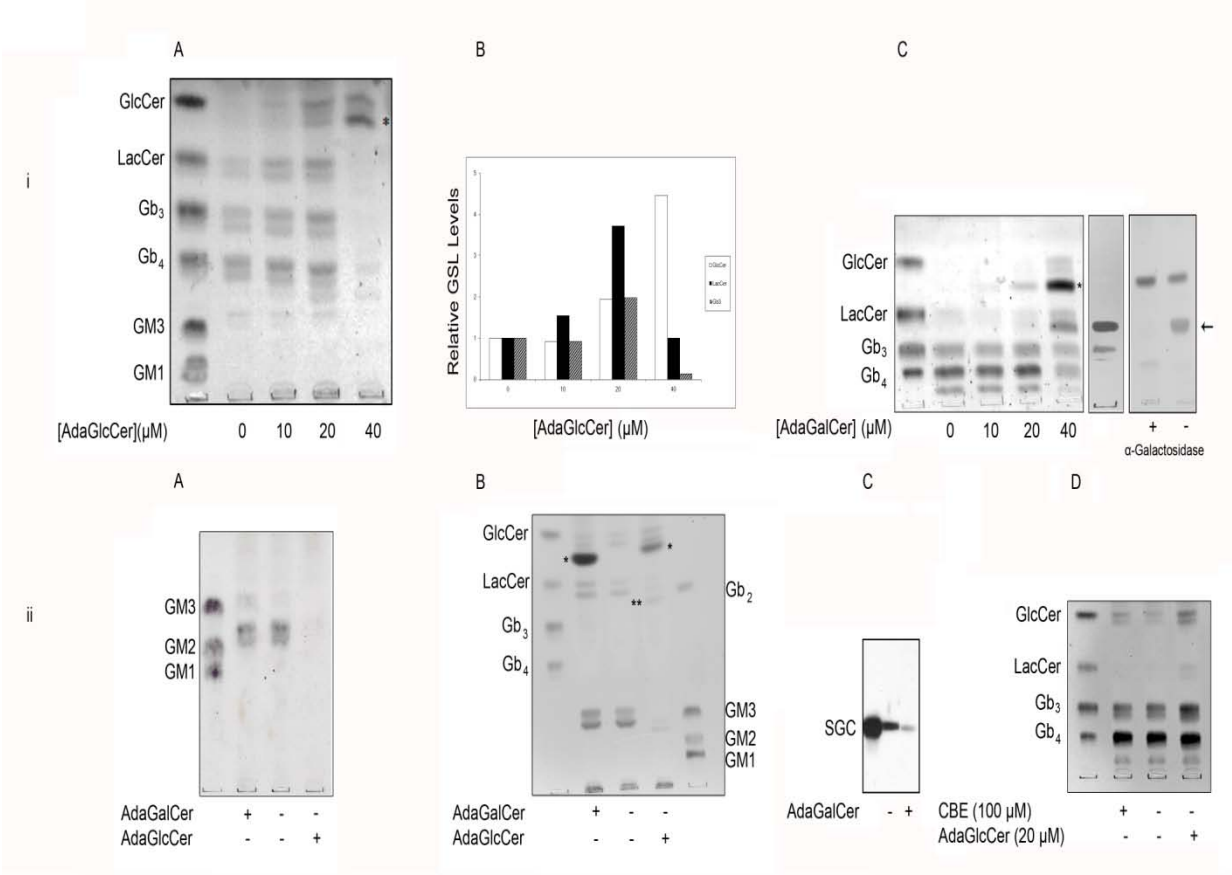



Figure. 3.3. **AdaGSL effects on cellular GSLs.** Vero cells were treated with 10, 20 or 40 μM adaGlcCer (i.A) or adaGalCer (i.C) for four days, followed by neutral GSL extraction and separation by TLC. Ethanol (0.4% v/v final concentration) was used as a vehicle-only control. GSL levels for adaGlcCer treated cells were quantified and compared by densitometry (i.B). Low doses of adaGlcCer increased cellular GSLs, while 40 μM treatment reduced all GSLs downstream of GlcCer, which was further increased. 40 μM adaGalCer reduced globo series GSLs and resulted in formation of a new GSL species. Middle panel in (i.C) shows VT1/TLC overlay of 40 μM extract. Right panel is an orcinol-stained TLC following incubation of conditioned media (containing $adaGb_2$) ± α-galactosidase. α -galactosidase was able to cleave $adaGb_2$. (ii.A) Resorcinol staining of acidic GSLs from Daoy cells treated ± adaGlcCer or adaGalCer. Gangliosides are eliminated after adaGlcCer, but unaffected by adaGalCer, treatment (ii.B) Total GSL extract from BHK cells treated ± 40 μM adaGlcCer or adaGalCer for 3 days. Neutral and acidic GSLs are unaltered by adaGalCer ,but significantly reduced following adaGlcCer treatment, (ii.C) Anti-sulfatide binding to the TLC-separated GSLs from BHK cells treated ± 25 μM adaGalCer for 3 days. AdaGalCer treatment reduces cellular sulfatide levels. *Lane 1: sulfatide standard*. Asterisks (*) and (**) indicate adaGSL substrate and adaGSL product, respectively, and arrow marks $adaGb_2$. (ii.D) Comparison of the effect of adaGlcCer and CBE on GSL levels. Treatment of cells with 100 μM CBE or 20 μM adaGlcCer elevates GlcCer levels. In the case of the latter, LacCer and $Gb_3$ are also increased, while the increase in GlcCer is greater than with CBE treatment.

AdaGalCer had little effect below 20 μM, but at 40 μM, a marked reduction of globo series GSLs ($Gb_3$, $Gb_4$) was seen, while GlcCer and LacCer accumulated (Figure 3.3i, C).

Furthermore, the presence of a new species was observed at this dose. This new GSL was

bound by VT, and susceptible to α-galactosidase, indicating a terminal galactose α1-4 galactose motif. This was identified by mass spectrometry as ada$Gb_2$ (Figure 3.2C). AdaGalCer was also found to have no effect on gangliosides in Daoy cells (Figure 3.3ii, A) or BHK cells (Figure 3.3ii, B), whereas 40 μM adaGlcCer resulted in ablation of both neutral and acidic GSLs. In BHK cells, an alternative product consistent with adaLacCer was detected. In addition, 25 μM adaGalCer lowered BHK cell levels of sulfatide, (3'-sulfogalactosylceramide, SGC), but no alternative product was detected (Figure 3.3ii, C) (In contrast, adaSGC (Park et al., 2009) increased cell sulfatide levels; data not shown).

The ability of conduritol β-epoxide (CBE), a covalent inhibitor of the lysosomal glucosylceramidase (Grabowski et al., 1986), to cause GSL accumulation was compared with that of 20 μM adaGlcCer (Figure 3.3ii, D). 20 μM adaGlcCer showed a much stronger ability to elevate levels of GlcCer, LacCer and $Gb_3$ than 100 μM CBE.

### 3.4.3 AdaGlcCer and adaGalCer inhibit microsomal LacCer and $Gb_3$ synthase respectively

Vero cell microsomes were incubated in the presence or absence of adaGSL and GlcCer/LacCer. Synthesis of LacCer and $Gb_3$ were monitored by incorporation of radiolabeled galactose. LacCer was the major endogenous microsomal GSL product (Figure 3.4A). Synthesis of LacCer was reduced in the presence of adaGlcCer and an alternative product, likely adaLacCer, was made. Exogenous GlcCer increased LacCer synthesis, which was competed out by adaGlcCer. Likewise, adaLacCer synthesis from adaGlcCer was competed out by addition of exogenous GlcCer. Pre-treatment of microsomes with adaGlcCer increased production of adaLacCer and reduced synthesis of LacCer from exogenous GlcCer.

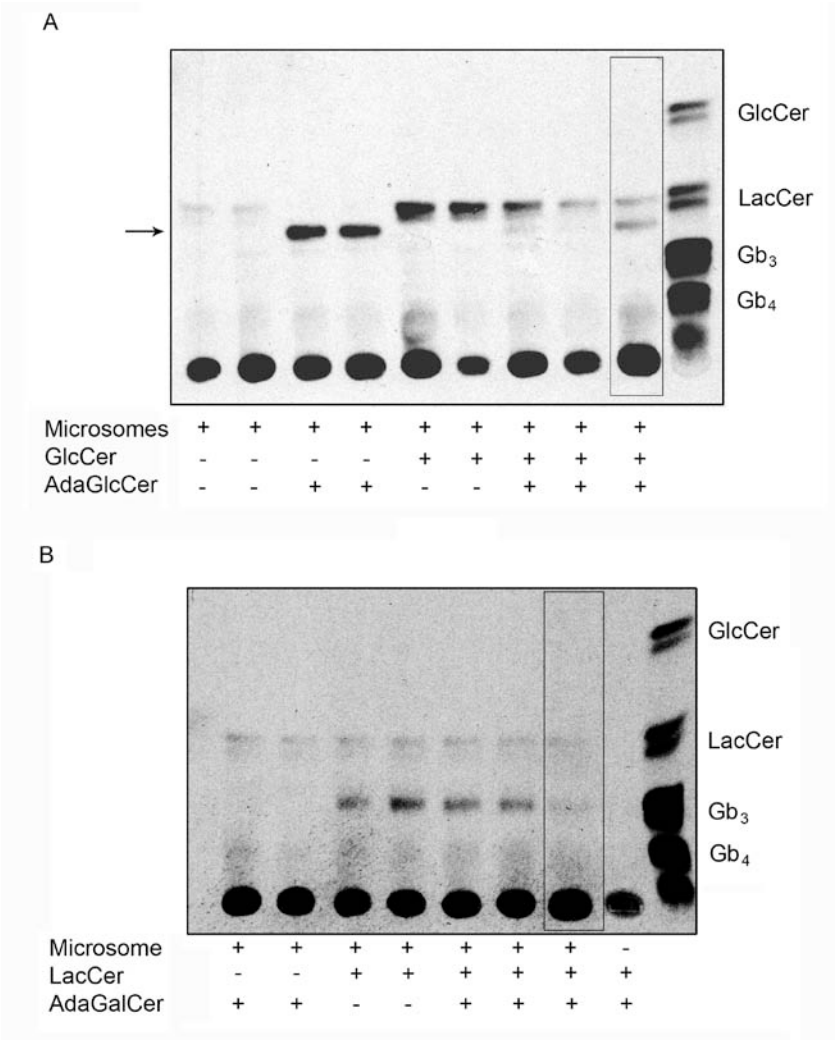



Figure. 3.4. **Effect of adaGSLs on cell-free GSL synthesis.** Microsomes prepared from Vero cells were incubated with 30 µg exogenous GlcCer and/or 40 µM adaGlcCer (A) or 30 µg exogenous LacCer and/or 40 µM adaGalCer (B), and the synthesis of LacCer and $Gb_3$, respectively, monitored by $^3$H-[UDP]-galactose incorporation for 3 h. Outlined lane denotes pre-treatment of microsomes with adaGSL for 3 h, prior to addition of radiolabel. LacCer was synthesized from endogenous GlcCer. A new radiolabelled product (arrowed, likely adaLacCer) was formed from adaGlcCer, and LacCer synthesis was inhibited. Exogenous GlcCer increased LacCer synthesis, while its synthesis was reduced in the presence of adaGlcCer. Conversely, product from adaGlcCer was less in the presence of exogenous GlcCer, but pre-incubation with adaGlcCer in part, restored this product formation. (B) AdaGalCer did not significantly reduce $Gb_3$ synthesis from exogenous LacCer, unless microsomes were pre-treated with adaGalCer. $AdaGb_2$ formation was not detected.

In contrast, adaGalCer did not reduce endogenous LacCer synthesis, but reduced $Gb_3$ synthesis from exogenous LacCer after pre-treatment of microsomes with adaGalCer (Figure 3.4B). However, microsomal $adaGb_2$ synthesis was not detected.

### 3.4.4 AdaGlcCer inhibits, but adaGalCer stimulates, glucocerebrosidase activity in vitro

Using recombinant enzyme, we tested, *in vitro*, if adaGlcCer prevents GlcCer catabolism by GCC inhibition. AdaGlcCer had no effect on GCC activity at pH 5, but inhibited GCC activity at pH 7 at adaGlcCer concentrations > 5 µM (Figure 3.5A). In contrast, adaGalCer had no effect at pH 7, but stimulated GCC activity at pH 5. Increased enzyme activity was seen at adaGalCer concentrations as low as 2 µM and continued through the entire range of concentrations examined. CBE showed dose-dependent inhibition at both pH 5 and 7. GlcCer was cleaved more effectively at pH 7, whereas adaGlcCer was deglucosylated by recombinant enzyme equally well at pH 5 and pH 7 (Figure 3.5B).

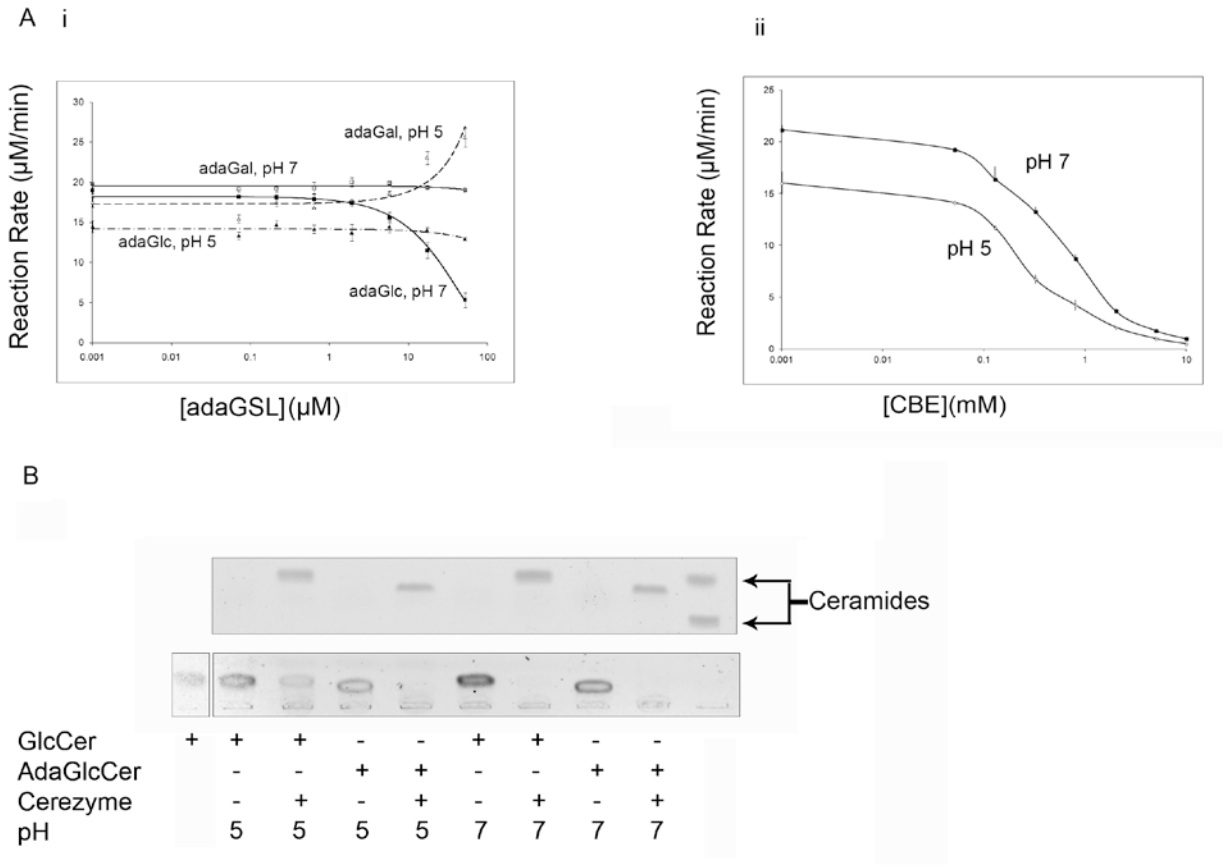



Figure. 3.5. **Effect of adaGSLs on *in vitro* glucocerebrosidase (GCC).** GCC was assayed using pH 5 or pH 7 citrate-phosphate buffer, cerezyme ®, a fluorescent substrate analogue (4-methylumbelliferyl glucopyranoside) and adaGlcCer, adaGalCer or conduritol β-epoxide. Fluorescent substrate was excited at 365 nm and emission recorded at 450 nm. AdaGlcCer had no effect on enzyme activity at pH 5, but showed inhibition at pH 7, while adaGalCer had a stimulatory effect at pH 5 and no effect at pH 7. ▲*: adaGlcCer, pH 5.0;* ■*: adaGlcCer, pH 7.0;*△*: adaGalCer, pH 5.0;* □*: adaGalCer, pH 7.0.* (A.ii) Inhibition assay was performed with CBE at both pH 5 and pH 7. A dose-dependent decrease in activity is observed at each pH. ■: pH 7; ◇: pH 5. (B) GlcCer or adaGlcCer were incubated in the absence or presence of cerezyme ® at pH 5 or 7 and the lipid extracts from the reaction separated by TLC and stained with iodine (upper), followed by orcinol (lower). AdaGlcCer and GlcCer were degraded by the enzyme at both pH, but adaGlcCer cleavage was more effective than GlcCer at pH 5

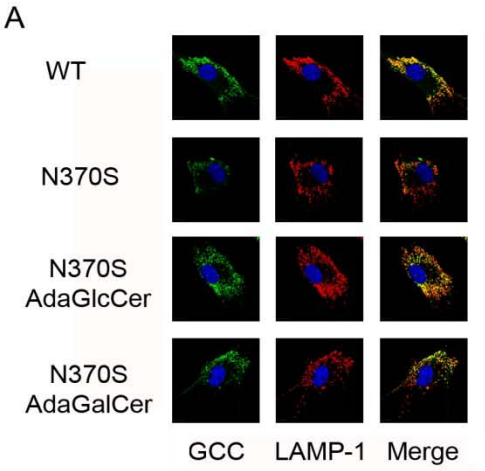
## 3.4.5 AdaGSLs facilitate transport of N370S glucocerebrosidase to the lysosome

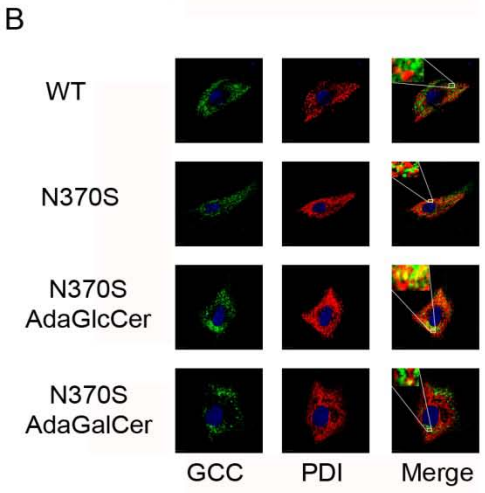
To determine if adaGlcCer or adaGalCer can chaperone mutant glucocerebrosidase, GD (homozygous N370S) patient skin fibroblasts were treated with 40 μM adaGalCer, 40 μM adaGlcCer or mock-treated with ethanol for three days, transferred to cover slips and

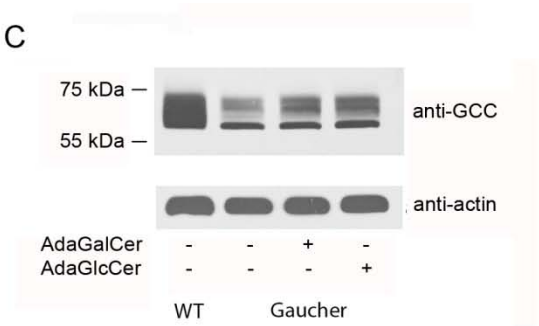
immunostained for GCC, LAMP-1 or PDI (Figure 3.6). Normal human skin fibroblasts were used as a control. Confocal microscopy showed considerable overlap between GCC fluorescence signal and the lysosomal marker LAMP-1 in normal fibroblasts (Figure 3.6A). Signal intensity was substantially less in mock-treated GD cells, with noticeable separation between the intracellular localization of GCC and LAMP-1. AdaGalCer or adaGlcCer treatment enhanced overall GCC signal intensity and markedly increased the coincidence of GCC and LAMP-1 to a pattern indistinguishable from WT cells. The increased GCC-LAMP1 colocalization was measured by quantitative analysis of 11 confocal microscopy images. In adaGalCer or adaGlcCer treated GD cells, GCC lysosomal targeting was greater than in WT cells (Figure 3.6D).

In WT cells, the fluorescence signals for GCC and the ER marker PDI did not colocalize (Figure 3.6B). However, in N370S GD fibroblasts, GCC signal colocalizes with PDI. After adaGlcCer treatment, a significant amount of GCC signal in GD remains colocalized with PDI; in contrast, after adaGalCer treatment, GCC signal localization becomes distinct from that of PDI, similar to the pattern observed in WT.

A Western blot for GCC in normal and N370S GD fibroblasts was performed to assess whether the relocation of N370S GCC by adaGSL treatment protects against ERAD. Consistent with co-chaperone function, the level of GCC protein (immature and mature, as determined by glycosylation-dependent molecular weight differences) in N370S fibroblasts was increased following cell treatment with 40 μM adaGalCer or adaGlcCer (Figure 3.6C). However, GCC activity in the lysate of adaGalCer-treated Gaucher or wildtype lymphoblasts using 4 methyl umbelliferyl glucose substrate, was not increased.


A
WT
N370S
N370S AdaGlcCer
N370S AdaGalCer
GCC
LAMP-1
Merge


B
WT
N370S
N370S AdaGlcCer
N370S AdaGalCer
GCC
PDI
Merge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75 kDa
55 kDa
anti-GCC
anti-actin
AdaGalCer - - + -
AdaGlcCer - - - +
WT
Gaucher

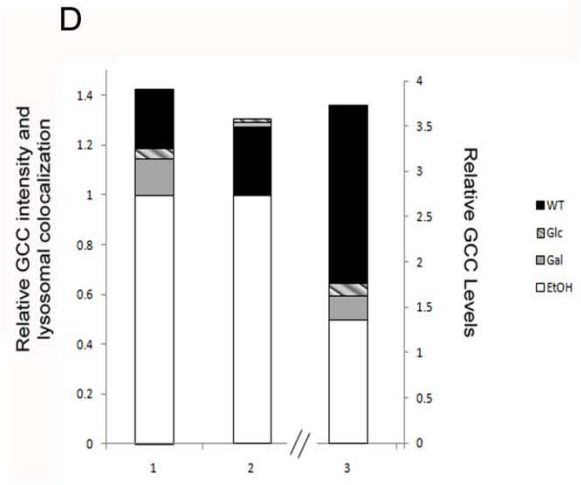
D
Relative GCC intensity and lysosomal colocalization
Relative GCC Levels
WT
Glc
Gal
EtOH
1
2
3

Figure. 3.6. **AdaGlcCer, adaGalCer correction of N370S GCC trafficking in Gaucher disease cells.** (A) Comparison of intracellular localization of GCC (green) relative to the lysosomal compartment marker LAMP-1 (red) in WT and N370S GD fibroblasts: WT fibroblasts show strong colocalization of both proteins in lysosomes as seen by the degree of punctuated yellow pattern in the merged image (right panels). In contrast, N370S display a lower level of GCC (decreased green signal) and of colocalization (decreased yellow signal). Treatment of N370S with either adaGSL increases the level of GCC (green signal levels-quantitated in panel D) and its localization to the lysosomal compartment as denoted by the level of yellow in the two lower merge panels (quantitated in panel D). (B) Comparison of intracellular localization of GCC (green) relative to the ER compartment marker PDI (red) in WT and N370S GD fibroblasts: in N370S GD fibroblasts, GCC colocalizes with PDI (degree of yellow signal in merge panel), but not wild-type fibroblasts. Treatment of N370S with adaGalCer increases the level of GCC (intensity of green levels) and strongly decreases its colocalization with PDI (compare level of yellow in merge panels). AdaGlcCer treatment shows some colocalization of GCC and PDI. (C) Western blot for GCC in GD and normal fibroblasts. Cells were treated for 4 days with 40 μM adaGlcCer or 40 μM adaGalCer. GCC levels were increased in treated GD fibroblasts, but remained considerably lower than normal fibroblasts (quantitated in panel D). (D) Relative change in GCC staining, lysosomal colocalization and expression. Intensity of GCC staining in WT and adaGSL treated N370S cells was measured, normalized for area, and compared to staining in untreated N370S cells using Volocity 5 (Improvision Inc.) (column 1). GCC staining was greater for adaGalCer (grey) and adaGlcCer (hatched) compared to untreated cells (open). Staining was significantly higher in WT ($t(20) = 3.43$, $p<0.05$). GCC colocalization with LAMP-1 was quantitated by applying Manders' coefficient, $M_x$ (column 2). Lysosomal GCC colocalization for WT ($M_x$= 0.773, SD= 0.0871) as well as adaGlcCer ($M_x$=0.793, SD=0.0840) and adaGalCer ($M_x$=0.783, SD=0.0512) treated mutant GD cells was significantly higher than untreated mutant cells ($M_x$=0.605, SD=0.0716) at $p<0.05$. GCC expression examined by Western blot was quantitated (column 3, secondary y-axis). Enzyme expression was increased following adaGSL treatment, but still lower than WT.

### 3.4.6 AdaGalCer reduces GlcCer in Gaucher disease cells

Since adaGalCer was found to activate GCC, correct the N370S GCC lysosomal trafficking defect, and decrease N370S GCC depletion by ERAD, the effect of adaGalCer on GlcCer accumulation in GD cells was examined. A reduction in GlcCer levels was seen following treatment of GD fibrobasts (Figure 3.7A) or lymphoblasts (Figure 3.7, B and C) with 40 μM adaGalCer. This effect on GlcCer levels was quantitated by image analysis and the data was pooled to show an overall 50% inhibition by adaGalCer (Figure 3.7D). As for Vero cells (and FD cells; see below) $Gb_3$ and $Gb_4$ were also depleted. AdaGalCer had no effect on ganglioside content (Figure 3.7A). AdaGlcCer depleted GD cells of all GSLs except GlcCer, which was further elevated.

To further verify adaGalCer reduction of GlcCer, [$^{14}$C]-GlcCer accumulated in GD or FD lymphoblasts by adaGlcCer treatment was determined after adaGlcCer wash out and treatment

with either adaGalCer or adaGlcCer (Figure 3.7E). GlcCer accumulation was far more marked in GD compared with FD cells. AdaGalCer reduced accumulated GlcCer levels in FD but not GD cells, whereas adaGlcCer further increased GlcCer in both cell types.

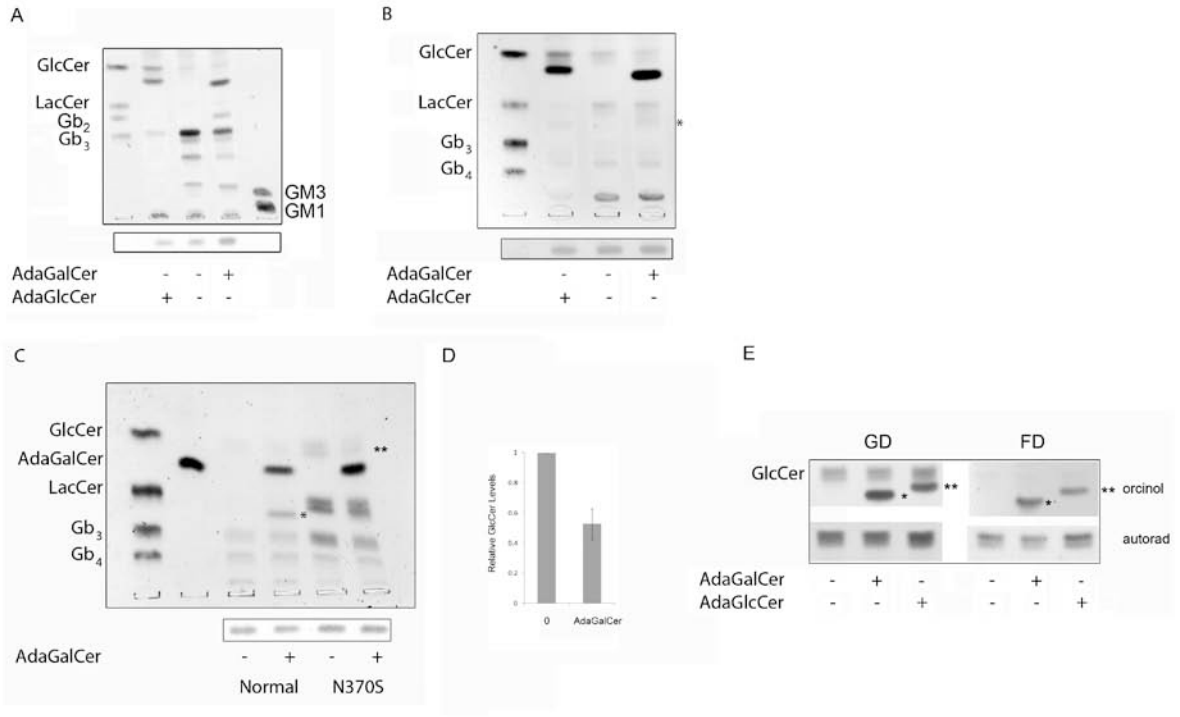


Figure. 3.7. **AdaGalCer reduces GlcCer in Gaucher disease cells.** GD fibroblasts (A) or lymphoblasts (B,C) were treated ± 40 µM adaGlcCer or adaGalCer for four days, followed by GSL extraction, TLC separation, sphingomyelin detection by iodine (lower bar, A-C) and GSL staining by orcinol. AdaGlcCer substantially reduced GSL levels except GlcCer, which was elevated. AdaGalCer treatment reduced cellular GlcCer and resulted in ada$Gb_2$ formation to reduce $Gb_3$. (C) Normal (lanes 3-4) and GD lymphoblasts (lanes 5-6) were left untreated or treated with 40 µM adaGalCer. GlcCer levels are reduced in GD cells treated with adaGalCer. (D) GlcCer levels in all treated and untreated GD cells were compared by densitometry. Single (*) and double (**) asterisks indicate ada$Gb_2$ and GlcCer, respectively. (E) AdaGalCer depletion of accumulated cellular GlcCer. Gaucher (left) and Fabry (right) disease cells were treated for 2 days with 40 µM adaGlcCer and 1 µCi $^{14}$C-galactose. Cells were washed extensively and cultured overnight in either fresh medium alone (lane 1), 40 µM adaGalCer (lane 2*) or 40 µM adaGlcCer (lane 3**). GSLs were extracted, separated by TLC and detected by autoradiography (lower panels) and orcinol (upper panels). AdaGlcCer was rapidly lost from treated cells. More GlcCer accumulated in GD than FD cells but adaGalCer reduced GlcCer levels only in FD cells (§). AdaGlcCer further increased GlcCer in both GD and FD cells. Admantyl ceramide was synthesized and found to be cell growth inhibitory but without effect on relative GSL synthesis.

## 3.4.7 AdaGalCer prevents $Gb_3$ synthesis in Fabry disease (FD) cells

Since adaGalCer provides an alternative substrate for Vero cell $Gb_3$ synthase to reduce cellular globo series GSL content, the utility of this approach as a strategy to lower $Gb_3$ in FD cells was

assessed. However, total $Gb_3$ levels from FD lymphoblasts treated with adaGalCer were only slightly reduced (Figure 3.8, A and B), although increasing levels of $adaGb_2$ were detected with increasing adaGalCer. As a positive control, cells were also treated with the GlcCer synthase inhibitor, P4 (32), resulting in a substantial decrease in $Gb_3$.

Metabolic labeling was used to assess whether $Gb_3$ synthesis is inhibited by adaGalCer. Normal and FD lymphoblasts were treated with adaGalCer for three days and then [$^{14}$C]-galactose and fresh adaGalCer were added for 24 h. Neutral GSLs were extracted and detected by TLC and autoradiography (Figure 3.8C). [$^{14}$C]-$Gb_3$ and [$^{14}$C]-$Gb_4$ levels in treated cells were significantly reduced relative to untreated cells. The additional VT reactive species ($adaGb_2$) seen in Vero cell treatments was also detected in FD lymphoblasts and fibroblasts (Figure 3.8, C and D) treated with adaGalCer, indicating competitive inhibition of $Gb_3$ synthase. FD lymphoblasts have high synthesis rates for GlcCer and LacCer which were reduced by adaGalCer, indicating GCC activation also occurs in these cells. The effect of adaGlcCer on FD fibroblast GSL synthesis was also determined by a 3 h pre-incubation, followed by culture with radiolabel ± adaGSL overnight. Synthesis of GSLs downstream of GlcCer was completely prevented by this brief adaGlcCer treatment (Figure 3.8D).

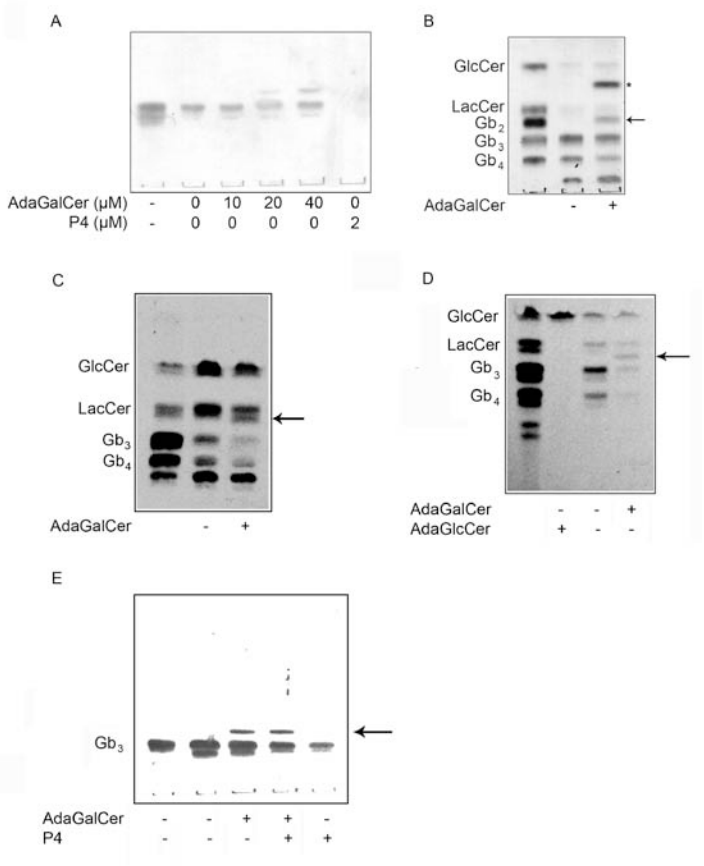


Figure. 3.8. **AdaGalCer reduces $Gb_3$ synthesis in Fabry disease cells.** (A) Verotoxin TLC overlay of GSLs extracted from $10^6$ untreated FD lymphoblasts treated for 3 days with 10, 20 or 40 μM adaGalCer or 2 μM P4. VT-bound $Gb_3$ levels were unaltered by adaGalCer and ada$Gb_2$ was detected, while P4 treatment eliminated $Gb_3$. (B) Orcinol-stained GSLs from $10^6$ control or 40 μM adaGalCer treated FD lymphoblasts. $Gb_3$ levels were reduced, but less effectively than $Gb_4$. (C) Metabolic labeling of GSLs from FD lymphoblasts. Untreated radiolabelled cellular GSLs were compared by autoradiography to GSLs from cells treated with 40 μM adaGalCer for 3 days, followed by addition of $^{14}$C-galactose and fresh adaGalCer for 24 h. Synthesis of $Gb_3$ and $Gb_4$ were significantly reduced (by ~70%). (D) Metabolic labeling of GSLs from FD fibroblasts. GSLs from FD cells ± 40 μM adaGlcCer or adaGalCer were separated by TLC and compared by autoradiography. Cells were left untreated or treated with adaGSL for 3 h prior to addition of $^{14}$C-galactose and fresh adaGSL overnight. AdaGlcCer completely inhibits GSL synthesis and GlcCer accumulates. In adaGalCer-treated cells, ada$Gb_2$ is produced and $Gb_3$/$Gb_4$ synthesis reduced by approximately 90%. (E) Co-treatment of FD fibroblasts with adaGalCer and P4. $Gb_3$ was detected by VT TLC overlay. Co-treatment results in FD cell $Gb_3$ levels intermediate between P4 and adaGalCer treatment. Asterisk (*) denotes adaGalCer and arrow indicates ada$Gb_2$.

## 3.4.8 AdaGalCer reduces $Gb_3$ turnover

Since FD cell $Gb_3$ synthesis was inhibited by adaGalCer treatment, without equivalent reduction in total $Gb_3$ levels, the possible inhibition of the (defective) $Gb_3$ catabolism in these cells was examined by combining adaGalCer treatment with P4. P4 severely diminished FD cell $Gb_3$

levels but co-treatment of FD fibroblasts (Figure 3.8E) or lymphoblasts (data not shown) with adaGalCer decreased the extent of $Gb_3$ reduction by P4. This is consistent with adaGalCer inhibition of residual α-galactosidase activity in these cells.

### 3.4.9 Soluble adamantylGSL products are preferably shed from cells

The ability of the alternative adaGSL product, $adaGb_2$, to be released from cells was addressed by examining the GSL content of the conditioned media from adaGalCer treated cells. Figure 3.9 shows the presence of a prominent $adaGb_2$ species by TLC of the extracellular GSLs, for both normal and GD lymphoblasts. $AdaGb_2$ was present in three-fold excess in the extracellular medium relative to intracellular levels, while $Gb_3$ was found to be in three-fold excess intracellularly. Thus, there is at least a nine-fold preference for adamantylGSL shedding.

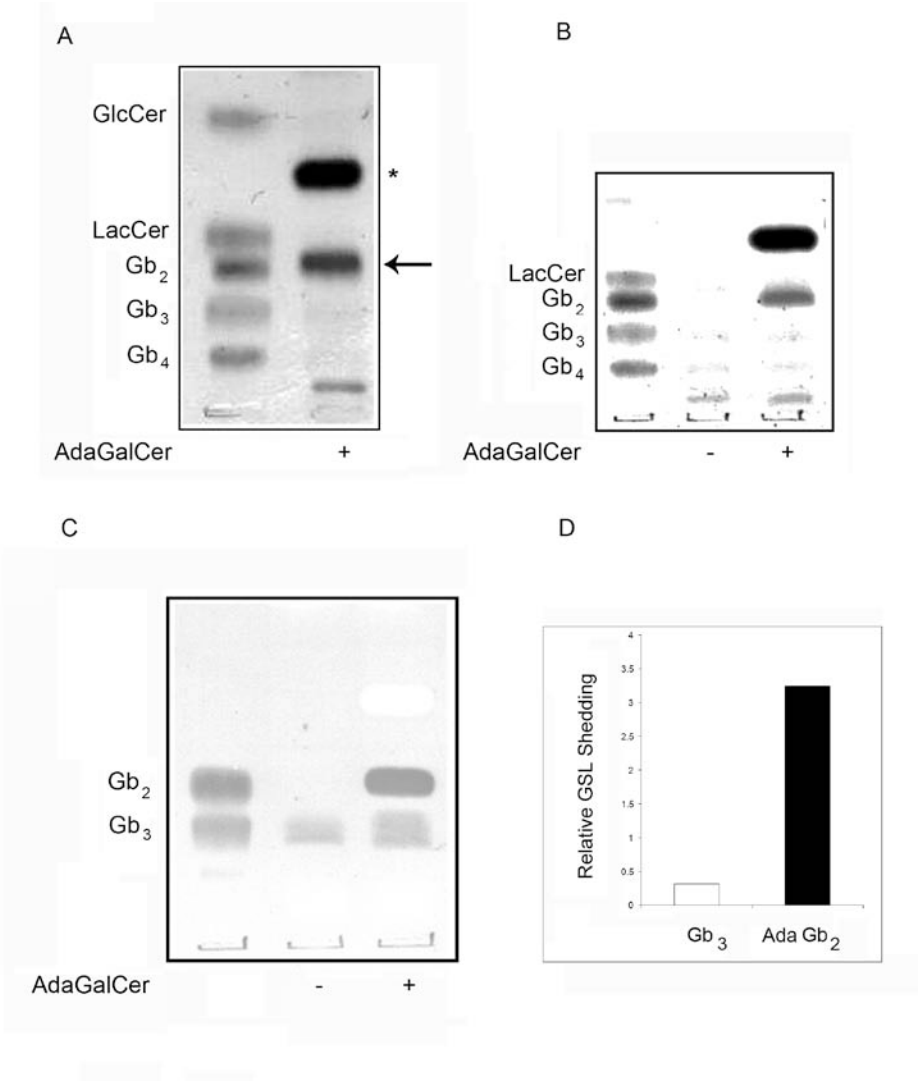


Figure 3.9. **AdaGb$_2$ is preferentially shed into the extracellular medium**. Conditioned medium was lyophilized and the neutral GSLs extracted and separated by TLC for both normal (A) and GD (B,C) lymphoblasts cultured with adaGalCer. GSLs were visualized by orcinol staining (A, B) or VT overlay (C). Cellular and media adaGb$_2$ and Gb$_3$ levels for normal lymphoblasts were compared by densitometry (D). Although more non-polar, as monitored by TLC migration, accumulation of adaGb$_2$ in the medium was approximately10-fold that of Gb$_3$. Asterisk (*) and arrow indicate adaGalCer and adaGb$_2$, respectively. Lower species in panels A and B is likely GM3 ganglioside.

## 3.4.10 AdaLacCer reduces globo-series GSLs

Vero cells were treated with 10, 20 or 40 µM adaLacCer and the effect on GSL content was determined (Figure 3.10). A significant reduction in Gb$_3$ was observed at 20 µM. Gb$_4$ levels were also reduced. 40 µM adaLacCer showed minimal change from the 20 µM dose.

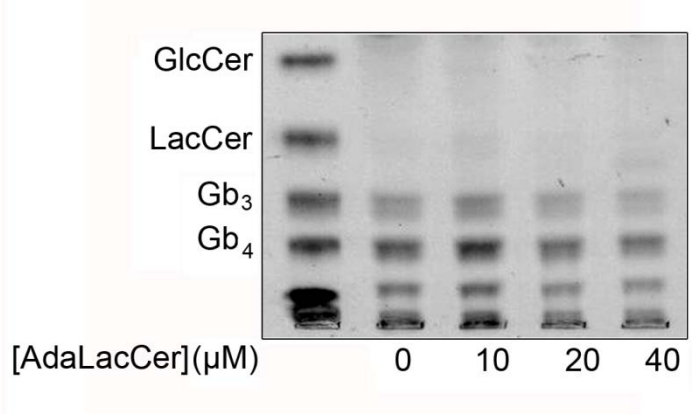


Figure 3.10. **AdaLacCer inhibits neutral GSL synthesis.** Vero cells were treated with adaLacCer for four days followed by extraction of neutral GSLs, TLC separation and orcinol staining. 20 μM adaLacCer reduced $Gb_3$ and $Gb_4$ levels. At 40 μM, another band appears, which likely corresponds to adaLacCer itself.

## *3.5 Discussion*

Using adaGalCer and adaGlcCer, we have found that adamantyl GSL mimics offer powerful new physiologically-based tools to more selectively regulate cellular GSL levels.

### 3.5.1 AdaGalCer selectively inhibits globo-series GSL synthesis in normal and Fabry disease cells

For adaGalCer, there was no obvious effect on Vero cell GSL levels < 20 μM. At 40 μM, however, there was a selective reduction in globo-series GSLs and a variable change in GlcCer and LacCer levels, while GM3 ganglioside levels were largely unaltered. In addition, a new dihexoside species was identified. Since this species was bound by VT1 (which binds GSLs containing terminal galactose α1-4 galactose) and was susceptible to α-galactosidase, this new GSL was considered to be adamantylgalabiosylceramide ($adaGb_2$), which was corroborated by mass spectrometry. The decrease in globo series GSLs can, therefore, be ascribed to substrate competition for $Gb_3$ synthase by adaGalCer, i.e. adaGalCer competes with LacCer. This is based on the premise that the α-galactosyl transferase which synthesizes $Gb_3$ from LacCer is the same that uses GalCer as a substrate to make galabiosylceramide ($Gb_2$) (Kojima et al., 2000),

and that adaGalCer can serve as an alternative substrate to GalCer, to form the new GSL, ada$Gb_2$. In this way, adaGalCer reduces synthesis of $Gb_3$ in favour of ada$Gb_2$. Importantly, alternative product formation suggests an increased substrate efficacy of adaGalCer for the α-galactosyl transferase.

Indeed, we have seen ada$Gb_2$ production in cells with little detectable $Gb_3$. Levels of sulfatide, the 3'sulfate ester of GalCer, were also reduced by adaGalCer, further attesting to the ability of adaGalCer to modulate GSL metabolic enzymes based on GalCer. In this case, no alternative product was formed.

The adamantane frame in our analogues plays a central role. Treating cells with lysoGalCer (deacylated GalCer) < 26 μM had no effect on GSL levels (data not shown). 40 μM lysoGalCer was toxic, while adaGSLs showed no toxicity. The adamantane confers increased water solubility, but membrane solubility of the analogues is retained. Thus, the adaGSL is able to partition into the plasma membrane and traffic to the Golgi (and lysosome) to provide an enzyme inhibitor or alternative glycosyltransferase substrate. Products from such adaGSL substrates retain the amphipathic character of the substrate analogue and are preferentially lost from the cell. In normal lymphoblasts, ada$Gb_2$ was preferentially released from cells and was present in three-fold excess in the growth medium relative to cells, compared to at least a three-fold excess of $Gb_3$ in cells relative to the extracellular medium. Thus, ada$Gb_2$ is approximately 10-fold more easily shed from cells than GSLs such as $Gb_3$, which, according to TLC migration, is more polar than ada$Gb_2$ (but not water soluble). The ease of shedding may also reflect reduced interaction of adaGSLs with cholesterol, which is known to associate with GSLs in membrane microdomains.

This offers the potential for the development of a titratable ‘safety valve’ approach to substrate reduction therapy of GSL storage diseases, allowing the alternative product to ‘bleed’ out of the cell without intracellular accumulation, while retaining partial, or even normal, synthesis of the native GSL to maintain normal function.

The reduction of $Gb_3$ levels, by adaGalCer treatment of FD lymphoblasts was less effective than in Vero cells, despite synthesis of $adaGb_2$. Since FD cell $Gb_3$ was lost after P4 treatment, these cells must contain sufficient residual α- galactosidase activity for this catabolism. Since metabolic labelling showed $Gb_3$ and $Gb_4$ synthesis were significantly reduced by adaGalCer in FD cells, adaGalCer may further reduce the low level of α-galactosidase activity in these cells, perhaps mediated by the $adaGb_2$ generated. Concurrent incubation of FD fibroblasts with adaGalCer and P4 restores $Gb_3$ to an intermediate level, consistent with adaGalCer inhibition of both synthesis and degradation of $Gb_3$. α-Galactosidase inhibition can also provide a chaperone approach to Fabry disease rescue (Fan, 2008).

The inhibitory effects of both adaGSLs are quite rapid—3 h pre-incubation of FD fibroblasts with the analogues, followed by overnight labelling, showed major reduction of GSL synthesis downstream of GlcCer using adaGlcCer, and inhibition of $Gb_3$ (and $Gb_4$) synthesis, together with formation of $adaGb_2$, using adaGalCer.

### 3.5.2 AdaGlcCer both stimulates and inhibits GSL synthesis

At lower doses (10-20 μM), adaGlcCer resulted in elevated levels of all cellular GlcCer-based GSLs. A method for the stimulation of cellular GSL synthesis has not been previously

described, and should be a useful adjunct to study GSL function. For example, GSL synthesis provides an escape from ceramide-induced apoptosis (Gouaze-Andersson and Cabot, 2006) for increased cell survival (Bieberich, 2004). The mechanism of this stimulation remains to be defined. Since adaGlcCer inhibited GCC at pH 7 but not pH 5, inhibition of GlcCer catabolism by Golgi GCC en route to lysosomes, could be an explanation.

However, at 40 μM adaGlcCer, a complete reversal and major reduction in all GSLs downstream of GlcCer was seen. This provides a new benign means to shut down GSL synthesis. We attributed this dichotomy to competitive inhibition of the catabolic GCC by low adaGlcCer concentrations, and competitive inhibition of the anabolic LacCer synthase at higher concentration, in addition to continued catabolic inhibition. At the lower doses, decreased degradation of GlcCer would provide increased substrate for synthesis of downstream GSLs. In fact, 20 μM adaGlcCer more effectively accumulates GSLs than the well-characterized GCC inhibitor CBE. At higher dose, inhibition of LacCer synthesis would reduce synthesis of these downstream GSLs, and further increase GlcCer. Thus, at high adaGlcCer, GlcCer becomes essentially the only cellular GSL. AdaGlcCer should, therefore, also provide a useful tool to study the role of GlcCer in intracellular vesicular traffic (Sillence et al., 2002).

Vero cell-free micosomal GSL synthesis confirmed 40 μM adaGlcCer as a competitive LacCer synthase inhibitor, and as far as we are aware, the first to be described. In our microsomal assay, adaGlcCer was converted to an alternative product, likely adaLacCer. This could be outcompeted by addition of more LacCer, verifying the reversibility of LacCer synthase inhibition. AdaLacCer was not detectable in Vero cells but if made, it would be preferentially secreted, similar to ada$Gb_2$. In contrast, microsomal ada$Gb_2$ synthesis from adaGalCer was not

detected, despite its ready detection in cells. The basis of this clear distinction between the formation of adamantyl ceramide dihexosides in cells versus microsomes treated with adaGlcCer or adaGalCer remains to be resolved, but could result from adaGSL access to the GSL biosynthetic machinery due to a differential Golgi location of $Gb_3$ synthase and LacCer synthase. In BHK cells, adaLacCer was formed, suggesting that product formation from the adaGlcCer-LacCer synthase complex is variable. Microsomal synthesis of $Gb_3$ was only decreased by pre-treatment of microsomes with adaGalCer, without ada$Gb_2$ detection. Lack of alternative product synthesis may be due to the short incubation time, since $Gb_3$ synthesis was reduced only by adaGalCer pre-treatment. It is plausible that adaGalCer binds to $Gb_3$ synthase, but reaction kinetics for conversion to ada$Gb_2$ may be slow in a cell-free environment. Also, the presence of Triton in the microsome assay may alter the relative availability of native and adamantyl GSL substrates.

### 3.5.3 Both adaGalCer and adaGlcCer affect GCC

AdaGlcCer inhibition of GCC was tested with recombinant enzyme. Our results show inhibition at pH 7, but no effect at pH 5. GCC trafficking in cells involves Limp-2 and is mannose-6-phosphate independent (Reczek et al., 2007). Mutation in the enzyme may lead to misfolding and degradation by the endoplasmic reticulum associated degradation pathway (Ron and Horowitz, 2005; Sawkar et al., 2006), to define the severity of Gaucher disease. Our results suggest that adaGlcCer may affect GCC during traffic from the ER to the lysosome. The pH dependent inhibition by adaGlcCer might be useful in enzyme enhancement therapy. AdaGlcCer could potentially bind to (and inhibit) mutant GCC in the ER to stabilize the enzyme and allow traffic to the lysosome, as found for other glycohydrolase inhibitors (Asano et al.,

2000; Fan et al., 1999; Steet et al., 2006), where inhibition is lost, an optimum therapeutic stratagem.

Recombinant GCC (Cerezyme®) was able to degrade adaGlcCer to adaCer and glucose at both pH 7 and pH 5, but GlcCer degradation was not quantitative at pH 5. Thus, adaGlcCer is a better GCC substrate than GlcCer. This could be a function of the increased solubility of adaGlcCer. How the elevated cellular GlcCer level following adaGlcCer treatment relates to its selective inhibition of GCC at neutral pH, is unclear. Inhibition of LacCer synthase may be the primary effect.

In the GCC assay, CBE provided a positive inhibition control and adaGalCer was tested as a negative control. While CBE showed the expected inhibition, adaGalCer surprisingly enhanced GCC activity at pH 5, suggesting a potential allosteric activation site. This remains to be defined, but is consistent with reduced GlcCer levels found in adaGalCer treated normal, GD and FD cells. An allosteric activation of Cerezyme ® GCC by adaGalCer is consistent with our finding that adaGalCer could rescue N370S GD GCC from ERAD and remedy its mislocation within the ER to traffic to lysosomes. Accumulation of N370S GCC in the ER was effectively reduced by adaGalCer. Indeed, the N370S GD GCC colocalization with the LAMP-1 lysosomal marker after adaGalCer treatment was, if anything, greater than that seen for wild-type GCC. However, though the mature N370S GCC, a function of glycosylation-dependent apparent molecular weight change, was increased by either adaGalCer or adaGlcCer cell treatment, the level remained less than wild-type. The fact that lysosomal targeting of N370S GCC after adaGalCer treatment became greater than wildtype indicates that “immature” glycosylated GCC

is also redirected to lysosomes, consistent with mannose-6-phosphate independence (Reczek et al., 2007).

Since adaGalCer activated cerezyme® at pH 5, and rescued N370S GCC from ERAD to target it to lysosomes, the potential of adaGalCer to reverse the GlcCer accumulation characteristic of Gaucher disease (Brady et al., 1965; Grabowski, 2008), was assessed by treating GD cells. AdaGalCer reduced GD GlcCer levels by approximately half, (in addition to inhibition of $Gb_3$ synthesis). This effect, in fact, was more notable in FD fibroblasts in which abnormally high levels of labeled GlcCer (and LacCer) were markedly reduced by adaGalCer. This indicates that adaGalCer traffics to both the Golgi—to inhibit $Gb_3$ synthase—and the lysosome, the acidic site for GCC activation.

Thus, after adaGalCer rescue of N370S GD GCC from ERAD and redirection to the lysosome, adaGalCer should activate N370S GD GCC. This would provide an optimum combination of benefits to reduce GlcCer levels for potential GD therapy. However, we were unable to show adaGalCer activation of N370S or WT GCC activity in treated cell lysates or by direct addition to cell lysate, using the 4-MU-glucose substrate assay (data not shown), despite induced N370S GCC lysosomal tageting and reduced GlcCer levels in adaGalCer treated cells. AdaGlcCer induced accumulated GlcCer was reduced by adaGalCer in FD, but not GD cells, suggesting that adaGalCer enhancement may be greater for cerezyme ® >WT GCC> N370S GCC. The increased efficacy of adaGalCer to reduce GlcCer levels in FD cells may relate to the abnormal trafficking of exogenous GSLs to lysosomes in FD, but not GD cells (Chen et al., 1999), increasing Fabry lysosome adaGalCer content. AdaGalCer may stabilize, rather than activate, GCC in cells and the artificial assay may be inadequate to detect enhanced activity. The

distinction between the effect on cerezyme® and WT cell enzyme remains to be clarified but identifies potentially useful allosteric regulation.

We have also performed an initial experiment with adaLacCer. We observed reduction of $Gb_3$ at a lower concentration than with adaGlcCer (20 μM versus 40 μM); however, we do not see complete ablation at 40 μM, as we do with adaGlcCer. Only neutral GSLs were examined in this experiment. We expect to see a similar effect on gangliosides as well. A more comprehensive characterization of this analogue is pending.

We have shown that cell membrane GSLs can be, in large part, masked from ligand binding by cholesterol (Lingwood et al., 2011; Mahfoud et al., 2010), due to a cholesterol-induced conformational change in GSL carbohydrate to become parallel to the membrane (Lingwood et al., 2011; Yahi et al., 2010). AdaGSLs do not interact with cholesterol (studies in progress) and should therefore, be resistant to such cholesterol masking. Cholesterol present in the Golgi (Mukherjee et al., 1998) might similarly affect GSL enzyme substrate presentation to restrict/regulate GSL metabolism, providing an advantage for adamantylGSLs, to explain the preferential substrate/inhibitor properties we have observed for these GSL mimics in cells.

The ability of adaGSLs to regulate different steps in GSL biosynthesis presents a more selective means to evaluate the biological function or pathology of a GSL than the elimination of all glucosylceramide-based GSLs achieved by glucosylceramide synthase inhibitors, the only current alternative.

## *3.6 Conclusion*

Our study shows the potential for adamantylGSLs as novel, benign means to selectively modulate cellular GSL metabolism, with potential clinical utility. We have probed this potential with adaGlcCer and adaGalCer, primary precursor GSL ananlogues. These provide the means to target selective early steps in GSL metabolism to investigate GSL function and prevent pathologic GSL accumulation. AdaGalCer could be a viable strategy for Fabry disease, if initiated early, by serving as an alternative substrate for $Gb_3$ synthase, generating a readily eliminated alternative product while maintaining "normal" $Gb_3$ levels, and for Gaucher disease, by chaperoning mutant glucocerebrosidase and activating cerezyme®. Adamantyl mimics of branch point GSLs may similarly divert GSL metabolism for other GSL storage diseases and pathologies characterized by aberrant GSL metabolism.

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# Chapter 4: Globotriaosylceramide metabolism in MDR1 knockout/Fabry crossbred mice

Project was initiated by M. Kamani and N. Pacienza. Mouse breeding, maintenance and genotyping were done by N. Pacienza. X. Fan performed enzyme and HPLC assays. A. Novak performed histochemistry experiments. Tissue harvesting was done by M. Kamani, N. Pacienza and B. Binnington. TLC analysis was performed by M. Kamani. Manuscript is in preparation.

Globotriaosylceramide metabolism in MDR1 knockout/Fabry crossbred mice

**Mustafa Kamani, Natalia Pacienza, Xin Fan, Anton Novak, Cindy Guo, Beth Binnington, Clifford Lingwood and Jeffrey A. Medin**

## *4.1 Abstract*

P-glycoprotein (P-gp, ABCB1, MDR1) transmembrane drug efflux pump has been implicated in glycosphingolipid (GSL) biosynthesis. Pharmacological inhibition has been reported to decrease neutral GSL synthesis. It has been suggested that P-gp is responsible for translocating glucosylceramide (GlcCer) from its site of synthesis on the cytosolic leaflet of the Golgi membrane to the lumenal leaflet for access to downstream glycosyltransferases. This is the key missing step in the understanding of GSL metabolism in eukaryotes. Fabry disease is a lysosomal storage disorder characterized by deficient activity or expression of α-galactosidase A that leads to progressive accumulation of globotriaosylceramide ($Gb_3$). To assess the involvement of P-gp in $Gb_3$ metabolism *in vivo*, we have generated a triple deficient (TKO) mouse by crossbreeding MDR1 knockout mice with Fabry mice. HPLC and histochemistry analyses reveal tissue-specific reduction in $Gb_3$ in TKO mice relative to Fabry mice. TLC analysis, however, showed no difference in $Gb_3$ for the various tissues examined. Reasons for the discrepancy are unclear.

## *4.2 Introduction*

P-glycoprotein (P-gp, ABCB1, MDR1) is a member of the ATP-binding cassette (ABC) transporter superfamily of proteins responsible for conferring resistance to cells from hydrophobic cytotoxic compounds. P-gp mediates the efflux of a broad range of hydrophobic substrates that can penetrate into cells to potentially cause toxic effects. Owing to its protective

function, P-gp is appropriately expressed at several locations, including the apical surface of intestinal cells, in the kidney, liver and at blood-tissue barriers—blood-brain-barrier and blood-testis-barrier (Cascorbi, 2011; Fromm, 2004).

A flippase model for P-gp was proposed whereby substrate within the lipid bilayer interacts with P-gp, which flips the substrate from the cytosolic to the exoplasmic leaflet of the membrane (Higgins and Gottesman, 1992). Clinical implications of such a model include the necessity to shield drug molecules—such as within liposomes—from exposure to the lipid bilayer where they might be extracted and flipped by P-gp (Higgins and Gottesman, 1992). This would prevent detection of the drug by P-gp. This model is supported by the finding that ABCB4 (MDR2) is a flippase for phosphatidylcholine (PC) in liver (Ruetz and Gros, 1994). ABCA1 (Ueda, 2011), ABCA2 (Sakai et al., 2007a), ABCA3 (Nagata et al., 2004), ABCG5 (Berge et al., 2000), ABCG8 (Berge et al., 2000), ABCD1 (van Meer et al., 2006) and ABCA12 (Sakai et al., 2007b) have all either been shown or suggested to be involved in the transport of particular lipids. In addition, P-type ATPases maintain membrane asymmetry by flipping aminophospholipids toward the cytoplasmic membrane leaflet (Pomorski and Menon, 2006), and opsin is a phospholipid flippase in photoreceptor discs (Menon et al., 2011).

Importantly, P-gp was shown to be involved in lipid translocation across membranes (Van Helvoort et al., 1996). Epithelial cells transfected with the ABCB1 gene are capable of translocating the GlcCer analogues NBD-GlcCer, $C_8C_8$-GlcCer and $C_6$-GlcCer, as well as short-chain fluorescent analogues of PC and phosphatidylethanolamine (PE) to the exoplasmic leaflet of the apical plasma membrane (Van Helvoort et al., 1996). Flipping of substrates was prevented by inhibitors of P-gp drug efflux (Van Helvoort et al., 1996). Reconstitution of P-gp

into liposomes similarly translocates GlcCer analogues, showing a flippase function of the protein (Eckford and Sharom, 2005).

Furthermore, we have previously shown that transfection of the human ABCB1 gene into MDCK cells markedly elevates neutral GSL expression (Lala et al., 2000) and pharmacological inhibition of P-gp prevents neutral GSL synthesis (De Rosa et al., 2004). We proposed that such GSL inhibition is achieved by preventing P-gp-dependent translocation of GlcCer from its site of synthesis on the cytosolic leaflet of the Golgi to the lumenal leaflet. All glycosyltransferases downstream of GlcCer are found within the Golgi lumen. Not every cell line examined showed GSL sensitivity to P-gp inhibition (De Rosa et al., 2004). HeLa cells were among the insensitive cell lines, suggesting alternative mechanisms for GlcCer Golgi lumenal access. Although $Gb_3$ is unaffected by cyclosporine (CsA) treatment of HeLa cells, GlcCer is reduced (Smith et al., 2006), possibly by GlcCer exposure on the cytosolic Golgi membrane to non-lysosomal glucosylceramidases (Hayashi et al., 2007; Van Weely et al., 1993).

Fabry disease is a lysosomal storage disorder (LSD) caused by insufficient expression or activity of α-galactosidase A (α-gal A, GLA) leading to progressive accumulation of globotriaosylceramide ($Gb_3$) (Brady et al., 1967). Fabry disease is the only sphingolipid disorder that is X-linked (Kolter and Sandhoff, 2006). Patients typically suffer from renal impairment, skin lesions, cardiomyopathy and cerebral vascular disease (Lidove et al., 2007; Motabar et al., 2010). At present, the only clinically approved treatment for Fabry disease is enzyme replacement therapy in which patients are supplemented recombinant wild-type enzyme to compensate for mutant enzyme that is defective (Garman and Garboczi, 2004). ERT,

however, is expensive and the enzyme suffers from inability to cross the blood-brain-barrier (Lidove et al., 2007). Thus, alternative options have been explored such as enzyme enhancement therapy using pharmacological chaperones to help stabilize the defective protein and substrate reduction therapy by preventing synthesis of $Gb_3$ (Fan et al., 1999).

In a previous study, we showed that following a short ERT course of Fabry mice with α-gal A to deplete serum and tissue $Gb_3$, serum and liver $Gb_3$ levels recovered to lower levels in mice that were subsequently treated with CsA, an inhibitor of P-gp (Mattocks et al., 2006). Kidney $Gb_3$ levels were not significantly altered, possibly due to the CsA dose in the study being insufficient to reduce the high renal $Gb_3$ levels in Fabry mice. This points towards a novel substrate reduction therapeutic approach in Fabry disease, and potentially other disorders characterized by neutral GSL accumulation, by targeting P-gp to prevent synthesis of neutral GSLs, including $Gb_3$.

In this study, we assessed the *in vivo* involvement of ABCB1 in GSL metabolism by generating a novel knockout mouse model through crossbreeding the MDR1 knockout mouse with the Fabry mouse. Mice contain two genes encoding two P-gps that, together, likely accomplish the same role as the one human P-gp (Schinkel et al., 1997). The resulting triple knockout (TKO) mice generated were evaluated for $Gb_3$ levels in order to assign a direct role for P-gp in *in vivo* GSL metabolism. The ambiguity that has arisen from our results has made such a conclusion difficult to confirm. Using a standard HPLC assay, we observed an ~50% decrease in $Gb_3$ accumulation in TKO mice relative to Fabry mice that was specific to the kidney, liver and spleen, while no difference was seen in heart and lung. Subsequent TLC analysis, however, revealed no difference in $Gb_3$ in any tissue examined. Reasons for the discrepancy between the

two methods are unclear. Regardless of which dataset is a more accurate depiction of the *in vivo* role of P-gp, it is clear that alternative mechanisms must exist for GlcCer to access the Golgi lumen.

## *4.3 Experimental Procedures*

### 4.3.1 Generation of MDR1/Fabry mice

Fabry mice (Ohshima et al., 1997) were bred at the Animal Resource Centre, University Health Network (UHN). MDR1a/b mice were purchased from Taconic (Albany, NY, USA) and bred in a colony maintained at the UHN. Animal experimentation protocols were approved by the UHN Animal Care Committee (ACC). The parental generation ($F_0$) involved in the generation of the MDR1/Fabry mice consisted of Fabry female (AABBxx) and Mdr1a/b male (aabbXY) mice. In order to acquire the four different genotypes analyzed in the present study, the F1-triple heterozygous mice were mated (AaBbXx x AaBbxY). At 23 weeks of age, mice were euthanized under conditions approved by the UHN ACC and tissue analyses performed.

### 4.3.2 Mouse Genotyping

Mouse genotype was analyzed by PCR from tail DNA. Following Taconic's recommendations, one PCR reaction using three different primers (MDR1AS2, 5'-CTCCTCCAAGGTGCATAGACC- 3'; MDR1AW2, 5'-CCCAGCTCTTCATCTAACTACCCTG-3', and MDR1AKO2, 5'-CTTCCCAGCCTCTGAGCCCAG-3') was sufficient to identify the two possible mdr1a alleles—wild-type (269 bp) and mutant (461 bp). The wild-type mdr1b (540 bp) as well as the wild-type α-gal A (1050 bp) alleles were assayed following the protocols of Taconic and

Ohshima et al. (Ohshima et al., 1997), respectively. A new set of primers was designed to determine the mutated-*mdr1b* (455'TGTCAAGACCGACCTGTCCG3' and NeoB-Reverse 5'ACGCGTCGCGACGCGTCT AG3') and mutated α-gal A alleles (Fabry Neo-Forward 5'TCGCCTTCTTGACGAGTTCTTCTG3' and I3-Reverse 5'GGCGATTTCCAGGCAGTGTG3'), 1127 bp and 1150 bp, respectively. The specificity of these new amplicons was confirmed by sequencing. Thus, three separate PCR reactions were performed to establish the mdr1a/b or Fabry genotype (Figure 4.1B).

### 4.3.3 Tissue GSL extraction for HPLC analysis

Tissue was homogenized in 9 volumes (9 times tissue weight) of homogenization buffer (28 mM citric acid, 44 mM $Na_2HPO_4$, sodium taurocholate, pH4.4). An aliquot was saved for protein assay. 5.5. volumes of 2 propanol was added to 400 µL homogenate and mixed for 1 h, followed by addition of 3.5 volumes $CHCl_3$ per volume of sample and mixing for 1 h. Samples were passed through glass wool to filter out debris, dried under nitrogen and saponified with 0.6 N methanolic NaOH. Extracts were neutralized with 0.5 N methanolic HCl. $CHCl_3$ and water were added to create a Folch partition (2:1:0.6, $CHCl_3$:$CH_3OH$:$H_2O$). The upper phase was removed and the lower phase was washed with theoretical upper phase (6:96:94, $CHCl_3$:$CH_3OH$:$H_2O$). Samples were dried under nitrogen.

### 4.3.4 HPLC quantitation of $Gb_3$

Mouse organ $Gb_3$ content was determined by HPLC as described (Yoshimitsu et al., 2007). Each measurement was assessed in triplicate, normalized to total protein concentration (DCTM Protein Assay, Bio-Rad Laboratories, ON, Canada), and expressed as the mean ± SD.

### 4.3.5 α-Galactosidase assay

Specific α-gal A activity was determined by fluorometric assay as previously described (Yoshimitsu et al., 2004). Briefly, plasma or organ extracts were incubated with 4-methylumbelliferyl-α-D-galactopyranoside (5mM) (RPI Corp., Mount Prospect, USA) in the presence of the α-Nacetylgalactosaminidase inhibitor, N-acetyl-D-galactosamine (100mM) (Sigma, ON, Canada). The product of the enzymatic reaction was quantified by comparison with known concentrations of 4-methylumbelliferone.

### 4.3.6 Tissue $Gb_3$ staining

Tissue $Gb_3$ was also evaluated by verotoxin 1 (VT1) staining and immunohistochemistry. VT1 staining was performed as described (Mattocks et al., 2006). Briefly, frozen tissue sections were air-dried overnight, blocked with endogenous peroxidise blocker (Universal Block, KPL Inc., Gaithersburg, MD), and then stained with VT1-B (200 ng/mL) purified in our lab as described (Petric et al., 1987). For some sections, cholesterol was extracted by treatment with 10 mM methyl-β-cyclodextrin (MCD) (Ottico et al., 2003) for 30 mins at 37°C prior to staining. After rinsing, sections were incubated with rabbit anti-(VT1B 6869) also purified in our lab (Boyd et al., 1991), washed and then incubated with goat anti-rabbit IgG conjugated with HRP (Bio-Rad, Hercules, CA). After washing, sections were developed using the DAB substrate (Vector Labs, Inc., Burlingame CA). For immunodetection of $Gb_3$, an additional avidin/biotin blocking step was added: tissue sections were incubated for 1h with mAb anti-$Gb_3$ (clone 38.13), washed and then incubated with biotin anti-rat IgM (Jackson Immunoresearch, West Grove, PA). Staining was developed using ABC Elite DAB stain (Vector Labs). Specificity of $Gb_3$ detection by VT1

or 38.13 was verified by preparing control sections in which VT1 was omitted, or isotype control rat IgM (eBioscience, San Diego, CA ) was substituted, respectively. Following DAB staining, sections were counterstained with hematoxylin then mounted with Permount (Fisher Scientific, Ottawa, ON).

### 4.3.7 Tissue GSL extraction for TLC analysis

Frozen tissue section in OCT blocks were popped out of the blocks and incubated in PBS on ice for OCT to thaw. Tissue was separated from the OCT and washed twice with PBS. The tissue fragment was homogenized in 1 mL PBS on ice using a hand-held homogenizer. An aliquot was saved for protein and enzyme assays. Homogenate was added to $CHCl_3$:$CH_3OH$ (2:1, v/v) for GSL extraction overnight with shaking. Cell and tissue debris were filtered with glass wool and the extracts dried under nitrogen. To remove glycerophospholipids, extracts were saponified with 0.5 M NaOH (methanolic) overnight at room temperature or for approximately 2 h at 37°C, followed by neutralization and de-salting by $C_{18}$ column chromatography. Salts were washed with 30 mL $H_2O$, and lipids eluted with 5 mL $CH_3OH$, followed by 3 mL $CHCl_3$:$CH_3OH$ (2:1). Extracts were dried and re-suspended in 98:2 ($CHCl_3$:$CH_3OH$). Cholesterol, ceramides and fatty acids were separated by passing extracts through a silica column equilibrated in 98:2. Hydrophobic molecules were eluted with 98:2 and GSLs first with 9:1 (acetone:methanol), followed by methanol.

### 4.3.8 TLC

Total tissue GSLs were separated by TLC, first in 98:2 ($CHCl_3$:$CH_3OH$) and then 60:40:9 ($CHCl_3$:$CH_3OH$:$H_2O$). Brief iodine staining to visualize sphingomyelin was used as an internal

loading control. Iodine stain was removed by brief heating. GSLs were then visualized by spraying TLC plates with orcinol and heating at 110°C until bands appeared. For detection of acidic GSLs only, TLC plates were sprayed lightly with resorcinol, tightly clamped between glass plates and heated at 110°C until bands appeared.

For VT1 overlays, GSLs were separated by TLC in 98:2 ($CHCl_3$:$CH_3OH$) and then 60:40:9 ($CHCl_3$:$CH_3OH$:$H_2O$). Plates were air-dried and blocked with 1% coldwater fish gelatin in 50 mM Tris-buffered saline (TBS), pH 7.4, for 1 h at room temperature or overnight at 4°C. Plates were washed with TBS and then incubated with 0.35 μg/mL VT1 B subunit in TBS for 1 h at room temperature or overnight at 4°C. After washing in TBS, plates were incubated with polyclonal rabbit anti-$VT1_B$ antibody. Plates were washed in TBS and then incubated with HRP-conjugated goat anti-rabbit antibody. VT1-bound GSLs were visualized with a 3 mg/mL solution of 4-chloro-1-naphthol in methanol mixed with 5 volumes of TBS and 0.015% $H_2O_2$.

## *4.4 Results*

### *4.4.1 Generation and characterization of MDR1/Fabry mice*

Mice deficient in both α-galactosidase A and ABCB1 (MDR1) were generated by crossbreeding Fabry female mice (AABBxx) with the ABCB1 knockout male mice (aabbXY) (Figure 4.1A). Four genetically distinct groups of male mice were used for GSL analyses: Wild-type (AABBXY), Fabry (AABBxY), MDR1 (aabbXY) and MDR1/Fabry (aabbxY, TKO). Since the phenotype of female Fabry disease patients varies considerably and does not necessarily correlate with $Gb_3$ levels (Aerts, 2008; Kolter and Sandhoff, 2006), only males were used in this study. Genotypes were confirmed by measuring plasma α-galactosidase A activity (Figure

4.1C). Fabry and TKO mice had significantly reduced serum activity, and essentially no tissue enzyme activity (Figure 4.2A), in comparison to WT and MDR1 mice, as expected.

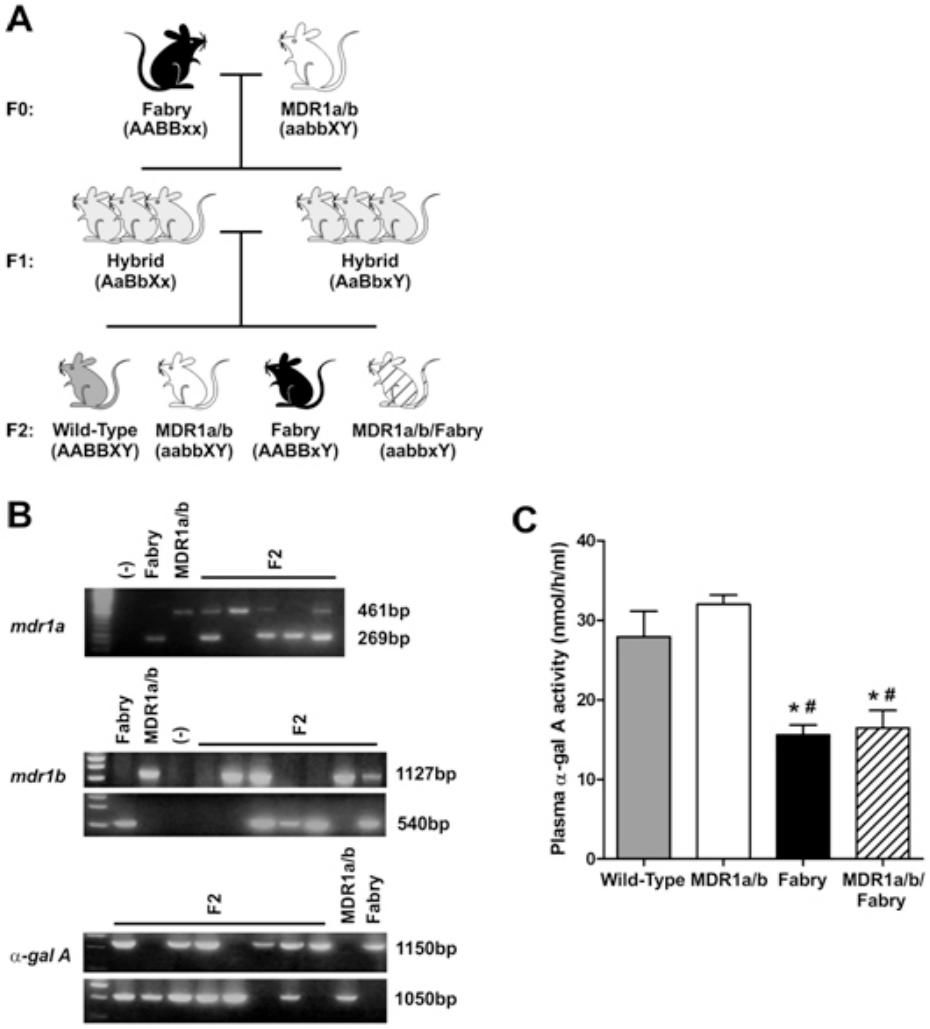



Figure 4.1. **MDR1a/b/Fabry mouse model and its characterization.** (A) The breeding scheme used to generate four genotypes of interest. Fabry females were mated with MDR1 males. The resulting hybrid offspring were mated with one another to generate mice of the four different genotypes (wild-type, MDR1, Fabry and MDR1/Fabry). (B) Genotyping of the genes of interest (*mdr1a/mdr1b/*α-gal A). Mouse genotype was assessed by PCR amplification of tail DNA. Wild-type mdr1a corresponds to a 269 bp species, while the size of the mutant allele is 461 bp. For mdr1b, wild-type and mutant alleles are 540 bp and 1127 bp, respectively. A 1050 bp species corresponds to wild-type α-gal A, while 1150 bp represents the mutant allele. A representative PCR is shown for each gene of interest. (C) Plasma α-gal A enzyme activity was assessed for the mice of interest. Activity was significantly lower in the Fabry and MDR/Fabry mice relative to normal and MDR1 mice. Data represent mean ± SD of 5 individuals (F2) per group. *,# $p<0.01$ to wild type and MDR1a/b control groups.

## 4.4.2 Reduced $Gb_3$ in TKO liver, kidney and spleen by HPLC

We evaluated $Gb_3$ levels in various tissues by two different methods: HPLC and TLC. HPLC analysis revealed considerably elevated $Gb_3$ levels in all Fabry mouse tissues relative to normal and MDR1 mice, with levels in the latter two being comparable (Figure 4.2B). $Gb_3$ levels were significantly reduced by approximately 30-60% in the liver, spleen and kidney of TKO mice relative to Fabry mice. $Gb_3$ levels were unaltered in heart, while in lung, TKO $Gb_3$ was slightly, but not significantly, less than in normal mouse lung.

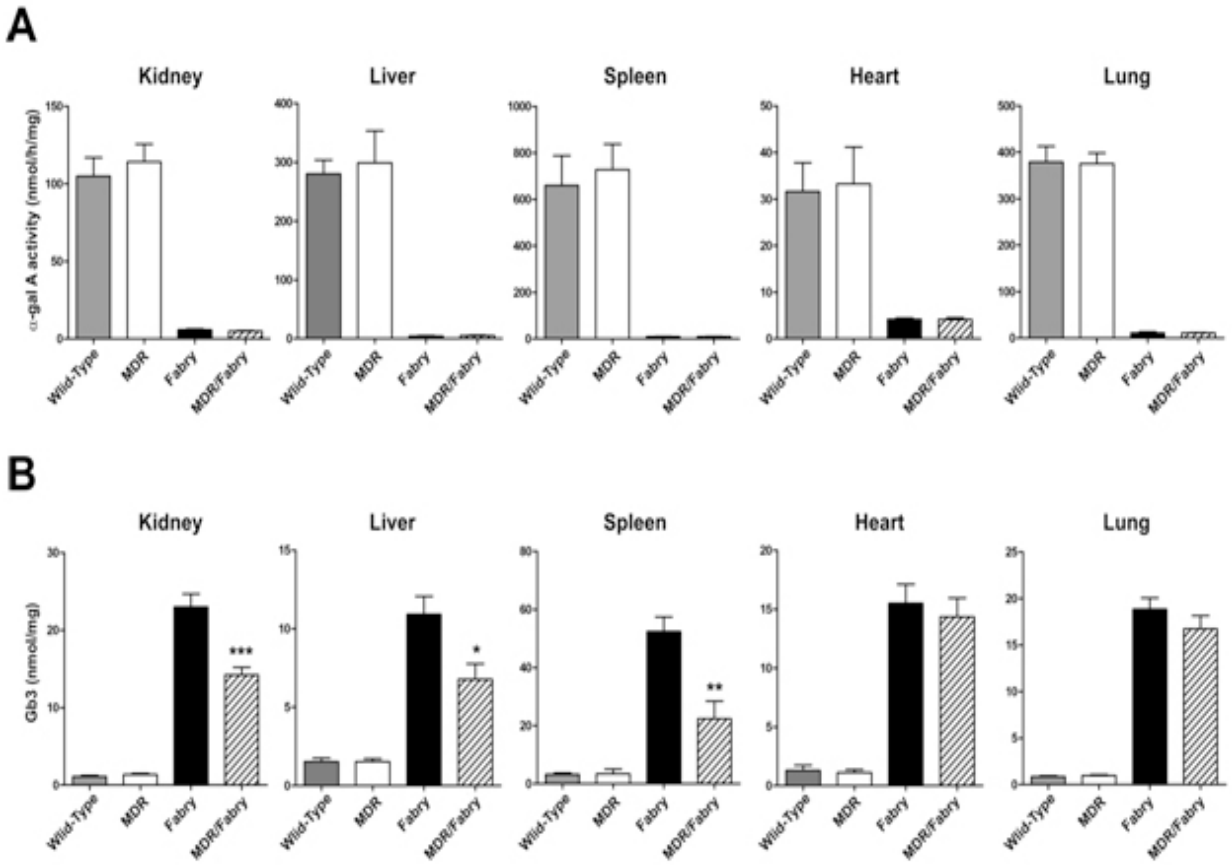



Figure 4.2. **Tissue α-galactosidase A activity and HPLC quantitation of $Gb_3$.** Tissue α-galactosidase A activity was measured. Wild-type and MDR mice had similar enzyme activities across all tissues examined. Fabry mice had virtually no tissue enzyme activity. MDR/Fabry mice had similar activities to Fabry mice. (B) $Gb_3$ was quantitated by HPLC in the heart, kidney, lung, liver and spleen of 22-23 week-old mice. Fabry mouse tissue $Gb_3$ levels were considerably higher than wild-type and MDR mice across all tissues. MDR/Fabry mouse $Gb_3$ levels were higher than wild-type and MDR mice for all tissues, but were significantly lower than Fabry mouse $Gb_3$ levels in the kidney, liver and spleen. Heart and lung MDR1/Fabry $Gb_3$ were similar to levels in Fabry mice. Data represent the mean ± SEM (n=5-10). *$p$<0.05, **$p$<0.01 and ***$p$<0.001 compared to Fabry control group.

### 4.4.3 Tissue GSLs are unaltered by TLC analysis

We extracted and purified the GSLs from cryo-frozen tissues in OCT blocks to confirm the total GSL content of the four groups of mice. Unfortunately, the conclusions drawn between Fabry and TKO mice from HPLC detection of $Gb_3$ could not be corroborated by TLC analysis of total GSLs, including $Gb_3$. Extraction of tissue GSLs from cryo-frozen blocks and subsequent TLC analysis revealed no significant difference in $Gb_3$ levels between Fabry and TKO mice in all tissues examined (Figure 4.3).

In each tissue examined by TLC, $Gb_3$ was markedly higher in Fabry relative to normal mice. Expression was similar between MDR1 and normal mice, and between Fabry and TKO mice. $Gb_3$ was the main GSL expressed in lung and heart, with levels essentially unaltered between Fabry and TKO mice. This result agrees with data obtained from HPLC analysis. TLC analysis of heart also reveals a band that likely corresponds to GM3 that is expressed similarly in the four mice examined.

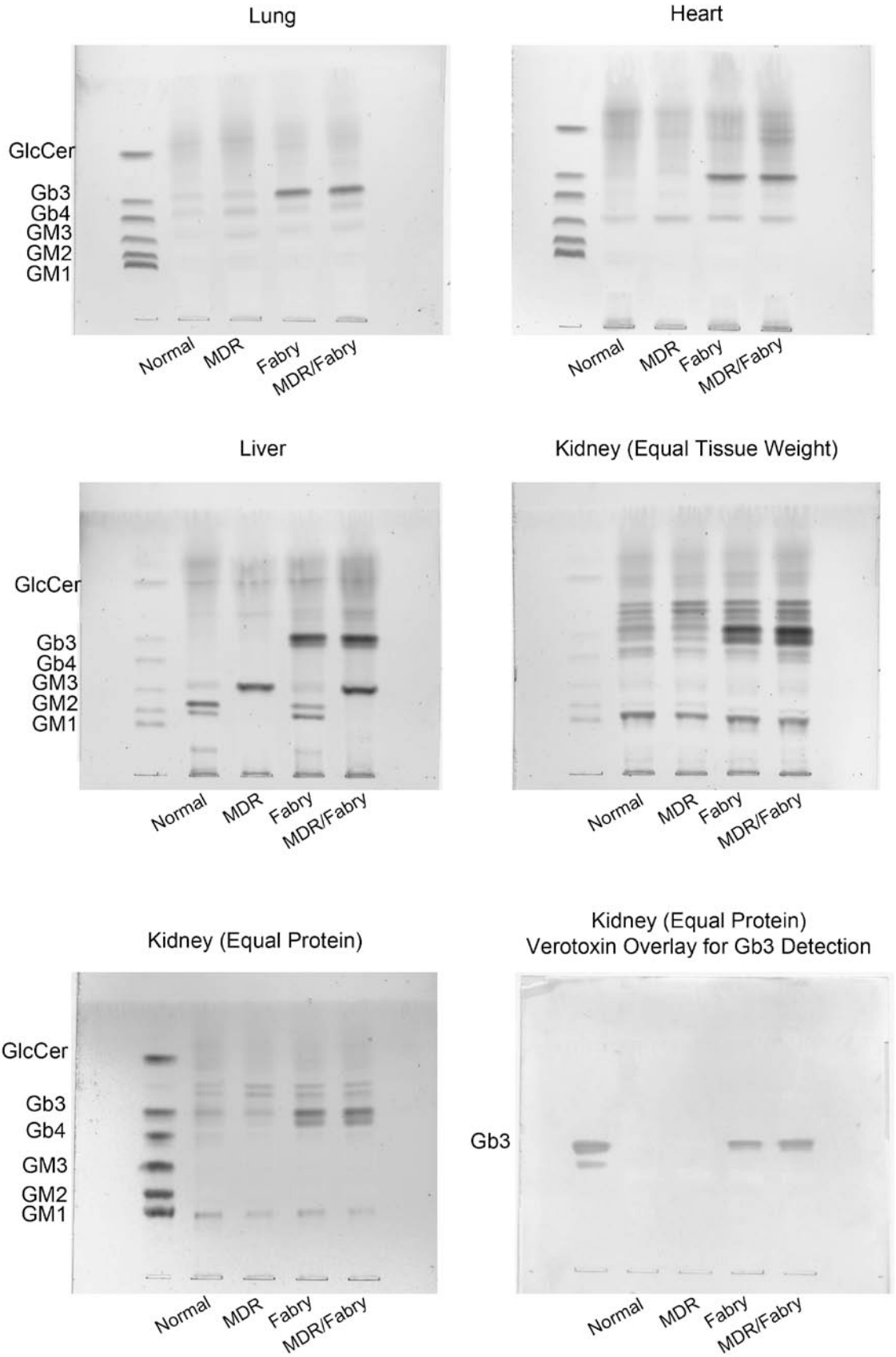

Lung
GlcCer
Gb3
Gb4
GM3
GM2
GM1
Normal
MDR
Fabry
MDR/Fabry
Heart
Normal
MDR
Fabry
MDR/Fabry
Liver
GlcCer
Gb3
Gb4
GM3
GM2
GM1
Normal
MDR
Fabry
MDR/Fabry
Kidney (Equal Tissue Weight)
Normal
MDR
Fabry
MDR/Fabry
Kidney (Equal Protein)
GlcCer
Gb3
Gb4
GM3
GM2
GM1
Normal
MDR
Fabry
MDR/Fabry
Kidney (Equal Protein)
Verotoxin Overlay for Gb3 Detection
Gb3
Normal
MDR
Fabry
MDR/Fabry

Figure 4.3. **TLC analysis of tissue GSLs shows no change in GSL levels.** Total GSLs were extracted from tissue fragments embedded in frozen OCT blocks. GSLs were purified, separated by TLC and either stained with orcinol or stained for VT binding (kidney). No difference in $Gb_3$ was observed in any tissue examined. In the liver, normal and Fabry mice expressed GM2, while MDR1 and MDR1/Fabry mice expressed GM3.

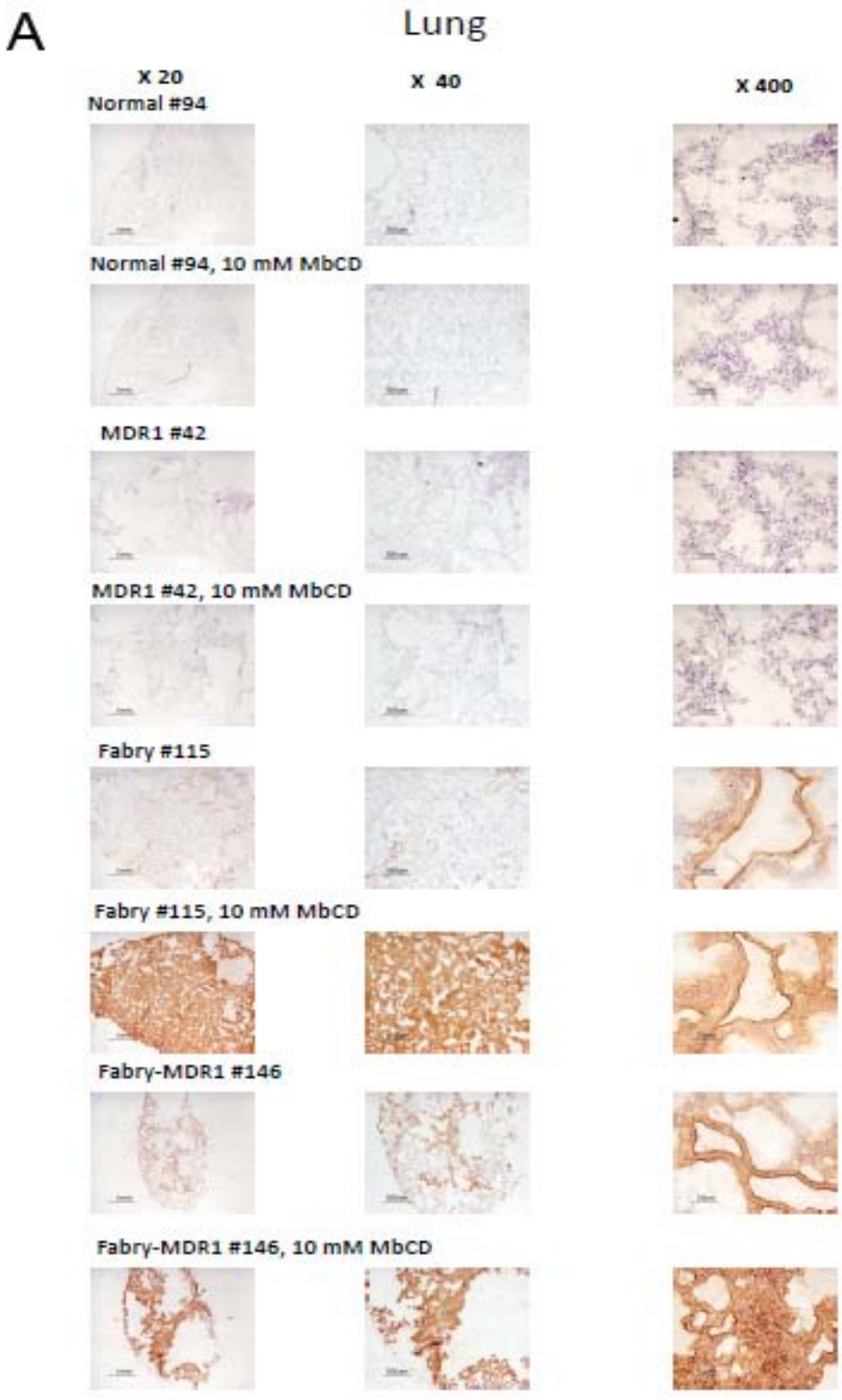
In kidney, $Gb_3$ appears to be reduced in MDR1 relative to normal mice, but unchanged in TKO versus Fabry mice. A band that migrates with GM1 is also reduced in MDR1 mice; however, this band does not appear to be GM1, as TLC overlay with cholera toxin, a known GM1 ligand, was unreactive. Although $Gb_3$, $Gb_4$ and the slower migrating species are reduced in MDR1 mice, LacCer levels are elevated.

Liver $Gb_3$ was virtually undetectable in normal and MDR1 mice. $Gb_3$ was the main GSL in Fabry and TKO liver with levels being similar in these mice. Interestingly, normal and Fabry mice appear to express GM2, with levels slightly lower in the faster migrating species in the latter and the slower species in the former. In contrast, MDR1 and TKO mice contain a band that migrates with GM3 rather than GM2.

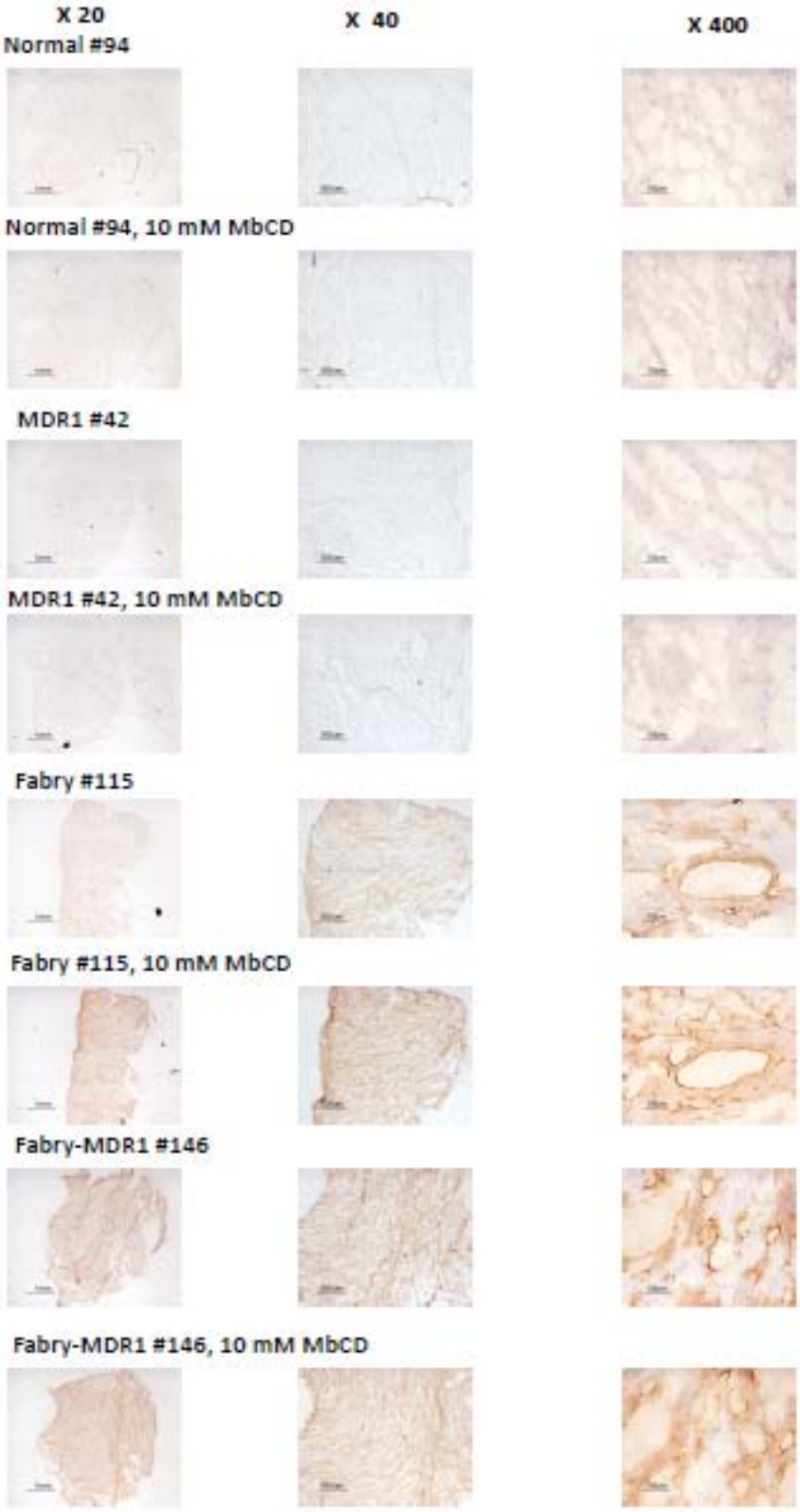
### 4.4.4 $Gb_3$ is reduced in Kupffer cells of TKO mice

We evaluated tissue $Gb_3$ levels by histochemistry to examine regional differences between the four groups of mice (Figure 4.4). Both VT1 and a monoclonal antibody against $Gb_3$ were used for $Gb_3$ binding. Cholesterol extraction from sections by methyl-β-cyclodextrin (MCD) prior to staining typically exhibited greatly increased $Gb_3$ staining in Fabry mice. Cholesterol has been shown to "mask" GSL receptors from their ligands by conferring a membrane parallel conformation of the glycan (Lingwood et al., 2011; Mahfoud et al., 2010). Such a conformation is not detected by ligands, as opposed to the membrane perpendicular conformation seen upon cholesterol depletion, which allows for ligand binding to GSL receptors (Lingwood et al., 2011).

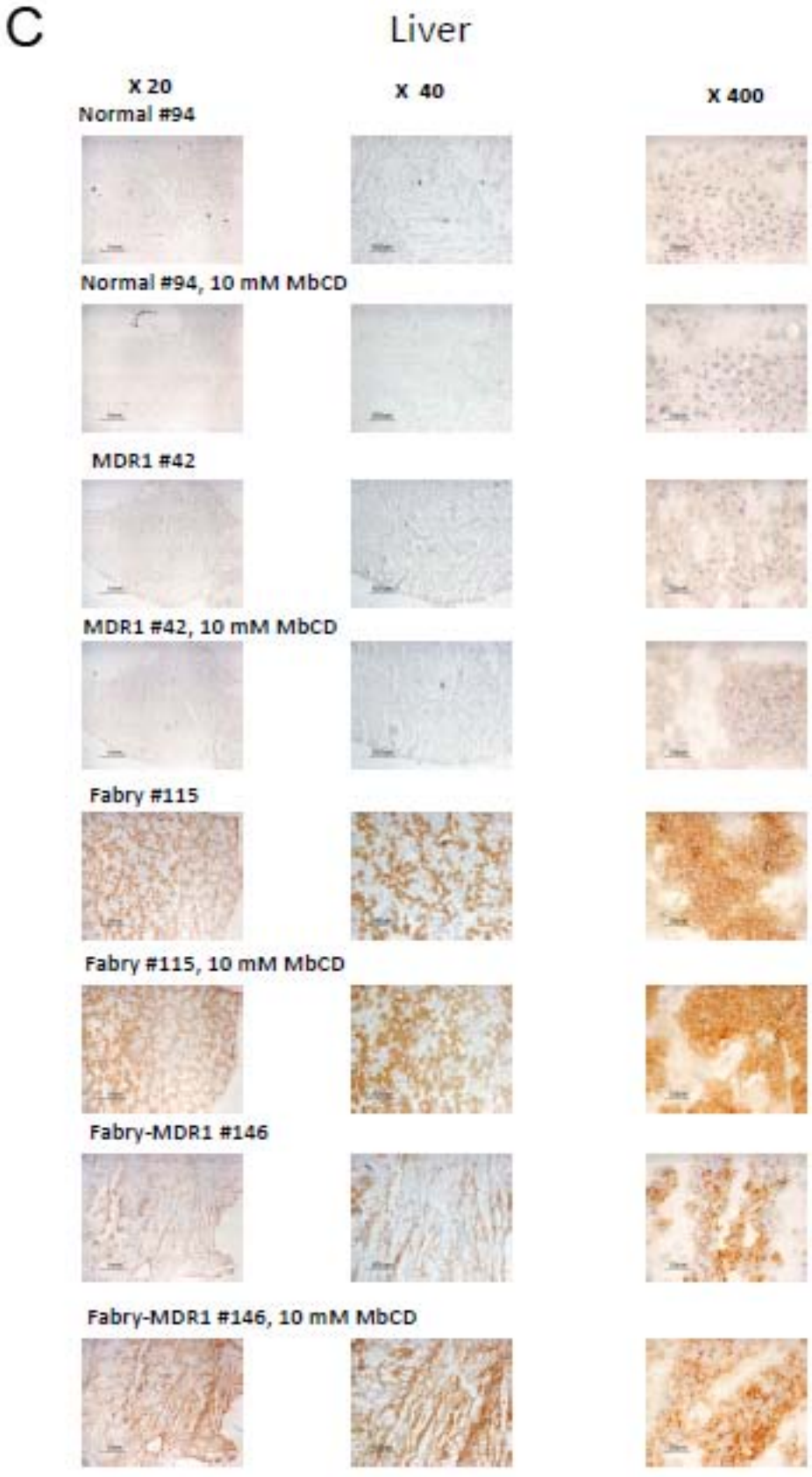
Any reduction in staining seen in TKO mice was emphasized under these cholesterol-depleted conditions.



B


Heart
X 20
X 40
X 400
Normal #94
Normal #94, 10 mM MbCD
MDR1 #42
MDR1 #42, 10 mM MbCD
Fabry #115
Fabry #115, 10 mM MbCD
Fabry-MDR1 #146
Fabry-MDR1 #146, 10 mM MbCD

C


Liver
X 20
X 40
X 400
Normal #94
Normal #94, 10 mM MbCD
MDR1 #42
MDR1 #42, 10 mM MbCD
Fabry #115
Fabry #115, 10 mM MbCD
Fabry-MDR1 #146
Fabry-MDR1 #146, 10 mM MbCD

D

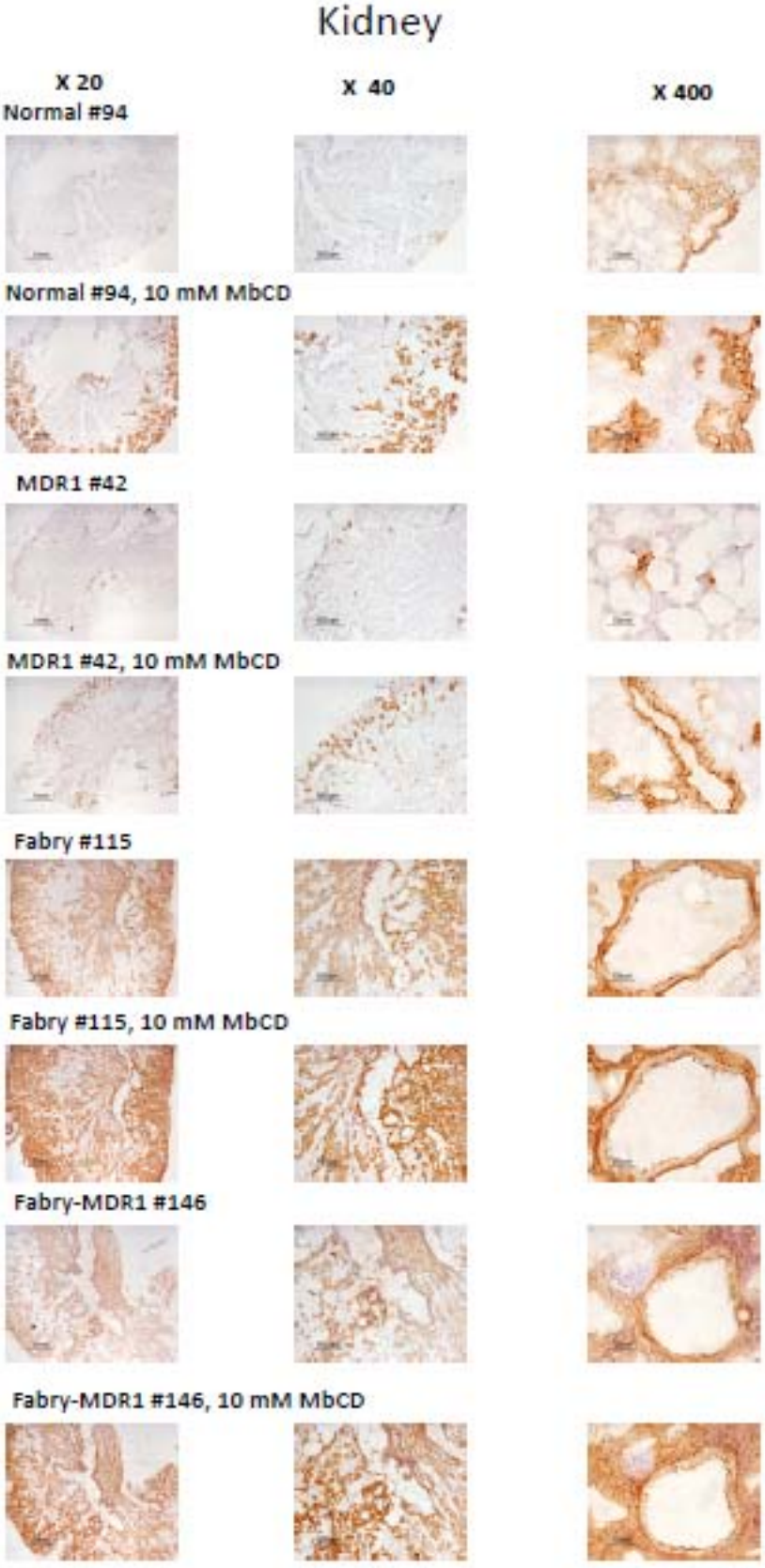

Kidney
X 20
X 40
X 400
Normal #94
Normal #94, 10 mM MbCD
MDR1 #42
MDR1 #42, 10 mM MbCD
Fabry #115
Fabry #115, 10 mM MbCD
Fabry-MDR1 #146
Fabry-MDR1 #146, 10 mM MbCD

Figure 4.4. **Liver and kidney show reduced Gb3 staining in MDR1/Fabry mice.** Tissue Gb3 levels were examined by immunohistochemistry. Fabry mouse Gb3 staining was significantly higher than wild-type and MDR1 mice for all tissues. MDR/Fabry mice showed reduced staining relative to Fabry mice in the liver (C) and kidney (D), but staining was still significantly higher than in wild-type and MDR mice Staining was similar between Fabry and MDR/Fabry mice in the lung (A) and heart (B).

Normal and MDR1 mouse lung (Figure 4.4A) and heart (Figure 4.4B) were essentially negative for $Gb_3$ staining with or without MCD treatmetnt. MCD treatment significantly enhanced $Gb_3$ staining in Fabry and TKO lung, with intensities being similar between the two. In heart, MCD treatment did not significantly increase staining in Fabry and TKO mice. Both mice showed a similar level of staining.

In the liver, we observed punctate $Gb_3$ staining in Fabry mice corresponding to Kupffer cells (Figure 4.4C). Staining was markedly lower in TKO mice, while normal and MDR1 mice were essentially negative. $Gb_3$ expression is high in the kidney of Fabry mice compared to normal mice, in which tubules mostly appear to be $Gb_3$-negative (Figure 4.4D). A reduction in $Gb_3$ staining by both methods was observed in TKO mice kidney tubules, but staining was still greater than for normal mice. MCD treatment revealed less staining in MDR1 mice relative to normal mice.

## *4.5 Discussion*

Our lab has previously shown that P-gp is involved in GSL biosynthesis and proposed that this protein is a key flippase of GlcCer, translocating it from the cytosolic leaflet of the Golgi to the lumenal leaflet (De Rosa et al., 2004). In this regard, transfection of the human MDR1 gene encoding P-gp into MDCK cells greatly increases neutral GSL content (Lala et al., 2000).

Inhibition of P-gp with cyclosporine A reduces neutral GSL levels by preventing synthesis of LacCer (De Rosa et al., 2004).

This view of P-gp as a major flippase for GlcCer has been challenged. It has been reported that P-gp is only capable of translocating short-chain GlcCer analogues and not natural GlcCer (Halter et al., 2007); however our lab has shown that microsomal synthesis of LacCer from natural GlcCer liposomes is also prevented by cyclosporine A treatment. In addition, the conclusions of the study opposing a role for P-gp in GSL biosynthesis have been drawn from experiments in HeLa cells, a cell line which has been shown to be resistant to cyclosporine-mediated inhibition of GSL synthesis, except for GlcCer, which is reduced (De Rosa et al., 2004; Smith et al., 2006).

To re-affirm the involvement of P-gp in GSL metabolism, we have generated, herein, a mouse model deficient in both MDR1 and α-galactosidase A by crossbreeding MDR1 KO mice with Fabry mice. Given our previous results with CsA-mediated MDR1 inhibition in cultured cells and Fabry mice (De Rosa et al., 2004; Lala et al., 2000; Mattocks et al., 2006), we expected to observe decreased neutral GSLs in TKO mice relative to Fabry mice. This was indeed the case in the liver, spleen and kidney using HPLC quantitation of tissue $Gb_3$. Lung and heart showed no significant differences in $Gb_3$.

These findings were corroborated for the liver and kidney using histochemistry. Spleen, however, did not show differences in $Gb_3$ staining by this method. Interestingly, cholesterol depletion by MCD markedly enhances staining such that those tissues for which TKO mice showed reduced $Gb_3$ relative to Fabry mice were more emphasized under these conditions.

Cholesterol, therefore, appears to mask $Gb_3$ from ligand detection. Decreased binding of cholera toxin and verotoxin to GM1 and $Gb_3$, respectively, in the presence of cholesterol has been recently shown and has been attributed to modulation of the GSL glycan to a more membrane parallel conformation that escapes ligand detection (Lingwood et al., 2011).

In taking tissue fragments for histology, an effort was made to acquire similar sections so reasonable comparisons between mice can be made. It is possible that the fragments taken represent regions of elevated GSL and low ABCB1 expression such that knockout of ABCB1 does not influence total GSL levels. This might explain the discrepancy between HPLC and TLC observations.

It is unclear why the livers of normal and Fabry mice express what appears to be GM2, while MDR1 and TKO mice apparently express GM3. This may be attributed to differences in the genetic backgrounds of the mice, as Fabry and MDR1 mice were from different backgrounds, with Fabry mice being C57BL/6 and MDR1 mice FVB/N. In order to correct for this and to make more representative comparisons between mice of different strains, we also bred MDR1 mice into the B6 background. GSL profiles were similar between these hybrid mice and normal B6 mice.

MDR1 a/b expression has been previously observed in the tissues we analyzed (Cui et al., 2009); however, subcellular distribution of the protein has not been assessed. A previous study by our lab in which Fabry mice were treated with cyclosporine A also revealed tissue-specific inhibition of $Gb_3$ synthesis (Mattocks et al., 2006). CsA inhibited synthesis in the liver, but not in the lung, heart and kidney. In this study, a reduction in $Gb_3$ was also observed in the kidney

by HPLC and histochemistry. Based on this, it is possible that pharmacological intervention is insufficient to overcome the high $Gb_3$ expression in kidney.

Regardless of whether the HPLC or TLC analyses are correct, it is clear that alternative mechanisms for GlcCer Golgi lumenal access exist. This is particularly true for those tissues that showed no reduction in $Gb_3$—if the HPLC analyses are correct. If P-gp was the only protein responsible, MDR/Fabry mice would be expected to lack $Gb_3$ expression. Assuming that HPLC analysis is correct, the alternative means for GlcCer Golgi lumenal access is unable to entirely compensate for loss of MDR1, indicating that it is independently regulated and not part of the same pathway.

Recently, the phosphatidylinositol-four-phosphate-adaptor protein 2 (FAPP2) has been described as a cytosolic transporter of GlcCer from its site of synthesis to a membrane region for subsequent translocation to the Golgi lumen (D'Angelo et al., 2007; Halter et al., 2007). However, there are inconsistencies between FAPP2-mediated GlcCer transport models as to whether transport is anterograde towards a distal Golgi membrane region (D'Angelo et al., 2007) or retrograde to the ER (Halter et al., 2007). In either scenario, a translocation event is still necessary for access to the lumen. This is thought to be achieved by an energy-independent mechanism in the retrograde model.

Assuming the HPLC results are correct, FAPP2 retrograde GlcCer transport may represent an MDR1-independent means for GSL biosynthesis. If any compensatory mechanisms have been activated, it is also possible that any effects of P-gp KO may have been underestimated. Thus,

we intend to use our mouse model to address levels of other proteins that may mediate GlcCer access to the Golgi lumen in the absence of P-gp.

Enzyme replacement therapy for Fabry disease patients has been used clinically with success in ameliorating some disease symptoms, including decreasing plasma $Gb_3$, reducing heart mass and decreasing pain (Pintos-Morell et al., 2010; Schiffmann et al., 2000). This treatment, however, suffers from high cost of the enzyme (Moore et al., 2007) as well as the inability to cross the blood-brain-barrier, rendering the therapy ineffective against neurological manifestations. This has stimulated interest in alternative therapeutic approaches, including enzyme enhancement therapy via pharmacological chaperones. 1-deoxygalactonojirimycin (1-DGJ, migalastat, Amigal $^{TM}$) is currently in phase III clinical trials for treatment of Fabry disease (Motabar et al., 2010). However, given that a number of different mutations in the α-galactosidase gene have been identified, each of which can potentially give rise to distinct effects on protein folding, many different compounds will likely be required to address the different mutations.

Preventing synthesis of the offending substrate is, therefore, an attractive treatment option and this can be accomplished by different approaches, involving direct inhibition of the biosynthetic enzyme responsible for synthesizing the accumulating substrate or preventing GlcCer access to the Golgi lumen by inhibiting P-gp, FAPP2 or other potential GlcCer flippases. Thus identifying the mechanism of GlcCer Golgi lumenal access is also of clinical importance.

A close relationship has been suggested to exist between P-gp-induced drug resistance in cancer cells and GSL synthesis; in particular, overexpression of GCS is accompanied by increased P-gp

expression and downregulation of GCS reduces P-gp (Gouazé et al., 2005). GSLs with a short acyl-chain (Veldman et al., 1999), GSL analogues (De Rosa et al., 2008) and GCS inhibitors can inhibit P-gp (Chai et al., 2011). Based on our HPLC analyses, depending on the cancer tissue origin, it may be prudent to consider that P-gp and GCS are part of a common metabolic pathway.

Our novel triple knockout (mdr1a/mdr1b/α-gal A) mouse model allows us to study the role of MDR1 in GSL metabolism and further investigate the alternative mechanisms involved in GlcCer access to the Golgi lumen in tissues where MDR1 shows no effect. Unfortunately, given our data, we cannot yet decisively state the involvement of MDR1 in specific tissues.

# Chapter 5: Glucosylceramide access to Golgi glycosyltransferases involves multiple mechanisms

Project was initiated by M. Kamani. Technical assistance was provided by Marko Drobac and Delowar Hossain. Manuscript is in preparation.

# Chapter 5: Glucosylceramide access to Golgi glycosyltransferases involves multiple mechanisms

**Mustafa Kamani, Beth Binnington, Zhenbo Zhang and Clifford A. Lingwood**

## *5.1 Abstract*

The topology of GCS has posed a problem for 30 years. A cytosolic-oriented catalytic centre requires translocation of GlcCer from the cytosolic leaflet of the Golgi to the lumenal leaflet for access to all downstream glycosyltransferases, whose catalytic sites are lumenally-oriented. Two predominant theories have been proposed to achieve this objective: one involves direct translocation of GlcCer by P-glycoprotein; the other is a more complex process involving retrograde transport of GlcCer by FAPP2 from the site of synthesis at the Golgi back to the ER, followed by vesicular traffic to allow Golgi lumenal access. We show that knockdown of each of these proteins reduces a particular subset of GSLs in DU-145 cells—selected because of their extensive neutral and acidic GSL composition—and that double knockdown has a slight additive effect. In particular, ABCB1 gene knockdown preferentially reduces GlcCer, LacCer and ganglioside levels, while FAPP2 knockdown selectively reduces neutral GSLs. However, neither protein appears to be involved in ACHN cell GSL biosynthesis. We also show knockdown of the aminophospholipid flippase ATP8B1 to elevate P-gp levels and total GSLs. Our results indicate that multiple mechanisms are likely involved in GlcCer Golgi lumenal access, involving P-gp, FAPP2 and possibly other proteins, according to cell type. Identifying the cell-selective mechanisms by which GlcCer can access the Golgi lumen will be useful therapeutically as a means for targeted regulation of GSL biosynthesis.

## *5.2 Introduction*

Glucosylceramide (GlcCer) is the prescursor glycosphingolipid (GSL) for most GSLs. The enzyme responsible for synthesizing GlcCer, GlcCer synthase (GCS) is unique in that its catalytic activity is cytoplasmically oriented, whereas the catalytic site of all downstream glycosyltransferases (type 2 membrane glycoproteins) involved in the formation of more complex GSLs is localized to the Golgi lumenal membrane (Coste et al., 1986; Jeckel et al., 1992; Maccioni et al., 2011b). Thus, the topological difference necessitates a mechanism to transport or translocate GlcCer from the cytoplasmic to the lumenal leaflet of the Golgi for complex GSL synthesis. Two different mechanisms have been proposed to carry out this function: one involves direct translocation of GlcCer by the ABC transporter P-glycoprotein (P-gp, ABCB1, MDR1, EC 3.6.3.44) (De Rosa et al., 2004). P-gp is a 170 kDa protein that is responsible for conferring drug resistance to cells, particularly tumour cells, which overexpress the protein at the cell surface and elsewhere (Molinari et al., 1994). Thus, P-gp has been a major target of pharmacological inhibition for several years in order to overcome resistance to drugs (Hennessy and Spiers, 2007).

In addition to its role at the surface to limit entry of toxic hydrophobic compounds into cells, P-gp is involved in the translocation of GlcCer analogues from the cytosolic to the exoplasmic leaflet of the plasma membrane (Van Helvoort et al., 1996). Transfection of the ABCB1 gene into epithelial cells enables translocation of the GlcCer analogues NBD-GlcCer, $C_8C_8$-GlcCer and $C_6$-GlcCer, as well as short-chain fluorescent analogues of PC and phosphatidylethanolamine (PE) to the exoplasmic leaflet of the apical plasma membrane (Van Helvoort et al., 1996). This flipping of substrates was prevented by inhibitors of P-gp drug

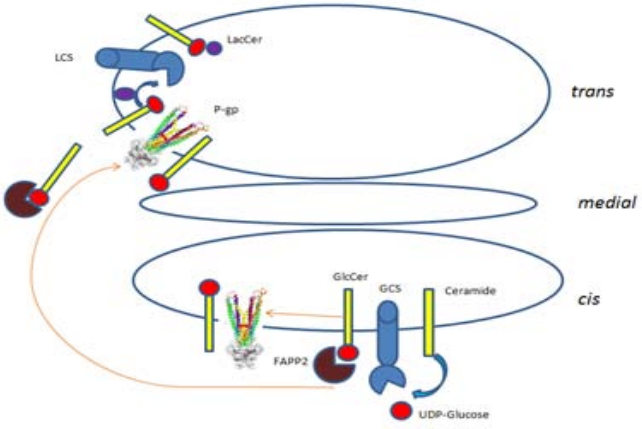
efflux (Van Helvoort et al., 1996). Reconstitution of P-gp into liposomes similarly translocates GlcCer analogues, showing a flippase function of the protein (Eckford and Sharom, 2005).

P-gp is also found at the Golgi (Molinari et al., 1994). We have previously shown that transfection of the human MDR1 gene encoding P-gp into MDCK cells markedly elevates GSL levels (Lala et al., 2000), while pharmacological inhibition of P-gp using cyclosporine A or ketoconazole inhibits neutral GSL synthesis (De Rosa et al., 2004). Inhibition of the multidrug resistance-associated protein (MRP) did not cause such inhibition, suggesting that this phenomenon is specific to P-gp (De Rosa et al., 2004). Thus, P-gp appears to be involved in GlcCer translocation across the Golgi membrane.

A second model for GlcCer access to the Golgi lumen involves transport of GlcCer by the phosphatidylinositol four-phosphate adaptor protein 2 (FAPP2) (D'Angelo et al., 2007; Halter et al., 2007). FAPP2 is a cytosolic protein recruited to the Golgi via interaction between its PH domain and the Golgi localized phosphatidylinositol-4-phosphate (PI4P) and ADP-ribosylation factor 1 (ARF1) (Godi et al., 2004). The protein also contains a glycolipid transfer protein (GLTP) domain. FAPP2 transports GlcCer either to a distal Golgi membrane region for subsequent flipping into the Golgi lumen (D'Angelo et al., 2007), or back to the ER for non-specific flipping to the ER lumen, followed by vesicular trafficking to the Golgi (Halter et al., 2007). Knockdown of FAPP2 in HeLa cells decreases all GSLs downstream of GlcCer (D'Angelo et al., 2007).

It is possible that the two mechanisms are not independent; rather, they may be part of the same pathway such that GlcCer on the cytosolic Golgi leaflet is carried by FAPP2 to a region of the

Golgi that contains P-gp for subsequent translocation of GlcCer across the Golgi membrane (Scheme 5.1). We have, therefore, initiated a comprehensive study examining the involvement of both P-gp and FAPP2 in GSL metabolism by means of a gene knockdown (KD) approach. This method has allowed us to compare the relative contributions of each of these proteins to GSL metabolism. As a positive control, we also generated a GlcCer synthase (GCS) KD. We show KD of each protein to affect GSL levels in prostate carcinoma DU-145 cells. KD of ABCB1 and FAPP2 moderately reduce $Gb_3$ in A431 cells, while no GSL reduction is observed in ACHN cells.




Scheme 5.1. **Proposed model for GlcCer Golgi lumenal access dependent on FAPP2 and P-gp.** In the hypothesized model, FAPP2 transports GlcCer from its site of synthesis to P-gp for translocation to the lumenal leaflet of the Golgi and access to downstream glycosyltransferases. In addition, GlcCer synthesized at the *cis* Golgi can also be translocated by P-gp in a FAPP2-independent mechanism. Thus, P-gp and FAPP2 are proposed to function within the same pathway.

## *5.3 Experimental Procedures*

### 5.3.1 Cell Culture

DU-145 (prostate carcinoma) cells were maintained in DMEM supplemented with 10% FBS. ACHN (renal adenocarcinoma) cells were maintained in EMEM supplemented with 10% FBS. MDCK cells transfected with the human MDR1 gene (MDR-MDCK) and A431 cells were cultured in DMEM supplemented with 5% FBS.

### 5.3.2 siRNA sequences

Negative Control:

Sense 5’-UUC UCC GAA CGU GUC ACG UdTdT-3’

Anti-sense 5’-ACG UGA CAC GUU CGG AGA AdTdT-3’

GAPDH Position 519

Sense 5’-GUA UGA CAA CAG CCU CAA GdTdT-3’

Anti-sense 5’-CUU GAG GCU GUU GUC AUA CdTdT-3’

MDR1 Position 657

Sense 5`-CUU UGG CUG CCA UCA UCC AdTdT-3’

Anti-sense: 5’-UGG AUG AUG GCA GCC AAA GdTdT-3’

MDR1 Position 2187

Sense 5’- GCG AAG CAG UGG UUC AGG UdTdT-3’

Anti-sense 5’- ACC UGA ACC ACU GCU UCG CdTdT-3’

MDR1 Position 3323

Sense 5’- CAC CCA GGC AAU GAU GUA UdTdT-3’

Anti-sense 5’-AUA CAU CAU UGC CUG GGU GdTdT-3’

GCS Position 386

Sense 5’-CCC GAU UAC ACC UCA ACA AdTdT-3’

Anti-sense 5’-UUG UUG AGG UGU AAU CGG GdTdT-3’

GCS Position 833

Sense 5’-CCA CCU UAG AGC AGG UAU AdTdT-3’

Anti-sense 5’-UAU ACC UGC UCU AAG GUG GdTdT-3’

GCS 1354

Sense 5’-CGC GAA UCC AUG ACA AAU UdTdT-3’

Anti-sense 5’-AUA UUG UCA UGG AUU CGC GdTdT-3’

FAPP2 229

Sense 5’-GGC AGU CUG UGA AAU UCA AdTdT-3’

Anti-sense 5’-UUG AAU UUC ACA GAC UGC CdTdT-3’

FAPP2 265

Sense 5’-CAC GCA UGG ACC UGA UAA UdTdT-3’

Anti-sense 5’-AUU AUC AGG UCC AUG CGU GdTdT-3’

FAPP2 1163

Sense 5’-GAU GGA UCU UGU UGG AAA UdTdT-3’

Anti-sense 5’-AUU UCC AAC AAG AUC CAU CdTdT-3’

β4GalT5 560

Sense 5’-GCC CAA UAG ACA UAA ACA UdTdT-3’

Anti-sense 5’-AUG UUU AUG UCU AUU GGG CdTdT-3’

β4GalT5 731

Sense 5’-CCU GUU CAG ACA CCU GCU UdTdT-3’

Anti-sense 5’-AAG CAG GUG UCU GAA CAG GdTdT-3’

β4GalT5 1199

Sense 5’-CCA GUU UCU UGG AAG GUA UdTdT-3’

Anti-sense 5’-AUA CCU UCC AAG AAA CUG GdTdT-3’

β4GalT6 430

Sense 5’-GGU ACA AGC UCG AGG UAU A dTdT-3’

Anti-sense 5’-UAU ACC UCG AGC UUG UAC C dTdT-3’

β4GalT6 514

Sense 5’-GCU CAA CGG UAC AGA UUA UdTdT-3’

Anti-sense 5’-AUA AUC UGU ACC GUU GAG CdTdT-3’

β4GalT6 951

Sense 5’-GUG UCU GGG ACU GUG UAA UdTdT-3’

Anti-sense 5’-AUU ACA CAG UCC CAG ACA C dTdT-3

ATP8B1 645

Sense 5’-GGA CGU GUG AAG UCA UUA AdTdT-3’

Anti-sense 5’-UUA AUG ACU UCA CAC GUC CdTdT-3’

ATP8B1 1381

Sense 5’-GGA CCU GCA AAU GUA CUA UdTdT-3’

Anti-sense 5’-AUA GUA CAU UUG CAG GUC CdTdT-3’

ATP8B1 3630

Sense 5’-GGC CAU CAG AAA GUG AUA AdTdT-3’

Anti-sense 5’-UUA UCA CUU UCU GAU GGC CdTdT-3’

### 5.3.3 siRNA Transfection

Cells were seeded into 6-well plates (for protein and RNA isolation) or 10 cm dishes (for GSL extraction) overnight. Immediately prior to transfection, culture medium was replaced with serum-free medium. As per the manufacturer’s (Life Technologies) protocol, 100 pmol siRNA was diluted in 250 µL Optim-MEM® I Reduced Serum Medium (600 pmol siRNA in 1.5 mL Opti-MEM® for 10 cm dishes) and 4 µL Lipofectamine™ 2000 was diluted in 250 µL Opti-MEM® (24 µL Lipofectamine in 1.5 mL Opti-MEM® for 10 cm dishes). The solutions were incubated at room temperature for approximately 5 min, followed by mixing the two together and incubating at room temperature for 20-25 min. Cells were transiently transfected with siRNA/lipofectamine complexes in a total volume of 2 mL (15 mL for 10 cm dishes). After approximately five hours, cell medium was replaced with fresh medium supplemented with FBS and cells were grown for 48 h.

### 5.3.4 Primer Sequences

MDR1 Forward: 5’- GCT CAT CGT TTG TCT ACA GTT CGT-3’

MDR1 Reverse: 5’- GCT CCA GTG GTG TTT TTA GGG TC-3’

PCR Product Size: 683 bp

GCS Forward: 5’- TAT CAT CTA CAC CCG ATT ACA CCT C -3’

GCS Reverse: 5’- GAA ACC AGT TAC ATT GGC AGA GAT A -3’

PCR Product Size: 529 bp

FAPP2 Forward: 5’- CTA TTA TGA TTC TCC TGA AGA TGC C-3’

FAPP2 Reverse: 5’- TCT TTC TCC TTC TGG GTC CTA CT -3’

PCR Product Size: 231 bp

GAPDH Forward: 5’- AAC TTT GGT ATC GTG GAA GGA CT -3’

GAPDH Reverse: 5’- GCT TCA CCA CCT TCT TGA TGT C-3’

PCR Product Size: 292 bp

β4GalT5 Forward: 5’- GGC GCC CGG CAT AGT GAA CA -3’

β4GalT5 Reverse: 5’- GAG GTG CTC GTG GCG GTT CC -3’

PCR Product Size: 430 bp

β4GalT6 Forward: 5’- GCA GCG GCT GGA ATT TGC GT -3’

β4GalT6 Reverse: 5’- CAG AGC AGG GAG GAC GGG TGA -3’

PCR Product Size: 292 bp

### 5.3.5 RNA Isolation

Cells in 6-well plate were washed with PBS. TRIzol® (Invitrogen) was added (1 mL per well) and the plate was incubated on ice for 5 min. Cells were collected in RNAse-free microfuge tubes, followed by addition of 200 µL $CHCl_3$ and gently mixing by inverting. Tubes were centrifuged at 4°C for 15 min at 12,000 rpm. The transparent upper layer was transferred to a new RNAse-free tube, taking care not to take any of the loose precipitated interface. Pre-cooled isopropanol was added (0.5 mL per mL of TRIzol®) and the contents were mixed by inverting, followed by incubation on ice for 15 min or overnight at -20°C. Tubes were centrifuged at 4°C and 12,000 rpm for 10 min. The supernatant was discarded and the pellet washed with pre-cooled 75% ethanol. Tubes were centrifuged at 10,000 rpm, 4°C for 5 min. The supernatant was discarded and the pellet was dissolved in DEPC $H_2O$. RNA concentration was measured using a spectrophotometer.

### 5.3.6 PCR

100-200 ng cDNA, 20-40 pmol of forward and reverse primers and autoclaved water to a total volume of 20 µL were added to PCR tubes containing a Taq polymerase pre-mix. PCR conditions were as follows: 1) 95°C strand separation for 5 min, 2) 95°C denaturation for 50 s, 3) 52°C to 59°C annealing for 50 s (temperature depends on primers being used), 4) 72°C extension for 40 s, 5) Steps 2-4 were repeated 19 to 34 times (cycle number varies for each gene), 6) 72°C completion of extension for 10 min. PCR was optimized to produce a band of appropriate density so as to be able to observe the effect of gene knockdown by siRNA (semi-quantitative analysis).

### 5.3.7 Membrane protein isolation

Cells from 10 cm dishes or one well of a 6-well plate were scraped and collected in PBS. The cell pellet was obtained by centrifugation at 7000 rpm, for 7 min at 4°C, followed by re-suspending in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1mM $MgCl_2$, protease inhibitor cocktail) as per Romiti et al. (2002) and incubation on ice for 15 min. Cells were lysed by passing through a 25 3/8 guage syringe approximately 20 times, followed by a low-speed (600x g, 10 min) centrifugation. The supernatant was transfered to new tubes. The pellet was re-suspended in a small volume of supernatant and passed through the syringe 20 times, centrifuged and supernatant pooled with the first round. Supernatants were transferred to high-pressure microfuge tubes and centrifuged on a table-top ultracentrifuge at 28,000 rpm to collect the membrane fraction. Supernatants were discarded and pellets re-dissolved in 50 µL lysis buffer (+/- 1% Triton-X100). Proteins were quantitated by BCA assay.

### 5.3.8 Western blot

For P-gp Western blots, 5 µg of total protein were incubated with SDS-loading buffer containing β-mercaptoethanol at room temperature for 20-30 min. Protein samples were loaded on either a 6% or 7.5% SDS-PAGE. Gels were run at a constant voltage of 150 V (for Next Gel system) or a constant current of 0.40 Amps (conventional gel system). Gels were rinsed in water and transferred to a nitrocellulose membrane at 100 V for 72 min. Membranes were washed with TBST and blocked overnight at 4°C, followed by washing twice in TBST. Membranes were incubated with 1 in 1000 anti P-gp C219 or 1 in 5000 $NH_2$11 (kindly provided by Dr. U.S. Rao, Texas Tech University Health Sciences Center, USA) for 2 h at room

temperature. For GCS blots, membranes were incubated with 1 in 1000 anti-GCS 6.2 (kindly provided by Dr. David Marks, Mayo Clinic College of Medicine, Minnesota). For GAPDH blots, membranes were incubated with 1 in 8000 anti-GAPDH for 1 h at room temperature. Membranes were washed three times in TBST, incubated with 1 in 6000 to 1 in 8000 goat anti-rabbit or goat anti-mouse HRP for 60-75 min. Membranes were washed four times in TBST and developed by ECL detection.

### 5.3.9 GSL Extraction

Adherent cells were washed, trypsinized, counted, then pelleted at 1300 x *g* for 10 min and washed in PBS. Total cellular lipids were extracted in $CHCl_3$:$CH_3OH$ (2:1, v/v) with shaking for 3 h or 24 h. Extracts were passed through glass wool to filter cellular debris, and dried under nitrogen. Phospholipids were saponified with 0.5 mL of 0.5 M methanolic NaOH for 1 h at 37°C or overnight at room temperature. Extracts were neutralized with 0.5 mL of 0.1 M methanolic $NH_4OAc$ and 0.5 mL of 0.5 M $HCl_{(aq)}$. Methanol was diluted to < 20% with water and the preparation was applied to a $C_{18}$ column. Salts were removed with water and GSLs eluted with 5 mL $CH_3OH$, followed by 4 mL $CHCl_3$:$CH_3OH$ (2:1). Eluates were dried and re-suspended in $CHCl_3$:$CH_3OH$ (2:1) equivalent to $10^5$ cells/μl.

### 5.3.10 TLC

The GSL extract from an equivalent of $10^6$ adherent cells or $2x10^6$ lymphoblasts was applied to a TLC plate and separated in $CHCl_3$:$CH_3OH$ (98:2), followed by $CHCl_3$:$CH_3OH$:$H_2O$ (60:40:9 v/v/v). Brief iodine staining to visualize sphingomyelin was used as an internal loading control.

Iodine stain was removed by brief heating. GSLs were then visualized by spraying TLC plates with orcinol and heating at 110°C until bands appeared.

## *5.4 Results*

In order to evaluate the effect of ABCB1 knockdown on GSLs, a cell line expressing a full complement of both acidic and neutral GSLs is preferable. In addition, the selected cell line must express the protein of interest. Having examined our history of TLCs for GSL expression by various cell lines, we decided to conduct our experiments on the prostate cancer DU-145 cell line. These cells have also been reported to express detectable levels of P-gp by Western blot (Theyer et al., 1993). We first optimized RT-PCR conditions for each of our target genes to determine the appropriate concentration of reagents and assay cycle number for semi-quantitative analysis of potential differences in gene expression. Target gene sequences are shown in Figure 5.1.

**A**

Homo sapiens UDP-glucose ceramide glucosyltransferase (UGCG)
NCBI Reference Sequence: NM_003358.1
>gi|4507810|ref|NM_003358.1| Homo sapiens UDP-glucose ceramide glucosyltransferase (UGCG), mRNA 291-1475

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**B**

Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), mRNA
NCBI Reference Sequence: NM_000927.4

>gi|318037598|ref|NM_000927.4| Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), mRNA

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**c**

Homo sapiens FAPP2
NCBI Reference Sequence: NM_001197026.1
>gi|308153326|ref|NM_001197026.1| Homo sapiens pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 8 (PLEKHA8), transcript variant 1, mRNA

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AAAAAAAAAAA

**D**

Homo sapiens GAPDH
NCBI Reference Sequence: NM_002046.3
gi|83641890|ref|NM_002046.3| Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA (103-1110)

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Figure 5.1. **Gene sequences of human GCS, ABCB1, FAPP2 and GAPDH.** The mRNA sequences of human GCS (A), ABCB1 (B), FAPP (C) and GAPDH (D) are shown. ATG start codon is indicated in bold font and highlighted in yellow. The selected siRNA sequences for each gene are highlighted in blue with red text. Sequences were obtained from the NCBI database, with accession numbers indicated.

## 5.4.1 siRNAs specifically knockdown target genes

We performed transient siRNA knockdowns of four different gene products: ABCB1, FAPP2, GCS and GAPDH, along with a negative control sequence not complementary to any region in the human genome. RT-PCR was performed after 48 h transient transfection of siRNAs. Since different siRNA sequences targeting the same gene have variable effects on gene knockdown, three different siRNAs were designed (except for GAPDH) and used in an effort to obtain a sequence with maximal gene knockdown. Results confirm the varying ability of different siRNA sequences to knockdown target genes (Figure 5.2 to 5.4). The name given to each siRNA corresponds to its position in the genomic sequence. For each gene, we selected the siRNA that produced the greatest knockdown for use in subsequent experiments.

### 5.4.2 GCS knockdown reduces total GSLs

At least one of the three siRNA sequences for each gene showed significant knockdown by RT-PCR (Figure 5.2A). RNA results were then correlated to protein expression by Western blot. The antibody against GCS (kindly provided by Dr. David Marks, Mayo Clinic College of Medicine, Minnesota) detects a 38 kD species corresponding to GCS (Marks et al., 1999). Each of the three siRNAs against GCS shows reduction in intensity of this band relative to the negative control (Figure 5.2B).

Having verified successful knockdown of target genes, we examined the effect on GSL levels. Following transient transfection of cells with siRNA against GCS, total GSLs were extracted, purified and separated by TLC. Knockdown of GCS, our positive control, should result in ablation of total GlcCer-based GSLs since GlcCer is the precursor for all downstream GSLs. Results confirmed our hypothesis, with each of the three siRNAs reducing levels of all observed GSLs on the TLC (Figure 5.2C). GCS 833 showed the greatest reduction by RT-PCR and the strongest decrease in GSLs. Correspondingly, GCS 1354 had the weakest effect on gene and GSL reduction.

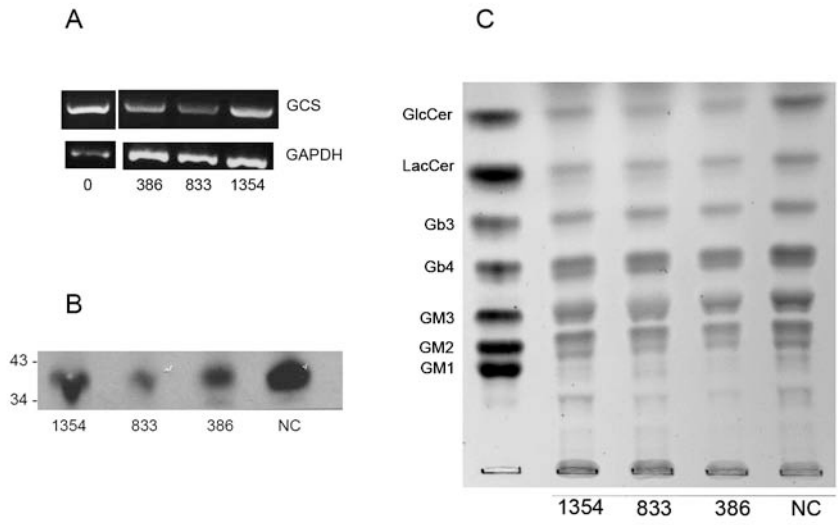


Figure 5.2. **GCS knockdown reduces GCS transcript, protein and total GSLs.** Three different siRNA, along with a negative control siRNA, were examined for their ability to knockdown GCS transcript (A), protein (B) and GSLs (C). Three separate experiments were conducted for assessment of gene, protein and GSL levels. DU-145 cells were transiently transfected with siRNA for 48 h, followed by extraction of RNA, protein or GSLs. (A) RNA was reverse transcribed and the resulting cDNA was PCR-amplified using primers for amplification of a portion of the GCS gene. The products of the reaction were separated on an agarose gel. GCS siRNA 833 showed the greatest reduction at the gene level, followed by GCS 386 and then 1354. (B) Western blot with anti-GCS 6.2 anitbody (kindly provided by Dr. Marks) showed a similar pattern of reduction. (C) An equivalent of $10^6$ cells were separated by TLC and detected with orcinol. Total GSLs were significantly reduced relative to the negative control transfection in a pattern corresponding to the extent of gene and protein reduction.

### 5.4.3 ABCB1 knockdown preferentially reduces gangliosides in DU-145 cells

We performed a Western blot to examine ABCB1 protein levels in control and ABCB1 KD cells. Unfortunately, we were unable to detect endogenous P-gp levels using the standard C219 anti-P-gp antibody. We attempted to obtain an enriched membrane protein fraction to concentrate P-gp in hope of detecting endogenous levels. However, this approach was also unsuccessful in detecting P-gp.

To confirm that our siRNA is, in fact, able to suppress synthesis of P-gp, we used MDCK cells that have been transfected with the human ABCB1 gene (MDR1-MDCK). These cells

overexpress P-gp; thus, it was thought that knockdown of a protein from highly expressing cells may not be achievable. Western blots, however, revealed significant KD of P-gp by approximately 60% relative to negative control siRNA using two different antibodies that target distinct epitopes in the protein (Figure 5.3B).

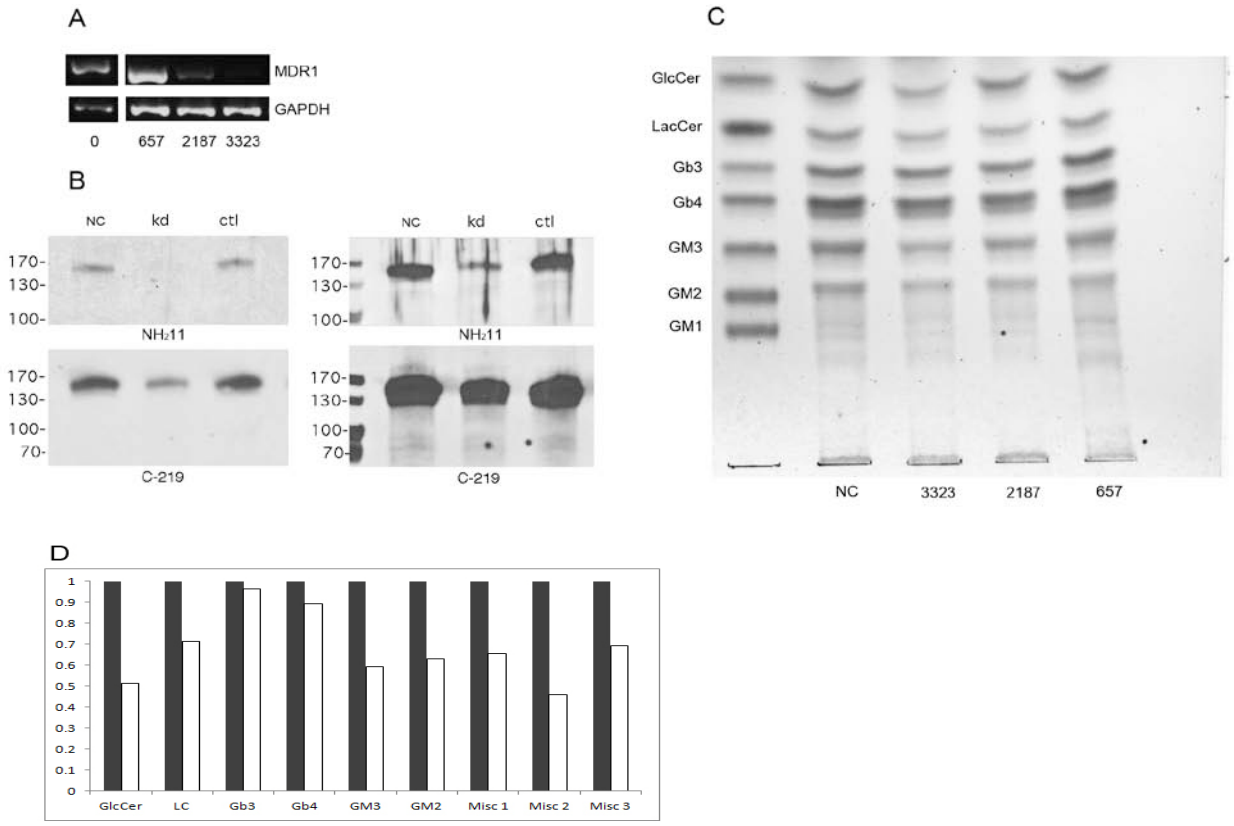


Figure 5.3. **Effects of ABCB1 knockdown.** DU-145 cells were transiently transfected for 48h with three different siRNA targeting the *ABCB1* gene (A and C). (A) RNA was reverse transcribed and the resulting cDNA was PCR-amplified using primers for amplification of a portion of the ABCB1 gene. Products were run on an agarose gel. ABCB1 3323 markedly reduces transcript, while 657 has little effect. (B) MDCK cells transfected with human *ABCB1* were transiently transfected with ABCB1 3323 for 48 h, followed by protein extraction and Western blotting with anti-P-gp C219 or $NH_2$11 (kindly provided by Dr. Rao). ABCB1 levels are significantly reduced by 3323 treatment. The right panel is the same Western blot with exposed for longer. (C) Orcinol-sprayed TLC of total GSLs from an equivalent of $10^6$ cells from siRNA treatments. Strong reduction of GlcCer, LacCer and gangliosides is observed with 3323. 657 marginally decrease these GSLs, while 2187 shows an intermediate effect. (D) Quantitation of TLC. Intensities of GSL species in NC (dark bars) and 3323 KD (open bars) were measured by ImageJ. Unidentified complex GSLs are labelled as "Misc".

At the level of GSLs, KD of ABCB1 appeared to preferentially reduce ganglioside levels (Figure 5.3C). In knocked down cells, GlcCer and LacCer levels were reduced to approximately 50% and 70% of control, respectively, while $Gb_3$ and $Gb_4$ were essentially

unaltered. GM3 and GM2 were reduced by 40 and 37%, respectively. Other higher GSLs whose identity has not been confirmed were also reduced by 30 to 55%. Knockdown of GAPDH had no affect on GSLs.

### 5.4.4 FAPP2 knockdown preferentially reduces neutral GSLs in DU-145

Unfortunately, due to limited studies on FAPP2, suitable antibodies against the protein are scarcely available, if at all present. We were unable to identify the FAPP2 species amongst the numerous bands using a commercially available antibody. We have, therefore, been unable to show the effects of FAPP2 siRNA on actual protein levels. In terms of GSLs, KD of FAPP2 reduced GlcCer levels to less than 50% of control, with LacCer also diminished to approximately 70% of control (Figure 5.4B). $Gb_3$ and $Gb_4$ levels were reduced by less than 20%, while gangliosides were reduced by 0 to 13% of control, with the exception of GM1, which was reduced by 81%. Importantly, the extent of reduction in each case corresponded to the level of reduction seen at the mRNA level; the greater the effect of siRNA on mRNA, the greater the reduction of GSLs.

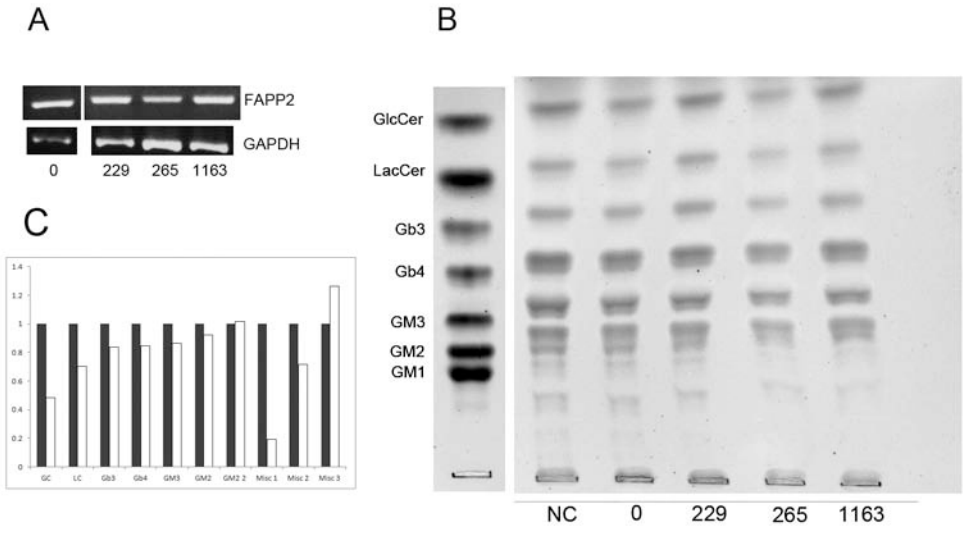


Figure 5.4. **Effects of FAPP2 siRNA.** DU-145 cells were transiently transfected for 48 h with three different siRNA targeting the FAPP2 gene. (A) RNA was extracted and reverse transcribed, and the resulting cDNA was PCR-amplified using primers for amplification of a segment of the FAPP2 gene. Products were separated by agarose gel separation of. FAPP2 265 shows the strongest reduction at the gene level, followed by 229. (B) TLC separation of total GSLs from an equivalent of $10^6$ cells from siRNA treatments. 265 shows reduction of neutral GSLs, while 1163 has little effect on GSL levels. (C) ImageJ Quantitation of GSL species from NC (dark bars) and 265 KD (open bars). Unidentified GSLs are labelled as "Misc".

### 5.4.5 ABCB1 and FAPP2 double knockdown enhances GSL reduction in DU-145 cells

To examine whether ABCB1 and FAPP2 act within the same biosynthetic pathway or they are independent of one another, we performed a double knockdown using siRNA against both ABCB1 and FAPP2. ABCB1 and FAPP2 transcript levels are both reduced in double knockdown cells, while GCS is unaltered. Cells doubly knocked down yielded a slightly greater reduction in both neutral and acidic GSLs than individual KDs (Figure 5.5).

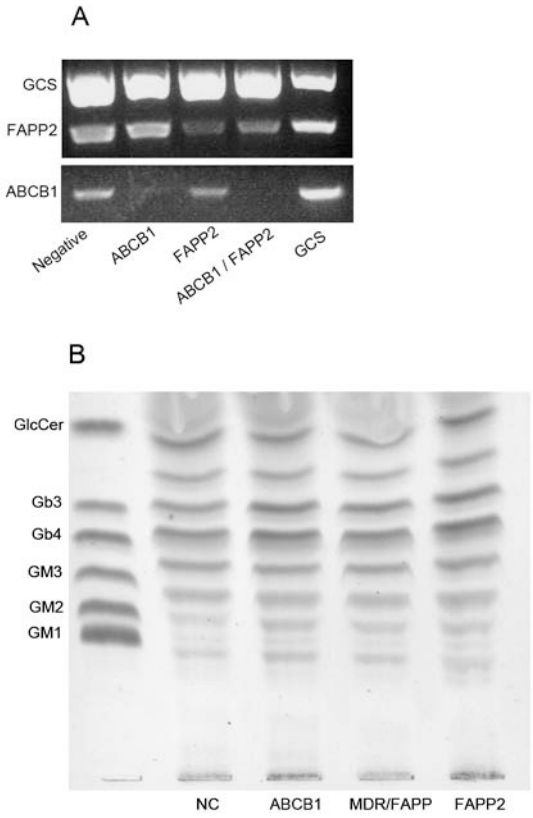


Figure 5.5. **Double knockdown of ABCB1 and FAPP2 has an additive effect.** DU-145 cells were transiently transfected with siRNA 3323 and 265. (A) RNA was extracted and reverse transcribed. The resulting cDNA was PCR amplified using primers for amplification of ABCB1 and FAPP2. Double knockdown shows reduction in ABCB1 and FAPP2, while single siRNA transfections knockdown levels of only the corresponding gene. (B) Orcinol-stained TLC of total GSLs. Double knockdown has a mildly additive effect of GSL reduction in comparison to single siRNA transfections.

### 5.4.6 Neutral GSL synthesis is reduced in MDR1-transfected MDCK cells treated with ABCB1 siRNA

Having established reduction in ABCB1 protein using siRNA 3323, we performed a metabolic labeling study using two different radiolabels: [$^{14}$C]-galactose and [$^{14}$C]-palmitate. Cells were treated with ABCB1 siRNA or negative control (NC) RNA, or left untreated (NT) for 48 h, following which radiolabel was added for 5 h. The experiment was performed in duplicate. LacCer and $Gb_3$ synthesis were markedly reduced by ABCB1 siRNA treatment using both radiolabels (Figure 5.6). An additional (unidentified) prominent band is seen in the non-treated lane beneath $Gb_3$.

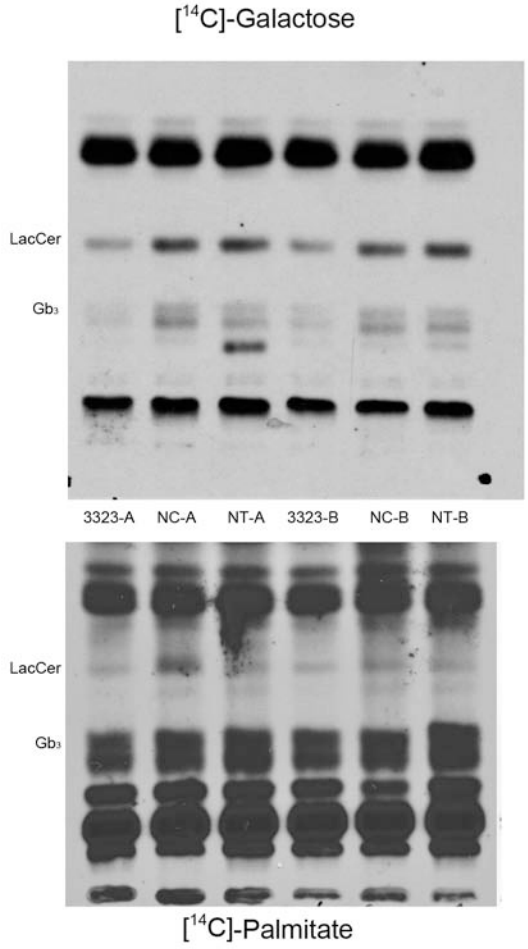


Figure 5.6. **ABCB1 3323 siRNA decreases GSL biosynthesis.** Autoradiogram of TLC separated GSLs. MDR-MDCK cells were transiently transfected with negative control or ABCB1 3323 siRNA for 48 h. Experiment was performed in duplicate. Prior to collection of cells, radiolabel (either [$^{14}$C]-palmitate or [$^{14}$C]-galactose) was added to cells for 5 h. Cells were collected and washed, and the GSLs were extracted. Both labels show reduced synthesis of LacCer and $Gb_3$ for ABCB1-siRNA treated cells. NT: cells not treated with siRNA; NC: cells treated with negative control siRNA.

### 5.4.7 ABCB1 knockdown reduces $Gb_3$ in A431 cells but not in ACHN cells

To examine whether the observed effects of knockdowns are selective for DU-145 cells, A431 cells and ACHN cells were subjected to the various siRNAs. These cells were selected based on ABCB1 transcript expression; a test RT-PCR revealed A431 to possess little *ABCB1*, while ACHN cells transcribe a considerable amount of the gene. Surprisingly, knockdown of ABCB1 showed 25% reduction of $Gb_3$, the major GSL expressed, in A431 cells along with a 45% reduction in GlcCer (Figure 5.7A). FAPP2 knockdown decreased $Gb_3$ and GlcCer by approximately 15% and 25%, respectively. No reduction was observed in any GSL in ACHN cells by ABCB1 KD or FAPP2 KD (Figure 5.7B).

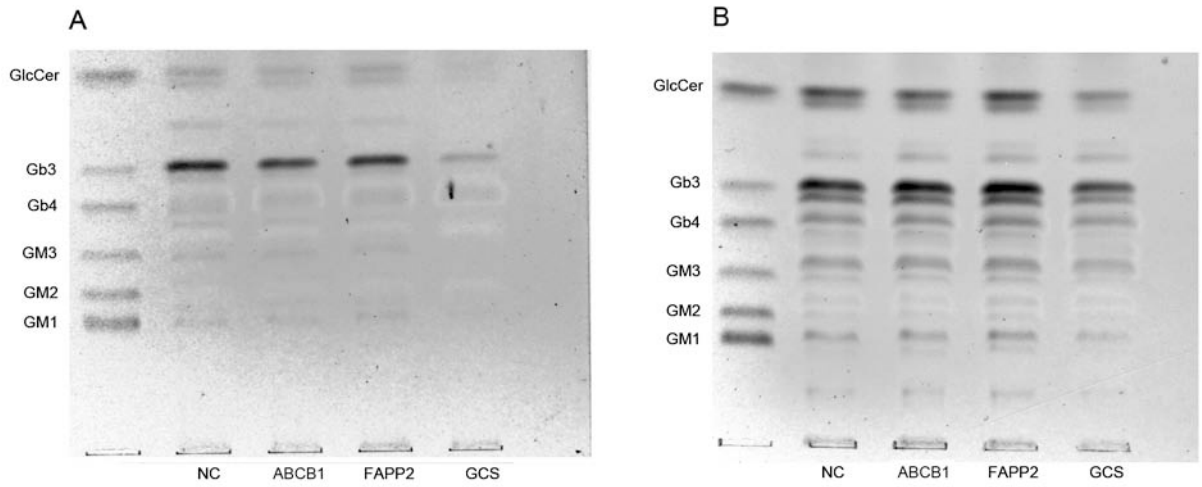


Figure 5.7. **Effects of ABCB1 and FAPP2 knockdowns in A431 and ACHN cells.** A431 (A) and ACHN (B) cells were transiently transfected with siRNA 3323, 265, 833 or negative control (NC). ABCB1 KD caused a 25% reduction in $Gb_3$, while FAPP2 KD caused a modest 15% reduction. In addition, GlcCer and LacCer levels were reduced by ABCB1 KD. Only GCS KD reduced GSL levels in ACHN cells, while other siRNA had no effect on neutral GSLs or gangliosides.

### 5.4.8 Knockdown of ATP8B1 increases ABCB1 expression and GSL levels in DU-145 cells

Following a report that knockdown of the aminophospholipid flippase TAT-2 rescues the lethality associated with inhibiting sphingolipid synthesis in *C.elegans* (Seamen et al., 2009), we examined the effect on GSLs of KD of the TAT-2 human orthologue, ATP8B1. KD of this protein using three different siRNA targeting the gene increased *ABCB1* mRNA as seen by RT-PCR (Figure 5.8A) and elevated total GSLs (Figure 5.8B).

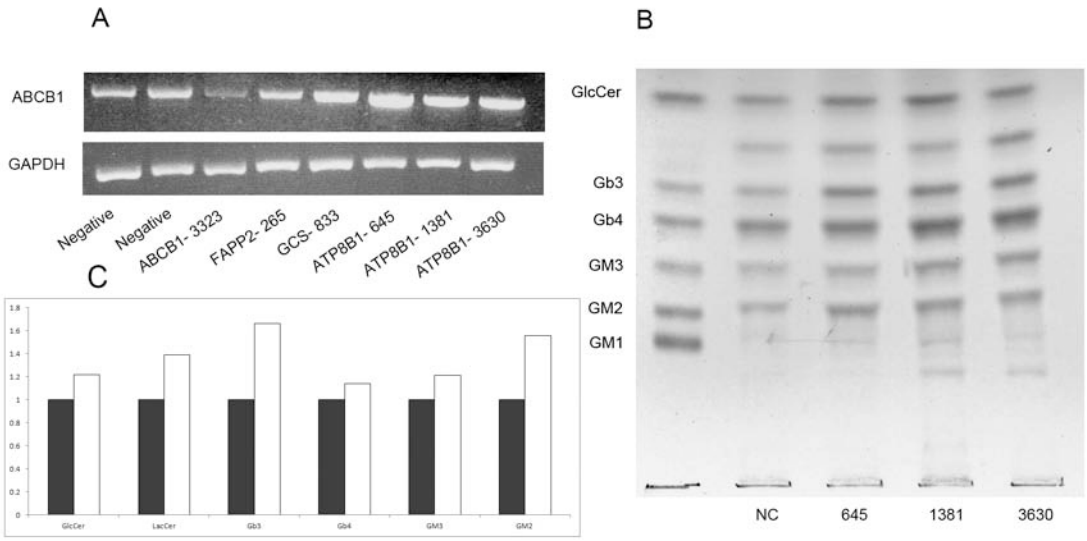


Figure 5.8. **ATP8B1 silencing increases ABCB1 transcript and GSL levels.** DU-145 cells were transfected with three different siRNA against ATP8B1. (A) RNA was reverse transcribed and the resulting cDNA was PCR-amplified using primers for amplification of a portion of the ABCB1 gene. ABCB1 gene levels are increased by ATP8B1 treatment. (B) Total GSLs were extracted, purified, TLC-separated and orcinol-stained. GSLs from an equivalent of $10^6$ GSLs were separated by TLC. Total GSL levels are elevated by siRNA transfection. (C) Image J quantitation of GSLs from NC (dark bars) and ATP8B1 KD (open bars).

## 5.4.9 β4GalT5 and β4GalT6 knockdowns decrease total GSLs downstream of GlcCer

Both enzymes that mediate the synthesis of LacCer were knocked down to identify whether they preferentially affect particular GSL sub-classes. Three different siRNA against each gene were used. By comparing the siRNAs that produced the strongest reduction in GSLs, it appears that decrease in either LacCer synthases decrease all GSLs downstream of GlcCer, without preference for particular subclasses (Figure 5.9). GSLs were reduced by 10% to 75%. Although LacCer synthase expression is reduced, GlcCer is not elevated; in fact, GlcCer levels are actually decreased by β4GalT6 430.

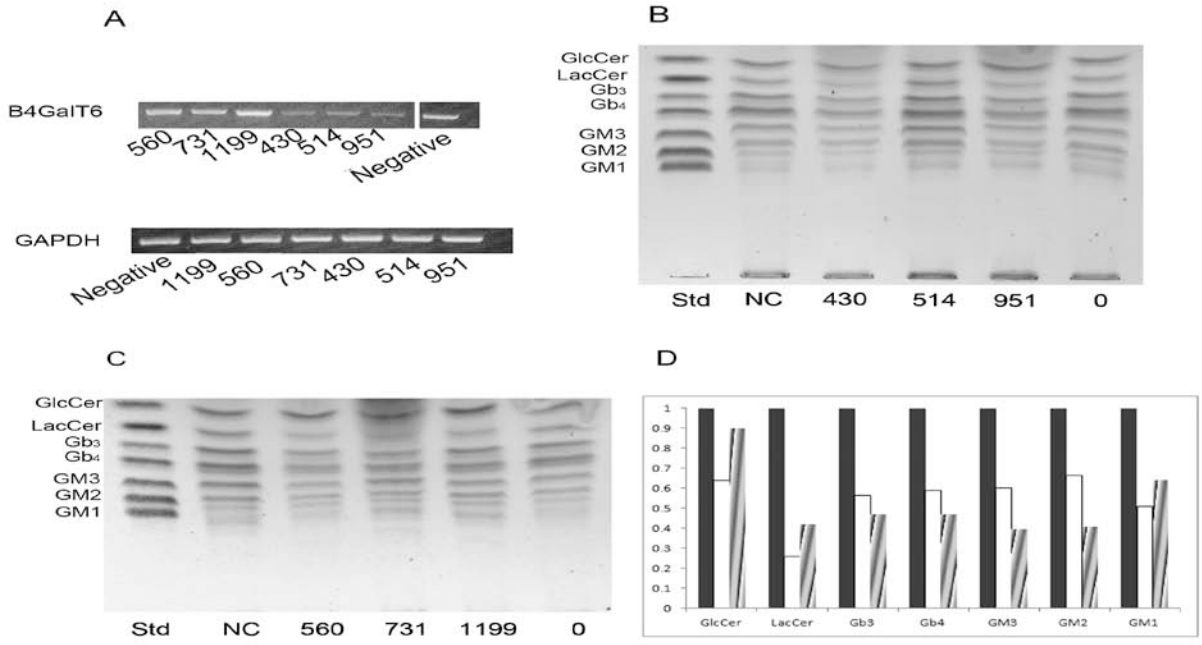


Figure 5.9. **LacCer synthase siRNA reduces total GSLs downstream of GlcCer.** DU-145 cells were transiently transfected with three different siRNA against each of the two LacCer synthases: β4GalT5 and β4GalT6. Two separate experiments were conducted, one for RNA extraction and the other for extraction of total GSLs. (A) RNA from treatments was extracted, reverse transcribed and the resulting cDNA was PCR-amplified using primers designed for amplification of a fragment of β4GalT6 or GAPDH. Levels of the β4GalT6 transcript were reduced relative to negative control treatment for the three siRNA against β4GalT6 (430, 514, 951), but not for siRNA targeting β4GalT5 (560, 731, 1199). GAPDH was not changed in any of the treated cells. (B and C) Total GSLs were extracted, separated by TLC and orcinol stained for cells treated with siRNA targeting β4GalT6 (B) and β4GalT5 (C). All GSLs downstream of GlcCer were reduced using all the siRNA, except 514. 430 and 560 had the greatest effect on GSL reduction. The '0' lane in (C) corresponds to cells not treated with any siRNA sequence. (D) ImageJ quantitation of GSLs from NC (dark bars), β4GalT6 KD (open bars) and β4GalT5 (diagonal bars).

## *5.5 Discussion*

Previous studies have shown, independently, the involvement of FAPP2 and ABCB1 in GSL biosynthesis (D'Angelo et al., 2007; De Rosa et al., 2004; Halter et al., 2007; Lala et al., 2000). The view of P-gp as a major GlcCer flippase responsible for complex GSL synthesis has, however, been challenged. Importantly, the study of Halter et al (Halter et al., 2007) disregards the involvement of P-gp based on conclusions from CsA experiments conducted with HeLa cells, a cell line already shown to be resistant to CsA-induced $Gb_3$ reduction (De Rosa et al., 2004). While HeLa cell treatment with CsA does not affect $Gb_3$ levels, GlcCer is reduced

(Smith et al., 2006). In addition, knockout of mouse *mrp1 and mdr1* results in decreased GSL levels in NPC1 fibroblasts, but brain GSLs are unaltered, indicating that the effect of ABCB1 on GSLs is likely cell-type dependent (Sillence, 2007).

We hypothesized that FAPP2 and P-gp may act in within a common pathway such that GlcCer synthesized on the cytosolic leaflet of the *cis* Golgi is transported by FAPP2 to the *trans* Golgi, where ABCB1 translocates it to the lumenal leaflet. FAPP2 retrograde GlcCer transit may be a P-gp independent neutral GSL source in addition to FAPP2-independent translocation by P-gp, while FAPP2 forward GlcCer transit may preferentially make gangliosides. This is an extension to the model proposed by D'Angelo et al (2007), in which FAPP2 is simply shown to transport GlcCer to the *trans* Golgi without any indication as to how it traverses the membrane.

Since pharmacological inhibition of target proteins often includes off-target effects, we decided to utilize a gene knockdown approach to specifically gauge the impact of diminished levels of particular proteins. KD of ABCB1 and FAPP2 each reduces GSL levels in DU-145 cells. Previous results reveal pharmacological inhibition of P-gp to specifically affect neutral GSL levels, without an effect on gangliosides (De Rosa et al., 2004; Mattocks et al., 2006). Our KD of ABCB1, however, shows preferential reduction of gangliosides (in addition to reduction of GlcCer and LacCer), with little to no effect on neutral GSLs downstream of LacCer. Metabolic labelling to examine GSL synthesis in MDR1 transfected MDCK cells, however, revealed $Gb_3$ (and LacCer) synthesis to be reduced upon ABCB1 siRNA treatment. In the previous study, HeLa cells were also shown to be resistant to cyclosporine-mediated depletion of neutral GSLs, although ganglioside levels were not examined (De Rosa et al., 2004). Transfection of HeLa cells with *CerS1*, which encodes the ceramide synthase responsible for C18 ceramide synthesis,

sensitizes HeLa cells to cyclosporine A (De Rosa and Lingwood, 2009). DU-145 cells, therefore, may represent another cell-type in which CerS1 expression is relatively low, thereby producing little effect of ABCB1 silencing on neutral GSL synthesis.

Based on previous investigations with FAPP2, it was expected that FAPP2 KD would reduce total GSLs downstream of GlcCer (D'Angelo et al., 2007). Not only do our results show preferential reduction of neutral GSLs (with the exception of GM1), levels of GlcCer itself are also decreased. This discrepancy between our results and those of the published study may reflect cell-type selectivity of the effects of knockdowns of these proteins. Since cell lines differ in their expression of various proteins, differences may also exist specifically in expression of MDR1 and FAPP2. In the previously published studies, HeLa cells were used (D'Angelo et al., 2007; Halter et al., 2007). Since HeLa cells are unaffected by inhibition of P-gp (De Rosa et al., 2004), it is possible, that FAPP2 plays a more significant role in these cells.

It is clear that ABC transporter expression varies by cell-type (Hennessy and Spiers, 2007). We, therefore, examined the effects of our knockdowns in different cell lines. Based on RT-PCR, A431 cells express very low endogenous levels of ABCB1 transcript. KD of this protein was expected to have little effect on GSL levels. However, ABCB1 KD reduced $Gb_3$ levels by 25% and FAPP2 KD decreased $Gb_3$ by approximately 15%. Although ABCB1 expression is low compared to other cell lines, it is possible that this is sufficient to carry out its function as a flippase in GSL synthesis, and knockdown of inherently low levels thereby reduces GSL levels. ABCB1 and FAPP2 knockdowns additionally revealed a 45% and 25% reduction in GlcCer, respectively. Such a reduction in GlcCer was also observed in DU-145 cells and may be

indicative of non-lysosomal glucosylceramidase-mediated degradation of GlcCer that is exposed on the cytosolic leaflet of the Golgi.

Unlike the effects of ABCB1 siRNA on DU-145 and A431 cells, ACHN cells, which do express ABCB1 transcript, did not show reduction in any GSL. The GCS siRNA control did indeed show reduction of all GSLs, indicating that the lack of an effect of both ABCB1 and FAPP2 siRNA is not due to cellular resistance to the introduction of siRNA or limited trasnfection efficiency of these cells. A second observation from the results of siRNA transfection of A431 and ACHN cells is the lack of an effect (or minimal effect) of FAPP2 knockdown on GSLs. With FAPP2 KD (and ABCB1 KD in ACHN cells) having minimal effect, it is clear that these proteins do not play a universal role and other mechanisms exist for GlcCer access to the Golgi lumen. Identifying other flippases or transporters of GlcCer is the subject of future studies in this laboratory.

In one of the proposed FAPP2 models, GlcCer is transported by FAPP2 to a distal Golgi compartment, where it is then translocated to the lumenal leaflet of the Golgi by an unknown mechanism (D'Angelo et al., 2007). We proposed that FAPP2 carries GlcCer to ABCB1 for translocation into the Golgi lumen. We, therefore, performed a double knockdown of FAPP2 and ABCB1 in DU-145 cells. According to our hypothesis, no difference in GSL levels is expected if one protein is knocked down or if both are knocked down. If, however, the two proteins act in distinct pathways, we can anticipate the effect of a double KD to be additive. Our results reveal a marginally greater reduction in GSLs for the double KD relative to each of the singly siRNA transfections. This, in conjunction with the observation that FAPP2 KD and ABCB1 KD affect different GSLs suggests that the two proteins do not only function within the

same pathway. Since the effect on GSLs of double knockdown of FAPP2 and ABCB1 was additive, it is possible that FAPP2 and ABCB1 actually deliver GlcCer to distinct LacCer synthases. Thus, the effects from double knockdowns of FAPP2 with each of the LacCer synthases (β4GalT5 and β4GalT6), and likewise for ABCB1, will be examined in the future.

In addition, it is possible that the different LacCer synthases are responsible for the generation of the distinct GSL classes, suggesting the presence of distinct pools of LacCer. To examine this possibility in DU-145 cells, we knocked down the two LacCer synthases and examined the effect on GSLs. GSLs downstream of GlcCer were markedly reduced in both knockdowns (and GlcCer was also reduced for β4GalT6 KD), without any preferential effect on neutral GSLs versus gangliosides. Thus, it appears that the LacCer synthases, at least in DU-145 cells, are not responsible for determining the pathway downstream of LacCer. We intend to simultaneously knockdown both LacCer synthases and examine if the effect of knockdown is additive and whether the two enzymes are redundant for GSL synthesis. Identifying the mechanism by which the different GSL classes are regulated would be a profound discovery, providing the ability to target therapeutics towards only the affected pathway.

That the GSL levels in cells doubly knocked down of FAPP2 and ABCB1 are still not entirely depleted suggests either that the low residual levels of these proteins are sufficient for GSL biosynthesis, or that other mechanisms are also involved in GlcCer translocation. GlcCer in *C.elegans* is composed of branched-chain sphingoid bases (Seamen et al., 2009). Depletion of monomethyl branched-chain fatty acids (mmBCFAs) causes developmental arrest. It has been shown that the P-type ATPase TAT-2 functions in opposition to the developmental role of mmBCFAs in *C. elegans* such that downregulation of TAT-2 overcomes the developmental

arrest associated with a deficiency in these fatty acids (Seamen et al., 2009). TAT-2 is a 1313 amino acid protein that is a member of the aminophospholipid flippase family responsible for maintaining asymmetry in the phospholipid bilayer. Importantly, the same study shows downregulation of TAT-2 rescues defects caused by sphingolipid depletion (Seamen et al., 2009). This could indicate that TAT-2 is involved in GSL metabolism in a manner which inhibits development.

We, therefore, thought that the human orthologue of TAT-2, ATP8B1, may also function as a flippase for GlcCer and decided to examine the effects of ATP8B1 knockdown. Indeed, P-type ATPases have been identified in the TGN (Pomorski and Menon, 2006). Surprisingly, KD caused an increase in ABCB1 RNA. Furthermore, levels of all GSLs were increased. From this experiment, we are unable to identify a GlcCer flippase function of ATP8B1. Given the results, if the protein did exhibit such activity, it would have to mediate translocation of GlcCer from the lumenal leaflet back to the cytosolic leaflet of the Golgi, thereby preventing downstream GSL synthesis or transport GlcCer to a pool unavailable for GSL synthesis. In such a scenario, KD of ATP8B1 would prevent removal of the GlcCer substrate from access to downstream GSL glycosyltransferases. However, it is also plausible that the increase in GSLs observed is simply a consequence of elevated ABCB1 expression observed after ATP8B1 KD. Thus, we intend to co-transfect DU-145 cells with siRNA against both ATP8B1 and ABCB1.

When comparing the effects of genetic or pharmacological manipulations on cellular GSL levels, assessing equal loading on the TLC is not a trivial issue. For protein analysis using Western blots, several housekeeping proteins can be examined to ensure an equal amount of protein has been loaded and that the treatment is specific for the intended target protein. For

GSL analysis, however, this becomes complicated because of the highly interconnected nature of GSL metabolism. Our quantitative analyses have been performed by normalizing band intensity with that of sphingomyelin (SM). We have traditionally used SM as an internal loading control for our TLCs of cellular GSL extracts. From past experience, SM levels are unaltered upon various treatments that elevate or deplete GSLs. However, treatments that prevent GlcCer access to the Golgi lumen may redirect ceramide towards synthesis of SM rather than GlcCer.

The topology of GCS has posed a problem for 30 years. Although two predominant theories have been previously proposed to explain the mechanism by which GlcCer accesses the Golgi lumen, our results indicate that GlcCer Golgi lumenal access is not as clear-cut as one pathway operating or the other. Rather, it is likely that multiple mechanisms—probably involving both ABCB1 and FAPP2, as well as others—are at play. Elucidating the various mechanisms involved in GlcCer access to the Golgi lumen will prove pivotal, not only for our understanding of GSL biosynthesis, but also for identifying alternative approaches to regulate this process for therapeutic purposes.

# Chapter 6: Discussion

Glycosphingolipid homeostasis is a complex process, depending not only on glycosyltransferases and hydrolases and their topology, but also on translocators of sugar-nucleotides and activator proteins among other molecules. Even the ultrastructural organization of organelles is important to maintain the balance in GSL levels (Maccioni et al., 2011b). As an example, mutations in tethering proteins involved in retrograde transport from the Golgi can mis-localize GSL biosynthetic enzymes and lead to a disease phenotype (Maccioni et al., 2011b). This adds another layer of complexity by implying that glycosyltransferases are cycled within the Golgi and between the Golgi and ER (Maccioni et al., 2011b). As noted by Mullen et al regarding the role of particular ceramides in apoptosis (Mullen et al., 2012), a GSL implicated in a particular process may likewise be dependent on subcellular location of the GSL species, structural features (eg. acyl-chain length, hydroxylation, etc) that give rise to over 10,000 possible structures (Merrill, 2011), and source pathway for synthesis (i.e. *de novo* or salvage pathway). Alterations in any of these processes can lead to cellular dysfunction and disease.

GSLs are involved in various cell functions; thus, maintaining an appropriate level of each individual GSL may be necessary to avoid cellular dysfunction, which may lead to disease pathologies. Many diseases are characterized by aberrant GSL expression, either as the primary cause of clinical symptoms or a secondary response that contributes to the particular pathology. The GSL-specific LSDs are characterized by defects in the catabolism of particular GSLs, leading to their accumulation.

Although the primary cause of diabetes is not known to be aberrant GSL expression, elevated levels of GM3 facilitates increased interaction with the insulin receptor, thereby dissociating it

from caveolae, where it is required for insulin signalling to take place (Kabayama et al., 2007). The GM3-IR interaction inhibits insulin signalling by preventing phorphorylation of the receptor. Although elevated GM3 may not cause diabetes, it contributes to the insulin-resistant state.

In both primary and secondary GSL-related pathologies, modulation of the offending GSL can alleviate clinical symptoms. In the case of LSDs, inhibitors of GSL biosynthesis have been used clinically to reverse symptoms (Elstein et al., 2004; Pastores et al., 2005); for diabetes, inhibiting GSL synthesis has been shown to lower blood glucose and increase muscle and liver insulin sensitivity (Aerts et al., 2007). Thus, the development of strategies to manipulate GSL levels is of clinical relevance. For this reason, a great deal of research has been conducted into the design, development and characterization of inhibitors of GSL metabolism. Since knockout studies show GSLs are necessary at the tissue level (Liang et al., 2010; Pontier and Schweisguth, 2012; Yamashita et al., 1999b), inhibitors must be used at a dose that will not entirely prevent their synthesis.

When designing enzyme inhibitors, a common starting point is to make minor chemical modifications in the target enzyme's substrate. While it is true that any number of structural changes can be made to the molecule, it is good to have a rationale that will exploit characteristics of the enzyme-substrate (or enzyme-inhibitor) interaction.

For reasons not completely understood, adamantane appears to be a relatively popular moiety for incorporation into candidate drug molecules. As of 2011, seven adamantyl-containing compounds were being used clinically, while several others are in the developmental pipeline

(Liu et al., 2011). Adamantane is used in the composition of various inhibitors for the treatment of several diseases (Liu et al., 2011): diabetes (vildagliptin, (S)-1-[*N*-(3-hydroxy-1-adamantyl)glycyl]pyrrolidine-2-carbonitrile; saxagliptin ((1S,3S,5S)-2-[(2S)-2-amino-2-(3-hydroxy-1-adamantyl)acetyl]-2-azabicyclo[3.1.0]hexane-3-carbonitrile) (Augeri et al., 2005; Villhauer et al., 2003), viral infections (amantadine , 1-aminoadamantane; rimantadine, 1-(1-adamantyl)ethanamine; tromantadine, *N*-1-adamantyl-*N*-[2-(dimethylamino)ethoxy]acetamide) (Dawkins Jr. et al., 1968; Grunert et al., 1965; Rosenthal et al., 1982); acne (adapalene, 6-[3-(1-adamantyl)-4-methoxy-phenyl]naphthalene-2-carboxylic acid) (Hensby et al., 1990) and Alzheimer's disease (memantine, 3,5-dimethyladamantan-1-amine) (Svensson, 1973). These compounds have unique (and in the case of amantadine, multiple) targets, including ion channels, neurotransmitter receptors, inflammatory molecules and dipeptidyl peptidases.

Perhaps the highly hydrophobic adamantane group is favoured due to the restrictions imparted by it on the entire compound; for example, adamantane does not have any conformational isomers and is rotationally restricted. In addition, it has been suggested that the bulk and rigidity of the adamantane group prevents binding of esterases and amidases, thereby increasing the half-life of drugs that may otherwise be susceptible to these enzymes (Liu et al., 2011). The adamantane group is also able to increase the membrane solubility of highly water-soluble compounds, without entirely losing water solubility (Liu et al., 2011).

Our adamantylGSLs were initially designed to inhibit verotoxin binding to $Gb_3$ (Mylvaganam and Lingwood, 1999). The first compound, ada$Gb_3$, was designed with the rationale that the rigid adamantane frame will retain the hydrophobicity of the substituted group; it will mimic the liquid-ordered membrane environment by resembling the GSL-cholesterol intercalation; it will

minimize lateral lamellar interaction in aqueous medium, thereby increasing water solubility (Lingwood and Mylvaganam, 2003; Mylvaganam and Lingwood, 1999).

Although we have replaced a hydrophobic fatty acid moiety with an equally hydrophobic adamantane frame, adaGSLs surprisingly show increased water-solubility over their natural counterparts (Mylvaganam and Lingwood, 1999). GalCer and $Gb_3$ partition entirely into the organic phase of a chloroform-water mixture; in contrast, 5% adaGalCer and virtually 100% ada$Gb_3$ partitions into the aqueous phase (Mylvaganam and Lingwood, 1999). AdaSGC similarly partitions entirely into the aqueous phase, while the natural counterpart is found in the organic phase.

We have shown that adaGlcCer and adaGalCer are capable of selectively interfering with GSL metabolism such that 40 µM adaGlcCer prevents synthesis of GSLs downstream of GlcCer, while 40 µM adaGalCer inhibits synthesis of $Gb_3$ and the globo-series GSLs. Interestingly, with adaGlcCer, we observed two distinct effects: elevation of GSLs using 20 µM adaGlcCer and substantial reduction downstream of GlcCer at 40 µM. At each concentration examined, GlcCer itself is increased. We attributed this dichotomy of effects to glucocerebrosidase inhibition throughout the range of concentrations used, and the added inhibition of LacCer synthase at 40 µM. This hypothesis was confirmed by inhibition of microsomal LacCer synthesis and *in vitro* inhibition of cerezyme®.

The elevation of GlcCer observed with adaGlcCer treatment is intriguing, as this can be exploited as a potential means to assess the effects of increased GSLs on various cellular functions and signalling pathways. This increase in GSLs at 20 µM is greater than the elevation

seen by 100 µM CBE treatment. Interestingly, adaGlcCer more effectively increases GSLs and at lower concentrations than CBE. Low concentrations of adaGlcCer, therefore, may be a better, and more specific, alternative to CBE as a model for elevated GSLs. Furthermore, 40 µM adaGlcCer is an effective means to study the effects of GlcCer elevation alone, as all GSLs downstream of GlcCer are depleted at this dose.

While adaGlcCer can potentially be used as an investigative tool, adaGalCer may have therapeutic potential. A successful therapeutic compound should be specific for its intended target with minimal off-target effects. Our data show that adaGalCer specifically inhibits globo-series GSL synthesis. Since galabiosylceramide synthase and $Gb_3$ synthase are in fact the same enzyme, synthesis of galabiosylceramide is also inhibited. AdaGalCer may be a viable substrate reduction therapeutic option for Fabry disease patients, who suffer from defective α-galactosidase A, leading to $Gb_3$ accumulation. In preventing $Gb_3$ synthesis, it turns out that adaGalCer actually serves as a substrate for $Gb_3$ synthase and is correspondingly converted to adagalabiosylceramide. The identity of this artificial GSL was confirmed by TLC overlay, mass spectrometry and *in vitro* α-galactosidase A assay (Figure 3.2C, 3.3i, C). Compared to $Gb_3$, ada$Gb_2$ is preferentially lost from the cell into the extracellular medium; thus, adaGalCer can be titrated to reduce $Gb_3$ storage while allowing alternative product (ada$Gb_2$) to exit the cell and simultaneously retaining normal synthesis of $Gb_3$. The latter point is an important consideration, as $Gb_3$ is the first GSL within a larger sub-class, the globo-series; drastic reduction in $Gb_3$ will similarly affect downstream GSLs within this pathway, including stem cell markers (such as SSEA-3 and SSEA-4), possibly with deleterious developmental consequences. The release of ada$Gb_2$ into the serum can be exploited as a marker for therapeutic efficacy. In

this way, formation of adaGb$_2$ is a direct consequence of preventing Gb$_3$ synthesis and should reflect reduced Gb$_3$ storage.

Since we observed formation of adaGb$_2$ in Fabry disease lymphoblasts without the corresponding reduction in Gb$_3$, we proposed that adaGb$_2$ likely inhibits residual α-galactosidase A activity, as it is a substrate for the enzyme *in vitro*. This finding does not necessarily exclude adaGalCer as a therapeutic candidate; rather it may be a viable treatment strategy if initiated early enough such that the effect of Gb$_3$ synthesis inhibition supersedes inhibition of catabolism by adaGb$_2$ (i.e. before Gb$_3$ accumulates to unmanageable levels).

Inhibition of α-galactosidase A by adaGb$_2$ can potentially be exploited for chaperoning capability. This needs to be tested using purified adaGb$_2$. It is possible that this artificial GSL is able to increase enzyme localization in the lysosome; however, based on our data, it appears to maintain continual inhibition of the enzyme. Thus, chemical modifications of adaGb$_2$ may yield a compound that is also capable of binding the enzyme, but with reduced affinity at the acidic pH of the lysosome.

AdaGalCer also has therapeutic utility in Gaucher disease, both as a pharmacological chaperone and as an activator for glucocerebrosidase. In characterizing the *in vitro* effect of adaGlcCer on glucocerebrosidase, we used adaGalCer as a control. Surprisingly, adaGalCer stimulated enzyme activity. Subsequent analysis of Gaucher disease and Fabry disease lymphoblasts revealed reduction in GlcCer. Our results indicate that this reduction is facilitated by a three-fold effect of adaGalCer: (1) binding to the mutant enzyme and stabilizing it to prevent its

degradation via ERAD; (2) chaperoning the enzyme to its correct destination in the lysosome; (3) stimulating enzyme activity.

Our results indicate that the stimulatory effect on glucocerebrosidase is not limited to adaGalCer; rather, lysoGalCer, which lacks the acyl chain of GalCer, similarly stimulates enzyme activity. In contrast, GalCer itself was unable to induce such increase in activity. It is possible that lysoGalCer and adaGalCer act to perturb the membrane, allowing for easier access of glucocerebrosidase to its GlcCer substrate. In essence, this would resemble the function of sphingolipid activator proteins. These findings need to be elaborated upon to determine the best chemical substitutions that will induce the highest activity.

Interestingly, glucosylsphingosine (lysoGlcCer) has been reported to be significantly elevated in Gaucher disease patients (Dekker et al., 2011; Nilsson and Svennerholm, 1982; Orvisky et al., 2002). Formation of lysoGlcCer appears to occur primarily via de-acylation of accumulated GlcCer by acid ceramidase, with glucosylation of sphingosine contributing to lysoGlcCer levels to a lesser extent (Yamaguchi et al., 1994). It is unclear why these molecules are not simply re-acylated, but it may simply be that lysoGSLs are not good substrates for the ceramide synthases. In our cell treatments with adaGSLs, we did not detect any lysoGlcCer or lysoGalCer by TLC or mass spectrometry; thus, the adaGSLs may not be substrates for acid ceramidase or the lysoGSLs are present in undetectable quantities.

Our results show lysoGlcCer and lysoGalCer to be toxic above 20 µM. In accordance with our findings, several studies have shown the toxic effects of lysoGlcCer: LysoGlcCer causes red blood cell hemolysis (Schueler et al., 2003); Incubation of LA-N-2 neuron-like cells with 10 µM

lysoGlcCer decreased, reversibly, activity of glucocerebrosidase, β-galactosidase and sphingomyelinase, reduced acetylcholine levels and eliminated neurite outgrowth (Schueler et al., 2003); lysoGalCer shows cytotoxicity in cells and upon intracerebral injection (Hannun and Bell, 1989). This highlights the importance of the adamantane frame in our analogues in mitigating the toxicity associated with lysoGSLs. Perhaps lysoGlcCer, due to its positive charge, exerts detergent-like effects in damaging the cell membrane (Schueler et al., 2003). Alternatively, the positive charge may interact with negatively charged lipids, thereby disrupting the membrane (Schueler et al., 2003).

LysoGSLs have also been reported to accumulate in other GSL LSDs, including lysoGalCer (galactosylsphingosine, psychosine) in Krabbe disease, lysosulfatide (sulfogalactosylsphingosine) in metachromatic leukodystrophy, lysoGM2 in GM2 gangliosidosis and lysoGb$_3$ in Fabry disease (Aerts, 2008). These findings give credence to the "Psychosine Hypothesis" from 1972, when it was proposed that lysoGalCer accumulation was the cause of the pathogenicity of Krabbe disease (Suzuki, 1998). It has also been suggested that lysoGSLs may be important physiologically as second messengers, with their formation linked to a cellular signal, similar to the generation of diacylglycerol from phospholipids (Hannun and Bell, 1989). A cell signal may cause deacylation of a particular GSL, yielding the corresponding lysoGSL, which may act to inhibit protein kinase C and, possibly, other proteins as well (Hannun and Bell, 1989).

Having characterized the effects of our adaGSLs, their utility can be extended to several different applications. Since aberrant GSL expression is characteristic of several diseases, our compounds can be used to elevate or reduce GSLs for therapeutic purposes. We have initiated

studies into the effect of adaGlcCer on EGFR activation. EGFR is overexpressed in several cancers (Ciardiello and Tortora, 2008). Importantly, GM3 has been shown to interact with the receptor in a glycan-dependent manner (Yoon et al., 2006) and via electrostatic interaction between the sialic acid of GM3 and a juxtramembrane lysine in EGFR (Coskun et al., 2011). This interaction prevents dimerization and phosphorylation of EGFR upon EGF stimulation, thereby keeping the protein in an inactive state and preventing the downstream signalling cascade that leads to proliferation and differentiation. We have shown GSL depletion, via P4 or 40 µM adaGlcCer, treatment increases EGF-independent phosphorylation of EGFR. In contrast, 20 µM adaGlcCer, which elevates total GSLs, reduces EGF-independent phosphorylation. Further studies using adaGSLs in this way may lead to clinical applications for this and other adaGSL compounds.

## *6.1 GlcCer access to the Golgi lumen*

The mechanism by which glucosylceramide accesses the Golgi lumen, the compartment in which subsequent glycosylation events take place to form complex GSLs, is intricate and varies according to cell-type. Although ABCB1 and FAPP2 have been proposed to be the possible proteins responsible for flipping or transporting GlcCer, resepectively, for access to the Golgi lumen, our results demonstrate the process is not so straightforward. First, HPLC analysis of $Gb_3$ in our MDR1/Fabry knockout mouse model revealed $Gb_3$ reduction to be tissue-specific; decrease was observed in the liver, spleen and kidney, while the heart and lungs were insensitive to ABCB1 knockout. This indicates the presence of at least one other mechanism (possibly FAPP2-mediated transport) in the heart and lung to allow for complex GSL synthesis. Second, in tissues sensitive to the decrease, $Gb_3$ levels are still significantly higher than in MDR1 and normal mice. If ABCB1 is solely responsible for flipping GlcCer to the lumenal leaflet of the

Golgi, we would expect $Gb_3$ levels in responsive tissues to be reduced to background levels (i.e. comparable to normal mice). The residual $Gb_3$ indicates the presence of alternative mechanisms for GlcCer to access the subsequent GSL biosynthetic machinery. Third, siRNA knockdown of FAPP2 reveals selective reduction of neutral GSLs in DU-145 cells, while marginal reduction is observed in A431 cells. Another mechanism must, therefore, be responsible for directing GlcCer towards ganglioside synthesis in DU-145 cells and for both neutral and acidic GSLs in A431 cells. In addition, a previous study on the requirement of FAPP2 for GSL synthesis showed reduction in total GSLs downstream of GlcCer upon silencing FAPP2 (D'Angelo et al., 2007) in HeLa cells. Thus, the relative contributions to GSL synthesis of proteins responsible for GlcCer Golgi lumenal access varies by cell type. Fourth, ABCB1 knockdown selectively reduces gangliosides in DU-145 cells. A previous study by our lab showed ABCB1 involvement in neutral GSL synthesis (De Rosa et al., 2004), suggesting that the particular role of ABCB1 in GSL synthesis is also cell-type specific. Fifth, knockdown of neither ABCB1 nor FAPP2 affects GSLs in ACHN cells. Thus, an alternative mechanism must exist for GlcCer to access the Golgi lumen.

The cell-type specificity seen with regards to ABCB1 and FAPP2 involvement in GSL biosynthesis may be due to differential expression and, possibly, localization of these proteins. The relative endogenous levels of these proteins in various cells remain to be assessed. As part of our gene knockdown study, we attempted to evaluate endogenous protein levels; however, expression was not detectable by Western blot. Using MDR1-MDCK cells, which greatly overexpress ABCB1, we were clearly able to detect ABCB1 and observe its knockdown upon siRNA treatment. Use of overexpressing cell lines is enticing but may confound studies of subcellular localization.

Taken together, it is clear that a single protein does not account for the delivery of GlcCer for complex GSL synthesis. At least two proteins contribute to this process, but given the reasons above and that GSL levels are not abolished in our FAPP2/ABCB1 double knockdown, it appears that other proteins are also involved, although the presence of GSLs could also be due to incomplete knockdown of target proteins. We have shown that the aminophospholipid flippase ATP8B1 antagonizes GSL synthesis, such that knockdown of this protein elevates total GSLs, although it remains to be confirmed whether the increase in GSLs is directly attributed to reduction in ATP8B1 or due to the accompanying increase in ABCB1 we show. It is possible that other P-type ATPases function similarly. Perhaps elevated levels of complex GSLs stimulate ATP8B1 activity to flip GlcCer out of the Golgi, thereby reducing precursor availability for downstream GSL synthesis. This seems unlikely, as the process would be highly energy-consuming.

Another candidate flippase is ABCA12, an essential protein responsible for GlcCer flipping into the lamellar granules of the epidermis in skin (Sakai et al., 2007b). ABCA12 is expressed from the Golgi to the lamellar granules to carry out this function. Since ABCA12 is localized to the Golgi, it is possible it may facilitate translocation across the Golgi membrane in other cell-types.

Modulating levels of proteins involved in GlcCer Golgi lumenal access may shift the balance of synthesized GSLs. This can be exploited for substrate reduction therapeutic purposes to limit synthesis of particular GSLs, while allowing normal synthesis of others. As an example, inhibitors targeting ABCB1 can be used to selectively decrease ganglioside synthesis in DU-145

cells, while essentially leaving neutral GSLs unaltered. Conversely, inhibitors targeting FAPP2 can selectively reduce neutral GSL synthesis, while allowing normal synthesis of gangliosides.

Most studies involving ABC transporter lipid flippase activity make use of short-chain and fluorescent GSL analogues. However, this may be a confounding factor, as it remains unclear whether the corresponding natural lipids can be similarly translocated (Pomorski and Menon, 2006). Although the lipid analogues used in experiments conducted with short-chain GSLs or phospholipids are artificial, short-chain lipids do exist endogenously. As an example, platelet-activating factor (PAF) is a 1-alkyl-2-acetyl phospholipid that is secreted at sites of inflammation (Van Helvoort et al., 1996). However, experiments conducted with short-chain and fluorescent analogues must be interpreted with care, so as not to ignore the possibility that natural lipids may show different behaviours.

Since two different LacCer synthases exist (β4GalT5 and β4GalT6), there still remains the possibility that FAPP2, ABCB1 and other GlcCer flippases deliver GlcCer to distinct LacCer synthases. Our preliminary evidence indicates that the LacCer synthases do not preferentially lead to synthesis of particular GSL sub-classes. Knockdown of each protein did not selectively reduce gangliosides or neutral GSLs; rather, a general decrease in all GSLs downstream of GlcCer was observed. However, we have not yet examined the acyl chains of GSLs synthesized upon knockdown of the proteins. It is possible that the two LacCer synthases discriminate between the particular acyl chain of GlcCer. As described in the introduction, the GSL aglycone is equally important functionally as the glycan. Such a relationship may, therefore, hint at functional roles for each of the LacCer synthases.

# Chapter 7: Future Studies

## *7.1 AdamantylGSLs*

Our work in generating and characterizing adaGlcCer and adaGalCer as modulators of GSL metabolic enzymes has facilitated a licensing agreement with Matreya, LLC (http://www.matreya.com) to synthesize our compounds and other adaGSLs. Synthesis has begun, making our compounds and other adaGSLs commercially available to anyone interested. This should yield a wealth of information on the effects of these compounds in various cells. Studies can range from the use of adaGSLs to specifically inhibit the synthesis of particular GSLs or GSL sub-classes to examine effects of such downregulation, the potential of adaGSLs to reverse the cellular effects caused by various diseases and the *in vivo* effects of adaGSLs in healthy and disease animal models.

Having shown modulation of GSL levels with our adaGSL compounds, *in vivo* mouse studies will be performed, preferably in Fabry and Gaucher disease models. The interest in pursuing a Fabry disease model stems from the fact that adaGalCer selectively inhibits the globo-series GSLs. $Gb_3$, one of the globo-series GSLs, is the GSL that accumulates in Fabry disease. The intrigue in studying a Gaucher disease model arises from the ability of adaGalCer to act as a pharmacological chaperone, allowing more N370S mutant enzyme to reach its correct destination in the lysosome and, more importantly, to stimulate GCC activity in cells and *in vitro*. Since only a marginal increase in lysosomal enzyme activity is required to alleviate the disease phenotype (Fan et al., 1999), the beneficial effects of adaGalCer observed in our experiments may be sufficient to alleviate disease symptoms.

The primary matter of concern with administering adaGlcCer or adaGalCer to animals is the compounds' limited water solubility. While ethanol was used as the solvent in our experiments, injecting compounds in ethanol into an animal is not feasible. One way to circumvent this might be to encapsulate the molecules within nanocarriers. Nanocarriers have recently become a trendy area of research, particularly for targeted drug delivery in diseases such as cancer. Nanocarriers are typically liposomes or nanoparticles that form a shell around a molecule of interest, for instance, a drug (Malam et al., 2009). The nanocarrier can be functionalized on its surface to contain antibody or ligand to specifically target the nanoparticle to cells containing the corresponding receptor (Corbin et al., 2007).

Drug delivery has traditionally been a complicated process due to the difficulty in tailoring molecules to cross the cell's plasma membrane and the inability to selectively target a subset of cells (Hillaireau and Couvreur, 2009). Nanoparticle vehicles offer several important advantages over carrier-free drug delivery, including longer circulation times, targeted delivery and the ability to penetrate into cells (Budhian et al., 2005). These particles can also carry a wide array of molecules (Kumari et al., 2010) and be used for targeting purposes in diagnostic imaging, including magnetic resonance imaging (Leiner et al., 2005).

Following endocytosis, the encapsulated molecules are able to escape from the nanoparticle, usually through a pH-sensitive mechanism, and bind their respective targets (Baigude and Rana, 2009). Encapsulating adaGalCer into a nanocarrier that has been modified to express mannose at the surface for macrophage targeting may yield improved expression and activity of glucocerebrosidase in targeted cells, thereby reducing GlcCer storage.

We have initiated preliminary studies on the ability of adaGlcCer to inhibit EGFR activation. This was based on the premise that GM3 interacts with EGFR to prevent phosphorylation and activation of the receptor and adaGlcCer at low dose increases total GSLs. An initial experiment has shown 20 µM adaGlcCer to decrease phosphorylation of EGFR (Figure 7.1). This work needs to be elaborated to confirm that the effect is due to elevated GM3 levels and not to a direct interaction between adaGlcCer and the receptor. By depleting cells of total GSLs using P4, followed by addition of 20 µM adaGlcCer, we should be able to decipher whether a direct interaction is likely if we observe inhibition of EGFR phosphorylation. Similar studies will also be performed with the insulin receptor, except in this case, the high dose (40 µM) will be used to reduce GM3 (and all GSLs downstream of GlcCer) to release the receptor from interaction with GM3. This should increase insulin signalling.

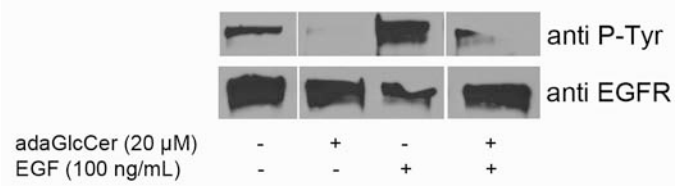


Figure 7.1. **AdaGlcCer inhibits phosphorylation of EGFR.** A431 cells were treated in duplicate with or without 20 µM for three days, after which the cell culture medium was replaced with serum-free medium and fresh adaGlcCer and the incubation was continued for one day. Prior to collection, one set of replicates was stimulated with 100 ng/mL EGF for 15 min. Cells were collected, and proteins were extracted. Two separate SDS-PAGE gels were run, followed by transferring to nitrocellulose membranes. One membrane was probed with anti-phosphotyrosine 100 antibody and the other with anti-EGFR (both kindly gifted to us by Dr. Michael Moran, Research Institute, The Hospital for Sick Children). AdaGlcCer treatment reduces EGF-independent and EGF-dependent phosphorylation of EGFR. Project initiated by M. Kamani and experiment conducted by Qazi Zain Sohail.

We have also performed a single experiment with adaCer. This molecule proved toxic to cells at a concentration of 40 µM. While the purpose of its use was to serve as a control for the effects of our adaGSLs, the observation is not entirely unexpected due to the link between free

ceramides and cell death (Mullen et al., 2012). Our adaCer, therefore, may be an important ceramide analogue due to its bioactivity. As it turns out, a similar compound has previously been shown to selectively target breast cancer cells for cytotoxicity (Crawford et al., 2003). Our adaCer differs from this compound by the presence of a methylene group between the adamantane and the carbonyl carbon. Further characterizing our adaCer in terms of its effect on sphingolipid metabolism may reveal its utility as an inhibitor.

While our adaGSLs show considerable promise not only as tools to study GSL metabolism, but also as potential therapeutic agents for diseases characterized by GSL anomalies, these are only first generation compounds. Additional modifications—some of which have already been synthesized by our lab—can be made to impart particular properties to the compounds. These include the addition of functional groups to increase solubility; introduction of carboxy or amino groups to introduce negative or positive charge, respectively. Some of these analogues have been developed for ada$Gb_3$ (Saito et al., 2012). It is possible that introducing phosphate groups may partially mimic the phospholipid bilayer environment (Lingwood and Mylvaganam, 2003). In this way, phosphorylated adaGSLs would in itself mimic a phospholipid-cholesterol-GSL network. As is often the case with the generation of enzyme inhibitors, slight refinements in the original compound may yield molecules with enhanced specificities and/or affinities.

## *7.2 GlcCer Golgi lumenal access*

At present, we have only monitored the effects of transient GCS, ABCB1 and FAPP2 knockdowns on gene, protein and GSL levels. A great deal of cell biology remains to be done to attain a more firm grasp of the specific roles of these proteins in GSL metabolism. To begin with, since two different studies have reported different locations of GCS—one report

identifying GCS in the *trans* Golgi (Halter et al., 2007), while the other has reported GCS in the *cis* Golgi (D'Angelo et al., 2007), a more comprehensive investigation into the localizations of GCS, ABCB1 and FAPP2 will be undertaken. We intend to use immunoelectron microscopy to examine the relative localizations of GCS, ABCB1 and FAPP2 under normal and KD conditions. It is possible that KD of a protein leads to a reorganization such that another GlcCer tranlocating or transporting protein is expressed to compensate for the loss. These studies will need to be expanded as other flippases and transporters of GlcCer are identified.

The ultimate experiment would be to monitor events in real-time; for instance, using GFP-labeled FAPP2, fluorescent ABCB1 and fluorescent GlcCer. The problem, however, is designing a fluorescent GlcCer analogue that retains chemical and physical properties that are essentially identical to natural GlcCer. Since most fluorescent tags are relatively bulky chemical groups attached to glucosylsphingosine, the physiological properties of GlcCer are easily altered by such substitution. Perhaps using the recently described polyene fluorescent tag approach to image lipids (Kuerschner et al., 2005) and generating a polyene ceramide analogue will represent a more physiological means for imaging GSLs.

It has been suggested that FAPP2 may be a GlcCer sensor as previously proposed for GLTP such that elevated LacCer levels or downstream GSLs trigger a feedback response to prevent GlcCer accessing the Golgi lumen (Tuuf and Mattjus, 2007). FAPP2 would then bind to GlcCer exposed on the cytosolic Golgi leaflet and induce tubule formation for GlcCer delivery to the cell surface (Cao et al., 2009). It is possible that FAPP2 also binds to another protein capable of communicating with the nucleus (i.e. a transcriptional activator or suppressor). It may be worth performing a co-immunoprecipitation of FAPP2 and monitor other proteins pulled down.

With regards to our knockdowns of proteins responsible for GlcCer Golgi lumenal access, we intend to develop stable cell lines by lentiviral transduction of short hairpin RNA (shRNA). Our hope is to develop several different cells lines with ABCB1, FAPP2 or GCS (and other candidate flippases after evaluating effects on transient gene silencing) knocked down. We will examine whether the expression levels and localization of the other proteins is affected by knockdown of one, and whether knockdowns selectively affect particular GSL biosynthetic pathways or the entire GSL ensemble.

The identification of two distinct LacCer synthases (β4GalT5 and β4 GalT6) raises the intriguing possibility of multiple pools of substrate for contribution to different GSL pathways. While preliminary data has not shown specificity for a particular GSL class (knockdown of either gene reduces both neutral GSLs and gangliosides), it remains to be seen whether the enzymes actually discriminate between particular substrate subtypes or whether there is some redundancy. Previous work has shown differential tissue expression of the genes, which may have functional relevance (Chatterjee and Pandey, 2008). As an initial line of investigation, the cellular localization of the two LacCer synthases will be examined by immunofluorescence—and possibly immunoelectron microscopy. A differential Golgi distribution may be indicative of involvement in different GSL synthesis pathways or different GlcCer substrate sources—specific GSL subtype or *de novo* versus recycled GlcCer. In addition, we intend to expand our knockdown studies by examining not only the levels of each GSL synthesized upon KD of each LacCer synthase, but also the particular acyl chain incorporated in these GSLs since it is possible that the two LacCer synthases have distinct ceramide backbone preferences.

## *7.3 MDR1/Fabry Mice*

Given the discrepancy between our HPLC and TLC analyses of tissue $Gb_3$ levels in TKO mice, we will repeat the crossbreeding study, this time employing mass spectrometry to more precisely quantify GSL levels. In addition, we will re-examine all tissues by HPLC and TLC as well; however, we will isolate GSLs from the same tissue or tissue fragment, rather than using distinct tissue fragments for the different assays. This should circumvent any possible confounding effects of differential GSL expression within tissues. We also intend to expand our mouse study by performing Western blots for FAPP2 and other candidate flippases in normal and KO mice to examine if there is compensation *in vivo* for loss of ABCB1. It is possible that the results we obtained in our study may underestimate the effects of ABCB1 KO due to activation of compensatory mechanisms.

In the current study, we only assessed GSL content from males due to the known variability of Fabry disease parameters in females (Aerts, 2008; Kolter and Sandhoff, 2006). Although there may be a larger range of $Gb_3$ levels, we intend to assess tissue GSL levels in females as well. Differences in GSL expression profiles have been previously observed between male and female mice (McCluer et al., 1983). It will be interesting to note whether similar trends are observed in TKO females, or whether they are more or less sensitive to ABCB1 KO than males. This may provide insight into other disease parameters that should be considered.

As an extension to this study, and in parallel to mass spectroscopic analysis of GSLs, we can assess the aglycones of $Gb_3$ in TKO mice to see if the expression of particular subtypes is altered. A previous study in mice has shown that fibroblasts derived from triple KO of the two ABCB1 genes and the gene encoding the multidrug resistance-associated protein (MRP1)

express GSLs with a greater proportion of C24:1 fatty acid relative to normal mice (Sillence, 2007).

## *7.4 GSL aglycone*

From analyses of the effects of ceramides with different acyl-chain types on biological function, and their subsequent implication on disease (Mullen et al., 2012), it is apparent that a more thorough analysis of GSL subtype needs to be performed when examining increases and decreases in particular GSLs. Importantly, care must be taken in interpreting the results, as simple MALDI mass spectrometry will be unable to distinguish between a species that contains, for example, a dihydro sphingoid base and an unsaturated acyl-chain with one that possesses a desaturated sphingoid base and a saturated acyl-chain. Thus, it is worth performing a comprehensive analysis on the GSL species whose expression has been altered to better ascertain potential upstream regulatory targets and functional relevance.

A second aspect of future studies with the aglycone involves identification of GlcCer acyl chain lengths preferred as substrates for ABCB1-mediated flipping. In this regard, we will generate GlcCer with varying acyl chain lengths and examine flippase function in ABCB1-containing microsomes. Having generated the various GlcCer acyl chain lengths, we can also assess LacCer synthase substrate preference.

# Chapter 8: Conclusions

GSL homeostasis is imperative to the normal functioning of cells. Aberrations in GSL metabolism often lead to disease pathologies, thereby necessitating approaches to specifically modulate GSL levels. The approaches described herein have utilized both pharmacological and genetic means in working towards this goal. Our adaGSLs show considerable promise both as tools for the study of particular GSLs in cells as well as therapeutic agents by reducing levels of offending GSLs that may accumulate in disease. Our crossbreeding and gene knockdown studies are crucial for enhancing our understanding of GSL synthesis and the development of new therapeutic approaches targeting the mechanisms responsible for GlcCer access to the Golgi lumen. The intrinsic and extrinsic approaches used, therefore, have been pivotal in working towards the goal of selectively regulating GSL metabolism.

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