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Ceramide Glycosylation Catalyzed by Glucosylceramide Synthase and Cancer Drug Resistance

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Abstract

Glucosylceramide synthase (GCS), converting ceramide to glucosylceramide, catalyzes the first reaction of ceramide glycosylation in sphingolipid metabolism. This glycosylation by GCS is a critical step regulating the modulation of cellular activities by controlling ceramide and glycosphingolipids (GSLs). An increase of ceramide in response to stresses, such as chemotherapy, drives cells to proliferation arrest and apoptosis or autophagy; however, ceramide glycosylation promptly eliminates ceramide and consequently, these induced processes, thus protecting cancer cells. Furthermore, persistently enhanced ceramide glycosylation can increase GSLs, participating in selecting cancer cells to drug resistance. GCS is overexpressed in diverse drug-resistant cancer cells and in tumors of breast, colon, and leukemia that display poor response to chemotherapy. As ceramide glycosylation by GCS is a rate-limiting step in GSL synthesis, inhibition of GCS sensitizes cancer cells to anticancer drugs and eradicates cancer stem cells. Mechanistic studies indicate that uncoupling ceramide glycosylation can modulate gene expression, decreasing MDR1 through the cSrc/β-catenin pathway and restoring p53 expression *via* RNA splicing. These studies not only expand our knowledge in understanding how ceramide glycosylation affects cancer cells, but also provide novel therapeutic approaches for targeting refractory tumors.

Keywords

Ceramide glycosylation; glucosylceramide synthase; glycosphingolipids; cancer; drug resistance; cancer stem cells; MDR1; p53 tumor suppressor; gene expression

I. INTRODUCTION

Sphingolipids are mainly present in eukaryote membranes and are lipids sharing similar structures that consist of sphinganine linked to a fatty acid (Hannun and Obeid, 2008; Merrill, 2011). Ceramide is the simplest in structure, and other complex sphingolipids possess additional hydrophilic domains, such as phosphate, phosphorylcholine and sugar

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moieties attached to their sphingoid bases. C_{18}-ceramide is the major one (Fig. 1), even though the generic "ceramide" is a family of more than 50 distinct molecular species that are synthesized by six ceramide synthases (CerS1-6, also known as the longevity assurance gene products, LASS1-6) (Pewzner-Jung et al., 2006; Rabionet et al., 2008). In the first glycosylation, glucose or galactose becomes attached to the 1-hydroxy group of ceramide, yielding a simple glycosphingolipid (GSL), glucosylceramide (GlcCer) or galactosylceramide (GalCer), respectively (Fig. 1). From these, more complex GSLs, such as lactosylceramide (LacCer), globotriaosylceramide (Gb3), monosialoganglioside (GM3) and others can be synthesized by incorporation of additional sugar residues (Hannun and Obeid, 2008; Yu et al., 2009).

Ceramide and GSLs are important biological molecules in cellular processes of cancer progression, and key modulators of the outcome of cancer treatments. Besides providing structural integrity in membranes, ceramide and GSLs play critical roles in modulating cellular signaling and gene expression (Hakomori, 2010; Hannun and Obeid, 2008; Patwardhan and Liu, 2011). Through these, they alter various aspects of cell functions, notably including apoptosis, proliferation, autophagy, endocytosis, transport, migration, senescence, and inflammation. These sphingolipid-modulated processes in turn are crucial in tumorigenesis, cancer progression, and the efficacies of cancer therapies (Ogretmen, 2006; Ogretmen and Hannun, 2001, 2004; Patwardhan and Liu, 2011; Senchenkov et al., 2001). The balance between ceramide and GlcCer or other GSLs can induce cells to undergo malignant growth, or rescue cancerous cells to normal. The rate-limiting enzymes in ceramide glycosylation, particularly GCS, actively participate in the cell biology of cancer progression by shifting reactions to generate metabolites in favor of cancer (Hakomori, 2010; Liu et al., 2001; Ogretmen and Hannun, 2004; Patwardhan and Liu, 2011). Ceramide can modulate cellular processes directly through interactions with effectors, such as in ceramide-induced mitochondria activation to initiate apoptosis (Chipuk et al., 2012; Hannun and Obeid, 2008; von Haefen et al., 2002). GSLs mainly form lipid rafts, or GSL-enriched microdomains (GEMs), in the plasma membrane, thus supporting or modulating definite signaling cascades (Hannun and Obeid, 2008; Patwardhan and Liu, 2011; Sonnino et al., 2006). Several comprehensive reviews have summarized the progress on dysregulated sphingolipids and cancers (Ogretmen and Hannun, 2004; Pyne and Pyne, 2010). Here, we address evidence showing that cancer drug resistance is attributed to ceramide glycosylation. Glucosylation is one critical step controlling ceramide levels, and also the synthesis of GSLs in cells responding to stresses such as chemotherapy or radiation therapy. As an increase of ceramide after treatments initiates processes of proliferation arrest, apoptosis and autophagy, this ceramide glucosylation can promptly arrest these cellular processes, and thereby protect cancer cells. Furthermore, persistently enhanced ceramide glycosylation can facilitate cancer progression by modulating the expression of genes involved in tumor metastasis, altering the status of cancer stem cells, and facilitating drug resistance (Modrak et al., 2006; Ogretmen et al., 2001a; Ogretmen et al., 1998; Ogretmen et al., 2002; Ogretmen and Safa, 1996, 1997; Ogretmen et al., 2001b; Patwardhan and Liu, 2011). To face the challenge of understanding how ceramide glycosylation by GCS confers drug resistance in cells, we examine these findings with relation to ABC transporters, cancer stem cells and p53 mutations. We also

consider the treatment of drug-resistant cancers through the inhibition of GCS-mediated processes.

II. CERAMIDE GLYCOSYLATION AND GLYCOSPHINGOLIPID-ENRICHED MICRODOMAINS (GEMs)

Ceramide is mainly generated in the endoplasmic reticulum (ER), but its glycosylation is primarily conducted by GCS in the Golgi apparatus of mammalian cells. Via a cascade of enzymatic reactions, more than 3,000 different GSLs can be generated in the ER. In addition to variations in the activities of enzymes involved in glycosylation, the transport of ceramide from the ER to the Golgi, and of GSLs from the Golgi to other membranes, also modulates the distribution of GSLs in the membrane microdomains, thus altering cellular processes (Gault et al., 2010) (Fig. 2).

II. A, Ceramide synthase and its translocation from the ER to Golgi

In mammal cells, ceramide is synthesized predominantly by the *de novo* pathway from serine and palmitoyl-CoA in the ER and ER-associated membrane (Hannun and Obeid, 2008; Merrill, 2011). Ceramide can also be produced from sphingomyelin breakdown catalyzed by sphingomyelinases (SMases) in the inner leaflet of the plasma membrane (neutral SMase) or the outer leaflet of lysosomal membrane (acid SMase) (Hannun and Obeid, 2008; Kolesnick et al., 1994) (Fig. 2). The cells employ two major mechanisms to mobilize ceramide, either ceramide transfer (CERT) or vesicular transport (Gault et al., 2010; Halter et al., 2007; Yamaji et al., 2008). CERT is a cytosolic protein that transfers ceramide from the ER to the Golgi, where it can be modified into sphingomyelin, and possibly GSLs, given that both sphingomyelin synthases (SMS1) and GCS have been localized biochemically to the *cis*-medial Golgi (Futerman and Pagano, 1991). The CERT protein is composed of at least three functional domains that determine its function: pleckstrin homology (PH) domain, FFAT domain, and START domain, sequentially from the N-terminus to the C-terminus (Kudo et al., 2008). The PH domain is able to recognize phosphatidylinositol 4-monophosphate (PI4P) on acceptor Golgi membranes, thereby allowing for directed transport to the Golgi. The FFAT domain is thought to enable binding to ER-resident VAMP-associated proteins (VAP), therefore CERT can only accept ceramide from the ER (Derre et al., 2011; Hanada et al., 2009). The START domain provides a hydrophobic pocket enabling ceramide transport through the aqueous environment of the cytoplasm for delivery to the Golgi. *In vitro* studies have shown that phosphorylation of CERT at multiple serine residues, by casein kinase I or others, results in inhibitory interaction between the START and PH domains that inactivates the PI4P binding and ceramide transfer (Kumagai et al., 2007). It is as of yet unclear if oligomerization or phosphorylation constitutes a general mechanism by which cellular stresses can inactivate CERT (Charruyer et al., 2008). CERT displays a preference for ceramide species with acyl chains less than C22. Although CERT still transfers C_{22}- and $C_{24:1}$-ceramide, it does so with 40% of the efficiency of shorter-chain species (Kudo et al., 2008; Kumagai et al., 2007). In addition, CERT shows minimal to no transfer of $C_{24:0}$-ceramide. Ceramide transported by CERT is preferentially incorporated into sphingomyelin rather than into GSLs (Hanada et al., 2003; Kudo et al., 2010). Because CERT has preference for specific ceramide chain-

lengths, this may at least partially govern which forms of ceramide are preferentially utilized for sphingomyelin synthesis versus which ceramide species are preferred for GSL incorporation.

An alternative pathway for the transport of ceramide species to the Golgi is coat protein dependent, and makes use of vesicular transport (Watson and Stephens, 2005). The principal driving force behind the formation of vesicular carriers is the multi-subunit coat protein complex (COPII); however, little is known about how this pathway is regulated with respect to ceramide transport. Vesicular transport is thought to be the major pathway delivering ceramide to the *cis*-Golgi for GSL synthesis (Gault et al., 2010) (Fig. 2).

II. B, Ceramide galactosylation catalyzed by galactosylceramide synthase (GalCerS), and the synthesis of sulfatide and GM4

Human galactosylceramide synthase (GalCerS), also known as UDP-galactose:ceramide galactosyltransferase (UGT8 or CGT) (E.C. 2.4.2.62), transfers the galactose residue from UDP-galactose to ceramide at the 1-hydroxyl moiety and forms galactosylceramide (GalCer) (Schulte and Stoffel, 1993; Stahl et al., 1994) (Fig. 1). GalCerS (61.1 kDa), encoded by human *UGT8* (2906 bp, accession# NM_001128174), is an ER transmembrane protein, and has its catalytic site facing the lumen of the ER (Bosio et al., 1996; Kapitonov and Yu, 1997; Sprong et al., 1998). GalCerS is structurally related to the UDP-glucuronyltransferases, enzymes critical to type II biotransformation of xenobiotics and to porphyrin metabolism (Stahl et al., 1994). GalCerS has a limited tissue distribution, and its expression is detected primarily in Schwann cells, oligodendrocytes, kidneys, testes, intestine and milk (Bouhours and Bouhours, 1979; Vos et al., 1994). In the central nervous system, GalCer and its subsequent metabolites (sulfatide and GM4) are highly enriched in myelin. *UGT8* knockout mice display a tremor phenotype, severe motor weakness due to loss of nerve conduction, male infertility, and premature death (Fujimoto et al., 2000). Interestingly, the neuronal phenotype in mice lacking *UGT8* can be rescued by expression of an oligodendrocyte-specific *UGT8* gene; this suggests that GalCer is extremely important for oligodendrocyte function (Zoller et al., 2005). GalCer is a precursor for sulfatide (3-sulfogalactosylceramide) and GM4, both of which are synthesized in the Golgi. CLN3 (neuronal ceroid-lipofuscinosis 3) protein, which has five transmembrane domains, including a GalCer lipid raft-binding domain, is crucial for lysosomal function, and is responsible for the transport of GalCer from ER/Golgi to lipid rafts in membrane (Persaud-Sawin et al., 2004; Rusyn et al., 2008) (Fig. 2). Sulfatides are synthesized from GalCer via sulfation by GalCer sulfotransferase, which transfers a sulfate residue from an activated sulfate donor, 3-phosphoadenosine-5′-phosphosulfate (PAPS) (Benjamins et al., 1982; Honke et al., 1997). Sulfatides are enriched in myelin, and many of the known myelination defects may be due to deficiency of sulfatide production. Evidence for this assertion comes from the observation that mice deficient in GalCer sulfotransferase have major defects in myelination, although their pathology is less severe than in an outright *UGT8* knockout mouse (Marcus et al., 2006). Following its biosynthesis, a fraction of GalCer reaches the lumen of the Golgi and is reacted with cytidine-5′-monophospho-*N*-acetylneuraminic acid by the action of sialyltransferase to form *N*-acetylneuraminyl-GalCer (GM4; sialyl-GalCer) (Shanker and Pieringer, 1983) (Fig. 2).

It has been reported that GalCerS expression levels are strongly associated with histological typing in human oligodendrogliomas and astrocytomas; GalCerS can be used as molecular marker with those of other myelin proteins (MBP, CNP, PLP) to distinguish these tumors (Popko et al., 2002). Transcriptome profiling of prostate cancer cell lines showed that cells with metastatic properties express much higher GalCerS mRNA in comparison with non-metastatic cells (Oudes et al., 2005). GalCerS is one of six genes whose elevated expression levels are correlated with increasing risk of lung metastasis in breast cancer patients (Landemaine et al., 2008). Dziegiel Giel *et al.* have furthermore reported that expression of GalCer is higher in breast tumors metastasized to the lung than in matched primary tumors, and that increased amounts of GalCerS enzyme in cancerous tissue are associated with the progression to a more malignant phenotype (Dziecedil Giel et al., 2010). The expression of GalCerS and GalCer appears only in those breast cancer cell lines observed to form metastases in a nude mice model (Dziecedil Giel et al., 2010).

II. C. Glucosylceramide synthase (GCS) and GlcCer translocation

Human GlcCer synthase (GCS) (EC2.4.1.80), also known as UDP-glucose:ceramide glucosyltransferase (GlcT-1), transfers glucose from UDP-glucose to ceramide, thereby producing GlcCer (Basu et al., 1968; Ichikawa et al., 1996; Shukla and Radin, 1990) (Fig. 1). GCS (44.9 kDa), encoded by human *UGCG* (1730 bp; accession# BC038711), is a transmembrane protein present on the *cis*-Golgi, and has its catalytic site facing the cytosol, where newly produced GlcCer can be recognized by the four-phosphate adaptor protein 2 (FAPP2) (Fig. 2) (D'Angelo et al., 2007; Ichikawa et al., 1996; Jeckel et al., 1992). Unlike GalCer, GlcCer is a precursor for more than 3,000 GSLs, the majority of all GSLs that can be produced by mammalian cells, and GCS is the first rate-limiting enzyme in the synthesis of these GSLs (Merrill, 2011; Radin, 1994). Ceramide substrate is transported by vesicles from the ER or by CERT (see section II. A.), and GlcCer synthesized on a cytosolic surface of the Golgi is then translocated across the Golgi membrane for higher GSL synthesis in the *trans*-Golgi (Fig. 2) (D'Angelo et al., 2007; Halter et al., 2007). Studies with rat liver Golgi membrane have found that transbilayer movement of spin-labeled GlcCer is rapid, saturable, and inhibited by protease treatment, suggesting the membranes contain a GlcCer flippase (Buton et al., 2002). GlcCer appears to be synthesized on the cytosolic side of the Golgi, and requires flipping to the inside of the Golgi for the synthesis of complex GSLs, possibly with the aid of the flippase and MDR1 has this activity (De Rosa et al., 2004; Eckford and Sharom, 2005).

FAPP2 is a cytosolic protein consisting of an N-terminal PH domain recognizing the Golgi marker, PI4P, followed by a central proline-rich region, and a glycolipid transfer protein (GLTP)-like domain toward the C terminus (Halter et al., 2007). The FAPP2 has transfer activity for GlcCer both *in vitro* and in cells (D'Angelo et al., 2007; Halter et al., 2007). Knocking down FAPP2 by RNAi reduces the conversion of GlcCer to LacCer, and to downstream high-order GSLs. It has been suggested that FAPP2 functions directly in the formation of apical carriers in the *trans*-Golgi network (TGN). Evidence suggesting that FAPP2 regulates membrane transport from the Golgi by its glycolipid transfer function has also been brought forward. D'Angelo *et al*. favor a transfer of GlcCer from the *cis*-Golgi to the *trans*-Golgi (D'Angelo et al., 2007), whereas Halter *et al*. suggest that FAPP2 takes

GlcCer from the *trans*-Golgi membrane to the ER (Halter et al., 2007). FAPP2 is a dimeric protein that has the capability to form tubules from membrane sheets (an activity that is dependent on the PI4P binding activity of the PH domain of FAPP2). Cao *et al* report that FAPP2 exerts membrane tubulating activity, by binding the small GTPase, Arf1, to induce membrane deformations leading to tubulation at the TGN (Cao et al., 2009). The function of the GLTP domain in FAPP2 remains unclear, but it could be involved in transferring GlcCer to the cellular site where GlcCer can be translocated across the membrane to function as a precursor luminally for complex GSL synthesis, either at the TGN or in the ER, as suggested previously (D'Angelo et al., 2007; Halter et al., 2007). It is also possible that FAPP2 functions as a sensor for regulating glycolipid levels in the cell. The presence of GlcCer on the cytoplasmic sides of the TGN membrane could serve as a signal for FAPP2 to bind. It would do so by coincidence, binding to PI4P, Arf1, and potentially other factors. This ensemble would contribute to the formation and tubulation of transport carriers, which exit from the TGN to deliver both protein and glycolipid cargos to the cell surface. A feedback mechanism would limit GlcCer translocation from the cytosolic to the luminal leaflet when LacCer and other downstream GSLs accumulate in the luminal leaflet of the TGN (Cao et al., 2009). Such a function would be in keeping with the proposition that lipid transfer proteins in general could function as biosensors regulating lipid levels in the cell (D'Angelo et al., 2012; Mattjus, 2009).

II. D. The synthesis of complex GSLs and the formation of membrane microdomains

In mammals, GlcCer and GalCer are initial monohexosylceramides for the synthesis of complex GSLs, but almost all the high-order GSLs are produced from GlcCer following additional reactions catalyzed by glycosyltransferases (Fig. 2) (Merrill, 2011). Human LacCer synthase (LacCerS), also known as UDP-galactose:glucosylceramide β-1→4-galactosyltransferase, is encoded mainly by *β4GalT-V* (3931 bp; accession# AF097159) or *β4GalT-VI*. LacCerS transfers galactose from UDP-galactose to GlcCer to produce LacCer in the Golgi (Fig. 2) (Takizawa et al., 1999). β4GalT-V is also implicated in the synthesis of N-glycans of cell surface glycoproteins. Some of the factors reported to regulate LacCerS include growth factors, cytokines, lipids, lipoproteins, and hemodynamic factors, such as fluid shear stress (Chatterjee et al., 2008).

LacCer is the precursor for synthesis of ganglio-series, globo-series, lacto-series and neolacto-series GSLs (Fig. 1) (Merrill, 2011; Sandhoff and Kolter, 2003). For the ganglio-series GSLs, the enzyme responsible for the first neutral metabolite, GalNAcβ1→4Galβ1→4Glcβ1Cer (GA2, also called asialo-GM2), is GM2 synthase (β4GalNAcT, β1→4-*N*-acetylgalactosylaminyltransferase), or GM2/GD2 synthase because it additionally converts gangliosides GM3 to GM2, GD3 to GD2, and so forth (Furukawa and Takamiya, 2002). LacCer is sialylated to ganglioside GM3 by ST3Gal-V (SAT-I, CMP-*N*-acetyl-neuraminate:lactosylceramide α2→3-sialyltransferase, also known as GM3 synthase) (Yu et al., 2004). Biosynthesis of the globotriosylceramide Gb3 (Galα1→4Galβ1→4Glcβ1Cer), the initial step for the globo-series GSLs, is catalyzed by Gb3 synthase (α1→4-galactosyltransferase, α1→4GalT) (Kojima et al., 2000). Gb3 is converted to Gb4 by Gb4 synthase (β3GalNAcT). Next in this series is Gb5, synthesized by the action of β3GalT-V. Gb5 is also known as the stage-specific embryonic antigen-3

(SSEA-3), a frequently used stem cell marker (Yu and Yanagisawa, 2006; Zhou et al., 2000). Biosynthesis of lacto-/neolacto-series GSLs begins with the formation of GlcNAcβ1→3Galβ1→4Glcβ1Cer (also referred to as Lc3, or amino-ceramide trihexoside, amino-CTH) by β-1→3-N-acetylglucosaminyltransferase (UDP-*N*-acetylglucosamine:β-galactose β1→3-N-acetylglucosaminyltransferase, amino-CTH synthase) (Togayachi et al., 2001).

GSLs synthesized in the Golgi are clustered with sphingolipids and other membrane components to form GSL-enriched microdomains (GEMs), or lipid rafts, and glycosynapses found within cell membranes (Gupta and Surolia, 2010; Hakomori, 2010; Sonnino et al., 2006). GSLs are inclined towards formation of lipid-ordered phases in membranes, both with and without cholesterol; they are therefore prime players in microdomain formation. Lipid rafts are small, heterogeneous and dynamic domains enriched in GSLs, sphingolipids, cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins or other proteins (tetraspanins, caveolins, growth factor receptors, integrins) (Hakomori, 2010; Hancock, 2006). These specialized membrane microdomains profoundly influence membrane organization, and are known to compartmentalize cellular processes by serving as organizing centers for the assembly of signaling molecules, influencing membrane fluidity and membrane protein trafficking, and regulating neurotransmission and receptor trafficking (Lingwood and Simons, 2010; Simons and Ikonen, 1997). Lipid rafts modulate membrane transport, signal transduction, and cell-cell interactions, thus modulating cell responses to stress, and playing key roles in cellular development of drug resistance (Lingwood and Simons, 2010; Liu et al., 2010b).

III. CERAMIDE GLYCOSYLATION AND CANCER DRUG RESISTANCE

Drug resistance is a characteristic detected in 40–80% of solid tumors, and constitutes a serious barrier to successful treatment of cancer patients. Resistance to treatment with anticancer drugs results from a variety of factors. Frequently, resistance is intrinsic to the cancer, but as therapy becomes more and more effective, acquired resistance become common (Gottesman, 2002). Due to genetic instability and survival responses to stress, cancer cells develop multiple mechanisms to evade drug toxicity (Dean et al., 2005; Liu, 2011; Senchenkov et al., 2001). A growing body of evidence indicates that ceramide glycosylation catalyzed by GCS is one of the causes of cancer drug resistance (Liu et al., 2010b; Liu et al., 2001; Reynolds et al., 2004; Senchenkov et al., 2001). These studies show that GCS, through its ability to increase levels of GlcCer, globo-series GSLs and others, modulates drug transport, reduces cell apoptosis, favors proliferation and promotes enrichment of tumors with drug resistance.

III. A, GCS and drug resistance

GCS overexpression is a cause of acquired drug resistance of cancer cells. This has been proven primarily by using drug-resistant cell models, as cells lines selected by stepwise exposure to drugs are a cornerstone for investigating molecular mechanisms underlying cellular resistance (Calcagno et al., 2010; Fairchild et al., 1987; Rogan et al., 1984). Lavie *et al.* reported that GlcCer was accumulated in NCI/ADR/RES ovarian cancer and KB-V-1 cervical cancer cells, indicating a correlation of cellular drug resistance and alterations in

GlcCer metabolism (Lavie et al., 1996). Subsequently, excessive GCS that is responsible for GlcCer production has been detected as a cause of drug resistance in more than 14 different cancer cell lines of human breast, ovarian, colon, and cervical cancers, and leukemia (Baran et al., 2011; Chai et al., 2011; Itoh et al., 2003; Liu et al., 2001; Liu et al., 2008; Song et al., 2012; van Vlerken et al., 2007; Xie et al., 2008; Zhang et al., 2009). These multidrug-resistant cells, selected by diverse agents (doxorubicin, paclitaxel, vinblastine, imatinib), overexpress GCS at levels two- to fourfold higher than their sensitive counterparts (Table 1). Additionally, GCS mRNA levels are significantly increased in drug-resistant HL-60/VCR and MeWo-Etol cells, as compared with their drug-sensitive counterparts (HL-60, MeWo) (Gouaze et al., 2004). Furthermore, silencing GCS expression (using siRNA or antisense oligonucleotide) or inhibition of GCS (using PDMP, Genz-123346) sensitizes these resistant cells, up to 100-fold, to more than 20 anticancer agents of diverse types including doxorubicin (Dox), paclitaxel (Tax), cisplatin (CDDP), vinblastine (Vin) and imatinib (Ima) (Table 1). Furthermore, introduction of the GCS gene into drug-sensitive cells confers cellular resistance to doxorubicin, tumor-necrosis factor-α, daunorubicin and C_6-ceramide in human MCF-7 breast cancer, A549 lung cancer and HL-60 leukemia cells (Itoh et al., 2003; Liu et al., 1999a; Liu et al., 2000; Liu et al., 1999b; Ogretmen et al., 2001a). It has also been noted that GCS could not protect such cell lines as mouse melanoma GM95 and human T-lymphoblastoid Jurkat J16 cells against Dox or CD95 (Tepper et al., 2000; Veldman et al., 2003), indicating that drug resistance is an outcome of sophisticated cellular processes, and is cell-type or cancer dependent. Enhancing cellular ceramide via GCS inhibition (Liu et al., 2001; Patwardhan et al., 2009; Weiss et al., 2003), or delivery of ceramide by using polymeric nanoparticles (van Vlerken et al., 2007), overcomes multidrug resistance, providing further evidence that GCS-mediated abolishment of ceramide-induced apoptosis is one mechanism underlying acquired drug resistance in these resistant cells.

GCS is expressed at diverse levels in normal tissues and cells; however, it has been found that *increased expression of GCS* (rather than any particular absolute expression level) is correlated to the progression of breast cancer, urinary cancer, ovarian cancer and leukemia (Liu et al., 2011b). Itoh *et al.* reported that decreases of cellular ceramide concentrations serves as an indicator of chemoresistance in leukemia (Itoh et al., 2003). Expression levels of GCS, as well as of sphingomyelin synthase (which converts ceramide to sphingomyelin), were found to be twofold higher in chemoresistant leukemia (n=14) than in chemosensitive leukemia (n=9). This finding has been corroborated by another study, wherein it was found that GCS mRNA was elevated by twofold in leukemia patients who displayed nonresponse to chemotherapy (n=30), as compared to the complete-response group (n=35) (Xie et al., 2008). In the same study, overexpression of GCS was also accompanied with increased MDR1 and Bcl-2 expression levels in leukemia that was unresponsive to chemotherapy. Additionally, retrospective analyses of microarray data in clinical trials indicate that elevated GCS expression is associated with ER-positive breast cancer and with poor response to paclitaxel in breast cancer patients (Juul et al., 2010; Ruckhaberle et al., 2009). Other groups reported that, based on microarray data, upregulated GCS expression is a genetic signature for the progression and metastasis of renal cell cancer (Jones et al., 2005), and is associated with lymphatic metastasis in penile carcinoma (Kroon et al., 2008). In bladder cancers, GCS overexpression is associated with lymph node metastasis; the overall

5-year survival and disease-free survival rates are reduced to 75% (45.1 *vs.* 60.3 months and 27.3 *vs.* 36.2 months, respectively) in patients with tumors exhibiting high levels of GCS (Sun et al., 2010a). The levels of GCS mRNA or protein are elevated by fourfold in approximately 80% of metastatic breast tumors in state III (n=5/7) or lymph node-positive (n=7/8) and ER-positive tumors (n=7/9) (Liu et al., 2011b). Different from previous reports, Tomioka *et al.* recently showed that the 9q31 (UGCG, encodes GCS) is one of four loci deleted in primary gastric cancers (n=56) after whole-genome array screening. If any one of these four loci was deleted, the prognosis of the patient was significantly worse (P= 0.0019) (Tomioka et al., 2010).

III. B, GSLs and ABC Transporters

Our understanding of how cancer cells acquire drug resistance during the course of chemotherapy remains incomplete. It is understood though, that in addition to inducing cell death, ceramide generated in cells exposed to anticancer drugs actively participates in modulating gene expression (Patwardhan and Liu, 2011). Possibly, certain species of ceramide modulate the expression of genes contributing to drug resistance of cancers. We at least know that doxorubicin (0.5 μM, a concentration below its IC_{50}) elicits increases in cellular ceramide levels in MCF-7 cells, and activates the GCS promoter via the transcription factor Sp1 to amplify GCS expression in a positive feedback regulation (Fig. 3) (Liu et al., 2008; Uchida et al., 2004). Furthermore, disruption of ceramide synthesis by the CerS inhibitor fumonisin B1 (FB1) prevents the transactivation doxorubicin of GCS expression by doxorubicin; exogenous C_6-ceramide (5 μM, a concentration below its IC_{50}) or SMase can mimic doxorubicin's ability to induce GCS expression (Liu et al., 2008). Cell-permeable ceramide (C_8-ceramide) selection or GlcCer treatments can lead cancer cells to develop resistance to anticancer drugs (Gouaze-Andersson et al., 2007). Alterations of sphingolipids and GSLs in cancer cells exposed to less-toxic concentrations of anticancer drugs may favor cancer cell survival.

GCS overexpression is frequently correlated with MDR1 levels in drug-resistant cells and tumors. Our recent work has shown that globo-series GSLs, synthesized downstream of ceramide glycosylation by GCS, upregulate MDR1 expression via activation of cSrc signaling and TCF4/β-catenin recruitment on the MDR1 gene promoter (Fig. 3) (Liu et al., 2010b). Increases or decreases of various GSLs will alter lipid-lipid interactions or lipid-protein interactions, and affect the action of protein kinases (cSrc kinases) in GEMs of the plasma membrane. As noted above, doxorubicin increases ceramide generation via the *de novo* synthesis pathway and transactivates GCS expression via the Sp1 transcription factor; the consequent increase in concentrations of certain globo-series GSLs (Gb3, Gb5) in turn activates cSrc kinases, increases nuclear β-catenin by diminishing its degradation after phosphorylation, and transactivates MDR1 gene expression. In this way, sphingolipids (ceramide, globo-series GSLs) upregulate GCS and MDR1 expressions in response to anticancer drugs, and thereby confer cell resistance by preventing ceramide-induced apoptosis and MDR1-mediated drug efflux (Fig. 3) (Liu et al., 2010b; Liu et al., 2008). In accord with these findings, we also found that MBO-asGCS, which silences GCS in the nanomolar range, reverses cell resistance by suppression of MDR1 in drug-resistant cells and tumors (Liu et al., 2010b; Patwardhan et al., 2009).

III. C, GSLs and cancer stem cells

Sphingolipids play crucial roles in determining stem cell fate, including self-renewal, proliferation and differentiation; accordingly, sphingolipids can potentially be developed as therapeutic agents to eliminate cancer stem cells (Bieberich, 2004; Hakomori, 2008; Yu et al., 2010). The cell-surface GSLs globopentaosylceramide (Gb5) and monosialyl-Gb5 (MSGb5) are also known as SSEA-3 and SSEA-4 (stage-specific embryonic antigen-3, -4), in recognition of their usefulness as markers on human ES cells (Bieberich, 2004; Pera and Tam, 2010; Stewart et al., 2006). Several studies have shown that alterations of GSLs, as compared with non-stem cells, are associated cancer stem cells (CSCs). As an example, SSEA-3 and Globo H are markers for a subpopulation of CSCs in breast cancer patients (Chang et al., 2008). Breast CSCs with CD55 are highly resistant to ceramide- or serum deprivation-induced apoptosis; and exposure to ceramide (nano-liposomal C_6-ceramide, 3 μM) prevents premature human ES cell differentiation and maintains pluripotent stem cell populations *in vitro* (Salli et al., 2009; Xu et al., 2007). Addition of serum (10% fetal bovine serum, 24 hr) or inhibition of STAT3 phosphorylation with WP1193 (5 μM, 24 hr) significantly decreases the numbers of human GCS11 glioblastoma CSCs ($CD133^+$), accompanied with decreased GCS expression (He et al., 2010). On the other hand, Deoxycholate promotes the survival of mouse breast CSC cells ($CD44^+$/Flk-1^+) by reducing ceramide levels (Krishnamurthy et al., 2008). CSCs isolated from drug-resistant breast cancer cells (MCF-7/Dox) highly express GCS and other stem cell markers (Calcagno et al., 2010); silencing of GCS by using MBO-asGCS (100 nM for 6 days) significantly decreases the CSC numbers in human MCF-7/Dox cells (Gupta et al., 2011). Our unpublished works indicate that ceramide glycosylation by GCS, and the levels of globo-series GSLs in breast CSCs are significantly higher than in non-cancer stem cells or in $ABCG2^+$ bone marrow stem cells. Collectively, these observations suggest that ceramide glycosylation by GCS plays one of the key mechanistic roles in maintaining CSCs in their de-differentiated state.

III. D. Ceramide glycosylation and the expression of p53 mutants

p53 is a key tumor suppressor in preventing tumorigenesis and cancer progression; however, mutant p53, detected in over 50% cases of cancers, promotes tumor progression and resistance to therapies, and such mutants have become the most common prognostic indicator for both tumor recurrence and cancer death (Brosh and Rotter, 2009; Hollstein et al., 1991; Olivier et al., 2006). The majority of p53 mutants in human cancers abrogate their transactivation effects to the p53-responsive genes, such as $p21^{Waf1/Cip1}$, *PUMA*, and *Bax*. Moreover, the mutants confer a dominant-negative activity over the remaining wild-type allele, and also gain new oncogenic properties (Brosh and Rotter, 2009; Bullock and Fersht, 2001). Restoration of wild-type p53 function has succeeded in bringing about regression of tumors, and in fact, this represents a promising approach for treating cancers (Brosh and Rotter, 2009; Ventura et al., 2007).

Disrupting ceramide glycosylation is a new approach to target mutant p53 for cancer treatments (Liu, 2011). Various strategies have been developed to reconstitute p53 functions in suppressing tumor progression and improving treatments. These mainly include replacing wild-type p53 by gene therapy, augmenting wild-type p53 by inhibiting MDM2/MDMX-mediated p53 degradation, and converting mutant p53 into a wild-type mimic by altering its

protein conformation (Brosh and Rotter, 2009; Chen et al., 2010; Wiman, 2010). A recent advance, in addition to these approaches, was the discovery that suppression of GCS could restore wild-type p53 expression and induce p53-dependent apoptosis in p53-mutant cancer cells (Liu et al., 2011a). Human NCI/ADR-RES and OVCAR-8 cancer cells dominantly express p53 mutants with a deletion of seven and six amino acids (encoded by exon 5), respectively, within the DNA binding domain of p53 (Liu et al., 2011a; Ogretmen and Safa, 1997). Silencing of GCS with MBO-asGCS substantially increases the levels of phosphorylated p53, and of the products of p53-responsive genes, including $p21^{Waf1/Cip1}$, *Bax* and *Puma*, consequently directing "mutant p53" cells to apoptosis. Conversely, inhibition of ceramide synthase with fumonisin B1 prevents p53 restoration induced by MBO-asGCS, while addition of exogenous C_6-ceramide reactivates p53 function in p53-mutant cells (Liu et al., 2011a). Furthermore, assessment of hnRNA shows the wild-type p53 hnRNA transcribed in both wild-type and mutant p53 cell lines, although the latter only expresses mutant mRNA and protein, suggesting that silencing GCS may restore p53 at the level of posttranscriptional processing. This study, as proof of concept, indicates that dysfunctional regulation in transcription and associated post-transcriptional processes is an important cause of p53 mutants in cancer cells.

IV. TARGETING CERAMIDE GLYCOSYLATION TO REVERSE DRUG RESISTANCE

Based on the body of work reviewed above, GCS, a rate-liming enzyme of ceramide glycosylation, constitutes a new therapeutic target for reversing drug resistance. Blocking ceramide glycosylation by the inhibition of GCS in drug-resistant cancers can result in increased levels of ceramide and decreased levels of GSLs, thus sensitizing cancer cells to chemotherapy. Compounds drawn from several groups of small molecules have been used to inhibit GCS activity, and gene-based agents, including oligonucleotides and siRNA, have shown promise in specifically targeting cancer cells that overexpress GCS (Fig. 4).

IV. A. GCS inhibitors

Ceramide glucosylation can be blocked by GCS inhibitors (Abe et al., 2001). Two classes of GCS inhibitors have been described, including analogs of D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-propanol (PDMP), and a group of imino sugars (Shayman et al., 2000). PDMP is the parent compound of "P" drugs, including PPMP, PPPP, and Genz-123346 (Fig. 4) (Chai et al., 2011; McEachern et al., 2007). The sensitizing effects of PDMP, PPMP and PPPP have been observed in several types of cancer cells (di Bartolomeo and Spinedi, 2001; Lavie et al., 1997; Nicholson et al., 1999; Sietsma et al., 2000) and in tumor-bearing mice (Huang et al., 2011). Unfortunately, pharmacological interpretation with PDMP is confounded because in addition to inhibiting GCS, PDMP reportedly can inhibit other enzymes involved in GSL metabolism (Lee et al., 1999; Liour and Yu, 2002), and can also affect calcium homeostasis and membrane fluidity (Kok et al., 1998). PPPP is at least tenfold more potent than PDMP or PPMP, and pharmacological interpretation is cleaner because PPPP can much more selectively inhibit human GCS (Hillig et al., 2005; Lee et al., 1999; Liour and Yu, 2002). Genz-123346 is another novel analog of PDMP also having improved selectivity for inhibiting GCS (Chai et al., 2011; McEachern et al., 2007).

Genz-123346 can sensitize drug-resistant KBV-1 cells to vinblastine, and its chemosensitizing activity appears to be mediated primarily through the type of suppression of MDR1 function (Chai et al., 2011). A new compound, CCG-203586, has been found to inhibit GCS at low-nanomolar concentrations, with little to no direct inhibition of MDR1 (Larsen et al., 2012). This compound may be a useful tool for clarifying the mechanistic association between GCS and MDR1 (vide supra) in modulating drug resistance of cancer cells.

The imino sugar, *N*-butyl-deoxynojirimycin (*N*B-DNJ, also known as miglustat, Zavesca®, OGT918) is a reversible and competitive inhibitor of ceramide, but not UDP-glucose, in the reaction catalyzed by GCS (Fig. 4) (Butters et al., 2005). *N*B-DNJ inhibits GCS with modest potency ($K_i \approx 5$ μM) and inhibits α-galactosidase only at much higher concentrations (K_i >100 μM) (Butters et al., 2005). OGT2378, C_4DGJ (*N*-butyl-deoxygalactonojirimycin, also known as OGB-1) and C_9DGJ (*N*-nonyl-deoxygalactonojirimycin, also known as OGB-2) are more selective GCS inhibitors in this class (Norris-Cervetto et al., 2004; Weiss et al., 2003). Genz-529468 is a new and more potent imino sugar-based inhibitor of GCS (Fig. 4) (Nietupski et al., 2012). NB-DNJ inhibition of GCS and, consequently, ganglioside synthesis delayed murine melanoma onset (Guerrera and Ladisch, 2003; Weiss et al., 2003). C_9DGJ can sensitize glioblastoma TMZ-R and PCL-R cells to paclitaxel or temozolomide (Giussani et al., 2012), and colon carcinoma HCT-15 cells to (Chai et al., 2011). C_4DGJ and C_9DGJ sensitize chronic lymphatic leukemia (CLL) patients that overexpress MDR1 (Gerrard et al., 2009). It is also observed that C_4DGJ and C_9DGJ could not reverse drug resistance in NCI/ADR-RES ovarian cancer cells and MES-SA/DX-5 uterine sarcoma cells (Norris-Cervetto et al., 2004). The applications of this group of inhibitors is limited by their micromolar-level inhibitory activity, and a low-specificity against GCS (Larsen et al., 2012).

The medical safety and therapeutic efficacy of GCS inhibitors, including *N*B-DNJ and "P" drugs, have been tested in clinical trials of Fabry disease, HIV, diabetes, and type 1 Gaucher disease (Butters et al., 2005; Larsen et al., 2012); however, their chemosensitizing effects on tumors have not yet been tested in clinical trials. Several therapeutic agents including tamoxifen, mifepristone, cyclosporine A, and arsenic trioxide are also known to nonspecifically inhibit GCS, and sensitize cancer cells to anticancer agents (Dbaibo et al., 2007; Senchenkov et al., 2001).

IV. B. Agents silencing GCS

Silencing of the GCS gene, which directly proves GCS to be a cause of drug resistance, offers a specific approach for sensitizing tumors that poorly respond to chemotherapy due to GCS overexpression (Fig. 4). Antisense gene transfection (full-length) demonstrates the concept that suppression of GCS can sensitize drug-resistant cells, such as NCI/ARE-RES, to anthracyclines, *Vinca* alkaloids, taxanes and other anticancer drugs (Liu et al., 2001; Liu et al., 2000). Oligonucleotides (20-mer) that target the open reading frame of GCS (ORF 18–37), as in a phosphorothioate DNA (Liu et al., 2004; Liu et al., 2008), or a 2′-*O*-methyl RNA with phosphorothioate DNA (mixed backbone oligonucleotide, MBO-asGCS) (Liu et al., 2010b; Patwardhan et al., 2009), sensitize drug cytotoxicity in resistant human NCI/ADR-RES, A2780AD, KB-A1, SW620/AD, and murine EMT6/AR1 cancer cells. RNA

interference by treatment of siRNA duplex (Gouaze et al., 2005) or vector-mediated transfection of short hairpin DNA (pSUPER-GCSshRNA) (Liu et al., 2010a; Sun et al., 2010b; Sun et al., 2006; Zhang et al., 2009; Zhang et al., 2011) sensitizes drug-resistant human breast cancer cells and leukemia cells; however, these siRNA agents have not yet been tested *in vivo*. MBO-asGCS directly administered in animal models has relatively higher uptake by tumors than other tissues, and less nonspecific toxicity (Patwardhan et al., 2009).

V. Prospective

Instead of pinpointing a single target, current studies in this field provide compelling evidence that ceramide glycosylation is highly associated with cancer drug resistance, particularly acquired resistance. As a rate-limiting enzyme in ceramide glycosylation, GCS is essential for many cellular processes in normal physiological as well as pathological conditions. Sensitizing cancer cells, not normal tissues, to chemotherapy requires that we gain further understanding as to how ceramide glycosylation by GCS specifically alters cancerous processes, including drug transport, induced apoptosis, mutant expression of tumor suppressors, and CSC formation. Identifying the roles of particular species of ceramide and GSLs (notably GlcCer, LacCer, Gb3, Gb5), and their effects on lipid-lipid and lipid-protein interactions in these processes, should help us discover approaches to reverse drug resistance, while sparing normal cells and tissues of adverse effects. Elucidating details of GCS protein structure and its conformational changes when interacting with inhibitors will assist in designing and synthesizing more-potent and more-selective GCS inhibitors. Finally, clinical studies designed specifically to assess the association of GCS and the responses of cancer patients to chemotherapy would pave the way for subsequent trials investigating the seemingly great promise of GCS inhibitors or modulating agents for reversing drug resistance in cancer patients.

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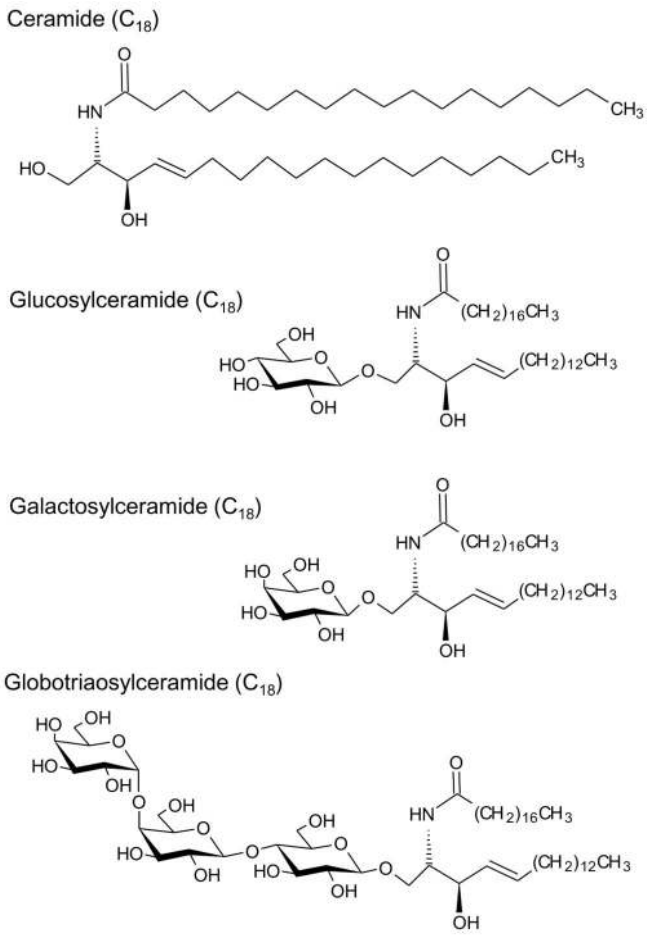

Fig. 1.

Basic structures of ceramide, glucosylceramide, galactosylceramide and globotriosylceramide. In mammals, the prevalent ceramide is C_{18}-ceramide, which has a sphinganine chain length of 18 carbon atoms, with an *E* double bond between C4 and C5 and a C18 fatty acid acylating its C-2 amino. Glucose or galactose is attached to the 1-hydroxy group of ceramide to form glucosylceramide or galactosylceramide. A series of glycosylations transfer galactose units to the glucose moiety of glucosylceramide to generate varies GSLs, such as globotriaosylceramide.

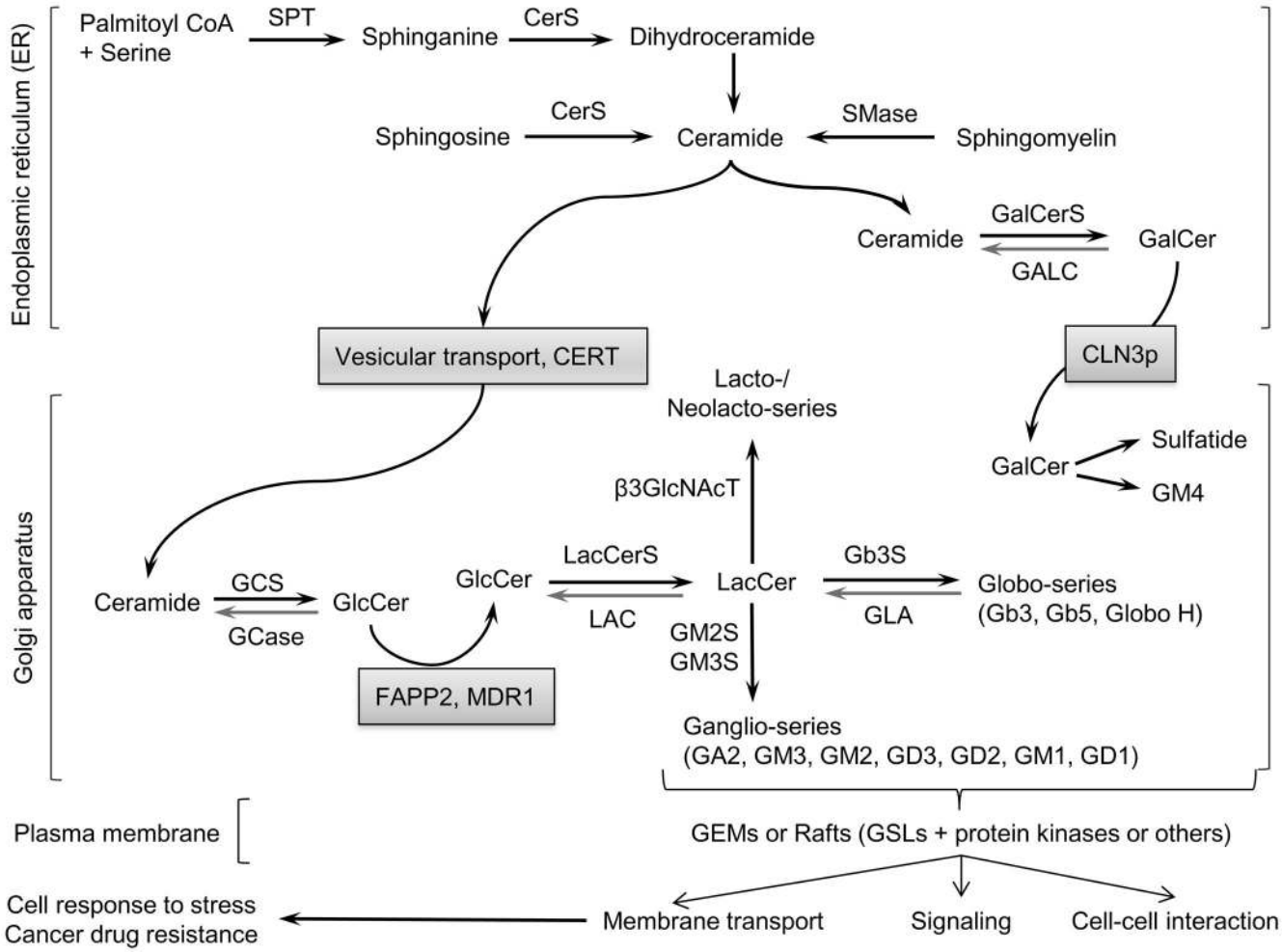

Fig. 2.

Glycosphingolipid biosynthesis and its cellular functions. SPT, serine-palmitoyl transferase; CerS, ceramide synthase; CERT, ceramide transporter; GCS, glucosylceramide synthase; GalCerS, galactosylceramide synthase; GALC, galactocerebrosidase (β-galactosidase); LacCerS, lactosylceramide synthase; Gb3S, globotriaosylceramide synthase; GCase, glucocerebrosidase (β-glucosidase); GLA, α-galactosidase A; GM2S, GM2 synthase; GM3S, GM3 synthase; GEMs, GSL-enriched microdomains.

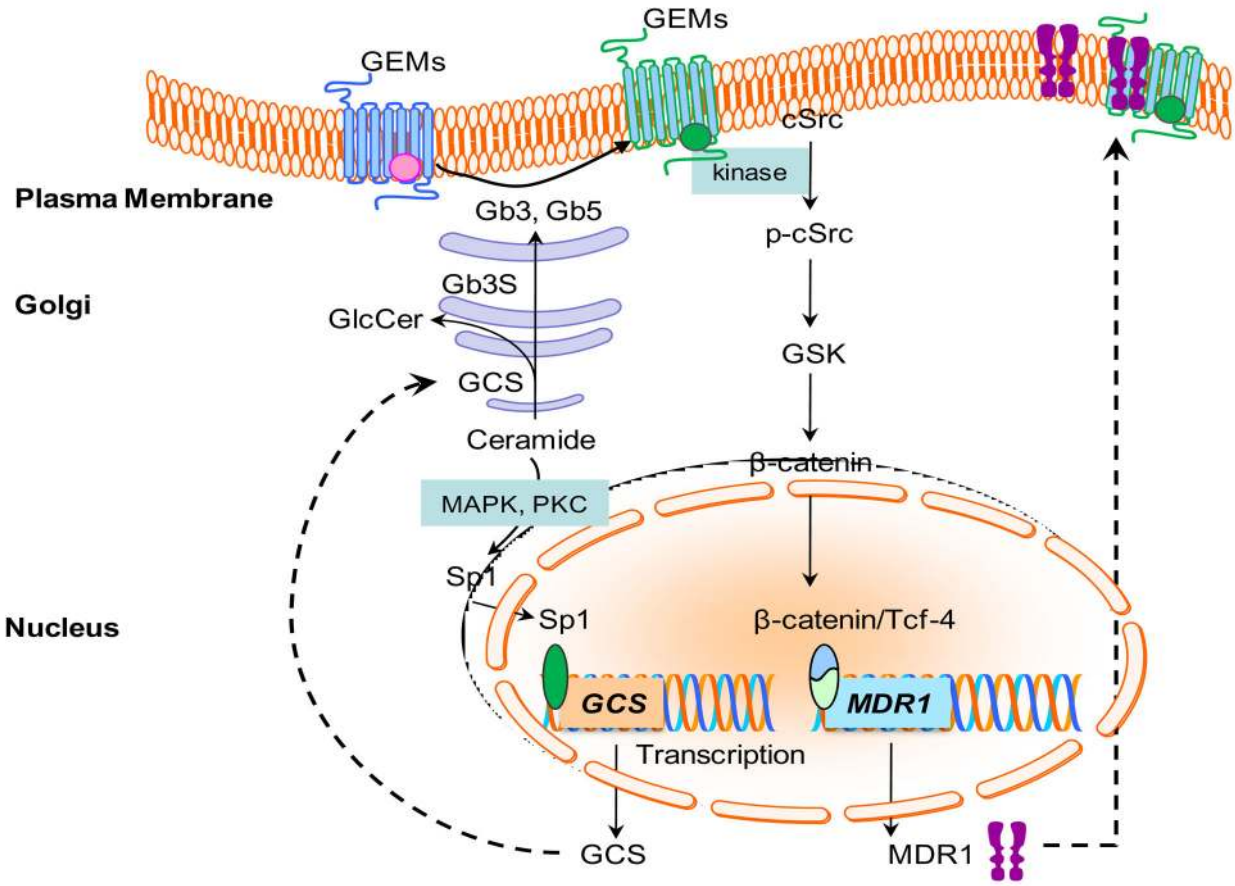

Fig. 3.

Cells exposed to drugs upregulate drug-resistant genes via actions of ceramide and GSLs. Ceramide, generated by *de novo* synthesis in response to stresses, transactivates GCS expression, possibly by way of the MAPK or PKC cascades and the Sp1 transcription factor; globo-series GSLs (Gb3, Gb5) interact with lipids/protein on GEMs and activate the cSrc-GSK cascade, consequently increasing the recruitment of β-catenin/Tcf-4 to upregulate MDR1. MAPK, mitogen-activated protein kinase; GEMs, GSL-enriched microdomains; GSK, glycogen synthase kinase-3.

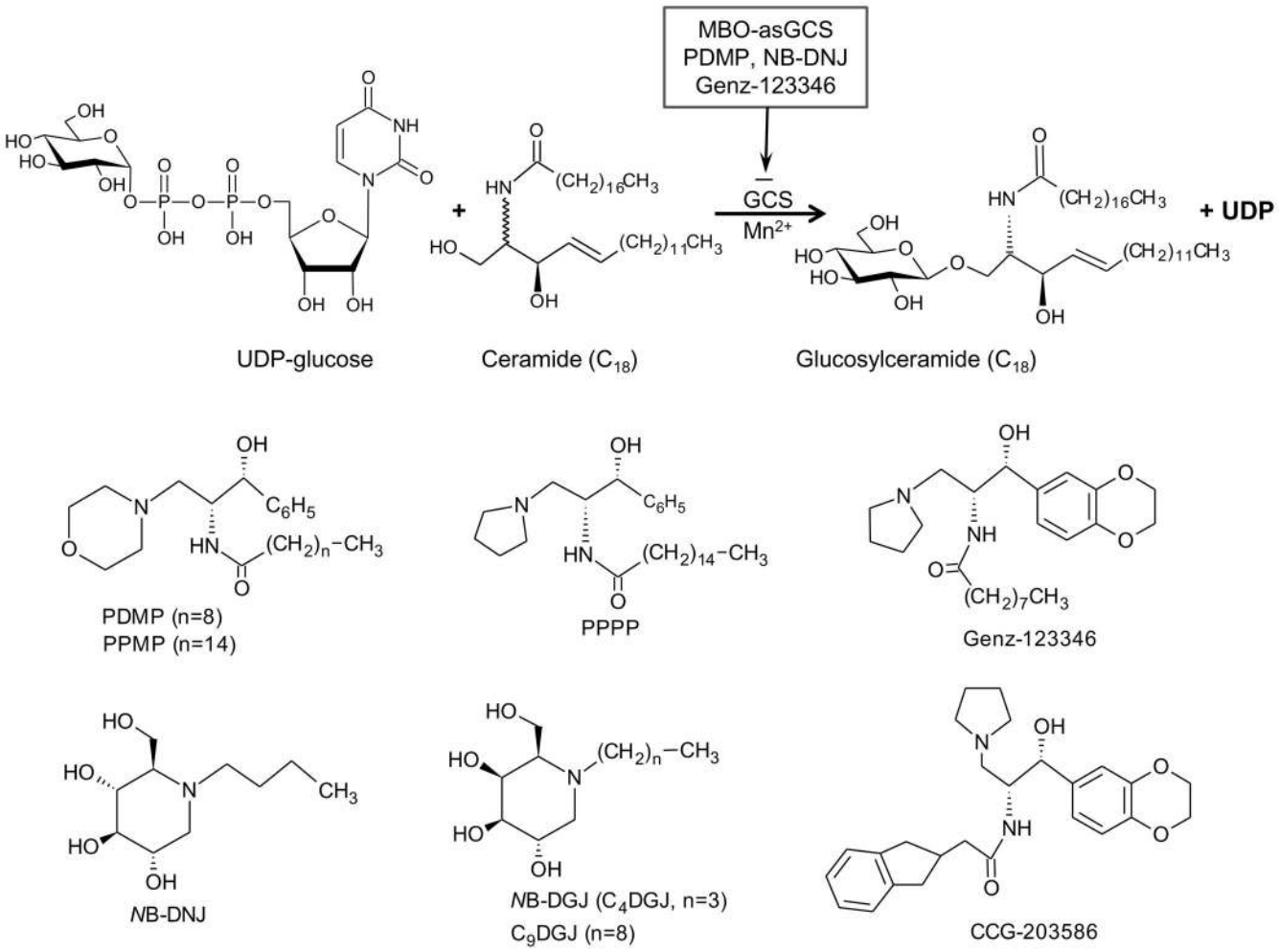

Fig. 4.
Targeting ceramide glycosylation by GCS.

Table 1

GCS and acquired drug resistance in cancer cells.

Cell lines	Acquired resistance (fold)	GCS/GlcCer level (fold)	Other markers	Increased sensitivity (fold)
Breast cancer				
MCF-7	1	1		
MCF-7 P500	55 (Dox)	3	MDR1	35 (oligo)
MCF-7/Dox	22 (Dox)	3	MDR1, CSC	20 (oligo)
MCF-7/ADM	192 (Dox)	2	MDR1	8 (shRNA)
Ovarian cancer				
OVCA8	1	1		
NCI/ADR-RES	33 (Dox)	4	MDR1, mp53	36 (oligo)
A2780	1	1		
A2780-AD	194 (Dox)	3	MDR1	4 (oligo)
SKOV3	1			
SKOV3TR	>100 (Tax)		MDR1	100 (nano-Cer)
Colon Cancer				
SW620	1	1		
SW620AD	121 (Dox)	4	MDR1	62 (oligo)
HCT-8	1	1		
HCT-8/VCR	4.5 (cisplatin)	1.33	MDR1	2 (shRNA)
HCT-15	n/a	n/a	MDR1	>30 (Genz-123346)
$HT29G^{+}$	1			
$HT29^{col}$	25 (Col)	2	MRP1	
Cervical cancer				
KB-3-1	1	1		
KB-A1	121 (Dox)	4	MDR1	76 (siRNA)
KB-V1	213 (Vin)	2	MDR1	17 (Genz-123346)
Leukemia				
K562	1	1		
K562/IMA	19 (imatinib)	2	n/a	4 (PDMP)
K562/A02	50 (Dox)	4	MDR1, Bcl-2	4.5 (PDMP)
HL-60	1	1		
HL-60/ADR	16 (Dox)	2–3	MDR1	
Glioblastoma				
T98G	1	1		
TMZ-R	6.1 (TMZ)	2	MGMT	2 (PPMP/NB-DGJ)
PCL-R	15 (Tax)	2	MDR1	2 (PPMP/NB-DGJ)

Cited from published reports (Baran et al., 2011; Chai et al., 2011; Gouaze et al., 2004; Itoh et al., 2003; Liu et al., 2001; Liu et al., 2008; Song et al., 2012; van Vlerken et al., 2007; Xie et al., 2008; Zhang et al., 2009). GCS levels have been reported in as mRNA or protein levels. Acquired resistance was evaluated in cell viability assay or apoptosis markers. Resistance to a particular anticancer drug or the identity of an agent used to inhibit GCS, is indicated in parentheses. Dox., doxorubicin; Vin, vinblastine; Col, colchicine; Tax, paclitaxel; TMZ, temozolomide; MGMT, O^6-methylguanine-DNA methyltransferase; shRNA, small hairpin RNA; siRNA, small interfering RNA; PDMP, PPMP, NB-DGJ, see main text.