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Letter to the GlycoForum

Glucosylceramide synthase inhibitors differentially affect expression of glycosphingolipids

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Abstract

Glucosylceramide synthase (GCS) catalyzes the first committed step in the biosynthesis of glucosylceramide (GlcCer)-related glycosphingolipids (GSLs). Although inhibitors of GCS, PPMP and PDMP have been widely used to elucidate their biological function and relevance, our comprehensive literature review revealed that the available data are ambiguous. We therefore investigated whether and to what extent GCS inhibitors affect the expression of lactosylceramide (LacCer), neolacto (nLc4 and P_1), ganglio (GM1 and GD3) and globo (Gb3 and SSEA3) series GSLs in a panel of human cancer cell lines using flow cytometry, a commonly applied method investigating cell-surface GSLs after GCS inhibition. Their cell-surface GSL expression considerably varied among cell lines and more importantly, sublethal concentrations (IC_{10}) of both inhibitors preferentially and significantly reduced the expression of Gb3 in the cancer cell lines IGROV1, BG1, HT29 and T47D, whereas SSEA3 was only reduced in BG1. Unexpectedly, the neolacto and ganglio series was not affected. LacCer, the precursor of all GlcCer-related GSL, was significantly reduced only in BG1 cells treated with PPMP. Future research questions addressing particular GSLs require careful consideration; our results indicate that the extent to which there is a decrease in the expression of one or more particular GSLs is dependent on the cell line under investigation, the type of GCS inhibitor and exposure duration.

Key words: cancer cell lines, flow cytometry, PDMP, PPMP

Introduction

The altered glycan moiety on glycosphingolipids (GSLs) has been connected to malignant transformation (Hakomori 2002). GSLs are made of a hydrophobic ceramide (Cer) lipid tail to which an oligosaccharide head group such as glucose (Glc) or galactose (Gal) is attached to form either glucosylceramide (GlcCer) or GalCer. GSLs are constituents of each eukaryotic cell located at the outer layer of the plasma membrane and play fundamental roles in cell adhesion, cell proliferation and differentiation, protein and lipid trafficking, signaling events, and even function as binding ligands of bacterial toxins and viruses (Hakomori 1992; D'Angelo et al. 2013; Ishibashi et al. 2013; Jennemann and Grone 2013).

GlcCer is the precursor to the synthesis of lactosylceramide (LacCer) that is, in turn, the key precursor for further branching into the neolacto, ganglio and globo series (Figure 1A). The transfer of Glc to Cer is catalyzed by the glucosylceramide synthase [UDP-glucose *N*-asylsphingosine glucosyl transferase (EC 2.4.1.80), GCS] and is the first committed and rate-limiting step in GSL biosynthesis (Kan and Kolesnick 1992). GlcCer and GCS are therefore crucial players in many biological processes and competitive GCS inhibitors have been used to study the biological functions of GSLs in more detail (Park et al. 2012).

We have previously shown that blood plasma-derived anti-glycan antibodies discriminate ovarian cancer patients from healthy controls

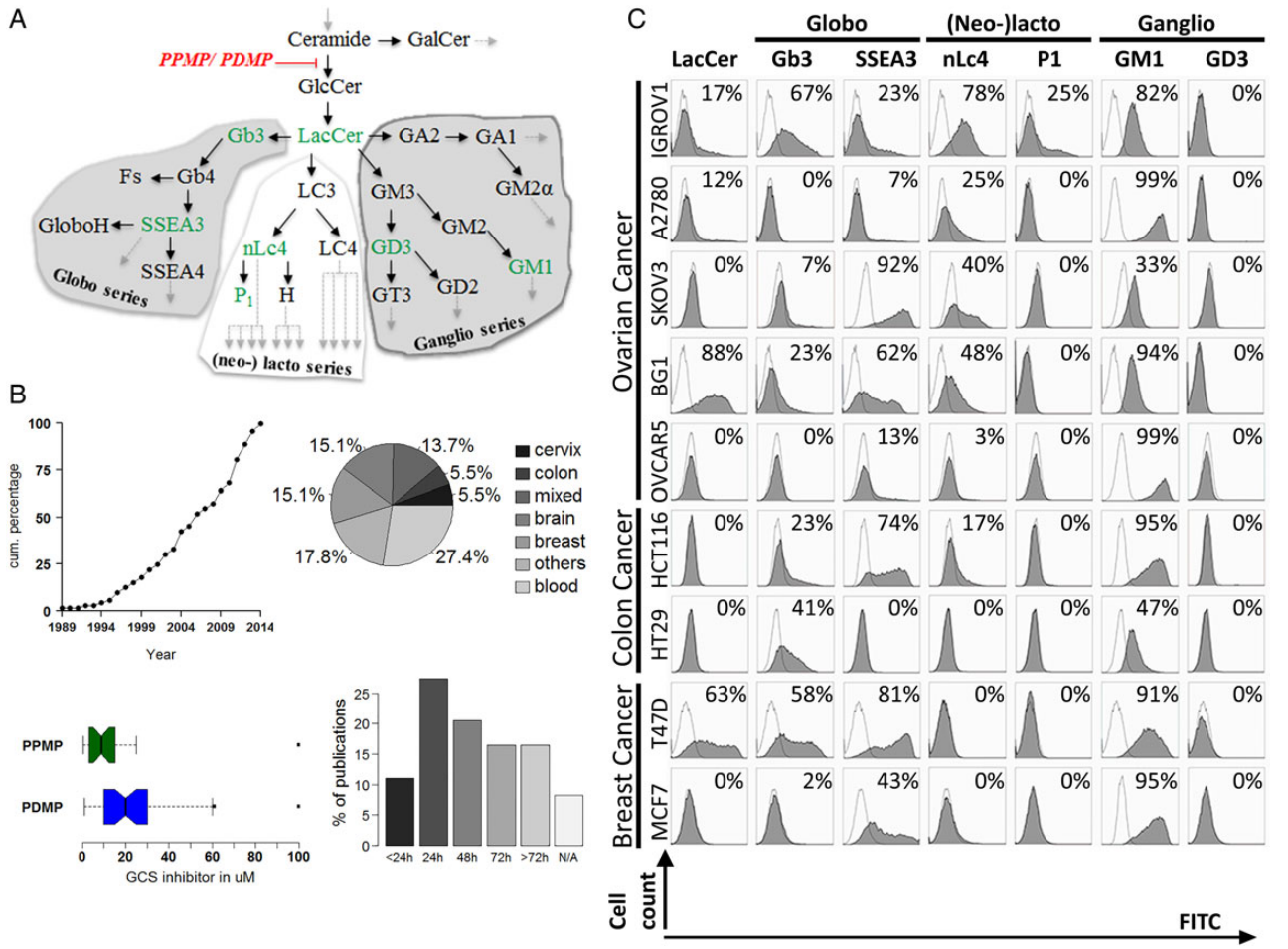

Fig. 1. Glucosylceramide-related biosynthesis, the use of GCS inhibitors in the literature and the differential expression of GlcCer–GSL in a pool of cancer cell lines. (**A**) Depiction of biosynthetic pathway of the globo, neolacto and ganglio series GSLs (respective shaded areas) from GlcCer and LacCer. The key step in this biosynthesis of GlcCer-related GSLs is catalyzed by GCS and is competitively inhibited by PPMP and PDMP (red). GSLs of interest are highlighted in green. Gray arrows indicate further extensions of GSLs. (**B**) Summarized literature showing cumulative increase of publications per year using either both or single inhibitor (top left). Pie chart (top right) presenting the number of studies subdivide according to the origin of the cell lines investigated; >2 organs in one study (mixed) and ≤3 publications on one organ investigated summarized by "others". Box plot (bottom left) showing the median and interquartile range of PPMP (green) and PDMP (blue) concentrations used in the literature. Bar graph depicting the percentage of all studies for categorized time of treatments. (**C**) Flow cytometry data on nine cancer cell lines shown as histogram for negative control (white area under the curve) and GSL-specifically stained sample (dark gray). The value provided in each histogram refers to the percentage of positively stained cells and is a representative of three independent experiments.

using glycan-based immunoassays (Pochechueva et al. 2011; Jacob et al. 2012). More recently, the neolacto series GSL P_1 (Figure 1A) was identified on malignant tissue and cultured cancer cells and therefore may be considered as a cancer-associated carbohydrate (Jacob et al. 2014). To investigate the still poorly understood cellular and molecular functions of neolacto series P_1 and globo series P^k (Gb3), another tumor-associated carbohydrate described in the literature (Kovbasnjuk et al. 2005; Johansson et al. 2009) and encouraged by previous studies showing that GCS inhibitors such as PPMP deplete or reduce the expression of particular GSLs, we evaluated the possibility of depleting cancer cell lines from various cell-surface GSLs using commercially available GCS inhibitors PPMP and PDMP. In order to carefully design our study, we performed a systematic review of the currently available literature in this context and investigated the effect of both inhibitors on representative GSLs of the globo, neolacto and ganglio series in various cell lines.

Results

Literature search

A total of 137 studies have been published since 1989. We identified 73 studies reporting on human cancer cell lines of different tissue that were treated with either with PPMP or PDMP. Since 1989 a continuously growing number of reports have been observed (Figure 1B). In general, both GCS inhibitors were shown to cause cytotoxic, antiproliferative, antiangiogenic and tumor-volume-reducing effects, modulate drug responses in cell lines both cytodestructive (as a drug sensitizer) and -protective (Morjani et al. 2001; Gouaze et al. 2005; Ma et al. 2011; Basu et al. 2012), modulate the level of LacCer and GlcCer and particular reduces the expression of Gb3 in cells (Johansson et al. 2009), modulated the transcription of a set of glycogenes (Ma et al. 2009; Basu et al. 2012), affect protein and lipid trafficking and membrane metabolism (Stefanic et al. 2010) and affect signaling

pathways in cells (Park et al. 2012). Most of the studies investigated cancer cell lines originated from blood (27.5%; Figure 1B). PDMP was generally used in higher concentrations than PPMP (Figure 1B). Information on the mock control [dimethyl sulfoxide (DMSO), isopropanol or water] was provided in only one-fourth (24.7%) of the studies and only 15.0% provided the information (IC_{10-50}) on how the concentration of the inhibitor(s) was determined. The duration of the treatment varied from 30 min up to 168 h (Figure 1B). Particularly interesting to us we found that less than half of the studies (45.2%) addressed the issue of GCS inhibitor-mediated accumulation of Cer or reduction of mainly gangliosides and globosides.

Expression of GSLs in different cancer cell lines

Before addressing the effect of PPMP and PDMP on GSL expression, we measured the GSL content on the cell surface of nine cancer cell lines (Figure 1C). The investigated glycan epitopes include GSLs such as LacCer, globo (Gb3 and SSEA3), neolacto (nLc4 and P_1) and ganglio series (GM1 and GD3) (Figure 1C). We observed a distinct expression of cell-surface GSLs in tested cell lines (Figure 1C). The precursor for all GlcCer-related GSL, LacCer, was highly expressed on BG1 (88%) and T47D (63%), moderately on IGROV1 and A2780 (12 to 30%) and absent on SKOV3, OVCAR5, HCT116, HT29 and MCF7. All cell lines except for HT29 were positive for a globo series GSL, SSEA3. Another member of this series, Gb3, was absent on A2780 and OVCAR5, weakly expressed on SKOV3, HCT116 and MCF7 (2 to 20%) and highly expressed on IGROV1, HT29 and T47D (41 to 67%) (Figure 1C). With regards to neolacto series GSL, more than half of the cell lines were positive for paragloboside (nLc4) except for HT29, T47D, MCF7 and OVCAR5. The second GSL of this series, P_1, was exclusively present on IGROV1 confirming our previous findings (Jacob et al. 2011, 2014). The ganglio series member GM1 was highly expressed in all cell lines while GD3 was completely absent. In summary, all cancer cell lines expressed at least two GSL on their cell surface but had significant differences in GSL distribution, ranging from 6 positive out of 7 measured GSL (IGROV1) to cancer cells expressing only 2 GSL (OVCAR5 and HT29).

Effect of GCS inhibitors on the survival of cancer cell lines and determination of optimal concentration and incubation period

Based on the flow cytometry data, we selected IGROV1, BG1, HT29 and T47D for further analysis according to the following criteria: (i) at least one cell line from each cancer type, (ii) glycan expression, (iii) moderate or high GSL expression and (iv) whether P_1 and/or Gb3 were expressed in cell lines as these are GSL of our primary interest. HT29 was favored due to its higher expression of Gb3 than HCT116. T47D and MCF7 were negative for the neolacto series but T47D had generally higher expression of GSL compared with MCF7 (Figure 1C).

In order to avoid the effects of PPMP and PDMP on the GSL expression, which are compromised by a reduction in cell survival, we first determined optimal inhibitor concentrations. MTT assay was performed in order to obtain the inhibitory concentrations, i.e. the "sublethal" concentrations for PPMP and PDMP (reduces cell survival by no >10%, i.e. IC_{10}) in combinations with different incubation times (24–72 h). The IC_{10} was chosen since concentrations were previously shown to be sufficient to inhibit the GCS activity leading to accumulation of ceramides in the cells (Baran et al. 2007). We also determined the IC_{50} concentrations in order to compare the potency of both inhibitors to influence cell viability. PPMP substantially decreased the cell viability (80–90%) and was more cytotoxic in all cell lines than PDMP (50–85%). This observation was independent of the incubation time (Figure 2A). Both inhibitors significantly differed in their molarity based on IC_{10} and IC_{50} among three cell lines. The only exception was T47D, in which case the treatment time over 24 and 72 h did not result in different cell viabilities.

PPMP and PDMP selectively reduce GSLs in a cell line-dependent manner

Both inhibitors were used to treat selected cell lines (IGROV1, BG1, HT29 and T47D) using IC_{10} (Figure 2A) for 48 h following immuno staining and flow cytometry analysis on viable cells. We expected a reduction in all GlcCer-related GSLs on the cell surface. However, our findings clearly demonstrates that Gb3 is the only GSL reduced on the cell surface of almost all investigated cell lines ($n = 4$) and for both inhibitors. The only exception was observed in case of PDMP treatment on T47D (Figure 2B). In contrast, the expression of the other globo series GSL SSEA3 was only affected in BG1.

While PPMP significantly reduced LacCer expression in BG1 cells, PDMP did not affect LacCer expression in all cell lines. Unexpectedly, remaining GSL levels (nLc4, GM1 and P_1) were not changed by either one or both GCS inhibitors.

Discussion

Chemical compounds PDMP and PPMP are structural analogs of ceramide and were designed to inhibit the GCS, the only known glycosyltransferase in the biosynthesis of GlcCer–GSLs. Consequently, an inhibition of this enzyme is expected to result in reduction of GlcCer–GSLs. We have demonstrated that the extent of the inhibitor-induced decrease of GSL expression is different for each cell line. Some GSLs are even not affected. Gb3 seems to be the only GSL which can be generalized, exhibiting in significant reduction in almost all cell lines for both inhibitors (IC_{10}). This is also in concordance with the literature. However, we would expect a similar reduction for SSEA3, a pentasaccharide GSL comprising of Gb3 in its carbohydrate moiety. This was, however, not observed in our experiments, which may be explained by SSEA3 being mentioned to be linked to *N*-glycopeptides (Shevinsky et al. 1982). Another major finding is that we could not observe a marginal extent of reduction within the ganglio (GM1) and the neolacto series (P_1 and nLc4) GSLs. Therefore, our data suggest that both inhibitors are not suitable to reduce GSL of these series in the investigated cell lines.

The unexpected finding on selective reduction of GSLs using GCS inhibitors could possibly be explained by the complex cellular turnover of GSLs. The GSL turnover is dependent on several factors such as metabolic pathways and fluxes, the flow of the endo- and exocytotic routes, the rate of plasma membrane turnover dependent on functional status of a cell, and the occurrence of external stimuli that may influence neosynthesis or degradation. Therefore, the half-life of a certain GSL can vary from only a few hours to several days (Tettamanti 2004). In the case of globosides, Gb3 may has a shorter turnover compared with SSEA3 in our investigated cell lines and is therefore affected by GCS inhibitors. This may also account for neolacto and ganglio series. In addition, the latter may be mostly affected by GCS inhibitors due to the fact that large amounts of gangliosides are expressed in human tumors of the neuroectodermal origin and only minor in normal tissue (Hakomori 1985).

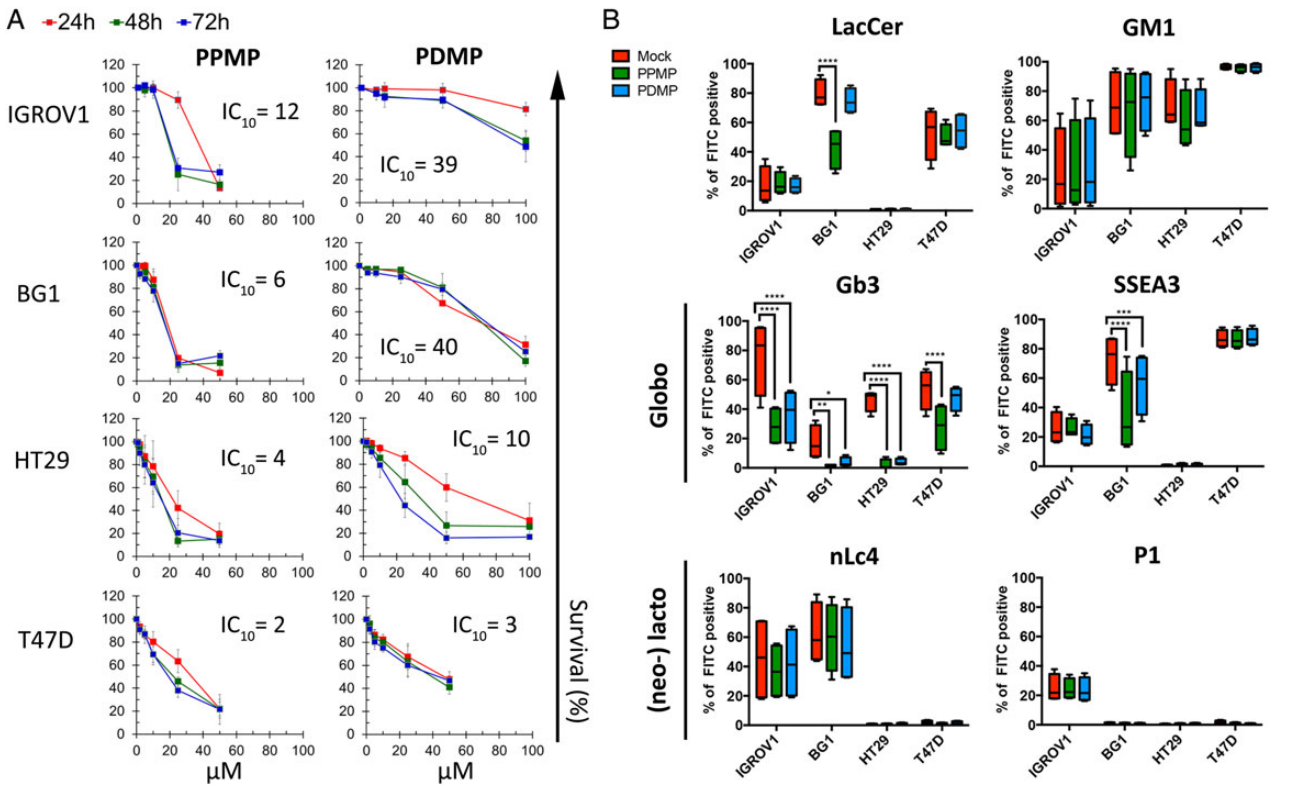

Fig. 2. Effect of PPMP and PDMP on cell survival and on expression of GSLs in IGROV1, BG1, HT29 and T47D cells. (**A**) MTT assay showing cell survival (ordinate) a function of the indicated concentrations and treatment duration [24 h (red), 48 h (green) and 72 h (blue)] for PPMP (left panel) and PDMP (right panel). Results are presented as percentage of the respective mock-treated control. Mean ± SD of three independent experiments performed in quadruplet cultures. The IC_{10} used for further flow cytometry experiments is shown for each cell line and inhibitor. (**B**) Box and Whisker plots representing inhibitor-induced (IC_{10}) changes in expression of individual GSLs (percentage of FITC-positive cells, ordinate) for each cell line (abscissa) after a 48 h treatment with DMSO (red), PPMP (green) or PDMP (blue). Data are representative of four independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$ were obtained by two-way ANOVA.

Another explanation for our observations may be found in the collected data of our systematic review of the currently available literature for the GCS inhibitor PPMP and PDMP affecting the expression of members of the GlcCer-related GSL in human cancer cells. The literature search revealed that in most cases only one or two cell lines and only a small set of GSL (mostly confined to gangliosides) were investigated within a single study. None of the studies investigated three GSL series in parallel. Moreover, the effect on the expression of GlcCer, the direct product of GCS and LacCer, the branching point for three major GlcCer-related GSL series was only determined in a few cases. In contrast, our experimental setup studied the effect of both inhibitors on a large panel of GSLs representing three GlcCer–GSL series in four cell lines, all measured at the same time. Further discrepancies among studies such as inhibitor concentrations, the treatment duration and the type of the vehicle/mock (if provided) may also cause variable results.

Chemical modifications at various sites of these inhibitors were described in order to enhance their inhibitory effect and to increase the extent of ceramide accumulation or of GlcCer-related GSL reduction. A longer acyl chain in PDMP homologs consisting of various lengths (C6–C18) more efficiently inhibited GlcCer synthesis in MDCK cells. Chemical modifications at the fatty acid chain as well as replacement of the morpholino with a pyrrolidoni ring forming D,L-threo-P4 enhanced the inhibitory activity (Abe et al. 1992). Phenyl group substitutions resulting in the compound EtDO-P4 inhibited GCS at an IC_{50} of 90 nM (Lee et al. 1999) and therefore was more potent compared with respective IC_{50} values in the current literature and to our data. However, the same inhibitor displayed an IC_{50} at 4 µM in murine myeloma cells (Manning and Radin 1999). Another important aspect is off-target effects. Enantiomers of GCS inhibitors were shown to raise ceramide levels by inhibition of a pathway forming 1-O-acylceramide that does not involve glucosylceramide synthesis (Shayman et al. 2004). Other GCS inhibitors, Genz-123346 (Silberstein et al. 2011) and EXEL-0346 (Richards et al. 2012), have been recently introduced and may enhance the impact on all GlcCer GSLs.

The initial purpose of our study was to reduce Gb3 and P_1 using PPMP or PDMP in ovarian cancer cell lines. However, a reduction was only seen in case of Gb3 suggesting that both inhibitors can be applied. In case of P_1, we consider the tested GCS inhibitor strategy not suitable to deplete cells of the neolacto series. We therefore conclude that a careful consideration of the type of the inhibitor, of its appropriate concentration, exposure duration and the type of cell line is recommended for experimental studies in which the depletion of a particular GSL is used to address the research question of interest. This, of course, applies to all inhibitor-based studies.

Material and methods

A systematic literature search was performed for the identification of scientific papers addressing human cell lines in combination with PPMP and PDMP using PubMed (http://www.ncbi.nlm.nih.gov/pubmed). The following key words were used: “PPMP” and/or “PDMP”, “PPMP and cancer” or “PDMP and cancer”. The retrieval

was limited from 1989 to August 2014 and included research papers and scientific reports written in English.

Ovarian (IGROV1, A2780, SKOV3, BG1 and OVCAR5), colon (HCT116 and HT29) and breast (T47D and MCF7) cancer cell lines were grown in RPMI-1640 media (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma-Aldrich) at 37°C in a 95% humidified atmosphere containing 5% CO_2.

Primary antibodies to detect the cell-surface-associated GSL were applied as follows: mouse IgM anti-LacCer (CD17) conjugated to biotin (Ancell, USA, 1 : 200), rat IgM anti-P^k (Gb3) antibody (ABDserotec, Germany, 1 : 200), rat IgM anti-SSEA3 antibody (eBioscience, Austria, 1 : 200), mouse IgM anti-nLc4 (paragloboside) antibody [clone 1B2 kindly provided by Prof. Mandel and Prof. Clausen (Young et al. 1981), 1 : 10], human IgM anti-P_1 antibody (Millipore, Germany, 1 : 10), mouse IgG anti-GD3 (BD Pharmingen, Switzerland, 1 : 200) and Cholera toxin B subunit (1 mg/mL, Sigma, 1 : 200) to detect GM1. Secondary antibodies (obtained from BD Pharmingen) were used in 1 : 100 dilutions and include: mouse anti-rat IgM antibody conjugated to biotin, rat anti-mouse IgM conjugated to biotin, mouse anti-human IgM conjugated to biotin and anti-mouse IgG/IgM antibody conjugated to fluorescein isothiocyanate. Cell-surface expressed GSL was detected with FITC streptavidin (1 : 200). Non-viable cells were positively stained with 7-amino-actinomycin (BD Pharmingen, 1 : 50). Isotype controls for each primary antibody were used including purified rat IgM (BD Pharmingen), purified mouse IgM and chrompure human IgM (JacksonImmunoResearch, USA).

D,L-Threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP; Sigma-Aldrich) and D,L-*erythro*-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol•HCl (PDMP; Matreya, USA) were aliquoted in DMSO to 20 and 50 mM stocks, respectively, and stored at −20°C.

The MTT assay was performed to define the inhibitor concentration (IC_{50}) and working concentration (IC_{10}) for flow cytometry experiments. Cells seeded in 96-well plates (BD Falcon, Switzerland) were incubated with various concentrations of PPMP, PDMP or respective DMSO concentrations (mock control) for 24, 48 and 72 h. Cell cultures were incubated with MTT-dye (Sigma) at final concentration 500 µg/mL for 3 h. After removal of the supernatant 200 µL DMSO was added to dissolve the crystals. The optical density (OD, absorbance at 540 nm) was measured with a SynergyH1 Hybrid Reader (Biotek, Switzerland). The data (mean ± SD of three independent experiments performed in quadruplets) were presented as the survival (percentage of control) calculated from the OD after subtraction of the contribution of DMSO mock as a function of PPMP and PDMP concentration and incubation time. IC_{10} and IC_{50} concentrations of PPMP and PDMP were calculated by linear extrapolation.

To evaluate the distribution of GSL LacCer, Gb3, SSEA3, nLc4, P_1, GD3 and GM1, cells grown in T25 cm^2 flask to 80% confluence were washed with PBS and harvested with 1× Cell Dissociation Solution non-enzymatic (Sigma-Aldrich) at 37°C. Cells were transferred to a 96-well V-bottom micro test plate (10^5 cells/well), and pelletized at 400 × *g* for 5 min.

Primary antibodies were added to the cells, resuspended and incubated on ice for 1 h. Cells were then washed twice with FACSWASH (1%, w/v, BSA in PBS) and incubated with the respective secondary antibodies. After additional washing, cells were incubated with streptavidin conjugated to FITC on ice for 30 min. Removal of unbound streptavidin was accomplished by additional washing. Following final washing, cells were re-suspended in 200 µL of FACSWASH and immediately analyzed with the flow cytometer (BD Accuri C6, BD Pharmingen). Each cell line was gated individually to exclude debris (FSC-A vs. SSC-A) and subjected to single cell gating FSC-A vs. FSC-H. Doublets and non-viable cells were excluded from analysis by gating width vs. 7-AAD. Data acquisition were performed using (BD Accuri C6 software, BD Pharmingen), while data analysis were performed using FlowJo v10.0.4 (Tree Star Inc., Ashland, USA).

All comparisons for the MTT assay were statistically evaluated with two-tailed Student's *t*-test. *P*-values of < 0.05 were considered statistically significant. Flow cytometry data were evaluated with two-way ANOVA comparing mock control with GCS-inhibitor treatments.

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Conflict of interest statement

None declared.

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Abbreviations

Cer, ceramide; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; Gal, galactose; Gb3, globotriaosylceramide; Gb4, globotetraose; GCS, glucosylceramide synthase; GD3, *N*-acetylneuraminyl-galactosylglucosylceramide; Glc, glucose; GlcCer, glucosylceramide; GSL, glycosphingolpid(s); IQR, interquartile range; LacCer, lactosylceramide; nLc4, paragloboside; OD, optical density; PDMP, D, L-erythro-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PPMP, D, L-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; SSEA3, stage-specific embryonic antigen 3.

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