Gangliosides do not affect ABC transporter function in human neuroblastoma cells

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Abstract **Previous studies have indicated a role for glucosylceramide synthase (GCS) in multidrug resistance (MDR), either related to turnover of ceramide (Cer) or generation of gangliosides, which modulate apoptosis and/or the activity of ABC transporters. This study challenges the hypothesis that gangliosides modulate the activity of ABC transporters and was performed in two human neuroblastoma cell lines, expressing either functional P-glycoprotein (Pgp) or multidrug resistance-related protein 1 (MRP1). Two inhibitors of GCS, D,L-*threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (*t*-PPPP) and *N*-butyldeoxynojirimycin (*N*B-dNJ), very efficiently depleted ganglioside content in two human neuroblastoma cell lines. This was established by three different assays: equilibrium radiolabeling, cholera toxin binding, and mass analysis. Fluorescence-activated cell sorting (FACS) analysis showed that ganglioside depletion only slightly and in the opposite direction affected Pgp- and MRP1-mediated efflux activity. Moreover, both effects were marginal compared with those of well-established inhibitors of either MRP1 (i.e., MK571) or Pgp (i.e., GF120918). *t*-PPPP slightly enhanced cellular sensitivity to vincristine, as determined by 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide analysis, in both neuroblastoma cell lines, whereas *N*B-dNJ was without effect. MRP1 expression and its localization in detergent-resistant membranes were not affected by ganglioside depletion. Together, these results show that gangliosides are not relevant to ABC transporter-mediated MDR in neuroblastoma cells.**—Dijkhuis, A-J., K. Klappe, W. Kamps, H. Sietsma, and J. W. Kok. **Gangliosides do not affect ABC transporter function in human neuroblastoma cells.** ***J. Lipid Res.*** **2006.** 47: **1187–1195.**

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The majority of children diagnosed with neuroblastoma already have an advanced stage of the disease and require chemotherapy as the primary approach for treatment. A complicating factor in the treatment of neuroblastoma is the occurrence of multidrug resistance (MDR) (1, 2). A number of molecular mechanisms responsible for MDR have been discovered (3), including drug removal by drug efflux pumps and changes in plasma membrane lipid composition (4). A key molecule in drug removal is P-glycoprotein (Pgp, or ABCB1). Pgp belongs to the family of ABC transporters, which couple the hydrolysis of ATP to drug efflux. Multidrug resistance-related protein 1 (MRP1, or ABCC1) also belongs to the ABC transporter family and couples ATP hydrolysis to organic ion transport.

In recent years, a regulatory role for sphingolipids in the MDR phenotype of tumor cells has emerged. The initial observation was that the adriamycin-resistant MCF-7 human breast cancer cell line MCF-7-AdrR accumulated glucosylceramide, which is the product of ceramide (Cer) glycosylation by glucosylceramide synthase (GCS) (5). Glucosylceramide accumulation was subsequently shown in other Pgp-overexpressing tumor cell lines as well as in MRP1-overexpressing cells (6, 7). D,L-*Threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (*t*-PDMP), an inhibitor of GCS, sensitized neuroblastoma cells to cytostatics (8–10). Inhibition of GCS may have a dual effect (i.e., Cer accumulation as well as depletion of glyco-

Abbreviations: Cer, ceramide; CFDA, 5-carboxyfluorescein diacetate; CSA, cyclosporin A; CTB, cholera toxin, B subunit; DRM, detergent-resistant membrane; FCS, fetal calf serum; GCS, glucosylceramide synthase; HPTLC, high-performance thin-layer chromatography; MDR, multidrug resistance; MRP1, multidrug resistance-related protein 1; MTT, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide; *N*B-dNJ, *N*-butyldeoxynojirimycin; Pgp, P-glycoprotein; Rh123, rhodamine 123; TNE, Tris-NaCl-EDTA buffer; *t*-PDMP, D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; *t*-PPPP, D,L-*threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol; TRITC, tetramethyl rhodamine iso-thiocyanate.

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sphingolipids, including gangliosides). Gangliosides have numerous physiological functions, especially in neuronal tissue, where they are relatively abundant. Characteristic ganglioside patterns are associated with aggressive neuroblastoma (11–13). Neuroblastoma cells shed high amounts of gangliosides from the plasma membrane into the surroundings of the cells, where they can inhibit cellular immune responses as well as hemopoiesis (14–16). Gangliosides are enriched in the plasma membrane of cells and more strongly so in certain plasma membrane domains, to which they confer particular physical characteristics. In recent years, the concept of membrane microdomains has been established, relating to a novel organization of sphingolipids, cholesterol, and specific (glycosylphosphatidylinositol-linked) proteins in the plasma membrane (17, 18). In Neuro-2a murine neuroblastoma cells, these membrane microdomains mediate the effects of gangliosides on cell adhesion, differentiation, and signal transduction. Administration of gangliosides (e.g., GM_3) stimulated neurite outgrowth in neuroblastoma cells, and this process was mediated by microdomains (19).

Sphingolipid-mediated and ABC transporter-mediated MDR may be coupled. It is likely that for optimal functioning, ABC transporters are dependent on their immediate lipid environment. Close association of Pgp or MRP1 with sphingolipids may occur in membrane microdomains. Indeed, ample evidence indicates the presence of ABC transporters in membrane domains, either classical lipid rafts or caveolae (20–22). When closely associated, sphingolipids could directly modulate Pgp or MRP1 efflux function. In this context, evidence has been obtained for gangliosides GD_3 and GM_3 as Pgp regulators through the modulation of Pgp phosphorylation in acute myeloid leukemia cells (23).

Thus, it is highly relevant to test whether in neuroblastoma cells gangliosides have a function as ABC transporter regulators, given the abundance of gangliosides in these cells and the importance of membrane domains in neuroblastoma cell biology. We recently established a good model system to study the contribution of specific gangliosides to drug resistance of neuroblastoma. Human SK-N-FI neuroblastoma cells specifically express functional Pgp, whereas SK-N-AS cells specifically express functional MRP1. Moreover, the two cell lines exhibit a specific ganglioside expression profile (24). In this study, we efficiently inhibited ganglioside biosynthesis in parallel using two different inhibitors of GCS, D,L-*threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (*t*-PPPP) and *N*-butyldeoxynojirimycin (*N*B-dNJ). This resulted in only marginal modulation of ABC transporter efflux function, which was statistically significant depending on the type of assay. Ganglioside depletion did not correlate with ABC transporter-dependent cell survival. Ganglioside depletion, which was also very efficient in membrane domains, did not affect MRP1 localization in membrane domains. More strongly, gangliosides and MRP1 appeared to be in different subsets of lipid rafts. In conclusion, we have established that in human neuroblastoma cells, gangliosides do not significantly modulate ABC transporter function.

EXPERIMENTAL PROCEDURES

Materials

MK571 was a gift from Prof. A. W. Ford-Hutchinson (Merck-Frosst, Inc., Kirkland, Canada), and GF120918 was a gift from Glaxo Wellcome (Les Ulis, France). All cell culture plastic was from Costar (Cambridge, MA). Dulbecco's modified Eagle's medium, HBSS, antibiotics, L-glutamine, nonessential amino acids, and trypsin were from Gibco (Invitrogen, Paisley, UK). Fetal calf serum (FCS) was from Bodinco (Alkmaar, The Netherlands). *t*-PPPP was purchased from Matreya LLC (Pleasant Gap, PA). *N*B-dNJ was purchased from Biomol (Plymouth Meeting, PA). Sep-Pak C18 cartridges were from Waters (Milford, MA). L-[U-^{14}C]serine was from Amersham Pharmacia Biotech UK, Ltd. (Buckinghamshire, UK). High-performance thin-layer chromatography (HPTLC) plates were from Merck (Amsterdam, The Netherlands). Cholera toxin, B subunit, type Inaba 569B, peroxidase conjugate (CTB-HRP) was from Calbiochem, Merck Biosciences, Ltd. (Nottingham, UK). 3,3′,5,5′-Tetramethylbenzidine, rhodamine 123 (Rh123), 5-carboxyfluorescein diacetate (CFDA), 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide (MTT), Triton X-100, cholera toxin, subunit B, FITC conjugate (CTB-FITC), and the mouse monoclonal anti-β-actin antibody were from Sigma-Aldrich (St. Louis, MO). Cyclosporin A (CSA) was purchased from Alexis (Carlsbad, CA). Lubrol was obtained from Serva (Heidelberg, Germany). The rat monoclonal anti-MRP1 (MRPr1) antibody was from Signet Laboratories (Dedham, MD). Rhodamine [(tetramethyl rhodamine iso-thiocyanate (TRITC)] goat anti-rat antibody was from Miles-Yeda (Rehovot, Israel).

Cell culture

Human neuroblastoma cell lines SK-N-AS and SK-N-FI were purchased from the American Type Culture Collection (Manassas, VA). They were grown as adherent monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and nonessential amino acids under standard incubator conditions (humidified atmosphere, 5% CO_2, 37°C). To deplete gangliosides, cells were incubated with 1 μM *t*-PPPP or 200 μM *N*B-dNJ for 7 days, unless stated otherwise.

Mass analysis of gangliosides

Gangliosides were isolated from 40×10^6 cells, as described (25, 26). Briefly, pelleted cells were extracted in $CHCl_3/CH_3OH$ (1:1, v/v) and $CHCl_3/CH_3OH$ (2:1, v/v). The supernatants were pooled and dried (N_2), and lipids were redissolved and sonicated in $CHCl_3/CH_3OH$ (1:1, v/v). After centrifugation and overnight storage at −20°C, the supernatants were collected and dried, and their phospholipid content was determined (27). Aliquots containing equal amounts of phospholipid were redissolved in diisopropylether-1-butanol (3:2, v/v), and 17 mM NaCl was added. The aqueous phase was reextracted with diisopropylether-1-butanol and subsequently lyophilized. Samples were dissolved in CH_3OH/water (1:1, v/v) and loaded onto prewashed Sep-Pak C18 cartridges. After rinsing (water), gangliosides were eluted with CH_3OH and $CHCl_3/CH_3OH$ (1:1, v/v). The eluate was concentrated and loaded onto HPTLC plates, which were developed in $CHCl_3/CH_3OH/0.2\%$ (w/v) $CaCl_2$ (11:9:2, v/v/v) and stained with Ehrlich reagent. Gangliosides were quantified using Scion Image Beta 4.0.2 software (Scion Corp., Frederick, MD).

Equilibrium radiolabeling and analysis of gangliosides

Cells were first incubated in the presence or absence of *t*-PPPP or *N*B-dNJ for 2 days and trypsinized. Subsequently, cells were

cultured in the presence of L-[U-^{14}C]serine (0.5 μCi/ml) for 5 days, again in the presence or absence of *t*-PPPP or *N*B-dNJ. Gangliosides were extracted according to the protocol described above, and aliquots containing equal amounts of phospholipid were loaded onto HPTLC plates, which were developed and exposed to film. Gangliosides were scraped, and radioactivity was measured by scintillation counting (Topcount microplate scintillation counter; Packard, Meriden, CT). The ganglioside content was expressed as a fraction of the total lipid-incorporated radioactivity, which was measured after the initial lipid extraction. The ganglioside content of treated cells was then compared with that of untreated cells, which was set at 100%.

Quantification of cellular GM1 levels

After trypsinization, 1×10^6 cells were pelleted and resuspended in 10 μl of HBSS and incubated in the presence of CTB-HRP (0.009 units) for 30 min at 4°C. Cells were washed with ice-cold HBSS, pelleted, and resuspended in 100 μl of ice-cold HBSS. Of each sample, 10 μl was used to determine protein content (28), and to a second 10 μl of each sample, 100 μl of 3,3′,5,5′-tetramethylbenzidine was added on a 96-well plate. After 10 min of incubation at room temperature, 100 μl of 0.5 M H_2SO_4 was added to stop the reaction, and the extinction at 450 nm was measured. To calculate GM1 levels, the extinction values were adjusted to equal protein levels. GM95 cells, which lack glycosphingolipids, were used for background subtraction.

Detection of MRP1- and Pgp-mediated efflux by FACS analysis

SK-N-AS and SK-N-FI cells (0.5×10^6 in HBSS), which were harvested by trypsinization, were incubated with the MRP1 substrate CFDA (0.5 μM) and the Pgp substrate Rh123 (10 μM), respectively, at 10°C for 60 min. Cells were washed twice with ice-cold HBSS and incubated in the presence or absence of the MRP1 inhibitor MK571 (20 μM) or the Pgp inhibitor CSA (10 μM) at 37°C for 0, 5, 10, or 30 min. Efflux of fluorescent substrate was stopped by washing cells with ice-cold buffer, followed by resuspension in buffer containing MK571 or CSA. Retention of fluorescence was determined by flow cytometric analysis using an Elite™ flow cytometer (Beckman Coulter, Miami, FL). For each sample, 10,000 events were collected and analyzed using Win-list 5.0 software (Verity Software House, Inc., Topsham, ME). To determine the efflux-blocking factor (BF), cells were incubated at 37°C in either the presence (+/+) or absence (−/−) of MK571 or CSA, during both loading (60 min) and efflux (10 min) of fluorescent substrate. The BF is defined as follows, where F.U. indicates median relative fluorescence units:

$$BF = \frac{[\text{F.U. in } (+/+) \text{ cells} - \text{F.U. in untreated cells}]}{[\text{F.U. in } (-/-) \text{ cells} - \text{F.U. in untreated cells}]}$$

Measurement of cellular sensitivity to cytotoxic drugs (MTT assay)

Fifteen thousand cells per well were plated on microtiter plates. Seventy-two hours after plating, the amount of viable cells was determined as described previously (29). Briefly, 100 μg of MTT was added to each well, and cells were incubated for 3.5 h at 37°C. Plates were then centrifuged (15 min at 900 *g*), and the supernatants were removed. Pellets were dissolved in DMSO, and absorbence was measured with a microtiter plate reader (μQuant; Bio-Tek Instruments, Winooski, VT) at a λ of 570 nm. The background absorbence was subtracted from all values, and data were expressed as percentages compared with untreated control values (=100%). From these data, the EC_{50} (concentration at which cell viability was reduced by 50%) was determined.

Isolation of detergent-resistant membranes

Detergent-resistant membrane (DRM) fractions were isolated from cells as described (30). For each isolation, confluent cells from two 75 cm^2 flasks were washed once with HBSS, harvested by scraping in 2 ml of ice-cold Tris-NaCl-EDTA buffer (TNE; 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and protease inhibitors) containing 1% (w/v) Triton X-100 or 0.5% (w/v) Lubrol. After 30 min of incubation on ice, cells were homogenized further by passing the lysate at least 10 times through a 21 gauge needle. Two milliliters of the lysate was transferred to a centrifuge tube and mixed with 2 ml of 80% (w/v) sucrose in TNE. On top of this, 4 ml of 35% (w/v) and 3 ml of 5% (w/v) sucrose in TNE were loaded successively, resulting in a discontinuous gradient. Gradients were centrifuged in a Beckman SW41 swing-out rotor (Beckman Coulter, Inc., Fullerton, CA) at 40,000 rpm for 18–20 h at 4°C. Eleven fractions of 1 ml each were collected (from top to bottom), vortexed, and stored at −80°C. The protein content (28) of all fractions was measured using BSA as a standard.

Immunoblot analysis

Protein from the gradient was TCA-precipitated and resuspended in sample buffer. TCA-precipitated proteins were resolved on SDS-PAGE (10%) minigels and subsequently electrotransferred onto a nitrocellulose membrane (Trans-Blot Transfer Medium membrane; Bio-Rad, Hercules, CA). The membranes were rinsed with PBS and incubated (1–2 h at room temperature) with 5% (w/v) nonfat dry milk in PBS. Membranes were rinsed in washing buffer [PBS containing 0.3% (v/v) Tween 20] and incubated (at least 2 h at room temperature) with a primary antibody against MRP1 (1:1,000) or β-actin (1:1,000) in washing buffer containing 1% (w/v) nonfat dry milk. Membranes were rinsed in washing buffer and subsequently incubated for 2 h with the appropriate horseradish peroxidase-conjugated secondary antibody (1:300) (ECL; Amersham Biosciences UK, Buckinghamshire, UK) in washing buffer containing 1% (w/v) nonfat dry milk (2 h at room temperature). Membranes were incubated in chemiluminescence substrate solution (ECL; Amersham Biosciences UK, Buckinghamshire, UK) according to the manufacturer's instructions, and immunoreactive complexes were visualized by exposure to Konica Minolta medical film (Tokyo, Japan). β-Actin was detected using phosphatase-conjugated sheep anti-mouse antibodies (AP) and *p*-nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates in 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl and 5 mM $MgCl_2$. The reaction was terminated by adding 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, and blots were scanned.

GM1 dot blot

The fractions of DRM isolation were screened for GM1 content by means of a dot blot with CTB-HRP. Five microliters of each fraction was spotted onto a nitrocellulose membrane. After a 1 h incubation with 5% (w/v) nonfat dry milk in PBS, the membrane was incubated with CTB-HRP (0.2 units per 10 ml) in PBS containing 0.3% Tween 20 for 1 h. After extensive washing with PBS/0.3% Tween 20, the membrane was incubated with ECL solution and exposed to Konica Minolta medical film. For quantification, different dilutions of the fractions were spotted on the membrane and different exposure times were used. The spots were quantified using Scion Image Beta 4.0.2, and only data in the linear range were used for further analysis.

Confocal laser scanning fluorescence microscopy

For microscopy, SK-N-AS cells were grown on glass coverslips on 12-wells plates. Cells were first blocked with 10% FCS in PBS

and incubated in the presence of FITC-conjugated CTB (1:100) on ice for 30 min before fixation. Cells were fixed with 4% paraformaldehyde on ice for 20 min, permeabilized with 0.1% Triton X-100 at room temperature for 5 min, and blocked with 10% FCS in PBS at room temperature for 30 min before antibody incubation. Primary (overnight at 4°C) and secondary (0.5 h at room temperature) antibody incubations were carried out in PBS containing 2% FCS. Cells were stained with rat anti-MRP1 (1:300) and rhodamine (TRITC) goat anti-rat antibody. After antibody incubations, cells were washed three times with 2% FCS in PBS. Analysis of the samples was performed using a TCS Leica SP2 Confocal Laser Scanner Microscope (Leica, Heidelberg, Germany) equipped with a HCX PL APO 63× 1.32-0.6 oil CS objective in combination with Leica Confocal Software. Pictures were processed using Corel Graphics Suite 11 (Corel, Minneapolis, MN).

Statistical analysis and conversion keys

Results are presented as means ± SD ($n \geq 3$). Statistical analysis was performed with Student's *t*-test, considering $P < 0.05$ significant. To compare results with different normalization, the following keys apply: SK-N-AS cells, 0.26 nmol phospholipid/μg protein (SD = 0.06; n = 4); SK-N-FI cells, 0.33 nmol phospholipid/μg protein (SD = 0.08; n = 4). It should be noted that *t*-PPPP and *N*B-dNJ treatment did not affect phospholipid levels in cells, as determined by lipid phosphorus determination (27). In *t*-PPPP-treated cells, the values were 0.29 nmol phospholipid/μg protein (SD = 0.11; n = 3; $P = 0.60$ compared with control) for SK-N-AS cells and 0.31 nmol phospholipid/μg protein (SD = 0.06; n = 3; $P = 0.73$ compared with control) for SK-N-FI cells. In *N*B-dNJ-treated cells, the values were 0.34 nmol phospholipid/μg protein (n = 2) for SK-N-AS cells and 0.34 nmol phospholipid/μg protein (n = 2) for SK-N-FI cells.

RESULTS

Both *t*-PPPP and *N*B-dNJ efficiently deplete gangliosides

Ganglioside biosynthesis and content were efficiently depleted in the human neuroblastoma cell lines SK-N-AS and SK-N-FI after a 7 day incubation with the GCS inhibitors *t*-PPPP (1 μM) or *N*B-dNJ (200 μM). Both inhibitors reduced ganglioside biosynthesis and content to ~10%, as determined by equilibrium radiolabeling of sphingolipids (**Fig. 1**). Endogenous ganglioside levels of both SK-N-AS and SK-N-FI cells showed a corresponding decrease to ~15% upon treatment with *t*-PPPP (**Fig. 2**). A third assay was used to determine the efficiency of ganglioside content depletion in the SK-N-AS cell line. This assay was based on the detection of GM1 levels using HRP-conjugated cholera toxin. Relative GM1 levels correlated well to those of the total ganglioside pool upon inhibition of GCS with *t*-PPPP (16 ± 9%; n = 3) or *N*B-dNJ (17 ± 10%; n = 3). In conclusion, three different assays show a depletion of gangliosides by *t*-PPPP or *N*B-dNJ of ~85%.

Ganglioside depletion has statistically significant but marginal effects on MRP1 and Pgp activity

We next determined whether the efficient depletion of gangliosides had any impact on the activity of the membrane proteins MRP1 and Pgp. SK-N-AS cells were loaded with CFDA, a fluorescent substrate of MRP1, and SK-N-FI cells were loaded with Rh123, a fluorescent substrate of Pgp. Efflux activity was determined on the basis of fluorescence retention after cells were placed at 37°C in the presence or absence of a specific MRP1 or Pgp inhibitor. Inhibition of ganglioside biosynthesis with *t*-PPPP or *N*B-dNJ appeared to have a stimulatory effect on MRP1 activity (**Fig. 3A**). In contrast, an inhibitory effect on Pgp activity was observed (Fig. 3B). However, under these experimental conditions, the observed effects were not significantly different from the ABC transporter activity of untreated cells. A different experimental setup of the efflux activity assay was used to examine whether depletion of gangliosides truly affected ABC transporter activity. In this setup, one batch of cells was loaded with a fluorescent substrate in the presence of an efflux inhibitor. Then, fluorescence retention was determined by cytometric analysis 10 min

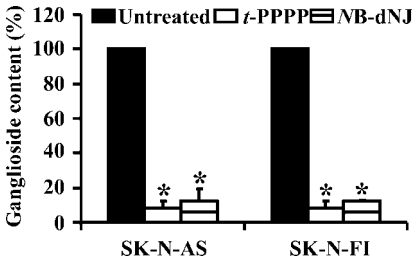

Fig. 1. Ganglioside depletion in SK-N-AS and SK-N-FI cells by D,L-*threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (*t*-PPPP) or *N*-butyldeoxynojirimycin (*N*B-dNJ). SK-N-AS and SK-N-FI cells were incubated in the absence or presence of *t*-PPPP or *N*B-dNJ for 7 days and in the presence of L-[U-^{14}C]serine for 5 days. Gangliosides were extracted and quantified. Both inhibitors reduced ganglioside biosynthesis to ~10% in both cell lines. Data represent means ± SD of three to five independent experiments. * Values are significantly ($P < 0.05$) different from those of untreated cells as determined by Student's *t*-test.

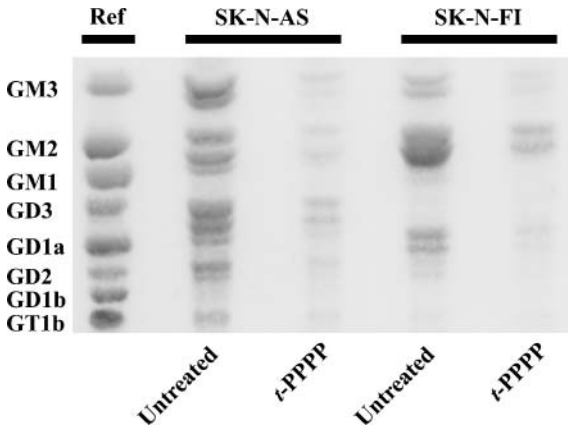

Fig. 2. *t*-PPPP significantly reduces endogenous ganglioside content. Incubation of SK-N-AS and SK-N-FI cells in the presence of *t*-PPPP (1 μM) for 7 days reduced the endogenous ganglioside content to 15 ± 2% (n = 3) and 14 ± 12% (n = 3), respectively. Gangliosides were isolated from aliquots of cells with equal phospholipid content, separated on high-performance thin-layer chromatography plates, and quantified by densitometry. Shown are representative results of three independent experiments.

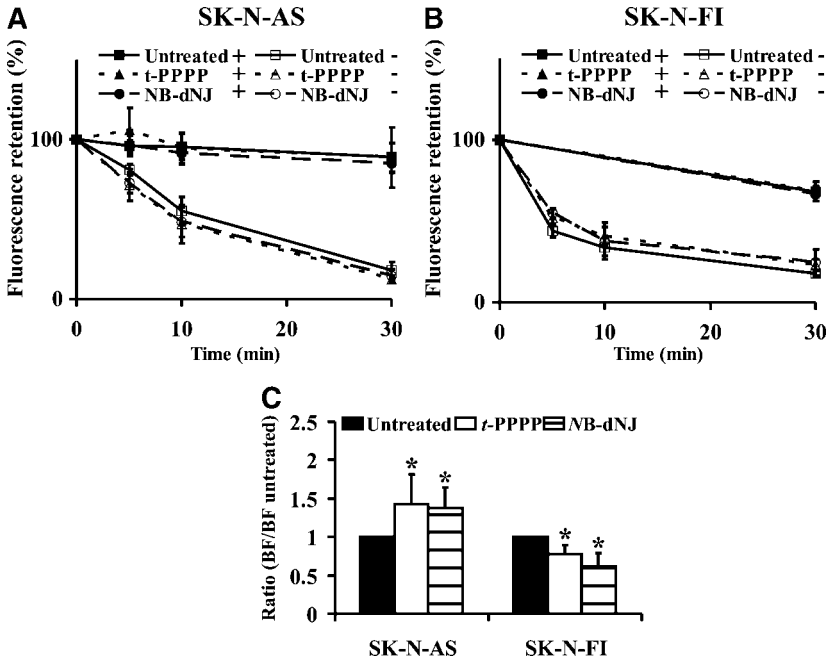

Fig. 3. Efflux activity of multidrug resistance-related protein 1 (MRP1) and P-glycoprotein (Pgp) is marginally affected by depletion of gangliosides. A, B: After a 7 day incubation with *t*-PPPP (1 μM) or *N*B-dNJ (200 μM), SK-N-AS (A) and SK-N-FI (B) cells were loaded with 5-carboxyfluorescein diacetate (0.5 μM) and rhodamine 123 (10 μM), respectively, for 1 h at 10°C. Retention of fluorescence was determined by cytometric analysis at several time points after cells were placed at 37°C in the presence (+) or absence (−) of MK571 (20 μM; SK-N-AS) or cyclosporin A (CSA; 10 μM; SK-N-FI). The data show that ganglioside depletion has a small and not statistically significant stimulatory effect on MRP1 activity (A) and an inhibitory effect on Pgp (B). C: Cells were incubated at 37°C either in the presence or the absence of MK571/CSA, during both loading (1 h) and efflux (10 min) of fluorescent substrate. Blocking factors (BF) were calculated and presented as a ratio, which is defined as the blocking factor of untreated, *t*-PPPP-treated, or *N*B-dNJ-treated cells divided by the blocking factor of untreated cells. In this setup, the effects of ganglioside depletion on MRP1 and Pgp activity reached statistical significance. Data represent means ± SD of three to five independent experiments. * Values are significantly ($P < 0.05$) different from those of untreated cells as determined by Student's *t*-test.

after cells were transferred to 37°C, again in the presence of an efflux inhibitor. Loading and efflux in the other batch of cells was performed in the absence of an efflux inhibitor. From these data, efflux-blocking factors were calculated (see Experimental Procedures), which were then converted to ratios, defined as the efflux-blocking factor of *t*-PPPP- or *N*B-dNJ-treated cells divided by the efflux-blocking factor of untreated cells. Thus, a ratio >1 indicates a higher efflux activity compared with untreated cells, as is the case for both *t*-PPPP- and *N*B-dNJ-treated SK-N-AS cells (Fig. 3C). MRP1 activity was stimulated as a consequence of *t*-PPPP as well as *N*B-dNJ pretreatment, whereas Pgp activity was inhibited under these conditions (Fig. 3C). Thus, in this setup (Fig. 3C), a similar trend was observed as in the previous setup (Fig. 3A, B), but now significance was reached. In conclusion, depletion of gangliosides does affect MRP1 and Pgp activity. However, these effects are marginal compared with the inhibitory actions of MK571 and CSA, respectively.

Ganglioside depletion does not affect chemosensitivity

One of the major obstacles for the successful treatment of cancer is the innate or acquired resistance to a wide range of chemotherapeutic drugs, which is often attributable to the expression of MRP1 or Pgp in tumor cells. Impaired transporter activity could thus restore tumor cell chemosensitivity. We used vincristine, a microtubule-destabilizing drug and substrate of both MRP1 and Pgp, to determine the chemosensitizing effect of ganglioside depletion. MK571 drastically sensitized SK-N-AS cells to vincristine, as indicated by a reduction in EC_{50} (**Fig. 4**), and CSA similarly sensitized SK-N-FI cells (Fig. 4). This indicates MRP1- and Pgp-dependent resistance to vincristine in SK-N-AS and SK-N-FI cells, respectively. Based on the efflux data (Fig. 3), an increased resistance to vincristine would be expected in SK-N-AS cells pretreated with *t*-PPPP or *N*B-dNJ, whereas a decrease in vincristine resistance would be expected in SK-N-FI cells pretreated with *t*-PPPP or *N*B-dNJ. However, *t*-PPPP pretreatment of both SK-N-AS and SK-N-FI cells reduced resistance to vincristine, whereas *N*B-dNJ had no effect on resistance in either cell line (Fig. 4). In conclusion, ganglioside depletion does not sensitize neuroblastoma cells to vincristine, whereas *t*-PPPP has a specific effect on cell survival independent of MRP1- or Pgp-mediated resistance.

Ganglioside depletion does not affect DRM localization of MRP1

To this point, we have shown that a 7 day incubation of the human neuroblastoma cell lines SK-N-AS and SK-N-FI

with either *t*-PPPP or *N*B-dNJ did not significantly affect MRP1 or Pgp activity, despite a dramatic decrease of ganglioside content in these cells. Because both ABC transporter proteins and gangliosides are enriched in lipid rafts, the possibility exists that a selective retention of residual gangliosides in these rafts could facilitate ABC transporter localization and activity. To exclude this possibility, we compared the GM1 content of Lubrol- and Triton X-100-insoluble membrane domains of untreated and *t*-PPPP-treated SK-N-AS cells. In agreement with the GM1 levels in whole cells, *t*-PPPP treatment significantly decreased the GM1 levels in Lubrol- and Triton X-100-insoluble membrane domains to 7 ± 3% (n = 3) and 16 ± 7% (n = 3), respectively. To test whether depletion of gangliosides from these membrane domains has any consequences for the localization of MRP1, we assessed the association of MRP1 to Lubrol- and Triton X-100-insoluble membrane domains upon *t*-PPPP-mediated ganglioside depletion. There was no effect of *t*-PPPP on the distribution of MRP1 in DRMs and detergent-soluble membranes (**Fig. 5A**). The total expression of MRP1 in the SK-N-AS cells also was not affected (Fig. 5B). In conclusion, gangliosides are efficiently depleted from DRMs, but this depletion does not affect MRP1 expression and its localization in DRMs.

MRP1 and GM1 do not colocalize

The observation that the depletion of DRM gangliosides did not have a significant impact on MRP1 activity or its DRM localization could imply that they are localized in different subsets of membrane domains. Supportive evidence for this notion came from colocalization studies. After staining for MRP1 and GM1 in SK-N-AS cells, confocal laser scanning microscopy revealed the absence of colocalization of the ganglioside and the ABC transporter (**Fig. 6**). Together, our results indicate the absence of a functional relationship or of direct contact between gangliosides and MRP1.

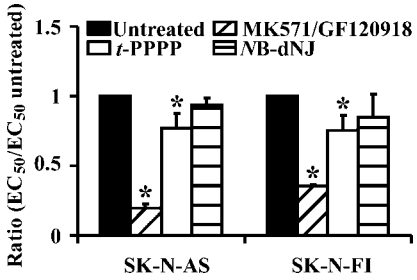

Fig. 4. Ganglioside depletion does not affect chemosensitivity. Treatment of SK-N-AS and SK-N-FI cells with the cytostatic vincristine in the presence of the MRP1 inhibitor MK571 (50 μM; SK-N-AS) or the Pgp inhibitor GF120918 (1 μM; SK-N-FI) significantly reduced EC_{50} values. This indicates an ABC transporter-dependent resistance to vincristine in both cell lines. Ganglioside depletion by *N*B-dNJ did not affect vincristine sensitivity, whereas depletion by *t*-PPPP resulted in a small reduction of the EC_{50} value in both cell lines. Results are presented as a ratio, which is defined as the EC_{50} of untreated, MK571/GF120918-treated, *t*-PPPP-treated, or *N*B-dNJ-treated cells divided by the EC_{50} of untreated cells. Data represent means ± SD of three to five independent experiments. * Values are significantly ($P < 0.05$) different from those of untreated cells as determined by Student's *t*-test.

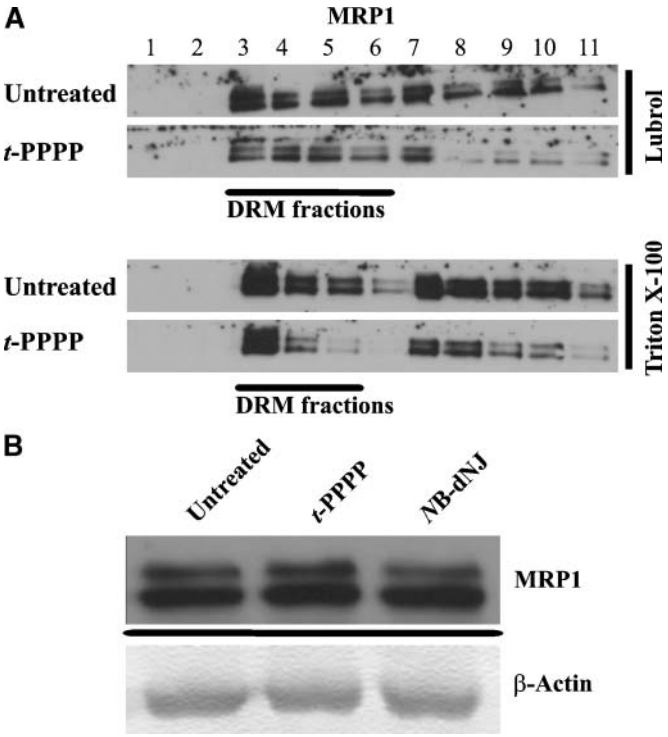

Fig. 5. Detergent-resistant membrane (DRM) localization of MRP1 in SK-N-AS cells is not affected by *t*-PPPP-mediated ganglioside depletion. A: The localization of MRP1 in Lubrol- and Triton X-100-insoluble membrane domains (DRM fractions 3–6 and 3–5, respectively) is not affected by *t*-PPPP-mediated depletion of gangliosides in SK-N-AS cells. Lubrol and Triton X-100 lysates were fractionated by flotation in a discontinuous sucrose density gradient. Aliquots of each fraction, containing equal protein levels, were subjected to SDS-PAGE and immunoblotting (see Experimental Procedures). B: Total cellular MRP1 expression is not affected by incubation of SK-N-AS cells with *t*-PPPP or *N*B-dNJ. MRP1 and β-actin were detected as described in Experimental Procedures. Shown are representative results of three to five independent experiments.

DISCUSSION

Sphingolipid-mediated and ABC transporter-mediated MDR may be coupled. First, in view of the fact that a general property of drug efflux protein substrates is their amphipathic nature, it is conceivable that lipid molecules could also be subjected to ABC transporter-mediated translocation (31–33). Second, it is likely that for optimal functioning, ABC transporters are dependent on their immediate lipid environment. One interesting option for a close association of ABC transporters with sphingolipids is their colocalization in membrane microdomains. Indeed, ample evidence indicates the presence of Pgp and MRP1 in membrane domains, either classical lipid rafts or caveolae (20–22). In this way, sphingolipids may modulate ABC transporter function irrespective of a

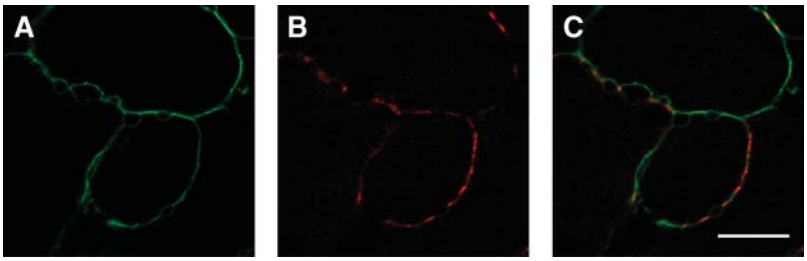

Fig. 6. MRP1 and GM1 do not colocalize in SK-N-AS cells. The raft-associated ganglioside GM1 (A) and the raft-associated ABC transporter protein MRP1 (B) do not colocalize in SK-N-AS cells (C), suggesting that they are localized in different membrane domains. Cells were stained for GM1 using the FITC-conjugated B subunit of cholera toxin and for MRP1 using a monoclonal antibody against MRP1 and a TRITC-labeled secondary antibody. Shown are representative results of three independent experiments. Bar = 10 μm.

potential substrate function. Third, when closely associated, sphingolipids could directly modulate ABC transporter efflux function. In this context, evidence has been obtained for gangliosides GD_3 and GM_3 as Pgp regulators through the modulation of Pgp phosphorylation in acute myeloid leukemia cells (23). Finally, gangliosides have been shown to be upregulated in MDR human ovarian and hepatic tumor cells, the latter overexpressing Pgp (34, 35).

We argued that it would be highly relevant to study the effect of gangliosides on ABC transporter function in neuroblastoma cells, given the high abundance and many established cell biological functions of gangliosides in these cells (36). Moreover, gangliosides as well as ABC transporters are enriched in membrane domains. MRP1 is usually overexpressed in MDR cell lines lacking Pgp and sometimes coexpressed with Pgp. In this study, we used two human neuroblastoma cell lines, one of which expresses functional Pgp and the other functional MRP1. This allowed us to separately study the modulation of these two ABC transporters by gangliosides. Moreover, the two neuroblastoma cell lines express distinct ganglioside patterns (24). Gangliosides were efficiently depleted in *t*-PPPP- as well as *N*B-dNJ-treated cells. First, ganglioside biosynthesis was severely reduced with both treatments, as indicated by the incorporation of radioactive serine. This does not necessarily imply that the ganglioside content is depleted, because there is the possibility of incorporation of (nonradioactive) gangliosides from the culture medium. Therefore, we measured gangliosides in two other ways (i.e., total mass and the amount of GM1 present as indicated by cholera toxin binding). Both of these methods also measure gangliosides that are incorporated from the medium, yet both lead to the same conclusion as the biosynthesis measurements: the efficient depletion of gangliosides. This shows that *1*) absolute ganglioside content is severely reduced in treated cells, and *2*) uptake of gangliosides from the medium is very limited and cannot replenish the original ganglioside pool.

Surprisingly, this study showed the absence of significant modulation of ABC transporter function in spite of a very efficient depletion of gangliosides. Statistically significant differences in drug efflux activity could only be obtained with a specific drug efflux assay protocol and clearly were marginal compared with the effects of established inhibitors of Pgp or MRP1. Moreover, ganglioside depletion did not correlate with ABC transporter-dependent cell survival, which again was highly susceptible to established Pgp/MRP1 inhibitors. *N*B-dNJ was without effect on cell survival, whereas *t*-PPPP slightly inhibited cell survival in both cell lines, which did not correlate with a higher efflux activity of MRP1 in SK-N-AS cells. Therefore, the effect of *t*-PPPP on cell survival was apparently not related to the modulation of efflux pump activity. In conclusion, ganglioside depletion only marginally affected ABC transporter activity in neuroblastoma cells, and this effect was not reflected in changes in cell survival. In contrast, established inhibitors of Pgp/MRP1 strongly inhibited drug efflux and reduced cell survival.

In agreement with these results, ganglioside depletion did not affect the membrane domain localization of MRP1; in fact, based on in situ localization studies, the ABC transporter and gangliosides appeared to be localized in different subsets of lipid rafts. Our results are in contrast with those of Plo et al. (23), who show modulation of Pgp activity by the gangliosides GD_3 and GM_3 in acute myeloid leukemia cells. In their study, they used only PDMP to reduce ganglioside content and showed that an ~50% reduction is sufficient to exert an effect on Pgp activity, whereas in our study, a stronger reduction of ganglioside content by either of two different GCS inhibitors was without effect. The different outcome may be attributable to cell type-dependence or the fact that the myeloid leukemia cells used by Plo et al. (23) highly overexpress Pgp as a result of drug selection or transfection with the *mdr-1* gene, whereas the human neuroblastoma cell lines used in our study were neither selected nor transfected.

It is difficult at this time to make general statements concerning the role of gangliosides in ABC transporter-mediated drug resistance. There is ample speculation on this topic, but very little data are available. Apart from the study by Plo et al. (23), two recent studies report the upregulation of gangliosides in drug-resistant ovarian and hepatic tumor cells, the latter overexpressing Pgp (34, 35). Our study is the first systematic approach using different GCS inhibitors in combination with cell lines expressing different functional ABC transporters and provides a clear answer in the case of neuroblastoma cells. More studies are needed, including those in other tumor models, to be able

to draw firm conclusions regarding whether the absence of modulation of ABC transporters by gangliosides is the rule or the exception. However, we conclude from our study that gangliosides are not essential to the regulation of ABC transporter activity and do not appear to represent a universal target for therapeutic potential. In this context, it should be mentioned that all studies discussed were performed in cell lines, so extrapolation to the in vivo situation should be done with care. The effects of ganglioside depletion on ABC transporters in tumor cells in vivo may be different as a result of systemic alterations occurring during harsh chemotherapy treatment in patients with neuroblastoma.

In addition, our results show that GCS inhibition did not sensitize human neuroblastoma cells to chemotherapy (i.e., vincristine treatment). This is in agreement with recently published studies using iminosugars to inhibit GCS and appears to contrast with the effects of PDMP as a GCS inhibitor and chemosensitizer (37, 38). It remains to be established whether PDMP-dependent chemosensitization is truly mediated by GCS (36, 39). Recent observations in our laboratory indicate that PDMP but not *t*-PPPP or *N*B-dNJ chemosensitizes murine Neuro-2a cells to taxol, whereas the effects of PDMP appear to be independent of GCS inhibition (40). Iminosugars especially offer the advantage of limited side effects and low toxicity. Therefore, when studying the potential involvement of GCS, Cer, and glycolipids in MDR, the choice of GCS inhibitors should be made with care. When, in addition to PDMP, other PDMP analogs such as *t*-PPPP, and more importantly also iminosugars, show similar biological effects, then the likelihood is increased that the observed effects can be ascribed to GCS inhibition.

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REFERENCES

1. Ling, V., N. Kartner, T. Sudo, L. Siminovitch, and J. R. Riordan. 1983. Multidrug-resistance phenotype in Chinese hamster ovary cells. *Cancer Treat. Rep.* **67:** 869–874.
2. Biedler, J. L. 1994. Drug resistance: genotype versus phenotype. Thirty-second G. H. A. Clowes Memorial Award Lecture. *Cancer Res.* **54:** 666–678.
3. Nooter, K., and G. Stoter. 1996. Molecular mechanisms of multidrug resistance in cancer chemotherapy. *Pathol. Res. Pract.* **192:** 768–780.
4. Ferte, J. 2000. Analysis of the tangled relationships between P-glycoprotein-mediated multidrug resistance and the lipid phase of the cell membrane. *Eur. J. Biochem.* **267:** 277–294.
5. Lavie, Y., H. Cao, S. L. Bursten, A. E. Giuliano, and M. C. Cabot. 1996. Accumulation of glucosylceramides in multidrug-resistant cancer cells. *J. Biol. Chem.* **271:** 19530–19536.
6. Lucci, A., W. I. Cho, T. Y. Han, A. E. Giuliano, D. L. Morton, and M. C. Cabot. 1998. Glucosylceramide: a marker for multiple-drug resistant cancers. *Anticancer Res.* **18:** 475–480.
7. Kok, J. W., R. J. Veldman, K. Klappe, H. Koning, C. M. Filipeanu, and M. Muller. 2000. Differential expression of sphingolipids in MRP1 overexpressing HT29 cells. *Int. J. Cancer.* **87:** 172–178.
8. Sietsma, H., R. J. Veldman, D. Kolk, B. Ausema, W. Nijhof, W. Kamps, E. Vellenga, and J. W. Kok. 2000. 1-Phenyl-2-decanoylamino-3-morpholino-1-propanol chemosensitizes neuroblastoma cells for taxol and vincristine. *Clin. Cancer Res.* **6:** 942–948.
9. Olshefski, R. S., and S. Ladisch. 2001. Glucosylceramide synthase inhibition enhances vincristine-induced cytotoxicity. *Int. J. Cancer.* **93:** 131–138.
10. Di Bartolomeo, S., and A. Spinedi. 2001. Differential chemosensitizing effect of two glucosylceramide synthase inhibitors in hepatoma cells. *Biochem. Biophys. Res. Commun.* **288:** 269–274.
11. Kaucic, K., N. Etue, B. LaFleur, W. Woods, and S. Ladisch. 2001. Neuroblastomas of infancy exhibit a characteristic ganglioside pattern. *Cancer.* **91:** 785–793.
12. Kushner, B. H., K. Kramer, and N. K. Cheung. 2001. Phase II trial of the anti-G(D2) monoclonal antibody 3F8 and granulocyte-macrophage colony-stimulating factor for neuroblastoma. *J. Clin. Oncol.* **19:** 4189–4194.
13. Pagnan, G., D. D. Stuart, F. Pastorino, L. Raffaghello, P. G. Montaldo, T. M. Allen, B. Calabretta, and M. Ponzoni. 2000. Delivery of c-myb antisense oligodeoxynucleotides to human neuroblastoma cells via disialoganglioside GD(2)-targeted immunoliposomes: antitumor effects. *J. Natl. Cancer Inst.* **92:** 253–261.
14. Li, R., N. Villacreses, and S. Ladisch. 1995. Human tumor gangliosides inhibit murine immune responses in vivo. *Cancer Res.* **55:** 211–214.
15. Floutsis, G., L. Ulsh, and S. Ladisch. 1989. Immunosuppressive activity of human neuroblastoma tumor gangliosides. *Int. J. Cancer.* **43:** 6–9.
16. Sietsma, H., W. Nijhof, B. Dontje, E. Vellenga, W. A. Kamps, and J. W. Kok. 1998. Inhibition of hemopoiesis in vitro by neuroblastoma-derived gangliosides. *Cancer Res.* **58:** 4840–4844.
17. Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1:** 31–39.
18. Brown, D. A., and E. London. 2000. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* **275:** 17221–17224.
19. Prinetti, A., K. Iwabuchi, and S. Hakomori. 1999. Glycosphingolipid-enriched signaling domain in mouse neuroblastoma Neuro2a cells. Mechanism of ganglioside-dependent neuritogenesis. *J. Biol. Chem.* **274:** 20916–20924.
20. Lavie, Y., G. Fiucci, and M. Liscovitch. 1998. Up-regulation of caveolae and caveolar constituents in multidrug-resistant cancer cells. *J. Biol. Chem.* **273:** 32380–32383.
21. Demeule, M., J. Jodoin, D. Gingras, and R. Beliveau. 2000. P-glycoprotein is localized in caveolae in resistant cells and in brain capillaries. *FEBS Lett.* **466:** 219–224.
22. Hinrichs, J. W. J., K. Klappe, I. Hummel, and J. W. Kok. 2004. ATP-binding cassette transporters are enriched in non-caveolar detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs) in human multidrug-resistant cancer cells. *J. Biol. Chem.* **279:** 5734–5738.
23. Plo, I., G. Lehne, K. J. Beckström, N. Maestre, A. Bettaïeb, G. Laurent, and D. Lautier. 2002. Influence of ceramide metabolism on P-glycoprotein function in immature acute myeloid leukemia KG1a cells. *Mol. Pharmacol.* **62:** 304–312.
24. Dijkhuis, A. J., J. Douwes, W. Kamps, H. Sietsma, and J. W. Kok. 2003. Differential expression of sphingolipids in P-glycoprotein or multidrug resistance-related protein 1 expressing human neuroblastoma cell lines. *FEBS Lett.* **548:** 28–32.
25. Senn, H. J., M. Orth, E. Fitzke, H. Wieland, and W. Gerok. 1998. Gangliosides in normal human serum. Concentration, pattern and transport by lipoproteins. *Eur. J. Biochem.* **181:** 657–662.
26. Ladisch, S., and B. Gillard. 1985. A solvent partition method for microscale ganglioside purification. *Anal. Biochem.* **146:** 220–231.
27. Böttcher, C. J. F., C. M. van Gent, and C. Pries. 1961. A rapid and sensitive submicro phosphorus determination. *Anal. Chim. Acta.* **24:** 203–204.
28. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Chem.* **150:** 76–85.
29. Carmichael, J., W. G. DeGraff, A. F. Gazdar, J. D. Minna, and J. B. Mitchell. 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* **47:** 936–942.
30. Lisanti, M. P., Z. Tang, P. E. Scherer, and M. Sargiacomo. 1995. Caveolae purification and glycosylphosphatidylinositol-linked protein sorting in polarized epithelia. *Methods Enzymol.* **250:** 655–668.
31. Smith, A. J., J. L. Timmermans-Hereijgers, B. Roelofsen, K. W.

Wirtz, W. J. van Blitterswijk, J. J. Smit, A. H. Schinkel, and P. Borst. 1994. The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. *FEBS Lett.* **354:** 263–266.
32. Raggers, R. J., A. van Helvoort, R. Evers, and G. van Meer. 1999. The human multidrug resistance protein MRP1 translocates sphingolipid analogs across the plasma membrane. *J. Cell Sci.* **112:** 415–422.
33. Eckford, P. D. W., and F. J. Sharom. 2005. The reconstituted P-glycoprotein multidrug transporter is a flippase for glucosylceramide and other simple glycosphingolipids. *Biochem. J.* **389:** 517–526.
34. Prinetti, A., L. Basso, V. Appierto, M. G. Villani, M. Valsecchi, N. Loberto, S. Prioni, V. Chigorno, E. Cavadini, F. Formelli, et al. 2003. Altered sphingolipid metabolism in N-(4-hydroxyphenyl)-retinamide-resistant A2780 human ovarian carcinoma cells. *J. Biol. Chem.* **278:** 5574–5583.
35. Hummel, I., K. Klappe, and J. W. Kok. 2005. Up-regulation of lactosylceramide synthase in MDR1 overexpressing human liver tumour cells. *FEBS Lett.* **579:** 3381–3384.
36. Sietsma, H., A. J. Dijkhuis, W. Kamps, and J. W. Kok. 2002. Sphingolipids in neuroblastoma: their role in drug resistance mechanisms. *Neurochem. Res.* **27:** 665–674.
37. Norris-Cervetto, E., R. Callaghan, F. M. Platt, R. A. Dwek, and T. D. Butters. 2004. Inhibition of glucosylceramide synthase does not reverse drug resistance in cancer cells. *J. Biol. Chem.* **279:** 40412–40418.
38. Klappe, K., J. W. J. Hinrichs, B. J. Kroesen, H. Sietsma, and J. W. Kok. 2004. MRP1 and glucosylceramide are coordinately over expressed and enriched in rafts during multidrug resistance acquisition in colon cancer cells. *Int. J. Cancer.* **110:** 511–522.
39. Kok, J. W., and H. Sietsma. 2004. Sphingolipid metabolism enzymes as targets for anti-cancer therapy. *Curr. Drug Targets.* **5:** 375–382.
40. Dijkhuis, A. J., K. Klappe, S. Jacobs, B. J. Kroesen, W. Kamps, H. Sietsma, and J. W. Kok. 2006. PDMP sensitizes neuroblastoma to paclitaxel by inducing aberrant cell cycle progression leading to hyperploidy. *Mol. Cancer Ther.* **5:** 593–601.