Sphingolipids, ABC transporters and chemosensitivity in neuroblastoma
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CHAPTER 3

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 activity of ATP binding cassette (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) or N-butyldeoxynojirimycin (NB-dNJ), very efficiently depleted ganglioside content in two human neuroblastoma cell lines. This was established by three different assays, i.e. equilibrium radiolabelling, cholera toxin binding and mass analysis. FACS analysis showed that ganglioside depletion only slightly and in opposite direction affected Pgp- and MRP1-mediated efflux activity. Moreover, both effects were marginal compared to 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 MTT analysis, in both neuroblastoma cell lines, while NB-dNJ was without effect. MRP1 expression and its localisation in detergent-resistant membranes (DRMs) were not affected by ganglioside depletion. Altogether, these results show that gangliosides are not relevant to ABC transporter-mediated MDR in neuroblastoma cells.
Introduction

The majority of children diagnosed with neuroblastoma already has advanced stage of the disease and requires chemotherapy as the primary approach for treatment. A complicating factor in the treatment of neuroblastoma is the occurrence of multidrug resistance (MDR) (Ling et al., 1983; Biedler, 1994). A number of molecular mechanisms responsible for MDR have been discovered (Nooter and Stoter, 1996), including drug removal by drug efflux pumps and changes in plasma membrane lipid composition (Ferte, 2000). A key molecule in drug removal is P-glycoprotein (Pgp or ABC B1). Pgp belongs to the family of ATP-binding cassette (ABC) transporters, which couple the hydrolysis of ATP to drug efflux. Multidrug resistance protein 1 (MRP1 or ABC C1) 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 tumour cells has emerged. The initial observation was that the adriamycin resistant MCF-7 human breast cancer cell line MCF-7-AdrR accumulated glucosylceramide (GlcCer), which is the product of ceramide (Cer) glycosylation by GlcCer synthase (GCS) (Lavie et al., 1996). GlcCer accumulation was subsequently shown in other Pgp over-expressing tumour cell lines as well as in MRP1 over-expressing cells (Lucci et al., 1998; Kok et al., 2000). D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (t-PDMP), an inhibitor of GCS, sensitised neuroblastoma cells to cytostatics (Sietsma et al., 2000; Olshefski and Ladisch, 2001; Di Bartolomeo and Spinedi, 2001). Inhibition of GCS may have a dual effect, i.e. Cer accumulation as well as depletion of glycosphingolipids, including gangliosides. Gangliosides have numerous physiological functions, especially in neuronal tissue, where they are relatively abundant. Characteristic ganglioside patterns are associated with aggressive neuroblastoma (Kaucic et al., 2001; Kushner et al., 2001; Pagnan et al., 2000). 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 haemopoiesis (Li et al., 1995; Floutsis et al., 1989; Sietsma et al., 1998). 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 organisation of sphingolipids, cholesterol and specific (GPI-linked) proteins in the plasma membrane (Simons and Toomre, 2000; Brown and London, 2000). 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. GM3) stimulated neurite outgrowth in neuroblastoma cells, and this process was mediated by microdomains (Prinetti et al., 1999).

Sphingolipid-mediated and ABC transporter-mediated MDR may well 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 (Lavie et al., 1998; Demeule et al., 2000; Hinrichs et al., 2004). When closely associated, sphingolipids could directly modulate Pgp or MRP1 efflux function. In this context, evidence has been obtained for gangliosides GD3 and GM3 as Pgp regulators through modulation of Pgp phosphorylation in acute myeloid leukaemia cells (Plo et al., 2002).

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 have 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 (Dijkhuis et al., 2003). In this study, we efficiently inhibited ganglioside biosynthesis in parallel employing two different inhibitors of GCS, i.e. D,L-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (t-PPPP) or N-butyldeoxynojirimycin (NB-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 localisation in membrane domains. More strongly, gangliosides and MRP1 appeared to be in a different subset of lipid rafts. In conclusion, we have established that in human neuroblastoma cells, gangliosides do not significantly modulate ABC transporter function.

Materials and methods

Materials

MK571 was a gift from Prof. A.W. Ford-Hutchinson (Merck-Frosst, Inc., Kirkland, Canada) and GF120918 from Glaxo Wellcome (Les Ulis, France). All cell culture plastic was from Costar (Cambridge, MA). Dulbecco’s modified Eagle medium, Hank’s balanced salt solution
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(HBSS), antibiotics, L-glutamine, non-essential amino acids and trypsin were from Gibco (Invitrogen, Paisley, UK). Fetal calf serum (FCS) was from Bodinco (Alkmaar, The Netherlands). D,L-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (t-PPPP) was purchased from Matreya LLC (Pleasant Gap, PA). N-butyldeoxyojirimycin (NB-dNJ) was purchased from Biomol (Plymouth Meeting, PA). Sep-Pak C$_{18}$ cartridges were from Waters (Milford, MA). L-[$U^{14}$C]serine was purchased from Amersham Pharmacia Biotech UK Limited (Buckinghamshire, UK). 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 (TMB), 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 (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 ATCC (Manassas, VA). They were grown as adherent monolayer cultures in Dulbecco’s modified Eagle medium supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and non-essential amino acids, under standard incubator conditions (humidified atmosphere, 5% CO$_2$, 37°C). In order to deplete gangliosides, cells were incubated with 1 µM t-PPPP or 200 µM NB-dNJ for seven days, unless stated otherwise.

Mass analysis of gangliosides

Gangliosides were isolated from $40 \times 10^6$ cells, as described (Senn et al., 1989; Ladisch and Gillard, 1985). Briefly, pelleted cells were extracted in CHCl$_3$/CH$_3$OH (1:1; v/v) and CHCl$_3$/CH$_3$OH (2:1; v/v). The supernatants were pooled, dried (N$_2$) and lipids were redissolved and sonicated in CHCl$_3$/CH$_3$OH (1:1; v/v). After centrifugation and overnight storage at -20°C, the supernatants were collected, dried and their phospholipid content was determined (Böttcher et al., 1961). 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 re-extracted with diisopropylether/1-butanol and subsequently lyophilised. Samples were dissolved in CH$_3$OH/H$_2$O (1:1; v/v) and loaded onto pre-washed Sep-Pak C$_{18}$ cartridges. After rinsing (H$_2$O), gangliosides were eluted with CH$_3$OH and CHCl$_3$/CH$_3$OH (1:1; v/v). The eluate was concentrated and loaded onto HPTLC plates, which were developed in CHCl$_3$/CH$_3$OH/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 (Scion Corporation, Frederick, MD) software.

**Equilibrium radiolabelling and analysis of gangliosides**

Cells were first incubated in the presence or absence of t-PPPP or NB-dNJ for three days and trypsinised. Subsequently, cells were cultured in the presence of L-[U-$^{14}$C]serine (0.5 µCi/ml) for five days, again in the presence or absence of t-PPPP or NB-dNJ. Gangliosides were extracted according to the protocol described above and aliquots containing equal amounts of phospholipid were loaded on HPTLC plates, which were developed and exposed to film. Gangliosides were scraped and radioactivity was measured by scintillation counting (Packard Topcount microplate scintillation counter, 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 to those of untreated cells, which was set at 100%.

**Quantification of cellular GM1 levels**

After trypsinisation 1x10$^6$ cells were pelleted and resuspended in 10 µl HBSS and incubated in the presence of CTB-HRP (0.009 U) for 30 min at 4°C. Cells were washed with ice-cold HBSS, pelleted and resuspended in 100 µl ice-cold HBSS. Of each sample 10 µl was used to determine protein content (Smith et al., 1985) and to a second 10 µl 100 µl TMB was added in a 96 wells plate. Upon 10 min incubation at room temperature 100 µl 0.5 M H$_2$SO$_4$ 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 (0.5x10$^6$ in HBSS), which were harvested by trypsinisation, were incubated with the MRP1 substrate CFDA (0.5 µM) or 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 analysed using Win-list 5.0 software (Verity Software House Inc., Topsham, ME). In order to determine the efflux-blocking factor (BF), cells were incubated at 37°C in either the presence (+/+ or absorption (-/-) of MK571 or CSA, both during loading (60 min) and efflux (10 min) of fluorescent substrate. The BF is defined as (F.U. are median relative fluorescence units):

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

Measurement of cellular sensitivity to cytotoxic drugs (MTT assay)
Fifteen thousand cells/well were plated in microtiter plates. Seventy-two hours after plating, the amount of viable cells was determined as previously described (Carmichael et al., 1987). Briefly, 100 µg MTT was added to each well and cells were incubated for 3.5h at 37°C. Plates were then centrifuged (15 min 900x g) and the supernatants were removed. Pellets were dissolved in DMSO and absorbencies were measured in microtiter plate reader (µQuant, Bio-Tek Instruments, Winooski, VT) at a λ of 570 nm. The background absorbency was subtracted from all values and data were expressed as percentage compared to untreated control cells (=100%). From these data the EC₅₀ (concentration at which the cell viability was 50% reduced) was determined.

Isolation of DRM
DRM fractions were isolated from cells as described (Lisanti et al., 1995). For each isolation, confluent cells from two 75 cm² 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 incubation on ice, cells were homogenised further by passing the lysate at least ten times through a 21 Gauge needle. Two ml 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 successively loaded, 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 18-20h at 4°C. Eleven fractions of 1 ml each were collected (from top to bottom), vortexed and stored at -80°C. The protein content (Smith et al., 1985) of all fractions was measured using bovine serum albumin as 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 (1-2h at room temperature) with 5% (w/v) non-fat dry milk in PBS. Membranes were rinsed in washing buffer (PBS containing 0.3% (v/v) Tween 20) and incubated (at least 2h at room temperature) with a primary antibody against MRP1 (1:1000) or β-actin (1:1000) in washing buffer containing 1% (w/v) non-fat dry milk. Membranes were rinsed in washing buffer and subsequently incubated for 2h with the appropriate horseradish peroxidase-conjugated secondary antibody (1:300) (ECL, Amersham Biosciences UK, Buckinghamshire, UK) in washing buffer containing 1% (w/v) non-fat dry milk (2h 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 visualised by exposure to a 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 MgCl₂. 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. 5 µl of each fraction was spotted onto a nitrocellulose membrane. Upon a 1h incubation with 5% (w/v) non-fat dry milk in PBS, the membrane was incubated with CTB-HRP (0.2 U per 10 ml) in PBS containing 0.3 % Tween 20 for 1 hour. After extensive washing with PBS/0.3% Tween 20 the membrane was incubated with ECL solution and exposed to a 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 was used for further analysis.

Confocal laser scanning fluorescence microscopy
For microscopy SK-N-AS cells were grown on glass coverslips in 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 prior to fixation. Cells were fixed with 4% paraformaldehyde (PFA) on ice for 20 min, permeabilised 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 prior to antibody incubation. Primary (overnight at 4°C) and secondary (0.5h 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 63x 1.32-0.6 oil CS objective in combination with Leica Confocal Software. Pictures were processed using Corel Graphics Suite 11 (Minneapolis, MN).

Statistical analysis and conversion keys
Results are presented as mean ± S.D. (n ≥ 3). Statistical analysis was performed with the Student’s t-test, considering P < 0.05 significant. In order to compare results with different normalisation, the following keys apply: SK-N-AS cells: 0.26 nmol phospholipid/µg protein (S.D.=0.06; n=4); SK-N-FI cells: 0.33 nmol phospholipid/µg protein (S.D.=0.08; n=4). It should be noted that t-PPPP- and NB-dNJ-treatment did not affect phospholipid levels in cells, as determined by lipid phosphorus determination (Böttcher et al., 1961). In t-PPPP-treated cells, the values were 0.29 nmol phospholipid/µg protein (S.D.=0.11; n=3; P=0.60 compared to control) for SK-N-AS and 0.31 nmol phospholipid/µg protein (S.D.=0.06; n=3; P=0.73 compared to control) for SK-N-FI cells. In NB-dNJ-treated cells, the values were 0.34 nmol phospholipid/µg protein (n=2) for SK-N-AS and 0.34 nmol phospholipid/µg protein (n=2) for SK-N-FI cells.
Chapter 3

Results

Both t-PPPP and NB-dNJ efficiently deplete gangliosides

Ganglioside biosynthesis and content were efficiently depleted in the human neuroblastoma cell lines SK-N-AS and SK-N-FI upon a seven-day incubation with the GCS inhibitors t-PPPP (1 μM) or NB-dNJ (200 μM). Both inhibitors reduced ganglioside biosynthesis and content to about 10%, as determined by equilibrium radiolabelling of sphingolipids (Fig. 1). Endogenous ganglioside levels of both SK-N-AS and SK-N-FI cells showed a corresponding decrease to about 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 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 NB-dNJ (17% ± 10, n=3). In conclusion, three different assays show a depletion of gangliosides by t-PPPP or NB-dNJ of about 85%.

![Ganglioside depletion in SK-N-AS and SK-N-FI cells by t-PPPP or NB-dNJ](image.png)

Figure 1. Ganglioside depletion in SK-N-AS and SK-N-FI cells by t-PPPP or NB-dNJ

SK-N-AS and SK-N-FI cells were incubated in the absence or presence of t-PPPP or NB-dNJ for seven days, and in the presence of L-[U-14C]serine for five days. Gangliosides were extracted and quantified. Both inhibitors reduced ganglioside biosynthesis to about 10% in both cell lines. Data represent the mean ± S.D. of 3-5 independent experiments. *Values are significantly (P < 0.05) different from untreated cells as determined by the Student’s t-test.

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

We next determined whether the efficient depletion of gangliosides had impact on the activity of the membrane proteins MRP1 and Pgp. SK-N-AS cells were loaded with CFDA, a fluorescent substrate of MRP1, while SK-N-FI cells were loaded with Rh123, a fluorescent...
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Inhibition of ganglioside biosynthesis with t-PPPP or NB-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 ABC transporter activity of untreated cells (‘Untreated -’). A different experimental setup of the efflux activity assay was used to examine if 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 minutes 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 ‘Materials and methods’), which were then converted to ratios, defined as the efflux blocking factor of t-PPPP- or NB-dNJ-treated cells divided by the efflux blocking factor of untreated cells. Thus, a ratio larger than “1” indicates a higher efflux

Figure 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 seven 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 HPTLC and quantified by densitometry. Shown are representative results of 3 independent experiments.

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.
activity compared to untreated cells, as is the case for \( t \)-PPPP as well as NB-dNJ-treated SK-N-AS cells (Fig. 3C). MRP1 activity was stimulated as a consequence of \( t \)-PPPP as well as NB-dNJ pre-treatment, while 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 to the inhibitory actions of MK571 and CSA, respectively.

![Figure 3. Efflux activity of MRP1 and Pgp is marginally affected by depletion of gangliosides](image)

A, B. After a seven-day incubation with \( t \)-PPPP (1 \( \mu \)M) or NB-dNJ (200 \( \mu \)M), SK-N-AS (A) and SK-N-FI (B) cells were loaded with CFDA (0.5 \( \mu \)M) and Rh123 (10 \( \mu \)M), respectively, for 1h 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 \( \mu \)M, SK-N-AS) or CSA (10 \( \mu \)M, SK-N-FI). The data show that ganglioside depletion has a small and not statistically significant stimulatory effect on MRP1 activity (A) and inhibitory effect on Pgp (B).

C. Cells were incubated at 37°C either in the presence or absence of MK571/CSA, both during loading (1h) and efflux (10 min) of fluorescent substrate. Blocking factors were calculated and presented as a ratio, which is defined as: the blocking factor of untreated, \( t \)-PPPP- or NB-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 the mean ± S.D. of 3-5 independent experiments. *Values are significantly (\( P < 0.05 \)) different from untreated cells as determined by the Student’s \( t \)-test.
Ganglioside depletion does not affect chemosensitivity

One of the major obstacles for 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 tumour cells. Impaired transporter activity could thus restore tumour cell chemosensitivity. We used vincristine, a microtubule destabilising drug and substrate of both MRP1 and Pgp, to determine the chemosensitising effect of ganglioside depletion. MK571 drastically sensitised SK-N-AS cells to vincristine, as indicated by a reduction in EC$_{50}$ ratio (Fig. 4), while CSA similarly sensitised 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 pre-treated with $t$-PPPP or NB-dNJ, while a decrease in vincristine resistance would be expected in SK-N-FI cells pre-treated with $t$-PPPP or NB-dNJ. However, $t$-PPPP pre-treatment of both SK-N-AS and SK-N-FI cells reduced resistance to vincristine, while NB-dNJ did not have any effect on resistance in either cell line (Fig. 4). In conclusion, ganglioside depletion does not sensitise neuroblastoma cells to vincristine, while $t$-PPPP has a specific effect on cell survival independent of MRP1- or Pgp-mediated resistance.

![Figure 4. Ganglioside depletion does not affect chemosensitivity](image)

Figure 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 NB-dNJ did not affect vincristine sensitivity, while 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-, $t$-PPPP- or NB-dNJ-treated cells divided by the EC$_{50}$ of untreated cells. Data represent the mean ± S.D. of 3-5 independent experiments. *Values are significantly ($P < 0.05$) different from untreated cells as determined by the Student’s $t$-test.
Figure 5. DRM localisation of MRP1 in SK-N-AS cells is not affected by t-PPPP-mediated ganglioside depletion
A. The localisation 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 “Materials and Methods”).
B. The total cellular MRP1 expression is not affected by incubation of SK-N-AS cells with t-PPPP or NB-dNJ. MRP1 (ECL) and β-actin (AP) were detected as described in the “Materials and Methods” section.
Shown are representative results of 3-5 independent experiments.
Ganglioside depletion does not affect DRM localisation of MRP1

So far, we have shown that a seven-day incubation of the human neuroblastoma cell lines SK-N-AS and SK-N-FI with either t-PPPP or NB-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 localisation and activity. In order 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 line with the results on 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 localisation 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 was not affected either (Fig. 5B). In conclusion, gangliosides are efficiently depleted from DRMs, but this depletion does not affect MRP1 expression and its localisation in DRMs.

Figure 6. MRP1 and GM1 do not co-localise in SK-N-AS cells

The raft-associated ganglioside GM1 (A) and the raft-associated ABC transporter protein MRP1 (B) do not co-localise in SK-N-AS cells (C), suggesting that they are localised 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-labelled secondary antibody. Bar, 10 µm. Shown are representative results of 3 independent experiments.
MRP1 and GM1 do not co-localise

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

Discussion

Sphingolipid-mediated and ABC transporter-mediated MDR may well be coupled. Firstly, 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 (Smith et al., 1994; Raggers et al., 1999; Eckford and Sharom, 2005). Secondly, 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 co-localisation in membrane microdomains. Indeed, ample evidence indicates the presence of Pgp and MRP1 in membrane domains, either classical lipid rafts or caveolae (Lavie et al., 1998; Demeule et al., 2000; Hinrichs et al., 2004). In this way, sphingolipids may modulate ABC transporter function irrespective of a potential substrate function. Thirdly, when closely associated, sphingolipids could directly modulate ABC transporter efflux function. In this context, evidence has been obtained for gangliosides GD3 and GM3 as Pgp regulators through modulation of Pgp phosphorylation in acute myeloid leukaemia cells (Plo et al., 2002). Finally, gangliosides have been shown to be up-regulated in MDR human ovarian and hepatic tumour cells, the latter over-expressing Pgp (Prinetti et al., 2003; Hummel et al., 2005).

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 (Sietsma et al., 2002). Moreover, gangliosides as well as ABC transporters are enriched in membrane domains. MRP1 is usually over-expressed in MDR cell lines lacking Pgp and sometimes co-expressed with Pgp. In this study we employed two human neuroblastoma cell lines, one of which expresses functional Pgp and the other functional MRP1. This allows us to separately study modulation...
of these two ABC transporters by gangliosides. Moreover, the two neuroblastoma cell lines express distinct ganglioside patterns (Dijkhuis et al., 2003). Gangliosides were efficiently depleted in PPPP- as well as NB-dNJ-treated cells. First, ganglioside biosynthesis was severely reduced with both treatments, as indicated by incorporation of radioactive serine. This does not necessarily imply that the ganglioside content is depleted, since there is the possibility of incorporation of (non-radioactive) gangliosides from the culture medium. Therefore, we have measured gangliosides in two other ways, i.e. total mass and the amount of GM1 present as indicated by cholera toxin binding. Both these methods also measure gangliosides which are incorporated from the medium, yet both lead to the same conclusion as biosynthesis measurements, i.e. 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 to 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. NB-dNJ was without effect on cell survival, while 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 PPPP on cell survival was apparently not related to 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 membrane domain localisation of MRP1 and in fact the ABC transporter and gangliosides appeared to be localised in a different subset of lipid rafts based on in situ localisation studies. Our results are in contrast with those of Plo et al. (2002) that show modulation of Pgp activity by the gangliosides GD3 and GM3 in acute myeloid leukaemia cells. In their study they used only PDMP to reduce ganglioside content and show that about 50% reduction is sufficient to exert an effect on Pgp activity, while in our study a stronger reduction of ganglioside content by either of two different GCS inhibitors is without effect. The different outcome may be due to cell type dependency or the fact that the myeloid leukaemia cells used in the study of Plo et al. (2002) highly over-express Pgp as a result of
drug selection or transfection with the mdr-1 gene, while the human neuroblastoma cell lines in our study were neither selected nor transfected. It is difficult at this point in 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. (2002), two recent studies report up-regulation of gangliosides in drug resistant ovarian and hepatic tumour cells, the latter over-expressing Pgp (Prinetti et al., 2003; Hummel et al., 2005). 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 tumour models, to be able to draw firm conclusions as to whether the absence of modulation of ABC transporters by gangliosides is the rule or the exception. We can however conclude from our study that gangliosides are not essential to 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 and extrapolation to the in vivo situation should be done with care. The effects of ganglioside depletion on ABC transporters in tumour cells in vivo may be different due to systemic alterations occurring during harsh chemotherapy treatment in patients with neuroblastoma.

In addition, our results show that GCS inhibition did not sensitise human neuroblastoma cells to chemotherapy, i.e. vincristine treatment. This is in agreement with recently published studies employing iminosugars to inhibit GCS and appears to contrast the effects of PDMP as GCS inhibitor and chemosensitiser (Norris-Cervetto et al., 2004; Klappe et al., 2004). It remains to be established whether PDMP-dependent chemosensitisation is really GCS mediated (Sietsma et al., 2002; Kok and Sietsma, 2004). Recent observations in our lab indicate that PDMP but not PPPP or NB-dNJ chemosensitise murine Neuro-2a cells to paclitaxel, while the effects of PDMP appear to be independent of GCS inhibition (Dijkhuis et al., 2006a). Especially iminosugars 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 analogues, such as PPPP, and more importantly also iminosugars show similar biological effects, than this increases the likelihood that the observed effects can be ascribed to GCS inhibition.