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Influence of Ceramide Metabolism on P-Glycoprotein Function in Immature Acute Myeloid Leukemia KG1a Cells

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ABSTRACT

Previous studies have emphasized the role of glucosylceramide (Glu-Cer) synthase in multidrug resistance (MDR) regulation. However, the mechanism by which the inhibition of this enzyme results in increased drug retention and cytotoxicity remains unclear. In this study, we investigated the respective role of ceramide (Cer) accumulation and Glu-Cer derivatives depletion in MDR reversal effect of 1-phenyl-2-decanoylamino-3-morpholino-1-propanolol (PDMP), a Glu-Cer synthase inhibitor. We show here that treatment with PDMP resulted in increased rhodamine 123 (Rh123) retention and potent chemosensitization of P-glycoprotein (P-gp)-expressing cells, including KG1a cells, KG1a/200 cells, K562/138 cells, and K562/mdr-1 cells. Metabolic studies revealed that PDMP induced not only time-dependent Cer accumulation but also reduction of all glycosylated forms of Cer, including Glu-Cer, lactosylceramide (Lac-Cer), monosialo ganglioside (GM3) and disialo ganglioside (GD3). The influence of these metabolites on P-gp function was investigated by measuring Rh123 retention in PDMP-treated cells. P-gp function was found to be stimulated only by the addition of gangliosides in all resistant cell lines, whereas Glu-Cer, Lac-Cer, and Cer had no effect. Moreover, in KG1a/200 cells, GD3 and, to a lesser extent, GM3 were found to phosphorylate P-gp on serine residues. Altogether, these results suggest that, at least in leukemic cells, gangliosides depletion accounts for PDMP-mediated MDR reversal effect, and that gangliosides are important P-gp regulators perhaps through their capacity to modulate P-gp phosphorylation.

The anthracycline daunorubicin (DNR) is widely used in the treatment of acute myeloid leukemia (AML). In first line therapy, DNR is most often administered in association with the antimetabolite cytosine arabinoside. Although the DNR/cytosine arabinoside combination induces a relatively high rate of complete remission, the high incidence of relapse indicates that this regimen is ineffective in completely eradicating leukemic progenitors. This clinical observation connotes the inherent chemoresistance of immature myeloid leukemic cells. Recent studies have phenotypically characterized leukemic stem cells as $CD34^+$ $CD38^-$ cells (Lapidot et al., 1994). This discrete cellular compartment proceeds to limited differentiation and provides the blast cells in terminal division, which represent the vast majority of the malignant cell population in AML patients. Among different mechanisms responsible for the chemoresistance of immature leukemic cells to DNR, the multidrug resistance (MDR) phenotype may play an important role. Indeed, CD34 expression has been correlated with both the *mdr-1* gene and P-glycoprotein expression (P-gp) (Campos et al., 1992). Moreover, further studies have shown that $CD34^+$ $P\text{-}gp^+$ AML cells present higher P-gp–mediated drug efflux capacity than $CD34^-$ $P\text{-}gp^+$ AML cells (Leith et al., 1995) and that leukemic progenitors display more efficient P-gp than blast cells in terminal division (Demur et al., 1998). In a previous study, we have found that, among a panel of P-gp–expressing $CD34^+$ and $CD34^-$ AML cell lines, the $CD34^+$ $CD38^-$ KG1a cells exhibited, despite low P-gp expression, the highest P-gp–mediated drug efflux capacity (Bailly et al., 1995). Moreover, KG1a cells display a high level of DNR resistance, similar to that of well-established drug-selected MDR cells, whereas DNR cytotoxicity is dramatically improved by P-gp

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ABBREVIATIONS: DNR, daunorubicin; AML, acute myeloid leukemia; MDR, multidrug resistance; P-gp, P-glycoprotein; Cer, ceramide; Glu-Cer, glucosylceramide; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanolol; VCR, vincristine; Lac-Cer, lactosylceramide; GM3, monosialo ganglioside; GD3, disialo ganglioside; FCS, fetal calf serum; Rh123, rhodamine 123; TLC, thin-layer chromatography; DMS, dimethylsphingosine; MTT, [4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium; PBS, phosphate-buffered saline; MRP, multidrug resistance protein; PSC833, cyclosporin derivative.

inhibitors (Bailly et al., 1995). Altogether, these results suggest that P-gp function is enhanced in immature AML cells and that KG1a cells may represent a relevant cellular model for investigating the regulation of P-gp function in leukemic immature myeloid cells.

In recent studies, metabolites of ceramide (Cer) have emerged as important MDR regulators. Indeed, accumulation of glucosylceramide (Glu-Cer), a simple glycosylated form of Cer, has been shown to be a characteristic of some MDR cells (Lavie et al., 1996; Lucci et al., 1998; Lala et al., 2000), whereas MDR cells are more sensitive to depletion of Glu-Cer than their non-MDR counterparts (Nicholson et al., 1999). Inhibition of the Cer glycosylation pathway has been shown to increase MDR cell sensitivity to cytotoxics (Lavie et al., 1997, 1999; Lucci et al., 1999b). Moreover, overexpression of Glu-Cer synthase confers doxorubicin (Adriamycin) resistance in human breast cancer cells (Liu et al., 1999), whereas transfection of Glu-Cer synthase antisense reverses doxorubicin resistance in MDR cells (Liu et al., 2000). Moreover, a number of compounds, including PSC833, that have been previously documented as P-gp inhibitors were found to facilitate Cer accumulation by stimulating Cer synthase-mediated de novo Cer synthesis (Cabot et al., 1999; Lucci et al., 1999b). Together, these results suggest that Cer and Cer metabolites may play an important role in regulating P-gp function. From this perspective, it is interesting to note that mifepristone, one of Glu-Cer synthase inhibitors used in these reports (Lucci et al., 1999a), had been previously reported in an independent study to inhibit P-gp activity in KG1a cells (Fardel et al., 1996). More recently, 1-phenyl-2-decanoylamino-3-morpholino-1-propanolol (PDMP), a Glu-Cer synthase inhibitor, has been described to sensitize neuroblastoma cells to paclitaxel (Taxol) and vincristine (VCR) by reducing drug efflux presumably through P-gp inhibition (Sietsma et al., 2000). These observations strongly support a role for endogenous Cer in regulating P-gp capacity.

However, in addition to facilitating Cer accumulation by inhibiting its conversion to Glu-Cer, prolonged PDMP treatment results also in the reduction of all glycosylated forms of Cer, including Glu-Cer, lactosylceramide (Lac-Cer), and gangliosides. The latter are widely distributed in AML cells, mostly monosialo ganglioside (GM3) and disialo ganglioside (GD3) forms (Anh-Tuan et al., 1986). Therefore, we reasoned that the inhibitory effect of PDMP on P-gp function could rather be related to depletion of glycosylated Cer derivatives including GM3 or GD3 than Cer accumulation. This hypothesis was supported by a recent study that showed that Cer had no effect on P-gp function (Veldman et al., 1999). The present study aimed to evaluate in KG1a cells the role of GM3 and GD3 in PDMP-induced P-gp inhibition. We show here for the first time that GD3 enhanced P-gp function in both KG1a and KG1a/200, a DNR- and VCR-selected KG1a MDR variant. This study suggests that GD3 plays an important role in the chemoresistance of immature myeloid leukemic cells.

Experimental Procedures

Materials. RPMI 1640 medium, penicillin, streptomycin and fetal calf serum (FCS) were from Invitrogen (Cergy-Pontoise, France). DNR was a gift from Aventis (Strasbourg, France). Rhodamine 123 (Rh123), VCR, exogenous Glu-Cer, Lac-Cer, GM3, GD3 were provided by Sigma-Aldrich (St-Quentin-Fallavier, France). Sphingosine was purchased from CalBiochem (San Diego, CA). [9,10(n)-^{3}H]palmitic acid (53 Ci/mmol) was purchased from Amersham Biosciences (Les Ulis, France). Silica gel 60 thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). Monoclonal antibody IgG2a C219, directed against P-gp, C6-Cer, and dihydro-Cer were purchased from Coger (Paris, France). Monoclonal antibody IgG2a anti–P-gp UIC2-PE, IgG2a-PE isotype U7.27 were from Immunotech (Marseille, France). The anti-phosphoserine antibody was from Zymed (Montrouge, France). Monoclonal antibody anti-GD3, D-*threo*-PDMP, D,L-*threo*-PDMP, and dimethylsphingosine (DMS) were from Biovalley (Marne La Vallée, France).

Cell Culture and Transfection. The human myeloblastic KG1a and KG1a/200 cell lines were cultured as described previously (Lehne and Rugstad, 1998). Briefly, they were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. KG1a/200 cells (DNR- and VCR-selected KG1a cells) were maintained with DNR (100 ng/ml) and VCR (100 ng/ml) in the same medium. KG1a cells constitutively express low levels of P-gp but display high P-gp–mediated efflux capacity (Bailly et al., 1995), whereas KG1a/200 cells exhibit typical MDR characteristics with high P-gp expression and activity (Lehne and Rugstad, 1998). Human myeloblastic K562, K562/138, and K562/mdr-1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 40 U/ml nystatin, and HEPES. K562/138 cells (VCR-selected K562 cells) were maintained with 138 ng/ml VCR. K562/mdr-1 cells (K562 variant cells stably transfected with mdr-1) were maintained with DNR (50 ng/ml). The pGEM 2 vector containing the *mdr-1* gene, MDR2000XS, was kindly provided by Dr. Michael M. Gottesman (National Cancer Institute, Bethesda, MD). The *mdr-1* gene was excised with *Xho*I and *Bam*HI restriction enzymes (Roche Applied Science, Germany) and ligated into the corresponding sites of pcDNA3 vector (Invitrogen, Carlsbad, CA). K562 cells were transfected with mdr-1 using DMRIE-C reagent (Invitrogen) and resulting clones were isolated in medium containing a combination of 30 ng/ml DNR and 400 μg/ml G418 (Geneticin). Finally, the K562/mdr-1 cells were sorted using a FACSvantage cell sorter (BD Biosciences, San Jose, CA) and maintained as described. Cell stocks were screened for mycoplasma (mycoplasma polymerase chain reaction kit; Stratagene, La Jolla, CA).

Analysis of Cellular Phospholipids. Evaluation of phospholipids, Cer, Glu-Cer, Lac-Cer, GM3, and GD3 were performed by labeling cells to isotopic equilibrium with [9,10(n)-^{3}H]palmitic acid (0.5 μCi/ml). After 48-h incubation, cells were washed and resuspended in serum-free medium for kinetic experiments and exposed to PDMP for the indicated times. Cellular lipids were extracted from aliquots (5×10^6 cells) by the method of Bligh and Dyer (1959) for Cer and Glu-Cer and were separated by TLC using chloroform/methanol/acetic acid/formic acid/water (65:30:10:4:2, v/v) followed by a second step using chloroform/methanol/acetic acid (95:5:5, v/v) as developing solvent system. Cellular lipids were extracted from aliquots (5×10^6 cells per point) by the method of Folch et al. (1957) for Lac-Cer, GM3, and GD3 and were separated by TLC using chloroform/methanol/acetic acid/water (100:60:20:5, v/v) followed by a second step using chloroform/methanol/acetic acid (95:5:5, v/v) as developing solvent system. Radioactive spots were scraped off and the amount of radioactivity was determined by liquid-scintillation counting. Statistical analyses were performed by Student's t test.

Analysis of P-gp Expression. Cell suspensions were washed with phosphate-buffered saline (PBS) and incubated at room temperature for 15 min with UIC2-PE (5 μl) in 250,000 cells/50 μl suspension. The IgG2a-PE isotype U7.27 was used as nonspecific control. After cell wash with PBS, the cells were suspended in PBS/bovine serum albumin 1% and fluorescence distributions were generated by Coulter Epics XL-MCL flow cytometry (Beckman Coulter, Inc., Fullerton, CA).

Rh123/DNR Uptake and Retention Studies. Cells were washed, resuspended at a concentration of 1×10^6 cells/ml in serum-free medium, and incubated with Rh123 (0.4 μM) for 30 min or DNR (1 μM) for 60 min at 37°C. At these times, aliquots were removed and kept on ice to evaluate the levels of intracellular Rh123 and DNR accumulation. The remaining cells were then centrifuged at 4°C and resuspended in drug-free medium. Cells were incubated at 37°C and aliquots were removed at various times, and kept on ice. The fluorescence associated with the different aliquots was analyzed using flow cytometry.

Phosphorylation of P-gp. Phosphorylation of P-gp was investigated using immunoprecipitation with anti-phosphoserine followed by Western blotting with anti–P-gp (C219). Briefly, after cell treatment with PDMP and/or exogenous sphingolipids, cells were incubated for at least 5 min with radioimmunoprecipitation assay solution (50 mM Tris, pH 8, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM dithiothreitol, 1 mM orthovanadate, 10 mM β-glycerophosphate, 50 mM NaF, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride). Cell extracts were then sonicated and centrifuged for 5 min at 10,000*g* at 4°C. Supernatants were taken and the protein content was calculated. Anti-phosphoserine (3 μg) was added to the same amount of protein and incubated at 4°C overnight. Immune complexes were collected by incubation with G-protein beads for 2 h at 4°C, eluted by boiling for 5 min in 20 μl of denaturation solution [Laemmli buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.04% bromphenol blue, and 4% β-mercaptoethanol)]. The extracts were resolved by migration in a 7.5% polyacrylamide gel, and proteins were transferred onto nitrocellulose membrane and probed with C219 (1/50) followed by anti-mouse horseradish peroxidase-conjugated IgG (1/5000).

Cytotoxicity Studies. Cells (5×10^5 cells/ml) were preincubated with PDMP or PSC833, treated with DNR for 1 h. After washing, cells (30,000 cells/100 μl) were seeded in 96-well plates; 48 h later, 100 μl of MTT (2 mg/ml) was added for 2 h. After centrifugation, dimethyl sulfoxide (100 μl) was added and optical density was read at 540/690 nm.

Results

Effect of PDMP on Cer Metabolism in KG1a and KG1a/200 Cells. In these experiments, KG1a cells and KG1a/200 cells were labeled with [^{3}H]palmitoyl acid, washed, then treated with various doses ranging from 0.5 to 20 μM PDMP for 24, 48, and 72 h. As shown in Table 1, treatment with PDMP resulted in time-dependent Cer accumulation in both KG1a and KG1a/200 cells. Maximum Cer accumulation was achieved at 72 h. At that time point, Cer accumulation had increased by 317 ± 45% in the KG1a/200 cells and by 188 ± 6% in the parental cells. In parallel, PDMP treatment resulted in a 50% decrease in Glu-Cer, Lac-Cer, and GM3 levels in both KG1a and KG1a/200 cells, whereas it reduced GD3 content in KG1a/200 but not in KG1a cells. Maximum depletion of Glu-Cer and Glu-Cer derivatives was achieved at 24 to 48 h.

Effect of PDMP on DNR- and VCR-Induced Cytotoxicity. The effect of PDMP on DNR- and VCR-induced cytotoxicity was studied in a panel of cell lines with diverse expression levels of P-gp. The flow cytograms in Fig. 1 demonstrate the constitutive P-gp level in each cell population. In these experiments, KG1a and KG1a/200 cells were treated with PDMP (20 μM for 48 h), cotreated with PDMP and various doses of DNR or VCR for 48 h, then assessed for viability using MTT. Pretreatment with PDMP increased DNR-induced cytotoxicity in both cell lines (Fig. 2, A and B). In KG1a cells, the IC_{50} of DNR decreased from 1 to 0.3 μM, whereas in KG1a/200 cells, the IC_{50} decreased from 4 to 0.9 μM. In both KG1a and KG1a/200 cells, PDMP reversal effect was similar to that of PSC833 (2 μM for 15 min), a highly potent P-gp inhibitor. Pretreatment with PDMP increased VCR-induced cytotoxicity in both cell lines (Fig. 2, C and D). In KG1a cells, the IC_{50} of VCR decreased from 0.09 to 0.05 μM, whereas in KG1a/200 cells, the IC_{50} decreased from 20 to 6 μM. To investigate the capacity of PDMP to overcome MDR in other cellular models, we measured the reversal effect of PDMP in K562/138 cells, a VCR-selected MDR variant, as well as in K562/mdr-1, a K562 variant stably transfected with mdr-1. As shown in Fig. 3, treatment with PDMP resulted in a 5-fold increase in DNR-induced cytotoxicity in resistant cell lines, whereas it has no effect on parental K562 cells.

Based on previous studies, which indicated that P-gp plays a critical role in DNR and VCR resistance in these cells (Bailly et al., 1995; Lehne and Rugstad, 1998), we hypothesized that PDMP could act through modulation of P-gp expression or function. The fact that PDMP increased drug-induced cytotoxicity in mdr-1–transfected cells supported this hypothesis. Flow cytometry analysis with anti–P-gp monoclonal UIC2 antibody revealed that treatment with PDMP at 20 μM for various times (24, 48, and 72 h) had no effect on P-gp expression in both KG1a and KG1a/200 cells

TABLE 1

Cer and gangliosides levels after PDMP treatment of KG1a and KG1a/200 cells

KG1a and KG1a/200 cells were labeled with [^{3}H] palmitic acid for 48 h and treated for various times with 20 μM PDMP. The levels of Cer and gangliosides were analyzed as described under *Experimental Procedures*. Results are calculated as percentages compared with untreated cells and are mean ± S.D. of at least three independent experiments. Statistical analyses were performed using the Student's *t* test.

	Cer	Glu-Cer	Lac-Cer	GM3	GD3
			%		
KG1a					
Control	100 ± 7	100 ± 6	100 ± 7	100 ± 10	100 ± 3
PDMP 24 h	124 ± 5*	37 ± 2*	46 ± 8*	80 ± 10*	132 ± 35
PDMP 48 h	129 ± 6*	N.T.	50 ± 23*	82 ± 8*	92 ± 20
PDMP 72 h	188 ± 6*	N.T.	52 ± 23*	67 ± 9*	116 ± 27
KG1a/200					
Control	100 ± 7	100 ± 6	100 ± 4	100 ± 5	100 ± 4
PDMP 24 h	183 ± 18*	32 ± 2*	56 ± 22*	74 ± 15*	51 ± 17*
PDMP 48 h	176 ± 9*	N.T.	55 ± 27*	55 ± 11*	67 ± 18*
PDMP 72 h	317 ± 45*	N.T.	45 ± 10*	59 ± 20*	50 ± 2*

N.T., not tested.
* Significant differences ($P < 0.05$) compared with untreated cells.

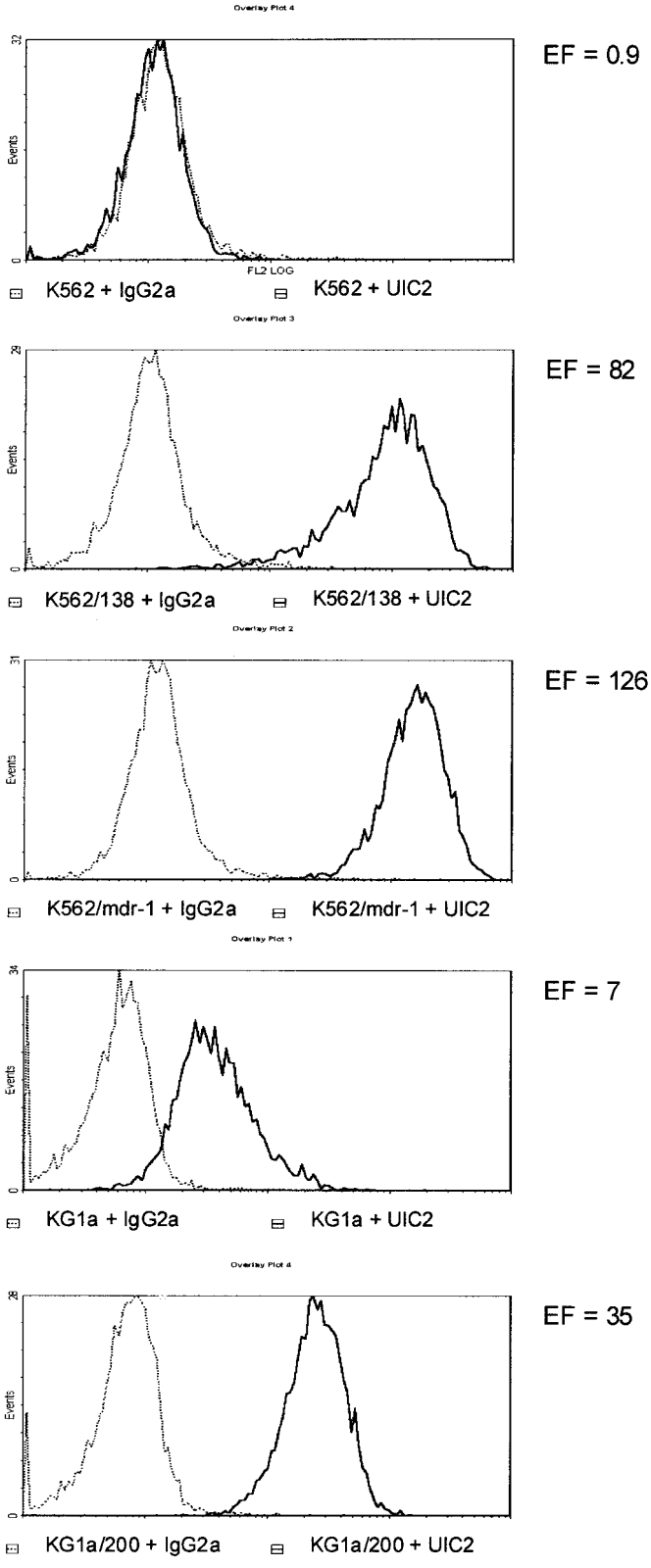

Fig. 1. The expression levels of P-gp in human leukemia cell lines. A panel of wild type and MDR subtype of human leukemia cell lines were tested for P-gp expression using flow cytometry with the P-gp specific monoclonal antibody UIC2. The expression factor (EF) for P-gp represents the relative level in terms of mean channel fluorescence for UIC2-labeled cell population (——) divided by mean channel fluorescence for controls labeled with IgG2a irrelevant antibody (. . . .). Each cell type is represented by corresponding EF value and overlay histograms.

(data not shown). Therefore, we have investigated the effect of PDMP on P-gp function in these cells.

Effect of PDMP on P-gp Function in KG1a and KG1a/200 Cells. KG1a and KG1a/200 cells were treated with PDMP (20 μM for 24 h) and incubated in the presence of Rh123 for 30 min or in the presence of DNR for 60 min. Cells were then washed and flow cytometry analysis of fluorescence intensity was performed at various times. As shown in Table 2, PDMP significantly increased Rh123 retention in both KG1a and KG1a/200 cells with a maximum of 200% increase in KG1a/200 cells at 15 min. The chemosensitizing effect of PDMP was compared with verapamil and PSC833, two other P-gp inhibitors. However, previous studies have described these agents also to possibly interfere with Cer metabolism when they are used at high doses or over a long period (Cabot et al., 1996, 1999; Lavie et al., 1997). For these reasons, verapamil and PSC833 was used for 15 min at doses of 10 and 2 μM, respectively. Under these conditions, these agents had no effect on intracellular ceramide concentration (data not shown) but significantly increased Rh123 retention (Table 2). As shown in Table 2, it seemed that PDMP was as potent as verapamil and only slightly less potent than PSC833 for increasing Rh123 retention. Moreover, as shown in Fig. 4, pretreatment with PDMP results in dramatic increase in DNR retention in both KG1a and KG1a/200 cells. PDMP was found to be as potent as PSC833 for limiting DNR release (Fig. 4). However, when the incubation time for PDMP was reduced (1–3 h), no effect was seen in both Rh123 and DNR retention (data not shown). This result suggests that, in KG1a cells, PDMP did not act directly on P-gp but

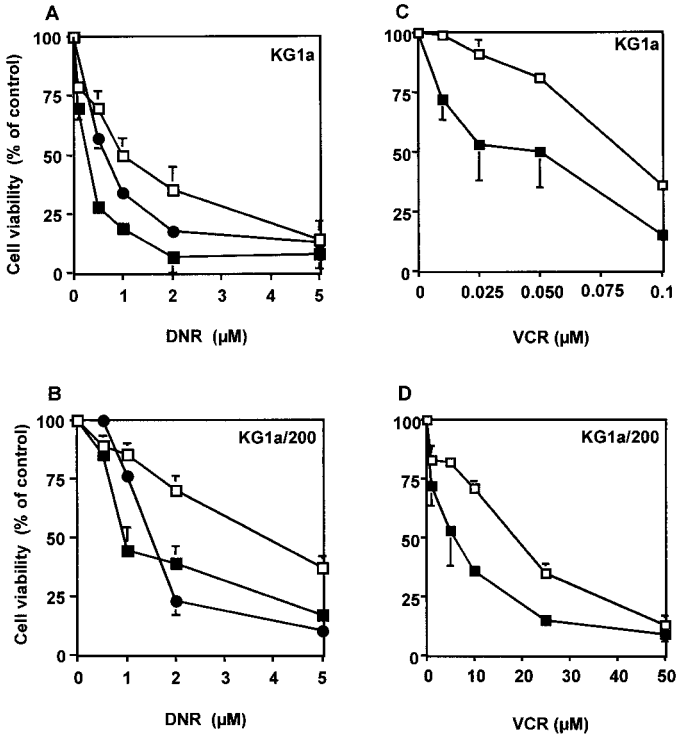

Fig. 2. Effect of PDMP on DNR- and VCR-induced cytotoxicity in KG1a and KG1a/200. KG1a (A, C) and KG1a/200 (B, D) cells were pretreated with PSC833 (2 μM, 15 min; ●) or PDMP (20 μM, 24 h; ■) or not pretreated (□), then treated with DNR (A, B) or VCR (C, D) for 48 h. Cell viability was assayed by MTT assay as described under *Experimental Procedures*. Results are mean ± S.D. of at least three independent experiments.

exerted its modulating effect through an indirect mechanism. Therefore, based on our metabolic studies, we hypothesized that PDMP effect could be mediated either by Cer or sphingosine accumulation or by depletion of glycosylated Cer derivatives.

Effect of Exogenous C6-Cer and Sphingosine on P-gp Function. To evaluate the role of endogenous Cer accumulation in PDMP-induced P-gp function inhibition, we investigated the effect of exogenous cell-permeant C6-Cer on P-gp function in both KG1a and KG1a/200 cells. As shown in Table 2, neither C6-Cer (25 μM for 1.5 h) nor DMS (50 μM for 1 h) had any effect on Rh123 retention in KG1a and KG1a/200 cells. No influence of C6-Cer was found when cells were treated with higher doses (up to 50 μM), for prolonged pretreatment time period (up to 14 h), or when Cer was added after wash-up (data not shown). Taken together, these results suggested that Cer or sphingosine did not mediate P-gp function inhibition induced by PDMP. However, these results raised the possibility that Glu-Cer derivatives, including Glu-Cer, Lac-Cer, or gangliosides, were positive regulators of P-gp function, and that PDMP-induced depletion of these compounds resulted in decreased P-gp mediated efflux capac-

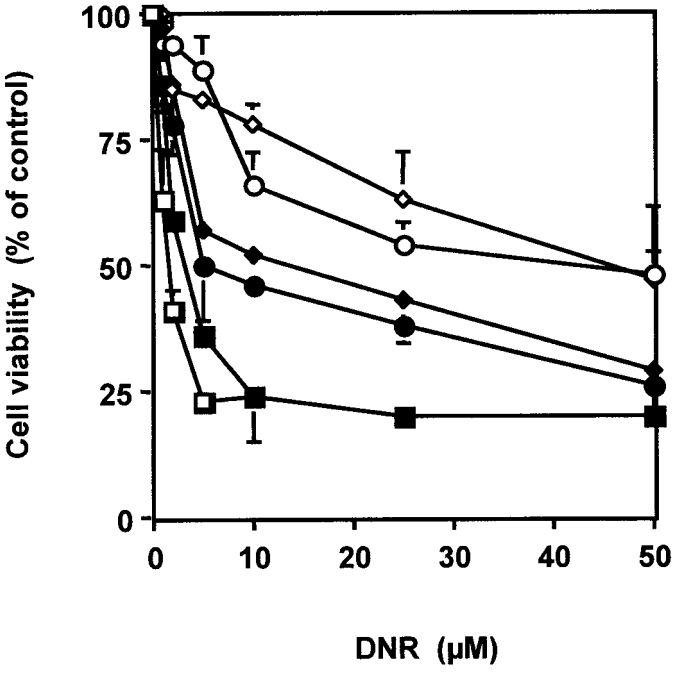

Fig. 3. Effect of PDMP on DNR–induced cytotoxicity in K562, K562/138, and K562-mdr-1 cells. K562 (□, ■), K562/138 (○, ●), and K562/mdr-1(◇, ◆) were pretreated with PDMP (20 μM, 24 h) (■, ●, ◆) or not pretreated (□, ○, ◇), then treated with DNR for 48 h. Cell viability was assayed by MTT assay as described under *Experimental Procedures*. Results are mean ± S.D. of at least three independent experiments.

TABLE 2

Effect of PDMP, Cer, and Cer metabolites on Rh123 retention

KG1a and KG1a/200 cells were untreated or pretreated with PDMP, C6-Cer, DMS, PSC833, and verapamil. Cells were then incubated with Rh123 (0.4 μM, 30 min), washed and Rh123 retention was evaluated 15 min later by flow cytometry analysis of fluorescence intensity as described under *Experimental Procedures*. Results are calculated as percentages compared with Rh123 accumulation at 30 min and are mean ± S.D. of at least three independent experiments. Statistical analyses were performed using the Student's *t* test.

Treatment	KG1a	KG1a/200
	%	
Control	57 ± 7	20 ± 3
PDMP (20 μM, 24 h)	72 ± 1*	40 ± 7*
C6-Cer (25 μM, 1.5 h)	61 ± 2	21 ± 2
DMS (50 μM, 1 h)	53 ± 2	16 ± 2
PSC833 (2 μM, 15 min)	86 ± 3*	86 ± 6*
Verapamil (10 μM, 15 min)	89 ± 4*	56 ± 6*

* Significant differences ($P < 0.05$) compared with cells treated with Rh123 alone.

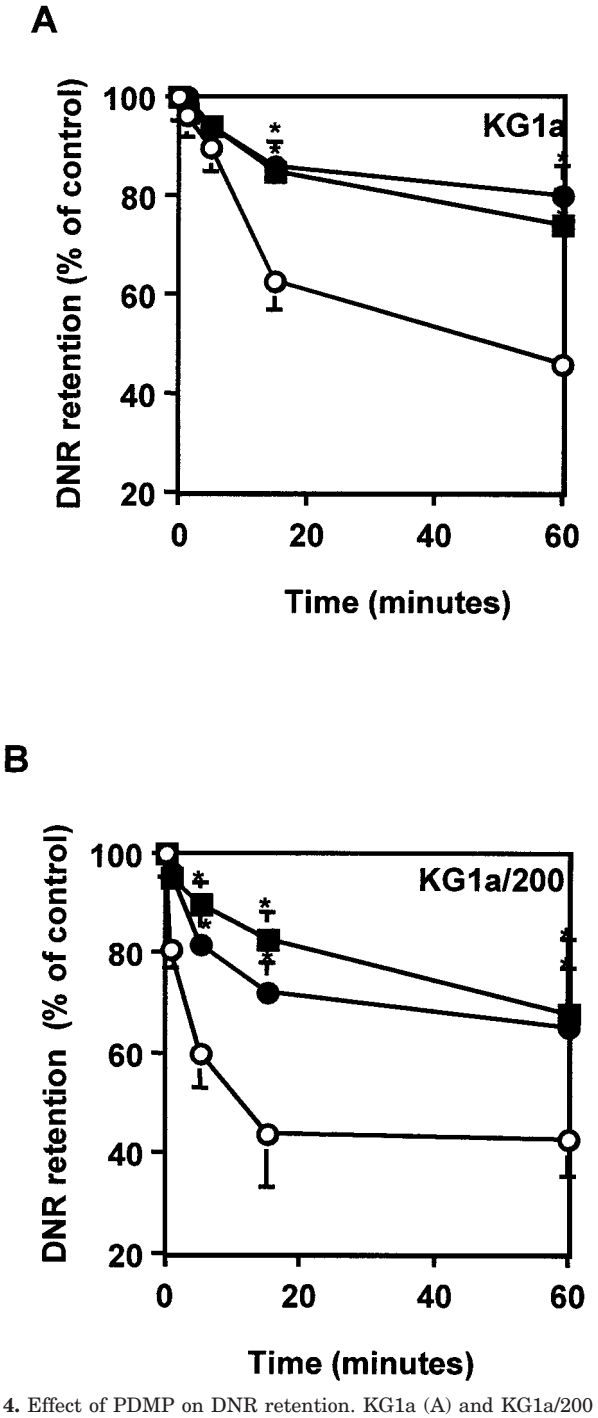

Fig. 4. Effect of PDMP on DNR retention. KG1a (A) and KG1a/200 (B) cells were untreated (○) or treated with PDMP (20 μM, 24 h; ●) or PSC833 (2 μM, 15 min; ■), then incubated with DNR (1 μM, 1 h), washed and DNR retention was evaluated at various times by flow cytometry analysis as described under *Experimental Procedures*. Results are expressed as percentage of DNR accumulation at 60 min and are mean ± S.D. of at least five independent experiments. Statistical analyses were performed using the Student's *t* test; ⋆, significant differences ($P < 0.05$) compared with cells treated with DNR alone.

ity. For this reason, we directly investigated the influence of these compounds on P-gp function in KG1a cells.

Effects on Glu-Cer, Lac-Cer, and Gangliosides on P-gp Function. KG1a cells and KG1a/200 cells were treated with PDMP (20 μM for 72 h) to deplete Glu-Cer derivatives, then exposed to exogenous Glu-Cer (50 μM for 1 h), Lac-Cer (25 μM for 1 h), GM3 (50 μM for 3 h), or GD3 (50 μM for 3 h). The influence of these compounds on P-gp function was evaluated by measuring Rh123 retention at 15 min. In KG1a cells, we found that Glu-Cer or Lac-Cer had a negligible effect on Rh123 retention (Fig. 5A), whereas pretreatment with GM3 resulted in a significant decrease in Rh123 retention compared with PDMP-treated cells (Fig. 5A). In PDMP-treated KG1a/200 cells, we found that, as we observed in KG1a cells, treatment with Glu-Cer and Lac-Cer had no effect on Rh123 retention, whereas treatment with either GM3 or GD3 resulted in a significant decrease in Rh123 retention, compared with PDMP-untreated control cells (Fig. 5B). It is interesting to note that the relative effect of each ganglioside in KG1a and KG1a/200 cells on P-gp function correlated with PDMP-induced ganglioside depletion (see Table 1). These results suggested that the inhibitory effect of PDMP on P-gp function was caused by ganglioside depletion and that GM3 and GD3 stimulated P-gp function in KG1a/200 cells. These results were extended to K562 MDR cells. Indeed, whereas PDMP increased Rh123 retention in K562/138 cells and in K562/mdr-1 cells, GM3 and GD3 were found to restore Rh123 retention in ganglioside-depleted cells (Fig. 6).

Effect of PDMP and Gangliosides on P-gp Phosphorylation Status in KG1a/200 Cells. Among different mechanisms that are believed to interfere with P-gp drug efflux capacity, it has been established that serine phosphorylation events play a major role (for review, see Gottesman and Pastan, 1993; Fine et al., 1996). Based on these considerations, we speculated that pretreatment with PDMP could result in changes in the P-gp serine phosphorylation profile. The present study was conducted in KG1a/200 cells but not in KG1a cells because P-gp was not sufficiently expressed to be detected by immunoblotting, as described previously (Bailly et al., 1995). When KG1a/200 cell extracts were immunoprecipitated with anti-phosphoserine and anti-phosphothreonine antibodies, and then immunoblotted with C219, we found that P-gp displayed constitutively phosphorylated phosphoserine residues but not threonine residues. As shown in Fig. 7A, treatment with 20 μM PDMP for 24 h dramatically decreased anti-phosphoserine antibody reactivity. Based on the effect of PDMP on Cer metabolism, these results suggested that P-gp phosphorylation changes could be related to either endogenous Cer accumulation or Glu-Cer derivative depletion. Therefore, we investigated the effect of these compounds on P-gp phosphorylation. To investigate the capacity of Cer to modulate P-gp serine phosphorylation, KG1a/200 cells were treated with cell-permeant C6-Cer. In repeated experiments ($n = 5$) performed with KG1a/200 cells, C6-Cer induced no significant change in anti-phosphoserine antibody reactivity, compared with either untreated cells or cells treated with dihydro-Cer, a biologically inactive Cer (Fig. 7A). The effect of GM3 and GD3 on P-gp phosphorylation was also investigated. In these experiments, KG1a/200 cells were treated with 20 μM PDMP for 24 h, then treated with either GM3 (50 μM for 3 h) or GD3 (50 μM for 3 h), and P-gp phosphoserine immunoreactivity was examined. These experiments showed that GD3 and to a lesser extent GM3 significantly increased P-gp serine phosphorylation (Fig. 7B).

Discussion

Previous studies have established that treatment with PDMP leads to extensive depletion of endogenous glycosphin-

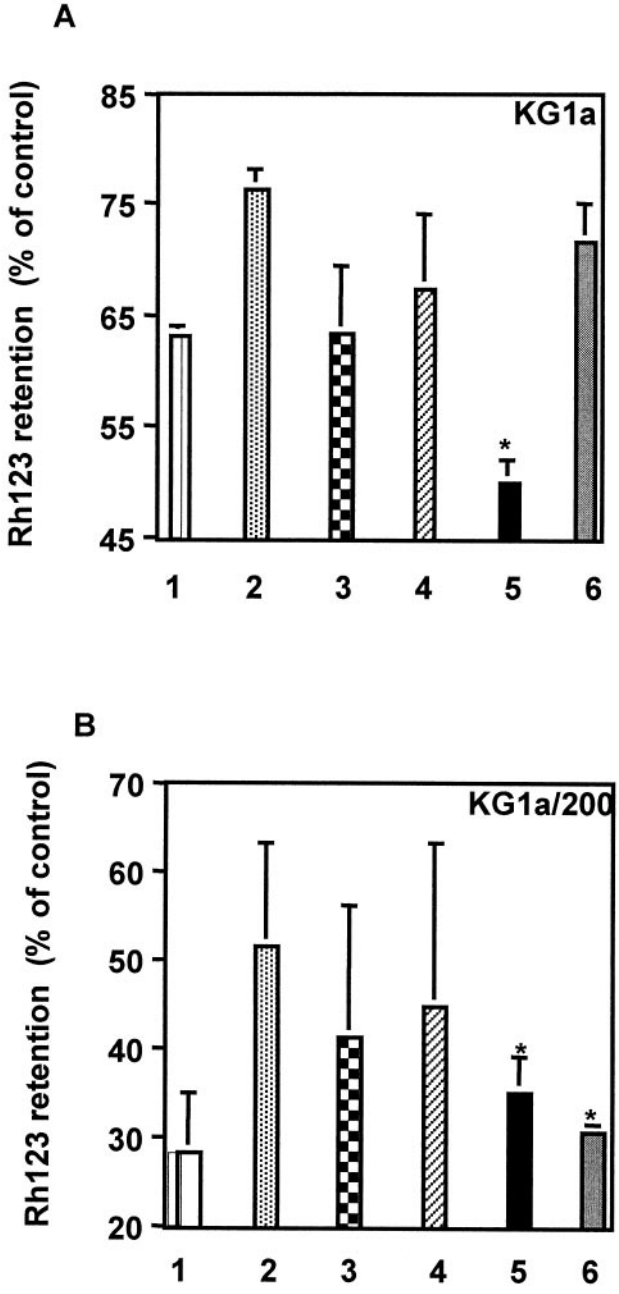

Fig. 5. Effect of Glu-Cer, Lac-Cer, and gangliosides on P-gp function in PDMP-treated cells. KG1a (A) and KG1a/200 (B) cells were untreated (1) or treated with PDMP (20 μM, 72 h) (2), and complemented with Glu-Cer (50 μM, 1 h) (3), Lac-Cer (25 μM, 1 h) (4), GM3 (50 μM, 3 h) (5), or GD3 (50 μM, 3 h) (6), then incubated with Rh123 (0.4 μM, 30 min), washed, and Rh123 retention was evaluated 15 min later by flow cytometry analysis as described under *Experimental Procedures*. Results are expressed as percentage of Rh123 accumulation at 30 min and are mean ± S.D. of at least three independent experiments. Statistical analyses were performed using the Student's t test; ⋆, significant differences ($P < 0.05$) compared with cells treated with PDMP alone.

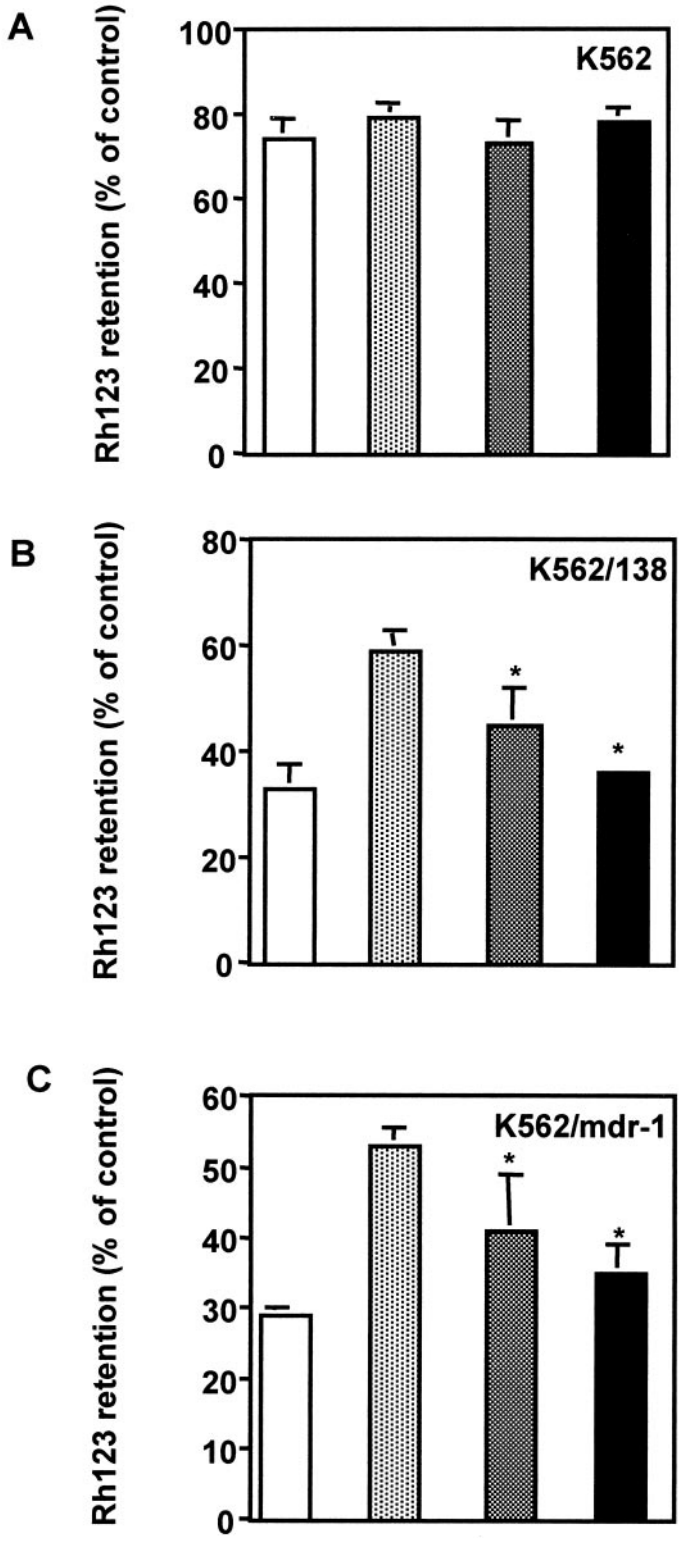

Fig. 6. Effect of gangliosides on P-gp function in PDMP-treated cells. K562 (A), K562/138 (B), and K562/mdr-1 (C) cells were untreated (□) or treated with D-PDMP (20 μM, 72 h) (▨), and complemented GM3 (50 μM, 3 h) (▩), or GD3 (50 μM, 3 h) (■), then incubated with Rh123 (0.4 μM, 30 min), washed and Rh123 retention was evaluated 15 min later by flow cytometry analysis as described under *Experimental Procedures*. Results are expressed as percentage of Rh123 accumulation at 30 min and are mean ± S.D. of three independent experiments. Statistical analyses were performed using the Student's *t* test; ⋆, significant differences ($P < 0.05$) compared with cells treated with PDMP alone.

golipids, including gangliosides biosynthesized from Glu-Cer, and causes accumulation of Cer and sphingosine. For this reason, it has proven to be a useful tool for studying various functional roles of endogenous glycosphingolipids (Radin et al., 1993; Mutoh et al., 1998). In a previous study, Sietsma et al. (2000) reported that PDMP treatment resulted in a decreased efflux of both paclitaxel and VCR, similar to treatment with PSC833, a P-gp inhibitor, or with MK571, a multidrug resistance protein (MRP) inhibitor, in murine neuroblastoma cells. From these results, the authors have proposed that PDMP treatment may interfere with P-gp and/or MRP function. Our study shows that, in both KG1a and KG1a/200 cells, PDMP increased the retention of Rh123, a specific P-gp substrate, suggesting that PDMP does act on P-gp function. Moreover, it has been documented that KG1a and KG1a/200 cells do not express MRP (Fardel et al., 1998; Lehne et al., 2000). Therefore, PDMP was very unlikely to have any effect on MRP but presumably acted through P-gp inhibition. The fact that PDMP increased both DNR-induced cytotoxicity and Rh123 retention in cells transfected with mdr-1 supports this hypothesis.

Sietsma et al. (2000) provided no information about the effect of PDMP on Glu-Cer metabolism; therefore, the mech-

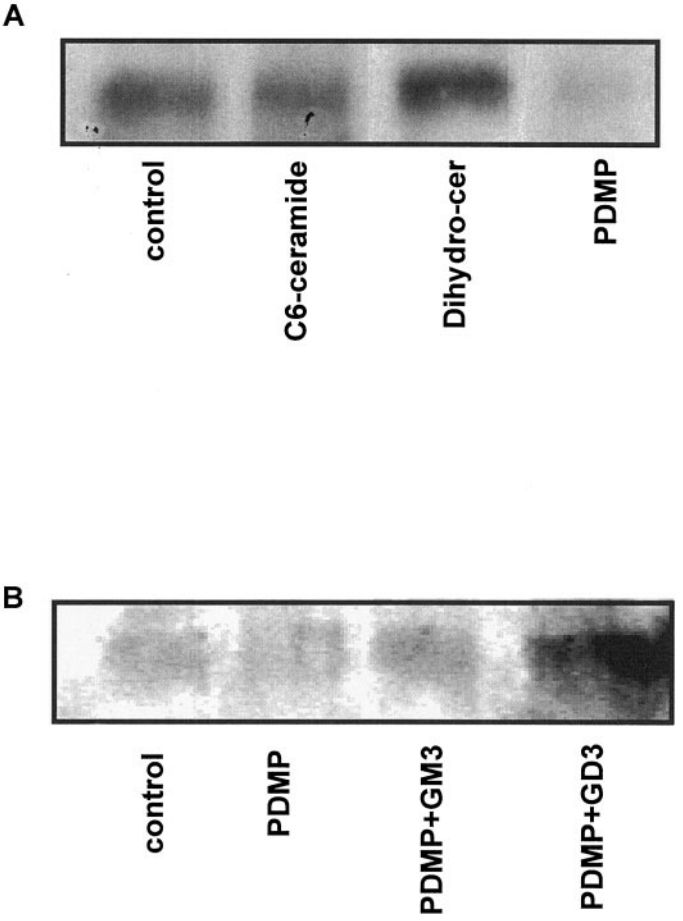

Fig. 7. Effect of Cer, GM3, or GD3 on P-gp phosphorylation status in KG1a/200 cells. A, KG1a/200 cells were treated or not with PDMP (20 μM, 24 h), Cer (20 μM, 1.5 h), or dihydro-Cer (20 μM, 1.5 h), and P-gp serine phosphorylation was analyzed after immunoprecipitation with anti-phosphoserine antibody and revelation with an anti–P-gp (C219) antibody. B, KG1a/200 cells were treated or not with PDMP (20 μM, 24 h), complemented with GM3 (50 μM, 3 h) or GD3 (50 μM, 3 h) and P-gp serine phosphorylation was detected as described previously.

anism by which PDMP alters drug transport remained unclear. For this reason, we have evaluated the influence of PDMP on intracellular concentrations of Cer, Glu-Cer, Lac-Cer, GM3, and GD3. Our experiments revealed that treatment with PDMP resulted not only in increased intracellular Cer concentration but also in Glu-Cer, Lac-Cer, and GM3 or GD3 depletion. From this result, we first hypothesized that Cer accumulation was responsible for P-gp function inhibition. However, we found that exogenous cell-permeant Cer influenced neither Rh123 nor DNR retention in KG1a cells. This result was not totally unexpected because it has been recently reported that C6-Cer did not affect Rh123 retention in the 2780AD ovarian carcinoma MDR cells (Veldman et al., 1999). Moreover, we found that DMS had no effect on Rh123 efflux. This result suggests that sphingosine does not play any role in regulating P-gp function. Previous studies have documented that sphingosine or L-*threo*-dihydrosphingosine, an analog of sphingosine, may enhance drug accumulation in MDR cells (Sachs et al., 1995, 1996). These results do not necessarily conflict with ours. Indeed, these studies provided no direct evidence that sphingosine (or L-*threo*-dihydrosphingosine) influenced drug retention. Therefore, it remains possible that sphingosine could facilitate drug uptake through a P-gp–independent mechanism.

Based on these findings and considerations, we reasoned that PDMP-induced loss of P-gp function could be related to Glu-Cer, Lac-Cer, or ganglioside depletion. Whereas Glu-Cer or Lac-Cer did not influence Rh123 retention, we observed that GM3 and GD3 restored P-gp function in PDMP-treated P-gp–expressing cells but not in K562 parental cells. This result suggests that PDMP-induced P-gp inhibition could be caused by ganglioside depletion. The role of ganglioside in MDR has been suspected from previous studies that showed changes in MDR cell ganglioside composition (Biedler et al., 1994). More recently, it has been reported that tamoxifen, a well-documented MDR reversal agent, inhibits ganglioside formation (Cabot et al., 1996). However, the influence of gangliosides on P-gp function had never been examined; our study shows for the first time that, in leukemic cells, gangliosides contribute to P-gp function regulation. This finding may have important clinical implication because ganglioside distribution may differ among leukemic cell population (Anh-Tuan et al., 1986). For example, it is conceivable that $CD34^+$ AML cells, which generally display very efficient P-gp-mediated drug efflux capacity, display different ganglioside distribution than $CD34^-$ AML cells, in which P-gp is much less efficient (Bailly et al., 1995; Leith et al., 1995). This hypothesis is under current investigation in our laboratory.

The fact that gangliosides interfere with P-gp–mediated transmembrane drug transport raises the more general question about the role of glycosphingolipids in modulating the function of membrane proteins. Indeed, it is now admitted that gangliosides are not only structural components of plasma membrane but are also involved in signaling events. Gangliosides were found to influence lipid order and hydration of the lipid bilayer, and it has been proposed that such changes could play an important role in the modulation of transmembrane molecular events (Ravichandra and Joshi, 1999). For example, it has been recently shown that gangliosides influence phosphorylation status of membrane signaling proteins by facilitating protein-protein interaction in glycosphingolipid-enriched membrane microdomain (Prinetti et al., 1999). Based on previous findings that established phosphorylation as an important regulator of P-gp function (Gottesman and Pastan, 1993; Fine et al., 1996), we have investigated the influence of PDMP and Cer derivatives on P-gp phosphorylation status in KG1a/200 cells. In fact, we found that treatment with PDMP resulted in decreased P-gp phosphorylation, whereas GD3 and GM3 exerted the opposite effect. Therefore, our study suggests that the influence of gangliosides on P-gp phosphorylation status represents the critical mechanism by which PDMP modulates P-gp function.

The mechanism by which gangliosides stimulate P-gp phosphorylation remains to be determined. Previous studies have emphasized the role of some PKC isoforms on P-gp function (Blobe et al., 1993; Ahmad et al., 1994). Therefore, we raised the possibility that gangliosides could stimulate the activity of some critical PKC isozymes. This hypothesis seems to be unlikely, because we found that, in PDMP-treated cells, GM3 and GD3 influenced neither PKC nor PKCα activity (data not shown), which is still considered to be critical for P-gp activity (Chambers et al., 1993; Gupta et al., 1996). Moreover, calphostin C, an inhibitor of classical and novel PKC isozymes, did not abrogate the stimulatory effect of gangliosides on P-gp function (data not shown). Alternatively, it is conceivable that gangliosides may facilitate the interaction between P-gp and its regulator. The latter hypothesis is supported by the fact that P-gp was found to be located, at least partially, in detergent insoluble glycosphingolipids-membrane domains (Lavie et al., 1998), which contained significant amounts of gangliosides as well as a number of signaling molecules, including PKC isoforms (Hakomori, 1997).

In conclusion, this study provides for the first time evidence of the regulatory effect of ganglioside on P-gp function of leukemic cells and suggests that inhibition of GM3 or GD3 synthesis may represent a promising approach for MDR reversal.

Acknowledgments

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