1-Phenyl-2-decanoylamino-3-morpholino-1-propanol Chemosensitizes Neuroblastoma Cells for Taxol and Vincristine[1]

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ABSTRACT

In this study, we show that an inhibitor of glycosphingolipid biosynthesis, D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), increases the chemosensitivity of neuroblastoma tumor cells for Taxol and vincristine. At noneffective low doses of Taxol or vincristine, the addition of a noneffective dose of PDMP resulted in 70% cytotoxicity, indicating synergy. Such an effect was not observed for etoposide (VP16). PDMP caused an early (6 h) increase in ceramide (Cer) levels, but the excess Cer was metabolically removed in the long-term (96 h). However, upon incubation with PDMP in combination with Taxol, but not with etoposide, Cer levels remained elevated at 96 h. These results suggest that neuroblastoma cells are normally able to metabolically remove excess Cer, but lose this capacity upon exposure to microtubule modulating anticancer agents (Taxol or vincristine). In addition, PDMP treatment resulted in a decreased efflux of [^{14}C]Taxol and [^{3}H]vincristine from neuroblastoma cells, similar to treatment with PSC833 or MK571, suggesting an effect of PDMP on the transporter proteins P-glycoprotein and/or multidrug resistance protein. PDMP did not further reduce [^{14}C]Taxol or [^{3}H]vincristine efflux in PSC833-treated cells, although it did further diminish cell survival under these conditions. We conclude that a combined administration of nontoxic concentrations of PDMP and either Taxol or vincristine results in highly sensitized neuroblastoma cells. This appears to involve a sustained elevation of Cer levels, possibly in concert with increased drug accumulation.

INTRODUCTION

Neuroblastoma is a common solid tumor in childhood (1). The majority of children with neuroblastoma present with advanced disease and require chemotherapy as the primary approach for treatment. Treatment failure is largely attributable to resistance to a diverse range of structurally and functionally unrelated drugs called MDR[3] (2, 3). A variety of cellular mechanisms have been shown to play a role in the clinical response of neuroblastoma to chemotherapy (4–6). Increased drug efflux by transporter proteins P-gp, which is encoded by the *MDR1* gene and MRP, enhanced intracellular drug detoxification, and altered drug targets are well known mechanisms of drug resistance. After the observation that etoposide (VP16) induces programmed cell death (7, 8), which was later extended to other chemotherapeutic agents, the inability of tumor cells to activate the apoptotic response has been proposed as a fundamental drug resistance mechanism. Interestingly, many chemotherapeutic agents, such as vincristine, daunorubicin, and 1-β-D-arabinofuranosylcytosine, have also been shown to induce apoptosis through accumulation of Cer (9–11). In addition, it has been recently established that MDR cells accumulate GlcCer, a metabolic product of Cer and a precursor for glycosphingolipid biosynthesis (12). Furthermore, agents that reverse MDR were shown to inhibit GlcCer synthetase (13). In conclusion, MDR cells have the ability to metabolically remove Cer by a high rate of conversion into GlcCer.

PDMP is a well-known inhibitor of Cer glycosylation, resulting in decreased GlcCer levels and often an accumulation of Cer (14). In addition, PDMP has been shown to inhibit protein transport through the Golgi complex and from the Golgi to the plasma membrane (15), possibly as a result of elevated levels of Cer (16). Recently, we have shown that PDMP blocks membrane transport in the endoplastic reticulum /Golgi system, not through its effects on sphingolipid metabolism, but rather by modulation of calcium homeostasis. Interestingly, this effect of PDMP could be mimicked by the MRP1 inhibitor MK571 (17). These results indicate that disruption of intracellular membrane transport may contribute to decreased drug resistance in MDR cells.

In addition to decreasing levels of GlcCer, long-term PDMP treatment results in the reduction of complex glycolipids, including gangliosides. Neuroblastoma cells contain relatively

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[3] The abbreviations used are: MDR, multidrug resistance; BF, efflux-blocking factor; Cer, ceramide; GlcCer, glucosylceramide; CF, 5-carboxyfluorescein; CFDA, CF diacetate; MRP, multidrug resistance protein; PDMP, D, L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; P-gp, P-glycoprotein; Rho123, rhodamine 123.

high amounts of these sialic acid-containing glycosphingolipids (18). Recently, we have shown that gangliosides shed by these and other tumor cells have inhibitory effects on the hemopoietic system (19, 20), whereby blocking the biosynthesis of gangliosides and their shedding with PDMP results in abrogation of their inhibitory effects on hemopoiesis (19). The present study was aimed to establish PDMP also as a direct reducer of neuroblastoma cell survival. We show that PDMP highly sensitizes neuroblastoma cells to treatment with microtubule-affecting cytostatics and provides evidence for the involvement of sustained Cer accumulation and possibly increased drug accumulation in this process.

MATERIALS AND METHODS

Cell Lines. Murine neuroblastoma cells (Neuro-2a or C1300) were grown as adherent monolayer cultures in 75-cm^2 Falcon flasks in DMEM (Life Technologies, Breda, the Netherlands) supplemented with 10% FCS (Hyclone, Logan, Utah) and 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 units/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies) under standard incubator conditions (humidified atmosphere; 95% air and 5% CO_2; 37°C).

Measurement of Cellular Sensitivity to Cytotoxic Drugs. Drug sensitivities of the Neuro-2a and C1300 cells were determined with the tetrazolium salt WST-1 test (Boehringer Mannheim, Almere, the Netherlands). Neuro-2a cells (1000/well) or C1300 (5000/well) were seeded into 96-microtiter plates (six replicate) and incubated for 24 h to adhere. Thereafter, cytotoxic drugs (Taxol/vincristine/VP16) in various concentrations, either with or without the inhibitors MK571 (a kind gift from Professor A.W. Ford-Hutchinson; Merck-Frosst Inc., Kirkland, Canada) or PSC833 (provided by Novartis Pharma Inc., Basel, Switzerland) and either with or without PDMP (Matreya Inc., Pleasant Gap, PA), were added to a total volume of 100 μl. After 72 h of incubation, 10 μl of WST-1 was added to each well. WST-1 tetrazolium salt is cleaved to formazan by the succinate-tetrazolium reductase system, which belongs to the respiratory chain of the mitochondria, and this only occurs in viable cells. The formazan dye was quantified using an ELISA reader (450 nm).

MRP1 and P-gp Expression Measured by Flow Cytometry. Cells (0.5×10^6) were permeabilized in 10% (v/v) lysing solution (Becton Dickinson Medical Systems, Sharon, MA) in H_2O and incubated for 15 min in PBS/BSA (2%) containing 1% normal goat serum. The cells were incubated for 1 h at 4°C in 200 μl PBS/BSA (2%) with the monoclonal antibody UIC2 (1 μg/ml; IgG2; Immunotech, Mijdrecht, the Netherlands) or MRPm6 (10 μg/ml; IgG1; kindly donated by Professor R. J. Scheper, Amsterdam, the Netherlands) or the mouse isotype-matched control monoclonal antibodies. Antibody binding was detected with R-Phycoerythrin-labeled rabbit antimouse immunoglobulins (Dako, Glostrup, Denmark). Fluorescence was measured by flow cytometry (FACStar, Becton Dickinson Medical Systems, Sharon, MA). For each sample, 10,000 events were collected. Protein expressions are given as the ratio of mean fluorescence of UIC2/IgG2 control or MRPm6/IgG1 control.

Flow Cytometric Detection of P-gp and MRP1-mediated Drug Efflux. The efflux by tumor cells of Rho123 (Sigma Chemical Co., St. Louis, MO) or CF, in the absence or presence of the P-gp inhibitor PSC833 or the MRP1 inhibitor MK571, respectively, was measured flow cytometrically, as previously described (21). Neuro-2a or C1300 cells (1×10^6) were incubated for 20 min at 37°C in RPMI 1640 medium, containing either 200 ng/ml Rho123 or 0.1 μM CFDA (Sigma), with or without inhibitor (2 μg/ml PSC833 or 20 μM MK571, respectively). Cells were washed with ice-cold medium, resuspended in medium with or without inhibitor, and incubated for 60 min at 37°C (efflux). Efflux was stopped by the substitution with ice-cold medium, followed by the measurement of Rho123/CF fluorescence by flow cytometry (FACStar, Becton Dickinson Medical Systems, Sharon, MA). The Rho123/CF fluorescence of 10,000 events was logarithmically measured at a wavelength of 488 nm through a 530-nm band-pass filter. These signals were converted into linear values and expressed as relative fluorescence units (rel. F.U.) using win-list 2.0 software (Verity Software House Inc. Topsham, ME). Values from cells incubated with Rho123/CFDA only, *i.e.*, without inhibitors, served as baseline Rho123/CFDA uptake. The BF was defined as follows:

$$\mathrm{BF} = \frac{[\text{rel. F.U. in inhibitor-blocked cells} - \text{baseline rel. F.U.}]}{[\text{rel. F.U. in unblocked cells} - \text{baseline rel. F.U.}]}$$

The doxorubicin-resistant P-gp-overexpressing subclone 2780AD (22), derived from the A2780 human ovarian carcinoma cell line, served as a positive control for PSC833 blocking of Rho123. The doxorubicin-resistant MRP1-overexpressing subclone GLC4/ADR (21), derived from the GLC4 human small cell lung cancer cell line, served as a positive control for MK571 blocking of CF.

[^{14}C]Taxol or [^{3}H]vincristine Efflux Studies. Neuro-2a cells were incubated with 3 ml of Hanks' solution, containing either 0.01 μCi [^{14}C]Taxol (44.5 mCi/mmol; Sigma Chemical Co., St. Louis, MO) or 1 μCi [^{3}H]vincristine (5.7 Ci/mmol; Amersham, Buckinghamshire, England) for 20 min at 37°C, followed by washing with ice-cold Hanks' solution. Thereafter, the cells were incubated for 60 min at 37°C in the absence (control) or presence of PDMP (5 μM), PSC833 (2 μg/ml), MK571 (20 μM), or combinations of these drugs. Cells and incubation medium were then separated, and the radioactive content was measured in a scintillation counter. The efflux was expressed as a percentage and defined as (radioactivity in efflux medium)/total radioactivity × 100%.

Radiolabeling and Analysis of Cellular Cer Levels. The total pool of sphingolipids was radiolabeled by growing the cells in the presence of 3 μCi/ml [^{3}H]L-serine (30 Ci/mmol; Amersham), a precursor for sphingolipid biosynthesis. After 48 h of incubation, the medium was removed, and the cells were fixed in ice-cold CH_3OH, followed by lipid extraction from the cells (23). Aliquots of the lipid extracts were taken for the determination of the total amount of lipid-incorporated radioactivity. Acylglycerolipids were hydrolyzed during 1 h of incubation at 37°C in $CHCl_3/CH_3OH$ (1:1, v/v) containing 0.1 M KOH. The remaining lipids were re-extracted and applied on high performance thin-layer chromatography plates. Plates were de-

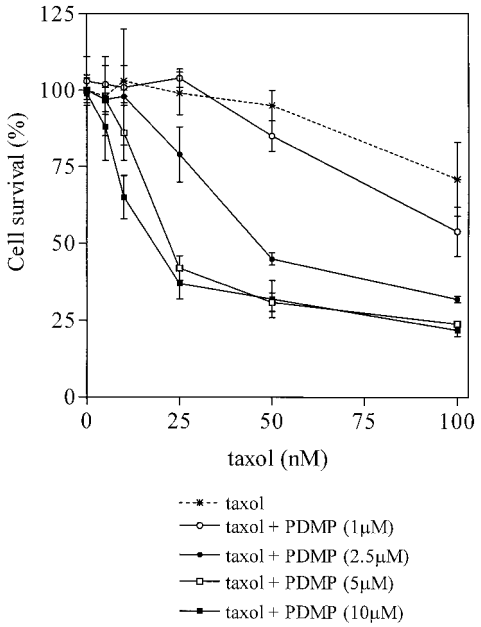

Fig. 1 Effect of PDMP on the Taxol dose-response curve for Neuro-2a cytotoxicity. Cell survival of Neuro-2a cells was determined in the presence of various concentrations (0–100 nM) of Taxol (*dashed line*) and increasing doses of PDMP (*continuous lines*; ○, 1 μM; ●, 2.5 μM; □, 5 μM; ■, 10 μM). Cell survival was determined with a cell proliferation assay (WST-1 test), as described in "Materials and Methods". Data represents the mean ± SD of more than three experiments, each consisting of six replicate determinations.

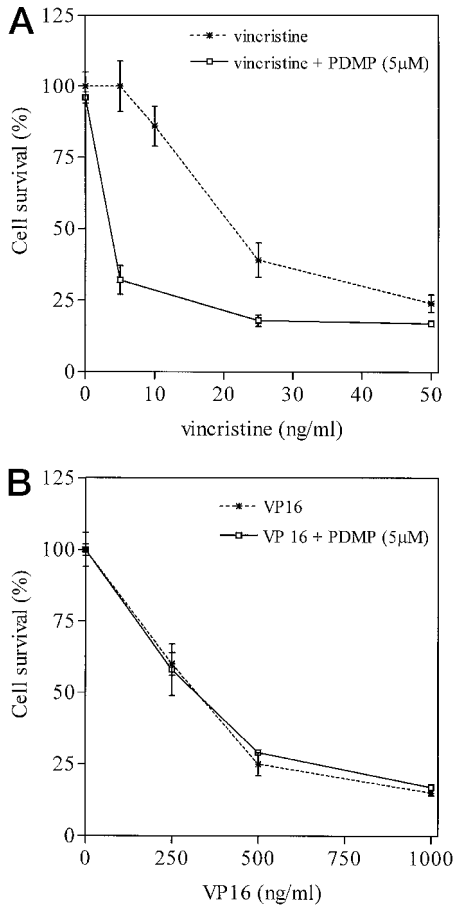

Fig. 2 Effect of PDMP on the vincristine and VP16 dose-response curves for Neuro-2a cytotoxicity. *A*, cell survival of Neuro-2a cells was determined in the presence of various concentrations (0–50 ng/ml) of vincristine (*dashed line*) and one dose (5 μM) of PDMP (*continuous line*). *B*, cell survival of Neuro-2a cells was determined in the presence of various concentrations (0–1000 ng/ml) of VP16 (*dashed line*) and one dose (5 μM) of PDMP (*continuous line*). Cell survival was determined with a cell proliferation assay (WST-1 test), as described in "Materials and Methods". Data represents the mean ± SD of more than three experiments, each consisting of six replicate determinations.

veloped in $CHCl_3/CH_3OH/H_2O$ (14:6:1, v/v) in the first dimension and in $CHCl_3/CH_3COOH$ (9:1, v/v) in the second dimension to resolve Cer. Cer-containing spots were scraped and subjected to scintillation counting. The effects of cytotoxic agents and PDMP on Cer levels were determined by growing the cells, after overnight adherence, for 4 days in medium containing [^{3}H]serine. Drugs were present for the entire 4-day period or only the final 6 h before the end of the incubation. Treatment with 0.1 units/ml bacterial sphingomyelinase (Sigma) for the final 2 h of the incubation served as positive control (maximal Cer formation from sphingomyelin; see Ref. 23).

Statistical Analysis. The unpaired two tailed Student's *t* test was used for statistical analysis. Values of $P < 0.05$ were considered to indicate statistically significant differences between data sets.

RESULTS

PDMP Chemosensitizes Neuro-2a Cells to Taxol. The ability of PDMP to enhance the cytotoxic effect of Taxol was evaluated by exposing Neuro-2a cells to increasing concentrations of Taxol in combination with a range of nontoxic doses (1–10 μM) of PDMP. In the presence of Taxol, a dose-dependent cytotoxic effect of PDMP was observed (Fig. 1). The IC_{50} value for Taxol was well above 100 nM, but shifted to 46.4 and 22.4 nM in the presence of 2.5 and 5 μM PDMP, respectively.

PDMP Also Acts as a Chemosensitizer for Vincristine, but not for VP16. A similar chemosensitizing effect of PDMP was observed in the case of vincristine-induced cytotoxicity of Neuro-2a cells (Fig. 2*A*). Cell survival decreased from 100% (±9; $n > 3$) to 32% (±5; $n > 3$) with the addition of 5 μM PDMP at a low dose of vincristine (5 ng/ml). The IC_{50} value for vincristine decreased from 21.3 to 3.7 ng/ml at 5 μM PDMP. In contrast to Taxol and vincristine, PDMP had no additional effect on VP16-induced cytotoxicity of Neuro-2a cells. The IC_{50} value for VP16 was 260 ng/ml in both VP16- and VP16/PDMP-treated cells (Fig. 2*B*).

The Synergistic Effect of PDMP on Taxol Cytotoxicity Appears to Involve Sustained Generation of Cer. PDMP is known for its inhibitory action on glycosphingolipid biosynthesis (14). PDMP treatment results in reduced levels of GlcCer, more complex glycolipids, and increased levels of Cer. Cer is a mediator of programmed cell death (24, 25). Therefore, we investigated whether Cer levels were enhanced under conditions where PDMP showed a chemosensitizing effect. When Neuro-2a cells were treated with Taxol, VP16, PDMP, or com-

Table 1 Cer levels after treatment of Neuro-2a cells with chemotherapeutic agents and/or PDMP[a]

Additions	Cer (% of control) 6-h treatment	Cer (% of control) 96-h treatment
Control	100 ± 7.8	100 ± 9.6
PDMP	132.2 ± 19.1	100.6 ± 15.0
Taxol	99.5 ± 11.8	122.3 ± 8.9
Taxol + PDMP	126.2[b] ± 7.3	151.2[b] ± 4.4
VP16	95.8 ± 12.0	72.9[b] ± 6.6
VP16 + PDMP	140.6[b] ± 15.1	74.1[b] ± 3.3

[a] Cellular Cer pools were labeled by growing the cells for 4 days in the presence of [^{3}H]serine. Taxol (25 nM), VP16 (0.25 μg/ml), and/or PDMP (5 μM) were present during the entire 4-day period (96 h) or during the final 6 h of this period. Cer pools were measured as described in "Materials and Methods". Data are expressed as percentage compared to untreated cells. The 100% (control) value corresponds to 4.8 d.p.s./10^3 d.p.s. in the total lipid extract. Bacterial sphingomyelinase treatment (0.1 unit/ml), performed during the final 2 h of the incubation period, resulted in an increase to 713.3% (±6.2) of control. Values are the means of three independent experiments ± SD.

[b] Values that were statistically different from the control according to Student's *t* test ($P < 0.05$).

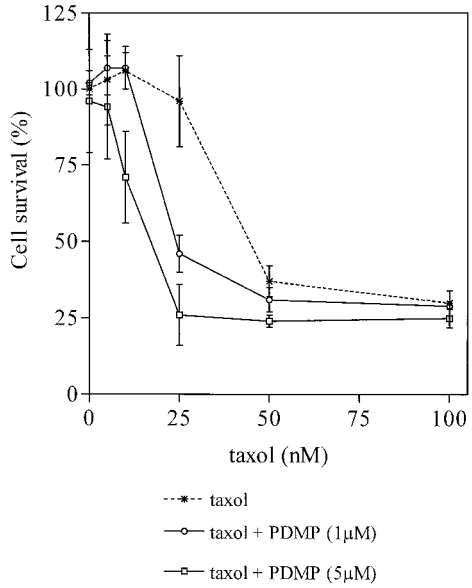

Fig. 3 Effect of PDMP on the Taxol dose-response curve for C1300 cytotoxicity. Cell survival of C1300 cells was determined in the presence of various concentrations (0–100 nM) of Taxol (*dashed line*) and two doses of PDMP (*continuous lines*; ○, 1 μM; □, 5 μM). Cell survival was determined with a cell proliferation assay (WST-1 test), as described in "Materials and Methods". Data represents the mean ± SD of more than three experiments, each consisting of six replicate determinations.

binations for 6 h, Cer levels increased ~33% when PDMP was present (Table 1). No synergistic effect of PDMP in combination with either Taxol or VP16 on the Cer level was observed. On the other hand, long-term Taxol treatment resulted in a marginally significant increase of 22% ($P = 0.058$) in Cer levels, whereas it decreased by 27% in the case of VP16. Interestingly, although PDMP was ineffective alone, when it was in combination with Taxol, a synergistic effect was observed on increasing Cer levels. In contrast, PDMP did not affect the reduction of Cer levels as a consequence of VP16 treatment. Thus, the chemosensitizing effect of PDMP on Taxol cytotoxicity correlated with a synergistic effect on increasing Cer levels. In the case of VP16, neither chemosensitization nor Cer increase was observed after PDMP cotreatment.

PDMP-induced Chemosensitization Is Not Restricted to Neuro-2a Cells. C1300 cells, with a different plasma membrane ganglioside composition, were more sensitive to Taxol (Fig. 3), with a cell survival of 37% (± 5; $n > 3$) at a concentration of 50 nM Taxol, whereas Neuro-2a cells remained insensitive at this concentration (cell survival of 95% ± 5%; $n > 3$; see Fig. 1). Still, PDMP acted as a chemosensitizer for Taxol cytotoxicity in C1300 cells. The IC_{50} value for Taxol decreased from 43.3 to 16.7 nM at 5 μM PDMP (Fig. 3). Similar to Neuro-2a cells, a synergistic effect of PDMP on vincristine cytotoxicity was observed in C1300 cells, whereas PDMP again had no effect on VP16 cytotoxicity (data not shown).

PDMP Affects Drug Efflux from Neuroblastoma Cells. We first analyzed whether MDR-related efflux pumps (P-gp and/or MRP1) were functionally expressed in Neuro-2a/C1300 cells. When measured by flow cytometry, P-gp and MRP1 expression in Neuro-2a/C1300 cells were low compared to their expression in 2780AD (158 ± 18) and GLC4/ADR (4.1 ± 0.2), respectively (Table 2). Rho123/CFDA efflux studies showed that functional activities of P-gp and MRP were low as well based on the low BF values in both Neuro-2a and C1300 cells as compared to 2780AD (BF = 93 ± 10.8) and GLC4/ADR cells (BF = 45.9 ± 5.8; Table 3).

Table 2 P-gp and MRP1 expression in Neuro-2a and C1300 cells[a]

	P-gp expression	MRP1 expression
Neuro-2a	1.02 ± 0.05	1.3 ± 0.1
C1300	0.97 ± 0.03	1.3 ± 0.2

[a] Neuro-2a and C1300 cells were permeabilized and incubated with the monoclonal antibodies UIC2, MRPm6, or the mouse isotype-matched control monoclonal antibodies. Antibody binding was detected with R-Phycoerythrin-labeled rabbit antimouse immunoglobulins and measured on a flow cytometer. Protein expression is given as the ratio of mean fluorescence of UIC2/IgG2 control (P-gp) or MRPm6/IgG1 control (MRP1). The results represent the mean ± SD of three independent experiments.

To test whether PDMP had an effect on the efflux of cytotoxic drugs, Neuro-2a cells were loaded with either [^{14}C]-Taxol or [^{3}H]vincristine. The efflux of the radiolabeled drugs was measured either in the presence or absence of PDMP and/or the MDR inhibitors PSC833 or MK571 (Table 4). The efflux of [^{14}C]Taxol was reduced by all compounds, with PSC833 being most effective. Similar results were obtained with [^{3}H]vincristine, although in this case, MK571 was most effective. Additive effects of PDMP and MDR inhibitors were not observed.

PDMP Increases Cytotoxicity When Used in Combination with Taxol and PSC833. A chemosensitizing effect of PDMP was still observed when Neuro-2a cells were cotreated with Taxol (10 nM), PSC833 (2 μg/ml), and PDMP (10 μM). Cell survival decreased to 17% (±1; $n > 3$) compared to 25% (±12; $n > 3$) of cells treated with a combination of Taxol and

Table 3 Efflux studies in murine neuroblastoma cell lines[a]

	Rho123-BF (PSC833)	CFDA-BF (MK571)
Neuro-2a	2.0 ± 0.4	2.8 ± 0.5
C1300	1.5 ± 0.3	5.1 ± 0.8

[a] Neuro-2α and C1300 cells were loaded with Rho123 (200 ng/ml) or CFDA (0.1 μM) for 20 min at 37°C, followed by efflux for 60 min at 37°C, as described in "Materials and Methods." Values indicate the BF for PSC833 (2 μg/ml) or MK571 (20 μM), calculated as described. The results represent the mean ± SD of at least three independent experiments.

Table 4 Effect of PDMP and MDR inhibitors on [^{14}C]Taxol and [^{3}H]vincristine efflux in Neuro-2a cells[a]

Neuro-2a cells were incubated with 3 ml of Hanks' solution, containing either 0.01 μCi [^{14}C]taxol (44.5 mCi/mmol) or 1 μCi [^{3}H]vincristine (5700 mCi/mmol) for 20 min at 37°C, followed by washing with ice-cold Hanks' solution. Thereafter, the cells were incubated for 60 min at 37°C in the absence (control) or presence of PDMP (5 μM), PSC833 (2 μg/ml), MK571 (20 μM), or combinations of these drugs. Cells and incubation medium were then separated, and the radioactive content was measured in a scintillation counter.

Treatment	[^{14}C]taxol efflux	[^{3}H]vincristine efflux
Control	32.4 ± 2.1	72.1 ± 6.7
PDMP	21.2 ± 2.1	53.3 ± 7.5
PSC833	17.1 ± 0.8	50.5 ± 11.3
PSC833 + PDMP	18.1 ± 0.8	53.3 ± 9.9
MK571	24.7 ± 1.2	47.1 ± 4.9
MK571 + PDMP	24.1 ± 2.0	46.8 ± 9.5

[a] The efflux was expressed as percentage, defined as (radioactivity in efflux medium)/total radioactivity × 100%. The results represent the mean ± SD of three independent experiments. All values are significantly different from control, according to Student's *t* test ($P < 0.001$). There were neither significant differences between cells treated with PSC833 alone or in combination with PDMP nor between cells treated with MK571 alone or in combination with PDMP.

PSC833 only (Fig. 4). There is a clear relation between Cer levels and cell survival. Increased Cer levels were measured with the use of the diglyceride kinase assay. A 10-fold increase compared to the control was found in the combined treatment of Taxol, PDMP, and PSC833, whereas the combination of Taxol and PSC833 resulted in a 7-fold increase (data not shown).

DISCUSSION

We have previously shown that PDMP could abrogate the inhibitory effects of neuroblastoma-derived gangliosides on hemopoiesis *in vitro* by inhibiting their biosynthesis and shedding (19). In the present work, we investigated whether PDMP also directly reduced the survival of neuroblastoma cells. This was studied by analyzing whether PDMP, in a nontoxic dose, exerts synergistic effects on chemotherapeutic treatment of neuroblastoma cells using an *in vitro* cell proliferation (WST) assay. In the case of the microtubule-effecting antimitotic agents, Taxol and vincristine, PDMP showed a strong synergistic reduction of neuroblastoma cell survival. In contrast, synergism was not observed with the topoisomerase II inhibitor VP16.

PDMP, an inhibitor of Cer glucosylation, is known to result in the accumulation of Cer (14), at least in the initial period

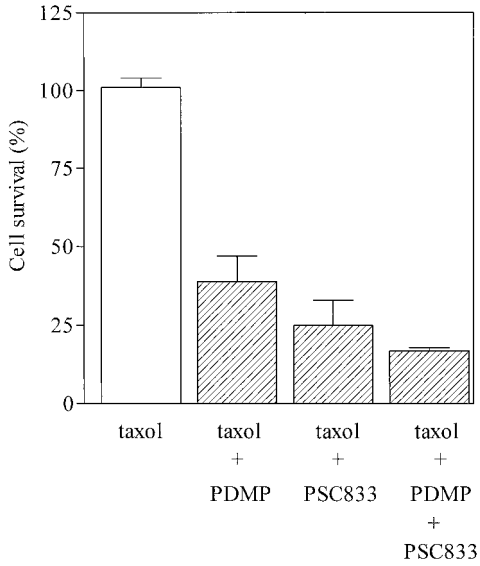

Fig. 4 PDMP chemosensitizes PSC833-blocked Neuro-2a cells to Taxol cytotoxicity. Cell survival of Neuro-2a cells was determined in the presence of Taxol (10 nM) alone or in combination with PDMP (10 μM), PSC833 (2 μg/ml), or both PDMP and PSC833. Cell survival was determined with a cell proliferation assay (WST-1 test), as described in "Materials and Methods". Data represents the mean ± SD of more than three experiments, each consisting of six replicate determinations.

following treatment. At longer term, cells are capable of removing excess Cer through diverse metabolic pathways (23, 26). Cer is a well-known mediator of programmed cell death (24, 25). Daunorubicin-induced cell death has been shown to be mediated by Cer generated either through sphingomyelin hydrolysis (11) or by *de novo* biosynthesis (10). Recently, Myrick *et al.* (27) demonstrated a potentiation of Taxol-mediated apoptosis in leukemic cells by Cer. Therefore, we investigated whether Cer accumulation occurred only under those conditions resulting in the negative synergistic effect of PDMP on cell survival. Our results show that PDMP elevates Cer levels after relatively short-term (6 h) incubations, but Cer levels have returned to normal after long-term (4 days) treatment. The short-term increase in Cer levels also occurs in the presence of VP16 (as well as Taxol). Clearly, if Cer accumulation is involved in the synergistic negative effect of combined treatment with PDMP/Taxol on cell survival, a short-term increase in the level of this sphingolipid is not sufficient. Interestingly, Neuro-2a cells treated with both PDMP and Taxol showed a significant long-term accumulation of Cer. On the other hand, long-term VP16 treatment did not raise the level of Cer, but rather caused a decrease, which was similar in the absence or presence of PDMP. These results are in agreement with the lack of synergistic effects on cell survival with PDMP/VP16 in contrast to PDMP/Taxol. Taken together, short-term treatment with PDMP results in a Cer excess, which upon metabolic removal does not result in long-term loss of cell viability. However, in the case of cotreatment with the microtubule-affecting agent, Taxol, the decrease in cell survival correlated with long-term Cer accumulation.

PDMP also inhibits the formation of gangliosides in neu-

roblastoma cells (19). Gangliosides are implicated in the regulation of proliferation, differentiation, tumor progression, immunosuppression, and MDR (28–30). Although heterogeneity in ganglioside composition in drug-resistant and drug-sensitive cells has been reported, no direct correlation with drug resistance has been demonstrated (31). We consider the involvement of specific gangliosides in chemosensitization of neuroblastoma cells by PDMP unlikely for two reasons. First, the ganglioside composition of Neuro-2a and C1300 cells is very different (19), and second, both cells are susceptible to PDMP-enhanced Taxol cytotoxicity.

In addition to a role for long-term Cer accumulation, the chemosensitizing effect of PDMP could (partly) be mediated by increased accumulation of the cytotoxic drugs. This effect could result from PDMP-inhibited drug efflux mediated by the MDR proteins P-gp and/or MRP. Flow-cytometric assays employing a fluorescent P-gp or MRP substrate revealed that the functional activity of these transporters was low in Neuro-2a/C1300 cells as compared to cell lines abundantly expressing P-gp (2780AD) or MRP1 (GLC4/ADR). This argues against a major impact on cell survival of inhibition of the activity of these transporters. However, PDMP did appear to cause some inhibition of [^{14}C]Taxol and [^{3}H]vincristine efflux, similar to the P-gp and MRP inhibitors PSC833 and MK571, respectively. Yet, we consider the contribution of such an effect to the observed long-term synergistic action of PDMP on cell survival limited for the following reasons: (*a*) If PDMP treatment resulted in a significant increase in the intracellular concentration of Taxol by modulation of MDR activity, it would also be expected to occur for VP16 because removal of structurally unrelated compounds is a characteristic of MDR proteins; (*b*) PDMP did not have an additional effect on [^{14}C]Taxol efflux in PSC833-treated cells (Table 4). However, under similar conditions, PDMP further reduced cell survival (Fig. 4).This decreased cell survival correlated with increased Cer levels (data not shown).

In summary, we have obtained evidence for a chemosensitizing effect of PDMP on neuroblastoma cells treated with the microtubule-affecting antimitotic agents, Taxol and vincristine. Although a contribution of drug efflux inhibition by PDMP cannot be excluded, this synergistic effect correlates with long-term Cer accumulation. After an initial rise of Cer (6 h) in PDMP-treated cells, the levels return to normal at the long term (4 days). This most likely involves metabolism of Cer to other sphingolipids, such as galactosylceramide and sphingomyelin, and possibly breakdown via sphingosine. However, when microtubules were stabilized by Taxol or disrupted by vincristine, Cer levels remained elevated. This suggests that due to blocking of transport processes in the cell, which require dynamic microtubular function, Cer is less efficiently metabolized. In the case of VP16, which reduces cell viability by an entirely different mechanism, long-term Cer accumulation does not occur even after cotreatment with PDMP. Thus, the present study provides evidence for a chemosensitizing effect of PDMP in neuroblastoma cells and opens new perspectives in improving the therapeutic efficacy of cytotoxic agents against malignancies. In fact, PDMP exerts a dual effect: In addition to strongly reducing the viability of Taxol/vincristine-treated neuroblastoma cells, it diminishes the adverse effects of neuroblastoma-derived gangliosides on hemopoiesis by inhibiting ganglioside biosynthesis and shedding.

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