

P-Glycoprotein Modulates Ceramide-mediated Sensitivity of Human Breast Cancer Cells to Tubulin-binding Anticancer Drugs¹

Jennifer A. Shabbits and Lawrence D. Mayer²

Department of Advanced Therapeutics, British Columbia Cancer Research Centre, Vancouver, British Columbia, V5Z 1L3 Canada, and Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, V6T 1Z3 Canada

Abstract

Alterations in metabolism of ceramide (Cer) to the noncytotoxic metabolite glucosylceramide have been implicated in the multidrug resistance (MDR) phenomenon. This observation has been made with tumor cells that also overexpress P-glycoprotein (Pgp), raising the possibility that Pgp plays a role in regulating Cer metabolism. We investigated the effect of the glucosylceramide synthase inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) on the chemosensitivity of two wild-type and multidrug-resistant human breast tumor cell lines. Subtoxic concentrations of PDMP sensitized drug-selected MCF7/AdrR and Pgp-overexpressing MDA435/LCC6^{MDR1} (MDR1 gene-transfected) cell lines to Taxol and vincristine but did not alter the chemosensitivity of the wild-type cells. Evaluation of Taxol uptake indicated that the effect of PDMP was not due to membrane permeability alterations because anticancer drug accumulation was unaffected by PDMP. Whereas both multidrug-resistant cell lines overexpress Pgp, only the MCF7/AdrR cell line overexpresses the glucosylceramide synthase enzyme. This difference enabled us to distinguish between sensitization effects associated with Cer metabolism versus Pgp-mediated transport. Interestingly, when Pgp function was blocked, the PDMP effect was reduced 3-fold in MCF7/AdrR cells and was no longer observed in the MDA435/LCC6^{MDR1} cells. These observations imply that Cer metabolism and apoptosis effects are regulated not only by enzymes that convert Cer to nontoxic metabolites but also by Pgp-mediated transport. Given the intracellular distribution patterns of Pgp, we propose that this effect is related to glucosylceramide translocation across the Golgi

bilayer. We have applied this model to the situation of Cer metabolism-based chemosensitization and demonstrate that MDR modulation strategies aimed primarily at altering drug transport mechanisms can influence other MDR mechanisms such as glycosphingolipid metabolism. This work highlights the relationship between drug transport and Cer metabolism in the context of chemosensitization and cautions against making oversimplified assumptions that these mechanisms act independently.

Introduction

Overexpression of the drug efflux pump Pgp³ has long been regarded as the major cause of MDR in a number of human malignancies. MDR reversal agents typically act by blocking the drug efflux activity of Pgp, thereby increasing intracellular drug levels and inducing cell death. As more has been learned about Pgp, its functional role has expanded to include activity as a drug flippase or phospholipid translocator (1), and it has been shown to transport short-chain fluorescent analogues of sphingomyelin and GlcCer across membranes (2). In addition, Pgp may play a role in regulating some caspase-dependent apoptotic pathways, a function completely independent of its drug/lipid transport properties (3). In light of these expanding roles, it is possible that Pgp may also be involved in regulating Cer-based apoptosis and metabolism.

Cer lipids have recently emerged as intracellular signaling molecules involved in mediating cell death. Increases in intracellular Cer levels and subsequent apoptosis have been observed in response to treatment with agents such as tumor necrosis factor (4) and Fas ligand (5, 6), environmental insults such as UV and ionizing radiation (7, 8), and exposure to cytotoxic agents such as chemotherapy drugs (9–12). Although Cer is a proapoptotic lipid, its GlcCer metabolite is not. Thus, conversion of Cer to GlcCer by the GCS is a mechanism by which cells can lower intracellular Cer levels and avoid apoptosis. This has been correlated with the MDR phenomenon both in cultured tumor cells and in clinical samples (13). In fact, the clinical utility of this Cer metabolite as a marker for drug-resistant tumors was investigated using tumor specimens from melanoma and breast cancer patients (14). In this study, GlcCer was detected in all patients who had failed chemotherapy, but the marker was absent in pa-

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² To whom requests for reprints should be addressed, at Department of Advanced Therapeutics, British Columbia Cancer Research Centre, 601 West 10th Avenue, Vancouver, British Columbia, V5Z 1L3 Canada. Phone: (604) 877-6098, ext. 3153; Fax: (604) 877-6011; E-mail: lmayer@bccancer.bc.ca.

³ The abbreviations used are: Pgp, P-glycoprotein; MDR, multidrug resistance; GlcCer, glucosylceramide; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; GCS, glucosylceramide synthase; Cer, ceramide; wt, wild type; PBSBT, PBS plus 0.1% BSA plus 0.5% Tween 20; PE, phycoerythrin; PBSB, PBS plus 0.1% BSA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

tients who showed a clinical response. Liu *et al.* (15) also demonstrated elevated GlcCer levels in patients who exhibited poor response to chemotherapy.

These observations suggest that inhibiting Cer glycosylation should keep intracellular levels of the proapoptotic lipid elevated, making it a potential approach for circumventing MDR. PDMP is a well-known inhibitor of the GCS enzyme that has been shown to decrease GlcCer production and promote Cer accumulation (16). Previous work has shown that PDMP sensitizes murine neuroblastoma cells to treatment with microtubule-affecting cytostatics (17). In the present study, we evaluate the involvement of Pgp in Cer-mediated cell death and chemosensitivity in two human breast cancer cell lines. We have specifically compared cells that overexpress both Pgp and GCS, which have developed resistance through drug selection, *versus* cells that were transfected with the *MDR1* gene and thus overexpress only Pgp. Comparisons made between these cells enabled us to better elucidate the specific role of Pgp-based transport in Cer-mediated chemosensitization and apoptosis.

Materials and Methods

Cell Lines. Human estrogen receptor-negative breast cancer cell lines MDA435/LCC6 and MDA435/LCC6^{MDR1} were a generous gift from Dr. Robert Clarke (Georgetown University, Washington, D.C.). Human estrogen receptor-positive breast adenocarcinoma MCF7 cells were obtained from the American Type Culture Collection (Manassas, VA). The Adriamycin-resistant MCF7/AdrR cells were obtained from Dr. Gerald Batist (Lady Davis Research Institute, Montreal, Quebec, Canada). All cell lines were grown as adherent monolayer cultures in 25-cm² Falcon flasks in DMEM (MDA435/LCC6 cells) or RPMI 1640 (MCF7 cells) culture medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% L-glutamine (Life Technologies, Inc., Burlington, Ontario, Canada). Cells were maintained at 37°C in humidified air with 5% CO₂ and subcultured weekly using 0.25% trypsin with 1 mM EDTA (Life Technologies, Inc.).

Fixation and Anti-Pgp Staining for Flow Cytometry. Cells were harvested with 0.25% trypsin + 1 mM EDTA and counted with trypan blue. We placed 6×10^6 cells of each cell type in 1.7-ml Eppendorf tubes (VWR, West Chester, PA). After centrifugation, the pellets were resuspended in 0.3 ml of 2% formaldehyde (Polysciences, Warrington, PA) in PBS and left to incubate at room temperature for 30 min for fixation. To permeabilize cells, 1% Tween 20 (Sigma Chemical Co., St. Louis, MO) was added for the last 15 min of fixation. Fixed cells were centrifuged, and pellets were resuspended in 0.1 ml of 10% BSA in PBS, followed by two washes with 1 ml of PBSBT. Pellets were resuspended in 0.75 ml of blocking reagent containing PBSBT with 100 μ g/ml human IgG (ICN ImmunoBiologicals, Lisle, IL), 500 μ g/ml RNase (Sigma Chemical Co.), and 20% normal human serum. Samples were placed at 4°C overnight for blocking. The next day, each sample was equally distributed to three Eppendorf tubes. The first aliquot of each cell line was used as an autofluorescence control; the second aliquot was

555743; BD Biosciences, Mississauga, Ontario, Canada) at a 1:5 dilution; and the third aliquot was stained with mouse antihuman Pgp-PE antibody (catalogue number 557003; BD Biosciences) at a 1:5 dilution. Cells were incubated for 2 h at room temperature. Cells were washed twice with 1 ml of PBSBT and once with PBSB, allowing a 30-min incubation at room temperature in the wash buffer before the second spin. All centrifuge steps were performed using a microcentrifuge (Biofuge pico; Geraeus, Osterode, Germany) set at 7,000 rpm/15 s for live cells and 11,000 rpm/25 s for fixed cells. Finally, cells were resuspended in 0.3 ml of PBSB containing 0.1 μ g/ml 4',6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) and transferred to 352063 Falcon tubes for flow cytometry analysis.

Flow Cytometry. Cells were harvested with 0.25% trypsin + 1 mM EDTA, and the concentration was adjusted to 10^7 cells/ml. Aliquots of 10^6 cells/cell line were resuspended in 0.1 ml of PBSB and 20% human serum and incubated on ice for 15 min. Antihuman Pgp-PE antibody (BD Biosciences) and isotype control-PE antibody (BD Biosciences) were added at a 1:5 dilution. Samples were incubated for 45 min on ice, followed by three washes in PBSB. Flow cytometric analysis was performed using the EPICS Elite^{ESP} flow cytometer (Becton-Coulter, Miami, FL).

Cytotoxicity Assays. Cells were counted with a hemocytometer and seeded into 96-well microtiter Falcon plates (Becton Dickinson, Franklin Lakes, NJ) at $1.5\text{--}2.5 \times 10^3$ cells/well in 0.1 ml of complete medium. The perimeter wells of the 96-well plates were not used and contained 0.2 ml of sterile water. After 24 h at 37°C, the medium was replaced with 0.2 ml of fresh medium containing Taxol (Bristol-Myers Squibb, Montreal, Quebec, Canada), vincristine sulfate [Faulding (Canada) Inc., Vaudreuil, Quebec, Canada], cisplatin (Faulding Inc.), or doxorubicin-HCl (Faulding Inc.), with or without PDMP (Matreya Inc., Pleasant Gap, PA), C₆-Cer (Avanti Polar Lipids, Alabaster, AL), or valsopodar (Novartis, Dorval, Quebec, Canada) at the appropriate concentrations. After 72 h, the cell viability was assessed using a conventional MTT dye reduction assay. Fifty μ l of 5 mg/ml MTT reagent in PBS were added to each well. Viable cells with active mitochondria reduce the MTT to an insoluble purple formazan precipitate that is solubilized by the subsequent addition of 150 μ l of DMSO. The formazan dye was measured spectrophotometrically using a Dynex plate reader (570 nm). All assays were performed in triplicate. The cytotoxic effect of each treatment was expressed as a percentage of cell viability relative to untreated control cells (percentage of control) and is defined as $[(A_{570\text{ nm}}^{\text{treated cells}}) / A_{570\text{ nm}}^{\text{nontreated cells}}] \times 100$.

[³H]Taxol Uptake Studies. Cells were seeded into 6-well Falcon plates at 1.5×10^6 cells/ml in 2 ml of complete medium. After 24 h at 37°C, the old medium was aspirated, and cells were incubated with 2 ml of complete medium containing the indicated concentrations of Taxol + 0.25 μ Ci/ml [³H]Taxol (Moravek Biochemicals Inc., Brea, CA) in the absence or presence of 5 μ M PDMP for the times indicated. Culture medium was then removed, and cells were washed twice with 0.2 ml of ice-cold PBS (pH 7.2). Cells were harvested by scraping into 0.5 ml of PBS, and radioactivity

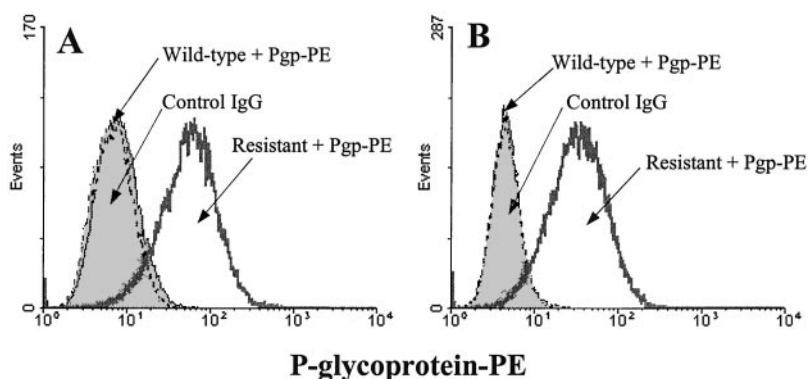


Fig. 1. Pgp expression in MCF7 (A) and MDA435/LCC6 (B) breast cancer cells as shown by flow cytometry.

was measured by liquid scintillation counting. Uptake was expressed as picograms of Taxol/ 10^6 cells.

Cell Radiolabeling and Lipid Extraction. Cells were seeded into 75-cm² Falcon flasks in complete medium and grown to ~70% confluence. Plating medium was replaced by 15 ml of complete medium containing 1.0 μ Ci/ml [³H]palmitic acid (New England Nuclear, Boston, MA) with or without 5 μ M PDMP and/or 1 μ g/ml valsopodar for 24 h. Cells were washed twice with ice-cold Hank's medium (Stem Cell) and harvested by scraping into 2.5 ml of ice-cold methanol containing 2% acetic acid. The collected cells were transferred to glass vials with Teflon-lined screw caps. Lipids were extracted from the methanol layer by adding equal volumes of water and chloroform, vortexing, and centrifuging at 3000 rpm for 5 min. The lipid-containing lower organic phase was collected into preweighed tubes. Three 10- μ l aliquots were removed for scintillation counting to determine the total amount of radiolabeled lipid. The remaining organic phase was dried under a stream of nitrogen, and tubes were reweighed to determine the total mass of extracted lipid.

Lipid Detection by TLC. The dried lipid film was dissolved in chloroform:methanol (2:1), and 300 μ g of total lipid were spotted onto TLC plates (Silica Gel G; Analtech, Newark, DE). Plates were developed in chloroform:acetic acid (90:10) for Cer and chloroform:methanol:ammonium hydroxide (70:20:4) for GlcCer. Commercial C₁₆-Cer (Avanti Polar Lipids) and GlcCer (Gaucher's spleen; Matreya, Inc.) standards were chromatographed. TLC plates were visualized with iodine vapor, and spots corresponding to Cer or GlcCer were scraped into scintillation vials for quantitation by liquid scintillation counting. The radioactivity of the selected spots was expressed as a percentage of the total amount of radiolabeled lipid extracted from the cells.

Fixation and Anti-Pgp Staining for Fluorescence Microscopy. Cells were harvested, fixed, and permeabilized as described elsewhere (18). Cells were blocked for 30 min at 37°C with 100 μ g/ml human IgG (ICN ImmunoBiologicals), 500 μ g/ml RNase (Sigma Chemical Co.), and 20% normal human serum in PBSBT, and aliquots of 2×10^6 cells of each type were used for antibody staining. Cells were incubated for 45 min at room temperature with mouse antihuman Pgp C219 primary antibody (catalogue number 8710-01; Signet Laboratories, Dedham, MA) at a 1:40 dilution. Cells were then washed three times in PBSB, allowing 30-min and 15-min

incubations at room temperature in the wash buffer between the second and third washes, respectively. Cells were resuspended in PBSBT + 20% goat serum, and a goat antimouse IgG FITC secondary antibody (BD Biosciences) was added at a 1:200 dilution to samples stained with a primary antibody. Goat antimouse IgG FITC antibody was added to unstained cells to control for nonspecific fluorescence. Cells were incubated for 45 min at room temperature, followed by three washes in PBSBT as described above. The cells were resuspended in PBSB + 0.1 μ g/ml 4',6-diamidino-2-phenylindole (Molecular Probes). Cells were spun onto glass slides, dried for 10 min at room temperature, and mounted in 10 μ l of 2.5% 1,4-diazabicyclo[2.2.2]octane anti-fade (Sigma Chemical Co.) in 90% glycerol. All images were acquired with a Leica DC 100 fluorescence microscope [Leica Microsystems (Canada), Richmond Hill, Ontario, Canada] and Image Database V4.01 software for 9 s. Images were later processed with ADOBE Photoshop 4.0 in an identical manner.

Results

Chemosensitization Effects of PDMP in Human Breast Cancer Cells.

The ability of PDMP to sensitize multidrug-resistant MCF7/AdrR and MDA435/LCC6^{MDR1} cells to Taxol and vincristine was evaluated by exposing cells to increasing concentrations of anticancer drug in the presence of a nontoxic concentration of PDMP (5 μ M). Pgp overexpression in both multidrug-resistant cell lines was confirmed by flow cytometry using the fluorescent Pgp-PE antibody. The fluorescence intensity of the MCF7/AdrR cell line was 9.5 times greater than that of the wt, and the fluorescence intensity of the MDA435/LCC6^{MDR1} cell line was 6.8 times greater than that of the wt (Fig. 1). Chemosensitization was evaluated by comparing the anticancer drug IC₅₀ values in the presence and absence of PDMP. PDMP sensitized both drug-resistant cell lines to the cytotoxic effects of Taxol and vincristine. In the MCF7/AdrR cell line, PDMP decreased the Taxol IC₅₀ from 3.24 ± 0.06 to 0.50 ± 0.20 μ M and decreased the vincristine IC₅₀ from 11.02 ± 2.16 to 1.82 ± 0.16 μ M (Fig. 2; Table 1). In the MDA435/LCC6^{MDR1} cell line, PDMP decreased the Taxol IC₅₀ from 30.01 ± 4.13 to 2.21 ± 1.14 nM and decreased the vincristine IC₅₀ from 39.25 ± 7.90 to 1.04 ± 0.40 nM (Fig. 3; Table 1). Similar results were observed on treatment with Taxotere and vinblastine (data not

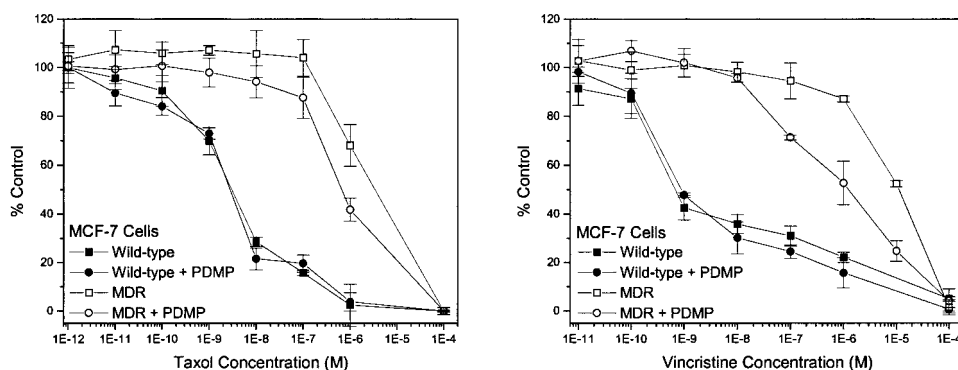


Fig. 2. Effect of 5 μM PDMP on Taxol and vincristine cytotoxicity in wt and multidrug-resistant MCF7 cells. Cells were incubated with the indicated anticancer drug concentrations \pm PDMP for 72 h, and cell viability was measured using the MTT assay. Each value represents the mean from at least three independent experiments. Bars, SE.

Table 1 Effect of 5 μM PDMP and 2 μM C₆-Cer on anticancer drug cytotoxicity in wt and multidrug-resistant MCF7 and MDA435/LCC6 breast cancer cells

| Cell line | IC ₅₀ ^a \pm SE | | | |
|--|--|--------------------------------|-------------------------------|---------------------------------|
| | Taxol | Vincristine | Doxorubicin | Cisplatin |
| MCF7 | 12.52 \pm 3.64 nM | 0.76 \pm 0.18 nM | 0.17 \pm 0.06 μM | 33.99 \pm 3.29 μM |
| MCF7 + PDMP | 12.08 \pm 0.11 nM | 0.77 \pm 0.16 nM | 0.18 \pm 0.07 μM | 34.38 \pm 4.55 μM |
| MCF7/AdrR | 3.24 \pm 0.06 μM | 11.02 \pm 2.16 μM | 0.34 \pm 0.12 μM | 27.57 \pm 9.08 μM |
| MCF7/AdrR + PDMP | 0.50 \pm 0.20 μM | 1.82 \pm 0.16 μM | 0.34 \pm 0.12 μM | 28.75 \pm 12.52 μM |
| MCF7/AdrR + PDMP + C ₆ -Cer | 0.68 \pm 0.08 μM | 1.48 \pm 0.01 μM | N/D ^b | N/D |
| MDA435/LCC6 | 1.81 \pm 1.37 nM | 0.22 \pm 0.02 nM | 0.25 \pm 0.14 μM | 10.17 \pm 3.57 μM |
| MDA435/LCC6 + PDMP | 1.27 \pm 1.06 nM | 0.18 \pm 0.02 nM | 0.23 \pm 0.16 μM | 9.95 \pm 5.02 μM |
| MDA435/LCC6 ^{MDR1} | 30.01 \pm 4.13 nM | 39.25 \pm 7.90 nM | 3.87 \pm 1.57 μM | 14.25 \pm 1.06 μM |
| MDA435/LCC6 ^{MDR1} + PDMP | 2.21 \pm 1.14 nM | 1.04 \pm 0.40 nM | 3.41 \pm 1.51 μM | 14.25 \pm 1.06 μM |
| MDA435/LCC6 ^{MDR1} + PDMP + C ₆ -Cer | 2.40 \pm 1.97 nM | 1.18 \pm 0.38 nM | N/D | N/D |

^a Cells were incubated with increasing drug concentrations \pm 5 μM PDMP and 2 μM C₆-Cer over 72 h, and viability was analyzed by the MTT assay. The IC₅₀ value was taken as the anticancer drug concentration that inhibits cell growth by 50% relative to untreated control cells.

^b N/D, not determined.

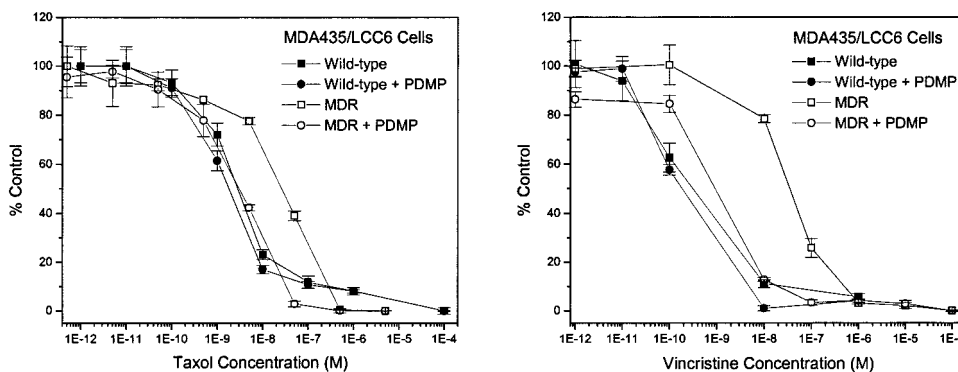


Fig. 3. Effect of 5 μM PDMP on Taxol and vincristine cytotoxicity in wt and multidrug-resistant MDA435/LCC6 cells. Cells were incubated with the indicated anticancer drug concentrations \pm PDMP for 72 h, and cell viability was measured using the MTT assay. Each value represents the mean from at least three independent experiments. Bars, SE.

shown). PDMP had no effect on the IC₅₀ values for the wt counterparts of either cell line (Table 1). Interestingly, PDMP did not sensitize the drug-resistant cells to the cytotoxic effects of doxorubicin or cisplatin (Table 1).

To demonstrate that the chemosensitization effect was not due to PDMP-induced alterations in cellular drug uptake, cells were incubated with 10 nM Taxol (MDA435/LCC6 cells) or 500 nM Taxol (MCF7 cells) + 0.25 $\mu\text{Ci}/\text{ml}$ [³H]Taxol for 1 and 4 h in the absence and presence of 5 μM PDMP. The presence of PDMP did not affect cellular drug accumulation because the percentage accumulation of total [³H]Taxol after 1 and 4 h was the same in the

presence and absence of PDMP (Fig. 4). Although the IC₅₀ values for the wt and multidrug-resistant counterparts of each cell line are different, we felt that it was important to incubate cells with the same Taxol concentration so that direct comparisons between uptake levels in the wt *versus* multidrug-resistant cells could be made. A Taxol concentration intermediate between the respective IC₅₀ values was chosen. Our results show increased Taxol accumulation in both wt cell lines compared with their multidrug-resistant counterparts. Specifically, Taxol accumulation was 2.5–3-fold greater in the MCF7^{wt} cells compared with MCF7/AdrR cells and approximately 4-fold greater in

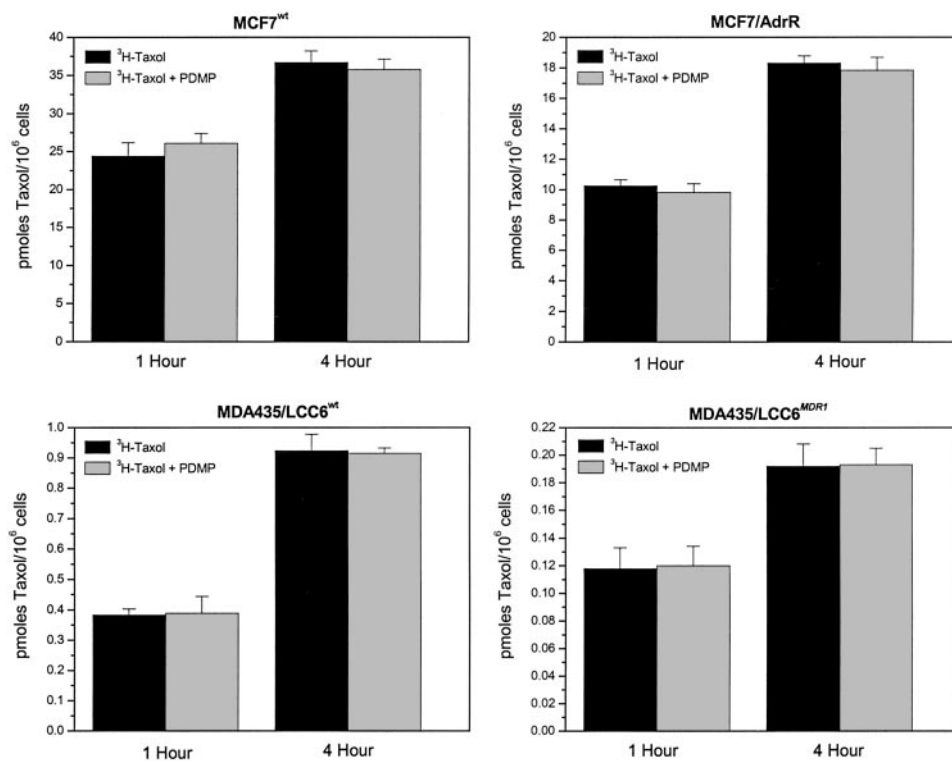


Fig. 4. Accumulation of [³H]-Taxol in wt and multidrug-resistant MCF7 and MDA435/LCC6 cells. Cells were incubated with 0.25 μ Ci/ml [³H]-Taxol (10 nM Taxol for MDA435/LCC6 cells and 500 nM Taxol for MCF7 cells) in the presence and absence of 5 μ M PDMP for the times indicated. Culture medium was removed, and cells were washed twice with ice-cold PBS. Cells were harvested by scraping into PBS, and radioactivity was measured by liquid scintillation counting. Uptake was expressed as picograms of Taxol/10⁶ cells. Each value represents the mean from three experiments. Bars, SE.

MDA435/LCC6^{wt} cells compared with MDA435/LCC6^{MDR1} cells.

Influence of Exogenous C₆-Cer on PDMP-induced Chemosensitization. Based on studies to date, PDMP chemosensitization effects are presumably attributed to increased intracellular Cer accumulation because its metabolism to GlcCer is blocked. On this basis, we speculated that the addition of exogenous short-chain Cer, which is itself cytotoxic (19), may further enhance this chemosensitization effect. To test this, cytotoxicity experiments with the various anticancer drugs were repeated in the presence of 2 μ M C₆-Cer. This Cer concentration reflected the approximate IC₂₀ (20% reduction of cell growth) that provided significant intracellular Cer levels without causing high cell death by itself. Combined treatment with Taxol or vincristine plus PDMP and Cer did not enhance chemosensitivity beyond that achieved by exposure to Taxol or vincristine with PDMP in the absence of exogenous Cer (Table 1). PDMP had no effect on the cytotoxicity of C₆-Cer alone in the wt or multidrug-resistant cells of either cell line (IC₅₀ values of 5.7 μ M for MCF7^{wt} \pm PDMP, 7.7 μ M for MCF7/AdrR \pm PDMP, 3.5 μ M for MDA435/LCC6^{wt} \pm PDMP, and 5.5 and 6.0 μ M for the MDA435/LCC6^{MDR1} cells \pm PDMP, respectively).

Effect of Pgp Inhibition on PDMP-induced Chemosensitization. To determine whether the PDMP effect could be directly attributed to Pgp, the cytotoxicity experiments were repeated in the presence of 1 μ g/ml valsopodar, a potent Pgp inhibitor. Specifically, we conducted side-by-side cytotoxicity experiments to assess the degree of PDMP-induced shift in Taxol/vincristine IC₅₀ values compared with the degree of PDMP-induced shift in the Taxol/vincristine + 1 μ g/ml val-

sopodar IC₅₀ values. Although valsopodar itself induces Taxol and vincristine chemosensitization by virtue of Pgp blockade, we were interested in measuring the degree of any further chemosensitization on subsequent addition of PDMP. The PDMP effect was evaluated in terms of fold sensitization, which was expressed as the ratio shown below.

$$(\text{Drug IC}_{50} \pm \text{PDMP} / \text{Drug IC}_{50} + 1 \mu\text{g/ml valsopodar} \pm \text{PDMP})$$

Under these conditions, we observed a 3-fold reduction in the PDMP-induced effect in the MCF7/AdrR cell line (6.5-fold Taxol and 6.1-fold vincristine shifts in the absence of valsopodar compared with only a 2-fold shift in the presence of valsopodar) and no shift in the MDA435/LCC6^{MDR1} cell line (13.6-fold Taxol and 37.7-fold vincristine shifts in the absence of valsopodar compared with no shift in the presence of valsopodar; Table 2). These results enabled us to identify a role for Pgp in the regulation of Cer metabolism to GlcCer and suggest that Pgp function is indeed related to the PDMP response. Valsopodar had no effect on the chemosensitivity of the wt, non-Pgp-expressing cell lines.

Correlation of Chemosensitization Effects with GlcCer Levels. Baseline and treatment-induced Cer and GlcCer levels in wt and multidrug-resistant cells were analyzed by TLC. GlcCer levels were approximately equal in the wt MDA435/LCC6 and the MDR1 gene-transfected MDA435/LCC6^{MDR1} cells (1.52 \pm 0.21% and 1.54 \pm 0.09% total [³H]lipid, respectively), which is consistent with lack of GCS overexpression in these multidrug-resistant cells. By contrast, GlcCer was elevated 2-fold in the drug-selected MCF7/AdrR cells compared with wt cells (Table 3). Elevated GlcCer in this cell line is consistent with previous reports (20, 21) and

Table 2 Effect of Pgp blockade on the degree of PDMP-induced chemosensitization in MCF7/AdrR and MDA435/LCC6^{MDR1} cells

| | Fold sensitization ^{a,b} | |
|--|-----------------------------------|-------------|
| | Taxol | Vincristine |
| MCF7/AdrR ± 5 μM PDMP | 6.48 | 6.07 |
| MCF7/AdrR + 1 μg/ml valsopodar ± 5 μM PDMP | 2.00 | 1.97 |
| MDA435/LCC6 ^{MDR1} ± 5 μM PDMP | 13.57 | 37.74 |
| MDA435/LCC6 ^{MDR1} + 1 μg/ml valsopodar ± 5 μM PDMP | 1.11 | 1.16 |

^a Cells were incubated with increasing drug concentrations ± 5 μM PDMP and valsopodar over 72 h, and viability was analyzed by the MTT assay.

^b Fold sensitization was calculated as: (Drug IC₅₀ ± PDMP/Drug IC₅₀ + 1 μg/ml valsopodar ± PDMP).

confirms that GCS is overexpressed in this multidrug-resistant cell line. Treatment of the MCF7/AdrR cells with 5 μM PDMP reduced GlcCer to levels comparable with the wt counterpart, indicating that PDMP indeed inhibits GlcCer formation. This decrease in GlcCer was correlated with a 1.7-fold increase in proapoptotic Cer, which provides the basis for PDMP-induced chemosensitization. Treatment with PDMP did not alter GlcCer levels in the wt MCF7 cells.

Treatment with 1 μg/ml valsopodar increased Cer production by approximately 4-fold and GlcCer production by approximately 1.5-fold in the MCF7/AdrR cells. This was expected, based on previous demonstration that valsopodar does elevate intracellular Cer (22). Because these cells overexpress the GCS enzyme, it would be expected that much of the Cer formed in response to valsopodar would be converted to GlcCer. Combined treatment with valsopodar + PDMP returned GlcCer back to wt levels but increased Cer an additional 1.5-fold. This supports the finding that PDMP-induced chemosensitization was still observed in the presence of Pgp blockade, although to a lesser degree.

Intracellular Localization of Pgp. Pgp expression in cells was shown using a two-step staining procedure with an anti-Pgp primary antibody and an IgG FITC secondary antibody (Fig. 5). Low-level background staining of the primary antibody was observed in the wt cells, as was low-level nonspecific staining with the FITC secondary antibody controls. However, the anti-Pgp staining in the multidrug-resistant cells was increased, and aggregates of high-intensity fluorescence were observed, which is indicative of intracellularly distributed Pgp.

Discussion

The sphingolipid Cer has become the subject of considerable interest as an intracellular signaling molecule involved in mediating apoptosis. In addition to its role as a mediator of cell death, recent studies have demonstrated that alterations in Cer metabolism whereby proapoptotic Cer is converted to the noncytotoxic GlcCer metabolite contribute to the MDR phenomenon (14, 21, 23). Elevated levels of GlcCer have been found in a number of multidrug-resistant human cancer cell lines (21), and overexpression of the GCS enzyme has been shown to impart doxorubicin resistance in MCF7 breast cancer cells *in vitro* (24). Analysis of human tumor specimens revealed elevated GlcCer levels in patients who failed con-

ventional chemotherapy, but GlcCer levels were low in those who responded to treatment (14). These findings demonstrate the potential clinical utility of GlcCer as a marker for MDR and suggest that the ability to modulate Cer metabolism should provide a new avenue by which drug sensitivity can be increased in multidrug-resistant cells.

The small molecule PDMP is a competitive inhibitor of the GCS enzyme that blocks the conversion of Cer to GlcCer at doses that are nontoxic to cells. It has previously been demonstrated that PDMP sensitizes murine neuroblastoma cells to Taxol and vincristine (17). In this work, we extended the study of PDMP-induced chemosensitization to two human multidrug-resistant cell lines. We compared the chemosensitization effects of PDMP-altered Cer metabolism in multidrug-resistant MCF7/AdrR and MDA435/LCC6^{MDR1} cell lines, which were developed for resistance by drug selection and *MDR1* gene transfection, respectively. The rationale for choosing these cell lines was based on the fact that although both cell lines overexpress Pgp, the transfected MDA435/LCC6^{MDR1} cell line does not exhibit elevated GlcCer, whereas GlcCer levels in the MCF7/AdrR cell line are increased. Consequently, these differences allowed for the differentiation of sensitization effects associated with Cer metabolism *versus* Pgp transport.

We first identified that a nontoxic dose of PDMP induced a significant chemosensitization effect for both multidrug-resistant breast cancer cell lines in response to treatment with Taxol and vincristine, but not in response to treatment with doxorubicin or cisplatin. Because Taxol and vincristine have both been demonstrated to induce Cer formation in cancer cells (10, 25), it might initially seem that the PDMP effect is simply due to inhibition of the metabolism of proapoptotic Cer induced by the cytotoxic drugs to nonapoptotic GlcCer. However, if this were the case, we would only expect to see PDMP-induced sensitization in the GlcCer-overexpressing MCF7/AdrR cells. Similar to wt MDA435/LCC6 cells, which are not affected by PDMP, the MDA435/LCC6^{MDR1} cells do not display elevated GlcCer, yet PDMP still induced a chemosensitization response. The commonality between the MCF7/AdrR and MDA435/LCC6^{MDR1} cell lines, however, is elevated Pgp expression, and this led us to investigate a possible role for Pgp in Cer signaling. This was further supported by the fact that PDMP had no effect on the chemosensitivity of non-Pgp-expressing wt MCF7 or MDA435/LCC6 cells. The fact that the PDMP-induced effect is specific for tubulin-binding drugs is interesting in itself and suggests that intracellular transport mechanisms/microtubule assembly may be involved in the processes of Cer signaling and/or metabolism. Additional experiments will be necessary to elucidate these relationships.

To rule out the possibility that PDMP induces chemosensitivity via nonspecific alterations in cellular drug uptake, we measured the accumulation of radiolabeled Taxol in the presence and absence of PDMP. PDMP had no effect on the cellular uptake of Taxol. Consequently, chemosensitization in the multidrug-resistant cell lines was not attributable to increased intracellular anticancer drug concentrations.

To assess whether the combination of PDMP plus exogenous Cer would further enhance the PDMP effect, the cy-

Table 3 Incorporation of [³H]palmitic acid into Cer and GlcCer of treatment and control MCF7 cells

| Lipid | Percentage ³ H-lipid ^a | | | | | |
|--------|--|-------------------------------------|-----------------|----------------------------|-------------------------------------|--|
| | MCF7 ^{wt} | MCF7 ^{wt} + 5 μ M PDMP | MCF7/AdrR | MCF7/AdrR + 5 μ M PDMP | MCF7/AdrR + 1 μ g/ml valsopodar | MCF7/AdrR + 1 μ g/ml valsopodar + 5 μ M PDMP |
| Cer | 0.54 \pm 0.09 | 0.25 \pm 0.01 | 0.21 \pm 0.03 | 0.35 \pm 0.05 | 0.86 \pm 0.01 | 1.33 \pm 0.08 |
| GlcCer | 1.19 \pm 0.07 | 1.16 \pm 0.18 | 2.19 \pm 0.15 | 1.32 \pm 0.12 | 3.02 \pm 0.35 | 1.16 \pm 0.09 |

^a Cells were incubated with 1.0 μ Ci/ml [³H]palmitic acid in complete medium for 24 h, washed, and harvested by scraping. Total lipids were extracted using equal volumes of methanol-2% acetic acid/chloroform/water. The radioactivity of total extracted lipids was measured by scintillation counting. Lipids were spotted onto TLC plates and developed in chloroform/acetic acid (90:10) for Cer or chloroform/methanol/ammonium hydroxide (70:20:4) for GlcCer. Lipid spots corresponding to cochromatographed standards were scraped into scintillation vials for quantitation by liquid scintillation counting. The lipid amounts presented are expressed as a percentage of the total tritiated lipid extracted from the cells.

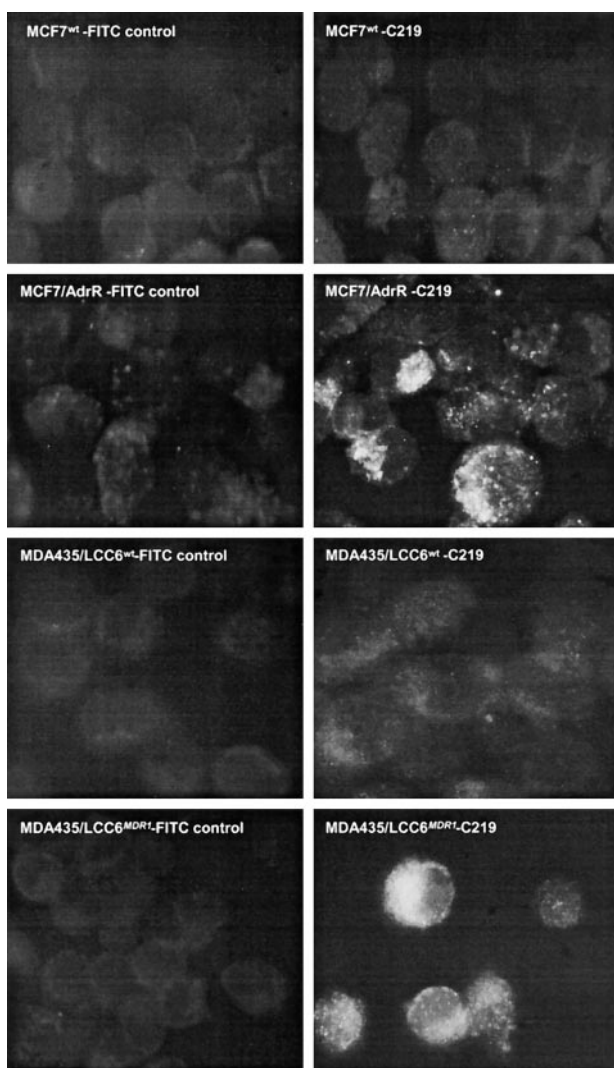


Fig. 5. Fluorescence microscope images of MCF7 (wt and Adriamycin-resistant) and MDA435/LCC6 (wt and *MDR1*-transfected) cells after two-step staining with anti-Pgp primary and IgG FITC secondary antibodies.

toxicity assays were repeated in the presence of 2 μ M C₆-Cer. Because this is a cell-permeable Cer, it readily and rapidly distributes to various membranes within the cell. The observation that combination treatment with Taxol or vincristine plus PDMP and Cer did not further enhance chemosen-

sitivity may be explained by the fact that short-chain, cell-permeable Cers can readily distribute to numerous intracellular locations due to their relatively hydrophilic nature. It has been reported that short-chain Cers can be glycosylated (26). However, the GCS enzyme is specifically localized to the cytoplasmic face of the Golgi (27, 28). Therefore, a significant proportion of exogenously added Cer would be expected to localize in GCS-poor regions. Consequently, addition of PDMP may not significantly impact overall Cer-induced apoptosis because the pools of Cer being modulated by PDMP are small relative to the overall amount of Cer that is distributed throughout the cell. This may also explain why PDMP did not shift the C₆-Cer IC₅₀ values for the multidrug-resistant cell lines.

The specific involvement of Pgp in Cer-mediated apoptosis was examined by evaluating the chemosensitization effect of PDMP in the presence of 1 μ g/ml valsopodar, a potent Pgp inhibitor. Interestingly, under conditions of Pgp blockade, we no longer observed a PDMP effect in the MDA435/LCC6^{MDR1} cells, and the PDMP effect was decreased 3-fold in the MCF7/AdrR cells. This suggests that Pgp function is directly involved in regulating Cer metabolism to GlcCer.

Fig. 6 provides an illustration of the proposed relationship between Pgp, GlcCer, and PDMP that is consistent with our observations. The presence of intracellularly distributed Pgp has been demonstrated by a number of groups (29–33) and was confirmed in our two multidrug-resistant cell lines by fluorescence microscopy studies. Functional Pgp activity has been demonstrated previously in the Golgi of multidrug-resistant cells (30, 31), including the same doxorubicin-resistant MCF7 cell line used in our studies. It has also been shown that Pgp can mediate the translocation of GlcCer from the cytoplasmic face of the Golgi to the lumen, where further processing to higher glycosphingolipid species is known to occur (34–36). The wt (drug-sensitive) cells do not overexpress Pgp or the GCS enzyme. Exposure of these cells to anticancer agents results in increased intracellular Cer and subsequent apoptosis. PDMP has negligible chemosensitizing effects in these cells because relatively little Cer is metabolized to GlcCer, and an equilibrium exists between the Cer and GlcCer that is generated. Multidrug-resistant cells that overexpress Pgp are capable of translocating GlcCer across the Golgi membrane. In these cells, the loss of GlcCer from the cytoplasmic face of the Golgi provides a driving force for further Cer-to-GlcCer conversion. Continuous removal of GlcCer by Pgp removes the negative feedback

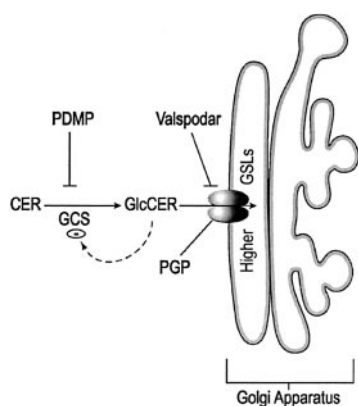


Fig. 6. A model to explain the influence of Pgp on Cer metabolism in the context of chemosensitization. The wt (drug-sensitive) cells that do not overexpress Pgp or GCS do not display PDMP-induced chemosensitization because very little Cer is converted to GlcCer. Multidrug-resistant cells that overexpress Pgp translocate newly formed GlcCer across the Golgi membrane. This provides a driving force for further Cer-to-GlcCer conversion and reduces negative feedback on the GCS enzyme. Inhibition of Pgp promotes GlcCer accumulation in the cytoplasm and reduces GlcCer formation via negative feedback, which in turn reduces PDMP-induced chemosensitization.

control on the GCS enzyme and promotes further Cer metabolism. This could explain why a PDMP-induced chemosensitization effect was observed in the MDA435/LCC6^{MDR1} cells, although they do not display elevated GlcCer. The loss of sensitization in the presence of valsopodar further supports this theory.

In the case of the MCF7/AdrR cells that additionally overexpress the GCS enzyme, Cer generated by anticancer drug exposure is rapidly converted to GlcCer before apoptosis pathways can be activated. We see chemosensitization in these cells because PDMP is inhibiting the conversion of Cer to GlcCer. When PDMP is coincubated with valsopodar, the Pgp-mediated translocation of GlcCer across the Golgi should be blocked. This causes a relative accumulation of GlcCer on the cytoplasmic face of the Golgi, which in turn reduces the Cer-to-GlcCer conversion via negative feedback. In this case, PDMP is still acting as an inhibitor of the GCS enzyme, but the PDMP effect is diminished due to GlcCer product feedback inhibition.

The research presented here provides an increased understanding of the relationship between Cer, Cer metabolism, and Pgp in the context of MDR. As more information is gained about Cer metabolism and the intracellular trafficking of Cer and glycosphingolipids, we will be better able to design therapeutic strategies that combine Pgp and Cer-based approaches to improve the treatment of multidrug-resistant tumors. Research in our laboratory is currently under way to elucidate intracellular distribution patterns of Cer and its metabolites in multidrug-resistant tumor cells under various treatment conditions. This will provide further insight into the relationships between these two resistance mechanisms.

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Molecular Cancer Therapeutics

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Jennifer A. Shabbits and Lawrence D. Mayer

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