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Agents that Reverse Multidrug Resistance, Tamoxifen, Verapamil, and Cyclosporin A, Block Glycosphingolipid Metabolism by Inhibiting Ceramide Glycosylation in Human Cancer Cells*

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We have previously shown that multidrug-resistant cancer cells display elevated levels of glucosylceramide (Lavie, Y., Cao, H., Bursten, S. L., Giuliano, A. E., and Cabot, M. C. (1996) *J. Biol. Chem.* 271, 19530–19536). In this study we used the multidrug-resistant human breast cancer cell line MCF-7-Adriamycin-resistant (AdrR), which exhibits marked accumulation of glucosylceramide compared with the parental MCF-7 wild type (drug-sensitive) cell line, to define the relationship between glycolipids and multidrug resistance (MDR). Herein it is shown that clinically relevant concentrations of tamoxifen, verapamil, and cyclosporin A, all circumventors of MDR, markedly decrease glucosylceramide levels in MCF-7-AdrR cells (IC_{50} values, 1.0, 0.8, and 2.3 μM, respectively). In intact cells, tamoxifen inhibited glycosphingolipid synthesis at the step of ceramide glycosylation. In cell-free assays for glucosylceramide synthase, tamoxifen (1:10 molar ratio with ceramide) inhibited glucosylceramide formation by nearly 50%. In cell cultures, inhibition of glucosylceramide synthesis by tamoxifen is correlated with its ability to sensitize MCF-7-AdrR cells to Adriamycin toxicity. Moreover, treatment of cells with 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, an inhibitor of glucosylceramide synthesis, likewise sensitized MCF-7-AdrR cells to Adriamycin. It is concluded that high cellular levels of glucosylceramide are correlated with MDR, and that glycolipids are a target for the action of MDR-reversing agents such as tamoxifen. The data entertain the notion that drug resistance phenomena are aligned with cell capacity to metabolize ceramide.

Multidrug resistance (MDR),[1] believed to be the basis for tumor cell survival, exhibits intrinsic resistance to multiple drugs on primary exposure to a single drug (1). Of the various biological mechanisms associated with MDR, overexpression of P-gp, a plasma membrane glycoprotein proposed to act as a drug efflux pump, has been most studied (2, 3). We have recently observed that multidrug-resistant cancer cells characteristically display elevated levels of a glycolipid identified as glucosylceramide (4).

Some associations have been drawn regarding the role of lipids in MDR. Reports show that P-gp ATPase activity is dependent on the lipid environment (5), and lipids interact with P-gp substrates (6). Differences in glycerolipid and sphingomyelin compositions of multidrug-resistant and drug-sensitive cells have been reported (7–11), and ganglioside composition of multidrug-resistant and drug-sensitive cells has been examined. Whereas diversity in ganglioside composition was revealed, no definitive correlation with drug resistance was demonstrated (7, 12). Our recent work (4) revealed a correlation between the cellular content of glycosphingolipids and MDR. This indicates a potential role for glycosphingolipids in MDR.

Circumvention of MDR carries major clinical importance. A battery of diverse agents has been shown to inhibit MDR, rendering cells sensitive to chemotherapy (13). These MDR-reversing agents include the calcium channel blockers verapamil and SR33557 (13, 14), antiarrhythmic agents such as quinidine (15), the immunosuppressant cyclosporin A (15, 16), and the antiestrogen anticancer drug tamoxifen (17, 18). The mechanism by which these drugs influence MDR is thought to be via direct binding to P-gp (19, 20), but MDR reversers may also modify cellular components that regulate P-gp. For example, selective expression of protein kinase C isozymes has been correlated with MDR (21), and studies have suggested that P-gp activity may be regulated by protein kinase C (22). An association between inhibition of protein kinase C activity by safingol, a sphingoid base, and reversal of cellular doxorubicin resistance has been demonstrated (23). Other works have revealed a link between sphingomyelinase activity and MDR (24).

Sphingolipids and glycosphingolipids have obligatory functions in cell proliferation (25–27), neuronal growth (28, 29), cell transformation (26) and tumor progression (30). Glucosylceramide is the precursor of all glucosphingolipids. The enzyme that catalyzes the synthesis of glucosylceramide, glucosylceramide synthase, is central in glycosphingolipid metabolism. Studies on inhibition of glucosylceramide synthase by PPMP, a synthetic inhibitor that acts as a ceramide analog, have revealed a diversity of physiological processes affected by depleting cells of glucosylceramide and higher glycosphingolipids (27, 28, 31, 32). Deficiencies in β-glucosidase, the degrading enzyme, are the cause of Gaucher's disease (33).

In this work we show that multidrug-resistant cells, as opposed to drug-sensitive cells, glycosylate ceramide with enhanced capacity. Of particular biological relevance are the di-

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[1] The abbreviations used are: MDR, multidrug resistance; P-gp, P-glycoprotein; PPMP, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; FBS, fetal bovine serum; AdrR, Adriamycin-resistant.

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verse effects of ceramide and glycosphingolipids on cell homeostasis. Whereas ceramide is suggested to serve as a second messenger for programmed cell death (34), glycosphingolipids are demonstrated to have a role in cell growth (27) and survival (29) and in escape from onset of apoptosis (35). Data from several studies have linked inhibition of glycosphingolipid synthesis to an array of cellular dysfunctions (29, 31, 36–38), thereby highlighting a role for glycolipids in cell health and stressing the importance of enzymes that regulate glycolipid metabolism. We show that accumulation of glucosylceramide in MCF-7-Adriamycin-resistant (AdrR) breast cancer cells is potently blocked by a myriad of chemically unrelated drugs that are known to circumvent MDR. An association between reduction in cellular glucosylceramide content and sensitization of MCF-7-AdrR cells to Adriamycin toxicity is demonstrated. In summation, these results reveal a new action of tamoxifen, pinpoint glucosylceramide synthase as a target for MDR-reversing agents, and define a potential role for glycosphingolipids and their metabolites in MDR.

EXPERIMENTAL PROCEDURES

Materials—Sphingosine, sphingomyelin, and ceramides were purchased from Avanti Polar Lipids (Alabaster, AL). C_6-ceramide was purchased from LC Laboratories (Woburn, MA). Glucosylceramide (Gaucher's spleen), and DL-*erythro*-PPMP were from Matreya, Inc. (Pleasant Gap, PA). DL-*Threo*-PPMP was from Biomol (Plymouth Meeting, PA). Triphenylbutene was kindly provided by Prof. Michael Jarman (Center for Cancer Therapeutics, The Institute of Cancer Research, Sutton, Surrey, United Kingdom), and SDZ PSC 833 was from Sandoz Pharmaceutical Corp. EN^{3}HANCE, L-[^{3}H]serine (21.7 Ci/mmol), [9,10-^{3}H]palmitic acid (56.5 Ci/mmol), and D-[6-^{3}H(N)]galactose (29.5 Ci/mmol) were purchased from DuPont NEN. UDP-[6-^{3}H]glucose (15 Ci/mmol) was from American Radiolabeled Chemicals, Inc. Liquid scintillation mixture (EcoLume) was from ICN Biomedical. Silica Gel G TLC plates were from Analtech (Newark, DE). Solvents were from Fisher Scientific. RPMI 1640 medium (Cellgro™) was purchased from Mediatech (Herndon, VA). FBS was from HyClone (Logan, UT), and culture ware was from Corning-Costar. All other biochemicals were from Sigma.

Cell Culture—MCF-7 wild-type and MCF-7-AdrR cells were kindly provided by Dr. Kenneth H. Cowan and Dr. Merrill E. Goldsmith (National Cancer Institute). Cells were maintained in RPMI 1640 medium containing 10% (v/v) FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 584 mg/liter L-glutamine. Cells were cultured in a humidified, 6.5% CO_2 atmosphere tissue culture incubator and subcultured once a week, using a 0.05% trypsin, 0.53 mM EDTA solution.

Cell Radiolabeling and Analysis of Lipids—MCF-7 cells, grown in medium containing 10% FBS, were switched to medium containing 5% FBS. Cell lipids were radiolabeled by adding [^{3}H]serine (2.0 μCi/ml), [^{3}H]palmitic acid (1.0 μCi/ml), or [^{3}H]galactose (1.0 μCi/ml) to the culture medium for the indicated times. After labeling, cell monolayers were rinsed twice with phosphate-buffered saline (pH 7.4), and 2 ml of ice-cold methanol containing 2% acetic acid was added. The cells were scraped free and transferred to glass test tubes (13 × 100 mm), and lipids were extracted (39). After brief centrifugation, the resulting organic lower phase was withdrawn and evaporated under a stream of nitrogen. Lipids were resuspended in 100 μl of chloroform/methanol (1:1, v/v), and aliquots were applied to TLC plates. When using [^{3}H]galactose, radiolabeled cells were washed twice with phosphate-buffered saline and transferred to glass tubes with methanol (2 ml), and glucosylceramides and gangliosides (2.5 μg of each) were added to aid recovery. Lipids were extracted by the addition of water (2 ml) and 2 ml of chloroform. The lower phase was withdrawn, and the upper phase was washed two times with consecutive additions of chloroform. The pooled organic lower phase was treated as above. Lipid analysis was carried out by TLC using solvent system I (chloroform/methanol/ammonium hydroxide; 40:10:1, v/v/v), for glucosylceramide separation, solvent system II (chloroform/methanol/water; 60:40:8, v/v/v), for glycosphingolipid separation, or solvent system III (chloroform/methanol/acetic acid/water; 50:30:7:3, v/v/v/v) for sphingomyelin separation. For determination of ceramides, an aliquot of the chloroform-soluble lipid was base-hydrolyzed in 0.1 N KOH in methanol for 1 h at 37 °C. The lipids were re-extracted, and ceramide was separated by TLC using solvent system IV (hexane/diethyl ether/formic acid; 60:40:1, v/v/v).

Radiochromatograms were sprayed with EN^{3}HANCE and exposed for 3–7 days for autoradiography. TLC areas, aligned with bands on the autoradiographs or with iodine-stained commercial lipid standards, were scraped from the plate. Water (0.5 ml) was added to the plate scrapings, followed by 4.5 ml of EcoLume counting fluid, and the samples were quantitated by liquid scintillation spectrometry.

Lipid Mass Analysis—Cell lipids were analyzed by TLC separation and charring of the chromatogram. Briefly, total cellular lipids were extracted by the method of Bligh and Dyer (39), and equal aliquots (by weight) from each sample were spotted on TLC plates. Plates were developed in the desired solvent system, air dried for 1 h, and sprayed using a 35% (v/v) solution of sulfuric acid in water. The lipids were charred by heating in an oven at 180 °C for 30 min.

Glucosylceramide Synthase Assay—The assay was performed according to the method of Shukla and Radin (40) with minor modifications. Components of the lipoidal substrate were freed of solvent under a stream of nitrogen (in borosilicate glass tubes) and sonicated as described, omitting the overnight lyophilization step. The enzymatic assay was performed with 100 μM UDP-glucose (230,000 cpm/tube), 2 mM β-NAD, 1 mM dithiothreitol, 2 mM EDTA, 10 mM $MgCl_2$, 0.1 M Tris buffer (pH 7.4), 0.2 mg of MCF-7-AdrR cell homogenate protein/tube, and liposomal substrate (containing 200 nmol of ceramide), in a total volume of 0.2 ml. Drugs (20 nmol) were added to components of the lipoidal substrate before solvent evaporation and sonication. The reaction was incubated at 37 °C for 90 min, lipids were extracted, and radiolabeled glucosylceramide formed was analyzed by TLC (solvent system I) and liquid scintillation spectrometry.

Cytotoxicity Assay—Cells were seeded in 96-well plates (2000 cells/well), in 0.1 ml of RPMI 1640 medium containing 5% FBS, and incubated at 37 °C for 24 h before drug addition. A drug was added in medium (0.1 ml), and the cells were incubated at 37 °C for an additional 4 days. The cytotoxic activity of a drug was determined using the Promega CellTiter 96™ AQueous cell proliferation assay kit. Each experimental point was performed in six replicates. Solution (20 μl) was aliquoted to each well, and the cells were incubated for 2–3 h, or until an optical density of 0.9–1.0 was obtained as a highest reading. Absorbance at 490 nm was recorded using an enzyme-linked immunosorbent assay plate reader (Molecular Devices, San Diego, CA).

Vehicles for Reagents—Tamoxifen and triphenylbutene were prepared as 20 mM stock solutions in acetone. Cyclosporin A was prepared as a 1.0 mM stock solution in ethanol. Verapamil was prepared as a 10 mM stock solution in water. Adriamycin was prepared as a 1.0 mM stock solution in water, and PPMP was prepared as a 2.0 mM stock solution in ethanol/water (1:1, v/v). All stock solutions were stored at −20 °C until use. Media containing drugs were prepared just prior to use. Vehicle was present in control (minus drug) cultures.

RESULTS

Tamoxifen Lowers Glycosphingolipid Levels in MCF-7-AdrR Cells—In a previous article (4) we showed that glucosylceramide accumulates in multidrug-resistant cancer cells. This work has been extended to assess the relationship of glycolipids to MDR. The effect of tamoxifen, an antiestrogen with MDR-reversing properties (17, 18), on glucosylceramide metabolism was examined in MCF-7-AdrR cells. Initial experiments revealed that inclusion of tamoxifen in the culture medium largely depressed cellular glucosylceramide levels, as determined by mass analysis (TLC and charring). Verification of this effect is shown in cells labeled with [^{3}H]galactose (Fig. 1). Cells were preincubated, without or with tamoxifen, and the extent of glycosphingolipid formation was surveyed by TLC autoradiography. As shown (Fig. 1), tamoxifen treatment caused a reduction in labeling of glycosphingolipids, subduing glucosylceramide, lactosylceramide, and ganglioside levels by 69, 74, and 33%, respectively (data based on TLC analysis of tritium).

MDR-reversing Drugs Inhibit Cellular Glucosylceramide Formation—In addition to tamoxifen, we evaluated two other MDR-reversing agents, verapamil and cyclosporin A, and assessed a structural analog of tamoxifen, triphenylbutene, which is devoid of the basic amino side chain. [^{3}H]Glucosylceramide formation was markedly inhibited by all MDR-reversing drugs but not by the tamoxifen analog (Fig. 2). Based on cpm of tritium, the order of potency for inhibition of glucosylceramide formation in intact cells was tamoxifen > cyclosporin

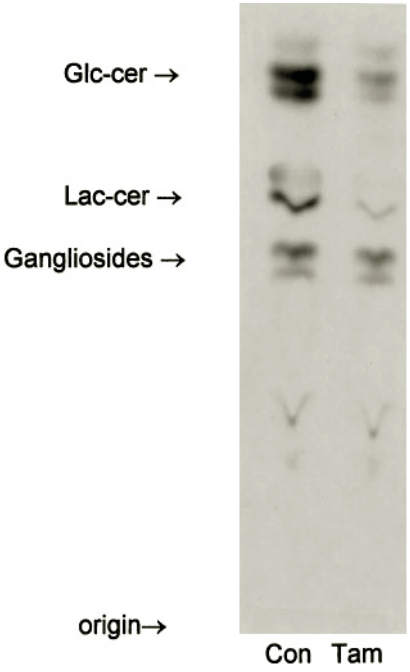

FIG. 1. **Influence of tamoxifen on glucosylceramide and ganglioside levels in multidrug-resistant cells.** MCF-7-AdrR cells, preincubated without or with tamoxifen (5.0 μM) for 30 min, were then given [^{3}H]galactose (1.0 μCi/ml) for 24 h. Glycosphingolipids were analyzed by TLC autoradiography as described under "Experimental Procedures," using equal aliquots of extracted lipids. *Glc-cer,* glucosylceramide; *Lac-cer*, lactosylceramide; *Con*, control; *Tam*, tamoxifen.

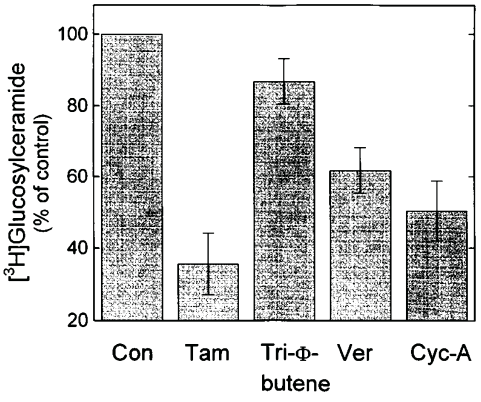

FIG. 2. **MDR-reversing drugs inhibit glucosylceramide formation in MCF-7-AdrR cells.** Cells, preincubated for 30 min with 5.0 μM of each of the indicated drugs, were then labeled with [^{3}H]serine (2.0 μCi/ml) in medium containing the indicated drugs for 24 h. Quantitation of [^{3}H]glucosylceramide levels in cells following treatment was conducted as described in the legend to Fig. 1. Each value represents the mean ± S.E. (*bars*) of determinations from three separate experiments. *Con*, control; *Tam*, tamoxifen; *Tri-ϕ-butene,* triphenylbutene; *Ver*, verapamil; *Cyc-A*, cyclosporin A.

A ≥ verapamil (65, 49, and 38% inhibition, respectively). Triphenylbutene had only a minor effect (13% inhibition), indicating that the basic amino side chain is essential. Inhibition of glucosylceramide formation by agents that circumvent MDR is not restricted to MCF-7-AdrR cells, as similar results have been obtained in KB-V-1 (vinblastine-resistant) epidermoid carcinoma cells (data not shown).

The concentration dependence of drugs for inhibition of glucosylceramide formation in MCF-7-AdrR cells is shown in Fig. 3. During a 24-h incubation, tamoxifen, verapamil, and cyclosporin A induced half-maximal inhibition (IC_{50}) of cellular [^{3}H]glucosylceramide formation at 1.0, 0.8, and 2.3 μM, respectively. Tamoxifen was the most efficient inhibitor of glucosylceramide formation, with the highest maximal effect and a low IC_{50} value. The effective concentrations used here are within the range of clinical use, since treatment with these drugs typically results in 0.5–5 μM drug concentrations in sera of patients (15, 41, 42). The time frame for tamoxifen-induced inhibition of glucosylceramide formation is shown in Fig. 4. Uptake and incorporation of the radiolabeled precursor, [^{3}H]palmitic acid, was similar in control and tamoxifen-treated cells (Fig. 4*A*), showing that tamoxifen does not interfere with transport or overall use of palmitic acid. Fig. 4*B* shows that tamoxifen retards glucosylceramide synthesis as early as 15 min (572 ± 102 cpm in tamoxifen-treated cells *versus* 1237 ± 53 cpm in control cells). In contrast, sphingomyelin formation was not altered by tamoxifen during the 4-h incubation period (Fig. 4*C*).

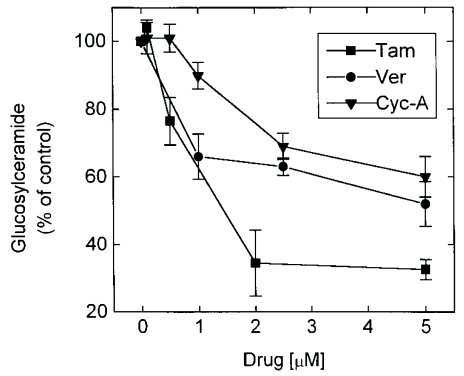

FIG. 3. **Dose response of tamoxifen, verapamil, and cyclosporin A for inhibition of glucosylceramide formation in MCF-7-AdrR cells.** Cells were labeled with [^{3}H]palmitic acid (1.0 μCi/ml) for 24 h in the presence of drugs at the indicated concentrations. [^{3}H]Glucosylceramide was quantitated as described in the legend to Fig. 1*A*. Data are from one of three experiments that gave similar values. *Tam,* tamoxifen; *Ver,* verapamil; *Cyc-A,* cyclosporin A. *Bars,* ± S.E.

Mechanism of Tamoxifen Action—Pulse-chase experiments using [^{3}H]galactose-labeled MCF-7-AdrR cells revealed that the degradation rates of [^{3}H]glucosylceramide were similar in the presence and absence of tamoxifen. The data suggest that tamoxifen-governed changes in glycosphingolipid levels result from inhibition of synthesis. Tamoxifen inhibition of glucosylceramide synthesis may result from influences on ceramide generation; however, experiments revealed that levels of radiolabeled ceramide paralleled one another in tamoxifen-treated and tamoxifen-naive cultures during the 4-h time frame (data not shown), similar to the experiment of Fig. 4. This raised the possibility that tamoxifen action is targeted to glycosylation of ceramide. A short chain analog of ceramide, C_6-ceramide, which is readily transported into cultured cells (43), was used to evaluate the influence of tamoxifen on ceramide glycosylation. Fig. 5 shows the spectrum, by autoradiograph, of cellular [^{3}H]galactose-labeled glycosphingolipids formed in the presence (*middle lane*) or absence (*left lane*) of C_6-ceramide. The formation of C_6-glucosylceramide, migrating just below the natural glucosylceramide doublet, was clearly visible in cells incubated with C_6-ceramide (Fig. 5, *middle lane*). In the presence of tamoxifen (Fig. 5, *right lane*), conversion of C_6-ceramide to C_6-glucosylceramide was inhibited by 54% (based on tritium incorporation). These results imply that tamoxifen inhibits ceramide glycosylation, a reaction catalyzed by glucosylceramide synthase (25). Cell-free assays of glucosylceramide synthase demonstrated that tamoxifen, at a 1:10 molar ratio with ceramide, inhibited glucosylceramide formation by 45% (1,467 ± 104 *versus* 809 ± 114 pmol [^{3}H]glucosylceramide synthesized/mg protein; n = 3). The tamoxifen analog triphenylbutene was devoid of inhibitory activity. The *threo* enantiomer of

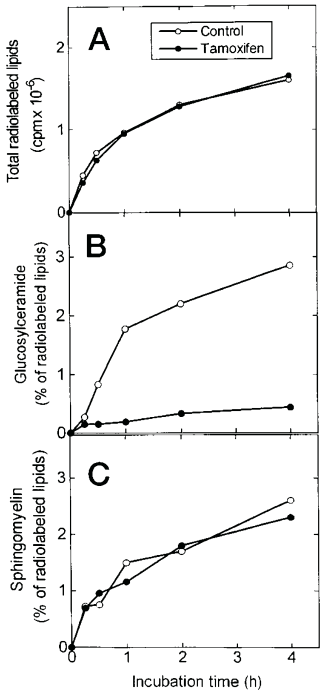

FIG. 4. **Temporal pattern for the influence of tamoxifen on total lipid, glucosylceramide, and sphingomyelin formation in MCF-7-AdrR cells.** Cells, preincubated in the absence or presence of tamoxifen (20 μM) for 30 min, were labeled by addition of [^{3}H]palmitic acid (1.0 μCi/ml) in RPMI 1640 medium containing 5% FBS for the times shown. At each time point, cells were washed with phosphate-buffered saline, and lipids were extracted. Aliquots of the labeling medium were counted directly by liquid scintillation spectrometry to determine [^{3}H]palmitic acid uptake and incorporation into total cell lipids. Glucosylceramide and sphingomyelin were evaluated by TLC of total cell lipids using solvent systems I and II, respectively, followed by quantitation of radiolabel in the relevant regions of the TLC plate. Data are the means of duplicate determinations from two separate experiments.

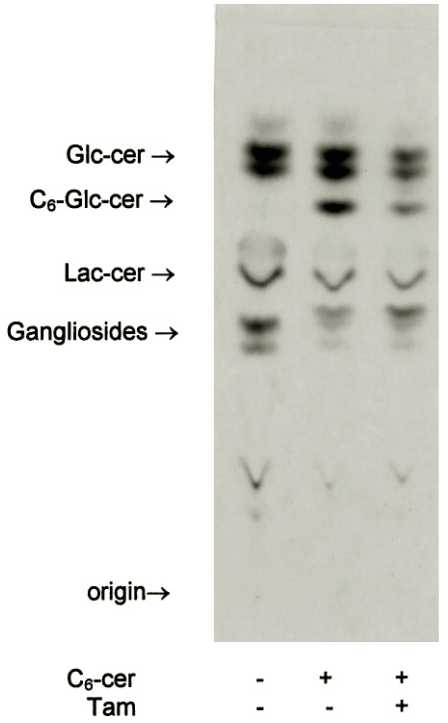

FIG. 5. **Effect of tamoxifen on ceramide glycosylation in intact MCF-7-AdrR cells.** Cells, in 100 × 20-mm culture dishes, were preincubated without or with tamoxifen (5.0 μM) for 30 min, followed by incubation without or with C_6-ceramide (5.0 μM), prepared at a 1:1 molar ratio with bovine serum albumin, for an additional 30 min. Thereafter, the cells were labeled with [^{3}H]galactose (1.0 μCi/ml) for 24 h. [^{3}H]Galactose uptake was found to be similar under all experimental conditions. The total lipid extract was applied to TLC plates for separation, and [^{3}H]glycosphingolipids were analyzed as described in the legend to Fig. 1. *Glc-cer,* glucosylceramide; *Lac-cer,* lactosylceramide; *C-cer,* C_6-ceramide; *Tam,* tamoxifen.

PPMP, known to be the active form (44), inhibited glucosylceramide synthase activity by 85% in the cell-free reaction, whereas the *erythro* isomer was ineffective.

Correlation between Recovery of Adriamycin Toxicity and Modified Glucosylceramide Metabolism—The ability of tamoxifen to reverse MDR was evaluated by exposing MCF-7-AdrR cells to increasing concentrations of Adriamycin in the absence or presence of a sublethal concentration of tamoxifen (or analog). The effect of tamoxifen and triphenylbutene on Adriamycin toxicity in MCF-7-AdrR cells is shown in Fig. 6*A*. In the presence of tamoxifen, the dose-response curve for Adriamycin toxicity was shifted to lower concentrations. The maximal cytotoxic effect of Adriamycin alone was achieved at 5.0 μM (28% cell death), whereas in the presence of tamoxifen, the same concentration of Adriamycin (5.0 μM) caused 60% cell death. In contrast, triphenylbutene had no effect on Adriamycin toxicity (Fig. 6*A*). Cellular [^{3}H]glucosylceramide levels were analyzed at 48 h under the same conditions as the experiment in Fig. 6*A*. As shown in Fig. 6*B*, Adriamycin alone (2.5 μM) caused minor diminution (17%) of glucosylceramide; this is similar with the minor effect of Adriamycin (2.5 μM) on MCF-7-AdrR cell survival (Fig. 6*A*). Tamoxifen at 5.0 μM markedly retarded glucosylceramide synthesis (Figs 1–5) but alone was not toxic (Fig. 6*A*). However, addition of tamoxifen to the Adriamycin regimen caused 72% inhibition of glucosylceramide production (Fig. 6*B*), and together these agents were cytotoxic (Fig. 6*A*). Triphenylbutene, when mixed with Adriamycin, did not enhance cell killing, nor was this combination effective in inhibiting glucosylceramide production.

PPMP Sensitizes MCF-7-AdrR Cells to Adriamycin—There is currently no evidence that PPMP, a chemical inhibitor of glucosylceramide synthase, possesses MDR-reversing activity. Fig. 7*A* shows the dose response for PPMP influence on glucosylceramide synthesis in MCF-7-AdrR cells. The enzyme inhibitor PPMP shows a strikingly similar dose-response relationship with tamoxifen (Fig. 3) for inhibition of glucosylceramide synthesis in intact cells. Maximal reduction in cellular glucosylceramide levels (86% inhibition) occurred at 5.0 μM PPMP, with a calculated IC_{50} of 0.9 μM. A concurrent effect of PPMP as a chemosensitizer is revealed by the data of Fig. 7*B*, demonstrating an enhancement of Adriamycin toxicity in MCF-7-AdrR cells when used in combination. Whereas Adriamycin was largely without influence on diminishing cell survival, the addition of PPMP to the Adriamycin regimen effectively decreased cell survival.

Ceramide Metabolism—Although at 4 h after tamoxifen addition, the levels of radiolabeled ceramide were not altered, compared with control, combination treatment of MCF-7-AdrR cells with tamoxifen and Adriamycin for extended time led to an elevation in ceramide. Following the protocol of Fig. 6, a 2-day exposure of cells to tamoxifen (5.0 μM) and Adriamycin (2.5 μM) caused a 300% increase in cellular radiolabeled ceramide. Therefore, the increased cell death, elicited by tamoxifen plus Adriamycin, correlated with a depletion of glucosylceramide (Fig. 6*B*) and a concomitant increase in ceramide. Preliminary data using the cyclosporin A analog SDZ PSC 833 solely shows that it too increased the levels of ceramide in MCF-7-AdrR cells.

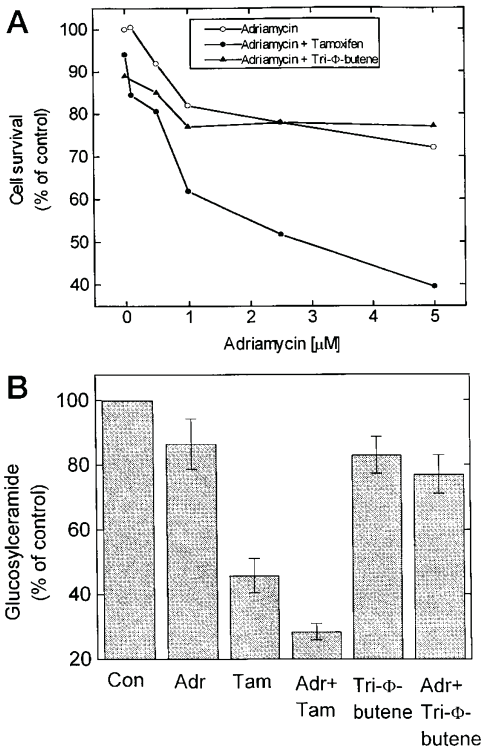

FIG. 6. **Correlation between MDR reversal and reduction in glucosylceramide levels in MCF-7-AdrR cells.** *A,* influence of tamoxifen on chemosusceptibility of multidrug-resistant cells to Adriamycin. MCF-7-AdrR cells were exposed to 0–5.0 μM Adriamycin for 72 h in the presence or absence of tamoxifen or triphenylbutene (5.0 μM). Cell survival was determined using a cell proliferation assay. Each point represents the mean of six replicate determinations. *B,* glucosylceramide levels in multidrug-resistant cells during MDR reversal. MCF-7-AdrR cells, preincubated without or with Adriamycin (2.5 μM), tamoxifen (5.0 μM), triphenylbutene (5.0 μM), or the indicated combination of drugs, were labeled with [^{3}H]serine (2.0 μCi/ml) for 48 h, similar to the experiment described in *A.* [^{3}H]Glucosylceramide was quantitated by TLC as described. Data are from one of two experiments that gave similar values. *Con,* control; *Tam,* tamoxifen; *Tri-ϕ-butene,* triphenylbutene. *Bars,* ± S.E.

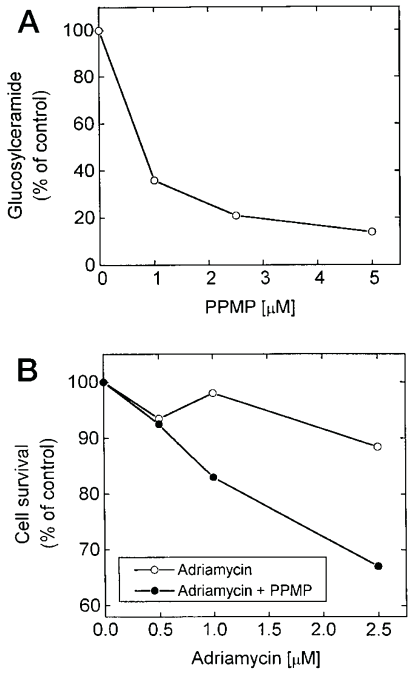

FIG. 7. **PPMP inhibits glucosylceramide synthesis and increases sensitivity to Adriamycin toxicity in multidrug-resistant cells.** *A,* effect of PPMP on cellular glucosylceramide metabolism. MCF-7-AdrR cells were labeled with [^{3}H]serine (2.0 μCi/ml) for 24 h in the presence of the indicated PPMP concentrations. [^{3}H]Glucosylceramide was quantitated as described. Data are from one of two experiments that gave similar values. PPMP had no influence on cell viability. *B,* effect of PPMP in combination with Adriamycin on MCF-7-AdrR cell survival. Cells were exposed to increasing concentrations of Adriamycin for 72 h in the presence or absence of PPMP (5.0 μM). Cell survival was determined by cell proliferation assay as described under "Experimental Procedures." Each point represents the mean of six replicate determinations.

DISCUSSION

Multidrug-resistant cells are characterized by high resistance to drug toxicity (1). The resistance is mediated at the cellular level by several mechanisms, including overexpression of proteins (*i.e.* P-gp), alteration of drug transport and metabolism, and repair of drug-induced damage (2). The present study is the first to introduce alteration of glucosylceramide metabolism with the action of MDR-reversing agents. The concept of glycosphingolipid involvement in cellular MDR was put forth in our recent study that identified glucosylceramide as the principle lipid accumulating in a number of multidrug-resistant cell types (4).

Chemosensitizers, agents that increase the sensitivity of multidrug-resistant cells to the toxic influence of previously less effective drugs, are intriguing in their mode of action. One challenge in cancer chemotherapy is to understand the molecular mechanisms by which chemosensitizers circumvent drug resistance. Tamoxifen has been reported to reverse MDR via direct binding to P-gp (18, 20). Here a new cellular target of tamoxifen, the glycosphingolipid pathway, has been identified. Tamoxifen is shown to potently inhibit production of glucosylceramide and concomitantly to suppress synthesis of some higher gangliosides in MCF-7-AdrR cells. Tamoxifen may be a general modulator of glycosphingolipid metabolism, as a similar inhibitory effect was elicited by the drug in KB-V-1, a vinblastine-resistant epidermoid carcinoma cell line (data not shown). In addition, verapamil and cyclosporin A, two well known MDR-reversing agents, also retarded glucosylceramide formation, suggesting that this mechanism of action is a common denominator in the chemosensitizing process.

To examine the mechanism of glucosylceramide depletion by tamoxifen, a cell-permeable short chain analog of ceramide (C_6-ceramide) was incubated with intact cells. Glycosylation of C_6-ceramide to C_6-glucosylceramide clearly occurs in MCF-7-AdrR cells; however, this glycosylation step was inhibited by tamoxifen. Additionally, direct measurements of cell-free glucosylceramide formation revealed inhibition by tamoxifen. Structural specificity for inhibition was demonstrated using triphenylbutene, a tamoxifen analog devoid of the dimethylethanolamine moiety.

An objective of the present study was to determine whether inhibition of glucosylceramide synthesis was associated with MDR circumvention. To assess the MDR reversal efficacy of tamoxifen, the sensitivity of MCF-7-AdrR cells to Adriamycin toxicity was studied in an *in vitro* growth assay (45, 46). Tamoxifen sensitized MCF-7-AdrR cells to Adriamycin and strongly inhibited glucosylceramide synthesis. These experiments also afforded the opportunity to investigate glycolipid metabolism at longer times after drug addition. Although at early times after tamoxifen addition (up to 4 h), ceramide metabolism was similar in control and tamoxifen-treated cultures, data from preliminary experiments demonstrated that at extended times cellular ceramide increased when Adriamycin and tamoxifen were used in combination. An increase in cell ceramide was likewise elicited by the MDR-reversing agent SDZ PSC 833 (data not shown). In light of recent work showing that daunorubicin promotes cellular ceramide elevation and

apoptosis (47), our data present the intriguing theory that Adriamycin and tamoxifen work together to enhance the levels of cellular ceramide. Our results align the capacity of drugs that modify glucosylceramide metabolism with circumvention of MDR. This idea was extended using PPMP, an inhibitor of glucosylceramide synthase (31). In our study, PPMP inhibited glucosylceramide synthesis in the low micromolar range (IC_{50}, 0.9 μM). Intriguingly, PPMP, at a concentration shown to maximally inhibit glucosylceramide synthesis, although having no influence on cell viability, induced sensitization of MCF-7-AdrR cells to Adriamycin (Fig. 7). It should be noted that at high concentrations (>30 μM), PPMP acts as a toxic lipophilic amine (48).

Although the phenomenon of MDR involves multiple cellular adjustments, it is now compelling to assign, as an important facet, the interplay of glycosphingolipids. Glucosylceramide may play a role in acquiring and/or maintaining MDR (4), as reducing the levels of glucosylceramide in multidrug-resistant cells treated with tamoxifen appears to render cells sensitive to the toxic insult of chemotherapeutic agents (Figs. 6 and 7). In addition, increases in ceramide, which accompanied glucosylceramide depletion when cells were treated with Adriamycin and tamoxifen, ascribe a glycolipid relationship in MDR reversal of a more complex nature. With regard to neoplasia, expression of various glycosphingolipids on the cell surface has been correlated with mechanisms of acquiring and maintaining a cancer phenotype and tumor progression (26, 30–32, 49). In work divorced from MDR, the glucosylceramide content of cells has been shown to be influential on epidermal homeostasis (50).

Drug resistance continues to be a major obstacle to successful chemotherapy. Clues to the molecular aspects of drug resistance will supply valuable tools to combat MDR. At present it is not known whether glycolipids influence P-gp activity or whether P-gp, in some fashion, regulates glycolipid metabolism. On the other hand, regarding estrogen receptor-independent actions (51–54), tamoxifen has long been viewed with curiosity, particularly with reference to reversal of MDR (55) and synergy in combination chemotherapy (56, 57). In view of the data presented herein, it is suggested that tamoxifen can increase cellular susceptibility to chemotherapeutic agents via a glycosphingolipid-governed avenue, and we propose that tamoxifen-induced MDR reversal is, in part, dependent on fine-tuned regulation of ceramide metabolism.

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