Role of Multiple Drug Resistance Protein 1 in Neutral but Not Acidic Glycosphingolipid Biosynthesis*

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Transfection studies have implicated the multiple drug resistance pump, MDR1, as a glucosyl ceramide translocase within the Golgi complex (Lala, P., Ito, S., and Lingwood, C. A. (2000) J. Biol. Chem. 275, 6246–6251). We now show that MDR1 inhibitors, cyclosporin A or ketoconazole, inhibit neutral glycosphingolipid biosynthesis in 11 of 12 cell lines tested. The exception, HeLa cells, do not express MDR1. Microsomal lactosyl ceramide and globotriaosyl ceramide synthesis from endogenous or exogenously added liposomal glucosyl ceramide was inhibited by cyclosporin A, consistent with a direct role for MDR1/glucosyl ceramide translocase activity in their synthesis. In contrast, cellular ganglioside synthesis in the same cells, was unaffected by MDR1 inhibition, suggesting neutral and acid glycosphingolipids are synthesized from distinct precursor glycosphingolipid pools. Metabolic labeling in wild type and knock-out (MDR1a, 1b, MRP1) mouse fibroblasts showed the same loss of neutral glycosphingolipid (glucosyl ceramide, lactosyl ceramide) but not ganglioside (GM3) synthesis, confirming the proposed role for MDR1 translocase activity. Cryo-immunoelectron microscopy showed MDR1 was predominantly intracellular, largely in rab7-containing Golgi vesicles and Golgi cisternae, the site of glycosphingolipid synthesis. These studies identify MDR1 as the major glucosyl ceramide flipase required for neutral glycosphingolipid anabolism and demonstrate a previously unappreciated dichotomy between neutral and acid glycosphingolipid synthesis.

GlcCer is the precursor of the majority of eukaryotic GSLs (1). The UDP galactose β-1-4 glucosyl ceramide glycosyltransferase (LacCer synthase) within the Golgi lumen then provides the common precursor, LacCer for the synthesis of most complex GSL. Drug-resistant tumor cells have been shown to increase GlcCer synthesis (2, 3), and this has been postulated as a mechanism for drug resistance by providing an alternative biosynthetic destination for ceramide, which might otherwise induce apoptosis (4). GlcCer is synthesized from ER-derived ceramide, on the cytosolic cis-Golgi surface (5–7), and may be transported to the plasma membrane from this site (8). However, the active sites of the glycosyltransferases responsible for further elongation of GSLs are located within the Golgi lumen (9, 10). Thus, a mechanism for the translocation of GlcCer from the cytosolic surface of the Golgi to the lumen must exist. We have shown that transfection of cells with the mdr1 gene results in a marked elevation of GlcCer, LacCer, and Gb3 levels with a concomitant increase in cell sensitivity to the Gb3-binding verotoxin 1. Both effects were reversed in the presence of MDR1 inhibitors (11). We proposed that MDR1 can function as a GlcCer translocase to flip GlcCer to the inner Golgi surface to enhance LacCer and subsequent Gb3 synthesis. MDR1 has been shown to function as a membrane translocase for chemically modified short chain fatty acid GlcCer (8). However such derivatized, indicator GSLs have been considered possible drug substrates for MDR1, and the ability of MDR1 to translocate natural, long-chain GSLs has been questioned on the basis of lack of overt phenotype in MDR1 knockout mice (12). In the present study, we show that fibroblasts from such mice are indeed defective in GSL synthesis, in the manner predicted from the in vitro MDR1 inhibition studies now reported.

In this study, we address the question as to whether MDR1, which is widely expressed in normal tissues (13), is involved in GSL synthesis in cultured cells in general. These studies fill a major gap in understanding the mechanism of GSL biosynthesis and demonstrate a new, unsuspected distinction between acidic and neutral GSL synthesis.

EXPERIMENTAL PROCEDURES

**Cell Culture**

MDCK cells transfected with the human MDR1 cDNA were a gift from Dr. M. Gottesman (National Institutes of Health, Bethesda, MD) (14). The human astrocytoma cell line SF-539, and IOMM Lee and CH157 MN meningioma cell lines were kindly provided by Dr. J. Rutka (Hospital for Sick Children, Toronto, Canada). Vero, HEp-2, SF-539 astrocortoma, ovarian carcinoma SK VLB (MDR variant of the parental SKOV3 cell line (15)), MDR1-MDCK medium also contained 80 μg/ml cycloheximide, and SK VLB medium contained 1 mg/ml vinblastine. HeLa cells, NIH 3T3 cells, and meningioma IOMM Lee and CH157 MN cell lines were maintained in Dulbecco’s modified Eagle’s medium (with 4.5 gm/liter of glucose for HeLa and 3T3, and with 1 gm/liter of glucose for meningioma cells) with 10% FBS. Daudi Burkitt’s lymphoma and Jurkat E6.1 cells were maintained in RPMI 1640 medium also supplemented with 10% FBS. ECV 304 bladder carcinoma cell line (16) was maintained in M199 medium with 10% FBS and 40 μg/ml gentamicin. MDR1-MDCK medium also contained 80 μg/ml colchicine, and SK VLB medium contained 1 mg/ml vinblastine. HeLa cells, NIH 3T3 cells, and meningioma IOMM Lee and CH157 MN cell lines were maintained in Dulbecco’s modified Eagle’s medium (with 4.5 gm/liter of glucose for HeLa and 3T3, and with 1 gm/liter of glucose for meningioma cell lines) with 10% FBS. Daudi Burkitt’s lymphoma and Jurkat E6.1 cells were maintained in RPMI 1640 medium also supplemented with 10% FBS. ECV 304 bladder carcinoma cell line (16) was maintained in M199 medium with 10% FBS and 40 μg/ml gentamicin. KOT

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† The abbreviations used are: GlcCer, glucosyl ceramide; MDR1, multi-drug resistance protein 1 (P-glycoprotein); MRP1, multidrug resistance protein 1; CsA, cyclosporin A; GSL, glycosphingolipid; Cer, ceramide; LacCer, lactosyl ceramide (Galβ1–4Glc ceramide); GM1, monosialoganglioside (SAα2-Galβ1–4Glc ceramide); Glcα1–4Glcα1–4Galβ1–4Glc ceramide; GM3, globotriaosyl ceramide (Galβ1–4Glcβ1–4Glc ceramide); Gb3, globotetraosyl ceramide (Galα1–3Galα1–4Glcβ1–4Glc ceramide); GM2, CTH, ceramide trihexoside; ER, endoplasmic reticulum; KOT, knock-out; VT1, verotoxin 1; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MDCK, Madin-Darby canine kidney cells.

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cells (KOT11 and KOT51 subclones) are epidermal fibroblasts derived from a triple knock-out (mdr1a−/−/−/mrp1−/−/−) mouse (17, 18) and were a generous gift from Dr. J. Wijnholds (Department of Ophthalmogenetics, Netherlands Ophthalmic Research Institute). Subclones were taken after 25 passages, after which the cells were grown for another 25 passages prior to use in the present studies. For MDR1 inhibition, cells were grown in medium containing inhibitor at the highest concentration (≤ 8 μM CsA for 4 days, or 5, 10, or 15 μM ketoconazole for 5 days), which our prior studies had shown to have no effect on cell growth.

**Verotoxin 1 (VT1) Cytotoxicity**

VT1 was purified as described previously (19). Target cells in logarithmic growth phase were cultured in microtiter plates and incubated in 1% Trypan Blue dye exclusion. For MDR1 inhibition studies, CsA or ketoconazole was added to cells 4 or 5 days, respectively, prior to VT1 and maintained during the cytotoxicity assay.

**GSL Extraction**

GSLs were extracted from 5 × 10⁸ exponential growth phase cells. Adherent cells were scraped, and all cells were pelleted by centrifugation at 1000 × g for 10 min. The cell pellet was extracted with 20 volumes of CHCl₃/CH₃OH (2:1) as described (21). The extract was partitioned against water, and the lower phase was applied to a silica column in CHCl₃/CH₃OH (98:2). The column was washed extensively with CHCl₃, and the glycolipid fraction eluted with CH₃COCH₃/CH₃OH (9:1) (22). Gangliosides were separated from pooled upper and lower phases by DEAE-Sephadex G-25 chromatography and eluted using 0.4 M ammonium acetate in methanol (29).

**VT1/ITLC Overlay**

Equivalent GSL aliquots, based on cell number, were separated by TLC, and the plates were dried and blocked with 1% gelatin in water at 37°C overnight. After washing with 50 mM Tris-buffered saline, pH 7.4, plates were incubated with 0.1 μg/ml VT1 for 1 h at room temperature. After washing, the plates were incubated with monoclonal anti-VT1 antibody (24) (2 μg/ml) followed, after washing, by peroxidase-conjugated goat anti-mouse antibody. VT1 bound was visualized with 4-chloro-1-naphthol (22).

**Microsomal Preparation**

Eighty percent confluent MDR1-MDCK cells were washed, scraped into PBS, and centrifuged for 5 min at 300 × g. The pellet was resuspended in buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 10 mM dithiothreitol, 0.5% sucrose, and 10 mM phenylmethanesulfonyl fluoride) and homogenized using a Dounce homogenizer. The homogenate was centrifuged at 800 × g for 10 min at 4°C, and the supernatant was further centrifuged at 10,000 × g for 10 min at 4°C. The resulting supernatant was centrifuged at 100,000 × g for 10 min, and the pellet was resuspended in 10% of the supernatant (to give a crude, cell-free supernatant was centrifuged at 100,000 g for 1 h). The extract was further centrifuged at 10,000 g for 10 min at 4°C (23).

**Autoradiography**

 Counts were further centrifuged at 100,000 g for 1 h. Following extensive washing with 50 mM PBS, the coverslips were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature, mounted with DABCO, and examined and photographed using a Leica DMIRE 2 (Leica Canada, Ontario) fluorescence microscope under incident UV illumination. All images were recorded at the same exposure.

**Cell-free Metabolic Labeling**

UDP-[14C]galactose. 100 μCi of the microsomal fraction (100 μg of protein) was incubated in Tris-HCl buffer, pH 7.4 (10 mM), and MnCl₂ (10 mM) ± 4 μM CsA at 37°C for 2 h, with shaking. 0.2 μCi of UDP-[14C]galactose was then added to a 300-μl final volume and incubated for a further 3 h at 37°C with shaking. For some assays, exogenous liposomal GlcCer (60 μmol sonicated in 20 μl of H₂O) was added during the latter incubation.

**Metabolic Labeling of Cellular GSLs**

MDR1-MDCK Cells—GSLs of exponential growth phase cells were metabolically labeled with [14C]sulfate or 1 μCi [14C]serine in 60 h in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. Medium was removed and replaced with 3 ml of CHCl₃, CH₃OH·H₂O (1:2:2:0.2, v/v), and extraction was allowed to proceed for 10 min. 3 ml was then transferred to an 11-ml glass tube, and 0.88 ml of CHCl₃, 0.78 ml of Hank's balance salt solution, and 0.78 ml of 10 mM acetic acid were added. Cells were then vortexed, and the lower phase was removed. The lower phase was dried and separated by two-dimensional TLC (first dimension, CHCl₃, CH₃OH·25% NH₄OH·H₂O (65:35:4.4, v/v); second dimension, CHCl₃, CH₃OH·COOH·H₂O (50:20:10:5, v/v)) and radioactive species quantitated by phosphorimaging and identified relative to standards. Wild type and KOT 51 fibroblasts were similarly labeled with [14C]sulfate ± 20 μg of indomethacin, a selective inhibitor of MRPs (25). In the presence of indomethacin, the dried lipid extract was resuspended in MeOH for 3 h at 37°C, neutralized with 1.6 ml of 10 mM H₂, 2 ml of CHCl₃, 1.8 ml of MeOH, and partitioned. Both phases were recombined, and polar products were removed on a SepPak C18 column. The methanol-eluted fraction was then separated by two-dimensional TLC as above.

**Immunostaining of Cell Surface MDR1—**HeLa, MDR1-MDCK, Vero, SKVLB, IOMM Lee, and CH157 MN cells were grown on glass coverslips. Washed cells were incubated with MRK-16 monoclonal anti-MDR1 antibody (10 μg/ml; Kamiya) for 1 h at 4°C, followed by fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody, also at 4°C for 1 h. Following extensive washing with 50 mM PBS, the coverslips were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature, mounted with DABCO, and examined and photographed using a Leica DMI 2 (Leica Canada, Ontario) fluorescence microscope under incident UV illumination. All images were recorded at the same exposure.

**Post-embedding Immunogold Cryo-electronmicroscopy—**Logarithmic phase MDR1-MDCK cells were scraped and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1M Sorenson phosphate buffer, pH 7.4, for 2–4 h at room temperature. Cells were then pelleted by centrifugation at 1000 × g for 5 min, washed thoroughly in phosphate buffer, embedded in 15% gelatin, cut into mm³-pieces, and incubated with 2.5 μM sucrose for several hours. The blocks were then mounted on aluminum cryo-ultramicrotomy pins and frozen in liquid nitrogen. Ultrathin cryosections were cut with a diamond knife at −95°C using a Leica Ultratome R cryo-ultramicrotome (Leica Canada, Ontario). Sections were transferred to Formvar-coated nickel grids in a loop of molar sucrose, and grids were washed thoroughly in PBS containing 15% sucrose and 0.5% BSA and PBS containing BSA alone. Sections were then incubated with a polyclonal rabbit antibody against Golgi marker rab6 (32) for 1 h. Following a thorough rinse in PBS/BSA, samples were incubated in a goat anti-rabbit IgG 5-nm gold complex (Amersham Biosciences, Quebec, Canada) for 1 h. This procedure was repeated on the same specimens except MRK-16 anti-MDR1 antibody was used as the primary antibody and goat anti-mouse IgG 10-nm gold complex as the secondary antibody. Sections were then rinsed thoroughly with PBS followed by distilled water and stabilized in a thin film of methylcellulose containing 0.2% uranyl acetate. Controls included the omission of primary and secondary antisera and the use of an irrelevant antibody to rabbit- or mouse-specific non-fibrillar acidic GSLs. Samples were then examined in a JEM 1230 transmission electron microscope (JEOL, Peabody, MA), and images were recorded using a charge-coupled device camera (AMT Corp.). Controls were uniformly negative for gold particles. Image analysis was performed on a minimum of 100 images from each group at a nominal magnification of 100,000× using an NIH image analysis program (Image Pro Plus). Median threshold was determined to determine cell particle density and percentage particle colocalization (where colocalization was defined as 5- and 10-nm particles being within 20 nm of each other).

**Data Analysis and Statistics—**Differences in CD50 values for each of the cell lines, obtained before and after treatment with CsA and ketoconazole, were compared by analysis of variance (ANOVA). Means and standard deviations for each experimental point, repeated at least three times, as well as the statistical analysis were performed by GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA). A value of p < 0.05 was considered significant.
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RESULTS

MDR1 Inhibitors Inhibit Gb3 Synthesis and Protect Cells against VT1 Cytotoxicity—The Gb3-containing cell lines, MDR1-MDCK (canine epithelial), Vero (monkey epithelial), Hep-2 (human epithelial), HeLa (human cervical carcinoma), Daudi (human B lymphoid), SF-539 (human astrocytoma), SK VLB (human ovarian carcinoma), Iomm Lee (human meningioma), CH157 MN (human meningioma), and ECV 304 (human bladder carcinoma) cell lines were analyzed for neutral GSL and Gb3 content and VT1 sensitivity, before and after ketoconazole (not shown). The effect of CsA on Jurkat cell Gb3, the only neutral GSL made, and ganglioside content, was compared. In Vero cells cultured with ketoconazole and MDR1-MDCK cells cultured with CsA, Gb3 levels were reduced by >95%, whereas the level of GM3, the only ganglioside expressed, was unaffected. In Jurkat cells, Gb3 was reduced by >90%, whereas GM3, GM1, and one unidentified, more polar, ganglioside were unaltered after CsA treatment (Fig. 3).

Autoradiography of the metabolically labeled neutral and acidic GSL fraction of MDR1-MDCK cells cultured with or without CsA, shows that, although Gb3 synthesis was significantly reduced, the synthesis of GM3 and the more complex gangliosides was not inhibited at all by CsA (Fig. 3b). Sphingomyelin was identified in the [14C]serine-labeled neutral GSL fraction (Fig. 3a), and labeling was not inhibited, even slightly enhanced, in CsA-treated cells.

Selective CsA Inhibition of Neutral Glycolipid as Opposed to Ganglioside Synthesis—GleCer is the precursor of both gangliosides and neutral GSLs. We therefore compared the effect of ketoconazole or CsA on the synthesis of Gb3 and GM3, the simplest ganglioside, in MDR1-MDCK and Vero cells. The effect of CsA on Jurkat cell GleCer, the only neutral GSL made, and ganglioside content, was compared. In Vero cells cultured with ketoconazole and MDR1-MDCK cells cultured with CsA, Gb3 levels were inhibited by >95%, whereas the level of GM3, the only ganglioside expressed, was unaffected. In Jurkat cells, GleCer was reduced by >90%, whereas GM3, GM1, and one unidentified, more polar, ganglioside were unaltered after CsA treatment (Fig. 3a).

Effect of CsA on Cell-free Glycolipid Synthesis—GSL synthesis (LacCer and some Gb3) in a crude MDR1-MDCK microsomal fraction, monitored by [14C]UDP-galactose incorporation, was inhibited by CsA (Fig. 2). Addition of exogenous GleCer increased LacCer labeling, but CsA inhibition was maintained.

Gb4 were run on each TLC (from the top) GlcCer, LacCer, Gb3, and Gb4 were run on each TLC (lane 1). A GM3 standard is included in NIH 3T3 and Jurkat panels, but GM3 cannot be quantitated within the lower phase (cf. Fig. 3a, panels E and F). In each panel, the GSL fraction of untreated cells corresponds to lane 2, cells treated with CsA are in lane 3 (4 μM for Vero, MDR1-MDCK, SF 539, and Daudi; 8 μM for SK VLB, HeLa, Hep-2, ECV 304, 3T3, and Jurkat), and cells treated with ketoconazole (not determined for all cell lines) are in lane 4 (5 μM for MDR1-MDCK and Daudi; 10 μM for SK VLB and ECV 304; and 15 μM for SF 539, HeLa, and Hep-2). Each experiment was repeated at least twice. MDR1-MDCK and HeLa analyses were repeated four times.
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The effect of MDR1 inhibition on the sensitivity of exponentially growing cell cultures to VT1 was determined. The VT1 dose required to kill 50% of cells (CD_{50}) calculated from the dose response was increased for all cells (except HeLa), after culture of cells with CsA or ketoconazole as described in Fig. 1. For each experiment, the effect on cell sensitivity to vinblastine was simultaneously assayed to confirm efficacy to prevent MDR1-mediated drug efflux.

| Cell type* | None, CD_{50} | CsA | Ketocazole | None, CD_{50} | CsA | Ketocazole |
|------------|---------------|-----|------------|---------------|-----|------------|
| MDCK-MDR1  | 3.00 × 10^{-6}| 0.42| 10^{3}    | <0.0001       | 4.50 × 10^{-2}| 10^{3}    | 0.0001       |
| Vero       | 3.95 × 10^{-6}| 0.19| 5 × 10^{4} | <0.0001       | 2.60 × 10^{-3}| 6 × 10^{4} | <0.0001       |
| SF 539     | 0.043         | 1.69| 39        | 0.0001        | 3.16 × 10^{-1}| 7.3       | <0.0001       |
| HEP-2      | 4.58 × 10^{-6}| 0.014| 3 × 10^{2} | 0.0011        | 2.40 × 10^{-4}| 5 × 10^{2} | 0.0011       |
| HeLa       | 2.54 × 10^{-6}| 2.8 × 10^{-6}| 1 | 0.22 NS     | 3.32 × 10^{-6}| 1 | 0.08 NS     |
| IOMM Lee   | 4.00 × 10^{-4}| 76.80| 2 × 10^{8} | <0.0001       | ND         | ND         |
| CH157 MN   | 0.38          | 26.47| 70        | 0.0005        | ND         | ND         |
| Daudi      | 4.39          | 1.78 × 10^{2} | 4 × 10^{2} | 0.044         | 9.94 × 10^{3}| 2 × 10^{3} | <0.0001       |
| SK VLB     | 7.86 × 10^{-9}| 7.0 × 10^{-9}| 1 | 0.35 NS     | 7.93 × 10^{-7} | 100       | <0.0001       |
| ECV 304    | 7.85 × 10^{-9}| 7.7 × 10^{-9}| 1 | 0.49 NS     | 4.80 × 10^{-2}| 6 × 10^{3} | 0.0001       |

* Dose-dependent VT1 cytotoxicity assayed in triplicate, repeated at least twice.

** NS, not significant.

* ND, not determined.

DISCUSSION

**MDR1 Plays a General Role in Neutral GSL Biosynthesis**—GlcCer synthase is the only GSL glucosyltransferase located at the cytosolic surface of the Golgi, rather than within the lumen (6). This requires a mechanism, until now undescribed, for GlcCer translocation into the Golgi for synthesis of the more complex GSLs. GlcCer synthase has been implicated in drug resistance, because its activation may metabolically prevent ceramide accumulation via the action of cytotoxic drugs (3, 33) and thereby avoid apoptosis induction. MDR1-mediated GlcCer translocation has been implicated to augment this process (34). In addition to its drug pump activity, MDR1 has been shown to be a phospholipid/GSL “flipase,” monitored using non-physiological short-chain lipid probes (8). MDR1 can traffic natural short-chain phospholipids (35), but defining a role for MDR1 in long-acyl-chain lipid transport has proved more elusive (12). Knockout mice are viable, and no GSL or phospholipid phenotype has been reported. The related MDR2 and MDR3 are phosphatidylcholine translocators (36), and MDR3, at least, has no activity for GSLs (8). Bacterial MDR1 homologues function as lipid flippases in phospholipid and lipid A trafficking (37, 38).

We suggested that MDR1 flipping GlcCer from the cytosolic to the luminal Golgi face mediated the increased GlcCer, LacCer, and Gb$_3$ observed following MDCK cell transfection with MDR1 (11). This increase was reversed by CsA. Our present studies show that MDR1 is within Golgi stacks/vesicles and carries out this function in most cultured cells. This defines a new function for “normal” cell MDR1 and fills a significant gap in understanding the steps involved in GSL biosynthesis. MDR1 inhibition resulted in the loss of lower phase neutral

...effect on GSL labeling with $^{14}$C]serine in wild type or knockout fibroblasts (Table III).

**Cell Expression of MDR1**—Because HeLa cell GSL biosynthesis and VT1 sensitivity were unaffected by MDR1 inhibitors, we assessed whether MDR1 was expressed by these cells. By anti-MDR1 Western blot (Fig. 5), a 170-kDa MDR1 band was clearly detected in the MDR1-MDCK cell positive control, but no immunoreactivity was found in the HeLa cell extract. HeLa cells showed no surface immunostaining for MDR1 as compared with a selection of cell lines in which MDR1 inhibitors reduce neutral GSL synthesis (MDR1-MDCK, Vero, SF-539, IOMM Lee, SK VLB, and CH157 MN cells), which served as positive controls (Fig. 5). mRNA screening has shown a lack of MDR1 expression in HeLa cells (30). In some cells (e.g. MDR1-MDCK, SF 539), surface MDR1 expression is remarkably punctate, potentially consistent with a caveolar location (31).

**Subcellular Localization of MDR1, Rab6 by Immunoelectron Microscopy**—Cry-immunoelectron microscopy of MDR1-MDCK cells showed the majority of MDR1 is intracellular (Fig. 6a) and membrane-associated (Fig. 6, a-h). Little MDR1 is expressed in the plasma membrane as monitored by immunoelectron microscopy (none seen in Fig. 6a) possibly due to the punctate expression of MDR1 (Fig. 5b), which might be missed in electron microscopy sectioning. This would be consistent with the surface staining seen in some electron microscopy sections (not shown). Significant MDR1 staining is clearly associated with the Golgi marker rab6 (32). In some instances, a limiting vesicular membrane can be seen around the colocalized MDR1 and rab6 (Fig. 6, b and c). These vesicles are primarily located at the termini of the Golgi cisternae (Fig. 6, d and e). MDR1 is also found within these termini (Fig. 6, f and g) and to a lesser degree, within the lamellar stacks of the Golgi cisternae (Fig. 6, g and h).
FIG. 3. Comparison of the effect of MDR1 inhibition on GM3/ganglioside and Gb₃/neutral GSL synthesis. a, cellular GSL content. Panels A, C, and E: neutral GSL fractions; panels B, D, and F: ganglioside fractions; panels A and B correspond to Vero cells, panels C and D to MDR1-MDCK cells, and panels E and F to Jurkat cells. Lane 1, standard GSLs: panels A, C, and E: GlcCer, LacCer, Gb₃, and Gb₄; panels B and D: GM3; panel F: GM3 (upper) and GM1 (lower); lane 2: GSLs from untreated cells; and lane 3: GSLs from ketoconazole (Vero)- or CsA (MDR1-MDCK and Jurkat)-treated cells. Neutral GSLs were visualized by orcinol and VT1 overlay for Gb₃. Gangliosides were visualized using resorcinol staining. Similar results were obtained in three experiments. b, metabolic labeling MDR1-MDCK cells were metabolically labeled with [14C]serine/H₁₁₀₀₆/H₉₂₆₂ for 4 days, and the neutral and acidic GSLs were extracted, separated, and resolved by TLC and visualized by autoradiography. Panel A, neutral GSLs: lanes 1 and 4, stds; lane 2: control cells; and lane 3: CsA-treated cells. Panel B, gangliosides, lane 1: control cells; lane 2: CsA-treated cells, and lane 3: stds. The [14C]serine-labeled doublet in the neutral GSL fraction comigrates with sphingomyelin and is reactive with the molybdenum stain for phosphate (not shown). This experiment was repeated three times.
GSLs, GlcCer, LacCer, Gb3, and Gb4 (if present), in all but (MDR1-negative) HeLa cells. (GM3, detected in some cell extracts, appears reduced, but GM3 partitions into both upper and lower phases and cannot therefore be quantitated in the lower phase. Isolation of the ganglioside fraction showed GM3 levels were unaffected.) The reduction in GlcCer following MDR1 inhibition can be attributed to cytosolic glucocerebrosidase (39). CsA inhibition of GlcCer-dependent LacCer biosynthesis in a microsomal assay supports a direct translocase role for MDR1. This assay is complex however, requiring transfer of the GlcCer from the exogenous liposome to the outer microsomal membrane for MDR1 translocation, as well as nucleotide luminal transit (40). The microsomal inhibitory effect of CsA is more rapid than in cell culture, consistent with a more immediate effect on GlcCer translocation. LacCer synthesis is affected in <5 h, whereas several days of culture are required for intact cells.

An alternative explanation might be that MDR1 inhibitors, in some way, promote neutral GSL catabolism. However, CsA was found to have no effect on cellular α-galactosidase activity in vitro (not shown) and did not affect LacCer synthesis from C6-GlcCer (41). Moreover, it is unlikely that the lack of MDR1 in the KOT fibroblasts would have a similar effect on GSL catabolism.

**FIG. 4.** Metabolic radiolabeling of lipids in MDR1 knockout fibroblasts. Autoradiogram of radiolabeled lipids from wild type (panels 1 and 3) and KOT 11 (mdr 1a−/−, 1b−/−, mnrp 1−/−) (panels 2 and 4) mouse fibroblasts, separated by two-dimensional TLC after metabolic labeling with [14C]acetate (panels 1 and 2) or [14C]serine (panels 3 and 4). Labeled species are quantitated in Table II.

**FIG. 5.** Cell expression of MDR1. In a: panel A, anti-MDR1 (C219) Western blot; lane 1, MDR1-MDCK cells; lane 2, HeLa cells, position of MW standards (210, 96 kDa) are indicated by arrows; panel B, HeLa cells immunostained with anti-MDR1 (MRK 16) at 4 °C; panel C, same HeLa cell field observed by differential interference contrast microscopy (results from one of three similar experiments are shown). In b: cell lines responsive to MDR1 inhibitors were also stained with anti-MDR1 at 4 °C: A, MDR1-MDCK; B, SF 539; C, Vero; D, CH157MN; E, IOMM Lee; and F, SKVLB cells. Bar = 100 μm.
Quantitation of metabolic labeling of GSL of MDR1 knockout fibroblasts with \[^{14}C\]acetate or \[^{14}C\]serine (shown in Fig. 4)

The metabolically radiolabeled lipids extracted from cells (in duplicate) and separated by two-dimensional TLC (Fig. 4) were quantitated by PhosphorImager analysis. To confirm that the lack of MRP1 in KOT cells plays no role in the effect of MDR1 inhibitors on ganglioside biosynthesis (except for HeLa cells). However the effect was not always proportional to the reduction in Gb3, likely due to the differential efficacy of Gb3 lipid isoforms to bind VT (42, 43), to be effectively presented within the phospholipid bilayer (44, 45) and traffic within the cell (46). Significantly, verapamil, another MDR1 inhibitor (47), has been shown both to protect cells against VT1 (48) and reduce GSL synthesis (49). In the latter study on tamoxifen, verapamil, and CsA MDR1 inhibition, the levels of GlcCer and LacCer, but not GM3 ganglioside, were primarily reduced. This is consistent with our results, as is the anecdotal report of the independence of GM3 ganglioside synthesis and MDR1 (50).

Although CsA and ketoconazole are inhibitors of MDR1 (51, 52), these inhibitors have other effects (53), most notable are the immunosuppressive effects of CsA (54). However, the lack of effect of these inhibitors on neutral GSL biosynthesis in HeLa cells may, in effect, provide a specificity control in which the effects of CsA and ketoconazole are MDR1-mediated. HeLa cells show that alternative mechanisms for GlcCer translocation must exist. Such redundancy may explain the lack of effect of these inhibitors on neutral GSL biosynthesis in HeLa cells.
lenges the paradigm of a single lactosyl (hence glucosyl) ceramide pool as the common precursor for neutral and acidic GSLs. Despite the fact that GlcCer (+LacCer, +Gb3 if present) levels were depleted to background values by CsA, GM3 synthesis was unaffected within the same cells. Higher gangliosides were not degraded to maintain GM3 levels. In the KOT cells, GlcCer and LacCer synthesis were reduced by 80–90%, yet the level of GM3 in the same cells was not reduced, compared with wild type cells. Both these data sets infer that GM3 and Gb3 (neutral GSLs) must be synthesized from separate LacCer (and hence GlcCer) pools. Moreover, because the GlcCer/LacCer pool from which GM3 is synthesized in the presence of CsA must be small, the LacCer substrate K_m for the sialyl transferase must be low or the GM3 synthase associated with GlcCer/LacCer synthase to provide a topological product → substrate advantage (55). In Jurkat cells, LacCer was undetectable, yet the gangioside content was significant, confirming that GM3 synthase requires only a low substrate pool. Inhibition of cellular GlcCer synthase using product analogues (56, 57) inhibits both neutral and acidic GSL biosynthesis (58–61) and only a single GlcCer synthase transcript has been detected (62). Thus, the separate regulation of ganglioside and Gb3 synthesis. The metabolic labeling of the neutral lipid fraction was significant, con- pared with wild type cells. Both these data sets infer that GM3 synthesis was unaffected within the same cells. Higher gangliosides were not degraded to maintain GM3 levels. In the KOT cells, GlcCer and LacCer synthesis were reduced by 80–90%, yet the level of GM3 in the same cells was not reduced, compared with wild type cells. Both these data sets infer that GM3 and Gb3 (neutral GSLs) must be synthesized from separate LacCer (and hence GlcCer) pools. Moreover, because the GlcCer/LacCer pool from which GM3 is synthesized in the presence of CsA must be small, the LacCer substrate K_m for the sialyl transferase must be low or the GM3 synthase associated with GlcCer/LacCer synthase to provide a topological product → substrate advantage (55). In Jurkat cells, LacCer was undetectable, yet the gangioside content was significant, confirming that GM3 synthase requires only a low substrate pool. Inhibition of cellular GlcCer synthase using product analogues (56, 57) inhibits both neutral and acidic GSL biosynthesis (58–61) and only a single GlcCer synthase transcript has been detected (62). Thus, the separate regulation of ganglioside and Gb3 synthesis. The metabolic labeling of the neutral lipid fraction was significant, compared with wild type cells. Both these data sets infer that GM3 synthesis was unaffected within the same cells. Higher gangliosides were not degraded to maintain GM3 levels. In the KOT cells, GlcCer and LacCer synthesis were reduced by 80–90%, yet the level of GM3 in the same cells was not reduced, compared with wild type cells. Both these data sets infer that GM3 and Gb3 (neutral GSLs) must be synthesized from separate LacCer (and hence GlcCer) pools. Moreover, because the GlcCer/LacCer pool from which GM3 is synthesized in the presence of CsA must be small, the LacCer substrate K_m for the sialyl transferase must be low or the GM3 synthase associated with GlcCer/LacCer synthase to provide a topological product → substrate advantage (55). In Jurkat cells, LacCer was undetectable, yet the gangioside content was significant, confirming that GM3 synthase requires only a low substrate pool. Inhibition of cellular GlcCer synthase using product analogues (56, 57) inhibits both neutral and acidic GSL biosynthesis (58–61) and only a single GlcCer synthase transcript has been detected (62). Thus, the separate regulation of ganglioside and neutral GSL synthesis is not at the level of GlcCer synthesis but must occur via some later sorting process.

CsA has no direct effect on the activity of enzymes involved in Gb3 biosynthesis (11). The LacCer K_m for Gb3 synthesis is 50 μM (63), whereas that for GM3 synthesis is 270 μM (64), suggesting that, in the absence of other factors, when LacCer is limiting, Gb3 should be synthesized in preference to GM3. This is consistent with studies, with fumonisin B inhibition of de novo GSL biosynthesis, that showed the selective enhancement of Gb3 synthesis from the remodeling of other GSLs (28). Gb3 synthesis was favored over GM3 at low LacCer levels. The synthesis of LacCer may be uncoupled from GlcCer synthesis in MDR cells (41); i.e., not all GlcCer is available for LacCer synthesis, indicating different GlcCer pools. Gb3 and GM3 synthases could be located within different Golgi stacks, using different LacCer pools, differentially affected by MDR1 inhibitors. GM3 synthase has been located to the medial Golgi/Golgi network (65, 66). The location of Gb3 synthase has yet to be reported, but a cis/medial Golgi location would not be unreasonable. However, our location of MDR1 primarily in the medial and cis-Golgi would argue against gross spatial separation. The different LacCer pools could require different GlcCer translocators. Our HeLa cell results indicate the existence of such species. Distinct precursor GSL pools might arise from differential ER ceramide delivery within the Golgi stacks, as proposed (67). In addition, because inhibition of GlcCer synthesis can result in relocation of LacCer (68), such redistributed LacCer pools may be more effectively available for GM3 than Gb3 synthesis.

An intriguing possibility is that the synthesis of gangliosides and neutral GSLs from LacCer might be cell cycle-selective. The receptors for cholera toxin (GM1 ganglioside) and vero-toxin (Gb3) have been shown to be synthesized at the G1 and G2/M phases, respectively (69). Although LacCer is a common precursor, Gb3 and GM1 synthesis were not competitive (69). Although the flipase activity of MDR1 for natural lipids has been questioned, largely on the basis of a lack of a phospholipid phenotype in knockout mice, the present studies with KOT cells demonstrate a selective role for MDR1 in GlcCer translocation for neutral GSL synthesis. Although murp1 is also lacking in KOT cells, inhibitors of MRP1 do not affect wild type (or KOT) fibroblast (present work) or MDR1-MDCK cell (11) GSL synthesis. The difference in phosphatidyl choline labeling we observe in KOT cells implies a role for MDR1 in endogenous phospholipid translocation, which has been overlooked. The lack of an overt phospholipid phenotype in knockout animals (70) is likely a function of redundancy such as we see for HeLa cells in the present study. The increased labeling of sphingomyelin in KOT cells could result from an increased ceramide content, subsequent to inhibition of neutral GSL synthesis.

The metabolic labeling of the neutral lipid fraction was sig-
nificantly reduced in the KOT cells. This fraction should con-
tain, among other species, cholesterol esters. The reduction may therefore relate to the role for MDR1 proposed in chole-
sterol homeostasis (71–73) and trafficking (74).

In our earlier report (11), we showed MDR1 activity corre-
lated with increased globoseries GSLs, primarily containing C16, and C18 fatty acids. In the present study, we see that for cells expressing Gb3 as a doublet by TLC, due to lipid hetero-
geneity, both bands are susceptible to MDR1 inhibitors, indi-
cating that under these conditions MDR1 may mediate glucosylceramide transport.

In the KOT cells, however, the more slowly migrating (shorter fatty acid chains) GlcCer and CTH were more severely reduced, consistent with an MDR1 bias for the more polar GlcCer lipid isomers.

The involvement of MDR1 in neutral, but not acidic, GSL synthesis is relevant to studies on the regulation of ceramide synthesis by the ouab1 gene (75). Transfection of cells with this gene resulted in the selective increase in C18 fatty acid-con-
taining dihydroceramide. This increased ceramide pool was utilized in neutral GSL, but not ganglioside, biosynthesis. This was proposed to result from an unusual fatty acid specificity of the synthetic enzymes (75), but this also requires, as do our studies, that gangliosides and neutral GSL are synthesized from different GlcCer/LacCer pools. It is possible that MDR1 is preferentially responsible for the translocation of C16 and C18 GlcCer, whereas long-chain fatty acid GlcCer is translocated by another mechanism in the later Golgi/endomembrane frac-
tions, in which the cholesterol content and, hence, bilayer thickness, is increased. Gangliosides containing short fatty acid chains may be synthesized via a recycling, as opposed to a de novo pathway (76).

The present studies show that MDR1 is a Golgi component involved in neutral GSL biosynthesis. This shows for the first time, that gangliosides and neutral GSLs can be synthesized via separate pathways that can be differentially regulated. The involvement of MDR1 in Gb3 synthesis supports the contention (77–81) that VTI1 can be an effective antineoplastic.

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