Inhibition of Multidrug Resistance by AdamantylGb₃, a Globotriaosylceramide Analog*

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Multidrug resistance (MDR) via the ABC drug transporter (ABC1), P-glycoprotein (P-gp/MDR1) overexpression, is a major obstacle in cancer chemotherapy. Many inhibitors reverse MDR but, like cyclosporin A (CsA), have significant toxicities. MDR1 is also a translocase that flips glucosylceramide inside the Golgi to enhance neutral glycosphingolipid (GSL) synthesis. We observed partial MDR1/globotriaosylceramide (Gb₃) cell surface co-localization, and GSL removal depleted cell surface MDR1. MDR1 may therefore interact with GSLs. AdamantylGb₃, a water-soluble Gb₃ mimic, but not other GSL analogs, reversed MDR1-MDCK cell drug resistance. Cell surface MDR1 was up-regulated 1 h after treatment with CsA or adaGb₃, but at 72 h, cell surface expression was lost. Intracellular MDR1 accumulated throughout, suggesting long term defects in plasma membrane MDR1 trafficking. AdaGb₃ or CsA rapidly reduced rhodamine 123 cellular efflux. MDR1 also mediates gastrointestinal epithelial drug efflux, restricting oral bioavailability. Vinblastine apical-to-basal transport in polarized human intestinal C2BBBe1 cells was significantly increased when adaGb₃ was added to both sides, or to the apical side only, comparable with verapamil, a standard MDR1 inhibitor. Disulfide cross-linking of mutant MDR1s showed no binding of adaGb₃ to the MDR1 verapamil/cyclosporin-binding site between surface proximal helices of transmembrane segments (TM) 6 and TM7, but rather to an adjacent site nearer the center of TM6 and the TM7 extracellular face, i.e. close to the bilayer leaflet interface. Verotoxin-mediated Gb₃ endocytosis also up-regulated total MDR1 and inhibited drug efflux. Thus, a functional interplay between membrane Gb₃ and MDR1 provides a more physiologically based approach to MDR1 regulation to increase the bioavailability of chemotherapeutic drugs.

Multidrug resistance (MDR) is one of the major obstacles for successful chemotherapy in patients with cancer. The MDR phenotype has been associated with overexpression of P-glycoprotein in cancer cells (1). MDR1 is a 170-kDa plasma membrane glycoprotein that uses ATP to pump hydrophobic molecules out of the cell (2, 3). Its efflux function decreases drug concentration in tumor cells that results in chemotherapy failure. MDR1 is expressed in relatively high levels in the apical membranes of epithelial cells of intestine, kidney, liver, and blood-brain/testes barriers (4). Thus, the presence of MDR1 within the endothelial cells’ epithelium can affect the bioavailability and the oral availability of therapeutic drugs (5).

Various attempts have been made to reverse this resistance using MDR1 inhibitors that interact with MDR1 and block drug efflux. First generation modulators such as calcium channel blockers, calmodulin inhibitors, and cyclosporin were developed for pharmacological uses other than reversal of MDR and were relatively nonspecific and weak inhibitors that were also substrates of MDR1, and their deleterious toxicities with their use at required concentrations to inhibit MDR1 have limited their clinical use (6, 7). Clinical trials with second generation modulators such as the cyclosporin analog, PSC 833, or dextramethasone V—710 have demonstrated some clinical benefits in terms of enhancement of pharmacokinetics but also increased toxicity of cytokotoxic drugs when administered with these modulators (8, 9). To date, a third generation of more potent and specific modulators or inhibitors such as GG918 and LY335979 have overcome toxicity side effects but exhibited no significant pharmacokinetic interaction with doxorubicin, etoposide, and paclitaxel in animal studies to make them suitable for co-administration in cancer therapy (10, 11). The development of new MDR1 inhibitors with higher selectivity and stronger potency remains a major goal for this field of research.

In recent years, links have been established between MDR1 and glycosphingolipids (GSL). Many cells expressing MDR1 show elevated levels of glucosylceramide (GlcCer) and sphingomyelin (12–15), and inhibitors of GlcCer synthase kill MDR cells (16). Retroviral transduction of MDCK cells with human MDR1 gene results in a major increase in neutral GSLs (17). Our previous studies showing that CsA inhibits neutral GSL biosynthesis lead us to propose MDR1 as a Golgi glucosylcerame-
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mide flipase that enhances neutral GSL synthesis (18). We also observed partial MDR1 and globotriaosylceramide (Gb₃) cell surface co-localization and that inhibition of GSL biosynthesis depleted cell surface MDR1. MDR1 may therefore interact with Gb₃. In this study, the effect of a water-soluble derivative of Gb₃, adamantylGb₃ (19), on MDR/MRD1 was analyzed. Verotoxin was used to probe the potential role of Gb₃ in MDR1 function.

EXPERIMENTAL PROCEDURES

Materials—AdamantylGb₃ was prepared as described previously (19). Dimethylformamide was added to a solution of oxalyl chloride in dichloromethane. Adamantane acetic acid was then slowly added over 30 min. After stirring at room temperature for 2 h, oxalyl chloride in excess and solvent were washed under the action of N₂, and residual adamantane acetic acid was dissolved in dichloromethane. LysoGb₃, prepared by hydrolysis of Gb₃, was suspended in dichloromethane and pyridine, and then 2 aliquots of the adamantane acetic acid solution were added at 30-min intervals. After the reaction, the mixture was dried under N₂ (TLC; chloroform:methanol:water, 80:20:2 (v/v/v)) and the product purified on a mini silica column. Adamantylgalactosylceramide (adaGalCer) was similarly made from lysogalactosylceramide, prepared by hydrolysis of galactosylceramide. Verotoxin 1 B subunit (VT1 B subunit) (20) was purified by affinity chromatography as described (21). VT1B was conjugated with fluorescein isothiocyanate (Invitrogen) as described (25). Rabbit polyclonal antiserum against purified VT1 B sub unit was prepared as described previously (22). Cyclosporin A and vinblastine were purchased from Sigma. Verapamil was a gift from A. Rasymas (Faculty of Pharmacy, Ohio State University). [3H]Vinblastine (11.2 Ci/mmol) was purchased from Amersham Biosciences. [14C]Mannitol (55.1 mCi/mmol) was purchased from DuPont.

Cell Culture—MDCK cells transfected with the human MDR1 cDNA were a gift from Dr. M. Gottesman (National Institutes of Health, Bethesda) (23). SKVLB ovarian carcinoma cell line (MDR variant of the parental SKOV3) and C2BBe1 cell line (“brush border-expressing”), subclone of human colorectal epithelial Caco-2 cell line, were obtained from the American Type Culture Collection (Manassas, VA). MDR1-MDCK and SKVLB cells were maintained in α-minimal essential medium (Multicell, Wisent Inc., Quebec, Canada) supplemented with 5 and 15% fetal bovine serum, respectively, as well as 100 units penicillin/ml and 100 μg of streptomycin/ml (Multicell, Wisent Inc., Quebec, Canada). MDR1-MDCK medium also contained 80 ng/ml colchicine, and SKVLB medium contained 1 μg/ml vinblastine to maintain a continuous selection pressure to express MDR1. C2BBe1 cell line is cultured in Dulbecco’s modified Eagle’s medium (Multicell, Wisent Inc.) supplemented with 10% fetal bovine serum and 0.01 mg/ml human transferrin (Sigma). All cell lines were incubated at 37 °C in an atmosphere of 5% CO₂, 95% air.

Immunostaining of Cell Surface MDR1—MDR1-MDCK and SKVLB cells were grown on 12-mm diameter glass coverslips in a 24-well plate (BD Biosciences). For inhibition of glycolipid biosynthesis, MDR1-MDCK and SKVLB cells were grown in the presence of 5 μM of 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP; Matreya Inc., Pleasant Gap, PA) for 5 days (24). For MDR1 inhibition studies, MDR1-MDCK cells were grown in the presence of 4 μM CsA or 50 μM adaGb₃ for 1, 3, 72 h and 4 days. For cell surface distribution of MDR1, cells previously washed with 50 mM phosphate-buffered saline (PBS) were incubated with MRK-16 monoclonal antibody anti-MDR1 (10 μg/ml; Kamiya Biomedical Co., Seattle, WA) for 1 h at 4 °C, followed by incubation with TRITC-conjugated goat anti-mouse secondary antibody (Sigma) 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 Dako Cytomation fluorescent mounting medium (Dako Cytomation, CA), and examined by Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss Ltd., Toronto, Ontario, Canada). Fluorescent images were captured, stored, and analyzed with LSM software (26). Digital images were transferred to Adobe Photoshop 8.0 for image handling.

Post-embedding Immunogold Cryo-electron Microscopy—Logarithmic phase MDR1-MDCK and SKVLB cells were washed twice with PBS and treated ±5 μg/ml VT1 B subunit at 4 °C for 1 h. Cells were then harvested by scraping, pelleted by centrifugation at 1,000 × g for 5 min, and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M Sorenson’s phosphate buffer, pH 7.4, for 2–4 h. Cells were then lightly pelleted and washed thoroughly in phosphate buffer. The cells were then embedded in 15% gelatin, cut into mm³ pieces, and infused with 2.3 m sucrose for several hours. The blocks were then mounted on aluminum cryo-ultramicrotomy pins and frozen in liquid nitrogen. Ultrathin cryosections were then cut on a diamond knife at −95 °C using a Leica Ultracut R cryo-ultramicrotome (Leica Canada). Sections were transferred to Formvar-coated nickel grids in a loop of molten sucrose and the grids washed thoroughly in PBS containing 0.15% glycine and 0.5% bovine serum albumin and PBS containing bovine serum albumin alone. Sections were then incubated with a polyclonal antibody against VT1 B subunit (1:2000) for 1 h. Following a thorough rinse in PBS/bovine serum albumin, samples were incubated in goat anti-rabbit IgG 5 nm gold complex (Amersham Biosciences) for an hour. The procedure was repeated on the same specimens except monoclonal antibody anti-MDR1 (MRK-16, 5 μg/ml) 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 antiserum and the use of an irrelevant antibody (either poly- or monoclonal anti glial fibrillary acidic protein). Samples were then examined in a JEOL JEM 1230 transmission electron microscope (JEOL USA) and images recorded either on photographic plates or with a CCD camera (AMT Corp.). Image analysis was performed on a minimum of 100 images from each group at a nominal magnification of ×100,000 using an image analysis program (Image Pro Plus, Media Cybernetics).

Cytotoxicity Assay—MDR1-MDCK and SKVLB cells were cultured in the presence of either 4 μM CsA, 20 or 50 μM of
adaGb₃, or 20 μM of adaGC, and tested for sensitivity to vinblastine over 3 days relative to untreated cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (DTTB) assay (27). The parental drug-sensitive cell line, MDCK-1, was also cultured in the presence of either 4 μM CsA or 50 μM of adaGb₃ as a control. Briefly, cells pretreated after 4 days with CsA, adaGb₃, or adaGalCer were plated into 96-well plates in α-minimal essential medium with 5% fetal bovine serum and incubated at 37 °C in 5% CO₂ with increasing dilutions of vinblastine. After 72 h, 20 μl of a 5 mg/ml DTTB solution was added to the wells and incubated at 37 °C in 5% CO₂ for 4 h. Supernatants were removed, and 100 μl of acidified isopropanol alcohol was added and incubated in the dark at room temperature for 30 min. Plates were agitated for 2–3 min, then read on an enzyme-linked immunosorbent assay reader at 490 nm. The concentration required to inhibit growth by 50% (IC₅₀ values) was calculated from the cytotoxicity curves using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego). The fold reversal of multidrug resistance was calculated by dividing the IC₅₀ values in the absence of the MDR1 inhibitors by those in the presence of the MDR1 inhibitors.

**Drug Transport**—Transport of 0.1 μM [³H]vinblastine, a P-glycoprotein substrate, was measured across a C2BB1 intestinal epithelial cell monolayer (a polarized subclone of Caco2 cells) grown on a permeable membrane (Costar Transwell: 0.2 μm pore size; polycarbonate filter). After preincubation for 45 min in the incubation media (Dulbecco’s modified Eagle’s media) without vinblastine, transport experiments were conducted at 37 °C. At time 0, labeled vinblastine was added to either the basal or apical side of the monolayer in the presence or absence of an inhibitor on both sides. Time course of basal-to-apical transport was examined by monitoring the appearance of radioactivity in the apical side after adding vinblastine to the basal side. Apical-to-basal transport was examined by monitoring appearance of radioactivity from the apical to the basal side. As an extracellular marker, [¹⁴C]mannitol was used with [³H]vinblastine to ensure integrity of the monolayer. The data obtained from the filter preparation, which showed more than 4% of mannitol concentration over 2 h (relative to the initial concentration in the opposite side at time 0), were excluded.

**Rhodamine 123 Efflux Assay**—For this assay, all the steps were performed at 37 °C on cells grown in chambers with borosilicate coverglass at a density of 5 × 10⁴ cells/ml. MDRI-MDCK and SKVLB cells were preincubated for 30 min with assay buffer (10 mM Hepes, 0.4 mM K₂HPO₄, 25 mM NaHCO₃, 3.0 mM KCl, 1.2 mM MgSO₄, 1.4 mM CaCl₂, 122 mM NaCl, 10 mM glucose) (28, 29). After the preincubation period, the assay buffer was replaced and replaced with 2.5 ml of assay buffer containing 100 μg/ml of rhodamine 123 (rhod123) (Sigma). After 20 min, the wells were washed three times for 5 min with assay buffer. For inhibition studies, 50 μM adamantylGb₃ or 10 μM cyclosporin A for positive control in assay buffer were added 5 min prior to the addition of rhod123 and maintained in all the solutions until visualization. MDRI-MDCK and SKVLB cells were also tested when treatment with 50 μM adamantylGb₃ was for 24 h. The efflux of rhod123 was directly visualized in these chambers by Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss Ltd.). Fluorescent images were captured, stored, and analyzed with LSM software.

**Disulfide Cross-linking Analysis**—Construction of the human P-glycoprotein cross-linkable mutants F343C(TM6)/F728C(TM7), F343C(TM6)/Q725C(TM7), and L339C(TM6)/F728C(TM7) was described previously (30, 31). The mutant cDNAs were transiently expressed in HEK 293 cells (25 10-cm plates). The cells were harvested and washed three times with PBS, and membranes were prepared as described (32). The membranes were suspended in 0.3 ml of Tris-buffered saline, pH 7.4, and preincubated for 15 min at 22 °C in the presence of different concentrations of the Gb₃ analog, verapamil, or no drug. Samples were then cross-linked by incubation with 0.2 mM of the methanethiosulfonate cross-linker, 3,6,9,12-tetraoxatetradecane-1,14-diyl bismethanethiosulfonate (M14M, 2.8%). Intramolecular disulfide cross-linking between TMD1 and TMD2 can be detected because of slower mobility of the cross-linked product on SDS-polyacrylamide gels (33). The amount of cross-linking was quantitated by scanning the gel.
lanes followed by analysis with the NIH Image Program ImageJ 1.34s (available at www.rsb.info.nih.gov).

Western Blot Analysis of MDR1—Untreated MDR1-MDCK cells or cells pretreated overnight with 10 μM CsA, or 4 μg/ml VT1B, or for 1 h, 3 h, overnight, 3 days, or 4 days with 50 μM adaGb₃, or for 5 days with PPMP were pelleted by centrifuging at 500 × g for 5 min. Cell pellets were washed three times with cold 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and solubilized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% NaN₃, 5% Nonidet P-40, 5 μg/ml aprotinin, 2 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) for 1 h with shaking at 4 °C. After spinning for 20 min at 12,000 × g at 4 °C, protein was determined in supernatants using a BCA-protein assay (Pierce). SDS-PAGE and Western blotting were performed by loading 5 μg of protein per sample onto a 8% polyacrylamide gel using a standard procedure (34). The transferred nitrocellulose blot was blocked with 5% skim milk powder in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20 at 37 °C for 1 h. The membrane was then immunoblotted with monoclonal antibody C219 (1 μg/ml) (DAKO, Glostrup, Denmark) in 1% skim milk overnight and was washed three times with 50 mM Tris-buffered saline, 0.05% Tween 20. Following incubation with horseradish peroxidase-conjugated goat anti-mouse IgG 1:1000 (Sigma) for 2 h at room temperature, the blot was developed using Supersignal West PICO enhanced chemiluminescence (ECL) system (Pierce).

RESULTS

MDR1 in Part Co-localizes with Gb₃ at Cell Surface—Our previous immunofluorescence study (17) had suggested that Gb₃ and MDR1 were, in part, located in similar regions of the plasma membrane. The possible cell surface co-localization of MDR1 and Gb₃ was therefore more precisely defined by double labeling cryo-immunoelectron microscopy (Fig. 1A). Morphometry established that 33% of MDR1 was within 25 nm of VT1 in MDR1-MDCK cells. This is a conservative estimate of co-localization, because the theoretical maximum distance for co-localized antigens by indirect gold labeling is 50 nm. Pro-
longed inhibition of GSL synthesis prevented the cell surface detection of MDR1 by confocal immunomicroscopy (Fig. 1B). Similarly, treatment with the MDR1 inhibitor, CsA, resulted in the loss of MDR1 surface staining.

AdaGb₃ Prevents Multidrug Resistance in MDR1-MDCK and SKVLB Cells—MDR1-MDCK cells are resistant to vinblastine compared with the parental cell line MDCK. The ability of adaGb₃ to reverse the drug resistance phenotype of MDR1-MDCK and SKVLB was examined and compared with that of CsA, and with adaGalCer to test specificity. Concentrations of 50 μM of either adaGb₃ or adaGalCer had no cytotoxic effects on MDR1-MDCK cells within the experimental period. AdaGb₃ at 50 μM significantly reversed the resistance of MDR1-MDCK cells to vinblastine (Fig. 2A) and reduced the IC₅₀ values similar to CsA (IC₅₀ MDRI = 12.6 μg/ml versus IC₅₀ MDR1CSA = 8.8 × 10⁻⁶ μg/ml and IC₅₀ MDR1adaGb₃ = 7.2 × 10⁻⁶ μg/ml). Even treatment with adaGb₃ at 20 μM reduced the IC₅₀ value (IC₅₀ MDR1adaGb₃ = 9.6 × 10⁻⁷ μg/ml). Treatment with adaGalCer did not reverse the drug resistance phenotype of MDR1-MDCK cells (IC₅₀ MDR1adaGalC = 13.9 μg/ml) showing a specific effect for adaGb₃ on the modulation of MDR1 function (Fig. 2A). Both CsA and adaGb₃, if anything, slightly reduced vinblastine sensitivity of the highly sensitive, parental untransfected MDCK1 cells.

In SKVLB cells, naturally resistant to vinblastine (Fig. 2B), treatment with 50 μM adaGb₃, but not 50 μM adaGalCer, reversed the multidrug resistance phenotype by reducing the IC₅₀ for vinblastine by 10-fold (IC₅₀ SKVLB = 7.8 μg/ml and IC₅₀ SKVLB adaGb₃ = 7.8 μg/ml), similar to CsA. The effect of AdaGb₃ and CsA on vinblastine sensitivity was also tested in the parental drug-sensitive cell line MDCK1, with no significant effect, as a control.

and CsA for 1, 3, and 72 h showed that adaGb₃ up-regulates MDR1 cell surface expression at 3 h, but by 72 h, it is completely lost compared with the untreated control (Fig. 2B, i, panels b–d, versus panel a). This short term up-regulation has been described for CsA (35), and CsA up-regulation of MDR1 was seen within 1 h as for adaGb₃ (Fig. 3B, ii, panels b–d versus panel a).

The total cellular MDR1 content was monitored by Western blot after treatment of MDR1-MDCK cells with either CsA, adaGb₃, VT1 B overnight and compared with control (Fig. 3C, i, lanes 2–4 versus lane 1). Despite or perhaps because of MDR1 inhibition, MDR1 synthesis is increased in the presence of CsA, adaGb₃, or VT1B (Fig. 3C, i, lane 2) (CsA < adaGb₃ < VT1B). Total cellular MDR1 increased rapidly after inhibitor addition (2×fold within 1 h) (Fig. 3, C, ii, lane 3, and D) to reach a maximum within 3 h (Fig. 3, C, ii, lane 4, and D) that was maintained during prolonged inhibitor exposure (Fig. 3C, ii, lanes 5 and 6). Cellular MDR1 is similarly increased when GSL synthesis is prevented by PPMP (Fig. 3C, ii, lane 2). The loss of cell surface MDR1 expression when intracellular MDR1 is increased suggests that cell surface trafficking of MDR1 may be an unappreciated target of these inhibitors and GSL deficiency.

AdaGb₃ Inhibits Cell Surface MDR1 Expression in the Long Term in MDR1-MDCK and SKVLB Cells but Increases Intracellular MDR1 as PPMP, VT1B, and CsA—MDR1-MDCK cells pretreated with adaGb₃ for 4 days and stained with anti-MDR1 at 4 °C showed complete loss of cell surface MDR1 expression (Fig. 3A, i, panel b) compared with the control with no pretreatment (Fig. 3A, i, panel a). Treatment with another adamantylGSL analog, adamantylgalactosylceramide (adaGal-Cer) had no effect on MDR1 staining (Fig. 3A, i, panel d). This absence of MDR1 cell surface expression was also observed when the cells were pretreated with CsA, a standard inhibitor of MDR1 (Fig. 3A, i, panel c). Similar prevention of cell surface MDR1 expression by adaGb₃ and CsA was seen for SKVLB cells (Fig. 3A, ii, panels a–d).

A time course pretreatment of MDR1-MDCK cells with adaGb₃

AdaGb₃ Inhibits the Efflux of Rhodamine 123 in MDR1-MDCK and SKVLB Cells—AdaGb₃ inhibition of MDR1 was tested in MDR1-MDCK cells and in SKVLB cells with the fluorescent substrate rhod123 in a confocal laser scanning microscope assay. After exposure to rhod123, cells were washed and incubated for 20 min at 37 °C before images were recorded. Confocal microscopy showed that rhod123 effluxed from MDR1-MDCK and SKVLB control cells (Fig. 4A, i, panel A, and ii, panel A). Treatment of MDR1-MDCK cells with 10 μM CsA or 50 μM adaGb₃ for 1.5 h completely inhibited the rhod123

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Inhibition of MDR1 by AdamantylGb3

Verotoxin Treatment Inhibits MDR1-mediated Rhodamine 123 Efflux—If the efficacy of adaGb3 to inhibit MDR1-dependent drug efflux were related to alteration in the membrane organization of Gb3 containing lipid rafts, internalization of cell surface Gb3, induced by verotoxin 1B subunit endocytosis might also affect MDR1-mediated cellular drug efflux. Fig. 4B shows that pretreatment of MDR1-MDCK or SKVLB cells with VT1B or VT1 for 30 min at 37 °C inhibits rhodamine efflux. This was not observed for a non-Gb3-binding VT1B subunit mutant (36). VT1 and VT1B were not as effective inhibitors as CsA or adaGb3 blocked the pumping activity of MDR1. This same inhibition pattern was observed when MDR-MDCK and SKVLB cells were treated with adaGb3 (Fig. 4A, i and ii, panels C and D). Cell treatment with adaGalCer did not prevent rho123 efflux (Fig. 4A, i and ii, panel E).

In the control or nontreated cells, most of the rho123 was pumped out and washed away, with little residual intracellular fluorescent staining. Any fluorescence retained within the cells showed a patchy distribution within the cytoplasm (Fig. 4A, i and ii, panels A and E) in these cells, whereas both CsA-treated MDR1-MDCK cells and CsA-treated SKVLB cells were intensively and homogeneously labeled (Fig. 4A, i and ii, panel B), as CsA blocked the pumping activity of MDR1. This same inhibition pattern was observed when MDR-MDCK and SKVLB cells were treated with adaGb3 (Fig. 4A, i and ii, panels C and D). Cell treatment with adaGalCer did not prevent rho123 efflux (Fig. 4A, i and ii, panel E).

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AdaGb₃ in MDR1-MDCK cells but were as effective in SKVLB cells. VT2 had a similar but smaller inhibitory effect than VT1 (not shown). TheGb₃ content of SKVLB cells is significantly less than that of MDR1-MDCK cells.

AdaGb₃ Inhibits MDR1-mediated Vinblastine Efflux in Polarized Gastrointestinal Epithelial Cells—The ability of adaGb₃ to inhibit MDR1 transport was analyzed by studying the flux of radiolabeled vinblastine in the apical:basal (A:B) and in the basal:apical (B:A) direction, as compared with verapamil, a well known inhibitor of MDR1 (Fig. 5). Apical/basolateral dependence of inhibition was also evaluated by adding adaGb₃, or verapamil as positive control, either on the apical side only or on both sides. Polarized C2BBe1 cells grown on filters were used for these experiments. Vinblastine basal-to-apical flux was higher than apical-to-basal flux, consistent with the unidirectional net transport mediated by MDR1 into the gastrointestinal lumen. 20 µM verapamil, a positive inhibitory control, decreased vinblastine basal-to-apical flux and increased apical-to-basal flux, showing inhibition of MDR1. 5 µM and 50 µM adaGb₃ showed effects similar to those of verapamil, indicating similar inhibition of MDR1. A dose response was also apparent. When the inhibitors were added only to the apical side, similar results were obtained. As shown in Fig. 5A, there was an increase in the A:B transport of [³H]vinblastine across C2BBe1 cells in the presence of inhibitor in the following order: 20 µM verapamil (3-fold) < 5 µM adaGb₃ (3.4-fold) < 50 µM adaGb₃ (4.5-fold) (Table 1). Similar results are shown in Fig. 5B, where the inhibitor was only added on the apical side as follows: 20 µM verapamil (1.5-fold) < 5 µM adaGb₃ (3-fold) < 50 µM adaGb₃ (3.6-fold) (Table 1). There was also a decrease in the B:A transport of [³H]vinblastine across C2BBe1 cells when they were treated with 50 µM adaGb₃ (1.4-fold) and similar to 20 µM verapamil (1.6-fold) treatment, even when adaGb₃ was added to the apical side only (Fig. 5, C and D, and Table 1). Pretreatment of C2BBe1 cells with 5 µM adaGb₃ showed no significant difference in the B:A transport of [³H]vinblastine (Fig. 5, C and D, and Table 1). Results are expressed as a percentage of radioactivity accumulated in the recipient side at each time point (100% is the initial concentration in the donor side of the monolayer). C2BBe1 cells are derived from theGb₃ expressing CaCo2 cell line (37) and were confirmed to express Gb₃ (not shown).

AdaGb₃ Differentially Binds the MDR1 Drug Binding Pocket as Compared with Verapamil and Cyclosporin A—It was proposed (38) that a common drug binding pocket lies at the interface between the two six-member transmembrane domains of MDR1, and that the diverse substrates bind through a “substrate-induced fit” mechanism. The common drug binding pocket is relatively large (39) and can accommodate different substrates simultaneously. We evaluated the possible binding of adaGb₃ to this common drug binding pocket by cross-linking analysis (33). If adaGb₃ binds to this drug binding pocket, cross-linking between cysteine residues of mutants prepared for binding analysis (33) would be inhibited. Membranes prepared from HEK 293 cells transfected with MDR1 expressing these cysteine substitution mutations (33) were preincubated ± adaGb₃. These mutants contain the cross-linking cysteines at different “depths” within the 6th and 7th TMDs. After the mixtures were treated with M14M cross-linker, they were subjected to Western blot analysis. Fig. 6 shows that pretreatment of the membranes with verapamil, a positive control, completely prevented cross-linking in all three mutations combinations. In contrast, no inhibition with adaGb₃ pretreatment was seen for the F343C containing mutants. However, when the mutation was earlier in TM6
(F339C), adaGb3 prevented cross-linking, indicating that adaGb3 binding overlaps but is distinct from CsA/verapamil within the common drug binding pocket.

**DISCUSSION**

MDR1 is associated with drug failure in various cancers and reduced oral drug bioavailability because the intestinal epithelial cell apical membrane is a prominent MDR1 expression site (4). Therefore, an understanding of multidrug resistance is of wide clinical importance. Although defining the mechanism of MDR1 remains a major challenge, many newer MDR1 inhibitors have been developed. However, few of these achieve clinical success because of intrinsic toxicities and effects on normal tissues expressing MDR1. MDR1 inhibitors based on physiological substrates have not been reported. Although it is not established that Gb3 is a substrate for MDR1, its precursor GSL, glucosylceramide, is a substrate (18, 40).

AdaGb3 reversed MDR in the MDR1-transfected MDCK cell line (23) and the naturally vinblastine-resistant ovarian carcinoma SKVLB cell line (41). AdaGb3 is a semi-synthetic analog of Gb3, in which the fatty acid is replaced with a rigid globular hydrocarbon frame (19). AdaGb3 inhibits Gb3 also plays a role in human immunodeficiency virus infection (43), within cholesterol-enriched lipid rafts (44). Cholesterol enhanced the interaction of gp120 and Gb3, but adaGb3 proved an even more effective receptor (45). AdaGb3 inhibits

![FIGURE 5. Effect of adamantylGb3 on gastrointestinal epithelial cell [3H]vinblastine transport.](image)

*Polarized C2BBe1 cells were preincubated for 45 min with 5 μM (—■—) or 50 μM (—▲—) adaGb3, or 20 μM verapamil (—○—) as positive control in serum-free medium on both apical and basal sides, and apical side only. [3H]Vinblastine was added at time = 0 min to the apical side only for apical-to-basal (A:B) transport, or to the basal side only for basal-to-apical (B:A) transport. Each point is the mean ± S.D. (n = 3) expressed as a percentage (%) of flux over time. [14C]Mannitol was used as an extracellular marker. A and B, A:B [3H]vinblastine transport; C and D, B:A [3H]vinblastine transport. A and C, treatment with inhibitors (adaGb3 or verapamil) on both sides; B and D, treatment with inhibitors (adaGb3 or verapamil) on apical side only. Each point represents mean value and S.D. of three independent filter preparations. Untreated cells, —.*

**TABLE 1**

**Summary of effect of adaGb3 on MDR1-mediated [3H]vinblastine transport**

| Direction | Treatment on apical and basal sides (A+B) | Treatment on apical side only (A only) |
|-----------|------------------------------------------|---------------------------------------|
|           | 20 μM verapamil | 5 μM adaGb3 | 50 μM adaGb3 | 20 μM verapamil | 5 μM adaGb3 | 50 μM adaGb3 |
| A:B       | 3-Fold increase | 3.4-Fold increase | 4.5-Fold increase | 1.5-Fold increase | 3-Fold increase | 3.6-Fold increase |
| B:A       | 1.6-Fold decrease | 1.1-Fold decrease | 1.4-Fold decrease | 1.4-Fold decrease | 1.2-Fold decrease | 1.3-Fold decrease |
Inhibition of MDR1 by AdamantylGb3

The prevention of MDR1 cell surface expression by GSL depletion, partial VT1/MDR1 cell surface co-localization, and the inhibitory effect of VT1/VT1B on MDR1 infer a link between Gb3 and MDR1, in addition to its flippase role in GSL biosynthesis already reported (17, 18, 47). Studies on the Gb3-binding SAL protein from *Silurus asotus* show that this lectin can activate MDR1, only in Gb3-expressing cell lines (48).

Our findings that adaGb3 abrogated cell surface expression of MDR1 in both MDR1-MDCK and SKVLB cells, increased intracellular MDR1, sensitized MDR1-MDCK and SKVLB cells to vinblastine cytotoxicity similar to CsA, increased apical-to-basal transport of vinblastine, even more effectively than verapamil, in C2BBe1 cells, and blocked rhodamine 123 efflux, similar to CsA, show that adaGb3 is an effective inhibitor of MDR1.

MDR1 is localized in the apical plasma membrane, enriched in cholesterol, GSLs, and sphingomyelin (49, 50). Although the role MDR1 plays in the transport and esterification of cholesterol (51, 52) has been questioned (53), membrane cholesterol binds and modulates MDR1 function (54). Cholesterol homeostasis also plays a significant role in GSL trafficking (55) and vice versa. MDR1 is predominantly localized in low density GSL/cholesterol-enriched domains (rafts) (56–59). MDR1 within rafts showed optimal ATPase activity and was activated by verapamil, but non-raft MDR1 had lower ATPase and was inhibited by verapamil (60). GSL depletion switched MDR1 from lipid raft fractions to more dense non-raft areas (60).

Because MDR1 function is dependent on cholesterol-enriched lipid rafts (60, 61), which are also GSL-enriched, and Gb3 is in rafts (62), modulation of the MDR1/raft/GSL environment may be a feature of adaGb3 MDR1 inhibition. Our data show that adaGb3 or CsA treatment prevents cell surface MDR1 immunostaining, whereas intracellular MDR1 accumulates. Thus, Gb3-containing lipid rafts may be important for intracellular MDR1 surface trafficking. Inhibitor-induced loss of plasma membrane MDR1 expression has not been reported previously, but the increased intracellular accumulation of MDR1 after CsA treatment, although counterintuitive, is often found (63). The binding of inhibitor to the drug site between TMD6 and TMD7 to prevent MDR1 surface expression, and the similar effect of GSL depletion, implies that this drug-binding site may be a lipid (GSL)-binding site, involved in MDR1 trafficking. GSLs can play roles in protein intracellular transport (64). The lipid flipase activity of MDR1 (65) may provide a basis for drug efflux (66) and surface expression.

The effect of VT1B/VT1 to prevent rhodamine123 efflux further highlights the importance of Gb3 containing lipid rafts for plasma membrane MDR1 function. Unlike adaGb3, VT1B internalization/cell surface Gb3 depletion did not result in loss of cell surface MDR1 staining, although intracellular MDR1 synthesis was nevertheless increased (but less than for adaGb3). A significant fraction of surface MDR1 is not co-localized with Gb3, and this could therefore be VT1B-insensitive. This would be consistent with the more temporary VT1/VT1B inhibition of MDR1-mediated drug efflux. VT1 internalization selectively depletes cellsurfaceGb3 (67), and this can affect other cholesterol-dependent cell surface antigens (68). After internalization of the toxin-Gb3 complex, the plasma membrane Gb3 raft complement is reduced for at least 1 h (69). The eventual recovery of cell surface Gb3 rafts after VT1 treatment may explain the lack of effect of VT1B treatment on long term MDR1-MDCK vin-

human immunodeficiency virus infection in vitro and prevents gp120-mediated host cell fusion, opening a new therapeutic route (46).
blastine resistance we observed (not shown). Treatment of mice with VT2 has been shown to compromise the MDR1-dependent blood/brain barrier and to induce MDR1 overexpression (70). Our studies may provide a cellular counterpart to these results.

The increase in cell surface expression of MDR1 seen in short time course with MDR1 inhibitors has been described, but this is nonfunctional because rhodamine efflux is inhibited at this time. The down-regulation of surface MDR1 expression following more prolonged cell treatment with an MDR1 inhibitor has not been generally reported, but this could be a significant component in the mechanism of inhibitor action. This clearly occurs while total cellular MDR1 is markedly elevated, implying a differential effect on cell surface MDR1 trafficking. This may be a self-amplification phenomenon. If GSL/cholesterol-enriched lipid microdomains are a key component in MDR1 trafficking/maturation/function, inhibitors will prevent Golgi MDR1-mediated GlcCer translocation to inhibit neutral GSL biosynthesis (18) and thus further compromise MDR1 membrane organization/function. If MDR1 is also involved in cholesterol homeostasis (51), MDR1 inhibition could also restrict raft function to further deplete MDR1 activity.

The interface between the TM6 and TM7, which are the C-terminal and N-terminal membrane spanning sections of the 1st and 2nd TMD hexamers, has been implicated as the position of the MDR1 common drug-binding site (71). Cross-linking binding analysis of adaGb3 within this common drug binding pocket at this interface between the transmembrane domains of the two homologous halves of MDR1 using Cys mutants in TM6 and -7 (72) surprisingly showed that adaGb3 (unlike CsA or verapamil) did not inhibit disulfide cross-linking when the TM6 cysteine was close to the bilayer surface and the TM7 in the exofacial region. However, when the TM6 cysteine was moved toward the cytosolic face by one α-helix turn, adaGb3 prevented cross-linking (IC_{50} < 5 μM). Only this central region of TM6 (Leu-339) is directly involved in the drug-stimulated conformational change required for the NBD-mediated ATP hydrolysis, dependent MDR1 function (73). Therefore, adaGb3 binding, in part, overlaps that of CsA/verapamil/vinblastine but is more restricted to a functional domain deeper within the bilayer. Modification of the L339C residue altered signal transduction from several distinct MDR1 drug-binding sites (73), suggesting adaGb3 should prove effective against many MDR1 substrates. AdaGb3/MDR1 binding may be similar to colchicine, demecolcine, Hoescht 33342, or flupentixol that similarly do not inhibit TM6 F343C disulfide cross-linking (33).

It is clear that MDR1 can be expressed in cells lacking Gb3. In such cases, other GSLs may substitute (74). However, drug-resistant metastatic ovarian tumor cells have a particularly high Gb3 content (75), and Gb3 is highly expressed in metastatic colon carcinoma (76).

The strong MDR1 reversal effects of adaGb3, as well as its favorable in vivo features make it a possible choice for inhibition of MDR1 to increase bioavailability of drugs across the intestinal epithelium. Although adaGb3 MDR1 inhibition is selective, because adaGalCer was unable to sensitize either MDR1-MDCK or SKVLB cell lines to vinblastine or to abrogate cell surface expression of MDR1, a hexanoyl derivative of GlcCer has been shown to inhibit MDR1 in a drug-resistant ovarian cancer cell line (77). Interestingly, GlcCer has been implicated in the maintenance of Gb3 within lipid rafts (78). Thus, specific GSL analogs provide a new approach to MDR reversal.

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