Award Number: W81XWH-04-1-0491

TITLE: Mechanisms of Chemoresistance in Breast Cancer Cells

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REPORT DATE: May 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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**Mechanisms of Chemoresistance in Breast Cancer Cells**

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**ABSTRACT (Maximum 200 Words)**

Our previous studies showed that both glucosylceramide synthase (GCS) and P-glycoprotein (P-gp) are overexpressed in Adriamycin-resistant human breast cancer cells, MCF-7-AdrR cells. When these cells were transfected with GCS antisense (asGCS), a stable 30% decrease in GCS activity was obtained. Experiments with paclitaxel (Taxol) showed that intracellular levels of drug were 8.6-fold greater in the asGCS-transfected cell line, MCF-7-AdrR/asGCS, compared to MCF-7-AdrR cells. In assessing P-gp, we observed a dramatic decrease in the level of MDR1 expression (80%) by RT-PCR that translated into a similar decrease in P-gp protein levels. To confirm the influence of GCS on MDR1 expression, we inhibited GCS. Treatment of MCF-7-AdrR cells with GCS inhibitor, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), or with GCS siRNA, produced a significant decrease in MDR1 mRNA levels compared to untreated. These results were used in manuscript for publication, which showed that P-gp expression can be downregulated by either GCS antisense transfection or chemical inhibition of GCS. In order to determine whether overexpression of GCS is a general characteristic of chemotherapy resistance, we assessed GCS expression and glycolipid levels in Adriamycin-, cisplatin-, etoposide-, and paclitaxel-resistant breast cancer cells.

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**Subject Terms**
Clucosylceramide synthase, P-glycoprotein, chemotherapy

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**Supplementary Notes**

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**Security Classification**

- **Report**: Unclassified
- **Page**: Unclassified
- **Abstract**: Unclassified

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**DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

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**Security Classification of ABSTRACT**: Unlimited

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**Number of Pages**: 21

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**Price Code**: Unlimited

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**Form Approved**
OMB No. 074-0188

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INTRODUCTION

Poor response to chemotherapy is a major clinical problem, and in most instances drug resistance is underlying cause (1). This is a most undesirable situation, and patients and oncologists would welcome its possible correction. In breast cancer nearly 50% of patients demonstrate primary and/or secondary resistance to Adriamycin (2). Several mechanisms of drug resistance are being examined, and avenues to reverse resistance are being sought. Research strategies in this area have become increasingly aimed at molecular targets such as P-glycoprotein (P-gp), multidrug resistance associated protein (MRP), topoisomerase, and Bcl-2 protooncogene, to name a few. Overexpression of the membrane efflux transporter, P-gp is one of the most consistent biological alterations in drug resistance (1,3). P-gp (170 kDa) is the product of the mdr1 gene, an energy-dependent pump that reduces the intracellular concentration of specific anticancer drugs, and it has been studied extensively. Our approach to drug resistance is new and involves ceramide metabolism. The area has been reviewed (4,5). Several front-line anticancer agents elicit the formation of ceramide, a proapoptotic lipid messenger (6,7), by activating either the de novo or sphingomyelinase pathways of ceramide production (4,7). Drugs that induce cellular ceramide generation include anthracyclines (Adriamycin), Vinca alkaloids, etoposide (VP-16), paclitaxel, and fenretinide (4-HPR). If ceramide formation in response to drug treatment is blocked, then the cytotoxic impact of the drug is largely reduced (8,9). This demonstrates ceramide's role in drug responses. Ceramide added directly to cells circumvents the enzyme route of ceramide formation and promotes an apoptotic cascade directly (9,10). Our group showed that increased cellular capacity for ceramide glycosylation, catalyzed by glucosylceramide synthase (GCS), is associated with chemotherapy resistance in cancer cells (11-15). In cultured breast cancer cells, sensitivity to anthracyclines and taxanes can be decreased or increased simply by manipulation of GCS activity (13,16,17). For example, transfection of drug-sensitive MCF-7 breast cancer cells with GCS cDNA confers resistance to Adriamycin (16), and tranfection of multidrug-resistant MCF-7-AdR breast cancer cells with antisense GCS (asGCS) increases cell sensitivity to chemotherapy by a factor of 28-fold for Adriamycin, more than 100-fold for vinblastine, and more than 200-fold for paclitaxel (13).
Training
- Real-Time RT-PCR Analysis
During the past year, I have learned the technique of real-time RT-PCR. I have worked to determine the best conditions to quantify gene expression in our system. I have designed sequences of primers and probes that we actually use, and I have also compared different reagents. Using this technique, I have accurately measured the levels of GCS and MDR1 expression in various cancer cell lines, under different experimental conditions.

- Deviations from the original statement of work
The aim of the study is to understand the role of glucosylceramide synthase (GCS) in the mechanism of drug resistance in breast cancer. We used a slightly different approach than we planned when we wrote the project, namely, we incorporated the use of another cell line, MCF-7-AdrR/asGCS. This cell line is an Adriamycin-resistant breast cancer cell line stably transfected with GCS antisense. In this cell line, GCS activity is knocked down by 30%. This tool helped us to understand the implications of GCS in the mechanisms of drug resistance in breast cancer cells, in particular as regards P-gp. This research allowed us to make important discoveries on the connection between GCS and P-gp, and this work has been reported in a publication (18). Moreover, this work can be related to task three of the statement of work: "Determine whether overexpression of GCS and overexpression of P-gp are allied or dissociated".
Key Research Accomplishments

1. Influence of asGCS transfection on paclitaxel-induced ceramide accumulation.
   Using mass analysis, we found that ceramide production in response to paclitaxel (1.0 μmol/L) was enhanced 3-fold in MCF-7-AdrR/asGCS cells compared with parental MCF-7-AdrR cells after 24 hour treatment. This shows that ceramide glycosylation is retarded by antisense GCS (because of high free ceramide levels).

2. Chemotherapy uptake in MCF-7-AdrR and MCF-7-AdrR/asGCS cells.
   Experiments with radiolabeled chemotherapy drugs showed that after 60 minutes, intracellular levels of paclitaxel were 8.6-fold greater in MCF-7-AdrR/asGCS cells compared with MCF-7-AdrR cells. This means that either drug uptake is enhanced or efflux is subdued.

3. P-gp expression in MCF-7-AdrR and MCF-7-AdrR/asGCS cells.
   Because of pronounced differences in drug levels in the two cell lines, we assessed P-gp expression by mRNA and protein determinations. The level of MDR1 mRNA, evaluated by reverse RT-PCR was dramatically lower in MCF-7-AdrR/asGCS cells compared with MCF-7-AdrR cells. We confirmed this by Western blot. Whereas, MCF-7-AdrR cells contained characteristically elevated levels of P-gp, MCF-7-AdrR/asGCS cells displayed a dramatic decrease (~80%) in P-gp levels.

4. Influence of PPMP on MDR1 gene expression in MCF-7-AdrR cells.
   To determine whether decreased MDR1 expression in asGCS cells was really due to GCS inhibition and/or downregulation, we evaluated the influence of D-L-threo-PPMP, a chemical inhibitor of GCS, on MDR1 expression in MDR1-rich MCF-7-AdrR cells. PPMP greatly diminished the expression of MDR1 in MCF-7-AdrR cells, with demonstrated stereospecificity. Unlike D-L-threo-PPMP, D-L-erythro-PPMP is not a GCS inhibitor, and this stereoisomer had no influence on MDR1 expression.
   Real-time RT-PCR showed that MDR1 expression in MCF-7-AdrR cells treated with D-L-threo-PPMP and D-L-erythro-PPMP was reduced by 58% and 12%, respectively, compared with untreated MCF-7-AdrR cells.

5. Influence of GCS siRNA on MDR1 gene expression in MCF-7-AdrR cells.
   To reinforce the results obtained with PPMP and to confirm that changes in MCF-7-AdrR/asGCS cellular MDR1 expression were not due to clonal artifacts, we used GCS siRNA to treat MCF-7-AdrR cells. siRNA was introduced into cells using lipofectAMINE in serum-free medium for 4 hours. After 48 hours, both GCS and MDR1 mRNA were dramatically decreased by GCS siRNA compared with lipofectAMINE only controls. The siRNA had no effect on expression levels of α-actin.
6. **GCS expression in chemotherapy-resistant breast cancer cells.**

For this study, we used several breast cancer cell lines:
- Adriamycin-resistant cells, MCF-7-AdrR; paclitaxel-resistant cells MCF-7/PtxxR30, and the matched wild type MCF-7.
- cisplatin-resistant cells, MCF-7/CDDP, and the matched wild-type, MCF-7/P.
- etoposide-resistant cells, MCF-7/VP, and the matched wild-type, MCF-7/F.

The levels of GCS mRNA were surveyed by RT-PCR. The highest GCS expression was observed in MCF-7-AdrR cells compared to wild-type breast cancer cells, whereas aGCS expression in MCF-7/CDDP cells was only slightly higher compared to MCF-7/P. A decrease in GCS mRNA was observed in MCF-7/VP cells compared to MCF-7/F cells.

To better quantitate GCS expression, we employed realtime RT-PCR.

GCS mRNA was 2.2- and 1.6-fold higher in MCF-7-AdrR and MCF-7/CDDP cells, respectively, compared to their wild-types. GCS expression in etoposide resistant cells, MCF-7/VP, was lowered by 36% compared to MCF-7/F.

7. **Determination of glucosylceramide (GC) mass in drug-resistant breast cancer cells.**

For GC quantitation, total lipids were extracted and analyzed by thin-layer chromatography (TLC) using a solvent system containing chloroform/methanol/NH₄OH (70:20:4, v/v/v). Approximately 250 µg of lipid were loaded per lane. After development, TLC plates were sprayed with sulfuric acid and heated (30 min, 180°C). The GC chars were quantitated by scanning densitometry, using a GC standard curve (0.5-5.0 µg). The results showed that GC mass was 3-fold higher in MCF-7-AdrR cells compared to the wild-type counterpart (3.95 versus 1.25 µg GC), and 1.76-fold higher in MCF-7/CDDP compared to wild-type counterpart (0.75 versus 0.425 µg). We found that GC mass decreased by 40 and 84% respectively, in etoposide- and paclitaxel-resistant cells, compared to the wild-type cells.

Key Research Accomplishments
- Mastered technique of real-time RT-PCR.
- Determined that paclitaxel induces ceramide accumulation in MCF-7-AdrR/asGCS cells.
- Determined that chemotherapy uptake is much higher in MCF-7-AdrR/asGCS cells compared to MCF-7-AdrR cells.
- Demonstrated that P-gp expression is lower in MCF-7-AdrR/asGCS cells compared to MCF-7-AdrR cells.
- Determined that PPMP diminishes the MDR1 expression in MCF-7-AdrR cells.
- Demonstrated that GCS siRNA decreases MDR1 gene expression in MCF-7-AdrR cells.
- Determined that GCS is overexpressed in Adriamycin- and cisplatin-resistant breast cancer cells, but not in etoposide-resistant cells, compared to wild type.
- Determined that GC mass is elevated in Adriamycin-, and in cisplatin-resistant cells, compared to wild-type cells, but it is diminished in etoposide- and paclitaxel-resistant cells compared, to wild-type cells.
Reportable Outcome

Oral Presentation

Publication
CONCLUSION

In conclusion, our work shows that limiting GCS activity by either GCS antisense transfection, or PPMP treatment down-regulates the expression of P-gp. Moreover, in certain breast cancer cells resistant to chemotherapy (13), we found that overexpression of GCS correlated with an elevation of GC mass. This suggests that lipids play a role in multidrug resistance and that targeting glycolipid biosynthesis could be a promising approach for enhancing chemotherapy.
REFERENCES

APPENDICES
Glucosylceramide Synthase Blockade Down-Regulates P-Glycoprotein and Resensitizes Multidrug-Resistant Breast Cancer Cells to Anticancer Drugs

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Abstract

Overexpression of glucosylceramide synthase (GCS), a pivotal enzyme in glycolipid biosynthesis, contributes to cancer cell resistance to chemotherapy. We previously showed that transfection of doxorubicin-resistant MCF-7-AdrR cells with GCS antisense restored cell sensitivity to doxorubicin and greatly enhanced sensitivity to vinblastine and paclitaxel. In that study, doxorubicin promoted generation of ceramide in MCF-7-AdrR/GCS antisense cells; the present study implicates factors in addition to ceramide that augment sensitivity to chemotherapy. Although GCS antisense cells showed enhanced ceramide formation compared with MCF-7-AdrR when challenged with paclitaxel, GCS antisense cells also showed a 10-fold increase in levels of intracellular drug (paclitaxel and vinblastine). In addition, transfected cells had dramatically decreased expression (60%) of P-glycoprotein and a 4-fold decrease in the level of cellular gangliosides. Chemical inhibition of GCS produced the same effects as antisense transfection: exposure of MCF-7-AdrR cells to the GCS inhibitor 1-phenyl-2-palmitoylamino-3-morphollno-I-propanol (PPMP, 5.0 μmol/L, 4 days) decreased ganglioside levels, restored sensitivity to vinblastine, enhanced vinblastine uptake 3-fold, and diminished expression of MDR1 by 55%, compared with untreated controls. A similar effect was shown in vinblastine-resistant KB-VO.O1 cells; after 7 days with PPMP (10 μmol/L), MDR1 expression fell by 84% and P-glycoprotein protein levels decreased by 50%. MCF-7-AdrR cells treated with small interfering RNAs to specifically block GCS also showed a dramatic decrease in MDR1 expression. This work shows that limiting GCS activity down-regulates the expression of MDR1, a phenomenon that may drive the chemosensitization associated with blocking ceramide metabolism. The data suggest that lipids play a role in the expression of multidrug resistance. (Cancer Res 2005; 65(9): 3861-7)

Introduction

Development of resistance to chemotherapeutic agents is a major concern in cancer patients. Resistance to chemotherapy is associated with myriad mechanisms that decrease drug cytotoxicity. Two members of the large family of ABC transporters confer multidrug resistance (MDR) in human cancer cells: P-glycoprotein and multidrug resistance protein (MRP). P-glycoprotein, a membrane-resident glycoprotein encoded by the MDR1 gene, decreases the intracellular concentration of anticancer agents by acting as a drug efflux pump (1, 2). P-glycoprotein exports many types of chemotherapy drugs, including Vinca alkaloids, anthracyclines, paclitaxel, actinomycin D, and epipodophyllotoxins. Like P-glycoprotein, MRP is a transport protein (3); however, the transport of unconjugated chemotherapeutic agents by MRP seems to require glutathione. MDR in tumors can also be caused by overexpression of proteins such as antiapoptotic proteins belonging to the Bcl-2 family (4, 5) and by loss of tumor suppressor gene p53 (6, 7). Others factors responsible for chemotherapy resistance include reduction of topoisomerase II activity (8), modification of glutathione S-transferase activity (9), and up-regulation of rafts and caveolae, which are glycosphingolipid-enriched constituents of microdomains (10).

Glucosylceramide synthase (GCS) catalyzes the first glycosylation step in the biosynthesis of glycosphingolipids (11, 12). This central enzyme of ceramide metabolism has also been implicated in MDR (13). Glycosphingolipids, including glucosylceramide, lactosylceramide, and gangliosides, play an essential role in cell development, cell death, tumor progression, and pathogen/host interaction (13, 14). In addition, membrane gangliosides can decrease the sensitivity of human melanoma cells to ionizing radiation (15). In that study, doxorubicin made radiosensitive by exposure to either fumonisin B1, which blocks ganglioside biosynthesis at the juncture of ceramide synthesis, or Vibrio cholerae neureaminidase, which cleaves cell surface gangliosides. Conversely, adding bovine brain GM1 to radiosensitive melanoma cells conferred radioresistance (16). Targeting glycolipid metabolism has proven useful in altering chemotherapy responses in numerous human tumor cell lines (15, 16–18).

In previous studies, we increased the level of MDR by transfecting doxorubicin-resistant human breast cancer cells (MCF-7-AdrR) with GCS, and we enhanced cellular sensitivity to anthracyclines, Vinca alkaloids, and taxanes by transfecting MCF-7-AdrR cells with GCS antisense (16, 19). Although doxorubicin treatment of GCS antisense transfectants increased intracellular levels of ceramide (16), which is a second messenger of apoptosis, the extremely high sensitivity of MCF-7-AdrR/GCS antisense cells to Vinca alkaloids and taxanes suggested the participation of mechanisms other than ceramide signaling in cellular responses. We have observed equivalent intracellular levels of rhodamine-123 in MCF-7-AdrR/GCS antisense cells and in rhodamine-123–exposed MCF-7 parental cells, which indicates that GCS antisense transfection reverts drug retention in MCF-7-AdrR cells on a par with the drug-sensitive phenotype (16, 19). Because rhodamine-123 is a substrate for P-glycoprotein, we began to investigate the influence of glycolipid metabolism on function and expression of MDR1 and P-glycoprotein. The present study shows that MDR1 and
P-glycoprotein expression can be down-regulated by GCS antisense transfection or chemical inhibition of GCS. We suggest that this containing 0.1% Tween 20, at room temperature for P-glycoprotein expression can be down-regulated by which is resistant to doxomycin (20), was kindly provided by Dr. Kenneth mmol/L could be a promising approach for enhancing chemotherapy.

Cancer Research

Materials and Methods

Cell cultures. The MCF-7-AdrR human breast adenocarcinoma cell line, which is resistant to doxorubicin (20), was kindly provided by Dr. Kenneth mmol/L Biochemical, Bra. CA), or 500 nmol/L vinblastine plus 0.25 pCi 

RNA analysis. Total RNA was isolated using the RNAprotect Mini Kit from Qiagen (Valencia, CA, USA). MDR1 reverse transcription-PCR (RT-PCR) was carried out by a one-step method (SuperScript One-Step RT-PCR with Platinum Taq; Invitrogen, Chicago, IL). Total RNA (5 μg) was added to containing 0.2 mM deoxynucleotide triphosphates, 1.5 mmol/L MgSO4, 0.6 μL of SuperScript II H/Ptumgene, Copenhagen, NJ). In the presence of 20% SDS, intracellular radioactivity was measured by liquid scintillation counting.

Western blot for P-glycoprotein. Confident monoclonals of MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells were rinsed, harvested in PBS, and lysed in a PBS buffer containing 10% glycerol, 1% Triton X-100, 10 mmol/L Na2VO4, 10 μmol/L P-glycerophosphate, 50 mmol/L NaF, 0.1 μmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, and 10 μg/mL aprotinin for 30 minutes on ice. The mixture was centrifuged at 11,000 × g for 15 minutes at 4°C. Equal aliquots of protein (25 μg) were resolved using 4% to Western blot for P-glycoprotein. Confident monoclonals of MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells were rinsed, harvested in PBS, and lysed in a PBS buffer containing 10% glycerol, 1% Triton X-100, 10 mmol/L Na2VO4, 10 μmol/L P-glycerophosphate, 50 mmol/L NaF, 0.1 μmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, and 10 μg/mL aprotinin for 30 minutes on ice. The mixture was centrifuged at 11,000 × g for 15 minutes at 4°C. Equal aliquots of protein (25 μg) were resolved using 4% to 20% gradient SDS-PAGE (Invitrogen, Chicago, IL). The transferred nitrocellulose blot was blocked with 5% fat-free milk powder in PBS containing 0.1% Tween 20, at room temperature for 1 hour. The membrane was incubated, blocked with 0.2 μCi/mL of [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 pCi [3H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblas...
MDR1 induction by glycolipids. KB-V0.01 cells were seeded into 6.0-cm
plates in complete medium. After 24 hours, medium was removed and cells
were incubated in 5% FBS DMEM medium containing either 50 μg/mL Cs
β-β-D-glucosylceramide (Avanti Polar Lipids, Alabaster, AL), 0.5 μM/L
doxorubicin (JKT Laboratories, Ste Paul, MN) as positive control, or 10 μg/mL
palmitic acid (Sigma) as negative control. Cells were treated for 48 hours,
and RNA was analyzed by real-time RTPCR.

Chemical inhibition of glucosylceramide synthase. α-Threo-1-
phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) was from Bio-
mol Research Laboratories (Plymouth Meeting, PA) and α-erythro-PPMP
and β-threo-PPMP were from Matreya (Pleasant Gap, PA). These reagents
were used as described in the figure legends.

Statistical Analyses. Student's t test was used for statistical analysis.

Results

Previously, we showed that GCS antisense transfection of multichick agent-resistant MCF-7-AdrR cells enhanced cell sensitivity to
doxorubicin, vinblastine, and paclitaxel (19). A doxorubicin-
induced increase in ceramide levels and caspase activity is in
keeping with ceramide-mediated cytotoxic responses to chem-
otherapy (16), but it is not clear whether ceramide is the only factor
involved in the significantly (>100-fold) increased sensitivity of GCS
antisense-transfected cells to vinblastine and paclitaxel. We confirmed this by Western blot; whereas MCF-7-AdrR cells
were Incubated in PBS DMEM medium containing either 30 μg/mL Cs
intracellular drug levels and on P-glycoprotein expression. For this
reason, we investigated whether partial inhibition of
drug efflux is the most widely characterized drug resistance
mechanism in cancer cells (28), and it is highly expressed in
MCF-7-AdrR cells (19). We previously reported that levels of
rhodamine-123, a substrate of P-glycoprotein, were ~5-
fold higher in MCF-7-AdrR/GCS antisense compared with
MCF-7-AdrR cells (19). This suggests that GCS antisense transfection
alters drug uptake and/or retention. Experiments with
chemotherapy drugs showed that after 60 minutes, intracellular
levels of vinblastine were 12-fold greater in MCF-7-AdrR/GCS antisense compared with MCF-7-AdrR cells (Fig. 2). Similarly,
uptake of paclitaxel increased 8.6-fold in GCS antisense trans-
fectedants compared with MCF-7-AdrR cells (Fig. 2).

Because of the pronounced differences in drug levels in the two
cell lines, we assessed P-glycoprotein expression, by mRNA
and protein. As shown in Fig. 3, the level of MDR1 mRNA, evaluated
by reverse transcription-PCR (RT-PCR), was dramatically lower
less than 30% in P-glycoprotein expression. Thus,
the stable 30% decrease in GCS activity of MCF-7-AdrR/GCS antisense compared with MCF-7-AdrR cells. We
confirmed this by Western blot; whereas MCF-7-AdrR cells
contained characteristically elevated levels of P-glycoprotein,
MCF-7-AdrR/GCS antisense cells were nearly devoid of P-
glycoprotein. These data suggest that high drug levels attainable
in MCF-7-AdrR/GCS antisense cells are a consequence of the
expression (Fig. 2). Of particular interest is membrane lipid
composition.

Steady-state [H]palmitic acid radiolabeling (24 hours) of MCF-
7-AdrR/GCS antisense and MCF-7-AdrR cells showed, in the
former, a 30% decrease in sphingomyelin levels and a 44%
decrease in the level of phosphatidylglycerol (data not shown).

Figure 1. Influence of chemotherapy on ceramide formation in MCF-7-AdrR and MCF-7-AdrR/GCS antisense (asGCS) cells. A, assays using radioactivity. Cells
were treated with vinblastine (0.5 μmol/L) or paclitaxel (1.0 μmol/L) for the times shown in medium containing [H]palmitic acid. Trifluorinated ceramide was evaluated in the
lipid extract by TLC and liquid scintillation spectroscopy as detailed in Materials and Methods. For comparison purposes, ceramide counts in MCF-7-AdrR control
(no drug) were 11,860 ± 3,752 cpm/500,000 cpm total lipid and 11,084 ± 2,948 cpm/500,000 cpm total lipid in control (no drug) MCF-7-AdrR/GCS antisense cells.
Points, mean from three experiments; bars, ±SD. B, ceramide assay using mass analysis. Cells were treated ± paclitaxel (1.0 μmol/L) for 24 hours and ceramide
was evaluated by TLC sulfuric acid staining and photodensitometry as detailed in Materials and Methods. Ceramide mass in both MCF-7-AdrR control
and MCF-7-AdrR/GCS antisense control and MCF-7-AdrR/GCS asGCS paclitaxel treated was 1.0 and 3.35 μg, respectively. Total lipid (80 h) was loaded in each lane.

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There were no significant differences in cholesterol esters or other glycerophospholipids between transfected and parent cells. Because GCS is pivotal in the genesis of cerebrosides and gangliosides, we also looked for changes in glycolipid content. Although MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells expressed a similar ganglioside pattern (GM3, GM2, GD3, and GD1a; data not shown), sialic acid assay showed that the level of gangliosides was 4-fold lower in GCS antisense transfected cells (data not shown).

To determine whether depletion of gangliosides and reduced expression of P-glycoprotein were strictly a consequence of GCS down-regulation by antisense transfection, we evaluated the influence of DL-threo-PPMP, a chemical inhibitor of GCS (29–31), on ganglioside synthesis and P-glycoprotein expression in MCF-7-AdrR cells. A 4-day exposure to DL-threo-PPMP produced a 34% decrease in ganglioside levels in MCF-7-AdrR cells (Fig. 4A). Moreover, Pglycoprotein expression was diminished by 70% in MCF-7-AdrR cells, with shown stereospecificity (Fig. 4B). Unlike DL-threo-PPMP, DL-erythro-PPMP is not a GCS inhibitor (32), and this stereosomer had no influence on MDRI expression.

Real-time RT-PCR showed that MDRI expression in MCF-7-AdrR cells treated with DL-threo-PPMP and DL-erythro-PPMP was reduced by 58% and 12%, respectively, compared with untreated MCF-7-AdrR cells (Fig. 4B). To reinforce the results obtained with PPMP and to confirm that changes in MCF-7-AdrR/GCS antisense cellular MDRI expression were not due to clonal artifacts, we used GCS siRNA to treat MCF-7-AdrR cells. As shown in Fig. 4C, after 48 hours, both GCS and MDRI mRNA were dramatically decreased by GCS siRNA compared with LipofectAMINE only controls. The siRNA had no effect on β-actin expression levels.

Whether chemical lowering of MDRI expression affects cellular response to chemotherapy was next evaluated. Treatment of MCF-7-AdrR cells for 4 days with DL-threo-PPMP enhanced vinblastine uptake by ~3-fold at 30 and 60 minutes (Fig. 4D), and as illustrated in Fig. 4E, vinblastine cytotoxicity, even at low concentrations (0.1 μmol/L), was enhanced ~60% in cells that had been cultured with DL-threo-PPMP. Thus, like GCS antisense transfection, inhibition of GCS by chemical means reversed resistance of MCF-7-AdrR cells to vinblastine. To examine the generality of this response, we used KB-V0.01 cells, a head/neck multidrug-resistant epidermoid carcinoma cell line that expresses both GCS and MDRI (33). As shown in Fig. 5A, MDRI expression in KB-V0.01 cells was lowered 70% by DL-threo-PPMP (10 μmol/L) and 38% by DL-erythro-PPMP (15 μmol/L). Therefore, the DL-threo isomer is the most effective inhibitor of GCS compared with the racemic mixture. KB-V0.01 cell treatment with DL-threo-PPMP for a prolonged period (7 days) induced a dramatic decrease (84%) in MDRI mRNA levels (Fig. 5B). Moreover, P-glycoprotein protein levels in these cells diminished by 50%, compared with the untreated control (Fig. 5C). We next investigated the effect of glycolipid supplementation on MDRI expression. Growth of KB-V0.01 cells with cell-permeable C8-glucosylceramide (30 μg/mL) elicited a 2-fold increase in MDRI mRNA levels (Fig. 5D), a response nearly comparable to the influence of Adriamycin (0.5 μmol/L) on MDRI expression (Fig. 5E). Palmitic acid, used as a lipid control, had no influence on MDRI expression.
Modulation of P-Glycoprotein by Glycolipids

Figure 4. Influence of GCS blockade via PPMP and siRNA on ganglioside levels, MDR1 expression, and drug uptake and chemosensitivity in multidrug-resistant MCF-7-AdrR cells. A and B, Influence of PPMP on ganglioside levels and MDR1 expression. A, MCF-7-AdrR cells were incubated with D-threo-PPMP (5.0 μM/L, 96 hours). Gangliosides were extracted and quantified by alicyclic acid derivatization. PPMP treatment did not alter cell doubling time. B, MDR1 mRNA levels in MCF-7-AdrR cells incubated in the absence and presence of either D-threo-PPMP or D-erythro-PPMP (5.0 μM/L, 48 hours). D-erythro-PPMP did not influence cell growth; the threo form retarded cell growth 20%, compared with control. RNA was extracted and quantified by real-time RT-PCR. C, Influence of GCS siRNA (100 nM/L, 48 hours) on ganglioside levels and MDR1 mRNA expression in MCF-7-AdrR cells. LipofectAMINE alone was used as control. D, Vinblastine uptake was determined in MCF-7-AdrR cells preincubated with D-threo-PPMP (5.0 μM/L, 96 hours). PPMP did not affect cell growth. E, Vinblastine cytotoxicity in MCF-7-AdrR cells preincubated with D-threo-PPMP (5.0 μM/L, 96 hours) and exposed to vinblastine at the indicated concentrations for 3 days. Cell viability was determined by MTS assay. Columns, mean of triplicates from two independent experiments; bars, ±SD. * P < 0.05; ** P < 0.01.

Discussion

The present study shows that GCS antisense transfection of multidrug-resistant human breast cancer cells modifies cellular lipid composition, reduces MDR1 expression, and enhances the cytotoxic effect of chemotherapeutic drugs. GCS antisense transfection decreased the levels of sphingomyelin in MCF-7-AdrR cells. Sphingomyelin is a major constituent of the external leaflet of the plasma membrane (34). Sphingomyelin, phosphatidylcholine, and proteins are laterally organized in biological membranes (35–37). These organized domains have been suggested to participate in cellular processes, such as signal transduction, membrane trafficking, and protein sorting (38). Expression of the principal component of caveolae, caveolin-1, in MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells, determined by Western blot, was not affected by antisense down-regulation of GCS (data not shown), although it should be mentioned that this assay is not a good estimate of the status of cellular lipids in rafts/caveolae. However, we found that transfected cells had lower levels of gangliosides, the alicyclic acid–containing glycosphingolipids. Gangliosides have been shown to influence lipid order and hydration of the lipid bilayer; such changes could play an important role in modulation of transmembrane molecular events (39). Moreover, gangliosides have been shown to influence membrane fluidity (40–42). Cellular ganglioside levels decreased 4-fold in MCF-7-AdrR/GCS antisense cells compared with MCF-7-AdrR cells. Such a change could modify membrane permeability and facilitate entrance of natural-product chemotherapeutic agents such as vinblastine and paclitaxel.

In addition to their role as a structural component of the plasma membrane, gangliosides might regulate signaling events. In melanoma cells, transient ganglioside depletion by GCS inhibition reduced tumorigenic capacity (43). Gangliosides can also induce production of nitric oxide, tumor necrosis factor-α, and cyclooxygenase 2 and activate extracellular signal-regulated kinase and c-Jun/stress-activated protein kinase kinase, p38, and nuclear factor κB (NF-κB; ref. 44). Our work showed that inhibiting the activity of GCS severely limited the expression of MDR1 and its product, P-glycoprotein. Studies have shown that the MDR1 promoter can be activated directly by anticancer agents such as vincristine, daunorubicin, doxorubicin, and colchicine (45);
limits the expression of MDR1 in human leukemia cells (53). Our independent experiments, in KB-V0.01 cells (50), and in the decreased efflux of Fgue metabolism. Other support for a link between glycolipids and A glycoprotein function through phosphorylation (47). Shabbits et al. and GD3 are thought directly Involved via modulation of P- for PDMP-mediated reversal of multidrug resistance, and GM3 leukemia cells, ganglioside depletion is believed to account has been correlated with fenretinide resistance (46). In human multidrug resistance. For example, the up-regulation of GM3 change, Increased cell doubling time were extracted and used for real-time RT-PCR (B) and Western blot analysis cellular P-glycoprotein are likely contributory to heightened real-time o,L-ftmo FRT-PCFR. In conclusion, our work shows that limiting GCS activity by however, an association between glycolipids and the MDR1 promoter has not been clearly established. Some studies have suggested that glycolipids, in particular gangliosides, modulate multidrug resistance. For example, the up-regulation of GM3 biosynthesis in ferretinide-adapted A2780 ovarian cancer cells has been correlated with ferritinide resistance (46). In human leukemia cells, ganglioside depletion is believed to account for PDMP-mediated reversal of multidrug resistance, and GM3 and GD3 are thought directly involved via modulation of P-glycoprotein function through phosphorylation (47). Shabbits et al. (48) showed a relationship between drug transport and ceramide metabolism. Other support for a link between glycolipids and multidrug resistance may be found in the PPMP-modulated expression of MDR1 mRNA in SKOV3/AdrR human ovarian cancer cells (49), in KBV200 cells (50), and in the decreased efflux of \[^{14}C\]paclitaxel and \[^{3}H\]vincristine in a neuroblastoma cell model (51). Results of other studies show that verapamil, an antihyper- sensitive formerly used clinically as a P-glycoprotein antagonist (52), limits the expression of MDR1 in human leukemia cells (63). Our group showed that verapamil, tamoxifen, and cyclosporin A block glucosylceramide formation and resultant downstream cerebrosides and ganglioside biosynthesis in drug-resistant cancer cells (54).

Previously, we showed that doxorubicin treatment of MCF-7-AdrR/GCS antisense cells enhanced the production of ceramide (16). In the present study using radiolabeling, ceramide buildup was not evident in MCF-7-AdrR/GCS antisense cells challenged with either vinblastine or paclitaxel (Fig. 1A); however, lipid mass analysis by TLC char clearly showed elevated ceramide levels in drug-challenged GCS antisense transfectants (Fig. 1B). Failure of radiolabeling techniques to accurately portray mass is not uncommon. More importantly however is the apparent dual role that GCS antisense transfection and/or GCS blockade play in sensitizing multidrug-resistant cancer cells to chemotherapy. From our experiments, it is evident that GCS antisense (i) down-regulates expression of MDRI and (ii) promotes ceramide buildup in cells that would otherwise scavenge ceramide via elevated GCS activity. This one-two punch could be of benefit in cancer treatment.

The present results suggest that glycolipids participate in MDR1 expression directly or via activation of a specific transcription factor. In a recent study, Bentires-Alj et al. (55) showed that NF-κB inhibition increased cellular uptake of daunorubicin and reduced expression of MDR1 mRNA and protein (P-glycoprotein) in colon cancer cells. NF-κB complexes can bind at a consensus NF-κB binding site in the first intron of the human MDR1 gene. Moreover, NF-κB can transactivate an MDR1 promoter luciferase construct (55).

In conclusion, our work shows that limiting GCS activity by either GCS antisense transfection, siRNA transfection, or PPMP treatment down-regulates the expression of P-glycoprotein. It should be noted, however, that drug resistance through enforced overexpression of GCS in wild-type MCF-7 cells, using a retroviral tetracycline-on expression system, did not rely on P-glycoprotein induction (13) but rather on ceramide scavenging. In addition, GCS antisense transfection retards clearance of ceramide generated in response to chemotherapeutics such as paclitaxel. Therefore, ceramide-signalled death cascades and depletion of cellular P-glycoprotein are likely contributory to heightened chemosensitivity in MCF-7-AdrR/GCS antisense. We propose that overexpression of GCS contributes to chemotherapy resistance by
enhancing levels of ceramides and/or gangliosides that could promote the expression of MDR1. Doxorubicin-activated expression of GCS in MCF-7 drug-sensitive cells (56) lends support to this novel slant on the metabolism of ceramide.

Acknowledgments

Received 6/30/2004; revised 2/18/2005; accepted 2/24/2005.

References

Glucosylceramide synthase blockade downregulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs

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Overexpression of glucosylceramide synthase (GCS), a pivotal enzyme in glycolipid biosynthesis, contributes to cancer cell resistance to chemotherapy. We previously demonstrated that transfection of doxorubicin-resistant MCF-7-AdrR cells with GCS antisense (asGCS) restored cell sensitivity to doxorubicin and greatly enhanced sensitivity to vinblastine and paclitaxel. In that study, doxorubicin promoted generation of ceramide in MCF-7-AdrR/asGCS cells; the present study implicates factors other than ceramide in the sensitivity of asGCS-transfected cells to chemotherapy. Although asGCS cells demonstrated enhanced ceramide formation compared to MCF-7-AdrR when challenged with paclitaxel, asGCS cells also demonstrated a 10-fold increase in levels of intracellular drug (paclitaxel, vinblastine). In addition, transfected cells had dramatically decreased (80%) expression of P-glycoprotein (P-gp), and a 4-fold decrease in the level of cellular gangliosides. Chemical inhibition of GCS produced the same effects as antisense transfection: exposure of MCF-7-AdrR cells to the GCS inhibitor PPMP (1-phenyl-2-palmitoylamino-3-morpholino-1-propanol) (5.0 μM; 4 days) decreased ganglioside levels, restored sensitivity to vinblastine, enhanced vinblastine uptake 3-fold, and diminished expression of MDR1 by 58%, compared to untreated controls. A similar effect was demonstrated in vinblastine-resistant KB-V0.01 cells; after 7 days with PPMP (10 μM), MDR1 expression fell by 84% and P-gp protein levels decreased by 50%. MCF-7-AdrR cells treated with small interfering RNAs to specifically block GCS also showed a dramatic decrease in MDR1 expression. This is the first evidence that limiting GCS activity downregulates the expression of MDR1, a mechanism that may drive the chemosensitization associated with targeting ceramide metabolism. The data suggest that lipids play a role in multidrug resistance, and that limiting ganglioside biosynthesis could be a promising approach for reversing multidrug resistance in cancer.