

# Overexpression of glucosylceramide synthase and P-glycoprotein in cancer cells selected for resistance to natural product chemotherapy

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## Abstract

Resistance to natural product chemotherapy drugs is a major obstacle to successful cancer treatment. This type of resistance is often acquired in response to drug exposure; however, the mechanisms of this adverse reaction are complex and elusive. Here, we have studied acquired resistance to Adriamycin, *Vinca* alkaloids, and etoposide in MCF-7 breast cancer cells, KB-3-1 epidermoid carcinoma cells, and other cancer cell lines to determine if there is an association between expression of glucosylceramide synthase, the enzyme catalyzing ceramide glycosylation to glucosylceramide, and the multidrug-resistant (MDR) phenotype. This work shows that glucosylceramide levels increase concomitantly with increased drug resistance in the KB-3-1 vinblastine-resistant sublines KB-V.01, KB-V.1, and KB-V1 (listed in order of increasing MDR). The levels of glucosylceramide synthase mRNA, glucosylceramide synthase protein, and P-glycoprotein (P-gp) also increased in parallel. Increased glucosylceramide levels were also present in Adriamycin-resistant KB-3-1 sublines KB-A.05 and KB-A1. In breast cancer, detailed analysis of MCF-7 wild-type and MCF-7-AdrR cells (Adriamycin-resistant) demonstrated enhanced glucosylceramide synthase message and protein, P-gp message and protein, and high levels of glucosylceramide in resistant cells. Similar

results were seen in vincristine-resistant leukemia, etoposide-resistant melanoma, and Adriamycin-resistant colon cancer cell lines. Cell-free glucosylceramide synthase activity was higher in lysates obtained from drug-resistant cells. Lastly, glucosylceramide synthase promoter activity was 15-fold higher in MCF-7-AdrR compared with MCF-7 cells. We conclude that selection pressure for resistance to natural product chemotherapy drugs selects for enhanced ceramide metabolism through glucosylceramide synthase in addition to enhanced P-gp expression. A possible connection between glucosylceramide synthase and P-gp in drug resistance biology is suggested. [Mol Cancer Ther 2004;3(5):633–9]

## Introduction

Inherent or acquired chemotherapy resistance, which can include development of simultaneous resistance to multiple drugs, is a frequent phenomenon in cancer cells. It is difficult to predict and to manage. Forty percent of cancer patients with resectable disease and 80% of cancer patients with unresectable disease have poor response to chemotherapy and radiotherapy. Several mechanisms of drug resistance have been examined. Overexpression of a membrane efflux transporter, P-glycoprotein (P-gp), is one of the most consistent alterations in drug resistance (1–3). P-gp has become an important clinical target and the object of numerous studies (4, 5). Drug resistance can also be caused by overexpression of multidrug resistance (MDR)-associated protein (6, 7), changes in topoisomerase II activity (8, 9), modifications in glutathione S-transferase (10, 11), and altered expression of apoptosis-associated protein Bcl-2 (12) and tumor suppressor protein p53 (13). Synthesis of vaults (14) and the overexpression of caveolae (15), although lesser known, have also been studied in connection with MDR.

Apoptosis is an essential element in the cytotoxic effect of many anticancer agents (16) and the neutral lipid, ceramide, has been shown to play a role in this response (17). Data suggest that chemotherapy resistance in cancer cells is in some instances associated with an enhanced cellular capacity to glycosylate ceramide (18–20). This implies that chemotherapy toxicity would be blunted in cancer cells that up-regulate ceramide clearance. This places glucosylceramide synthase, the enzyme that catalyzes ceramide glycosylation, in a key position, especially when considering the large number of drugs that elicit ceramide formation (reviewed in Ref. 19).

The present work was undertaken to test the hypothesis that selection of cancer cells for resistance to natural product chemotherapy drugs promotes accelerated metabolism of ceramide through enhanced expression of

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glucosylceramide synthase. Using a series of drug-resistant human epidermoid carcinoma, breast, leukemia, melanoma, and colon cancer cell lines developed through selection pressure cloning with various anticancer drugs, we show that increases in drug resistance are accompanied by increases in the expression and activity of glucosylceramide synthase. Moreover, the increases in glucosylceramide synthase expression mirrored the increases in P-gp expression in the KB-3-1 drug-resistant cell line series and the drug-resistant breast, leukemia, melanoma, and colon cancer cell lines.

## Materials and Methods

### Materials

KB-3-1, the parent wild-type human epidermoid carcinoma cell line, and the vinblastine sulfate-resistant (KB-V.01, KB-V.1, and KB-V1) and Adriamycin-resistant (KB-A.05 and KB-A1) sublines were grown as monolayer cultures in high glucose (4.5 g/l) DMEM with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and additives as described (18, 21). The human breast cancer cell lines MCF-7 and MCF-7-AdrR (Adriamycin-resistant) were provided by Drs. Kenneth Cowan (Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE) and Merrill E. Goldsmith (National Cancer Institute, Bethesda MD) and grown as described (18). A description of these cell lines is given in Table 1. The human melanoma cell line MeWo derived from lymph node metastasis and the etoposide selected resistant subline MeWoEto1 (22, 23) were gifts from Dr. Dirk Schadendorf (German Cancer Research Center, Heidelberg, Germany). MeWoEto1 cells were selected to grow in 1  $\mu$ g/ml etoposide. The human colon cancer cell line SW620 and the Adriamycin-selected SW620Ad1000 (24) were gifts from Drs. Susan Bates and Antonio Fojo (National Cancer Institute). HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD) and

HL-60/VCR, a vincristine-resistant line, was a gift from Dr. Arthur E. Frankel (Wake Forest University School of Medicine, Winston-Salem, NC). The melanoma, colon cancer and leukemia cell lines were cultured in RPMI 1640 with 10% FBS, 50 units/ml penicillin, 50  $\mu$ g/ml streptomycin, and 584 mg/l L-glutamine. All cell lines were subcultured using 0.05% trypsin/0.53 mM EDTA solution. The SW620Ad1000 cells were maintained in medium containing 1000 ng/ml Adriamycin and the MeWoEto1 cells were maintained in medium containing 1.0  $\mu$ g/ml etoposide. HL-60/VCR cells were grown with 1.0  $\mu$ g/ml vincristine in the medium.

RPMI 1640 and DMEM were from Life Technologies, Inc. (Grand Island, NY). Vinblastine, Adriamycin (doxorubicin hydrochloride), etoposide, and other chemicals were purchased from Sigma Chemical Co (St. Louis, MO). [9,10-<sup>3</sup>H(N)]Palmitic acid (50 Ci/mmol) and EN<sup>3</sup>HANCE were purchased from DuPont NEN Research Products (Boston, MA) and [<sup>3</sup>H]UDP-glucose (40 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). C219, the monoclonal antibody against human P-gp, was from Calbiochem (Pasadena, CA). GCS-1.2 antiserum from rabbit was a gift from Drs. Richard Pagano and David Marks (Mayo Clinic and Foundation, Rochester, MN). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and EcoLume (liquid scintillation cocktail) was purchased from ICN (Costa Mesa, CA). Silica Gel G prescored TLC plates were purchased from Analtech (Newark, DE).

RNA was isolated using the RNeasy Protect Mini Kit (Qiagen, Inc., Los Angeles, CA) following instructions from the manufacturer. Reverse transcription-PCR (RT-PCR) was carried out using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA). Housekeeping primer,  $\beta$ -actin, was purchased from Stratagene (Cedar Creek, TX). The thermocycler (Mastercycler Gradient) was from Eppendorf Scientific (Westbury, NY).

**Table 1. Characteristics of cell lines used in experiments**

Cell line	Media additions <sup>a</sup>	MDR status <sup>b,c</sup>		GC
		Vinblastine	Adriamycin	Content <sup>d</sup>
KB-3-1	None	(-)	(-)	7
KB-V.01	Vinblastine, 10 ng/ml	2.8	1.8	21
KB-V.1	Vinblastine, 100 ng/ml	23	5	54
KB-V1	Vinblastine, 1.0 $\mu$ g/ml	213	422	66
KB-A.05	Adriamycin, 50 ng/ml	38	31	59
KB-A1	Adriamycin, 1.0 $\mu$ g/ml	43	97	92
MCF-7	None	(-)	(-)	6
MCF-7-AdrR	None	ND	33	65

<sup>a</sup>Cells continually maintained in culture medium containing drugs at concentrations indicated.

<sup>b</sup>MDR status shown as relative resistance and calculated from  $D_{10}$  values (concentration of drug that reduced the cloning efficiency of the sublines to 10% of the control without drugs; Ref. 21) by dividing  $D_{10}$  of the resistant line by the  $D_{10}$  of the KB-3-1 parental drug-sensitive cell line.

<sup>c</sup>MDR status of MCF-7-AdrR derived by dividing Adriamycin  $EC_{50}$  in MCF-7-AdrR by Adriamycin  $EC_{50}$  in MCF-7 cells.

<sup>d</sup>Glucosylceramide content of cell lines given in densitometric values relative to corresponding wild-type cell lines (KB-3-1 and MCF-7). For the KB-3-1 cell lines, glucosylceramide levels were measured by densitometric scanning of the autoradiogram, and in the MCF-7 lines, glucosylceramide levels were measured by densitometric scanning of the sulfuric acid chars. ND, not determined; (-), no drugs present.

### Cell Radiolabeling and Glucosylceramide Analysis

Cells at ~60% confluence in 10 cm dishes were given fresh 5% FBS containing 1.0  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]palmitic acid for 48 h. Total cellular lipids were extracted from PBS-washed monolayers according to previous methods (18, 20). Autoradiographic analysis of glucosylceramide in the KB-3-1 series of cell lines was conducted by applying equal aliquots based on radioactivity (500,000 cpm/lane) to the origin of TLC plates. Radiolabeled glucosylceramide was resolved from the total lipid extract using a solvent system containing 80:20:2 (v/v/v) chloroform/methanol/ammonium hydroxide. Radiochromatograms were sprayed with EN $^3$ HANCE and exposed for 40 h at  $-8^\circ\text{C}$  for autoradiography. Lipids (nonradiolabeled) were also charred by heating  $\text{H}_2\text{SO}_4$  sprayed TLC plates in an oven at  $180^\circ\text{C}$  for 30 min, and resultant black bands were quantitated by densitometry. This method of detection was used with MCF-7 and MCF-7-AdrR cells.

### Glucosylceramide Synthase Assay

Cells harvested in log-phase growth were homogenized by sonication in lysis buffer [50 mM Tris-HCl (pH 7.4), 1.0  $\mu\text{g/ml}$  leupeptin, 10  $\mu\text{g/ml}$  aprotinin, 25  $\mu\text{M}$  phenylmethylsulfonyl fluoride]. Microsomes were isolated by centrifugation ( $129,000 \times g$  for 60 min). The enzyme assay, modified from a previous method (25), contained 50  $\mu\text{g}$  microsomal protein in a final volume of 0.2 ml and was performed in a shaking water bath at  $37^\circ\text{C}$  for 60 min. The reaction contained liposomal substrate composed of 1.0 mM  $\text{C}_6$ -ceramide, 3.6 mM phosphatidylcholine ( $M_r$  786), and 0.9 mM brain sulfatides ( $M_r$  563). The liposomal substrate was prepared by mixing the components, evaporating the solvents under a stream of nitrogen, and sonicating in water over ice for 1 min using a microtip at 50% output (Micro Ultrasonic Cell Disrupter, Kimble Kontes, Vineland, NJ). Other reaction components included 0.1 M sodium phosphate buffer (pH 7.8), 2.0 mM EDTA, 10 mM  $\text{MgCl}_2$ , 1.0 mM DTT, 2.0 mM  $\beta$ -oxidized  $\text{NAD}^+$ , and 0.5 mM [ $^3\text{H}$ ]UDP-glucose. Radiolabeled and unlabeled UDP-glucose were diluted to achieve the desired radiospecific activity (5000 dpm/nmol). To terminate the reaction, tubes were placed on ice, and 0.5 ml isopropanol and 0.4 ml  $\text{Na}_2\text{SO}_4$  were added. After brief vortex mixing, *t*-butylmethyl ether (3 ml) was added and the tubes were mixed for 0 s. After centrifugation, 0.5 ml of the upper phase, which contained glucosylceramide, was withdrawn and mixed with 4.5 ml EcoLume for analysis of radioactivity by liquid scintillation spectroscopy.

### Western Blot

Confluent cells were washed, harvested in PBS, and lysed in a PBS containing 10% glycerol, 1% Triton X-100, 1.0 mM  $\text{Na}_3\text{VO}_4$ , 10 mM  $\beta$ -glycerophosphate, 50 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 2  $\mu\text{g/ml}$  leupeptin, and 10  $\mu\text{g/ml}$  aprotinin for 30 min on ice. The mixture was centrifuged at  $11,000 \times g$  for 15 min at  $4^\circ\text{C}$ . Equal aliquots of protein (25  $\mu\text{g}$ ) were resolved using 4–20% gradient PAGE. For SW620, MeWo, and HL-60 cells, equal aliquots of protein (20  $\mu\text{g}$ ) were resolved using 6% Tris-glycine PAGE (Invitrogen). The transferred nitrocellulose blot

was blocked with 5% fat-free milk powder in PBS-0.1% Tween 20 at room temperature for 1 h. The membrane was immunoblotted with murine monoclonal antibody C219 against human P-gp (0.7  $\mu\text{g/ml}$ ) or with GCS-1.2 antiserum (diluted 1:1000) in 5% fat-free milk in PBS-0.1% Tween 20. Detection was performed using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

### RNA Analysis

cDNA synthesis and glucosylceramide synthase and *mdr1* RT-PCR were achieved using a one-step method (SuperScript One-Step RT-PCR). Total RNA (25 ng for *mdr1*, 50 ng for glucosylceramide synthase) was added to buffer containing 0.2 mM dNTP, 1.2 mM  $\text{MgSO}_4$ , 1.0  $\mu\text{l}$  SuperScript II RT/Platinum Taq mix (containing reverse transcriptase and Platinum Taq DNA polymerase), and 0.2  $\mu\text{g}$  of each specific glucosylceramide synthase primer (upstream 5'-CCT TTC CTC TCC CCA CCT TCC TCT-3' and downstream 5'-GGT TTC AGA AGA GAG ACA CCT GGG-3') or *mdr1* primer (*umdr1* 5'-CCA TCA TTG CAA TAG CAG G-3' and *dmdr1* 5'-GAG CAT ACA TAT GTT CAA ACT TC-3'). RT-PCR was performed in a total volume of 50  $\mu\text{l}$ . The three-step PCR was performed for 38 cycles in a thermocycler (Mastercycler Gradient, Eppendorf Scientific): denaturation at  $95^\circ\text{C}$  for 30 s, annealing at  $60^\circ\text{C}$  for 30 s, and elongation at  $68^\circ\text{C}$  for 2 min. RT-PCR products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide.  $\beta$ -actin primer (Stratagene) was used for even loading.

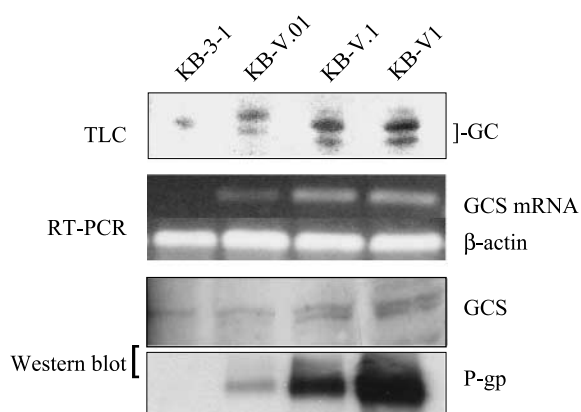
### Glucosylceramide Synthase Promoter Transfection

The full-length glucosylceramide synthase promoter (pGCS-P1) was a gift from Dr. Y. Hirabayashi (Institute of Chemical and Physical Research, Saitama, Japan). MCF-7 and MCF-7-AdrR cells ( $10^6$  cells/well of six-well plates) were seeded and grown overnight in 10% FBS RPMI 1640. After rinsing cells in serum-free medium, the luciferase reporter plasmid of pGCS-P1 was introduced by incubation with LipofectAMINE in serum-free medium for 5 h. After 48 h growth in 10% FBS, cells were harvested in lysis buffer and 25  $\mu\text{l}$  soluble fraction was incubated with luciferase substrate at room temperature for 1 min. Light intensity catalyzed by luciferase was measured using scintillation spectroscopy.

## Results

KB-3-1 cells and the corresponding drug-resistant sublines were first evaluated for glucosylceramide content. Cells were radiolabeled to steady state using [ $^3\text{H}$ ]palmitic acid, and glucosylceramide levels were measured in the total lipid extract using TLC autoradiography. As shown in Fig. 1 (*top*), glucosylceramide levels increased concomitantly with increasing vinblastine selection pressure. KB-V1 cells, subcloned to grow with 1.0  $\mu\text{g/ml}$  vinblastine, had the highest glucosylceramide content. By comparison, the parent KB-3-1 drug-sensitive cell line was nearly devoid of glucosylceramide, whereas KB-V.01 and KB-V.1 cells showed incrementally increased glucosylceramide levels,

respectively. Relative values for glucosylceramide levels in the KB-3-1 series are given in Table 1. All of the KB-3-1 sublines selected for growth in vinblastine demonstrated the characteristic glucosylceramide doublet, with the lower band corresponding to predominantly saturated *N*-palmitoyl ceramides and the upper band corresponding to a mixture of longer amide chains (lignoceroyl and nervonyl; Ref. 18). To determine whether the glucosylceramide increases were associated with enhanced expression of glucosylceramide synthase, glucosylceramide synthase mRNA and protein levels were assessed. As shown in Fig. 1, both glucosylceramide synthase message and protein increased in a complementary fashion with the level of drug resistance (Table 1). Because drug resistance in KB-V1 cells has been attributed to drug efflux, we evaluated the levels of P-gp in the cells. As shown in Fig. 1 (bottom), whereas P-gp could not be detected by our methods in KB-3-1 cells, the levels of P-gp increased in resistant sublines in a pattern very similar to the glucosylceramide synthase and glucosylceramide increases. Analysis of KB-3-1 cells selected for growth in Adriamycin, KB-A.05 (50 ng/ml Adriamycin) and KB-A1 (1  $\mu$ g/ml Adriamycin), also showed increases in glucosylceramide that corresponded with the degree of Adriamycin resistance (Fig. 2; Table 1). These data show that both *Vinca* alkaloids and anthracyclines enhance cellular glucosylceramide synthase and/or glucosylceramide levels in a pattern consistent with the severity of chemotherapy resistance. It should be noted that densitometric scanning of autoradiograms may overestimate differences in glucosylceramide levels due to the 48 h radiolabeling period and



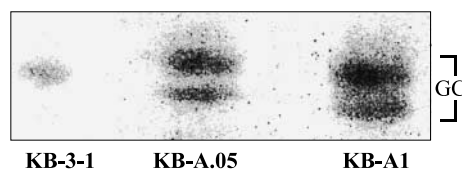
**Figure 1.** Glucosylceramide (GC) levels and glucosylceramide synthase (GCS) and P-gp expression in drug-sensitive and drug-resistant KB-3-1 epidermoid carcinoma cells. KB-3-1 and vinblastine-resistant sublines were cultured to 70% confluence, after which RNA and protein were extracted and used in RT-PCR and Western blotting as detailed in Materials and Methods. Amplified product was analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide.  $\beta$ -actin was employed as housekeeping primer. C219 (0.7  $\mu$ g/ml) murine monoclonal antibody against human P-gp was used in Western blotting and GCS-1.2 antiserum (rabbit) was used to detect glucosylceramide synthase as detailed in Materials and Methods. To assess glucosylceramide content, cells were grown in 10 cm dishes in the presence of [ $^3$ H]palmitic acid for 48 h and total lipids were extracted and analyzed by TLC autoradiography. Each lane was spotted with 500,000 cpm total lipid from the corresponding cell line. The glucosylceramide (doublet) area of the autoradiograph is shown.

recycling and metabolism of tritiated palmitate that occur. When the glucosylceramide area of the TLC plate was scraped and analyzed by liquid scintillation spectroscopy, glucosylceramide in the KB-V1 and KB-A1 was 2.7-fold and 8.2-fold higher, respectively, than in KB-3-1 cells.

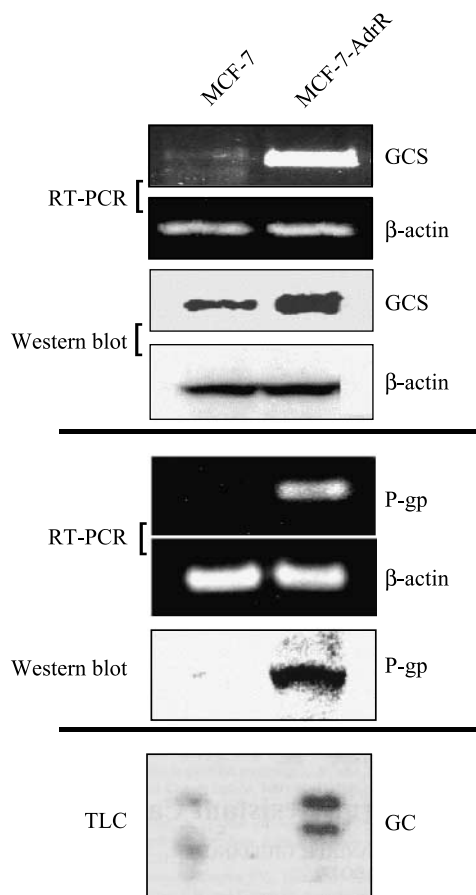
We next sought to determine whether glucosylceramide synthase expression was up-regulated in other chemotherapy-resistant types of human cancer. Should this be a more global response, the findings would have added significance. Data in Fig. 3 reveal a detailed analysis of glucosylceramide synthase expression in wild-type MCF-7 human breast cancer cells and the isogenic Adriamycin-resistant counterpart, MCF-7-AdrR. Resistant cells exhibited a 20-fold increase over wild-type in glucosylceramide synthase mRNA and a 4-fold increase in glucosylceramide synthase protein (Fig. 3, top panel). Similarly, P-gp expression measured by mRNA and protein was also greatly elevated in drug-resistant breast cancer cells compared with drug-sensitive cells (Fig. 3, middle panel). Further, TLC analysis using  $H_2SO_4$  charring for lipid visualization based on carbon mass showed glucosylceramide predominance in MCF-7-AdrR cells with MCF-7 cells being nearly devoid. To learn more about glucosylceramide synthase expression in the breast cancer cell lines, we transiently transfected pGCS-P1 promoter plasmid into MCF-7 and MCF-7-AdrR cells. pGCS-P1 contains the full-length sequence of the human glucosylceramide synthase promoter with a luciferase reporter. Based on luciferase activity controlled by pGCS-P1, the experiments showed that glucosylceramide synthase promoter activity was 15-fold higher in MCF-7-AdrR cells compared with MCF-7 cells.

We further investigated whether elevated glucosylceramide synthase expression was associated with drug resistance and P-gp in other types of cancer cells. Vincristine-resistant HL-60 human leukemia cells, MeWoEto1 etoposide-resistant human melanoma cells, and SW620Ad1000 Adriamycin-resistant human colon cancer cells and their isogenic wild-type drug-sensitive counterparts were evaluated. Data in Fig. 4 demonstrate that elevated expression of glucosylceramide synthase (mRNA) was a characteristic of the drug-resistant phenotype in each of the cell lines investigated. P-gp levels were also measured, and as shown in Fig. 4, overexpression corresponded with enhanced expression of glucosylceramide synthase.

Cell-free assays using microsomes as enzyme source were carried out to measure glucosylceramide synthase activity under defined *in vitro* conditions. For this, [ $^3$ H]UDP-glucose and  $C_6$ -ceramide were employed as reactants and the product, [ $^3$ H]glucosyl- $C_6$ -ceramide, was



**Figure 2.** Glucosylceramide levels in KB-3-1 cells and Adriamycin-resistant sublines. Cellular radiolabeling (48 h) and lipid analysis were carried out as detailed in Materials and Methods and Fig. 1.



**Figure 3.** Glucosylceramide synthase (*GCS*) and P-gp expression and glucosylceramide (*GC*) levels in MCF-7 and MCF-7-AdrR human breast cancer cells. RT-PCR and Western blotting were carried out as detailed in Materials and Methods and Fig. 1. Glucosylceramide (100  $\mu$ g total lipid spotted/lane) was analyzed by mass using sulfuric acid charring. *Top panel*, glucosylceramide synthase expression; *middle panel*, P-gp expression; *bottom panel*, glucosylceramide analysis.

quantitated. Table 2 demonstrates that cell-free glucosylceramide synthase activity was higher in cells selected to grow in the presence of the natural product anticancer agents as opposed to chemotherapy naive cells. A comparison of KB-3-1 cells with KB-V1 cells showed that glucosylceramide synthase activity was approximately twice as high in the latter ( $10.2 \pm 0.6$  and  $21.8 \pm 1.5$  nmol glucosylceramide/mg protein/h, respectively). Glucosylceramide synthase activity was also slightly higher in Adriamycin-resistant cells KB-A.05 and KB-A1 compared with KB-3-1 cells; however, the levels of enzyme activity were not fully reflective of the Adriamycin resistance pattern (see Table 1).

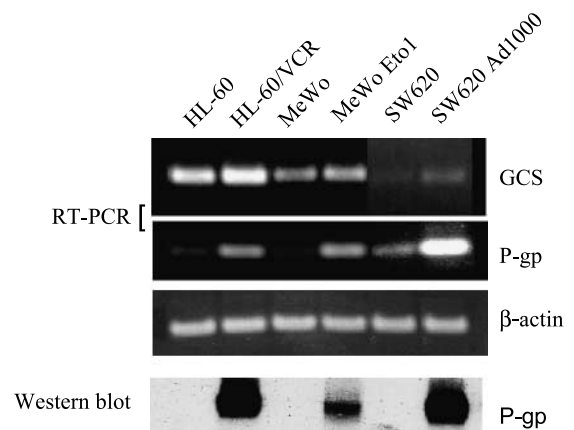
## Discussion

Ceramide serves a signal transduction function for apoptosis in response to a variety of frontline anticancer agents (26, 27). Formation of glucosylceramide by glucosylceramide synthase, on the other hand, is thought to be a lipid shunting pathway for bypassing cell death, providing a

mechanism for chemotherapy resistance (19). Accelerated conversion of ceramide to glucosylceramide was first observed in MDR MCF-7-AdrR and KB-V1 cells (18). Data suggested that modified glucosylceramide metabolism could be indicative of cellular changes that accompany drug resistance; however, the mechanism by which anticancer drugs enhance ceramide glycosylation is poorly understood.

In the present work, we sought to determine whether enhanced ceramide metabolism by the glycosylation pathway accompanied the selection pressure exerted by natural product chemotherapy drugs. Such a mechanism could be operable *in vivo*, as repeated exposure to natural product drugs can result in acquired resistance in patients. Use was made of two well-established cell line families, KB-3-1 and MCF-7. The epidermoid carcinoma series was appropriate for this study because the MDR subtypes were selected to grow with graded increases of vinblastine. This model is useful in determining whether the changes in ceramide metabolism that accompany drug resistance are related to up-regulated expression in proportion to selection pressure mediated by drug concentration as opposed to an all-or-none phenomenon.

Our work demonstrates that graded increases in cellular glucosylceramide content and glucosylceramide synthase mRNA levels mirror increases in cellular tolerance to vinblastine and are accompanied by incremental increases in P-gp expression. Similar findings were shown with Adriamycin resistance in the KB-3-1 Adriamycin series, although this was only based on lipid analysis (Fig. 2). To determine whether enhanced glucosylceramide synthase expression could be translated into enhanced enzyme activity *in vitro*, we examined glucosylceramide synthase in a cell-free system. Although we noted a slight discrepancy between gradedness of glucosylceramide synthase activity (Table 2) and levels of glucosylceramide synthase



**Figure 4.** Glucosylceramide synthase (*GCS*) and P-gp expression in drug-sensitive and in drug-resistant human leukemia, melanoma, and colon cancer cell lines. Cells were grown to 70% confluence and total RNA (100 ng) was used in RT-PCR analysis. Vincristine-resistant leukemia (HL-60/VCR), etoposide-resistant melanoma (MeWoEto1), and Adriamycin-resistant colon cancer cells (SW620Ad1000) are shown along with wild-type counterparts.

**Table 2. Cell-free glucosylceramide synthase activity in wild-type, vinblastine-resistant, and Adriamycin-resistant cells**

Cell line	GCS activity (nmol GC/mg protein/h)
KB-3-1	10.2 ± 0.6
KB-V.1	14.6 ± 0.6
KB-V1	21.8 ± 1.5
KB-A.05	14.7 ± 0.3
KB-A1	12.4 ± 0.7

Note: Glucosylceramide synthase assays were conducted as detailed in Materials and Methods using microsomes (50 µg protein) as enzyme source.

expression, higher glucosylceramide synthase activity *in vitro* was nevertheless a trend in the drug-resistant sublines of KB-3-1 compared with wild-type. The nature of *in vitro* enzymology and possible differences in endogenous lipid composition of the microsomal preparations may explain the irregular data. Additionally, the KB-A1 cell line was found by fluorescence-activated cell sorting analysis to be a somewhat heterogeneous population of cells as regards P-gp expression (C. Cardarelli and G. Leiman, personal communication). This could explain the lower than expected glucosylceramide synthase activity in KB-A1 cells (Table 2).

In this work, we also assessed glucosylceramide synthase expression and its relationship to drug resistance in human leukemia, melanoma, colon, and breast cancer cells. Our work showed that glucosylceramide synthase is up-regulated in response to drug exposure and suggests that resistance to natural product chemotherapy may involve glycolipids. Previous work shows that enforced expression of glucosylceramide synthase by transfection of drug-sensitive MCF-7 cells conferred resistance to Adriamycin and to ceramide supplements (28). This gives support to the idea that glucosylceramide synthase regulates cellular sensitivity to chemotherapy. Whereas the up-regulation of glucosylceramide synthase and its association with drug resistance is a relatively new area of study, the importance of glycolipid metabolism in cellular responses to anticancer agents should not be overlooked. Uncoupling ceramide glycosylation by transfection with glucosylceramide synthase antisense has been shown to restore sensitivity to Adriamycin, *Vinca*, and taxanes in MDR cancer cells (20, 29). Glucosylceramide synthase antisense transfection has also been demonstrated to inhibit melanoma formation in mice (30). Similarly, blocking glucosylceramide synthase by chemical means can restore chemotherapy cytotoxicity in drug-resistant cells (31–35). That tamoxifen, cyclosporine A, and verapamil, all P-gp antagonists (5), also block glucosylceramide formation in P-gp-expressing cells (36, 37) remains a curious point for study.

The relationship between P-gp and lipid metabolism is intriguing. It has been shown that P-gp can function as a lipid translocator (38, 39), and work by Shabbits and Mayer (40) indicates that there may be a link between ceramide metabolism and P-gp. Lipids have also been shown to influence P-gp function. Plo *et al.* (41) have shown that depletion of cellular ganglioside levels produces enhanced

retention of rhodamine 123 in P-gp-expressing acute myeloid leukemia cells, prompting the authors to conclude that gangliosides such as GD3 regulate P-gp activity. Although high levels of glucosylceramide have been shown to be associated with MDR cancer cells harboring P-gp (18, 37, 42, 43), the relationship of the two remains illusive. In a previous study, we demonstrated that whereas *MDR1* transfection was alone sufficient to greatly enhance P-gp expression in MCF-7 cells, glycolipid metabolism was not changed (44). It remains a question whether glucosylceramide synthase influences P-gp expression or vice versa. Here, we have shown for the first time that P-gp expression increases in parallel with increases in glucosylceramide synthase expression in epidermoid carcinoma cells, demonstrating graded increases in vinblastine resistance. We have also shown in chemotherapy-resistant breast cancer, leukemia, melanoma, and colon cancer cells that glucosylceramide synthase and P-gp expression are enhanced together in conjunction with selection pressure. These data present the intriguing possibility that cerebroside and/or gangliosides participate in *MDR1* expression.

There appears to be a strong case for the role of lipids, particularly glycolipids, in cell response to Adriamycin and vinblastine. It is interesting to note that glucosylceramide synthase promoter activity is greatly elevated in MCF-7-AdrR cells compared with MCF-7 cells. We recently demonstrated that Adriamycin treatment up-regulated glucosylceramide synthase promoter activity in promoter-transfected MCF-7 cells (45) and we are currently evaluating responses to the other anthracyclines and to taxanes. Our work shows for the first time that up-regulated expression of glucosylceramide synthase occurs in response to selection pressure for anticancer agents of the natural products class of drugs. Although only preliminary, results from lipid analysis of MCF-7 and MCF-7/CDDP (cisplatin-resistant) cells showed glucosylceramide levels to be nearly identical (M. Cabot, unpublished data). This suggests that ceramide metabolism is not a factor in the mechanism of platinum-based chemotherapy resistance and that accelerated ceramide clearance may only be a property that is characteristic to natural product drug resistance.

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