Uncoupling Ceramide Glycosylation by Transfection of Glucosylceramide Synthase Antisense Reverses Adriamycin Resistance*

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Yong-Yu Liu, Tie-Yan Han, Armando E. Giuliano, Nora Hansen, and Myles C. Cabot‡

From the John Wayne Cancer Institute at Saint John’s Health Center, Santa Monica, California 90404

Previous work from our laboratory demonstrated that increased competence to glycosylate ceramide conferred adriamycin resistance in MCF-7 breast cancer cells (Liu, Y. Y., Han, T. Y., Giuliano, A. E., and M. C. Cabot. (1999) J. Biol. Chem. 274, 1140–1146). This was achieved by cellular transfection with glucosylceramide synthase (GCS), the enzyme that converts ceramide to glucosylceramide. With this, we hypothesized that a decrease in cellular ceramide glycosylation would result in heightened drug sensitivity and reverse adriamycin resistance. To down-regulate ceramide glycosylation potential, we transfected adriamycin-resistant breast cancer cells (MCF-7-AdrR) with GCS antisense (asGCS), using a pcDNA 3.1/his A vector and developed a new cell line, MCF-7-AdrR/asGCS. Reverse transcription-polymerase chain reaction assay and Western blot analysis revealed marked decreases in both GCS mRNA and protein in MCF-7-AdrR/asGCS cells compared with the MCF-7-AdrR parental cells. MCF-7-AdrR/asGCS cells exhibited 30% less GCS activity by *in vitro* enzyme assay (19.7 ± 1.1 versus 27.4 ± 2.3 pmol GC/h/μg protein, p < 0.001) and were 28-fold more sensitive to adriamycin (EC₅₀ 0.44 ± 0.01 versus 12.4 ± 0.7 μM, p < 0.0001). GCS antisense transfected cells were also 2.4-fold more sensitive to C₆-ceramide compared with parental cells (EC₅₀ 4.0 ± 0.03 versus 9.6 ± 0.5 μM, p < 0.0005). Under adriamycin stress, GCS antisense transfected cells compared with parental cells displayed time- and dose-dependent increases in endogenous ceramide and dramatically higher levels of apoptotic effector, caspase-3. Western blotting showed that adriamycin sensitivity, introduced by asGCS gene transfection, was independent of P-glycoprotein and Bcl-2 expression. In summary, this work shows that transfection of GCS antisense tempers the expression of native GCS and restores cell sensitivity to adriamycin. Therefore, limiting the potential to glycosylate ceramide, which is an apoptotic signal in chemotherapy and radiotherapy, provides a promising approach to combat drug resistance.

Ceramide, now recognized as a second messenger in cellular apoptotic signaling events, has been shown to play a role in chemotherapy and radiotherapy of cancer (1, 2). Loss of ceramide production is one cause of cellular resistance to apoptosis induced by either ionizing radiation or tumor necrosis factor-α and adriamycin (2–7). Accumulation of glucosylceramide (GC), a simple glycosylated form of ceramide, is a characteristic of some multidrug-resistant cancer cells and tumors derived from patients who are less responsive to chemotherapy (8, 9). The study of GC metabolism, as a molecular determinant of the drug-resistant phenotype, has been a subject of recent attention. Modification of ceramide metabolism by blocking the glycosylation pathway has been shown to increase cancer cell sensitivity to cytotoxics (10–12). Further, drug combinations that enhance ceramide generation and limit glycosylation have been shown to enhance kill in cancer cell models (11, 12). Other work has shown that ceramide toxicity can be potentiated in experimental metastasis of murine Lewis lung carcinoma and human neuroepithelioma cells by inclusion of a glucosylceramide synthase inhibitor (13, 14). These findings assign biological significance to ceramide metabolism as it relates to circumvention of resistance to antineoplastic agents.

The increased capacity for ceramide glycosylation in GC-transfected human breast cancer cells conferred resistance to adriamycin and to tumor necrosis factor-α (7, 15). Both agents are known to activate ceramide generation and potentiate apoptosis (1, 2, 7, 15). From this, we hypothesized that transfection of asGCS, to limit cellular ceramide glycosylation, would overcome adriamycin resistance. By introducing asGCS to modulate GC activity in adriamycin-resistant human breast cancer cells, we successfully decreased native GC expression and restored cellular sensitivity to adriamycin and to C₆-ceramide. The present study shows further that ceramide generation is a major factor in the cytotoxicity of adriamycin and suggests that asGCS would be a novel force to overcome adriamycin resistance.

EXPERIMENTAL PROCEDURES

Materials—[3H]UDP-glucose (40 Ci/μmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). C₆-Ceramide (N-hexanoylsphingosine) was purchased from LC Laboratories (Woburn, MA). Sulfatides (ceramide galactoside 3-sulfate) were from Matreya (Pleasant Gap, PA), and phosphatidylcholine (1,2-dioleoyl-sn-glycero-3-phosphocholine) was from Avanti Polar Lipids (Alabaster, AL). Adriamycin (doxorubicin hydrochloride) and other chemicals were purchased from Sigma. FBS was purchased from HyClone (Logan, UT). RPMI medium 1640 and Dulbecco’s modified Eagle’s medium (high glucose) were from Life Technologies, Inc., and cultureware was from Corning Costar (Cambridge, MA). GCS antisemur (from rabbit) was kindly provided by Drs. D. L. Marks and R. E. Pagano (Mayo Clinic and the National Institutes of Health and grants from the Fashion Footwear Association of New York Shoes on Sale, the Strauss Foundation, Sandra Krause, Trustee, and The Streisand Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.‡ To whom correspondence should be addressed: John Wayne Cancer Inst., 2200 Santa Monica Blvd., Santa Monica, CA 90404. Tel.: 310-998-3924; Fax: 310-998-3995; E-mail: Cabot@jwci.org.

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1 The abbreviations used are: GC, glucosylceramide; GCS, glucosylceramide synthase (ceramide glucosyltransferase, UDP-glucose:N-acetylsphingosine 1-glucosyltransferase, EC 2.4.1.80); asGCS, GCS antisense; PBS, fetal bovine serum; MCF-7-AdrR, MCF-7 adriamycin-resistant cells; MCF-7-AdrR/asGCS, MCF-7-AdrR GCS antisense-transfected cells; RT, reverse transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.
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Foundation, Rochester, MN). Anti-Xpress tag antibody was from Invitrogen (Carlsbad, CA). C219, the monoclonal antibody against P-glycoprotein, was from Signet Laboratories (Dedham, MA), and Bcl-2 monoclonal antibody (Ab-1) against human Bcl-2 was from Oncogene Research Products, Beverly, MA.

Cell Line and Culture Conditions—The human breast adenocarcinoma cell line, MCF-7-AdrR, which is resistant to adriamycin (16), was kindly provided by Dr. Kenneth Cowan and Dr. Merrill Goldsmith (NCI, National Institutes of Health, Bethesda, MD). Cells were maintained in RPMI 1640 medium containing 10% (v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 584 mg/liter L-glutamine. Cells were cultured in a humidified, 5% CO₂, atmosphere tissue culture incubator and subcultured weekly using trypsin-EDTA (0.05%, 0.53 mEq/l solute). The stably transfected cells, MCF-7-AdrR/asGCS, were cultured in RPMI 1640 medium containing 400 μg/ml G418 (geneticin) in addition to the above components.

Giemsa staining was performed as described (17). Cells were seeded in 60-mm dishes (10⁶ cells/dish) in 10% FBS RPMI 1640 medium and grown for 2 days at 37 °C. After rinsing with PBS, cells were fixed with 50% methanol/PBS, followed by methanol, and stained with KaryOMAX Giems stain stock solution (Life Technologies, Inc.). Following washing and deionized water, cells were photomicrographed. The population doubling time of each cell line was measured. Briefly, cells were seeded in 24-well plates (10⁶ cells/well) in 10% FBS RPMI 1640 medium and grown for 24-, 48-, 72-, and 96-h periods. After rinsing with PBS, cells were detached with trypsin/EDTA, suspended in medium, and counted by hemocytometer.

pcDNA 3.1/His A-asGCS and pcDNA 3.1/His-GCS Expression Vectors and Transfection—pcDNA-2, a BlueScript II KS containing GclT 1 (Ref. 18; terminology for GCS) in the EcoRI site, was kindly provided by Dr. Shinichi Ichikawa and Dr. Yoshio Hirabayashi (The Institute of Chemical and Physical Research, Saitama, Japan). The full-length cDNA of human GCS was cloned into the EcoRI site in the pcDNA 3.1/His A vector with Xpress tag (Invitrogen) in the upstream region. Xpress tag fuses at the N terminus of the cloned gene; therefore, GCS will be expressed as Xpress-GCS. The antisense and sense orientation of GCS cDNA was analyzed with Vector NTI 4.0 and double checked by restriction digestion. When MCF-7-AdrR cells reached 20% GCS will be expressed as Xpress-GCS. The antisense and sense orientation of GCS cDNA was cloned into the pcDNA 3.1/GCS expression vector (Ref. 18; terminology for GCS) in the XbaI site, just downstream from the antisense peptide of the pcDNA 3.1/His A-asGCS and pcDNA 3.1/His-GCS expression vectors. The pcDNA 3.1/His A plasmid, without GCS DNA, was used in control transfection.

Glucosylceramide Synthase Assay—To determine the levels of GCS in the G418-resistant clones, a modified radioenzymatic assay was utilized (7, 19). Cells were homogenized by sonication in lysis buffer (50 mm Tris-HCl, pH 7.4, 150 m M NaCl, 0.05% Tween-20) and centrifuged at 12,000 × g for 60 min. The enzymatically active microsomal fraction, in a final volume of 0.2 ml, was performed in a shaking water bath at 37 °C for 60 min. The reaction contained liposomal substrate composed of C₇ ceramide (1.0 μM), phosphatidylcholine (3.6 μM), and brain sulfatides (0.9 μM). Other reaction components included sodium phosphate buffer (0.1 M), pH 7.8, EDTA (2.0 μM), MgCl₂ (10 μM), dithiothreitol (1.0 μM), β-glycerophosphate (2.0 μM), and [³H]UDP-glucose (0.5 μM). Radiolabeled and unlabeled UDP-glucose were diluted to achieve the desired radiospecific activity (4,700 dpm/nmol). To terminate the reaction, tubes were placed on ice, and 0.5 M of isopropanol and 0.4 M of Na₂SO₄ were added. After brief vortex mixing, 3 ml of t-butyl methyl ether was added, and the tubes were mixed for 30 s. After centrifugation, 0.5 ml of upper phase, which contained GCS, was withdrawn and mixed with 4.5 ml of EcoLume for analysis of radioactivity by liquid scintillation spectrometry.

RNA Analysis—Cellular mRNA was purified using a mRNA isolation kit (Roche Molecular Biochemicals). Equal amounts of mRNA (5.0 ng) were used for RT-PCR. Under upstream primer (5′-CCCCATCTCTCC- CCCCACCTTCTC-3′) and downstream primer conditions (5′-GGTT TTTAGACCGAGAAGCTGCG-3′), a 302-base pair fragment from the 5′-terminal region of the GCS gene was produced using the ProlongSTAR HF single-tube RT-PCR system (High Fidelity, Stratagene) in a thermocycler (Masterecycler Gradient, Eppendorf). A 40-cycle PCR reaction, using the following conditions: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 68 °C for 120 s. RT-PCR products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide. β-Actin (Life Technologies, Inc.) was used as control for even loading.

Cytotoxicity Assay—Assays were performed as described previously (7, 9). Briefly, cells were seeded in 96-well plates (2,000 cells/well) in 0.1 ml RPMI 1640 medium containing 10% FBS and cultured at 37 °C for 24 h before addition of drug. Drugs were added in FBS-free medium (0.1 ml), and cells were cultured at 37 °C for the indicated periods. Drug cytotoxicity was determined using the Promega 96 Aqueous cell proliferation assay kit (Promega, Madison, WI). Absorbance at 490 nm was recorded using a Microplate Fluorescent Reader, model FL600 (Biotek Instruments). Absorbance at 490 nm was recorded using a Microplate Fluorescent Reader, model FL600 (Biotek Instruments). Absorbance at 490 nm was recorded using a Microplate Fluorescent Reader, model FL600 (Biotek Instruments).

Analysis of Ceramide—Analysis was performed as described previously (7, 8). Cells were seeded in 6-well plates (60,000 cells/well) in 10% FBS RPMI 1640 medium. After 24 h, cells were shifted to 5% FBS medium with or without adriamycin and grown for the indicated times. Cellular lipids were radiolabeled by adding [³H]palmitic acid (2.5 μCi/ml culture medium) for 24 h. After removal of medium, cells were rinsed twice with PBS (pH 7.4), and total lipids were extracted as described (8). The resulting organic lower phase was dried and evaporated under a stream of nitrogen. Lipids were resuspended in 100 μl of chloroform/methanol (1:1, v/v), and aliquots were applied to TLC plates. Ceramide was resolved using a solvent system containing chloroform/acetic acid (90:10, v/v). Commercial lipid standards were cochromatographed. After development, lipids were visualized with iodine vapor staining, and the ceramide area was scraped into 0.5 ml of water. EcoLume counting fluid (4.5 ml) was added, the samples were mixed, and radioactivity was quantitated by liquid scintillation spectrometry.

Caspase-3 Assay—Caspase-3 activity was assayed by DEVD-AFC cleavage, using the ApoAlert Caspase-3 assay kit (CLONTECH, Palo Alto, CA). The assay was performed as described previously (15). Cells were seeded in 100-mm dishes (500,000 cells/dish) in 10% FBS RPMI 1640 medium. After 24 h, cells were shifted to 5% FBS RPMI 1640 medium without or with adriamycin and grown for 24 and 48 h. Following harvest, cells (10⁶/ml) were lysed on ice for 10 min with 50 μl of lysis buffer, and cell debris was removed by centrifugation at 4 °C at 10,000 × g for 5 min. The soluble fraction was incubated with 50 μl of a substrate DEVD-AFC in a 100-μl reaction volume at 37 °C for 60 min. The free AFC fluorescence was measured at λmaxexcitation, 405 nm and λmaxemission, 505 nm using a FL600 Microplate Fluorescence Reader. The caspase-3 inhibitor, acetyl-Asp-Glu-Val-asp-aldehyde, was used to exclude nonspecific background in the enzymatic reaction.

Western Blot Analysis—Western blots were performed using a modified procedure (7, 15, 20). Confluent cell monolayers were washed twice with PBS containing 0.1% sodium dodecyl sulfate, which contained 25 μm phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 μm phenylmethylsulfonyl fluoride). Microsomes were isolated by centrifugation, pcDNA 3.1/GCS or pcDNA 3.1-GCS (10 μg/ml, 100-mm dish) was introduced by co-preparation with calcium phosphate (Mammalian Transfection Kit, Stratagene, La Jolla, CA). The transfected cells were selected in RPMI 1640 medium containing 10% FBS and 400 μg/ml G418. Each G418-resistant clone, isolated utilizing cloning cylinders, was propagated and later screened by GCS enzyme assay. pcDNA 3.1/His A plasmid, without GCS DNA, was used in control transfection.

Expression of GCS Antisense—The structure of pcDNA 3.1/His A-asGCS is shown in Fig. 1A. The GCS antisense was cloned into the EcoRI site just downstream from the anti-Xpress tag sequence in pcDNA 3.1/His A. This plasmid was introduced into MCF-7-AdrR cells by calcium phosphate coprecipitation. G418 was used to select transfecants. We found that the number of G418-resistant clones in MCF-7-AdrR as- GCS transfected cells was much lower than in MCF-7-AdrR cells transfected with pcDNA3.1/His A vector (54/10⁶ versus 251/10⁶). G418-resistant clones were further selected by meas-
uring GCS activity using the cell-free radioenzymatic assay. In all, fifty-four G418-resistant clones of MCF-7-AdrR asGCS-transfected cells were obtained, and we identified one clone that exhibited a stable 30% decrease in GCS activity (Fig. 1B). Compared with 27.4 ± 2.3 pmol of GC synthesized by MCF-7-AdrR parental cells, GCS activity in MCF-7-AdrR/asGCS was decreased to 19.7 ± 1.1 pmol of GC (Fig. 1B, p < 0.001). There were no differences in GCS activities between the pcDNA 3.1/ his A vector-transfected cells and parental MCF-7-AdrR cells (Fig. 1B).

The asGCS-transfected and parental MCF-7-AdrR cells were stained with Giemsa. Representative photomicrographs are shown in Fig. 1C. MCF-7-AdrR/asGCS cells, including nuclei, are flatter and larger than the dome-shaped, more stellate MCF-7-AdrR cells. The asGCS cell line is also more cuboidal with less dense cytoplasm. The population doubling times for both cell lines were similar, 32 and 30 h for MCF-7-AdrR/ asGCS and MCF-7-AdrR cells, respectively.

Consistent with diminished GCS activity, GCS mRNA and GCS protein were reduced in MCF-7-AdrR/asGCS cells, compared with MCF-7-AdrR cells. Total mRNA was isolated from both cell lines and reverse transcribed and amplified by RT-PCR. A representative RT-PCR gel electropherograph is shown in Fig. 2A. As with that revealed by densitometric scanning, the mRNA in MCF-7-AdrR/asGCS cells was reduced 3-fold compared with that in MCF-7-AdrR cells (25.4% versus 77.5% of β-actin). GCS protein in cell lysates was resolved by SDS-polyacrylamide gel electrophoresis and identified using GCS antisera. Western blotting showed that the total amount of GCS protein in MCF-7-AdrR/asGCS cells decreased by 32% compared with MCF-7-AdrR parental cells (77,520 and 112,860 optical density units, respectively) (Fig. 2B, right and center bands). However, MCF-7-AdrR cells that were transfected with pcDNA 3.1/ his A GCS expressed greater amounts of GCS (Fig. 2B, left band, AdrR/GCS). MCF-7-AdrR/GCS cells were developed by stable transfection of sense orientation pcDNA 3.1/ his A GCS vector in MCF-7-AdrR cells. This GCS-transfected cell line displays 80% higher GCS activity than MCF-7-AdrR cells as measured by radioenzymatic assay. After transfection with pcDNA 3.1/ his A GCS vector, although the expressed GCS was fused with Xpress tag (-Asp-Leu-Tyr-Asp-Asp-Asp-Lys-), the upward shift in molecular mass (about 800 daltons) was undetectable by Western blot (Fig. 2B).

Asp-Asp-Lys-), the upward shift in molecular mass (about 800 daltons) was undetectable by Western blot (Fig. 2B). To evaluate the expression of transfected GCS antisense gene, we employed a Xpress antibody to detect the production of Xpress-fused protein (Fig. 1A). We did not find the Xpress-tag in either MCF-7-AdrR or MCF-7-AdrR/asGCS cells (Fig. 2C). However, the tag protein was highly expressed in MCF-7-AdrR GCS transfected cells (Fig. 2C, center band). In MCF-7-AdrR/asGCS cells, what appears to be the Xpress-asGCS protein (Fig. 2C, faint band) had a higher molecular mass compared with Xpress-GCS protein of MCF-7-AdrR/GCS and was present at only 15% of the level of the latter (Fig. 2C, center band).
To further elucidate the dynamics of ceramide metabolism, ceramide levels in MCF-7-AdrR/asGCS cells increased dramatically elevated ceramide levels in GCS antisense-transfected cells. The same conditions cited above were employed, except that C6-ceramide was used in place of adriamycin. *, p < 0.0001, compared with MCF-7-AdrR cells. Adr, adriamycin; C6-Cer, C6-ceramide.

Ceramide Generation and Caspase-3 Activity under Adriamycin Stress—To further elucidate the dynamics of ceramide metabolism in drug sensitivity, we measured ceramide generation in the two cell lines. We found that adriamycin exposure dramatically elevated ceramide levels in GCS antisense-transfected cells. As shown in Fig. 4, adriamycin treatment increased the levels of ceramide in MCF-7-AdrR/asGCS cells in a time- and dose-dependent manner. At 24 and 48 h post-treatment, ceramide levels in MCF-7-AdrR/asGCS cells increased 200 and 250%, respectively (Fig. 4A). In sharp contrast, adriamycin treatment did not greatly modify ceramide levels in MCF-7-AdrR cells, which at 48 h increased only 16% above control. The result of increasing adriamycin dose on ceramide metabolism in the cell lines is shown in Fig. 4B. Adriamycin at 0.5, 1.0, and 2.5 μM enhanced ceramide levels by 181, 188, and 246%, respectively, in MCF-7-AdrR/asGCS cells (Fig. 4B), whereas MCF-7-AdrR cells displayed minimal response over the same dose range.

In mammalian cells, ceramide induces apoptosis directly through effector caspases, such as caspase-3 (21, 22). To identify whether an alteration in ceramide metabolism in GCS cells is related to adriamycin sensitivity via signal cascades, we analyzed caspase-3 activity in the parental and transfected cell lines. The data demonstrate that increased effector caspase-3 activity is consistent with changes in ceramide metabolism. At 10 μM adriamycin, the EC50 in MCF-7-AdrR cells, caspase-3 activity in MCF-7-AdrR/asGCS cells increased 290 and 980% over control, at 24 and 48 h, respectively (Fig. 5). In contrast, adriamycin treatment increased caspase-3 by 160% in MCF-7-AdrR cells, albeit only at 48 h (Fig. 5). In summary, caspase-3 activity in the GCS antisense-transfected cells was 3- and 6-fold greater in response to adriamycin treatment than observed in parental cells (p < 0.0001). This suggests that impaired GCS activity permits cells to maintain high levels of ceramide under adriamycin stress, activating caspase-3 for progression of programmed cell death.

Because GCS antisense transfection resulted in enhanced drug sensitivity, we evaluated the expression of P-glycoprotein and Bcl-2. A representative Western blot of P-glycoprotein is shown in Fig. 6A. P-glycoprotein was found only in trace amounts in MCF-7 cells (adriamycin sensitive). Decreased expression of P-glycoprotein was not evident in MCF-7-AdrR/asGCS cells, when compared with the parent MCF-7-AdrR cell line (Fig. 6A). Bcl-2 was found only in trace amounts in MCF-7-AdrR and in MCF-7-AdrR/asGCS cells (Fig. 6B), although Bcl-2 was highly expressed in MCF-7 cells, consistent with our prior finding (7).

DISCUSSION

We have introduced GCS antisense DNA into chemotherapy-resistant cancer cells and revealed that this transfection reverses cellular resistance to adriamycin and to C6-ceramide in the resulting MCF-7-AdrR/asGCS cell line. The parent line, MCF-7-AdrR was selected from MCF-7 cells by culturing in the presence of adriamycin (16, 23). These cells exhibit cross-resistance to a wide range of antineoplastic agents including Vinca alkaloids, anthracyclines, and epipodophyllotoxins (11, 16, 23–26). The MCF-7 human breast cancer cell line (27), in contrast, is drug-sensitive (10, 11, 16, 23–27). Previously, using an inducible retroviral Tet-on expression system, we showed that overexpression of GCS in MCF-7 cells conferred resistance to adriamycin and tumor necrosis factor-α (7, 15). In an opposing scenario, a chemical inhibitor of GCS, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, blocks GCS activity in intact MCF-7-AdrR cells and reverses adriamycin resistance (11).

Based on these findings and the capacity of ceramide to drive apoptosis (1, 2, 5, 7, 10, 12, 13), it was hypothesized that GCS antisense would restore adriamycin toxicity in an otherwise adriamycin-insensitive model, such as MCF-7-AdrR cells.

After transfection with pcDNA 3.1/His A-asGCS plasmid, we found that MCF-7-AdrR/asGCS cells expressed lower levels of GCS, based upon both mRNA and protein (Fig. 2). GCS enzy-
more prevalent in apoptosis signaling (30). Cellular ceramide response to DNA damage has been shown to rely on mitochon- 
drion-dependent caspases (31).

Ceramide can be generated by de novo biosynthesis and 
 sphingomyelin degradation via the action of sphingomyelins-
ases (1, 32, 33). Intracellular levels of ceramide are elevated by 
a variety of stimuli and/or agents that induce apoptosis, 
 including Fas ligand engagement of CD95, ionizing radiation, 
 ultraviolet radiation, chemotherapeutic drugs and genotoxic 
 chemicals, and several cytokines (1–7, 15, 33–35). Ceramide-
 induced cellular death is one mechanism of adriamycin-in-
duced toxicity (7, 8, 12, 14). Cellular ceramide impacts a vari-
y of signaling molecules and pathways (33). Of these various 
effects, ceramide induction of the stress-activated protein ki-
 nase cascade and inhibition of complex III activity in the 
 mitochondrial respiratory chain have been linked to the induc-
tion of apoptosis (36–38). Caspase-3, one of the effector caspases in 
 the stress-activated protein kinase apoptotic signaling path-
 way, is activated by cell-permeable ceramide as well as en-
dogenous ceramide generated in response to extracellular 
 stimuli (15, 39, 40). In present study, adriamycin treatment 
 increased cellular ceramide with activation of caspase-3 in 
 the GCS antisense transfected cells but not in parental cells. 
 Therefore, the diminished capacity for glycosylation promotes 
adriamycin-induced cytotoxicity via ceramide-linked activation 
of caspase-3.

P-glycoprotein, a well characterized drug resistance mecha-
nism (41), is highly expressed in MCF-7-AdrR cells (18). In 
 previous work on the conversion of cells toward drug resist-
ance, increased expression of P-glycoprotein in MCF-7 cells 
 transfected with GCS sense was not observed (7). Much in line, 
 in the present study we did not observe decreased expression of 
P-glycoprotein in chemosensitive MCF-7-AdrR/asGCS cells 
 (Fig. 6). This suggests that the reversal of adriamycin resist-
 ance conferred by asGCS is not related to P-glycoprotein. Bel-2 
in dephosphorylated form is a strong anti-apoptosis effector 
involved in ceramide-induced apoptosis signaling pathways 
 (42–44). We did not find that increased Bel-2 in GCS modulates 
 MCF-7 cells (7), nor in this study was altered Bel-2 expression 
 found in GCS antisense-transfected MCF-7-AdrR cells. These 
data reinforce the idea that up-regulation and down-regulation of 
 GCS regulates adriamycin sensitivity by a mechanism di-
 vored from Bel-2.

In keeping with our previous report (7), the GCS gene knock-
 out data presented here further demonstrate that GCS is one 
 cause of adriamycin resistance. This positions antisense tech-
nology as a promising tool for reversal of certain forms of 
 chemotherapy resistance.

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FIG. 5. Caspase-3 activity under adriamycin stress. Cells were 
treated without or with adriamycin (10 μM) for 24 and 48 h. After 
harvest, the soluble fraction obtained after cell lysis (10⁶ cell/tube) was 
incubated with DEVD-AFC substrate at 37 °C for 60 min as detailed 
under “Experimental Procedures.” The fluorescence of cleaved AFC was 
measured at 505 nm, * p < 0.0001, compared with MCF-7-AdrR cells 
treated with adriamycin for each corresponding treatment period.

FIG. 6. P-glycoprotein and Bel-2 expression in MCF-7-AdrR 
and MCF-7-AdrR/asGCS cells. Detergent-soluble cellular protein 
was isolated from the respective cell lines and subjected to SDS-poly-
acrylamide gel electrophoresis (50 μg/lane). Protein was transferred 
in nitrocellulose, and the immunoblot was incubated with the specified 
antibody. A, P-glycoprotein Western blots. C219 monoclonal antibody 
was used to recognize P-glycoprotein. B, Bel-2 Western blots. Ab-1 
monoclonal antibody was utilized to blot Bel-2 protein. MCF-7 cells 
were used as a positive control for Bel-2.
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