Oligonucleotides blocking glucosylceramide synthase expression selectively reverse drug resistance in cancer cells

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Abstract **High glucosylceramide synthase (GCS) activity is one factor contributing to multidrug resistance (MDR) in breast cancer. Enforced GCS overexpression has been shown to disrupt ceramide-induced apoptosis and to confer resistance to doxorubicin. To examine whether GCS is a target for cancer therapy, we have designed and tested the effects of antisense oligodeoxyribonucleotides (ODNs) to GCS on gene expression and chemosensitivity in multidrug-resistant cancer cells. Here, we demonstrate that antisense GCS (asGCS) ODN-7 blocked cellular GCS expression and selectively increased the cytotoxicity of anticancer agents. Pretreatment with asGCS ODN-7 increased doxorubicin sensitivity by 17-fold in MCF-7-AdrR (doxorubicin-resistant) breast cancer cells and by 10-fold in A2780-AD (doxorubicin-resistant) ovarian cancer cells. In MCF-7 drug-sensitive breast cancer cells, asGCS ODN-7 only increased doxorubicin sensitivity by 3-fold, and it did not influence doxorubicin cytotoxicity in normal human mammary epithelial cells. asGCS ODN-7 was shown to be more efficient in reversing drug resistance than either the GCS chemical inhibitor D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol or the P-glycoprotein blocking agents verapamil and cyclosporin A. Experiments defining drug transport and lipid metabolism parameters showed that asGCS ODN-7 overcomes drug resistance mainly by enhancing drug uptake and ceramide-induced apoptosis. This study demonstrates that a 20-mer asGCS oligonucleotide effectively reverses MDR in human cancer cells.**—Liu, Y-Y., T. Y. Han, J. Y. Yu, A. Bitterman, A. Le, A. E. Giuliano, and M. C. Cabot. **Oligonucleotides blocking glucosylceramide synthase expression selectively reverse drug resistance in cancer cells.** ***J. Lipid Res.*** **2004.** 45: **933–940.**

Supplementary key words ceramide • antisense oligonucleotides • apoptosis • chemotherapy • breast cancer • doxorubicin • drug uptake • D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol

Glucosylceramide synthase (GCS; EC 2.41.80) is a transmembrane protein with the C-terminal catalytic domain located in the cytoplasm (1). GCS transfers a glucose residue from UDP-glucose to ceramide for the synthesis of glucosylceramide. This mainly occurs on the cytoplasmic surface of the Golgi (2). In the Golgi lumen, glucosylceramide is further modified by a series of glycosyltransferases that produce higher order glycosphingolipids. Glycosphingolipids are composed of a group of membrane lipids in which the lipid portion is embedded in the outer leaflet of the plasma membrane with the sugar chain extending to the extracellular space. Glycosphingolipids are integral components of plasma membrane microdomains known as rafts, caveolae, and glycosignaling domains that are rich in sphingolipids and cholesterol (3, 4). These lipid domains assemble receptors and glycosylphosphatidylinositol-anchored proteins on their external surface and signaling molecules, including Src family kinases, G proteins, and nitric oxide synthase, on their internal surface. Glycosignaling domains in membranes have been proposed to couple cell adhesion interactions with signaling (4). With regard to cerebrosides, the accumulation of glucosylceramide has been shown to be highly consistent with chemotherapy resistance in breast, ovarian, and colon cancer cell lines and in some patients with melanoma and breast cancer (5–7). Overproduction of gangliosides that are derived from glucosylceramide at the cell surface has been shown to be strongly associated with antagonism of host immune function in cancer (8).

Ceramide has been recognized as a second messenger involved in the induction of apoptosis and in cell growth arrest (9, 10). Ceramide generation can occur in response to the postreceptor action of a variety of cytokines, hor-

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Abbreviations: asGCS, antisense glucosylceramide synthase; GCS, glucosylceramide synthase (ceramide:UDP-glucosyltransferase); HMEC, human mammary epithelial cells; NB-DNJ, *N*-butyldeoxynojirimycin; OD, optical density; ODN, oligodeoxyribonucleotide; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol.

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mones, and growth factors (9, 10). These include members of the tumor necrosis factor superfamily, Fas/Apo-1 ligand, interleukin-1, and 1,25-α-dihydroxy vitamin D_3 (9, 10). Ceramide can also be generated in cancer cells in response to treatment with anticancer drugs that are commonly used in the clinic, such as doxorubicin, paclitaxel, vinblastine, etoposide, and actinomycin D (10, 11). The loss of ceramide generation can cause cellular resistance to apoptosis in response to ionizing radiation, tumor necrosis factor-α, and doxorubicin (12–14). Our previous work showed that overexpression of GCS by gene transfection conferred cellular resistance to chemotherapy and to tumor necrosis factor-α (14–16). Recently, we found that GCS expression is upregulated in metastatic breast cancer and in multidrug-resistant cancer cell lines (our unpublished data). Overall, these studies indicate that enhanced expression of GCS contributes to poor chemotherapy response.

Inhibition of GCS activity is being evaluated as a possible treatment for several lipid-storage diseases and some types of cancer (17–19). Among the existing inhibitors of GCS, clinical trials of *N*-butyldeoxynojirimycin [NB-DNJ (Miglustat)] in patients with Gaucher's disease demonstrate the therapeutic potential of such inhibitors in glycolipid storage diseases (18, 20); D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) has been used to inhibit tumor formation in mice (19) and to increase the cytotoxicity of anticancer drugs in tumor cells (10, 21). However, undesirable side effects and the low specificity of these GCS inhibitors have hampered further application. Using a genetic approach, gene transfection with full-length antisense GCS has been shown to reverse multidrug resistance in breast cancer cells (16, 22), decrease human neuroepithelioma cell growth (23), and inhibit melanoma formation in mice (24). These studies indicate that blocking GCS gene expression has the potential to suppress ceramide glycosylation and induce apoptosis and/or cell growth arrest. The present study was undertaken to determine whether a small antisense oligonucleotide (20-mer) of GCS can act as effectively as the full-length antisense GCS (11,182-mer) to suppress GCS expression and overcome drug resistance in cancer, the idea being that such an agent would have therapeutic applications. To this end, we have designed and tested antisense oligodeoxyribonucleotides (ODNs) to GCS and found that these agents suppress GCS expression and selectively enhance doxorubicin cytotoxicity in multidrug-resistant human cancer cells.

MATERIALS AND METHODS

ODNs and chemicals

Eleven antisense ODNs that targeted GCS mRNA (GenBank accession number D50840) (25) were designed based on selection criteria described elsewhere (26). Their sequences and hybridization strength parameters are given in **Table 1**. Each ODN was synthesized as a 20-mer, modified with phosphorothioate, and purified by reverse-phase HPLC (Integrated DNA Technologies, Inc., Coralville, IA). Scrambled ODNs for each antisense GCS (asGCS) DNA were also synthesized and used as non-sequence-specific controls in this study. OligofectAMINE was purchased from GIBCO BRL (Grand Island, NY). C_6-ceramide (*N*-hexanoyl-sphingosine) was from LC Laboratories (Woburn, MA), and doxorubicin hydrochloride was from Sigma. [9,10-^{3}H]palmitic acid (50 Ci/mmol) was purchased from DuPont/NEN. GCS antiserum was kindly provided by Dr. D. L. Marks and Dr. R. E. Pagano (Mayo Clinic and Foundation, Rochester, MN).

Cell culture

The human breast adenocarcinoma cell line MCF-7-AdrR (NCI/ADR-Res), which is resistant to doxorubicin (27), was kindly provided by Dr. Kenneth Cowan (UNMC Eppley Cancer Center, Omaha, NE) and Dr. Merrill Goldsmith (National Cancer Institute, Bethesda, MD). Normal human mammary epithelial cells (HMECs) were purchased from Cambrex (Walkersville, MD). The ovarian cancer cell line A2780-AD, which is resistant to doxorubicin (28), was kindly provided by Dr. Thomas C. Hamilton (Fox Chase Cancer Institute, Philadelphia, PA). Cells were maintained in RPMI 1640 medium containing 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 584 mg/l L-glutamine. A2780-AD cells were cultured in medium containing 100 nM doxorubicin in addition to the above components. HMECs were cultured in mammary epithelial growth medium supplied by Cambrex.

TABLE 1. Characteristics of antisense oligonucleotides against the human glucosylceramide synthase gene

Oligomer	Sequence	Target	Hybridization Strength Parameter			
			−dG	Hairpin	Dimer	Percentage GC
			kcal/mol			
ODN-1	GCCAGGTCCAGCAGCGCCAT	Start code (1–20)	29.1	2.3	−6.2	70
ODN-2	CCATAATAT CCCATCTGA AC	ORF (929–938)	21.1	3.4	−1.4	40
ODN-3	GCAGAGATA TAGTATCTT GG	ORF (579–598)	20.6	2.2	−3.2	40
ODN-4	GATTAAGTT AGGATCTAC CC	ORF (181–200)	21.1	2.6	−3.0	40
ODN-5	GCTGTAGTT ATACATCTA GG	ORF (1172–1191)	20.4	2.9	−3.0	40
ODN-6	CCACCTATA AACAATCTA GC	ORF (327–346)	21.4	3.0	−2.3	40
ODN-7	ACGGCCATT CCCTCCAAG GC	ORF (18–37)	28	0.95	−5.5	65
ODN-8	CTGCTGTAC CCCCACAGC GT	ORF (1146–1166)	27.2	−1.5	−5.8	65
ODN-9	TATCTTGGA TGTGAAGTT CC	His^{193} (568–585)	22.5	1.3	−3.5	45
ODN-10	GACATTGCA AACCTCCAA CC	Exon-7 (739–756)	25.2	2.2	−6.8	50
ODN-11	ATTCCTGTC ACACAAAAG AA	Cyc^{207} (613–632)	22.9	2.0	−4.2	35

ODN, oligodeoxyribonucleotide; ORF, open reading frame. Oligonucleotides were analyzed by HYBsimulator program.

RNA extraction and RT-PCR mRNA analysis

Cells were pretreated with OligofectAMINE alone (vehicle) or OligofectAMINE containing oligonucleotide (50–400 nM) for 4 h in serum-free medium and then incubated for another 20 h in 5% FBS medium. Cellular RNA was purified using a total RNA isolation RNeasy mini kit (Qiagen, Valencia, CA). Equal amounts of RNA (100 ng) were used for RT-PCR, as previously described (16). Under upstream primer (5′-CCATCGATGGCTGGAAACATTCTTTGAATTGGAT-3′) and downstream primer (5′-CCATCGATGATGGCTCATTAAACAAGACATTCCTGTC-3′) conditions, a 421 bp fragment in the 5′ terminal region of the GCS gene was produced using a high-fidelity single-tube RT-PCR system (ProSTAR HF; Stratagene, La Jolla, CA).

Cytotoxicity assay

Assays were performed as previously described (5, 14, 16). To assess the cytotoxic influence of the ODNs, cells were pretreated with OligofectAMINE alone (vehicle) or with OligofectAMINE plus ODN at increasing concentrations in serum-free medium for 4 h and then incubated for 72 h in medium containing 5% FBS. To test the influence of ODNs on cell response to drug, cells were pretreated with OligofectAMINE alone (vehicle) or with OligofectAMINE plus ODN (100 nM in MCF-7-AdrR cells, 200 nM in A2780-AD cells) for 4 h and then incubated for another 72 h in 5% FBS medium containing increasing concentrations of doxorubicin. Cytotoxicity was determined using the Promega 96 Aqueous cell proliferation assay kit (Promega, Madison, WI).

Apoptotic cell death detection by ELISA and terminal deoxynucleotide transferase-mediated dUTP nick end labeling

The presence of mononucleotides and oligonucleotides, a feature of cells undergoing apoptosis (29), was evaluated by Cell Death Detection ELISA (Boehringer Mannheim, Indianapolis, IN) performed according to the manufacturer's instructions. Briefly, cells were pretreated with asGCS ODN in serum-free medium and cultured in 5% FBS medium containing doxorubicin (2.5 μM) for 48 h; 10^4 cells from each sample were then lysed in 200 μl of lysis buffer. After centrifugation (1,000 *g*, 10 min), a 20 μl aliquot of lysate supernatant (10^3 cells/tube) was incubated with DNA histone antibody and anti-DNA conjugated antibody for 2 h at 24°C and then with substrate for 15 min. Absorbance was measured at 405 nm.

Immunohistochemical detection of apoptosis was performed by terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) staining using the FragEL DNA Fragmentation detection kit (Oncogen, Boston, MA). Terminal deoxynucleotidyl transferase labeling with fluorescein-dUTP was done according to the manufacturer's recommendations. Briefly, cells (2×10^4 per chamber) were cultured overnight in 10% FBS RPMI 1640 medium using chamber slides (Nalge Nunc, Inc., Naperville, IL). Cells were then pretreated with OligofectAMINE alone or OligofectAMINE containing the indicated asGCS ODNs (100 nM, 4 h) and then incubated with doxorubicin (2.5 μM) for 48 h. Cells were fixed with methanol (50% in TBS for 10 min, 100% for 10 min, and 50% in TBS for 10 min) and finally rinsed with TBS. The cells on slides were digested for 20 min with 0.2 mg/ml proteinase K in 10 mM Tris-HCl, pH 8, and labeled for 90 min with Fluorescein-FragEL terminal deoxynucleotide transferase reaction mixture at 37°C in a humidified chamber. After mounting, the cells were visualized using a standard fluorescein filter (465–495 nm).

Ceramide and glucosylceramide analysis

Analysis was performed as previously described (5, 14). Cells were seeded in six-well plates (6×10^4 cells/well) in 10% FBS RPMI 1640 medium. After pretreatment with ODNs (100 nM, 4 h) and culture for another 20 h, cells were shifted to 5% FBS medium and grown for the indicated times. Cellular lipids were radiolabeled by adding [^{3}H]palmitic acid (2.5 μCi/ml) to the culture medium for 24 h. Tritium-labeled ceramide and glucosylceramide were isolated by thin-layer chromatography of the total cellular lipid extract. Ceramide was resolved using a solvent system containing chloroform-acetic acid (90:10, v/v), and glucosylceramide was resolved using a solvent system containing chloroform-methanol-ammonium hydroxide (70:20:4, v/v). Commercial lipid standards were cochromatographed. After separation, lipids were visualized with iodine vapor staining and identified by migration. The ceramide and glucosylceramide areas were 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. Radiochromatograms were sprayed with EN^{3}HANCE (DuPont/NEN) and exposed for 2–3 days for autoradiography.

Rhodamine assay

After pretreatment with ODNs (100 nM, 4 h) and culture for another 20 h, cells (2.0×10^6) were incubated with rhodamine-123 (0.1 mg/ml) at 37°C for 30 min. After centrifugation at 500 *g* for 15 min, supernatants were discarded and the cells were washed twice in RPMI 1640 medium. Uptake of rhodamine-123 was measured at 485 nm/530 nm (excitation/emission) using the FL-600 fluorescent microplate reader (16). For fluorescence photomicrographs, cells were fixed with cold acetic acid-methanol (1:3, v/v) and photographed using an Olympus IX70 fluorescence microscope equipped with a digital photomicrographic system (16).

RESULTS

asGCS ODN selection

Analyzed by the HYBsimulator program (RNAture, Inc., Irvine, CA), a series of ODNs were identified as potential antisense candidates. Eleven ODNs were generated directed against different regions of human GCS mRNA, including the start and stop codons, the activity-associated sites corresponding to the sites of rat His^{193} and Cys^{207} (30, 31) and mouse exon 7 (32), and other open reading frame regions (Table 1). Antisense GCS ODN-1, ODN-7, and ODN-8 had the highest hybridization strengths. Comparing sequences of GCS in human, rat, and mouse by gene alignment analysis, the homology values in target regions of asGCS ODN-1 and ODN-7 were 100% and 85%, respectively, and the homology values for asGCS ODN-5, ODN-9, ODN-10, and ODN-11 were 90%. BLAST analysis showed that only asGCS ODN-7 and ODN-1 had high similarity to human GCS (score of 40 bits; E value of 0.008).

The 20-mer phosphorothioate-modified asGCS ODNs were evaluated for effects on cell growth and gene expression. asGCS ODNs were delivered to cells by incubation in serum-free medium containing OligofectAMINE. All asGCS ODNs inhibited the growth of MCF-7-AdrR cells, albeit variably; the EC_{50} values ranged from 0.3 μM (asGCS ODN-7) to 2.2 μM (asGCS ODN-6). A scrambled ODN (ODN-SC) that has the same base composition as ODN-7,

but in random sequence, was used as a nonspecific oligonucleotide control. As shown in **Fig. 1A**, the influence of asGCS ODN-7 on cell viability was dose dependent in MCF-7-AdrR breast cancer cells. At a concentration of 100 nM, cell viability in MCF-7-AdrR was 75%, and it decreased to ~45% at 400 nM. In contrast, asGCS ODN-7 treatment elicited only minor cytotoxic responses in normal HMECs: with 100 and 400 nM asGCS ODN-7 treatment, cell viability was 94% and 84% of untreated HMECs, respectively. ODN-SC treatment produced minor cytotoxic responses in HMECs and MCF-7-AdrR cells. Of the ODNs tested, only asGCS ODN-7 (Table 1) was effective in reducing GCS expression. RT-PCR analysis showed that the inhibitory effect of asGCS ODN-7 on gene expression was dose dependent (Fig. 1B). GCS mRNA levels decreased to 21% compared with that in untreated MCF-7-AdrR cells [optical density (OD) 6,612 vs. 30,972 normalized with GADPH; Fig. 1B] in the presence of 100 nM ODN-7. As examined by tritium incorporation and TLC analysis of glucosylceramide, asGCS ODN-7 (100 nM) reduced ceramide glycosylation by 25% compared with untreated cells (3,639 vs. 4,405 cpm). A representative autoradiograph of glucosylceramide is shown in Fig. 1C. These data indicate that the cytotoxicity of the asGCS ODNs is associated with the suppression of GCS expression and enzyme activity.

Influence on doxorubicin cytotoxicity

We used a ceramide analog, C_6-ceramide, and doxorubicin to assess the influence of asGCS ODNs on the cellular response to chemotherapy. The doxorubicin-resistant human breast cancer cell line MCF-7-AdrR and the human ovarian cancer cell line A2780-AD were selected by passage of the drug-sensitive wild-type counterparts in medium containing increasing concentrations of doxorubicin (Adriamycin) (27, 28). Both MCF-7-AdrR and A2780-AD cells exhibit a multidrug-resistant phenotype and are cross-resistant to a wide range of antineoplastic agents, including *Vinca* alkaloids, anthracyclines, and epipodophyllotoxins (5, 16, 27, 28). We previously found that GCS expression is upregulated in MCF-7-AdrR (16, 22) and in A2780-AD cells (our unpublished data) compared with their wild-type counterparts. From this, we hypothesized that suppression of GCS gene expression would overcome drug resistance by increasing the cytotoxicity of chemotherapeutic agents that are known to generate ceramide (10, 16). asGCS ODN-7 exposure increased C_6-ceramide sensitivity by 2-fold in MCF-7-AdrR cells (EC_{50}, 6.4 vs. 12.4 μM) and by 4-fold in A2780-AD cells (EC_{50}, 3.4 vs. 16.0 μM). As shown in **Fig. 2**, asGCS ODN-7 (100 nM, 4 h) increased doxorubicin cytotoxicity in MCF-7-AdrR cells by ~19-fold (EC_{50}, 0.34 vs. 6.4 μM) and in A2780-AD cells by 10-fold (EC_{50}, 0.6 vs. 6.0 μM). In contrast, asGCS ODN-7 enhanced doxorubicin cytotoxicity by 2.8-fold in drug-sensitive MCF-7-AdrR cells (EC_{50}, 0.11 vs. 0.31 μM) and did not influence doxorubicin cytotoxicity in normal HMECs (EC_{50}, 0.85 vs. 0.72 μM).

To compare and contrast other modes of GCS inhibition for influence on cell sensitivity to chemotherapy, we used PDMP, a GCS inhibitor. PDMP exposure enhanced doxorubicin sensitivity by ~2.3-fold (EC_{50}, 2.8 vs. 6.4 μM) (**Fig. 3A**) at a relatively high concentration (5 μM). We next investigated verapamil and cyclosporin A, which block P-glycoprotein drug effluxing and reverse drug resistance in a number of systems (28, 33, 34). However, both verapamil and cyclosporin A (1.0 μM; **Fig. 3B**) increased doxorubicin sensitivity only minimally [1.1-fold (EC_{50}, 6.0 vs. 6.4 μM) and 1.3-fold (EC_{50}, 5.0 vs. 6.4 μM), respectively] in MCF-7-AdrR cells. These data show that asGCS ODN-7 is a more potent reverser of doxorubicin resistance than agents with previous clinical histories.

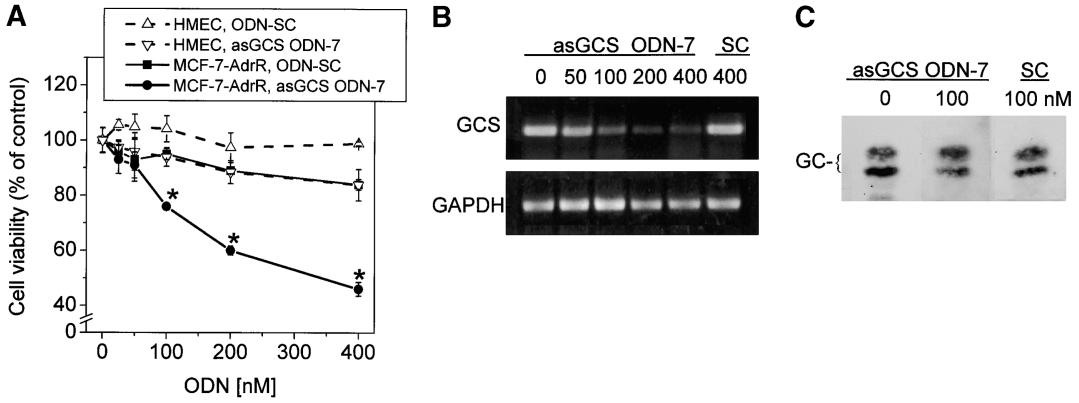

Fig. 1. Influence of antisense glucosylceramide synthase (asGCS) oligodeoxyribonucleotides (ODNs) on cell viability and GCS expression. A: Influence of asGCS ODN-7 concentration on cell viability. MCF-7-AdrR cells (3,000 cells/well) and normal human mammary epithelial cells (HMECs; 8,000 cells/well) were seeded in 96-well plates, treated the next day with increasing concentrations of ODNs, and cultured for an additional 72 h. Cell viability was determined using the Promega 96 Aqueous cell proliferation assay kit. Data shown are means ± SD from three experiments in triplicate. ODN-SC, scrambled ODN control. * $P < 0.001$. B: Influence of asGCS ODN-7 on GCS expression. MCF-7-AdrR cells were treated with ODNs for 4 h and cultured for an additional 48 h. Isolated total RNA (100 ng) was analyzed by high-fidelity RT-PCR and 1% agarose gel electrophoresis. Housekeeping GAPDH, glyceraldehyde-3-phosphate dehydrogenase was used as an endpoint control. C: Thin-layer autoradiograph of glucosylceramide. MCF-7-AdrR cells were treated with ODNs for 4 h and cultured for an additional 48 h. Cellular lipids were radiolabeled by adding [^{3}H]palmitic acid (2.5 μCi/ml culture medium) during the last 24 h. The autoradiograph was developed using EN^{3}HANCE (exposed for 2 days).

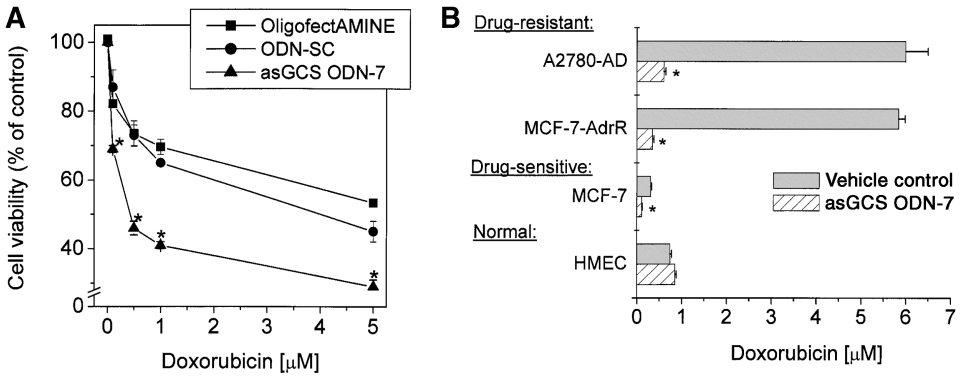

Fig. 2. Influence of asGCS ODN on the cellular response to doxorubicin. Cells were pretreated with the indicated ODNs (100 nM, 4 h) and exposed to doxorubicin as detailed in Materials and Methods. Data shown are means ± SD from three experiments in triplicate. A: Influence of asGCS ODN-7 on doxorubicin cytotoxicity in MCF-7-AdrR cells. * $P < 0.001$ compared with pretreatment with OligofectAMINE (vehicle control). B: The influence of asGCS ODN-7 on doxorubicin EC_{50} in drug-resistant and drug-sensitive cancer cells and normal cells. * $P < 0.001$, compared to vehicle control.

Influence on drug uptake and apoptosis

To further elucidate the mechanism by which asGCS ODNs sensitize cancer cells to chemotherapy, drug uptake and ceramide-induced apoptosis were analyzed. To assess the influence of asGCS ODNs on drug uptake, we used rhodamine-123. As shown by fluorescence photomicrographs, asGCS ODN-7 treatment (100 nM, 4 h) substantially enhanced the uptake of rhodamine-123 in MCF-7-AdrR cells compared with ODN-SC (**Fig. 4A**). Quantitative fluorescence measurements (Fig.4B) showed that asGCS ODN-7 doubled rhodamine-123 uptake.

Ceramide is a lipid second messenger in the apoptotic pathway initiated by anticancer drugs, cytokines, and ionizing radiation (10, 11). The apoptotic impact of doxorubicin, daunorubicin, paclitaxel, etoposide, and actinomycin D depends in part on the cellular generation of ceramide (10, 11). Therefore, suppressing GCS gene expression and enhancing the levels of intracellular ceramide should quell the poor responses to ceramide-generating agents (10, 16). As shown in **Fig. 5A**, doxorubicin treatment alone or in combination with ODN-SC did not significantly increase [^{3}H]ceramide in MCF-7-AdrR cells; however, doxorubicin combined with asGCS ODN-7 increased cellular [^{3}H]ceramide by 165% (477 ± 5 cpm vs. 290 ± 45 cpm, $P < 0.001$). This suggests that asGCS ODN-7 significantly depresses ceramide glycosylation and causes ceramide accumulation in MCF-7-AdrR cells in response to doxorubicin treatment. Further characterization revealed that doxorubicin elicited apoptosis only in cells pretreated with asGCS ODN-7 (Fig. 5B, C). Experi-

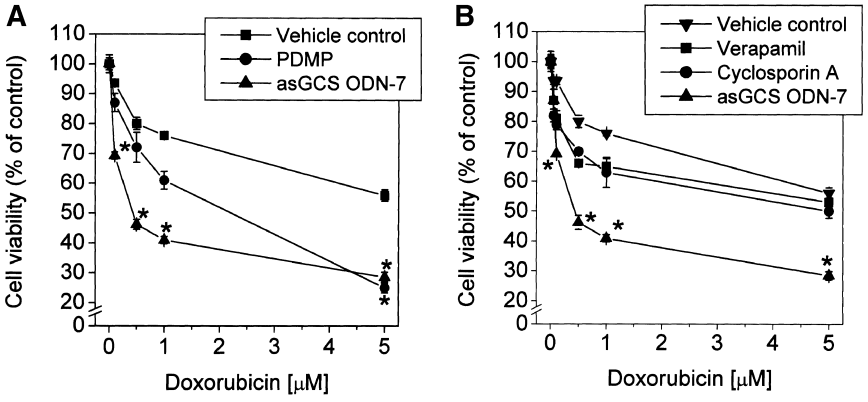

Fig. 3. Influence of the GCS inhibitor D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and P-glycoprotein blocking agents on doxorubicin cytotoxicity. A: Influence of PDMP. MCF-7-AdrR cells were pretreated with either ODN-7 (100 nM, 4 h) or PDMP (5 μM, 4 h) in serum-free medium containing OligofectAMINE and exposed to doxorubicin as detailed in Materials and Methods. For vehicle control, cells were pretreated in serum-free medium containing OligofectAMINE. B: Influence of verapamil and cyclosporin A. MCF-7-AdrR cells were pretreated with asGCS ODN-7 (100 nM, 4 h), verapamil (1.0 μM, 4 h), or cyclosporin A (1.0 μM, 4 h) in serum-free medium containing OligofectAMINE and exposed to doxorubicin. Data shown are means ± SD from three experiments in triplicate. * $P < 0.001$ compared with the vehicle control.

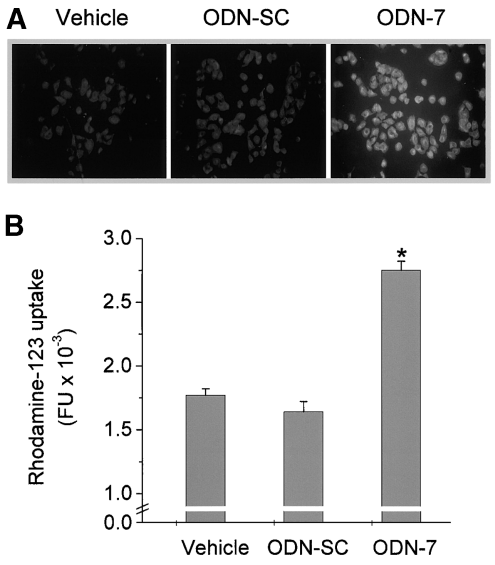

Fig. 4. Influence of asGCS ODN-7 on the uptake of rhodamine-123. A: Fluorescence photomicrographs. MCF-7-AdrR cells were pretreated with asGCS ODN (100 nM, 4 h) and cultured overnight. The pretreated cells were incubated with rhodamine-123 (0.1 mg/ml) for 30 min at 37°C and fixed as detailed in Materials and Methods. B: Cellular uptake of rhodamine-123. The pretreated MCF-7-AdrR cells were incubated with rhodamine-123 (0.1 mg/ml) for 30 min at 37°C, and intracellular rhodamine-123 was measured. * $P < 0.001$, compared to pretreatment with ODN-SC. FU, fluorescent units.

ments showed an apoptotic index of 200% (OD 0.43 vs. 0.22) with asGCS ODN-7 treatment and 267% (OD 0.59 vs. 0.22) with the asGCS ODN-7/doxorubicin combination (Fig. 5B). However, ODN-SC did not significantly increase apoptosis with or without doxorubicin. TUNEL fluorescence imaging confirmed that apoptosis was highest in cells treated with the asGCS ODN-7/doxorubicin regimen (Fig. 5C).

DISCUSSION

asGCS ODN-7 is a novel GCS inhibitor that decreases enzyme activity through the suppression of GCS gene expression. The GCS gene is composed of nine exons and encodes 1,182 nucleotides with 98% homology in human and mouse (25, 35). Recently, several groups have demonstrated that transfection of full-length antisense GCS DNA depresses GCS expression, reduces drug resistance in breast cancer cells (22), inhibits neuroepithelioma cell growth (23), and retards melanoma growth in mice (24). These works suggest that a gene sequence approach could be used to develop a specific GCS inhibitor. Antisense oligonucleotides represent a genre of effective small molecule inhibitors (26, 36). Zeng et al. (37) have used antisense DNA techniques to target GD3 synthase (CMP-sialic acid:α-2,8-sialytransferase; EC 2.4.99.8) and GM2 synthase (UDP-*N*-acetylgalactosamine:β-1,4-*N*-acetylgalactosaminyltransferase; EC 2.499.8) to manipulate glycolipid synthesis in HL-60 human leukemia cells. Antisense oligonucleotide-type drugs have been shown to be safe and more effective than other types of anticancer agents in animal models and in patients with cancer (38). asGCS ODN-7 is a 20-mer phosphorothioate oligonucleotide (Table 1), but we show that it is as efficient as full-length antisense GCS gene transfection (22, 24). We found that even at low concentration (100 nM) ODN-7 displays a suppressive influence on GCS gene expression and cellular growth. With the advantage of a low molecular weight, asGCS ODN-7 is easily amenable for knocking down GCS expression and studying the role of GCS in health and disease.

Although several amino acids in human GCS, including His^{193} and Cys^{207}, are essential for GCS activity (30–32), the design of new inhibitors is still hampered by a lack of knowledge concerning GCS active sites and spatial structure-catalytic mechanisms. Polyclonal antibodies produced

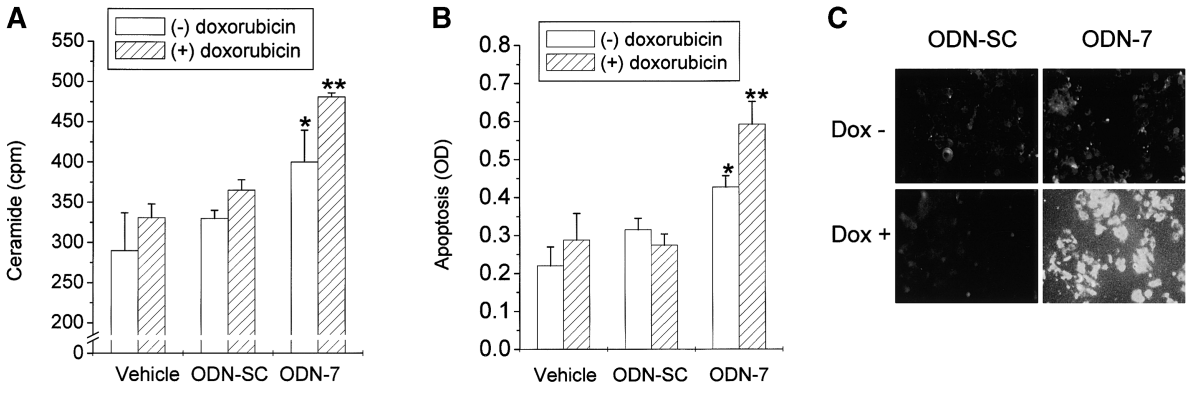

Fig. 5. Influence of asGCS ODN-7 on ceramide generation and apoptosis. A: Ceramide generation. MCF-7-AdrR cells were pretreated with ODNs (100 nM, 4 h) and exposed to doxorubicin (2.5 μM) as detailed in Materials and Methods. Ceramide values are given as cpm tritium per 10^5 cpm total lipid. Data represent means ± SD of triplicate measurements from three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with pretreatment with OligofectAMINE with or without ODN control (ODN-SC). B: Apoptosis, ELISA method. MCF-7-AdrR cells were pretreated with the indicated ODN (100 nM, 4 h) and cultured with doxorubicin (2.5 μM, 48 h). Data represent means ± SD of triplicate measurements from two independent experiments. * $P < 0.05$, ** $P < 0.001$ compared with pretreatment with OligofectAMINE with or without ODN control. OD, optical density. C: Apoptosis, terminal deoxynucleotide transferase-mediated dUTP nick end labeling staining. MCF-7-AdrR cells were pretreated with ODN (100 nM, 4 h) and cultured with doxorubicin (2.5 μM, 48 h). Dox, doxorubicin.

in recent years do not display GCS inhibitory activity (30, 31). Among the existing inhibitors of GCS, NB-DNJ has been shown to decrease glycosphingolipid accumulation in Tay-Sachs disease and Sandhoff disease in mice and in a clinical trial of patients with Gaucher's disease (39, 40). However, this compound also inhibits α-glycosidase I and II and is less effective in inhibiting GCS (21, 40). PDMP and related compounds reduce glycolipid buildup in a mouse model (41), increase the cytotoxicity of anticancer drugs in cultured tumor cells (10, 33, 42), decrease tumor formation in melanoma models (19), and inhibit metastasis in lung carcinoma (43). However, the clinical application of these agents is limited because of the lack of oral availability, their rapid elimination and degradation, and reported neurological side effects (21). In the present study, we found that asGCS ODN-7 is more effective than PDMP in reversing chemotherapy resistance (Fig. 3A). As a new specific inhibitor of GCS, asGCS ODN-7 should be further studied in other cell lines and in vivo.

Overexpression of GCS is one factor that contributes to drug resistance in cancer; therefore, suppression of GCS could substantially reverse drug resistance and sensitize multidrug-resistant cancer cells to chemotherapy. Drug-resistant MCF-7-AdrR and A2780-AD cells overexpress GCS, MDR1, and other molecules associated with drug resistance (5, 16, 28). In this study, asGCS ODN-7 increased doxorubicin cytotoxicity in drug-resistant cancer cells but had little influence on doxorubicin cytotoxicity in drug-sensitive breast cancer cells and in normal HMECs. In MCF-7-AdrR cells, asGCS ODN-7 was more efficient than the P-glycoprotein blockers verapamil and cyclosporin A in reversing doxorubicin resistance (Fig. 3B). asGCS ODN-7 also enhanced drug uptake and increased ceramide-induced apoptosis (Figs. 4, 5). These results indicate that asGCS ODN-7 has potential as a chemosensitizer in cancer treatment. Developing an agent that could efficiently enhance chemotherapy cytotoxicity in the drug-resistant setting is a huge challenge. The effectiveness of an antisense oligonucleotide may be influenced by many factors, including chemical modification of the oligonucleotide backbone and the kinetics of cellular uptake, especially in in vivo models (26). Optimal backbone modification and testing in vitro and in vivo are the next steps in the development of asGCS ODN-7 into an agent of clinical utility.

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REFERENCES

1. Marks, D. L., K. Wu, P. Paul, Y. Kamisaka, R. Watanabe, and R. E. Pagano. 1999. Oligomerization and topology of the Golgi membrane protein glucosylceramide synthase. *J. Biol. Chem.* **274:** 451–456.
2. Jeckel, D., A. Karrenbauer, K. N. Burger, G. van Meer, and F. Wieland. 1992. Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. *J. Cell Biol.* **117:** 259–267.
3. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature.* **387:** 569–572.
4. Hakomori, S., K. Handa, K. Iwabuchi, S. Yamamura, and A. Prinetti. 1998. New insights in glycosphingolipid function: "glycosignaling domain," a cell surface assembly of glycosphingolipids with signal transducer molecules, involved in cell adhesion coupled with signaling. *Glycobiology.* **8:** XI–XIX.
5. Lavie, Y., H. Cao, S. L. Bursten, A. E. Giuliano, and M. C. Cabot. 1996. Accumulation of glucosylceramides in multidrug-resistant cancer cells. *J. Biol. Chem.* **271:** 19530–19536.
6. Lucci, A., W. I. Cho, T. Y. Han, A. E. Giuliano, and M. C. Cabot. 1998. Glucosylceramide: a marker for multiple-drug resistant cancers. *Anticancer Res.* **18:** 475–480.
7. Kok, J. W., R. J. Veldman, K. Klappe, H. Koning, C. M. Filipeanu, and M. Muller. 2000. Differential expression of sphingolipids in MRP1 overexpressing HT29 cells. *Int. J. Cancer.* **87:** 172–178.
8. McKallip, R., R. Li, and S. Ladisch. 1999. Tumor gangliosides inhibit the tumor-specific immune response. *J. Immunol.* **163:** 3718–3726.
9. Jarvis, M. D., S. Grant, and R. N. Kolesnick. 1996. Ceramide and the induction of apoptosis. *Clin. Cancer Res.* **2:** 1–6.
10. Senchenkov, A., D. A. Litvak, and M. C. Cabot. 2001. Targeting ceramide metabolism—a strategy for overcoming drug resistance. *J. Natl. Cancer Inst.* **93:** 347–357.
11. Ogretmen, B., and Y. A. Hannun. 2001. Updates on functions of ceramide in chemotherapy-induced cell death and in multidrug resistance. *Drug Resist. Updat.* **4:** 368–377.
12. Santana, P., L. A. Pena, A. Haimovitz-Friedman, S. Martin, D. Green, M. McLoughlin, C. Cordon-Cardo, E. H. Schuchman, Z. Fuks, and R. Kolesnick. 1996. Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. *Cell.* **86:** 189–199.
13. Cai, Z., A. Bettaieb, N. E. Mahdani, L. G. Legres, R. Stancou, J. Masliah, and S. Chouaib. 1997. Alteration of the sphingomyelin/ceramide pathway is associated with resistance of human breast carcinoma MCF7 cells to tumor necrosis factor-α-mediated cytotoxicity. *J. Biol. Chem.* **272:** 6918–6926.
14. Liu, Y. Y., T. Y. Han, A. E. Giuliano, and M. C. Cabot. 1999. Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers Adriamycin resistance in human breast cancer cells. *J. Biol. Chem.* **274:** 1140–1146.
15. Liu, Y. Y., T. Y. Han, A. E. Giuliano, and M. C. Cabot. 1999. Glycosylation of ceramide potentiates cellular resistance to tumor necrosis factor-α induced apoptosis. *Exp. Cell Res.* **252:** 464–470.
16. Liu, Y. Y., T. Y. Han, A. E. Giuliano, and M. C. Cabot. 2001. Ceramide glycosylation potentiates cellular multidrug resistance. *FASEB J.* **15:** 719–730.
17. Tifft, C. J., and R. J. Proia. 2000. Stemming the tide: glycosphingolipid synthesis inhibitors as therapy for storage diseases. *Glycobiology.* **10:** 1249–1258.
18. Lachmann, R. H. 2003. Miglustat. Oxford GlycoSciences/Actelion. *Curr. Opin. Invest. Drugs.* **4:** 472–479.
19. Deng, W., R. Li, and S. Ladisch. 2000. Influence of cellular ganglioside depletion on tumor formation. *J. Natl. Cancer Inst.* **92:** 912–917.
20. Pastores, G. M., and N. L. Barnett. 2003. Substrate reduction therapy: miglustat as a remedy for symptomatic patients with Gaucher disease type 1. *Expert Opin. Invest. Drugs.* **12:** 273–281.
21. Radin, N. S. 1994. Rationales for cancer chemotherapy with PDMP, a specific inhibitor of glucosylceramide synthase. *Mol. Chem. Neuropathol.* **21:** 111–127.
22. Liu, Y. Y., T. Y. Han, A. E. Giuliano, N. Hansen, and M. C. Cabot. 2000. Upcoupling ceramide glycosylation by transfection of glucosylceramide synthase antisense reverses Adriamycin resistance. *J. Biol. Chem.* **275:** 7138–7143.
23. Di Sano, F., S. Di Bartolomeo, C. Fiorentini, P. Matarrese, A. Spinedi, and M. Piacentini. 2002. Antisense to glucosylceramide

synthase in human neuroepithelioma affects cell growth but not apoptosis. *Cell Death Differ.* **9:** 693–695.
24. Deng, W., R. Li, M. Guerrera, Y. Liu, and S. Ladisch. 2002. Transfection of glucosylceramide synthase antisense inhibits mouse melanoma formation. *Glycobiology.* **12:** 145–152.
25. Ichikawa, S., H. Sakiyama, G. Suzuki, K. I. Hidari, and Y. Hirabayashi. 1996. Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyzes the first glycosylation step of glycosphingolipid synthesis. *Proc. Natl. Acad. Sci. USA.* **93:** 4638–4643.
26. Agrawal, S., and E. R. Kandimalla. 2000. Antisense therapeutics: is it as simple as complementary base recognition? *Mol. Med. Today.* **6:** 72–81.
27. Fairchild, C. R., S. P. Ivy, C. S. Kao-Shan, J. Whang-Peng, M. A. Israel, P. W. Melera, K. H. Cowan, and M. E. Goldsmith. 1987. Isolation of amplified and overexpressed DNA sequences from Adriamycin-resistant human breast cancer cells. *Cancer Res.* **47:** 5141–5148.
28. Rogan, A. M., T. C. Hamilton, R. C. Young, R. W. Klecker, and R. F. Ozols. 1984. Reversal of Adriamycin resistance by verapamil in human ovarian cancer. *Science.* **224:** 994–996.
29. Bonfoco, E., D. Krainc, M. Ankarcrona, P. Nicotera, and S. A. Lipton. 1995. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. USA.* **92:** 7162–7166.
30. Wu, K., D. L. Marks, R. Watanabe, P. Paul, N. Rajan, and R. E. Pagano. 1999. Histidine-193 of rat glucosylceramide synthase resides in a UDP-glucose- and inhibitor (D-threo-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol)-binding region: a biochemical and mutational study. *Biochem. J.* **341:** 395–400.
31. Marks, D. L., M. Dominguez, K. Wu, and R. E. Pagano. 2001. Identification of active site residues in glucosylceramide synthase: a nucleotide-binding/catalytic motif conserved with processive beta-glycosyltransferases. *J. Biol. Chem.* **276:** 26492–26498.
32. Yamashita, T., R. Wada, T. Sasaki, C. Deng, U. Bierfreund, K. Sandhhoff, and R. Proia. 1999. A vital role for glycosphingolipid synthase during development and differentiation. *Proc. Natl. Acad. Sci. USA.* **96:** 9142–9147.
33. Lavie, Y., H. Cao, A. Volner, A. Lucci, T. Y. Han, V. Geffen, A. E. Giuliano, and M. C. Cabot. 1997. Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells. *J. Biol. Chem.* **272:** 1682–1687.
34. Page, R. L., C. S. Hughes, S. Huyan, J. Sagris, and M. Trogdon. 2000. Modulation of P-glycoprotein-mediated doxorubicin resistance in canine cell lines. *Anticancer Res.* **20:** 3533–3538.
35. Ichikawa, S., K. Ozawa, and Y. Hirabayashi. 1998. Molecular cloning and characterization of the mouse ceramide glucosyltransferase gene. *Biochem. Biophys. Res. Commun.* **253:** 707–711.
36. Crooke, S. T. 2000. Potential roles of antisense technology in cancer chemotherapy. *Oncogene.* **19:** 6651–6659.
37. Zeng, G., T. Ariga, X. B. Gu, and R. K. Yu. 1995. Regulation of glycolipid synthesis in HL-60 cells by antisense oligodeoxynucleotides to glycosyltransferase sequences: effect on cellular differentiation. *Proc. Natl. Acad. Sci. USA.* **92:** 8670–8674.
38. Jansen, B., V. Wacheck, E. Heere-Ress, H. Schlagbauer-Wadl, C. Hoeller, T. Lucas, M. Hoermann, U. Hollenstein, K. Wolff, and H. Pehamberger. 2000. Chemosensitisation of malignant melanoma by Bcl-2 antisense therapy. *Lancet.* **356:** 1728–1733.
39. Jeyakumar, M., T. D. Butters, M. Cortina-Borja, V. Hunnam, R. L. Proia, V. H. Perry, R. A. Dwek, and F. M. Platt. 1999. Delayed symptom onset and increased life expectancy in Sandhoff disease mice treated with N-butyldeoxynojirimycin. *Proc. Natl. Acad. Sci. USA.* **96:** 6388–6393.
40. Cox, T., R. Lachmann, C. Hollak, J. Aerts, S. van Weely, M. Hrebicek, F. Platt, T. Butters, R. Dwek, C. Moyses, I. Gow, D. Elstein, and A. Zimran. 2000. Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. *Lancet.* **355:** 1481–1485.
41. Abe, A., S. R. Wild, W. L. Lee, and J. A. Shayman. 2001. Agents for the treatment of glycosphingolipid storage disorders. *Curr. Drug Metab.* **2:** 331–338.
42. Olshefski, R. S., and S. Ladisch. 2001. Glucosylceramide synthase inhibition enhances vincristine-induced cytotoxicity. *Int. J. Cancer.* **93:** 131–138.
43. Inokuchi, J., M. Jimbo, K. Momosaki, H. Shimeno, A. Nagamatsu, and N. S. Radin. 1990. Inhibition of experimental metastasis of murine Lewis lung carcinoma by an inhibitor of glucosylceramide synthase and its possible mechanism of action. *Cancer Res.* **50:** 6731–6737.