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13. Abstract *(Maximum 200 Words) (abstract should contain no proprietary or confidential information)*
Glucosylceramide synthase (GCS) catalyzes ceramide glycosylation, disrupts ceramide-induced apoptosis elicited by chemotherapy, and appeared to be a major cause of multidrug resistance (MDR) in cancer. Previous studies pinpoint GCS as a therapeutic target for MDR. In this work, we have synthesized antisense GCS oligodeoxyribonucleotides (asGCS ODNs) to block GCS mRNA transcription, and tested several of the oligos for chemotherapy-enhancing properties in drug resistant cancer cell models. Antisense GCS ODN-7 suppressed GCS mRNA expression (RT-PCR) by 80%, and GCS protein (Western blot) by 40%, and affected 30-fold increases in sensitivity to Adriamycin in drug resistant breast cancer MCF-7-AdrR (EC_{50} 0.25 *vs.* 7.8 μM). Further, asGCS ODN-7 increased MCF-7-AdrR cell sensitivity to Taxol, Vinblastine, and Actinomycin D by 3-, 9- and 11-fold, respectively. Compared to asGCS ODN-7, the GCS chemical inhibitor, PDMP (D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), was less efficient and increased Adriamycin sensitivity approximately 4-fold. Subsequent studies revealed that asGCS ODN-7 overcomes drug resistance by enhancing ceramide-induced apoptosis and drug uptake. In conclusion, antisense GCS oligonucleotides effectively depress GCS expression, enhance apoptosis and drug uptake, and increase chemotherapy sensitivity, making them promising agents for cancer therapy.

14. SUBJECT TERMS
antisense oligonucleotide, multidrug resistance, glucosylceramide synthase, synthase, breast cancer, chemotherapy

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A. INTRODUCTION

Glucosylceramide synthase (GCS) catalyzes the first glycosylation step leading to the formation of higher order glycosphingolipids, important membrane constituents of mammalian cells that influence cell signaling, development, differentiation, and host-pathogen interactions (1-3). In human cancer cells, accumulation of glucosylceramide is highly consistent with resistance to anticancer drugs (4-6); furthermore, enhancement of gangliosides at the cell surface is tightly associated with antagonism of host immune function in cancer (7,8). Recently, several studies have shown that targeting the GCS gene modulates ceramide-induced apoptosis (9-11), indicating that enhanced GCS gene expression contributes to poor chemotherapy response (12,13). Inhibition of GCS activity *in vivo* is being evaluated as a possible treatment for several lipid storage diseases and certain types of cancer (12,14-16). Among the existing inhibitors for GCS, PDMP and related compounds have shown promising results for the reduction of glycolipid storage in Fabry's disease and have been used to increase the cytotoxicity of anticancer drugs in tumor cells (3,15,16). However, side effects and low specificity of GCS inhibitors have hampered further application (16). The present work was aimed at developing specific GCS inhibitory agents that could be used in cancer therapy (17,18). Our previous study using antisense glucosylceramide synthase (asGCS) cDNA transfection, which showed that drug resistance could be reversed by this avenue (10), was the stimulus for developing asGCS oligonucleotides.

B. BODY

We hypothesized that GCS is a cause of multidrug resistance in breast cancer, and antisense oligonucleotides (ODNs) of GCS will reverse breast cancer resistance to many classes of chemotherapeutic agents. To determine weather the hypothesis is feasible, we have done the following works during funded period:

1. Designed and synthesized oligodeoxyribonucleotides: Oligodeoxyribonucleotides (ODNs) targeting human GCS mRNA (GenBank accession number D50840) were designed by us based on criteria previously described (17). Using a computer program (HYBsimulator, Version 4), we selected 11 ODNs based on high hybridization strength (ΔG, GC content) which target the start codon, open reading frame, and stop codon. The target region of each of our ODNs is depicted in Fig. 1. Hybridization strength parameters of each ODN were analyzed (Table 1). Antisense oligos 9, 11, and 10 target the regions in human GCS mRNA corresponding to His^{193}, Cys^{207} in rat, and exon-7 in the mouse GCS gene, which are associated with GCS activity (19-21). Each ODN was synthesized as a 20-mers, modified with phosphorothioate backbone and purified by reverse-phase HPLC.

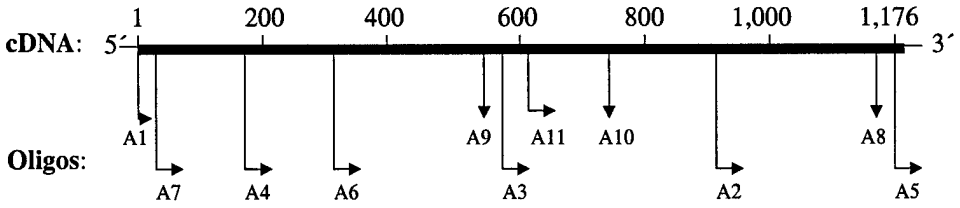

Fig. 1. Schematic of Human GCS cDNA Sequence and asGCS Oligonucleotides. The target position of each oligonucleotide in human GCS mRNA is indicated in the cDNA sequence (top). A1 to A11 represent asGCS ODN-1 through asGCS ODN-11; Oligos, oligonucleotides.

Table 1. Oligonucleotide Sequences and Hybridization Strength Parameters. All sequence entries written in the 5′ to 3′ direction. ORF, open reading frame; AS, antisense oligonucleotide.

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

2. Screen for an Effective Antisense Oligodeoxyribonucleotide to GCS. To screen for activity, we assessed the influence of each agent on cell viability and on GCS mRNA expression. Of the eight asGCS ODNs screened, only ODN-1 and ODN-7 decreased MCF-7-AdrR cell viability (Fig. 2A), with ODN-7 being slightly more potent. ODN-6 did not have any influence on cell viability, and was designed as asGCS ODN-control for further study. RT-PCR analysis indicated that asGCS ODN-1 and –7 (400 nM, 4 hr) substantially depressed GCS mRNA expression, compared with the ODN control (Fig. 2B).

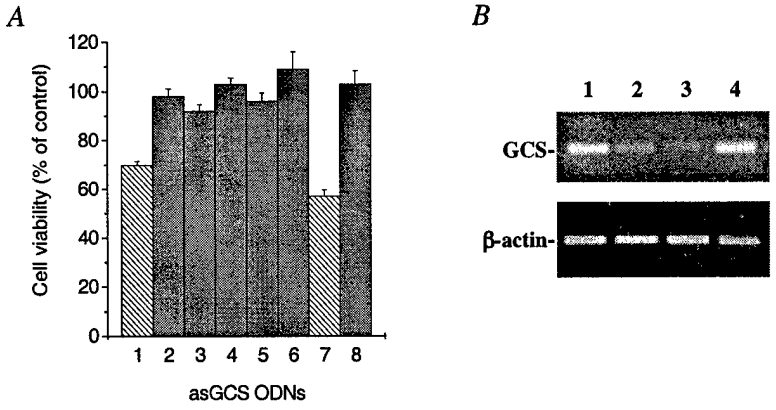

Fig. 2. Preliminary Screening of asGCS ODNs in MCF-7-AdrR Cells. *A.* The influence of asGCS ODNs on cell viability. Cells were pretreated in RPMI-1640 serum-free medium containing OligofectAMINE (GibcoBRL, 0.5 μl/well) with indicated ODNs (100 nM) for 4 h, and grown in 5% FBS medium for 72 h. Data are the mean ± SD from three experiments in triplicate. ***B.*** Influence of asGCS ODNs on GCS mRNA expression. Cells were treated with 400 nM asGCS ODN in serum-free medium for 4 h. After 24 h culture, mRNA was isolated and analyzed by RT-PCR. Lanes 1 to 4 represent MCF-7-AdrR cells treated with OligofectAMINE, asGCS ODN-1, asGCS ODN-7, and ODN-control, respectively.

3. Influence of asGCS ODN-7 on multidrug resistance in MCF-7-AdrR cells. Drug resistant human MCF-7-AdrR breast cancer cells display multiple resistance to several types of anticancer drugs (22). asGCS ODN-7 exposure increased C_6-ceramide sensitivity 2-fold in MCF-7-AdrR cells (EC_{50} 6.4 *vs.* 12.4 μM). Ceramide is a lipid second messenger in the apoptotic pathway participating in cell death initiated by anticancer drugs, cytokines, and ionizing radiation (23,24). The apoptotic impact of Adriamycin, depends, in part, on cellular ceramide generation (12,23-26). Therefore, suppressing GCS gene expression should diminish poor response to ceramide-generating agents (11,12). Pretreatment

with asGCS ODN-7 (100 nM, 4 h) substantially increased Adriamycin sensitivity in MCF-7-AdrR cells. The cell viability of Adriamycin combined with asGCS ODN-7 is shown in Fig. 3. asGCS ODN-7 increased Adriamycin sensitivity 30-fold (EC_{50} 0.25 *vs.* 7.8 μM).

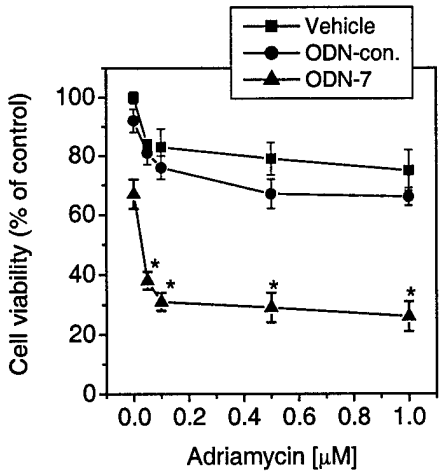

Fig. 3. Influence of asGCS ODN-7 on Adriamycin Cytotoxicity. MCF-7-AdrR cells were pretreated with indicated ODNs (100 nM, 4h). Data are the mean±SD from three experiments in triplicate, sensitivity is designated numerically by fold decrease in drug EC_{50}, compared with ODN-control treatment. ODN-con, ODN-control; *p<0.001, compared with the OligofectAMINE (vehicle) or ODN-control pretreatment.

4. Comparison of asGCS ODN-7 and GCS Chemical Inhibitors on Chemosensitization. PDMP (D, L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) is typical chemical inhibitor of GCS (8,14). As expected, PDMP increased Adriamycin cytotoxicity in MCF-7-AdrR cells (Fig. 4). Sensitivity to Adriamycin increased 4.4-fold (EC_{50} 2.8 vs. 12.4 μM) with PDMP. By contrast, asGCS ODN-7 treatment (100 nM, 4h) increased Adriamycin sensitivity 30-fold in MCF-7-AdrR cells (Fig.11C), making it 10-fold more effective than PDMP.

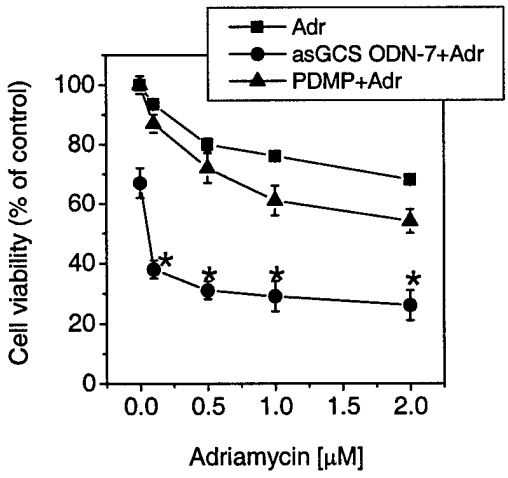

Fig. 4. Effects of asGCS and GCS Inhibitors on the Cytotoxicity of Adriamycin in MCF-7-AdrR Cells. Cells were pretreated with indicated agents (PDMP, 5 μM; asGCS ODN-7, 100 nM), for 4 h. After 72 h exposure to drugs, cell viability was determined using the Promega 96 Aqueous cell proliferation assay kit. *p<0.001, compared with Adriamycin treatment in MCF-7-AdrR cells. Adr, Adriamycin.

5. asGCS ODN-7 on ceramide-induced apoptosis. To further elucidate the mechanism by which asGCS ODNs sensitize cells, ceramide generation and apoptosis were analyzed under various treatment conditions. Adriamycin treatment alone or combined with ODN-control did not increase cellular ceramide levels (Fig. 5A); however, Adriamycin combined with asGCS ODN-7 increased the levels of ceramide by 165% (477 vs. 290 cpm, $p<0.001$). Under like conditions, we did not find a difference in glucosylceramide levels (data not shown). Further characterization revealed that Adriamycin elicited apoptosis only in cells pretreated with asGCS ODN-7 (Fig. 5B, C). The apoptotic index was 200 % (0.43 vs. 0.22 OD) and 267 % (0.59 vs. 0.22 OD), compared to vehicle groups, respectively (Fig. 5B). However, ODN-control did not statistically increase apoptosis with or without Adriamycin. TUNEL fluorescence imaging also showed that apoptosis was highest in cells treated with the combination of asGCS ODN-7 and Adriamycin (Fig. 2C).

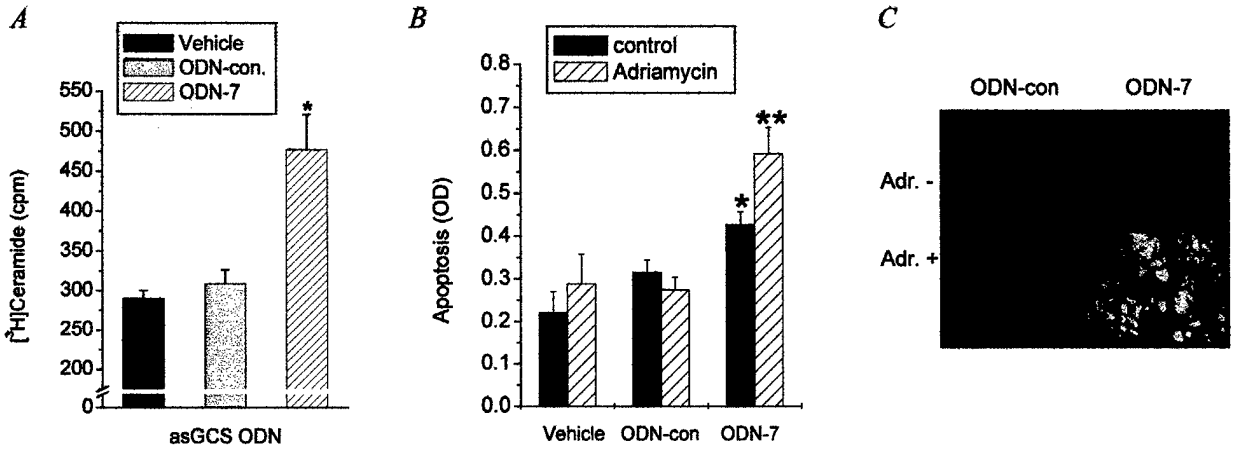

Fig. 5. Influence of Adriamycin combined with asGCS ODN-7 on ceramide-induced apoptosis. *A*. Ceramide Generation. MCF-7-AdrR cells were pretreated with ODNs (100 nM, 4h) and grown with Adriamycin (2.5 μM). Ceramide values are given as cpm per 10^5 cpm total lipid. Data represent the mean±SD of triplicates from three independent experiments; Adr, Adriamycin; *$p<0.05$, **$p<0.01$ compared with the OligofectAMINE or ODN-control pretreatment. ***B.*** Apoptosis. MCF-7-AdrR cells were pretreated with indicated ODN (100 nM, 4h) and cultured with Adriamycin (2.5 μM, 48 h). Data represent the mean ± SD of triplicates from two independent experiments; Adr, Adriamycin; *$p<0.05$, **$p<0.001$, compared with the OligofectAMINE or ODN-control pretreatment. ***C.*** Apoptosis Cells in TUNEL Staining. MCF-7-AdrR cells were pretreated with ODN (100 nM, 4h) and cultured with Adriamycin (2.5 μM, 48h).

6. asGCS ODN-7 on drug uptake. As integral components of plasma membrane microdomains, such as caveolae and GM_3-enriched microdomains, glycosphingolipids are intimately involved in mediating membrane trafficking, drug transport, signal activity, and tumor immunity (2,3,8,11). To assess the influence of asGCS ODNs on drug transport, we evaluated rhodamine-123 uptake in MCF-7-AdrR cells. As captured by fluorescence photomicrographs, asGCS ODN-7 treatment substantially increased rhodamine-123 uptake (Fig. 6A). Quantitative fluorescence measurements (Fig.6B) showed that asGCS ODN-7 doubled Rhodamine-123 uptake compared to ODN-control and untreated cells (vehicle).

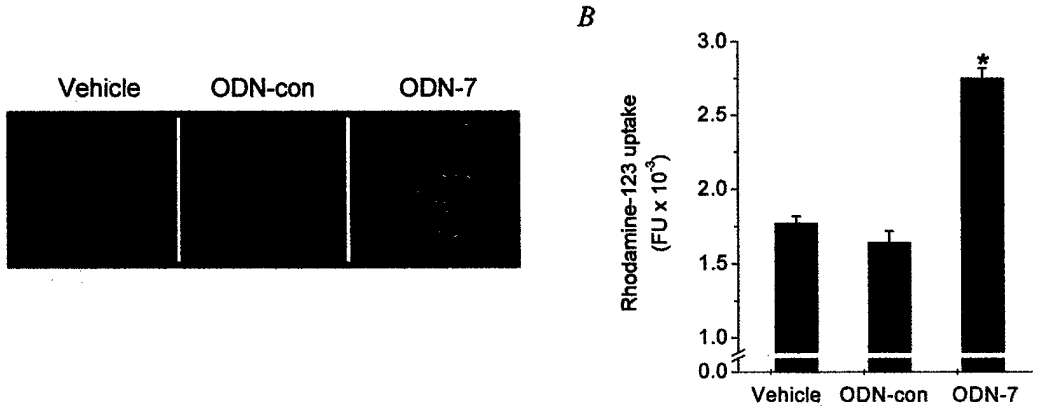

Fig. 6. Influence of asGCS ODN-7 on the uptake of Rhodamine-123. ***A***. Fluorescence Photomicrographs. Pretreated MCF-7-AdrR cells were incubated with rhodamine-123 (0.1 mg/ml, 30 min) at 37°C, and fixed with methanol. ***B***. Cellular uptake of Rhodamine-123. After incubation with rhodamine-123, uptake of rhodamine-123 was determined in the SDS-lysate, by fluorescence. *p<0.001, compared with ODN-control treatment.

C. KEY RESEARCH ACCOMPLISHMENTS:

1. Identified the asGCS ODN-7 depress GCS expression in MCF-7-AdrR cells. The 20-mer phosphorothioate-modified asGCS ODNs were evaluated for their effects on cell growth and gene expression. All asGCS ODNs inhibited growth of MCF-7-AdrR cells, albeit variably. The EC_{50} values ranged between 0.3 μM (asGCS ODN-7) and 2.2 μM (asGCS ODN-6). The influence of asGCS ODN-7 on cell viability was dose-dependent (Fig. 1A). Antisense ODN-7 was substantially more cytotoxic, compared to asGCS-control. We next evaluated the influence of selected ODNs on GCS expression. RT-PCR and Western blot analysis demonstrated a significant reduction in GCS expression after treatment with asGCS ODN-7. As shown in Fig. 1B (top), asGCS ODN-7 reduced GCS mRNA by 80%. GCS protein was reduced by 40%, Fig. 1B (bottom), whereas untreated (vehicle) or ODN-control treated cells showed little change.

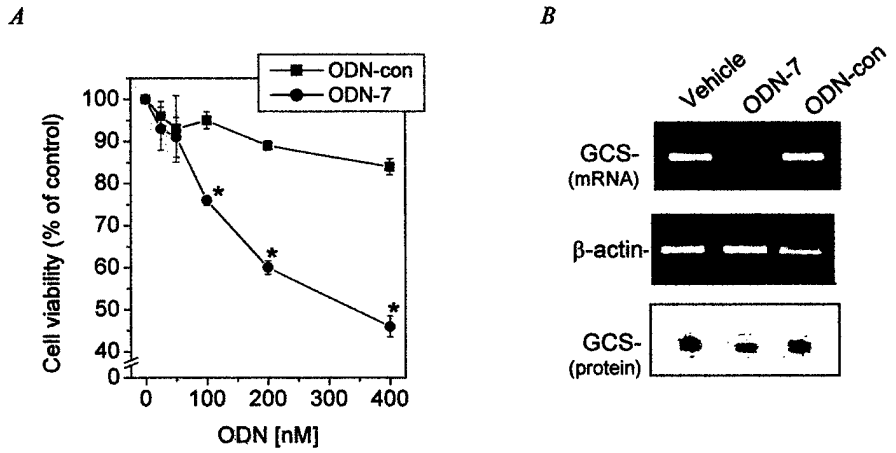

Fig. 7. Influence of asGCS ODN on Cell Viability and GCS Expression. ***A***. Influence of the asGCS ODN-7 doses on cell viability. MCF-7-AdrR cells were treated with increased concentrations of ODNs, and grown for another 72 h. Cell viability was determined using Promega 96 Aqueous cell proliferation assay kit. Data are the mean±SD from three experiments in triplicate. ODN-con, ODN-control; *p<0.01. ***B***. Influence of asGCS ODN-7 on GCS Expression. MCF-7-AdrR cells were treated with ODNs (400 nM) for 4 h and cultured another 24 h. Isolated mRNA (5 ng) was analyzed by high-fidelity RT-PCR and 1% agarose gel electrophoresis; detergent-soluble proteins (50 μg/lane) was resolved and recognized with anti-GCS serum. β-actin was used as endpoint control.

2. Determined asGCS ODN-7 reverse multidrug resistance in MCF-7-AdrR cells. MCF-7-AdrR cells, asGCS ODN-7 increased Adriamycin cytotoxicity approximately 30-fold (EC_{50} 0.25 *vs.* 7.8 μM), the most significant effects appearing at lower concentrations (<0.2 μM) (Fig. 2*A*). Further, asGCS ODN-7 increased the sensitivity to Daunorubicin by 16-fold (0.16 vs. 2.67 μM) and Actinomycin D by 9-fold (0.008 *vs.* 0.07 μM), respectively. These evidences indicate that asGCS ODN-7 has potential to reverse drug resistance in breast cancer cells.

Table 2. Influence of antisense GCS oligonucleotides on drug sensitivity in drug-resistant cells of human breast cancer (MCF-7-AdrR). * p<0.001, compared with control group.

Treatment	Adriamycin		Actinomycin D		Daunarobicin	
	EC_{50} (μM)	Sensitivity (fold)	EC_{50} (μM)	Sensitivity (fold)	EC_{50} (μM)	Sensitivity (fold)
Control	7.8		0.07		2.67	
asGCS ODN-7	0.25*	30	0.008*	9	0.16*	16

3. Demonstrated asGCS ODN-7 increase ceramide-induced apoptosis (Fig. 5) and drug uptake (Fig. 6).

D. REPORTABLE OUTCOMES (4 abstracts; one manuscript):

1. Liu Y. Y. et al. Antisense oligonucleotides to glucosylceramide synthase sensitize drug-resistance cancer cells to Adriamycin. Tenth International Conference on Gene Therapy of Cancer, San Diego, CA, December 2001. Cancer Gene Therapy, Vol 8, Suppl 2:O-63, 2001
2. Liu Y. Y., et al. Antisense oligonucleotides targeting glucosylceramide synthase sensitize multidrug resistant breast cancer cells to Adriamycin. Era of Hope-Department of Deference Breast Cancer Research Program Meeting (submitted), Orlando, Florida, September, 2002
3. Liu Y. Y., et al. Antisense transfection of glucosylceramide synthase modulates gene profiles in response to doxorubicin treatment in drug resistant human breast cancer cells. 25th Annual San Antonio Breast Cancer Symposium (submitted). San Antonio, Texas, December 11-14, 2002
4. Liu Y. Y. et al. Antisense oligonucleotides targeting ceramide glycosylation overcome multidrug-resistance in cancer cells. 14th EORTC-AACR Symposium on "Molecular Targets and Cancer Therapeutics" (submitted), Frankfurt, Germany, November 19-22, 2002
5. Liu Y. Y. et al. Antisense oligonucleotides targeting glucosylceramide synthase reduce Adriamycin resistance in cancer cells. Cancer Res. (manuscript, submitted) 2002

E. CONCLUSIONS:

This funded study demonstrated that GCS is a cause of multidrug resistance in breast cancer, and antisense oligonucleotides (ODNs) of GCS can reverse drug resistance in breast cancer cells. The asGCS ODN-7 identified in our study has more effective than chemical inhibitor for GCS (PDMP, Verabermal) on reversal of drug resistance. Wherefore, asGCS ODN-7 may be a new effective inhibitor for GCS. Further animal studies will be necessary to reveal whether asGCS ODN-7 has potential for cancer treatment in clinic.

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Antisense oligonucleotides to glucosylceramide synthase sensitize drug-resistant cancer cells to adriamycin

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Glucosylceramide synthase (GCS) disrupts ceramide-induced apoptosis elicited by chemotherapy and is thus a major cause of multidrug resistance (MDR) in cancer. Previous studies with antisense GCS cDNA transfection show complete reversal MDR in breast cancer cells, indicating GCS is a select target for cancer therapy [Liu, Y. Y., Han, T. Y., Giuliano, A. E., and M. C. Cabot. *FASEB J.* 15:719–730, 2001]. Here we show for the first time that antisense GCS oligodeoxyribonucleotides (asGCS ODNs) chemosensitize MDR breast and ovarian cancer cells to anticancer agents. More than 12 ODNs (20-mer) targeting human GCS mRNA (GenBank accession number D50840) have been designed and synthesized (phosphorothioate backbone). The target regions of these asGCS ODNs cover the start codon, the corresponding sites to His^{193}, Cys^{207} in rat and exon-7 in mouse GCS, and the stop codon in the human GCS sequence. In screening experiments, asGCS ODN-7 significantly decreased cell viability, GCS mRNA levels, and enzyme protein. Consistent with this, pretreatment of MCF-7-AdrR cells (Adriamycin-resistant breast cancer) with asGCS ODN-7 (200 nM, 4 hours) increased cell sensitivity to C_6-ceramide by 2-fold (EC_{50} 12.4 *vs.* 6.4 μM), and in A2780-AD (Adriamycin-resistant ovarian cancer), sensitivity was increased 4-fold (EC_{50} 16 *vs.* 3.4 μM). More importantly, asGCS ODN-7 overcame Adriamycin resistance in both cell lines. Adriamycin sensitivity increased 30-fold in MCF-7-AdrR (EC_{50} 7.8 *vs.* 0.25 μM), and 10-fold in A2780-AD cells (EC_{50} 6.0 *vs.* 0.6 μM), when asGCS ODN-7 was given. Further studies show that asGCS ODNs sensitize drug-resistant cancer cells through increased ceramide-apoptosis signaling. This work suggests that asGCS ODNs will be a useful tool for treating MDR in cancer.

Era of Hope
Department of Defense Breast Cancer Research Program Meeting

Antisense Oligonucleotides Targeting Glucosylceramide Synthase Sensitize Multidrug Resistant Breast Cancer Cells to Adriamycin

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Glucosylceramide synthase (GCS) disrupts ceramide-induced apoptosis elicited by chemotherapy and is thus a major cause of multidrug resistance (MDR) in cancer. Previous studies with antisense GCS DNA transfection show complete reversal MDR in breast cancer cells, indicating GCS is a therapeutic target for cancer treatment [Liu, Y. Y., Han, T. Y., Giuliano, A. E., and M. C. Cabot. FASEB J. 15:719-730, 2001]. Here we show for the first time that antisense GCS oligodeoxyribonucleotides (asGCS ODNs) chemosensitize MDR breast and ovarian cancer cells to Adriamycin. More than 12 ODNs (20-mer) targeting human GCS mRNA (GenBank accession number D50840) have been designed and synthesized (phosphorothioate backbone). The target regions of these asGCS ODNs cover the start codon, the corresponding sites to His^{193}, Cys^{207} in rat, and exon-7 in mouse GCS, and the stop codon in the human GCS sequence. In screening experiments, asGCS ODN-7 significantly decreased cell viability, GCS mRNA levels, and enzyme protein. Consistent with this, pretreatment of MCF-7-AdrR cells (Adriamycin-resistant breast cancer) with asGCS ODN-7 (100 nM, 4 h) increased cell sensitivity to C_6-ceramide by 2-fold (EC_{50} 12.4 *vs.* 6.4 μM); in A2780-AD (Adriamycin-resistant ovarian cancer) cells, asGCS ODN-7 pretreatment (200 nM, 4 h) increase the sensitivity to C_6-ceramide 4-fold (EC_{50} 16 *vs.* 3.4 μM). More importantly, asGCS ODN-7 overcame Adriamycin resistance in both cell lines. Adriamycin sensitivity increased 30-fold in MCF-7-AdrR (EC_{50} 7.8 *vs.* 0.25 μM), and 10-fold in A2780-AD cells (EC_{50} 6.0 *vs.* 0.6 μM), when asGCS ODN-7 was given. In addition our studies show that asGCS ODNs sensitize drug-resistant cancer cells through increased ceramide-apoptosis signaling and by enhancing drug uptake. This work suggests that asGCS ODNs will be a useful tool for treating MDR in cancer.

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Title: Antisense transfection of glucosylceramide synthase modulates gene profiles in response to doxorubicin treatment in drug resistant human breast cancer cells

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BACKGROUND: Doxorubicin (Adriamycin) is first-line treatment for breast cancer; however, multidrug resistance occurring after treatment greatly limits overall effectiveness. In doxorubicin-selected drug resistant human breast cancer cells (MCF-7-AdrR), several drug resistance-associated molecules, including *mdr1*, mutant p53, and GCS (glucosylceramide synthase) are highly expressed. GCS converts ceramide to glucosylceramide, thus blunting ceramide-induced apoptosis. Antisense GCS (asGCS) blocks GCS gene expression and decreases GCS enzyme activity, and we have shown that asGCS transfection overcomes multidrug resistance in human breast cancer MCF-7-AdrR cells [Liu, Y. Y., Han, T. Y., Giuliano, A. E., and Cabot, M. C. FASEB J. 15, 719-730, (2001)].
MATERIALS AND METHODS: Using cDNA array (Human Apoptosis Q Series), we analyzed apoptosis-associated gene profiles in response to doxorubicin treatment in MCF-7-AdrR and in asGCS transfected MCF-7-AdrR/asGCS cells. GCS and p53 expression levels were also evaluated using RT-PCR and Western blot. Ceramide was measured by [^{3}H]palmitic acid radiolabeling of cells and thin-layer chromatography.
RESULTS: Antisense GCS transfection alone significantly increased the expression of apoptosis-associated genes, including those encoding NFκB, mdm2, Fas (Apo-1), p53, p-21, Caspase-7, and Trail receptor (DR5) in MCF-7-AdrR/asGCS cells, compared to the parent MCF-7-AdrR cells. Doxorubicin treatment (2.5 μM, 48hr) increased ceramide levels in MCF-7-AdrR/asGCS cells by 227% (857 *vs.* 376 cpm, $p<0.001$), compared with MCF-7-AdrR. Consistent with the chemotherapy-sensitizing effect of asGCS transfection, enhanced expression of the apoptosis-associated genes TRAF4, TNFRSF10D, Caspase-6 and Caspase-3 occurred in MCF-7-AdrR/asGCS cells in response to doxorubicin treatment.
DISCUSSION: Antisense GCS transfection modulates gene profiles in MCF-7-AdrR/asGCS cells. Further, doxorubicin treatment enhances cellular ceramide levels and results in enhanced expression of several important apoptosis-associated genes. This study suggests that in addition to inducing apoptosis, ceramide produced via chemotherapy can augment gene expression.

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Antisense Oligonucleotides Targeting Ceramide Glycosylation Overcome Multidrug Resistance in Cancer Cells

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Glucosylceramide synthase (GCS) catalyzes ceramide glycosylation, disrupts ceramide-induced apoptosis elicited by chemotherapy, and appears be a major cause of multidrug resistance (MDR) in cancer. Previous studies pinpoint GCS as a therapeutic target for MDR [Liu, Y. Y., Han, T. Y., Giuliano, A. E., and Cabot, M. C. *FASEB J.* 15, 719-730, (2001)]. In this work, we have synthesized antisense GCS oligodeoxyribonucleotides (asGCS ODNs) to block GCS mRNA transcription, and tested several of the oligos for chemotherapy-enhancing properties in drug resistant cancer cell models. Of the eleven reagents generated, asGCS ODN-7 at low concentrations (EC_{50} 0.3 μM) displayed a dramatic inhibitory influence on cell growth. Antisense GCS ODN-7 suppressed GCS mRNA expression (RT-PCR) by 80%, and GCS protein (Western blot) by 40%. Consistent with down-regulation of GCS and the ceramide mode of anthracycline action, asGCS ODN-7 affected 30- and 10-fold increases in sensitivity to Adriamycin in drug resistant breast cancer MCF-7-AdrR (EC_{50} 0.25 *vs.* 7.8 μM), and in drug resistant ovarian cancer A2780-AD cells (EC_{50} 0.6 *vs.* 6.0 μM), respectively. Further, asGCS ODN-7 increased MCF-7-AdrR cell sensitivity to Taxol, Vinblastine, and Actinomycin D by 3-, 9- and 11-fold, respectively. Compared to asGCS ODN-7, the GCS chemical inhibitor, PDMP (D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), was less efficient and increased Adriamycin sensitivity approximately 4-fold. Subsequent studies revealed that asGCS ODN-7 overcomes drug resistance by enhancing ceramide-induced apoptosis and drug uptake. In conclusion, antisense GCS oligonucleotides effectively depress GCS expression, enhance apoptosis and drug uptake, and increase chemotherapy sensitivity, making them promising agents for cancer therapy.

Antisense Oligonucleotides Targeting Glucosylceramide Synthase Reduce Adriamycin Resistance in Cancer Cells[1]

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Running title: asGCS Oligos reduce drug resistance

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[2]The abbreviations used are: GCS, glucosylceramide synthase, ceramide:UDP-glucosyltransferase, EC 2.4.1.80; asGCS, antisense glucosylceramide synthase; ODN, oligodeoxyribonucleotide; RT-PCR, reverse transcription polymerase chain reaction; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol.

ABSTRACT

Glucosylceramide synthase (GCS) disrupts ceramide-induced apoptosis elicited by chemotherapy and is thus one cause of multidrug resistance (MDR) in cancer. Our previous studies with antisense GCS (asGCS) transfection strongly suggest that GCS is a therapeutic target for MDR. Here we show for the first time that asGCS oligodeoxyribonucleotides (asGCS ODNs) chemosensitize MDR breast and ovarian cancer cells to Adriamycin. Eleven ODNs (20-mer's) were synthesized to target GCS mRNA. One, asGCS ODN-7, significantly suppressed cellular GCS expression. Consistent with a ceramide mode of Adriamycin action, in cells preexposed to asGCS ODN-7, Adriamycin sensitivity increased 30-fold in drug resistant MCF-7-AdrR breast cancer cells (EC_{50} 0.25 *vs.* 7.8 μM) and 10-fold in drug resistant A2780-AD ovarian cancer cells (EC_{50} 0.6 *vs.* 6.0 μM). C_6-Ceramide cytotoxicity was also enhanced by asGCS ODN-7. Compared to the GCS chemical inhibitor PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), asGCS ODN-7 was 7-fold more effective in increasing Adriamycin sensitivity. Mechanism studies revealed that asGCS ODN-7 reduced drug resistance by enhancing ceramide-induced apoptosis and drug uptake.

INTRODUCTION

GCS[2] catalyzes the first glycosylation step leading to the formation of higher order glycosphingolipids, important membrane constituents of mammalian cells that influence cell signaling, development, differentiation, and host-pathogen interactions (1-3). In human cancer cells, accumulation of glucosylceramide is highly consistent with resistance to anticancer drugs (4-6); furthermore, enhancement of gangliosides at the cell surface is tightly associated with antagonism of host immune function in cancer (7,8). Recently, several studies have shown that targeting the GCS gene modulates ceramide-induced apoptosis (9-11), indicating that enhanced GCS gene expression contributes to poor chemotherapy response (12,13).

Inhibition of GCS activity *in vivo* is being evaluated as a possible treatment for several lipid storage diseases and certain types of cancer (12,14-16). Among the existing inhibitors for GCS, PDMP and related compounds have shown promising results for the reduction of glycolipid storage in Fabry's disease and have been used to increase the cytotoxicity of anticancer drugs in tumor cells (3, 15, 16). However, undesirable side effects and low specificity of GCS inhibitors have hampered further application (16). The present work was aimed at developing specific GCS inhibitory agents that could be used in cancer therapy (17, 18). Our previous study using asGCS cDNA transfection, which showed that drug resistance could be reversed by this avenue (10), was the stimulus for developing asGCS oligonucleotides.

MATERIALS AND METHODS

Chemicals and Oligonucleotides. asGCS ODNs targeting GCS mRNA (GenBank accession number D50840) were designed based on the selection criteria described earlier (17). The sequences and hybridization strength parameters of the 11 selected asGCS ODNs 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). ODN-6, displayed a little cytotoxicity (EC_{50} 2.2 μM), and was designed as ODN-control in this study. OligofectAMINE was purchased from GIBCO BRL (Grand Island, NY). C_6-Ceramide (*N*-hexanoylsphingosine) was from LC Laboratories (Woburn, MA), and Adriamycin (doxorubicin hydrochloride) was from Sigma.

Cell Culture. The human breast adenocarcinoma cell line, MCF-7-AdrR (NCI/ADR-Res), which is resistant to Adriamycin (19), was kindly provided by Dr. Kenneth Cowan (UNMC Eppley Cancer Center, Omaha, NE) and Dr. Merrill Goldsmith (National Cancer Institute, Bethesda, MD). The ovarian cancer cell line, A2780-AD, which is resistant to Adriamycin (20), 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 units/ml penicillin, 100 μg/ml streptomycin, and 584 mg/liter L-glutamine. A2780-AD cells were cultured in medium containing 100 nM Adriamycin in addition to the above components.

RNA Extration and RT-PCR mRNA Analysis. Cells were pretreated with OligofectAMINE alone (vehicle) or OligofectAMINE containing oligonucleotide (400 nM) for 4 h in serum-free medium, and then incubated another 20 h in 5% FBS medium. Cellular mRNA was purified using a mRNA isolation kit (Boehringer Mannheim, Indianapolis, IN). Equal amounts of mRNA (5.0 ng) were used for RT-PCR, as previously described (10). Under upstream primer (5′-CCTTTCCTCTCCCCACCTTCCTCT-3′) and downstream primer conditions (5′-

GGTTTCAGAAGAGAGACACCTGGG-3′), a 302 bp fragment in the 5′-terminal region of the GCS gene was produced using the ProSTAR HF single-tube RT-PCR system, High Fidelity, Stratagene (La Jolla, CA).

Western Blot Analysis. Cells were pretreated with OligofectAMINE alone (vehicle) or OligofectAMINE plus indicated ODNs (400 nM) for 4 h in serum-free medium, and then incubated another 20 h in 5% FBS medium. The detergent-soluble fraction from cells was used for Western blots, as previously described (10).

Cytotoxicity Assay. Assays were performed as previously described (4, 9, 10). To assess the cytotoxic influence of the ODNs, cells were pretreated with OligofectAMINE alone (vehicle) or OligofectAMINE plus ODN at increasing concentrations in serum-free medium for 4 h, and then incubated another 72 h in 5% FBS medium. To test the influence of ODNs on cell response to drug, cells were pretreated with OligofectAMINE alone (vehicle) or OligofectAMINE plus ODN (100 nM, MCF-7-AdrR; 200 nM, A2780-AD) for 4 h, and then incubated another 72 h in 5 % FBS medium containing increasing concentrations of Adriamycin. Cytotoxicity was determined using the Promega 96 Aqueous cell proliferation assay kit (Promega, Madison, WI).

Apoptotic Cell Death Detection by ELISA and TUNEL. The presence of mono- and oligonucleotides, a feature of cells undergoing apoptosis (17, 18), was evaluated by Cell Death Detection ELISA, performed following kit instructions (Boehringer Mannheim). Briefly, cells were pretreated with asGCS ODN in serum-free medium and cultured in 5% FBS medium containing Adriamycin (2.5 μM) for 48 h, after which 10^4 cells from each sample were lysed in 200 μl lysis buffer. After centrifugation (1000 x *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 incubated with substrate for 15 min. Absorbance was measured at 405 nm. Immunohistochemical detection of apoptosis was performed by TUNEL (terminal

deoxynucleotide-transferase-mediated dUTP nick end labeling) staining using the FragEL DNA Fragmentation detection kit, Oncogen (Boston, MA). Terminal deoxynucleotidyl transferase labeling with fluorescein-dUTP was done according to manufacturer recommendations. Briefly, cells (2 x 10^4 cells/chamber) cultured overnight in 10% FBS RPMI-1640 medium using chamber slides (Nalge Nunc, Inc. Naperville, IL), were pretreated with OligofectAMINE alone or OligofectAMINE containing the indicated asGCS ODNs (100 nM, 4h), and then incubated with Adriamycin (2.5 μM) for 48 h. Cells were fixed with methanol (50% in TBS, 10 min; 100%, 10 min; 50% in TBS, 10 min), and finally rinsed with TBS. The cells on slides were digested with 0.2 mg/ml Proteinase K in 10 mM Tris-HCl, pH 8, for 20 min, and labeled with Fluorescein-FragEL TdT reaction mixture at 37 °C for 90 min 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 (4, 11). Cells were seeded in 6-well plates (6 x 10^4 cells/well) in 10% FBS RPMI-1640 medium. After pretreatment with ODNs (100 nM, 4h) and culture for another 20 h, cells were shifted to 5% FBS medium with or without agents, 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 lipid extraction and measured as described (9, 11).

Rhodamine Assay. After pretreatment with ODNs (100 nM, 4h) and culture for another 20 h, cells (2.0 x 10^6) were incubated with rhodamine-123 (0.1 mg/ml) at 37 °C for 30 min. Following centrifugation at 500 x *g* for 15 min, supernatants were discarded, and the cells were washed twice in RPMI-1640 medium. Uptake of rhodamine-123 was measured at $\lambda_{excitation}$ 485 nm/$\lambda_{emission}$ 530 nm using the FL-600 fluorescent microplate reader (11). For fluorescence photomicrographs, cells were fixed with cold acetic acid/methanol (1:3, v/v), and

photomicrographed using an Olympus IX70 fluorescence microscope equipped with a digital photomicrographic system (11).

RESULTS AND DISCUSSION

asGCS ODN Selection. Analyzed with an HYBsimulator program (RNAture, Inc., Irvine, CA), a series of ODNs was identified as potential antisense candidates. Eleven ODNs were generated directed against different regions of human GCS mRNA, and these covered the start and stop codon, the activity-associated sites corresponding to the rat His^{193} and Cys^{207} (21,22) and mouse exon-7 (23), and other open reading frame regions (Table 1). Antisense GCS ODN-1, ODN-7, and ODN-8 had the highest hybridization strengths. Comparing these sequences in human, rat, and mouse by gene alignment analysis, the homology values in target regions of asGCS ODN-1, ODN-7, were 100 and 85 %, respectively, and homology values for asGCS ODN-5, -9, -10, and -11 were 90%.

The 20-mer phosphorothioate-modified asGCS ODNs were evaluated for their effects on cell growth and gene expression. All asGCS ODNs inhibited growth of MCF-7-AdrR cells, albeit variably. The EC_{50} values ranged between 0.3 μM (asGCS ODN-7) and 2.2 μM (asGCS ODN-6). The influence of asGCS ODN-7 on cell viability was dose-dependent (Fig. 1*A*). Antisense ODN-7 was substantially more cytotoxic, compared to asGCS-control. We next evaluated the influence of selected ODNs on GCS expression. RT-PCR and Western blot analysis demonstrated a significant reduction in GCS expression after treatment with asGCS ODN-7. As shown in Fig. 1*B* (top), asGCS ODN-7 reduced GCS mRNA by 80%. GCS protein was reduced by 40%, Fig. 1*B* (bottom), whereas untreated (vehicle) or ODN-control treated cells showed little change.

Adriamycin Sensitivity and Apoptosis. We employed Adriamycin to assess the influence of asGCS ODNs on cellular response to chemotherapy. The human breast and ovarian cancer cell lines, MCF-7-AdrR and A2780-AD, were selected by passage of the drug-sensitive wild-type counterparts in media containing increasing concentrations of Adriamycin (19, 20). 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 (10, 19, 20). We have previously shown that the levels of glucosylceramide, the product of GCS, are higher in MCF-7-AdrR and in drug resistant ovarian cancer cells, compared to wild-type (4, 9, 10). From this we hypothesized that suppressing GCS gene expression would decrease drug resistance by increasing the cytotoxicity of chemotherapeutic agents that are known to generate ceramide (11,12). In MCF-7-AdrR cells, asGCS ODN-7 increased Adriamycin cytotoxicity approximately 30-fold (EC_{50} 0.25 *vs.* 7.8 μM), the most significant effects appearing at lower concentrations (<0.2 μM) (Fig. 2*A*). In contrast, pretreatment MCF-7-AdrR cells with the GCS inhibitor, PDMP (5 μM, 4h), enhanced Adriamycin sensitivity by approximately 4-fold (EC_{50} 2.8 *vs.* 12.4 μM). It suggests that asGCS ODN-7 is 7-fold more effective than PDMP (sensitivity, 30-fold *vs.* 4-fold). OligofectAMINE decreased the Adriamycin EC_{50} from 12.4 to 7.8 μM, although alone it did not display cytotoxicity even at high concentrations, indicating that OligofectAMINE slight enhances Adriamycin cytotoxicity. Antisense GCS ODN-7 also produced a 10-fold enhancement of Adriamycin sensitivity (EC_{50} of 0.6 *vs.* 6.0 μM) in drug resistant A2780-AD ovarian cancer cells.

To further elucidate the mechanism by which asGCS ODNs sensitize cells to anticancer agents, ceramide generation and apoptosis were analyzed under various treatment conditions. Adriamycin treatment alone or combined with ODN-control did not increase cellular ceramide levels (Fig. 2*B*); however, Adriamycin combined with asGCS ODN-7 increased the levels of ceramide by 165% (477 *vs.* 290 cpm, $p<0.001$). Under like conditions, we did not find a difference in glucosylceramide levels (data not shown). Further characterization revealed that Adriamycin elicited apoptosis only in cells pretreated with asGCS ODN-7 (Fig.2C, D). The

apoptotic index was 200 % (0.43 *vs.* 0.22 OD) and 267 % (0.59 *vs.* 0.22 OD), compared to vehicle groups, respectively (Fig. 2*C*). However, ODN-control did not significantly increase apoptosis with or without Adriamycin. TUNEL fluorescence imaging also showed that apoptosis was highest in cells treated with the combination of asGCS ODN-7 and Adriamycin (Fig. 2*D*).

Ceramide is a lipid second messenger in the apoptotic pathway participating in cell death initiated by anticancer drugs, cytokines, and ionizing radiation (24,25). The apoptotic impact of Adriamycin, Daunorubicin, Taxol, etoposide and Actinomycin D depends, in part, on cellular ceramide generation (12, 24-27). Therefor, suppressing GCS gene expression should alleviate poor responses to ceramide-generating agents (11, 12). Our work shows that asGCS ODN-7 in combination with Adriamycin, enhances apoptosis in drug resistant cancer cells, and demonstrates that cellular buildup of free ceramide under Adriamycin stress potentiates programmed cell death under conditions of GCS impairment. As expected, asGCS ODN-7 exposure increased C_6-ceramide sensitivity 2-fold in MCF-7-AdrR cells (EC_{50} 6.4 *vs.* 12.4 μM) and 4-fold (EC_{50} 3.4 *vs.* 16.0 μM) in A2780-AD cells.

Drug Uptake. As integral components of plasma membrane microdomains, such as caveolae and GM_3-enriched microdomains, glycosphingolipids are intimately involved in mediating membrane trafficking, drug transport, signal activity, and tumor immunity (2,3,8,11). To assess the influence of asGCS ODNs on drug transport, we evaluated rhodamine-123 uptake in MCF-7-AdrR cells. As captured by fluorescence photomicrographs, asGCS ODN-7 treatment substantially increased rhodamine-123 uptake (Fig. 3*A*). Quantitative fluorescence measurements (Fig.3*B*) showed that asGCS ODN-7 doubled Rhodamine-123 uptake compared to ODN-control and untreated cells (vehicle).

Targeting ceramide metabolism through impairment of GCS may be a novel strategy for cancer treatment (8,11,12,16). Suppression of GCS by antisense DNA transfection overcomes drug resistance in breast cancer cells (11) and reduces melanoma growth in mice (8). In addition to the influence on ceramide metabolism, asGCS ODN-7 increased drug uptake, significantly. These data are consistent with previous finding with asGCS transfection (11) and help to establish the mechanism of action of asGCS ODN-7 in reversing drug resistance. The present work shows that asGCS ODNs may be effective inhibitor of GCS, enhancing both ceramide-induced apoptosis and increasing drug uptake. Animal studies will reveal whether asGCS ODNs have potential for cancer treatment in the clinic.

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Figure Legends:

Fig 1. Influence of asGCS ODNs on Cell Viability and GCS Expression. ***A***. Influence of asGCS ODN-7 concentration on cell viability. MCF-7-AdrR cells (4,000 cells/well) were seeded into 96-well plates and treated the following day with increasing concentrations of ODNs, and grown for another 72 h. Cell viability was determined using Promega 96 Aqueous cell proliferation assay kit. Data are the mean ± SD from three experiments in triplicate. ODN-con, ODN-control; *, $p<0.01$. ***B***. Influence of asGCS ODN-7 on GCS Expression. MCF-7-AdrR cells were treated with ODNs (400 nM) for 4 h and cultured another 24 h. Isolated mRNA (5 ng) was analyzed by high-fidelity RT-PCR and 1% agarose gel electrophoresis; detergent-soluble proteins (50 μg/lane) were resolved and recognized with anti-GCS serum. β-actin was used as endpoint control.

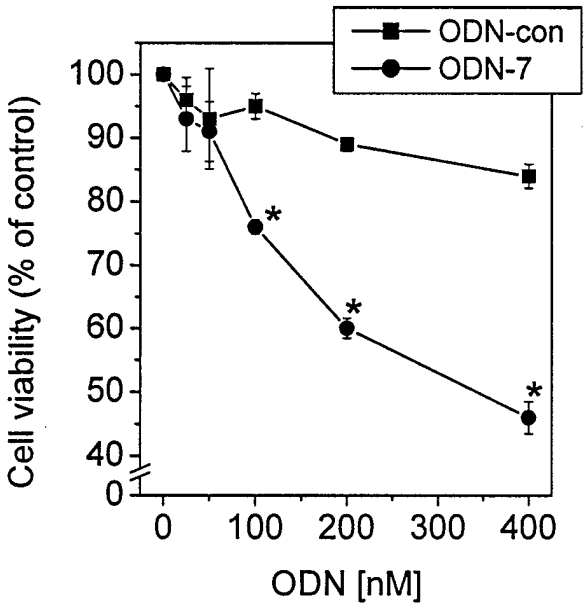
Fig 2. Influence of asGCS ODN on Cell Response to Adriamycin. ***A***. Influence of asGCS ODN on Adriamycin cytotoxicity. MCF-7-AdrR cells were pretreated with the indicated ODNs (100 nM, 4h) and exposed to Adriamycin as detailed in Methods. Data are the mean ± SD from three experiments in triplicate. ODN-con, ODN-control; *, $p<0.001$, compared with OligofectAMINE (vehicle) or ODN-control pretreatment. ***B***. Ceramide generation. MCF-7-AdrR cells were pretreated with ODNs (100 nM, 4h) and exposed to Adriamycin (2.5 μM) as detailed in Methods. Ceramide values are given as cpm per 10^5 cpm total lipid. Data represent the mean ± SD of triplicates from three independent experiments. Adr, Adriamycin; *, $p<0.05$, **, $p<0.01$ compared with the OligofectAMINE or ODN-control pretreatment. ***C***. Apoptosis, ELISA method. MCF-7-AdrR cells were pretreated with the indicated ODN (100 nM, 4h) and cultured with Adriamycin (2.5 μM, 48 h). Data represent the mean ± SD of triplicates from two independent experiments; Adr, Adriamycin; *, $p<0.05$, **, $p<0.001$, compared with the OligofectAMINE or ODN-control pretreatment. ***D***. Apoptosis, TUNEL staining. MCF-7-AdrR cells were pretreated with ODN (100 nM, 4h) and cultured with Adriamycin (2.5 μM, 48h).

Fig 3. Influence of asGCS ODN-7 on Uptake of Rhodamine-123. MCF-7-AdrR cells were pretreated with asGCS ODN (100 nM, 4h), further cultured and then incubated with Rhodamine-123 as detailed in Methods.

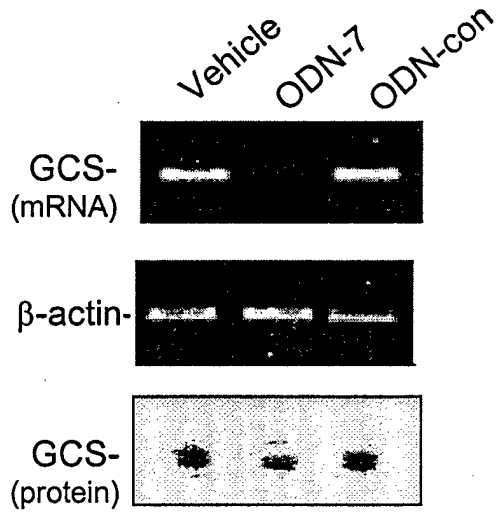
Table 1. The characteristics of antisense oligonucleotides against glucosylceramide synthase.

Oligomer	Sequence	Target	Hybridization Strength Parameter*			
			-dG (Kcal/mol)	Hairpin (Kcal/mol)	Dimer (Kcal/mol)	% GC
ODN-1	GCCAGGTCC AGCAGCGCC AT	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 CCCCACAGCGT	ORF (1146-1166)	27.2	-1.5	-5.8	65
ODN-9	TATCTTGGA TGTGAAGTT CC	H^{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	C^{207} (613-632)	22.9	2.0	-4.2	35

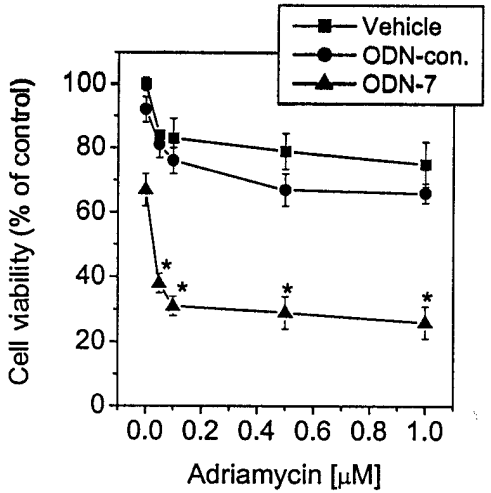
A


ODN-con
ODN-7
Cell viability (% of control)
100
90
80
70
60
50
40
0
0
100
200
300
400
ODN [nM]
*
*
*

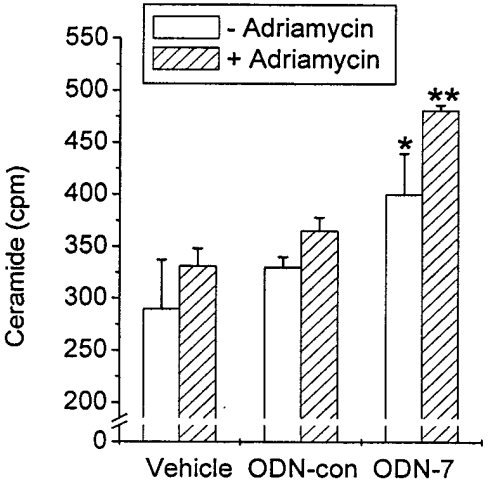
B


Vehicle
ODN-7
ODN-con
GCS-
(mRNA)
β-actin-
GCS-
(protein)

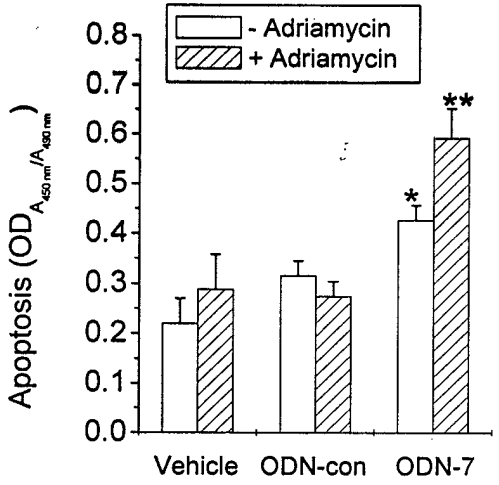
A


Vehicle
ODN-con.
ODN-7
Cell viability (% of control)
100
80
60
40
20
0
0.0 0.2 0.4 0.6 0.8 1.0
Adriamycin [μM]
*
*
*
*

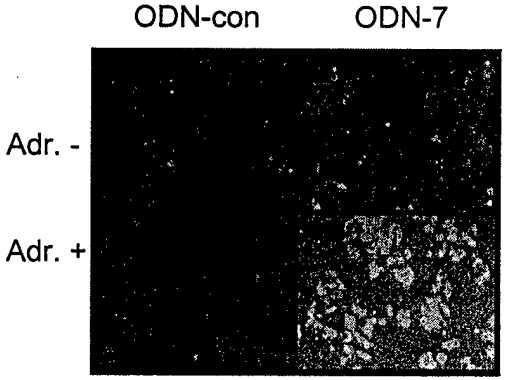
B

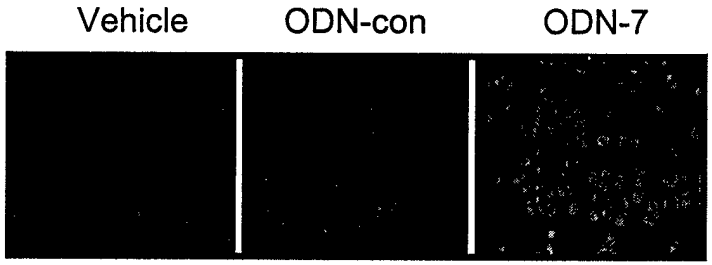

- Adriamycin
+ Adriamycin
Ceramide (cpm)
550
500
450
400
350
300
250
200
0
Vehicle ODN-con ODN-7
*
**

C


- Adriamycin
+ Adriamycin
Apoptosis (OD A450 nm/A490 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Vehicle ODN-con ODN-7
*
**

D


ODN-con
ODN-7
Adr. -
Adr. +


Vehicle
ODN-con
ODN-7