The chemosensitizing activity of inhibitors of glucosylceramide synthase is mediated primarily through modulation of P-gp function

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Abstract. Glucosylceramide synthase (GCS) is a key enzyme engaged in the biosynthesis of glycosphingolipids and in regulating ceramide metabolism. Studies exploring alterations in GCS activity suggest that the glycolase may have a role in chemosensitizing tumor cells to various cancer drugs. The chemosensitizing effect of inhibitors of GCS (e.g. PDMP and selected analogues) has been observed with a variety of tumor cells leading to the proposal that the sensitizing activity of GCS inhibitors is primarily through increases in intracellular ceramide leading to induction of apoptosis. The current study examined the chemosensitizing activity of the novel GCS inhibitor, Genz-123346 in cell culture. Exposure of cells to Genz-123346 and to other GCS inhibitors at non-toxic concentrations can enhance the killing of tumor cells by cytotoxic anti-cancer agents. This activity was unrelated to lowering intracellular glycosphingolipid levels. Genz-123346 and a few other GCS inhibitors are substrates for multi-drug reisistance efflux pumps such as P-gp (ABCB1, gP-170). In cell lines selected to over-express P-gp or which endogenously express P-gp, chemosensitization by Genz-123346 was primarily due to the effects on P-gp function. RNA interference studies using siRNA or shRNA confirmed that lowering GCS expression in tumor cells did not affect their responsiveness to commonly used cytotoxic drugs.

Introduction

Treatment failure with certain anti-cancer agents can be associated with the expression of multidrug resistance (MDR) efflux pumps by malignant cells including the ATP-dependent drug efflux proteins that belong to the super family of ATP-binding cassette (ABC) transporter proteins, such as ABCB1 (P-gp) or MDR1 (1,2). Moreover, there is emerging evidence that lipids, particularly sphingolipids, glycosphingolipids and cholesterol, may also play a role in drug resistance, possibly in concert with ABC transporter proteins (3,4). Much of the evidence for a relationship between MDR and sphingolipids comes from studies using anti-cancer drug selected cancer cell lines which have multiple alterations in sphingolipid levels compared with the parental drug-sensitive cells. Glucosyl-ceramide (GL1), a precursor of all complex glycosphin-golipids and a direct metabolic product of ceramide (CER), is consistently elevated in several P-gp-over-expressing cell lines (5). It has been proposed that the accumulation of GL1 is a characteristic biomarker of some multidrug-resistant cancer cells and tumors derived from patients whose tumors are weakly responsive to chemotherapy (6).

The enzyme responsible for the synthesis of GL1 is glucosylceramide synthase (GCS or UDP-glucose ceramide glucosyltransferase, UGCG). GCS catalyzes the first step in the biosynthesis of glycosphingolipids, and its cellular activity modulates the levels of the other lipids as well as ceramide (7-9). Early studies demonstrated that increasing the capacity for ceramide glycosylation in GCS-transfected human breast cancer MCF-7 cells conferred greater resistance to doxorubicin and to TNF-α (10,11). In contrast, exposing multi-drug-resistant tumor cell lines to antisense oligonucleotides directed toward GCS mRNA increased sensitivity to doxorubicin (12,13). Furthermore, basic cellular functions, such as growth and morphology, are affected in cells treated

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Abbreviations: MDR, multidrug resistance; GSLs, glycosphingo-lipids; GCS, glucosylceramide synthase; GM3S, GM3 synthase; PDMP, DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; CER, ceramide; GL1, glucosylceramide; GL2, lactosylce-ramide; GL3, triglycosylceramide

with different GCS inhibitors (14-16). Moreover, these compounds have been reported to sensitize tumor cells to the cytotoxic effects of various anti-cancer compounds (15,17). However, DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), a widely used GCS inhibitor in numerous studies, reportedly exhibits many non-specific actions, such as inhibition of other enzymes involved in glycolipid metabolism (18,19), as well as affects on calcium homeostasis, membrane fluidity (20) and thymidine transport (21). The chemosensitizing effects of PDMP and/or its analogues in different types of tumor cells have been observed in various studies (9,15,22-24). In general, the assumption has been that the sensitizing effect was due to inhibition of GCS, thereby resulting in increased ceramide levels and induction of apoptosis. However, recent studies suggest that the chemosensitizing effect of PDMP and its analogues may not be related to GCS inhibition (25-27). Iminosugar-based GCS inhibitors which are from a different chemical class than the ceramide-based inhibitor PDMP were observed to be ineffective in chemosensitizing multidrug-resistant tumor cells (25). Moreover, the chemosensitizing effects of PDMP on neuroblastoma cells were GCS- and ceramide-independent (27). Therefore, questions exist regarding the role and mechanism of action associated with inhibition of GCS in chemosensitizing tumor cells.

Genz-123346 is a novel analogue of PDMP with improved specificity for inhibiting glucosylceramide synthase (19,28,29). In this study, we examined the chemosensitizing effect of Genz-123346 on tumor cells and provide evidence suggesting that modulation of P-gp function may be the primary mode of action. Supporting this notion are studies using siRNA and shRNA showing that lowering of GCS levels did not alter tumor cell response to commonly used cytotoxic drugs which are efflux pump substrates.

Materials and methods

Reagents and cell culture. PBS, fetal bovine serum (FBS), RPMI medium, trypsin-EDTA, penicillin G sodium/streptomycin were purchased from Invitrogen, Inc. (Carlsbad, CA). PDMP (DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), vinblastine, vincristine, docetaxel, doxorubicin, 5-fluorouracil (5-FU) and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO). The C9DGJ (N-nonyl-deoxygalactonojirimycin) and C4DGJ (N butyl-deoxygalactonojirimycin) were from Toronto Research Chemicals (Toronto, Canada). Genz-123346 was from Genzyme Corp. (Waltham, MA). Anti P-gp antibody was obtained from Cell Signaling Technology (Danvers, MA). Human colon carcinoma cell lines HCT-15 and human hepatocellular carcinoma cell line HepG2 were obtained from American Type Culture Collection (Manassas, VA). Human cervical carcinoma cell line KB3-1 and a vinblastine-selected subline KBV-1 were a gift from Dr E. J. LaVoie (Rutgers, New Jersey). All tumor cell lines are maintained in RPMI supplemented with 5% fetal bovine serum (FBS), penicillin G sodium (100 U/ml), and streptomycin (100 μg/ml). Vinblastine (1 μg/ml) was added only to the KBV-1 culture medium periodically to maintain drug-resistant phenotype (30). Cell lines were maintained in a humidified atmosphere at 37˚C/5%CO$_2$ for growth.

mRNA extraction and quantitative real-time PCR (qRT-PCR) analysis. Total RNA was extracted using the RNeasy mini kit (Qiagen GmbH, Valencia, CA) and first-strand cDNA was synthesized using random hexamer primers and Superscript II reverse transcriptase (Invitrogen). Purified total RNA was subjected to formaldehyde gel electrophoresis and spectrophotometry (A260 nm and A280 nm) to assess integrity and purity. qRT-PCR analysis was performed using an ABI-PRISM 7700 Sequence Detector System and the SYBR-Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Primer and probe mixtures for genes of interest were pre-validated from ABI's 'assay on demand' (Applied Biosystems). The ribosomal protein RPLP0 (NM_001002) was used as internal standard.

RNA interference and generation of stable GCS RNAi HepG2 cells. siRNA (pool) for GCS was purchased from Dharmacon (Lafayette, CO) and resuspended to a concentration of 20 μM in buffer. The scrambled non-targeting siRNA (NT siRNA siRNAs) was purchased from Qiagen (Valencia, CA).

HepG2 cells were cultured in RPMI media supplemented with 5% FBS. RNAintro™ pSM2 retroviral vector (Open Biosystems, Huntsville, AL) was used to sub-clone non-silencing or GCS shRNAs. The GCS shRNA sequence was designed using the online software from Open Biosystems. This vector contains a puromycin-resistant marker site for positive colony selection. The specificity of GCS shRNA was verified by transient transfection using Arrest-In transfect reagent (Open Biosystem) followed by Western blotting. To generate stable cell lines, shRNAs targeted to GCS or non-silencing controls were transfected into HepG2 cells. Stable transfectants were selected in the presence of 2 μg/ml puromycin (Invitrogen), a working concentration determined previously by kill curves. Cells derived from these transfectants were used for qRT-PCR to confirm the decrease of GCS mRNA expression. GCS knockdown stable HepG2 cells were maintained in RPMI media supplemented with 5% FBS containing 1 μg/ml puromycin.

Cell viability/proliferation assay. Cells were resuspended in growth media at a concentration of 20,000 cells/ml. One hundred μl cell suspension (2,000 cells) was seeded into each well of a 96-well flat bottom, tissue culture treated Corning PN 3595 plate (Corning, Corning, NY). The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) was used to measure viable cells. The protocol was followed according to the manufacturer's instructions. ATP content of the wells was measured using a BioTek Synergy™ 2-plate reader (Winooski, VT). Cells were treated for 72 h in assays and the effect of treatment on cell proliferation was calculated based on the National Cancer Institute drug screen assay (31).

Cell-free P-gp ATPase activity assay. Changes in Pg-p ATPase activity were detected by the Pg-p-Glo™ assay system (Promega). According to the manufacturer's instructions, the activity of P-gp ATPase was measured in the presence or absence of 250 μM sodium orthovanadate (Na$_3$VO$_4$), 400 μM verapamil (as a positive control) and various concentrations of PDMP, Genz-123346, C4DGJ diluted with assay buffer. Control or test compounds were incubated in 25 mM MgATP

Table I. The GSL levels in human cervical carcinoma KB3-1 and drug-resistant subline KBV-1 cells.

Cells	Cer	GL1	GL3	GL2	GM3	GM1
KB3-1	182.0±11.8[a]	40.5±1.4	321.0±0.6	126.1±3.5	57.8±1.6	27.8±1.9
KB3-1 +Genz-123346[b]	160.7±17.2	15.8±1.6	23.3±17.0	59.1±7.9	10.2±2.4	10.5±2.0
KBV-1	201.8±10.8	64.4±5.4	265.1±11.7	247.8±12.8	190.5±9.7	154.4±44.4
KBV-1 +Genz-123346[b]	190.0±12.4	31.3±5.8	36.2±11.0	99.5±33.4	19.6±0.4	30.6±13.4

[a]The quantity of all the sphingolipid species is expressed as mean ± S.D. (ng/10^6 cells) from two independent samples. [b]Genz-123346 (1 μM) was used to treat cells for 72 h.

and 25 μg recombinant human P-gp membranes at 37˚C for 40 min. The plate was moved to room temperature and the reactions were stopped by adding 50 μl of ATP detection reagent. The plate was incubated at room temperature for 20 min to allow the luminescent signal to develop. Samples were transferred to a white opaque 96-well plate (Corning) for detection of luminescence. The level of luminescence was measured using a BioTek Synergy 2-plate reader. The measurement reflects the ATP level in the sample and is negatively correlated with the activity of P-gp ATPase. Changes in relative luminescence units (ΔRLU) were determined by comparing Na_3VO_4-treated samples with test compounds and verapamil combination-treated samples.

Calcein-AM retention/efflux assay. Calcein-AM (Invitrogen) was used to determine P-gp-mediated efflux functions in HCT-15 cells in the presence of Genz-123346 and other compounds. Briefly, HCT-15 cells were grown in a low fluorescence, black 96-well plates (Whatman Inc, Piscataway, NJ) to 70-90% confluence. Cells in 100 μl volumes of tissue culture medium were exposed to various concentrations of Genz-123346 or other compounds in 50 μl culture medium for 15 min at 37˚C. Calcein-AM was added to each well in 50 μl culture medium giving a final concentration of 0.25 μM and the cells were incubated for 15 min at 37˚C. Then cells were gently agitated. Fluorescence intensity was measured using a BioTek Synergy 2-plate reader with filters appropriate for detection (excitation 485/20, emission 528/20). The fluorescence values were plotted.

Quantitation of cell glucosylceramide levels by mass spectrometry. Glucosylceramide was quantified by mass spectrometry as described (32). Briefly, sphingolipids were analyzed using high-performance liquid chromatography (HPLC) tandem mass spectrometry (32). Cell samples were removed from the -80˚C freezer and allowed to thaw. An internal standard cocktail was added to microcentrifuge tubes and dried under nitrogen gas. Cells were added to the tubes and vortexed to help incorporate the internal standards into the cell suspension. Acetonitrile was then used to precipitate proteins from the cell sample, and the resulting suspension was sonicated. The tubes were centrifuged and an aliquot of the supernatant was

then transferred to an autosampler for LC/MS/MS analysis. Samples were analyzed on a system consisting of an HTC PAL autosampler, Agilent 1200 HPLC, and API-4000 mass spectrometer. During analysis, samples were stored at 9˚C in the autosampler cool stack. The HPLC was run in isocratic mode with a normal-phase silica column, and MS/MS was performed in MRM mode.

Flow cytometry analysis of GM1 level on cell surface. GCS inhibition by either Genz-123346 or GCS si/shRNA was determined indirectly by measuring the effect on the cell surface gangliosides GM1 on HCT-15 cells using flow cytometry analysis. GM1 levels on the HCT-15 cells were determined by incubating the cells with Genz-123346 or siRNA transfection for the indicated time after which the cells were harvested and stained using 10 μg of recombinant cholera toxin-FITC (Sigma) in 100 μl phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) for 30 min on ice. Cells were washed, resuspended in PBS containing 0.5% BSA and the fluorescence was quantified using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). CellQuest software (BD Biosciences) was used to analyze the GM1 levels on HCT-15 cell membrane.

Results

Exposure of drug-resistant cancer cells to Genz-123346 lowers GSL levels and increases sensitivity to cytotoxic drugs. Altered composition of GSL and an increase in GL1 levels are characteristic of multidrug-resistant cancer cells such as KBV-1 and NCI/ADR-RES cells (5,33,34). Quantitative analysis of GCS and GM3S (GM3 synthase) mRNA levels by real-time PCR and measurement of GL1 levels by mass spectrometry confirmed KBV-1 cells exhibit increased expression of GCS and GM3S mRNAs and higher GL1 levels when compared to parental KB3-1. As shown in Fig. 1A, the mRNA level of GCS in the multi-drug-resistant KBV-1 cells was increased 2-fold and that for GM3 by >5-fold. Concomitant with these increases in GCS and GM3S mRNA levels were higher levels of GL2, GM3 and GM1 (measured by mass spectrometry, see Materials and methods) when compared to the parental KB3-1 cells (Table I). Exposure of KBV-1 cells to the GCS
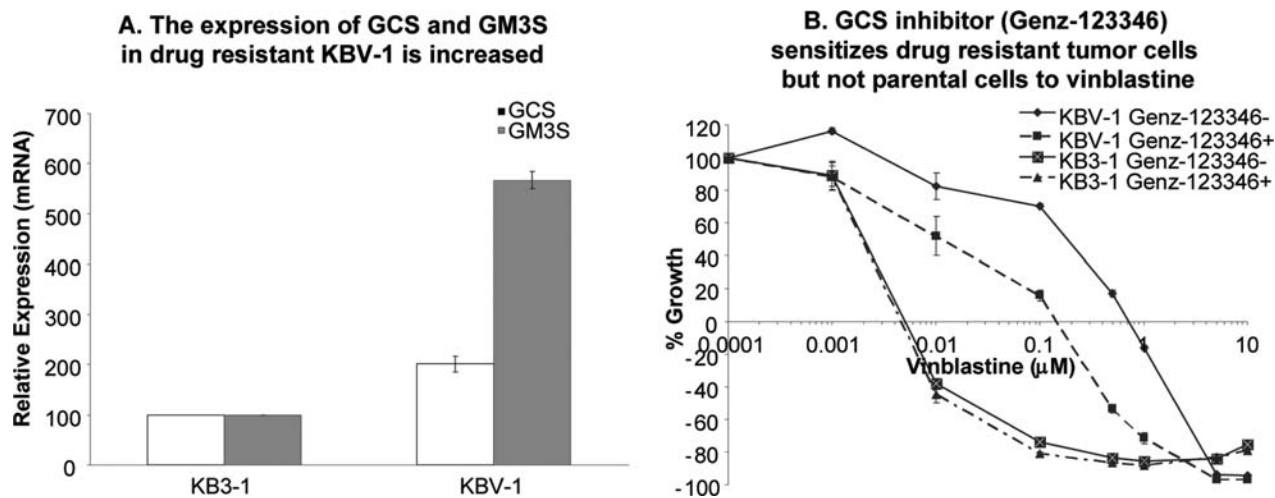
Figure 1. (A) The expression of GCS and GM3S mRNA is increased in drug-resistant KBV-1. The GCS and GM3S mRNA from KBV-1 and KB3-1 cells were analyzed by quantification real-time reverse transcription-PCR. For each gene, mRNA levels were presented relative to the levels of KB3-1 sample (equal to 100). (B) GCS inhibitor (Genz-123346) sensitizes drug-resistant tumor cells but not parental cells to vinblastine. A representative result of GCS inhibitor on cell proliferation. KBV-1 and KB3-1 cells were grown in RPMI medium supplemented with 5% FBS. Cells (n=2,000) in 100 μl growth media were seeded into each well of a 96-well plate. The cells were allowed to attach and grow overnight. The media were then replaced with fresh growth media containing indicated concentrations of vinblastine with (Genz-123346+; 1 μM) or without (Genz-123346-) GCS inhibitor. The cells were returned to 5% CO_2 humidified atmosphere at 37˚C. Cell proliferation was determined 72 h after drug addition (Materials and methods). The % of growth is compared to the cell number at zero concentration of vinblastine.

inhibitor, Genz-123346 (1 μM), decreased the levels of all the GSLs downstream of GCS examined indicating effective inhibition of GCS activity. Interestingly, the level of GCS substrate, ceramide (CER), was unchanged.

Having established the conditions for Genz-123346-mediated reduction of GSL in KBV-1 cells, the ability of the inhibitor to sensitize the drug-resistant cells to vinblastine was evaluated. Exposure of KBV-1 cells to concentrations of Genz-123346 up to 20 μM for 72 h did not alter cell proliferation (data not shown). KBV-1 cells were more sensitive to vinblastine in the presence of a non-toxic concentration (1 μM) of Genz-123346 (Fig. 1B). The GI_{50} of KBV-1 in the absence of Genz-123346 was ~255 nM. In the presence of Genz-123346, the GI_{50} was reduced to 15 nM. This enhancement in cytotoxicity was not observed with the parental KB3-1 cells treated in the same manner.

Chemosensitizing effects of varied GCS inhibitors on HCT-15 cells. To test if the observed potentiation of cytotoxicity was restricted to MDR cancer cells generated by selection in cell culture, we examined cell lines with an endogeneous MDR phenotype. HCT15 colon cancer cells, which endogenously express P-gp, were used to test the chemosensitization activity of Genz-123346 as well as other GCS inhibitors (35). Four GCS inhibitors including PDMP, PDMP analogue Genz-123346, iminosugar-based C4DGJ and C9DGJ were tested, as shown in Fig. 2A. An increased sensitivity to vinblastine was observed with the ceramide-based inhibitors of GCS (e.g. 1 μM PDMP and Genz-123346). In contrast, cells exposed to 1 μM of the iminosugar-based GCS inhibitor, C9DGJ and C4DGJ, had only slight changes in sensitivity to vinblastine. HCT15 cells treated with increasing concentrations of Genz-123346 showed a concentration-dependent increase in sensi-

tivity to 8 nM vinblastine (Fig. 2B). Proliferation of HCT15 cells were effectively inhibited in the presence of 1.25 μM Genz-123346 (27.7% of control) in combination with vinblastine.

To determine whether GCS function was inhibited, lipid compositions at the end of the cell proliferation assays were measured. As illustrated in Fig. 2C, a marked decrease in GSLs was observed in cells treated with Genz-123346, PDMP and C9DGJ for 72 h. C4DGJ treatment also resulted in a reduction of GSLs but to a lesser extent than with the other inhibitors. Consistent with these results, flow cytometry measurements also showed a reduction of cell surface GM1 levels (data not shown).

The ability of Genz-123346 to sensitize HCT-15 to other cytotoxic anti-cancer agents that are P-gp substrates was also determined. Genz-123346 was equally effective at increasing the sensitivity of HCT-15 cells to vincristine, docetaxel and doxorubicin (Fig. 3).

The ceramide-based inhibitors of GCS (PDMP and Genz-123346) but not the iminosugar-based inhibitors (C4 and C9 DGJ) are substrates of P-gp. The observed difference in chemosensitization activity between the ceramide-based inhibitors of GCS (PDMP and Genz-123346) and the iminosugar-based inhibitors (C4 and C9 DGJ) implied that inhibition of GCS activity alone was unlikely to be responsible for the enhanced sensitivity of HCT-15 cells to cytotoxic drugs. We speculated whether discrete interactions between P-gp and the structurally distinct inhibitors of GCS may have a role in driving the observed sensitization effect.

To test this, cell-free P-gp ATPase activity assays were performed (Fig. 4A). The P-gp-Glo assay relies on the ATP dependence of the light-generating reaction of luciferase

A. Influence of GCS inhibitors on Vinblastine sensitivity in HCT-15 cells

B. Concentration-response curve for Genz-123346 to sensitize HCT-15 cells to vinblastine

C. The effects of GCS inhibitors on the sphingolipid composition of HCT-15 cells
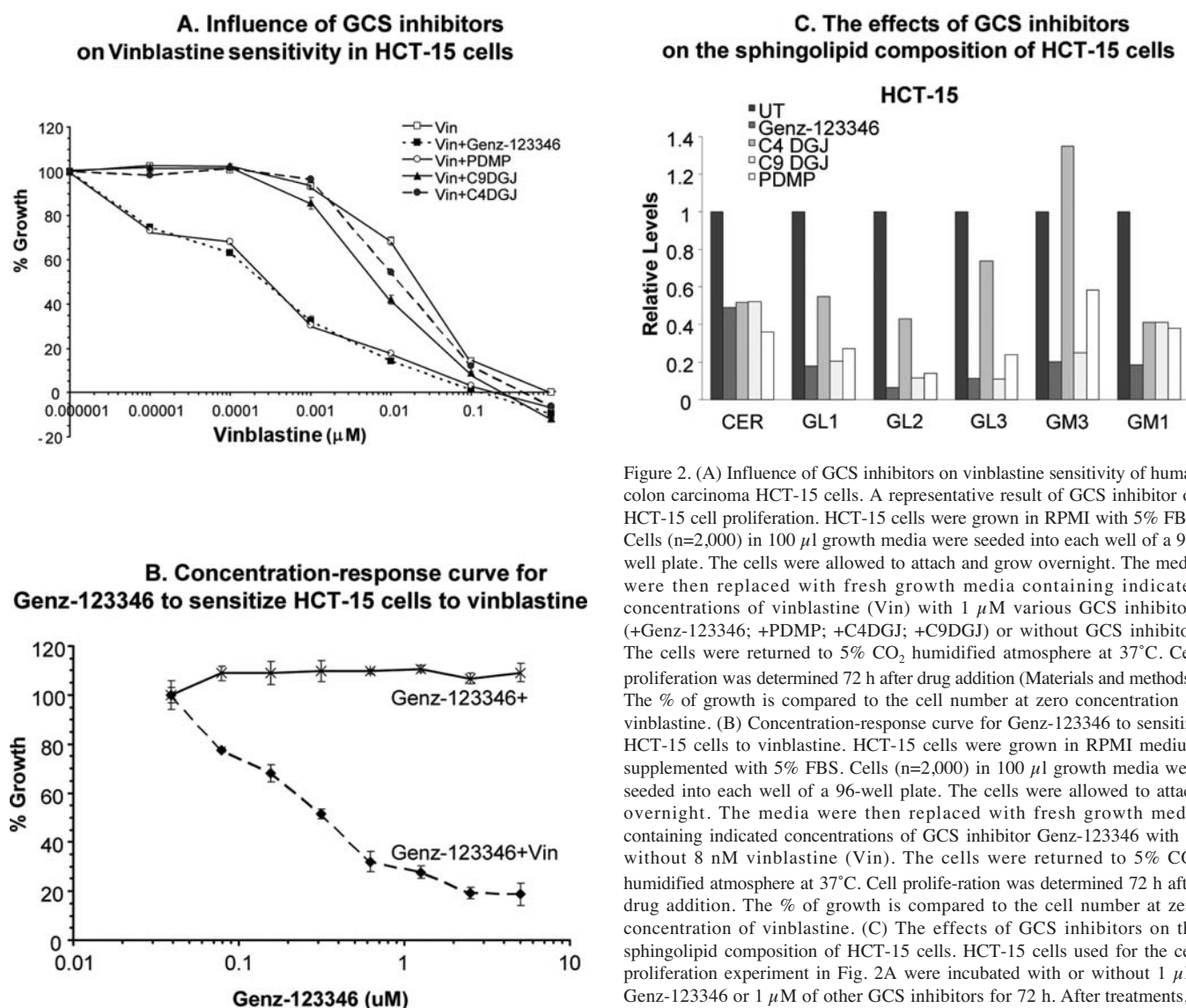

Figure 2. (A) Influence of GCS inhibitors on vinblastine sensitivity of human colon carcinoma HCT-15 cells. A representative result of GCS inhibitor on HCT-15 cell proliferation. HCT-15 cells were grown in RPMI with 5% FBS. Cells (n=2,000) in 100 μl growth media were seeded into each well of a 96-well plate. The cells were allowed to attach and grow overnight. The media were then replaced with fresh growth media containing indicated concentrations of vinblastine (Vin) with 1 μM various GCS inhibitors (+Genz-123346; +PDMP; +C4DGJ; +C9DGJ) or without GCS inhibitor. The cells were returned to 5% CO_2 humidified atmosphere at 37˚C. Cell proliferation was determined 72 h after drug addition (Materials and methods). The % of growth is compared to the cell number at zero concentration of vinblastine. (B) Concentration-response curve for Genz-123346 to sensitize HCT-15 cells to vinblastine. HCT-15 cells were grown in RPMI medium supplemented with 5% FBS. Cells (n=2,000) in 100 μl growth media were seeded into each well of a 96-well plate. The cells were allowed to attach overnight. The media were then replaced with fresh growth media containing indicated concentrations of GCS inhibitor Genz-123346 with or without 8 nM vinblastine (Vin). The cells were returned to 5% CO_2 humidified atmosphere at 37˚C. Cell prolife-ration was determined 72 h after drug addition. The % of growth is compared to the cell number at zero concentration of vinblastine. (C) The effects of GCS inhibitors on the sphingolipid composition of HCT-15 cells. HCT-15 cells used for the cell proliferation experiment in Fig. 2A were incubated with or without 1 μM Genz-123346 or 1 μM of other GCS inhibitors for 72 h. After treatments, a variety of GSL levels were measured by mass spectrometry analysis (Materials and methods).

where ATP consumption is detected as a decrease in lumines-cence; the greater the decrease in signal, the higher the P-gp activity. Sodium orthovanadate was used as a P-gp ATPase inhibitor, and verapamil was used as a positive control. All compounds were tested at 400 and 200 μM. Genz-123346 (and PDMP to a lesser extent) treatment resulted in a change in luminescence that was similar to or higher than that obtained with the positive control (verapamil), implying that Genz-123346 is a P-gp substrate. Vinblastine, a known P-gp substrate, also showed positivity as a P-gp substrate in this assay.

P-gp efflux activity in the HCT-15 cells, using Calcein-AM, a substrate for both P-gp and MRP1 (ABCC1) transporters, was also determined (36). Increased intracellular calcein fluorescence was observed in the presence of the P-gp inhi-bitor, verapamil (10-100 μM) and vinblastine (10-100 μM), a known P-gp substrate (Fig. 4B). An increase in intracellular calcein fluorescence was also detected in the cells exposed to Genz-123346, indicating that Genz-123346 is a P-gp/MRP substrate. In contrast, C4DGJ and 5-FU did not affect

intracellular calcein fluorescence, consistent with previous reports that these compounds are not P-gp substrates (25,35,37).

In a subsequent study, the chemosensitizing effect of Genz-123346 was measured in the presence of the P-gp inhibitor verapamil. As shown in Fig. 4C, inhibition of P-gp by verapamil (10 μM) resulted in sensitization of HCT-15 cells to vinblastine. A similar response was observed in Genz-123346 (10 μM)-treated cells. However, Genz-123346 did not further sensitize the tumor cells to vinblastine in the presence of verapamil, suggesting that the mechanism of action of Genz-123346 was primarily through P-gp, and not GCS inhibition.

Reducing GCS expression did not alter the sensitivity of HCT-15 and HepG2 tumor cells to vinblastine and docetaxol. The results above suggest a less important role of GCS activity in mediating chemosensitization of HCT-15 cells to anti-cancer drugs. To further investigate the role of GCS in chemosensitizing this cell line, siRNA-mediated reduction in
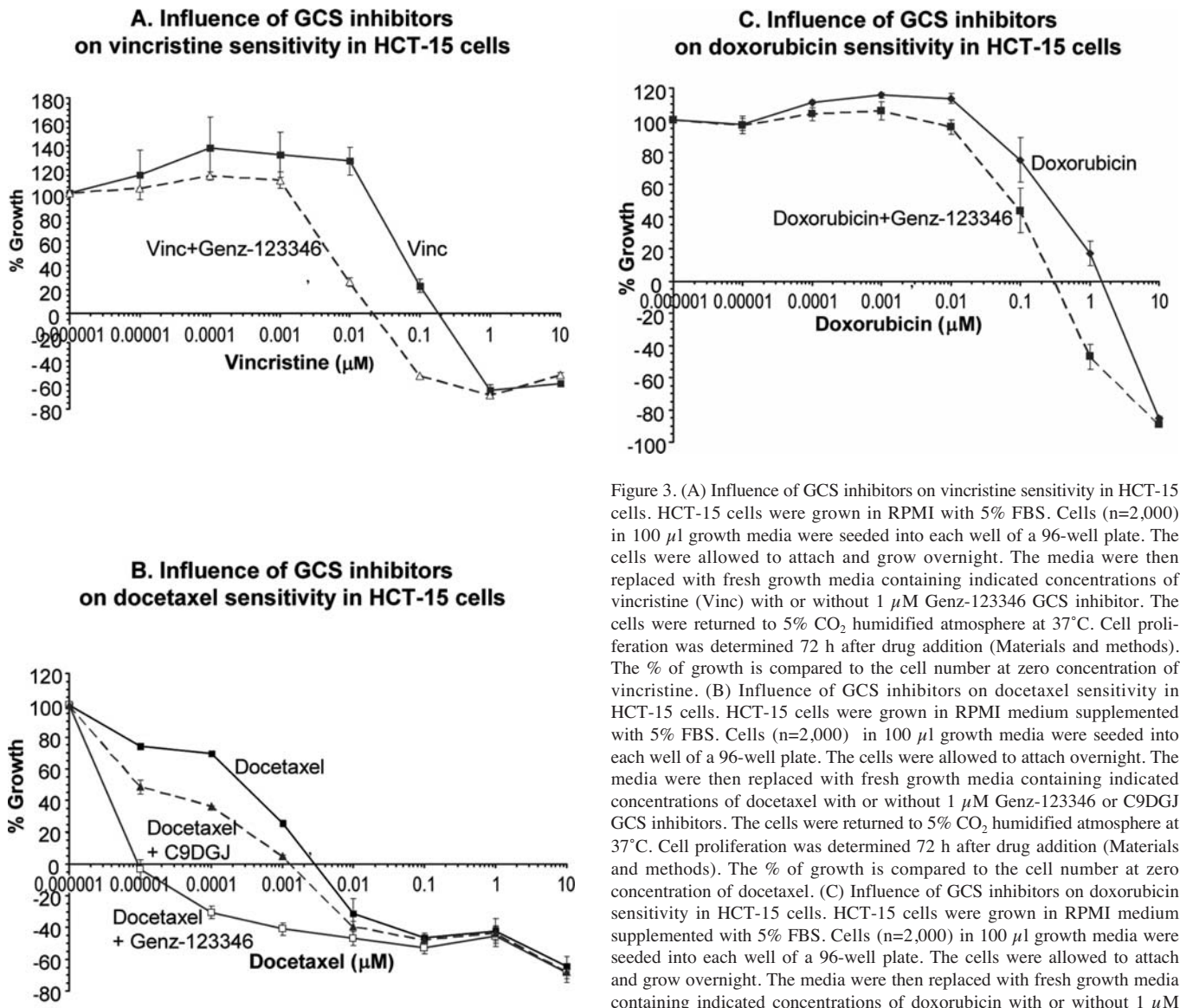
A. Influence of GCS inhibitors on vincristine sensitivity in HCT-15 cells

B. Influence of GCS inhibitors on docetaxel sensitivity in HCT-15 cells

C. Influence of GCS inhibitors on doxorubicin sensitivity in HCT-15 cells

Figure 3. (A) Influence of GCS inhibitors on vincristine sensitivity in HCT-15 cells. HCT-15 cells were grown in RPMI with 5% FBS. Cells (n=2,000) in 100 μl growth media were seeded into each well of a 96-well plate. The cells were allowed to attach and grow overnight. The media were then replaced with fresh growth media containing indicated concentrations of vincristine (Vinc) with or without 1 μM Genz-123346 GCS inhibitor. The cells were returned to 5% CO_2 humidified atmosphere at 37˚C. Cell proliferation was determined 72 h after drug addition (Materials and methods). The % of growth is compared to the cell number at zero concentration of vincristine. (B) Influence of GCS inhibitors on docetaxel sensitivity in HCT-15 cells. HCT-15 cells were grown in RPMI medium supplemented with 5% FBS. Cells (n=2,000) in 100 μl growth media were seeded into each well of a 96-well plate. The cells were allowed to attach overnight. The media were then replaced with fresh growth media containing indicated concentrations of docetaxel with or without 1 μM Genz-123346 or C9DGJ GCS inhibitors. The cells were returned to 5% CO_2 humidified atmosphere at 37˚C. Cell proliferation was determined 72 h after drug addition (Materials and methods). The % of growth is compared to the cell number at zero concentration of docetaxel. (C) Influence of GCS inhibitors on doxorubicin sensitivity in HCT-15 cells. HCT-15 cells were grown in RPMI medium supplemented with 5% FBS. Cells (n=2,000) in 100 μl growth media were seeded into each well of a 96-well plate. The cells were allowed to attach and grow overnight. The media were then replaced with fresh growth media containing indicated concentrations of doxorubicin with or without 1 μM Genz112638C9 GCS inhibitor. The cells were returned to 5% CO_2 humidified atmosphere at 37˚C. Cell proliferation was determined 72 h after drug addition (Materials and methods). The % of growth is compared to the cell number at zero concentration of doxorubicin.

GCS expression was studied. To ensure GCS activity was reduced, two sequential GCS siRNA transfections were performed (Materials and methods) and resultant mRNA levels were quantified as a measure of siRNA efficiency (Fig. 5A). The mRNA level of GCS was significantly reduced 48 h post siRNA tranfection [22% of non-targeting (NT) siRNA control] and remained low until four days post-transfection (45% of NT siRNA control). GM1 ganglioside level, a downstream product of GCS was used to indirectly assess the decrease in GCS expression (28). Results from flow cytometry analysis revealed that the GM1 level on HCT-15 cells decreased as indicated by the shift in fluorescence intensity 48 h post-the second transfection with siRNA as compared to that of NT siRNA-transfected cells (Fig. 5B). The GM1 level remained low at 72 h post-second transfection but returned close to the control level of NT siRNA transfected cells at 96 h post-transfection. Cells were also tested for their

response to vinblastine in cell proliferation assays (Fig. 5C). There was no significant difference between GCS siRNA- and NT siRNA-transfected cells in response to vinblastine.

To further assess the role of GCS in chemosensitizing tumor cells, a second approach at reducing GCS expression that entails the use of short hairpin RNA (shRNA) against GCS in a different cell line (HepG2) was also assessed. GCS mRNA levels as well as GSL levels were measured post-transfection with shRNAs directed against GCS. Two stable cell lines exhibiting lower levels of GCS mRNA generated using two shRNAs that targeted different GCS sequences (HepG2 GCS-c1 and GCS-c2) were characterized with qRT-PCR. As shown in Fig. 6A, GCS mRNA levels in both cell lines were reduced (33% of non-silencing control (NS) for GCS-c1 and 29% of NS control for GCS-c2). Lipid analysis of the two cell lines indicated that GL1, GL2 and GM3 were decreased as well (Fig. 6B). In general, the growth
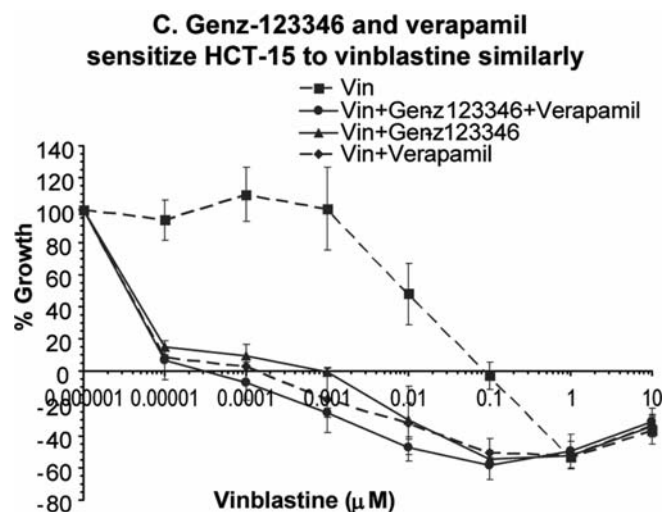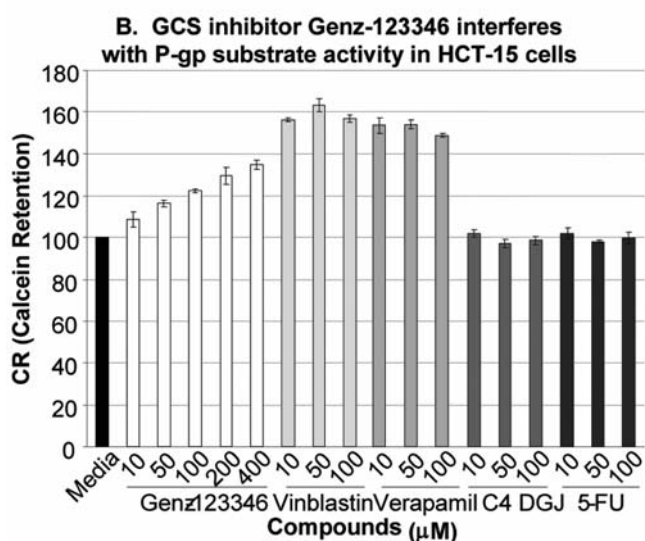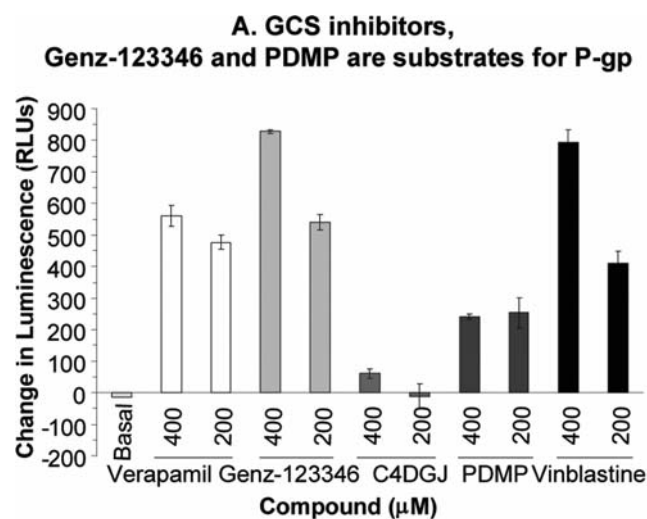
A. GCS inhibitors, Genz-123346 and PDMP are substrates for P-gp

B. GCS inhibitor Genz-123346 interferes with P-gp substrate activity in HCT-15 cells

C. Genz-123346 and verapamil sensitize HCT-15 to vinblastine similarly

Figure 4. (A) GCS inhibitors, Genz-123346 and PDMP are substrates for P-gp. Untreated, 250 μM Na_3VO_4, 200-400 μM verapamil and 200-400 μM Genz-123346, PDMP, C4DGJ or vinblastine treated cell-free P-gp ATPase activity assay were performed according to the manufacturer's protocol (Materials and methods). The decrease change in luminescence of untreated samples compared to samples plus Na_3VO_4 represents basal P-gp ATPase activity (Basal). The decrease change in luminescence of Verapamil, Genz-123346, PDMP, C4DGJ or vinblastine-treated samples represent P-gp ATPase activity. The diagram shows decreased change in luminescence of Genz-123346-treated sample as compared with Na_3VO_4 or verapamil, PDMP and other drugs-treated samples. (B) GCS inhibitor Genz-123346 interferes with P-gp substrate activity in HCT-15 cells. Calcein-AM was used to determine P-gp-mediated efflux functions in HCT-15 cells in the presence of Genz-123346 and other compounds. HCT-15 cells were grown to 70-90% confluence. Cells in 100 μl volumes of tissue culture medium were exposed with various concentrations of Genz-123346 or other compounds in 50 μl culture medium for 15 min at 37˚C. Calcein-AM was added to each well in 50 μl culture medium with final concentration of 0.25 μM) and the cells were incubated for 15 min at 37˚C. Fluorescence intensity was measured using a BioTek Synergy 2-plate reader with filters appropriate for detection (excitation 485/20, emission 528/20). The fluorescence values (Calcein retention) were plotted. (C) Genz-123346 and verapamil sensitize HCT-15 to vinblastine similarly. Cell proliferation assays for HCT-15 cells were carried out in the presence of 10 μM Genz-123346 with or without 10 μM verapamil and the cell proliferation inhibition and for vinblastine was determined as described under Materials and methods. The % of growth is compared to the cell number at zero concentration of vinblastine.

characteristics of the two cell lines expressing lower levels of GCS mRNA, the NS control and parental cells were similar (data not shown). Examination of the response of shRNA-knockdowned HepG2 cells and the control cells to vinblastine and docetaxel (Fig. 6C and D) showed they all responded similarly to the drugs, although the GCS-c2 clone showed a moderate increase in sensitivity to docetaxel.

Discussion

Elevated GL1 has been found in a number of multidrug-resistant human cancer cell lines and this feature has been proposed as a marker for MDR in the clinic (5,6,12). GSC is the primary ceramide glycosylase and decreased GCS expression with RNA interference or small molecule inhibitors was thought to result in a buildup of ceramide thereby enhancing the cell chemosensitivity to certain cytotoxic anti-cancer agents (8,12,13). However, most data generated on GCS-related MDR have been obtained using cells that over-

expressed ABC transporters, and from cells that had been artificially selected for multi-drug resistance (e.g. NCI/ADR-RES cells) (4). Thus, the relationship between GSL metabolism via GCS activity and ABC transporter expression and function needed further elucidation.

P-gp over-expression has long been regarded as the major cause of MDR in a number of human malignancies. In addition, P-gp acts as a drug flippase or phospholipid translocator (38) and P-gp has been shown to transport short-chain fluorescent analogues of sphingomyelin and GL1 across membranes (39). Additionally, it has been proposed that P-gp mediates GL1 translocation across the lipid bilayer, from the cytosolic face of the golgi to the lumen, to provide increased substrate for the luminal synthesis of GL2 and Gb3 (40).

Reduction of P-gp expression was observed in NCI/ADR-RES cells transfected with an antisense RNA to GCS or treated with the GCS inhibitor, PPMP (1-phenyl-2-palmitoylamino-3-morpholino-1-propanol) (41). The chemosensitizing effect of the GCS inhibitior, PDMP, was observed in MDR cell
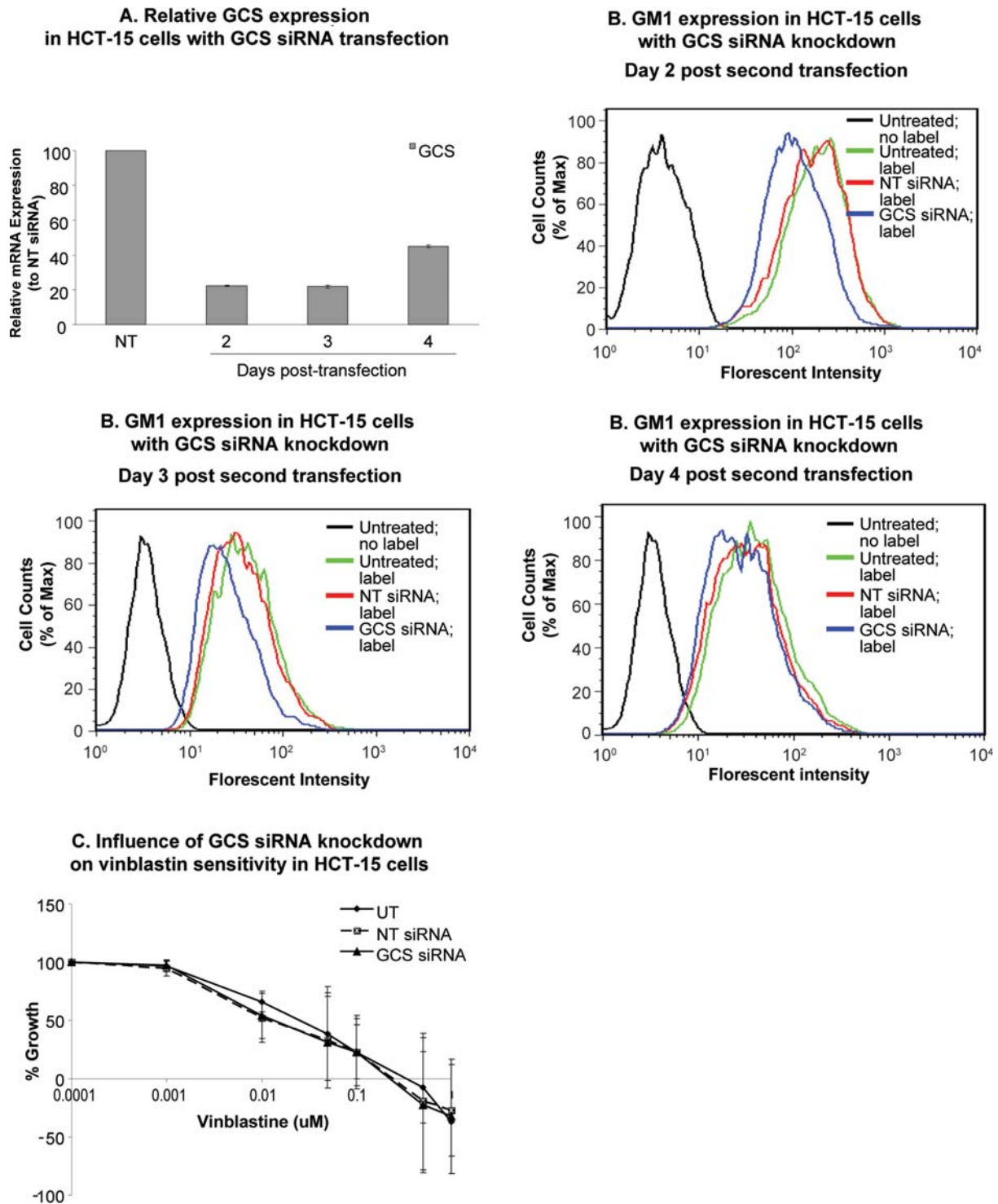
Figure 5. (A) Relative GCS expression in HCT-15 cells with GCS siRNA transfection. The GCS mRNA levels from HCT-15 cells transfected with GCS siRNA for various time were analyzed with quantification real-time reverse transcription-PCR. The mRNA levels were presented relative to the levels of HCT-15 cells transfected with scramble non-targeting siRNA (NT siRNA equal to 100). (B) GM1 expression in HCT-15 cells with GCS siRNA knockdown. Flow cytometry analysis of GM1 level on cell surface was performed to determine indirectly the inhibitory activity of GCS siRNA transfection. HCT-15 cells were cultured under standard conditions and two-time sequential GCS siRNA transfections were performed (Materials and methods). The cells were harvested and stained using recombinant cholera toxin-FITC (Materials and methods). The fluorescence was quantified using a FACSCalibur flow cytometer. (C) Influence of GCS siRNA knockdown on vinblastine sensitivity in HCT-15 cells. The GCS activity was indirectly determined by examining GM1 level on cell surface by flow cytometry (B) for cells used for this assay. The cell proliferation inhibition and for vinblastine was determined as described in Materials and methods. The % of growth is compared to the cell number at zero concentration of vinblastine.

lines either with P-gp and GCS over-expressing NCI/ADR-RES line or with P-gp gene-transfected cell lines but not in P-gp low expressing wild-type cells (22). In a recent review,

Sietsma *et al* discussed GCS-mediated drug resistance dependent on ABC transporter proteins and hypothesized that GSL-mediated drug resistance may be dependent on the
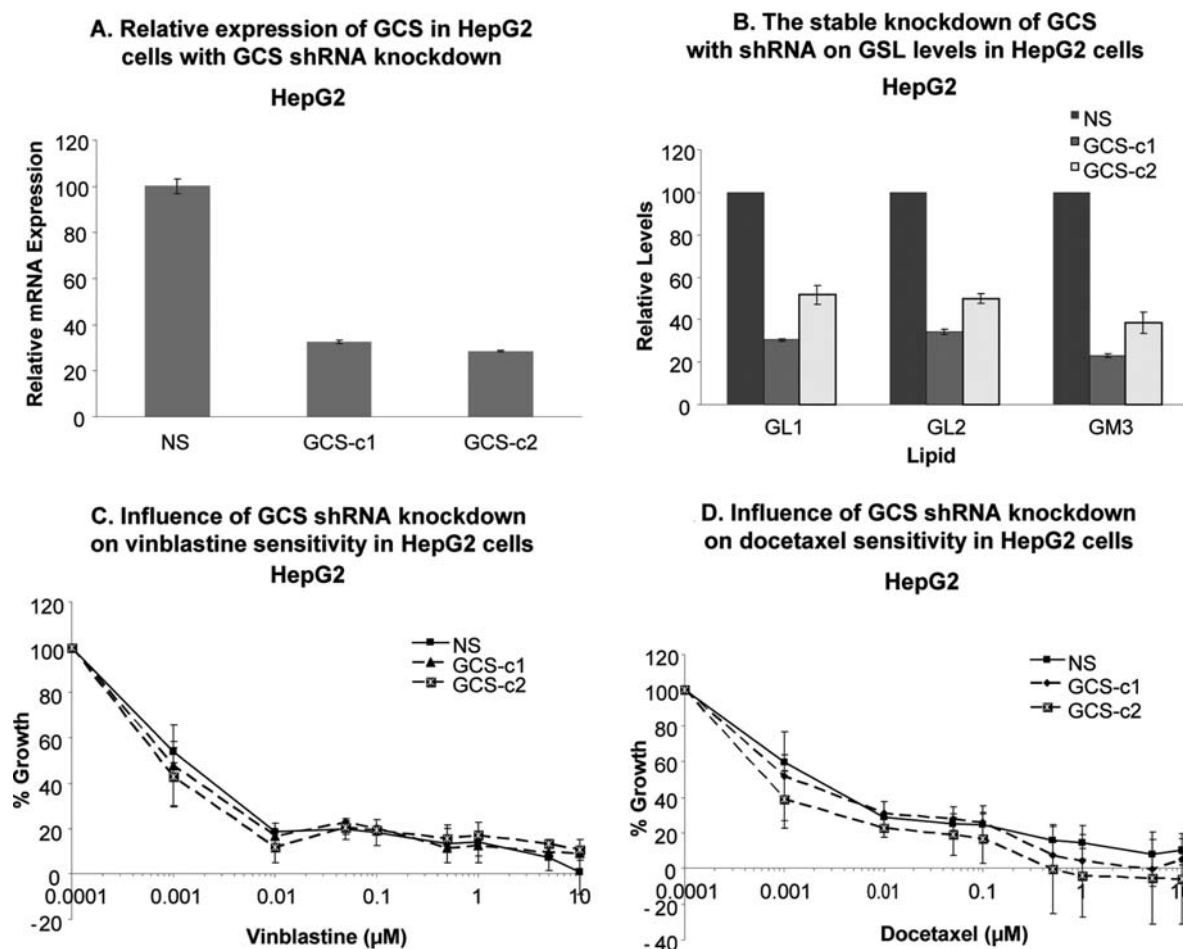
Figure 6. (A) Relative expression of GCS in HepG2 cells with GCS shRNA knockdown. The GCS mRNA levels from HCT-15 cells with GCS shRNA or NT shRNA (NS) were analyzed with quantification real-time reverse transcription-PCR. The mRNA levels were presented relative to the levels of HCT-15 cells with non-targeting shRNA (NS shRNA sample equals to 100). (B) The stable knockdown of GCS with shRNA on GSL levels in HepG2 cells. Analysis of GM1 level on cell by mass spectrometry was performed to determine indirectly the inhibitory activity of GCS in GCS shRNA cells. HCT-15 cells with shRNA knockdown were cultured under standard conditions and analyses were performed as described in Materials and methods. (C) Influence of GCS shRNA knockdown on vinblastine sensitivity in HepG2 cells. The cell proliferation inhibition and for vinblastine was determined as described in Materials and methods. The % of growth is compared to the cell number at zero concentration of vinblastine. (D) Influence of GCS shRNA knockdown on docetaxel sensitivity in HepG2 cells. The cell proliferation inhibition and for docetaxel was determined as described in Materials and methods. The % of growth is compared to the cell number at zero concentration of docetaxel.

presence of drug transporter proteins (4). Of note, many previous studies relied on the use of the GCS inhibitor PDMP. Our current study showed that Genz-123346, like PDMP, could sensitize various tumor cells to multiple cancer drugs. We attempted to define whether the chemosensitization effect of PDMP and Genz-123346 could be explained at least partially because PDMP and Genz-123346 are modulaters, or substrates, of P-gp. A study evaluating PDMP chemosensitization of neuroblastoma cells with paclitaxel and vincristine demonstrated that PDMP treatment resulted in a decreased efflux of [14C]paclitaxel and [3H]vincristine from neuroblastoma cells, similar to treatment with P-gp inhibitors, suggesting an effect of PDMP on the transporter proteins P-gp and/or multidrug resistance protein (15). Consistent with this result, we found that PDMP and its analogue Genz-123346 are potential substrates of P-gp using the cell-free P-gp ATPase activity assays (Fig. 4A). We did not observe Genz-123346 chemosensitization effect to non P-gp substrate anti-cancer agent, such as 5-FU (data not shown). In addition, ABC transporter efflux assays employing a fluorescent P-gp

or MRP substrate calcein-AM in HCT-15 cells revealed that the functional activity of these transporters was affected in the presence of Genz-123346 (Fig. 4B). Genz-123346 did not further sensitize HCT-15 cells to vinblastine in the presence of a P-gp inhibitor (Fig. 4C). Nevertheless, cellular drug accumulation in P-gp over-expressing NCI/ADR-RES cells treated with PDMP was not observed in previous studies (22,37). Therefore, the modulation of P-gp function by PDMP seems, at the very least, to be cell line-dependent.

In this study, we further explored the biological role of GCS in chemosensitization of tumor cells using RNA interference. Consistent with prior genetic studies in GM95, CHP-100 human neuroepithelioma and drug-selected multidrug-resistant cells (25,42,43), we found siRNA and shRNA knockdown of GCS in HCT-15 and HepG2 cancer cells, respectively, did not alter their response to anti-cancer cytotoxic drugs. Veldman *et al* studied GM95 mouse melanoma cells which lack functional GCS and do not express detectable P-gp or MRP1. Compared to non-mutant cells, the mutant cells showed no difference in viability upon exposure to various

drugs (42). Furthermore, potent and non-toxic iminosugar based GCS inhibitors had no chemosensitizing effect on wild-type GM95 and multidrug-resistant tumor cells (25,42). Altogether, the evidence from both genetic and pharmacological studies suggested that alterations of the cellular ceramide metabolism through GCS inhibition may have limited importance in chemosensitization.

Not withstanding these observations, we cannot exclude the possibility that GCS inhibition may have contributed to the chemosensitization effect within alternative cellular context. When iminosugar-based GCS inhibitors were applied to chronic lymphocytic leukaemia (CLL) cells to reduce GSL levels, Gerrard *et al* observed that the GCS inhibitors sensitized CLL cells to the conventional cytotoxic drug 2-chlorodeoxyadenosine (CdA) and the cytostatic drugs chlorambucil and fludarabine. In addition, they did not appear to have any effect on P-gp functional activity (44). Recent study demonstrated that with NCI/ADR-RES cells were sensitized to cytotoxic drugs when cancer cells were treated with a mixed-backbone anti-sense oligos against GCS (MBO-asGCS) both *in vitro* and *in vivo* (45). We also observed that in HCT-15 cells, C9DGJ demonstrated a chemosensitization effect with docetaxel, although it was not as robust as Genz-123346 (Fig. 3B). Nevertheless, the selectivity of GCS inhibition of iminosugar based GCS inhibitors is subjective to D-*gluco*-, D-*galacto* and L-*ido*-stereochemistry in combination with a hydrophobic substituent on the endocyclic nitrogen inhibited GCS (29). Again, the sensitization effect of GCS inhibitors should be interpreted with caution.

Our study provides clarity for the basis for by which PDMP-based GCS inhibitors mediate sensitization of tumor cells to anti-cancer cytotoxic drugs. The data from our current study suggest that the chemosensitizing effect of PDMP-based GCS inhibitor Genz-123346 is primarily mediated through P-gp modulation. If GCS inhibition is important for multidrug-resistance, at the very least it is likely to be cell line-dependent. These observations suggest that chemosensitization data obtained using these inhibitors should be interpreted with caution.

Acknowledgments

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