Inhibition of Glucosylceramide Synthase Sensitizes Head and Neck Cancer to Cisplatin

Jong-Lyel Roh,* Eun Hye Kim, Jin Young Park, Ji Won Kim

Department of Otolaryngology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

*Corresponding author: Jong-Lyel Roh, MD, PhD

Department of Otolaryngology, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 138-736, Republic of Korea. Phone: +82-2-3010-3965; Fax: +82-2-489-2773; E-mail: rohjl@amc.seoul.kr.

Running title: GCS inhibition in HNC

Key words: head and neck cancer, cisplatin resistance, glucosylceramide synthase, P-glycoprotein.

Disclosure of Potential Conflicts of Interest: The authors declare no conflicts of interest.

Acknowledgements: This study was supported by a grant (NRF-2012R1A1A2002039) the Basic Science Research Program through the National Research Foundation (NRF), the Ministry of Education, Science and Technology, and a grant (HI14C23050000) of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Seoul, Republic of Korea (J.-L. Roh).

Word counts: 216 words in the abstract; 3,441 words (10 pages) in the text; 36 references; 0 table; 6 figures (gray scale); supplementary methods.
Abstract

Glucosylceramide synthase (GCS) overexpression is associated with multidrug resistance in several human cancers. GCS blockade, which overcomes multidrug resistance by downregulating P-glycoprotein (P-gp), has not been tested in head and neck cancer (HNC). This study investigates whether GCS is targetable in HNC by assessing whether GCS inhibition sensitizes HNC to cisplatin. The effect of genetic or pharmacological GCS inhibition (using GCS siRNA/shRNA or D,L-threo-PPMP, respectively) on cisplatin sensitivity was assessed in several human HNC cells and acquired cisplatin-resistant HNC cells by measuring cell viability, cell cycle, death, mRNA and protein expression, ceramide production and in preclinical tumor xenograft mouse models. GCS and P-gp expression were significantly associated with cisplatin resistance in several HNC cell lines ($P = 0.007$). Both were significantly increased in HN9-cisR cells, which display acquired cisplatin resistance ($P < 0.001$). Genetic or pharmacological inhibition of GCS induced accumulation of increased ceramide levels. GCS inhibition increased cisplatin-induced cell death in HNC cells via P-gp downregulation and pro-apoptotic protein activation, which were abrogated by siPUMA transfection. Genetic and pharmacological GCS inhibition sensitized resistant HNC cells to cisplatin in vitro and in vivo. GCS and P-gp overexpression is associated with acquired cisplatin resistance, suggesting a role for these molecules as therapeutic targets for HNC. Genetic or pharmacological GCS blockade may have therapeutic benefit in cisplatin-resistant HNC.
Introduction

Cisplatin or *cis*-diaminedichloroplatinum (II) (CDDP) is a platinum-based compound widely used in various types of human solid neoplasms, including bladder, testicular, ovarian, colorectal, lung and head and neck cancer (HNC) (1). Cisplatin remains a first-line chemotherapeutic agent and has been continuously used as a primary treatment modality in HNC in combination with radiotherapy in organ preservation strategies (2, 3). It interferes with DNA repair mechanisms by crosslinking with the DNA purine bases, causing DNA damage and inducing apoptosis in cancer cells (4). Unfortunately, the numerous side effects of cisplatin, which affect the kidneys, the gastrointestinal tract, the immune system, peripheral nerves and the inner ear, have led to the greater use of other platinum-containing anticancer agents such as carboplatin, oxaliplatin and others (4). In addition, the main limitation to the clinical usefulness of cisplatin is the high incidence of chemoresistance, a major cause of treatment failure in cancer management (1). Combining cisplatin with other drugs may reduce toxicity and circumvent chemoresistance.

Several mechanisms of resistance to chemotherapy have been reported. P-glycoprotein (P-gp), a plasma membrane glycoprotein encoded by the *MDR1* gene, acts as a drug efflux pump, thereby resulting in the reduced intracellular accumulation and decreased cytotoxicity of anticancer drugs including cisplatin (5). Further, P-gp regulates cancer proliferation, invasion and caspase-dependent cell death (6, 7). P-gp overexpression in tumors is associated with poor prognosis in several types of cancers (8–10). The upregulation of rafts and caveolae, including that of the glucosphingolipid-enriched constituents of microdomains, represents another multidrug resistance (MDR) mechanism (11). Ceramide, as a second messenger of apoptosis, participates in cell death signaling, autophagy and cell cycle arrest, and defects in ceramide generation and metabolism in cancer cells contribute to resistance to chemotherapy (12, 13).

Glucosylceramide synthase (GCS), a transmembrane protein encoded by the *UGCG* gene in humans, is the rate-limiting enzyme that controls the first glycosylation step in the biosynthesis of glucosphingolipids; it catalyzes ceramide glycosylation, converting ceramide to glucosylceramide (14). GCS overexpression confers resistance to apoptosis (15, 16), suggesting a potential role as a
biomarker predicting tumor response to chemotherapy (17). Growing evidence shows that a persistent increase in ceramide glycosylation facilitates cancer progression and drug resistance (15, 16). GCS blockade by gene silencing or pharmacological inhibition leads chemoresistant cancer cells to apoptosis via downregulation of P-gp and restoration of p53-dependent apoptosis (18, 19). GCS blockade overcomes MDR in several cancers but has not been tested in HNC. This study investigates whether GCS is targetable in HNC by assessing whether genetic or pharmacological GCS inhibition sensitizes chemoresistant HNC cells to cisplatin in vitro and in vivo.

**Materials and methods**

**Cell culture and establishment of cisplatin-resistant HNC cells**

Our experiments utilized five human HNC cells, AMC-HN2, -HN3, -HN4, -HN5 and -HN9 cell lines previously established from the primary tumors of head and neck in our institution (20). All cancer cell lines were authenticated by DNA (short-tandem-repeat, STR) profiling provided by the Korean Cell Line Bank (Seoul, Korea). HNC cells were cultured in Eagle’s minimum essential medium (Life Technologies™, Carlsbad, CA) supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cisplatin-resistant AMC-HN9 (HN9-cisR) cells were derived from the parental cisplatin-sensitive AMC-HN9 cells by exposure to increasing concentrations of cisplatin (cis-platinum (II) diamine dichloride [CDDP], Sigma-Aldrich, Louis, MO) (21). The cisplatin resistance was evaluated by cell viability assays performed on both the resistant and the parental cells.

**Cell viability assay**

Cell viability was assessed by trypan blue exclusion and MTT assays. Trypan blue exclusion was performed in HNC cells seeded at 1 × 10⁵ in 6-well plates, allowed to reach 60–70% confluence, and exposed to D,L-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) (Sigma-Aldrich) for 72 h. The cells were then trypsinized, stained with 0.4% trypan blue (Life Technologies™) and counted using a hemocytometer. MTT assays was performed with HNC cells seeded at 3–5 × 10³
cells/well in 96-well plates, incubated overnight and exposed to D,L-threo-PPMP and cis-platinum (II) diamine dichloride (cisplatin; Sigma-Aldrich), alone or in combination, for 72 h. The cells were then exposed to the tetrazolium compound 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich) for 4 h, after which solubilization buffer was added for 2 h. The absorbance in each well was measured at 570 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

Cisplatin cytotoxicity was assessed by MTT assay after 72 h and the half maximal inhibitory concentration (IC50) of this drug for each HNC cell line was calculated. The interaction of two drugs was considered synergistic when growth suppression was greater than the sum of the suppression induced by either drug alone combination index (CI) was used to evaluate drug interaction: CI = 1, additive interaction; CI <1, synergistic interaction; CI >1, antagonistic interaction (22).

Cell cycle and cell death assays

For cell cycle assays, the cells were exposed to D,L-threo-PPMP for 72 h and then trypsinized, fixed overnight in ice-cold ethanol, and stained for 30 min with propidium iodide (Sigma-Aldrich) at 37°C. The cellular DNA content was measured using a FACScalibur flow cytometer (BD Bioscience, San Jose, CA). For cell death assays, cells were assessed by flow cytometry after staining with annexin V-FITC (fluorescein isothiocyanate) and propidium iodide, using an annexin V-FITC apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ). For caspase activity assays, cells were assessed in triplicate wells using the fluorimetric Homogeneous Caspase Assay (Roche, Basel, Switzerland) after treatment with cisplatin and D,L-threo-PPMP, alone or in combination, for 72 h. All data were analyzed using the Cell Quest software (BD Biosciences). All assays were performed with triplicate samples in three separate experiments. For the measurement of mitochondrial membrane potential (ΔΨm), HN9-cisR cells were seeded in a 96-well plate and then exposed to 100 µL medium containing cisplatin and D,L-threo-PPMP, alone or in combination, for 36 h. The cells were stained with 200 nM tetramethylrhodamine ethyl ester (TMRE, Life Technologies™) for 20 min and then analyzed by flow cytometry. The median fluorescent intensity (MFI) of each treatment group was
normalized to the control group.

Ceramide production measurements

Total ceramide levels were measured in HNC cells and tumors of the transplanted mice. After treatment, cells were homogenized, and total protein concentrations were measured, and lipids were then extracted from samples. Sphingolipids were separated on an Agilent 1200 high performance liquid chromatography system (Agilent Technologies, Santa Clara, CA) and analyzed by electrospray ionization-tandem mass spectrometry on a 400 QTRAP (AB Sciex, Framingham, MA). The peak areas for different sphingolipid subspecies were quantified by internal standards and then normalized to protein concentrations (23). Total ceramide levels in the cells exposed for 24 h to 10 μM PPMP, 10 μM cisplatin or their combination were calculated as the percentage of those in untreated HNC controls and compared between groups. Total ceramide levels were also measured in the tumors of each group in mice after implantation of tumor cells.

Transfection and infection

For knockdown experiments of GCS and PUMA, AMC-HN2 with mutant p53 and HN9-cisR were seeded onto 60 mm plates in medium without antibiotics, and 18 h later were transfected with 100 nmol/L small interfering RNA (siRNA) targeting human UGCS or PUMA or a scrambled control siRNA (Life Technologies™). After 72 h, the cells were exposed to D,L-threo-PPMP for an additional period of 72 h and then analyzed for protein expression. For knockdown of GCS gene, AMC-HN9-cisR cells were stably transfected with small hairpin RNA (shRNA) directed against UGCS or control shRNA in a lentiviral vector (Life Technologies™). At 72 h after transfection, cells at 60–70% confluence were infected with virus-containing media supplemented with 4 μg/mL polybrene (EMD Millipore) and incubated overnight. Selection was performed using 2 μg/mL puromycin (Sigma-Aldrich). Protein expression and knockdown was confirmed by western blotting using anti-GCS and anti-PUMA antibodies.
Quantitative real time PCR

Total cellular RNA was extracted using the QIAzol lysis reagent and the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was generated from the purified RNA using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. The cDNAs corresponding to GCS, MDR1 and ACTB were amplified by PCR using the primers (supplementary data). Quantitative real-time PCR was performed using SYBR Green Mix (Qiagen) on a 7900HT Fast Real-time PCR System (Applied Bioscience, Foster, CA). Relative target mRNA levels were normalized to β-actin transcript levels. Quantification was performed using GCS DNA standard curves generated by serial dilution of pcDNA 3.1-GCS plasmid (GenScript, Piscataway, NJ) (24).

Immunoblotting

Cells were lysed and immunoblotting was then performed according to standard procedures. Briefly, 50 µg protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10–12% gels, transferred to nitrocellulose or polyvinylidene difluoride membranes, and probed with primary and secondary antibodies. The following primary antibodies were used: GCS (H-300) and p53 (DO1) (Santa Cruz Biotechnology), P-gp (EMD Millipore) and p21<sup>WAF1/CIP1</sup>, PUMA, cleaved poly(ADP-ribose) polymerase (PARP), phospho-p53-Ser15, cleaved caspase-3, BAX, and Bcl-2 (Cell Signaling Technology, Danvers, MA). β-actin (Sigma-Aldrich) was used as the loading control. All antibodies were diluted at between 1:250 and 1:5,000.

Preclinical studies

All mouse study procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of our institution. Six-week-old athymic male BALB/c nude mice (nu/nu) were purchased from Central Lab Animal Inc. (Seoul, Republic of Korea). AMC-HN9-cisR cells (5 × 10<sup>6</sup>) with or without control or GCS shRNA transfection were injected subcutaneously into each flank. Treatment began when the cell implants became palpable nodules (day 0). HN9-cisR cells transfected with control or GCS shRNA were exposed to vehicle or cisplatin.
The tumors were extracted after sacrifice, weighted, and compared between groups. In the other in vivo experiments, AMC-HN9-cisR cells were injected subcutaneously into the flank of nude mice. Mice were randomized into four treatment groups: vehicle, cisplatin, D,L-threo-PPMP, and cisplatin plus D,L-threo-PPMP. Mice were treated by intraperitoneal (i.p.) injection of 5 mg/kg cisplatin once per week, by i.p. injection of 5 mg/kg D,L-threo-PPMP once per day, or with a combination of cisplatin and D,L-threo-PPMP according to the same schedules. Tumor volume and body weights were measured every three days. Tumors were measured using a caliper, and volume was calculated as (length × width²)/2. The mice were sacrificed, and tumors were harvested and analyzed by immunoblotting, ceramide production measurements and in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (R&D Systems, Minneapolis, MN). The number of apoptotic bodies was counted blindly in ten randomly selected high-power fields. The statistical significance of differences observed between treatment groups was assessed by using the two-tailed Mann-Whitney U-test or Student’s t-test.

Results

GCS and P-gp expression is associated with cisplatin resistance in HNC cells

The cytotoxic effect of cisplatin was tested in cultured human HNC cells (Fig. 1A). The half maximal inhibitory concentration (IC₅₀) of cisplatin in different HNC cell lines was positively correlated with the GCS mRNA copy number [correlation coefficient (r²) = 0.796, P = 0.017] (Fig. 1B). Cisplatin-resistant HN9 (HN9-cisR) cells showed an 18-fold increase in IC₅₀ compared to parental HN9 cells and a 4-fold increase in GCS mRNA copy number compared to HN9 cells. The expression of GCS and P-gp proteins varied between HNC cell lines, but the two values remained inversely correlated (Fig. 1C). In addition, GCS and MDR1 gene expression significantly increased in HN9-cisR cells compared to parental HN9 cells (P < 0.001, Fig. 1D).

Genetic or pharmacological inhibition of GCS increases cisplatin-induced cell death in HNC cells
When AMC-HN2 cells, which express mutant p53, were transfected with scrambled or GCS siRNA, the protein and mRNA expression of both GCS and P-gp decreased significantly (Fig. 2A–B). PPMP also inhibited GCS mRNA and protein expression and subsequently increased the expression of the pro-apoptotic protein BAX. GCS suppression appeared to be associated with decreased expression of P-gp. Further, GCS inhibition upon shRNA transfection in HN9-cisR cells was confirmed by Western blotting (Fig. 2C). Both the genetic and pharmacological (D,L-threo-PPMP) blockade of GCS induced P-gp inhibition and increased cisplatin cytotoxicity in HN9-cisR cells. GCS inhibition increased ceramide production in both HN9 and HN9-cisR cells. Ceramide production much also increased by both GCS inhibition and cisplatin treatment (Fig. 2D).

**GCS blockade promotes cell cycle arrest and apoptosis in HNC cells**

D,L-threo-PPMP induced phospho-p53 (ser 15) in mutant p53-bearing HN2 cells and increased the p53-responsive proteins PUMA, p21 and cleaved PARP in a concentration- and time-dependent manner (Fig. 3A). These findings were abrogated by the suppression of PUMA transcription (Fig. 3B). PUMA siRNA transfection also inhibited the expression of the pro-apoptotic protein BAX and increased the expression of the anti-apoptotic protein Bel-2 induced by D,L-threo-PPMP. The cell cycle was arrested by D,L-threo-PPMP, but resumed upon transfection of PUMA siRNA (Fig. 3C). Apoptosis was induced by D,L-threo-PPMP or cisplatin and increased by the combination of both drugs; this effect was abrogated by PUMA siRNA (Fig. 3D).

**GCS inhibition sensitized cisplatin-resistant HNC cells to cisplatin in vitro and in vivo**

Cisplatin (10 μM) did not induce significant cytotoxicity or apoptotic protein expression in cisplatin-resistant HNC (HN9-cisR) cells compared to parental cisplatin-sensitive HN9 cells (Fig. 4A). D,L-threo-PPMP induced the expression of apoptotic proteins and increased cisplatin-induced cytotoxicity and apoptotic protein expression in HN9-cisR cells. Further, genetic blockade of GCS also increased cisplatin cytotoxicity and the induction of apoptotic proteins; that effect was weakened by PUMA gene inhibition (Fig. 4B). In combination, cisplatin and GCS inhibition (genetic or pharmacological)
increased the cytotoxicity of cisplatin in HN9-cisR cells by increasing caspase activity to an extent greater than the sum of the effects of either agent alone (Fig. 4C). The mitochondrial membrane potential (ΔΨm) was higher in the cisplatin-resistant HN9-cisR cells than in the cisplatin-sensitive parental HN9 cells (median fluorescent intensity [MFI] of ΔΨm: 1 ± 0.42 vs. 0.70, P < 0.001), and reduced by PPMP or by the combination of cisplatin and PPMP; the effect was abrogated by PUMA gene inhibition (Fig. 4D).

These findings were further examined in vivo. BALB/c athymic nude mice received AMC-HN9-cisR xenograft cells transfected with GCS shRNA or control shRNA. The tumors were treated with i.p. injections of vehicle or cisplatin. GCS genetic blockade increased the cisplatin sensitivity of HN9-cisR cells (Fig. 5A). GCS inhibition increased ceramide production in HN9-cisR cells in vivo, and ceramide production much increased by both GCS inhibition and cisplatin treatment (Fig. 5B). This was further examined by pharmacological inhibition of GCS using D,L-threo-PPMP in HN9-cisR tumors. D,L-threo-PPMP alone decreased the growth rate of HN9-cisR tumors (Fig. 6A). Notably, the combination of cisplatin and D,L-threo-PPMP synergistically suppressed in vivo tumor growth. In situ apoptosis assays showed that TUNEL-positive apoptotic bodies were more frequently seen in tumors treated with D,L-threo-PPMP and cisplatin plus D,L-threo-PPMP than in those treated with vehicle (Fig. 6B). Ceramide production much increased by both PPMP and cisplatin treatment (Fig. 6C). Western blot analyses of tumor tissues showed that apoptotic protein levels were increased to a greater extent in HN9-cisR cells treated with the combination of cisplatin and D,L-threo-PPMP than in cells treated with single agents (Fig. 6D).

Discussion

The present study shows that GCS and P-gp overexpression is associated with an aggressive HNC phenotype showing cisplatin resistance. Increased expression of GCS is correlated with the progression of glioblastoma, melanoma, leukemia, and ovarian, lung, colon, urinary and breast cancers (13, 15–19). The levels of GCS mRNA and protein are elevated four-fold in approximately 80% of metastatic breast cancers, and GCS overexpression is associated with lymph node metastasis.
in bladder cancers (25, 26). In addition, P-gp overexpression is associated with unfavorable prognosis in leukemia, lymphoma and breast, colon, pancreatic and gastric cancers (8–10). The clinical significance of P-gp overexpression has also been studied in HNC. P-gp accumulation is associated with chemotherapy resistance and progression of HNC (27, 28). Unlike that of P-gp, the prognostic value of GCS overexpression in HNC patients has been rarely studied. Our study demonstrates GCS overexpression and its association with chemoresistance in vitro and in vivo. GCS and P-gp are closely linked with each other and predictive of response to chemotherapy in HNC cells, which has been noted in several types of human cancers (7, 12, 13). These findings suggest that GCS and P-gp may be suitable therapeutic targets in HNC.

In our study, blockade of GCS by gene silencing or PPMP increased the cisplatin-induced cell death of HNC cells. GCS overexpression is frequently correlated with P-gp (MDR1) levels in chemotherapy-resistant cells and tumors. Glycosphingolipids, produced by ceramide glycosylation, upregulate MDR1 expression via cSrc signaling and TCF4/β-catenin recruitment (29). Increased GCS and P-gp expression in response to anticancer drugs confers cancer cell resistance by augmenting ceramide glycosylation and MDR1-mediated drug efflux (24, 30). Further, GCS silencing, achieved by transfection of GCS siRNA or mixed-backbone oligonucleotides, delays the clearance of ceramide generated in response to chemotherapeutic drugs and reverses cell resistance by suppression of MDR1 (18, 31). Close correlation between GCS and P-gp expression was also observed in our study. In addition, limiting GCS activity by either GCS siRNA/shRNA transfection or PPMP treatment down-regulates P-gp expression and restores the ceramide-mediated death cascade, thereby heightening chemosensitivity in cisplatin-resistant HNC cells. Although cisplatin is a substance that is untransportable by P-gp, the sensitivity of cisplatin is affected by P-gp expression via inhibition cisplatin-induced apoptosis without any contribution the drug efflux activity of P-gp (32). GCS blockade down-regulates P-gp and restores p53-dependent apoptosis in chemoresistant cancer cells (18, 19). In addition to targeting GCS, PPMP appeared to down-regulate P-gp by acting as an antagonist (18, 33), contributing to resensitize cisplatin-resistant HNC cells to cisplatin.

The present study shows that downregulation of P-gp and ceramide generation by GCS inhibition
drives the ceramide-induced apoptosis pathway and consequently caspase-dependent mitochondrial apoptosis. Cisplatin-resistant HNC cells present elevated expression of the anti-apoptotic protein Bcl-2, commonly linked to cancer cell survival and resistance to chemotherapy (34), but decreased Bcl-2 expression upon blockade of ceramide glycosylation via inhibition of GCS (35). The blockade of GCS elevates the pro-apoptotic protein BAX and reverses the Bcl-2/BAX ratio (35). Further, silencing of GCS by siRNA or PPMP treatment substantially increases the levels of phosphorylated p53 in both cisplatin-sensitive and -resistant HNC cells. This recovered the transactivation of p53-responsive genes, such as p21<sub>WAF1/CIP1</sub>, PUMA and BAX. This is supported by the recent finding that suppression of GCS can restore p53-dependent apoptosis in drug-resistant ovarian cancer cells with p53 mutants (19). Ceramide accumulation promotes p53 activation and inhibits BcL-2 phosphorylation through the activation of protein phosphatase 2A (PP2A), leading to increased p53/Bcl2 binding and apoptotic cell death (36). Our study suggests that the accumulation of p53 triggered by GCS inhibition is followed by a decrease in BcL-2 levels and an increase in BAX levels, leading to apoptotic cell death in cancer cells. This is further supported by the fact that the expression of p53-responsive proteins induced by GCS silencing is abrogated by siPUMA transfection. Therefore, suppression of GCS is associated with restoration of p53 tumor suppression in mutant p53-bearing cancer cells (19, 31). Mutant p53 proteins not only lose the tumor suppressive function of wild-type p53 but also gain new functions promoting tumorigenesis (37). Mutation of <i>TP53</i> is associated with aggressive cancer, poor prognosis and drug resistance in a growing array of malignancies (37, 38). Our and prior studies indicates that GCS inhibition may restore p53 function at the level of posttranscriptional processing (19), supporting a new approach to targeting mutant p53 for cancer therapy.

Our study revealed that the pharmacological GCS inhibitor PPMP or GCS siRNA/shRNA transfection synergized with cisplatin and thereby circumvented resistance to cisplatin in HNC cells. Since cisplatin is a first-line chemotherapeutic agent used in HNC, the combination of cisplatin and PPMP may be effective in the clinical setting at reducing toxicity and overcoming drug resistance. The present study is the first to show that PPMP or GCS genetic silencing recovers the cytotoxic effect of cisplatin in drug-resistant HNC cells <i>in vitro</i> and <i>in vivo</i>. PPMP induced a robust increase in
cisplatin-mediated apoptosis via BAX, PUMA and PARP activation in cisplatin-resistant HNC cells. Genetic or pharmacological inhibition of GCS sensitizes drug-resistant HNC cells to cisplatin, leading to increased cytotoxicity and more effective therapy for aggressive HNC. Taken together, these findings may be of paramount clinical significance: by inducing the death of resistant cells with GCS inhibition, PPMP treatment or siRNA/shRNA transfection could reduce the dose of cisplatin required in the clinical setting and thereby minimize the potential adverse effects of cisplatin chemotherapy.

In conclusion, our data suggest that GCS and P-gp overexpression is significantly associated with cisplatin resistance in several HNC cell lines, suggesting that GCS and P-gp may be suitable therapeutic targets in HNC. Genetic or pharmacological inhibition of GCS increases cisplatin-induced cell death in cisplatin-resistant HNC cells via downregulation of P-gp and activation of pro-apoptotic proteins \textit{in vitro} and \textit{in vivo}. This study supports the need for further investigation of GCS inhibition as a potential cancer therapy, particularly in HNC with aggressive phenotypes.
References


**Figure Legends**

**Figure 1.** GCS and P-gp expression is associated with cisplatin resistance. (A) Cell viability was assessed by trypan blue exclusion after exposure to cisplatin for 72 h. (B) GCS mRNA was measured by quantitative real-time PCR and cisplatin cytotoxicity was assessed by MTT at 72 h. The correlation between GCS mRNA copy numbers and the half maximal inhibitory concentration (IC₅₀) was examined by Pearson’s correlation coefficient ($r^2$). $P = 0.017$. (C) Western blot analysis showing different levels of GCS, P-gp and p53 proteins in untreated AMC-HNC cells and cisplatin-resistant HN9 (HN9-cisR) cells. β-actin was assessed as a loading control. (D) Change in gene expression of GCS and MDR1 between HN9 and HN9-cisR. The error bars represent s.d. from three independent experiments, each performed with triplicate samples. * denotes $P < 0.001$ relative to HN9 cells.

**Figure 2.** Genetic or pharmacological inhibition of GCS increases cisplatin-induced cell death in HNC cells. (A, B) GCS inhibition with siRNA or D,L-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP). AMC-HN2 cells with mutant p53 were transfected with scrambled (scr) or GCS siRNA for 72 h, or exposed to 10 μM PPMP. The graph indicates relative expression of GCS or MDR1/β-actin gene in HN2 cells with relative resistance to cisplatin. * denotes $P < 0.05$ versus control. (C) Increased cisplatin sensitivity by GCS gene knockdown or PPMP treatment. GCS was stably transfected in cisplatin-resistant AMC-HN9 cells using a lentiviral vector. The knockdown was confirmed by Western blotting using a GCS antibody. Cell viability was determined by MTT assay in AMC-HN9-cisR and sublines treated with 5 μM PPMP, 10 μM cisplatin (cis), or the combination of both drugs for 72 h. (D) Change in ceramide production by GCS inhibition. Total ceramide levels were measured by mass spectroscopy in AMC-HN9-cisR cells and parental HN9 cells treated for 72 h with 10 μM PPMP, 10 μM cisplatin, or their combination. The error bars represent s.d. from three independent experiments, each performed with triplicate samples. * and ** denote $P < 0.05$ and $P < 0.001$ versus untreated HN9 control cells, respectively.

**Figure 3.** GCS inhibition promotes cell cycle arrest and apoptosis in HNC cells bearing mutant p53. AMC-HN2 were exposed to D,L-threo-PPMP. PUMA siRNA (100 nM) and scrambled control (scr,
100 nM) were introduced to cells for 72 h. (A, B) Western blotting of p53 and p53-responsive proteins after PPMP treatment. (C) Cell cycle analysis after exposure to PPMP and PUMA siRNA. AMC-HN2 cells exposed to PPMP for 72 h were stained with propidium iodide and subjected to flow cytometry analysis. (D) Apoptosis assays after exposure to PPMP, cisplatin and PUMA siRNA. Cells were exposed to PPMP and/or cisplatin for 72 h and the annexin V-positive apoptotic fractions were measured. * and ** denote $P < 0.01$ compared to untreated control and scrambled (scr) siRNA, respectively. *** denotes $P < 0.001$ compared to PPMP or cisplatin treatment alone.

**Figure 4.** GCS inhibition sensitizes cisplatin-resistant HNC cells to cisplatin. (A) Western blotting showing increased cisplatin (CDDP) sensitivity by PPMP. Cisplatin-resistant AMC-HN9 (HN9-cisR) and parental HN9 cells were treated with PPMP, cisplatin, or both for 48 h. (B) Western blotting after cisplatin treatment in HNC cells with GCS genetic inhibition. HN9-cisR cells with stable transfection of GCS or control shRNA lentiviral vector were exposed to PUMA siRNA and/or 10 μM cisplatin or vehicle control. (C) Elevation of caspase after exposure to PPMP, cisplatin and PUMA siRNA. HN9-cisR cells were exposed to PPMP, cisplatin or the combination of both drugs, and caspase activity was measured. (D) Changes in mitochondrial membrane potential ($\Delta \Psi_m$) of HN9-cisR cells after a 36 h-exposure to PPMP, cisplatin or the combination of both drugs. The $\Delta \Psi_m$ was measured using tetramethylrhodamine ethyl ester and analyzed by flow cytometry. The median fluorescent intensity (MFI) of each treatment group was normalized to the control group. The error bars represent s.d. from three independent experiments, each performed with triplicate samples. * and ** denote $P < 0.01$ compared to vehicle control and scrambled (scr) siRNA treatment, respectively. *** denotes $P < 0.001$ compared to PPMP or cisplatin treatment alone.

**Figure 5.** GCS genetic inhibition sensitizes cisplatin-resistant HNC cells to cisplatin *in vivo.* (A) Increased cisplatin sensitivity by GCS genetic inhibition in a tumor xenograft mouse model. Balb/c nude mice were implanted with $5 \times 10^6$ AMC-HN9 cells or HN9-cisR cells with control or GCS shRNA in flanks. Treatments with vehicle or cisplatin began once the implanted tumor cells formed palpable nodules. Each group included eight mice. The tumors were extracted after sacrifice and
weighted. The error bars represent standard errors (right panel). Two-tailed Student’s t-test, * and ** denote $P < 0.01$ versus HN9 control mice and HN9-cisR-shGCS mice treated with vehicle, respectively. Left panel shows representative photographs of mice and tumors with implantation of stably control or GCS shRNA-transfected HN9-cisR cells and cisplatin treatment. (B) Change in ceramide production to cisplatin treatment in vivo. Total ceramide levels were measured by mass spectroscopy in tumors of each group. * denotes $P < 0.01$ versus shRNA control.

**Figure 6.** GCS pharmacological inhibition sensitizes cisplatin-resistant HNC cells to cisplatin in vivo. (A) Increased cisplatin sensitivity by GCS pharmacological inhibition in vivo. Nude mice were injected with $5 \times 10^6$ AMC-HN9-cisR cells in the flank. Treatments with vehicle (control), cisplatin, PPMP or the combination of both drugs. Each group included eight mice. (B) Quantification from in situ TUNEL assays in tumor sections from each group. TUNEL-positive apoptotic bodies were counted blindly in ten randomly selected high-powered fields. The error bars represent standard errors. Two-tailed Student’s t-test, * denotes $P < 0.01$. (C) Change in ceramide production to cisplatin and/or PPMP in vivo. Total ceramide levels were measured by mass spectroscopy in tumors of each group. * denotes $P < 0.001$. (D) Western blot analysis of cleaved PARP, PUMA and GCS proteins obtained from tumors treated with vehicle control, cisplatin, PPMP or the combination of both drugs. β-actin served as internal loading control.
Figure 3

**A**

<table>
<thead>
<tr>
<th>PPMP (μM)</th>
<th>0</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>pp53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PUMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cPARP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PPMP (10 μM)</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>pp53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PUMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cPARP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>PPMP (μM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>10</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>siPUMA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>scr</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(kDa)</th>
<th>53</th>
<th>53</th>
<th>23</th>
<th>21</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cPARP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cCasp3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C**

% Cells

- PPMP (μM) – 5 10 10 10
- siPUMA – – – + scr

- G2/M
- S
- G1/G0
- SubG1
- *
- **

**D**

% Apoptotic cells

- PPMP (μM) – 5 10 10 5 10 5 5
- Cisplatin (μM) – – 5 5 – –
- siPUMA – – – + + scr

- *
- **
- ***
- ****
Figure 5

**A**

Tumor weight (Relative to control)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle</th>
<th>Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN9-cisR (ctr shRNA)</td>
<td>1.0 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>HN9-cisR (GCS shRNA)</td>
<td>1.0 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

**B**

Ceramide production (% of control)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle</th>
<th>Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN9-cisR (ctr shRNA)</td>
<td>100 ± 10</td>
<td>150 ± 15</td>
</tr>
<tr>
<td>HN9-cisR (GCS shRNA)</td>
<td>200 ± 20</td>
<td>500 ± 50</td>
</tr>
</tbody>
</table>

* indicates a statistically significant difference.
Figure 6

A

![Graph showing the effect of different treatments on tumor volume](image)

- Control
- Cisplatin
- PPMP
- Cisplatin + PPMP

Tumor volume (cm³) vs. Treatment time (days)

B

![Bar graph showing apoptotic bodies](image)

- Control
- Cisplatin
- PPMP
- Cisplatin + PPMP

Apoptotic bodies (per high-power field)

C

![Bar graph showing ceramide production](image)

- Control
- Cisplatin
- PPMP
- Cisplatin + PPMP

Ceramide production (% of control)

D

![Western blot analysis](image)

- cPARP
- PUMA
- GCS
- β-actin

Control
Cisplatin
PPMP
PPMP + Cisplatin

Downloaded from mct.aacrjournals.org on June 21, 2017. © 2015 American Association for Cancer Research.
Inhibition of Glucosylceramide Synthase Sensitizes Head and Neck Cancer to Cisplatin

Jong-Lyel Roh, Eun Hye Kim, Jin Young Park, et al.

*Mol Cancer Ther* Published OnlineFirst June 10, 2015.

Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-15-0171

Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2015/06/10/1535-7163.MCT-15-0171.DC1

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.