Cancer Therapeutics Insights

Tunicamycin Potentiates Cisplatin Anticancer Efficacy through the DPAGT1/Akt/ABCG2 Pathway in Mouse Xenograft Models of Human Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma is highly chemoresistant, and ATP-binding cassette subfamily G member 2 (ABCG2) is thought to play a critical role in this drug resistance. The present study aims to develop effective therapeutic strategies to decrease ABCG2 expression level and to surmount drug resistance in hepatocellular carcinoma chemotherapy. First, we verified a positive correlation between the ABCG2 protein level and the drug resistance of hepatocellular carcinoma cell lines. ABCG2 was preferentially expressed in highly chemoresistant hepatocellular carcinoma cancer stem cells (CSC) enriched with CD133. In addition, ABCG2 was N-linked glycosylated in hepatocellular carcinoma cells, and this modification was involved in sustaining its protein stability. The N-linked glycosylation (NLG) inhibitor tunicamycin dramatically reduced ABCG2 expression, altered its subcellular localization, and reversed its drug efflux effect in multiple hepatocellular carcinoma cell lines. Furthermore, tunicamycin reduced the expression levels of several CSC markers and suppressed the tumorigenicity of CD133+ CSCs. Tunicamycin combined with cisplatin (CDDP) inhibited proliferating cell nuclear antigen (PCNA) expression and increased the cleavage of PARP; this effect was partially rescued by the overexpression of ABCG2 or Akt-myr. The combination therapy more effectively suppressed tumor growth in xenograft mice than did single-agent therapy with either drug. Finally, the CDDP treatment combined with UDP-GlcNAc-dolichol-phosphate N-acetylglucosamine-1 phosphate transferase (DPAGT1) knockdown recapitulated the effect observed when CDDP was used in combination with tunicamycin. In summary, our results suggest that tunicamycin may reverse the drug resistance and improve the efficacy of combination treatments for hepatocellular carcinomas by targeting the DPAGT1/Akt/ABCG2 pathway.

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Introduction

Resistance to chemotherapy is common in malignant tumors and ultimately leads to unsuccessful treatment (1). Hepatocellular carcinoma is the fifth most common cancer worldwide. Hepatocellular carcinoma is highly chemoresistant, leaving patients with this disease with no effective therapeutic options. Numerous lines of evidence imply that a small cellular subpopulation with stem cell–like characteristics, commonly referred to as cancer stem cells (CSC), is critical for tumorigenicity and tumor maintenance (2). CSCs appear to be protected from routine chemotherapeutic drugs through various mechanisms, such as high expression levels of ATP-binding cassette (ABC) drug transporters (3). Drug-resistant CSCs have been shown to survive treatment with several widely used chemotherapeutic agents (4, 5).

Our previous studies showed that CD133 is an effective marker for identifying hepatocellular carcinoma CSCs (6). This cell population is further characterized by the expression of CD133 and CD44 (7). Importantly, a significant difference in ABCG2 expression was observed between CD133+/CD44+ cells and a corresponding double negative subpopulation. ABCG2+ tumor cell populations are often enriched in cells with cancer stem–like phenotypes, and ABCG2 activity reportedly underlies the drug resistance of cancer cells (8, 9). Therefore, the elucidation of the mechanisms through which ABC drug transporters, especially ABCG2, are regulated in hepatocellular carcinomas may facilitate the development of targeted agents to restore or increase chemosensitivity.

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The detailed molecular mechanisms by which ABCG2 expression is controlled in tumor cells and CSCs are currently unclear (10). Early studies suggested that stemness-related factors are directly linked to ABCG2 expression and drug resistance (11). Additional studies showed that growth signaling pathways also affect ABCG2 expression. The inhibition of PI3K/Akt signaling in leukemia cells causes a downregulation of total ABCG2 expression (12). In hepatocellular carcinomas, a triterpene in fruits and vegetables has been found to suppress the self-renewal ability of CSCs and to enhance the effect of chemotherapeutic agents through the PTEN/Akt/ABCG2 pathway (13).

In addition to the above mechanisms, ABCG2 post-translational modification is now drawing increasing attention. In glioma CSCs, the PI3K/Akt pathway has been shown to modulate ABCG2 activity by inhibiting its transport to the plasma membrane (14). We also found that Akt signaling can regulate efflux activity in SP cells by altering the subcellular localization of the ABCG2 transporter (15). The inhibition of Akt signaling can attenuate the efflux of doxorubicin from MHCC-97L hepatocellular carcinoma cells and increase drug efficacy.

N-linked glycosylation (NLG) is believed to play a central role in sustaining protein stability, trafficking, and function (16). Disruption of NLG accelerates the proteosomal degradation of ABCG2 (17). Tunicamycin potently inhibits NLG by competitively inhibiting DPAGT1 activity and can target several types of tumors by reducing angiogenesis (18), inhibiting colony formation (19), and enhancing TRAIL-induced apoptosis (20). A combination of tunicamycin and anticancer drugs synergistically enhanced the toxicity of each in a multidrug-resistant (MDR) human ovarian cancer cell line (21).

Swainsonine is a natural α-mannosidase inhibitor that inhibits Golgi α-mannosidase II activity in the N-glycan biosynthesis pathway (22) and potentially increases the antitumor efficacy of CDDP in Ehrlich ascites carcinoma cells (23). Previous studies have also shown that 2-deoxy-α-glucose (2-DG) interferes with the process of NLG in viral coat glycoprotein synthesis, and this interference can be reversed by the addition of exogenous mannose (24). 2-DG increased the efficacy of adriamycin and paclitaxel in human osteosarcoma and non–small cell lung cancers in vivo (25). The significant role of the glycosylation modification in ABCG2 regulation suggests the utility of glycosylation inhibitors in combination with other conventional anticancer agents to overcome MDR in hepatocellular carcinomas.

In this study, we found that several glycosylation inhibitors and Akt were able to influence the protein expression or activity level of ABCG2. The glycosylation inhibitor tunicamycin reduced the expression of CSC markers and suppressed the tumorigenicity of CD133+ CSCs in vivo. Through the Akt/ABCG2 pathway, tunicamycin potentiated the cytotoxicity of routinely used chemotherapy drugs. DPAGT1 protein expression was significantly higher in hepatocellular carcinomas than in corresponding noncancerous surrounding tissues. Moreover, the CDDP sensitization effect was reproduced by a DPAGT1 knockdown instead of tumicamycin treatment. The development of additional drugs that inhibit glycosylation to decrease ABCG2 expression will potentially improve the efficacy of combination treatments for hepatocellular carcinomas in the future.

Materials and Methods

Cell culture and reagents

The HuH7 cell line was obtained from the Riken Cell Bank; PLC/PRF/5 and HEK 293T were purchased from the American Type Culture Collection; SMMC-7721 was obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology, China Academy of Sciences (Shanghai, China); and human HCC-LY5 was a primary hepatocellular carcinoma cell line established in our laboratory. (This cell line was routinely screened for Mycoplasma species with Mycoplasma Stain Kit purchased from Beyotime; and its purity was confirmed with STR genomic analysis.) All above cell lines used were always less than 40 passages from the stocks tested for purity. MHCC-97L, MHCC-97H, and MHCC-LM3 were kindly provided by the Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China); and human HCC-LY5 was a primary hepatocellular carcinoma cell line (except for HCC-LY5, which was cultured in Williams’ Medium E) used in this study were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich) containing 10% heat-inactivated FBS (HyClone) and incubated at 37°C in a humidified atmosphere with 5% CO2. The major drugs and reagents used in this study are listed in Supplementary Table S1. The chemicals and other reagents were purchased from Sigma-Aldrich unless otherwise specified.

Cell sorting by fluorescence-activated cell sorting or magnetic-activated cell sorting

PLC/PRF/5 cells were labeled directly with a phycoerythrin (PE)-conjugated anti-human CD133/1 antibody (AC133, Miltenyi Biotech) according to the manufacturer’s instructions and sorted by fluorescence-activated cell sorting (FACS) to obtain CD133+ and CD133− cell subpopulations. SMMC-7721, MHCC-97L, and HCC-LY5 cells, which include less than 1% CD133+ cells, were magnetically isolated to produce populations of CD133+ and CD133− cells using the EasySep PE Selection Kit (Stem Cell Technologies) according to the manufacturer’s instructions.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Corporation) and reverse transcribed using the
PrimeScript RT Reagent Kit (Perfect Real Time; TaKaRa Biotechnology). Real-time PCR was subsequently carried out using SYBR Premix Ex Taq (TaKaRa Biotechnology) as described previously (26). The primers used to amplify the target genes are listed in Supplementary Table S2, and the expression levels were normalized against those of the internal reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blotting
Immunoreactive blots were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce). Information for both the primary and horseradish peroxidase (HRP)-conjugated secondary antibodies used is given in Supplementary Table S3.

Vector constructs, lentivirus production, and cell transfection
The ABCG2 cDNA clone (SC-117127) was purchased from OriGene. The Asn596-encoding codon AAT was converted to CAA to generate the N596Q variant of ABCG2 using the megaprimer PCR method as described previously (27). The N596Q mutant primer is listed in Supplementary Table S2. Akt-myr, the constitutively active myristoylated form of Akt, was kindly provided by Prof. Yongzhong Liu. The above cDNA sequences were cloned into the lentiviral expression vector pWPXL (Addgene) by replacement of the GFP fragment. Virus packaging and cell transfection were conducted as previously described (26).

RNA interference–based gene knockdown experiment
The siRNA oligos targeting DPAGT1 and a negative control (Cat. No. B01001) were synthesized and annealed by GenePharma. Two fragments were designed to target the corresponding gene transcripts, and the silencing effects of the sequences were validated by Western blotting. The effective siRNA sequences are shown in Supplementary Table S2. RNA interference was conducted as described previously (26).

Tumor xenograft assay
Six- to 8-week-old BALB/c (nu/nu) mice were maintained under standard conditions according to protocols developed by the Shanghai Medical Experimental Animal Care Commission. At the end of the experimental period, the mice were euthanized, and the excised tumors were weighed and fixed in formalin. Frozen tumor samples were analyzed by Western blotting and real-time PCR. Details can be found in the Supplementary Data.

Statistical analysis
The experimental data are presented as the mean ± SEM and were analyzed using the two-tailed Student t test. P < 0.05 was considered statistically significant.

Results
Glycosylation inhibitors affected ABCG2 protein levels
The MTT analysis revealed that the MHCC-97L and MHCC-97H cells, which exhibit high ABCG2 protein levels, were more resistant to common chemotherapeutic drugs than Huh7 and SMMC-7721 cells, which express low ABCG2 protein levels (Supplementary Fig. S1A–S1D). Thus, we used real-time PCR to examine the mRNA levels of several ABC drug transporters in 4 CD133-selected hepatocellular carcinoma cell populations. Only ABCG2 was universally elevated in CD133+ CSCs compared with CD133− subpopulations in these 4 cell lines (Supplementary Fig. S1E). Both CD133+ MHCC-97L and PLC/PRF/5 cells were more resistant to doxorubicin than their corresponding CD133− subpopulations as shown in Supplementary Fig. S1F. These data further verified that the ABCG2 expression level was well correlated with the drug resistance of hepatocellular carcinoma.

ABCG2 is an N-linked glycosylated protein localized to the apical domain of plasma membranes (28). In particular, the NLG site corresponding to Asn596 in human ABCG2 is highly conserved. Following digestion with the glycosidase PNGase F, Western blotting using an anti-ABCG2 antibody revealed the appearance of a new band of approximately 60 kDa, showing that ABCG2 is glycosylated in hepatocellular carcinoma cells (Fig. 1A and B). To examine the potential role of NLG in stabilizing and degrading the ABCG2 protein, we established the N596Q variant of human ABCG2, in which Asn596, the amino acid for NLG, was substituted by Gln596. Both wild-type (WT) ABCG2 and the N596Q variant were stably transfected into Huh7 cells. A Western blot analysis revealed that the protein expression level of the human ABCG2 N596Q variant in Huh7 cells was much lower than that of WT ABCG2 (Fig. 1C), whereas the mRNA expression levels of the WT and N596Q variant ABCG2 were nearly identical (Fig. 1D). Because NLG is involved in maintaining ABCG2 protein stability, several NLG inhibitors that possess antitumor activity were selected to treat the hepatocellular carcinoma cell lines. We found that tunicamycin dramatically reduced the ABCG2 protein level in the MHCC-97L cells, and this result was further confirmed in the MHCC-97H cell line (Fig. 1E). In addition, tunicamycin decreased ABCG2 expression in another 2 hepatocellular carcinoma cell lines (Supplementary Fig. S2A and S2B). These findings prompted us to choose tunicamycin to conduct further experiments because it may represent a valuable drug for reversing MDR in hepatocellular carcinomas.

Tunicamycin altered ABCG2 subcellular localization and reduced its activity
We next asked whether tunicamycin could alter the cellular localization or reverse the drug efflux activity of ABCG2. Akt signaling can reportedly control ABCG2
subcellular localization (14, 15). Tunicamycin inhibited Akt phosphorylation in several hepatocellular carcinoma cell lines (Fig. 2A). In MHCC-97L cells, Akt phosphorylation was inhibited in a time- and dose-dependent manner (Fig. 2B). ABCG2 was primarily localized at the plasma membrane in the control group, whereas tunicamycin caused abnormal intracellular aggregation of ABCG2 (Fig. 2C). Similar results were obtained in the MHCC-97H cell line (data not shown). Moreover, the SP rate, which is related to ABCG2 efflux activity, decreased after the tunicamycin treatment in MHCC-97L and MHCC-97H cells (Supplementary Fig. S3A–S3C). This result suggests that tunicamycin potentially reverses ABCG2 activity by decreasing its expression and altering its subcellular localization.

The antitumor effect of tunicamycin on hepatocellular carcinoma CSCs

In hepatocellular carcinomas, the PTEN/Akt/ABCG2 pathway plays a crucial role in the self-renewal and chemoresistance of CD133+ CSCs (13). We therefore sought to determine whether tunicamycin could effectively target hepatocellular carcinoma CSCs, which are thought to be highly drug-resistant. As shown in Fig. 3A, the tunicamycin treatment dramatically reduced the expression of well-known CSC markers, including CD133, CD44, CD13, EpCAM, and CK-19. The expression of CD44, ABCG2, and p-Akt simultaneously decreased in magnetic-activated cell-sorted (MACS) CD133+ MHCC-97L cells in response to tunicamycin treatment (Fig. 3B). This result suggests that tunicamycin may be able to eliminate the CD133+ hepatocellular carcinoma CSC subpopulation to some degree. The effect of tunicamycin on the tumorigenicity of CD133+ cells was assessed using a nude mouse xenograft model. To ensure the generation of CD133+/− cells by the xenograft tumors, a much greater quantity of CD133+/− cells was used relative to CD133+ cells. After 4 weeks of treatment with 0.5 mg/kg tunicamycin, CD133+ MHCC-97L cells formed fewer (3 of 6 mice) and smaller tumors than did the control group (Fig. 3C). Total protein was then extracted from frozen tumor tissues taken from the CD133+/− xenografts (Supplementary Fig. S4). These
results suggested that tunicamycin potentially targets hepatocellular carcinoma CSCs by the Akt/ABCG2 pathway.

**Tunicamycin can promote the antitumor effect of CDDP both in vitro and in vivo**

Because tunicamycin was able to reduce ABCG2 expression and eliminate hepatocellular carcinoma CSCs, it may enhance the sensitivity to routine chemotherapy drugs and reduce drug resistance in hepatocellular carcinomas. To support this hypothesis, PLC/PRF/5 cells were first treated with tunicamycin alone or in combination with common anticancer drugs. The expression levels of CD133 and ABCG2 were both notably reduced following the tunicamycin treatment and in the combined therapy groups (Supplementary Fig. S5). Interestingly, these 2 markers were upregulated in the single common anticancer drug group. Tunicamycin in combination with 5-fluorouracil (5-FU) or CDDP decreased the proliferating cell nuclear antigen (PCNA) protein levels but enhanced the cleavage of PARP, which are indicators of cellular proliferation and apoptosis, respectively. However, tunicamycin in combination with doxorubicin or vincristine did not produce similar effects (Supplementary Fig. S5).

In 2 additional hepatocellular carcinoma cell lines, the combination of tunicamycin with 5-FU or CDDP reduced ABCG2 protein levels and inhibited Akt phosphorylation (Fig. 4A and B). In addition, the combination treatment reduced the expression of the CSC markers CD44 and CD133. It also dramatically decreased PCNA expression but enhanced the cleavage of PARP compared with the single-agent 5-FU or CDDP treatment group (Fig. 4A and B). Several drugs have been reported to influence ABCG2 activity or expression in vitro; however, few of these drugs have been verified in animal experiments (29). Therefore, we investigated the therapeutic effect of tunicamycin in 2 hepatocellular carcinoma nude mouse models using MHCC-97L and Huh7 cells. Tunicamycin exerted a synergistic effect with a low dose of CDDP that resulted in dramatic tumor shrinkage in both animal models (Fig. 4C).
and D; Supplementary Fig. S6A and S6B). We also examined the expression of ABCG2 and p-Akt in both MHCC-97L and Huh7 cells xenografts. The results showed that tunicamycin in combination with CDDP treatment dramatically declined the protein level of ABCG2 and p-Akt. Furthermore, the combination treatment reduced PCNA expression and noticeably increased cleaved PARP protein level compared with either single drug group (Supplementary Fig. S6C and S6E). These findings were consistent with in vitro studies. Enrichment of the CSC population, based on increased expression of stemness-related genes, was observed following treatment with CDDP. Conversely, tunicamycin combined with CDDP treatment significantly reduced mRNA expression levels of the stemness-related genes OCT4, Nanog, and Notch1 compared with the CDDP alone group, indicating that tunicamycin can reverse the resistance of CSCs to CDDP (Supplementary Fig. S6D and S6F).

**Tunicamycin sensitized cells to CDDP partially through the DPAGT1/AKT/ABCG2 pathway**

We sought to determine whether tunicamycin enhanced the antitumor effect of CDDP through an Akt/ABCG2-dependent pathway by conducting 2 rescue experiments. As expected, a combined tunicamycin and CDDP treatment reduced PCNA expression and noticeably increased cleaved PARP protein levels, whereas the overexpression of either ABCG2 or a constitutively active myristoylated form of Akt (Akt-myr) attenuated the relative effect of the combination therapy (Fig. 5A–D). These data were consistent with previous reports indicating that the Akt/ABCG2 pathway contributes to chemotherapeutic resistance (13, 30). As a tightly binding competitive inhibitor, tunicamycin inhibits NLG by inhibiting the DPAGT1-catalyzed transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P. This result urged us to examine the role of DPAGT1 in hepatocellular carcinoma progression. Because no available antibody is suitable for immunohistochemical analysis, we detected the protein expression of DPAGT1 in hepatocellular carcinomas and adjacent noncancerous tissue samples by Western blotting (Fig. 6A). Of the 30 pairs of hepatocellular carcinoma samples detected, DPAGT1 expression was found to be upregulated in the tumor tissues (53.3%) in 16 cases, indicating that DPAGT1 may play a role in hepatocellular carcinoma biology. We also found that DPAGT1 was upregulated in the hepatocellular carcinoma cell lines compared with an immortalized normal liver cell line
L02 (Fig. 6B). To better understand the significance of DPAGT1 expression in chemoresistance, Huh7 and MHCC-97L cells were transiently transfected with DPAGT1 siRNAs. These cells showed efficient DPAGT1 silencing and reduced ABCG2 and p-Akt protein levels as judged by Western blotting (Fig. 6C). Concomitant DPAGT1 knockdown and CDDP treatment dramatically inhibited PCNA and induced cleaved PARP expression. In contrast, individual DPAGT1 knockdown or CDDP treatment produced much weaker effects (Fig. 6D). These results indicate that DPAGT1 may be a potential target to reverse chemoresistance in hepatocellular carcinoma therapy.

Discussion

Most cases of hepatocellular carcinomas are detected at advanced stages and are therefore inoperable. The overall prognosis of this disease is very poor due to the inherently chemoresistant character of hepatocellular carcinomas and the toxicity of therapeutic drugs (31). Therefore, there is an urgent need to elucidate the detailed molecular events governing MDR in hepatocellular carcinomas, which may aid in the development of effective agents that can enhance chemosensitivity.

Compared with CD133− cells, CD133+ hepatocellular carcinoma CSCs are resistant to chemotherapy in vivo as detected in real-time by color-coded imaging (32). High expression levels of ABC transporter family members are critical for the resistance of CSCs to widely used chemotherapeutic agents (3). We confirmed that CD133+ hepatocellular carcinoma CSCs displayed higher ABCG2 expression levels and were much more drug-resistant than CD133− cells. Furthermore, single-agent treatment with tunicamycin or CDDP dramatically suppressed tumor growth in the Huh7 tumor xenograft model but not in the MHCC-97L cell xenograft model. This result may be attributed to a lower level of ABCG2 expression in Huh7 cells. Zen and colleagues reported that cancer cells with ABCG2 expression may play a central role in
hepatocarcinogenesis and the maintenance of the cancer cell hierarchy of human hepatocellular carcinomas (8). New therapeutic strategies targeting ABCG2 expression or ABCG2\(^+\) CSCs may effectively eliminate CSCs and overcome current chemotherapeutic resistance.

We discovered that tunicamycin dramatically reduced ABCG2 expression in hepatocellular carcinomas. However, the inhibitory effect of tunicamycin on ABCG2 expression was not restricted to hepatocellular carcinomas. Tunicamycin also clearly inhibited ABCG2 expression in A549 lung cancer and MCF-7 breast cancer cell lines (Supplementary Fig. S2C). Therefore, the present study provided the first direct evidence that tunicamycin can reduce ABCG2 expression in several tumor cell types.

In addition to changes at the protein level, the subcellular localization of ABCG2 was also altered by tunicamycin. A previous report showed that NLG is not essential for routing ABCG2 to the plasma membrane because the N596A mutation had little effect on ABCG2 subcellular localization (28). However, the study used site-specific mutations, and these genetic modifications differ significantly from tunicamycin treatment, which was able to inhibit Akt phosphorylation. The EGF receptor (EGFR) is a glycoprotein, and the inhibition of EGFR glycosylation by tunicamycin alters conformation and phosphorylation of EGFR (33). Consistent with the aforementioned findings, we found that digestion with PNGase F or treatment with tunicamycin generated a nonglycosylated form of EGFR in MHCC-97L cells (Supplementary Fig. S7A). Concomitantly, the phosphorylation levels of EGFR’s downstream effectors (including Akt, ERK1/2, and STAT3) were impaired in response to tunicamycin (Fig. 2B; Supplementary Fig. S7B). We therefore speculated that tunicamycin may inhibit Akt phosphorylation by altering EGFR glycosylation.

Common chemotherapies have been shown to enrich the CSC subpopulations in some studies (4, 34). CD133\(^+\) hepatocellular carcinoma CSCs may confer chemoresistance by preferentially expressing the Akt/Bcl-2 or PTEN/Akt/ABCG2 pathway (13, 30). We observed that tunicamycin was able to suppress the tumorigenicity of CD133\(^+\) hepatocellular carcinoma CSCs and enhance the therapeutic effect of CDDP through the Akt/ABCG2 pathway. More importantly, tunicamycin reversed the CDDP-induced high expression of stemness-related genes. This result indicated that tunicamycin could reverse the resistance of hepatocellular carcinoma CSCs to CDDP chemotherapy. A recent report stated that treatment of ovarian cancer cells with CDDP results in Akt activation \textit{in vitro} and \textit{in vivo}. The therapeutic utility of Akt inhibition in combination with CDDP enhances the induction of apoptosis and improves tumor control compared

Figure 5. The sensitization effect of tunicamycin could be rescued by forced expression of ABCG2 or Akt-myr. A, overexpression of ABCG2 in Huh7 and SMMC-7721 cells was validated by Western blotting. B, Western blotting analysis of ABCG2, PCNA, and cleaved PARP in Huh7 cells stably transfected with empty vector (pWPXL) or ABCG2 and exposed for 48 hours to tunicamycin (1.0 \(\mu\)g/mL) and CDDP (3.5 \(\mu\)g/mL), alone or in combination. C, overexpression of Akt-myr in Huh7 and SMMC-7721 cells was validated by Western blotting. D, Western blotting analysis of p-Akt, PCNA, and cleaved PARP in Huh7 cells stably transfected with the empty vector (pWPXL) or Akt-myr and exposed for 48 hours to tunicamycin (1.0 \(\mu\)g/mL) and CDDP (3.5 \(\mu\)g/mL), alone or in combination.
with either agent alone (35). Other research has shown that low molecular weight heparin ablates lung cancer CDDP resistance by inducing proteasome-mediated ABCG2 protein degradation (36). Tunicamycin combined with CDDP clearly inhibited proliferation and enhanced apoptosis in human nasopharyngeal carcinoma cells (37). Our findings were consistent with these reports.

DPAGT1 is a key regulator of the metabolic pathway of NLG, and its activity can be inhibited by tunicamycin, a potent competitive NLG inhibitor. The function of DPAGT1 in tumor biology remains to be elucidated. DPAGT1 overexpression leads to aberrant NLG of E-cadherin and cellular discohesion in oral cancer (38). The partial inhibition of DPAGT1 reduced canonical Wnt signaling, indicating that DPAGT1 and canonical Wnt signaling function in a positive feedback loop (39). In many other types of cancer, the aberrant activation of the canonical Wnt/β-catenin signaling pathway is an important contributor to tumorigenicity in hepatocellular carcinomas (40). It is still unclear whether DPAGT1 influences the activation of Wnt signaling in hepatocellular carcinomas. The detailed role of DPAGT1 in hepatocellular carcinoma tumorigenesis and drug resistance should be further studied. In this study, we revealed that DPAGT1 is upregulated in hepatocellular carcinoma cell lines and tissues. The knockdown of DPAGT1 reduced ABCG2 expression, inhibited Akt phosphorylation, and enhanced the antitumor effect of CDDP. On the basis of the above results, we hypothesized that tunicamycin can sensitize resistant cells to CDDP, at least partially, through the DPAGT1/Akt/ABCG2 pathway.

Sorafenib is a multikinase inhibitor that has shown efficacy against a wide variety of tumors in preclinical models, including hepatocellular carcinomas (41). Clinical trials have shown the survival benefits of sorafenib treatment, making it the new standard therapy for patients with advanced hepatocellular carcinomas (42). We observed antitumor effects with tunicamycin in combination with sorafenib. However, tunicamycin did not enhance the cellular toxicity of sorafenib, as shown in Supplementary Fig. S8A and S8B. On the basis of a previous report, sorafenib may promote invasiveness and the metastatic potential of orthotopic tumors from hepatocellular carcinoma cells in animal experiments via the JAK/STAT3 signaling pathway (43). Both tunicamycin and sorafenib abolished Akt and Erk1/2 phosphorylation; however, tunicamycin showed a lesser inhibitory effect on STAT3 phosphorylation compared with sorafenib.

Figure 6. DPAGT1 knockdown enhanced the antitumor effect of CDDP in hepatocellular carcinomas. A, Western blotting assays and quantification of DPAGT1 expression in 30 paired hepatocellular carcinoma tissue samples. The representative immunoblots of DPAGT1 in 10 paired tissue samples are shown. B, protein levels of DPAGT1 in hepatocellular carcinoma cell lines and the normal liver cell line L02 were detected using Western blotting. C, DPAGT1 knockdown reduced the expression of ABCG2 and p-Akt as confirmed by Western blotting. D, knockdown of DPAGT1 in combination with CDDP (5.5 μg/mL) reduced PCNA expression and induced the protein level of cleaved PARP compared with either single treatment.
In conclusion, we found that the NLG inhibitor tunicamycin was able to reverse ABCG2 drug efflux activity by decreasing its protein level and altering its subcellular localization. Tunicamycin reduced the expression of CSC markers and suppressed the tumorigenicity of CD133+ hepatocellular carcinoma CSCs. Tunicamycin sensitized hepatocellular carcinoma to common chemotherapeutic drugs and impaired tumor growth at least partially through the DPAGT1/Akt/ABCG2 pathway. In the future, tunicamycin can potentially be used to attenuate the treatment of hepatocellular carcinomas when combined with common chemotherapeutic drugs. Additional drugs disrupting the NLG protein modification remain to be discovered or designed to reduce the drug resistance of hepatocellular carcinomas.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Study supervision: J. Li

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References
4. Lee TK, Castilho A, Cheung VC, Tang KH, Ma S, Ng IO. CD24+ hepatocellular carcinoma cells are more sensitive to tunicamycin than the immortalized liver cell line L02 (data not shown). Therefore, tunicamycin could likely be used to sensitize cancer cells to common chemotherapeutic drugs at a much lower concentration that has minor side effects on normal cells. Alternatively, chemoembolization is a valuable strategy for improving the survival of patients with unresectable hepatocellular carcinomas (31), and tunicamycin can be used in this way to attenuate its systemic toxicity. Furthermore, NLG inhibitors possessing lower toxicities may be explored on the basis of DPAGT1 inhibition.

In conclusion, we found that the NLG inhibitor tunicamycin could reverse the sorafenib-induced expression of CD44, N-cadherin, and vimentin (Supplementary Fig. S8D), implying that tunicamycin and sorafenib may be used together to attenuate sorafenib-enhanced metastasis. It is necessary to acknowledge that tunicamycin possesses systemic toxicity (44). However, experiments in normal cells show no reduction of receptor tyrosine kinase-dependent signaling following tunicamycin treatment (45). Furthermore, hepatocellular carcinoma cells were more sensitive to tunicamycin than the immortalized liver cell line L02 (data not shown). Therefore, tunicamycin could likely be used to sensitize cancer cells to common chemotherapeutic drugs at a much lower concentration that has minor side effects on normal cells. Alternatively, chemoembolization is a valuable strategy for improving the survival of patients with unresectable hepatocellular carcinomas (31), and tunicamycin can be used in this way to attenuate its systemic toxicity. Furthermore, NLG inhibitors possessing lower toxicities may be explored on the basis of DPAGT1 inhibition.

In conclusion, we found that the NLG inhibitor tunicamycin was able to reverse ABCG2 drug efflux activity by decreasing its protein level and altering its subcellular localization. Tunicamycin reduced the expression of CSC markers and suppressed the tumorigenicity of CD133+ hepatocellular carcinoma CSCs. Tunicamycin sensitized hepatocellular carcinoma to common chemotherapeutic drugs and impaired tumor growth at least partially through the DPAGT1/Akt/ABCG2 pathway. In the future, tunicamycin can potentially be used to attenuate the treatment of hepatocellular carcinomas when combined with common chemotherapeutic drugs. Additional drugs disrupting the NLG protein modification remain to be discovered or designed to reduce the drug resistance of hepatocellular carcinomas.

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