

# A Novel Podophyllotoxin-Derived Compound GL331 Is More Potent than Its Congener VP-16 in Killing Refractory Cancer Cells

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**Purpose.** GL331 is a new homolog of VP-16, and has demonstrated more efficacious anti-cancer activity in both the *in vitro* and *in vivo* lymphoma systems. To extensively explore GL331's clinical value, we furthermore evaluate the cytotoxicity and apoptosis-inducing activity of GL331 in several human cell lines from cancers that are not normally treated with VP-16.

**Methods.** By MTT and clonogenic survival assays, the cytotoxicities of GL331 and VP-16 were evaluated in a variety of cell lines including nasopharyngeal, hepatocellular, gastric, colon, cervical, and neuroblastoma cancer types. Western blot analysis was performed to detect the MDR-1 level in these cell lines. By Annexin V-staining flow cytometry and detection of DNA ladders, the apoptosis-inducing activities of GL331 and VP-16 were also evaluated.

**Results.** GL331 showed more efficacy than its congener VP-16 in killing cancer cells. The estimated ID<sub>50</sub> of GL331 were 2.5 to 17-fold lower than those of VP-16. GL331 possessed more cell-killing activity even in MDR-1-overexpressing cell lines such as HCC36 and SW620. Its higher cytotoxicity could be attributed by the elevated ability to induce apoptotic cell death.

**Conclusion.** GL331's overriding drug resistance and higher cancer cell-killing activity suggest its superiority in clinical cancer therapy.

**KEY WORDS:** GL331; VP-16; apoptosis; cytotoxicity.

## INTRODUCTION

GL331 is a new semisynthetic compound derived from a naturally occurring plant toxin podophyllotoxin (reviewed in

Ref. 1). The molecular formula is C<sub>27</sub>H<sub>24</sub>N<sub>2</sub>O<sub>9</sub> with a molecular weight of 520.48, and the chemical formula is 4'-demethyl-4β-(4"-nitroanilino)-4-desoxypodophyllotoxin. GL331 shares many physico-chemical and biochemical properties with its congener compound, VP-16. Like VP-16, GL331 causes apoptosis in several types of cancer cell lines (2). Overexpression of Bcl-2 attenuates GL331-induced apoptosis by down-regulation of poly(ADP-ribose)polymerase (PARP) activity (3). Although GL331 was originally identified as a topoisomerase II (Topo II) inhibitor, it could induce a plethora of cellular responses which are qualitatively or quantitatively different from those induced by VP-16. During induction of apoptosis, protein tyrosine kinase (PTK) activities are decreased in the cancer cells treated with GL331 or VP-16, while protein tyrosine phosphatase (PTP) activities are induced only in GL331-treated cancer cells (2). In a further study, cellular CDC 25 phosphatase was activated after GL331 exposure, which was responsible for the subsequent abnormal increase in the activity of cyclin B-associated CDC 2 kinase and ultimate apoptotic cell death (4,5). Therefore, activation of PTPs could be an important event in the cytotoxic mechanisms elicited by GL331.

Clinically, VP-16 has been widely used to treat lymphoma, testicular carcinoma and other malignancies (reviewed in Refs. 6 and 7). However, the therapeutic efficacy of VP-16 is compromised owing to the rise of multi-drug resistance in cancer cells, indicating a necessity to develop more effective chemotherapeutic agents and schedules (8). It has been demonstrated that the cytotoxicity of GL331 was approximately 40 times more potent than VP-16 in adriamycin-resistant murine lymphoma cells, and the life span of lymphoma cells-inoculated mice could be greatly increased by GL331 treatment (1). In the human promyelocytic leukemia xenograft model, GL331 also demonstrated the anti-tumor activity at dose levels significantly lower than VP-16 (1). GL331 is therefore expected to treat the lymphoma cells resistant to conventional agents. To explore whether GL331 is also more efficacious than VP-16 in treating other cancer types, we herein evaluated the cytotoxicity and apoptosis-inducing activity of GL331 in several cancer cell lines derived from tumors that are not normally treated with VP-16 (nasopharyngeal, hepatocellular, gastric, colon, cervical, and neuroblastoma cancers). We found GL331 exhibited more efficacy than its congener VP-16 in killing these selected cancer cells. The estimated ID<sub>50</sub> of GL331 were 2.5- to 17-fold lower than those of VP-16. GL331 possessed an elevated ability to induce cancer cell apoptosis even in MDR-1-overexpressing cell lines. The information presented here would provide some important basis for further animal experiments and future clinical application.

## MATERIALS AND METHODS

### Cell Lines

Seventeen human cancer cell lines, including nasopharyngeal (NPC-TW01, NPC-TW04, and KB), hepatocellular (HCC36, HA22T, HA59T, Tong, HepG2, and Hep3B), gastric (SC-M1, TSGH-9201, AGS, and NUGC-3), colon (SW620 and B77), cervical (HeLa), and neuroblastoma (HTB-11) cancer types, were grown in MEM supplemented with 10% fetal bovine

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**ABBREVIATIONS:** Topo II, topoisomerase II; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; PARP, poly(ADP-ribose)polymerase; NPC, nasopharyngeal carcinoma; 5-FU, 5-fluorouracil; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; PI, propidium iodide; HCC, hepatocellular carcinoma.

serum, 2 mM of L-glutamine, 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin (2,9–11).

### Cell Lysate Extraction and Western Blot Analysis

Cell lysates for Western blot analyses were prepared according to the method described previously (5). Aliquots (30  $\mu$ g protein) of cell lysates were separated on 8% SDS-polyacrylamide gels, and electrotransferred onto polyvinylidene difluoride membranes. After blocking with PBST (phosphate-buffered saline plus 0.1% Tween-20) and 5% non-fat milk, the membranes were incubated with anti-MDR-1 antibody (JSB-1 clone, Signet Co.) in PBST and 5% milk at 4°C for 12 hours. These membranes were then washed three times with PBST, and incubated with horseradish peroxidase-conjugated secondary antibody for one hour at room temperature. After washing three times with PBST, the band representing the antibody-recognized MDR-1 was detected by enhanced chemiluminescence (ECL, Amersham Co.).

### Cytotoxicity Assay

Cytotoxicities of GL331 and VP-16 were evaluated by MTT and clonogenic survival assays. In MTT assay, one to three thousand cancer cells were seeded on each well of 96-well dishes and incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere for overnight. Adherent cells were then subjected to GL331 or VP-16 treatments in 200  $\mu$ l of medium. After the treatment periods, 50  $\mu$ l of 0.25% MTT in PBS was added and incubated for four hours. Then, medium was removed from each well, and formazan was extracted with 50  $\mu$ l of dimethyl sulfoxide and quantitated by an ELISA reader at 550 nm. In the clonogenic survival assay, five hundred cancer cells were seeded on each 10-cm petri-dish and incubated for overnight. After subjecting treated cells to GL331 or VP-16 treatments as performed in MTT assay, the cells were incubated for 10–14 days in the culture medium which was refreshed at 3-day intervals. Finally, colonies were fixed with 20% methanol/20% acetic acid (in water) and stained with 0.1% crystal violet. Colonies containing more than 50 cells were scored.

### Annexin V-FITC/PI Double-Staining Flow Cytometry

This technique, adopted to quantitatively detect the apoptosis ratio (12), was performed according to the manual of Apoptosis Detection Kit (R & D Systems Co.). After drug treatments, approximate  $4 \times 10^5$  cells were trypsinized, collected and washed twice with 4°C pre-cold PBS. Cells were further resuspended in 100  $\mu$ l of calcium-containing binding buffer, and added with 10  $\mu$ l of Annexin V-FITC and 10  $\mu$ l of PI. The mixed cell suspension was incubated at 4°C and kept in dark for 15 min. After diluted with 400  $\mu$ l of binding buffer, the samples were measured by FITC/PI flow cytometry (FAC-Star, Becton Dickinson, CA).

### Internucleosomal Cleavage Assay

Apoptotic DNA fragments were isolated by the method described previously (13). Briefly, the logarithmically growing cancer cells were treated as indicated for 24 hours, and then adherent and nonadherent cells were collected together for lysis

by using 4°C pre-cold lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 20 mM EDTA, and 1% NP-40. The cell lysates were centrifugated at 3200  $\times$ g for 1 minute, and the supernatants were brought to 1% SDS and treated for 1 hour with 0.2  $\mu$ g/ $\mu$ l of RNase A at 45°C followed by digestion with 2.5  $\mu$ g/ $\mu$ l of proteinase K at 37°C for overnight. After the addition of ammonium acetate and glycogen, DNA was further extracted from the cell lysates with phenol-chloroform twice and chloroform once and precipitated by ethanol. The pattern of DNA ladders was examined after 1% agarose gel electrophoresis and ethidium bromide staining, and the image was printed by Alpha-InnoTech IS500 digital Imaging System (Avery Dennison, CA).

### RESULTS

Multi-drug resistance is a major obstacle to drug treatment of patients with cancer. Overexpression of MDR-1 is one of mechanisms cancer cells use to escape death induced by chemotherapeutic agents. Western blot analysis was therefore performed to evaluate the MDR-1 levels in 17 cancer cell lines, including several cancer types such as hepatocellular carcinoma (HCC), gastric carcinoma, nasopharyngeal carcinoma (NPC), etc. We found only the SW620 colon cancer cell line and HCC36 hepatocellular carcinoma cell line had higher MDR-1 expression levels. Other cell lines, including HCC (Hep3B, HepG2, Tong, HA22T, and HA59T), gastric cancer (SC-M1, NUGC-3, TSGH-9201, and AGS), NPC (NPC-TW01, NPC-TW04, and KB), colon cancer (B77), cervical carcinoma (HeLa), and neuroblastoma (HTB-11), exhibited very low or even non-detectable MDR-1 expression (Fig. 1 and data not shown).

To compare the cytotoxic potencies of GL331 and VP-16, ten cancer cell lines were used to generate the survival curves in response to GL331 or VP-16 exposure. The survival rates were obtained by MTT assays after incubation with GL331 or VP-16 for 72 h. As four examples shown in Fig. 2, GL331 exhibited higher cytotoxicities than its homologous compound VP-16. Depending on different cell lines, the estimated ID<sub>50</sub> of GL331 ranged from 0.07 to 1.5  $\mu$ M, which were 2.5- to 17-fold lower than those of VP-16 (Table I). The phenomenon that GL331 was more cytotoxic than VP-16 could be confirmed by using the clonogenic survival assay (Fig. 3). To take SC-M1

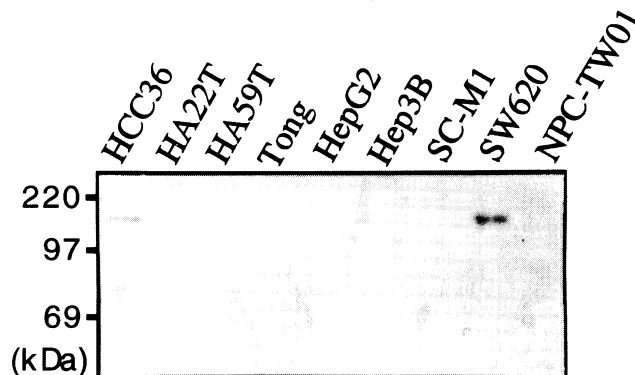
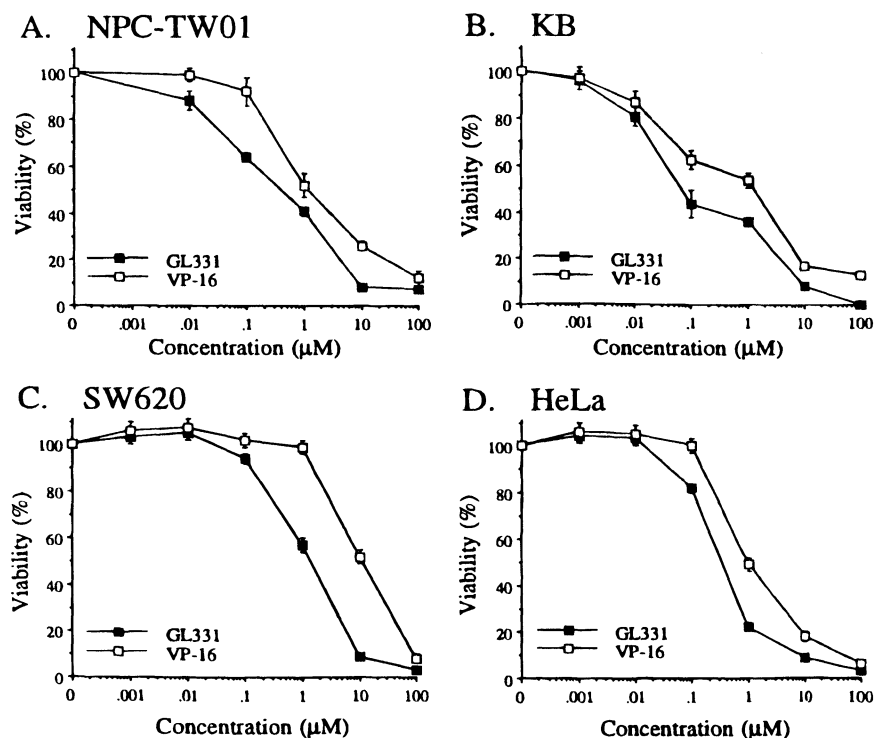


Fig. 1. Western blot analysis of MDR-1 expression in HCC (HCC36, HA22T, HA59T, Tong, HepG2 and Hep3B), gastric carcinoma (SC-M1), colon cancer (SW620), and NPC (NPC-TW01) cell lines. The representative data from two independent experiments is shown.



**Fig. 2.** Survival curves of NPC-TW01 (A), KB (B), SW620 (C) and HeLa (D) cell lines in response to GL331 and VP-16 treatments. Viabilities of the cells treated with GL331 and VP-16 for 72 h were evaluated at the same time by MTT assay. The data represent the means of three independent experiments. Each experiment was performed in quintuple. Error bars are shown when they are larger than the symbol.

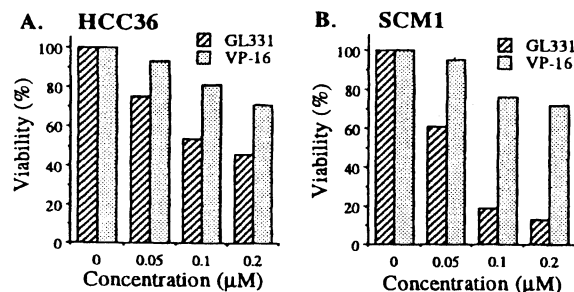
**Table I.** Estimation of the ID<sub>50</sub> of GL331 and VP-16 in Various Cancer Cell Lines<sup>a</sup>

Cell Lines	ID <sub>50</sub> of GL331 (µM)	ID <sub>50</sub> of VP-16 (µM)
Nasopharyngeal Carcinoma		
NPC-TW01	0.4	1
NPC-TW04	1	5
KB	0.07	1.2
Hepatocellular Carcinoma		
Hep3B	0.9	8
HCC36	1.5	10
Colon Carcinoma		
SW620	1.5	10
B77	2	10
Gastric Carcinoma		
SC-M1	2	10
Cervical Carcinoma		
HeLa S3	0.3	1
Neuroblastoma		
HTB-11	1	5

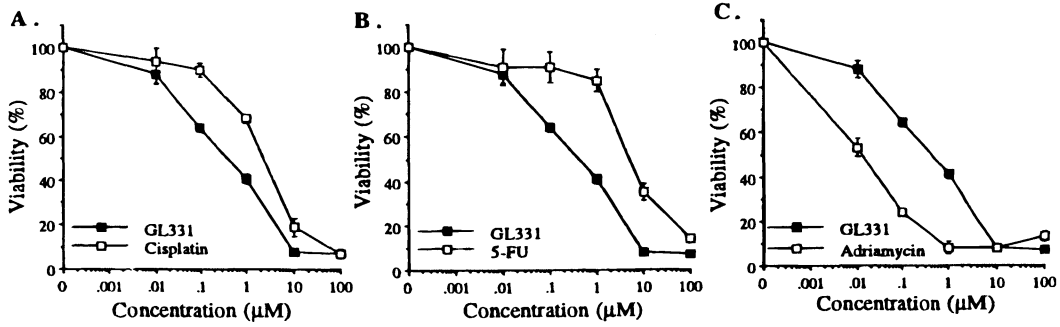
<sup>a</sup> The cytotoxicities of GL331 and VP-16 in each cell line were determined at the same time by using MTT assays after 72-h drug exposure as described in Materials and Methods. The concentrations required for 50% inhibition of cell viability (ID<sub>50</sub>) were estimated from the survival curves as shown partly in Fig. 2.

cell line as an example, 80% of cells were killed by 0.1 µM of GL331; however, 75% of SC-M1 cells were still alive in the parallel experiments using VP-16 instead. In addition, we compared the cytotoxicities induced by GL331 and other cancer chemotherapeutic agents such as cisplatin, 5-FU, and adriamycin. By MTT assays, GL331's concentration required for 50% inhibition of NPC-TW01 cell viability was 5- and 15-fold lower than that of cisplatin and 5-FU, respectively (Fig. 4A and 4B), but was 20 times higher than the dose of adriamycin to have the same killing effect (Fig. 4C).

Apoptosis is an important pattern of cell death induced by cancer chemotherapeutic agents. To address whether or not the higher cytotoxicity of GL331 was attributed by the increased level of apoptotic induction, we first observed the apoptotic



**Fig. 3.** Viabilities of HCC36 (A) and SC-M1 (B) cells treated with GL331 and VP-16 for 72 h. The results were obtained by clonogenic survival assay, and each data represents the mean of two independent experiments. Each experiment was performed in triplicate.



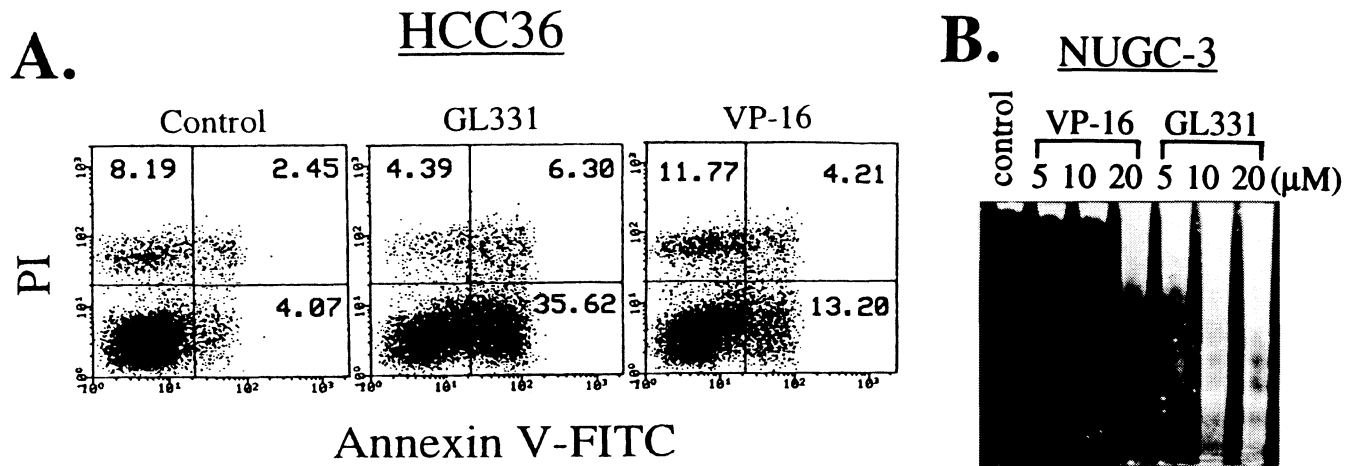
**Fig. 4.** Survival curves of NPC-TW01 cells in response to GL331 vs. cisplatin (A), GL331 vs. 5-FU (B), and GL331 vs. adriamycin (C) treatments. The viabilities were determined at the same time by MTT assay, and the data represent the means of three independent experiments. Each experiment was performed in quintuple.

morphology appeared in NPC-TW01, SC-M1 and HCC36 cells after their exposure to GL331 or VP-16 for 36 h (ref. 2 and data not shown). Although more cells became blebbed and died with increasing concentrations of GL331 and VP-16, the number of VP-16-affected cells was obviously fewer than those with GL331 treatment. Moreover, we performed the Annexin V-FITC/PI double-staining flow cytometry to quantitate the apoptosis level induced by GL331 and VP-16, respectively. As shown in Fig. 5A, the apoptotic cell fraction of HCC36 cells significantly increased from 4.07% to 35.62% after incubation with 1 µM of GL331 for 72 h. Comparatively, only 13.20% was seen in VP-16-treated HCC36 cells. Cellular DNA was extracted and analyzed by agarose gel electrophoresis, GL331 was also more efficient than VP-16 to induce apoptotic DNA fragmentation. To take NUGC-3 cell line as an example, the pattern of DNA ladders could be detected from the cells treated for 24 h with 10 µM of GL331 but not with VP-16 even at 20 µM (Fig. 5B).

**DISCUSSION**

By acting as a Topo II inhibitor, VP-16 has been widely used to treat lymphoma, testicular carcinoma, and other malignancies (reviewed in Refs. 6 and 7). However, its therapeutic

efficacy has been compromised with the rising of multi-drug resistance in cancer cells (8). GL331 is a new homolog of VP-16 and also has the ability to inhibit Topo II (1). This study has demonstrated that GL331 causes higher cytotoxicities than VP-16 in several human cell lines derived from various cancer types such as nasopharyngeal, hepatocellular, colon, gastric and cervical carcinomas, and neuroblastoma. Depending on different cell lines, the estimated ID<sub>50</sub> of GL331 ranged from 0.07 to 1.5 µM, which were 2.5- to 17-fold lower than those of VP-16. In the cell lines tested, overexpression of MDR-1 was found in the hepatocellular carcinoma HCC36 and colon carcinoma SW620 cells. Although MDR-1 was not highly expressed in gastric carcinoma SC-M1 and colon carcinoma B77 cells, it remains to be determined whether other MDR-related proteins render these two cell lines more resistant to VP-16. GL331's more cell-killing activity still could be detected in these cell lines both by MTT and clonogenic survival assays, suggesting the possibility of clinical application in these cancer types. In addition, GL331's overcoming drug resistance and its higher anti-neoplastic activity than VP-16 have also been confirmed in various animal models (1). The life span of P388 lymphoma cells-inoculated BDF1 mice could be greatly increased by GL331 treatment. In the human promyelocytic



**Fig. 5.** Different apoptosis levels induced by GL331 and VP-16. (A). Measurement of the apoptotic cell ratio in VP-16 or GL331-treated HCC36 cells. Logarithmically growing HCC36 cells were treated with 1 µM of GL331 or VP-16 for 72 h, and subjected to Annexin V-FITC/PI double-staining flow cytometry as described in the "Materials and Methods" section. The lower right population at each data represents the cells undergoing apoptosis. (B). Agarose gel electrophoresis of the DNA extracted from NUGC-3 cells treated with varying doses of VP-16 or GL331 for 24 h.

leukemia HL-60 xenograft model, GL331 also demonstrated the anti-tumor activity at dose levels significantly lower than VP-16. These data suggest GL331's superiority in clinical cancer therapy.

Apoptosis is an important form of cell death induced by cancer chemotherapeutic agents. This study has also demonstrated that GL331 induced apoptosis in the cancer cell lines tested and its more ability to induce apoptosis than VP-16 was shown by detection of the internucleosomal DNA fragmentation and phosphatidyl serine externalization. Although GL331 was more potent than VP-16 in Topo II inhibition (14,15), the concentration of GL331 required for induction of apoptosis was considerably lower than that for inhibition of Topo II, suggesting other biomolecules related to apoptosis could be affected by GL331. During the induction of apoptosis, GL331 and VP-16 decreased cellular PTK activities, while the PTP activities were in contrast induced by GL331 but not VP-16 treatment (2). Recently, cellular CDC 25 phosphatase was found to be activated after GL331 exposure for 3 h, and this activation seemed to be required for the subsequent abnormal change in the activity of cyclin B-associated CDC 2 kinase and ultimate apoptotic cell death (4,5). On the other hand, MDR-1 is a cell membrane P-glycoprotein which acts as a drug transport pump and therefore confers drug resistance on cancer cells, and the increase of MDR-1 phosphorylation was accompanied by a decrease in the accumulation or cytotoxicity of anticancer drugs (16–18). The efflux of GL331 and VP-16 was assessed in parental and vincristine-resistant KB head-and-neck cancer cell lines. GL331 and VP-16 were pumped-out of KB cells at a similar rate, but the efflux of VP-16 in vincristine-resistant cells was significantly higher than that of GL331 (1). It is possible GL331 would override multiple drug resistance by affecting protein kinases and phosphatases and thus modulating MDR-1 phosphorylation. This supposition could be supported by the finding that *O*-phospho-L-tyrosine, an inducer of cellular PTP activity, can synergistically sensitize the renal carcinoma ACHN cells to killing by VP-16 (19). Collectively, the ability to activate cellular PTP could contribute to GL331's overriding drug resistance and its higher cytotoxicity in comparison with VP-16.

Our data showed GL331's concentration required for 50% inhibition of NPC-TW01 cell viability was 5- and 15-fold lower than that of cisplatin and 5-FU, respectively, but was 20 times higher than the dose of adriamycin to have the same killing effect. In clinical use, the single agent cisplatin or adriamycin has been shown to be active for NPC, whereas the activity of 5-FU is not evident (20). These drugs have individual mechanisms of action, and are metabolized via different pathways. Cisplatin is excreted by kidney and can cause renal failure. Adriamycin is metabolized by liver, and administration to jaundiced patients can cause severe toxicity. The phase I clinical trial of GL331 has defined the maximum tolerated dose as 300 mg/m<sup>2</sup>, and the toxicity is dose-dependently limited to neutropenia and thrombocytopenia (1). Therefore, further clinical trial may be performed to show the possible use of GL331 in treating NPC patients who have liver or kidney dysfunction.

In conclusion, a better understanding of GL331's modes of action has promoted considerable interest in exploring its possibility of clinical application, and our *in vitro* evaluation of GL331's cytotoxic potency would provide some information for further animal experiments and future clinical trials.

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