GL3, a Novel 4β-Anilino-4′-O-Demethyl-4-Desoxypodophyllotoxin Analog, Traps Topoisomerase II Cleavage Complexes and Exerts Anticancer Activities¹

Abstract

A novel VP-16 derivative, 4β-[N-(4‴-acetyloxyl-phenyl-1‴-carbonyl)-4″-aminoanilino]-4′-O-demethyl-4-desoxypodophyllotoxin (GL3), displayed a wide range of cytotoxicity in a panel of human tumor cell lines, with half-maximal inhibitory concentration (IC₅₀) values ranging from 0.82 to 4.88 μM, much less than that of VP-16 (4.18–39.43 μM). Importantly, GL3 induces more significant apoptosis and cell cycle arrest than VP-16. The molecular and cellular machinery studies showed that GL3 functions as a topoisomerase II (Top 2) poison through direct binding to the enzyme, and the advanced cell-killing activities of GL3 were ascribed to its potent effects on trapping Top 2–DNA cleavage complex. Moreover, GL3-triggered DNA double-strand breaks and apoptotic cell death were in a Top 2–dependent manner, because the catalytic inhibitor aclarubicin attenuated these biologic consequences caused by Top 2 poisoning in GL3-treated cells. Taken together, among a series of 4β-anilino-4′-O-demethyl-4-desoxypodophyllotoxin analogs, GL3 stood out by its improved anticancer activity and well-defined Top 2 poisoning mechanisms, which merited the potential value of GL3 as an anticancer lead compound/drug candidate deserving further development.

Introduction

DNA topoisomerase II (Top 2) solves the topological problems of DNA in replication, transcription, recombination, chromosome condensation, and decondensation by mediating the ATP-dependent induction of coordinated nicks in both strands of the DNA duplex, followed by crossing of another double-strand DNA through the transiently broken duplex [1,2]. Given the ability to interfere with this essential enzyme has been proved to be effective strategy for cancer therapy, Top 2 is regarded as an excellent target of anticancer drugs [2,3].

A wide range of Top 2–targeted inhibitors are commonly classified as Top 2 poisons and catalytic inhibitors [3,4]; among them, the poisons that are recognized to interrupt the breakage/reunion reaction of the enzyme, resulting in the accumulation of Top 2–DNA covalent intermediate, the cleavage complex, and causing cancer cell death, attracts more attention in terms of cancer-killing capability [2,3]. One of the most widely used Top 2 poisons, etoposide (VP-16), acts by trapping the Top 2 cleavage complex, induces a high level of DNA damage and subsequent apoptosis [5], and was officially approved for clinical use against various types of cancers including breast cancer, testicular cancer, small cell lung cancer, lymphoma, Kaposi’s sarcoma, and childhood leukemia [6,7]. In contrast, those agents that functioned as Top 2 catalytic inhibitors generally imposed limited anticancer activity against the
cancer cells but displayed abrogation effects on Top 2 poison(s)—caused cleavage complex formation, DNA double-strand breaks (DSBs), cell cycle, and apoptosis [4]. Consequently, all the Top 2–targeted anticancer drugs clinically used for their antitumor activities belong to Top 2 poisons. Over the past decades, increasing research is focused on the development of novel Top 2 poisons, for their superiority in apoptotic induction and cancer cell–killing capabilities.

4β-[N-(4‴-acetyloxyl-phenyl-1‴-carbonyl)-4‴-aminoanilino]-4″-O-demethyl-4′-desoxypodophyllotoxin (GL3) was developed as a novel 4β-anilino-4′-O-demethyl-4-desoxypodophyllotoxin analog with potential anticancer activities and undefined mechanism(s) (Figure 1A) [8]. Preliminary data showed that GL3 was a promising anticancer agent that can be orally administered and significantly arrested the growth of human lung cancer 95-D xenografted tumors on nude mice at the dosage of 50 mg/kg.

In the present study, we report the antitumor effects of GL3 on human oral squamous carcinoma KB cells and human non–small cell lung cancer A549 cells in vitro. Importantly, we examined the inhibitory profiles of GL3 on Top 2 and its subsequent events, including G2/M arrest and apoptosis through the ataxia telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) and mitochondria signaling pathways, respectively. Comparing with its parent compound VP-16, GL3 displayed improved anticancer activity, highly active Top 2 inhibitory effects. Taken together, these results suggest the potential value of GL3 as a novel anticancer drug candidate that deserves further development.

Figure 1. GL3 was a novel 4β-anilino-4′-O-demethyl-4-desoxypodophyllotoxin analog with anticancer activities and Top 2–targeting effects. (A) Chemical structure of GL3. (B) The half-maximal inhibitory concentration (IC50) values of GL3 against a variety of cancer cells. Cells were treated with serial diluted concentrations of GL3 for 72 hours, and the viability of cells was calculated using MTT assay. (C) GL3 exhibited Top 2–targeting activity as determined by kDNA decatenation assay. (D) Top 1 catalytic activity was not impacted by GL3. MONCPT is used as a positive control. (E) The interaction between GL3 or VP16 and Top 2 was predicted by automated molecular docking.
Materials and Methods

Reagents

GL3 was synthesized by Dr Yongzhou Hu (Zhejiang University–Ecole Normole Superiener Joint Laboratory of Medicinal Chemistry, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China) [8]. The structure was shown in Figure 1A, and its purity was more than 99%. Etoposide (VP-16), aclarubicin, and caffeine were purchased from Sigma (St Louis, MO). GL3, VP-16, and aclarubicin were dissolved in DMSO (50 mM) as stock solutions. Caffeine was dissolved in sterilized water. The stock solutions were kept frozen in aliquot at −20°C. The primary antibodies to chk1, chk2, phospho-cdk25c, cdc2, cyclin B1, p53, phospho-p53, caspase-8, caspase-9, caspase-3, polyADP-ribose polymerase (PARP), and β-actin and HRP-labeled secondary anti-goat, anti-mouse, and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies to cleaved caspase-3, γ-H2AX, phospho-chk2, phospho-chk1, and phospho-cdc2 (T14/T15) were purchased from Cell Signaling Inc (Danvers, MA). ECL was purchased from Pierce (Rockford, IL).

Cell Line and Cell Culture

The human ovarian cancer A2780 cells, human prostate cancer PC-3 cells, oral squamous carcinoma KB cells, and human gastric carcinoma SGC-7901 cells were maintained in RPMI-1640 medium (Invitrogen, Grand Island, NY). Human hepatocellular liver carcinoma HepG2 cells and colorectal carcinoma HCT-116 cells were maintained in high-glucose Dulbecco modified eagle medium (HG-DMEM) (Life Technologies, Grand Island, NY; 2 g/l glucose). Non-small cell lung cancer A549 cells were maintained in F12 medium (Invitrogen). All media were supplemented with heat-inactivated FBS plus 2 mM glutamine and 50 units/ml penicillin. All the cell lines were endowed by Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China) and incubated at 37°C in 5% CO2.

Cell Proliferation Assay

Cells were seeded in 96-well plates at 4 × 10³ cells/well. After 24 hours, GL3 or VP-16 at a series of concentrations (0–50 μM) was added to each well, and then the cells were incubated for 72 hours. At the end of the treatment, the cells were incubated for another 4 hours after 5.0 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added. A quantity of 100 μl of DMSO was added to each well to dissolve the purple formazan crystals. Then, cell viability was obtained by measuring the absorbance on an automated microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 570 nm. The growth inhibition was calculated according to the following formula: The Growth Inhibition Ratio (IR%) = (1 – OD570 treated/OD570 control) × 100%.

kDNA Decatenation Assay

The detection of Top 2–mediated cleavage-religation complex stabilization was performed using the Topoisomerase II Assay Kit (TopoGEN, Inc, Port Orange, FL; Catalog No. TG1001-1). The Top 2 assay reaction buffer consisting of 2 mM ATP, 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, 10 mM MgCl2, and 0.5 mM dithiothreitol was mixed with 0.1 μg of kDNA. Reactions are assembled in tubes with water, buffer, and kDNA. The test fractions were added last and the reactions incubated at 37°C for 30 minutes. After termination with stop buffer, samples were run on electrophoresis in a 2% agarose gel in 1× TAE at 50 V for 2 hours and stained with 0.5 mg/ml ethidium bromide to facilitate visualization by fluorescence under UV light. Images were photographed by Bio-Rad GD2000 (Bio-Rad, Hercules, CA).

Top 1–Mediated Supercoiled pBR322 Relaxation

DNA relaxation assay was done to evaluate the effects on Top 1 activity. The reaction buffer contained 35 mM Tris-HCl (pH 8), 72 mM KCl, 5 mM MgCl2, 5 mM DTT, 5 mM spermidine, 0.01% BSA, 0.5 μg of supercoiled pBR322 DNA, and 1 unit of Top 1 (GE Healthcare, Buckinghamshire, England) in a total volume of 20 μl. Reaction mixtures were incubated at 37°C for 30 minutes in the presence of drugs and terminated by adding 10% sodium dodecyl sulfate.

Molecular Docking of Top 2–DNA Complex and GL3/VP-16

In an attempt to understand the molecular interaction between GL3 and Top 2, a molecular docking study was performed using the Discovery Studio 2.1/CDOCKER protocol. The crystal structure of Top 2/G-segment DNA complex (PDB ID: 2RGR) was used as the template. Docking and subsequent scoring studies were performed using default parameters.

Western Blot Analysis

Proteins of A549 and KB cells were extracted in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 0.5% deoxycholic acid, 0.02% sodium azide, 1% NP-40, 2.0 μg/ml aprotinin, and 1 mM PMSF). For each lane, 40.0 to 80.0 μg of total protein (equal amount) was loaded. After being fractionated on 8% to 15% Tris-glycine gels, proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Billerica, MA) and probed with primary antibodies and then HRP-labeled secondary antibodies. Finally, proteins were visualized using ECL.

Neutral Single-Cell Gel Electrophoresis Assay

Cells (5 × 10⁶/ml) were treated with GL3 for indicated times. DNA DSBs were evaluated by neutral single-cell gel electrophoresis assay, which was done according to the method with slight modifications [9]. Images were captured using fluorescent microscope (Leica Microsystems, Wetzlar, Germany).

Flow Cytometry Analysis of DNA Content

Exponentially growing cells (2 × 10⁶) were seeded at 4 × 10⁵ cells/flask in 75-cm² flasks. The cells were cultured at 37°C in a 5% CO2 atmosphere for 24 hours before they were exposed to GL3 or VP-16. Cells were harvested, washed, and resuspended in 500 μl of phosphate-buffered saline containing 50.0 μg/ml RNaseA (Ameresco, Solon, OH). After incubation for 30 minutes, the cells were stained with 10.0 μg/ml propidium iodide (PI; Sigma) in the dark for 30 minutes. Flow cytometry was performed on FACSCalibur (BD Biosciences, San Jose, CA), and then the data were analyzed using CellQuest software (BD Biosciences).

Results

GL3 Arrests Cancer Cell Proliferation In Vitro

The antiproliferative activity of GL3 was determined on various human tumor cell lines. As shown in Figure 1B, GL3 displayed wide
potent concentration-dependent cytotoxicity in a diversity of human cancer cell lines including ovarian cancer, prostate cancer, colorectal cancer, gastric carcinoma, oral squamous carcinoma, hepatocellular liver carcinoma, and lung cancer without significant tissue specificity. Different degrees of antiproliferative effect on these cells based on IC₅₀ values of GL3 were shown as a range from 0.82 to 4.88 μM, much less than VP-16 (4.18–39.43 μM). In particular, among all the tested tumor cells, GL3 exhibited most potent growth inhibitory effects against KB cells, and in A549 cells, the IC₅₀ values for GL3 and VP-16 achieved a maximum difference; thus, these two cell lines were chosen for our further research.

**GL3 Targets Top 2 through Binding with the Active Site and Inhibiting the Catalytic Activity**

To determine the effects of GL3 on Top 2, the putative cellular target of VP-16, kDNA decatenation assays was first employed to examine the Top 2 catalytic activity with or without GL3 and VP-16. kDNA would be catalytically degraded into nicked circular DNA and relaxed circular DNA in the presence of Top 2 and ATP. As shown in Figure 1C, both GL3 and VP-16 inhibited the activity of Top 2 when measured by a loss in its capacity to decatenate kDNA. Intensity of nicked circular DNA diminished in a concentration-dependent manner in both VP-16 and GL3 groups, indicating the comparable inhibitory effects of these two compounds on the Top 2 catalytic activity.

To help clarify the mechanism(s) that GL3 inhibits Top 2 catalytic activity, we used automated molecular docking to predict the conformation of GL3 and VP-16 when bound to Top 2. The most probable binding conformations of these two compounds were obtained, and we noticed that GL3 made several important interactions along the active site of Top 2 with different mode in comparison with VP-16 as shown in Figure 1E. The binding energy of compound GL3 was stronger than that of VP-16. Ranking according to Dock energy and conformational analyses together indicate that comparing with VP-16, the different binding modes and the higher affinity for the Top 2 of GL3 may explain the observation that GL3 showed more potent antiproliferative activity than that of VP-16.

Collectively with our finding that GL3 or VP-16 imposed no effects on Top 1-mediated DNA relaxation (MONCPT, a reported CPT derivative [10] served as a positive control; Figure 1D), these results indicated that GL3 exhibited Top 2–targeted activity as indicated by the comparable inhibition of catalytic activity and the distinct interactive binding mode with Top 2.

**GL3 Induces DNA DSBs**

As demonstrated by previous report that VP-16 trapped Top 2–DNA complexes and lead to the subsequent DNA DSBs, we were inspired to speculate that GL3 may also exert Top 2 poisoning effects and result in DNA DSBs.

Though there are a lot of experimental results that point to the validity of certain models, the phosphorylation of histone H2AX (noted as γ-H2AX) within seconds after occurrence of DSBs is generally accepted. As a result, the levels of γ-H2AX were monitored to detect the generation of DNA DSBs. As shown in Figures 2A and 3A, γ-H2AX was upregulated in A549 and KB cells after incubation with GL3 for 2 hours.

The neutral single-cell gel electrophoresis assay (also known as neutral comet assay) is also widely employed to detect DNA damage at the level of the individual eukaryotic cell. Using this assay, we found that the exposure of A549 and KB cells to GL3 within 2 hours generated longer comet tails than that of VP-16 groups (Figures 2B and 3B), suggesting that GL3 induced extensive DNA DSBs, with higher extent than that of VP-16.

In contrast, VP-16 elicited much less phosphorylation of H2AX or comet tails under our experimental conditions (20 μM, 2 hours), which was probably owing to the lower concentration we used than the previous report [9]. For DNA damage detection, the reason we

![Figure 2](image-url) **Figure 2.** GL3 induced DNA DSBs and G₂/M arrest in A549 cells. (A) GL3 (5–20 μM, 2 hours) triggered phosphorylation of H2Ax (γ-H2Ax) in A549 cells. (B) GL3 induced DNA DSBs in A549 cells. Cells were untreated or treated with GL3 (20 μM) for 2 hours. Detection of DSBs was done using the neutral comet assay. Representative comet images were shown. (C) Cell cycle distribution histogram of A549 cells treated with GL3 from the concentration of 125 to 500 nM for 24 hours, representative of three independent experiments. (D) Cell cycle regulating pathways were activated by GL3 treatment in A549 cells.
exposed cells with higher concentration than the IC_{50} values of GL3 achieved from cell proliferation assay was that when detecting DNA damage, we exposed cells to GL3 for much shorter time than that in cell proliferation assays (2 hours for DNA damage detection and 72 hours for cell proliferation assay).

**GL3 Induces DSB-Mediated G2/M Arrest at Low Concentrations**

Top 2 poisons generally induce cell cycle arrest owing to the generation of DNA DSBs. In the current study, the cell cycle distribution of GL3-treated A549 and KB cells was determined. GL3 induced G2/M arrest in a concentration-dependent manner (Figures 2C and 3C). Most of the cells were arrested in the G2/M phase in A549 and KB cells after a 24-hour treatment (51.20% for 500 nM in A549 cells and 84.83% for 100 nM in KB cells).

Furthermore, the levels of proteins involved in the regulation of the G2/M transition in A549 and KB cells were examined to explore the underlying mechanism of GL3-induced G2/M arrest. GL3 increased the levels of the phosphorylated Chk2 (T68) and did not change the protein level of Chk2 (Figure 2D), implying the activation of Chk2 pathway(s). In addition, in a concentration-dependent manner, GL3 increased the protein level of cyclin B1 and the putative inhibitory phosphorylation on Cdc2 (T14/T15) (Figures 2D and 3D).

Taken together, these results indicated that the predominant change in the expression of G2/M regulatory proteins caused by GL3 treatment were induced through the Chk2 signaling pathway, which subsequently lead to G2/M arrest in A549 and KB cells.

**GL3 Causes Apoptosis in a Caspase-Dependent Manner**

Most chemotherapeutic agent including Top 2-targeted drugs kill tumor cells through apoptosis induction. In this context, flow cytometry analysis after PI staining was applied to investigate the apoptosis-inducing effects of GL3. We found that GL3 induced apoptosis in A549 and KB cells in a concentration-dependent manner. More than 40% of A549 cells were detected to be apoptotic following the 24-hour treatment of GL3 at 20 μM, whereas VP-16 at 20 μM drove only about 10% of A549 cells to experience apoptosis (Figure 4A). In KB cells, 10 μM GL3 caused more than 60% apoptosis in treated cells, whereas 10 μM VP-16 elicited 40% of apoptosis (Figure 4C).

The aforementioned result indicated that GL3 induced apoptosis in A549 and KB cells; we were thus encouraged to explore how GL3 is involved in the activation of apoptosis. After exposure to GL3, the levels of p53, phospho-p53, and Bax were elevated in a concentration-dependent manner, indicating a possible role for p53 and Bax in a high-concentration GL3-induced apoptosis. Furthermore, GL3 decreased the protein levels of procaspase-8, procaspase-9, and procaspase-3 and induced the cleavage of PARP (a major substrate of caspases) and caspase-3 in a concentration-dependent manner (Figure 4B). The similar results were also observed in KB cells (Figure 4D). Cleaved caspase-3 and cleavage of PARP increased visibly in a concentration-dependent manner, accompanied with the decline trend of PARP expression (Figure 4D).

**GL3-Triggered DNA DSB Is Attenuated by Top 2 Catalytic Inhibitor Aclarubicin**

Aclarubicin, a classic Top 2 catalytic inhibitor, specifically inhibits the catalytic activity of Top 2 without significantly elevating the level of cleavage complexes but is able to abrogate Top 2 poison-induced DSBs via disrupting the formation of cleavage complexes. In this context, aclarubicin was widely used to help validate the Top 2 poisons and the involvement of Top 2 in compound-induced effects including DNA DSBs, cell cycle, and apoptosis. In this study, we found that aclarubicin significantly antagonized GL3-induced DNA DSBs in A549 and KB cells, as indicated by the reduction of γ-H2AX level in aclarubicin-pretreated cells, comparing with GL3 monotreated group (Figure 5, A and B). Using the single-cell gel electrophoresis assay, we further demonstrated that aclarubicin could attenuate the generation of DNA DSBs triggered by GL3 (data not shown). In addition, as expected, aclarubicin pretreatment counteracted GL3-triggered apoptosis in KB cells as demonstrated by the attenuated cleavage of caspase-3 (Figure 5C). Taken together, these results indicated that GL3 acted as a Top 2 poison and inducing DNA DSBs as well as apoptosis by stabilizing Top 2 cleavage complexes.
Figure 4. GL3 elicited apoptosis in A549 and KB cells. (A) Percentage of apoptotic A549 cells after treatment of GL3 (5 to 20 μM) for 24 hours (mean ± SD, n = 3). (B) Pro–caspase-3, pro–caspase-8, and pro–caspase-9 were cleaved in GL3-treated A549 cells. A549 cells were cultured with medium or serial diluted concentrations of GL3 (5–20 μM) for 24 hours. The expressions of PARP, pro–caspase-3, cleaved caspase-3, pro–caspase-8, pro–caspase-9, p53, and Bax were tested. (C) GL3 caused apoptosis in KB cells. Cells were exposed to GL3 (2.5–10 μM) for 24 hours (mean ± SD, n = 3), subjected to PI staining and fluorescence-activated cell sorting (FACS) analyses. (D) PARP and pro–caspase-3 cleavage were detected in GL3 (2.5–10 μM, 24 hours)-treated KB cells.

Figure 5. DNA DSBs generated by Top 2 poisoning by GL3 mediated the G2/M arrest and apoptosis. (A) Top 2 catalytic inhibitor aclorubicin abated γ-H2Ax upregulated by GL3. A549 cells were exposed to GL3 (20 μM) for 2 hours with or without pretreatment with aclorubicin (50 nM) for 0.5 hour. (B) Aclorubicin (50 nM, 0.5-hour pretreatment) attenuated γ-H2Ax up-regulation in KB cells treated with GL3 (10 μM) for 2 hours. (C) Aclorubicin (50 nM, 0.5-hour pretreatment) antagonized the cleavage of pro–caspase-3 induced by GL3 (10 μM, 24 hours) in KB cells. (D) Caffeine preincubation (2 mM, 0.5 h) abrogated the G2/M accumulation in GL3 (500 nM, 24 hours)-treated A549 cells. G2/M population of GL3 group versus that of GL3 + caffeine group: **P < .01.
**GL3-Induced G2/M Arrest Is Antagonized by ATM/ATR Inhibitor Caffeine**

ATM/ATR, members of the phosphoinositide kinase-related protein family, is critical for the cellular response including cell cycle arrest and apoptosis upon genotoxic stresses, especially DNA DSBs. To clarify whether ATM/ATR pathway was involved in GL3-induced G2/M arrest, caffeine, a widely used ATM/ATR inhibitor, was employed. As expected, preincubation with caffeine (2 mM, 30 minutes) caused an obvious decrease of G2/M population from 56.78 ± 3.99% to 26.59% ± 1.60% in GL3 (500 nM, 24 hours)—treated A549 cells (Figure 5D), with significant differences. Thus, a possible relationship between ATM/ATR pathway and GL3-induced G2/M arrest was established, further supporting the notion that the biologic consequences of GL3 treatment were attributed to GL3-caused DNA DSBs.

**Discussion**

VP-16 was first synthesized in the 1960s and officially approved for clinical use against various types of cancers since 1983. Despite the crucial role in clinical cancer therapy for decades, etoposide (VP-16) has been challenged with toxic side effects (bone marrow depression and increased risk of secondary acute myelogenous leukemia), which make it urgent to further optimize the structure of the analogs to overcome the drawbacks and improve the anticancer activities. On the basis of the structure-activity analyses, several critical features for the Top 2–targeted activity were summarized to orient the chemical modification of podophyllotoxin: modification on rings A, B, D, and E made little contribution to improve Top 2 inhibitory activity or had different biologic activities and β-D-glucopyranose substituent on ring C is not essential, but the 4β configuration is important for the Top 2 inhibitory activity. Thus, C4 becomes an optimal position to significant structural modification. CoMFA model further demonstrated that bulky substituents at C4 might be favorable for Top 2 inhibition.

Our efforts focus on the design and screening of novel analogs of VP-16 based on the structure-activity relationship analyses, and Hu et al. synthesized several derivatives bearing bulky nitrous tails at C4 side chain to conquer the limitations of the previous compounds, of which compounds containing the 4β-anilino moiety exhibited improved anticancer activities. On the basis of that structural feature,amide groups were introduced to optimize activities of the compound. Among these compounds, 4β-anilino-4’-O-demethyl-4-desoxypodophyllotoxin derivative named as 26c (GL3) exhibits potent cell-killing and tumor growth–inhibiting capabilities against human lung cancer cells, human oral squamous carcinoma cells, and human lung cancer xenografted tumors and imposes less toxicity compared with the other derivative [8]. In the present study, we further examined the effects of this compound on cancer cell proliferation, apoptosis, cell cycle, and importantly, the Top 2–targeting activities and the subsequent biologic consequences, aiming to explore the mechanism(s) by which its anticancer abilities were exerted. We found that GL3 showed a higher antiproliferative activity than that of VP-16, with the average IC50 value about 10-fold lower than that for VP-16 (Figure 1B). Together with the previous findings that GL3 (26c) displayed potent growth inhibition on 95-D xenografted tumors when administrated intragastrically at 50 mg/kg two to three times per week, without significant effects on mouse body weight [8], GL3 was demonstrated as a promising anticancer agent both active in vitro and in vivo. Extensive evidences reveal the impact of VP-16 and its derivatives on Top 2. Two groups of Top 2 inhibitors are defined. Those agents able to stabilize the covalent DNA Top 2 cleavage complex are traditionally called Top 2 poisons, whereas agents acting on any of the other steps in the catalytic cycle are called catalytic inhibitors. VP-16 functions as a Top 2 poison, trapped the double-stranded DNA cleavage normally catalyzed by Top 2, inhibits the reigation of DNA breaks, and stabilized the DNA–Top 2 cleavage complexes, subsequently triggering the desired anticancer effects, i.e., cell cycle arrest, apoptosis, and proliferation inhibition [5]. Most derivatives of VP-16 share the same target and the similar mechanisms with their parental structure [11–13], by stabilizing the Top 2 cleavage complex. To achieve maximum cancer-killing efficiency, the compounds designed to target Top 2 are generally preferred to exert more potent Top 2 poisoning effects. In our study, GL3 exerted differential antiproliferative activities against various cancer cell lines, in a similar pattern with VP-16 (except that in HCT-116 and SGC-7901); whether this differing effect was related to the expression level of Top 2 needs to be investigated in further studies.

Catalytic inhibitors, the other group of Top 2–targeted agents, are a heterogeneous group of compounds that might interfere with the binding between DNA and Top 2 (aclarubicin and suramin), stabilize non-covalent DNA Top 2 complexes (merbarone, ICRF-187, and bisdioxopiperazine derivatives), or inhibit ATP binding (novobiocin). In contrast with the observations that poisons could induce apoptosis and act as anticancer agents, the catalytic inhibitors generally caused limited apoptosis and accordingly regarded as cardioprotectors (ICRF-187) or modulators to increase the efficacy of other agents (suramin and novobiocin) [14], and interestingly, most catalytic inhibitors (ICRF-187, ICRF-159, aclarubicin, etc.) can antagonize the DNA damage, apoptosis, and/or cytotoxicity of Top 2 poisons in cultured mammalian cells [15–18]. Thus, the attenuation of DNA DSBs, apoptosis, or cytotoxicity by Top 2 catalytic inhibitors could be used to validate that these cell-killing effects are imposed by Top 2 poisons [9,18].

A typical catalytic inhibitor aclarubicin, which is known to intercalate into DNA thereby preventing the binding of Top 2 to DNA and subsequently protecting Top 2 poison–induced DNA damage and apoptosis [9,19,20], is introduced in the present study. Our study showed that GL3-triggered DNA DSBs and subsequent apoptosis could be abolished by aclarubicin pretreatment as denoted by the reduction of comet tails, phosphorylated H2AX, and caspase-3 cleavage, respectively, indicating the inhibition of Top 2 catalytic activity could abate the DNA DSBs and subsequent apoptosis caused by GL3, which was in accordance with the aforementioned reports that Top 2 catalytic inhibitors could abrogate the effects of poisons [16,17,19–21]. Thus, the mechanisms by which GL3 targeting Top 2 could be speculated as trapping Top 2–DNA cleavage complexes, and in this context, the formation of the cleavage complex was critical for GL3-triggered DNA DSBs and apoptotic cell death. In accordance with this, greater interaction between Top 2 and GL3 predicted by automated molecular docking further aids to explain the superior anticancer activity in vitro achieved by GL3 than that of VP-16.

In our previous study [8], we have shown that GL3 (26c), a novel 4β-anilino-4’-O-demethyl-4-desoxypodophyllotoxin derivative, was a promising agent that can be orally administered and significantly inhibited human lung cancer growth but imposed limited toxicity in vivo. This study aimed to explore the mechanisms of action of GL3 and has shown for the first time that GL3 targets Top 2 by
trapping cleavage complex, which drives DNA DSBs and leads to cell cycle arrest and apoptosis. The appreciable pharmacologic profiles of GL3, including well-defined antitumor activities and less toxicity, are probably attributed to the delicate C-4 substitution of the anilino group at C4 position, with a favorable amide manipulation. This superiority to the parent compound VP-16 favors GL3, a potential antitumor drug candidate deserving of further development, and more importantly, in turn offers a favorable approach in the exploration of VP-16 structure optimization.

References