Adva-27a, a Novel Podophyllotoxin Derivative Found to Be Effective against Multidrug Resistant Human Cancer Cells

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Abstract. *Background/Aim: Multidrug resistance poses a serious challenge in cancer therapy. To address this problem, we designed and synthesized Adva-27a, a novel non-ester GEM-difluorinated C-glycoside derivative of podophyllotoxin. Materials and Methods: Adva-27a activity was evaluated in a variety of assays including inhibition of topoisomerase IIα, cytotoxic activity in drug-sensitive and drug-resistant cancer cell lines, metabolic stability in human liver microsomes and pharmacokinetic properties in rats. Results: Adva-27a exhibited dose-dependent human topoisomerase IIα inhibitory activity and dose-dependent growth inhibitory activity in several drug-sensitive and two multidrug-resistant cancer cell lines. In the multidrug-resistant cell lines, MCF-7/MDR (breast cancer) and H69AR (small-cell lung cancer), Adva-27a was significantly more potent than etoposide. The metabolic stability of Adva-27a in human liver microsomes and its pharmacokinetic properties in rats were better than those of etoposide. Conclusion: Our studies have identified Adva-27a as a novel topoisomerase II inhibitor with superior cytotoxic activity against multidrug-resistant human cancer cells and more desirable pharmacokinetic properties than etoposide.*

Etoposide (VP-16), which is a derivative of podophyllotoxin (Figure 1A and B), is widely used in human cancer chemotherapy, *i.e.* in small-cell lung cancer and testicular cancers (1, 13). It acts as a topoisomerase II "poison" by stabilizing the cleavage complex formed between the enzyme and its DNA substrate, leading to the inhibition of relegation, accumulation of chromosomal breaks and triggers cell death (23). However, similar to many other chemotherapeutic agents, the clinical utility of etoposide has been hampered by the lack of activity of the drug on certain tumor types, particularly multidrug resistant tumors (reviewed in (13)). In fact, etoposide has been found to be a substrate of multidrug efflux transporters, such as P-glycoprotein (P-gp) (2, 12).

Several strategies have emerged to design and synthesize analogs of etoposide or podophyllotoxin with improved pharmaceutical and clinical activities (13, 31). These include glycosylated etoposide derivatives, such as teniposide, phosphorylated etoposide, such as etopophos, and non-glycosylated podophyllotoxin derivatives containing nitrogen, such as GL331 (14, 15) and TOP-53 (26). Interestingly, teniposide and etopophos appear to be less toxic and are currently used in clinics, though they show little improvement of activity on drug resistant tumors compared to etoposide (13, 31). Furthermore, the non-glycoside, non-ether derivatives GL331 and TOP-53 have shown improved activity against topoisomerase II although still limited activity against multidrug resistant cells (7, 14, 16, 17, 26).

In the search for novel etoposide analogs with improved efficacy and pharmacokinetic properties, which can overcome multidrug resistance of human cancer cells, we designed and synthesized Adva-27a, a non-ester, nitrogen-containing glycoside analog of etoposide (Figure 1C). This study describes the evaluation of Adva-27a using a battery of biochemical, cell biological, and pharmacological analyses. Compared to etoposide, Adva-27a was found to be substantially more potent against two multidrug-resistant human cancer cell lines, and has a better metabolic stability and pharmacokinetic properties than etoposide. Taken

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Key Words: Adva-27a, topoisomerase II, cancer, multidrug-resistant, multidrug efflux transporters.

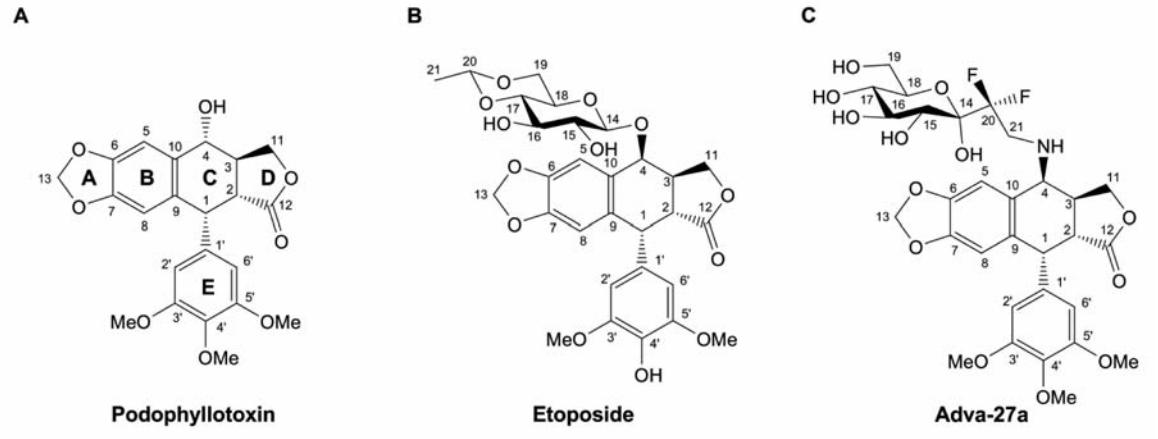

Figure 1. *Chemical structures of podophyllotoxin (A), etoposide (B) and Adva-27a (C).*

together, these results suggest that further development of Adva-27a is warranted as a novel topoisomerase II inhibitor for use in human cancer therapy with multidrug-resistant tumors.

Materials and Methods

Synthesis of Adva-27a. Synthesis of Adva-27a (a-D-gluco-3-octulopyranose, 1, 2-dideoxy-2, 2-difluoro-1, [[(5S, 5aS, 8aR, 9R)-5, 5a,6,8,8a,9-hexahydro-8-oxo-9-(3,4,5-trimethyoxyphenyl) furo [3',4':6,7] naphtha[2,3-d]-1,3-dooxol-5-yl]amino]) was performed by TFChem (Val de Reuil, France). The details of the synthesis were as described for molecule 32a in US Patent Application US20090318675A1. The molecular mass of Adva-27a is 655.59 Dalton. The chemical structure of Adva-27a is shown in Figure 1C.

Topoisomerase II enzyme inhibition assay. Analysis of the activity of human topoisomerase IIα was done using the Human Topoisomerase II DNA Decatenation Assay Kit Plus (Catalog number HDD96KE) from Profoldin Inc. (Hudson, Massachusetts, USA). The assay uses a spin column to separate concatenated from decatenated DNA. Briefly, Adva-27a or etoposide (MP Biomedical LLC, Ohio, USA) was dissolved in 100% DMSO to make a 100 mM stock solution. Stock solutions were further diluted in DMSO to 10 mM, followed by 2-fold serial dilutions. The final concentration of Adva-27a or etoposide was in the range of 0.781 μM to 200 μM. The activity of human topoisomerase IIα to catalyze the production of decatenated DNA from concatenated DNA (provided in the kit) was monitored in 96-well assay plates following the manufacturer's instructions. The reaction mixture (50 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.5 mM EDTA, 10 mM $MgCl_2$, 6.5 μg/mL concatenated DNA, 1 mM ATP and 5 U/mL recombinant human topoisomerase IIα enzyme) with various drug amounts was incubated at ambient temperature for 20 min and loaded onto filter plates. After filtration, plates were washed (10 mM Tris-HCl, pH 7.5 and 10 mM NaCl). In each well of the receiver plate containing decatenated DNA, SYBR Green II dye (provided with the kit) was added and fluorescence on a microplate reader was monitored at 535 nm using an excitation wavelength of 485 nm.

Cell culture. Human prostate carcinoma cell line, PC-3, human non-small cell lung carcinoma cell line, A549, and multi-drug-resistant human small-cell lung cancer cell line, H69AR, were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). All cell lines were cultured in a 37˚C CO_2 incubator. PC-3 cells were cultured in RPMI-1640 media with 10% FBS, while A549 cells were cultured in DMEM media with 10% FBS. Glutamine (2 mM), penicillin (100 I.U.) and streptomycin (100 μg/mL) were added to the media. The breast cancer cell lines, MCF-7 and MCF-7/MDR, the latter being a doxorubicin-resistant derivative of the former (30), were grown in RPMI-1640 supplemented with L-glutamine and HEPES (ATCC), 0.1% insulin (Sigma Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (Thermo Scientific, Logan, UT, USA) and penicillin/streptomycin (5000 U/mL and 5000 μg/mL, respectively) (Lonza Biowhittaker, Walkersville, MD, USA) at 37˚C in 5% CO_2 with the MCF-7/MDR cells maintained in media containing 1 μM doxorubicin. One week prior to starting the drug treatment, the MCF-7/MDR cells were transferred to media without doxorubicin. Cells of the human multidrug-resistant small-cell lung cancer cell line, H69AR, which was derived from the parental cell line, NCI-H69 (19) were cultured in RPMI-1640 media with L-glutamine (Lonza Biowhittaker, Walkersville, MD, USA). For the H69AR cells, 20% FBS was added. Penicillin (100 I.U.) and streptomycin (100 μg/mL), HEPES buffer (10 mM) and sodium pyruvate (1 mM) were added to the media. Cells were split twice per week in T75 tissue culture flasks using 0.25% trypsin.

Cytotoxicity assay in human cancer cell lines. Two different methods were used to analyze the cytotoxicity of the drugs. PC-3 cells and A549 cells were plated at 4000 cells/well and 3000 cells/well, respectively while H69AR cells were plated at 6500 cells/well in 96-well tissue culture plates in triplicates. Twenty-four h post-plating, cells were treated with DMSO, or Adva-27a or etoposide in a serial dilution. In assays with PC3 and A549 cells, the final concentration of DMSO was 1% for all wells treated with Adva-27a or etoposide. In assays with H69AR cells, the final concentration of DMSO was 2.5%. At 68 h post-treatment, 20 μL of Alamar Blue (Invitrogen Canada Inc., Burlington, Ontario, Canada) were added to each well, and 4 h later, the plates were read on a microtiter plate reader (SpectraMax 190, Molecular Devices) at

two wavelengths (570/600 nm). The percentage of reduction of Alamar Blue was calculated using the following formula as suggested by the manufacturer:

*% reduction of Alamar Blue=(117216A$_{570}$-80586A$_{600}$)/(155677A'$_{600}$-14652A'$_{570}$)*100*

Where A is the absorbance at 570 nm or 600 nm of test wells and A' is the absorbance at 570 nm or 600 nm of negative control wells (media-only, no cells with Alamar Blue). The cell growth % was calculated as follows:

*% Growth=(% of reduction of Alamar Blue in a treated well)/(the mean of % of reduction of DMSO-treated wells)*100*

For analysis of the cytotoxicity in the MCF-7 and MCF-7/MDR cells, 6000 cells were plated per well in 24-well plates. The cells were allowed to incubate for 18-24 hours, then DMSO (0.5%) or Adva-27a or etoposide (Sigma Aldrich, St. Louis, MO, USA) were added at various concentrations. The cells were then monitored for cell viability 1-9 days later by adding Alamar Blue (10% of media) and reading the fluorescence at 2 and 4 h in a fluorescence microtiter plate reader (Cytofluor II Biosearch, Bedford, MA, USA) with excitation filter at 530/25 and emission filter at 620/40 and a gain of 48. Growth curves data were calculated as % fluorescence relative to the value detected in comparable cells in the presence of DMSO-only. For statistical analysis, unpaired Student's *t*-test was used to compare the group treated with a drug *vs*. the group treated with DMSO. Standard errors of the mean (SEM) were used to represent the variation of the data.

Quantitative PCR (qPCR) analysis of mdr1 mRNA in MCF-7/MDR cells. RNA extraction was performed using the RNA XS® extraction kit from Macherey-Nagel Inc. (Bethlehem, PA, USA) according to the manufacturer's protocol. Total RNA was quantified and RNA integrity was evaluated by the ratio of 28S/18S ribosomal RNA using the Agilent BioAnalyzer 2100 (Agilent Technologies, Mississauga, ON, Canada), following the manufacturer's protocol. A total of 1 μg purified RNA was reverse-transcribed in a final volume of 20 μL using the First Strand cDNA Transcriptor Kit (Roche Diagnostics, Laval, Quebec, Canada) with oligo-dT primers as recommended by the supplier. For *mdr1* (or ABCB1) mRNA expression level, the primers and probes were TaqMan Gene Expression Assays-on-Demand products from Applied Biosystems. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. The ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) was used to detect the amplification level and was programmed with an initial step of 3 min at 95˚C, followed by 45 cycles of 5 sec at 95˚C and 30 sec at 60˚C. All reactions were performed in triplicate and the average values of Ct (threshold cycle) were used for quantification. GAPDH was used as endogenous control. The relative quantification of target genes was determined using the $2^{-\Delta\Delta CT}$ method. Briefly, the Ct values of target genes were normalized to an endogenous control gene (endogenous control) [ΔCT=Ct (target gene) – Ct (endogenous control)] and compared with a calibrator: ΔΔCT=ΔCt target – ΔCt calibrator. Relative expression (RQ) was calculated using the Sequence Detection System 2.2.2 software using the $RQ=2^{-\Delta\Delta CT}$ formula. The measurements data were collected and expressed as mean values±standard deviation (s.d.) and were analyzed with the Statistica 9.0 software (STATSOFT; Statistica, Tulsa, OK, USA). Statistical significance was determined by one-way ANOVA followed by Tukey post hoc test. Differences were considered highly significant at $^{**}p<0.01$.

Western blot analysis of P-gp. The level of the P-gp protein in both MCF-7 and MCF-7/MDR cell lines was assessed using western blot analysis. Cells (2×10^6) were harvested by centrifugation and washed twice with PBS, and then lysed in 200 mL RIPA buffer (50 mM Tris–HCl, pH7.4, 1% NP-40, 50 mM NaCl, 0.1% SDS, 1% Na-deoxycholic acid, 1 mM sodium orthovanadate, 2 mM PMSF) for 30 min on ice with gentle shacking. After centrifugation at 12,000 ×g, 4˚C for 10 min, the total protein concentration was determined by the BCA protein assay with BSA as standard. An aliquot of protein (30 μg) was separated by 5% SDS-PAGE and then transferred to a PVDF membrane. The membrane was blocked for 2 h at room temperature with 5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBST), then, the membranes were incubated with primary a antibody anti-P-gp (CALBIOCHEM, Anti-P-Glycoprotein Mouse mAb C219, Cat#517310) at 1:5000 dilutions in 5% non-fat milk overnight at 4˚C, then the membranes were washed with PBST for three times, and incubated with the secondary rabbit anti-mouse IgG-HRP at 1:10000 dilution for 1 h at room temperature. Finally, the signals were visualized using enhanced chemiluminescence detection (Thermo Scientific, Rockford, IL).

Human liver microsomal clearance assay. Adva-27a and etoposide (at a final concentration of 1 μM) were incubated in duplicates with 0.3 mg/mL human liver microsomes (Invitrogen) at 37˚C. Two control compounds were tested at the same concentration: warfarin as a low-metabolized compound and verapamil as a high-metabolized compound (5, 10). The reaction contained microsomal proteins in 100 mM potassium phosphate, 2 mM NADPH, 3 mM $MgCl_2$, pH 7.4. A control was run for each test agent omitting NADPH, to detect for NADPH-free metabolism. At the indicated times (0, 10, 20, 30 and 60 min), an aliquot was removed from each reaction and mixed with an equal volume of ice-cold Stop Solution (acetonitrile containing 1 μM propanol). The stopped reactions were incubated at least 10 min at –20˚C. The samples were centrifuged to remove precipitated proteins (3500 rpm, 20 min at room temperature) and the supernatants were analyzed by LC-MS/MS (liquid chromatography-tandem mass spectrometry) using an Agilent 6410 mass spectrometer, coupled with an Agilent 1200 HPLC (high pressure liquid chromatography) and a CTC PAL chilled autosampler. After separation on a C18 reverse phase HPLC column (Agilent) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode. Data were converted to % drug remaining by dividing the current amount by the time zero concentration value and were fitted to a first-order decay model to determine half-life ($T_{1/2}$). Intrinsic clearance (CL_{int}) was calculated from the $T_{1/2}$ and microsomal protein concentration using the formula:

$CL_{int}=ln(2)/(T_{1/2}[microsomal\ protein\ concentration])$

Pharmacokinetic analysis of Adva-27a and etoposide in rats. Adva-27a or etoposide was weighed in an airtight container and dissolved in 40% HP-β-CD by vortexing, followed by sonication and homogenization until a uniform stock solution of 1.0 mg/mL was obtained. The pH value of the stock solution was 7.0. The dose used for injection was 2.5 mg/kg. Five-to six-week-old male Sprague-Dawley Rats (Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) were used. The body weight of rats was between 240 and 255 g at the beginning of dosing. Animals were examined for their general health condition and acclimatized for 5 days prior to compound administration. Animals were housed 3 per cage with

an automatic watering system. Cage card and tail masking was used to identify the rats. The animal room environment was controlled (temperature 20-25˚C, relative humidity 40-70%, and 12 h light/dark cycle with fresh air change). A standard certified commercial rodent chow (Beijing Keaoxieli) irradiated by Co-60 and sterile water were provided to the animals *ad libitum*. Animals were fasted overnight prior to dosing. Adva-27a or etoposide was given under one single bolus injection *via* the jugular vein. Blood (300 μL at each time point) was collected at 5, 15, 30 min, and 1, 2, 4, 6, 8 and 24 h post-injection. After each blood collection, 0.3 mL saline was given through a cannulated tube in the carotid artery. Food was returned 2 h post-dosing. The animals were monitored up to 2 h post-injection and again at 24 h post-injection. Blood samples were collected from carotid artery into a tube containing EDTA-K2 (2.5%) (Beijing Chemical Reagent Company, Beijing, China) and were centrifuged at ×2000 g for 5 min at 4°C. Obtained plasma was transferred into a polyethylene microcentrifuge tube and stored at –80˚C until analysis. At the end of the study, all rats were euthanized. For detection of etoposide in rat plasma, LC-MS/MS was used. The chromatographic system consisted of an Agilent 1200 Series LC System and a GRACE Alltima HP C18 column (5 μm, 2.1×50 mm) was connected to an AB API 4000 tandem mass spectrometer. Data were acquired *via* the multiple reactions monitoring (MRM) system. The MS/MS ion transitions were monitored at m/z of 589.2/229.0 for etoposide. A gradient HPLC method was employed for the separation. Mobile phase A consisted of 0.1% formic acid and 10% acetonitrile in water, and mobile phase B consisted of 0.1% formic acid in 100% acetonitrile. The gradient profile was as follows (minute, %A/%B): 0, 95/5; 0.1, 95/5; 0.2, 5/95; 1.8, 5/95; 1.9, 95/5; 3.0, 95/5. The flow rate was 0.4 mL/min with an injection volume of 10 μL. Plasma concentrations were analyzed using a non-compartmental method. Plasma concentration of Adva-27a was analyzed by LC-MS/MS, similarly to the method for etoposide except that the MS/MS ion transitions were monitored at m/z of 656.3/397.3 and the gradient profile was shown as follows (minute, %A/%B): 0, 90/10; 0.1, 90/10; 0.2, 5/95; 1.4, 5/95; 1.5, 90/10; 2.6, 90/10.

Results

Dose-dependent inhibition of topoisomerase IIα in vitro. A new derivative of etoposide, Adva-27a, was designed and synthesized (Figure 1C). Adva-27a was found to induce a dose-dependent inhibition of recombinant human topoisomerase IIα activity *in vitro,* as monitored by the production of decatenated DNA (Figure 2). The IC_{50} of Adva-27a for inhibition of topoisomerase IIα was determined to be 13.7 μM. Under the same experimental conditions, etoposide was confirmed to be a topoisomerase IIα inhibitor with an IC_{50} of 4.0 μM. Compared to etoposide, the replacement on the C4 position with an aminoalkyl chain and on the E4' position with a methoxyl group in Adva-27a resulted in a ~3-fold reduction in enzyme inhibitory activity *in vitro*.

Growth inhibition of drug-sensitive and multidrug-resistant breast cancer cells. We analyzed the growth inhibitory activity of Adva-27a compared to etoposide in the drug-sensitive breast cancer cell line, MCF-7, and in a multidrug-resistant variant of MCF-7 derived by doxorubicin selection, MCF-7/MDR. With the drug-sensitive MCF-7 cell line, the concentration-dependent growth inhibitory activity of Adva-27a was similar to that of etoposide starting at approximately 20 μM (Figure 3A). Similarly, the time-dependence study performed at 100 μM Adva-27a or etoposide yielded nearly superimposable curves (Figure 3B). With the MCF-7/MDR cell line however, quite the opposite was observed. In these cells, etoposide even at 100 μM had little effect on cell growth, while the new compound, Adva-27a, caused a dramatic reduction in cell growth starting at a concentration between 20 and 30 μM (Figure 3A). The results of the time-dependence study were also dramatic in terms of the much greater inhibitory effect of Adva-27a on the MCF-7/MDR cells relative to etoposide (Figure 3B). With etoposide, some MCF-7/MDR cell growth inhibition was observed between days 2 and 5, but after that, the cells quickly recovered and regrew to the same level as the DMSO-treated control population. This apparent cell growth slowdown and recovery phenomenon was not seen with Adva-27a. Instead, Adva-27a limited cell growth very effectively starting at day 3 and continued the inhibitory effect progressively through day 9 (Figure 3B). We further studied the mechanisms underlying the sensitivity of the MCF-7/MDR cells to Adva-27a by analyzing the level of expression of the *mdr1* gene using qPCR and the P-gp protein using western blotting. The *mdr1* gene which encodes for P-gp was the first ABC transporter identified to be overexpressed in breast cancer cell lines displaying multidrug resistance (28). P-gp is a broad spectrum efflux

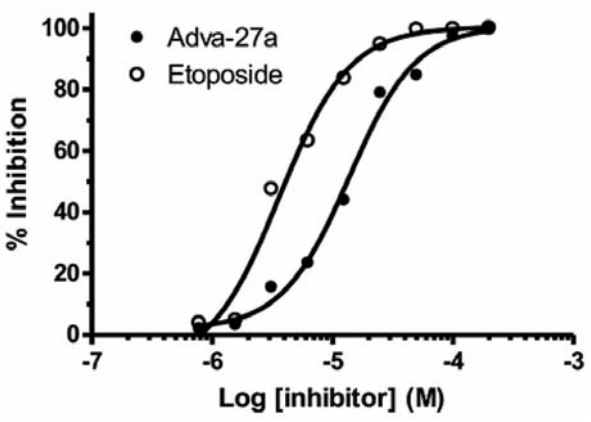

Figure 2. *Dose-dependent inhibition of recombinant human topoisomerase IIα by Adva-27a or etoposide in vitro. Adva-27a or etoposide in the range of 0.781 μM to 200 μM was used in human topoisomerase IIα activity assay, as described in the methods section. The production of decatenated DNA from concatenated DNA was monitored by fluorescence in a 96-well plate providing the percentage of enzyme inhibition data shown.*

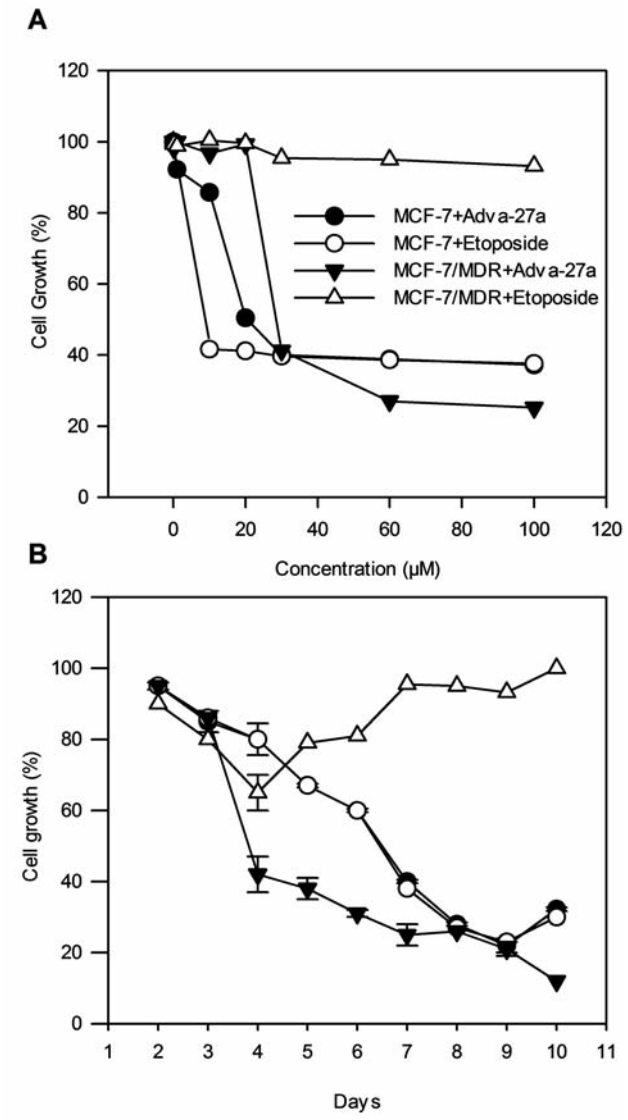

Figure 3. *Dose- and time-dependent cytotoxic activity of Adva-27a and etoposide in MCF-7 and MCF-7/MDR breast cancer cells. MCF-7 or MCF-7/MDR cells were cultured in the presence of different concentrations of Adva-27a or etoposide and the cells were assayed for metabolic activity using the fluorescence of Alamar Blue, as described in the methods section. The fluorescence relative to that detected in 1% DMSO-treated control cells is plotted versus the concentration of the compound after 7 days of treatment (panel A) and versus the days of treatment with 100 μM of the compounds (panel B). The data are representative of an experiment performed 3 times.*

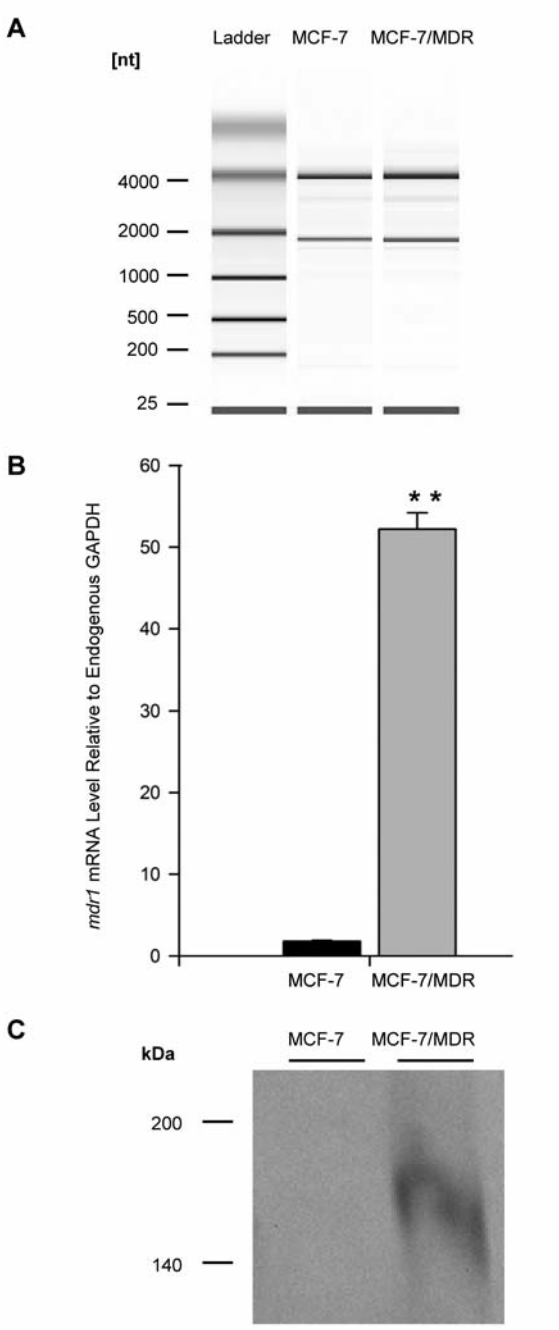

Figure 4. *Levels of mdr1 mRNA and P-gp protein in MCF-7 and MCF-7/MDR cells. (A) Total RNA extraction was performed on MCF-7 and MCF-&/MDR cells. Total RNA was quantified and RNA integrity measured using the Agilent BioAnalyzer 2100 (Agilent Technologies, Mississauga, ON). (nt)=Nucleotide, L=standard ladder, the green band is a lower marker, which allows sample alignment and permits comparison for RIN calculation. RIN=RNA integrity number, is an algorithm-based numbering system that calculates-RNA integrity with 10 being the most intact and 1 being fully-degraded. (B) mRNA level of mdr1 was detected with real-time PCR; **p<0.01. (C) Protein levels of P-gp in MCF-7 and MCF-7/MDR cell lines were assessed by western blot analysis using 30 μg total protein from each cell line.*

pump and is known to be involved in the transport of many drugs including doxorubicin and etoposide (28). Both *mdr1* mRNA and P-gp protein levels in MCF-7/MDR cells were significantly higher than in MCF-7 cells (Figure 4). More specifically, the expression level of *mdr1* mRNA was 29-times greater in MCF-7/MDR than in MCF-7. Based on the

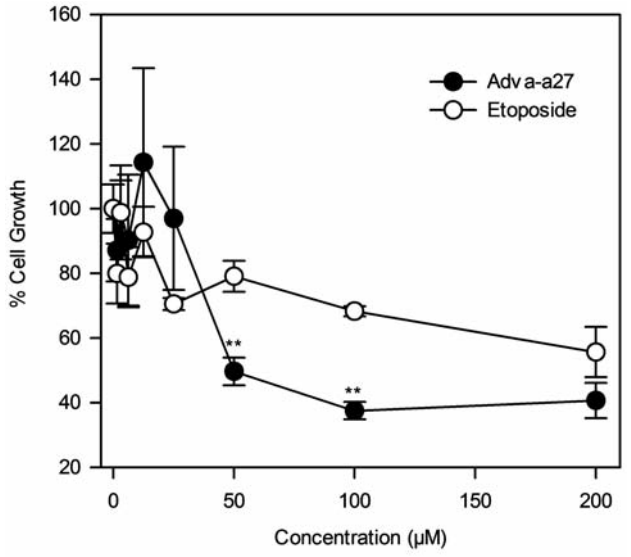

Figure 5. *Anti-proliferative activity of Adva-27a and etoposide at equal molar concentrations in the multidrug-resistant human small-cell lung cancer cell line, H69AR, following a 72-hour incubation. The Alamar Blue reduction was determined using absorbance at 570 and 600 nm and used as an indicator of cell growth, as described in the methods section. "**", p<0.01 compared to the values of etoposide at equal molar concentration.*

fact that Adva-27a is very effective at killing the MCF-7/MDR cells, it appears that this compound is not transported by this efflux protein.

Growth inhibition of multidrug-resistant small-cell lung cancer cells. To investigate if Adva-27a could also overcome multidrug resistance in another human cancer cell line of a different cancer type and tissue origin, we performed cytotoxicity studies using the human small-cell lung cancer cell line, H69AR. This multidrug resistant cell line was derived from the parental cell line, NCI-H69 by using Adriamycin (doxorubicin) selection (19). Figure 5 shows that Adva-27a can reduce the growth of H69AR cells more significantly than etoposide starting from 50 μM ($p<0.01$). The doxorubicin-selected H69AR cells used in this study are known to overexpress ABCC1 (MRP1), one of the three most frequently encountered members the ATP-binding cassette family of drug transporters involved in multidrug resistance (4, 9). It is interesting to observe that Adva-27a can overcome multidrug resistance in cancer cells associated with different ABC transporters.

Cytotoxicity of Adva-27a in other cancer cells. We also analyzed the growth inhibitory activity of Adva-27a, compared to etoposide in PC-3, a prostate cancer cell line, and A549, a non-small-cell lung cancer cell line. Adva-27a exhibited dose-dependent growth inhibitory activity in PC-3

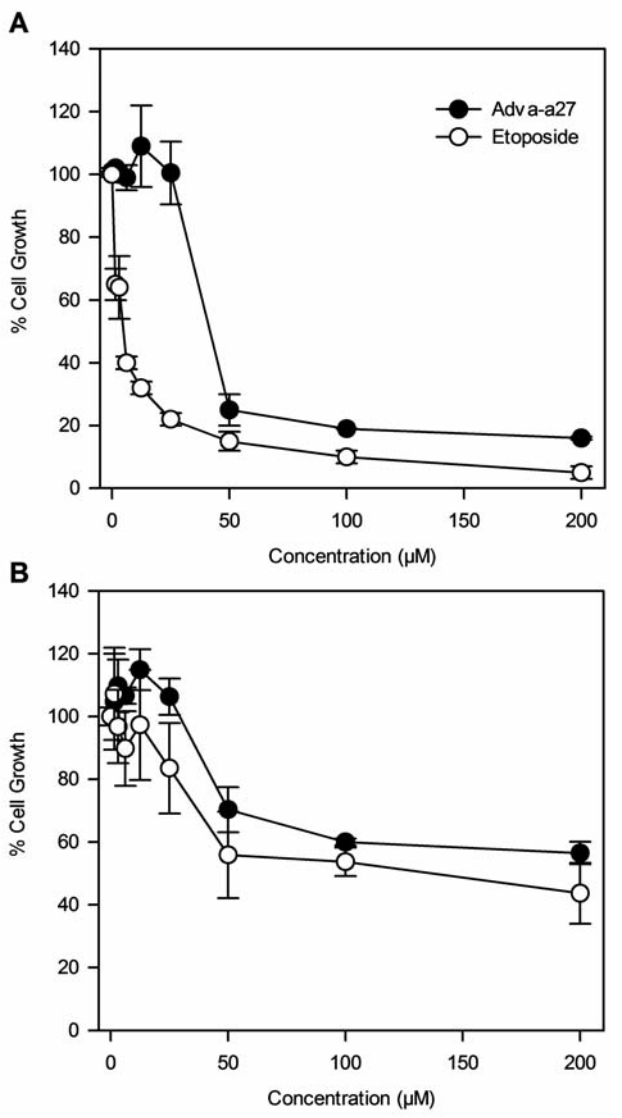

Figure 6. *Dose-dependent anti-proliferative activity of Adva-27a and etoposide in the PC-3 prostate cancer cell line (A) and the A549 lung cancer cell line (B) following a 72-hour incubation. The Alamar Blue reduction was determined using absorbance at 570 and 600 nm and used as an indicator of cell growth, as described in the methods section.*

and A549 cells following 72 h of incubation (Figure 6A and B). In PC-3 cells, the growth inhibitory activity of Adva-27a appeared to be dose-dependent but weaker than that of etoposide with IC_{50}'s of 3 μM and 42 μM, for etoposide and Adva-27a, respectively (Figure 6A). In the A549 cell line, the growth-inhibitory activity of Adva-27a was also dose-dependent and largely similar to that of etoposide (Figure 6B). Adva-27a had previously been shown to inhibit the growth of other cancer cell lines including KB (pharyngeal cancer), SF-268 (brain cancer), HL-60 (leukemia) and HT-29 (colon cancer) (US Patent Application: US20090318675A1).

Table I. *Microsomal intrinsic clearance (CL_{int}) and half-life ($T_{1/2}$) of Adva-27a and etoposide in human liver microsomes in vitro.*

	With NADPH		Without NADPH	
	CL_{int} (μL/min*mg)	$T_{1/2}$, min	CL_{int} (μL/min*mg)	$T_{1/2}$, min
Adva-27a	60	38.7	43	54
Etoposide	64	35.8	48	48
Verapamil	168	13.7	3	>200
Warfarin	1	>200	ND	ND

ND, Not determined.

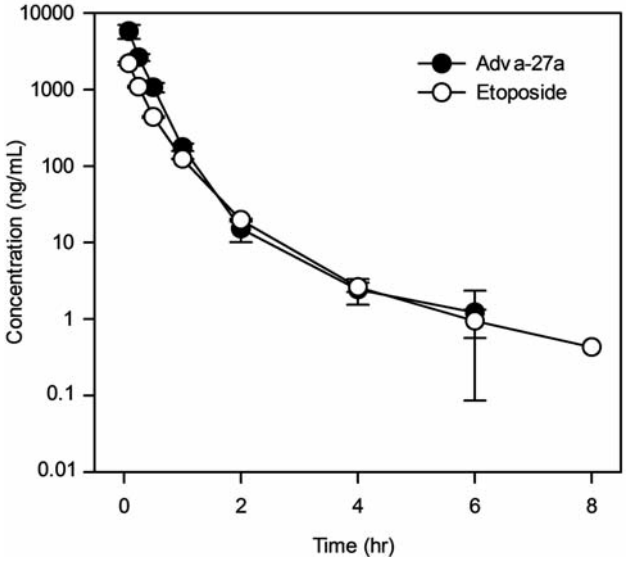

Figure 7. *Time-dependent change in plasma concentration of Adva-27a (open diamond) or etoposide (filled square) in rats after a single intravenous bolus injection at a dose of 2.5 mg/kg. Mean values and standard error of mean (SEM) are presented (n=3 for each compound). The large variation at 6 h was represented by the rats treated with Adva-27a (concentration 1.22 ng/mL with SEM of 1.13 ng/mL).*

Metabolic stability of Adva-27a in human liver microsomes in vitro. Non-renal clearance of etoposide accounts for 60% of elimination of etoposide in humans (8). Though the metabolism of etoposide is not fully elucidated, it is known that the NADPH-dependent cytochrome *P450* system and the NADPH-independent glucuronidation process are partly involved in the human non-renal clearance of etoposide (22, 27). In the present study, we analyzed the metabolic stability of Adva-27a and etoposide *in vitro* using human liver microsomes. Similar to etoposide, Adva-27a was found to have a desirable medium-low clearance rate in human liver microsomes *in vitro via* both NADPH-dependent and NADPH-independent mechanisms (Table I). The estimated $T_{1/2}$ of Adva-27a was 38.7 min when NADPH was added and 54 min without the addition of NADPH. For etoposide, the estimated $T_{1/2}$ with and without NADPH was 35.8 and 48 min, respectively (Table I). Under the same experimental conditions, verapamil, a high clearance control, exhibited an estimated $T_{1/2}$ of ~14 min in the presence of NADPH and a $T_{1/2}$ of >200 min without NADPH. In contrast, warfarin, a low clearance control, had a clearance rate in the presence of NADPH of more than 200 min (Table I).

Pharmacokinetic properties of Adva-27a and etoposide. A new analog of etoposide with increased plasma concentration and slower clearance may lead to improved efficacy of cancer treatment. The pharmacokinetic properties of etoposide in rats had previously been analyzed using a simple HPLC-based method with a detection limit of 10 ng/mL of plasma. For the present study, we developed a more sensitive method of bioanalysis using LC-MS/MS with a detection limit for etoposide is 1 ng/mL (see Materials and Methods). Using this 10-fold more sensitive detection technique, we tested whether Adva-27a had an improved pharmacokinetic profile compared to etoposide in rats under similar experimental conditions. Adva-27a and etoposide were administered to rats at 2.5 mg/kg by a single intravenous (*i.v.*) bolus injection and the plasma concentration of the drugs was analyzed over time (Figure 7). The pharmacokinetic parameters of Adva-27a and etoposide were determined by non-compartmental modeling and are presented in Table II. After *i.v.* administration, both compounds were cleared from the plasma in a *bi*-phasic mode and had a similar terminal plasma half-life ($T_{1/2}$) of 1.13-1.16 h and a mean plasma resident time (MRT) of 0.27-0.37 h. Interestingly, however, Adva-27a produced a mean initial plasma concentration (C_0) of 8779 ng/mL, which was approximately 2.8-times the value for etoposide (3183 ng/mL) (Table II). When converted to molar concentration, the value for Adva-27a was found to be approximately 2.5- times that of etoposide. The area under the plasma concentration-*versus*-time curve (AUC_{inf}) for Adva-27a (2211 hr*ng/mL) was approximately 2.4-times that of etoposide (933 hr*ng/mL), while the plasma clearance rate (CL) for Adva-27a (19.0 mL/min/kg) was 2.4-fold lower than that for etoposide (44.7 mL/min/kg) (Table II). On the other hand, etoposide had a larger volume of distribution at steady state (Vss) in plasma (1.04 L/kg) than that of Adva-27a (0.323 L/kg) but both values were far greater than the plasma volume of 0.03 L/kg for rats, suggesting the distribution of both compounds in compartments other than plasma, namely tissues and organs (Table II).

Table II. *Pharmacokinetic parameters of Adva-27a and etoposide in rat plasma.*

	Animal #	$T_{1/2}$ (h)	C_0 (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{inf} (h*ng/mL)	AUC_{Extr} (%)	V_z (L/kg)	V_{ss} (L/kg)	CL (mL/min/kg)	MRT (h)
Adva-27a	1	1.09	5659	1985	1987	0.085	1.98	0.385	21.0	0.30
	2	1.30	9276	2113	2116	0.117	2.21	0.311	19.7	0.25
	3	0.99	11404	2527	2529	0.072	1.42	0.272	16.5	0.27
	Mean	1.13	8779	2208	2211	0.091	1.87	0.323	19.1	0.28
	SD	0.16	2904	283	283	0.023	0.41	0.057	2.3	0.02
	CV%	13.8	33.1	12.8	12.8	25.0	21.9	18.0	12.2	8.6
Etoposide	4	1.16	3106	905	906	0.104	4.63	1.070	46.0	0.379
	5	1.28	2715	939	940	0.142	4.90	1.070	44.3	0.386
	6	1.03	3727	952	954	0.207	3.89	0.967	43.7	0.354
	Mean	1.16	3183	932	933	0.151	4.47	1.036	44.7	0.373
	SD	0.13	510	24	25	0.052	0.52	0.059	1.2	0.017
	CV%	10.8	16	2.58	2.63	34.5	11.7	5.7	2.7	4.7

$T_{1/2}$, Half-life; C_0, initial plasma concentration; AUC, area under the curve; V_z, volume of distribution; V_{ss}, volume of distribution at steady state; CL, intrinsic clearance; MRT, mean plasma resident time; SD, standard deviation; CV, co-efficient of variation.

Discussion

We have designed and synthesized a new epipodophyllotoxin which bears the laboratory name Adva-27a. The structure of Adva-27a differs from that of the most commonly known epipodophyllotoxin, etoposide, in that it has an amino linked GEM-difluoro glycoside at the C4 position and a methoxyl group at the E4' position (Figure 1). The latter appears to be responsible for the observed ~3-fold reduction in topoisomerase II inhibitory activity compared to etoposide, as the C4 substitutions have previously been shown not to be involved in the enzyme inhibition function (3, 21, 29). According to recent reports, the glycoside moiety of etoposide rests in a spacious binding pocket of the topoisomerase with relatively few interactions with the enzyme (3, 21, 29). Thus, the C4 substitution in Adva-27a should generally be allowed and is not likely responsible for our observed reduction in topoisomerase IIα inhibitory activity. We suspect that the substitution of the E4' hydroxyl group with a methoxyl group in Adva-27a may have resulted in the observed reduction in topoisomerase II inhibitory activity. As reported in the literature, the hydroxyl group on the E4' position imparts crucial hydrogen bonding and Van der Waals interactions between the E ring and certain amino acid side chains of the topoisomerase II enzyme (3, 21, 29).

Interestingly however, the C4 and E4' changes in Adva-27a have resulted in a remarkable cytotoxic activity against multidrug-resistant breast and lung cancer cells (Figures 3 and 5). At present, the precise mechanism by which these substitutions have allowed Adva-27a to overcome multidrug resistance in these cells is not known. However, it appeared that Adva-27a is able to evade recognition by both P-gp (overexpressed in MCF-7/MDR cells) and MRP-1 (H69AR cells). It is also possible that Adva-27a may be able to evade BCRP, as it is likely that this drug resistance protein is also expressed to some extent in both MCF-7/MDR and H69AR (4, 6, 28). To date, it is still unknown what drives the ability of a drug to evade recognition by the ATP-binding cassette family of proteins. The apparent ability of Adva-27a to evade these efflux pumps will allow it to be used as a monotherapy without any requirement for the addition of inhibitors of the ATP-binding cassette family of proteins. The sensitivity of different cell lines and cancers to etoposide is well-known and is consistent with the cell killing observed in this work (14, 18, 20, 25, 26). The fact that Adva-27a is equally potent in killing drug-sensitive human cancer cells (MCF-7, A549, PC3, KB, SF-268, HL-60 and HT-29), but more effective than etoposide in killing multidrug-resistant cancer cells (MCF-7/MDR and H69AR) implies that Adva-27a could form the basis of a new therapy to overcome multidrug resistance in human cancer. It would be interesting to test directly whether Adva-27a is a weaker substrate of the drug transporters, P-gp, MRP1 and BCRP, compared to etoposide.

In addition to cytotoxic effects, we studied the metabolic stability of Adva-27a in human liver microsomes *in vitro*. Our results showed that the clearance rate of Adva-27a in human liver microsomes is similar to that of etoposide and involves both NADPH-dependent and NADPH-independent pathways (Table I). The NADPH-dependent cytochrome *P450* family of enzymes comprises the major enzymes involved in drug metabolism, accounting for approximately 75% of all drug clearance reactions (11). Many drugs may increase or decrease the activity of the cytochrome *P450* isozymes. In addition, the level of cytochrome *P450* expression varies across individuals due to genetic variations depending on ethnic backgrounds (24). These are a major source of adverse drug interactions and general toxicity (11,

24). It remains to be determined whether the cytochrome *P450*-mediated metabolism of Adva-27a will be different from that of etoposide, considering that Adva-27a has an extra O-methyl group at the E4' position (Figure 1).

Finally, we analyzed the pharmacokinetic properties in rats of Adva-27a and etoposide under similar experimental conditions. Collectively, the results indicate that Adva-27a had much better plasma accumulation and slower plasma clearance rate in rats compared to etoposide. Since Adva-27a had not only a lower clearance but also a lower plasma distribution than etoposide, we speculate that Adva-27a may have distributed into tissues and organs to a greater extent than etoposide. It will be interesting to compare the tissue distribution of the two compounds by direct measurements. The difference in tissue distribution may help to target Adva-27a to other tumor types, which are resistant to etoposide. Another analog of etoposide, TOP-53, was found to have superior anti-tumor activity in non-small cell lung cancer, a tumor type resistant to etoposide, partly due to its superior tissue distribution in xenograft models (26). If the larger area under the curve (AUC) and higher plasma concentration of Adva-27a in rats, and its superior growth-inhibitory activity towards multidrug-resistant cancers *in vitro* relative to etoposide can be extrapolated to humans, it may be that Adva-27a has greater clinical efficacy.

Overall, our investigations indicate that Adva-27a is able to kill multidrug-resistant breast cancer cells *in vitro* and has more desirable metabolic stability and pharmacokinetic properties *in vitro* and *in vivo* than etoposide. Design and development of non-ester, N-glycoside analogs of podophyllotoxin, such as Adva-27a, may prove to be a practical strategy for cancer therapy, especially for overcoming multidrug-resistant cancers.

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References

1 Arnold AM: Podophyllotoxin derivative VP 16-213. Cancer Chemother Pharmacol *3*: 71-80, 1979.

2 Bates SE, Wilson WH, Fojo AT, Alvarez M, Zhan Z, Regis J, Robey R, Hose C, Monks A, Kang YK and Chabner B: Clinical Reversal of Multidrug Resistance. Oncologist *1*: 269-275, 1996.

3 Bender RP, Jablonksy MJ, Shadid M, Romaine I, Dunlap N, Anklin C, Graves DE, and Osheroff N: Substituents on etoposide that interact with human topoisomerase IIalpha in the binary enzyme-drug complex: contributions to etoposide binding and activity. Biochemistry *47*: 4501-4509, 2008.

4 Brock I, Hipfner DR, Nielsen BS, Jensen PB, Deeley RG, Cole SP and Sehested M: Sequential coexpression of the multidrug resistance genes MRP and mdr1 and their products in VP-16 (etoposide)-selected H69 small cell lung cancer cells. Cancer Res *55*: 459-462, 1995.

5 Brown HS, Griffin M and Houston JB: Evaluation of cryopreserved human hepatocytes as an alternative *in vitro* system to microsomes for the prediction of metabolic clearance. Drug Metab Dispos *35*: 293-301, 2007.

6 Calcagno AM and Ambudkar SV: Molecular mechanisms of drug resistance in single-step and multi-step drug-selected cancer cells. Methods Mol Biol *596*: 77-93, 2010.

7 Chen Y, Su YH, Wang CH, Wu JM, Chen JC and Tseng SH: Induction of apoptosis and cell cycle arrest in glioma cells by GL331 (a topoisomerase II inhibitor). Anticancer Res *25*: 4203-4208, 2005.

8 Clark PI and Slevin ML: The clinical pharmacology of etoposide and teniposide. Clin Pharmacokinet *12*: 223-252, 1987.

9 Cole SP, Chanda ER, Dicke FP, Gerlach JH and Mirski SE: Non-P-glycoprotein-mediated multidrug resistance in a small cell lung cancer cell line: evidence for decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II. Cancer Res *51*: 3345-3352, 1991.

10 Giuliano C, Jairaj M, Zafiu CM and Laufer R: Direct determination of unbound intrinsic drug clearance in the microsomal stability assay. Drug Metab Dispos *33*: 1319-1324, 2005.

11 Guengerich FP: Cytochrome p450 and chemical toxicology. Chem Res Toxicol *21*: 70-83, 2008.

12 Guo A, Marinaro W, Hu P and Sinko PJ: Delineating the contribution of secretory transporters in the efflux of etoposide using Madin-Darby canine kidney (MDCK) cells overexpressing P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP1), and canalicular multispecific organic anion transporter (cMOAT). Drug Metab Dispos *30*: 457-463, 2002.

13 Hande KR: Etoposide: four decades of development of a topoisomerase II inhibitor. Eur J Cancer *34*: 1514-1521, 1998.

14 Huang TS, Lee CC, Chao Y, Shu CH, Chen LT, Chen LL, Chen MH, Yuan CC and Whang-Peng J: A novel podophyllotoxin-derived compound GL331 is more potent than its congener VP-16 in killing refractory cancer cells. Pharm Res *16*: 997-1002, 1999.

15 Lee KH: Novel antitumor agents from higher plants. Med Res Rev *19*: 569-596, 1999.

16 Lin S, Huang HC, Chen LL, Lee CC and Huang TS: GL331 induces down-regulation of cyclin D1 expression *via* enhanced proteolysis and repressed transcription. Mol Pharmacol *60*: 768-775, 2001.

17 Liu JM, Chen LT, Chao Y, Li AF, Wu CW, Liu TS, Shiah HS, Chang JY, Chen JD, Wu HW, Lin WC, Lan C and Whang-Peng J: Phase II and pharmacokinetic study of GL331 in previously treated Chinese gastric cancer patients. Cancer Chemother Pharmacol *49*: 425-428, 2002.

18 Mimeault M, Venkatraman G, Johansson SL, Moore E, Henichart JP, Depreux P, Lin MF and Batra SK: Novel combination therapy against metastatic and androgen-independent prostate cancer by using gefitinib, tamoxifen and etoposide. Int J Cancer *120*: 160-169, 2007.

19 Mirski SE, Gerlach JH, and Cole SP: Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin. Cancer Res *47*: 2594-2598, 1987.

20 Olie RA, Simoes-Wust AP, Baumann B, Leech SH, Fabbro D, Stahel RA, and Zangemeister-Wittke U: A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res *60*: 2805-2809, 2000.

21 Pitts SL, Jablonksy MJ, Duca M, Dauzonne D, Monneret C, Arimondo PB, Anklin C, Graves DE and Osheroff N: Contributions of the D-Ring to the activity of etoposide against human topoisomerase IIalpha: potential interactions with DNA in the ternary enzyme – drug – DNA complex. Biochemistry *50*: 5058-5066, 2011.

22 Relling MV, Evans R, Dass C, Desiderio DM and Nemec J: Human cytochrome P450 metabolism of teniposide and etoposide. J Pharmacol Exp Ther *261*: 491-496, 1992.

23 Ross W, Rowe T, Glisson B, Yalowich J and Liu L: Role of topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage. Cancer Res *44*: 5857-5860, 1984.

24 Rostami-Hodjegan A and Tucker GT: Simulation and prediction of *in vivo* drug metabolism in human populations from *in vitro* data. Nat Rev Drug Discov *6*: 140-148, 2007.

25 Shimada K, Nakamura M, Ishida E, Kishi M, Yonehara S and Konishi N: c-Jun NH2-terminal kinase-dependent Fas activation contributes to etoposide-induced apoptosis in p53-mutated prostate cancer cells. Prostate *55*: 265-280, 2003.

26 Utsugi T, Shibata J, Sugimoto Y, Aoyagi K, Wierzba K, Kobunai T, Terada T, Oh-hara T, Tsuruo T and Yamada Y: Antitumor activity of a novel podophyllotoxin derivative (TOP-53) against lung cancer and lung metastatic cancer. Cancer Res *56*: 2809-2814, 1996.

27 Watanabe Y, Nakajima M, Ohashi N, Kume T and Yokoi T: Glucuronidation of etoposide in human liver microsomes is specifically catalyzed by UDP-glucuronosyltransferase 1A1. Drug Metab Dispos *31*: 589-595, 2003.

28 Wind NS and Holen I: Multidrug resistance in breast cancer: from *in vitro* models to clinical studies. Int J Breast Cancer *2011*: 967419, 2011.

29 Wu CC, Li TK, Farh L, Lin LY, Lin TS, Yu YJ, Yen TJ, Chiang CW and Chan NL: Structural basis of type II topoisomerase inhibition by the anticancer drug etoposide. Science *333*: 459-462, 2011.

30 Yeh GC, Lopaczynska J, Poore CM and Phang JM: A new functional role for P-glycoprotein: efflux pump for benzo(alpha) pyrene in human breast cancer MCF-7 cells. Cancer Res *52*: 6692-6695, 1992.

31 You Y: Podophyllotoxin derivatives: current synthetic approaches for new anticancer agents. Curr Pharm Des *11*: 1695-1717, 2005.

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