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Preclinical report

Characterization of human lung cancer cells resistant to 4′-*O*-demethyl-4β-(2″-nitro-4″-fluoroanilino)-4-desoxypodophyllotoxin, a unique compound in the epipodophyllotoxin antitumor class

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A new semi-synthetic podophyllotoxin derivative, 4′-*O*-demethyl-4β-(2″-nitro-4″-fluoroanilino)-4-desoxypodophyllotoxin (compound 1), an analog of GL-331 (compound 2), is a potent and broad-spectrum inhibitor of cultured human cancer and drug-resistant cell growth. In general, 4′-demethylepipodophyllotoxin analogs, including 2, exert anti-tumor activity by targeting the nuclear enzyme DNA topoisomerase II, but 1 is not an enzyme inhibitor. Unlike the cytotoxic activity of compound 2, cell killing by 1 is dose-limiting and a significant fraction of cells (30–40%) survive treatment. As an approach to investigate mechanism of action, 1-resistant A549 (human lung cancer) sub-lines were selected and characterized. Results of the work show that 1-resistant cells: (i) are moderately cross-resistant (2- to 3-fold) to various cytotoxic drugs via a P-glycoprotein-independent mechanism, (ii) have an altered growth habit, (iii) are deficient in normal attachment on plastic and collagen substrata, and (iv) have an altered plasma membrane protein composition including several proteins in the 140–>200 kDa molecular mass range and a doublet of phosphoserine-containing proteins of about 135 kDa. Since 1 treatment of cells affects neither cellular attachment or membrane-protein phosphorylation, the changes observed in 1-resistant cells are interpreted as a survival response to drug action. [© 2000 Lippincott Williams & Wilkins.]

Key words: Cell attachment, drug resistance, epipodophyllotoxin analog, growth inhibition, protein phosphorylation.

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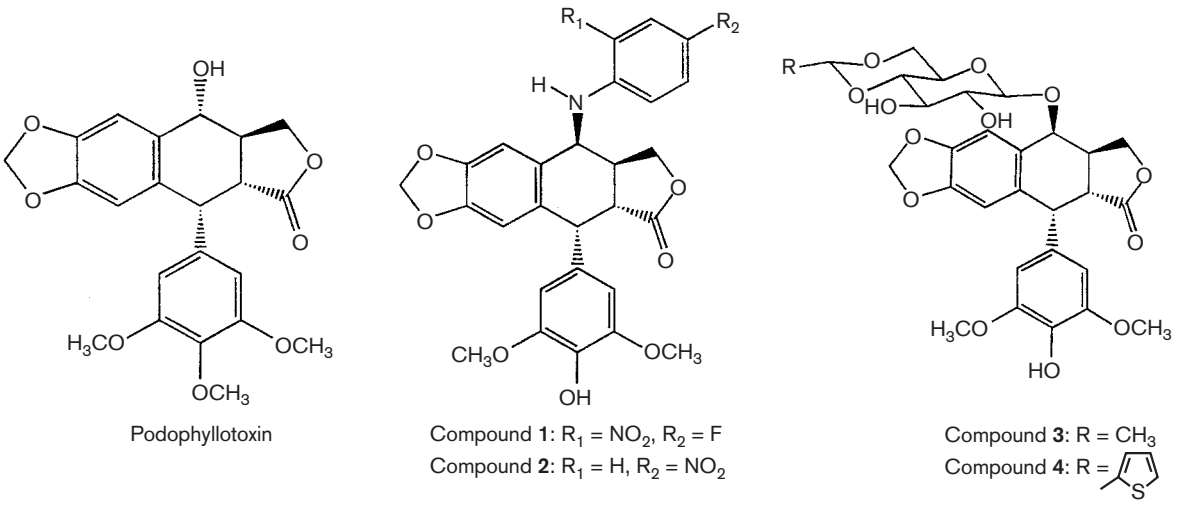
Introduction

Etoposide (VP-16) (**3**) and teniposide (VM-26) (**4**) (structures are shown in Figure 1) are semi-synthetic derivatives of podophyllotoxin, an antimitotic cytotoxic agent isolated from *Podophyllum peltatum* or *P. emodii* (Berberidaceae). Unlike podophyllotoxin, **3** and **4** inhibit the nuclear enzyme DNA topoisomerase II and, as drugs, they are first-line treatments for small cell lung cancer, testicular carcinoma, lymphoma and Kaposi's sarcoma.[1,2] GL-331 (**2**), a new 4′-*O*-demethyl-epipodophyllotoxin derivative, is currently in phase II clinical trial for treatment of various cancers including gastric carcinoma and non-small cell lung cancer.[3] Compound **2** is more potent than **3** and has improved activity against various types of drug-resistant tumor cells. In addition to their primary action as DNA topoisomerase II inhibitors, compounds **2** and **3** have other biochemical effects in cells. They decrease cellular protein tyrosine kinase (PTK) activities and **2** can activate protein tyrosine phosphatase (PTP), which induces apoptotic cell death.[4] An extensive series of epipodophyllotoxin derivatives, including **2**, has been prepared and evaluated as antitumor topoisomerase II inhibitors.[5–7] In general, 4′-demethyl-epipodophyllotoxin analogs inhibit tumor cell growth primarily by a topoisomerase II-dependent mechanism. However, as reported herein, 4′-*O*-demethyl-4β-(2″-nitro-4″-fluoroanilino)-4-desoxypodophyllotoxin (**1**), an analog of **2**, is a potent and broad-spectrum inhibitor of cancer cell growth but is not a topoisomerase II inhibitor. Compound **1** is also an interesting lead molecule since, like **4**, the drug's cytotoxic action is not affected by various mechanisms of drug



Podophyllotoxin

Compound **1**: $R_1 = NO_2$, $R_2 = F$
Compound **2**: $R_1 = H$, $R_2 = NO_2$

Compound **3**: $R = CH_3$
Compound **4**: R =

Figure 1. Structures of podophyllotoxin and 4′-*O*-demethylepipodophyllotoxin derivatives.

resistance including those mediated by P-glycoprotein (P-gp), multidrug-resistance protein (MRP) and reduced levels of DNA topoisomerase II (at MDR). This paper also reports the selection and the characterization of A549 (human lung cancer) sub-lines that are resistant to the action of compound **1**.

Materials and methods

Chemicals and reagents

Compound **1** was synthesized by the method described previously[7] and will be reported in detail elsewhere. Compounds **2**, **3** and camptothecin were obtained from the Natural Products Laboratory, UNC-CH. Verapamil, colchicine, cytochalasin D, sulforhodamine B (SRB), trichloroacetic acid (TCA), collagen type I (calf skin) and poly-L-lysine were purchased from Sigma (St Louis, MO). Concentrated drug solutions were prepared and stored (−70°C) in dimethylsulfoxide (DMSO) and were diluted aseptically into culture medium immediately before use. Human DNA topoisomerase II (p170 isoform) was obtained from TopoGen (Columbus, OH). Monoclonal antibodies to phosphoserine (PSR-45), phosphotyrosine (p-Tyr(Ab-2)) and the anti-mouse IgG(H+L)AP conjugate were obtained from Sigma, Oncogene Research Products (Cambridge, MA) and Promega (Madison, WI), respectively. Protease inhibitors were purchased from Boehhringer Mannheim (Indianapolis, IN).

Cell lines

Human cancer cell lines KB (nasopharyngeal carcinoma), A549 (lung carcinoma), HCT-8 (ileocecal), CAKI-1 (kidney), MCF-7 (breast adenocarcinoma) and SK-MEL-2 (malignant melanoma) were obtained from ATCC (Rockville, MA). KB-7d (**3** resistant), KB-VCR (vincristine resistant and cross-resistant to compound **3**) and KB-CPT (camptothecin resistant) were a generous gift of Dr Y-C Cheng (Yale University), and their isolation and characterization were reported in detail elsewhere.[8,9] The 1A9 (ovarian cancer) cell line and PTX 10 and PTX 22 (paclitaxel resistant) sub-lines were a generous gift of Dr P Ginnakakou.[10]All cell lines were cultured in RPMI 1640 supplemented with 25 mM HEPES, 0.2% (w/v) sodium bicarbonate, 10% (v/v) fetal bovine serum (FBS) and 100 μg/ml kanamycin. Cultures were maintained in a 5% CO_2 and humidified atmosphere at 37°C.

Selection of A549 cells resistant to compound **1**

Resistant A549 cells were isolated by stepwise selection with compound **1** in the medium at the concentration of 3 times the IC_{50} for 3 months, at 5 times the IC_{50} for 2 months (A549(5×)), at 10 times the IC_{50} for 2 months (A549(10×)) and 20 times the IC_{50} for 2 months (A549(20×)). Clonal isolates at each level of selection were not derived. With the exception of the A549(20×) sub-line, A549 cells and **1**-resistant variants grew at comparable rates. Consequently, biological and biochemical studies were carried out using the lower-level resistant sub-lines to avoid spurious effects arising from dissimilar rates of cell replication. For all biological and biochemical assays, resistant cells were cultured in the absence of **1** for 1 day prior to use.

Growth inhibition assay

Antiproliferative effects were evaluated by SRB assay as described in detail by Rubinstein *et al.*[11] Freshly trypsinized cell suspensions (5×10^4–2×10^5/ml) were seeded in 50 μl medium in 96-well plates (Costar, Cambridge, MA). Compounds from DMSO-diluted stock were added in 50 μl aliquots to wells at twice the desired final concentration. Micro cultures were treated in triplicate. After 3 days in culture, attached cells were fixed with 50% (w/v) TCA and then stained with 0.4% (w/v) SRB in 1% acetic acid. The absorbency at 562 nm was measured using a microplate reader (Molecular Devices, Menlo Park, CA) after solubilizing the bound dye. The IC_{50} value interpolated from dose–response graphs is the concentration of compound that causes a 50% reduction in absorbance relative to the untreated cells. Absorbance values between triplicate treatments typically varied no more than 5%.

DNA topoisomerase assay

Inhibition of topoisomerase II was determined by DNA relaxation assay using p170 human enzyme as described previously.[12] The IC_{100} value is the concentration (μM) that completely inhibits enzyme activity relative to control. Cellular protein–DNA complex formation was determined by the SDS–potassium precipitation method as described by Caldecott *et al.*[13]

Clonogenic survival assay

Drug toxicity was evaluated in treatments with suspended and plated cells. Freshly trypsinized (suspension) A549 cells (4×10^4/ml) were treated with compounds for various times as indicated, and then the suspension was diluted with fresh medium and cultured in six-well plates (Falcon, Franklin Lakes, NJ); the final concentrations of drugs after dilution were not themselves growth inhibitory. Plated cells (250 cells/0.5 ml) in six-well plates were treated with compounds and then carefully washed with phosphate-buffered saline (PBS). Cells were cultured for a minimum of 10 days after treatment. After fixing and staining with 5% (w/v) crystal violet in 50% (v/v) ethanol, macroscopic colonies clearly visible by eye were scored. The LD_{50} value interpolated from dose–response graphs is the drug concentration that caused a 50% reduction in colonies relative to untreated controls. The LD_{100} value is the concentration that completely prevented colony formation. The plating efficiencies of control cells were in the range of 32–40% in all experiments.

Attachment assay

Ninety-six-well tissue culture plates were coated with 6 μg/cm^2 of collagen type I or 4 μg/cm^2 poly-L-lysine according to the manufacturer's recommendations. Freshly trypsinized cells were plated at 1×10^4 cells/well. After incubation for various times at 37°C, unattached cells were removed by washing twice with PBS. Attached cells were then fixed with 50% TCA and stained with 0.4% SRB in 1% acetic acid. The absorbance at 562 nm was subsequently measured. The rate of attachment defined as the half-time ($t_{1/2}$) is the time taken for 50% of cells to not be affected by the washing treatment.

Isolation of plasma membranes

Plasma membranes were prepared according to the method described by Yang *et al.*[14] Briefly, scraped and pelleted cells were washed 3 times with ice-cold PBS and resuspended in a hypotonic lysing buffer [1 mM Tris–HCl, pH 7.2, containing 10 μg/ml phenylmethylsulfonylfluoride (PMSF) freshly added before use]. After incubation at room temperature for 30 min, nuclei were removed by centrifugation at 400 *g* for 10 min at 4°C. The supernatant was collected and centrifuged at 95 000 *g* for 1 h. Crude membrane pellets were resuspended in 10 mM Tris–HCl, pH 8.0, containing 75 mM sucrose, 25 mM $MgCl_2$, 1.5 mM EDTA, 5 mM dithiothreitol, 0.15 M NaCl, 0.15 M KCl and 10 μg/ml each of the following protease inhibitors: PMSF, leupeptin and pepstatin. Resuspended pellets were subjected to discontinuous sucrose gradient centrifugation (37 500 r.p.m., 4°C, 3.5 h) using a Beckman SW41 rotor. Plasma membrane-rich bands were visualized by light scattering and collected by side-puncture. Using this method, the 20/34% interface (upper layer) is enriched in broken plasma membrane particulates and the 34/40% interface (lower layer) is a largely intact plasma membrane preparation.

SDS–PAGE and Western blot analysis

Protein constituents of plasma membrane–enriched fractions purified from A549 and **1**-resistant cells were separated using SDS–PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 12 V for 30 min using an electrophoretic transfer cell and buffers recommended by the manufacturer (Trans-Blot SD; BioRad, Hercules, CA). Before immuno-staining,

Table 1. Human tumor cell growth inhibition

Compound	Cell line/IC_{50} (μM)[a]						
	KB	A549	HCT-8	CAKI-1	MCF-7	SK-MEL-2	1A9
1	0.19	0.30 ± 0.02[b] (*n*=10)	0.34	0.93	0.26	0.22	0.11
2	2.25	0.25 ± 0.05[b] (*n*=7)	3.17	2.15	9.65	4.66	0.20

[a]Cytotoxicity as IC_{50} for each cell line is the concentration that causes a 50% reduction in adsorbance at 562 nm relative to untreated cells using SRB assay.
[b]Average ± SE (*n*=numbner of independent experiments).

Table 2. Drug-resistant cell growth inhibition

Compound	Cell line/IC_{50}[a]						
	KB	KB-7d	KB-VCR	KB-CPT	1A9	PTX10	PTX22
1	0.19	0.15	0.15	0.22	0.11	0.54	>1.8 (47)[b]
2	2.25	3.32	5.13	1.86	0.20	1.60	2.60
3	0.16[c]	23.80[c]	30.60[c]	0.20	0.60	>9.6	>9.6

[a]Cytptoxicity as IC_{50} for each cell line is the concentration (μM) that causes a 50% reduction in adsorbance at 562 nm relative to untreated cells using SRB assay.
[b]When the inhibition was below 50% at the highest test concentration, the value is indicated in the brackets.
[c]See reference 8.

membranes were blocked by incubation with 5% BSA in buffer (TBST; 0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated with 1:5000 dilution of monoclonal anti-phosphoserine antibody or 0.25 μg/ml anti-phosphotyrosine antibody, overnight at 4°C. Bands of antibody-reactive proteins were visualized using alkaline phosphatase-conjugate anti-mouse IgG(H+L) and NBT/BCIP as substrates for color development. Proteins were visualized directly in the gel using silver-staining as described by Merril *et al.*[15]

Results

Cell growth inhibition

Compound **1** was evaluated and compared to **2** as an inhibitor of human tumor cell line replication (Table 1). The IC_{50}s of **1** were less than 1 μM (0.54 μg/ml) against all lines tested. These values are significantly lower (about 2- to 3-fold) as compared to those of compound **2** in all cell lines except A549, against which both compounds were equipotent. A similar study was carried out using a panel of drug-resistant cell lines (Table 2). In general, KB-resistant sub-lines were not highly cross-resistant to either **1** or **2**. Interestingly, the paclitaxel-resistant sub-clones derived from 1A9 cells displayed varying degrees of cross-resistance to **1** (4.9- to 16.4-fold) and **2** (8.0- to 13.0-fold). The overall results show that **1** has broad-spectrum activity against tumor cell replication. Compound **1** is typically more active than **2** and the patterns of activities against drug-resistant cells are similar for both analogs. These results establish compound **1** as a new antitumor lead molecule in the epipodophyllotoxin class.

Topoisomerase inhibition assay

Since compound **2** is a DNA topoisomerase II-directed antitumor drug, as are 4′-*O*-demethylepipodophyllotoxin analogs, compound **1** was evaluated as an enzyme inhibitor. Two assays were used: inhibition of enzyme-dependent relaxation *in vitro* and measurement of intracellular protein-linked DNA breaks (PLDB; a signature of enzyme inhibitors exemplified by compounds **2** and **3**). Results are shown in Table 3. While **2** inhibited *in vitro* topoisomerase II completely at 50 μM, compound **1** was not active up to 150 μM. In addition, PLDB induced by compound **1** was only 5% of levels induced by **2**, even at a concentration 263-fold higher than the IC_{50} for KB cell growth inhibition. Other experiments using lower concentrations of **1** showed no significant induction of PLDB by the drug (data not shown). These results suggest that the biochemical target of compound **1** is not DNA

topoisomerase II and imply the mechanism of action of **1** is different from topoisomerase inhibitors like **2** and **3**.

Cytotoxic activity

To compare cytotoxic activity between compounds **1** and **2**, the clonogenic survival of treated A549 cells was measured and compared to control. As shown in Table 4, the LD_{50}s of compound **1** were 0.52 μM (when treating suspended cells) and 0.50 μM (when plated cells were treated). Interestingly, compound **1** up to 2.5 μM exhibited a plateau dose–response with 32% survival. Compound **2** was more active under both assay conditions. For comparison, the LD_{100}s of compound **2** were 1.25 and 2.5 μM for suspension and for plated cells, respectively. Time-dependence of **1**'s cytotoxic action was also investigated and a plateau dose–response was observed even after 2.5 μM **1** treatment for 24 h (40% survival, data not shown). The difference in cytotoxic activities of compounds **1** and **2** prompted isolation of **1**-resistant cell lines in order to investigate mechanism of action.

Table 3. DNA topoisomerase II inhibition

Compound	Inhibition of topoisomerase II (IC_{100})[a]	Cellular protein–DNA complex formation (%)[b]
1	NA[c]	5.0 ± 0.5[d] (n=2)
2	50	100
3	100	76 ± 3[d] (n=2)

[a]IC_{100} is the concentration (μM) that completely inhibited the enzyme relative to control enzyme reaction.
[b]Percent values are levels induced by drug treatment (50 μM) relative to compound **4** (compound **4** induced protein-linked DNA breaks 86-fold relative to mock-treated levels, a value arbitrarily set at 100%).
[c]Not active.
[d]Average $\pm$ SE (n=number of independent experiements).

Growth properties of **1**-resistant cells

A series of **1**-resistant A549 sub-lines were isolated using stepwise selection and continuous exposure to escalating drug concentrations. Details are given in Materials and methods. During the isolation work, a noticeable difference in growth habit of **1**-resistant cells was observed. As shown in Figure 2, the A549 cell line grows as evenly dispersed cells on the surface of the flask, ultimately forming a near-homogeneous cell monolayer (Figure 2A). However, cultures established from mono-dispersed suspensions of resistant cells grew as compact colonies (Figure 2B and C). The potential significance of this difference in growth habit will be discussed.

Sensitivity of **1**-resistant cells to antiproferative agents

To explore possible biochemical changes in **1**-resistant cells, the effects of various antiproferative agents on cell replication were studied. The test agents selected included prototypical inhibitors of DNA topoisomerases I and II, and cytoskeleton-targeting drugs. The results in Table 5 show that the A549(10 $\times$) sub-line is 8.5-fold resistant to compound **1** and is 2.6-fold cross-resistant to compound **2**. Cross-resistance to compound **3** (2.3-fold) and the tubulin polymerization inhibitor, colchicine (2.8-fold), was also observed using the A549(10 $\times$) sub-line. In contrast, no significant differences in sensitivity of drug-resistant cells to camptothecin (DNA topoisomerase I inhibitor) and cytochalasin D (microfilament polymerization inhibitor) were detected.

Based on the limited drug-sensitivity profile obtained, it is possible that the A549(10 $\times$) sub-line has a pleiotrophic mechanism of drug resistance. One component confers moderate cross-resistance to structurally and mechanistically dissimilar drugs,

Table 4. Cytotoxic activities of **1** and **4** against A549 and **1**-resistant sub-line [A549(10 $\times$)]

Compound		A549		A549(10 $\times$)	
		Suspended	Plated	Suspended	Plated
1	LD_{50}[a]	0.52	0.50	2.07	2.50
	LD_{100}[b]	>10 (32.1)[c]	>5 (17.0)[c]	>10 (30.2)[c]	>5 (42.2)[c]
2	LD_{50}[a]	<16 (27.2)[d]	0.26	0.35	0.32
	LD_{100}[b]	1.25	2.50	2.50	2.50

Cytotoxicity was determined by a clonogenic assay as described in Materials and methods.
[a]The LD_{50} is the drug concentration (μM) that reduced plating efficiency by 50% relative to control.
[b]The LD_{100} is the drug concentration (μM) that reduced plating efficiency by 100% relative to control.
[c]When the reduced plating efficiency was below 100% at the highest test concentration, the survival rate (%) is indicated in brackets.
[d]When the reduced plating effiency was above 50% at the lowest test concentration, the survival rate (%) is indicated in brackets.

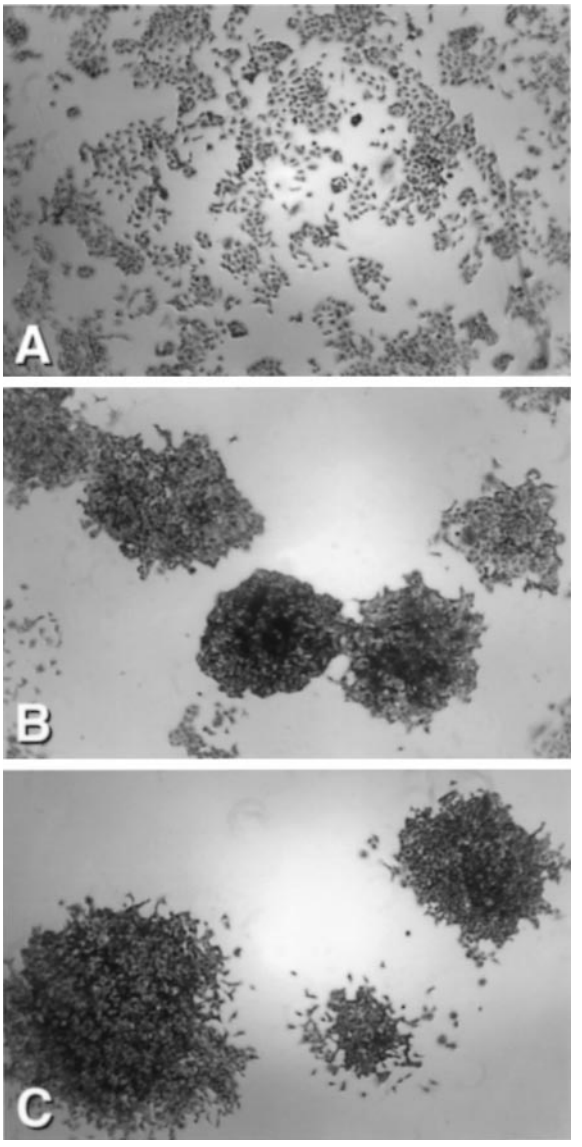


Figure 2. Growth habit of A549 and **1**-resistant sub-lines. A549 cells and drug-resistant sub-lines were seeded at different densities (8×10^3 and 1.5×10^4/cm^2, respectively) and cultured for different amounts of time (2 and 14 days, respectively) as described in Materials and methods. Cells were fixed with 10% formalin/PBS and stained 0.1% w/v toluidine blue prior to photography. A549 cells, A549(10 ×) cells and A549(20 ×) cells are shown in panels (A), (B) and (C), respectively (×400).

and another confers specific resistance to compound **1**.

Verapamil effects on drug sensitivity of resistant cells

One established mechanism of MDR involves overexpression of P-gp, an ATP-dependent drug-efflux pump. This mechanism reduces intercellular accumulation of a variety of cytotoxic agents that differ in both structure and mechanism.[16,17] In order to examine the contribution of P-gp to **1** resistance, verapamil, a modulator of P-gp-mediated drug-efflux,[18] was used. Results in Table 6 show the susceptibility of A549 to compound **1** [potentiation index (PI)=0.49], and the sensitivity to both compound **3** (PI=0.28) and colchicine (PI=0.36) increased when cells were co-cultured with drug and verapamil. In contrast, verapamil co-treatment has less effect on the activity of camptothecin (PI=0.88). These results suggest P-gp is expressed

Table 5. Growth inhibition of resistant cells by antiproliferative agents and compound **1**

Compound	A-549	A549(10 ×)	
	IC_{50}[a]	IC_{50}[a]	Fold resistance[b]
1	0.30 ± 0.02[c] (*n*=10)	2.56 ± 0.22[c] (*n*=6)	8.5
2	0.25 ± 0.05[c] (*n*=7)	0.65 ± 0.18[c] (*n*=4)	2.6
3	5.54 ± 0.29[c] (*n*=2)	12.52	2.3
Camptothecin	0.044 ± 0.004[c] (*n*=3)	0.041	0.9
Colchicine	0.057 ± 0.001[c] (*n*=3)	0.16 ± 0.05[c] (*n*=2)	2.8
Cytochalasin D	0.23 ± 0.06[c] (*n*=3)	0.19 ± 0.01[c] (*n*=2)	0.8

[a]IC_{50} is the concentration of compound (μM) that reduced the absorbance by 50% relatve to control using SRB assay as described in Materials and methods.
[b]Resistance index is the ratio of IC_{50} values [A549(10 ×)/A549].
[c]Average ± SE (*n*=number of independent experiments).

Table 6. Modulatory effects of verapamil of drug sensitivity of **1**-resistant cells

Compound	PI[a]	
	A549	A549(10 ×)
1	0.49 ± 0.01[b] (*n*=2)	0.48
3	0.28	0.34
Camptothecin	0.88 ± 0.20[b] (*n*=2)	1.00
Colchicine	0.36	0.29

Cell growth inhibition was tested as desribed in Materials and methods except agents were used alone or co-incubated with 10 μM verapamil, a concentration without apparent effect on cell proliferation.
[a]Potentiation index (PI) is expressed as the ratio of IC_{50}'s measured with verapamil versus without verapamil.
[b]Average ± SE (*n*=number of independent experiments).

in parental A549 cells and decreases effectiveness of known drug substrates (**3** and colchicine) and to a lesser extent of compound **1**. The potentiating effects of verapamil in the resistant line for compound **1** (PI=0.48), compound **3** (PI=0.34), colchicine (PI=0.29) and camptothecin (PI=1.00) were similar to those observed using the parental line. These results suggest that P-gp overexpression does not play a significant role in the resistant phenotype of the A549(10×) sub-line.

Attachment property of **1**-resistant cells

During the selection of resistant cell lines, a difference was observed in the growth habit of the variants. To explore the basis for the different behavior, cell–substrate adhesion was measured using an attachment assay. These studies were conducted using tissue culture plates as substrates with or without supplemental coating. As shown in Table 7, the adhesion rates to tissue culture plastic, on poly-L-lysine and on collagen-coated substrates were different within and between cell lines. The resistant A549(10×) cell sub-line required more than 3-fold more time ($t_{1/2}$=39.5 min) to adhere to plastic than the parent A549 ($t_{1/2}$=12.5 min). Both cell lines attached equally to the poly-L-lysine coated plate ($t_{1/2}$ =3.4 and 3.7 min for parent and resistant sub-line, respectively). Poly-L-lysine treatment creates a positively charged surface for attachment, which enhances electrostatic interaction with the negatively charged cell membrane. The similar attachment rates on poly-L-lysine-coated wells suggest the net negative charges on membranes of A549 and A549(10×) are similar. Collagen also decreased half-time of attachment of both cell lines relative to the behavior on tissue culture plastic. However, the resistant cell line attached less readily to a collagen substrate than parental cells. Similar studies were conducted with other **1**-resistant sub-lines. A549(20×) was markedly deficient in attachment to plastic, the $t_{1/2}$ being more than 120 min (data not shown). Other experiments were conducted to examine whether treatment of A549 and A549(10×) cells with compound **1** directly affected attachment. No effects of the drug were observed (data not shown). In summary, these results suggests that the cell adhesion machinery is altered in **1**-resistant cells and this change is not a direct effect of compound **1**.

Table 7. Cell attachment activity

Plate	$t_{1/2}$ (min)[a]	
	A549	S549(10×)
TC[b]	12.5	39.5
PL[c]	3.4	3.7
COL[d]	8.8	14.0

[a]The time taken for 50% of cells to attach.
[b]Tissue culture plate.
[c]Poly-L-lysine-coated plate.
[d]Collagen-coated plate.

Membrane proteins in resistant cell lines

In order to investigate whether changes in membrane-associated protein composition correlated with differences in the attachment behavior of **1**-resistant cells, plasma membrane fractions were purified from parent and two resistant sub-lines. Analysis of protein composition using SDS–PAGE and silver-staining showed several unique proteins were present in membrane preparations of resistant cells. Five high molecular mass species (>200 kDa) were more abundant and clearly detected in membranes of resistant sub-lines (Figure 3, lanes 4, 5, 6 and 7). In addition, two sharp bands, 155 and 165 kDa apparent mass, in the intact membrane preparation (Figure 3, lanes 5 and 7) and one 140 kDa species, in both intact and particulate samples (Figure 3, lanes 4, 5, 6 and 7) were detected and were specific for resistant cell lines. These results demonstrate that specific changes in the composition of plasma membrane proteins have occurred in **1**-resistant cell lines.

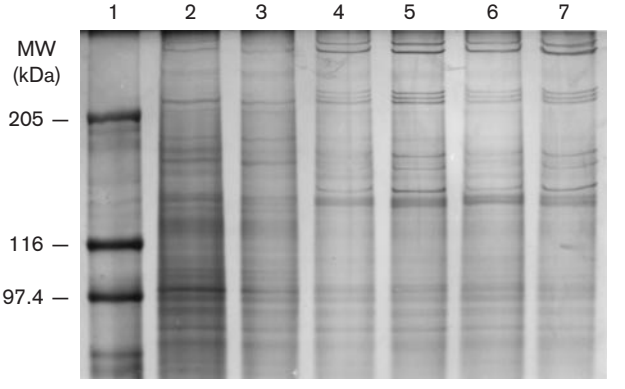


Figure 3. Plasma membrane protein composition of A549 and **1**-resistant sub-lines. Membrane fractions isolated as described in Materials and methods were electrophoresed on 7% SDS–polyacrylamide gels and silver stained. Lane 1, protein marker; lanes 2 and 3, upper and lower boundary layers, respectively, isolated from A549 cells; lanes 4 and 5 and lanes 6 and 7, upper and lower boundary layers prepared from A549(5×) cells and from A549(10×) cells, respectively.

Protein phosphorylation in resistant cell lines

It is known that phosphorylation of membrane proteins occurs through various signal transduction pathways (including PKC, PKA, Cdc42 kinase, LIM kinase, Rho kinase and tyrosine kinase), and these modifications are associated with the regulation of cell motility and cell adhesion.[19–26] Therefore it was of interest to determine if the novel species detected in membranes of resistance cells were substrates of protein kinase activities. Western blot analysis using anti-phosphoserine antibody identified two polypeptides, around 135 kDa, which were specifically phosphorylated in resistant cell sub-lines (Figure 4, lanes 3 and 4). Neither of the phosphorylated species were over-produced in drug-resistant cell membranes based on silver-stained protein gels (Figure 3, lanes 4, 5, 6 and 7). In order to examine whether **1** treatment induced phosphorylation of the 135 kDa doublet, A549 cells were treated with 5 μM of compound **1** for 2 h, and membrane fractions were isolated and analyzed by Western blot immuno-localization. The results in Figure 5 show that the doublet around 135 kDa was not hyperphosphorylated by **1** treatment and no other changes in the composition of phosphoserine-containing proteins was detected. Similar experiments using a phosphotyrosine antibody showed tyrosine phosphorylation both in membrane and whole cell preparation was not altered in cells **1**-resistant and treated with compound **1** (data not shown). These results demonstrate specific changes in the composition of phosphoserine-containing proteins have occurred in the plasma membrane of **1**-resistant cells. The changes did not occur as a direct result of drug treatment.

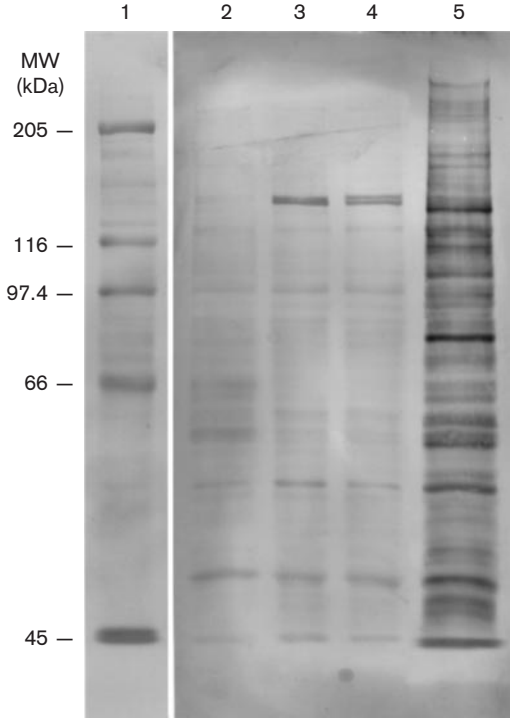


Figure 4. Phosphoserine-containing proteins in plasma membranes of A549 and resistant sub-lines. Membrane fractions (lower boundary layer) isolated as described in Materials and methods were electrophoresed on 7% SDS–polyacrylamide gels and analyzed by Western blotting using an anti-phosphoserine antibody. Lane 1, protein marker; lane 2, membrane fraction of A549 cells; lane 3, membrane fraction of A549(5×); lane 4, membrane fraction of A549(10×); lane 5, whole extract of A549 cells.

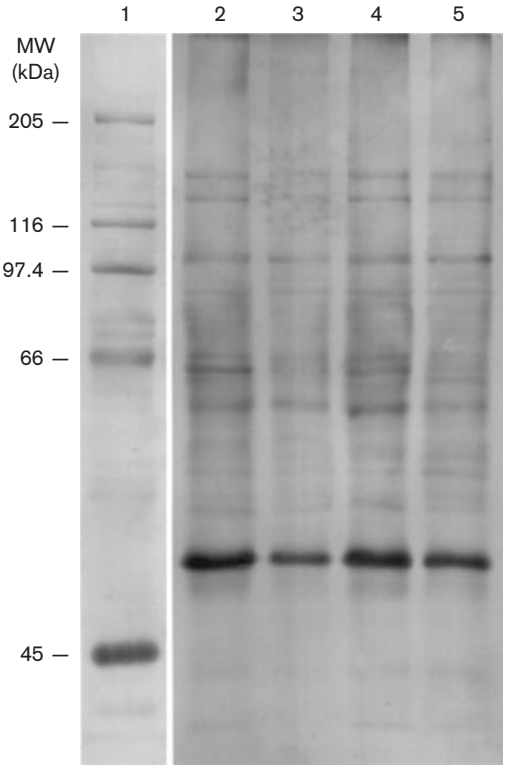


Figure 5. Phosphoserine-containing proteins in plasma membranes of A549 cells treated with compound **1**. Membrane fractions were isolated from A549 and **1**-treated cells as described in Materials and methods. Samples were electrophoresed on 8% SDS–polyacrylamide gels and analyzed by Western blotting with an anti-phosphoserine antibody. Lane 1, protein marker; lanes 2 and 3, upper and lower boundary layers of A549 cells; lanes 4 and 5, upper and lower boundary layers of A549 cells treated with compound **1** (5 μM, 2 h).

Discussion

The works reported establish compound **1** as a new lead in the 4′-*O*-demethylepipodophyllotoxin drug class. Compound **1** is more active than **2** at both killing cultured tumor cells and inhibiting their replication. Moreover, the biochemical mechanism of **1** action, when compared to **2** and **3**, is clearly different. This claim is based on the inability of **1** to (i) inhibit DNA topoisomerase II catalytic activity, (ii) induce enzyme–DNA covalent reaction intermediates (PLDB) and (iii) completely prevent clonogenic survival either as a function of dose or treatment duration. The finding that compound **1** does not act as a topoisomerase II inhibitor is particularly intriguing since the 'variable' domain of the composite pharmacophore can tolerate substantial molecular diversity in terms of functional groups and substitution patterns.[5–7,27] A simple interpretation of the result is that the 2-nitro-4-fluoroanilino moiety of compound **1** cannot be accommodated in the critical drug-interaction domain of the enzyme. This domain has been crudely mapped using biochemical approaches and modeled using computational technique but is not yet resolved at the structural level.[27,28] Moreover, even though it is established that mammalian type II enzymes function during DNA replication, transcription and chromosome segregation, their biological roles in these and other critical cellular processes are not fully understood.[29] Additional studies will be necessary to determine whether **1** interacts with DNA topoisomerase II and what biochemical consequences, if any, might ensue as a result of drug binding.

Work undertaken to explore and establish the mechanism of **1** action used a traditional approach of isolating and characterizing drug-resistant cell lines. Variants were established with relative ease following a step-wise selection protocol of continuous drug exposure and without prior mutagenic treatment. In general, **1**-resistant cells were not obviously defective in their ability to replicate under standard culture conditions. However, significant biological and biochemical changes had occurred in response to drug selection. Specific differences between the parent A549 cells and drug-resistant sub-lines are illustrated by the following observations. Interactions between **1**-resistant cells and substrata, either tissue-culture plastic or collagen coated, were abnormal based on prolonged times and altered kinetics (data not shown) of attachment. The growth habit of resistant variants was abnormal under standard culture conditions, with them having a clear preference to proliferate as compact and dense 'aggregates' rather than colonize the culture surface as a dispersed layer, typically one cell in thickness. Plasma membrane protein composition of drug-resistant variants was altered both in terms of increased complexity (seven unique polypeptides detected by silver staining) and chemical modifications (specifically a restricted hyperphosphorylation of serine residues). These changes in membrane proteins may be more dramatic in scope than currently defined since low-resolution SDS–PAGE was used for analysis and only at a single gel concentration. At this time it is not possible to define how the stated differences accommodate cellular growth in the presence of **1** or say whether the biological changes are dependent on the biochemical alterations. Furthermore it is unclear whether any properties of the **1**-resistant phenotype arose, either directly or indirectly from changes in the biochemical target of the drug. Resolution of these important issues awaits additional research. Nevertheless, it is possible to draw general conclusions from current results. The **1**-resistant phenotype is compatible with alteration in adhesive properties (cell–cell and/or cell–substrate) and associated behaviors. It is known that fundamental cellular behaviors including adherence and spreading, motility, proliferation differentiation, and survival depend on complex signal transduction pathways that converge at the plasma membrane. The biochemical machinery is understood in some detail and delineation of the pathways, i.e. cross-talk, as well as the associated molecules are areas of intensive investigations. Developments are covered in recent reviews.[30,31] Interestingly, cellular adhesion molecules can also contribute both directly and indirectly to acquired multicellular drug resistance.[32] It is speculative but (some of) the plasma membrane-associated proteins identified in **1**-resistant cells could be components of the cellular adhesion machinery and may thereby participate in the resistance of A549 variants to multiple drugs including compound **1**. For example, the five high molecular mass (>200 kDa) membrane proteins of resistant cells could be epithelial mucin gene products, some of which are known to have distinct roles in adhesion modulation and cell signaling.[33] However, based on the apparent molecular weights of the remaining proteins, the following known adhesion molecules, catenin, paxillin,[34] adducin[22] and integrins (other than α_5 and β_1), can be excluded as a possible candidates.

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