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Podophyllotoxin Analogs: Effects on DNA Topoisomerase II, Tubulin Polymerization, Human Tumor KB Cells, and Their VP-16-Resistant Variants

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SUMMARY

Several derivatives of podophyllotoxin with modifications at the C-4 position of ring C, in addition to demethylation at the C-4′ position of ring E, were examined for inhibitory activity against DNA topoisomerase II and tubulin polymerization, generation of protein-linked DNA breaks, and cytotoxicity against KB cells and VP-16-resistant KB variants. Substitution of podophyllotoxin with a group in the β configuration at the C-4 position of ring C resulted in compounds with greater inhibitory activity against DNA topoisomerase II and lower inhibitory activity against tubulin polymerization than those with an α configuration. These active analogs exhibited the same mechanism of DNA topoisomerase II inhibition as the epipodophyllotoxin derivative VP-16, which causes protein-linked DNA breaks *in vitro* as well as in cells. Two analogs selectively inhibited DNA topoisomerases II to a greater extent than tubulin polymerization. These analogs were cytotoxic towards KB cells in addition to VP-16-resistant KB cell lines, which indicated limited cross-resistance with VP-16 in VP-16-resistant KB variants.

An epipodophyllotoxin derivative, VP-16, is currently used for the clinical treatment of cancer (1, 2). This compound inhibits cell growth by freezing the covalent complex of DNA with DNA topoisomerase II, an enzyme that causes double-stranded DNA breaks (3–5). VP-16 does not inhibit tubulin polymerization (6); however, its parental compound podophyllotoxin, an effective inhibitor of mitosis, blocks the assembly of microtubules (7, 8) but has no inhibitory activity against DNA topoisomerase II (4). The severe toxic side effects of podophyllotoxin caused by inhibition of microtubule polymerization have limited its application in cancer treatment (9, 10).

Results from this laboratory demonstrate that important biochemical determinants of VP-16 action include DNA topoisomerase II content and behavior (11–14), as well as drug uptake (11). In view of these results, new analogs that have an uptake mechanism different from that of VP-16 but that interact with DNA topoisomerase II and tubulin in a manner similar to that of VP-16 would be of chemotherapeutic interest. The spectrum of anticancer activity for these newly developed analogs may differ from that of VP-16. A group of new podophyllotoxin analogs with systematic modifications was recently synthesized (15, 16). The behavior of these analogs towards DNA topoisomerase II, tubulin polymerization, and VP-16-resistant cells are described in this manuscript.

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Materials and Methods

Cells. KB cells from ATCC were maintained, in RPMI 1640 medium that contained 5% fetal bovine serum and 100 μg/ml kanamycin, under humidified air with 5% CO_2 at 37°.

The VP-16-resistant cell lines KB1.0c and KB20a were maintained in the same medium supplemented with 1.0 or 20 μM VP-16, respectively. The characteristics of these cell lines were described previously (11).

Chemicals. VP-16 was a gift from Bristol-Myers Pharmaceuticals (Syracuse, NY). Podophyllotoxin and its derivatives were synthesized (15, 16) in Dr. K. H. Lee's laboratory in the School of Pharmacy at the University of North Carolina (Chapel Hill, NC). P-11 phosphocellulose was obtained from Whatman Bio-Systems Ltd. (Maidstone, England). Phenyl-Sepharose was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). [α-^{32}P]dATP (specific activity, 3000 Ci/mmol) and [^{14}C]thymidine (specific activity, 50.5 mCi/mmol) were purchased from ICN Radiochemicals (Costa Mesa, CA). GTP was purchased from Sigma Chemical Co. (St. Louis, MO). Proteinase K was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). *Eco*RI

ABBREVIATIONS: VP-16, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside); ATCC, American Type Culture Collection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OEN, 4′-demethyl-4-[2″-aminoethyl]-epipodophyllotoxin; NEO, 4′-demethyl-4β-[2″-hydroxyethylamino]-4-desoxypodophyllotoxin; OEO, 4′-demethyl-4-[2″-hydroxyethyl]-epipodophyllotoxin; **6**, 4′-demethyl-4β-(amino)-4-desoxypodophyllotoxin; **7**, 4′-demethylepipodophyllotoxin; **8**, 4′-demethyl-4α-(amino)-4-desoxypodophyllotoxin; **9**, 4′-demethylpodophyllotoxin; **10**, 4′-demethyl-4α-[2″-hydroxyethylamino]-4-desoxypodophyllotoxin; VM-26, 4′-demethylepipodophyllitoxin thenylidene-β-D-glucoside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

restriction enzyme and the Klenow fragment of *Escherichia coli* DNA polymerase I were purchased from Bethesda Research Laboratories (Gaithersburg, MD). All other chemicals were of standard analytical grade.

Partial purification of DNA topoisomerase II from human acute lymphocytic leukemia cells. All buffers were potassium phosphate containing 10% glycerol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.5 μg/ml leupeptin, and pepstatin A. Human acute lymphocytic leukemia cells were collected during leukophoresis and were stored at −70° after hypotonic lysis of red blood cells. The cells were suspended in 75 mM potassium phosphate buffer (pH 7.0) and were lysed by three cycles of freezing and thawing. The potassium phosphate concentration was increased to 0.3 M (pH 7.0), and the lysate was extracted for 1 hr at 4°. This preparation was centrifuged at 15,000 rpm for 20 min at 4°, and the supernatant was applied to a P-11 phosphocellulose column that had been preequilibrated with 0.3 M potassium phosphate buffer (pH 7.0). The column was eluted with a linear gradient of 0.3 to 0.55 M potassium phosphate buffer (pH 7.0). The fractions that contained DNA topoisomerase II, as determined by the P4 DNA unknotting assay, were pooled. The potassium phosphate concentration of the pooled fractions was adjusted to 1 M, and the solution was applied to a phenyl-Sepharose column that was preeqililbrated with 1 M potassium phosphate (pH 7.0). The bound proteins were then eluted with a linear gradient of 1 to 0.02 M potassium phosphate. DNA topoisomerase II eluted at approximately 0.2 M potassium phosphate, and the pooled active fractions had a specific activity of 1×10^5 units/mg. One unit was defined as the amount of enzyme required to unknot 50% of 0.4 μg of P4 knotted DNA. Purified enzyme was stored at −70° with bovine serum albumin added to a final concentration of 100 μg/ml.

Preparation of P4 knotted DNA and P4 DNA unknotting assay. Methods for the preparation of P4 knotted DNA and the unknotting assay were those described by Liu *et al.* (17). Various concentrations of the drugs were incubated with 3 units of DNA topoisomerase II and 0.4 μg of P4 knotted DNA. The effects of the drugs were estimated by comparing the unknotting activity of serially diluted enzyme alone. The knotted forms stay diffused and migrate into the gel, whereas the product of the reaction, the unknotted forms, stay close to the origin. The inhibition of the unknotting activity is determined by comparison with untreated controls.

Topoisomerase II-dependent double-stranded DNA breakage assay. Linear pBR 322 DNA was 3′ end-labeled, as described by Nelson *et al.* (18). The topoisomerase II-dependent double-stranded DNA breaks were quantitated using 3′ end-labeled pBR 322 DNA as a substrate as described previously (5).

Isolation of tubulin and tubulin polymerization assay. The tubulin was isolated from rabbit brain and the polymerization was monitored by turbidimetry, following the methods of Hwang *et al.* (19). The ID_{50} was determined by 50% inhibition of complete polymerization.

Protein-linked DNA breaks in KB ATCC cells. Log phase KB ATCC cells were labeled with [^{14}C]thymidine (0.05 μCi/ml) for 18 hr. After labeling, the cells were trypsinized, resuspended in fresh medium at a density of 5×10^5 cells/ml, and shaken gently for 1 h. After 1 hr in suspension, various concentrations of drugs were added and incubation was continued for an additional hour. The cells (5×10^5/sample) were then collected and analyzed for protein-linked DNA breaks by the potassium-sodium dodecyl sulfate precipitation method developed by Rowe *et al.* (20).

Toxicity of drugs in KB and VP-16-resistant KB cell variants. The KB, KB1.0c and KB20a cells were seeded at 10^4 cells/16-mm² well and exposed to various concentrations of drugs for 72 hr at 37°. The cell number was estimated using an MTT assay to establish the ID_{50} (21).

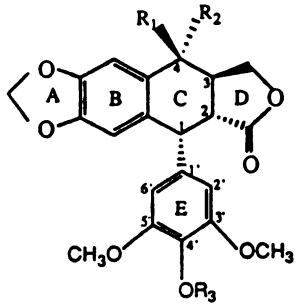
Results

The structures of the podophyllotoxin derivatives studied are shown in Fig. 1. In addition to demethylation at the 4′ position of ring E, modifications were made at the C-4 position on ring C. These modifications include α and β epimerization, amino and aminoalkyl substitutions, and hydroxy and hydroxyalkyl substitutions.

Because podophyllotoxin is a potent inhibitor of tubulin polymerization (ID_{50}, 0.5 μM), the action of these derivatives on tubulin polymerization was examined (Table 1). The derivatives with a hydroxy or an amino group in the α configuration at the C-4 position were more potent than those with a similar substitution in the β configuration. When the length of the side chain at this position increased, the inhibitory effect on tubulin polymerization tended to decrease. Therefore, substitutions at the C-4 position on ring C, particularly in the β configuration, as well as the removal of the methoxyl group at the C-4′ position on ring E of podophyllotoxin will decrease the potency of the inhibition of tubulin polymerization.

When the effects of these compounds on DNA topoisomerase II unknotting activity were examined, only the β forms were inhibitory (Fig. 2). Their order of potency was as follows: OEN > NEO > OEO > **6**, **7** (Table 1). At concentrations up to 100 μM, compounds **8**, **9**, and **10** did not inhibit DNA topoisomerase II unknotting activity. None of these derivatives inhibited the DNA topoisomerase I relaxation activity at concentrations of 100 μM except OEN, which resulted in less than 50% inhibition (data not shown).

VP-16 stabilizes DNA topoisomerase II and DNA covalent complexes, which are intermediates of topoisomerase activity



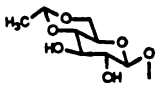
Compound	R_1	R_2	R_3	Abbreviation
1	 (etoposide)	H	H	VP-16
2	H (podophyllotoxin)	OH	CH_3	
3	$OCH_2CH_2NH_2$	H	H	OEN
4	$NHCH_2CH_2OH$	H	H	NEO
5	OCH_2CH_2OH	H	H	OEO
6	NH_2	H	H	
7	OH	H	H	
8	H	NH_2	H	
9	H	OH	H	
10	H	$NHCH_2CH_2OH$	H	
11	 (teniposide)	H	H	VM-26

Fig. 1. Chemical structures of the podophyllotoxin analogs. The R_1 substitutions are the β forms and the R_2 substitutions are the α forms.

TABLE 1

Inhibition of tubulin polymerization and DNA topoisomerase II by podophyllotoxin derivatives

Compound	Tubulin Polymerization ID_{50}[a]	DNA Topoisomerase II Inhibition[b]
	μM	%
VP-16	>100	75
Podophyllotoxin (2)	0.5	0
OEN	>100	>75
NEO	50	75
OEO	15	50
6	7	<50
7	4	<50
8	4	0
9	1	0
10	>200	0

[a] ID_{50} is the concentration that caused 50% inhibition of complete polymerization, as compared with the control.

[b] Represents the percentage of inhibition of the unknotting activity of topoisomerase II at 100 μM, as compared with serial dilutions of enzyme activity. If the unknotting activity in the presence of the drug is equal to 1.5 units of the enzyme alone, it is defined as 50% inhibition. If the activity is equal to 0.75 units, it is defined as 75% inhibition.

(5). We examined whether these analogs behave in a manner similar to that of VP-16 in this regard. The effect of the analogs on DNA topoisomerase II-dependent double-stranded DNA breaks was examined *in vitro*. The order of potency to induce topoisomerase II-dependent DNA breaks was: OEN > NEO > **6** > OEO > **7** > **8** > **9** > **10** (Fig. 3). The β forms were more potent than the α forms.

Because VP-16 generates protein-linked DNA breaks in cells (22), the ability of these analogs to generate protein-linked DNA breaks in cells after 1 hr of treatment was examined, using the potassium-sodium dodecyl sulfate precipitation method (20). The β forms of these compounds generated protein-linked DNA breaks in a dose-dependent manner (Fig. 4A), whereas the α forms, some of which are very toxic, do not. Therefore, DNA breaks do not necessarily correlate with cytotoxicity, and other factors such as inhibition of tubulin polymerization could be involved. However, the apparent quantity of breaks *in vivo* does correspond to the ability of the compounds to inhibit the topoisomerase II unknotting activity and to cause DNA breaks *in vitro*. Because protein-linked DNA breaks caused by short term VP-16 treatment are rapidly repaired in HeLa and SV-40 virus-infected BSC-1 cells after drug removal (5, 23), the rate of repair in KB cells treated with OEN or NEO at a 5 μM concentration for 1 hr was examined (Fig. 4B). Thirty minutes after removal of NEO or OEN, the cells had repaired more than 50% of the protein-linked DNA breaks. Four hours after removal of OEN, the cells retained a significant number of protein-linked DNA breaks. OEN was 2 times as toxic as VP-16 (Table 2). NEO and VP-16 were equipotent in all the aspects studied here, but NEO was only about 1/10 as toxic to the cells.

The sensitivity of two VP-16-resistant KB cell lines to the cytotoxic effects of NEO and OEN was examined (Table 1). KB1.0c is resistant to VP-16 as a result of a 50% decrease in both the VP-16 uptake and the DNA topoisomerase II content. KB20a is resistant as a result of a 50% decrease in VP-16 uptake and a 75% decrease in DNA topoisomerase II (11). Both cell lines were resistant to NEO and OEN; however, the degree of this resistance was less than that for VP-16.

Discussion

The cytotoxicity induced by VP-16 and VM-26 is thought to be mediated through DNA topoisomerase II-dependent double-stranded DNA breaks (3, 23, 24), inhibition of active nucleoside transport at high doses (6), and the production of free radicals during metabolic activation in the oxidation/reduction reaction (25–27). Structural features of VP-16 critical for the induction of DNA breaks are the presence of a hydroxy group at the C-4′ position of ring E (3) and the β configuration of the substituent at the C-4 position on ring C (3, 15). Because the substitution of the glycosidic moiety by a 4-alkoxy group, as in 4′-demethyl-epipodophyllotoxin ethyl ether, still yields some DNA topoisomerase II inhibitory activity (16), the presence of the glycosidic ethylidene cyclic acetial moiety at the C-4 position may not be as critical as previous results have suggested (28, 29). Several analogs that have a mechanism of action more similar to that of VP-16 than the microtubule assembly-inhibiting parental compound podophyllotoxin were synthesized

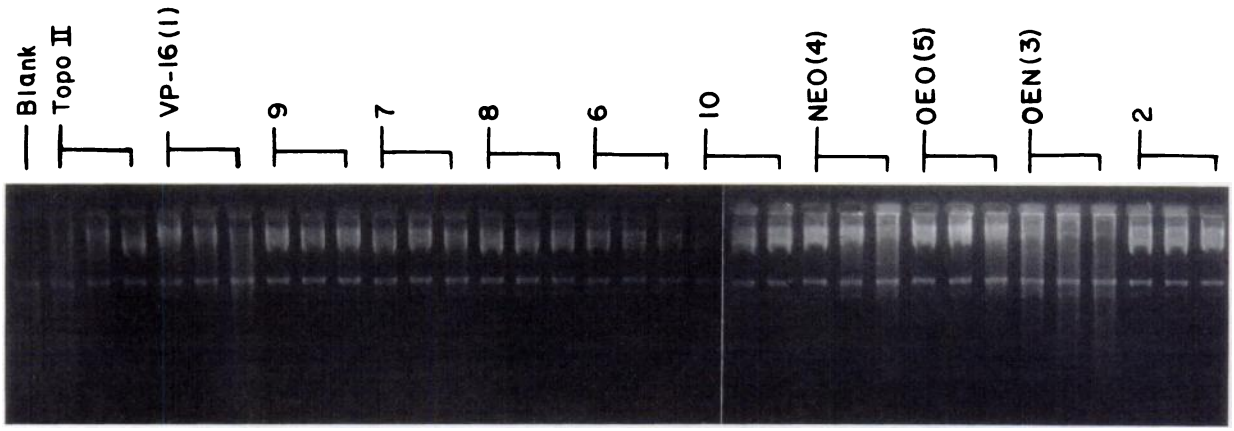


Fig. 2. Inhibition of the unknotting activity of DNA topoisomerase II by podophyllotoxin analogs. The reaction mixtures contain 50 mM HEPES, pH 7.0, 50 mM KCl, 0.1 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$, 50 μg/ml bovine serum albumin, 1 mM ATP, 0.4 μg of P4 knotted DNA, 3 units of DNA topoisomerase II, and 25, 50, or 100 μM concentrations of the analogs. The reactions were performed at 37° for 30 min and were terminated by the addition of sodium dodecyl sulfate to a final concentration of 1%. The samples were then electrophoresed on a 1% agarose gel in 90 mM Tris borate, 2 mM EDTA, pH 8.0. The DNA topoisomers were visualized by ethidium bromide staining and photographed under UV light using Polaroid type 55 film. From *left* to *right*, *lane 1* is the DNA blank and contains no enzyme. *Lanes 2* through *4* contain 0.75, 1.5, and 3 units of DNA topoisomerase II, respectively. The remainder of the lanes contain 3 units of the enzyme, and 25, 50, or 100 μM concentrations of VP-16 (*lanes 5–7*), **9** (*lanes 8–10*), **7** (*lanes 11–13*), **8** (*lanes 14–16*), **6** (*lanes 17–19*), **10** (*lanes 20–22*), NEO (*lanes 23–25*), OEO (*lanes 26–28*), OEN (*lanes 29–31*), and **2** (*lanes 32–34*), respectively.

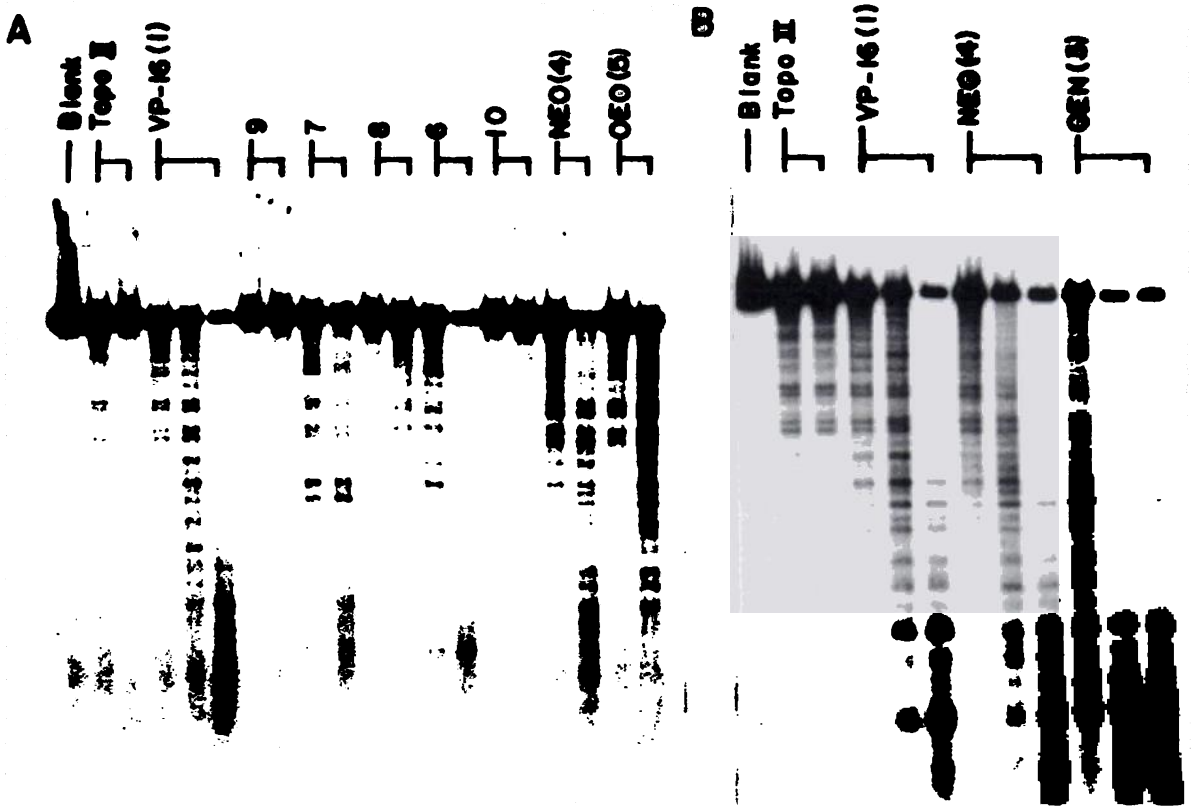


Fig. 3. The effects of the analogs on DNA topoisomerase II-dependent double-stranded DNA breaks. The reaction mixture contained 40 mM Tris·HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, 0.5 mM dithiothreitol, 30 μg/ml bovine serum albumin, 3′-^{32}P-end-labeled pBR 322 DNA, DNA topoisomerase II, and either 2 or 10 μM concentrations of the analogs. The reactions proceeded at 37° for 30 min. Then the samples were brought to 0.5 mg/ml protease K and 1% sodium dodecyl sulfate and incubated for 1 hr at 50°. Samples were electrophoresed in a 1% agarose gel with 90 mM Tris borate, 2 mM EDTA, pH 8.0, and the dried gel was autoradiographed with Kodak X-OMAT AR X-ray film. A, From *left* to *right*, *lane 1* is the DNA blank and contains no enzyme. *Lanes 2* and *3* contain 60 and 30 units of DNA topoisomerase II, respectively. The rest of the lanes contain 30 units of DNA topoisomerase II in addition to 0.4, 2, or 10 μM concentrations of VP-16 (*lanes 4–6*), and 2 or 10 μM concentrations of **9** (*lanes 7* and *8*), **7** (*lanes 9* and *10*), **8** (*lanes 11* and 12), **6** (*lanes 13* and *14*), **10** (*lanes 15* and *16*), NEO (*lanes 17* and 18), and OEO (*lanes 19* and *20*), respectively. B, From *left* to *right*, *lane 1* is the DNA blank and contains no enzyme. *Lanes 2* and *3* contain 60 and 30 units of DNA topoisomerase II, respectively. The rest of the lanes contain 30 units of DNA topoisomerase II and 0.4, 2, or 10 μM concentrations of VP-16 (*lanes 4–6*), NEO (*lanes 7–9*), and OEN (*lanes 10–12*), respectively.

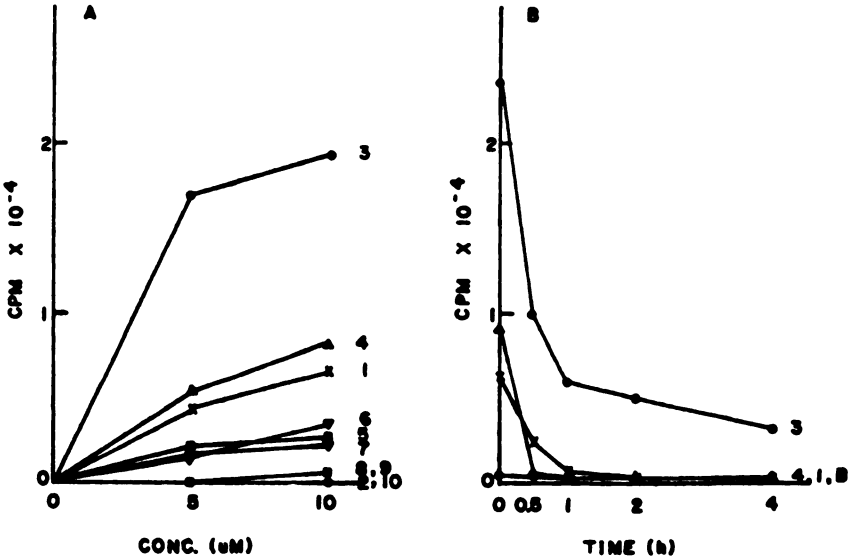


Fig. 4. Protein-linked DNA breaks produced by the drugs in KB-ATCC cells. A total of 5 × 10^5 cells were treated with 0, 5, or 10 μM concentrations of the analogs for 1 hr at 37°. Then the samples were precipitated by the potassium-sodium dodecyl sulfate method for analysis of the quantities of protein-linked DNA breaks. ×, VP-16 (**1**); △, NEO (**4**); ○, OEN (**3**); □, OEO (**5**); ▼, **6**; ▽, **7**; ■, **8**; ◨, **9**; ●, **10**; ◑, **2**. B, Repair of protein-linked DNA breaks in cells after removal of the analogs. A total of 5 × 10^5 KB cells were treated with 5 μM concentrations of either VP-16, NEO, or OEN for 1 hr at 37°. The samples were then washed with fresh medium and incubated further at 37° for 0, 0.5, 1, 2, or 4 hr. The cells were harvested and the protein-linked DNA was precipitated by the potassium-sodium dodecyl sulfate method. ●, Blank (B); ○, OEN (**3**); △, NEO (**4**); ×, VP-16 (**1**).

with the features mentioned above. The β-hydroxyalkylated NEO and β-aminoalkylated OEN inhibited DNA topoisomerase II with a potency similar to, or greater than, that of VP-16. These results further demonstrate that the glycosidic moiety at the C-4′ position is not important for topoisomerase II inhibition. NEO and VP-16 caused a similar amount of protein-linked DNA breaks both *in vitro* and *in vivo*, but NEO is 10-fold less cytotoxic against KB cells. One possibility is that, in addition to DNA topoisomerase II inhibition, VP-16 has other mechanisms of action in cells, including free radical production (25, 26); another possibility is that NEO may be metabolized to an inactive form faster than VP-16.

TABLE 2

Cytotoxicity of drugs towards KB and VP-16-resistant cells

ID_{50} is the concentration that resulted in 50% growth inhibition during continuous exposure of the cells to various concentrations of drugs for 72 hr. KB1.0C, VP-16-resistant cells that are maintained in 0.1 μM VP-16-containing medium; KB20a, VP-16-resistant cells that are maintained in 20 μM VP-16-containing medium.

Compound	ID_{50}		
	KB ATCC	KB1.0c	KB20a
	μM		
VP-16	0.1	4.7	30.6
OEN	0.1	0.5	1.0
NEO	1.6	2.5	6.5
Vincristine	0.0015	0.020	0.021
Podophyllotoxin (2)	0.042		
OEO	0.73		
6	1.0		
7	0.34		
8	0.42		
9	0.045		
10	>10 (70% growth)		

The VP-16-resistant cell lines KB1.0c and KB20a contain about one half and one fourth as much DNA topoisomerase II as the parental KB cell line, respectively. The accumulation of [^{3}H]VP-16 in both resistant lines is approximately 50% that of KB cells (11). Comparative cytotoxicity studies using these two resistant variants together with the parental KB cell line provide an excellent system to select drugs that can overcome the VP-16 uptake defect but still target DNA topoisomerase II as VP-16 does. NEO and OEN were studied because they have a potency similar to, or higher than, that of VP-16 against DNA topoisomerase II, but they have a lower potency than podophyllotoxin against tubulin polymerization. The ID_{50} values of NEO for KB1.0c and KB20a are 1.5- and 4-fold higher, respectively, than for the parental cell line. The ID_{50} values of OEN for KB1.0c and KB20a are 5- and 10-fold higher, respectively, than for the parental cell line. The 30-fold decrease in the sensitivity of KB1.0c cells to VP-16 could result from a 50% decrease in the intracellular accumulation of VP-16, as well as a 50% decrease in DNA topoisomerase II content. Because KB1.0c and 20a cells were less resistant to NEO and OEN than to VP-16, the cellular uptake system for these derivatives could be different from VP-16. This also suggests the importance of the glycosidic moiety of VP-16 in its own uptake. The resistance of KB1.0c and 20a cells to these two drugs could be primarily due to the decrease of DNA topoisomerase II. There was no difference between KB1.0c and KB20a cells in VP-16 uptake, but there was approximately a 2-fold difference in DNA topoisomerase II content. This could explain the difference between the sensitivity of these cell lines to VP-16. Because these two analogs target DNA topoisomerase II, the fact that a 2-fold difference in resistance to NEO and OEN is observed between the KB1.0c and the KB20a cells is not surprising.

In conclusion, two podophyllotoxin analogs, NEO and OEN, are potent inhibitors of DNA topoisomerase II but are not potent inhibitors of tubulin polymerization. Both cause DNA topoisomerase II-linked DNA breaks *in vitro* and protein-linked DNA breaks in cells, as VP-16 does. Based on their behavior toward KB cells and VP-16-resistant variants, these two compounds could have a spectrum of antitumor activity different from that of VP-16, because they may use a different cellular uptake system than VP-16. The lack of tubulin polymerization inhibition, specific targeting of DNA topoisomerase II, and the use of an uptake system that differs from that of VP-16 may render these compounds useful in cancer chemotherapy when VP-16 resistance occurs due to uptake deficiency.

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