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Unique Biochemical, Cytotoxic, and Antitumor Activity of Camptothecin and 4β-Amino-4′-*O*-demethylepipodophyllotoxin Conjugates

Jang-Yang Chang,† Xin Guo,* Hong-Xing Chen,* Zaoli Jiang,* Qin Fu,* Hui-Kang Wang,‡ Kenneth F. Bastow,‡ Xiao-Kang Zhu,‡ Jian Guan,‡ Kuo-Hsiung Lee‡ and Yung-Chi Cheng*§*

*Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06520, U.S.A.; ‡School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, U.S.A.; and †Cancer Cooperative Ward at National Taiwan University Hospital, Division of Cancer Research, National Health Research Institutes, Taipei, Taiwan, R.O.C.

ABSTRACT. Two compounds having a camptothecin (CPT) analog conjugated to the 4β-amino-4′-*O*-demethylepipodophyllotoxin analog were evaluated for their biochemical and biological activities. W1 [camptothecin-(*para*)-4β-amino-4′-*O*-demethylepipodophyllotoxin] had no activity against topoisomerase II (TOP II), but inhibited topoisomerase I (TOP I) with an IC_{50} value 2-fold higher than CPT. W2 [camptothecin-(*ortho*)-4β-amino-4′-*O*-demethylepipodophyllotoxin] had inhibitory activity against TOP I and TOP II with IC_{50} values 1.5-fold higher than either CPT or etoposide (VP-16). Both conjugates had similar cytotoxicity against the KB cell line, although the protein-linked DNA breaks (PLDBs) generated by W2 in KB cells were about 4-fold more than those of W1. No cross-resistance with the two conjugates was seen in a VP-16-resistant KB subline, which showed down-regulation of TOP II and overexpression of the multiple drug resistance-associated protein, or in a vincristine-resistant KB subline with overexpression of gp-170/*mdr*-1. The CPT-resistant KB variant (KB CPT 100), which has a reduction in TOP I content and another mechanism that occurs post-PLDB formation, was partially resistant to both compounds. W1 was not affected by this post-PLDB resistance mechanism. Cell cycle analysis demonstrated that W1 and W2 had similar cell cycle effects on KB and KB CPT 100 cells, which accumulated in S-phase upon drug treatment. These results suggested that W1 and W2 exerted their cytotoxicity through TOP I. In CPT-resistant cells, however, an unidentified target also may be involved in the cytotoxic action of W1, and TOP II may still be a target for W2. *In vivo*, W1 was more effective against the growth of human prostate cancer cells in nude mice than VP-16, CPT, or W2. Given its antitumor activity and unique biochemical mechanism of action, W1 warrants exploration as an antitumor compound. BIOCHEM PHARMACOL **59**;5:497–508, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. topoisomerase; camptothecin; VP-16; conjugates; antitumor

The plant alkaloid CPT‖ and several of its more water-soluble derivatives have shown promising activity against human tumors [1–3]. The primary intracellular target for CPT is the DNA unwinding enzyme TOP I, which introduces a transient, single-stranded break in the phosphodiester backbone of duplex DNA [4–6]. CPT acts by stabilizing the TOP I–DNA reaction intermediate, resulting in a high level of steady-state PLDBs and subsequently leading to cell death when replication occurs [7, 8]. However, no direct correlation between the level of PLDBs and CPT cytotoxicity has been observed. It has been demonstrated that CPT, at high concentrations, can kill both S-phase and non-S-phase cells [9]. The mechanism of cytotoxicity of CPT in non-S-phase cells is still not clear. Furthermore, a number of studies showed that cellular events other than the formation of PLDBs were also essential for CPT cytotoxicity [10–12]. The mechanisms of CPT resistance characterized to date include (a) reduced drug accumulation, (b) reduced TOP I content, and (c) altered TOP I resulting in decreased formation of PLDBs [13–18]. Beidler *et al.* [19] further demonstrated that the steps subsequent to CPT-induced formation of TOP-I linked DNA breaks and independent of TOP I could potentially be altered to confer resistance to CPT cell killing. Collaterally increased sensitivity to etoposide (VP-16) or elevated TOP II levels also were observed in some CPT-resistant cell lines [14, 17, 19], although this is not a general phenomenon for all CPT-resistant cell lines.

§ Corresponding author: Professor Yung-Chi Cheng, Department of Pharmacology, Yale University School of Medicine, Sterling Hall of Medicine, P.O. Box 208066, New Haven, CT 06520-8066. Tel. (203) 785-7119; FAX (203) 785-7129.

‖ *Abbreviations*: CPT, camptothecin; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein; PLDB, protein-linked DNA strand break; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; TOP I, topoisomerase I; and TOP II, topoisomerase II.

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In contrast to the limited number of drugs that act on TOP I, TOP II is a target for several structurally disparate antitumor agents, including epipodophyllotoxins, anthracyclines, *m*-AMSA, and ellipticines [20–23]. VP-16, the prototypical epipodophyllotoxin, has been used widely in the treatment of human cancers [24, 25]. It inhibits cell growth by stabilizing TOP II in a covalent complex with DNA, resulting in double-stranded DNA breaks and subsequent cell death [23, 26]. Three mechanisms have been proposed to be involved in the development of VP-16 resistance: (a) decreased cellular uptake of VP-16, (b) quantitative change of TOP II, and (c) qualitative change of TOP II [27]. It has been suggested that compounds with substitutions in the glycosidic moiety of VP-16 are unaffected by the mechanism responsible for the decreased uptake of VP-16 in VP-16-resistant cells, but still exert cytotoxicity through the action of TOP II [28].

Contradictory reports on the interaction between CPT, or its derivatives, and TOP II-directed anticancer compounds in different cell lines have been reported. Antagonistic interactions were seen in human HL-60 and HT-29 cell lines [29, 30]. However, additive or subadditive effects were seen in other cells [31–33]. These interactions likely depend on the compounds being tested as well as concentration, scheduling, and the cell line being used. We were interested in the development of compounds that can act on both TOP I and TOP II with different potency and still be active when resistance is developed due to alteration of one target.

We demonstrated previously that compounds in which the glycosidic moiety of VP-16 at the C-4 position is replaced with a 4β-*N*-aromatic ring via the *para*-position have enhanced inhibitory activity against TOP II [28]. VP-16-resistant KB/7D cells, which show a decrease in the amount of TOP II and overexpression of MRP [27, 34], are only partially resistant to this class of compounds. Several lines of evidence suggest that modifications at the 9, 10, and 11 positions of the A-ring and the 7-position of the B-ring of CPT generally are well tolerated and in many cases enhance the potency of CPT analogues in both *in vitro* and *in vivo* studies [35]. Based on these observations, two conjugates of camptothecin and 4β-4′-*O*-demethylepipodophyllotoxin were synthesized. The preliminary studies showed that one of these two conjugates inhibits both TOP I and II but with less potency than that of the precursors [36]. Here, we describe more detailed studies of these two conjugates in terms of their behaviors toward TOP I and II and their cytotoxicity against human KB cells and their VP-16-, CPT-, and vincristine-resistant variants. The activity of W1 [camptothecin-(*para*)-4β-amino-4′-*O*-demethylepipodophyllotoxin] and W2 [camptothecin-(*ortho*)-4β-amino-4′-*O*-demethylepipodophyllotoxin] against a variety of tumor cell lines and the results of animal studies also are presented. The structures of these two conjugates and their precursors are shown in Fig. 1. The C-4 position of VP-16 was substituted with an *N*-aromatic ring, with the CPT analogue at either the *para*- (W1) or *ortho*- (W2) position.

MATERIALS AND METHODS

Cell Lines

All cell lines, except the gastric cancer cell line (AGS), were maintained in RPMI 1640 medium containing 5% fetal bovine serum. AGS was maintained in the same medium with 10% fetal bovine serum. The VP-16 resistant cell line KB/7D, the CPT-resistant cell line KB CPT 100, and the vincristine-resistant cell line KB Vin 20 were maintained in growth medium supplemented with 7 μM, 100 nM, and 20 nM drug, respectively. Revertant KB CPT 100^{rev} cells also were used for studies. The characteristics of these cell lines have been described previously [19, 27, 34]. KB CPT 100 cells display a decrease in TOP I levels and an unidentified post-PLDB resistance mechanism to TOP I poisons; KB/7D cells overexpress MRP and display a decrease in TOP II; and KB Vin 20 cells overexpress P-gp. All the cell lines were tested periodically for mycoplasma by the Gen-Probe rapid detection system (Gen-Probe, Inc.).

Animals

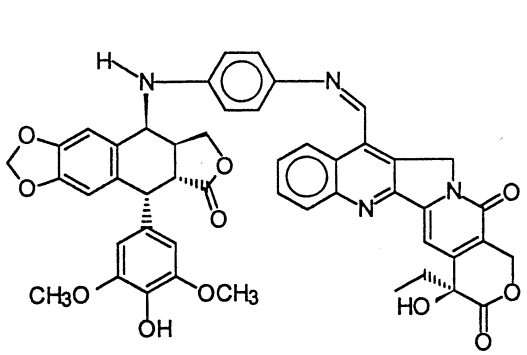
Male NCR nude mice (4- to 6-weeks-old) weighing 16–20 g were obtained from the National Institutes of Health. Animals were used for toxicity studies and for evaluation of the antitumor activity of these compounds.

Chemicals

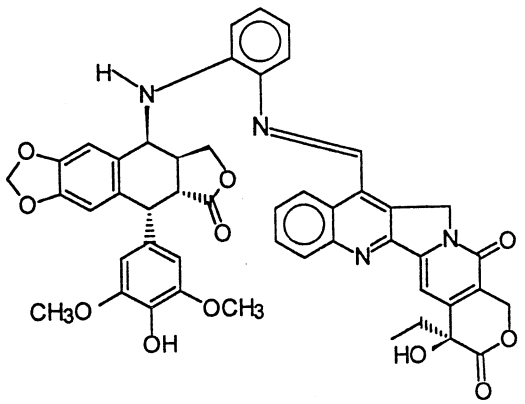
W1, W2, and CPT were provided by Dr. K. H. Lee of the School of Pharmacy at the University of North Carolina. The synthesis of W1 and W2 has been described previously [36]. VP-16, topotecan, *m*-AMSA, actinomycin D, and daunorubicin were purchased from the Sigma Chemical Co. Stock solutions of these drugs, except water-soluble drugs, were dissolved in dimethyl sulfoxide at 20 mM, stored at −20°, and diluted in water immediately before use. Bacteriophage T4 ligase and restriction endonucleases *Eco*RI and *Hin*dIII were purchased from New England Biolabs, Inc. [α-^{32}P]dATP and [^{14}C]thymidine were purchased from ICN.

Enzymes

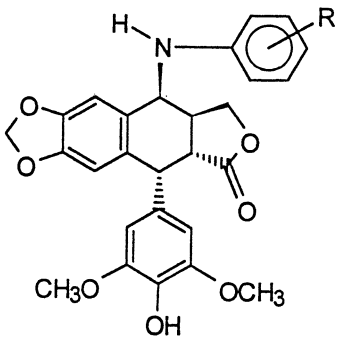
The methods for the partial purification of TOP I and II were described previously [37]. Briefly, HepG2 cells were collected and homogenized in buffer containing 75 mM potassium phosphate, 10% glycerol, 1 mM EDTA, 0.4 M phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.5 μg/mL of leupeptin, and 0.5 μg/mL of pepstatin. The lysate was adjusted to 0.35 M potassium phosphate. After standing for 1 hr at 4°, the extract was centrifuged, and the supernatant was applied sequentially to DEAE and P-11 columns. TOP I and II activities were determined by



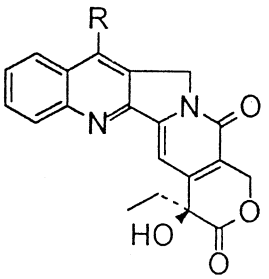
W1



W2



W-P-1 (R= *p*-NH2)
W-P-2 (R= *o*-NH2)



CPT (R= H)
CPT analogue (R= CHO)

FIG. 1. Chemical structures of two conjugates (W1 and W2), their precursors (W-P-1 and W-P-2), and CPT and its analogue.

pBR322 relaxation and P4 DNA unknotting assays, respectively. The active fractions were pooled and stored at −20°.

Inhibition of the Catalytic Activity of Topoisomerases

TOP I catalytic activity was assayed by the ATP-independent relaxation of pBR322 supercoiled DNA, as described previously [38]. One unit of enzyme was defined as the amount of enzyme able to relax 50% of 0.4 μg of supercoiled DNA. TOP II catalytic activity was assayed by the ATP-dependent unknotting of P4 phage DNA, as described previously [39]. One unit of enzyme was defined as the amount unknotting 50% of 0.3 μg of knotted P4 DNA. Various concentrations of drugs were used to determine the inhibitory activity against topoisomerases. Photographs of the resulting ethidium bromide-stained agarose gels were taken under UV light, and the resulting negatives were used for densitometric scans to quantitate band densities. The IC_{50} was defined as the drug concentration required for 50% inhibition of the enzyme activity.

Topoisomerase-Induced DNA Cleavage

STIMULATION OF DNA CLEAVAGE BY TOP I IN PBR322 DNA. The method for evaluation of TOP I-induced single-stranded DNA breaks was described previously [40]. Linear pBR322 DNA was 3′-end labeled, as described previously [41]. The reaction was performed at 37° for 15 min and stopped by the addition of 5 μL of 5% SDS and 0.75 mg/mL of proteinase K followed by incubation for 30 min at 50°. Prior to loading onto a 1% agarose gel in 1X TBE (Tris base, boric acid, and EDTA) with 0.1% SDS, 10-μL samples were denatured with 0.45 N NaOH, 30 mM EDTA, 15% (v/w) sucrose, and 0.1% bromophenol blue.

TABLE 1. Inhibition of TOP I and II catalytic activity and induction of PLDBs by these compounds

	IC_{50}(μM)		Apparent K_d for PLDBs in KB cells
	TOP I	TOP II	
VP-16	NA*	36 ± 2	16 ± 3
CPT	27 ± 3	NA	4.5 ± 0.5
W1	62	>100†	8 ± 2
W2	40 ± 2	50 ± 6	6.5 ± 1.2
CPT-A‡	20 ± 5	NA	4 ± 1
W-P-1	NA	10 ± 4	4.3 ± 0.7
W-P-2	NA	30 ± 3	8.3 ± 1.5

Values are means ± SD. K_d values were determined by three independent experiments (average ± SD); IC_{50} values were determined by three independent experiments (average ± SD) except for W1, which is the average of two experiments.

* Not assayed.

† No inhibitory activity was observed at the concentration tested.

‡ CPT-A: CPT analogue (see Fig. 1).

After electrophoresis, the gel was dried and autoradiographed for 24 hr.

STIMULATION OF DNA CLEAVAGE BY TOP II. The cleavage reaction was performed as described above, except for the addition of 1 mM ATP and 10 mM $MgCl_2$ to the reaction mixture. Samples were incubated with 5 μL of sample buffer [50 mM EDTA, 50% (v/w) sucrose, and bromophenol blue] prior to loading onto the gel.

Ethidium Bromide Displacement Assay

DNA binding capacity of W1 and W2 was monitored by an ethidium bromide displacement assay, as described previously [42, 43]. Different concentrations of drugs were incubated with 2.5 μM ethidium bromide and 20 μM calf thymus DNA in a buffer containing 9.4 mM NaCl, 20 mM EDTA, and 2 mM HEPES. Fluorescence intensity of the solution was measured by a spectrophotometer (SPF 500 spectrofluorometer); the excitation wavelength was 546 nm, and the emission wavelength was 595 nm for all compounds.

DNA Unwinding Measurement

The DNA unwinding effect of drugs was assayed using a method described previously [44]. The DNA circle-ligation assay, using nicked DNA as substrate, was performed based on the method of Montecucco *et al.* [45].

Measurement of PLDBs In Vivo

A potassium–SDS co-precipitation (K–SDS) assay was used for quantitation of PLDBs *in vivo*, as described previously [46]. The apparent K_d values for each compound were determined graphically from double-reciprocal plots of the saturation curves.

Growth Inhibition Assay

Cells in logarithmic phase were cultured at a density of 5000 cells/mL in a 24-well plate. The resistant cells were maintained in drug-free medium for 3 days prior to use. The cells were exposed to various concentrations of the drugs for 72 hr. The methylene blue dye assay was used to evaluate the effects of the drugs on cell growth, as described previously [47], and to determine the concentration of drug that inhibited 50% of cell growth (IC_{50}).

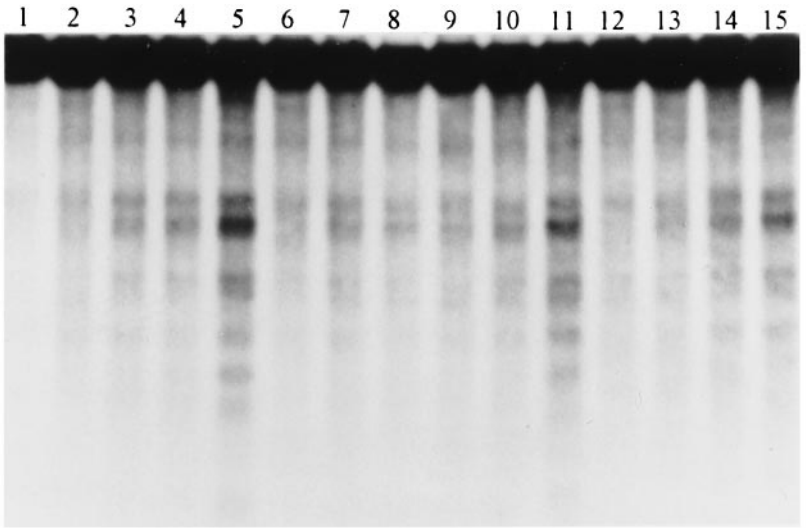


FIG. 2. DNA cleavage patterns induced by W1, W2, and CPT in the presence of TOP I. The DNA cleavage assay was done as described in Materials and Methods, and reactions were terminated by SDS–proteinase K treatment. Samples were analyzed by alkali gel electrophoresis. Lane 1, control DNA; lane 2, DNA plus 20 units of TOP I; lanes 3–5, enzyme plus 0.1, 0.3, and 1 μM CPT, respectively; lanes 6–8, enzyme plus 1, 5, and 25 μM W1, respectively; lanes 9–11, enzyme plus 1, 5, and 25 μM W2, respectively; and lanes 12–15, enzyme plus 0.1, 0.3, 1, and 5 μM CPT analogue, respectively.

TABLE 2. Growth inhibition assay

Drug	KB	IC_{50} (nM) KB CPT 100	KB CPT^{rev}	KB/7D	KB Vin 20*
VP-16	560 ± 50	450 ± 50	400 ± 15	33,500 ± 2,500	2,000
CPT	13 ± 4	436 ± 28	70 ± 3	17 ± 1	18
Topotecan	17 ± 2	510 ± 27	50 ± 2	16 ± 3	34
W1	50 ± 10	216 ± 30	250 ± 30	75 ± 5	70
W2	43 ± 7	230 ± 30	80 ± 20	81 ± 11	100
Vin	5 ± 0	7 ± 3	NA†	119 ± 1	107

Experiments were performed in five cell lines: KB, KB CPT 100, KB CPT^{rev}, KB/7D, and KB Vin 20. Values are means ± SD; all values are the average of at least three separate experiments, unless noted otherwise.

* Average of two experiments.

† Not assayed.

Clonogenic Assays

Cells in logarithmic phase were cultured in 6-well plates for 24 hr. Next, the cells were treated with different concentrations of drugs for one generation. Then cells were washed with pre-warmed PBS and maintained in drug-free fresh medium for 10–14 days. The colonies were fixed and stained with 50% methanol containing 0.5% methylene blue. The LC_{50} was calculated as the drug concentration that inhibited colony formation to 50% of control values.

Cell Cycle Analysis

Flow cytometric analysis of propidium iodide-stained cells was performed with a FACS 20 flow cytometer (Becton Dickinson). Cell cycle analysis was performed according to the mathematical method of Jett [48].

In Vivo *Toxicity Studies*

Male NCR nude mice were administered various dosages of drugs by i.p. injection on a schedule of once daily for 5 consecutive days. Control and treated animals were observed and weighed daily until termination of the experiments at day 30. Toxicity was determined by the death/survival ratio of the treated versus untreated animals, and the drug dose lethal to 50% of the mice (LD_{50}) was determined.

In Vivo *Therapeutic Activity Against Human Tumor Xenografts*

Male NCR nude mice were inoculated s.c. with 5×10^6 DU-145 cells over bilateral inguinal regions. Eighteen days after implantation, when the tumor weight reached 100–

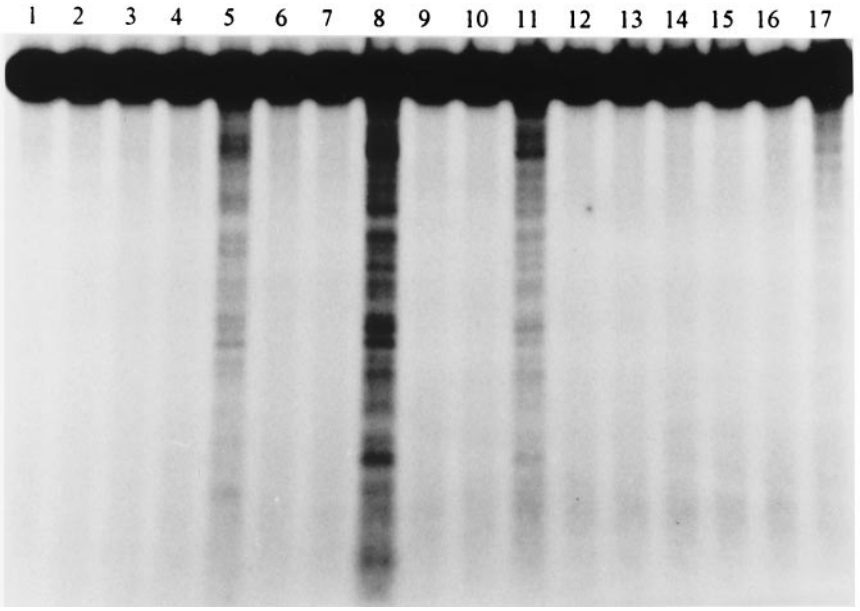


FIG. 3. Effect of compounds on TOP II-dependent DNA double-stranded breaks. This assay was done as described in Materials and Methods, stopped by SDS–proteinase K treatment, and then analyzed by neutral gel electrophoresis. Lane 1, control DNA; lane 2, DNA plus 30 units of TOP II; lanes 3–5, enzymes plus 5, 10, and 50 μM VP-16, respectively; lanes 6–8, enzyme plus 5, 10, and 50 μM W-P-1, respectively; lanes 9–11, enzyme plus 5, 10, and 50 μM W-P-2, respectively; lanes 12–14, enzyme plus 10, 50, and 100 μM W1, respectively; and lanes 15–17, enzyme plus 10, 50, and 100 μM W2, respectively.

250 mg, drugs were administered at one-third of the LD_{50} by i.p. injection on a schedule of once daily for 5 consecutive days. The tumor volume was determined by caliper measurement and calculated according to the following formula:

$$\text{Tumor weight (mg)} = [\text{Length (mm)} \times \text{width}^2\ (\text{mm}^2)]/2$$

The tumor growth curves were generated as described previously [49]. Toxicity was evaluated by the change in body weight.

RESULTS

Interaction with TOP I

The effects of W1, W2, CPT, and a CPT analog (Fig. 1) against TOP I catalytic activity were examined. All of the compounds showed inhibitory activity against TOP I (Table 1). The IC_{50} of CPT was 27 ± 3 μM; that of the CPT analogue was 20 ± 5 μM; that of W1 was 62 μM; and that of W2 was 40 ± 2 μM. When the effects of those compounds on TOP I-induced single-stranded DNA cleavage of linearized pBR322 DNA were assessed (Fig. 2), it was found that both conjugates stimulated TOP I-induced DNA cleavage, but with much less potency than that of CPT and its analogue. CPT and W2 stimulated TOP I-induced single-stranded DNA breaks in a concentration-dependent manner. However, the amount of cleaved complex generated by W1 reached a plateau at 5 μM and was higher than at 25 μM. W2 generated more single-stranded DNA breaks than W1. The cleavage patterns induced by W1 and W2 were similar to that of CPT.

Interaction with TOP II

We previously demonstrated that substitution of the glycosidic moiety of VP-16 enhanced the inhibitory activity against TOP II [28]. One of the precursors, W-P-1, showed 3-fold more potency than VP-16 against TOP II [28]. Another precursor, W-P-2, displayed activity against TOP II similar to that of VP-16 (Table 1). To our surprise, W1, which is a conjugate of a CPT analogue with W-P-1, displayed very poor inhibitory activity against TOP II (Table 1). VP-16 was about 1.4-fold more potent against TOP II than W2. Furthermore, VP-16, its analogs, and W2 induced the same pattern of TOP II-induced double-stranded DNA breaks. W1 did not induce any TOP II-mediated double-stranded DNA breaks, even at 100 μM (Fig. 3).

Interaction of W1 and W2 with DNA

Most of the antitumor drugs that stabilize the cleavable complex are DNA intercalators, such as *m*-AMSA, daunorubicin, and ellipticines. To investigate whether these two conjugates intercalated into DNA, an ethidium bromide displacement assay and an unwinding assay with linearized pBR322 DNA and T4 DNA ligase were performed (Fig. 4). In contrast to the strong intercalator actinomycin D and the weak intercalator *m*-AMSA, W1 and W2 did not reduce the fluorescence intensity of intercalated ethidium bromide (Fig. 4A). In the DNA unwinding measurement, *m*-AMSA and daunorubicin were included as controls. In contrast to *m*-AMSA and daunorubicin, which produced a concentration-dependent DNA shift, neither W1 nor W2 caused any DNA shifting. Daunorubicin, as a control, produced positively supercoiled DNA at 30 μM (Fig. 4B).

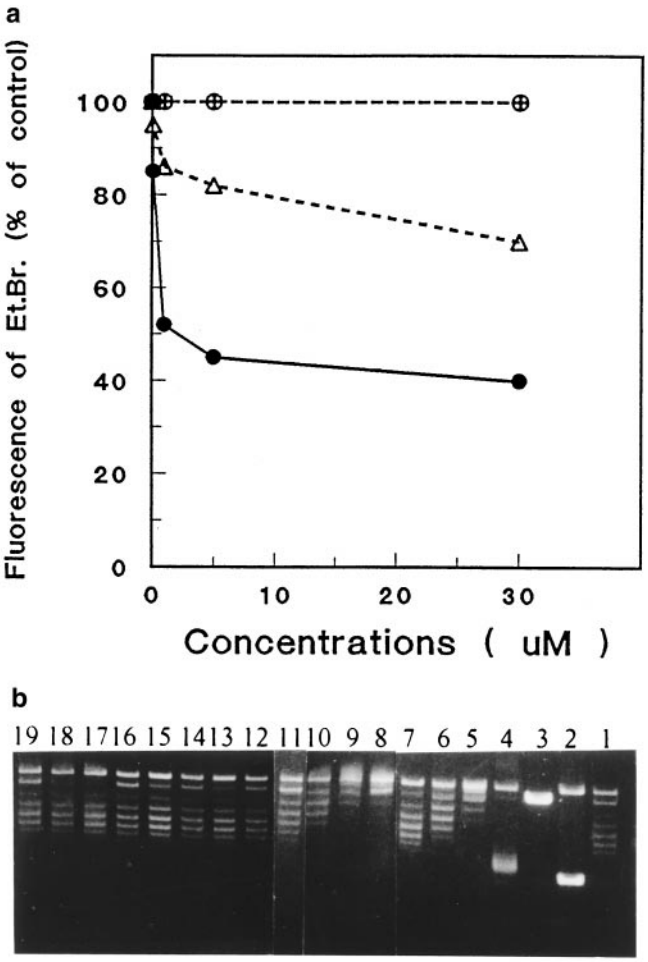


FIG. 4. (A) Ethidium bromide displacement assay. Ethidium bromide (2.5 μM) and calf thymus DNA (20 μM) were incubated with 0.1, 1, 5, and 30 μM actinomycin D (filled circles), *m*-AMSA (open triangles), W1 (open circles), and W2 (+ signs), respectively. The percentage of fluorescence intensity was calculated by setting the intensity of 2.5 μM ethidium bromide and 20 μM calf thymus DNA as 100%. No decrease in fluorescence was detected in the W1 and W2 samples, and the lines are superimposed. (B) Unwinding of DNA by W1, W2, *m*-AMSA, and daunorubicin. Unwinding measurement was done as described before. Lane 1, no drug control; lane 2, supercoiled DNA control; lane 3, linear DNA; lanes 4–7, 30, 10, 5, and 2.5 μM daunorubicin; lanes 8–11, 30, 10, 5, and 2.5 μM *m*-AMSA; lanes 12–15, 2.5, 5, 10, and 30 μM W1; and lanes 16–19, 2.5, 5, 10, and 30 μM W2.

TABLE 3. Clonogenic assay

Drug	LC_{50} (nM)			
	KB	KB CPT 100	KB CPT^{rev}	KB/7D
VP-16	198 ± 27	340 ± 77	400 ± 40	18,200 ± 1,600
CPT	15 ± 3	3,750 ± 250	65 ± 7	18 ± 2
Topotecan	15 ± 8	1,260 ± 115	50 ± 3	20 ± 9
W1	44 ± 10	620 ± 85	250 ± 45	42 ± 18
W2	75 ± 18	580 ± 103	100 ± 12	68 ± 24

Experiments were performed in four cell lines: KB, KB CPT 100, KB CPT^{rev}, and KB/7D. Values are means ± SD for three independent experiments.

Cytotoxicity

Based on *in vitro* studies, it was concluded that W1 was a TOP I inhibitor with less potency than CPT, and W2 was a dual TOP I and II inhibitor. To further evaluate the cytotoxic effects of these conjugates in cells, KB cells and their VP-16-, CPT-, and vincristine-resistant variants were used. The results of growth inhibition and clonogenic assays are shown in Tables 2 and 3. To our surprise, W1 and W2 displayed almost identical cytotoxic effects against these cells. Both of these conjugates showed no cross-resistance in VP-16-resistant cells and showed partial cross-resistance with CPT-resistant cells. However, in CPT partial revertant cells, due to the loss of the post-PLDB resistance mechanism, W2 increased its activity, whereas W1 activity was almost the same as in the resistant cells. W1 and W2 displayed no cross-resistance in KB Vin 20 or KB/7D cells, which showed over-expression of MDR1 and MRP, respectively. We further tested the cytotoxicity of these compounds against different human tumor cell lines. As shown in Table 4, these two conjugates displayed similar cytotoxic effects against different human tumor cell lines. W1 and W2 were not altered by incubation with KB cells or with PBS at 37° for 3 days, demonstrating that the compounds were stable over the course of the experiment (data not shown).

Induction of PLDBs

After a 30-min exposure to increasing concentrations of these compounds, the level of PLDBs increased in a concentration-dependent manner, and reached a plateau (Fig. 5A). In KB cells, the maximal level of PLDBs generated by CPT, W2, and VP-16 was 4-, 4-, and 2-fold higher than by W1. The apparent K_d, which reflects the apparent binding affinity of the compound in stabilizing PLDBs associated with TOP I, TOP II, or both enzymes, was determined by double-reciprocal plots of the concentration–response relationship for these compounds. CPT, W1, and W2 exhibited 2- to 4-fold better binding affinity in stabilizing PLDBs than did VP-16 (Table 1). In the VP-16 resistant cells, which displayed down-regulation of TOP II and overexpression of MRP, CPT, W1, and W2 generated similar concentration–response curves of PLDBs (Fig. 5B), whereas VP-16 caused only 10% of the maximal PLDBs seen in KB cells. In a CPT-resistant cell line (CPT 100), which showed a reduced amount of TOP I and a post-PLDB mechanism responsible for CPT resistance, the maximal amount of PLDBs generated by CPT, W1, and W2 was decreased by 50% in comparison with that in KB cells. VP-16-induced maximal PLDBs in KB CPT 100 cells were about 1.5-fold of those in KB cells (Fig. 5C). To test whether W1- and W2-induced PLDBs were reversible, KB cells were incubated with drug-free medium for specified periods of time after treating the cells with 5 μM CPT, 10 μM W1, and 5 μM W2 (Fig. 6). The PLDBs induced by all three compounds were found to be reversible, with the order of rates of reversibility being W2 > W1 > CPT.

Effect on Cell Cycle

The impact of different concentrations of CPT, W1, and W2 on cell cycle progression of KB cells was studied after one generation of drug exposure (Fig. 7). CPT-, W1-, and W2-treated cells displayed almost identical effects on KB cell progression. Cells accumulated in S-phase with concomitant losses from the G_0/G_1 and G_2/M phases, especially at the higher drug concentrations.

In Vivo *Toxicity and Therapeutic Activity*

The *in vivo* toxicity of CPT, VP-16, W1, and W2 was examined in NCR immunodeficient nude mice by administering each compound at different dosages, i.p., for 5

TABLE 4. *In vitro* cytotoxicity of various compounds against human tumor cell lines

Cell line	Origin	IC_{50} (nM)			
		VP-16	CPT	W1	W2
KB	Epidermoid carcinoma	560 ± 50	13 ± 4	50 ± 10	43 ± 7
HepG2	Liver carcinoma	1334 ± 100	11 ± 1	28 ± 3	35 ± 4
HeLa	Cervical carcinoma	410 ± 27	14 ± 2	40 ± 3	28 ± 2.5
DU-145	Prostate carcinoma	800 ± 45	8 ± 2	38 ± 2	36 ± 3
HCT116	Colon carcinoma	700 ± 23	1 ± 0	20 ± 3	25 ± 3
SKOV3	Ovarian tumor	> 1000	22 ± 4	60 ± 7	90 ± 5
AGS	Stomach carcinoma	180 ± 12	4 ± 1	5 ± 1	6 ± 0.6
CEM	T cell lymphoma	450 ± 25	8 ± 1	12 ± 2	9 ± 1

Values are averages ± SD of at least three separate experiments.

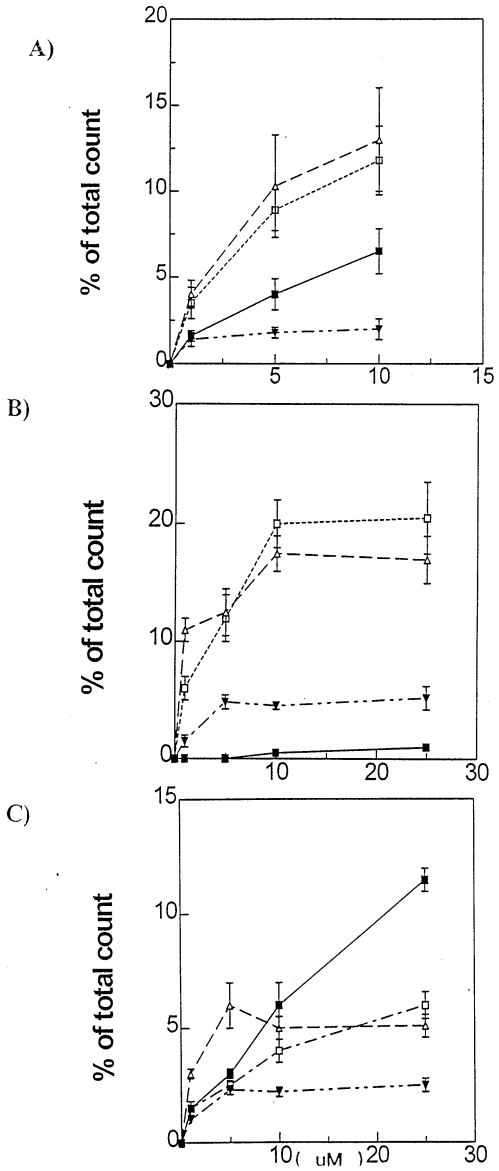


FIG. 5. Measurement of PLDBs *in vivo* by a K–SDS co-precipitation assay. The graphs show steady-state levels of PLDBs in KB (A), KB/7D (B), and KB CPT 100 (C) cells after 30 min of incubation with 1, 5, and 10 μM VP-16 (filled squares), CPT (open triangles), W1 (filled inverted triangles), and W2 (open squares) for KB cells and 1, 5, 10, and 25 μM concentrations of these compounds for KB/7D and KB CPT 100 cells. Data represent means ± SD from three independent experiments performed in duplicate. Note that the left-hand scales differ.

consecutive days. Survival of the mice was monitored for 30 days. The LD_{50} was estimated to be 3.6, 36, 40, and 75 mg/kg for CPT, VP-16, W1, and W2, respectively. One-third of the LD_{50} of CPT, VP-16, or W1 and one-fifth or two-thirds of the W2 LD_{50} was used to treat NCR nude mice bearing DU-145 human prostate tumor cells. The results are shown in Fig. 8. No drug-related deaths were observed. Interestingly, although the *in vitro* cytotoxicity of W2 was equal to that of W1 (Table 4), W2 was much less effective than W1 in this animal model against the DU-145 tumor. W1 caused

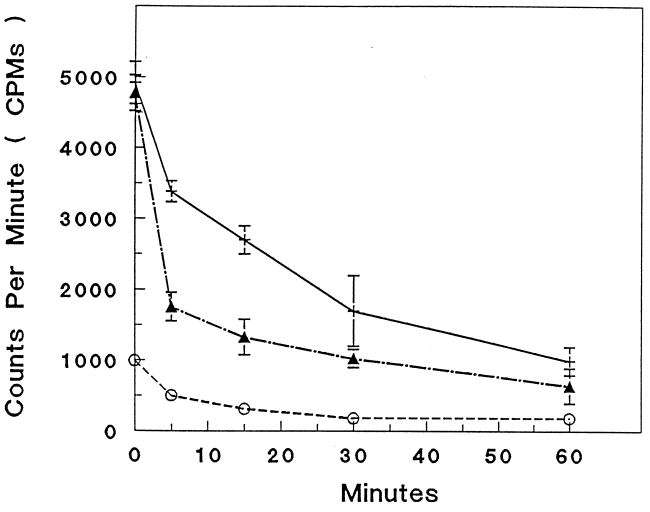


FIG. 6. Reversibility of CPT-, W1-, and W2-induced PLDBs. KB cells were treated with 5 μM CPT (+ signs), 10 μM W1 (open circles), and 5 μM W2 (filled triangles) for 60 min, and then the cells were incubated in drug-free medium for the indicated times. The K–SDS assay was performed as described in Materials and Methods. Bars = SD, N ≥ 3, within a representative experiment, which was repeated at least once.

approximately a 50% regression of tumor size by day 15, but the tumor did begin to grow back to the original size 25 days after the last treatment. At approximately equitoxic dosages of VP-16 and CPT (one-third of the LD_{50}), no therapeutic effect was apparent.

DISCUSSION

Evaluation of the activity of these two conjugates against TOP I and II revealed some unexpected findings. Modification at the B-7 position of CPT generally was well-tolerated. W1 and W2 were inhibitory to TOP I with IC_{50} values 2- and 1.5-fold higher than that of CPT, based on DNA relaxation assays. Using a TOP I-induced DNA cleavage assay, it was observed that both W1 and W2 could cause DNA breaks *in vitro*. The maximal PLDBs generated by W2 were higher than those generated by W1, and W1 at high concentrations generated fewer DNA breaks than at lower concentrations. One explanation is that W1 has two modes of action against TOP I. The stabilization of PLDBs required lower concentrations of W1 than those required for the inhibition of a step before PLDB formation. Thus, at high concentration W1 generated fewer DNA breaks than were induced at low concentration. Further experiments are required to verify this hypothesis.

Previously, we demonstrated that compounds that replace the glycosidic moiety of VP-16 with an *N*-aromatic ring or other nonglycosidic moieties retain their activity against TOP II [28, 50]. This was applicable to W2, which had about the same activity against TOP II as W-P-1. Unexpectedly, W1 lost almost all its activity against TOP II. This suggests that TOP II cannot tolerate such a bulky addition to the *para*-position of W-P-1. Since some TOP

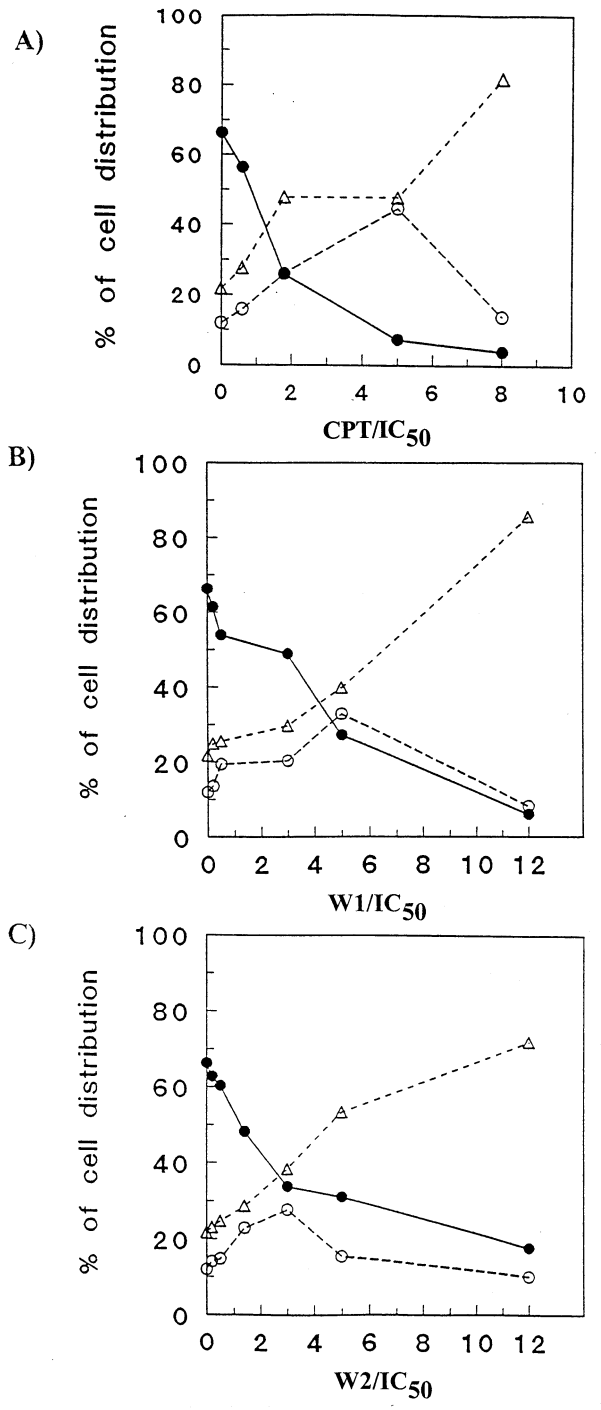


FIG. 7. Effects of CPT, W1, and W2 on cell cycle distribution of KB cells. The cell cycle analysis was done as described in Materials and Methods. Cells were treated with different ratios of drugs to IC_{50}, as indicated, for one generation time. IC_{50} was based on the growth inhibition assay. G_0/G_1 (filled circles); S (open triangles); and G_2+M (open circles). Data displayed are from a representative experiment, which was repeated at least once.

inhibitors exert their action through the interaction with DNA, and a growing number of dual TOP I and II inhibitors including TOP-53, intoplicine, and saintopin interact with DNA [51–53], we assessed the interaction of W1 and W2 with DNA using an ethidium bromide displacement assay and a DNA unwinding assay. The dual inhibitory activity of W2 against TOP I and TOP II as well as the inhibitory activity of W1 against TOP I was not due to their interaction with DNA.

To further study the action of these two conjugates with TOP I and TOP II in cells, cell-based drug-induced PLDB assays using KB cells and their CPT- or VP-16-resistant variants were performed. In KB cells, the amount of PLDBs induced by W2 was 4-fold more than that induced by W1. In VP-16-resistant cells, which overexpressed MRP and had lower TOP II than KB cells, the maximal amount of PLDBs induced by W2 was indistinguishable from that induced by CPT but still higher than W1. Furthermore, in KB CPT 100 cells, which had lower TOP I activity, the formation of PLDB induced by W1, W2, and CPT was lower than that in KB cells. This difference in PLDB formation induced by W1 and W2 in cells could not be due to a faster rate of dissociation of PLDBs in cells treated with W1, since the reversal of W2-induced PLDBs was faster than that of W1-induced PLDBs. Since W2 was shown to induce TOP I- and TOP II-mediated DNA breaks, one would expect that W2 would induce maximal PLDBs equivalent to the summation of CPT- and VP-16-induced PLDBs. This was not observed. One possible explanation is that the formation of drug-induced TOP I PLDBs hindered drug-induced TOP II PLDB formation in cells. Indeed, when we combined a VP-16 analogue with CPT, we did not observe more PLDB formation than that of CPT alone [36]. This will require further exploration.

Another related issue was the relationship between cytotoxicity and cellular PLDB formation by drugs. W1 and W2 were equitoxic toward KB cells and their drug-resistant variants, but a dramatic difference in the ability of W1 and W2 to generate PLDBs in those cells was found. This indicated that no direct relationship existed between the amount of maximal PLDBs induced and their cytotoxicity. Other factors, such as the quality of PLDBs induced by the compounds or the rate of repair of replication-related double-stranded DNA breaks, are likely to be critical in determining the cytotoxicity of TOP I and TOP II inhibitors.

Several resistant variants of KB cells, with different resistance mechanisms, were chosen to study the impact of resistance mechanisms on these two conjugates. This study also could provide a system to obtain information about the principal cellular target for these two conjugates. W1 and W2 displayed equal cytotoxicity toward KB cells in spite of the disparity of their effects on TOP I and II. KB CPT 100 was partially resistant to W1 and W2, whereas KB/7D (VP-16-resistant) was as sensitive as the KB cell line to these two compounds. In KB CPT 100^{rev} cells, which have lost the post-PLDB CPT resistance mechanism [19], the sensitivity to W1 was not altered much from that of CPT 100 cells, whereas W2 exerted more activity against CPT 100^{rev} cells than against CPT 100 cells (Table 2). Taking these observations together, we propose that (a) the main

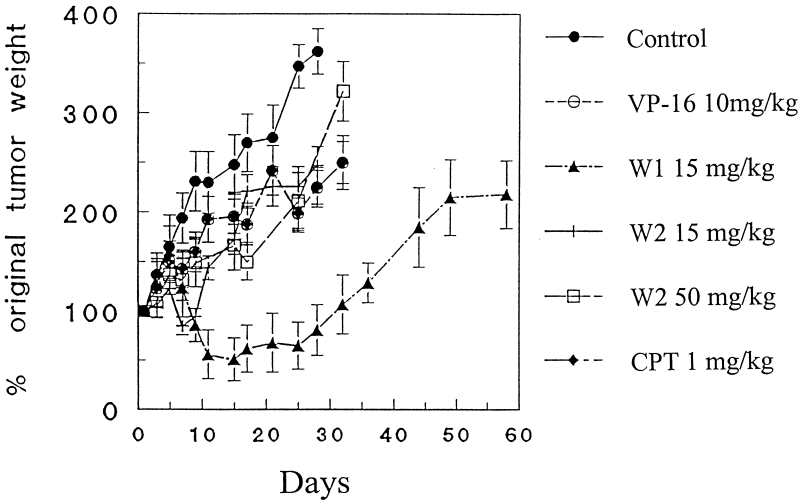


FIG. 8. Inhibition of human tumor growth by CPT, VP-16, W1, and W2. Four- to six-week-old NCR nude mice were inoculated s.c. in bilateral inguinal regions with 5×10^6 DU-145 cells. Treatment was started when the tumors were measurable. Drugs, as indicated, were administered once daily on days 0 through 4, and then the sizes of the tumors were measured frequently. Each point represents the mean of 10 tumors; bar = SD.

target for W1 is TOP I, but W1 at higher concentrations may act on a new, unidentified target that is unrelated to a post-PLDB CPT resistance mechanism; (b) the main cellular target for W2 is TOP I, and a post-PLDB formation mechanism responsible for CPT resistance also could cause resistance to W2, but W2 can act on TOP II when cells become resistant to CPT; and (c) W1 and W2 are not substrates for P-gp or MRP.

Several reports have shown that CPT exposure at low concentrations results in the accumulation of cells in the G_2-phase of the cell cycle [54, 55]. Our KB cell line had similar responses at low concentrations of CPT. With an increase in the concentration of CPT, a greater accumulation of cells in S-phase was observed. Similar responses were demonstrated in W1- and W2-treated KB cells. Their impact on cell cycle progression was the same as that of CPT in KB cells.

There were differences in sensitivity to W1 and W2 among the different tumor cell lines examined. We chose DU-145 and KB cells for further *in vivo* study. Preliminary results of antitumor activity of W1, W2, VP-16, and CPT in DU-145 tumor-bearing nude mice are depicted in Fig. 8. Using approximately one-third of the LD_{50} of these compounds, only W1 markedly delayed DU-145 tumor growth. There was a lack of correlation of cytotoxicity in cell culture and antitumor activity *in vivo* for these two compounds. This could have been due to many factors. Since both W1 and W2 delayed the growth of KB and its VP-16-resistant tumor in nude mice (data not shown), it is unlikely to be due to simple differences in pharmacokinetics or metabolism.

In conclusion, two conjugates composed of CPT and a VP-16 semi-synthetic analogue were synthesized. A large disparity between these two conjugates with respect to their interactions with TOP I and II was observed both *in vitro* and *in vivo*. However, they exhibited the same cytotoxic effect against KB cells, their drug-resistant variants, and other human cell lines, except for KB CPT^{rev} cells. The major site of action of the conjugates was TOP I. When cells become resistant to CPT, another target unrelated to the action of CPT or VP-16 may be involved in the cytotoxicity of W1, whereas W2 may act on TOP II to exert its cytotoxicity in CPT-resistant cells. W1, but not W2, inhibited the growth of human prostate tumor cells in the nude mouse model, which is resistant to VP-16 and CPT. The optimal dose and schedule of W1 for the treatment of tumors need to be explored further.

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