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Antitumor Agents—CLXXIII.† Synthesis and Evaluation of Camptothecin-4β-amino-4′-*O*-demethyl Epipodophyllotoxin Conjugates as Inhibitors of Mammalian DNA Topoisomerases and as Cytotoxic Agents

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Abstract—Two conjugates composed of a camptothecin and a 4′-*O*-demethyl epipodophyllotoxin derivative joined by an imine linkage were prepared and evaluated as inhibitors of mammalian DNA topoisomerases I and II. Target compounds stimulated cleavable complex formation with both types of enzyme in vitro although activities were reduced at least twofold relative to the activity of unconjugated constituents. The behavior of the most active conjugate as an inhibitor of cell growth closely resembled both topoisomerase I- and II- inhibitory components in that the compound displayed a combined spectrum of activity against various drug-resistant KB sublines. Cytotoxic activity and selectivity were largely retained through conjugation, the exception being a lower than expected activity against a pleiotrophic multidrug-resistant subline. The induced levels and the properties of cellular protein-associated DNA complexes were consistent with topoisomerase involvement and with the in vitro cleavage assay results. Based on the present findings, conjugation afforded cleavable complex-forming topoisomerase inhibitors which display dual target specificity and a broad spectrum of cytotoxic activity against drug-resistant cells. © 1997 Published by Elsevier Science Ltd.

Introduction

Etoposide, (VP-16, **1**, Fig. 1) is a widely-used antineoplastic agent that inhibits mammalian DNA topoisomerase II isoenzymes.[2–4] Various **1**-derivatives have been developed in order to improve antitumor activity, cytotoxicity against drug resistant cells and drug-formulation characteristics including the 4′-*O*-demethyl epipodophyllotoxins bearing C-4β-N-linked substituents (**4a** and **4b**, Fig. 1).[5] The other mammalian DNA topoisomerase, a type I enzyme, is also considered to be a useful therapeutic target. Several selective inhibitors have been identified to date including the antitumor alkaloid, camptothecin, (CPT, **2**, Fig. 1) and two analogues are currently approved for clinical use in the United States.[3,6] However, not all topoisomerase inhibitors of potential clinical value are topoisomerase-type specific. For example, a 7-*H*-benzopyrido (4,3-*b*) indole-derivative (inotoplicine), inhibits topoisomerases I and II simultaneously and can circumvent topoisomerase-mediated mechanisms of drug-resistance.[7]

All of the above-mentioned compounds share a common inhibitory mechanism, which is understood in some depth at the biochemical level.[6] Enzyme inhibition involves the trapping of a putative covalent reaction intermediate called a reversible 'cleavable complex'. The intracellular lesion, presumably a topoisomerase–DNA–drug ternary complex, ultimately leads to cell death. Although the cytotoxic events depend on the particular type of topoisomerase involved, the precise biochemical pathway(s) to cell killing remains to be defined.[3,6,8]

The aim of the present work was to investigate whether a topoisomerase inhibitor displaying dual target specificity could be prepared by conjugating derivatives of the prototypical drug **1**, with an analogue of compound **2**. For this purpose, a linkage position was chosen based on the known tolerance of bulky C-4β-substituents in 4′-*O*-demethyl epipodophyllotoxin derivatives and recent SAR studies of 7-substituted camptothecin analogues.[5,9,10] Subsequent evaluation of target conjugates revealed biological and biochemical properties consistent with their action as cleavable complex-forming inhibitors of both DNA topoisomerase I and II.

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†For Part 172, see ref 1.
Abbreviations: THF, tetrahydrofuran; SDS, sodium dodecylsulfate; PBS, 140 mM NaCl, 4 mM KCl, 0.5mM Na_2HPO_4, 150 mM KH_2PO_4; TBE, 95 mM Tris–borate, 2 mM EDTA pH 8; mAMSA, 4′-(9-acridinylamino)-methanesulfon-m-anisidide; EDTA, Disodium(ethylenedinitrilo) tetraacetic acid; RNase, bovine pancreatic ribonuclease; PFGE, pulsed-field gel electrophoresis.

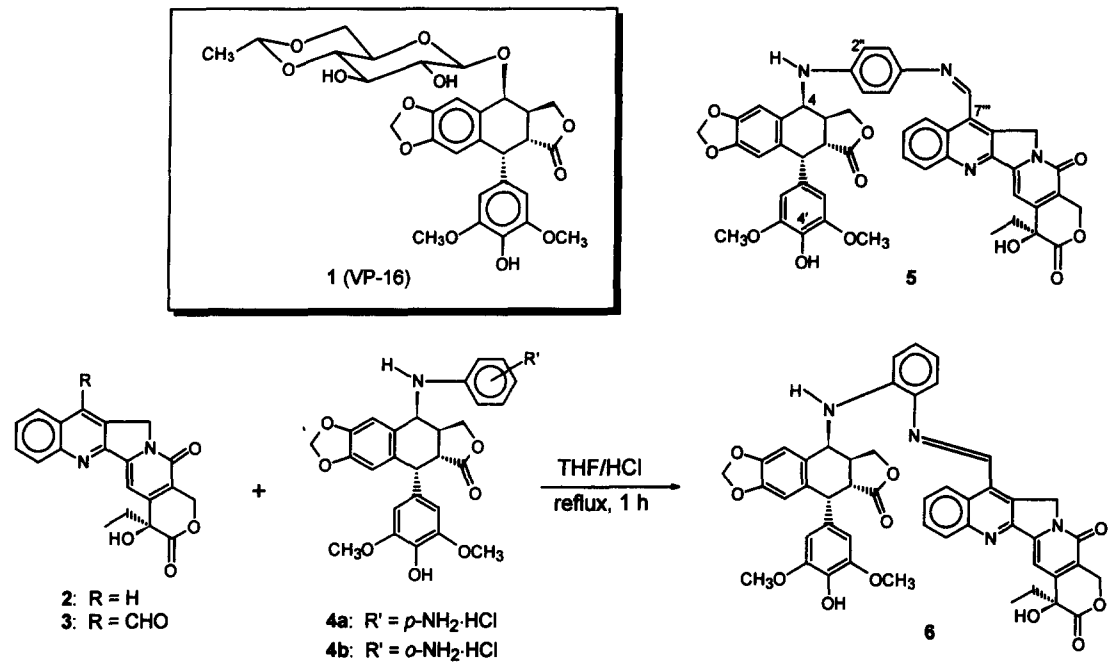

Figure 1. Structures of compounds and synthetic scheme for compounds **5** and **6**.

Results

Stimulation of in vitro cleavable-complexes by conjugated inhibitors

Compounds were evaluated as cleavable complex-forming inhibitors of calf thymus DNA topoisomerases I and II in vitro. The prototypical inhibitors etoposide (**1**) and camptothecin (**2**) were included as reference standards. Activities and specificities of compounds tested at 10 μM are shown in Figure 2A. The enzyme specificity and activity of three unconjugated inhibitors (**1–3**) were consistent with measurements made using human enzymes and cellular assay systems.[9,10] In contrast, compound **4a** was less active than **1** at stimulating topoisomerase II-mediated DNA cleavage in vitro and not threefold more active based on an earlier study with a cell-based assay system.[10] Compound **4b** has not been evaluated previously but it displayed similar properties as the *meta*-substituted congener.[10] The conjugated inhibitors, **5** and **6**, induced cleavable complexes with both types of enzyme however, their activities were reduced about twofold relative to the unconjugated components **3**, **4a** and **3**, **4b**, respectively (Fig. 2A). These results indicate that **5** and **6** displayed the combined selectivity of the unconjugated inhibitors but had lower relative activities at the single concentration tested. Compound **6** was selected for further evaluation since the levels of **6**- and etoposide-induced DNA cleavage were similar. Concentration-dependent in vitro cleavage results using topoisomerase II as well as gel analysis of cleavage products are shown in Figure 2B. Etoposide (**1**) stimulated enzyme induced cleavage 1.5-, 3-, and 12-fold at 1, 10, and 50 μM, respectively. A different activity profile was displayed by compounds **4b** and **6** in that induced levels of cleavage were maximal at 10 μM, being about sevenfold and fourfold increased over enzyme controls, respectively. Compound **4b** was about four times more active than **1** at 1 μM and three times less active at 50 μM. For similar treatments with compound **6**, only marginal stimulation of enzyme-mediated DNA cleavage was detected. Interestingly, some enzyme cleavage sites induced by compounds **4b** and etoposide were also distinguishable because at least two unique cleavage fragments were produced upon **4b**-treatment. Similar fragments were faintly visible as cleavage products induced by 10 μM treatment with **6** (Fig. 2B). Additional studies with topoisomerase I showed that the stimulation of cleavage activity by both **3** and **6** was not inhibited at higher test concentrations, however, the alkaline-treated reaction products were too numerous and heterogeneously-sized on autoradiographs to allow meaningful comparisons of cleavage site-specificies (data not shown). Based on the overall findings, activity as cleavable complex-forming inhibitors was reduced by conjugation yet specific interactions in the presumed ternary complexes appeared to be unaffected.

Cell growth inhibition by a conjugated inhibitor

Compounds **3**, **4b**, and **6** were evaluated as cell growth inhibitors of KB, KB-7d, KB-CPT100, and KB-VIN20c and IC_{50} values were established. The −7d cell line is a pleiotrophic multidrug resistant KB-subclone that exhibits 145-fold resistance to etoposide (IC_{50} for KB, 0.16 ± 0.04 μM), in part because of reductions in both drug uptake and intracellular levels of topoisomerase II. Furthermore, KB-7d cells are cross-resistant to

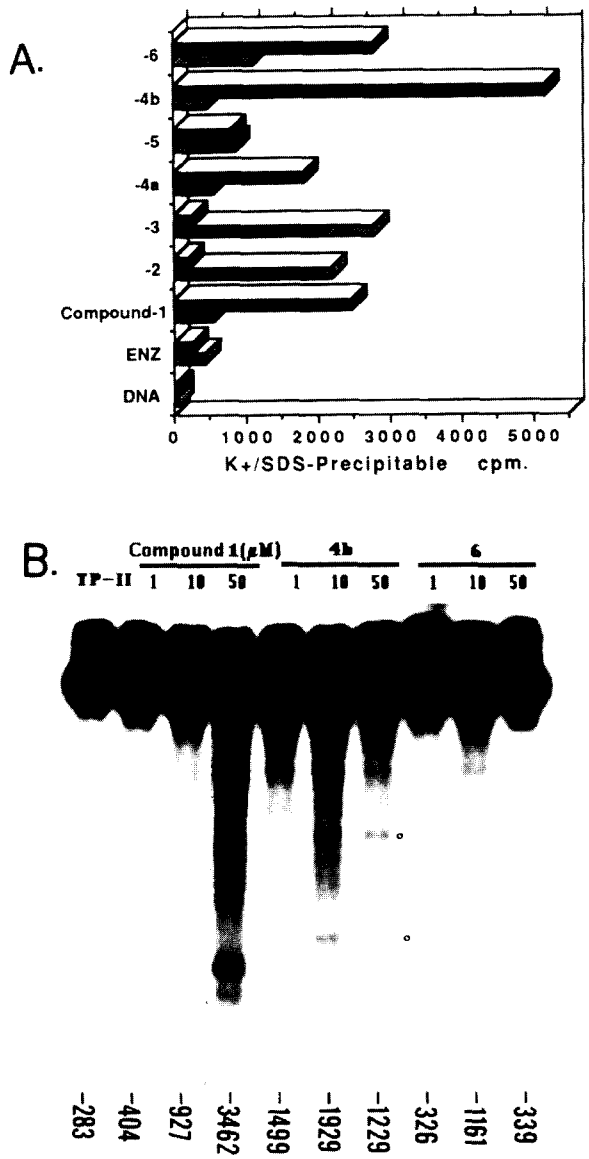

Figure 2. Topoisomerase–DNA complex formation in vitro. (**A**) Potassium–SDS precipitable radioactivity recovered from cleavage reactions containing topoisomerase I (stippled boxes) and topoisomerase II (shaded boxes), alone or with compounds **1–6** at 10 μM. Input radioactivity was about 7000 P^{32} cpm. Data are single determinations from the same experiment. (**B**) Agarose gel autoradiogram of topoisomerase II-induced DNA cleavage products. Treatment conditions are indicated above the lanes and potassium–SDS precipitable radioactivity recovered are given below. Input radioactivity was about 5000 ^{32}P cpm. DNA fragments discussed in the text are indicated by small open circles in the right margin of lane 7. Nonadjacent lanes from a single gel autoradiogram were juxtaposed to prepare this figure.

structurally diverse topoisomerase II-targeting drugs including doxorubicin, mAMSA and mitoxanthrone but are not cross-resistant to 10-hydroxy-camptothecin, an analogue of **2**.[11] The −CPT100 cell line exhibits a 32-fold resistance to the growth-inhibitory effect of **2** (IC_{50} for KB, 9.8 ± 3.2 nM) and a reduced intercellular level, about twofold, of topoisomerase I; these cells are not cross-resistant to **1**. Interestingly, the resistance phenotype of −CPT100 cells appears to be independent of topoisomerase I but the biochemical mechanism remains to be fully characterized.[12] Results from growth inhibition assays are given in Table 1. Compound **4b** was about 1.5-fold more active than **1** at inhibiting KB cell growth with an estimated IC_{50} of 1 μM and KB-7d cells were only about fourfold cross-resistant, in contrast to the cells marked resistance to etoposide. This type of improvement in activity against **1**-resistant cells has been reported for other C-4β-substituted epipodophyllotoxin derivatives.[5] The relative growth inhibitory activities of **4b**, like **1**, were similar for KB parent and −CPT100 cells and the KB-VIN20c subclone was about twofold cross-resistant (Table 1). The unconjugated CPT analogue, **3**, inhibited KB cell growth with an estimated IC_{50} value of 0.007 μM; it was equally active against the −7d subclone, and the −CPT100 cell line was about 58-fold cross-resistant. The vincristine-resistant subline (−VIN20c) was marginally cross-resistant to **3**, no more than twofold. In contrast, a unique cell growth inhibition profile was displayed by **6**, the conjugated inhibitor. The relative inhibitory activities of compounds **6** and **3** against the KB, −VIN20c, and −7d cell lines were similar but the KB-CPT100 derivative was only sixfold cross-resistant to **6**. In fact, the conjugated inhibitor was marginally more active at inhibiting the growth of −CPT100 cells than compound **4b**. Based on these findings, the growth inhibitory properties of compound **6** closely resembles the behaviors of both the topoisomerase I- and topoisomerase II-inhibitory components.

Cell toxicity of a conjugated inhibitor

Compounds **3**, **4b**, and **6** were evaluated as cytotoxic agents and LD_{50} values for a 3-h exposure were established (Table 2). Compound **4b** was equipotent at killing all KB cell lines. The conjugated inhibitor more closely resembled the topoisomerase II-inhibitory component in that KB, −CPT100, and −VIN20c cells were killed by **6** and **4b** at equimolar doses. However, KB-7d cells appeared cross-resistant to the cytotoxic action of the conjugate, which more closely resembled the behavior of **3** (Table 2).

Stimulation of intracellular protein–DNA complexes by a conjugated inhibitor

Intracellular activities of compounds **3**, **4b**, and **6** were compared to co-treatments with unconjugated inhibitors **3** and **4b** using the potassium–SDS precipitation assay method. Etoposide (**1**) was included as the reference standard for **4b** and **6**. Concentration-dependent protein–DNA complex formation in KB cells is shown in Figure 3. Analysis of cotreated cultures showed that **3** and **4b** gave additive effects at low test concentrations (0.5–2.5 μM, Fig. 3A) but at higher concentrations, cotreatment effects were indistinguishable from levels of protein–DNA complexes induced by **4b**-treatment alone (Fig. 3B). At high test concentrations (7.5–60 μM), the intracellular activity of the conjugated derivative, **6**, was different from either **3** or **4b** alone and in combination but was quite similar to the

Table 1. Cell growth inhibition

Cell line	IC_{50} (nM)		
	Compound 6	**Compound 3**	**Compound 4b**
KB	14 (10, 18)[a]	7 (5, 9)	100 (72, 128)
KB-7d	15 (11, 19)	9 (5, 13)	388 (306, 460)
KB-CPT100	85 (71, 99)	575 (435, 705)	134 (112, 156)
KB-VIN20c	27 (21, 33)	18 (14, 22)	234 (144, 309)

[a]Dose–responses were calculated by linear regression with significance 95% confidence ($p \geq 0.01$). IC_{50} values, the concentration which inhibits growth by 50% relative to control, were interpolated from graphed data. Values in parentheses are taken from the confidence interval of the

profile for **1** (Fig. 3B). However, at concentrations of one μM and lower, the intracellular activity of **6** and **4b** were indistinguishable from each other but different from **1**, which illicited no measurable response (Fig. 3A). Based on these comparisons, the relative intracellular activities of **1**, **4b**, and **6** correlated well with the in vitro measurements except for one noticeable difference: the activities of **4b** and **6** in cells were clearly not dose-inhibitory at high concentration. Also of interest was the relationship between KB cell growth inhibition and cellular protein–DNA complex formation by compound **6**. This conjugate was as effective as etoposide (**1**) in cleavage assays but was about tenfold more active at inhibiting cell growth. To further characterize intracellular activities, compounds **1**, **3**, **4b**, and **6** were compared using a gel lysis assay method. Since this type of assay system is only sensitive enough to detect widespread double-stranded genomic breaks, the effect of topoisomerase II inhibitors like etoposide (**1**) will be readily observed whereas topoisomerase I inhibitors like camptothecin (**2**) will be marginally effective. Furthermore, genomic DNA integrity will generally be rapidly restored in cells on drug removal if the causitive lesions involve topoisomerases II-cleavable complexes.[13] All compounds were evaluated at 50 times the IC_{50} concentration for KB cell growth inhibition and for **3** and **6**, additional treatments at 20-fold higher concentrations were examined. Results from a representative experiment are shown in Figure 4. Control DNAs were intact and remained in the agarose plug at the gel origin (lanes 1 and 2). High molecular weight DNA fragments were detected after treatment with 8 μM etoposide (lane 3) and the integrity of the DNA was restored intracellularly on drug removal (lane 4), consistent with the known properties of **1**-induced topoisomerase II cleavable-complexes.[13] Resolution of similar DNA species using PFGE has shown the induced fragments to be 50 kbp and, under some circumstances 300 kbp.[15] Fragmentation was also detected following treatment with 4 μM **4b** (lane 5) and the lesions were reversible (lane 6), again consistent with the compounds known pharmacological action.[10] The camptothecin derivative, **3**, was inactive at 0.4 μM (lane 10). At 16 μM, marginal fragmentation was detected (compare lane 11 to the control in lane 1), which was not affected by reversing treatment (compare lane 12 to the control in lane 2). These effects of **3** are consistent with known actions of cleavable complex-forming topoisomerase I inhibitors.[13,14] By comparison, marginal DNA fragmentation was detected for cells treated with the conjugated inhibitor **6** at 0.8 μM (compare lanes 7 and 1) but at a 20-fold elevated concentration, high molecular mass genomic fragments were detected (lane 8) and the integrity of the DNA was only partially restored following treatment reversal (compare lanes 8, 9, and 2). Based on these findings, intermediate levels of protein–DNA complexes were induced by compound **6** relative to cotreatments with unconjugated components. A high concentrations, the majority of intracellular complexes induced by **6** behaved as topoisomerase II-mediated DNA lesions.

Discussion and Conclusion

This paper describes cleavable complex-forming inhibitors of mammalian DNA topoisomerases I and II that are composed of a camptothecin and a 4′-*O*-demethyl epipodophyllotoxin derivative joined by an imine linkage. These novel inhibitors displayed lower activity

Table 2. Cytotoxicity measured as reduced plating efficiency

Cell line	LD_{50} (μM)		
	Compound 6	**Compound 3**	**Compound 4b**
KB	4 (1, 7)[a]	10 (9, 11)	3 (1.5, 4.5)
KB-7d	>15[b]	>16[c]	5 (3.5, 6.5)
KB-CPT100	2 (1.1, 2.9)	ND	2 (0.2, 3.8)
KB-VIN20c	4.6 (2.6, 6.6)	12 (6, 18)	3 (1.5, 4.5)

[a]Dose–responses were calculated by linear regression with significant 95% confidence ($p \leq 0.01$) except for 'b' and 'c', $p = 0.47$ and 0.2, respectively. LD_{50} values, the concentration which inhibits plating by 50% relative to control, were interpolated from graphed data. Values in parentheses are taken from the confidence intervals of the regression line.

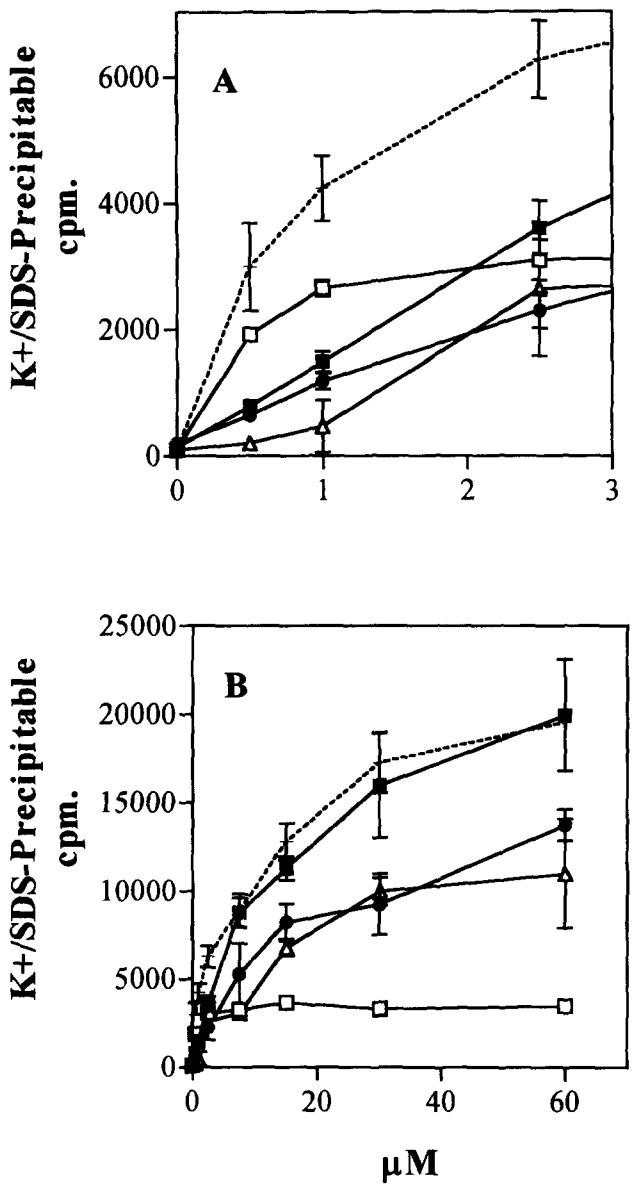

Figure 3. Protein-associated DNA breaks in KB cells. Treatments were for 1 h with test concentrations indicated on the ordinate. Etoposide (**1**, Δ); Compounds **3** (□), **4b** (■), **6** (●), and cotreatments with **3** and **4b** (dotted line). Data obtained from triplicate treatments in a single experiment. Bars, SD. The effect of treatments at low concentration (0.5–2.5 μM) in A are results from B replotted for clarity.

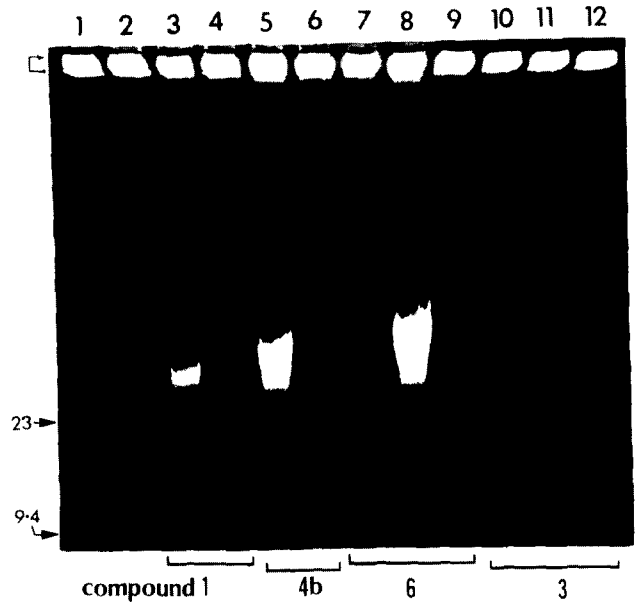

Figure 4. Reversible genomic DNA fragmentation detected by a gel lysis assay method. KB cells were immobilized in agarose plugs, lysed and analyzed as described in the Experimental section. Cells were prepared immediately following 3 h of treatment (lanes 3, 5, 7, 8, 10, and 11) or following an additional hour of incubation in fresh growth medium lanes 4, 6, 9, and 12). Treatments are indicated under the sample lanes and concentrations used are given in the text in the Results section. The first two lanes are control samples from KB cells mock-treated with 0.5% (v/v) dimethylsulfoxide as a carrier control; lane 1 is DNA from cells treated for 3 h and the other sample was prepared following a 1 h treatment reversal. Arrows in the left margin indicate the plug position at the gel origin and the mobility of phage lambda DNA molecular mass markers in kb.

in vitro relative to their unconjugated constituents, about twofold at 10 μM, but they retained target recognition specificity based on limited analysis of the activity profiles and induced cleavage products. These findings were not entirely unexpected. Previous evaluation of semisynthetic 4′-*O*-demethyl epipodophyllotoxins with modifications at the C-4β-position revealed that bulky substituents are well tolerated.[5,10] This same region corresponds to the 'variable-substituent' domain in the composite pharmacophore model for topoisomerase II inhibitors proposed by MacDonald et al.[15] It is also known that camptothecin analogues can bear C7-(aminoacylhydrazone)-formyl substituents without markedly affecting the ability to induce cleavable complexes.[9] In view of the current finding that conjugates bearing a substituent of substantial bulk also retained activity as topoisomerase I inhibitors, this suggests the interaction domain(s) within the presumed ternary complex is not proximal to the C7-position in **2**.

Evaluation of compounds **4b** and **6** as topoisomerase II inhibitors revealed an unexpected finding. The stimulation of in vitro cleavable-complexes was dose-inhibitory for **4b** and **6** but not for **1**, which is known to act saturably (Anyanwutaku I.O., Guo X., Chen H.-X., Zheng J., Lee K.H., Cheng Y.C.; manuscript submitted for publication). Self-suppressive effects seen with cleavable complex-forming inhibitors, ascribed to unfavorable topoisomerase drug template interactions, have been reported for DNA intercalators acting both in vitro and in cellular assay systems.[16–18] As the induction of intracellular protein-associated DNA complexes by **4b** and **6** was clearly not dose inhibitory, the variant behavior seen in vitro must be dependent on enzyme and/or template interaction(s) which are peculiar to the in vitro assay conditions. Interestingly, the inhibition of 'background' cleavage typical of DNA-interactive agents[18] was not seen with **4b.** Additional investigation will be required to define the mechanism(s) responsible for self-suppression of cleavable complexes in the in vitro assay.

The experimental support presented for the dual target specificity of compound **6** was based in part on measurements of cleavable complexes induced in vitro and in treated cells. Activity of the conjugated inhibitor relative to reference treatments was similar in the two assay systems and stimulatory effects of **6** on cellular protein–DNA complexes were distinguishable from the response to co-treatment controls. These observations suggest that the conjugate remained intact under cell culture conditions, which is consistent with the reported stability of the imine linkage.[19] Additional indirect support for intracellular topoisomerase I and II targeting by **6** came from growth inhibition and cytotoxicity studies, as a correlation was noted between these results and in vitro cleavage assay data when compounds **6**, **3**, and **4b** were compared. It has been reported that topoisomerase-selective drugs, including **1, 2**, and mAMSA can either give antagonistic cytotoxic effects when used simultaneously in combination[4,8,20] or synergistic effects,[21] so potential therapeutic benefits of combining such agents through conjugation are unclear. Nevertheless, the unique cytotoxic spectrum of **6** and the biochemical properties of conjugated inhibitors suggest that it would be worthwhile to explore the antitumor potential of these, and similar types of compounds.

Experimental

Chemistry: synthesis

Compounds **5** and **6** were synthesized according to the procedure shown in Figure 1. In general, 7-formyl camptothecin (**3**, 1 equiv) and 4-demethyl epipodophyllotoxin derivatives (**4a** or **4b**, 1 equiv) were dissolved in dry THF, then 1.0 M hydrogen chloride ether solution was added to adjust pH to 1–2. The solution was refluxed for 1 h and evaporated to dryness. The residue was purified via column chromatography (silica gel with chloroform methanol, 100:1 (v/v) as the eluent) to give target compounds in 70–80% yield. The structures of final products were secured by spectroscopic and analytical data.

Compound (**5**), red crystals, mp >300 °C (decomp.), IR (KBr) cm^{-1} 3350 (broad, OH, NH), 1770 (sh, δ-lactone), 1650 (carbonyl of amide); ^1H NMR ($CDCl_3$) δ 1.06 (t, J = 7.5 Hz, 3H, 18‴-H), 1.97 (q, J = 7.5 Hz, 2H, 19‴-H), 3.14 (m, 1H, 3-H), 3.28 (dd, J = 4.9, 14.0 Hz, 1H, 2-H), 3.80 (s, 6H, $OCH_3 \times 2$), 4.05, 4.48 (t, J = 8.7 Hz, 1H each, 11-H), 4.62 (d, J = 4.9 Hz, 1H, 1-H), 4.86 (d, J = 3.9 Hz, 1H, 4-H), 5.35, 5.69 (ABq, J = 16.0 Hz, 1H each, 17‴-H), 5.72 (s, 2H, 5‴-H), 5.97 (d, J = 5.1 Hz, 2H, OCH_2O), 6.36 (s, 2H, 2′, 6′-H), 6.54 (s, 1H, 8-H), 6.76 (d, J = 8.7 Hz, 2H, 2″, 6″-H), 6.85 (s, 1H, 5-H), 7.56 (d, J = 8.7 Hz, 2H, 3″, 5″-H), 7.76 (s, 1H, 13‴-H), 7.79, 7.89 (t, J = 8.0 Hz, 1H each, 10‴, 11‴-H), 8.25, 8.61 (d, J = 8.0 Hz, 1H each, 9‴, 12‴-H), 9.60 (s, 1H, N=CH); anal. calcd for $C_{48}H_{40}N_4O_{11}\cdot H_2O$: C 66.50, H 4.88, N 6.46; found C 66.81, H 4.90, N 6.50.

Compound (**6**), red crystals, mp 255–256 °C (decomp.), IR (KBr) cm^{-1} 3350 (broad, OH, NH), 1770 (sh, δ-lactone), 1650 (carbonyl of amide); ^1H NMR ($CDCl_3$) δ 1.05 (t, J = 7.5 Hz, 3H, 18‴-H), 1.91 (m, 2H, 19‴-H), 3.10 (m, 1H, 3-H), 3.37 (dd, J = 4.9, 14.2 Hz, 1H, 2-H), 3.81 (s, 6H, $OCH_3 \times 2$), 4.08 (dd, J = 2.9, 8.0 Hz, 1H, 11-H), 4.46 (t, J = 8.0 Hz, 1H, 11-H), 4.74 (d, J = 4.9 Hz, 1H, 1-H), 4.84 (t-like, 1H, 4-H), 5.28 (s, 1H, 2″-H), 5.23, 5.42 (ABq, J = 20.5 Hz, 1H each, 17‴-H), 5.30, 5.74 (ABq, J = 16.6 Hz, 1H each,, 5‴-H), 5.96 (d, J = 5.5 Hz, 2H, OCH_2O), 6.37 (s, 2H, 2′, 6′-H), 6.49 (s, 1H, 8-H), 6.71, 6.90 (both d, J = 7.5 Hz, 1H each, 2″, 5″-H), 6.86 (s, 1H, 5-H), 7.33, 7.35 (t, J = 7.5 Hz, 1H, 3″, 4″-H), 7.62 (s, 1H, 13‴-H), 7.71, 7.87 (t, J = 8.0 Hz, 1H each, 10‴, 11‴-H), 8.28, 8.57 (d, J = 8.0 Hz, 1H each, 9‴, 12‴-H), 9.55 (s, 1H, N=CH); anal. calcd for $C_{48}H_{40}N_4O_{11}\cdot 0.5H_2O$: C 67.18, H 4.82, N 6.53; found C 67.04, H 4.88, N 6.41.

Biology

Enzymes and reagents. Calf thymus DNA topoisomerases were purchased from TopoGen (Columbus, OH). Tritium-labeled thymidine (60–90 Ci/mmol) for labeling cellular DNA to measure protein–DNA complexes and [α^{32}P]-labeled deoxycytidine triphosphate (>3000 Ci/mmol) were from ICN Biochemicals Inc. (Irvine, CA). The latter radiochemical was used with a T4-DNA polymerase polymerase-labeling system (BRL, Gaithersburg, MD) to prepare ^{32}P-plasmid DNA for in vitro cleavage assays. Tissue culture reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Gibco-BRL. All other chemicals were reagent grade.

Drugs. Etoposide and camptothecin were from the Natural Products Laboratory (UNC, Chapel Hill). The derivatives **3** , **4a**, and **4b** were prepared according to published methods.[9,10] All compounds were dissolved to 20 mM final concentration in dimethylsulfoxide and stored at −70 °C. Immediately before use compounds were diluted in water for biochemical studies or in culture medium under sterile conditions for cell-based assays.

Cell lines. The KB oral carcinoma cell line was provided by M. Fisher (Pharmacology, UNC, Chapel Hill). The isolation and properties of the etoposide-resistant subclone, KB-7d, and the camptothecin-resistant line, KB-CPT100, have been reported elsewhere,[11,12] and are discussed briefly in the Results section. The vincristine-resistant subclone, KB-VIN20c was isolated by selection in 20 nM vincristine and over-expresses the P-glycoprotein multidrug-resistant drug-efflux protein (unpublished results). All cell types were propagated in RPMI-1640 medium supplemented with 5% (v/v) fetal calf serum and 100 μg/mL kanamycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Over the course of the experiments, doubling times of cells were 24 ± 4 h.

Cell growth inhibition. Fifty thousand cells were plated in 25 cm^2 flasks (Falcon, NJ) and incubated overnight. The medium was supplemented with test compounds at various concentrations and cultures were incubated for 48–51 h (for about two cell doublings) before cells were trypsinized and then counted using a hemacytometer. The number of treated cells which excluded 0.5% (v/v) trypan blue were expressed as a percentage of the growth measured in control cultures. Compounds were evaluated in at least two independent experiments and the combined results were analyzed as scatter plots with dose-responses being computer fitted by linear regression at the 95% confidence level (Graphpad Software, San Diego, CA). The IC_{50} concentrations which inhibited cell growth by 50% relative to control were interpolated from graphed results.

Cytotoxicity assay. LC_{50} concentrations were determined by treating cells for 3 h with different concentrations of compounds then replating cells to measure colony formation and plating efficiency, the percentage of total cells forming colonies. Compounds were evaluated in three independent experiments. The plating efficiences of untreated cells varied between experiments with values ranging from 14–31% for KB, 31–56% for KB-7d, 16–38% for KB-VIN20c, and 21–36% for KB-CPT100. A concentration of compound that reduced plating efficiency by 50% relative to control, the LC_{50}, was determined graphically as stated in the preceding section.

Cellular protein–DNA complex formation. Stimulation of intracellular protein-associated DNA breaks was measured using a standard assay method.[27] Briefly, KB cells were labeled overnight with tritiated thymidine (0.5 μCi/mL), chased for 2 h and then treated in triplicate with test compounds at various concentrations. After 1 h, samples were processed and protein–DNA complexes were measured as potassium–SDS precipitable radioactivity.

In vitro topoisomerase–DNA cleavage assay. The detailed method used to measure stimulation of cleavable complexes in vitro has been published.[23] Briefly, incubated cleavage reactions containing 1 ng of ^{32}P end-labeled linearized PBR322 DNA, one unit of enzyme and test compound were divided equally for analysis. One portion was prepared for gel electrophoresis with subsequent autoradiography and protein–DNA complexes in the remaining sample were analyzed using the potassium–SDS precipitation method and scintillation spectrometry.

Gel lysis assay method for DNA breaks. The procedure used to detect inhibitor-induced cleavable-complexes as genomic DNA fragmentation on agarose gels is based on a published report[24] with suggested modifications (Goz B, LaBiche R; personal communication) as follows. One million KB cells in 25 cm^2 flasks were treated for 3 h with test compound, the medium was discarded then cells were scraped into ice-cold PBS and harvested by centrifugation. Washed cell pellets were resuspended in eighty microliters of a 37 °C gel solution [1% (w/v) low-melting point agarose in PBS], and were cast in a mold and refrigerated to form 6 mm × 7 mm × 2 mm agarose plugs. Plugs of cells were incubated in lysis-digestion buffer (0.4 M EDTA, 0.01 M Tris (HCl, pH 8.0), 1% (w/v) *N*-lauryl-sarcosine and 100 μg/mL proteinase K) at 37 °C overnight or 50 °C for 1 h. The treated plugs were equilibrated in TBE buffer and placed against a gel comb in a horizontal gel casting tray (BRL Inc., H4 system format). A 55 °C gel solution containing 0.8% (w/v) agarose in TBE was poured and allowed to gel. Electrophoresis was at 3.5 V/cm (measured between electrodes) for 16 h at 4 °C without buffer recirculation. The gel was stained with 1 μg/mL ethidium bromide in water, treated overnight at 25 °C with RNase (1 μg/mL), and then photographed under UV illumination. For the reversibility studies, treated cells were washed with PBS and incubated with fresh media for an additional hour before harvesting for plug preparation and gel analysis.

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