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Design of two etoposide-amsacrine conjugates: topoisomerase II and tubuline polymerization inhibition and relation to cytotoxicity

Paola Arimondo, Chawki Boukarim[1], Christian Bailly, Daniel Dauzonne[1] and Claude Monneret[1]

Laboratoire de Pharmacologie Antitumorale du Centre Oscar Lambret, et INSERM U-524, IRCL, Place de Verdun, 59045 Lille and [1]Laboratoire de Pharmacochimie, UMR 176 CNRS/Institut Curie, Section Recherche, 26 rue d'Ulm, 75248 Paris cedex 05, France

Summary

Topoisomerase II represents the main target for the antitumour drugs etoposide and amsacrine, which are both used clinically. Previous studies have shown that the glycoside moiety of etoposide is not necessary for cytotoxicity or DNA topoisomerase II inhibition. For this reason, we designed two epipodophyllotoxin derivatives for which the dispensable sugar moiety of etoposide has been replaced by a *m*-methoxy-methanesulfonamide-anilino group analogous to the topoisomerase II-targeted domain of amsacrine. We report the synthesis of the hybrid molecules that have the epipodophyllotoxin and anilino groups directly linked (ICP-114) or connected by an ethylene spacer (ICP-147). Plasmid DNA relaxation and kinetoplast DNA decatenation assays were used to evaluate the effects of the drug on the catalytic activity of human topoisomerase II. We found that the hybrid ICP-147 was significantly more potent than both etoposide and amsacrine at stimulating DNA cleavage by the enzyme, whereas the hybrid ICP-114 lacking the linker chain was less potent. ICP-147 produces ~3 times more double-stranded breaks than ICP-114, suggesting that an ethylene spacer between the epipodophyllotoxin and amsacrine moieties is highly effective at inhibiting topoisomerase II. Sequencing data also supported the idea that the two moieties of ICP-147 participate to the interaction with topoisomerase II–DNA covalent complexes. Both hybrid compounds are more cytotoxic than etoposide but much less toxic than amsacrine toward L1210 leukemia cells. In addition to its effect on topoisomerase II, ICP-114 can inhibit tubulin polymerization, whereas ICP-147 is almost totally inactive in this assay. The unexpected capacity of ICP-114 to interfere with the polymerization of tubulin suggests that this compound can target tubulin dimers, as it is the case with certain antitumor sulfonamides. The design of etoposide–amsacrine hybrids may thus represent an opportunity for the discovery of dual inhibitors that target both topoisomerase II and tubulin.

Key words

etoposide/amsacrine/topoisomerase II/DNA cleavage/cytotoxicity/antitumour drugs

Introduction

Etoposide (VP-16, Figure 1) is a semisynthetic glycoside derivative of podophyllotoxin first synthesized in 1966, tested clinically as an anticancer agent from 1971, and officially approved for clinical use in 1983 (Slevin, 1991; Hande, 1998). Eighteen years after its introduction in medical practice, etoposide remains one of the most extensively used antitumor agents. It is administered in combination chemotherapy for the treatment of many forms of cancer including breast cancer, testicular cancer, small cell lung cancer, lymphoma and chilhood leukemia, and also Kaposi's sarcoma associated with AIDS (Chabner and Longo, 1996).

In contrast to the podophyllotoxin, etoposide neither binds to tubulin nor inhibits microtubule assembly (Damayanthi and Lown, 1998). The mechanism by which etoposide exerts its antineoplastic effects was elucidated in the 1980s (i.e. at the same time the drug was being brought into clinical use) after the discovery of the DNA-cleaving enzyme topoisomerase II. Etoposide stimulates double-stranded cleavage and inhibits DNA religation mediated by topoisomerase II. The drug stabilizes a transient covalent topoisomerase II–DNA complex, preventing it from dissociation (Burden and Osheroff, 1998). Trapping of these cleavable complexes by the drug induces multiple DNA lesions which ultimately lead to cell death. Etoposide poorly interacts with naked DNA (Chow *et al.*, 1988) but binds avidly to the enzyme–DNA complex and can also interact

Correspondence to: C. Bailly, e-mail: bailly@lille.inserm.fr and C. Monneret, e-mail: Claude.Monneret@curie.fr. Received 16 October 2000; accepted 27 February 2001

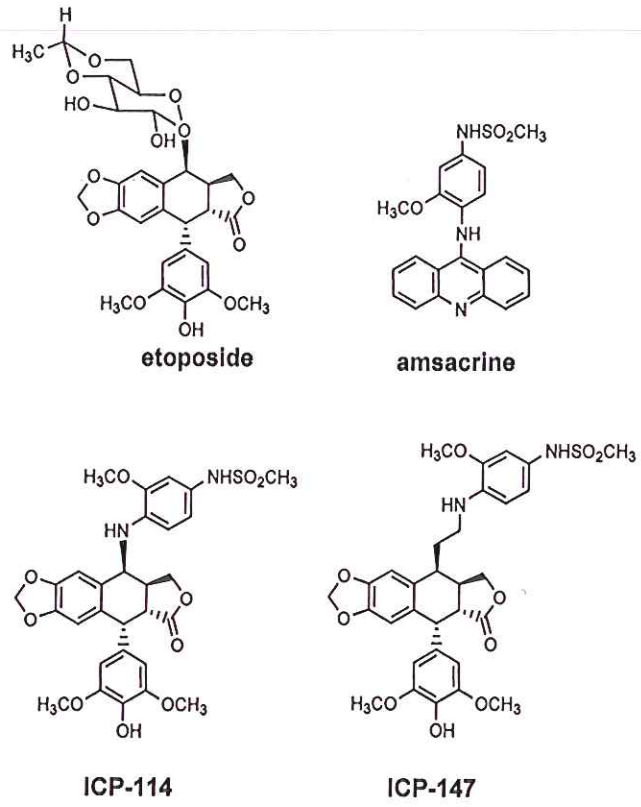

Figure 1
Structure of etoposide, amsacrine and the two hybrids.

directly with topoisomerase II in the absence of DNA (Kingma *et al.*, 1999).

Poisoning of topoisomerase II is not the only mechanism by which etoposide induces DNA strand cleavage. In the presence of several enzymatic systems, such as cytochrome P450/NADPH, horseradish peroxidase/H_2O_2 or prostaglandin synthetase/arachidonic acid, etoposide undergoes *O*-demethylation to yield reactive, DNA-damaging metabolites (Haim *et al.*, 1987; Maanen *et al.*, 1987; Kalyanaraman *et al.*, 1989). Metal- and photo-induced cleavage of DNA by etoposide have also been reported (Sakurai *et al.*, 1991). It has been suggested that metabolic activation of etoposide is essential for its cytotoxicity (Usui and Sinha, 1990). More recent studies suggest that etoposide phenoxyl radicals are responsible for the genotoxic/leukemogenic effects of the drug (Kagan *et al.*, 1999).

Despite its extensive use, etoposide is not devoid of toxic side-effects. Bone-marrow depression is a frequent dose-limiting toxicity encountered in patients receiving etoposide, and the efficacy of the drug is calamitously associated with an increased risk of secondary acute myelogenous leukemia (Pedersen-Bjergaard, 1992; Felix, 1998). A causative link between treatment of cancer with etoposide and the development of secondary leukemia in children and adults has been firmly established (Felix, 1998). For this reason, the development of non-leukemogenic etoposide analogs would be highly valuable.

The replacement of the sugar unit could afford therapeutically active etoposide analogs with different biochemical determinants for DNA binding, enzyme inhibition, cellular uptake or drug resistance. Numerous glycoside analogs of etoposide have been synthesized and a few of them, such as NK611 (Saito *et al.*, 1986), or 3-*N*,*N*-dimethylamino-2-deoxy analogs (Daley *et al.*, 1998) elicit promising antitumor activities. On the other hand, extensive structure–activity relationship studies in the epipodophyllotoxin series have revealed that the glycoside moiety of etoposide is not necessary for cytotoxicity or DNA topoisomerase II inhibition (Anyanwutaku *et al.*, 1996). The glycoside moiety of etoposide has thus also been replaced with non-sugar groups such as pyrrolecarboxamidino elements (Zheng *et al.*, 1997) and, above all, by a *p*-nitroanilino substituent (Zhu *et al.*, 1999). This latter substituent gives the drug GL-331, which is currently undergoing phase II clinical trial for treatment of various cancers (Lee and Wang, 1995).

In this context, we report here the synthesis, effect on topoisomerase II and cytotoxic activities of two new epipodophyllotoxin derivatives that incorporate a methanesulfonamide-anilino group in place of the dispensable glycoside moiety of etoposide. The two designed drugs, ICP-114 and ICP-147 in Figure 1, both bear a *m*-methoxy and a methanesulfonamide substituent on an anilino group. This unit derives from the anticancer drug amsacrine (*N*-[4-(9-acridinylamino)-3-methoxyphenyl]methanesulfonamide, *m*-AMSA in Figure 1), which is also a potent inhibitor of topoisomerase II (Finlay *et al.*, 1996) and is occasionally used for the treatment of acute leukemia (Arlin, 1983; Miller *et al.*, 1991). The two test drugs can thus be considered as hybrids of etoposide and amsacrine. They differ by the nature of the linkage between the two portions of the drug. The epipodophyllotoxin and anilino groups are directly linked in ICP-114 whereas they are connected by an ethylene spacer in ICP-147.

Materials and methods

Drugs and chemicals

Etoposide and amsacrine were purchased from Sigma Chemical Co. (La Verpillière, France). The drugs were dissolved in dimethylsulfoxide (DMSO) at 5 mM and then further diluted with water. The final DMSO concentration never exceeded 0.3 % (v/v) in the cleavage reactions. Under these conditions DMSO, which is also used in the controls, does not affect the topoisomerase activity. The stock solutions of drugs were kept at –20°C and freshly diluted to the desired concentration immediately prior to use. All other chemicals were analytical grade reagents and all solutions were prepared using doubly deionized, Millipore-filtered water.

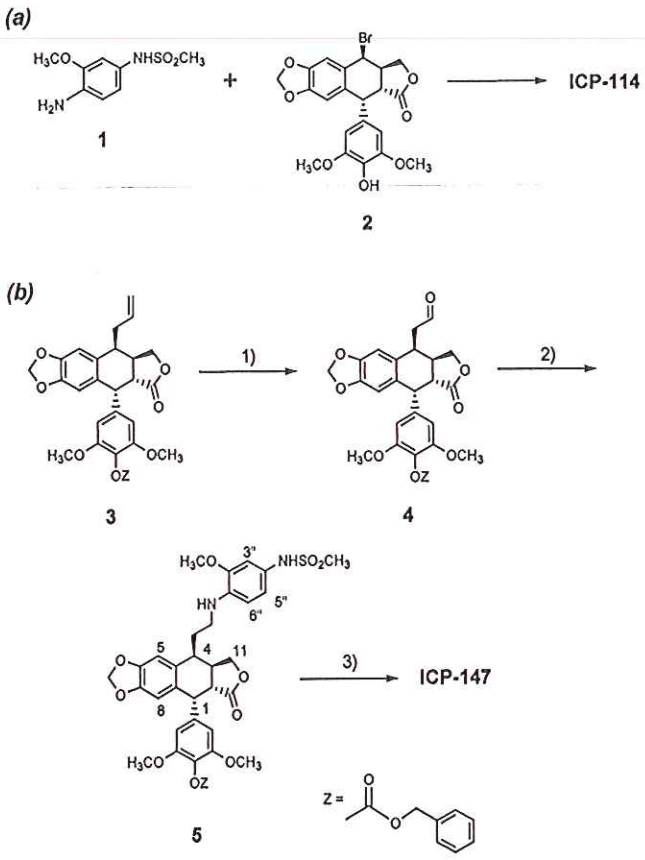

Figure 2

Synthesis of (a) ICP-114 and (b) ICP-147. (a) $BaCO_3$, CH_2Cl_2, 24 h, room temp.; (b) (1) (i) cat. OsO_4–NMO/CH_3COCH_3, room temp.; (ii) $Pb(OAc)_4$/benzene; (2) *N*-(4-amino-3-methoxyphenyl)methanesulfonamide (1), $NaCNBH_3$, AcOH–MeOH, room temp., 1 h; (3) 10% Pd-C, H_2 (1 atm), overnight.

Chemical synthesis (Figure 2)

Melting points were measured on a Köfler hot stage apparatus and are uncorrected. Mass spectra were obtained with a Nermag–Ribermag R10-10C spectrometer applying a desorption chemical ionization (CI) technique using ammonia as the reagent gas. Infrared spectra were obtained with a Perkin–Elmer 1710 spectrophotometer for chloroform solutions. The ^{1}H-NMR (300 MHz) spectra were recorded on a Bruker AC 300 spectrometer. Chemical shifts are expressed as parts per million downfield from tetramethylsilane. Splitting patterns have been designated as follows: s (singlet), d (doublet), dd (doublet of doublet), m (multiplet), t (triplet) and br (broad signal). Coupling constants (*J* values) are listed in hertz (Hz). Optical rotations were measured with a Perkin–Elmer 241 polarimeter. Reactions were monitored by analytical thin layer chromatography and products were visualized by exposure to UV light. Merck Silica gel (230–400 Mesh ASTM) was used for column chromatography.

N-(4-Amino-3-methoxyphenyl)methanesulfonamide (1)

This starting sulfonamide was prepared on a 0.15 mol scale in a 81% overall yield according to a previously reported four-step procedure starting from commercially available 2-nitro-4-methoxyaniline (Brennan *et al.*, 1989), mp 146–147°C (from ethanol).

4′-O-Demethyl-4β-(2″-methoxy-4″-methanesulfonylamidoanilino)-4-deoxypodophyllotoxin (ICP-114)

This compound was synthesized on a 2 mmol scale from 4′-*O*-demethyl-4β-bromo-4-deoxypodophyllotoxin (**2**) and *N*-(4-amino-3-methoxyphenyl)methanesulfonamide (**1**) according to the method involving barium carbonate previously described by Lee and co-workers for other 4′-*O*-demethyl-4β-anilino-4-deoxypodophyllotoxins (Wang *et al.*, 1990) but using dry dichloromethane instead of 1,2-dichloroethane as solvent. Yield 62%; mp 216–219°C (recrystallized from an isopropanol/ethanol mixture); $[\alpha]^{22}_D$ 96° (*c* = 1.0, $CHCl_3$); IR ($CDCl_3$) 3539 (OH), 3420, 3362 (NH), 1775 (lactone), 1608, 1519, 1484 (aromatic C=C), 1328, 1153 (SO_2) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 2.92–3.10 (m, 1H, 3-H), 2.98 (s, 3H, SO_2CH_3), 3,18 (dd, *J* = 4.7, 14.0 Hz, 1H, 2-H), 3.80 (s, 6H, 3′,5′-OCH_3), 3,82 (s, 3H 2″-OCH_3), 3.86 (br s, 1H exchangeable with D_2O, NH), 3.90 (t, *J* = 7.8 Hz, 1H, 11-H), 4.36 (t, *J* = 7.8 Hz, 1H, 11-H), 4.47 (d, *J* = 5.9 Hz, 1H, 1-H), 4.59–4.70 (m, 1H, 4-H), 5.46 (br s, 1H exchangeable with D_2O, OH), 5.96 (d, *J* = 6.0 Hz, 1H, OCH_2O), 5.99 (d, *J* = 6.0 Hz, 1H, OCH_2O), 6.35 (s, 2H, 2′,6′-H), 6.40 (d, *J* = 8.1 Hz, 1H, 6″-H), 6.54 (s, 1H, 8-H), 6.74 (s, 1H, 5-H), 6.79 (dd, *J* = 8.1, 2.1 Hz, 1H, 5″-H), 6.81 (d, 1H, *J* = 2.1 Hz, 3″-H); MS *m/z* 599 $(M + H)^+$, 616 $(M + NH_4)^+$. Anal. calcd for $C_{29}H_{30}N_2O_{10}S$: C, 58.19; H, 5.05; N, 4.68. Found: C, 58.40; H, 5.11; N, 4.55.

4′-O-Demethyl-4′-O-benzyloxycarbonyl-4β-allyl-4-deoxypodophyllotoxin (3) and 4′-O-demethyl-4′-O-benzyloxycarbonyl-4β-(oxo-2-ethyl)-4-deoxypodophyllotoxin (4)

These compounds were prepared in comparable yields from 4′-*O*-demethyl-4-deoxypodophyllotoxin on a 4 mmol scale as formerly reported (Terada *et al.*, 1993). The synthesis of the new anilino derivatives **5** and **ICP-147** is described below.

4′-O-Demethyl-4′-O-benzyloxycarbonyl-4β-[2-(2″-methoxy-4″-methanesulfonylamidoanilino) ethyl]-4-deoxypodophyllotoxin (5)

To a stirred solution of **4** (560 mg; 1 mmol) in methanol (30 ml) at 0°C was added *N*-(4-amino-3-methoxyphenyl)-methanesulfonamide **1** (222.5 mg; 1.03 mmol), AcOH (0.617 ml) and sodium cyanoborohydride (72.6 mg; 1.17 mmol). The mixture was stirred at room temperature for 1 h, then diluted with AcOEt (500 ml). The obtained solution was successively washed with cold saturated $NaHCO_3$ (10 ml) and water (to pH 6–7). The organic extract was dried over $MgSO_4$, then concentrated *in vacuo* maintaining the temperature below 30°C. Purification of the obtained residue by silica gel chromatography (40 g) using a dichloromethane/acetone (95/5) mixture as eluent provided **5** as a white solid (581 mg). Yield 76%; mp 165–170°C; $[\alpha]^{22}_D$ –55°

(c = 1.0, $CHCl_3$); IR ($CDCl_3$) 3375 (NH), 1770 (C=O), 1603 1521, 1485 (aromatic C=C), 1338, 1151 (SO_2) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.80–1.98 (m, 1H, CH_2CH_2NH), 2.04–2.17 (m, 1H, CH_2CH_2NH), 2.95 (s, 3H, SO_2CH_3), 3.02 (m, 1H, 3-H), 3.15–3.30 (m, 3H, 2-H and CH_2N), 3.68 (s, 6H, 3′,5′-OCH3), 3.82–3.90 (m, 1H, 11-H), 3.87 (s, 3H, 2″-OCH3), 4.09–4.20 (m, 1H, 11-H), 4.32–4.42 (m, 1H, 4-H), 4.60 (d, 1 H, J = 4.2 Hz, 1-H), 5.26 (s, 2H, $PhCH_2OCO$), 5.94 (br s, 2H, OCH_2O), 6.30 (s, 2H, 2′,6′-H), 6.47 (s, 1H, 8-H), 6.50 (d, 1H, J = 8.4 Hz, 6″-H), 6.66 (s, 1H, 5-H), 6.73 (dd, 1H, J = 2.1, 8.4 Hz, 5″-H), 6.81 (d, 1H, J = 2.1 Hz, 3″-H), 7.30–7.45 (5H, m, $PhCH_2OCO$); MS *m/z* 761 $(M + H)^+$. Anal. calcd for $C_{39}H_{40}N_2O_{12}S$: C, 61.57; H, 5.30; N, 3.68. Found: C, 61.28; H, 5.45; N, 3.50.

4′-O-Demethyl-β-[2-(2″-methoxy-4″-methanesulfonylamido-anilino)ethyl]-4-deoxypodophyllotoxin (ICP-147)

A solution of **5** (259 mg; 0,34 mmol) in a chloroform/methanol (3/15 ml) mixture was stirred overnight, under hydrogen, in the presence of 10% Pd–C (50 mg), at atmospheric pressure and at room temperature. After filtration on a pad of Celite and rinsing of the solid with chloroform, the filtrate was evaporated under reduced pressure. The residue was chromatographed over a silica gel column (40 g) employing a dichloromethane/acetone (85/15) mixture as eluent to afford ICP-147 as a white solid (170 mg) satisfactorily pure as judged by microanalysis. Yield 80%; mp 198–200°C; $[\alpha]^{22}_D$ –74° (c = 1.0, $CHCl_3$); IR ($CDCl_3$) 3539 (OH), 3372 (NH), 1775 (C=O), 1618, 1520, 1485 (aromatic C=C), 1326, 1153 (SO_2) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.80–1.98 (m, 1H, CH_2CH_2NH), 2.02–2.16 (m, 1H, CH_2CH_2NH), 2.95 (s, 3H, SO_2CH_3), 2.80–3.08 (m, 1H, 3-H), 3.18–3.02 (m, 3H, 2-H and CH_2N), 3.77 (s, 6H, 3′,5′-OCH_3), 3.87 (s, 3H, OCH_3-2″), 3.82–3.90 (m, 1H, 11-H), 4.08–4.20 (m, 1H, 11-H), 4.32–4.42 (m, 1H, 4-H), 4.57 (d, 1-H, J = 4.0 Hz, 1-H), 5.43 (s, 1H exchangeable with D_2O, 4′-OH), 5.92–5.96 (m, 2H, OCH_2O), 6.28 (s, 2H, 2′,6′-H), 6.47 (s, 1H, 8-H), 6.50 (d, 1H, J = 8.3 Hz, 6″-H), 6.66 (s, 1H, 5-H), 6.73 (dd, 1 H, J = 2.1, 8.3 Hz, 5″-H), 6.81 (d, 1H, J = 2.1 Hz, 3″-H); MS *m/z* 628 $(M + H)^+$. *Anal.* calcd for $C_{31}H_{34}N_2O_{10}S$: C, 59.41; H, 5.47; N, 4.47. Found: C, 59.25; H, 5.63; N, 4.38.

Biochemicals

The nucleoside triphosphate labeled with ^{32}P (γ-ATP) was obtained from Amersham. Restriction endonucleases *Pvu*II, *Hin*dIII and *Eco*RI, alkaline phosphatase and T4 polynucleotide kinase were purchased from Roche Biochemicals and used according to the supplier's recommended protocol in the activity buffer provided. Human topoisomerase II and kinetoplast DNA were from TopoGEN Inc. (Columbus, OH).

DNA purification and labeling

Plasmids pBS (Stratagene) was isolated from *Escherichia coli* by a standard sodium dodecyl sulfate–sodium hydroxide lysis procedure and purified by banding in CsCl–ethidium bromide gradients. Ethidium was removed by several isopropanol extractions followed by exhaustive dialysis against Tris–EDTA-buffered solution. The purified plasmid was then precipitated and resuspended in appropriate buffered medium prior to digestion by the restriction enzymes. The 117 bp pBS DNA fragment was prepared by 5′-^{32}P-end labeling of the *Eco*RI/alkaline phosphatase treated plasmid using [γ-^{32}P]ATP and T4 polynucleotide kinase followed by treatment with *Pvu*II. Plasmid digestion products were separated on a 6% polyacrylamide gel under native conditions in TBE-buffered solution (89 mM Tris-borate, pH 8.3, 1 mM EDTA). After autoradiography, the band of DNA was excised, crushed and soaked in water overnight at 37°C. This suspension was filtered through a Millipore 0.22 μm filter and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum drying of the precipitate, the labeled DNA was resuspended in 10 mM Tris adjusted to pH 7.0 containing 10 mM NaCl.

Topoisomerase II-mediated DNA relaxation assay

Supercoiled pAT DNA (0.5 μg) or kinetoplast DNA kDNA (0.3 μg) was incubated for 15 min at 30°C in a 50 mM Tris–HCl buffer, pH 7.5, containing 1 mM ATP, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA and 30 μg BSA, in the presence of the drug at the indicated concentration (total reaction volume 20 μl). Human DNA topoisomerase II (6 units) was added to the DNA substrate and incubated for 30 min at 30°C. DNA–topoisomerase II cleavage complexes were dissociated by addition of SDS (final concentration 0.5%) and proteinase K (500 μg/ml), followed by incubation for 30 min at 55°C. DNA samples were then added to the electrophoresis dye mixture (5 μl) and electrophoresed (35 V/cm) in a 1% agarose gel in Tris–borate–EDTA buffer containing ethidium bromide (1 μg/ml) at room temperature for 2 h. Gels were washed and photographed under UV light.

Topoisomerase II cleavage assay

The radiolabeled 117 bp DNA fragment (50 nM) was incubated for 15 min at 30°C, in 50 mM Tris–HCl, pH 7.5, 1 mM ATP, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA and 30 μg BSA, in the presence of the drug at the indicated concentration (total reaction volume 20 μl). Ten units of topoisomerase II were added to the sample prior to incubation for 30 min at 30°C. DNA–topoisomerase II cleavage complexes were dissociated by addition of SDS (final concentration 0.5%) and of proteinase K to 500 μg/ml, followed by incubation for 30 min at 55°C. After ethanol precipitation, all samples were resuspended in 6 μl of formamide, heated at 90°C for 4 min and then chilled on ice for 4 min, before being loaded onto a denaturing 8% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 7.5 M urea in 0.5× TBE buffer (50 mM Tris base, 55 mM boric

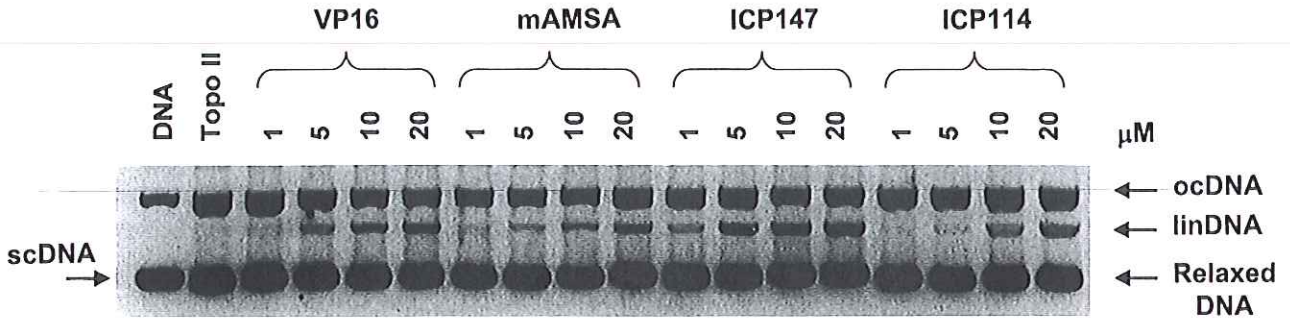

Figure 3

Stimulation of the topoisomerase II-mediated DNA double-stranded cleavage. Native supercoiled pAT (0.5 μg, lane DNA) was incubated for 30 min at 30°C with 6 units of topoisomerase II in the absence (lane Topo II) or in the presence of increasing concentrations of the drugs at the indicated concentration (μM). Reactions were stopped with SDS and treatment with proteinase K. Samples were analyzed by native agarose gel eletrophoresis in the presence of ethidium bromide (1 μg/ml) and the gels were photographed under UV light. The positions of supercoiled (form I), open-circular (form II), linear (form III) and relaxed DNA species are indicated.

acid, 1 mM EDTA). To quantitate the extent of cleavage, gels were scanned with a Molecular Dynamics 445SI Phosphorimager. For the determination of cleavage levels a normalization relative to total loading was performed.

Tubulin polymerization

This was determined as reported previously (Zavala, 1980).

Cell culture and cytotoxicity

L1210 cells were cultivated in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES buffer (pH 7.4). Cytotoxicity was measured by the microculture tetrazolium assay (MTA) as described elsewhere (Leonce *et al.*, 1992, Pierré *et al.*, 1992). Cells were exposed to graded concentrations of drug (nine serial dilutions in triplicate) for 48 h. Results are expressed as IC_{50}, the concentration which reduced by 50% the optical density of treated cells with respect to the optical density of untreated controls.

Results

Topoisomerase II inhibition

Supercoiled DNA was treated with human DNA topoisomerase II in the absence and in the presence of increasing concentrations of test compounds (Figure 3). Etoposide and amsacrine were used as reference inhibitors. In the presence of both ICP-114 and ICP-147, a band corresponding to linear DNA (form III) can be clearly seen attesting that these two epipodophyllotoxin–amsacrine hybrid molecules have inhibited the religation of DNA once the double helix is cleaved by the enzyme. The extent of DNA cleavage is proportional to the drug concentration. ICP-147 produces much more double-stranded DNA cleavage than ICP-114. For example, the linear DNA form is already pronounced with 1 μM ICP-147 whereas no effect was detected with 1 μM ICP-114. The incorporation of a connecting chain between the two drug units thus seems beneficial for topoisomerase II inhibition. Interestingly, the intensity of the linear DNA band is significantly stronger with ICP-147 than with etoposide and amsacrine.

To investigate further the inhibitory effect of the drugs on topoisomerase II, we used an assay based upon decatenation of kinetoplast DNA, which is specific for topoisomerase II activity (Figure 4A). Topoisomerase II decatenates kinetoplast DNA (lane Topo II) and the presence of an inhibitor such as etoposide induces the formation of linear DNA species as a result of the stabilization of the topoisomerase II–DNA covalent complexes. A band corresponding to linear DNA fragments can be detected with both the reference inhibitors and the test compounds. In this decatenation assay, ICP-147 appeared considerably more potent than ICP-114, confirming the idea that an ethylene spacer between the epipodophyllotoxin and amsacrine moieties is highly profitable for inhibition of topoisomerase II. A quantitative analysis of the extent of linear DNA fragments (Figure 4B) indicates that the effect of ICP-114 is not significantly higher than that of etoposide. In contrast, ICP-147 is approximately twice as efficient as etoposide at stimulating double-strand breaks by topoisomerase II.

The above results confirm that ICP-114 and ICP-147 effectively function as topoisomerase II poisons that stabilize DNA–topoisomerase II covalent complexes. Next we investigated the sequence specificity of the cleavage reaction. The 117 bp *Eco*RI–*Pvu*II restriction fragment from pBS was incubated with each drug at 50 μM prior to cleavage by topoisomerase II. The cleavage patterns observed with the hybrids were almost identical to those seen with etoposide and amsacrine. DNA cleavage occurs at the same nucleotide positions but the intensity of cleavage slightly differs from one drug to another. For example, ICP-147 stimulates DNA cleavage by the enzyme on the 3′ side of cytosine residues at positions 37, 47 and 73. At the latter site the cleavage intensity is more pronounced with the hybrid than with etoposide. In

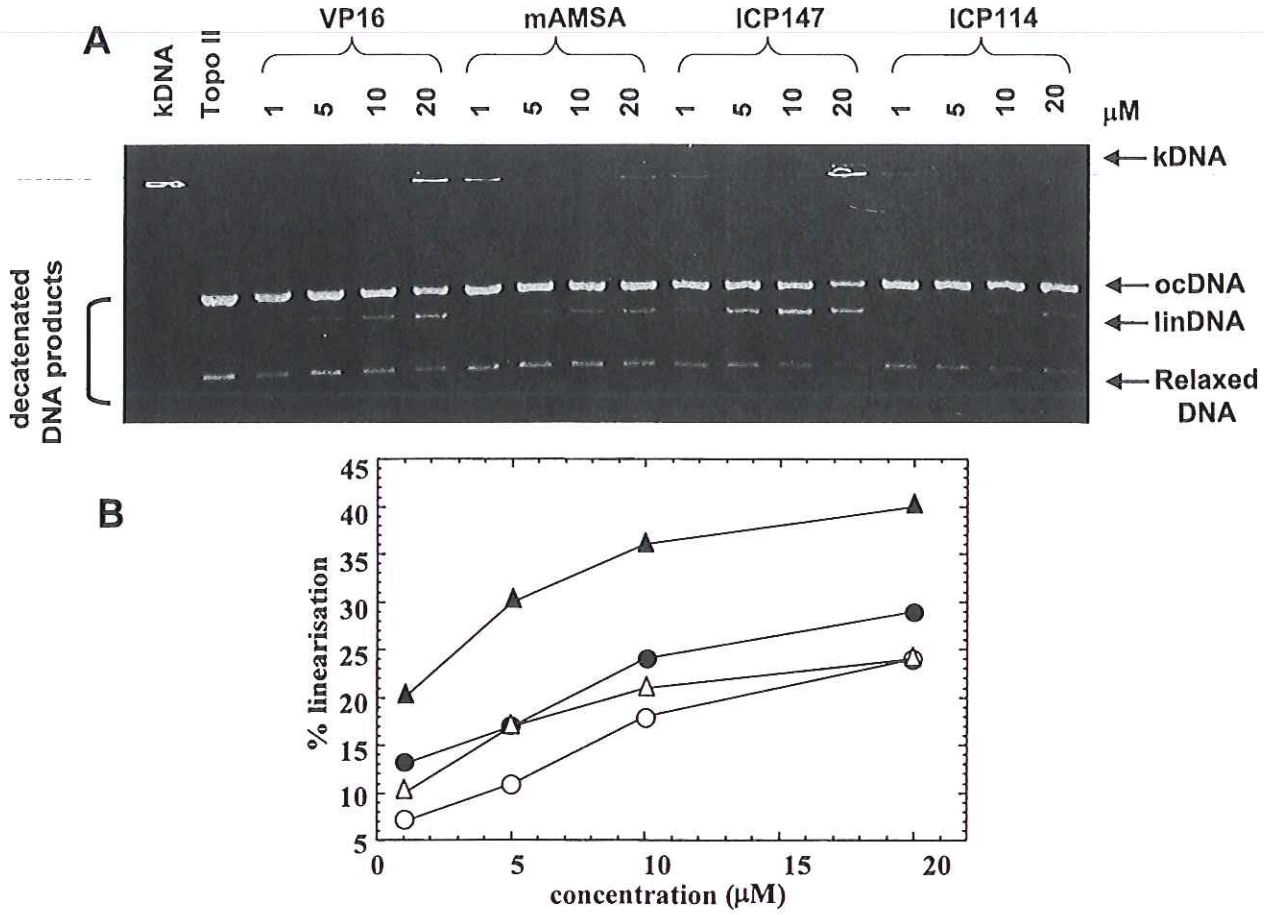

Figure 4

Decatenation assay. (A) Kinetoplast DNA (0.3 μg, lane kDNA) was incubated for 30 min at 30°C with 6 units of topoisomerase II in the absence (lane Topo II) and in the presence of increasing concentrations of the drugs. Other details as in Figure 3. (B) The plots show the formation of linear DNA as a function of the drug concentration. Data were compiled from quantitative analysis of four gels. •, Etoposide; △, amsacrine; ○, ICP-114; and ▲, ICP-147.

addition to the etoposide-type sites, we also detected a few sites typical of amsacrine such as at the A63 position.

Antiproliferative activity and inhibition of tubulin polymerization

The L1210 murine leukemia cell line was employed to evaluate the antiproliferative activity of the drugs. IC_{50} values are collated in Table 1. Surprisingly, the two hybrid compounds proved to be 3–4 times more cytotoxic than etoposide but considerably less potent than amsacrine. ICP-114 was slightly more toxic than ICP-147 despite its reduced capacity to inhibit topoisomerase II. These observations led us to suggest that the two hybrid drugs may have different biological targets. Beyond topoisomerase II, one potential target for these types of compounds is tubulin, which is the primary target for most podophyllotoxin derivatives. For this reason, tubulin polymerization inhibition (TPI) was investigated with the two hybrids. In the *vitro* assay, ICP-114 showed a modest but consistent inhibition of TPI (IC_{50} = 23 μM), inferior to that of podophyllotoxin (IC_{50} = 3 μM) but considerably superior to that of ICP-147 which was almost totally inactive in this assay. A marginal 17% inhibition was detected with ICP-147 at 16 mM (10 mg/ml).

Table I
Cytotoxicity

	IC_{50} (μM)[a]
Amsacrine	0.0187
Etoposide	0.83
ICP-114	0.19
ICP-147	0.26

[a]Drug concentration that inhibits L1210 cell growth by 50% after incubation in liquid medium for 48 h.

Discussion

The newly synthesized compounds ICP-114 and ICP-147 both contain an epipodophyllotoxin moiety but lack the

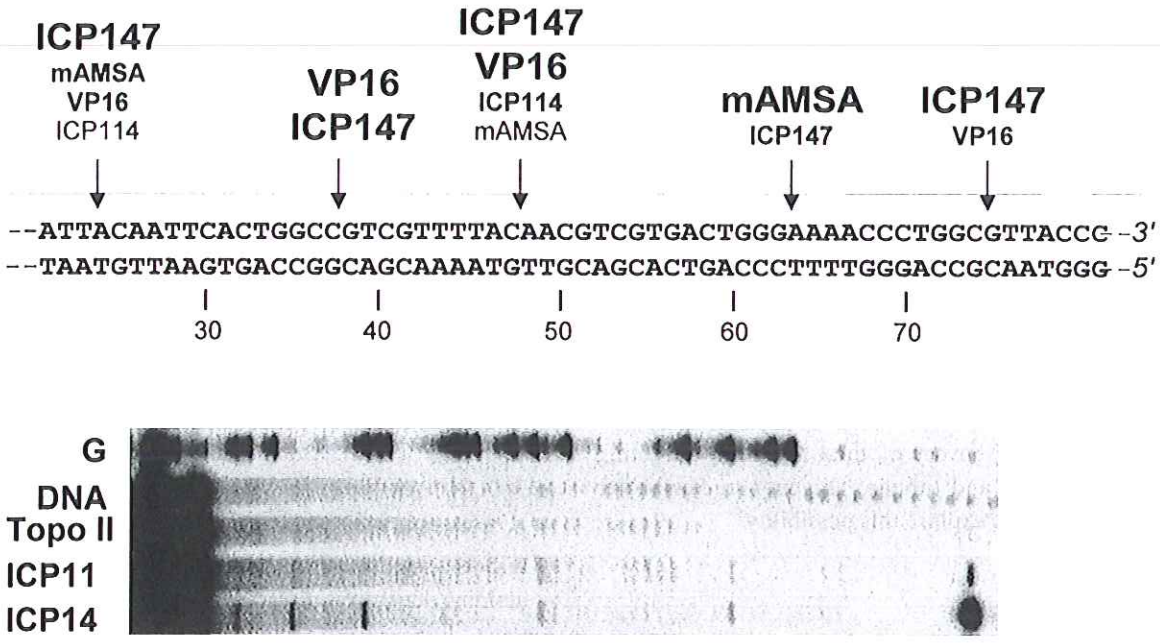

Figure 5

Diagrammatic representation of topoisomerase II-mediated cleavage sites in the presence of etoposide, amsacrine and ICP-147 on a portion of the 117-mer DNA fragment. Only the region of the restriction fragment which was analyzed by densitometry is shown. Arrows indicate sites of DNA cleavage detected with each drug, with small and large letters denoting weak and strong cleavage respectively (the bigger the font, the stronger the cleavage). Numbers below the sequence refer to the standard numbering scheme for this 117 bp fragment. A typical sequencing gel obtained with ICP-114 and ICP-147 (10 μM each) is presented.

glycoside unit characteristic of etoposide. Because the carbohydrate unit of etoposide is known to be dispensable, we envisaged introducing a methanesulfon-*m*-anisidide group as found in amsacrine. The reason for this approach comes from previous modeling studies with amsacrine and anilinoacridine derivatives which suggest that the methanesulfonamide group at position 1′ on the anilino ring represents the topoisomerase-targeted domain (Macdonald *et al.*, 1991). In the amsacrine series, this substituent is required to permit the drug to interfere with the catalytic activity of topoisomerase II (Baguley *et al.*, 1990). Amsacrine is composed of two functional domains: (i) a tricyclic acridine moiety which represents the DNA-binding domain and (ii) a methanesulfonamide group which constitutes the topoisomerase II-targeted domain. The two domains are connected by a skeletal core represented by the anilino ring. This model was validated by structure–activity relationship studies with anilinoacridine derivatives bearing different groups at the 1′ position (Fossé *et al.*, 1994; René *et al.*, 1995).

The demonstration that the methanesulfon-*m*-anisidide group of amsacrine is directly responsible for the interference with topoisomerase II activity (Finlay *et al.*, 1999) prompted us to incorporate this unit in place of the dispensable glycoside unit of etoposide. Two etoposide–amsacrine hybrids were thus designed and synthesized. The results presented above suggest that the strategy is valid because the designed drugs do inhibit topoisomerase II, and one of the drugs, ICP-147, appears more potent than etoposide and amsacrine at stimulating DNA cleavage by topoisomerase II. The sequencing data indicated that ICP-147 shares features of both amsacrine and etoposide, thus suggesting that both moieties participate in the interaction with topoisomerase II–DNA covalent complexes. In addition, this compound turned out to be ~3 times more cytotoxic than etoposide toward L1210 leukemia cells. It is possible that the anilino side-chain of ICP-147 occupies a similar topoisomerase II-binding pocket to that of amsacrine. *N*-Phenylmethanesulfonamide can mimic the anilino side-chain of amsacrine so as to inhibit its cytotoxicity (Finlay *et al.*, 1999). However, at present, we cannot conclude whether or not the enhanced cytotoxic potential is linked to topoisomerase II inhibition. Further hybrids of this type thus need to be designed in order to develop potent topoisomerase II poisons. The length and/or chemical nature of the linker chain between the two connected species must be carefully considered. Indeed, the results also indicate that the drug ICP-114 with no linking chain between the epipodophyllotoxin and anilino groups is much less efficient at inhibiting topoisomerase II than the hybrid ICP-147 which has an ethylene spacer. A systematic analysis of the influence of the connecting chain would be useful to guide the rational design of potent topoisomerase II poisons.

Surprisingly, the hybrid compound ICP-114 is slightly more cytotoxic than ICP-147 despite its reduced capacity to

inhibit topoisomerase II. The high cytotoxicity of ICP-114 perhaps derives from its unexpected capacity to interfere with the polymerization of tubulin, at least *in vitro*. In addition to topoisomerase II, this compound may also target tubulin dimers. We found in the literature that certain antitumor sulfonamide derivatives act at the tubulin level (Yoshino *et al.*, 1992; Owa *et al.*, 1999). For example, the promising antitumor agent E7010, which incorporates a benzenesulfonamide moiety similar to that found in the present ICP compounds, was shown to inhibit microtubule assembly by reversible binding to the colchicine site on tubulin (Koyanagi *et al.*, 1994; Yoshimatsu *et al.*, 1997). The design of etoposide–amsacrine hybrids may thus represent an opportunity for the discovery of dual inhibitors targeting both topoisomerase II and tubulin. Ongoing studies in our laboratories will seek to explore this possibility.

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