0026-895X/93/040767-08$03.00/0

MOLECULAR PHARMACOLOGY, 44:767-774

Dual Topoisomerase I and II Inhibition by Intoplicine (RP-60475), a New Antitumor Agent in Early Clinical Trials

BRUNO PODDEVIN, JEAN-FRANÇOIS RIOU, FRANCOIS LAVELLE, and YVES POMMIER

Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 (B.P., Y.P.), and Rhône-Poulenc-Rorer S.A., 94403 Vitry-sur-Seine, France (J.-F.R., F.L.)

Received April 28, 1993; Accepted July 16, 1993

SUMMARY

The mechanisms of action of intoplicine (RP-60475), a 7*H*-benzo[*e*]pyrido[4,3-*b*]indole derivative that is presently in early clinical trials, have been investigated. Intoplicine induced both topoisomerase I- and II-mediated DNA strand breaks, using purified topoisomerases. The topoisomerase cleavage site patterns induced by intoplicine were unique, relative to those of camptothecin, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), and other known topoisomerase inhibitors. Both topoisomerase I- and II-induced DNA breaks decreased at drug concentrations higher than 1 μM, which is consistent with the DNA-intercalating activity of intoplicine. DNA damage was investigated in KB cells in culture by using alkaline elution. Intoplicine induced single-strand breaks (SSB) in a bell-shaped manner with respect to drug concentration (maximum frequency at 1 μM ≈ 220 rad-equivalents). SSB formation was fast, whereas reversal after drug removal was slow. Similar bell-shaped curves were obtained for DNA double-strand breaks (DSB) and DNA-protein cross-links. SSB and DNA-protein cross-link frequencies were approximately equal, and no protein-free breaks were detectable, indicating the protein concealment of the breaks, as expected for topoisomerase inhibition. Comparison of SSB and DSB frequencies indicated that intoplicine produced a significant amount of SSB not related to DSB, which is consistent with concomitant inhibition of both DNA topoisomerases I and II in cells. Data derived from resistant cell lines indicated that multidrug-resistant cells were cross-resistant to intoplicine but that *m*-AMSA- and camptothecin-resistant cells were sensitive to intoplicine. Hence, intoplicine might circumvent topoisomerase I-mediated and topoisomerase II-mediated resistance by poisoning both enzymes simultaneously.

DNA topoisomerases I and II are important targets in cancer chemotherapy (1, 2). Inhibitors of topoisomerases comprise a variety of structurally diverse compounds that interfere with the DNA nicking-closing reactions catalyzed by these enzymes. Clinically active drugs include inhibitors of topoisomerase I such as CPT (3, 4) and its water-soluble derivatives (5, 6) and inhibitors of topoisomerase II such as anthracyclines (7, 8), acridines (9–11), ellipticines (11, 12), anthracenediones (7), and epipodophyllotoxins (13). All of these drugs, although acting through the stabilization of cleavable complexes, have different experimental and clinical antitumor activities. This could be due partly to specific sites of DNA interactions between the different classes of drugs (1, 14, 15). Based on this observation, the synthesis of new inhibitors of topoisomerases is an objective actively pursued by many groups.

New polycyclic intercalating compounds, 1-amino-substituted 5*H*-pyrido[4,3-*b*]indoles and 5*H*-benzo[*e*]- or benzo[*g*]pyrido[4,3-*b*]indoles, have been prepared (16, 17). Among the last series, one compound, intoplicine (RP-60475) (Fig. 1), has been selected for clinical trials because of its potent activities in various cellular and animal models (18, 19). The aim of the present study was to further characterize the cytotoxic activity of intoplicine and to investigate its mechanisms of action in cultured cells.

B.P. was the recipient of a Rhône-Poulenc-Rorer fellowship.

Materials and Methods

DNA, Enzymes, and Chemicals

The c-*myc* DNA (plasmid pJB327) was a gift from Dr. Jim Battey (Laboratory of Neurochemistry, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, MD). Klenow polymerase and pBR322 DNA were purchased from Boehringer Mannheim (Meylan, France). Restriction endonucleases, T4 polynucleotide kinase, calf intestine phosphatase, agarose, and polyacrylamide/bisacrylamide were purchased from GIBCO BRL (Gaithersburg, MD) or from New England Biolabs (Beverly, MA). DNA topoisomerase II was purified from mouse leukemia L1210 cell nuclei as described previously (20, 21) and was stored at −70° in 40% (v/v) glycerol, 0.35 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM KH_2PO_4, 0.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 6.4. The purified

ABBREVIATIONS: CPT, camptothecin; DPC, DNA-protein cross-link(s); DSB, double-strand break(s); *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; NMHE, 2-methyl-9-hydroxyellipticinium acetate; SDS, sodium dodecyl sulfate; SSB, single-strand break(s); BSA, bovine serum albumin; kDNA, kinetoplast DNA; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

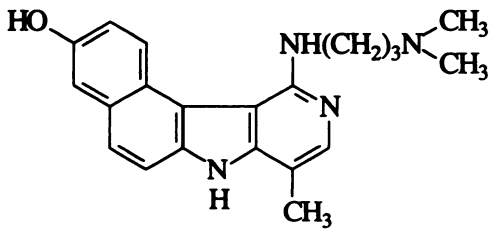

Fig. 1. Structure of intoplicine (RP-60475).

enzyme yielded a single 170-kDa band after silver staining of SDS-polyacrylamide gels (20, 21). Calf thymus DNA topoisomerases I and II were purified from frozen glands (100 g) using an adaptation of the procedures described previously (22, 23). Briefly, nuclei were prepared and lysed with 0.35 M NaCl, and nucleic acids were precipitated by the addition of 0.1% (final concentration) polymine P. The supernatant was successively applied to phosphocellulose, hydroxyapatite, and DNA-cellulose columns for chromatography. Topoisomerase I or topoisomerase II active fractions were combined independently at each step. Then, topoisomerase I or topoisomerase II was purified by glycerol gradient centrifugation and was stored at −70° in 50% glycerol, 10 mM Tris·HCl, pH 7.5, 20 mM 2-mercaptoethanol, 0.5 mM EDTA. The purified topoisomerase I yielded a single 70-kDa band and topoisomerase II a single 165-kDa band after silver staining of SDS-polyacrylamide gels.

[α-^{32}P]ATP and [γ-^{32}P]ATP were purchased from New England Nuclear Research Products (Boston, MA). DNA fragments were 5′-end-labeled as described previously (15).

m-AMSA was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). RP-60475 is 11-(3-dimethylaminopropylamino)-3-hydroxy-8-methyl-7*H*-benzo[*e*]pyrido[4,3-*b*]indole, dimethanesulfonate, and it was prepared according to the method of Nguyen *et al.* (17). The following reference compounds were commercially available: etoposide (Sandoz Laboratory, Rueil-Malmaison, France), CPT (Sigma Chemical Co., La Verpillière, France), doxorubicin (Roger Bellon Laboratory, Neuilly-sur-Seine, France), NMHE (Sanofi, Toulouse, France), and vinblastin (Lilly France S.A., Saint-Cloud, France). All drugs were kept frozen at −20° in 1 mM (in water) or 10 mM (in dimethylsulfoxide) solutions. Final dimethylsulfoxide concentrations never exceeded 2%, and all control experiments were carried out with an equal volume of drug solvant.

Cell Culture and Drug Treatment

Murine leukemia P388 and P388/DX cell lines were obtained from the tumor bank of the National Cancer Institute. These cell lines were grown in RPMI 1640 medium containing 10 μM 2-mercaptoethanol, 2 mM L-glutamine, 200 units/ml penicillin, and 200 μg/ml streptomycin and supplemented with 10% (v/v) fetal calf serum. Doxorubicin (1 μM) was added to the medium of P388/DX cells. P388/CPT-5 is a stable CPT-resistant subclone, isolated in soft agar, that was derived from the P388/CPT-0.3 cell line, which is resistant to CPT (24). Cells were grown in the same medium as P388 cells.

KB human epidermoid carcinoma cells were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle medium with 2 mM L-glutamine and 10% fetal calf serum. The KB-V1 cell line, with acquired resistance to vinblastin, was a gift from Dr. M. Gottesman (National Cancer Institute). Cells were grown in the same medium as KB cells but with 1 μg/ml vinblastin (25). Calc18 human breast carcinoma cells and Calc18/AM cells, with acquired resistance to *m*-AMSA, were a gift from Dr. G. Riou (Institut Gustave Roussy, Villejuif, France) and were grown as monolayers in Dulbecco's modified Eagle medium with 2 mM L-glutamine and 10% fetal calf serum (26). CEM human leukemia cells and CEM/Adr cells, with acquired resistance to doxorubicin, were a gift from Dr. M. C. Chevallier-Multon (Rhône-Poulenc Rorer, Vitry-sur-Seine, France) and were grown in suspension in RPMI 1640 medium with 2 mM L-glutamine and 10% fetal calf serum (27). Doxorubicin (0.5 μg/ml) was added to the medium of CEM/Adr cells. DC3F Chinese hamster lung fibroblasts and DC3F/C-10 cells with acquired resistance to CPT were grown as described previously (28).

Cytotoxicity Assays

Resistant cells maintained in culture in the presence of drug were grown without drug for 7–9 days before the tests. For the evaluation of antiproliferative properties, the concentrations of drug giving 50% growth inhibition (IC_{50}) were determined from three separate experiments, in 96-well microculture plates. Cell lines seeded at 3×10^4 to 3×10^5 cells/ml (0.2 ml/well) were grown for 96 hr in the presence of various drug concentrations in a volume of 20 μl (each point measured in quadruplicate). Cells were then incubated for 16 hr with 0.02% neutral red.[1] The cells were washed and lysed with 1% SDS, and the incorporation of the dye, reflecting cellular growth and viability, was evaluated by measurement of the absorbance of each well at 540 nm, using a Titertek multiwell spectrophotometer. For the evaluation of anticlonogenic properties, KB cells from exponential and plateau-phase cultures were incubated with different concentrations of intoplicine at 37° for various periods of time (1, 3, or 24 hr). Exponential cultures were obtained by seeding 25-cm^2 flasks at 2400 cells/cm^2, 1 day before experiments. Plateau-phase culture were obtained by seeding 25-cm^2 flasks at 16,000 cells/cm^2, 4 days before experiments. After incubation, the cells were washed with phosphate-buffered saline and collected by trypsinization. Cells were diluted in complete medium to a final concentration of 500 viable cells/ml. Noble Difco agar (2.4%, 0.4 ml) maintained at 45° was added to 2.5 ml of cell suspension. The mixture was immediately poured into Petri dishes (four dishes/concentration). The number of cellular clones (>60 cells) was measured after 15 days of incubation at 37° under 5% CO_2. Results were expressed as percentage inhibition of clonogenicity, relative to control cells. IC_{50} values were determined graphically from semilogarithmic plots. Determination of the relative resistance index of DC3F/C-10 cells to intoplicine was performed as described previously (28).

Alkaline Elution Technique

DNA damage was quantitated by alkaline elution filter methods, as described in detail by Kohn (29). KB cells were labeled with 0.04 μCi/ml [*methyl*-^{14}C]thymidine (53.6 mCi/mmol; New England Nuclear, Boston, MA) for 1.5–2 cell doublings at 37°. KB internal standard cells were labeled with 0.2 μCi/ml [*methyl*-^{3}H]thymidine (80.9 Ci/mmol; New England Nuclear) also for 1.5–2 cell doublings at 37°. All cells were then chased by incubation in nonradioactive fresh medium for at least 4 hr before drug treatment. Experimental procedure to measure DNA SSB under deproteinization and nondeproteinization conditions, DSB, and DPC were described previously (30).

SSB frequencies were expressed in SSB rad-equivalents and calculated from the formula:

$$\text{SSB (SSB rad-equivalent)} = \frac{K - K_0}{K_1 - K_0} \times 300 \qquad (1)$$

where K is the slope of the elution curve for cells treated with the drug, K_0 the elution slope of untreated control cells, and K_1 the elution slope of cells irradiated with 300 rad (29).

DPC, expressed in DPC rad-equivalents, were quantitated using the bound to one terminus model (31), from the formula:

$$\text{DPC (DPC rad equivalent)} = [(1 - R)^{-1} - (1 - R_0)^{-1}] \times 3000 \qquad (2)$$

where R and R_0 are the fractions of ^{14}C-DNA remaining on the filter for drug-treated and untreated cells, respectively.

[1] J. F. Riou, A. Naudin, and F. Lavelle. Effects of Taxotere on murine and tumor cell lines. Submitted for publication.

DSB were expressed in DSB rad-equivalents and calculated from the formula:

$$\text{DSB (DSB rad-equivalent)} = \frac{K - K_0}{K_1 - K_0} \times 5000 \qquad (3)$$

where K is the slope of the elution curve for cells treated with the drug, K_0 the elution slope of untreated control cells, and K_1 the elution slope of cells irradiated with 5000 rad (29).

Topoisomerase-Induced DNA Cleavage

Stimulation of DNA cleavage by calf thymus topoisomerase II in pBR322 DNA. Cleavage reactions were performed in a 20-μl final reaction volume containing 25 ng of 5′-end-labeled pBR322 DNA (*Eco*RI to *Hind*III), 20 mM Tris, pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 30 μg/ml BSA, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM ATP, 10 decatenating units of calf thymus topoisomerase II (1 unit is able to decatenate 50% of 0.25 μg of kDNA at 37° in 30 min in the same buffer), and drug or water (2-μl volume). Reactions were performed at 37° for 10 min and stopped by addition of 2 μl of 2.5% SDS, 2.5 mg/ml proteinase K, followed by incubation for 30 min at 50°. Five microliters of loading buffer (50 mM EDTA, 50%, v/w, sucrose, 0.1% bromphenol blue) were added to each sample before loading onto a 1% agarose gel made in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8) plus 0.1% SDS. Electrophoresis was at 11–12 V/cm for 3 hr. Gels were dried and autoradiographed for 12 hr.

Stimulation of DNA cleavage by calf thymus topoisomerase I in pBR322 DNA. Cleavage reactions were performed as described above, using the same reaction buffer without $MgCl_2$ and ATP, with 20 relaxation units of calf thymus topoisomerase I (1 unit is able to relax 50% of 0.25 μg of pBR322 DNA at 37° in 30 min in the same buffer). Before gel electrophoresis, samples were resuspended in 10 μl of 0.45 M NaOH, 30 mM EDTA, 15% (v/w) sucrose, 0.1% bromocresol green.

Sequencing of topoisomerase II cleavage sites induced by intoplicine in the promoter region of the human c-*myc* proto-oncogene first exon. DNA fragments (15) were equilibrated with or without drug in 0.01 M Tris·HCl, pH 7.5, 0.05 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, 15 μg/ml BSA, for 5 min before addition of purified mouse L1210 leukemia cell topoisomerase II (40–70 ng in 20-μl final reaction volume). Reactions were performed at 37°. They were stopped by addition of SDS to a final concentration of 1% and proteinase K to 100 μg/ml, followed by incubation for 1 hr at 42°. Samples were precipitated with ethanol and resuspended in 2.5 μl of loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue). Samples were heated to 90° and immediately loaded onto DNA-sequencing gels (8% polyacrylamide; 29:1, acrylamide/bisacrylamide) containing 7 M urea in 1× TBE buffer. Electrophoresis was at 1500 V (60 W) for 2–3 hr.

Inhibition of Topoisomerase II-Mediated Decatenation

Trypanosoma cruzi kDNA (0.1 μg), a gift from Dr. G. Riou (Institut Gustave Roussy, Villejuif, France), was reacted with 4 decatenation units of P388 topoisomerase II in 20 mM Tris, pH 7.5, 80 mM KCl, 30 μg/ml BSA, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP. Reactions were performed at 37° for 30 min and were stopped on ice by addition of 5 μl of loading buffer (50 mM EDTA, 50%, v/w, sucrose, 0.1% bromphenol blue). Gel analysis was performed on 2% agarose gels made in 1× TBE buffer. Electrophoresis was at 11–12 V/cm for 3 hr, and gels were stained with ethidium bromide (10 μg/ml) for 10 min, washed with distilled water, and photographed under UV light.

DNA Unwinding Assay

The assay was performed as described previously (32). Briefly, a 20-μl final reaction volume containing 0.25 μg of supercoiled pBR322 DNA, 20 mM Tris, pH 7.5, 60 mM KCl, 30 μg/ml BSA, 0.5 mM EDTA, 0.5 mM dithiothreitol, and drugs or water (2-μl volume) was reacted with 2 (catalytic) or 20 (excess) relaxation units of calf thymus topoisomerase. Reactions were performed at 37° for 30 min and stopped by addition of 2 μl of 2.5% SDS and 2.5 mg/ml proteinase K, followed by incubation for 30 min at 50°. Five microliters of loading buffer (50 mM EDTA, 50%, v/w, sucrose, 0.1% bromphenol blue) were added to each sample before loading onto a 1% agarose gel made in 1× TBE buffer plus 0.1% SDS. Electrophoresis was at 11–12 V/cm for 3 hr. Gels were stained with ethidium bromide (10 μg/ml), washed with distilled water, and photographed under UV light.

Results

Cytotoxicity

The cytotoxicity of intoplicine was assessed by colony formation assays or cell growth inhibition assays using different cell lines, i.e., Calc18 (human breast adenocarcinoma cells), KB-3.1 (human oral cavity epidermoid cells), CEM (human lymphoblastic cells), P388 (murine leukemia cells), and DC3F (Chinese hamster lung fibroblasts). Corresponding drug-resistant cell lines were also used, i.e., Calc18/AM (resistant to *m*-AMSA), KB-V.1 (resistant to vinblastin), CEM/Adr and P388/DX (resistant to doxorubicin), and P388/CPT-5 and DC3F/C-10 (resistant to CPT). Intoplicine was compared with a variety of potent antitumor drugs including *m*-AMSA, doxorubicin, vinblastin, etoposide, CPT, and NMHE (Table 1). The IC_{50} was approximately approximately 60 nM in P388 and KB cells and 550 nM in Calc18 cells for a 4-day continuous exposure to intoplicine. The other antitumor drugs gave IC_{50} values in the same concentration range in P388 cells (Table 1). However, intoplicine was more active than the ellipticine derivative NMHE in all three cell lines, P388, KB, and Calc18. Intoplicine cytotoxicity increased with duration of exposure, and proliferating KB cells appeared to be more sensitive than quiescent cells after 24-hr exposure (data not shown). Cross-resistance to intoplicine was observed in KB-V.1 cells, which are resistant to vinblastin, in P388/DX cells, which are resistant to doxorubicin, and in CEM/Adr cells, which are also resistant to doxorubicin. These three cell lines overexpress the *mdr* gene, suggesting that intoplicine is a substrate for P-glycoprotein and that its activity is reduced in multidrug-resistant cells (Table 2). No cross-resistance to intoplicine was observed either in Calc18/AM cells resistant to *m*-AMSA or in P388/CPT-5 and DC3F/C-10 cells resistant to CPT, suggesting that a decrease in the intracellular topoisomerase II activity (Calc18/AM) or a specific mutation in the topoisomerase I gene (P388/CPT-5 and DC3F/C-10) is not by itself a decisive parameter regarding intoplicine cytotoxicity.

TABLE 1

Cytotoxicity of intoplicine, compared with other antitumor drugs

IC_{50} values (drug concentrations that reduced the cell survival fraction to 50%) for different cell lines, after a 4-day continuous exposure to the drugs (see Materials and Methods), are shown.

	IC_{50}		
	P388	KB-3.1	Calc18
	nM		
m-AMSA	15	14	387
Doxorubicin	105	12	80
Vinblastin	36	10	<10
Etoposide	178	130	7,500
CPT	66	20	12
NMHE	195	436	>10,000
Intoplicine	57	62	551

TABLE 2

Relative resistance to intoplicine of different cell lines resistant to other topoisomerase inhibitors and vinblastine

Cell line		Relative resistance to intoplicine[a]	Characteristics of the resistant cell line
Sensitive	Resistant		
KB-3.1	KB-V.1	290	*mdr* overexpression (25)
P388	P388/DX	200	*mdr* overexpression, decreased topoisomerase II (25, 27)
CEM	CEM/Adr	30	*mdr* overexpression (27)
Calc18	Calc18/AM	1.1	Decreased topoisomerase II, increased topoisomerase I, (26)
P388	P388/CPT-5	1.1	Mutant topoisomerase (24)
DC3F	DC3/C-10	1	Mutant topoisomerase I (39)

[a] IC_{50} ratios for resistant and sensitive cells.

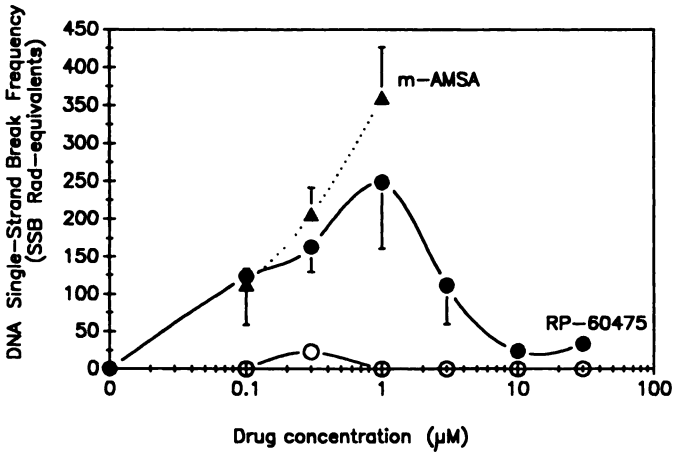

Fig. 2. Dependence of DNA SSB frequency on drug concentration in KB cells. Cells were exposed for 75 min at 37° to *m*-AMSA (▲) or intoplicine (○, ●). After drug exposure cells were washed once at 4° and then assayed by alkaline elution under deproteinizing (▲, ●) or nondeproteinizing (○) conditions. *Vertical bars*, standard errors from five independent experiments.

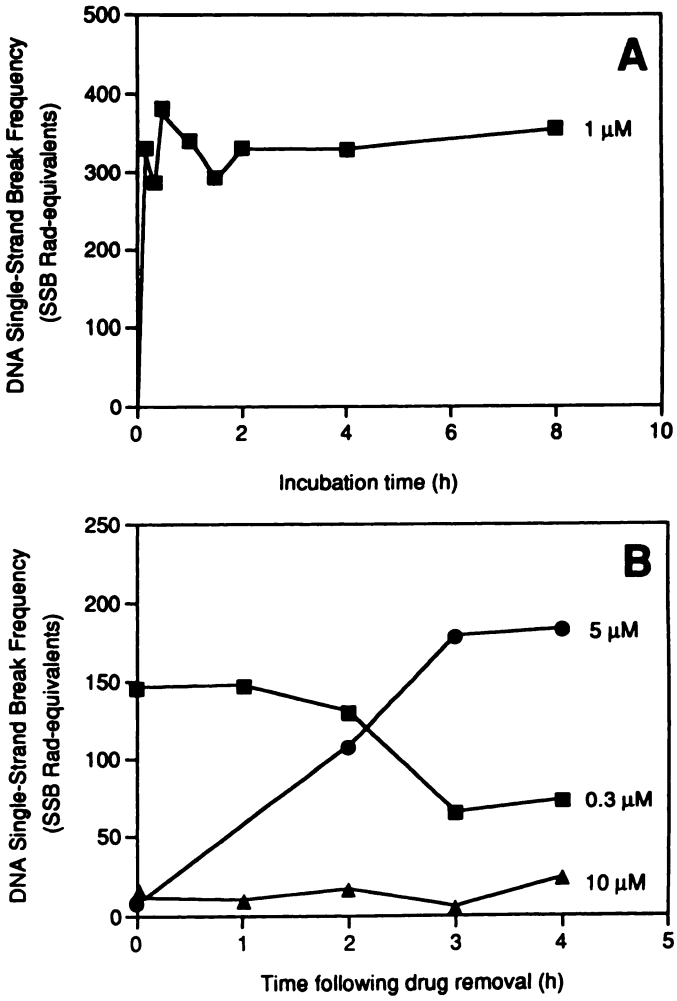

Fig. 3. Kinetics of appearance and disappearance of DNA SSB in KB cells. A, Kinetics of formation of SSB. Cells were treated with 1 μM intoplicine for the indicated times. After drug exposure, cells were washed once at 4° and then assayed by alkaline elution under deproteinizing conditions. B, Kinetics of disappearance of SSB after drug removal. Cells were treated for 75 min with intoplicine. Drug was removed by rinsing the cell cultures twice and replating the cells in drug-free medium (time 0). Cells were then incubated at 37° in drug-free medium for periods from 30 min to 4 hr before being assayed by alkaline elution under deproteinizing conditions.

DNA Damage Induced by Intoplicine in KB Cells

DNA SSB. The production of SSB by intoplicine was measured by alkaline elution using proteinase K and was compared with that induced by *m*-AMSA (Fig. 2). As shown previously, *m*-AMSA-induced SSB increased with increasing drug concentrations. Their frequency was, however, relatively low (360 SSB rad-equivalents at 1 μM), compared with previous results in other cell lines (9, 33, 34). In contrast, intoplicine-induced SSB yielded a bell-shaped curve (Fig. 2). Their frequency increased to a maximum at 1 μM (≈220 rad-equivalents) and decreased at higher concentrations. In all cases, elution curves were first order, and no significant fraction of the DNA was found in the lysis fraction. We also performed SSB frequency measurements under nondeproteinizing conditions; no breaks were detectable (Fig. 2), which indicates that the drug-induced DNA fragments were protein-linked.

Kinetics of DNA SSB. The breaks induced by 1 μM RP-60475 appeared very rapidly (Fig. 3A). They were already maximal after 10 min of drug treatment and their frequency remained constant for the next 8 hr.

The reversal of the SSB induced by 75-min treatments with various concentrations of intoplicine was measured during the first 4 hr after drug removal (Fig. 3B). Two concentration ranges were studied, 0.3 and 1 μM, which correspond to the peak region for maximal SSB frequency, and 5 and 10 μM, which inhibit SSB production (see Fig. 2). The breaks induced after the 0.3 μM treatment reversed slowly, and approximately 50% of the breaks persisted after 4 hr (Fig. 3B). The same results were observed at 1 μM (data not shown). In the case of 5 μM intoplicine treatment, SSB appeared slowly after drug removal, and in the case of 10 μM SSB frequency remained low, close to control levels, even 4 hr after drug removal. These results suggest that intoplicine enters the cells rapidly but exhibits a slow efflux. Therefore, intoplicine-induced SSB are persistent long after drug wash-out from the cell medium. The frequency of DNA lesions appeared to decrease slowly when the initial drug concentration was lower than the self-inhibitory threshold (1–3 μM). However, more DNA lesions appeared after drug removal when the initial drug concentration was 5 μM. With 10 μM, no SSB appeared after drug removal, which may be due to the slow egress of intoplicine from KB cells and the

persistence of a drug concentration above the self-inhibitory threshold.

DPC. As in the case of SSB, a bell-shaped curve was obtained in cells treated with increasing concentrations of intoplicine (Fig. 4). The concentration-DPC response curve exhibited a maximum at 3 μM, whereas SSB exhibited a maximum at 1 μM. However, SSB and DPC frequencies were comparable within experimental error at each of the concentrations tested, indicating that this difference was probably due to experimental variability (Figs. 2 and 4). These observations, taken together with the finding that all the DNA fragments generated by the drug were protein linked, strongly suggest that the breaks induced by intoplicine result from topoisomerase I or II inhibition. To investigate this point further, drug-induced DNA DSB were measured.

DNA DSB. Intoplicine induced DSB, as measured by filter elution with proteinase K at nondenaturing pH (pH 9.6). As in the case of SSB and DPC, DSB production yielded a bell-shaped curve when plotted against the intoplicine concentration; maximum DSB were achieved at 1 μM (approximately 2200 DSB rad-equivalents) and no DSB were detectable at 10 μM and higher concentrations (data not shown). Because SSB can arise both from true SSB and from DSB, the ratios of true SSB and DSB were calculated (9). These ratios were compared with those for *m*-AMSA and NMHE (Table 3). To perform these calculations, SSB and DSB frequencies were always measured from the same cell cultures. The S/D ratios were calculated according to the equation described by Zwelling *et al.* (9), using a S/D ratio of 23 for γ radiation (35). At 0.3 μM, the S/D ratio for intoplicine was around 8, which is 2.7 greater than in the case of *m*-AMSA. Therefore, intoplicine produces a large number of true SSB (almost 10 for each DSB). At higher drug concentrations, the S/D ratios decreased for both intoplicine and *m*-AMSA, indicating that the breaks tended to become DSB. Our finding that the S/D ratio was nearly 0 for NMHE is consistent with previous results describing this compound as a "pure" DSB inducer. For both *m*-AMSA and intoplicine this ratio was significantly greater than 0, indicating that these two compounds induce SSB not related to DSB.

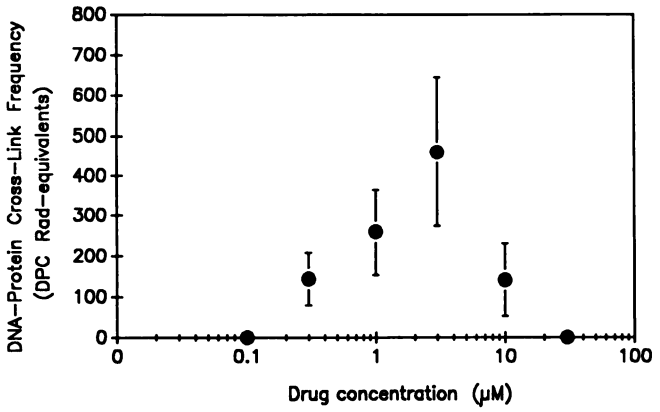

Fig. 4. Induction of DPC in KB cells. Cells were exposed to various intoplicine concentrations for 75 min at 37° (see Fig. 2). DPC were assayed by alkaline elution (pH 12.1) without proteinase K. *Vertical bars*, standard errors from three independent experiments.

TABLE 3

Estimates of true SSB to DSB ratios in KB cells treated with intoplicine, *m*-AMSA, or NMHE

Values are mean ± standard deviation for at least three independent experiments.

Treatment	SSB/DSB[a]	S/D[b]
Intoplicine (0.3)[c]	0.44 ± 0.14	8.0 ± 3.3
Intoplicine (1.0)	0.20 ± 0.05	2.6 ± 1.2
m-AMSA (0.3)	0.22 ± 0.05	3.0 ± 1.1
m-AMSA (0.6)	0.10 ± 0.02	0.2 ± 0.5
MNHE (15)	0.09 ± 0.03	0.1 ± 0.1
NMHE (30)	0.08 ± 0.02	0

[a] SSB/DSB, ratio of the frequency of DNA SSB and DSB.
[b] S/D, ratio of "true SSB" and DSB, calculated according to the equation $S/D = [(KR_S/KR_D) \times (SSB/DSB) - 2]$, by assuming $KR_S/KR_D = 23$ (see Ref. 35).
[c] Concentration in micromolar.

Dual Inhibition of DNA Topoisomerases I and II by Intoplicine

Induction of cleavable complexes. To test directly whether the protein-linked DNA breaks produced by intoplicine were due to topoisomerase inhibition, we studied the effect of the drug on both purified calf thymus DNA topoisomerases I and II, using the *Eco*RI to *Hind*III restriction fragment of pBR322 as a DNA substrate (Fig. 5). Intoplicine stimulated the formation of cleavable complexes both with topoisomerase I and with topoisomerase II (Fig. 5). Topoisomerase I-mediated cleavage was induced at low concentrations (0.01 μM) (Fig. 5A, *lane 7*) and was suppressed at 3 μM (Fig. 5A, *lane 10*). Intoplicine also induced topoisomerase II-mediated DNA cleavage at low concentrations and suppressed cleavage at high concentrations. These results show the same type of bell-shaped concentration-response curve as in cells. Optimum drug concentrations for stabilization of cleavable complexes appeared slighly lower for topoisomerase I than for topoisomerase II with this pBR322 fragment, because topoisomerase II cleavage persisted at nearly maximal levels with 3 μM intoplicine (Fig. 5B, *lane 3*), whereas topoisomerase I cleavage was inhibited at 1 μM (Fig. 5A, *lane 10*). In comparison with *m*-AMSA or CPT, intoplicine appears to be a potent inducer of topoisomerase-

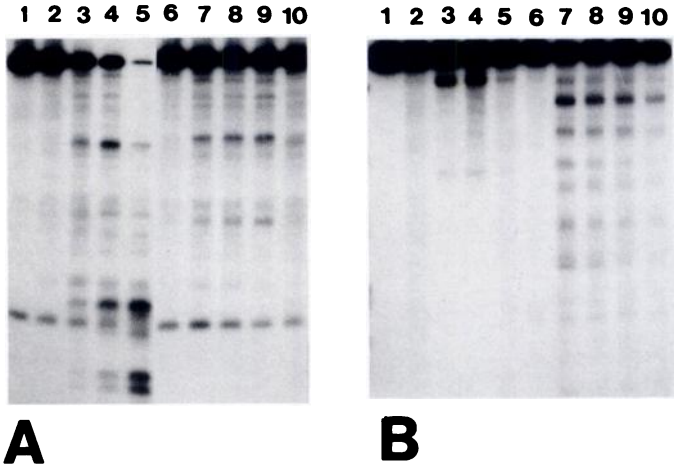

Fig. 5. Stimulation by intoplicine of topoisomerase-induced DNA cleavage in the *Eco*RI/*Hind*III restriction fragment of pBR322 (^{32}P-labeled at the *Eco*RI site). A, Topoisomerase I cleavage. Reactions were carried out for 10 min at 37° and were stopped with SDS-proteinase K treatment. Samples were analyzed by alkaline agarose gel electrophoresis. *Lane 1*, control DNA; *lane 2*, enzyme without drug; *lanes 3-5*, enzyme plus 0.01, 0.1, and 1 μM CPT, respectively; *lanes 6-10*, enzyme plus 0.01, 0.03, 0.1, 0.3, and 1 μM intoplicine, respectively. B, Topoisomerase II cleavage. Reactions were carried out for 10 min at 37° and were stopped with SDS-proteinase K treatment. Samples were analyzed by neutral agarose gel electrophoresis. *Lane 1*, control DNA; *lane 2*, enzyme without drug; *lanes 3-6*, enzyme plus 3, 1, 0.3, and 0.1 μM intoplicine, respectively; *lanes 7-10*, enzyme plus 30, 10, 3, and 1 μM *m*-AMSA, respectively.

mediated cleavage, because the minimal effective concentrations for these agents were comparable, i.e., 1 μM for *m*-AMSA and intoplicine with topoisomerase II and 0.01 μM for CPT and 0.03 μM for intoplicine with topoisomerase I. The cleavage sites enhanced by intoplicine were a subset of the enzyme sites detected in the absence of drug and are different for topoisomerases I and II. The specificity of intoplicine was, however, different from those of CPT (Fig. 5A) and *m*-AMSA (Fig. 5B).

The topoisomerase II cleavage sites induced by intoplicine were also determined at the nucleotide level by DNA sequencing in the 5′ region of the first exon of the human c-*myc* protooncogene and were compared with the cleavage sites induced by *m*-AMSA (Fig. 6). In this particular DNA sequence, intoplicine induced fewer cleavage sites than did *m*-AMSA. Both drugs enhanced specifically a subset of the cleavage sites induced by the enzyme alone. The patterns of enhancement were clearly different for intoplicine and *m*-AMSA. The major cleavage site induced by *m*-AMSA in the P2 promoter, at position 2499, was much weaker in the case of intoplicine. In contrast, some cleavage sites enhanced by intoplicine did not appear (or appeared much more weakly) in the *m*-AMSA cleavage site pattern, such as those at positions 2583 and 2585 or the three cleavage sites located near position 2639. The topoisomerase II cleavage pattern for intoplicine was also different from those for other topoisomerase II inhibitors, including etoposide, doxorubicin, and NMHE.[2] The topoisomerase I cleavage sites induced by intoplicine were also determined by DNA sequencing in a fragment of the Chinese hamster topoisomerase I gene and were compared with the cleavage sites induced by CPT (data not shown). We observed that in this particular DNA sequence intoplicine induced fewer cleavage sites than did CPT, that all the sites were also induced by the enzyme alone but were stronger in the presence of the drug, and that the cleavage site pattern was significantly different from that of CPT. Taken together, these results show that intoplicine is a dual topoisomerase inhibitor with unique DNA cleavage patterns.

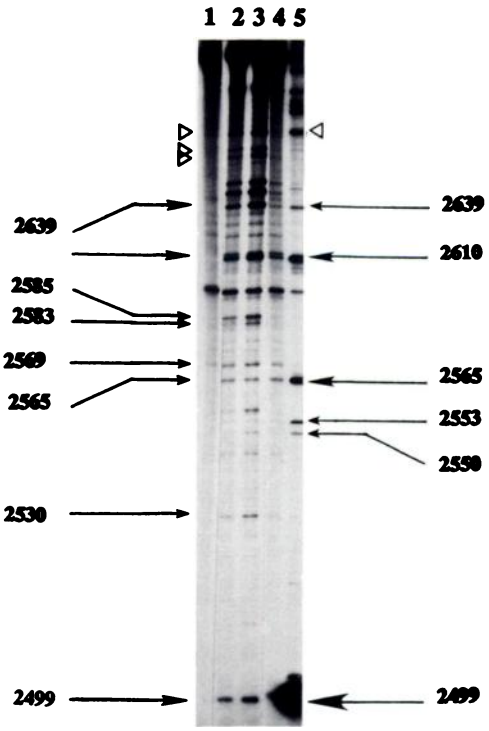

Fig. 6. Sequencing of topoisomerase II cleavage sites induced by intoplicine and *m*-AMSA in the promoter region of the human c-*myc* protooncogene first exon. *Lane 1*, control DNA; *lane 2*, enzyme plus 0.1 μM intoplicine; *lane 3*, enzyme plus 0.5 μM intoplicine; *lane 4*, enzyme without drug; *lane 5*, enzyme plus 10 μM *m*-AMSA. Electrophoresis was from *top* to *bottom*. *Numbers to the right*, genomic positions of *m*-AMSA main cleavage sites on the coding strand. *Numbers to the left*, genomic positions of intoplicine main cleavage sites. *Open triangles*, cleavage sites whose precise genomic positions could not be determined.

[2] Fosse and Riou. Manuscript in preparation.

Inhibition of topoisomerase II decatenation. Fig. 7 shows that intoplicine inhibits topoisomerase II catalytic activity. Inhibition of decatenation was complete at 3 μM (Fig. 7, *lane 5*) and only partial at 1 μM (Fig. 7, *lane 4*). Therefore, enzyme inhibition was achieved at concentrations corresponding to those that stimulated cleavable complex formation.

DNA interaction. Using the topoisomerase I unwinding assay, we compared the DNA unwinding potencies of intoplicine, *m*-AMSA, CPT, and NMHE (Fig. 8). The catalytic reac-

Fig. 7. Inhibition by intoplicine of topoisomerase II-mediated decatenation. Decatenation reactions were performed in the presence of 4 units of P388 topoisomerase II, as described in Materials and Methods. *Lane 8*, control *T. cruzi* kDNA (0.1 μg); *lane 7*, control topoisomerase II; *lanes 1-6*, as in *lane 7* with 0.03, 0.1, 0.3, 1, 3, and 10 μM intoplicine, respectively. *mC*, minicircles.

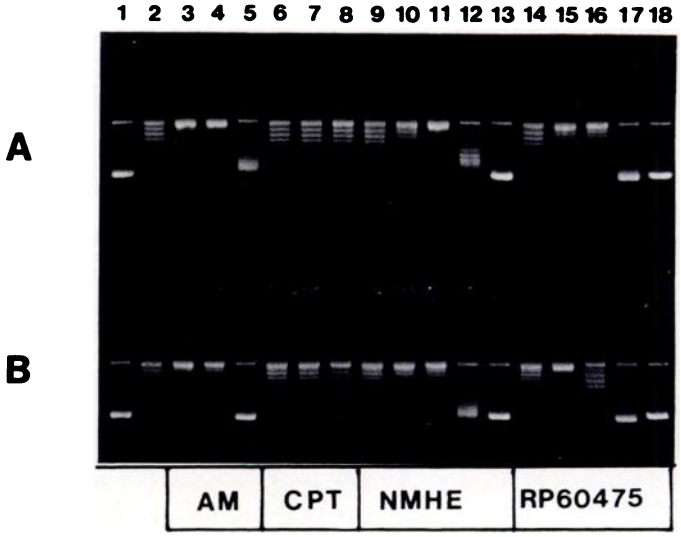

Fig. 8. DNA unwinding by intoplicine. The unwinding assay was performed in the presence of supercoiled pBR322 DNA and 20 units of calf thymus topoisomerase I (excess enzyme) (A) or 2 units of calf thymus topoisomerase I (catalytic amount) (B), as described in Materials and Methods. A and B, *Lane 1*, control supercoiled pBR322 DNA; *lane 2*, DNA and control topoisomerase I; *lanes 3-5*, as in *lane 2* with 1, 10, and 100 μM *m*-AMSA (*AM*), respectively; *lanes 6-8*, as in *lane 2* with 3, 30, and 300 μM CPT, respectively; *lanes 9-13*, as in *lane 2* with 0.1, 0.3, 1, 3, and 10 μM NMHE, respectively; *lanes 14-18*, as in *lane 2* with 0.1, 0.3, 1, 3, and 10 μM intoplicine, respectively.

tion of topoisomerase I was fully inhibited at 3 μM intoplicine under either catalytic or excess enzyme conditions (Fig. 8, A and B, *lanes 17*). In this assay, intoplicine appeared to be as potent as NMHE and more potent than *m*-AMSA. As expected, CPT did not show any unwinding activity. These results indicate that intoplicine is a DNA unwinding agent like DNA intercalators.

Discussion

Our results demonstrate that intoplicine inhibits both topoisomerase I and topoisomerase II by trapping cleavable complexes. Intoplicine is thus, after actinomycin D (36, 37) and saintopin (38), one of the few known agents reported as a "dual topoisomerase" inhibitor. The cytotoxic activity of intoplicine upon sensitive cell lines is comparable to those of other well known anti-topoisomerase II agents. The cross-resistance exhibited against intoplicine by different cell lines overexpressing the *mdr* gene indicates that intoplicine, like other anti-topoisomerase II inhibitors, is likely to be recognized and carried out of the cell by gp170, which is responsible for the multidrug resistance phenotype. However, intoplicine cytotoxicity also exhibits features that are somewhat unusual, compared with those of other agents that inhibit topoisomerase II. For instance, a decrease in the topoisomerase II intracellular activity does not inhibit the cell sensitivity to intoplicine, as is observed in Calc18/AM cells, indicating that intoplicine cytotoxicity is likely to be correlated with parameters other than the anti-topoisomerase II activity, one of which may be the anti-topoisomerase I activity. It is also interesting to note that P388/CPT-5 and DC3F/C-10 cells (28, 39), which exhibit a topoisomerase I mutation leading to CPT resistance, are not cross-resistant to intoplicine. Therefore, intoplicine appears to exhibit a broad spectrum of activity and to be cytotoxic in cells that are resistant to either topoisomerase II or topoisomerase I inhibitors.

Cellular damage was analyzed using alkaline elution. Intoplicine produces SSB, DSB, and DPC in whole cells. As expected for a topoisomerase II inhibitor, a significant fraction of the breaks are DSB, the frequencies of SSB and DPC are similar (within a factor of 2), and all the breaks are protein linked (see the protein concealment experiments). SSB, DSB, and DPC are produced with the same bell-shaped effect, just as with other ellipticine derivatives, aza-ellipticines (BD40) (30), or γ-carbolines (40). These results are consistent with the hypothesis that, like these intercalating agents (BD40 or γ-carbolines), high concentrations of intoplicine may destabilize topoisomerase II cleavable complexes. Using purified systems, it is usual to observe this kind of self-inhibition of anti-topoisomerase II activity in the case of intercalators (7, 11, 41). Indeed, our *in vitro* experiments clearly show the self-inhibition of intoplicine anti-topoisomerase I and II activity above 3 μM. Nonetheless, it is less common to observe it in whole cells, because usually the net influx of drug into the cell is limited and the intracellular drug concentration barely reaches the inhibitory threshold. This limitation seems not to apply to intoplicine. The results concerning the kinetics of formation of the breaks are consistent with the hypothesis of a quick influx of intoplicine into the nucleus, because exposure of cells to the drug for as short a time as 10 min leads to the same number of SSB as does an exposure of several hours. Kinetic experiments also show slow reversal of the breaks, which could be consistent with slow efflux of the drug from the cell. Interestingly, after the drug is washed out of the extracellular medium, the number of breaks increases in the case of 5 μM treatment with intoplicine, probably because the intracellular drug concentration slowly decreases toward the optimum concentration for induction of SSB. A similar phenomenon has been noted previously for a related derivative (BD40) (30).

We performed specific experiments to measure the ratio between "true SSB" and DSB formation (S/D in Table 3). To calculate this ratio we had to take into account the fact that the SSB frequency measured by alkaline elution assays might include SSB arising from DSB (9) (each DSB gives rise to two SSB) as well as true SSB (those arising from DSB). Making this distinction might allow us to detect SSB formed by different mechanisms. For instance, in the case of ellipticine, which is reported to be a "pure" DSB inducer (42), every SSB is related to one DSB, which leads to a ratio equal to zero. We confirmed this result in our experiments using NMHE. In the case of intoplicine the ratio is significantly different from zero and decreases from 8 to 2 when drug concentration goes from 0.3 to 1 μM. This result is consistent with the assumption that, in the case of intoplicine, SSB may be a mixture of both topoisomerase I-mediated SSB and topoisomerase II-mediated DSB. However, we cannot rule out the possibility that at least some of the true SSB arise from topoisomerase II inhibition, because a specific topoisomerase II inhibitor, like *m*-AMSA, produces true SSB (9) (see Table 3). However, the SSB/DSB ratio values measured for intoplicine are much higher than those measured for *m*-AMSA or NMHE, which suggests that another source of true SSB may be involved in the formation of SSB and that this source may be topoisomerase I mediated (43, 44).

In conclusion, intoplicine is a cytotoxic agent that interacts with DNA and inhibits both DNA topoisomerases I and II *in vitro*, by stabilizing cleavable complexes. Intoplicine induces protein-linked DNA SSB and DSB in cells, consistent with concomitant inhibition of both cellular topoisomerases I and II. Intoplicine easily and quickly enters cells, where it seems to stay active for several hours. It is probably recognized by gp170 and pumped out of the cells that express this protein. Finally, intoplicine stands as a new agent of great clinical interest that may exhibit cytotoxic effects on a variety of tumor cells, including those altered in either topoisomerase I or topoisomerase II and against which pure topoisomerase I or topoisomerase II inhibitors are inactive. Also, structure-activity studies performed with this kind of dual inhibitor should help to elucidate the common features shared by topoisomerase I and II inhibitors.

Acknowledgments

The authors wish to thank Dr. Kurt W. Kohn for valuable discussion and support during the course of this work.

References

1. Pommier, Y., and K. W. Kohn. Topoisomerase II inhibition by antitumor intercalators and demethylepipodophyllotoxins, in *Developments in Cancer Chemotherapy* (R. I. Glazer, ed.). CRC Press, Inc., Boca Raton, FL, 175–196 (1989).
2. Schneider, E., Y.-H. Hsiang, and L. F. Liu. DNA topoisomerases as anticancer drug targets. *Adv. Pharmacol.* **21**:149–183 (1990).
3. Hsiang, Y. H., R. Hertzberg, S. Hecht, and L. F. Liu. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J. Biol. Chem.* **260**:14873–14878 (1985).
4. Jaxel, C., K. W. Kohn, M. C. Wani, M. E. Wall, and Y. Pommier. Structure-activity study of the actions of camptothecin derivatives on mammalian

topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity. *Cancer Res.* **49**:1465–1469 (1989).
5. Kunimoto, T., K. Nitta, T. Tanaka, N. Uebuara, H. Baba, M. Takeuchi, T. Yokokura, S. Sawada, T. Miyasaka, and M. Mutai. Antitumor activity of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin, a novel water-soluble derivative of camptothecin, against murine tumors. *Cancer Res.* **47**:5944–5947 (1987).
6. Kingsbury, W. D., J. C. Boehm, D. R. Jakas, K. G. Holden, S. M. Hecht, G. Gallagher, M. J. Caranfa, F. McCabe, L. F. Faucette, R. K. Johnson, and R. P. Hertzberg. Synthesis of water-soluble (aminoalkyl)camptothecin analogues: inhibition of topoisomerase I and antitumor activity. *J. Med. Chem.*, **34**:98–107 (1991).
7. Tewey, K. M., T. C. Rowe, L. Yang, B. D. Halligan, and L. F. Liu. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science (Washington D. C.)* **226**:466–468 (1984).
8. Capranico, G., F. Zunino, K. W. Kohn, and Y. Pommier. Sequence-selective topoisomerase II inhibition by anthracycline derivatives in SV40 DNA: relationship with DNA affinity and cytotoxicity. *Biochemistry* **29**:562–569 (1990).
9. Zwelling, L. A., S. Michaels, L. C. Erickson, R. S. Ungerleider, M. Nichols, and K. W. Kohn. Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and Adriamycin. *Biochemistry* **20**:6553–6563 (1981).
10. Nelson, E. M., K. M. Tewey, and L. F. Liu. Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)methanesulfon-*m*-anisidide. *Proc. Natl. Acad. Sci. USA* **81**:1361–1365 (1984).
11. Pommier, Y., J. K. Minford, R. E. Schwartz, L. A. Zwelling, and K. W. Kohn. Effects of the DNA intercalators 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and 2-methyl-9-hydroxyellipticinium on topoisomerase II mediated DNA strand cleavage and strand passage. *Biochemistry* **24**:6410–6416 (1985).
12. Tewey, K. M., G. L. Chen, E. M. Nelson, and L. F. Liu. Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* **259**:9182–9187 (1984).
13. Chen, G. L., L. Yang, T. C. Rowe, B. D. Halligan, K. M. Tewey, and L. F. Liu. Non-intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* **259**:13560–13566 (1984).
14. Gewirtz, D. A. Does bulk damage to DNA explain the cytostatic and cytotoxic effects of topoisomerase II inhibitors? *Biochem. Pharmacol.* **42**:2253–2258 (1991).
15. Pommier, Y., A. Orr, K. W. Kohn, and J. F. Riou. Differential effects of amsacrine and epipodophyllotoxins on topoisomerase II cleavage in the human c-*myc* proto-oncogene. *Cancer Res.* **52**:3125–3130 (1992).
16. Bisagni, E., C. H. Nguyen, A. Pierre, O. Pepin, P. de Cointet, and P. Gros. 1-Amino-substituted 4-methyl-5*H*-pyrido[4,3-*b*]indoles (γ-carbolines) as tricyclic analogues of ellipticines: a new class of antineoplasic agents. *J. Med. Chem.* **31**:398–405 (1988).
17. Nguyen, C. H., J. M. Lhoste, F. Lavelle, M. C. Bissery, and E. Bisagni. Synthesis and antitumor activity of 1-[(dialkylamino)alkyl]amino-4-methyl-5*H*-pyrido[4,3-*b*](benzo[*e*]- and benzo[*g*])indoles: a new class of antineoplasic agents. *J. Med. Chem.* **33**:1519–1528 (1990).
18. Bissery, M. C., C. H. Nguyen, E. Bisagni, and F. Lavelle. Preclinical evaluation of RP-60475, a pyrido-benzo-indole antitumor agent. *Proc. Am. Assoc. Cancer Res.* **31**:2747 (1990).
19. Lavelle, F., C. H. Nguyen, M. C. Bissery, J. F. Riou, and E. Bisagni. Synthesis and antitumor properties of new derivatives of the pyrido-benzo-indole series. *Proc. Am. Assoc. Cancer Res.* **32**:2411 (1991).
20. Minford, J., Y. Pommier, J. Filipski, K. W. Kohn, D. Kerrigan, M. Mattern, S. Michaels, R. Schwartz, and L. A. Zwelling. Isolation of intercalator-dependent protein-linked DNA strand cleavage activity from cell nuclei and identification as topoisomerase II. *Biochemistry* **25**:9–16 (1986).
21. Pommier, Y., D. Kerrigan, R. E. Schwartz, J. A. Swack, and A. McCurdy. Altered DNA topoisomerase II activity in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Res.* **46**:3075–3081 (1986).
22. Halligan, B. D., K. A. Edwards, and L. F. Liu. Purification and characterization of a type II DNA topoisomerase from bovine calf thymus. *J. Biol. Chem.* **260**:2475–2482 (1985).
23. Riou, J. F., M. Gabillot, M. Philippe, J. Schrevel, and G. Riou. Purification and characterization of *Plasmodium berghei* DNA topoisomerases I and II: drug action, inhibition of decatenation and relaxation, and stimulation of DNA cleavage. *Biochemistry* **25**:1471–1479 (1986).
24. Madelaine, J., S. Prost, A. Naudin, G. Riou, F. Lavelle, and J. F. Riou. Sequential modifications of topoisomerase I activity in a camptothecin resistant cell line established by progressive adaptation. *Biochem. Pharmacol.* **45**:339–348 (1993).
25. Riou, J.-F., A. Naudin, and F. Lavelle. Effect of taxotere on murine and human tumor cell lines. *Biochem. Biophys. Res. Commun.* **187**:164–170 (1992).
26. Lefevre, D., J.-F. Riou, J. C. Ahomadege, D. Zhou, J. Benard, and G. Riou. Study of molecular markers of resistance to *m*-AMSA in a human breast cancer cell line: decrease of topoisomerase II and increase of both topoisomerase I and acidic glutathione-*S*-transferase. *Biochem. Pharmacol.* **41**:1967–1979 (1991).
27. Chevallier-Multon, M.-C., A. Jacquemin-Sablon, R. Besselievre, H.-P. Husson, and J. B. Le Pecq. Comparative cytotoxicities of a series of ellipticine and olivacine derivatives on multidrug resistant cells of human and murine origins. *Anticancer Drug. Des.* **5**:319–335 (1990).
28. Tanizawa, A., and Y. Pommier. Topoisomerase I alterations in a camptothecin-resistant cell line derived from Chinese hamster DC3F cells in culture. *Cancer Res.* **52**:1848–1854 (1992).
29. Kohn, K. W. Principles and practice of DNA filter elution. *Pharmacol. Ther.* **49**:55–77 (1991).
30. Pierson, V., A. Pierre, Y. Pommier, and P. Gros. Production of protein-associated DNA breaks by 10-[diethylaminopropylamino]-6-methyl-5*H*-pyrido[3′,4′:4,5]pyrrolo[2,3-*g*]isoquinoline in cultured L1210 cells and in isolated nuclei: comparison with other topoisomerase II inhibitors. *Cancer Res.* **48**:1404–1409 (1988).
31. Ross, W. E., D. Glaubiger, and K. W. Kohn. Qualitative and quantitative aspects of intercalator-induced DNA strand breaks. *Biochim. Biophys. Acta* **562**:41–50 (1979).
32. Pommier, Y., J. M. Covey, D. Kerrigan, J. Markovits, and R. Pham. DNA unwinding and inhibition of mouse leukemia L1210 DNA topoisomerase I by intercalators. *Nucleic Acids Res.* **15**:6713–6731 (1987).
33. Pommier, Y., R. E. Schwartz, L. A. Zwelling, D. Kerrigan, M. R. Mattern, J. Y. Charcosset, A. Jacquemin-Sablon, and K. W. Kohn. Reduced formation of protein-associated DNA strand breaks in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Res.* **46**:611–616 (1986).
34. Estey, E. H., L. Silberman, M. Beran, B. S. Andersson, and L. A. Zwelling. The interaction between nuclear topoisomerase II activity from human leukemia cells, exogenous DNA, and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) or 4-(4,6-*O*-ethylidene-beta-D-glucopyranoside) (VP-16) indicates the sensitivity of the cells to the drugs. *Biochem. Biophys. Res. Commun.* **144**:787–793 (1987).
35. Blöcher, D. DNA double-strand breaks in Erhlich ascites tumour cells at low doses of X-rays. I. Determination of induced breaks by centrifugation at reduced speed. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* **42**:317–328 (1982).
36. Trask, D. K., and M. T. Muller. Stabilization of type I topoisomerase-DNA covalent complexes by actinomycin D. *Proc. Natl. Acad. Sci. USA* **85**:1417–1421 (1988).
37. Wassermann, K., J. Markovits, C. Jaxel, G. Capranico, K. W. Kohn, and Y. Pommier. Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerases I and II. *Mol. Pharmacol.* **38**:38–45 (1990).
38. Yamashita, Y., S.-Z. Kawada, N. Fujii, and H. Nakano. Induction of mammalian topoisomerase I and II mediated DNA cleavage by saintopin, a new antitumor agent from fungus. *Biochemistry* **30**:5838–5845 (1991).
39. Tanizawa, A., A. Tabuchi, R. Bertrand, and Y. Pommier. Cloning of Chinese hamster DNA topoisomerase I cDNA and identification of a single point mutation responsible for camptothecin resistance. *J. Biol. Chem.*, in press.
40. Fruehauf, J. P., C. E. Myers, and B. K. Sinha. Synergistic activity of suramin with tumor necrosis factor *alpha* and doxorubicin on human prostate cancer cell lines. *J. Natl. Cancer Inst.* **82**:1206–1209 (1990).
41. Pommier, Y., R. E. Schwartz, K. W. Kohn, and L. A. Zwelling. Formation and rejoining of deoxyribonucleic acid double-strand breaks induced in isolated cell nuclei by antineoplastic intercalating agents. *Biochemistry* **23**:3194–3201 (1984).
42. Zwelling, L. A., S. Michaels, D. Kerrigan, Y. Pommier, and K. W. Kohn. Protein-associated deoxyribonucleic acid strand breaks produced in mouse leukemia L1210 cells by ellipticine and 2-methyl-9-hydroxyellipticinium. *Biochem. Pharmacol.* **31**:3261–3267 (1982).
43. Mattern, M. R., S. M. Mong, H. F. Bartus, C. K. Mirabelli, S. T. Crooke, and R. K. Johnson. Relationship between the intracellular effects of camptothecin and the inhibition of DNA topoisomerase I in cultured L1210 cells. *Cancer Res.* **47**:1793–1798 (1987).
44. Covey, J. M., C. Jaxel, K. W. Kohn, and Y. Pommier. Protein-linked DNA strand breaks induced im mammalian cells by camptothecin, an inhibitor of topoisomerase I. *Cancer Res.* **49**:5016–5022 (1989).

Send reprint requests to: Yves Pommier, Building 37, Room 5C25, National Institutes of Health, Bethesda, MD 20892.