Biochemical Pharmacology, Vol. 45, No. 2, pp. 339–348, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

SEQUENTIAL MODIFICATIONS OF TOPOISOMERASE I ACTIVITY IN A CAMPTOTHECIN-RESISTANT CELL LINE ESTABLISHED BY PROGRESSIVE ADAPTATION

ISABELLE MADELAINE,* SANDRINE PROST,† ANNETTE NAUDIN,* GUY RIOU,†
FRANÇOIS LAVELLE* and JEAN-FRANÇOIS RIOU*‡

*Rhône-Poulenc Rorer, Unité de Cancérologie, Département Biologie, Centre de Recherche de Vitry-Alfortville, 94403 Vitry-sur-Seine Cedex; and
†Institut Gustave Roussy, Laboratoire de Pharmacologie Clinique et Moléculaire, 94800 Villejuif, France

(*Received* 6 *July* 1992; *accepted* 19 *October* 1992)

Abstract—The DNA–topoisomerase I (Topo I) inhibitor, camptothecin (CPT), is a plant alkaloid with an important antitumor activity. In order to investigate the cellular mechanism leading to the development of the resistance to this agent, we have established by progressive adaptation a P388 subline resistant to CPT. After 5 months of continuous drug exposure, the resistance index reached a value of 20 and the resistant cell line, P388CPT0.3, was maintained in the presence of CPT. CPT-induced single strand breaks measured by alkaline elution were found drastically reduced in the resistant cell line. Topo I activity and CPT-induced DNA cleavage were measured on cells at different steps of resistance. We first observed that the Topo I activity was strongly decreased. In addition, the resistant cells recovered the ATP-independent relaxation activity after 3 months of exposure to CPT, but still kept a reduced CPT-induced DNA cleavage. Further evaluations at the final stage of the resistance induction have indicated that cells presented a CPT-resistant form of Topo I. Rearranged Topo I gene on one allele and a reduced Topo I transcription were also observed in resistant cells. The putative role of the rearrangement was discussed. These data show that the resistance mechanism has evolved from a decreased Topo I activity to an altered form of the enzyme, and suggest that multiple mechanisms of Topo I modifications could contribute to CPT resistance.

Topoisomerases are enzymes that catalyse the topological changes of DNA [1, 2]. In eukaryotes, two types of enzymes (topoisomerases I and II; Topo I and II§) have been shown to play a crucial role in replication, transcription and chromosomal segregation processes. Topo II was identified for several years as an important target for different anticancer drugs commonly used in the treatment of human cancer [3]. Recently, it was shown that camptothecin (CPT), a plant alkaloid presenting an important preclinical antitumor activity, was a specific inhibitor of Topo I [4, 5]. CPT inhibits RNA and DNA synthesis and is associated with a protein-concealed fragmentation of DNA in cell lines [6–9]. CPT acts by stabilizing an intermediary form of the Topo I–DNA complex [4]. Two previous studies have shown that CPT-resistant cell lines (CPTK5, CHO-CPT^R) selected by mutagenesis displayed a mutant form of Topo I [10, 11]. These results provided strong evidence that Topo I is one cellular target of CPT.

Recently, several other cell lines resistant to CPT or derivatives, have been established and their mechanism of resistance was characterized [12–15]. The major feature of these cell lines was a reduced Topo I activity. Differences in the Topo I sensitivity towards CPT were observed. One of these cell lines, P388/CPT+, displayed a rearrangement of the Topo I gene on one allele which results in reduced mRNA level [12]. In addition, the relative resistance indexes of CPT in these cell lines displayed important variations. These data strongly suggest that cells have the faculty to acquire resistance by several mechanisms.

We have investigated during the various stages of resistance induction the different changes which led to CPT resistance in P388 subline. The acquisition of resistance is associated at first with a strong decrease of the cellular Topo I activity and then with the presence of a CPT-resistant form of Topo I. Both rearrangements and reduced transcription of the Topo I gene were also detected in the resistant cells.

MATERIALS AND METHODS

Drug. Camptothecin was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Stock solutions at 1 mg/mL in dimethyl sulfoxide were stored at −20°. Drug dilutions in sterile distilled water were made just before use.

Cell line and culture conditions. The murine P388 leukemia wild type cells (P388wt) were grown in RPMI 1640 medium containing 0.01 mM β-mercaptoethanol, 10 mM L-glutamine, 10% (v/v) fetal calf serum, 100 IU/mL penicillin, 2 μg/mL

‡ Corresponding author. Tel. (33) 1 45 73 74 70; FAX (33) 1 45 73 74 71.

§ Abbreviations: BSA, bovine serum albumin; CPT, camptothecin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; SSB, single-strand break; TBE, Tris-borate-EDTA buffer; Topo I, topoisomerase I; P388wt, P388 wild type cells.

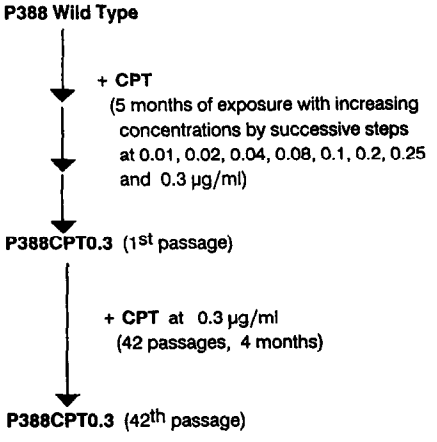

Fig. 1. Selection of the CPT-resistant cell line P388CPT0.3.

streptomycin, 50 μg/mL gentamycin and 50 μg/mL nystatin, at 37° in a humidified atmosphere containing 5% CO_2. CPT-resistant cells were selected by successive step exposure with increased concentrations of CPT added in the culture medium as described in Fig. 1. Finally, cells were resistant to 0.3 μg/mL of CPT (P388CPT0.3). Fetal calf serum concentration was increased to 15% (v/v) for the current culture of the resistant sublines.

Evaluation of the antiproliferative activity. The concentrations of CPT giving 50% of growth inhibition (IC_{50}) were determined from four separate experiments in 96-well microculture plates on P388wt and resistant cell lines. Cell lines seeded at 10^5 cells/mL (0.2 mL/well) were grown for 96 hr in the presence of various CPT concentrations (each point in quadruplicate). Cells were then incubated for 16 hr with 0.02% neutral red. The cells were washed and lysed with 1% sodium dodecyl sulfate (SDS). The incorporation of the dye reflecting cellular growth and viability was evaluated by the measurement of the optical density for each well at 540 and 346 nm, using a Titertek multiwell spectrophotometer. Cell viability was expressed as the per cent of cell surviving fraction relative to the untreated control cells.

Topoisomerase preparations. Nuclear extracts were prepared in a parallel assay from 10^8 wild type and resistant P388 cells in exponential phase of growth [16]. Nuclear extracts, adjusted to the same protein concentration, were stored at −20° for 1 month.

Relaxation assay. Nuclear extracts were assayed for the Topo I relaxation activity as already described [17]. Various amounts of nuclear extracts were incubated for 30 min at 37° with 0.1 μg of supercoiled pBR322 DNA (Boehringer, Mannheim, Germany) in a final reaction volume of 20 μL containing 25 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 30 mg/mL bovine serum albumin (BSA), 100 mM KCl and 10 mM $MgCl_2$. The reaction was stopped on ice by the addition of 5 μL containing 0.1% bromophenol blue, 50 mM EDTA, 50% (v/v) glycerol. The samples were loaded onto a 1% agarose gel in 1× Tris-borate-EDTA buffer (TBE) and electrophoresed at 40 V for 16 hr. The gel was stained with 10 μg/mL ethidium bromide for 10 min, then washed with distilled water and photographed on a UV table and negative scanned with a Pharmacia Ultroscan® densitometer. One relaxation unit corresponds to the minimal amount of protein (mg) necessary to relax 50% of the supercoiled pBR322 DNA in the assay conditions. Topo I specific activity in the nuclear extracts was expressed as relaxation units per mg of protein. Topo I relaxation determinations were done in parallel in sensitive and in resistant cells.

Inhibition of the Topo I relaxation by CPT was done under the same experimental conditions using 2 μg (1 unit) of nuclear extracts from P388wt or P388CPT0.3 (30th passage) cells, in the presence of various concentrations of CPT. Negatives of the gel pictures were scanned with a Pharmacia Ultroscan® densitometer in order to quantify the relaxation reaction. CPT-induced inhibition of topoisomerase I-mediated DNA relaxation was computed from the formula:

$$\% \text{ of inhibition} = 100 \times (S_c - S_t)/(S_o - S_t)$$

where S_c and S_t represent the fraction of supercoiled DNA measured with 1 U enzyme (2 μg), in the presence or absence of drug, respectively, and S_o represents the fraction of supercoiled DNA in untreated DNA.

CPT-induced cleavage assay. Various amounts of nuclear extracts were incubated for 10 min at 37° with 25 ng of ^{32}P end-labeled pBR322 DNA, prepared as described previously [16], in 20 μL of the relaxation reaction mixture and with or without 100 μg/mL CPT. The reaction was stopped by the addition of 2 μL of 2.5% SDS, 2.5 mg/mL proteinase K; then samples were further incubated for a period of 30 min at 50° and DNA was denatured by the addition of 10 μL of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, 0.1% bromocresol green. Samples were loaded onto a 1% agarose gel in 1× TBE, 0.1% SDS buffer. After electrophoresis, gels were dried and autoradiographed for 1 or 2 days (Hyperfilms MP, Amersham).

Alkaline elution. CPT-induced DNA single-strand breaks (SSB) were estimated by the alkaline elution assay [18]. Briefly, cellular DNA from exponentially growing cells was labeled by incubation with [^{14}C]thymidine (0.02 μCi/mL) or [^{3}H]thymidine (0.05 μCi/mL) for 24 hr. Such a period corresponds to about one and a half cell cycles. ^{14}C-Labeled experimental cells (2×10^5) were treated in culture medium for 1 hr with various concentrations of CPT, then washed twice with 1× phosphate-buffered saline. Tritiated internal standard cells (2×10^5) were irradiated by a ^{60}Co source (300 rad) and were combined with the experimental cells; then layered on polycarbonate membrane filter, lysed with 2 mL of 25 mM Na_2 EDTA, 2% SDS, pH 9.7 and with 0.5 mg/mL proteinase K. Elution was performed with tetrapropylammonium hydroxide, EDTA, 0.1% SDS pH 12.2 using a peristaltic pump to control the flow rate at a speed of 0.03–0.04 mL/min. Fractions were collected at 3 hr intervals for 15 hr. Calculations

were performed and drug-induced break frequencies were expressed in rad equivalents [18].

RNA, DNA preparations and hybridizations. Total cellular RNA from exponentially growing cells were prepared by the guanidinium isothiocyanate/CsCl density gradient fractionation method [19]. For northern blot analysis, 10 μg of each RNA sample were electrophoresed on a formaldehyde containing 1% agarose gel and transferred to Hybond N^+ membrane (Amersham). For slot blot analysis, serial dilutions of RNAs (10, 5, 2.5 and 1 μg) were loaded on Hybond N^+ filter using a Schleicher and Schuell apparatus. DNAs were extracted and resuspended in 10 mM Tris pH 7.5, 0.5 mM EDTA as described previously [19]. Ten micrograms of each DNA sample were digested overnight by the different restriction enzymes (Eco RI, Pvu II, Hind III, Xba I, Bgl II), electrophoresed in a 1.2% agarose gel, transferred on Hybond N^+ membrane and hybridized with the different probes. Washings were carried out under stringent conditions as described previously [19].

Probes. The probes used were: the 3.4 kbp Bam HI–Eco RI cDNA fragment of the human Topo I gene [20], provided by Dr J. C. Wang (Harvard University, Boston, MA, U.S.A.); the 1.15 kbp *Pst* I DNA fragment of the mouse β-actin gene [21].

RESULTS

Selection of CPT-resistant cells

The parental P388 cell line (P388wt) was exposed for 5 months to increased concentrations of CPT (see Fig. 1). At the initial step, CPT was used at 0.01 μg/mL, corresponding to the concentration inhibiting cell growth by 20% (IC_{20}). Cells became resistant to 0.3 μg/mL (P388CPT0.3) and were maintained on this drug concentration for 42 passages (Fig. 1). The cell doubling times were found to be equal to 12 and 19 hr for P388wt and P388CPT0.3, respectively. IC_{50} values of CPT for the sensitive and resistant cells were determined for 96 hr of drug exposure (see Materials and Methods) and were found to be equal to 0.035 and 0.70 μg/mL, respectively (Fig. 2). These data indicated that the relative index of resistance to CPT is equal to 20. When resistant cells were grown for 21 passages in the absence of CPT, the IC_{50} measured was equal to 0.50 μg/mL, showing that the P388CPT0.3 cell line displayed a stable resistant phenotype.

Determination of CPT-induced DNA strand breaks

Analysis of the eventual DNA alterations induced by CPT was studied using the alkaline elution technique which allows the production of DNA SSB to be quantified [18]. In the case of P388wt cells, CPT treatment for 1 hr caused a dose-dependent increase in the DNA SSB frequencies (Fig. 3). In contrast, CPT used at a concentration as high as 50 μg/mL did not produce detectable DNA SSB in the P388CPT0.3 cells. Similar results were obtained on isolated nuclei (results not shown).

These data indicate that CPT induces lower cellular DNA damage in the resistant cells.

In vitro DNA relaxation and CPT-induced cleavage activities of nuclear extracts

Topo I ATP-independent relaxation activities of nuclear extracts from P388wt and resistant cells were determined (Fig. 4). When compared with Topo I activity in P388wt extracts, the activities found in P388CPT0.3 underwent great variations according to the number of passages in the presence of CPT. At passages 16, 23 and 27, the relaxation activities were 5.5, 10 and 4-fold lower than in P388wt extract, respectively. From passage 30, in the P388CPT0.3 cells, this activity was recovered and found to be identical to that measured in P388wt extract (Fig. 4). At an early stage of the resistance (P388CPT0.08), the Topo I activity was decreased by 2.6-fold (see Fig. 4).

The CPT-induced DNA cleavage activities of nuclear extracts were also measured. DNA breaks are generated by the stabilization of the Topo I–DNA cleavable complex. In these experiments, DNA breaks induced by CPT could depend on the total activity of Topo I, or to its sensitivity to CPT. Compared to the P388wt cell line, the nuclear extracts from P388CPT0.3 cells contained a considerably lower CPT-induced DNA breakage activity (Fig. 5). The DNA cleavage was quantified by densitometric scanning of the autoradiogram. CPT-induced DNA cleavage decreased by more than 50-fold at the 9th passage (data not shown) and decreased by 16-fold at the 30th passage (Fig. 5).

These data show that both *in vitro* relaxation and cleavage activities were decreased in the resistant extracts up to the 27th passage. From the 30th passage, the relaxation activity was recovered, while the cleavage activity remained decreased.

Inhibition of the relaxation activity by CPT

The inhibitory effect of CPT on the catalytic activity of Topo I was analysed in extracts from P388wt and in P388CPT0.3 cells (30th passage). The relaxation of supercoiled DNA was measured using the same amount of nuclear protein obtained from parental and resistant cells (Fig. 6). DNA relaxation, using the Topo I preparation from P388wt cells, was significantly inhibited by increasing concentrations of CPT. In contrast, the CPT inhibitory effect was markedly less with the Topo I from resistant cells (Fig. 6). At a concentration of 100 μg/mL of CPT, the relaxation reactions were inhibited by 74 and 23%, using P388wt and P388CPT0.3 preparations of Topo I, respectively. These data indicate that, for identical specific activities, the enzyme from P388CPT0.3 cells is 3-fold resistant to CPT inhibition.

Topo I gene transcript

The variations of the Topo I activity in the resistant cells could be the result of a transcriptional process, therefore we have examined the Topo I gene transcript level in resistant and sensitive cells at their exponential phase of growth. Equal amounts of total RNAs from P388wt and P388CPT0.3 (42nd passage) were analysed by northern blot hybridization, using sequentially the Topo I and the actin probes (see Materials and Methods). The Topo I probe detected a 4.0 kbp mRNA band in both preparations (Fig. 7,

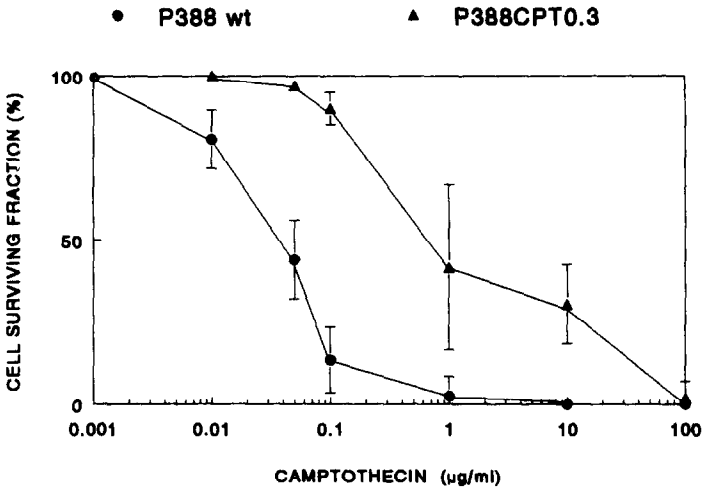

Fig. 2. Growth inhibition of P388wt (●) and P388CPT0.3 (▲) cell lines incubated with various concentrations of CPT. Cells were grown in liquid medium for 96 hr in the presence of CPT. Viability was determined after incorporation of neutral red and expressed as the per cent of cell surviving fraction, relative to the control untreated cells (100%). Values are the mean (±SD) of four separate determinations.

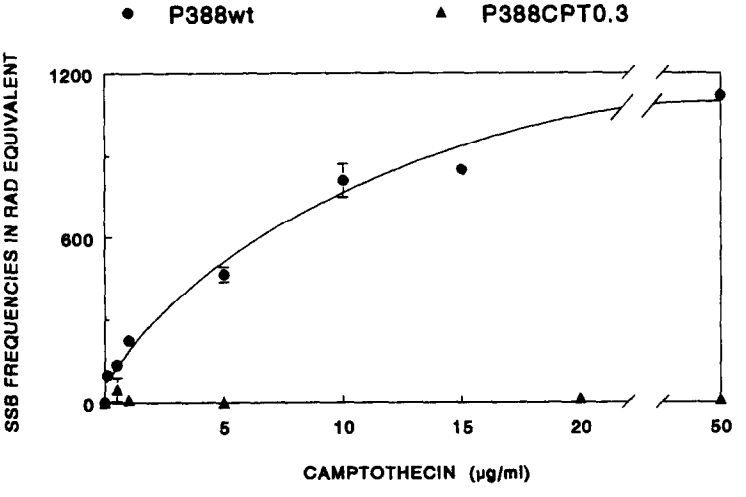

Fig. 3. DNA SSB frequencies induced by CPT in sensitive (●) and resistant (▲) P388 cell lines. SSB frequencies were determined as described in Materials and Methods on P388wt and P388CPT0.3 cells (42nd passage), after exposure for 1 hr to various CPT concentrations. Values represent the mean (± SD) of three or four independent experiments.

lanes 1 and 2). The actin signal was used as an internal control for the quantity of RNA loaded in each lane. The intensity of the Topo I transcript band decreased in the resistant cells (lane 2), and was found to be 2-fold lower than in the P388wt cells (lane 1).

The Topo I gene transcript level was also determined in sublines with lower degrees of resistance to CPT (P388 cells resistant to 0.08, 0.1, 0.2 and 0.25 µg/mL of CPT). The Topo I transcript level was decreased in all the resistant sublines studied, whatever their degree of resistance (Table 1).

These data indicate that the diminution of the Topo I transcript level is an early event.

Topo I gene locus

In order to research alterations of the Topo I gene structure which could be associated with the resistance phenotype, genomic DNAs, after digestion with several restriction enzymes (Bgl II, Hind III, Eco RI, Pvu II, Xba I), were analysed by Southern

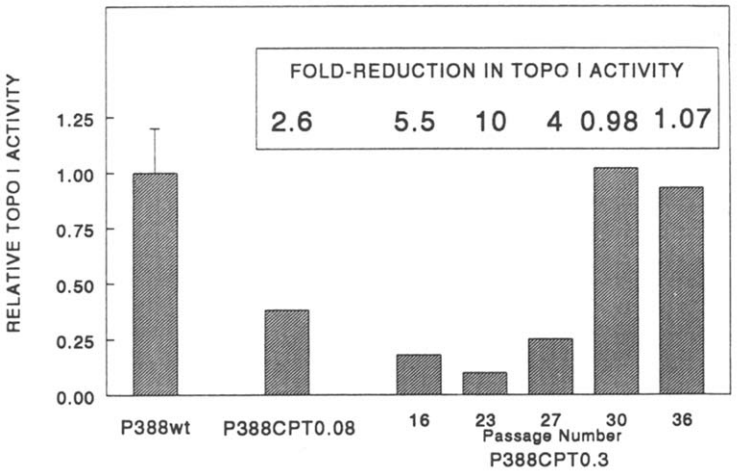

Fig. 4. Variations of the Topo I relaxation activity in the resistant cell line at different passages. The Topo I relaxation activities were determined as described in Materials and Methods on sensitive or resistant nuclear extracts prepared in parallel and adjusted to the same protein concentrations. Results correspond to single determinations performed in parallel on sensitive and resistant cells and are expressed relative to the specific activity of Topo I measured in P388wt (defined as 1). Error bar for P388wt reflects the reproducibility of the experiments (sp. act. = 3385 ± 689 U/mg protein, N = 6).

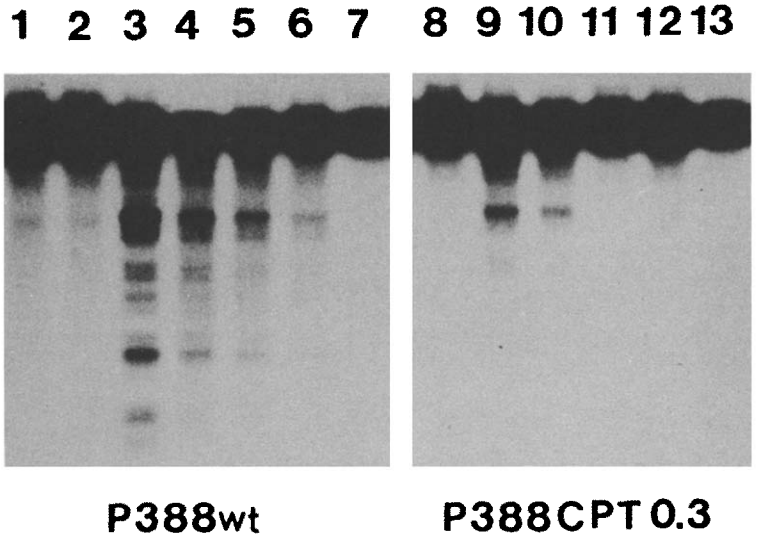

Fig. 5. CPT-induced DNA cleavage activities of nuclear extracts from P388wt and P388CPT0.3 cells. Cleavage reactions were carried out as described in Materials and Methods on sensitive or resistant nuclear extracts prepared in parallel and adjusted to the same protein concentration. Lane 1: control pBR322 DNA. Lane 2: DNA and P388wt nuclear extract at dilution 1:1. Lanes 3–7: DNA and P388wt nuclear extract at dilutions 1:1, 1:2, 1:5, 1:10 and 1:50, respectively, in the presence of 100 μg/mL CPT. Lane 8: DNA and P388CPT0.3 nuclear extract (30th passage) at dilution 1:1. Lanes 9–13: DNA and P388CPT0.3 nuclear extract at dilutions 1:1, 1:2, 1:5, 1:10 and 1:50, respectively, in the presence of 100 μg/mL CPT.

blot hybridization, using the Topo I cDNA probe (see Materials and Methods). A rearrangement of the Topo I gene was detected in the resistant cell line when DNA was digested with Pvu II and Hind III, but not with the other enzymes. Typical Pvu II and Xba I cleavage patterns are presented in Fig. 8A. In resistant cells, the Topo I rearrangement detected with Pvu II was characterized by the presence of a new DNA fragment (arrow **b**) and a diminution of a germline DNA band (arrow **a**).

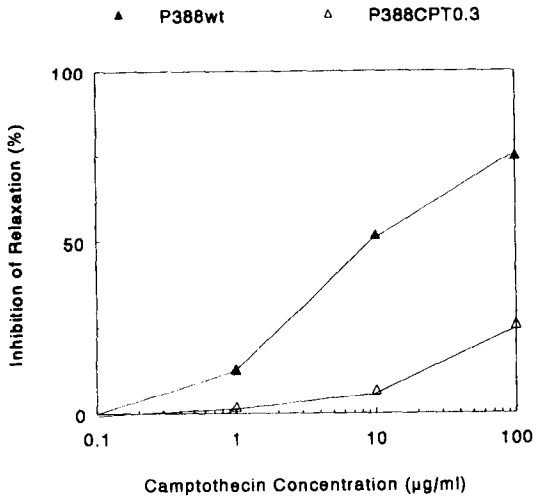

Fig. 6. Inhibition of the relaxation reaction by CPT. Relaxation reactions were carried out and electrophoresed as described in Materials and Methods in the presence of 2 μg of nuclear extract from P388wt (▲) or P388CPT0.3, at 30th passage (△). Relaxed DNA was quantified by densitometric scanning and normalized relative to the total amount of DNA loaded for each sample. Results are expressed in per cent inhibition of the relaxation reaction catalysed without CPT.

Table 1. Relative amount of Topo I transcript in P388 sublines resistant to CPT

Cell lines*	Topo I relative transcript level†
P388wt	1
P388CPT0.08	0.66
P388CPT0.1	0.5
P388CPT0.2	0.45
P388CPT0.25	0.3
P388CPT0.3	
16th passage	0.7
42nd passage	0.5

* The origin of the sublines studied is indicated in Fig. 1.

† The Topo I transcript level was determined by slot blot hybridization using serial dilutions of total RNAs (10, 5, 2.5 and 1 μg). Blots were sequentially hybridized with the human Topo I and the mouse actin probe (see Materials and Methods). Autoradiograms were scanned by a densitometric analyser (Ultroscan, Pharmacia) and Topo I values corrected by normalization with the actin values. Results are expressed relative to the P388wt value defined as 1.

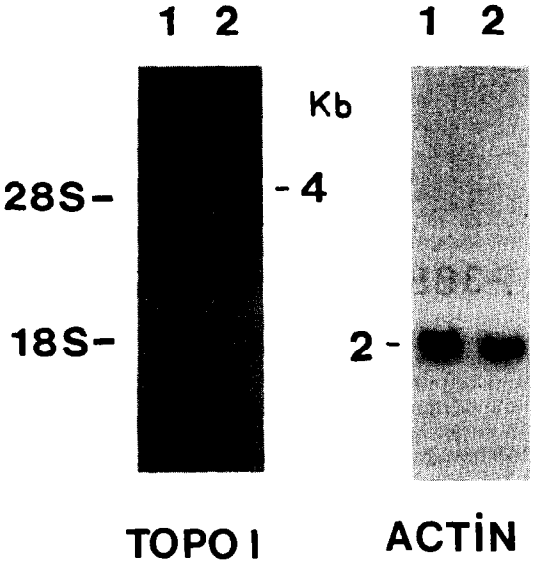

Fig. 7. Northern blot analysis of the Topo I transcripts in P388wt and P388CPT0.3 cell lines. Total RNAs (10 μg) were analysed by agarose gel electrophoresis, then transferred on membrane and sequentially hybridized with the human Topo I and the mouse β actin probes (see Materials and Methods). Lane 1: RNA from P388wt cells. Lane 2: RNA from P388CPT0.3 cells (42nd passage).

Densitometric analysis of autoradiograms showed that the new band **b** and the reduced band **a** in the resistant cells each represented approximately 50% of the area value measured for the band **a** in the P388wt cells (Fig. 8B). Similar results were found for the Hind III digestion (data not shown). Sublines with lower degrees of resistance to CPT (P388 cells resistant to 0.08, 0.1 and 0.2 μg/mL), were also found to present the Pvu II rearrangement (data not shown).

These results suggest that the Topo I gene from resistant cells displayed a rearrangement of one allele. The rearrangement was found to be an early event, during the resistance acquisition.

DISCUSSION

Resistance to CPT has already been established in several cell lines by different methods that have generated broad cellular resistance phenotypes, in particular resistant indexes varying from 2 to 333-fold, and different events at the Topo I level (modified Topo I content and/or resistant Topo I form) [10–15]. The characteristics of all these resistant cell lines are summarized in Table 2. Cell mutagenesis or *in vivo* adaptation to CPT derivatives were the most often used methods of selection. In this study, we have established *in vitro* a P388 subline resistant to CPT and analysed some cellular, biochemical and gene parameters which could be associated with the resistance phenotype.

The resistant cell line P388CPT0.3 was obtained after 5 months of progressive adaptation to increased concentrations of CPT. Cells were found to be 20-fold resistant to CPT and displayed a stable resistance phenotype.

CPT induced the formation of SSB in the genomic DNA of intact P388wt cells, as well as in isolated

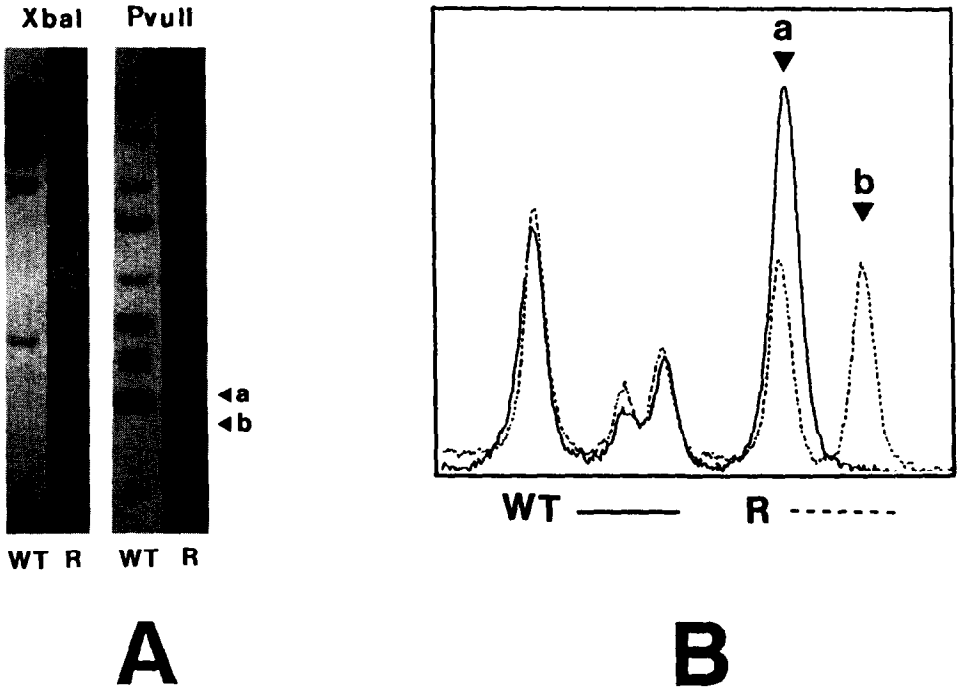

Fig. 8. Southern blot analysis of the genomic DNA from sensitive and resistant cells. (A) Genomic DNA (10 μg) from P388wt and P388CPT0.3 (40th passage) cells was digested with Xba I or Pvu II and analysed by Southern hybridization using the human Topo I probe (see Materials and Methods). Arrow a: unrearranged allele, arrow b: rearranged allele. (B) Densitometric scan of the Pvu II digestion shown in (A): (——) P388wt DNA, (---) P388CPT0.3 DNA.

nuclei. These lesions could not be detected in the genomic DNA of resistant cells and in the corresponding nuclei. It has been shown that CPT-induced Topo I SSB reverse rapidly upon drug removal [22], indicating that SSB in the resistant cells could be underestimated. Since alkaline elution was performed in parallel for sensitive and resistant cells, a rapid reversion of the SSB does not change the interpretation of the results: i.e. a strong decrease of SSB in resistant compared to sensitive cells. In addition, northern blot analysis of the P388CPT0.3 cells did not reveal any MDR gene overexpression (data not shown). These findings are in agreement with the nuclear mechanism of action of CPT on Topo I [3, 4] and with the lack of cross resistance of this compound on cell lines exhibiting the P-glycoprotein multidrug resistance phenotype [23, 24].

Biochemical studies of the Topo I activity in the sensitive and resistant P388 cells revealed two steps which are associated with an evolution of the resistant phenotype. Firstly, at passages 16, 23 and 27, the total Topo I activity was found to be 4–10-fold reduced in the resistant cells. At an early step of the resistant cell line establishment (P388CPT0.08), the Topo I activity was also decreased by 2.6-fold, indicating that such reduction of Topo I seems to be an early event in the resistance process. Quantitative reduction of the Topo I was also observed (2–8-fold) in all the other resistant cell lines established, except A549/CPT (see Table 2). These data indicated that the quantitative decrease of the Topo I activity seems to be a common mechanism of resistance to CPT.

Secondly, we observed that, after passages 30–36, the Topo I in resistant cells had recovered the activity found in P388wt cells. It could be suggested that an additional cellular event induced by CPT led to the restoration of the Topo I activity.

Another type of modification of the Topo I, related to a qualitative alteration of the enzyme, was described in the cellular CPT resistance. CPTK5, CHO-CPT^R and PC7/CPT cells [10, 11, 13] and more recently, DC3F/C-10 cells [15] were shown to present a CPT-resistant Topo I (Table 2). In the CPTK5 cell, the Topo I alteration was attributed to the presence of punctual DNA mutations which caused in Topo I a protein substitution of glycine into aspartic acid [25]. The mutant enzyme presented less CPT-induced cleavable formation and less inhibition of the relaxation reaction by CPT. Therefore, we have measured the amount of CPT-induced DNA cleavage in nuclear extracts from P388wt and P388CPT0.3 cells. CPT-induced cleavage in resistant cells was found to be decreased by 50-fold at passage 9 and 16-fold at passage 30. Such decreased cleavage could reflect either the lower level of Topo I activity in the resistant cells or an alteration of the Topo I sensitivity to CPT. Since the Topo I relaxation activity was decreased between passages 9 and 27, no conclusion could be deduced from the cleavage experiment with regard to the presence of a Topo I mutant form. On the other

Table 2. Characteristics of different cell lines resistant to CPT derivatives

Method of selection	Cell line	Origin	Resistance index	Cellular DNA-SSB	Cellular Topo I activity*	*In vitro* CPT-induced DNA-cleavage*	Resistant Topo I†	Topo I gene transcription*	Topo I gene alteration	Refs
In vitro mutagenesis adaptation	CPTK5§	Human T cell ALL	300	ND	0.33–0.5	0.008	+	Unchanged	Base codon mutation	[10]
	CHO-CPT^R	Chinese hamster ovary	333	Strongly decreased	0.5	0.1	+	ND	ND	[11]
	A-549/CPT	Human lung cancer	2	ND	Unchanged	ND	ND	ND	ND	[14]
	HT-29/CPT	Human colon cancer	7	ND	0.125–0.25	ND	–	ND	ND	
	St-4/CPT	Human gastric cancer	9	ND	0.125	ND	ND	ND	ND	
	DC3F/C-10	Chinese hamster fibroblast	130	Strongly decreased	0.125‡	Strongly decreased	+	Increased	Base codon mutation	[15]
In vivo adaptation	P388/CPT§	Murine leukemia	45	ND	0.33	ND	ND	ND	ND	[14]
	P388/CPT+	Murine leukemia	5	ND	0.25	ND	–	0.3	Rearrangement on one allele	[12]
In vitro adaptation	PC7/CPT§	Human lung carcinoma	10	ND	0.25	ND	+	ND	ND	[13]
	P388CPT0.3									
	9–27th passages	Murine leukemia	20	ND	0.1–0.25	0.02	ND	0.7	Rearrangement on one allele	This study
	30–42nd passages	Murine leukemia	20	Strongly decreased	Unchanged	0.062	+	0.5	Rearrangement on one allele	

* Expressed as a function of WT cell level, defined as 1.
† Topo I resistant to inhibition of catalytic activity and induction of DNA cleavage by CPT.
‡ Immunoreactive Topo I protein is increased 2-fold.
§ The agent of selection is CPT-11.
ND, not determined.

hand, at the 30th passage, the 16-fold reduced cleavage indicated a variation of the Topo I sensitivity for CPT, since the total Topo I relaxation activities at this passage were equal in both P388wt and P388CPT0.3 cells. Furthermore, at this passage, CPT was found to inhibit with 3-fold less efficiency the catalytic activity of the Topo I from resistant cells. In terms of concentration, 25–50-fold less CPT was needed to inhibit the Topo I from sensitive cells as compared to the resistant enzyme. Therefore, our results indicated that the P388CPT0.3 cells possessed a CPT-resistant form of Topo I which is detected during the second step of the establishment of the resistance.

All the CPT-resistant cell lines presenting a resistant Topo I, also displayed a reduced Topo I activity (Table 2). Furthermore, in the DC3F/C-10 cell line, the purified Topo I presented a lower specific activity, but the immunoreactive Topo I was found increased by 2-fold [15]. The resistance to CPT of the DC3F/C-10 cells was due to a mutation in the Topo I gene, that might be responsible for the lower specific activity of the enzyme [26]. The P388CPT0.3 cell line at 30–36 passages differs from these cell lines as it has a CPT-resistant Topo I but no quantitative decrease in the Topo I activity, suggesting that the Topo I may have unique biochemical properties. A cellular clone from the P388CPT0.3 cell line, at the 42nd passage and named P388CPT5, was isolated in soft agar which displayed the following characteristic for Topo I : decreased mRNA level, allele rearrangement, normal Topo I activity and mutated Topo I (J.F. Riou, manuscript in preparation). However, the resistance index to CPT in P388CPT5 increased to 80-fold, suggesting that the cell population is heterogeneous at the 42nd passage.

Variations in the Topo I activity may also result from a transcriptional process. By northern blot analysis, we have found a reduced Topo I transcript level in the P388CPT0.3 cell line as well as in less resistant sublines. These results indicated that the decreased level of the Topo I transcript is an early and constant event associated with the resistance phenotype. During the first steps of the resistance establishment, the reduced Topo I activity may be explained by the decreased level of Topo I transcript. However, at the 30–36th passages, the Topo I activity was found to be equal. In CPTK5, DC3F/C-10 and P388/CPT+ cell lines, the Topo I transcript levels were found unchanged, increased or decreased, respectively, while the Topo I activity was decreased in all these cell lines (Table 2), indicating that the Topo I activity is not always related to the Topo I transcript level. A possible explanation for these discrepancies could result from the methodology used for RNA analysis. Northern blot experiments allow measurement of the steady state level of mRNA but not the rate of transcription of the gene nor the mRNA stability which could be different in these cell lines.

On the other hand, different post-transcriptional mechanisms could also explain the variation of the Topo I activity in the P388CPT0.3. Phosphorylation and polyADPribosylation of the Topo I are known to modulate the enzyme activity [27, 28]. In etoposide-resistant mutants of KB cells, Takano *et al.* [29] have described an increased phosphorylation on serine of the Topo II, which explains a decreased Topo II content but a similar level of the enzyme activity. The existence of phosphorylation defects for Topo I has not been investigated in the P388CPT0.3 cells, nor in the other cell lines resistant to CPT.

A decrease of the Topo I transcript level has also been reported in the P388/CPT+ resistant cells and was thought to result from one allele rearrangement and a hypermethylation of the Topo I gene [12]. An investigation of the genetic alterations in the P388CPT0.3 cells has been undertaken and has shown that the Topo I gene locus was also rearranged on one allele (for Pvu II and Hind III restriction enzymes). By comparison with the results of Eng *et al.* [12], it resulted that the rearrangements between these two cell lines were different by their restriction enzymes. The Topo I gene rearrangement was found in sublines with lower resistance indexes, indicating that it results from an early process. A possible consequence of the Topo I gene rearrangement in the P388CPT0.3 cell line could be the reduced gene transcript level. It would be of interest to determine the exact nature of the genetic alteration in the resistant cells, i.e. whether the coding or the flanking regions of the gene are affected by the rearrangement, and whether the formation of the CPT-resistant Topo I is due to the rearrangement.

Recently, two CPT derivatives, CPT11 and Topotecan, have undergone clinical trials [30–34]. In addition, several compounds such as Actinomycin D, Saintopin, morpholinyl-doxorubicin and indoloquinolinediones derivatives, have been reported to inhibit Topo I activity [16, 35–37]. The examination of the properties of these agents, and all the new Topo I inhibitors, in the CPT-resistant cell line is of interest to discover and design new chemical entities able to overcome CPT resistance.

Acknowledgements—We wish to thank to Dr Yves Pommier for critical review of the manuscript. We also thank Mrs Lucile Grondard for technical assistance and Mrs Lisette Ramdani for secretarial assistance.

REFERENCES

1. Wang JC, DNA topoisomerases. *Annu Rev Biochem* **54**: 665–697, 1985.
2. Wang JC, Recent studies of DNA topoisomerases. *Biochim Biophys Acta* **909**: 1–9, 1989.
3. D'Arpa P and Liu LF, Topoisomerase targeting antitumor drugs. *Biochim Biophys Acta* **989**: 163–177, 1989.
4. Hsiang YH, Hertzberg R, Hecht S and Liu LF, Camptothecin induces protein-linked DNA breaks mediated via mammalian DNA topoisomerase I. *J Biol Chem* **260**: 14873–14878, 1985.
5. Wall ME, Wani MC, Cook CE, Palmar KH, MacPhail AT and Sim GA, Plant antitumor agents. 1. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from Camptotheca acuminata. *J Am Chem Soc* **88**: 3888–3890, 1966.
6. Bosmann HB, Camptothecin inhibits macromolecular synthesis in mammalian cells but not in isolated mitochondria or *E. coli*. *Biochem Biophys Res Commun* **41**: 1412–1420, 1970.

7. Kessel D, Effects of camptothecin on RNA synthesis in leukemia L1210 cells. *Biochim Biophys Acta* **246**: 225–232, 1971.
8. Horwitz SB, Chang CK and Grollman AP, Studies on camptothecin. I. Effects on nucleic acid and protein synthesis. *Mol Pharmacol* **7**: 632–644, 1971.
9. Mattern MR, Mong SM, Bartus HF, Mirabelli CK, Crooke ST and Johnson RK, Relationship between the intracellular effects of camptothecin and the inhibition of DNA topoisomerase I in cultured L1210 cells. *Cancer Res* **47**: 1793–1798, 1987.
10. Andoh T, Ishii K, Suzuki Y, Ikegami Y, Kusunoki Y, Takemoto T and Okada K, Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. *Proc Natl Acad Sci USA* **84**: 5565–5569, 1987.
11. Gupta RS, Gupta R, Eng B, Lock RB, Ross WE, Hertzberg RP, Caranfa MJ and Johnson RK, Camptothecin-resistant mutants of chinese hamster ovary cells containing a resistant form of topoisomerase I. *Cancer Res* **48**: 6404–6410, 1988.
12. Eng WK, McCabe FL, Tan KB, Mattern MR, Hofman GA, Woessner RD, Hertzberg RP and Johnson RK, Development of a stable camptothecin-resistant subline of P388 leukemia with reduced topoisomerase I content. *Mol Pharmacol* **38**: 471–480, 1990.
13. Kanzawa F, Sugimoto Y, Minato K, Kasahara K, Bungo M, Nakagawa K, Fujiwara Y, Liu LF and Saijo N, Establishment of a camptothecin analogue (CPT-11)-resistant cell line of human non-small cell lung cancer: characterization and mechanism of resistance. *Cancer Res* **50**: 5919–5924, 1990.
14. Sugimoto Y, Tsukahara S, Oh-hara T, Isoe T and Tsuruo T, Decreased expression of DNA topoisomerase I in camptothecin-resistant tumor cell lines as determined by a monoclonal antibody. *Cancer Res* **50**: 6925–6930, 1990.
15. Tanizawa A and Pommier Y, Topoisomerase I alteration in a camptothecin-resistant cell line derived from Chinese hamster DC3F cells in culture. *Cancer Res* **52**: 1848–1845, 1992.
16. Riou JF, Helissey P, Grondard L and Giorgi-Renault S, Inhibition of eukaryotic DNA topoisomerase I and II activities by indoloquinolinedione derivatives. *Mol Pharmacol* **40**: 699–706, 1991.
17. Lefevre D, Riou JF, Ahomadegbe JC, Zhou D, Bernard J and Riou G, 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.
18. Kohn KW, Elution methods in anticancer drug development. In: *Advances in Cancer Chemotherapy*. (Ed. Muggia F), pp. 3–38. Martinus Nijhoff, Boston, 1987.
19. Maniatis T, Fritsh EF and Sambrook J, *Molecular Cloning*. Cold Spring Harbor Laboratory Press, New York, 1982.
20. Bjornsti MA, Benedetti P, Viglianti GA and Wang JC, Expression of human DNA topoisomerase I in yeast cells lacking yeast DNA topoisomerase I: restoration of sensitivity of the cells to the antitumor drug camptothecin. *Cancer Res* **49**: 6318–6323, 1989.
21. Alonso S, Minty A, Bourlet Y and Buckingham M, Comparison of three actino-coding sequences in the mouse; evolutionary relationships between the actin genes of warm-blooded vertebrates. *J Mol Evol* **23**: 11–12, 1986.
22. Covey JM, Jaxel C, Kohn KW and Pommier Y, Protein-linked DNA strand breaks induced in mammalian cells by camptothecin, an inhibitor of topoisomerase I. *Cancer Res* **49**: 5016–5022, 1989.
23. Tsuruo T, Matsuzaki T, Matsushita M, Saito H and Yokokura T, Antitumor effect of CPT-11, a new derivative of camptothecin, against pleiotropic drug-resistant tumors *in vitro* and *in vivo*. *Cancer Chemother Pharmacol* **21**: 71–74, 1988.
24. Chen AY, Yu C, Potmesil M, Wall ME, Wani MC and Liu LF, Camptothecin overcomes MDR1-mediated resistance in human KB carcinoma cells. *Cancer Res* **51**: 6039–6044, 1991.
25. Tamura H, Kohchi C, Yamada R, Ikeda T, Koiwai O, Patterson E, Keene JD, Okada K, Kjeldsen E, Nishikawa K and Andoh T, Molecular cloning of a cDNA of a camptothecin-resistant human topoisomerase I and identification of mutation sites. *Nucleic Acids Res* **19**: 69–75, 1991.
26. Tanizawa A, Tabuchi A, Bertrand R and Pommier Y, Mutation in DNA topoisomerase cDNA from a camptothecin resistant chinese hamster cell line. *Proc Am Assoc Cancer Res* **33**: 2691, 1992.
27. Jongstra-Bilen J, Ittel ME, Niedergang C, Vosberg HP and Mandel P, DNA topoisomerase I from calf thymus is inhibited *in vitro* by poly(ADP-ribosylation). *Eur J Biochem* **136**: 391–396, 1983.
28. Durban E, Mills JS, Roll D and Busch H, Phosphorylation of purified Novikoff hepatoma Topoisomerase I. *Biochem Biophys Res Commun* **111**: 897–905, 1983.
29. Takano H, Kohno K, Ono M, Uchida Y and Kuwano M, Increased phosphorylation of DNA topoisomerase II in etoposide-resistant mutants of human cancer KB cells. *Cancer Res* **51**: 3951–3957, 1991.
30. Kunimoto T, Nitta K, Tanaka T, Uehara N, Baba H, Takeuchi M, Yokokura T, Sawada S, Miyasaka T and Mutai M, 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.
31. Bissery MC, Mathieu-Boué A and Lavelle F, Preclinical evaluation of CPT-11, a camptothecin derivative. *Proc Am Assoc Cancer Res* **32**: 402, 1991.
32. Fukoaka M, Niitani H, Suzuki A, Motomiya M, Hasegawa K, Nishiwaki Y, Kuriyama T, Ariyoshi Y, Negoro S, Masuda N, Nakajima S and Taguchi T, A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer. *J Clin Oncol* **10**: 16–20, 1992.
33. Kingsbury WD, Boehm JC, Jakas DR, Holden KG, Hecht SM, Gallagher G, Caranfa MJ, McCabe FL, Johnson RK and Hertzberg RP, Synthesis of water-soluble aminoalkylcamptothecin analogues: inhibition of topoisomerase I and antitumor activity. *J Med Chem* **34**: 98–107, 1991.
34. Recondo G, Abbruzzese J, Newman B, Kuhn J, Von Hoff D, Garteiz D and Raber M, A phase I trial of topotecan administered by a 24-hour infusion. *Proc Am Assoc Cancer Res* **32**: 206, 1991.
35. Trask DK and Muller MT, Stabilization of type I topoisomerase-DNA covalent complexes by actinomycin D. *Proc Natl Acad Sci USA* **85**: 1417–1421, 1988.
36. Yamashita Y, Kawada SZ, Fujii N and Nakano H, Induction of mammalian DNA topoisomerase I and II mediated DNA cleavage by Saintopin, a new antitumor agent from fungus. *Biochemistry* **30**: 5838–5845, 1991.
37. Wassermann K, Markovits J, Jaxel C, Capranico J, Kohn K and Pommier Y, Effects of Morpholinyl Doxorubicin, and Actinomycin D on mammalian DNA Topoisomerases I and II. *Mol Pharmacol* **38**: 38–45, 1990.