Pergamon

Biochemical Pharmacology, Vol. 48, No. 6, pp. 1145–1154, 1994.
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0006-2952/94 $7.00 + 0.00

0006-2952(94)00275-4

ANTIPROLIFERATIVE ACTIVITY OF THE TOPOISOMERASE I INHIBITORS TOPOTECAN AND CAMPTOTHECIN, ON SUB- AND POSTCONFLUENT TUMOR CELL CULTURES

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(*Received* 17 *December* 1993; *accepted* 3 *June* 1994)

Abstract—We have assessed the antiproliferative effects of a 24-hr exposure to the topoisomerase I inhibitors, topotecan and camptothecin, on two colon and one ovarian human tumor cell lines, cultured as subconfluent and as multilayered postconfluent cultures. Chemosensitivity was measured by the sulforhodamine B assay. In general, postconfluent cultures were less sensitive to these agents, yielding GI_{50}s (drug concentrations inhibiting growth by 50%) from 1.2 to more than 6000 times higher than those of subconfluent cultures. Both compounds displayed similar effects on subconfluent cells, inducing complete growth inhibition at concentrations ranging from 0.03 to 0.5 μM. Topotecan, however, was more potent than camptothecin in two out of the three cell lines tested as multilayered postconfluent cultures. Topoisomerase I mRNA expression on postconfluent cultures was 50% lower than on subconfluent cultures in the three cell lines studied. However, we did not detect any reproducible differences in topoisomerase I protein expression and in relaxation activity of supercoiled DNA between the two types of cultures. From accumulation experiments it appeared that the peak concentration of the lactone form of topotecan as well as the area under the concentration–time curve (AUC) were 2-fold higher in the monolayer than in the multilayer cultures. Therefore, the differences in the activity of topoisomerase I inhibitors under our experimental conditions were likely due to a decreased rate of proliferation of postconfluent cells, associated with a reduction in drug uptake.

Key words: topoisomerase I; camptothecin; topotecan; cytotoxicity; postconfluent cultures; gene expression

Topoisomerase I inhibitors are potent anticancer agents undergoing extensive clinical investigation [1]. They are derivatives of CPT‡, originating from an extract of *Camptotheca acuminata* and were discovered in the 1960's. Despite its remarkable preclinical potency, CPT underwent limited clinical testing because of its unpredictable non-hematological toxicity, including haemorrhagic cystitis. The clinical studies were performed with the sodium salt of CPT, due to its poor water solubility. Only recently was it discovered that the integrity of the E lactone ring, which is pH dependent, is essential for topoisomerase I inhibitors to be active (Fig. 1), and therefore that the initial studies conducted with the sodium salt of CPT were likely performed with a less than optimal drug formulation. Once the mechanism of action of CPT was discovered, derivatives which were more water soluble than the parent compound and devoid of the unpredictable non-hematological side effects were synthesized [1]. TT (9-dimethyl aminomethyl-10-hydroxycamptothecin; NSC 609669) is a semisynthetic, water-soluble CPT analog. It showed significant activity both *in vitro* and *in vivo* against murine and human tumor xenografts (e.g. colon and lung cancer) [2–4]. During Phase I studies, TT displayed neutropenia as limiting toxicity and the non-hematologic side effects observed with CPT did not occur with TT. Moreover, a number of objective responses were recorded in several tumor types [5–8].

Topoisomerase I is a nuclear enzyme that catalyses topological changes in DNA by transiently breaking one of its strands. The proposed reaction mechanism for activity of topoisomerase I inhibitors involves the formation of a reversible covalent complex between enzyme and DNA, known as the cleavable complex [9, 10]. Topoisomerase I inhibitors stabilize this cleavable complex by interfering with the rejoining reaction catalysed by the enzyme. This eventually leads to cell death in a process which appears to involve the block of replication forks in proliferating cells [11].

We are assessing the use of multilayered postconfluent tumor cell cultures as an alternative

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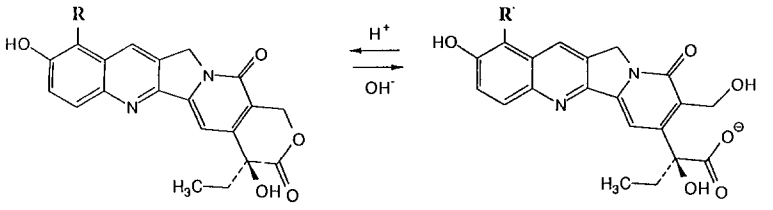
‡ Abbreviations: CPT, camptothecin; TT, topotecan; FCS, fetal calf serum; AUC, area under the concentration–time curve; PBS, phosphate buffered solution; DMEM, Dulbecco's Modified Eagle's Medium; SRB, Sulforhodamine B; PMS, phenylmethylsulfonyl fluoride; MDR, multidrug resistance.



A B

Fig. 1. Chemical structures and equilibrium of camptothecin (R = H) and TT [R = CH_2-N(CH_3)$_2$]. A = parent drug with intact lactone moiety; B = the lactone ring opened hydroxy acid.

model to screen for new anticancer drugs. This model might be more appropriate than the systems using cell monolayers, as it may more closely mimic the microenvironment of solid tumors. As topoisomerase I inhibitors have shown remarkable *in vitro* and *in vivo* activity against solid tumors, we wanted to investigate the activity of TT and CPT against traditionally cultured cell lines as well as those growing in a postconfluent state. We have previously demonstrated the feasibility of chemosensitivity tests on postconfluent cultures using the SRB assay [12]. In the present study, we have evaluated the antiproliferative profile of CPT and TT on colon and ovarian tumor cell lines growing as subconfluent and multilayered postconfluent cultures.

MATERIALS AND METHODS

Chemicals. DMEM was purchased from Flow Laboratories (Irvine, U.K.); FCS was from Gibco (New York, U.S.A.). TT as the hydrochloride salt, was kindly provided by the EORTC New Drug Development Office (Free University Hospital, Amsterdam, The Netherlands) and kept at −20°. CPT was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) in its lactone form. It was dissolved in dimethyl sulfoxide and stored at −20°. Test concentrations of drugs were prepared in culture medium immediately before addition to the plates. All other chemicals were of standard analytical grade.

Cell culture and chemosensitivity tests. Detailed description of maintenance culture and plating procedures is given elsewhere [12, 13]. Briefly, the human colon adenocarcinoma cell lines, HT29 and SW620, and the human ovarian carcinoma cells, A2780, were cultured in DMEM supplemented with 5% FCS and 1 mM L-glutamine, and incubated in a humidified 5% CO_2–air atmosphere at 37°. Exponentially growing cells were trypsinized from culture flasks and single cell suspensions were seeded at 15,000 cells/50 μL/well in "V"-bottomed, 96-well microtiter plates (Greiner Labortechnik, Solingen, Germany).

On day 1 (D1; subconfluent cells) or on day 5 (D5; multilayered postconfluent cells) after plating, triplicate wells received 150 μL of medium with or without drugs. On day 5 all the cell lines used were shown to be in an advanced plateau phase of growth [12]. Following 24 hr drug exposure, wells were washed once with culture medium and re-fed. Plates were incubated for 4 additional days with medium renewal every 24 hr and then cellular growth and cytotoxicity were assessed by the SRB assay as described elsewhere [12]. Wells without cells, but containing medium or drug-containing medium, were included in every experiment for background O.D. determination. As part of the assay, additional plates were assessed for cellular growth control just before drug exposure (time 0).

In this study, we evaluated cellular response using the assay parameters currently employed by the anticancer drug screening program of the National Cancer Institute, as reported elsewhere [14]. Thus, the measured effect was defined as percentage growth (PG). Taking into account the mean control O.D. (C), the mean test O.D. (T) and the O.D. at time 0 (T_0):

If $(T - T_0) \geq 0$, then percentage growth = $100 \times (T - T_0/(C - T_0)$;

if $(T - T_0) \leq 0$, then percentage growth = $100 \times (T - T_0)/T_0$.

The GI_{50}, TGI and LC_{50} represent the concentrations at which the percentage growth is +50, 0 and −50, respectively. For a given parameter, differences between results of D1 and D5 treatments were considered significant if $P < 0.05$ (Student's *t*-test).

Northern blotting. D1 and D5 cells were trypsinized, resuspended in ice-cold culture medium, washed twice in PBS at 4° and pelleted. Total RNA was extracted after cell lysis with guanidine isothiocyanate and centrifugation in cesium chloride gradient [15]. Poly(A) RNA was purified from total RNA using oligo(dT)-cellulose chromatography [15]. Ten micrograms of total RNA or 5 μg of purified poly(A) RNA were electrophoresed on a denaturating 1% agarose-formaldehyde gel and transferred to a Nylon membrane (GeneScreen-*Plus*™; NEN Research Products, Boston, MA, U.S.A.). Prehybridization, hybridization with [α-^{32}P]dCTP labeled (Random Primer Labelling System; Bethesda Research Laboratory, Rockville, MD, U.S.A.) cDNA probes, and washing of the membrane were performed according to the instructions supplied by the manufacturer. Expression of a given gene was normalized by the

expression of the GAPDH gene on the same northern blot. A 0.7-Kb human topoisomerase I cDNA fragment [16] and a 1.8-Kb human p170 topoisomerase II-α cDNA fragment (ZII-1.8) [17] were kindly provided by Dr L.F. Liu (Baltimore, MD, U.S.A.). A *c-myc* cDNA fragment was donated by Dr B. Johnson [18] and a 1.3 fragment of rat GAPDH cDNA [19] was provided by Dr C. Thiele (Bethesda, MD, U.S.A.).

RNase protection assay. RNase protection assay for MDR1 was performed as described earlier [20]. Briefly, 10 μg of total RNA were hybridized with a [α-^{32}P]CTP-labeled anti-sense RNA probe. A specific RNA probe for MDR1 was obtained by transcription of a 301 nucleotide cDNA fragment (positions 3500–3801) with SP6 RNA polymerase. MRP cDNA, obtained as described previously [21], was kindly provided by G. Zaman. MRP mRNA sequences were transcribed from Eco-RI site of pGEM3-Zf(-) (Promega), using SP6 RNA polymerase. A probe for γ-actin was included as an internal control for determination of RNA loading. The hybridized probe was visualized by autoradiography after electrophoresis through a denaturing 6% acrylamide gel followed by autoradiography.

Topoisomerase I catalytic activity. Nuclei from D1 and D5 cultures (2×10^7 cells/sample) harvested simultaneously were isolated as described elsewhere [22]. The nuclei pellets corresponding to D1 and D5 samples were then resuspended in a nucleus buffer containing 150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol and 1 mM PMSF, at pH 6.4; an equal volume of the nucleus buffer with 0.35 M NaCl (final concentration) was added. Nuclear protein extraction was for 30 min on ice before centrifugation (14,000 *g*/20 min at 4°). The enzyme solution was diluted with an equal volume of 87% glycerol and immediately assayed for topoisomerase I activity. The method of Bradford [23] was used to determine protein concentrations.

Topoisomerase I activity on nuclear extracts of D1 and D5 cells was evaluated by relaxation of supercoiled DNA as described previously [22]. The reaction mixture contained 50 mM Tris–HCl (pH 7.5), 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA and BSA (0.03 mg/mL). Relaxation was for 30 min at 37°, by incubation of 5 μL of serial dilutions of the nuclear extract with 0.9 μg supercoiled pBR329 DNA in a final volume of 25 μL reaction mixture. The reaction was stopped by the addition of 5 μL of 3% SDS, 0.03% bromophenol blue and 30% glycerol. Samples were then electrophoresed in a 1% agarose gel at 20 V for 16 hr. The gel was stained with ethidium bromide (1 μg/mL) and photographed. Negatives were used for scanning densitometry.

Western blotting. SDS–PAGE was performed according to Laemmli [24] with a 7.5% gel, utilizing 10 mg of nuclear extracts of D1 and D5 cells. Proteins were then stained with 0.1% Coomassie brilliant blue R250 in 10% methanol and 10% acetic acid. Proteins from a non-stained acrylamide gel were transferred onto nitrocellulose paper at 125 mA for 2 hr at 4°, using a semidry-blot system (Novablot, Pharmacia; Brussels, Belgium). Topoisomerase I expression was detected in western blots by overnight incubation at room temperature with serum from a scleroderma patient [25], kindly provided by Dr E. de Vries (Groningen, The Netherlands), followed by incubation with rabbit anti-human IgG-horseradish peroxidase (Dakopatts, Denmark) and staining with 3,3′-diaminobenzidine and hydrogen peroxide.

TT accumulation studies. For measurement of TT accumulation, cells were cultured as mono- or multilayers in 24-well (flat bottomed plates) or 96-well (V-shaped) microtiter plates, respectively. Cells were plated at such a density that at the time of exposure, cell numbers per well were comparable for monolayers and multilayers ($0.12–0.24 \times 10^6$ cells/well). On D1 or D5, medium was replaced by 150 μL of 1 or 10 μM TT in medium. At the time points 2, 10, 20, 30, 60 min, 2 hr and 3 hr, the plates were placed on ice and the medium was removed and mixed with 600 μL cold methanol (−20°). After vortexing, mixing and centrifugation, the clear supernatant was stored at −20° until analysis. The cells were subsequently washed twice with cold PBS and after addition of 100 μL cold methanol, were harvested by mechanical scraping to detach the cells from the bottom. The cell extracts of two or three wells were pooled and treated as described for the medium samples.

The samples were analysed according to the method described by Beijnen *et al.* [26]. The parent TT and its lactone ring opened form were separated on a Lichrosorb RP-18 analytical column (125×4 mm i.d., particle size: 5 μm, Merck, Darmstadt, Germany). The analytical column was protected by a pre-column (20×4 mm i.d.), dry-packed with 325 mL methanol, 215 mL water, 20 mL sodium dioctylsulfosuccinate, 11.5 mL phosphate buffer pH 6, and 1.5 mL triethylamine; the apparent pH of this mixture was adjusted to pH 6 with phosphoric acid. The flow rate was maintained at 1.0 mL/min and the column effluent monitored spectrofluorometrically using a Perkin–Elmer LS 40 fluorescence detector (Perkin–Elmer, Beaconsfield, U.K.) with excitation wavelength set at 381 nm and emission wavelength at 527 nm.

Care was taken to prevent hydrolysis of the closed form of TT during the analysis, since TT is not stable in aqueous solutions at physiological pH. The drug is stable in acid aqueous solutions (pH lower than 4). At physiological pH, at room temperature, 50% of the lactone ring is hydrolysed into the inactive open ring form within 50 min [26]. TT is stable, however, in cold methanol (MeOH); only 10% is hydrolysed at 4° in 1 hr and samples can be stored for weeks at −30° without conversion[26].

RESULTS

Chemosensitivity tests

The effects of a 24-hr treatment with TT and CPT on D1 and D5 cultures (subconfluent and postconfluent cells, respectively) are illustrated in Fig. 2. This type of graph permits the discrimination between the regions of the dose–response curves that represent growth inhibitory (PG varying from 0 to +100) and cytotoxic effects (PG varying from 0 to −100). It also makes possible the extrapolation of the chemosensitivity parameters studied (Table

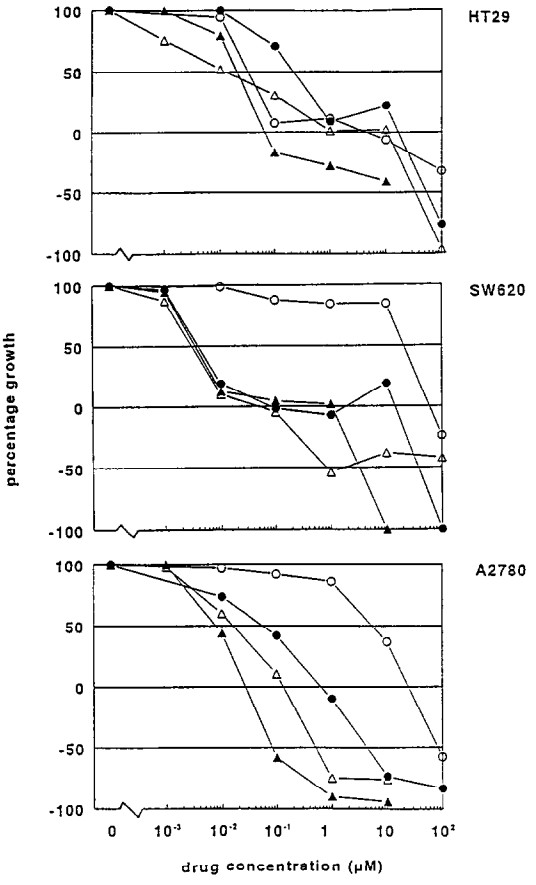


Fig. 2. TT (closed symbols) and CPT (open symbols) dose–response curves determined by the SRB assay in HT29, SW620 and A2780 microcultures 4 days after 24 hr drug exposure. Cells were treated on day 1 (△, ▲) or on day 5 (○, ●) following plating, when they were structured as mono- or multilayered cultures, respectively. The measured effect is defined as percentage growth (PG), as described in Materials and Methods. The extrapolated chemosensitivity parameters are: GI_{50} (PG = + 50), TGI (PG = 0) and LC_{50} (PG = − 50). Means of triplicate wells per drug concentration of a representative experiment are shown. Means of standard deviation (SD) varied between 0 and 40%. SD bars were omitted for the sake of clarity.

Table 1. Chemosensitivity parameters of CPT and TT

	HT29		SW620		A2780	
Parameter	CPT	TT	CPT	TT	CPT	TT
D1-GI_{50}*	0.01	0.02	0.003	0.0025	0.015	0.008
D5-GI_{50}	0.05	0.3	20	0.003	4	0.08
(D5:D1)	(5)	(15)	(6666)	(1.2)	(266.6)	(10)
D1-TGI†	0.5	0.075	0.05	0.1	0.15	0.03
D5-TGI	0.5	15	60	0.1	15	0.5
(D5:D1)	(1)	(200)	(1200)	(1)	(100)	(16)
D1-LC_{50}‡	25	10	0.8	3	0.4	0.1
D5-LC_{50}	>100	65	>100	40	80	4
(D5:D1)	(>4)	(6.5)	(>125)	(13.3)	(200)	(40)

Cells were treated for 24 hr on D1 or D5 after seeding, when they were organized as subconfluent or multilayered postconfluent cultures, respectively. Values are means of three experiments expressed in μM. SD was ≤30% of the mean.

* Drug concentration inhibiting growth by 50%.

† Drug concentration inhibiting growth by 100%.

‡ Drug concentration causing a 50% reduction in the number or mass of cells present in the control wells just before the beginning of drug exposure.

1). Against D1 cultures, TT and CPT in general produced steep dose–response curves and induced complete growth inhibition at concentrations ranging from 0.03 to 0.5 μM. All the chemosensitivity parameters derived from TT exposure on D1 did not differ significantly from those observed after CPT treatment.

The magnitude of the growth inhibitory effects of CPT on D5 HT29 cells was not significantly different from that seen on D1, but, CPT cytotoxic effects were less pronounced on D5 than on D1 cultures. In fact, a D5-LC_{50} could not be determined for this cell line for it was higher than the maximum CPT concentration tested. In the other two cell lines, SW620 and A2780, CPT growth inhibitory and cytotoxic effects on D5 were much smaller than on D1. In these cell lines, the ratios between D5 and D1 chemosensitivity parameters of CPT varied between 100 and 6666, while for TT they varied between 1 and 40 (Table 1).

The effects of TT on D5 HT29 and A2780 cells produced chemosensitivity indexes which were higher than on D1, indicating that this agent was significantly less active overall against postconfluent cultures than subconfluent cells. On SW620 cells, TT treatment on D5 resulted in the same degree of growth inhibition as with D1. Nevertheless, in order to achieve the same degree of cytotoxicity as seen on D1 cells, an approximately 10-fold higher TT concentration had to be used against SW620 cultures on D5. TT results with D5 cultures of the colon tumor lines, but not of A2780 cells, showed a plateau in cellular response effects at levels corresponding to total growth inhibition (Fig. 2). In these cases, cytocidal effects could, however, still be observed in the colon lines when TT was used at the highest concentrations tested (100 μM).

Gene expression

Figure 3 displays topoisomerase I and II, *c-myc* and GAPDH gene expression on D1 and D5 as determined by northern blotting. The expression of the topoisomerase I gene in HT29 cells was virtually identical to that in SW620 and A2780 cells on D1 (Table 2). Compared to D1 levels, topoisomerase I gene expression on D5 was decreased by 50% in all three cell lines tested.

On D1, the expression of the topoisomerase II gene in HT29 and A2780 cells was 40% higher than in SW620 cells. As for topoisomerase I, topoisomerase II gene expression on D5 was reduced by 25 to 40% compared to D1.

The expression of the *c-myc* oncogene in D1 A2780 cells was higher than in D1 SW620 and HT29

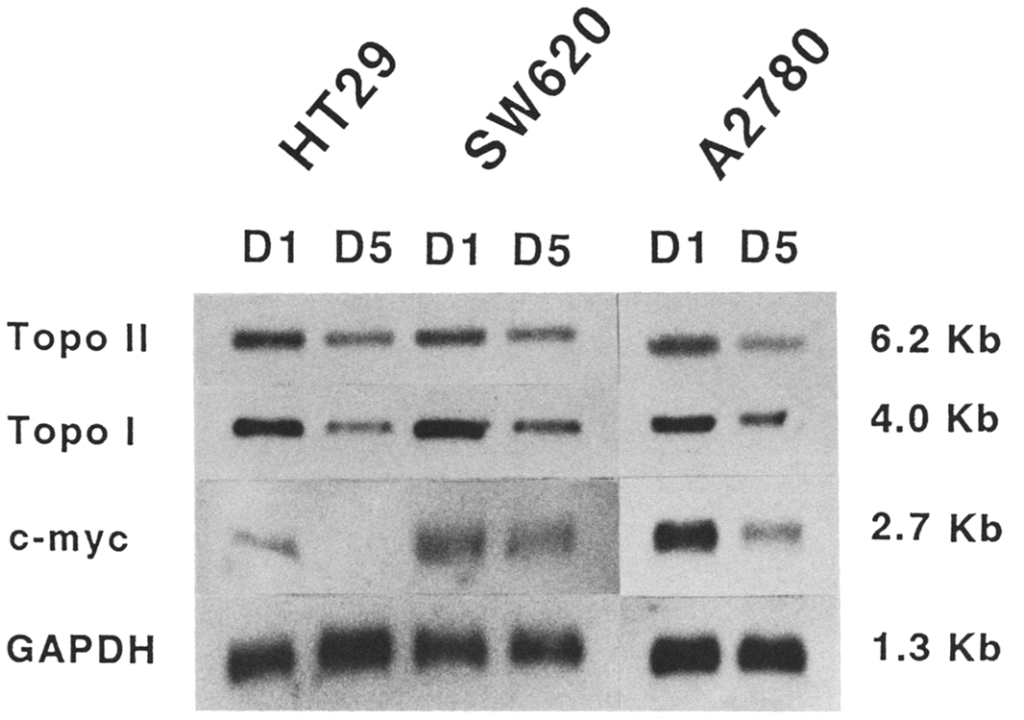


Fig. 3. Representative northern blot autoradiograms showing the expression of topoisomerase (Topo) I and II, *c-myc* and GAPDH genes of D1 and D5 cells cultured in "V"-bottomed wells. Total RNA was electrophoresed, transferred to a Nylon membrane and hybridized with a GAPDH probe as described in Materials and Methods. The same membrane was "stripped" and rehybridized with *c-myc* and topoisomerase I and II probes.

Table 2. Relative gene expression of topoisomerases I and II and *c-myc*

	D1/D5 relative gene expression		
Gene	HT29	SW620	A2780
Topoisomerase I	1.0/0.5	0.9/0.45	0.9/0.45
Topoisomerase II	1.0/0.75	0.6/0.45	1.0/0.6
c-myc	1.0/ND	2.3/1.9	2.8/1.2

Gene expression on D1 and D5 cells was assessed on total RNA by densitometry of northern blot autoradiograms (see also Fig. 3), as described in Materials and Methods. For each gene, expression is quantitated in arbitrary units relative to HT29 levels, which were considered as 1. ND = not detectable.

cells. In all three cell lines *c-myc* expression was reduced on D5 as compared to D1 (Table 2). The difference was small in SW620 cells although more pronounced in A2780 cells; no *c-myc* gene expression was detectable in total RNA extracted from HT29 cells (Fig. 3). However, *c-myc* gene expression was detectable on 5 μg of purified poly(A) RNA extracted from D5 HT29 cells (not shown).

Expression of MDR1 and MRP genes has been evaluated by a sensitive and specific RNase protection assay. In two cell lines (HT29 and A2780) no MDR1 expression was detectable, while a barely visible transcript was present in RNA extracted from SW620 cells (not shown). MRP expression was present in similar amounts in all three cell lines at a level comparable to GLC4, a small cell lung cancer cell line known not to overexpress MRP [21] (not shown).

Topoisomerase I activity and protein amounts

We investigated whether the differences in TT sensitivity were correlated to changes in topoisomerase I catalytic activity and/or nuclear protein amounts. The activities of this enzyme in nuclear extracts of D1 and D5 cells were compared as a function of the highest dilution of nuclear extract which still produced complete relaxation of supercoiled DNA. Topoisomerase I catalytic activity was approximately the same on D1 and D5 for the 3 cell lines studied (Fig. 4).

In western blotting experiments, two forms of topoisomerase I (100 and 67 kDa), both representing active enzymes [27], were visible in all cell lines and on both D1 and D5 (Fig. 5). Densitometry of both forms was performed and the signals of the two bands were added up for each cell line. As with the results of topoisomerase I catalytic activity, no significant differences in protein expression between D1 and D5 were found in any of the cell lines tested.

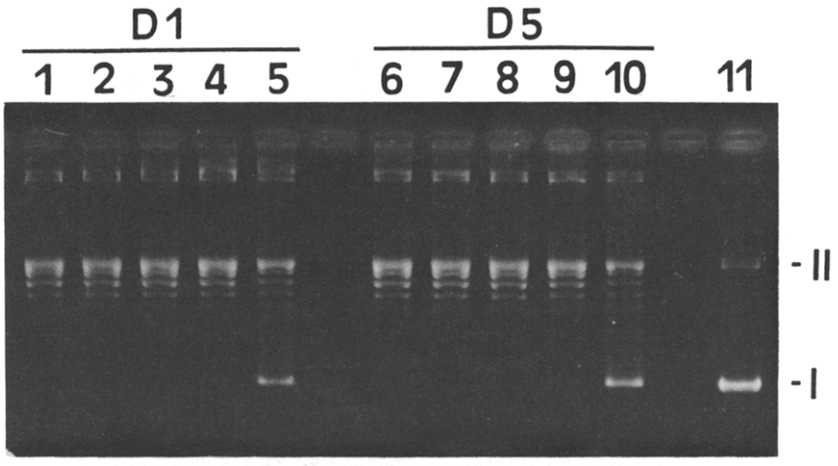


Fig. 4. Topoisomerase I activity in cellular extracts of D1 and D5 HT29 cells, assayed as described in Materials and Methods. The reaction mixture containing supercoiled pBR329 DNA was incubated in the presence of different amounts of the nuclear protein extract, namely: 100 ng (lanes 1 and 6), 50 ng (lanes 2 and 7), 25 ng (lanes 3 and 8), 12.5 ng (lanes 4 and 9), 6.2 ng (lanes 5 and 10). Lane 11 = control containing no protein extract. Supercoiled (I) and relaxed (II) forms of DNA are indicated.

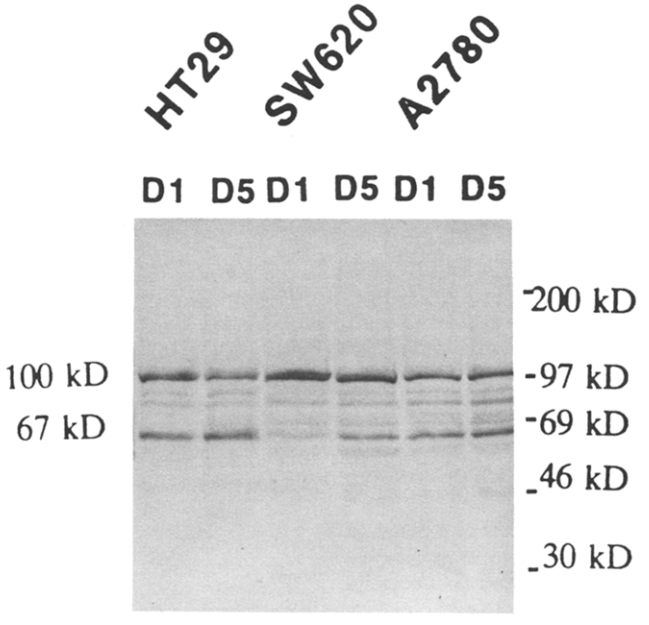


Fig. 5. Western blot analysis. Ten milligrams of nuclear proteins extracted from D1 and D5 HT29, SW620 and A2780 cells were electrophoresed in a SDS–polyacrylamide gel and immunostained with human polyclonal topoisomerase I antibodies as described in Materials and Methods. Two forms of topoisomerase I are visible (100 and 67 kDa).

TT accumulation studies

Initial accumulation experiments were performed at the low TT concentrations of 0.1 or 1 μM. The half-life for the lactone form of the drug in the medium was 49 min, similar to that in plasma [26]; after 40–50 min 50% of the drug was present in the open-ring form (Fig. 6). Exposure of the cells to a higher concentration, 10 μM, resulted in an apparent saturation, since 50% conversion was only observed after more than 2 hr.

The uptake of TT by cells was faster than the release. The peak was reached within the first 20 min and the concentration in the cells decreased to 10–30% of this peak level after 2–3 hr. The concentration of open form was around the detection limit (0.2 ng/mL = 0.5 pmol/mL) in some samples. The decrease in the concentration of the closed form

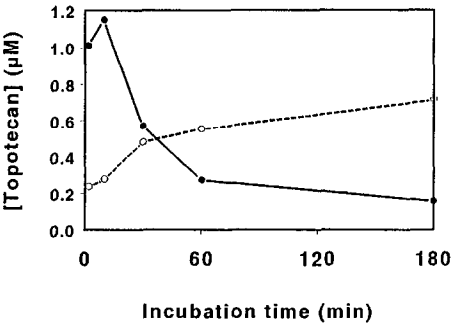


Fig. 6. TT concentration in medium of monolayered HT29 cells, treated with 1 μM TT for 3 hr. The closed lactone form (●) and the open-ring form (○) have both been measured.

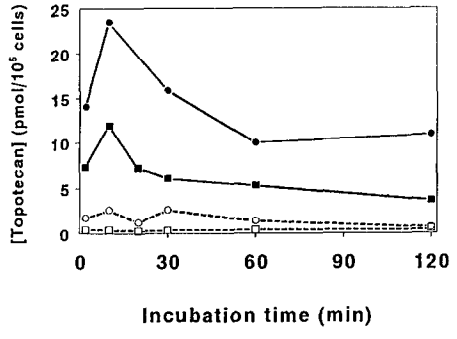


Fig. 7. TT uptake by SW620 cells treated with 10 μM TT for 2 hr. Circles: monolayers; squares: multilayers. Filled symbols: closed form of TT; open symbols: ring opened form of TT.

Table 3. TT cell accumulation results

Cell line	Peak level mono/multi	AUC mono/multi
HT29	1.9	1.3
A2780	2.0	1.5
SW620	1.7	2.5

TT uptake by mono- and multilayered cells, expressed as ratio peak level in mono-/multilayers (see also Fig. 7). mono = monolayer culture; multi = multilayer culture.

Exposure was to 1 μM TT for HT29 cells and to 10 μM for A2780 and SW620.

in the cell was not accompanied by a similar increase of the open form. Therefore, it was speculated that either the closed form is excreted before hydrolysis takes place or that the open form is excreted faster. It is also clear that the open form from the medium (present after 1 hr) does not enter the cell to the same extent as the closed form (Fig. 7).

The amount of TT found in the cell extracts was between 0.44 and 2.7% of the amount added in medium to the cells (1500 pmol). In general, the peak concentration of the closed form per million cells was 2-fold higher in the monolayers than the multilayers (Table 3). In addition to comparing the peak concentrations we also estimated the exposure of the cells by calculating the AUC until 2 hr, and the same results were found as for the peak concentrations.

The peak levels of TT in A2780 and SW620 were 10 and 5 times higher than in HT29, respectively. This is in line with the chemosensitivity data for TT, as shown in Table 1, where HT29 is the least sensitive cell line, with respect to GI_{50} and LC_{50}.

DISCUSSION

We have demonstrated that the antiproliferative effects of TT and CPT varied according to the point in time at which the cultures were treated. Overall, cells which were allowed to organize as multilayered postconfluent cultures were less sensitive to these agents than subconfluent cells. Our data also indicated that the magnitude of these differences varied across cell lines depending on the compound tested and the level of effects analysed (growth inhibition or cell kill). It has recently been reported that confluent HT29 cells showed markedly reduced sensitivity to six conventional anticancer agents as compared to exponentially growing monolayers [28]. In this study, the authors observed that drug resistance in confluent cells was associated with a decreased intracellular accumulation in the case of doxorubicin, vincristine and etoposide, but not of fluorouracil, melphalan and cisplatin. Confluent HT29 cells remained less sensitive to doxorubicin and etoposide, but not to vincristine, when drug levels in culture medium were adjusted to produce intracellular drug concentrations similar to those of non-confluent cells. These authors suggested as mechanisms: confluency-dependent changes in membrane fluidity and intrinsic alterations of DNA sensitivity.

Part of the relative resistance of TT in the multilayers could be the result of the lower intracellular concentration of active drug in the postconfluent cells. A 2-fold intracellular concentration difference was found between monolayers and multilayers. This difference is probably too small to be totally responsible for the large difference in cytotoxicity with this drug; on the other hand, given the rapidity of interconversion of the lactone form into the open-ring form and the rapid reversibility of the DNA single strand breaks induced by topoisomerase I inhibitors, it cannot be excluded that this limited accumulation difference may be largely responsible for the different effect observed. The importance of TT drug uptake is evident when the level of TT in the cells within the first hour is compared with the chemosensitivity of the three cell lines: HT29 was in fact the least sensitive cell line and had lower TT uptake than the other two cell lines, while the A2780 cell line had the highest TT peak uptake and was in general the most sensitive (lowest LC_{50}).

Because TT has been shown to be a substrate for P-glycoprotein [29], differences in accumulation of this drug might have been due to extrusion by active efflux. We therefore investigated the expression of MDR1 gene in all three cell lines. Only SW620 cells had a barely visible MDR1 transcript, while no expression was present in the other two cell lines. This makes it unlikely that MDR1 expression could have played a major role in determining differences of TT drug accumulation and cytotoxicity between cell lines. A recent study suggests that MDR in non-P-glycoprotein expressing cells may possibly be due to overexpression of MDR-associated protein (MRP), along with P-glycoprotein another gene product member of the ATP binding cassette transporter proteins [30]. Although it has not been reported that TT might be a substrate of MRP, we also investigated the expression of MRP; however, no differences in MRP gene expression between the cell lines could be found which might explain the different results of TT accumulation or cytotoxicity.

Both TT and CPT displayed similar effects on subconfluent cells; however, TT clearly performed better than CPT against two out of the three cell lines tested as multilayered postconfluent cultures. A similar trend has been reported when TT and CPT were compared in preclinical tumor models *in vivo* [2, 4]. TT has better water solubility and reduced protein binding as compared to CPT [31], which may have contributed to its greater potency in our assay. It was striking that CPT did not have substantially different activity in HT29 cells growing in mono or multilayers, while a very large difference was observed in the other two cell lines tested. As for TT there was no large difference in any of the three cell lines tested. Interestingly, while CPT was unable to cross the blood–brain barrier, TT appears to be present in cerebrospinal fluid (CSF) at 30% of the concentration obtained in plasma in non-human primates [32]. This could be partially due to the lesser protein binding and higher water solubility of TT in comparison with CPT.

Dimanche-Boitrel *et al.* [28] reported that as HT29 cells became confluent, the prevalence of cells in the G_0/G_1 phase of the cell cycle increased by 30%. We have previously demonstrated that the growth curves of HT29, SW620 and A2780 cells, cultured under the same conditions as in the present study, tend to level off after 5 days in "V"-bottomed wells [12]. Using DNA flow cytometry, we have also found that the G_0/G_1 compartment on D5 cultures of these three cell lines was significantly higher than on D1 cultures. The percentage of S-phase cells in HT29 and SW620 cultures on D5 was 50 to 60% lower than on D1 (also observed in [^{3}H]thymidine incorporation assays). In postconfluent A2780 cultures, we found a decrease in the G_2/M compartment in association with a small (20%) but significant increase in the number of S-phase cells as compared to subconfluent cultures. Hsiang *et al.* [33] have shown that active DNA synthesis is a pre-requisite for CPT cytotoxicity. Therefore, a decreased rate of cellular proliferation associated with an increase in the number of quiescent cells in multilayered postconfluent cultures may well be responsible for their lower sensitivity to CPT and TT. These results are consistent with the plateaus that we observed in dose–response effects of the colon tumor lines. The fact that reduction in cell numbers below these plateaus could still be achieved at higher TT concentrations suggests that concentration-dependent differences may be operative in the mechanisms of the cytostatic/cytotoxic activity of TT. Since this mechanism of "resistance" to TT is likely to occur in slowly-growing human solid tumors, multilayered postconfluent cells might represent a more suitable model than monolayers for preliminary studies using alternative treatment schedules, drug combinations and new camptothecin analogs, which could eventually circumvent this limitation. Based on these assumptions, prolonged infusion of lower doses appears to be more active than short infusion of higher doses of TT, indicating a significant schedule dependency of TT [3].

D5 HT29 cells express features of a differentiated phenotype, forming dome-like structures in "V"-bottomed wells [12]. Our results are in agreement with other reports showing that a change to a less proliferative status or a more differentiated phenotype can lead to a lower expression of *c-myc* and topoisomerase II genes [34–36]. In contrast, changes in cell-cycle distribution and differentiation status are not usually associated with significant alterations in topoisomerase I expression [35–37]. Differences in the effects of TT can also be the result of differences in the gene expression and catalytic activity of the target enzyme [38–40]. Indeed, we found that topoisomerase I mRNA expression on D5 was lower than on D1 in the three cell lines studied. However, we did not detect any reproducible differences in topoisomerase I protein amounts nor in the enzyme-dependent relaxation of supercoiled DNA between D1 and D5 samples. Although this discrepancy might be accounted for by the relatively lower sensitivity of activity assays and western blotting in comparison to northern blotting, they might also reflect changes in the turnover rates of topoisomerase I protein and/or mRNA between D1 and D5 which might not be detectable by the methods applied. However, it has been shown that topoisomerase I protein stability in MSB1 chicken lymphoblastoid cells did not vary significantly during cell-cycle progression [35]. These results suggest that factors other than target enzyme expression might have influenced the performance of TT and CPT in our chemosensitivity assays (e.g. intracellular accumulation of TT and CPT). Our experiments, nevertheless, did not rule out the possibility that differences between the chemosensitivity patterns observed on sub- and postconfluent cells might be due to qualitative changes in topoisomerase I, resulting in altered interactions between the enzyme and drug/DNA. In addition, the activity of topoisomerase I, and hence the efficacy of its inhibitors, can in fact be modulated by post-translational processes such as protein kinase C-dependent enzyme activation [41, 42]. It has also been reported that confluent HT29 cells present an increase in the lipid order of the plasma membrane as compared to non-confluent cells [28]. This condition may limit membrane permeability to drugs.

Acknowledgements—This work was supported by the Dutch Cancer Society (grant IKA-VU 88-18). G.J.P. is the recipient of a senior research fellowship from the Royal Netherlands Academy of Sciences. The authors are grateful to M. Sarks (Groningen, The Netherlands) for technical advice, and to H. Rozing for performing HPLC topotecan analysis.

REFERENCES

1. Slichenmyer WJ, Rowinsky EK, Donehouwer RC and Kaufmann SH, The current status of camptothecin analogues as antitumor agents. *J Natl Cancer Inst* **85**: 271–291, 1993.
2. Kingsbury WD, Boehm JC, Jakas DR, Holden KG, Hecht SM, Gallagher G, Caranfa MJ, McCabe FL, Faucette LF and Johnson RK, Synthesis of water-soluble aminoalkylcamptothecin analogues: inhibition of topoisomerase I and antitumor activity. *J Med Chem* **34**: 98–107, 1991.
3. Houghton PJ, Cheshire PJ, Myers L, Stewart CF, Synold TW and Houghton JA, Evaluation of 9-dimethylaminomethyl-10-hydroxycamptothecin against xenografts derived from adult and childhood solid tumors. *Cancer Chemother Pharmacol* **31**: 229–239, 1992.
4. Johnson RK, McCabe FL, Gallagher G, Wood J, Galef J and Hertzberg RP, Comparative efficacy of topotecan, irinotecan, camptothecin and 9-aminocamptothecin in preclinical tumor models. *Ann Oncol* **3**(Suppl. 1): 85, 1992.
5. Rowinsky EK, Grochow LB, Hendricks CB, Ettinger DS, Forastiere AA, Hurowtiz LA, McGuire WP, Sartorius SE, Lubejko BG, Kaufmann SH and Donehower RC, Phase I and pharmacologic study of topotecan: a novel topoisomerase I inhibitor. *J Clin Oncol* **10**: 647–656, 1992.
6. Blaney SM, Balis FM, Cole DE, Craig C, Reid JM, Ames MM, Krailo M, Reaman G, Hammond D and Poplack DG, Pediatric Phase I trial and pharmacokinetic study of topotecan administered as a 24-hour continuous infusion. *Cancer Res* **53**: 1032–1036, 1993.
7. Kantarjian HM, Beran M, Ellis A, Zwelling L, O'Brien S, Cazenave L, Koller C, Rios MB, Plunkett W, Keating MJ and Estey EH, Phase I study of topotecan, a new topoisomerase I inhibitor, in patients with refractory or relapsed acute leukemia. *Blood* **81**: 1146–1151, 1993.
8. Wall JG, Burris III HA, Von Hoff DD, Rodriguez G, Kneuper-Hall Rayna, Shaffer D, O'Rourke T, Brown T, Weiss G, Clark G, McVea S, Brown J, Johnson R, Friedman C, Smith B, Mann WS and Kuhn J, A phase I clinical and pharmacokinetic study of the topoisomerase I inhibitor topotecan (SK&F 104864) given as an intravenous bolus every 21 days. *Anti-Cancer Drugs* **3**: 337–345, 1992.
9. Liu LF, DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* **58**: 351–375, 1989.
10. Wang WC, DNA topoisomerases. *Annu Rev Biochem* **54**: 665–697, 1985.
11. Zhang H, D'Arpa P and Liu LF, A model for tumor cell killing by topoisomerase poisons. *Cancer Cells* **2**: 23–27, 1990.
12. Pizao PE, Lyaruu DM, Peters GJ, van Ark-Otte J, Winograd B, Giaccone G and Pinedo HM, Growth, morphology and chemosensitivity studies on post-confluent cells cultured in "V"-bottomed microtiter plates. *Br J Cancer* **66**: 660–665, 1992.
13. Keepers YP, Pizao PE, Peters GJ, van Ark-Otte J, Winograd B and Pinedo HM, Comparison of the sulforhodamine B protein and the tetrazolium (MTT) assays for *in vitro* chemosensitivity testing. *Eur J Cancer* **27**: 897–900, 1991.
14. Boyd MR, Paull KD and Rubinstein L, Data display and analysis strategies for the NCI disease-oriented *in vitro* drug screen. In: *Cytotoxic Anticancer Drugs: Models and Concepts for Drug Discovery and Development* (Eds. Valeriote FA, Corbett TH and Baker LH), pp. 11–34. Kluwer Academic Publishers, Boston, 1992.
15. Davis LG, Dibner MD and Battey JF, *New Methods in Molecular Biology*. Elsevier Science Publishing, New York, 1988.
16. Juan CC, Hwang J, Liu AA, Whang-Peng J, Knutsent T, Huebner K, Croce CM, Zhang H, Eang JC and Liu LF, Human DNA topoisomerase I is encoded by a single-copy gene that maps to chromosome region 20q12–13.2. *Proc Natl Acad Sci USA* **85**: 8910–8913, 1988.
17. Tsai-Pflugfelder M, Liu LF, Liu AA, Teweg KM, Whang-Peng J, Knutsen T, Huebner K, Croce CM and Wang JC, Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21–22. *Proc Natl Acad Sci USA* **85**: 7177–7181, 1988.
18. Battey J, Modding C, Taub R, Murphy W, Stewart T, Potter H, Lenoir G and Leder P, The human *c-myc* oncogene: structural consequences of translocation into the IgH locus in Burkitt lymphoma. *Cell* **34**: 779–787, 1983.
19. Fort P, Marty L, Piechaczyk M, Sabrouty SE, Dani C, Jeanteur P and Blanchard JM, Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res* **13**: 1431, 1985.
20. Baas F, Jongsma APM, Broxterman HJ, Arceci RJ, Housman D, Scheffer GL, Riethorst A, van Groeningen M, Nieuwint AWM and Joenje H, Non-P-glycoprotein mediated mechanism for multidrug resistance precedes P-glycoprotein expression during *in vitro* selection for doxorubicin resistance in a human lung cancer cell line. *Cancer Res* **50**: 5392–5398, 1990.
21. Zaman GJR, Versantvoort CHM, Smit JJM, Eijdems WHM, de Haas M, Smith AJ, Broxterman HJ, Mulder NH, de Vries EGE, Baas F and Borst P, Analysis of the expression of MRP, the gene for a new putative transmembrane drug transporter, in human multidrug resistant lung cancer cell lines. *Cancer Res* **53**: 1747–1750, 1993.
22. De Jong S, Zijlstra JG, De Vries EG and Mulder NH, Reduced DNA topoisomerase II activity and drug-induced DNA cleavage activity in an Adriamycin-resistant human small cell lung carcinoma cell line. *Cancer Res* **50**: 304–309, 1990.
23. Bradford MM, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
24. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (Lond) **227**: 680–685, 1970.
25. Shero JH, Bordwell B, Rothfield NF and Earnshaw WC, High titers of autoantibodies to topoisomerase I (ScL-70) in sera from scleroderma patients. *Science* **231**: 737–740, 1986.
26. Beijnen JH, Smith BR, Keijer WJ, van Gijn R, ten Bokkel Huinink WW, Vlasveld LTh, Rodenhuis S and Underberg WJM, High-performance liquid chromatographic analysis of the new anticancer drug SK&F 104864-A (NSC 609699). *J Pharm Biomed Anal* **8**: 789–794, 1990.
27. Tanizawa A and Pommier Y, Topoisomerase I alteration in a camptothecin-resistance cell line derived

from chinese hamster DC3F cells in culture. *Cancer Res* **52**: 1848–1854, 1992.
28. Dimanche-Boitrel M-T, Pelletier H, Genne P, Petit JM, Le Grimellec C, Canal P, Ardiet C, Bastian G and Chauffert B, Confluence-dependent resistance in human colon cancer cells: role of reduced drug accumulation and low intrinsic chemosensitivity of resting cells. *Int J Cancer* **50**: 677–682, 1992.
29. Hendricks CB, Rowinsky EK, Grochow LB, Donehower RC and Kaufmann SH, Effect of P-glycoprotein expression on the accumulation and cytotoxicity of topotecan (SK&F 104864), a new camptothecin analogue. *Cancer Res* **52**: 2268–2278, 1992.
30. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurtz EU, Duncan AMV and Deeley RJ, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* (Washington DC). **258**: 1650–1654, 1992.
31. Johnson RK, Hertzberg RP, Eng W-K, McCabe FL, Kingsbury WD and Hecht SM, Discovery and design of topoisomerase I inhibitors. *Proc Am Assoc Cancer Res* **33**: 593–594, 1992.
32. Blaney SM, Cole DE, Balis FM, Godwin K and Poplack DG, Plasma and cerebrospinal fluid pharmacokinetic study of topotecan in nonhuman primates. *Cancer Res* **53**: 725–727, 1993.
33. Hsiang YH, Lihou MG and Liu LF, Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res* **49**: 5077–5082, 1989.
34. Penn LJZ, Laufer EM and Land H, *c-myc*: Evidence for multiple regulatory functions. *Semin Cancer Biol* **1**: 69–80, 1990.
35. Heck MM, Hittelman WN and Earnshaw WC, Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. *Proc Natl Acad Sci USA* **85**: 1086–1090, 1988.
36. Bodley AL, Wu H-Y and Liu LF, Regulation of DNA topoisomerases during cellular differentiation. *NCI Monogr* **4**: 31–35, 1987.
37. Hsiang Y-H, Wu H-Y and Liu LF, Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. *Cancer Res* **48**: 3230–3235, 1988.
38. 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.
39. 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.
40. Tan B, Mattern MR, Eng W-K and McCabe FI, Nonproductive rearrangement of DNA topoisomerase I and II genes: correlation with resistance to topoisomerase inhibitors. *J Natl Cancer Inst* **81**: 1732–1735, 1989.
41. Samuels DS, Shimizu Y and Shimizu N, Protein kinase C phosphorylates DNA topoisomerase I. *FEBS Lett* **259**: 57–60, 1989.
42. Pommier Y, Kerrigan D, Hartman KD and Glazer RI, Phosphorylation of mammalian DNA topoisomerase I and activation by protein kinase C. *J Biol Chem* **265**: 9418–9422, 1990.