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DNA Topoisomerase: the Mechanism of Resistance to DNA Topoisomerase II Inhibitor VP-16

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ABSTRACT

K6-1 and 50B-3 cell lines, resistant to VP-16, a DNA topoisomerase II inhibitor, were established from two different types of cells respectively: human T-cell derived acute lymphoblastic leukemia cell line RPMI8402 and mouse mammary tumor cell line FM3A. IC_{50} values of K6-1 and 50B-3 cells to VP-I6, evaluated by the colony forming ability on methyl cellulose medium, were 11- and 84-fold higher than their sensitive parental cell lines, respectively. Membrane permeability of the drug was not responsible for the resistance in K6-1 and 50B-3 cells. Quantitative analysis of drug-induced DNA cleavage (so called cleavable complex formation) was performed using ^{32}P end-labeled pBR322 restriction fragments. The formation of the topoisomerase II-DNA cleavable complex stimulated by VP-16 in 50B-3 cells was approximately 1/5 compared with that of FM3A wild-type cells. Dot blot analysis of RNA extracted from these cell lines showed that the levels of mRNA for DNA topoisomerase II in 50B-3 cells were markedly decreased and that catalytic activity was reduced to 1/2—1/3 compared with that of parent cells. There was a slight reduction of DNA topoisomerase II mRNA in K6-1 cells. However, DNA topoisomerase II activities were similar in wild-type and K6-1 cells. In addition, 50B-3 cells showed cross resistance to VM-26, m-AMSA and adriamycin, whereas K6-1 cells exhibited increased resistance only to VM-26. These resistant cell lines did not show collateral sensitivity to CPT-11, a DNA topoisomerase I inhibitor. Southern blot analysis of genomic DNA did not show any change in the restriction pattern of the DNA topoisomerase II gene between the parental and their resistant lines. These findings suggest that the reduced levels in DNA topoisomerase II contribute to the drug resistance of 50B-3 cells.

Key words: *DNA topoisomerase II, VP-16, Drug resistance*

DNA topoisomerases (Topo) are enzymes which control and modify the topological state of DNA[48]. These enzymes are classified into two types according to the mechanism of actions, and isolated from a wide variety of organisms ranging from viruses to mammalian cells[18,19,]. In eukaryotes, these enzymes relax supercoiled DNA and mediate the strand passage by introducing transient single strand (Topo I) or double strand (Topo II) breakage on duplex DNA[48]. The functional role and mechanism of action of these enzymes have been studied extensively. Eukaryotic Topo II catalyses in vitro catenation and decatenation of kinetoplast DNA, knotting and unknotting of P4 DNA, and relaxation of supercoiled DNA[28,34]. Both human Topo I[9] and Topo II[33] cDNA have been cloned. The human Topo I and Topo II genes are single copy genes located on the chromosome regions 20q12-13-13.2 and 17q21—22, respectively[24,33]. These genes encode for 100 (Topo I:monomer) and 170 kDa (Topo II:homodimer) proteins[32,34]. Topo II has been implicated in several processes of DNA metabolism including replication[8], transcription[4,5], sister chromatid exchange[13], chromatin condensation and decondensation[22,45], and illegitimate recombination[3,4]. Topo II is also an important structural protein which appears to be a major component of the metaphase chromosome scaffold and nuclear matrix in interphase[2,14,21].

The importance of DNA topoisomerases in cancer chemotherapy has been emphasised since it has been shown that eukaryotic Topo II and Topo I are the targets of antitumor agents such as 4'-(9-acridinylamino)methanesulfon-m-anisidide (m-AMSA) and camptothecin, respectively[20,36]. Recent studies have shown that DNA intercalators, such as m-AMSA[37] and adriamycin[31,40], and DNA non-intercalating antitumor agents, VP-16, VM-26[25,30] and ellipticine derivative[15], inhibit eukaryotic Topo II by blocking the reaction[36,46]. These drugs inhibit mammalian Topo II by stabilizing the Topo II-DNA complex, called the cleavable complex, in which the enzyme is covalently bound to the 5'-terminus of enzyme catalyzed DNA breaks[7,36] (Fig. 1)[1]. The

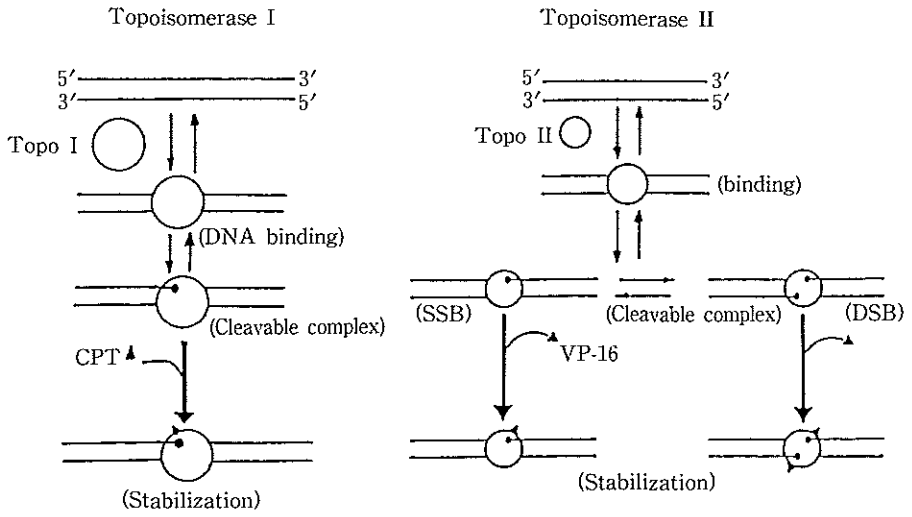

Fig. 1. Mechanism of action of topoisomerase-targeting-drugs.

Table 1. Mammalian cell lines resistant to DNA topoisomerase II inhibitors

Investigator (Year)	Cell Line (Parent)	Cross Resistance	Degree of Resistance	Drug Uptake	Catalytic Activity	DNA Strand Breaks	Cleavable Camplex	Level of Topo I, II
Glisson, B.[24] (1986)	Vpm^r-5 (CHO) VP-16^r	ADR, MTX, m-AMSA	×20	−	−	+	+	ND
Ishida, R.[39] (1987)	Nov A2 (BHK) Novobiocin^r	ADR, VP-16, m-AMSA VBL, Ara-C	×4	−	−	+	−	ND
Ferguson, P. J.[22] (1988)	KB/1c etc. (Human KB) VP-16^r	m-AMSA, VCR, Doxorubicin MTX	×29—287	+	+	ND	ND	Topo II↓ Topo I↑
Sinha, B. K.[62] (1988)	MCF-7/ADR ADR^r	VP16	×125—200	1/2—1/3 +	1/2—1/3 +	+	+	ND

ADR: adriamycin, MTX: methotrexate, VCR: vincristin, VBL: vinblastine, 9-OHE: 9-hydroxyellipticine.
− : Singificant difference was detected.
+ : Significant difference was not detected.
↓ : reduced; ↑: inreased.
ND : Not determined.

mechanism by which drugs stabilize the cleavable complex remains unknown. In the presence of strong protein denaturants such as sodium dodesyl sulfate or alkali[26], Topo II is trapped in a cleavable complex which may be detected experimentally as protein bound DNA strand breaks[27,28].

Recently, some studies have shown a relationship between altered DNA topoisomerase activity and cleavable complex formation[29,38,39,42]. Similar studies have been conducted on drug resistant cell lines[10,11,23,43]. However, there is insufficient knowledge about the multifactoral nature of the resistant mechanism (Table 1). Resistance to antitumor agents could be due to many cellular changes. For example, an alteration of the drug transport process or a modification of the drug may lead to drug resistance.

A DNA non-intercalating agent, VP-16, is one of the most important antitumor agents and studies of the mechanisms responsible for resistance to this drug are of particular importance. The present study characterized two different types of VP-16 resistant cell lines isolated from human T-cell derived lymphoblastic leukemia RPMI8402 and mouse mammary tumor cell line FM3A.

MATERIALS AND METHODS

1. Materials. VP-16 was obtained from Nippon Kayaku and CPT-11 from Yakult Co. 4'-(9-acridinylamino)methanesulfon-m-anisidide (m-AMSA: Warner Lambert Co.) and teniposide (VM-26:Brystol Myers Ger.) were generous gifts from Dr. M. Oguro

(Chiba Cancer Center, Japan) and Dr. M. Kobayashi (Hiroshima Univ, Japan), respectively. [^{3}H]-VP-16 (388 mCi/nmol) was obtained from Chemicin Sci. Lab. through the courtesy of Nippon Kayaku. Methyl-[^{3}H]-thymidine (70—85 Ci/nmol), 5,6-[^{3}H]-uridine (35—50 Ci/nmol), 4,5-[^{3}H]-leucine (120—190 Ci/nmol), [α-^{32}P]dATP (-3000 Ci/nmol) and [α-^{32}P]dCTP (-3000 Ci/nmol) were purchased from Amersham. Hydroxylapatite HT and Cellulose Phosphate P-11 were obtained from Bio-Rad and Whatman, respectively. Methyl cellulose was obtained from Shinets Chem. Co. pC15 DNA containing 1.8 kilobase (kb) human Topo II cDNA fragment[47] was a generous gift from Dr. T. Andoh (Aichi Cancer Center, Japan).

2. Cell maintenance. FM3A and RPMI8402, and their drug resistant variants were maintained in RPMI1640 (Gibco) supplemented with 10% fetal calf serum (FCS), 125 μg/ml of streptomycin and 125 U/ml of pencillin.

3. Establishment of VP-16-resistant cells. K6-1 cells were isolated by stepwise increment of VP-16 concentrations from 0.2 μg/ml in RPMI1640 during about a period of 6 months. 50B-3 cells were isolated by treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Wild-type cells of 1×10^6 were treated with MNNG at 0.75 μg/ml for 4 hr (survival rate < 10%) and washed 3 times with drug free medium. The cells then cultured until the survived cells grew to about 5×10^6 cells, in RPMI1640 supplemented with 2% FCS and antibiotics. The cells were suspended at 1×10^5 cells per 9-cm plate in a 0.2% agar medium made in RPMI1640 and then overlayered onto a 0.45% agar medium containing 10 μg/ml of VP-16. The dishes were incubated for 10—14 days. Five clones were obtained from 2 plates made independently. The degree of resistance to VP-16 was not the same for those clones. Among the clones, 50B-3 was chosen for further study.

4. Syntheses of DNA, RNA and Proteins. Syntheses of cellular DNA, RNA and proteins were estimated by the incorporation of [^{3}H]-thymidine (1 μCi/ml), [^{3}H]-uridine (2 μCi/ml), and [^{3}H]-leucine (2 μCi/ml), respectively, during 1 hr incubation of the cells (1×10^6 cells/ml) in the absense or presence of VP-16.

5. Drug uptake and efflux. a. Uptake. Drug sensitive and resistant cells were seeded at 2×10^6 cells/well in a 24 well plate and exposed to various concentrations of [^{3}H]-VP-16 for 30 min at 37°C. The cells were then harvested and washed 3 times with phosphate buffered saline (PBS(-)). The cells were solubilized in 0.2 ml of 1 N NaOH for 2 hr at 70°C[23]. The sample was neutralized with 0.2 ml of 1 N HCl and its radioactivity was determined by liquid scintillation counter after addition of ACS II (Amersham). b. Efflux. Drug sensitive and resistant cells were exposed to [^{3}H]-VP-16 for 30 min at 37°C. They were washed 3 times with medium and allowed to release the drug in RPMI1640 at 37°C. Intracellular radioactivity was determined at 3 and 7 min (for the mouse cells) or 5 and 10 min (for the human cells) after the start of the release as described[23].

6. Large scale preparation of the cells and partial purification of Topo II from FM3A and 50B-3 cells. The cells were injected intraperitoreally at 5×10^6 into 6-weeks old ddY mice irradiated with 400 rad of ^{60}Co γ-ray. The cells were harvested while in an exponential phase of the growth at 8—9 days after transplantation. Cells were suspended in PBS(-), collected by centrifugation and then stored quickly in a -80°C freezer until use. Frozen cells were thawed at 0°C and washed 2 times with PBS(-), and were then resuspended in buffer A (20 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 1 mM phenylmethanesulfonyl-fluoride (PMSF), 0.1% Triton X-100 and 0.25 mM 2-mercaptoethanol), sonicated for 20 sec by the TOMY ultra disrupter, and homogenized 10 strokes by Dounce homogenizer. A 1/10 volume of buffer A containing 3.3 M KCl was added to the homogenate. The extraction was carried out for 30 min at 0°C. The extracts were centrifuged at 21,000g for 20 min and the supernatant was removed for recentrifugation at the same condition. A half volume of 60% ethylene glycol-buffer A containing 0.3 M KCl was added to the extracts and applied onto a cellulose phosphate column (bed volume:20ml). The Topo II activity was eluted by linear gradient from 0.3 to 1.0 M KCl in 20% ethylene glycol-buffer A. Each fraction was diluted 10 times with dilution buffer and assayed for the unknotting activity. The active fractions were then applied onto hydroxylapatite HT column (bed volume:5ml) equilibriated with buffer A containing 0.3 M KCl and then were eluted in linear gradient from 20 to 500 mM phosphate buffer containing 0.3 M KCl. The fractions eluted around 300 mM phosphate contained Topo II activity, and these fractions were collected and dialysed against 20 mM phosphate buffer containing 50% glycerol, 100 mM KCl, 0.25 mM 2-mercaptoethanol, 1 mM PMSF, 0.1% Triton X-100 and stored at -80°C until use.

7. Preparation of P4 knotted DNA. The procedure is a modification of that used by Liu et al[29].

8. Topo II assay. The standard reaction mixture for the assay of Topo II contained 50 mM Tris/HCl, pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothreitol (DTT), 0.25 mM EDTA and 30 μg/ml of bovine serum albumin (BSA). The unknotting activity was assayed with 0.2 μg of P4 knotted DNA incubated at a final volume of 20 μl for 30 min at 30°C. The reaction was then terminated by the addition of 4 μl of a stop solution containing 5% sodium dodesyl sulphate (SDS), 50% glycerol and 0.25% bromophenolblue. The samples were then subjected to electrophoresis in 0.9% agarose gel with TAE (40 mM Tris-acetate, 1 mM

EDTA, pH 8.0) buffer for 40 min at 100 volt by Mupid mini-electrophoresis apparatus. The gel was then stained with ethidium bromide (0.5 μg/ml) and photographed under UV light.

9. DNA strand breaks. The cells (2×10^6) a log phase of growth were labeled with 0.5 μCi/ml [^{3}H]-thymidine for 16—20 hr and collected by centrifugation at 800g for 5 min. The pellets were resuspended in fresh medium and then treated with 10 μg/ml (for the human cells) and 100 μg/ml (for the mouse cells) of VP-16 for 1 hr at 37°C. After the treatment, the cells were washed with PBS(-) and resuspended in PBS(-). The cells were then lysed in 0.6 ml of lysis solution containing 2 M KCl, 10 mM EDTA, 0.6 M KOH and 1% sarkosyl (sodium salt) and layered onto a 5—20% alkaline sucrose gradient solution containing 2 M KCl, 1 mM EDTA, 0.3 M KOH, 0.1% sarkosyl with a cushion of 0.5 ml of 80% sucrose at the bottom and allowed to lyse overnight at 4°C. The sample was then centrifuged at 8,500 rpm for 12.5 hr at 4°C with a Beckman SW40 Ti rotor. Twenty fractions of 0.5 ml each were collected from the bottom of the tubes. Each fraction was added with 150 μl of 1 N HCl, 6.7 μl of yeast tRNA (5 mg/ml) and then 50 μl of 100% trichloroacetic acid (TCA). The samples were trapped on DE81 filter (Whatman) and the filters were washed with 40 ml of 3% TCA and 10 ml of cold ethanol and then dried. The radioactivities were counted by liquid scintillation counter.

10. DNA cleavage activity (single-strand breakage). Quantitative analysis of the DNA cleavage activity of Topo II (the cleavable complex formation) was assayed using the method by Liu et al[27].

11. Preparation of enzyme extracts. Crude nuclear extracts were prepared by the methods of Glisson et al[17]. Protein concentration in the extracts was determined by the method of Braford[6].

12. Colony forming ability. Drug induced cytotoxicity was determined by the colony formation assay. For the wild-type and resistant cells, the drug treated cells was seeded in fresh RPMI1640 containing 0.75% methyl cellulose onto 24 well plate (Coster). Cells were then incubated for 6—7 days at 37°C, and colonies appearing on the medium were scored using a microscope. IC_{50} value was calculated from the relative survival rate of the drug treated cells after correction of the plating efficiency. All the experiments were done in triplicate.

13. Southern blot analysis. Genomic DNA was extracted from the log phase cells by CsCl density gradient ultracentrifugation. DNA digested with restriction endonucleases was subjected to electrophoresis in 1% agarose gel and was transferred onto Zeta-probe filter. The procedures of Southern blot were based on the methods described by Reed[41]. After the blotting, the filter was prehybridized overnight at 43°C in 50% formamide, 4 × SSC (1× SSC = 150 mM NaCl, 15 mM sodium acetate), 1% SDS, 0.5% skim milk and 0.5 mg/ml salmon sperm DNA. The filter was then hybridized overnight at 43°C in the same buffer as for prehybridization with the nick translated cDNA probe at $1-2 \times 10^6$ cpm/ml. After hybridization, the filters were washed 2 times for 10 min each with 0.1 × SSC containing 1% SDS at room temperature followed by two 30 min washes with the same buffer at 60°C and rinsed briefly with 0.1 × SSC.

14. Dot blot analysis[44]. Zeta-probe nylon filters were presoaked in 2 × SSC. Total RNA of 15, 5, 1 μg of each were applied to each well of Bio-Rad dot blot apparatus. After fixation in 0.05 N NaOH, the filters were prehybridized for 5—6 hr at 43°C and hybridized overnight at 43°C. The composition of pre- and hybridization buffer was the same as that for the Southern analysis.

RESULTS

1. Establishment of resistant cells. K6-1 cells were established as described in the Materials and Methods. As shown in Fig. 2 and in Table 2, K6-1 cells were 11-fold more resistant to VP-16 than RPMI8402, the parental (wild-type:WT) cells. IC_{50} values of WT and K6-1 cells were 0.12 and 1.3 μg/ml, respectively. 50B-3 cells were isolated from MNNG treated FM3A cells. 50B-3 cells showed 84-fold resistance to VP-16 than their parental cell line FM3A (Table 3).

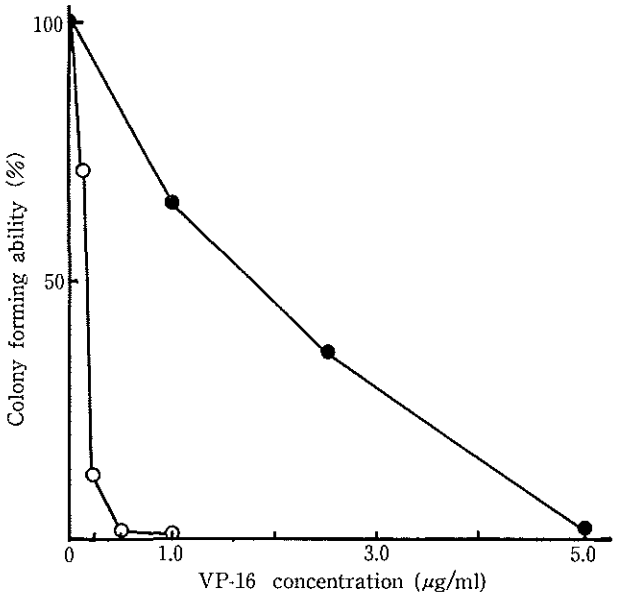

Fig. 2. Colony forming ability of RPMI8402 (○) and K6-1 (●) cells.

2. Effects of VP-16 on cell growth. The effects of VP-16 on syntheses of DNA, RNA and proteins in WT and resistant cells were estimated. As shown in Fig. 3, VP-16 inhibited synthesis of DNA and RNA synthesis in WT as well as in K6-1 cells. There was a significant inhibition of DNA and RNA

Table 2. IC_{50} values of various topoisomerases inhibitors for RPMI8402 and K6-1 cells

	IC_{50}[1]		relative resistance[2]
	RPMI8402	K6-1	
VP-16	0.12 ± 0.02	1.13 ± 0.10[3]	11
VM-26	24 ± 3.5	95 ± 12	4
ADR	13.0 ± 4.50	12.5 ± 3.20	1
m-AMSA	3.90 ± 0.20	4.20 ± 0.20	1
CPT-11	0.23 ± 0.04	0.21 ± 0.03	1

1: IC_{50} values are concentration causing 50% inhibition of colony forming ability relative to that of untreated cells. Values are shown in μg/ml (VP-16, CPT-11) or ng/ml (VM-26, m-AMSA and adriamycin).
2: Ratio of IC_{50} values for K6-1 cells to that for RPMI8402.
3: Mean ± SD for 2-3 separate experiments.

Table 3. IC_{50} values of various topoisomerases inhibitors for WT and 50B-3 cells

	IC_{50}[1]		relative resistance[2]
	WT	50B-3	
VP-16	0.19 ± 0.04	15.9 ± 1.20[3]	84
VM-26	25.5 ± 4.30	950 ± 230	37
ADR	76 ± 13	224 ± 29	3
m-AMSA	1.60 ± 0.18	16.0 ± 2.40	10
CPT-11	3.0 ± 0.2	3.8 ± 0.2	1

1: IC_{50} values are concentrations causing 50% inhibition of colony forming ability relative to that of untreated cells. Values are shown in μg/ml (VP-16, CPT-11) or ng/ml (VM-26, m-AMSA and adriamycin).
2: Ratio of IC_{50} values for 50B-3 cells to that for FM3A cells.
3: Mean ± SD 2-3 separate experiments.

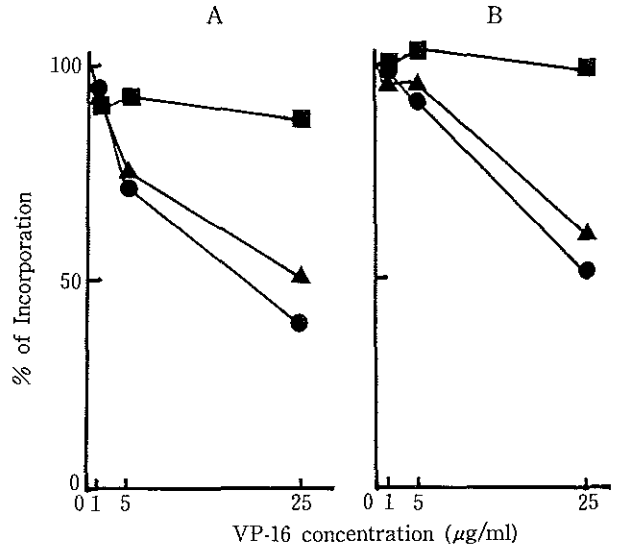

Fig. 3. Effect of VP-16 on synthesis of DNA (●), RNA (▲) and proteins (■) in RPMI8402(A) and VP-16 K6-1 (B) cells. 1 × 10^6 cells were incubated with radiolabeled precursors and various concentrations on VP-16 for 1 hr and harvested of glass filter. The filters were dried at 80°C for 2 hr and counted for the radiactivities.

syntheses in WT cells even at low concentrations of about 5 μg/ml compared with K6-1 cells. For protein synthesis, both WT and K6-1 cells were not inhibited even up to the 25 μg/ml level.

3. DNA strand breaks induced by VP-16. K6-1 and 50B-3 cells were examined as to whether the resistance is attributed to a reduction of DNA breaks induced by the drug. WT and resistant cells labelled with [^{3}H]-thymidine were incubated with VP-16 for 1 hr and analysed by the sucrose gradient centrifugation. As shown in Fig. 4, the extensive fragmentation of DNA was detected in WT cells but not in K6-1 cells. This was also the case for FM3A and 50B-3 cells exposed to the drug (data not shown).

4. Uptake and efflux of [^{3}H]-VP-16. In order to know whether the membrane permeability is changed in the resistant cells, drug uptake was examined as a function of VP-16 concentration. Cellular drug contents were determined as described in the Materials and Methods. From the results shown in Fig. 5, drug uptake increased linearly with the extracellular concentration of VP-16. There was no difference in the drug uptake between WT and K6-1 cells (Fig. 5A). The rate of drug release from WT and K6-1 cells was also tested. There was again no difference in the rate of drug release from WT and K6-1 cells (Fig. 5B). Similar results were obtained for FM3A and 50B-3 cells (data not shown).

5. Effect of VP-16 on the catalytic activity of Topo II. The strand passing activity of Topo II is inhibited by various anti-topoisomerase agents in a concentration dependent manner. It is possible that the resistant cells produce mutant Topo II which may be resistant to VP-16. Therefore, Topo II activity of crude nuclear extracts from WT and K6-1 cells was examined in the presence of the

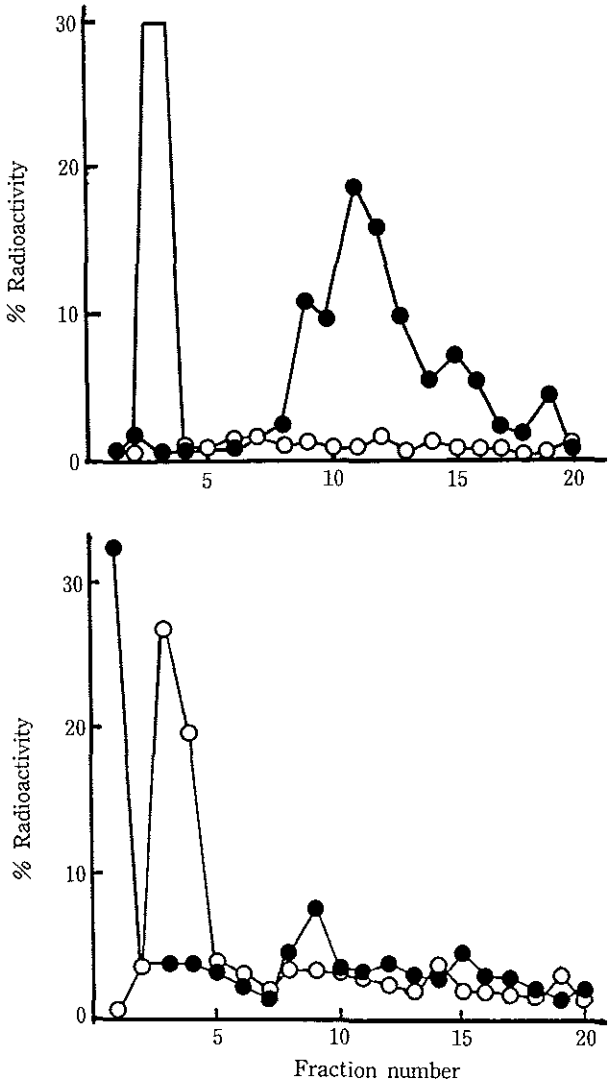

Fig. 4. DNA strand breaks in RPMI8402 and K6-1 cells treated with VP-16. Cells were incubated in the absence or presence of VP-16 for 1 hr. Untreated control (○) and with 10 μg/ml of VP-16 (●). Upper panel, RPMI8402; lower panel, K6-1. Sedimentation is from right to left.

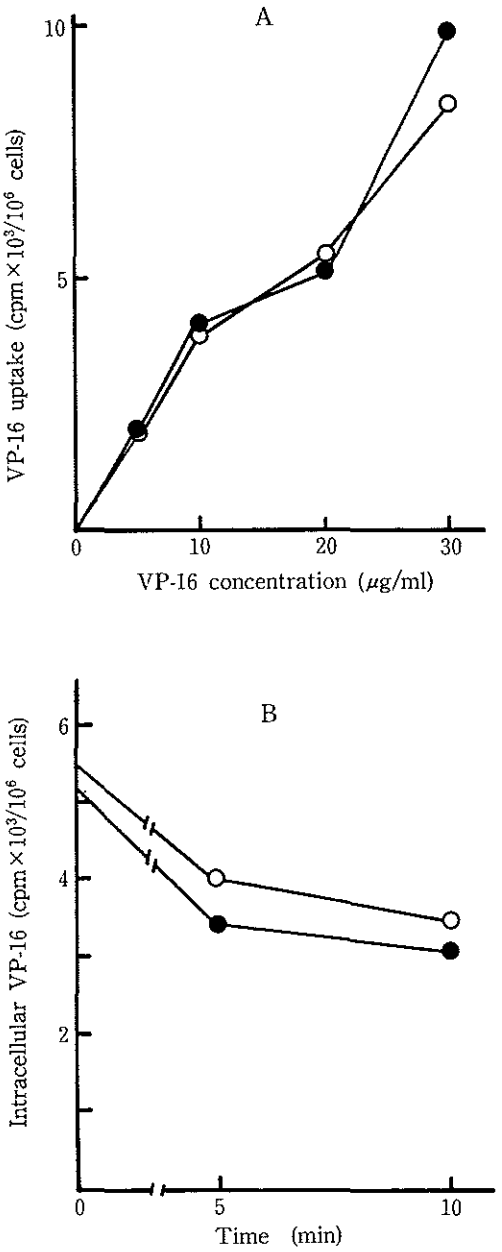

Fig. 5. A, Uptakes of [^{3}H]-VP-16 into RPMI8402 (○) and K6-1 (●) cells. B, Release of [^{3}H]-VP-16 from RPMI8402 (○) and K6-1 (●) cells. Cells were incubated with 20 μg/ml of VP-16 for 30 min and washed 3 times with PBS(−) then allowed to release the drug in the drug free medium for the indicated time at 37°C.

drug. It was found that no difference existed between WT and K6-1 cells in the VP-16-induced inhibition of the strand passing activity of Topo II (Fig. 6). Partially purified Topo II was also prepared from 12 grams of frozen FM3A or 50B-3 cells. Both the enzymes from WT and 50B-3 cells showed no difference in the pattern of elution in column chromatography. Active fractions were eluted at about 0.6 M as a [KCl] (Fig. 7). Three units of partially purified Topo II were used in the assay. However, the sensitivities of partially purified Topo II to VP-16 were not significantly different between FM3A and 50B-3 cells as in those from RPMI8402 and K6-1 cells (data not shown).

6. VP-16 stimulated DNA cleavage activity. Quantitative analysis of cleavable complex formation was carried out with crude preparation of Topo II from each cell line, using ^{32}P-labeled restriction fragments of pBR322 DNA as substrates. Protein concentration was adjusted to 2.5 μg/ml in each reaction. The catalytic activities of enzyme preparations were not significantly different among Topo II preparations. The amount of the ^{32}P-labeled DNA precipitated by the treatment of SDS-KCl solution increased dramatically with concentration of the drug up to 50 μg/ml in WT and 50B-3 Topo II. However, the plateau level of the precipitate was greatly reduced for Topo II of 50B-3 cells. A comparison of slopes from the initial linear portion of the curves shows that Topo II activity from

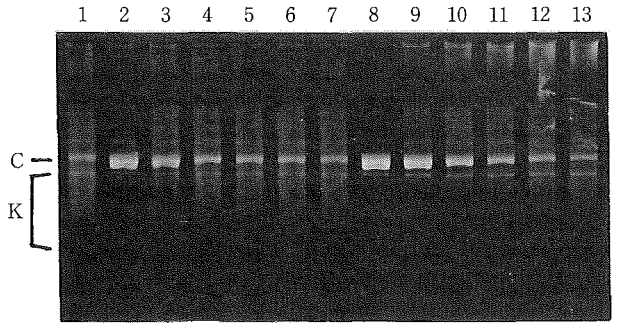

Fig. 6. Inhibition of strand passing activity of topoisomerase II in crude nuclear extracts from RPMI8402 and K6-1 cells by VP-16. Lane 1, knotted P4 DNA; lane 2-7, extract of RPMI8402 and lane 8-13, extract of K6-1. Lane 2 and 8, no drug added; lane 3 and 9, 25 μg/ml of VP-16; lane 4 and 10, 50 μg/ml of VP-16; lane 5 and 11, 100 μg/ml of VP-16; lane 6 and 12, 200 μg/ml of VP-16; lane 7 and 13, 400 μg/ml of VP-16. K, knotted P4 DNA; C, circular P4 DNA.

FM3A cells was approximately 5-fold greater than that from 50B-3 cells (Fig. 8A). On the other hand, there was no difference in the DNA cleavage activity between the crude extracts from RPMI8402 and K6-1 cells (Fig. 8B).

7. Cross resistance. Several studies have shown that cross resistance is expressed in drug-resistant cells. To verify whether K6-1 and 50B-3 cells show cross resistance or not, the cells were tested against Topo II inhibitors such as VM-26, adriamycin and m-AMSA, and a Topo I inhibitor, CPT-11 (camptothecin derivative). It was found that K6-1 cells showed increased resistance only to VM-26. IC_{50} value to VM-26 of K6-1 cells was higher by 4-fold than that of WT cells (Table 2). 50B-3 cells expressed cross resistance to VM-26, m-AMSA and adriamycin (Table 3). K6-1 and 50B-3 cells did not show a change in the sensitivity to CPT-11.

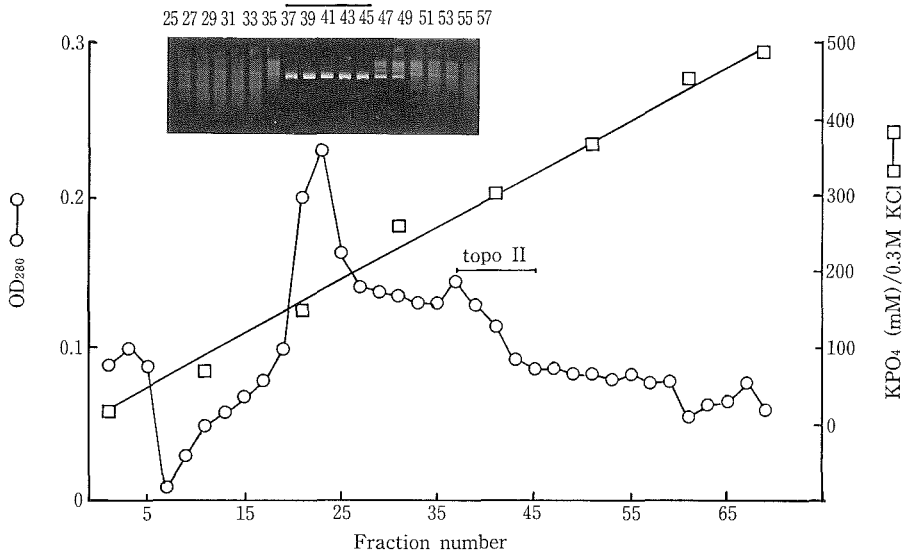

Fig. 7. Hydroxylapatite chromatography of FM3A topoisomerase II. OD_{280}, (○), and concentration of KPO4 in elution buffer (□). Fraction number 37—45, active fractions of topoisomerase II.

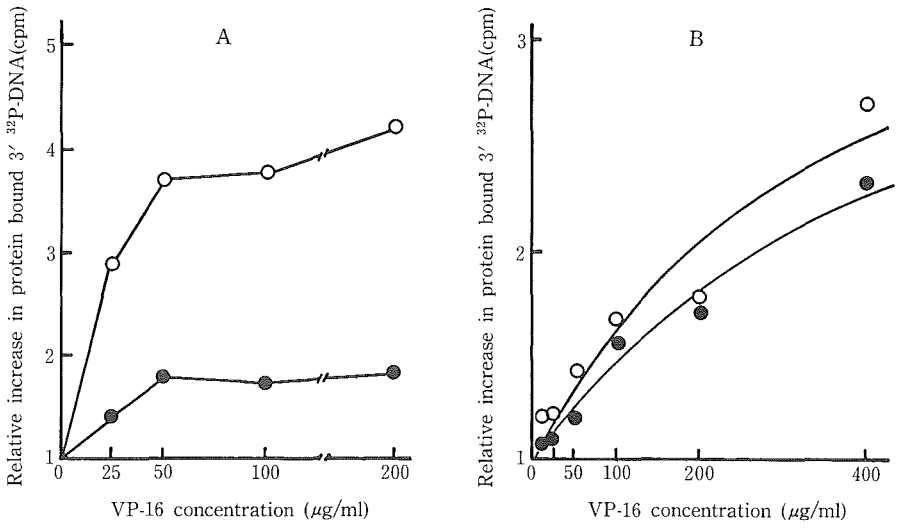

Fig. 8. Quantitative precipitation assay for the formation of the cleavable complex in the presence of VP-16. A, FM3A (○) and 50B-3 (●). B, RPMI8402 (○) and K6-1 (●). Each point is a mean of 3 separate experiments.

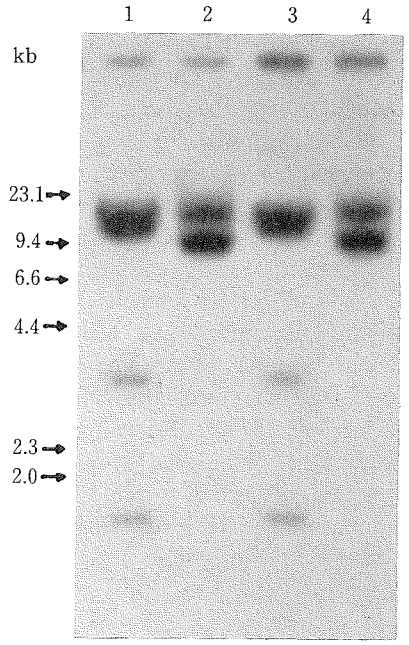

Fig. 9. Southern blot analysis of topoisomerase II. ^{32}P labeled pC15 DNA fragment containing 1.8 kb of human topoisomerase II cDNA clone was used as the probe. Lane 1, RPMI8402/EcoRI; lane 2, RPMI8402/BamHI; lane 3, K6-1/EcoRI and lane 4, K6-1/BamHI, respectively. Lengths of size markers (in kb) are indicated on the left side of the autoradiogram.

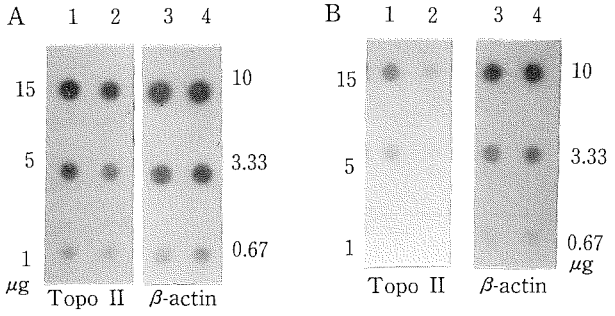

Fig. 10. Dot blot analysis of topoisomerase II. Serial dilutions of 15, 5, 1 μg of total RNA were applied to each well. Hybridization of blot with beta-actin probe demonstrated comparable amount of RNA loaded in all wells (column 3 and 4 of each panel). A, RPMI8402 and K6-1. Column 1 and 3, RPMI8402 and column 2 and 4, K6-1. B, FM3A and 50B-3. Column 1 and 3, FM3A and column 2 and 4, 50B-3.

8. Southern blot analysis. The restriction patterns of genomic DNA by EcoRI or BamHI were identical for RPMI8402 and K6-1 cells (Fig. 9). Similar results were obtained for FM3A and 50B-3 cells (data not shown).

9. Dot blot analysis of Topo II. It was found that Topo II mRNA levels were reduced by 10—20% in K6-1 cells (Fig. 10A) and 75—85% in 50B-3 cells (Fig. 10B).

DISCUSSION

VP-16 inhibited the DNA synthesis in wild-type cells (Fig. 3). The result indicates the functional role of Topo II in proliferating cells. Woynarowski et al[47] have shown that VP-16 and VM-26 induced DNA-protein cross link in nascent DNA. It had been shown also that anti-topoisomerase II agents are most cytotoxic to cells in the S phase[35].

Intracellular drug concentration was measured to determine whether the reduction in the intracellular concentration of the drug is responsible for resistance in K6-1 and 50B-3 cells. These results showed no significant difference in the incorporation and release of VP-16 in wild-type and resistant cell lines. There have been several reports which are consistent with these results. No difference was observed in the intracellular concentration of various antitumor drugs such as m-AMSA, VM-26 and novobiocin between wild-type and resistant cells[17,23,38].

Since Topo II has been identified as the intracellular target for certain antitumor agents, a study of catalytic and cleavage activity was carried out in VP-16 sensitive and resistant cell lines. Both 50B-3 and K6-1 cells were resistant to the induction of DNA strand breaks by the drug. It was shown that Topo II mediated DNA cleavage activity was reduced by about 5-fold in 50B-3 cells. The Topo II catalytic activity determined by unknotting reaction was also decreased by about 2—3-fold in 50B-3 cells (data not shown). These results suggest a good relationship between drug sensitivity and enzyme activity. This agrees with Deffie et al[12], who showed a relationship between Topo II catalytic activity and DNA cleavage activity in adriamycin resistant P388 leukemia cell lines. Dot blot analysis also showed reduced Topo II mRNA in 50B-3 cells compared with FM3A wild-type cells. Reduced enzyme levels may lead to the decrease in cleavable complex formation and catalytic activity in 50B-3 cells. The result are consistent with those of Ferguson et al[16] who reported that, the level of Topo II protein determined by immunoblotting analysis was reduced in accordance with the relative resistance in VP-16 resistant KB cell lines.

K6-1 cells contrasted with 50B-3 cells in that the catalytic and DNA cleavage activity did not change significantly as compared with parent cells, despite a slight reduction in Topo II mRNA levels (Fig. 8 and 10). The specific activity of Topo II was probably increased in K6-1 cells, consistent with Pommier et al[38] who reported reduced enzyme molecules in ellipticine-resistant Chinese hamster cells with a Topo II activity similar to WT cells. In the DNA cleavage activity analysis, the disagreement between K6-1 cells and 50B-3 cells might reflect a methodological problem involved in discriminating a slight change of cleavable complex formation possibly due to low resistance of K6-1 cells. However, the slightly reduced mRNA levels of Topo II in K6-1 cells corresponded to their low resistance to the drug. The increased resistance to

DNA strand breaks induced by VP-16 was also observed in K6-1 cells and may be due to the reduction of Topo II expression. 50B-3 cells exhibited cross resistance to VM-26, m-AMSA and adriamycin, whereas K6-1 cells exhibited cross resistance only to VM-26, a congener of VP-16. There seems to be a different mechanism between K6-1 and 50B-3 cells. If Topo I compensated for the lack of Topo II in resistant cell lines, there could have been an increased Topo I activity, resulting in an increased sensitivity to CPT-11 in resistant cell lines. CPT-11 showed a similar cytotoxicity in wild-type and resistant cell lines. Therefore, there seems to be no compensatory increase in the level of Topo I in resistant cell lines.

The inhibition patterns of strand passing activity by VP-16 were indentical in wild-type and resistant cell lines. Southern blot analyses also showed that the restriction patterns of genomic DNA were indentical in wild-type and resistant cell lines. These results may exclude the possibility of large mutation in Topo II gene itself. The present study suggests that a major factor in the resistance is the reduced Topo II levels in one of the resistant cell lines.

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REFERENCES

1. **Andoh, T., Ishii, K., Ikekami, Y., Kusunoki, Y., Takemoto, Y. and Okada, K.** 1987. Characterization of mammalian mutant with a camptothecin resistant DNA topoisomerase I. Proc. Natl. Acad. Sci. USA 84: 5565–5569.
2. **Berrios, M., Osheroff, N. and Fisher, P.A.** 1985. In situ localization of DNA topoisomerase II, a major polypeptide component of the Drosophila nuclear matrix fraction. Proc. Natl. Acad. Sci. USA 82: 4142–4146.
3. **Bae, Y. S., Kawasaki, I., Ikeda, H. and Liu, L. F.** 1988. Illegitimate recombination mediated by calf thymus DNA topoisomerase II in vitro. Proc. Natl. Acad. Sci. 85: 2076–2080.
4. **Brill S.J., DiNardo, S., Voelkel-Meiman, K. and Sternglanz, R.** 1987. Need for DNA topoisomerase activity as a swivel for DNA replication for transcription of ribosomal RNA. Nature 326: 414–416.
5. **Brill, S. J. and Sternglanz, R.** 1989. Transcription-dependent DNA supercoiling in yeast DNA topoisomerase mutants. Cell 54: 403–411.
6. **Braford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.
7. **Chen, G. L., Yang, L., Rowe, T. C. and Halligan, B. D.** 1984. Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. J. Biol. Chem. 259: 13560–13566.
8. **Champoux, J. J. and Dulbecco, R.** 1972. An activity from mammalian cells that untwists superhelical DNA - a possible swivel for DNA replication. Proc. Natl. Acad. Sci. USA 69: 143–146.
9. **D'Arpa, P., Machlin, P. S., Ratrie, H., Rothfield, N. F., Cleveland, D. W. and Earnshaw, W. C.** 1988. cDNA cloning of human DNA topoisomerase I: catalytic activity of a 67.7-kDa carboxylterminal fragment. Proc. Natl. Acad. Sci. 85: 2543–2547.
10. **Drake, F. H., Zimmerman, J. P., McCabe, F. L., Bartus, H. F., Per, S. R., Sullivan, D. H., Ross, W. E., Mattern, M. R., Johnson, R. K., Crooke, S. T. and Mirabelli, C. K.** 1987. Purification of topoisomerase II from amsacrine-resistant P388 leukemia cells. J. Biol. Chem. 262: 16739–16747.
11. **Danks, M. K., Yalowich, J. C. and Beck, W. T.** 1987. Atypical multiple drug resistance in a human leukemic cell line selected for resistance to teniposide (VM-26). Cancer Res. 47: 1297–1301.
12. **Deffie, A. M., Batra, J. K. and Goldenberg, G. J.** 1989. Direct correlation between DNA topoisomerase II activity and cytotoxicity adriamycin-sensitive and -resistant P388 leukemia cell lines. Cancer Res. 49: 58–62.
13. **Dillehay, L. E., Denstman, S. C. and William, J. R.** 1987. Cell cycle dependence of sister chromatid exchange induction by DNA topoisomerase II inhibitors in chinese hamster V79 cells. Cancer Res. 46: 206–209
14. **Earnshaw, W. C., Halligan, B., Cooke, C. A., Heck M. M. S. and Liu, L. F.** 1985. Topoisomerase II is a structural component of mitotic chromosome scaffolds. J. Cell Biol. 100: 1706–1715.
15. **Filipski, J. and Kohn, K. W.** 1982. Ellipticine-induced protein associated DNA breakes in isolated L1210 nuclei. Biochim. Biophys. Acta 698: 280–286.
16. **Ferguson, P. J., Fisher, M. H., Stephenson, J., Li, D. H., Zhou, B. S. and Cheng, Y. -C.** 1988. Combined modalities of resistance in etoposide-resistant human KB cell lines. Cancer Res. 48: 5956–5964.
17. **Glisson, B., Gupta, R., Smallwood-kentro, S. and Ross, W.** 1986. Characterization of acquired epipodophyllotoxin resistance in a chinese hamster overy cell line: loss of drug-stimulated DNA cleavage

activity. Cancer Res. **46**: 1394–1398.
18. **Gellert, M.** 1981. DNA topoisomerases. Ann. Rev. Biochem. **50**: 897–910.
19. **Geider, K. and Hoffmann-Berling, H.** 1981. Proteins controlling the helical structure of DNA. Ann. Rev. Biochem. **50**:233–260.
20. **Hsiang, Y. H., Hertzberg, R., Hecht, S. and Liu, L. F.** 1985. Camptothecin induces protein-linked DNA breaks via mammlian DNA topoisomerase I. J. Biol. Chem. **260**: 14873–14878.
21. **Heck, M. M. S. and Earnshaw, W. C.** 1986. Toposomerase II: a specific marker for cell proliferation. J. Cell. Biol. **103**: 2569–2581.
22. **Holm, C., Steraus, T. and Botstein, D.** 1989. DNA topoisomerase II must act at mitosis to prevent nondisjunction and chromosome breakage. Mol. Cell. Biol. **9**: 159–168.
23. **Ishida, R., Nishizawa, M., Nishimoto, T. and Takahashi, T.** 1988. Cross-resistance of novobiocin-resistant BHK cell line to topoisomerase II inhibitors. Somatic Cell and Molecular Genetics **14**: 489–497
24. **Juan, C. -C., Hwang, J., Liu, A. A., Whang-Peng, J., Knutsen, T., Huebner, K., Croce, C. M., Zhang, H., Wang, J. C. and Liu, L. F.** 1988. 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.
25. **Kerrigan, D., Pommier, Y. and Kohn, K. W.** 1987. Protein-linked DNA strand breaks produced by etoposide and teniposide in mouse L1210 and human VA-13 and HT-29 cell lines: relationship to cytotoxicity. NCI Monographs 4: 117–121.
26. **Kohn, K. W., Erickson, L. C., Ewing, R. A. G. and Friedman, C.** 1976. Fractionation of DNA from mammalian cells by alkaline elution. Biochem. **15**: 4629–4637.
27. **Liu, L. F., Rowe, T. C., Yang, L., Tewey, K. M. and Chen, G. L.** 1983. Cleavage of DNA by mammalian DNA topoisomerase II. J. Biol. Chem. **258**: 15365–15370.
28. **Long, B. H., Musial, S. T. and Brattain, M. G.** 1986. DNA breakage in human lung carcinoma cells and nuclei that are naturally sensitive or resistant to etoposide and teniposide. Cancer Res. **46**: 3809–3816.
29. **Liu, L. F. and Davis, J.** 1981. Novel topologically knotted DNA bacteriophage P4 capsids: studies with DNA topoisomerase. Nucleic Acids Res. **9**: 3979–3989.
30. **Long, B. H., Musial, S. T. and Brattain, M. G.** 1984. Comparison of cytotoxicity and DNA breakage activity of congeners of podophylotoxin including VP16-213 and VM26: a quantitative structure-activity relationship. Biochem. **23**: 1183–1188.
31. **Levin, M., Silver, R., Israel, M., Goldstein, A., Khetarpal, V. K. and Potmesil, M.** 1981. Protein-associated DNA breaks and DNA-protein cross-links caused by DNA nonbinding derivatives of adriamycin in L1210 cells. Cancer Res. **41**: 1006–1010.
32. **Liu, L. F. and Miller, K. G.** 1981. Eukaryotic DNA topoisomerases: two form of type I DNA topoisomerases from HaLa cell nuclei. Proc. Natl. Acad. Sci. USA **76**: 3487–3491.
33. **Monika, T. P., Liu, L. F., Liu, A. A., Tewey, K. M., Jacqueline, W. -P., Knutsen, T., Huebner, K., Croce, C. M. and Wang, J. C.** 1988. Cloning and sequencing of cDNA encoding human DNA topoisomerase II and location of the gene to chromosome region 17q21—22. Proc. Natl. Acad. Sci. USA **85**: 7177–7181.
34. **Miller, K. B., Liu, L. F. and Englund, P. T.** 1981. A homogeneous type II DNA topoisomerase from HeLa cell nuclei. J. Biol. Chem. **256**: 9334–9335.
35. **Nelson, W. G., Liu, L. F. and Coffey, D. S.** 1986. Newly replicated DNA is associated with DNA topoisomerase II in cultured rat prostatic adenocarcinoma cells. Nature **322**: 187–198.
36. **Nelson, E. M., Tewey, K. M. and Liu, L. F.** 1984. Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4'-(9-aridinylamino)-methanesulfon-m-anisidide. Proc. Natl. Acad. Sci. USA **81**: 1361–1365.
37. **Pommier, Y., Minford, J. K., Schwartz, R. E., Zwelling, L. A. and Kohn, K. W.** 1985. 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. Biochem **24**: 6410–6416.
38. **Pommier, Y., Schwartz, R. E., Zwelling, L. A., Kerrigan, D., Mattern, M. R., Charcososset, J. Y., Jacquemin-sablon, A. and Kohn, K. W.** 1986. Reduced formation of protein-associated DNA strand breaks in chinese hamster cells resistant to topoisomerase II inhibitors. Cancer Res. **46**: 611–616.
39. **Potmesil, M., Hsiang, Y. H., Liu, L. F., Bank, B., Grossberg, H., Kirschenbaum, S., Kanganis, D., Knowles, D., Traganos, F. and Silber, R.** 1988. Resistance of human leukemic and normal lymphocytes to drug-induced DNA cleavage and low levels of DNA topoisomerase II. Cancer Res. **48**: 3537–3543.
40. **Potmesil, M., Kirschenbaum, S. and Silber, R.** 1983. Relationship of adriamycin concentrations to the DNA lesions induced in hypoxic and euoxic L1210 cells. Cancer Res. **43**:3528–3533.
41. **Reed, K. C. and Mann, D. A.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13**: 7207–7221.
42. **Sinha, B. K., Haim, N., Dusre, L., Kerrigan, D. and Pommier, Y.** 1988. DNA strand breaks produced by etoposide (VP-16, 213) in sensitive and resistant human breast tumor cells: implication for the mechanism of action. Cancer Res. **48**: 5096–5100.
43. **Spiridonidis, C. S., Chatterjee, S., Petzold, S. J. and Berger, N. A.** 1989. Topoisomerase II-dependent and -inde-pendent mechanisms of etoposide resistance in chinese hamster cell lines. Cancer Res. **49**: 644–650.
44. **Sakai, M.** 1987. Northern and dot blot analysis. Experimental Medicine (Japan) **5**: 81–86.
45. **Uemura, T., Ohkura, H., Adachi, Y., Morino, K., Shiozaki, K. and Yanagida, M.** 1987. DNA topoisomerase II is required for condensation and separation of mitotic chromosome in S. pombe. Cell **50**: 917–925.
46. **Wang, J. C.** 1987. Recent studies of DNA

topoisomerases. Biochemica et Biophysica Acta **909**: 1–9.

47. **Woynarowski, J. M., Sigmund, R. D. and Beerman, T. A.** 1988. Topoisomerase II-mediated lesions in nascent DNA: Comparison of the effects of epipodophyllotoxin derivatives, VM-26 and 9-anilinoacridine derivatives, m-AMSA and o-AMSA. Biochim. Biophys. Acta **950**: 21–29.
48. **Wang, J. C.** 1985. DNA topoisomerases. Ann. Rev. Biochem. **54**:665–697.