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CHARACTERIZATION OF CAMPTOTHECIN-RESISTANT CHINESE HAMSTER LUNG CELLS

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Abstract—Three camptothecin-resistant sublines (V79^r, IRS-1^r and IRS-2^r) of V79 cells and their irradiation-sensitive mutants, IRS-1 and IRS-2, were developed by stepwise, continuous exposure to camptothecin (CPT). The degree of resistance varied among these cells. Based on the biochemical characterizations of these resistant cell lines, the mechanisms which could be responsible for the resistance to CPT were proposed to be: (a) a decrease in the intracellular accumulation of CPT with or without alteration of DNA topoisomerase I, (b) a decrease in the amount of DNA topoisomerase I, or (c) a decrease in the sensitivity of DNA topoisomerase I to CPT. The resistant cells which exhibited down-regulation of DNA topoisomerase I were collaterally sensitive to etoposide (VP-16) and its analogue, 4′-demethy-4β-(4″-fluoroanilino)-4-desoxypodophyllotoxin, despite the fact that there were equal amounts of DNA topoisomerase II in the parental and in the resistant cell lines. Alternating the usage of CPT and VP-16 for the treatment of cancer is indicated.

Camptothecin (CPT§), a plant alkaloid isolated from *Camptotheca acuminata*, is an antitumor agent which is effective against several murine tumors [1–5]. It exhibits several cellular effects, including inhibition of RNA and DNA syntheses, and causes a rapid and reversible fragmentation of chromosomal DNA in mammalian cultured cells [6–8]. It was suggested recently that mammalian DNA topoisomerase I is the primary intracellular target of CPT. It acts by blocking the rejoining step of the DNA breakage–reunion reaction of topoisomerase I, subsequently leading to permanent DNA damage and cytotoxicity when replication occurs [9]. Structure–activity relationship studies on analogues of CPT demonstrated that the derivatives do not inhibit topoisomerase I and lack antitumor activity [10, 11].

A number of studies have shown that quantitative and qualitative changes in topoisomerase I can lead to resistance to CPT [12–14]. CPT is not cross-resistant to multiple drug-resistant cells which possess increased ability to pump out intracellular drugs [15–17]. Decreases in intracellular accumulation of CPT, which could theoretically cause resistance, have not been demonstrated. Recently, three CPT-resistant cell lines from V79 cells and their irradiation sensitive mutants, IRS-1 and IRS-2, were developed independently in this laboratory. The properties and phenotypes of these CPT-resistant cells are described in this study. In addition to the quantitative and qualitative change in topoisomerase I, the decrease in intracellular accumulation of CPT could also play a role in the development of resistance to CPT. The compensatory interaction between DNA topoisomerase I and II has been suggested [14, 18]. Thus, the response of these cell lines to etoposide (VP-16) and its analogue, 4′-demethyl-4β-(4″-fluoroanilino)-4-desoxypodophyllotoxin (NPF), which exerts its action through DNA topoisomerase II [19, 20], were also examined.

MATERIALS AND METHODS

Cells. V79 cells (Chinese hamster lung cell) and their irradiation-sensitive mutants, IRS-1 and IRS-2, were maintained in RPMI 1640 with 10% fetal bovine serum and 100 μg/mL kanamycin, under humidified conditions with 5% CO_2 at 37°. The characteristics of IRS-1 and IRS-2 cells have been described previously [21–23]. The CPT-resistant cells (V79^r, IRS-1^r and IRS-2^r) were established by stepwise, continuous exposure to CPT in Dr. Dethlefsen's laboratory, Department of Radiology, University of Utah. These resistant cells were maintained in medium supplemented with different concentrations of CPT (5 nM for IRS-1^r and 20 nM for both IRS-2^r and V79^r). The generation time (10–14 hr) and cell cycle distribution for all cell lines were similar. All cell lines were tested periodically for mycoplasma by the Gen-Probe Rapid Detection System (Gen-Probe Inc., San Diego, CA).

Chemicals. CPT was provided by Dr. Zang-chao Liu, Cancer Institute of Sun Yat-sen University of Medical Science, Guang-zhou, China. VP-16 was the gift of Bristol-Myers Pharmaceuticals (Syracuse, NY). NPF was provided by Dr. K. H. Lee's

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§ Abbreviations: CPT, camptothecin; NPF, 4′-demethyl-4β-(4″-fluoroanilino)-4-desoxypodophyllotoxin; PLDBs, protein-linked DNA breaks; K-SDS, potassium-sodium dodecyl sulfate; DTT, dithiothreitol; PMSF, phenylmethyl sulfonyl fluoride; PCR, polymerase-chain reaction; and 1 × SSC, sodium citrate buffer, pH 7.0, containing 150 mM NaCl.

laboratory in the School of Pharmacy at the University of North Carolina (Chapel Hill, NC). [α and γ-^{32}P]-dATP and -dCTP (specific activity, 6000 Ci/mmol) and [^{14}C]-thymidine (specific activity, 50.5 mCi/mmol) were purchased from ICN Radiochemicals (Costa Mesa, CA). Verapamil was purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were of standard analytical grade or higher.

Antibodies. The monoclonal antibody of human topoisomerase I was established and characterized in this laboratory, as reported previously [24]. Rabbit serum containing anti-topoisomerase II antibody (p180) was a gift from Dr. Fred H. Drake, Smithkline Beecham Pharmaceuticals, King of Prussia, PA.

Drug sensitivity test. All drugs, except verapamil which was dissolved in water, were dissolved in dimethyl sulfoxide at a concentration of 20 mM and stored at −20°. Different dilutions of drugs were made in water just before use. The resistant cells were maintained in drug-free medium for 3 days prior to use. Briefly, cells with logarithmic growth were plated at a density of 2500 cells/well in a 24-well plate. The cells were exposed continuously to different concentrations of drugs for 72 hr at 37°. The final concentration of dimethyl sulfoxide was less than 1%. The MTT-dye [3-(4,5-dimethylthiazol-zyl)-2,5-diphenyltetrazolium] assay was used to evaluate the effects of the drugs on cell growth and to determine the concentration of the drugs which inhibit 50% of cell growth (IC_{50}) [25].

Measurement of protein-linked DNA breaks. Parental and resistant cells in log phase growth were labeled with [^{14}C]-thymidine for 24 hr. After labeling, the cells were trysinized, resuspended in fresh medium at a density of 5×10^5 cells/mL, and shaken gently in a 37° water bath for 1 hr in suspension, various concentrations of CPT and VP-16 in the presence or absence of 10 μM verapamil were added and incubation was continued for an additional 0.5 hr. The cells were then collected and analyzed for protein-linked DNA breaks (PLDBs) by the potassium-sodium dodecyl sulfate (K-SDS) precipitation method, as described previously by Rowe *et al.* [26]. The percentage of DNA breaks was determined by dividing the amount of radioactivity detected in potassium-precipitated DNA by the amount of radioactivity detected in the total trichloroacetic acid-precipitated DNA.

Preparation of whole cell and nuclear extracts. Crude cellular extracts were prepared as described previously by Ferguson *et al.* [18]. Briefly, cells were harvested and washed twice with cold phosphate-buffered saline. The cells were resuspended in a buffer containing 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethyl sulfonyl fluoride (PMSF), 10% glycerol (v/v) and 300 mK KH_2PO_4 (pH 7.0). Cell pellets were subjected to three cycles of freezing and thawing. Supernatants were collected after centrifugation at 13,000 *g* for 15 min. Crude nuclear extracts were prepared as reported previously by Deffie *et al.* [27]. Cells were collected and resuspended in 1 mL of cold nuclear buffer (2 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA and 0.1 mM DTT), and 9 mL of cold nuclear buffer containing 0.35% Triton X-100 and 1 mM PMSF was added. The cells were put on ice for 10 min and washed twice with Triton-free cold nuclear buffer. Nuclear protein was extracted from the nucleus for 30 min at 4° with ice-cold nuclear buffer containing 0.35 M NaCl. Nuclear protein was collected by centrifugation at 13,000 *g* for 15 min. Protein concentration of both whole cell and nuclear extracts was determined by the method of Bradford [28].

DNA topoisomerase activity. DNA topoisomerase I activity was determined by relaxation of supercoiled *Escherichia coli* DNA (pBR322), as described previously by Liu and Miller [29]. One unit of topoisomerase I activity was defined as the amount of enzyme which causes the relaxation of 50% of 0.5 μg pBR322 DNA. DNA topoisomerase II catalytic activity was assayed by ATP-dependent unknotting of P4 knotted DNA, as described previously [30]. One unit of topoisomerase II activity was defined as the amount of enzyme which causes unknotting of 50% of 0.5 μg of P4 knotted DNA. Both crude whole cell and nuclear extracts were used for DNA topoisomerase I and II activity assay. Specific activity was determined by dividing the topoisomerase I and II activity by the total amount of the protein in the extracts.

Western blot analysis. Cellular extracts were analyzed on a 7.5% sodium dodecyl sulfate–polyacrylamide electrophoresis gel (SDS–PAGE gel). The samples which contained equilvalent amounts of protein were then loaded to the gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with human topoisomerase I monoclonal antibody and topoisomerase II antibody. Antibody staining was performed with the use of alkaline phosphatase-conjugated secondary antibody [31]. The quantitative difference of DNA topoisomerase I and II was determined by serially diluted protein of parental cells to assess the degree of change. Ten and three independent experiments, respectively, were done for DNA topoisomerase I and II analysis.

Measurement of the sensitivity of DNA topoisomerase I to CPT. Partially purified DNA topoisomerase I was used to determine the sensitivity of DNA topoisomerase I to CPT. The method of partial purification of DNA topoisomerase I was described previously [32]. Approximately 2×10^8 cells in logarithmic growth were harvested and resuspended in 75 mM potassium phosphate buffer (pH 7.0). All buffers consisted of potassium phosphate containing 10% glycerol, 1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 0.5 μg/mL leupeptin and pepstatin. Cells were then lysed by three cycles of freezing and thawing. Potassium phosphate buffers were added to a final concentration of 0.3 M (pH 7.0) and the lysates were extracted for 1 hr at 4°. The supernatants were obtained by centrifugation and applied to DEAE cellulose and P-11 phosphocellulose columns, which were preequilibrated with 0.3 M potassium phosphate buffer, sequentially. The P-11 phosphocellulose column was then eluted with a linear gradient of 0.3 to 1 M of potassium phosphate buffer. The fractions that contain DNA topoisomerase I, as determined by the relaxation of supercoiled pBR322 DNA, were pooled and used for *in vitro* K-SDS assay to determine the sensitivity

of DNA topoisomerase I to CPT. Linear pBR322 DNA was 3'-end labeled, prepared as described previously [33], and used as a substrate for this assay.

Preparation of Chinese hamster topoisomerase I cDNA. To get specific Chinese hamster topoisomerase I cDNA probe for Northern blot analysis of DNA topoisomerase I mRNA, polymerase-chain reaction (PCR) technology was used with two primers based on the sequence of human topoisomerase I. Briefly, 30 μg of total cellular RNA isolated from V79 cells was mixed with 100 pmol of 3'-ended human topoisomerase I primer (-GTCTGTGGCTTGAACTTCCAGCTTC-), incubated in a buffer containing 50 mM Tris–HCl, pH 8.2, 6 mM $MgCl_2$, 100 mM NaCl, 6 mM DTT and 2.5 mM dNTP at 90° for 1 min, and then put on ice immediately. Twenty-five units of Avian myeloblastosis virus reverse transcriptase was added later. The reaction mixture was incubated at 42° for 1 hr, following which 100 pmol of 5'-ended human topoisomerase I primer (-CTATTTATGGAG-AACAAGCAGCCCG-) and 10 μL of 10X PCR buffer were added. The reaction mixture was heated to 97° for 7 min and 2.5 units of Taq DNA polymerase was added after the reaction mixture had cooled to room temperature. The reaction scheme included heating at 72° for 2 min followed by 30 cycles of PCR. Each cycle included reaction for 30 sec at 95°, 30 sec at 55°, and 2 min at 72°. The products of PCR were separated on a 1.5% low melting agarose gel. The desired band was recognized by Southern blot analysis with human topoisomerase I cDNA and followed by extraction with phenol, phenol/chloroform and chloroform, as described in the report of Maniatis *et al.* [34]. To determine whether this probe is a real Chinese hamster topoisomerase I cDNA, direct sequencing of the PCR product was performed. The method of generation of ssDNA template and the annealing of [^{32}P]-rATP-labeled primer to ssDNA template were described previously [35]. The sequence reaction was performed by following the protocol of the U.S.B. Co. (Cleveland, OH). The sequence was analyzed using the Beckman Microgenie Sequence Analysis program.

RNA isolation and Northern blot analysis. The method for the isolation of total cellular RNA was described previously [36]. Total cellular RNA (30 μg) was electrophoresed in a 1% agarose gel and then transferred to a Hybond-N membrane. Next, the filter was probed with Chinese hamster topoisomerase I cDNA, β-actin or MDR 1 [37]. The filter was washed with 2 × SSC with 0.2% SDS at room temperature for 1 hr and followed by washing with 0.1 × SSC with 0.2% SDS at 55° for another hour. The filter was then exposed to Kodak X-Omat AR film. mRNA was quantitated by densitometry of the autoradiogram.

CPT uptake study. The method for determination of intracellular accumulation of CPT was described previously [18, 38]. The parent and CPT-resistant cells were harvested with a rubber scraper, and 1×10^6 cells in 3 mL of culture medium were then incubated with different concentrations of CPT for 20 min at 37°. The cell pellets were resuspended in PBS with 0.01 N HCl and homogenized by sonication, using a Branson model Sonifier 450 for 30 sec at an output control setting of 2. The intensity of CPT-induced fluorescence was determined by a spectrofluorometer, and the concentrations of CPT by extrapolation from a standard curve. The excitation wavelength was 370 nm and the emission detection wavelength was 430 nm.

RESULTS

Sensitivity to CPT, epipodophyllotoxin and vincristine. The parental and resistant cell lines were exposed to various concentrations of different anticancer agents to determine the concentration required for inhibition of 50% of cell growth. The V79^r, IRS-1^r and IRS-2^r were, respectively, 140-, 25- and 340-fold more resistant to CPT than their parental cell lines (Table 1). Verapamil, which could reverse P-glycoprotein-170 associated multidrug resistant phenotype, did not alter the cytotoxicity of CPT toward either parental or resistant cell lines. V79^r and IRS-2^r were about 3- to 5-fold more sensitive to VP-16 and its analogue, NPF, when compared with their respective parental cell lines, while IRS-1 and IRS-1^r showed less sensitivity to VP-16 than the other lines examined. Verapamil could sensitize all the cell lines to VP-16, but there was no substantial difference in sensitivity to vincristine.

PLDBs in cells induced by CPT and VP-16. CPT was capable of inducing PLDBs in both parental and resistant cells (Fig. 1A). The number of PLDBs induced by CPT reached a plateau in cells within 30 min of drug treatment and this number was dependent on the dose of CPT employed. All the resistant cell lines were less susceptible to CPT-induced PLDBs than their respective parental cell lines. At 10 μM, the number of CPT-induced PLDBs in V79^r, IRS-1^r and IRS-2^r was about one-seventh, one-half and one-sixth of that of their respective parental cell lines. Verapamil did not alter the number of CPT-induced PLDBs in these cells (Fig. 1B). BP-16 could also induce PLDBs in these cell lines as shown in Fig. 2A. The number of PLDBs induced by VP-16 in V79 and IRS-2 cell lines was greater than that induced in the IRS-1 cell line. The number of PLDBs induced by VP-16 in V79^r cells was markedly greater than that in V79 cells at all concentrations of VP-16 examined. It was only at the concentration of 20 μM VP-16 that the PLDBs in IRS-2^r was more than in IRS-2. In contrast, neither IRS-1 nor IRS-1^r could be induced to generate PLDBs by 10 μM VP-16 (data not shown). PLDBs were induced at 20 μM VP-16 but the number of PLDBs in IRS-1^r cells was only one-quarter of that in IRS-1 cells. Verapamil could markedly increase the generation of PLDBs induced by VP-16 (Fig. 2B).

Alteration in CPT uptake. The decrease in the number of CPT-induced PLDBs in these CPT-resistant cells could be due to a decrease in the uptake of drug and/or an alteration of the intracellular target: DNA topoisomerase I. Thus, the uptake of CPT was examined by taking advantage of the fluorescent property of CPT and using the same method described previously by others [34].

Table 1. Cytotoxicity of various drugs toward parental and camptothecin (CPT)-resistant cell lines

Cell line	CPT	CPT + verapamil*	VP16	VP16+ verapamil	NPF	Vincristine†
			IC_{50} (μM)			
V79	0.05 ± 0.006	0.05 ± 0.01	0.7 ± 0.03	0.26 ± 0.1	0.12 ± 0.04	0.04 ± 0.004
V79^r	7.2 ± 1	7.0 ± 0.5	0.16 ± 0.04	0.05 ± 0.01	0.04 ± 0.01	0.02 ± 0.002
IRS-1	0.03 ± 0.004	0.03 ± 0.002	1.8 ± 0.2	0.7 ± 0.05	0.4 ± 0.1	0.05 ± 0.01
IRS-1^r	0.7 ± 0.06	0.75 ± 0.05	2.9 ± 0.7	1.0 ± 0.3	0.5 ± 0.13	0.04 ± 0.005
IRS-2	0.01 ± 0.002	0.01 ± 0.003	0.7 ± 0.1	0.2 ± 0.06	0.15 ± 0.05	0.04 ± 0.002
IRS-2^r	3.4 ± 0.1	3.5 ± 0.2	0.15 ± 0.05	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01

All values are means ± SD of at least three experiments, except where noted.
* The concentration of verapamil used was 10 μM.
† Each value is the average ± range of two experiments.

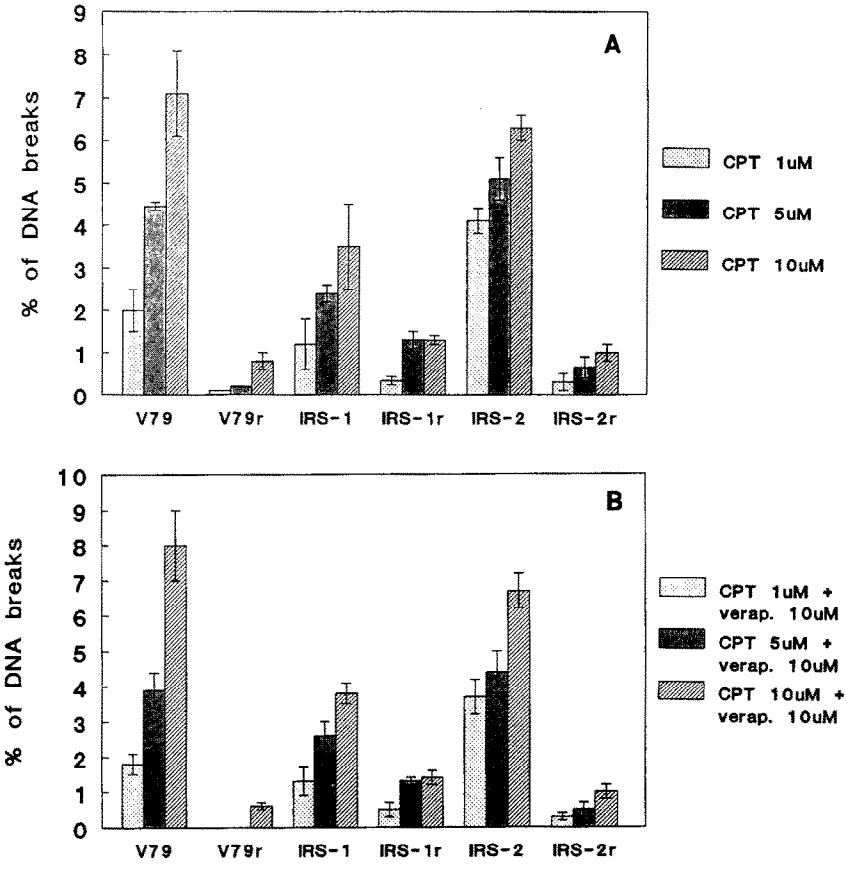

Fig. 1. Camptothecin-induced protein-linked DNA breaks in the absence (A) or presence (B) of 10 μM verapamil. The parental and resistant cells were incubated with 1, 5 and 10 μM CPT for 30 min at 37°. The experimental procedures are described in Materials and Methods. Each value is the average ± range of duplicate determinations from a representative of three experiments.

The uptake of CPT reached a steady state in 20 min (data not shown) and the concentration of CPT intracellularly was similar in all three parental cell lines. There was no difference in uptake of CPT between V79 and V79^r; however, there was a substantial decrease in the uptake of CPT in IRS-1^r and IRS-2^r cells when compared with their respective parental cell lines (Fig. 3).

Alteration of DNA topoisomerase I and II activity. To determine whether there was a change of the intracellular target, DNA topoisomerase I, in these resistant cells, the activity of the enzymes in crude

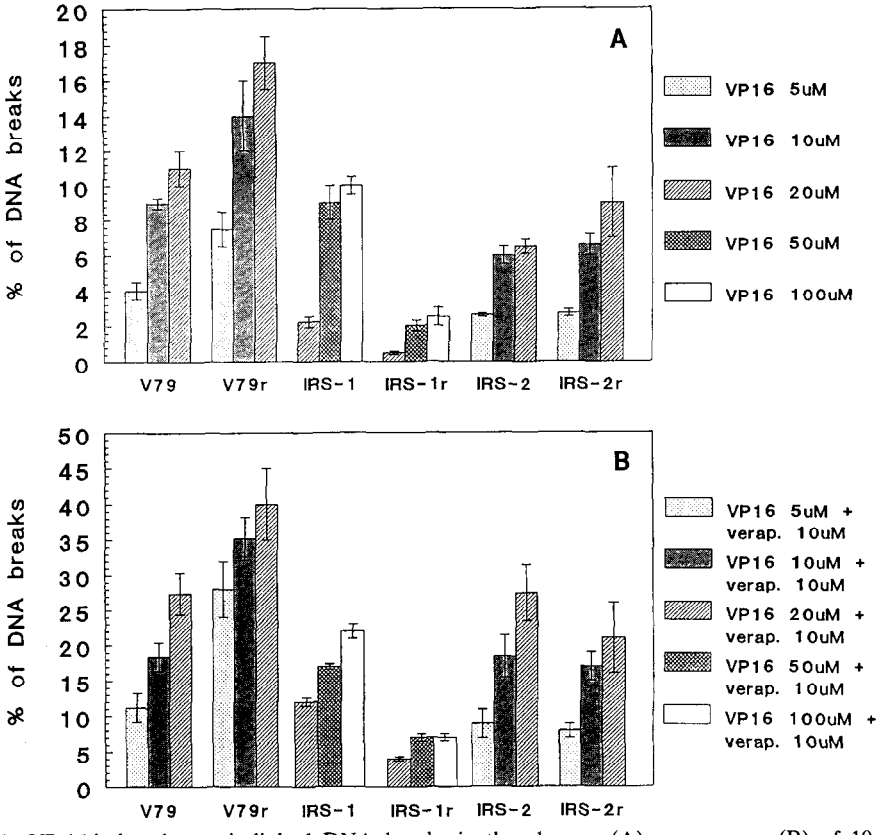

Fig. 2. VP-16-induced protein-linked DNA breaks in the absence (A) or presence (B) of 10 μM verapamil. (A) The parental and resistant cells were incubated with 5, 10, 20, 50 and 100 μM VP-16 for 30 min at 37°. (B) The parental and resistant cells were incubated with: 5 μM VP16 + 10 μM verapamil; 10 μM VP16 + 10 μM verapamil; 20 μM VP16 + 10 μM verapamil; 50 μM VP16 + 10 μM verapamil; and 100 μM VP16 + 10 μM verapamil. The procedures are described in Materials and Methods. Each value is the average ± range of duplicate determinations from a representative of three experiments.

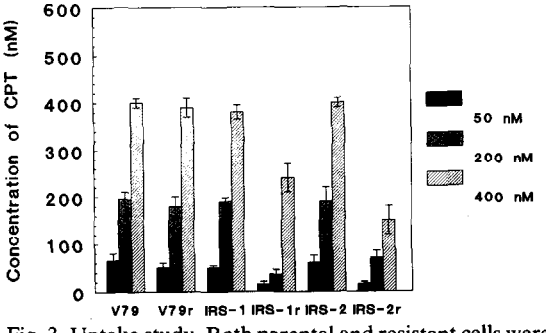

Fig. 3. Uptake study. Both parental and resistant cells were incubated with 50, 200 and 400 nM CPT for 20 min at 37°. The experimental procedures are described in Materials and Methods. Each value is the average ± range of two independent experiments.

cellular and nuclear extracts was measured (Table 2). There was 16- and 2-fold less topoisomerase I activity in both cellular and nuclear extracts from V79^r and IRS-2^r than their respective parental cell lines. The activity of DNA topoisomerase I in IRS-1 and IRS-1^r was identical.

The activity of topoisomerase II in these cell lines was also examined. They all had similar activity with the exception of IRS-1 and IRS-1^r cell lines which exhibited about half and one-third, respectively, the activity of the other cell lines.

Western blot analysis was used to assess the amount of DNA topoisomerase I and II protein in these cells. There was a strong correlation between the activity and the amount of enzyme, suggesting that the alteration of DNA topoisomerase I and II activity was due to the change in the amount of both enzymes (Fig. 4, A and B).

In all cases, except V79^r, two separate bands of about 100,000 daltons (100 kDa) in size were recognized by the human topoisomerase I monoclonal antibody. A larger protein with the molecular weight of 150 kDa in V79^4 cells, which has very low topoisomerase I activity, was also recognized by this

Table 2. Specific activity of topoisomerase I and II in cell extracts and the relative amount of topoisomerase I mRNA*

Cell line	Topoisomerase I† (U/μg protein)		Relative amount of topo. I mRNA	Topoisomerase II‡ (U/μg protein)
	Whole cell	Nuclear		Whole cell
V79	4	6	1	2
V79^r	0.25	0.4	0.03	2
IRS-1	4	6	1.38	1
IRS-1^4	3.9	5.8	1.40	0.6
IRS-2	4	6	1	2
IRS-2^r	2	2.8	0.48	2

* These data are based on one result of three independent experiments.

† One unit of topoisomerase I activity was defined as the amount of enzyme which causes the relaxation of 50% of 0.5 μg of pBR322 DNA.

‡ One unit of topoisomerase II activity was defined as the amount of enzyme which causes unknotting of 50% of 0.5 μg of P4 knotted DNA.

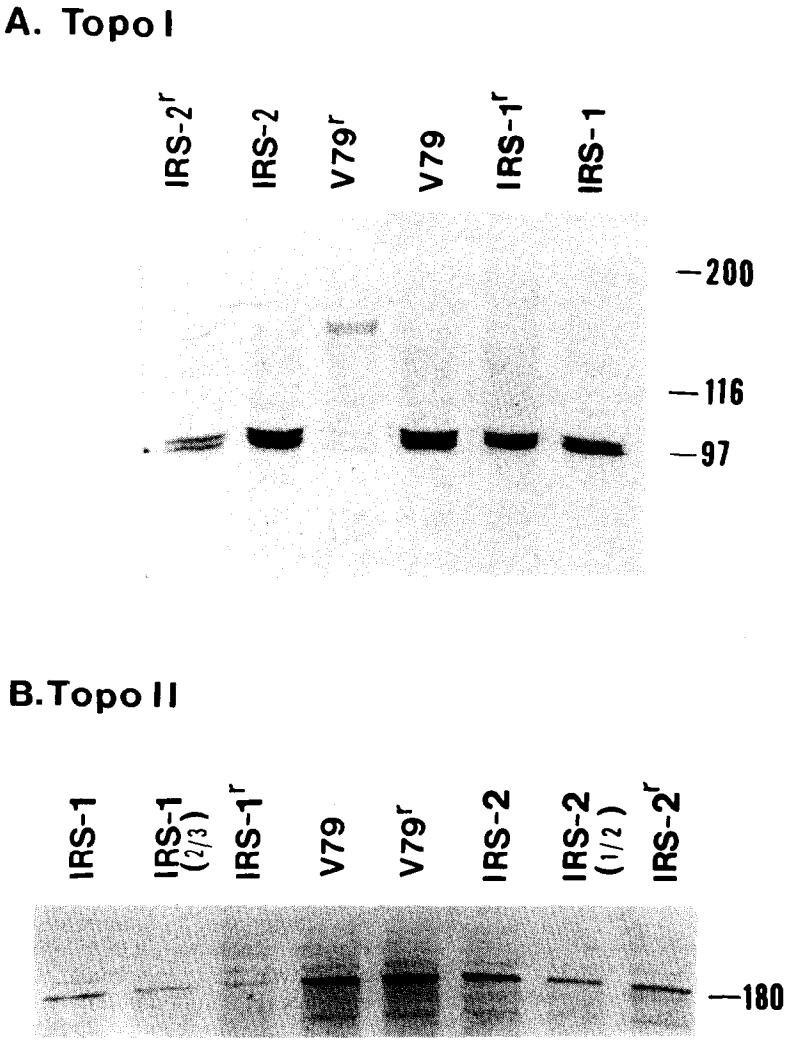

Fig. 4. Assay for topoisomerase I and II by antibody staining. The methods for this assay are described in the text. (A). From left to right, 100 μg of protein was applied to each lane. (B). From left to right, 100 μg of protein was applied to each lane except lane 2 (66.7 μg) and lane 7 (50 μg).

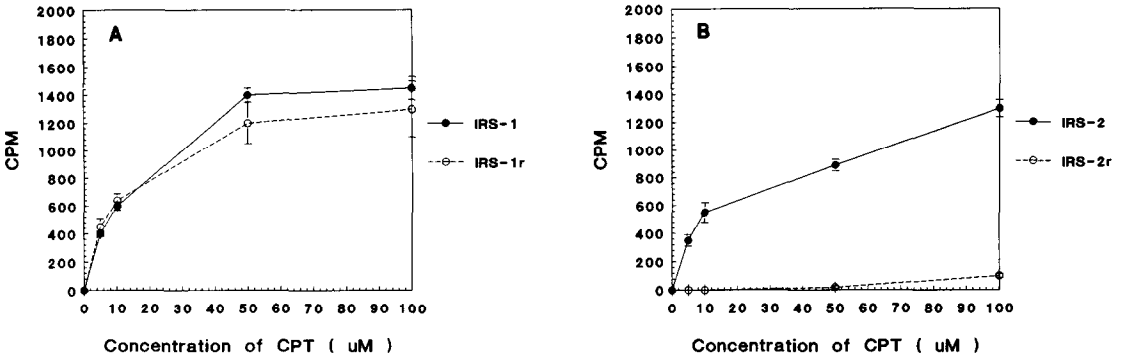

Fig. 5. Sensitivity of the topoisomerase I from IRS-1 and IRS-1^r (A), and IRS-2 and IRS-2^r (B) to CPT. 3′-Ended ^{32}P-labeled pBR322 DNA and partially purified topoisomerase I (10 units) from parental and resistant cells were incubated with 5, 10, 50 and 100 μM CPT. The samples were then precipitated by the potassium-sodium dodecyl sulfate method for analysis of the quantities of protein-linked DNA breaks. Each point is the average ± range of two independent duplicated experiments.

antibody. It is unclear whether this large protein is responsible for the low activity detected in V79^r cells.

The sensitivity of DNA topoisomerase I to CPT was measured by using partially purified enzyme extracted from all these cell lines, with the exception of V79^r which was excluded because of low topoisomerase I activity. There was no difference in sensitivity to DNA topoisomerase I among IRS-1, IRS-1^r and IRS-2 cell lines; however, the DNA topoisomerase I of IRS-2^r cells was resistant to CPT when compared to that of other cell lines (Fig. 5, A and B).

Cellular RNA was extracted from those cells and the amount of topoisomerase I mRNA was measured by Northern blot analysis using Chinese hamster topoisomerase I cDNA. This probe is 0.47 Kb. Sequence analysis indicated a 90% homology to that of the corresponding region in human topoisomerase I mRNA. The amino acid sequence had 95% homology to the corresponding region of topoisomerase I protein (data not shown). Two RnAs, 4.1 Kb and 2.3 Kb, could be identified in Chinese hamster cells, in contrast to 4.1 Kb in human cells (SK cells). (Fig. 6). Meanwhile, a larger-sized RnA, about 7.4 Kb, was detected in V79^r cells by this probe. No expression of MDR1 mRNA was found (data not shown). After normalization with the density of β-actin, the relative amount of mRNA (4.1 Kb) in V79^r, IRS-1^r and IRS-2^r cells was one-thirteenth, the same and one-half, respectively, as that of their parental cell lines. The relative amount of mRNA in IRS-1 cells was about 40% higher than that in V79 and IRS-2 cells.

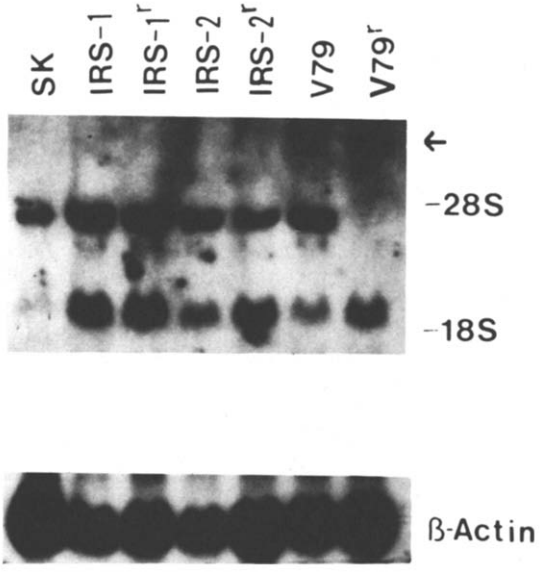

Fig. 6. Analysis of topoisomerase I mRNA expression by Northern blot hybridization. Thirty micrograms of total cellular RNA was separated in agarose-formaldehyde gels and hybridized to ^{32}P-labeled topoisomerase I cDNA probe as described in Materials and Methods. The size of the RNA band was determined relative to the position of 28 S and 18 S rRNA.

DISCUSSION

CPT exerts its cytotoxicity mainly through the formation of DNA topoisomerase I-linked DNA breaks in drug-treated cells [11]. The amount of DNA topoisomerase I-linked DNA breaks depends on the rate of uptake, the amount of intracellular accumulation and the location of the drug, as well as the activity and the properties of the DNA topoisomerase I. For the IRS-1^r cell line, there was a 45–65% decrease in uptake of CPT and no change of DNA topoisomerase I when compared with its parental cell line. This decrease in the uptake of CPT could be responsible for the decrease in the formation of CPT-induced PLDBs. This could be the cause of a 25-fold decrease in IRS-1^r sensitivity to CPT as compared to its parental cell line. For V79^r cells, a 16-fold decrease in the DNA

topoisomerase I activity without an alteration in the uptake rate of CPT when compared with its parental cell line, V79, was observed. This difference in enzyme level could be the major mechanism of CPT resistance in the V79^r cell line. The mechanism of resistance to CPT in the IRS-2^r cell line could be due to a combination of the 60% decrease in uptake of CPT, the change of sensitivity, and the decrease in the amount of DNA topoisomerase I. The irradiation-sensitive mutants were more sensitive to CPT than the parent V79 cell line, although DNA topoisomerase I activity and uptake of CPT were similar among these three cell lines. These data suggested that additional factors may contribute to the sensitivity of cells to CPT [23, 39].

Since a change in the activity of DNA topoisomerase I could be accompanied by a change in DNA topoisomerase II activity, the sensitivity of these cells to VP-16 and its analogue, NPF, was examined. CPT-resistant cell lines with down-regulated DNA topoisomerase I (V79^r and IRS-2^r cell lines) were about 3- to 5-fold more sensitive to VP-16 and NPF. VP-16 could induce more PLDBs in V79^r and IRS-2^r cell lines than in their parental cell lines, respectively, although the unknotting activity and amount of DNA topoisomerase II (180 kDa) were identical between the parental and resistant cell lines. It is conceivable that the decrease in DNA topoisomerase I activity may result in an increased reliance on a fraction of DNA topoisomerase II and that not all the DNA topoisomerase II activity in cells is required for cellular function in normal cells. More DNA topoisomerase II activity may be required for cells to function in DNA topoisomerase I deficient or altered cell lines. This phenomenon could result in hypersensitivity to an inhibitor of topoisomerase II, such as VP-16 or amasacrine. This hypothesis is also consistent with the observation that in the CPT-resistant cell line, IRS-1^r, there was no significant change in its sensitivity to BP-16 when compared with that of IRS-1. There was no difference in the activity of DNA topoisomerase I. Sugimoto *et al.* [40] reported that this CPT-resistant cell line (A549/CPT) was cross-resistant to DNA topoisomerase II inhibitors, although the DNA topoisomerase II mRNA and enzymatic activity were elevated. It is not clear whether the uptake of VP-16 was altered in their CPT-resistant cell line.

It is still unclear what mechanism could be responsible for the difference of CPT uptake in different cell lines. Unpublished results indicated that uptake was temperature sensitive. Since verapamil could not alter the uptake of CPT and no expression of MDR1 mRNA could be detected in all cell lines, the decrease of the uptake of CPT is unlikely to be related to MDR/Pg-170. It should be noted that verapamil could enhance the sensitivity of all studied cell lines to VP-16. However, this enhancement of the sensitivity to VP-16 by verapamil may also be unrelated to MDR/Pg-170. It has been observed that verapamil could enhance uptake of VP-16 into cells which have no MDR/Pg-170 expression [41].

In the cell lines studied by Western blot analysis using topoisomerase I antibody, two proteins with molecular weights of about 100 kDa were detected in all cell lines except the V79^r cell line. It is unclear whether both forms have topoisomerase I activity and what the interrelationship of these two forms is. It was observed that the relative amount of these two forms is about the same in all cell lines and their amount is proportional to the enzyme activity detected. In the V79^r cell line, a unique protein of 150 kDa was detected by Western blot analysis using topoisomerase I antibody. This protein could be responsible for the residual low topoisomerase I activity detected in the V79^r cell line. Attempts to purify this activity were not successful.

Northern blot analysis of RNA which could hybridize with a topoisomerase I probe was performed. Two RNAs, 4.1 Kb and 2.3 Kb, were detected in all cell lines except V79^r. The amount of 4.1 Kb RNA, but not 2.3 Kb, was directly related to the amount of topoisomerase I activity or protein in the cells. It is conceivable the 4.1 Kb RNA is the mRNA of topoisomerase I, and it is about the same size as the human topoisomerase I mRNA. The functional role of 2.3 Kb is unclear. The decrease of topoisomerase I activity in IRS-2^r cells is probably due to the decrease of mRNA and not the modification of the post-transcriptional process or the alteration of enzyme as evidenced by the changing sensitivity to CPT. The regulation of the expression of DNA topoisomerase I has not been clearly defined. A nonproductive gene rearrangement and/or hypermethylation of topoisomerase I gene could result in down-regulation of topoisomerase I expression [42]. The post-transcriptional regulation of topoisomerase I was also suggested. Romig and Richter [43] observed a continuous increase in the amount of topoisomerase I mRNA in serum-stimulated human fibroblasts, but the specific enzymatic activity of topoisomerase I remained constant. In the V79^r cell line, a unique 7.4 Kb RNA was detected instead of the 4.1 Kb RNA seen in the other cell lines. The 150 kDa protein detected by topoisomerase I antibody in Western blot analysis of V79^r cells could be coded by this RNA. The appearance of this transcript in V79^r cells may be due to a mutation in the topoisomerase I gene which will synthesize the transcript but is unable to process the normal Kb transcript. The decrease of topoisomerase I activity in V79^r cells could be due to this mechanism. This theory is still under investigation.

In summary, by using a stepwise method of selection, three Chinese hamster lung cell lines resistant to CPT, which may have different mechanisms of resistance, were established. The mechanisms include reduction in the intracellular accumulation of CPT and alteration of the amount or nature of the drug target, DNA topoisomerase I. In these cell lines which have down-regulation of DNA topoisomerase I, collateral sensitivity to VP-16 was observed. More cell lines are required to determine whether this collateral sensitivity is a general phenomenon. Based on the possible collateral sensitivity and lack of cross-resistance between CPT and VP-16, an alternating use of CPT or its analogues and VP-16 or NPF should be considered for further clinical protocol design. The

simultaneous use of VP-16 and CPT may not be advantageous since an antagonism between CPT and topoisomerase II-directed chemotherapeutic agents has been observed [44].

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