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Characterization of an etoposide-resistant human small-cell lung cancer cell line

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Summary. We established an etoposide (VP-16)-resistant human small-cell lung cancer cell line (H69/VP) by stepwise exposure to VP-16. The resistance of H69/VP to VP-16 was 9.4-fold that of the parent cell line (H69/P). H69/VP showed cross-resistance to Adriamycin (ADM), (4S)-4,11-diethyl-4-hydroxy-9-[(4-piperidinopiperidino) carbonyloxy]-1H-pyrano[3′,4′:6,7]indolizino [1,2-b]quinoline-3,14(4H,12H)-dionehydrochloride trihydrate (CPT-11), teniposide (VM-26), vindesine (VDS) and vincristine (VCR). The amount of DNA topoisomerase II (topo.II) was nearly the same in H69/P and H69/VP cells. The catalytic activity of topo.II in H69/VP cells was lower than that in the H69/P line. Accumulation of [^{3}H]-VP-16 in H69/VP was 6.1–7.5 times lower than that in H69/P. According to Northern blot analysis, the *mdr*-1 mRNA level in H69/VP was markedly higher than that in H69/P. These findings suggest that H69/VP has a typical multidrug resistance (MDR) phenotype and that alteration of the drug accumulation mediated by P-glycoprotein may play an important role in resistance to VP-16. Reduced topo.II activity may also be associated with VP-16 resistance.

Introduction

Etoposide (VP-16) is one of the more effective drugs against human small-cell lung cancer. Recently, combination treatment consisting of cisplatin and etoposide has come to be considered a "standard" chemotherapy for small-cell lung cancer. Although the rate of response to this regimen is high, the majority of patients die due to recurrence of the tumor. One of the main reasons for the failure of chemotherapy is the development of drug resistance. Therefore, it is very important that the mechanism of drug resistance be elucidated such that this resistance can be overcome.

The possible mechanisms of VP-16 resistance include a decrease in drug accumulation, detoxification by intracellular peptides and alterations in DNA damage or repair [9]. The target of VP-16 is thought to be DNA topoisomerase II (topo.II), which cleaves the tangling double-strand DNA and rejoins it [2, 12, 17]. Alterations in this activity or in the level of topo.II might also be associated with VP-16 resistance [12].

The purpose of this study was to establish a VP-16-resistant human small-cell lung cancer cell line (H69/VP) using the method of stepwise exposure to increasing drug concentrations and to investigate the mechanism of resistance to VP-16.

Abbreviations used: VP-16, etoposide; ADM, Adriamycin; CPT-11, (4S)-4,11-diethyl-4-hydroxy-9-[(4-piperidinopiperidino)carbonyloxy]-1H-pyrano[3′,4′:6,7]indolizino[1,2-b]quinoline-3,14(4H,12H)-dionehydrochloride trihydrate; VM-26, teniposide; VDS, vindesine; VCR, vincristine; MDR, multidrug resistance; topo.II, DNA topoisomerase II; CDDP, *cis*-diamminedichloroplatinum(II); L-PAM, L-phenylalanine mustard; BLM, bleomycin; kDNA, kinetoplast DNA; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; EDTA, ethylendiaminetetraacetic acid; topo.I, DNA topoisomerase I; CHO, Chinese hamster ovary

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Materials and methods

Chemicals. [^{3}H]VP-16 was obtained from Moravek Biochemicals (Brea, Calif.) at a radiochemical purity of 90% as measured by high performance liquid chromatography. VP-16, teniposide (VM-26) and cisplatin (CDDP) were donated by Bristol-Myers (Japan) Ltd. (Tokyo, Japan). (4S)-4,11-Diethyl-4-hydroxy-9-[(4-piperidinopiperidino)carbonyloxy]-1H-pyrano[3′,4′:6,7]indolizino[1,2-b]quinoline-3,14(4H,12H)-dionehydrochloride trihydrate (CPT-11) was obtained from Yakult Co. Ltd. (Tokyo, Japan). L-Phenylalanine mustard (L-PAM) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Bleomycin (BLM) was obtained from Nihon Kayaku Co., Ltd. (Tokyo, Japan); vindesine (VDS) and vincristine (VCR), from Shionogi Co., Ltd. (Tokyo, Japan); and Adriamycin (ADM), from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Plasmid DNA pBR322 was purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Kinetoplast DNA (kDNA) was kindly donated by Dr. M. Kuwano (Oita Medical School, Oita, Japan), who obtained *Crithidia fasciculata* from Dr. P. T. Englund (Johns Hopkins Medical School).

Topo.II antiserum was generously provided by Dr. L. F. Liu (Johns Hopkins Medical School).

Establishment of an etoposide-resistant human lung cancer cell line. We established a VP-16-resistant cell line by stepwise exposure to increasing concentrations of the drug. H69, a human small-cell lung cancer cell line, established at the United States National Cancer Institute and obtained from Dr. Y. Shimosato (National Cancer Center Research Institute, Tokyo, Japan), was used to establish the resistant cell line. The cells were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Immuno-Biological Laboratories, Fujioka, Japan), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (RPMI-FBS) at 37°C in a highly humidified incubator with an atmosphere containing 5% CO_2. The VP-16-resistant cell line was selected by stepwise and continuous exposure to the drug; the starting dose was 0.1 μg/ml, which was increased stepwise to 3 μg/ml. The established VP-16-resistant cell line could grow in the medium containing 3 μg VP-16/ml (H69/VP). The cells were used in experiments after being cultivated in drug-free medium for 7 days.

Drug sensitivity test. We determined the sensitivity of the parent and VP-16-resistant cell lines to anticancer agents by a growth inhibition assay [7]. Briefly, exponentially growing cells were collected and adjusted to 6×10^4 cells/ml. The cells were plated in 25-cm^2 flasks and incubated for 7 days at 37°C in an atmosphere containing 5% CO_2. Various concentrations of the agents were added to the culture medium. After incubation for 7 days, the cells were counted with a cell counter (TOA Microcellcounter CC-108; TOA Medical Electronics Co. Ltd., Kobe, Japan). IC_{50} values (the drug concentration that gives 50% growth inhibition) were then calculated. The nine drugs used in this study included VP-16, VM-26, ADM, VDS, VCR, CPT-11, L-PAM, CDDP and BLM.

Accumulation study. The accumulation, net uptake and efflux of [^{3}H]-VP-16 in the cell lines were determined by the method previously described by Ferguson and Cheng [5]. The parent and VP-16-resistant cells were harvested and 2×10^6 cells/ml were incubated with 10 or 30 μ*M* [^{3}H]-VP-16 at 37°C for up to 60 min. For the study of net uptake, 0.5 ml cell suspension was transferred to other tubes and washed three times with ice-cold phosphate-buffered saline (PBS) at each time point. Subsequently, 0.5 ml 1 *N* NaOH was added to dissolve the cell pellet, then 0.5 ml 1 *N* HCl was added to neutralize the NaOH. The protein concentration of part of the sample was measured by the method of Lowry et al. [13], and the rest was transferred to ACS II solution (Amersham Japan Co., Tokyo, Japan). The radioactivity was measured with a Beckman liquid scintillation counter (LS 3801, Beckman Instruments Inc., Irvine, Calif.). For the efflux study, after incubation of the cells with RPMI-FBS containing 10 or 30 μ*M* [^{3}H]-VP-16 at 37°C for 60 min, they were washed twice with drug-free RPMI-FBS medium. The subsequent process was carried out as described above.

Preparation of nuclear extracts. Crude nuclear extracts were prepared as previously reported by Deffie et al. [4]. Cells were collected by centrifugation and washed twice with cold NB [2 m*M* K_2HPO_4, 5 m*M* $MgCl_2$, 150 m*M* NaCl, 1 m*M* ethylene glycol-bis(β-aminoethyl ether), *N,N,N',N'*-tetraacetic acid, and 0.1 m*M* dithiothreitol, adjusted to a pH of 6.5]. The cells were resuspended in 1 ml cold NB, and 9 ml cold NB containing 0.35% Triton-X 100 and 1 m*M* phenylmethylsulfonyl fluoride was added. The cell suspension was put on ice for 10 min and then washed with Triton-X 100-free, cold NB. Nuclear protein was eluted for 1 h at 4°C with cold NB containing 0.35 *M* NaCl. A solution of nuclear protein was obtained by centrifugation at 17,000 *g* for 10 min. Protein concentration was determined by the method of Bradford [1].

DNA topoisomerase II content. Using Western blot analysis with topo.II antiserum, we determined the topo.II content. In brief, 0.5 vol. sampling buffer, which consists of 187.5 m*M* TRIS-phosphate buffer, 6% sodium dodecyl sulfate (SDS), 3% 2-mercaptoethanol, 1.5 m*M* ethylendiaminetetraacetic acid (EDTA), 30% glycerin and 0.01% bromophenol blue, was added to the eluted nuclear protein solution. Electrophoresis was then carried out on a 5%–15% linear-gradient polyacrylamide gel. Proteins were electrically transferred to a nitrocellulose membrane (Nihon Millipore Ltd, Tokyo, Japan) by the method of Towbin et al. [19], using an LKB-Nova blot apparatus. The membrane was incubated with blocking buffer for 2 h and was then allowed to react with topo.II antiserum for 16 h at 4°C. After incubation, the membrane was washed four times with PBS containing 0.05% Tween-20 and then incubated with biotinylated anti-rabbit IgG antibody at room temperature for 30 min. The bands were detected with an alkaline phosphatase-avidin-biotin system kit (Vectastain ABC-AP Kit; Funakoshi Pharmaceutical Co. Ltd., Tokyo, Japan). We determined the topo.II content of the parent and VP-16-resistant lines by this method. The cells were collected and disrupted by the sonicator, and the subsequent process was carried out as described above.

DNA topoisomerase II activity. The reaction mixture consisted of 50 m*M* TRIS-HCl (pH 8), 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 0.5 m*M* EDTA, 10 μg bovine serum albumin/ml, and 1 m*M* adenosine 5'-triphosphate (pH 7.7). Topo.II catalytic activity was determined by decatenation of kDNA ([14]; Matsuo et al., submitted for publication). Crude nuclear extracts from parent and VP-16-resistant cells, 1 μg kDNA and the reaction mixture were incubated at 30°C for 30 min, followed by the addition of 5 μl dye solution containing SDS, bromophenol blue and glycerol. The samples were then electrophoresed on 1% agarose gel in TRIS-acetate-EDTA at 60 V for 3 h. The gels were stained with ethidium bromide and photographed under UV light.

RNA extraction and Northern blotting. RNA was extracted from the parent and VP-16-resistant cell lines by the acid guanidinium thiocyanate-phenol-chloroform extraction method [3]. Then, 20 μg total RNA was electrophoresed on a 1% agarose-6% formaldehyde gel at 50 V for 6 h. The RNA was transferred to a positively charged nylon membrane (Hybond-N+, Amersham Japan), which was then hybridized overnight with a ^{32}P-labeled DNA probe at 42°C. The probe was labeled with [α-^{32}P]-dCTP by means of a multiprime labeling system kit (Amersham Japan). After hybridization, the membrane was washed three times with 2 × SSC (1 × SSC consists of 0.15 *M* NaCl and 0.015 *M* sodium citrate) and 0.1% SDS for 10 min. Next, it was washed three times with 0.1 × SSC and 0.1% SDS for 15 min at 65°C, followed by several washes with 0.1 × SSC. Thereafter, the membrane was exposed to X-ray film (Amersham Hyperfilm-MP) for 5 days. The probe used, pMDR-1 (coding for human *mdr*-1), was kindly provided by I. B. Roninson (University of Illinois) [16].

DNA topoisomerase I activity. DNA topoisomerase I (topo.I) activity was determined by the relaxation of supercoiled *Escherichia coli* DNA (pBR322) [11]. The reaction mixture consisted of 100 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 0.1 m*M* EDTA, 10% glycerol and 50 m*M* TRIS-HCl (pH 7.4). The reaction mixture, 0.7 μg pBR322 and the crude nuclear extract were incubated at 37°C for 10 min. After incubation, the dye solution was added and the mixture was electrophoresed on a 0.7% agarose gel at 60 V for 4 h. The gels were stained with ethidium bromide and photographed under UV light.

Results

Drug sensitivity

Table 1 shows the cytotoxic effects of various anticancer agents in parent and VP-16-resistant cells. The IC_{50} value for VP-16 was 0.52 μg/ml in the parent line and 4.9 μg/ml in the resistant line. H69/VP was 9.4 times more resistant to the drug than was H69/P. The resistant cell line showed cross-resistance to VM-26, ADM, VCR, VDS and CPT-11, with particularly high resistance to VCR and VDS. In contrast, no cross-resistance to CDDP or L-PAM was observed. H69/VP showed collateral sensitivity to BLM (relative resistance, 0.23).

Table 1. Response of parent (H69/P) and VP-16-resistant human small-cell lung cancer (H69/VP) cell lines to various anticancer agents

Anticancer agent	IC_{50} (μg/ml)		Relative resistance
	H69/P	H69/VP	
VP-16	0.52±0.15[a]	4.9 ±0.4	9.4
VM-26	0.019±0.003	0.15±0.01	7.9
ADM	0.0071±0.0009	0.15±0.01	19.7
VCR	0.000045±0.000001	0.11±0.03	2,518
VDS	0.00000044±0.00000018	0.15±0.03	340,909
CPT-11	0.47±0.04	1.4 ±0.2	3.0
I-PAM	0.18±0.06	0.18±0.04	1.0
CDDP	0.15±0.04	0.15±0.04	1.0
BLM	0.29±0.06	0.067±0.038	0.23

[a] Mean ± SD of three determinations

Table 2. Steady-state accumulation of VP-16 in H69/P and H69/VP cells

Concentration of extracellular VP-16	H69/P (nmol/g protein)	H69/VP	H69/P / H69/VP
10 μ*M*	188	31	6.1
30 μ*M*	459	61	7.5

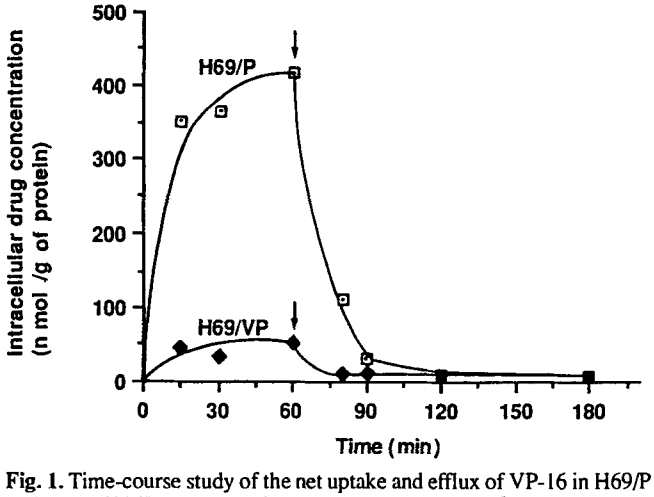

Fig. 1. Time-course study of the net uptake and efflux of VP-16 in H69/P (□) and H69/VP (■) cells after the addition of 30 μ*M* [^{3}H]-VP-16 to the medium. After a 60-min incubation (*arrow*), the cells were cultured in VP-16-free medium

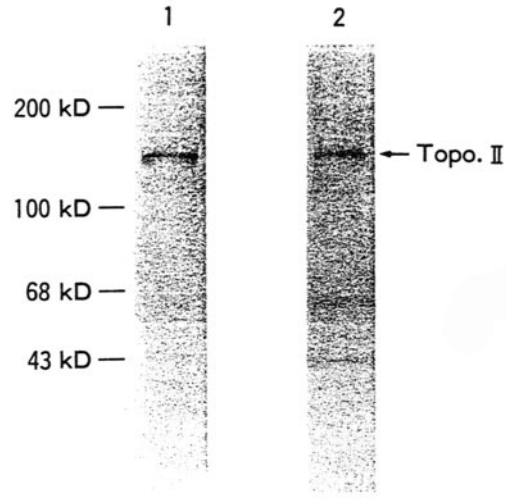

Fig. 2. Western blot analysis of DNA topoisomerase II (*Topo.II*) from nuclear extracts of H69/P and H69/VP. Proteins (100 μg) were electrophoresed on a 5%–15% linear-gradient polyacrylamide gel. *Lane 1*, H69/P; *lane 2*, H69/VP

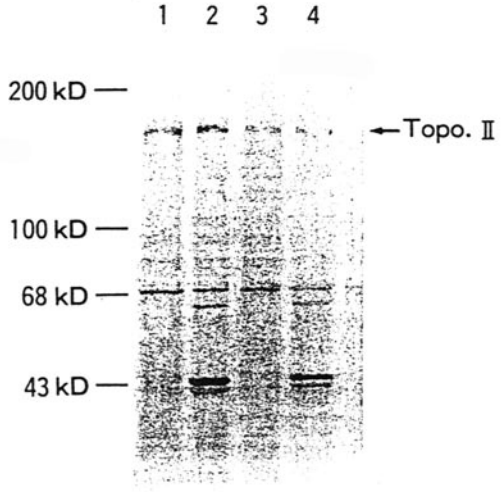

Fig. 3. Western blot analysis of DNA topoisomerase II (*Topo.II*) in H69/P and H69/VP. The proteins were electrophoresed on a 5%–15% linear-gradient polyacrylamide gel. *Lanes 1, 3*, whole-cell lysate of H69/P; *lanes 2,4*, whole-cell lysate of H69/VP; *lanes 1,2*, 100 μg proteins; *lanes 3,4*, 50 μg proteins

Accumulation study

Table 2 shows the intracellular drug concentrations after the addition of 10 or 30 μ*M* [^{3}H]-VP-16 to the culture medium. In the steady state, intracellular VP-16 concentrations in H69/P and H69/VP were 188 and 31 nmol/g protein, respectively, after 10 μ*M* [^{3}H]-VP-16 had been added to the medium. When 30 μ*M* tritiated VP-16 was used, VP-16 concentrations in H69/P and H69/VP were 459 and 61 nmol/g protein, respectively. Accumulation of VP-16 in H69/P was about 6.1- to 7.5-fold that in H69/VP.

Figure 1 shows the data of the net drug uptake and efflux in H69/P and H69/VP. The net uptake of VP-16 reached a plateau within 15 min. At 15 min, the concentration of VP-16 in H69/P was 7.5-fold that in H69/VP. In the efflux study, intracellular VP-16 levels decreased steeply in both cell lines after removal of the drug. Within 30 min, most of the intracellular VP-16 had been released from the cells.

Topoisomerase II content

The DNA topo.II content of the parent and VP-16-resistant cell lines was determined by Western blot analysis using topo.II rabbit antiserum. The level of DNA topo.II homodimer subunit relative mol. wt., (170,000 daltons) in the nuclear extract of the parent and VP-16-resistant cell lines was almost the same, as shown in Fig. 2. Also, the amount of topo.II in the whole-cell extract was almost identical in the two lines (Fig. 3). On the other hand, 43- and 68-kDa proteins were obviously overexpressed in H69/VP as com-

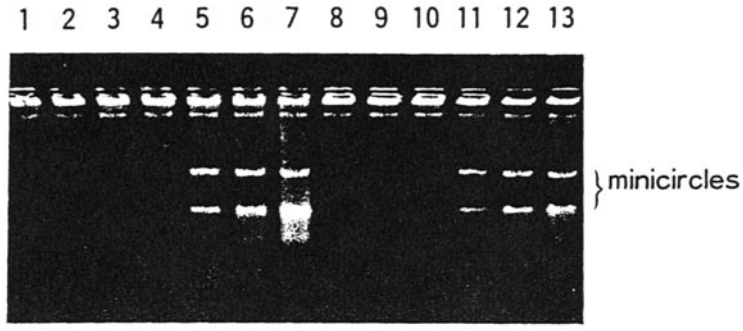

Fig. 4. Decatenation assay of DNA topoisomerase II in H69/P and H69/VP. The reaction mixture, kDNA and the nuclear extract were incubated at 30° C for 30 min. *Lane 1*, no nuclear extract; *lanes 2–7*, H69/P; *lanes 8–13*, H69/VP. Amounts of nuclear extract: *lanes 2,8*, 0.005 μg; *lanes 3,9*, 0.024 μg; *lanes 4,10*, 0.12 μg; *lanes 5,11*, 0.6 μg; *lanes 6,12*, 3 μg; *lanes 7,13*, 6 μg

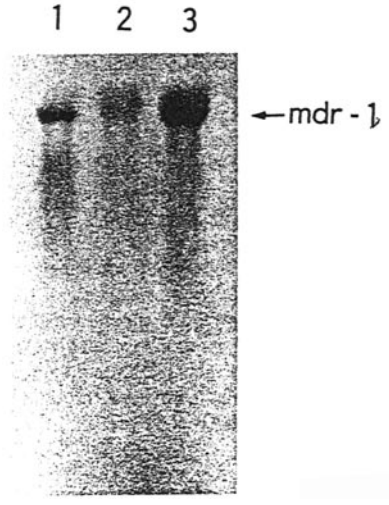

Fig. 5. Northern blot analysis of *mdr*-1 in H69/P and H69/VP. The K562 ADM-resistant cell line (K562/ADM) was used as the positive control (*lane 1*). *Lanes 2 and 3* represent the expression of *mdr*-1 mRNA in H69/P and H69/VP, respectively

pared with H69/P, although their role could not be explained. These proteins were observed in the whole-cell extract of H69/VP, but not in the nuclear extract.

Topoisomerase II activity

DNA topo.II activity in H69/P and H69/VP was determined by decatenation of kDNA. In the decatenation assay (Fig. 4), free minicircles were not obtained in the sample that did not contain the nuclear extract (lane 1). The formation of minicircles increased in both cell lines with increases in the amount of crude nuclear extract. However, fewer free minicircles were formed in H69/VP than in H69/P at the same amount of nuclear extract.

mdr-1 mRNA levels

Human *mdr*-1 (multidrug resistance phenotype 1) mRNA levels were determined by Northern blot analysis. The RNA level of an ADM-resistant human leukemia cell line (K562/ADM), which was kindly provided by Dr. T. Tsuruo (Institute of Applied Microbiology, University of Tokyo, Japan) was used as the positive control. The human *mdr*-1 mRNA level in the VP-16-resistant cell line was markedly higher than that in the parent cell line (Fig. 5).

Topoisomerase I activity

Bands of supercoiled DNA were observed only in samples containing only a small amount, if any, of the nuclear extract (Fig. 6, lanes 1, 2, 7). Relaxed forms were observed in both cell lines in the presence of >0.2 μg nuclear extract; however, relaxation of supercoiled DNA was nearly the same in the two cell lines.

Discussion

We established a VP-16-resistant human small-cell lung cancer cell line by stepwise selection with VP-16. Other VP-16-resistant cell lines selected by stepwise exposure to the drug include a human nasopharyngeal cancer (human KB cell) cell line established by Ferguson et al. [6]; human squamous carcinoma of the tongue (HN-1), established by Hill et al. [10]; and Ehrlich ascites tumor, established by Seeber et al. [18]. These VP-16-resistant cell lines show

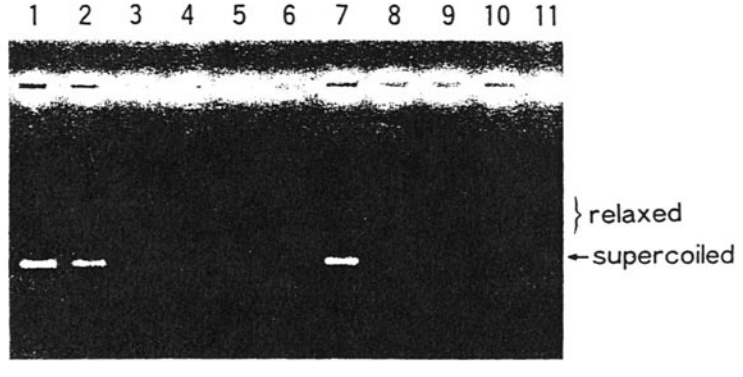

Fig. 6. Relaxation of pBR322 in nuclear extract from H69/P and H69/VP. The reaction mixture, 0.7 μg pBR322 and the nuclear extract were incubated at 37° C for 10 min; electrophoresis was performed on 0.7% agarose gel. *Lane 1*, no nuclear extract; *lanes 2–6*, H69/P; *lanes 7–11*, H69/VP. Amounts of nuclear extract: *lanes 2,7*, 0.04 μg; *lanes 3,8*, 0.2 μg; *lanes 4,9*, 0.5 μg; *lanes 5,10*, 1 μg; *lanes 6,11*, 1.8 μg

cross-resistance to ADM and VCR. Our VP-16-resistant line showed cross-resistance to VM-26, ADM, VCR, VDS and CPT-11 and have the typical multidrug resistance (MDR) phenotype.

The proposed mechanisms of resistance to VP-16 include: (1) defective drug transport, (2) modulation of cytotoxicity by intracellular detoxifying peptides and (3) alterations in DNA damage and DNA topo.II activity [9].

Seeber et al. [18] have reported that steady-state accumulation of [^{3}H]-VP-16 is reduced in Ehrlich ascites tumor cells resistant to VP-16. On the other hand, Hill et al. [10] have indicated that modification of the permeability of cells to the drug is not the main cause of VP-16 resistance because drug uptake is not changed in cells that are selected by continuous exposure to VP-16. Glisson et al. [8] suggested that the VP-16 resistance of Chinese hamster ovary (CHO) cells selected by VM-26 might be caused by the *mdr*-1 gene. In the present study, accumulation of [^{3}H]-VP-16 was reduced and overexpression of *mdr*-1 mRNA was shown in the VP-16-resistant cell line, suggesting that the mechanism of resistance in H69/VP might be a P-glycoprotein-mediated increase in drug efflux.

Some studies [2, 12, 17] have shown that VP-16 acts as an inhibitor of DNA topo.II. Glisson et al. [8] have reported that the formation of a drug-stimulated cleavage complex is decreased in VP-16-resistant CHO cells and suggested that resistance to VP-16 is caused by a mutant topo.II enzyme (qualitative alteration of topo.II). Deffie et al. [4] reported that the catenation and cleavage activity of topo.II were lower in an ADM-resistant P388 cell line than in the parent cell line, as was the topo.II content. These authors suggested that the quantitative and qualitative alterations were associated with resistance to VP-16. The mechanisms of VP-16 resistance mediated by topo.II were thought to involve the qualitative alteration of topo.II, a quantitative change in topo.II content, or the extrinsic regulation of topo.II. In our study, the topo.II content was the same in H69/P and H69/VP, but the catalytic activity of topo.II in H69/VP was lower than that in H69/P. These results suggest that the qualitative alteration of topo.II may be associated with resistance to VP-16 in H69/VP.

In addition, 43- and 68-kDa proteins were overexpressed in the whole-cell extract of H69/VP according to Western blot analysis for topo.II, but the role of these proteins could not be explained from this study.

Topo.I activity in the VP-16-resistant KB cell line was slightly increased as compared with that in the parent KB cell line [6]. However, Pommier et al. [15] reported that the parent and 9-hydroxyellipticine-resistant cell lines had similar topo.I activity. In our study, topo.I activity was nearly the same in H69/P and H69/VP, suggesting that topo.I activity is not associated with VP-16 resistance.

At least two mechanisms were considered to be involved in the MDR of H69/VP cells: (1) reduced accumulation of drug, which is thought to result from a P-glycoprotein-mediated increase in drug efflux, and (2) reduced topo.II activity.

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