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Establishment and characterization of an etoposide-resistant human small cell lung cancer cell line.

Nagio Takigawa* Taisuke Ohnoshi† Hiroshi Ueoka‡

Katsuyuki Kiura** Ikuro Kimura††

*Okayama University,
†Okayama University,
‡Okayama University,
**Okayama University,
††Okayama University,

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Abstract

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KEYWORDS: small cell lung cancer, etoposide-resistant cell line, P-glycoprotein, topoisomerase

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Nagio Takigawa*, Taisuke Ohnoshi, Hiroshi Ueoka, Katsuyuki Kiura and Ikuro Kimura

Second Department of Medicine, Okayama University Medical School, Okayama 700, Japan

An etoposide-resistant subline, SBC-3/ETP, from a human small cell lung cancer cell line, SBC-3, was developed by continuous exposure to increasing concentrations of etoposide in culture. The SBC-3/ETP was 52.1-fold more resistant to etoposide than the parent cell line. The SBC-3/ETP was highly cross-resistant to teniposide, adriamycin, vinca alkaloids, 4-hydroperoxycyclophosphamide, CPT-11 and mitomycin C, and marginally cross-resistant to cisplatin, while the subline showed a collateral sensitivity to bleomycin. Topoisomerase I activity in the SBC-3/ETP was reduced to an extent of one half and topoisomerase II activity to an extent of one eighth in comparison with those of the SBC-3. Intracellular accumulation of [^{3}H]-etoposide in the SBC-3/ETP was significantly lower in comparison to the SBC-3. An overexpression of MDR1 mRNA, and the presence of its product, P-glycoprotein, were detected in the SBC-3/ETP by Northern blotting and flowcytometry using a monoclonal antibody of the protein, MRK16. These results indicate that a decreased activity of topoisomerase II is the major factor for the development of etoposide resistance, and that an overexpression of the MDR1 gene is responsible, in part, for the development of resistance to the drug and some structurally unrelated compounds such as adriamycin and vinca alkaloids.

Key words : **small cell lung cancer, etoposide-resistant cell line, P-glycoprotein, topoisomerase**

Etoposide (ETP) is an anticancer agent with a substantial activity against a wide variety of human malignancies, and is regarded to be one of the most active agents used in the treatment of small cell lung cancer (SCLC). ETP has achieved overall response rates in SCLC ranging from 15% to 84% when used singly. The overall response rate of the agent in combination with cisplatin (CDDP), adriamycin (ADM), and/or cyclophosphamide (CPA), is over 90%, including 30-50% of complete response (1). However, the response was temporary. Almost all the patients eventually relapsed and long-term survivors still remain within 10%. The emergence of multi-drug resistance (MDR) is considered to be a major obstacle to improving the treatment results of SCLC. MDR in the chemotherapy for SCLC was defined as a striking difference in response rates to a certain agent between previously untreated and treated patients (2). Therefore, one strategy to achive a better outcome in SCLC treatment is either to circum-

* To whom correspondence should be addressed.

vent the emergence of MDR or to overcome the acquired MDR. As the most possible explanation for the mechanism of MDR, P-glycoprotein (P-gp), the product of MDR1 gene, is considered to act as a transmembrane energy-dependent pump for drug efflux.

One contribution of P-gp has been observed in a ETP-resistant subline, while, an activation of the drug detoxification system by intracellular peptides or an altered topoisomerase II activity has also been shown to affect the ETP resistance (3,4). Nevertheless, the exact mechanism of resistance to ETP still remains obscure. The aim of this study were to elucidate the exact mechanism of resistance to ETP by establishing an ETP-resistant SCLC subline and to search a way for overcoming the ETP resistance.

Materials and Methods

Chemical agents. Drugs were obtained from the following sources: ETP and carboplatin (CBDCA) from Bristol-Myers Squibb, Tokyo, Japan; teniposide (VM-26) from Bristol-Myers Squibb, New York, USA; (5R, 5aR, 8aR, 9S)-9-[[2-Deoxy-2-(dimethylamino)-4, 6-O-(R)-ethylidene-β-D-glucopyranosyl]oxy]-5, 8, 8a, 9-tetrahydro-5-(4-hydroxy-3, 5-dimethoxyphenyl) furo [3', 4': 6, 7] naphtho-[2, 3-d]-1, 3-dioxol-6 (5aH)-one hydrochloride dihydrate (NK611), CDDP and bleomycin (BLM) from Nippon Kayaku Kogyo Co., Ltd, Tokyo, Japan; vincristine (VCR), vindesine (VDS), vinblastine (VLB), 4-hydroperoxycyclophosphamide (4-HC), and cisdiammine (glycolate)-platinum (254-S) from Shionogi Co., Ltd. Osaka, Japan; ADM, navelbine (NVB), mitomycin C (MMC), and 5-fluorouracil (5-Fu) from Kyowa Hakko, Tokyo, Japan; 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin (CPT-11) and 7-ethyl-10-hydroxy-camptothecin (SN-38) from Yakult, Tokyo, Japan; methotrexate (MTX) from Lederle, Tokyo, Japan. 3-[4, 5-dimethyl-thiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co., St. Louis, MO, USA.

Cell culture and isolation of an ETP-resistant subline. The parent cell line, SBC-3, was established in our laboratory from bone marrow aspirates of an untreated patient with SCLC. The growth medium (RPMI-FBS) was RPMI-1640 supplemented with 10 % fetal bovine serum (FBS), penicillin-G (100 units/ml), and streptomycin (100 μg/ml). The ETP-resistant subline was derived from the parent SBC-3 by continuous exposure to increasing concentrations of ETP with subsequent cloning procedures. Initially, the SBC-3 cells were passaged continuously in RPMI-FBS containing 3×10^{-8} M ETP. The drug concentration was gradually elevated every 2 or 3 weeks. Finally cells growing vigorously in the medium containing 5×10^{-6} M ETP were obtained 24 months later. Two hundred and fifty cells were then plated in soft agar containing 5×10^{-6} M ETP. Two weeks later, growing colonies were selected and distributed in 24-multiwell tissue culture plates containing RPMI-FBS without ETP. After continuous growth was established, we allowed the cells to grow in 35 mm tissue culture flasks (Costar 3055) and passaged continuously. The cloning procedure was subsequently repeated again. This ETP-resistant clonal cell line was designated SBC-3/ETP and used in the current studies.

Assay of drug sensitivity. Drug sensitivity was evaluated by the MTT assay, which was modified from the original method described by Mossman (5). Fifty microliters RPMI-FBS containing serial concentrations of chemotherapeutic agents or 50 μl RPMI-FBS without agents were plated in 96-well flat bottomed microplates (Costar). Fifty microliters of tumor cell suspension containing approximately 5,000 cells in RPMI-FBS was plated to each well of the microplates, and incubated at 37 °C for 96 h in a humidified atmosphere with 5 % CO_2. Thereafter, 10 μl phosphate buffer saline (PBS) containing 50 μg MTT was added to the each well and incubated for 4 h more. After the addition of 125 μl fresh isopropanol with 0.04 N HCl to the each well, the microplates were vigorously shaken using a Direct Mix Model TS-50 (Thermal Kagaku Sangyo Co, Ltd, Tokyo) for 2 min. The absorbance of the wells at 560 nm was measured using a Model 3550 microplate reader (Bio-Rad Laboratories, CA, USA). The absorbance of wells containing drug-free medium without tumor cells was measured as background, and the absorbance of wells containing drug-free medium with tumor cells as a control. The experiment was repeated at least 3 times for each drug. The surviving cell fraction was calculated by the following formula:

> [(Mean absorbance in four test wells − absorbance in background wells)/(mean absorbance in control wells − absorbance in background wells)] × 100.

The concentration of each drug necessary to inhibit the growth of tumor cells by 50 % (IC50) was determined

by plotting the surviving cell fraction to a drug concentration on a semi-log section paper.

Cell growth rate. The growth rate of cells was determined using the MTT assay (6). Cells growing in the exponential phase were seeded in 96-well microplates. The doubling time of each cell line was estimated from the time course of cell increments determined by measuring the mean absorbance of 8 wells for 7 successive days.

Intracellular glutathione and glutathione S-transferase-π. Cells in the exponential growth phase were washed 3 times in cold PBS and sonicated with a 30 min burst using a Bioruptor (model UC100-D; Olympus, Tokyo). Glutathione (GSH) and glutathione S-transferase-π (GST-π) concentrations in the supernatant were determined after centrifuging the sonicates at 7,000 *g* for 5 min. GSH was assayed by the method reported by Tietze (7) and GST-π was assayed using a GST-π EIA kit (one step sandwich EIA; Dainihon Seiyaku, Osaka, Japan). The GSH and GST-π concentration were expressed as the ratio to mg protein determined by the method of Bradford (8).

Uptake study of [^3H]-ETP. The SBC-3 and SBC-3/ETP cells were incubated at a cell density of 1×10^6/ml at 37°C in RPMI-FBS containing 100 μM ETP supplemented with 10 % [^{3}H]-ETP (Moravek Biochemicals, Brea CA, USA). At various time intervals, triplicate 0.2 ml samples were removed from the culture and filtered with a Labo Mesh cell harvester. The cells on the filter were solubilized by Clear-sol I (Nakarai Chemicals, Ltd. Japan) and the radioactivity in the cells was determined by an Aloka LSC-700 liquid scintillation counter.

The efflux of the drug was measured subsequently. The cells incubated for 30 min in the drug containing medium were washed twice with ice cold PBS and resuspended in drug-free medium at 37°C. At appropriate time intervals, triplicate 0.2 ml samples were withdrawn, and the residual radioactivity in the cells was determined in the manner described above.

Flowcytometry. One million cells in the exponential growth phase were reacted for 30 min on ice with 20 μg/ml of MRK16 monoclonal antibody, which were kindly provided by Dr. T. Tsuruo, the Applied Microbial Institute, Tokyo University, or non-immune mouse serum IgG_{2a} as a negative control. The cells were analyzed with a FACStar flowcytometer (Becton Dickinson, CA, USA). The degree of positivity for MRK16 monoclonal antibody was expressed as the mean fluorescence intensity relative to the negative control.

RNA preparation and Northern blots. Total cellular RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction as reported by Chomczynski P *et al.* (9). Ten micrograms of total cellular RNA from each cell line was electrophoresed on a 1.5 % agarose-formaldehyde gel and transferred to a nylon membrane (Hybond-N, Amersham Corporation, IL) under vacuum (Vacu-Gene XL, Pharmacia LKB, NJ), and hybridized as described by Maniatis *et al.* (10). Probes used for hybridization were a polymerase chain reaction generated 0.8-kilobase fragment of MDR1, and a 0.77-kilobase chicken NcoI-TaqI restriction fragment of β-actin as an internal control. The probes were labelled by random priming using [α-^{32}P] dCTP (Random primed DNA Labelling Kit, Boehringer Manhaim, W. Germany). Hybridization was performed at 65°C for 24 h in 6X SSC, 5X Denhardt'reagent, 0.5 % sodium dodecyl sulfate (SDS) and 200 μg/ml denatured salmon sperm DNA. The membrane was washed stringently and exposed to Fuji RX film at −72°C for 7 days using intensifying screens.

Assay of DNA Topoisomerase Activity

Preparation of crude nuclear extracts. Crude nuclear extracts were prepared according to the method of Tsutsui *et al.* (11). All procedure were performed on ice. The cells were incubated in 1 ml of cold PBS containing 10 mM $MgCl_2$ and 0.35 % Triton X-100, for 10 min. Nuclei were pelleted by centrifugation at 1,000 *g* for 10 min, resuspended to a final concentration of 10 mg DNA/ml in an extraction buffer consisting of 20 mM Tris-HCl (pH 7.5), 0.35 M NaCl, 140 mM 2-mercaptoethanol, 50 μg/ml BSA (DNase free, Takara Shuzo, Kyoto, Japan), then incubated at 4°C for 30 min with periodic mixing. Nuclear extracts were obtained by centrifugation at 12,000 *g* for 5 min.

DNA topoisomerase I (topo I) activity. Topo I assay was performed as described by Tsutsui *et al.* (11). Plasmid DNA pBR322 was kindly provided by Dr. K. Tsutsui, Department of Molecular Biology, Institute of Cell Molecular Biology, Okayama University Medical School, Japan. The reaction proceeded at 30°C for 40 min in a 20 μl mixture containing 10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA (pH 8.0), 0.5 μg of pBR322 DNA and 1 μl of nuclear extracts. The mixture was treated with 0.66 % SDS and 0.33 mg/ml proteinase K (Sigma) prior to the analysis of DNA products by 0.8 % agarose gel electrophoresis. The gels were stained with 0.5 μg/ml ethidium bromide and photographed under UV light.

DNA topoisomerase II (topo II) activity. Topo II was assayed modifying the technique described by Miller *et al.* (12). Kinetoplast DNA (kDNA) was also kindly provided by Dr. K. Tsutsui who obtained Crithidia

fasciculata from Dr. M.T. Muller as a sance of kDNA. After incubation in a total of 20 μl containing 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA (pH 8.0), 0.5 mM ATP, 30 μg/ml BSA, 0.5 μg of kDNA and 1 μl of nuclear extracts at 30°C for 40 min, the reaction mixture was electrophoresed, and the gels were stained and photographed as mentioned above.

Results

Drug sensitivity of the SBC-3/ETP. Cell survival curves of the SBC-3 and SBC-3/ETP to ETP determined by the MTT assay are shown in Fig. 1. The value for each point represents the mean ± standard deviation of four independent experiments. The IC50 value of ETP was 0.275 ± 0.0438 μM for the parent SBC-3 and 14.3 ± 2.49 μM for the etoposide-resistant SBC-3/ETP, indicating that the SBC-3/ETP was 52.1-fold more resistant to ETP than the SBC-3 in terms of IC50. Further continuous cultivation in a drug-free medium for 2 months or longer did not lead the SBC-3/ETP to any decrease in the degree of resistance.

Patterns of cross-resistance of the SBC-3/ETP are shown in Table 1. The SBC-3/ETP was highly resistant to VM-26, VCR, VDS, VLB, NVB and ADM, showing a relative resistance index (IC50 of SBC-3/ETP/IC50 of SBC-3) of 147, 172, 290, 59.2, 94.3, and 39.5, respectively. However, the cells were not so resistant to NK611, a new epipodophyllotoxin analogue, as to ETP and VM-26. The SBC-3/ETP showed a moderately less resistance to certain drugs such as topo I inhibitors and alkylators: the cells were only 2.9 to 3.8-fold more resistant to CPT-11 and its active metabolite, SN-38; 4-HC, an active metabolite of CPA; and MMC. The cells retained a substantial sensitivity to CDDP, CBDCA, and 254-S, a new platina analogue, MTX and 5-Fu. Of note, the SBC-3/ETP was significantly more sensitive to BLM than the parent cells ($p < 0.05$), indicating a collateral sensitivity to the drug in the resistant cells.

Characteristics of the SBC-3/ETP. The doubling time of the SBC-3/ETP cells, 38.0 h, was longer than that of the SBC-3, showing a

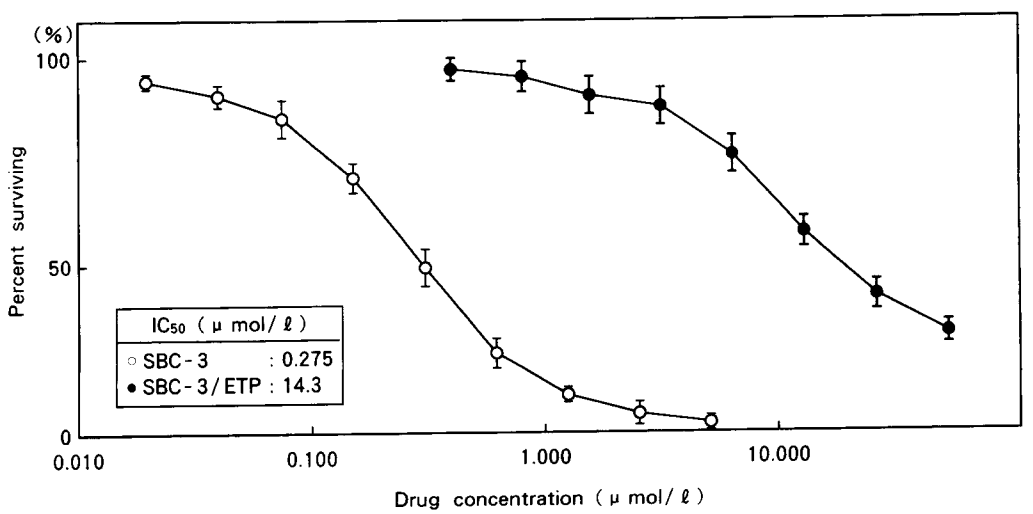


Fig. 1 Dose response curves of the SBC-3 (○) and SBC-3/ETP (●) cells to etoposide determined by the MTT assay. Drug exposure was continuous for 96 h in 96-well microplates. Each point represents the mean of four experiments ± SD.

Table 1 Patterns of cross-resistance of the ETP-resistant subline (SBC-3/ETP).

Drugs	IC50 value for (μM; $\pm$ SD)		Relative resistance index ($\pm$ SD)
	SBC-3	SBC-3/ETP	
ETP	0.275 $\pm$ 0.0438	14.3 $\pm$ 2.49***	52.1 $\pm$ 5.11
VM-26	0.0357 $\pm$ 0.0239	4.87 $\pm$ 0.751***	147 $\pm$ 7.71
NK611	1.39 $\pm$ 0.471	24.8 $\pm$ 7.80***	18.6 $\pm$ 4.03
VCR	0.00275 $\pm$ 0.000531	0.475 $\pm$ 0.103***	172 $\pm$ 5.83
VDS	0.00235 $\pm$ 0.000500	0.765 $\pm$ 0.199***	290 $\pm$ 36.6
VLB	0.00220 $\pm$ 0.000200	0.128 $\pm$ 0.0189***	59.2 $\pm$ 14.1
NVB	0.00372 $\pm$ 0.00144	0.322 $\pm$ 0.0491***	94.3 $\pm$ 30.0
ADM	0.0274 $\pm$ 0.00185	1.09 $\pm$ 0.246***	39.5 $\pm$ 7.36
CPT-11	0.236 $\pm$ 0.0531	0.903 $\pm$ 0.266***	3.79 $\pm$ 0.535
SN-38	0.000530 $\pm$ 0.000121	0.00170 $\pm$ 0.000283***	3.29 $\pm$ 0.494
4-HC	0.943 $\pm$ 0.373	2.99 $\pm$ 0.982**	3.34 $\pm$ 1.27
MMC	0.0343 $\pm$ 0.0118	0.0968 $\pm$ 0.0175***	2.93 $\pm$ 0.479
CDDP	0.660 $\pm$ 0.156	1.01 $\pm$ 0.180*	1.56 $\pm$ 0.213
CBDCA	4.67 $\pm$ 1.33	6.90 $\pm$ 1.28	1.50 $\pm$ 0.265
254-S	1.17 $\pm$ 0.126	1.67 $\pm$ 0.306	1.40 $\pm$ 0.265
MTX	0.0270 $\pm$ 0.00374	0.0320 $\pm$ 0.00163	1.20 $\pm$ 0.141
5-Fu	1.55 $\pm$ 0.404	1.54 $\pm$ 0.0304	1.02 $\pm$ 0.214
BLM	0.0438 $\pm$ 0.00995	0.0240 $\pm$ 0.0143*	0.540 $\pm$ 0.240

ETP, etoposide; VM-26, teniposide; NK611, (5R, 5aR, 8aR, 9S)-9-[[2-Deoxy-2-(dimethylamino)-4, 6-0-(R)-ethylidene-β-D-glucopyranosyl] oxy]-5, 8, 8a, 9-tetrahydro-5-(4-hydroxy-3, 5-dimethoxyphenyl) furo [3', 4': 6, 7] naphtho-2, 3-d]-1, 3-dioxol-6 (5aH)-one hydrochloride dihydrate; VCR, vincristine; VDS, vindesine; VLB, vinblastine; NVB, navelbin; ADM, adriamycin; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyl oxycamptothecin; SN-38, 7-ethyl-10-hydroxy-camptothecin (an active metabolite of CPT-11); CDDP, cisplatin; CBDCA, carboplatin; 254-S, cis-diammine (glycolate)-platinum; 4-HC, 4-hydroperoxycyclophosphamide (an active metabolite of cyclophosphamide); MMC, mitomycin C; MTX, methotrexate; 5-Fu, 5-fluorouracil; BLM, bleomycin.
Relative resistance index: IC50 of SBC-3/ETP/IC50 of SBC-3.
Statistical significance between SBC-3 and SBC-3/ETP by Student's t test: ***, $p < 0.01$; **, $p < 0.02$; *, $p < 0.05$; No asterisks, not significant.

Table 2 Characteristics of the parent cell line (SBC-3) and the etoposide-resistant subline (SBC-3/ETP) of human small cell lung cancer

Cell lines	SBC-3	SBC-3/ETP
Doubing time (h)	21.6	38.0
Intracellular GSH (μg/mg protein; $\pm$ SD)	< 0.14	< 0.14
Intracellular GST-π (μg/mg protein; $\pm$ SD)	3.15 $\pm$ 1.44	4.80 $\pm$ 0.644

doubling time of 21.6 h. Amount of intracellular GST-π in SBC-3/ETP was slightly higher than that in SBC-3. Intracellular GSH was undetectable either in SBC-3 or in SBC-3/ETP (Table 2).

Uptake study of ETP. Fig. 2 illustrates the time course of uptake and efflux of [^{3}H]-ETP in the SBC-3 and SBC-3/ETP. The net uptake of ETP reached a plateau within 5 min for SBC-3/ETP and 20 min for SBC-3. The peak intracellular accumulation of [^{3}H]-ETP was 4.0×10^{-18} mol/cell for the parent cells and 1.8×10^{-18} mol/cell for the resistant cells. The net uptake of the drug was significantly decreased in the resistant cells compared with the parent cells at 10 min ($p < 0.05$) and 20 min ($p < 0.05$).

The accumulated ETP was rapidly released not only from the SBC-3/ETP cells but also in the SBC-3 cells after replacing with the drug-free medium. However, the intracellular retention of [^{3}H]-ETP in the resistant cells was significantly

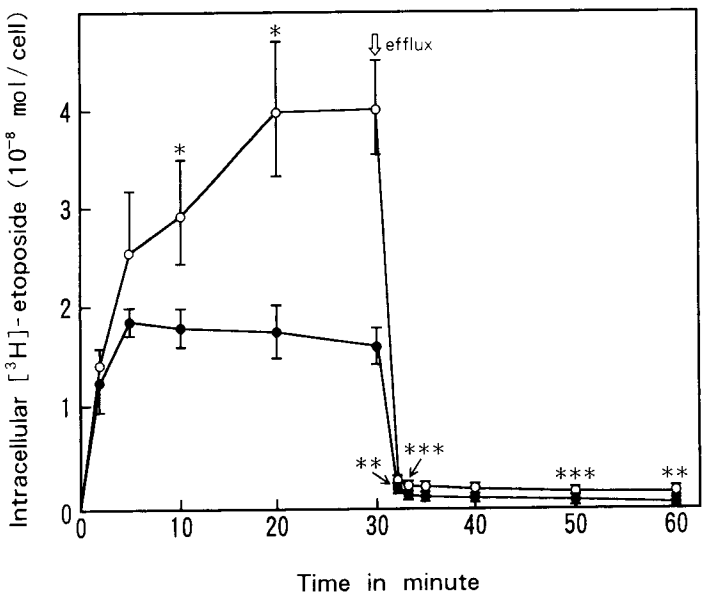


Fig. 2 Time course of the uptake and efflux of [^{3}H]-ETP in the SBC-3 (○) and the SBC-3/ETP (●) cells. After addition of [^{3}H]-ETP to cell suspensions, serial samples were withdrawn at the time indicated, and intracellular drug content was determined as described in Materials and Methods. Each point represents the mean of three experiments ± SD. Statistical significance, by Student's *t*-test: ***, $p < 0.01$; **, $p < 0.02$; *, $p < 0.05$.

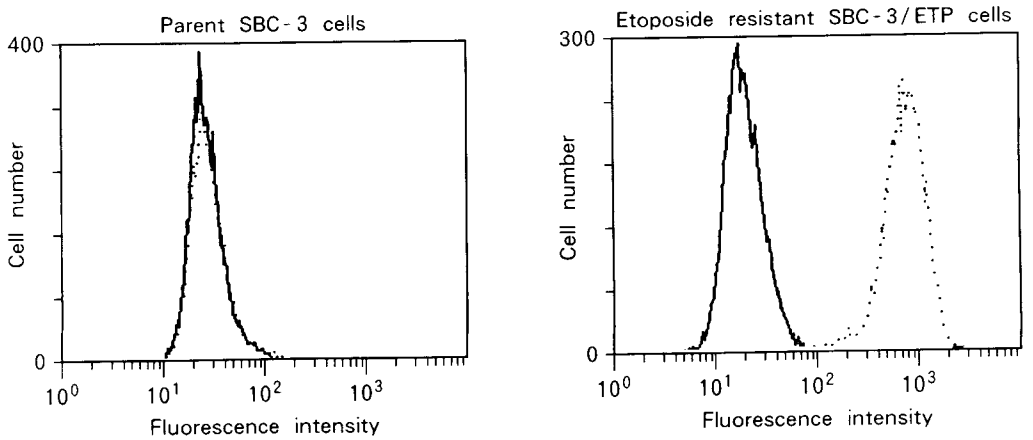


Fig. 3 Flowcytometry of the SBC-3 and the SBC-3/ETP cells. The SBC-3/ETP cells were immunostained with MRK16 monoclonal antibody. Solid lines in the figures represent a fluorescence intensity of mouse IgG_{2a}, and broken lines represent that of MRK16.

less than in the parent cells at 2, 3, 20, and 30 min after replacement with the drug-free medium ($p < 0.02$).

P-gp and MDR1 mRNA expression. P-gp was markedly demonstrated in the SBC-3/ETP cells by flowcytometry using MRK16, but it was not detected in the parent SBC-3 cells (Fig. 3). Northern blot hybridization revealed that a significant MDR1 mRNA expression was demonstrated in the SBC-3/ETP cells, but not in the SBC-3 cells (Fig. 4).

Topoisomerase I activity. The appearance of relaxed DNA bands and the disappearance of supercoiled forms are regarded as the evidence for an adequate topo I activity in the nuclear extracts. In this experiment, supercoiled forms disappeared in the presence of nuclear extracts of over 0.05 μg of the SBC-3 (Fig. 5A, lanes 1–3) and 0.1 μg of the SBC-3/ETP (Fig. 5B, lanes 1, 2). Accordingly, topo I activity of the resistant cells was considered to be one half of the parent cells.

Topoisomerase II activity. DNA topo II activity in SBC-3 and SBC-3/ETP was determined by using kDNA decatenation assay. The formation of minicircles increased in the presence of over 0.0625 μg of nuclear extract of the SBC-3 (Fig. 6, lanes 2–6). However, it increased in the presence of over 0.5 μg nuclear extract of the SBC-3/ETP (lanes 9, 10), indicat-

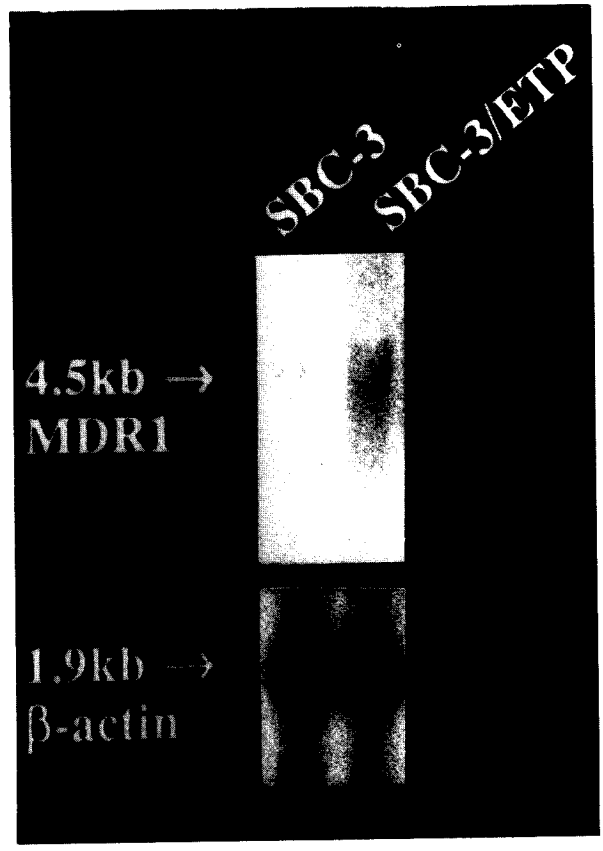


Fig. 4 Northern blot hybridization of MDR1 in the SBC-3 and the SBC-3/ETP. The MDR1 mRNA expression was detectable only in the SBC-3/ETP cells.

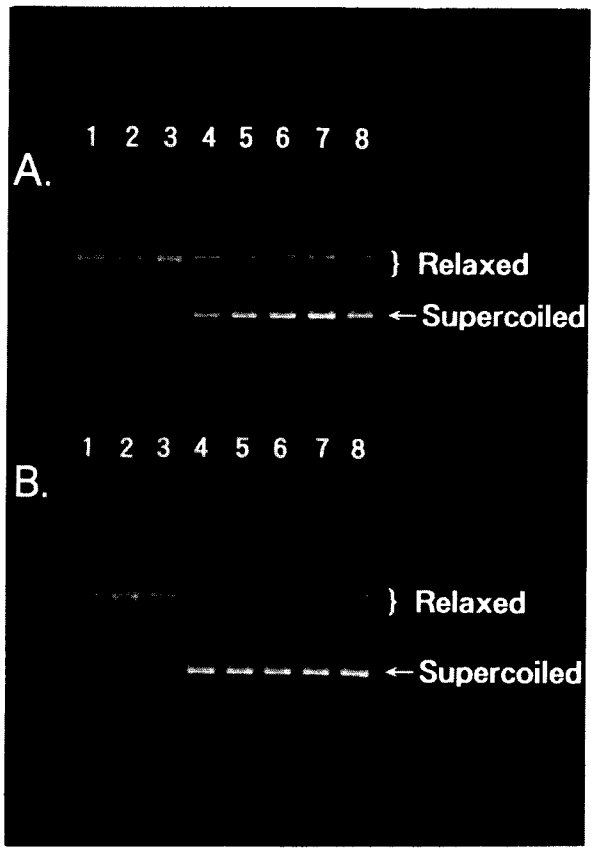
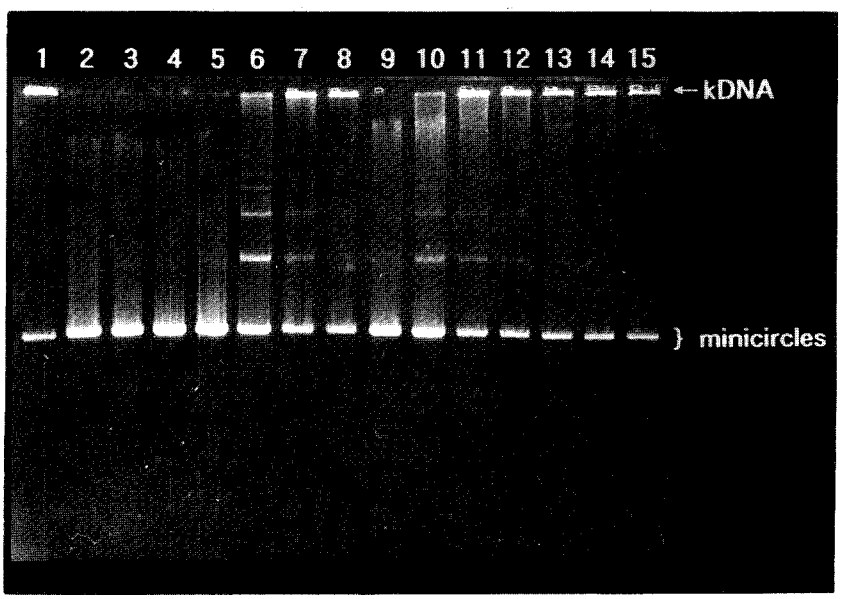


Fig. 5 (Left) Topo I activity determined by relaxation assay of pBR322 showing a decrease of topo I activity in the SBC-3/ETP. Lanes 1–8 show relaxation of pBR322 in nuclear extracts from SBC-3 and SBC-3/ETP. A indicates the SBC-3 cells and B indicates SBC-3/ETP cells. The amount of nuclear extracts was 0.2 μg for lane 1, 0.1 μg for lane 2, 0.05 μg for lane 3, 0.025 μg for lane 4, 0.0125 μg for lane 5, 0.00625 μg for lane 6, 0.00312 μg for lane 7, and 0.00156 μg for lane 8.

Fig. 6 (Bottom) Topo II activity determined by decatenation assay of kDNA showing a marked decrease in topo II activity in the SBC-3/ETP. Lane 1 represents no nuclear extract, lanes 2–8 represent nuclear extracts from the SBC-3 cells, lanes 9–15 represent those from the SBC-3/ETP cells. The amount of nuclear extract was 1 μg for lanes 2 and 9, 0.5 μg for lanes 3 and 10, 0.25 μg for lanes 4 and 11, 0.125 μg for lanes 5 and 12, 0.0625 μg for lanes 6 and 13, 0.0312 μg for lanes 7 and 14, and 0.0156 μg for lanes 8 and 15.



ing that the topo II activity of the resistant cells was one eighth of the SBC-3.

Discussion

Many ETP-resistant sublines have been established by a stepwise elevation of ETP concentration. For example, human nasopharyngeal carcinoma KB cell lines by Ferguson *et al.* (13), human squamous cell carcinoma of the tongue and testicular teratoma by Hill *et al.* (14), Ehrlich ascites tumor by Seeber *et al.* (15) and human SCLC cell line by Minato *et al.* (16) have been reported to date. These ETP-resistant sublines showed a cross-resistance to anthracyclines, vinca alkaloids and VM-26. However, they generally showed less cross-resistance or sometimes a collateral sensitivity to CDDP. An ETP-resistant human SCLC cell line, SBC-3/ETP was 52.1-fold more resistant to ETP than the parent cell line, SBC-3. This resistant subline showing a typical MDR phenotype, was highly cross-resistant to VM-26, vinca alkaloids and adriamycin, and was moderately cross-resistant to CPT-11, 4-HC and MMC. The SBC-3/ETP is distinctive from the previously reported cell lines, showing a marginal cross-resistance to CDDP, and a significant collateral sensitivity to BLM. The latter finding indicates that breaking through ETP resistance may be possible by introducing a chemotherapy regimen containing BLM in clinical trials.

As to the mechasism of ETP resistance, a reduced accumulation of [^{3}H]-ETP, a reduction of ETP-induced DNA strand breaks mediated via topo II, and an elevation of GST and/or glutathione peroxidase activity have been reported (3). Seeber *et al.* (15) have reported a reduced accumulation of ETP in an ETP-resistant subline of Ehrlich ascites tumor. Minato *et al.* (16) also demonstrated a reduced accumulation of ETP and an overexpression of the MDR1 mRNA in an ETP-resistant SCLC subline. In the present study, the uptake of ETP was markedly reduced and P-gp and MDR1 mRNA overexpression was demonstrated only in the SBC-3/ETP cells. In contrast, Hill *et al.* (3) and Glisson *et al.* (17) failed to show an impaired uptake of ETP in an ETP-resistant subline. Thus, the contribution of reduced drug accumulation in the developement of ETP resistance is still controversial.

Deffie *et al.* (18) reported a decreased topo II content, and reduced catenation and cleavage activity of the enzyme in an ADM-resistant subline. Minato *et al.* (16) and Glisson *et al.* (17) suggested that the ETP resistance in their subline is associated with a qualitative alteration of topo II. In this study, we demonstrated a marked decline in topo II activity of the ETP-resistant cell subline. In respect to topo I activity, Minato *et al.* (16) reported that the ETP-resistant subline and the parent cell line showed nearly equal activity, while Ferguson *et al.* observed an increment of topo I activity in the ETP-resistant KB subline (13). On the contrary, the SBC-3/ETP showed a little decline in topo I activity with a marked decline in topo II activity.

Regarding intracellular GSH and GST levels, no remarkable difference was noted between a squamous cell carcinoma cell line of the tongue and its ETP-resistant subline (3). In the present study, GSH levels could not be determined because of the low sensitivity of this assay. However, in an ADM-resistant subline (SBC-3/ADM100) (19) and a CDDP-resistant subline (SBC-3/CDDP) (20) established in our laboratory, an elevated GSH level has been clearly shown with a significant reproductivity (date not shown). Therefore, the contribution of GSH to the ETP resistance was considered to be minimum. On the other hand, an elevated GST-π level was demonstrated in the SBC-3/ETP suggesting a contribution of resistance to CDDP.

The decreased topo II activity in the SBC-3/ETP appears to be the major cause of resistance to ETP. And the overexpression of MDR1 mRNA is responsible, in part, for resistance to the agent and some structurally unrelated compounds such as ADM and vinca alkaloids.

Furthermore, a cross-resistance to CPT-11, a topo I inhibitor, would be due to the decreased activity of the enzyme in the resistant cells. Finally, the resistant subline described here would be useful in the screening of new drugs showing a sensitivity to ETP-resistant tumors.

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