

Characterization of a Human Small-cell Lung Cancer Cell Line Resistant to a New Water-soluble Camptothecin Derivative, DX-8951f

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DX-8951f, a water-soluble and non-pro-drug analogue of camptothecin, exhibits a strong inhibitory action on DNA topoisomerase I (Topo I) and *in vitro* cytotoxicity against various human cancer cell lines. In order to elucidate the mechanisms of its cytotoxicity, we established a DX-8951f-resistant cell line, SBC-3/DXCL1, from human small cell lung cancer cells (SBC-3) by stepwise exposure to DX-8951f. SBC-3/DXCL1 cells were approximately 400 times more resistant to DX-8951f than parent cells. The SBC-3/DXCL1 cells showed a high degree of cross-resistance to other Topo I inhibitors such as CPT-11, SN-38 and camptothecin, but not to non-Topo I targeting agents such as cisplatin, adriamycin, etoposide, and vincristine. The mechanisms of resistance of SBC-3/DXCL1 cells to DX-8951f were examined. Intracellular accumulation of DX-8951f by SBC-3 and SBC-3/DXCL1 cells did not differ significantly. Although the Topo I activity of nuclear extracts obtained from SBC-3/DXCL1 cells was the same as that of the parent cells, the Topo I of SBC-3/DXCL1 cells was resistant to the inhibitory effects of DX8951f and SN-38. Immunoblotting using anti-Topo I antibody demonstrated similar protein levels of Topo I in SBC-3 and SBC-3/DXCL1 cells. The active Topo I protein of SBC-3/DXCL1 was eluted by a high concentration of NaCl (0.4 N) compared with that of SBC-3 (0.3 N). DX-8951f stabilized the DNA-Topo I cleavable complex from SBC-3 cells, as measured by Topo I-mediated cleavage assay. In SBC-3/DXCL1 cells, DX-8951f also stabilized the DNA-Topo I complex, but with a 10-fold lower efficiency. These results suggest that a qualitative change in Topo I contributes, at least partially, to the resistance to DX-8951f in SBC-3/DXCL1 cells. Therefore, SBC-3/DXCL1 cells may have a unique mechanism of resistance to Topo I-directed antitumor drugs.

Key words: DX-8951f — Drug-resistance — Topoisomerase I — Lung cancer — Camptothecin

The DNA topoisomerases are nuclear enzymes that catalyze the concerted breaking and rejoining of DNA strands, thereby controlling the topological states of DNA. DNA topoisomerase I (Topo I) catalyzes the passage of DNA strands through transient single-strand breaks, whereas DNA topoisomerase II (Topo II) catalyzes the passage of DNA double strands through transient double-strand breaks. These topoisomerases are known to be involved in many DNA metabolic processes, including replication, recombination, transcription, and chromosome segregation at mitosis.¹ Both enzymes are considered to be potential targets for cancer chemotherapy. Eukaryotic Topo I is the target of the antitumor plant alkaloid camptothecin,²⁻⁴ and its synthetic derivatives, such as 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy camptothecin (CPT-11)⁵ and topotecan.⁶ High levels of Topo II in proliferating cells and very low levels in quiescent cells appear to explain the selective sensitivities of proliferative tumor cells to the cytotoxic effects of Topo II-targeting drugs.³ In contrast, the intracellular levels of Topo I have been reported to be largely unaffected by growth

conditions of cultured cells. However, the findings of Giovanella *et al.*⁷ that Topo I levels were elevated in advanced stage human colon cancer tissues compared with those in normal colon tissues lend support to the possibility that this enzyme is also an important target for antitumor drugs. In agreement with the above results, recent clinical trials of camptothecin derivatives have shown that these drugs are potentially promising new antitumor agents.⁸⁻¹⁰

(1*S*,9*S*)-1-Amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1*H*,12*H*-benzo[de]pyrano[3',4':6,7]indolizino[1,2-*b*]quinoline-10-13(9*H*,15*H*)-dione methanesulfonate dihydrate, DX-8951f (the chemical structure of which is shown in Fig. 1), a water-soluble and non-pro-drug analogue of camptothecin, was shown to inhibit strongly the growth of various experimental tumors, not only *in vitro*, but also *in vivo*.¹¹ The mechanism of the action of DX-8951f is through the inhibition of Topo I activity.

We have established DX-8951f-resistant cell lines, because development of drug resistance is likely to be major limiting factor in determining the clinical success of DX-8951f. The possible mechanisms of Topo I inhibitor-resistance were considered to be; 1) decreased drug accumulation mediated by ATP-dependent active drug efflux, 2) decreased Topo I activity due to decreased Topo

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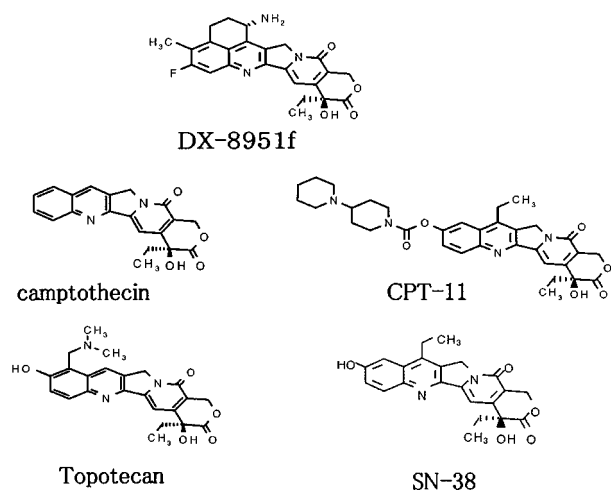


Fig. 1. Chemical structure of DX-8951f and other camptothecin derivatives.

I expression, 3) decreased Topo I activity due to *Topo I* gene mutation, or 4) decreased activity of the intracellular converting enzyme to generate the active form.¹²⁻¹⁴⁾ Decreased activity of Topo I-mediated DNA relaxation activity is generally thought to be the mechanism of acquired resistance to Topo I inhibitors. However, the DX-8951f-resistant cells described in this report have a similar activity of Topo I to the parental cells, and a novel mechanism of resistance appears to operate in them.

MATERIALS AND METHODS

Materials The camptothecin derivative DX-8951f was obtained from New Product Laboratories IV, Daiichi Pharmaceutical Co., Ltd. (Tokyo). CPT-11 and SN-38 (7-ethyl-1-hydroxycamptothecin) were obtained from Yakult Honsha Co., Ltd. (Tokyo). Cisplatin and etoposide were gifts from Bristol Myers Squibb Japan (Tokyo). Adriamycin, vincristine, and camptothecin were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo), Shionogi Pharmaceutical Co. (Osaka), and Sigma Chemical Co. (St. Louis, MO), respectively. Plasmid pBR322 DNA was purchased from Takara Shuzo Co., Ltd. (Kyoto).

Cell culture and isolation of DX-8951f-resistant cell lines The parental cell line used to obtain DX-8951f-resistant sublines was SBC-3, which is sensitive to DX-8951f and CPT-11, and was derived from a human small cell lung carcinoma (provided by Professor Kimura, Okayama University, School of Medicine, Okayama). The cells were propagated in RPMI1640 supplemented with 10% fetal bovine serum, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 units penicillin/ml in humidified air containing 5% CO_2 at 37°C. The DX-8951f-resistant cell line was selected from

a subculture that had acquired resistance to DX-8951f as a result of stepwise exposure to DX-8951f. Cultured SBC-3 cells were exposed to DX-8951f at an initial concentration of 0.1 ng/ml (levels are expressed in terms of the methanesulfonate form of DX-8951f), for 72 h and were then washed and cultured in drug-free medium for 7 days. This treatment was repeated at least three times and the resulting cells were exposed to a 2-fold higher concentration of the drug. After treatment with 2.0 ng/ml DX-8951f, the cultured cells were cloned by limiting dilution, which resulted in the establishment of three DX-8951f-resistant SBC-3 sublines (designated SBC-3/DXCL1, DXCL2, and DXCL3).

Growth-inhibition assay The cytotoxicity of the drugs was determined by means of the tetrazolium-dye assay of Mosmann.¹⁵⁾ Briefly, 1 to 3×10^3 cells were seeded and incubated overnight in each well of a 96-well plate and then 20 μl of drug solution of the required concentration was added at time zero, to produce a final volume of 200 μl in each well. After incubation for 72 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) solution (5 mg/ml in phosphate-buffered saline, PBS) was added to each well. The plates were incubated at 37°C for 4 h, then centrifuged at 800g for 5 min, and the medium was discarded. The color reaction was induced by adding 200 μl of dimethylsulfoxide to each well, and the resulting solutions were assayed colorimetrically by measuring the absorbances at 562 and 566 nm on an EL340 microplate reader (Bio-Tec, Winooski, VT). Wells containing identical amounts of medium and MTT only were used as drug-free wells. Each experiment was performed using 6 replicate wells for each drug concentration, and at least 3 independent experiments were carried out for each cell line. The IC_{50} was defined as the drug concentration required to reduce the optical density in each test by 50%, and was calculated as; [(mean absorbance of 6 wells containing a drug - mean absorbance of 6 drug-free wells)/(mean absorbance of 6 drug-free wells - mean absorbance of 6 drug-free wells)] $\times 100$. Dose levels of CPT-11 and SN-38 were expressed in terms of the hydrochloride trihydrate forms.

Relative resistance value (RR) was defined as the IC_{50} of the resistant cell line/ IC_{50} of the parental cell lines.

Accumulation study The intracellular uptake of DX-8951f in the cell lines was determined by the method of Tsuruo *et al.*¹⁶⁾ as modified by Horichi *et al.*¹⁷⁾ In brief, exponentially growing SBC-3 and SBC-3/DXCL1 cells were collected and adjusted to a concentration of 1×10^7 cells/ml. These cells were incubated with 1 $\mu\text{g}/\text{ml}$ DX-8951f for 10 to 120 min and then washed with cold PBS. The absorbed DX-8951f was extracted with 200 μl of dimethyl sulfoxide, and then cellular protein was precipitated by the addition of 1.8 ml of absolute methanol. The fluorescence intensity of the extracts was measured with a

fluorescence spectrophotometer (Spectrofluorometer FP-770f; Japan Spectroscopic, Tokyo) at excitation and emission wavelengths of 375 and 443 nm, respectively.

Preparation of nuclear extracts and DNA Topo I activity assay Crude nuclear extracts were prepared as described by Deffie *et al.*¹⁸⁾ The cells were collected by centrifugation, washed twice with ice-cold nuclear buffer (NB) [pH 6.5, 2 mM K₂HPO₄, 5 mM MgCl₂, 1 mM ethyleneglycol-bis(α -aminoethylether)-*N,N,N',N'*-tetraacetic acid, and 0.1 mM dithiothreitol (DTT)], re-centrifuged, and resuspended in 1 ml of cold NB, then 9 ml of cold NB containing 0.35 % Triton X-100 and 1 mM phenylmethylsulfonyl fluoride was added. The cell suspension was kept on ice for 10 min, and washed with Triton X-100-free cold NB. The nuclear protein was eluted for 1 h at 4°C with cold NB containing 0.35 N NaCl. In order to determine whether the Topo I was affected by the concentration of NaCl, the nuclear protein samples were eluted with several concentrations of NaCl (0–0.4 N). A nuclear protein solution was obtained by centrifugation of each eluted fraction at 18,000g for 10 min, and the protein concentration of the supernatant was determined using the method of Bradford with bovine serum albumin (Sigma) as a standard.

The DNA Topo I activity was determined by measuring the relaxation of supercoiled *Escherichia coli* plasmid (pBR322), essentially as described by Liu and Miller.¹⁹⁾ The reaction mixtures for measuring the total Topo I activities in both cell lines comprised 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 50 mM Tris-HCl (pH 7.4), 0.5 μ g of pBR322 plasmid, and crude nuclear extracts (0.01–1.0 μ g protein). The reaction mixtures for measuring the inhibition of DNA relaxation by DX-8951f contained the specified concentrations of drug and 0.5 μ g of nuclear extract in addition to the above components. The reaction mixtures were incubated at 37°C for 20 min, and the reactions were terminated by adding 3 μ l of dye solution comprising 2.5% (w/v) sodium dodecyl sulfate (SDS), 0.01% (w/v) bromophenol blue, and 50% glycerol. These mixtures were applied to a 1% (w/v) agarose gel and electrophoresed with a running buffer of Tris-acetate EDTA, after which the gel was stained with 2 μ M ethidium bromide and photographed under transillumination with 300 nm UV light.

Immunoblotting of Topo I protein The total nuclear protein and samples fractionated by the NaCl gradient were obtained from each cell line as described above. The proteins of each fraction were co-precipitated with bovine serum albumin by addition of trichloroacetic acid at a final concentration of 15% (w/v) followed by incubation on ice for 15 min and centrifugation at 15,000 rpm for 10 min. The pellets were resolved with dye solution and denatured at 95°C for 3 min. Samples were analyzed

using polyacrylamide slab gels containing 10% SDS. Nuclear protein (10 μ g) from each type of cell was applied to the gel, electrophoresed, and then transferred to polyvinylidene-difluoride membranes (Immobilon, 0.45- μ m pore diameter, Nihon Millipore Kogyo, Tokyo). The membranes were blocked by incubation in 5% (w/v) skim milk in PBS for 24 h at 4°C, incubated at room temperature for 2 h with human anti-Topo I antibody (TopoGEN, Columbus, OH) diluted with PBS to the required concentration, and then rinsed with PBS containing 0.1% (v/v) Triton X-100. Next, the membranes were incubated with anti-human immunoglobulin G (Fc)-horseradish-peroxidase conjugate diluted with PBS containing 5% skim milk for 1 h at room temperature, and finally washed with PBS containing 0.1% Triton X-100. The membranes were processed with chemiluminescence detection reagents (Amersham, Aylesbury, UK).

Formation of Topo I-DNA-DX-8951f complex Crude nuclear extracts (0.7 μ g) from each cell line were incubated with 0.5 μ g of pBR322 DNA and DX-8951f in the reaction buffer (100 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 10% glycerol, 50 mM Tris-HCl) at 37°C for 20 min. Reactions were terminated by the addition of SDS (final concentration, 1%), and Topo I was digested for 30 min at 37°C with 50 μ g of proteinase K. After digestion, samples were mixed with dye solution (2.5% SDS, 0.01% bromophenol blue, and 50% glycerol), and these mixtures then were applied to a 1% (w/v) agarose gel containing 0.5 μ g/ml ethidium bromide and electrophoresed with a running buffer of Tris-acetate EDTA containing 0.5 μ g/ml ethidium bromide. DNA was visualized by transillumination with 300 nm UV light.

RESULTS

Establishment of DX-8951f-resistant SBC-3 cells Cultured SBC-3 cells were exposed to DX-8951f at an initial concentration of 0.1 ng/ml for 72 h and were then washed and cultured in drug-free medium for 7 days. This treatment was repeated at least three times and the resulting cells were exposed to a 2-fold greater concentration of the drug. After the final treatment with 2.0 ng/ml DX-8951f, the cultured cells were cloned by limiting dilution and cultured in drug-free medium for 10 weeks. We obtained three DX-8951f-resistant SBC-3 sublines (designated SBC-3/DXCL1, DXCL2, and DXCL3). Of these, SBC-3/DXCL1 was selected for comparative studies because of its high degree of resistance to Topo I inhibitors and its stability. The doubling times of SBC-3 and SBC-3/DXCL1 cells were 14.0 and 33.3 h, respectively. The cloning efficiency and cell size of SBC-3/DXCL1 did not differ from those of the parental cells.

Sensitivities of SBC-3/DXCL1 cells to antitumor drugs Table I shows the cytotoxic effects (IC₅₀; μ g/ml or ng/ml)

Table I. Cytotoxicity of Various Antitumor Drugs against SBC-3 and DX-8951f-resistant Clones in the MTT Assay

Drugs	<i>In vitro</i> cytotoxicity (IC ₅₀ ±SD µg/ml ^{a)})						
	SBC-3	SBC-3/DXCL1	RR ^{b)}	SBC-3/DXCL2	RR	SBC-3/DXCL3	RR
DX-8951f (ng/ml)	0.078±0.028	31.2±5.79 ^{c)}	397.2	36.3±18.9 ^{c)}	462.6	7.70±1.84	98.1
CPT-11	0.343±0.037	46.9±0.7	136.7	35.5±7.07	103.5	27.1±1.98	79.0
SN-38 (ng/ml)	0.391±0.272	176.7±100.1	1722.5	113.5±4.95	1741.7	129.0±26.8	1237.7
cisplatin	0.087±0.022	0.423±0.27	4.88	0.397±0.20	4.57	0.418±0.023	4.82
adriamycin	0.0043±0.0013	0.0185±0.0019	4.13	0.0245±0.0207	5.71	0.0192±0.0103	4.49
vincristine	0.0015±0.0011	0.0019±0.0012	1.30	ND	—	ND	—
etoposide	0.048±0.035	0.035±0.024	0.73	ND	—	ND	—

- a) Drug concentration inhibiting cell growth by 50%. Dose levels of DX-8951f were expressed in terms of the methanesulfonate form. Dose levels of CPT-11 and SN-38 were expressed as hydrochloride trihydrate forms.
- b) Relative resistance value equals the IC₅₀ value of the resistant cell line divided by that of the parent cell lines.
- c) Each value is the mean±SD of 3 independent experiments.

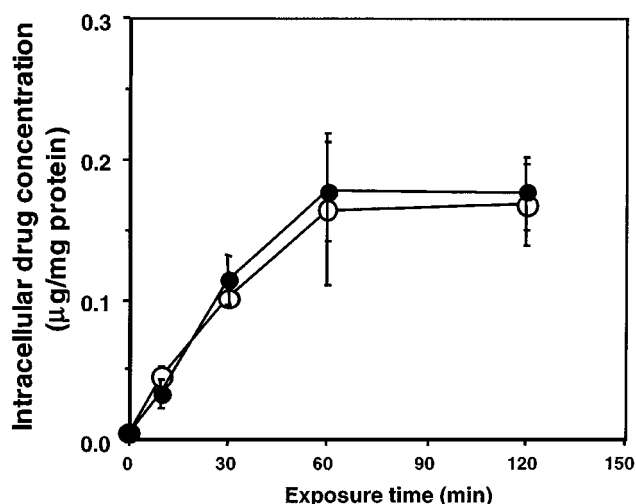


Fig. 2. Time course study of intracellular accumulation of DX-8951f in SBC-3 (●) and SBC-3/DXCL1 (○) cells; 1×10⁷ cells in 10 ml of culture medium were exposed to 1 µg/ml DX-8951f at 37°C for 0–120 min. Intracellular concentrations of DX-8951f at each time point were determined by spectrofluorometry. Dose levels of DX-8951f are expressed in terms of the methanesulfonate form.

of various antitumor drugs against SBC-3 and its resistant sublines. SBC-3/DXCL1 was approximately 400 times more resistant to DX-8951f than the parental cells, and showed a high degree of cross-resistance to Topo I inhibitors, such as CPT-11 (×~140), SN-38 (×~450), and camptothecin (×~1700). Only a relatively small degree of cross-resistance to cisplatin and adriamycin was observed. SBC-3/DXCL1 showed no definite cross-resistance to etoposide or vincristine. Therefore SBC-3/DXCL1 cells are highly and specifically resistant to Topo I inhibitors.

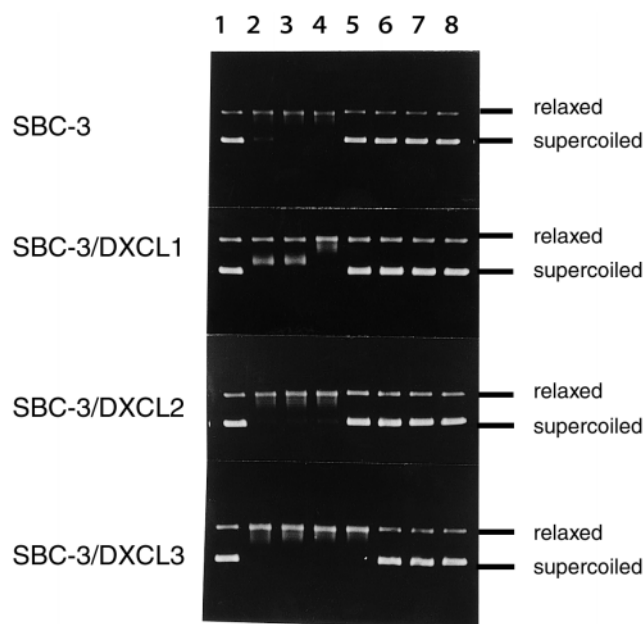


Fig. 3. Topo I activities of DX-8951f-sensitive and -resistant SBC-3 cells. The enzyme activity was determined by measuring the relaxation of supercoiled DNA (pBR322) during incubation with nuclear extract at 37°C for 10 min, using electrophoresis on a 0.7% agarose gel for 4 h. Lane 1, no nuclear extract (control); lane 2, 1.0 µg; lane 3, 0.5 µg; lane 4, 0.2 µg; lane 5, 0.1 µg; lane 6, 0.05 µg; lane 7, 0.02 µg; lane 8, 0.01 µg nuclear protein from SBC-3 cells and SBC-3/DXCL1 cells were added.

Intracellular accumulation of DX-8951f To determine whether the resistance of SBC-3/DXCL1 cells to DX-8951f was related to the decreased drug accumulation in cells, we examined the intracellular accumulation of DX-8951f in SBC-3 and SBC-3/DXCL1 cells (Fig. 2). SBC-3

and SBC-3/DXCL1 cells were incubated with 1 $\mu\text{g/ml}$ DX-8951f. The intracellular drug concentrations increased time-dependently in both cell lines, reaching saturation at 60 to 90 min. There was no significant difference in the accumulation of DX-8951f between SBC-3 and SBC-3/DXCL1 cells at any of the time points.

Activity of Topo I and inhibitory effects of DX-8951f and SN-38 on Topo I The total cellular activities of DNA Topo I of SBC-3 and SBC-3/DXCL1 in the crude nuclear extracts eluted with 0.35 *N* NaCl were measured. The relaxation of pBR322 DNA incubated with different amounts of SBC-3 and SBC-3/DXCL1 cells nuclear protein extracts is shown in Fig. 3. In this experiment, the relaxation of supercoiled DNA catalyzed by Topo I was monitored using gel electrophoresis. Relaxed forms were observed in the presence of 0.2 μg of nuclear extract from both cell lines. Surprisingly, the Topo I activity of nuclear extract obtained from SBC-3/DXCL1 cells was the same as that of the parent cells (Fig. 3). The effect of DX-8951f on the catalytic activities of DNA Topo I extracted from SBC-3 and SBC-3/DXCL1 cells was determined using 0.5

μg of their nuclear extracts. The DNA relaxation catalyzed by Topo I isolated from SBC-3 and SBC-3/DXCL1 cells was inhibited by DX-8951f in a concentration-dependent manner and was completely inhibited at 20 and 200 $\mu\text{g/ml}$, respectively (Fig. 4). Topo I catalytic activity

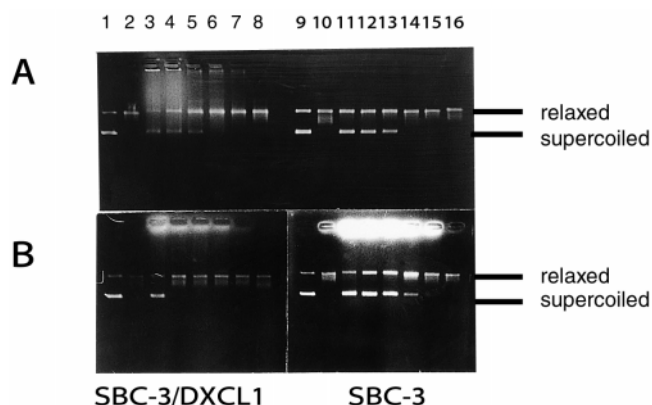


Fig. 4. Inhibitory effects of DX-8951f on DNA relaxation catalyzed by Topo I from SBC-3 and SBC-3/DXCL1 cells. The experiments are the same as those described in the legend to Fig. 3. A) Lanes 1 and 9, no nuclear extract (negative control). Lanes 2–8 and 10–16 were loaded in the presence of 0.5 μg of SBC-3/DXCL1 and SBC-3 nuclear extracts, respectively. Lanes 2 and 10, no drug (positive controls); lanes 3, 4, 5, 6, 7 and 11, 8 and 12, 13, 14, 15, and 16 contained 800, 400, 200, 100, 60, 40, 20, 10, 5, and 1 $\mu\text{g/ml}$ DX-8951f, respectively. B) Lanes 1 and 9, no nuclear extract (negative control). Lanes 2–8 and 10–16 were loaded in the presence of 0.5 μg of SBC-3/DXCL1 and SBC-3 nuclear extracts, respectively. Lanes 2 and 10, no drug (positive controls); lanes 3 and 11, 4 and 12, 5 and 13, 4 and 14, 5 and 15, and 6 and 16 contained 200, 120, 80, 40, 20, and 10 $\mu\text{g/ml}$ SN-38, respectively. Dose levels of DX-8951f are expressed in terms of the methanesulfonate form. Dose levels of CPT-11 and SN-38 are expressed in terms of the hydrochloride trihydrate forms.

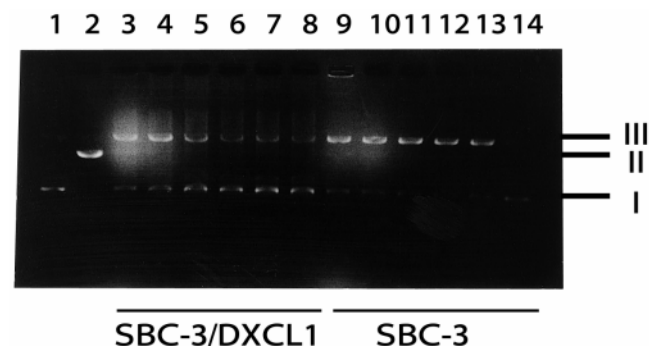


Fig. 5. DX-8951f-induced cleavage activity of Topo I in nuclear extracts from SBC-3 and SBC-3/DXCL1 cells. The DNA cleavage assay was performed as described in the text. Lane 1, pBR322 DNA (control); lane 2, linear form (control); lanes 3–8 and 9–14 were loaded in the presence of 0.7 μg of SBC-3/DXCL1 and SBC-3 nuclear extracts, respectively. Lanes 8 and 14, no drug (positive controls); lanes 3 and 9, 4 and 10, 5 and 11, 6 and 12, and 7 and 13 contained 200, 100, 60, 20, and 10 $\mu\text{g/ml}$ DX-8951f, respectively. The topological isomers of pBR322 are marked I (supercoiled), II (linear), and III (nicked) forms.

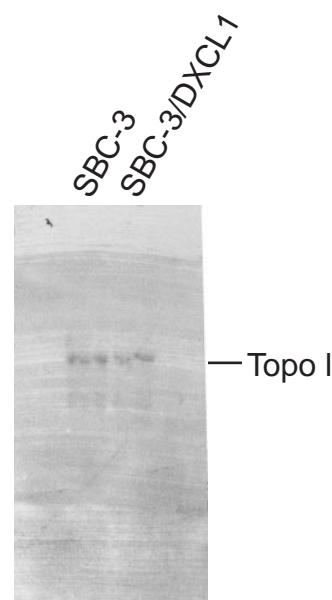


Fig. 6. Immunoblotting of Topo I in nuclear extracts from SBC-3 cells and SBC-3/DXCL1. Ten micrograms of protein was loaded onto each lane.

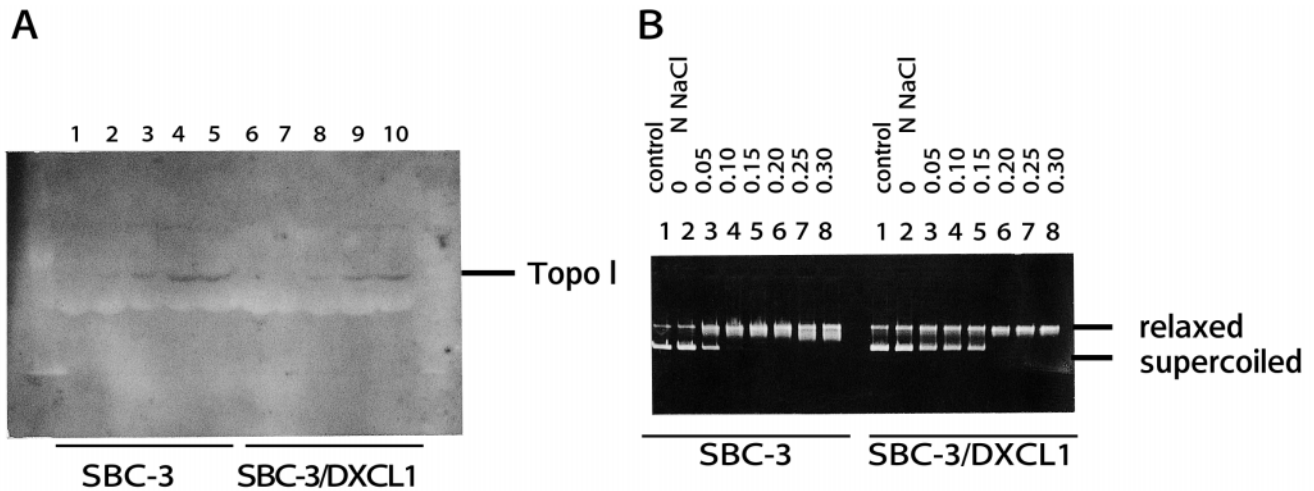


Fig. 7. Protein content and relaxation activity of Topo I in nuclear extracts eluted with increasing concentrations of NaCl. A) Immunoblotting with anti-Topo I anti-serum; lanes 1–5 and 6–10 were loaded with the eluted fractions of SBC-3 and SBC-3/DXCL1 nuclear extracts, respectively. Lanes 1 and 6, 2 and 7, 3 and 8, 4 and 9, and 5 and 10 were loaded with the fractions eluted with NB buffer containing 0, 0.1, 0.2, 0.3, and 0.4 N NaCl, respectively. B) Relaxation of supercoiled DNA by each eluted sample; lane 1, no nuclear extract (negative control). Lanes 2–8 were loaded with eluted fractions of SBC-3 (left) and SBC-3/DXCL1 (right) nuclear extracts, respectively. Lanes 2, 3, 4, 5, 6, 7, and 8 were loaded with the fractions eluted with NB buffer containing 0, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 N NaCl, respectively.

was also inhibited by 40 and 200 $\mu\text{g/ml}$ SN-38 in SBC-3 and SBC-3/DXCL1 cells, respectively. These results suggest that the Topo I of SBC-3/DXCL1 cells is resistant to the inhibitory effect of Topo I inhibitors and retains catalytic activity.

Effect of DX-8951f on DNA-Topo I cleavable complexes The formation of Topo I-DNA-DX-8951f complexes was assayed by measuring the amount of nicked DNA generated from pBR322 DNA in the presence of 0.7 μg of crude nuclear extract from each cell line (Fig. 5). The amount of nicked DNA corresponds directly to the quantity of the Topo I-DNA-DX-8951f complexes. Formation of Topo I-DNA-DX-8951f complexes was detected in a drug concentration-dependent manner, but Topo I from the nuclear extract of SBC-3/DXCL1 cells showed decreased formation of Topo I-DNA-DX-8951f complexes compared to SBC-3 cell Topo I. In SBC-3/DXCL1 cells, nicked DNA was detected at 60 $\mu\text{g/ml}$ DX-8951f, whereas in the parental SBC-3 cells nicked DNA was detected at 10 $\mu\text{g/ml}$ DX-8951f.

Qualitative change of Topo I in the SBC-3/DXCL1 cells We compared the Topo I protein content in nuclear extracts from SBC-3 and SBC-3/DXCL1 by immunoblotting (Fig. 6). The Topo I content of SBC-3 and SBC-3/DXCL1 cells did not differ significantly. In order to determine whether the Topo I in SBC-3/DXCL1 cells had undergone a qualitative change compared with that in the parental SBC-3 cells, we measured the amount and activ-

ity of Topo I in each fraction eluted with gradually increasing concentrations of NaCl (0–0.4 M). In SBC-3 cells, Topo I was detected in the 0.1–0.4 M NaCl fractions, whereas 0.2–0.4 M NaCl eluted the Topo I in SBC-3/DXCL1 cells (Fig. 7A). There was no obvious difference of protein concentration between the fractions eluted at the various concentrations of NaCl. On the other hand, the Topo I content was differently eluted by the NaCl, as shown in Fig. 7A. The Topo I in the SBC-3/DXCL1 cells appeared to elute under conditions of high salt concentration, suggesting that some qualitative change in the Topo I had occurred in the SBC-3/DXCL1 cells. The same samples were used to determine Topo I activity in each eluted fraction, as indicated in Fig. 7B. The samples in which Topo I was detected by immunoblotting possessed Topo I-mediated DNA relaxation activity. These results suggested that a qualitative change in Topo I was responsible for the decreased formation of cleavable complex in SBC-3/DXCL1 cells.

DISCUSSION

DX-8951f, a water-soluble and non-prodrug analogue of camptothecin, exhibits strong *in vitro* cytotoxicity against various human cancer cell lines.¹¹ It is more potent than SN-38 or topotecan in terms of cytotoxicity. The cellular determinants of sensitivity to Topo I inhibitors may include Topo I protein levels, the activity of the

enzyme, Topo I gene expression, and/or the formation of drug-stabilized cleavable complexes. Other cellular factors of importance for sensitivity to camptothecins may be the uptake of the drugs, the cell-cycle distribution and, for CPT-11, the endogenous carboxylesterase activity.²⁰⁾ In this study, we established and characterized the DX-8951f-resistant cell line, SBC-3/DXCL1 from a human small lung cancer cell line, SBC-3.

Changes in the cellular uptake/efflux of drugs are known to be common drug resistance mechanisms. Previous reports have suggested that the sensitivity of cells to some camptothecin analogues, in particular topotecan,²¹⁾ might be affected by classical P-glycoprotein-mediated multidrug resistance, whereas the sensitivity to the parent camptothecin was not affected by this mechanism. Taken together, these reports indicate that intracellular drug accumulation is a determinant of cellular resistance to camptothecin analogues. In this experiment, we observed that intracellular accumulation of DX-8951f by SBC-3 and SBC-3/DXCL1 cells did not differ significantly. Some studies have suggested a role for P-glycoprotein in resistance to CPT-11. For example, Mattern *et al.* have shown that the expression of P-glycoprotein may contribute to the resistance to topotecan, 9-aminocamptothecin, CPT-11, and SN-38 *in vitro*.²²⁾ *In vivo*, however, the various camptothecins were equally effective against the multidrug-resistant tumors and the parental tumors. These results indicate that the mechanism of resistance of these cells is unlikely to be related to that of typical multidrug resistance. Resistance to topoisomerase inhibitors has been found to correlate with decreased topoisomerase activity.²³⁾ Jansen *et al.* have described a positive correlation between the DNA Topo I activity and cellular sensitivity to activated CPT-11, SN-38.²⁴⁾ Topoisomerase inhibitors are cytotoxic by virtue of producing DNA lesions as a result of inhibiting topoisomerases during the course of DNA replication, and therefore drug sensitivity is directly proportional to enzyme activity. SBC-3/DXCL1 was highly resistant to DX-8951f compared to the paren-

tal cells, but the Topo I activity of SBC-3/DXCL1 cells was equivalent to that of the parental SBC-3 cells (Fig. 3).

Furthermore, Topo I in the nuclear extracts of SBC-3/DXCL1 showed decreased formation of Topo I-DNA-DX-8951f complex compared with that of SBC-3-derived Topo I (Fig. 5). Goldwasser *et al.* reported a positive correlation between camptothecin-induced Topo I-cleavable complexes and camptothecin sensitivity.²⁵⁾ We demonstrated a qualitative change in Topo I by eluting the nuclear extracts from both cell lines with increasing concentrations of NaCl. We hypothesized, based on these results, that some qualitative change in Topo I in the SBC-3/DXCL1 cells had altered the binding affinity of the Topo I-DNA-drug complex.

We and others have previously reported that a point mutation in the *Topo I* gene was present in resistant cell lines and that this *Topo I* gene mutation caused decreased Topo I catalytic activity.¹²⁻¹⁴⁾ However, in the SBC-3/DXCL1 cells, the Topo I activity remained similar to that of the parental SBC-3 cell-derived Topo I. One possible explanation is that, while the former cell lines may contain a mutation at the catalytic site of the enzyme, SBC-3/DXCL1 has a mutation at a different region, perhaps at the drug-binding site. Another possibility is altered intracellular metabolism of DX-8951f. Further study is required to elucidate the nature of the qualitative changes in the Topo I protein in these resistant cells.

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