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## Report

# Growth inhibitory effect of a new camptothecin analog, DX-8951f, on various drug-resistant sublines including BCRP-mediated camptothecin derivative-resistant variants derived from the human lung cancer cell line PC-6

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**DX-8951f, a new water-soluble camptothecin (CPT) derivative, has been reported to show potent antitumor effects against various tumors *in vitro* and *in vivo*. We further evaluated the cytotoxic effect of DX-8951f against eight drug-resistant sublines derived by stepwise exposure of human oat cell carcinoma PC-6 to various drugs. In paclitaxel-, adriamycin-, vincristine- and etoposide-resistant cells, overexpression of P-glycoprotein (P-gp) and a correlative reduction in drug accumulation and typical drug-sensitivity pattern were confirmed. The etoposide-resistant line with the highest P-gp level was cross-resistant also to SN-38, CPT-11 and topotecan (TPT), but not to 9-aminocamptothecin (9-AC), CPT and DX-8951f. SN-38- and CPT-11-resistant cells, of which topoisomerase I activities and levels were similar to those of the parent cells, showed cross-resistance clearly to TPT, 9-AC and mitoxantrone, but hardly to DX-8951f. In these two resistant sublines, the intracellular topotecan level was significantly lower than that in parental PC-6 and the reduced accumulation was found to be mediated by breast cancer resistant protein (BCRP). The cisplatin-resistant variant, which had a 2-fold increase in glutathione content, showed no cross-resistance and the 5-fluorouracil-resistant variant, which had a 50% decrease in glutathione content, exhibited collateral sensitivity to most of the other anticancer agents including DX-8951f. We concluded that DX-8951f showed a potent cytotoxic effect on various types of drug-resistant cells. [© 2000 Lippincott Williams & Wilkins.]**

*Key words*: BCRP, camptothecin, DX-8951f, drug resistance, human lung cancer, P-glycoprotein.

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## Introduction

DX-8951f, a non-pro-drug-type water-soluble camptothecin (CPT) derivative, exhibits the highest antitumor activity *in vitro* against 32 malignant cell lines among the CPT analogs that have been clinically developed, i.e. topotecan (TPT), 9-aminocamptothecin (9-AC) and CPT-11, and its active metabolite SN-38.[1] The potent antitumor effects of DX-8951f were also confirmed with a variety of human tumor xenografts in nude mice.[1,3,4] Based on these encouraging preclinical data, DX-8951f is now under phase I and phase II clinical trials in Japan, Europe and the USA.

In the clinic, the development of anticancer drug resistance, especially multidrug resistance (MDR), is believed to be one of the main reasons for the failure of cancer chemotherapy. Therefore, it is important to develop anticancer drugs to overcome this resistance. Previous reports[1–4] indicated that DX-8951f was effective against certain CPT derivative-resistant cell lines and MDR cell lines. In the present study, we established various drug-resistant cell lines from PC-6 and partially characterized their resistance mechanism by investigating several well-known resistance factors, such as two major drug transporters [P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP)] and a recently reported novel drug transporter [breast cancer resistant protein (BCRP)], as well as intracellular drug accumulation, drug detoxification molecules [glutathione (GSH) and GSH transferase-$\pi$ (GST-$\pi$)], and certain target molecules [topoisomerase (Topo) I, and $\alpha$- and $\beta$-tubulin]. We further compared the antitumor effects of DX-8951f with those of other

antitumor drugs including some CPT analogs on these cell lines.

## Materials and methods

### Anticancer drugs

Paclitaxel (Tax), CPT, TPT, 9-AC and DX-8951f were synthesized in our laboratory. SN-38 and CPT-11 were provided by Yakult Honsya (Tokyo, Japan). Adriamycin (ADM) and 5-fluorouracil (5-FU) were purchased from Kyowa-Hakko Kogyo (Tokyo, Japan). Etoposide (VP-16) and cisplatin (*cis*-diaminedichloroplatium; CDDP) were purchased from Nippon Kayaku (Tokyo, Japan). Vincristine (VCR) and mitoxantrone (MIT) were obtained from Shionogi Pharmaceutical (Osaka, Japan) and Takeda-Yakuhin (Tokyo, Japan), respectively. [G-$^3$H]vincristine sulfate ([$^3$H]VCR) was obtained from Amersham (Little Chalfont, UK). [$^3$H]VP-16 and [$^3$H]5-FU were obtained from Moravek Biochemicals, Brea, CA).

### Establishment of drug-resistant cell lines

Human oat cell lung cancer cell line PC-6 was obtained from Immuno-Biological Laboratories (Gunma, Japan). Various drug-resistant cell lines, i.e. PC-6/Tax1-1, -/ADM2-1, -/VCR29-9, -/VP1-1, -/SN2-5, -/CPT2-2, -/CDDP2-7 and -/FU26-23, were established as results of stepwise exposure to Tax, ADM, VCR, VP-16, SN-38, CPT-11, cisplatin and 5-FU, respectively. The cells were cultured in RPMI 1640 (Immuno-Biological Medical Laboratories, Kyoto, Japan) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 10 $\mu$g/ml kanamycin. The resistant variants were maintained with the drugs at the highest concentrations indicated in Table 1. The cultures were grown in 5% $CO_2$ at 37°C and passaged once or twice a week.

### Detection of P-gp and MRP by Western blotting

Cells were washed with cold phosphate-buffered saline (PBS) and solubilized with lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100 and 0.2 mM PMSF). The lysate was centrifuged for 5 min at 8500 *g* to remove debris. The resulting supernatant was mixed with SDS sample buffer [125 mM Tris–HCl, pH 6.8, 2.3% (w/v) SDS, 10% (w/v) glycerol, 5% 2-mercaptoethanol, 10 $\mu$g/ml BPB] and boiled for 5 min. Samples were separated in 4–20% graduation acrylamide gel. Protein fractions from the gels were electrophoretically transferred onto nitrocellulose membranes.

For the detection of P-gp, the membrane was blocked with 1% skim milk in PBS containing 0.1% Tween 20 (PBS-T), and then exposed to 1 $\mu$g/ml of murine anti-P-gp antibody (C219; Centocor, Malvern, PA) overnight at 4°C. The membrane was washed with PBS-T and incubated with secondary antibody, 1:1000 diluted horseradish peroxidase-linked anti-mouse IgG (Amersham) for 45 min at room temperature. For the detection of MRP, rat monoclonal antibody MRPr1 (Nichirei, Tokyo, Japan) was used as the primary antibody. The membrane was blocked with 1% BSA containing 0.3% Tween 20 and then was incubated with 1 $\mu$g/ml of MRPr1 overnight at 4°C. The membrane was next washed with PBS-T and incubated with the secondary antibody, 1:500 peroxidase-linked anti-rat Ig (Amersham) for 45 min at room. T98G, a human glioma cell line, was used as a MRP-expressing control cell line.[5]

After a final wash with PBS-T, the specific bands of P-gp or MRP were detected by the enhanced chemoluminescence (ECL) Western blotting method (Amersham) with fluorography on Hyperpaper-ECL Western (Amersham). Relative levels of P-gp were

**Table 1.** Cell lines used in *in vitro* experiments

| Cell lines | Drugs for selection | Drug concentration used (initial→final) | r.f.[a] |
|---|---|---|---|
| PC-6 (human oat cell line) | – | – | – |
| PC-6/Tax1-1 | Tax | 1→8 ng/ml | 299 |
| PC-6/ADM2-1 | ADM | 3→10 ng/ml | 18.7 |
| PC-6/VCR29-9 | VCR | 0.2→10 ng/ml | 842 |
| PC-6/VP1-1 | VP-16 | 0.07→1 $\mu$g/ml | 19.9 |
| PC-6/SN2-5 | SN-38 | 0.04→2 ng/ml | 38.0 |
| PC-6/CPT2-2 | CPT-11 | 0.6→3 $\mu$g/ml | 14.0 |
| PC-6/CDDP2-7 | cisplatin | 0.2→3 $\mu$g/ml | 28.6 |
| PC-6/FU26-23 | 5-FU | 0.1→1.5 $\mu$g/ml | 24.8 |

[a]Resistance factor, $GI_{50}$ of the drug for selection for the resistant cell line/that for the parent cell line; $GI_{50}$ was determined with the MTT assay, as described in Materials and methods.

determined with an image analyzer (BIO PROFILE; MS Instruments, Tokyo, Japan).

### Detection of Topo I by Western blotting

Approximately $1 \times 10^7$ cells were washed with cold PBS, to which was added 10% trichloroacetic acid (TCA). The TCA-precipitable fraction containing the protein was recovered, solubilized with SDS sample buffer and then neutralized with 2 M Tris. The protein concentration of the samples was determined by Bradford's method using a protein assay kit (BioRad, Melville, NY). Twenty-five micrograms of protein was subjected to 7.5% SDS–PAGE and electroblotted onto a nitrocellulose membrane. Detection of Topo I was carried out as previously described.[2]

### Determination of GSH, GSSG and cellular GST-$\pi$ contents

Cellular GSH contents were determined by an enzymatic assay utilizing GSH reductase.[6] Ten million cells suspended in 1 ml of EDTA solution (1 mM EDTA·2 Na, 0.4 M $HClO_4$) were sonicated and the resulting whole cell lysates were centrifuged at 1800 *g* for 10 min at 4°C to obtain protein-free lysates. Then the lysates were neutralized with 3 M $KHCO_3$ and equilibrated for 5 min, after which 5,5′-dithiobis-nitrobenzoic acid was added. In the case of GSH disulfide (GSSG) determination, reductive GSH was removed from the protein-free lysates with nitrous and ammonium sulfamate, and then the supernatant neutralized with 3 M $KHCO_3$ was incubated with GSH reductase, 5,5′-dithiobis-nitrobenzoic acid and 0.3 mM NADPH. After a definite time, the absorbance of the supernatant at 450 nm was determined with Sjeia auto reader (Sanko Jyunyaku, Tokyo, Japan) and the GSH content was calculated by comparison to a standard curve obtained with known amounts of GSH.

For determination of cellular GST-$\pi$ contents, $2 \times 10^7$ cells were suspended in 1 ml of 1% Triton-X100/PBS, sonicated and then centrifuged at 10 000 *g* for 1 h at 4°C. The GST-$\pi$ content of the supernatant was measured by use of a GST Pi ELISA (Immunodiagnostik, Benheim, Germany).

### Growth inhibition assay

Growth inhibitory effects of drugs on cancer cell lines were determined by the MTT assay.[7] Briefly, 150 $\mu$l aliquots of an exponentially growing cell suspension ($5 \times 10^3$ cells/well) were seeded in 96-well flat-bottomed microplates (Falcon, Oxnard, CA). Fifty microliter aliquots of drugs at various concentrations or the medium alone as a control was added 24 h later. After incubation for 3 days at 37°C in 5% $CO_2$, 20 $\mu$l of MTT solution (5 mg/ml in PBS) was added to each well and the plates were incubated at 37°C for a further 4 h. Then the medium was removed from each well as completely as possible and 150 $\mu$l of dimethyl sulfoxide was added to each well to dissolve the formazan. The optical density was measured at 540 and 655 nm. Each experiment was performed with four replicates for each drug concentration. The inhibitory effects of drugs were expressed as the $GI_{50}$ value, which is the drug concentration inhibiting total cell growth by 50%. The resistance factor was defined as a ratio of the $GI_{50}$ for the resistant subline to the $GI_{50}$ for the parental cell line. When the resistance factor was above 5.0, the cell line was considered to had acquired resistance.

To confirm the effect of GSH depletion on the cytotoxicity of cisplatin, we preincubated the cells with 1 or 25 $\mu$M DL-buthionine-(*S*,*R*)sulfoximine (BSO), an inhibitor of $\gamma$-glutamylcysteine synthetase ($\gamma$-GCS). After an overnight incubation, the cells were then exposed to cisplatin in the presence of BSO for 3 days.[8]

### Measurement of cellular drug accumulation

Cellular accumulation of ADM or TPT was measured by flow cytometry (FCM). Cells ($2 \times 10^6$ cells/ml) were incubated at 37°C with 2 $\mu$g/ml of ADM or 4.9 $\mu$g/ml of TPT. Fluourescence was analyzed with a FACScan analyzer (Becton Dickinson, Mountain View, CA) with excitation at 488 nm (argon laser). The peak channel number of each sample was recorded as intensity of fluorescence as described elsewhere.[2]

For the determination of accumulation of [$^3$H]VCR, [$^3$H]VP-16 or [$^3$H]5-FU, cells ($2 \times 10^6$ cells/ml) were incubated at 37°C with 37 ng/ml (15.7 kBq/tube) of [$^3$H]VCR, 10 $\mu$g/ml (48.3 kBq/tube) of [$^3$H]VP-16 or 86.7 ng/ml (225.7 kBq/tube) of [$^3$H]5-FU. The cells were washed with cold PBS and cell pellets obtained after centrifugation were dissolved in 500 $\mu$l of 4% SDS. The radioactivity was measured with a liquid scintillation system (LSC900; Aloka, Tokyo, Japan).

### Detection of BCRP by RT-PCR

The isolation of poly(A)$^+$ RNA was performed with a QuickPrep Micro mRNA Purification Kit (Amersham).

The RNA (40 ng) was reverse transcribed by MuLV reverse transcriptase and random hexamer, following the protocol provided with the RNA PCR Core Kit (Perkin-Elmer Cetus, Norwalk, CT) used for the procedure. BCRP and control $\beta$-actin gene sequences were co-amplified in the same reaction, by use of the following gene-specific primers: 5′-GTGTTTCAGCAGTGTTTCAGCCGTG-3′ (BCRP, forward); 5′-GGCCACGTGATTCTTCCACAAGCCC-3′ (BCRP, reverse); 5′-GACTACCTCATGAAGATCCT-3′ ($\beta$-actin, forward); 5′-ACTCCTGCTTGCTGATCCAC-3′ ($\beta$-actin, reverse). The expected sizes of the PCR products with the sets of primers used were 586 (BCRP) and 526 ($\beta$-actin) bp. Aliquots of cDNA corresponding to 20 ng of RNA were subjected to PCR in a final volume of 50 $\mu$l with the RNA PCR Core Kit. An initial denaturation of 5 min at 94°C was followed by 25 cycles of a 30 s denaturing step at 94°C, a 30 s annealing step at 57°C and a 1 min extension step at 72°C. Following the PCR, 13.5 $\mu$l of the PCR reaction mixture was subjected to electrophoresis on a 2% agarose gel.

## Results

### Overexpression of P-gp and MRP

At first, expression of P-gp and MRP in all resistant variants was compared by Western blotting analysis and compared with that in parental cells.

As Figure 1(A) shows, P-gp was overexpressed weakly in PC-6/Tax1-1, moderately in PC-6/ADM2-1 and -/VCR29-9, and strongly in PC-6/VP1-1 cells, but not at all in parental PC-6 cells. No P-gp was overexpressed in the other four resistant variants, PC-6/SN2-5, -/CPT2-2, -/CDDP2-7 and -/FU26-23 (data not shown).

In contrast, MRP protein was slightly expressed in parental cells and moderately enhanced in CPT derivative-resistant variants PC-6/SN2-5 and -/CPT2-2 (Figure 1B). The levels of MRP protein in these cell lines, however, were significantly lower than the level in T98G, as a positive control. No overexpression of MRP was observed in PC-6/CDDP2-7 or in the other five variants, i.e. the above four variants overexpressing P-gp and PC-6/FU26-23 (data not shown).

### Intracellular GST-$\pi$, GSH and GSSG contents

Contents of drug detoxification molecules, GST-$\pi$ and GSH and its oxidized form, GSSG, were determined in PC-6 and all resistant sublines. As shown in Table 2, there were no significant differences in GST-$\pi$ content among the cell lines. Intracellular GSH and GSSG, and thereby also total GSH (sum of GSH and GSSG), increased 2-fold only in PC-6/CDDP2-7 cells, but decreased by half in PC-6/FU26-23 and -/Tax1-1 cells, and did not change in the other variants in comparison with the value for the parental cells.

**Table 2.** GSH, GSSG and GST-$\pi$ contents

| Cell lines | Content ($\mu$g/1 $\times$ $10^7$ cells) (relative value[a]) | | |
|---|---|---|---|
| | GSH | GSSG | GST-$\pi$ |
| PC-6 | 14.3 (1.0) | 2.6 (1.0) | 3.5 (1.0) |
| PC-6/CDDP2-7 | 27.4 (1.9) | 5.5 (2.1) | 3.5 (1.0) |
| PC-6/TAX1-1 | 8.3 (0.6) | 1.2 (0.5) | 2.9 (0.8) |
| PC-6/ADM2-1 | 14.4 (1.0) | 2.3 (0.9) | 3.4 (1.0) |
| PC-6/VCR29-9 | 11.9 (0.8) | 1.8 (0.7) | 2.7 (0.8) |
| PC-6/VP1-1 | 11.1 (0.8) | 1.9 (0.7) | 3.4 (1.0) |
| PC-6/SN2-5 | 15.7 (1.1) | 2.5 (1.0) | 3.1 (0.9) |
| PC-6/CPT2-2 | 13.9 (1.0) | 1.9 (0.7) | 3.4 (1.0) |
| PC-6/FU26-23 | 8.1 (0.6) | 1.1 (0.4) | 3.9 (1.1) |

GSH, GSSG and GST-$\pi$ were determined as described in Materials and methods.
[a]Relative value was defined as value for the resistant cells/value for the parental PC-6.

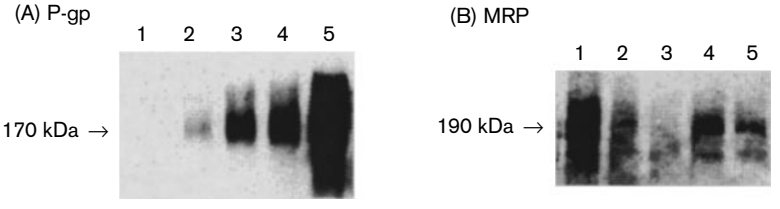


**Figure 1.** Comparison of levels of P-gp or MRP in PC-6 and the resistant sublines by Western blotting. Crude membrane extracts (30 $\mu$g) were added to each lane and separated by 4–20% SDS–PAGE. (A) P-gp. The monoclonal anti-P-gp C219 antibody was used at 1 $\mu$g/ml. Lane 1, PC-6; lane 2, PC-6/Tax1-1; lane 3, PC-6/ADM2-1; lane 4, PC-6/VCR29-9; lane 5, PC-6/VP1-1. (B) MRP. The monoclonal anti-MRP MRPr1 antibody was used at 0.4 $\mu$g/ml. Lane 1, T98G as positive control; lane 2, PC-6; lane 3, PC-6/CDDP2-7; lane 4, PC-6/SN2-5; lane 5, PC-6/CPT2-2.

### Drug sensitivity of the cell lines overexpressing P-gp

Sensitivity to various anti-cancer agents of the P-gp-overexpressing variants was assessed (Table 3). PC-6/Tax1-1, -/ADM2-1 and -/VCR29-9 exhibited a classical P-gp-mediated MDR phenotype, i.e. a marked resistance to Tax, ADM and VCR, but no resistance to cisplatin, 5-FU or camptothecin derivatives. PC-6/VCR29-9 also showed weak cross-resistance to VP-16. PC-6/VP1-1, with the highest P-gp level, displayed cross-resistance to Tax, ADM, VCR and VP-16, as well as to SN-38, CPT-11 and TPT, but it remained sensitive to 9-AC, CPT and DX-8951f. These MDR cell lines, respectively, showed approximately >300-fold higher resistance to Tax and VCR than their parental PC-6 cells. In the MDR cell lines, the resistance rates to VCR, ADM and VP-16 were proportional to their levels of P-gp (Figure 2). The resistance rates of these cell lines to Tax were also positively correlated with the P-gp levels (data not shown).

### Accumulation of ADM, VCR, VP-16 and TPT in the cell lines overexpressing P-gp

ADM accumulation in the variants overexpressing P-gp, i.e. PC-6/Tax1-1, -/ADM2-1, -/VCR29-9 and -/VP1-1, was determined by FACScan and compared with that in the parental PC-6. The ADM content in parental cells increased continuously in a time-dependent fashion, but that in the resistant cells reached a plateau within 120 min. The maximum level of ADM content was apparently lower in the resistant cells than in the parental cells (Figure 3A).

A decrease in the cellular accumulation of VCR in PC-6/VCR29-9 or of VP-16 in PC-6/VP1-1 was also confirmed by use of the respective radiolabeled compound (data not shown). In PC-6/VP1-1, which was also cross-resistant to TPT, a significant reduction in TPT accumulation was also confirmed by FACScan analysis (Figure 3B).

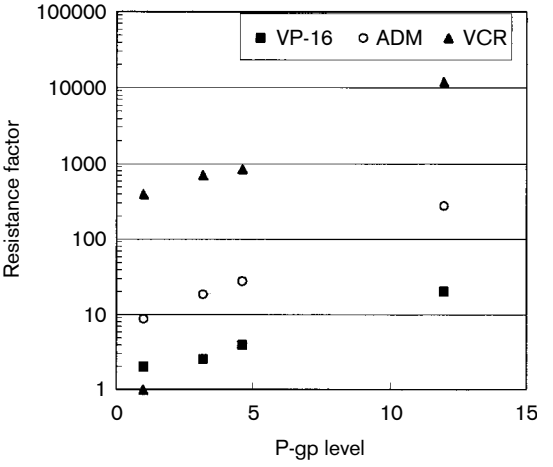


**Figure 2.** The correlation between P-gp levels and resistance factors in the resistant sublines. P-gp levels in Figure 1 were quantified using an image analyzer and expressed as relative values to that of PC-6/Tax1-1. There were excellent correlations between P-gp levels and resistance factors: VCR, $r^2$=0.9874; ADM, $r^2$=0.9995; VP-16. $r^2$=0.9913.

**Table 3.** Drug sensitivity of the cell lines overexpressing P-gp

| Drugs | PC-6 [$GI_{50}$[a] (ng/ml)] | r.f.[b] | | | |
|---|---|---|---|---|---|
| | | PC-6/Tax1-1 | PC-6/ADM2-1 | PC-6/VCR29-9 | PC-6/VP1-1 |
| Taxol | 0.307 | 299 | 365 | 580 | >10000 |
| ADM | 12.6 | 8.65 | 18.7 | 27.9 | 277 |
| VCR | 0.101 | 398 | 691 | 842 | 12200 |
| VP-16 | 201 | 2.00 | 2.55 | 3.95 | 19.9 |
| Cisplatin | 203 | 0.660 | 1.17 | 0.542 | 1.90 |
| 5-FU | 420 | 0.776 | 1.09 | 0.852 | 1.09 |
| DX-8951f | 0.0938 | 0.821 | 1.15 | 0.591 | 4.33 |
| SN-38 | 0.285 | 1.05 | 1.27 | 0.779 | 9.26 |
| CPT-11 | 612 | 1.99 | 2.78 | 2.03 | 13.2 |
| TPT | 1.76 | 1.43 | 2.12 | 1.69 | 19.2 |
| 9-AC | 0.669 | 1.01 | 0.955 | 0.806 | 2.80 |
| CPT | 1.33 | 0.759 | 0.697 | 0.597 | 1.80 |

[a]$GI_{50}$ was determined with the MTT assay, as described in Materials and methods.
[b]Resistance factor was determined as $GI_{50}$ of each drug for the resistant cell line/that for the parent cell line.

### Drug sensitivity and Topo-I levels of CPT derivative-resistant variants

Table 4 shows the sensitivity of PC-6/SN2-5 and PC-6/CPT2-2 cell lines to various anticancer agents. These cell lines showed high resistance to most of the CPT derivatives, i.e. SN-38, TPT and 9-AC, but relatively weak resistance to DX-8951f and CPT. Interestingly, both cells were also resistant to MIT ($\times 25$ and $\times 31$), but not to ADM, which has a similar chemical structure to MIT, but not to other non-CPT-type anticancer agents.

Topo-I expression, determined by Western blotting analysis, revealed that there were no significant differences between these two variants and the parental cells (Figure 4).

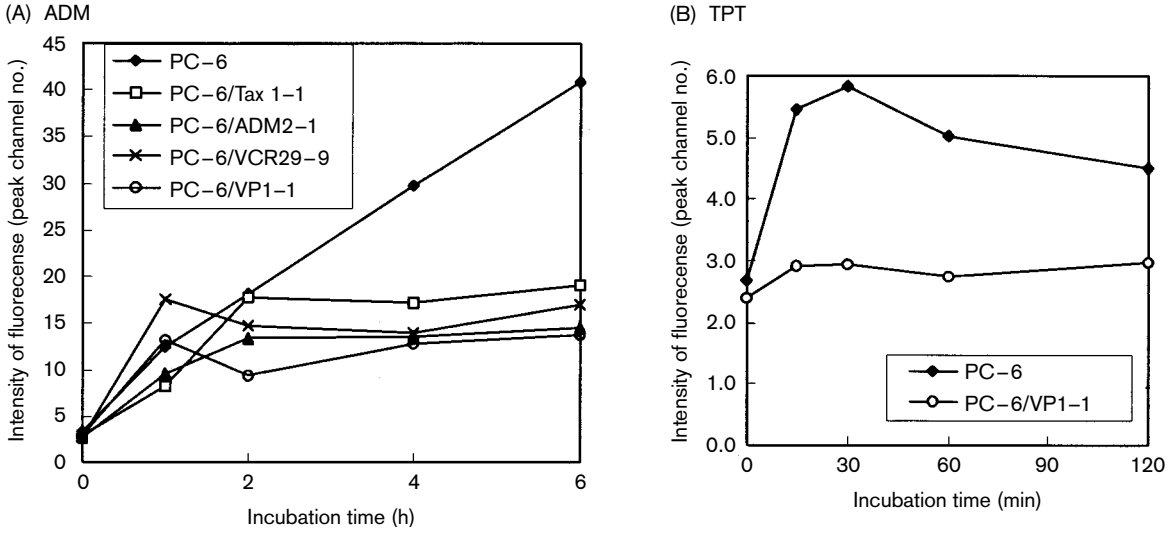


**Figure 3.** Accumulation of drugs in parental PC-6 and P-gp-positive sublines. The intracellular ADM and TPT contents were determined by FCM as described in Materials and methods. (A) Accumulation of ADM (2 $\mu$g/ml). (B) Accumulation of TPT (4.9 $\mu$g/ml). PC-6 (◆), PC-6/VP1-1 (○), PC-6/VCR29-9 (×), PC-6/ADM2-1 (▲) and PC-6/Tax1-1 (□). Intensity of fluorescence is indicated as the peak channel number of each sample.

**Table 4.** Drug sensitivity in non-P-gp cell lines

| Drugs | No. of experiments | PC-6 [$GI_{50}$[a] (ng/ml)] | r.f.[b] | | | |
|---|---|---|---|---|---|---|
| | | | PC-6/SN2-5 | PC-6/CPT2-2 | PC-6/CDDP2-7 | PC-6/FU26-23 |
| DX-8951f | 2 | 0.0938, 0.127 | 4.39 | 13.8 | 1.45 | 0.174 |
| SN-38 | 2 | 0.285, 0.765 | 38.3 | 126 | 2.06 | 0.267 |
| CPT-11 | 2 | 612, 657 | 4.57 | 13.6 | 2.19 | 0.645 |
| TPT | 2 | 1.76, 1.59 | 24.7 | 86.2 | 2.60 | 0.484 |
| 9-AC | 2 | 0.669, 0.974 | 12.3 | 53.0 | 2.11 | 0.426 |
| CPT | 2 | 1.33, 1.32 | 1.92 | 2.98 | 1.47 | 0.336 |
| Taxol | 1 | 0.712 | 0.917 | 0.966 | 1.08 | 1.02 |
| ADM | 3 | 6.85–7.49 | 1.66 | 1.57 | 1.02 | 0.458 |
| VCR | 3 | 0.203–0.318 | 1.19 | 1.21 | 2.58 | 1.60 |
| VP-16 | 3 | 159–166 | 1.52 | 1.46 | 1.09 | 0.421 |
| Cisplatin | 2 | 203, 295 | 0.769 | 0.892 | 28.6 | 0.108 |
| 5-FU | 2 | 420, 330 | 0.924 | 0.618 | 0.717 | 24.8 |
| Tiotepa | 2 | 643, 384 | 1.33 | 2.04 | 1.87 | 0.288 |
| MMC | 3 | 24.7–58.7 | 1.17 | 1.72 | 1.85 | 0.370 |
| MIT | 3 | 0.551–1.43 | 24.8 | 31.2 | 2.40 | 0.562 |

[a]$GI_{50}$ was determined with the MTT assay, as described in Materials and methods.
[b]Resistance factor: $GI_{50}$ of each drug for the resistant cell line/that for the parent cell line.

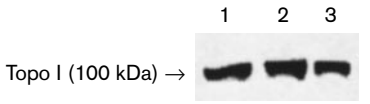


**Figure 4.** Immunoblotting of Topo I in PC-6 (lane 1), PC-6/SN2-5 (lane 2) and PC-6/CPT2-2 (lane 3). Whole cell lysates (25 $\mu$g) were added to each lane and separated by 7.5% SDS–PAGE. Topo I protein was then detected with Topo I antibody.

### Accumulation of TPT in CPT derivative-resistant variants

Accumulation of TPT in PC-6/SN2-5 and -/CPT2-2 was compared with that in the parental PC-6, again by use of the FACScan. The cellular concentrations of TPT in PC-6 increased rapidly within the first 15 min and then decreased slowly thereafter (Figure 5). TPT concentrations in PC-6/SN2-5 and -/CPT2-2 rose slowly, and were significantly lower than the concentration in the parental PC-6 cells.

### Expression of BCRP in CPT-derivative-resistant variants

Since BCRP, a novel drug transporter, was recently reported to mediate the mechanism of resistance to MIT and CPT derivatives, we further determined whether BCRP was expressed or not in PC-6/SN2-5 and -/CPT2-2 cells. RT-PCR revealed that a substantial level of BCRP mRNA was observed in both of the resistant variants, whereas the transporter was not detectable in the parental PC-6 (Figure 6).

### Drug sensitivity of cisplatin-resistant cells and the effect of BSO treatment

In comparison with the parental cells, PC-6/CDDP2-7 cells showed about a 28.6-fold higher resistance to cisplatin but no resisitance to other agents (Table 4). Because of a 1.9-fold higher GSH content as shown in Table 2, we examined the effect of GSH depletion by BSO on the sensitivity of this variant. PC-6/CDDP2-7 cells had 145-fold higher resistance to BSO than PC-6 cells, i.e. $GI_{50}$ values for BSO were 218 and 1.5 $\mu$M against the resistant and parent cell lines, respectively. Effects of BSO were compared at the maximum BSO concentration at which 90% of the cells remained (1 $\mu$M for PC-6 and 25 $\mu$M for PC-6/CDDP2-7). Treatment with BSO did not significantly alter the sensitivity to cisplatin of PC-6 or that of PC-6/CDDP2-7 to cisplatin (Table 5).

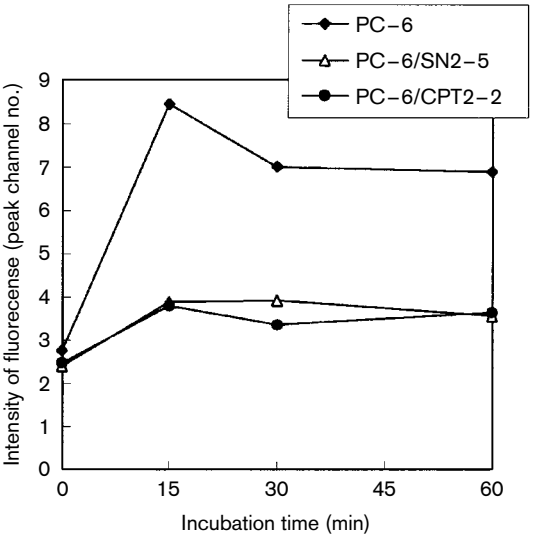


**Figure 5.** Accumulation of TPT (4.9 $\mu$g/ml) in PC-6, -/SN2-5 and -/CPT2-2. The intracellular TPT content was determined by FCM as shown in Materials and methods. PC-6 (◆), PC-6/SN2-5 (△) and PC-6/CPT2-2 (●). Intensity of fluorescence is indicated as the peak channel number of each sample.

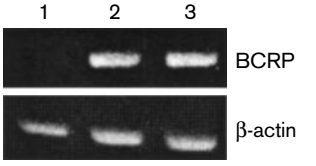


**Figure 6.** The result of RT-PCR analysis for the expression of the BCRP and control genes. The PCR products were separated on a 2% agarose gel. The PCR products sizes are 586 (BCRP) and 526 ($\beta$-actin) bp. Lane 1, PC-6; lane 2, PC-6/SN2-5; lane 3, PC-6/CPT2-2.

### Drug sensitivity of 5-FU-resistant cells and 5-FU accumulation

PC-6/FU26-23 had 24.8-fold higher resistance to 5-FU, but showed a collateral sensitivity to various other drugs (Table 4).

Accumulation of 5-FU measured with a labeled compound was not significantly altered in this variant in comparison with that in the parent PC-6 (data not shown).

## Discussion

### Characterization of the resistant cell lines

In the present study, we selected drug-resistant sublines from PC-6 with seven commonly used anticancer agents and SN-38, and found different mechanisms of resistance among them.

**Table 5.** Effects of BSO on sensitivity to cisplatin of PC-6 and its cisplatin-resistant variants

| Cell lines | BSO concentration ($\mu$M) | Effect of BSO treatment | | | BSO sensitivity [$GI_{50}$[c] ($\mu$M)] |
|---|---|---|---|---|---|
| | | Cell growth T/C (%) | Cisplatin sensitivity | | |
| | | | r.f.[a] | r.v.[b] | |
| PC-6 | 0 | 100 | 1 | 1 | 1.5 |
| | 1 | 87.8 | | 0.91 | |
| PC-6/CDDP2-7 | 0 | 100 | 24.8 | 1 | 218 |
| | 1 | 104.6 | 20.5 | 0.83 | |
| | 25 | 87.8 | 22.3 | 0.90 | |

[a]Resistance factor: $GI_{50}$ of cisplatin for the cells with or without BSO treatment/that for the parental cells without BSO treatment.
[b]Relative value: effect of BSO on the sensitivity of cisplatin; $GI_{50}$ of cisplatin for the cells with BSO treatment/that for the cells without BSO treatment.
[c]$GI_{50}$ of BSO was determined with the MTT assay.

*PC-6/Tax1-1, -/ADM2-1, -/VCR29-9 and -/VP1-1.* The acquisition of multidrug resistance is often associated with the overexpression of P-gp, whose apparent function is to pump various hydrophobic agents.[9,10] Therefore we first examined the expression of P-gp in all of the resistant sublines established. Among these variants, PC-6/Tax1-1, -/ADM2-1, -/VCR29-9 and -/VP1-1 showed overexpression of P-gp (Figure 1A). The resistance pattern of these variants was similar to a typical multidrug resistance phenotype (Table 3). Their relative resistance values were well correlated with their P-gp levels (Figure 2). In addition, the drug accumulation in these variants was confirmed to be significantly lower than that in parental PC-6 (Figure 3). PC-6/VCR29-9 and -/Tax1-1 were selected with drugs that interact with microtubules, but no difference in total $\alpha$- and $\beta$-tubulin contents was observed between these variants and parental PC-6 (data not shown). As there was no overexpression of MRP (Figure 1B), we conclude that the acquired drug resistance in PC-6/Tax1-1, -/ADM2-1, -/VCR29-9 and -/VP1-1 is attributable to the elevation of P-gp levels.

*PC-6/SN2-5 and -/CPT2-2.* Both PC-6/SN2-5 and -/CPT2-2 showed high resistance to SN-38, TPT and 9-AC, but relatively weak resistance to DX-8951f or CPT (Table 4). These cell lines were also significantly resistant to MIT but not to the other anticancer agents. We have already reported that the level and activity of Topo I in PC-6/SN2-5 were similar to those in PC-6,[2] and confirmed in this study that the Topo I level of PC-6/CPT2-2 was also similar to that of PC-6 (Figure 4). These findings suggest that their resistance was not caused by Topo I-related changes. The intracellular concentration of TPT in these variants was significantly lower than that in the parental cells (Figure 5), suggesting that the resistance was mediated by a certain drug-efflux pump. However, the resistance phenotype of these variants was apparently different from that mediated by P-gp or MRP (Table 4), and no overexpression of P-gp in these resistant cells was confirmed (data not shown). MRP levels were moderately higher in the resistant cells than in the parental ones, but treatment with probenecid, a chemosensitizer for MRP-mediated multidrug resistance,[11] did not reverse the resistance (data not shown), indicating no critical role of MRP in the resistant mechanisms. Recently, a TPT-resistant ovarian cancer cell line and MIT-resistant breast cancer cell lines were independently reported to be resistant to both TPT and MIT.[12,13] Of interest, the resistance of these cell lines was commonly reported to be accompanied by reduced accumulation and also shown to be independent of P-gp or MRP. Ross *et al.* further found that mRNA of BCRP, a novel drug transporter, was frequently overexpressed in multidrug-resistant cell lines selected with MIT.[14] The features of the above resistant cell lines established by others were very similar to those of PC-6/SN2-5 and -/CPT2-2, and we confirmed the overexpression of the BCRP in the latter lines (Figure 6).

*PC-6/CDDP2-7.* The GSH concentration in PC-6/CDDP2-7 was approximately 2 times higher than that in the parental cells (Table 2). This result is consistent with the findings of previous studies indicating a positive correlation between the cellular GSH level and cisplatin resistance.[15] Intracellular GSH depletion, which occurred when cells were exposed to BSO, however, had no effect on the sensitivity of PC-6/CDDP2-7 to cisplatin (Table 5). The cisplatin-resistant lung cancer cell line SBC-3/GCS, which was transfected with the $\gamma$-GCS gene, was recently reported to show increased levels of GSH, high resistance to BSO

and no sensitization by GSH depletion with BSO treatment, and was concluded to have increased GS-X pump activity as a main mechanism of resistance.[8] Interestingly, PC-6/CDDP2-7 exhibited similar features, such as high resistance to BSO ($\times$ 145). Since MRP, a well-known GS-X pump, was not overexpressed in PC-6/CDDP2-7 (Figure 1B), there is a possibility that the resistant cells overexpress a novel GS-X pump.

*PC-6/FU26-23.* PC-6/FU26-23 exhibited a 24.8-fold higher resistance to 5-FU but showed collateral sensitivity to various drugs such as CPT analogs, cisplatin, tiotepa, MMC, MIT, ADM and VP-16 (Table 4). A recent study by Esaki *et al.*[16] demonstrated that pretreatment with 5-FU augmented the cytotoxicity of cisplatin by inhibiting the repair of platinum–DNA interstrand crosslinks as well as by reducing the cellular GSH contents in cells. The effect seems to be mediated by down-regulation of the excision repair cross-complementing rodent repair deficiency gene 1 (ERCC1) and $\gamma$-GCS gene. Since the cellular GSH content was reduced in PC-6/FU26-23 (Table 2), the enhanced sensitivity of this subline may be attributable to a similar mechanism such as down-regulation of the $\gamma$-GCS gene and/or other repair genes. Further investigation is required to clarify the mechanism of resistance and collateral sensitivity of this variant.

### Antitumor effects of DX-8951f

For prediction of the clinical usefulness of DX-8951f, a water-soluble and non-prodrug analog of CPT, we compared the antitumor activity of DX-8951f against these drug-resistant sublines with that of various drugs used in clinical therapy. In *in vitro* chemosensitivity tests, DX-8951f showed the highest effect among the drugs examined, even against the drug-resistant variants. In other words, most of the drug-resistant sublines showed no cross-resistance to DX-8951f. Although cross-resistance to DX-8951f was observed in PC-6/VP1-1 and -/SN2-5, the degree of resistance to DX-8951f was markedly lower than that to SN-38 and TPT. Previous studies demonstrated that DX-8951f exhibits strong *in vitro* cytotoxicity against various human cancer cells and that it is a far more potent Topo I inhibitor than other CPT derivatives.[1] *In vivo* evaluation also revealed that this compound is superior to CPT-11 and TPT.[17] Based on the results of this and previous studies, DX-8951f appears to be relatively insensitive to various types of drug resistance and therefore it is expected to show antitumor effects even on tumors refractory to other agents. The results of phase IIa clinical trials progressing in Europe and the USA are eagerly awaited.

In conclusion, the PC-6 variants described appear to be useful for the evaluation of various potential anticancer drugs for their ability to overcome the various types of drug resistance.

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