ANTICANCER RESEARCH *24*: 3911-3916 (2004)

Establishment of a 7-Ethyl-10-hydroxy-camptothecin-resistant Small Cell Lung Cancer Cell Line

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Abstract. *Irinotecan is one of the most active drugs used in the treatment of small cell lung cancer (SCLC). 7-Ethyl-10-hydroxy-camptothecin (SN-38) is an active metabolite of irinotecan. We established an SN-38-resistant subline (SBC-3/SN-38) by continuous exposure of SN-38 to a human SCLC cell line, SBC-3. Using the 3-[4, 5-dimethyl-thiazol-2-yl] 2, 5-diphenyltetrazolium bromide assay, we evaluated the cytotoxicity of 17 anticancer agents. The SBC-3/SN-38 cells were 73-fold more resistant than the parental SBC-3 cells to SN-38 and showed cross-resistance not only to topoisomerase (topo) I inhibitors (irinotecan and topotecan), but also to topo II inhibitors (adriamycin and etoposide), antimicrotubule agents (vincristine, vindesine, vinorelbine and docetaxel), alkylating agents (cyclophosphamide and ifosfamide), platinum (cisplatin and carboplatin) and antifolate (methotrexate). Interestingly, the resistant subline reserved the sensitivity to bleomycin and 5-fluorouracil. The SBC-3/SN-38 cells had decreased topo I and II activity compared to the parent cells. The SN-38-resistant cell line, SBC-3/SN-38, will be useful to elucidate the mechanism of action of the topo I inhibitors.*

The role of chemotherapy in the treatment of small cell lung cancer (SCLC) was established in the past decade. More than 80% of patients receiving current intensive chemotherapy regimens achieve an objective response. However, most responders eventually relapse and less than 20% survive longer than 3 years (1). The development of drug resistance in tumor cells is assumed to play a major role in these disappointing outcomes (2).

Irinotecan is a semi-synthetic analogue of camptothecin. In serum and tumor cells, it is converted to an active metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38), which has a specific mechanism of action *via* inhibition of a nuclear enzyme, topoisomerase (topo) I (3, 4). Irinotecan has exerted high activity as a single agent for SCLC (5). A recent phase III study, in extensive disease (ED) SCLC, demonstrated that a combination cisplatin and irinotecan regimen yielded a highly significant improvement in survival over the standard cisplatin and etoposide regimen (6). Accordingly, the combination is considered the standard treatment for ED SCLC. Even using a combination of irinotecan and cisplatin, the median survival and two-year survival rate were only 12.8 months and 19.5%, respectively. The emergence of irinotecan resistance has become a concern in patients with refractory ED SCLC.

Our objectives were to elucidate the mechanism of resistance to irinotecan by establishing an SN-38-resistant human SCLC cell line and to find anticancer agents to overcome the resistance.

Materials and Methods

Chemicals and reagents. The drugs used in this study were provided by the following sources: irinotecan and SN-38 from Yakult Honsha, Tokyo, Japan; topotecan from Smithcline Beecham, Tokyo, Japan; etoposide and carboplatin from Britol-Myers Sqibb, Tokyo, Japan; cisplatin and bleomycin from Nippon Kayaku Kogyo Co., Ltd, Tokyo, Japan; docetaxel from Rhone-Poulenc Rorer,

* Died on October 8, 1996

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Key Words: Irinotecan, small cell lung cancer, drug resistance, topoisomerase.

Antony, France; adriamycin, 5-fluorouracil, mitomycin C and vinorelbine from Kyowa Hakko Kogyo, Tokyo, Japan; active metabolite of cyclophosphamide: 4-hydroperoxycyclophosphamide (4-HC), active metabolite of ifosfamide: 4-hydroperoxyifosfamide (4-HI), vincristine and vindesine from Shionogi & Co., Ltd. Osaka, Japan; methotrexate from Lederle, Tokyo, Japan. 3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co., St Louis, MO, USA.

Cell cultures. The parent cell line, the SBC-3 cell line, was established from the bone marrow aspirate of a previously untreated SCLC patient (7). The growth medium (RPMI-FBS) was RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin-G (100 units/ml) and streptomycin (100 μg/ml). The SN-38-resistant cell line was established by continuous exposure of the SBC-3 cell line to increasing concentrations of SN-38, with subsequent cloning procedures. Initially, the SBC-3 cells were cultured continuously in RPMI-FBS containing 0.1nM SN-38. The drug concentration was gradually increased every 2 to 4 weeks. Finally, the cells growing vigorously in medium containing 10nM SN-38 were obtained 24 months later. Two weeks later, growing colonies were harvested and distributed in 24-multiwells; the cells were allowed to grow in a T25 tissue culture flask. The SN-38-resistant cell line was designated as SBC-3/SN-38.

Assay of drug sensitivity. Drug sensitivity was determined by MTT assay (8). Briefly, 50μl of RPMI-FBS containing serial concentrations of each chemotherapeutic agent was prepared in 96-well flat-bottomed microplates (Coster, Cambridge, MA, USA). Then 50μl of RPMI-FBS containing 2,000 cells for SBC-3 and 5,000 cells for SBC-3/SN-38 was added to each well. The cells were incubated for 96 h in a highly humidified incubator with 5% CO_2 and 95% air. Then 50μl of MTT (5 mg/ml phosphate-buffered saline [PBS], pH 7.2) was added to each well. After incubation at 37˚C for 4 h, 125μl of fresh isopropanol with 0.04 M HCl was added to each well. The 96-well microplates were vigorously shaken by the Direct Mix Model TS-50 (Thermal Kagaku Sangyo, Tokyo, Japan). Absorbance of each well was measured at 560nm with the model 3550 microplate reader (Bio-Rad laboratories, Richmond, CA, USA). The absorbance of a well without chemotherapeutic agents was used as the control, while that of a well containing only RPMI-FBS, MTT and isopropanol was used as the background. The percent of surviving cells 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)] x 100. The drug concentration required to inhibit the growth of tumor cells by 50% (IC_{50}) was determined by plotting the logarithm of drug concentration *versus* the percent of surviving cells. Determinations were carried out in quadruplicate in each experiment and the results were confirmed by three or more separate experiments. Relative resistance was calculated by dividing the IC_{50} value of the SBC-3/SN-38 cells by the IC_{50} value of the SBC-3 cells.

Cell growth rate. The growth rate of cells was determined using the MTT assay. 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 (9).

Table I. *Drug sensitivity in the parent (SBC-3) and the SN-38-resistant cell lines (SBC-3/SN-38).*

	IC_{50} value (nM; mean±SD)		Relative resistance value (mean±SD)	*P*
	SBC-3	SBC-3/SN-38		
SN-38	0.83±0.11	60±9.6	73±11	0.001
Irinotecan	21±4.2	570±120	27±2.8	0.014
Topotecan	4.2±0.075	130±5.1	32±0.68	0.001
Adriamycin	20±1.6	68±8.8	3.4±0.50	0.011
Etoposide	110±36	580±260	5.5±1.6	0.071
Vincristine	1.6±0.50	5.1±1.4	3.2±0.9	0.039
Vindesine	1.1±0.18	2.6±0.80	2.5±1.1	0.111
Vinorelbine	3.8±0.99	8.7±2.7	2.3±0.14	0.038
Docetaxel	0.75±0.14	1.3±0.015	1.8±0.30	0.016
4-HC	1000±270	1500±457	1.5±0.098	0.049
4-HI	1300±46	1900±83	1.4±0.11	0.018
Cisplatin	390±67	860±43	2.3±0.68	0.026
Carboplatin	4200±940	9900±3200	2.4±0.96	0.111
Mitomycin C	39±6.0	69±22	1.8±0.38	0.129
Bleomycin	39±4.8	32±8.8	0.81±0.13	0.096
Methotrexate	20±0.71	92±7.2	4.6±0.20	0.003
5-fluorouracil	2600±660	3100±750	1.2±0.39	0.331

IC_{50}: 50% inhibitory concentration, SD: standard deviation, relative resistance value (IC_{50} value of SBC-3/SN-38 cells / IC_{50} value of SBC-3 cells) was calculated from each experiment. 4-HC: 4-hydroperoxycyclophosphamide, 4HI: 4-hydroperoxyifosfamide. *P*-value is evaluated using paired Student's *t*-test.

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, Japan). The glutathione (GSH) and glutathione-S-transferase-π (GST-π) concentration in the supernatant were determined after centrifuging the sonicates at 7,000g for 5 min. GSH was assayed by the method reported by Tietze (10) while GST-π was assayed using a GST-π EIA kit (Dainihon Seiyaku, Osaka, Japan). GSH and GST-π concentration were expressed as the ratio to mg protein determined by the method of Bradford (11).

Flow cytometry. A monoclonal antibody against P-glycoprotein, MRK16 was kindly provided by Dr. Tsuruo, the Applied Microbial Institute, the Tokyo University, Japan. As a negative control, mouse IgG2a was used. The cells were stained as described previously. Flow cytometric analysis was performed on a FACStar (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Data were analyzed according to Consort 30 software (Becton Dickinson Immunocytometry Systems).

DNA topo activity. Crude nuclear extract was prepared according to the method of Tsutsui *et al.* (12). DNA topo I activity was determined as described by Tsutsui *et al.* (12). Plasmid DNA pBR322 was kindly provided by Dr. Tsutsui. The reaction proceeded at 30˚C for 40 min in a 20μl mixture containing 10mM Tris-HCl, 0.1M NaCl, 1mM EDTA (pH 8.0), 0.5 μg of pBR322 DNA and 1μl of nuclear extract. The mixture was then treated with 0.66% SDS and 0.3mg/proteinase K prior to the analysis of DNA products by 0.8% agarose gel electrophoresis. The gels were

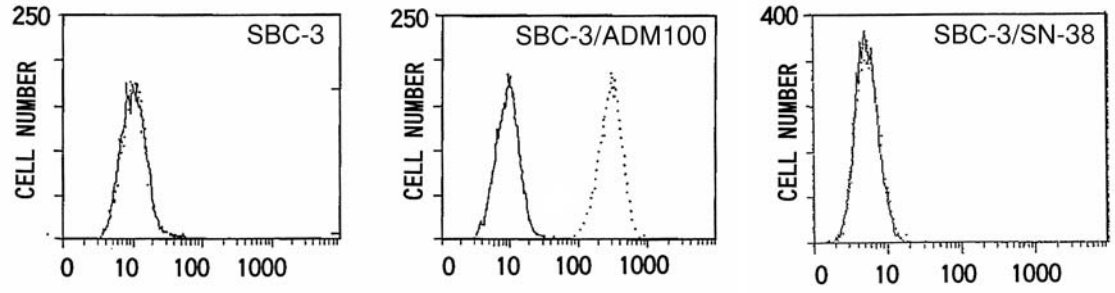

Figure 1. *Expression of P-glycoprotein in the SBC-3, SBC-3/ADM100 and SBC-3/SN-38 cells was analyzed by flow cytometry. A solid line represents a fluorescence histogram by control antibody (mouse IgG2a), while a dotted line represents a fluorescence histogram by MRK16 monoclonal antibody.*

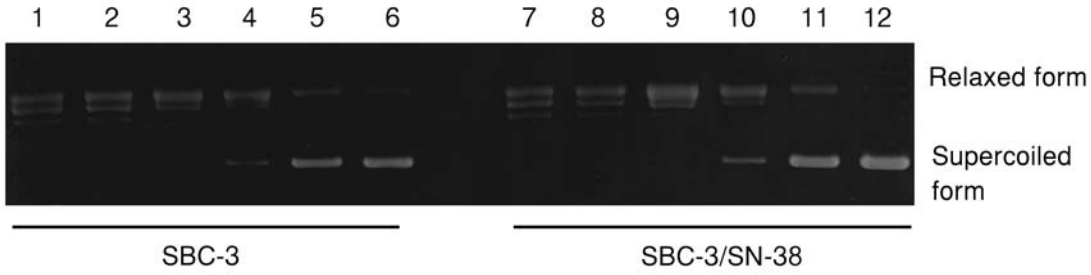

Figure 2. *DNA topo I activity determined by relaxation assay of pBR322 showing a decreased topo I activity in the SBC-3/SN-38. The amount of nuclear extracts was 3 μg for lanes 1 and 7, 1.5 μg for lanes 2 and 8, 0.75 μg for lanes 3 and 9, 0.375 μg for lanes 4 and 10, 0.18 μg for lanes 5 and 11, and 0.09 μg for lanes 6 and 12. Lanes 1-6: SBC-3; lanes 7-12: SBC-3/SN-38.*

stained with 0.5 μg/ml ethidium bromide and photographed under UV light. DNA topo II activity was assayed according to a modified technique described by Miller *et al.* (13). Kinetoplast DNA (kDNA) was also kindly provided by Dr. Tsutsui. After incubation in a total of 20-μl mixtures containing 50mM Tris-HCl (pH 8.0), 120mM KCl, 10mM $MgCl_2$, 0.5mM dithiothreitol, 0.5mM EDTA (pH 8.0), 0.5mM ATP, 30 μg/ml BSA, 0.5 μg of kDNA and 1μl of nuclear extract at 30˚C for 40 min, the reaction mixture was electrophoresed. The gels were stained and photographed as mentioned above.

Statistical analysis. Values are given as means ± standard deviation. Statistical analyses were performed using the SPSS Base System™ programs (SPSS, Chicago, IL, USA). The significance of difference between two paired groups was determined by the Student's *t*-test. *P*-values less than 0.05 in two-tailed analyses were considered significant.

Results

The IC_{50} and relative resistance to 17 anticancer agents of SBC-3 and SBC-3/SN-38 cells are shown in Table I. The SBC-3/SN-38 cells were 73-fold more resistant than the SBC-3 cells to SN-38 and showed high cross-resistance: 27-fold to irinotecan and 32-fold to topotecan. The resistant subline revealed moderate cross-resistance to topo II inhibitors (adriamycin and etoposide), to antimicrotubule agents (vincristine, vindesine, vinorelbine and docetaxel), to platinum (cisplatin and carboplatin), to mitomycin C and to methotrexate. The SBC-3/SN-38 cells were less, but significantly cross-resistant to 4-HC (1.5-fold) and 4-HI (1.4-fold). The SBC-3/SN-38 cells retained the sensitivity to bleomycin and 5-fluorouracil.

The doubling-time of the SBC-3/SN-38 cells, 23.6 h, was close to that of the SBC-3 cells, 21.6 h. The intracellular GST-π was similar in the two cell types (4.24 μg/mg protein for the SBC-3 cells and 4.39 μg/mg protein for the SBC-3/SN-38 cells). Intracellular GSH was undetectable in both cell lines. Although P-glycoprotein was demonstrated in the SBC-3/ADM100 cells as positive control, it was not detected in the SBC-3/SN-38 cells (Figure 1).

The appearance of a relaxed DNA band and the disappearance of supercoiled forms are regarded as evidence for an adequate topo I activity in the nuclear extracts. In this experiment (Figure 2), supercoiled forms disappeared in the presence of nuclear extracts of over 0.375 μg of the SBC-3 (lanes 1-4) and over 0.75 μg of the SBC-3/SN-38 (lanes 7-9). Accordingly, the topo I activity of

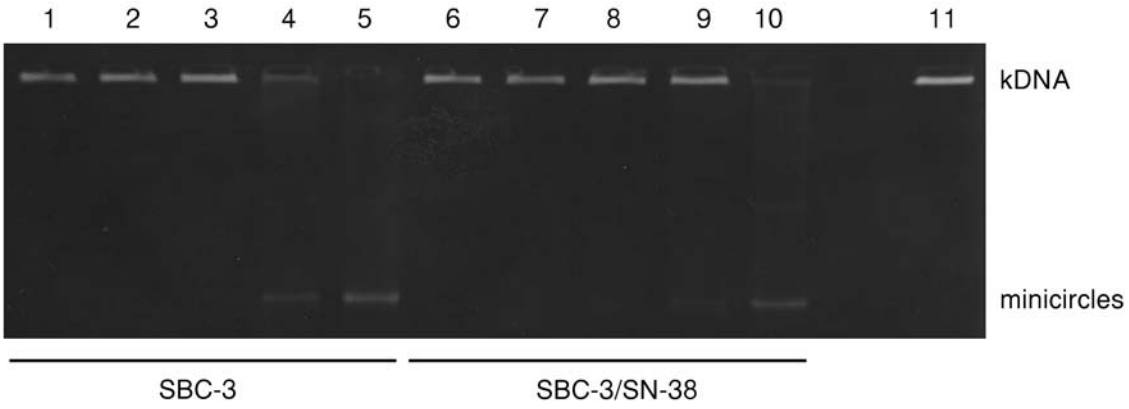

Figure 3. *DNA topo II activity determined by decatenation assay of kDNA showing a decreased topo II activity in SBC-3/SN-38. The amount of nuclear extracts was 0.00025 μg for lanes 1 and 6, 0.0005 μg for lanes 2 and 7, 0.001 μg for lanes 3 and 8, 0.002 μg for lanes 4 and 9, 0.004 μg for lanes 5 and 10, and none for lane 11 as a negative control. Lanes 1-5: SBC-3; lanes 6-10: SBC-3/SN-38.*

the SBC-3/SN-38 cells was considered to be half that of the SBC-3 cells. Topo II activity was determined by a kDNA decatenation assay (Figure 3). The formation of minicircles increased and kDNA disappeared in the presence of over 0.002 μg of the SBC-3 (lanes 4-5) and over 0.004 μg of the SBC-3/SN-38 (lane 10). This indicates that the topo II activity of the resistant cells is half that of the parent cells.

Discussion

We established an SN-38-resistant SCLC cell line *ex vivo* derived from SBC-3 cells. Several sublines resistant to a topo I inhibitor, such as camptothecin-resistant leukemia cell lines (14,15), a camptothecin-resistant Chinese hamster ovary cell line (16), a camptothecin-resistant non-small cell lung cancer, colon cancer and gastric cancer cell lines (17,18), an irinotecan-resistant non-small cell lung cancer cell line (19), an SN-38-resistant SCLC cell line (20) and a topotecan-resistant ovarian cancer cell line (21), have been reported. Although there is a slight difference in the cross-resistance pattern among these sublines, they are generally non-cross-resistant or collaterally sensitive to topo II inhibitors and non-cross-resistant to platinum, alkylating agents, antimicrotubule agents or methotrexate. On the contrary, the SBC-3/SN-38 cells were resistant to these anticancer agents. In addition, the relative resistance values of bleomycin and 5-fluorouracil were 0.81-fold and 1.2-fold, respectively. Bleomycin has not been examined in topo I inhibitors-resistant sublines to our best knowledge. A CPT-11-resistant non-small cell lung cancer cell line was cross-resistant to 5-fluorouracil (20), but an SN-38-resistant SCLC cell line was not (21).

Several mechanisms of resistance to topo I inhibitors have been reported (22). P-glycoprotein, which contributes to reduced accumulation of adriamycin, etoposide or antimicrotubule agents in the cells, is not overexpressed in the topo I inhibitor-resistant sublines as confirmed in our study. Another transporter, breast cancer resistance protein (BCRP), is responsible for the enhanced efflux of SN-38 (22). Another SN-38-resistant SCLC cell line (23) overexpressed BCRP, which has been confirmed in the SBC-3/SN-38 cells (24). A decrease in topo I activity and/or content also contributes to the resistance. In this study, we demonstrated that topo I activity in the SBC-3/SN38 cells was approximately half of the parent cell line. However, the 73-fold resistance value of SN-38 could not be explained by the reduced activity alone. On the other hand, topo II activity was elevated in the topo I inhibitor-resistant sublines (25, 26). In the present study, the decline of topo II activity in SBC-3/SN-38 was demonstrated and is responsible, in part, for the development of resistance to adriamycin (3.4-fold) and etoposide (5.5-fold). Regarding the drug detoxification system, Goto *et al.* (27) reported that irinotecan induced an increase in intracellular GST-π level. GST-π level was elevated in the cisplatin-resistant subline (SBC-3/CDDP) (28), adriamycin-resistant subline (SBC-3/ADM100) (29) and etoposide-resistant subline (SBC-3/ETP) (30), compared to that of the parent cell line. However, it was not elevated in the SBC-3/SN-38 cells. In addition, the GSH level was lower than the detection level in the SBC-3/SN-38, although it was elevated in the SBC-3/CDDP (29) and SBC-3/ADM100 (30). Accordingly, GST-π and GSH were not responsible for the resistance to platinum, alkylating agents and anthracyclines in the SBC-3/SN-38 cells.

Other mechanisms of resistance to topo I inhibitors, such as cellular localization of topo I, stabilization of DNA-topo I complexes, ubiquitin/26S proteasome-dependent degradation of topo I, DNA repair activity and regulation of NF-Î B, *etc.*, have also been reported (reviewed in Ref No. 22). Further studies are needed to

clarify the cross-resistance pattern in the SBC-3/SN-38 cells. However, the resistant subline described here would be useful in the screening of anticancer agents showing sensitivity to irinotecan-resistant SCLC. Jensen *et al.* (31) reported that the different cytotoxicity patterns for a panel of acquired drug-resistant cells could enable the selection of non-cross-resistant drugs. The drugs that are cytotoxic to both SBC-3/SN-38 and SBC-3/CDDP cells might be effective in refractory SCLC patients previously treated with irinotecan and cisplatin. The SBC-3/CDDP cells were significantly more sensitive than the parent cells to 5-fluorouracil (29) and were equally sensitive to bleomycin (unpublished data).

There were no sets of adriamycin-, etoposide-, cisplatin- and SN-38- (or irinotecan)-resistant cell lines derived from the same parent cell line. Adriamycin-resistant SBC-3/ADM, SBC-3/ADM100, etoposide-resistant SBC-3/ETP and cisplatin-resistant SBC-3/CDDP cells were established in our laboratory and now SN-38-resistant SBC-3/SN-38 cells are presented here. Using these resistant cell lines, the drug-resistant mechanisms induced by each drug can be compared and reported (24).

In conclusion, the irinotecan-resistant cell line selected by continuous exposure of SBC-3 cells to SN-38 will be useful to elucidate the mechanism of irinotecan resistance and to explore new drugs for irinotecan-resistant SCLC.

Acknowledgements

The authors wish to thank Dr. K Tsutsui, Department of Molecular Biology, Okayama University Medical School, Japan, for his kind assistance in measuring the topo activity and providing kDNA and plasmid DNA pBR322. The authors also acknowledge Dr. T Tsuruo, the Applied Microbial Institute, the Tokyo University, Japan, for providing MRK16.

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Received May 13, 2004
Revised September 29, 2004
Accepted October 13, 2004