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In vitro Antitumor Activity of TAS-103, a Novel Quinoline Derivative That Targets Topoisomerases I and II

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TAS-103 is a novel anticancer agent targeting both topoisomerase (Topo) I and Topo II, that stabilizes cleavable complexes of Topo-DNA at the cellular level. In this study, the *in vitro* antitumor effects of TAS-103 were compared with those of other known Topo I and Topo II inhibitors. TAS-103 inhibited DNA synthesis more strongly than RNA and protein synthesis, and induced an increase of cell population in the S-G2/M phase. The cytotoxicity of TAS-103 was strongest against S-phase cells, but its cell cycle phase specificity was not clear, and depended on drug concentration and exposure time. The cytotoxicity of TAS-103 (IC_{50}: 0.0030–0.23 μM) against various tumor cell lines was much stronger than that of VP-16 and comparable to that of SN-38. The cytotoxicity of TAS-103 seemed to be more related to the amount of protein-DNA complexes than to the accumulation of TAS-103 in the cells. P-Glycoprotein (P-gp)-mediated MDR, CDDP-resistant and 5-FU-resistant cell lines did not show cross-resistance to TAS-103. Although PC-7/CPT cells bearing a *Topo I* gene mutation showed cross-resistance to TAS-103, the sensitivity of P388/CPT, HT-29/CPT and St-4/CPT cells, showing decreased Topo I expression, was not changed. KB/VM4 and HT-29/Etp cells, showing decreased Topo II expression, were slightly cross-resistant to TAS-103. These results suggest that TAS-103 may act as an inhibitor of both Topo I and Topo II at the cellular level. This property may be responsible for its strong antitumor effect and broad-spectrum, growth-inhibitory effect on drug-resistant cell lines.

Key words: TAS-103 — Quinoline derivative — Topoisomerase — Cytotoxicity — Drug resistance

DNA topoisomerases (Topo) are important target molecules for cancer chemotherapy. Topo I inhibitors, CPT-11 and Topotecan (camptothecin derivatives), and Topo II inhibitors, VP-16, VM-26 and doxorubicin, are frequently used in the clinic to treat solid tumors, and their effectiveness has been confirmed. Two factors are considered to be important in determining cellular resistance to Topo inhibitors; the first is a decrease in the amount and activity of Topo[1–4] and the second is gene mutation of Topo.[5–7] Further, a decreased drug uptake[2, 8] and in the case of CPT-11, a decreased activity of the enzyme that converts CPT-11 into SN-38, its active metabolite,[9] have been reported.

As an approach to overcome drug resistance to Topo I and II targeting agents and to potentiate the antitumor effects of anticancer drugs, combination therapy using Topo I and II inhibitors has been considered. The reasoning is based on the following findings: (1) lack of cross-resistance of Topo I inhibitor-resistant cells to Topo II inhibitors,[1, 6] (2) lack of cross-resistance of Topo II inhibitor-resistant cells to Topo I inhibitors,[2–4] and (3) an increase of Topo II expression in cell lines *a priori* resistant to Topo I inhibitor,[10] and in cells after treatment with Topo I inhibitor.[11, 12] However, the results of basic experiments[13–15] and clinical studies[16, 17] led to widely different interpretations of the outcome of combined treatment with the inhibitors. Our interest in this area led us to develop TAS-103, a compound possessing Topo I and Topo II inhibitory activity, as shown by *in vitro* enzyme assay, and stabilizes cleavable complexes of Topo-DNA at the cellular level.[18] In this study, we compared TAS-103 with other inhibitors of Topo I and Topo II from the viewpoint of antitumor effects on cultured cells, i.e., inhibition of macromolecular synthesis, induction of changes in cell cycle, cell cycle phase-specific cytotoxic effects, and cytotoxicity towards various cancer cells and their drug-resistant lineage. Furthermore, we examined whether TAS-103 accumulation or the induction of cleavable complexes formation in the cells correlates better with the overall cytotoxic effects of TAS-103.

MATERIALS AND METHODS

Chemicals TAS-103 (6-[[2-(dimethylamino)ethyl]amino]-3-hydroxy-7H-indeno[2,1-*c*]quinolin-7-one dihydrochloride) (Fig. 1) and SN-38 were synthesized by Taiho Pharmaceutical Co., Ltd. (Tokyo). The following drugs were purchased : VP-16 and CDDP from Nippon Kayaku, Co., Ltd. (Tokyo), Adriamycin (ADR) from Kyowa Hakko

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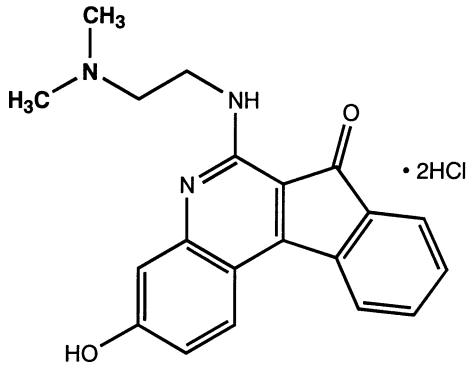

Fig. 1. Chemical structure of TAS-103.

Kogyo Co., Ltd. (Tokyo), vincristine (VCR) from Eli Lilly, Ltd. (Tokyo), 5-fluorouracil (5-FU) from Wako Pure Chemical Industries, Ltd. (Tokyo), and camptothecin (CPT) from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-Thymidine, [^{3}H]uridine and [^{3}H]leucine were purchased from Amersham (Tokyo).

Cells A549, Lu-99, LX-1, AZ-521, DLD-1, HT-29, HLF and HuH-7 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo). KM12SM, MDA-MB-231, SN12CL1, A375SM, K1735M2, Renca and UV2237M were kindly supplied by Dr. I.J.Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX). TMK-1 was a gift from Dr. N. Saijo (National Cancer Center Research Institute). MCF-7 was supplied by Dr. E. Chu (NIH, Bethesda). B16BL6 was supplied by Dr. Kobayashi (Hokkaido University School of Medicine). LLC cell line was maintained as an *in vivo* growing tumor. The origins of these tumor cell lines are shown in Table II. The drug-resistant cancer cell lines, PC-7/CPT, PC-9/CDDP and PC-14/CDDP were provided by Dr. N. Saijo (National Cancer Center Research Institute), P388/CPT, P388/ADR and HT-29/CPT, HT-29/Etp, St-4/CPT and St-4/Etp were kindly provided to us by Dr. T. Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo), and KB/VM4 was supplied by Dr. M. Kuwano (Department of Biochemistry, Oita Medical School). KB/VCR was supplied by Dr. S. Sone (Tokushima University School of Medicine). MCF-7/Ad10 and H630-R10 were supplied by Dr. E. Chu (NIH, Bethesda). These cells were passaged in appropriate media, according to information provided to us by a supplying investigator.

Drug sensitivity test The tumor cells were plated at the density of 1–10×10^3 cells/0.1 ml/well in 96-well flat-bottomed microplates. After overnight incubation at 37°C under 5% CO_2, 0.1 ml aliquots of the medium containing serially diluted test compounds were added to the wells in triplicate, and incubation was continued for 72 h. Cell growth was assessed using the sulforhodamine B dye-staining (SRB) method[19] for HT-29/CPT, HT-29/Etp, St-4/CPT St-4/Etp and their parental cell lines, the crystal violet staining method[20] for P388/CPT, P388/ADR, KB/VM4, KB/VCR, H630-R10 and their parental cell lines, and the colorimetric tetrazolium-formazan (MTT) assay[21] for the other cell lines. The IC_{50} was defined as the drug concentration needed to produce a 50% reduction of growth relative to the control.

Macromolecular synthesis in intact cells A549 cells plated at a cell density of 4×10^4 cells/well were cultured for 2 days. After 6 h incubation with the test compounds at varying concentrations, the cells were labeled with [^{3}H]thymidine (0.5 μCi/well), [^{3}H]uridine (0.5 μCi/well) and [^{3}H]leucine (2 μCi/well) for 1 h at 37°C. Then the cells were washed twice with cold PBS, treated with trypsin/EDTA and suspended in PBS. The cell suspensions were loaded on glass filter plates (GF/C96, Packard, California), and washed 4 times with distilled water and once with ethanol. The radioactivity was counted using a microplate scintillation counter (Top Count, Packard). The radioactivity in each well was considered to be proportional to the rate of synthesis of the respective macromolecules.

Cell cycle analysis A549 cells, in the logarithmic phase of growth, were treated with the test drug for 8 or 24 h. The cells were collected, and treated with trypsin in a spermine tetrahydrochloride detergent buffer, then exposed to trypsin inhibitor and RNase treatment in citrate-stabilizing buffer, and finally stained with 125 μg/ml of propidium iodide (PI) (performed using a Cycle TEST PLUS DNA Reagent kit, Becton Dickinson, San Jose, CA). Cell distribution according to the cycle phase was determined by measuring the DNA content using a FACScan flow cytometer (Becton Dickinson).

Cell cycle phase specificity of the drug KB cells were synchronized by double thymidine block. Briefly, exponentially growing cells were incubated for 24 h in medium containing 1 m*M* thymidine, followed by incubation for 8 h in thymidine-free medium. Synchronization of cells at the G1/S boundary was then achieved by further incubation for 16 h in thymidine-containing medium. The thymidine block was released by washing the cells twice with PBS, followed by incubation in a fresh medium. At 0, 2, 4, 6, 8 and 10 h after release from the thymidine block, the test drugs were applied. Following 1 h exposure to a drug, the cells were washed with PBS, treated with trypsin/EDTA, and suspended in culture medium. Then the cells were plated at a cell density of 2×10^3 cells/well and incubated for 3 days. The number of viable cells was determined by MTT assay. Cell cycle synchronization was assessed by flow cytometry as described above.

Colony-forming assay DLD-1 cells were plated into 35 mm dishes at a cell density of 200 cells/well. One day

after seeding, the test drug solutions were added to the wells at varying concentrations in triplicate. One to 72 h after addition of the drug, the cells were washed twice with PBS, and fresh culture medium was added. On the 7th day after seeding, the cells were fixed with 25% glutaraldehyde, and stained with 0.05% crystal violet. The number of colonies was counted using a colony analyzer (PCA-11, System Science Co., Tokyo). The surviving fraction was calculated by dividing the number of colonies formed by cells exposed to the drug by that of the control colonies. The IC_{90} value (the drug concentration causing 90% inhibition of colony formation) was determined from the dose-response curve.

Accumulation of TAS-103 in various cells Exponentially growing cells (1×10^6 cells/ml) were incubated in RPMI1640 medium containing 0.2 μM [^{3}H]TAS-103 for 2 h. An aliquot of 0.2 ml of cell suspension was layered on warmed silicon oil (0.2 ml) and centrifuged (12,000g, 5 min). The cell pellet was dissolved by sonication in Soluene (1.5 ml) and HIONIC-FLUOR (10 ml) was added to each tube. The radioactivity was measured in a liquid scintillation counter.

Protein-DNA complexes in whole cells The production of DNA-protein complexes in various tumor cells following 1 h treatment with TAS-103 was assessed using an SDS-KCl precipitation assay.[22] Cells (2×10^6 cells/0.5 ml) radiolabeled with [^{3}H]thymidine (1 μCi/ml) and [^{14}C]leucine (0.5 μCi/ml) were treated with various concentrations of TAS-103 (0.05 ml). After 1 h treatment the cells were lysed by the addition of 61 μl of a lysis solution (15% SDS, 5 mM EDTA) preincubated at 65°C, followed by pipetting. Then 68 μl of 650 mM KCl was added and the tubes were placed on ice for 5 min. The SDS-K precipitates containing protein/DNA complexes were collected by centrifugation (15,000g, 5 min). The DNA/protein-containing pellet was dissolved in 500 μl of deionized water (65°C) and the radioactivity was counted to determine ^{14}C and ^{3}H. The data were expressed as the ratio of dpm ^{3}H-DNA/dpm ^{14}C-protein.

RESULTS

Inhibitory effect on macromolecular synthesis in A549 cells After 6 h exposure, TAS-103 predominantly inhibited the synthesis of DNA; the effect on RNA synthesis was one order of magnitude weaker and synthesis of proteins was only slightly affected (Table I). This effect was the same as that of SN-38 on DNA synthesis. VP-16 had a strong inhibitory effect on DNA synthesis, and also inhibited RNA synthesis, though its overall inhibitory effect on macromolecular synthesis was much weaker than those of TAS-103 and SN-38.

Table I. Inhibitory Effect of TAS-103 on Macromolecular Synthesis in A549 Cells

Compound	IC_{50} (μM)		
	DNA	RNA	Protein
TAS-103	0.020	0.49	3.7
SN-38	0.036	4.1	>25
VP-16	3.9	22	95

Effects on the cell cycle of A549 cells Changes in the cell cycle of A549 cells treated with TAS-103, SN-38 and VP-16 for 8 h and 24 h are shown in Fig. 2. The drugs caused about 20–40% inhibition of cell growth compared to controls after 24 h (approximately one doubling time) of treatment at the concentration used. SN-38 induced an increase of cell population in the early S phase after 8 h of treatment. After 24 h, SN-38 mainly induced an increase of the number of cells in the S and G2/M phases, at low concentrations (Fig. 2c). VP-16 induced a slight increase of the number of cells in the S phase after 8 h of treatment. After 24 h, VP-16 induced an increase of the number of cells mainly in the G2/M phase and S phase, however, the latter effect was observed at high concentrations (Fig. 2d). When cells were treated with TAS-103 for 8 h, the number in the early S phase increased, following the same pattern as that after treatment with SN-38, although the increase was less pronounced than that caused by SN-38. After 24 h, however, TAS-103 mainly induced an increase of the number of cells in the G2/M and S phases at high concentrations, following the pattern of VP-16 (Fig. 2b).

The cell cycle changes induced by treatment with TAS-103 show the same pattern as those induced after treatment with SN-38 and VP-16.

Cell cycle phase specificity of the cytotoxic effect of drugs The cell sensitivity to the drugs and cell distribution according to the cell cycle phase in relation to time after release from the thymidine block are shown in Fig. 3. The open circles indicate the effect of the compound on nonsynchronized cells, while the closed circles show the effect of the compound on synchronized cells.

The cytotoxicity of SN-38 was strongest against cells in S phase. However, this drug did not exert cytotoxic effects against G2/M-phase cells. Thus, it is concluded that the cytotoxicity of SN-38 is S phase-specific. The cytotoxicity of VP-16 was strongest against S-phase cells, as well. Its cytotoxic effect against cells in the G2/M phase was comparable to that against nonsynchronized cells. Thus, the cytotoxicity of VP-16 was not cell cycle phase-specific. TAS-103 also showed an increased cytotoxicity against S-phase cells. Its cytotoxic effect against G2/M-phase cells was comparable to that observed in nonsynchronized cells. Therefore, we conclude that the cytotoxic effect of TAS-103 is not cell cycle phase-specific.

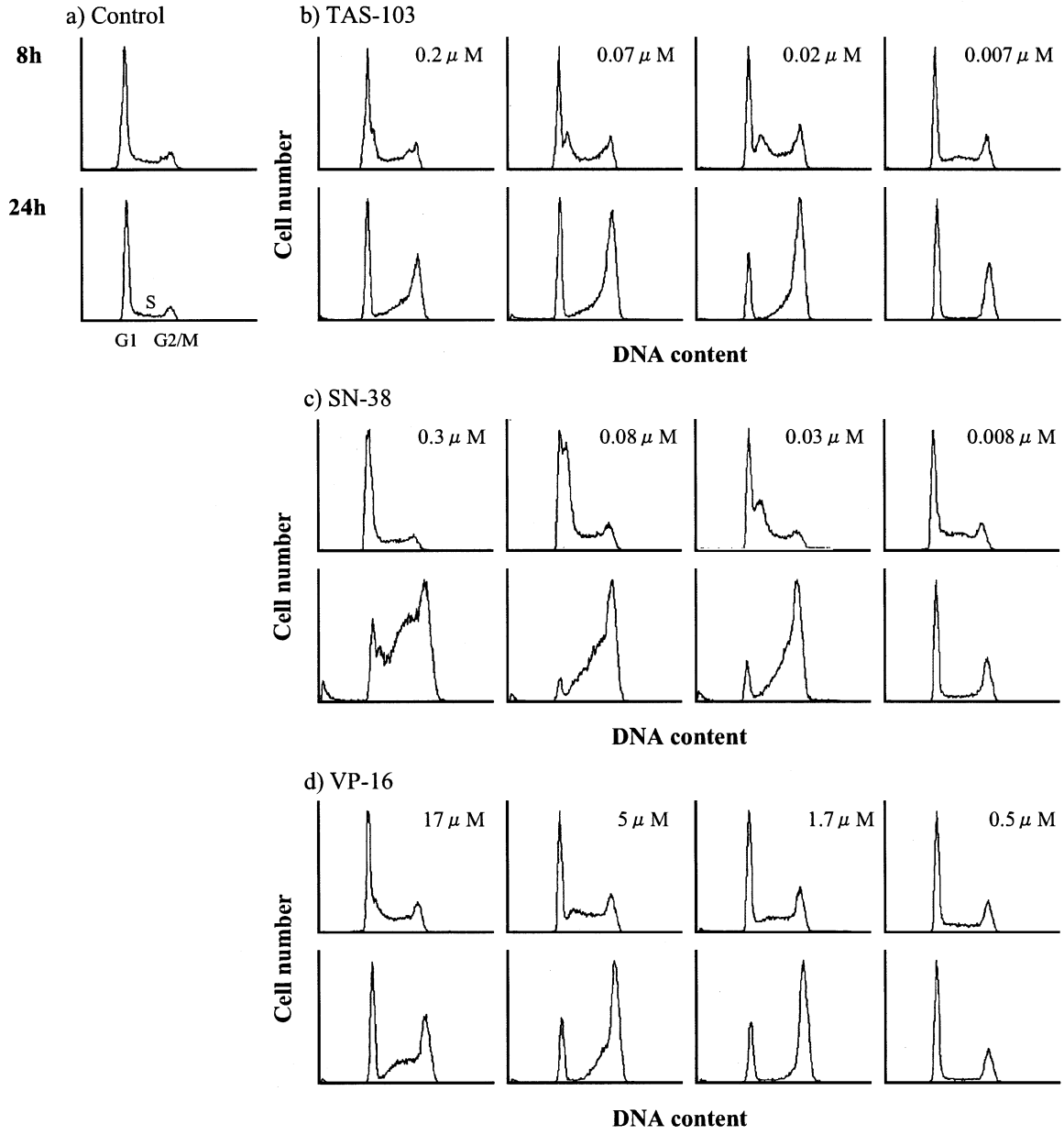

Fig. 2. Effects of TAS-103, SN-38 and VP-16 on cell cycle progression. A549 cells were treated with the indicated concentration of the drugs for 8 or 24 h. The DNA content was analyzed by flow cytometry.

Because the cell cycle phase-specificity of drugs is assumed to affect cell-kill kinetics,[23, 24] we determined the cell-killing ability of the tested compounds.

Cell-kill kinetics of TAS-103 against DLD-1 cells The IC_{90} values, as a function of the exposure time, were plotted on a log scale as shown in Fig. 4. The cell-killing ability of TAS-103 depended on both time and concentration; the IC_{90} values constantly shifted to a low concentration as the exposure time was prolonged. TAS-103 showed an almost linear relationship, with a slope of −1. The shape of this curve was similar to that of VP-16, a cell cycle phase-nonspecific agent. The cell-killing effect of this class of agents depends on drug concentration and exposure time (area under the concentration curve, AUC). On the other hand, the cell-killing effect of SN-38 seemed to depend more on time than on concentration. A potent cell-killing effect was observed after continuous exposure for more than 24 h. This very steep curve of SN-38 was

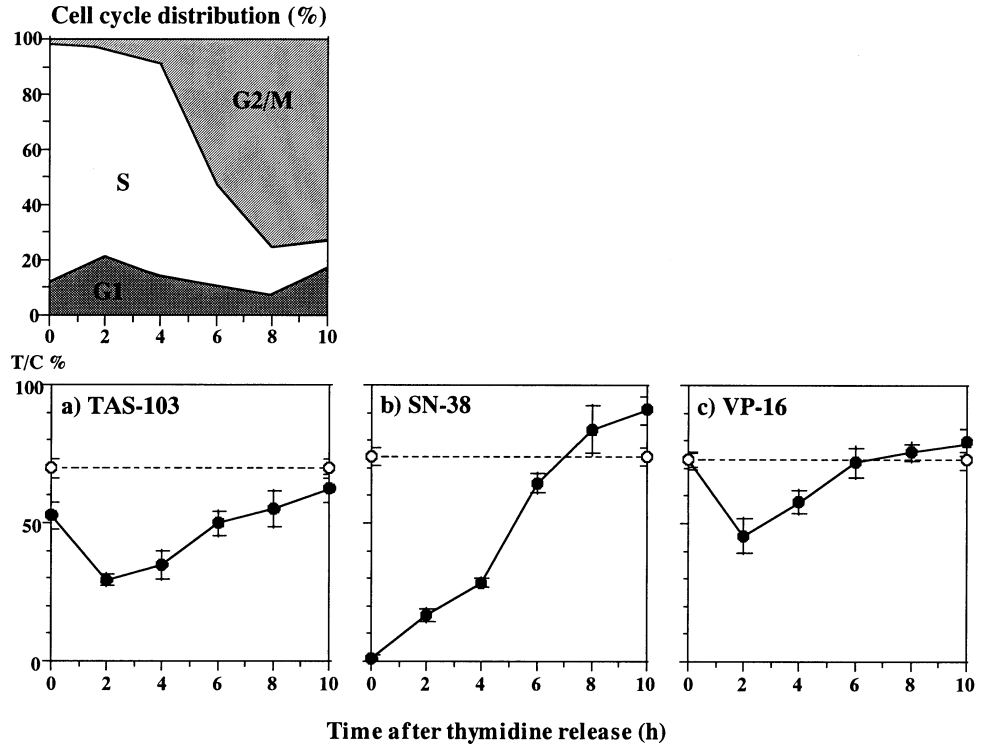

Fig. 3. Cell cycle phase specificity of cytotoxic effects. After release from thymidine block, KB cells were treated with TAS-103 (0.07 μM), SN-38 (0.8 μM) or VP-16 (1.7 μM) for 1 h at the indicated time. Cells were washed and reseeded into 96-well plates. After 3 days of culture, the number of viable cells was determined by MTT assay. Open circles show the effect on nonsynchronized cells and closed circles show the effect on synchronized cells.

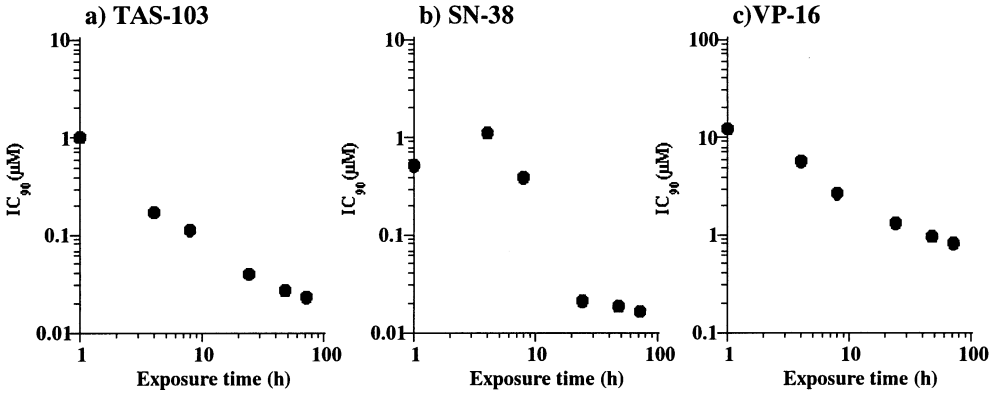

Fig. 4. Log-log relationship between IC_{90} and exposure time. DLD-1 cells were exposed for various periods of time, and the surviving fractions were determined by colony-forming assay.

regarded as being characteristic of cell cycle phase-specific agents. In the case of this class of agents, long-term exposure is indispensable for efficient cell-killing.

Cytotoxicity of TAS-103 against various tumor cell lines The cytotoxicity of TAS-103 against various tumor cell lines was very strong (IC_{50}: 0.0030–0.23 μM), as shown in Table II. The effect was stronger than that of VP-16 (IC_{50}: 0.41–13 μM) and was comparable to that of SN-38 (IC_{50}: 0.0028–0.66 μM).

Accumulation of TAS-103 in cells We measured the

Table II. Cytotoxicity of TAS-103 against Various Tumor Cell Lines

Cell line	Origin	IC_{50} (μM)[a]		
		TAS-103	SN-38	VP-16
Human cell lines				
A549	Lung	0.047	0.14	4.4
Lu-99	Lung	0.010	0.0028	0.46
LX-1	Lung	0.074	0.025	2.9
DLD-1	Colon	0.084	0.056	1.4
HT-29	Colon	0.23	0.028	3.6
KM12SM	Colon	0.071	0.024	1.6
AZ-521	Stomach	0.013	0.0031	0.41
TMK-1	Stomach	0.019	0.017	1.1
MDA-MB-231	Breast	0.14	0.15	4.1
MCF-7	Breast	0.052	0.0043	1.3
SN12CL1	Kidney	0.098	0.041	4.8
HLF	Liver	0.020	0.012	1.1
HuH-7	Liver	0.17	0.66	13
A375SM	Melanoma	0.079	0.0074	1.4
Murine cell lines				
B16BL6	Melanoma	0.064	0.025	0.58
K1735M2	Melanoma	0.0059	0.082	0.63
Renca	Kidney	0.066	0.66	2.2
UV2237M	Fibrosarcoma	0.0049	0.061	0.75
LLC	Lung	0.0030	0.099	0.73

a) The cell growth inhibition was determined by MTT assay after 72 h drug treatment.

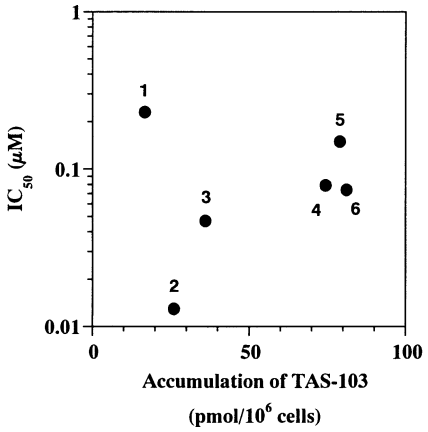

Fig. 5. Accumulation of TAS-103 in tumor cells and its cytotoxicity. 1, HT-29; 2, AZ-521; 3, A549; 4, A375SM; 5, LC-11; 6, LX-1. Abscissa, the amount of intracellular TAS-103 in cells after 2 h incubation; ordinate, the cytotoxicity of TAS-103 on 72 h treatment.

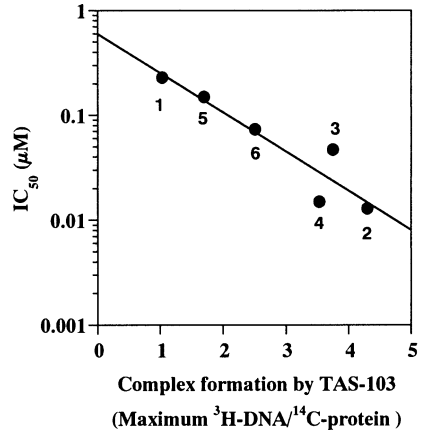

Fig. 6. Correlation between the formation of DNA-protein complexes in tumor cells treated with TAS-103 and cytotoxicity. 1, HT-29; 2, AZ-521; 3, A549; 4, SBC-5; 5, LC-11; 6, LX-1. Abscissa, the maximum formation of DNA-protein complexes in cells after 1 h incubation; ordinate, the cytotoxicity of TAS-103 on 72 h treatment.

accumulation of TAS-103 in various cell lines using radiolabeled TAS-103. In this experiment, cancer cell lines of different sensitivity to TAS-103 were selected. As shown in Fig. 5, the sensitivity to TAS-103 appeared not to correlate with the amount of TAS-103 taken up by the tested cell lines.

Formation of protein-DNA complexes in cells Formation of TAS-103-induced cleavable complexes in the cells was measured using the SDS-KCl precipitation assay. In each cell line, cleavable complex formation was induced by TAS-103 in a concentration-dependent manner (0.1–30 μM) (data not shown). The maximum increase in formation of cleavable complexes was observed in the concentration range of 1–3 μM. The cytotoxicity of TAS-103 toward the tumor cells appeared to be closely related to the amount of cleavable complex formed in the cells treated with TAS-103 (Fig. 6).

Cross-resistance to TAS-103 of drug-resistant cell lines Table III shows the cross-resistance of various resistant cell lines. Typical multi-drug resistant cells, showing an excessive expression of P-glycoprotein (P-gp), P388/ADR, KB/VCR[25] and MCF-7/Ad10,[26] did not show cross-resistance to TAS-103. These three cell lines did not show resistance to CPT, while they showed partial resistance to VP-16. The cisplatin-resistant cells, PC-9/CDDP[27, 28] and PC-14/CDDP,[27, 29] and the 5-FU-resistant cells, H630-R10,[30] showed no cross-resistance to TAS-103, CPT or VP-16. Table IV shows the cytotoxicity of TAS-103 to cells resistant to Topo inhibitors. PC-7/CPT cells (resistant to CPT-11 owing to a mutation in the *Topo I* gene) showed cross-resistance to TAS-103 (relative resistance: 9.5), while P388/CPT, HT-29/CPT and St-4/CPT cells (resistant to CPT and having reduced Topo I expression) showed no cross-resistance to TAS-103. KB/VM4 and HT-29/Etp cells (resistant to VM26 and VP-16 and having reduced Topo

Table III. Relative Resistance to TAS-103 of Various Drug-resistant Cell Lines

Drug-resistant cell line	Characteristics of resistant cell line	Relative resistance[a]				
		Reference compounds[b]		TAS-103	CPT	VP-16
P388/ADR	P-gp MDR	ADR	71	1.6	1.6	22
KB/VCR	P-gp MDR	VCR	169	0.46	0.33	3.1
MCF-7/Ad10	P-gp MDR	ADR	1250	8.3	1.5	51
PC-9/CDDP	Decreased uptake	CDDP	13	1.8	1.2	8.5
PC-14/CDDP	Decreased uptake	CDDP	3.6	0.45	0.48	0.31
H630-R10	Increased TS	5-FU	21	2.4	4.0	0.39

a) Degree of resistance represents the ratio of IC_{50} value for the resistant line to IC_{50} value for the sensitive line.
b) ADR, adriamycin; VCR, vincristine; CDDP, *cis*-diamminedichloroplatinum (II); 5-FU, 5-fluorouracil.

Table IV. Cytotoxicity of TAS-103 to Topo Inhibitor-resistant Cell Lines

Cell line	Characteristics of resistant cell line	IC_{50} (μM)				
		Reference compound			TAS-103	
PC-7			0.77		0.094	
PC-7/CPT	Mutant Topo I	CPT	>29	(>38)[a]	0.89	(9.5)
P388			0.017		0.00084	
P388/CPT	Decreased Topo I	CPT	1.2	(71)	0.00034	(0.40)
HT-29			0.043		0.32	
HT-29/CPT	Decreased Topo I	CPT	0.75	(17)	0.11	(0.34)
St-4			0.20		0.47	
St-4/CPT	Decreased Topo I	CPT	1.5	(7.5)	0.12	(0.26)
KB			0.44		0.059	
KB/VM4	Decreased Topo II	VP-16	29	(66)	0.22	(3.7)
HT-29			4.4		0.32	
HT-29/Etp	Decreased Topo II	VP-16	68	(15)	1.1	(3.4)
St-4			8.0		0.47	
St-4/Etp	Decreased Topo II	VP-16	88	(11)	0.74	(1.6)

a) Relative resistance represents the ratio of IC_{50} for the resistant cell line to IC_{50} for the parental cell line.

II expression) showed partial resistance to TAS-103 (relative resistance values: 3.7 and 3.4), but their resistance was weaker than that to VP-16. St-4/Etp cells showed no cross-resistance to TAS-103. These results provide indirect evidence that the mechanism of the cytotoxic effect of TAS-103 is mechanistically associated with the inhibition of both Topo I and Topo II.

DISCUSSION

We have investigated the antitumor effect of TAS-103, a Topo I, II-dual inhibitor, on cultured cells. Topo I is considered to play an important role in DNA replication and transcription,[31, 32] and its expression does not change during the cell cycle. Topo II, having the ability to catenate/decatenate DNA, is also important for DNA replication and for chromosomal condensation and segregation.[33, 34] It is reported that the expression of Topo II increases along with DNA replication, reaches a peak in cells in the G2/M phase, then sharply decreases.[35, 36] As shown in Table I, Topo I inhibitor SN-38 strongly inhibited DNA synthesis in A549 cells and mainly induced an increase of cell number in the S phase. The cytotoxic effect of SN-38 was specific for cells in the S phase and depended on the time of exposure to the drug. On the other hand, the Topo II inhibitor VP-16 predominantly inhibited DNA synthesis and also exerted an inhibitory effect on RNA synthesis. These effects were associated with the induction of an increase of cell population in the G2/M and S phases after 24 h treatment at high concentrations of VP-16. The cytotoxic effect of VP-16 was the strongest on cells in the S phase, but its effect was cell cycle phase non-specific and depended on drug concentration and time (AUC). These results are in agreement with previous

reports,[24, 37, 38] and are consistent with the role and expression of Topo during the cell cycle. The Topo I, II-dual inhibitor TAS-103 inhibited DNA synthesis most strongly and induced an increase in the number of cells in the early S phase (6 h treatment), thus showing a similar pattern to SN-38. On the other hand, the accumulation of cells in the G2/M phase after 24 h treatment with TAS-103 was similar to that induced by VP-16. The cytotoxic effect of TAS-103 was the strongest against cells in the S phase, but appeared to be cell cycle phase non-specific and dependent on drug concentration and exposure time (AUC), as found for VP-16. These results suggest that the changes induced in cells by TAS-103, which are partly common with those induced by SN-38 and VP-16, were probably due to its dual inhibitory effect on Topo I and Topo II.

As shown in Table II, TAS-103 exhibited potent antitumor effects on a broad spectrum of tumor cell lines; its effects were much stronger than those of VP-16 but comparable to those of SN-38. To elucidate the factors determining the sensitivity of cells to TAS-103, we first measured the accumulation of TAS-103 to see whether the biological effects depend on the uptake or occur via other mechanisms. A low cytotoxicity was often associated with a decreased accumulation, especially in cell lines exerting multidrug resistance. However, TAS-103 accumulation in the cells tested did not correlate with the cytotoxicity of TAS-103, indicating that the cytotoxic effects are due to other mechanisms. Therefore, we next examined whether formation of cleavable complexes induced by TAS-103 was related to the cytotoxicity. Recently, the cytotoxic effect of a Topo inhibitor has been reported to be related to the content or activity of Topo or to its ability to form cleavable complexes. In this study, the cytotoxicity of TAS-103 could be partly explained by the increased formation of cleavable complexes in the cells. TAS-103 did not show cross-resistance in various drug-resistant cell lines. MDR cell lines, showing excessive expression of P-gp, did not show cross-resistance to TAS-103 or CPT. Two CDDP-resistant cell lines and one 5-FU-resistant cell line also did not show cross-resistance to TAS-103, CPT, or VP-16. Among the cell lines resistant to Topo inhibitors, PC-7/CPT, bearing a *Topo I* gene mutation, showed cross-resistance to TAS-103, but P388/CPT and HT-29/CPT, exhibiting decreased Topo I expression, did not. KB/VM4, HT-29/Etp and St-4/Etp, showing reduced Topo II expression, had weak or no cross-resistance to TAS-103. These results suggested that the mechanisms of action of TAS-103 involve the inhibition of both Topo I and Topo II, and that TAS-103 can be effective against cell lines with already acquired resistance to an inhibitor of Topo. One may expect TAS-103 to show anti-tumor effects in patients when CPT-11 and VP-16 are no longer effective, since it was reported that the resistance of clinical cancers is correlated with a low expression of either Topo,[39] rather than with a mutation of the *Topo* gene.[40]

One of the possible approaches to overcome drug resistance to either Topo I or Topo II inhibitor is combination therapy with a Topo I inhibitor and a Topo II inhibitor. Several studies have noted potentiation of their combined effects when the Topo I inhibitor was administered first, followed by treatment with the Topo II inhibitor.[15, 16] The increase of Topo II expression and the number of cells in the S phase after treatment with the Topo I inhibitor were also considered to increase the number of cells highly sensitive to the Topo II inhibitor. Using a reverse sequence of administration may cause an increase of the population of cells with low sensitivity to the Topo I inhibitor, namely, cells in the G2/M phase. The combination schedule for the administration of Topo I and Topo II inhibitors is therefore important to achieve an additive effect.[13–15] Some clinical studies on combination therapy have also been performed. For example, the combination of Topotecan with doxorubicin was reported to be effective.[16] But, combination therapy using CPT-11 and VP-16 induced severe diarrhea and lung toxicity with no objective response.[17] Therefore, the potentiation of antitumor effects by combining Topo I and Topo II inhibitors cannot be obtained by a simple combination and it is necessary to establish the most appropriate timing for the administration of the drugs. However, at that stage, it is very difficult to establish a proper chemotherapeutic regimen. As indicated in this study, TAS-103 is a compound which functions both as a Topo I and a Topo II inhibitor. TAS-103 is strongly cytotoxic to a variety of parental tumor cell lines, as well as their drug-resistant congeners. The fact that TAS-103 inhibits both Topo I and Topo II appears to be highly advantageous.

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