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Antitumor Effect of DX-8951, a Novel Camptothecin Analog, on Human Pancreatic Tumor Cells and Their CPT-11-resistant Variants Cultured *in vitro* and Xenografted into Nude Mice

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DX-8951 is a novel water-soluble derivative of camptothecin. We evaluated the effects of DX-8951 on the growth of several pancreatic tumor cell lines *in vitro* and *in vivo*. *In vitro* cytotoxic activity of DX-8951 against SUIT-2 and KP-1N cells, as indicated by IC_{50} value, was several times more potent than that of SN-38, an active metabolite of CPT-11, and dozens of times more potent than that of SK&F104864 (topotecan). DX-8951 also showed the greatest cytotoxicity against CPT-11-resistant variants, SUIT-2/CPT-11 and KP-1N/CPT-11 cells, and the cross-resistance of these cells to DX-8951 was lower than that to SN-38 and SK&F104864. Topoisomerase I inhibitory activity of DX-8951 was about three-fold stronger than that of SN-38, as measured in crude nuclear extract obtained from SUIT-2 cells. DX-8951 induced DNA fragmentation, a specific feature of apoptosis, in SUIT-2 cells more effectively than SN-38. DX-8951 exhibited potent antitumor effects against SUIT-2 in a solid tumor model and in a liver metastasis model, in which tumor cells were xenografted subcutaneously and intrasplenically, respectively, into nude mice. The *in vivo* effects were closely similar to or somewhat superior to those of CPT-11. DX-8951 also showed significant antitumor effects against SUIT-2/CPT-11 solid tumors, against which CPT-11 had no effect. These results suggest that, on the basis of its strong antitumor activity and effectiveness against CPT-11-resistant tumors, DX-8951 may be a useful therapeutic agent in the treatment of human cancer. The potent cytotoxicity of DX-8951 may result from strong inhibition of topoisomerase I, which may then trigger apoptotic cell death.

Key words: DX-8951f — Camptothecin — Pancreatic cancer — Drug resistance — Nude mice

Camptothecin, isolated from the plant *Camptotheca acuminata*,[1] has been shown to have antitumor activity in various experimental tumor models.[2] However, the clinical therapeutic index of this compound was not sufficient to justify further development because of severe adverse effects.[3] 7-Ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin hydrochloride trihydrate (CPT-11, irinotecan hydrochloride), a semi-synthetic, water-soluble derivative, was found to exhibit higher antitumor activity with less toxicity than camptothecin in experimental models[4–6] and in clinical studies.[7–12] CPT-11 is a prodrug with marginal effects *in vitro* and is hydrolyzed *in vivo* to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38).[13] Therefore, the clinical activity of CPT-11 may strongly depend on its conversion to SN-38. Camptothecins act mainly by inducing DNA breaks by inhibiting topoisomerase I through the stabilization of the enzyme-DNA complex.[14,15] SN-38 has also been confirmed to interact directly with the DNA-topoisomerase I cleavable complex,[16] and finally to induce apoptotic cell death.[17,18]

DX-8951, (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, is a synthetic derivative of camptothecin with a unique hexacyclic structure[19] (Fig. 1). The compound is water-soluble and does not require metabolic activation. DX-8951 is reported to show stronger antiproliferative activity than camptothecin, SN-38, and another non-prodrug-type analog, SK&F104864 (generally called topotecan), against human cancer cell lines including breast cancer, gastro-intestinal cancer, lung cancer, ovarian cancer, and leukemia.[20] The compound has been confirmed to exhibit more potent therapeutic effects *in vivo* than CPT-11 against human gastric cancer xenografts in nude mice.[20] In addition, DX-8951 is highly effective against a multidrug-resistant tumor overexpressing P-glycoprotein.[20] These experimental data suggest that DX-8951 is a promising candidate drug for the treatment of cancer in patients. Phase I clinical trials of DX-8951 are planned to start in the near future.

In the present study, we examined the antitumor activity of DX-8951 against human pancreatic cancers, in which CPT-11 has been reported to show moderate but insufficient antitumor effects in clinical studies.[21,22] Two

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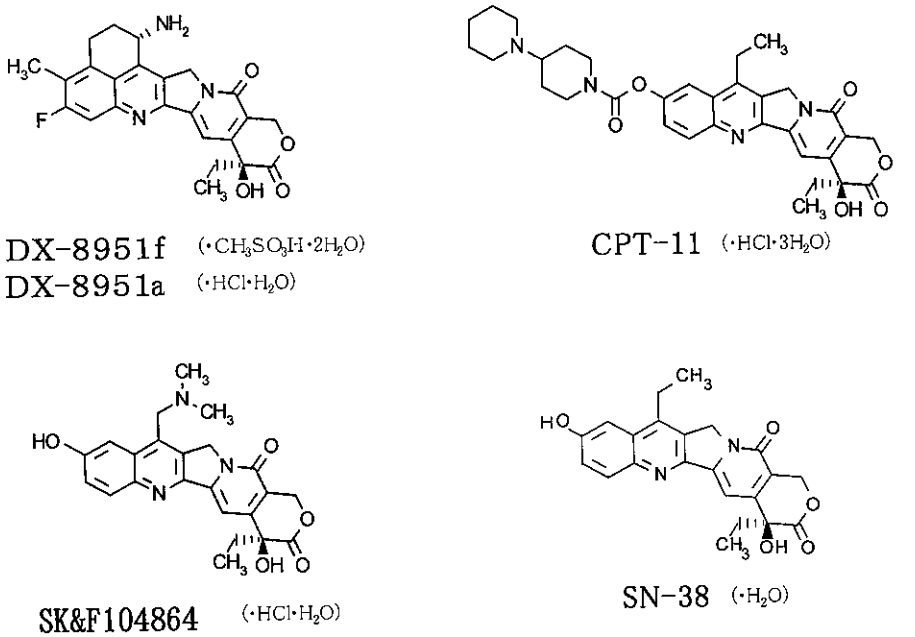

Fig. 1. Chemical structures of camptothecin analogs. Empirical formula of the salt and hydrate of each compound is indicated in parenthesis.

human pancreatic tumor cell lines[23, 24] and their variants with acquired CPT-11 resistance[25] were used. We first evaluated cytotoxic activity *in vitro*, then confirmed the drug's topoisomerase I-inhibiting and apoptosis-inducing activities, and finally compared the *in vivo* antitumor activity of DX-8951 with that of CPT-11 in solid tumor models and a liver metastasis model[6, 24, 26] in nude mice.

MATERIALS AND METHODS

Tumor cell lines Both SUIT-2 and KP-1N tumor cell lines were derived from a metastatic liver tumor of human pancreatic carcinoma.[23, 24] The cells were maintained in a humidified atmosphere of 5% CO_2 in air. Daigo's T Medium (Nihon Seiyaku Co., Ltd., Tokyo) supplemented with 7% fetal bovine serum (Hazaleton Research Products, Inc., Lenexa, KS) was used as growth medium. SUIT-2/CPT-11 and KP-1N/CPT-11 cells were obtained from the respective parental tumor cells through continuous exposure to CPT-11[25]: the concentration of CPT-11 added to the growth medium was increased stepwise for 35 weeks. These resistant variants were finally maintained in culture in the presence of 5 μg/ml of CPT-11.

Camptothecin analogs Chemical structures of the camptothecin analogs used in the present study are shown in Fig. 1. CPT-11 and SN-38 were provided by Yakult Honsha Co., Ltd., Tokyo. SK&F104864, 20-(*S*)-9-dimethylaminomethyl-10-hydroxycamptothecin, was synthesized as described by others.[27] DX-8951 was used in the form of the hydrochloride (DX-8951a) and the methanesulfonate (DX-8951f). These camptothecin derivatives were each dissolved in dimethyl sulfoxide (DMSO) and then diluted with the growth medium for *in vitro* use, or dissolved in distilled water for *in vivo* experiments. Dose levels are all expressed in terms of the anhydrous free base.

***In vitro* cytotoxicity test** Tumor cells seeded into 96-well plates (180–300 cells/well) were grown for 24 h, and then exposed to a camptothecin analog (DX-8951a, DX-8951f, SK&F104864, CPT-11 or SN-38) for 48 h. They were washed twice with the medium and cultured for an additional 5 days. Cell growth was measured by MTT assay.[28] The concentration of the compound required for 50% inhibition of cell growth (IC_{50}) was determined from the dose-response curve.

DNA topoisomerase I activity assay DNA topoisomerase I activity was determined from the relaxation of supercoiled plasmid DNA, pBR322,[29] essentially as described by Liu and Miller.[30] For measurement of total activity of topoisomerase I in cells, the reaction mixture (40 μl) consisted of 25 m*M* Tris HCl (pH 7.5), 50 m*M* KCl, 5 m*M* $MgCl_2$, 25 m*M* EDTA·2Na, 0.25 m*M* dithiothreitol, 5% glycerol, 0.5 μg of pBR322 and crude nuclear extract of SUIT-2 cells. To measure the inhibition of DNA relaxation induced by the camptothecin analogs,

DX-8951a and SN-38, reaction mixtures contained the specified amount of crude nuclear extract (0.053 μg) and drug solution (1 μl), diluted serially with DMSO. The reaction mixtures were incubated at 37°C for 10 min, and reaction was terminated by the addition of dye solution (12 μl) consisting of 1.5% SDS, 22.5 m*M* EDTA·2Na, 25% sucrose and 0.02% bromophenol blue. The mixtures were applied to 1% agarose gel and electrophoresed. The gel was stained with 1 μg/ml ethidium bromide and photographed on a Polaroid type 55 film under UV light of 302 nm. The regions of supercoiled DNA in the negatives of the films were densitometrically analyzed with a computer scanner and the NIH image program controlled by a Macintosh computer.

Analysis of DNA fragmentation SUIT-2 cells were incubated in the presence of DX-8951a or SN-38 for 24 h. Analysis of DNA fragmentation was performed essentially as described by Smith *et al.*[31] Briefly, the cells were lysed in phosphate-buffered saline containing 0.1% EDTA·4Na, 5% SDS and 0.2 mg/ml proteinase K (Boehringer Mannheim, Germany) for 18 to 24 h at 37°C. Samples were extracted with phenol once and chloroform/isoamyl alcohol once, precipitated with ethanol, treated with DNA-free RNase for 5 h at 37°C and finally dissolved in TE buffer (10 m*M* Tris·Cl, 1 m*M* EDTA·2Na, pH 8.0). Equal amounts of DNA were electrophoresed on 1.2% agarose gel. The gel was stained and photographed, and the regions of fragmented DNA in the photo were densitometrically scanned in the same manner as above.

Microscopic examination of cultured tumor cells SUIT-2 cells were cultured in the growth medium containing 0.05 μg/ml of DX-8951a or SN-38 for 24 h. The cells were washed and Giemsa-stained with Diff-Quik (International Reagents Corp., Kobe) according to the manufacturer's instructions. Microscopic pictures were taken using a Microphot-FXA (Nikon, Tokyo).

Animals Male athymic nude mice (BALB/c-*nu/nu*) aged 6 weeks were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu). They were housed in an exclusive experimental room and given sterilized food and water *ad libitum*.

Evaluation of antitumor effects in solid tumor models *in vivo* SUIT-2 and SUIT-2/CPT-11 cells cultured *in vitro* were subcutaneously inoculated into nude mice, and passaged *in vivo* for 11 and 9 months, respectively, by further subcutaneous transplantation. To maintain and enhance resistance to CPT-11, the mice transplanted with SUIT-2/CPT-11 cells were intravenously administered 20 mg/kg of CPT-11 every 10 days for the first 2 months and 60 mg/kg CPT-11 weekly for the last 4 months. Tumor masses thus maintained *in vivo* were excised, cut into fragments, and transplanted into nude mice for antitumor tests (day 0). On day 14, when the mean tumor volume (calculated by multiplying the length and width of the tumor mass) reached 100 mm^3, the mice were divided into experimental groups. Groups of 6 mice were treated intravenously with DX-8951f at 3.125 to 18.75 mg/kg (as the anhydrous free base) or CPT-11 at 40 to 80 mg/kg (as the anhydrous free base) every 4 days for a total of four doses (q4d×4). Tumor volume and body weight of the mice were measured two or three times a week for 28 days and then the tumor masses were excised and weighed. The tumor growth inhibition rate (IR, %) was calculated from $IR=(1-TWt/TWc)\times 100$, where TWt indicates the mean tumor weight of a treated group and TWc represents that of the control group. When IR was 58% or over, the drug was considered effective.[32] The statistical significance of IR between control and other groups was obtained by Dunnett's test, and that between treated groups was determined by Tukey-Kramer's test. The rate of body weight loss (BWL, %) was obtained from $BWL=(1-BW_n/BW_0)\times 100$, where BW_n and BW_0 represent the mean body weights of mice on day n and day 0, respectively. The maximum BWL value was designated as BWLmax.

Evaluation of antitumor effects in a liver metastasis model *in vivo* SUIT-2 cells cultured *in vitro* were collected, washed and finally suspended in Hanks' balanced salt solution (HBSS, Gibco Lab., NY) at a concentration of 4×10^7 cells/ml. Nude mice were anesthetized with sodium pentobarbital for animal use (Abott Lab., North Chicago, IL) and intrasplenically inoculated with 50 μl of the tumor cell suspension (2×10^6 cells/mouse) as described by Fidler *et al.*,[26] since certain tumor cells, including SUIT-2 cells, have been found to express their liver metastatic capacity by intrasplenic injection, but not by intravenous injection.[6,24,26] DX-8951f at 2.5 to 10 mg/kg as the anhydrous free base or CPT-11 at 15 to 60 mg/kg as the anhydrous free base was administered intravenously 4 times at 5-day intervals (q5d×4) from the day after cell injection. Twenty-one days after cell injection, mice were killed and their abdominal cavities were examined for the presence of tumor nodules in the spleen and liver, and other signs. Tumor nodule formation in the liver was scored on a scale of 0 to 4, with score 0 defined as no visible nodules; score 1, a few pinpoint nodules localized at the perimeter of liver lobes; score 2, moderate numbers of nodules in liver lobes; score 3, numerous nodules in liver lobes or liver lobes partially invaded with tumor masses; score 4, all liver lobes fully invaded with tumor masses. The tumor nodule score and ascites volume were statistically analyzed by the use of Dunnett's test, and the incidence of tumor nodule formation in the liver and spleen and the occurrence of ascites, by Fisher's exact probability test.

Table I. *In vitro* Cytotoxicity of Camptothecin Derivatives on Human Pancreatic Tumor Cells, SUIT-2 and KP-1 and Their Sublines with Acquired CPT-11 Resistance

Exp.	Compound	IC_{50} values[a] SUIT-2 ng/ml	(Ratio)[c]	SUIT-2/CPT-11 ng/ml	(Ratio)[c]	Degree of resistance[b]	IC_{50} values[a] KP-1N ng/ml	(Ratio)[c]	KP-1N/CPT-11 ng/ml	(Ratio)[c]	Degree of resistance[b]
1	DX-8951[d]	0.12	(1)	0.35	(1)	2.9	0.10	(1)	0.69	(1)	6.9
	CPT-11	1100	(9167)	7000	(20000)	6.4	1600	(16000)	17000	(24638)	10.6
	SN-38	0.47	(4)	14	(40)	29.8	1.2	(12)	29	(42)	24.2
	SK&F104864	3.8	(32)	50	(143)	13.2	5.2	(52)	100	(145)	19.2
2	DX-8951[d]	0.070	(1)	nd		nd	0.12	(1)	nd		nd
	DX-8951[e]	0.079	(1)	nd		nd	0.12	(1)	nd		nd

a) IC_{50} values were determined by MTT assay, as described in "Materials and Methods." Dose levels of all compounds are expressed as the anhydrous free base.
b) IC_{50} for resistant cell line/IC_{50} for parental cell line.
c) IC_{50} of the compound/IC_{50} value of DX-8951 hydrochloride.
d) DX-8951 hydrochloride (DX-8951a) was used.
e) DX-8951 methanesulfonate (DX-8951f) was used.

RESULTS

***In vitro* cytotoxicity of camptothecin analogs against human pancreatic tumor cell lines** Table I shows the IC_{50} values of four camptothecin analogs against SUIT-2, KP-1N and their CPT-11-resistant variants, SUIT-2/CPT-11 and KP-1N/CPT-11. Among the four analogs, DX-8951 exhibited the strongest antiproliferative activity. In terms of the IC_{50} values of the analogs against SUIT-2 and KP-1N parental cell lines, DX-8951 was 9000 and 16000 times more potent than CPT-11, 4 and 12 times more potent than SN-38, and 32 and 52 times more potent than SK&F104864, respectively (Exp. 1). DX-8951a and DX-8951f were confirmed to show equal inhibitory effects against these parental cell lines (Exp. 2). SUIT-2/CPT-11 and KP-1N/CPT-11 cells showed about 6- and 11-fold greater resistance to CPT-11 than the respective parental cells. These resistant cells exhibited cross-resistance towards SN-38 and SK&F104864, with 30- and 24-fold greater resistance to SN-38, and 13- and 19-fold greater resistance to SK&F104864. Cross-resistance was also observed to DX-8951, but the resistance was low. The activity of DX-8951 against these resistant cells was about 25000, 40 and 150 times stronger than those of CPT-11, SN-38 and SK&F104864, respectively.

Inhibitory effects of DX-8951 and SN-38 on topoisomerase I obtained from SUIT-2 In order to elucidate the reason for stronger cytotoxic activity of DX-8951, inhibition of topoisomerase I activity in the crude extract from SUIT-2 was measured. For the inhibition test, the precise amount of nuclear cell extract of SUIT-2 cells required to convert closed-circular DNA (RFI) to relaxed-circular DNA (RFIr) was used (Fig. 2A). The drugs were titrated in the reaction mixture to a final concentration of 0.16 to 10 μg/ml. The IC_{50} values of DX-8951 and SN-38 for topoisomerase activity were 0.82 and 2.3 μg/ml, respectively (Fig. 2B), with DX-8951 therefore showing a 2.8 times stronger inhibitory effect than SN-38.

DNA fragmentation induced by DX-8951 and SN-38 in SUIT-2 cells The effect of DX-8951 and SN-38 on induction of DNA fragmentation in SUIT-2 cells was examined. As shown in Fig. 3, A and B, both drugs dose-dependently induced DNA fragmentation. In an extract from treated cells, about 60% of DNA was fragmented at the DX-8951 concentration of 20 ng/ml, which was about a 5 times lower concentration than that for SN-38. This fragmentation of DNA was induced in a time-dependent manner by both drugs (data not shown).

Apoptotic cell death of SUIT-2 cells following DX-8951 treatment The specific features of apoptosis, namely chromatin condensation, nuclear fragmentation and cytoplasmic vacuolation were apparent in SUIT-2 cells exposed to DX-8951 at the concentration of 0.05 μg/ml for 24 h (Fig. 4). In contrast, SN-38 at the same concentration had no effect on the cells (data not shown).

Antitumor effects of DX-8951 and CPT-11 against solid tumor models of SUIT-2 and SUIT-2/CPT-11 *In vivo* antitumor effects of DX-8951 were compared with those of CPT-11 using solid tumor models of SUIT-2 and its CPT-11-resistant variant, SUIT-2/CPT-11. The highest doses were set as the maximum tolerable dosages (MTD) based on preliminary results. The drug was considered effective when the IR value was 58% or over.[32]

As shown in Table II, DX-8951 suppressed the tumor growth of SUIT-2 with IR of 68% to 79% over a wide dose range (12.5 to 75 mg/kg total dose, i.e., 1/6 MTD to MTD). CPT-11 was similarly effective against SUIT-2 solid tumors at total doses of 320 and 240 mg/kg, i.e.,

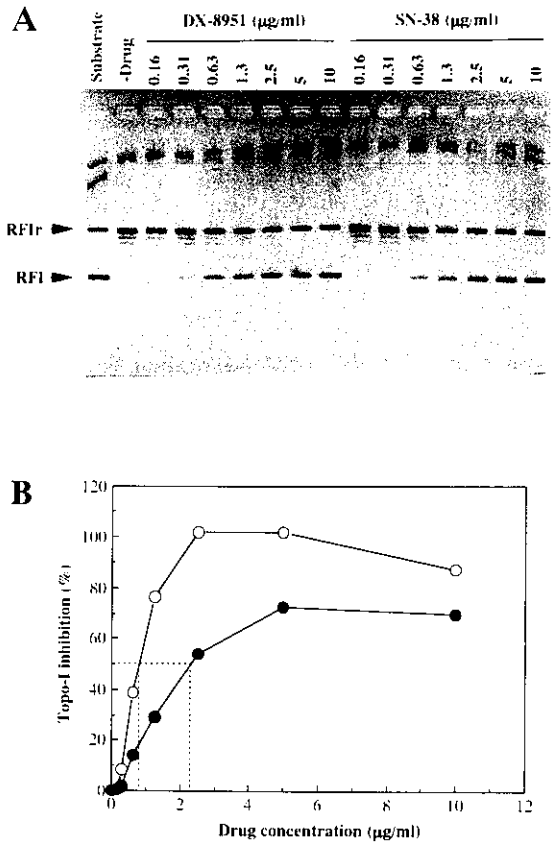

Fig. 2. Inhibition of catalytic activity of SUIT-2 topoisomerase I by DX-8951 and SN-38. Supercoiled pBR322 DNA was treated with nuclear extract from SUIT-2 in the presence of DX-8951a or SN-38. Agarose gel analysis was performed (A) and the bands at the supercoiled (RFI) position in the picture were densitometrically scanned and plotted (B). The 50%-inhibitory concentrations of DX-8951 and SN-38 were 0.82 and 2.3 μg/ml, respectively. ○ DX-8951a, ● SN-38. Dose levels of DX-8951a are expressed as the anhydrous free base.

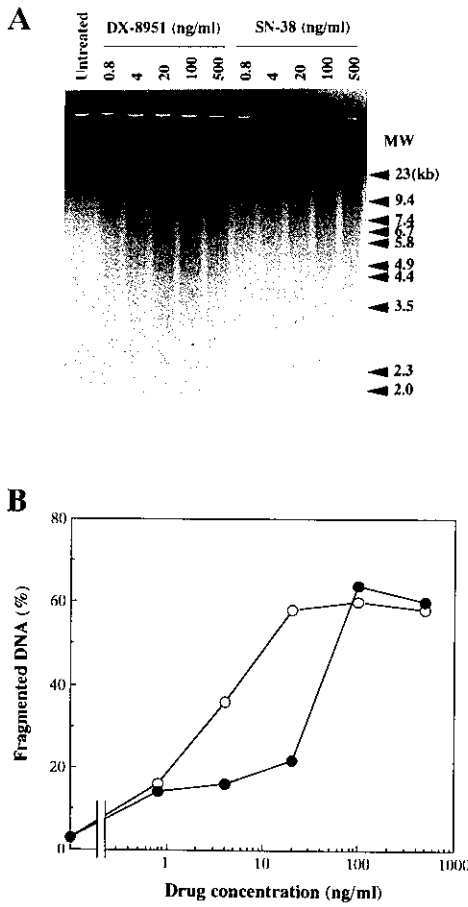

Fig. 3. DNA fragmentation in SUIT-2 cells induced by DX-8951 or SN-38. SUIT-2 cells were incubated in the presence of DX-8951a or SN-38 for 24 h. Cellular DNA was isolated and analyzed by agarose gel analysis (A). A mixture of lambda DNA digested with either *Eco*R I or *Hin*d III was used as a size marker. The regions of fragmented DNA in the photograph were densitometrically scanned and plotted on the graph (B). ○ DX-8951a, ● SN-38. Dose levels of DX-8591a are expressed as the anhydrous free base.

MTD and 2/3 MTD, though it was not effective at lower doses. No statistically significant difference was observed between DX-8951- and CPT-11-treated groups at the MTD level.

Against SUIT-2/CPT-11 solid tumors, DX-8951 exhibited an antitumor effect with an IR of 72% at the MTD, but CPT-11 failed to show any antitumor effect even at the MTD (Table III). A statistically significant difference ($P<0.05$) was found between the DX-8951- and CPT-11-treated groups at the MTD level.

Antitumor effects of DX-8951 and CPT-11 in a liver metastasis model of SUIT-2 Table IV shows the inhibitory effect of DX-8951f or CPT-11 on the growth of spleen and liver of nude mice following intrasplenic implantation of SUIT-2 cells. The drugs were intravenously injected four times at 5-day intervals from the day after implantation. In the control group, 4 of 8 mice died on days 18 to 21, and ascites and severe tumor growth in the liver and spleen were observed in all mice. DX-8951 at total doses of 10 to 40 mg/kg exhibited potent antitumor effects in a dose-dependent fashion. In particular, the growth of tumor cells in the liver and spleen and the degree of ascites were markedly inhibited in mice treated with the highest dose ($P<0.05$–0.001 vs. control), with no visible tumor nodules or ascites observed in 3 of 7 mice ($P<0.10$ vs. control). CPT-11 at total doses of 60 to 240 mg/kg dose-dependently in-

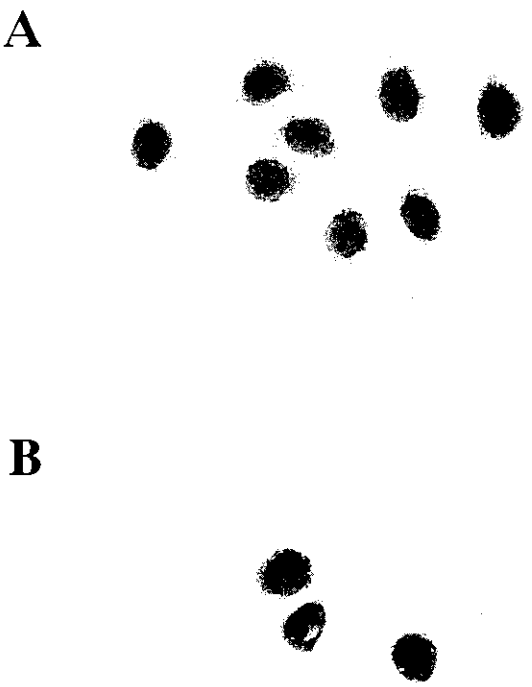

Fig. 4. Morphological changes in SUIT-2 cells cultured *in vitro*. Untreated SUIT-2 cells (A) and SUIT-2 cells cultured in medium containing 0.05 μg/ml of DX-8951a for 24 h (B) ($\times$400). The dose level of DX-8591a is expressed as the anhydrous free base.

hibited tumor growth and ascites accumulation, with the effects being closely similar to or somewhat inferior to those of DX-8951. No statistically significant difference was observed between the effects of DX-8951 and those of CPT-11 at the highest dose level. No significant decrease in body weight was observed in either DX-8951- or CPT-11-treated mice.

DISCUSSION

In this study, SN-38, an active metabolite of CPT-11, exhibited potent antitumor activity against human pancreatic cancer cell lines, SUIT-2 and KP-1N (Table I). CPT-11 was confirmed to show antitumor effects against SUIT-2 in both a solid tumor model (Table II) and, as we previously reported, in a liver metastasis model[6] (Table IV). However, the *in vitro* activity of SN-38 was markedly reduced against two variants which had acquired CPT-11 resistance (Table I), while CPT-11 failed to inhibit the growth of SUIT-2/CPT-11 *in vivo* even at the MTD (Table III). In contrast, DX-8951 showed stronger cytotoxic activity than SN-38 *in vitro* against all human pancreatic tumor cell lines used (Table I). The cells with acquired CPT-11 resistance also showed marked cross resistance to SK&F104864, but less resistance to DX-8951, with the resistance rates to DX-8951 being the lowest among current camptothecin derivatives (Table I). Of note was the observation that DX-8951 was as active against SUIT-2/CPT-11 xenografts as it was against the parental tumors (Table III).

Table II. Antitumor Effects of DX-8951 and CPT-11 against Human Pancreas Cancer SUIT-2 Subcutaneously Xenografted in Nude Mice

Compound	Total dose[a] mg/kg	Tumor weight			BWLmax[d] %	N[e]
		Mean±SE, g	IR, %[b]	Sig.[c]		
Control	—	0.738±0.368	0		<0	0/6
DX-8951[f]	75 (18.75×4)	0.154±0.027	79	*, #	<0	0/6
	50 (12.5×4)	0.236±0.100	68		<0	0/6
	25 (6.25×4)	0.164±0.053	78	#	<0	0/6
	12.5 (3.125×4)	0.235±0.057	68		<0	0/6
CPT-11	320 (80×4)	0.175±0.039	76	#	<0	0/6
	240 (60×4)	0.284±0.097	62		<0	0/6
	160 (40×4)	0.460±0.102	38		<0	0/6
	80 (20×4)	0.744±0.261	−1		<0	0/6

a) Compounds were administered 4 times at 4-day intervals. Dose levels are expressed as the anhydrous free base.
b) (1−mean tumor weight of treated group/that of control group)×100.
c) $P<0.05$ level of significance vs. control by Dunnett's test (*) or vs. CPT-11 80 mg/kg by Tukey-Kramer's test (#).
d) Maximum value for rate of body weight loss. <0: no body weight loss was observed.
e) Number of mice that died of toxicity/number of mice used.
f) DX-8951 methanesulfonate (DX-8951f) was used.

Table III. Antitumor Effect of DX-8951 and CPT-11 against Human Pancreas Cancer SUIT-2/CPT-11 Subcutaneously Xenografted in Nude Mice

Compound	Total dose[a] mg/kg	Tumor weight			BWLmax[d] % [Day]	N[e]
		Mean±SE, g	IR, %[b]	Sig.[c]		
Control	—	0.892±0.295	0		<0	0/6
DX-8951f[f]	75 (18.75×4)	0.250±0.061	72	*, ##	14.6 [31]	0/6
	50 (12.5×4)	0.595±0.130	33		6.1 [21]	0/6
	25 (6.25×4)	0.776±0.157	13		8.7 [15]	0/6
	12.5 (3.125×4)	0.712±0.065	20		3.2 [15]	0/6
CPT-11	320 (80×4)	1.088±0.259	−22		1.0 [15]	0/6
	240 (60×4)	0.930±0.205	−4		5.7 [15]	0/6
	160 (40×4)	1.074±0.142	−20		3.0 [15]	0/6

a) Compounds were administered 4 times at 4-day intervals. Dose levels are expressed as the anhydrous free base.
b) (1−mean tumor weight of treated group/that of control group)×100.
c) $P<0.05$ level of significance vs. control by Dunnett's test (*) or vs. CPT-11 320 mg/kg and 160 mg/kg by Tukey-Kramer's test (##).
d) Maximum value for rate of body weight loss, numbers in parentheses denoting the day of BWLmax. <0: no body weight loss was observed.
e) Number of mice that died of toxicity/number of mice used.
f) DX-8951 methanesulfonate (DX-8951f) was used.

Table IV. Antitumor Effects of DX-8951 and CPT-11 in a Liver Metastasis Model of SUIT-2[a]

Compound	Total dose[b] mg/kg	Liver tumor							Spleen tumor		Ascites				Tumor-free mice[e]	
		Tumor growth score[c]			Incidence[d]		Liver weight (mg)		Incidence[d]		Volume (ml)			Incidence[d]		
		Mean±SD	IR%[f]	Sig.[g]		Sig.[h]	Mean±SD	Sig.[g]		Sig.[h]	Mean±SD	IR%[f]	Sig.[g]		Sig.[h]	
Control	—	3.3±1.0	0		8/8		2.253±0.815		8/8		3.1±0.8	0		8/8	0/8	
DX-8951[i]	40 (10×4)	0.7±0.9	78	***, #	3/7	$$, &&, £	1.594±0.154	*	2/7	$$$, &&	0.0±0.0	100	***	0/7 $$$$	3/7	$
DX-8951	20 (5×4)	1.0±0.6	69	***	5/6		1.594±0.163	*	2/6	$$, &&	0.0±0.0	100	***	0/6 $$$$	1/6	
DX-8951	10 (2.5×4)	2.3±1.1	28		6/6		1.780±0.197		3/6	&	0.1±0.1	97	***	2/6 $$	0/6	
CPT-11	240 (60×4)	0.7±0.5	79	***, #	4/6		1.662±0.116		3/6	&	0.0±0.0	100	***	0/6 $$$$	1/6	
CPT-11	120 (30×4)	1.4±0.5	57	**	5/5		1.592±0.131	*	3/5		0.4±0.5	88	***	2/5 $$	0/5	
CPT-11	60 (15×4)	2.3±0.5	28		6/6		1.727±0.260		6/6		0.6±1.0	81	***	2/6 $$	0/6	

a) Male nude mice were injected with 2×10^6 cells of SUIT-2 tumor cells into their spleen on day 0, and intravenously administered with each compound on days 1, 6, 11 and 16. Four mice in the control group died on days 18 to 21. Others were killed on day 21. The abdominal cavity of all mice was inspected.
b) Dose levels are expressed as the anhydrous free base.
c) Tumor nodule formation in liver was scored on a scale of 0 to 4 (see "Materials and Methods" for details).
d) No. of mice with visible tumor colonies in the organs or with ascites/No. of mice used.
e) No. of mice without visible tumor colonies or ascites/No. of mice used.
f) Inhibition rate (IR%)=(1−mean value in treated group/that in control group)×100.
g) *, **, ***: $P<0.05$, 0.01 and 0.001 levels of significance vs. control determined by Dunnett's test. #: $P<0.05$ level of significance vs. DX-8951 10 mg/kg and CPT-11 60 mg/kg by Tukey-Kramer's test.
h) $, $$, $$$ and $$$$: $P<0.10$, 0.05, 0.01 and 0.001 levels of significance vs. control; & and &&: $P<0.10$ and 0.05 levels of significance vs. CPT-11 60 mg/kg; £: $P<0.10$ and 0.05 levels of significance vs. CPT-11 120 mg/kg and DX-8951 10 mg/kg, respectively, determined by Fisher's exact probability test.
i) DX-8951 methanesulfonate (DX-8951f) was used.

Several kinds of resistance mechanisms in CPT-11-resistant tumor cells have been reported,[25,33–38] including a decrease in the cellular level and/or in the activity of topoisomerase I,[33–35] alteration in the topoisomerase I gene,[36] a low population of S-phase cells,[34] a decrease in intracellular uptake of CPT-11[37] and a decrease in the activity of intracellular carboxylesterase, which converts CPT-11 to SN-38.[34,38] In the cases of SUIT-2/CPT-11 and KP-1N/CPT-11, we previously reported that reduced levels of topoisomerase I mRNA and protein may contribute to the resistance.[25] Considering the grade of resistance of our cells, it is unlikely that they have a mutation of their topoisomerase I gene. A one-third decrease in the uptake of CPT-11 was found in KP-1N/CPT-11 as compared with its parental cell line, while no difference in the uptake of CPT-11 was detected between SUIT-2 and SUIT-2/CPT-11.[25] We did not examine the uptake of SN-38, and this factor may also be involved in the resistance mechanisms of our cells on the basis of their higher resistance to SN-38 than to CPT-11 (Table

I). The reason why DX-8951 shows sufficient antitumor activity against resistant cells is not presently clear, but it is possible that its uptake is not influenced even in resistant cells. This idea seems to be supported by the previous findings that DX-8951 overcame P-glycoprotein-mediated multidrug resistance[20] and that this effect might be attributed to good membrane permeability.[39] Further detailed investigation on this matter is required.

Camptothecins have been shown to inhibit topoisomerase I by stabilizing the enzyme-DNA cleavable complex[30, 40] and thereby to induce lethal impairment of cells through the strong inhibition of DNA synthesis. Although the mechanisms of their cytotoxicity have not been precisely determined, it was recently reported that CPT-11 and SN-38, like other anticancer agents, finally induce apoptotic cell death.[41] As mentioned in "Results," DNA fragmentation, a process believed to be one outcome of cell apoptosis, was dose-dependently induced by DX-8951 in SUIT-2 cells (Fig. 3), and its effect was more potent than that of SN-38. Fragmentation of nuclei, a specific feature of apoptosis, was observed in SUIT-2 cells treated with DX-8951 (Fig. 4). These results indicate that DX-8951 has the potential to induce apoptosis, and this seems to be the major pathway by which the drug kills cells following topoisomerase I inhibition.

Comparative study of molecular and cellular pharmacology by Tanizawa *et al.* revealed that SN-38 is the most potent compound among the various camptothecin derivatives, including those previously evaluated and currently under evaluation in clinical trials.[40] They demonstrated that SN-38 is superior to camptothecin, SK&F104864, 9-aminocamptothecin, 10-hydroxycamptothecin and 10,11-methylenedioxycamptothecin in the formation and stabilization of cleavable complexes, inhibition of topoisomerase I, DNA-damaging effect and resulting cytotoxic activity.[40] Since the results presented here clearly demonstrate the superiority of DX-8951 to SN-38 in terms of topoisomerase I inhibition (Fig. 2), apoptosis induction (Figs. 3 and 4) and cytotoxicity (Table I), we conclude that DX-8951 is the most potent camptothecin derivative currently available.

Clinical evaluation of CPT-11 has revealed that it has promising activity against lymphoma,[7] small-cell lung cancer,[8] non-small-cell lung cancer,[9] colorectal cancer,[10] gastric cancer,[11] breast cancer, ovarian cancer, and cervical cancer.[12] CPT-11 has been reported to show moderate antitumor activity against pancreas cancer,[21, 22] but this effect was concluded to be insufficient. On the basis that it has the strongest activities of topoisomerase I inhibition, apoptosis induction and growth inhibition *in vitro* and *in vivo* among currently known camptothecin derivatives, DX-8951 seems to have potential as a therapeutic agent for the treatment of various human cancers including pancreatic cancer.

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