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A New Water-soluble Camptothecin Derivative, DX-8951f, Exhibits Potent Antitumor Activity against Human Tumors *in vitro* and *in vivo*

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CPT-11, a semisynthetic derivative of camptothecin, exhibited strong antitumor activity against lymphoma, lung cancer, colorectal cancer, gastric cancer, ovarian cancer, and cervical cancer. CPT-11 is a pro-drug that is converted to an active metabolite, SN-38, *in vivo* by enzymes such as carboxylesterase. We synthesized a water-soluble and non-pro-drug analog of camptothecin, DX-8951f. It showed both high *in vitro* potency against a series of 32 malignant cell lines and significant topoisomerase I inhibition. The anti-proliferative activity of DX-8951f, as indicated by the mean GI_{50} value, was about 6 and 28 times greater than that of SN-38 or SK&F 10486-A (Topotecan), respectively. These three derivatives of camptothecin showed similar patterns of differential response among 32 cell lines, that is, their spectra of *in vitro* cytotoxicity were almost the same. The antitumor activity of three doses of DX-8951f administered i.v. at 4-day intervals against human gastric adenocarcinoma SC-6 xenografts was greater than that of CPT-11 or SK&F 10486-A. Moreover, it overcame P-glycoprotein-mediated multi-drug resistance. These data suggest that DX-8951f has a high antitumor activity and is a potential therapeutic agent.

Key words: Camptothecin — Topoisomerase I — P-Glycoprotein — CPT-11 — Topotecan

Camptothecin (CPT) was isolated from the Chinese tree, *Camptotheca acuminata* and its structure was identified by Wall *et al.*[1] It inhibits type I DNA topoisomerase (topoisomerase I)[2–4] and has strong antitumor activity against several experimental tumors,[5] but in clinical trials in the early 1970s, failed to show meaningful activity and proved to cause severe and unpredictable cystitis.[6, 7]

The outcome of a great amount of effort to find new derivatives of CPT with higher antitumor activity and less toxicity has been the production of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin hydrochloride trihydrate (CPT-11), a semisynthetic analogue of CPT which has significant antitumor activity against various experimental tumor models in mice.[8] Clinical evaluation of CPT-11 has revealed that it is active against lymphoma,[9] small-cell lung cancer,[10] non-small-cell lung cancer,[11] colorectal cancer,[12] gastric cancer,[13] ovarian cancer, and cervical cancer.[14] CPT-11 is a pro-drug that is converted *in vivo* to 7-ethyl-10-hydroxycamptothecin (SN-38), which has an antitumor activity at least 100 times that of CPT-11 *in vitro*[15, 16] and is responsible for much of the efficacy and toxicity of CPT-11. CPT-11 has exhibited high antitumor activity in clinical studies, but side effects such as leukopenia and diarrhea have been observed with a large interpatient variation.[17] The polymorphism of enzymes such as carboxylesterase, which has been demonstrated to convert CPT-11 to SN-38 in rat serum,[18] may cause the interpatient variation.

We intended to synthesize water-soluble CPT derivatives retaining potent topoisomerase I-inhibitory activity and broad-spectrum antitumor activity, and having little or no interpatient variation in efficacy or toxicity. We have already reported that hexacyclic derivatives of CPT showed superior topoisomerase I inhibitory activity.[19] Also, we have synthesized about 150 hexacyclic CPT analogues and selected a new non-pro-drug compound of camptothecin, DX-8951f, as a candidate for further development (Fig. 1).

In this study, we examined the antitumor activities of DX-8951f *in vitro* and *in vivo*, and its inhibitory activity against topoisomerase I.

MATERIALS AND METHODS

Cell lines *In vitro*: Human cell lines (Table I) were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah) and 2 m*M* L-glutamine (Sanko Junyaku Co., Ltd., Tokyo). A mouse cell line, P388, was maintained in the former medium supplemented with 20 μM 2-mercaptoethanol (Nacalai Tesque, Inc., Kyoto). Cell cultures were passaged once or twice weekly.

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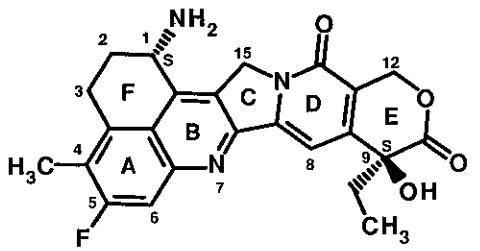

Fig. 1. Molecular structure of DX-8951f. The absolute configuration at position 1 of DX-8951f is *S*.

In vivo: Poorly differentiated gastric adenocarcinoma SC-6 was supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo). It was maintained by sequential s.c. transplantations into nude mice.

Animals Male athymic nude mice (BALB/c-nu/nu), aged 5 or 6 weeks, were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu). They were housed in an exclusive experimental room and were given sterilized food and water *ad libitum*.

Drugs SK&F 104864-A (SK&F) was synthesized as described previously.[20] Camptothecin (CPT), CPT-11 and SN-38 were provided by Yakult Honsha Co., Ltd. (Tokyo). DX-8951f, (1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1*H*,12*H*-benzo[*de*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione methanesulfonate was totally synthesized in our laboratory.[21] For *in vitro* usage, SK&F, CPT, SN-38 and DX-8951f were each dissolved in dimethyl sulfoxide (DMSO) and diluted with the medium described above, and for the *in vivo* experiment, SK&F, CPT-11 and DX-8951f were each dissolved in distilled water. Dose levels of SK&F, CPT, CPT-11, SN-38 and DX-8951f were expressed in terms of the free bases. Vincristine (VCR) was purchased from Shionogi Co., Ltd. (Osaka). Cisplatin (CDDP) was purchased from Nippon Kayaku Co., Ltd. (Tokyo). Verapamil was purchased from Eisai Co., Ltd. (Tokyo).

***In vitro* cytotoxicity** Growth inhibition experiments were carried out in 96-well flat-bottomed microplates (Falcon, Oxnard, California), and the amount of viable cells at the end of the incubation was determined by MTT assay, essentially as described by Mosmann.[22] Thus, 500–20,000 cells/well in 150 μl of medium were plated and grown for 24 h (P388, CCRF-CEM and K562 cells for 4 h), the drug (in 50 μl medium/well), or the medium alone as a control, was added, and the cells were cultured for an additional 3 days. After addition of MTT (20 μl/well, 5 mg/ml in phosphate-buffered saline), the plates were incubated for 4 h and centrifuged at 800*g* for 5 min, then the medium was removed and the blue dye formed was dissolved in 150 μl of DMSO. The absorbance was measured at 540 nm using a Microplate Reader model 3550 (Bio-rad, Richmond, California). Each data point on the growth curves represents the average of results from four wells. The cell population density at time 0 (the time at which drugs were added) was also measured. Growth inhibition of 50% (GI_{50}) was calculated from $100\times[(T-T_0)/(C-T_0)]=50$, which is the drug concentration causing a 50% reduction in optical density in control cells during the drug incubation (C: control optical density, T: test optical density, T_0: optical density at time zero). Differential growth inhibition was designated by Δ (Fig. 2), which was calculated from $\log(1/GI_{50})-\text{mean}[\log(1/GI_{50})]$ as described previously.[23]

***In vivo* antitumor activity** Tumor masses maintained in nude mice were excised, cut into fragments (ca. 3-mm

Table I. Cell Lines Used in *in vitro* Experiments

Breast cancer (human)		
DU-4475[a]	MCF-7[a]	MDA-MB-157[a]
MDA-MB-231[a]	MDA-MB-361[a]	MDA-MB-415[a]
MDA-MB-435S[a]	MDA-MB-468[a]	SK-BR-3[a]
Colon cancer (human)		
HCT116[a]	SK-CO-1[a]	SW480[a]
SW620[a]	T84[a]	WiDr[a]
Stomach cancer (human)		
AGS[a]	HS746T[a]	MKN28[b]
NUGC-3[c]		
Non-small cell lung cancer (human)		
A549[d]	PC-10[e]	PC-12[d]
PC-13[e]	PC-14[e]	PC-6[e]
PC-7[e]	PC-9[e]	
Small cell lung cancer (human)		
QG90[d]		
Ovarian cancer (human)		
HOC-21[f]		
Leukemia (human)		
CCRF-CEM[a]	K562[g]	
Leukemia (mouse)		
P388[h]		
MDR overexpressing cell (human)		
PC-6/VCR (VCR-selected MDR variant of PC-6)[i]		

a) Obtained from the American Type Culture Collection, Rockville, MD.
b) Supplied by Dr. A. Hoshi, National Cancer Center, Tokyo.
c) Supplied by Japanese Cancer Research Resources Bank, Tokyo.
d) Supplied by Toray Co., Ltd., Tokyo.
e) Obtained from Immuno-Biological Laboratories, Gunma.
f) Supplied by Dr. S. Sekiya, Chiba University Medical School, Chiba.
g) Supplied by Dr. J. Yata, Tokyo Medical and Dental University, Tokyo.
h) Supplied by Dr. T. Tashiro, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo.
i) Established in our laboratory.

cubes), and transplanted s.c. into nude mice. When the estimated tumor volume (TV) in the mice had grown to between 100 and 300 mm^3, the animals were divided into experimental groups of 6/cage and were treated i.v. with a test drug every 4 days for a total of three doses. The TV was calculated from $TV=L\times W^2/2$ where L and W represent the length and the width of the tumor mass, respectively. After the first administration on day 0, the TV and body weight of the mice were measured two or three times a week for 26 days. Then the tumor masses were excised and weighed. The rate of inhibition of tumor growth on the basis of TV (IR) was calculated from $IR=(1-RV_t/RV_c)\times 100$, where RV_t represents the mean ratio of TV on day n to that on day 0 of a treated group and RV_c indicates that of the control group. The largest value for IR was designated as IR_{max}, which indicates the greatest effect of each drug. The tumor growth inhibition rate on the basis of tumor weight (IR_{tw}) was calculated from $IR_{tw}=(1-TW_t/TW_c)\times 100$, where TW_t indicates the mean tumor weight of a treated group and TW_c represents that of the control group. IR_{tw} was statistically analyzed using the Williams-Wilcoxon test (a nonparametric test). The rate of body weight reduction (ΔBW) was calculated from $\Delta BW=(1-BW_n/BW_0)\times 100$, where BW_n and BW_0 represent the mean body weights of mice on day n and day 0, respectively. The maximum value of ΔBW was designated as ΔBW_{max}.

Topoisomerase I assay Topoisomerase I was prepared from P388 cells by the methods of Ishii *et al.*[24] with minor modifications as described elsewhere in detail.[25] Assay of topoisomerase I activity and calculation of the concentration of each test compound required for 50% inhibition of topoisomerase I activity (IC_{50}) were performed as previously described.[25]

RESULTS

***In vitro* potency against a series of 32 malignant cell lines** The cytotoxicity of DX-8951f, SN-38, SK&F and CPT against 31 human cancer cell lines, including breast cancer, gastro-intestinal cancer, lung cancer, ovarian cancer and leukemia, and against mouse leukemia P388 was assessed. DX-8951f showed strong antiproliferating activity, and the mean GI_{50} values against breast cancers, colon cancers, stomach cancers, and lung cancers were 2.02 ng/ml, 2.92 ng/ml, 1.53 ng/ml, and 0.877 ng/ml, respectively (Table II). Comparing the mean GI_{50} values of the compounds, DX-8951f was about 6 times as potent as SN-38, and about 28 times as potent as SK&F. DX-8951f also showed superior cytotoxicity against human ovarian cancer, leukemia, and mouse leukemia.

To confirm that the spectrum of anticellular activity of DX-8951f was similar to that of SN-38, we also evaluated the patterns of cytotoxicity of the drugs in terms of the value of Δ that was calculated from $\log(1/GI_{50})-\text{mean}[\log(1/GI_{50})]$ (Fig. 2). The three camptothecin derivatives had similar patterns.

Table II. *In vitro* Cytotoxicity against a Series of 32 Cell Lines

Cell line	GI_{50} (ng/ml)			
	DX-8951f	SN-38	SK&F	CPT
Breast				
DU4475	0.808	1.55	13.8	4.41
MCF-7	0.322	0.64	3.01	1.98
MDA-MB-157	0.163	1.11	2.79	1.31
MDA-MB-231	8.74	35.3	211	45.6
MDA-MB-361	2.52	7.56	263	18.1
MDA-MB-415	3.64	4.32	12.4	14.5
MDA-MB-435S	1.16	5.33	24.0	10.8
MDA-MB-468	0.328	1.36	7.73	5.45
SK-BR-3	0.518	1.94	39.5	10.3
Colon				
HCT116	0.347	1.29	9.39	3.86
SK-CO-1	0.125	0.592	2.22	0.651
SW480	3.00	13.8	65.5	30.7
SW620	0.375	1.78	5.86	3.41
T84	12.1	66.4	435	86.1
WiDr	1.55	6.82	37.3	6.06
Stomach				
AGS	0.335	1.45	6.69	2.84
HS746T	4.47	10.2	57.6	26
MKN28	1.07	14.7	52.6	20.6
NUGC-3	0.240	0.841	5.39	2.66
Lung				
A549	0.914	14.9	45.5	5.69
PC-10	0.778	14.0	50.8	7.89
PC-12	1.15	12.5	40.8	8.34
PC-13	0.368	1.76	8.47	4.04
PC-14	1.82	13.7	110	29.8
PC-6	0.269	0.828	3.19	1.53
PC-7	1.43	2.98	28.2	16.5
PC-9	0.324	2.43	11.1	4.22
QG90	0.844	3.23	19.6	11.9
Others				
CCRF-CEM	0.382	0.901	4.08	3.02
K562	10.4	108	191	54.3
P388	0.728	2.91	12.7	5.73
HOC-21	5.80	32.8	124	41.2
Mean				
Breast	2.02	6.57	64.1	12.5
Colon	2.92	15.1	92.5	21.8
Stomach	1.53	6.80	30.6	13.0
Lung	0.877	7.37	35.3	9.99
Others	4.33	36.2	82.9	26.1
All	2.09	12.1	59.5	15.3

GI_{50} was determined by MTT assay, as described in "Materials and Methods."

Topoisomerase I-inhibitory activity In comparison with CPT and the other CPT analogues, SN-38 and SK&F,

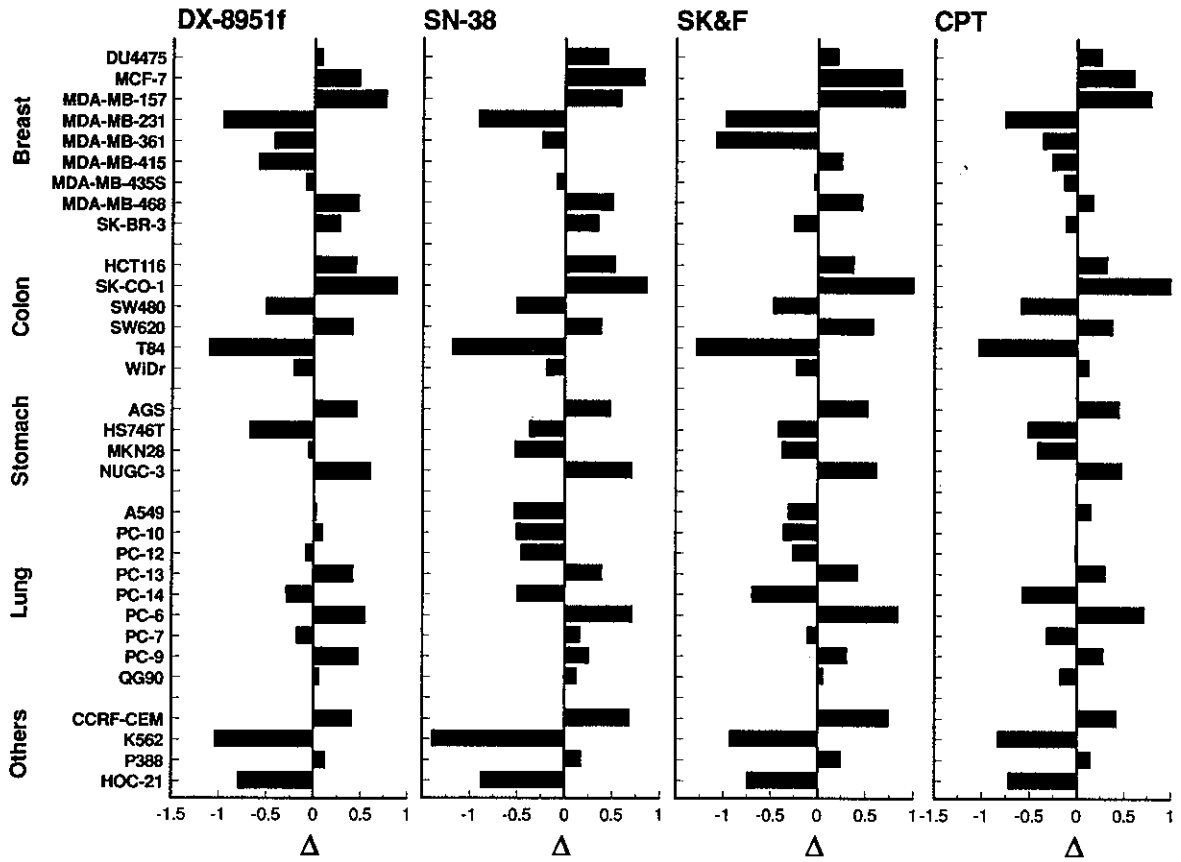

Fig. 2. Pattern of activity against a panel of 32 malignant cell lines. GI_{50} was determined by MTT assay, as described in "Materials and Methods." Δ was calculated from $\log(1/GI_{50}) - \text{mean}[\log(1/GI_{50})]$. Δ indicates the relative sensitivity of each cell line among the 32 cell lines. A cell line having a larger Δ value is more sensitive to the agent, and Δ 0 is the average sensitivity.

DX-8951f was the most potent topoisomerase I inhibitor. DX-8951f was about 3 times as potent as SN-38, and about 10 times as potent as SK&F (Table III).

DX-8951f can overcome P-glycoprotein-mediated resistance To investigate the effect of P-glycoprotein (Pgp) overexpression on the cytotoxicity of DX-8951f, we assessed the cytotoxicity of DX-8951f against a human non-small cell lung cancer cell line, PC-6, and its variant, PC-6/VCR, which was isolated from PC-6 by stepwise selection with increasing VCR concentrations. Overexpression of Pgp was detected by Western blotting analysis with anti-Pgp monoclonal antibody C219 (data not shown). Two independent experiments were done. PC-6/VCR was 380 to 530 times as resistant to VCR, and 3 times as resistant to SK&F as PC-6 (Table IV). On the other hand, Pgp overexpression had little or no effect on the cytotoxicity of SN-38, DX-8951f and CDDP. We also investigated the effect of verapamil on Pgp-overexpressing cells. Verapamil (5 μg/ml) was used with each agent. It greatly affected the cytotoxicity of VCR against PC-6/VCR cells, and enhanced the cytotoxicity of SK&F by about twice. However, verapamil did not affect the cytotoxicity of DX-8951f. DX-8951f had the same degree of activity as the non-MDR agent CDDP against PC-6/VCR.

Table III. Inhibitory Effects on Topoisomerase I Activity

Agent	IC_{50} (μg/ml)
DX-8951f	0.975
SN-38	2.71
SK&F	9.52
CPT	23.5

Topoisomerase I was prepared from P388 cells.

Antitumor activity of DX-8951f against human tumor xenograft As Table V shows, DX-8951f suppressed the growth of human gastric adenocarcinoma SC-6 xeno-

Table IV. Cytotoxicity against Pgp-overexpressing Cells

Agent	Exp. 1			Exp. 2				
	VR (−)[a] (GI_{50} ng/ml)		Degree of resistance[c]	VR (−)[a] (GI_{50} ng/ml)		Degree of resistance[c]	VR (+)[b] (GI_{50} ng/ml)	VR (+)/ VR (−)[d]
	PC-6	PC-6/VCR		PC-6	PC-6/VCR		PC-6/VCR	
DX-8951f	0.0896	0.0690	0.8	0.179	0.182	1.0	0.156	0.86
SN-38	0.655	0.751	1.1	0.723	1.23	1.7	0.800	0.65
SK&F	1.73	4.36	2.5	2.20	6.37	2.9	2.40	0.38
VCR	0.284	107	380	0.215	114	530	0.225	0.002
CDDP	138	69.0	0.5	113	93.9	0.83	86.5	0.92

GI_{50} was determined by MTT assay, as described in "Materials and Methods."
a) Cells were exposed to each agent in the absence of verapamil.
b) Cells were exposed to each agent in the presence of 5 μg/ml verapamil.
c) (GI_{50} for PC-6/VCR)/(GI_{50} for PC-6) in the absence of verapamil.
d) (GI_{50} for PC-6/VCR in the presence of verapamil)/(GI_{50} for PC-6/VCR in the absence of verapamil).

Table V. Antitumor Activity against Human Tumor Xenograft, Gastric Adenocarcinoma SC-6

Agent	Total (mg/kg)	IR_{max}[a] (%)	IR_{tw}[b] (%)	ΔBW_{max}[c] (%)	No. of toxic deaths
DX-8951f	3.325	54 (12)	43	3.8 (6)	0/6
	6.25	72 (14)	58*	2.6 (6)	0/6
	12.5	82 (17)	73**	5.9 (6)	0/6
	25	95 (17)	90**	7.0 (6)	0/6
	50	96 (17)	92**	12.5 (12)	0/6
CPT-11	173	82 (17)	66**	2.5 (6)	0/6
	260	89 (17)	75**	4.8 (6)	1/6
SK&F	26.6	69 (14)	49*	10.4 (9)	1/6
	40	92 (14)	81**	22.3 (8)	3/6

a) Maximal tumor growth-inhibition rate calculated from the estimated tumor volume; numbers in parentheses indicate the number of days after initial drug administration on which IR_{max} was reached.
b) Tumor growth-inhibition rate calculated from the tumor weight: * $P<0.01$, ** $P<0.001$ as compared with untreated controls.
c) Maximal rate of body weight reduction, the numbers in parentheses denoting the day of ΔBW_{max}.

grafted into nude mice. When mice were treated i.v. with DX-8951f three times at 4-day intervals, significant inhibition of tumor growth was observed over a wide dose range (3.325 mg/kg to 50 mg/kg of total dose) without toxic death. Its antitumor activity was dependent on the total dose, and at the highest dose of 50 mg/kg, the tumor growth inhibition rate was 92%, while the inhibition rate of CPT-11 at around the maximum tolerated dose (MTD), a total dose of 260 mg/kg, was 75%. The tumor growth inhibition rate of SK&F was only 49% at a total dose of 26.6 mg/kg, which was thought to be close to the MTD.

DISCUSSION

In an investigation of water-soluble and non-pro-drug CPT derivatives with strong antitumor activity, we have succeeded in obtaining DX-8951f, a hexacyclic compound having an amino group and a fluorine atom at positions 1 and 5, respectively. It is more potent than SN-38 or SK&F in terms of cytotoxicity. The superior efficacy of DX-8951f has led to its becoming a candidate for further development.

To determine the anticancer spectrum of DX-8951f, its anticellular activity against a series of 32 malignant cell lines was tested *in vitro*. The *in vitro* activities of DX-8951f were stronger than those of the CPT derivative SN-38, the active metabolite of CPT-11, or of SK&F, and showed the smallest GI_{50} values against all of the 32 cell lines used. This series of 32 malignant cell lines was used to test various compounds, and similar patterns of sensitivity were seen for compounds that had similar chemical structures or mechanisms of action to those found in the National Cancer Institute's drug screening program[23] (data are not shown). The pattern of activity of DX-8951f was similar to that of SN-38, and DX-

8951f had strong topoisomerase I-inhibitory activity. These data suggest that DX-8951f may have the same mechanism of action as CPT-11.

It was reported that SK&F is subject to Pgp-mediated resistance.[26, 27] In our case, although the degree of resistance was low, SK&F was considered to be affected by Pgp, but SN-38 and DX-8951f were not. Overexpression of Pgp has been commonly observed in colorectal carcinoma.[28] In clinical trials CPT-11 was active against colorectal cancer[12] but SK&F was not.[29, 30] Though there might be a considerable difference beween CPT-11 and SK&F, we consider that overexpression of Pgp is an important factor influencing the response in patients with colorectal cancer. DX-8951f was obviously less affected by Pgp compared to SK&F, and equally or less affected compared to SN-38. Therefore, DX-8951f may exhibit activity against Pgp-overexpressing tumors.

Three i.v. doses of DX-8951f administered at 4-day intervals showed strong antitumor activity against human gastric adenocarcinoma SC-6 xenografts transplanted into nude mice. Although both DX-8951f and CPT-11 were significantly effective against SC-6, the effective total dose range of DX-8951f was much wider than that of CPT-11. Moreover, the tumor growth inhibition ratio of DX-8951f was greater than those of CPT-11 and SK&F. The maximum body weight reduction and number of toxic deaths indicate that the toxicity of DX-8951f may be weaker than that of CPT-11 or SK&F at effective dose levels.

In conclusion, DX-8951f exhibits potent antitumor activity both *in vitro* and *in vivo*. Furthermore, it may overcome Pgp-mediated multi-drug resistance, and is expected to reduce the interpatient variations in side effects and efficacy since it is not a pro-drug. DX-8951f is therefore considered to be a promising candidate drug for the treatment of various cancers.

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