DE-310, a novel macromolecular carrier system for the camptothecin analog DX-8951f: Potent antitumor activities in various murine tumor models

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DE-310 is a novel macromolecular conjugate composed of DX-8951f, a camptothecin analog, and a carboxymethyldextran polyalcohol carrier, which are covalently linked via a peptidyl spacer. In a murine Meth A (fibrosarcoma) solid tumor model, once daily×5 treatments (qd×5) with DX-8951f at the maximum tolerated dose (MTD) were required to shrink the tumor, and DX-8951f (qd×5) at 1/4 MTD was required to inhibit tumor growth. A single treatment (qd×1) with DE-310 at the MTD or 1/4 MTD shrank the tumor, with no body weight loss occurring at 1/4 MTD. Even at 1/16 MTD, DE-310 inhibited tumor growth. In a long-term assay, Meth A solid tumors disappeared in mice treated with DE-310 (qd×1) at the MTD and 1/2 MTD, and all 6 mice remained tumor-free on the 60th day after administration. Repeated injection (4 times) on schedules of every 3 days, 7 days or 14 days demonstrated that multiple treatment with DE-310 produced greater tumor growth delay than a single treatment with DE-310. Against 5 human tumor (colon and lung cancer) xenografts in mice, DE-310 (qd×1) was as effective as DX-8951f administered once every 4 days, 4 times. The life-prolonging activity of DE-310 was assessed in lung (3LL, Lewis lung carcinoma) and liver (M5076, histiocytoma) metastasis models. Against 3LL, DE-310 (qd×1) at the MTD to 1/3 MTD significantly prolonged survival, with an increase in life span (ILS) of 4.8- to 1.6-fold, respectively, over that in untreated control mice. Also, DE-310 (qd×1) significantly prolonged survival in the liver metastasis model of M5076. These results demonstrate that DE-310 is a promising agent for the treatment of cancer. (Cancer Sci 2004; 95: 168–175)

The effect of current therapeutic agents appears when the agent acts on the target organ in the body. Since drug molecules that are distributed to organs that are not targeted may cause side effects, treatment is generally performed using a dose at which efficacy is obtained without unacceptable side effects. However, most antitumor chemotherapeutic agents show clinical effects only at doses that cause side effects such as digestive toxicity and myelosuppression. Therefore, in the development of new antitumor chemotherapeutic agents, it is important to discover compounds having a stronger antitumor effect and weaker toxicity compared with current antitumor chemotherapeutic agents. For example, CPT-11 was found to exhibit higher antitumor activity with less toxicity than camptothecin (CPT) and showed promising activity in clinical evaluation.[1–8] However, it causes side effects such as severe watery diarrhea and there are marked inter-patient variations in both its effects and toxicity.[7, 9, 10] To obtain a more effective analogue with a lower toxicity, DX-8951f was designed.[11] DX-8951f is the most active inhibitor of topo I and tumor growth both *in vitro* and *in vivo* among the currently available CPT derivatives.[12–14] However, even with DX-8951f, there is no apparent increase in the selectivity to tumor tissue versus normal tissue and the half-life of the compound is relatively short ($t_{1/2}$ of 0.3 h mice).[15]

The ideal antitumor drug would have high antitumor activity but no toxicity, i.e., it would be a compound for which efficacy and side effects are completely separate, but it is difficult to achieve this with low-molecular compounds, as the history of antitumor chemotherapeutics shows. Various approaches for superior targeting of antitumor compounds have been investigated by many researchers, and there have been trials of macromolecular pro-drugs.[16, 17] Based on the results obtained from examinations of macromolecular conjugates using doxorubicin in DDS Institute, Ltd., we started to study drug delivery using a macromolecular carrier based on a DX-8951-conjugate, in order to enhance the antitumor effect and to reduce the hematological toxicity of DX-8951f.

A novel macromolecular carrier system, based on the well-known enhanced permeability and retention (EPR) effect,[18] was designed to target DX-8951 to tumors and to provide sustained release of DX-8951 in tumors. We conducted extensive optimization studies of the peptidyl spacer, molecular weight of the carrier, DX-8951 content and degree of carboxymenthylation.[19] DE-310 is composed of DX-8951 and a biodegradable carrier, carboxymethyldextran polyalcohol (CM-Dex-PA), which are covalently linked by a peptidyl spacer (Gly-Gly-Phe-Gly, GGFG).[19] It has an average molecular weight of 340,000 (DX-8951 content: less than 8%, degree of carboxymethylation: ca. 0.3–0.4 per sugar residue).[19]

In the present study, we examined the therapeutic efficacy of DE-310 against a variety of human and murine tumors in mice.

Materials and Methods

Compounds. DE-310 (CM-Dex-PA-GGFG-DX-8951, Fig. 1A), a CM-Dex-PA adduct of the antineoplastic agent, DX-8951 [(1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-1,2,3,9,12,15-hexahydro-9-hydroxy-4-methyl-10*H*,13*H*-benzo[*de*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13-dione]. The carboxymethylated polyalcohol is covalently bonded to the DX-8951 moiety via a tetrapeptidyl spacer (Gly-Gly-Phe-Gly, GGFG). DX-8951f (Fig. 1B) is [(1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-1,2,3,9,12,15-hexahydro-9-hydroxy-4-methyl-10*H*,13*H*-benzo[*de*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13-dione monomethanesulfonate (salt) dihydrate. DE-310 and DX-8951f was synthesized in our laboratory. DE-310 and DX-8951f were dissolved in pyrogen-free distilled water and diluted using the same water. Dose levels of DE-310 and DX-8951f are expressed as those of DX-8951. Cisplatin (CDDP, Nippon Kayaku Co., Ltd., Tokyo) was diluted with pyrogen-free physiological saline. Cyclophosphamide (CPA, Shionogi Co., Ltd., Osaka) was dissolved in pyrogen-free distilled water and diluted using the same water.

Animals. Male, 6-week-old BALB/c mice, BALB/c-*nu*/*nu* nude mice, C57BL/6 mice were purchased from Japan SLC,

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Inc. (Shizuoka). They were housed in an exclusive experimental room under specific pathogen-free conditions, and given food sterilized by irradiation with γ-rays, in pelleted form (Oriental Yeast Co., Tokyo) or non-sterilized food, in pelleted form (Funabashi Farms, Chiba). Water treated with hypochloride or filtered water was available *ad libitum*.

Tumors. HCT116 was obtained from the American Type Culture Collection, Rockville, MD. PC-6 was obtained from Immuno-Biological Laboratories, Gunma. PC-12 was supplied by Toray Co., Ltd., Tokyo. HCT116/SN2-3 and PC-6/SN2-5S are drug-resistant cell lines established in our laboratory and isolated from HCT116 and PC-6, respectively, by stepwise selection with increasing concentrations of SN-38 (Yakult Honsha Co., Ltd., Tokyo). SN-38 is an active metabolite of CPT-11, a clinically available CPT analog. Differences in the cytotoxic effects of SN-38 against parental and resistant cell lines are indicated as "degree of resistance" and were calculated as follows: Degree of resistance=(GI_{50} for resistant cell line)/(GI_{50} for parental cell line). The degrees of resistance of HCT116/SN2-3 and PC-6/SN2-5S are 129 and 243, respectively. PC-12 is a cell line overexpressing P-glycoprotein.[13] HCT116, PC-6, HCT116/SN2-3 and PC-6/SN2-5S were cultured *in vitro* in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT or Bocknek, Canada), and then inoculated s.c. into nude mice and maintained as solid tumors by monthly or bi-monthly subcutaneous transplantation. Meth A was obtained from Hokkaido University, Sapporo. M5076[20] and 3LL[21, 22] were obtained from the Japanese Foundation for Cancer Research, Tokyo. Meth A and M5076 were maintained by serial intraperitoneal passage in syngeneic mice. Ascitic fluid was collected 7 or 14 days after implantation, and tumor cells were washed twice with Hanks' balanced salt solution (HBSS). The cell suspension was inoculated into the peritoneal cavity of syngeneic mice. 3LL was serially maintained as a solid tumor by subcutaneous passage every 2 weeks in syngeneic mice.

Experimental designs. 1) Antitumor activity against murine Meth A. Meth A cells (1×10^6 cells/0.1 ml) were inoculated s.c. into the right flank of syngeneic BALB/c mice and compounds were injected using various schedules from 7 or 11 days after tumor inoculation. For the study of tumor growth-inhibitory effects, the antitumor activity was evaluated in terms of the tumor weight. Tumor masses were excised and weighed on the 21st day after tumor inoculation. Differences in tumor weight were statistically analyzed by using Dunnett's test. For tumor growth delay effects, growth delay, expressed as $T-C$, was defined as the difference in days between the median times (MT) required for the tumors of treated (T) and control (C) animals to reach an established tumor weight (ETW) approximately 3 or 5 times greater than that measured at the time of the original treatment.[23] The tumor growth delay was statistically analyzed by use of the Williams-Wilcoxon test. 2) Tumor growth-inhibitory effects against human cancer xenografts. Various human tumors maintained in nude mice were excised and cut into pieces approximately 2 to 3 mm in diameter in HBSS. A piece of tumor was transplanted s.c. into the right flank of nude mice using a trocar. When the mean ETW reached 100 to 170 mg between days 13 and 35 after tumor transplantation, the mice were randomly divided into experimental groups (5 or 6 mice per group) and were treated i.v. with DE-310 as a single dose (qd×1) or DX-8951f every 4th day for a total of 4 doses (q4d×4). After the first administration on day 0, the ETW and body weight of the mice were measured 2 to 5 times per week for 21 or 28 days. The tumor masses were then excised and weighed. The tumor weight was statistically analyzed by using Dunnett's test. 3) Life-prolonging effects in a liver metastasis model of M5076. M5076 cells (1×10^5 cells/0.2 ml) were inoculated i.v. via the tail vein of syngeneic C57BL/6 mice (day 0). Following an intravenous inoculation, the M5076 cells disseminated widely in the whole body and formed tumor colonies in various host organs. These tumor cells have been shown to be retained initially for 3 to 4 days in the lungs, then they detach, re-circulate, and grow mainly in the liver. Occasionally tumors form in other organs such as the spleen, ovary, bone marrow and brain.[20] Tumor colonies established in the liver grow rapidly and host mice usually die of hepatic dysfunction within 2 to 3 weeks after tumor cell inoculation. In the present experiment, mice were i.v. administered either a single dose of DE-310 or an intermittent dose (every 14th day for a total of 4 doses, q14d×4) of DE-310 beginning on day 8. A single dose of CDDP was i.v. administered on day 8. It was confirmed by preliminary histopathological examinations that tumor colonies were already established in the liver and other organs on day 8 after inoculation of M5076 cells (1×10^5 cells/0.2 ml). Survival time was monitored daily until termination of the study on day 130. Dead mice were immediately necropsied, and surviving mice were sacrificed and autopsied on day 130. Survival time was statistically analyzed by use of the Williams-Wilcoxon test. 4) Life-prolonging effects in a lung metastasis model of 3LL. For the preparation of single cell suspensions from 3LL solid tumors, the tumors were excised, and rinsed several times in HBSS. The necrotic portion was removed, and the tumors were cut into pieces and dispersed using 0.14% collagenase type I (Type I, Clostridiopeptidase A; EC3.4.24.3 from *Clostridium histolyticum*, Lot 80H0518, Sigma, St. Louis, MO) and 0.03% DNase (deoxyribonuclease I; EC3.1.21.1, Lot 33H9598, Sigma), as previously described in detail.[24, 25] 3LL cells (5×10^5 cells/0.2 ml) were inoculated i.v. via the tail vein of syngeneic C57BL/6 mice (day 0). Following intravenous inoculation, the 3LL cells disseminated to the lungs and consistently formed tu-

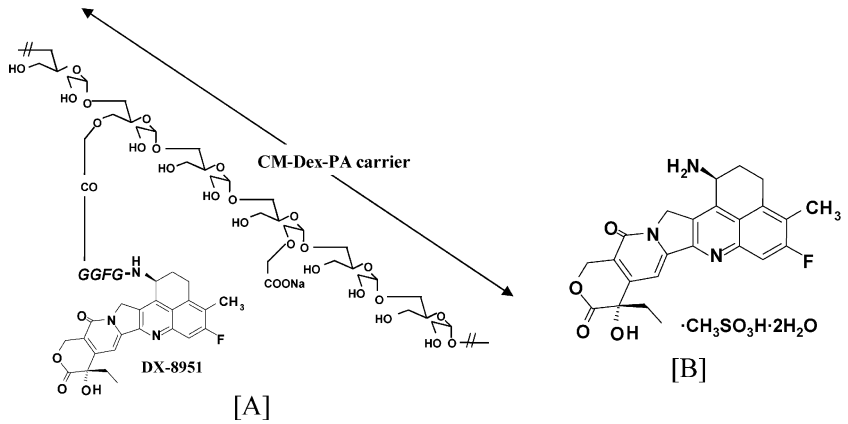

Fig. 1. Chemical structures of DE-310 (partial structure) [A] and DX-8951f [B].

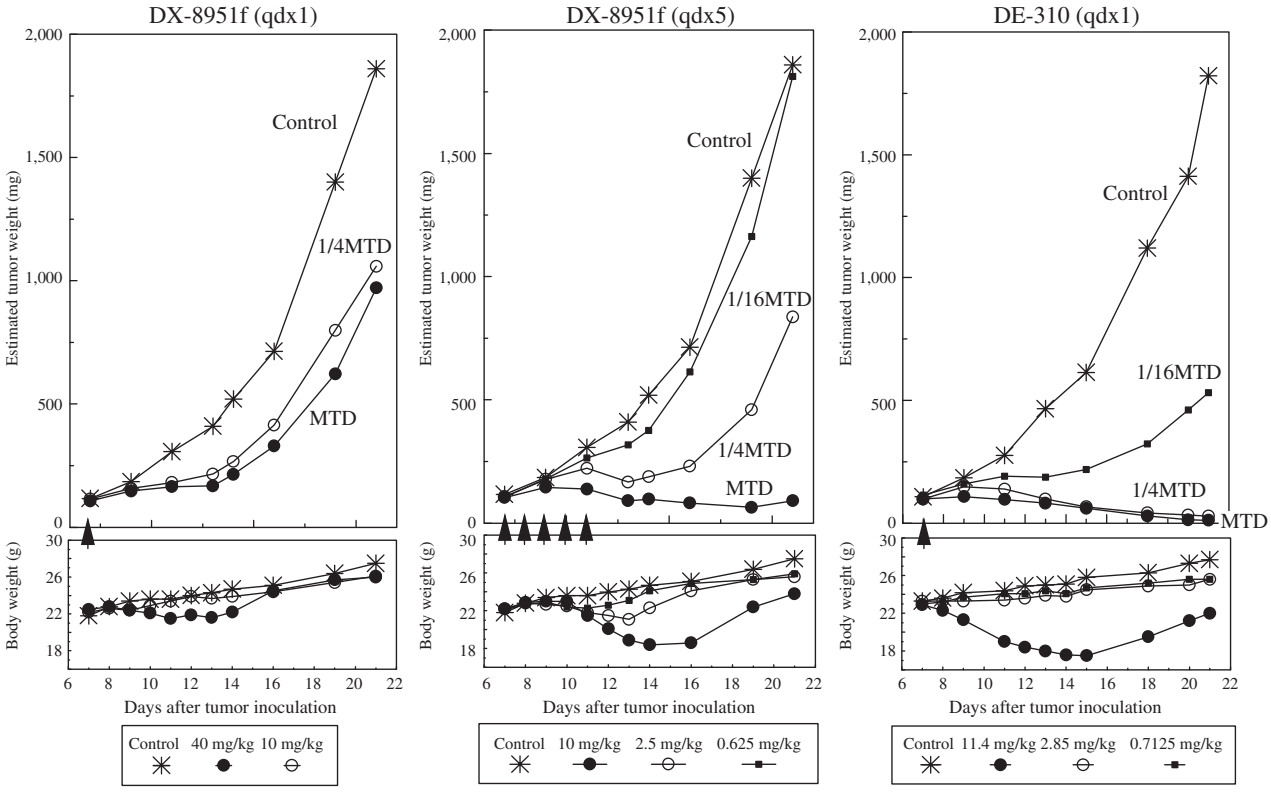

Fig. 2. Tumor growth curves and body weight changes in animals given DE-310 and DX-8951f as a single dose (qd×1) or once daily×5 doses (qd×5). The mice were divided into several groups (6 mice per group) on day 7. Estimated tumor weight and body weight for the MTD, 1/4 MTD and 1/16 MTD of each compound are plotted. Arrows indicate the days of treatment on each schedule. Points represent the mean value of surviving mice.

mor colonies. As a result of rapid tumor growth in the lungs, the host animals died of bleeding in the pleural cavity within 3 weeks after tumor cell inoculation.[21, 22, 24, 25] In the present experiment, mice were i.v. administered either a single dose or intermittent doses (every 7th day for a total of 4 doses, q7d×4) of DE-310, once daily times 5 doses (qd×5) of DX-8951f, or a single dose of CPA beginning on day 8. Survival time was monitored daily until termination of the study on day 123. Dead mice were immediately necropsied, and surviving mice were sacrificed and autopsied on day 123. Survival time was statistically analyzed by use of the Williams-Wilcoxon test.

Evaluations of activity and toxicity. The ETW was calculated using the formula[13]: $ETW=L\times W^2/2$, where L and W represent the length and the width of the tumor mass, respectively. The growth inhibition rate (IR) on the basis of tumor weight was calculated using the formula: $IR=(1-TW_t/TW_c)\times 100$ (%), where TW_t and TW_c represent the mean tumor weight of a treated group and that of the control group, respectively. When the IR was 58% or higher, the drug was considered effective.[26] Survival rates (SR) on day n were calculated from the following formula: SR (%)=(number of mice that survived on day n/number of mice used)×100. Increase in life span (ILS) was calculated from the following formula: $ILS\ (\%)=[(MST_t/MST_c)-1]\times 100$, where MST_t and MST_c represent the median survival time of a treated group in days and that of the control group in days, respectively. When the ILS was above 50%, the drug was considered effective.[27] The rate of body weight loss (BWL) was calculated based on the following formula: $BWL\ (\%)=(1-BW_n/BW_s)\times 100$, where BW_n and BW_s represent the mean body weight of mice on day n and that of mice on the day when administration started, respectively. BWL_{max} indicates the maximum value of BWL. BWL_{max} of less than zero (0) indicates no body weight loss. In the tables, the notations D and U indicate the number of mice that died of toxicity and the number of mice used, respectively.

Animal welfare. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co., Ltd.

Results

Comparison of antitumor effects of DE-310 and DX-8951f. As shown in Fig. 2, DE-310 with qd×1 at the maximum tolerated dose (MTD) and 1/4 MTD shrank the tumor, with no body weight loss occurring at 1/4 MTD. Even at 1/16 MTD, DE-310 inhibited tumor growth. In contrast, DX-8951f with qd×1 at the MTD and 1/4 MTD inhibited tumor growth moderately. In qd×5, DX-8951f at the MTD (10 mg/kg as total dose) and 1/4 MTD shrank the tumor and inhibited tumor growth, respectively.

Tumor growth delay effects of DE-310 with qd×1. Antitumor activity of DE-310 was examined in a longer-term study (about 3 months) because the short-term evaluation (14 days) showed substantial antitumor activity. As shown in Table 1, tumors in mice treated at the MTD and 1/2 MTD disappeared by the 15th day after administration, and all mice treated with those doses remained tumor-free up to day 67. Additionally, the rates of complete disappearance of tumor masses by DE-310 at 1/4 MTD and 1/8 MTD was 67%. DE-310 at 1/16 MTD and lower

doses exhibited dose-dependent tumor growth delay effects.

Tumor growth delay effects of DE-310 with various schedules. DE-310 with qd×1 caused the complete disappearance of tumor masses at several doses, as shown in Table 1. Therefore, in this examination with various schedules, treatment was started from 11 days. The size of tumor masses on day 11 was 1.6 times larger than in the case of Table 1 and Fig. 2. As shown in Fig. 3, DE-310 at 5.7 mg/kg with intermittent schedules exhibited a higher ratio of complete response or produced a greater tumor growth delay than when given with qd×1. BWL in the schedules of q3d×4 and q7d×4, which gave complete response, was very severe (25.1%) or moderate (11.1%), respectively. DE-310 at 2.85 mg/kg also resulted in a significant tumor growth delay in inverse proportion to the length of the administration interval.

Antitumor activity against colon cancer xenografts. As shown in Table 2, against parental HCT116, DE-310 and DX-8951f exhibited strong antitumor activity at the MTD to 1/3 MTD and at the MTD to 1/6 MTD, respectively. Antitumor activity of DE-310 at 1/6 MTD was similar to that of DX-8951f at 1/12 MTD. Against the resistant variant HCT116/SN2-3, DE-310 at 1/3 MTD and DX-8951f at 1/6 MTD produced moderate antitumor activity.

Antitumor activity against lung cancer xenografts. As shown in Table 3, DE-310 at the MTD and 1/6 MTD against parental PC-6 exhibited exceptional antitumor activity without any death from toxicity. Antitumor activity of DE-310 at the MTD against PC-6/SN2-5S was moderate. DX-8951f at the MTD against PC-6 showed strong antitumor activity, but was not effective at 1/6 MTD. Antitumor activity of DX-8951f at the MTD against PC-6/SN2-5S was moderate. Results against PC-

Table 1. Tumor growth delay effect of DE-310 with a single dose (qd×1) against Meth A solid tumors

Dose[1] (mg/kg)	Time to reach estimated tumor weight of 500 mg (days)			BWL_{max}[4] (%) [days]	*F/U*[5]
	Range	MT[2]	*T−C*[3]		
0	13–18	15.13	0	<0	0/6
11.4 (MTD)	>67	>67.08**	NA[6]	22.6 [13]	6/6
5.7	>67	>67.08**	NA	9.2 [11]	6/6
2.85	32–>67	>66.88**	NA	1.8 [9]	4/6
1.425	32–>67	>66.88**	NA	0.3 [8]	4/6
0.7125	18–>67	31.75*	16.63	<0	1/6
0.3563	15–20	19.67	4.54	<0	0/6
0.1781	13–18	15.25	0.13	0.2 [8]	0/6

The mice were randomized on day 7 and received an i.v. injection of DE-310. ** $P \leq 0.01$, * $P \leq 0.05$ vs. control (Williams-Wilcoxon test). Mean of estimated tumor weights of each group at the start of treatment were 107–118 mg.

1) Dose levels of DE-310 were expressed as those of DX-8951. The content of DX-8951 present in DE-310 was determined by HPLC.

2) MT indicates median time taken for tumors to reach estimated tumor weight of 500 mg.

3) *T−C*, tumor growth delay, is the difference between the median time required for tumors in treated (*T*) and control (*C*) mice to reach an estimated tumor weight of 500 mg as approximately 5 times their untreated estimated tumor weight on day 7.

4) BWL_{max} is the maximum rate of body weight loss, with numbers in parentheses denoting the day. <0 indicates no body weight loss.

5) *F/U* indicates number of tumor-free mice on day 67/number of mice used.

6) NA means "not applicable."

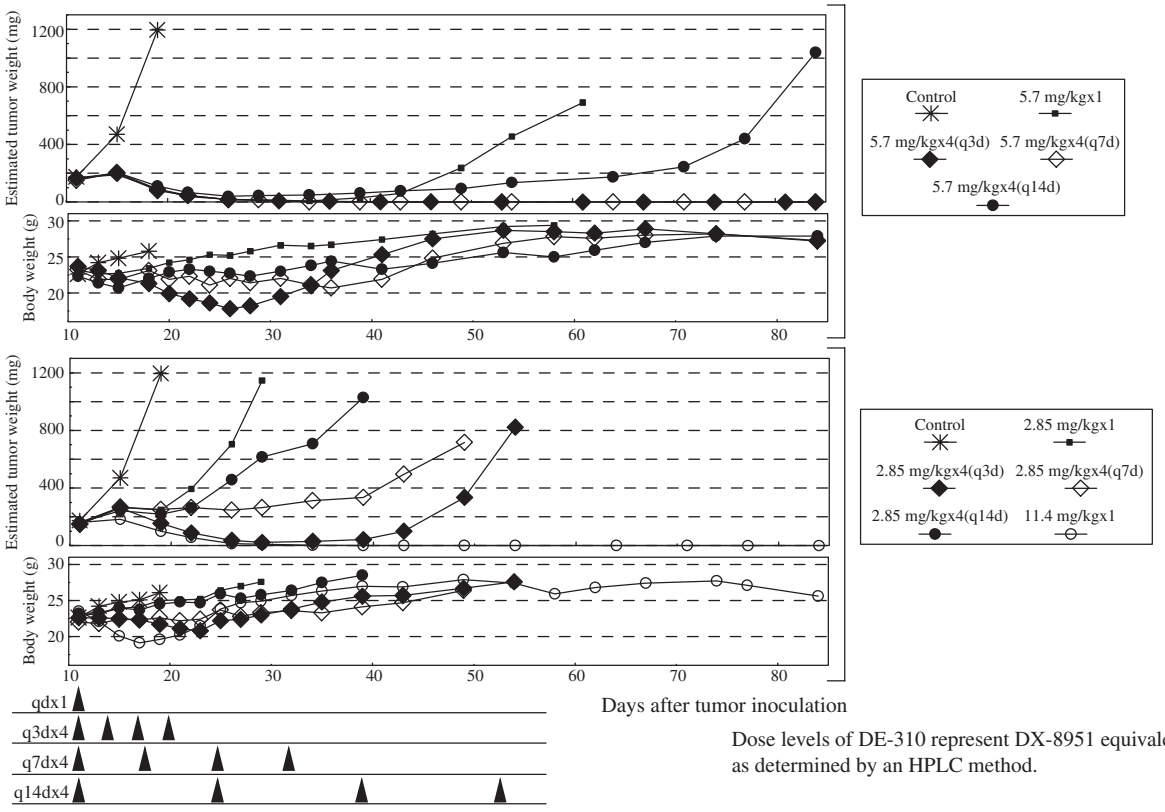

Fig. 3. Tumor growth curves and body weight changes in animals given DE-310 as a single dose and several repeated doses. The mice were divided into several groups (6 mice per group) on day 11 and received an i.v. injection of DE-310 with the schedule of a single dose (qd×1), every 3 days 4 times (q3d×4), every 7 days 4 times (q7d×4) or every 14 days 4 times (q14d×4). Arrows indicate the day of treatment. Points represent the mean value for 6 mice.

12 are shown in Table 4. DE-310 produced antitumor activity with IR values of 80% or higher at the MTD and 2/3 MTD. However, DX-8951f showed similar activity only at the MTD.

Antitumor activity in a liver metastasis model of murine histiocytoma. The results are shown in Table 5. Because two mice died following a single dose administration of DE-310 at 22.8 mg/kg, this dose was considered to be the MTD in this tumor model. DE-310 at the MTD to 1/2 MTD exhibited significant life-prolonging effects. The death of one mouse at 1/2 MTD was secondary to a poor nutritional state due to abnormal incisors. DE-310 at 1/4 MTD to 1/32 MTD also exhibited significant life-prolonging effects with ILS values of over 700%. Neither toxic death nor severe BWL was observed in these groups. Furthermore, 5 to 7 mice survived until day 130 and the ratio of surviving mice showing no macroscopic evidence of tumor tissues or nodules was 80 to 100%. Even at 1/64 MTD, the life-prolonging effect of DE-310 was statistically significant compared with the controls. DE-310 given intermittently for a total dose of 11.4 mg/kg exhibited a superior life-prolonging effect and more tumor-free survivors than did a single dose of 11.4 mg/kg. CDDP as positive control at the MTD to 1/3 MTD exhibited significant life-prolonging effects with ILS values of over 700%.

Antitumor activity in a lung metastasis model of murine lung carcinoma. The results are shown in Table 6. Because 2 mice died at 17.1 mg/kg, this dose was considered to be the MTD of DE-310 in this tumor model. DE-310 at the MTD to 1/12 MTD exhibited life-prolonging effects in a dose-dependent manner. DE-

Table 2. Anti-tumor effects of DE-310 and DX-8951f against human colon cancer HCT116 and HCT116/SN2-3 xenografts in nude mice

Compound	Schedule	Total dose[1] (mg/kg)	HCT116				HCT116/SN2-3			
			Tumor weight		BWL_{max}[2] (%) [day]	*D/U*[3]	Tumor weight		BWL_{max} (%) [day]	*D/U*
			Mean±SE (g)	IR (%)			Mean±SE (g)	IR (%)		
Control	—	0	2.810±0.408	0	0.3 [19]	0/6	1.944±0.457	0	7.6 [41]	0/6
DE-310	qd×1	11.4	0.012±0.004***	100	24.3 [20]	3/6[5a]	0.445±0.073**	77	30.4 [26]	3/6[5c]
	qd×1	8.55 (MTD)	0.024±0.007***	99	27.0 [23]	1/6[5b]	ND[4]			
	qd×1	5.70	0.051±0.014***	98	17.4 [19]	0/6	0.747±0.077**	62	14.0 [21]	0/6
	qd×1	2.85	0.277±0.040***	90	8.7 [20]	0/6	0.912±0.120*	53	7.1 [41]	0/6
	qd×1	1.425	0.589±0.093***	79	5.1 [17]	0/6	1.438±0.157	26	6.1 [43]	0/6
	qd×1	0.7125	1.028±0.189***	63	9.2 [41]	0/6	1.395±0.182	28	9.1 [41]	0/6
	qd×1	0.3563	1.742±0.184**	38	11.0 [41]	0/6	ND			
DX-8951f	q4d×4	75 (MTD)	0.007±0.003***	100	13.8 [26]	0/6	0.552±0.092***	72	16.6 [33]	0/6
	q4d×4	50	0.010±0.001***	100	10.4 [22]	0/6	0.603±0.086**	69	13.7 [33]	0/6
	q4d×4	25	0.016±0.005***	99	9.5 [25]	0/6	0.700±0.073**	64	10.1 [33]	0/6
	q4d×4	12.5	0.069±0.021***	98	4.7 [22]	0/6	0.872±0.180*	55	10.2 [43]	0/6
	q4d×4	6.25	0.477±0.064***	83	10.7 [22]	0/6	ND			

The mice were divided into several groups when the mean estimated tumor weight reached approximately 140 mg on day 13 (HCT116) or approximately 100 mg on day 15 (HCT116/SN2-3). Tumor weight was assessed on 28 days after the first administration (HCT116: day 41, HCT116/SN2-3: day 43). *** $P<0.001$, ** $P<0.01$, * $P<0.05$ vs. control (Dunnett's test).

1) Dose levels of DE-310 and DX-8951f were expressed as those of DX-8951. The content of DX-8951 present in DE-310 was determined by HPLC.

2) Maximum rate of body weight loss, with numbers in parentheses denoting the day. <0 indicates no body weight loss.

3) Number of mice that died of toxicity/number of mice used.

4) Not done.

5a, *5b*, *5c*) Death days were as follows: days 19, 19 and 21 (*5a*), days 25 (*5b*), days 20, 21 and 30 (*5c*).

Table 3. Anti-tumor effects of DE-310 and DX-8951f against human lung cancer PC-6 and PC-6/SN2-5S xenografts in nude mice

Compound	Schedule	Total dose[1] (mg/kg)	PC-6				PC-6/SN2-5S			
			Tumor weight		BWL_{max}[2] (%) [day]	*D/U*[3]	Tumor weight		BWL_{max} (%) [day]	*D/U*
			Mean±SE (g)	IR (%)			Mean±SE (g)	IR (%)		
Control	—	0	2.016±0.473	0	<0	0/5	0.799±0.146	0	4.8 [41]	0/6
DE-310	qd×1	8.55 (MTD)	0.002±0.001***	100	27.7 [33]	0/5	0.306±0.181	62	25.9 [41]	0/6
	qd×1	5.70	ND[4]				0.425±0.183	47	12.6 [42]	0/6
	qd×1	2.85	ND				0.705±0.176	12	0.9 [39]	0/6
	qd×1	1.425	0.045±0.013***	98	2.5 [30]	0/5	0.796±0.287	0	<0	0/6
	qd×1	0.7125	ND				0.818±0.176	–3	<0	0/6
DX-8951f	q4d×4	75 (MTD)	0.263±0.098***	87	18.5 [45]	0/5	0.381±0.161	52	16.0 [48]	0/6
	q4d×4	50	ND				0.299±0.101	63	11.3 [50]	0/6
	q4d×4	25	ND				0.410±0.141	49	5.6 [48]	0/6
	q4d×4	12.5	1.282±0.145	36	0.8 [29, 34]	0/5	0.559±0.184	30	0.6 [48]	0/6

The mice were divided into several groups when the mean estimated tumor weight reached approximately 150 mg on day 28 (PC-6) or 140–160 mg on day 35 (PC-6/SN2-5S). Tumor weight was assessed on 28 days after the first administration (PC-6: day 56, PC-6/SN2-5S: day 63). *** $P<0.001$, ** $P<0.01$ vs. control (Dunnett's test).

1) Dose levels of DE-310 and DX-8951f were expressed as those of DX-8951. The content of DX-8951 present in DE-310 was determined by HPLC.

2) Maximum rate of body weight loss, with numbers in parentheses denoting the day. <0 indicates no body weight loss.

3) Number of mice that died of toxicity/number of mice used.

4) Not done.

Table 4. Anti-tumor effects of DE-310 and DX-8951f against human lung cancer PC-12 xenografts in nude mice

Compound	Schedule	Total dose[1] (mg/kg)	Tumor weight		BWL_{max}[2] (%) [day]	D/U[3]
			Mean±SE (g)	IR (%)		
Control	—	0	1.928±0.424	0	0.1 [18]	0/6
DE-310	qd×1	8.55 (MTD)	0.299±0.056***	85	24.5 [22]	0/6
	qd×1	5.70	0.378±0.057***	80	10.1 [22]	0/6
	qd×1	3.80	0.725±0.181***	62	4.6 [21]	0/6
	qd×1	2.53	1.179±0.151*	39	0.9 [19]	0/6
	qd×1	1.70	1.014±0.156*	47	<0	0/6
DX-8951f	q4d×4	75 (MTD)	0.353±0.050***	82	9.7 [32]	0/6
	q4d×4	50	0.682±0.181**	65	8.4 [31]	0/6

The mice were divided into several groups when the mean estimated tumor weight reached approximately 170 mg on day 17. Tumor weight was assessed on 21 days after the first administration (day 38). *** $P<0.001$, ** $P<0.01$, * $P<0.05$ vs. control (Dunnett's test).
1) Dose levels of DE-310 and DX-8951f were expressed as those of DX-8951. The content of DX-8951 present in DE-310 was determined by HPLC.
2) Maximum rate of body weight loss, with numbers in parentheses denoting the day. <0 indicates no body weight loss.
3) Number of mice that died of toxicity/number of mice used.

Table 5. Anti-tumor effects of DE-310 and CDDP in a liver metastasis model of murine histiocytoma M5076

Compound	Schedule	Total dose[1] (mg/kg)	Survival (day)			BWL_{max}[2] (%) [day]	D/U[3]	N/S[4]
			Range	MST	ILS (%)			
Control	—	0	16–18	16.19	0.0	<0	0/10	—
DE-310	qd×1	34.2	16–18	17.60	8.7	31.2 [16]	10/10	—
	qd×1	22.8 (MTD)	26–>130	76.00**	369.4	11.6 [20]	2/10	2/2
	qd×1	17.1	50–>130	75.75**	367.9	5.4 [11, 20]	0/10	1/1
	qd×1	11.4	55–>130	81.00**	400.3	3.4 [9]	1/10	2/2
	qd×1	5.70	52–>130	>129.60**	>700.5	2.3 [9]	0/10	4/5
	qd×1	2.85	49–>130	>129.75**	>701.4	<0	0/10	6/6
	qd×1	1.425	48–>130	>129.86**	>702.1	<0	0/10	7/7
	qd×1	0.7125	70–>130	>129.86**	>702.1	<0	0/10	7/7
	qd×1	0.3563	47– >130	116.00**	616.5	<0	0/10	3/4
	qd×1	0.1781	21–127	33.00	103.8	<0	0/10	—
	q14d×4	11.4	81–>130	>129.94**#	>702.6	<0	0/10	8/8
	q14d×4	2.85	57–>130	>130.00**	>703.0	<0	0/5	4/4
CDDP	qd×1	15 (MTD)	16–>130	>129.94**	>702.6	20.9 [13]	1/10	7/8
	qd×1	10	52–>130	>129.94*	>702.6	6.5 [12]	0/10	5/8
	qd×1	5.0	74–>130	>130.00**	>703.0	2.3 [9]	0/10	7/9
	qd×1	2.5	20–>130	26.00**	60.6	<0	0/10	1/1
	qd×1	1.25	18–32	19.00	17.4	1.2 [9]	0/10	—

** $P\leq0.01$, * $P\leq0.05$ vs. control, # $P\leq0.05$ vs. single dose injection of the same total dose (Williams-Wilcoxon test).
1) Dose levels of DE-310 were expressed as those of DX-8951. The content of DX-8951 present in DE-310 was determined by HPLC.
2) Maximum value for rate of body weight loss (%), with numbers in parentheses denoting the day, <0 indicates no body weight loss.
3) Number of mice that died of toxicity/number of mice used.
4) Number of tumor-free mice/number of mice that survived by day 130 after tumor inoculation.

310 given intermittently for a total dose of 22.8 mg/kg exhibited a significant life-prolonging effect without toxic death. On the other hand, the life-prolonging effect of DX-8951f at the MTD was only 60% as the ILS value. CPA as a positive control exhibited a significant life-prolonging effect.

Discussion

Passive tumor-targeting with a macromolecular carrier system is based on the EPR effect. The EPR effect is a well-known feature of tumor tissue, including tumor blood vessels.[18] Moreover, the lymphatic clearance of macromolecules from tumor tissues does not occur. Accordingly, macromolecules remain in tumor tissues for a long period of time. DE-310 has a structure in which DX-8951 is combined with the biodegradable CM-Dex-PA carrier by a peptidyl spacer. The average molecular weight of DE-310 is 340,000 and the compound has a "stealthy" property.[19] These characteristics of DE-310 result in decreased excretion through the kidneys and decreased endocytosis in the liver/RES.[19] Accordingly, DE-310 exhibits prolonged high plasma-levels, and this phenomenon increases the EPR effect. After the distribution of DE-310 to the tumor tissue, DX-8951 is continuously generated from DE-310.[19]

In order to clarify the change in characteristics obtained by applying DX-8951 to drug delivery system (DDS), a comparison of the antitumor effects of DE-310 and DX-8951f was carried out using the Meth A model. Although DX-8951f at the MTD by qd×1 showed IR 50% at most, DX-8951f at the MTD

Table 6. Anti-tumor effects of DE-310, DX-8951f and CPA in a lung metastasis model of murine lung carcinoma 3LL

Compound	Schedule	Total dose[1] (mg/kg)	Survival (day)			BWL_{max}[2] (%) [day]	*D/U*[3]	*N/S*[4]
			Range	MST	ILS (%)			
Control	—	0	17–19	17.7	0.0	<0	0/10	—
DE-310	qd×1	22.8	15–16	15.2	−14.0	34.3 [15]	10/10	—
	qd×1	17.1 (MTD)	26–>123	84.0**	375.5	23.4 [12]	2/10	1/1
	qd×1	11.4	35–49	42.8**	142.0	17.6 [12]	0/10	—
	qd×1	5.70	26–32	28.4**	60.6	8.6 [12]	0/10	—
	qd×1	2.85	21–25	23.3*	32.1	3.7 [11]	0/10	—
	qd×1	1.425	18–23	21.0	18.9	0.8 [9]	0/10	—
	q7d×4	22.8	45–53	47.3**##	167.5	14.8 [35]	0/10	—
	q7d×4	11.4	30–45	42.0**	137.7	28.0 [35]	0/10	—
DX-8951f	qd×5	10 (MTD)	26–37	28.4**	60.8	11.0 [15]	0/10	—
	qd×5	5.0	24–29	27.7**	56.6	5.6 [12]	0/10	—
	qd×5	2.5	23–28	25.0**	41.5	3.3 [15]	0/10	—
	qd×5	1.25	21–26	23.4*	32.3	2.1 [11]	0/10	—
CPA	qd×1	400 (MTD)	74–>123	>122.8**	>594.8	17.0 [11]	0/10	6/6
	qd×1	200	29–>123	43.0**	143.4	6.2 [9]	0/10	1/1
	qd×1	100	22–29	23.7*	34.0	2.4 [9]	0/10	—

** $P \leq 0.01$, * $P \leq 0.05$ vs. control, ## $P \leq 0.01$ vs. single dose injection of the same total dose (Williams-Wilcoxon test).
1) Dose levels of DE-310 and DX-8951f were expressed as those of DX-8951. The contents of DX-8951 present in DE-310 was determined by HPLC.
2) Maximum value for rate of body weight loss (%), with numbers in parentheses denoting the day, <0 indicates no body weight loss.
3) Number of mice that died of toxicity/number of mice used.
4) Number of tumor-free mice/number of mice that survived by day 123 after tumor inoculation.

Table 7. Comparison of the effective dose-ranges of DE-310 and DX-8951f against Meth A solid tumors

Compound	Schedule	MTD (mg/kg)	MED (mg/kg)	Therapeutic index
DE-310	qd×1	11.4	0.7125	16
DX-8951f	qd×1	40	>40	1>
	q4d×4	60	15	4
	qd×4	15	1.875	8
	qd×2/q4d×4	60	3.75	16
	q3h×3/q4d×4	60	3.75	16

Dose levels of DE-310 and DX-8951f were expressed as DX-8951. MTD: the maximum tolerated dose (total dose). MED: the minimum effective dose (total dose) which exhibited IR ≥58%. Therapeutic index: MTD/MED. qd×1: a single dose, q4d×4: every 4th day for a total of 4 doses, qd×4: once daily×4 treatments. qd×2/q4d×4: 4 cycles of daily doses for 2 days every 4th day, q3h×3/q4d×4: 4 cycles of 3 doses per day every 4th day.

and at 1/4 MTD by qd×5 showed a tumor regression effect and a tumor-inhibitory effect, respectively. Since DX-8951f has a time-dependent mode of cytotoxic action, it is possible to improve its antitumor action by changing the administration method. We were able to obtain an improvement in therapeutic index expressed as MTD/MED (dose which shows an antitumor effect of IR 58% and higher), as shown in Table 7.[28] That is, DX-8951f showed a therapeutic index of 16 when given by multiple administrations.

On the other hand, DE-310 at the MTD and at 1/4 MTD by qd×1 showed tumor regression, and even at 1/16 MTD, DE-310 showed a tumor growth-inhibitory effect. The therapeutic index of 16 obtained with multiple administrations of DX-8951f was achieved by a single dose of DE-310 corresponding to 1/5th of the dose of DX-8951f (compared as DX-8951 equivalents). Moreover, no body weight loss of Meth-A-bearing mice treated with DE-310 at 1/4 MTD was observed. These findings clearly suggest that the pharmacokinetics, antitumor effects and toxicity of DX-8951 are improved, enhanced and reduced, respectively, by applying to DDS.

The pharmacokinetics of DE-310 in a Meth A model have already been reported, as follows[5]: (I) The half-life times in the blood of ^{14}C-DX-8951f and ^{14}C-DE-310 (carrier-labeled and DX-labeled) given by qd×1 are about 20 min and 2.6 days, respectively. (II) The AUC of free DX-8951 in tumors following the administration of DE-310 is 3 times and 28 times higher than those in the liver and lung, respectively. (III) The half-life of free DX-8951 in tumors following the administration of DE-310 is 2 or 3 days. (IV) In mice treated with ^{14}C-DE-310 (carrier-labeled), 95% or more of the radioactivity is excreted within 42 days after administration. These results suggest that DE-310 has desirable characteristics, such as high retention in the blood, preferential accumulation in the tumor and sustained and low release of free DX-8951, and that the carrier of DE-310 is biodegradable. It is considered that the above pharmacokinetic results account well for the difference in antitumor effect between DX-8951f and DE-310.

Because DX-8951f administered every 4th day for a total of 4 injections showed an excellent antitumor effect against various human tumors xenografted in nude mice,[13] the antitumor effects of this schedule of DX-8951f and a single dose of DE-310 were compared. Considering that the half-life in the blood of the conjugated DX-8951 was 2 days (Meth A-bearing mice), the administration schedule of DE-310 may have been slightly disadvantageous compared with that of DX-8951f. DE-310, however, showed a remarkable effect against PC-6. In other human tumor xenografts too, the antitumor effect of DX-8951f given by intermittent administration was similar to that achieved by qdx1 of DE-310.

On the other hand, tumors in the clinical setting exist within organs and thus differ from human tumor xenograft models. Therefore, prolongation of the survival period with DE-310 was examined in a lung metastasis model and a liver metastasis model involving intravenous transplantation of tumor cells. In both metastasis models, DE-310 showed significant prolonga-

tion of survival. These findings showed that DE-310 was effective not only against s.c. transplanted tumors, but also against tumors in organs.

We have already obtained some results using Meth A ascites cells. Experiments suggest that DE-310 is internalized in tumor cells and macrophages by endocytosis as the conjugate, and endosomes containing DE-310 transfer to lysosomes, whereupon DX-8951 is released from DE-310 by lysosomal enzymes.[29] It was also observed that M5076 characteristically exhibits higher uptake of DE-310 into the cell and higher release of DX-8951 from DE-310 compared with Meth A or various human tumor cell lines (unpublished results). Therefore, tumor cell lines with high pinocytosis, such as histiocytoma, are likely to be good candidate tumors for DE-310 treatment.

Finally, the main dose-limiting toxicity of DE-310 was hematological toxicity, consistent with that previously reported for DX-8951f.[13, 28] However, DE-310 induced far less myelotoxicity than DX-8951f at doses that showed similar degrees of antitumor activity.[28] Moreover, we evaluated the antigenic potential of DE-310 in guinea pig models of active systemic anaphylaxis reaction (ASA) and passive cutaneous anaphylaxis reaction (PCA). The results suggest that DE-310 may have weak antigenic potential (positive ratio: 5%) (unpublished results).

The findings presented in this paper suggest that the pharmacokinetics of the CM-Dex-PA carrier are favorable, and this carrier may also be applicable to develop conjugates of other currently used antitumor drugs, in addition to DX-8951f.

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