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Improved *in vivo* Antitumor Efficacy and Reduced Systemic Toxicity of Carboxymethylpullulan-peptide-doxorubicin Conjugates

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The antitumor efficacy of the conjugate of doxorubicin (DXR) and carboxymethylpullulan (CMPul) with Phe-Gly spacer (CMPul-FG-DXR) was evaluated using murine tumor models and compared with that of DXR. The conjugate exhibited higher antitumor efficacy against Lewis lung carcinoma than DXR. Complete tumor regression followed by long-term tumor-free survival was frequently observed when CMPul-FG-DXR was administered i.v. three times at a dose equivalent to 10 mg/kg of DXR. The superior survival as well as anti-metastatic effect of CMPul-FG-DXR in comparison with DXR was also demonstrated with the M5076 murine reticulosarcoma model. Body weight loss in mice treated with the conjugate was less than that in the DXR-treated group, indicating lower systemic toxicity of CMPul-FG-DXR. Simply mixing CMPul with DXR did not enhance the antitumor activity of DXR, showing that the conjugation of DXR with CMPul is necessary for improved antitumor activity. However, no enhanced antitumor efficacy of the conjugates was observed against a non-solid tumor model such as P388 leukemia. In summary, improved antitumor efficacy with reduced systemic toxicity of CMPul-FG-DXR was demonstrated in the present study. CMPul-FG-DXR may be useful as a cancer chemotherapy agent against solid tumors and metastases.

Key words: Carboxymethylpullulan — Doxorubicin — Polymeric drug — Enhanced permeability and retention

Most current anticancer drugs have been developed in the past half century. Although some combination therapies have proved to be active against human malignancies, the fact remains that their efficacy is still limited, especially against solid cancers. Recently developed chemotherapeutic agents have been found to show better response rates and survival benefits against solid cancers. However, these compounds are highly toxic to a wide spectrum of normal tissues, including the gastrointestinal tract, bone marrow, heart, lung, kidney and brain, and the frequent induction of systemic toxicity restricts their clinical efficacy.

To enhance the therapeutic efficacy of anticancer agents while reducing systemic toxicity, the drug should be selectively administered to the tumor tissue. Thus, various types of macromolecules have been proposed as drug carriers of anticancer agents, because macromolecules administered i.v. are known to accumulate preferentially and be retained more in solid tumors than in normal tissues (enhanced permeability and retention, EPR).[1, 2] For example, PK1, which consists of N-(2-hydroxypropyl)methacrylamide bound to doxorubicin (DXR) through a Gly-Leu-Phe-Gly spacer, has entered clinical trials in the UK.[3]

Pullulan, an α-1,6-linked linear polymer of maltotriose, has many advantages as a macromolecular drug carrier, including high water solubility, multiple hydroxyl groups that can readily be modified chemically, lack of immunogenicity, and usefulness as a plasma expander.[4] Our previous report described carboxymethylpullulan (CMPul)-DXR conjugate via Gly-Gly-Phe-Gly as being more effective than DXR in rats bearing Walker 256 carcinosarcoma and Yoshida sarcoma.[5, 6] We have further developed CMPul-DXR conjugates with a short spacer and have found that the conjugate via Phe-Gly showed good distribution in tumor tissue with a high content of free DXR.[7]

The objectives of the present study were to evaluate the *in vivo* antitumor activity of CMPul-Phe-Gly-DXR conjugates (CMPul-FG-DXR) in the murine tumor model in comparison with that of DXR. Our results demonstrated that CMPul-FG-DXR has greater antitumor efficacy than DXR, with less toxicity.

MATERIALS AND METHODS

Animals BDF_1, DBA/2 and C57BL/6 mice (female, 7–9 weeks old) used in this study were produced in our breeding colony and maintained in a specific pathogen-free facility at Aburahi Laboratory of Shionogi & Co., Ltd. (Kohka, Shiga).

Tumors Lewis murine lung carcinoma was provided by the National Cancer Institute (Bethesda, MD) and maintained by serial s.c. transplantation of tumor fragments in C57BL/6 mice. P388 murine leukemia was provided by

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Dr. T. Tsuruo (Tokyo Univ., Tokyo) and maintained by serial i.p. transplantation in DBA/2 mice. M5076 murine reticulosarcoma was provided by the Cancer Chemotherapy Center (Tokyo) and maintained by serial s.c. transplantation of tumor fragments in C57BL/6 mice.

Chemicals CMPul-FG-DXR was synthesized at the Drug Delivery System Institute, Ltd. (Noda, Chiba) using pullulan with a molecular weight of 65 000 (M_w/M_n=1.2) according to a procedure described elsewhere.[6, 8] DXR was obtained from Kyowa Hakko Kogyo (Tokyo). The conjugate had a degree of substitution of carboxymethyl groups of 0.6 per glucose unit and a DXR content of 6.0%. Fig. 1 shows the structure of the CMPul-FG-DXR conjugate. All drugs were dissolved in saline immediately before use.

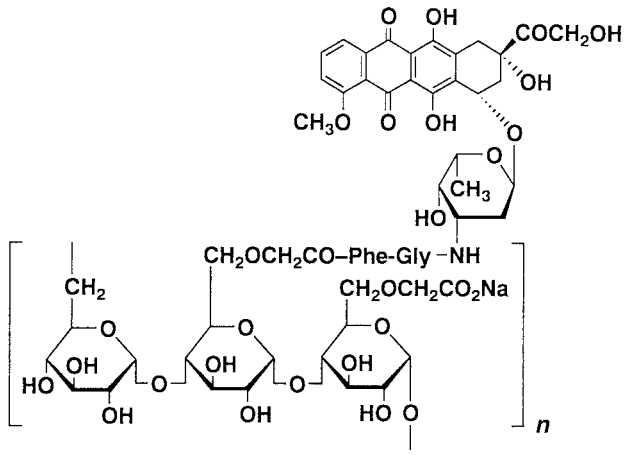

Fig. 1. Chemical structure of CMPul-FG-DXR.

***In vivo* therapeutic experiments** The experimental procedure was described previously.[9, 10] All experiments consisted of 6 to 10 mice per group. A tumor fragment (8 mm^3) of Lewis murine lung carcinoma was implanted s.c. into the back of BDF_1 mice. M5076 reticulosarcoma (1×10^6) was injected i.v. to BDF_1 mice. P388 murine leukemia (1×10^6) was injected i.p. to BDF_1 mice. CMPul-FG-DXR and DXR were administered i.v. once a day after tumor implantation (day 1) or three times (days 1, 5 and 9). Based on the calculated contents of DXR in conjugates, doses were adjusted in terms of DXR. All studies were performed with the approval of Shionogi Animal Care and Use Committee.

Evaluation of antitumor efficacy Tumor size, body weight and survival were assessed throughout each experiment. The endpoint of survival was considered to be the onset of moribundity, such as hypoactivity or hypothermia, and the mice were then sacrificed. Growth-inhibitory effect and prolonged survival were estimated from the treated/control ratio (T/C) and increased life span (ILS%), respectively.[9, 10] In the experiments using M5076 cells, liver weights were measured on day 21. T/C was determined as follows: $T/C=(W_t-W_i)/(W_v-W_i)$, where W_t, W_v and W_i are the liver weight in the test group, vehicle control and intact mice, respectively.

Statistics In this study, the statistical significance of differences from the non-treated group or among treated groups was evaluated using Welch's test and Dunnett's test, respectively.[11, 12]

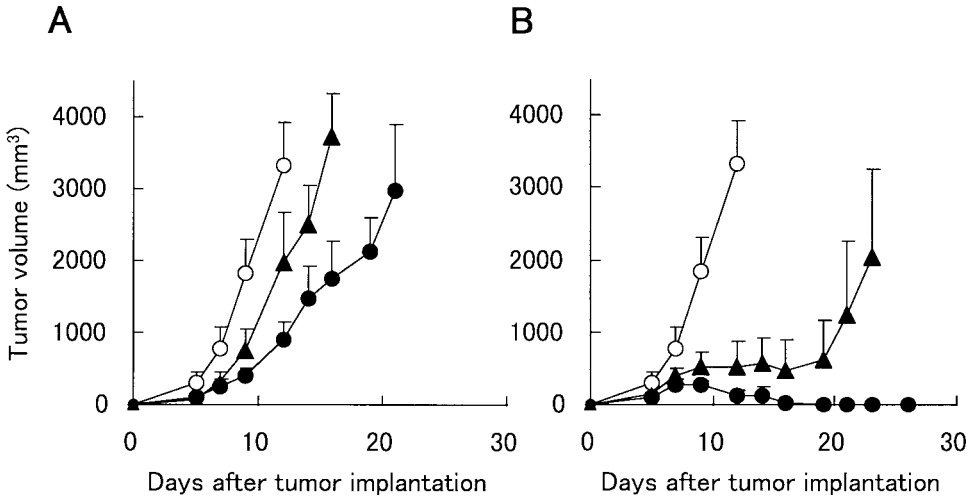

Fig. 2. Growth inhibition of Lewis lung carcinoma by DXR or CMPul-FG-DXR. Mice bearing Lewis lung carcinoma were treated i.v. with vehicle only (○), DXR (A) or CMPul-FG-DXR (B) three times (days 1, 5 and 9) at 5 mg/kg (▲) or 10 mg/kg (●). Bars show standard deviations.

RESULTS

Improved antitumor efficacy of CMPul-FG-DXR against Lewis lung carcinoma We compared the *in vivo* antitumor efficacy of CMPul-FG-DXR with that of unconjugated DXR against s.c. implanted Lewis lung carcinoma. Both CMPul-FG-DXR and DXR were administered three times (days 1, 5 and 9) at 5 or 10 mg/kg. Dosing of CMPul-FG-DXR (Fig. 2B) resulted in significantly superior ($P<0.01$) antitumor activity compared with unconjugated DXR (Fig. 2A) at both doses. The survival of the tumor-bearing mice was also improved (Fig. 3). In particular, established tumors showed regression after treatment of CMPul-FG-DXR at 10 mg/kg and finally, 6 out of 6 mice survived more than 60 days (more than 3 times the mean survival period of the vehicle control) without recurrent tumor (Fig. 3B). In addition, maximum body weight loss in the group treated with the conjugate at 10 mg/kg was 4.1% of the initial body weight, which represented a smaller loss than in the DXR-treated group (13.5%).

Anti-metastatic efficacy of CMPul-FG-DXR against M5076 carcinoma When M5076 murine reticulosarcoma was implanted via the tail vein of mice, metastases formed, particularly in the liver.[13] The anti-metastatic

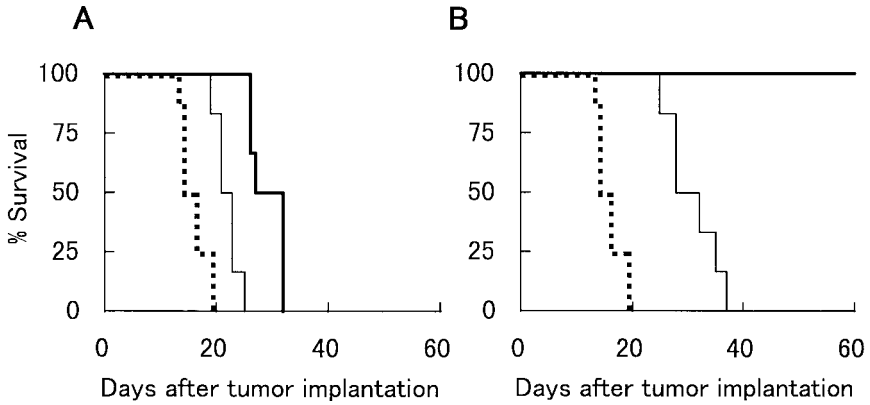

Fig. 3. Survival effect of DXR or CMPul-FG-DXR against Lewis lung carcinoma. Mice bearing Lewis lung carcinoma were treated i.v. with vehicle only (dotted lines), DXR (A) or CMPul-FG-DXR (B) three times (days 1, 5 and 9) at 5 mg/kg (thin lines) or 10 mg/kg (thick lines).

Table I. Inhibition of Liver Metastasis of M5076 Reticulosarcoma by CMPul-FG-DXR

Compound	Dose[a] (mg/kg)	Liver weight (mg)[b] (Mean±SD)	T/C[c]	MBW loss[d]
Vehicle control	0	3661±804		0.0
DXR	5	2749±688[e]	0.63	4.6 (2)[h]
	10	1667±178[f]	0.18	3.5 (2)
	20	1308±108[f]	0.03	7.4 (5)[i]
CMPul-FG-DXR	2.5	1222±114[f]	0.00	0.0
	5	1253±89[f, g]	0.01	1.5 (2)
	10	1274±96[f, g]	0.02	2.5 (2)
	20	1350±88[f]	0.05	3.0 (2)

a) i.v.×1 (day 1).
b) On day 21.
c) (Test groups − intact mice)/(vehicle group − intact mice), weight of intact mice; 1225±35.
d) Maximum body weight loss, % of initial.
e, *f*) $P<0.05$, 0.01 for vehicle by Welch's test.
g) $P<0.01$ for DXR-treated group by Dunnett's test.
h) Day of nadir.
i) $P<0.01$ for initial body weight by Welch's test.

Table II. Survival Effect of CMPul-FG-DXR in M5076-bearing Mice

Compound	Dose[a] (mg/kg)	Survival days (Mean±SD)	ILS (%)[b]	MBW loss[c]
Vehicle	0	18.4±2.6		0.0
DXR	5	17.5±1.2	0	4.4 (2)[g]
	10	21.2±1.8[d]	15	1.7 (2)
	20	21.7±1.8[d]	18	12.4 (7)[h]
	30	16.8±8.1	0	29.0 (9)[h]
CMPul-FG-DXR	5	30.8±1.9[e, f]	51	2.8 (2)
	10	37.3±2.6[e, f]	103	3.7 (2)
	20	44.3±3.3[e, f]	141	3.2 (2)
	30	55.0±10.2[e, f]	199	3.7 (2)
	40	56.5±5.2[e]	207	3.2 (9)

a) i.v.×1 (day 1).
b) Increased life span.
c) Maximum body weight loss, % of initial.
d, *e*) $P<0.05$, 0.01 for vehicle by Welch's test.
f) $P<0.01$ for DXR-treated group by Dunnett's test.
g) Day of nadir.
h) $P<0.01$ for initial body weight by Welch's test.

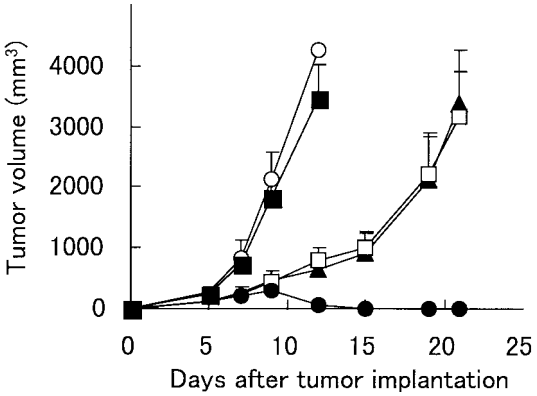

Fig. 4. Effect of CMPul on the antitumor activity of DXR. Mice bearing Lewis lung carcinoma were treated i.v. with vehicle only (○), DXR (▲), CMPul (■), DXR plus CMPul (□) or CMPul-FG-DXR (●) three times (days 1, 5 and 9). The doses used were 10 mg/kg for DXR, 230 mg/kg for CMPul and 10 mg/kg as DXR for CMPul-FG-DXR. Bars show standard deviations.

effects of CMPul-FG-DXR conjugate and DXR were tested by measuring the liver weight. As shown in Table I, dosing of DXR at 10 mg/kg resulted in marked inhibition of hepatic growth of M5076. However, the therapeutic index of CMPul-FG-DXR against hepatic growth was clearly superior to that of DXR, because 2.5 mg/kg of CMPul-FG-DXR was found to afford nearly complete growth inhibition of M5076 in the liver, whereas 20 mg/kg DXR was needed for a similar effect. The improved anti-metastatic effect of CMPul-FG-DXR was remarkable when the survival effect was assessed. Whereas even high-dose administration of DXR did not contribute to the survival of M5076-bearing mice, dosing with CMPul-FG-DXR resulted in significantly ($P<0.01$) prolonged survival of the treated mice at all doses tested (Table II). The body weight of mice given 30 mg/kg of DXR decreased after the treatment, followed by toxic death. In contrast, body weight loss in the CMPul-FG-DXR groups was found to be small, and dosing up to 40 mg/kg was tolerated with increased survival benefits. These *in vivo* therapeutic experiments clearly indicated that conjugation of DXR with CMPul improved the antitumor efficacy and reduced the systemic toxicity of DXR.

Requirement of molecular conjugation for antitumor activity of CMPul-FG-DXR To demonstrate that the conjugation of DXR with CMPul is essential for improved antitumor efficacy and to rule out the possibility of a direct or bystander antitumor activity of CMPul, a comparative study of a mixture of CMPul and DXR, CMPul given alone, and the conjugate was conducted. As shown in Fig. 4, CMPul did not exert any antitumor activity when administered alone. The mixture of CMPul and DXR showed neither augmentation nor inhibition of the antitumor activity of DXR. In contrast, the CMPul-FG-DXR conjugate showed potent antitumor efficacy. Again, all tumors regressed and 6 out of 6 mice survived without recurrent tumor.

Table III. Survival Effect of CMPul-FG-DXR against P388 Leukemia-bearing Mice

Compound	Dose[a] (mg/kg)	Survival days (Mean±SD)	ILS (%)[b]	MBW loss[c]
Vehicle	0	7.8±1.3		0.0
CMPul-PG	230	8.0±1.2	3	0.0
DXR	1.25	8.8±1.0	13	0.0
	2.5	13.3±3.9[d]	71	0.0
	5	15.5±4.3[e]	99	0.0
	10	18.8±2.6[e]	141	1.7 (6)[f]
	20	>27.5±2.9[e]	>253	13.7 (8)[g]
CMPul-FG-DXR	1.25	9.5±1.6	22	0.0
	2.5	13.5±3.0[e]	73	0.0
	5	14.5±1.2[e]	86	0.0
	10	18.3±2.5[e]	159	0.0
	20	>29.0±2.4[e]	>272	0.0

a) i.v.×1 (day 1).
b) Increased life span.
c) Maximum body weight loss, % of initial.
d, *e*) $P<0.05$, 0.01 for vehicle by Welch's test.
f) Day of nadir.
g) $P<0.01$ for initial body weight by Welch's test.

Absence of improved antitumor activity of CMPul-FG-DXR against P388 leukemia cells To examine the possibility that an EPR effect due to polymer conjugation may contribute to the improved antitumor activity of CMPul-FG-DXR, we finally investigated the efficacy of CMPul-FG-DXR against a non-solid tumor model in which both P388 leukemia cells and drugs were i.p. injected. In terms of survival, no significant difference was observed between the antitumor activities of CMPul-FG-DXR and DXR in this model (Table III). Both the conjugate and DXR were active (increased life span >30%) at 2.5 mg/kg. In spite of the similar antitumor efficacy, treatment with CMPul-FG-DXR produced a smaller reduction of body weight loss than that with DXR at 20 mg/kg dosing.

DISCUSSION

In the present study, we evaluated the antitumor effects of CMPul-FG-DXR using three murine experimental tumor models and compared them with those of DXR. Superior tumor growth inhibition and survival effects of CMPul-FG-DXR in comparison to DXR were demon-

strated against both Lewis lung carcinoma and M5076 reticulosarcoma. In particular, chemotherapy with CMPul-FG-DXR showed an improvement in the survival period and the rate of complete regression in the Lewis lung carcinoma model. Six out of six mice treated with CMPul-FG-DXR, but no mice given DXR, were found to be tumor-free survivors. It was also found that CMPul-FG-DXR showed potent antitumor activity against human lung carcinoma (unpublished results).

When M5076 cells were administered i.v., they formed metastases in various tissues, particularly in the liver.[13] The growth-inhibitory effect of CMPul-FG-DXR conjugate and DXR on metastases in the liver was examined by monitoring the liver weight. The conjugate showed greatly enhanced inhibition against hepatic metastases of M5076 cells, compared with DXR. Although CMPul-FG-DXR exerted anti-metastatic activity in other tissues, such as lung, spleen and ovary, the efficacy in the liver was found to be most potent (unpublished results). We did not conduct a pharmacokinetic study with this model, but have previously demonstrated that CMPul-Gly-Gly-Phe-Gly-DXR yielded an 8.3 times higher area under the curve (AUC) value of free DXR in the liver than did DXR itself, while it gave only 2.0 times higher AUC in the spleen in Walker 256 tumor bearing rats.[11] The much higher anti-metastatic activity of CMPul-FG-DXR in the liver might be due to the greater accumulation of free DXR in the liver than in other organs.

CMPul itself may potentiate an antitumor activity of DXR by stimulating the host's immune response. In order to test the direct effect of CMPul on tumor growth or the modulator effect on the cytotoxicity of DXR, the antitumor activity of CMPul alone or a mixture of CMPul and DXR was examined. The data clearly demonstrated that CMPul did not show antitumor activity when it was administered alone and did not modulate the activity of DXR. This strongly indicates that the augmented antitumor effect of CMPul-FG-DXR is due to the conjugation of CMPul with DXR via a dipeptide spacer.

The conjugation itself, however, might mask the cytotoxic activity of DXR. We previously demonstrated that the IC_{50} value of CMPul-FG-DXR conjugates was higher by a factor of 10^3 than that of DXR against Walker 256[4] or other human cancer cells *in vitro* (unpublished results). It has also been demonstrated that high-molecular-weight CMPul-FG-DXR was incorporated into tumor cells less efficiently than DXR after becoming attached to the cell surface.[4] These results suggest that DXR exhibits its activity after being released from the conjugates. Thus, it is important to examine whether drug release takes place at the tumor tissue. Further studies are needed to detect quantitatively free DXR in tumor or normal tissues and to identify enzymes involved in drug release.

The EPR effect may explain the mechanism of the improved antitumor efficacy of CMPul-FG-DXR. If this is the case, CMPul-FG-DXR would be effective against solid tumor, but not non-solid tumor such as leukemia cells. The results in the P388 leukemia model showed no difference in antitumor activity against P388 leukemia between CMPul-FG-DX and DXR, supporting this idea. It is curious that CMPul-FG-DXR showed less toxicity than DXR even with similar antitumor efficacy. Our previous study demonstrated that the pharmacokinetic behavior of DXR was markedly changed when CMPul was conjugated with DXR.[7] Further study is necessary to clarify the relation between toxicity and tissue disposition.

In conclusion, the present study demonstrated that CMPul-FG-DXR can be more effective than DXR, with reduced systemic toxicity, in murine tumor models. CMPul-FG-DXR appears to be a good candidate for chemotherapy of solid tumors in the clinical setting.

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