***In Vivo* Properties of the Conjugates of Mitomycin C with Estradiol Benzoate and Estradiol: Pharmacokinetics and Antitumor Characteristics against P388 Leukemia and Sarcoma 180**

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Conjugates of mitomycin C (MMC) with estradiol benzoate and estradiol *via* glutaric acid, abbreviated to EB-glu-MMC and E-glu-MMC, respectively, as previously reported, were examined concerning their pharmacokinetic behaviors and antitumor effects against two kinds of general and popular tumors, P388 leukemia and Sarcoma 180. EB-glu-MMC and E-glu-MMC were dissolved in propylene glycol. Their solution was administered intraperitoneally to rats and mice in order to examine their plasma concentration–time profiles and antitumor characteristics, respectively. After the administration of EB-glu-MMC, EB-glu-MMC was detected slightly in blood only in the initial stage, while E-glu-MMC and MMC were observed there for a prolonged period. In the administration of E-glu-MMC, a similar phenomenon was observed but the drug retention effect seemed lower than that in EB-glu-MMC. In the antitumor test against P388 leukemia, E-glu-MMC exhibited a better effect than EB-glu-MMC; however, neither conjugate surpassed the effect of MMC. The toxic side effect was improved in each conjugate. As to the growth inhibition against Sarcoma 180, EB-glu-MMC and E-glu-MMC produced good effect and improved the toxic side effect. Especially, in the administration of EB-glu-MMC at the dose of 30 mg eq MMC/kg, a decrease in tumor volume was observed in the latter stage. EB-glu-MMC and E-glu-MMC were demonstrated to produce prolonged retention, to enter the systemic circulation to a fair extent, and to exhibit a good effect against the general solid tumor, Sarcoma 180, *in vivo*.

Key words EB-glu-MMC; E-glu-MMC; pharmacokinetic behavior; antitumor effect; P388 leukemia; Sarcoma 180

Hormone-antitumor agent conjugates have been developed for improving the therapeutic effects of the parent agents or for lowering their toxic side effects. Some are known to work as active targeting systems.¹⁾ Estradiol and some of its derivatives show specific binding with the estrogen receptor, and the effects of some estradiol analogues are associated with their receptor binding specificities.^{2,3)} Antiestrogenic agents against breast cancer exhibit action through binding with the estrogen receptor. Therefore, the estrogen receptor is of possible use as a target site. Further, estradiol analogues with a high affinity to the estrogen receptor are localized much higher to the target tissues such as the uterus or ovary than to non-target tissues such as the liver, kidney or spleen.^{4,5)} Also, they seem to show a long residence in the target tissues and to be quickly eliminated from the non-target tissues.⁶⁾ In these phenomena, the estradiol receptor seems to play an important role.^{4–6)} In addition, some derivatives of antitumor drugs with estradiol derivatives or other steroids are known to improve the chemotherapeutic activity of the parent drugs in various tumors. Bestrabucil, which is a conjugate of chlorambucil with estradiol benzoate, exhibited a higher effect and lower toxicity in various tumors compared with the parent compound, chlorambucil.^{7–9)} This was because Bestrabucil showed a higher affinity to various tumors than chlorambucil and was distributed less in other tissues, where the possibility of mechanism other than receptor-mediated localization was proposed.^{7,9,10)} Thus, the conjugates of mitomycin C (MMC) with estradiol and estradiol derivatives have been proposed to be of possible use as agents to improve the chemotherapeutic activity of MMC or to reduce its toxicity. In the previous work, the conjugates of MMC with estradiol and estradiol benzoate, abbreviated to E-glu-MMC and EB-glu-MMC, respectively, were synthesized.¹¹⁾ The kinetics of their conversion to the parent compound, MMC, and receptor

binding characteristics were examined *in vitro*.¹²⁾ The conversion demonstrated that the conjugates could act as prodrugs of MMC. The receptor binding indicated that E-glu-MMC showed a high affinity ($K_d \approx 10^7 \text{ M}^{-1}$) to the estrogen receptor, which suggested the ability of the conjugate to operate as an active targeting system to the estrogen receptor-positive tumor cells. However, since the conjugates are small molecules but have very low aqueous solubility, the *in vivo* analysis of their pharmacokinetics and chemotherapeutic effect is crucial for the detailed evaluation of their drug action.

In this work, the pharmacokinetics and effect against two general tumors, P388 leukemia and Sarcoma 180, are examined. The pharmacokinetics are investigated in rats after the i.p. administration of E-glu-MMC and EB-glu-MMC in a solution dosage form prepared using propylene glycol as a solvent. The chemotherapeutic activity and toxic side effect are also examined using mice bearing P388 leukemia intraperitoneally and Sarcoma 180 solid tumor subcutaneously by the i.p. administration of E-glu-MMC and EB-glu-MMC in the solution dosage form using propylene glycol. The estrogen receptor was found to exist not only in target tissues such as the uterus, ovary or estrogen receptor-positive breast cancer cells, but also in several other tissues or cells; for example, osteoblast-like cells, blood mononuclear cells, thymocytes and L1210 cells have been reported to possess some estrogen receptors.^{13–15)} However, these cells seem to be ruled biologically by estradiol to some extent. Information on the estrogen receptor of P388 leukemia and Sarcoma 180 could not be obtained in related literature, according to our research. Since the localization of estradiol-like compounds is more marked in the tissues or cells ruled more strongly by estradiol, P388 leukemia and Sarcoma 180 are presumed not to be affected so much by an estradiol-like compound, even if the estrogen receptor may exist in them. This study aims to elu-

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cidate the effect of the conjugates against the general tumors considered to be slightly or not ruled by estradiol.

MATERIALS AND METHODS

Chemicals Mitomycin C (MMC) was used after extraction by tetrahydrofuran (THF) from Mitomycin Kyowa S (Kyowa Hakko Kogyo Co., Japan) which contains only NaCl in addition to MMC. Estradiol benzoate and estradiol were purchased from Wako Pure Chemical Industries, Ltd. (Japan). The conjugates of MMC with estradiol benzoate and estradiol, abbreviated to EB-glu-MMC and E-glu-MMC, respectively, were synthesized according to the previous report.¹¹⁾ Briefly, first their glutaric esters, 4-[3-benzoyloxy-1,3,5(10)-estratrien-17 β -oxycarbonyl]butyric acid (EB-glu) and 4-[3-hydroxy-1,3,5(10)-estratrien-17 β -oxycarbonyl]butyric acid (E-glu), were synthesized. Next, EB-glu-MMC was synthesized by condensation between the carboxyl group of EB-glu and 1a-NH of MMC. E-glu-MMC was also synthesized by the conjugation of MMC with E-glu in the same way.

Animals Female Wistar rats weighing 200 g and male and female ddY mice, 6 weeks old, weighing 26–29 g were purchased from Saitama Experimental Animal Supply Co. (Japan). Male DBA/2 mice, 6 weeks old, weighing 19–23 g and female BDF₁ mice, 6 weeks old, weighing 19–23 g were obtained from Clea Japan, Inc. (Japan).

Tumors P388 leukemia cells were maintained by weekly transplantation of 1×10^6 cells suspended in Hanks' balanced solution (0.1 ml) into the peritoneal cavity of each male DBA/2 mouse. In the *in vivo* antitumor activity tests, 1×10^6 P388 cells suspended in Hanks' balanced solution (0.1 ml), which were obtained from the above tumor-bearing male DBA/2 mice, were inoculated intraperitoneally into each female BDF₁ mouse. Sarcoma 180 cells were maintained by weekly transplantation of 1×10^6 cells suspended in Hanks' balanced solution (0.1 ml) into the peritoneal cavity of each male ddY mouse. In the *in vivo* antitumor activity tests, 1×10^7 Sarcoma 180 cells suspended in Hanks' balanced solution (0.1 ml), which were obtained from the above tumor-bearing male ddY mice, were inoculated subcutaneously into each female ddY mouse at the axillary region.

Pharmacokinetics after I.P. Administration Wistar rats were anesthetized by the i.p. injection of 25 % (w/v) urethane saline solution at 1 g urethane/kg, then fixed on their backs during the experiment. EB-glu-MMC and E-glu-MMC were dissolved in propylene glycol to give a concentration of 2 mg/ml. Each solution was administered intraperitoneally to the rats at the dose of 5 mg eq MMC/kg. After administration, the 0.5 ml of blood was taken from the jugular vein at 0.5, 1, 2, 4, 7 and 11 h. Immediately after each withdrawal of blood, the plasma was separated by centrifugation of the blood at 3000 rpm for 10 min. Immediately after that, 2 ml of a mixture of chloroform and 2-propanol (1:1, w/w) was added to 0.2 ml of the plasma. After the obtained mixture was vigorously shaken, it was centrifuged to obtain the supernatant. The whole of the supernatant was transferred to a glass tube and evaporated to dryness at 40 °C under nitrogen gas. The residue was dissolved in 100 μ l of methanol, and 20 μ l of the solution was injected in HPLC, which is described in the next part, in order to determine the plasma concentration of MMC, EB-glu-MMC and E-glu-MMC.

From the recovery check, the recovery ratios from the plasma by the above extraction method were more than 80% for all three compounds, EB-glu-MMC, E-glu-MMC and MMC. The pharmacokinetic parameters such as area under the plasma concentration–time curve (*AUC*), *etc.* were calculated using the program MULTI which had been reported by Yamaoka *et al.*¹⁶⁾

HPLC Assay The HPLC assay was executed at room temperature using a Shimadzu LC-6A (Japan) with a Shimadzu SPD-6A detector (Japan) set at 364 nm. The reversed phase column, SUMIPAX Nucleosil 5C₁₈ (4 mm i.d. $\times$ 250 mm) (Japan), with a guard column, Applied Biosystems RP-18 NEW GUARD 7 micron (3.2 mm i.d. $\times$ 15 mm) (U.S.A.), was used as an analytical column. Mixtures of 10 mM phosphate buffer of pH 6.0 and methanol at 13:7, 1:9 and 1:4 (v/v) were used as mobile phases for the determination of MMC, EB-glu-MMC and E-glu-MMC, respectively. The flow rates were set at 0.6, 0.9 and 0.7 ml/min for the determination of MMC, EB-glu-MMC and E-glu-MMC, respectively. Each was determined by the absolute calibration curve method.

Antitumor Tests (i) Antitumor activity against P388 leukemia: At 24 h after i.p. inoculation, MMC, EB-glu-MMC and E-glu-MMC were administered intraperitoneally as a propylene glycol solution to the tumor-bearing mice. At that time, EB-glu-MMC and E-glu-MMC were administered at the concentration of 2 mg/ml for the doses of 5 and 10 mg eq MMC/kg, or administered at the concentration of 5 mg/ml for the doses of 15 and 20 mg eq MMC/kg. As to the control mice, propylene glycol alone was injected at a volume equivalent to the mean one of the tested samples. All the mice were observed for survival time for 2 months after inoculation. The antitumor effect was measured by comparing the mean survival time of the treated mice after inoculation (*T*) with that of the control mice after inoculation (*C*); that is, from the increase in life span (*ILS*) calculated as follows:

$$ILS = (T/C - 1) \times 100 (\%) \quad (1)$$

At the same time, the change in body weight of each group was measured to evaluate the toxic side effects.

(ii) Antitumor activity against Sarcoma 180: At 4 d after s.c. inoculation, MMC, EB-glu-MMC and E-glu-MMC were administered intraperitoneally to the tumor-bearing mice as a propylene glycol solution. At that time, EB-glu-MMC was administered at the concentration of 20 mg/ml for the doses of 5, 10, 20 and 30 mg eq MMC/kg, and E-glu-MMC was administered at the concentration of 5 mg/ml for the dose of 5 mg eq MMC/kg, at the concentration of 10 mg/ml for the doses of 10 and 20 mg eq MMC/kg, and at the concentration of 20 mg/ml for the dose of 30 mg eq MMC/kg. As to the control mice, propylene glycol alone was injected with a similar volume. All the mice were investigated for tumor volume. The length (*L*, cm) of the tumor major axis, the longest axis, and width (*W*, cm) of the tumor minor axis, the vertical axis to the major axis, were measured with slide calipers, and the tumor volume (*V*, cm³) was calculated as follows:¹⁷⁾

$$V = L \times W^2 / 2 \quad (2)$$

At the same time, the change in body weight of each group was measured to evaluate the toxic side effects.

Fig. 1. Plasma Concentration–Time Profiles of MMC (●), E-glu-MMC (▲) and EB-glu-MMC (■) after I.P. Administration of EB-glu-MMC (A) and E-glu-MMC (B) to Rats

Each point represents the mean $\pm$ S.D. ($n=3$ for EB-glu-MMC, $n=5$ for E-glu-MMC).

Table 1. Pharmacokinetic Parameters of MMC, E-glu-MMC and EB-glu-MMC after I.P. Administration of EB-glu-MMC and E-glu-MMC

Compound	Species in plasma	$C_{\max}$ ($\mu\text{g}/\text{ml}$) (mean $\pm$ S.D.)	$T_{\max}$ (h) (mean $\pm$ S.D.)	$AUC_{0-\infty}$ ($\mu\text{g} \cdot \text{h}/\text{ml}$) (mean $\pm$ S.D.)	$MRT_{0-\infty}$ (h) (mean $\pm$ S.D.)	$VRT_{0-\infty}$ (h^2) (mean $\pm$ S.D.)
EB-glu-MMC	EB-glu-MMC	0.033 ± 0.036	0.917 ± 0.144	0.039 ± 0.041	0.542 ± 0.470	0.039 ± 0.034
	E-glu-MMC	0.135 ± 0.050	2.333 ± 0.577	0.521 ± 0.170	2.469 ± 0.262	1.567 ± 0.159
	MMC	0.137 ± 0.057	2.667 ± 1.155	0.974 ± 0.369	5.680 ± 1.996	22.91 ± 19.50
E-glu-MMC	E-glu-MMC	0.255 ± 0.141	0.650 ± 0.224	0.494 ± 0.260	1.220 ± 0.239	0.396 ± 0.332
	MMC	0.177 ± 0.059	1.100 ± 0.224	0.771 ± 0.200	3.231 ± 0.352	5.409 ± 4.910
MMC (i.v.) ^{a)}	MMC	$6.223 \pm 0.674^b)$	$0.000 \pm 0.000^b)$	2.867 ± 0.647	0.410 ± 0.146	0.224 ± 0.132
MMC (i.p.) ^{a)}	MMC	3.357 ± 0.949	0.250 ± 0.000	3.917 ± 1.316	1.077 ± 0.283	0.954 ± 0.589

When the concentration was above zero at 11 h after administration, the concentration curve was extrapolated to the infinite by monoexponential decline best fitted. ^{a)} These are based on the results in male rats at the dose of 5 mg/kg, previously reported (Sato *et al.*⁹⁾). ^{b)} These are the values calculated from the linear decline curves best fitted to the log(plasma concentration)–time plots.

RESULTS AND DISCUSSION

Plasma Concentration after I.P. Administration The plasma concentration–time profiles of MMC, E-glu-MMC and EB-glu-MMC after the i.p. administration of EB-glu-MMC are described in Fig. 1A. EB-glu-MMC was observed in plasma at a low level only for a short time immediately after the injection. E-glu-MMC and MMC were observed for a much longer period in plasma. Although E-glu-MMC was not detected after 6 h after the administration, MMC was still observed at 11 h after the administration. The pharmacokinetic parameters were calculated from the data of Fig. 1A (Table 1). EB-glu-MMC showed very small AUC , mean residence time (MRT) and variance of residence time (VRT), but E-glu-MMC and MMC exhibited much larger values. Namely, the AUC values of E-glu-MMC and MMC were more than 10 times and more than 30 times larger than that of EB-glu-MMC. The AUC of MMC, that is, 0.974 ($\mu\text{g} \cdot \text{h}/\text{ml}$), corresponded to almost one-fourth of that obtained at the i.p. injection of free MMC to male rats¹⁸⁾ (Table 1). This indicated a good transfer of the drug to the blood circulation. The MRT values of E-glu-MMC and MMC were more than 4 times and more than 10 times larger than that of EB-glu-MMC, respectively. The VRT values of E-glu-MMC and MMC also expressed their long retention in plasma. These

indicated that EB-glu-MMC could sustain drug release *in vivo*.

Figure 1B shows the plasma concentration–time profiles of MMC and E-glu-MMC after the i.p. administration of E-glu-MMC. E-glu-MMC and MMC were observed for a fairly long period in plasma. The pharmacokinetic parameters were calculated from the data of Fig. 1B (Table 1). E-glu-MMC was detected until 2 h after the administration, and MMC was observed at 7 h after the administration. E-glu-MMC showed a higher $C_{\max}$ value, shorter $T_{\max}$ value and faster elimination than in the administration of EB-glu-MMC. MMC also exhibited similar phenomena on the drug appearance and elimination. Namely, the retention of MMC tended to be shorter than in the administration of EB-glu-MMC, though the difference was not marked. Although the AUC of E-glu-MMC was similar to that in the administration of EB-glu-MMC, the AUC value of MMC, that is, 0.771 ($\mu\text{g} \cdot \text{h}/\text{ml}$), was smaller to some extent than that in the administration of EB-glu-MMC. The MRT and VRT values expressed a fairly long retention of the drug *in vivo*, but they were smaller than those in the administration of EB-glu-MMC. This indicated that E-glu-MMC could not produce as a good retention effect as EB-glu-MMC. According to the previous paper,^{11,12)} when both conjugates are dissolved completely in a mixture of propylene glycol and 1/15 M phosphate buffer, pH 7.4 (1 : 1,

v/v), the MMC release rates from both conjugates are similar. Thus, under the dissolved condition, the conversion from EB-glu-MMC to E-glu-MMC does not influence the total release of MMC from EB-glu-MMC and E-glu-MMC. Therefore, some reason other than the difference between their conversion schemes may cause EB-glu-MMC to produce an *in vivo* slower release of MMC than E-glu-MMC. As discussed in the next section, after the injection of the solution of the conjugates, some part may be precipitated around the injected site because of the dilution of propylene glycol by the surrounding aqueous body fluid. Actually, the addition of an aqueous buffer, pH 7.4, to the propylene glycol solution of the conjugates makes them easily precipitated. Namely, it was considered that such precipitation or suspension formation might be caused after injection and it should affect the *in vivo* drug release rate.

These results showed the abilities of MMC and E-glu-MMC to transfer to various organs *via* blood circulation with the i.p. administration of EB-glu-MMC and E-glu-MMC. Since E-glu-MMC possesses the ability to bind the estrogen receptor, this suggests the possibility for the drug to be homed to estrogen receptor-positive tumor cells. Further, the results demonstrated that the i.p. administration of EB-glu-MMC and E-glu-MMC could produce sustained drug release *in vivo*.

Antitumor Effect against Peritoneal P388 Leukemia after I.P. Administration At 24 h after inoculation, MMC, EB-glu-MMC and E-glu-MMC were administered intraperitoneally at a single dose as a clear solution in propylene glycol. Their dose-dependent effects are shown in Table 2. MMC produced a significantly higher survival time than the control and the highest *ILS* value at the dose of 5 mg/kg. However, it exhibited a lethally toxic effect at the dose of 10 mg/kg, that is, some of this group died considerably earlier than the control group. Although E-glu-MMC did not produce a significantly larger lifespan than the control at the dose of 5 mg eq MMC/kg, it was significantly effective at 10–20 mg eq MMC/kg. E-glu-MMC acts as a prodrug of MMC, but it might not give a sufficient exposure of the drug to the tumor site at the low dose. The drug will be supplied

effectively to the tumor site at the high dose. The toxic side effect was measured by the change in body weight (Fig. 2). MMC produced a marked decrease in body weight at 10 mg/kg, and mice started to die from 6 d after inoculation. At the dose of 10–15 mg eq MMC/kg, E-glu-MMC showed a slight decrease in body weight only at the initial stage, and the body weight increased after then. E-glu-MMC showed a decrease in body weight to some extent at the dose of 20 mg eq MMC/kg, but the body weight increased from 4 d after the administration. Such a lower toxic side effect in E-glu-MMC than in MMC could be also proposed from the above plasma concentration data, that is, because it generated MMC at the lower level and slower rate.

On the other hand, EB-glu-MMC exhibited a significantly larger lifespan only at the dose of 10 mg eq MMC/kg. The slight effect at the lower dose was considered to be due to a reason similar to that with E-glu-MMC. However, no significant effect was achieved at 15–20 mg eq MMC/kg. This might be because EB-glu-MMC was administered at a different concentration, 2 and 5 mg/ml, for the low (5–10 mg eq MMC/kg) and high (15–20 mg eq MMC/kg) doses, respectively. Namely, in this administration, a drug solution with a

Table 2. Effect of MMC, E-glu-MMC and EB-glu-MMC on the Survival Time of Mice Bearing P388 Leukemia Intraperitoneally

Compound	Dose (mg eq. MMC/kg)	Survival days of the tested (mean±S.D.)	Survival days of the control (mean±S.D.)	<i>ILS</i> (%)
MMC	2.5	19.3±2.9***	8.5±2.4	126.47
	5	24.3±2.8***	8.5±2.4	185.29
	10	11.5±8.4	8.5±2.4	35.29
E-glu-MMC	5	11.3±1.0	8.5±2.4	32.52
	10	12.8±1.0*	8.5±2.4	50
	15	15.8±3.4*	8.5±2.4	85.29
	20	16.0±2.2**	8.5±2.4	88.24
EB-glu-MMC	5	10.8±1.0	10.0±0.0	7.5
	10	14.3±1.7*	10.0±0.0	42.5
	15	13.0±2.2	10.0±0.0	30
	20	12.5±2.7	10.0±0.0	25

The survival time of each group is determined with $n=4$. * $p<0.05$ vs. control; ** $p<0.01$ vs. control; *** $p<0.001$ vs. control; by Student's *t*-test.

Fig. 2. Change in Mean Body Weight of Mice Bearing P388 Leukemia Intraperitoneally after I.P. Administration of MMC (A), EB-glu-MMC (B) and E-glu-MMC (C)

(A) MMC control ($\diamond$), 2.5 mg/kg ($\blacktriangle$), 5 mg/kg ($\bullet$), 10 mg/kg ($\blacksquare$); (B) EB-glu-MMC control ($\diamond$), 5 mg eq MMC/kg ($\blacklozenge$), 10 mg eq MMC/kg ($\blacktriangle$), 15 mg eq MMC/kg ($\bullet$), 20 mg eq MMC/kg ($\blacksquare$); (C) E-glu-MMC control ($\diamond$), 5 mg eq MMC/kg ($\blacklozenge$), 10 mg eq MMC/kg ($\blacktriangle$), 15 mg eq MMC/kg ($\bullet$), 20 mg eq MMC/kg ($\blacksquare$). The horizontal axis is time (d) after inoculation. Administration was executed at 24 h after inoculation as indicated by arrows. The initial body weight means the weight measured immediately after inoculation. Each point represents the mean value ($n=3-4$).

Fig. 3. Growth Inhibitory Effect on Sarcoma 180 Solid Tumor Implanted Subcutaneously after I.P. Administration of MMC (A), EB-glu-MMC (B) and E-glu-MMC (C)

(A) MMC control ($\diamond$), 2.5 mg/kg ($\blacktriangle$), 5 mg/kg ($\bullet$), 10 mg/kg ($\blacksquare$); (B) EB-glu-MMC control ($\square$), 5 mg eq MMC/kg ($\blacklozenge$), 10 mg eq MMC/kg ($\blacktriangle$), 20 mg eq MMC/kg ($\bullet$), 30 mg eq MMC/kg ($\blacksquare$); (C) E-glu-MMC control for 5–20 mg eq MMC/kg ($\diamond$), control for 30 mg eq MMC/kg ($\square$), 5 mg eq MMC/kg ($\blacklozenge$), 10 mg eq MMC/kg ($\blacktriangle$), 20 mg eq MMC/kg ($\bullet$), 30 mg eq MMC/kg ($\blacksquare$). The horizontal axis is time (d) after inoculation. Administration was executed at 4 d after inoculation as indicated by arrows. The initial tumor volume means the volume measured just before administration. Each point represents the mean of the ratio to the initial volume ($n=3-4$).

high concentration was used for the purpose of avoiding the influence of propylene glycol on the antitumor effect. However, since the aqueous solubility of EB-glu-MMC is very low, the administered EB-glu-MMC may precipitate more in the peritoneal cavity or surrounding tissues because of the dilution of propylene glycol by the aqueous body fluid. For example, although both conjugates showed a similar MMC rate in the dissolved form in the buffer, pH 7.4, containing 50% propylene glycol,^{11,12} E-glu-MMC exhibited a release rate more than 10 times faster than EB-glu-MMC under the suspension condition in the buffer, pH 7.4, containing 10% propylene glycol; the latter phenomena are not shown here but will be described in another report. This suggests that E-glu-MMC should show better release under a suspension condition in 10% propylene glycol because of its higher solubility; however, the aqueous solubility of each conjugate was not determined due to the extreme low value and to its adsorption to the filtration membrane. The solubility will have to be solved correctly in the future. Thus, it is supposed that EB-glu-MMC should precipitate more easily to a greater extent from the higher concentration solution than from the lower concentration one. This should be true in E-glu-MMC, but the influence of the change in solubility may be smaller in E-glu-MMC. Consequently, EB-glu-MMC administered at 5 mg/ml (15–20 mg eq MMC/kg) might not produce a significant effect and the significant difference in the survival time was not observed among 10, 15 and 20 mg eq MMC/kg ($p>0.3$). Simultaneously, the drug release rate should be lowered very much by such precipitation, and the much slower release should make the drug effect lower. Actually, although the kind of tumor is different, in the i.p. treatment of Ehrlich ascites tumor, even when the total dose of MMC is higher, consecutive administration was reported to be less effective compared with intermittent administration.¹⁹ Such a consideration is also supported by the fact that the suspended dosage form in the buffer, pH 7.4, containing 10% propylene glycol exhibited much lower *ILS* values, which will be described in another paper (data not shown in this report). The body weight changed slightly (Fig. 2), which indicated the low toxic side effect of EB-glu-MMC at 15–20 mg eq MMC/kg. This also suggests that the small values of *ILS* at

15–20 mg eq MMC/kg should not be due to the toxic side effect.

Antitumor Effect against Subcutaneous Sarcoma 180 Solid Tumor after I.P. Administration At 4 d after s.c. inoculation of Sarcoma 180, MMC, EB-glu-MMC and E-glu-MMC were administered intraperitoneally at a single dose as a clear solution in propylene glycol. The tumor growth profiles after administration are shown in Fig. 3. MMC produced marked tumor growth inhibition at the dose of 5 mg/kg. Although it exhibited extensive inhibition at the dose of 10 mg/kg, the mice experienced a lethally toxic side effect as seen in the tests against P388 leukemia. The toxic side effect was recognized from the decrease in body weight (Fig. 4). The effect of E-glu-MMC increased as the dose was raised. Especially, it caused similar or greater tumor growth inhibition at the dose of 30 mg eq MMC/kg compared with MMC at the dose of 5 mg/kg. E-glu-MMC was found to act very effectively at a high dose. The toxic side effect was not observed from the change in body weight. Since the plasma levels of E-glu-MMC and MMC were achieved to a certain extent in the i.p. administration of E-glu-MMC as stated above, the high dose of E-glu-MMC might permit the drugs to transfer well to the sites of Sarcoma 180 solid tumor located apart from the administered site. The long retention of plasma concentration indicated the sustained supply of the drugs to the tumor, which was considered to cause marked tumor growth inhibition. However, it is not necessarily easy to explain how the retention effect of the conjugate influences the efficacy. Although the kind of tumor is different, according to the literature using Ehrlich ascites and solid tumors,¹⁹ the consecutive administration of MMC tends to lower the effect more than the intermittent administration of MMC. As to the i.v. treatment of Ehrlich solid tumor, the higher total dose of MMC seems to be required to obtain a similar or better effect in the consecutive administration compared with that in the intermittent administration. Therefore, characteristics other than the drug release rate, such as the lipophilicity of the conjugate or tumor localization, may also have to be considered in order to clear the drug action. The influence of the drug concentration of the administered sample on efficacy was considered as follows: In the study of

Fig. 4. Change in Mean Body Weight of Mice Bearing Sarcoma 180 Solid Tumor Subcutaneously after I.P. Administration of MMC (A), EB-glu-MMC (B) and E-glu-MMC (C)

(A) MMC control ($\diamond$), 2.5 mg/kg ($\blacktriangle$), 5 mg/kg ($\bullet$), 10 mg/kg ($\blacksquare$); (B) EB-glu-MMC control ($\square$), 5 mg eq MMC/kg ($\blacklozenge$), 10 mg eq MMC/kg ($\blacktriangle$), 20 mg eq MMC/kg ($\bullet$), 30 mg eq MMC/kg ($\blacksquare$); (C) E-glu-MMC control for 5–20 mg eq MMC/kg ($\diamond$), control for 30 mg eq MMC/kg ($\square$), 5 mg eq MMC/kg ($\blacklozenge$), 10 mg eq MMC/kg ($\blacktriangle$), 20 mg eq MMC/kg ($\bullet$), 30 mg eq MMC/kg ($\blacksquare$). The horizontal axis is time (d) after inoculation. The initial body weight means the weight measured just before administration. Each point represents the mean value ($n=3-4$).

effect against P388 leukemia, the drug concentration of the administered sample seemed not to influence the efficacy of E-glu-MMC as much as that of EB-glu-MMC. Therefore, although the drug concentration of the administered samples of E-glu-MMC was different among the doses, the influence of the concentration on efficacy was not considered marked.

EB-glu-MMC was dissolved in propylene glycol and administered at an equivalent concentration for all the doses in order to avoid the dependency of concentration on efficacy, which was observed in the treatment of intraperitoneal P388 leukemia. Thus, in this treatment by EB-glu-MMC against Sarcoma 180 solid tumor, the dependency of the concentration on efficacy may be relatively avoided. The tumor growth inhibitory profile of EB-glu-MMC was similar to that of E-glu-MMC. At the dose of 30 mg eq MMC/kg, EB-glu-MMC tended to show a superior effect to that of E-glu-MMC. Namely, the tumor volume was recognized to decrease from 10 d after administration. The dose of 30 mg eq MMC/kg was considered to cause the distribution of a considerable amount of EB-glu-MMC in the peritoneal cavity or the surrounding tissues, and to supply the drugs for a long period. Thus, EB-glu-MMC produced a marked growth inhibitory effect against the tumor located distant from the administered site. This consideration is supported by the results of the examination of the plasma level after the i.p. administration. Although the sustained systemic drug supply surely causes a marked drug action against Sarcoma 180 solid tumor, the interpretation is not necessarily easy, as stated above regarding E-glu-MMC. Namely, the localization of the conjugate to the tumor may also have to be considered. As stated in the following section, total exposure by the drug, which is closely related to the *AUC* of MMC, seems to the effect against Sarcoma 180 solid tumor. Only a scarce toxic side effect was found according to the change in body weight. These suggested that EB-glu-MMC should exhibit good efficacy against the Sarcoma 180 solid tumor.

In the i.p. treatment stated above, a relatively higher effect of the conjugates against Sarcoma 180 may be obtained when compared with that against P388 leukemia. Although

the type of tumor is different, the consecutive i.p. injection of MMC was reported not to be so effective against Ehrlich ascites tumor even when the total dose was higher, but the consecutive i.v. administration of MMC reportedly showed a higher effect against Ehrlich solid tumor when the total dose was increased¹⁹; that is, the total dose seems to be important for the effect against the solid tumor. Similarly, in this study, the conjugates gave a better effect against the Sarcoma 180 solid tumor at a higher dose. At that time, considering the *AUC* of MMC, the extent of the *AUC* of MMC may play an important role in its antitumor effect against the Sarcoma 180 solid tumor. For example, although the clearance of MMC is known to be fairly dose-dependent,²⁰ simple calculation based on Table 1 suggests that a several-fold dose should be required in the i.p. administration of the conjugate to obtain the equivalent *AUC* value in the i.p. injection of free MMC. Actually, although the administration condition in the antitumor test was rather different from that in the plasma concentration measurement, when compared with free MMC at 5 mg/kg, the conjugate showed a similar or better effect against Sarcoma 180 solid tumor at a 6-fold dose, 30 mg eq MMC/kg. Thus, the *AUC* value of MMC is considered to be one of the important factors for the effect against Sarcoma 180 solid tumor; however, in order to evaluate the effect in more detail, factors other than pharmacokinetics in plasma, such as tissue distribution, will have to be further examined.

In the present study, E-glu-MMC and EB-glu-MMC, administered intraperitoneally in a solution form, were demonstrated to transfer to systemic circulation to a fair extent and to act effectively on general and popular inoculated tumors, especially the Sarcoma 180 solid tumor existing apart from the administered site. Good retention of each conjugate in the body is considered to be due to their low aqueous solubility and high lipophilicity. The good transfer of the conjugates to the systemic circulation and their sustained supply may be related to the high efficacy against Sarcoma 180 solid tumor. This work suggested the ability for the two synthesized conjugates to work effectively against the general tumors, especially a solid tumor. In order to grasp the detailed mechanism

of the effect against the solid tumor, it will be important to elucidate the tissue distribution or localization characteristics of both conjugates. Moreover, such a biodistribution study will also be needed to make clear the mechanism of the effect against the estradiol-ruled and estradiol-non-ruled tumors.

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