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Evaluation of antitumor and toxic side effects of mitomycin C–estradiol conjugates

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Abstract

The antitumor and toxic side effects of mitomycin C–estradiol conjugates (EB-glu-MMC and E-glu-MMC) were evaluated in detail for solutions in propylene glycol and suspensions in 10% (v/v) propylene glycol. Tumor growth, body weight and number of leukocytes were examined after i.p. administration to sarcoma 180 solid tumor-bearing mice. Body weight and number of leukocytes were also examined in normal mice after i.p. administration of the solution. In solution dosage forms, the two conjugates had almost the same suppressive effect on tumor growth at 30 mg MMC eq./kg as MMC at 5 mg/kg, did not lower body weight significantly, but reduced the number of leukocytes at 30 mg MMC eq./kg. MMC, lethally toxic at 10 mg, significantly lowered the body weight and leukocyte number. In the suspension dosage forms, these conjugates had a greater suppressive effect on tumor growth at 50 mg MMC eq./kg than MMC at 5 mg/kg, and reduced the body weight and leukocyte number, with E-glu-MMC more toxic than EB-glu-MMC. The presence of the tumor itself influenced the body weight and leukocyte number. However, toxic side effects could be evaluated from the body weight and leukocyte number to almost the same extent between tumor-bearing and normal mice.
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Keywords: Mitomycin C–estradiol conjugate; Antitumor and toxic side effect; Tumor growth; Body weight; Leukocyte number

1. Introduction

As many antitumor agents lack tumor selectivity, a large dose is needed to achieve a therapeutic effect. However, a high dose often causes toxic side effects, which limits the use of the agent. The targeting of antitumor agents to tumor sites or surrounding tissues is one approach to overcoming their drawbacks. Hormone-dependent tumors possess a hormone receptor, and anti-hormonal agents or hormone–drug conjugates have been developed for treatment. Anti-estradiol agents, one anti-hormonal agent form, suppress the binding of estradiol to its receptor and its function; some are already clinically available (Sutherland et al., 1980; Benz et al., 1983; Malet et al., 1988; Buzdar and Hortobagyi, 1998; Ellmen et al., 2000; Brigger et al., 2001; Chawla and Amiji, 2002). In addition, hormone–drug conjugates have been developed for the targeting of antitumor agents to malignant tissues (Jimbow et al., 1984; Ohsawa et al., 1984; Varga, 1985; Kuduk et al., 2000; Kasiotis et al., 2001). These conjugates can be selectively delivered to hormone-dependent tumors through the specific binding of hormonal ligands to the receptor, which is the basic concept behind hormone–drug conjugates. The utilization of steroid

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hormones as homing ligands has been actively studied. Estramustine phosphate disodium, a conjugate of nitrogen mustard and estradiol disodium, is delivered selectively to prostate tumor via a specific estramustine binding protein, leading to good efficacy (Mittelman et al., 1976; Ozaki et al., 1980). Although, bestarbucil, a conjugate of chlorambucil and estradiol benzoate, hardly showed specific binding to the estrogen receptor (Wada et al., 1986), it localizes well to many solid tumors, resulting in a good antitumor effect and low toxic side effect (Tamura et al., 1986). Conjugation with estradiol and its analogues, therefore, might be useful to improve the pharmacokinetic properties of anticancer agents separately from the specific delivery to the estradiol receptor. Thus, we synthesized the conjugates of MMC with estradiol benzoate and estradiol via glutaric acid, abbreviated to EB-glu-MMC and E-glu-MMC, respectively, and investigated their possible usefulness in vitro and in vivo. These conjugates worked as prodrugs of mitomycin C, and showed reduced binding affinity for the estrogen receptor (Ishiki et al., 1997a, 1997b). E-glu-MMC had a relatively strong antitumor effect on murine P-388 ascitic tumor in the i.p.–i.p. system, while the antitumor activity of EB-glu-MMC against murine P-388 ascitic tumor was very weak in the same system (Ishiki et al., 1998). The low aqueous solubility of EB-glu-MMC, leading to a slow conversion from EB-glu-MMC to MMC in ascitic fluid, was considered to be associated with the poor efficacy. On the other hand, E-glu-MMC and EB-glu-MMC were highly potent against sarcoma 180 solid tumor in the s.c.–i.p. system (Ishiki et al., 1998, 2002). This finding might be due to pharmacokinetics of the conjugates; that is, the lipophilic properties of the conjugates might be adequate for the treatment of solid tumors. This needs to be clarified by examining the biodistribution characteristics of the conjugates. In studies having been performed for the conjugates, the relation between efficacy and toxic side effects were not necessarily clear. In particular, their toxic side effects have been evaluated based only on a decrease in body weight. For a more detailed evaluation of toxic side effects, other methods such as an investigation of the leukocyte number are needed. Myelosuppression and leukopenia are known as major toxic side effects of MMC (Kobayashi et al., 1981; Futamura and Matsumoto, 1995). Thus, in the present study, in order to evaluate the relation between efficacy and toxic side effects in detail, antitumor effects against sarcoma 180 solid tumor were examined using solution and suspension dosage forms, and simultaneously, toxic side effects were investigated based on change in body weight and reduction in the leukocyte number in peripheral blood vessels. Also, the body weight and leukocyte number were monitored after i.p. administration of the conjugates in solution to normal mice. Furthermore, biodistribution was examined after i.p. administration of EB-glu-MMC to sarcoma 180 solid tumor-bearing mice.

2. Materials and methods

2.1. Materials

Mitomycin Kyowa S (Kyowa Hakko Kogyo Co., Japan), which is composed of mitomycin C (MMC) and sodium chloride, was used to obtain MMC. MMC was extracted with tetrahydrofuran, and used in the following experiment. Estradiol benzoate, estradiol and Turk's solution were purchased from Wako Pure Chemical Industries, Ltd., Japan. The conjugates of MMC with estradiol benzoate and estradiol (EB-glu-MMC and E-glu-MMC, respectively) were synthesized as described previously. Briefly, glutaric anhydride was reacted with estradiol benzoate and estradiol to give 4-[3-bezoyloxy-1,3,5(10)-estratrien-17β-oxycarbonyl]butyric acid (EB-glu) and 4-[3-hydroxy-1,3,5(10)-estratrien-17β-oxycarbonyl]butyric acid (E-glu), respectively. EB-glu-MMC and E-glu-MMC were obtained by the condensation of MMC with EB-glu and E-glu, respectively, at the 1a-N position of MMC using carbonyldiimidazole. All other chemicals were of reagent grade.

2.2. Animals and tumor

Male and female ddY mice (6-week-old) weighing 26–29 g were purchased from Saitama Experimental Animal Supply Co., Japan. The animals were kept on the breeding diet MF (Oriental Yeast, Japan) with water ad libitum at $23 \pm 1\,^{\circ}C$ and a relative humidity of $60 \pm 5\%$. The experimental protocol was approved by the Committee on Animal Research of Hoshi University, Tokyo, Japan, and the animal experiments were

performed in compliance with Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan.

Sarcoma 180 cells, kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, were maintained by weekly intraperitoneal transplantation of 1×10^6 sarcoma 180 cells suspended in Hanks' balanced solution (0.1 ml) per male ddY mouse. In the in vivo antitumor experiment, 1×10^7 sarcoma 180 cells, obtained from the above tumor-bearing male ddY mice, were suspended in Hanks' balanced solution (0.1 ml), and inoculated subcutaneously per female ddY mouse in the axillary region. Female mice were used in all experiments on antitumor and/or toxic side effects.

2.3. Drug preparations for administration

2.3.1. Solution dosage form

MMC was dissolved in propylene glycol at a concentration of 5 mg/ml. EB-glu-MMC and E-glu-MMC were dissolved in propylene glycol at 20 mg/ml. The MMC solution was administered intraperitoneally to mice at 5 and 10 mg/kg. The solutions of EB-glu-MMC and E-glu-MMC were administered intraperitoneally to mice at 10 and 30 mg MMC eq./kg. As a control, each mouse received propylene glycol (0.1 ml) intraperitoneally.

2.3.2. Suspension dosage form

MMC was dissolved at a concentration of 2 mg/ml in a 10% (v/v) propylene glycol solution in saline, and injected intraperitoneally at doses of 2.5 and 5 mg/kg. EB-glu-MMC and E-glu-MMC were suspended at 10 mg/ml in a 10% (v/v) propylene glycol solution in saline by homogenization using a glass homogenizer with a Teflon pestle, and administered intraperitoneally at doses of 25, 50, and 75 mg MMC eq./kg. As a control, 0.22 ml of a 10% (v/v) propylene glycol solution in saline was injected intraperitoneally per mouse.

2.4. Antitumor tests

Each drug was administered intraperitoneally at 4 days after inoculation, and tumor volume was monitored. The length (L, cm) of the longest tumor axis and the length (W, cm) of the axis vertical to the longest axis (width) were measured with slide calipers, to obtain the tumor volume (V, cm^3), which was calculated using the following equation (Takakura et al., 1987):

$$V = \frac{L \times W^2}{2} \tag{1}$$

The tumor volume immediately before administration was used as the initial tumor volume, and the tumor growth ratio was calculated as a ratio of the tumor volume to the initial volume. Further, taking the mean tumor growth ratios of the control and treated groups at a given day after administration as $G(C)$ and $G(T)$, respectively, tumor growth inhibition was calculated using the following equation (Tokunaga et al., 1988):

$$\text{Tumor growth inhibition (\%)} = \left\{ \frac{G(C) - G(T)}{G(C)} \right\} \times 100 \tag{2}$$

2.5. Measurement of body weight and number of leukocytes after i.p. administration in tumor-bearing mice

The body weight and the number of leukocytes were monitored during the antitumor tests. The body weight immediately before administration was used as the initial body weight. Change in body weight was calculated as a ratio to the body weight to the initial weight. A blood sample (10 μl) was taken via the tail vein at 4, 11 and 18 days post-inoculation, that is, at 0, 7 and 14 days after administration. The blood sample and Turk's solution were mixed at a ratio of 1:9 (v/v), and number of leukocytes per 0.1 μl of the mixture was counted in four different areas of the plate using a Burker–Turk hemocytometer. The number of leukocytes per μl of the blood was then obtained by multiplying the mean value by 100.

2.6. Measurement of body weight and number of leukocytes after i.p. administration in normal mice

After normal mice received the solution dosage forms intraperitoneally, the body weight and the number of leukocytes were monitored. The body weight immediately before administration was used as the initial body weight. Blood sampling, its treatment and

count of leukocyte number were performed in the same manner as in described in tumor-bearing mice.

2.7. Biodistribution

After female mice were inoculated with sarcoma 180 cells in the same manner as described in antitumor tests, EB-glu-MMC suspended in a 10% (v/v) propylene glycol solution in saline at 10 mg/ml was injected intraperitoneally at a dose of 10 mg MMC eq./kg at 10 days post-inoculation. The mice were sacrificed by decapitation at 1, 3 and 6 h after administration, and blood, liver, heart, kidney, lung, spleen, thymus, ovary, uterus, muscle and tumor were taken. Plasma was obtained by centrifugation of the blood at 3000 rpm for 10 min. One-fifteenth M phosphate buffer, pH 7.4, was added to the liver at half the volume of the liver, and to the thymus at 10-fold volume of the thymus. For other tissues, the phosphate buffer was added at three-fold the volume of each tissue. The mixture was homogenized using a glass homogenizer with a Teflon pestle. Four hundred microliters of 0.1 M phosphate buffer, pH 9.0, was mixed with 100 μl of the plasma or homogenate. To the mixture, 5 ml of a mixture of chloroform and methanol (1:1, v/v) was added, shaken vigorously and centrifuged at 3000 rpm for 10 min. The whole supernatant was transferred to a glass tube, and evaporated to dryness at 35 °C under nitrogen gas. The residue was dissolved in 100 μl of methanol, and 20 μl of the solution was analyzed by high performance liquid chromatography (HPLC). The recovery ratio from each tissue was determined by adding a specified amount of MMC, EB-glu-MMC or E-glu-MMC to fresh plasma or tissue homogenate taken from untreated mice, dealing with the samples as above, and analyzing the final sample in the same manner as described in the test sample. As a result, MMC, EB-glu-MMC and E-glu-MMC were completely recovered.

2.8. HPLC assay

HPLC analysis was performed at room temperature using a Shimadzu LC-6A with a Shimadzu SPD-6A detector set at 364 nm. The reverse-phase column (SUMIPAX Nucleosil $5C_{18}$, 4 mm ϕ × 250 mm), connected to a guard column (Applied Biosystems RP-18 NEW GUARD 7 μm, 3.2 mm ϕ × 15 mm), was adopted as an analytical column. Mixtures of 0.1 M phosphate buffer, 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 analyses of MMC, EB-glu-MMC and E-glu-MMC, respectively.

2.9. Statistical analysis

Statistical analyses were performed using the unpaired *t*-test. Differences were considered statistically significant at $P < 0.05$.

Table 1
Tumor growth ratio and inhibition of solution of EB-glu-MMC, E-glu-MMC and MMC in 100% propylene glycol in sarcoma 180 solid tumor-bearing mice

Substance	Dose (mg MMC eq./kg)	Tumor growth ratio after inoculation[a] (mean ± S.E.)			Tumor growth inhibition at 18 days post-inoculation (%)
		4 days[b]	11 days	18 days	
Control	–	1 ± 0	17.2 ± 4.9	46.9 ± 13.0	0
EB-glu-MMC	10	1 ± 0	23.1 ± 8.1	35.1 ± 14.4	25.0
	30	1 ± 0	10.8 ± 1.5	15.6 ± 2.6	66.8
E-glu-MMC	10	1 ± 0	18.9 ± 5.4	42.9 ± 12.6	8.4
	30	1 ± 0	10.7 ± 2.2	20.5 ± 1.7	56.2
MMC	5	1 ± 0	7.3 ± 1.6	17.0 ± 2.2	63.4
	10	1 ± 0	7.4 ± 2.9	–	–

[a] $n = 4$ for 4, 11 and 18 days except for MMC (10 mg/kg) at 18 days.
[b] As this is an initial day, the tumor growth ratio is 1.

3. Results

3.1. Tumor growth inhibition, change in body weight and reduction in leukocyte number after i.p. administration of solution dosage forms to tumor-bearing mice

Table 1 shows tumor growth and its inhibition after i.p. administration of the solution dosage forms. EB-glu-MMC and E-glu-MMC suppressed the tumor growth dependent on the dose, and showed the tumor growth inhibitions of 67 and 56% at 30 mg MMC eq./kg, respectively. MMC showed the tumor growth inhibition of 64% at 5 mg/kg, and was lethally toxic at 10 mg/kg, in which every mouse died within 14 days after the inoculation. These results were consistent with previous findings (Ishiki et al., 1998). However, the difference in the tumor growth ratio was not significant between the tested and control groups.

Change in body weight and number of leukocytes, which were measured during the antitumor tests, are described in Figs. 1 and 2, respectively. For EB-glu-MMC and E-glu-MMC at 10 and 30 mg MMC eq./kg, the change in body weight was scarcely significant different from the control value. However, for EB-glu-MMC and E-glu-MMC at 30 mg MMC eq./kg, the number of leukocytes was significantly reduced as compared with the control. MMC at 5 mg/kg caused a significant decrease in body weight at 9–11 days post-inoculation as compared with the control, but not after that. Leukocyte number did not differ significantly between the mice given MMC at 5 mg/kg and the control. Following the administration of MMC at 10 mg/kg, a significant and rapid decrease in body weight occurred from 2 days post-administration until death. One mouse survived at 7 days post-administration of MMC at 10 mg/kg, when the number of leukocytes in this animal was only 1600 cells/μl.

3.2. Tumor growth inhibition, change in body weight change and reduction in leukocyte number after i.p. administration of suspension dosage forms to tumor-bearing mice

Table 2 shows the tumor growth ratio and tumor growth inhibition after administration of the suspension dosage forms. EB-glu-MMC significantly suppressed tumor growth at each dose. E-glu-MMC also

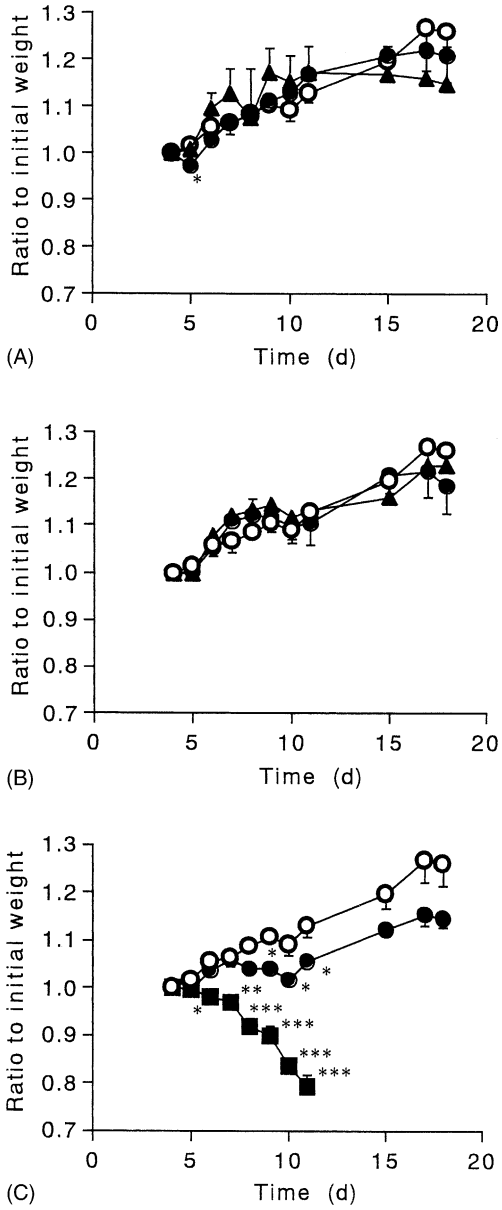

Fig. 1. Change in body weight in sarcoma 180 solid tumor-bearing mice after i.p. administration of solution dosage forms of EB-glu-MMC (A), E-glu-MMC (B) and MMC (C). (○) control; (▲) 10 mg MMC eq./kg; (●) 30 mg MMC eq./kg in (A) and (B). (○) control; (●) 5 mg/kg; (■) 10 mg/kg in (C). Each point represents the mean ± S.E. ($n = 3$–4). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control.

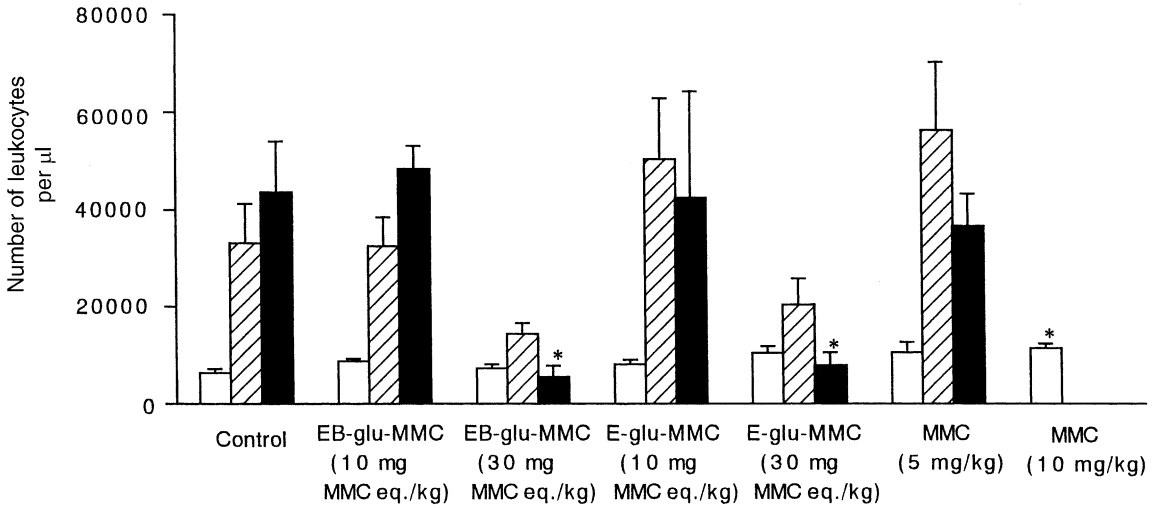

Fig. 2. Number of leukocytes in sarcoma 180 solid tumor-bearing mice after i.p. administration of solution dosage forms of EB-glu-MMC, E-glu-MMC and MMC. Open, hatched and closed columns show the values at 4, 11 and 18 days post-inoculation, respectively (0, 7 and 14 days post-administration, respectively). Each column represents the mean ± S.E. ($n = 3$–4). $^{*}P < 0.05$ vs. control.

inhibited tumor growth significantly except 25 mg MMC eq./kg at 18 days post-inoculation. MMC significantly suppressed tumor growth at 2.5 and 5 mg/kg. Tumor growth inhibitions by EB-glu-MMC at 18 days post-inoculation were 76 and 70% at 50 and 75 mg MMC eq./kg, respectively. E-glu-MMC showed the greatest tumor growth inhibition of 88% at 50 mg MMC eq./kg at 18 days post-inoculation. The tumor growth inhibitions by MMC at 18 days post-inoculation were 52 and 61% at 2.5 and 5 mg/kg, respectively.

Change in body weight and number of leukocytes, which were monitored during the antitumor tests, are shown in Figs. 3 and 4, respectively. EB-glu-MMC

Table 2
Tumor growth ratio and inhibition of suspension of EB-glu-MMC, E-glu-MMC and MMC in 10% aqueous propylene glycol in sarcoma 180 solid tumor-bearing mice

Substance	Dose (mg MMC eq./kg)	Tumor growth ratio after inoculation[a] (mean ± S.E.)			Tumor growth inhibition at 18 days post-inoculation (%)
		4 days[b]	11 days	18 days	
Control	–	1 ± 0	20.3 ± 1.1	42.6 ± 4.9	0
EB-glu-MMC	25	1 ± 0	14.4 ± 1.7*	–	–
	50	1 ± 0	10.7 ± 1.1***	13.3 ± 0.8**	76.2
	75	1 ± 0	10.2 ± 1.3**	12.4 ± 1.1***	69.6
E-glu-MMC	25	1 ± 0	15.9 ± 0.5*	41.4 ± 20.3	16.3
	50	1 ± 0	6.2 ± 0.7***	7.2 ± 1.4***	88.4
	75	1 ± 0	6.1 ± 0.8***	–	–
MMC	2.5	1 ± 0	11.6 ± 1.8**	22.7 ± 1.8**	52.0
	5	1 ± 0	6.8 ± 1.2***	19.2 ± 4.5*	61.1

[a] $n = 4$ for 4 and 11 days except for E-glu-MMC at 75 mg MMC eq./kg ($n = 3$). At 18 days, $n = 4$ except for E-glu-MMC at 50 and 75 mg MMC eq./kg ($n = 3$) and E-glu-MMC at 50 mg MMC eq./kg ($n = 3$).
[b] As this is an initial day, the tumor growth ratio is 1.

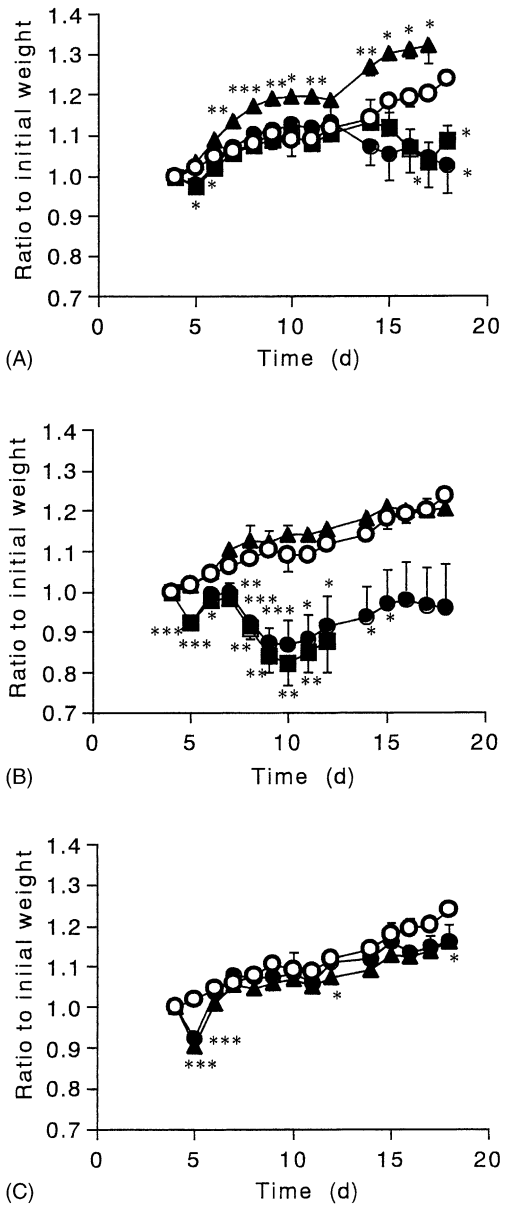

Fig. 3. Change in body weight in sarcoma 180 solid tumor-bearing mice after i.p. administration of suspension dosage forms of EB-glu-MMC (A), E-glu-MMC (B) and MMC (C). (○) control; (▲) 25 mg MMC eq./kg; (●) 50 mg MMC eq./kg; (■) 75 mg MMC eq./kg in (A) and (B). (○) control; (▲) 2.5 mg MMC eq./kg; (●) 5 mg/kg in (C). Each point represents the mean ± S.E. ($n = 3$–4). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control.

caused a significant decrease in body weight in the later period (17–18 days post-inoculation) at 50 and 75 mg MMC eq./kg; body weight was rather high after administration of EB-glu-MMC at 25 mg MMC eq./kg. EB-glu-MMC reduced the number of leukocytes at 50 and 75 mg MMC eq./kg. E-glu-MMC did not lower the body weight significantly at 25 mg MMC eq./kg, but caused a significant decrease in body weight over almost the entire observation period at 50 and 75 mg MMC eq./kg. E-glu-MMC reduced the number of leukocytes at 18 days post-inoculation even at 25 mg MMC eq./kg. MMC did not significantly lower the number of leukocytes at 2.5 and 5 mg/kg.

3.3. Change in body weight and number of leukocytes in administration of solution dosage forms to normal mice

The toxic side effects of EB-glu-MMC, E-glu-MMC and MMC were examined by monitoring change in body weight and number of leukocytes after i.p. injection of solution dosage forms to normal mice. The results are shown in Figs. 5 and 6. The body weight increased gradually in the control. Number of leukocytes in the control did not change significantly during the observation period. EB-glu-MMC and E-glu-MMC caused no decrease in body weight at each dose. EB-glu-MMC actually increased body weight, as did E-glu-MMC at a low dose. EB-glu-MMC and E-glu-MMC did not significantly reduce the number of leukocytes at each dose at 7 days post-administration but significantly lowered the number of leukocytes at 30 mg MMC eq./kg at 14 days post-administration. MMC caused no decrease in body weight at 5 mg/kg, but significantly reduced the body weight from 4 days post-administration at 10 mg/kg. Three of four mice died within 11 days post-administration of MMC at 10 mg/kg. MMC did not cause a significant decrease in number of leukocytes at 5 mg/kg. MMC exhibited lethal toxicity at 10 mg/kg, and only one mouse remained alive at 7 days post-administration, when the number of leukocytes in this mouse was just 980 cells/μl. For all the administrations except MMC at 10 mg/kg, all mice survived until at least 26 days post-administration.

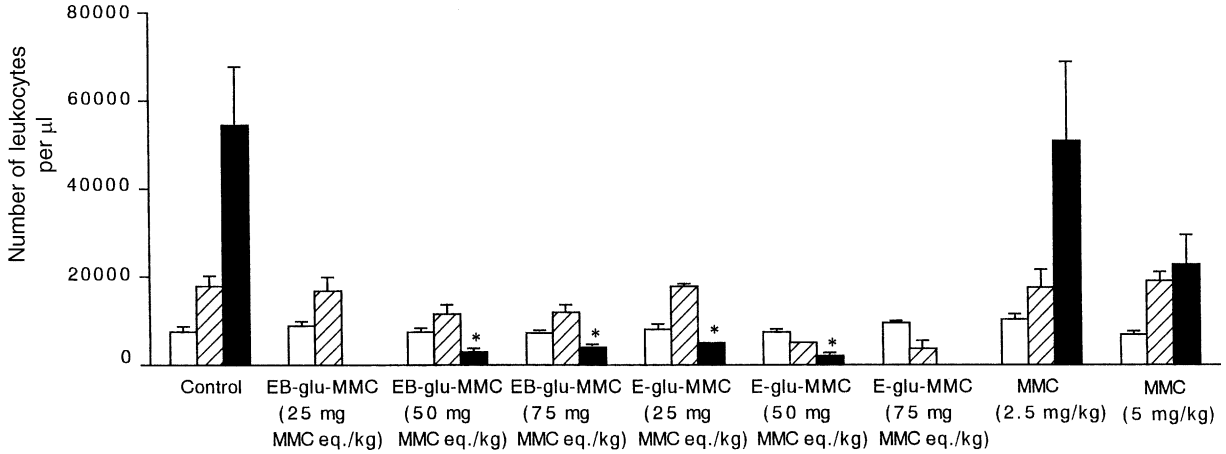

Fig. 4. Number of leukocytes in sarcoma 180 solid tumor-bearing mice after i.p. administration of suspension dosage forms of EB-glu-MMC, E-glu-MMC and MMC. Open, hatched and closed columns show the values at 4, 11 and 18 days post-inoculation, respectively (0, 7 and 14 days post-administration, respectively). Each column represents the mean ± S.E. ($n = 3$–4). $^*P < 0.05$ vs. control.

3.4. Biodistribution after i.p. administration of EB-glu-MMC suspension

The distribution of EB-glu-MMC, E-glu-MMC and MMC was investigated after i.p. administration of EB-glu-MMC suspension at 10 mg MMC eq./kg in 10% (v/v) propylene glycol in saline. Their plasma levels were very low. EB-glu-MMC exhibited a maximal plasma level of 0.04 μg/ml (mean) at 3 h post-administration, while E-glu-MMC and MMC were detected at levels of 0.01 and 0.04 μg/ml (mean), respectively, only at 1 h post-administration. Fig. 7 shows tissue distribution profiles of each compound at 1, 3 and 6 h post-administration. EB-glu-MMC was detected to the greatest extent at 3 h post-administration; in particular, it was observed in spleen, lung, thymus, uterus and kidney at concentrations ranging from several μg to 10 μg/g of tissue. The distribution of the drugs to the liver, heart, tumor and ovary was relatively limited. The MMC level was detected highest in spleen through the studies.

4. Discussion

In solution dosage forms in propylene glycol, EB-glu-MMC and E-glu-MMC at 30 mg MMC eq./kg showed a similar tumor growth inhibition to MMC at 5 mg/kg. However, all the compounds were not significantly effective as compared with the control, probably due to the large variance in the tumor growth ratio of the control (Table 1). At a later stage after administration, EB-glu-MMC and E-glu-MMC tended to exhibit a lower increase in body weight than the control (Fig. 1). MMC exhibited a significant decrease in body weight at 5 mg/kg at 9–11 days post-inoculation. The tumor growth ratio and change in body weight suggest that EB-glu-MMC and E-glu-MMC should be almost as effective and toxic at 30 mg MMC eq./kg as MMC at 5 mg/kg. On the other hand, EB-glu-MMC and E-glu-MMC caused a significant reduction in the number of leukocytes at 30 mg MMC eq./kg at 18 days post-inoculation, while MMC did not lower leukocyte number significantly at 5 mg/kg (Fig. 2). These results suggest that toxic side effect on number of leukocytes might be greater for EB-glu-MMC and E-glu-MMC at 30 mg MMC eq./kg than MMC at 5 mg/kg, though overall toxicities shown by a decrease in body weight were similar among the preparations.

In suspension dosage forms, EB-glu-MMC, E-glu-MMC and MMC significantly suppressed tumor growth at each dose. Significant difference was found in suspension dosage forms though it was not ob-

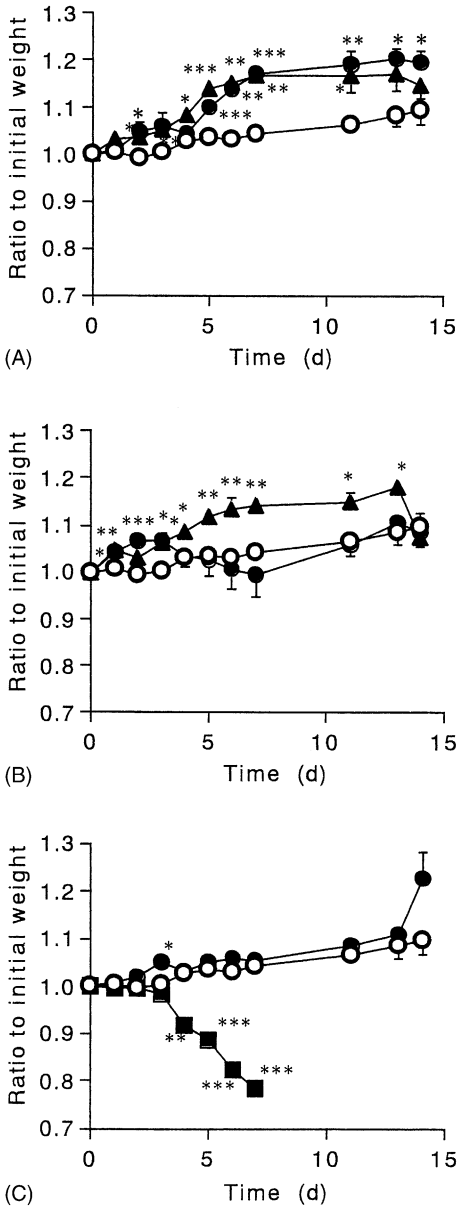

Fig. 5. Change in body weight in normal mice after i.p. administration of solution dosage forms of EB-glu-MMC (A), E-glu-MMC (B) and MMC (C). (○) control; (▲) 10 mg MMC eq./kg; (●) 30 mg MMC eq./kg in (A) and (B). (○) control; (●) 5 mg/kg; (■) 10 mg/kg in (C). Each point represents the mean ± S.E. (n = 3–4). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

tained in solution dosage forms; probably because the variance in the tumor growth ratio of the control was much smaller in suspension dosage forms than in solution dosage forms (Tables 1 and 2). EB-glu-MMC and E-glu-MMC exhibited greater tumor growth inhibition at 50 mg MMC eq./kg than MMC at 5 mg/kg, and E-glu-MMC appeared to be more effective than EB-glu-MMC. EB-glu-MMC did not lower but rather raised body weight at 25 mg MMC eq./kg, and caused a decrease in body weight at 50 and 75 mg MMC eq./kg only in the later period (Fig. 3). E-glu-MMC little affected body weight at 25 mg MMC eq./kg, but lowered it markedly at 50 and 75 mg MMC eq./kg (Fig. 3). These profiles of change in body weight indicated that E-glu-MMC suspension would be more toxic than EB-glu-MMC suspension. Actually, three of four mice died within 12 days post-administration of E-glu-MMC at 75 mg MMC eq./kg. As reported previously, E-glu-MMC releases MMC faster than EB-glu-MMC in suspension; probably because E-glu-MMC is more water-soluble than EB-glu-MMC (Ishiki et al., 2002). This appears to be related to the fact that, in suspension dosage form, E-glu-MMC is more toxic than EB-glu-MMC. EB-glu-MMC and E-glu-MMC reduced the number of leukocytes at 50 and 75 mg MMC eq./kg (Fig. 4), and the reduction tended to be greater in E-glu-MMC than in EB-glu-MMC. For MMC at 2.5 and 5 mg/kg, the body weight decreased significantly immediately after administration, but recovered soon to the control level. MMC did not cause a significant decrease in number of leukocytes. Thus, for all the preparations, the decrease in body weight almost paralleled the reduction in number of leukocytes. However, for E-glu-MMC at 25 mg MMC eq./kg, the body weight was not significantly lowered, but the number of leukocytes was reduced significantly at 18 days post-inoculation. These results suggested that number of leukocytes might be a more sensitive indicator of toxic side effects than body weight.

In normal mice, EB-glu-MMC and E-glu-MMC solution in propylene glycol exhibited no significant decrease in body weight at 10 and 30 mg MMC eq./kg (Fig. 5). For MMC, the body weight did not decrease significantly at 5 mg/kg, but did so at 10 mg/kg. The number of leukocytes was not reduced significantly at 7 days post-administration by any preparation

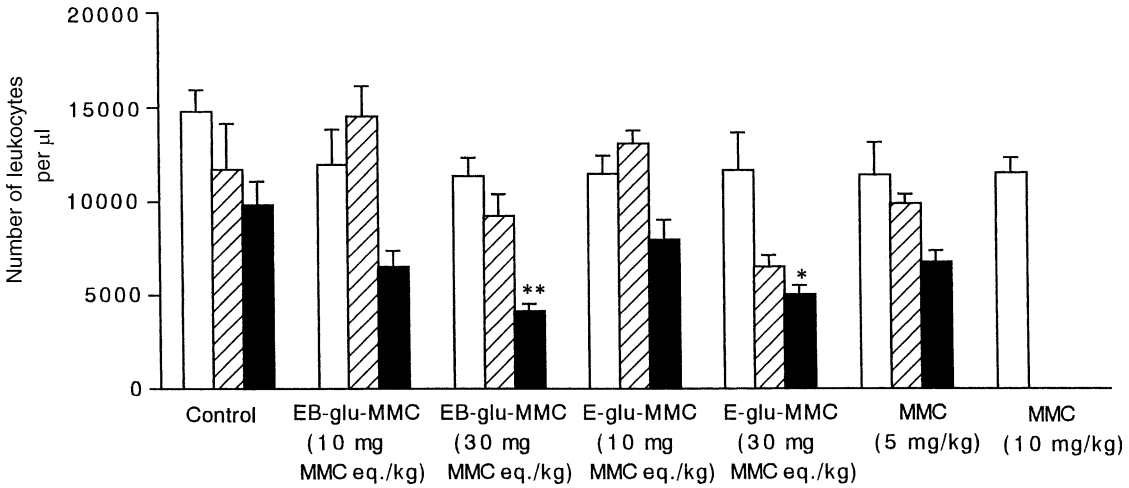

Fig. 6. Number of leukocytes in normal mice after i.p. administration of solution dosage forms of EB-glu-MMC, E-glu-MMC and MMC. Open, hatched and closed columns show the values at 0, 7 and 14 days post-administration, respectively. Each column represents the mean ± S.E. ($n = 3$–4). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control.

except for MMC at 10 mg/kg (Fig. 6). At 14 days post-administration, number of leukocytes was lowered significantly by EB-glu-MMC and E-glu-MMC at 30 mg/kg. MMC did not reduce the number of leukocytes significantly at 5 mg/kg. These results were similar to those obtained in tumor-bearing mice.

The distribution of a drug in the body is important to any discussion of efficacy and toxicity. As reported previously (Ishiki et al., 1998), when EB-glu-MMC was administered intraperitoneally to rats at 5 mg MMC eq./kg as a solution dosage form in propylene glycol, the maximal plasma levels of EB-glu-MMC, E-glu-MMC and MMC were 0.3 μg/ml at 0.9 h, 0.14 μg/ml at 2.3 h, and 0.14 μg/ml at 2.7 h after administration, respectively. As compared with these results, the present plasma levels after i.p. administration of EB-glu-MMC suspension at 10 mg MMC eq./kg to tumor-bearing mice were much lower. This low plasma levels of drugs might be related to the reduced toxicity of the suspension dosage form. However, EB-glu-MMC and E-glu-MMC were found in spleen, lung, thymus, uterus and kidney at the concentrations of several μg to 10 μg/g tissue (Fig. 7). The tissue distribution of MMC after i.v. administration of MMC itself to sarcoma 180-bearing mice had already been reported by Fujita (1982). That is, after i.v. administration of MMC at 8 mg/kg to sarcoma 180-bearing mice, MMC was eliminated quickly, and distributed in plasma and tissues to a slight extent at 1 h post-administration, when the concentration of MMC was less than 0.3 μg/ml even in lung and kidney showing a higher biodistribution. Therefore, EB-glu-MMC and/or E-glu-MMC were considered to localize MMC to the above tissues, especially lymphatic tissues. However, the elimination of EB-glu-MMC and E-glu-MMC from the tissues appeared to be fairly fast because their levels in each tissue were very low at 6 h post-administration. EB-glu-MMC might transfer relatively easily to tissues because of its lipophilic characteristics and change fast into E-glu-MMC and/or MMC in the tissues. The higher level of EB-glu-MMC in the uterus will not be due to the receptor-mediated uptake, because the ability of EB-glu-MMC to bind estrogen receptors is very low (Ishiki et al., 1997b). This also suggests that EB-glu-MMC might be useful for localizing MMC to uterine tumor, because E-glu-MMC and MMC will be supplied by the conversion of EB-glu-MMC. Further, EB-glu-MMC and E-glu-MMC were observed at a higher level in the lymphatic tissues such as spleen and thymus, which might explain the fairly strong toxic side effect of EB-glu-MMC on number of leukocytes.

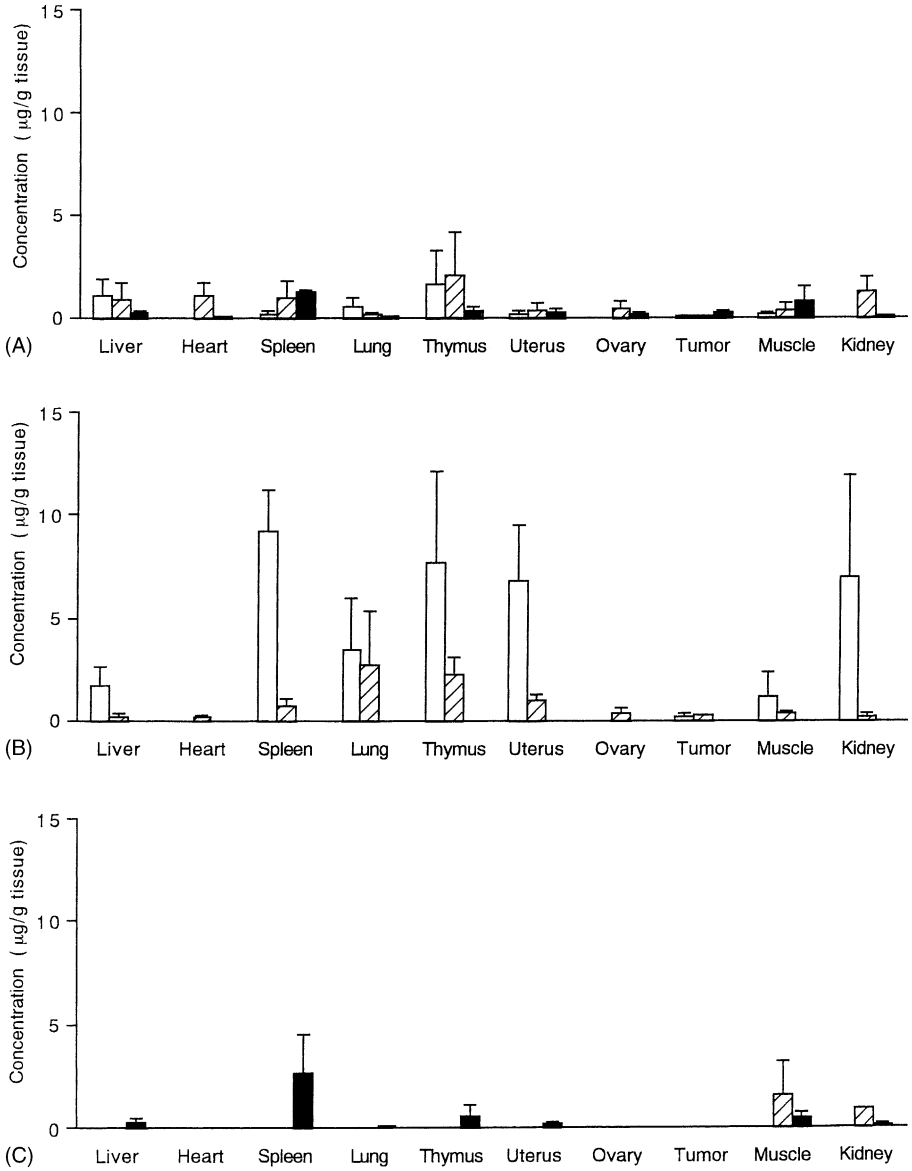

Fig. 7. Tissue distribution at 1 h (A), 3 h (B) and 6 h (C) after i.p. administration of EB-glu-MMC suspension to sarcoma 180 solid tumor-bearing mice. Administration was performed at 10 days post-inoculation. Open, hatched and closed columns show the concentrations of EB-glu-MMC, E-glu-MMC and MMC, respectively. Each column represents the mean ± S.E. ($n = 3$–4) except for EB-glu-MMC in kidney ($n = 2$) in (A), and for EB-glu-MMC and E-glu-MMC in uterus ($n = 2$), ovary ($n = 2$), muscle ($n = 2$) and kidney ($n = 1$) in (C). The results are expressed as the mean ± difference/2 when $n = 2$, and the result of one point is shown when $n = 1$.

5. Conclusion

The antitumor and toxic side effects of EB-glu-MMC and E-glu-MMC were examined. The toxic side effects were evaluated based on change in body weight and reduction in leukocyte number using sarcoma 180 solid tumor-bearing mice and normal mice. EB-glu-MMC and E-glu-MMC at 50 mg MMC eq./kg

were highly effective in suspension dosage forms, and exhibited greater inhibition of tumor growth than MMC at 5 mg/kg. However, they showed fairly strong toxic side effect at higher doses (50 and 75 mg MMC eq./kg). In both tumor-bearing and normal mice, the reduction in leukocyte number appeared to be slightly more sensitive as an indicator of toxic side effects than the decrease in body weight. The toxic side effects evaluated based on comparison of the body weight and number of leukocytes between tested and control groups were almost the same between tumor-bearing and normal mice. Namely, tumor growth hardly influenced the evaluation of toxic side effects based on decrease in body weight. Also, although the presence of the tumor itself increases the number of leukocytes due to the immune response, it was found not to prevent evaluation of the toxic side effects based on a reduction in number of leukocytes.

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