Antitumor Activities of Conjugates of Mitomycin C with Estradiol Benzoate and Estradiol via Glutaric Acid in Suspension Dosage Form

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Conjugates of mitomycin C (MMC) with estradiol benzoate and estradiol via glutaric acid (EB-glu-MMC and E-glu-MMC, respectively) were examined for their antitumor activities against P388 leukemia and sarcoma 180. EB-glu-MMC and E-glu-MMC were suspended in 10% (v/v) propylene glycol in saline and administered intraperitoneally to mice bearing P388 leukemia intraperitoneally or to mice bearing sarcoma 180 subcutaneously. The antitumor effect against P388 leukemia was greater in the order MMC > E-glu-MMC > EB-glu-MMC, and only the former two compounds significantly increased life span. On the other hand, EB-glu-MMC and E-glu-MMC showed suppression of sarcoma 180 growth at higher doses close to or better than MMC. In the mixture of 1/15 mM phosphate buffer (pH 7.4, ionic strength (μ) adjusted to 0.3 with NaCl)–propylene glycol (9 : 1, v/v) at 37°C, MMC was released much more slowly from EB-glu-MMC suspension than from E-glu-MMC suspension. With regard to chemotherapy against sarcoma 180, both conjugates were considered to supply MMC slowly but effectively at higher doses.

Key words EB-glu-MMC; E-glu-MMC; suspension dosage form; antitumor activity

Estradiol-antitumor drug conjugates have been examined for improvement or modification of the therapeutic efficacies of the parent antitumor drugs.1–6) For example, estramustine and bestrabucil show strong antitumor effects.1,5) These agents show specific tissue distribution, which is a characteristic favorable for the chemotherapy of tumors.3,5,6) Bestrabucil, a conjugate of estradiol benzoate and chlorambucil, was developed in an attempt to treat estrogen receptor-positive tumors, but various findings demonstrated that bestrabucil could accumulate well in various tumors.5,6) This agent exhibited strong antitumor effects against many solid tumors such as Walker 256 and sarcoma 180.4,5) Therefore, conjugation of antitumor drugs with estradiol or estradiol bezoate was considered to be a good approach to improve drug action. Mitomycin C (MMC) has been derivatized to prepare its prodrugs, and water-soluble macromolecular prodrugs of MMC have been examined extensively.7–12) The prodrugs obtained have been shown to be effective against many tumors. Further, although many lipophilic prodrugs of MMC were prepared and evaluated, prodrugs of MMC with moieties of estradiol benzoate and estradiol have not yet been reported. Thus, conjugates of MMC with estradiol benzoate and estradiol via glutaric acid (EB-glu-MMC and E-glu-MMC, respectively) were prepared as novel conjugates of MMC and their physicochemical and biological properties and antitumor characteristies were investigated.13–15)

EB-glu-MMC and E-glu-MMC released MMC directly by nonenzymatic hydrolysis of the amide bond at the 1a-N position of MMC in both nonbiological and biological media.13,14) As the conjugates showed only very slight affinities to estrogen receptors,14) interest was focused on their antitumor efficacy against many tumors not limited to estrogen receptor-positive tumors. Bestrabucil exhibited low affinities to estrogen receptors but good antitumor effect against various tumors such as Walker 256 and sarcoma 180.4,5,16) So far, EB-glu-MMC and E-glu-MMC, administered in solution form dissolved in propylene glycol (PG), exhibited less antitumor effects against P388 leukemia but equivalent or slightly better effects against sarcoma 180 as compared with MMC.5,15) The suspension forms of EB-glu-MMC and E-glu-MMC are expected to supply MMC more slowly due to their low solubility. In the present study, the drug actions of EB-glu-MMC and E-glu-MMC in suspension form were examined.

MATERIALS AND METHODS

Materials Mitomycin Kyowa S (Kyowa Hakko Kogyo Co., Japan), which is composed of MMC and sodium chloride, was used to obtain MMC. MMC was used after extraction with tetrahydrofuran from Mitomycin Kyowa S. Estradiol benzoate and estradiol 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.7) Briefly, glutaric anhydride was reacted with estradiol benzoate and estradiol to give 4-[3-3'-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 synthesized by condensation of MMC with EB-glu and E-glu, respectively, at the 1a-N position of MMC using carbonyldiimidazole.

Animals Male DBA/2 mice (6 weeks old) weighing 19–23 g and female BDF 1 mice (6 weeks old) weighing 19–23 g were obtained from Clea Japan, Inc., Japan. Male and female ddY mice (6 weeks old) weighing 26–29 g were purchased from Saitama Experimental Animal Supply Co., Japan.

Tumors P388 leukemia cells were maintained by weekly intraperitoneal transplantation of 1 × 10^6 cells suspended in Hanks’ balanced solution (0.1 ml) per male DBA/2 mouse. In the in vivo antitumor experiment, 1 × 10^6 P388 leukemia cells, obtained from the above tumor-bearing BDF/2 mice, suspended in Hanks’ balanced solution (0.1 ml) were inoculated intraperitoneally into each female BDF/1 mouse. Sarcoma 180 cells were maintained by weekly intraperitoneal transplantation of 1 × 10^6 sarcoma 180 cells suspended in

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Hanks’ balanced solution (0.1 ml) into each male ddY mouse. In the in vivo antitumor experiment, 1×10^7 sarcoma 180 cells, obtained from the above tumor-bearing male ddY mice, suspended in Hanks’ balanced solution (0.1 ml), were inoculated subcutaneously into each female ddY mouse in the axillary region.

**Antitumor Experiment against P388 Leukemia** Each drug was administered intraperitoneally at 24 h after i.p. inoculation. MMC was dissolved in a mixture of saline and propylene glycol (PG) (9 : 1, v/v) (10% PG saline), and injected at doses of 2.5, 5, and 10 mg/kg (0.1 ml per mouse) at 24 h after inoculation. In controls, the same volume of 10% PG saline was injected at 24 h after inoculation. EB-glu-MMC and E-glu-MMC were suspended in 10% PG saline using a glass homogenizer with a Teflon pestle. EB-glu-MMC suspension was injected at 10, 25, and 50 mg MMC eq./kg (0.4 ml per mouse) and 75 mg MMC eq/kg (0.35 ml per mouse) at 24 h after inoculation. In controls, the same volume of 10% PG saline was injected at 24 h after inoculation. E-glu-MMC suspension was injected at 10, 25, and 50 mg MMC eq./kg (0.35 ml per mouse) and 75 mg MMC eq/kg (0.3 ml per mouse) at 24 h after inoculation. In controls, the same volumes of 10% PG saline were injected at 24 h after inoculation. The survival time of the mice after inoculation was observed for 2 months. The antitumor effect was measured by comparing the mean survival time of treated mice (T) with that of control mice (C), i.e., from increase in life span (ILS) calculated as follows:7)

\[ ILS(%) = \left( \frac{T}{C} - 1 \right) \times 100 \]  

(1)

At the same time, the body weight change was measured as an index of toxic side effects. The body weight immediately before inoculation was used as the initial body weight. Statistical analysis was performed using the unpaired t-test.

**Antitumor Experiment against Sarcoma 180** Each drug was administered intraperitoneally at 4 d after s.c. inoculation. MMC was dissolved at a concentration of 2 mg/ml in 10% PG saline, and injected at doses of 2.5 and 5 mg/kg. EB-glu-MMC and E-glu-MMC were suspended at the concentration of 10 mg/ml in 10% PG saline, and administered at doses of 25, 50, and 75 mg MMC eq/kg. Immediately before administration, all suspensions of EB-glu-MMC and E-glu-MMC were homogenized using a glass homogenizer with a Teflon pestle. In controls, 0.22 ml of 10% PG saline was injected per mouse. All the mice were examined for tumor volume. The length (L, cm) of the longest tumor axis and the length (W, cm) of the vertical axis (width) were measured with slide calipers, and the tumor volume (V, cm^3) was calculated as follows:12)

\[ V = L \times W^{1/2} \]  

(2)

The tumor volume immediately before administration was used as the initial tumor volume. The tumor growth ratio was calculated as a ratio of the tumor volume to the initial tumor volume. When the tumor growth ratios of the control and treated groups on a specified day after administration were G(C) and G(T), respectively, the growth inhibition was estimated using the following equation:

\[ \text{growth inhibition (\%) = } \left( 1 - \frac{G(T)}{G(C)} \right) \times 100 \]  

(3)

At the same time, the body weight change was measured as an index of toxic side effects. The body weight immediately before administration (4 d post-inoculation) was used as the initial body weight. Statistical analysis was performed using the unpaired t-test.

**MMC Release** EB-glu-MMC and E-glu-MMC were dissolved in PG at a concentration of 20 μg/ml. To 1 ml of each solution was added 9 ml of 1/15 M phosphate buffer, pH 7.4, in which ionic strength (μ) was adjusted to 0.3 with NaCl to form a suspension. This suspension was incubated at 37°C. Aliquots (200 μl) were withdrawn at appropriate times after sufficient stirring. These samples (20 μl) were directly injected without filtration on a high-performance liquid chromatography (HPLC) column. HPLC analysis was performed at room temperature using a Shimadzu LC-6A with a Shimadzu SPD-6A detector set at 264 nm. The reverse-phase column (SUMIPAX Nucleosil 5C18, 4 mmφ×250 mm), connected with a guard column (Applied Biosystems RP-18 NEW GUARD 7 micron, 3.2 mmφ×15 mm), was used as an analytical column. A mixture of 0.1 M phosphate buffer, pH 6.0, and methanol (13:7, v/v) was used as the mobile phase at a flow rate of 0.6 ml/min for determination of MMC.

**RESULTS**

**Antitumor Effects against P388 Leukemia** The effects of MMC, EB-glu-MMC, and E-glu-MMC on the survival time were examined in mice bearing P388 leukemia intraperitoneally. As the i.p.–i.p. system was adopted in this experiment, the injection volume was adjusted similarly (0.3—0.4 ml) to reduce the influence of PG on tumor cells. The results are shown in Table 1. MMC exhibited the highest ILS value at a dose of 5 mg/kg. EB-glu-MMC scarcely increased life span at doses of 10—75 mg MMC eq/kg. E-glu-MMC increased life span significantly at doses of 50—75 mg MMC eq/kg. MMC was lethally toxic at a dose of 10 mg/kg; marked toxic side effects were also exhibited with a great decrease in body weight (Table 1). EB-glu-MMC did not notably decrease body weight. On the other hand, E-glu-MMC reduced body weight at the dose of 75 mg MMC eq/kg (Table 1).

**Antitumor Effects against Sarcoma 180** The antitumor effects of MMC, EB-glu-MMC, and E-glu-MMC were examined in mice bearing sarcoma 180 solid tumor subcutaneously. As the tumor site was located apart from the site of drug administration, each drug was administered at a similar concentration in each dose. MMC was injected at a concentration of 2 mg/ml in 10% PG saline, and EB-glu-MMC and E-glu-MMC were administered at a concentration of 10 mg/ml in 10% PG saline. Therefore, the volume of 10% PG saline was different among the doses. The results are shown in Table 2. Figure 1 shows tumor growth profiles for MMC (5 mg/kg), EB-glu-MMC (50 mg MMC eq/kg), and E-glu-MMC (50 mg MMC eq/kg), which exhibited the greatest suppression at 21 d post-inoculation in the tested doses. Growth inhibition was compared for 3 weeks after inoculation (Fig. 1) because animal deaths occurred in each group with further observation. EB-glu-MMC tended to show more suppression of tumor growth at later periods, that is, at 21 d post-inoculation (Table 2). E-glu-MMC exhibited the greatest suppression of tumor growth at doses of 50—75 mg MMC eq/kg. However, E-glu-MMC induced marked body weight loss at doses of 50—75 mg MMC eq/kg, and a
The results are expressed as the mean 6 eq./kg.

lethal toxicity was observed at the dose of 75 mg MMC eq./kg.

Release of MMC from Suspension When 9 ml of 1/15

Table 1. Effect of MMC, EB-glu-MMC, and E-glu-MMC on the Survival Time of Mice Bearing P388 Leukemia

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg MMC eq./kg)</th>
<th>Survival days of control mice (mean 6 S.D.)</th>
<th>Survival days of treated mice (mean 6 S.D.)</th>
<th>ILS (%)</th>
<th>Change in mean body weight (%) 4 d – 0 d&lt;sup&gt;a&lt;/sup&gt;</th>
<th>7 d – 0 d&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC</td>
<td>2.5</td>
<td>10.3 6 0.5</td>
<td>19.5 6 3.1***</td>
<td>89.3</td>
<td>+0.7</td>
<td>+3.0</td>
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<tr>
<td></td>
<td>5</td>
<td>10.3 6 0.5</td>
<td>24.5 6 3.8***</td>
<td>137.9</td>
<td>–6.5</td>
<td>+0.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.8 6 1.3</td>
<td>12.4 6 7.0</td>
<td>26.5</td>
<td>–3.8</td>
<td>–11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>9.5 6 1.0</td>
<td>10.8 6 1.5</td>
<td>13.7</td>
<td>–4.8</td>
<td>+18.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>9.5 6 1.0</td>
<td>11.0 6 2.5</td>
<td>15.8</td>
<td>+2.8</td>
<td>+26.6</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>10.5 6 0.6</td>
<td>11.5 6 1.0</td>
<td>9.5</td>
<td>–7.4</td>
<td>+6.6</td>
</tr>
<tr>
<td>EB-glu-MMC</td>
<td>10</td>
<td>9.8 6 1.3</td>
<td>10.0 6 1.4</td>
<td>2.0</td>
<td>+8</td>
<td>+26.4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>9.5 6 1.0</td>
<td>10.8 6 1.5</td>
<td>13.7</td>
<td>+2.1</td>
<td>–2.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>9.5 6 1.0</td>
<td>11.3 6 1.3</td>
<td>18.9</td>
<td>+1.9</td>
<td>+13.8</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>10.5 6 0.6</td>
<td>12.8 6 1.0***</td>
<td>34.7</td>
<td>–8.2</td>
<td>+6.6</td>
</tr>
<tr>
<td>E-glu-MMC</td>
<td>10</td>
<td>9.8 6 1.3</td>
<td>10.0 6 1.7</td>
<td>2.0</td>
<td>+1.0</td>
<td>–2.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>9.5 6 1.0</td>
<td>11.3 6 1.3</td>
<td>18.9</td>
<td>+1.9</td>
<td>+13.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>9.5 6 1.0</td>
<td>12.8 6 1.0***</td>
<td>34.7</td>
<td>–8.2</td>
<td>+6.6</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>10.5 6 0.6</td>
<td>14.0 6 1.4***</td>
<td>33.3</td>
<td>–5.5</td>
<td>–2.2</td>
</tr>
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</table>

The survival days are determined with n=4—5. *p<0.01 vs. control; **p<0.001 vs. control. a) Change ratio of mean body weight at 4 d post-inoculation to that immediately before inoculation. b) Change ratio of mean body weight at 7 d post-inoculation to that immediately before inoculation. c) One mouse died before 7 d post-inoculation.

Table 2. Growth Inhibitory Effect of MMC, EB-glu-MMC and E-glu-MMC on Sarcoma 180 Solid Tumor Inoculated Subcutaneously to Mice

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Dose (mg MMC eq./kg)</th>
<th>Tumor volume (cm&lt;sup&gt;3&lt;/sup&gt;)&lt;sup&gt;c&lt;/sup&gt; 4 d after inoculation</th>
<th>14 d after inoculation</th>
<th>21 d after inoculation</th>
<th>Growth inhibition at 21 d (%)</th>
<th>Change in mean body weight (%) 8 d – 4 d&lt;sup&gt;e&lt;/sup&gt;</th>
<th>11 d – 4 d&lt;sup&gt;e&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0</td>
<td>0.13 6 0.03</td>
<td>4.56 6 1.42</td>
<td>7.27 6 0.59</td>
<td>0</td>
<td>–8.1</td>
<td>+9.1</td>
</tr>
<tr>
<td>MMC</td>
<td>2.5</td>
<td>0.22 6 0.08</td>
<td>3.71 6 1.05</td>
<td>5.64 6 1.34</td>
<td>54.2</td>
<td>+4.9</td>
<td>+5.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.18 6 0.09</td>
<td>1.70 6 0.79*</td>
<td>3.85 6 1.02**</td>
<td>61.8</td>
<td>+7.5</td>
<td>+6</td>
</tr>
<tr>
<td>EB-glu-MMC</td>
<td>25</td>
<td>0.17 6 0.09</td>
<td>3.90 6 1.29</td>
<td>7.36 6 0.99**</td>
<td>82.3</td>
<td>+17.5</td>
<td>+19.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.18 6 0.08</td>
<td>2.89 6 0.61</td>
<td>2.26 6 0.30***</td>
<td>77.5</td>
<td>+10.2</td>
<td>+12.1</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.20 6 0.10</td>
<td>2.78 6 0.87</td>
<td>3.28 6 0.40***</td>
<td>70.7</td>
<td>+7.8</td>
<td>+8.4</td>
</tr>
<tr>
<td>E-glu-MMC</td>
<td>25</td>
<td>0.12 6 0.01</td>
<td>3.26 6 0.76</td>
<td>5.52 6 2.81</td>
<td>17.1</td>
<td>+12.9</td>
<td>+14.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.12 6 0.03</td>
<td>1.20 6 0.84**</td>
<td>0.74 6 0.32***</td>
<td>89.0</td>
<td>–7.5</td>
<td>–11.5</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.15 6 0.06</td>
<td>–60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–8.9</td>
<td>–10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The preparations were administered intraperitoneally at 4 d post-inoculation. a) The tumor volumes at 4, 14, and 21 d post-inoculation are shown as described under the line. The results are expressed as the mean 6 S.D. (n=3—4). *p<0.05 vs. saline; **p<0.01 vs. saline; ***p<0.001 vs. saline. b) The number of remaining mice <3. c) Change ratio of mean body weight at 8 d post-inoculation to that immediately before administration. d) Change ratio of mean body weight at 11 d post-inoculation to that immediately before administration. e) One mouse died before 11 d post-inoculation.

![Fig. 1. Growth Inhibitory Effect at 1p. Administration against Sarcoma 180 Solid Tumor Inoculated Subcutaneously in Mice](image1)

Fig. 1. Growth Inhibitory Effect at 1p. Administration against Sarcoma 180 Solid Tumor Inoculated Subcutaneously in Mice

O, control; ▲, MMC 5 mg/kg; ●, EB-glu-MMC 50 mg MMC eq./kg; ■, E-glu-MMC 50 mg MMC eq./kg. Tumor was inoculated at 0 d, and administration was performed at 4 d post-inoculation. Each compound was mixed in 10% PG saline and injected. The initial tumor volume means the tumor volume obtained immediately before administration. Each point represents the mean value (n=3—4).

Fig. 2. Release of MMC from EB-glu-MMC (●) and E-glu-MMC (■) in the Mixture of 1/15 M Phosphate Buffer (pH 7.4, μ=0.3 with NaCl) and PG (9:1, v/v) at 37°C

Each point represents the mean 6 S.D. (n=3).

M phosphate buffer (pH 7.4, μ=0.3) was added to 1 ml of solution of EB-glu-MMC and E-glu-MMC (20 μl/ml) in PG, a very fine suspension was formed. The filtration study with a membrane filter (0.45 μm pore diameter) indicated that both conjugates were almost insoluble in the mixture of the phosphate buffer and PG (9:1, v/v). The MMC release profiles from these suspensions at 37°C are shown in Fig. 2. The
amount of MMC released was 2.7% and 35% at 48 h after the start of incubation of EB-glu-MMC and E-glu-MMC, respectively.

**DISCUSSION**

When administered in the suspension form using 10% PG saline, EB-glu-MMC did not significantly increase life span as compared with the control, but E-glu-MMC exhibited a small increase in life span at higher doses (Table 1). In comparison with injection in the solution form using 100% PG saline, the *ILS* values of both conjugates were smaller. For example, although E-glu-MMC showed an *ILS* of more than 80% at a dose of 15 mg MMC eq./kg in the solution form, its *ILS* was less than 35% even at doses of 50—75 mg MMC eq./kg in the suspension form. MMC was released in vivo more slowly in the suspension form than the solution form (Fig. 2). As to the release of MMC from the solution form, when the solution of E-glu-MMC in the mixture of 1/15 M phosphate buffer (pH 7.4, *μ* = 0.3) and PG (1 : 1, v/v) was incubated at 37 °C, approximately 50% of the MMC was released at 24 h after the start of incubation. For EB-glu-MMC, approximately 50% of the MMC was released in the mixture of 1/15 M phosphate buffer (pH 7.4, *μ* = 0.3) and PG (1 : 1, v/v) at 37 °C at 48 h after the start of incubation. These release characteristics were possibly related to the low or slight antitumor effect against intraperitoneal P388 leukemia in the suspension form. That is, the slower release was considered to make the concentration of MMC lower in the intraperitoneal site and to reduce the supply of MMC from the intraperitoneal site. The MMC release rate was markedly reduced from the EB-glu-MMC suspension, resulting in slight antitumor effect. Further, the low or slight effect of the conjugates against intraperitoneal P388 leukemia suggested that specific affinities of the conjugates to P388 leukemia would be unlikely. MMC was lethally toxic at a dose of 10 mg/kg, when the body weight decreased and was not recovered, and one mouse died at 5 d post-inoculation (Table 1). One mouse died before 7 d post-inoculation in the E-glu-MMC at a dose of 10 mg MMC eq./kg (Table 1); the disease might have caused the death because no body weight loss was observed. E-glu-MMC decreased body weight at 75 mg MMC eq./kg (Table 1). E-glu-MMC was probably more toxic than EB-glu-MMC.

On the other hand, EB-glu-MMC and E-glu-MMC suppressed sarcoma 180 tumor growth markedly at doses of 50—75 mg MMC eq./kg. A higher dose was required to obtain good tumor suppression in the suspension form as compared with the solution form. This might be due to the slower and/or smaller supply of MMC in the suspension form. Although EB-glu-MMC exhibited no significant effect on P388 leukemia, it markedly suppressed tumor growth of sarcoma 180, especially at later periods. MMC is known to exhibit high antitumor effects against sarcoma 180 solid tumor as well as ascitic P388 leukemia. The effect of MMC against sarcoma 180 solid tumor appears to depend heavily on drug distribution; good localization and retention of MMC in the tumor site enabled more extensive suppression of tumor growth. This biodisposition effect appeared to be observed with greater suppression of tumor growth at later periods after inoculation. These facts suggest that tumor localization of MMC is important for effective and long-term suppression of tumor growth. The biodisposition mechanism of reported macromolecular compounds is considered different from that of EB-glu-MMC and E-glu-MMC. Bestribucil is distributed to tumor based on its own biodisposition properties. Considering these drug characteristics, it is suggested that the conjugates did not only act as prodrugs of MMC, but that they exhibited appropriate biodisposition properties for the suppression of sarcoma 180 solid tumor. In fact, the conjugates exhibited very different pharmacokinetics from MMC, which were reported previously for the solution form, suggesting a difference in biodisposition characteristics between the conjugates and MMC. In a preliminary study, EB-glu-MMC administered intraperitoneally exhibited good localization to lymphatic tissues including the thymus and spleen (data not shown), which might represent the specific biodisposition of EB-glu-MMC. However, for exact evaluation of the in vivo efficacy, further detailed investigations on tissue distribution are needed. More body weight loss was observed in the E-glu-MMC than in the EB-glu-MMC. Therefore, simple comparison of body weight loss indicated that E-glu-MMC would be more toxic than EB-glu-MMC. In the mouse that died at 21 d post-inoculation after receiving EB-glu-MMC at 10 mg MMC eq./kg, disease or toxicity was considered as the cause, though the reason was ambiguous. On the other hand, in the administration of E-glu-MMC at a dose of 75 mg MMC eq./kg, the mice died following a marked decrease in body weight. E-glu-MMC exhibited the greatest inhibition of tumor growth (nearly 90% growth inhibition at 21 d post-inoculation) at the dose of 50 mg MMC eq./kg (Table 2), though it showed strong toxic side effects at the dose of 75 mg MMC eq./kg. The different evaluation of the toxic side effects in addition to measurement of body weight changes might be required in the treatment tests against sarcoma 180 solid tumor because prolonged or delayed drug toxicity might appear with longer-term examination.

Overall, the present study suggested that suspensions of EB-glu-MMC and E-glu-MMC in 10% PG saline at higher doses might be adequate for the treatment of sarcoma 180 solid tumor. Although E-glu-MMC exhibited the greatest suppression of tumor growth, it appeared to be more toxic than EB-glu-MMC. The EB-glu-MMC suspension was less toxic, probably due to its reduced solubility and very slow conversion to MMC. From the viewpoint of safety, EB-glu-MMC might be preferable in the treatment of sarcoma 180 solid tumor. Further examination such as biodisposition studies will make possible more exact in vivo evaluation of the conjugates.

**REFERENCES**