***In Vivo* Drug Release and Antitumor Characteristics of Water-Soluble Conjugates of Mitomycin C with Glycol-Chitosan and *N*-Succinyl-Chitosan**

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Received January 23, 1996; accepted June 3, 1996

The water-soluble conjugates of mitomycin C (MMC) with *N*-succinyl-chitosan (*N*-Suc-chitosan) and glycol-chitosan (Gly-chitosan), named *N*-Suc-chitosan-glu-MMC and Gly-chitosan-glu-MMC, respectively, were characterized mainly by the plasma concentration–time profiles of MMC after intraperitoneal administration and their *in vivo* antitumor effect against P388 leukemia and Sarcoma 180. Before *in vivo* evaluation, polymer-drug binding characteristics were checked by gel-chromatography. Gel-chromatographs proposed the covalent binding of 1a-(4-carboxybutyryl)-MMC (glu-MMC) with both the polymer supports. The plasma concentration of MMC showed that each conjugate released MMC *in vivo* at a similar rate. Kinetic analysis suggested that the *in vivo* drug release should be considerably faster than the *in vitro* release in the buffer, pH 7.4, alone. In the treatment against P388 leukemia inoculated intraperitoneally, Gly-chitosan-glu-MMC showed the highest increase in life span (ILS) at 10 mg MMC eq/kg. It was lethally toxic at the dose of 20 mg MMC eq/kg, while *N*-Suc-chitosan-glu-MMC gave the highest ILS value at this dose. Each conjugate exhibited a little larger ILS value than MMC. For the Sarcoma 180 solid tumor inoculated subcutaneously, the polymer characteristics affected the antitumor effect. Namely, with the intravenous injection, Gly-chitosan-glu-MMC hardly exhibited any tumor growth inhibition, but *N*-Suc-chitosan-glu-MMC showed significant tumor growth suppression. As to the intratumoral administration, the tendency to suppress tumor growth was observed in MMC and both the conjugates.

Key words Gly-chitosan-glu-MMC; *N*-Suc-chitosan-glu-MMC; *in vivo* antitumor effect; plasma concentration–time profile; gel-chromatography

We have already reported on the conjugates prepared from the direct coupling of mitomycin C (MMC) with *N*-succinyl-chitosan and 6-*O*-carboxymethyl-chitin by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), which were water-insoluble and partially water-soluble, respectively.^{1–5} These insoluble properties should affect the biopharmaceutical behaviors of the polymer supports as well as their physicochemical properties. Actually, their body distribution after intravenous administration was influenced by the particle size.⁵ Therefore, in order to make use of the physicochemical polymer characteristics for controlling the conjugate biopharmaceutically, the preparation of water-soluble conjugates will be needed. As stated in the previous work,⁶ novel water-soluble conjugates of MMC using two kinds of chitosan derivatives, *N*-succinyl-chitosan-glutaryl-MMC conjugate (*N*-Suc-chitosan-glu-MMC) and glycol-chitosan-glutaryl-MMC conjugate (Gly-chitosan-glu-MMC), were prepared and investigated for their *in vitro* release characteristics. They showed a gradual drug release but their release rates were different from those of the directly coupled conjugates. The difference in such physicochemical characteristics as solubility and drug release rate should affect the antitumor activity. Further, since their polymer supports have opposite electrostatic characteristics, it is meaningful to investigate their effect on the antitumor characteristics. In this study, the binding between the drug and the polymer support was checked by gel-chromatography, and further *in vivo* release and antitumor characteristics were investigated.

MATERIALS AND METHODS

Materials MMC, produced by Kyowa Hakko Kogyo Co., was used. *N*-Succinyl-chitosan sodium salt (*N*-Suc-chitosan: M.W., 3×10^5 ; *N*-succinylation degree per glucosamine unit, 0.72) was obtained from Katakura Chikkarin Co., Ltd. Glycol-chitosan (Gly-chitosan: M.W., 1.6×10^5 ; deacetylation degree, 77%) was purchased from Wako Pure Chemical Industries, Ltd. All other chemicals were of a reagent grade.

N-Suc-chitosan-glutaryl-MMC conjugate (*N*-Suc-chitosan-glu-MMC) and Gly-chitosan-glutaryl-MMC conjugate (Gly-chitosan-glu-MMC) were prepared as reported previously.⁶ Briefly, first, 1a-[4-(*N*-succinimidoxycarbonyl)butyryl]-MMC (MMC-glu-OSU) was synthesized according to the method by Kato *et al.*⁷ Next, the conjugates were prepared by the reaction of MMC-glu-OSU with *N*-Suc-chitosan and Gly-chitosan. Conjugates with a low drug content, the preparation procedure of which had been reported before,⁶ were prepared as follows: *N,N'*-Dimethylformamide solution (0.2 ml) containing MMC-glu-OSU (8.2 mg) was added to the aqueous solution (9.5 ml) with 100 mg of *N*-Suc-chitosan or Gly-chitosan, and the solution pH was adjusted to 6 with 0.1 N HCl aqueous solution. After stirring the solution at 4 °C for 24 h, the conjugate was separated and purified by ultrafiltration, with a molecular weight cut-off limit of 50000, and by subsequent washing according to the previous report.⁶ Finally, the conjugates were obtained as a powder by lyophilization. The drug contents of the conjugates were also determined following the previous study.⁶ Namely, they were calculated spectrophotometrically from the ultraviolet (UV) absorption maxima around

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Table 1. Reaction Conditions and MMC Contents of Conjugates with Low and High Drug Contents

Conjugate ^{a)}	Carrier (mg)	MMC-glu-OSU (mg)	MMC content (%)
Gly-chitosan-glu-MMC (low drug content)	100	8.2	4.1
Gly-chitosan-glu-MMC (high drug content)	100	35.0	10.7
N-Suc-chitosan-glu-MMC (low drug content)	100	8.2	1.3
N-Suc-chitosan-glu-MMC (high drug content)	100	20.0	4.7

a) The preparation was done as follows: After *N,N'*-dimethylformamide (0.2 ml) containing MMC-glu-OSU was added to purified water (9.5 ml) containing a carrier, the pH of the mixture was adjusted to 6 by 0.1 N HCl aqueous solution, and then stirred for 24 h at room temperature.

364 nm of their solutions in 1/15 M phosphate buffer of pH 7 based on the extinction coefficient of MMC in the same buffer. The resulting contents of MMC were 4.1 and 1.3% for Gly-chitosan-glu-MMC and N-Suc-chitosan-glu-MMC, respectively (Table 1). Conjugates with a high drug content were prepared in the same way, except that the amounts of MMC-glu-OSU added in the reaction were 35 mg for Gly-chitosan-glu-MMC and 20 mg for N-Suc-chitosan-glu-MMC. Then, the MMC contents were 10.7 and 4.7% for Gly-chitosan-glu-MMC and N-Suc-chitosan-glu-MMC, respectively (Table 1).

Gel-Filtration The binding characteristics between the polymer supports and MMC were checked using gel-filtration. A Sephadex G50 column (2.8 × 20 cm) was used with 0.2 M NaCl aqueous solution as an elution solvent, and each fraction volume was kept at 6 ml. Conjugates (10 mg) with a low drug content were dissolved in 1 ml of the elution solvent and the solution was chromatographed. In addition, 1a-(4-carboxybutyryl)-MMC (glu-MMC) (1.7 mg) and chitosan derivatives (10 mg) were dissolved in 1 ml of the elution solvent, then eluted. The eluted fractions were measured spectrophotometrically at 364 nm. Characterization of the column was executed by the elution of the 0.2 M NaCl aqueous solution (2 ml) containing blue-dextran 2000 (M.W., 2 × 10⁶) (4 mg) and isonicotinamide (1 mg), and each eluted fraction was measured spectrophotometrically at 260 nm.

Animals Male Wistar rats weighing 180–200 g and male ddY mice (6 weeks old) weighing 19–23 g were purchased from Saitama Experimental Animal Supply Co. Male DBA/2 (6 weeks old) weighing 19–23 g and male BDF₁ (C57BL/6 female × DBA/2 male) mice (6 weeks old) weighing 19–23 g were obtained from Clea Japan, Inc. These animals were kept on the breeding diet MF (Oriented Yeast Co., Ltd., Tokyo) with water *ad libitum* in a room maintained at 23 ± 1 °C and a relative humidity of 60 ± 5%, and were used soon after purchase.

Tumors P388 leukemia cells were maintained by weekly transplantation of 1 × 10⁶ cells suspended in Hanks' balanced solution (0.1 ml) into the peritoneal cavity of each DBA/2 mouse. In the test of *in vivo* antitumor effect, 1 × 10⁶ P388 leukemia cells suspended in Hanks' balanced solution (0.1 ml) were inoculated intraperitoneally to each

BDF₁ mouse. Sarcoma 180 cells were maintained by weekly transplantation of 1 × 10⁷ cells suspended in Hanks' balanced solution (0.1 ml) into the peritoneal cavity of each ddY mouse. In the test of *in vivo* antitumor effect, 1 × 10⁷ Sarcoma 180 cells suspended in Hanks' balanced solution (0.1 ml) were inoculated subcutaneously at the axillary region of each ddY mouse.

Plasma Concentration of MMC after the Intraperitoneal Administration Wistar rats were anesthetized by the intraperitoneal injection of 25% urethane saline solution at 3 ml/kg, and were then fixed on their backs during the experiment. MMC was injected intravenously *via* the jugular vein (0.5 ml) and intraperitoneally (1 ml) into the rats at 5 mg/kg as saline solution. Conjugates with a high drug content were injected intraperitoneally as saline solution (1 ml) to the rats at 10 mg MMC eq/kg. Each blood sample (0.5 ml) was withdrawn from the jugular vein at appropriate times. The plasma was separated by centrifugation of the blood at 3000 rpm for 5 min. The extraction of MMC from the plasma sample was executed according to the method of Hartigh *et al.*⁸⁾ This operation allowed the complete recovery of MMC in the plasma sample. The operation was as follows: Two milliliter 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 shaken vigorously for 3 min, it was centrifuged to obtain the supernatant. All the supernatant was transferred to a glass tube and evaporated to dryness at 40 °C under nitrogen gas. The residue was dissolved in 100 μl of methanol, and then 20 μl of the solution was injected in high performance liquid chromatography (HPLC).

HPLC Assay The HPLC assay was done using the absolute calibration curve method reported by Hartigh *et al.*⁸⁾ Namely, the determination of MMC by HPLC was executed using a Shimadzu LC-6A apparatus equipped with an SPD-6AV detector set at 364 nm. A Neo-Pack 120-5C₁₈ reversed phase column (4.6 × 250 mm) to which a guard column, an Applied Biosystems RP-18 column (4.6 × 30 mm), was connected was used as an analyzing column. The mobile phase was a mixture of 10 mM phosphate buffer, pH 6, and methanol (65 : 35, v/v), and was flowed at the rate of 0.8 ml/min. The concentration of MMC was calculated using a standard curve. All the operations were done at room temperature.

Antitumor Tests In antitumor tests, conjugates with a high drug content were used. 1) Antitumor activity against P388 leukemia: At 24 h after intraperitoneal inoculation, the drugs were injected intraperitoneally as saline solution (0.5 ml) at a single dose, while saline (0.5 ml) was injected intraperitoneally as a control. For all the mice, the survival time after inoculation was observed for 35 d 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 by the following equation:

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

At the same time, the body weight of each group was measured for the initial 8 d after inoculation. And also,

for each group, the ratio of the body weight to that measured immediately before inoculation (initial weight) was investigated for the initial 8 d after inoculation. The mean weight and mean ratio were used for the evaluation of toxic side effects. 2) Antitumor effect against Sarcoma 180: At 4 d after subcutaneous inoculation, the drugs were administered as saline solution (0.2 ml) into the tail vein or the center of the solid tumor. The control mice underwent no administration. The tumor volume immediately before administration was used as the initial volume, and the tumor volume of each group was measured for 13 d after administration. The ratio of the tumor volume to the initial volume was investigated for 13 d after administration. The mean volume and mean ratio were used to evaluate the antitumor effect. The tumor size was measured with slide calipers at appropriate times, and the tumor volume was calculated by the following equation which had been used by Takakura *et al.*⁹⁾:

$$\text{tumor volume} = L \times W^2 / 2 \quad (2)$$

where L and W are the tumor length and width, respectively.

RESULTS AND DISCUSSION

Conjugates As the amount of added MMC-glu-OSU was increased, the drug content was heightened for each conjugate. As described in the experimental section, for the conjugates with a low drug content, Gly-chitosan-glu-MMC and *N*-Suc-chitosan-glu-MMC had MMC contents of 4.1 and 1.3 %, respectively. For the conjugates with a high drug content, Gly-chitosan-glu-MMC and *N*-Suc-chitosan-glu-MMC had MMC contents of 10.7 and 4.7%, respectively. The preparation conditions of the conjugates with a high drug content were selected for the following reason: Gly-chitosan-glu-MMC with a relatively high drug content could be obtained by increasing the amount of MMC-glu-OSU added in the reaction. Namely, when MMC-glu-OSU of 35 mg was used, a drug content of 10.7% was obtained. However, the binding ratio was then observed to be lowered to a certain extent. As a result, considering that the yield decreased in raising the added amounts of MMC-glu-OSU and that the content of 10.7% was a considerably high value, the added amount of MMC-glu-OSU was set at 35 mg for the preparation of Gly-chitosan-glu-MMC with a high drug content, though the drug content might increase by using more MMC-glu-OSU. For *N*-Suc-chitosan-glu-MMC, even though MMC-glu-OSU of more than 20 mg was added in the conjugation reaction, the obtained drug content was not much different from that (4.7%) obtained using MMC-glu-OSU of 20 mg. Thus, for the preparation of *N*-Suc-chitosan-glu-MMC with a high drug content, MMC-glu-OSU of 20 mg was used. This is probably because Gly-chitosan possesses more amino groups as reactive groups to MMC-glu-OSU than *N*-Suc-chitosan. The drug content is important for suppressing the toxicity by the drug carrier. As reported previously,⁶⁾ *N*-Suc-chitosan and Gly-chitosan did not cause lethal toxicity at 2 and 0.4 g/kg in intraperitoneal administration to mice (data not shown). Simple calculation suggested the

possibility of administering *N*-Suc-chitosan-glu-MMC and Gly-chitosan-glu-MMC at more than 90 and 40 mg/kg dose to mice, respectively, without lethal toxicity by the polymer supports.

The binding of glu-MMC with Gly-chitosan or *N*-Suc-chitosan was supposed to be covalently formed from the HPLC study in the previous report.⁶⁾ Namely, glu-MMC and MMC were hardly detected by HPLC for the solution of each conjugate before the incubation. The binding of glu-MMC with polymer supports was further checked by gel-filtration. The elution profiles of *N*-Suc-chitosan-glu-MMC and Gly-chitosan-glu-MMC are described in Figs. 1 and 2, respectively. The ultraviolet (UV)

Fig. 1. Elution Profiles of Gly-chitosan-glu-MMC (●), the Mixture of Glu-MMC and Gly-chitosan (○) and the Mixture of Blue Dextran and Isonicotinamide (□) on a Sephadex G50 Column

For Gly-chitosan-glu-MMC and the mixture of glu-MMC and Gly-chitosan, the eluted sample was measured at 364 nm. For the mixture of blue dextran and isonicotinamide, the eluted sample was checked at 260 nm.

Fig. 2. Elution Profiles of *N*-Suc-chitosan-glu-MMC (●), the Mixture of Glu-MMC and *N*-Suc-chitosan (○) and the Mixture of Blue Dextran and Isonicotinamide (□) on a Sephadex G50 Column

For *N*-Suc-chitosan-glu-MMC and the mixture of glu-MMC and *N*-Suc-chitosan, the eluted sample was measured at 364 nm. For the mixture of Blue dextran and isonicotinamide, the eluted sample was checked at 260 nm.

Fig. 3. Proposed Chemical Structures of Gly-chitosan-glu-MMC (a) and *N*-Suc-chitosan-glu-MMC (b)

absorption at 364 nm was detected only around the void volume and not in the other fractions. When the mixture of glu-MMC and the polymer support was eluted, glu-MMC was detected only in the small molecular fractions. This indicated that each polymer support hardly adsorbed glu-MMC, which might be caused by the hydrolysis of MMC-glu-OSU in the conjugation reaction, and also that glu-MMC in the conjugates was combined tightly to the polymer supports. Further, since the hydroxysuccinimide ester of a carboxyl group generally reacts with an amino group in a 1:1 ratio as an amide coupling reagent, unreacted amino groups should remain unchanged. Therefore, amide coupling between glu-MMC and the amino group of *N*-Suc-chitosan was proposed. In fact, Tanaka *et al.* reported on the reaction of MMC-glu-OSU with a macromolecule (bovine serum albumin) in which the amino groups of the macromolecule decreased by the amounts of the introduced glu-MMC.¹⁰ Further, it was also consistent with the above proposition on the covalent binding between glu-MMC and the amino groups of the polymer support that the binding yield was higher in the reaction with Gly-chitosan, which contained more amino groups than *N*-Suc-chitosan. Thus, the chemical structures of *N*-Suc-chitosan-glu-MMC and Gly-chitosan-glu-MMC were proposed to be as shown in Fig. 3. The purification and washing using an ultrafiltration method was found to be satisfactory by this experiment, which had been suggested by the HPLC study reported before. The gel-filtration study was not performed for the conjugates with a high drug content. However, in this case, since the washing was done until the filtrate became clear and colorless, MMC and such MMC moiety-containing compounds as MMC-glu-OSU, glu-MMC were supposed to be washed out sufficiently from the conjugates. Therefore, conjugates with a high drug content were also supposed to be formed by the covalent binding.

Plasma Concentration–Time Profiles Figure 4 shows the plasma concentration–time profiles of MMC after the intravenous administration of MMC and after the intraperitoneal injections of MMC, *N*-Suc-chitosan-glu-MMC and Gly-chitosan-glu-MMC. Each curve-fitting was

Fig. 4. Plasma Concentration–Time Profiles of MMC after Intravenous Injection of MMC (●) and Intraperitoneal Injections of MMC (▲), Gly-chitosan-glu-MMC (○) and *N*-Suc-chitosan-glu-MMC (△)

MMC was administered at 5 mg/kg. The conjugates were administered at 10 mg MMC eq/kg. Each point represents the mean value ± S.D. ($n=4$ for intravenous and intraperitoneal injections of MMC; $n=3$ for administration of each conjugate). S.D. is shown unless smaller than the point plotted. The broken lines show the calculated profiles based on Eq. 5., for *in vitro* k_3 (0.015) of Gly-chitosan-glu-MMC; ---, for *in vitro* k_3 (0.025) of *N*-Suc-chitosan-glu-MMC; ---, for *in vivo* k_3 (0.08) of Gly-chitosan-glu-MMC; ---, for *in vivo* k_3 (0.07) of *N*-Suc-chitosan-glu-MMC.

done to the mean of the observed values using the first order kinetics Eqs. 3–5 shown below, when the non-least squares program, MULTI¹¹ was used as a program for the curve-fitting. For determination of the pharmacokinetic parameters, the elimination rate of MMC from blood circulation was first calculated from the study of the intravenous administration of MMC. Second, the transfer rate of MMC from the peritoneal cavity to the blood circulation was estimated from the study of the intraperitoneal administration of MMC, when the above elimination rate from the blood circulation was used. Further, the *in vivo* drug release rate of MMC from the conjugate was calculated from the study of the intraperitoneal administration of the conjugate, when the above elimination and transfer rates were used. The

simultaneous curve fitting in which multiple equations were used at the same time was not executed. The elimination rate constant (k_1) of MMC from blood circulation was calculated by fitting the mono-exponential elimination Eq. 3 to the observed plasma concentration at intravenous administration of MMC.

$$C(t) = C(0) \times \exp(-k_1 t) \quad (3)$$

where $C(t)$ and $C(0)$ represent the plasma concentration of MMC at the time t and 0 after administration. As a result, k_1 was 2.04 h^{-1} . The plasma concentration-time profiles after the intraperitoneal administration of MMC and the conjugates were analyzed by referring to the previous approach.²⁾ Namely, each AUC was parallel to that with the intravenous administration, which suggested a good transfer of MMC to the blood circulation in the intraperitoneal administration of MMC and the conjugates. Therefore, the following schemes were proposed, when k_1 was the elimination rate constant of MMC stated in Eq. 3, and k_2 and k_3 represented, respectively, the transfer rate constant of MMC from the peritoneal cavity to the blood circulation and the *in vivo* release rate constant of MMC from the conjugate in the peritoneal cavity. These schemes had also been used in the previous report.²⁾

For the intraperitoneal administration of MMC,

For the intraperitoneal administration of the conjugates,

The plasma concentrations for schemes 1 and 2 are given by the Eqs. 4 and 5, respectively.

$$C(t) = C(0)k_2 \times (\exp(-k_2 t) - \exp(-k_1 t)) / (k_1 - k_2) \quad (4)$$

$$\begin{aligned} C(t) = C(0)k_2k_3 \times &(\exp(-k_3 t) / ((k_2 - k_3)(k_1 - k_3)) \\ &+ \exp(-k_2 t) / ((k_2 - k_3)(k_2 - k_1)) \\ &+ \exp(-k_1 t) / ((k_1 - k_3)(k_1 - k_2))) \end{aligned} \quad (5)$$

where $C(t)$ and $C(0)$ represent the plasma concentrations of MMC at time t and 0 after administration. As a result of the curve-fitting using (4) with k_1 fixed, k_2 was 3.31 h^{-1} . For the *in vivo* release from the conjugates, since the plasma concentration of MMC was considerably higher from the first stage than expected from the *in vitro* release, the drug release was considered to be fairly accelerated *in vivo*. Actually, in the curve-fitting using (5), k_1 and k_2 were fixed, and the k_3 values were then calculated to be 0.080 h^{-1} for Gly-chitosan-glu-MMC and 0.070 h^{-1} for *N*-Suc-chitosan-glu-MMC. In each conjugate, the *in vivo* release rate presented by k_3 was much higher than the *in vitro* release rate at pH 7.4. However, the calculated plasma concentration-time profiles did not fit sufficiently to the observed ones. The complete depot model shown by scheme 2 did not seem to exactly reflect the actual state

of the *in vivo* release and transfer. It is thus proposed that a more complicated analysis is necessary for more exact evaluation.

Antitumor Activity against P388 Leukemia The antitumor activities of MMC and the conjugates against P388 leukemia are shown in Table 2. MMC exhibited the highest ILS, 61%, at 5 mg/kg and lethal toxicity at 10 mg/kg. On the other hand, Gly-chitosan-glu-MMC and *N*-Suc-chitosan-glu-MMC showed the highest ILS values, 68.2 and 73.9%, at 10 and 20 mg MMC eq/kg, respectively. At 20 mg MMC eq/kg, Gly-chitosan-glu-MMC was lethally toxic but *N*-Suc-chitosan-glu-MMC produced the largest extension of survival time in the tested doses, 1–20 mg MMC eq/kg. *N*-Suc-chitosan-glu-MMC might be more effective at a certain dose greater than 20 mg MMC eq/kg. As stated in the "Conjugates" section, for the conjugates with a high drug content, if the toxicity of the conjugates is determined by that of the polymer supports, doses of 40 and 90 mg/kg are proposed to be tolerable for Gly-chitosan-glu-MMC and *N*-Suc-chitosan-glu-MMC, respectively. Therefore, in addition to toxicity by the polymer support, other side effects were considered to be related to the acute toxicity of Gly-chitosan-glu-MMC. These were likely due to MMC released from the conjugate. Actually, it had been found in the two insoluble conjugates, *N*-Suc-chitosan-MMC conjugate and carboxymethyl-chitin-MMC conjugate, that the drug release rate seemed to be related to the drug action or acute toxicity.²⁾ The dose-antitumor effect curves were similar between Gly-chitosan-glu-MMC and *N*-Suc-chitosan-glu-MMC except their maximum tolerable doses. This similar antitumor effect paralleled the similarity of their plasma concentration-time profiles stated above. Thus, it was considered that the *in vivo* drug release rate might play an important role in the drug action. Figure 5 shows the change in body weight after administration. Figure 6 shows the ratio of body weight to that measured immediately before inoculation (initial weight). They are often both used as indices of toxic side effects. The decrease in body

Table 2. Effect of MMC, Gly-chitosan-glu-MMC and *N*-Suc-chitosan-glu-MMC on the Survival Time of Mice Bearing P388 Leukemia^{a)}

Drug	Dose (mg equivalent MMC/kg)	Survival days (mean ± S.D.)	ILS (%)	Control ^{b)}
MMC	1.0	11.3 ± 1.3	2.3	A
	2.5	15.3 ± 2.2 ^{c)}	38.6	A
	5.0	17.8 ± 1.5 ^{d)}	61.0	A
	10.0	6.3 ± 0.5 ^{d)}	–43.2	A
Gly-chitosan-glu-MMC	1.0	11.8 ± 1.3	6.8	A
	2.5	13.8 ± 1.7 ^{c)}	25.0	A
	5.0	16.3 ± 2.9 ^{c)}	47.7	A
	10.0	18.5 ± 1.3 ^{d)}	68.2	A
	20.0	5.0 ± 0.0 ^{d)}	–56.5	B
<i>N</i> -Suc-chitosan-glu-MMC	1.0	11.8 ± 1.3	6.8	A
	2.5	14.0 ± 2.2	27.3	A
	5.0	16.5 ± 1.9 ^{c)}	50.5	A
	10.0	18.0 ± 1.4 ^{d)}	63.6	A
	20.0	20.0 ± 3.8 ^{d)}	73.9	B

^{a)} P388 leukemia was inoculated intraperitoneally, and drug administration was executed at 24 h after that inoculation ($n=4$ for each group). ^{b)} The survival days (mean ± S.D.) of the control groups: A, 11.0 ± 0.0 ; B, 11.5 ± 1.9 . ^{c)} $p < 0.05$, versus the control group. ^{d)} $p < 0.01$, versus the control group.

Fig. 5. Change in Body Weight for P388 Leukemia-bearing Mice after Intraperitoneal Administration of Saline (●), MMC (□), Gly-chitosan-glu-MMC (○) and *N*-Suc-chitosan-glu-MMC (△)

a, 5 mg MMC eq/kg; b, 10 mg MMC eq/kg; c, 20 mg MMC eq/kg. Each point represents the mean value $\pm$ S.D. ($n=4$). S.D. is shown unless smaller than the point plotted. In each dose, the observed time was the same, but the plotted points were slightly moved in order to make each S.D. distinguishable. The body weight at 0 d was the body weight measured immediately before inoculation. Comparison with the control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig. 6. Change in the Ratio of the Body Weight to the Initial One for P388 Leukemia-bearing Mice after Intraperitoneal Administration of Saline (●), MMC (□), Gly-chitosan-glu-MMC (○) and *N*-Suc-chitosan-glu-MMC (△)

a, 5 mg MMC eq/kg; b, 10 mg MMC eq/kg; c, 20 mg MMC eq/kg. Each point represents the mean value $\pm$ S.D. ($n=4$). S.D. is shown unless smaller than the point plotted. In each dose, the observed time was the same, but the plotted points were slightly moved in order to make each S.D. distinguishable. The initial body weight was the body weight measured immediately before inoculation (body weight at 0 d). Comparison with the control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

weight was large at 10 mg/kg of MMC, which indicated strong toxicity. Figures 5c and 6c indicated that Gly-chitosan-glu-MMC was strongly toxic at 20 mg MMC eq/kg. Further, they indicated that *N*-Suc-chitosan-glu-MMC caused side effects to a certain extent at 20 mg MMC eq/kg. Therefore, *N*-Suc-chitosan-glu-MMC might not be much safer than Gly-chitosan-glu-MMC. So, the MMC released from the conjugate might be related to the side effects of *N*-Suc-chitosan-glu-MMC. The physicochemical properties of the conjugates can be discussed as follows: The molecular weights of both the polymer supports are not much different and are large enough to escape glomerular excretion; also, they are linear polymers. Since their molecular weights were considered hardly to be changed by the reaction with MMC-glu-OSU because of its small binding ratio, each conjugate will possess a similar molecular weight. As stated above in the 'conjugates' section, since a hydroxysuccinimide ester of a carboxyl group generally reacts with an amino group at a 1:1

ratio, unreacted amino groups should remain intact. Actually, Tanaka *et al.* reported that the amino groups of a macromolecule decreased by the amount of the glu-MMC introduced.¹⁰⁾ Further, MMC is neutral under a physiological condition.^{12,13)} Therefore, as to the electric charge of the conjugate, considering the conjugation reaction and the drug content, many amino groups are probably maintained in Gly-chitosan-glu-MMC, so the conjugate should be cationic. On the other hand, for *N*-Suc-chitosan, the amino groups are considered to decrease only to small extent by the conjugation, and the conjugate should be anionic because many carboxyl groups remain. Therefore, the main difference in physicochemical characteristics between Gly-chitosan-glu-MMC and *N*-Suc-chitosan-glu-MMC should be their electrostatic property. Since the MMC release from *N*-Suc-chitosan-glu-MMC was similar *in vitro* and *in vivo* (this work) to that from Gly-chitosan-glu-MMC, the difference in their electrostatic properties might be considered to be one of

Fig. 7. Periodic Change in the Tumor Volume for the Sarcoma 180 Solid Tumor Implanted Subcutaneously in Mice with the Intravenous (○) and Intratumoral (□) Administration of MMC (a), Gly-chitosan-glu-MMC (b) and *N*-Suc-chitosan-glu-MMC (c)

●, control (non-treated). Each point represents the mean value $\pm$ S.E. ($n=3-4$). S.E. is shown unless smaller than the point plotted. In each material, the observed time was the same, but the plotted points were slightly moved in order to make each S.E. distinguishable. The initial volume was measured immediately before administration at 4 d after inoculation. Significantly different points in comparison with the control: (a) □, $p<0.05$ at 5 d; ○, $p<0.05$ at 10 d, $p<0.01$ at 9, 15, 17 d, $p<0.001$ at 7, 11, 12 d. (b) □, $p<0.05$ at 4, 6 d; ○, $p<0.05$ at 6 d, $p<0.01$ at 7 d. (c) □, $p<0.05$ at 16 d; ○, $p<0.05$ at 10, 13, 17 d, $p<0.01$ at 16 d.

Fig. 8. Periodic Change in the Ratio of the Tumor Volume to the Initial One for the Sarcoma 180 Solid Tumor Implanted Subcutaneously in Mice at the Intravenous (○) and Intratumoral (□) Administration of MMC (a), Gly-chitosan-glu-MMC (b) and *N*-Suc-chitosan-glu-MMC (c)

●, control (non-treated). Each point represents the mean value $\pm$ S.E. ($n=3-4$). S.E. is shown unless smaller than the point plotted. In each material, the observed time was the same, but the plotted points were slightly moved in order to make each S.E. distinguishable. The initial volume was measured immediately before administration at 4 d after inoculation. Significantly different points in comparison with the control: (a) ○, $p<0.05$ at 9, 10, 11, 12, 15, 16 d, $p<0.01$ at 7, 17 d. (c) ○, $p<0.05$ at 6, 9, 11, 13 d, $p<0.01$ at 16, 17 d.

the reasons for the difference in acute toxicity between the two conjugates. Since cationic polymers generally show high affinity to tissues,¹⁴⁻¹⁹ Gly-chitosan-glu-MMC which is cationic might have a higher affinity to tissues than *N*-Suc-chitosan-glu-MMC, and consequently Gly-chitosan-glu-MMC might be more toxic because of the closer contact of regenerated MMC to the tissues. However, for more detailed discussion, such properties will have to be made clear.

Antitumor Characteristics against Sarcoma 180 The effect of the physicochemical, and especially the electrostatic, characteristics of the polymer support on the efficacy of the conjugates against the solid tumor, Sarcoma 180, inoculated subcutaneously at the axillary part of mice was investigated. Figure 7 shows a periodic change in tumor volume following the intravenous and intratumoral administration of MMC and the conjugates. Figure 8 shows a periodic change in the ratio of tumor volume to

that measured immediately before the administration at 4 d after inoculation (initial volume). The tumor was ellipsoidal in shape, almost spherical. For each material, the initial mean tumor volumes were similar among the control, intravenously and intratumorally administered groups, and the initial individual volumes did not deviate largely around the mean value. That is to say, in MMC, the initial tumor volumes measured immediately before administration were 0.075–0.126, 0.04–0.126 and 0.04–0.126 cm³ for the control, intravenously and intratumorally administered groups, respectively. In Gly-chitosan-glu-MMC, the initial volumes were 0.032–0.048, 0.04–0.075 and 0.048–0.0875 cm³ for the control, intravenously and intratumorally administered groups, respectively. In *N*-Suc-chitosan-glu-MMC, the initial volumes were 0.075–0.126, 0.048–0.126 and 0.0625–0.126 cm³ for the control, intravenously and intratumorally administered groups, respectively. In the intravenous

administration of *N*-Suc-chitosan-glu-MMC, no lethal toxicity was shown at 2.5 mg MMC eq/kg but lethal toxicity was exhibited at a higher dose. This was probably due to the side effect caused by the high viscosity of *N*-Suc-chitosan-glu-MMC. Therefore, it was administered at 2.5 mg MMC eq/kg at which no lethal toxicity was observed with intravenous or intratumoral administration. In this experiment, since the effect was not checked at different doses, their utilities could not be discussed in detail. However, since the tumor growth inhibition represents the degree of antitumor effect, the results in Figs. 7 and 8 should indicate the degree of antitumor effect of each material. The mean tumor volume and the mean tumor growth ratio gave the similar profiles. MMC exhibited significant growth inhibition with the intravenous administration. With the intratumoral injection, MMC exhibited the tendency to inhibit the tumor growth, but showed no significant effect. Gly-chitosan-glu-MMC exhibited a tendency to suppress tumor growth with the intratumoral injection, while with the intravenous injection no inhibitory effect was detected. *N*-Suc-chitosan-glu-MMC showed a tendency to suppress tumor growth with both intravenous and intratumoral administration, and tumor growth was significantly inhibited only with the intravenous administration. Thus, MMC, Gly-chitosan-glu-MMC and *N*-Suc-chitosan-glu-MMC showed different characteristics of tumor growth inhibition. One of the reasons why none of these materials were highly effective with the intratumoral injection could have been that the injected sample leaked out of the tumor solid because the initial tumor volume was very small. As to MMC, as reported previously,²⁰⁾ the growth inhibitory effect against the subcutaneous Sarcoma 180 solid tumor with the subcutaneous administration was equivalent to or better than that with intratumoral injection. Namely, for MMC, intratumoral injection does not necessarily seem to be a superior administration route. Even though MMC solution is injected into the tumor solid, the access of MMC to the tumor cells might be difficult because of the obstruction of tissue walls, etc., which prohibits MMC from contacting to the tumor cells. MMC administered intravenously might gain better access to the tumor cells. For the intravenous administration, the results suggested that *N*-Suc-chitosan-glu-MMC should be effective, whereas Gly-chitosan-glu-MMC should be almost non-effective. This result might be accounted for by the physicochemical characteristics stated in the section, Antitumor Activity against P388 Leukemia. The main difference in physicochemical characteristics between both the conjugates seemed to be an electrostatic property. The effect of the electrostatic characteristics of a conjugate on the accumulation and retention of the conjugate in the tumor site or injected part has been reported before.¹⁴⁻¹⁹⁾ It is generally stated that a cationic polymer possesses the property of exhibiting good retention at the injected site, which is explained by its high affinity to the tissue or organ having anionic charge. On the other hand, the anionic

polymer shows long retention in a body fluid because of its low interaction to tissues or organs, and consequently it accumulates in the diseased part by the enhanced permeability of blood vessels there. Our results on the intravenous administration nearly agreed with such general findings. In order to evaluate the *in vivo* behaviors of the conjugates more minutely, investigation of the effect at the different doses and the body distribution with each administration route will be necessary.

In this work, the *in vivo* characteristics of Gly-chitosan-glu-MMC and *N*-Suc-chitosan-glu-MMC were mainly investigated. Namely, each showed gradual *in vivo* release and good antitumor activity. Further, physicochemical properties, especially the electrostatic characteristics, of the polymer support were suggested to influence the drug action of the conjugate in changing the administration route.

Acknowledgements The authors are very grateful to the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research for the supply of P388 leukemia cells, and express many thanks to the Cancer Cell Repository (CCR) of Tohoku University for the supply of Sarcoma 180 cells.

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