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Conjugate of mitomycin C with *N*-succinyl-chitosan: In vitro drug release properties, toxicity and antitumor activity

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Summary

Macromolecular conjugates between *N*-succinyl-chitosan (Suc-chitosan) and mitomycin C (MMC), referred to as Suc-chitosan-MMC, were prepared. Conjugates with different drug contents were investigated with respect to in vitro drug regeneration rates. Suc-chitosan, MMC and Suc-chitosan-MMC were evaluated for acute toxicity using normal mice, and antitumor activity was investigated using mice bearing P388 leukemia. Some treatment schedules were also studied for the conjugate. The percent rates of MMC release were similar among the conjugates with different drug contents. Suc-chitosan was not toxic on intraperitoneal administration at 2 g/kg. The LD_{50} of the conjugate was about 3-times of that of MMC. Suc-chitosan alone exhibited no antitumor activity. The conjugate showed a higher maximum ILS value than MMC. A mixture of MMC and Suc-chitosan-MMC displayed a much higher ILS value than the conjugate alone at equivalent dose (5 mg eq MMC/kg).

Introduction

In the field of cancer chemotherapy, it is desirable that antitumor drugs are delivered to the tumor sites in sufficient amounts for a long period of time. Many attempts have been made to deliver drugs to the target sites by means of various drug delivery system (Gregoriadis, 1977; Widder et al., 1979; Zaharko et al., 1979). Conjugation of drugs with appropriate macromolecules is known as a useful method for the prolonged retention of the drugs at the target sites or the delivery of the drugs to the target sites. Macromolecular derivatives of MMC such as dextran-MMC conjugate (Kojima et al., 1980; Hashida et al., 1981, 1983; Takakura et al., 1984; Matsumoto et al., 1985, 1986), poly(L-glutamic acid)-MMC conjugate (Kato et al., 1982; Roos et al., 1984), albumin-MMC conjugate (Kaneo et al., 1990) and antibody-MMC conjugate (Kato et al., 1983; Greenfield et al., 1989) have been developed in recent years.

In a previous study, we proposed the possible use of derivatives of chitin and chitosan with carboxyl groups as novel drug carriers for a chemical drug delivery system of MMC, and reported on the preparation procedures and in vitro drug

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release characteristics for the conjugates between the derivatives and MMC (Song et al., 1992).

In the present work, the in vitro drug release characteristics of Suc-chitosan-MMC conjugates with different contents of MMC at physiological pH (pH 7.4) have been examined. Further, the toxicity and antitumor activity of the conjugate against P388 leukemia have been investigated in detail for a thorough evaluation of the usefulness of Suc-chitosan-MMC.

Materials and Methods

Materials

Mitomycin C (MMC), produced by Kyowa Hakko Kogyo Co., was used throughout this work. *N*-Succinyl-chitosan (Suc-chitosan: molecular weight, 3×10^5; degree of *N*-succinylation per glucosamine unit of chitosan, 0.72) was supplied by Katakura Chikkarin Co., Ltd. All other chemicals were reagent-grade products.

Conjugate

Three kinds of *N*-succinyl-chitosan-MMC conjugates were synthesized in the same manner as reported before (Song et al., 1992). Namely, concerning the first conjugate, Suc-chitosan (480 mg), MMC(60 mg) and EDC (1.2 g) were mixed in 150 ml of purified water, and the solution pH was adjusted to 5.0 using 1% HCl aqueous solution. The mixture was stirred for 45 min at room temperature. Subsequently, the resulting precipitate was isolated by filtration, washed sufficiently with water and dried in vacuo. The resulting conjugate was used as *N*-succinyl-chitosan-MMC conjugate (Suc-chitosan-MMC). Two other kinds of conjugates were obtained from the mixture of Suc-chitosan (48 mg), MMC (12 mg) and EDC (240 mg) in 15 ml of purified water, and from that of Suc-chitosan (48 mg), MMC (30 mg) and EDC (600 mg) in 15 ml of purified water. Their reaction and purification were carried out following the same procedure as in the case of the first conjugate except that the solvent volume was different. The conjugates obtained were denoted Suc-chitosan-MMC_M and Suc-chitosan-MMC_H, respectively. The drug content of every conjugate was determined by the method of Song et al. (1992). Namely, the reaction mixture for conjugation was ultrafiltered at the end of the conjugation reaction using an ultrafilter unit (USY-5; Advantec Toyo), with a molecular weight cut-off limit of 5×10^4. Then, the amount of MMC combined with Suc-chitosan was calculated from the concentration of MMC of the filtrate. The drug content of the conjugate (w/w) was estimated as the ratio of the amount of combined MMC to the total amount of combined MMC and Suc-chitosan used. Suc-chitosan-MMC, Suc-chitosan-MMC_M and Suc-chitosan-MMC_H showed MMC contents of 12.2, 21.3 and 33.3% (w/w), respectively. All studies on the toxicity and antitumor activity of the conjugate were carried out using Suc-chitosan-MMC with an MMC content of 12.2% (w/w), which was prepared from a mixture of Suc-chitosan (480 mg), MMC (60 mg) and EDC (1.2 g).

Preparation of drug samples for administration

Unconjugated MMC was dissolved in sterile normal saline. Suc-chitosan and the mixture of MMC and Suc-chitosan were dissolved in the sterile normal saline and air in the resultant solutions was removed by ultrasonication. Suc-chitosan-MMC was ground to a very fine powder in sterile normal saline, using a glass homogenizer with a Teflon pestle, and suspended by sufficient stirring. The solution or suspension obtained was used as a drug sample for administration. Sterile normal saline was used for the control group in the animal experiments. In the acute toxicity studies, Suc-chitosan at a concentration of 4% (w/v) was injected at 0.5 ml/10 g body weight and MMC was administered at 0.14–0.22 ml/10 g body weight. In all other administrations, MMC was injected at 0.1 ml/10 g body weight, and Suc-chitosan-MMC, Suc-chitosan, saline, the mixture of MMC and Suc-chitosan and the mixture of Suc-chitosan-MMC and MMC were injected at 0.2 ml/10 g body weight.

Animals

Male DBA/2 mice, male BDF_1 hybrid mice (C57BL/6, female × DBA/2, male) were obtained from Clea Japan, Inc. Male ddY mice

were purchased from Saitama Experimental Animal Supply Co. These animals were kept on a breeding diet (MF) (Oriental Yeast Co., Ltd, Tokyo), with water ad libitum in a room maintained at 23 ± 1°C and a relative humidity of 60 ± 5%.

In vitro drug release experiment

This test was operated in the same manner as reported previously (Song et al., 1992), as decribed in the following. Each conjugate obtained (10 mg) was suspended in 1/15 M phosphate buffer, pH 7.4 (20 ml), and stirred sufficiently at 37°C. The aliquot samples were withdrawn at appropriate times and were ultrafiltered using the USY-5 ultrafilter unit mentioned above. The amounts of released MMC were determined from the UV absorption of the filtrates at 364 nm using a Jasco Ubest-30 UV/Vis spectrophotometer. This analytical method has been described in detail in the previous report (Song et al., 1992). Namely, the previous study demonstrated that MMC was very stable on incubation in 1/15 M phosphate buffer, pH 7.4, at 37°C for 72 h and that the MMC release profile determined via UV spectroscopy was recognized to be almost equal to that evaluated by HPLC for incubation in 1/15 M phosphate buffer, pH 7.4, at 37°C for 72 h. Therefore, in this work, UV spectroscopy was applied to the determination of the amount of released MMC for convenience of measurement.

Acute toxicity to mice

To evaluate the toxicity of Suc-chitosan, since its aqueous solution was highly viscous, the maximum possible amount for injection was dissolved in about 1 ml of sterile normal saline and administered intraperitoneally to normal ddy mice (6 weeks old) weighing 18–22 g. Namely, Suc-chitosan was injected at a concentration of 4% (w/v) at 0.5 ml/10 g body weight, as stated under *Preparation of drug samples for administration*. The survival of the mice was monitored for 35 days after administration. Acute toxicity of Suc-chitosan-MMC and unconjugated MMC was evaluated from the number of survivors on the 35th day after the single intraperitoneal administration to normal ddY mice, which were 6 weeks old and weighed 18–22 g. The LD_{50} values were determined according to previously reported methods (Van der Waerden, 1940; Zhang, 1985; Kimura et al., 1987; Chujyo, 1988).

Evaluation of antitumor activity

Male DBA/2 mice (5–6 weeks old) were used for maintaining cells. Male BDF_1 mice (6 weeks old) weighing 19–23 g were employed for investigating the antitumor effects of drugs. Ascitic fluid of male DBA/2 mice containing tumor cells was diluted to 1×10^7 P388 leukemia cells/ml with Hanks' balanced salt solution. 1×10^6 P388 leukemia cells, corresponding to a diluted suspension of 0.1 ml, were injected intraperitoneally per mouse for both maintaining cells and investigating antitumor effects. Drug samples were administered intraperitoneally according to the chemotherapeutic schedules described as follows.

First, the chemotherapeutic activities of MMC and Suc-chitosan-MMC were investigated by single administration at 24 h after tumor inoculation. Next, the antitumor activities of Suc-chitosan and the mixture of MMC and Suc-chitosan were examined by single administration at 24 h after tumor inoculation. Moreover, chemotherapy following three different treatment schedules was carried out. This consisted of a single administration of Suc-chitosan-MMC at 5 mg eq MMC/kg at 2 h after tumor inoculation, or the administration of Suc-chitosan-MMC at 5 mg eq MMC/kg was repeated 1, 6 and 11 days after tumor inoculation, or a single administration of a mixture of Suc-chitosan-MMC (4 mg eq MMC/kg) and MMC (1 mg/kg) was performed 24 h after tumor inoculation.

Antitumor activity was evaluated from the ratio of the mean survival time of the treated mice (T) to that of the control mice (C), i.e., by calculating the increase in life span (ILS) given by the equation $((T/C) - 1) \times 100$ (%). The observation period was 60 days.

Results

In vitro drug release rate

Fig. 1 shows a semi-logarithmic plot of MMC remaining in the conjugate vs incubation time for

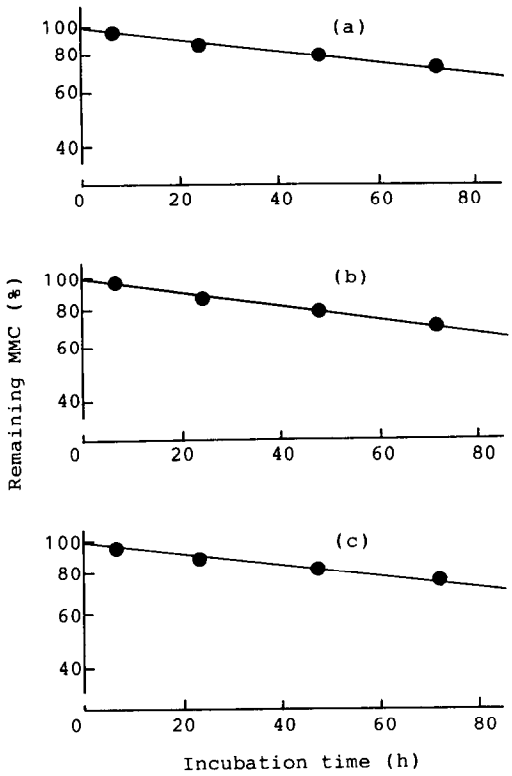

Fig. 1. Semi-logarithmic plots of the amounts of MMC remaining in the conjugates vs incubation time for in vitro drug release in 1/15 M phosphate buffer of pH 7.4 at 37°C. (a) Suc-chitosan-MMC; (b) Suc-chitosan-MMC_M; (c) Suc-chitosan-MMC_H.

the in vitro drug release experiment. All of the conjugates showed monoexponential liberation of MMC. Each drug release rate constant was calculated from the slope of the linear equation fitted to each plot in Fig. 1 using the least-squares technique. The resulting values of the release rate constants were 3.77×10^{-3}, 4.61×10^{-3} and 3.85×10^{-3} h^{-1} for the conjugates containing 12.2, 21.3 and 33.3% (w/w) MMC, respectively, as shown in Table 1. That is to say, in spite of different contents of MMC in the conjugates, their percent drug release rates were of similar magnitude. This meant that the absolute amount of MMC released from conjugates of equal weights over the same time period increased almost proportionally with increasing content of MMC in the conjugate. The absolute amount of MMC released from 1 g of every conjugate at 7 h after incubation is also listed in Table 1.

Acute toxicity to mice

The results obtained on the tolerable dose for intraperitoneal injection of Suc-chitosan to mice were as follows. The maximum injectable single dose of Suc-chitosan was 2 g/kg due to the high viscosity of the sample solution. All tested mice (six animals) were alive 35 days after intraperitoneal injection. Therefore, the maximum tolerable dose for the intraperitoneal injection of Suc-chitosan to mice was determined to be more than 2 g/kg. Fig. 2 illustrates the dose-toxicity relationship in normal ddY mice receiving a single intraperitoneal injection of MMC and Suc-chitosan-MMC. Table 2 shows the results on the acute toxicities of MMC and Suc-chitosan-MMC, which were calculated for the data in Fig. 2. The LD_{50} values of MMC and Suc-chitosan-MMC were 9.0 mg/kg and 25.0 mg eq MMC/kg, respectively.

TABLE 1

Drug release characteristics of the conjugates with different contents of MMC in the physiological pH (pH 7.4) [a]

Conjugate [b]	MMC content of the conjugate (%) (w/w)	Regeneration rate constant of MMC from the conjugate (h^{-1})	Half-life of the conjugate (h)	Absolute amount of MMC released per g of the conjugate at 7 h (μg)
Suc-chitosan-MMC	12.2	3.77×10^{-3}	184	4.4
Suc-chitosan-MMC_M	21.3	4.61×10^{-3}	150	7.8
Suc-chitosan-MMC_H	33.3	3.85×10^{-3}	180	13.0

[a] 1/15 M phosphate buffer (pH 7.4) was used as incubation medium.

[b] Suc-chitosan-MMC_M and Suc-chitosan-MMC_H denote the Suc-chitosan-MMC conjugates with medium and high contents of MMC, respectively. Only Suc-chitosan-MMC with an MMC content of 12.2% (w/w) was used in the in vivo study.

TABLE 2

Acute toxicity of MMC and Suc-chitosan-MMC to normal ddY mice [a]

Material	Number of dose levels	Number of mice in each dose level	LD_{50} (mg eq MMC/kg)	95% confidence interval (mg eq MMC/kg)	Days observed
MMC	4	6	9.00	8.09–10.0	35
Suc-chitosan-MMC	4	6	25.0	20.2 –30.9	35

[a] This evaluation was performed on the basis of the lethal toxicity following a single intraperitoneal administration.

The 95% confidence intervals of LD_{50} are also given in Table 2.

Antitumor activity against P388 leukemia

Table 3 describes the effects of Suc-chitosan-MMC and unconjugated MMC on the life span of mice bearing P388 leukemia after single intraperitoneal administration. Suc-chitosan-MMC showed a maximum ILS value of 119.7% at a dose of 20 mg/kg. In this case, on omitting the data for the mouse surviving for more than 60 days after tumor inoculation from the calculation of ILS, the ILS value was 72.7% and significantly different from the control group ($p < 0.05$). Unconjugated MMC showed a maximum ILS value of 87.1% at a dose of 2.5 mg/kg. In this case, again excluding the results for the mouse surviving for more than 60 days after tumor inoculation, the ILS value was 33.3% and significantly different from the control group ($p < 0.05$). MMC was observed to be toxic at a dose of 10 mg/kg. The antitumor activity of Suc-chitosan and that of the mixture of MMC and Suc-chitosan are detailed in Table 4. The mixture of MMC and Suc-chitosan gave a maximum ILS value of 213.3% at a dose of 5 mg MMC/kg and 37 mg Suc-chitosan/kg, and two animals (out of a total of six mice) survived for more than 60 days. In this case, also omitting the data for the mice surviving longer than 60 days after tumor inoculation, the ILS value was calculated to be 92.6%. Suc-chitosan alone was unable to exhibit any significant antitumor effect at any dose.

TABLE 3

Antitumor effect of MMC and Suc-chitosan-MMC on the survival time of mice bearing P388 leukemia [a]

Material	Dose (mg eq MMC/kg)	Survival days (mean ± S.D.)	ILS (%)	Survivors at 60 days
Control		13.2 ± 2.6		0/6
MMC	1.0	14.3 ± 2.3	8.3	0/6
	2.5	24.7 ± 17.4	87.1	1/6
		(17.6 ± 2.4 [c]) [b]		
	5.0	24.0 ± 9.3 [c]	81.8	0/6
	10.0	14.3 ± 5.6	8.3	0/6
Suc-chitosan-MMC	2.5	16.5 ± 2.8	25.0	0/6
	5.0	18.5 ± 5.0 [c]	40.2	0/6
	10.0	19.0 ± 5.3 [c]	43.9	0/6
	20.0	29.0 ± 16.5	119.7	1/6
		(22.8 ± 7.3 [c]) [b]		
	25.0	23.0 ± 3.6 [d]	74.2	0/6

[a] Chemotherapy was carried out by single intraperitoneal administration at 24 h after tumor inoculation. Sterile normal saline was injected at 24 h after tumor inoculation for the control group.

[b] The values in parentheses show the mean survival days calculated from mice dying with 60 days.

[c] $p < 0.05$, vs the control group.

[d] $p < 0.001$, vs the control group.

TABLE 4

Antitumor effect of Suc-chitosan and the mixture of MMC and Suc-chitosan on the surival time of mice bearing P388 leukemia [a]

Material	Dose			Survival days (mean ± S.D.)	ILS (%)	Survivors at 60 days
	MMC (mg/kg)	+	Polymer (mg/kg)			
Control				10.8 ± 1.0		0/6
Suc-chitosan	0	+	18.5	11.0 ± 1.1	1.9	0/6
	0	+	37.0	11.8 ± 2.1	9.6	0/6
	0	+	74.0	10.7 ± 0.5	−1.2	0/6
	0	+	148.0	12.2 ± 2.6	12.6	0/6
MMC + Suc-chitosan	2.5	+	18.5	17.5 ± 2.7 [b]	62.0	0/6
	5.0	+	37.0	33.8 ± 20.9 [c] (20.8 ± 6.6) [d]	213.3	2/6
	10.0	+	74.0	12.5 ± 6.4	15.7	0/6
	20.0	+	148.0	5.5 ± 0.6	−49.1	0/6

[a] Chemotherapy was carried out by single intraperitoneal administration at 24 h after tumor inoculation. Sterile normal saline was injected at 24 h after tumor inoculation for the control group.
[b] $p < 0.05$, vs the control group.
[c] $p < 0.01$, vs the control group.
[d] The values in parentheses show the mean survival days calculated from mice dying within 60 days.

The effect of the treatment schedules was also examined, the results being summarized in Table 5. At a dose of 5 mg eq MMC/kg, the treatment with Suc-chitosan-MMC at 2 h after tumor inoculation led to a much higher ILS value, compared with that at 24 h after tumor inoculation. Namely, the ILS value of mice treated at 2 h after tumor inoculation was 142.5%, corresponding to a 3.5-fold greater value than that of mice treated at 24 h after inoculation with the same dose (5 mg eq MMC/kg). Repeated administration of the conjugate at a dose of 5 mg eq MMC/kg at 1, 6 and 11 days after tumor inoculation was observed to yield an ILS value similar to that for the treatment at 2 h after inoculation. Further, when the mixture of the conjugate (4 mg eq MMC/kg) and unconjugated MMC (1 mg/kg) was administered at 24 h after inoculation, the ILS value was also observed to be similar to that in the case of treatment using Suc-chitosan-MMC at 2 h after tumor inoculation at a dose of 5 mg eq MMC/kg or of that using Suc-chitosan-MMC 1, 6 and 11

TABLE 5

Antitumor effect of treatment schedules of Suc-chitosan-MMC on the survival time of mice bearing P388 leukemia [a]

Material	Dose (mg eq MMC/kg)	Treatment schedule [b]	Survival days (mean ± S.D.)	ILS (%)	Surivors at 60 days
Control			9.8 ± 0.8		0/6
Suc-chitosan-MMC	5.0	2 h	23.8 ± 9.3 [d]	142.5	0/6
	5.0, 5.0, 5.0	1, 6, 11 d	23.3 ± 3.6 [e]	137.4	0/6
Suc-chitosan-MMC + MMC	4.0 + 1.0 [c]	24 h	23.2 ± 2.9 [e]	135.6	0/6

[a] Chemotherapy was carried ou in the i.p.–i.p. system. Sterile normal saline was injected at 2 h after tumor inoculation for the control group.
[b] Expressed as the time for the drug to be administered after tumor inoculation.
[c] Mixture of Suc-chitosan-MMC (4 mg eq MMC/kg) and MMC (1 mg/kg).
[d] $p < 0.05$, vs the control group.
[e] $p < 0.001$, vs the control group.

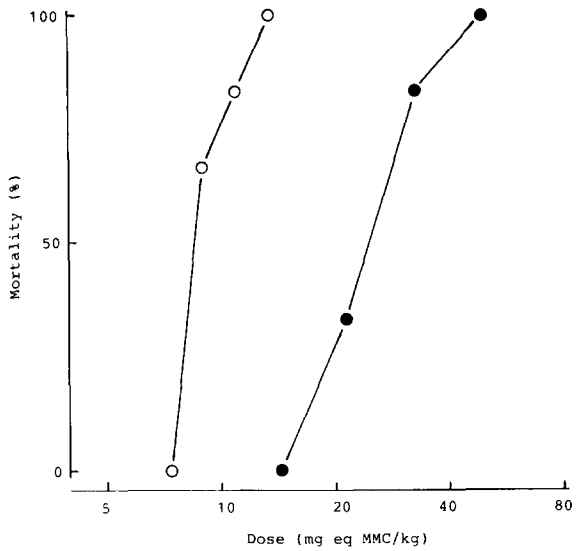

Fig. 2. Relationship between dose and mortality. Each point represents the result of six mice receiving a single administration of the drug. For each dose, mortality (%) on the 35th day after administration is exhibited. (○) MMC; (●) Suc-chitosan-MMC.

days after tumor inoculation at a dose of 5 mg eq MMC/kg.

Discussion

The clinical use of MMC is limited due to its toxic effect to host normal cells. Recently, in order to overcome this problem, various kinds of macromolecules have been utilized as carrier moieties for the purpose of obtaining useful prodrugs of MMC. Some macromolecular prodrugs of MMC have previously been reported to possess suitable pharmacological activity and to suppress toxicity (Kojima et al., 1980; Hashida et al., 1981, 1983; Kato et al., 1982; Roos et al., 1984; Takakura et al., 1984; Matsumoto et al., 1985, 1986).

In previous work it was found that Suc-chitosan scarcely passed the USY-5 ultrafilter membrane, that the adsorption of MMC to Suc-chitosan and the ultrafilter membrane was slight, and that the decomposition of MMC was considerably small in extent during the conjugation reaction (Song et al., 1992). Therefore, MMC combined with Suc-chitosan could be estimated as MMC unfiltered following ultrafiltration, and the MMC content could be established based on the ratio of the amount of combined MMC to the total amount of combind MMC and Suc-chitosan used. The conjugate was wholly water-insoluble. Since the only washing of the conjugate was simply with purified water in the final step of preparation, the MMC content remained at the value calculated above. The resulting powder of the conjugate was considered to have a uniform MMC content, since it was prepared via uniformly mixing the reactants. Therefore, dispersion of the density of MMC in the conjugate was considered to be slight. Moreover, for further uniformity of the density of MMC in the conjugate, the conjugate powder was used after it had been sufficiently mixed. Thus, unless a small quantity of conjugate was being measured, it was considered feasible to estimate the amount of MMC contained in the conjugate from the MMC content calculated above. In this work, the minimum amount of conjugate measured was not less than 10 mg, and all samples for every in vivo test were prepared by the dilution of the samples for the highest dose. Therefore, the amounts of MMC contained in all the prepared samples were considered to be estimable from the MMC content calculated above in the ultrafiltration study.

Previous work revealed that the drug content could be changed by altering the ratio of the reaction components and that the rate of hydrolysis of the bond between Suc-chitosan and MMC followed pseudo-first order kinetics for the conjugate with a drug content of 12% (w/w) (Song et al., 1992). Since the conjugate with a higher drug content was expected to be similarly bound, it was expected to display a comparable drug release rate. This proposition was confirmed for more exact characterization of the conjugate in this work. Namely, the in vitro drug release rates of conjugates with MMC contents of 12.2, 21.3 and 33.3% (w/w) were investigated in 1/15 M phosphate buffer, pH 7.4 at 37°C. As a result, the percent ratio of MMC released to the total loaded MMC was similar for each conjugate irrespective of whether the drug content of Suc-chitosan-MMC was changed (Fig. 1 and Table 1). From

these results, the absolute amount of MMC released per g of conjugate at any time point was considered to increase almost proportionally with its MMC content. Therefore, it could be stated that the amount of MMC released over a definite period could be controlled varying the drug content in the conjugate as well as the amount of Suc-chitosan-MMC. The in vitro results showed that MMC was liberated slowly from Suc-chitosan-MMC. Therefore, it was suggested that Suc-chitosan-MMC could provide a sustained supply of free MMC.

On the basis of the biodegradation of the structure of Suc-chitosan being proposed to be negligible and since Suc-chitosan-MMC was a water-insoluble product, it was expected that the retention of the conjugate in the body fluid would be considerable. The release of MMC from Suc-chitosan-MMC was very slow and Suc-chitosan was considered to be of low toxicity, being an anionic derivative of chitosan. From these considerations, it was suggested that Suc-chitosan-MMC should be less toxic and act effectively at a higher dose than MMC alone. Thus, in order to clarify the above propositions, evaluation of the in vivo characteristics of Suc-chitosan and Suc-chitosan-MMC, i.e., their toxicity and antitumor effects, was performed. It was found that Suc-chitosan was non-toxic at a dose of 2 g/kg. Further, it was observed that the toxicity of Suc-chitosan-MMC was only about one-third of that of free MMC (Table 2). Considering Suc-chitosan to be non-toxic at a dose of 2 g/kg, the toxicity of Suc-chitosan-MMC could be estimated to be due to the free MMC liberated from the conjugate. Table 3 shows that the optimum dose of Suc-chitosan-MMC was 20 mg eq MMC/kg. The optimum dose of unconjugated MMC was observed to be 2.5 mg/kg. Suc-chitosan-MMC showed a higher maximum ILS value than unconjugated MMC. This suggested that Suc-chitosan-MMC at a dose of 20 mg eq MMC/kg could deliver MMC for a prolonged period, thereby exerting a stronger therapeutic effect. On the other hand, since unconjugated MMC was superior to the conjugate at a dose of 2.5 or 5 mg eq MMC/kg, it could be supposed that the amount of MMC liberated from the conjugate in the low dose range was insufficient to produce strong chemotherapeutic activity.

Suc-chitosan alone did not show any significant antitumor activity (Table 4). The ILS value for the mixture of MMC (5 mg/kg) and Suc-chitosan (37 mg/kg) given in Table 4 was more than 2-fold that for MMC (5 mg/kg) alone in Table 3. When the in vivo activity was examined using the mixture of MMC and Suc-chitosan at the same time as MMC alone, their effects could be compared directly and more clearly. Although the control in Table 4 shows a survival time more than 2 days shorter that in Table 3, that of the mixture of MMC (5 mg/kg) and Suc-chitosan (37 mg/kg) in Table 4 is more than 9 days longer than that of MMC (5 mg/kg) alone in Table 3. Therefore, the former was considered to exert a better effect than the latter. Thus, Suc-chitosan was suggested to strengthen the effect of MMC. It was hypothesized that the higher maximum ILS value was attained partly because spreading of MMC into the body fluid might be suppressed by the interaction with Suc-chitosan or by the viscosity of Suc-chitosan and subsequently the retention of MMC might increase. Also, although the biological effect of Suc-chitosan, other than the physical effect stated above, could be supposed, it was ambiguous. In order to elucidate this point, further detailed investigation would be needed. However, the decrease in ILS, considered to be due to the toxic effect of MMC, was observed on administration of the mixture of MMC (10 mg/kg) and Suc-chitosan (74 mg/kg). This phenomenon was analogous to that in the case of administration of MMC alone at a dose of 10 mg/kg (Table 3). Therefore, simple addition of Suc-chitosan to MMC was found to be ineffective at decreasing the toxic effect of MMC.

When the treatment schedules of Suc-chitosan-MMC were changed, marked antitumor effects were observed to result (Table 5). The treatments scheduled in Table 5 produced more than 3-fold greater ILS values than that for Suc-chitosan-MMC (5 mg eq MMC/kg) in Table 3. The control group in Table 5 was treated with normal saline at 2 h after inoculation, while that in Table 3 was carried out at 24 h after inoculation. However, the difference in the time of injection of

normal saline was considered to lack any significant influence on the survival time due to its inertness. When the treatments scheduled in Table 5 were carried out at the same time as treatment with Suc-chitosan-MMC (5 mg eq MMC/kg), their effects could be compared directly and more clearly. The control in Table 5 shows a survival time more than 3 days shorter than that in Table 3. Neverthless, each treatment listed in Table 5 was found to show a survival time more than 4 days longer than that in the case of Suc-chitosan-MMC (5 mg eq MMC/kg) in Table 3. Therefore, the treatments scheduled in Table 5 were considered to have a greatter effect than Suc-chitosan-MMC (5 mg eq MMC/kg). The treatment with Suc-chitosan at a dose of 5 mg eq MMC/kg at 2 h after inoculation produced a marked prolongation of life span. The early treatment using Suc-chitosan-MMC was suggested to be very useful. Further, the treatment with repeated Suc-chitosan-MMC doses of 5 mg eq MMC/kg at 1, 6 and 11 days after tumor inoculation resulted in a markedly high ILS value. The treatment with Suc-chitosan-MMC according to the multiple dose regimen was considered to be very useful. Regarding the use of the mixture of the conjugate and free MMC, a higher ILS value was obtained than with the conjugate alone or unconjugated MMC alone in Table 3 at the equivalent dose (5 mg eq MMC/kg). The good chemotherapeutic activity of the mixture of the conjugate and MMC could be presumed to be due to the initial killing effect on tumor cells by unconjugated MMC and the sustained action on the residual tumor cells by MMC delivered gradually from the conjugate. The treatment with the mixture of the conjugate and free MMC could be evaluated to be very useful for achieving high antitumor activity.

As a result of the present work, it can be concluded that Suc-chitosan-MMC exerts an action as a macromolecular prodrug of MMC, delivering free MMC for a prolonged period, reducing the toxicity of MMC, and maintaining the therapeutic potency of the parent drug. Further, the development of a treatment schedule using Suc-chitosan-MMC would make it possible to enhance the chemotherapeutic effect of Suc-chitosan-MMC. Taking into consideration the fact that Suc-chitosan-MMC is an insoluble material, it is supposed that the conjugate should be capable of remaining at a site of administration and act there as a potential source of MMC for a long period. Therefore, Suc-chitosan-MMC can be concluded to be of value not only as a source of MMC, to provide prolonged release, but also as a local targeting system to a localized cancer.

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