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Efficacy of lactosaminated and intact *N*-succinyl-chitosan–mitomycin C conjugates against M5076 liver metastatic cancer

Yoshinori Kato, Hiraku Onishi and Yoshiharu Machida

Abstract

In this study, lactosaminated *N*-succinyl-chitosan (Lac-Suc) was investigated for its liver targeting ability in the early metastatic stage of liver cancer, and subsequently Lac-Suc–mitomycin C conjugate (Lac-Suc-MMC) and highly-succinylated *N*-succinyl-chitosan (Suc(II))–MMC conjugate (Suc(II)-MMC) were examined for efficacy against the liver metastasis. Mice into which M5076 cells were inoculated intravenously were used as liver metastatic models. Fluorescently labelled Lac-Suc (Lac-Suc-FTC) was intravenously administered at a daily dose of 0.2 mg/mouse for 4 days or at a single dose of 0.8 mg/mouse at 3 days post-inoculation. At a dose of 0.2 mg/mouse for 4 days, liver accumulation of Lac-Suc-FTC was increased after all except the fourth injection, indicating that the capacity of accumulation might be limited to around 110 μg per mouse with repeated daily administration at 0.2 mg/mouse. As to the efficacy of intravenous administration at 7 days post-inoculation, Lac-Suc-MMC was less effective at a dose of 1 mg kg^{-1} for 4 days than a single dose of 4 mg kg^{-1}. This result was not in accordance with that expected from the biodistribution study. On the other hand, with intravenous administration at 3 days post-inoculation, Suc(II)-MMC was more effective on repeated administration, and it showed higher efficacy than Lac-Suc-MMC at both 1 mg kg^{-1} for 4 days and 4 mg kg^{-1} as a single dose. Further, with intravenous administration at 3 days post-inoculation, Suc(II)-MMC exhibited a much higher survival effect at a dose of 4 mg kg^{-1} for 4 days.

Department of Drug Delivery Research, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan

Yoshinori Kato, Hiraku Onishi, Yoshiharu Machida

Correspondence: Y. Kato, Department of Drug Delivery Research, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan. E-mail: yk-no.1@jcom.home.ne.jp

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Introduction

We have been studying the possible usefulness of the chitosan derivative *N*-succinyl-chitosan as a drug carrier (Song et al 1993, 1996; Sato et al 1996; Kamiyama et al 1999; Kato et al 2000a, b, c, 2001a, b). Since it is retained for long periods in the systemic circulation after intravenous administration (Kamiyama et al 1999; Kato et al 2000a) and has low toxicity (Song et al 1993; Izume 1998), *N*-succinyl-chitosan is expected to be useful as a safe systemic long-circulating carrier. Recently, there has been a great deal of interest in liver targeting using various glycosylated macromolecules (Pimm et al 1996; Fiume et al 1997; Mahato et al 1997; Akamatsu et al 1998; Di Stefano et al 2000; Hashida et al 2000), microparticles with suitable diameter (Ogawara et al 1999a, b) and liposomes (Shimizu et al 1998; Yamamoto et al 2000) as drug carriers. Similarly, it was confirmed that connecting lactose to *N*-succinyl-chitosan enabled selective distribution to the liver in mice (Kato et al 2001a).

It is important for cancer chemotherapy to determine the administration schedules that present higher efficacy and lower toxicity (Sokoloff et al 1959; Hata

et al 1961; Kojima et al 1972). In the case of polymer–drug conjugates, biodistribution of the polymer is importantly related to the therapeutic efficacy. In the liver metastatic tumour model used in this study, M5076 cells (Hart et al 1981; Talmadge et al 1981; Perez-Soler et al 1987), differences in the timing of injection following inoculation influenced the biodistribution of *N*-succinyl-chitosan and lactosaminated *N*-succinyl-chitosan (Lac-Suc) (Kato et al 2001b). Therefore, biodistribution characteristics dependent on the administration schedule (i.e. single or repeated administration) have to be elucidated. We investigated differences in biodistribution of Lac-Suc between single and repeated administration. Further, the antitumour effects of Lac-Suc–mitomycin C conjugate (Lac-Suc-MMC), against M5076 cells as a liver metastatic tumour model, were examined after single or repeated intravenous administration. Similarly, the antitumour effect of water-soluble highly-succinylated *N*-succinyl-chitosan–mitomycin C conjugate (Suc(II)-MMC) against M5076 cells was also examined. Finally, administration schedules of Lac-Suc-MMC and Suc(II)-MMC were evaluated based on these results from the viewpoint of efficacy.

Materials and Methods

Materials

Mitomycin C (MMC) was purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan). *N*-Succinyl-chitosan sodium salt (succinylation degree 0.81 mol/sugar unit, deacetylation degree 1.0 mol/sugar unit) was kindly provided by Katakura Chikkarin Co. Ltd. (Tokyo, Japan). Its degrees of succinylation and deacetylation were measured using proton nuclear magnetic resonance (Kato et al 2000a), and the average molecular weight was determined to be 3.4×10^5 (range: 5×10^4 to 1.5×10^6) by size exclusion chromatography–multi angle light scattering. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Dojindo Laboratories (Kumamoto, Japan). Lactose and fluorescein isothiocyanate (FITC) were obtained from Sigma Chemical Company (St Louis, MO). Sodium cyanoborohydride ($NaBH_3CN$) was obtained from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). All other chemicals were purchased as reagent-grade products.

Animals and tumours

Male C57BL/6 mice weighing approximately 20 g at the age of 6 weeks were purchased from Tokyo Laboratory Animals Science Co. Ltd (Tokyo, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hoshi University. All mice were housed in a pathogen-free environment. Five or six mice were used in each group to examine in-vivo antitumour effects (n = 5, 6). Four or five mice were used at each time point in distribution experiments (n = 4, 5).

Murine reticulum cell sarcoma M5076 cells were used as tumour cells. M5076 cells were maintained in C57BL/6 mice by intraperitoneal transfer of 1×10^5 cells obtained from ascitic fluid every other week. In the in-vivo antitumour activity tests, 1×10^5 M5076 cells suspended in 0.1 mL of Hanks' balanced salt solution, which were obtained from tumour-bearing mice, were inoculated intravenously into each male C57BL/6 mouse.

Accumulation of Lac-Suc-FTC into the liver at repeated administration

Lac-Suc was prepared by reductive amination between *N*-succinyl-chitosan and lactose using $NaBH_3CN$, and Lac-Suc-FTC was prepared by reaction of Lac-Suc and FITC as described previously (Kato et al 2001a). The lactosamine residue content of Lac-Suc was determined to be approx. 0.30 mol/sugar unit by elemental analysis (Yanako Analytical Industrial Co., Japan). Lac-Suc-FTC was used throughout the biodistribution studies. The distribution of Lac-Suc-FTC in M5076-bearing mice was examined as follows. After intravenous inoculation with M5076 cells, 0.8 mg of Lac-Suc-FTC was administered intravenously on day 3 (single dose) or 0.2 mg of Lac-Suc-FTC was intravenously injected repeatedly on each of days 3–6 (repeated dose). In the case of repeated administration, the mice were sacrificed at 1, 8 or 24 h after each injection, blood samples were withdrawn and the liver was enucleated. The liver was washed with phosphate buffered saline, pH 7.4 (PBS), gently blotted using filter paper and weighed. A threefold volume of PBS was added, and the mixture was homogenized using a glass homogenizer with a Teflon pestle. The supernatant was obtained by centrifugation (3000 rev min^{-1}, 10 min). Plasma was obtained by centrifugation of the blood. The supernatant and plasma were diluted appropriately with PBS, and their fluorescence intensities were investigated (Ex = 495 nm, Em = 520 nm). The blank sample was obtained by injecting normal saline alone into mice instead of Lac-Suc-FTC solution. The concentration of Lac-Suc-FTC in the sample was determined from the net fluorescence in-

tensity obtained by subtracting the fluorescence intensity of the blank from that of each sample based on the standard calibration curve. The concentration was corrected by the recoveries calculated in advance (Kato et al 2001a). The distributed amount was calculated from the concentration and tissue weight. The amount of Lac-Suc-FTC in plasma was calculated using the reported volume of mouse plasma, 48.8 mL kg^{-1} (Tajima 1989). To determine the biodistribution of Lac-Suc-FTC administered as a single dose, blood and liver samples were taken at 1, 8, 24, 48, 72 and 96 h after intravenous injection. The distributed Lac-Suc-FTC was measured and determined in the same manner as described for the repeated administration schedule.

The cumulative collection of urine was executed simultaneously in the biodistribution studies. Mice were placed separately in metabolite cages immediately after administration. Urine was collected for 24 h after each intravenous injection (repeated dose) or 24, 48, 72 and 96 h after intravenous administration (single dose), and then urine volume was measured. The subsequent procedure was performed as reported previously (Kato et al 2000a). The total amount excreted in urine was calculated from the concentration and urine volume.

Pharmacokinetic data analysis

The areas under the plasma or liver concentration–time curves for $t_1 - t_2$ h ($AUC_{t1-t2\,h}$) and the mean residence time ($MRT_{t1-t2\,h}$) were calculated by the trapezoidal method (Yamaoka et al 1981). Equation 1 was employed to determine relative effectiveness of liver targeting (R_{et}):

$$R_{et} = AUC^{liver}_{t1-t2\,h}/AUC^{plasma}_{t1-t2\,h} \quad (1)$$

In-vitro release characteristics of Lac-Suc-MMC

Lac-Suc-MMC conjugate was synthesized as reported previously except that Lac-Suc was used instead of highly-succinylated *N*-succinyl-chitosan (Kato et al 2000b). The release of mitomycin C from Lac-Suc-MMC in 1/15 M phosphate buffer (pH 6.0 and 9.0) and in the mixture of 1/15 M phosphate buffer (pH 7.4) and mouse plasma (4:1, v/v), named 20% (v/v) mouse plasma, were investigated according to the method described in the previous report (Kato et al 2000b). Briefly, the amount of free mitomycin C released was measured using high-performance liquid chromatography (HPLC), which was carried out using a Shimadzu LC-6AD apparatus equipped with a SUMIPAX Nucleosil $5C_{18}$ reversed-phase column (4 × 250 mm) and an SPD-10AV UV detector (Shimadzu) set at 365 nm. The mobile phase was a mixture of 0.01 M phosphate buffer, pH 6.0, and methanol (65:35, v/v). The samples obtained from the buffers were directly injected onto the HPLC system. The samples obtained from 20% (v/v) mouse plasma were firstly mixed with a 10-fold volume of the mixture of chloroform and 2-propanol (1:1, w/w). After centrifuging the mixture, the whole supernatant was decanted and evaporated to dryness below 40°C under nitrogen gas. The residue was dissolved in methanol, and the solution was analysed for mitomycin C in the HPLC system stated above. All determinations were performed using three samples.

Antitumour effects of Suc(II)-MMC or Lac-Suc-MMC against M5076-bearing mice

Highly-succinylated Suc (Suc(II)) was prepared by reaction of Suc with succinic anhydride and then a water-soluble Suc(II)–MMC conjugate (Suc(II)-MMC) was also prepared (Kato et al 2000b). Mitomycin C contents of Suc(II)-MMC and Lac-Suc-MMC, measured in the manner previously reported (Kato et al 2000b), were about 12 and 20% (w/w), respectively. The doses reported for mitomycin C conjugates refer to the quantity of parent mitomycin C contained in the conjugate.

Antitumour effects were examined using mice intravenously inoculated with M5076 cells 7 days previously. Namely, at 7 days post-inoculation, mitomycin C, Suc(II)-MMC and Lac-Suc-MMC were intravenously administered at a single dose of 4 mg kg^{-1} or repeatedly at a dose of 1 mg kg^{-1} on each of days 7–10 following inoculation (total dose of 4 mg kg^{-1}). Controls were injected with a similar volume of saline alone according to the same schedules. For all mice, the survival time after inoculation was observed for 50 days after inoculation. The antitumour effects were measured by comparing the mean survival time of the treated mice (T) with that of controls (C); the increase in life span (ILS) was calculated by equation 2:

$$ILS = (T/C - 1) \times 100\,(\%) \quad (2)$$

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

Antitumour effects using mice intravenously inoculated with M5076 cells 3 days previously were also investigated in the same way as stated above for treatment at 7 days post-inoculation. In the mice treated at 3 days post-inoculation, the efficacies of Suc(II)-MMC and Lac-Suc-MMC were also examined by intravenous administration at a dose of 4 mg kg^{-1} given on 4 days.

Statistical analysis

With the exception of the survival tests, the statistical analysis was performed using Student's *t*-test for unpaired data. For the survival tests, Kaplan–Meier curves were constructed and the survival ratios were compared by the Mantel–Cox log-rank test. Differences were considered significant when the *P* value was less than 0.05.

Results

Accumulation of Lac-Suc-FTC in the liver

The distribution profiles in the liver and the elimination from plasma of Lac-Suc-FTC after intravenous administration are shown in Figure 1. The amount of Lac-Suc-FTC in plasma was calculated using the reported volume of mouse plasma, 48.8 mL kg^{-1} (Tajima 1989). The distribution of Lac-Suc-FTC to the liver at a single dose reached 66 μg per mouse at 8 h after injection, and remained at this level throughout the 96-h observation period. Following repeated administration, however, the plasma level of Lac-Suc-FTC declined rapidly at a similar rate after each injection, and accumulation in the liver showed a similar profile until 72 h. The distribution profile after the fourth injection suggested that the accumulation might reach a plateau (approximately 110 μg per mouse).

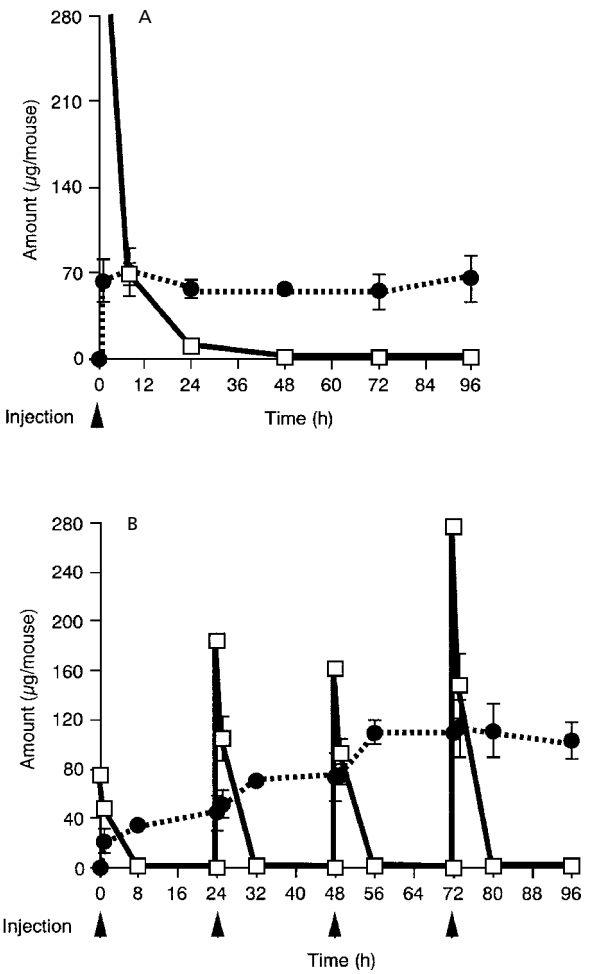

Figure 1 Plasma level (□) and accumulation of Lac-Suc-FTC in the liver (●) after intravenous administration to M5076-bearing mice at a dose of 0.8 mg (single dose) (A) or 0.2 mg given on each of 4 days (B) per mouse. Arrow shows intravenous injection. At 3 days after intravenous inoculation, test substance (0.2 mL) was injected intravenously. Each point represents the mean±s.d. (n = 4–5).

Table 1 Pharmacokinetic parameters of Lac-Suc-FTC after intravenous administration of a single dose of 0.8 mg (0.2 mL) or repeated injection of 0.2 mg (0.2 mL) given on each of 4 days per M5076-bearing mouse at 3 days post-inoculation.

		$AUC_{0-96\,h}$ (h μg mL^{-1} or g^{-1})	$MRT_{0-96\,h}$ (h)	R_{et}
Single dose	Liver	5330	47.0	1.6
	Plasma	3400	5.5	
Repeated administration	Liver	8010	59.0	3.2
	Plasma	2530	5.0	

Since mice were sacrificed at each time point for measurement of plasma concentration, the s.d. for AUC and MRT could not be calculated. Therefore, the mean values of the data are shown. R_{et} values indicate the relative effectiveness of liver targeting and were calculated by the following equation: $R_{et} = AUC^{liver}_{0-96\,h}/AUC^{plasma}_{0-96\,h}$.

Table 2 Urinary excretion of Lac-Suc-FTC after intravenous administration of a single dose of 0.8 mg (0.2 mL) or repeated injection of 0.2 mg (0.2 mL) given on each of 4 days per M5076-bearing mouse at 3 days post-inoculation.

	Urinary excretion (μg)			
	24 h	48 h	72 h	96 h
Single dose	20.1±9.4	26.9±11.5	32.8±6.9	38.0±6.9
Repeated administration	3.1±2.4	2.6±1.4	7.1±5.0	9.5±6.7

Each value represents the mean±s.d. (n = 4 or 5).

Pharmacokinetic parameters of Lac-Suc-FTC administered as a single dose or by repeated injection are summarized in Table 1. $AUC^{liver}_{0-96\,h}$ following injection four times at a dose of 0.2 mg was larger than that after a single injection at a dose of 0.8 mg. Conversely,

$AUC^{plasma}_{0-96\,h}$ of the former was lower than that of the latter. R_{et} following repeated administration was double that following a single injection; repeated administration of Lac-Suc-FTC was thus considered more effective for liver targeting than a single injection.

Table 2 shows the cumulative urinary excretion of Lac-Suc-FTC. The urinary excretion of Lac-Suc-FTC was much lower with repeated administration.

In-vitro release characteristics of Lac-Suc-MMC

Figure 2 shows the in-vitro release properties of Lac-Suc-MMC. The release profiles from Suc(II)-MMC were described in the same figure by referring to the previous paper (Kato et al 2000b). Lac-Suc-MMC showed a pH-dependent release similar to Suc(II)-MMC. Lac-Suc-MMC showed faster release than Suc (II)-MMC. Fifty-percent mitomycin C release time of Lac-Suc-MMC was about 1 day in 20% (v/v) mouse plasma. Under each condition, similar release profiles were observed for both the conjugates.

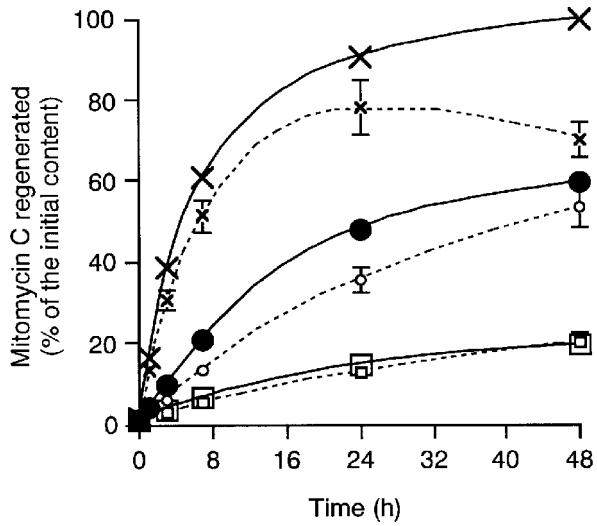

Figure 2 Release of mitomycin C from Lac-Suc-MMC and Suc(II)-MMC in 1/15 M phosphate buffer (pH 6.0 (□) and 9.0 (×)) and the mixture of 1/15 M phosphate buffer (pH 7.4)–mouse plasma (4:1, v/v) (●, ○) at 37°C. Each point represents the mean±s.d. (n = 3). Large symbols show the release of mitomycin C from Lac-Suc-MMC and small symbols show that from Suc(II)-MMC. The data relating to Suc(II)-MMC are from our previous report (Kato et al 2000b).

Antitumour effects of Suc(II)-MMC and Lac-Suc-MMC against M5076

Table 3 shows the therapeutic efficacy of Suc(II)-MMC and Lac-Suc-MMC at a single or repeated dose using mice inoculated 7 days previously with M5076 cells. The best ILS value was observed after a single dose of 4 mg kg^{-1} of mitomycin C; however, this dose caused the greatest loss of body weight, which did not lead to death (data not shown). A single dose of Suc(II)-MMC, 4 mg kg^{-1}, showed a similar good ILS value, with no significant loss of body weight. Lac-Suc-MMC exhibited a lower ILS value than single 4 mg kg^{-1} doses of mitomycin C or Suc(II)-MMC. On the whole, the groups treated with a single injection (4 mg kg^{-1}) exhibited better survival than those given repeated injections. No drug produced high ILS at a dose of 1 mg kg^{-1} given on 4 days.

The therapeutic efficacy of Lac-Suc-MMC and Suc (II)-MMC after single (4 mg kg^{-1}) or repeated doses (1 mg kg^{-1} given for 4 days) using mice inoculated 3 days previously with M5076 cells is also shown in Table 3. The ILS value of the mixture of Lac-Suc and mitomycin C after a single dose of 4 mg kg^{-1} was over 100%, which was observed as the best ILS. The survival patterns given by mitomycin C at 3 days post-inoculation were similar to those at 7 days post-inoculation; that is, a single dose showed a high degree of efficacy but repeated administration was hardly effective. On the other hand, for Lac-Suc-MMC, a moderate ILS was obtained following repeated injection and was similar to that following a single injection. The best ILS value was observed in the group treated with Suc(II)-MMC at a dose of 1 mg kg^{-1} given on 4 days, and two of five mice in this group survived for over 50 days. Suc(II)-MMC showed better therapeutic efficacy than mitomycin C. Thus, the pattern of efficacy of Suc(II)-MMC at 3 days post-inoculation was markedly different from that at 7 days post-inoculation. Loss of body weight was observed in the groups treated with mitomycin C, Lac-Suc plus mitomycin C, Suc(II) plus mitomycin C and Suc(II)-MMC at a single dose of 4 mg kg^{-1}, but it was not lethal (data not shown). In the group treated with Suc(II)-MMC at a dose of 1 mg kg^{-1} given for 4 days, at which the best ILS value was observed, loss of body weight was slight.

The therapeutic efficacy of Lac-Suc-MMC and Suc (II)-MMC against M5076-bearing mice when test solutions were administered intravenously at a dose of 4 mg kg^{-1} on each of days 3–6 following inoculation (i.e. at a dose of 4 mg kg^{-1} × 4 days) is also shown in Table 3 and Figure 3. The ILS values of both groups treated with both conjugates were over 100%. Four of six mice in the group treated with Suc(II)-MMC survived for over 50 days. Control mice died within 17 days post-inoculation. No loss of body weight was observed in the

Table 3 Increase in life span (ILS values) in M5076-bearing mice treated with mitomycin C, Suc(II)-MMC or Lac-Suc-MMC.

Administration schedule	Substance	Single administration[a]		Multiple administration[b]	
		Dose	ILS (%)	Dose	ILS (%)
7 Days post-inoculation	Control	—	—	—	—
	Mitomycin C	4 mg kg^{-1} ×1 day	59.5**	1 mg kg^{-1} ×4 days	0
	Suc(II)-MMC	4 mg kg^{-1} ×1 day	54.8**	1 mg kg^{-1} ×4 days	3.6
	Lac-Suc-MMC	4 mg kg^{-1} ×1 day	23.8	1 mg kg^{-1} ×4 days	−6.0
3 Days post-inoculation	Control	—	—	—	—
	Mitomycin C	4 mg kg^{-1} ×1 day	98.6**	1 mg kg^{-1} ×4 days	18.8**
	Lac-Suc-MMC	4 mg kg^{-1} ×1 day	56.5**	1 mg kg^{-1} ×4 days	42.0*
	Lac-Suc	16 mg kg^{-1}[c]	8.7		
	Lac-Suc+mitomycin C	4 mg kg^{-1} ×1 day	156.5**		
	Control	—	—	—	—
	Mitomycin C	4 mg kg^{-1} ×1 day	37.8**		
	Suc(II)-MMC	4 mg kg^{-1} ×1 day	>97.3**	1 mg kg^{-1} ×4 days	>185.1**
	Suc(II)	29 mg kg^{-1}[c]	16.2		
	Suc(II)+mitomycin C	4 mg kg^{-1} ×1 day	>91.9**		
	Control			—	—
	Suc(II)-MMC			4 mg kg^{-1} ×4 days	>192.5**
	Lac-Suc-MMC			4 mg kg^{-1} ×4 days	118.8**

[a]At 3 or 7 days post-inoculation, each substance was intravenously administered at a dose of 4 mg kg^{-1}. [b]Each substance was intravenously administered repeatedly at a dose of 1 mg kg^{-1} on each of days 3–6 or 7–10 post-inoculation. [c]Lac-Suc or Suc(II) was administered at a dose of 16 or 29 mg polymer equiv. per kg, respectively. *$P < 0.05$, $P < 0.01$ vs control (Mantel–Cox log-rank test).

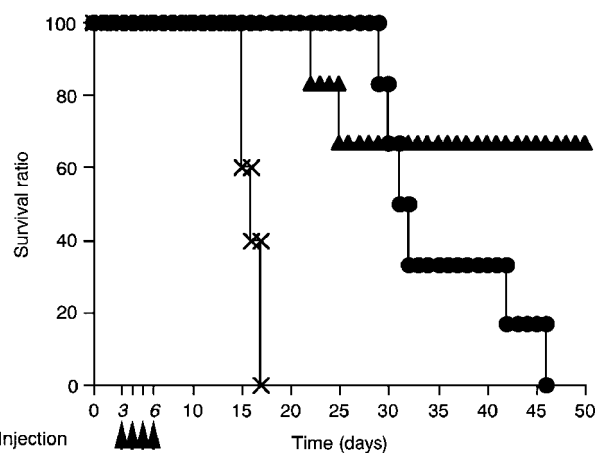

Figure 3 Effects of Suc(II)-MMC and Lac-Suc-MMC on the survival of M5076-bearing mice after injection of 4 mg equiv. mitomycin C per kg given on each of 4 days). ×, Control; ▲, Suc(II)-MMC 4 mg kg^{-1} (single dose); ●, Lac-Suc-MMC 4 mg kg^{-1} given on each of 4 days. Arrow shows intravenous injection. n = 6 for groups treated with conjugates; n = 5 for control. At 3–6 days after inoculation, substance (0.2 mL) was injected intravenously.

group treated with Lac-Suc-MMC (data not shown). In the group treated with Suc(II)-MMC, an initial slight loss of body weight was observed, but the body weight recovered to the initial level at 1 week after treatment (data not shown).

Discussion

As shown in Figure 1B, accumulation of Lac-Suc-FTC in the liver, according to the repeated administration schedule, implied that the capacity of accumulation might be limited to around 110 μg per mouse in the case of daily repeated administration of Lac-Suc-FTC at 0.2 mg/mouse. Table 1 also suggested that repeated administration was superior to a single injection with regard to liver distribution. Lac-Suc-MMC and Suc(II)-MMC were shown to act as prodrugs, and both showed a similar release profile (Figure 2). Intravenous administration of mitomycin C at 2–5 mg kg^{-1} is known to be effective in-vivo (Hata et al 1961; Kato et al 2000b, c); therefore, in this study, a dose of 4 mg kg^{-1} of mitomycin C was selected. The dose of Suc(II)-MMC and Lac-Suc-MMC was chosen as 4 mg equiv. mitomycin C per kg for comparison with free mitomycin C (Table 3). For repeated administration, this dose was divided into 1 mg equiv. mitomycin C per kg per day for the consecutive 4 days (Table 3). The subsequent trial was planned based

on these results (Figure 3). With administration at 7 days post-inoculation, the therapeutic efficacy of Lac-Suc-MMC was in contrast to the results of the biodistribution study. Further, the observations that the antitumour effect of Lac-Suc-MMC was less than that of mitomycin C suggested that Lac-Suc-MMC was taken up by hepatic cells but not targeted to tumour cells, resulting in insufficient drug concentration in tumour sites. The antitumour effect of Suc(II)-MMC, acting as a prodrug of mitomycin C, was similar to that of mitomycin C. However, mitomycin C caused a decrease in body weight, while Suc(II)-MMC did not (data not shown). Therefore, Suc(II)-MMC was considered to be a better antitumour agent due to its reduced side effects. Further, the timing of administration was considered to be an important factor because chemotherapy was hardly effective in the highly advanced metastasis stage (Kato et al 2001b). Namely, since the disease severity is lower in the early stage, earlier treatment should result in better efficacy of the drugs. In the groups treated at 3 days post-inoculation, Lac-Suc-MMC showed similar survival-enhancing effects with both single and repeated injections (Table 3). In these experiments, the mixture of mitomycin C and Lac-Suc showed a very high ILS value (over 100%). This phenomenon may be explained by a synergistic effect between the immunostimulatory effect of Lac-Suc and antitumour activity of mitomycin C because some chitin and chitosan derivatives tend to stimulate the immune system, including macrophages (Peluso et al 1994; Tokura et al 1999). Mice treated with Suc(II)-MMC showed longer survival when treated by repeated administration rather than with a single dose. Suc(II)-MMC showed a much higher ILS value than mitomycin C and Lac-Suc-MMC when administered according to the repeated treatment schedule. These results suggested the better availability of Suc(II)-MMC at a multiple dose in the early metastasis stage. To pursue further therapeutic efficacy, Lac-Suc-MMC and Suc(II)-MMC were intravenously administered repeatedly at a daily dose of 4 mg kg^{-1} on days 3–6 post-inoculation (i.e. at a dose of 4 mg $kg^{-1} \times 4$ days) in M5076-bearing mice. The survival of mice treated with each conjugate was markedly improved in comparison with those treated with a dose of 1 mg kg^{-1} on each of 4 days or with a single dose of 4 mg kg^{-1}. This indicated increases in dose raised the therapeutic efficacy. Especially, Suc(II)-MMC exhibited a very strong antitumour effect. This conjugate may become a useful prodrug for metastatic cancer if the loss of body weight can be improved. A good result was also obtained with Lac-Suc-MMC, which was associated with no loss of body weight; Lac-Suc-MMC was considered to be less toxic than Suc(II)-MMC, which might be due to the suppression of the systemic flow of mitomycin C in Lac-Suc-MMC.

On the whole, the antitumour effects of Lac-Suc-MMC against M5076-bearing mice were inferior to those of Suc(II)-MMC. Duncan et al (1992) reported that hepatic targeting via asialoglycoprotein receptors of the liver was not necessarily effective against liver metastasis, and indicated that a simple macromolecular conjugate not containing galactose might act better in animal models of liver metastasis. This was considered to be because the conjugate was not targeted directly to the metastatic cells but to the parenchymal cells. For effectiveness against metastasis, the drug targeted to the parenchymal cells must escape from inactivation and diffuse to the diseased region. Therefore, the following considerations are proposed based on the results described above. Lac-Suc-MMC is rapidly eliminated from the systemic flow and distributed to the liver parenchymal cells by specific binding to the asialoglycoprotein receptors. Drug released in or on the liver parenchymal cells is related to the efficacy. Therefore, the biological stability of the drug will influence its effects on the surrounding tumour tissue. Since mitomycin C is very unstable in the liver (Hashida et al 1983; Song et al 1996), that released from Lac-Suc-MMC in the liver parenchymal cells is thought not to be able to reach the tumour cells sufficiently. Intact *N*-succinyl-chitosan is much less interactive with body tissues and is maintained for long periods in the systemic circulation after intravenous injection (Kamiyama et al 1999; Kato et al 2000a), which is considered to be due to the lack of specific ligands and its physicochemical properties such as anionic charge. Therefore, drugs combined with *N*-succinyl-chitosan can be liberated on or in various cells around the sites at which the carrier is distributed. The systemic long-term circulation at higher levels of Suc(II)-MMC may permit its effective and long-term access to the whole liver and consequently facilitate the supply of liberated mitomycin C or endocytosis of the conjugate by liver metastatic M5076 cells. Thus, the antitumour characteristics of both the conjugates could be evaluated by the physiological behaviour of their carriers.

Conclusions

Following administration at 7 days post-inoculation, the therapeutic efficacy of repeated injection of Lac-Suc-MMC (1 mg kg^{-1} on each of 4 days) was inferior to that of a single dose of Lac-Suc-MMC (4 mg kg^{-1}), and Lac-Suc-MMC was much less effective than mitomycin

C. At 3 days post-inoculation, single and repeated injection schedules using Lac-Suc-MMC demonstrated similar antitumour effects. These results were not in accordance with those of the biodistribution studies. In contrast, at both 7 days and 3 days post-inoculation, Suc(II)-MMC showed similar or higher therapeutic efficacy in comparison with mitomycin C and Lac-Suc-MMC. Further, the efficacy of Suc(II)-MMC at a repeated dose was better than that at a single dose except for the administration at 7 days post-inoculation. At 3 days post-inoculation, Suc(II)-MMC resulted in very high survival at a dose of 4 mg kg^{-1} given on each of 4 days. These results demonstrated that high therapeutic efficacy could be achieved with repeated administration of Suc(II)-MMC in the early metastatic stage.

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