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Development of a novel polymeric prodrug of mitomycin C, mitomycin C–dextran conjugate with anionic charge. I. Physicochemical characteristics and in vivo and in vitro antitumor activities

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Summary

A novel polymeric prodrug of mitomycin C (MMC), mitomycin C–dextran conjugate with anionic charge (MMC-Dan.) was developed employing 6-bromohexanoic acid as a spacer. Three types of MMC-Dan., conjugates with dextran with molecular weights of 10,000, 70,000 and 500,000 were synthesized, and physicochemical characteristics, such as molecular size, electrical charge and drug release rate, and in vitro and in vivo antitumor activities were investigated. All types of MMC-Dan. were estimated to contain about 8% MMC (w/w) and MMC was released from the conjugate by a chemical hydrolysis. In vitro antitumor activity was evaluated by L1210 cell culture system. All types of MMC-Dan. showed less cytotoxicity than MMC in continuous or one-hour drug exposure experiments, regardless of molecular size. In vivo antitumor activity was tested in mice bearing P388 leukemia. Intravenous and intraperitoneal injection of MMC-Dan. prolonged the survival of mice inoculated tumor cells by the same route. Especially, large MMC-Dan. exhibited a superior effect and a larger therapeutic index as compared with MMC. The usefulness of mitomycin C–dextran conjugates with anionic charge as a potent reservoir which supplies active MMC in the body was thus suggested.

Introduction

One of the major limitations of cancer chemotherapy is that most of the antitumor drugs have indiscriminate cytotoxicity towards both neoplastic cells and proliferating normal cells. Therefore, optimization of antitumor drug administration has been advocated and has led to a number of reports on drug delivery systems for antitumor agents. In our laboratory, various kinds of delivery systems have been developed from the viewpoints of utilizing the physical devices (Yoshioka et al., 1981; Sezaki et al., 1982), chemical transformation of the drug molecules (Sasaki et al., 1983; Takakura et al., 1985), and combinations of physical and chemical approaches (Hashida and Sezaki, 1984).

Among these approaches, application of macro-

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molecular compounds such as carriers for antitumor agents seem to be promising and various kinds of macromolecules have been utilized and evaluated as candidates for targeted cancer chemotherapy (for reviews, see Sezaki and Hashida, 1984; Poznansky and Cleland, 1980). In our series of investigations, we have developed several polymeric prodrugs of mitomycin C, such as agarose beads conjugate (Hashida et al., 1977), dextran conjugate with cationic charge (MMC-Dcat.) (Kojima et al., 1980), and polyamino acid conjugate (Roos et al., 1984), and evaluated their physicochemical, pharmacokinetical and pharmacodynamic properties. Especially, systematic studies were carried out for MMC-Dcat. (Kato et al., 1982; Hashida et al., 1983, 1984; Matsumoto et al., 1985; Takakura et al., 1985) and the usefulness in local application was established (Takakura et al., 1984, 1986) involving clinical trials (Honda et al., 1985).

In the present study, a novel polymeric prodrug of MMC, MMC–dextran conjugate with anionic charge (MMC-Dan.) was developed in order to assess the feasibility of dextran conjugate in cancer drug delivery systems. Physicochemical properties and in vivo and in vitro antitumor activities of MMC-Dan. were evaluated.

Materials and Methods

Chemicals

MMC was kindly supplied by Kyowa Hakko Kogyo Co. (Tokyo, Japan). Dextran with various molecular weights were purchased from Pharmacia (Uppsala, Sweden), and had average molecular weights of about 10,000 (T-10), 70,000 (T-70), and 500,000 (T-500). All other chemicals were reagent grade products obtained commercially.

Animals and tumors

Male DBA/2 mice, male C57bl/6 × DBA/2F_1 (B6D2F_1) mice, and male *ddY* mice were obtained from Sizuoka Agricultural Co-operate Association for Laboratory Animals (Shizuoka, Japan). L1210 mouse leukemia cells were maintained by serial culture every 3 or 4 days in RPMI 1640 containing 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY). P388 leukemia was supplied from Shionogi Pharmaceutical Co. (Osaka, Japan), and maintained in DBA/2 mice by weekly i.p. transfer of 10^6 cells obtained from ascitic fluid. Ehrlich ascites carcinoma (EAC) cells were maintained by weekly i.p. transplantation to male *ddY* mice.

Synthesis of MMC-Dan.

MMC-Dan. was synthesized by following two steps. (1) Dextran (1 g) was dissolved in 4 M NaOH solution (10 ml) and 6-bromohexanoic acid (2.8 g) was added. The mixture was kept at about 80 °C for 3 h with occasional stirring. The product, spacer-introduced dextran, was dialyzed against water. (2) MMC was conjugated to the spacer-introduced dextran by a carbodiimide-catalyzed reaction. One gram of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added to the solution of spacer-introduced dextran, and the pH of the solution was maintained between 5.0 and 5.5 with 1 N HCl. The MMC (150 mg) was stepwise dissolved in this solution, and the reaction was allowed to proceed for 12 h at room temperature. The product was washed with water, concentrated by ultrafiltration, and then precipitated by acetone to yield purple solid. The doses reported for MMC-Dan. refer to the quantity of MMC contained in the conjugate. The compounds were prepared in saline solution at an adequate concentration for injection.

In vitro release experiment

The release of MMC from the conjugate was determined with a dialysis system. A Visking dialysis tube containing 2 ml of pH 7.4 isotonic phosphate buffer solution of the conjugate was immersed in 50 ml of the same buffer maintained at 37 °C, and was shaken continuously. At fixed time intervals, 1 ml of sample was taken from the outer medium and the amount of MMC was measured spectrophotometrically at 364 nm. The release rate and half-life were calculated by the least-squares method.

Molecular size estimation

A Sephadex G-200 or Sepharose 4B column (1.7 cm × 65 cm) was employed for molecular size

estimation. A 2 mg of sample dissolved in 1 ml of 0.5 M NaCl solution was applied on a column and eluted with 0.5 M NaCl solution. For each sample, K_{av} value was calculated from the following equation:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = the elution volume of the sample; V_o = the column void volume; and V_t = the total bed volume. The Stokes' radii of the sample were determined from the calibration curves obtained from K_{av} values of 8 kinds of protein of a known molecular radii (ribonuclease A = 16.4 Å; chymotrypsinogen A = 20.9 Å; ovalbumin = 30.5 Å; albumin = 35.5 Å; aldolase = 48.1 Å; catalase = 52.2 Å; ferritin = 61.0 Å; thyroglobulin = 85.0 Å) (Gel filtration calibration kit, Pharmacia Fine Chemicals Co., Sweden).

Molecular charge estimation and cellular interaction

The molecular charge of the conjugate was estimated by a batch method using DEAE-Sephadex A-50 anion exchanger and CM-Sephadex C-50 cation exchanger (Pharmacia Fine Chemicals, Uppsala, Sweden). An ion exchanger (25 mg) was suspended in the 5 ml of 20 mM N-2-hydroxyethylpiperadine-N'-2-ethanesulfonic acid (Hepes)-buffered saline (pH 7.2) and shaken with the 0.5 ml of the conjugate solution (160 μg equiv. MMC/ml). After 30 min, the samples were centrifuged. The amount of the conjugate in the supernatant was determined spectrophotometrically at 364 nm and the adsorption percent on the ion exchanger was calculated. For the determination of cellular interaction, EAC cells (10^7 cells/ml) were incubated in 20 mM Hepes-buffered Hanks' balanced salt solution (pH 7.2) containing MMC-Dan. (10 μg equiv. MMC/ml). Cells were separated by centrifugation at 3000 rpm for 5 min. Drug concentration remaining in the supernatant was determined spectrophotometrically and the percentage of adsorption was calculated.

Analytical method

The amount of conjugated MMC as well as free MMC was determined spectrophotometrically at 364 nm. The amount of dextran was determined by the anthrone method (Scott and Melvin, 1953).

In vitro antitumor activity

In vitro antitumor activity was evaluated by two different methods using L1210 cell culture system (Matsumoto et al., 1986). In the continuous drug exposure system, L1210 cells were suspended in a RPMI 1640 medium supplemented with 10% fetal bovine serum containing various concentrations of drugs and seeded on a multiwell tissue culture plate (Becton, Dickinson and Co., CA) at a density of 10^5 cells/ml/well. After incubation in a humidified atmosphere containing 5% CO_2 at 37°C for 3 days, viable cells were counted by the Trypan blue exclusion method. The growth inhibition was calculated as follows:

$$\text{Growth Inhibition } (\%) = (1 - T/C) \times 100$$

where T and C represent the number of surviving cells in the treated group and that in the untreated group, respectively. Experiments were carried out in triplicate. The IC_{50} values, concentration exhibiting the 50% growth inhibition, were calculated from the growth inhibition curves. In the 1-h exposure system, L1210 cells were exposed to the drug at various concentrations in a Hanks' solution (pH 7.2) for 1 h at 37°C and then washed twice with the same medium by the centrifugation. Drug-treated cells were resuspended in the growth medium and incubated for 4 days and the growth inhibition and IC_{50} value were determined as above.

In vivo antitumor activity

$B6D2F_1$ mice weighing 18–20 g were intraperitoneally or intravenously inoculated with 1×10^6 P388 leukemia cells. Chemotherapy was given intraperitoneally or intravenously 24 h after tumor inoculation. Six mice were used per each group. The activities of the compounds were indicated as the percentage of increase in life span (*ILS* %), i.e.,

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

where T and C represent the mean survival time of the treated animals and that of the control

animals, respectively. The therapeutic index, defined as the ratio of the dose showing a maximal effect (ILS_{max}) to that giving a 30% increase in life span (ILS_{30}), was also used for the evaluation. The ILS_{30} was calculated from the regression line between the dose and the *ILS* value obtained by the least-squares method.

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by the paired *t*-test.

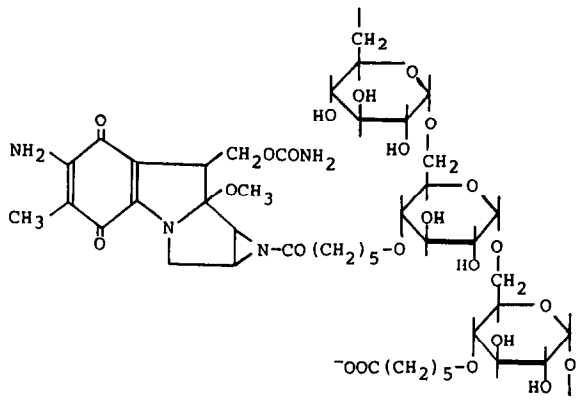

Fig. 1. Proposed molecular structure of MMC-Dan.

Results

Synthesis of MMC-Dan.

The conjugation of MMC to carrier dextran was confirmed by gel filtration for each type of MMC-Dan. MMC-Dan. was estimated to contain about 8% MMC (w/w) regardless of molecular weight (Table 1). The degree of substitution by MMC of the dextran was estimated to be one molecule per approximately 20–25 glucose units. The proposed molecular structure of MMC-Dan. is shown in Fig. 1.

Molecular size estimation

The molecular size of MMC-Dan. was determined by a gel filtration method. Representative patterns are shown in Fig. 2 for MMC-Dan. (T-70) and dextran (T-70). The conjugate was eluted faster than the original dextran, indicating that molecular size was increased as a result of the coupling reaction. The calculated effective molecular sizes of 3 types of MMC-Dan. and original dextran are summarized in Table 2. These values were determined from the calibration curve obtained from the K_{av} values of 8 molecular markers. All MMC-Dan.s had the same order of size as original dextrans, although their molecular sizes were larger than those of the original dextrans.

In vitro release

Fig. 3 shows the in vitro release of MMC from MMC-Dan. All types of MMC-Dan. showed the monoexponential liberation of MMC under the present condition (pH 7.4, 37 °C). The release rates were not affected by the addition of rat plasma (data not shown). The release half-lives were 35 h observed for MMC-Dan. (T-10) and MMC-Dan. (T-70) and 50 h for MMC-Dan. (T-500) (Table 1).

Molecular charge estimation and cellular interaction

MMC-Dan. was adsorbed on the anion exchanger DEAE-Sephadex to an almost equal extent but was not adsorbed on the cation exchanger

TABLE 1

Physicochemical characteristics of mitomycin C–dextran conjugates

	Mol. wt. (Carrier)	Mitomycin C Content (weight %)	In vitro release $t_{1/2}$ (h)	Adsorption % at pH 7.2		
				CM-Sephadex	DEAE-Sephadex	EAC cells
MMC-Dan. (T-10)	9900	9.43	35.0	0	29.2	0
MMC-Dan. (T-70)	64,400	8.15	35.4	0	32.0	0
MMC-Dan. (T-500)	487,000	8.47	50.0	0	28.4	0

TABLE 2

Molecular sizes of dextrans and MMC-Dan. estimated by gel filtration chromatography

Compound	K_{av} value	Effective radius (Å) [c]
Dextran (T-10) [a]	0.631	19.7
MMC-Dan. (T-10) [a]	0.535	23.3
Dextran (T-70) [b]	0.404	58.2
MMC-Dan. (T-70) [b]	0.382	61.7
Dextran (T-500) [b]	0.359	65.7
MMC-Dan. (T-500) [b]	0.292	80.2

[a] Chromatographed on a Sephadex G-200 column.
[b] Chromatographed on a Sepharose 4B column.
[c] Effective molecular sizes were calculated from the standard curve of Stokes' radii of marker proteins.

CM-Sephadex at pH 7.2 regardless of the molecular weight (Table 1). MMC-Dan. did not interact with the EAC cells.

In vitro antitumor activity

Fig. 4 shows the in vitro antitumor activities of MMC and MMC-Dan. in the continuous (A) or the 1-h (B) drug exposure system. In both systems, MMC-Dan. showed less activity than MMC regardless of molecular weight.

In vivo antitumor activity

Fig. 5 shows the effect of MMC and 3 types of

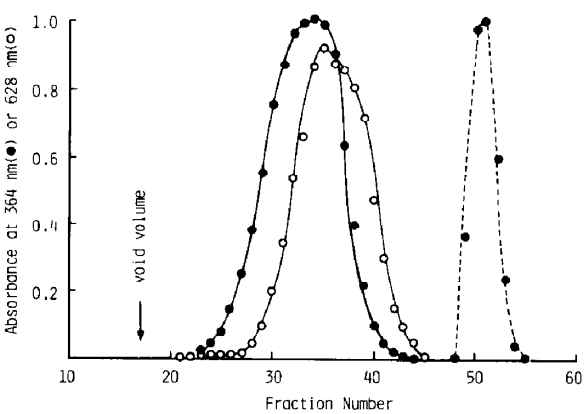

Fig. 2. Gel filtration patterns of MMC, dextran (T-70) and MMC-Dan. (T-70) on a Sepharose-4B column. Key: ●— — —●, MMC; ○———○, dextran (T-70); ●———●, MMC-Dan. (T-70).

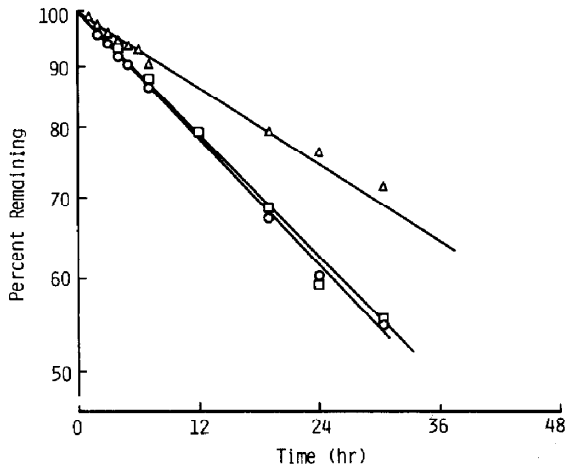

Fig. 3. In vitro release of MMC from MMC-Dan. (T-10) (○), MMC-Dan. (T-70) (□), and MMC-Dan. (T-500) (△) in a pH 7.4 buffer at 37 °C.

MMC-Dan. on the life-span of mice bearing P388 leukemia. Single i.p. injections of MMC-Dan. prolonged the survival of mice with i.p. tumor inoculation. The maximum *ILS* values were increased with the increase in molecular weight, i.e., 84%, 106%, and 139% for MMC-Dan. (T-10) (40 mg/kg), MMC-Dan. (T-70) (20 mg/kg), and MMC-Dan. (T-500) (30 mg/kg), respectively. Free

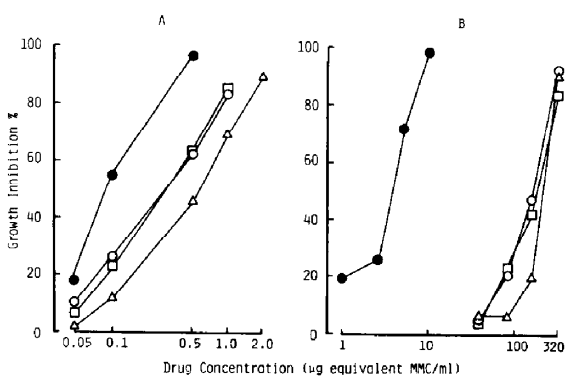

Fig. 4. In vitro growth inhibition effect of MMC and MMC-Dan. with various molecular weights on L1210 leukemia in continuous (A) and 1-h (B) drug exposure system. Key: ●, MMC; ○, MMC-Dan. (T-10); □, MMC-Dan. (T-70); △, MMC-Dan. (T-500).

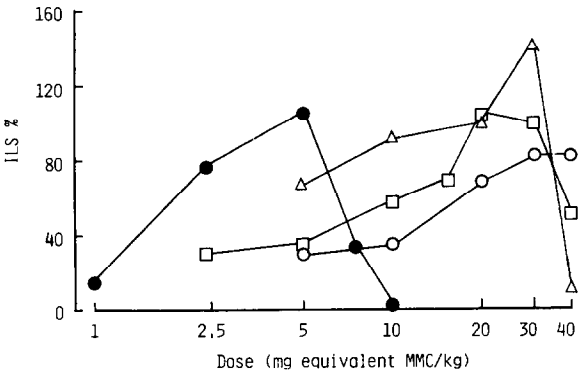

Fig. 5. Effect of MMC and MMC-Dan. on the survival of mice bearing P388 leukemia (i.p.–i.p. system). Tumor cells were inoculated intraperitoneally and chemotherapy was given intraperitoneally 24 h after inoculation. Key: ●, MMC; ○, MMC-Dan. (T-10); □, MMC-Dan. (T-70); △, MMC-Dan. (T-500).

MMC gave the maximum *ILS* (129%) at the dose of 5 mg/kg. The dose–response curve of MMC-Dan. was shifted to the higher dose region and MMC-Dan. (T-500) exhibited a superior activity to free MMC. Fig. 6 shows the effect of i.v. injections of MMC-Dan., MMC-Dcat., and MMC on the survival of mice after being inoculated with

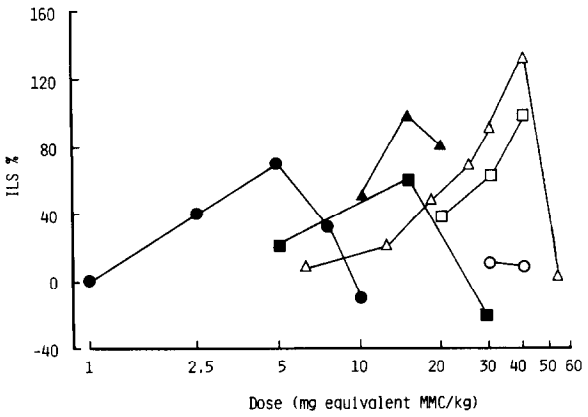

Fig. 6. Effect of MMC, MMC-Dan., and MMC-Dcat. on the survival of mice bearing P388 leukemia (i.v.–i.v. system). Tumor cells were inoculated intravenously and chemotherapy was given intravenously 24 h after inoculation. Key: ●, MMC; ○, MMC-Dan. (T-10); □, MMC-Dan. (T-70); △, MMC-Dan. (T-500); ■, MMC-Dcat. (T-70); ▲, MMC-Dcat. (T-500).

P388 leukemia intravenously. Remarkable antitumor effect was observed in the case of MMC-Dan. ILS_{max} was increased as the molecular weight increased, and MMC-Dan. (T-500) was more effective ($ILS = 134\%$ at 40 mg/kg) than MMC-Dcat. and MMC.

Discussion

Dextran has been used as a plasma expander for many years in the clinical field. It has excellent physicochemical properties such as high water solubility and has numerous reactive hydroxyl groups available for drug fixation. Therefore, dextran has been tested as a candidate for macromolecular carrier of various drugs (Molteni, 1979; Hurwitz et al., 1980; Takakura et al., 1984; Larsen and Johansen, 1985). Several methods such as direct esterification, periodate oxidation and cyanogen bromide activation have been reported for the conjugation of drugs to dextrans. In the papers on dextran conjugates, however, little attention has been focused on the physicochemical properties of the conjugates, which are defined by the synthetic method, although they might affect the in vivo behaviour and the biological activities of the conjugates.

In our series of investigations, we have developed a polymeric prodrug of mitomycin C, mitomycin C–dextran conjugate with cationic charge (MMC-Dcat.) (Kojima et al., 1980). In this case, dextran was activated by cyanogen bromide and then a spacer, ε-aminocaproic acid, was coupled. Three structures were proposed for the linkage between the spacer and the dextran (Schaar et al., 1977), and two of them have the possibility to give a cationic charge in MMC-Dcat. In the present study, 6-bromohexanoic acid was introduced to the hydroxyl group in the dextran chain through ether linkage with no electric charge and MMC was coupled to a spacer-introduced dextran by a carbodiimide-catalyzed reaction. Since all spacer arms were not used for coupling, the remaining free carboxyl group might give an anionic charge in MMC-Dan.

MMC-Dcat. exhibited superior antitumor activities against various murine tumors as compared

with MMC (Hashida et al., 1981) and the size of the carrier dextran was proved to affect their efficiency (Kato et al., 1982). A stability study revealed that MMC-Dcat. was converted to free MMC by a chemical hydrolysis with a half-life of 24 h, and the conversion was not accelerated by the tissue homogenate (Hashida et al., 1983). In general, polymeric antitumor drugs are considered to enter the tumor cells by endocytosis and to be cleaved to free forms by the lysosomal enzymes (lysosomotropic agents) (De Duve et al., 1974; Trouet et al., 1972). In contrast to this mechanism, free form of MMC regenerated by a chemical hydrolysis in the extracellular space should contribute to the activity of the MMC–dextran conjugate. Our previous study showed that MMC-Dcat. was adsorbed on the tumor cell surface with negative charge by an electrostatic force and liberated intact MMC which then exhibited cytotoxicity (Matsumoto et al., 1985). This interaction is important for the antitumor activity of MMC-Dcat., as well as the modified pharmacokinetic properties.

Table 3 summarizes the IC_{50} values of MMC, MMC-Dcat. and MMC-Dan. MMC-Dan. showed less in vitro growth-inhibitory effect than MMC and MMC-Dcat. In the continuous drug exposure system, growth inhibitory effect of MMC-Dan. was significantly weaker than those of free MMC and MMC-Dcat. The slow release rate of MMC from MMC-Dan. (Table 1) seemed to be responsible for these results, and the minimum effect was observed in the case of MMC-Dan. (T-500) with the longest release half-life. In the 1-h drug exposure system, MMC-Dcat. showed stronger activity than free MMC owing to its binding capacity to the cell surface (Matsumoto et al., 1986). However, MMC-Dan., which could not be adsorbed on the cell surface (Table 1), showed much less activity regardless of molecular weight.

On the contrary, MMC-Dan. exhibited a remarkable in vivo antitumor effect in i.p.–i.p. and i.v.–i.v. systems as shown in Figs. 5 and 6. In Table 4, the antitumor activities of MMC, MMC-Dan., and MMC-Dcat. in both systems are summarized in terms of the therapeutic indices and maximum *ILS* values. MMC showed maximum activities at the dose of 5 mg/kg, while the optimal doses were in the dose range between 20 mg/kg and 40 mg/kg in the case of MMC-Dan. MMC-Dcat. exhibited the maximum *ILS* at the dose of 15 mg/kg in the i.v.–i.v. system. These results suggested that MMC-Dan. has reduced toxicity as compared with MMC and MMC-Dcat. owing to its slow release rate of MMC. Maximum *ILS* value was increased as the molecular weight of MMC-Dan. increased, and MMC-Dan. (T-500) afforded a superior maximum *ILS* value than MMC in both systems. Furthermore, an increased therapeutic index was obtained by MMC-Dan. (T-500). MMC-Dan. (T-500) can be considered to act as a potent reservoir which supply active MMC in the peritoneal cavity or systemic circulation. Therefore, the main factor dominating the antitumor effects of MMC-Dan. is considered to be the altered pharmacokinetic behaviour whereas cellular interaction contributes little.

Thus, the usefulness of MMC-Dan. as a potent polymeric prodrug in systemic administration was suggested. The present investigation has shown that physicochemical properties, especially electric charge and molecular weight, affect the pharmacological effect of polymer–drug conjugate. This in-

TABLE 3

In vitro growth inhibition effect of MMC, MMC-Dan. and MMC-Dcat. against L1210 leukemia cells

	IC_{50} (μg equiv. MMC/ml) [a]	
	Continuous exposure	1-h exposure
1 MMC	0.091 ± 0.013	3.60 ± 0.27
2 MMC-Dan. (T-10)	0.271 ± 0.012	166.8 ± 1.83
3 MMC-Dan. (T-70)	0.283 ± 0.021	182.7 ± 16.6
4 MMC-Dan. (T-500)	0.557 ± 0.063	214.0 ± 2.99
5 MMC-Dcat. (T-10)	0.158 ± 0.011	30.0 ± 2.17
6 MMC-Dcat. (T-70)	0.153 ± 0.011	0.803 ± 0.0564
7 MMC-Dcat. (T-500)	0.134 ± 0.006	0.424 ± 0.0836

[a] Mean ± S.D. The values were compared among the groups by means of a one-way ANOVA, followed by the paired *t*-test. Statistical significance ($P < 0.05$) was recognized between the groups (1,2), (1,3), (1,4), (1,5), (1,6), (1,7), (2,4), (2,5), (2,6), (2,7), (3,4), (3,5), (3,6), (3,7), (4,5), (4,6), (4,7), (5,7) and (6,7) in the continuous exposure and between the groups (1,2), (1,3), (1,4), (1,5), (1,6), (1,7), (2,4), (2,5), (2,6), (2,7), (3,4), (3,5), (3,6), (3,7), (4,5), (4,6), (4,7), (5,6), (5,7) and (6,7) in the 1-h exposure.

TABLE 4

Therapeutic evaluation of MMC, MMC-Dan. and MMC-Dcat. in P388 leukemia systems

System	Compound	ILS_{30} (mg/kg)	ILS_{max} (mg/kg)	Therapeutic Index	Maximum [a] *ILS*(%)
i.p.–i.p. 1	MMC	1.22	5.0	4.1	105.9 ± 22.6
2	MMC-Dan. (T-10)	5.99	30.0	5.0	84.1 ± 19.6
3	MMC-Dan. (T-70)	3.44	20.0	25.8	105.5 ± 37.9
4	MMC-Dan. (T-500)	11.37	30.0	10.6	139.1 ± 38.5
i.v.–i.v. 1	MMC	1.90	5.0	2.63	71.3 ± 4.9
2	MMC-Dan. (T-10)	–	30.0	–	10.6 ± 4.6
3	MMC-Dan. (T-70)	18.64	40.0	2.15	99.5 ± 10.1
4	MMC-Dan. (T-500)	11.37	40.0	3.52	134.1 ± 13.2
5	MMC-Dcat. (T-70)	10.79	15.0	1.39	60.6 ± 6.8
6	MMC-Dcat. (T-500)	8.38	15.0	1.79	99.5 ± 22.6

[a] Mean ± S.D. The values were compared among the groups by means of a one-way ANOVA, followed by the paired *t*-test. Statistical significance ($P < 0.05$) was recognized between the groups (2,4) in the i.p.–i.p. system and between the groups (1,2), (1,3), (1,4), (1,5), (1,6), (2,3), (2,4), (2,5), (2,6), (3,4), (3,5), (4,5), (4,6) and (5,6) in the i.v.–i.v. system.

formation seems to be useful for the design of macromolecular prodrugs in cancer drug delivery systems.

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