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Plasma Disposition and *in Vivo* and *in Vitro* Antitumor Activities of Mitomycin C–Dextran Conjugate in Relation to the Mode of Action

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In order to clarify the factors dominating the antitumor effects of mitomycin C–dextran conjugates (MMC-D), the plasma disposition of MMC-D in rats, and the *in vivo* and *in vitro* antitumor activities were studied in comparison with those of mitomycin C. Three types of MMC-D, conjugates with dextran having molecular weights of 10000, 70000 and 500000, were administered to rats by intravenous, intramuscular and intraperitoneal injection, and the plasma concentrations of MMC were determined by bioassay. Similar sustained plasma levels of MMC after injection of MMC-D were obtained regardless of carrier size or administration route. Intraperitoneal injection of MMC-D significantly improved the survival time of mice bearing L1210 leukemia, while intravenous or intramuscular injection did not. MMC-D showed higher maximum activity at a lower dose as its molecular size increased. L1210 leukemia cells were exposed to MMC-D for 1 h *in vitro* and cytocidal activities were evaluated in terms of the survival time of mice inoculated with treated cells, or the growth rate of cells in a cell culture system. MMC-D exhibited different cytocidal activities depending on its molecular weight in these systems whereas MMC-D was considered to liberate MMC at the same rate regardless of molecular weight *in vitro*. These results suggest that MMC-D has some direct interaction with tumor cells and this interaction plays an important role in the manifestation of antitumor activity *in vivo*, as well as a modified pharmacokinetic behavior in the body.

Keywords——mitomycin C; high-molecular-weight prodrug; dextran; plasma disposition; L1210 leukemia; *in vivo* antitumor activity; *in vitro* antitumor activity; cell culture system

Mitomycin C (MMC) is widely used in cancer chemotherapy, but its utility is limited by the side effects of severe bone marrow suppression and gastrointestinal damage.[1] To overcome these defects, it is beneficial to concentrate its cytotoxicity at the tumor site and to minimize the burden on other tissues by modifying the biological and pharmacokinetic properties.[2] One possible approach to alter the biopharmaceutical properties of MMC might be conjugation to a high molecular weight compound.[3] On the basis of this consideration, various kinds of macromolecules, such as polysaccharides,[4] polyamino acid[5,6] and proteins[5] including biospecific ligands such as antibodies, have been utilized and evaluated as candidates for tumor-specific carriers.

In a series of investigations, we have developed a macromolecular derivative of MMC, MMC–dextran conjugate (MMC-D) (Fig. 1),[7] and examined its physicochemical, pharmacodynamic and pharmacokinetic properties. MMC-D exhibited improved antitumor activity against various murine tumors, and the size of the carrier dextran affected the efficiency.[5,7,8] Stability studies revealed that MMC-D was converted to MMC by hydrolysis, but the conversion was not accelerated by tissue homogenate.[9] Pharmacokinetic analysis elucidated that MMC-D acts as a reservoir which behaves as a macromolecule while supplying active

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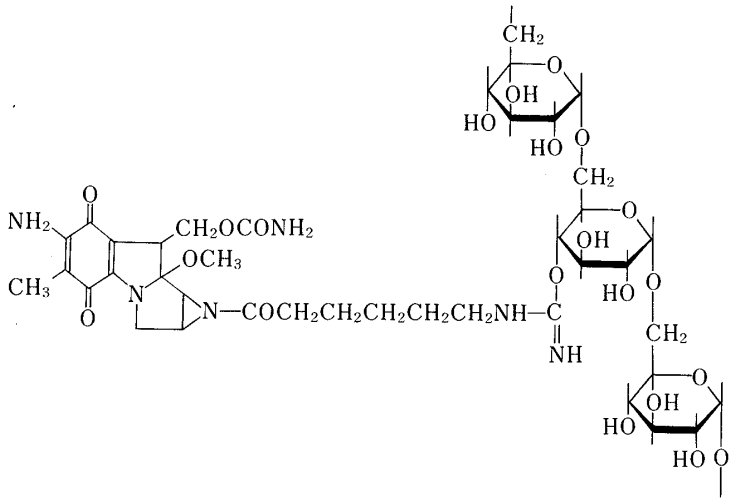

Fig. 1. Representative Chemical Structure of Mitomycin C–Dextran Conjugate

MMC in the body.[10] Furthermore, MMC-D showed sustained retention at the injection site and enhanced lymphatic transport after local injection.[11] Based on these findings, a clinical study is in progress and MMC-D has shown remarkable effects on advanced cancer in several cases.[12]

In the present work, we investigated the plasma disposition characteristics of MMC-D of different molecular weights after injection through various routes, and the antitumor activities in tumor-bearing mice and a cell culture system in order to elucidate the mode of action.

Experimental

Materials——MMC was kindly supplied by Kyowa Hakko Kogyo Co. Dextrans of various molecular weights were purchased from Pharmacia Fine Chemicals Co., Sweden, and have average molecular weights of about 10000 (Dextran T-10), 70000 (T-70) and 500000 (T-500). All other chemicals were reagent-grade products obtained commercially.

Preparation of MMC-D——MMC-D was synthesized as reported previously.[7] In brief, dextran was activated with cyanogen bromide at pH 10.7 and ε-aminocaproic acid was coupled covalently to a glucose chain as a spacer. The product was washed repeatedly by ultrafiltration and precipitated with acetone. MMC was conjugated to the spacer-introduced dextran by means of a carbodiimide-catalyzed reaction. The product was washed, concentrated by ultrafiltration and precipitated with acetone to yield a purple solid. All conjugates were estimated to contain MMC to about the same extent, about 10% (w/w), and the degree of substitution of dextran by MMC was one molecule per 14—17 glucose units. The doses reported for MMC-D refer to the quantity of MMC in the conjugates.

Animal Experiment——Male Wistar albino rats weighing between 200 and 250 g were anesthetized by intraperitoneal injection of sodium pentobarbital and fixed on their backs during the course of the experiment. After intravenous (*i.v.*), intramuscular (*i.m.*) or intraperitoneal (*i.p.*) injection of MMC-D, blood samples were withdrawn from the carotid vein or aorta periodically. *I.v.* and *i.m.* injections were performed into the femoral vein and the thigh muscle, respectively. The dose of MMC-D was 5 mg eq MMC per kg and the drug was given in the form of saline solution (100 μl).

Analytical Method——Blood samples were centrifuged at 3000 rpm for 5 min and the obtained plasma was stored in a freezer until used for assay. The plasma concentration of liberated MMC was determined by microbiological assay using *E. coli* B as a test organism. Assay was performed by the disk-plate method and the results were calculated using a standard curve. The contribution of conjugated MMC to the antimicrobial activity of the plasma sample was confirmed to be less than 10% of total activity by this procedure.

Evaluation of Antitumor Activity——L1210 leukemia cells were maintained by weekly transplantation into the peritoneal cavity of male DBA/2 mice or by serial culture of tumor cells in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO, U.S.A.). Antitumor activities of MMC-D were evaluated by the following methods.

(1) *In Vivo* Antitumor Activity Evaluation: BDF_1 mice (C57Bl/6, female × DBA/2, male) weighing 19—24 g were inoculated intraperitonealy with 1×10^6 L1210 leukemia cells suspended in 0.2 ml of Hanks' balanced salt solution (HBSS). Drugs were given intravenously, intramuscularly or intraperitoneally at 24 h after inoculation. All

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activities of compounds were recorded as $T/C\%$ values, calculated as the ratio of the mean survival time of the treated groups to that of the control group.

(2) *In Vitro* Antitumor Activity Evaluation Using BDF_1 Mice: L1210 leukemia cells were incubated with drugs at various concentrations for 1 h at 37 °C in HBSS and then washed twice with the same medium by centrifugation. Cytocidal effects of the drug on the tumor cells were evaluated by recording the survival time of BDF_1 mice given an *i.p.* inoculation of drug-treated cells.

(3) *In Vitro* Antitumor Activity Evaluation in Cell Culture System: Cells treated with drugs by the same procedure as described above were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and seeded on a multiwell tissue culture plate (Becton, Dickinson and Company, U.S.A.) at a density of 10^5 cells/ml/well. Experiments were carried out in triplicate. After incubation in a humidified atmosphere containing 5% CO_2 at 37 °C for 4 d, viable cells were counted with a Bürker-Turk hemocytometer by trypan blue exclusion. 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 a treated group and that in an untreated group, respectively.

Results

Plasma Concentration

Figure 2 shows the plasma concentration–time courses of MMC after *i.v.*, *i.m.* and *i.p.* injections of unconjugated MMC. The concentrations were highest for all administration routes at 5 min after injection, then decreased rapidly in a roughly monoexponential fashion. Figures 3, 4 and 5 illustrate the time courses of plasma levels of MMC following injection of MMC-D (T-10), MMC-D (T-70) and MMC-D (T-500), respectively, through the same administration routes as above. In contrast with MMC injection, all types of MMC-D gave lower concentrations of MMC initially, but the concentrations decreased more gradually and significant antimicrobial activities could be detected even at 8 h after injection, regardless of the administration route. However, *i.v.* injections of MMC-D showed somewhat lower plasma levels of MMC than *i.m.* and *i.p.* injections with all types of MMC-D.

In Vivo Antitumor Activity

Figures 6, 7 and 8 show the antitumor activities of MMC and MMC-D against L1210 leukemia by *i.v.*, *i.m.* and *i.p.* injection, respectively. Only in the case of *i.p.* injection did MMC and MMC-D exhibit significant therapeutic effects. In this system, MMC-D (T-500) showed

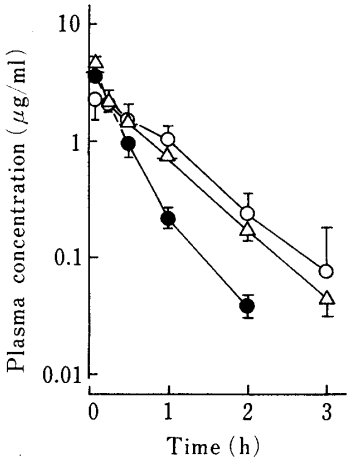

Fig. 2. Plasma Concentrations of MMC in Rats Following Intravenous (●), Intramuscular (△) and Intraperitoneal (○) Injection

Each point represents the mean value with S.D. of at least three rats.

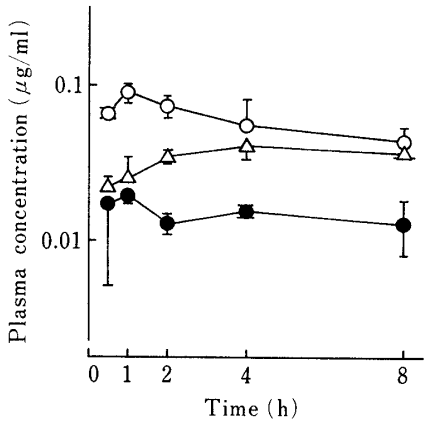

Fig. 3. Plasma Concentration of Free MMC in Rats Following Intravenous (●), Intramuscular (△) and Intraperitoneal (○) Injection of MMC-D (T-10)

Each point represents the mean value with S.D. of at least three rats.

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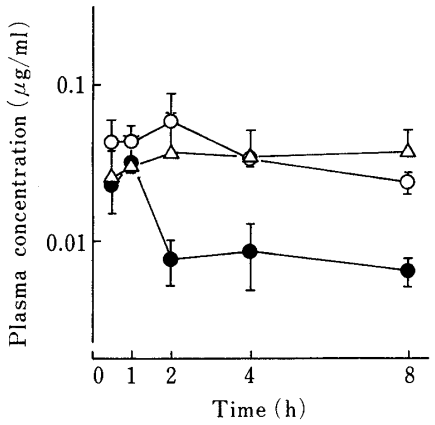

Fig. 4. Plasma Concentrations of Free MMC in Rats Following Intravenous (●), Intramuscular (△) and Intraperitoneal (○) Injection of MMC-D (T-70)

Each point represents the mean value with S.D. of at least three rats.

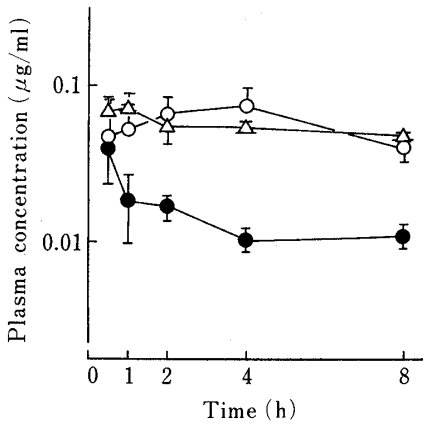

Fig. 5. Plasma Concentrations of Free MMC in Rats Following Intravenous (●), Intramuscular (△) and Intraperitoneal (○) Injection of MMC-D (T-500)

Each point represents the mean value with S.D. of at least three rats.

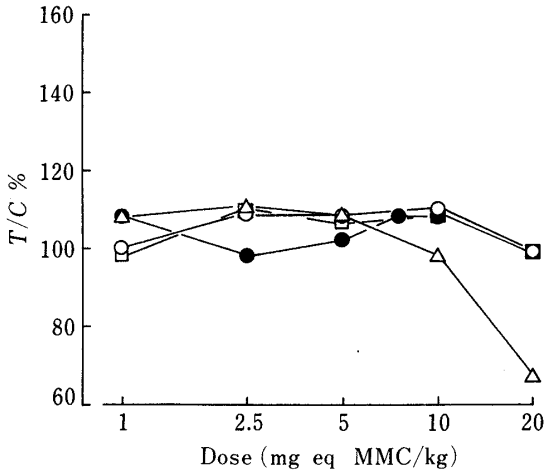

Fig. 6. Effects of Intravenous Administration of MMC and MMC-D on the Survival Time of Mice Inoculated Intraperitoneally with L1210 Leukemia

●, MMC; ○, MMC-D (T-10); □, MMC-D (T-70); △, MMC-D (T-500).

Each point represents the mean value of six mice. The mean survival time of the control group was 8.14 d.

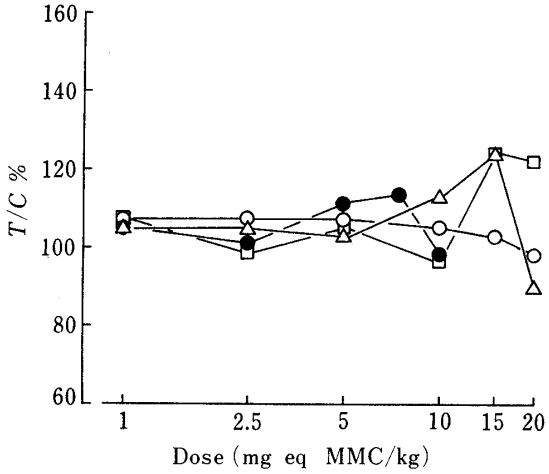

Fig. 7. Effects of Intramuscular Administration of MMC and MMC-D on the Survival Time of Mice Inoculated Intraperitoneally with L1210 Leukemia

●, MMC; ○, MMC-D (T-10); □, MMC-D (T-70); △, MMC-D (T-500).

Each point represents the mean value of six mice. The mean survival time of the control group was 7.92 d.

the largest $T/C\%$ value of 141.8 at the lowest dose (10 mg/kg) among the three types of conjugates. The maximum $T/C\%$ value decreased as the molecular weight of dextran decreased. The maximum effect of MMC was found at a dose of 5 mg/kg ($T/C\% = 152.4$), and was essentially equal to that of MMC-D (T-500).

In Vitro Antitumor Activity

Figures 9 and 10 illustrate the *in vitro* cytocidal effects of MMC and MMC-D on L1210 leukemia cells. As shown in Fig. 9, the exposure of leukemia cells to MMC at a concentration between 2.5 and 10 μg/ml caused a remarkable prolongation of the survival time of mice inoculated with the treated cells. MMC-D exhibited cytocidal activities from one-thirtieth

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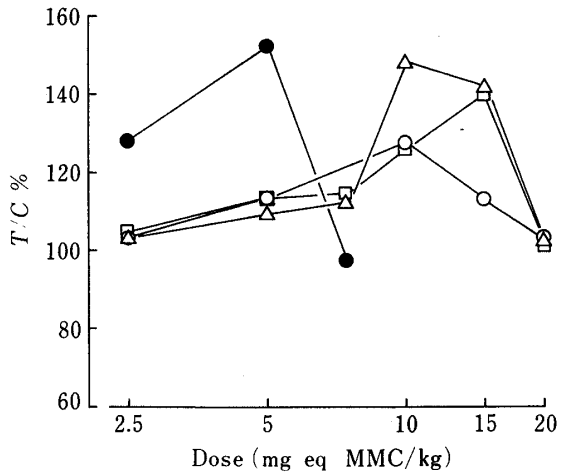

Fig. 8. Effects of Intraperitoneal Administration of MMC and MMC-D on the Survival Time of Mice Inoculated Intraperitoneally with L1210 Leukemia

●, MMC; ○, MMC-D (T-10); □, MMC-D (T-70); △, MMC-D (T-500).

Each point represents the mean value of six mice. The mean survival time of the control group was 7.3 d.

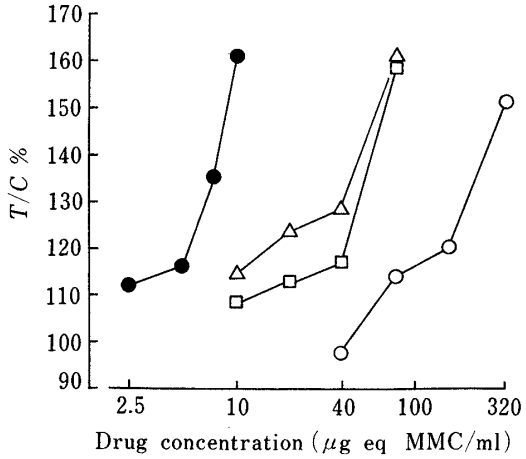

Fig. 9. *In Vitro* Cytocidal Effects of MMC and MMC-D on L1210 Leukemia Cells Evaluated in the Intraperitoneal Inoculation System

●, MMC; ○, MMC-D (T-10); □, MMC-D (T-70); △, MMC-D (T-500).

L1210 leukemia cells were exposed to drugs for 1 h at 37 °C, washed twice and inoculated into BDF_1 mice. Cytotoxicity was evaluated by comparing the survival time of mice inoculated with treated cells (*T*) with that of control mice given drug-untreated cells (*C*).

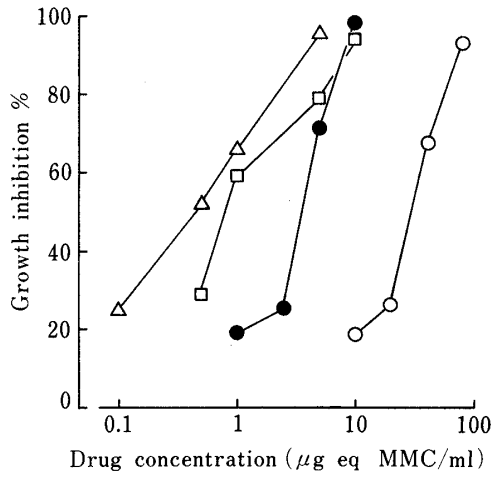

Fig. 10. *In Vitro* Cytocidal Effects of MMC and MMC-D on L1210 Leukemia Cells Evaluated in the Cell Culture System

●, MMC; ○, MMC-D (T-10); □, MMC-D (T-70); △, MMC-D (T-500).

L1210 leukemia cells were exposed to drugs for 1 h at 37 °C, washed twice and cultured for 4 d. Cytotoxicity was evaluated by comparing the number of viable cells in the treated group with that in the control group.

(MMC-D (T-10)) to one-sixth (MMC-D (T-500)) of that of MMC. The dose–response curve shifted to lower dose as the molecular weight of MMC-D increased. These shifts of the dose–response curve depending on the molecular size were also observed in the tissue culture system as shown in Fig. 10. The IC_{50} values (the drug concentration showing 50% growth inhibition) were 3.5 μg/ml for MMC, 30.0 μg/ml for MMC-D (T-10), 0.8 μg/ml for MMC-D (T-70) and 0.4 μg/ml for MMC-D (T-500). In this case, MMC-D (T-70) and MMC-D (T-500) exhibited about 5 and 9 times higher cytocidal activities than MMC, respectively.

Discussion

Recently, the use of macromolecular carriers for the selective targeting of antitumor agents has been advocated with increasing frequency and had led to numerous reports on the conjugation of agents such as anthracyclines,[13-15] alkylating agents[16] and methotrexate[17,18] with carriers such as deoxyribonucleic acid (DNA),[19] polypeptides,[13,17,20] polysac-

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charides,[15] and antibodies.[14,16,18] In the case of polymeric anthracyclines and methotrexate, which gave the most successful results among the polymeric antineoplastics, drugs are considered to enter the tumor cells with the carrier by endocytosis and to be cleaved to free forms by lysosomal enzymes (these agents are called lysosomotropic agents).[21] MMC-D has been developed in an attempt to improve pharmacokinetic behavior and cellular access of MMC. MMC-D was proved to act as a macromolecular prodrug of MMC and to exhibit antitumor activity after being converted to MMC.[9,10] In contrast to the lysosomotropic agents, however, regeneration of MMC was considered to proceed by chemical hydrolysis at the same rate ($T_{1/2}$ = *ca.* 24 h) both *in vitro* and in the body. Thus, regenerated MMC in plasma may contribute to the activity of MMC-D, and our attention was first focused on this point.

As shown in Figs. 3, 4 and 5, the plasma concentrations of MMC after injection of MMC-D are fairly similar regardless of molecular size in each administration route. However, *i.m.* and *i.p.* injections showed higher plasma levels than *i.v.* injection. This is probably because MMC-D administered systemically accumulates predominantly in the reticuloendothelial organs such as the liver and spleen, and is directly inactivated as such, while MMC-D injected locally remains at the injection site and supplies active MMC for a long period.

However, no direct relationship between plasma MMC levels and antitumor activities was observed in the administrations of MMC-D through different routes and with different molecular weights. The *i.p.* injection of MMC-D significantly improved the survival time of mice given *i.p.* inoculation of L1210 leukemia, whereas the *i.m.* injection, which gave almost the same plasma concentration–time profiles of MMC as the *i.p.* injection, did not show significant antitumor activities; *i.v.* injection was also ineffective. These results suggest that the contact of the drug with tumor cells in the peritoneal cavity was important in exhibiting the antitumor effect. Furthermore, in the *i.p.* administration of MMC-D, antitumor activities were enhanced as the molecular weight of the conjugates increased, though the plasma concentrations of liberated MMC were almost equal irrespective of the molecular size. Possible explanations for these results are as follows: 1) larger MMC-D remains and supplies a larger amount of MMC for a longer period in the peritoneal cavity, 2) MMC-D has a molecular weight-dependent interaction with tumor cells. The results of *in vitro* antitumor activity evaluation suggested a contribution of the second mechanism, though the first mechanism cannot be disregarded.

In Figs. 9 and 10, the shifts of the dose–response curve depending on the molecular weight of MMC-D were in good accordance with the *in vivo* antitumor activity. Considering that all types of MMC-D released MMC at the same rate even during incubation with tumor cells (unpublished data), MMC liberated from MMC-D during incubation with tumor cells might be responsible for essentially the same cytotoxicity regardless of the molecular weight of MMC-D, at least *in vitro*. From these results and discussions, it may be considered that MMC-D interacts with tumor cells in conjugated form, and this interaction increases with an increase of the molecular weight of dextran.

Concerning the linkage between the spacer and dextran, three structures can be considered.[22] MMC-D has a cationic charge at pH 7.4 probably due to structures such as isourea (Fig. 1) and N-substituted imidocarbonate, though a carbamate linkage is also possible. The cellular interaction study revealed that MMC-D was adsorbed on the tumor cell surface having an anionic charge by electrostatic interaction, suggesting that MMC-D adsorbed on the cell surface liberates intact MMC which then exhibits cytotoxicity (unpublished data). In the cell culture system, MMC released from MMC-D on the tumor cell surface should spread only in the culture medium (1 ml), whereas in the *i.p.* inoculation system, it might spread not only in the peritoneal cavity but also into the blood. Therefore, cells should be exposed to a higher concentration of MMC in the former system than in the

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latter system after incubation with MMC-D. In accordance with this, MMC-D (T-70) and MMC-D (T-500) exhibited higher antitumor activities than MMC in the cell culture system, but lower activities in the *i.p.* inoculation method, as shown in Figs. 9 and 10. MMC, which is hardly adsorbed on tumor cells, exhibited almost the same activity in both experimental systems.

The present results indicate that MMC-D has some interaction with tumor cells, and this interaction is important for the antitumor activity, as well as the modified pharmacokinetic properties. A further study on the cellular interaction is in progress to elucidate whether some part of MMC-D passes into the cell and how physicochemical characteristics such as electric charge influence the interaction between the cells and macromolecular prodrugs.

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