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Physico-chemical and Antitumor Characteristics of High Molecular Weight Prodrugs of Mitomycin C[1]

Akira Kato, Yoshinobu Takakura, Mitsuru Hashida, Toshikiro Kimura, and Hitoshi Sezaki*

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto, 606, Japan

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High molecular weight derivatives of mitomycin C (MMC) were synthesized by using dextrans of various molecular weights (MMC–D), poly-L-glutamic acid (MMC–PGA), and bovine serum albumin (MMC–BSA) as carrier moieties, and their physico-chemical characteristics and antitumor activities were examined. MMC was liberated from MMC–D *in vitro* with a half-life of about 24 h regardless of the size of dextran, while the liberation half-lives of MMC–PGA and MMC–BSA were 35.5 h and 20.2 h, respectively. The molecular sizes of conjugates determined by gel filtration chromatography were slightly larger than those of the original carrier molecules. MMC–D showed significant antitumor activities in BDF_1 mice bearing P388 leukemia or B16 melanoma in an *i.p.-i.p.* system, depending on their molecular sizes; *i.e.*, higher maximum activity was obtained at lower dose as the molecular weight increased. MMC–PGA exhibited a superior effect against B16 melanoma in spite of its low molecular weight. These observations suggest the existence of some relationship between the physico-chemical properties of carrier moieties and the antitumor activities of the conjugates.

Keywords——mitomycin C; prodrug; high molecular weight prodrug; dextran; protein; physico-chemical characteristics; molecular size; release rate; antitumor activity; murine tumor

A possible approach for improving the chemotherapeutic activity of anticancer agents would be to concentrate their cytotoxicity at the tumor site by altering their biological properties. We have previously reported the covalent attachment of anticancer agents to agarose beads and the consequent enhancement of effects on transplanted tumors in mice.[2–4] These conjugates act as an immobile molecular depot form of their parent agents, reducing acute toxic and immuno-supressive effects on the host while maintaining local therapeutic potency.

We next synthesized soluble high molecular weight derivatives of mitomycin C(MMC), mitomycin C-dextran conjugate (MMC–D),[5] and evaluated their antitumor activities in various tumor systems having different physiological characteristics.[6] The ability to remain in a specific locality (*e.g.* the peritoneal cavity) as a potential sourse of free MMC for a long period had suggested the usefulness of these derivatives in the treatment of solid tumors and malignancies thriving in isolated body cavities. The potential value of derivatives which behave differently from the parent compound in biological environments has thus been demonstrated.

Although various conjugates of cytotoxic agents with high molecular weight materials have been synthesized as candidates for tumor-specific agents, as summarized by Poznansky and Cleland,[7] little is known about the relationship between their efficiency and physico-chemical characteristics, which appear to be one of the most important factors governing their *in vivo* fate and consequent therapeutic potency. In the present investigation, we synthesized high molecular weight derivatives of MMC by using dextrans of various molecular weights, poly-L-glutamic acid (PGA), and bovine serum albumin (BSA) as carrier moieties. Herein, we describe the physico-chemical properties and antitumor activities of these conjugates and discuss their relationship.

Experimental

Material——MMC was kindly supplied by Kyowa Hakko 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). PGA and BSA were purchased from Sigma Chemicals Co., U.S.A., and have average molecular weights of 14000 and 66000, respectively. All other chemicals were reagent grade products obtained commercially.

Preparation of MMC Derivatives——MMC-dextran conjugates (MMC–D) were synthesized in the same mannar as described previously.[5,6] The conjugate of MMC and PGA (MMC–PGA) was synthesized by reacting 200 mg of PGA in 50 ml of aqueous solution with 600 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 40 mg of MMC. The pH of the mixture was kept between 5.0 and 6.0 and the reaction was allowed to proceed for 4 h at room temperature. MMC–PGA was isolated by precipitation with cold acetone. The conjugate of MMC and BSA (MMC–BSA) was synthesized in the same manner as MMC–PGA but the product was purified by gel filtration (Sephadex G-25, column 3.1 cm × 30 cm) and obtained as a lyophilized form. All compounds were prepared in saline solution and injected intraperitoneally at 0.1 ml/10 g body weight in animal experiments. The dose of MMC conjugate was expressed in terms of amount of parent MMC.

***In Vitro* Release Experiment**——The release of MMC from conjugates was determined by means of modified dynamic dialysis system of Meyer and Guttman.[8] A Visking dialysis tube (8/32) containing 10 ml of pH 7.4 isotonic phosphate buffer solution of MMC derivative was immersed in 100 ml of the same buffer maintained at 37°C. The inner solution was stirred continuously and 1 ml of sample was withdrawn from the outer medium at fixed time intervals for analyses. The release rate and half-life were calculated by the least-squares method. Adsorption of MMC on the cellulose tube was negligible.

Molecular Size Estimation——A Sephadex G-200 or Sepharose 4B column (1.7 cm × 65 cm) was employed for molecular size determination. A 2 mg sample dissolved in 1 ml of NaCl solution was chromatographed and each column was eluted with 0.5 M NaCl solution at 5°C. The flow rate was kept at 8—9 ml/h and fractions of 3 ml were collected for analysis.

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 = elution volume of the sample; V_o = column void volume; and V_t = total bed volume. The Stokes' radii of the samples were determined from the calibration curves obtained from K_{av} values of eight kinds of protein of known molecular radii (Gel filtration calibration kit, Pharmacia Fine Chemicals Co., Sweden).

Analytical Method——In the *in vitro* release experiment, the amount of released MMC was determined by measuring antimicrobial activity against *Escherichia coli* B using the disc-plate method. In the gel filtration experiment, the amount of conjugated MMC was determined spectrophotometrically at λ_{max} = 364 nm. The amount of dextran was measured by the anthrone mehtod[9] and PGA and BSA were assayed spectrophotometrically by measuring the absorbance at 212 and 280 nm, respectively.

Animal Experiment——Male DBA/2 mice and male BDF_1 hybrid mice (C57Bl/6, female × DBA/2, male) were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals, Shizuoka. These animals were kept on the breeding diet NMF (Oriental Yeast Co.) with water *ad libitum* in a room maintained at 23 ± 1°C and a relative humidity of 55 ± 5%. P388 leukemia cells were maintained by weekly transplantation of tumor cells into the peritoneal cavity of male DBA/2 mice. B16 melanoma cells were maintained by biweekly subcutaneous implantation into C57Bl/6 mice.

Evaluation of Antitumor Activity——BDF_1 mice weighing 19—24 g were inoculated intraperitoneally with 1×10^6 P388 leukemia or 5×10^8 B16 melanoma cells suspended in 0.2 ml of Hanks' solution. Chemotherapy was given intraperitoneally at 24 h after inoculation. All activities of compounds were recorded as T/C % values, calculated as the ratio of the mean survival time of the treated group divided by that of the control group. The observation period was 60 days.

Results

Synthesis of MMC-macromolecule Conjugates

On gel filtration chromatography of the three types of MMC–D, MMC was proved to be coupled to the dextran backbone and conjugates were estimated to contain about 10% MMC (w/w) regardless of molecular weight (Table I). The degree of substitution by MMC of the dextran was estimated to be one molecule per approximately 14—17 glucose units. The contents of MMC in MMC–PGA and MMC–BSA were about 21 and 5% (w/w), respectively.

In the case of MMC–PGA, one MMC molecule was estimated to be coupled per about eight glutamic acid units.

TABLE I. Characteristics of Mitomycin C–Macromolecule Conjugates studied in the Present Investigation

Compound	Mitomycin C content (w/w %)	Molecular weight of carrier ($\bar{M}_w$)	*In vitro* release[a]	
			Rate (h^{-1})	Half-life (h)
MMC–D (T-10)	10.8	9900	0.0284	24.36
MMC–D (T-70)	11.8	64400	0.0287	24.19
MMC–D (T-500)	10.1	487000	0.0292	23.75
MMC–PGA	21.2	14000	0.0193	35.50
MMC–BSA	5.0	66000	0.0343	20.22

a) *In vitro* release rate and half-life were calculated by the least-squares method. The mean value of two experiments is given in each case.

Molecular Size Estimation

The molecular sizes of MMC derivatives were determined by the gel filtration method. Fig. 1 shows elution patterns of MMC–D (T-70) and dextran T-70 chromatographed on a Sepharose 4B column. There is fair agreement between the elution peak of MMC–D (T-70) determined by the anthrone method and that measured spectrophotometrically based on the maximum absorption peak of MMC. The original dextran T-70 was eluted somewhat later. This indicates that the molecular size of the conjugate is increased as a result of the coupling reaction. The calculated effective molecular sizes of all derivatives and original carriers are summarized in Table II. These values were determined from the calibration curve obtained from the K_{av} values of eight molecular size markers. The molecular sizes of all derivatives

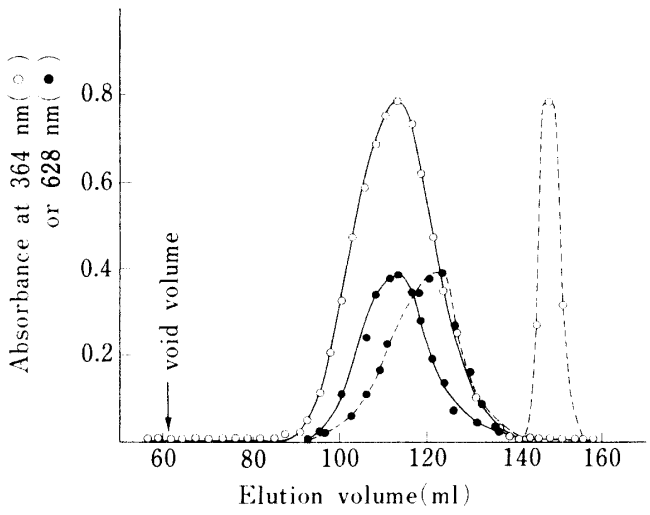

Fig. 1. Gel Filtration Patterns of Mitomycin C (---○---), Dextran (T-70) (---●---), and Mitomycin C–Dextran (T-70) Conjugate (—○—, —●—) on a Sepharose 4B Column

Chromatography was carried out on a Sepharose 4B column (1.7 × 65 cm) with 0.5 M NaCl at 5°C and fractions of 3 ml were collected automatically. Mitomycin C was detected spectrophotometrically at 364 nm and dextran was determined by the anthrone method at 628 nm.

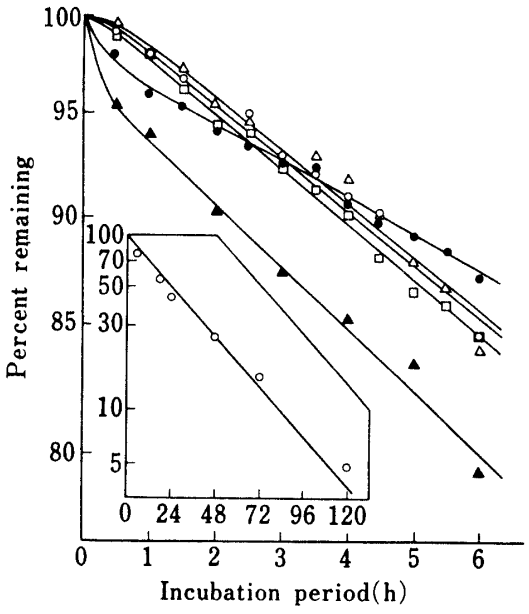

Fig. 2. *In Vitro* Release of Mitomycin C from MMC–D (T-10) (△), MMC–D (T-70) (○), MMC–D (T-500) (□), MMC–PGA (●), and MMC–BSA (▲)

Data are means of two experiments, and are shown as semi-logarithmic plots of percent remaining-mitomycin C *versus* incubation time.

except MMC–D (T-500) were larger than those of the original carriers, but all of them except for MMC–BSA were of the same order of size as the original carriers.

Table II. Molecular Sizes of Mitomycin C–Macromolecule Conjugates and Their Carrier Moieties estimated by Gel Filtration Chromatography

Compound	K_{av} value	Estimated effective radius (Å)[c]
Dextran T-10[a]	0.697	20.05
MMC–D (T-10)[a]	0.598	25.36
Dextran T-70[b]	0.715	58.06
MMC–D (T-70)[b]	0.598	74.51
Dextran T-500[b]	0.479	96.03
MMC–D (T-500)[b]	0.503	91.24
PGA[a]	0.970	10.49
MMC–PGA[a]	0.863	13.52
BSA[a]	0.432	37.61
MMC–BSA[a]	0.024	99.05

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 macromolecules.

In Vitro Release Rate

Fig. 2 shows a semilogarithmic plot of percent of MMC remaining in the conjugate *versus* time during the *in vitro* release experiment. All MMC derivatives showed monoexponential liberation of biologically active MMC which continued for several days, as shown in the case of MMC–D (T-70). MMC–D all showed half-lives of about 24 h regardless of their molecular sizes, as shown in Table I. MMC–PGA and MMC–BSA showed rapid disappearance of MMC from inside the Visking tube at the initial period because of slight contamination by free MMC, but after this period MMC–PGA and MMC–BSA showed monoexponential release of MMC with half-lives of 35.5 and 20.2 h, respectively.

Antitumor Activity against P388 Leukemia and B16 Melanoma

Fig. 3 shows the effect of three types of MMC–D on the life-span of mice bearing P388

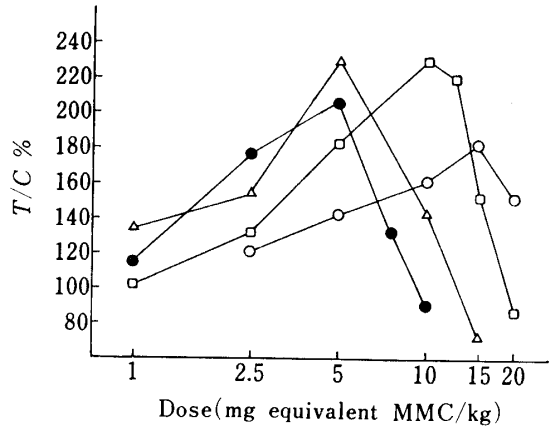

Fig. 3. Effects of Mitomycin C and Mitomycin C–Dextran Conjugates on the Survival Time of Mice bearing P388 Leukemia

●, Mitomycin C; ○, MMC–D (T-10); □, MMC–D (T-70); △, MMC–D (T-500). Each point represents the mean value of six mice. The mean survival period of the control group was 10.1 d.

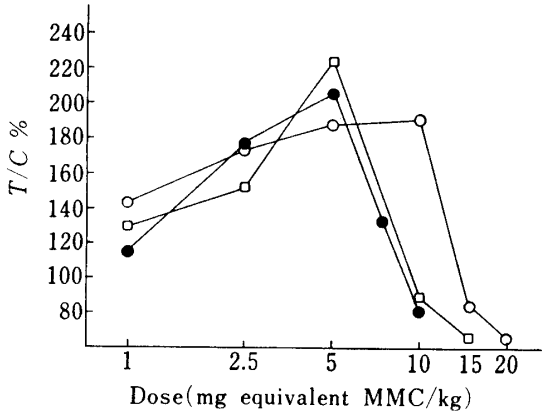

Fig. 4. Effect of Mitomycin C and Its Poly-L-glutamic Acid and Bovine Serum Albumin Conjugates on the Survival Time of Mice bearing P388 Leukemia

●, Mitomycin C; ○, MMC–PGA; □, MMC–BSA. Each point represents the mean value of six mice. The mean survival period of the control group was 10.1 d.

leukemia. Single intraperitoneal injection of MMC–D (T-70) and MMC–D (T-500) showed the same maximum T/C value of 224.9% at doses of 10 mg/kg and 5 mg/kg, respectively. MMC–D (T-10) showed a maximum T/C value of 183.2% at the dose of 15 mg/kg; it was less effective than T-500 and T-70 ($p<0.1$). From these results, shifts of dose-response curve with change of molecular size of the conjugates were clearly apparent, *i.e.*, the dose-response curve shifts to lower dose as the molecular weight increases.

In Fig. 4, the antitumor activities of MMC–PGA and MMC–BSA against P388 leukemia are compared with that of free MMC. MMC–PGA showed the maximum T/C value of 189.2% at the dose of 10 mg/kg, which is similar to that of MMC–D (T-10) ($P<0.1$), but over this dose MMC–PGA exhibited marked toxicity. MMC–BSA exhibited higher antitumor activity and maximal effective dose, 5 mg/kg, affording 224.4% T/C value. The dose-response relation of MMC–BSA resembled that of MMC–D (T-500) indicating some effect of molecular size on antitumor efficiency.

The effects of various conjugates on the survival time of mice bearing B16 melanoma are summarized in Table III in comparison with that of free MMC. Free MMC exhibited a T/C value of 194.4% at the dose of 5 mg/kg. MMC–D (T-10), MMC–D (T-70), and MMC–D (T-500) showed T/C values of 164.5, 175.2, and 205.1%, respectively at the dose of 10 mg/kg.

Table III. Effect of Mitomycin C and Mitomycin C–Macromolecule Conjugates on the Survival Time of Mice bearing B16 Melanoma[a)]

Compound	Dose (mg equivalent MMC/kg)	Mean survival days	T/C %[b)]	Median survival days	Survivors at 60 days
Control		15.6	100.0	15.5	0/6
MMC	5.0	30.3[c)]	194.4	28.0	0/6
MMC–D (T-10)	5.0	20.2[c)]	129.3	19.0	0/6
	10.0	25.7[c)]	164.5	24.5	0/6
MMC–D (T-70)	5.0	21.5[c)]	137.8	20.5	0/6
	10.0	27.3[c)]	175.2	27.5	0/6
MMC–D (T-500)	5.0	19.0	121.8	19.0	0/6
	10.0	>32.0[c)]	>205.1	27.5	1/6
MMC–PGA	5.0	>39.5[c,d)]	>253.2	40.5	1/6
	10.0	30.3[c)]	194.4	34.5	0/6
MMC–BSA	5.0	17.7	113.3	15.5	0/6

a) B16 melanoma cells were inoculated intraperitoneally and intraperitoneal treatment was given at 24 h after inoculation.
b) The ratio of the mean survival time of the treated group divided by that of the control group.
c) $p<0.05$, *versus* the control group.
d) $p<0.05$, *versus* the free MMC (5 mg/kg) group.

Discussion

Recently, various kinds of carrier moieties, especially macromolecular compounds, have been utilized for targeting cytotoxic agents to cancer cells.[7,10)] In these cases, effective interaction of the cytotoxic agents with the target cells *in vivo* depends on the following two prerequisites; 1) localization in the vicinity of the tumor site and 2) accessibility to cancer cells. Thus not only affinity to cancer cells but also the biopharmaceutical characteristics of the conjugate can affect the target specificity, and the physico-chemical properties of the conjugates should be closely related to both factors.

The antitumor antibiotic MMC was isolated by Wakaki *et al.*[11)] as the most effective among three fractions from *Streptomyces caespitosus* in 1958. Although MMC has been found to have a broad spectrum of activity against transplanted tumors, and has been used to treat neoplasia

including chronic myelogenous leukemia and solid tumors of various organs, its use is limited by the manifestation of severe bone marrow depression and gastrointestinal damage.[12] To overcome these defects, it may be possible to optimize the concentration-time profile of MMC at the target organ and to minimize the burden to other tissues by modifying its biopharmaceutical characteristics.

MMC–D has thus been developed in the hope that this compound would act as a latent form of MMC which would show lower toxicity than the parent drug. By tracing the *in vivo* fate of MMC conjugated in MMC–D, it was suggested that MMC–D behaved characteristically as a high molecular weight prodrug in the body of mice, supplying potential free MMC.[5] Further, pharmacokinetical analysis of the *in vivo* disposition of MMC–D revealed that MMC was liberated from MMC–D at a rate similar to that obtained *in vitro* (manuscript in preparation). On the other hand, the direct effect of MMC–D (T-70) on tumor cells *in vitro* has been proved to be about one-tenth of that of free MMC (in preparation). These results suggest the importance of liberated MMC as the active form.

As shown in Fig. 3, MMC–D showed a shift of the dose-response curve according to its molecular size. On the other hand, the three types of MMC–D have generally similar characteristics, such as MMC content and release rate, except for the molecular size, as summarized in Table I. On the basis of these facts, it can be speculated that change of molecular weight between 1×10^4—5×10^6 affects the antitumor activity of MMC–D by modifying its pharmacokinetic behavior after intraperitoneal injection and thus affecting the bioavailability of free MMC.

Although the ultimate action site at which growth of malignant cells leads to the death of the host animal is not known for the present tumor systems, smaller MMC–D would undergo rapid elimination from the peritoneal cavity and thus, the total exposure of tumor cells to MMC, at least in the peritoneal cavity, would decrease. Conversely, large MMC–D would remain in the injection site for a considerable period supplying active MMC, as shown in a previous report.[6] The findings of Barlogie and Drewinco[13] that the cytotoxicity of MMC is determined by the given exposure dose support the present speculation.

MMC–PGA showed high activity on B16 melanoma in spite of its smaller molecular size as compared with MMC–D (T-10). MMC–PGA showed the highest MMC content and the slowest release rate, as shown in Table I. Furthermore, electrophoresis revealed that MMC–PGA has anionic charge under physiological conditions, *i.e.*, at pH 7.4, whereas all MMC–Ds behave as polycationic compounds under the same conditions. These factors may complicate its pharmacological activity in combination with the molecular size effect. BSA has both carboxyl and amino groups in its molecular structure which makes it convenient for use as the carrier moiety, but BSA readily aggregates during the carbodiimide reaction, as demonstrated in the gel filtration chromatography. This is considered to be partly responsible for the observed dose-response profile of MMC–BSA, which is almost the same as that of MMC–D (T-500).

The present investigation has thus shown that physico-chemical properties, especially molecular size, significantly affect the activity of a macromolecular prodrug. Further experiments are in progress to elucidate whether some components of MMC derivatives pass into the the cell and how physico-chemical characteristics such as electric charge influence the interaction between the cells and macromolecular prodrugs. Studies on the relation between the physico-chemical properties, pharmacokinetic properties, and pharmacological activities of conjugates should aid in the development of new marcomolecular prodrugs and cancer drug delivery systems.

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