J. Pharm. Pharmacol. 1980, 32: 30–34 Received April 6, 1979

Mitomycin C-dextran conjugate: a novel high molecular weight pro-drug of mitomycin C

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A high molecular weight derivative of mitomycin C (MMC), mitomycin C-dextran conjugate (MMC-D) has been synthesized and its biological and pharmacological properties investigated. MMC is released from MMC-D in vitro with a half-life of 24 h. After intraperitoneal injection of MMC-D, free MMC could be detected in plasma and urine of mouse for 5–8 h, while MMC administered as a free form was eliminated rapidly. After MMC-D, given to mice bearing Ehrlich ascites carcinoma or B16 melanoma there was a reduction in toxicity and an increase in survival time compared with MMC. These observations suggest that the high molecular weight MMC-dextran derivative is a kind of pro-drug which persists in the body giving a sustained release of free MMC thus significantly increasing the antitumour activity of the parent drug.

A possible approach for improving the chemotherapeutic activity of anticancer agents would be to concentrate their cytotoxicity at the tumour site by altering their biological properties. We have previously reported the covalent attachment of anticancer agents to agarose beads and the enhanced effect of the resulting conjugates on transplanted tumours in mice (Hashida et al 1977, 1978; Kojima et al 1978). These conjugates exhibited their efficiency as an immobile molecular depot form of their parent agents while reducing acute toxic and immunosuppressive effects towards the host yet maintaining local therapeutic potency. We have now synthesized a high molecular weight derivative of mitomycin C (MMC), mitomycin C-dextran conjugate (MMC-D), as a macromolecular mobile pro-drug which might be expected to have behave differently from the parent compound in biological environments. Herein, we present biological, toxicological, and chemotherapeutic properties of the conjugate.

MATERIALS AND METHODS

Materials

MMC was supplied from Kyowa Hakko Ltd. Dextran was purchased (Dextran T-70, Pharmacia Fine Chemicals). All other chemicals were of reagent grade quality and obtained commercially (Nakarai Chemicals Ltd.).

Animals and tumour

Animals used were male ddY mice and male hybrid BDF_1 mice (C57BL/6 × DBA/2). Ehrlich ascites carcinoma (EAC) was supplied by Shionogi Pharmaceutical Ltd. and B16 melanoma was kindly supplied by the Cancer Chemotherapy Center, Tokyo, Japan.

Preparation of MMC-D

MMC-D was synthesized by following two steps. (a) Dextran was activated with cyanogen bromide according to Axén & Ernback (1971). To a stirred solution of dextran (1 g) in water (100 ml), cyanogen bromide was added in three portions (0·2, 0·2 and 0·15 g) and the pH of the mixture was kept at 10·7 with 1 M NaOH. Then ϵ-aminocaproic acid (1 g) was added maintaining the pH at 9·0 with 1 M HCl and coupling reaction was allowed to proceed for 24 h at room temperature (20 °C). The product, spacer-introduced dextran, was dialysed against Na_2CO_3 solution (pH 9·0) and washed repeatedly with the same solution by ultrafiltration (Pellicon PSED 043 10). (b) MMC was conjugated through a carbodiimide-catalysed reaction. MMC (100 mg) was dissolved into a stirred solution of spacer-introduced dextran (100 ml) and then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (2 g) was added. The pH of the mixture was kept between 5·0 and 6·0, and the reaction was allowed to proceed for 24 h at room temperature. The product was washed with water and concentrated by ultrafiltration and then precipitated with an addition of acetone. The precipitate was washed on a glass filter with acetone under suction and evaporated.

In vitro release experiment

MMC-D (4 mg) was dissolved in pH 7·4 isotonic phosphate buffer (10 ml) and the solution was incubated at 37 °C with moderate shaking. Both liberated MMC and MMC remaining in the conju-

* Correspondence

gate at several incubation periods were determined after separation by ultrafiltration (Pellicon PSED 043 10).

Determination of plasma concentration and urinary excretion

Male ddY mice (23–30 g) were injected intraperitoneally with 5 mg (equivalent MMC) kg^{-1} of MMC-D or unbound MMC. For the measurement of plasma MMC concentration, blood was removed from the heart at various times after injection and plasma obtained by centrifugation. For the measurement of urinary MMC excretion, mice were housed in a metabolic cage and urine was collected at varying times for 5 days.

Analytical method

In the in vitro release experiment, the amount of MMC and MMC-D were determined spectrophotometrically based on $\epsilon = 22000$, $\lambda_{max} = 364$ nm. The antimicrobial activity of MMC in plasma or urine sample was determined by the disc-plate method using *Escherichia coli* B as a test microorganism.

Growth inhibitory effect on EAC cells

Male ddY mice (20–22 g) were inoculated intraperitoneally with $1{\cdot}4 \times 10^7$ EAC cells and the drug, MMC-D or MMC, was injected intraperitoneally 24 h after inoculation. At 7 days after inoculation, ascitic fluid was removed from each mouse and the total number of harvested cells was counted using a haemocytometer.

Acute toxicity to mice

Toxicities of MMC-D and MMC were evaluated with survivors on the 35th day after intraperitoneal single administration to ddY mice.

Therapeutic efficiencies on tumour-bearing mice

To examine the antitumour efficiency of the compounds, ddY mice (20–22 g) and BDF_1 mice (18–20 g) were inoculated intraperitoneally with $1{\cdot}4 \times 10^7$ EAC cells and 8×10^6 B16 melanoma cells, respectively. Chemotherapy was given intraperitoneally at 24 h after inoculation. All activities of the drugs were recorded in T/C% values, calculated as the ratio of the mean survival time of the treated group divided by that of the control group.

RESULTS

Synthesis of MMC-D

On gel filtration chromatography (Sephadex G-75), approximately 90% of MMC was proved to be coupled to the dextran (Fig. 1) and (1 mg) conjugate was estimated to contain about 100 μg MMC from spectrophotometric analysis.

In vitro sustained release

Fig. 2 shows a semilogarithmic plot of percent of drug remaining in the conjugate vs time. MMC-D showed a successive monoexponential liberation with a half-life of 24 h. In Table 1, the amounts of released MMC during the 24 h of the first and the

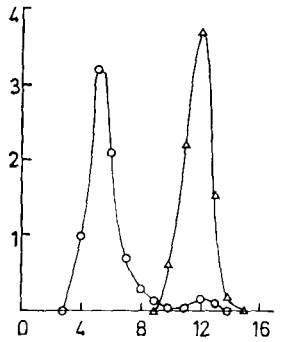

FIG. 1. Gel filtration patterns of mitomycin C-dextran conjugate (MMC-D) (○) and mitomycin C (MMC) (△) on Sephadex G-75 column. Chromatography was carried out on Sephadex G-75 column (20 × 1·7 cm) with isotonic phosphate buffer (pH 7·4) and each fraction (5 ml) was collected automatically. MMC-D and MMC were detected spectrophotometrically. Ordinate: absorbance at 364 nm. Abscissa: fraction number.

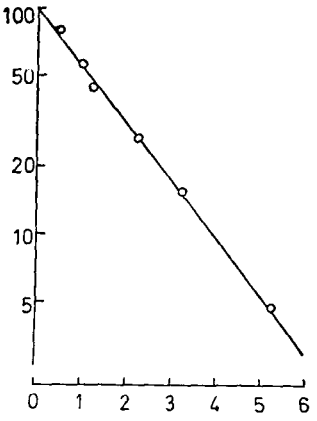

FIG. 2. In vitro release of mitomycin C (MMC) from mitomycin C-dextran conjugate (MMC-D). MMC-D (4 mg) was dissolved in isotonic phosphate buffer (pH 7·4, 10 ml) and incubation was carried out at 37 °C. Results are expressed as semi-logarithmic plots of percent remaining of MMC versus incubation time. Ordinate: percent remaining of MMC. Abscissa: incubation time (days).

Table 1. Amount of mitomycin C released from mitomycin C-dextran conjugate (1 mg) during 24 h incubation in vitro. Mitomycin C-dextran conjugate was dissolved in a isotonic phosphate buffer (pH 7·4) and incubated at 37 °C with moderate shaking. At the end of each 24 h incubation, liberated mitomycin C and conjugate were separated by ultrafiltration and amount of mitomycin C in the filtrate was determined.

Incubation period	Amount of released mitomycin C (μg) Spectrophotometric analysis†	Microbial analysis‡
First 24 h	64·6	56·6
Third 24 h	13·3	11·3

† Determined based on $\epsilon = 22000$ at $\lambda_{max} = 364$ nm.
‡ Antimicrobial activity was determined by disc-plate method using *Escherichia coli* B as a test microorganism.

third day, determined by spectrophotometric analysis, are compared with the values of antimicrobial activity. The results show that the MMC liberated from MMC-D had retained almost all of its original antimicrobial activity.

Fate of MMC-D after intraperitoneal injection

Fig. 3 illustrates plasma concentration of MMC after intraperitoneal injection of MMC-D, together with the results following injection of unbound MMC. Unbound MMC showed a maximum concentration of 1·4 μg ml^{-1} 10 min after injection, but the concentration decreased rapidly and was no longer measurable after 3 h. In contrast, although MMC-D gave a lower concentration of MMC initially, this decreased gradually and a significant antimicrobial activity could be detected even at 8 h after injection.

Fig. 4 represents the urinary excretion rate of MMC after intraperitoneal injection of MMC-D or unbound MMC. After injection of unbound MMC, MMC was excreted at a rate of $6{\cdot}3 \times 10^{-1}$ μg h^{-1} in the urine for the first 6 h, but the rate was rapidly reduced and no antimicrobial activity could be detected in urine after 24 h. On the contrary, when MMC-D was injected, the excretion rate of MMC during the first 6 h was $2{\cdot}7 \times 10^{-2}$ μg h^{-1} but this was maintained above $2{\cdot}9 \times 10^{-5}$ μg h^{-1} for 5 days.

On ascending paper chromatography using *n*-propanol–1% ammonium hydroxide, (2:1 v/v), as solvent system of the plasma and urine samples after injection of MMC-D showed major spot having antimicrobial activity, with R_F value identical to that of original MMC (R_F 0·65). Consequently, it is suggested that the antimicrobial activities of these samples correspond approximately to the amounts of unbound MMC existing in these samples.

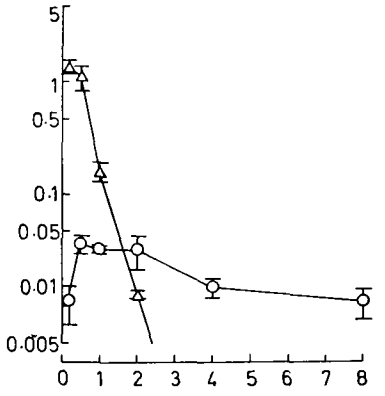

FIG. 3. Plasma mitomycin C concentration after intraperitoneal administration of mitomycin C-dextran conjugate (○) or mitomycin C (△) in mice. Dose was 5 mg equivalent mitomycin C kg^{-1}. Plasma concentration was determined by microbiological analysis and values are expressed as that of corresponding amount of mitomycin C. Each result represents the mean value of 3 mice and vertical bar indicates s.e. Ordinate: concentration (μg equivalent mitomycin C kg^{-1}). Abscissa: time after administration (h).

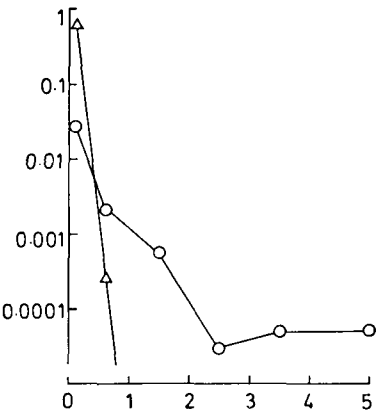

FIG. 4. Urinary excretion rate of mitomycin C after intraperitoneal administration of mitomycin C-dextran conjugate (○) or mitomycin C (△) in mice. Dose was 5 mg equivalent mitomycin C kg^{-1}. Urinary concentrations were determined by microbiological analysis and values are represented as that of corresponding amount of mitomycin C. Each point represents the mean value of 2 groups (10 mice). Ordinate: excretion rate (μg equivalent mitomycin C h^{-1}). Abscissa: time after administration (days).

Growth inhibitory effect on EAC cells and toxicity to mice

Fig. 5 shows an in vivo growth-inhibitory effect of MMC-D and unbound MMC on EAC cells. The growth-inhibitory effect is expressed as a percentage of the cell population of the treated group per control group in which the mean total cell number at 7 days

after inoculation was $7{\cdot}8 \times 10^8$ cells. Since both MMC-D and MMC showed dose-dependent activities, a dose of the drug required to inhibit the cell growth by 90% (ED90) was calculated from the regression line. The ED90 values of MMC-D and unbound MMC were 4·6 and 2·6 mg kg^{-1} respectively.

Fig. 6 illustrates the dose-toxicity relationship in normal mice receiving a single injection of MMC-D or unbound MMC. From these results, LD50 values were determined according to Litchfield & Wilcoxon (1949). The LD50 values of MMC-D and MMC were 15·6 mg kg^{-1} and 7·7 mg kg^{-1}, respectively.

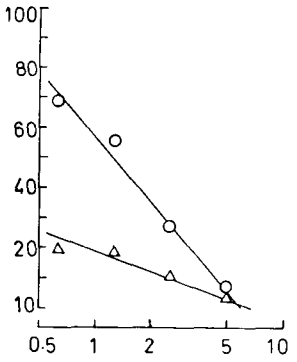

FIG. 5. Growth inhibitory effect of mitomycin C-dextran conjugate (○) or mitomycin C (△) on Ehrlich ascites carcinoma cells in ddY mice. Male ddY mice were inoculated intraperitoneally with $1{\cdot}4 \times 10^7$ cells and chemotherapy was given intraperitoneally at 24 h after inoculation. Results are expressed as percent ratio of harvested cell number of the treated group to the control group (T/C %) (Ordinate) at 7 days after inoculation. Each point represents the mean value of 6 experiments. Abscissa: dose (mg equivalent mitomycin C kg^{-1}).

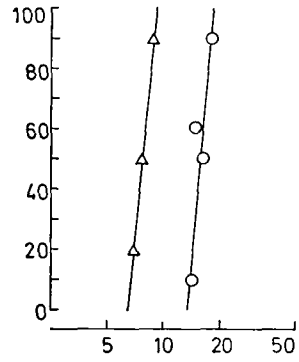

FIG. 6. Toxicity of mitomycin C-dextran conjugate (○) or mitomycin C (△) to normal ddY mice. Each point represents the result of ten mice receiving single administration of drug. Survivors at the 35th day after administration were exhibited. Ordinate: percent of mortality. Abscissa: dose (mg equivalent mitomycin C kg^{-1}).

Antitumour activity

In Table 2, the antitumour activities of MMC-D and MMC on EAC- and B16 melanoma-bearing mice are summarized. Unbound MMC exhibited maximum antitumour activities on both tumours at a dose of 5 mg kg^{-1}, and T/C% was 197% for EAC and 169% for B16 melanoma, respectively. Over this dose, MMC exhibited a marked toxicity. On the contrary, MMC-D had a slightly inferior activity than unbound MMC at the doses less than 5 mg equivalent MMC kg^{-1}. Over this dose, however, MMC-D exhibited marked antitumour activity and three of eight mice bearing B16 melanoma given 8 mg kg^{-1} and one of six mice bearing EAC given 10 mg kg^{-1} were still surviving 60 days after inoculation.

DISCUSSION

MMC has been one of the most extensively used antitumour agents both in the clinic and laboratory since its isolation by Wakaki et al (1958). Although its therapeutic responses have been reported in a variety of cancers, its treatment has been palliative because it is always necessary to halt therapy short of a cancer-destroying dose because of its toxicity. It is possible, however, that if the biological properties of MMC could be modified by substitution into a polymeric compound of high molecular weight, the resultant derivative might overcome this disadvantage. In the present investigation, dextran was chosen as a carrier that might be linked to MMC with a high yield.

Our results must be considered to be of a preliminary nature but from the in vitro release experiment, it is seen that MMC is slowly liberated from MMC-D while its original activity is maintained, and after intraperitoneal injection of MMC-D, unbound MMC was detected in plasma and urine for 5–8 h (Figs 3, 4). It could be considered that the sustained release of MMC also occurs in the body circulation before excretion or metabolic degradation thus MMC-D is a novel kind of pro-drug for MMC.

As is apparent in Table 2, MMC-D exhibited higher chemotherapeutic activity towards transplanted murine tumours than did the parent drug. This advantage was observed only at doses above 5 mg equivalent MMC kg^{-1}, whereas at doses of 1–5 mg kg^{-1} the free drug was superior to the conjugate. The reduction of the overall toxicity of the dextran-linked drug, shown in Fig. 6, should be

Table 2. Effect of mitomycin C-dextran conjugate (MMC-D) and mitomycin C (MMC) on survival time of mice bearing Ehrlich ascites carcinoma (EAC) or B16 melanoma*. EAC ($1{\cdot}4 \times 10^7$ cells) was inoculated intraperitoneally into ddY mice and B16 melanoma (8×10^6 cells) was inoculated intraperitoneally into BDF_1 mice. Chemotherapy was given with intraperitoneal single injection at 24 h after inoculation.

Compound	Dose (mg equiv. MMC kg^{-1})	Mean survival time (days)	T/C %†	Survivors at 60th day
EAC				
Control*		14·4	100·0	0/20
MMC	1·0	19·2	133·3	0/6
	2·5	23·3	162·2	0/6
	5·0	28·3	197·0	0/6
	10·0	10·3	71·8	0/6
MMC-D	1·0	16·7	115·9	0/6
	2·5	20·3	141·3	0/6
	5·0	21·5	149·5	0/6
	10·0	>27·7	>192·4	1/6
MMC + dextran (50 mg kg^{-1})	5·0	27·5	191·0	0/6
B16 melanoma				
Control		11·7	100·0	0/15
MMC	2·5	13·3	114·2	0/6
	5·0	19·7	168·6	0/6
	8·0	11·2	95·7	0/6
MMC-D	5·0	16·0	137·1	0/6
	8·0	>33·1	>283·9	3/8
MMC + dextran (50 mg kg^{-1})	5·0	19·1	163·3	0/6

* Control group had no chemotherapy.
† Calculated as the ratio of the mean survival time of the treated group divided by that of the control group.

responsible for this increased chemotherapeutic effect at doses above 5 mg kg^{-1} at which unbound MMC exerts its optimal activity. Therapeutic indices, calculated as the ratio of ED90 divided by LD50 are 3·4 for MMC-D and 3·0 for unbound MMC suggesting that MMC-D improved the original chemotherapeutic utility of the parent drug.

The principle of introducing small amounts of cytotoxic agents into cells in the form of macromolecular complexes is well known and various attempts have been made to use this approach with covalently linked (Szekerke et al 1972; Ryser & Shen 1978) and non-covalent combinations of complexes (Trouet et al 1972, 1974), as summarized by Gregoriadis (1977). With MMC-D, therefore, dextran is also expected to play a role of endocytable carrier. In our previous reports (Hashida et al 1977, 1978; Kojima et al 1978), however, MMC and cytosine arabinoside linked covalently to agarose beads, a non-endocytable carrier, also exhibited increased therapeutic efficiency, and it was suggested that an alteration in biological behaviour resulted in the significant changes in their activities. Consequently, persistent retention of MMC-D in the body and slow release of active drug from the conjugate shown in the present investigation are considered to be one of the most important factors for increasing therapeutic effectiveness.

On the basis of the evidence presented in this investigation, it can be concluded that the attachment of MMC to dextran changed the biological and pharmacological properties of the parent drug. The ability to remain in a specific locality as a potential source of free MMC suggests its use both in the treatment of solid tumours and malignancies thriving in isolated body cavities.

REFERENCES

Axén, R., Ernback, S. (1971) Eur. J. Biochem. 18: 351–360

Gregoriadis, G. (1977) Nature (London) 265: 407–411

Hashida, M., Kojima, T., Takahashi, Y., Muranishi, S., Sezaki, H. (1977) Chem. Pharm. Bull. 25: 2458–2460

Hashida, M., Kojima, T., Muranishi, S., Sezaki, H. (1978) Gann 69: 839–843

Kojima, T., Hashida, M., Muranishi, S., Sezaki, H. (1978) Chem. Pharm. Bull. 26: 1818–1824

Litchfield, J. T., Wilcoxon, F. (1949) J. Pharmacol. Exp. Ther. 96: 99–113

Ryser, H. J., Shen, W. (1978) Proc. Natl. Acad. Sci. USA 75: 3867–3870

Szekerke, M., Wade, R., Whisson, M. E. (1972) Neoplasma 19: 199–209

Trouet, A., Deprez-de Campeneere, D., De Duve, C. (1972) Nature (London) 239: 110–112

Trouet, A., Deprez-de Campeneere, D., De Smedt-Malengreaux, M. Atassi, G. (1974) Eur. J. Cancer 10: 405–411

Wakaki, S., Marumo, H., Tomioka, K., Shimazu, G., Kato, E., Hamada, H., Kudo, S., Fujimoto, Y. (1958) Antibiot. Chemother. 8: 228–236