Biliary Excretion of Mitomycin C Dextran Conjugates in Relation to Physicochemical Characteristics of Carrier Dextran

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(Received January 16, 1990)

Biliary excretion characteristics of polymeric prodrugs of mitomycin C (MMC), mitomycin C-dextran conjugates with cationic or anionic charge (MMC-D_{cat}, MMC-D_{an}) and with different molecular weights of dextran (10000, 70000, and 500000) were studied in rat. Following intravenous injection, bile was periodically collected and concentrations of free and dextran-conjugated MMC in it were determined by bioassay. MMC administered as a free form was excreted rapidly into bile and 1.8% of the dose was recovered within 2 h. A small amount of MMC was gradually excreted into bile after administration of all MMC-Ds and total recovery at 8 h was less than 1% of dose. In this case, a major part of excreted MMC was recovered as a conjugated form. MMC-D_{cat} gave a larger total excretion of MMC than MMC-D_{an} and excretion was also affected by the molecular weight of carrier dextran. The biliary recovery of MMC-D_{cat} labeled with ¹⁴C at a spacer moiety was significantly higher than that of conjugated MMC determined by bioassay suggesting release and/or inactivation of MMC in MMC-D during the circulation in the body. These results were compared with biliary excretion of model macromolecules with the same molecular weight but different electric charge in order to clarify the effect of electric charge on the biliary excretion of macromolecules. Cationic macromolecules exhibited higher biliary excretion in relation to greater hepatic uptake.

Keywords — mitomycin C; mitomycin C-dextran conjugate; biliary excretion; physicochemical property; molecular weight; electric charge; model macromolecule

Introduction

Recently, various macromolecular drug carrier systems have been developed in order to control the *in vivo* behavior of drugs. In previous studies,¹⁻⁵⁾ we have developed polymeric prodrugs of mitomycin C (MMC), MMC-dextran conjugates having cationic (MMC-D_{cat}) and anionic (MMC-D_{an}) charges, and examined their physicochemical, pharmacokinetic, and pharmacological characteristics. The *in vivo* disposition study in rats and mice demonstrated remarkable accumulation of MMC-D_{cat} in the liver, while MMC-D_{an} showed small plasma clearance and slight accumulation in the liver. The effect of molecular charge on the hepatic uptake of macromolecules was also studied using the model macromolecules with the same molecular weight but different electric charge, and the electric charge of macromolecules was found to play an important role in their hepatic uptake.⁶⁾

After taken up by the liver, macromolecules

should undergo various processes and biliary excretion may play a significant role in them. Excretion of biologically active molecular species would be important from therapeutic and toxicity viewpoints. However, little is known about the relationship between physicochemical properties and biliary excretion of macromolecules including MMC-D.

In the present study, we investigated the biliary excretion characteristics of MMC-D with different molecular sizes and electric charges in rats after intravenous injection in order to elucidate their effects.

Materials and Methods

Materials — MMC was kindly supplied by Kyowa Hakko Kogyo Co. Dextran was purchased from Pharmacia, Uppsala, Sweden, and had average molecular weights of about 10000(T-10), 70000(T-70), and 500000(T-500). Polybuffer exchanger 94 resin, Polybuffer 96 elution buffer, and Sephadex G-25 were also ob-

TABLE I. Structures and Physicochemical Characteristics of MMC-D Conjugates

	M_r (carrier)	Mitomycin C content (weight %)	<i>In vitro</i> release $T_{1/2}$ (h)	Adsorption % at pH 7.2	
				CM-Sephadex	DEAE-Sephadex
MMC-D _{cat} (T-10)	9900	10.80	24.4	51.3	0
MMC-D _{cat} (T-70)	64400	8.46	23.6	61.7	0
MMC-D _{cat} (T-500)	487000	10.10	23.8	50.0	0
MMC-D _{an} (T-10)	9900	9.43	35.0	0	29.2
MMC-D _{an} (T-70)	64400	8.15	35.4	0	32.0
MMC-D _{an} (T-500)	487000	7.80	50.0	0	28.4

tained from Pharmacia, Uppsala, Sweden. Radiolabeled γ -amino[U- 14 C]butyric acid with a specific radioactivity of 74 MBq/mg was purchased from New England Nuclear, Boston, MA. [111 In]Cl₃ (74 MBq/ml) was kindly supplied by Nihon Medipysics Co., Takarazuka. Potassium[14 C]cyanide (30 MBq/mg) was supplied from Amersham Japan, Tokyo, Japan. All other chemicals were reagent grade products obtained commercially.

MMC-D_{cat} and MMC-D_{an} were synthesized as reported previously.^{1,7)} Radiolabeled MMC-D_{cat} was synthesized by coupling γ -amino[U- 14 C]butyric acid to dextran with ϵ -aminocaproic acid at molar ratio of 1:1000.⁶⁾ Radiolabeled MMC-D_{an} was synthesized by coupling γ -amino[U- 14 C]butyric acid together with MMC to dextran at molar ratio of 1:2000. All conjugates contained almost equal amounts of MMC, about 10%(w/w), and the degree of substitution of dextran by MMC was 1 mol MMC/14–17 glucose units. Table I shows the structures and physicochemical properties of MMC-D_{cat} and MMC-D_{an}.^{1,8)}

Diethylaminoethyl-dextran (DEAE-dextran) was synthesized by the method of Mckerman *et al.*⁹⁾ Carboxymethyl-dextran (CM-dextran) was synthesized according to Peterson *et al.*¹⁰⁾ Radiolabeled DEAE-dextran and CM-dextran were synthesized in the same manner as for unlabeled DEAE-dextran and CM-dextran, respectively from [carboxyl- 14 C]dextran(T-70), which was prepared using potassium[14 C]cyanide according to the method of Isbell *et al.*¹¹⁾ with slight modification.

Cationized bovine serum albumin (BSA) was synthesized according to the method of Pardridge *et al.*¹²⁾ The product was then purified by chromatofocussing using the Polybuffer exchanger 94 resin and the Polybuffer 96 elution buffer system. The major protein peak eluted at pI higher than 9.0 was collected. Cationized BSA was labeled with 111 In using a bifunctional chelating agent diethylenetriaminepentaacetic acid (DTPA) anhydride according to the method of Hnatowich *et al.*¹³⁾

Procedure of Animal Experiment — Male Wistar albino rats weighing 200–240 g were

Fig. 1. Cumulative Amount of Free, Dextran-Conjugated, and Total MMC Excreted into Bile after Intravenous Injection of Free MMC (A), MMC-D_{cat}(T-10)(B), MMC-D_{cat}(T-70)(C), MMC-D_{cat}(T-500)(D), MMC-D_{an}(T-10)(E), MMC-D_{an}(T-70)(F), and MMC-D_{an}(T-500)(G) (●, ▲, MMC; ○, □, dextran conjugated MMC; ▽, total MMC. Results are expressed as the mean of at least three rats and vertical bars indicate S.D.

used. Rats were anesthetized with pentobarbital and injected with drug solution (50 mg/kg). Their body temperature was kept at 37 °C by a heat lamp during the experiment. The bile duct was cannulated with polyethylene tubing (i.d. 0.28 mm), and the bile sample was collected in test tubes for 8 h. At 8 h after the administration, the liver was excised, just after the animal was killed.

Analytical Method — The concentration of MMC was determined by microbiological assay. Assay was performed by a disk-plate method using *E.coli* B as a test organism.⁶⁾ The concentration of MMC-D was determined as for MMC, after the bile sample was heated in order to hydrolyze MMC-D to MMC. The procedure employed for the determination of ¹⁴C-radioactivity was a modification of the method of Mahin and Loftberg.¹⁴⁾ Bile sample (0.1 ml) was put into a counting vial with 10 ml of scintillation medium (Clear-Sol, Nacalai Tesque) and radioactivity was determined in a liquid scintillation system. The ¹¹¹In radioactivity were counted with a well NaI scintillation counter without any special procedure.

Fractionation of Radioactivity Recovered from Bile — Bile sample was applied to a Sephadex G-25 fine column (45 × 0.90 cm) and eluted with 0.2 M acetic acid. Fractions of 3 ml were collected in tubes and the radioactivity of

each fraction was determined.

Results

Biliary Excretion of MMC after Intravenous Injection of Free MMC and MMC-D

Figure 1 shows the cumulative amount of MMC excreted in bile as free and conjugated form after intravenous injection of MMC-D_{cat} and MMC-D_{an} with various molecular sizes at a dose of 5 mg MMC/kg. Results of free MMC administration is shown for comparison (Fig.1A). Free MMC was excreted rapidly and 1.8% of dose was recovered in the bile sample of initial 2 h. On the other hand, only a small amount (<1% of dose) of MMC (free plus conjugated form) was excreted into bile following the injection of MMC-D_{cat} and MMC-D_{an} (Fig.1B—G). The excreted total amount of MMC within 8 h differed with the molecular weight; that is, MMC-D_{cat}(T-500) > MMC-D_{cat}(T-70) > MMC-D_{cat}(T-10), and MMC-D_{an}(T-70) > MMC-D_{an}(T-10) > MMC-D_{an}(T-500). MMC was excreted into bile in conjugated form rather than free form following injection of MMC-D irrespective of its electric charge and molecular weight. The ratio of excreted amount of free to conjugated MMC differed with the molecular weight, but each value was almost constant during the experiment.

Fig. 2. Bile Concentration Time Course of MMC and MMC-D after Intravenous Injection of MMC(A), MMC-D_{cat} (B), and MMC-D_{an} (C)

●, MMC; ○, T-10; □, T-70; △, T-500. Results are expressed as the mean of at least three rats.

Fig. 3. Concentration (A) and Cumulative Amount (B) of MMC-D_{cat}(T-500) Excreted into Bile Calculated from ¹⁴C-Radioactivity Determination (○) and Bioassay (●)

Results are expressed as the mean of at least three rats.

In Fig.2A—C, time courses of biliary concentration of MMC after intravenous injection of free MMC(A), MMC-D_{cat}(B), and MMC-D_{an}(C) are illustrated. For MMC-D_{cat} and MMC-D_{an}, the concentration of conjugated MMC are shown. Comparing with the steep excretion-time profile of free MMC, MMC-D_{cat} and MMC-D_{an} showed delayed appearance of conjugated MMC after intravenous administration. Gradual excretion of MMC-D_{cat} continued until 8 h after administration.

Biliary Excretion of ¹⁴C-Labeled MMC-D_{cat}

Figure 3 shows the concentration time course and accumulation of ¹⁴C-radioactivity of MMC-D_{cat}(T-500) excreted into bile after intravenous injection. Results are compared with those of conjugated MMC determined by bioassay after injection of cold MMC-D_{cat}(T-500).

The ¹⁴C-radioactivity showed a similar excretion pattern to that of the conjugated MMC but the recovered amount of the former was larger than that of the latter throughout the experiment. Molecular species of ¹⁴C-radioactivity was examined with gel filtration method. Pooled bile sample gave two peaks of radioactivity corresponding to macromolecular MMC-D_{cat}(T-500) and low molecular weight fraction. In the case of first 1 h bile, about 70% was recovered in the macromolecular fraction.

Biliary Excretion of Model Macromolecules

Biliary excretion of model macromolecules with the same molecular weight (*M_r* 70000) but different electric charges was investigated in order to examine the effect of electric charge on it. Table II summarizes the cumulative radioactivity of model macromolecules excreted in the bile

TABLE II. Biliary Excretion of MMC-Ds, DEAE-Dextran, CM-Dextran, Dextran, and Cationized BSA in 8 h after Intravenous Administration

Compounds	a) Biliary excretion (% of dose)	b) Hepatic retention (% of dose)	a/b
[¹⁴ C]Dextran	0.55 ± 0.20	3.44 ± 0.71	0.160
[¹⁴ C]DEAE-dextran	0.75 ± 0.19	6.58 ± 2.43	0.114
[¹⁴ C]CM-dextran	0.07 ± 0.02	1.62 ± 0.09	0.043
[¹⁴ C]MMC-D _{cat} (T-70)	2.73 ± 0.89	39.30 ± 8.37	0.069
[¹⁴ C]MMC-D _{an} (T-70)	0.42 ± 0.03	3.48 ± 0.74	0.121
[¹¹¹ In]cat.BSA	1.03 ± 0.13	86.03 ± 9.98	0.012

within 8 h after intravenous injection in the rat. The radioactivities recovered in the liver at 8 h after injection and the ratio of radioactivities recovered in the bile to that in the liver (a/b) are also listed in Table II. In general, excreted amount of cationic macromolecules such as [^{14}C]-MMC- D_{cat} (T-70), [^{14}C]DEAE-dextran, and [^{111}In]cationized BSA was larger than that of anionic macromolecules. The recovery ratios were fairly small in all cases and there was not much difference among them.

Discussion

The biopharmaceutical characteristics such as distribution, metabolism, and excretion of polymeric prodrugs of antitumor agents seem to be the most important factors which determine their therapeutic potency. Based on this consideration, we have studied the disposition and pharmacokinetics of MMC-D in various animal models. The pharmacokinetic analysis revealed that MMC-D acts as a reservoir of MMC which behaves in the body as macromolecules while supplying active MMC.⁶⁾ In these experiments, hepatic uptake was concluded to be the major route of elimination for MMC- D_{cat} ⁶⁾ and also for polycationic model macromolecules.¹⁵⁾ However, no information has been obtained about biliary transport of them while biliary excretion should play an important role in successive disposition of compounds accumulated in the liver.

In our present study, the biliary excretion of MMC- D_{cat} and MMC- D_{an} after bolus intravenous injection was evaluated by means of two kinds of analytical method; that is, biological assay of parent drug MMC with or without dextran carrier and ^{14}C -radioactivity counting of spacer-labeled MMC-D.

About 1.8% of the biologically active MMC was rapidly recovered in the bile after injection of free MMC (Fig. 1A). In contrast, total recovery of MMC in MMC- D_{cat} and MMC- D_{an} until 8 h after injection was less than 1% at the same MMC doses (Fig. 1B—G). Although biliary excretion rate profiles of free MMC are not shown, similar sustained excretion of free MMC was observed in all types of MMC-D as is also suggested in gradual increase in cumula-

tive amount from 2 to 6 h after intravenous injection. In a previous study on urinary excretion of MMC-Ds after intravenous injection, sustained excretion of free MMC was obtained regardless of their molecular weight.¹⁶⁾ It was concluded that the excretion patterns reflected the fairly constant plasma concentration of MMC which was regenerated from MMC-Ds at almost similar rates in the body.⁸⁾ In the case of biliary excretion, similarly, the sustained biliary concentration of MMC can be attributed to the plasma concentrations at least in part. These results revealed that polymeric modification decreases and retards the biliary excretion of drug as well as urinary excretion. Comparing with free MMC, the access and transport to the bile of MMC-D is expected to be considerably restricted.

Molecular sizes gave various effects on the biliary excretion of MMC- D_{cat} and MMC- D_{an} ; that is, MMC-Ds with a molecular weight of 500000 showed the largest and the smallest excretion among three molecular sizes in each type, respectively. Concerning an electric charge, total excreted amounts of free and conjugated forms of MMC for MMC- D_{cat} were slightly larger than those for MMC- D_{an} but differences were not as large as was seen in the hepatic uptake.^{2,6)} The molecular charge should have some complicated effects on biliary excretion of MMC-D.

Appearance of ^{14}C -radioactivity in bile after injection of ^{14}C -labeled MMC- D_{cat} (T-500) was larger than that of MMC in a conjugated form determined by bioassay (Fig. 3). The major part of the radioactivity was recovered in the macromolecular fraction and this amount also exceeded that of conjugated MMC. This suggested that MMC was released and/or inactivated during the circulation in the body as a conjugated form. Since some part of ^{14}C -radioactivity was recovered in the low molecular weight fraction, release of the spacer arm or degradation of the dextran backbone also should be considered.

In order to clarify the effect of electric charge on the biliary excretion of macromolecules in detail, biliary excretion of model macromolecules was investigated following intravenous administration (Table II). Generally, cationic macro-

molecules gave larger hepatic uptake and biliary excretion than anionic ones. Thus the effect of electric charge on the biliary excretion of macromolecules was suggested. In the present study, the contribution of trans- and paracellular route to the total biliary excretion of macromolecules could not be distinguished, much less each process of the former route such as adsorption on the cell surface, endocytosis, diffusion in cytoplasm, and transport through the bile canalicular membrane could not be evaluated. Further investigation must be done on this point, and the *in situ* liver perfusion and isolated hepatocyte systems are expected to offer effective means of investigation.

The present investigation has thus shown the biliary excretion of the polymeric prodrugs and effect of the physicochemical properties on it. This information may be useful for the development of new macromolecular prodrugs and a cancer drug delivery system.

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