Preparation and Properties of a Mitomycin C–Albumin Conjugate

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Mitomycin C, an anti-neoplastic agent, was covalently attached to bovine serum albumin through various kinds of spacers such as glutaryl, succinyl, *trans*-aconityl, methylsuccinyl and the trimellityl group. The prior acylation of albumin not only prevented protein polymerization in the presence of carbodiimide, but also increased the extent of conjugation of the drug. The conjugate of mitomycin C–glutarylated albumin showed the best properties among the conjugates prepared in meeting the requirements for a high yield of nonpolymerized product with an adequately high mitomycin C content and stability as a macromolecular prodrug.

Keywords macromolecular-drug conjugate; mitomycin C; albumin; acylation; physico-chemical characteristics; molecular size; stability; release rate

In the field of cancer chemotheraphy, macromolecular drug carrier systems have been developed in an attempt to enhance the selectivity action of cytotoxic agents.[1,2] Macromolecules such as albumin, globulins and synthetic polymers markedly accumulate in tumor tissues because these tissues have a vascular network characterized by a lack of the lymphatic recovery system.[3,4]

Mitomycin C (MMC) has the potential to act against a number of human neoplasms. However, it presents the problems of severe myelosuppression and gastrointestinal complications. In an attempt to improve these side-effects, we have synthesized an MMC–albumin conjugate with the idea that MMC might be effectively delivered to the tumor tissues by taking advantage of the tumor accumulation of albumin.

The preparation of drug–protein conjugates is usually dependent on the employment of bifunctional cross-linking reagents. This approach, in addition to yielding reaction products of considerable heterogeneity in molecular size, affords relatively low levels of drug conjugation. Furthermore, insufficient attention has been paid to the nature of the link between the drug and the protein. For a drug–protein conjugate to be effective, the link between drug and protein must remain stable under the physiological conditions.

We have developed a method of preparing an MMC–albumin conjugate with various spacer arms. This report presents details of the method which produces high-conjugating yields without causing polymerization of the protein carrier.

Experimental Method

Reagents Bovine serum albumin (BSA) was purchased from Sigma Chemical Company (St. Louis, U.S.A.). BSA monomer was purified by the gel filtration of a commercial BSA preparation of Sephadex G-150. MMC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 2,4,6-trinitrobenzene sulfonic acid sodium salt (TNBS) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals and reagents were of the highest grades commercially available.

Preparation of MMC-Alubumin Conjugates Dicarboxylic acid anhydrides, glutaric anhydride, succinic anhydride, methylsuccinic anhydride, *trans*-aconitic anhydride and trimellitic anhydride were used to introduce a spacer arm between MMC and BSA. The reaction of BSA with the dicarboxylic anhydrides resulted in an acylated BSA (Acyl-BSA). To a stirred solution of BSA (100 mg) in 1 M $NaHCO_3$ (10 ml), dicarboxylic acid anhydride (400 mg) was added slowly in small portions. The pH of the reaction mixture was maintained within a range of 7.8—8.2 by the addition of 1 M NaOH for 1 h and gentle stirring at 4 °C for 16 h. The reaction mixture was washed repeatedly with a cold saline solution and concentrated to the volume of 20 ml by filtration through an ultrafilter (UP-20 mounted in UHP-43, Toyo, Japan) under high nitrogen pressure. Added to the solution (10 ml) of Acyl-BSA were MMC (5 mg) and EDC (10 mg). The pH was maintained at 6.5, and the conjugating reaction was allowed to proceed for 1 h at room temperature. The reaction mixture was applied on a column of Sephadex G-25 (50 × 200 mm) developed with 0.9% NaCl. MMC–Acyl-BSA was eluted in a void volume, and free MMC was retarded by the gel. Fractions (4 ml) containing MMC–Acyl-BSA were collected and lyophilized, producing a water-soluble powder.

Measurement of MMC Content of the Conjugates The MMC content of the conjugates was calculated by the ultraviolet (UV) absorbance at 363 nm in saline with reference to the absorbance rate of the MMC standard. The protein content of the conjugate was determined by the method of Lowry *et al.*[5] with a standard of BSA. The MMC content of the conjugate was calculated by dividing the content of MMC by that of the protein.

Determination of Amino Groups Free amino groups were determined according to the method of Fields.[6] The sample solution (0.5 ml) was diluted with 0.5 ml of a borate buffer (0.1 M $Na_2B_4O_7$ in 0.1 M NaOH). Then, 0.2 ml of a solution of 0.11 M TNBS was added. After exactly 5 min, the reaction was stopped by the addition of 2 ml of 0.1 M NaH_2PO_4 containing 0.0015 M Na_2SO_3. The absorption at 420 nm correlated with the number of free amino groups.

Size Exclusion Chromatography A high performance liquid chromatography (HPLC) system (CCPD, Tosoh, Tokyo, Japan) equipped with a spectrophotometric detector (SPD-6A, Shimadzu, Kyoto, Japan) was used with a column of TSKgel G-3000SW (4.6 × 600 mm, Tosoh). The mobile phase was 0.2 M NaCl in a 0.05 M phosphate buffer solution (pH 7.0), and the flow rate was 1.0 ml/min. The sample was dissolved in the mobile phase, then 80 μl of the solution was injected into the HPLC. The absorbance of the eluted solution was monitored at 280 and 350 nm.

Determination of MMC by HPLC The amount of MMC released from the conjugate was determined by HPLC. Chromatography was carried out using a Shimadzu liquid chromatographic system (LC-6A) with a variable-wavelength UV detector (SPD-6A) operated at 350 nm. A 4.6 × 250 mm, 5-μm particle size, C_{18} reversed-phase column (TSK gel 120T, Tosoh) was used at ambient temperature. The mobile phase was 20% acetonitrile in a 0.05 M phosphate buffer (pH 7.0). The injection volume was 80 μl, and the flow rate was 1.0 ml/min. It was confirmed that the determination of MMC was not affected by the presence of MMC–Acyl-BSA, and no MMC was liberated from the conjugate during the determination process.

***In Vitro* Release Experiment** The release of MMC from the conjugate was determined in a phosphate buffer system (pH 5.0, 7.4 and 9.0, 0.1 M, μ = 0.3, 37 °C) according to the method of Hashida *et al.*[7] The experiment was initiated by dissolving the conjugate in a preheated buffer solution to produce a concentration of 200 μg/ml in protein equivalent. At a fixed time interval, the amount of MMC released was determined by the method of HPLC. The stability of MMC and MMC–Acyl-BSA was also measured photometrically under the same conditions.

Results and Discussion

The conjugation of MMC to carrier albumin was confirmed by size exclusion chromatography for each spacer type of MMC–Acyl-BSA. Acyl-BSA and MMC–Acyl-BSA

TABLE I. Characteristics of MMC–Acyl-BSA

Acyl spacer		MMC content (%(w/w))	Percent of acylation	$T_{1/2}$ of MMC release (h)[a]
Glutaryl (G)	$-CO(CH_2)_3CO-$	6.0	95.6	66.0
Succinyl (S)	$-CO(CH_2)_2CO-$	4.9	81.8	56.0
trans-Aconityl (A)	$-CO(CHCCOOHCH_2)CO-$	1.7	32.6	0.656
Methylsuccinyl (MS)	$-CO(CH_2CHCH_3)CO-$	2.4	90.0	35.4
Trimellityl (T)	$-CO(C_6H_3COOH)CO-$	2.2	71.8	39.3

a) The data were calculated from the value of k_2 at pH 7.4 (see text for details).

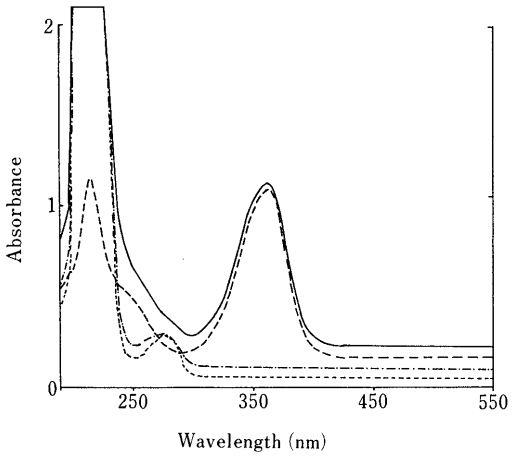

Fig. 1. UV Spectra of MMC (----), BSA (------), G-BSA (-·-·-) and MMC–G-BSA (——) in 0.9% NaCl

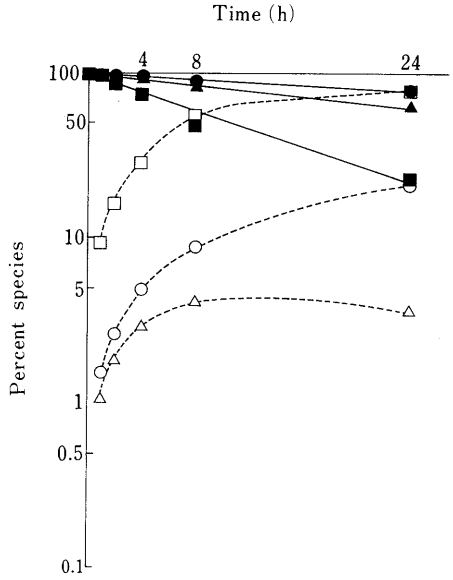

Fig. 2. Stability of MMC–G-BSA (——) and Release of MMC (------) from the Conjugate in 0.1 M Phosphate Buffer Solutions (μ=0.3) of pH 5.0 (▲, △), 7.4 (●, ○) and 9.0 (■, □) at 37 °C

$$\text{MMC–Acyl-BSA} \xrightarrow{k_2} \text{MMC} \xrightarrow{k_1} \text{decomposition products}$$
$$\downarrow k_3$$
$$\text{decomposition products}$$

Chart 1

were eluted faster than the BSA, indicating that molecular size was increased as a result of the acylation. Considerable conformational changes have been reported to occur after acylation of the lysine residues of various proteins with mono- and dicarboxylic acid anhydrides.[8-10] These conformational changes have generally been attributed to electrostatic destabilization of the native protein conformation.[9,10] In this study, the acylation of BSA by the use of dicarboxylic acid anhydrides caused a decrease in the number of free ε-amino groups of lysine residues, consequently increasing number of carboxyl groups derived from dicarboxylic acid (Table I), so that conformational changes occured in Acyl-BSA and MMC–Acyl-BSA. MMC–Acyl-BSA was eluted somewhat slower than the precursor, Acyl-BSA. The degree of retardation of the elution was proportional to the MMC content of the conjugates. This may be explained by a conformational change due to the introduction of MMC molecules.

Figure 1 shows the UV absorption of MMC–glutarylated BSA (MMC–G-BSA) as representative patterns. MMC–G-BSA showed an absorbance spectrum which contains the pattern of free MMC as shown in Fig. 1. The MMC content of MMC–Acyl-BSA was estimated by the UV absorbance at 363 nm with reference to the absorbance rate of MMC. MMC content and the percentage of acylation are summarized in Table I. Although BSA was highly acylated (72—96%) except by *trans*-aconitic anhydride, the MMC content varied, with values ranging from 2.2—6.0% (w/w). This may be explained in part by different reactivity among the carboxyl groups. The MMC content of MMC–G-BSA was largest in the conjugates, at 6.0% (w/w) (Table I). This corresponds to approximately 12 molecules of MMC per one molecule of BSA. Kato and co-workers have prepared an albumin conjugate of MMC (MMC–BSA, 5.0% (w/w) MMC content) by binding MMC directly to BSA using a large amount of carbodiimide.[11] The effective molecular size of the MMC–BSA they made (99.05Å) was much larger than that of BSA (37.6 Å). BSA is readily polymerized during the carbodiimide reaction because it has both carboxyl and amino groups in its molecule. The prior acylation of BSA was found to result in a decrease of protein polymerization in the presence of carbodiimide. The MMC–G-BSA prepared in this study showed a much smaller Stokes radius (45.1 Å) according to size exclusion chromatography using a Sephadex G-150 column.[12] The acylation of BSA before conjugation of MMC not only prevented protein polymerization, but also increased the extent of conjugation of the drug. MMC–Acyl-BSA produced an anionic charge due to the remaining free carboxyl group, because all spacer arms were not used for the conjugation of MMC.

The degradation of MMC–Acyl-BSA conjugates was investigated in aqueous phosphate buffer solutions. A representative example of MMC–G-BSA is depicted in Fig. 2. The release of MMC was observed during the degradation of MMC–G-BSA at pH values of 5.0, 7.4, and 9.0. The disappearance of MMC–G-BSA in buffered solutions followed pseudo first-order kinetics. The degradation of MMC at each pH followed apparent first-order kinetics as did MMC–G-BSA.

On the basis of the present results, the overall reactions may be described by Chart 1, where k_1—k_3 are the pseudo first-order rate constants for the depicted reactions.[7] Thus, the concentration of the conjugate ([MMC–Acyl-BSA])

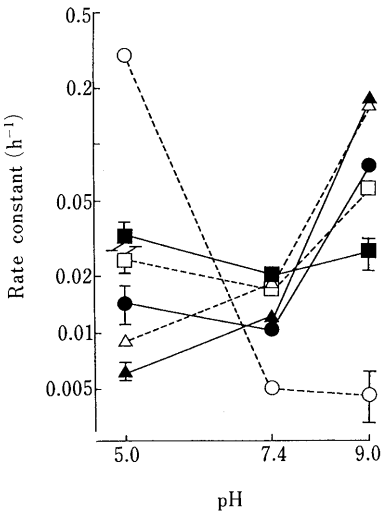

Fig. 3. pH Dependence of the Rate Constants in the Formation of MMC from the Conjugate (k_2) and the Degradation of MMC (k_1) in 0.1 M Phosphate Buffer Solutions at 37 °C and $\mu = 0.30$

—●—, MMC–G-BSA; —▲—, MMC–S-BSA; —■—, MMC–A-BSA; ---△---, MMC–MS-BSA; ---□---. MMC–T-BSA; ---○---, MMC. Vertical bars represent S.D. calculated from 15—20 sets of experimental data by the least squares method.

and that of the MMC released ([MMC]) in the reaction mixture have a time dependence given by the following equations:

$$[\text{MMC–Acyl-BSA}] = [\text{MMC–Acyl-BSA}]_0 \times \exp(-(k_2+k_3)t) \quad (1)$$

$$[\text{MMC}] = k_2/(k_1-(k_2+k_3)) \times [\text{MMC}]^* \times (\exp(-(k_2+k_3)t) - \exp(-k_1 t)) \quad (2)$$

where $[\text{MMC–Acyl-BSA}]_0$ and [MMC]* respectively represent the initial concentration of the conjugate and that of MMC covalently bound to MMC–Acyl-BSA.

Based on these equations, curve fitting was done using a non-linear least-squares program MULTI.[13] The values of k_1 and k_2 showed a good convergence. The degradation of MMC bound to albumin was regarded as negligible ($k_3 \simeq 0$) because a small negative value of k_3 with an extraordinarily large standard deviation was obtained.

The pH-rate profiles of k_1 and k_2 are shown in Fig. 3. MMC was very stable above neutral pH, but decomposition increased markedly in acidic conditions (k_1, Fig. 3). MMC–Acyl-BSA was markedly stable at pH 5.0, contrary to MMC, but it released predominant MMC at pH 9.0 (k_2, Fig. 3). These results were similar to those obtained in the experiment using an MMC–dextran conjugate.[7] However, the pH-rate profile of the release of MMC from the conjugate markedly depended upon the kind of spacer arm, as shown in Fig. 3.

Half-lives of the release of MMC from MMC–Acyl-BSA under the physiological conditions (pH 7.4 and 37 °C) are listed in Table I. MMC–G-BSA showed the highest stability with a half-life of 66.0 h in the conjugates prepared (Table I). The MMC–BSA conjugate synthesized by Kato *et al.* showed monoexponential release of MMC with a half-life of 20.2 h.[11] The introduction of a glutaric spacer arm resulted in the highest MMC content and the slowest release rate of MMC from the conjugate. Sezaki showed that the release rate of MMC from MMC–dextran conjugates decreased as the length of the spacer arm increased.[14] In the present study, MMC–Acyl-BSA tended to release MMC slower with a glutaric spacer arm (C_3) than with a succinic spacer arm (C_2).

MMC–G-BSA showed the best properties among the conjugates tested in meeting the requirements for a high yield of a nonpolymerized product which has adequately high MMC content and stability as a macromolecular prodrug. Further experiments are in progress to determine whether the conjugates accumulate in tumor tissues and liberate MMC there, showing antitumor activities.

Acknowledgments The authors are grateful to T. Morichika for his technical assistance.

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