Cellular Interaction and in Vitro Antitumor Activity of Lipophilic Mitomycin C Prodrugs

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Cellular interaction and in vitro antitumor activities of lipophilic prodrugs of mitomycin C (MMC) were studied in order to clarify their mode of action. Five lipophilic derivatives with various lipophilic promoieties (benzylcarbonyl, benzyloxycarbonyl, pentyloxycarbonyl, nonyloxycarbonyl, and cholesteryloxycarbonyl groups) were tested. All the derivatives except for cholesteryloxycarbonyl MMC were converted to MMC in the supernatant of tumor cell homogenate.

Lipophilic derivatives, especially nonyloxycarbonyl MMC and cholesteryloxycarbonyl MMC, associated with Ehrlich ascites carcinoma (EAC) cells more readily than MMC. While the association percentage remained almost constant during the course of incubation at 4 °C, it increased with incubation period at 37 °C, suggesting metabolic consumption of lipophilic derivatives in the tumor cells. Association percentages of derivatives at 4 °C were closely correlated to their partition coefficients between chloroform and water. The apparent distribution ratio of benzyloxycarbonyl MMC between EAC cells and the incubation medium also correlated with their volume ratio. These results suggested that lipophilic derivatives were incorporated into tumor cells through partition equilibrium between lipid components of tumor cells and the medium.

In vitro antitumor activities of lipophilic MMC derivatives were studied using EAC and L1210 leukemia cell culture. In the continuous exposure experiment, lipophilic derivatives which were converted to MMC in tumor cells showed equal or somewhat lower growth inhibitory activity as compared with MMC. In the case of 1 h or 5 min exposure, nonyloxycarbonyl MMC was more active than MMC, indicating that the growth-inhibitory effects of lipophilic derivatives are closely related to both cellular interaction and conversion rate to MMC.

Keywords—mitomycin C; lipophilic prodrug; L1210 leukemia; Ehrlich ascites carcinoma; cellular interaction; in vitro antitumor activity; cell culture system

Introduction

In cancer chemotherapy, it is desirable to deliver anticancer drugs selectively to the tumor site and to minimize the drug exposure of normal tissues in order to obtain high therapeutic efficacy. In the pharmaceutical field, the prodrug approach is one of the most promising means of improving drug delivery, by altering the biopharmaceutical characteristics through the introduction of promoieties with suitable physicochemical properties.

On the basis of this consideration, we have synthesized various kinds of prodrugs of mitomycin C (MMC) by coupling it with polymer beads, polysaccharide, and polyamino acid, and examined the therapeutic characteristics of the products. In a series of investigations, some lipophilic MMC prodrugs were also developed by substituting the la-N-position of MMC with lipophilic carrier moieties. These compounds showed significant in vivo antitumor activities when given by intraperitoneal injection and a stability study revealed that they were converted to MMC mostly by enzymatic hydrolysis.

Enhanced dermal absorbabilities, which resulted in improvement of the efficacy in topical use, were observed in
these compounds.\textsuperscript{9} Chemical modification of a lipophilic prodrug was also shown to be a useful approach to improve the applicability of MMC to liposome and O/W emulsion formulations.\textsuperscript{10–12} Combined delivery systems of lipophilic prodrugs with physical devices showed improved pharmacokinetic behavior such as sustained retention in the injection site and enhanced lymphatic transport after local injection, and significant anticancer activities against murine tumors were observed.\textsuperscript{10,12} However, detailed information has not been reported about their mode of action at the cellular level.

In the previous papers, we dealt with the cellular interaction of polymeric prodrugs of MMC such as dextran conjugate and a polyamino acid conjugate in relation to their physicochemical properties.\textsuperscript{13} In this investigation, the cellular interaction and \textit{in vitro} antitumor activity of five lipophilic derivatives of MMC were studied in order to clarify their mode of action. The physicochemical characteristics of the derivatives are discussed from the viewpoint of prodrug design.

### Experimental

**Material**—MMC was kindly supplied by Kyowa Hakko Kogyo Co. Five lipophilic derivatives of MMC (Table I) were synthesized as described previously.\textsuperscript{6,7} Other chemicals were reagent-grade products obtained commercially.

**Cellular Interaction of Lipophilic MMC Derivatives**—Tumor cells were suspended in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered Hanks' balanced salt solution (10\(^7\) cells/ml), and a drug solution in ethanol (160 \(\mu\)g eq MMC/ml) was added to give a final concentration of 10 \(\mu\)g eq MMC/ml. The mixture incubated for a definite time at 37 or 4 °C. After centrifugation at 3000 rpm for 5 min, the drug concentration in the supernatant was measured spectroscopically and the association percentage was calculated. The effects of temperature, drug concentration and cell density on cellular interaction were examined.

**In Vitro Antitumor Activity with Continuous Drug Exposure**—Murine L1210 leukemia or Ehrlich ascites carcinoma (EAC) cells were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (Grand Island Biological Company, NY), then the drug solution in ethanol was added, and the mixture was seeded on a multiwell tissue culture plate (Becton, Dickinson and Co., CA) at a density of 10\(^5\) cells/well. After incubation in a humidified atmosphere containing 5% CO\(_2\) at 37°C for 72 h, viable cells were counted with a Bürker–Türk hemocytometer by the trypan blue exclusion method. The growth inhibitory effect was assessed as follows: 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 control group, respectively. Experiments were carried out in triplicate.

**In Vitro Antitumor Activity with 1 h or 5 min Drug Exposure**—Tumor cells were exposed to various concentrations of drugs in Hanks' solution for 1 h or 5 min at 37°C and then were washed twice with the same medium by centrifugation, as described previously.\textsuperscript{14} Drug-treated cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated for 4 d. Growth inhibition was determined as above.

**Conversion of Lipophilic MMC Derivatives to MMC**—EAC cells (10\(^6\) cells/ml) were homogenized in HEPES-buffered Hanks' solution (pH 7.2) with a glass-Teflon homogenizer under ice-cooling. The homogenate was centrifuged at 3000 rpm for 5 min. MMC derivatives were added to the supernatant of the cell homogenate at a final concentration of 30 \(\mu\)g eq MMC/ml and incubated at 37°C. The incubation was stopped by addition of ice-cold acetonitrile (4 volumes with respect to the sample). After being shaken for 10 min, the mixture was filtered through a Micro Filter FR-20 (Fuji Photo Film Co.). Derivatives and regenerated MMC in the filtrate were determined by high performance liquid chromatography (system LC-5A, Shimadzu) with a variable-wavelength UV detector (SPD-2A, Shimadzu) and a Chromatopack CR2AX (Shimadzu) in a reverse-phase mode. The stationary phase used was Cosmosil 5C18 (Nakarai Chemicals) and a short column packed with RP-2 (E. Merck, Germany) was used to guard the main column. Methanol–water was used as the mobile phase at a flow rate of 0.8 ml/min. Standard solutions were chromatographed and calibration lines were constructed on the basis of peak-area measurements.

### Results

**Conversion of Lipophilic MMC Derivatives to MMC**

Table I summarizes the structures and physicochemical characteristics of lipophilic MMC derivatives. All the derivatives were degraded conforming to first-order kinetics in the supernatant of EAC cell homogenate. Under the condition used, the conversion of cholesterol-
yloxycarbonyl MMC into MMC was not observed, but all other derivatives were converted to MMC, as they are in the liver homogenate or plasma of rodents.\textsuperscript{7,9} The conversion rates in EAC cell homogenate were slower than those in the liver homogenate or plasma.

\begin{table}
\centering
\caption{Structures and Physicochemical Characteristics of Lipophilic Mitomycin C Derivatives}
\begin{tabular}{llcc}
\hline
Compound & R & PC\textsuperscript{a)} & Degradation rate constant (h\textsuperscript{-1}) \\
\hline
MMC & -H & 0.259 & 0.046 \\
Benzylicarbonyl MMC & -COCH\textsubscript{2}C\textsubscript{6}H\textsubscript{5} & 163 & 0.036\textsuperscript{b)  } & 0.841\textsuperscript{b)  } \\
Benzyloxy carbonyl MMC & -COOCH\textsubscript{2}C\textsubscript{6}H\textsubscript{4} & 1643 & 0.050\textsuperscript{c)  } & 25.61\textsuperscript{c)  } \\
Pentyloxy carbonyl MMC & -COOC\textsubscript{11}H\textsubscript{11} & 1785 & 0.015\textsuperscript{c)  } & 4.35\textsuperscript{c)  } \\
Nonyloxy carbonyl MMC & -COOC\textsubscript{19}H\textsubscript{19} & 29195 & 0.036\textsuperscript{c)  } & 2.62\textsuperscript{c)  } \\
Cholesteryloxy carbonyl MMC & -COOC\textsubscript{27}H\textsubscript{45} & 8216 & 0.015 & 0.073 \\
\hline
\end{tabular}
\begin{flushleft}
a) Partition coefficient between chloroform and water (from ref. 11). b) Values are cited from refs. 7) and 9). c) MMC was regenerated during the incubation period.
\end{flushleft}
\end{table}
Cellular Interaction of Lipophilic MMC Derivatives

Figure 1 shows association–time profiles of lipophilic MMC derivatives with EAC cells. At 4°C, lipophilic derivatives, especially nonyloxycarbonyl and cholesteryloxycarbonyl MMC, associated with EAC cells more efficiently than MMC. Association percentages of drugs remained almost constant during the incubation period. On the other hand, association of lipophilic derivatives at 37°C increased with increasing incubation period and was higher than that at 4°C.

Figure 2 illustrates the relation between association percentage of lipophilic derivatives

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**Fig. 2. Relationship between Partition Coefficient and Association Percentage with Ehrlich Ascites Carcinoma Cells of Lipophilic Prodrugs**

Abscissa; partition coefficient between chloroform and water cited from ref. 11. Ordinate; association percentage obtained from Fig. 1 (4°C, 15 min).

△, benzyloxycarbonyl MMC; ○, benzyloxy carbonyl MMC; ■, benzyloxy carbonyl MMC; □, nonyloxycarbonyl MMC; ▲, cholesteryloxycarbonyl MMC.

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**Fig. 3. Effects of Concentration of Benzyloxy carbonyl MMC on Its Association with Ehrlich Ascites Carcinoma Cells in 15 min at 4°C**

Uptake amount was determined as described in the legend to Fig. 1. The results are expressed as the mean ± S.D.

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**Fig. 4. Effect of Number of Ehrlich Ascites Carcinoma Cells on the Cellular Association of Benzyloxy carbonyl MMC**

Uptake amount was determined as described in the legend to Fig. 1. The results are expressed as the mean ± S.D.

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**Fig. 5. Relationship between the Ratio of Drug Amount Associated with EAC Cells (Xc) to That in the Medium (Xm) and the Ratio of Cellular Volume (Vc) to Medium Volume (Vm)**

Drug amount associated with EAC cells and that in the medium after incubation for 15 min at 4°C were determined as described in the legend to Fig. 1. Cellular volume was calculated by assuming the cell to be a sphere with a diameter of 20 μm.
at 4°C and their partition coefficients between chloroform and water; there is a linear relationship.

In order to characterize the cellular interaction in detail, association experiments were carried out under various conditions employing benzyloxy carbonyl MMC as a model compound. Figure 3 shows the effect of concentration of benzyloxy carbonyl MMC on the cellular association at 4°C. Association amount increased in proportion to drug concentration in the medium.

Figure 4 shows the effect of cell number on total drug association at 4°C. The amount of associated drug increased with increase in cell number. Based on the results shown in Fig. 4, the apparent distribution ratio of benzyloxy carbonyl MMC between the EAC cells and the incubation medium was calculated. A plot of apparent distribution ratio against the ratio of cell volume to medium volume gave a straight line (Fig. 5) which is consistent with the following equation;

\[ \frac{X_c}{X_m} = \left( \frac{C_c}{C_m} \right) \left( \frac{V_c}{V_m} \right) = P_c \left( \frac{V_c}{V_m} \right) \]

where \( X_c \) and \( X_m \) represent drug amount incorporated in the tumor cells and that remaining in the incubation medium, \( C_c \) and \( C_m \) represent drug concentration in the cells and medium, and \( V_c \) and \( V_m \) represent cellular volume and medium volume, respectively. \( P_c \) represents a partition parameter between the cells and medium and was calculated to be about 4 under the conditions used.

**In Vitro Antitumor Activity of Lipophilic MMC Derivatives**

Figure 6A, B, and C shows the growth inhibitory effects of MMC derivatives on L1210 cells in continuous, 1 h, and 5 min exposure experiments, respectively. MMC showed growth-inhibitory effects at the lowest concentration in the continuous exposure experiment. Nonyloxy carbonyl MMC and benzyloxy carbonyl MMC exhibited essentially equal activity to...
MMC but other derivatives were somewhat less active. Cholesteryloxycarbonyl MMC, which was not converted to MMC, failed to inhibit cell growth. In the 1 h exposure experiments, nonyloxycarbonyl MMC, which interacted strongly with tumor cells, showed higher activity than MMC. Other derivatives exhibited lower activity. In the 5 min exposure experiment, the growth-inhibitory activity of nonyloxycarbonyl MMC was much higher than that of MMC.

Figure 7 shows growth inhibitory effects of MMC derivatives on EAC cells during continuous exposure and 1 h exposure. Derivatives showed similar activities against EAC cells in each exposure experiment.

**Discussion**

Among various physicochemical parameters, molecular lipophilicity plays a dominant role in determining the biopharmaceutical characteristics of drugs. Many attempts have been made to avoid enzymatic degradation, enhance the uptake by tumor cells, improve the encapsulation efficiency into liposomes, and accelerate intestinal absorption by controlling the lipophilicity of the drug.

In previous studies, we developed lipophilic prodrugs of MMC, an amphiphilic anticancer drug, aiming at the improvement of its pharmacokinetic behavior and applicability to lipoidal dosage forms. We subsequently elucidated their pharmaceutical and pharmacological characteristics. In this paper, the cellular interaction and growth-inhibitory activity of lipophilic MMC derivatives were examined and compared with those of other types of MMC prodrugs.

Lipophilic MMC derivatives showed greater interaction with EAC cells both at 4 and 37 °C, and a good correlation was observed between the association percentage and partition coefficient in chloroform/water of these prodrugs. Cellular association at 37 °C increased with incubation time except in the case of nonyloxycarbonyl MMC, while the uptake percentages at 4 °C remained almost constant. These results suggested that the derivatives were metabolically consumed in the tumor cells at 37 °C. In the case of nonyloxycarbonyl MMC, which is also metabolically converted to MMC, the increase in association amount was not significant since 85% of the applied dose was already incorporated into tumor cells at the initial period of incubation. Cellular interaction of lipophilic derivatives with other cell lines including L1210 leukemia, P388 leukemia, and AH 66 ascites hepatoma was essentially similar to that with
EAC cells (data not shown). Therefore, we used EAC cells, which are easily obtainable from ascitic fluid of mice, for further examination of cellular interaction.

As shown in Fig. 3, the uptake of benzylxycarbonyl MMC by EAC cells at 4 °C was proportional to its concentration in the medium. The association of benzylxycarbonyl MMC also increased with increase of cell number (Fig. 4). The apparent distribution ratio between EAC cells and the incubation medium correlated well with the volume ratio (Fig. 5). These findings suggest that lipophilic derivatives are incorporated into tumor cells by partitioning to cellular lipid components. The possibility of enhancing cellular access by increasing lipophilicity through the prodrug approach is clear from these results.

MMC has been shown to cross-link double-helical deoxyribonucleic acid (DNA) after enzymatic reduction to the corresponding hydroquinone.\textsuperscript{21} These processes are accelerated under hypoxic conditions and result in selective toxicity to chronically hypoxic tumor cells\textsuperscript{22,23} Positions 1a and 10 appear to be the alkylating sites of MMC, and their alkylating ability is enhanced when methanol is eliminated from the produced hydroquinone to give the indolehydroquinone.\textsuperscript{24} Consequently it is considered that substitution at the 1a position leads to diminution of the biological activity.\textsuperscript{6,7} Lipophilic derivatives showed slightly lower cytotoxicities than MMC in the continuous exposure experiments (Figs. 6A and 7A). Cholesteryloxycarbonyl MMC was not converted to MMC even in the biological media (Table I) and did not show any cytotoxic activity. These results indicate that lipophilic MMC derivatives have to be converted to MMC in order to exhibit cytocidal effect. Thus, the lability of prodrugs should play an important role in the manifestation of their antitumor activities. The linkage structure between MMC and the lipophilic promoiety is the determinant of this property.\textsuperscript{8}

As reported previously, plasma and liver homogenate of rats or mice successfully catalyzed the hydrolysis of MMC derivatives having the carbamate linkage, except for cholesteryloxycarbonyl MMC.\textsuperscript{7} Nonspecific esterase or carbamidase might be responsible for this reaction. These compounds were also converted to MMC by EAC cell homogenate, as shown in Table I. In the 1 h or 5 min exposure experiment, nonyloxycarbonyl MMC, which strongly interacted with tumor cells, exhibited higher cytocidal activity than MMC, but other derivatives showed less activity. These results suggested that cellular interaction is also an important factor determining the cytotoxic activity. Extremely high lipophilicity (partition coefficient > 10\textsuperscript{4}) seems to guarantee sufficient retention of the active species in the tumor cell even after repeated washing. Similar considerations might apply \textit{in vivo} when the drug exposure is limited to a short period.

Lipophilic prodrugs must satisfy the following two criteria to exhibit potent activities: 1) high cellular association, 2) full conversion to the parent drug at an adequate rate. Lipophilic modification of an antitumor drug renders the drug more able to permeate into cells, and therefore such modification is a promising approach for further development of anticancer drugs. It might also be possible to overcome the drug resistance of tumor cells in which drug uptake is impaired.

Another possible approach to improve the pharmacokinetical properties and cellular access of MMC is conjugation to a high-molecular-weight compound. We have developed macromolecular derivatives of MMC, MMC–dextran conjugate (MMC-D)\textsuperscript{41} and MMC–polyamino acid conjugates,\textsuperscript{5} and investigated their physicochemical,\textsuperscript{25,26} biopharmaceutical,\textsuperscript{27,28} and chemotherapeutic characteristics.\textsuperscript{29} These macromolecular prodrugs of MMC were designed to liberate MMC by chemical hydrolysis in contrast with lipophilic MMC prodrugs.\textsuperscript{1} MMC-D with cationic charge and MMC–polylysine conjugate were strongly adsorbed on tumor cell surface through electrostatic interaction.\textsuperscript{5,13} Adsorption of MMC-D increased with increase of its molecular weight and conformed to Langmuir’s adsorption isotherm. A good correlation was observed between the growth-inhibitory effects of MMC-D
and the extent of its cellular interaction, and cationic MMC-D was more active than MMC in the 1 h exposure experiment. As with the lipophilic prodrugs of MMC demonstrated in this study, the cellular interaction and release rate of MMC were concluded to play an important role in the manifestation of the antitumor activity of macromolecular MMC prodrugs.

Improvement of the access of antitumor agents to target tumor cells is one of the most important aims in designing prodrugs. We have applied two approaches to one conventional drug, MMC, with considerable therapeutic success. In these studies, lipid components and anionic surface materials of the tumor cell were chosen as the target and lipophilic small molecules and cationic macromolecules were tested as carrier moieties of the prodrug. The close relationship of lipophilicity and electrostatic properties of prodrugs with their cellular interactions was explored from the viewpoints of physicochemistry. The significance of regeneration process also had been elucidated and the guide for designing linkage structures was given for each approach. Through these reporis, possibility and a rational avenue for improving cellular accessibility of antitumor agent by prodrug design have been demonstrated.

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References