Synergistic Anti-tumor Effects of Mitomycin C and Bile Salts against L1210 Cells

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The effects of various adjuvants on the cytotoxicity of mitomycin C (MMC) were studied in L1210 mouse leukemia cells. Adjuvants examined in this study were sodium glycocholate (Na-GC), sodium deoxycholate (Na-DC), O-n-dodecyl-β-D-maltopyranoside (LM), and sodium salicylate. Among various additives, bile salts such as Na-GC and Na-DC were the most effective for increasing the cytotoxicity of MMC against L1210 cells. A dose-dependent increase in cytotoxic effect of MMC was observed in the presence of these bile salts. To elucidate a possible mechanism for enhancing the cytotoxic effect of MMC by the bile salts, the cellular uptake of MMC with or without Na-GC was examined using L1210 cells. The cellular concentration of MMC was determined by a reversed-phase HPLC. When Na-GC was coadministered with MMC, the uptake of MMC into L1210 cells was significantly enhanced as compared with MMC alone. Furthermore, the membrane fluidity of L1210 cells, as determined by fluorescence polarization, was increased in the presence of Na-GC. These results suggested that the enhancement of cytotoxicity of MMC by the addition of Na-GC could be attributed to the increasing cellular uptake of MMC due to the increasing membrane fluidity of L1210 cells.

Key words mitomycin C; bile salt; anti-tumor effect; L1210 cell; membrane fluidity

In cancer chemotherapy, it is important to increase and maintain the concentration of anticancer drugs in tumor cells, since the enhancement of the antitumor effect of anticancer drugs is closely related to the increase in intracellular drug levels. Therefore, various approaches have been examined in order to accomplish this. One possible approach is to use additives which can decrease the drug efflux from the tumor cells. Tsuruo et al. reported that the cytotoxicity of vincristine and Adriamycin (ADR) was potentiated by verapamil, which could inhibit the efflux of these anticancer agents and thereby increase their intracellular accumulation. However, few studies have examined the use of additives which could increase the uptake of anticancer drugs to tumor cells. Akiyama et al. found that three polyene antibiotics remarkably enhanced the antitumor effect of bleomycin A2 by increasing the cellular uptake of this agent. In our previous reports, we demonstrated that the antitumor effects of bleomycin were greatly potentiated by solubilized lipids i.e., lipid-surfactant mixed micelles (MM) using oleic or linoleic acid against mouse Ehrlich ascite tumor, rat ascites hepatoma AH66 and bleomycin-resistant murine L1210 leukemia.

Bile salts, which are known to have surface active properties, have been used for enhancing the permeability of poorly absorbable drugs across intestinal or other mucous membranes. Gordon et al. reported that bile salts interact with the cell membranes and form a channel to increase the permeability of insulin. Recently, we demonstrated that the rectal permeability of insulin was remarkably enhanced by various bile salts. Therefore, considering these actions of bile salts, it may be possible that these bile salts may also affect tumor cell membranes as well as intestinal membranes, and may increase the influx of anticancer drugs to the tumor cells.

In this study, the effects of bile salts and other various adjuvants on the cytotoxic effect of mitomycin C (MMC) were investigated in L1210 mouse leukemia cells. Furthermore, to clarify the mechanisms by which these adjuvants potentiated the cytotoxic effect of MMC, we also examined the cellular accumulation of MMC into L1210 cells and the membrane fluidity of L1210 cells by using 1-4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (tma-DPH) as a probe in the presence or absence of sodium glycocholate (Na-GC).

MATERIALS AND METHODS

Chemicals MMC was kindly supplied by Kyowa Hakko Kogyo Co., Tokyo, Japan. Na-GC and O-n-dodecyl-β-D-maltopyranoside (LM) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium deoxycholate (Na-DC), sodium salicylate and tma-DPH were purchased from Wako Pure Chemicals, Osaka, Japan. All other chemicals were of the finest grade available without further purification.

Cell Culture L1210 mouse leukemia cells were a kind gift from Professor Tsuruo (University of Tokyo, Japan) and were maintained in culture in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (100 µg/ml) and penicillin (100 U/ml) under humidified air containing 5% CO2 at 37°C. Generally, L1210 cells in the exponential growth phase were used for the experiments.

In Vitro Cytotoxicity Assay Exponentially growing L1210 cells (1×10^6 cells) were exposed for 1 h to MMC with or without adjuvants in Hank's balanced salt solution (HBSS) at 37°C. Following treatment, the cells were washed three times with HBSS and resuspended in PRMI1640 medium supplemented with 10% fetal bovine serum, and then transferred to yield 1×10^6 cells per well (24-well plats). The cells were subsequently placed in an incubator for 3 d and the number of viable cells were determined in each well by WST-1 assay (Cell counting

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kit, Dojindo Laboratory, Kumamoto, Japan). Control cells were handled identically to the treated cells and plated into triplicated wells.

**Cellular Accumulation of MMC** L1210 cells (1 × 10^6 cells/ml) from exponentially growing culture were exposed for 1—60 min to MMC (10 μg/ml) with or without Na-GC (200 μM) at 37°C. At an appropriate time, cells were immediately centrifuged at 10000 rpm for 10 s, and the supernatant was carefully removed by aspiration. The resultant cells were lysed with 100 μl distilled water, and were then extracted with 1 ml chloroform–2-propanol (1:1 v/v). The extracts were evaporated to dryness under a stream of nitrogen gas at 40°C. Residues were reconstituted in 100 μl of HPLC mobile phase, and 50 μl were injected into HPLC. MMC was determined by HPLC (System LC-10A, Shimadzu, Kyoto, Japan) using a UV detector (SPD-10), Chromatopack C-R6A and C18 Cosmosil AR-300 column (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan). The HPLC conditions were as follows: mobile phase, 10 mM phosphate buffer (pH 6.0) and methanol (7:3 v/v); flow rate, 1 ml/min; detection at 365 nm. 

**Fluorescence Measurement** The membrane fluidity of L1210 cells was measured by the microviscosity of tma-DPH. L1210 cells (1 × 10^6 cells/ml) were labeled by mixing equal volumes of tma-DPH suspension (2 μM) and incubating the mixture for 10 min at 37°C. The labeled cells were washed twice with HBSS, then added to HBSS containing 200 μM Na-GC, and the suspensions were incubated for another 1—60 min at 37°C. The fluorescence polarization and microviscosity were obtained by simultaneous measurement of I_p and I_0, parallel and perpendicular to the direction of polarization of the excitation beam, with a Hitachi 650-10S spectrofluorophotometer (Hitachi, Tokyo, Japan) equipped with a fluorescence polarizer and a water jacketed cuvet chamber. The degree of fluorescence polarization (P) and apparent microviscosity (η) were calculated by the following equations:

\[
P = \frac{I_p - I_0}{I_p + I_0}, \quad \eta = \frac{2P}{0.42 - P}
\]

For the measurement of fluorescence of tma-DPH, excitation and emission wavelengths were 340 and 430 nm, respectively. In all experiments, temperature was controlled with a thermostat, NTT-1200 (Tokyo Rikakiki, Tokyo, Japan).

**Statistical Analysis** Results were expressed as the mean ± S.D. of at least 3 experiments. Statistical analyses were performed using the Student's t-test.

**RESULTS**

**Effects of Various Adjuvants on the Cytotoxicity of MMC against L1210 Cells** Effects of various adjuvants on the cytotoxicity of MMC were studied using L1210 cells. Na-GC and Na-DC significantly potentiated the cytotoxicity of MMC as compared with the control. In contrast, neither LM or sodium salicylate showed any marked effect on the cytotoxicity of MMC against L1210 cells (Fig. 1). We next examined the dose dependent effects of Na-GC and Na-DC on the potentiation of the cytotoxicity of MMC against L1210 cells. Figure 2 shows the survival of L1210 cells exposed to various concentrations of MMC in the presence of various concentrations of Na-GC. The cytotoxic effects of MMC were potentiated with increasing the applied concentration of Na-GC. However, above the concentration of 100 μM Na-GC, the cytotoxic effect of MMC was saturated. In Fig. 3, a dose-dependent increase in the cytotoxic effect of MMC was observed in the presence of various concentrations of Na-DC. However, the effective concentration of Na-DC on the potentiation of cytotoxicity of MMC was lower than that of Na-GC. Furthermore, the potentiated
Fig. 3. The Effects of Varying Concentrations of Na-DC on the Survival of Exponentially Growing L1210 Cells Exposed to MMC
Experimental procedure is similar to Fig. 1. Key: Control (■), 1 μM (○), 2 μM (□), 5 μM (●), 10 μM (▲), and 20 μM (△). Results are expressed as the mean ± S.D. of three independent experiments.

Fig. 4. Effects of Adjuvants on the Cytotoxicity of MMC against L1210 Cells
Concentrations producing 50% growth inhibition of L1210 in the presence of MMC (IC_{50}) were calculated from Figs. 1, 2 and 3. Results are expressed as the mean ± S.D. of three independent experiments. Significant differences compared with the control are represented as follows: ** p < 0.01; and *** p < 0.001.

cytotoxic effect of MMC with Na-DC was not saturated over the range of its concentration (1–20 μM).

The concentration producing 50% growth inhibition of L1210 in the presence of MMC (IC_{50}) was summarized in Fig. 4. As shown in Fig. 4, the IC_{50} values of MMC in the presence of bile salts were reduced with an increase in their applied concentrations.

Effects of Na-GC on the Uptake of MMC by L1210 Cells Figure 5 shows the cellular accumulation of MMC into L1210 cells in the presence or absence of Na-GC (200 μM). The amount of MMC taken up by L1210 cells was increased with the addition of Na-GC. Thus, Na-GC might enhance the permeability of MMC across the L1210 plasma membrane.

Effect of the Exposure of Na-GC on Membrane Fluidity of L1210 Cells The membrane fluidity of L1210 cells with or without Na-GC was investigated by measuring microviscosity determined using tma-DPH as a fluorescence probe. Figure 6 shows the apparent microviscosity (η)-time profiles in the presence or absence of Na-GC (200 μM). The η values were markedly reduced by the addition of Na-GC. Thus, it was suggested that the membrane fluidity of L1210 was increased by the exposure of Na-GC, and the enhancement of L1210 cells' membrane fluidity might result in an increase in the uptake of MMC into L1210 cells.

DISCUSSION
In our previous reports, we demonstrated that MM
improved the absorption of anticancer drugs such as bleomycin (BLM) and interferons from the gastrointestinal tract and potentiated the antitumor effect of BLM against mouse Ehrlich and rat hepatoma AH66 ascite tumors. Moreover, it has been reported that MM might potentiate the cytocidal action of BLM against BLM-natural resistant mouse L1210 leukemia cells.

On the other hand, we previously learned that bile acids such as Na-GC and Na-DC improved the absorption of poorly absorbed drugs via various mucosal tissues such as the small and large intestine, rectum, nose, and lung. In particular, Na-GC was demonstrated to enhance the membrane permeability of a drug across the mucosa in these tissues without local irritation or serious membrane damage. Various mechanisms of absorption enhancement by bile salts have been proposed; e.g., 1) the alteration of membrane perturbation, 2) reversed micelle formation in cell membrane, and 3) loosing of the tight junction due to the removal of $Ca^{2+}$ ions from intercellular region.

Based on these findings, we studied the effects of bile salts on the potentiation of the cytotoxicity of MMC against L1210 cells. In an in vitro cytotoxicity assay, Na-DC was more effective in enhancing the cytotoxicity of MMC against L1210 cells than Na-GC (Fig. 4). Gordon et al. indicated that there is a striking correlation between bile salt hydrophobicity, which is calculated by its HPLC retention time, and adjuvant activity. Therefore, the effective concentration of Na-DC to potentiate the cytotoxicity of MMC was about one-tenth lower than that of Na-GC because of the relatively high hydrophobicity of Na-DC (Figs. 3, 4).

As shown in Fig. 1, LM did not affect the cytotoxicity of MMC against L1210 cells. LM, an alkyl saccharide, has recently been found to lower surface tension and to have absorption enhancing activity in the gastrointestinal tract. In our previous study, it was demonstrated that LM is one of the most effective enhancers for improving the intestinal absorption of poorly absorbed drugs in both in vitro and in vivo experimental systems. In this study, however, we found no significant synergistic cytotoxicity of MMC in the presence of 10 $\mu M$ LM (Fig. 1). This negative effect of LM may be due to its different optimal concentration for enhancing the permeability of drugs across the membrane between tumor cells and intestinal mucosa. Similarly, the addition of salicylate into L1210 cells did not produce any synergistic effect of MMC. It was demonstrated that salicylate increased the transcellular transport of drugs by increasing the interaction of protein into the plasma membrane at a relatively high concentration ($\geq 750 \mu M$). At the concentration (10 $\mu M$) used in the present study, however, little interaction between salicylate and cell membrane was observed. Thus, this low interaction may be one of the main reasons salicylate did not affect the cytotoxicity of MMC.

There was a significant correlation between the cellular accumulation of MMC and its cytotoxic activity against L1210 cells (Figs. 2, 4, 5), suggesting that synergistic cytotoxic effects of MMC and Na-GC might result in an increased cellular accumulation of MMC. In our previous report, MMs using oleic and linoleic acids enhanced the cellular accumulation of ADR into P388/S cells and ADR resistant P388 cells. The enhancement mechanisms by MM are as follows: the fusogenic lipid which was solubilized by MM and incorporated into the membrane, interacted with the polar head groups of membrane lipids, increased the membrane fluidity, and thereby resulted in enhanced membrane permeability to drugs. As shown in Fig. 6, Na-GC also increased the membrane fluidity of L1210 cells, as well as did MM. Similarly, Matsunaga et al. demonstrated that the synergistic cytotoxicity of MMC and panaxytriol, an amphiphilic agent, may be due to the increasing cellular accumulation of MMC in MK-1 cells. On the other hand, MMC was reported to be taken up by HCT116 cells, a human colon carcinoma cell line, via a passive diffusion mechanism. It was reported that the increase in membrane fluidity was accompanied by an increase in membrane permeability to $K^+$ and $Na^+$ and to glycerol and erythritol, which were transported by passive transport mechanisms. Therefore, the increase in cellular accumulation of MMC into L1210 cells might enhance its uptake via passive transport by increasing the membrane fluidity of L1210 cells caused by Na-GC. In accordance with these findings, a good correlation was obtained between the effect of Na-GC on the cellular accumulation of MMC into L1210 cells and their membrane microviscosity in the present study.

In conclusion, we demonstrated that the cellular accumulation of MMC into L1210 cells could be enhanced by the addition of Na-GC, resulting in the increased cytotoxicity of MMC against L1210 cells. It was suggested that the enhanced cellular uptake of MMC by the addition of bile salts was attributed to an increase in the membrane fluidity of L1210 cells. We have started in vivo therapeutic studies of MMC and bile salts using a Sarcoma 180 bearing mice model, and promising therapeutic effects have been obtained for Na-GC and Na-DC. The therapeutic effectiveness of MMC, coadministered with bile salts, will be described in a future report.

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