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Potentiation of cytotoxicity of mitomycin C by a polyacetylenic alcohol, panaxytriol

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Abstract. Polyacetylenic alcohol, panaxytriol, which was isolated from *Panax ginseng* C. A. Meyer, has antiproliferative activity against several kinds of tumor cells. In this paper, the effect of panaxytriol on the cytotoxicity of mitomycin C (MMC) against a human gastric carcinoma cell line, MK-1, was investigated. The combination of a subthreshold concentration of MMC and panaxytriol produced a significant cytotoxic effect, which indicates that the effects of panaxytriol and MMC are synergistic. A synergistic effect was observed when MK-1 cells were treated with the mixture of MMC and panaxytriol or treated with MMC followed by panaxytriol. In contrast, when MK-1 cells were exposed to panaxytriol and then to MMC, only an additive effect was induced. With the aim of finding a possible mechanism, the effect of panaxytriol on the accumulation of MMC into the MK-1 cells was examined. Cellular concentrations of MMC were measured by high-performance liquid chromatography (HPLC). When MK-1 cells were treated with a mixture of panaxytriol and MMC or first with MMC and then with panaxytriol, the cellular level of MMC was significantly higher than that in MK-1 cells treated with MMC alone, but no significantly increased accumulation was found when MK-1 cells were treated with panaxytriol followed by MMC. These results suggest that synergistic effects of panaxytriol and MMC may be induced by acceleration of the effect of MMC on cellular accumulation by panaxytriol. In addition, they suggest that the enhanced accumulation of MMC in MK-1 cells treated with panaxytriol can probably be attributed to the decreased fluidity of the cell membrane caused by panaxytriol.

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Introduction

Mitomycin C (MMC), a reductive alkylating agent, is widely used in the treatment of various solid tumors [15]. When it is administered as a single agent, however, the clinical results are not as good as expected. Therefore, combinations of MMC with various biological response modifiers (biomodulators) are now being tested in the treatment of cancer [4–6, 14, 24]. It was recently demonstrated that the uptake and efflux of MMC and its analogues in tumor cells may be a passive diffusion process [25]. It was also indicated that the passive transport of non-electrolytes into cells can be retarded by altering the lipid composition of the surface membrane, for example, by the addition of cholesterol, in ways that decrease membrane fluidity [7–9, 11, 12, 23]. Aliphatic alcohols have been reported to enhance the permeability of artificial lipid membranes [13, 26]. These alcohols are believed to interact with the lipid domain of cell membranes and increase membrane fluidity [13, 26]. These findings indicate that it might be possible to control the passive diffusion transport of chemotherapeutic agents such as MMC by altering the membrane fluidity. Agents that can modify the membrane fluidity, therefore, may be suitable for use as biomodulators in chemotherapy for cancer patients.

As part of a program aimed at the development of new types of antiproliferative agents for solid tumors, work in our laboratories has examined substances toxic to tumor cells. Recently, we reported that polyacetylenic alcohol, panaxytriol, isolated from *Panax ginseng* C. A. Meyer has antitumor activity against both cultured tumor cells and tumors transplanted into mouse [16, 20, 21]. It has been also suggested that panaxytriol may directly influence the membrane fluidity of tumor cells [22]. These findings led us to postulate that panaxytriol alters the membrane fluidity and modifies the uptake and efflux of MMC in tumor cells. In the study reported here, effects of panaxytriol on surface membrane fluidity and accumulation of MMC in tumor cells were demonstrated. A possible combination of MMC with panaxytriol in the treatment of cancer patients is also discussed.

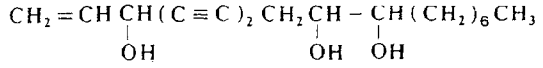

Fig. 1. Chemical structure of panaxytriol

Materials and methods

Reagents. MMC and 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (tma-DPH) were purchased from Wako Pure Chemicals (Osaka, Japan). Panaxytriol was isolated and purified from a powder of heat-treated roots of *Panax ginseng* C. A. Meyer, red ginseng, by the method used in our previous reports [16, 20, 21]. Red ginseng powder was provided from Nikkan Korai Ninjin (Kobe, Japan). Briefly, red ginseng powder was extracted with ethyl acetate, and the extracts were fractionated by silica gel chromatography. Panaxytriol-rich fractions were collected and panaxytriol was purified by crystallization from distilled water. The chemical structure of panaxytriol is shown in Fig. 1.

Cell culture. Nude mouse-transplantable human gastric adenocarcinoma cells (MK-1 cells) were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS).

Clonogenic assay. The in vitro cytotoxicity of MMC, with or without panaxytriol, was determined by clonogenic assay with MK-1 cells as target cells. Briefly, the growing cells were trypsinized and collected. MMC, panaxytriol, or a mixture of these drugs was added to MK-1 cell suspensions. The suspensions were plated (100 cells/wells, 34.6×17 mm) and incubated at 37°C for 8–10 days, after which surviving colonies (more than 50 cells) were stained with methylene blue and counted. Survival ratio of MK-1 cells = (No. of colonies in drug-containing medium/No. of colony in drug-free medium)×100.

Definition of the interaction in a quantitative manner. Interactions between panaxytriol and MMC were determined by means of an isobologram by a method introduced by Berenbaum [3]. The approaches for analyzing interactions are as follows;

$d_m/D_m + d_p/D_p = 1$; additive effect
$d_m/D_m + d_p/D_p < 1$; synergistic effect
$d_m/D_m + d_p/D_p > 1$; antagonistic effect

where d_m and d_p are the concentrations of MMC and panaxytriol combination, respectively. D_m and D_p are the concentrations of MMC and panaxytriol separately that are isoeffective with the combination.

MMC concentration. MMC concentration was determined by high-performance liquid chromatography (HPLC) analysis. HPLC analysis was performed using a Simadzu Model LC-6A pump, Shimadzu SPD-6A UV detector with 365 nm (Kyoto, Japan) and a 4×125 mm Lichrospher 100 RP-18 endcapped (4 μm) column (Merck, Darmstadt, Germany). MMC was eluted with a mobile phase of 0.01 M phosphate buffer pH 6.0: methanol (70:30, v/v).

Cellular uptake and efflux of MMC were determined by HPLC, with a modified version of Den Hartigh et al.'s technique [10]. MK-1 cells (5×10^5 cells/ml) were exposed to MMC in microtubes at 37°C. The resulting cell–drug incubation mixtures were further incubated from 30 s to 60 min. They were then centrifuged at 15,000 rpm for 10 s. Media containing the drug were carefully removed by aspiration, and the cells were rinsed with cold phosphate-buffered saline (PBS). Following rinsing, the cells were lysed with distilled water, scraped into a conical glass centrifuge tube, and extracted with a 8:2 (v/v) mixture of ethyl acetate and 2-isopropanol. The mixture was centrifuged at 2,000 rpm for 10 min. The upper, organic phase, was collected and dried at 40°C under a stream of nitrogen. The sediment was resuspended in 50 μl of methanol and analyzed by HPLC.

MMC concentration in culture medium was also determined by HPLC. To this end, 1 ml of MMC (10 μg/ml) was added to 1 ml of MK-1 cell suspensions (1×10^6 cells/ml), and the suspensions were incubated from 5 to 60 min at 37°C. From each suspension 100 μl was removed and transferred into microtubes followed by centrifuging at 10,000 rpm for 1 min; 50 μl of each the supernatant was collected into new microtubes, and 50 μl of acetonitril was added. The resulting mixtures were centrifuged at 10,000 rpm for 5 min and the supernatants were analyzed by HPLC.

Fluorescence polarization measurements. The membrane fluidity of MK-1 cells was measured by fluorescence polarization of tma-DPH, an extrinsic probe that specifically labels the surface membrane [17, 18, 27]. The cell density was adjusted to 1×10^6 cells/ml with RPMI 1640 containing 20% FCS. A 2 μM tma-DPH dispersion in RPMI 1640 was prepared from a 0.5 mM solution in 1,2-propanediol by vigorous mixing. Cells were labeled by mixing equal volumes of the probe dispersion and the cell suspension and incubating for 10 min at 37°C. The labeled cells were rinsed twice with PBS. Following rinsing, the labeled cells were added to each PBS solution containing agent, and the suspensions were incubated further for 5–60 min at 37°C. The fluorescence intensity of the labeled suspensions was determined with a Shimadzu RF-540 spectrofluorophotometer equipped with excitation and emission polarizers and a water-jacketed cuvette chamber. Temperature was controlled with a Lauda Compact Thermostats RMS6 (Lauda-Königshofen, Germany). The excitation wavelength was 340 nm and the emission wavelength was 430 nm. Polarization values were derived from intensities obtained with polarizer orientation in each of the four possible combinations, according to the formula:

$$P = (Iv - I\Uparrow)/(Iv + I\Uparrow)$$

where Iv is the intensity when excitation and emission polarizers are parallel and $I\Uparrow$ is the intensity when polarizers are perpendicular to each other. The correction for scattering from unlabeled cells was less than 1%. In this assay, increased fluorescence polarization of tma-DPH reflects a decrease in membrane fluidity.

Results

Potentiation of cytotoxicity of MMC against tumor cells by panaxytriol

The time- and dose-dependent effects of panaxytriol on the cytotoxicity of MMC were studied by clonogenic assay using MK-1 cells as targets. As single agents, low doses of MMC (1.25 ng/ml) and panaxytriol (3.13 ng/ml) produced only a limited killing of MK-1 clonogenic tumor cells. However, the survival ratio of MK-1 cells in a combination of MMC and panaxytriol was lower than the predicted additive effect, which indicates that the effects of MMC and panaxytriol are synergistic. The synergistic action was dose-dependent but more sensitive at low than at high concentrations (Fig. 2).

The effect of time sequencing on the interaction of MMC and panaxytriol in MK-1 clonogenic cells is shown in Fig. 3. The interactive effect was maximum when MK-1 cells were exposed to MMC for 1 h and then to panaxytriol for 1 h. Simultaneous exposure to both MMC and panaxytriol also induced a synergistic cytotoxic effect. However, the interactive effect was only additive when MK-1 cells were exposed to panaxytriol for 1 h and then to MMC for 1 h.

Effects of panaxytriol on uptake and efflux of MMC in MK-1 cells

The effects of panaxytriol on MMC accumulation into the MK-1 cells were investigated. MK-1 cells were exposed to

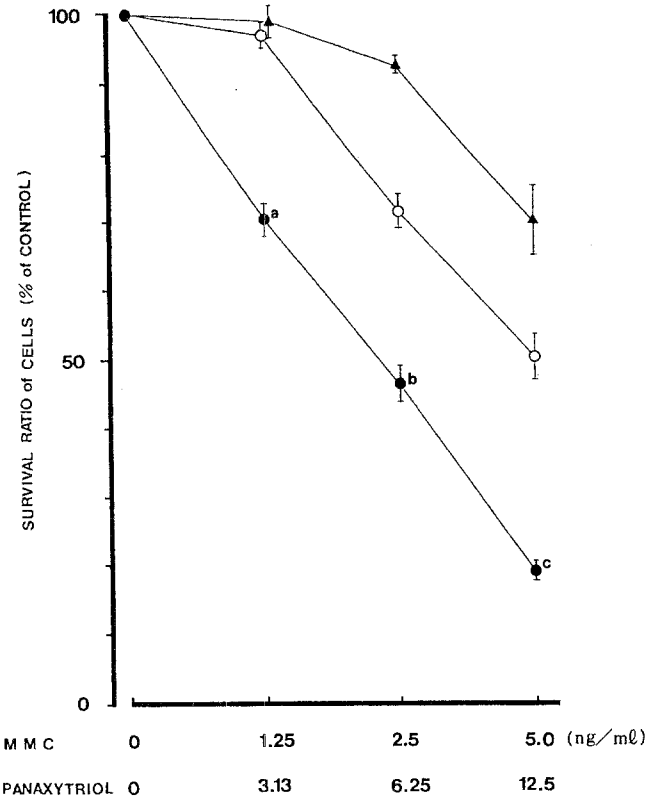

Fig. 2. Synergistic cytotoxic effect of MMC and panaxytriol on the growth of MK-1 cells. Cytotoxic activity was determined by clonogenic assay as described in "Materials and methods." MK-1 cells were continuously exposed to MMC (○), panaxytriol (▲), or a mixture of these two drugs (●). Means ± SD of three experiments. The values of $d_m/D_m + d_p/D_p$ (<1.0: synergistic) at *a*, *b*, and *c* were 0.628, 0.767 and 0.946, respectively

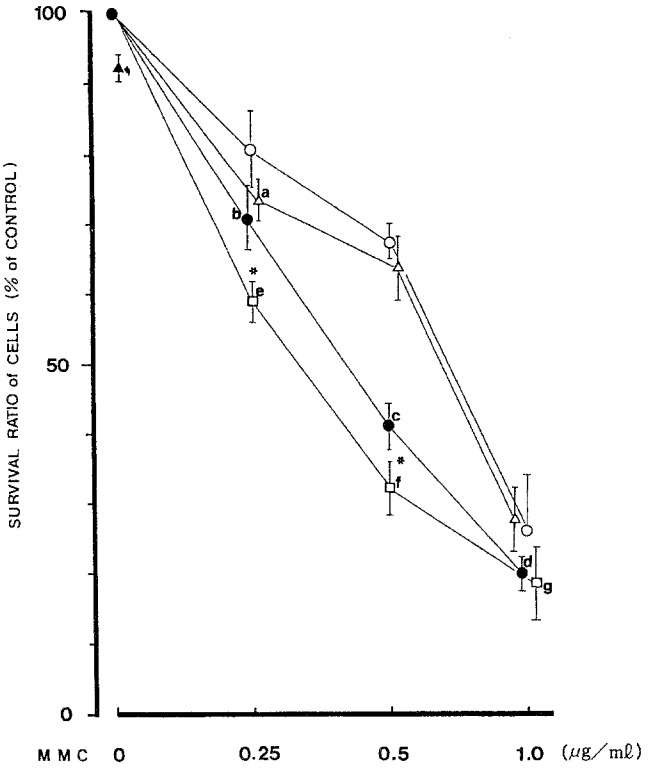

Fig. 3. Effect of sequencing on MMC-panaxytriol interaction in MK-1 cells. MK-1 cells were exposed to panaxytriol (1 μg/ml) for 1 h (▲), various concentrations of MMC for 1 h (○), mixtures of panaxytriol (1 μg/ml) and various concentrations of MMC for 1 h (●), panaxytriol (1 μg/ml) for 1 h and then to various concentrations of MMC for 1 h (△), or various concentrations of MMC for 1 h and then to panaxytriol (1 μg/ml) for 1 h (□). Those cells were rinsed with PBS, resuspended with drug-free medium and cultured for 10 days at 37°C. Means ± SD of three experiments. The values of $d_m/D_m + d_p/D_p$ (<1.0: synergistic) at *a*, *b*, *c*, *d*, *e*, *f*, and *g* were 0.986, 0.894, 0.698, 0.928, 0.631, 0.588, and 0.913, respectively. * $P<0.05$ by Student's *t*-test against simultaneous exposure to the mixture of MMC and panaxytriol

5 μg/ml MMC, with or without panaxytriol (5 μg/ml), and intracellular MMC levels were determined by HPLC at the times indicated between 30 s and 60 min (Fig. 4). The cellular levels of MMC were significantly higher in the cells exposed to the mixture of MMC and panaxytriol than in those exposed to MMC alone between 5 and 60 min. This finding indicates that panaxytriol may enhance the cellular accumulation of MMC in MK-1 cells.

To clarify the mechanism of increased MMC accumulation in MK-1 cells by panaxytriol, the following experiments were performed. MK-1 cells were exposed to 100 μg/ml MMC for 1 h, rinsed twice with PBS, and then overlaid with fresh medium with or without panaxytriol (5 μg/ml). Cellular levels of MMC in MK-1 cells were determined at the incubation times indicated between 0 and 20 min (Fig. 5). Cellular levels of MMC in MK-1 cells incubated with medium containing panaxytriol were significantly higher than those in the cells incubated with panaxytriol-free medium. This result indicates that panaxytriol may suppress the efflux of MMC from MK-1 cells and cause increased cellular accumulation of MMC.

Next, MK-1 cells were exposed to RPMI medium with or without panaxytriol at 37°C for 1 h and then washed twice with PBS. The washed cells were exposed to MMC at 37°C, and MMC concentrations in both MK-1 cells and cell-free medium were determined at varying incubation times. MMC levels in culture medium containing panaxytriol-treated MK-1 cells were similar to those in medium containing non-treated MK-1 cells (Fig. 6). And no significant difference in cellular levels of MMC was found between panaxytriol-treated MK-1 cells and non-treated MK-1 cells (Fig. 7). These data suggest that panaxytriol may suppress both uptake and efflux of MMC in MK-1 cells. Thus enhanced accumulation of MMC in MK-1 cells was induced when MK-1 cells were exposed to MMC and then to panaxytriol, but not when the cells were exposed to panaxytriol and then to MMC.

Effect of panaxytriol on membrane fluidity of MK-1 cells

It has been reported that the passive transport of non-electrolytes into cells can be retarded in ways that decrease membrane fluidity. Therefore, the effect of panaxytriol on membrane fluidity of MK-1 cells was investigated. MK-1 cells were exposed o panaxytriol (1 μg/ml) at 37°C and the membrane fluidity was determined at the times indicated. Increased fluorescence polarization of tma-DPH reflects a decrease in membrane fluidity. The membrane fluidity of

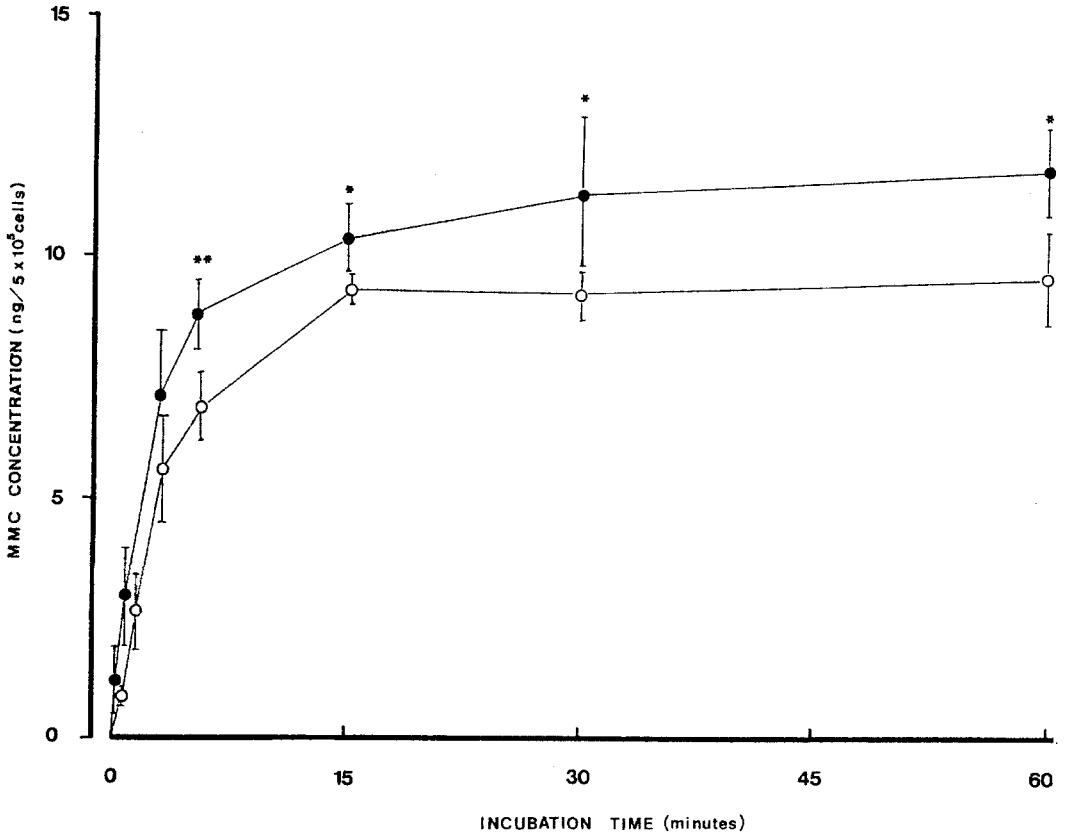

Fig. 4. Effect of panaxytriol on cellular accumulation of MMC in MK-1 cells. MK-1 cells were exposed to 5 μg/ml MMC with (●) or without (○) panaxytriol (5 μg/ml) for various times at 37° C. The cells were collected at various intervals and washed with cold PBS. Cellular concentration of MMC was determined by HPLC analysis as described in "Materials and methods" Means ± SD of three experiments. * $P<0.05$; ** $P<0.01$

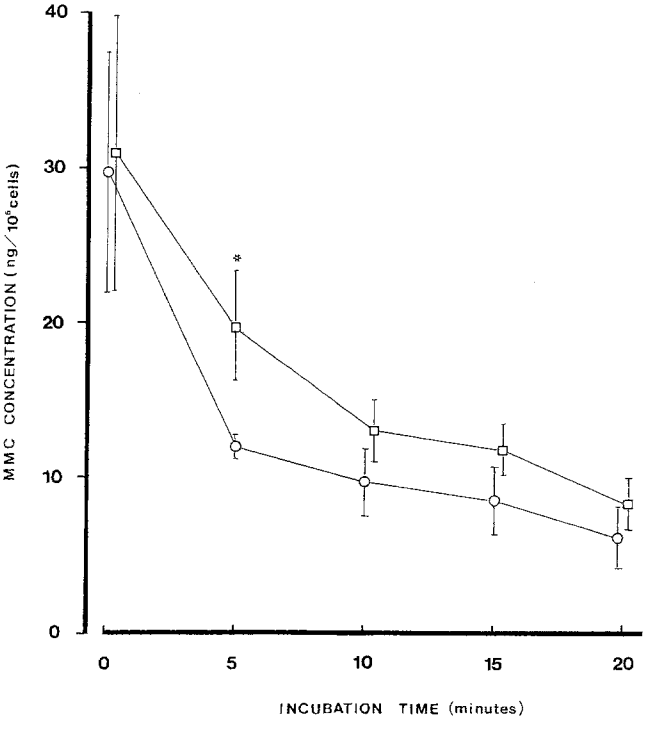

Fig. 5. Effect of panaxytriol on the efflux of MMC in MK-1 cells. MK-1 cells were exposed to 100 μg/ml MMC for 1 h at 37° C and then washed with cold PBS. The washed cells were resuspended in fresh medium with (□) or without (○) panaxytriol (5 μg/ml) and incubated at 37° C. At varying time intervals, MK-1 cells were collected and the cellular concentration of MMC was determined by HPLC analysis. Means ± SD of three experiments. * $P<0.05$

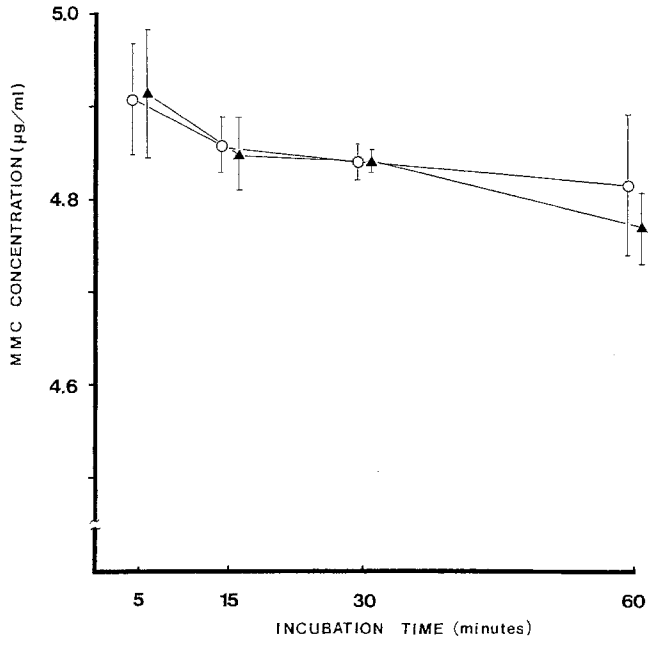

Fig. 6. Measurements of extracellular concentration of MMC. MK-1 cells were exposed to 5 μg/ml panaxytriol-containing (▲) or panaxytriol-free (○) medium for 1 h at 37° C and then washed with cold PBS. The washed cells were suspended with MMC (5 μg/ml)-containing medium and incubated for various times at 37° C. Culture medium was collected at various times and MMC concentration in the medium was determined by HPLC analysis. Means ± SD of three experiments

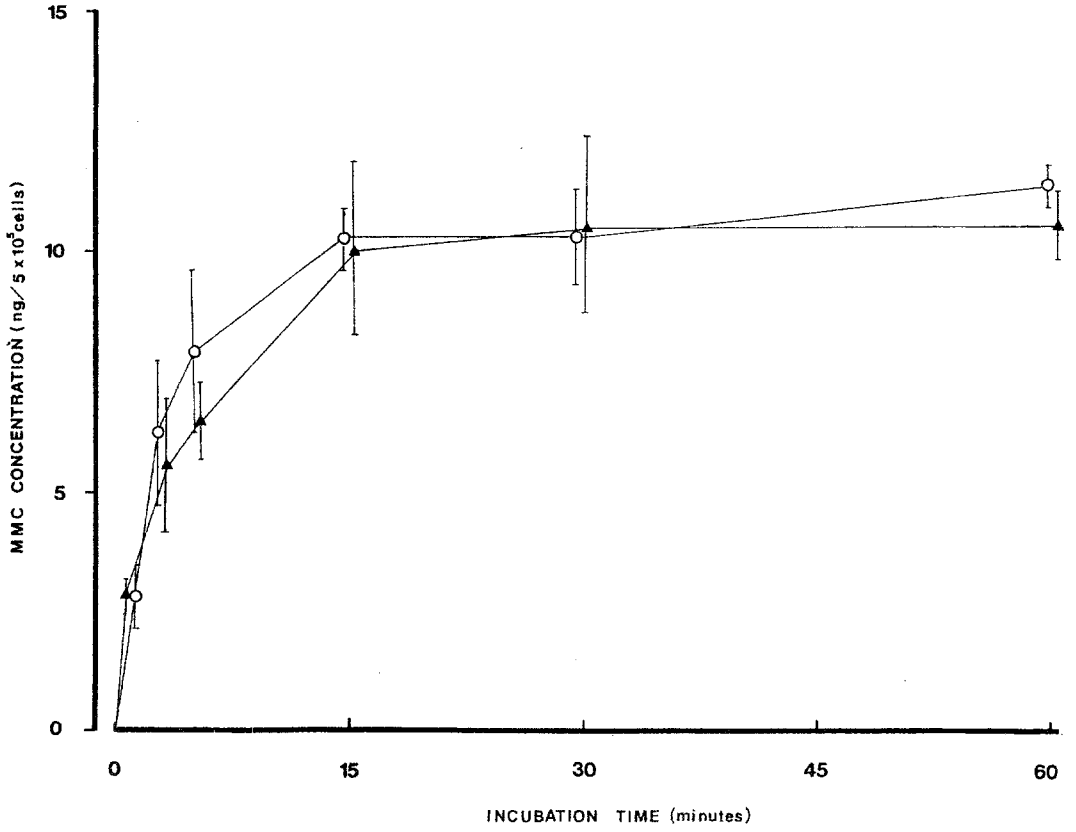

Fig. 7. Cellular accumulation of MMC in panaxytriol-treated MK-1 cells. MK-1 cells were treated with 5 μg/ml panaxytriol-containing (▲) or panaxytriol-free (○) medium for 1 h at 37°C. The cells were washed with cold PBS, suspended in MMC (5 μg/ml)-containing medium and incubated for various times at 37°C. At varying intervals, cells were collected and the cellular concentration of MMC was determined by HPLC analysis. Means ± SD of three experiments

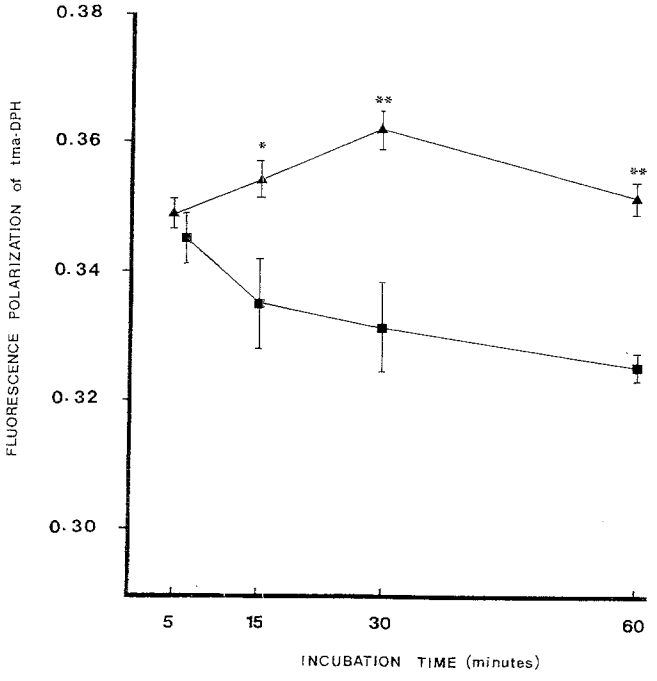

Fig. 8. Effect of panaxytriol on membrane fluidity of MK-1 cells. MK-1 cells were incubated with 2 μM tma-DPH for 10 min at 37°C. Labeled cells were incubated with 1 μg/ml panaxytriol-containing (▲) or panaxytriol-free (■) medium for varying incubation times at 37°C. The membrane fluidity of MK-1 cells was measured by the fluorescence polarization using tma-DPH as described in Materials and methods. Means ± SD of three experiments. Increased fluorescence polarization of tma-DPH reflects a decrease in membrane fluidity. * $P<0.05$; ** $P<0.01$

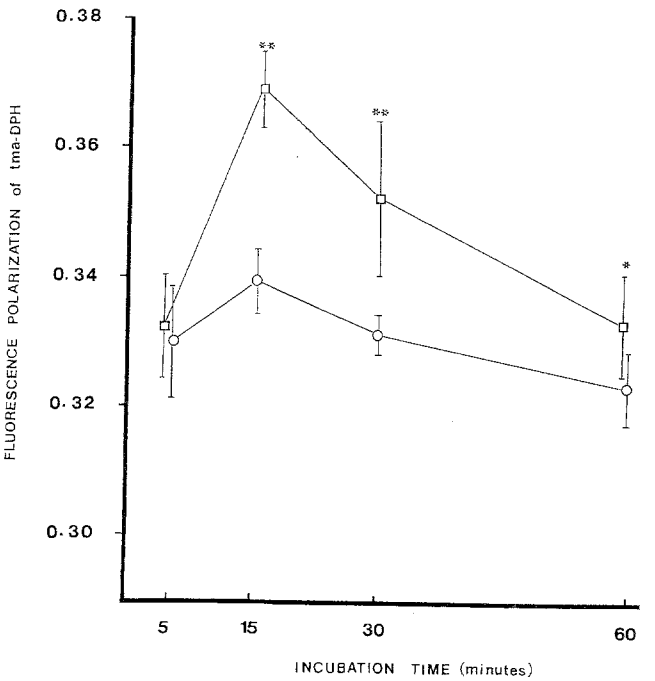

Fig. 9. Effect of panaxytriol on membrane fluidity of MMC-treated MK-1 cells. MK-1 cells were incubated with 1 μg/ml MMC for 1 h at 37°C, washed with warm PBS and labeled with 2 μM tma-DPH for 10 min at 37°C. The cells were resuspended in 1 μg/ml panaxytriol-containing (□) or panaxytriol-free (○) medium and incubated for various times at 37°C. After varying incubation times, membrane fluidity was measured. Means ± SD of three experiments. * $P<0.05$; ** $P<0.01$

MK-1 cells exposed to panaxytriol showed a time-dependent decrease as shown in Fig. 8. The decrease of membrane fluidity by panaxytriol was also found in MK-1 cells pretreated with MMC (Fig. 9). These results suggest that inhibition of uptake and efflux of MMC in panaxytriol-treated MK-1 cells can be, in part, attributed to the decrease of membrane fluidity caused by panaxytriol.

Discussion

In this study, we demonstrated that a polyacetylenic alcohol, panaxytriol, isolated from *Panax ginseng* C. A. Meyer promoted cellular accumulation of MMC into a human gastric carcinoma cell line, MK-1 cells, and enhanced the cytotoxicity of MMC against MK-1 cells. We also demonstrated a possibility that the increased MMC accumulation in MK-1 cells could result at least in part from decreased membrane fluidity of MK-1 cells induced by panaxytriol.

Pan et al. [25] demonstrated that the uptake and the efflux of porfiromycin (PFM), and *N*-methyl analogue of MMC, in HCT 116 cells, a human colon carcinoma cell line, are a passive diffusion process. It is shown that MMC and PFM share the same metabolic pathways and the same pattern of DNA alkylation. The time course of uptake and efflux of MMC is very similar to that of PFM reported by Pan et al. (Figs. 4, 5). Data obtained in our study indicate that the transport, both uptake and efflux, of MMC is a passive diffusion process for MK-1 cells.

Several investigators have shown that passive transport of nonelectrolytes in cells is closely related to their membrane fluidity [7–9, 11, 12, 23]. Amphiphilic molecules, such as soaps, alcohols, and detergents, partition between aqueous and lipid phases and have a fluidizing effect on membrane lipids. Panaxytriol is also an amphiphilic molecule (Fig. 1). Although it is demonstrated that aliphatic alcohols increase the membrane fluidity and accelerate the initial uptake rate of nonelectrolytes [13, 26], a polyacetylenic alcohol, panaxytriol, decreased the membrane fluidity and suppressed both uptake and efflux of MMC (Figs. 4, 5, 8). Why panaxytriol, in contrast to other aliphatic alcohols reported, decreases the membrane fluidity remains unknown. It is well known that both membrane fluidity and nonelectrolyte permeability of membranes are reduced by the incorporation of cholesterol [7–9, 11, 12, 23]. In addition, panaxytriol is rapidly incorporated into the membrane, and the incorporated panaxytriol can be extracted from the cell membrane (unpublished data). There is a possibility, therefore, that panaxytriol is incorporated into cell membrane and acts as if panaxytriol was cholesterol. At any rate, panaxytriol reduces both the uptake and the efflux of MMC in MK-1 cells and promotes cellular accumulation of MMC (Figs. 5, 6). The decreased uptake and efflux of MMC could also result in part from decreased membrane fluidity induced by panaxytriol.

There was a significant positive correlation between the cellular MMC levels and cytotoxic activity against MK-1 cells (Figs. 3–5, 7), suggesting that synergistic cytotoxic effect of MMC and panaxytriol may result from increased cellular accumulation of MMC. When MK-1 cells were exposed to MMC and then to panaxytriol, efflux of MMC from MK-1 cells was mainly suppressed because of decreased membrane fluidity induced by panaxytriol. As a result, cellular levels of MMC in MK-1 cells become higher than those in control MK-1 cells (Fig. 5). When MK-1 cells were first exposed to panaxytriol, both uptake and efflux of MMC are suppressed. As a result, cellular levels of MMC become almost equal to those in non-treated MK-1 cells (Fig. 7).

Panax ginseng C. A. Meyer has been widely used in Asian countries as a commercial medical drug. It is known that *Panax ginseng* contains other kinds of antiproliferative polyacetylenic compounds, such as panaxynol and panaxydol [1, 2 19]. In vitro studies such as those described here may provide a direction for further in vivo work and clinical applications of *Panax ginseng* for cancer patients.

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