Xanthones induce cell-cycle arrest and apoptosis in human colon cancer DLD-1 cells

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Abstract—We investigated the antiproliferative effects of four structurally similar prenylated xanthones, α-mangostin, β-mangostin, γ-mangostin, and methoxy-β-mangostin, in human colon cancer DLD-1 cells. These xanthones differ in the number of hydroxyl and methoxy groups. Except for methoxy-β-mangostin, the other three xanthones strongly inhibited cell growth at 20 μM and their anti-tumor efficacy was correlated with the number of hydroxyl groups. Hoechst 33342 nuclear staining and nucleosomal DNA-gel electrophoresis revealed that the antiproliferative effects of α- and γ-mangostin, but not that of β-mangostin, were associated with apoptosis. It was also shown that their antiproliferative effects were associated with cell-cycle arrest by affecting the expression of cyclins, cdc2, and p27; G1 arrest was by α-mangostin and β-mangostin, and S arrest by γ-mangostin. These findings provide a relevant basis for the development of xanthones as an agent for cancer prevention and combination therapy with anti-cancer drugs.
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1. Introduction

Mangosteen, *Garcinia mangostana* L., is a tree found in South East Asia, and its pericarps have been used as traditional medicine for treatment of skin infection and wounds for many years. Phytochemical studies have shown that they contain a variety of secondary metabolites, such as oxygenated and prenylated xanthones.[1,2] Recent studies have revealed that these xanthones exhibited a variety of biological activities containing anti-inflammatory,[3] antibacterial,[4] and anti cancer effects.[5–7] Previously, we reported that six xanthones from mangosteen exhibited potent cytotoxicity against human leukemia HL60 cells and that α-mangostin induced mitochondrial dysfunction.[6,8]

It is well-known that the cell-cycle machinery and checkpoint signaling pathway are dysregulated in most human tumor cells.[9] Thus, the cell cycle is thought to be a target for cancer therapeutics.[10,11] Recently, increasing

attention has been directed to cell-cycle regulation and its modulation by dietary factors and natural products.[12] To gain further insight into the mechanism underlying the growth inhibitory effect against tumor cells of xanthones, we investigated the relationship of the effects on the cell-cycle regulation by the four structurally similar xanthones, α-mangostin, β-mangostin, γ-mangostin, and methoxy-β-mangostin, in human colon cancer cell line DLD-1, and found that the cell-cycle arrest at different stages was induced depending on the type of xanthone.

2. Results and discussion

2.1. Antiproliferative effect of xanthones against DLD-1 cells

As shown in Figure 1, four xanthones contained in the pericarps and stem bark of mangosteen,[1,2] α-mangostin (αM), β-mangostin (βM), γ-mangostin (γM), and methoxy-β-mangostin (βM-OMe), used in the current study differ in the number of hydroxyl and methoxy groups. We examined the effects of these compounds on the
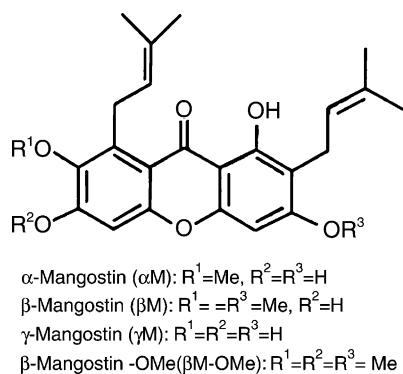
Figure 1. Chemical structures of xanthones used in this study.

antiproliferative effect against colon cancer DLD-1 cells (Fig. 2). Except for βM-OMe, other three xanthones inhibited the cell growth at 20 μM, and γM was effective even at 10 μM. Among the effective xanthones, the inhibitory activity of βM was lowest at 20 μM (αM and γM versus βM, $p < 0.005$–0.0001; αM versus γM, not significant, at 48, 72, and 96 h).

We have previously reported that antitumor efficacy was correlated with the number of hydroxyl groups of xanthone in HL60 cells.[8] Similar results were also observed in DLD-1 cells in the present study. Sato et al. examined the efficacy of eight xanthone derivatives including αM and γM on the viability of rat pheochromocytoma PC12 cells,[7] and showed stronger activity of αM than

γM at 10 μM and no efficacy of non-prenylated xanthones with three or four hydroxyl groups. It is thus suggested that the substitution of hydroxyl group for methoxy group seemed to attenuate the antiproliferative potency of prenylated xanthones, but some discrepancy seems to have set in on account of the difference in cell type. The exact explanation for this finding is not available, but formation of an intramolecular hydrogen bond via a hydroxy group could be involved in the antiproliferative potency of the prenylated xanthones.

Our previous study has show that αM induces apoptosis in human leukemia cell lines, HL60, K562, NB4, and U937,[6] but the potency of other xanthones was not examined. To examine the involvement of apoptosis in the antiproliferative effects of xanthones on DLD-1 cells, we carried out Hoechst 33342 nuclear staining and nucleosomal DNA-gel electrophoresis at 72 h after the treatment with each xanthone 20 μM (Fig. 3). αM and γM treatment induced nuclear condensation and fragmentation, and DNA ladder formation, but βM was not effective. These results suggest that the antiproliferative effects of αM and γM, but not that of βM, were associated with apoptosis in DLD-1 cells.

2.2. α- and β-Mangostin induce G1 arrest and γ-mangostin induces S arrest

To investigate further the mechanism underlying the antiproliferative effect of the xanthones tested, we analyzed the cell-cycle distribution of xanthone-treated cells
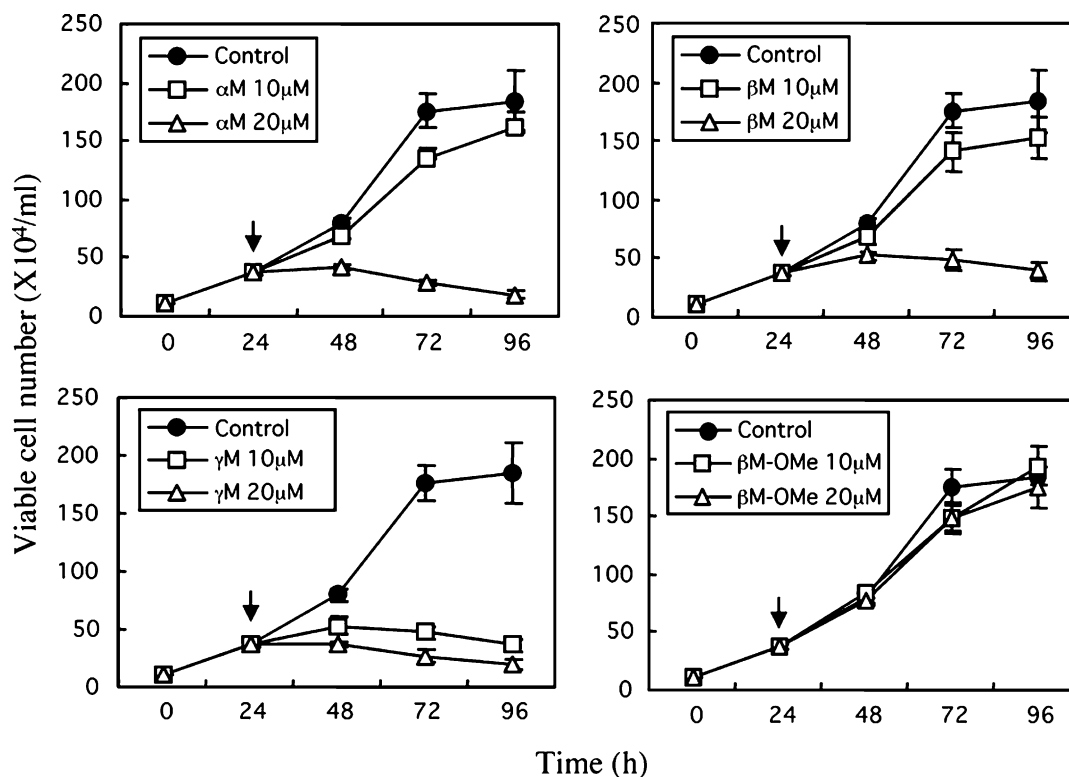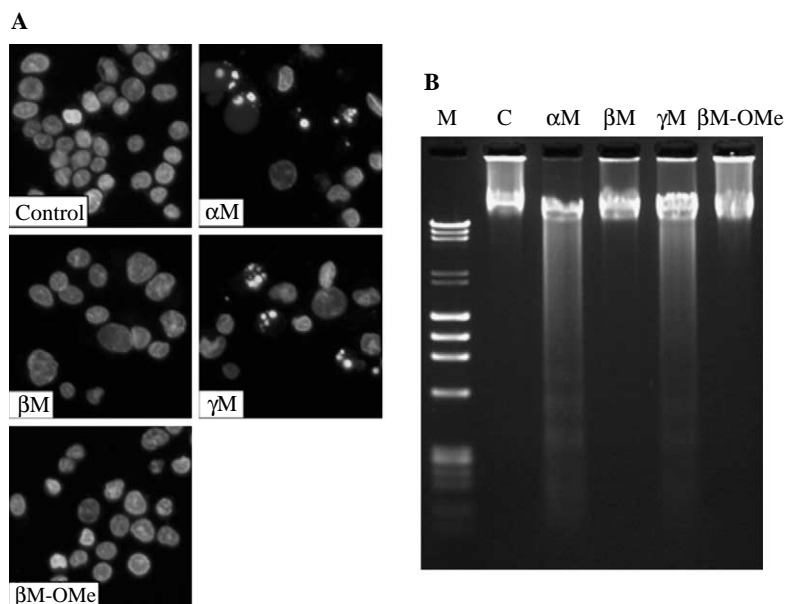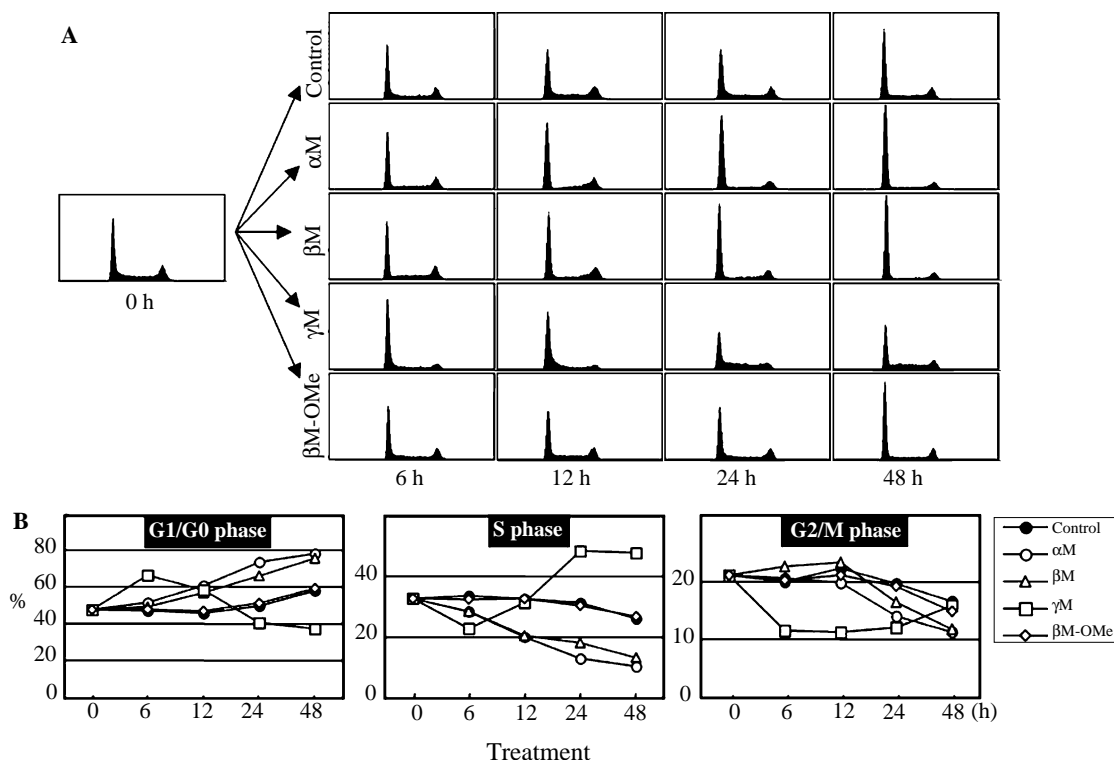

Figure 2. Effect of xanthones on the cell growth of DLD-1 cells. 1×10^5 cells/well were cultured in 24-well plate dishes, to which DMSO alone or 10 and 20 μM xanthones were added at 24 h (arrow). Viable cell numbers were counted by trypan-blue dye exclusion test at 24, 48, 72, and 96 h. Data are expressed as means ± SD from four different experiments.

Figure 3. Induction of apoptotic response by xanthone treatment. Cells were treated with each xanthone for 72 h. (A) Morphological examination was performed by Hoechst 33342 nuclear staining. (B) Nucleosomal DNA fragmentation was detected by agarose-gel electrophoresis using 3 μg DNA (M, DNA size marker).

(20 μM) using flow cytometry (Fig. 4A). Interestingly, the cell cycle was arrested at different stages depending on the type of xanthones. Treatment with αM and βM induced G1-phase cell-cycle arrest. Figure 4B shows that the G1-phase proportion gradually increased from 6 to 48 h after the treatment of αM and βM, concurrent with decreased S-phase proportion. These results indicated

that G1-phase arrest induced by αM and βM treatment was due to the inhibition of entry into S-phase. On the other hand, γM treatment induced S-phase cell-cycle arrest, which was almost complete by 24 h. The S-phase proportion was shown to be decreased at 6 h, but increased thereafter, while the G2/M-phase proportion was reduced (Fig. 4B). These results indicated that the


Figure 4. Effect of xanthones on cell-cycle progression in DLD-1 cells. (A) Flow cytometric analysis of DLD-1 cells treated with DMSO or 20 μM of each xanthone. (B) Time course of the cell-cycle distribution in the cells after the treatment. Experiments were repeated three times with similar results.

S-phase arrest induced by γM treatment was due to the inhibition of entry into the G2/M-phase.

Since the effects of xanthones on the cell cycle were observed, we then examined changes in expression of the cell-cycle regulatory proteins in DLD-1 cells treated with αM, βM, and γM at 20 μM (Fig. 5). Western blot analysis revealed that αM up-regulated the expression of p27 levels after 24–48 h of treatment, and down-regulated the levels of cyclin A, B1, D1, and E1, and cdc2, phospho-cdc2 (tyrosine 15) levels. βM treatment also caused down-regulation of cyclin A, B1, D1, and E1, and cdc2 levels, to less marked extent compared to αM. It has been reported that p27 overexpression induces apoptosis in several different cancer cell lines,[13,14] and cyclin and cdc2 suppression was implicated in apoptosis of cancer cells.[15–18] Thus, this difference in expression profiles of cell-cycle regulating proteins between αM and βM may be responsible for the different potency to induce apoptosis. On the other hand, the treatment with γM markedly down-regulated cyclin D1, but other proteins were not altered in the expression levels. Cyclin D1 plays as a G1 cyclin,[19] indicating that the other

mechanism probably relates to the S-phase arrest that was induced by γM. Although there are many other proteins associated with cell-cycle machinery, it can be concluded that αM, βM, and γM affect the expression levels of cyclins, cdc2, and p27, resulting in induction of cell-cycle arrest. To explore the precise molecular mechanism of cell-cycle arrest induced by αM, βM, and γM, further experiments are required and are currently under progress in our laboratory.

Recent studies with gene-targeted mice revealed the association of cell-cycle with tumor formation. Loss of p27, a multifunctional cyclin-dependent kinase inhibitor,[20] enhances the malignancy and frequency of tumor generation in cooperation with different oncogenic stimuli.[21] On the other hand, the deficiency in cyclin D1 suppressed the intestinal tumor in familial adenomatous polyposis model APC[Min] mice.[22] Thus, our findings showing the up-regulation of p27 treatment and the down-regulation of cyclin D1 by αM, βM, and γM provide a promising clue toward cancer preventive treatment or therapeutic agent. We previously reported that the mixture of αM (77.8%) and γM (15.9%) exhibited
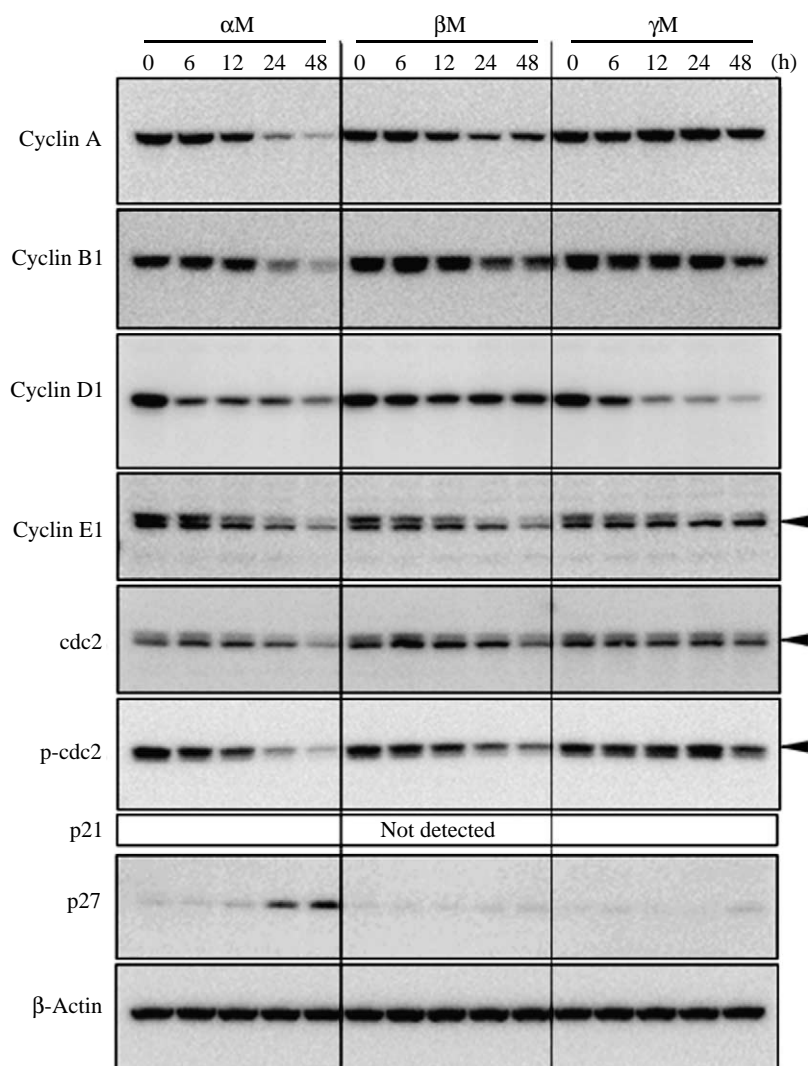
Figure 5. Western blot analysis of cell-cycle regulatory proteins in xanthone-treated (20 μM) DLD-1 cells. Data shown are representative of two separate experiments.

short-term chemopreventive effects on rat colon carcinogenesis model induced by 1,2-dimethylhydrazine.[23] Together, these data suggest that the xanthones tested here can be candidates for preventive and therapeutic application for cancer treatment.

3. Conclusion

The present study showed that the number of hydroxyl groups in xanthone was related to antiproliferative efficacy, which was associated with cell-cycle arrest by regulating the expression of cyclins and p27. The xanthones tested qualify for the development of preventive and therapeutic agents for cancers.

4. Experimental section

4.1. Materials

α-Mangostin, β-mangostin, and γ-mangostin were purified, as described in our previous study.[1] Methoxy-β-mangostin was obtained from partial methylation of β-mangostin. Their purity of over 98% was confirmed by a high-performance liquid chromatography using Develosil ODS-5 column (4.6 × 250 mm) (Nomura Chemical, Seto, Aichi, Japan); mobile phase, acetonitrile/0.05 M phosphate buffer (7:3); flow rate, 1.0 ml/min; column temperature, 40 °C, UV 365 nm (data not shown).

4.2. Cell culture and treatments

Human colon adenocarcinoma cell line DLD-1, carrying the mutated p53 gene,[24] was provided by Health Science Research Resources Bank (Sennan, Osaka, Japan). Cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum. Xanthones dissolved in DMSO were added to the cell cultures with a final DMSO concentration of 0.1% v/v, which had no significant effect on the growth and differentiation of DLD-1 cells (data not shown). DMSO (0.1%) treatment was used as control. Viable cell number was measured by trypan-blue dye test using Thoma type cell count chamber.

4.3. Hoechst 33342 staining

For morphological examination of apoptotic changes, cells were stained with Hoechst 33342 (Calbiochem, San Diego, CA, USA). Hoechst 33342 was added to cultured medium at a concentration of 5 μg/ml. After incubation for 30 min, cells were collected and washed with phosphate-buffered saline (PBS) and then observed under a fluorescence microscope, Olympus BX-51 (Olympus, Tokyo, Japan).

4.4. DNA extraction and agarose-gel electrophoresis

The cells treated with or without xanthones (20 μM) for 72 h were collected and washed with PBS. Cells were then incubated at 37 °C overnight with 100 mM Tris–HCl (pH 7.4), 5 mM EDTA, 200 mM NaCl, 0.2%

SDS, and 200 μg/ml proteinase K (Takara, Otsu, Shiga, Japan) and then extracted with phenol/chloroform. DNA was precipitated with ethanol and then treated with 0.1 mg/ml RNaseA (Sigma, St. Louis, MO, USA). Two micrograms of DNA was analyzed by electrophoresis on 2% agarose gel.

4.5. Flow cytometry

Cells were washed with PBS, treated with 100 μg/ml RNase A in PBS containing 0.1% Triton X-100 (Sigma), and stained with 20 μg/ml propidium iodide (Invitrogen, Carlsbad, CA, USA). To analyze for DNA content, flow cytometry was performed over 20,000 cells by using a FACS Calibur cytometer and CellQuest softoware (Becton–Dickinson, San Jose, CA, USA).

4.6. Antibodies and Western blotting

We used antibodies against: rabbit polyclonal anti-cyclin E1, anti-phospho-cdc2 (Tyr15), and anti-p27 Kip1, and mouse monoclonal anti-cyclin A, cyclin D1, and anti-p21 Waf1/Cip1 (Cell Signaling, Beverly, MA, USA), mouse monoclonal anti-cdc2 (A-17) anti-β-actin (AC-15) (Sigma), and rabbit polyclonal anti-cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells treated with or without xanthones were harvested and rinsed with ice-cold PBS. For the preparation of cell lysate, the cell pellet was resuspended in lysis buffer A containing 10 mM Tris–HCl, pH 7.4, 1% NP-40, 0.1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1% Phosphatase Inhibitor Cocktail I and II (Sigma), and 1% Protease Inhibitor Cocktail (Sigma) and allowed to stand on ice for 30 min. After centrifugation at 14,000 rpm for 20 min at 4 °C, the supernatant was collected as the cell lysate. Protein content was measured with a DC Protein assay kit (Bio-Rad, Hercules, CA, USA). Five micrograms of protein of each sample was analyzed, as previously described in our previous study.[6] For detection of β-actin used as an internal control, membranes were reprobed by using a Restore Western Blot Stripping Buffer (PIERCE, Rockford, IL, USA).

4.7. Statistics

In statistical analysis, we performed one-way ANOVA using a StatView software (SAS Institute Inc., Cary, NC).

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