Screening of mammalian DNA polymerase and topoisomerase inhibitors from *Garcinia mangostana* L. and analysis of human cancer cell proliferation and apoptosis

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Abstract. We purified and identified eight xanthones from mangosteen (*Garcinia mangostana* L.) and investigated whether these compounds inhibited the activities of mammalian DNA polymerases (Pols) and human DNA topoisomerases (Topos). β-Mangostin was the strongest inhibitor of both mammalian Pols and human Topos among the isolated xanthones, with 50% inhibitory concentration (IC_{50}) values of 6.4-39.6 and 8.5-10 μM, respectively. Thermal transition analysis indicated that β-mangostin did not directly bind to double-stranded DNA, suggesting that this compound directly bound the enzyme protein rather than the DNA substrate. β-Mangostin showed the strongest suppression of human cervical cancer HeLa cell proliferation among the eight compounds tested, with a 50% lethal dose (LD_{50}) of 27.2 μM. This compound halted cell cycle in S phase at 12-h treatment and induced apoptosis. These results suggest that decreased proliferation by β-mangostin may be a result of the inhibition of cellular Pols rather than Topos, and β-mangostin might be an anticancer chemotherapeutic agent.

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Abbreviations: Pol, DNA polymerase (E.C.2.7.7.7); Topo, DNA topoisomerase; dsDNA, double-stranded DNA; TdT, terminal deoxynucleotidyl transferase; dTTP, 2'-deoxythymidine-5'-triphosphate; dNTP, 2'-deoxynucleoside 5'-triphosphate; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; EtBr, ethidium bromide; ssDNA, single-stranded DNA; DAPI, 4',6-diamidino-2-phenylindole; SD, standard deviation; IC_{50}, 50% inhibitory concentration; Tm, melting temperature; LD_{50}, 50% lethal dose

Key words: β-mangostin, mangosteen, DNA polymerase inhibition, DNA topoisomerase inhibition, anticancer, cell cycle, apoptosis

Introduction

Cancer is a major public health problem worldwide. Epidemiologic and animal studies have demonstrated an association with the inclusion of vegetables and fruits containing chemopreventive compounds in the diet and a reduced risk of cancer development (1,2). Our laboratory has spent over 20 years screening vegetables and fruits for natural phytochemical products that inhibit DNA metabolic enzymes, primarily mammalian DNA polymerases (Pols) and human DNA topoisomerases (Topos).

Pols (DNA-dependent DNA polymerase, EC 2.7.7.7) catalyze the addition of deoxyribonucleotides to the 3'-hydroxyl terminus of primed double-stranded DNA (dsDNA) molecules (3). The human genome encodes at least 15 Pols that function in cellular DNA synthesis (4,5). Eukaryotic cells contain three replicative Pols (α, δ and ε), a single mitochondrial Pol (γ), and at least 11 non-replicative Pols [β, ζ, η, θ, ι, κ, λ, μ, ν, terminal deoxynucleotidyl transferase (TdT), and REV1] (6,7). Pol structure is highly conserved, and the catalytic subunit shows little variance among species. Sequence homology classifies eukaryotic Pols into four main families: A, B, X and Y (6). Family A includes mitochondrial Pol γ as well as Pols θ and ν; family B includes the three replicative Pols α, δ and ε and also Pol ζ; family X comprises Pols β, λ and μ, as well as TdT; and family Y includes Pols η, ι and κ, in addition to REV1 (7).

Topos are nuclear enzymes that alter DNA topology and are required for the replication, transcription, recombination and segregation of daughter chromosomes (8). Eukaryotic cells have two types of Topos, I and II. Topo I catalyzes the passage of a DNA strand through a transient single-strand break in the absence of any high-energy cofactor. In contrast, Topo II catalyzes the passage of dsDNA through a transient double-strand break in the presence of ATP.

Selective inhibitors of Pols and Topos can suppress the proliferation of normal and cancerous human cells or exhibit cytotoxic effects (9-11). These inhibitors are potentially useful as anticancer, antiviral, antiparasitic or antipregnancy agents. In screening for these enzyme inhibitors in the ethanol

extracts of various food materials, we found that the extract of mangosteen (*Garcinia mangostana* L.) from various parts of the fruit, such as the fruit, pericarp, leaves and rind, strongly inhibited the activities of mammalian Pols. We purified major components and identified eight xanthones by NMR and mass spectra analysis.

The mangosteen tree has been cultivated for centuries in tropical areas of the world. The tree is presumed to have originated in Southeast Asia or Indonesia and has largely remained indigenous to Malay Peninsula, Myanmar, Thailand, Cambodia, Vietnam and the Moluccas (12). The white, inner pulp of the mangosteen fruit is highly praised as one of the best tasting of all tropical fruits. This fruit has been dubbed the 'queen of fruit' in its native Thailand. The mangosteen rind, leaves and bark have been used as folk medicine for thousands of years (13). The thick mangosteen rind has been and is used for treating catarrh, cystitis, diarrhea, dysentery, eczema, fever, intestinal ailments, pruritis and other skin ailments. The mangosteen leaves are also used by some natives in teas and for diarrhea, dysentery, fever and thrush. Concentrates of mangosteen bark are used for genito-urinary afflictions and stomatosis.

Over 200 xanthones are currently known to exist in nature and ~50 of them are found in the mangosteen. The xanthones from mangosteen are gaining more interest because of their remarkable pharmacological effects, including not only anticancer activities (14,15), but also analgesic (16), antioxidant (17), anti-inflammatory (18), anti-allergy (19), antibacterial (20), anti-tuberculosis (21), antifungal (22), antiviral (23), cardioprotective (24), neuroprotective (25) and immunomodulation (26) effects. However, the underlying molecular mechanisms of these compounds and their effects on the activities of DNA metabolic enzymes have not been reported.

Therefore, the present study focused on the isolated eight xanthones from mangosteen and investigated these compounds for their inhibitory effects *in vitro* on mammalian Pol and human Topo activities.

Materials and methods

Materials. A chemically synthesized DNA template, poly(dA), and calf thymus DNA were purchased from Sigma-Aldrich Inc. and a customized oligo(dT)$_{18}$ DNA primer was produced by Sigma-Aldrich Japan K.K. (Hokkaido, Japan). Radioactive nucleotide [^{3}H]-labeled 2'-deoxythymidine-5'-triphosphate (dTTP; 43 Ci/mmol) was obtained from Moravek Biochemicals Inc. (Brea, CA, USA). Supercoiled pHOT-1 DNA was obtained from TopoGEN Inc. (Port Orange, FL, USA). All other reagents were analytical grade from Nacalai Tesque Inc. (Kyoto, Japan).

Isolation of xanthones from mangosteen. Some xanthone compounds were isolated from the leaves of mangosteen (*Garcinia mangostana* L.), which were collected in Vietnam and provided by Dr Duy Hoang Le (Kobe Pharmaceutical University). The chemical structures were determined by spectroscopic analyses and comparison with reported data (27-30). The identified eight compounds are α-mangostin **(1)**, β-mangostin **(2)**, 3,6-di-*O*-methyl-γ-mangostin **(3)**, fuscaxanthone C **(4)**, gartanin **(5)**, 8-deoxygartanin **(6)**, mangostanin **(7)** and morusignin I **(8)** (Fig. 1).

Enzymes. Pol α was purified from calf thymus by immunoaffinity column chromatography, as described by Tamai *et al* (31). Recombinant rat Pol β was purified from *Escherichia coli* JMpβ5, as described by Date *et al* (32). The human Pol γ catalytic gene was cloned into pFastBac, with the histidine-tagged enzyme expressed using the BAC-TO-BAC HT Baculovirus Expression System according to the supplier's instructions (Life Technologies, Inc., Gaithersburg, MD, USA), and purified using ProBound resin (Invitrogen Japan K.K, Tokyo, Japan) (33). Human Pols δ and ε were purified by nuclear fractionation of human peripheral blood cancer cells (Molt-4) using the second subunit of Pols δ and ε-conjugated affinity column chromatography, respectively (34). A truncated form of human Pol η (residues 1-511) tagged with His$_6$ at the C-terminus was expressed in *E. coli* and purified as described by Kusumoto *et al* (35). Recombinant mouse Pol ι tagged with His$_6$ at the C-terminus was expressed and purified by Ni-NTA column chromatography (36). A truncated form of Pol κ (residues 1-560) with a His$_6$-tag at the C-terminus was expressed in *E. coli* and purified as described by Ohashi *et al* (37). Recombinant human His-Pol λ was expressed and purified according to a method described by Shimazaki *et al* (38). Calf TdT, T7 RNA polymerase and T4 polynucleotide kinase were purchased from Takara Bio Inc. (Kyoto, Japan). Purified human placenta Topos I and II were purchased from TopoGEN Inc. Bovine pancreas deoxyribonuclease I was obtained from Stratagene Cloning Systems (La Jolla, CA, USA).

Measurement of Pol activity. The reaction mixtures for calf Pol α and rat Pol β have been described (39,40). Those for human Pol γ and for human Pols δ and ε were as described by Umeda *et al* (33) and Ogawa *et al* (41), respectively. The reaction mixtures for mammalian Pols η, ι and κ were the same as that for calf Pol α, and the reaction mixture for human Pol λ was the same as for rat Pol β. For Pols, poly(dA)/oligo(dT)$_{18}$ (A/T=2/1) and tritium-labeled 2'-deoxythymidine 5'-triphosphate ([^{3}H]-dTTP) were used as the DNA template-primer and nucleotide [i.e., 2'-deoxynucleoside 5'-triphosphate (dNTP)] substrates, respectively. For the TdT reactions, oligo(dT)$_{18}$ (3'-OH) and dTTP were used as the DNA primer substrate and nucleotide substrate, respectively.

The eight isolated xanthone compounds were dissolved in distilled dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec. Aliquots of 4 μl of sonicated samples were mixed with 16 μl of each enzyme (final amount, 0.05 units) in 50 mM Tris-HCl (pH 7.5), containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM ethylenediaminetetraacetic acid (EDTA), and kept at 0˚C for 10 min. These inhibitor-enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme standard reaction mixtures and incubation carried out at 37˚C for 60 min. Activity without the inhibitor was considered 100% and the remaining activity at each inhibitor concentration was determined relative to this value. One unit of Pol activity was defined as the amount of enzyme that catalyzed incorporation of 1 nmol dNTP [measured using [^{3}H]-dTTP] into synthetic DNA template-primers in 60 min at 37˚C under the normal reaction conditions for each enzyme (39,40).

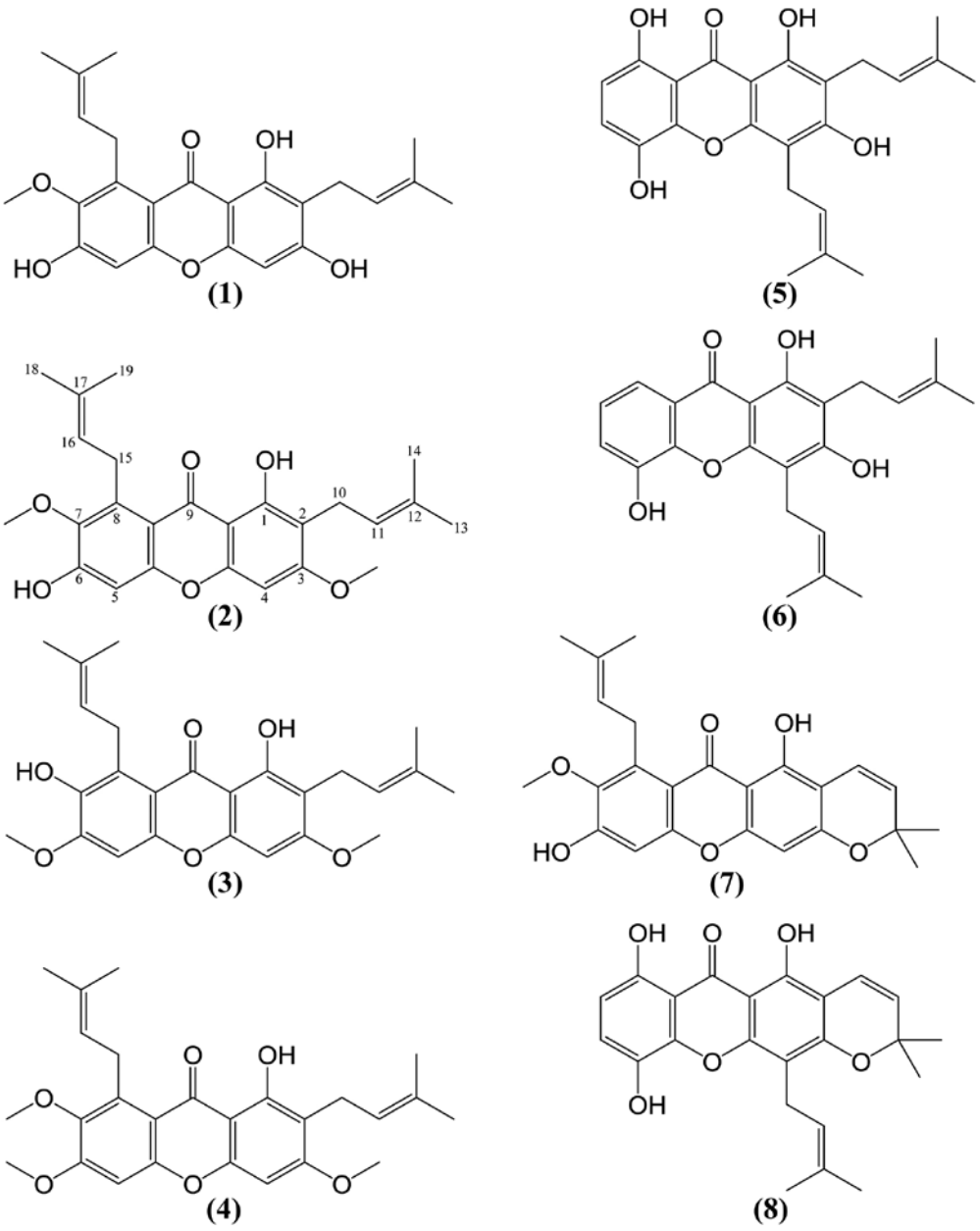

Figure 1. Structure of the identified xanthone compounds **(1-8)** from mangosteen (*Garcinia mangostana* L.). α-Mangostin **(1)**, β-mangostin **(2)**, 3,6-di-*O*-methyl-γ-mangostin **(3)**, fuscaxanthone C **(4)**, gartanin **(5)**, 8-deoxygartanin **(6)**, mangostanin **(7)** and morusignin I **(8)**.

Measurement of Topo activity. Purified human placental Topos I and II were purchased from TopoGen Inc. The catalytic activity of Topo I was determined by detecting supercoiled plasmid DNA (Form I) in its nicked form (Form II) (42). The Topo I reaction was performed in a 20-μl reaction mixture that contained 10 mM Tris-HCl (pH 7.9), pHOT-1 DNA (250 ng), 1 mM EDTA, 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, 2 μl of one of the eight isolated xanthone compounds dissolved in DMSO, and 2 units of Topo I. Topo II catalytic activity was analyzed in the same manner, except the reaction mixture contained 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, supercoiled pHOT-1 DNA (250 ng), and 2 units of Topo II (42). Reaction mixtures were incubated at 37˚C for 30 min, followed by digestion with 1% sodium dodecyl sulfate (SDS) and 1 mg/ml proteinase K. After digestion, 2 μl loading buffer (5% sarkosyl, 0.0025% bromophenol blue and 25% glycerol) was added. The same procedure was followed for mobility shift assay assessment of enzyme DNA binding, except SDS denaturation and proteinase K digestion were omitted. Reaction mixtures were subjected to 1% agarose gel electrophoresis in Tris/borate/EDTA buffer. Agarose gels were stained with ethidium bromide (EtBr) and DNA band shifts from Form I to Form II were detected using an enhanced chemiluminescence detection system (Perkin-Elmer Life Sciences Inc., Waltham, MA, USA). Zero-D scan (Version 1.0, M & S Instruments Trading Inc., Osaka, Japan) was used for densitometric quantitation.

Other enzyme assays. The activities of T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I were measured using standard assays according to the

manufacturer's specifications as described by Nakayama and Saneyoshi (43), Soltis and Uhlenbeck (44) and Lu and Sakaguchi (45), respectively.

Thermal transition of DNA. Thermal transition profiles of dsDNA to single-stranded DNA (ssDNA) with or without α-mangostin were obtained with a spectrophotometer (UV-2500; Shimadzu Corp., Kyoto, Japan) equipped with a thermoelectric cell holder was used as previously described (46). Calf thymus DNA (6 μg/ml) was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 1% DMSO. The solution temperature was equilibrated at 75˚C for 10 min, and then increased by 1˚C at 2-min intervals for each measurement point. Any change in the absorbance (260 nm) of the test compound itself at each temperature point was automatically subtracted from that of DNA plus the compound in the spectrophotometer.

Cell culture and measurement of cancer cell viability. The human cervical cancer cell line HeLa was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37˚C in a humidified incubator containing 5% CO_2/95% air. For assessment of cell growth, cells were plated at $2x10^3$ cells/well in 96-well microplates, cultured for 12 h and various concentrations of the eight isolated xanthone compounds added. The compounds were dissolved in DMSO to produce 10-mM stock solutions, which were further diluted to appropriate final concentrations with growth medium containing 0.5% DMSO immediately prior to use. Cell viability was determined by the WST-1 assay following 24-h incubation (47).

Cell cycle analysis. Cellular DNA content for cell cycle analysis was determined as follows: aliquots of $3x10^5$ HeLa cells were added to a 35-mm dish and incubated with the medium that contained the test compound for 0 to 24 h. Cells were then washed with ice-cold PBS three times, fixed with 70% (v/v) ethanol, and stored at -20˚C. DNA was stained with a PI (3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide) staining solution for at least 10 min at room temperature in the dark. Fluorescence intensity was measured using a BD FACSCalibur flow cytometer in combination with CellQuest software (Becton-Dickinson Co., Tokyo, Japan).

Measurement of caspase-3 activity. The enzymatic activity of caspase-3 was measured using EnzChek Caspase-3 Assay Kit #2 (Life Technologies Japan Co., Ltd., Tokyo, Japan), according to the instruction manual. HeLa cells ($1x10^6$) after inducing apoptosis with test compound were washed with PBS and lysed. Then, the caspase-3 activity in the extracts was determined with a fluorometric assay. The fluorescent product of the substrate Z-DEVD-Rhodamine 110 generated by caspase-3 in the cell extract was detected in a fluorescent microplate reader (SH-9000 Lab; Hitachi High-Technologies Corp., Tokyo, Japan) using an excitation of 496 nm and emission of 520 nm. To determine the background fluorescence of the substrate, test compound without the Z-DEVD-R110 substrate for negative controls, and mixture of activity buffer and Z-DEVD-R110 substrate for only substrate controls were also executed.

4',6-Diamidino-2-phenylindole (DAPI) staining. Aliquots of $2.5x10^4$ HeLa cells were plated in each well of an 8-well chamber slide (Thermo Fisher Scientific K.K., Tokyo, Japan). Then, the cells were incubated with or without the test compound (27.2 and 54.4 μM) for 12 and 24 h at 37˚C with 5% CO_2. After washed with PBS twice, the cells were fixed with 4% paraformaldehyde (pH 7.4) for 15 min. Then, the cells were stained with DAPI for 1 min, and the percentage of apoptotic cells was counted under a fluorescence microscope (Olympus IX70; Olympus, Tokyo, Japan).

Statistical analysis. The data are expressed as the mean value ± the standard deviation (SD) of the mean of at least three independent determinations for each experiment. Statistical significance between each experimental group was analyzed using Student's t-test, and a probability level of 0.01 and 0.05 was used as the criterion of significance.

Results

Effect of the isolated xanthone compounds 1-8 on the activities of mammalian Pols. Selective inhibitors of mammalian Pols have been investigated with the goal to develop chemotherapeutic drugs for anticancer, antivirus and anti-inflammation treatments (48). In our ongoing screening for natural inhibitors of mammalian Pols, we found that the extract of mangosteen (*Garcinia mangostana* L.) had inhibitory activity against mammalian Pols. The isolated xanthone compounds **1-8** (Fig. 1) were selected and prepared for this study.

The inhibitory activity of each compound toward mammalian Pols was investigated using calf Pol α, rat Pol β, and human Pols γ and κ. For mammalian Pols, Pols α, β, γ and κ were used as the representatives of Pol families B, X, A and Y, respectively (6,7). Assessment of the relative activity of each Pol at a set concentration (10 μM) of the eight test compounds showed that β-mangostin **(2)** was the strongest inhibitor of these Pol species among the compounds tested (Fig. 2). In particular, this compound showed the strongest inhibitory activity against Pol α among the four mammalian Pols. α-Mangostin **(1)** and 3,6-di-*O*-methyl-γ-mangostin **(3)** slightly inhibited Pols α, γ, and κ activities and Pol κ activity, respectively. These results suggested that the common backbone structure with prenyl groups at C-2 and C-8 of compounds **1-3** might be important for Pol inhibition, and both the hydroxyl group at C-6 and the methoxy group at C-3, which are present in β-mangostin **(2)**, must be much more important for Pol inhibitory activity.

Effect of the isolated xanthone compounds 1-8 on the activities of human Topos I and II. Next, the inhibitory effects of the eight isolated xanthones (10 μM each) were examined against human Topos I and II, which have ssDNA and dsDNA nicking activity, respectively (8). β-Mangostin **(2)** was the strongest inhibitor of both Topos I and II, and the inhibitory effect of this compound on Topo II was more potent than that on Topo I (Fig. 3). In contrast, mangostanin **(7)** and morusignin I **(8)**

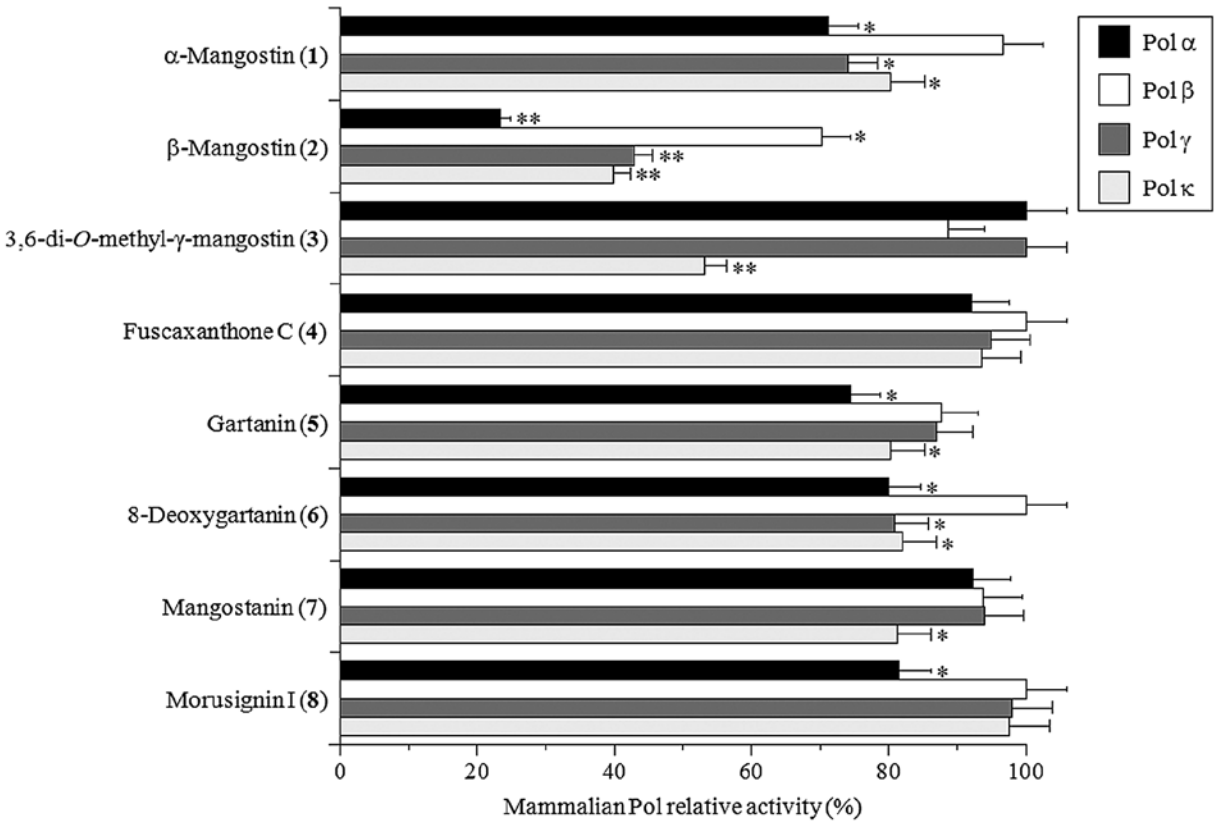

Figure 2. Effect of the isolated compounds **(1-8)** from mangosteen on the activities of mammalian Pols. Each compound at 10 μM was incubated with calf Pol α, rat Pol β, human Pol γ and human Pol κ (0.05 units each). Pol activity was measured as described in Materials and methods and is shown as a percentage of enzyme activity in the absence of the compound, which was taken as 100%. Data are the mean ± SD of three independent experiments. $^{**}P<0.01$ and $^{*}P<0.05$ vs. controls.

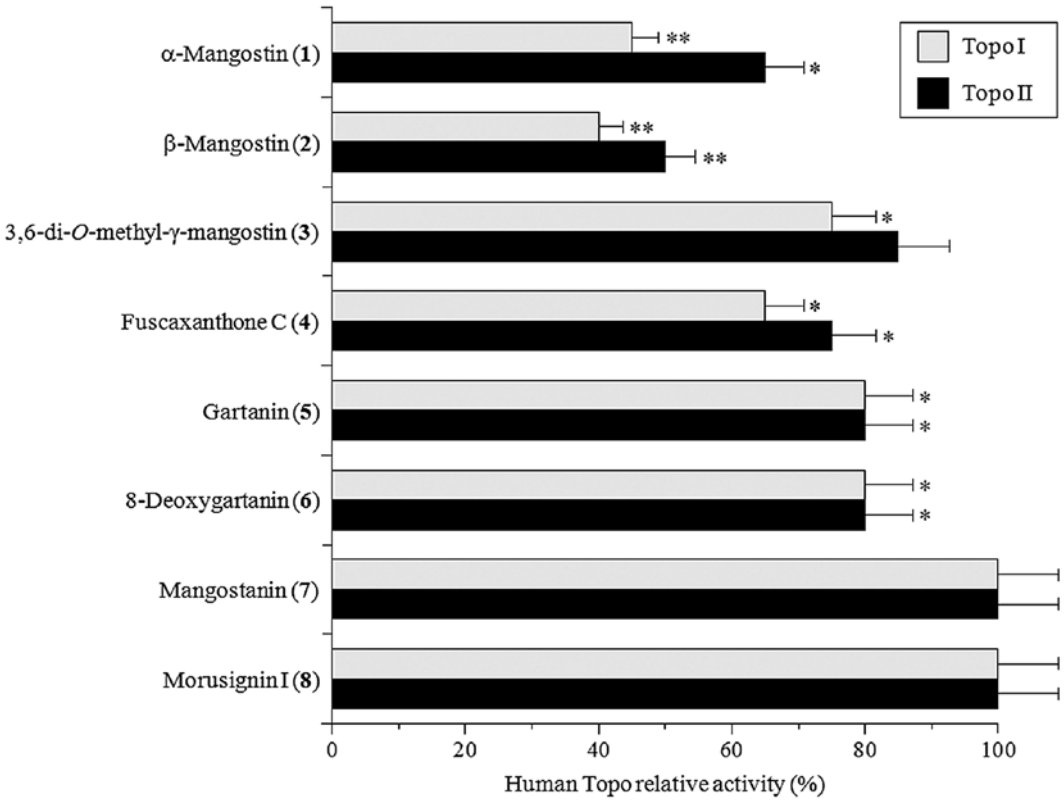

Figure 3. Effect of the isolated compounds **(1-8)** from mangosteen on the activities of human Topos I and II. Each compound at 10 μM was incubated with Topo I (gray bars) and Topo II (black bars). Topo activity was measured as described in Materials and methods, and is shown as a percentage of enzyme activity in the absence of the compound, which was taken as 100%. Data are the mean ± SD of three independent experiments. $^{**}P<0.01$ and $^{*}P<0.05$ vs. controls.

Table I. IC_{50} values of β-mangostin on the activities of mammalian Pols, human Topos and various DNA metabolic enzymes.

Enzyme	IC_{50} values (μM)
Mammalian Pols	
A-Family of Pol	
Human Pol γ	8.6±0.51
B-Family of Pols	
Calf Pol α	6.4±0.38
Human Pol δ	7.4±0.44
Human Pol ε	8.5±0.50
X-Family of Pols	
Rat Pol β	34.8±2.1
Human Pol λ	28.5±1.7
Human Pol μ	38.2±2.2
Calf TdT	39.6±2.3
Y-Family of Pols	
Human Pol η	9.3±0.55
Mouse Pol ι	8.7±0.52
Human Pol κ	9.2±0.54
Human Topos	
Human Topo I	10±1.0
Human Topo II	8.5±0.9
Other DNA metabolic enzymes	
T7 RNA polymerase	>200
T4 polynucleotide kinase	>200
Bovine deoxyribonuclease I	>200

Compounds were incubated with each enzyme (0.05 units). Data, mean ± SD (n=3).

did not influence the activities of these Topos. Topo inhibition could be ranked as β-mangostin **(2)** >α-mangostin **(1)** >3,6-di-*O*-methyl-γ-mangostin **(3)** = fuscaxanthone C **(4)** = gartanin **(5)** = 8-deoxygartanin **(6)** >mangostanin **(7)** = morusignin I **(8)**. Since β-mangostin **(2)** showed the strongest inhibitory activities on both mammalian Pols and human Topos, we focused on this compound in the subsequent study.

Effects of β-mangostin on the activities of various mammalian Pols and other DNA metabolic enzymes. Evaluation of the inhibition of DNA metabolic enzyme activities *in vitro* by β-mangostin revealed that this compound inhibited the activities of all eleven mammalian Pols tested from all four families. Calf Pol α and calf TdT were the strongest and weakest inhibited, respectively, with 50% inhibitory concentration (IC_{50}) values of 6.4 and 39.6 μM, respectively (Table I). The inhibition by β-mangostin against mammalian Pol families could be ranked as B-family Pols >A-family Pol >Y-family Pols > X-family Pols. By comparison, the IC_{50} values of aphidicolin, a known eukaryotic DNA replicative Pols α, δ and ε inhibitor, were 20, 13 and 16 μM, respectively (49), showing that the Pol inhibitory activity of β-mangostin was >1.5-fold

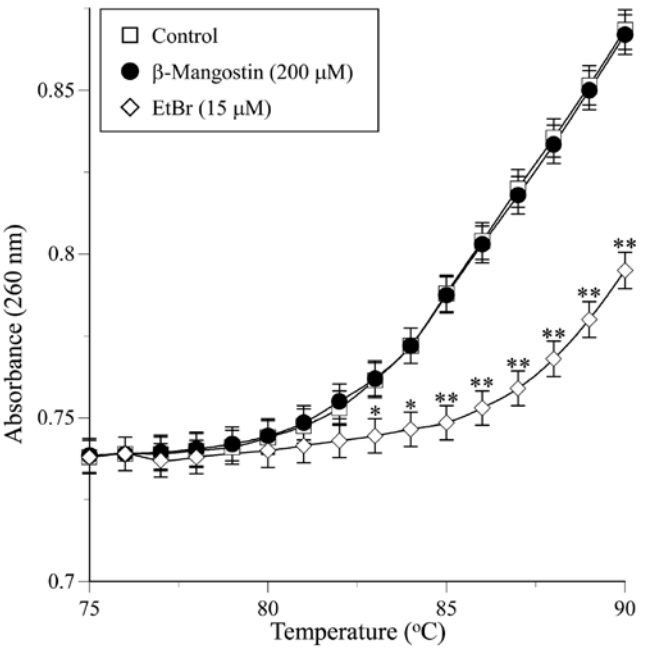

Figure 4. Effect of β-mangostin on the thermal transition of dsDNA. β-Mangostin (200 μM, closed circle) or EtBr (15 μM, open diamond) were incubated with 6 μg/ml of calf thymus dsDNA in 0.1 M Na-phosphate buffer (pH 7.0). Control is indicated by an open square. Data are the mean ± SD of three independent experiments. $^{**}P<0.01$ and $^{*}P<0.05$ vs. controls.

higher than that of aphidicolin. β-Mangostin inhibited the activity of human Topos I and II with IC_{50} values of 10.0 and 8.5 μM, respectively. Topotecan and doxorubicin, which are Topo I and Topo II inhibitors, respectively, also inhibited the nicking activities of Topos I and II with IC_{50} values of 45 and 60 μM, respectively (data not shown). The data show that the inhibitory effect on Topos I and II of β-mangostin was more potent than that of topotecan and doxorubicin, respectively. The inhibition of human Topos activities by β-mangostin was approximately the same order of potency compared with that of the activities of mammalian Pols except for X-family of Pols (Table I).

In contrast, β-mangostin did not influence the activities of other DNA metabolic enzymes, such as T7 RNA polymerase, T4 polynucleotide kinase, and bovine deoxyribonuclease I (Table I). These results indicate that β-mangostin can be specifically classified as an inhibitor of mammalian Pols and Topos.

Influence of β-mangostin on the hyperchromicity of dsDNA. Specific assays were performed to determine whether β-mangostin-induced inhibition resulted from the ability of the compound to bind to DNA or the enzyme. The interaction of β-mangostin with dsDNA was investigated by studying its thermal transition. The melting temperature (Tm) of dsDNA in the presence of an excess of β-mangostin (200 μM) was observed using a spectrophotometer equipped with a thermoelectric cell holder. As shown in Fig. 4, a thermal transition (i.e., Tm) from 75 to 90˚C was not observed within the concentration range used in the assay, whereas when a typical intercalating compound, such as EtBr (15 μM), was used as a positive control, an obvious thermal transition was observed.

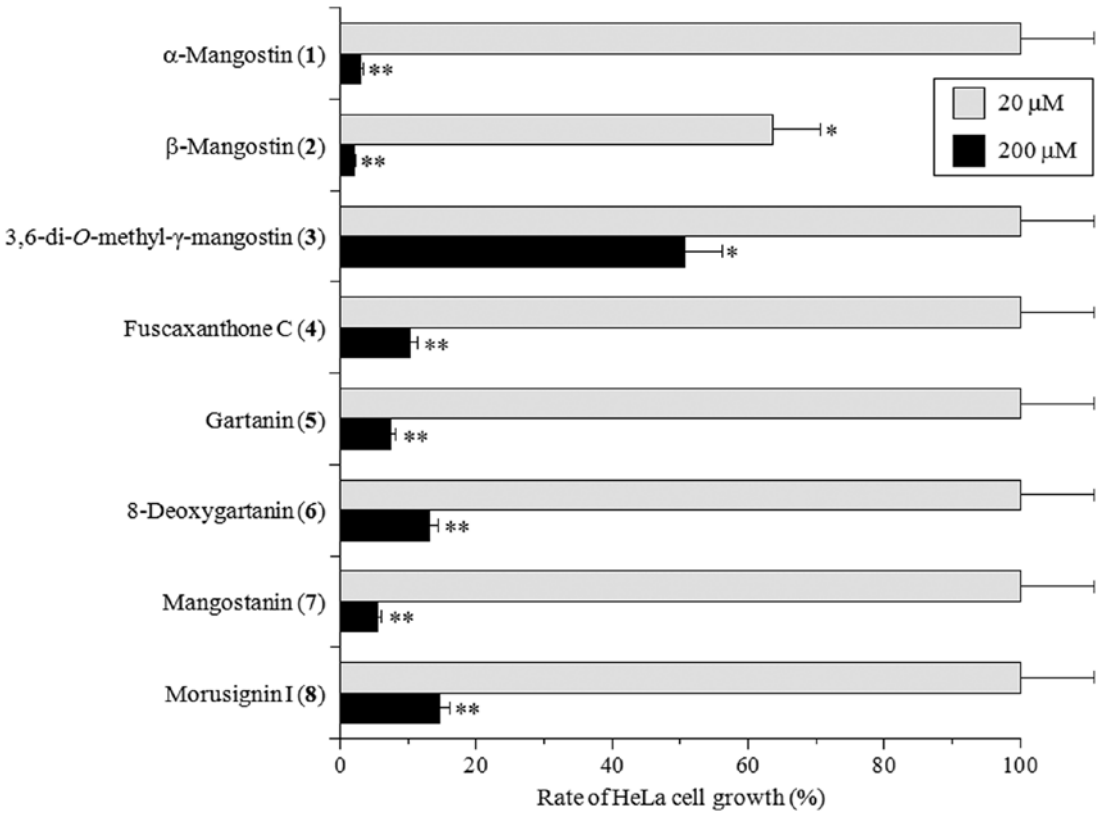

Figure 5. Effect of the isolated compounds **(1-8)** from mangosteen on HeLa human cervical cancer cultured cell growth. Cells were cultured for 24 h in media containing 10 μM (gray bars) or 100 μM (black bars) of each compound. Cell proliferation was determined by the WST-1 assay. Cell growth inhibition in the absence of the compound was taken as 100%. Data are the mean ± SD for five independent experiments. $^{**}P<0.01$ and $^{*}P<0.05$ vs. controls.

We then assessed whether the inhibitory effect of β-mangostin on mammalian Pols and Topos resulted from non-specific adhesion to the enzyme or from selective binding to specific sites. Neither excessive nucleic acid [in the form of Poly(rC)] nor protein (BSA) significantly influenced β-mangostin-induced both Pol and Topo inhibition (data not shown). This suggests that β-mangostin selectively bound to the Pol and Topo molecules. These observations reveal that β-mangostin does not act as a DNA intercalating agent, and rather it directly binds the enzyme to inhibit its activity.

Collectively, these results suggested that β-mangostin might be a potent and selective inhibitor of mammalian Pols and Topos. We next investigated whether Pol and/or Topo inhibition by β-mangostin resulted in decreases in human cancer cell proliferation.

Effect of the isolated xanthone compounds 1-8 on cultured human cancer cells. Pols and Topos have recently emerged as important cellular targets for the development of anticancer agents. Therefore, we also investigated whether the eight isolated xanthones had any cytotoxic effect on HeLa cells. At 100 μM, all the compounds except for 3,6-di-*O*-methyl-γ-mangostin **(3)** potently suppressed the rate of HeLa cell growth to <80% that of control (Fig. 5). At 10 μM, β-mangostin **(2)** could moderately suppress HeLa cell growth, but other compounds did not influence cell proliferation. The 50% lethal dose (LD_{50}) of β-mangostin **(2)** was 27.2 μM, which is ~2-fold higher than the IC_{50} for DNA replicative Pols (X-family Pols α, δ and ε) and Topos. This suggests that β-mangostin may be able to penetrate the cell membrane and reach the nucleus, where it may then inhibit Pols and/or Topos activities to suppress cell growth. Similar findings for all eight xanthones were observed in the HCT116 human colon carcinoma cell line (data not shown). These results reveal that Pol and Topo inhibition (Figs. 2 and 3) and human cancer cell cytotoxicity (Fig. 5) can be induced by xanthones from mangosteen. Therefore, Pol/Topo inhibition by β-mangostin represents a potentially useful anticancer effect.

Next we analyzed whether β-mangostin could affect the cell cycle distribution of HeLa cells. The cell cycle fraction was recorded after 0, 6, 12 and 24 h of treatment with a concentration of β-mangostin at 54.4 μM (the double density of LD_{50}). The ratio of cells in each of the three cell cycle phases (G1, S and G2/M) is shown in Fig. 6A. Treatment with β-mangostin significantly increased the population of cells in S and G1 phase at 12 and 24 h, respectively, but did not influence the rate of G2/M phase. Dehydroaltenusin, which is a mammalian Pol α specific inhibitor, arrested the cell cycle in the G1 and S phases (50), and etoposide, which is a classical Topo II inhibitor, arrested the cell cycle in the G2/M phase (data not shown). These results suggest that β-mangostin may be an effective inhibitor of DNA replicative Pols, such as Pol α, that arrests the cell cycle at the G1 and S phases.

To examine whether the cell cycle arrest of HeLa cells treated with β-mangostin was due to apoptosis, caspase-3 activity was analyzed using fluorescent substrate (Fig. 6B). When HeLa cells were treated with the compound at 27.2 (=LD_{50} value) and 54.4 μM, the cells maximally increased

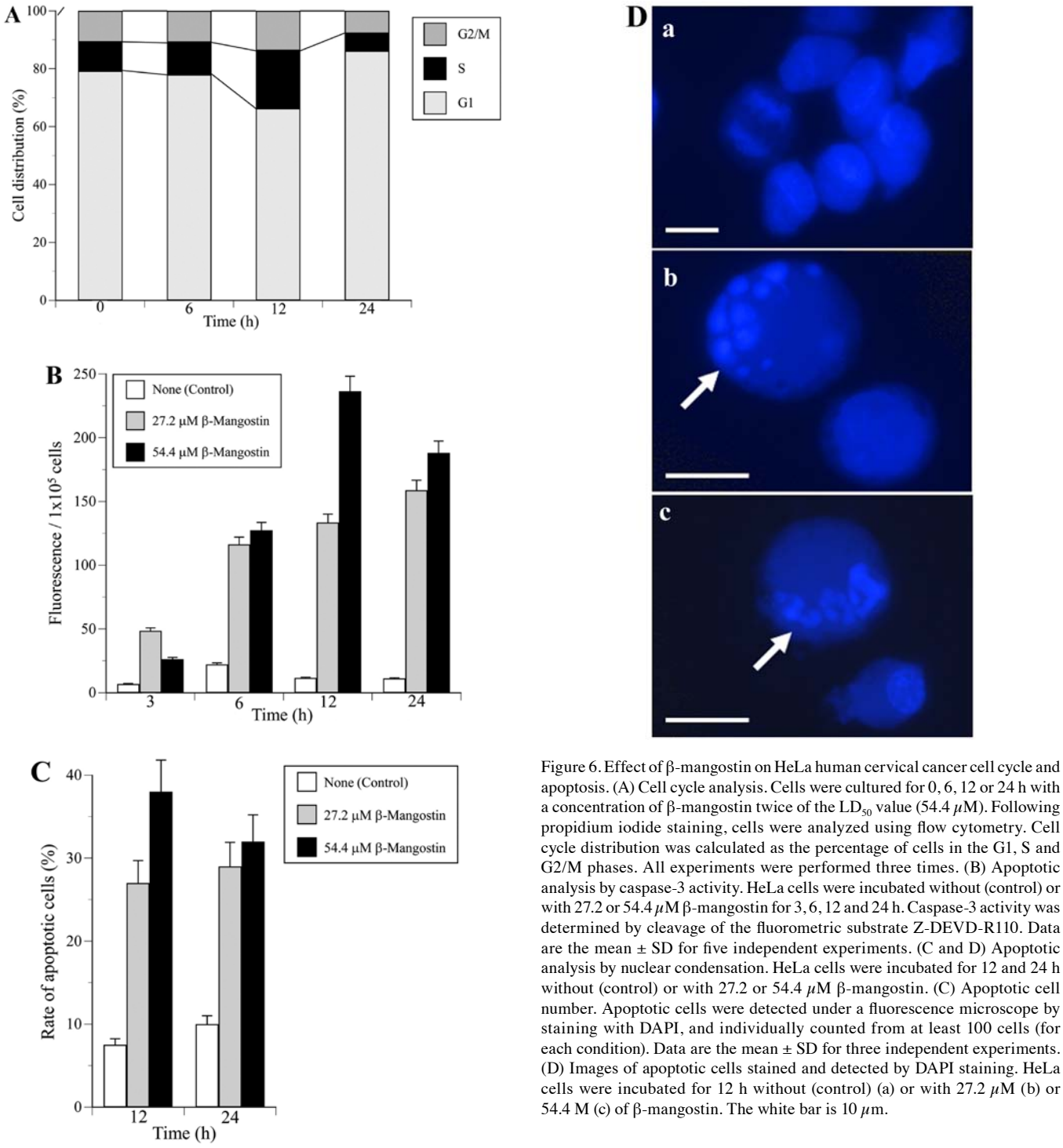

Figure 6. Effect of β-mangostin on HeLa human cervical cancer cell cycle and apoptosis. (A) Cell cycle analysis. Cells were cultured for 0, 6, 12 or 24 h with a concentration of β-mangostin twice of the LD_{50} value (54.4 μM). Following propidium iodide staining, cells were analyzed using flow cytometry. Cell cycle distribution was calculated as the percentage of cells in the G1, S and G2/M phases. All experiments were performed three times. (B) Apoptotic analysis by caspase-3 activity. HeLa cells were incubated without (control) or with 27.2 or 54.4 μM β-mangostin for 3, 6, 12 and 24 h. Caspase-3 activity was determined by cleavage of the fluorometric substrate Z-DEVD-R110. Data are the mean ± SD for five independent experiments. (C and D) Apoptotic analysis by nuclear condensation. HeLa cells were incubated for 12 and 24 h without (control) or with 27.2 or 54.4 μM β-mangostin. (C) Apoptotic cell number. Apoptotic cells were detected under a fluorescence microscope by staining with DAPI, and individually counted from at least 100 cells (for each condition). Data are the mean ± SD for three independent experiments. (D) Images of apoptotic cells stained and detected by DAPI staining. HeLa cells were incubated for 12 h without (control) (a) or with 27.2 μM (b) or 54.4 M (c) of β-mangostin. The white bar is 10 μm.

caspase-3 activity for 24 and 12 h, respectively (Fig. 6B). In addition, preincubation with a caspase-3 inhibitor, DEVD-CHO, completely blocked this activity by β-mangostin (data not shown), suggesting that the apoptosis by this compound was mediated through caspase-3 activation.

Fig. 6C and D shows the extent of apoptotic cells by DAPI staining. The percentage of HeLa apoptotic cells after treatment with 27.2 and 54.4 μM of β-mangostin was markedly high for 24 and 12 h, respectively (Fig. 6C). These findings suggest that the human cancer cytotoxic effect of β-mangostin must involve a combination of cell proliferation arrest and apoptotic cell death.

Discussion

This is the first study to examine the inhibitory effects of xanthones isolated from mangosteen (*G. mangostana* L.) (i.e., compounds **1-8** in Fig. 1) on the activities of mammalian Pol species. β-Mangostin was the strongest inhibitor of mammalian Pols α, β, γ and κ as the representatives of Pol families B, X, A and Y, respectively (Fig. 2), and human Topos I and II (Fig. 3) among the compounds investigated. Its human cancer cytotoxicity (Fig. 5) was realized through the inhibition of the DNA metabolic enzymes Pols and Topos, which are essential for DNA replication, repair and recombination as well as cell division.

Topo inhibitors, such as adriamycin, amsacrine, ellipticine, saintopin, streptonigrin and terpentecin, are intercalating agents that are thought to bind directly to the DNA molecule and subsequently indirectly inhibit both types of Topo activity (51). These chemicals inhibit the DNA chain rejoining reactions catalyzed by Topos by stabilizing a tight Topo protein-DNA complex termed the 'cleavable complex' (52,53). The possible binding of β-mangostin to DNA was examined by measuring the Tm of dsDNA, and no β-mangostin was found to bind to dsDNA (Fig. 4). Topo inhibitors are categorized into two classes, 'suppressors', which are believed to interact directly with the enzyme, and 'poisons', which stimulate DNA cleavage and intercalation (54,55). β-Mangostin may be considered a suppressor of Topo functions rather than a conventional poison as this compound does not appear to stabilize Topo protein-DNA covalent complexes, as do the above-mentioned agents. Therefore, β-mangostin could be a new type of Topo inhibitor.

The fifteen Pols encoded by mammalian genomes are specialized for different functions, including DNA replication, DNA repair, recombination and translesion synthesis (6). Some Pol inhibitors, such as cytosine arabinoside (AraC), are anticancer drugs, because these compounds inhibit the activities of DNA replicative Pols α, δ and ε, suppress DNA synthesis, arrest cells at S phase, and induce apoptosis in cancer cells that have higher cellular Pol activity than normal cells (56). β-Mangostin suppressed proliferation of HeLa cell growth (Fig. 5) and arrested the cell cycle at S phase (Fig. 6A). These effects occur via the inhibition of Pols, especially DNA replicative B-family Pol species (Fig. 2 and Table I), suggesting that this compound affects the cellular DNA synthesis of cancer cells. Together this suggests that foods containing β-mangostin have potential positive effects in the prevention of cancer and promotion of health.

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References

1. Liu RH: Potential synergy of phytochemicals in cancer prevention: Mechanism of action. J Nutr 134 (Suppl): 3479S-3485S, 2004.
2. Surh YJ: Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 3: 768-780, 2003.
3. Kornberg A and Baker TA: DNA replication. 2nd edition. W.D. Freeman and Co., New York, pp197-225, 1992.
4. Hubscher U, Maga G and Spadari S: Eukaryotic DNA polymerases. Annu Rev Biochem 71: 133-163, 2002.
5. Bebenek K and Kunkel TA: DNA repair and replication. In: Advances in Protein Chemistry. Yang W (ed). Elsevier, San Diego, pp137-165, 2004.
6. Lange SS, Takata K and Wood RD: DNA polymerases and cancer. Nat Rev Cancer 11: 96-110, 2011.
7. Loeb LA and Monnat RJ Jr: DNA polymerases and human disease. Nat Rev Genet 9: 594-604, 2008.
8. Wang JC: DNA topoisomerases. Annu Rev Biochem 65: 635-692, 1996.
9. Liu LF: DNA topoisomerase poisons as antitumor drugs. Annu Rev Biochem 58: 351-375, 1989.
10. Sakaguchi K, Sugawara F and Mizushina Y: Inhibitors of eukaryotic DNA polymerases. Seikagaku 74: 244-251, 2002 (In Japanese).
11. Berdis AJ: DNA polymerases as therapeutic targets. Biochemistry 47: 8253-8260, 2008.
12. Ji X, Avula B and Khan IA: Quantitative and qualitative determination of six xanthones in *Garcinia mangostana* L. by LC-PDA and LC-ESI-MS. J Pharm Biomed Anal 43: 1270-1276, 2007.
13. Wexler B: Mangosteen. Woodland Publishing, Utah, 2007.
14. Akao Y, Nakagawa Y, Iinuma M and Nozawa Y: Anti-cancer effects of xanthones from pericarps of mangosteen. Int J Mol Sci 9: 355-370, 2008.
15. Yoo JH, Kang K, Jho EH, Chin YW, Kim J and Nho CW: α- and γ-Mangostin inhibit the proliferation of colon cancer cells via β-catenin gene regulation in Wnt/cGMP signalling. Food Chem 129: 1559-1566, 2011.
16. Cui J, Hu W, Cai Z, Liu Y, Li S, Tao W and Xiang H: New medicinal properties of mangostins: Analgesic activity and pharmacological characterization of active ingredients from the fruit hull of *Garcinia mangostana* L. Pharmacol Biochem Behav 95: 166-172, 2010.
17. Jung HA, Su BN, Keller WJ, Mehta RG and Kinghorn AD: Antioxidant xanthones from the pericarp of *Garcinia mangostana* (Mangosteen). J Agric Food Chem 54: 2077-2082, 2006.
18. Chen LG, Yang LL and Wang CC: Anti-inflammatory activity of mangostins from *Garcinia mangostana*. Food Chem Toxicol 46: 688-693, 2008.
19. Nakatani K, Atsumi M, Arakawa T, Oosawa K, Shimura S, Nakahata N and Ohizumi Y: Inhibitions of histamine release and prostaglandin E2 synthesis by mangosteen, a Thai medicinal plant. Biol Pharm Bull 25: 1137-1141, 2002.
20. Sakagami Y, Iinuma M, Piyasena KG and Dharmaratne HR: Antibacterial activity of α-mangostin against vancomycin resistant Enterococci (VRE) and synergism with antibiotics. Phytomedicine 12: 203-208, 2005.
21. Suksamrarn S, Suwannapoch N, Phakhodee W, Thanuhiranlert J, Ratananukul P, Chimnoi N and Suksamrarn A: Antimycobacterial activity of prenylated xanthones from the fruits of *Garcinia mangostana*. Chem Pharm Bull (Tokyo) 51: 857-859, 2003.
22. Kaomongkolgit R, Jamdee K and Chaisomboon N: Antifungal activity of alpha-mangostin against *Candida albicans*. J Oral Sci 51: 401-406, 2009.
23. Chen SX, Wan M and Loh BN: Active constituents against HIV-1 protease from *Garcinia mangostana*. Planta Med 62: 381-382, 1996.
24. Devi Sampath P and Vijayaraghavan K: Cardioprotective effect of alpha-mangostin, a xanthone derivative from mangosteen on tissue defense system against isoproterenol-induced myocardial infarction in rats. J Biochem Mol Toxicol 21: 336-339, 2007.
25. Weecharangsan W, Opanasopit P, Sukma M, Ngawhirunpat T, Sotanaphun U and Siripong P: Antioxidative and neuroprotective activities of extracts from the fruit hull of mangosteen (*Garcinia mangostana* Linn.). Med Princ Pract 15: 281-287, 2006.
26. Tang YP, Li PG, Kondo M, Ji HP, Kou Y and Ou B: Effect of a mangosteen dietary supplement on human immune function: A randomized, double-blind, placebo-controlled trial. J Med Food 12: 755-763, 2009.
27. Ryu HW, Curtis-Long MJ, Jung S, Jin YM, Cho JK, Ryu YB, Lee WS and Park KH: Xanthones with neuraminidase inhibitory activity from the seedcases of *Garcinia mangostana*. Bioorg Med Chem 18: 6258-6264, 2010.
28. Ito C, Itoigawa M, Takakura T, Ruangrungsi N, Enjo F, Tokuda H, Nishino H and Furukawa H: Chemical constituents of *Garcinia fusca*: Structure elucidation of eight new xanthones and their cancer chemopreventive activity. J Nat Prod 66: 200-205, 2003.
29. Hano Y, Okamoto T, Suzuki K, Negishi M and Nomura T: Constituents of the Moraceae plants. 17. Components of the root bark of *Morus insignis* Bur. 3. Structures of three new isoprenylated xanthones morusignins I, J, and K and an isoprenylated flavone morusignin L. Heterocycles 36: 1359-1366, 1993.
30. Bennett GJ, Lee HH and Das NP: Biosynthesis of mangostin. Part 1. The origin of the xanthone skeleton. J Chem Soc, Perkin Trans 1 (10): 2671-2676, 1990.
31. Tamai K, Kojima K, Hanaichi T, Masaki S, Suzuki M, Umekawa H and Yoshida S: Structural study of immunoaffinity-purified DNA polymerase α-DNA primase complex from calf thymus. Biochim Biophys Acta 950: 263-273, 1988.

32. Date T, Yamaguchi M, Hirose F, Nishimoto Y, Tanihara K and Matsukage A: Expression of active rat DNA polymerase β in *Escherichia coli*. Biochemistry 27: 2983-2990, 1988.
33. Umeda S, Muta T, Ohsato T, Takamatsu C, Hamasaki N and Kang D: The D-loop structure of human mtDNA is destabilized directly by 1-methyl-4-phenylpyridinium ion (MPP^+), a parkinsonism-causing toxin. Eur J Biochem 267: 200-206, 2000.
34. Oshige M, Takeuchi R, Ruike T, Kuroda K and Sakaguchi K: Subunit protein-affinity isolation of *Drosophila* DNA polymerase catalytic subunit. Protein Expr Purif 35: 248-256, 2004.
35. Kusumoto R, Masutani C, Shimmyo S, Iwai S and Hanaoka F: DNA binding properties of human DNA polymerase *et al*: Implications for fidelity and polymerase switching of translesion synthesis. Genes Cells 9: 1139-1150, 2004.
36. Biertümpfel C, Zhao Y, Kondo Y, Ramón-Maiques S, Gregory M, Lee JY, Masutani C, Lehmann AR, Hanaoka F and Yang W: Structure and mechanism of human DNA polymerase eta. Nature 465: 1044-1048, 2010.
37. Ohashi E, Murakumo Y, Kanjo N, Akagi J, Masutani C, Hanaoka F and Ohmori H: Interaction of hREV1 with three human Y-family DNA polymerases. Genes Cells 9: 523-531, 2004.
38. Shimazaki N, Yoshida K, Kobayashi T, Toji S, Tamai K and Koiwai O: Over-expression of human DNA polymerase lambda in *E. coli* and characterization of the recombinant enzyme. Genes Cells 7: 639-651, 2002.
39. Mizushina Y, Tanaka N, Yagi H, Kurosawa T, Onoue M, Seto H, Horie T, Aoyagi N, Yamaoka M, Matsukage A, *et al*: Fatty acids selectively inhibit eukaryotic DNA polymerase activities in vitro. Biochim Biophys Acta 1308: 256-262, 1996.
40. Mizushina Y, Yoshida S, Matsukage A and Sakaguchi K: The inhibitory action of fatty acids on DNA polymerase β. Biochim Biophys Acta 1336: 509-521, 1997.
41. Ogawa A, Murate T, Suzuki M, Nimura Y and Yoshida S: Lithocholic acid, a putative tumor promoter, inhibits mammalian DNA polymerase β. Jpn J Cancer Res 89: 1154-1159, 1998.
42. Yonezawa Y, Tsuzuki T, Eitsuka T, Miyazawa T, Hada T, Uryu K, Murakami-Nakai C, Ikawa H, Kuriyama I, Takemura M, *et al*: Inhibitory effect of conjugated eicosapentaenoic acid on human DNA topoisomerases I and II. Arch Biochem Biophys 435: 197-206, 2005.
43. Nakayama C and Saneyoshi M: Inhibitory effects of 9-β-D-xylofuranosyladenine 5'-triphosphate on DNA-dependent RNA polymerase I and II from cherry salmon (*Oncorhynchus masou*). J Biochem 97: 1385-1389, 1985.
44. Soltis DA and Uhlenbeck OC: Isolation and characterization of two mutant forms of T4 polynucleotide kinase. J Biol Chem 257: 11332-11339, 1982.
45. Lu BC and Sakaguchi K: An endo-exonuclease from meiotic tissues of the basidiomycete *Coprinus cinereus*. Its purification and characterization. J Biol Chem 266: 21060-21066, 1991.
46. Mizushina Y, Murakami C, Ohta K, Takikawa H, Mori K, Yoshida H, Sugawara F and Sakaguchi K: Selective inhibition of the activities of both eukaryotic DNA polymerases and DNA topoisomerases by elenic acid. Biochem Pharmacol 63: 399-407, 2002.
47. Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y and Ueno K: A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol Pharm Bull 19: 1518-1520, 1996.
48. Mizushina Y: Specific inhibitors of mammalian DNA polymerase species. Biosci Biotechnol Biochem 73: 1239-1251, 2009.
49. Mizushina Y, Kamisuki S, Mizuno T, Takemura M, Asahara H, Linn S, Yamaguchi T, Matsukage A, Hanaoka F, Yoshida S, *et al*: Dehydroaltenusin, a mammalian DNA polymerase α inhibitor. J Biol Chem 275: 33957-33961, 2000.
50. Murakami-Nakai C, Maeda N, Yonezawa Y, Kuriyama I, Kamisuki S, Takahashi S, Sugawara F, Yoshida H, Sakaguchi K and Mizushina Y: The effects of dehydroaltenusin, a novel mammalian DNA polymerase α inhibitor, on cell proliferation and cell cycle progression. Biochim Biophys Acta 1674: 193-199, 2004.
51. Gatto B, Capranico G and Palumbo M: Drugs acting on DNA topoisomerases: Recent advances and future perspectives. Curr Pharm Des 5: 195-215, 1999.
52. Larsen AK, Escargueil AE and Skladanowski A: Catalytic topoisomerase II inhibitors in cancer therapy. Pharmacol Ther 99: 167-181, 2003.
53. Teicher BA: Next generation topoisomerase I inhibitors: Rationale and biomarker strategies. Biochem Pharmacol 75: 1262-1271, 2008.
54. Bailly C: Topoisomerase I poisons and suppressors as anticancer drugs. Curr Med Chem 7: 39-58, 2000.
55. Pommier Y, Leo E, Zhang H and Marchand C: DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. Chem Biol 17: 421-433, 2010.
56. Park JK, Lee JS, Lee HH, Choi IS and Park SD: Accumulation of polycyclic aromatic hydrocarbon-induced single strand breaks is attributed to slower rejoining processes by DNA polymerase inhibitor, cytosine arabinoside in CHO-K1 cells. Life Sci 48: 1255-1261, 1991.