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Ganoderic acid X, a lanostanoid triterpene, inhibits topoisomerases and induces apoptosis of cancer cells

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Abstract

Lanostanoid triterpenes isolated from *Ganoderma amboinense* were found to inhibit the growth of numerous cancer cell lines, and some of them inhibited the activities of topoisomerases I and IIα in vitro. Among the bioactive isolates, one of the most potent triterpene was identified to be 3α-hydroxy-15α-acetoxy-lanosta-7,9(11),24-trien-26-oic acid, ganoderic acid X (GAX). Treatment of human hepatoma HuH-7 cells with GAX caused immediate inhibition of DNA synthesis as well as activation of ERK and JNK mitogen-activated protein kinases, and cell apoptosis. Molecular events of apoptosis including degradation of chromosomal DNA, decrease in the level of Bcl-xL, the disruption of mitochondrial membrane, cytosolic release of cytochrome c and activation of caspase-3 were elucidated. The ability of GAX to inhibit topoisomerases and to sensitize the cancer cells toward apoptosis fulfills the feature of a potential anticancer drug.

Keywords: Ganoderma triterpene; Ganoderic acid X; Apoptosis; Topoisomerase inhibitor

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Introduction

Fruit bodies of ganoderma fungi have been used as folk medicine for thousands of years in the Far East. These medicinal fungi have attracted increasing attention as many ingredients such as polysaccharides, glycoproteins, triterpenes etc. are shown to be pharmacologically active (Shiao et al., 1994). Over a hundred ganoderma triterpenes have been isolated, and some of them were reported to mediate anticomplement activity (Min et al., 2001) or to influence the function of enzymes including β-glucuronidase (Kim et al., 1999), cholesterol synthase (Komoda et al., 1989), angiotension converting enzymes (Lee and Rhee, 1990) and phospholipases (Wang et al., 1994), thereby mediating hepato-protection, cholesterolstasis and antihypertension bioactivities. Several ganoderma triterpenes were reported to elicit cytotoxicity in cancer cell lines in yet unknown mechanism (Gao et al., 2002; Wu et al., 2001; Gan et al., 1998). The purpose of this study is to search for anticancer triterpenes from ganoderma fungi and to elucidate their anticancer mechanism in molecular level.

Some pentacyclic triterpenes from plant sources (Wada et al., 2001; Syrovets et al., 2000) were found to inhibit DNA topoisomerases, which are enzymes essential for the maintenance of chromatin structure, replication of DNA and mitosis/meiosis in eukaryotic cells. Topoisomerases mediate sequential breakage and religation of either one (topoisomerase I, Topo I) or both (topoisomerase II, Topo II) DNA strands, and strand passing associated with such breakage thereby alter the linking number of DNA. These topological changes are necessary for replication, transcription and repair of DNA to take place. Among different types of topoisomerases, Topo I and Topo IIα have been established as targets of many chemotherapeutic drugs in clinical usage (Li and Liu 2001). Inhibition of either enzyme can result aberrant mitosis in cancer cells and lead to mitotic catastrophe, which has been characterized as the main form of cell death caused by inhibitors of Topo I and Topo IIα (Roninson et al., 2001, Lock and Stribinskiene, 1996). In apoptosis-competent cells, the mitotic catastrophe frequently triggers signals commitment to apoptosis (Brantley-Finley et al., 2003; Lee et al., 2002).

We have previously reported that a triterpene enriched fraction from *Ganoderma lucidum* inhibits the growth of human hepatoma HuH-7 cells by modulating activities of protein kinase C and MAP kinases (Lin et al., 2003). In this study, triterpenes were isolated from the fruit bodies of *Ganoderma amboinense,* growth inhibitory effect of the triterpenes on cancer cells was studied. Molecular events triggered by ganoderic acid × (GAX), one of the most potent isolates, in the apoptosis of hepatoma HuH-7 cells are elucidated.

Materials and methods

Fruit bodies of *Ganoderma amboinense* were purchased from traditional Chinese medicine shop in Taipei, Taiwan, ROC during June 1999. Silica gel (MN Kiesel gel 60 M) was purchased from Macherey-Nagel Co. (Düren, Germany). HPLC grade methanol, n-hexane, ethyl acetate and acetonitrile were purchased from J. T. Baker (Phillipsburg, NJ). Human Topo I and Topo IIα were purchased from Amersham Pharmacia Biotech. (Uppsala, Sweden) and kinetoplast DNA (kDNA) was obtained from TopoGEN Inc. (Columbus, Ohio). Monoclonal antibodies for phosphorylated ERK, JNK and p38 were obtained from Cell Signaling (Beverly, MA). Polyclonal antibodies for detecting ERK, JNK, p38 and for Bcl-xL were purchased from Santa Crutz Biotech (Santa Crutz, CA). Antibodies for cytochrome c and

poly (ADP-ribose) polymerase (PARP) were purchased from BD Biosciences Clontech (Palo Alto, CA) and Upstate Biotechnology Inc. (Lake Placid, NY), respectively.

Isolation and characterization of anticancer triterpenes

Air-dried fruit bodies (100 g) of *G. amboinense* were threaded and extracted with one-liter methanol at 37 °C. The methanol extract was evaporated to dryness; about 10 g of crude methanol extract (ME) was obtained. ME was dissolved in methanol and subject to chromatography on a 4.2 × 56 cm column packed with 45 cm silica gel (MN Kiesel gel 60 M, Macherey-Nagel Co., Germany). The column was eluted sequentially with 400 mL of hexane/ethyl acetate (v/v = 1/1), hexane/ethyl acetate (1/2), ethyl acetate, ethyl acetate/methanol (1/1), and then methanol. Twelve fractions (200 mL/fraction) were collected and each of them was tested on HuH-7 cells for their drug efficacy. Fraction seven (about 0.06 g) was the most potent thus was separated further by another silica gel chromatography (2.7 cm × 45 cm column packed with 35 cm height of silica gel) eluted with hexane/ethyl acetate (1/3). Every 50 mL sub-fractions were collected. The fourth in ten sub-fractions shown the highest biological activity was subjected to reverse phase HPLC separation (Hypersphere C18 column, 250 × 4.6 mm, Thermo Hypersil-Keystone, Bellfonte, PA). The elution gradient was 50 to 75% acetonitrile in water. Peaks showing drug activity were isolated and characterized by atomic analysis, MALDI mass spectra and homo-nuclear and hetero-nuclear 2D NMR spectra analysis to determine the experimental formula and the structure. The purified triterpenes were also characterized by melting temperature and UV spectroscopy.

Assays for topoisomerase activity

The relaxation activities of Topo I and Topo IIα were determined by analyzing the conversion of supercoiled plasmid DNA into relaxed forms. The Topo I reaction was performed in 20 μL reaction buffer containing 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% BSA, 2 units Topo I and 400 ng supercoiled pRYG plasmid DNA. After incubation for 30 min at 37 °C, the reaction was stopped by adding 4 μL loading dye containing 7 mM EDTA, 1% SDS, 50% glycerol, and 0.02% each of bromophenol blue and xylene cyanol. The DNA was separated on 1% agarose gel at 50 mV for 2.5 h. The gels were then stained with ethidium bromide for 30 min and photographed using a gel-imaging system. The relaxation activity of Topo IIα was analyzed in the same manner, except that the reaction mixture contained 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL BSA, 1 mM ATP and 4 units Topo IIα. Decatenation of kDNA (TopoGEN Inc.) was performed in the same condition except that supercoiled plasmid DNA was substituted by 400 ng kDNA.

Effects of drugs on the growth of cell lines

Human hepatocellular carcinoma HuH-7 cells, colorectal carcinoma HCT-116 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM). Burkitt's lymphoma Raji cells and acute promyelocyte leukemia HL-60 cells were cultivated in RPMI-1641. The media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell lines were cultured at 37 °C in an atmosphere of humidified 5% CO_2. For drug efficacy experiments, the

cells were always seeded and cultured for 18 h before drug additions. After a 24-h incubation in the presence of the drug, cell viability was determined by ACP assay using the Abacus cell proliferation kit (BD Clontech, Palo Alto, CA), which measured cellular acid phosphatase activity (Lin et al., 2003, 2000). Cell viability as function of absorbance at 410 nm was plotted versus dosage of drug to obtain growth inhibition curves and IC_{50}.

Discontinuous drug exposure and clonogenic assay

HuH-7 cells were cultured in 10-cm dishes (1×10^6 cells in 6 mL DMEM) in the presence of 40 μM GAX for 24 h. The medium was removed and the cells were washed twice with PBS, trypsinized, resuspended in DMEM without the drug, and then subjected to clonogenic assay on two-layer soft agar plates (Wang et al., 2000). In the assay, 2 mL of 0.3% agar in DMEM containing 5×10^3 viable cells (indicated by the ability to exclude trypan blue) was plated over pre-formed base agar layers (3 mL, 0.8% agar in DMEM) in 3-cm petri dishes and cultured for 7 days. The colonies formed were inspected under an inverted microscope and photographed.

Assay for cellular DNA synthesis

HuH-7 cells were grown in 96-well plates (1×10^4 cells in 200 μL DMEM for each well) without GAX (control group) or in the presence of GAX for 0.5~3 h. Every 30 min, the medium was withdrawn from triplicate wells, the cells were washed with DMEM and then incubated with fresh DMEM containing 10% FBS and [^{3}H]-thymidine (2 μCi/well, Dupont NEN, Boston, MA). After a 4-h incubation, the cells were trypsinized and harvested on glass fiber filters using a Packard Filtermate 196 cell harvester (Downers Grove, IL). [^{3}H]-thymidine incorporated by cells and retained on the filters was measured in a Packard Direct Beta counter MATRIX™. The level of cellular DNA synthesis of each experimental group normalized to control group was calculated from the counts of [^{3}H]-thymidine.

Analysis of cell apoptosis by flow cytometry

HuH-7 cells were cultured in 6-cm petri dishes (2.5×10^4 cells in 3 mL DMEM for each well) and treated with 40 μM GAX for various time. For the measurement of sub-G1 cell populations, both adherent and floating cells were collected, washed with PBS, fixed in ice-cold absolute ethanol, treated with RNase, stained with propidium iodide (PI), and then analyzed by a flow cytometer (FACScan, Becton-Dickinson, San Jose, CA) as described previously (Lin et al., 2000). Cell Quest analysis software was used to quantify the sub-G1 cell population. For measurement of the mitochondrial membrane potential ($\Delta\psi m$), a mono-layer culture of HuH-7 cells was washed to remove drugs, incubated in DMEM containing 40 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, Molecular Probes, Eugene, OR) at 37 °C under 5% CO_2 for 15 min. The cells were then trypsinized, resuspended in 0.5 mL DMEM, and analyzed by flow cytometry. The fluorescence of mitochondrial membrane-bound $DiOC_6(3)$ was measured (Mathur et al., 2000) and the data were analyzed using the Cell Quest software. For the measurement of cell size and integrity of plasma membrane, the cells were washed with PBS (without Mg and Ca salt), incubated with PI (2 μg/mL in PBS) for 15 min at room temperature and then subjected to flow cytometric analysis for the forward scatter (representing cell size) and fluorescence of PI.

Western-blot analysis

For the analysis of ERK, JNK, p38, Bcl-xL and PARP, whole-cell lysates were prepared as described (Lin et al., 2003). For cytosolic cytochrome c analysis, cytosolic extracts (S-100) were prepared. Briefly, the cells were washed twice with PBS and lysed for 30 min in ice-cold lysis buffer containing 20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 1 mM PMSF. The suspensions were then centrifuged at 100,000 × g for 30 min; the supernatants obtained were stored at −80 °C until analysis. All lysates were subjected to BCA protein assay reagent (Pierce, Rockford, IL) for the quantification of protein concentration, and then Western blot analysis was performed using the ECL kit (NEN™ Life Science Products, Boston, MA) as previously described (Lin et al., 2003).

Caspase-3 activity assay

Whole-cell lysate of HuH-7 cells was prepared and caspase-3 activity was determined using the BD ApoAlert™ Caspase Colorimetric Assay Kit (BD Biosciences Clontech) according to the user manual. Briefly, the cell lysate (200 μg total protein) was incubated at 37 °C for 2 h in a reaction mixture (final volume 50 μL) containing DEVD-*p*NA. The liberated *p*NA was measured at 405 *nm* with an ELISA reader, and relative values of absorbance of GAX-treated groups to the control sample were calculated.

Results

Isolation and characteristics of GAX

The methanol extract (ME) of the fungi induced a dose-dependent decrease in cell viability of cancer cell lines including hepatoma HuH-7 cells, colorectal carcinoma HCT-116, Burkitt's lymphoma Raji cells and acute promyelocytic leukemia HL-60 cell with IC_{50} between 82.2 μg/mL ~ 135.3 μg/mL. The most marked inhibitory effect was observed in the HuH-7 cells (Table 1). Subsequently, efficacy in growth inhibition of the HuH-7 cells was used to guide the isolation of active components. After two runs of silica gel chromatography followed by a cycle of reverse phase HPLC, several bioactive peaks were obtained. The most potent isolate was from the fourth fraction of second silica gel chromatography, and was eluted at 34.2 min on HPLC by gradient 50~75% acetonitrile in water (Fig. 1). It showed an IC_{50} = 20.3 μg/mL (39.6 μM) for the HuH-7 cells and the structure was identified to be tetracyclic

Table 1
Effects of methanol extract (ME) and GAX on viability of cancer cells*

IC_{50} in μg/mL				
Cell line	HuH-7	HCT-116	Raji	HL60
ME	82.2	135.3	126.2	89.9
GAX	20.3	38.3	39.2	26.5

* ACP assay for cell viability was performed after 24 h of drug-treatment (n = 3). The cells were treated with 25~200 μg/mL of methanol extract or 5~50 μg/mL of GAX. Growth inhibition by the drug caused decreases in cell viability and reductions in $A_{410\ nm}$. The concentration for 50% growth inhibition (IC_{50}) was deduced from a dose-dependent inhibition curve.

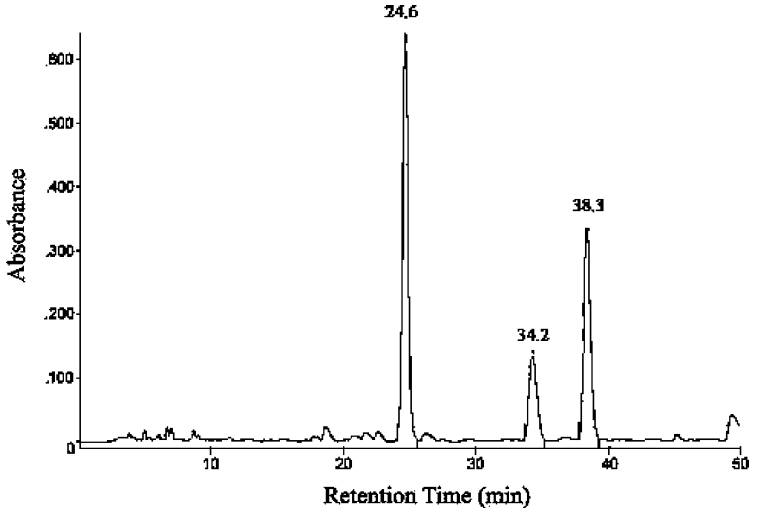

Fig. 1. HPLC chromatogram of the fourth sub-fraction eluted by 50~75% acetonitrile in water. Ganoderic acid X was eluted at 34.2 min.

lanostanoid GAX (Fig. 2). The purified GAX was obtained as a white powder. Mp 67–70 °C; UV (acetonitrile) λ_{max} (log ε) 218 (4.12), 234 (4.07), 243 (4.06), $\lambda_{shoulder}$ 251 nm; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.83 (1H, t, H-24), 5.46 (1H, m, H-7), 5.33 (1H, m, H-11), 5.05 (1H, t, H-15), 3.43 (1H, m, H-3), 2.30 and 2.07 (2H, m, H-12), 2.22 and 2.08 (2H, m, H-23), 2.10 and 1.70 (2H, m, H-16), 2.07 (3H, s, H-32), 1.99 (2H, m, H-6), 1.97 and 1.68 (2H, m, H-2), 1.82 (3H, s, H-27), 1.75 and 1.68 (2H, m, H-1), 1.67 (1H, t, H-17), 1.50 and 1.11 (2H, m, H-22), 1.48 (1H, t, H-5), 1.39 (1H, m, H-20), 1.00 (3H, s, H-28), 0.97 (6H, s, H-29 and H-19), 0.92 (3H, s, H-30), 0.90 (3H, d, H-21), 0.64 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 171.9 (C-26), 171.2 (C-31), 146.0 (C-9), 145.1 (C-24), 140.2 (C-8), 126.6 (C-25), 121.29 (C-7), 115.6 (C-11), 77.41 (C-15), 76.1 (C-3), 51.40 (C-14), 48.85 (C-17), 44.11 (C-13), 42.92 (C-5), 37.97 (C-12), 37.34 (C-10), 37.32 (C-4), 37.03 (C-16), 35.96 (C-20), 34.65 (C-22), 29.89 (C-1), 28.18 (C-29), 25.93 (C-23), 25.56 (C-2), 22.95 (C-6), 22.78 (C-30), 22.65 (C-19), 21.42 (C-32), 18.50 (C-28), 18.17 (C-21), 15.95 (C-18), 12.03 (C-27). EIMS m/z 512.72 $[M]^{+}$(calc. for $C_{32}H_{48}O_5$).

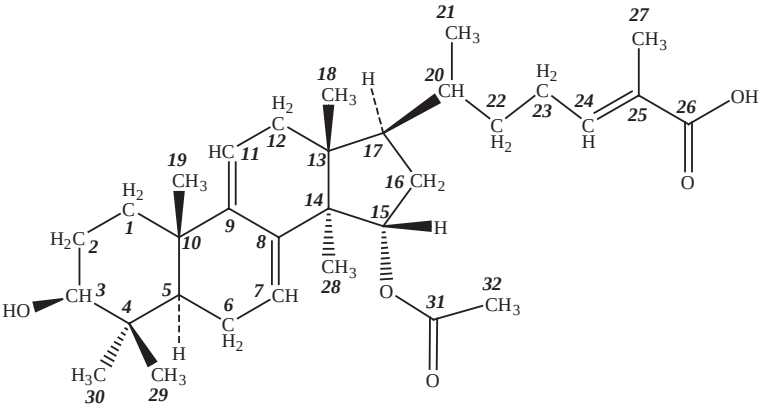

Fig. 2. Chemical structure of ganoderic acid X, 3α-hydroxy-15α-acetoxy-lanosta-7,9 (11),24-trien-26-oic acid.

Elemental analysis: C 74.0% (calc. 75.0%), H 9.40% (9.44%), O 16.6% (15.6%). The empirical formula for GAX is $C_{32}H_{48}O_5$ with a molecular weight of 512 Dalton. GAX is water-insoluble therefore is dissolved in methanol as a 20 mM stock solution. The solvent vehicle, up to 0.5%, exerts no effect on all the experiments.

Inhibition of Topo I and Topo IIα activities by GAX

Several triterpene isolates were found to inhibit relaxation activity of topoisomerases. The results of relaxation of supercoiled (SC) plasmid DNA by Topo I and Topo IIα as well as effects of GAX on these reactions were shown in Fig. 3A and B. Supercoiled plasmid was converted to relaxed forms (RLX) in a time-dependent manner by both enzymes. In the assay condition, 90% of supercoiled plasmid relaxation was achieved within 30 min by both enzymes (control). Dose-dependent inhibition of Topo I and Topo

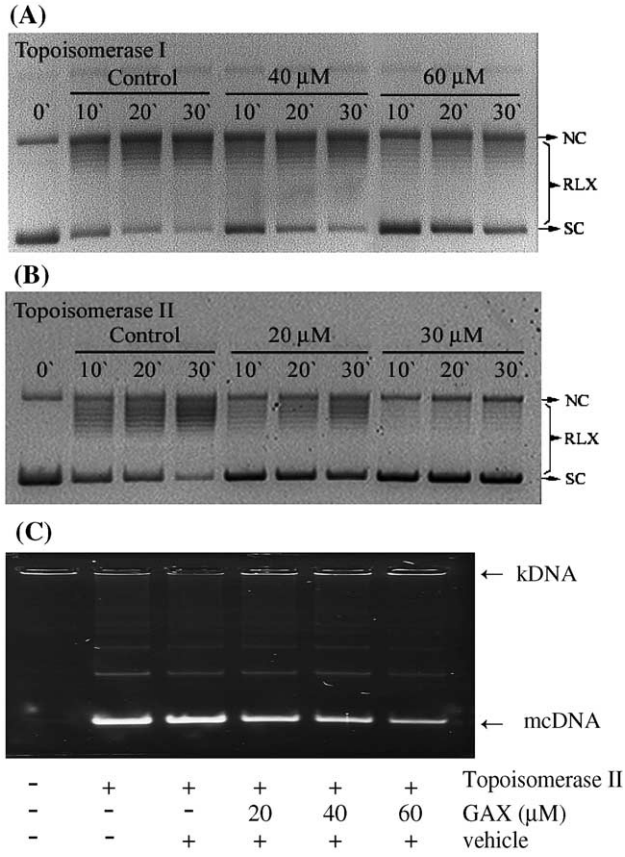

Fig. 3. Relaxation and decatenation of DNA by topoisomerases and effects of GAX. Relaxation reaction catalyzed by (A) Topo I and (B) Topo IIα. Nicked circular (NC), relaxed form (RLX) and supercoiled (SC) plasmid DNA were separated by agarose gel electrophoresis. (C) Decatenation of kinetoplast DNA (kDNA) by Topo IIα. As a massive network DNA, kDNA consisting thousands of catenated minicircle DNA (mcDNA) was too bulky to enter into matrix of agarose gel. Topo IIα catalyzed decatenation of kDNA to form mcDNA, which can migrate into the gel. The gels shown are representatives of at least three experiments that showed similar results.

IIα by GAX was shown at concentration 40~60 μM and 20~30 μM, respectively. As calculated from the reaction kinetics shown in Fig. 4A and B, IC_{50} of GAX was about 55 μM and 25 μM, respectively, for Topo I and Topo IIα. Inhibitory activity of GAX on Topo IIα was further confirmed in decatenation of kDNA. The activity of Topo IIα to decatenate massive network kDNA into minicircle DNA was also inhibited by GAX in a dose-dependent manner (Fig. 3C). In cultured HuH-7 cells, as shown in Fig. 4C, 40 μM GAX-treatment caused an immediate and time-dependent DNA synthesis inhibition; about one-third decrease in [^{3}H]-thymidine incorporation was observed within 30 min; the inhibition reached to two-thirds within 3 h. On the other hand, compounds from another HPLC peak, shown as control, that did not inhibit topoisomerases exerted no obvious effect on DNA synthesis.

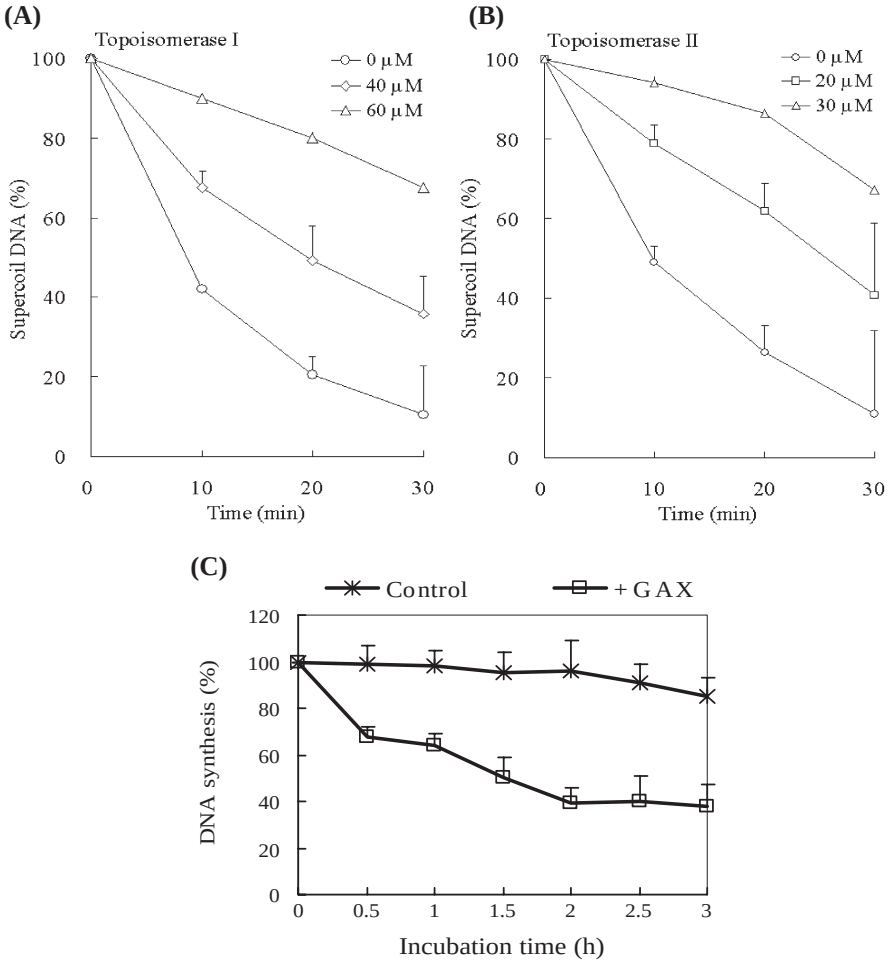

Fig. 4. Kinetic profiles of DNA relaxation reaction catalyzed by (A) Topo I and (B) Topo IIα, and effects of GAX on the reactions. Intensity of bands on each lane of Fig. 3A and B were quantified by a densitometer. The changes of SC form normalized to blank group (time = 0) were plotted. (C) Effect of GAX and a control triterpene fraction on DNA synthesis in HuH-7 cells. Cellular incorporation of [^{3}H]-thymidine was measured after incubation of the cells to the drug for the indicated time interval. The data presented were obtained from triplicate experiments.

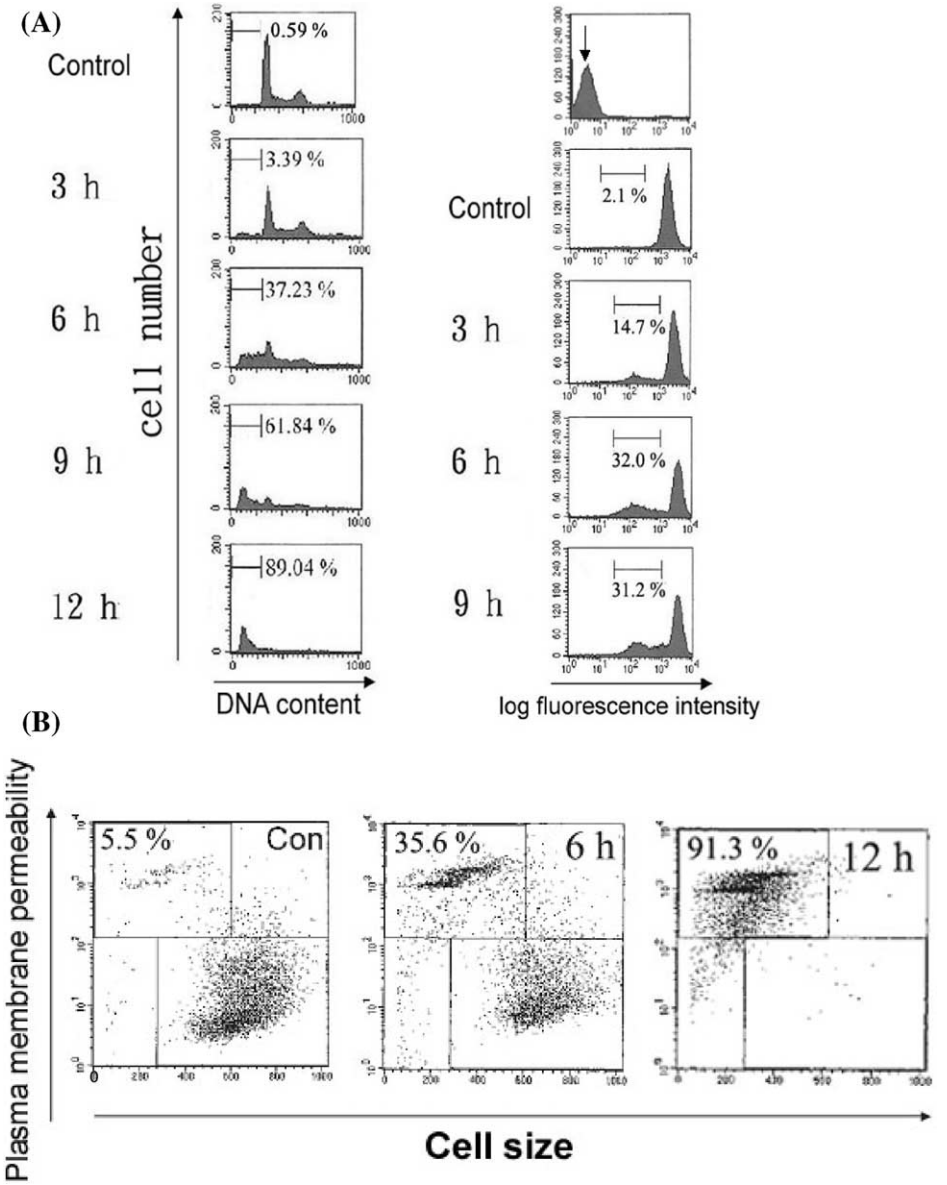

Fig. 5. Flow cytometric analysis of GAX-induced apoptosis. HuH-7 cells without drug treatment (Con) or treated with 40 μM GAX for the indicated time were processed as described in 'Methods' and then analyzed for (A) sub-G1 cell population (left panel) and integrity of mitochondrial membrane (right panel), and (B) shrunken cells and PI-permeable cells. In (A), percentage of sub-G1 cells or the percentage of cells with lower $DiOC_6(3)$ fluorescence is indicated in each histogram. The peak of auto-fluorescence of cells is indicated by an arrow. In (B), the number in each scatter plot shows the percentage of apoptotic cells. The figures shown are representative of three independent experiments with similar patterns.

Growth inhibition and induction of apoptosis in HuH-7 cells by GAX

HuH-7 cells treated with 40 μM GAX for 24 h decreased in number by about 50%. Interestingly, the treated cells lost tumorigenicity, which was shown by inability of anchorage-independent growth (Wang et al., 2000) and forming no colony on soft agar. While control cells without GAX treatment and the cells treated with the solvent vehicle grew to colonies on soft agar. The total number of colonies (with diameter >60 μm) in ten microscopic fields was 161 ± 16 and 170 ± 21 for the control and the vehicle-treated groups, respectively. This result suggests that GAX efficiently triggers the death pathway. As shown in Fig. 5A-left panel, flow cytometric analysis of HuH-7 cells treated with GAX showed a cell population with sub-G1 DNA content. The sub-G1 cell populations accounted for 37%, 62% and 89%, respectively, for the 6-, 9-and 12-h GAX-treated groups, while an insignificant amount was detected in the control and vehicle-treated groups. With $DiOC_6$(3)-staining (Fig. 5A-right panel), cell populations with suppressed fluorescence were increased in GAX-treated groups. These are the cells losing integrity of mitochondrial membrane (Mathur et al., 2000), accounting for 2%, 15%, 32% and 31%, respectively, for the control and the 3-, 6-and 9-h treatment groups. GAX treatment also caused cell shrinkage, one of

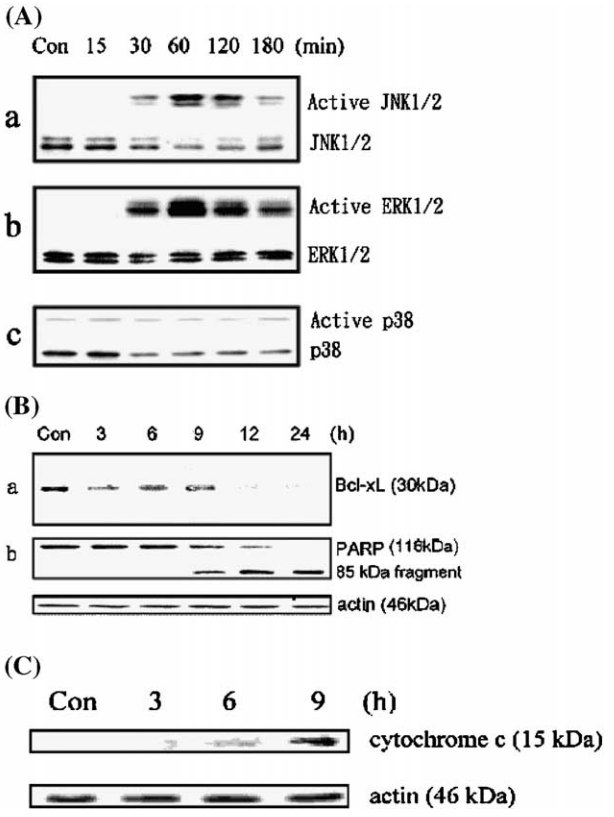

Fig. 6. Molecular responses in HuH-7 cells treated with GAX. HuH-7 cells without drug treatment (Con) or treated with 40 μM GAX for the indicated time. Western blot analysis of (A) protein level and phosphorylated (active) forms of JNK, ERK and p38 MAP kinases, (B) Bcl-xL protein, PARP and its degradation product (85 kDa) in whole cell lysates, and (C) cytochrome c in cytosolic extract. Actin was used as a loading control in each experiment.

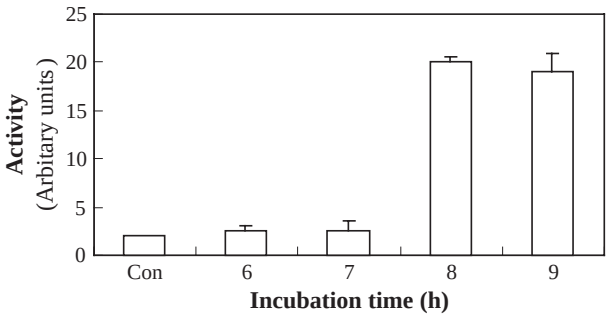

Fig. 7. Caspase-3 activity in HuH-7 cells treated with GAX. Whole cell lysates were prepared from HuH-7 cells without drug treatment (Con) or the cells incubated with 40 μM GAX for the indicated time interval. Caspase-3 activity in the lysates was determined. The data presented were obtained from triplicate experiments.

the characteristics of apoptotic cells (Bortner and Cidlowski, 1998). As shown in Fig. 5B, The results from flow cytometric analysis showed that the majorities of the shrunken cells were permeable to PI and appeared in the upper left section of each scatter plot. The amounts of these cells were 5.5%, 35.6% and 91.3%, respectively, for the control and the 6-and 12-h treatment groups. Taken together, our data suggest that GAX induces apoptotic cell death in the HuH-7 cells, and the mitochondrial pathway is likely involved (Parone et al., 2002).

Molecular events responding to GAX-treatment

GAX-treatment caused a prompt activation of ERK and JNK, as indicated by increases in the levels of the phosphorylated (active) forms of JNK and ERK, but not the JNK and ERK protein levels. On the other hand, the protein level and the activation status of p38 were not affected (Fig. 6A-a, b and c). Activations of JNK and ERK were observed within 30 min of GAX-treatment; they reached the maximum at 60 min and were sustained for at least 3 h. As to the involvement of apoptosis effectors, we found decreases of anti-apoptotic protein Bcl-xL (Fig. 6B-a) and increases of cytosolic cytochrome c (Fig. 6C). Both changes were seen within 3 h and became obvious after 9 h of GAX-treatment. Bcl-xL, like Bcl-2, is a member of the anti-apoptotic Bcl-2 family proteins. It has been found that the HuH-7 cells express Bcl-xL but not Bcl-2 protein (Takehara et al., 2001, unpublished data). It appears that Bcl-xL protects HuH-7 cells from apoptosis by inhibiting cytosolic release of cytochrome c (Kharbanda et al., 1997). GAX treatment caused the decrease in the level of Bcl-xL and loss of $\Delta\psi m$, thus cytochrome c was released and subsequently caspases were activated. About ten-fold increase in the activity of caspase-3, the downstream execution ercaspase, was detected after 8~9 h GAX-treatment (Fig. 7). Cleavage of PARP, a known substrate for caspase-3, was observed at the same time periods (Fig. 6B-b). As activation of caspase-3 is an irreversible commitment to cell death (Earnshaw et al., 1999), apoptosis of GAX-treated HuH-7 cells is thus confirmed.

Discussion

There are varieties of triterpenes present ubiquitously in plant kingdom. We have found that methanol extract (ME) of *Ganoderma amboinense* contains less than 1% GAX, but its efficacy of

growth inhibition on HuH-7 cells is about 25% of GAX (Table 1). This observation implies that GAX is not the sole anti-proliferative ingredient in ME. In fact, we found other triterpene isolates possessing similar anti-proliferative and topoisomerase inhibitory activities. Tetracyclic triterpenes seem to be common secondary metabolites of ganoderma fungi as GAX and its isomers and other lanostanoids have been isolated from *Ganoderma lucidum* (Lin et al., 1988) and from *Ganoderma tsugae* (Gan et al., 1998). In previous study, GAX and its stereo-or positional isomers were found to mediate activation of human platelets via complex signaling pathways involving the activation of phospholipases C and A2 (Wang et al., 1994). In this report, the anticancer activity of GAX and the underlying mechanism are elucidated for the first time. We demonstrate that GAX promptly inhibits DNA synthesis in the HuH-7 cancer cells likely due to inhibition of topoisomerases. Topo IIα is possibly the crucial target for the death of HuH-7 cells as inferred from the findings that cultured tumor cells can survive without much Topo I (Madelaine et al., 1993) and that the former enzyme seems able to functionally compensate the later (Sugimoto et al., 1990). It appears that mitotic catastrophe aroused by inhibition of Topo IIα provokes cell guardians sensing the stress and activating stress-associated signaling as well as apoptotic pathways (Earnshaw et al., 1999; Kaufmann, 1998). Among the distinct stress activated MAP kinases, JNK, ERK and p38 (Schaeffer and Weber, 1999), we find an early activation of JNK and ERK with a more potent and sustained activation of ERK in apoptosis of GAX-treated hepatoma HuH-7 cells, As compared to other topoisomerase targeting drugs, doxorubicin and etoposide induce apoptotic cell death in KB-3 carcinoma cells by activating only JNK (Brantley-Finley et al., 2003), and camptothecin induces death in gastric cancer cells by activating ERK, JNK, and p38 (Lee et al., 2002). Different topoisomerase inhibitors seem to function through diverse MAP kinases to trigger apoptosis. GAX inhibits the activities of topoisomerases, deregulates MAP kinase signaling, and induces cell apoptosis; its anticancer potency is thus expectable. The bioactivity of GAX may also account for the chemopreventive efficacy of ganoderma fungi. It has been indicated that chemically and virally transformed cells contain higher levels of topoisomerases (Crespi et al., 1988). There might be transformed cells of these types caused by food or environmental contaminants. The triterpenes may correct growth abnormality of these cells by suppressing activities of topoisomerases. The inhibition mechanism of topoisomerases by GAX is next issue to be investigated. As structure feature of GAX sounds similar to some Michael reaction acceptors, which inactivate enzymes by reacting with sulfhydryl (SH) group of proteins (Liu and Hanzlik 1992; Dinkova-Kostova et al., 2001), and Topo IIα is a SH-dependent enzyme therefore it is not ruled out that GAX acts as a Michael acceptor. On the other hand, the capability of inhibition on Topo I, which is not a SH-dependent enzyme, implicates that other mechanisms, such as DNA intercalating, protein interaction or topoisomerase poisoning (Pommier et al., 1998; Burden and Osheroff, 1998) maybe involved in drug activity of GAX.

Fruit bodies of the fungi used to be collected from forest where it grows parasitically on trees. Nowadays cultivation of ganoderma in the farms becomes popular. Mass production of these medicinal fungi in the farms can be a constant source of raw material for isolation of the anticancer triterpenes. Further research and development of ganoderma triterpenes towards anticancer drug is achievable.

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