JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Lucidenic Acid B Induces Apoptosis in Human Leukemia Cells via a Mitochondria-Mediated Pathway

CHIN-LIN HSU, YU-SHAN YU, AND GOW-CHIN YEN*

Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan

Ganoderma lucidum is known as a medicinal mushroom used in traditional Chinese medicine. In the present study, the effect of lucidenic acids (A, B, C, and N) isolated from a new *G. lucidum* (YK-02) on induction of cell apoptosis and the apoptotic pathway in HL-60 cells were investigated. The results demonstrated that lucidenic acids decreased cell population growth of HL-60 cells, assessed with the MTT assay. The cell cycle assay indicated that treatment of HL-60 cells with lucidenic acid A, C, and N caused cell cycle arrest in the G_1 phase. Lucidenic acid B (LAB) did not affect the cell cycle profile; however, it increased the number of early and late apoptotic cells but not necrotic cells. Treatment of HL-60 cells with LAB caused loss of mitochondria membrane potential. Moreover, the ratio of expression levels of pro- and antiapoptotic Bcl-2 family members was changed by LAB treatment. LAB-induced apoptosis involved release of mitochondria cytochrome *c* and subsequently induced the activation of caspase-9 and caspase-3, which were followed by cleavage of poly(ADP-ribose) polymerase (PARP). Pretreatment with a general caspase-9 inhibitor (Z-LEHD-FMK) and caspase-3 inhibitor (Z-DEVD-FMK) prevented LAB from inhibiting cell viability in HL-60 cells. Our finding may be critical to the chemopreventive potential of lucidenic acid B.

KEYWORDS: ***Ganoderma lucidum*; lucidenic acid; HL-60 cells; apoptosis**

INTRODUCTION

Ganoderma lucidum is known as a medicinal mushroom used in traditional Chinese medicine. It has been considered to be a panacea used traditionally in many Asian countries for the prevention and treatment of various diseases (*1*). Many studies have indicated that the extracts of *G. lucidum* have antitumor, anti-inflammatory, hepatoprotective, antivirus, and antioxidative activities (*2–6*). This mushroom contains a large variety of biologically active polysaccharides and triterpenes. Some reports indicated that lucidenic acids (A, C, D_2, E_2, F, O, P, Q, and N) were isolated from *G. lucidum* (*7–9*). Ganoderic acids T and X isolated from *G. lucidum* and *G. amboinense* are reported to inhibit cell growth and induce apoptosis in many cancer cells, such as HuH-7 cells, HCT-116 cells, Raji cells, HL-60 cells, and lung cancer cells (*10, 11*). Our previous study indicated that lucidenic acids isolated from *G. lucidum* (YK-02) are anti-invasive bioactive components on HepG2 cells (*12*). We also demonstrated that the anti-invasive effect of the lucidenic acid B on the PMA-induced HepG2 cells might be via inhibiting the phosphorylation of ERK1/2 and reducing AP-1 and NF-κB DNA binding activities, leading to down-regulation of MMP-9 expression (*13*).

Cell death under physiological conditions usually occurs via apoptosis. Apoptosis is characterized by activation of the caspases family of cysteine proteases followed by caspase-mediated specific morphological changes including cell shrinkage, chromatin condensation, nuclear DNA fragmentation, membrane blebbing, and breakdown of the cell into apoptotic bodies (*14*). It may be initiated through an intrinsic pathway including release of apoptotic signals from the mitochondria (*15*). Cory and Adams (*16*) indicated that mitochondrial release of cytochrome *c* can be controlled by the Bcl-2 family of proteins and may be activated by proteolytic cleavage and heterodimerization. Green and Reed (*17*) indicated that disruption of the mitochondrial membrane potential is defined as an early stage of apoptosis, where release of cytochrome *c* from the mitochondria is followed by caspase-3/caspase-9 cascade activation. However, the literature regarding the effect of lucidenic acids on cell population growth and apoptosis in human acute promyelocytic leukemia cells (HL-60 cells) remains unclear.

In the present study, we investigated the effects of lucidenic acids on cell growth inhibition and apoptosis in HL-60 cells. We discovered that lucidenic acid B decreased the cell viability of some cancer cell lines and induced apoptosis in the HL-60 cells. The results demonstrate the lucidenic acid B-induced apoptosis in HL-60 cells through the mitochondria pathway.

* To whom correspondence should be addressed. Phone: 886-4-22879755. Fax: 886-4-22854378. E-mail: gcyen@nchu.edu.tw.

10.1021/jf800006u CCC: $40.75 © 2008 American Chemical Society
Published on Web 05/16/2008

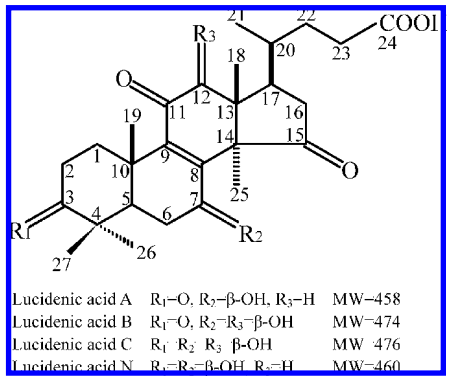

Figure 1. Chemical structures of lucidenic acids.

MATERIALS AND METHODS

Chemicals. Lucidenic acids (A, B, C, and N) were obtained from the Biotechnology Research and Development Institute (Double Crane Group, Tainan Hsien, Taiwan), which were isolated and identified from the fruiting body of a new strain of *G. lucidum* (YK-02) (*12*). MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], propidium iodide (PI), ribonuclease (RNase), DAPI (4,6-diamidino-2-phenylindole dihydrochloride), and sodium bicarbonate were purchased from Sigma Chemical (St. Louis, MO). Dimethylsulfoxide (DMSO) was purchased from Merck Co. (Darmstadt, Germany). Iscove's modified Dulbecco's medium, minimum essential medium (Eagle), McCoy's 5a medium, RPMI 1640 medium, fetal bovine serum, L-glutamine, nonessential amino acids, sodium pyruvate, and antibiotic mixture (penicillin−streptomycin) were purchased from the Invitrogen Co. (Carlsbad, CA). Anticaspase-3, anticaspase-9, anti-PARP, anti-Bcl-2, anti-Bcl-X_L, anti-Bax, anti-Bad, anti-Bak, and anti-β-Actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-cytochrome *c* was obtained from BioVision (Mountain View, CA). Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). Antirabbit and antimouse secondary horseradish peroxidase antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Polyvinyldifluoride (PVDF) membrane for Western blotting was obtained from Millipore (Bedford, MA). All other chemicals were reagent grade.

Culture. The human acute promyelocytic leukemia cells (HL-60 cells), human hepatoblastoma cell (HepG2 cells), and human colorectal carcinoma cells (HCT 116 cells) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). Human colorectal carcinoma cells (COLO 205 cells and HT-29 cells) were provided by Dr. Min-Hsiung Pan (National Kaohsiung Marine University, Kaohsiung, Taiwan). HL-60 cells were grown in Iscove's modified Dulbecco's medium and 20% fetal bovine serum supplemented with 1.5 g/L sodium bicarbonate and 100 U/mL penicillin−streptomycin. HepG2 cells were grown in 90% minimum essential medium (Eagle) and 10% fetal bovine serum supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate. HCT 116 cells were grown in 90% McCoy's 5a medium and 10% fetal bovine serum supplemented with 1.5 mM L-glutamine. COLO 205 cells and HT-29 cells were grown in RPMI 1640 medium and 10% fetal bovine serum. Human peripheral blood lymphocytes were obtained from healthy male donors and separated by the Ficoll-Paque premium (GE Healthcare, Uppsala, Sweden). The cell culture condition was 37 °C in humidified 5% CO_2 incubator.

MTT Assay. The MTT assay was performed according to the method of Mosmann (*18*). The human cancer cells and human peripheral blood lymphocytes were plated into 96-well microtiter plates at a density of 1×10^4 cells/well. After 24 h, culture medium was replaced by 200 μL serial dilutions (0, 5, 10, 25, 50, 100, 250, and 500 μM) of lucidenic acids (A, B, C, and N), and the cells were incubated for 24, 48, and 72 h. The final concentration of solvent was less than 0.1% in cell culture medium. Culture solutions were removed and replaced by 90 μL culture medium. Ten microliters of sterile filtered MTT solution (5 mg/mL) in phosphate-buffered saline (PBS, pH = 7.4) was added to each well reaching a final concentration of 0.5 mg MTT/mL. After 5 h, the unreacted dye was removed and then the insoluble formazan crystals were dissolved in 200 μL/well dimethyl sulfoxide and measured spectrophotometrically in a FLUOstar galaxy spectrophotometer (BMG Labtechnologies Ltd., Offenburg, Germany) at 570 nm. The relative cell viability (%) related to control wells containing cell culture medium without lucidenic acids were calculated by $A_{570\ nm}$(lucidenic acids)/$A_{570\ nm}$(control) × 100. The IC_{50} was calculated as lucidenic acid concentration, which inhibits growth of 50% of cells relative to untreated control cells.

Table 1. Effect of Lucidenic Acids on the Growth of Various Human Cancer Cells

	lucidenic acids IC_{50} (μM)			
cell line	A	B	C	N
COLO 205	154 ± 27	249 ± 38	195 ± 13	486 ± 26
HCT 116	428 ± 33	*a*		
HepG2	183 ± 4.3	112 ± 12	193 ± 17	230 ± 15
HL-60	61.0 ± 5.5	19.3 ± 1.4	45.0 ± 2.1	64.5 ± 1.7
HT-29		382 ± 21		

[a] The IC_{50} value was over 500 μM. Cells were treated with 0−500 μM lucidenic acids for 72 h. Reported values are the means ± SD (n = 3).

Analyses of Cell Cycle and Cell Apoptosis (PI Staining Method) by Flow Cytometry. The HL-60 human leukemic cells were plated into 100 mm culture dish at a density of 5×10^6 cells/dish. The HL-60 human leukemic cells stimulated with 0−100 μM lucidenic acids for 24, 48, and 72 h were assayed for cell cycle progression and/or apoptosis by the PI staining method (*19*). Briefly, cells were harvested, washed with PBS twice, and fixed in 80% ethanol at 4 °C for 30 min, followed by incubation with 100 μg/mL of RNase for 30 min at 37 °C. The cells were then stained with 40 μg/mL of PI for 15 min at room temperature and subjected to flow cytometric analysis of DNA content using a FACScan flow cytometry (Becton-Dickinson Immunocytometery Systems, San Jose, CA). Approximately 1×10^4 counts were made for each sample. The percentage of distribution of cell cycle phase and apoptosis were calculated by CELL Quest software. The nuclear morphology of cells was examined by fluorescence microscopy (Olympus, Tokyo, Japan).

Nuclear Staining with DAPI. Apoptosis was evaluated by staining with DAPI. The HL-60 human leukemic cells were plated into 100 mm culture dish at a density of 5×10^6 cells/dish. The HL-60 cells were stimulated with 0−100 μM lucidenic acid B for 24, 48, and 72 h. For this purpose, cells were fixed with 4% paraformaldehyde for 30 min and incubated with 1 μg/mL DAPI solution for 30 min in the dark condition. The nuclear morphology of cells was examined by fluorescence microscopy (Olympus, Tokyo, Japan). Typical apoptotic changes involved condensation of chromatin, its compaction along the periphery of the nucleus, and segmentation of the nucleus. The rate of apoptosis was determined as the percentage of apoptotic nuclei.

Annexin V-FITC/PI Assay. Annexin V-FITC/PI assay was performed using Annexin V-FITC Kit (ANNEX100F, SEROTEC, U.K.) according to the instructions of manufacturer. Briefly, 1×10^6 cells were plated in a 6 cm dish. Cells were treated with 0−100 μM lucidenic acid B for 72 h. The cells were labeled with Annexin V-FITC/PI according to the instructions of manufacturer. The Annexin V-FITC−/PI− population was regarded as normal healthy cells, while Annexin V-FITC+/PI− cells were taken as a measure of early apoptosis, Annexin V-FITC+/PI+ as late apoptosis, and Annexin V-FITC-/PI+ as necrosis. About 1×10^4 events were acquired and analyzed using a CELL Quest software.

Mitochondria Membrane Potential ($\Delta\Psi$m) Assay. Mitochondria membrane potential was determined using the MitoPT 100 Test Kit (Immunochemistry Technologies, Bloomington, MN). Cells were seeded in 12-well plates (1×10^5 cells/well). After 24 h, the cells were treated with 0−100 μM lucidenic acid B for 3, 6, and 12 h. Routine passage consisted of rinsing cells in 12-well plates once with phosphate-buffered saline followed by harvesting and thorough dispersion. Aliquots of the resultant cell suspensions were placed in eppendorf of 1×10^6 cells per eppendorf containing 1 mL of culture medium. After centrifugation, cells were incubated with 10 μg/mL of JC-1 at 37 °C

for 15 min in humidified 5% CO_2 incubator. Cells were collected and washed with 1 × assay buffer (MitoPT 100 Test kit). Cells were resuspended in an adequate amount of the same solution (0.5 mL) and analyzed by a FLUOstar galaxy fluorescence plate reader with an excitation wavelength of 485 nm and emission wavelength at 590 nm for red fluorescence. Apoptotic cells will generate a lower reading of red fluorescence, and the changes in the mitochondria membrane potential ($\Delta\Psi m$) can be most accurately assessed by comparing the red fluorescence of untreated and treated with lucidenic acid B. The cell morphology was examined by fluorescence microscopy (Olympus, Tokyo, Japan).

Western Blot Analysis and Measurement of Caspase-3 and -9 Activity. The HL-60 cells (1×10^7 cells/10 cm dish) were treated with 0−25 μM lucidenic acid B for 3 and 6 h. Cells were collected and lysed in ice-cold lysis buffer (20 mM tris-HCl (pH 7.4), 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin, and 1 mM PMSF). The Bcl-2, Bax, Bak, Bad, Bcl-X_L, caspase-9, caspase-3, and PARP proteins were assessed in HL-60 cells. The release of mitochondrial cytochrome *c* was determined by Western blotting. After the various treatments, the cytosolic fraction of cells was isolated using the Mitochondria/Cytosol Fractionation kit (BioVision, Mountain View, CA) as specified by the manufacturer. The protein concentration of the extracts was estimated with the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard. Total proteins (50−60 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel. The proteins in the gel were transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in PBST (0.05% v/v Tween-20 in PBS, pH 7.2) for 1 h. Membranes were incubated with primary

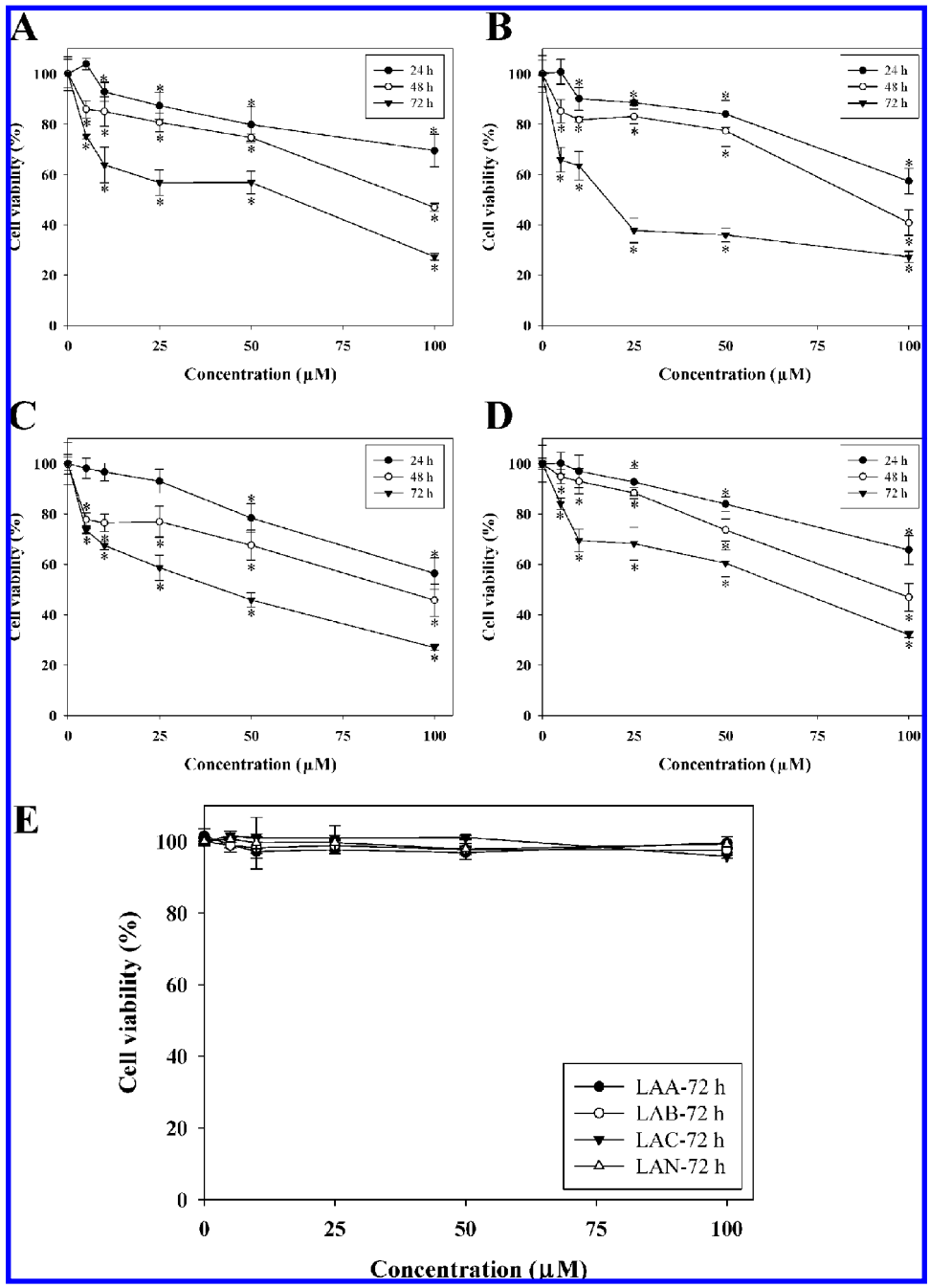

Figure 2. Effect of lucidenic acids on cell viability in HL-60 cells and normal peripheral blood lymphocytes. HL-60 cells were treated with 0−100 μM lucidenic acid A (**A**), lucidenic acid B (**B**), lucidenic acid C (**C**), and lucidenic acid N (**D**) for 24, 48, and 72 h. Normal peripheral blood lymphocytes were treated with 0−100 μM lucidenic acids (**E**) for 72 h. Reported values are the means ± SD ($n = 3$). *, $p < 0.05$ is significantly different from that of the control.

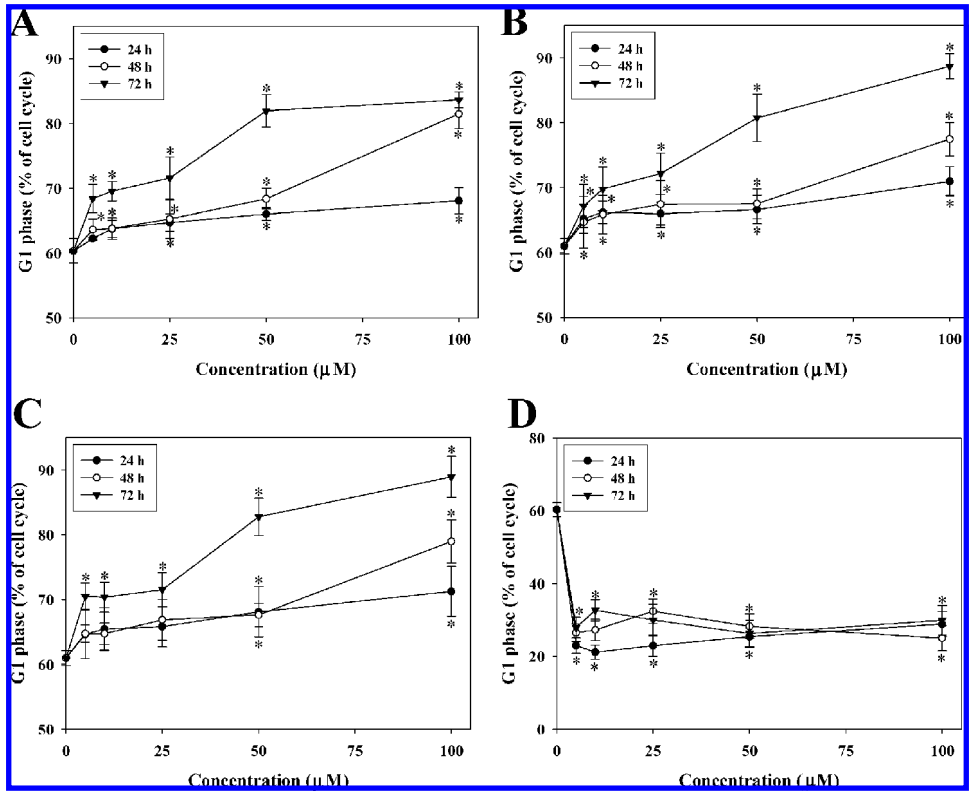

Figure 3. Effect of lucidenic acids-mediated G_1 phase of cell cycle distribution in HL-60 cells. Cells were treated with 0−100 μM lucidenic acid A (**A**), lucidenic acid C (**B**), lucidenic acid N (**C**), and lucidenic acid B (**D**) for 24, 48, and 72 h. Reported values are the means ± SD ($n = 3$). *, $p < 0.05$ is significantly different from that of the control.

antibody (1:5000) at 4 °C overnight and then with secondary antibody (1:5000) for 1 h. Membranes were washed three times in PBST for 8 min between each step. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). Relative protein expression was quantified densitometrically using the LabWorks 4.5 software and calculated according to the β-Actin reference bands. Caspase-3 and caspase-9 activities were assayed using commercial kits as specified by the manufacturers (Upstate Biotechnology, Lake Placid, NY; BioVision, Mountain View, CA). Fluorescence was measured with a FLUOstar galaxy fluorescence plate reader (BMG Labtechnologies Ltd., Offenburg, Germany).

Statistical Analysis. Differences between variants were analyzed by one-way analysis of variance using the Statistical Analysis System. Values of $p < 0.05$ were considered to be statistically significant. Each experiment was performed in triplicate.

RESULTS

Effect of Lucidenic Acids on Cell Population Growth. The chemical structures of lucidenic acids tested in the present study are shown in **Figure 1**. The inhibitory effects of lucidenic acids on cell population growth in cancer cells were determined by MTT assay. As shown in **Table 1**, lucidenic acids had the strongest inhibitory effect on HL-60 cell growth. The effect of lucidenic acids (A, B, C, and N) on cell population growth is shown in **Figure 2**. The results showed that addition of lucidenic acids A, B, C, and N to the growth medium decreased the cell population growth of HL-60 cells. The IC_{50} (inhibits growth of 50%) values of lucidenic acids A, B, C, and N on HL-60 cells were 61.0, 19.3, 45.0, and 64.5 μM, respectively. Moreover, lucidenic acids A, B, C, and N did not affect the survival of normal peripheral blood lymphocytes (**Figure 2E**).

Lucidenic Acids-Induced Cell Cycle Arrest and Apoptosis. Inhibition of cell population growth might be mediated through cell cycle arrest or cell cytotoxic effect. Cell cycle analysis of HL-60 cells was performed by flow cytometry after being treated with 0−100 μM lucidenic acids for 24−72 h. As shown in **Figure 3A**, lucidenic acid A resulted in a significant ($p < 0.05$) increase in cell population in the G1 phase from 60.3% (control) to 83.6% (100 μM) at 24−72 h. The exposure of HL-60 cells to lucidenic acid C resulted in a significant ($p < 0.05$) increase in the population of cells in the G1 phase from 61.0% (control) to 88.7% (100 μM) at 24−72 h (**Figure 3B**). In lucidenic acid N treated cells, a significant ($p < 0.05$) increase in the number of cells in the G1 phase from 61.0% (control) to 88.9% (100 μM) at 24−72 h was observed (**Figure 3C**). However, the presence of lucidenic acid B in the medium did not lead to cell cycle arrest at the G1 phase (**Figure 3D**). These results indicated that lucidenic acids A, C, and N led to cell cycle arrest at the G1 phase in a time- and dose-dependent manner. Addition of lucidenic acid B to HL-60 cells resulted in a markedly increased accumulation of the sub-G1 phase (apoptotic cells) in a time- and dose-dependent manner (**Figure 4A**). The nuclear morphology of untreated and treated cells is shown in **Figure 4B** by DAPI staining. DAPI staining revealed that apoptotic bodies appeared when the cells were treated with 50 μM lucidenic acid B for 24 h. Lucidenic acid B-mediated cell apoptosis of HL-60 cells indicated that the increase of apoptotic cells occurred in a time- and dose-dependent manner. **Figure 4C** shows the effect of lucidenic acid B on percentage of normal, early apoptotic, late apoptotic, and necrotic cells in HL-60 cells. The results indicated that treatment with 0−100 μM lucidenic acid B decreased the number of HL-60 cells in a dose-dependent manner. The apoptotic cells including early apoptotic (Annexin V-FITC+/PI−) and late apoptotic cells (Annexin V-FITC+/PI+) were increased in a dose-dependent manner.

Lucidenic Acid B Induces Apoptosis via a Mitochondrial-Mediated Pathway. Alterations in mitochondria function have been shown to play a crucial role in apoptosis, and thus, the effect of lucidenic acid B on $\Delta\Psi$m was investigated. Cell

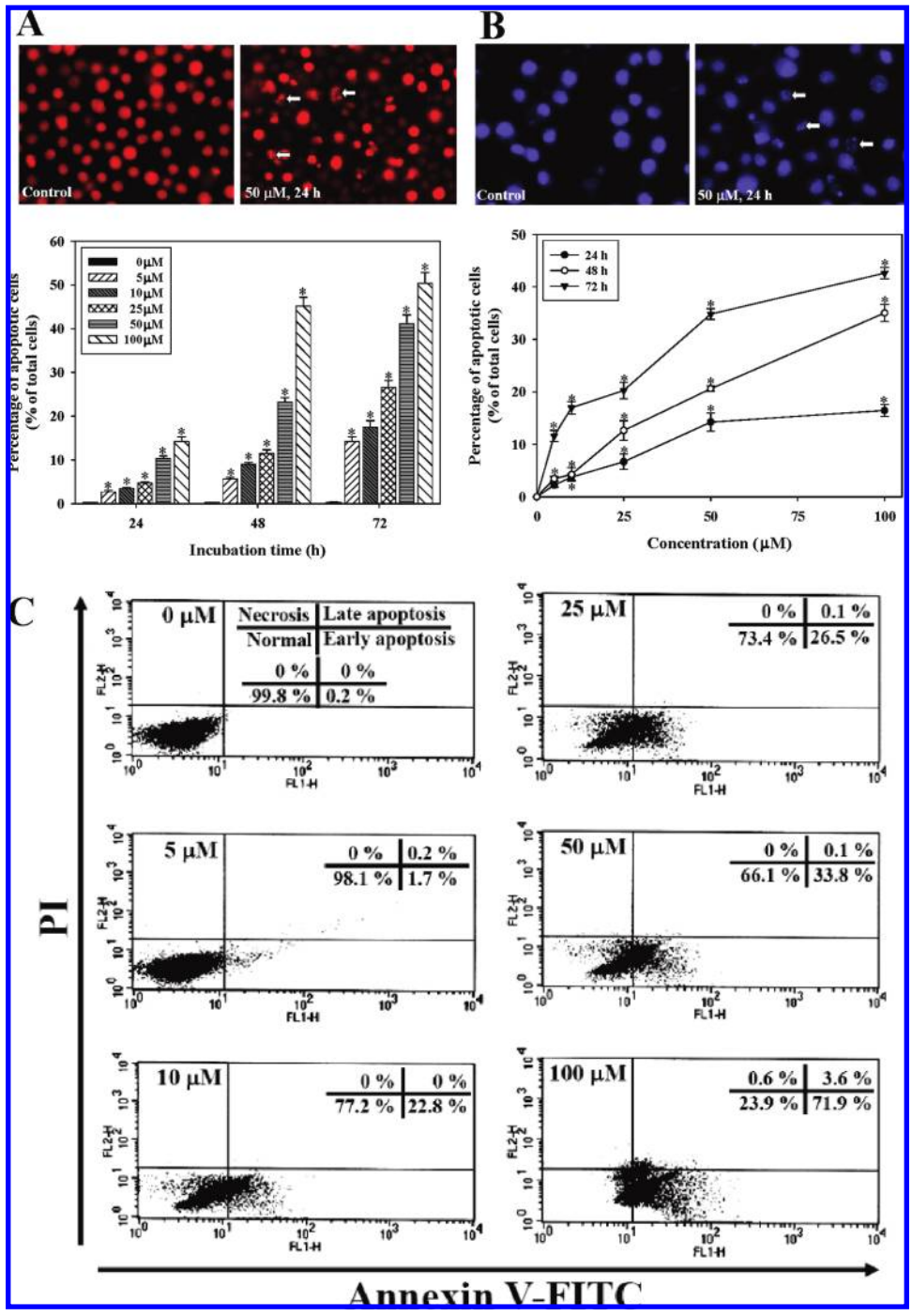

Figure 4. Effect of lucidenic acid B on induction of cell apoptosis in HL-60 cells. Cell morphological changes and percentage of apoptotic cells were analyzed by PI staining (**A**) and DAPI staining (**B**). Arrowheads indicate condensed chromatin. (**C**) Apoptosis and necrosis were analyzed by Annexin V-FITC/PI double staining. Cells were treated with 0−100 μM lucidenic acid B for 72 h. Reported values are the means ± SD ($n = 3$). ∗, $p < 0.05$ is significantly different from that of the control.

morphology indicated that nonapoptotic cells with healthy mitochondria appear as red fluorescent cells and apoptotic cells appear as green fluorescent cells. HL-60 cells showed a significant ($p < 0.05$) decrease in red fluorescence intensity when treated with 0−100 μM lucidenic acid B for 3−12 h (**Figure 5A**). The results demonstrated early damage to mitochondria membrane potential, which may further activate the intrinsic pathway of apoptosis. The effects of lucidenic acid B on the constitutive protein levels of Bax and Bcl-2 in HL-60 cells are shown in **Figure 5B**. Lucidenic acid B (25 μM, 6 h) resulted in a significant ($p < 0.05$) increase in Bax expression from 1.00- (control) to 2.02-fold. Treatment of cells with lucidenic acid B (25 μM, 6 h) significantly ($p < 0.05$) decreased Bcl-2 expression from 1.00- (control) to 0.13-fold. A significant time- and dose-dependent manner shift in the ratio of Bax and Bcl-2 was observed after lucidenic acid B treatment. **Figure 5C** shows the effect of lucidenic acid B on protein expression of Bak, Bad, Bcl-X_L, and cytochrome *c* in HL-60 cells. The pro-apoptotic protein expression of Bak and Bad was increased in a time- and dose-dependent manner from 1.00 (control) to 4.90- and 4.59-fold after being treated with 25 μM lucidenic acid B for 6 h, respectively. The antiapoptotic protein expression of Bcl-X_L was decreased in a time- and dose-dependent manner in cells treated with lucidenic acid B and decreased to 0.33-

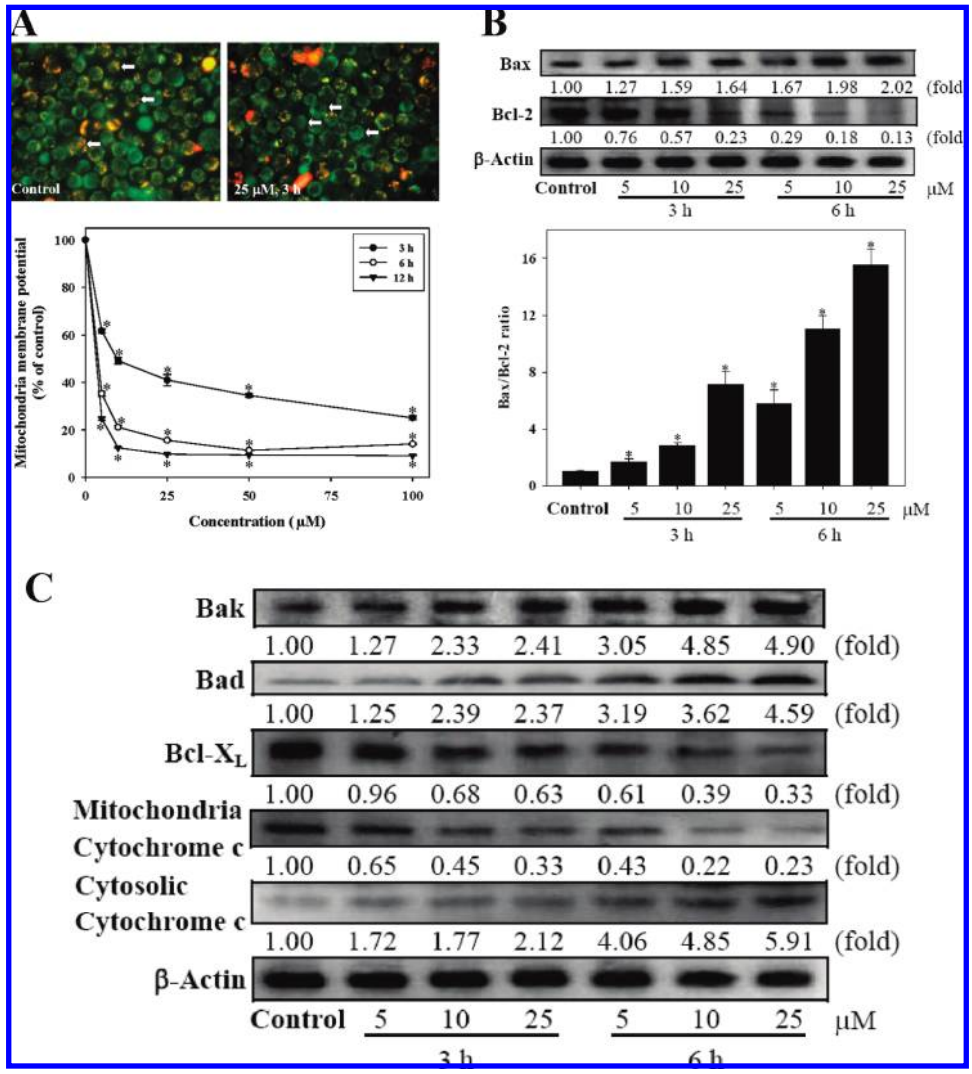

Figure 5. Effect of lucidenic acid B on mitochondria-dependent apoptosis in HL-60 cells. (**A**) Cell morphological changes and percentages of mitochondria membrane potential. (**B**) Protein expression of Bax and Bcl-2 and Bax/Bcl-2 ratio. (**C**) Protein expression of Bcl-2 family and cytochrome *c*. Relative expression was quantified densitometrically using the LabWorks 4.5 software and calculated according to the β-Actin reference bands. Reported values are the means ± SD ($n = 3$). ∗, $p < 0.05$ is significantly different from that of the control.

fold after being treated with 25 μM lucidenic acid B for 6 h. Cytochrome *c* release in the cytosolic fraction following lucidenic acid B treatment was then investigated. Lucidenic acid B (25 μM, 6 h) resulted in a significant ($p < 0.05$) increase in cytosolic cytochrome *c* expression from 1.00- (control) to 5.91-fold.

The effects of lucidenic acid B on the expression of caspase-9, caspase-3, and PARP in HL-60 cells are shown in **Figure 6A**. The results showed that exposure of HL-60 cells to lucidenic acid B caused the degradation of pro-caspase-9 and pro-caspase-3, which generated a fragment of caspase-9 and caspase-3 in a time- and dose-dependent manner. Treatment of cells with lucidenic acid B induced PARP cleavage in a time- and dose-dependent manner. To monitor the enzymatic activities of caspase-9 and caspase-3 during apoptosis induced by lucidenic acid B, we used the specific fluorogenic peptide substrate LEHD-AFC and Ac-DEVD-MCA for detection of caspase-9 and caspase-3 activities, respectively. The results indicated that lucidenic acid B treatment caused a significant time- and dose-dependent increase in caspase-9 and caspase-3 activities ($p < 0.05$) (**Figure 6B**). Our data indicated that lucidenic acid B induced cell apoptosis in HL-60 cells via expression of the Bcl-2 family, caspase-9 and caspase-3. The caspase-3/CPP32 inhibitor (Z-DEVD-FMK) and caspase-9/Mch6 inhibitor (Z-LEHD-FMK) were used to block intracellular proteases, and inhibition of cell viability by lucidenic acid B was analyzed by the MTT assay. The results indicated that both caspase-3 and caspase-9 inhibitors significantly reduced the inhibition of cell viability caused by lucidenic acid B in the HL-60 cells ($p < 0.05$) (**Figure 6C**).

DISCUSSION

The HL-60 cells are a useful model to study cell growth inhibition of leukemia cells by phytochemicals. The aim of the present study was to investigate the effect of lucidenic acids on cancer cell growth. We found that lucidenic acids caused a significant decrease ($p < 0.05$) in the cell population growth of HL-60 cells at 24−72 h (**Figure 2**). This concentration had no influence on human primary lymphocyte cells (data not shown). In order, to examine the mechanism responsible for cell proliferation and growth inhibition, cell cycle distribution was evaluated using flow cytometry. Our results showed that lucidenic acid A, C, and N could lead to cell cycle arrest at the G_1 phase in a time- and dose-dependent manner (**Figure 3**).

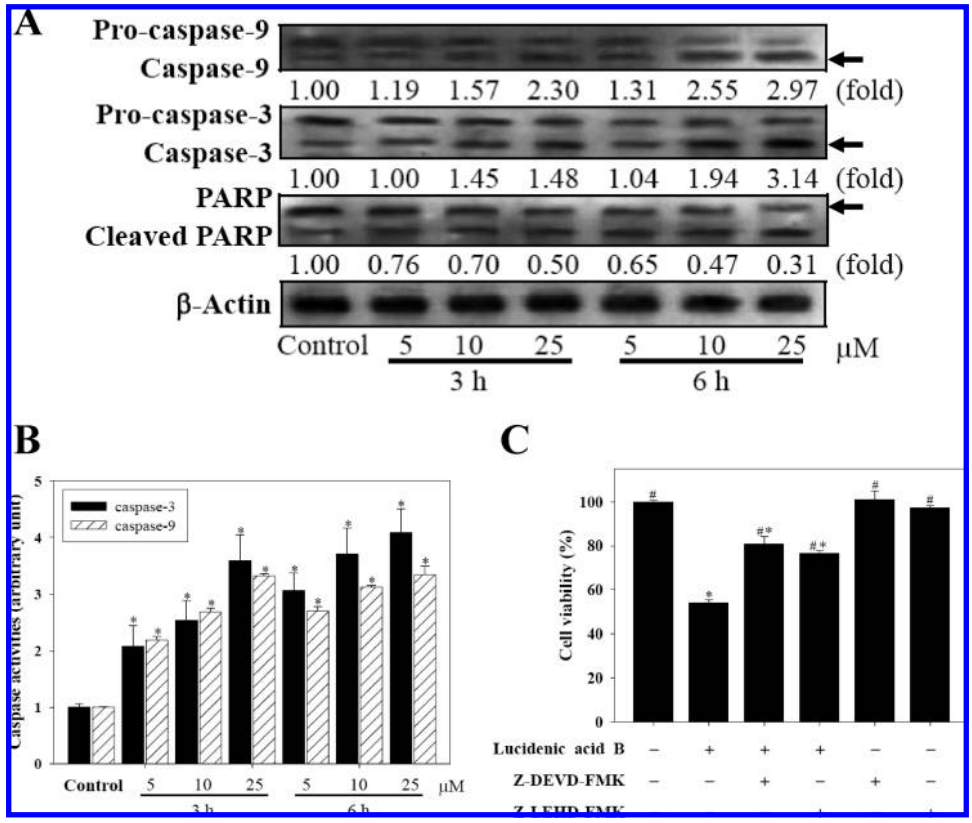

Figure 6. Effect of lucidenic acid B-induced caspases activation and cleaved PARP in HL-60 cells. (**A**) Protein expression of caspases and PARP. Relative expression was quantified densitometrically using the LabWorks 4.5 software and calculated according to the β-Actin reference bands. Arrowheads indicate the revealed fold of quantified protein. (**B**) Activities of caspase-9 and caspase-3. (**C**) Suppression of lucidenic acid B-induced cell dead by caspase-9 and caspase-3 inhibitors. Cells were pretreated with 25 μM caspase-3 (Z-DEVD-FMK) and caspase-9 (Z-LEHD-FMK) inhibitors for 3 h and then treated with 100 μM lucidenic acid B for 24 h. Reported values are the means $\pm$ SD ($n = 3$). $*$, $p < 0.05$ is significantly different from that of the control. #, $p < 0.05$ as compared to lucidenic acid B alone.

However, lucidenic acid B did not affect the cell cycle profile; it increased the number of apoptotic cells (**Figure 4A**,**B**) but not necrotic cells (**Figure 4C**). Cell death was assayed by quantification of plasma membrane damage.

Mitochondria play an essential role in the permeability of the transition pore opening, and collapse of the mitochondrial membrane potential resulted in the rapid release of caspase activators (*17, 20*). Changes in the membrane PS externalization are generally observed at a stage later than that of the loss of mitochondrial membrane potential (*21*). In the present study, treatment of HL-60 cells with lucidenic acid B decreased the mitochondrial membrane potential (**Figure 5A**). The Bcl-2 family plays a crucial role in apoptosis since it includes both antiapoptotic members such as Bcl-2 and Bcl-X_L and pro-apoptotic members such as Bax, Bad, and Bak (*22*). Our data indicated that the ratio of expression levels of pro- and antiapoptotic Bcl-2 family members was changed by lucidenic acid B treatment (**Figure 5B**,**C**). Lucidenic acid B also caused the release of mitochondrial cytochrome *c* into the cytosol (**Figure 5C**). Madesh et al. (*23*) indicated that the mitochondria undergoing permeability transition cause a loss of mitochondrial membrane potential and cytochrome *c* release in the cytosol. Cory and Adams (*16*) indicated that mitochondrial release of cytochrome *c* can be controlled by the Bcl-2 family of proteins and may be activated by proteolytic cleavage and heterodimerization.

Many models of apoptosis show a loss of the mitochondrial transmembrane potential mediated by opening of the mega-channel, which precedes caspase activation (*24*). Activation of caspase-3 is required for several typical hallmarks of apoptosis and is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types examined (*25*). Caspase-3 cleaves poly(ADP-ribose polymerase) (PARP), which responds to DNA fragmentation, and eventually leads to apoptosis (*26*). Treatment of HL-60 cells with lucidenic acid B revealed the activation of caspase-9 and caspase-3 (**Figure 6A**,**B**). We next examined whether caspase-9 and caspase-3 inhibitors significantly reduced the inhibition of cell viability caused by lucidenic acid B in HL-60 cells (**Figure 6C**). These data suggest that apoptosis induced by lucidenic acid B also involves caspase-9- and caspase-3-mediated mechanisms. Kobayashi et al. (*27*) indicated that the edible mushroom *Agaricus blazei* Murill induces apoptosis in human ovarian cancer HRA cells via a p38 MAPK and mitochondrial-mediated pathway.

In conclusion, the present study shows that lucidenic acid B leads to loss of the mitochondrial transmembrane potential, release of cytochrome *c* from the mitochondria into the cytosol, regulation of Bcl-2 family members, and subsequent activation of caspase-9 and caspase-3 followed by cleavage of PARP. These results provide a potential molecular mechanism for lucidenic acid B-induced apoptosis in HL-60 cells. The lucidenic acid B would provide selective killing of tumor cells relative to adjacent normal cells, suggesting it may have positive effects on human health. The lucidenic acids could be further tested by an in vivo model to justify if it is effective for prevention of human cancer.

ABBREVIATIONS USED

Annexin V-FITC, annexin V-fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DMSO,

4,6-diamidino-2-phenylindole dihydrochloride; IC_{50}, 50% growth inhibitory concentrations; LAB, lucidenic acid B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; PVDF, polyvinyldifluoride; RNase, ribonuclease; TE, trypsin-EDTA; $\Delta\Psi m$, mitochondria membrane potential.

ACKNOWLEDGMENT

We thank Kuang-Dee Chen (Biotechnology Research and Development Institute, Double Crane Group, Tainan Hsien, Taiwan) for a complimentary sample of lucidenic acids.

LITERATURE CITED

(1) Zhu, S.; Mori, M. *Research on Ganoderma*; Shanghai Medical University Press: Shanghai, 1994.

(2) Lin, J. M.; Lin, C. C.; Chiu, H. F.; Yang, J. J.; Lee, S. G. Evaluation of the anti-inflammatory and liver protective effects of *Anoectochilus formosamus*, *Ganoderma lucidum* and *Gunostemma pentaphyllum* in rats. *Am. J. Chin. Med.* **1993**, *21*, 59–69.

(3) Ahmad, S. *Oxidative Stress and Antioxidant Defences in Biology*; Chapman and Hall: New York, 1995; pp 62−88.

(4) Zhu, M.; Chang, Q.; Wong, L. K.; Chong, F. S.; Li, R. C. Triterpene antioxidants from *Ganoderma lucidum*. *Phytother. Res.* **1999**, *13*, 529–531.

(5) Xie, J. T.; Wang, C. Z.; Wicks, S.; Yin, J. J.; Kong, J.; Li, J.; Li, Y. C.; Yuan, C. S. *Ganoderma lucidum* extract inhibits proliferation of SW 480 human colorectal cancer cells. *Exp. Oncol.* **2006**, *28*, 25–29.

(6) Akihisa, T.; Nakamura, Y.; Tagata, M.; Tokuda, H.; Yasukawa, K.; Uchiyama, E.; Suzuki, T.; Kimura, Y. Anti-inflammatory and anti-tumor-promoting effects of triterpene acids and sterols from the fungus *Ganoderma lucidum*. *Chem. Biodivers.* **2007**, *4*, 224–231.

(7) Mizushina, Y.; Takahashi, N.; Hanashima, L.; Koshino, H.; Esumi, Y.; Uzawa, J.; Sugawara, F.; Sakaguchi, K. Lucidenic acid O and lactone, new terpene inhibitors of eukaryotic DNA polymerases from a basidiomycete *Ganoderma lucidum*. *Bioorg. Med. Chem.* **1999**, *7*, 2047–2052.

(8) Wu, T. S.; Shi, L. S.; Kuo, S. C. Cytotoxicity of *Ganoderma lucidum* triterpenes. *J. Nat. Prod.* **2001**, *64*, 1121–1122.

(9) Iwatsuki, K.; Akihisa, T.; Tokuda, H.; Ukiya, M.; Oshikubo, M.; Kimura, Y.; Asano, T.; Nomura, A.; Nishino, H. Lucidenic acids P and Q, methyl lucidenate P, and other triterpenoids from the fungus *Ganoderma lucidum* and their inhibitory effects on Epstein-Barr virus activation. *J. Nat. Prod.* **2003**, *66*, 1582–1585.

(10) Li, C. H.; Chen, P. Y.; Chang, U. M.; Kan, L. S.; Fang, W. H.; Tsai, K. S.; Lin, S. B. Ganoderic acid X, a lanostanoid triterpene, inhibits topoisomerases and induces apoptosis of cancer cells. *Life Sci.* **2005**, *77*, 252–265.

(11) Tang, W.; Liu, J. W.; Zhao, W. M.; Wei, D. Z.; Zhong, J. J. Ganoderic acid T from *Ganoderma lucidum* mycelia induces mitochondria mediated apoptosis in lung cancer cells. *Life Sci.* **2006**, *80*, 205–211.

(12) Weng, C. J.; Chau, C. F.; Chen, K. D.; Chen, D. H.; Yen, G. C. The anti-invasive effects of lucidenic acids isolated from a new *Ganoderma lucidum* strain. *Mol. Nutr. Food Res.* **2007**, *51*, 1472–1477.

(13) Weng, C. J.; Chau, C. F.; Hsieh, Y. S.; Yang, S. F.; Yen, G. C. Lucidenic acid inhibits PMA-induced invasion of human hepatoma cells through inactivating MAPK/ERK signal transduction pathway and reducing binding activities of NF-κB and AP-1. *Carcinogenesis* **2008**, *29*, 147–156.

(14) Johnstone, R. W.; Ruefli, A. A.; Lowe, S. W. Apoptosis: A link between cancer genetics and chemotherapy. *Cell* **2002**, *108*, 153–164.

(15) Vermeulen, K.; Van Bockstaele, D. R.; Berneman, Z. N. Apoptosis: Mechanisms and relevance in cancer. *Ann. Hematol.* **2005**, *84*, 627–639.

(16) Cory, S.; Adams, J. M. The Bcl-2 family: Regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* **2002**, *2*, 647–656.

(17) Green, D. R.; Reed, J. C. Mitochondria and apoptosis. *Science* **1998**, *281*, 1309–1312.

(18) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63.

(19) Takada, E.; Toyota, H.; Suzuki, J.; Mizuguchi, J. Prevention of anti-IgM-induced apoptosis accompanying G1 arrest in B lymphoma cells overexpressing dominant-negative mutant form of c-Jun N-terminal kinase 1. *J. Immunol.* **2001**, *166*, 1641–1649.

(20) Li, P.; Nijhawan, D.; Budihardjo, J.; Srinivasula, S. M.; Ahmad, M.; Alnewri, E. S.; Wang, X. Cytochrome c and ATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic proteasome cascade. *Cell* **1997**, *91*, 479–489.

(21) Raghuvar Gopal, D. V.; Narkar, A. A.; Badrinath, Y.; Mishra, K. P.; Joshi, D. S. Protection of Ewing's sarcoma family tumor (ESFT) cell line SK-N-MC from betulinic acid induced apoptosis by α-DL-tocopherol. *Toxicol. Lett.* **2004**, *153*, 201–212.

(22) Hunt, A.; Evan, G. Apoptosis. Till death us do part. *Science* **2001**, *293*, 1784–1785.

(23) Madesh, M.; Antonsson, B.; Srinivasula, S. M.; Alnemri, E. S.; Hajnoczky, G. Rapid kinetics of tBid-induced cytochrome c and Smac/ DIABLO release and mitochondrial depolarization. *J. Biol. Chem.* **2002**, *277*, 5651–5659.

(24) Zamzami, N.; Metivier, D.; Kroemer, G. Quantitation of mitochondiral transmembrane potential in cells and in isolated mitochondria. *Methods Enzymol.* **2000**, *322*, 208–213.

(25) Sánchez-Alonso, J. A.; López-Aparicio, P.; Recio, M. N.; Pérez-Albarsanz, M. A. Polychlorinated biphenyl mixtures (Aroclors) induce apoptosis via Bcl-2, Bax and caspase-3 proteins in neuronal cell cultures. *Toxicol. Lett.* **2004**, *153*, 311–326.

(26) Salvesen, G. S.; Dixit, V. M. Caspases: Intracellular signaling by proteolysis. *Cell* **1997**, *91*, 443–446.

(27) Kobayashi, H.; Yoshida, R.; Kanada, Y.; Fukuda, Y.; Yagyu, T.; Inagaki, K.; Kondo, T.; Kurita, N.; Suzuki, M.; Kanayama, N.; Terao, T. Suppressing effects of daily oral supplementation of beta-glucan extracted from Agaricus blazei Murill on spontaneous and peritoneal disseminated metastasis in mouse model. *J. Cancer Res. Clin. Oncol.* **2005**, *131*, 527–538.

Received for review January 1, 2008. Revised manuscript received March 23, 2008. Accepted March 25, 2008. This research work was partially supported by the Council of Agriculture, Republic of China, under Grant 97AS-3.1.3-FD-Z1(1).

JF800006U