JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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α-Mangostin Induces Apoptosis in Human Chondrosarcoma Cells through Downregulation of ERK/JNK and Akt Signaling Pathway

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ABSTRACT: Chondrosarcoma is a malignant primary bone tumor that is resistant to chemotherapy and radiation therapy. α-Mangostin, a component of *Garcinia mangostana* Linn, is a xanthone derivative shown to have antioxidant and antitumor properties. This study is the first to investigate anticancer effects of α-mangostin in the human chondrosarcoma cell line SW1353. We showed that α-mangostin inhibited cell proliferation of SW1353 cells in a time- and dose-dependent manner by using the trypan blue exclusion method. Hoechst 33342 nuclear staining and nucleosomal DNA-gel electrophoresis revealed that α-mangostin could induce nuclear condensation and fragmentation, typically seen in apoptosis. Flow cytometry using Annexin V/PI double staining assessed apoptosis, necrosis and viability. α-Mangostin activated caspase-3, -8, -9 expression, decreased Bcl-2 and increased Bax. This promotes mitochondrial dysfunction, leading to the release of cytochrome *c* from the mitochondria to the cytoplasm. In addition, total and phosphorylated ERK and JNK were downregulated in α-mangostin-treated SW1353 cells but no changes in p38. α-Mangostin also decreased phosphorylated Akt without altering total Akt. These results suggest that α-mangostin inhinbited cell proliferation and induced apoptosis through downregulation of ERK, JNK and Akt signaling pathway in human chondrosarcoma SW1353 cells.

KEYWORDS: α-mangostin, apotosis, chondrosarcoma, MAPK, Akt

INTRODUCTION

Chondrosarcoma is a malignant primary bone tumor that overproduces chondrocytes and subsequently cartilage matrix. Chondrosarcoma is the third most common primary malignancy of bone after myeloma and osteosarcoma.[1] This disease usually grows within bone or on its surface. The most common sites are pelvic bones, shoulder bones, and the upper part of the arms and legs. It develops from slow growing nonmetastasizing lesions to highly aggressive metastasizing sarcomas.[2] Patients are usually between 30 and 70 years old.[3] Chondrosarcoma is highly insensitive to conventional chemotherapy and radiation treatment, thereby the surgical resection remains the mainstay of treatment.[4] However, the development of effective adjuvant chemotherapy is still required. New therapeutic and preventive strategies targeted at cell growth inhibition and apoptosis induction may be another alternative of treatment for chondrosarcoma.

Mangosteen, *Garcinia mangostana* Linn., is a fruit found in South East Asia, for example, Thailand, Malaysia, Myanmar, Philippines, Sri Lanka and India. Mangosteen is known as "the queen of fruits" because it is one of the best tasting tropical fruits. The fruit hull of mangosteen has been used as traditional medicine for treatment of trauma, diarrhea, skin infection and wounds for many years.[5] Moreover, xanthones, the secondary metabolites from pericarp of mangosteen, possess biological activities, such as antioxidant,[6] antibacterial,[7] antifungal,[8] anti-inflammatory,[9] and cytotoxic activities.[10,11] Previous studies have reported the anticancer activities of α-mangostin, one of the major xanthones from mangosteen, on human breast cancer,[12] prostate cancer,[13] leukemia,[14] glioblastoma cells[15] and colon cancer.[16,17] However, the effects and related molecular mechanism of α-mangostin on human chondrosarcoma have not been reported.

In this study, we investigated the hypothesis that α-mangostin could inhibit the growth of human chondrosarcoma SW1353 cell line through induction of apoptosis. Our study shows expression of caspase-3, -8 and -9, increased expression of Bax, decreased expression of Bcl-2 and promoted cytochrome *c* release from mitochondria. The results also show downregulated phosphorylation of ERK, JNK and Akt in α-mangostin-treated chondrosarcoma cells. Altogether, our data demonstrate that α-mangostin could induce apoptosis involving mitochondrial-mediated pathway and caspase-8 activation through downregulating the phosphorylated ERK, JNK and Akt in human chondrosarcoma SW1353 cells.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS), penicillin/streptomycin and trypsin were purchased from PAA Laboratories (Pasching, Austria). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), trypan blue and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342 and Annexin V−FITC conjugate were purchased from Invitrogen Molecular Probes (Eugene, OR). Primary rabbit polyclonal anti-caspase-3, -caspase-9, -Bax, -BID, rabbit monoclonal

Received: February 14, 2011
Accepted: March 29, 2011
Revised: March 29, 2011
Published: March 29, 2011

anti-Bcl-2, -Bcl-xL, MAPK, Akt and mouse monoclonal anti-caspase-8 antibody were obtained from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody against human cytochrome *c* was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ECL reagent and X-ray film were purchased from Pierce (Rockford, IL)

α-Mangostin Extraction and Isolation. Mangosteen fruit (*G. mangostana*) was collected from Kombang District, Chanthaburi Province, Thailand, in April, 2007. A voucher specimen (Porntip Wongnapa No. 002) was deposited at the Faculty of Science, Ramkhamhaeng University. The dried and pulverized fruit hull of *G. mangostana* (0.5 kg) was thoroughly extracted with ethyl acetate (EtOAc) at 50 °C. The combined extract after filtration was concentrated under reduced pressure to yield the extract as a yellowish solid (285 g). A portion of the extract was subjected to repeated column chromatography over silica gel using a gradient of hexane/acetone and yielded the pure major compound, α-mangostin, including other minor xanthones. Purity of α-mangostin exceeded 98% as determined by LC analysis, and its spectroscopic data (NMR and MS) was consistent with the reported values.[18] α-Mangostin ($C_{24}H_{26}O_6$) was dissolved in dimethyl sulfoxide (DMSO; Amresco, OH) at the stock concentration of 100 μg/mL and further diluted to the desired working concentration before use.

Cell Culture. The chondrosarcoma cell line SW1353 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell line was maintained as a monolayer in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The medium was refreshed every 2−3 days. After about 90% of confluence, the cultured cells were detached with 0.25% trypsin−EDTA and subcultured.

Cell Proliferation and Cell Viability Assay. The cytotoxictiy of α-mangostin was initially determined by cell proliferation analysis using MTT assay. Cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and allowed to grow for 24 h. Cells were then treated with α-mangostin at various concentrations of 0.005, 0.05, 0.5, 5, 50, and 500 μg/mL, whereas the control group was treated with DMSO. After incubation for 24 h, 100 μL of 0.5 mg/mL MTT solution was added to each well and the plate was further incubated for 2 h at 37 °C. The supernatant was aspirated and 100 μL of DMSO was added to each well to solubilize water insoluble purple formazan crystals. The absorbance at 550 nm was measured using a microplate reader (Multiskan EX, Thermo electron corporation, Finland), and the IC_{50} value was calculated using the software GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA).

The effect of α-mangostin on the cell viability was analyzed by using a trypan blue exclusion method. Cells were seeded in a 24-well plate at 1×10^5 cells/well and incubated for 24 h. Then, the cells were treated with the indicated concentrations (0, 5, 10, 15, 20, and 30 μg/mL) of α-mangostin for 3, 6, 9, 12, and 24 h. Cells were harvested by trypsinisation, stained with trypan blue and counted with a hemocytometer. Cell survival was expressed as percentage of viable cells of treated cells to control cells. Cells were treated in triplicate, and the experiments were repeated three times.

Cell Cycle Analysis. Cells were seeded in a 6-well plate at 1×10^6 cells/well for 24 h. The cells were then treated with DMSO (control) and 20 μg/mL α-mangostin for 3 and 6 h. Cells were washed with ice-cold PBS once and resuspended in 0.5 mL of PI solution (50 μg/mL PI, 0.1% Triton X-100, 0.1% sodium citrate) and incubated at 4 °C overnight in the dark and analyzed for DNA content on a BD FACScan flow cytometer (Becton Dickinson, San Jose, CA) in linear scale using BD FACSDiva Software Version 4.1.2 (BD Science, Becton Dickinson, San Jose, CA

Nuclear Morphology Staining with Hoechst 33342. SW1353 cells (1×10^6 cells/well in a 6-well plate) were treated with 20 μg/mL α-mangostin for 0, 3, and 6 h. After trypsinization, cells were washed with 1× PBS and stained with 3 μg/mL of Hoechst 33342 for 15 min. Stained cells were examined using fluorescence microscope (Olympus, Tokyo, Japan) with an ultraviolet filter.

DNA Fragmentation Analysis. SW1353 cells (1×10^6 cells/well) were seeded in 6-well plates and treated with 0, 10, 20 μg/mL α-mangostin for 18 h. Both floating and attached cells were collected by trypsinisation and washed once with PBS, and DNA was extracted using a Genomic DNA extraction kit (Gentra Puregene cell kit, QIAGEN) according to the manufacturer's instructions. The sample DNA was analyzed using 1.7% agarose gel and visualized by ethidium bromide staining of the gel.

Phosphatidylserine Exposure. Cells (1×10^6) were treated with 20 μg/mL α-mangostin for 3 and 6 h. Then cells were harvested by low speed centrifugation and washed once with ice-cold PBS. Cell pellets were resuspended in 100 μL of binding buffer (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2$), 5 μL of Annexin V−FITC conjugate and 10 μL of PI were added. The cells were incubated at room temperature for 15 min in the dark. After adding 400 μL of binding buffer, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Western Blot Analysis. SW1353 cells were incubated for different times in the presence or absence of 20 μg/mL α-mangostin, harvested and washed once with ice cold PBS. Then, 2×10^6 cells were lysed for 30 min on ice in 50 μL of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl, 0.5% Triton X-100) containing complete mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Clear cell lysate supernatants were prepared by centrifugation, and the protein content was determined using Bio-Rad protein assay (Bio-Rad Laboratories, USA). Proteins were separated by 12% SDS−PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Pall Corporation, USA) for 1 h at 100 V with the use of a Mini Trans-Blot Cell (Bio-Rad). After blocking with TBST (10 mM Tris, pH 7.5, 150 mM NaCl and 0.1% Tween 20) containing 5% nonfat milk, the blots were incubated overnight at 4 °C with primary antibody. The membranes were washed in TBST, and the appropriate secondary antibody conjugated with horseradish peroxidase was added for 1 h at room temperature. Immunoreactive protein bands were detected by chemiluminescence using enhanced chemiluminescence reagent (ECL) and exposed to X-ray film. The membranes were stripped and reprobed with β-actin antibody to assess protein loading for each lane.

Detection of Cytochrome *c* Release. After incubating SW1353 cells for different times in the presence or absence of 20 μg/mL α-mangostin, cells were harvested and washed once with ice cold PBS. Cells were resuspended in 50 μL of S-100 lysis buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 250 mM sucrose), homogenized and centrifuged at 4 °C at 500*g* for 5 min to eliminate nuclei and unbroken cells. The resulting cell lysates were further centrifuged at 4 °C at 10000*g* for 30 min. The supernatants (cytosol fractions) were separated by 12% SDS−PAGE, and proteins were transferred onto PVDF membranes. The resulting blots were subjected to immunodetection of cytochrome *c*, which had been released from mitochondria, using a mouse monoclonal antibody against human cytochrome *c* with goat anti-mouse IgG, conjugated to horseradish peroxidase as secondary antibody. The peroxidase activity of bound secondary antibodies on the blots was detected by using ECL and exposed to X-ray film.

Statistical Analysis. All data presented were obtained from at least three independent experiments and are presented as mean ± standard deviation (SD). Statistical significance was assessed by Student's *t*-test, carried out using the software GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA).

RESULTS

α-Mangostin Inhibited Cell Proliferation and Induced Apoptosis in Human Chondrosarcoma Cells. Initially, the antiproliferative activity of α-mangostin in SW1353 cells was screened by using MTT assay and the IC_{50} value was estimated to be 10 μg/mL. Then, the effect of α-mangostin on cell viability

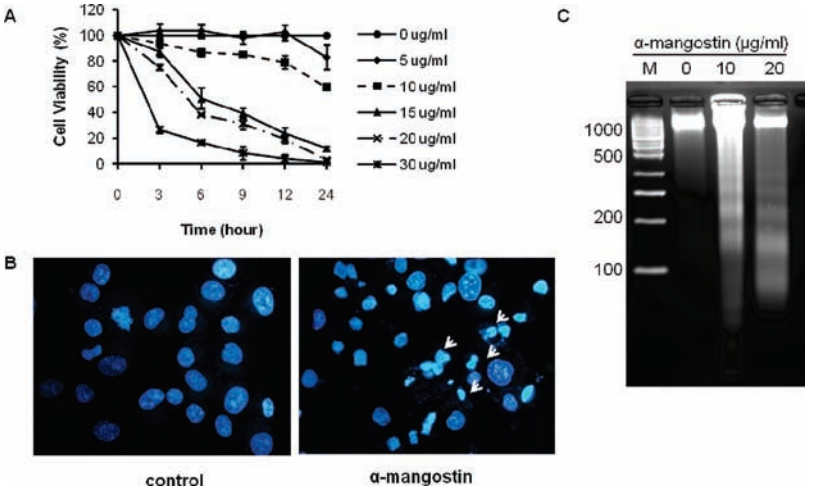

Figure 1. Effects of α-mangostin on cell viability and apoptosis induction in SW1353 cells. (A) Time- and dose-dependent effect of α-mangostin was performed when SW1353 cells were treated with various concentrations of α-mangostin for various times, and their viability was determined by MTT assay. Results are mean values ± SD of three independent experiments performed in triplicate ($n = 3$). (B) The morphologic changes of SW1353 treated with DMSO (control) or 20 μg/mL α-mangostin for 6 h, then stained with Hoechst 33342 and examined under a fluorescent microscope (40× magnification). (C) DNA fragmentation of SW1353 cells treated with 10, 20 μg/mL α-mangostin for 18 h visualized by gel electrophoresis (M, DNA size marker).

was reanalyzed for varying concentrations and times by using the trypan blue exclusion method. As shown in Figure 1A, inhibition of cell viability by α-mangostin occurred in a dose- and time-dependent manner. Treatment SW1353 cells with α-mangostin at 15 μg/mL or higher for 24 h reduced cell viability to approximately 10% or lower comparing with control cells.

To determine whether antiproliferation and cell death are associated with apoptosis, SW1353 cells were stained with Hoechst 33342 dye after exposure to α-mangostin and observed under fluorescence microscopy. The result showed that the nuclei of untreated SW1353 cell appeared round in shape, while α-mangostin caused nuclear condensation and simultaneously induced morphological changes in some of the cells at 6 h (Figure 1B). After prolonged exposure (18 h), DNA samples were extracted and analyzed by gel electrophoresis to determine DNA fragmentation. Result clearly showed that DNA from α-mangostin treated cells exhibited such fragments typical of apoptosis, whereas control cells did not provide ladders (Figure 1C). These results suggested that the proliferation inhibition and the death of target cells upon treatment with α-mangostin are consequent to the induction of apoptosis.

To further investigate the effects of α-mangostin on cell cycle distribution, we analyzed SW1353 cells treated with α-mangostin using flow cytometry. The sub-G1 peak formed with reduced DNA content represented the presence of apoptotic cells. The two major peaks represented the G0/G1 and G2/M phases of the cell cycle. It was observed that the mean apoptotic population of SW1353 cells was 3.6% under control condition, while it was increased to 5.3% and 14.9% after treatment with 20 μg/mL α-mangostin for 3 and 6 h respectively in a time-dependent manner (Figure 2).

Effect of α-Mangostin on Phosphatidyl Serine Exposure. One of the early indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane. Annexin V−PI double-labeling and FACS analysis were used for the detection of phosphatidylserine externalization. As shown in Figure 3, the four quadrants in each panel correspond, respectively, to necrotic cells (upper left), late apoptotic cells (upper right), viable cells (lower left), and early apoptotic cells (lower right). After treatment with α-mangostin, the results showed that control cells were mostly alive whereas the α-mangostin treated cells increased early and late apoptotic cells in a time-dependent manner.

Effect of α-Mangostin on Capase-3, Caspase-8 and Caspase-9 Activation. To investigate whether the induction of apoptosis by α-mangostin treatment is caspase-dependent and which caspase(s) is involved, we examined the formation of active forms of caspases in cell lysates by Western blot analysis. As shown in Figure 4, α-mangostin could induce caspase-3, caspase-8 and caspase-9 cleavage after 3 and 6 h of treatment. Thus, α-mangostin-induced apoptosis is mediated by caspase-3 and maybe associated with the activation of both extrinsic (via caspase-8) and intrinsic apoptosis pathways (via caspase-9).

Effect of α-Mangostin on the Expression of Bcl-2 Family Proteins and the Induction of Cytochrome *c* Release. The Bcl-2 family of proteins regulates cell death by controlling of the mitochondrial membrane permeability during apoptosis. The insertion of Bax into mitochondrial membrane induces the opening of the mitochondrial voltage-dependent anion channel (VDAC) and the release of cytochrome *c* from mitochondria to cytoplasm, which is a hallmark of the mitochondrial apoptosis pathway.[19] To determine whether α-mangostin induces apoptosis by triggering the mitochondrial apoptotic pathway, we measured the change in the expression of the Bcl-2 family of proteins. The results showed that treatment of SW1353 cells with α-mangostin induced Bax protein level and decreased Bcl-2 expression but no apparent changes in the expression level of Bcl-xL (Figure 5). In the case of Bid, its expression apparently decreased after the treatment, however, the cleaved active form (tBid) was not produced. In addition, the release of cytochrome *c* was detected in cytosolic extract of α-mangostin-treated SW1353 at 3 h and 6 h but the control SW1353 cells (Figure 6). These results indicate that α-mangostin induced apoptosis via mitochondrial pathway.

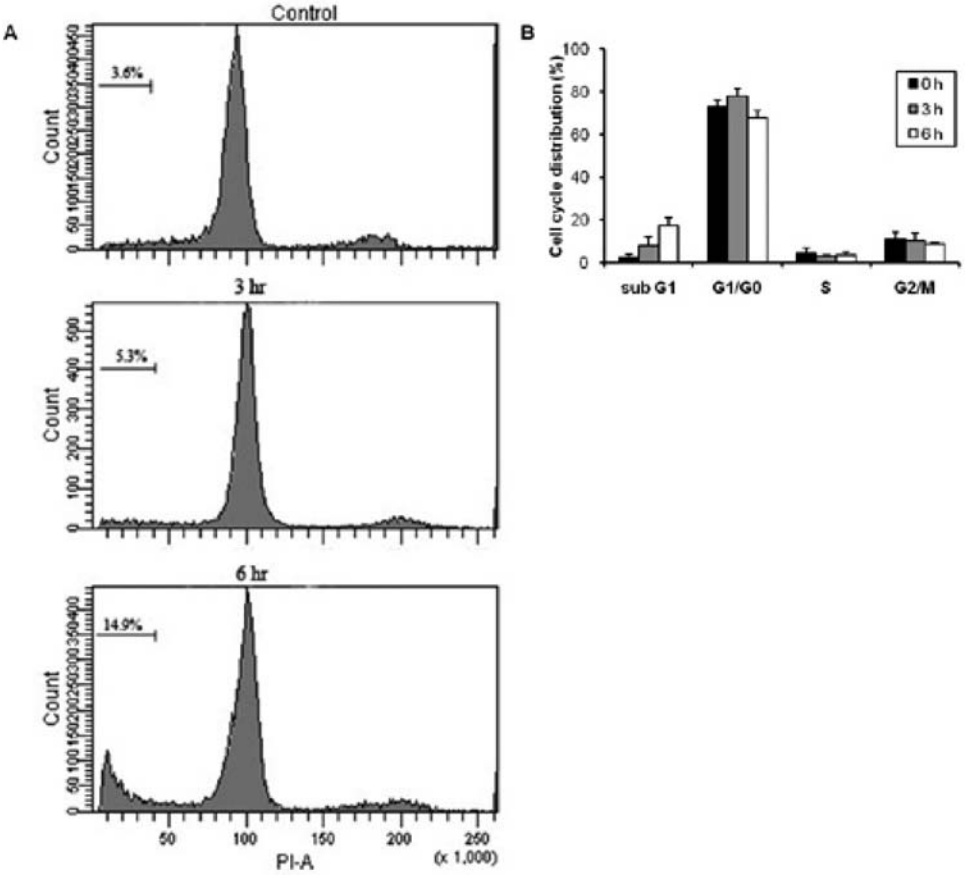

Figure 2. Cell cycle analysis of SW1353 cells treated with α-mangostin. The sub-G1 peak formed with reduced DNA content represented the presence of apoptotic cells. The two major peaks represented the G0/G1 and G2/M phases of the cell cycle. It was observed that the mean apoptotic population of SW1353 cells was 3.6% under control condition, while it was increased to 5.3% and 14.9% after treatment with 20 μg/mL α-mangostin for 3 and 6 h respectively.

Effect of α-Mangostin on the Expression of MAPKs and Akt. MAPKs are a widely conserved family of serine/threonine protein kinases involved in many cellular activities such as cell proliferation, differentiation, motility, survival and death.[20] MAPKs are activated by phosphorylation of specific tyrosine and threonine residues and the relative levels of phosphorylated MAPKs in total MAPKs represent the degree of MAPK activation. Figure 7 shows that α-mangostin decreased the level of total and phosphorylated ERK1/2 and JNK proteins but it did not affect p38 activity. It should be noted that the level of phosphorylated ERK1/2 was increased at 3 h and then decreased at 6 h after treatment with α-mangostin. The results suggested that ERK and JNK might be involved in α-mangostin-induced apoptosis in human chondrosarcoma cells.

Akt plays a key role in controlling survival and apoptosis. It can be activated by phospholipid binding and activation loop phosphorylation at threonine 308 by PDK1 and by phosphorylation within the carboxy terminus at serine 473.[21] We examined the phosphorylation of Akt and PDK1 after treatment of α-mangostin. The phosphorylated Akt and PDK1 decreased in a time-dependent manner, but α-mangostin treatment did not affect total Akt protein (Figure 8). Our results suggested that α-mangostin triggers apoptosis by downregulating phosphorylation of Akt pathway in human chondrosarcoma cells.

DISCUSSION

Chondrosarcoma is a malignant primary bone tumor with a poor prognosis and resistant to chemotherapy and radiation treatment. In this study, we investigated the potential of α-mangostin from pericarp of mangosteen for the treatment of human chondrosarcoma. We demonstrated the anticancer effect of α-mangostin in SW1335 cell line. The results show that α-mangostin could inhibit cell growth of SW1353 cells in a time- and dose-dependent manner with an IC_{50} value of 10 μg/mL. Several researchers have documented the cytotoxic and antiproliferative effects of α-mangostin at concentrations ranging from 1 to 10 μg/mL in various human cancer cell lines.[10−13] Our data are consistent with the IC_{50} value of 9 and 9.25 μg/mL in PC3 human prostate cancer cells and SKBR3 human breast cancer cells, respectively. The differences in IC_{50} values of α-mangostin in individual cell lines may be attributed to the differences in the duration of exposure and the differential sensitivities of cell lines to the cytotoxic effects of α-mangostin.

To confirm apoptosis, DNA fragmentation analysis and characteristic morphological changes, including membrane blebbling, cell shrinkage, chromatin condensation, and formation of apoptotic bodies were investigated. Hoechst 33342 staining clearly showed condensed and fragmented nuclei in SW1353 cells treated with α-mangostin (Figure 1B). Cell cycle analysis

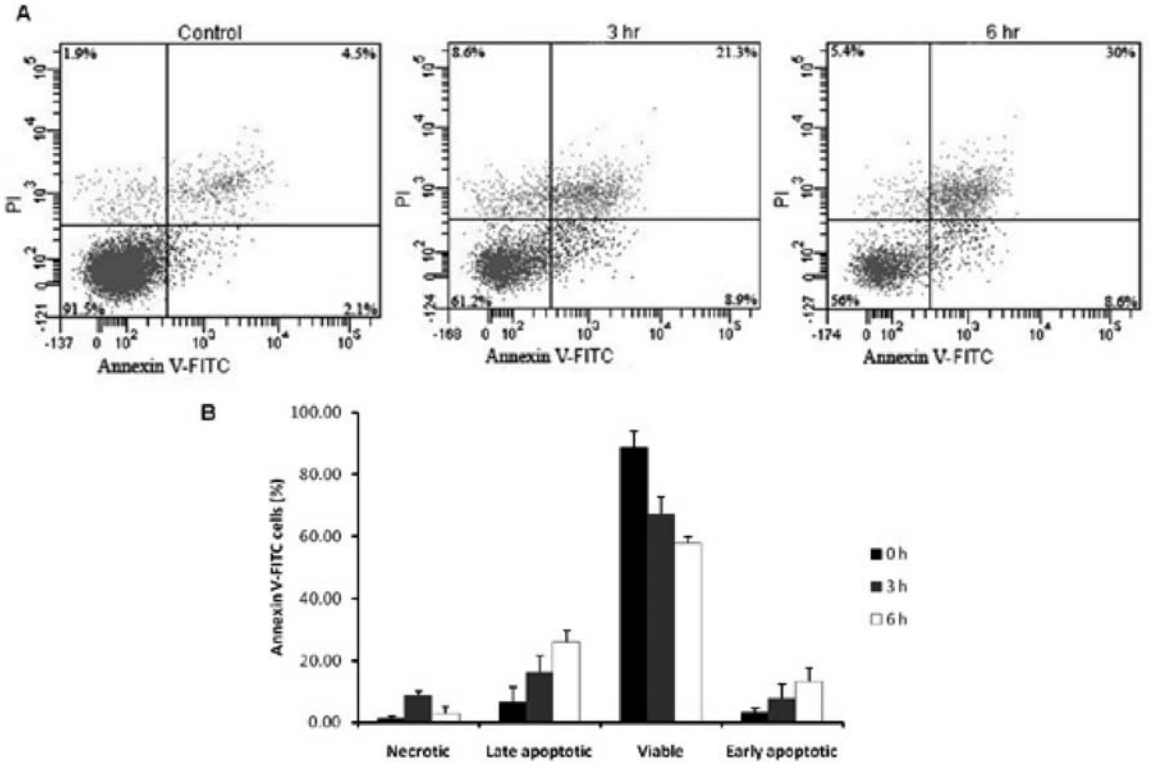

Figure 3. Flow cytometric of phosphatidylserine exposure for SW1353 cells. SW1353 cells were exposed DMSO (control) and 20 μg/mL α-mangostin for 3 and 6 h. (A) Representative dot plots of Annexin V/PI staining are shown. The upper left quadrant contains the necrotic (Annexin V−/PI+) population. The upper right quadrant contains the late apoptotic/necrotic (Annexin V+/PI+) population. The lower left quadrant contains the vital (double negative) population. The lower right quadrant contains the early apoptotic (Annexin V+/PI−) population. The result is from one experiment representative of three similar experiments. (B) The percentage of necrotic cells, early apoptotic cells, viable cells and late apoptotic cells at 0, 3, and 6 h are indicated. The experiment was performed in three independent experiments, and the results are expressed as mean ± SD.

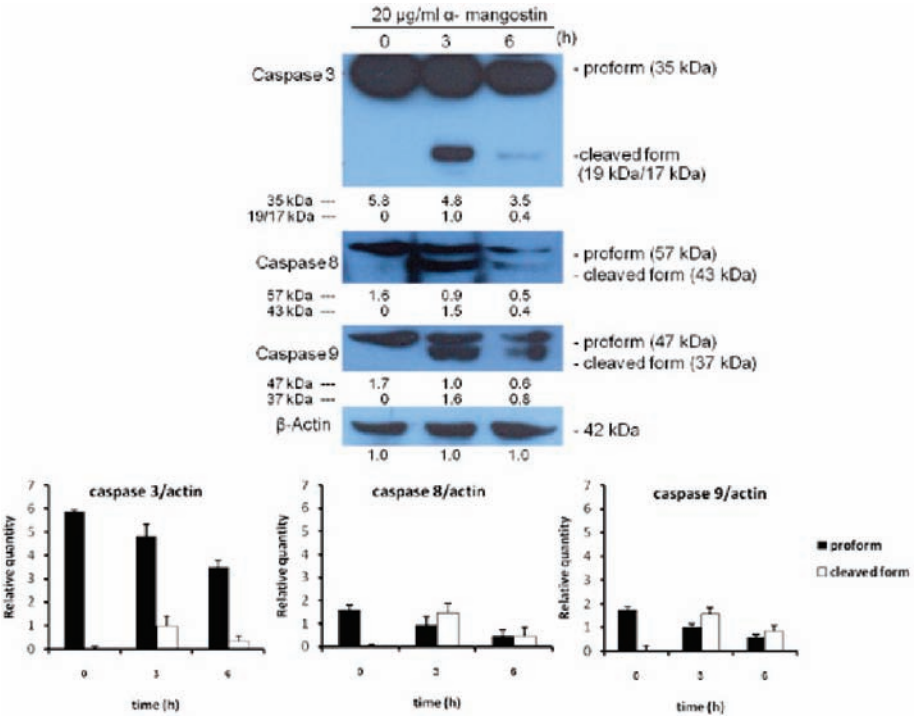

Figure 4. Effects of α-mangostin on activation of caspase-3, -8, and -9 in SW1353 cells. Cells were treated with 20 μg/mL α-mangostin for 3 and 6 h and examined by Western blot analysis. β-Actin was used as the internal control.

dx.doi.org/10.1021/jf200620n |*J. Agric. Food Chem.* 2011, 59, 5746–5754

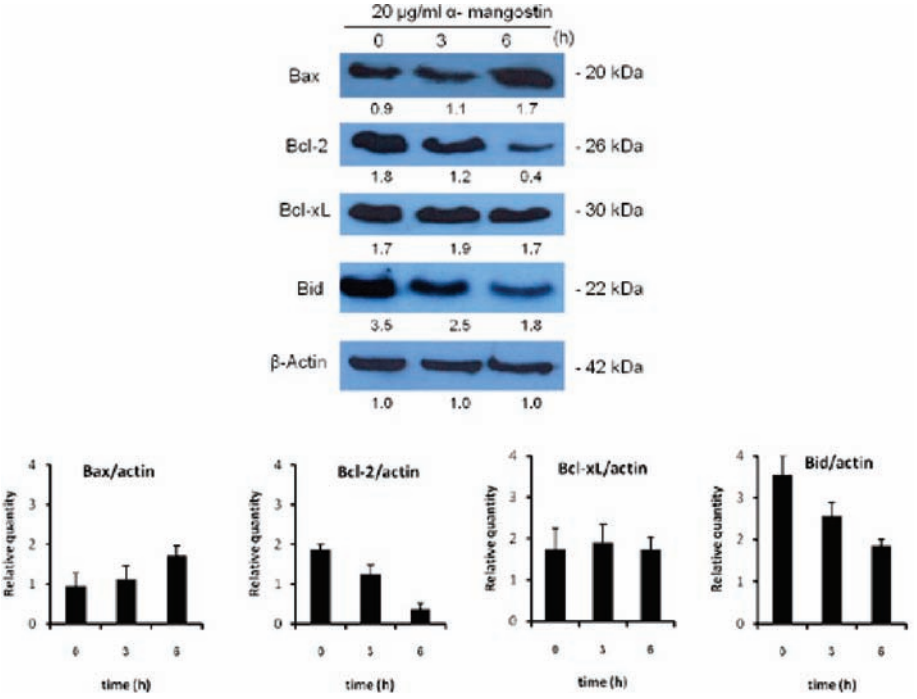

Figure 5. Effects of α-mangostin on expression of Bax, Bcl-2, Bcl-xL and Bid in SW1353 cells. Cells were treated with 20 μg/mL α-mangostin for 3 and 6 h and examined by Western blot analysis. β-Actin was used as the internal control.

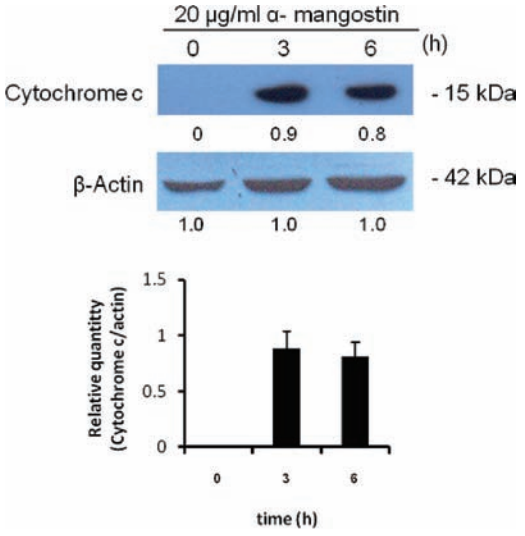

Figure 6. α-Mangostin induces cytochrome *c* release from the mitochondria in SW1353 cells. Cells were treated with 20 μg/mL α-mangostin for 3 and 6 h. Cytosol fractions were separated by SDS−PAGE and subjected to immunodetection of cytochrome *c*.

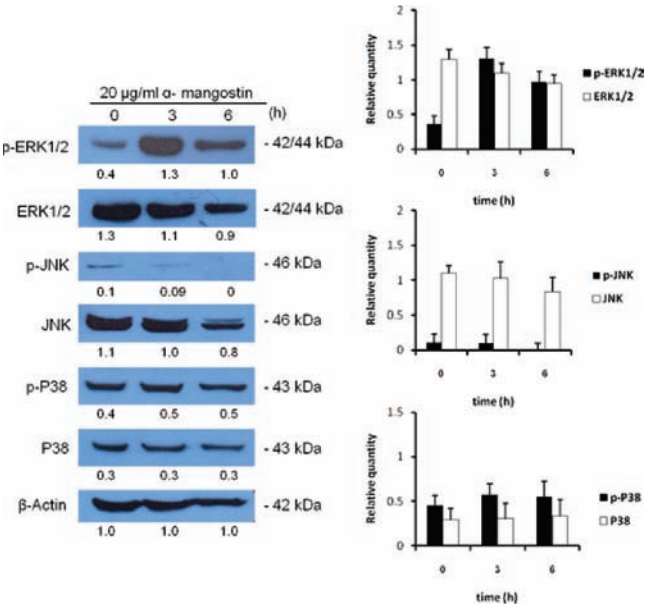

Figure 7. Effects of α-mangostin on expression of ERK1/2, phosphorylated ERK1/2 (p-ERK1/2), p38, phosphorylated p38 (p-p38), JNK, phosphorylated JNK (p-JNK) in SW1353. Cells were treated with 20 μg/mL α-mangostin for 3 and 6 h and examined by Western blot analysis. β-Actin was used as the internal control.

was also conducted (Figure 2). As shown in Figure 1C, cleavage of DNA at the internucleosomal linker sites yielding DNA fragments in multiple fragments (180−200 bp) is regarded as a biochemical hallmark of apoptosis.[22] Furthermore, in early apoptosis phosphatidylserine is translocated from the inner to outer surface of the plasma membrane due to the loss of membrane asymmetry which can be detected by Annexin V. Flow cytometry analysis demonstrated phosphatidylserine exposure in α-mangostin treated cells, and the percentage of early apoptotic and late apoptotic cells increased with the incubation time (Figure 3).

It is now well-known that apoptotic signaling proceeds through two main pathways, the extrinsic and intrinsic pathways. The extrinsic pathway is triggered through the binding of death

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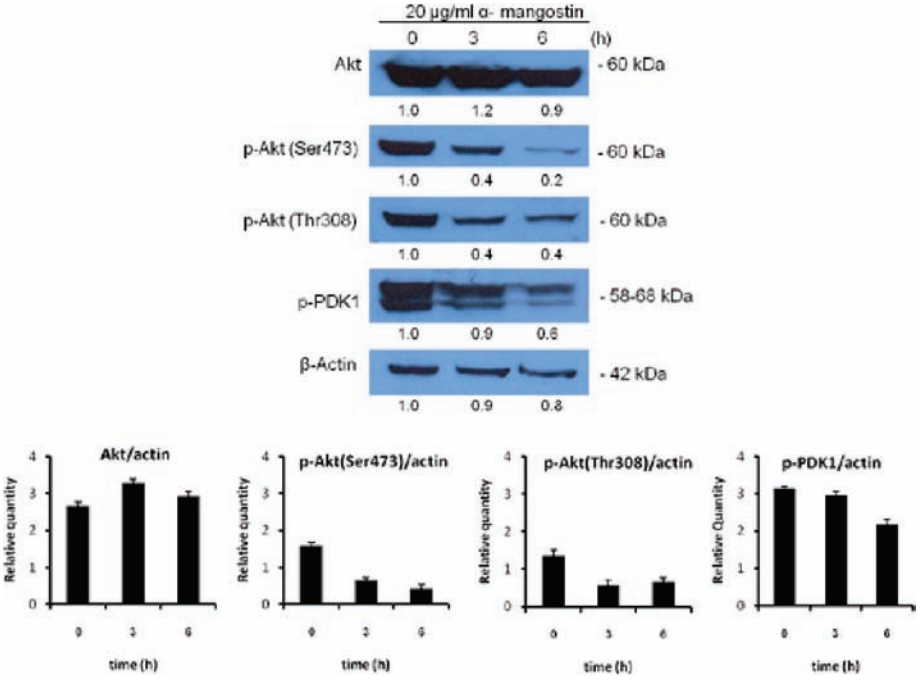

Figure 8. Effects of α-mangostin on expression of Akt and phosphorylated Akt (p-Akt) in SW1353 cells. Cells were treated with 20 μg/mL α-mangostin for 3 and 6 h and examined by Western blot analysis. β-Actin was used as the internal control.

ligands and death receptors on the cell surface resulting in activation of initiator caspase-8. The intrinsic or mitochondrial pathway is characterized by depolarization of mitochondrial membrane, leading to the release of cytochrome *c* and caspase-9 activation. The intrinsic pathway is regulated by Bcl-2 family of proteins which are composed of both pro- and antiapoptotic molecules.[23] Antiapoptotic Bcl-2 and proapoptotic Bax are two of the major members of the Bcl-2 family. During apoptosis, antiapoptotic Bcl-2 protects cells from many different apoptotic stimuli. On the other hand, the proapoptotic Bax interacts with the permeability transition pores to facilitate protein movement through the mitochondrial membrane, leading to the release of cytochrome *c* from the intermembrane space into cytoplasm. Subsequently, activated caspase-8 and -9 promote caspase-3 cleavage and activation, resulting in nuclear DNA degradation and apoptotic death of cells. In our study, activated caspase-3, -8, and -9 were detected in the presence of α-mangostin (Figure 4), implying that the induction of apoptosis in SW1353 cells by α-mangostin may be associated with the activation of both pathways. Previous studies report that caspase-8 can be activated by caspase-3 and caspase-8-mediated Bid cleavage appears to be downstream of mitochondria-mediated pathway.[24,25] As a result, tBid could not be detected (Figure 5), therefore further studies about the binding of death ligands and death receptors are needed to confirm the extrinsic pathway of apoptosis. However, α-mangostin could induce the release of cytochrome *c*, upregulation of Bax and downregulation of Bcl-2 (Figure 5-6). These results suggested that α-mangostin could induce apoptosis in SW1353 cells through the mitochondrial pathway.

The MAPK family plays critical roles in cell survival and death. Three major mammalian MAPK subfamilies, ERK, JNK and p38, were activated through a specific phosphorylation cascade. It is well-known that JNK and p38 induce apoptosis, while ERK promotes cell survival.[26] Previous studies have shown that upregulation of MAPK phosphorylation is involved in migration of chondrosarcoma cells.[27,28] Therefore, downregulation of MAPK phosphorylation may be an appropriate alternate therapy for chondrosarcoma patients. In our study, α-mangostin was shown to downregulate the total and phosphorylated ERK1/2 and JNK proteins but not p38 (Figure 7). However, the phosphorylated ERK1/2 was increased at 3 h and decreased at 6 h after treatment with α-mangostin. As reported,[17] the phosphorylated ERK1/2 indicated two peaks, one at the early phase (0.5−3 h) and the other at the late phase (24−48 h) of the treatment with α-mangostin in DLD-1 cells. ERK1/2 may play dual roles, as a cellular adaptive response during the initial phase and a cytotoxic response during the later stages of such stress. Therefore, the decline in the phosphorylated ERK1/2 may be associated with the apoptotic machinery.

Akt signaling pathway is essential for cell survival, and the expression of a constitutively active Akt or an increased activity of the PI3K/Akt pathway leads to multidrug resistance and prevent apoptosis in a variety of cell types.[29,30] Our results showed that α-mangostin could decrease the phosphorylation of Akt in chondrosarcoma cells (Figure 8). These results correspond with much research that reported downregulating of ERK/Akt pathway induced apoptosis in cancer cells. For instance, Lu et al. showed that sorafenib induced apoptosis via inhibition of the ERK pathway in human chondrosarcoma cells.[31] Fei et al. reported that perifosine induced apoptosis in human hepatocellular carcinoma cells by inhibition of ERK/AKT phosphorylation.[32] Chai et al. demonstrated that downregulation of ERK/Akt is associated with sorafenib-induced apoptosis in human neuroblastoma cells.[33]

In conclusion, we demonstrated that α-mangostin inhibited cell proliferation and induced apoptosis in chondrosarcoma cells, which may be through downregulating the ERK, JNK and Akt signaling pathway. These data suggest the potential clinical

application of α-mangostin in the treatment of human chondrosarcoma.

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Funding Sources

This work was supported by Srinakharinwirot Research Budget, Research Division, Srinakharinwirot University and "the program Strategic Scholarships for Frontier Research Network for the Join Ph.D. Program Thai Doctoral degree" from the Office of the Higher Education Commission, Thailand (to A.K.).

REFERENCES

(1) Gelderblom, H.; Hogendoorn, P. C.; Dijkstra, S. D.; van Rijswijk, C. S.; Krol, A. D.; Taminiau, A. H.; Bovee, J. H. The clinical approach towards chondrosarcoma. *Oncologist* **2008**, *13*, 320–329.

(2) Marco, R. A.; Gitelis, S.; Brebach, G. T.; Healey, J. H. Cartilage tumors: evaluation and treatment. *J. Am. Acad. Orthop. Surg.* **2000**, *8*, 292–304.

(3) Ollivier, L.; Vanel, D.; Leclere, J. Imaging of chondrosarcomas. *Cancer Imaging* **2003**, *4*, 36–38.

(4) Wesolowski, R.; Budd, G. T. Use of chemotherapy for patients with bone and soft-tissue sarcomas. *Cleveland Clin. J. Med.* **2010**, *77* s23–s26.

(5) Mahabusarakam, W.; Wiriyachtra, P.; Taylor, W. Chemical constituents of *Garcinia mangostana*. *J. Nat. Prod.* **1987**, *50*, 474–478.

(6) Jung, H. A.; Su, B. N.; Keller, W. J.; Mehta, R. G.; Kinghorn, A. D. Antioxidant xanthones from the pericarp of *Garcinia mangostana* (Mangosteen). *J. Agric. Food Chem.* **2006**, *54*, 2077–2082.

(7) Suksamrarn, S.; Suwannapoch, N.; Phakhodee, W.; Thanuhiranlert, J.; Ratananukul, P.; Chimnoi, N.; Suksamrarn, A. Antimycobacterial activity of prenylated xanthones from the fruits of *Garcinia mangostana*. *Chem. Pharm. Bull.* **2003**, *51*, 857–859.

(8) Gopalakrishnan, G.; Banumathi, B.; Suresh, G. Evaluation of the antifungal activity of natural xanthones from *Garcinia mangostana* and their synthetic derivatives. *J. Nat. Prod.* **1997**, *60*, 519–524.

(9) Chen, L. G.; Yang, L. L.; Wang, C. C. Anti-inflammatory activity of mangostins from *Garcinia mangostana*. *Food Chem. Toxicol.* **2008**, *46*, 688–693.

(10) Suksamrarn, S.; Komutiban, O.; Ratananukul, P.; Chimnoi, N.; Lartpornmatulee, N.; Suksamrarn, A. Cytotoxic prenylated xanthones from the young fruit of *Garcinia mangostana*. *Chem. Pharm. Bull.* **2006**, *54*, 301–305.

(11) Zhang, Y.; Song, Z.; Hao, J.; Qiu, S.; Xu, Z. Two new prenylated xanthones and a new prenylated tetrahydroxanthone from the pericarp of *Garcinia mangostana*. *Fitoterapia* **2010**, *81*, 595–599.

(12) Moongkarndi, P.; Kosem, N.; Kaslungka, S. Antiproliferation, antioxidation and induction of apoptosis by *Garcinia mangostana* (mangosteen) on SKBR3 human breast cancer cell line. *J. Ethnopharmacol.* **2004**, *90*, 161–166.

(13) Hung, S. H.; Shen, K. H.; Wu, C. H.; Liu, C. L.; Shin, Y. W. α-mangostin suppresses PC-3 human prostate carcinoma cell metastasis by inhibiting matrix metalloproteinase-2/9 and urokinase-plasminogen expression through the JNK signaling pathway. *J. Agric. Food Chem.* **2009**, *57*, 1291–1298.

(14) Matsumoto, K.; Akao, Y.; Emi, K.; Ohguchi, K.; Ito, T.; Tanaka, T.; Iinuma, M.; Nozawa, Y. Induction of apoptosis by xanthones from mangosteen in human leukemia cell lines. *J. Nat. Prod.* **2003**, *66*, 1124–1127.

(15) Chao, A. C.; Hsu, Y. L.; Liu, C. K.; Kuo, P. L. α-Mangostin, a dietary xanthone, induces autophagic cell death by activating the AMP-activated protein kinase pathway in glioblastoma cells. *J. Agric. Food Chem.* **2011**, *59*, 2086–2096.

(16) Matsumoto, K.; Akao, Y.; Ohguchi, K.; Ito, T.; Tanaka, T.; Iinuma, M.; Nozawa, Y. Xanthones induce cell-cycle arrest and apoptosis in human colon cancer DLD-1 cells. *Bioorg. Med. Chem.* **2005**, *13* 60646069.

(17) Nakagawa, Y.; Iinuma, M.; Naoe, T.; Nozawa, Y.; Akao, Y. Characterized mechanism of α-mangostin-induced cell death: Caspase-independent apoptosis with release of endonuclease-G from mitochondria and increased miR-143 expression in human colorectal cancer DLD-1 cells. *Bioorg. Med. Chem.* **2007**, *16*, 5620–5628.

(18) Watanapokasin, R.; Jarinthanan, F.; Jerusalmi, A.; Suksamrarn, S.; Nakamura, Y.; Sukseree, S.; Uthaisang-Tanethpongtamb, W.; Ratananukul, P.; Sano, T. Potential of xanthones from tropical fruit mangosteen as anti-cancer agents: caspase-dependent apoptosis induction in vitro and in mice. *Appl. Biochem. Biotechnol.* **2010**, *162*, 1080–1094.

(19) Shimizu, S.; Narita, M.; Tsujimoto, Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* **1999**, *399*, 483–487.

(20) Boutros, T.; Chevet, E.; Metrakos, P. Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. *Pharmacol. Rev.* **2008**, *60*, 261–310.

(21) Alessi, D. R.; Andjelkovic, M.; Caudwell, B.; Cron, P.; Morrice, N.; Cohen, P.; Hemmings, B. A. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **1996**, *15*, 6541–6551.

(22) Rello, S.; Stockert, J.; Moreno, V.; Gamez, A.; Pacheco, M.; Juarranz, A.; Canete, M.; Villanueva, A. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis* **2005**, *10*, 201–208.

(23) Gross, A.; McDonnell, J. M.; Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev.* **1999**, *13*, 1899–1911.

(24) Chen, C. J.; Makino, S. Murine coronavirus-induced apoptosis in 17Cl-1 cells involves a mitochondria-mediated pathway and its downstream caspase-8 activation and Bid cleavage. *Virology* **2002**, *302*, 321–332.

(25) Oh, S. H.; Lee, B. H. A ginseng saponin metabolite-induced apoptosis in HepG2 cells involves a mitochondria-mediated pathway and its downstream caspase-8 activation and Bid cleavage. *Toxicol. Appl. Pharmacol.* **2004**, *194*, 221–229.

(26) Xia, Z.; Dickens, M.; Raingeaud, J.; Davis, R. J.; Greenberg, M. E. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **1995**, *270*, 1326–1331.

(27) Chen, Y. J.; Wei, Y. Y.; Chen, H. T.; Fong, Y. C.; Hsu, C. J.; Tsai, C. H.; Hsu, H. C.; Liu, S. H.; Tang, C. H. Osteopontin increases migration and MMP-9 up-regulation via alphavbeta3 integrin, FAK, ERK, and NF-kappaB-dependent pathway in human chondrosarcoma cells. *J. Cell Physiol.* **2009**, *221*, 98–108.

(28) Tan, T. W.; Lai, C. H.; Huang, C. Y.; Yang, W. H.; Chen, H. T.; Hsu, H. C.; Fong, Y. C.; Tang, C. H. CTGF enhances migration and MMP-13 up-regulation via alphavbeta3 integrin, FAK, ERK, and NF-kappaB-dependent pathway in human chondrosarcoma cells. *J. Cell Biochem.* **2009**, *107*, 345–356.

(29) McCubrey, J. A.; Steelman, L. S.; Abrams, S. L.; Lee, J. T.; Chang, F.; Bertrand, F. E.; Navolanic, P. M.; Terrian, D. M.; Franklin, R. A.; D'Assoro, A. B.; Salisbury, J. L.; Mazzarino, M. C.; Stivala, F.; Libra, M. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv. Enzyme Regul.* **2006**, *46*, 249–279.

(30) Knuefermann, C.; Lu, Y.; Liu, B.; Jin, W.; Liang, K.; Wu, L.; Schmidt, M.; Mills, G. B.; Mendelsohn, J.; Fan, Z. HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. *Oncogene* **2003**, *22*, 3205–3212.

(31) Lu, X.; Tang, X.; Guo, W.; Ren, T.; Zhao, H. Sorafenib induces growth inhibition and apoptosis of human chondrosarcoma cells by blocking the RAF/ERK/MEK pathway. *J. Surg. Oncol.* **2010**, *102*, 821–826.

(32) Fei, H. R.; Chen, G.; Wang, J. M.; Wang, F. Z. Perifosine induces cell cycle arrest and apoptosis in human hepatocellular carcinoma cell lines by blockade of Akt phosphorylation. *Cytotechnology* **2010**, *62*, 449–460.

(33) Chai, H.; Luo, A. Z.; Weerasinghe, P.; Brown, R. E. Sorafenib downregulates ERK/Akt and STAT3 survival pathways and induces apoptosis in a human neuroblastoma cell line. *Int. J. Clin. Exp. Pathol.* **2010**, *3*, 408–415.