JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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

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α-Mangostin, a component of *Garcinia mangostana* Linn, is a xanthone derivative shown to have antioxidant and anticarcinogen properties. In this study, we first report the antimetastatic effect of α-mangostin in the human prostate carcinoma cell line PC-3. The results show that α-mangostin exhibited an inhibitory effect on the abilities of adhesion, migration, and invasion by cell-matrix adhesion assay, wound healing assay, and Boyden chamber assay. In the cancer cell metastasis process, matrix degrading proteinases are required. Results from zymography showed that α-mangostin treatment could decrease the expressions of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and urokinase-plasminogen activator (u-PA) in a concentration-dependent manner. Moreover, α-mangostin also exerted an inhibitory effect on phosphorylation of c-Jun N-terminal kinase 1 and 2 (JNK1/2) and inhibition of activation of nuclear factor kappa B (NF-κB), c-Fos, and c-Jun. Furthermore, the treatment of inhibitors specific for JNK (SP600125) to PC-3 cells could result in a reduced expression of MMP-2, MMP-9, and u-PA. These results demonstrated that α-mangostin could mediate PC-3 cells metastasis by reduction of MMP-2, MMP-9, and u-PA expression through the suppression of the JNK1/2 signaling pathway and inhibition of NF-κB and AP-1 binding activity. These findings proved that α-mangostin might be offered further application as an antimetastatic agent.

KEYWORDS: α-Mangostin; metastasis; MMP-2; MMP-9; u-PA; NF-κB; AP-1

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in older men and one of the major life-threatening diseases in Western countries (*1*). The incidence of prostate cancer has been increasing in Taiwan over the past 2 decades (*2*). Nearly 50% of prostate cancer patients present with pathologic or clinical evidence of bone metstasis (*3*). Thus, metastasis has been a major challenge for a successful cancer treatment to improve patient outcome.

Mangosteen (*Garcinia mangostana* Linn.) belongs to the family of Guttiferae and is named "the queen of fruits" (*4*). It is widely cultivated in the tropical rainforest of some Southeast Asian nations such as Thaliand, Sir Lanka, Malaysia, and Indonesia to produce a popular refreshing juicy fruit in the summer. The edible portion of mangosteen is milky white, whereas the pericarp is dark red and composes about two-thirds of the whole fruit weight as agricultural waste. In fact, the nonedible pericarps have been used for treating truma, diarrhea, and skin infections in Southeast Asia for many years (*5*, *6*). Moreover, the pericarps are rich in xanthones and anthocyanins. The xanthones, α-, *β*-, and *γ*-mangostins, are the major bioactive compounds found in the pericarps of the mangosteen (*7*, *8*). The biological activities of α-mangostin (**Figure 1**) have been

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10.1021/jf8032683 CCC: $40.75
Published on Web 01/29/2009

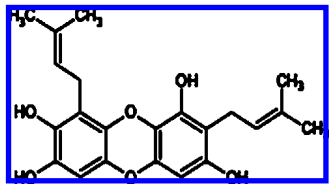

Figure 1. Chemical structure of α-mangostin isolated from the pericarps of mangosteen (*Garcinia mangostana* Linn).

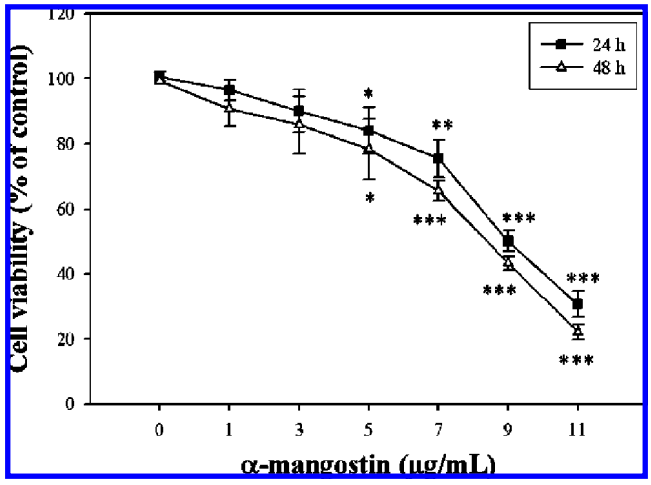

Figure 2. Effect of α-mangostin on the viability of PC-3 cells. PC-3 cells (3×10^4 cells/mL) were treated with various concentrations (0, 1, 3, 5, 7, 9, and 11 μg/mL) of α-mangostin for 24 and 48 h. Cell viability was determined by MTT assay. The survival cell number was directly proportional to that of formazan, which was measured spectrophotometrically at 563 nm. Values are expressed as mean ± SD of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the untreated control (dose 0).

confirmed to consist of antiproliferative and apoptotic effects on human hepatoma (*9*), breast cancer (*10*), and colorectal cancer (*11*). However, the precise impact and related molecular mechanism of α-mangostin on metastasis of cancer cells were still unclear.

Metastasis of cancer cells involves multistep processes and various cytophysiological changes, including changed adhesion ability between cells and the extracellular matrix (ECM) and damaged intercellular interaction. Excess ECM degradation is one of the hallmarks of tumor invasion and migration. The metastasis is involving an overexpression of proteolytic enzymes, such as matrix metalloproteinases (MMPs) and u-PA. MMP-2 and MMP-9 (also known as type IV collagenases or gelatinases) are capable of degrading most ECM components that form the basal membrane (*12*). In addition, u-PA may initiate the activation of an enzymatic cascade and convert the zymogen plasminogen to plasmin (*13*). Meanwhile, The activation of these enzymes enables the degradation of the extracellular matrix (ECM) by tumor cells, allowing their access to the vasculature, migration, and invasion into the target organ and development of tumor metastasis (*14*).

As well as MMPs and u-PA, the mitogen-activated protein kinase family members (MAPK) are also known to mediate metastasis. The MAPK serine/threonine kinase superfamily is activated by numerous extracellular stimuli and is involved in signal transduction cascades that play an important regulatory role in cell growth, differentiation, apoptosis, and metastasis (*15*). Three major mammalian MAP kinases have been described: ERK1/2 or p44/42 MAPK, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK. The diverse MAP kinase members are activated in response to different extracellular stimuli and have distinct downstream targets, thus serving different roles in cellular responses. ERK1/2, p38 MAPK, and JNK/SAPK play a central role in regulating the expression of MMPs and u-PA (*16*−*18*). Inhibition of the MAPK pathway might have the potential to prevent angiogenesis, proliferation, invasion, and metastasis for a wide range of tumors. In addition, the PI3K/Akt signal transduction pathway regulates cell metastasis of PC-3 and is closely associated with the development and progress of various tumors (*19*).

NF-κB is a multisubunit transcription factor involved in cellular responses to viral infection and inflammation. The active NF-κB consists of a dimer of a REL family/p65 subunit and a p50 or p52 subunit. NF-κB is maintained in the cytoplasm through interactions with an inhibitor of NF-κB (IκB), but upon dissociation, it moves into the nucleus and promotes cancer cell proliferation, angiogenesis, and metastasis. c-Fos and c-Jun also are important transcription factors and oncogenes, which form a heterodimer (AP-1 complex). They are associated with invasion and metastasis of cancer cells (*20*).

Cancer metastasis is highly related to degradation of ECM, intercellular adhesion, and cellular motility. In this study, we observed the impact of α-mangostin on several relevant proteases, including MMP-2, MM-9, and u-PA of human prostate carcinoma PC-3 cells. Therefore, we investigated whether α-mangostin inhibited PC-3 cell invasion and migration and explored the molecular mechanism by which α-mangostin inhibited tumor metastasis.

MATERIALS AND METHODS

Materials. α-Mangostin, DMSO, Tris-HCl, EDTA, SDS, phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), gelatin, casein, plasminogen, type I collagen, crystal violet, leupeptin, Nonidet P-40, deoxycholic acid, sodium orthovanadate, SP600125, and trypsin-EDTA were purchased from Sigma-Aldrich (St. Louis, MO); the protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Dulbecco's phosphate buffer solution (PBS) and F-12K medium were purchased from Gibco/BRL (Gaithersburg, MD). Matrigel was from BD Biosciences (Bedford, MA). Antibody against PKB/Akt, MAPK/ERK1/2, p38 MAPK, and JNK/SAPK, proteins, and phosphorylated proteins were purchased from Cell Signaling Tech. (Beverly, MA). NF-κB (p65), c-Fos, c-Jun, β-Actin, and C23 antibodies were from BD Transduction Laboratories (San Diego, CA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science (Amersham, U.K.).

Cell Culture and α-Mangostin Treatment. PC-3, a human prostate carcinoma cell line, was obtained from BCRC (Food Industry Research and Development Institute in Hsin-Chu, Taiwan). Cells were cultured in F-12K supplemented with 10% fetal calf serum, 100 U/mL of penicillin and 100 mg/mL streptomycin mixed antibiotics, and 1 mM sodium pyruvate. All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2—95% air. The culture medium was renewed every 2−3 days. Adherent cells were detached by incubation with trypsin. For α-mangostin treatment, the stock solution of α-mangostin was dissolved in dimethyl sulfoxide (DMSO) and sterilized by filtration through 0.2 μm disc filters. Appropriate amounts of stock solution (1 mg/mL in DMSO) of α-mangostin were added into the cultured medium to achieve the indicated concentrations (the final DMSO concentration was less than 0.2%) and then incubated with cells for the indicated time periods.

Analysis of Cell Viability (MTT Assay). To evaluate the cytotoxicity of α-mangostin, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed to determine the cell viability. Briefly, cells were seeded at a density of 3×10^4 cells/mL in a 24-well plate for 24 h. Then, the cells were treated with α-mangostin at various concentrations (0, 1, 3, 5, 7, 9, and 11 μg/mL) for various periods of time (24 and 48 h). Each concentration was repeated three times. After the exposure period, medium was removed that was followed by washing the cells with PBS. Then, the medium was

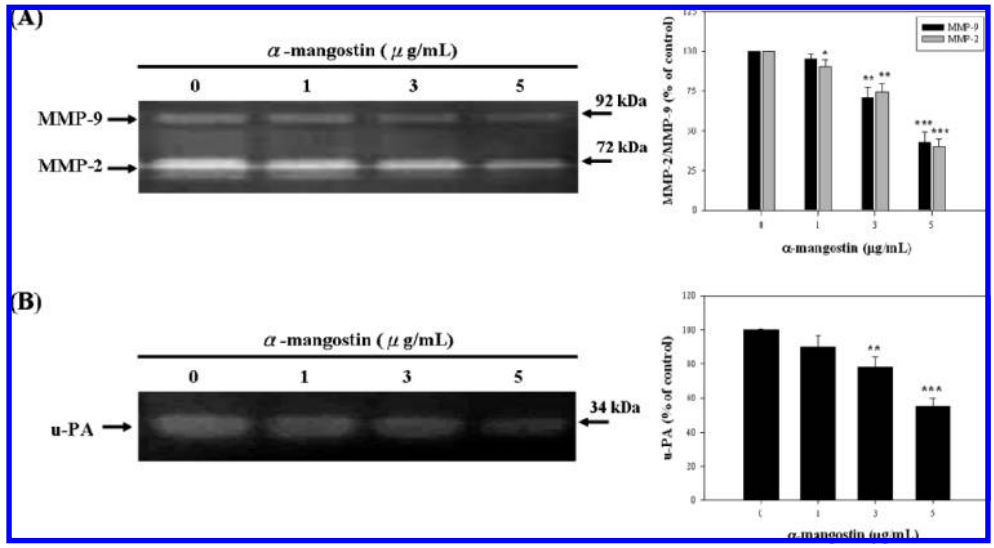

Figure 3. Effect of α-mangostin on the MMP-2/MMP-9 and u-PA activities of PC-3 cells. Cells were treated with various concentrations (0, 1, 3, and 5 μg/mL) of α-mangostine for 24 h. The conditioned media were collected, and MMP-2/MMP-9 and u-PA activities were determined by gelatin or casein zymography. MMP-2/MMP-9 and u-PA activity were quantified by densitomeric analysis. The densitomeric data were expressed as mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with the untreated control (dose 0).

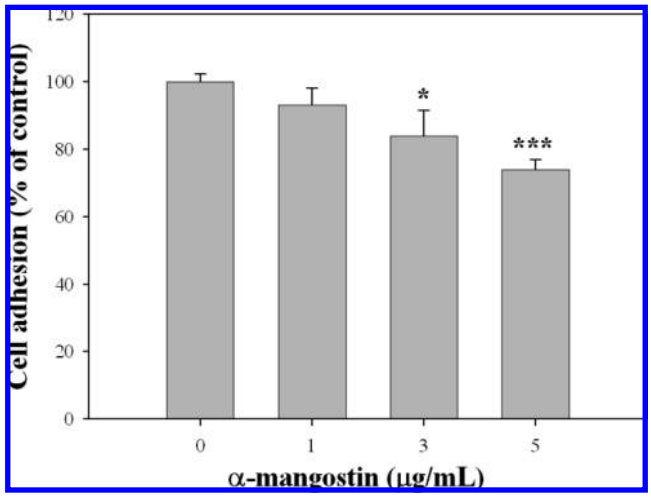

Figure 4. Effect of α-mangostin on the cell-matrix adhesion of PC-3 cells. Cells were treated with various concentrations (0, 1, 3, and 5 μg/mL) of α-mangostin for 24 h and were then subjected to analyses for cell-matrix adhesion as described in the MATERIALS AND METHODS. Values are expressed as mean ± SD of three independent experiments. $*p < 0.05$, $***p < 0.001$ compared with the untreated control (dose 0).

changed and incubated with MTT solution [(5 mg/mL)/well for 4 h]. The medium was removed, and formazan was solubilized in isopropanol and measured spectrophotometrically at 563 nm. The percentage of viable cells was estimated by comparison with untreated control cells.

Analysis of MMP-2, MMP-9, and u-PA Activity by Zymography. The activities of MMP-2 and MMP-9 were assayed by gelatin zymography. Briefly, conditioned media from cells cultured in the absence of serum for 24 h were collected. Samples were mixed with loading buffer and were electrophoresed on 8% SDS-polyacrylamide gel containing 0.1% gelatin. Electrophoresis was performed at 140 and 110 V for 3 h. Gels were then washed twice in zymography washing buffer (2.5% Triton X-100 in double-distilled H_2O) at room temperature to remove SDS, followed by incubation at 37 °C for 12−16 h in zymography reaction buffer (40 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$, 0.02% NaN_3), stained with Coomassie blue R-250 (0.125% Comassie blue R-250, 0.1% amino black, 50% methanol, 10% acetic acid) for 1 h, and destained with destaining solution (20% methanol, 10% acetic acid, 70% double-distilled H_2O). Nonstaining bands representing the levels of the latent form of MMP-2 and MMP-9 were quantified by densitometer measurement using a digital imaging analysis system.

Visualization of u-PA activity was performed by casein-plasminogen zymography. Briefly, 2% casein and 20 μg/mL plasminogen were added to 8% SDS-PAGE gel. Samples of about 20 μg total protein were then loaded onto the gels. The u-PA activity of cells treated or untreated with α-mangostin was measured as described in the gelatin zymography section.

Cell-Matrix Adhesion Assay. After a pretreatment with α-mangostin (0, 1, 3, and 5 μg/mL) for 24 h, cells were seeded on a 24-well plate and coated with 150 μL of type I collagen (10 μg/mL); then they were cultured for 30 min. Afterward, nonadherent cells were removed by PBS washes, and adherent cells were fixed in ethanol. After a staining with 0.1% crystal violet, fixed cells were lysed in 0.2% Triton-100 and measured spectrophotometrically at 550 nm.

Wound Healing Assay. For cell motility determination, PC-3 cells (1×10^5cells/mL) were seeded in a 6-well tissue culture plate and grown to 80−90% confluence. After aspirating the medium, in the center of the cell, monolayers were scraped with a sterile micropipet tip to create a denuded zone (gap) of constant width. Subsequently, cellular debris was washed with PBS, and PC-3 cells were exposed to various concentrations of α-mangostin (0, 1, 3, and 5 μg/mL). The wound closure was monitored and photographed at 0, 12, 24, 36, and 48 h with an Olympus CKX-41 inverted microscope and an Olympus E-410 camera. To quantify migrated cells, pictures of the initial wounded monolayers were compared with the corresponding pictures of cells at the end of the incubation. Artificial lines fitting the cutting edges were drawn on pictures of the original wounds and overlaid on the pictures of cultures after incubation. Migrated cells across the white lines were counted in six random fields from each triplicate treatment, and data are presented as mean ± SD.

Boyden Chamber Invasion and Migration Assay. The ability of PC-3 cells for passing through matrigel-coated filters was measured by the Boyden chamber invasion assay. Matrigel (BD Biosciences, Bedford, MA) was diluted to 200 μg/mL with cold filtered distilled water and applied to the top side of the 8-μm pore polycarbonate filter. Briefly, PC-3 cells were treated with various concentrations of α-mangostin. After 48 h, cells were detached by trypsin and resuspended in serum-free medium. Medium containing 10% FBS-medium was applied to the lower chamber as chemoattractant, and then cells were seeded on the upper chamber at a density of 1×10^5 cells/well in 50 μL of serum-free medium. The chamber was incubated for 8 h at 37 °C. At the end of incubation, the cells in the upper surface of the membrane were carefully removed with a cotton swab and cells that invaded across the matrigel to the lower surface of the membrane were

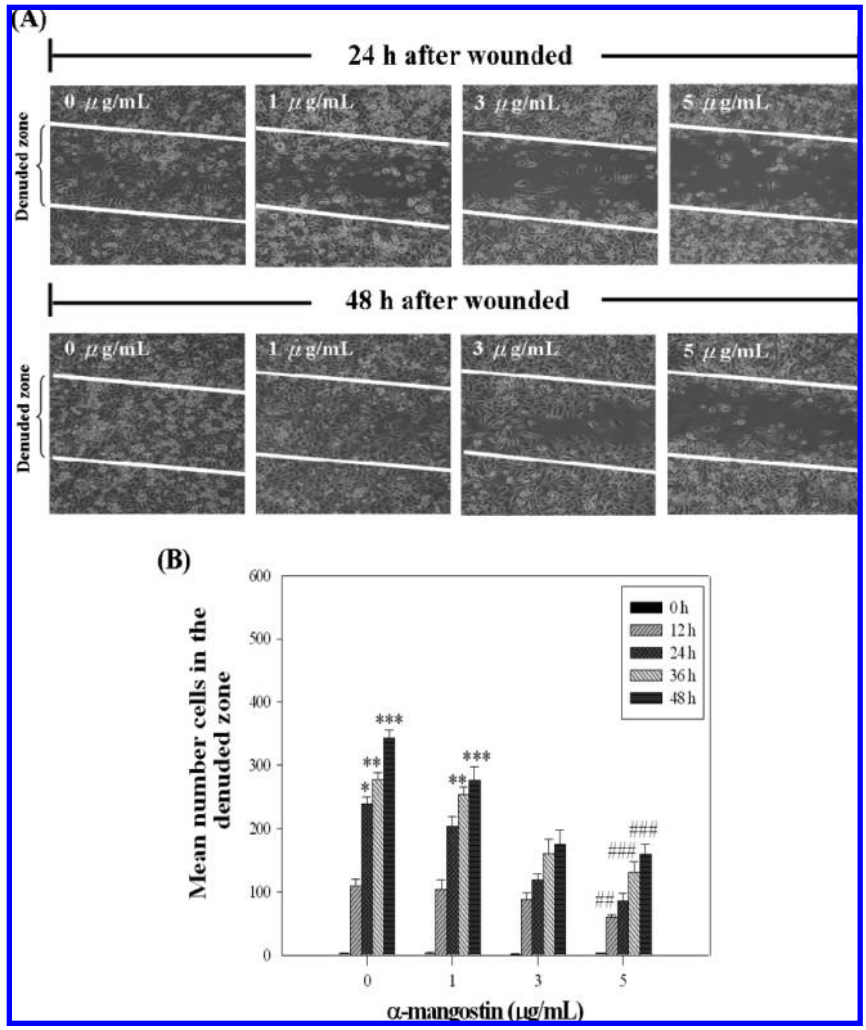

Figure 5. Effect of α-mangostin on the motility of PC-3 cells. (**A**) PC-3 cell monolayers were scraped by a sterile micropipet tip, and the cells were treated with various concentrations (0, 1, 3, and 5 μg/mL) of α-mangostin for 0, 12, 24, 36, and 48 h. The number of cells in the denuded zone was quantitated after indicated times (0, 12, 24, 36, and 48 h) by inverted microscopy. White lines indicate the wound edge. Pictures were presented at only 24 and 48 h. (**B**) Migrated cells across the white lines were counted in six random fields from each treatment. Quantitative assessment of the mean number of cells in the denuded zone is expressed as mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the untreated control (dose 0); $\#\#p < 0.01$, $\#\#\#p < 0.001$ compared with the 0 h treated time.

fixed with methanol and stained with 5% Giemsa solution. The invasive cells on the lower surface of the membrane filter were counted with a light microscope. The data are presented as the average number of cells attached to the bottom surface from randomly chosen fields. Each experiment was carried out in triplicate.

To measure the ability of PC-3 cells on migration, cells were seeded into a Boyden chamber with 8 μm pore polycarbonate filters which were not coated with matrigel. Migration of cells was treated with various concentrations of α-mangostin. The migration assay was measured as described in the invasion assay.

Preparation of Whole-Cell Lysates and Nuclear Extracts. The cells were lysed with iced-cold RIPA buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5), and then the following were added: phenylmethylsulfonyl fluoride (10 mg/mL), leupeptin (17 mg/mL), and sodium orthovanadate (10 mg/mL). The samples were vortex mixed for 30 min on ice and then centrifuged at 12000*g* for 10 min. Then the supernatants were collected, denatured, and subjected to SDS-PAGE and Western blotting. Nuclear extracts were prepared as previously described and then used for NF-κB, c-Fos, c-Jun, and AP-1 detection. Each nuclear pellet was resuspended in nuclear extract buffer (1.5 mM $MgCl_2$, 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 420 mM NaCl). The nuclear suspension was incubated on ice for 20 min and then centrifuged at 14000*g* for 5 min. The supernatant (corresponding to the soluble nuclear fraction) was saved, and the remaining pellet was solubilized by sonication in PBS. The protein content was determined with Bio-Rad protein assay reagent using bovine serum albumin as a standard.

Western Blotting Analysis. To analyze the metastasis-related proteins, Western blotting was performed as follows. The denatured samples (50 μg of purified protein) were resolved on 10−12% SDS-PAGE gels. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 (TBST) for more than 2 h. Membranes were washed with TBST three times for 10 min and incubated with appropriate dilution of specific primary antibodies in TBST overnight at 4 °C. Subsequently, the membranes were washed with TBST and incubated with appropriate secondary antibody (horseradish peroxidase-conjugated goat antimouse or antirabbit IgG) for 1 h. After washing the membrane three times for 10 min in TBST, the band detection was revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed ECL hyperfilm in a UVP luminescent image analyzer.

Analysis of NF-κB and AP-1 Binding Assay (Electrophoretic Mobility Shift Assay). Cell nuclear proteins were extracted with a nuclear extract buffer and measured by an electrophoretic mobility shift assay (EMSA). Cells (1×10^5/mL) were collected in PBS buffer (pH 7.4) and centrifuged at 2000*g* for 5 min at 4 °C. Cells were lysed with buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT,

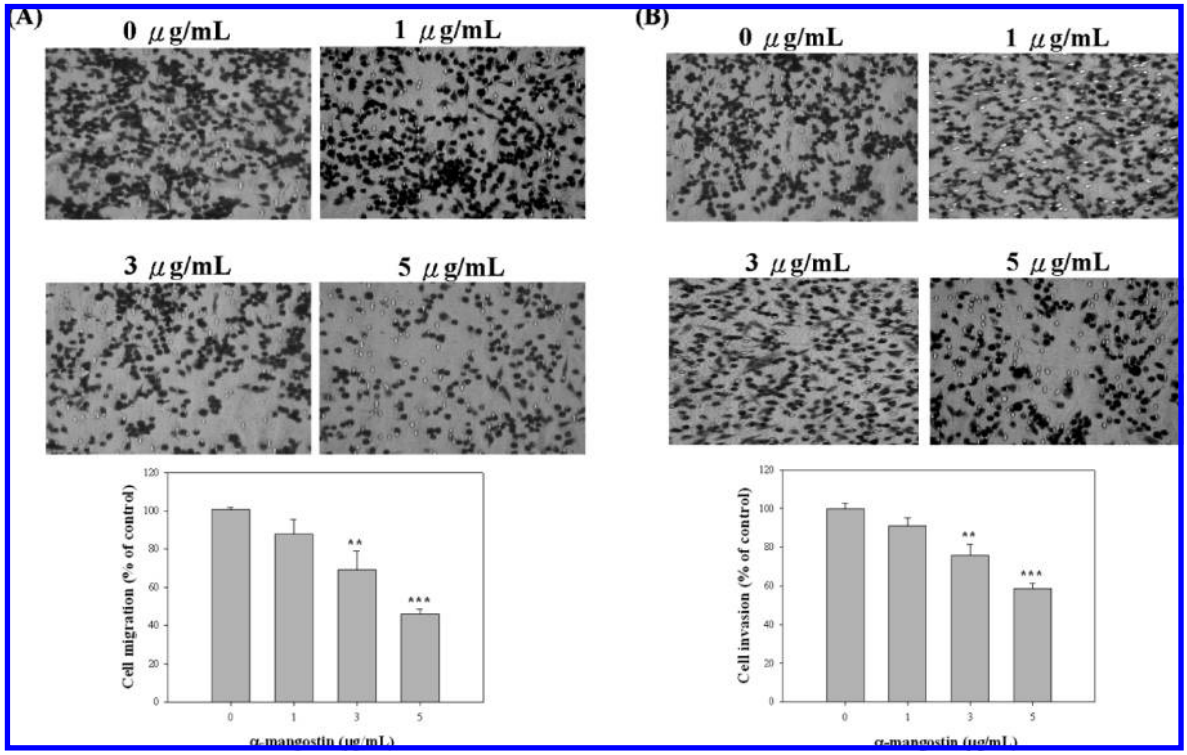

Figure 6. Effect of α-mangostin on migration and invasion of PC-3 cells. PC-3 cells were treated with various concentrations (0, 1, 3, and 5 μg/mL) of α-mangostin for 48 h. (**A**) Cell migration was measured in a Boyden chamber for 6 h with polycarbonate filters (pore size, 8 μm); (**B**) cell invasion was measured in a Boyden chamber for 8 h; polycarbonate filters (pore size, 8 μm) were precoated with matrigel. Migration and invasion ability of PC-3 cells were quantified by counting the number of cells that invaded the underside of the porous polycarbonate membrane under microscopy and represent the average of three experiments ± SD [$^{**}p < 0.01$, $^{***}p < 0.001$ compared with the untreated control (dose 0)].

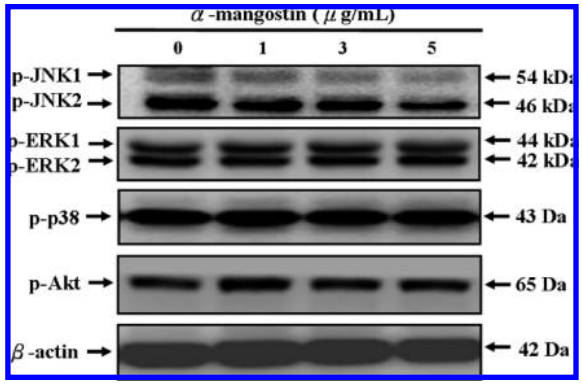

Figure 7. Inhibitory effect of α-mangostin on the phosphorylation of JNK1/2. Cells were treated with various concentrations (0, 1, 3, and 5 μg/mL) of α-mangostin for 3 h, and then cell lysates were subjected to SDS-PAGE followed by Western blotting and immunoprobing with antiphospho-JNK1/2, antiphospho-ERK1/2, antiphospho-p38, and antiphospho-Akt antibodies. β-Actin was used as a loading control. The relative densities of phosphorylated forms of JNK, ERK, p38, and Akt were normalized to total values of JNK, ERK, p38, and Akt, which were determined by densitometric analysis. Results from three repeated and separated experiments were similar.

and 0.5 mM PMSF (pH 7.9) containing 5% NP-40) for 10 min on ice, and this was followed by vortexing to shear the cytoplasmic membranes. The lysates were centrifuged at 2000*g* for 10 min at 4 °C. The pellet containing the nuclei was extracted with high salt buffer B (20 mM HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, and 25% glycerol) for 15 min on ice. The lysates were clarified by centrifuge at 13000*g* for 10 min at 4 °C. The supernatant containing the nuclear proteins was collected and frozen at −80 °C until use. The protein content of nuclear fractions was determined with a Bio-Rad protein assay. Five microgram aliquots of nuclear proteins were mixed with either biotin-labeled NF-κB or AP-1 oligonucleotide probes for 15 min at room temperature. Oligonucleotides containing (sense of NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, antisense of NF-κB, 3′-TCAACTCCCCTGAA AGGGTCCG-5′; sense of AP-1, 5′-CGCTTGATGACTCAGCCGGAA-3′, antisense of AP-1, 3′-G CGAACTACTGAGTCGGCCTT) DNA probes were added to 10 μL binding reactions containing double-distilled H_2O, 5 μg of nuclear protein, 1 μL of poly(dI-dC), 1 μL of biotin-labeled double-stranded NF-κB or AP-1 oligonucleotides, and 2 μL of 10-fold binding buffer into a microcentrifuge tube and were incubated for 15 min at room temperature. Specific competition binding assays were performed by adding a 200-fold excess of unlabeled probe as a specific competitor. Following protein−DNA complex formation, samples were loaded on a 6% nondenaturing polyacrylamide gel in 0.5 × TBE buffer and were then transferred to positively charged nitrocellulose membranes (Millipore, Bedford, MA) by a transfer blotting apparatus and cross-linked in a Stratagene cross-linker. Gel shifts were visualized with streptavidin-horseradish peroxidase followed by chemiluminescent detection.

Statistical Analysis. Data were expressed as means ± standard deviation of three independent experiments and were analyzed by Student's *t*-test (Sigmaplot 2001). Significant differences were established at $p \leq 0.05$.

RESULTS AND DISCUSSION

Cytotoxicity of α-Mangostin to PC-3 Cells. α-Mangostin is a natural antioxidant with pleiotropic activities against cancer growth. In this study, we first examined the cytotoxicity of α-mangostin by treating PC-3 cells with α-mangostin at various concentrations (0, 1, 3, 5, 7, 9, and 11 μg/mL) for 24 and 48 h followed by an MTT assay. In **Figure 2** it is shown that 24 and 48 h treatment of α-mangostin at various concentrations (0−5 μg/mL) exhibits no cytotoxicity in PC-3 cells. In the following experiments, these doses below 5 μg/mL of α-mangostin were applied in all subsequent experiments.

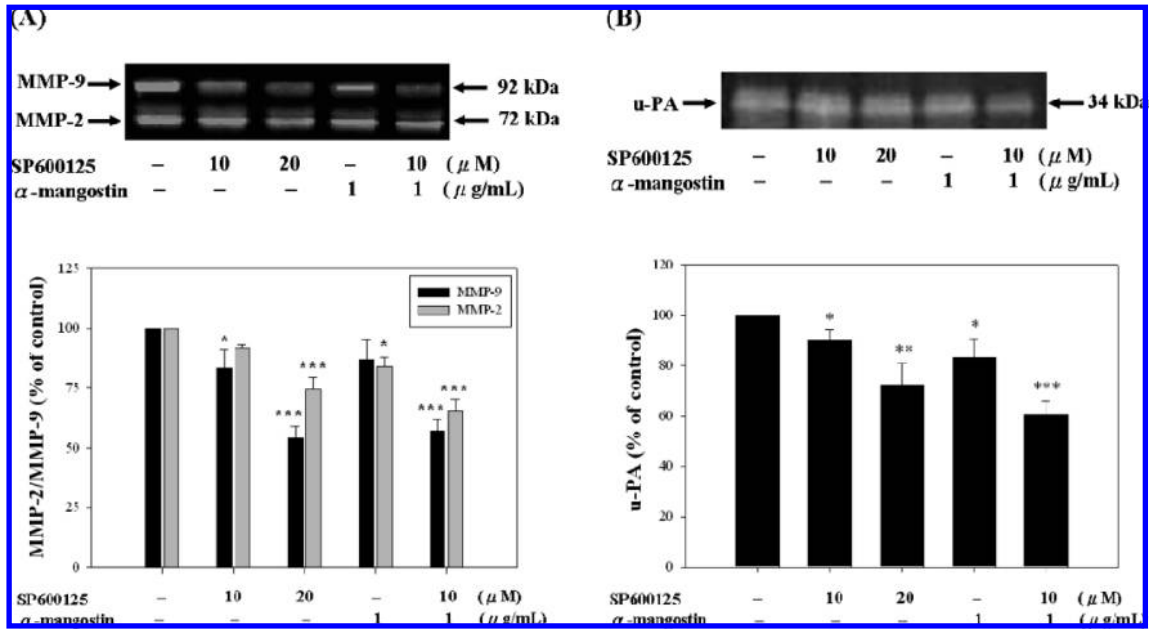

Figure 8. Effect of JNK inhibitor (SP600125) and α-mangostin on the activities of MMP-2, MMP-9, and u-PA. Cells were plated in 6-wells and pretreated with SP600125 (10 or 20 μM) for 1 h and then incubated in the presence or absence of α-mangostin (1 μg/mL) for 24 h. Afterward, the culture medium was subjected to gelatin and casein zymography to analyze the activities of (**A**) MMP-2/MMP-9 and (**B**) u-PA. Determined activities of these proteins were subsequently quantified by densitometric analysis, with that of control being 100%, as shown just below the gel data. Data represented the mean ± SD of three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001).

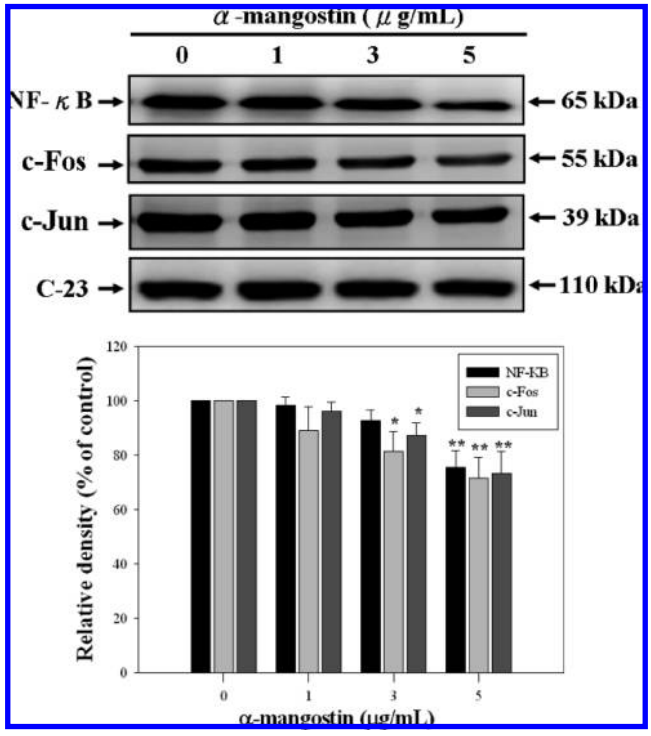

Figure 9. Effect of α-mangostin on the nuclear levels of NF-κB, c-Fos, and c-Jun. PC-3 cells were treated with various concentrations (0, 1, 3, and 5 μg/mL) of α-mangostin for 24 h. (**A**) Cell nuclear extracts were prepared and analyzed by Western blotting with anti-NF-κB (p65), c-Fos, and c-Jun antibodies. C23 was a nucleus protein loading control. Determined activities of NF-κB, c-Fos, and c-Jun were subsequently quantified by densitometric analysis with that of control being 1-fold. The densitometric results are expressed as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 compared with the untreated control.

The Inhibition of MMP-2, MMP-9, and u-PA Expression by α-Mangostin in PC-3 Cells. Many reports have addressed the importance of interations between cells and ECM, which could enhance cell migration, invasion, proliferation, and ECM degradation. Metastasis has been found to be accompanied by various physiological alterations involved in the degradation of ECM, such as the overexpression of proteolytic enzyme activity, such as MMPs or u-PA, as well as the migration and invasion of tumor cells into the bloodstream or lymphatic system to spread to another tissue or organ (*21*). To further investigate that matrix-degrading proteinases are required, the conditioned media were collected and concentrated, and the inhibition of metastasis was measured after PC-3 cells were treated for 24 h by α-mangostin. As shown in **Figure 3A**, dose-dependent and markedly reduced in MMP-2 and MMP-9 activities were observed in the serum-free medium treated with 5 μg/mL α-mangostin for 24 h. Similarly, the reduction in u-PA activity of PC-3 cells was also measured when α-mangostin treated concentrations were beyond 3 μg/mL (**Figure 3B**). There were 78% and 55% of u-PA activity in PC-3 cells after a 3 μg/mL and 5 μg/mL α-mangostin treatment when compared with those of the non-α-mangostin treated group. These results suggested that the antimetastasis effect of α-mangostin was related to the inhibition of enzymatically degradative processes of tumor metastasis. This was the first to demonstrate the biochemical mechanism(s) by which α-mangostin reduced the metastasis in human prostate carcinoma cells. The expressions of MMP-2, MMP-9, and u-PA have been shown to play a critical role in degrading the basement membrane in cancer invasion and migration.

Inhibition of Adhesion, Migration, and Invasion by α-Mangostin in PC-3 Cells. To investigate the inhibitory effect of α-mangostin on PC-3 cells adhesion, migration and invasion process, a cell-matrix adhesion assay, a wound healing assay, and a Boyden chamber assay were used. In the cell-matrix adhesion assay, α-mangostin showed a dose-dependent inhibitory effect on the cell adhesion ability of PC-3 cells (**Figure 4**). In the wound healing assay, according to a quantitative assessment, the cells were treated with various concentrations

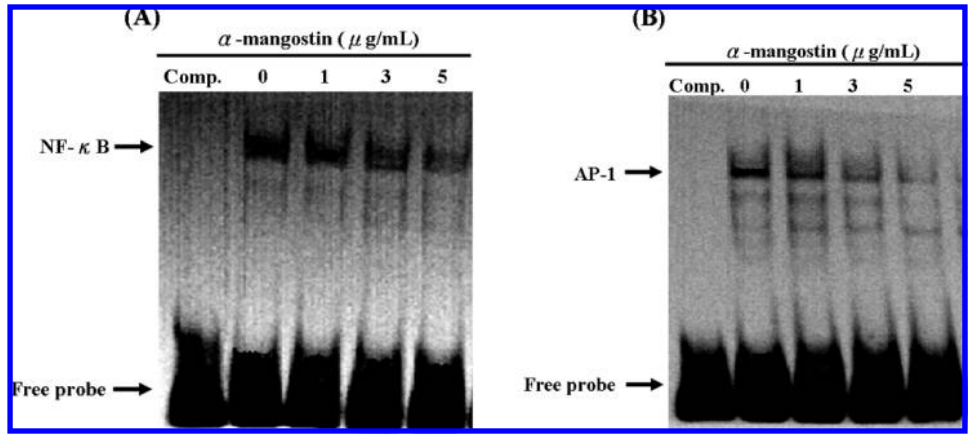

Figure 10. Effect of α-mangostin on the DNA binding activities of NF-κB and AP-1. PC-3 cells were treated with various concentrations (0, 1, 3, and 5 μg/mL) of α-mangostin for 24 h, and then nuclear extracts were prepared and analyzed for (**A**) NF-κB and (**B**) AP-1 DNA binding activities using biotin-labeled consensus NF-κB and AP-1 specific oligonucleotide. Then EMSA assays were performed as described in the MATERIALS AND METHODS. Lane 1: nuclear extracts incubated with 100-fold excess unlabeled consensus oligonucleotide (comp.) to confirm the specificity of binding. Excess free probe is indicated at the bottom. Results from three repeated and separate experiments were similar.

of α-mangostin for 0, 12, 24, 36, and 48 h. The results showed that 5 μg/mL of α-mangostin exhibited the greatest effect of inhibition on cell motility after 48 h of incubation (**Figure 5A and 5B**). Additionally, as compared with untreated cells, the level of the PC-3 cell number decreased almost 2.1-fold with the treatment of 5 μg/mL α-mangostin for 48 h. These results revealed that α-mangostin significantly inhibited the motility of PC-3 cells.

One important characteristic of metastasis is the migratory and invasive ability of tumor cells. We used Boyden chamber assay to quantify the migratory and invasive potential of PC-3 cells. The results showed that α-mangostin induced a dose-dependent decrease in migration with increasing concentration of α-mangostin (**Figure 6A**). At 3 μg/mL, the migration was reduced to 72%, and at 5 μg/mL, the migration was reduced to 46%. Subsequently, α-mangostin also induced a dose-dependent decrease in invasion with increasing concentration of α-mangostin (**Figure 6B**). At 3 μg/mL, the invasion was reduced to 75%, and at 5 μg/mL, the invasion was reduced to less than 59%. The results demonstrated that α-mangostin significantly inhibited the migration and invasion of PC-3 cells.

Inhibition of Phosphorylation of JNK by α-Mangostin in PC-3 Cells. Since we have shown that treatment of PC-3 cells with α-mangostin inhibited the cell metastasis and activities of MMP-2, MMP-9, and u-PA, the underlying mechanisms were further investigated. A major mechanism through which signals from extracellular stimuli are transmitted to the nucleus involves activation of kinase and mediates signals from cell membrane receptors triggered by growth factors, cytokines, and cell-matrix interactions. Several studies have indicated the transcription factors (for example, NF-kB, c-Fos, c-Jun) JNK1/2, ERK1/2, p38 MAPK, and Akt are involved in the activities of MMP-2, MMP-9, and u-PA on different cell types (*17*, *22*, *23*). To assess whether α-mangostin mediates and/or inhibits phosphorylation of JNK1/2, ERK1/2, p38, and Akt, we investigated the effect of α-mangostin on the phosphorylated status of MAPK family members (JNK1/2, ERK1/2, p38) and Akt in PC-3 cells which were treated with various concentrations of α-mangostin for 3 h. **Figure 7** showed that α-mangostin significantly inhibited the activation of JNK1 and JNK2, as shown by decreasing the phosphorylation of JNK1 and JNK2, whereas it had no significant effect on ERK1/2, p38, and Akt activity. Moreover, no significant change in the total amount of ERK1/2, JNK1/2, p38, and Akt proteins was observed (data not shown).

To further investigate whether the inhibition of α-mangostin was mainly through inhibition of the JNK1/2 signaling pathway, PC-3 cells were pretreated with a JNK inhibitor (SP600125; 10 or 20 μM) for 1 h and then incubated in the presence or absence of α-mangostin (1 μg/mL) for 24 h. Results of the gelatin zymography assay have shown that a sole treatment of SP600125 (10 or 20 μM) or α-mangostin (1 μg/mL), respectively, reduced the expressions of MMP-9 or MMP-2 by 16.7%, 46%, and 13% or 8.3%, 25.7%, and 16%, and the combination treatment (10 μM SP600125 + 1 μg/mL α-mangostin) could reduce MMP-9 or MMP-2 secretion even more dramatically, by 43% or 34.7% (**Figure 8A**). Similarly, in a casein zymography assay, a sole treatment of SP600125 (10 or 20 μM) or α-mangostin (1 μg/mL) reduced the expression of u-PA by 10%, 27.7%, and 16.7%, respectively, and the combination treatment could further reduce the secretion of u-PA by 39.7% (10 μM SP600125 + 1 μg/mL α-mangostin) (**Figure 8B**). Indeed, as shown in **Figures 7** and **8**, α-mangostin could inhibit the phosphorylation of JNK1/2, and the involvement of the MAPK pathway was further supported by the use of the JNK inhibitor in our experimental model, to show that a treatment with SP600125 could lead to an inhibition of MMP-2, MMP-9, or u-PA secretion, as well as a reduction in cell invasion.

Inhibition of the Nuclear Levels of NF-κB, c-Fos, and c-Jun by α-Mangostin. NF-κB and AP-1 families of transcriptional factors have been known to translocate to the nucleus and regulate the expression of multiple genes involved in MMPs or u-PA secretion. To further explore the nuclear protein levels of NF-κB, c-Fos, and c-Jun, the nuclear extracts were analyzed by Western blotting to assess the possible inhibitory effect of α-mangostin on NF-κB, c-Fos, and c-Jun. As explained in **Figure 9**, the nuclear levels of NF-κB, c-Fos, and c-Jun were tremendously diminished by treatment with α-mangostin in comparison to 0 μg/mL after 24 h. Especially, data was shown to be strongly inhibited by a treatment with 5 μg/mL α-mangostin.

Inhibition of the DNA Binding Activities of NF-κB and AP-1 by α-Mangostin. Previous reports have demonstrated that the MMP-2, MMP-9, and u-PA promoters have several transcription-binding motifs, including NF-κB and AP-1 (*24*−*27*). Thus, multiple pathways leading to activation of NF-κB and AP-1 binding factors in tumor cells may contribute to MMP-2, MMP-9, and u-PA transcription and metastasis enhancement. In an additional study, to clarify the involvement of NF-κB and

AP-1 proteins in the mechanism of α-mangostin's action, the effect of α-mangostin on the DNA binding activities of NF-κB and AP-1 in PC-3 cells was investigated by EMSA. As shown in **Figure 10**, PC-3 cells were treated with 0−5 μg/mL of α-mangostin for 24 h; α-mangostin inhibited NF-κB and AP-1 transcriptional activity in a dose-dependent manner. Especially, the binding activities of NF-κB and AP-1 were strongly inhibited by treatment with 5 μg/mL α-mangostin.

In conclusion, the results above imply the therapeutic potential of α-mangostin for controlling tumor metastasis based on the observation of its inhibitory effect on migration and invasion of carcinoma cancer cell line PC-3 cells. Characterization of the detailed mechanism of the inhibitory effects of α-mangostin showed that they inhibit PC-3 cells, maybe through inactivation of the JNK signaling pathway, exert inhibitory effects on NF-κB, c-Fos, and c-Jun transcriptional factors, inhibit NF-kB and AP-1 DNA binding activity, thereby decreasing the activities of MMP-2, MMP-9, and u-PA, and then exert an antimetastasis in the cells. As evidenced from the above results, α-mangostin may be a potential candidate for a preventive agent against prostate cancer metastasis.

ABBREVIATIONS

MMPs, matrix metalloproteinases; u-PA, urokinase-type plasminogen activator; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; ERK, extracellular signaling-regulating kinase; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; PI3K, phosphoinositide 3-kinase; NF-κB, nuclear factor kappa B; AP-1, activator protein-1, IκB, inhibitor of NF-κB.

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Received for review October 21, 2008. Revised manuscript received December 30, 2008. Accepted December 30, 2008. This work was supported by Grant DOH93-TD-1012 from the Department of Health, Taiwan.

JF8032683