

Silibinin suppresses human osteosarcoma MG-63 cell invasion by inhibiting the ERK-dependent c-Jun/AP-1 induction of MMP-2

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Silibinin is a natural flavonoid antioxidant with anti-hepatotoxic properties and pleiotropic anticancer capabilities. We tested the hypothesis that silibinin inhibits cellular invasiveness by down-regulating the focal adhesion kinase (FAK) and extracellular signal-regulated protein kinase (ERK)-dependent c-Jun/activator protein-1 (AP-1) induction, which leads to inhibition of urokinase-type plasminogen activator (u-PA) and matrix metalloproteinase-2 (MMP-2) expressions in human osteosarcoma MG-63 cells. We found that silibinin decreased cell adhesion and invasiveness, as well as inhibited u-PA and MMP-2 expressions. Silibinin reduced ERK 1/2 phosphorylation, but had no effects on the phosphorylation of c-Jun N-terminal kinases (JNKs) 1/2, p38 and Akt. Silibinin suppressed AP-1-binding activity and c-Jun levels and its phosphorylation without changes of c-Fos and Ets-1 levels. Silibinin also inhibited interleukin-6-induced ERK 1/2 and c-Jun phosphorylation, and cell invasiveness. Thus, silibinin may possess an anti-metastatic activity in MG-63 cells.

Introduction

Silibinin, a polyphenolic flavonoid, is a major pure bioactive component in silymarin, which is isolated mainly from the fruits or seeds of milk thistle (*Silybum marianum* (L.) Gaertn.). Silymarin has been widely used for its anti-hepatotoxic properties for more than three decades in Europe and recently in Asia and the USA (1). Recent evidence over the past decade has demonstrated that silibinin possesses pleiotropic anticancer capabilities in different tumor cells, including prostate, skin, colon, and bladder cancer models (2–4). In *in vitro* studies, silibinin strongly inhibits cell growth and DNA synthesis to cause cell cycle arrest and apoptotic cell death through an activated caspase cascade (5,6). Silibinin is exceptionally well tolerated and largely free of adverse effects (7) and mice fed with silibinin (up to 2 g/kg) do not show any apparent signs of toxicity (8). Thus, due to its excellent safety profile, anticancer activity, well-known pharmacokinetics and widespread tissue distribution, silibinin has been considered a suitable candidate for cancer chemotherapy and chemoprevention (9–11).

Metastasis is a complex process of tumor cell invasion to new tissues, involving the coordination of several signaling pathways that allow changes in cell morphology, changes in adhesion and migration capabilities between the cells and the extracellular matrix (ECM) and

Abbreviations: AP-1, activator protein-1; cDNA, complementary DNA; ECM, extracellular matrix; ERK, extracellular signal-regulated protein kinase; FAK, focal adhesion kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, minimum essential medium; MMP, matrix metalloproteinase; MEK, mitogen-activated protein kinase kinase; NF- κ B, nuclear factor- κ B; PAI, plasminogen activator inhibitor; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PCR, polymerase chain reaction; TIMP, tissue inhibitor of metalloproteinase; u-PA, urokinase-type plasminogen activator.

changes in cell–cell interaction. Degradation of the ECM by cancer cells is mediated via protease, such as matrix metalloproteinases (MMPs), serine proteinase, and cathepsins. Among these proteases, urokinase-type plasminogen activator (u-PA), which is an upstream enzyme of MMPs, MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) are the most vital enzymes for the degradation of the main constituent of the basement membrane, type IV collagen (12,13), and are therefore deeply involved in cancer invasion and metastasis (14,15). Plasminogen activator inhibitor-1 (PAI-1), the major circulating PAI, controls the rate of plasmin generation by forming irreversible inhibitory complexes with u-PA (16). MMP-2 is activated on the cell surface by a multimeric complex that is composed of MMP-2, membrane type 1 MMP and the tissue inhibitor of metalloproteinase-2 (TIMP-2). The activity of MMP-2 released by osteosarcoma MG-63 cells is significantly increased with interleukin (IL)-6 induction and it is involved in bone remodeling mechanisms (17). Thus, elucidation of u-PA, PAI-1, MMP-2 and MMP-9 activation mechanism will help understand the process of cell invasion and metastasis (18,19). However, the expression of different MMPs depends on unique combinations of different signal transduction pathways in various cells.

The mitogen-activated protein kinase (MAPK) family, the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 kinase are unique serine/threonine kinases that are activated via reversible phosphorylation and mediate signal transduction of a wide variety of extracellular stimuli (such as cell–cell and cell–ECM adhesion and soluble mediators) into intracellular cascades. MAPKs regulate a number of transcription factors such as activator protein-1 (AP-1) (20,21) and nuclear factor- κ B (NF- κ B) (20,22), which act independently or coordinately to regulate numerous genes involved in the regulation of u-PA and MMPs expression. Additionally, phosphatidylinositol 3-kinase (PI3K)–Akt signaling plays a prominent role in several processes considered the hallmark of cancer (23). We have reported previously that u-PA and MMP-2 activities, important determinants of lung cancer cellular invasiveness, were inhibited by silibinin via PI3K–Akt and MAPKs signaling pathways (20,24).

Hallmarks of the malignant phenotype are invasion and metastasis events that depend on cell–cell and cell–matrix interactions. Intercellular adhesion restricts cellular migration, invasion, and metastasis and is mainly regulated by the homophilic interaction of cadherin molecules. Malignant cells often down-regulate the levels of E-cadherin, which is anchored to the cytoskeleton via the associated cytoplasmic proteins α -, β - and γ -catenins (25). The cytoskeleton, a complex network of interconnected fibrillar elements, also has been recognized as an important factor in mediating cell–matrix adhesion-independent and -dependent signaling (26,27). In particular, changes in the organization of the actin cytoskeleton, which are implicated in adhesion-induced, integrin-mediated focal adhesion kinase (FAK) activation, lead to remarkable changes in the tyrosine phosphorylation of several signaling proteins localized at the focal adhesion complex (28). In addition to regulating cell adhesion to the ECM and regulating the activities of proteolytic enzymes to degrade the basement membrane, integrins activate kinases that phosphorylate cytoskeletal proteins, regulating stress-fiber formation, cellular shape and migration (29).

Osteosarcoma is the most common primary malignant tumor of the bone, especially in children. An outcome of osteosarcoma generally suggests that 80% of the patients have pulmonary and hepatic metastases (perhaps undetectable) at the time of presentation (30,31). Hence, chemotherapy is usually employed as an adjuvant to improve the prognosis and long-term survival. Combination chemotherapy has received more attention in order to find compounds with a known mechanism of action that could increase the therapeutic index of clinical anticancer drugs (19). In this regard, dietary supplements as well

as phytotherapeutic agents (such as silibinin) with high anticancer efficacy and with the least toxicity to normal tissues are suggested as possible candidates to be investigated for their synergistic efficacy in combination with anticancer drugs (32,33). The effect of silibinin on cancer invasion and metastasis of osteosarcoma and the underlying mechanisms of such effect remain unclear. Therefore, we tested the hypothesis that silibinin inhibits osteosarcoma MG-63 cellular invasiveness through an ERK/c-Jun-dependent induction of u-PA and MMP-2 expression and activity. We also tested the effect of silibinin on cellular adhesion, changes of cellular shape and suppression of FAK to Raf-ERK signaling pathway.

Materials and methods

Cells and silibinin treatment

Human osteosarcoma MG-63 cells (human male, 14 years old) obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan) were cultured in minimum essential medium (MEM) (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum, 1 mM glutamine, 1% penicillin/streptomycin, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate (Sigma, St Louis, MO). The cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. For silibinin treatment, appropriate amounts of stock solution [0.1 M in dimethyl sulfoxide (Merck, Darmstadt, Germany)] of silibinin (Sigma) were added into culture medium to achieve the indicated concentrations and then incubated with cells for indicated time periods, whereas dimethyl sulfoxide solution without silibinin was used as blank reagent.

Microculture tetrazolium assay

For cell viability experiment, a microculture tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was performed to determine the cytotoxicity of silibinin (34). MG-63 cells were plated in 24-well plates at a density of 3×10^4 cells per well and silibinin was added at different concentrations (0, 5, 10, 15, 20, 25 and 30 μ M) at 37°C for 24 h. After the exposure period, the media was removed, and cells were washed with phosphate-buffered saline (PBS). Then, the medium was changed and cells were incubated with 20 ml microculture tetrazolium (0.5 mg/ml) for 4 h. The viable cell number per dish is directly proportional to the production of formazan, which can be measured spectrophotometrically at 563 nm following solubilization with isopropanol.

Cell invasion and migration assays

Using a modified Boyden chamber invasion assay (24,35), we evaluated the effect of silibinin on the invasiveness of MG-63 cells *in vitro* with or without IL-6 treatment. Matrigel (Collaborative Biomedical Products, Bedford, MA) was diluted to 25 mg/50 ml with cold-filtered distilled water, and applied to 8 μ m pore size polycarbonate membrane filters. Cells treated with indicated concentrations of silibinin (0, 5, 10, 15 and 20 μ M of silibinin in 50 μ l of serum-free medium) were seeded into the upper section of the Boyden chamber (Neuro Probe, Cabin John, MD) at a density of 2.5×10^4 cells per well, and then incubated for 24 h at 37°C. The cells that had migrated to the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. Random fields were counted under a light microscope. For testing the effect of silibinin on cell migration, MG-63 cells were seeded into the Boyden chamber on membrane filters that were not coated with Matrigel. Migration of cells treated or untreated with different concentrations of silibinin was measured as described in the cell invasion assay.

Cell-matrix adhesion assay

MG-63 cells (5×10^4 cells per well) treated with different concentrations of silibinin (0, 5, 10, 15 and 20 μ M) for 24 h were plated on 24-well dishes coated with type IV collagen and cultured for 30 min. Then, non-adherent cells were

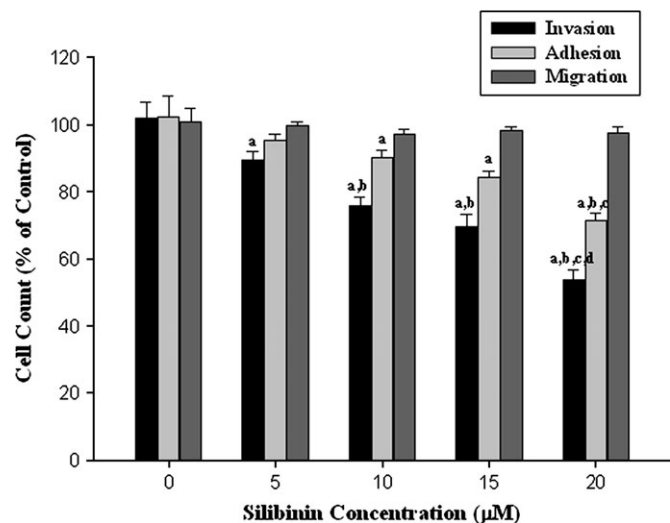


Fig. 1. Concentration effects of silibinin on the invasiveness, cell-matrix adhesion and migration of MG-63 cells. Each datum represents mean \pm standard deviation of triplicate experiments. Analysis of variance with Scheffe posteriori comparison was used. Cell invasion: $F = 87.801$, $P < 0.001$; cell adhesion: $F = 35.342$, $P < 0.001$; cell migration: $F = 1.406$, $P = 0.301$. ^aSignificantly different, $P < 0.05$, when compared with control. ^bSignificantly different, $P < 0.05$, when compared with 5 μ M. ^cSignificantly different, $P < 0.05$, when compared with 10 μ M. ^dSignificantly different, $P < 0.05$, when compared with 15 μ M.

removed by PBS washes and adherent cells were fixed with 1% formaldehyde. After a staining with 0.1% crystal violet, fixed cells were lysed in 0.2% Triton X-100 and the absorbance was measured at 550 nm (24,35).

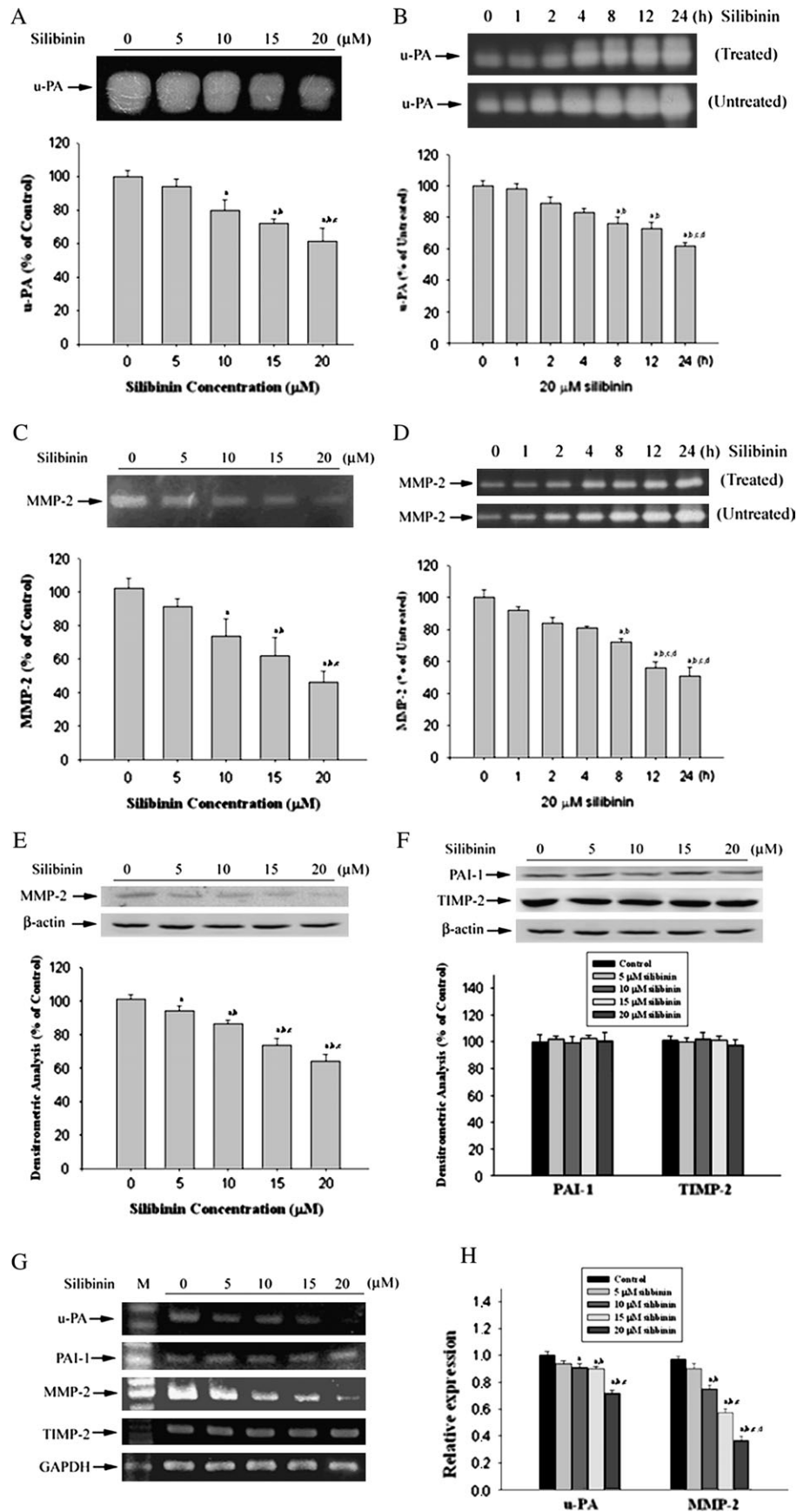
Immunofluorescence assay

To determine the effect of silibinin on cell morphology and actin stress fibers, MG-63 cells (8×10^4 cells per well) were plated in six-well plates and grown for 16 h so that they attached to the surface of the plates completely. Silibinin was added to cells at different concentrations (0, 5, 10, 15 and 20 μ M) and cells were grown at 37°C in humidified 5% CO₂ for 24 h. After the exposure period, media was removed, and cells were washed with Ca²⁺/Mg²⁺-free PBS. Cells were then fixed with 3.7% paraformaldehyde in Ca²⁺/Mg²⁺-free PBS for 15 min and incubated with 0.1% Triton X-100 in Ca²⁺/Mg²⁺-free PBS for 1 h. Cells were incubated with 1% bovine serum albumin in Ca²⁺/Mg²⁺-free PBS for 30 min (blocking) and then with 500 ng/ml tetramethylrhodamine isothiocyanate-phalloidin (Sigma) for 1 h to stain the actin filaments, and the culture plates were examined and photographed by immunofluorescence microscopy.

Casein and gelatin zymography

Casein zymography was used to evaluate u-PA activity in MG-63 cells treated with different concentrations (0, 5, 10, 15 and 20 μ M) of silibinin or with 20 μ M silibinin for different times (0, 1, 2, 4, 8, 12 and 24 h). Briefly, prepared samples containing 20 μ g of total protein were loaded onto a precast 8% sodium dodecyl sulfate-polyacrylamide gel containing 2% casein and 20 μ g/ml plasminogen. After electrophoresis, gels were processed as described previously (36,37). The non-staining band representing the level of u-PA was measured by spot density measurement using a densitometer (AlphaImager 2000, Alpha Innotech Corp., San Leandro, CA) (38,39). MMP-2 and MMP-9 activities were evaluated by gelatin zymography. Briefly, prepared samples containing 10 μ g of total protein were loaded onto a precast sodium dodecyl sulfate-polyacrylamide

Fig. 2. Effects of silibinin on secreted u-PA, PAI-1, MMP-2 and TIMP-2 as well as their messenger RNA (mRNA) levels. MG-63 cells were treated with different concentrations (0, 5, 10, 15 and 20 μ M) of silibinin for 24 h or with 20 μ M silibinin for different times (0, 1, 2, 4, 8, 12, and 24 h). The conditioned media were collected and then the activity of u-PA [(A) concentration effects: $F = 24.495$, $P < 0.001$; (B) time effects: $F = 18.225$, $P < 0.001$] and MMP-2 [(C) concentration effects: $F = 22.774$, $P < 0.001$; (D) time effects: $F = 25.582$, $P < 0.001$] were assessed by casein zymography and gelatin zymography, respectively. The cells were lysed and the cell lysates were electrophoresed and probed by western blotting with (E) anti-MMP-2 ($F = 66.096$, $P < 0.001$), (F) anti-PAI-1 ($F = 0.223$, $P = 0.919$) and anti-TIMP-2 ($F = 0.942$, $P = 0.479$) antibodies. (G) A reverse transcriptase-PCR analysis was carried out for u-PA, PAI-1, MMP-2 and TIMP-2 mRNA levels. (H) The mRNA levels of u-PA ($F = 22.568$, $P < 0.001$) and MMP-2 ($F = 197.191$, $P < 0.001$) were quantified with real-time PCR assay. The densitometric data shown are mean \pm standard deviation of triplicate experiments. Analysis of variance with Scheffe posteriori comparison was used. ^aSignificantly different, $P < 0.05$, when compared with control or 0 h. ^bSignificantly different, $P < 0.05$, when compared with 5 μ M or 1 h. ^cSignificantly different, $P < 0.05$, when compared with 10 μ M or 2 h. ^dSignificantly different, $P < 0.05$, when compared with 4 h.



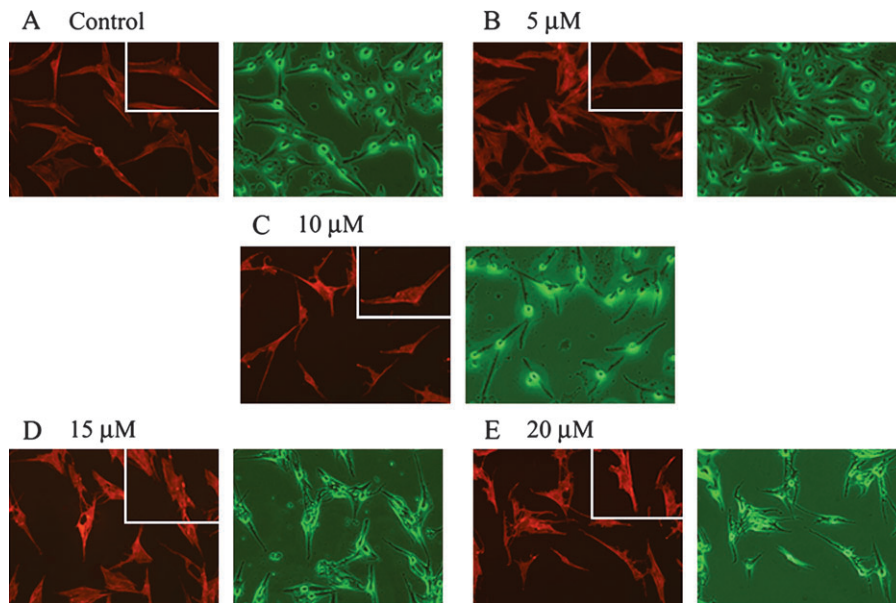


Fig. 3. Concentration effects of silibinin on MG-63 cell morphology and actin filaments. (A–E) Actin stress fibers of serum-starved MG-63 cells are stained with tetramethylrhodamine isothiocyanate–phalloidin (red) and observed with inverse fluorescence microscopy at the left and phase-contrast images of the treated cells are shown at the right. The cellular shape and actin cytoskeleton organization, i.e. stress fibers, are apparently changed upon 5–20 μM silibinin treatment, comparing with the control plate. Original magnification, $\times 200$; the larger magnification at right upper column of the left, $\times 800$.

gel containing 0.1% gelatin. MMP-2 and MMP-9 activities were measured in MG-63 cells treated with different concentrations (0, 5, 10, 15 and 20 μM) of silibinin or with 20 μM silibinin for different times (0, 1, 2, 4, 8, 12 and 24 h), as described in the casein zymography procedure (38,39).

Preparation of cell lysates and western blot analysis

MG-63 cells were treated with different concentrations (0, 5, 10, 15 and 20 μM) of silibinin for 24 h or with 20 μM silibinin for different times (0, 1, 2, 4, 8, 12 and 24 h). After treatment, cells were rinsed with PBS twice and added with 0.1 ml of cold radioimmunoprecipitation buffer and protease inhibitors, and then scraped. Cell lysates were subjected to a centrifugation of 10 000g for 10 min at 4°C, after a vortex at 0°C for 10 min. For western blot analysis, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted as described by Chen *et al.* (20). The blot was subsequently incubated with 5% non-fat milk in PBS for 1 h to block non-specific binding and then overnight with polyclonal antibodies against u-PA, PAI-1, MMP-2, TIMP-2, FAK, α -catenin, β -catenin, Raf, three MAPKs (ERK 1/2, JNK 1/2 and p38), Akt, c-Jun, c-Fos or Ets-1 or with the specific antibodies for unphosphorylated or phosphorylated activated forms of the corresponding ERK 1/2, JNK 1/2, p38 and Akt. Blots were then incubated with a horseradish peroxidase goat anti-rabbit or anti-mouse IgG for 1 h. All incubations were carried out at 37°C and intensive PBS washing was performed. After the final PBS washing, signal was developed using the ECL (enhanced chemiluminescence) plus detection kit (Amersham Life Sciences Inc., Piscataway, NJ), and relative photographic density was quantitated by scanning the photographic negatives on a gel analysis system (AlphaImager 2000, Alpha Innotech Corp.).

Reverse transcriptase–PCR and quantitative real-time PCR

Total RNA was extracted from MG-63 cells using a guanidinium chloride procedure. Complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR) amplification were performed as described elsewhere (38). For reverse transcription, 4 μg of total cellular RNA was used as template in a 20 μl reaction containing 10 μmol deoxynucleoside triphosphates, 25 pmol oligo dT and 200 U reverse transcriptase, and the reaction was performed at 42°C for 1 h. Afterward, 5 μl cDNA product was used as templates in PCR amplifications together with the appropriate primers as described previously (20,24). The final products were separated by electrophoresis on 1.8% agarose gels and detected by ethidium bromide staining. Real-time PCR assay was performed as described by Pedersen *et al.* (40) with the following modification. Specific primers and fluorogenic probes were used for u-PA and MMP-2 genes. For u-PA, MMP-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the following forward (F) primers, reverse (R) primers and probes (P) were used: u-PA—F: GAAACCCTACAATGCCACAGA, R: GACAAACTGCCTTAGGC-CATC, P: CACAATTACTGCAGGAACCCTGACAAC; MMP-2—F: TCACTCTGAGATCTGCAAAACAG, R: TCACAGTCCGCCAAATGAAC, P: CAAG-

CTTCCCCTTCTCAGCC; GAPDH—F: GAAGGTGAAGTCGGAGTC, R: GAAGATGGTGATGGGATTTC, P: CAAGCTTCCCGTTCTCAGCC. Primers and probes were designed using the Primer Express 1.0 Software (PE Applied Biosystems, Warrington, UK) and synthesized by PE Applied Biosystems. The cycle threshold (C_T) value was used as an indicator of the amount of target RNA in each sample. Normalized gene expression levels were then calculated using the starting levels of GAPDH in each sample to normalize for differences in total RNA content in the individual samples.

Preparation of nuclear fraction

Nuclear extracts were prepared as described previously (20,22). Harvested cells were lysed with buffer A (10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mmol/l KCl, 0.1 mmol/l ethylenediaminetetraacetic acid, 1.5 mmol/l MgCl_2 , 0.2% Nonidet P40, 1 mmol/l dithiothreitol and 0.5 mmol/l phenylmethylsulfonyl fluoride), followed by vortexing to shear the cytoplasmic membranes. Nuclei were pelleted by centrifugation at 3000 r.p.m. for 30 s at 4°C in a microcentrifuge, and then nuclear proteins were extracted with high-salt buffer B (20 mmol/l HEPES, 25% glycerol, 1.5 mmol/l MgCl_2 , 0.1 mmol/l ethylenediaminetetraacetic acid, 420 mmol/l NaCl, 1 mmol/l dithiothreitol and 0.5 mmol/l phenylmethylsulfonyl fluoride).

Electrophoretic mobility shift assay

AP-1- and NF- κB -binding assays in nuclear extracts were performed with biotin-labeled double-stranded AP-1 or NF- κB oligonucleotides (Promega, Madison, WI), and the electrophoretic mobility shift assay was carried out by using the Lightshift kit (Promega). Briefly, binding reactions containing 10 μg of nuclear protein, 10 mM Tris, 50 mM KCl, 1 mM dithiothreitol, 5 mM MgCl_2 , 2 μg poly (dI-dC) and 2 pmol of oligonucleotide probe were incubated for 20 min at room temperature. Protein DNA complexes were separated by electrophoresis on a 6% non-denaturing acrylamide gel, transferred to positively charged nylon membranes and then cross-linked in a Stratagene cross-linker. Gel shifts were visualized with a streptavidin–horseradish peroxidase followed by chemiluminescent detection.

Transient transfection

MG-63 cells were cultured in MEM with 10% fetal calf serum at 37°C in 5% CO_2 . Cell transfection was carried out using LipofectAMINE 2000 (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cells were plated in a 6 cm dish at a density of 3×10^5 cells per well and incubated overnight in MEM supplemented with 10% fetal calf serum. Vectors containing a constructively active mitogen-activated protein kinase kinase 1 (MEK1, designated MEK1EE) cDNA (5 μg), which was kindly provided by Dr Min-Liang Kuo (National Taiwan University, Taipei, Taiwan), were diluted in MEM (500 μl) and then mixed with the transfection solution for 30 min. After washing twice with PBS to remove sera, the cells were incubated

with the transfection mixture at 37°C for 4 h and then were allowed to grow in fresh media. Two days after transfection, the cells were used for the following experiment. Collected cells were processed for western blot analysis, gelatin zymography and cell invasion assay.

Statistical analysis

For all of the measurements, analysis of variance followed by Scheffe posteriori comparison was used to assess the differences between control and cells treated with various concentration of silibinin. The Student's *t*-test was used to analyze the difference between control and silibinin-treated cells after over-expression of constructively active MEK1. Statistical significance was set at $P < 0.05$.

Results

Cytotoxicity of silibinin on MG-63 cells

MG-63 cell viability in the presence of 20 μM silibinin was not significantly different to that of controls in the microculture tetrazolium assay (data not shown) ($F = 1.633$, $P = 0.241$). Thus, a 24 h treatment with silibinin up to 20 μM had no cytotoxic effect on MG-63 cells, which is consistent with *in vitro* studies from other laboratories (9–11). Thus, we used this concentration range for silibinin in all subsequent experiments.

Silibinin inhibited the invasiveness and adhesion of MG-63 cells

Silibinin significantly inhibited the invasive activity of MG-63 cells in a dose-dependent manner (>40% inhibition at 20 μM) ($P < 0.001$) (Figure 1). The cell–matrix adhesion assay also showed that silibinin significantly suppressed the adhesive activity of MG-63 cells in a dose-dependent manner (>20% inhibition at 20 μM) ($P < 0.001$). Unexpectedly, the migration potential of MG-63 cells in the Boyden chamber without Matrigel coating was not affected even when the concentration of silibinin was as high as 20 μM ($P = 0.301$).

Silibinin suppressed the expression and activity of u-PA and MMP-2 in MG-63 cells

Silibinin reduced the level of u-PA in casein zymography ($P < 0.001$) and MMP-2 in gelatin zymography ($P < 0.001$) and in western blotting ($P < 0.001$) in a dose- and time-dependent manner, but had no effect on PAI-1 ($P = 0.919$) and TIMP-2 levels ($P = 0.479$) in western blotting (Figure 2A–F). However, the impact of silibinin on MMP-9 activity was inconclusive because no MMP-9 was expressed in MG-63 cells, even in the absence of silibinin. Reverse transcriptase–PCR analysis showed that silibinin significantly suppressed the messenger RNA expression of u-PA and MMP-2 in MG-63 cells, which was further confirmed by quantitative real-time PCR ($P < 0.001$) (Figure 2G and H). The inhibitory effect of silibinin on MMP-2 was stronger than that on u-PA. Besides, silibinin treatment seemed to have more significant effect on the inhibition of MMP-2 enzyme activity than on its protein expression. Therefore, the regulation of u-PA and MMP-2 expression by silibinin in MG-63 cells was transcriptional and post-translational rather than translational. On the other hand, the messenger RNA expression of PAI-1 and TIMP-2 in MG-63 cells was not repressed by any concentration of silibinin examined.

Silibinin induced a change in cell morphology and in actin cytoskeleton arrangement in MG-63 cells

After treatment of MG-63 cells with different concentrations (0, 5, 10, 15 and 20 μM) of silibinin for 24 h, actin microfilaments, which were stained with tetramethylrhodamine isothiocyanate–phalloidin (red), distributed heterogeneously and aggregated depending on the treated concentrations (Figure 3). MG-63 cells changed morphology and became round and shrunken.

Silibinin inhibited the FAK and Raf expression in MG-63 cells

In western blot analysis, we found that silibinin significantly decreased FAK levels in MG-63 cells in a dose-dependent manner ($P < 0.001$), but had no effect on α -catenin ($P = 0.114$) and β -catenin

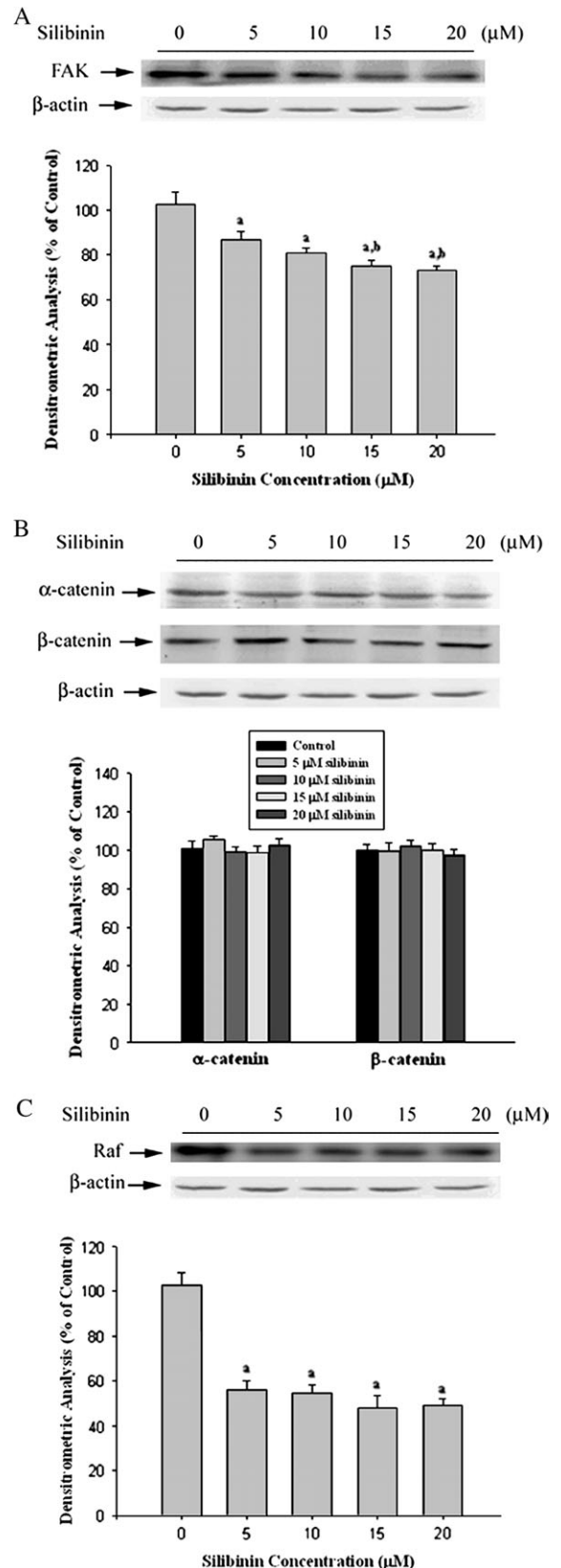


Fig. 4. Concentration effects of silibinin on FAK, α - and β -catenins and Raf. The densitometric data shown are mean \pm standard deviation of triplicate experiments. Analysis of variance with Scheffe posteriori comparison was used. (A) FAK: $F = 37.581$, $P < 0.001$; (B) α -catenin: $F = 2.450$, $P = 0.114$; β -catenin: $F = 0.734$, $P = 0.590$; (C) Raf: $F = 79.768$, $P < 0.001$. ^aSignificantly different, $P < 0.05$, when compared with control. ^bSignificantly different, $P < 0.05$, when compared with 5 μM .

levels ($P = 0.590$) (Figure 4A and B). Silibinin, as expected, significantly reduced the level of Raf in MG-63 cells ($P < 0.001$) (Figure 4C).

Silibinin inhibited the phosphorylation of ERK 1/2 in MG-63 cells

Western blots showed that silibinin significantly inhibited the phosphorylation of ERK 1/2 in MG-63 cells in a dose- and time-dependent manner ($P < 0.001$), but had no effect on the phosphorylation of JNK 1/2 (JNK 1: $P = 0.082$, JNK 2: $P = 0.078$), p38 ($P = 0.858$) and Akt ($P = 0.706$) (Figure 5).

Silibinin decreased c-Jun/AP-1 activation in MG-63 cells

Electrophoretic mobility shift assay showed that silibinin inhibited the DNA-binding activity of AP-1 in MG-63 cells, but not that of NF- κ B (Figure 6A and B). In western blotting, silibinin significantly inhibited c-Jun levels and its phosphorylation in nuclear extracts of MG-63 cells in a dose-dependent manner ($P < 0.001$), but had no effects on c-Fos ($P = 0.819$) and Ets-1 ($P = 0.195$) levels (Figure 6C and D). In contrast, we found that c-Jun levels significantly increased in

the cytosolic fractions after silibinin treatment in a dose- and time-dependent manner ($P < 0.001$) (Figure 6E and F). Silibinin did not affect the levels of c-Fos ($P = 0.947$) and Ets-1 ($P = 0.985$) in cytosolic fractions.

Silibinin inhibited the phosphorylation of ERK 1/2 and c-Jun, the activity of MMP-2 and the invasiveness of MG-63 cells with IL-6 induction

Western blots showed that IL-6 (50 ng/ml) gradually induced the phosphorylation of ERK 1/2 and reached the plateau at 10 min in MG-63 cells (data not shown). IL-6 also gradually increased the phosphorylation of c-Jun in nuclear extracts of MG-63 cells and reached the plateau at 45 min (data not shown). Thus, we used these two time points, respectively, for IL-6 induction in the subsequent experiments. As expected, silibinin significantly inhibited the phosphorylation of ERK 1/2 in MG-63 cells with 10 min of IL-6 induction in a dose-dependent manner ($P < 0.001$) (Figure 7A). Similarly, silibinin significantly decreased the phosphorylation of c-Jun in nuclear extracts of MG-63 cells with 45 min of IL-6

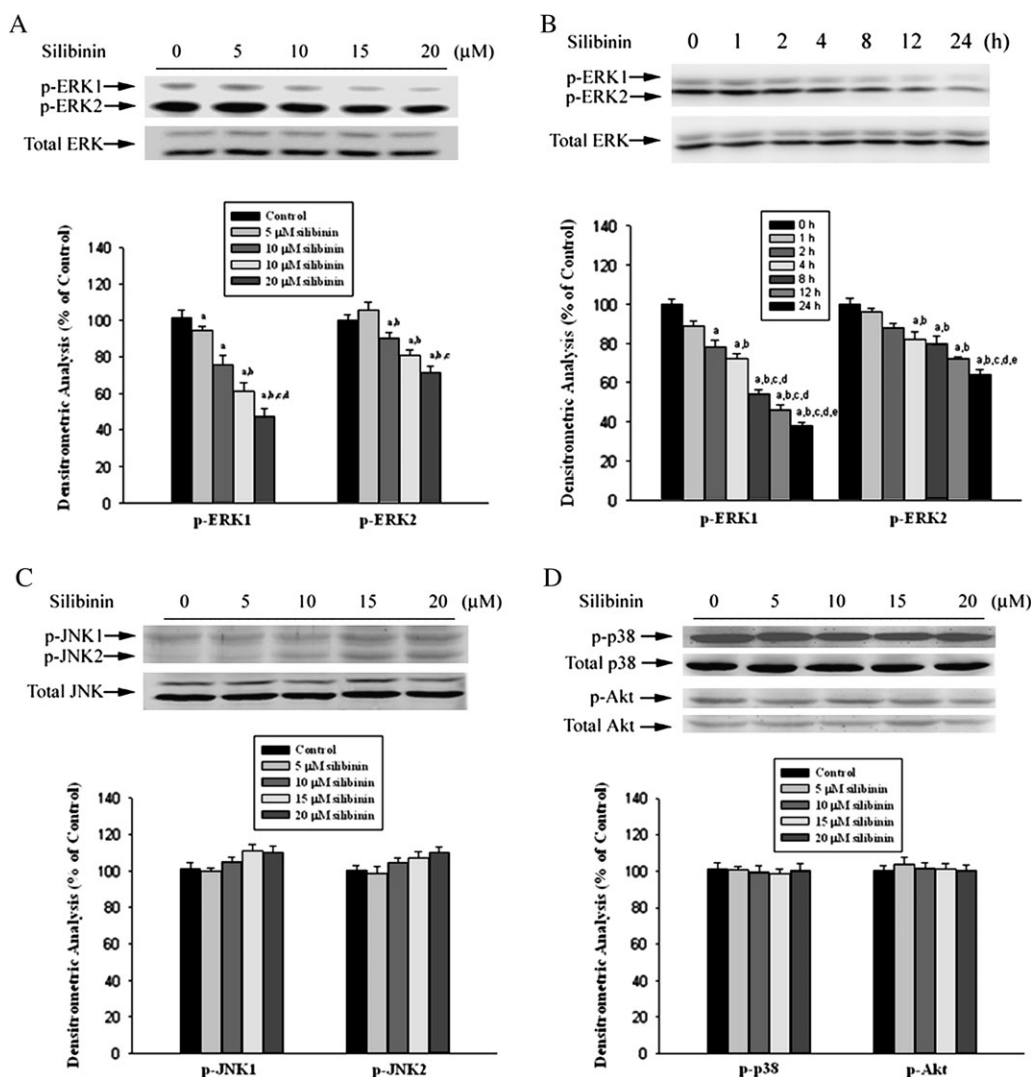


Fig. 5. Effects of silibinin on phosphorylation of ERK 1/2, JNK 1/2, p38 and Akt. The densitometric data shown are mean \pm standard deviation of triplicate experiments. Analysis of variance with Scheffe posteriori comparison was used. (A) Concentration effects (p-ERK 1: $F = 50.698$, $P < 0.001$; p-ERK 2: $F = 55.671$, $P < 0.001$); (B) time effects (p-ERK 1: $F = 73.948$, $P < 0.001$; p-ERK 2: $F = 26.383$, $P < 0.001$); (C) p-JNK 1: $F = 2.848$, $P = 0.082$; p-JNK 2: $F = 2.908$, $P = 0.078$; (D) p-p38: $F = 0.320$, $P = 0.858$; p-Akt: $F = 0.547$, $P = 0.706$. ^aSignificantly different, $P < 0.05$, when compared with control or 0 h. ^bSignificantly different, $P < 0.05$, when compared with 5 μ M or 1 h. ^cSignificantly different, $P < 0.05$, when compared with 10 μ M or 2 h. ^dSignificantly different, $P < 0.05$, when compared with 15 μ M or 4 h. ^eSignificantly different, $P < 0.05$, when compared with 8 h.

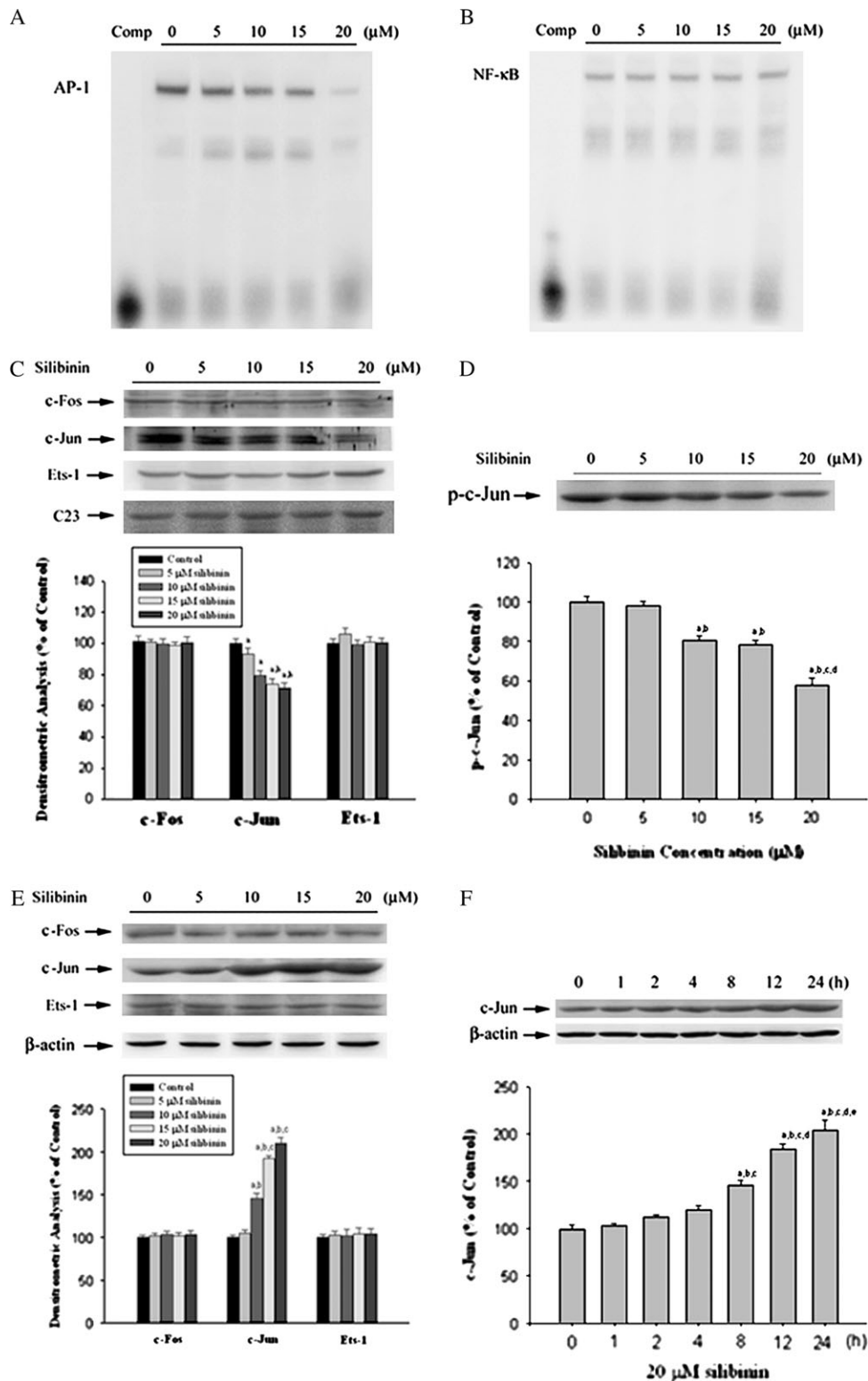


Fig. 6. Effects of silibinin on AP-1 and NF-κB DNA binding, c-Jun, c-Fos and Ets-1. MG-63 cells were treated with different concentrations (0, 5, 10, 15 and 20 μM) of silibinin for 24 h or with 20 μM silibinin for different times (0, 1, 2, 4, 8, 12 and 24 h), and then nuclear extracts were analyzed for (A) AP-1 and (B) NF-κB DNA-binding activity using biotin-labeled AP-1- and NF-κB-specific oligonucleotide by electrophoretic mobility shift assay. Lane 1 represents nuclear extracts incubated with unlabeled oligonucleotide (Comp) to confirm the specificity of binding. Results from three repeated and separated experiments were similar. Nuclear and cytosolic fractions were also electrophoresed and probed by western blotting with anti-c-Jun, anti-p-c-Jun, anti-c-Fos and anti-Ets-1 antibodies. The densitometric data shown are mean ± standard deviation of triplicate experiments. Analysis of variance with Scheffe posteriori comparison was used. (C) Concentration effects (nuclear—c-Jun: $F = 21.976$, $P < 0.001$; c-Fos: $F = 0.378$, $P = 0.819$; Ets-1: $F = 1.854$, $P = 0.195$); (D) concentration effects (nuclear—p-c-Jun: $F = 37.329$, $P < 0.001$); (E) concentration effects (cytosolic—c-Jun: $F = 26.383$, $P < 0.001$; c-Fos: $F = 0.173$, $P = 0.947$; Ets-1: $F = 0.085$, $P = 0.985$); (F) time effects (cytosolic—c-Jun: $F = 105.816$, $P < 0.001$). ^aSignificantly different, $P < 0.05$, when compared with control or 0 h. ^bSignificantly different, $P < 0.05$, when compared with 5 μM or 1 h. ^cSignificantly different, $P < 0.05$, when compared with 10 μM or 2 h. ^dSignificantly different, $P < 0.05$, when compared with 15 μM or 4 h. ^eSignificantly different, $P < 0.05$, when compared with 8 h.

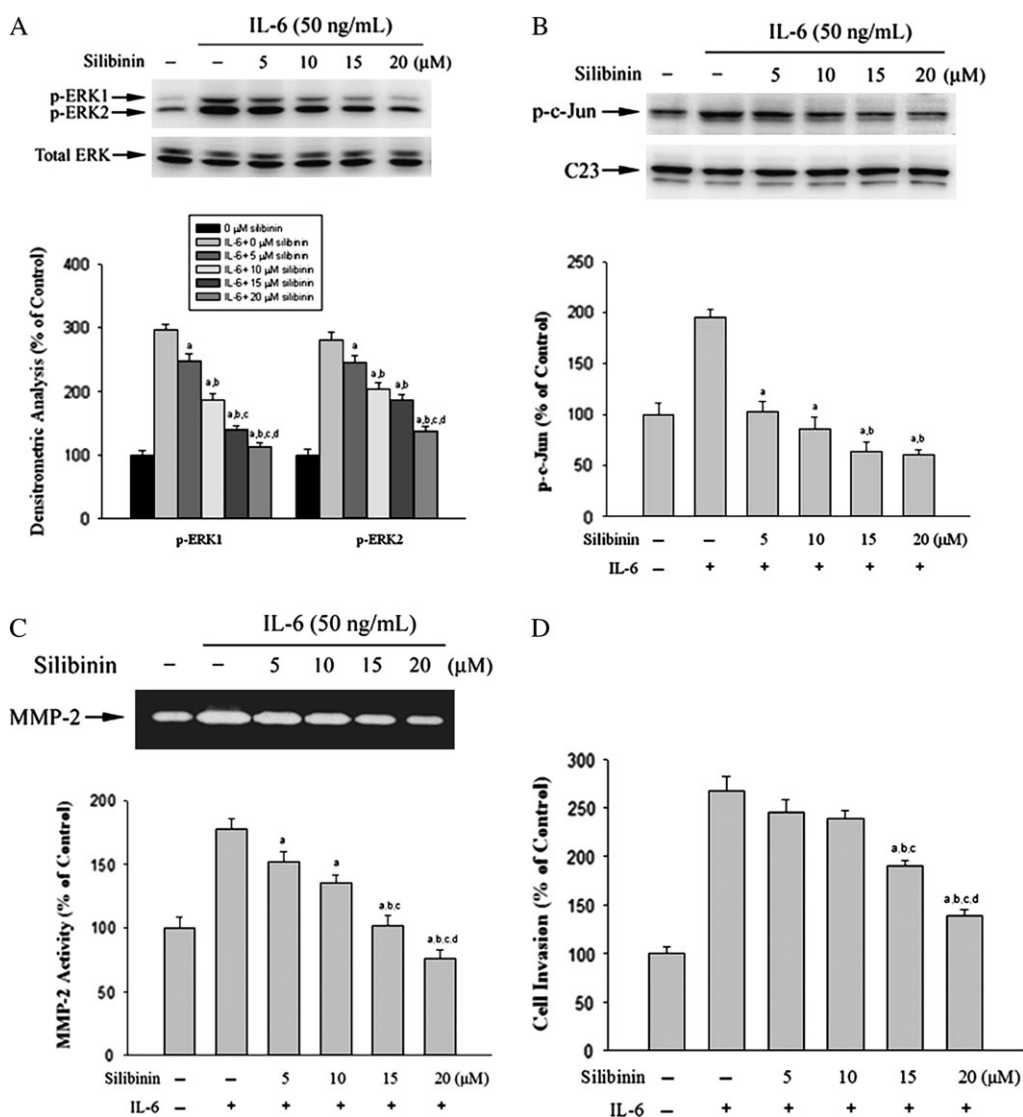


Fig. 7. Effects of 20 μM silibinin on phosphorylation of ERK 1/2, c-Jun, MMP-2 and the invasiveness after IL-6 induction. After pretreatment of 20 μM silibinin for 24 h, MG-63 cells were treated with 50 ng/ml IL-6 for the appropriate time. The densitometric data shown are mean \pm standard deviation of triplicate experiments. Analysis of variance with Scheffe posteriori comparison was used. (A) Concentration effects (p-ERK 1: $F = 243.109$, $P < 0.001$; p-ERK 2: $F = 92.198$, $P < 0.001$); (B) concentration effects (nuclear-c-Jun: $F = 118.604$, $P < 0.001$); (C) concentration effects (MMP-2: $F = 88.399$, $P < 0.001$); (D) concentration effects (cell invasion: $F = 72.020$, $P < 0.001$). ^aSignificantly different, $P < 0.05$, when compared with 0 μM silibinin with IL-6 induction. ^bSignificantly different, $P < 0.05$, when compared with 5 μM silibinin with IL-6 induction. ^cSignificantly different, $P < 0.05$, when compared with 10 μM with IL-6 induction. ^dSignificantly different, $P < 0.05$, when compared with 15 μM with IL-6 induction.

induction in a dose-dependent manner ($P < 0.001$) (Figure 7B). We also confirmed that silibinin significantly suppressed the activity of MMP-2 ($P < 0.001$) and the invasive activity ($P < 0.001$) of MG-63 cells with IL-6 induction in a dose-dependent manner (Figure 7C and D).

Overexpression of constructively active MEK1 prevented the suppression of silibinin on the phosphorylation of ERK 1/2 and the invasiveness of MG-63 cells

Transfection of vectors containing a constructively active MEK1 cDNA into MG-63 cells increased the phosphorylation of MEK1 and the phosphorylation of ERK 1/2 (Figure 8A). However, the increase of the phosphorylation of ERK 1/2 after transfection of constructively active MEK1 could not be affected with 24 h of silibinin treatment (p-ERK 1: $P = 0.702$; p-ERK 2: $P = 0.629$). Similarly, silibinin did not affect the invasive activity of MG-63 cells after 48 h of transfection ($P = 0.592$) (Figure 8B).

Discussion

The present study sought to determine the effect of silibinin on tumor cell adhesion to the ECM and on tumor cell migration and invasiveness *in vitro* by monitoring the regulation of u-PA, PAI-1, MMP-2 and TIMP-2 expressions and the possible signaling pathways in human osteosarcoma MG-63 cells. The major findings are as follows: (i) silibinin suppresses cellular adhesion and invasiveness of MG-63 cells, accompanied by a decrease in u-PA and MMP-2 expression; (ii) the mechanism may involve modifications of the cytoskeleton assembly and the cellular shape, as well as the inhibition of FAK production and Raf-ERK-dependent c-Jun/AP-1 induction, but does not involve JNK, p38 and PI3K-Akt pathways; (iii) silibinin abolishes the increase of the phosphorylation of ERK 1/2 and c-Jun in the nucleus, the level of MMP-2 and the invasiveness of MG-63 cells with IL-6 induction and (iv) silibinin cannot suppress the increase of the phosphorylation of ERK 1/2 and the invasiveness of MG-63 cells after overexpression of constructively active MEK1,

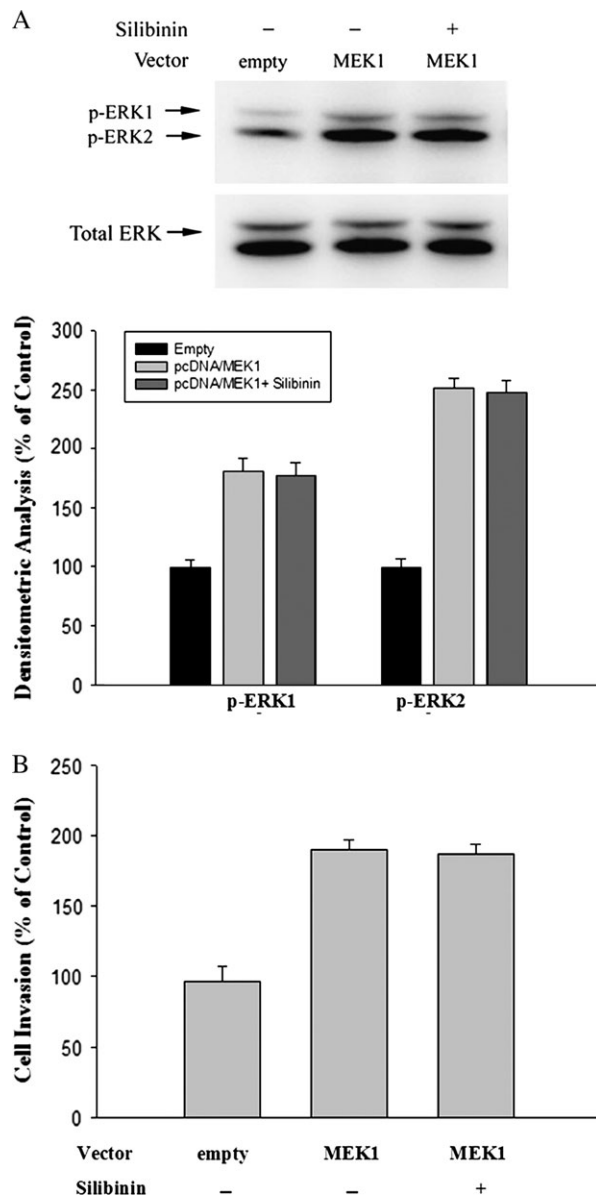


Fig. 8. Effects of 20 μ M silibinin on (A) phosphorylation of ERK 1/2 (p-ERK 1: $P = 0.702$; p-ERK 2: $P = 0.629$) and (B) the invasiveness ($P = 0.592$) after overexpression of constructively active MEK1. For overexpression of the phosphorylation of ERK 1/2, vectors containing a constructively active MEK1 cDNA were transfected into MG-63 cells. The densitometric data shown are mean \pm standard deviation of triplicate experiments. The Student's t -test was used.

which suggests that the target point is the upstream of ERK 1/2. Thus, learning more about the cellular and molecular basis of these different adhesion/invasion processes may help understand how bone tumor cells disseminate, which may lead to new treatment strategies. First, key components of the signaling pathways represent candidate biomarkers that may predict patient outcomes more reliably than traditional morphological criteria; second, understanding the pathways may suggest potential adjuvant targets for inhibiting invasion and metastasis, two major contributors to death among patients with osteosarcoma (30,31). In addition, these results are significant because silibinin anti-metastatic capabilities may provide an advantage in combination chemotherapy for osteosarcoma.

For tumor invasion of the basement membrane, MMP-2 is believed to be important because (i) it plays a critical role in the degradation of the ECM, including type IV collagen (12,13); (ii) it is activated in

a tumor-specific manner and (iii) its expression correlates with metastatic abilities and poor prognosis (14,15). In the present study, treatment with silibinin inhibited the expression of u-PA and MMP-2 in a dose- and time-dependent manner in MG-63, suggesting that this down-regulation of u-PA and MMP-2 contributes to the reduction of the invasiveness of MG-63 cells.

Cell-cell adhesion is an important factor to restrict cellular migration, invasion and metastasis (29), and reduced expressions of α - and β -catenins have been demonstrated in some cervical cancer cells and metastatic lesions (41). However, in this study, the level of α - and β -catenins and the migration potential of MG-63 cells were not affected by any concentration of silibinin examined. Intriguingly, silibinin suppressed the cell-matrix adhesion of MG-63 cells, which is the initial and critical step of invasion. After silibinin treatment, the actin microfilaments virtually distributed heterogeneously and aggregated and MG-63 cells became round and shrunken. We therefore wondered what types of adhesion-dependent signaling pathways were involved in these changes of cell adhesion and migration. In addition to its effect on cell migration, FAK is thought to be a key player in integrating both growth factors and adhesion-dependent signals (28). Because the arrangement of actin cytoskeleton (which anchors to the cell membrane at focal adhesions) was modified by silibinin treatment, we therefore hypothesized that FAK levels would be suppressed. Our findings indicated that FAK and actin filaments are associated in silibinin-mediated inhibition of cell adhesion; therefore, we further explored the downstream regulation of the FAK.

FAK, a 125 kDa protein tyrosine kinase, helps transduce integrin-generated signals to the Ras-MAPKs cascades (28,42) and is overexpressed in a variety of malignancies to regulate the transcription of many MMPs, including MMP-2. FAK autophosphorylation activates Ras, which, in turn, activates PI3K and Raf (43). PI3K regulates integrin-dependent cell migration by modulating integrin responses in both normal and neoplastic tissue. Raf can lead to MEK and ERK activation, which increases their transcriptional activity (19). ERK activation is likely to impact invasion and migration through many pathways by influencing gene transcription and survival, as well as by directly regulating the enzymes that are necessary for cell locomotion. Previous research on different cell types has demonstrated that MAPKs like ERK 1/2, JNK and p38 seem to play a central role in regulating the expression of u-PA and MMPs (20,44). Thus, inhibition of the MAPKs pathway might prevent angiogenesis, proliferation, invasion and metastasis in a wide range of tumors. In the present study, silibinin certainly inhibited cell invasiveness by reducing the expression and activity of u-PA and MMP-2 through a Raf-ERK-dependent pathway, which targeted the upstream of ERK 1/2, but not via the JNK and p38 signal pathways. FAK may have a dual effect on Raf-ERK-u-PA/MMP-2 signaling invasion and actin cytoskeleton-related adhesion in silibinin-treated MG-63 cells. Nevertheless, silibinin did not affect the cell migration potential through the PI3K-Akt signal pathway in this study.

Although the MMP-2 promoter does not contain binding sites for NF- κ B, c-Jun or c-Fos, it has been reported recently that MMP-2 activation occurs in tumor cells through an AP-1- or NF- κ B-dependent pathway (45,46). The transcription of MMPs or u-PA gene is regulated by upstream sequences, including motifs corresponding to AP-1-, NF- κ B- or Ets-1-binding sites (44,47). Our data showed that silibinin inhibits AP-1 DNA-binding activity but not that of NF- κ B, which is surprising since silibinin inhibits the invasiveness of human lung cancer cells by activating NF- κ B and inhibits angiogenesis of human endothelial cells by down-regulating NF- κ B (20,48). We also found that, in MG-63 cells, silibinin only depresses the phosphorylation and activation of c-Jun in the nucleus, without affecting c-Fos. This is different to what was observed in A549 cells where silibinin affects both c-Jun and c-Fos activation (20). These findings suggested that silibinin-mediated inhibition of the transcription factor AP-1 might play a role in the inhibition of MMP-2 or u-PA synthesis, and might occur via a down-regulation of ERK 1/2 activity and expression of c-Jun. Like AP-1 (20,21) and NF- κ B (20,22), Ets-1 protein is induced by MAPK signaling (49) and interacts with AP-1 and NF- κ B

(50–52). In this study, silibinin did not inhibit Ets-1 level in MG-63 cells, which suggests that there is no involvement of the ERK/Ets-1 signaling pathway.

Collectively, our data provide an extensive understanding of the effect of silibinin on cell adhesion and invasion, as well as the possible signaling pathway involved in these processes in MG-63 cells. Silibinin prevents MG-63 cell invasion by inhibiting u-PA and MMP-2 through down-regulation of the FAK to Raf-ERK/c-Jun signaling pathway. Silibinin also inhibits MG-63 cell–matrix adhesion by aggregating filamentous actin and changing cellular shape. The dual effect of silibinin may have synergetic effects on cancer invasion and metastasis. These results provide a mechanism for the observed relationship between u-PA and MMP-2 expression and cell invasiveness on the one hand and cell morphology and cell adhesion on the other hand. Targeting the FAK to Raf-ERK/c-Jun pathway may be a potential adjuvant strategy to inhibit the adhesion and invasiveness of this highly metastatic cancer; however, more studies are needed to further justify silibinin as a novel agent with anti-metastatic capabilities in combination chemotherapy against osteosarcoma and other cancers.

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