

α -Tomatine Suppresses Invasion and Migration of Human Non-Small Cell Lung Cancer NCI-H460 Cells Through Inactivating FAK/PI3K/Akt Signaling Pathway and Reducing Binding Activity of NF- κ B

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Abstract α -Tomatine, isolated from *Lycopersicon esculentum* Linn., is a naturally occurring steroidal glycoalkaloid in immature green tomatoes. Some reports demonstrated that α -tomatine had various anticarcinogenic properties. The purpose of this study is to investigate the anti-metastatic effect of α -tomatine in NCI-H460 human non-small cell lung cancer cells. First, the results showed that α -tomatine significantly suppressed the abilities of the adhesion, invasion, and migration of NCI-H460 cells under non-cytotoxic concentrations. Molecular data also showed α -tomatine could inhibit the activation of focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K)/Akt signal involve in the downregulation the enzyme activities, protein and messenger RNA levels of matrix metalloproteinase-7 (MMP-7). Next, α -tomatine also strongly inhibited the degradation of inhibitor of kappaB α (I κ B α) and the

nuclear levels of nuclear factor kappa B (NF- κ B). Also, a dose-dependent inhibition on the binding ability of NF- κ B by α -tomatine treatment was further observed. Furthermore, α -tomatine significantly decreased the levels of phospho-Akt and MMP-7 in *Akt1*-cDNA-transfected cells concomitantly with a marked reduction on cell invasion and migration. Presented results indicated α -tomatine might be further application for treating cancer metastasis.

Keywords α -Tomatine · Invasion · Migration · FAK · PI3K/Akt · MMP-7

Abbreviations

MMPs Matrix metalloproteinases
ECM Extracellular matrix
MAPK Mitogen-activated protein kinase

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ERK	Extracellular signaling-regulating kinase
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
PI3K	Phosphoinositide 3-kinase
NF- κ B	Nuclear factor kappa B
AP-1	Activator protein-1
I κ B	Inhibitor of NF- κ B

Introduction

Lung cancer is one of the most common types of cancer in the world, and accounts for around more than a million deaths yearly worldwide. This high mortality of probably attributable to early metastasis, principally through the spreading of malignant cells, especially non-small cell lung carcinoma (NSCLC) [1], to distant organs such as the brain, contralateral lung, and bone as a result of the hematogenous spread of cancer cells [2, 3]. Also, non-small cell lung cancers commonly develop resistance to radiation and chemotherapy, and often present at stages too late for surgical intervention. Thus, effective chemopreventive treatment for metastasis would have an important impact on lung cancer mortality rates.

FAK is a non-receptor tyrosine kinase primarily localized to cell–matrix adhesions which acts as a central regulator of the focal adhesion to influence cell survival, differentiation, proliferation, metastasis, and tissue remodeling [4–6]. Tumor metastasis occurs by a series of steps and various cytophysiological changes, including damaged intercellular interaction, increased cancer cells and extracellular matrix (ECM) interaction, damaged ECM components, vessel formation. Excess ECM degradation is one of the hallmarks of tumor invasion and migration, and is regulated by extremely complicated mechanisms [7]. PI3K/Akt signal transduction pathway was found to be necessary to mediate the proliferation and metastasis of human non-small cell lung cancer (NSCLC) and is closely associated with the development and progress of various tumors [8]. Akt kinase, an important component of postsurvival signaling pathways, is activated via PI3K signaling pathway. [9]. The mitogen-activated protein kinases superfamily members (MAPK) are associated with increased scattering/motility, invasion, proliferation, survival, and morphogenesis [10, 11]. Three major mammalian MAP kinases include extracellular signal-regulated kinase1 and 2 (ERK1/2), 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 expressions of MMPs [12–14]. The promoter of MMPs is highly conserved and shown to contain multiple functional elements, including NF- κ B, AP-1, and EST elements [15, 16].

Many NF- κ B activation pathways have been revealed, and all of them rely upon sequentially activated kinase cascades [17]. NF- κ B is a multisubunit transcription factor, which is involved in immune response, inflammation, and malignant transformation. The active NF- κ B consists of p50, p52, p65 (Rel A), Rel B, and c-Rel. Under normal condition, NF- κ B is maintained in the cytoplasm through interactions with an inhibitor of NF- κ B (I κ B), but upon dissociation, moves into the nucleus and promotes cancer cells proliferation, angiogenesis, and metastasis [18, 19]. AP-1 is a nuclear transcription, which is involved in cell proliferation, differentiation, apoptosis, and neoplastic transformation. AP-1 consists of homodimers and heterodimers of members from Fos (c-Fos, Fos B, Fra-1, and Fra-2) and Jun (c-Jun, Jun B, and Jun D) families [20].

α -Tomatine occurs naturally in tomatoes (*Lycopersicon esculentum*). Immature green tomatoes contain up to 500 mg α -tomatine/kg fresh fruit weight. The compound is partly degraded as the tomato ripens until at maturity levels in red tomatoes are about 5 mg/kg fresh fruit weight [21]. Figure 1a shows α -tomatine is constructed of an aglycon moiety (tomatidine), and a tetrasaccharide moiety (β -lycotetose) that contains two molecules of D-glucose and one each of D-galactose and D-xylose. Previous studies demonstrated α -tomatine has exhibited antiproliferative and apoptotic effects on the growth of cancer cells originating from the human colon and liver [22]. In addition, α -tomatine also induced T-cell-mediated regression of murine lymphoid experimental tumors, EG7-Ova [23], and acted as an anti-inflammatory agent by blocking NF- κ B and JNK signaling in mouse macrophages [24]. In this study, we employed human non-small cell lung cancer NCI-H460 cells to assess the molecular mechanism responsible for the antimetastatic effect of α -tomatine. We found that α -tomatine suppresses invasion and migration of NCI-H460 cells through inactivating PI3K/Akt signaling pathway.

Materials and Methods

Test Compound

α -Tomatine purchased from Extrasynthese (Genay, France), dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C . The purity was $>98\%$, as assessed by HPLC. Control cultures received the carrier solvent (0.1% DMSO).

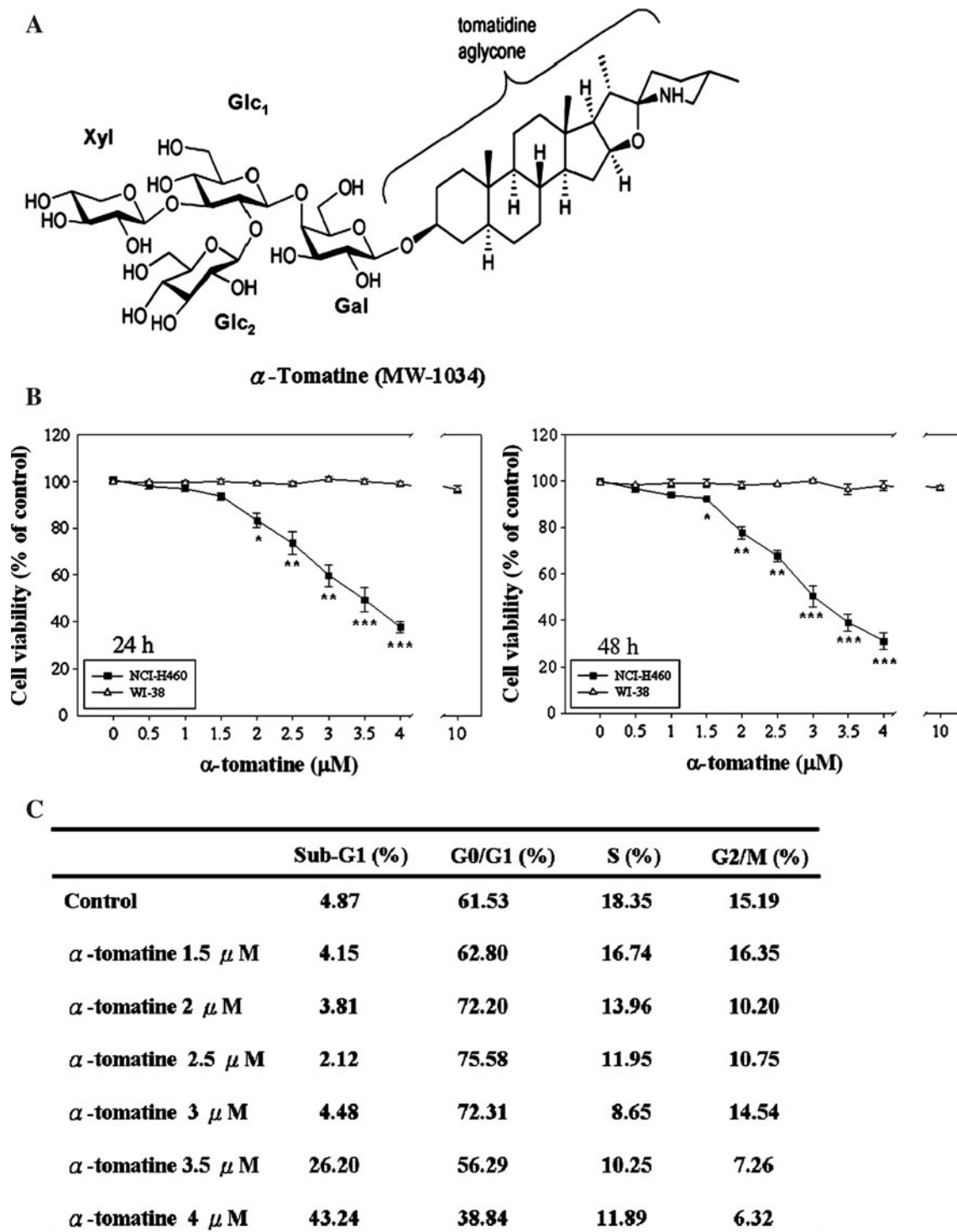


Fig. 1 The effects of α -tomatine on the viability and growth in NCI-H460 and WI-38 cells. **a** Chemical structure of α -tomatine. **b** Cultured cells were treated with or without α -tomatine under various concentration for 24 and 48 h. Thereafter, cell viability was determined by MTT assay. Values represent mean \pm S.D. of three independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared

with the untreated control (dose 0). **c** The cells were fixed and stained with PI, and the cell cycle distribution was then analyzed by flow cytometry (FACS). The data indicate the percentage of cells in sub-G1, G0/G1, S, and G2/M phases of the cell cycle. Data was presented from three independent experiments

Cell Culture

Non-small-cell lung cancer cell line NCI-H460 (human lung large cell carcinoma) was maintained in RPMI-1640 medium. Normal lung cell line WI-38 (human lung epithelial fibroblast) was maintained in MEM medium. Above-mentioned cell lines were obtained from BCRC (Bioresource Collection and Research Center in Hsin-Chu, Taiwan). All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂–95% air. In medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics (100 U/ml of penicillin and 100 mg/ml of streptomycin).

Cell Viability Assay

To measure the effect of α -tomatine on cell viability, the NCI-H460 and WI-38 cells (1×10^5 cells/ml) were seeded into a 24-well plate. Cells were treated with or without α -tomatine under various concentrations for various periods of time (24 and 48 h). At the end of the assay period, cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay, as described previously [25]. Each concentration was repeated three times. After the exposure period, the medium was removed and followed by washing the cells with PBS. Then, the medium was 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 comparing with the untreated control cells.

Cell-Cycle Assay

Flow cytometric analysis of NCI-H460 cells was performed using a FACScan (Becton Dickinson Immunocytometry Systems, UK). To analyze the cell-cycle distribution, the cells were first treated with various concentrations of α -tomatine for 24 h, and then were collected by trypsinization, fixed in 75% absolute ethanol, washed in PBS, resuspended in 1 ml of PBS containing 0.5 mg/ml ribonuclease (RNase) A and 0.01 mg/ml propidium iodide (PI) in the dark for 30 min at room temperature. The cell-cycle profiles were analyzed by a flow cytometer. The percentage of cell in the sub-G1, G0/G1, S, and G2/M phases of the cell cycle was analyzed by the ModFit LT 3.0 software (Verity Software, Topsham, ME).

Mitochondrial Membrane Potential Assay

We used mitochondrial-specific cationic dye JC-1 (5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolycarbocyanine iodide) (Molecular Probes, Inc.), which

undergoes potential-dependent accumulation in the mitochondria. Cells were seeded in a 96-well plate. Following treatment with α -tomatine (0, 1.5, 3, and 4 μ M) for the indicated times, cells were stained with 25 μ M JC-1 for 30 min at 37°C. Fluorescence was monitored with the fluorescence plate reader at wavelengths of 490 nm (excitation)/540 nm (emission) and 540 nm (excitation)/590 nm (emission) pairs. Changes in the ratio between the measurement at test wavelengths of 590 nm (red) and 540 nm (green) fluorescence intensities are indicative of changes in the mitochondrial membrane potential.

Measurement of Intracellular ROS Formation

Intracellular ROS was estimated using a fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA diffuses through the cell membrane readily and is enzymatically hydrolyzed by intracellular esterases to non-fluorescent dichlorofluorescein (DCFH), which is then rapidly oxidized to highly fluorescent DCF in the presence of ROS. Following exposure to various concentrations of α -tomatine, NCI-H460 cells (1×10^5 cells/ml) were incubated in a culture medium containing 20 μ M DCFH-DA for 30 min at 37°C, and then were washed with PBS. The cell suspensions were centrifuged at 412 \times g for 10 min and medium was removed. Cells were dissolved with 1% Triton X-100, and DCF fluorescence intensity was detected at different time intervals with excitation wavelength 503 nm and emission wavelength at 529 nm (slit width: 5 nm). The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly [26].

Measurement of Intracellular GSH Content

Intracellular levels of GSH were determined using the method of Hisin and Hilf [27]. Cells after treatment were washed with PBS and scrapped into 6.5% trichloroacetic acid. Phosphate–EDTA buffer (4.5 ml) of pH 8.0 was added to 0.5 ml of 12000 \times g supernatant. The final assay mixture (2 ml) contained 100 μ l of the diluted supernatant, 1.8 ml phosphate–EDTA buffer pH 8.0, and 100 μ l 0.1% o-phthalaldehyde solution containing 100 μ g of OPT. After thorough mixing and incubation at room temperature for 15 min fluorescence was read at wavelengths of 350 nm (excitation)/420 nm (emission) in FLUOstar OPTIMA spectrofluorophotometer. The reduced form of GSH was used as a standard. Data were expressed as nanomole GSH per 10⁶ cells.

Cell–Matrix Adhesion Assay

After pretreatment with α -tomatine (0, 0.5, 1, and 1.5 μ M) for 24 h, cells were seeded at a density of 1×10^5 cells/ml

in a 24-well plate and coated with 500 μ l type IV collagen (10 μ g/ml); then they were cultured for 30 min. Then, non-adherent 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.

Immunofluorescence Staining Assay

To determine the effect of α -tomatine on cell morphology and actin stress fibers, NCI-H460 cells (1×10^5 cells/ml) were plated in six-well plates and grown for 16–18 h and then incubated in the different concentrations of α -tomatine (0, 0.5, 1, and 1.5) for 24 h. After the exposure period, media were removed, and cells were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS. Cells were then fixed with 4% paraformaldehyde in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS for 15 min and incubated with 0.5% Triton X-100/in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS for 5 min. Cells were incubated with 1% bovine serum albumin in 0.5% Triton X-100/in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS for 1 h (blocking) and then with 200 units/ml Alexa flour 488-phalloidin (Invitrogen, Karlsruhe, Germany) for 1 h to stain the actin filaments. At last, the nucleus were stained with DAPI (1 μ g/ml) solution for 30 min at 25°C and examined by a fluorescence microscopy (BX51, Olympus, Tokyo, Japan).

Boyden Chamber Invasion and Migration Assay

The ability of NCI-H460 cells to pass through filters coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) was measured by Boyden chamber invasion assay [28]. Matrigel was diluted to 200 μ g/ml with cold filtered distilled water and applied to the top side of the 8 μ m pore polycarbonate filter. Briefly, NCI-H460 cells were treated with various concentrations of α -tomatine. After 24 h, cells were detached by trypsin and resuspended in serum-free medium. Medium containing 10% fetal bovine serum 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/ml in 50 μ l 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. Cells invading across the matrigel to the lower surface of the membrane were fixed with methanol and stained with 5% Giemsa solution. The invading 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 NCI-H460 cells on migration, cells were seeded into a Boyden chamber with 8 μ m pore polycarbonate filters which were not coated with matrigel.

Migrating cells were treated with various concentrations of α -tomatine. The migration assay was measured as described in the invasion assay.

Gelatin- and Casein-Zymography

The activities of MMP-2 and MMP-9 in the conditioned medium were measured by gelatin-zymography assay as described previously [29]. Samples were mixed with loading buffer and 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, 10 mM CaCl_2 , 0.02% NaN_3), stained with Coomassie blue R-250 (0.125% Coomassie 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). Proteolytic activity of MMP-7 was evaluated by β -casein zymography on 12–15% polyacrylamide gels containing 0.1% β -casein as well as by gelatin-zymography.

Western Blotting Analysis

The preparation of cytosolic and nuclear fractions of the cells was performed as described previously [30]. The Western blotting was performed as follows. The denatured samples (50 μ g purified protein) were resolved on 10–12% SDS-PAGE gels. The proteins were then transferred onto nitrocellulose membranes. Non-specific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 1% (w/v) non-fat 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 an appropriate dilution of specific primary antibodies in TBST overnight at 4°C. Subsequently, 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 bands detection were revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed ECL hyperfilm in FUJIFILM Las-3000 mini (Tokyo, Japan). Then proteins were quantitatively determined by densitometry using FUJIFILM-Multi Gauge V3.0 software.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted by using the total RNA Extraction Midiprep System (Viogene BioTek, Taiwan). Total

RNA (2 µg) was transcribed to 20 µl cDNA with 1 µl deoxynucleotide triphosphate (dNTP; dNTP set consists of 2.5 mM aqueous solutions at pH 7.0 of each of dATP, dCTP, dGTP, and dTTP), 1 µl Oligo dT (10 pmol/ml), 1 µl RTase (200 U), 1 µl RNase inhibitor, and 5 × reaction buffer. The appropriate primers (sense of MMP-2, 5'-GGCCCTGTCACCTCTGAGAT-3', nt 1337-1356; antisense of MMP-2, 5'-GGCATCCAGTTATCGGGGA-3', nt 2026-2007; sense of MMP-9, 5'-AGGCCTCTACAGAGTCTTTG-3', nt 1201-1220; antisense of MMP-9, 5'-CAGTCCAACAAGAAAGGACG-3', nt 1700-1683; sense of MMP-7, 5'-CTATGCGACTCACCGTGCTG-3' nt 147-166; antisense of MMP-7, 5'-CTTGAGATAGTCCTGAGCCTGTTC-3', nt 696-667; sense of GAPDH, 5'-CGGAGTCAACGGATTGGTGT-3' nt 94-126; antisense of GAPDH, 5'-AGCCTTCTCCATGGTTGGTGAAGAC-3', nt 399-375) were used for polymerase chain reaction (PCR) amplifications. PCR was performed with Platinum Taq polymerase (Invitrogen, San Diego, CA, USA) under the following conditions: 30 cycles of 94°C for 1 min, 59°C (MMP-2 and MMP-7) or 60°C (MMP-9 and GAPDH) for 1 min, 72°C for 1 min followed by 10 min at 72°C. PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

Electrophoretic Mobility Shift Assay (EMSA)

The DNA-binding activities of NF-κB and AP-1 in nuclear extracts were assessed by EMSA [31] using the Lightshift kit from Pierce (Rockford, Illinois, USA) with biotin-labeled double-stranded AP-1 and NF-κB oligonucleotides. A five microgram aliquot of nuclear proteins was mixed with either biotin-labeled NF-κB or AP-1 oligonucleotide probe for 15 min at room temperature or with oligonucleotides containing (sense of NF-κB, 5'-AGTTGAGGGGA CTTTCCCAGGC-3', antisense of NF-κB, 3'-TCAAC TCCCCTGAAAGGGTCCG-5; sense of AP-1, 5'-CGCTTGATGACTCAGCCGGAA-3', antisense of AP-1, 3'-GC GAACTACTGAGTCGGCCTT). DNA probes were added to 10 µl binding reactions containing double-distilled H₂O, 5 µg nuclear proteins, 1 µl poly (dI-dC), 1 µl biotin-labeled double-stranded NF-κB 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 200-fold excess of unlabeled probe as a specific competitor. Following protein–DNA complexes formation, samples were loaded on a 6% non-denaturing polyacrylamide gel in 0.5 × TBE buffer and then transferred to positively charged nitrocellulose membranes (Millipore, Bedford, MA, USA) and crosslinked in a Stratagene crosslinker. Gel shifts were visualized with streptavidin-horseradish peroxidase followed by chemiluminescent detection.

Transient Transfection with *Akt1* cDNA

Transient transfection assay was carried out as described previously [32]. Liposome-mediated transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) on NCI-H460 cells with a control pUSEamp empty vector (as the control group) or an expression construct for *Akt1* cDNA in pUSEamp (activated) (Upstate Biotechnology, Lake Placid, NY, USA). Briefly, NCI-H460 cells (2 × 10⁵ cells/well) were plated onto 6-well plates and transfected with the indicated plasmid at 60–70% confluence on the next day using Lipofectamine Plus reagent according to the manufacturer's instructions. Briefly, lipofectamine (4 µl) and DNA (2 µg) were diluted in 100 µl of RPMI-1640 followed by equilibration at room temperature for 15 min after mixing. The lipofectamine–DNA complex was added to NCI-H460 cells, and incubated for 12 h. Cells were then washed with PBS and replenished with RPMI-1640 containing 20% serum. Twelve hours after transfection, the cells were incubated with 1.5 µM α-tomatine for 24 h. After further 24 h incubation, the media were removed, and cells were washed once with cold PBS. At last, the transfected cells were collected and subjected to Western blot, invasion, and migration assay.

Statistical Analysis

Data were expressed as means ± standard deviation of three independent experiments. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences were established at $P \leq 0.05$.

Results

Cytotoxicity of α-Tomatine to NCI-H460 Cells

α-Tomatine is a natural steroidal glycoalkaloid with pleiotropic activities against cancer growth. In this study, we first examined the effect of α-tomatine on cell cytotoxicity in NCI-H460 cells. As shown in Fig. 1b, α-tomatine showed a dose- and time-dependent inhibitory effect on the cell viability of NCI-H460 cells. Compared to 0 µM (DMSO was treated alone, data not shown), after 24 and 48 h treatment with α-tomatine at a concentration between 0 and 1.5 µM was not significantly altered, indicating α-tomatine was not toxic to NCI-H460 cells at these dosages. When cells were treated with 2–4 µM α-tomatine for 24 and 48 h, the cell viability was significantly decreased. These results demonstrated treating with α-tomatine with doses higher than 1.5 µM for 24 and 48 h resulted in dose- and time-dependent loss of cell viability in NCI-H460

cells, but doses lower than 1.5 μM for 24 and 48 h did not cause cytotoxicity. In addition, same procedures were performed on a normal lung cell line WI-38, and result shown that the cytotoxicity effect of α -tomatine on WI-38 cells was not significant when at various concentrations (0–10 μM). In order to ascertain that the dose range was applied in all subsequent experiments to avoid the influence of cell growth and cytotoxicity on the observed parameters, a series of studies were performed to measure the cell growth and cytotoxicity upon α -tomatine stimulation. First, the effects of α -tomatine on NCI-H460 cell-cycle progression were measured by flow cytometry (Fig. 1c). It has revealed that a treatment with 1.5 μM α -tomatine had no effect on the cell-cycle distribution for 24 h. At higher concentrations (2, 2.5, 3, 3.5, and 4 μM), α -tomatine increased the G1 fraction (from 61.53 to 72.31%), and caused an apparent accumulation of the cells in the sub-G1 phase, while decreased the S fraction in a dose-dependent manner (Fig. 1d). Therefore, the results demonstrated that a 24 h treatment of α -tomatine at a concentration ranging from 0 to 1.5 μM had no cytotoxicity to NCI-H460 cells. In addition, α -tomatine had no

effect on the mitochondrial membrane potential in the concentration range 0.5–1.5 μM , while the NCI-H460 cells were treated with α -tomatine for dramatically reduced mitochondrial membrane potential in a time-dependent manner in the concentration range 3 or 4 μM (Fig. 2a). Oxidative reactions in the mitochondria result in the production of reactive oxygen species (ROS), which are converted to H_2O_2 by superoxide dismutase. The non-enzymatic antioxidant glutathione (γ -glu-cys-gly) is the most abundant weapon against the production of ROS. The depletion of glutathione has been associated with apoptotic cell death machinery [33], as a drop in the GSH levels and concomitant increase in ROS during the apoptosis process has been reported. It has been observed in our studies that there is an increase in the generation of intracellular ROS formation along with decreased levels of GSH in NCI-H460 cells following α -tomatine treatment (2–4 μM). While α -tomatine had no effect on the GSH depletion and ROS formation in the concentration range 0.5–1.5 μM (Fig. 2b, c). Thus, non-cytotoxic concentrations of α -tomatine (0.5–1.5 μM) were used for subsequent experiments.

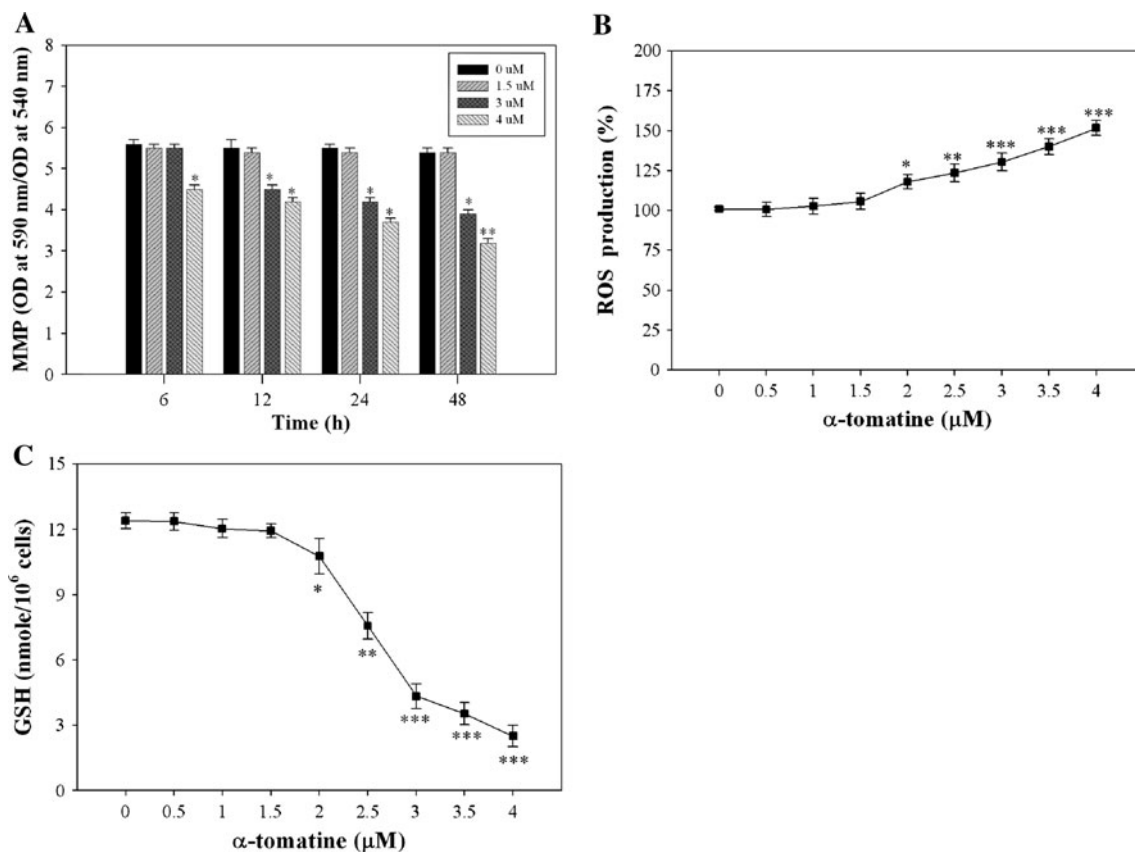


Fig. 2 The effects of α -tomatine on mitochondrial membrane potential, intracellular ROS formation, and intracellular GSH content. **a** Time course effect of various concentrations (0, 1.5, 3, and 4 μM) of α -tomatine on mitochondrial membrane potential. Changes in **b** ROS formation and **c** intracellular GSH content in human

NCI-H460 cells incubated with various concentrations (0–4 μM) of α -tomatine. Values represent mean \pm S.D. of three independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) compared with the untreated control (dose 0)

α -Tomatine Suppresses the Cell Adhesion, Morphology/Actin Cytoskeleton Arrangement, Invasion, and Migration in NCI-H460 Cells.

Previous studies have demonstrated that the cancer metastasis and invasion are highly related to the degradation of ECM, intercellular adhesion, and cellular motility. First, in the cell–matrix adhesion assay, the result showed the cell adhesion ability of NCI-H460 cells was significantly reduced by a 24 h treatment of α -tomatine. Such reduction was concentration-dependent with a 45% decrease ($P < 0.001$) when α -tomatine was at 1.5 μM (Fig. 3a). We next assessed the effect of α -tomatine on the cell morphology and actin cytoskeleton arrangement in NCI-H460 cells by immunofluorescence staining assay. The Fig. 3b shows that NCI-H460 cells were treated with 1.5 μM α -tomatine, the cells changed morphology and became elongated, spindle shaped, and shrunken. Furthermore, the effect of α -tomatine on cell invasion and migration were investigated using a Boyden chamber and the results showed α -tomatine induced a dose-dependent decrease in invasion and migration with an increasing concentration of α -tomatine. According to a quantitative assessment, treatment with 1.5 μM of α -tomatine inhibited 74 and 78% of cell invasion and migration after 48 h incubation, respectively, compared with those of the non- α -tomatine-treated group. The rounding form of cells might reduce the invasion and migration ability of cancer cell (Fig. 3c, d). The results demonstrated α -tomatine significantly inhibited the invasion and migration of highly metastatic NCI-H460 cells.

α -Tomatine Suppresses the MMP-7 Activity and Expression in NCI-H460 Cells

As ECM degradation is by overexpression of proteolytic enzyme activity, such as the matrix metalloproteinases. Meanwhile, locomotion of tumor cells into the extracellular matrix and invasion of lymph and blood vessels occur, and the migrated tumor cells concomitantly escape the immunologic system in the circulation, exit to the new tissue, and eventually colonize the distant site [7]. Thus, the effect of α -tomatine on MMPs activities was investigated by gelatin- and casein-zymography under a condition of serum starvation to explain the contribution of MMPs in the inhibitory effect of α -tomatine on the metastatic ability of NCI-H460 cells. As shown in Fig. 4a, treatment with α -tomatine for 24 h dramatically reduced MMP-7 activity in a dose-dependent manner in the concentration range 0.5–1.5 μM , while it had no significant effect on MMP-2 and MMP-9 activities. We next used an RT–PCR and Western blot assay to investigate the inhibitory effect of α -tomatine on the mRNA and protein levels of MMPs. The results of RT–PCR and Western blot showed that

α -tomatine could reduce the MMP-7 expression. The decrease in mRNA and protein expression was particularly strong when the α -tomatine concentration reached 1.5 μM , but not MMP-2 and MMP-9 (Fig. 4b, c). These results reveal that α -tomatine might regulate MMP-7 expression at transcription level. Furthermore, these results suggested that the antimetastatic effect of α -tomatine was related to the inhibition of enzymatically degradative processes of tumor metastasis.

α -Tomatine Suppresses the FAK/PI3K/Akt Signaling Pathway in NCI-H460 Cells

As we have shown that treatment of α -tomatine to NCI-H460 cells inhibited the cell invasion, migration, and activity of MMP-7, the underlying mechanisms were further investigated by studying the effect of α -tomatine on the constitutive activation status of the three major mammalian MAPKs and Akt. Figure 5 showed the treatment of α -tomatine reduced the phospho-FAK, phospho-Akt and PI3K levels at 6 h after incubation, whereas it had no significant effect on phosphorylated form of p38, JNK1/2, and ERK. Moreover, no significant change in the total amount of Akt, ERK1/2, p38, and JNK1/2 proteins was observed (data not shown).

α -Tomatine Suppresses the DNA-Binding Activity of NF- κ B and the Degradation and Phosphorylation of I κ B α in NCI-H460 Cells

It was known that the MMP-7 promoter has several transcription factor-response elements, including binding sequences for NF- κ B and AP-1 [16, 34]. Also, NF- κ B and AP-1 family of transcriptional factors have been known to translocate to the nucleus and regulate the expressions of multiple genes involved in MMP-7 secretion. NCI-H460 cells were treated with various concentrations of α -tomatine (0, 0.5, 1, 1.5 μM) for 12 h, and nuclear extracts were analyzed by the EMSA for NF- κ B and AP-1 DNA-binding activities. Figure 6a has shown a decrease in the DNA-binding activity of NF- κ B. By contrast, AP-1 binding activity was not affected by α -tomatine. Further, the expressions of NF- κ B, c-Fos, and c-Jun in nuclear extracts were analyzed by Western blotting to assess the possible inhibitory effect of α -tomatine. Figure 6b showed that the nuclear level of NF- κ B (p50 and p65) was gradually diminished at doses of 0.5, 1, and 1.5 μM α -tomatine when compared to the 0 μM after treatment for 12 h. However, there was no noticeable change in the translocation of c-Fos and c-Jun under the same treatment condition. Also, the activation of NF- κ B is through the phosphorylation of I κ B α to release the NF- κ B for nuclear translocation [35], and for binding to the promoter sites of target genes. To

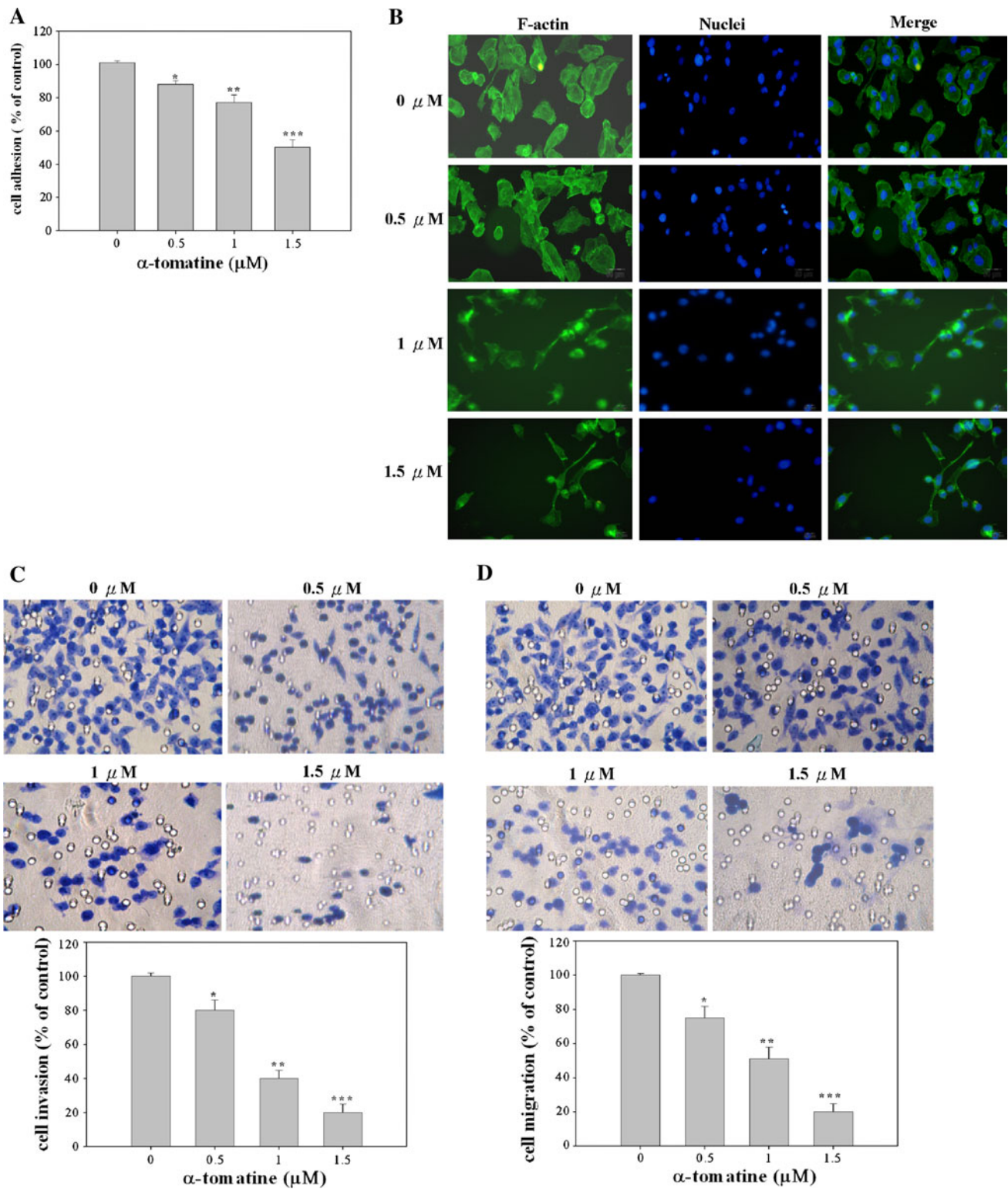


Fig. 3 The effects of α -tomatine on the cell–matrix adhesion, cell morphology/actin cytoskeleton arrangement, invasion, and migration in NCI-H460 cells. Cells were treated with various concentrations (0, 0.5, 1, and 1.5 μ M) of α -tomatine and were then subjected to analyses for **a** cell–matrix adhesion, **b** immunofluorescence, **c** invasion, and

d migration as described in “Materials and Methods”. Values are expressed as mean \pm S.D. of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the untreated control (dose 0)

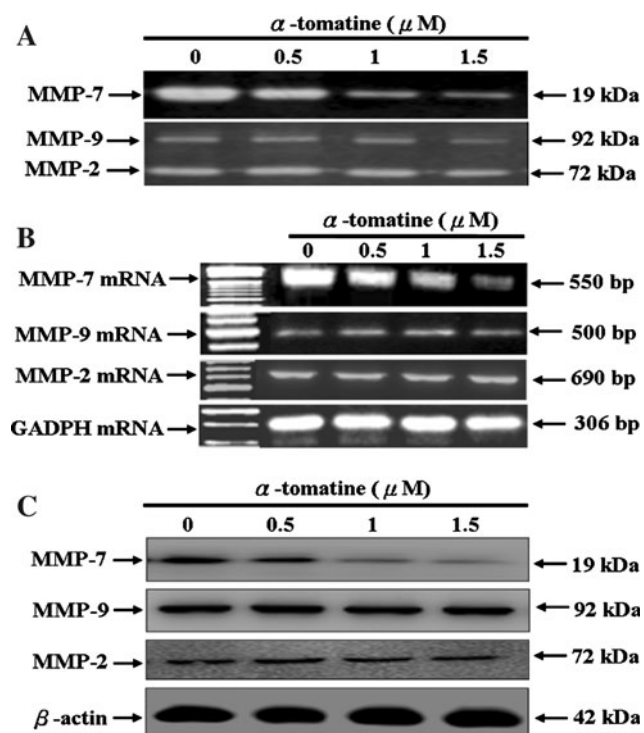


Fig. 4 The effects of α -tomatine on the activities and expressions of MMPs in NCI-H460 cells. **a** Cells in serum-free medium were treated with various concentrations (0–1.5 μ M) of α -tomatine for 24 h. The culture medium of cells after treatment was subjected to gelatin- and casein-zymography to analyze the activities of MMPs. **b** Real-time quantitative RT-PCR and **c** Western blot analysis of MMPs mRNA and protein expressions in cells treated with indicated doses of α -tomatine and harvested at 24 h. β -Actin and GADPH were used as internal controls

examine the effect of α -tomatine on $I\kappa B\alpha$ regulation, we investigated whether α -tomatine has inhibitory effects on $I\kappa B\alpha$ degradation and phosphorylation. As shown in Fig. 6c, α -tomatine blocked $I\kappa B\alpha$ degradation through inhibiting phosphorylation of $I\kappa B\alpha$. Also, the intensity of Western blotting reflected that the α -tomatine at a concentration >0.5 μ M could enhance $I\kappa B\alpha$ protein expression.

α -Tomatine Suppresses the Levels of Phospho-38, MMP-2/9, u-PA and Invasion, Migration in *Akt*-Transfected NCI-H460 Cells

Our results had demonstrated that α -tomatine could suppress cell invasion/migration, down-regulate FAK/PI3K/Akt signal, and reduce MMP-7 expression in NCI-H460 cells. To further investigate whether the inhibition of α -tomatine was mainly through inhibition of FAK/PI3K/Akt signaling pathway, NCI-H460 cells were transiently transfected with active Akt. The Western blotting results showed that the cells expressed as a control vector indeed had diminished the levels of phospho-Akt and MMP-7 when cells were treated with α -tomatine (Fig. 7a). The

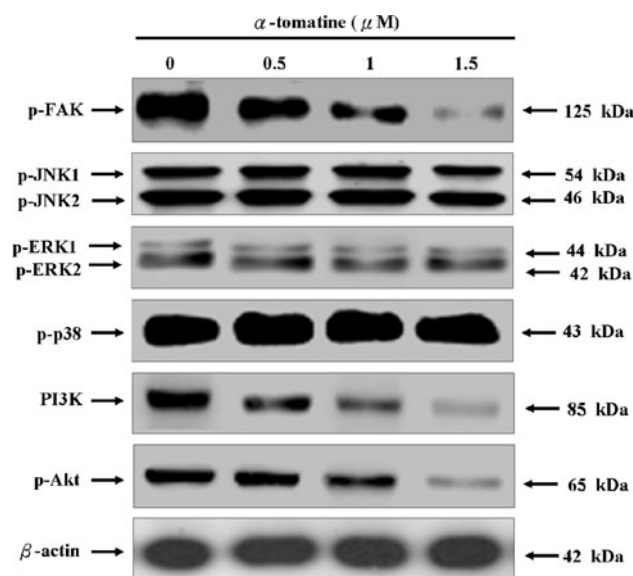


Fig. 5 The effects of α -tomatine on FAK, MAPK, and PI3K/Akt signaling in NCI-H460 cells. Cells were treated with various concentrations (0–1.5 μ M) of α -tomatine for 6 h, after which cells were harvested and analyzed for phospho-FAK, FAK, MAPK (phospho-JNK, JNK, phospho-ERK, ERK, phospho-p38, p38), PI3K, phospho-Akt, and Akt expressions. The protein levels were determined by Western blot. β -Actin was used as an internal control

expression of constitutively active Akt also improved the abilities of invasion and migration in NCI-H460 cells that were originally inhibited by α -tomatine as analyzed by Boyden chamber assay (Fig. 7b, c). After treatment with α -tomatine, the Akt phosphorylation, MMP-7 expression, and invasion/migration were decreased in the *Akt*-transfected NCI-H460 cells.

Discussion

Lung cancer is one of the most common malignancy, with a generally poor prognosis due to its tendency toward local invasion and subsequent metastasis. Recently, antimetastatic agents have been defined as a new class of cancer chemopreventive agent. α -Tomatine is a major glycoalkaloid compound of *Lycopersicon esculentum* Linn. Various studies have shown that α -tomatine exerts pleiotropic anticancer effects, preventive and anticarcinogenic as well as antiproliferative, in various in vivo and in vitro models. α -Tomatine is also reported to exhibit antibiotic activities against microorganisms (eg. *Herpes simplex* and *Herpes zoster* viruses) in human [36], to enhance the duration of action of anesthetics, which act by inhibiting acetylcholinesterase [37], and to potentiate the immune response of vaccines in mice [38].

To verify the anti-metastatic mechanism of α -tomatine on the invasion and migration of human NCI-H460 cells in

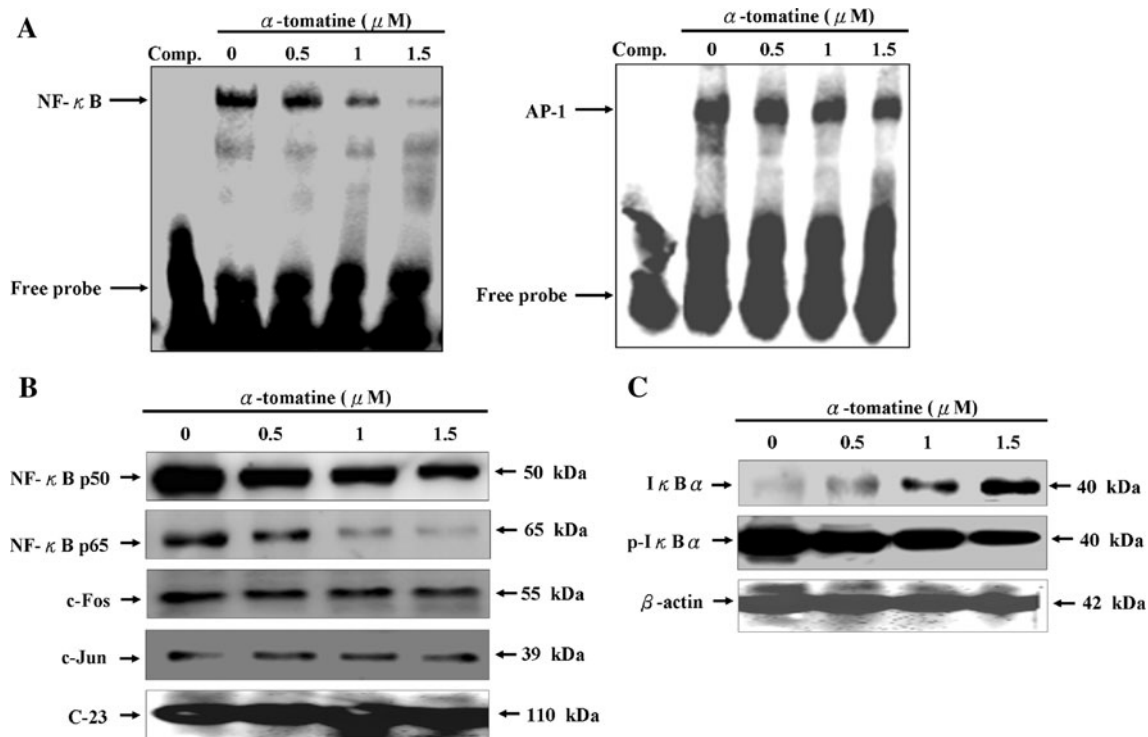


Fig. 6 The effects of α -tomatine on the NF- κ B and AP-1 DNA-binding activates/expressions of NF- κ B, c-Fos, and c-Jun/I κ B α phosphorylation and degradation NCI-H460 cells. Cells were treated with various concentrations (0–1.5 μ M) of α -tomatine for 12 h, and then nuclear extracts were prepared and analyzed for **a** NF- κ B and AP-1 DNA-binding activities by EMSA, as described in [Materials and Methods](#). *Lane 1*: nuclear extracts incubated with 100-fold excess unlabeled consensus oligonucleotide (comp.) to confirm the binding

in vitro. We excluded the effect of NCI-H460 cells from tumor cells growth by MTT assay which is showing that the cell viability was significantly altered by treatment of α -tomatine at concentrations less than 1.5 μ M. In addition, Flow cytometric analysis of NCI-H460 cells exposed to α -tomatine (at concentrations ranging from 2 to 4 μ M) showed that α -tomatine exhibited an anti-proliferation effect. Previous studies reported that GSH depletion and direct oxidative damage are involved in the regulation cell growth and death. Therefore, the data from the analyses of mitochondrial membrane potential, intracellular ROS formation, and GSH content have demonstrated that cell growth was not affected by α -tomatine at a concentration less than 5.0 μ M.

During cancer progression, some tumor cells become motile and gain the capacity to attack the host tissue leading to metastasis. FAK can be activated in response to diverse stimuli and plays an important role in the proliferation and metastasis of cells [39]. The overexpression and phosphorylation of FAK correlates with the increase of cell motility and invasion and alteration in the cytoskeleton [40]. Adhesion and spreading of cells on a variety of ECM proteins, including collagen type IV, leads to an increase in

specificity. **b** Nuclear extracts were subjected to SDS–PAGE followed by western blotting with anti-NF- κ B (p50), anti-NF- κ B (p65), anti-c-Jun, and anti-c-Fos. **c** Equal amounts of cytoplasmic proteins were resolved by SDS–PAGE, transferred to PVDF membrane and probed with specific antibodies (anti-p-I κ B α and anti-I κ B α). C23 and β -actin were used as internal controls. Results from three repeated and separated experiments were similar

tyrosine phosphorylation and activation of FAK [41]. In melanoma cells, the increased expression of FAK correlates with increased cell motility [42]. Overexpression of FAK has also been reported in breast cancer and sarcoma [43, 44]. Also, FAK is at the crossroad of several signaling pathways, including PI3K/Akt and MAPKs pathways [45, 46]. Recent study showed that α -tomatine caused a dose-dependent decrease in cellular level of phospho-Akt, phospho-FAK, and PI3K. Nevertheless, there was no significant change in total and phosphorylated levels of MAPK at the same dosage. When NCI-H460 cells were treated with α -tomatine (0–1.5 μ M) for 24 h, there were dramatically reduced the activity and expression of MMP-7, but not gelatinases of MMP isoforms (MMP-2 and MMP-9). It is of importance to note that MMP-7 plays roles in tumor progression, as over-expression of the MMP-7 gene in transgenic mice led to enhanced tumorigenesis in a breast cancer model [47]. Also, Sasaki et al. [48] reported that the survival rates of lung cancer patients were poor in those with an elevated MMP-7 mRNA expression on the tumor tissue. We have demonstrated that reduction of proteolytically active MMP-7 is involved in α -tomatine mediated-cell invasion and migration. In addition, the

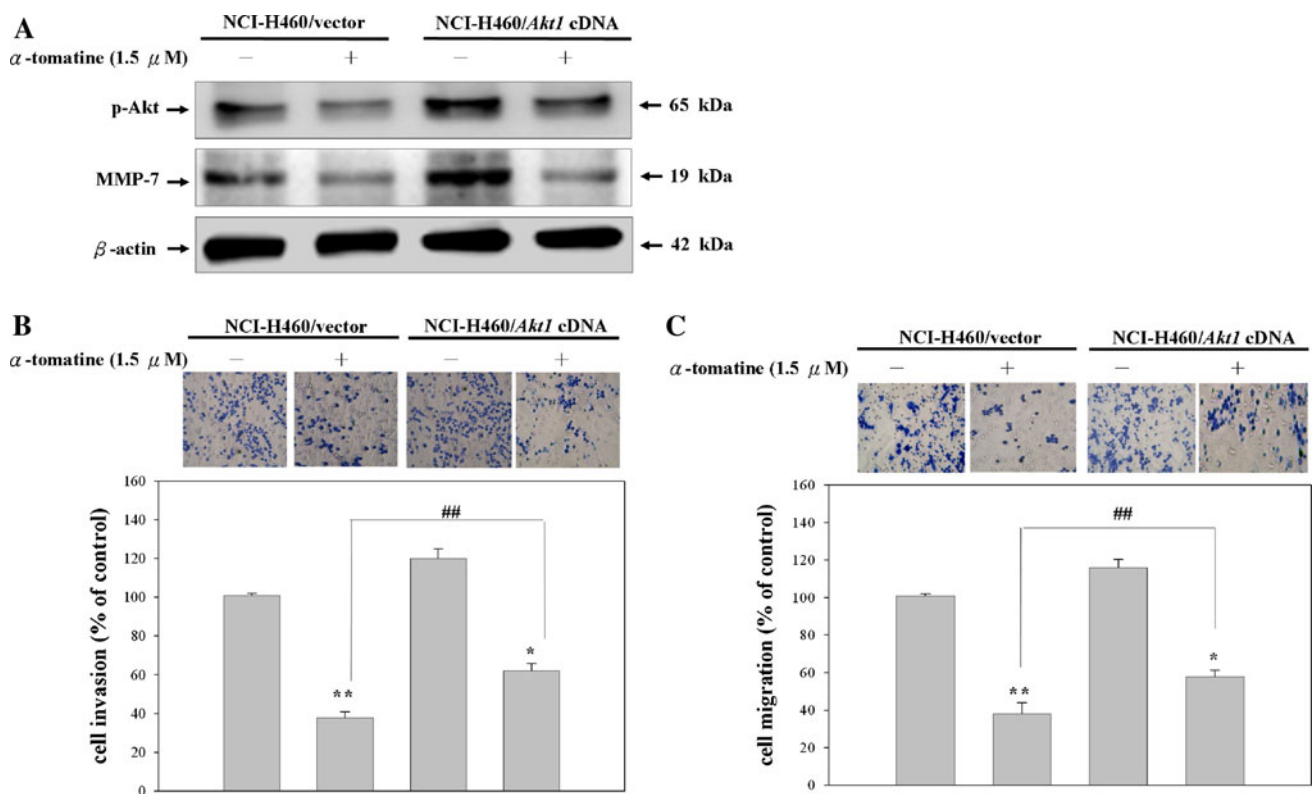


Fig. 7 The effects of α -tomatine inhibited the levels of phospho-Akt, MMP-7 and invasion, migration in *Akt*-transfected NCI-H460 cells. Cells were transfected with empty vector or *Akt1* cDNA (activated) and treated with or without 1.5 μ M of α -tomatine for 24 h. **a** The cellular levels of phospho-Akt and MMP-7 were analyzed by Western blot. β -Actin was used as an internal control. **b** Cell invasion and **c** migration were analyzed by Boyden chamber assay. The

quantitative data were presented as means \pm S.D. of three repeats from one independent study. * $P < 0.05$, ** $P < 0.01$ compared with the respective untreated group. (NCI-H460/vector or NCI-H460/*Akt1* cDNA). ## $P < 0.01$ compared with invasive/migrated cells in α -tomatine-treated NCI-H460/vector group compared with α -tomatine-treated NCI-H460/*Akt1* cDNA group

transcription of MMP-7 gene is regulated by upstream sequences, including motifs corresponding to NF- κ B and AP-1 binding elements [16, 49]. Here, we have also found that α -tomatine inhibited MMP-7 expression through preventing I κ B α being phosphorylated and enhancing I κ B α protein expression, both leading to inactivation of NF- κ B DNA-binding activity. A comprehensive analysis of MMP-7 expression in NCI-H460 cells will be necessary to fully understand the biological properties of NCI-H460 cells and to promote the development of new therapeutic strategies.

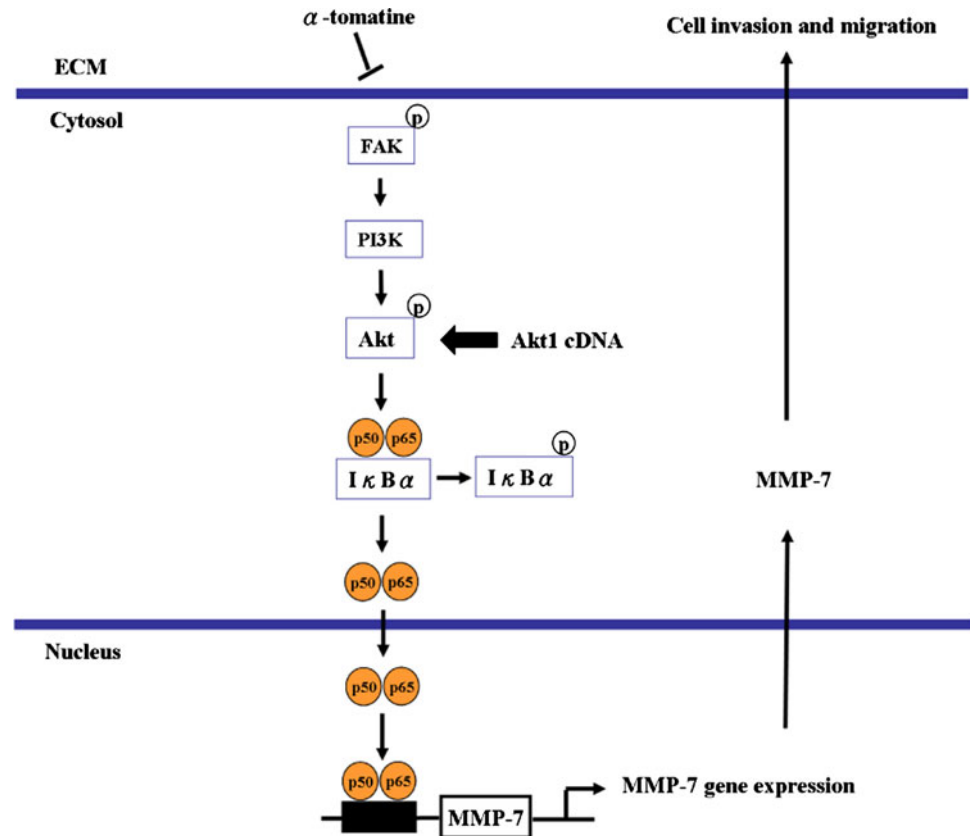
Owing to their roles in invasion and migration, Akt and MMP-7 were shown to be overexpressed in tumors with highly metastatic ability. NCI-H460 cells transfected with *Akt1* cDNA (active form) showed an increase in phospho-Akt and also an increase in MMP-7 expression. This Akt activation resulted in an increase in invasion and migration as measured by a Boyden chamber assay. Furthermore, our genetic evidence revealed that FAK/PI3K/Akt signaling played a crucial role in α -tomatine-inhibited invasion/migration in this study. Owing to the activation of FAK/PI3K/Akt will stimulate cis-acting regulatory element including the binding site of NF- κ B which play an

important role in controlling MMP-7 gene expression in NCI-H460 cells.

To investigate and establish the lung antimetastatic model of α -tomatine in the future, we can utilize NCI-H460 cells-induced C57BL6 mice, and further investigate α -tomatine whether inhibits metastatic model of NCI-H460 cells in mice. Additional *in vivo* studies are needed to define (a) whether the observed effects of α -tomatine on lung metastasis (b) possible the molecular mechanism for the antimetastatic effect of α -tomatine.

In summary, we proposed a schematic presentation of possible mechanisms for the inhibitory effect of α -tomatine on invasion and migration of NCI-H460 cells (Fig. 8). These results imply the antimetastatic effect of the α -tomatine on NCI-H460 cells might be through inactivating PI3K/Akt signaling, as well as enhancing I κ B α protein expression to reduce NF- κ B DNA-binding activity, leading to the downregulation of MMP-7 expression. Besides, α -tomatine can interfere with the rearrangement of the actin cytoskeleton by decreasing the expression of p-FAK and contribute to the inhibition of cell invasion and migration. The α -tomatine could be further tested by in

Fig. 8 Proposed mechanisms by which α -tomatine suppresses invasion and migration of NCI-H460 cells. α -Tomatine could inhibit cell invasion and migration through FAK/PI3K/Akt signaling pathway as well as enhance I κ B α protein expression to reduce NF- κ B DNA-binding activity and further suppress the expression of MMP-7



vivo model to justify if it is effective for prevention of lung cancer invasion or metastasis.

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