Inhibitory effects of terrein on lung cancer cell metastasis and angiogenesis

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Abstract. Cancer metastasis is the leading cause of mortality in cancer patients. Over 70% of lung cancer patients are diagnosed at advanced or metastatic stages, and this results in an increased incidence of mortality. Terrein is a secondary bioactive fungal metabolite isolated from *Aspergillus terreus*. Numerous studies have demonstrated that terrein has anticancer properties, but in the present study, the cellular mechanisms underlying the inhibition of lung cancer cell metastasis by terrein was investigated for the first time. Using MTT assays, the cytotoxic effects of terrein were first examined in human lung cancer cells (A549 cells) and then compared with its cytotoxic effects in three noncancer control cell lines (Vero kidney, L6 skeletal muscle and H9C2 cardiomyoblast cells). The results indicated that terrein significantly reduced the viability of all these cells but exhibited a different level of toxicity in each cell type; these results revealed a specific concentration range in which the effect of terrein was specific to A549 cells. This significant cytotoxic effect of terrein in A549 cells was verified using LDH assays. It was then demonstrated that terrein attenuated the proliferation of A549 cells using IncuCyte image analysis. Regarding its antimetastatic effects, terrein significantly inhibited A549 cell adhesion, migration and invasion. In addition, terrein suppressed the angiogenic processes of A549 cells, including vascular endothelial growth factor (VEGF) secretion, capillary-like tube formation and VEGF/VEGFR2 interaction. These phenomena were accompanied by reduced protein levels of integrins, FAK, and their downstream mediators (e.g., PI3K, AKT, mTORC1 and P70S6K). All these data indicated that terrein was able to inhibit all the major metastatic processes in human lung cancer cells, which is crucial for cancer treatment.

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Introduction

Lung cancer is a disease that is characterized by uncontrolled growth of abnormal cells in one or both of the lungs. Lung cancer is a common type of cancer that has been identified as the leading cause of cancer-related deaths worldwide. The data from GLOBOCAN 2018, a project of the International Agency for Research on Cancer (IARC), estimated that lung cancer caused 1.8 million deaths, resulting in a mortality rate of 18.4%; thus, lung cancer ranks first in mortality among all cancer types. In Thailand, 23,957 (15.1%) new lung cancer cases and 21,371 (20.1%) lung cancer-related deaths were reported; thus, lung cancer ranks second after liver cancer (1). The high mortality rate results from the late diagnosis of patients already in advanced or metastatic stages (2). Therefore, studying and understanding the molecular mechanisms underlying lung cancer metastasis are essential for the development of therapeutic strategies for lung cancer.

Cancer metastasis is a complex process in which cancer cells spread from a primary tumor site to distant sites. Metastasis includes several processes, including cell proliferation, adhesion, invasion, migration and angiogenesis (3). These processes involve the complex interactions of multiple crucial proteins and signaling pathways that lead to cancer cell movement and survival. Cancer metastasis is typically initiated by the attachment of cancer cells to extracellular matrix (ECM) proteins and then by the activation of integrins (4). Integrin activation contributes to the formation of focal adhesions (FAs) and the stimulation of signaling transduction pathways that regulate cell migration and invasion. FA proteins recruit focal adhesion kinase (FAK) to FAs, and then FAK directly binds to integrins, which subsequently leads to FAK autophosphorylation at Tyr397. FAK is a nonreceptor tyrosine kinase that regulates signaling related to cell adhesion and migration in various cell lines (5,6). For cell migration, FAK activates p85 subunit of phosphatidylinositol 3-kinase (PI3K) (7), which further increases the phosphorylation of the protein kinase-B (AKT) and mammalian target of rapamycin (mTOR) proteins. The involvement of PI3K/AKT/mTOR signaling in cancer

metastasis has been clearly observed in numerous works through the stimulation of cancer cell development, migration, invasion and motility (8). In addition, phosphorylating ribosomal p70S6 kinase (P70S6K) and eukaryotic translation factor 4E-binding protein 1 (4E-BP1), which are downstream effectors of mTORC1, can also promote cancer cell migration and invasion (9) and cell cycle progression (10).

Angiogenesis is another crucial process that promotes tumor growth and metastasis. This process is activated through the interaction of growth factors and their specific receptors. Vascular endothelial growth factors (VEGFs) are the main regulators of vascular development and angiogenesis initiation. VEGF subtype A (VEGF-A) has been widely studied and plays a major role in angiogenesis by acting through VEGF receptor tyrosine kinase type 2 (VEGFR2) (11,12). VEGFR2 is expressed in different types of cells, such as neuronal cells, megakaryocytes, hematopoietic stem cells and various cancer cells, including lung cancer cells (13-16). The Tyr951, Tyr1175 and Tyr1214 residues of VEGFR2 are the main sites of phosphorylation that stimulate cell migration during angiogenesis. Phosphorylation at Tyr1175 recruits the binding of SH2 domain-containing adaptor protein B (SHB), resulting in the activation of FAK and the promotion of actin polymerization, lamellipodia formation and cell migration (17,18).

For lung cancer treatment, standard treatments, such as radiotherapy and chemotherapy, are currently accepted for treating advanced stage lung cancer; however, there are still several issues that need to be considered, such as treatment side effects, drug resistance, and high treatment cost. The development of effective anti-lung cancer agents, especially from natural resources, is a promising approach to increase patient survival. Terrein (4,5-dihydroxy-3-[(*E*)-1'-propenyl]-2-cyclopenten-1-one, $C_8H_{10}O_3$) is a natural substance produced by microorganisms. Terrein is a secondary bioactive fungal metabolite that was first isolated from *Aspergillus terreus* by Raistrick and Smith in 1935 (19). Numerous studies have demonstrated that terrein exerts several potential effects, including anti-inflammation, melanogenesis inhibition and anticancer effects (20-22). Regarding its anticancer effect, terrein was revealed to inhibit cell proliferation through the induction of cell cycle arrest in human hepatoma Bel7402 (23) and human ovarian cancer cells (24). Terrein has also been revealed to induce the apoptosis of ABCG2-expressing breast cancer cells via the caspase-7 pathway and inhibit AKT signaling (25). Additionally, terrein induced apoptosis by regulating p53 and ERK in human cervical carcinoma cells (22). Furthermore, terrein has been revealed to suppress angiogenin production in head and neck cancer cells (26) and androgen-dependent prostate cancer cells (27), and both studies suggested that terrein has an inhibitory effect on angiogenesis. The inhibitory effect of terrein on cell migration has also been observed in human breast cancer cells (28). However, the anticancer effect of terrein on A549 human lung cancer cell metastasis and angiogenesis has not yet been fully elucidated. Therefore, the present study aimed to investigate the inhibitory effects of terrein on human lung cancer cell metastasis and angiogenesis as well as the important cellular mechanisms involved in both processes.

Materials and methods

Chemicals and reagents. Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (EMEM), α-minimum essential medium (α-MEM), fetal bovine serum (FBS), 0.25% trypsin-EDTA, and penicillin-streptomycin were purchased from Gibco/Thermo Fisher Scientific, Inc. 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) was purchased from USB Corporation. Matrigel was purchased from BD Biosciences. The VEGF-A Human ELISA Kit was purchased from Abcam. The lactate dehydrogenase (LDH) assay was purchased from G-Biosciences.

Cell culture. The A549 human NSCLC cell line (ATCC® CCL-185™) and normal African green monkey kidney (Vero) cell line (ATCC® CCL-81™) were purchased from ATCC. The L6 skeletal muscle cell line and H9C2 cardiomyoblast cell line were obtained from author Gary Sweeney, Department of Biology, York University, Toronto, Canada (29,30). The A549 cells and H9C2 cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin. The Vero cells were maintained in EMEM containing 10% FBS and 1% penicillin/streptomycin. The L6 cells were maintained in α-MEM containing 10% FBS and 1% antibiotic-antimycotic. All the cell types were cultured in an incubator at 37˚C in 5% CO_2 and a 95% humidified atmosphere. The cells were provided with fresh medium 2 to 3 times per week, and when the cells had grown to approximately 80% confluence, they were subcultured approximately 2 or 3 times per week.

Preparation of terrein. The fungus *Aspergillus terreus* CRI301 was cultivated in Sabouraud dextrose agar under stationary conditions at room temperature for 34 days. Then, the *Aspergillus terreus* CRI301 culture was filtered to separate the cells from the broth. The culture broth was extracted three times with an equal volume of ethyl acetate (EtOAc), and then, the EtOAc layers were combined and evaporated to dryness. The crude EtOAc extract was further purified by Sephadex LH-20 column chromatography (2-cm inner diameter and 125-cm length) and eluted with MeOH. The structure of terrein was characterized by ^{1}H NMR spectroscopy (AVANCE III HD; frequency, 400 MHz; TopSpin version 3.6.2 software; Bruker).

Cell viability assay. The cytotoxic effects of terrein in A549 cells and normal cells, including Vero cells, L6 cells and H9C2 cells, was determined using a colorimetric MTT assay. A549, Vero, L6 and H9C2 cells were harvested with 0.25% trypsin containing 1 mM EDTA, plated in 96-well plates at densities of $1x10^4$, $1.8x10^4$, $3x10^4$, and $2x10^4$ cells/well, respectively, and allowed to adhere overnight. Then, the cells were treated with various concentrations of terrein: 0-1 mM for A549 cells and 0-2 mM for all the normal cell lines. The highest concentration of dimethyl sulfoxide (DMSO; 0.1%) was used as the vehicle control. Then, all the plates were incubated for 24 h at 37˚C. Subsequently, the medium was removed, and 0.5 mg/ml MTT solution was added to each well. The plates were further incubated for 4 h at 37˚C, and the supernatants were discarded after the incubation. Then, the formazan crystals in each well were dissolved in 100 µl of DMSO. The amount of purple

formazan was determined by using a multimode microplate reader (Synergy; BioTek Instruments, Inc.) at 595 nm. All the measurements were carried out in triplicate. The cell viability is presented as the percentage of the control.

Cell proliferation assay. An IncuCyte proliferation assay was used to determine the effect of terrein on human lung cancer cell proliferation. Real-time live-cell imaging was conducted using IncuCyte S3 (Essen BioScience; Sartorius). A549 cells were seeded in 96-well plates at a density of $4x10^3$ cells/well and allowed to adhere overnight. Then, the cells were treated with various concentrations (0-1 mM) of terrein, and the plates were placed in the IncuCyte for imaging every 3 h for 3 days. Proliferation curves were generated using IncuCyte proliferation analysis with confluence as the parameter.

LDH enzyme assay. The cytotoxic effect of terrein on A549 cells was determined using an LDH assay kit, following manufacturer's instructions (G-Biosciences). Briefly, the cells ($1x10^4$ cells/well) were treated with various concentrations (0-1 mM) of terrein for 24 h. Then, 25 μl of supernatant from each sample was transferred to a new 96-well plate, and 25 μl reaction mixture was added to each well. After incubation at 37˚C for 30 min, 25 μl stop solution was added to each well, and then, the absorbance was measured using a multimode microplate reader (Synergy; BioTek Instruments, Inc.) at 490 nm.

Wound healing assay. The effect of terrein on A549 cell migration was determined by a monolayer wound healing assay. A549 cells were harvested with 0.25% trypsin containing 1 mM EDTA, plated in 6-well plates at a density of $5x10^5$ cells/well in serum-free medium and allowed to adhere overnight. After forming a confluent monolayer, the cells were scratched with a 200-μl sterile pipette tip from one side to the other of the wells of the 6-well culture plate. Subsequently, the cells were washed three times with DMEM to remove the cell debris. Then, the cells in each well were immediately given DMEM with or without 20, 40 and 80 μM terrein. Cell migration was monitored and imaged under a light inverted microscope (Olympus CKX41; Olympus Corporation) using a magnification of x10 for 0, 6, 12 and 24 h. The wound area was measured in three independent wound sites per group and compared with that in the vehicle control group at 0 h. Relative cell motility was calculated as the wound area at 6, 12 and 24 h in at least three independent experiments. The wound areas were quantified using ImageJ software [Java 1.8.0_112 (64_bit); National Institutes of Health].

Transwell migration and invasion assays. The effect of terrein on aggressive A549 cell migration and invasion was assessed using 24-well plate Transwell inserts with polycarbonate membranes with 8-μm pores (BD Biosciences). Briefly, the cells were harvested, and $5x10^4$ cells were resuspended in 200 μl serum-free medium with or without 20, 40 and 80 μM terrein. The cells were then plated into the Matrigel-coated upper chambers of Transwell inserts for the invasion assay or the noncoated upper chambers of Transwell inserts for the migration assay. In the lower chamber, 750 μl medium containing 10% FBS was used as a chemoattractant to stimulate cell invasion and migration. The plate was incubated at 37˚C for 24 h. The Transwell insert was separated from the 24-well plate and washed twice with cold PBS. Each sample was fixed with 100% ice-cold methanol for 20 min and washed twice with cold PBS. Then, the samples were stained with 0.5% crystal violet at room temperature for 15 min and washed with dH_2O several times until all the excess dye had been removed. The cells that did not invade or migrate from the upper chamber were removed using cotton swabs. The cells that invaded and migrated through the pores of the insert into the lower chamber were photographed under an light inverted microscope at a magnification of x10 and quantified from at least five randomly selected fields by ImageJ software [Java 1.8.0_112 (64_bit)]. The percentage of cell invasion and migration were compared to that in the vehicle control group, which was set to 100%.

Adhesion assay. The effect of terrein on A549 cell adhesion was determined. Briefly, 96-well plates were coated with 50 μl of 2.0 mg/ml Matrigel and incubated at 37˚C for 2 h. Then, the Matrigel was removed, and cells at a density of $3x10^4$ were plated in each well in 100 μl of serum-free medium with or without 20, 40 and 80 μM terrein. After incubation for 30 min at 37˚C, the nonadherent cells were removed by washing 4 times with 50 μl of PBS, and 0.5 mg/ml MTT solution was added and incubated for 4 h at 37˚C. The amount of adherent cells was determined by measuring the optical density using a multimode microplate reader at 595 nm. Cell adhesion is expressed as a percentage of that observed in the control group.

Gelatin zymography. A gelatin zymography assay was used to examine the effect of terrein on the activities of proteolytic enzymes released from human lung cancer cells. Briefly, A549 cells were seeded in 6-well plates at a density of $5x10^5$ cells/well in DMEM containing 10% FBS and incubated overnight. The cells were treated with or without 20, 40 and 80 μM terrein and incubated at 37˚C for 24 h. After treatment, the supernatants were collected and concentrated using Amicon Ultra-0.5 Centrifugal Filters 3K (Thermo Fisher Scientific, Inc.). The protein concentration was measured using a Bradford protein assay. Bovine serum albumin (BSA) was used as the standard protein sample. After the calculation of the protein concentration, each sample was adjusted to the same concentration.

The protein samples were separated in 10% SDS-polyacrylamide gels containing 0.73 mg/ml gelatin. After electrophoresis, the gels were washed with 1X renaturing buffer [Novex™ Zymogram Renaturing Buffer (10X) Invitrogen™; Thermo Fisher Scientific, Inc.] for 1 h at room temperature with gentle agitation on a benchtop rocker. The gels were washed 4 times with ddH_2O for 20 min each and then incubated with 1X developing buffer [Novex™ Zymogram Developing Buffer (10X) Invitrogen™; Thermo Fisher Scientific, Inc.] for 30 min at room temperature with gentle agitation on a benchtop rocker. Then, the gels were incubated with fresh 1X developing buffer at 37˚C overnight. Subsequently, the gels were stained with staining solution (0.5% Coomassie Brilliant Blue R-250 in methanol:acetic acid:water, 4:1:5, v/v/v) and then washed with destaining solution. Finally, the bands corresponding to matrix metalloproteinase (MMP)-2 (68 kDa) and MMP-9 (82 kDa) were clearly visible in contrast to the blue background and

were detected by a MiniBIS Pro DNR Bio-Imaging System (BioSciences). The intensities of the bands were quantified by ImageJ software [Java 1.8.0_112 (64_bit)]. The percentages of the MMP-2 and MMP-9 activities are expressed relative to those in the vehicle control group, which was set to 100%.

Reverse transcription-quantitative (RT-q)PCR. A549 cells were seeded in a 6-well plate at a density of $5x10^5$ cells/well in DMEM containing 10% FBS and incubated overnight. The cells were treated with or without 20, 40 and 80 μM terrein and incubated for 24 h at 37˚C. Total RNA was isolated using RNeasy Mini Kit following manufacturer's instructions (Qiagen, Inc.) and RNA concentration was measured using NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). First strand cDNA was synthesized from 400 ng/μl RNA using the Thermo Scientific RevertAid Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.). Quantitative real-time PCR was conducted using a Chromo4™ Detection system (Bio-Rad Laboratories, Inc.) according to cycling conditions outlined by the PCR array manufacturer. All genes were detected (listed in the Table I) and analyzed through real-time PCR. PCR cycle was performed with iTaq™ Universal SYBR Green mixture (Bio-Rad Laboratories, Inc.) using the following cycling conditions: 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec, 61˚C for 15 sec, 72˚C for 1 min, and then 72˚C for 2 min. Data were analyzed using Optical Monitor 3 software (Bio-Rad Laboratories, Inc.) and normalized to GAPDH mRNA expression. Relative gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (31).

Table I. Sequence-specific primers for qPCR.

Gene name	Primer sequence (5'-3')	Product size
MMP-2	F: CTCATCGCAGATGCCTGGAA	104
	R: TTCAGGTAATAGGCACCCTTGAAGA	
MMP-9	F: ATCCGGCACCTCTATGGTC	121
	R: CTGAGGGGTGGACAGTGG	
GAPDH	F: ATCTTCTTTTGCGTCGCCAG	51
	R: TTCCCCATGGTGTCTGAGC	

F, forward; R, reverse.

VEGF determination. To examine whether terrein could inhibit angiogenic processes, the function of VEGF was evaluated using the VEGF human enzyme-linked immunosorbent assay (ELISA) kit (product code ab100662; Abcam). Briefly, the cells were treated with or without 20, 40 and 80 μM terrein for 24 h. The supernatants were collected and concentrated using Amicon Ultra-4 Centrifugal Filters 10K (Thermo Fisher Scientific, Inc.). The protein concentrations were measured using a Bradford protein assay, and each sample was adjusted to the same concentration.

For the assay, as aforementioned, an ELISA kit (product code ab100662; Abcam), with an antibody specific for human VEGF was used to coat the wells of the plate prior to adding the standards and protein samples, according to the manufacturer's instructions. Then, the plate was incubated at 4˚C overnight with gentle shaking. The solution was discarded, and the plate was washed 4 times with 1X wash solution. Then, 100 μl of 1X biotinylated anti-human VEGF antibody was added to each well and incubated for 1 h at room temperature. Subsequently, the unbound biotinylated antibody was removed by washing, and 100 μl of 1X HRP-conjugated streptavidin was added to each well and incubated for 45 min at room temperature. Then, 100 μl of TMB substrate solution was added and incubated for 30 min at room temperature in the dark. The color developed in proportion to the bound VEGF in each sample. The stop solution was added to each well, and the color changed from blue to yellow. The intensity of the color was immediately measured at 450 nm.

Tube formation assay. The tube formation assay is a rapid and quantitative method for examining cell differentiation and changes in angiogenic processes (32). A 24-well plate was coated with 10 mg/ml Matrigel and incubated for 1 h at 37˚C. The remaining liquid was carefully removed from the culture plate without disrupting the layer of Matrigel matrix. Then, $5x10^4$ A549 cells in 1 ml of serum-free medium with or without 20, 40 and 80 μM terrein were added to each well and incubated for 24 h at 37˚C. Tube formation was photographed by inverted microscopy (Olympus CKX41), and the tubular structures in five randomly selected fields were quantified using the Angiogenesis Analyzer plugin for ImageJ software [Java 1.8.0_112 (64_bit)] (33). The percentage of tube length was compared to that in the vehicle control group.

Western blot analysis. A549 cells were seeded in 60-mm culture dishes at a density of $5x10^5$ cells/dish in DMEM containing 10% FBS and incubated overnight. The cells were treated with or without 20, 40 and 80 μM terrein and incubated for 24 h at 37˚C. After treatment, the cells were lysed with RIPA buffer (5 ml of 1 M Tris-HCl pH 7.4, 30 ml of 5 M NaCl, 5 ml of 20% NP-40, 5 ml of 10% sodium deoxycholate, 0.5 ml of 20% SDS, 50 ml of dH_2O and protease inhibitor cocktail) on ice. Subsequently, the cell lysates were centrifuged at 15,000 g at 4˚C for 10 min. The protein concentration was measured using a Bradford protein assay. Equal amounts (50 μg) of the total protein extracts were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were blocked with 5% BSA in TBS-T buffer (5% w/v BSA, 1X Tris-buffered saline solution and 0.1% Tween-20) at room temperature for 1 h and then incubated with primary antibodies against integrin αM (1:1,000; cat. no. sc-1186; Santa Cruz Biotechnology, Inc.), total AKT (1:1,000; product no. 9272) , pAKT (Ser473) (1:1,000; product no. 9271), pAKT (Thr308) (1:1,000; product no. 4056), total FAK (1:1,000; product no. 3285; all from Cell Signaling Technology, Inc.), pFAK (Tyr397) (1:1,000; cat. no. 44-624G; Invitrogen™; Thermo Fisher Scientific, Inc.), total mTOR (1:1,000; product no. 2972), pmTOR (Ser2448) (1:1,000; product no. 2971), total P70S6 Kinase (1:1,000; product no. 2708), pP70S6 Kinase (Thr389) (1:1,000; product no. 9234), total PI3K p85 (1:1,000; product no. 4292; all from Cell Signaling Technology, Inc.), pPI3K p85 (Tyr458) (1:1,000; cat. no. PA5-17387; Invitrogen™; Thermo Fisher Scientific, Inc.), total VEGFR2 (1:1,000; product no. 9698), pVEGFR2 (Tyr1175) (1:1,000; product no. 3770), and GAPDH (1:1,000; product no. 2118; all from Cell Signaling Technology,

Inc.) at 4˚C overnight. After washing 3 times with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse (1:5,000; product no. 7076) or anti-rabbit IgG antibodies (1:5,000; product no. 7074; both from Cell Signaling Technology, Inc.) for 1 h. The membranes were visualized by enhanced chemiluminescence using ECL plus™ western blotting detection reagents (Bio-Rad Laboratories) and recorded on gel documentation (UVITE,) or X-ray film. The intensities of the protein bands were quantified by ImageJ software [Java 1.8.0_112 (64_bit)].

Immunofluorescence microscopy. A549 cells were seeded on round micro cover glasses (VWR®) in 24-well plates at a density of $3x10^3$ cells/well in DMEM containing 10% FBS and incubated overnight. The cells were treated with or without 20, 40 and 80 μM terrein for 24 h. The cells were washed three times with PBS^{++} (calcium and magnesium) and fixed with 4% paraformaldehyde for 20 min at room temperature. After washing three times with PBS^{++}, the cells were permeabilized with 0.1% Triton X-100 for 5 min at room temperature, and then, the permeabilized cells were washed three times with PBS^{++}. The cells were blocked with blocking buffer (3% BSA in PBS^{++}) for 1 h at room temperature and then incubated with a pFAK (Tyr397) polyclonal antibody (cat. no. 44-624G; Invitrogen™; Thermo Fisher Scientific, Inc.) (1:200) at 4˚C overnight. Then, the cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (1:800; cat. no. A27034; Life Technologies; Thermo Fisher Scientific, Inc.) and rhodamine phalloidin (1:320; cat. no. R415; Life Technologies; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The stained cells were washed three times with PBS^{++}, and then, the cells on glass coverslips were mounted onto microscope slides (VWR®) in ProLong Gold Antifade Mountant (Life Technologies; Thermo Fisher Scientific, Inc.) and VECTASHIELD® Mounting Medium with DAPI (Vector Lab) (1:1). The stained cells were observed with a Nikon A1R confocal laser scanning microscope system (Nikon Corporation) with a 60X objective.

Statistical analysis. All the experiments were performed at least in triplicate in each group. The data are presented as the mean ± SEM and were analyzed by GraphPad Prism version 5.01 software (GraphPad Software, Inc.). Statistical significance was calculated using ANOVA with Dunnett's multiple comparison post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Purification of terrein. The fungus *Aspergillus terreus* CRI301 was cultured in Sabouraud dextrose agar under stationary conditions at room temperature for 34 days. As revealed in Fig. 1A, the increased number of fungi was associated with the days of culture. The culture (3.6 l) of *Aspergillus terreus* CRI301 was filtered to separate the cells from the broth. The culture broth was extracted three times with an equal volume of EtOAc. The EtOAc layers were combined and evaporated to dryness, yielding 2.535 g of crude extract. The crude EtOAc extract was purified by Sephadex LH-20 column chromatography and eluted with MeOH to yield 30 fractions (Fig. 1B). On the basis of their TLC characteristics, similar fractions were combined to yield 10 fractions (Fig. 1C-E). Fraction 4 was obtained as a pale yellow powder that was crystallized from dichloromethane (CH_2Cl_2) and contained terrein (258.7 mg) in the form of white needles. The structure of terrein was characterized by ^{1}H NMR spectroscopy and finally confirmed by comparison with data in the literature (Fig. 1F). Terrein was obtained in the form of white needles. The ^{1}H NMR spectrum of terrein (Fig. 1G) exhibited three methylene protons at δ_H 6.82 (^{1}H, *m*, H-2'), 6.43 (^{1}H, *d*, J = 15.9 Hz, H-1') and 5.96 (^{1}H, *s*, H-2), two oxygenated methine protons at δ_H 4.73 (^{1}H, s, H-4) and 4.07 (^{1}H, s, H-5) and a methyl group at δ_H 1.90 (^{3}H, *d*, J = 15.9 Hz, H-3'), as revealed in Table II.

Table II. ^{1}H NMR (400 MHz) data of terrein in Acetone-d6.

	Terrein
Position	δ_H (ppm)
1	-
2	5.96 (s)
3	-
4	4.73 (^{1}H, br *s*)
5	4.07 (^{1}H, br *s*)
1'	6.43 (^{1}H, *d*, J=15.9 Hz)
2'	6.82 (^{1}H, *m*)
3'	1.90 (^{3}H, *d*, J=15.9 Hz)

Cytotoxic effects of terrein on the viability and proliferation of lung cancer cells. First, the cytotoxic effects of terrein on different cell lines were examined, including A549 lung cancer cells, African green monkey kidney (Vero) cells, L6 skeletal muscle cells and H9C2 cardiomyoblast cells, using an MTT assay. All the cell lines were treated with various concentrations of terrein for 24 h, and the maximum final concentration of DMSO (0.1%) was used as the vehicle control. The results demonstrated that terrein significantly inhibited the viability of A549 cells, Vero cells, L6 cells and H9C2 cells with IC_{50} values of 229, 870, 1,240 and 579 μM, respectively (Fig. 2A-D). Terrein exhibited more toxicity in lung cancer cells than in all the representative normal cells. Moreover, the selective index (SI) of terrein on A549 cells compared with that of terrein on all the normal cell lines was calculated using the following equation: SI=IC_{50} of normal cells/IC_{50} of A549 cells. As a result, the SI values of terrein on A549 cells were 3.8, 5.4 and 2.5 compared to Vero cells, L6 cells and H9C2 cells, respectively, indicating that terrein has high cytotoxic selectivity against cancer cells compared with normal cells. In addition, LDH assays were performed to confirm the damaging effect of terrein. The LDH enzyme is normally used as a biomarker of cellular cytotoxicity and cytolysis, as it is released from damaged cells (34). As revealed in Fig. 2E, 50-1,000 μM terrein significantly induced cytotoxicity in A549 cells.

It was also determined whether these cytotoxic effects of terrein interfered with the process of cell proliferation using the IncuCyte assay. Cells were treated with different concentrations of terrein (1-1,000 μM). Cell proliferation

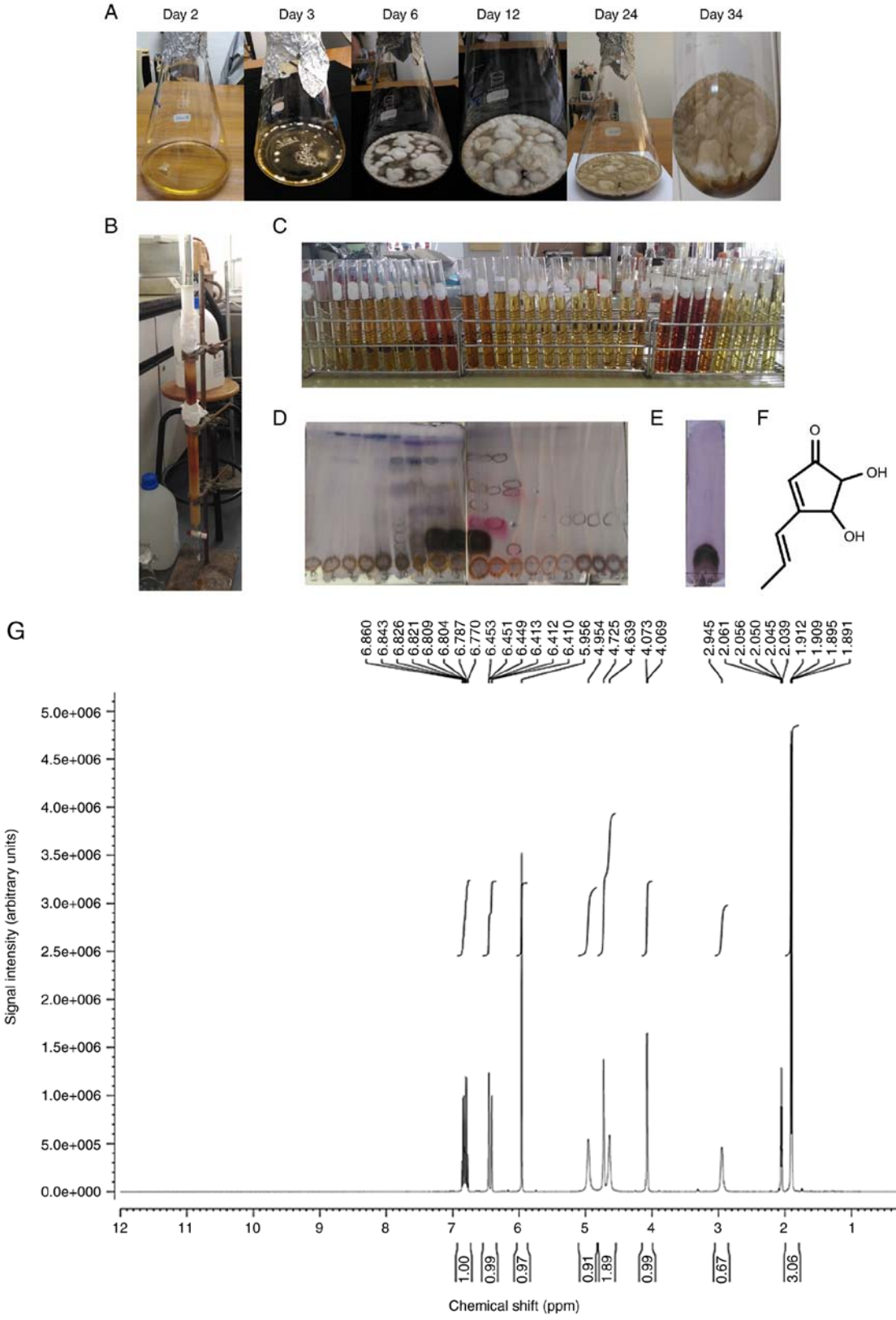


Figure 1. Characteristics of terrein. (A) *Aspergillus terreus* was cultured for 34 days. (B) Separation of the pure compound using Sephadex LH-20 column chromatography. (C) All fractions were collected. (D and E) Thin layer chromatography. (F) The structure of terrein was characterized by ^{1}H NMR spectroscopic data. (G) The 1H NMR spectrum of terrein.

was monitored every 3 h for 3 days. Proliferation curves were generated using IncuCyte proliferation analysis with cell confluence as the parameter. The results revealed that 20-1,000 μM terrein exhibited dose-dependent inhibitory effects on A549 cell proliferation, as revealed in Fig. 2F. The present results indicated that a high concentration of terrein inhibited A549 cell viability and proliferation by damaging the cells. Thus, to further investigate the effects of terrein on

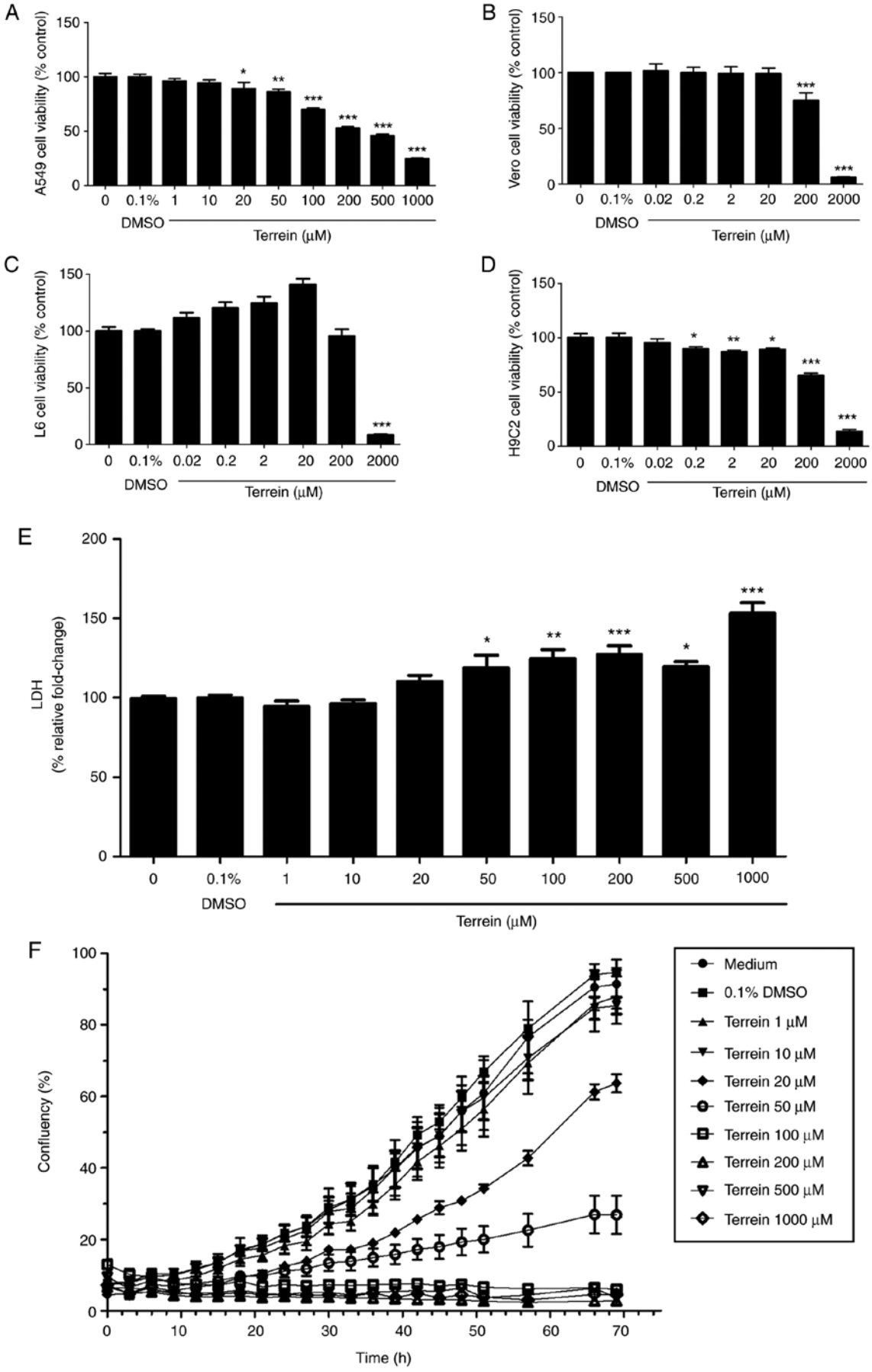


Figure 2. Terrein inhibits cell viability and proliferation in lung cancer cells. Cell viability was assessed by MTT assay, in which various concentrations of terrein were treated for 24 h. (A) A549 cells (B) Vero cells (C) L6 cells (D) H9C2 cells (E) LDH assay and (F) proliferation assay. Values are expressed as the mean ± SEM of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001, compared with the vehicle control (0.1% DMSO). LDH, lactate dehydrogenase.

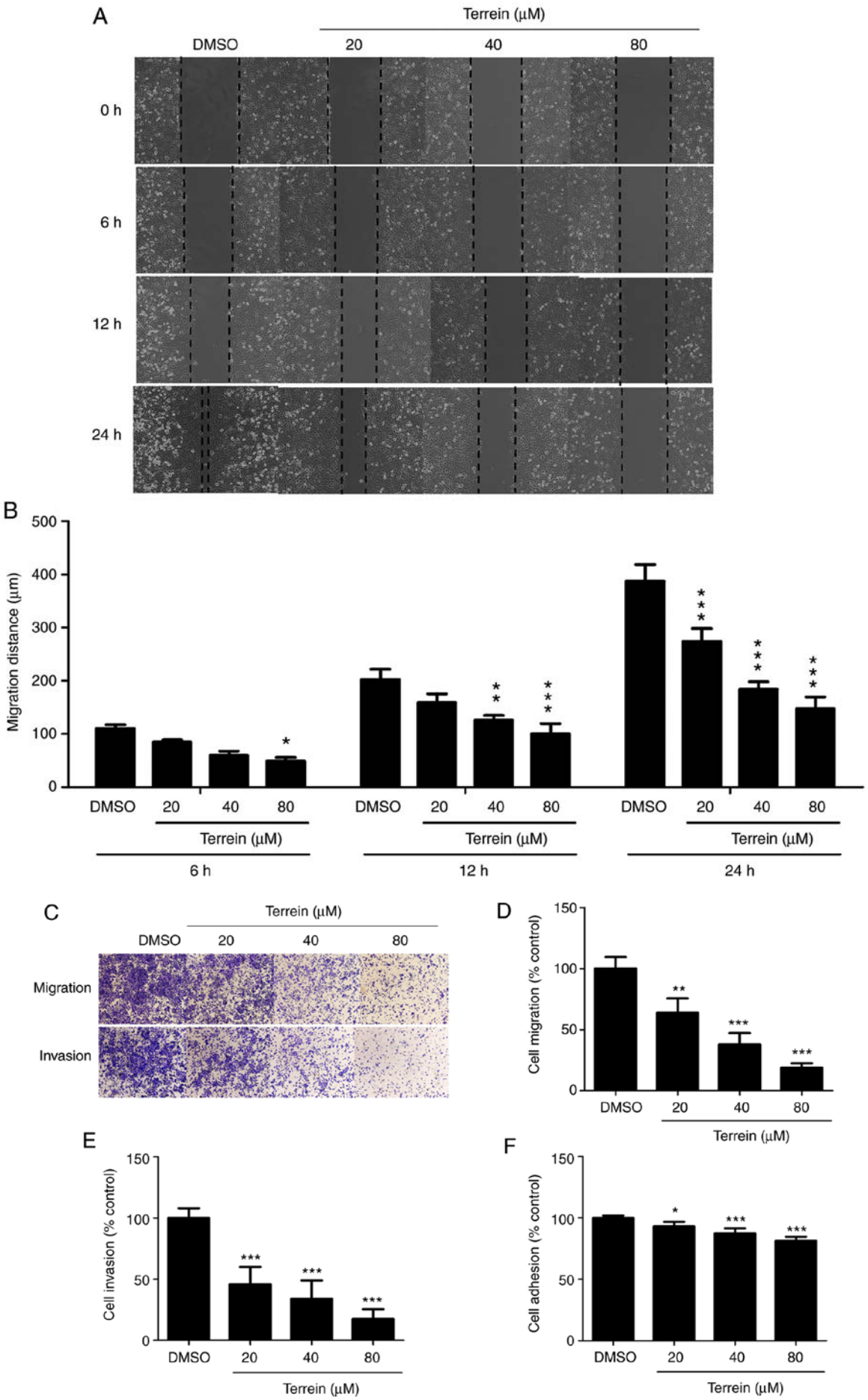


Figure 3. Continued.

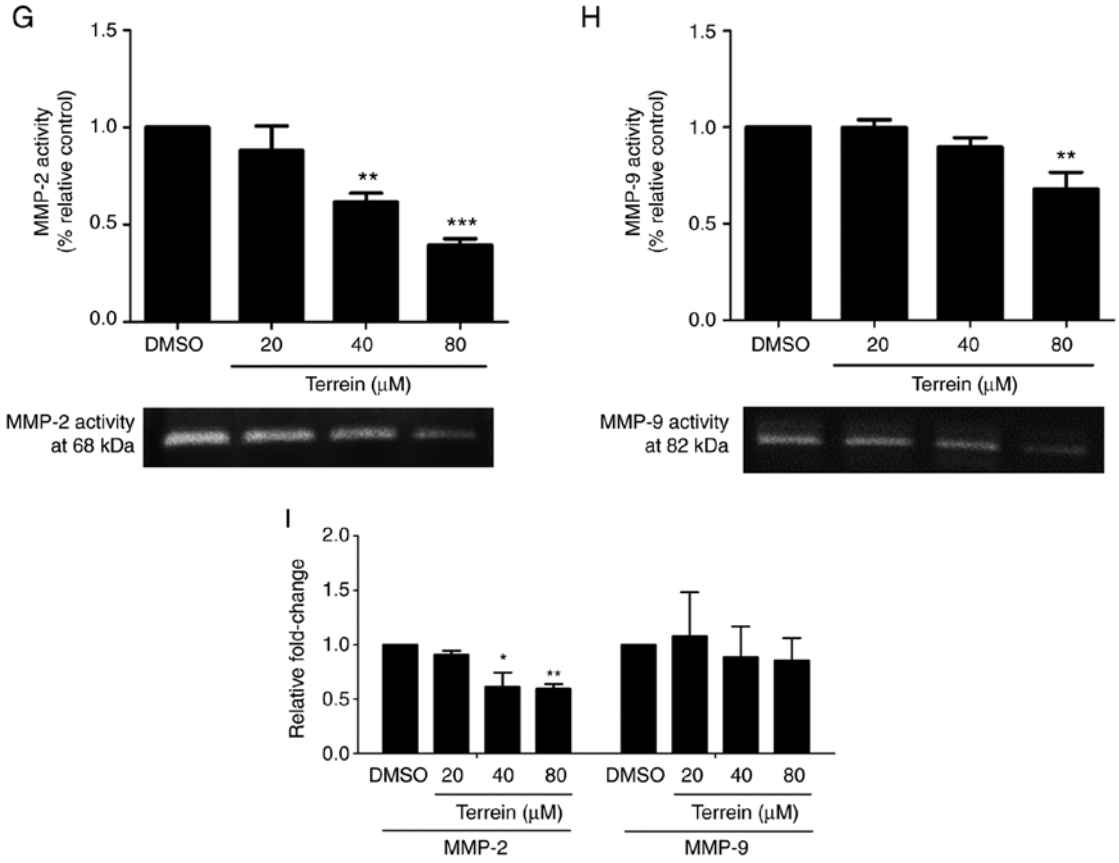


Figure 3. Effect of terrein on A549 cell metastatic processes. Wound healing assay was performed to assess the migration of A549 cells after 0, 6, 12 and 24 h of terrein treatment. (A) A representative image of the scratch-wound healing assay of A549 cells using a magnification of x10. (B) Migration distance of the treated and untreated samples were measured in at least three independent locations in each wound. (C) Effects of terrein on A549 lung cancer cell migration and invasion after 24 h of terrein treatment and crystal violet staining. Representative images were captured at a magnification of x10. (D) The migrated and (E) invasive cells were quantified by using ImageJ software. (F) Adhesion of A549 cells on Matrigel-coated plates. Effect of terrein on cell adherence were measured by MTT assay after 30 min of terrein incubation. (G) Representative gelatin zymography of MMP-2 after 24 h of terrein treatment. (H) Representative gelatin zymography of MMP-9 after 24 h of terrein treatment. The activity of MMP-2 and MMP-9 were quantified by ImageJ. (I) Expression of MMP-2 and MMP-9 was determined using qPCR after 24 h of terrein treatment. Significance was measured as the mean ± SEM of at least three separate experiments. *P<0.05, **P<0.01 and ***P<0.001, compared with the vehicle control. MMP, matrix metalloproteinase.

metastatic processes, the concentrations of 20, 40 and 80 μM terrein, which exhibit low levels of toxicity in cells, were selected.

Terrein inhibits metastatic processes of A549 lung cancer cells. To determine the effect of terrein on the metastatic processes of lung cancer cells, its inhibitory effect on lung cancer cell migration was first evaluated. The migration of cells was measured using a wound healing assay. In this method, the concentrations of terrein that exhibited low toxicity (20, 40 and 80 μM) were used to treat A549 cells for 0, 6, 12 and 24 h. The results revealed that terrein significantly inhibited cell migration at 6, 12 and 24 h (Fig. 3A and B).

The ability of terrein to suppress the migration and invasion of A549 lung cancer cells was further examined. A549 cells were treated with or without 20, 40 and 80 μM terrein for 24 h, and a Transwell assay was used to observe the effect of terrein on A549 cell migration and invasion. The results revealed that terrein significantly decreased the number of migrated and invasive cells by as much as 78 and 82%, respectively, compared with the vehicle control (Fig. 3C-E). The effect of terrein on the adhesion process, since it is associated with the early step of metastasis (35), was also determined. As revealed in Fig. 3F, the highest concentration of terrein decreased A549 cell adhesion by approximately 19% compared with the vehicle control.

To assess the effect of terrein on the process of cell invasion, the effect of terrein on the activities and expression of MMP-2 and MMP-9 was determined using gelatin zymography and qPCR, respectively. A549 lung cancer cells were treated with 20, 40 and 80 μM terrein for 24 h. The results revealed that terrein significantly suppressed the gelatinase activities of both MMP-2 and MMP-9. Bands corresponding to MMP-2 (68 kDa) and MMP-9 (82 kDa) were clearly observed, and 40 and 80 μM terrein significantly inhibited MMP-2 and 80 μM terrein significantly inhibited MMP-9, as revealed in Fig. 3G and H, respectively. In addition, terrein significantly inhibited MMP-2 expression and tended to inhibit MMP-9 expression as revealed in the Fig. 3I. These data suggested that terrein had the ability to inhibit the metastatic processes of lung cancer cells.

Terrein inhibits the angiogenesis process of A549 lung cancer cells. To examine whether terrein could inhibit the angiogenesis process, A549 cells were treated with or without 20, 40 and 80 μM terrein for 24 h. The VEGF-A that was

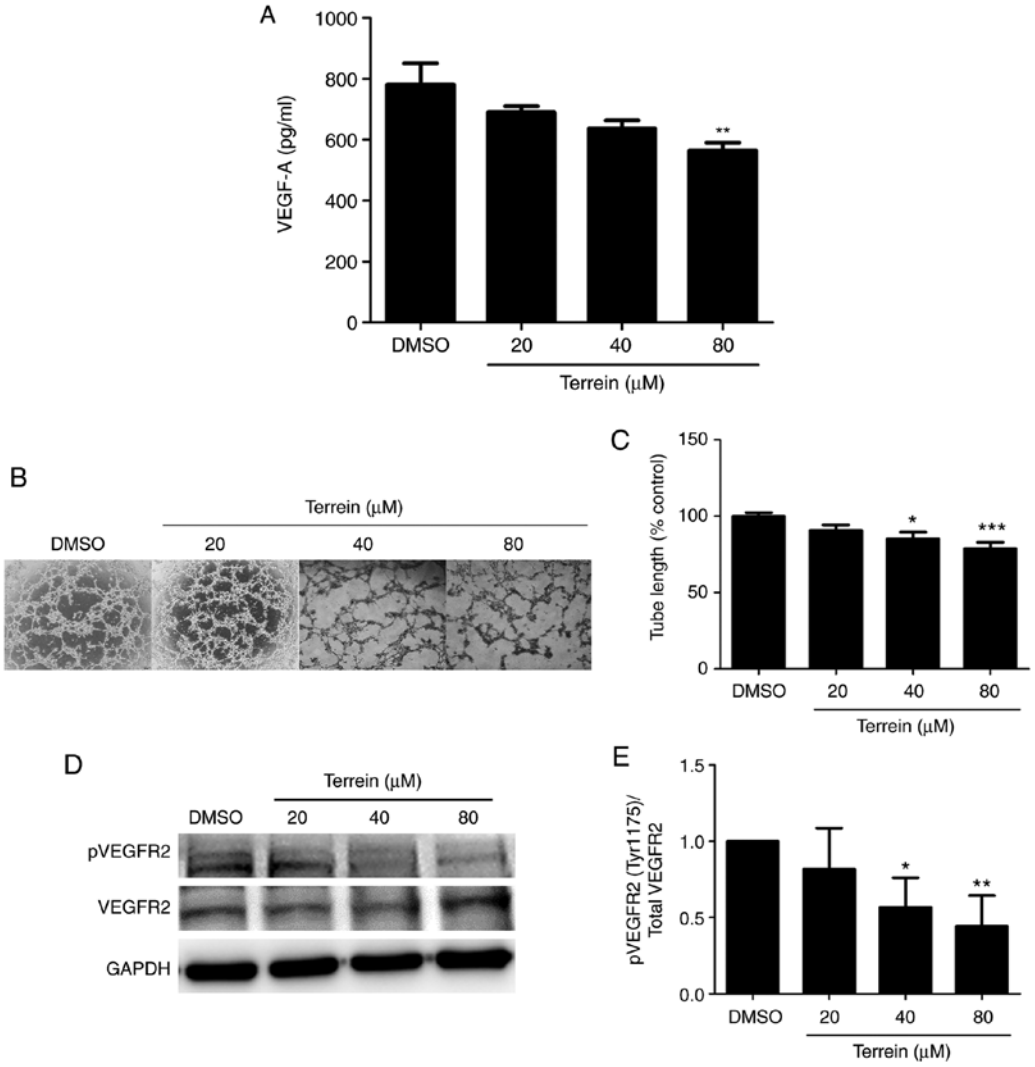
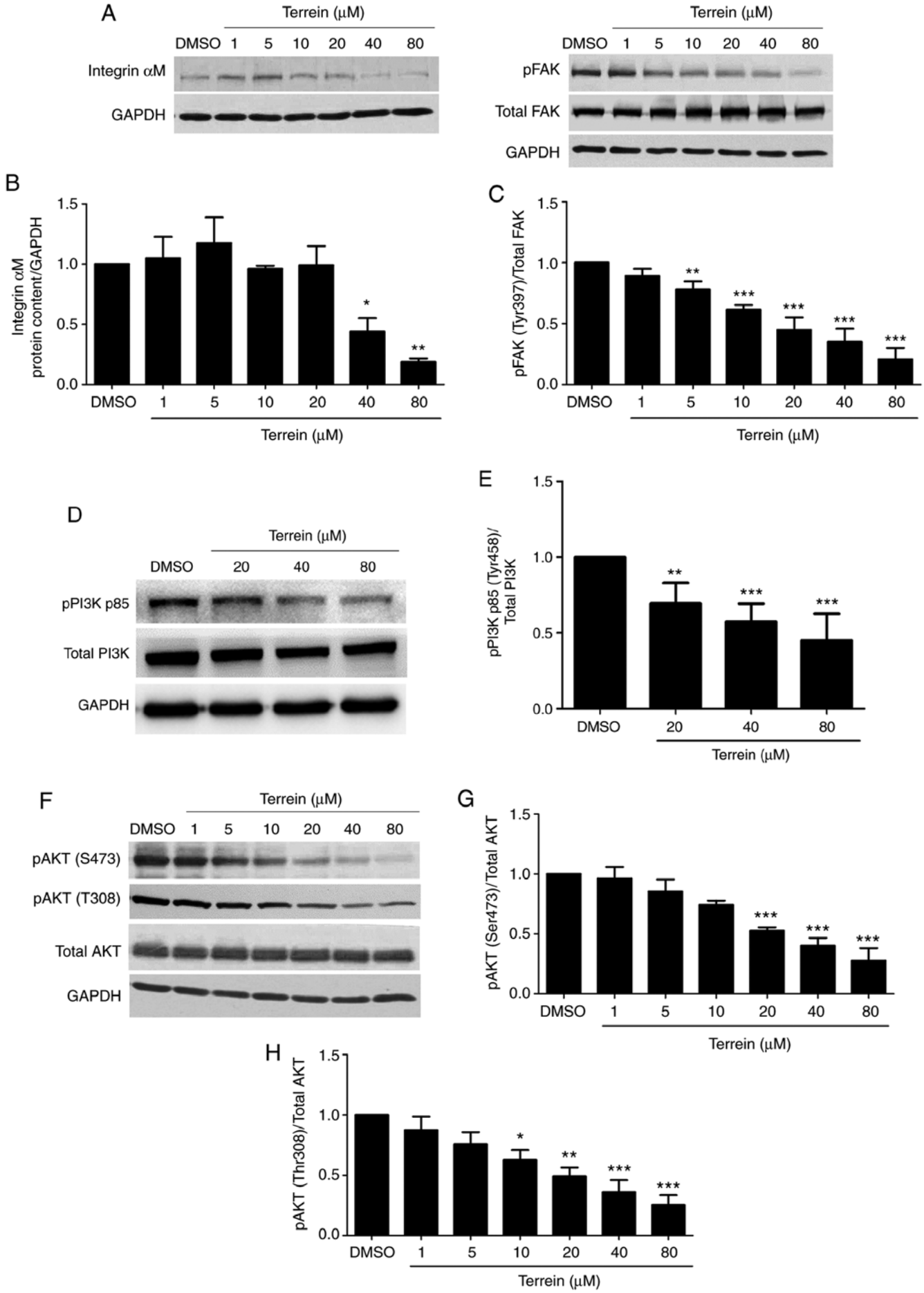


Figure 4. Terrein inhibits the angiogenesis process of A549 lung cancer cells. (A) Effects of terrein on release of VEGF-A from A549 lung cancer cells that was examined by VEGF-A Human ELISA assays after 24 h of terrein treatment. (B) Terrein inhibited A549 lung cancer cell tube formation. After 24 h of terrein treatment, tubular structures were captured under an inverted microscope at a magnification of x10. (C) Quantification graph by Angiogenesis Analyzer plugin for ImageJ software. (D) Representation of the protein level of VEGFR2 was measured by western blotting after 24 h of terrein treatment. (E) Quantification graph of western blotting by ImageJ software. Significance was measured as the mean ± SEM of at least three separate experiments. *P<0.05, **P<0.01 and ***P<0.001 compared with the vehicle control. VEGF, vascular endothelial growth factor; VEGF-A, VEGF subtype A; VEGFR2, VEGF receptor tyrosine kinase type 2; p, phosphorylated.

secreted by the A549 cells into the medium was collected, concentrated and detected by human VEGF-A ELISAs. The results revealed that terrein significantly reduced the secretion of VEGF-A from A549 lung cancer cells compared with the vehicle control (Fig. 4A). To further confirm this inhibitory effect, an *in vitro* capillary-like tube formation assay was performed. This is a rapid and quantitative method for examining cell differentiation and changes associated with the angiogenesis process. To perform the experiment, A549 cells were cultured in Matrigel-coated plates and then treated with or without 20, 40 and 80 μM terrein for 24 h. Tube formation or the characteristics of A549 cells that formed vessel-like channels were captured by inverted microscopy. As revealed in Fig. 4B and C, terrein significantly suppressed the formation of tube-like structures compared with the vehicle control. The inhibition of VEGFR2 phosphorylation at Tyr1175, which is a crucial site for the migration of cells during angiogenesis, was also detected (Fig. 4D and E).

Terrein inhibits the activity of metastasis mediators in A549 lung cancer cells. To investigate the effect of terrein on the expression of metastasis mediators, such as integrin αM, FAK, mTORC1, PI3K, AKT and P70S6K, western blotting was performed. A549 cells were treated with different concentrations of terrein for 24 h. As revealed in Fig. 5A-C, the results indicated that terrein significantly inhibited integrin αM expression and FAK phosphorylation at Tyr397 compared with the vehicle control. Notably, a low concentration of terrein clearly inhibited the phosphorylation of FAK. Active integrins recruit various proteins, including FAK, to FAs to stimulate cell signaling. When FAK is activated, it further stimulates the expression and activation of downstream proteins. The signaling proteins downstream of FAK are PI3K, AKT, mTORC1 and P70S6K. These proteins affect cell survival, proliferation, angiogenesis, invasion, migration and metastasis. The inhibition of FAK leads to the suppression of these downstream signaling mediators (36). The phosphorylation of



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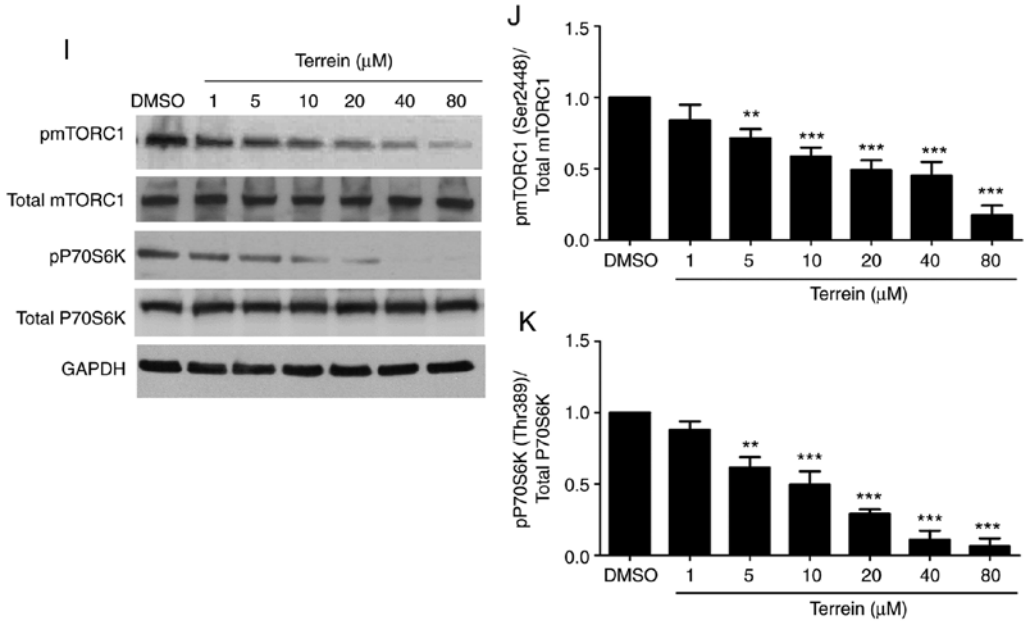

Figure 5. Effects of terrein on the expression of signaling mediators associated with metastatic processes in A549 lung cancer cells. Representative protein levels were examined by western blotting after 24 h of terrein treatment and the quantified bar graphs are presented. (A-C) Integrin αM and FAK phosphorylation. (D and E) PI3K p85 phosphorylation. (F-H) AKT phosphorylation. (I) mTORC1 and P70S6K as determined by western blotting. (J) mTORC1 phosphorylation and (K) P70S6K phosphorylation. Significance was measured as the mean ± SEM of at least three separate experiments. *P<0.05, **P<0.01 and ***P<0.001, compared with the vehicle control. FAK, focal adhesion kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase-B; mTOR, mammalian target of rapamycin; P70S6K, ribosomal p70S6 kinase; p, phosphorylated.

PI3K p85 at Tyr458 (Fig. 5D and E), AKT at Ser473 (Fig. 5F and G), AKT at Thr308 (Fig. 5F and H), mTORC1 at Ser2448 (Fig. 5I and J) and pP70S6K at Thr389 (Fig. 5I and K) was decreased by terrein in a dose-dependent manner compared with the vehicle control. Therefore, the inhibition of these proteins by terrein resulted in the suppression of metastatic and angiogenic processes in lung cancer cells.

Terrein inhibits the phosphorylation of FAK in A549 lung cancer cells. Immunofluorescence was used to further confirm the effect of terrein on the phosphorylation of FAK at Tyr397 in A549 cells. The representative and quantitative images (Fig. 6) revealed that 40 and 80 μM terrein significantly suppressed the phosphorylation of FAK at Tyr397 compared with the vehicle control. Combining these results with the western blot data clearly indicated that terrein had the ability to inhibit the phosphorylation of FAK at Tyr397, and FAK was revealed to be an upstream regulator of numerous proteins involved in cancer metastasis.

Discussion

Human lung cancer is the leading cause of cancer-related deaths worldwide (1). Most often, lung cancer is diagnosed at metastatic stages, and at these stages, cancer cells have spread to nearby tissues or other parts of the body (2). Therefore, the suppression of metastasis is required to reduce the premature death of cancer patients. Currently, the world has turned its attention to the use natural substances for cancer treatment because they typically have fewer side effects. Terrein is a bioactive natural substance that has been reported to exert various biological effects, including anti-inflammation, melanogenesis inhibition and anticancer effects (20-22). However, to the best of our knowledge, the effect of terrein on the molecular mechanisms that regulate human lung cancer metastasis has not been examined.

In the present study, the effects of terrein on metastatic processes, including cell proliferation, adhesion, migration, invasion and angiogenesis, were investigated using A549 human lung cancer cells. The present results revealed that terrein exhibited cytotoxic effects on different cell types, including A549 cells, Vero cells, L6 cells and H9C2 cells, by inhibiting cell viability with IC_{50} values of 229, 870, 1,240 and 579 μM, respectively. These results suggest that terrein has a specific effect on A549 lung cancer cells relative to normal cells with a relatively high selectivity index value. As revealed in other studies, terrein exhibited a cytotoxic effect on human cervical cancer cells (HeLa) with an IC_{50} value of 290 μM (22) and on the human breast cancer cell lines MCF-7 and MDA-MB-231 with IC_{50} values of 2,340 and 700 μM, respectively (28). LDH assays were used to measure cell viability (34) and further verified this conclusion. Overall, these data indicated that terrein was more toxic to A549 lung cancer cells than to other types of cancer cells.

Cancer metastasis includes several steps, including cell proliferation, adhesion, migration, invasion and angiogenesis. To determine the effects of terrein on these processes, low concentrations of terrein (20, 40 and 80 μM) that were not toxic to the normal cell lines tested were selected. It was first observed that a terrein concentration as low as 20 μM started to reduce the proliferative ability of A549 cells. The antimetastatic properties of terrein were observed upon analysis of MMP activity. Numerous studies have demonstrated that MMPs play important roles in tumor progression, invasion, metastasis and angiogenesis (37,38). Increased expression of MMPs has been revealed

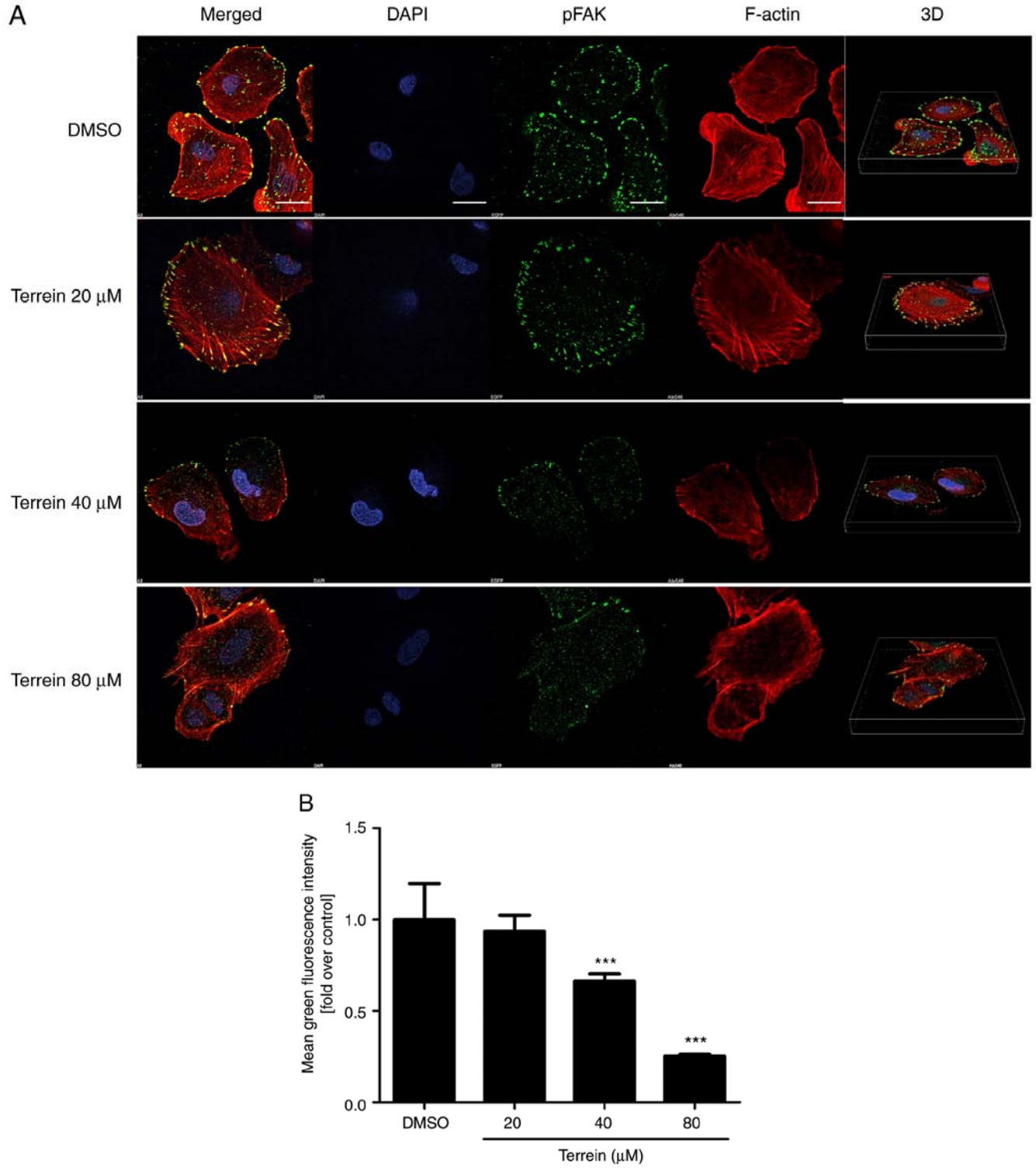

Figure 6. Effects of terrein on phosphorylation of FAK at Tyr397 in A549 cells. Cells were treated with terrein at 20, 40 and 80 μM for 24 h. (A) Representative confocal images of phosphorylation of FAK at Tyr397. (B) Its quantification. Significance was measured as the mean ± SEM of at least three separate experiments. ***P<0.001, compared with the vehicle control. Scale bar, 20 μm. FAK, focal adhesion kinase.

to be associated with poor prognosis in several types of tumors, including breast cancer, gastric cancer and osteosarcoma (39-41). All invasive malignant tumors, including lung cancer cells, are known to express high levels of MMPs, especially MMP-2 and MMP-9 (42). MMP-2 plays an essential role in the progression of cancer because it cleaves several ECM components and basement membranes (43); thus, the development of potential MMP-2 inhibitors has become an important goal in lung cancer therapy. The expression and activity of MMP-9 are increased in NSCLC and are associated with the pathological type and clinical stage of NSCLC (44). The expression levels of MMP-9 were higher in advanced stage III and IV tumors than in primary stage I and II tumors of NSCLC patients, and MMP-9 expression was higher in NSCLC patients with metastasis than in NSCLC patients

without metastasis and a reduced 5-year survival rate (45). Both MMP-2 and MMP-9 are classified as soluble enzymes that are secreted into the extracellular milieu. Therefore, blocking the activities of these enzymes has the potential to suppress lung cancer cell metastasis and increase the possibility of NSCLC patient survival. In the present study, it was revealed that terrein significantly suppressed both the activities and expression of MMP-2 and MMP-9. These findings indicated that terrein has the potential to act as an antimetastatic agent, which, to the best of our knowledge, to date, has never been reported.

Angiogenesis, or the creation of new blood vessels, is another important mechanism during cancer progression. New blood vessels support the growth of tumors by specifically feeding their hypoxic and necrotic areas to provide essential nutrients and oxygen (46). In the present study, the effect of terrein on angiogenesis was investigated by determining the expression of VEGF-A as a surrogate biomarker. VEGF is a major chemotactic factor during angiogenesis that initiates the migration and adhesion of cells, interactions between endothelial cells and ECM, and formation of a tubular network (47). In mammals, there are several types of VEGF, including VEGF-A, VEGF-B, VEGF-C, and VEGF-D; however, VEGF-A is widely studied and plays a major role in angiogenesis by acting through VEGFR2 (12). It was observed that 80 μM terrein significantly attenuated VEGF-A expression. Consistently, the ratio of phosphorylated VEGF-A/VEGFR2 was decreased by terrein compared to the vehicle control, thus indicating that the downstream signaling events induced by the addition of VEGF to the cell medium were inhibited. Furthermore, the effect of terrein on angiogenesis was investigated by examining the *in vitro* capillary-like tube formation of A549 cells. The characteristics of tube-like structures were reduced by terrein in a dose-dependent manner. These results suggest that terrein suppresses processes known to be involved in angiogenesis.

Thus, our collective dataset suggests that terrein can suppress multiple steps of cancer metastatic processes, including proliferation, wound healing, adhesion, migration and invasion. A more detailed mechanistic analyses was next conducted based on the rationale that focal adhesion complexes are known to be crucial nodes of signaling events that mediate cancer metastasis (48). Notably, the interaction of multiple proteins at focal adhesions is critical to promote the protrusion of the cell membrane leading edge, resulting in the development of invadopodia and lamellipodia (49). At focal adhesions, signal transduction is initiated by interactions of integrins with the ECM, which further promote the assembly of cytoplasmic scaffolds and the recruitment of kinase proteins (50). FAK, or focal adhesion kinase, is one of the principal integrin signaling regulators that is recruited to the site of adhesion, and FAK is autophosphorylated at the Tyr397 site. The autophosphorylation of FAK contributes to the further activation of its intrinsic kinase activity and creates docking sites for several downstream signaling molecules (51). FAK expression is increased in numerous highly malignant human cancers (52). Overexpression of FAK in cancer cells leads to resistance to the apoptotic process (53). In addition, an increase in FAK was revealed to contribute to the activation of the PI3K/AKT and MEK-ERK1/2 signaling pathways, resulting in increased cancer cell survival and proliferation (54). Therefore, to inhibit integrin signaling, the regulation of FAK expression and

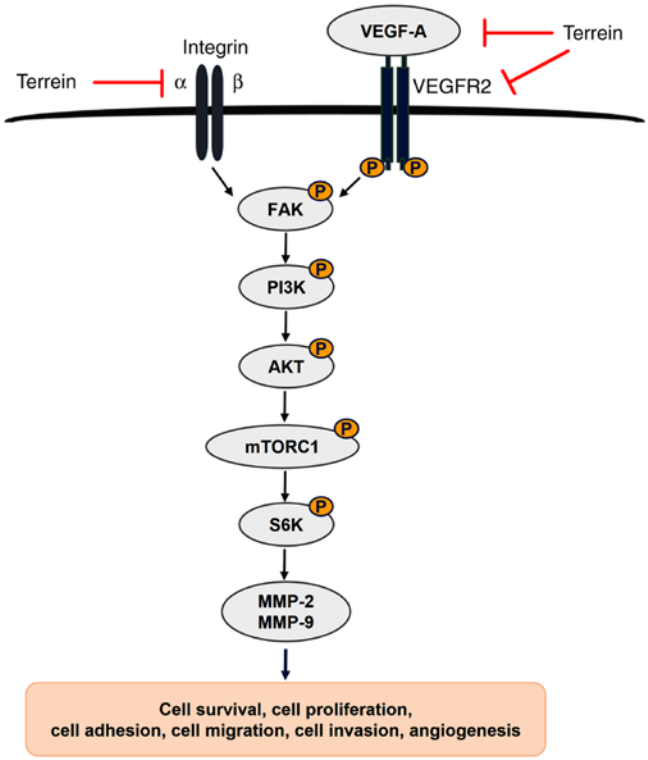

Figure 7. Summary of terrein effects on lung cancer cell metastasis and angiogenesis signaling pathways.

phosphorylation is a well-established goal for the development of effective pharmaceutical antimetastatic agents. As revealed in the present study, terrein could inhibit the expression of integrin αM and the phosphorylation of FAK at Tyr397, as demonstrated by western blot and immunofluorescence analyses. The present results revealed that terrein could reduce stress fiber formation. Normally, cell migration is associated with adhesion and actin cytoskeleton organization, which also involves a series of lamellipodia extension and actin polymerization. In addition, initial adhesions are formed through the engagement of integrin receptors by the ECM (55). The integrin-FAK signaling pathway is known to be associated with cell adhesion and migration, and these effects are regulated by downstream molecules. Inhibition of FAK leads to retraction of filopodia and lamellipodia in cell protrusions, resulting in stress fiber formation in retractile cell bodies (6). The present results suggested that the inhibition of FAK reduced the adhesion of cells to the ECM, which contributed to reduced cell migration.

Next, mediators downstream of FAK, including PI3K, AKT, mTOR and P70S6K, were further investigated. The signaling pathway of PI3K/AKT is well recognized to associate with multiple cellular processes such as cell proliferation, differentiation and motility (56). In addition, mTOR is one important downstream target of the PI3K/AKT signaling involved with cell movement and metastasis (57). The protein mTORC1 induces protein synthesis and cell growth by phosphorylating S6K1 and 4E-BP1 (58), while mTORC2 regulates the organization of the actin cytoskeleton through F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42 and PKCα (59). The inhibition of the PI3K/AKT/mTOR pathway by an mTOR inhibitor has

been demonstrated to suppress cancer cell invasion and migration and promote apoptosis in tumors (60,61). Recent research has demonstrated that inhibiting the FAK/PI3K/AKT/mTOR pathway and reducing MMP-2 and MMP-9 protein expression suppressed gastric cancer cell migration and invasion (62). Furthermore, the PI3K/AKT/mTOR pathway plays a pivotal role in regulating angiogenesis, increasing hypoxia-inducible factor 1α (HIF-1α) translation and promoting VEGF production (63). The results of the present study revealed that terrein significantly decreased the phosphorylation of PI3K p85 at Tyr458, AKT at Ser473 and Thr308, mTORC1 at Ser2448 and pP70S6K at Thr389. Therefore, all these data indicated that terrein has the ability to suppress the PI3K/AKT/mTOR/S6K1 pathway and thus affects cancer cell proliferation, survival, migration, invasion, metastasis and angiogenesis, as revealed in Fig. 7.

In conclusion, the present study demonstrated the novel finding that terrein acted as an anticancer agent in lung cancer. This occured via numerous effects, such as the inhibition of proliferation and metastatic processes, including adhesion, migration and invasion. Mechanistically, terrein regulated the integrin/FAK interaction and its downstream signaling pathway, PI3K/AKT/mTOR/S6K1. In addition, terrein could inhibit angiogenic processes by decreasing VEGF secretion and tube formation by reducing the VEGF-A/VEGFR2 interaction. All these data suggested that terrein is a potential new compound that is worthy of further development as an anticancer agent. Further experiments to assess the effect of terrein *in vivo* are further suggested.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

PB performed the experiments and data analysis and was the main author of the manuscript. WT and GS supervised the experiments and revised the manuscript for important intellectual content. MNA provided the materials needed for the preparation of terrein and validated the data analysis. HKS performed the immunofluorescence experiment and interpreted the data. CP participated in the experimental design and provided the reagents needed for the IncuCyte assay. All the authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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