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Ds-echinoside A, a new triterpene glycoside derived from sea cucumber, exhibits antimetastatic activity via the inhibition of NF-κB-dependent MMP-9 and VEGF expressions*

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Abstract: Ds-echinoside A (DSEA), a non-sulfated triterpene glycoside, was isolated from the sea cucumber *Pearsonothuria graeffei*. In vitro and in vivo investigations were conducted on the effects of DSEA on tumor cell adhesion, migration, invasion, and angiogenesis. In this study, we found that DSEA inhibited the proliferation of human hepatocellular liver carcinoma cells Hep G2, with a half-maximal inhibitory concentration (IC_{50}) of 2.65 μmol/L, and suppressed Hep G2 cell adhesion, migration, and invasion in a dose-dependent manner. DSEA also reduced tube formation of human endothelial cells ECV-304 on matrigel in vitro and attenuated neovascularization in the chick embryo chorioallantoic membrane (CAM) assay in vivo. Immunocytochemical analysis revealed that DSEA significantly decreased the expression of matrix metalloproteinase-9 (MMP-9), which plays an important role in the degradation of basement membrane in tumor metastasis and angiogenesis. DSEA also increased the protein expression level of tissue inhibitor of metalloproteinase-1 (TIMP-1), an important regulator of MMP-9 activation. From the results of Western blotting, the expressions of nuclear factor-kappa B (NF-κB) and vascular endothelial growth factor (VEGF) were found to be remarkably reduced by DSEA. These findings suggest that DSEA exhibits a significant antimetastatic activity through the specific inhibition of NF-κB-dependent MMP-9 and VEGF expressions.

Key words: Triterpene glycoside, Ds-echinoside A (DSEA), Metastasis, Angiogenesis, Nuclear factor-κB (NF-κB), Matrix metalloproteinase-9 (MMP-9), Vascular endothelial growth factor (VEGF)

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1 Introduction

Metastasis, which refers to the spread of cancer cells from primary neoplasms to distant sites, is often proven to be the most damaging aspect of cancer, and is one of the major causes of mortality in cancer patients. Tumor metastasis, which includes cell proliferation, proteolytic digestion of extracellular matrix (ECM), cell migration to the circulatory system, and tumor growth at metastatic sites, is a multistep and complex process (Kleiner and Stetler-Stevenson, 1999; Chambers *et al*., 2002; Steeg, 2006; Imanaka *et al*., 2008). When tumor cells metastasize, various proteolytic enzymes contribute to the degradation of ECM and basement membrane (BM) (Pasco *et al*., 2005). Matrix metalloproteinases (MMPs) play a crucial role in ECM degradation associated with both metastasis and angiogenesis of cancer (Westermarck and Kähäri, 1999; McCawley and Matrisian, 2000). Both the transcriptional and post-transcriptional

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levels of MMPs are closely related. MMP expression is controlled by various cytokines and growth factors at the transcriptional level. At the post-transcriptional level, MMP activity is controlled by proteolytic activation of latent proenzymes and by interactions with specific inhibitors (Rao, 2003). MMP-9, a member of the MMP family, has been suggested to play a key role in tumor invasion and angiogenesis (Nabeshima *et al*., 2002) and is overexpressed in many human cancers with invasive and metastatic capabilities (Sanceau *et al*., 2003). The expression of MMP-9 can be regulated by nuclear factor-kappa B (NF-κB) (Nagao *et al*., 2000; Choi *et al*., 2010). NF-κB activation has also been reported to be associated with metastatic phenotypes (La Porta and Comolli, 1998), and inhibition of NF-κB activation has been shown to suppress MMP-9 and increase tumor invasion (Alaniz *et al*., 2004).

Angiogenesis, which is required to sustain the growth of primary tumors and metastases, is mediated by an increased production of various angiogenic molecules released by both the tumor itself and neighboring host cells (Folkman, 2006). Among the known angiogenic growth factors and cytokines, vascular endothelial growth factor (VEGF) and its corresponding receptors are indispensable in regulating multiple facets of angiogenic processes (Leung *et al*., 1989). VEGF is an NF-κB-inducible protein that is regulated by activated NF-κB (Dai *et al*., 2009). Recently, several studies have reported that VEGF production and gene expression are compromised by NF-κB inhibition (Shibata *et al*., 2002; Ko *et al*., 2006; Dai *et al*., 2009).

The sea cucumber *Pearsonothuria graeffei* (*P. graeffei*), which is of little food value, is widely distributed in Taiwan, the Nansha Islands, Madagascar, the Maldives, the Philippines, Guam, and Fiji. It contains a high content of triterpene glycosides, up to 3.5% of the body wall dry matter. Triterpene glycosides with a wide spectrum of biological effects (Chludil *et al*., 2002; Liu *et al*., 2007; Zhang *et al*., 2007) are the predominant secondary metabolites of holothurians. Recently, the anti-tumor and anti-angiogenic activities of triterpene glycosides have attracted considerable attention. Different species of the sea cucumber show differences in the content and structure of triterpene glycosides, and differ according to the growing environment and food intake. Both frondoside A and cucumarioside A_2-2, which are sulfated triterpene glycosides, isolated from sea cucumbers *Cucumaria frondosa* and *Cucumaria japonica*, respectively, possessed cell apoptosis-inducing capability in a caspase-dependent or -independent manner, depending on the holothurian structure (Jin *et al*., 2009). Another two sulfated triterpene glycosides, philinopside A (PA) and philinopside E (PE), derived from the sea cucumber *Pentacta quadrangulari*, were both found to exhibit dual anti-angiogenic and anti-tumor effects through receptor tyrosine kinases (RTKs) or VEGF-induced kinase insert domain-containing receptor (KDR) signaling pathways, respectively (Tian *et al*., 2005; Tong *et al*., 2005). Ds-echinoside A (DSEA), a non-sulfated triterpene glycoside, was derived from the desulfurization reaction of echinoside A by Kitagawa in 1980, and so far, the only report on the bioactivity of DSEA has been about its antifungal effects (Kitagawa *et al*., 1980). To our knowledge, there is no report on the separation of DSEA from a natural source directly, and no information is available about the anticancer potential and related molecular mechanisms of nonsulfated triterpene glycosides. Here, for the first time, we directly isolated DSEA from the sea cucumber *P. graeffei* and demonstrated that DSEA exhibited a significant anti-metastatic activity both in vitro and in vivo. Regarding the underlying mechanism, the bioactivity of DSEA may involve the modulation of NF-κB, which, in turn, reduced the expressions of VEGF and MMP-9.

2 Materials and methods

2.1 Triterpene glycoside

DSEA was isolated and purified using the procedure described by Dong *et al*. (2008). Briefly, an ethanolic extract of *P. graeffei* was sequentially submitted to macroporous resin, Si-gel, and reversed Si-gel chromatographies, and the final purification of DSEA was achieved by preparative high-performance liquid chromatography (HPLC). The chemical structure of DSEA was determined by spectroscopic analysis (infrared spectroscopy, nuclear magnetic resonance spectroscopy, and electrospray ionization mass spectrometry). The molecular weight of DSEA was 1 104 Da, and the molecular formula was

deduced to be $C_{54}H_{88}O_{23}$ (Fig. 1). DSEA was dissolved in dimethyl sulfoxide (DMSO) and diluted to the desired concentrations before use. The final concentration of DMSO in the culture media was below 0.05% (v/v), and this concentration of DMSO showed no effect in the assay systems.

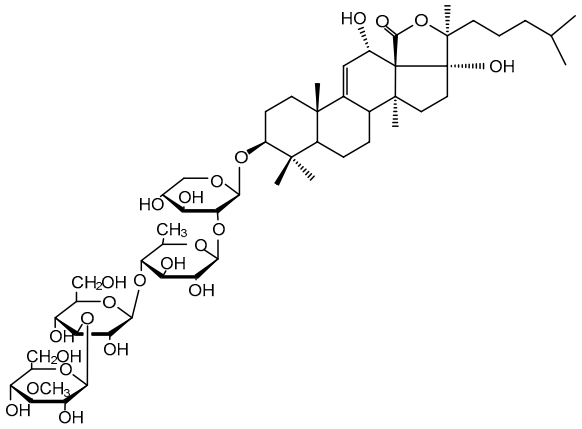

Fig. 1 Chemical structure of ds-echinoside A (DSEA)

2.2 Cell lines and cell culture

Human hepatocellular liver carcinoma cells Hep G2 and human umbilical vein endothelial cells ECV-304 were obtained from Shanghai Cell Bank (Shanghai, China) and grown in RPMI-1640 medium or Dulbecco's modified eagle medium (DMEM), supplemented with 10% (v/v) newborn calf serum (NCS), 100 μg/ml streptomycin, and 100 U/ml penicillin at 37 °C in a humidified atmosphere containing 5% CO_2. All experiments were repeated three times to ensure the reproducibility.

2.3 Cell proliferation assay

Hep G2 cells (8×10^3 well^{-1}) were seeded in a 96-well plate and incubated for 24 h. Then, the medium was replaced with fresh RPMI-1640 medium containing different concentrations of DSEA. After incubation for 6–24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml in RPMI-1640 medium) was added and further incubated for 4 h. Cell viability was determined by the MTT assay (Skehan *et al*., 1990).

2.4 Cell adhesion assay

Cell adhesion efficiency was determined by measuring the number of cells that adhered to a given substrate, as described by Liaw *et al*. (1994). Matrigel diluted to 50 μg/ml with DMEM was applied to 96-well plates and allowed to polymerize in a humidified incubator at 37 °C for 1 h. Hep G2 cells pretreated with various concentrations of DSEA (0, 1.35, and 2.70 μmol/L) for 12–24 h were collected and suspended at a final concentration of 2×10^5 cells/ml in serum-free medium; 100 μl of the cell suspension was seeded into each well and allowed to adhere at 37 °C for 1.5 h. Non-adherent cells were carefully rinsed off with phosphate-buffered saline (PBS) and the remaining cells were measured using the MTT assay.

2.5 Wound migration assay

Hep G2 cells (1.5×10^5 well^{-1}) were seeded into a 24-well plate for 24 h. The confluent monolayer was starved using serum-free medium for 8 h and wounded by scratching with a 1-ml pipette tip. After washed three times with PBS, cells were incubated in serum-free medium containing various concentrations of DSEA (0, 1.35, and 2.70 μmol/L). Photographs were taken at 0, 12, and 24 h after wounding. The width of the wound was measured using the Image Pro Plus 5 software.

2.6 Cell invasion assay

The cell invasion assay was performed using a Transwell Boyden chamber (diameter 6.5 mm) with polycarbonate filter (pore size 8 μm). Briefly, the upper culture chamber was coated with a uniform layer of matrigel (1:20, diluted in RPMI-1640), and 750 μl RPMI-1640 medium containing 20% (v/v) fetal bovine serum (FBS) was placed in each lower well. Hep G2 cells (5×10^4 cells) were loaded into each upper well in 100 μl RPMI-1640 along with various concentrations of DSEA (0, 1.35, and 2.70 μmol/L). After incubation at 37 °C for 12 h, nonmigrating cells on the upper surface of the filter were removed with a cotton swab. The filters were then fixed with ethanol and stained with 10 g/L crystal violet. The cells were visualized using an inverted microscope (IX51; Olympus, Japan) and the images were analyzed using the Image Pro Plus 5 software. Five random fields were counted for each filter. The rate of invasion was calculated as migrated cells of the treated/migrated cells of the control.

2.7 Tube formation assay

A tube formation assay was carried out to determine the effect of DSEA on angiogenesis in vitro. A 96-well plate coated with 50 μl of matrigel (1:4, diluted in DMEM) per well was allowed to solidify at 37 °C for 1 h. Each well was seeded with 1×10^4 ECV-304 cells, resuspended in DMEM with 2% (v/v) NCS, and cultured in a medium containing various concentrations of DSEA (0, 2.26, and 4.53 μmol/L) for 24 h. The networks of enclosed tubes were photographed from five randomly chosen fields under an inverted phase contrast microscope (IX51; Olympus, Japan). Images were captured under an Olympus DP72 microscope digital camera system using the Image Pro Plus 5 software.

2.8 Chicken embryo chorioallantoic membrane (CAM) assay

Inhibition of angiogenesis in vivo was determined using a modified CAM assay (Tan *et al*., 2001). Fertilized chicken eggs were allowed to grow for 9 d in an egg incubator. Then, a small hole was punched into the broad side of the egg and a 1-cm^2 window was carefully created in the eggshell. The shell membrane was removed to expose the CAM. Sterile filter-paper discs (diameter 5 mm) saturated with various concentrations of DSEA (0, 0.23, and 0.46 nmol/L per egg) were placed on the CAM. After further incubated for 24 h, the neovascular zones under the disks were photographed under a stereomicroscope (SZ61; Olympus, Japan) at 10× magnification.

2.9 Western blotting analysis

Hep G2 cells were plated onto 6-well plates and allowed to grow to confluence (1×10^6 cells). Dilutions of DSEA (0, 1.35, and 2.70 μmol/L) were added and incubated for 12 h at 37 °C. The cells were then lysed with lysis buffer (20 mmol/L Tris/HCl (pH 8.0), 2 mmol/L EDTA, 137 mmol/L NaCl, 1 mmol/L Na_3VO_4, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and 1 mmol/L phenylmethylsulfonyl fluoride). Lysates were clarified by centrifugation (12000×*g*, 10 min), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk overnight at 4 °C and incubated with primary antibodies against NF-κB p65, VEGF, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for 1.5 h at room temperature. Then, the membranes were washed (Tris-buffered saline and Tween 20 (TBS/T), three times, 5 min per time), incubated (1.5 h, room temperature) with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000), and washed three times with TBS/T. Antigen reactivity was detected using a Tiangen KC-420 chemiluminescent substrate kit (Beijing, China). Each experiment was repeated at least three times; representative blots are presented.

2.10 Immunocytochemical assay

Hep G2 cells (5×10^5 $well^{-1}$) were seeded in 6-well plates with sterilized coverslips and treated with various concentrations of DSEA (0, 1.35, and 2.70 μmol/L) for 12 h. Then the cells were washed in PBS and fixed with 4% (v/v) paraformaldehyde at 4 °C. The streptavidin biotin complex (SABC) immunohistochemistry kit was used for immunocytochemical analysis. Cells were incubated overnight with polyclonal MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) antibodies at 4 °C. After washing three times with PBS, cells were incubated with biotinylated goat anti-rabbit IgG for 40 min at 37 °C. Then cells were incubated with SABC at 37 °C for 40 min and colored with diaminobenzidine (DAB) for 15 min. Finally, the slides were counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted. Stained cells were examined and photographed using a light microscopy (BX41; Olympus, Japan). Data were analyzed using the Image Pro Plus 5 software.

2.11 Statistical analysis

Data are presented as mean±standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) and the least significant difference (LSD) test.

3 Results

3.1 DSEA inhibits proliferation of Hep G2 cells

To determine the cytotoxicity of DSEA, its inhibitory effect on the proliferation of Hep G2 cells was measured by the MTT assay. As shown in Fig. 2, DSEA had a dose-dependent inhibitory effect on the

viability of Hep G2 cells, with an average half-maximal inhibitory concentration (IC_{50}) of 2.65 μmol/L. These results indicate that DSEA was strongly cytotoxic to hepatocellular carcinoma cells.

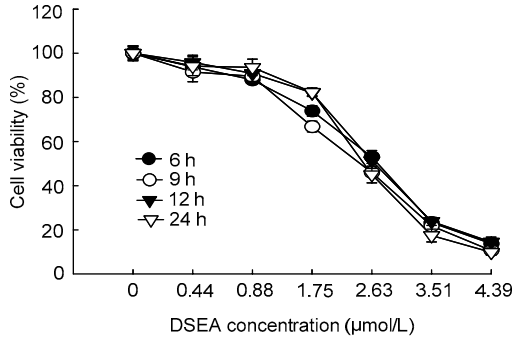

Fig. 2 Effect of DSEA on the proliferation of Hep G2 cells
Hep G2 cells were treated with various concentrations of DSEA (0, 0.44, 0.88, 1.75, 2.63, 3.51, and 4.39 μmol/L) for 6, 9, 12, and 24 h. Cell viability was estimated by MTT assay. Data represent the mean±SD of three independent experiments

3.2 DSEA suppresses Hep G2 cell adhesion

Cell adhesion is a prerequisite for tumor cell invasion and metastasis formation. Thus, we investigated whether DSEA affected the adhesion of tumor cells to matrigel, a common analog of ECM. After attaching efficiently to matrigel for 1.5 h, DSEA-treated Hep G2 cells showed a dose- and time-dependent decrease in cell adhesion. As shown in Fig. 3, DSEA decreased the average adhesion rate of Hep G2 cells to 67.6% and 39.5% at 1.35 and 2.70 μmol/L, respectively (P<0.01).

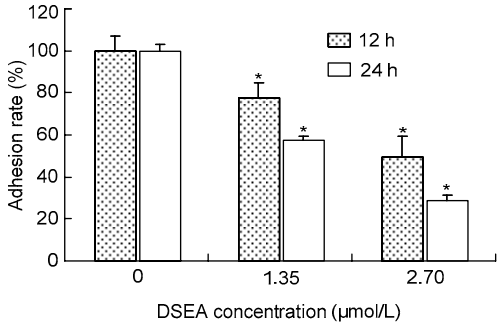

Fig. 3 Effect of DSEA on the adhesion of Hep G2 cells
Hep G2 cells were seeded on matrigel-coated wells with the indicated concentrations of DSEA for 1.5 h. The relative number of attached cells was assessed using the MTT assay. Data represent the mean±SD of three independent experiments. $^{*}P$<0.01 vs. control (0 μmol/L)

3.3 DSEA inhibits Hep G2 cell migration

As tumor cell migration is an essential process in cancer development and metastasis, we explored the effects of DSEA on Hep G2 cell motility using a scratch wound assay. As shown in Fig. 4, in the absence of DSEA, Hep G2 cells migrated along the edges of the wound and showed a large-scale migration, whereas a dose- and time-dependent inhibition of cell flattening and spreading was observed in the presence of DSEA. At 1.35 and 2.70 μmol/L, DSEA decreased the average migration rates of Hep G2 cells to 80.9% and 36.4%, respectively.

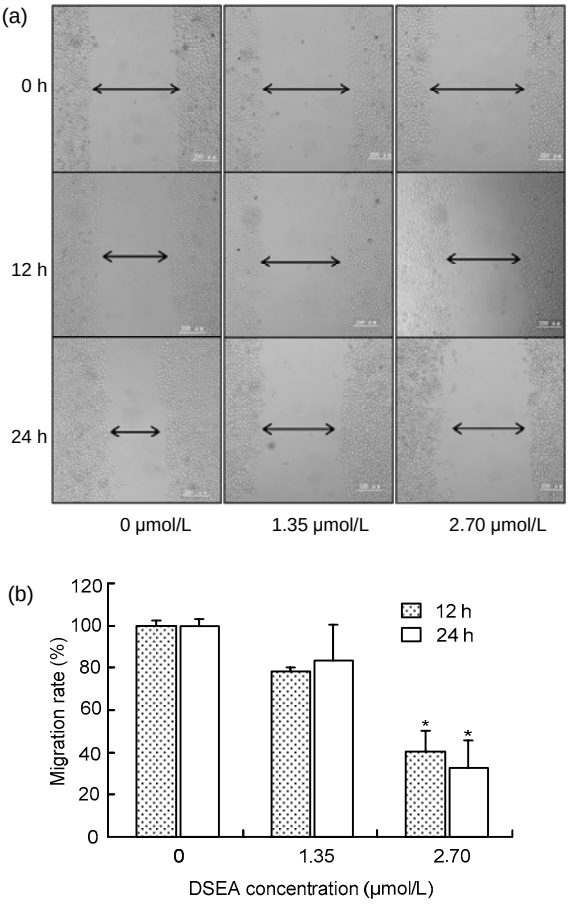

Fig. 4 Effect of DSEA on the migration of Hep G2 cells
Microphotographs of Hep G2 cells (a) and quantitative analysis (b) of the wound migration assay. Hep G2 cells were seeded in a 24-well plate and the confluent monolayers were wounded and then incubated in serum-free medium with 0, 1.35, and 2.70 μmol/L DSEA. At 0, 12, and 24 h after wounding, the cells were photographed under an inverted microscope. Migration rate was expressed as a percentage of control (0 μmol/L). Data represent the mean±SD of three independent experiments. $^{*}P$<0.01 vs. control (0 μmol/L)

3.4 DSEA hinders Hep G2 cell invasion

The effects of DSEA on Hep G2 cell migration were analyzed in a Transwell Boyden chamber assay. As shown in Fig. 5, incubation of control Hep G2 cells in the chamber for 12 h resulted in large-scale migration of cancer cells to the lower side of the filter. In contrast, treatment with DSEA caused a dose-dependent inhibition of Hep G2 invasion, with DSEA concentrations of 1.35 and 2.70 μmol/L yielding inhibition rates of 60.5% and 81.4%, respectively.

3.5 DSEA disrupts microvascular tube formation of endothelial cells

As capillary tube formation of endothelial cells is an essential process in angiogenesis, we studied the effect of DSEA on the formation of tube-like structures in the matrigel tube formation assay. As shown in Fig. 6, control ECV-304 cells differentiate into extensive networks of enclosed tubes. In contrast, the tube-forming ability of DSEA-treated ECV-304 cells decreased significantly. DSEA treatment disrupted tube structure, leading to the development of incomplete tube morphologies, in a dose-dependent manner.

3.6 DSEA inhibits angiogenesis in the CAM assay

To investigate the effect of DSEA on angiogenesis in vivo, a modified CAM assay was carried out. The results indicated that in control eggs, blood vessels formed dense branching vascular networks. In contrast, CAM neovascularization, presented as blood vessel numbers and branching patterns, was dose-dependently suppressed by DSEA (Fig. 7).

3.7 DSEA suppresses the expression level of MMP-9 via enhancement of TIMP-1 expression

MMP-9 is known to play an important role in cancer cell invasion by mediating the degradation of ECM proteins. Thus, we examined the effects of DSEA on the expression levels of MMP-9 and TIMP-1 by using immunocytochemical methods. As shown in Fig. 8, the expression of MMP-9 was decreased significantly by DSEA. At the concentration of 2.70 μmol/L, the expression of MMP-9 in DSEA-treated cells was reduced to 87.9%.

The protein expression level of TIMP-1 in Hep G2 cells treated with different concentrations of DSEA for 12 h is shown in Fig. 9. DSEA increased TIMP-1 expression in Hep G2 cells by 534.0% and 613.3% at 1.35 and 2.70 μmol/L, respectively.

3.8 DSEA down-regulates the protein expression level of VEGF in Hep G2 cells

VEGF plays pivotal roles in angiogenesis and subsequent cancer growth and progression. After exposure to DSEA, the expression of VEGF protein was determined by Western blotting; the result is shown in Fig. 10a. DSEA dose-dependently inhibited VEGF expression in Hep G2 cells. At 1.35 and 2.70 μmol/L, DSEA reduced VEGF expression in Hep G2 cells by 44.1% and 72.9%, respectively.

3.9 DSEA inhibits the protein expression level of NF-κB in Hep G2 cells

It is accepted that the expressions of MMP-9 and VEGF are regulated by NF-κB (Farina *et al*., 1999; Dai *et al*., 2009). We further examined the effects of DSEA on the expression level of NF-κB p65 by Western blotting analysis. As shown in Fig. 10b, the expression of NF-κB p65 was significantly suppressed by DSEA. After treatment with 2.70 μmol/L DSEA for 12 h, the expression of NF-κB p65 was decreased to 5.77% in Hep G2 cells.

4 Discussion

Metastasis is among the last great frontiers in cancer research, and preventing or eliminating metastasis is one of the biggest challenges for therapeutic intervention (Machesky, 2008). The discovery of novel natural products and their components to block cancer metastasis has attracted researchers' interests and attentions (Lee *et al*., 2008). Marine natural products contain a variety of chemotherapeutic compounds that have been shown to prevent the development of malignancies, and several marine-derived molecules are currently in or are entering clinical trials in cancer therapy (Tian *et al*., 2005). Here, we found that DSEA, a novel marine natural non-sulfated triterpene glycoside, is capable of inhibiting the main steps involved in metastasis, including suppressing Hep G2 cell migration, adhesion, and invasion, and blocking endothelial cell tube formation and angiogenesis in an in vivo CAM model. Our findings demonstrate that DSEA has a potent anti- metastatic activity both in vitro and in vivo.

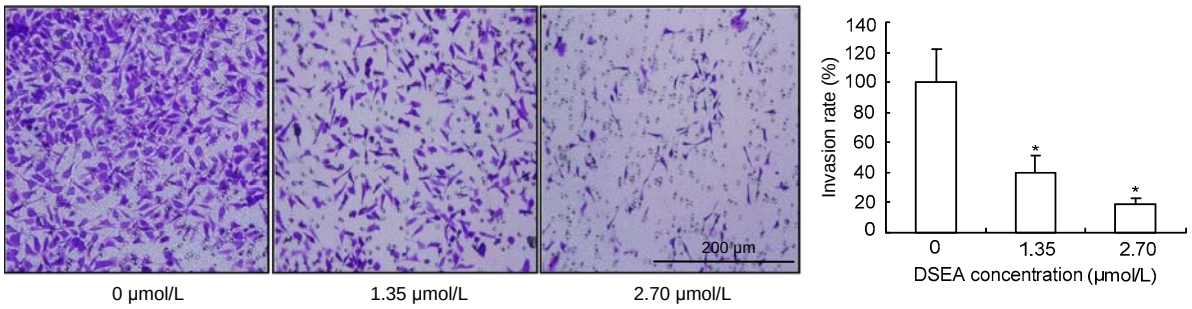

Fig. 5 Effect of DSEA on the invasion of Hep G2 cells

Microphotographs of filters (a) and quantitative analysis (b) of the matrigel chamber invasion assay are shown. Hep G2 cells seeded in Transwell Boyden chambers were incubated for 24 h with 0, 1.35, and 2.70 μmol/L DSEA. Invasion rate was expressed as a percentage of control (0 μmol/L). Data represent the mean±SD of three independent experiments. $^{*}P<0.01$ vs. control (0 μmol/L)

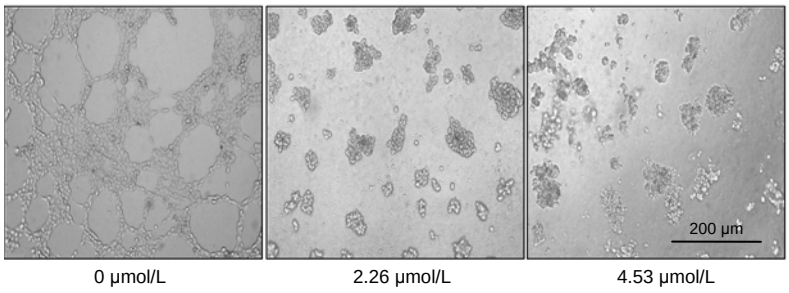

Fig. 6 Effect of DSEA on the tube formation ability of ECV-304 cells

ECV-304 cells seeded in matrigel-coated 96-well plates were incubated for 24 h with DSEA at the concentrations of 0, 2.26, and 4.53 μmol/L. The enclosed networks of tubes were photographed using an inverted microscope

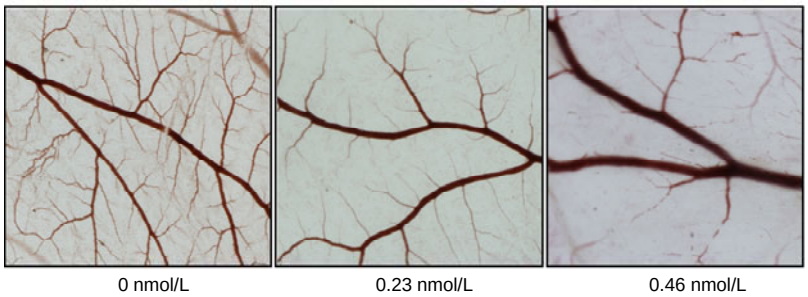

Fig. 7 Effect of DSEA on the angiogenesis of CAM

Various concentrations of DSEA (0, 0.23 and 0.46 nmol/L per egg) were loaded on the CAMs. After 24 h incubation, 10% (v/v) formaldehyde was added onto the surface of CAMs to fix the blood. The disc and surrounding CAMs were harvested and photographed at 10× magnification

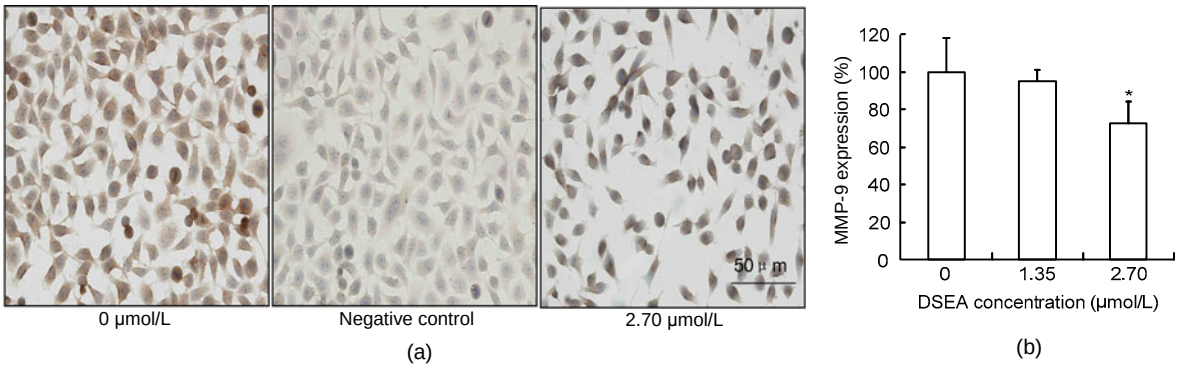

Fig. 8 Effect of DSEA on the protein expression of MMP-9

Hep G2 cells were treated with various concentrations of DSEA for 12 h. The protein expression level of MMP-9 was measured by immunocytochemical analysis. Microphotographs of Hep G2 cells (a) and quantitative analysis (b) of the MMP-9 protein expression are shown. Data represent the mean±SD of three independent experiments. $^{*}P<0.05$ vs. control (0 μmol/L)

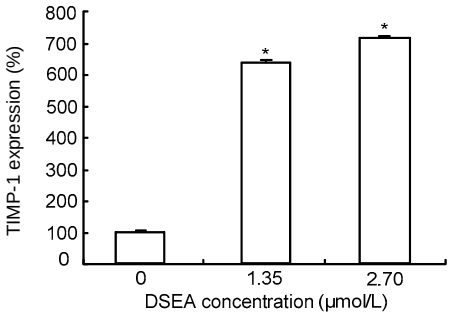

Fig. 9 Effect of DSEA on the protein expression of TIMP-1

Hep G2 cells were treated with various concentrations of DSEA (0, 1.35, and 2.70 μmol/L) for 12 h. The protein expression levels of TIMP-1 were measured by immunocytochemical analysis. Data represent the mean±SD of three independent experiments. $^{*}P<0.01$ vs. control (0 μmol/L)

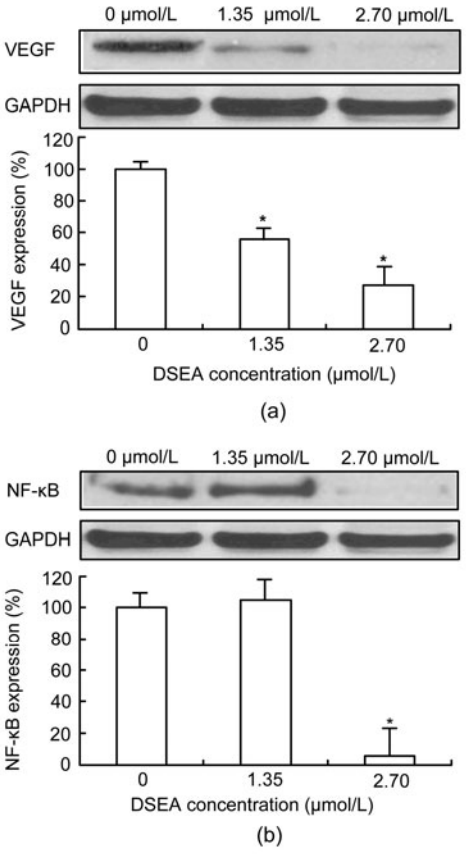

Fig. 10 Effect of DSEA on the protein expression of VEGF (a) and NF-κB (b)

Hep G2 cells were incubated with various concentrations of DSEA for 12 h at 37 °C. The levels of VEGF and NF-κB were measured by Western blotting. Densitometry ratios of VEGF and NF-κB were normalized to GAPDH. Data represent the mean±SD of three independent experiments. $^{*}P<0.01$ vs. control (0 μmol/L)

MMPs, which belong to a multigene family of zinc-dependent endopeptidases and are principal enzymes in ECM degradation, play a major role in promoting angiogenesis and tumor metastasis (McCawley and Matrisian, 2000). Among the MMPs, MMP-2 and MMP-9 are key enzymes in the process of tumor metastasis as a result of ECM degradation (Chang and Werb, 2001). Both MMP-2 and MMP-9 have been correlated with an aggressive, advanced invasive or metastatic tumor phenotype (Liotta *et al*., 1991). It has been demonstrated that MMP-9 is over-expressed in many human solid and hematological malignancies (Sanceau *et al*., 2003). Thus, an inhibitory effect on MMP-9 expression is important for anti-metastasis. Our data demonstrate that DSEA suppressed MMP-9 expression levels in a dose-dependent manner in Hep G2 cells. The levels of cancer invasiveness and malignancy are primarily determined by a sensitive balance between MMPs and their endogenous inhibitors, TIMPs (Hornebeck *et al*., 2005). TIMPs may display distinct influences on tumor progression. It is reported that up-regulation of TIMPs inhibits tumor growth, invasion, and metastasis (Jiang *et al*., 2002). Generally, regulation of MMP-9 is controlled by proenzyme activation and inhibition by TIMP-1 (Watanabe *et al*., 1993). Down-regulation of TIMP-1 was demonstrated to correlate with high cancer invasiveness (Zhang *et al*., 2003; Chan *et al*., 2005). In this study, we found that DSEA enhanced TIMP-1 expression significantly in Hep G2 cells. These results suggest that DSEA directly inhibits the metastatic ability of Hep G2 cells by depressing MMP-9 expression, with augmented TIMP-1 expression.

Two crucial events in tumor progression, tissue invasion and metastasis, are regulated by NF-κB-dependent genes, including *MMPs* (Farina *et al*., 1999). It has been reported that the *MMP-9* promoter contains DNA-binding sites for NF-κB, and that the activation of NF-κB is required to up-regulate the expression of MMP-9 (Nagao *et al*., 2000). In normal resting cells, NF-κB is sequestered in the cytoplasm by binding to a member of inhibitory proteins of the IκB family. NF-κB activation involves its liberation from IκB and its subsequent translocation to the nucleus, where it binds to specific sequences within the promoter region of target genes, and induces gene expression (Pahl, 1999). NF-κB activation has also

been found to induce resistance to various chemotherapeutic agents. Thus, NF-κB may be a good target for the anticancer drug development (Yu *et al*., 2005). Our results showed that the expression of NF-κB in Hep G2 cells was significantly decreased by DSEA. These findings suggest that DSEA suppresses the expression of MMP-9 by blocking NF-κB activation in Hep G2 cells.

NF-κB is also involved in the regulation of angiogenesis, by which tumor cells promote neovascularization for their growth and invasiveness. VEGF is one of the most potent and specific angiogenic factors of tumor-induced angiogenesis (Leung *et al*., 1989). Numerous reports have demonstrated that the metastatic potential of tumor cells is directly correlated with the VEGF expression level, and tumor growth and invasion were significantly suppressed through inhibition of VEGF-induced angiogenesis both in vitro and in vivo (Alferez *et al*., 2008; Wang *et al*., 2008). Here we showed that DSEA treatment markedly suppressed the expression of VEGF. VEGF is under transcriptional control by NF-κB (Kiriakidis *et al*., 2003). Although the VEGF promoter does not contain an NF-κB binding site (Forsythe *et al*., 1996), we and others have demonstrated that VEGF expression is inhibited by NF-κB inhibition (Shibata *et al*., 2002; Ko *et al*., 2006; Dai *et al*., 2009). These results suggest that DSEA down-regulates VEGF expression, in part through the NF-κB signaling pathway. There are various signal transduction pathways involved in the regulation of MMP-9 expression in tumor cells. Mitogen-activated protein kinases (MAPKs) are suggested to be intricately involved in the expression of the components involved in the induction of the promoter for MMP-9 via NF-κB (Kaomongkolgit *et al*., 2008). Further in-depth investigations are needed to better understand the complex mechanism involved.

In conclusion, the present study provides evidence that DSEA possesses marked anti-metastatic activity. We have shown that DSEA plays a role in the inhibition of Hep G2 cell migration, invasion, and angiogenesis through suppression of VEGF and MMP-9 and enhancement of TIMP-1 protein expression by blocking the NF-κB signaling pathway. To our knowledge, this is the first report demonstrating the anti-metastatic activity of a nonsulfated triterpene glycoside isolated from *P. graeffei*, and providing a scientific basis for its application in therapeutic intervention against tumor progression.

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