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# DS-1 Inhibits Migration and Invasion of Non-small-cell Lung Cancer Cells Through Suppression of Epithelial to Mesenchymal Transition and Integrin β1/FAK Signaling

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**Abstract.** *Background/Aim: Epithelial to mesenchymal transition (EMT), and focal adhesion kinase (FAK) facilitate lung cancer cell motility and survival. We, therefore, investigated the antimigratory effect of 3,4-dihydroxy-5,4'-dimethoxybibenzyl (DS-1) on human lung cancer cells. Materials and Methods: Cell viability and proliferation were examined by the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide assay. Filopodia formation, migration, and anchorage-independent growth assays were performed to assess metastatic behaviors while EMT-related proteins, integrins, and FAK-RhoA pathway were evaluated by western blot analysis. Results: We found that DS-1 significantly inhibited the proliferation of lung cancer cells compared to the control. The aggressive behavior of cancer cells, including migration and invasion, was significantly reduced by DS-1. Anchorage-independent growth analysis provided evidence that DS-1 suppressed the growth and survival of cancer cells in detached conditions as indicated by the significant reduction in size and number of colonies. With regard to the mechanisms involved, we found that DS-1-suppressed EMT, as indicated by the reduction of EMT markers, namely N-cadherin, SNAIL and SLUG, and increased levels of the epithelial marker, E-cadherin. In addition, DS-1 was shown to reduce the level of integrin β1 protein and FAK activation. Conclusion: DS-1 suppressed lung cancer metastasis via suppressing EMT, integrin β1 expression and FAK-related signaling.*

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Metastasis is the primary cause of cancer-related morbidity and mortality. Lung cancer is often diagnosed at a late stage with metastasis (1). Studies reported that 20-40% of advanced lung cancer patients develop brain metastases, which significantly reduces their quality of life (2, 3). Metastatic cancer cells resist therapy (4) and, due to their increased migration and invasion, actively spread, leading to the formation of secondary tumors (5).

The change of cancer cell phenotype from epithelial into motile mesenchymal cells through the epithelial–mesenchymal transition (EMT) is essential for migration, invasion, and survival in detached conditions during metastasis (6, 7). The major hallmarks of this process are the loss of cell polarity, dissociation of cell–cell junctions, and restructuring of the extracellular matrix (8). Along with EMT progression, expression of motility- and adhesion-regulatory proteins is altered, including the switch from E-cadherin to N-cadherin (9, 10). N-Cadherin facilitates the interaction of cancer and stromal cells, facilitating cancer cell migration and invasion (8, 11, 12). Both E-cadherin and N-cadherin are adherens junction molecules (13). However, E-cadherin expression is exhibited in epithelial tissue and contributes to maintaining cell integrity and homeostasis. Expression of E-cadherin has been correlated with better prognosis and long-term overall survival in patients with lung cancer (11). In contrast, N-cadherin is mostly expressed in mesenchymal cells and is associated with cell motility (14). Thus, cells expressing N-cadherin acquire the capacity to migrate and invade the surrounding stroma and subsequently spread through the blood and lymphatic vessels to distant sites (15). Equally important, cadherin alteration was found to be associated with zinc-finger-family transcriptional activity, including that of SNAIL and SLUG (16). SNAIL-overexpressing cells exhibited increased expression of N-cadherin while their E-cadherin level was reduced,

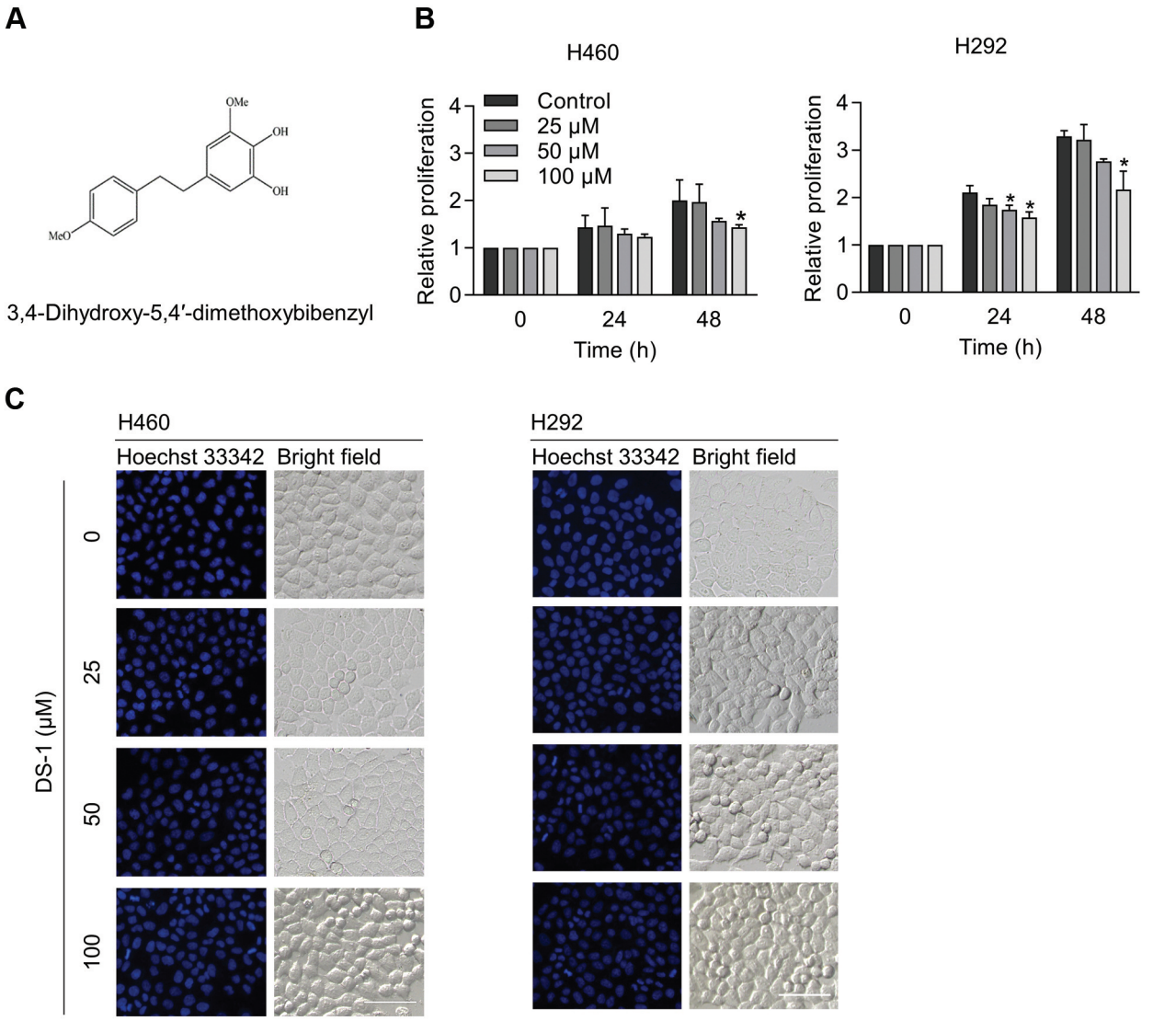


Figure 1. *Continued*

accomplishing the process of EMT (17). Inhibition of N-cadherin, SNAIL, and SLUG has been suggested as a possible way to inhibit cancer metastasis (15, 18).

Anoikis is a type of programmed cell death that occurs upon the detachment of cells, which are normally adherent (19). Resistance to anoikis is required for cancer cells to sustain their survival until establishing a tumor metastasis. Integrins serve as heterodimeric transmembrane receptors for adhesion of cells to the extracellular matrix (20, 21). Upon activation, the interaction between specific extracellular ligands and the outer domain of integrins causes dimerization of their α and β glycoprotein subunits to convey the ligand cues, this extracellular signal leading to intracellular transduction (22). This requires adaptor protein-mediated kinase activation, including those of the focal adhesion kinase (FAK) signaling pathway. It has been confirmed that the integrin-mediated FAK apparatus controls cell adhesion, mobility, phenotype and cell survival, and restricts induction of anoikis (20, 23). The common integrins in epithelial cells, such as αvβ3 and α5β1, have been found to be overexpressed in many cancer types (24). Although the functional activity of these integrins implies beneficial biological activity, integrin β3 has been shown to facilitate cancer cell survival in response to cellular stress within the circulation, and cancer cell adhesion to blood vessel endothelium at a secondary site (20). Moreover, the expression of integrin β1 has been correlated to poor prognosis in non-small-cell lung cancer (NSCLC) (25, 26). Previous studies showed positive

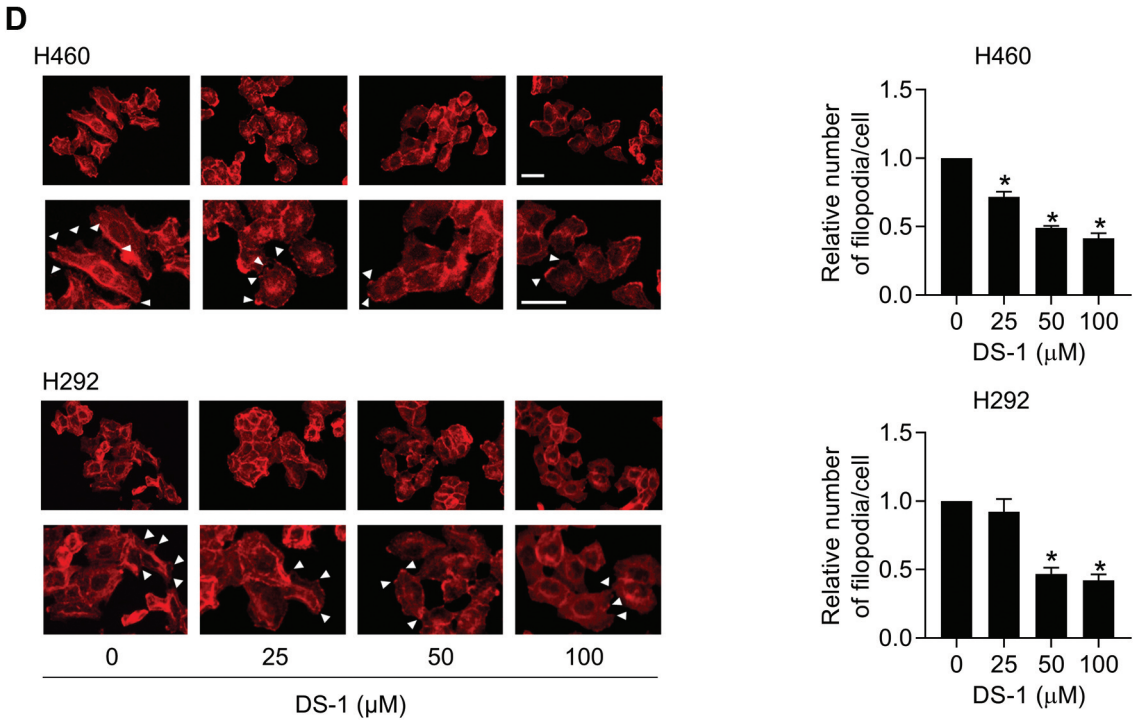


Figure 1. *(A) Chemical structure of DS-1. (B) Cells were treated with DS-1 (0-100 μM). Cell viability was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at 0, 24, 48 h. Proliferation was calculated relative to the control group. Data are presented as the mean±SD (n=3), *p<0.05 vs. control group. (C) Apoptotic cells were stained by Hoechst 33342. Scale bar represents 25 μm. (D) Effect of DS-1 on filopodia formation by H460 and H292 cells. After treatment with nontoxic concentrations of DS-1, cells were stained with phalloidin-rhodamine and examined using fluorescence microscopy. Features representing filopodia are indicated by arrowheads. Scale bar represents 100 μm. The number of filopodia per cell relative to the control are shown as the mean±SD (n=3) *p<0.05 vs. non-treated control.*

correlation between certain types of integrins and metastasis, suggesting that specific integrins may represent potential targets for anti-metastasis therapy (27, 28).

Since EMT is an essential step in cancer metastasis, the role of preventing the alteration of expression of epithelial factors to mesenchymal factors has attracted much interest (29). The present study aimed to investigate the potential effect of 3,4-dihydroxy-5,4'-dimethoxybibenzyl (DS-1) (Figure 1A), a compound isolated from *Dendrobium signatum* (30), on EMT of H460 NSCLC cells

## Materials and Methods

*Cell culture and reagents*. H460 and H292 human NSCLC cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute (RPMI) medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml streptomycin and penicillin, and maintained at 37˚C in a humidified incubator with 5% $CO_2$. RPMI 1640 cell growth medium, FBS, phosphate-buffered saline (PBS), and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), phalloidin-rhodamine, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Primary antibodies against E-cadherin, N-cadherin, SNAIL, SLUG, integrin-β1, integrin-β3, integrin-α5, RhoA, FAK, p-FAK (Try397) and glyceraldehyde 3-phosphate dehydrogenase, and horseradish peroxidase-linked secondary antibody against rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA, USA). DS-1 (30) with 95% purity was obtained from the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

*Preparation of DS-1 stock solution*. DS-1 was dissolved in dimethylsulfoxide (DMSO) as a stock solution. Different concentrations of DS-1 for each experimental group were prepared and diluted in cell culture media. The final concentration of DMSO (<0.2%) in experiments had no cytotoxic effect on H460 and H292 cells.

*Cell proliferation assay*. For testing the antiproliferative effect of DS-1, H460 lung cancer cells cultured in a 96-well plate ($3\times10^3$ cell/well) were treated with non-toxic doses of DS-1 (0-100 μM) for 24, and 48 h. At each time point during incubation, cell viability was measured by MTT assay. The cells were incubated with MTT reagent (0.4/ml in PBS) in the dark at 37˚C for 4 h. Formazan crystals were then dissolved by DMSO (100 μl/well) and the

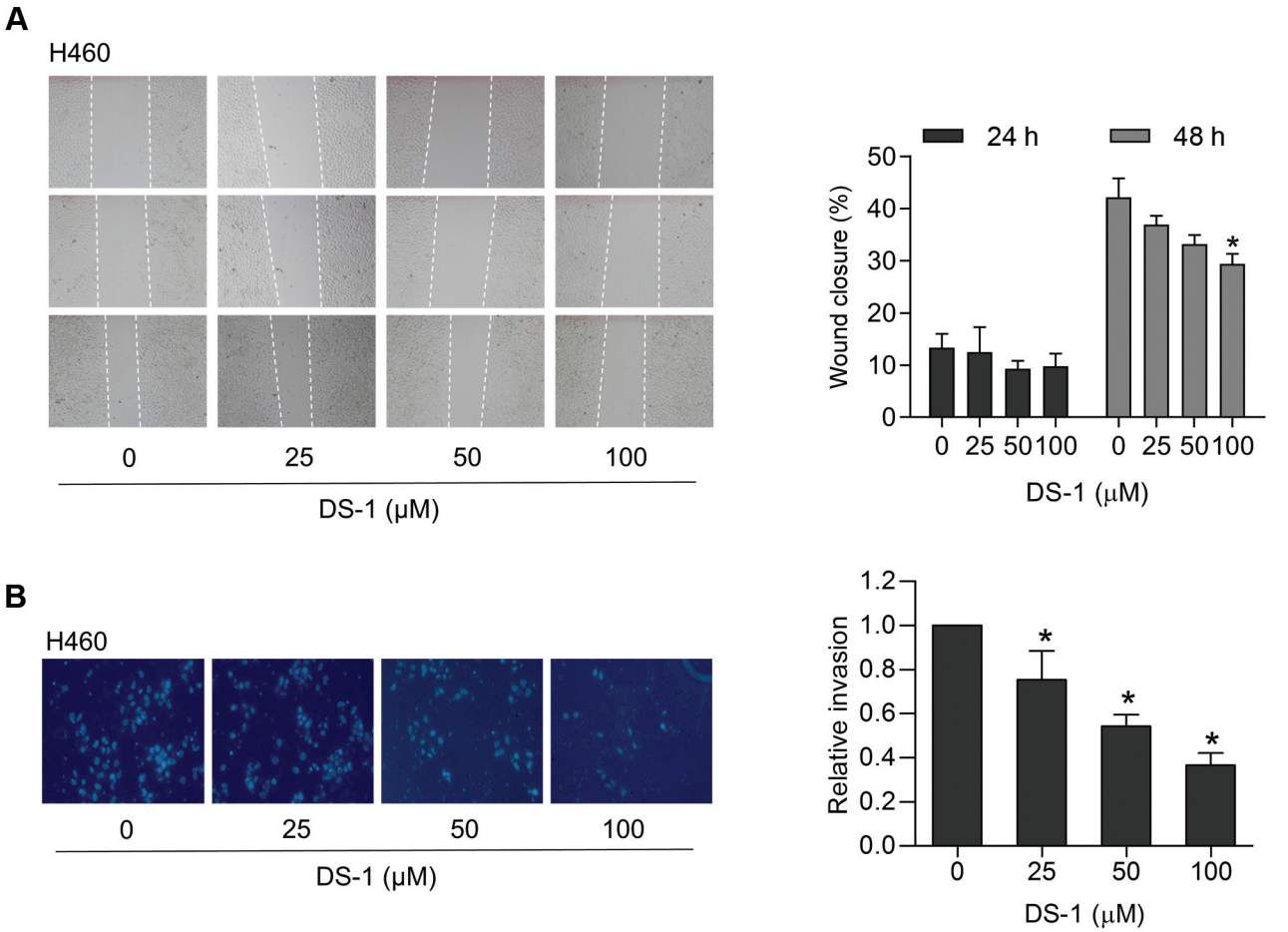


Figure 2. *Continued*

intensity was measured at 570 nm using a microplate reader (Anthros, Durham, NC, USA).

*Nuclear staining assay.* Apoptotic cells were visualized by staining cell nuclei with Hoechst 33342 (Sigma Chemical). Cells in 96-well plates were incubated with DS-1 for 24 h. After that, cells were washed with PBS and Hoechst 33342 solution was added for 30 min. The cells were then analyzed and captured under fluorescence microscopy (Nikon ECLIPSE Ts2; Nikon, Tokyo, Japan). Blue-fluorescent condensed or fragmented nuclei represent nuclei of apoptotic cells.

*Filopodia formation assay.* Cells at a density of $5\times10^3$ cells/well in a 96-well plate were treated with DS-1 (0-100 μM) for 24 h. The cells were then fixed with 4% paraformaldehyde for 10 min. After permeabilization with 0.1% Triton-X in PBS for 5 min, the cells were rinsed with PBS and blocked for nonspecific binding with 0.2% BSA in PBS for 30 min. The cells were then incubated with a 1:100 dilution of phalloidin-rhodamine in PBS for 30 min, rinsed in PBS three times and mounted in 50% glycerol in PBS. The morphology of untreated and treated cells, focusing particularly on the development of filopodia, was captured using a fluorescence microscope (Nikon Eclipse Ts2; 40× magnification).

*Wound-healing cell-migration assay.* To determine the antimigratory effect of DS-1, a wound-healing assay was performed. H460 and H292 cells were pretreated with DS-1 (0-100 μM) for 24 h. The cells were then resuspended and seeded ($1.5\times10^5$ cells/well) onto a 24-well plate. Monolayer cells were scratched by a 20-μl pipette tip creating a wound area, then rinsed with PBS to discard the cell debris. Cell movement was analyzed and photographed under microscopy (Nikon Eclipse Ts2; 10× magnification) at 0, 24, and 48 h. The wound area was measured using ImageJ software. Migration was calculated and is presented as a relative value.

*Invasion assay.* Cell invasion was evaluated using a transwell chamber (pore size: 8 μm). In the upper chamber, 0.5% Matrigel was added (100 μl/well) and the chamber was incubated at 37˚C overnight. Cells were treated with DS-1 (0-100 μM). After 24 h, the cells were then dispersed and placed onto the set Matrigel ($2\times10^4$). The lower chamber was filled with culture medium with 10% FBS. After 48 h of incubation, non-invading cells on the upper side of the chamber were cleaned by cotton swab while the invading cells were stained with Hoechst 33342 for 10 min, then captured under fluorescence microscopy (Nikon Eclipse Ts2; 20× magnification).

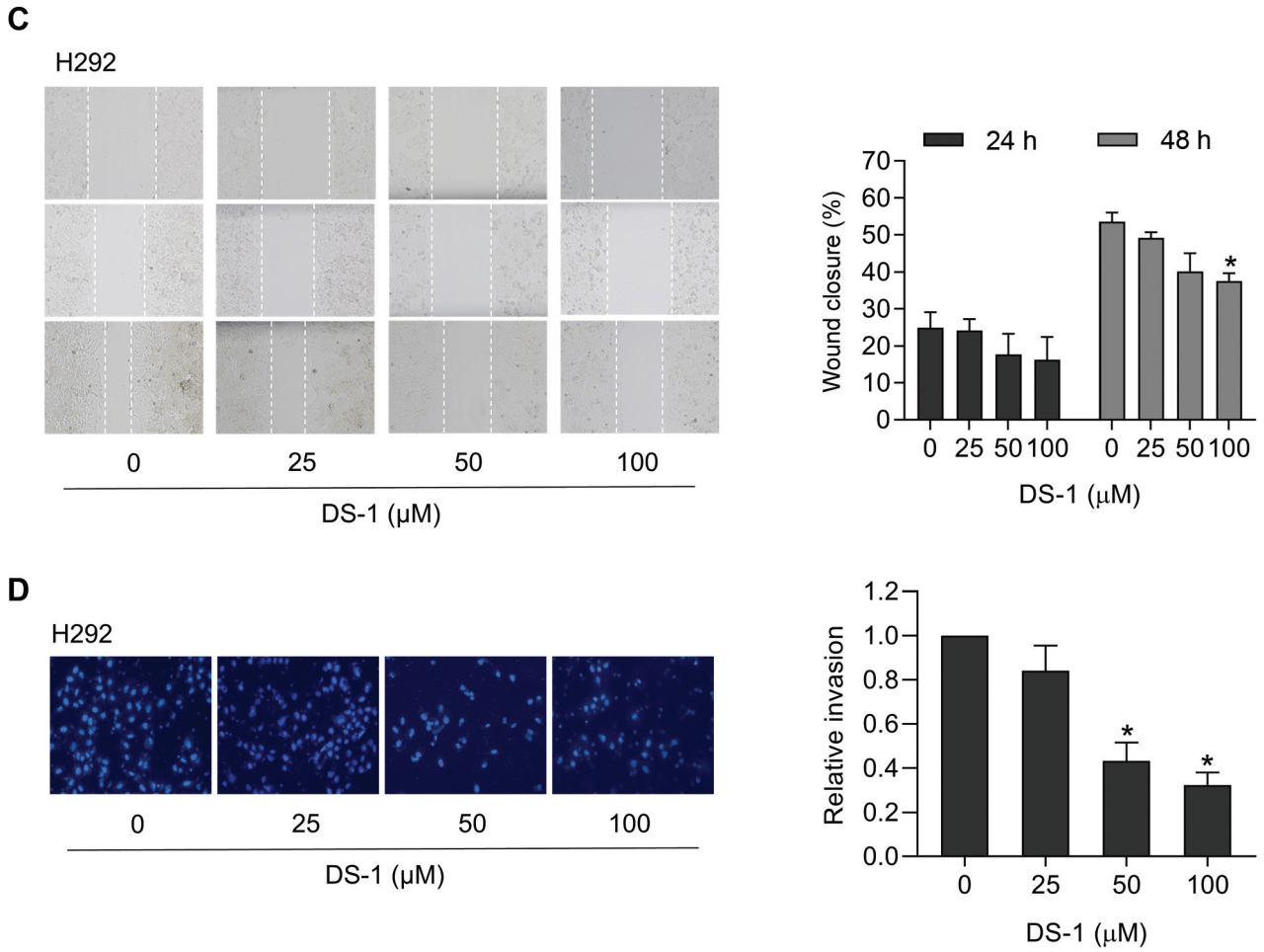


Figure 2. *DS-1 inhibits migration and invasion of H460 and H292 cells. (A and C) Cells were pretreated with DS-1 (0-100 μM) for 24 h. Cell migration was evaluated by wound-healing assay. The percentage of wound closure (migration) at 24 and 48 h was determined by comparison with the wound area at 0 h. (B and D) Cell invasion was investigated by transwell migration assay (pore size: 8 μm). The DS-1-treated cells were inserted into the upper chamber containing Matrigel and incubated for 48 h. The invading cells were stained by Hoechst 33342 and visualized by fluorescence microscopy (20× magnification). Relative invasion was calculated as the total invading cells of the treated group divided by that of the control group. Data are presented as the mean±SD (n=3) *p<0.05 vs. control group.*

*Anchorage-independent cell-growth assay.* Anchorage-independent cell growth was determined in an assay using two-layer soft-agar. Briefly, the first layer was made by preparing 1% agarose and RPMI culture medium at a 1:1 ratio. The mixture was added to a 24-well plate (500 μl/well) and kept at 4˚C. The second layer contained a mixture of treated cells in suspension in culture medium and 0.33% agarose gel. After the first 3 h incubation, and then every 3 days, culture medium with 10% FBS was added. Fourteen days later, colonies were photographed under microscopy (Nikon Eclipse Ts2; 10× and 20× magnification) and the colony number and size were measured using ImageJ software.

*Western blot analysis.* Cells were placed in 6-well plates ($1.5\times10^5$ cell/ml) overnight. After 24 h of DS-1 treatment (0-100 μM), cells were lysed with 1× RIPA buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 1 mM sodium orthovanadate, 50 mM 15 sodium fluoride, 100 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche Molecular Biochemical, Mannheim, Germany), and incubated at 4˚C for 30 min. Centrifugation was performed and the protein content in the supernatant was analyzed by BSA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Using the same amount for each sample, proteins were denatured at 95˚C for 5 min, then separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a membrane. Five percent skimmed milk in TBST was applied to the membrane for 1 h. Then the membrane was incubated with primary antibodies at 4˚C overnight. After washing three times with TBST for 5 min, the membrane was incubated with secondary antibody for up to 2 h. Chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize the protein expression.

*Statistical analysis*. The data are presented as the mean±standard deviation of three independent analyses. The differences between groups were analyzed by one-way analysis of variance followed by Tukey *post hoc* test using Prisma 8 (GraphPad Software, San Diego, CA, USA). A value of $p<0.05$ was considered as indicating statistical significance.

## Results

*Effect of DS-1 on proliferation of H460 and H292 cells*. Previous studies have demonstrated the cytotoxic effect of DS-1 on several types of cancer cell and revealed doses up to 100 μM as being non-toxic to H460 cells (30, 31). Accordingly, non-toxic doses of DS-1 were utilized in this study to ensure that findings were not due to cytotoxic effects.

Multiple cascades control the proliferation and metastasis of cancer cells. However, the unregulated proliferation of cancer cells can facilitate metastasis progression (32). Therefore, we first looked at proliferation of H460 and H292 cells in the presence of DS-1. Briefly, cells were incubated in 96-well plates with different doses of DS-1 (0-100 μM). The number of cells at different time points was measured by MTT assay. The results showed that relative cell proliferation at 24 h in H292 lung cancer cells and in both cell lines at 48 h was suppressed (Figure 1B). Moreover, apoptosis induction was also evaluated. Cells were treated with DS-1 (0-100 μM) for 24 h, then stained with Hoechst 33342. As shown in Figure 1C, DS-1 treatment did not induce apoptosis of H460 and H292 cells.

*DS-1 inhibited cell migration and invasion of H460 and H292 cells*. To examine the effect of DS-1 on cell behavior, we further assessed EMT-related morphological changes. Normally, epithelial cells are characterized as round, elongated, or spherical shape. Upon the development of a phenotype enabling migration and invasion, cells tend to exhibit morphological transformation from epithelial morphology to a spindle-shape mesenchymal morphology, which is characterized by the formation of filopodia or plasma membrane protrusions (33). In this study cells were treated with DS-1 (0-100 μM) for 24 h and filopodia formation was evaluated by phalloidin-rhodamine staining. As shown in Figure 1D, treatment with 100 μM DS-1 reduced the formation of filopodia by H460 and H292 cells, suggesting that DS-1 might prevent the development of mesenchymal-like morphology.

To determine the effect of DS-1 on regulating migration and invasion by H460 and H292 cells, wound-healing and transwell invasion assays were performed. Cells were left untreated or treated with DS-1 (25-100 μM) for 24 h then subjected to migration and invasion assays. The results showed that after 48 h, DS-1 prevented migration of H460 and H292 cells in a dose-dependent manner (Figure 2A and C). Similarly, transwell invasion assay results showed a reduced number of H460 and H292 cells invading through Matrigel at 25-100 μM of DS-1 compared to the control (Figure 2B and D). These findings show that DS-1 inhibited NSCLC cell migration and invasion.

*DS-1 inhibited tumor cell growth in an anchorage-independent model*. As tumor cells become resistant to anoikis, they are able to get into the systemic circulation to reach distant tissues and form metastatic colonies (34). The interaction of tumor cells with platelets mediates the survival cascade and protects tumor cells from shear stress whilst in the circulation (35). Thus, we further investigated the effect of DS-1 on H460 cell growth in an anchorage-independent model. Cells were pre-treated with DS-1 (0-100 μM) for 24 h, then subjected to soft-agar anchorage-independent growth assay as described in the Materials and Methods. Our results showed that DS-1 significantly reduced the number and the size of H460 and H292 colonies (Figure 3).

*DS-1 down-regulated EMT-associated proteins and integrin β1 in H460 cells*. Following our finding that DS-1 exhibited an inhibitory effect on NSCLC cell migration and invasion, we suspected that DS-1 might regulate EMT-associated proteins. Western blot assay was performed to detect EMT-related protein expression. As shown in Figure 4A, DS-1 down-regulated the expression of mesenchymal markers N-cadherin, SNAIL and SLUG but increased that of the epithelial marker E-cadherin. These findings indicate that DS-1 may inhibit the initiation of cell metastasis by regulating the cadherin switch. Integrin expression has also been associated with the EMT process (37, 38). Therefore we investigated the expression of integrin α5, β1, and β3. Integrin β1 expression was significantly reduced after 24 h of treatment with 100 μM DS-1. However, only a minimal effect was shown on integrin α5 and β3 expression in response to DS-1 exposure (Figure 4B). Furthermore, total FAK protein and p-FAK (Try397) were also determined. We found that DS-1 reduced the active form of p-FAK compared to the control, although not significantly. As FAK/p-FAK is one of the downstream pathways of integrin signaling that contributes to cancer metastasis, these results indicate the mechanism of action of DS-1 might be through diminishing integrin β1-mediated FAK activation facilitating cell migration.

## Discussion

The EMT process is important in the movement of normal cells during tissue development and wound healing. In cancer, it was shown that EMT is critical for the initial step of cancer metastasis and for chemoresistance (39, 40). A study demonstrated EMT as a hallmark of cancer in the initiation of cancer cell migration and invasion (7). EMT is

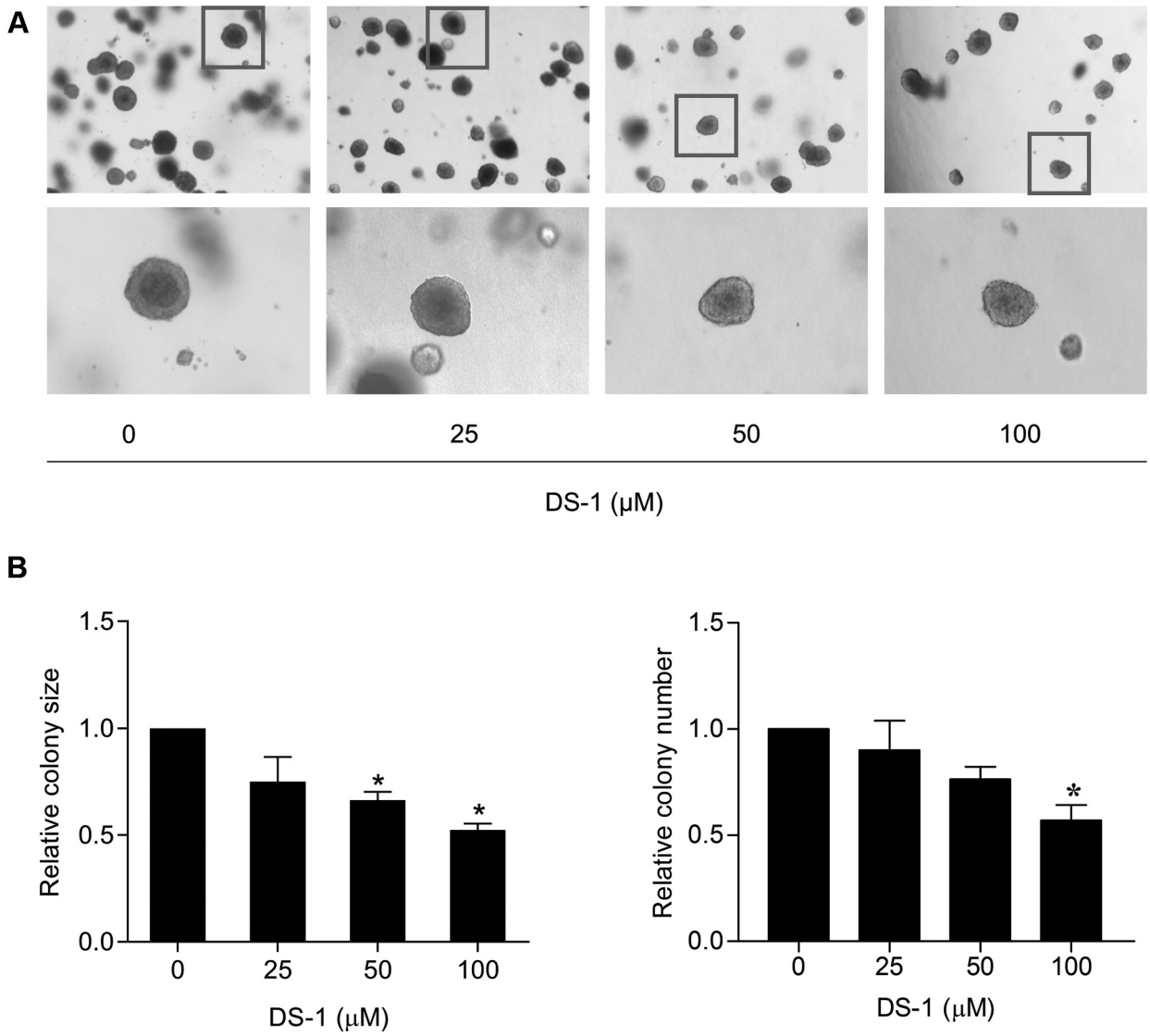


Figure 3. *DS-1 inhibits the cell growth of H460 cells in an anchorage-independent condition. (A) Cells were pretreated with DS-1 (0-100 μM) for 24 h and subjected to anchorage-independent assay. After 14 days of incubation, cells were captured under microscopy at 10× (upper) 20× (lower) magnification (B). The data are presented as mean±SD (n=3) *p<0.05 vs. control group.*

related to treatment failure in lung cancer (41), and suppressing the molecular mechanisms of EMT has been suggested as an anti-metastasis strategy (36, 42). Certain integrins are emerging as pivotal factors in preventing cancer cell apoptosis during metastasis. Integrins respond to extracellular signal transduction and generate signaling pathway activation of non-receptor tyrosine kinase, FAK (20). Activation of the FAK signaling pathway supporting cell proliferation and survival has also been reported to regulate transcription factors involved in EMT (43, 44). Therefore, inhibition of EMT-regulating properties is a possible strategy for preventing cancer metastasis. Herein, we demonstrated the anti-metastasis potential of DS-1, a compound isolated from *D. signatum*, on EMT involving the FAK pathway.

Studies investigating the active compounds of *Dendrobium* species have revealed their anti-metastasis effect on NSCLC cells (45, 46). In the present study, an expanded investigation of another compound derived from *D. signatum*, DS-1, aimed to determine its anti-metastasis potency in NSCLC cells. For cancer metastasis, it has been revealed that most cancer cells are able to generate survival signals once in contact with the basement membrane (34). With the loss of cell–cell adhesion leading to cell

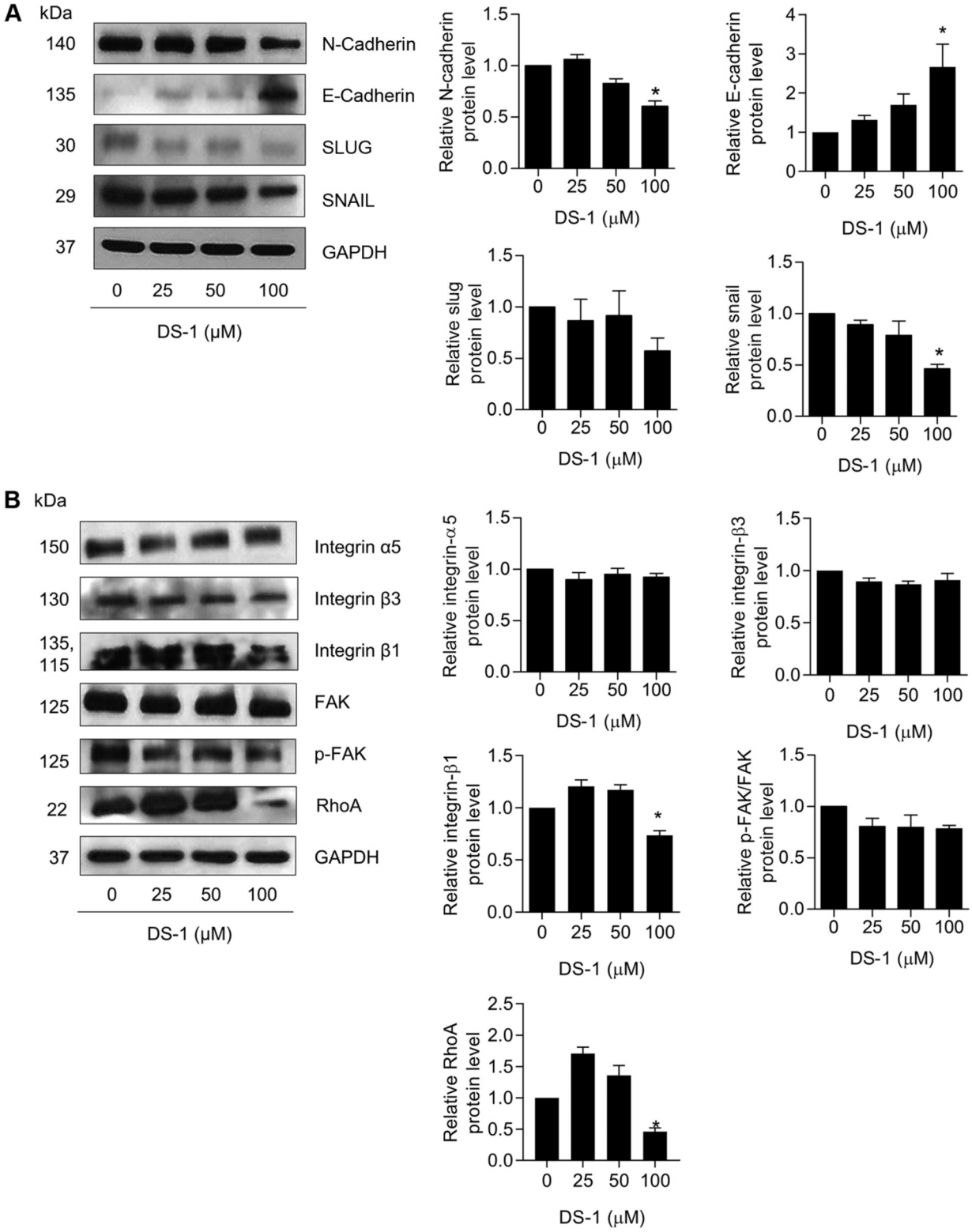


Figure 4. *Effect of DS-1 on epithelial–mesenchymal transition (EMT)-regulatory proteins in H460 cells. (A-B) Cells were treated with 25, 50, and 100 μM of DS-1. Protein expression was detected by western blot and quantified with ImageJ software. The relative protein level was calculated and compared to that of untreated cells. The data are presented as the mean±SD (n=3) *p<0.05 vs. control group. FAK: Focal adhesion kinase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.*

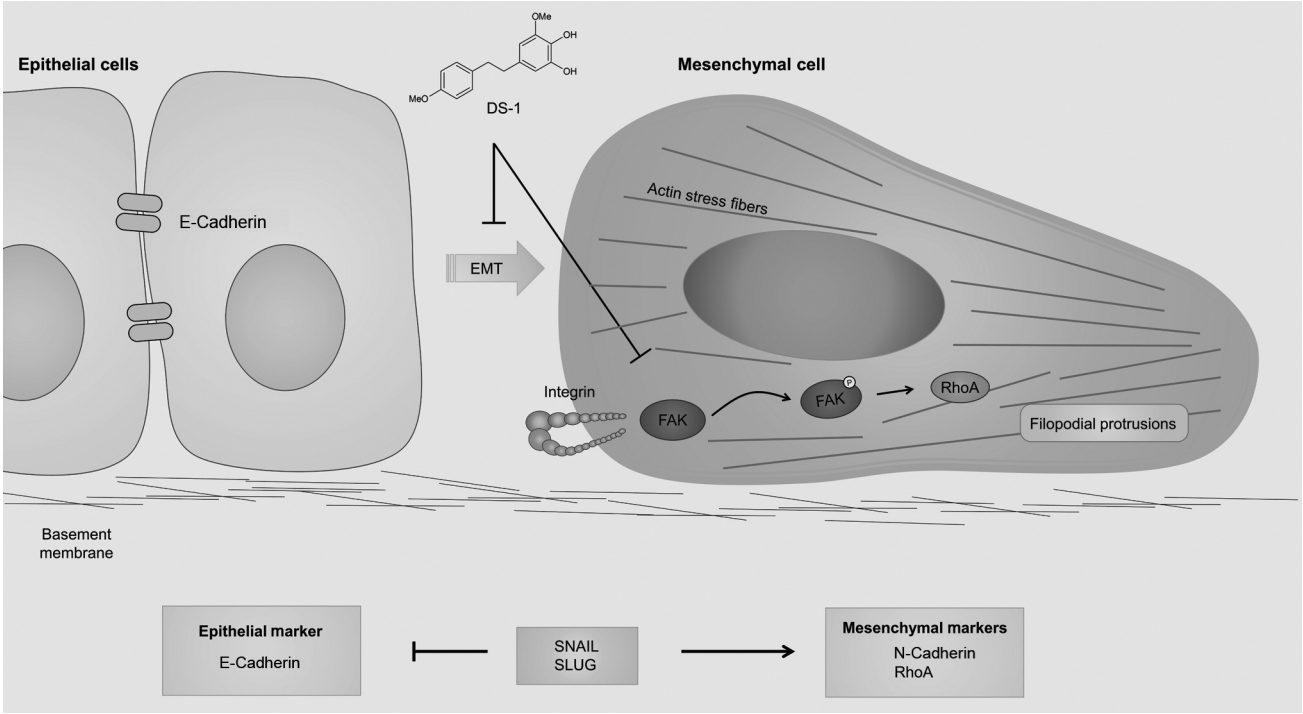


Figure 5. *Schematic mechanism of DS-1 in preventing the epithelial–mesenchymal transition (EMT) process as an initiation step of lung cancer cell migration and invasion. DS-1 inhibits SLUG and SNAIL together with alleviation of N-cadherin and enhancement of E-cadherin. DS-1 attenuation of integrin β1 expression leads to down-regulation of focal adhesion kinase (FAK) activation.*

dissemination, cancer cells need to develop anoikis resistance to survive in circulation in order to reach a secondary site (47). Interestingly, we found that DS-1 inhibited cell growth in the anchorage-independent condition (Figure 3). EMT, typically characterized by the reduction of epithelial markers such as E-cadherin, and enhancement of mesenchymal markers including N-cadherin, is controlled by activation of transcriptional factors including SNAIL and SLUG (8). Studies have demonstrated the correlation between SNAIL and N-cadherin, where the induction of SLUG/SNAIL in lung cancer cells caused an increase of N-cadherin as well as cancer cell migration and invasion (14). Moreover, SNAIL was shown to inhibit the expression of E-cadherin by blocking the binding of its promoter (48), which plays an important role in chemoresistance and radioresistance (49). Small molecules that inhibit these proteins may prevent development of metastasis (50).

DS-1 prevented migration and invasion by lung cancer cells (Figure 2) together with acquisition of mesenchymal properties through down-regulation of N-cadherin, SNAIL and SLUG, and up-regulation of E-cadherin (Figure 4). The increased expression of EMT-related proteins are mediated by the FAK signaling pathway. In the present study, the protein expression of integrin β1 and p-FAK was also reduced in response to DS-1 treatment. This demonstrated that DS-1 can inhibit migration and invasion through suppression of integrin β1-mediated FAK pathway and consequently reduce the expression of SNAIL and SLUG, thereby regulating the cadherin switch toward cell migration.

In conclusion, these results reveal the potential effect of DS-1 on suppressing integrin and activating the downstream pathway of FAK, showing it to have a promising role in blocking EMT of cancer cells towards a metastatic phenotype (Figure 5). Our study provides the basis for further investigation of DS-1 in anticancer therapy.

## Conflicts of Interest

The Authors declare that there are no conflicts of interest in this study.

## Authors' Contributions

HEP conducted experiments, performed data analysis, wrote the manuscript; BS contributed compound; PC participated in research design, performed data analysis, wrote the manuscript.

## Acknowledgements

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