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Pongamol Inhibits Epithelial to Mesenchymal Transition Through Suppression of FAK/Akt-mTOR Signaling

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Abstract. *Background/Aim: Cancer metastasis is the main cause of mortality in cancer patients. As lung cancer patients are mostly detected at metastatic stages, strategies that inhibit cancer metastasis may offer effective therapies. Activation of FAK and Akt/mTOR pathways promotes the highly metastatic phenotypes of epithelial to mesenchymal transition (EMT). We unraveled EMT inhibitory action of pongamol and the mechanism controlling cell dissemination in lung cancer cells. Materials and Methods: Cytotoxic and antiproliferative effects of pongamol were determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptosis and necrosis induction in response to pongamol treatment was observed and visualized by nuclei staining assay. Wound healing migration, invasion, and anchorage-dependent growth assay were conducted to evaluate metastatic behaviors. EMT protein expression and FAK pathway were detected by western blot analysis. Results: Pongamol at 0-100 μM exhibited significant inhibition on migration, and invasion of cancer cells. Regarding anoikis resistance potential, the compound significantly inhibited survival and growth of cancer cells in an anchorage-independent manner, as indicated by the depletion of growing colonies in pongamol-pretreated cells. Protein level analysis further showed that pongamol exerted its anti-metastasis effect by inhibiting EMT, as indicated by a decrease of several mesenchymal proteins (N-cadherin, vimentin, Snail, and Slug). Regarding the up-stream mechanisms, we found that pongamol inhibited activation of FAK and Akt/mTOR signaling pathways. Conclusion: Pongamol exhibits potent anti-metastatic activity through suppressing key potentiating factors of cancer metastasis EMT and FAK.*

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Lung cancer is an important cancer causing high incidence of cancer-related deaths, worldwide (1). The majority of lung cancer patients have been found with metastatic disease at the time of first diagnosis, and the success rate of treatment of metastatic lung cancer is very low (2, 3). The patients diagnosed with stage IV NSCLC presenting liver metastasis have the worst prognosis with the survival time of less than 3 months (4).

Cancer metastasis is the process of cells spreading to a different part of the body, which is responsible for 90% of deaths (5, 6). To prevent metastasis formation, the dissemination of cancer cells from a primary tumor could be inhibited at the fundamental metastatic processes including invading cells, escaping anoikis induction, modulating the tissue microenvironment, and establishing of secondary tumor (7). A number of studies have shown that the alteration of cells from epithelial phenotype to mesenchymal phenotype termed "epithelial to mesenchymal transition" (EMT), initiates the metastasis process and drives the progression (8, 9). During the transition, epithelial cells lose cell adhesion by the induction of mesenchymal regulators such as Snail family transcription factors facilitating the decrease of E-cadherin (10) and the increase of mesenchymal protein N-cadherin and vimentin (11, 12). Consequently, N-cadherin cooperates with fibroblast growth factor receptor to regulate the cell-surface receptor level and encourage Akt signaling, thus increasing cell migration and invasion (13), and elevated vimentin, the type III intermediate filament, stabilizes the cytoskeleton interactions, maintains cellular integrity, and promotes cell motility (14). Moreover, cancer cells must resist to anoikis. It has been reported that the

anoikis-resistant lung cancer cells had a high level of N-cadherin and vimentin (15).

Likewise, focal adhesion kinase (FAK) is a key regulator of metastasis by controlling cancer cell motility and survival (16, 17). FAK activation propagates a diverse array of signals, such as the Akt/mTOR cascade. mTOR can directly phosphorylate S6 ribosomal protein leading to activate the transcription factor used in metastasis process (18) and cell proliferation (19). A previous study demonstrated the use of FAK inhibitor in alleviation of tumor growth and metastasis (20). Accordingly, in the present study, we aimed at evaluating the novel anti-metastatic effect of pongamol, an isolated compound from the roof of *Millettia erythrocalyx* (21), and its potential inhibitory activity on EMT and FAK-related pathways in lung cancer cells.

Materials and Methods

Cell line and reagents. The H460 cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were maintained in Roswell Park Memorial Institute (RPMI) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium completed with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml streptomycin, and penicillin at 37˚C in a humidified atmosphere with 5% (v/v) CO_2 and 95% (v/v) air (10). The primary antibodies against E-cadherin, N-cadherin, vimentin, Slug, Snail, GAPDH, mTOR, p-mTOR, FAK, p-FAK, Akt, p-Akt, and the secondary antibody anti-rabbit IgG were acquired from Cell Signaling Technology (Danvers, MA, USA). Pongamol (Figure 1A) was received from the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. Pongamol was prepared in dimethylsulfoxide (DMSO) and diluted in culture media.

Cell viability assay. H460 cells were seeded at 1×10^4 cell/well in 96-well plate (Corning, Acton, MA, USA) overnight. The cells were incubated with fresh culture media containing pongamol for 24 h. The cell viability was assessed by MTT assay as following steps: MTT solution (0.4 mg/ml in PBS) was applied for 4 h in a dark room. DMSO 100 μl/well was applied to dissolve the purple formazan product. The intensity of the formazan product was measured by a microplate reader at 570 nm. The percentage of viable cells was calculated relative to the control group. The anti-proliferation effect of Pongamol was also confirmed. H460 cells in 96-well plate (3×10^3 cells/well) were treated with Pongamol (25-100 μM). To determine the relative cell proliferation, living cells were measured by MTT assay at 0, 24, and 48 h.

Nuclear staining assay. To determine the apoptosis induction in response to pongamol treatment, nuclear staining assay was conducted. Here, H460 cells were seeded on 96-well plates at the density of 1×10^4 cells/well and incubated overnight. The cells were then treated with various concentrations of pongamol (0-100 μM) for 24 h. After that, Hoechst 33342 and propidium iodide (PI) solution were applied for 30 min incubation. Then, cells were visualized under a fluorescence microscope (Nikon ECLIPSE Ts2, Nikon, Tokyo, Japan).

Migration and invasion assays. Cells were pretreated with pongamol (0-100 μM) for 24 h. The cells were seeded onto 24-well plate. The wound area was made by using a 200 μl pipette tip and the floating cells were rinsed with PBS. Images were captured under a phase-contrast microscope (Nikon ECLIPSE Ts2) at 0, 24, 48 h. The wound area was measured by the ImageJ software and calculated relative to the pre-migration wound area. For invasion, pongamol-treated cells (2×10^4 in 100 μl serum-free RPMI) were placed over the 0.5% matrigel in the upper transwell while the culture media with 10% FBS was inserted in the lower chamber. After 48 h of incubation, non-invaded cells on the upper side of the chamber were cleaned by a cotton swab, and the invaded cells underside of the polycarbonate membrane were stained with Hoechst 33342 and captured under fluorescent microscopy (20× magnification). The number of invaded cells in the treatment groups was calculated relative to the control group.

Anchorage-independent growth. Cells were pretreated with pongamol (0-100 μM) for 24 h. The cells were resuspended at a density of 3×10^3 and mixed with 0.3% agarose in 10% FBS-RPM culture media. A total of 200 μl of the mixture were added over a 0.6% agarose layer in a 24-well plate and incubated at 37˚C. The culture medium was added over the top layer every three days. After two weeks, colonies were photographed using a phase-contrast microscope.

Western blot assay. Cells were lysed with 1× RIPA buffer and incubated for 30 min at 4˚C. The cell lysate was centrifuged. the supernatant was collected for measurement of protein content using a BSA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked in 5% skim milk in TBST (25 mmol/l Tris-HCl, pH 7.4, 125 mmol/l NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated with the primary antibody at 4˚C overnight. After that, the membrane was washed with TBST three times and incubated with a secondary antibody for 2 h at room temperature. The protein was detected using Chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis. Values were presented as the mean±standard deviation (SD). Statistical comparisons between groups were conducted using one-way ANOVA and $p<0.05$ was considered to be statistically significant. All statistical analyses were conducted using Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Cytotoxic and anti-proliferative effect of pongamol. We first determined the cytotoxic effect of pongamol. H460 cells on 96-well plates were incubated with pongamol (0-100 μM) for 24 h. An MTT assay was performed to measure viable cells. We observed that pongamol reduced cell viability in a dose-dependent manner (Figure 1B). We also evaluated whether pongamol inhibits the proliferation of H460 cancer cells. The cells were cultured with pongamol (25-100 μM) for up to 2 doubling times. The result showed that pongamol significantly suppressed cell proliferation at 48 h compared to the control group (Figure 1B). Moreover, apoptosis induction after pongamol treatment was also

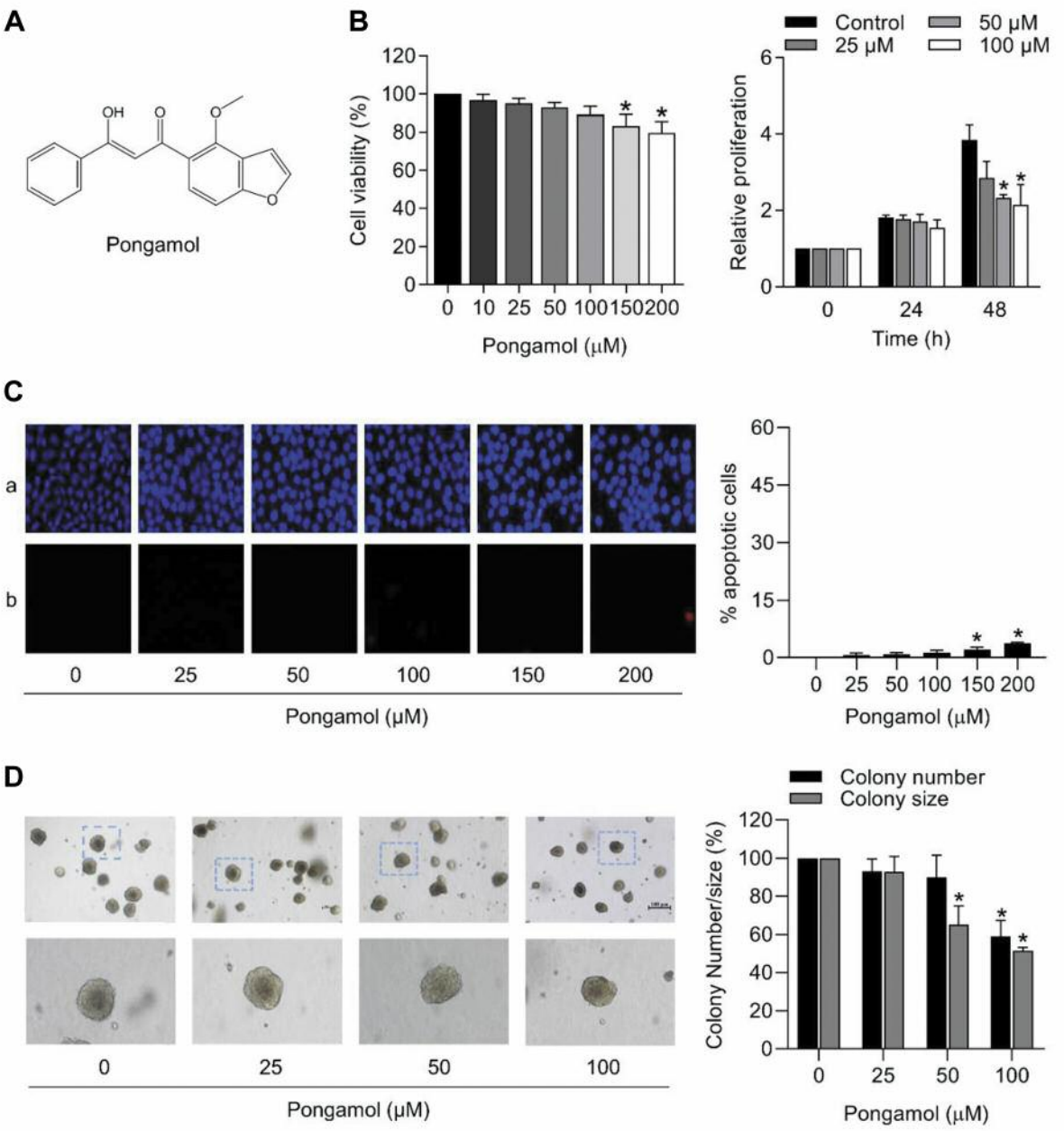

Figure 1. *(A) Chemical structure of Pongamol. (B) Cells were treated with pongamol (0-200 μM) and subjected to MTT assay. The percentage of cell viability was calculated relative to control. (C) The apoptotic and necrotic nuclei were stained by (a) Hoechst 33342 and (b) propidium iodide. Scale bar represents 25 μm. The percentage of apoptotic and necrotic nuclei was calculated relative to control. The data are presented as mean±SD (n=3). *p<0.05 vs. control group. (D) Cells were pretreated with pongamol (0-100 μM) for 24 h and subjected to an anchorage-independent assay. After 14 days of incubation, colonies were captured under phase bright 10× (upper) 20× (lower) magnification. The results were represented in the relative number and size of the colonies. Data are presented as mean±SD (n=3). *p<0.05 vs. control group.*

examined by nuclear staining assay. The cells were similarly treated for 24 h and the nuclei were stained with Hoechst 33342 and propidium iodide. Neither necrotic nor apoptotic cells were found in response to 25-100 μM of pongamol (Figure 1C).

Pongamol inhibits migration, invasion, and anchorage-dependent growth of H460 cells. The metastatic cancer cells are recognized to acquire anoikis resistance during circulation to reach the distance (22). To determine the inhibitory effect of pongamol on the ability of cancer cells

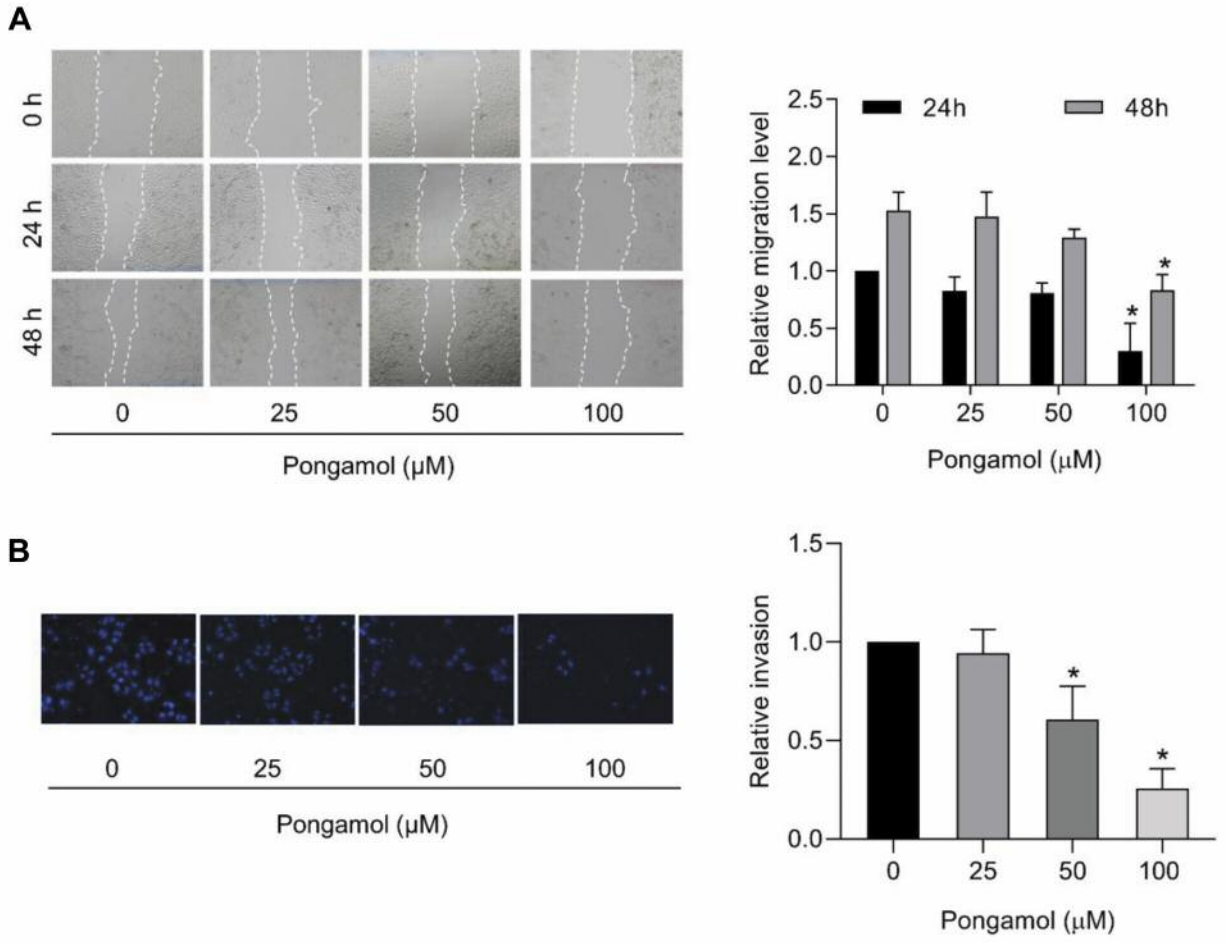

Figure 2. *Effect of pongamol on migration and invasion. (A) Cells were pretreated with pongamol (0-100 μM) for 24 h. Cell migration was evaluated by wound healing migration assay. The percentage of wound closure migration levels at 24 and 48 h was calculated relative to the wound area at 0 h. (B) Cell invasion was investigated using transwell chamber assay. After treatment, the cells were inserted onto the upper chamber containing the set of matrigel and incubated for 48 h. The invading cells were stained with Hoechst 33342 and visualized by fluorescence microscopy (20× magnification). The relative invasion was calculated as total invading cells of the treatment group divided by total invaded cells of control group. The data is presented as mean±SD (n=3). *p<0.05 vs. control group.*

to survive in absence of anchorage to the extracellular matrix (ECM) and their neighboring cells, termed anchorage independence of growth, we performed anchorage-independent growth by soft agar colony formation assay. Cells were pretreated with the compound for 24 h, then the treated cells were seeded in agarose, as described. After 14 days of incubation, colony formation of all groups was determined for the number and size. As shown in Figure 1D, pretreatment of the cells with pongamol significantly decreased the number and size of cancer colonies.

Cell migration and invasion were evaluated as described in the Materials and Methods section. The migration of cells was evaluated at 0, 24, and 48 h. The results showed that the concentration of 100 μM pongamol could inhibit cell migration at 48 h compared with non-treated control (Figure 2A). For cell invasion analysis, cells were pretreated with pongamol and seeded on the Matrigel layer, and allowed to invade through the transwell chamber (8 μm pore size). The results showed that pongamol at the concentrations of 50 and 100 μM significantly inhibited cell invasion through the matrigel layer (Figure 2B). These results indicated that pongamol potentially inhibits the cell metastasis behaviors of lung cancer cells.

Pongamol inhibits epithelial to mesenchymal transition. Having shown that pongamol has an inhibitory effect on metastatic behavior of H460 cells, we further questioned whether pongamol may affect the EMT process of lung cancer cells. In the present study, we monitored the EMT status of cells by evaluating the EMT markers including E-cadherin, N-cadherin, and vimentin. H460 cells were treated with pongamol

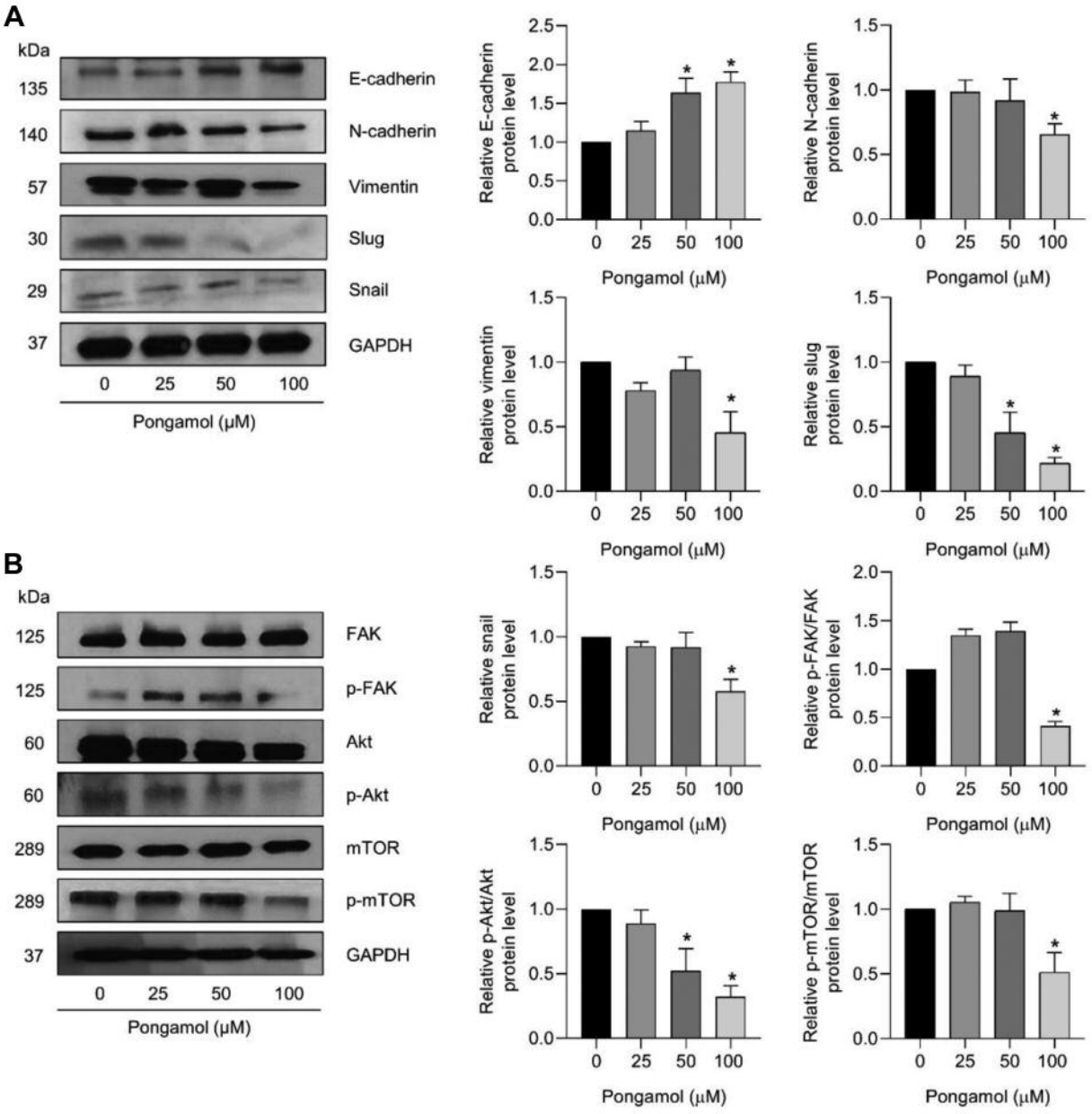

Figure 3. *Effect of pongamol on EMT regulatory proteins. (A-B) Cells were treated with 25, 50, and 100 μM of pongamol. The protein expression was detected by Western blotting. The membrane blots were reprobed with GAPDH to confirm equal loading of the sample. Band density was quantified by densitometry. Protein levels were calculated relative to the untreated group. The data are presented as mean±SD (n=3). *p<0.05 vs. control group.*

at concentrations of 0-100 μM for 24 h. Western blot was carried out to investigate the expression of EMT protein markers. The results showed that pongamol caused an increase in the expression of the epithelial marker, E-cadherin protein, and decrease of mesenchymal proteins, including N-cadherin and vimentin in a dose-dependent manner (Figure 3A). For further confirmation, we analyzed the functional proteins regulating EMT including mesenchymal transcription factors Snail and Slug in the pongamol-treated cells. Protein level analysis revealed that treatment of cells with non-toxic concentrations of pongamol significantly decreased the cellular level of mesenchymal transcription factors Snail and Slug.

Activation of several signaling systems has been reported to contribute to EMT progression (17). FAK pathway is identified as a key mediator of signaling in tumor progression and metastasis. We further tested whether the

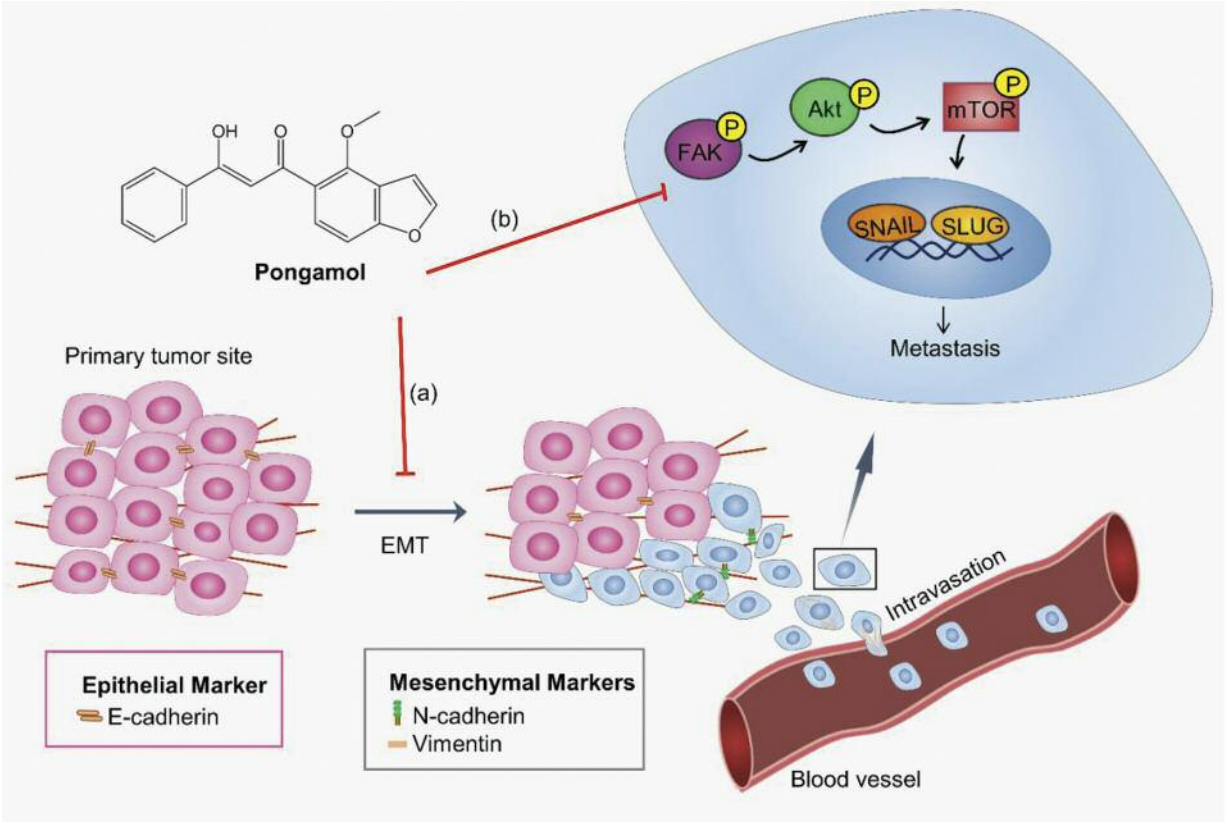

Figure 4. *Schematic representation of the mechanism through which pongamol inhibits progression of EMT in lung cancer cells. EMT is commonly characterized by losing epithelial marker (E-cadherin), which is accompanied by upregulation of N-cadherin expression. Treatment of lung cancer cells with pongamol leads to inhibition of metastasis progression via suppressing (a) EMT regulatory proteins including N-cadherin and vimentin and inhibition of (b) FAK signaling activation and as a consequence of inhibition of Akt/mTOR signals.*

compound inhibited the EMT process via suppression of upstream signals of FAK and Akt. We treated cells with pongamol at 0-100 μM for 24 h and the activated and total FAK and Akt were determined by western blotting. The results revealed that pongamol administration reduced the level of phosphorylated FAK (p-FAK), phosphorylated Akt (p-Akt) and phosphorylated-mTOR (p-mTOR), while pongamol had only minimal effect on the total form of FAK, Akt, and mTOR. These results showed that pongamol inhibited EMT by blocking the activation of FAK/Akt-mTOR mechanism.

Discussion

Constitutive activation or dysregulation of the FAK/Akt pathway is associated with metastasis and chemoresistance in advanced-stage lung cancer (23, 24). FAK/Akt signaling governs the transcriptional factor activation such as Snail and Slug for mesenchymal properties and driving EMT in cancers (25). Consequently, this encourages the initiation of cancer metastasis progression. EMT initiates the suppression of cell-cell adhesion protein E-cadherin and increases mesenchymal proteins including vimentin and N-cadherin (26). Previous studies have suggested that the inhibition of FAK/Akt network could be a target for metastasis prevention (16, 27). Recently, our research group has investigated several compounds that potentially inhibit lung cancer metastasis through EMT and FAK signaling intervention (28, 29). In line with previous studies, herein, we demonstrated for the first time the potential effect of pongamol isolated from the roof of *Millettia erythrocalyx* in inhibiting EMT by blocking FAK/Akt activation.

Previously, FAK activating Akt-mTOR signaling pathways have been widely accepted as pivotal effectors encouraging EMT progression (30, 31). The indicators of advancing EMT including N-cadherin and vimentin proteins have been related to the progression of various cancers (32). N-cadherin-positive tumor cells were related to high clinical stage and malignant degree (33, 34). In regards to FAK/Akt, N-cadherin acts on membrane as adherents junction that mediates the activation of mitogen-activated protein kinase controlled by FAK signaling

to enhance cell survival and migration (35). In contrast with N-cadherin, the interaction of E-cadherin between two cells causes contact inhibition of proliferation when cells reach confluence (36), that serves as a suppressor of PI3K pathway in epithelial cells to keep maintaining the epithelial phenotypes and regulating the homeostasis of tissues (36, 37). Thus, loss of E-cadherin expression is considered as part of the main molecular events driving loss of cell-cell adhesion facilitating cancer invasion and metastasis (38). In addition to cadherin function, E-cadherin controls the balance of cell survival and apoptosis (39). For instance, reduction of E-cadherin level is associated with anoikis induction. However, during EMT progression, N-cadherin-representing metastatic cancer cells facilitate the cell-cell contact upon detached conditions, which leads the cell to survive and cause anoikis resistance (15). Interestingly, we observed that cells treated with pongamol possess a high expression of E-cadherin, where the mesenchymal cells markers including N-cadherin, vimentin, Snail, and Slug have lower expression in response to pongamol (Figure 3B). Further studies demonstrated that knockdown of FAK could control N-cadherin expression and Snail family of EMT transcriptional factors (40), indicating that FAK is a potential target to suppress EMT regulatory proteins. The attempts at inhibition focus upon down-regulation of FAK expression or inhibition of the ATP binding site and phosphorylation of FAK. Several small-molecule FAK inhibitors have been clinically investigated and found to inhibit cancer cell migration (41, 42). In line with this study, we observed that pongamol suppressed migration behavior of lung cancer cells and mesenchymal proteins including N-cadherin, vimentin, snail, and slug. Moreover, the phosphorylation of FAK level was decreased in pongamol treatment together with the downregulation of its downstream signaling, Akt/mTOR pathway. This demonstrated that pongamol inhibits lung cancer migration and invasion through attenuating EMT regulatory proteins and FAK activating Akt-mTOR signaling pathway (Figure 3).

In conclusion, this study demonstrated that pongamol inhibited migration and invasion of H460 lung cancer cells with the underlying mechanism of its actions through the suppression of FAK/Akt cascade (Figure 4). This new finding of the pharmacological effect of pongamol provides the basic data for further development for alternative anticancer approaches.

Conflicts of Interest

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

Authors' Contributions

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

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