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Millettocalyxin B Inhibits Migratory Behavior of Lung Cancer Cells *via* Integrin α5 Suppression

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Abstract. *Background/Aim: Integrin-targeting compounds have shown clinically significant benefits in many patients. Here, we examined the activity of millettocalyxin B, extracted from the stem bark of Millettia erythrocalyx, in lung cancer cells. Materials and Methods: The viability of human lung cancer cells was investigated by the 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazoliumbromide (MTT) assay. Migration and invasion assays were performed. Phalloidin-rhodamine staining was used to determine the formation of filopodia. Western blot analysis and immunofluorescence staining were used to identify the signaling proteins involved in migration regulation. Results: Non-toxic concentrations (0-25 μM) of millettocalyxin B reduced migration and invasion of lung cancer A549 cells. Filopodia were significantly reduced in millettocalyxin B-treated cells. The migration regulatory proteins including integrin α5, active FAK, active Akt, and Cdc42 were significantly decreased in Millettocalyxin B-treated cells. Conclusion: Our findings revealed a novel anti-migration and anti-invasion effects and the underlying mechanism of millettocalyxin B, which may be exploited for cancer treatment.*

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Lung cancer has become a major public health issue worldwide, with a high incidence and mortality rate (1). Cancer metastasis is responsible for most of cancer mortality (2, 3). Cell migration is a multi-step process controlled through signaling molecules such as integrin, focal adhesion kinase (FAK), Akt and Rho-GTPases (4-7). In cancers, integrins are known to regulate cancer cell survival and motility and have been recognized as important anti-metastatic drug targets (8). Integrins α5, and integrin β1 have been shown to augment survival and metastatic potential of lung cancer cells (9). In lung cancer, integrin α5 and β1 has been demonstrated to induce FAK activation leading to cancer cell movement (10). In addition, integrin α5 and β1 promote cell invasion *via* the enhanced transmission and generation of contractile forces (11) and considered as markers of highly motile cancer cells (8). Integrin-dependent activation of FAK has been shown to trigger protein kinase B (Akt) activation *via* phosphorylation at serine 473 and this activation correlated with cancer metastasis (12-14). Rho-GTPase and cell division cycle 42 (Cdc42) are down-stream targets of FAK regulating cell migration and filopodia (15).

Millettocalyxin B is a phenolic compound from the stem bark of *Millettia erythrocalyx* Gagnep (16). This compound has a variety of pharmacological effects, including antioxidant, anti-herpes simplex virus, anti-allergy, and anti-inflammatory properties (17-19). To reveal the potential use of millettocalyxin B for anti-metastasis approaches, this study aimed at investigating its inhibitory effect on integrin-mediated lung cancer cell migration and invasion.

Materials and Methods

Cell culture. A549 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in

Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) at 37˚C in a humidified atmosphere of 5% CO_2. The culture media were supplemented with 10% (v/v) fetal bovine serum (FBS) (Merck, Darmstadt, Germany), 100 U/ml of penicillin and 100 μg/ml streptomycin (Gibco), and 2 mML-glutamine (Gibco).

Reagents. Millettocalyxin B was isolated from the stem bark of *Millettia erythrocalyx* Gagnep as previously described (16). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Hoechst 33342, propidium iodide (PI), and Phalloidin-Rhodamine were purchased from Invitrogen (Eugene, OR, USA). Antibodies against FAK (#3285), p-FAK (#3283), Akt (#4685), p-Akt (#4060), Cdc42 (#2466), β-actin (#4970), Integrin α5 (#4705), and Integrin β1 (#4706) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell viability assay and nuclear staining. After treatment, 100 μl of MTT solution were added and the cells were incubated at 37˚C for 4 h. The MTT solution was replaced with 100% DMSO (100 μl) to dissolve the formazan crystals. The optical density was measured by a microplate reader at 570 nm (Anthros, Durham, NC, USA). For nuclear staining, 10 μM of Hoechst 33342 and propidium iodide (PI) were added for 15 min at 37˚C. Cells were imaged and counted using a fluorescence microscopy in four random fields at 20× magnification (Nikon ECLIPSE Ts2, Tokyo, Japan).

Wound-healing migration assay. The monolayer of cells grown in 96-well plates (2.5×10^5 cells/well) was scratched by pipette tip. Cells were treated with millettocalyxin (0-25 μM) at 37˚C for 24, 48 and 72 h, and cell migration was determined under a phase contrast microscope (Olympus, Melville, NY, USA).

Transwell invasion assay. The upper chamber was coated with 0.5% Matrigel (BD Biosciences, San Jose, CA, USA). After pretreatment with the compound, cells (2.5×10^5 cells/well) were seeded in a serum free medium into the upper chamber with a 0.8 μm pore-sized membrane and complete medium containing 10% FBS (Merck, Darmstadt, Germany) was added into the lower chamber. After 24 h, the non-invading cells were removed and cells on the lower surface of the membrane were stained with 10 μM of Hoechst 33342 (Sigma, St. Louis, MO, USA) for 10 min. Finally, migrating cells were imaged using a fluorescence microscope.

Cell morphology and filopodia characterization. After treatment, cells were fixed with 4% paraformaldehyde (Sigma) in 1× PBS (Gibco) for 10 min at 37˚C. Cells were permeabilized with 0.1% Triton X (Sigma) in 1× PBS for 4 min and blocked with 0.2% bovine serum albumin (BSA) (Merck) for 30 min. The phalloidin-rhodamine123 solution (Sigma) was added and incubated for 15 min. Cell morphology and filopodia were observed under a fluorescence microscope (Nikon ECLIPSE Ts2, Tokyo, Japan).

Western blot analysis. Cells were lysed with lysis buffer protein concentration was determined by a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins were subjected to SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membrane was blocked, washed, and incubated with a specific primary antibody at 4˚C overnight. After washed, the membranes were probed with a secondary antibody. The proteins were detected by enhanced chemiluminescence Super signal West Pico (Pierce Biotechnology) and quantified using ImageJ software (NIH, Bethesda, MD, USA).

Immunofluorescence assay. Cells were fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.1%Triton-x for 20 min and blocked with 4% BSA for 30 min. The cells were incubated with primary antibodies at 4˚C for 12 h and incubated with secondary antibodies at room temperature for 2 h. Cells were visualized by using a fluorescence microscopy with a 40×objective lens (Nikon ECLIPSE Ts2) and analysis were performed using ImageJ software.

Statistical analysis. All data are presented as the mean±SD, derived from at least three independent experiments. GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA) was used to analyze all data. Statistical analysis was performed by one-way analysis of variance, followed by Turkey's *post-hoc* test at significance levels of $p<0.05$.

Results

Cytotoxic effect of millettocalyxin B on human non-small cell lung cancer A549 cells. We first determined the cytotoxicity of millettocalyxin B (Figure 1A) on A549 cells by the MTT assay. Cells were treated with millettocalyxin B (0-100 μM) for 24 h. Cell viability assay revealed that millettocalyxin B was not cytotoxic at concentrations less than 25 μM (Figure 1B). A nuclear co-staining assay was used to detect apoptotic and necrotic cells. Cells were treated with millettocalyxin B (0-100 μM) for 24 h. Hoechst/PI staining showed that 5-25 μM millettocalyxin B caused no apoptosis or necrosis, whereas 50-100 μM of the compound could induce significant apoptosis (Figure 1C). Non-toxic doses of millettocalyxin B were used for the further experiments.

Millettocalyxin B inhibits migration and invasion of the cells. Cell migration activity was determined by wound-healing assay in response to the treatment with 0-25 μM millettocalyxin B at 24, 48 and 72 h. The results showed that 5-25 μM millettocalyxin B significantly inhibited A549 cell migration at all time points, compared with the non-treated control (Figure 2A). Additionally, cell invasion was determined using the transwell Boyden chamber assay. A549 cells were treated with 0-25 μM millettocalyxin B for 24 h and then added to the upper chamber. Millettocalyxin B significantly inhibited cell invasion at 24 h (Figure 2B). These results indicate that millettocalyxin B possesses the ability to inhibit human lung cancer cell migration and invasion.

Millettocalyxin B suppresses filopodia formation and down-regulates Cdc42. The formation plasma membrane protrusion termed filopodia associates with increased cell movement (20). We next analyzed the effect of millettocalyxin B on filopodia formation. A549 cells were treated with 0-25 μM millettocalyxin B and phalloidin-

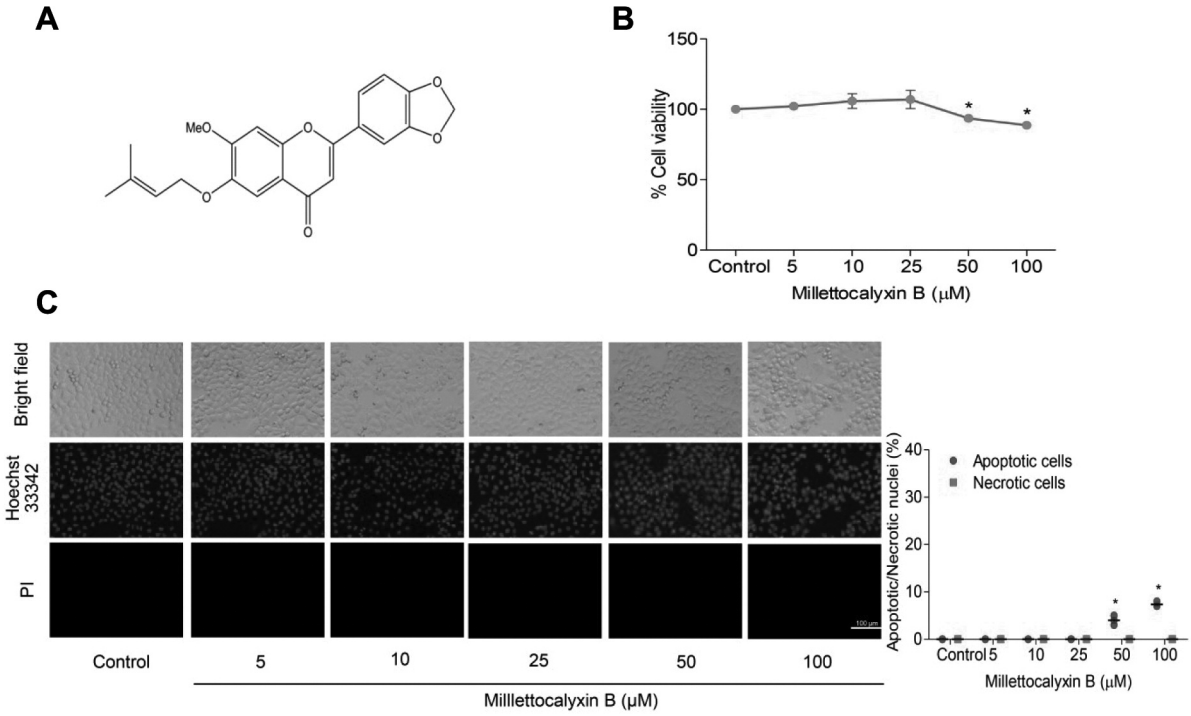

Figure 1. *(A) Chemical structure of millettocalyxin B. (B) Effect of millettocalyxin B on the viability of A549 cells. The cells were treated with 0-100 μM of millettocalyxin B for 24 h and cell viability was analyzed by the MTT assay. (C) Apoptosis and necrosis was determined with the Hoechst 33342 and propidium iodide staining assay. The values are means of triplicate measurements ± SD.; *p<0.05 versus control.*

labeled filopodia were determined. Figure 3A shows that the untreated control cells exhibited several membrane protrusions of filopodia which was significantly diminished following treatment with 5-25 μM millettocalyxin B. As Cdc42 is a known regulator of filopodia formation (20), we further investigated the expression levels of Cdc42 in millettocalyxin B-treated cells. Compared to untreated cells, Cdc42 was strongly down-regulated in cells treated with 5-25 μM millettocalyxin B (Figure 3B).

Millettocalyxin B suppresses integrin α5 and FAK-Akt signaling. Integrins control cancer cell invasion and migration *via* activating several pathways including FAK-Akt signaling. Cells were incubated with the compound (0-25 μM) for 24 h and migration-related proteins were examined by western blot analysis. Figure 4A shows that millettocalyxin B minimally affected integrin β1, while it significantly reduced the levels of integrin α5. The activation status of proteins regulating cell motility down-stream of integrins such as FAK and Akt was further elucidated by western blotting. In parallel with decreased integrin α5, millettocalyxin B significantly reduced the cellular levels of active FAK indicated by the reduction of p-FAK/FAK ratio at the concentrations of 5-25 μM. Figure 4A further reveals that millettocalyxin B could decrease the levels of active Akt (phosphorylation at Ser 473). We further confirmed the integrin α5 inhibitory effect of millettocalyxin B by immunofluorescence-based detection of integrin α5 and p-FAK. Figure 4B and C show that millettocalyxin B significantly decreased the levels of Integrin α5 and p-FAK. These results suggested that millettocalyxin B exhibits anti-migratory effect through the suppression of integrin α5 and its down-stream FAK-Akt signaling pathway.

Discussion

Cancer metastasis is known as important process resulting in the death of lung cancer patients (21). Previous studies have reported the anti-metastasis potential of several compounds *via* integrin targeting (22-24). In line with such studies, we showed the integrin α5 targeting activity of millettocalyxin B in lung cancer cells.

In lung cancer, it has been demonstrated that the expression levels of integrin α5 are important for tumor growth and cancer progression (25). It is interesting that integrin α5 could be promising target as it is not generally expressed in normal lung cells (26). Furthermore, integrin α5 overexpression is associated with cancer aggressiveness and poor prognosis (27). Cancer cell migration and invasion involve multiple pathways that are associated with integrins,

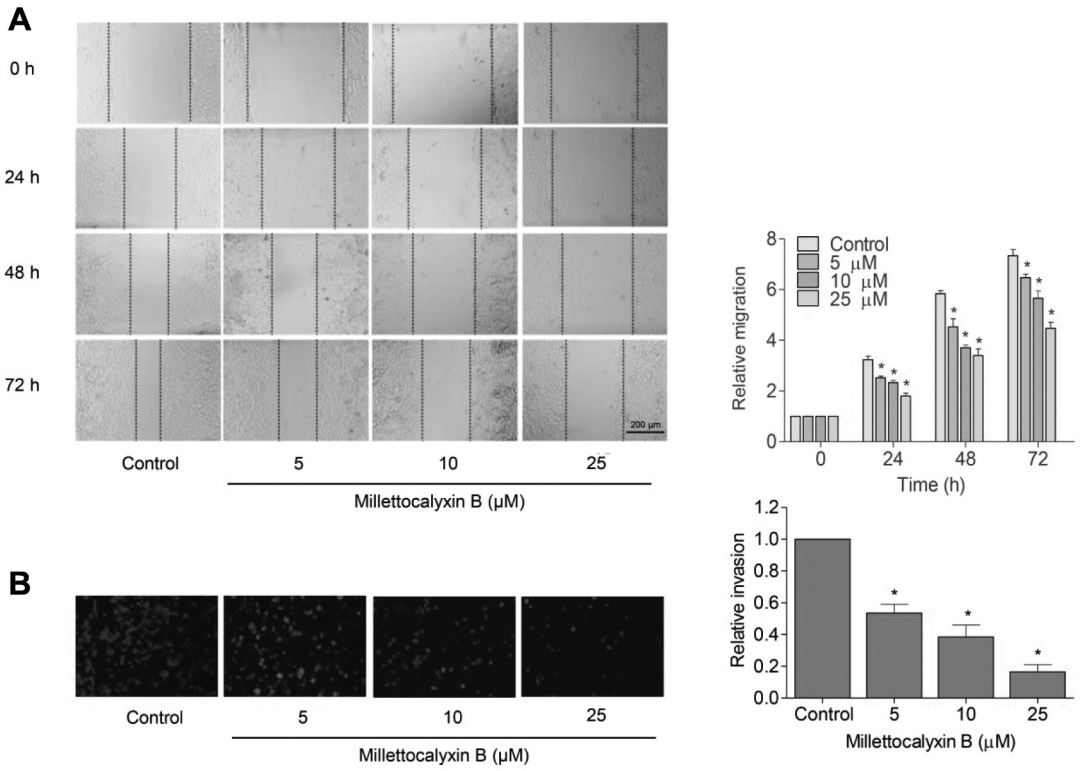

Figure 2. *The effect of millettocalyxin B on the migratory behavior of cancer cells. (A) The wound healing assay was performed to evaluate cell migration after treatment with millettocalyxin B for 24, 48, and 72 h. The relative cell migration was calculated by comparing with the control. (B) For the invasion assay, the cells were seeded onto Matrigel and treated with millettocalyxin B (0-25 μM) for 24 h. The values are means of triplicate measurements ±SD.; *p<0.05 versus control.*

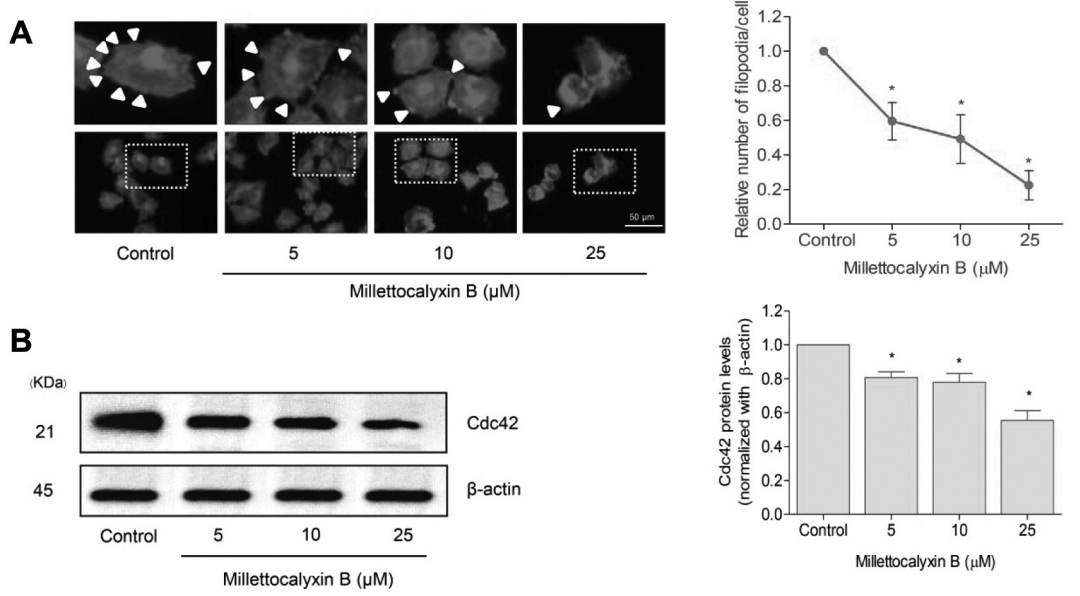

Figure 3. *Millettocalyxin B decreases filopodia formation and Cdc42 levels. (A) After treatment with millettocalyxin B for 24 h, the relative number of filopodia was evaluated. The values are means of triplicate measurements ±SD.; *p<0.05 versus control. (B) The effect of millettocalyxin B on Cdc42 protein levels. The levels of Cdc42 were determined by western blotting. The relative levels of the proteins were quantified by densitometry and mean data from three independent experiments are presented. The values are means of measurements ±SD. (n=3); *p<0.05 versus control.*

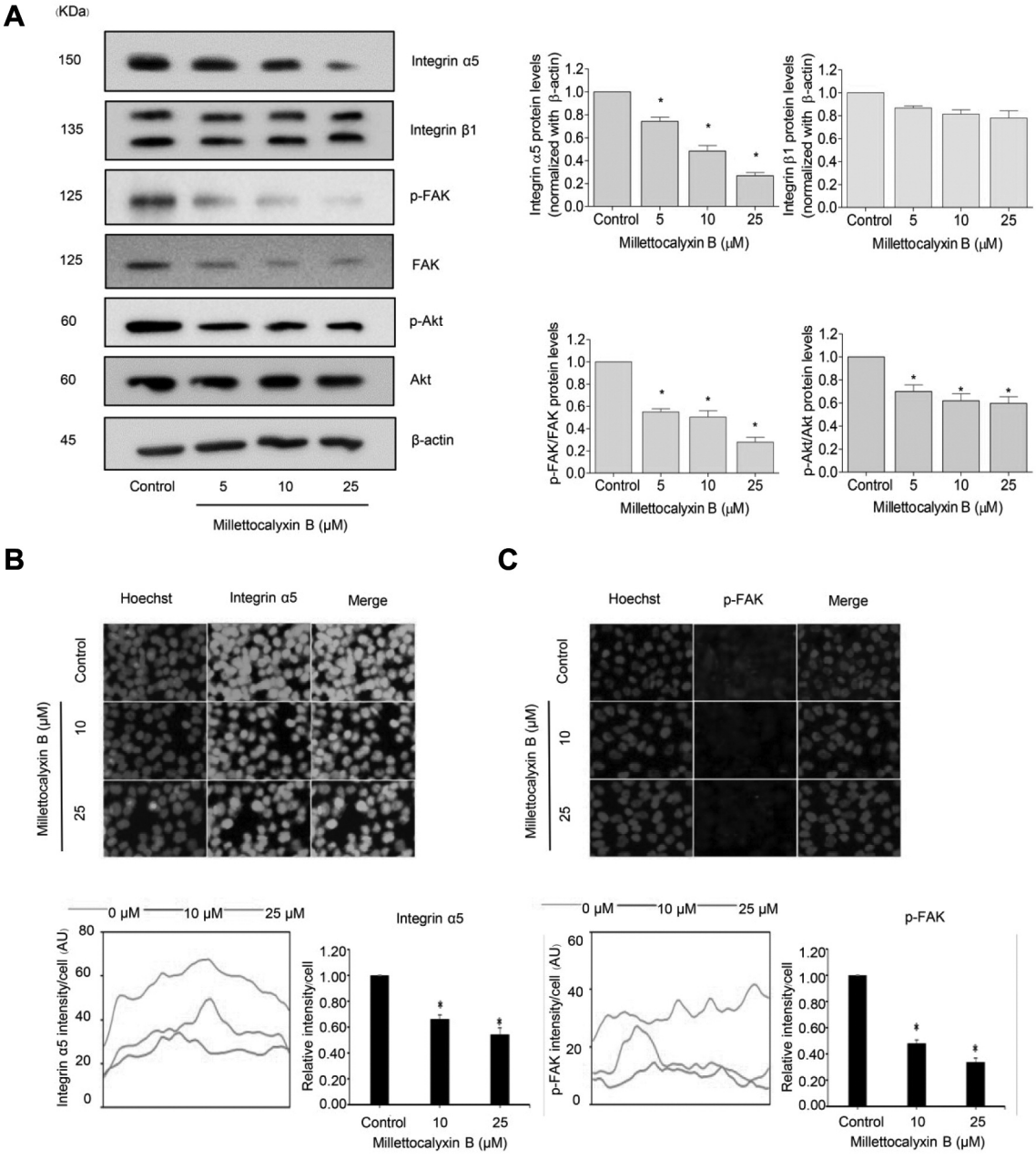

Figure 4. *The effect of millettocalyxin B on integrin α5, integrin β1 and migratory proteins. (A) The cells were treated with 0-25 μM millettocalyxin B for 24 h. The protein levels were evaluated by western blot analysis followed by densitometry. Values are means of measurements ±SD. (n=3); *p<0.05 compare with control. (B) The expression of integrin α5 and (C) p-FAK were examined using immunofluorescence. The nucleus of cells was stained with Hoechst33342. The fluorescence intensity was analyzed by ImageJ software. The values represent the mean±SD. *p<0.05 compare with control.*

such as FAK, Akt, and Cdc42 (28). It is known that integrins are important to elevate cell motility (29, 30). In this study, millettocalyxin B treatment suppressed integrin α5 (Figure 4A) and reduced the expression of p-FAK, p-Akt and Cdc42 (Figure 4A and 3B). Cdc42 belongs to the Rho GTPase family of proteins and is involved in the formation of filopodia, which facilitate migration and invasion (31, 32). In addition, decreased Cdc42 expression has been demonstrated to inhibit cell migration (33-35). Cycloartobiloxanthone has been reported to have an inhibitory effect on cancer migration and invasion by suppressing integrin α5, integrin αV, FAK and Cdc 42 (23). Also, phoyunnanin E and a bibenzyl have

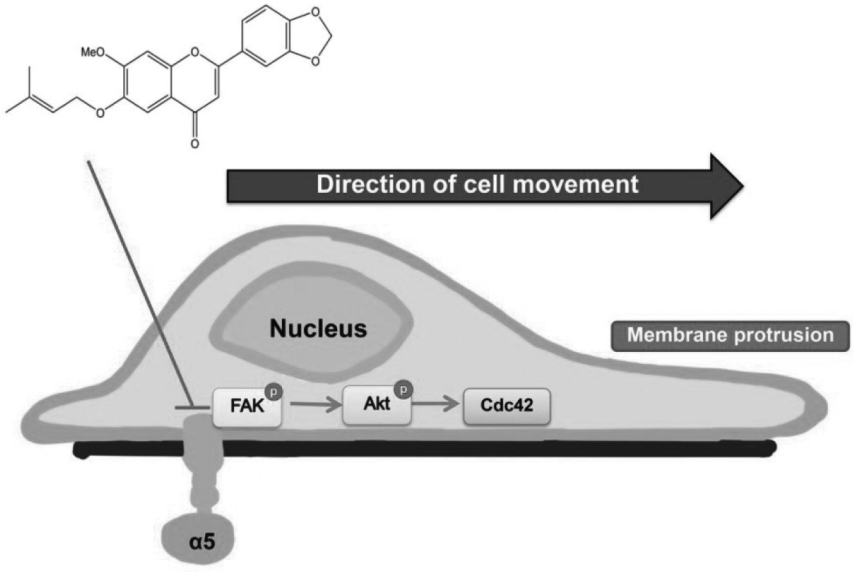

Figure 5. *Schematic figure showing the anti-migratory mechanism of millettocalyxin B. Integrin regulates the motility of the cells through FAK and Akt pathways. Here, we found that millettocalyxin B suppresses cell migration by suppressing integrin α5 and FAK/Akt pathway.*

been reported to have an inhibitory effect by inhibiting integrin α5, αV, α4, β3, FAK and Cdc 42 (22, 24).

In conclusion, the migration activity of the cells was inhibited by targeting integrin α5 leading to the downregulation of active FAK, active Akt and Cdc42 (Figure 5). We provided information regarding anti-migratory activity of millettocalyxin B that may benefit the future development of this compound for anti-metastasis therapy or its use as an adjuvant with standard therapies to improve clinical outcome.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding the publication of this article.

Authors' Contributions

Conceptualization, P.C.; Methodology, P.C. and P.L.; Validation, P.C.; Formal analysis, P.L. and P.C.; Investigation, A.S., P.L. and N.A.; Resources, B.S. and P.C.; Writing-original draft preparation, P.L. and P.C.; Writing-review and editing, P.C.; Funding acquisition, P.C. All Authors have read and agreed to the published version of the manuscript.

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