ANTICANCER RESEARCH *40*: 2583-2589 (2020)
doi:10.21873/anticanres.14229

Benzoxazine Dimer Analogue Targets Integrin β3 in Lung Cancer Cells and Suppresses Anoikis Resistance and Migration

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Abstract. *Background/Aim: Certain integrins including integrin β3 facilitate movement and survival of metastatic cancer cells. We examined whether benzoxazine dimer analogue N,N-bis(5-ethyl-2-hydroxybenzyl) methylamine (HM) has anti-metastatic effects. Materials and Methods: Cell viability was examined by the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. Wound healing and phalloidin-rhodamine assays were performed to evaluate the migration and filopodia formation, respectively. Anoikis resistance was studied by anchorage-independent growth assay. The expression of proteins regulating migration were examined by western blot. Results: HM treatment significantly inhibited growth and survival of detached lung cancer cells as indicated by the reduced colony number and size of anchorage-independent growth analysis. HM inhibited cell migration and suppressed filopodia formation. Protein analysis indicated that the compound dramatically decreased integrin β3 and its related downstream proteins including active focal adhesion kinase (FAK) and active protein kinase B (AKT); however, integrin β1 and α5 were found to be unaltered. Conclusion: HM shows a potential in targeting integrin β3 and could be a good candidate for further developed as an anti-metastatic therapy.*

Lung cancer is frequently diagnosed at advanced metastatic stages (1). Metastasis strongly associates with high mortality of cancer patients (2) and treatment failure (3, 4). During metastasis, cancer cells detach from the original site, and then move through blood or lymphatic vessels to distant tissues to form secondary and tertiary tumors (5). Escaping from anoikis, a programmed cell death induced by cell detachment from the basement is essential for survival of the cancer cells in the circulations (6). Cancer cells can avoid anoikis by activating or increasing survival pathways including integrin-mediated survival and transform to anchorage-independent cells for successful metastasis (7, 8).

In addition, the migration of cancer cells is an critical factor facilitating cancer metastasis (9, 10). This process involves the interaction of the cell with surrounding extracellular matrix (ECM), which induces changes in the cytoskeleton and cell adhesion (11). The transmembrane proteins of the integrin family link ECM to intracellular cytoskeletal proteins. The specific dimerization of α and β subunits can promote migration of cancer cells. A study has demonstrated that certain integrins, such as integrin β1, β3, α5 and αv, promote cancer cell migration (12). Moreover, integrin β3 has been shown to form a complex with integrin αv and play a role in angiogenesis (13) and to mediate microtubule-dependent cell migration (14). Activating signals from the integrin family need secondary messengers to transduce the signal such as tyrosine kinases, serine/threonine kinases, lipid mediators, low molecular weight GTPases, and intracellular calcium fluxes. The activation of focal adhesion kinase (FAK), one of the tyrosine kinases (15), induces a signaling cascade in a PI3K-dependent manner and supports cell migration (16). In the process of migration, protrusion of cell membrane is initiated to form a filopodium before a new focal adhesion formation on the attachment surface. This process is controlled by the Rho family of proteins (17). Therefore, de-regulation of this protein family can promote a more aggressive cell migration (18). Inhibition of the proteins that control the cell migration process will be a target of cancer therapy.

The *N,N*-bis(5-ethyl-2-hydroxybenzyl) methylamine (HM) is a benzoxazine dimer compound (19-21) with unknown

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Key Words: Anti-migration, benzoxazine dimer analogue, non-small cell lung cancer, integrins.

activity regarding cancer cell metastasis. Therefore, this study aimed at examining the effect of HM on migration as well as anoikis resistance in human lung cancer cells.

Materials and Methods

Reagents and antibodies. Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, phosphate-buffered saline (PBS), and trypsin-EDTA were obtained from Gibco (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phalloidin tetramethyl rhodamine B isothiocyanate, dimethyl sulfoxide (DMSO), Hoechst 33342 and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). The primary antibodies against integrin β1 (#4706), integrin β3 (#4702), integrin α5 (#4705), FAK (#3285), phosphorylated FAK or p-FAK (#3283), RhoA (#2117), Mcl-1 (#94296), Bcl-2 (#4223), Akt (#9272), phosphorylated Akt or p-Akt (#4060), β-actin (#4970) and the secondary antibody anti-rabbit IgG (#7074) were acquired from Cell Signaling Technology (Danvers, MA, USA). The tested compound, *N,N*-bis(5-ethyl-2-hydroxybenzyl) methylamine (HM) was received from the Department of Materials Engineering, Faculty of Engineering, Kasetsart University, Thailand. The chemical structure of HM is presented in Figure 1A.

Preparation of HM stock solution. HM was prepared as described in previous studies (19-21). The compound was prepared as a 40 mM stock solution by dissolving in dimethyl sulfoxide (DMSO) solution and then diluted to 2, 4 and 8 mM stock solution and stored at –20˚C. It was freshly diluted 200 times with complete RPMI medium. The final concentration of DMSO of all treatment conditions was not more than 0.5% DMSO.

Cell lines and culture. The non-small cell lung cancer cell line H292 (ATCC®CRL 1848™) was cultured in 10% FBS RPMI with 1% penicillin and streptomycin. The cells were stored at 37˚C in a humidified incubator of 5% carbon dioxide. The cells were used at about 75% confluence.

Cytotoxicity assay. For cytotoxicity assay, 1×10^4 cells/well were seeded onto 96-well plates and incubated overnight. Then, cells were treated with various concentrations of HM for 24 h at 37˚C and analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to manufacturer's protocol (Sigma-Aldrich). To calculate cell viability, the absorbance of the treated cells was divided by that of non-treated cells and is reported as percentage.

Nuclear staining assay. This method was applied to define apoptotic cell death by nuclear staining with Hoechst 33342. The cells were seeded on 96-well plates at the density of 1×10^4 cells/well and incubated overnight. The cells were treated with various concentrations of HM for 24 h at 37˚C. Afterwards, the cells were incubated with 10 μg/ml of Hoechst 33342 for 30 min at 37˚C. Then, they were visualized and imaged under a fluorescence microscope (Nikon ECLIPSE Ts2, Nikon, Tokyo, Japan). Results are reported as percentage of apoptotic cells.

Anchorage-independent growth assessment. For studying anchorage-independent cell growth, soft agar colony formation assay was used. H292 cells were seeded and incubated on 24-well plates at a concentration of 2.5×10^4 cells/well for 24 h at 37˚C. The cells were pre-treated with various non-toxic concentrations of HM for 24 h at 37˚C. Soft agar was prepared by using a 1:1 mixture of 10% FBS-RPMI culture media and 1% agarose gel. After 24 h, the 8x103 cells/ml were mixed with 10% FBS-RPMI culture media and 0.33% agarose gel, the cell mixture was added into the well and allowed to solidify for 3 h. The 10% FBS-RPMI culture medium was then added on the top layer. Colony formation was measured after two weeks using a phase contrast microscope (Nikon ECLIPSE Ts2). Relative colony number and diameter were calculated by dividing the values of the treated cells to those of the control cells.

$$Relative\ colony\ number = \frac{Number\ of\ colony\ from\ treated\ cells}{Number\ of\ colony\ from\ controlled\ cells}$$

$$Relative\ diameter\ of\ colony = \frac{Diameter\ of\ colony\ from\ treated\ cells}{Diameter\ of\ colony\ from\ controlled\ cells}$$

Wound healing cell migration assay. H292 cells were seeded on a 24-well plate at a density of 1.2×10^5 cells/well to create the monolayer within 24 h. Wound space was scratched by a 200 μl-tip. Medium was removed and the cells were washed with PBS before photographed under a phase contrast microscope (Nikon ECLIPSE Ts2). The cells were treated with various concentrations of HM and the images were captured at various time points. The wound area at each time point was measured by using Image J software (version 1.52, National Institutes of Health, Bethesda, MD, USA). The results are reported as a relative wound area.

Filopodia formation assessment. H292 cells were seeded on 96-well plate at a density of 5×10^3 cells/well. After HM treatment for 24 h, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at 37˚C. Then, the cells were permeabilized with 0.1% Triton-x100 for 5 min and non-specific binding was blocked with 0.2% BSA for 30 min. Phalloidin-rhodamine was diluted to 1:100 in PBS before staining for 30 min. Afterwards, the cells were mounted with 50% glycerol in PBS. They were visualized and imaged under a fluorescence microscope (Nikon ECLIPSE Ts2). The results are reported as relative numbers of filopodia per cell.

Western blot analysis. The cells were seeded on 6-well plate at the density of 4×10^5 cells/well and incubated overnight. After HM treatment, the supernatants were aspirated and the cells were detached by using a scrapper before incubated with RIPA lysis buffer containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS for 30 min at 4˚C. The lysates were collected and their protein contents were determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of proteins from each sample were separated by SDS-PAGE and transferred to 0.2 μm polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The separating blots were blocked with 5% skim milk in TBST (Tris-buffer saline with 0.1% tween containing 25 mM Tris-HCl pH 7.5, 125 mM NaCl and 0.1% tween 20) for 2 h and incubated with primary antibody against integrin β1, integrin β3, integrin α5, FAK, p-FAK, Akt, p-Akt, Bcl-2, Mcl-1 or beta-actin overnight at 4˚C. Then, the membranes were incubated with Secondary antibody for 2 h at 25˚C after washed by TBST three times. Finally, the protein

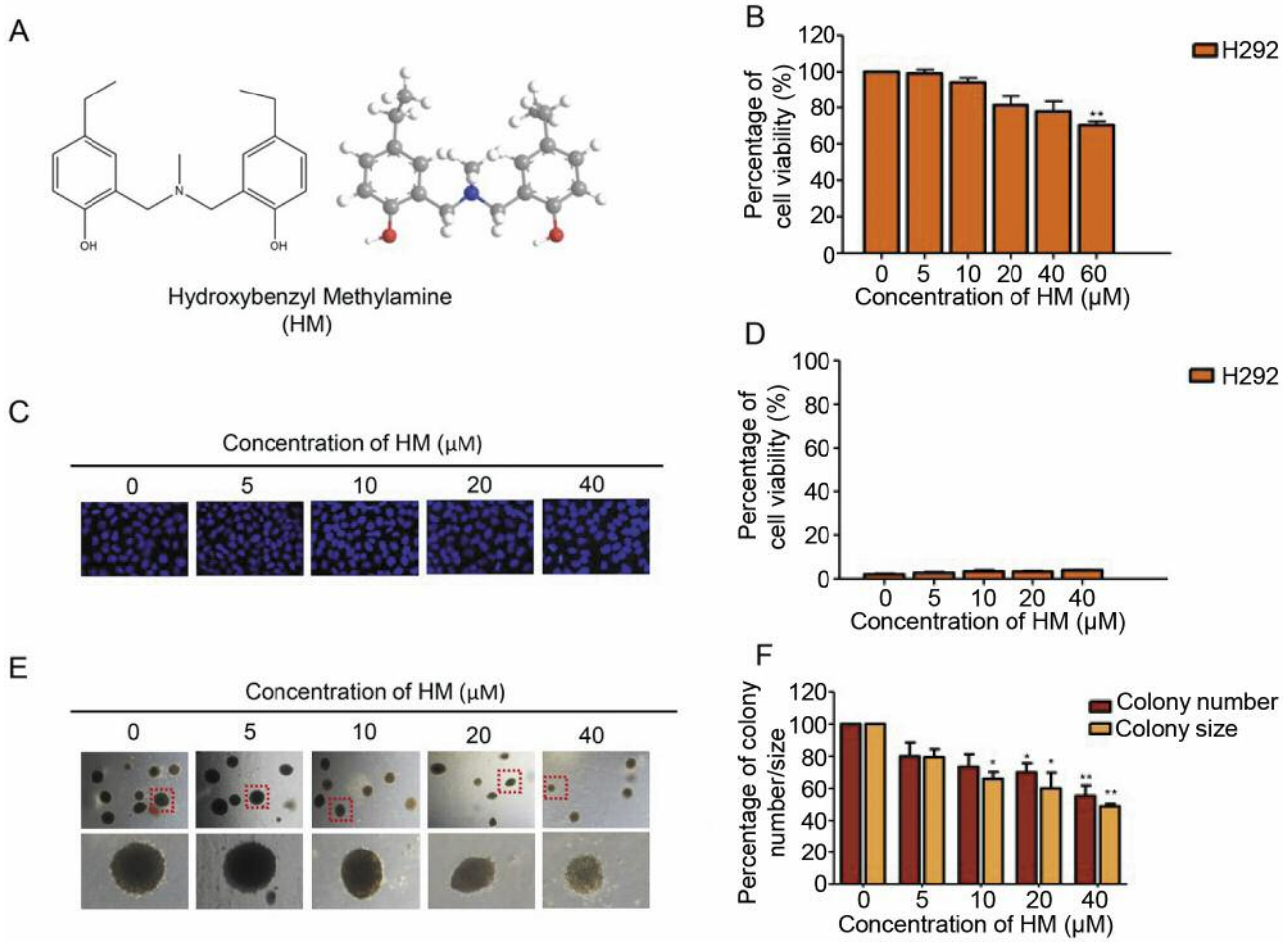

Figure 1. *Effect of HM on cell viability, apoptosis, and anchorage-independent growth of lung cancer cells. (A) The chemical structure of HM. (B) The effect of HM on cell viability was examined by the MTT assay. H292 cells were treated with various concentrations of HM (0-60 μM) for 24 h and the percentage of cell viability was calculated by comparison with the non-treated control. (C-D) Nuclear morphology of H292 cells was evaluated by Hoechst 33342 staining. The percentage of apoptotic cells was calculated. (E-F) H292 cells were treated with non-toxic doses of HM for 24 h before subjected to anchorage-independent growth assay. The results were represented in relative number of colonies and relative colony size. The data is presented as mean±SD. (n=3) (*0.01≤p<0.05, **p<0.01, compared with the non-treated control).*

bands were detected using chemiluminescence substrate and the Chemiluminescent ImageQuant LAS4000 image station. Protein band had been analyzed using Image J software (version 1.52, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The results are presented as mean±SD of at least 3 independent measurements. Multiple comparisons for statistically significant differences between multiple groups were performed by ANOVA using the SPSS software program version 16 (SPSS Inc., Chicago, IL, USA). Statistical significance was considered at $p<0.05$. GraphPad prism 5 was used for creating graphs in all experiments.

Results

Effect of HM on cell viability, survival, and anchorage-independent growth. H292 cell viability was examined following treatment with HM (0-60 μM) for 24 h by the MTT assay as described in Methods. HM treatment significantly reduced cell viability of H292 at the concentration of 60 μM (Figure 1B). To select the non-toxic concentrations, we further examined apoptosis. The cells were similarly treated with HM and nuclear morphology was monitored by Hoechst 33342 staining. The number of apoptotic cells representing fragmented or condensed nuclei were calculated. The results indicated that HM at the concentrations of 0-40 μM did not induce apoptotic cell death (Figure 1C and D).

Next, we examined whether HM, at non-toxic concentrations (indicated by cell viability and no apoptosis induction), inhibited the survival and anchorage-independent growth of cancer cells. As detachment-induced apoptosis or cell death termed anoikis is an important obstacle of cancer metastasis, and lung cancer cells are frequently found to be anoikis resistant, the compound that inhibits anchorage-

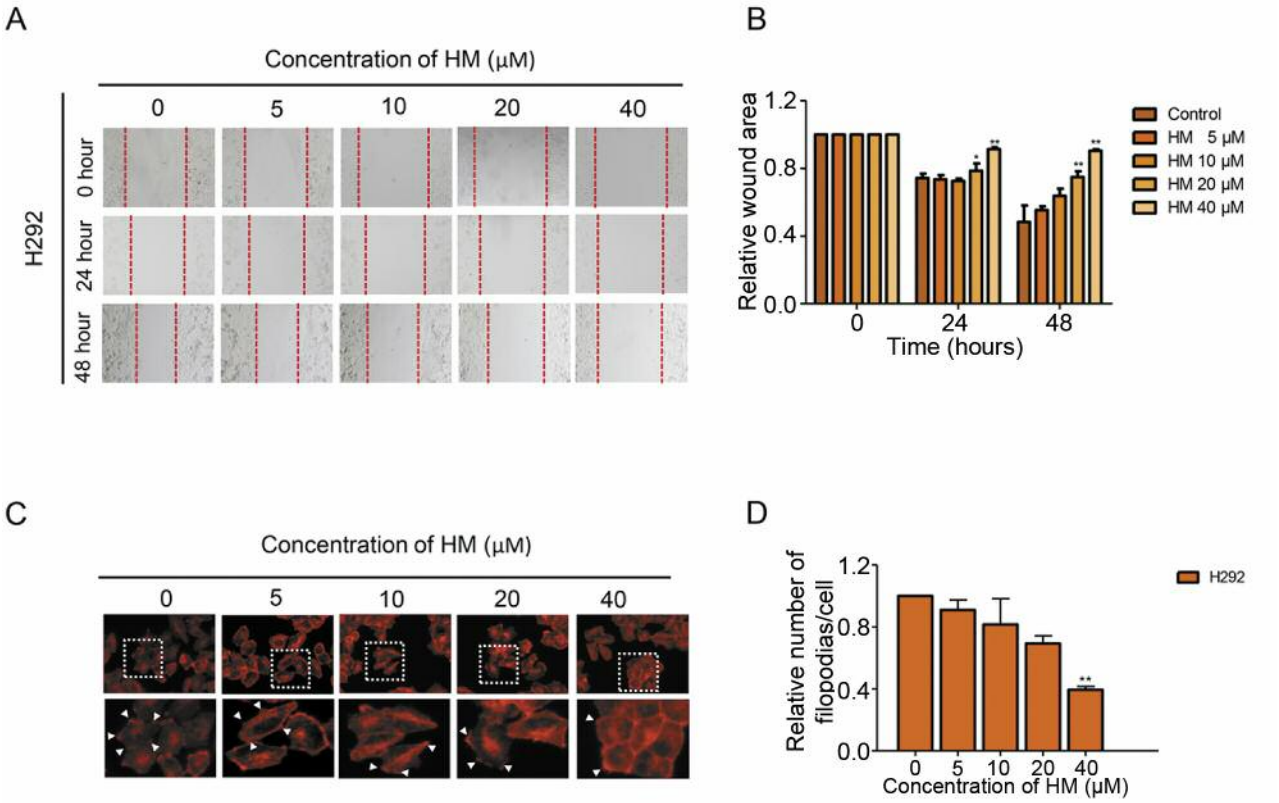

Figure 2. *HM inhibits cell migration. (A-B) Wound healing assay was performed to evaluate cell migration after treatment with various concentrations of HM (0-40 μM) for 24 and 48 h. Relative wound area at 24 and 48 h was calculated compared with that at 0 h. (C-D) Filopodia formation was examined by phalloidin rhodamine staining and the numbers of filopodia per cell were calculated compared with the non-treated control. The data is presented as mean±SD. (n=3) (*0.01≤p<0.05, **p<0.01, compared with the non-treated control).*

independent growth may have anti-metastatic activity. The cells were treated with non-toxic concentrations of HM (0-40 μM) for 24 h before subjected to the anchorage-independent growth assay. Colony formation of the cells was assayed following incubation on agarose gel for 14 days. The number and size of colonies were significantly reduced in response to HM treatment (Figure 1E and F).

HM inhibits migration and suppresses formation of filopodia. Having shown that HM potentially inhibits anchorage-independent cell growth, we further evaluated the effect of the compound on the migratory activity of cancer cells by the wound healing assay. The monolayer of H292 cells was scratched to make wound area and then treated with non-toxic concentrations of HM (0-40 μM) for 24 and 48 h. The relative wound area at 24 and 48 h was measured. Cell migration after treatment with HM was significantly reduced at concentrations of 20 and 40 μM when compared with the non-treated control (Figure 2A and B).

Furthermore, the formation of filopodia, the mandatory step of migration, which involves cell movement, was observed by the phalloidin-rhodamine staining assay. The cells were visualized after HM treatment at various concentrations (0-40 μM). The filopodia were stained with phalloidin-rhodamine and their numbers per cell were calculated. HM treatment significantly decreased the formation of filopodia at the concentration of 40 μM (Figure 2C and D).

HM down-regulates integrin signaling and the proteins involved in cell migration. Integrin generates survival and motility signals. In order to clarify the underlying mechanism of the inhibitory effect of HM on migration and anchorage-independent growth, essential integrins including α5, β1 and β3 were evaluated by western blot analysis. H292 lung cancer cells were treated with various concentrations of HM (0-40 μM) for 24 h. Treatment of cells with HM dramatically decreased integrin β3 levels in a dose-dependent fashion, while had no effect on the levels of integrins α5 and β1 (Figure 3A and B). The downstream integrin-mediated signaling was examined by assaying for the levels of focal adhesion kinase (FAK), phosphorylated FAK (p-FAK), Akt,

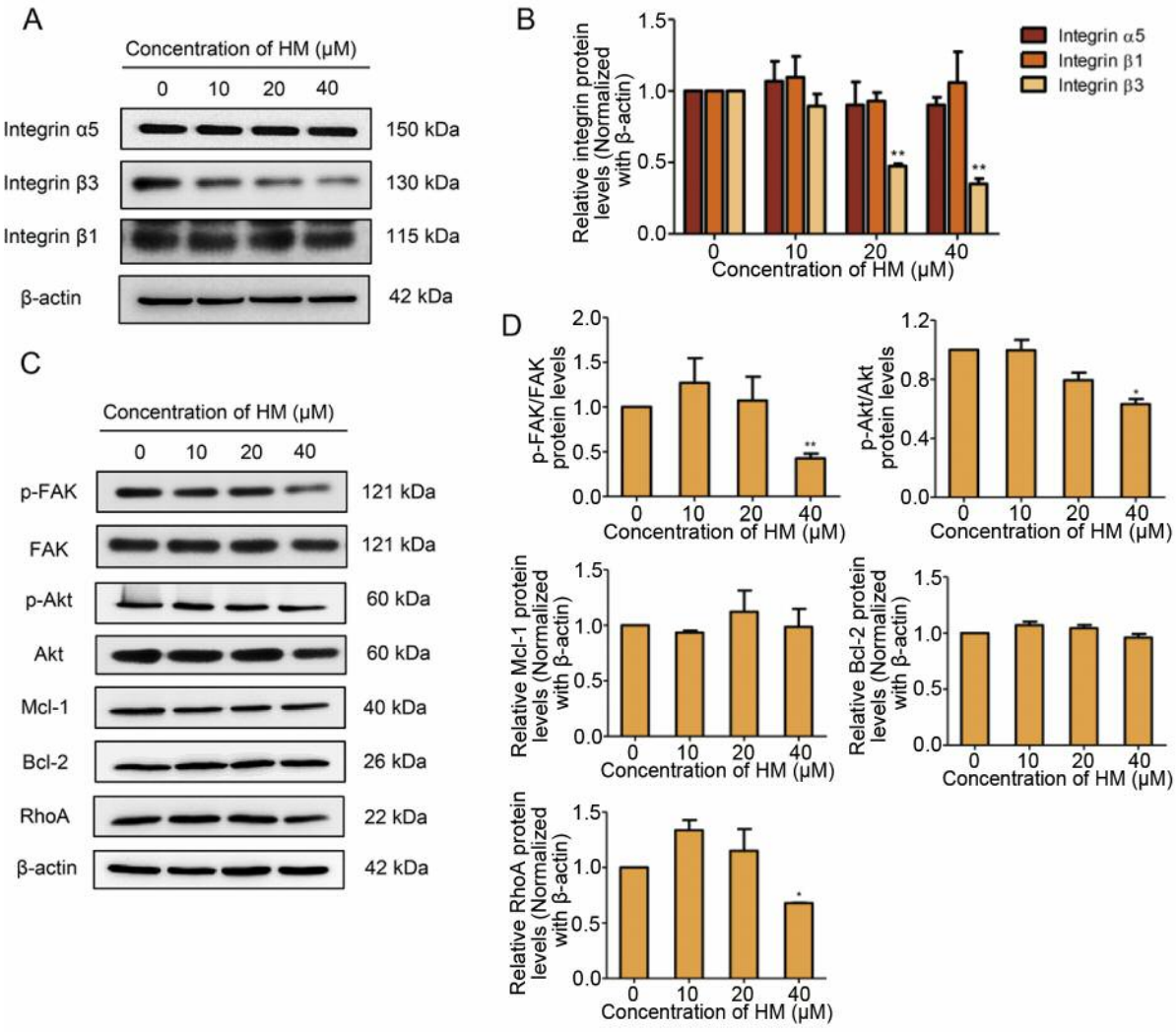

Figure 3. *HM decreases the levels of proteins involved in migration. Cells were treated with various concentrations of HM (0-40 μM) for 24 h. (A) and (C) western blot analysis was performed to evaluated the levels of integrins and migration signaling proteins. β-actin protein was examined to confirm equal loading of protein samples. (B) and (D) Densitometric analysis was performed and the results are presented as relative protein levels. The data is presented as mean±SD. (n=3) (*0.01≤p<0.05, **p<0.01, compared with the non-treated control).*

phosphorylated Akt (p-Akt), and RhoA. Mcl-1 and Bcl-2 in HM treated cells. The levels of p-FAK/FAK and p-Akt/Akt were significantly decreased in HM-treated cells compared with the non-treated control. Moreover, RhoA, which plays a role in filopodia formation was also evaluated. The protein levels of RhoA were notably decreased in response to 40 μM of HM. However, the levels of the pro-survival proteins Mcl-1 and Bcl-2 were not significantly changed after HM treatment (Figure 3C and D).

Discussion

Lung cancer is among the serious health problems worldwide. Metastasis plays an important impact on malignant lung cancer and in treatment failure (22). The process of metastasis involves cell detachment form original tumor, migration and invasion into circulation, and formation of new tumor at distant site (23). Researches have focused in finding a treatment, which can target these crucial steps of cancer cell dissemination. Cell migration involves movement by changing the morphology of the cells, detachment from one adhesion surface and adhesion to a new one (24). Migration requires integrin complexes to respond to extracellular signals, which activate intracellular signaling pathways, leading to the protrusion of membrane and formation of filopodia (25, 26). Focal adhesion kinase (FAK) is a secondary messenger in the integrin signaling pathway that activates other downstream targets such as AKT and Rho family of proteins (27). Thus, targeting key integrins could potentially be a strategy for inhibiting cell migration.

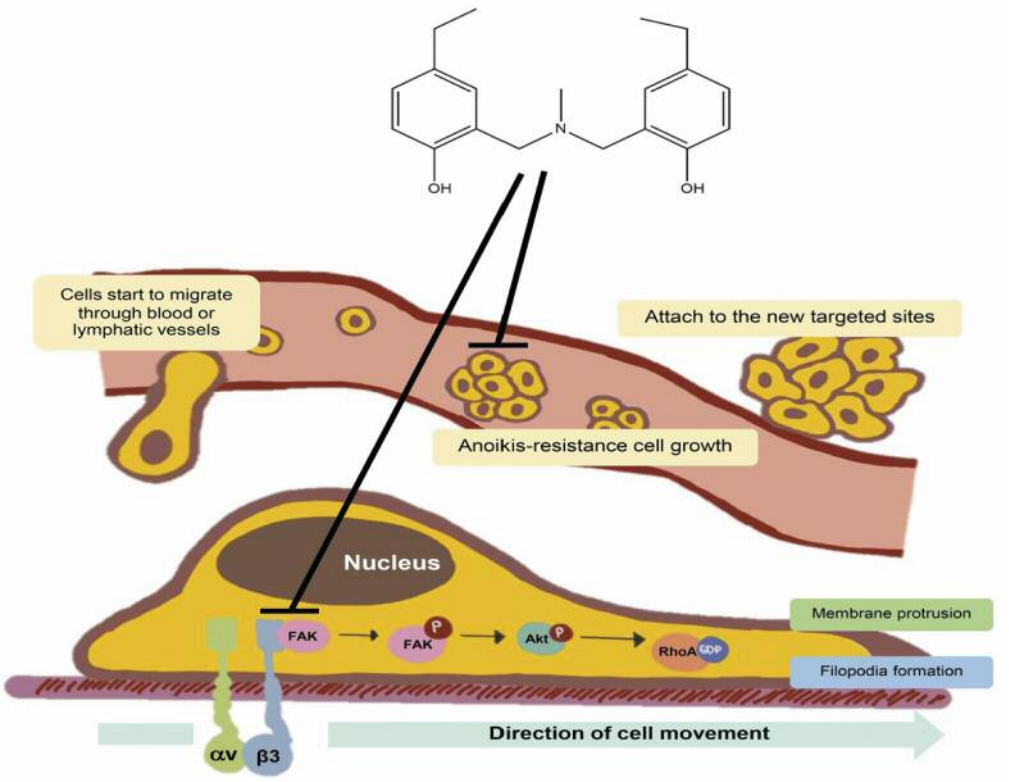

Figure 4. *Cell migration is one of the multiple steps of metastasis. HM suppresses integrin β3 which is involved in cell migration process through downstream signaling to FAK, Akt, and Rho A. In the absence of stimulated signals, filopodia formation is inhibited and cell migration process is arrested. HM inhibited cell movement and attenuated anoikis-resistant cell growth.*

During metastasis, most of the solid tumor cells will undergo apoptosis after detachment (7). The detachment-induced apoptosis termed anoikis has been shown to be a critical inhibitor of successful metastasis in many cancers (7), so metastatic cancer cells must acquire mechanisms to escape from anoikis (6, 28). Here, we demonstrated that HM inhibited cancer cell migration and anchorage-independent cell growth *via* suppression of integrin β3. In agreement with our finding, previous studies have demonstrated that down-regulation of integrin and integrin signaling pathway caused cell migration arrest (29, 30). We observed that HM-mediated suppression of integrins was specific to integrin β3, as integrins β1 and α5 were not affected. In addition, the integrin-associated downstream proteins, p-FAK, p-Akt and RhoA were found to be depleted (Figure 3). HM inhibited also anchorage-independent colony formation and reduced colony size (Figure 1E and F).

In summary, we evaluated the effect of HM against migration and anchorage-independent growth of lung cancer cells. Migration activity of the cells was inhibited by targeting integrin β3 leading to the downregulation of active FAK (p-FAK), active Akt (pAkt) and RhoA. Furthermore, HM reduced the ability of the cells to survive and grow in the detached condition when used at non-toxic concentrations (Figure 4). These data indicate HM as a candidate for anticancer approaches.

Conflicts of Interest

The Authors declare that we have no conflicts of interest regarding this study.

Authors' Contributions

NS performed experiments, analyzed the data, and wrote the manuscript; NN performed experiments; WW synthesized HM compound; PC designed the study, analyzed the data, and edit the manuscript.

Acknowledgements

The Scholarship from the Graduate School, Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibol Adulyadej is gratefully acknowledged. This project was financially supported by Chulalongkorn Academic Advancement under its second century fund (C2F).

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Received March 9, 2020
Revised March 18, 2020
Accepted March 19, 2020