ANTICANCER RESEARCH *37*: 615-622 (2017)
doi:10.21873/anticanres.11355

Renieramycin M Attenuates Cancer Stem Cell-like Phenotypes in H460 Lung Cancer Cells

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Abstract. *Background/Aim: Cancer stem cells (CSCs) are a subpopulation of cancer cells that possess self-renewal and differentiation capacities. CSCs contribute to drug-resistance, cancer recurrence and metastasis, thus development of CSC-targeted therapeutic strategies has recently received significant attention in cancer research. In this study, the potential efficacy of renieramycin M (RM) isolated from the sponge Xestospongia species, was examined against lung CSCs. Materials and Methods: Colony and spheroid formation assays, as well as western blotting analysis of lung CSC protein markers were employed to determine the CSC-like phenotypes of H460 lung cancer cells after treatment with RM at non-toxic concentrations. Results: RM treatment reduced significantly colony and spheroid formation of H460 cells. Moreover, the CSC markers CD133, CD44 and ALDH1A1 of CSC-enriched H460 cells were reduced significantly following RM treatment. Conclusion: RM could be a potent anti-metastatic agent by suppressing lung CSC-like phenotypes in H460 cells.*

Cancer stem cells (CSCs) are a subpopulation of cancer cells possessing self-renewal capability and pluripotency. The existence of CSCs was first proven in human acute myeloid leukemia in 1997 (1) and was further extended to a broad spectrum of solid tumor types, including lung cancer (2-7). In general, lung cancer has been accepted as one of leading causes of cancer deaths worldwide (8) with a high degree of metastasis (9-10). A number of studies show that CSCs within the tumors are major contributors towards drug-resistance, cancer recurrence and metastasis following chemotherapy (11-13). Since the clinical outcomes under current chemotherapeutic agents in non-small cell lung cancer (NSCLC) are not in a satisfactory level (5-year survival is less than 15%) (9), research for novel lung CSC-targeted therapies is highly needed in order to improve clinical outcome.

The main characteristics of CSCs are the abilities to form colonies in an anchorage-independent condition and grow indefinitely as detached tumor spheroids in a serum-free condition (4, 7, 14-16). Moreover, several studies have identified CD133, CD44 and aldehyde dehydrogenase (ALDH) as CSC markers, which are commonly used to distinguish CSCs from non-CSC populations (7, 11, 15-16). CD133 is a potential marker for clinical prognosis of lung cancer. CD133-positive NSCLC patients had worse 5-year overall survival compared to the CD133-negative expression (17). CD133-positive cancer cells have been found in several lung cancer subtypes (7) and shown higher tumorigenicity and chemoresistance than their CD133-negative counterparts (14). CD44 expression is commonly used as a marker for lung CSCs, and stem cell-like characteristics are enriched in CD44 positive cells of lung cancer cell lines (11, 15). Aldehyde dehydrogenase 1 (ALDH1) is another cell surface marker connected with stem cell-like properties in NSCLC cell lines (18). ALDH1A1 expression is associated with poor clinical outcome in NSCLC patients (16).

Most current anticancer agents are focused on the bulk of cancer cells. Interestingly, a number of natural products such as curcumin (19), piperine (19), sulforaphane (20), 6-shogaol (21) and gigantol (22) have recently been reported to target CSCs. Renieramycin M (RM) is a major *bis*tetrahydroiso-

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Key Words: Renieramycin M, cancer stem cell, lung cancer, anti-metastasis.

quinolinequinone alkaloid isolated from the Thai blue sponge *Xestospongia* sp., and it was reported to show potent cytotoxicity against several human cancer cell lines (23-25). In this study, the inhibitory effects that RM may have, were analyzed on CSCs of human H460 NSCLC cells. CSC-like phenotypes, including colony and spheroid formations as well as cell surface CSC markers, were evaluated after RM treatment.

Materials and Methods

Renieramycin M (RM). RM was isolated from the Thai blue marine sponge *Xestospongia* sp. as previously reported (23). RM was dissolved in dimethyl sulfoxide (DMSO, RCI Labscan, Bangkok, Thailand) and 10 mM stock solution of RM was further diluted in serum-free medium to achieve concentrations containing less than 0.2% DMSO.

Cells and reagents. Human non-small cell H460 lung cancer cells and immortalized hair follicle dermal papilla (DP) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and Applied Biological Materials Inc. (Richmond, BC, Canada), respectively. H460 and DP cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium and Dulbecco's modified Eagle's medium (DMEM) medium, respectively. The media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco, Carlsbad, CA, USA). Cell cultures were incubated in humidified atmosphere of 5% CO_2 at 37˚C.

Cell viability assay. Cell viability was determined by the water-soluble tetrazolium salt (WST) assay according to the manufacturer's instruction (Roche Diagnostic GmbH, Mannheim, Germany). H460 and DP cells were seeded at a density of 1×10^5 cells/ml in Costar 96-well plate (Corning, New York city, NY, USA) and allowed to adhere overnight. The cells were treated with different concentrations of RM (0-1 μM) for 24 h. The detailed experimental procedure was described in a previous study (26). The percentage of cell viability was calculated relative to the non-treated control value. Three concentrations of RM that were not significantly toxic to attached H460 cells were chosen for further experiments on lung CSC like-phenotypes.

Colony formation assay. Anchorage-independent cell growth was determined in two-layer soft agar (27). To prepare the lower layer, equal volumes of melted 1% agarose (Bio-Rad, Hercules, CA, USA) and the cultured RPMI medium were mixed (1:1), and then 500 μl of the mixture was put in a 24-well plate and allowed to solidify at 4˚C for 15 min. To prepare the upper layer, melted 1% agarose and cultured RPMI medium containing anoikis-resistant H460 cells at a density of 4×10^3 cells/ml and subtoxic concentrations of RM (0-0.1 μM) were mixed (1:2) and then 250 μl of the mixture was added as an upper layer. After the upper layer solidified, the cultured medium was added over the upper layer and incubated at 37˚C for two weeks. Fresh culture medium (200 μl/well) was fed to the system every three days. Colony formation was photographed at day seven and day fourteen using a Nikon inverted phase contrast light microscope (Tokyo, Japan) at ×40 magnification equipped with a Sony NEX-5 camera (Tokyo, Japan). Relative colony number and size were determined using ImageJ 1.48v software (http://imagej.nih.gov/ij/index.html, Bethesda, MD, USA) with respect to the non-treated control cells.

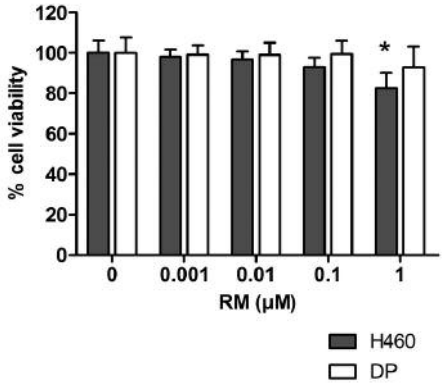

Figure 1. *Cytotoxic effect of renieramycin M (RM) on H460 lung cancer and normal dermal papilla (DP) cells under normal culturing conditions. Cells were treated with different concentrations of RM (0-1 μM) for 24 h. Cell viability was determined by WST assay. Values are means±SD calculated as percentages compared to the non-treated control value. Asterisk indicates statistically significance at p<0.05 threshold versus the non-treated control.*

Spheroids formation assay. H460 cells were seeded in a 24-well plate with ultra-low attachment surface at a density of 5×10^3 cells/ml in serum-free RPMI medium. Cells were treated with non-toxic concentrations of RM (0-0.1 μM) and incubated at 37˚C. Fresh serum-free medium was added to the system every three days. Primary spheroids were allowed to form and photographed at day seven after treatment using an inverted phase contrast light microscope (Nikon) at ×40 magnification equipped with a camera (Sony NEX-5). These primary spheroids were re-suspended into single cells and seeded in a 24-well ultra-low attachment plate. Secondary spheroids were allowed to form in the absence of RM and photographed at day fourteen, day twenty-one, and day thirty after RM treatment.

Western blot analysis. Anoikis-resistant H460 cells in a detached condition established by culturing H460 cells on an ultra-low attachment plate for 48 h (26) were treated with non-toxic concentrations of RM (0-0.1 μM), cultured and harvested at 24 h. Western blotting was analyzed according to method of our previous study (26). Primary antibodies to CD44 (Cell Signaling Technology, Danvers, MA, USA), CD133 (Cell Applications, San Diego, CA, USA), and ALDH1A1 (Cell Signaling Technology) were incubated with the membranes at 4˚C for two days. After washing the membranes with TBST, the secondary antibody, either horseradish peroxide-conjugated to anti-rabbit or to anti-mouse IgG (Cell Signaling Technology), was incubated for 2 h at room temperature. The immune complexes were detected using a SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA) and exposed to carestream medical X-ray blue/MXB film (Rochester, NY, USA). Relative protein levels were quantified by densitometric analysis using an ImageJ 1.48v software. The blots were reprobed with α-tubulin (Cell Signaling Technology) to confirm equal amounts of loaded proteins in each treatment.

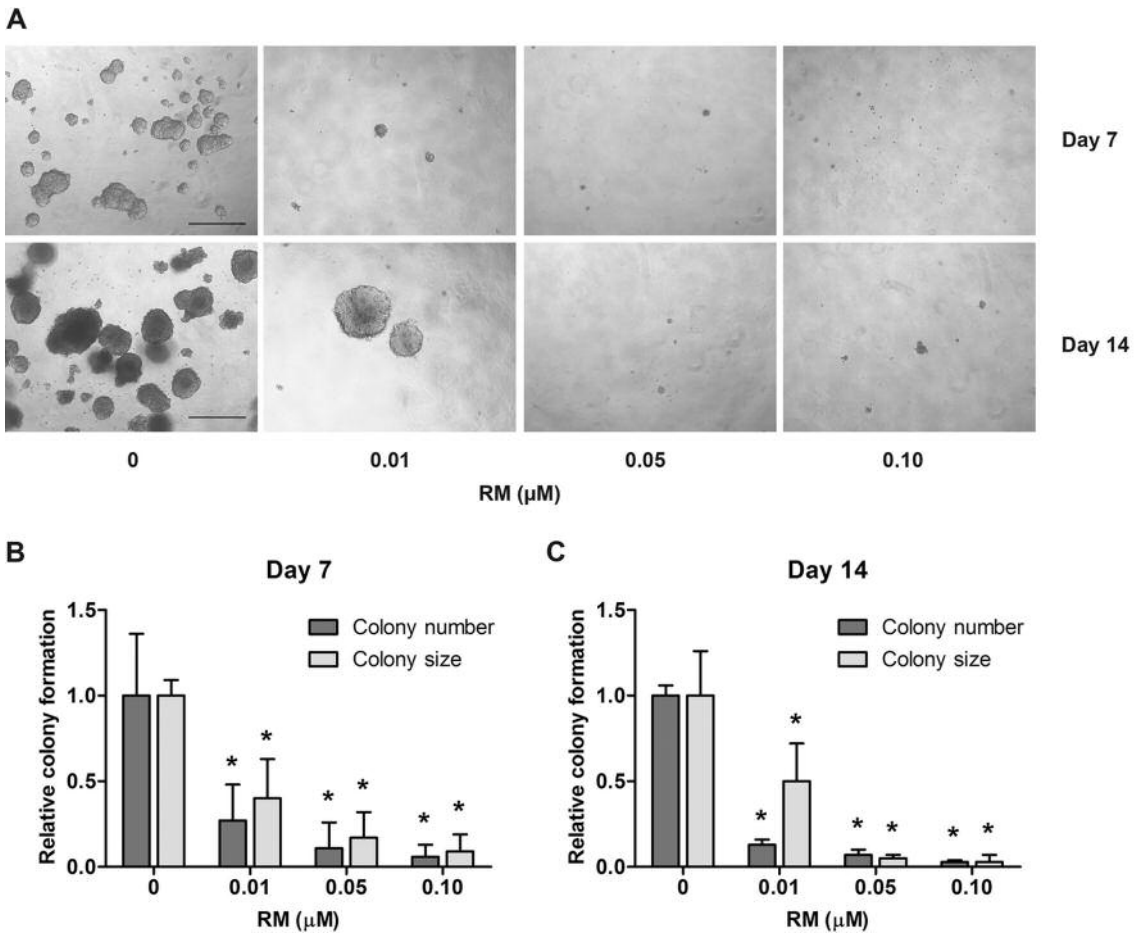

Figure 2. *Colony formation of anoikis-resistant H460 lung cancer cells treated with RM at non-toxic concentrations. (A) Cells were subjected to soft agar colony assay and treated with RM (0-0.1 μM). The colony formation was observed using an inverted phase contrast light microscope with ×40 magnification at day seven and day fourteen. Scale bar is 500 μm. Colony number and size at day seven (B) and day fourteen (C) were analyzed and calculated as relative values to the non-treated control value. Asterisks indicate statistically significance at p<0.05 threshold versus the non-treated control.*

Statistical analysis. Data were expressed in means±standard deviation (±SD) obtained from at least three independent experiments. Statistical differences were performed using one-way ANOVA with Turkey's *post hoc* test at a significance level of $p<0.05$. IBM SPSS statistics version 20 (IBM Company, New York city, NY, USA) was used for all statistical analyses.

Results

Effect of RM on viability of H460 lung cancer and DP normal cells under attached conditions. To evaluate the effects of RM on CSC-like phenotypes, a range of concentrations of the compound were initially evaluated on attached H460 lung cancer and DP normal cells (Figure 1). Cells were treated with different concentrations of RM (0-1 μM) for 24 h and cell viability was measured by the WST assay. The results showed that RM was considered not significantly toxic at the concentrations of 0-0.1 μM for both H460 and DP cells. However, RM at 1 μM was considered toxic to H460 cells (viability 83±8%) but non-toxic to DP cells (viability 93±10%).

RM treatment suppressed colony formation. Anoikis-resistant cells were established by culturing H460 cells on an ultra-low attachment plate for 48 h. Anoikis-resistant H460 cells were subjected to soft agar colony-formation assay as a single-stage cell and treated with non-toxic concentrations of RM (0-0.1 μM). Colony formation was observed at day seven and day fourteen after treatment and representative images are shown in Figure 2A. Colony number and size were counted and presented as relative values in comparison to those of non-treated control at day seven (Figure 2B) and day fourteen (Figure 2C). Even

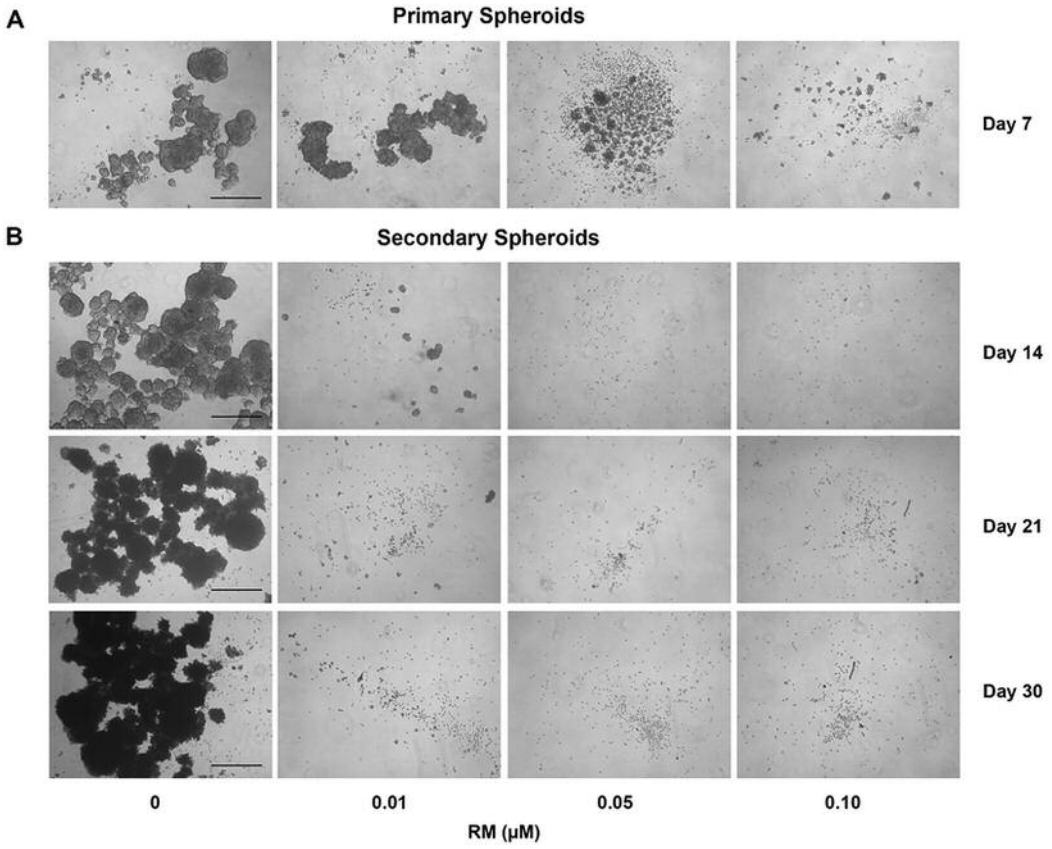

Figure 3. *Spheroid formation of H460 lung cancer cells treated with RM at non-toxic concentrations. Cells at a low density in a serum-free condition were subjected to spheroid formation assay and treated with RM (0-0.1 μM). (A) The primary spheroids were observed using an inverted phase contrast light microscope with ×40 magnification at day seven. (B) The secondary spheroids were allowed to form and observed at day fourteen, day twenty-one and day thirty. Scale bar is 500 μm.*

though non-treated control cells showed an ability to survive and grow in an anchorage-independent condition, treating cells with RM significantly suppressed colony formation in a dose-dependent manner in colony number and in size for both observed days.

RM suppressed spheroid formation. To evaluate whether RM could suppress spheroid formation, H460 cells were seeded as single-cells in low density and in serum-free RPMI medium treated with non-toxic concentrations of RM (0-0.1 μM). The primary spheroids were allowed to form for seven days in a detached condition and then re-suspended into single cells and cultured for an additional passage under the absence of RM. The secondary spheroids were allowed to form for thirty days in a detached condition. As shown in Figure 3, non-treated control cells have an ability to survive and form spheroids in a serum starvation condition for long-term period. The number of secondary spheroids represented the presence of CSCs in H460 cells regarding their self-renewal ability and pluripotency. Interestingly, RM at concentrations of 0.01-0.10 μM significantly suppressed the formation of primary spheroids in dose-dependent manner and completely abolished the formation of secondary spheroids after day thirty.

RM reduced cancer stem cells (CSCs) markers. Having shown that treatment of lung cancer cells with RM significantly suppressed the ability of cells to survive and grow in an anchorage-independent manner as well as to form spheroids, the expression of CSC-specific protein markers in these cells in response to RM were investigated. Anoikis-resistant cells were established by culturing H460 cells on an ultra-low attachment plate for 48 h and were treated with non-toxic concentrations of RM (0-0.1 μM) for 24 h. The expression of CSC markers including CD133, CD44, and ALDH1A1 was evaluated by western blot analysis (Figure 4). The results showed that the expression of CD133, CD44, and ALDH1A1 significantly decreased in RM-treated cells at 0.1 μM compared to those of non-treated control.

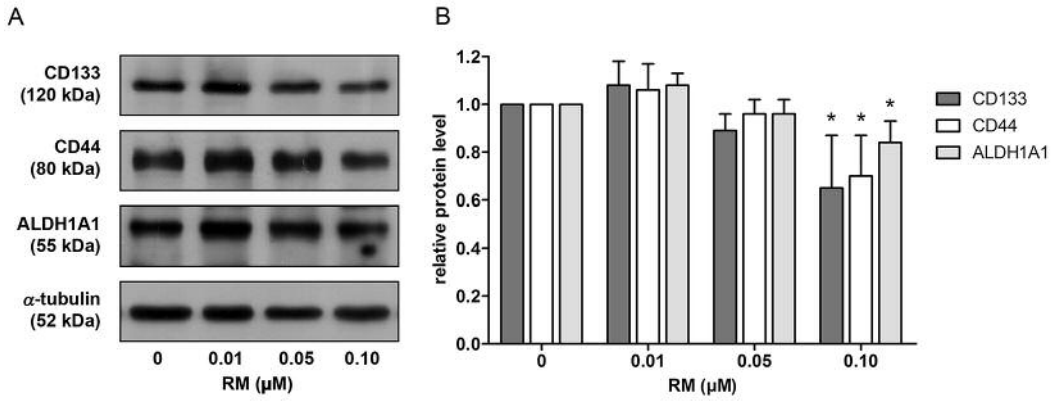

Figure 4. *Expression of the lung cancer stem cell markers of anoikis-resistant H460 lung cancer cells treated with RM at non-toxic concentrations. (A) Anoikis-resistant cells were established by culturing H460 cells on an ultra-low attachment plate for 48 h (26). Cells that resist to anoikis were collected for this experiment. After cells were treated with RM (0-0.1 μM) for 24 h, the indicated antibodies were analyzed by western blot analysis. (B) Relative protein levels were quantified by densitometric analysis. Values are means±SD calculated as relative values to the non-treated control value. Asterisks indicate statistically significance at p<0.05 threshold versus the non-treated control.*

Discussion

Lung cancer remains the most common cause of cancer-related deaths worldwide (8). Limited outcomes of current chemotherapies are related to the metastatic potential of lung cancer (9, 14, 28). These might be due to the presence of CSCs in lung cancer (29). A small subpopulations of cancer cells with CSC properties have been identified within various cancers (1-7). With a major role of CSCs in treatment failure and cancer recurrence (11-13), CSCs-targeting therapeutic strategy is an attractive approach for a more effective cancer treatment. Based on the self-renewal capability and pluripotency, CSCs can initiate tumor development and maintain tumor growth. The colony- and spheroid-forming assays are commonly used to demonstrate self-renewal of CSCs (4, 7, 14-16) and assess the inhibitory activity against CSCs of anticancer agents (19-22). CSCs possess high clonogenicity and produce more soft agar colonies compared to non-CSCs (4, 15-16). Long-term cultures of spheroid-forming cells in the serum-free medium enrich with CSCs and exhibit high tumorigenicity (7, 14-15). It has been demonstrated that the spheroids enhance the production of key angiogenic cytokines, preservation of extracellular matrix components, activation of survival signals as well as inhibition of apoptotic proteins leading to increase in survival and proliferation in culture time- and spheroid-size-dependent manners (30). Furthermore, lung CSCs can be characterized by specific cell surface markers including CD133, CD44, and ALDH1A1 (7, 11, 15-16). Lung cancer patients with high level of these CSC markers are related to poor prognosis and overall survival (7, 31). The identification of CSCs may provide a tool to investigate new anticancer drugs targeted to CSCs.

Previous research showed that RM, a major *bis*tetrahydroisoquinolinequinone alkaloid isolated from the blue sponge *Xestospongia* sp. (23), exhibited potent cytotoxicity against several human cancer cell lines, including H460 NSCLC (23-25). In addition, RM exhibited other related anticancer activities, including anoikis sensitization, inhibition of migration, invasion, and anchorage-independent growth in H460 NSCLC cells (32). Recently, our study has reported that RM is effective in targeting anoikis-resistant H460 NSCLC cells at concentrations of 0.01-0.10 μM, which are not significantly toxic to the cancer cells in a normal culturing condition. Anoikis resistance plays an important role in promoting the survival of circulating tumor cells, which is one of the keys to cancer metastasis (26). In the present investigation, the effects of RM on CSCs in H460 cells were examined. We found that treatment of H460 cells with RM at non-toxic concentrations (0.01-0.10 μM) resulted in the decrease of CSCs indicated by the reduction of colony and spheroid formations along with the down-regulation of lung CSC-specific markers. RM markedly decreased colony-forming activity of CSCs in terms of colony number and size at both observed day seven and day fourteen. RM also suppressed the formation of primary spheroids at day seven and completely abolished the formation of secondary spheroids after day thirty. Having shown that RM suppressed CSC-like phenotypes in H460 cells, we next confirmed these observations by using well-known lung CSC markers, including CD133, CD44, and ALDH1A1. The expression of these markers decreased in a statistically significant manner only when cells were treated with the highest non-toxic RM concentration of 0.1 μM. Even though, this result may not strongly support that RM decreases colony and spheroid-

forming ability via direct targeting of CSCs, it is indicating a potential role of RM in the treatment of CSCs in H460 cells at 0.1 μM.

In conclusion, RM plays a role in the attenuation of CSC-like phenotypes in H460 lung cancer cells by the reduction of colony- and spheroid-forming activities, along with a less significant decrease of lung CSC markers. Our findings indicated that RM might be a potential candidate for prevention and treatment of metastasis in patients with lung cancer.

Acknowledgements

Financial support from the Grant for International Research Integration: Chula Research Scholar, Ratchadaphiseksomphot Endowment Fund to P.C. and Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0276/2552) to N.S. and K.S.

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Received December 7, 2016
Revised January 17, 2017
Accepted January 19, 2017