Renieramycin M Sensitizes Anoikis-resistant H460 Lung Cancer Cells to Anoikis

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Abstract. Background/Aim: Anoikis resistance plays a crucial role in the promotion of survival of circulating tumor cells. This study aimed to evaluate the mechanistic pathways of anoikis resistance in human lung cancer cells and test the possible therapeutic effect of renieramycin M (RM) from the sponge Xestospongia sp. in conversion of anoikis resistance. Materials and Methods: Anoikis-resistant H460 (AR_H460) lung cancer cells in a detached condition were treated with RM at subtoxic concentrations for 24 h. Cell viability, cell morphology, and expression of the proteins involved in survival and apoptotic pathways were determined. Results: Anoikis resistance in H460 cells is mediated through the up-regulation of survival and anti-apoptotic proteins, namely phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated ATP-dependent tyrosine kinase (p-AKT), B-cell lymphoma-2 (BCL2), and myeloid cell leukemia-1 (MCL1). RM significantly reduced cell viability and inhibited spontaneous aggregation of AR_H460 cells. Western blot analysis revealed that RM suppressed the levels of survival proteins p-ERK and p-AKT and anti-apoptotic proteins BCL2 and MCL1. Conclusion: RM is a potential anti-metastatic agent by sensitizing anoikis-resistant lung cancer cells to anoikis by the suppression of anoikis-resistance mechanisms.

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In order to prevent cancer metastasis, our group focuses on searching for natural products with a capability of sensitizing anoikis-resistant cells to anoikis. Renieramycin M (RM), a bistetrahydroisoquinolinequinone alkaloid isolated from the Thai blue sponge Xestospongia sp., has been reported to possess highly potent cytotoxicity against several human cancer cell lines, including lung, colon, prostate, breast, pancreatic, and oral cancer cells (16-19). However, the effects on anoikis-resistant cells are still unknown. In this study, anoikis-resistant H460 lung cancer cells were established and used as a model of CTCs. The effects of RM on cell viability and cell morphology, and the role of survival and apoptotic proteins in the anoikis-resistant lung cancer cells were investigated.

Materials and Methods

Renieramycin M (RM). RM (Figure 1) was isolated from the Thai blue marine sponge Xestospongia sp. as previously reported, and its chemical structure was determined by comparison of the spectroscopic data with the literature (16). RM was dissolved in dimethyl sulfoxide (DMSO; RCI Labscan, Bangkok, Thailand) as a 10 mM stock solution and diluted with 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. Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Carlsbad, CA, USA) was used for the culture of H460 cells and Dulbecco’s modified Eagle’s medium (DMEM) used for the culture of DP cells 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 a humidified incubator at 37˚C in an atmosphere of 5% CO2. Primary antibodies to total ERK, phosphorylated ERK (p-ERK), total AKT, phosphorylated AKT (p-AKT), BCL2, MCL1, BAX, and α-tubulin and a secondary antibody: horseradish peroxidase (HRP)-linked anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA).

Anoikis-resistant cells. Anoikis-resistant H460 cells (AR_H460) were established according to the method of Winitthana et al (20). Briefly, attached H460 cells were trypsinized with 0.05% trypsin/0.02% EDTA (Gibco). Then the single-cell suspension in serum-free RPMI medium at a density of 2x10⁵ cells/ml was seeded in a Costar 6-well plate with ultra-low attachment surface (Corning) at a density of 1x10⁵ cells/ml and allowed to adhere overnight in cultured RPMI and DMEM media, respectively. After the culture media were removed, cells were treated with different concentrations of RM (0-1 μM) and cultured in the respective serum-free media for 24 h. Following the treatment, cells were incubated with 10% WST-1 for 2 h at 37˚C. The intensity of the formazan product was determined by a VICTOR3 multilabel plate reader (PerkinElmer, Waltham, MA, USA) at 450 nm. Cell viability was calculated from optical density (OD) readings and represented as percentages with respect to the non-treated control value. Three concentrations of RM subtoxic to attached H460 cells were used for cell viability, cell morphology, and western blot assays on AR_H460 cells.

Cell viability assay of detached cells and anoikis assay. Cell viability was determined by WST assay. Cells in serum-free RPMI medium were seeded in a Costar 96-well plate with ultra-low attachment surface (Corning) at a density of 1x10⁵ cells/ml and allowed to adhere overnight in cultured RPMI and DMEM media, respectively. After the culture media were removed, cells were treated with subtoxic concentrations of RM (0-0.1 μM) and cultured in the respective serum-free media for 24 h. H460 cells in a detached condition were cultured and harvested at 0, 6, 12, 24, and 48 h for cell viability assay. AR_H460 cells in a detached condition were treated with subtoxic concentrations of RM (0-0.1 μM) and harvested at 0, 6, 12, 24, and 48 h for cell viability assay. The harvested cells were incubated with 10% WST-1 for 2 h at 37˚C. The intensity of the formazan product was measured at 450 nm using a plate reader. Cell viability was calculated from OD readings and represented as percentages with respect to the non-treated control value.

Cell morphology. Cells in serum-free RPMI medium were seeded in a 24-well plate with ultra-low attachment surface at a density of 2x10⁵ cells/ml. H460 cells in a detached condition were cultured and observed at 0, 24, and 48 h. AR_H460 cells in a detached condition were treated with subtoxic concentrations of RM (0.01 μM) and cultured for 24 h. Cell morphology was observed using a Nikon inverted phase contrast light microscope (Nikon, Tokyo, Japan) at ×40 and ×100 magnification equipped with a Sony NEX-5 camera (Sony, Tokyo, Japan).

Figure 1. Chemical structure of renieramycin M.
Western blot analysis. Cells in serum-free RPMI medium were seeded in a 6-well plate with ultra-low attachment surface at a density of \(2 \times 10^5\) cells/ml. H460 cells in a detached condition were cultured and harvested at 0, 24, and 48 h. AR_H460 cells in a detached condition were treated with subtoxic concentrations of RM (0-0.1 μM), cultured and harvested at 24 h. The cells were harvested by centrifugation at \(2,690 \times g\) for 10 min at 4°C and incubated in lysis buffer for 1 h on an ice box. Cell lysates were collected by centrifugation at \(6,720 \times g\) for 1 min at 4°C, and protein contents were analyzed using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). An equal amount of protein from each sample (60 μg) was denatured by heating at 95°C for 5 min with Laemmli loading buffer and loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The transferred membranes were blocked with 5% skim milk (Sigma-Aldrich, St. Louis, MO, USA) in TBST [20 mM Tris–HCl (pH 7.5), 138 mM NaCl, and 0.1% Tween 20] for 1 h at room temperature. The membranes were then washed with TBS and incubated with specific primary antibodies at 4°C overnight. After washing with TBST for 5 min (three times) to remove non-specific binding of the antibody, the membranes were further incubated with HRP-linked anti-rabbit IgG for 2 h at room temperature. The immune complexes were detected using a SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology) 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 (http://imagej.nih.gov/ij/index.html, Bethesda, MD, USA). To confirm equal amounts of loaded proteins, α-tubulin was re-probed as a loading control in each treatment.

Statistical analysis. All data were obtained from at least three independent experiments and presented as means±standard deviations (SD). Statistical differences were performed using one-way ANOVA with Turkey’s post hoc test, unless otherwise stated, at a significance level of \(p<0.05\). IBM SPSS statistics version 20 (IBM Company, New York, NY, USA) was used for all statistical analyses.

Results

Characterization of anoikis-resistant H460 lung cancer cells. We first characterized the anoikis response of the lung cancer cells by culturing the cells in a detached condition for 0-48 h and cell viability at different times was determined. As shown in Figure 2A, cell viability gradually declined until 24 h after detachment and approximately 60% of cells remained viable for a further 24 h. Therefore, we isolated the live cells after the first 24 h, and these anoikis-resistant H460 cells were named as AR_H460. Cell morphology in detached AR_H460 cells at 24 and 48 h was observed and compared to that of their parental H460 cells at 0 h, as shown in Figure 2B. Interestingly, we found AR_H460 cells spontaneously formed multicellular aggregations while their parental H460 cells mostly exhibited a single-stage cell pattern.

To study the proteins involved in anoikis resistance, the expression of the survival and apoptotic proteins in H460 and AR_H460 cells at 24 and 48 h under detached conditions was examined as illustrated in Figure 3. It is postulated that the declining viability of H460 cells during 0-24 h might mainly involve the reduced expression of the survival protein p-AKT, while the levels of the anti-apoptotic protein MCL1 and pro-apoptotic protein BAX were unchanged. Interestingly, it is clear that anoikis-resistant response of AR_H460 cells was mediated through the significant increase of the activation of ERK and AKT as well as the anti-apoptotic proteins BCL2 and MCL1 at 48 h compared with that at 24 h.

Effect of RM on viability of H460 lung cancer and DP normal cells under attached conditions. To determine the appropriate concentrations for anoikis-resistant assay, concentrations of RM subtoxic to attached H460 lung cancer and DP normal cells were evaluated (Figure 4A). Cells were incubated with
different concentrations of RM (0-1 μM) for 24 h, and cell viability was analyzed by WST assay. The results showed that RM at concentrations of 0-0.1 μM was non-toxic to both attached H460 and DP cells. Furthermore, RM at 1 μM was considered toxic to H460 cells but non-toxic to DP cells.

**Effect of RM on viability of AR_H460 lung cancer cells.** AR_H460 cells in a detached condition were established by culturing H460 cells on an ultra-low attachment plate for 48 h. AR_H460 cells were treated with subtoxic concentrations of RM (0-0.1 μM) and harvested at different times. Cell viability was evaluated by the WST assay. Figure 4B shows that viability of non-treated AR_H460 cells was unchanged over the entire 48-h period. Interestingly, RM sensitized AR_H460 cells to anoikis in both time- and dose-dependent manners. RM at concentrations of 0.01-0.1 μM significantly reduced cell viability at 24 h, whilst cell viability was significantly decreased as early as 12 h after treatment with 0.1 μM RM, compared to the non-treated control.

**Effect of RM on cell morphology of AR_H460 lung cancer cells.** AR_H460 cells in a detached condition were treated with subtoxic concentrations of RM (0-0.1 μM) and cultured in a plate with ultra-low attachment surface for 24 h. Cell morphology was observed under an inverted phase-contrast light microscope (Figure 5). Non-treated AR_H460 cells spontaneously formed large aggregates. Treatment of AR_H460 cells with RM resulted in a dose-dependent suppression of aggregate formation.

RM sensitizes AR_H460 lung cancer cells to anoikis by decreasing anoikis-resistant pathways. To investigate the mechanisms of RM in sensitizing AR_H460 cells to anoikis, AR_H460 cells in a detached condition were treated with subtoxic concentrations of RM (0-0.1 μM) for 24 h. The expression of the survival proteins p-ERK, total ERK, p-AKT, and total AKT, anti-apoptotic proteins BCL2 and MCL1, and pro-apoptotic protein BAX was evaluated by western blot analysis. Figure 6 shows that the levels of p-ERK, p-AKT, total AKT, BCL2, and MCL1 were decreased in response to RM treatment in a dose-dependent manner. Treatment of RM at 0.01 μM resulted in a significant decrease of p-AKT and BCL2 levels, while a higher concentration of RM at 0.05 μM reduced the expression of total AKT and MCL1. Likewise, the protein level of p-ERK was only slightly reduced by RM at 0.1 μM. However, the expression of total ERK and BAX was not significantly altered.
Discussion

Lung cancer is a significant cause of cancer-related death worldwide, with the majority of such a mortality being due to metastasis (1, 2). To metastasize, cancer cells must have the capability to resist anoikis and survive within the circulatory system, and to initiate new tumors in distant secondary organs. Anoikis resistance plays an important role in promoting the survival of CTCs, which is one of the keys to cancer metastasis. Although fewer than 1% of cancer cells are still viable within 24 h after entering the circulation, very few surviving cells can give rise to metastases (21, 22). Not surprisingly, the presence of a high number of CTCs in blood is associated with poor survival in patients with lung metastatic cancer (23). Targeting anoikis resistance in CTCs might represent a promising approach to reducing metastasis, thereby improving patient survival.

In this study, we employed anoikis-resistant H460 (AR_H460) lung cancer cells as a model of CTCs. Acquisition of anoikis resistance can be successfully developed in H460 lung cancer (12, 20, 24) by cell culturing under detached conditions. AR_H460 cells exhibited characteristics of anoikis resistance with unchanged cell viability for 24-48 h period after detachment and increased aggregate formation. These results were consistent with a previous study reporting that spontaneous aggregate formation of detached cells correlated with cell survival and
Although the mechanisms of anoikis resistance are still largely unknown, many underlying mechanisms have been proposed, and the survival and apoptotic pathways were mentioned as being implicated most frequently (3-5, 10, 11). Our study showed that the survival proteins activated ERK and AKT, and anti-apoptotic proteins BCL2 and MCL1 were upregulated, and such proteins were likely responsible for anoikis resistance in these human lung cancer cells. Consistently, overexpression of BCL2 has been shown to be a marker of CTCs in patients with metastatic cancer (8, 13). The activation of ERK and AKT signals in these cells promotes cell survival, in part, through the increase of BCL2 protein. Furthermore, several studies indicated that the inhibition of ERK and AKT signals, resulting in the decrease of BCL2 and MCL1, is able to restore anoikis sensitivity in several cancer types (14, 26, 27).

Our investigations demonstrated that RM at subtoxic concentrations significantly reduced viability and suppressed spontaneous aggregate formation of AR_H460 cells. The molecular mechanisms indicate that RM sensitized AR_H460 cells to anoikis through suppression of the survival proteins activated ERK and AKT, resulting in reduction of expression of the anti-apoptotic proteins BCL2 and MCL1 (Figure 7). Inhibition of BCL2 and MCL1 protein expression of AR_H460 cells in a detached condition by RM were consistent with our previous finding for H460 cells in an attached condition (28).

In conclusion, our findings highlight the potent effects of RM, a bistetrahydroisoquinolinequinone alkaloid isolated from the Thai blue sponge *Xestospongia* sp., on sensitization of anoikis-resistant lung cancer cells to anoikis through reduction
of the survival proteins activated ERK and AKT and anti-apoptotic proteins BCL2 and MCL1. Interestingly, the concentrations used caused no effect on viability of attached H460 and normal DP cells. A better understanding of molecular mechanisms of RM involved in anoikis resistance would assist in the development of anticancer drugs to kill CTCs and prevent metastasis in patients with lung cancer.

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References