Anticancer and Antimetastatic Activities of Renieramycin M, a Marine Tetrahydroisoquinoline Alkaloid, in Human Non-small Cell Lung Cancer Cells

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Abstract. *Background: Renieramycin M, has been shown to exhibit promising anticancer activity against some cancer cell lines; however, the underlying mechanism remains unknown. Materials and Methods: Renieramycin M was isolated from the blue sponge Xestospongia sp. Anticancer and antimetastatic activities of renieramycin M were investigated in human non-small cell lung cancer cells. Results: Renieramycin M treatment caused p53 activation, which subsequently down-regulated anti-apoptotic MCL-1 and BCL-2 proteins, while the level of pro-apoptotic BAX protein was not altered. The subtoxic concentrations of renieramycin M significantly decreased invasion and migration abilities of cancer cells. In addition, this compound showed a strong inhibitory effect on anchorage-independent growth of the cells. Conclusion: These results reveal that renieramycin M induced lung cancer cells apoptosis through p53-dependent pathway and the compound may inhibit progression and metastasis of lung cancer cells.*

The efficient prevention of and therapy for cancer is a focus of modern cancer research. Strategies in the treatment of many types of cancer frequently fail because of the resistance of cancer cells to chemotherapeutic treatments.

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Additionally, cancer cell metastasis causes difficulty since the metastatic cancers cannot be completely cured by surgery. Metastasis is often responsible for cancer-related death which frequently occurs in lung cancer patients (1). In order to metastasize, tumor cells must have an ability to overcome anoikis or apoptosis induced by loss of cell adhesion (2). Since anoikis is a principle mechanism in inhibition of cancer cell metastasis, attenuating or suppressing this cellular event by anoikis resistance mechanism(s) occurring in some cancer cells will allow the detached cell to survive and facilitate the secondary tumor formation. In addition to anoikis resistance, the ability of tumor cells to migrate and invade through the extracellular matrix is an inherant feature of determining the aggressiveness of cancer. Therefore, the ability of anti-cancer compounds to inhibit anoikis resistance or sensitize cancer cells to anoikis and attenuate cancer cell aggressiveness might have a significant beneficial impact on cancer therapy.

As part of the investigation for biologically active natural products from Thai marine organisms, renieramycin M, a major *bis*tetrahydroisoquinolinequinone alkaloid, was recently isolated from the blue sponge *Xestospongia* sp. in a gram-scale supply (3). Renieramycins are a group of tetrahydroisoquinoline marine natural products which are structurally and biologically related to saframycins, naphthyridinomycins, quinocarcins, and ecteinascidins (4) (Figure 1). The most attractive member of this family, ecteinascidin 743 (ET-743, Yondelis™ , Trabectedin) is the first marine anticancer agent approved in the European Union for the treatment of patients with soft tissue sarcoma. ET-743 has a unique mechanism of action based on its binding to the minor groove of DNA to interfere with cell division, activated transcription, and DNA repair (5). Renieramycin M and other renieramycins have also been shown to possess a promising cytotoxicity, with IC_{50} values in the range of nanomolar concentrations against HCT116 human colon, DLD1 human

colon, QC56 human lung, NCI-H460 human non-small cell lung, MDA-MB-435 breast, T47D human ductal breast epithelial, and AsPC1 human pancreatic cancer cell lines (6-9). However, its mechanism(s) of action and related anticancer activities, such as anti-metastasis and anti-invasion activities, remain unknown. This study investigated the novel activities of renieramycin M on anoikis, invasion, migration, and anchorage-independent growth of NCI-H460 human non-small cell lung cancer cells. The study revealed the attenuating effect of this compound on anoikis, invasion, and tumor growth. Moreover, it elucidated the underlying mechanism by which renieramycin M induces lung cancer cell apoptosis by mediating MCL-1 and BCL-2 down-regulation. These findings should facilitate the development of renieramycin M for new strategies in overcoming cancer.

Materials and Methods

Renieramycin M. Renieramycin M was isolated from the Thai blue sponge *Xestospongia* sp. as previously described (3) and was dissolved in dimethyl sulfoxide (DMSO) and distilled water to achieve indicated concentrations containing less than 0.5% DMSO. The subtoxic concentrations of renieramycin M on H460 cells were used for anoikis, migration, anchorage-independent growth, and invasion assays.

Cells and reagents. Human non-small cell lung cancer cells H460 (NCI-H460) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin in a 5% CO_2 environment at 37°C. Hoechst 33342 was obtained from Molecular Probes, Inc (Eugene, OR, USA). Antibody for MCL-1, BCL-2, BAX, and P53 and peroxidase-conjugated secondary antibody were obtained from Abcam (Cambridge, MA, USA).

Cell viability and apoptosis assays. Cells were seeded onto a 96-well plate at a density of 1×10^4 cells/well for 24 h and then treated with various concentrations of renieramycin M for 24 h. Afterward, cells were incubated with 0.5 mg/ml of MTT for an additional 4 h at 37°C. The intensity of the MTT product was measured at 570 nm using a microplate reader. The cell viability was calculated from optical density (OD) readings and represented as percentage to the non-treated control value. For apoptosis and necrosis detection, cells were stained with 10 μM Hoechst 33342 and/or 5 μg/ml propidium iodide (PI) for evaluation of cell apoptosis and necrosis, respectively. The fluorescent dye stained in cells was visualized by a fluorescence microscope. PI stained only the DNA of cell membrane-damaged cells which were considered as necrotic cells. Hoechst 33342 stained the nuclei of all cells. Cells that displayed intensely condensed and/or fragment nuclei stained by Hoechst 33342 were considered as apoptotic cells.

Wound closure assay. Cells were seeded onto a 24-well plate at a density of 2×10^5/well for 24 h. The wounds were made by scraping with plastic tip across the cell monolayer. The wounded cells were treated with sub-toxic concentrations of renieramycin M. Phase-contrast images were recorded at the time of wounding (0 h) and 24 h thereafter.

Anoikis assay. To prevent cell adhesion, tissue culture 6-well plates were coated with 200 μl (6 mg/ml in 95% ethanol) of poly(2-hydroxyethylmethacrylate (poly-HEMA; Sigma) which was left to evaporate overnight in a laminar flow hood at room temperature. Adherent H460 cells in the culture plate were trypsinized into a single cell suspension in RPMI medium and then seeded in Poly-HEMA-coated plates at a density of 1×10^5 cells/ml. Suspended cells were incubated at 37°C for various times up to 24 h. Cells were harvested, washed, and stained with Hoechst 33342 and PI, then analyzed for fluorescence intensity by fluorescence microscopy. For Hoechst 33342 apoptosis assay, cells were incubated with 10 μM of the Hoechst dye for 30 min at 37°C. The apoptotic cells having condensed chromatin and/or fragmented nuclei were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70). For cell survival assay, cells were similarly treated, harvested, washed, and incubated with 20 μM of 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) for 4 h at 37°C. Optical density was then determined by V-max photometer (Molecular Devices Inc., Menlopark, CA, USA) at a wavelength of 450 nm.

Western blotting. After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor cocktail (Roche, IN, USA) for 30 min on ice. After insoluble debris was pelleted by centrifugation at 14,000 $\times g$ for 15 min at 4°C, the supernatants were collected and determined for protein content using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). An equal amount of protein of each sample (40 μg) were denatured by heating at 95°c for 5 min with Laemmli loading buffer, and subsequently loaded on 10% SDS-polyacrylamide gel electrophoresis. After separation, proteins were transferred onto 0.45 μM nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20) and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase–coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by enhanced with chemiluminescence substrate (Pierce, Rockford, IL, USA) and quantified using analyst/PC densitometry software (Bio-Rad).

Colony formation assay. Anchorage-independent growth was determined by colony formation assay in soft agar. Briefly, H460 cells from 6-well plate monolayer cultures were prepared into a single-cell suspension by treatment with a mixture of 350 μl trypsin and 1.5 mM EDTA. Cells were suspended in RPMI containing 10% FBS and 0.33% low melting temperature agarose, then 2 ml containing 2×10^4 cells were plated in a 35 mm dish over a 3 ml layer of solidified RPMI/10% FBS/0.6% agarose. The cells were fed every three days by adding 200 μl of RPMI/10% FBS. Colonies were attained by light microscope and fluorescence microscope together with the Hoechst 33342 nuclear staining and photographed after two weeks.

Matrigel invasion assay. The invasion assay was carried out using Transwell cell culture chambers (Corning Costar No. 3422, MA, USA) according to the manufacturer's recommendation with some modifications. Briefly, polyvinylpyrrolidone-free polycarbonate
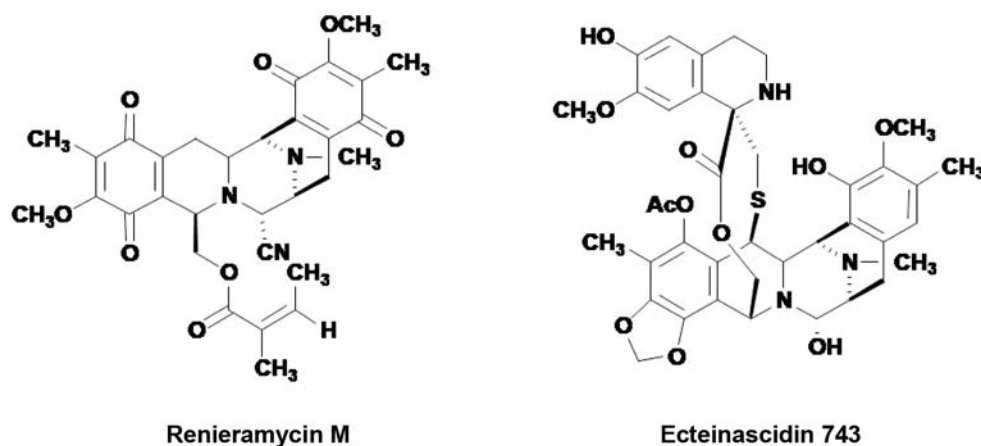
Renieramycin M

Ecteinascidin 743

Figure 1. *Structure of renieramycin M and Ecteinascidin 743.*

filters (8.0 mm pore size, Nuclepore, CA, USA) were pre-coated with 15 μl ice-cold Matrigel (BD Biosciences, MA, USA) on the upper surface for 60 min at room temperature. Conditioned media (200 μl RPMI-1640 media with 0.1% FBS) were added into the lower compartment of the chamber. H460 (at density of 10^5 cells in 100 μl RPMI-1640 per well) cells that treated renieramycin M at indicated concentration were added to the upper compartment of the chamber. After 24 h incubation, the top side of the insert membrane was scrubbed free of cells with a cotton swab and the bottom side was fixed with 3.7% paraformaldehyde and stained with Hoechst 33342 and pictures were taken.

Statistical analysis. Mean densitometric data from independent experiments were normalized to result in cells in the control. The data were presented as the mean±SD from three or more independent experiments and analyzed by the Student's *t*-test at a significance level of $p<0.05$.

Results

Renieramycin M induces human non-small cell lung cancer H460 cells apoptosis. The anticancer activity of renieramycin M against human lung carcinoma H460 cells was investigated by incubating the cells in the presence of renieramycin M (0-40 μM) for 24 h, and cell viability was analyzed using MTT assay. Figure 2 shows that treatment with renieramycin M caused a dose-dependent decrease in cell survival with ~50% of the cells remaining viable when the cells were treated with 40 μM renieramycin M. In order to investigate the mechanism of cell death, Hoechst 33342 and PI staining assay were performed. The results showed that the decrease in cell viability in response to renieramycin M treatment was mainly due to apoptosis, as determined by the increase in number of cells with intense nuclear fluorescence and chromatin condensation. Approximately 8% apoptosis was detected at a concentration of 5 μM and this reached ~40% at a concentration of 40 μM of renieramycin

M. PI-positive cells were not observed, indicating that apoptosis was the primary mode of cell death in response to renieramycin M.

Renieramycin M induces H460 cell death through a p53-dependent mechanism. In order to investigate the mechanism of renieramycin M on apoptosis induction, the expression level of apoptosis-related proteins including tumor suppressor protein P53, anti-apoptotic proteins BCL-2 and MCL-1, and pro-apoptotic protein BAX in response to renieramycin M treatment in concentration and time-dependent manners was determined. For dose-dependent experiments, the cells were incubated in the presence or absence of renieramycin M (5-20 μM) for 12 h and P53, BCL-2, MCL-1, and BAX protein expression was then determined by Western blotting. Figure 3A shows that renieramycin M significantly increased P53 level and this induction correlated with the decrease of anti-apoptotic BCL-2 and MCL-1 protein. However, alteration in pro-apoptotic BAX level was no detected. The P53 induction and MCL-1 and BCL-2 down-regulation were confirmed by time-dependent study. The cells were incubated with 20 μM renieramycin M and protein expression was determined at various times (0-16 h). Figure 3B demonstrates the consistent results with the previous finding that p53 was up-regulated while MCL-1 and BCL-2 were down-regulated. Notably, the induction of P53 was detected as early as 3 h after treatment. Moreover, the results indicate that BCL-2 and MCL-1 down-regulation was able to be first detected at 8 h and 10 h after renieramycin M treatment, while it was not possible to detect the induction of pro-apoptotic BAX (data not shown). These results reveal that the anticancer mechanism of renieramycin M involve P53-dependent down-regulation of anti-apoptotic proteins BCL-2 and MCL-1.
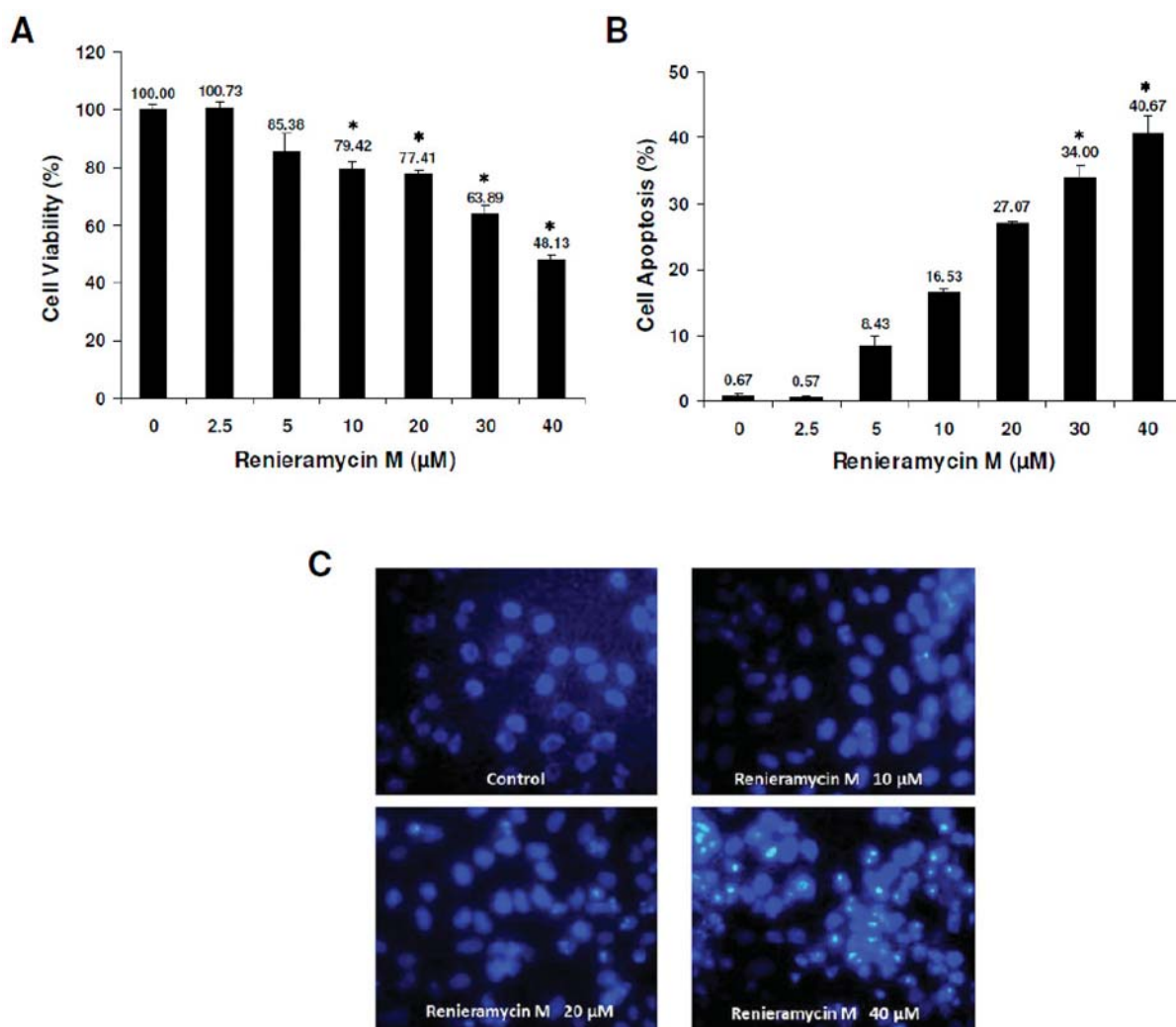
Figure 2. *Renieramycin M induces H460 cells apoptosis. H460 cells were treated with various concentrations of renieramycin M for 24 h. A: Cell viability was analyzed by MTT assay. B: Percentage of cell apoptosis was determined by Hoechst 33342 apoptosis detection. C: Morphology of apoptotic nuclei stained with Hoechst 33342 dye and propidium iodide. Values are means of triplicate samples±SD. *p<0.05 versus untreated control.*

Renieramycin M sensitizes H460 cells to anoikis. Anoikis, detachment-induced apoptosis, has been widely accepted to be one of the most important mechanisms in the inhibition of cancer metastasis. Having shown anticancer activity of renieramycin M on lung cancer H460 cells, it was further tested whether the subtoxic concentrations of this agent could sensitize lung cancer cell anoikis. Cytotoxic evaluation (MTT assay) was performed and it was found that renieramycin M at 2.5 μM had no toxic effect on H460 cells. The cells were detached and incubated in attachment-resistant poly-HEMA-coated plates for 6 h in the presence of 0-2.5 μM renieramycin M. Cell viability after detachment was then evaluated by XTT assay. Figure 4A shows that renieramycin M significantly decreased cell viability of the detached cells as compared to non-treated control. To

determine whether the observed reduction in cell viability was due to apoptotic cell death, the profile of chromosomal DNA in the cells was analyzed by Hoechst 33342 and PI staining. Figure 4B shows the increase of condensed nuclear fluorescent staining with Hoechst 33342 indicating the increase of apoptotic cells in treated detached cells. The induction of cells anoikis detected by Hoechst 33342 exceeded approximately 40% at the concentration of 2.5 μM renieramycin M, whereas no PI-positive nuclei in these cells were detected, suggesting that the main mode of cell death was apoptosis.

Renieramycin M inhibits migration, invasion, and anchorage-independent growth of H460 cells. Anchorage-independent growth is a key characteristic of metastatic cancer cells.
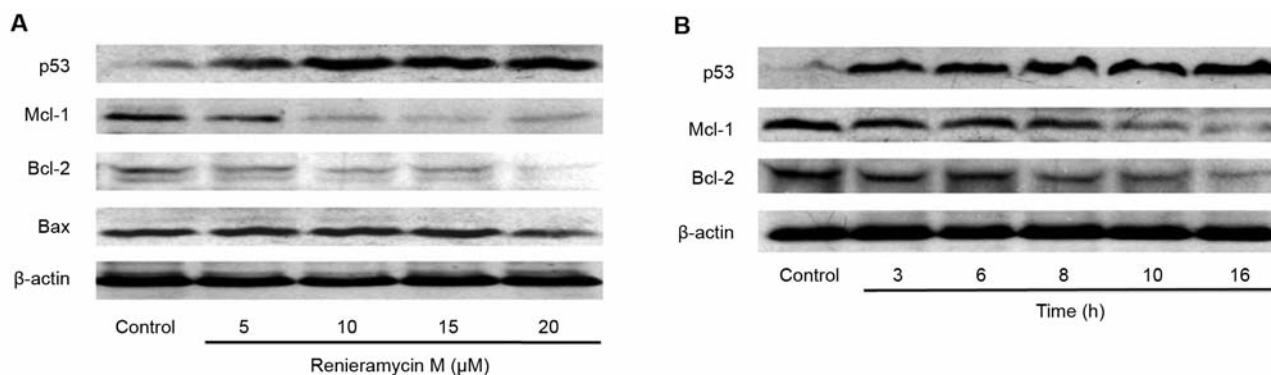
Figure 3. *Effect of renieramycin M on apoptosis related proteins. Cell extracts were prepared and separated on 10% polyacrylamide-SDS gels, transferred, and analyzed for P53, MCL-1, BCL-2, and BAX protein expression by Western blotting. Equal loading of proteins was confirmed by reprobing the blots with β-actin antibody. (A) H460 cells were treated with various concentrations of renieramycin M (0-20 μM) for 12 h. (B) H460 cells were treated with 10 μM renieramycin for various times (0-16 h). A typical immunoblot from three independent experiments with similar results is shown.*
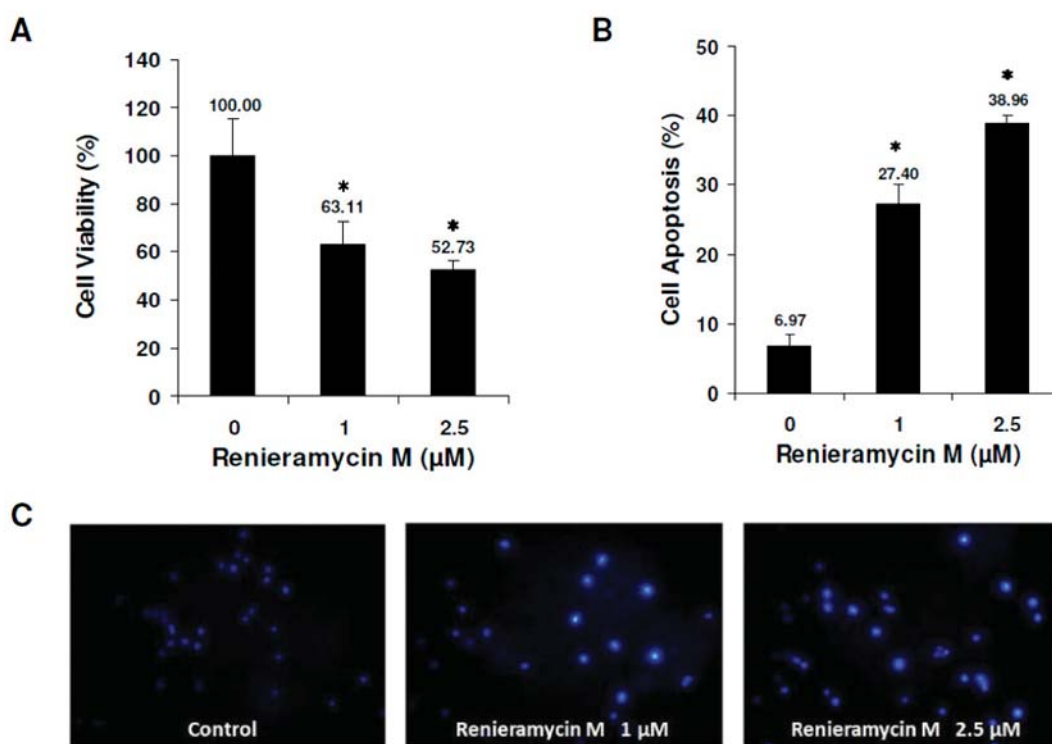

Figure 4. *Renieramycin M sensitizes H460 cells to anoikis. The cells were treated with various concentrations of renieramycin M for 6 h in detached conditions. A: Cell viability was analyzed by XTT assay. B: Percentage of cell apoptosis was determined by Hoechst 33342 apoptosis detection. C: Nuclear morphology was evaluated by Hoechst 33342 apoptosis detection and propidium iodide necrosis detection under fluorescence microscopy. Values are the means of triplicate samples±SD. *p<0.05 versus untreated control.*

Moreover, the capability of cancer cells to migrate and invade through the extracellular matrix has been shown to be an important factor in determining cancer aggressiveness and is associated with poor prognosis. To test whether renieramycin M might inhibit anchorage-independent growth, H460 cells were subjected to soft agar assay for two weeks as described in the Materials and Methods section. The number of cell colonies was determined by microscopy. Figure 5 shows that H460 cells had the ability to survive and grow under anchorage-independent conditions and that treatment of the cells with renieramycin M at subtoxic concentration (0.5 μM) significantly decreased cell growth as indicated by the absence
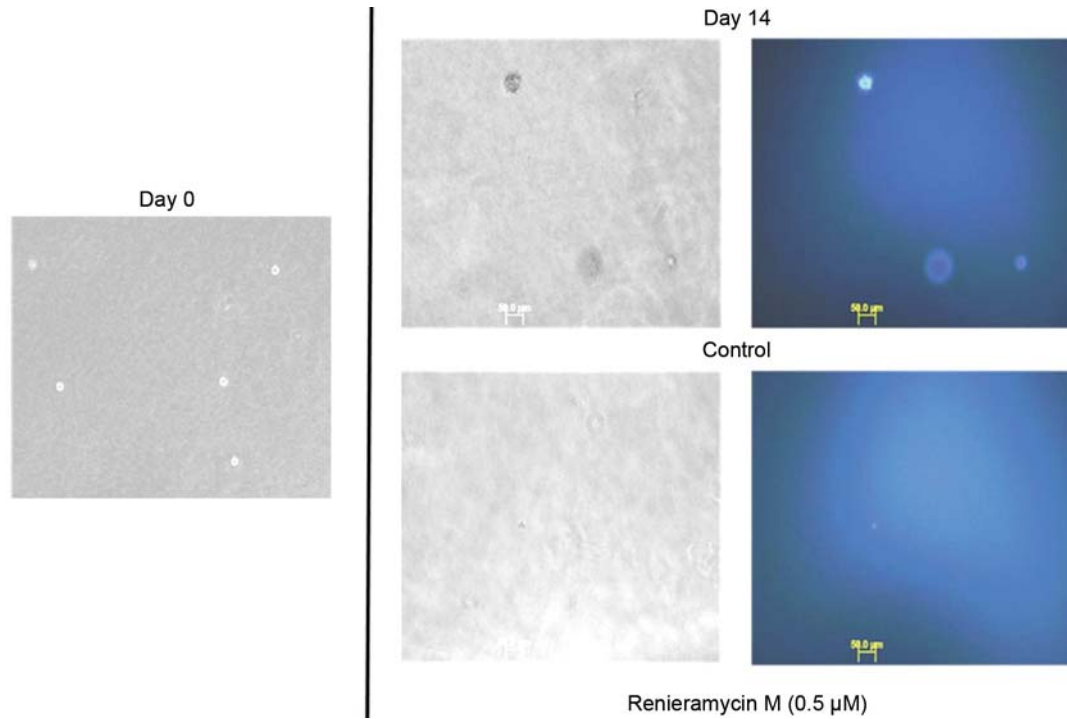
Figure 5. *Effects of renieramycin M on anchorage-independent growth of H460 cells. H460 cells were detached and left untreated (control) or treated with 0.5 μM renieramycin M in suspension in soft agar containing RPMI, 10% FBS, and 0.33% agarose as described in the Materials and Methods. Representative fields from three independent experiments were photographed at ×10 magnification.*
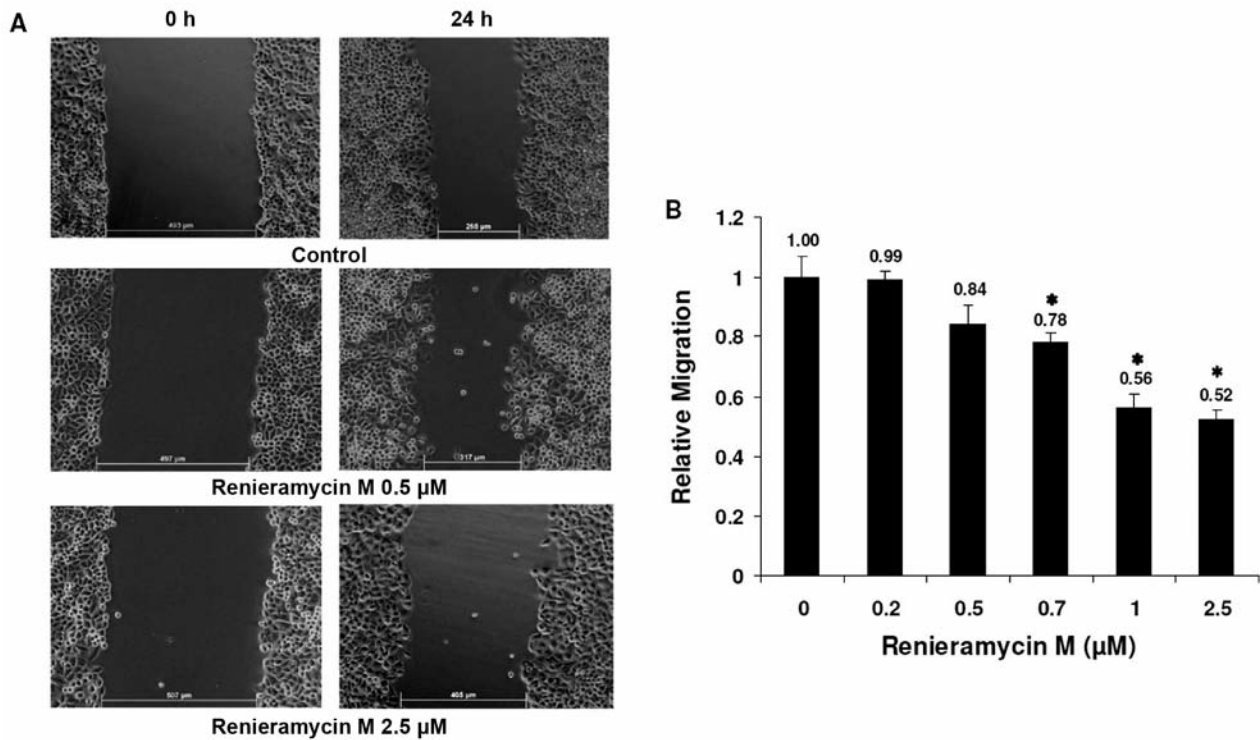

Figure 6. *Effect of renieramycin M on H460 cells migration in a scratch-wound assay. Wounds were made by scraping a plastic tip across the cell monolayer and then treating with subtoxic concentrations of renieramycin M. A: Phase-contrast image was photographed at time 0 h and 24 h after treatment. B: The relative cell migration was determined by dividing the length of cell migration in the treated cells by that of the control. Values are the means of triplicate samples±SD. *p<0.05 versus untreated control.*
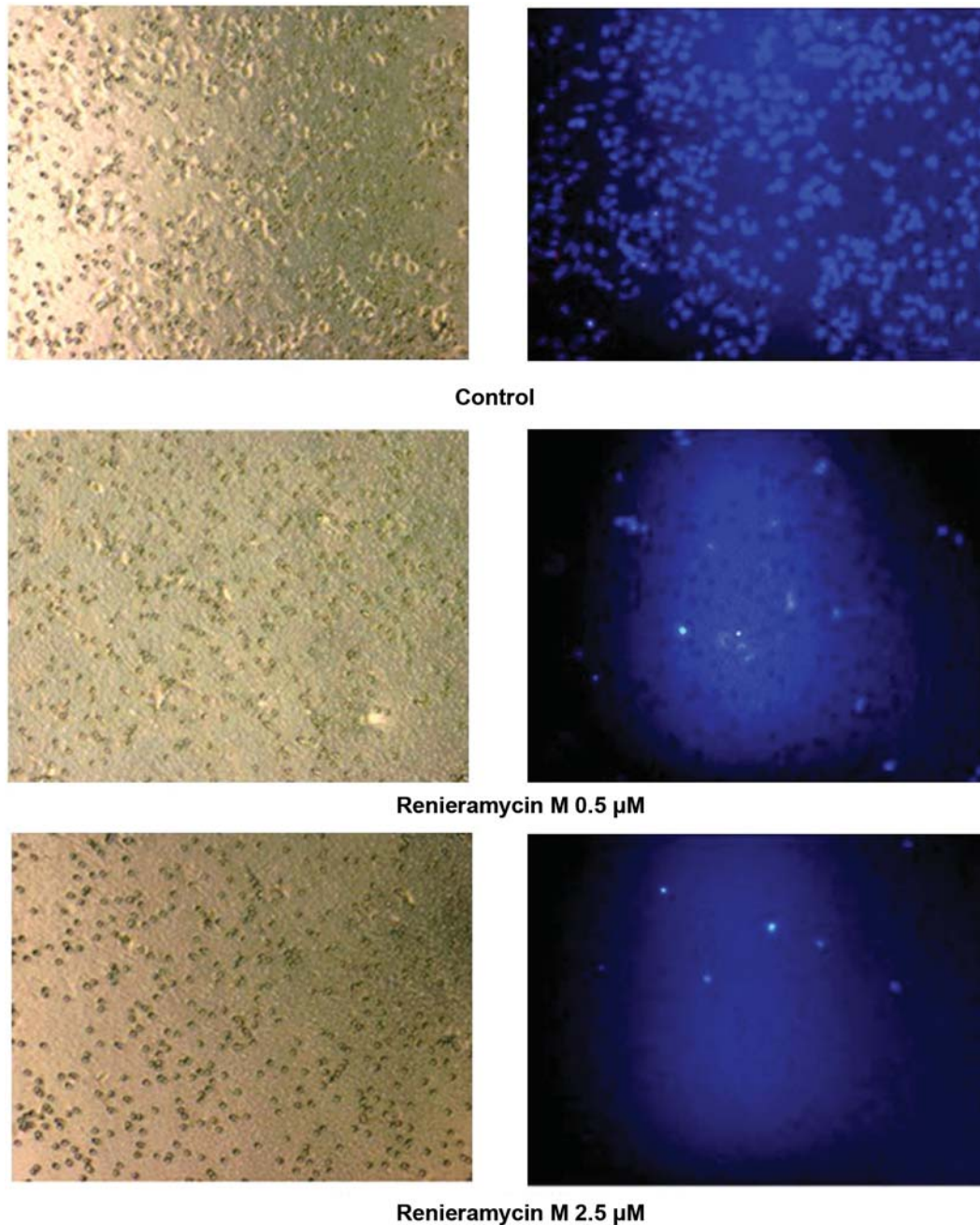
Figure 7. *Effect of renieramycin M on invasion of H460 in a Matrigel assay. The cells were seeded onto Matrigel-coated membrane and treated with subtoxic concentrations of renieramycin M. After 24 h, the invaded cells were fixed and visualized by light microscopy or stained by Hoechst 33342 and visualized by fluorescence microscopy.*

of cell colonies. Furthermore, a cell scratch-wound assay was performed to investigate the effect of renieramycin M on the migration of H460 cells. The confluence monolayer of H460 cells was scratched and incubated in the presence or absence of subtoxic concentrations of renieramycin M (0.5-2.5 μM) for 24 h. Figure 6 shows that treatment with renieramycin M reduced the spreading of the H460 cells along the edges of the wound compared to an untreated control. At a concentration of 1 μM, renieramycin M caused approximately 50% reduction in H460 cell migration. To examine the effects of renieramycin M on the invasion capacity of H460 cells, a modified Boyden chamber was used to determine the ability of cells to pass through a biological basement membrane *in vitro*. Cells were seeded into a reconstituted basement

membrane (Matrigel) in transwell cell culture chambers in the presence or absence of sub-toxic concentrations renieramycin M. After 24 h, the upper part of the chamber was removed and the cells that invaded the membrane were visualized under light microscopy. Nuclear staining of the cells by Hoechst 33342 was also performed to precisely detect invaded cells. The results show that in the non-treated control, H460 cells were able to invade the gel and the membrane. Treatment with renieramycin M caused the reduction of invaded cells in a dose-dependent manner. These results suggest that renieramycin M not only has a strong cytotoxic activity against lung carcinoma H460 cells, but also possesses potential anti-metastasis properties which might facilitate the development of this agent for cancer therapy.

Discussion

Despite advances in new therapeutic strategies for cancer patients, metastatic progression is responsible for approximately 90% of cancer-related deaths (10). Potential options for reducing cancer fatality focus on the strategy of metastasis prevention and novel potent chemotherapeutics. The results from this study are in agreement with a previous report (3) and further indicate that renieramycin M possesses capabilities to inhibit cancer cells through many approaches. This study reports, for the first time, that renieramycin M induced lung cancer cell apoptosis through P53 induction, which in turn down-regulated anti-apoptotic BCL-2 and MCL-1 levels. Coinciding with the decrease of cell viability, it was found that renieramycin M caused P53 induction early in treatment, which is generally associated with apoptosis initiation (11). Moreover, ecteinascidin-743 (E-743), another tetrahydroisoquinolinequinone alkaloid from marine natural products, also exhibited P53 induction activity (12, 13). It has been well-documented that P53 activation plays a key role in regulating cell death signal by altering the balance of BCL-2 family protein (14, 15). Anti-apoptotic proteins include BCL-2 and MCL-1 bind and sequester pro-apoptotic proteins and provoke apoptosis by competitively inhibiting the pro-apoptotic proteins form interaction (16). The decrease of these anti-apoptotic proteins turn the balance of pro-apoptotic and anti-apoptotic proteins toward apoptosis and induce the loss of the mitochondrial membrane potential followed by the opening of the transition pore, release of pro-apoptotic factors, caspase activation, and apoptosis (17-19).

Together with the fact that many cancer cells depend upon BCL-2 and other anti-apoptotic proteins for their survival, the approach of molecular therapies that are targeted to anti-apoptotic BCL-2 protein has shown potential success in killing many types of cancer. Several BCL-2 antagonists such as ABT-263 (Abbott Laboratories, Worcester, MA, USA), Obatoclax (Gemin X, Malvern, PA, USA), and Oblimersen (Genta, Berkeley Heights, NJ, USA) are currently under evaluation in the clinical phase for the treatment of lung cancer (16). Renieramycin M, which has the ability to suppress anti-apoptotic protein of the BCL-2 family, may become a promising candidate for the treatment of lung cancer. Increasing evidence indicates the role of anti-apoptotic MCL-1 as an important protein in inhibition of cell apoptosis (20) and anoikis (21). In particular, non-small cell lung cancer cells taken from patients have been shown to have elevated MCL-1 protein levels compared to the surrounding lung cells (22), and enhanced MCL-1 levels could protect cancer cells from various apoptotic stimuli (20, 23, 24). Amplification and over-expression of BCL-2 occurs in many malignancies, including small cell lung carcinomas, and impairs the intrinsic apoptotic signaling (25, 26). Moreover, the overexpression of BCL-2 has been shown to inhibit cisplatin-induced cell death in non-small cell lung cancer H460 cells (27). Therefore, the findings of the current study indicate that renieramycin M targeting MCL-1 and BCL-2 proteins may further support the use of this agent for the treatment of lung cancer.

Aggressiveness of cancer determines the prognosis of the disease and is frequently associated with the ability of cancer cells to migrate, invade, and resist anoikis. Anoikis resistance results in the survival of cancer cells in the blood or lymph circulation and allows them to establish secondary tumors at distant sites. The results from this study indicate that treatment with sub-toxic doses of renieramycin M may sensitize lung cancer cell to anoikis. Moreover, this compound showed many potential anti-metastatic activities in controlling cell migration and inhibiting cancer cell invasion. Cancer cell migration and invasion is necessary at several steps of the metastatic cascade, at which time the cancer cells invade the surrounding tissue and gain access to the blood or lymph circulations, and also at the end of metastasis, when cells reach the secondary site. The current results indicate that renieramycin M showed strong anti-migration and anti-invasion activities, which were not associated with its cytotoxic activity since the concentrations tested did not cause either toxic or proliferative effects on H460 cells (Figure 2). Although the underlying mechanisms explaining how renieramycin M may inhibit cancer cell migration and invasion are still under investigation, these findings reveal novel activities which will inform further investigations and developments of this agent.

Taken together, this study provides evidence supporting the observation that renieramycin M is a promising agent for cancer therapy. The mechanism of action of this compound on lung cancer cells was revealed and its capabilities in inhibition of lung cancer cells invasion, migration, and growth in an anchorage-independent condition were demonstrated. These finding might benefit the development of this agent to be used as an anticancer drug or other anti-metastasis therapies.

Acknowledgements

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