Bishydroquinone Renieramycin M Induces Apoptosis of Human Lung Cancer Cells Through a Mitochondria-dependent Pathway

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Abstract. Background: Renieramycin M (RM), a bistetrahydroisoquinolinequinone isolated from the Thai blue sponge, *Xestospongia* sp. was reported to be a potent anti-lung cancer agent. Modification at quinone ring enhanced apoptosis over necrosis. Thus, bishydroquinone renieramycin M (HQ-RM) was prepared and evaluated for apoptosis induction in lung cancer cells. Methods: HQ-RM was examined for cytotoxicity and apoptosis induction in human lung cancer H292 cells by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazoliumbromide and Hoechst/propidium iodide staining, respectively. The key molecular markers of mitochondrial apoptosis pathway were determined by western blot analysis. Results: HQ-RM exhibited stronger cytotoxicity than RM. HQ-RM reduced vitality of lung cancer cells in a dose-dependent manner. Nuclear staining assay indicated that apoptotic cell death was the main mechanism of toxicity caused by HQ-RM. Protein analysis revealed that HQ-RM-mediated apoptosis involved the increase of pro-apoptotic B-cell lymphoma 2 associated X (BAX) protein, and the decrease of anti-apoptosis myeloid cell leukemia 1 (MCL1) and B-cell lymphoma 2 (BCL2) proteins. Moreover, caspase-9 and -3 and Poly (ADP-ribose) polymerase (PARP) were dramatically cleaved in response to HQ-RM treatment. Conclusion: HQ-RM has highly potent anticancer activity,

greater than its parental RM, and induces lung cancer cell apoptosis through a mitochondrial apoptosis caspase-dependent mechanism. This information benefits the development of this compound for cancer therapy.

Due to the problem of drug resistance frequently found in patients with lung cancer, a much research is currently focused on novel drug discovery and efficacy testing (1, 2). Marine organisms have been documented as important sources of bioactive anticancer compounds. Renieramycins are a group of tetrahydroisoquinoline marine natural products which are structurally and biologically related to saframycins, naphthyridinomycins, quinocarcins, and ecteinascidins (3). Previously, we described anticancer and anti-metastatic activities of renieramycin M (RM), a bioactive compound isolated from a Thai blue sponge, *Xestospongia* sp. (4); however, its low solubility has limited its further development.

Renieramycin M (Figure 1) and other renieramycins have been shown to possess promising cytotoxicity, with 50% inhibitory concentration (IC₅₀) values in the range of nanomolar 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 (5-8). Interestingly, reduction of the quinone moiety at the left-handed side of the core structure followed by acetylation of C-5 position selectively controlled the cancer cell death mechanism through apoptosis without necrosis (8).

Herein, bishydroquinone renieramycin M (HQ-RM) (Figure 1) was prepared from hydrogenation of RM with 20% Pd(OH)₂/C (8). The present study aimed to elucidate

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Figure 1. The synthesis of bishydroquinone renieramycin M (HQ-RM). Hydrogenation of renieramycin M (RM) with 20% Pd(OH)₂/C in ethyl acetate for 1 h gave the colorless luco compound HQ-RM.

apoptosis-inducing effect and the underlying mechanism of action HQ-RM against lung cancer cells.

Materials and Methods

Materials. Human non-small cell lung cancer cells (H292) were purchased from the American Type Culture Collection (Manassas, VA, USA). Penicillin, streptomycin, glutamate, and phosphate-buffered saline (PBS) were purchased from GIBCO (Grand Island, NY, USA). Methanol, ethanol and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to caspase-3, caspase-9, Poly (ADP-ribose) polymerase (PARP), B-cell lymphoma 2 associated X (BAX) protein, Myeloid cell leukemia 1 (MCL1) and B-cell lymphoma 2 (BCL2) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). RM was extracted from Thai blue sponge, *Xestospongia* sp. and HQ-RM was prepared from RM by Pd(OH)₂/C catalyzed hydrogenation (8).

Cell culture conditions. H292 was cultured with Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/ml penicillin and streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Cells were seeded as recommended by the supplier and split at 70% confluence.

Cell viability assay. The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl-tetrazoliumbromide (MTT) assay was performed to determine the effect of HQ-RM and RM on the cellular viability of H292 cells. Briefly, approximately 1×10⁴ cells/well were plated in 96-well plates and kept in an incubator at 37°C under the previously described cell culture conditions. After overnight incubation to allow them to adhere, the cells were treated with different concentrations of HQ-RM and RM (0.5 μM to 100 μM) in fresh medium for 24 h. Each treatment at this time point was assayed in at least triplicate. At the stipulated time following treatment with HQ-RM or RM, the medium was aspirated and MTT (4 mg/ml stock solution in 1×PBS) was

added to each well of the 96-well culture plate. Incubation was continued at 37°C for an additional 4 h, then supernatants were discarded, and purple-colored precipitates of formazan were dissolved in 100 μl of DMSO. The color absorbance of each aliquot was recorded at 570 nm with a microplate reader and repeated three times. The cytotoxic effect was expressed as the percentage cell viability compared to that of non-treated cells.

Nuclear staining assay. H292 cells were seeded in 96-well plates at a density of 1×10⁴ cells/well, incubated overnight, and treated with HQ-RM (15, 30 and 60 μM) for 12 h. Next, the cells were rinsed in PBS and subsequently incubated with 10 μg/ml Hoechst 33342 and 5 μg/ml propidium iodide for 30 min. Nuclear condensation and DNA fragmentation of apoptotic cells and propidium iodide-positive necrotic cells were visualized and scored using a fluorescence microscope (Olympus IX5; ×40) equipped with a DP70 digital camera system (Olympus, Tokyo, Japan).

Western blot analysis. Proteolytic cleavage of pro-apoptotic proteins and pro-apoptotic proteins were detected by western blot analysis. H292 cells were treated with 15, 30 and 60 μM of HQ-RM for 12 h and harvested. All cells were collected by scraping and were lysed with lysis buffer for 45 min. The protein concentrations were measured with the Pierce™ BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The resultant lysates were boiled at 95°C for 5 min with Laemmli loading buffer and were subsequently loaded onto a 10% SDS-polyacrylamide gel. After separation, the proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl and 0.05% Tween 20) and incubated overnight with the appropriate primary antibodies at 4°C. The 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 enhancement with a chemiluminescence substrate (Pierce) and quantified using analyst/PC densitometry software (Bio-Rad).

Figure 2. Cytotoxic effect of bishydroquinone renieramycin M (HQ-RM). H292 Human non-small cell lung cancer cells were treated with HQ-RM (0-100 μ M) for 24 h. Cell viability was analyzed by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazoliumbromide assay. Data represent the mean $\pm$ SD ($n=3$). * $p<0.05$ versus renieramycin M (RM).

Statistical analysis. Data from three or more independent experiments are presented as the mean $\pm$ standard deviation (SD). Multiple comparisons for significant differences between multiple groups were performed using analysis of variance (ANOVA), followed by individual comparisons with Scheffe's *post-hoc* test. Statistical significance was considered at $p<0.05$.

Results

Cytotoxicity evaluation. The anticancer activity of HQ-RM and its parental compound RM were evaluated in human lung cancer H292 cells by MTT assay. Cells were treated with the compounds over a range of concentrations between 0.5 μ M and 100 μ M for 24 h. Cell viability was determined and is shown in Figure 2.

HQ-RM was found to be more potent than RM as it had a lower IC_{50} than RM ($n=3$). In order to further investigate the mode of cell death, the occurrence of apoptotic and necrotic cells was determined by nuclear staining. After 24 h of treatment, cells were incubated with Hoechst 33342 and propidium iodide and subsequently analyzed by fluorescence microscopy as shown in Figure 3. The control or untreated cells appeared to be intact with oval shape nuclei stained with a less bright blue fluorescence. However, the decrease in cell viability in response to HQ-RM treatment was mainly due to apoptosis (reflected by bright dots in Figure 3), as determined by the increase in number of cells with intense nuclear fluorescence and chromatin condensation. Approximately

~9% apoptosis was detected at a concentration of 0.05 μ M RM and this reached ~90% at a concentration of 100 μ M of HQ-RM. The results from propidium iodide staining show that HQ-RM induced necrosis at high concentrations (50 and 100 μ M) (Figure 3).

In order to evaluate the cytotoxicity of HQ-RM, the cells were collected after 12-h treatment with HQ-RM and the dead cells were quantified by TC20™ Automated Cell Counter. The results indicate increasing death of cells in response to increasing HQ-RM concentration from image-based cytometry (Figure 4).

HQ-RM induced apoptosis via mitochondrial pathway. Having shown that HQ-RM mediated lung cancer cell death via apoptosis, we next investigated the mechanism of such an apoptosis induction. The effect of HQ-RM on apoptosis-related proteins was determined by western blot analysis. Cells were treated with HQ-RM (0-60 μ M) and the pro- and anti-apoptotic protein of BCL2 family proteins, and the cleavage and total forms of caspase3 and -9, and PARP were evaluated after 12 h. Anti-apoptotic MCL1 and BCL2 proteins were down-regulated, while the expression of pro-apoptotic BAX protein was significantly up-regulated (Figure 5). The mitochondrial specific caspase-9 was found to be activated after HQ-RM treatment. Furthermore, the executive caspase-3, caspase-9 and PARP were found to be activated in HQ-RM-treated cells. Taken together, these

Figure 3. Apoptosis and necrosis inducing effects of bishydroquinone renieramycin M (HQ-RM). Cells were treated with HQ-RM at different concentrations for 24 h. A: Apoptotic and necrotic cell death after HQ-RM treatment were examined by Hoechst 33342/propidium iodide (PI) co-staining. B: Percentage of apoptotic and necrotic nuclei in HQ-RM-treated cells. Data represent the mean $\pm$ SD (n=3). * p <0.05 versus non-treated control.

results indicate that HQ-RM mediates lung cancer apoptosis by increasing the ratio of pro-apoptotic/anti-apoptotic proteins and activating the mitochondrial apoptosis pathway.

Discussion

The need for more effective chemotherapy is increasing each year, as the estimated number of patients with lung cancer continuously rises. More than 25% of drugs used during the last 20 years were developed directly from natural products, while the other 25% are chemically derived natural products (9). Based on the data gathered from this study, HQ-RM appears to be more cytotoxic than

its parental RM (Figure 2). HQ-RM was shown herein to mediate cell death through apoptosis. Indeed, apoptosis induction is an important cell death mechanism by which most cytotoxic drugs destroy cancer cells. Apoptosis can be triggered by two major pathways: the death receptor and mitochondrial pathways (10). While the death receptor pathway is mainly caused by the activity of immune cells, anticancer agents trigger mitochondrial pathway mediated apoptosis. As shown in Figure 6, the mechanism of action of HQ-RM is *via* the reduction of anti-apoptotic BCL2 and MCL1 proteins, and the increase of pro-apoptotic BAX protein. This altered balance of BCL2 family members initiates the release of mitochondrial substances and activates caspase-9 (11, 12).

Figure 4. Analysis of cell death. H292 cells were treated with different concentrations bishydroquinone renieramycin M (HQ-RM) for 12 h. Cell death was quantified by TC20™ Automated Cell Counter.

Figure 5. Evaluation of mitochondrial apoptosis proteins. H292 cells were treated with bishydroquinone renieramycin M (HQ-RM) for 12 h. The expression levels of anti-apoptotic proteins and pro-apoptotic proteins in H292 cells were determined by western blotting (A) and relative protein levels were quantified by densitometry (B). Data represent mean±SD (n=3). *p<0.05 versus non-treated control. MCL1: Myeloid cell leukemia 1; BCL2: B-cell lymphoma 2; BAX: B-cell lymphoma 2 associated X; PARP: Poly (ADP-ribose) polymerase.

Figure 6. Schematic overview of apoptosis pathway of bishydroquinone renieramycin M (HQ-RM) action. HQ-RM causes apoptosis by decreasing the anti-apoptotic member of the B-cell lymphoma 2 (BCL2) family, namely BCL2 and Myeloid cell leukemia 1 (MCL1), and increasing the pro-apoptotic B-cell lymphoma 2 associated X (BAX) protein. The balance between pro- and anti-apoptotic BCL2 family members shifts toward increased pro-apoptotic proteins, then the caspase cascade is activated and results in cell apoptosis. PARP: Poly (ADP-ribose) polymerase.

Many cancer cells depend upon BCL2 and other anti-apoptotic proteins for their survival. Several BCL2 antagonists such as ABT-263, Obatoclax, and Oblimersen, are currently under evaluation in the clinical phase for the treatment of lung cancer (13). Publications on cell death show that members of the BCL2 family such as BCL2, MCL1 and BAX, are major regulators of cell survival. MCL1, an important anti-apoptotic member of the BCL2 family, is one of the most frequently overexpressed anti-apoptotic genes in human cancer. The overexpression of MCL1 in cancer cells is implicated in resistance to multiple cancer therapies (14). Moreover, drug resistance in cancer was associated with an increased level of BCL2 and MCL1 (15). Thus, HQ-RM potentially targets these proteins and may not only induce apoptosis but also benefit the therapeutic strategy by overcoming drug-resistant cancer cells.

The caspases are a family of cysteine proteases whose main function is the central regulation of cell death. In our study, the cleavage of caspase-3, caspase-9 and PARP were significantly increased in HQ-RM-treated cells. When activated, caspase-9

in this complex activates caspase-3 to execute apoptosis (16). Caspase-3 then cleaves key substrates in the cell to produce many of the cellular and biochemical events of apoptosis. In addition, the activated caspases play a key role in the execution of the apoptotic program, including the cleavage of PARP during cell death to prevent the cells from repairing DNA (17).

Previous studies have shown that RM is a potential anti-metastatic agent by sensitizing lung cancer cells to anoikis by the suppression of anoikis-resistance mechanisms (18). Moreover, RM was shown to induce lung cancer cell apoptosis through the p53-dependent pathway (4). RM and derivatives exhibited anticancer activity against human colonic carcinoma (HCT116) and human lung carcinoma (QG56) cells (8). Moreover, 5-*O*-acetylated hydroquinone derivative of RM was reported to have a beneficial activity by causing low degree of necrosis (19). Our studies are in agreement with such reports that RM and its derivative are good candidates for further development and further provide evidence for the promising activity of HQ-RM in apoptosis induction (Figure 6).

Conclusion

Our results demonstrate that HQ-RM, a newly-synthesized RM derivative, exhibits potent anticancer activity against H292 lung cancer cells. HQ-RM appears to induce apoptosis by suppressing expression of anti-apoptotic BCL2 and MCL1 and increasing that of pro-apoptotic BAX. The altered balance of BCL2 family proteins leads to the activation of caspase-3 and -9 and ultimately to apoptosis (Figure 6). Understanding the mechanism of action of this compound may benefit its further development for anticancer approaches.

Conflicts of Interest

The Authors declare there is no conflict of interest with regard to this study.

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