ANTICANCER RESEARCH *37*: 6259-6267 (2017)
doi:10.21873/anticanres.12077

Apoptosis-inducing Effect of Hydroquinone 5-*O*-Cinnamoyl Ester Analog of Renieramycin M on Non-small Cell Lung Cancer Cells

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Abstract. *Background: A newly-synthesized derivative of renieramycin M (RM), an anticancer lead compound isolated from the blue sponge Xestospongia sp., hydroquinone 5-O-cinnamoyl ester (CIN-RM), was investigated here for its activity against non-small cell lung cancer cells. Materials and Methods: Cytotoxicity effects of CIN-RM and RM on H292 lung cancer cells were determined by the MTT assay. We also investigated the mechanism of CIN-RM-mediated apoptosis and mechanism of action of this compound by western blotting. Results: CIN-RM showed more potent cytotoxicity than its parental compound (RM) against H292 lung cancer cells. At concentrations of 15-60 μM, CIN-RM significantly induced apoptosis by increasing expression of apoptosis-inducing factor (AIF) and activation of caspase-3 and -9. For up-stream mechanism, CIN-RM mediated apoptosis through a p53-dependent mechanism, that consequently down-regulated anti-apoptotic B-cell lymphoma 2 (BCL2), while increasing the level of pro-apoptotic BCL2-associated X (BAX). In addition, phosphorylation of pro-survival protein AKT was found to be dramatically reduced. Conclusion: This study revealed the potential of CIN-RM for apoptosis induction and in the development of a novel anticancer agent.*

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Key Words: Renieramycin M, hydroquinone 5-*O*-cinnamoyl ester analog of renieramycin M, apoptosis, lung cancer, lead drug.

Lung cancer is the leading cause of cancer-mediated deaths, and 80-85% of all lung cancers are non-small cell lung cancer (1, 2). Importantly, resistance to anticancer drugs frequently found in patients with lung cancer has long been recognized as one of the most important obstacles to success of cancer therapy (3-5), leading to the urgent need for more effective chemotherapeutic agents.

The main mechanism of action of the currently effective chemotherapeutic drugs is to mediate cancer cell apoptosis. Apoptosic cell death can be triggered by the production of DNA adducts or damage signal that subsequently activates the p53-dependent cascade. The activation of p53 results in the increase of pro-apoptotic proteins which are members of B-cell lymphoma 2 (BCL2) family such as BCL2-associated X (BAX) accompanied by the reduction of anti-apoptotic proteins, including BCL2. The induction of pro-apoptotic signaling leads to the formation of mitochondrial pores, the release of cytochrome *c* into the cytosol, the activation of caspases, and finally cell apoptosis (6-8). In contrast, resistance to apoptosis in lung cancer can be caused by several mechanisms; however, the up-regulation of expression of pro-survival proteins including ATP-dependent tyrosine kinase (AKT), and anti-apoptotic proteins of the BCL2 family were shown to be the predominant mechanisms (9-11).

In order to overcome drug resistance problems, our group has focused on searching for natural products with anticancer capability. Renieramycin M (RM), a *bis*tetrahydroisoquinolinequinone alkaloid isolated from the Thai blue sponge *Xestospongia* sp., was reported to possess highly potent cytotoxicity against several human cancer cell lines, including lung, colon, prostate, breast, pancreatic, and oral cancer cells (12-15). Recently, RM was shown to have potent anti-metastatic activity by sensitizing anoikis-resistant lung cancer

cells to anoikis (16). In addition, RM was shown to suppress cancer stem-like phenotypes in lung cancer cells (17).

Recently, a series of 22-*O*-acyl and hydroquinone 5-*O*-acyl ester analogs of RM were synthesized. These RM derivatives were tested for structure–cytotoxicity relationship study in H292 and H460 non-small-cell lung cancer cell lines. (15, 18, 19). Interestingly, most 22-*O*-acyl ester and hydroquinone 5-*O*-acyl ester analogs of RM exhibited potent cytotoxicity at nanomolar concentrations and several of them exhibited higher potency than RM. Having a unique aromatic ester substituent, hydroquinone 5-*O*-cinnamoyl ester (CIN-RM) is structurally attractive as a potential anticancer candidate. CIN-RM was obtained *via* two-step chemical synthesis involving Pd-catalyzed hydrogenation and esterification and the structural characterization was matched with the reported data (19). Herein, we investigated the effect of CIN-RM on lung cancer cells. This information may benefit the further development of new anticancer agents.

Materials and Methods

Preparation of CIN-RM. A solution of RM (15.0 mg, 0.026 mmol) in EtOAc (10 ml) was added with 20% $Pd(OH)_2$ on carbon (7.5 mg, 50% w/w). A hydrogen balloon was attached to the reaction flask. The heterogeneous reaction was stirred vigorously at room temperature (25˚C) and 1 atm for 5 h. The reaction was filtered through a pad of celite and washed with EtOAc (10 ml, 3 times) and $CHCl_3$ (10 ml, 3 times). The filtrates were combined and concentrated *in vacuo* to yield HQ-RM as a colorless solid. The product was employed in the next step without further purification. Next, the resulted HQ-RM was placed in an oven dried-rounded bottom flask. To a solution of HQ-RM (15.0 mg, 0.026 mmol) in dry CH_2Cl_2 (5 ml) was stirred at room temperature (25˚C) under Ar atmosphere. The reaction was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI.HCl, 4.0 mg, 0.026 mmol) and 4-dimethylaminopyridine (DMAP, 3.2 mg, 0.026 mmol), followed by cinnamoyl chloride (4.3 mg, 0.026 mmol). The reaction mixture was stirred for 3 h. After completion, the reaction mixture was quenched by addition of water (5 ml). The organic layer was separated by separatory funnel and the aqueous layer was extracted with CHCl3 (10 ml, 3 times). The organic layers were combined, washed with brine (30 ml), dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. Purification by the silica gel flash chromatography eluting with hexanes:EtOAc (3:1) gave 7.9 mg (43%) of CIN-RM as a yellow amorphous solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.8 (1H, d, *J*=16.0 Hz, CH=CH_β), 7.6 (2H, m, H-3'and H-5'), 7.5 (3H, m, H-2', H-6', and H-4'), 6.7 (1H, d, *J*=16.0 Hz, CH_α=CH), 6.0 (1H, qq, *J*=7.4, 1.2 Hz, H-26), 5.8 (1H, s, OH-8), 4.5 (1H, d, *J*=11.2 Hz, H-22b), 4.3 (1H, br t, *J*=4.0 Hz, H-1), 4.1 (1H, d, *J*=2.0 Hz, H-21), 4.1 (1H, dd, *J*=11.2, 4.0 Hz, H-22a), 4.0 (1H, br d, *J*=3.6 Hz, H-11), 3.9 (3H, s, 17-OCH_3), 3.8 (3H, s, 7-OCH_3), 3.4 (1H, dt, *J*=7.2, 2.0 Hz, H-13), 3.2 (1H, d, *J*=12.0 Hz, H-3), 2.8 (1H, dd, *J*=20.8, 7.2 Hz, H-14*α*), 2.6 (1H, dd, *J*=14.0, 2.0 Hz, H-4*α*), 2.3 (1H, d, *J*=20.8 Hz, H-14*β*), 2.3 (3H, s, 12-NCH_3), 2.1 (3H, s, 6-CH_3), 1.9 (3H, dq, J=7.2, 1.4 Hz, H_3-27), 1.9 (3H, s, 16-CH_3), 1.7 (3H, dq, *J*=1.4, 1.2 Hz, H_3-28), 1.7 (1H, overlapped, H-4*β*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 186.0 (C-15), 182.6 (C-18), 167.1 (C-24), 164.7 (5-O*C*=O), 155.5 (C-17), 146.8 (CH=CH_β), 143.8 (C-7), 143.1 (C-8), 141.5 (C-20), 140.0 (C-26), 139.1 (C-5), 135.7 (C-19), 134.1 (C-1'), 131.0 (C-4'), 129.1 (C-2' and C-6'), 128.8 (C-16), 128.4 (C-3' and C-5'), 126.8 (C-25), 124.5 (C-10), 122.6 (C-6), 117.6 (21-CN), 117.2 (C, C-9), 116.4 (CH_α=CH), 64.5 (C-22), 61.2 (7-OCH_3), 60.6 (17-OCH_3), 59.5 (C-21), 56.5 (C-1), 55.3 (C-3), 54.9 (C-13), 54.6 (C-11), 41.5 (12-NCH_3), 27.8 (C-4), 21.1 (C-14), 20.6 (C-28), 15.9 (C-27), 10.1 (6-CH_3), 8.6 (16-CH_3). Chemicals were purchased from Tokyo Chemical Industry. NMR spectroscopy was performed on JEOL-JNM AL 400 FT-NMR spectrometer (19).

Chemicals. Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethysulfoxide (DMSO), trypsin and phosphate-buffered saline (PBS) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibodies to apoptosis-inducing factor (AIF), phosphorylated AKT (p-AKT), total AKT, p53, BCL2, BAX, poly ADP ribose polymerase (PARP), caspase-3, caspase-9, and β-actin were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture and treatment. H292 Non-small cell lung cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI medium supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine and 100 units/ml penicillin/streptomycin. Cells were maintained in a humidified incubator containing 5% CO_2 at 37˚C. Compounds were dissolved in DMSO and achieve the working concentrations (0, 15, 30 and 60 μM). DMSO in the working solution was less than 0.1% and showed no toxicity towards H292 cells.

Cell viability assay. Cell viability was determined by the MTT assay, which measures the cellular capacity to reduce MTT (yellow) to formazan crystals (purple) by mitochondria dehydrogenase. After 24 h treatment, the medium was replaced by 100 μl/well of MTT solution (0.4 mg/ml) and the cells were incubated for 4 h at 37˚C. Subsequently, the MTT solution was removed and a 100 μl/well of DMSO were added to dissolve the formazan crystals. The intensity was measured at 570 nm using a microplate reader (SpectraMax® M5, Molecular Devices, City, Ca, USA). All analyses were performed in at least three independent replicate cultures. The cell viability was calculated from the ratio of the optical density (OD) of treated to non-treated control cells and is presented as a percentage of the non-treated controls.

Nuclear staining assay. Cells were treated without or with different concentrations of CIN-RM in RPMI with serum for 24 h at 37˚C. Apoptotic and necrotic cell death was determined by Hoechst 33342 staining: cells were incubated with 10 μM of Hoechst 33342 dye for 30 min at 37˚C in the dark. Finally, cells were visualized under a fluorescence microscope (Olympus IX51 with DP70; Olympus, Center Valley, PA, USA). The blue fluorescent Hoechst dye was used for detection of nuclear condensation and DNA fragmentation in apoptosis cell. The data are presented as the percentage of apoptosis as follows: Apoptosis (%)= (number of apoptotic cells×100)/number of total cells.

Membrane integrity analysis. The cells collected after treatment with compounds were stained with trypan blue dye and subsequently morphologically visualized and quantified under an

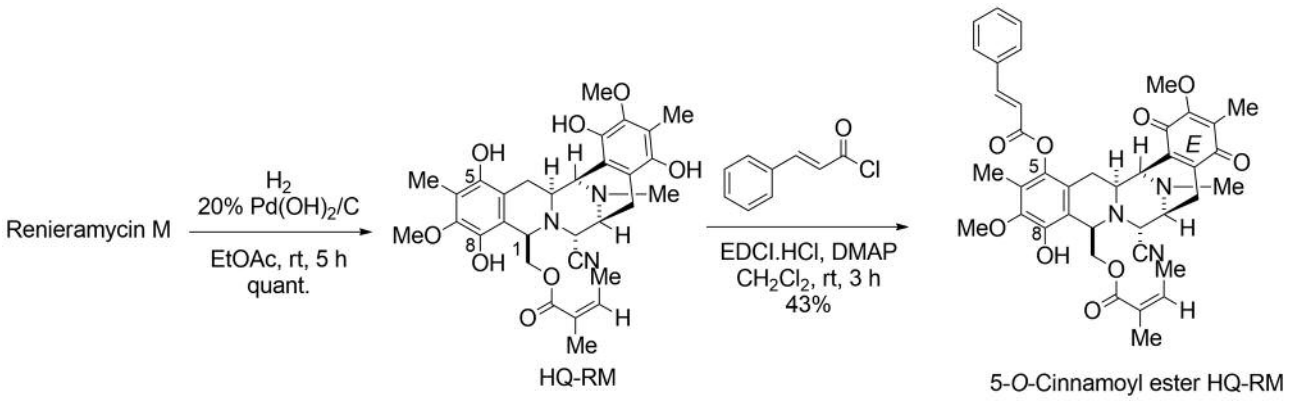

Figure 1. *The synthesis of 5-O-cinnamoyl ester analog of renieramycin M (CIN-RM). CIN-RM was derived from hydrogenation of renieramycin M with 20% Pd(OH)2/C in ethyl acetate for 5 h to obtain the bishydroquinonerenieramycin M (HQ-RM). HQ-RM was subjected to esterification with cinnamoyl chloride in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI.HCl) and 4-dimethylaminopyridine (DMAP) yielding CIN-RM.*

automated cell counter (TC10 automated cell counter; Bio-Rad, Hercules, CA, USA).

Western blot analysis. Treated and untreated cells were harvested and lysed on ice for 60 min. The protein content of cell lysate was determined using BSA protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein from each sample was separated by size using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred into nitrocellulose membranes. The membrane was blocked in 5% skim milk in 25 mmol/l Tris-HCl (pH 7.4), 125 mmol/l NaCl and 0.1% Tween 20 TBST) for 1 h at room temperature and then probed with appropriate primary antibodies at 4˚C overnight. Subsequently, the membrane was washed three times with TBST for 8 min then incubated with horseradish peroxidase-conjugated secondary antibodies according to the primary antibodies for 2 h at room temperature. The signal of immunoreactive proteins was detected by enhanced chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL, USA). Protein expression was investigated and β-actin was used as a loading of control in each treatment. Subsequently, the bands were then visualized using a film exposure with a chemiluminescence detection system and were quantified with analystPC densitometric software.

Statistical analysis. Mean data from at least three independent experiments were normalized to result for the non-treated control. All data are presented as the mean±standard error of the mean (S.E.M.). Statistical differences between means were determined using analysis of variance (ANOVA) and *post hoc* test at a significance level of $p<0.05$.

Results

Evaluation of the cytotoxic effect of CIN-RM and RM on H292 cells. CIN-RM was synthesized from RM as shown in Figure 1.

The cytotoxicity of CIN-RM and its parental compound RM was characterized by treating H292 cells with different concentrations of compounds (0-100 μM) for 24 h, then cell viability was measured by MTT assay. Figure 2A shows that a concentration of 100 μM of CIN-RM significantly reduced viability of H292 cells to approximately 40% when compared with 62% with RM at the same concentration. To confirm effect of CIN-RM in mediating cell death, after 24 h of treatment with 0-100 μM of CIN-RM, the cells were collected, stained with trypan blue dye, and subsequently morphologically visualized, and quantified by an automated cell counter (Figure 2B-C).

CIN-RM induces apoptotic cell death. In order to investigate the apoptosis inducing effect of CIN-RM, cells were exposed to CIN-RM and apoptosis was evaluated by Hoechst 33342 staining assay. The nuclear staining assay indicated that 15-60 μM of CIN-RM caused a significant increase in the number of cells with condensed or fragmented nuclei compared with non-treated control cells (Figure 3A and B). At a concentration of 60 μM, CIN-RM-induced cell apoptosis was approximately 55%.

To confirm the above apoptotic activity of CIN-RM, the hallmarks of apoptosis including cleavage of PARP, induction of AIF expression, and activation of caspase-3 and caspase-9 were investigated in cells treated with CIN-RM. Western blot analysis indicated that treatment of H292 cells with CIN-RM increased production of cleaved PARP, while reducing the total intact PARP. Consistent with this, CIN-RM up-regulated AIF and increased activation of caspase-3 and caspase-9 in a dose-dependent manner (Figure 3C and D). Taken together, these results show that CIN-RM induced apoptosis of H292 lung cancer cells.

CIN-RM triggers apoptosis via a p53-dependent mechanism and suppresses AKT. It is known that apoptosis induction

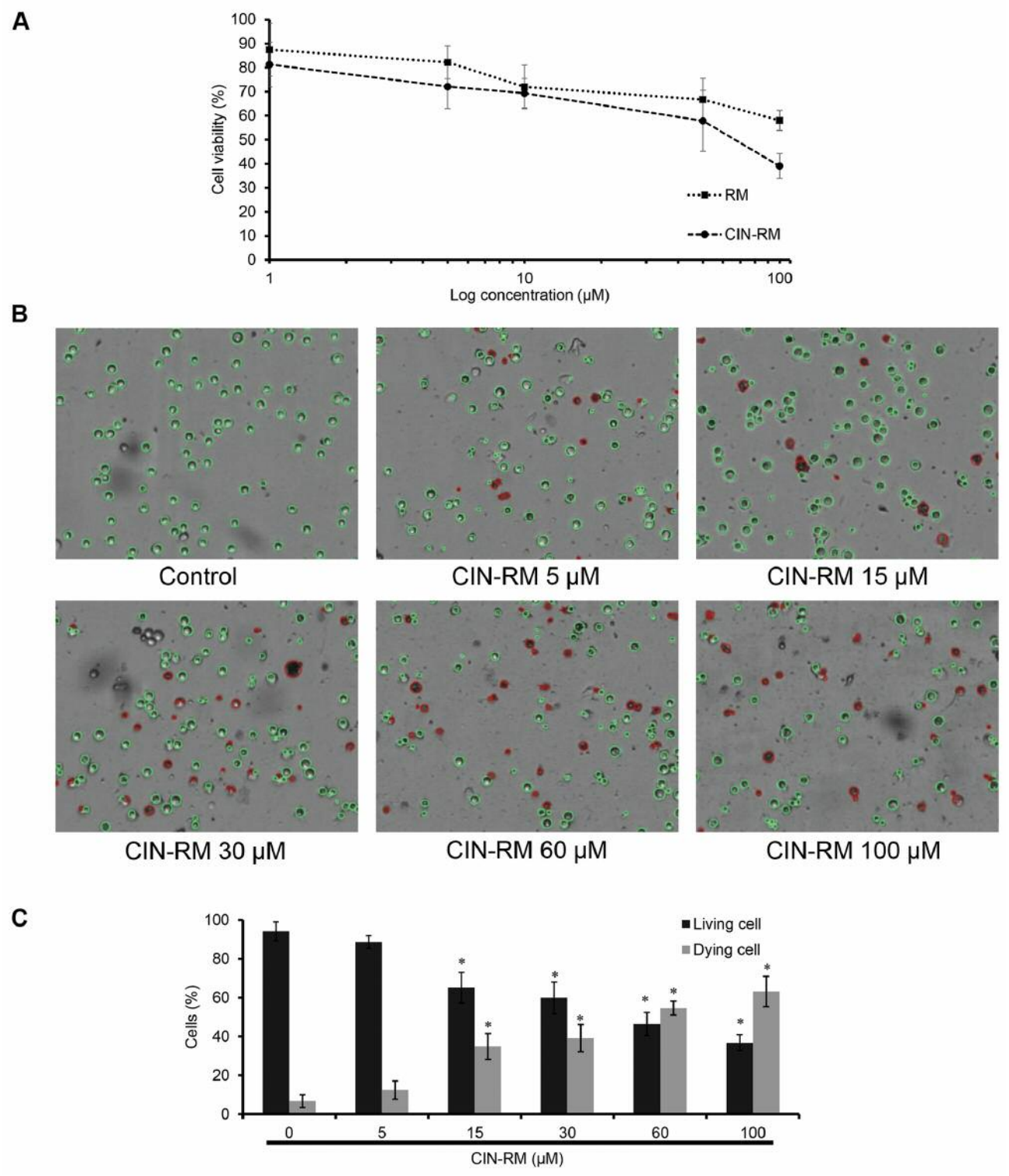

Figure 2. *Cytotoxic effect of 5-O-cinnamoyl ester analog of renieramycin M (CIN-RM) on H292 lung cancer cells. A: H292 Human lung cancer cells were treated with different concentrations (0-100 μM) of CIN-RM or RM for 24 h. The percentage of cell viability was analyzed. Data represent the mean±SD (n=3). Significantly different at *p<0.05 versus non-treated control. B: H292 cells collected after treatment with CIN-RM (0-100 μM) for 24 h were stained with trypan blue dye and subsequently analyzed cell membrane integrity under an automated cell counter. The green color indicates living cells, while the red color indicates dying cells. C: The percentage of living cells and dying cells after treatment with CIN-RM (0-100 μM) for 24 h were quantified. Data represent the mean±SD (n=3). Significantly different at *p<0.05 versus the non-treated control.*

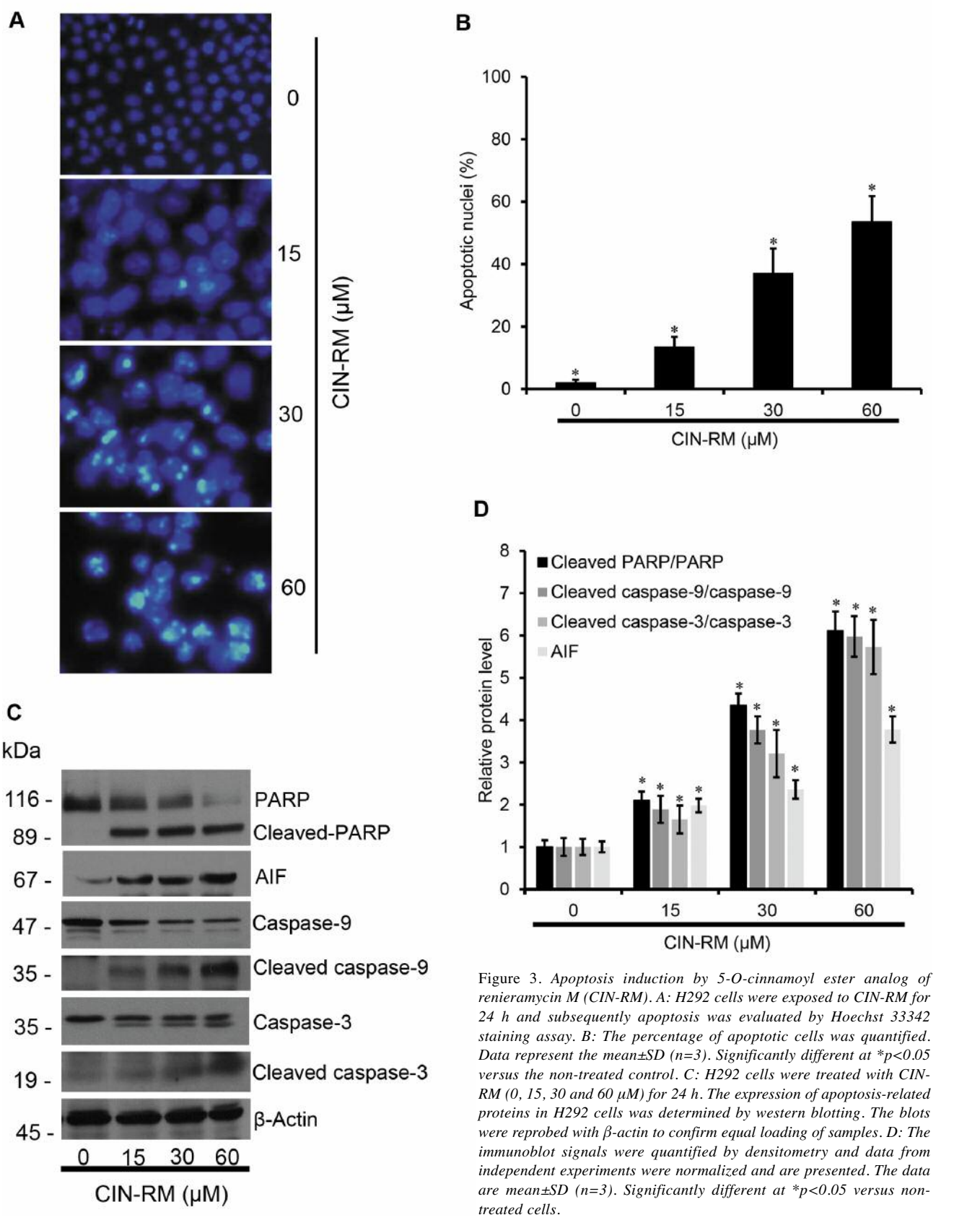

Figure 3. *Apoptosis induction by 5-O-cinnamoyl ester analog of renieramycin M (CIN-RM). A: H292 cells were exposed to CIN-RM for 24 h and subsequently apoptosis was evaluated by Hoechst 33342 staining assay. B: The percentage of apoptotic cells was quantified. Data represent the mean±SD (n=3). Significantly different at *p<0.05 versus the non-treated control. C: H292 cells were treated with CIN-RM (0, 15, 30 and 60 μM) for 24 h. The expression of apoptosis-related proteins in H292 cells was determined by western blotting. The blots were reprobed with β-actin to confirm equal loading of samples. D: The immunoblot signals were quantified by densitometry and data from independent experiments were normalized and are presented. The data are mean±SD (n=3). Significantly different at *p<0.05 versus non-treated cells.*

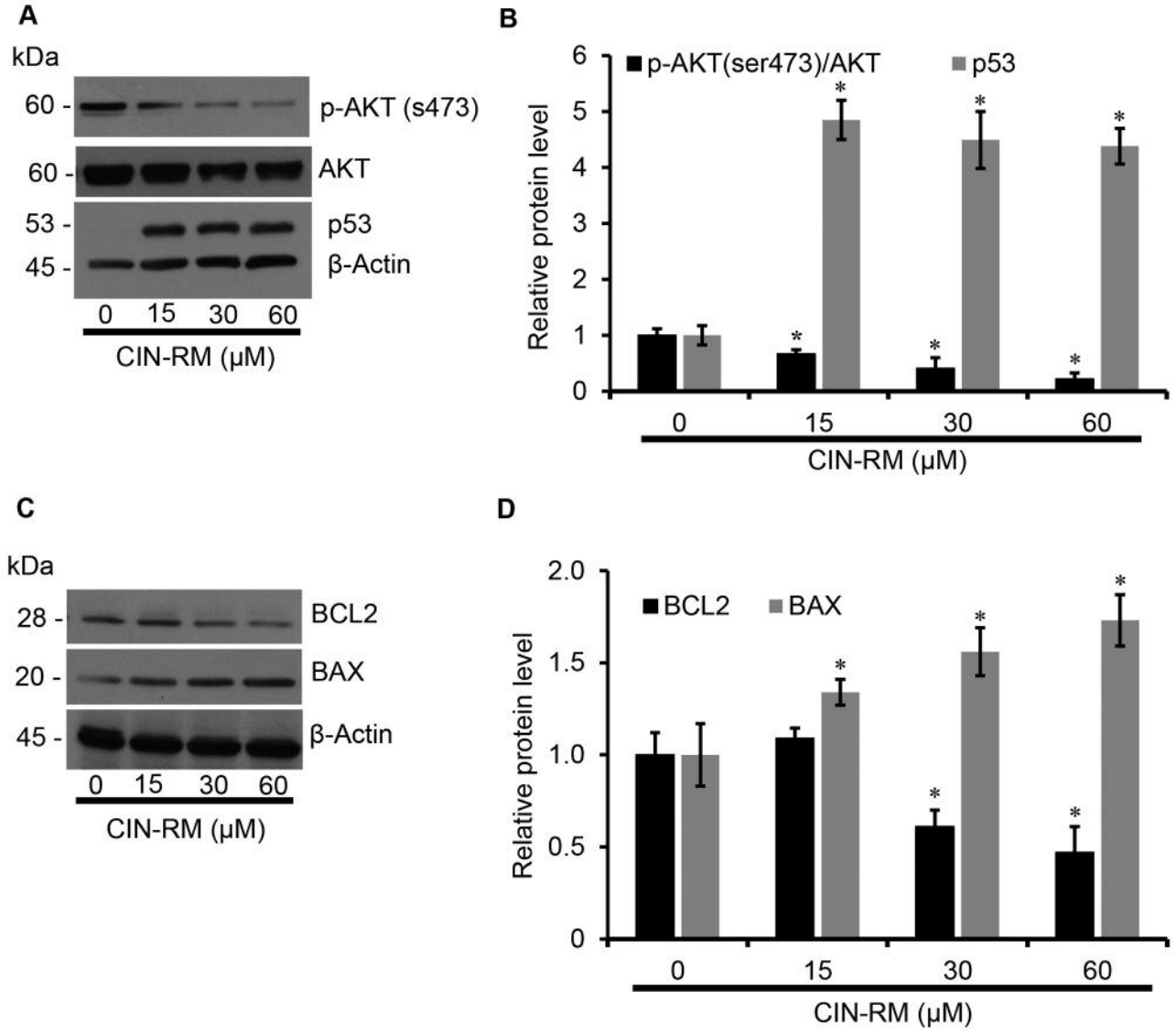

Figure 4. *5-O-Cinnamoyl ester analog of renieramycin M (CIN-RM) induces apoptosis through accumulation of p53 and suppresses ATP-dependent tyrosine kinase (AKT). H292 cells were treated with CIN-RM (0, 15, 30 and 60 μM) for 12 h. A: The expression of AKT and phosphorylated (active) AKT [p-AKT(s473)] was determined by western blot assay. The blots were reprobed with β-actin to confirm equal loading of samples. B: The immunoblot signals were quantified by densitometry and data from independent experiments were normalized and are presented. The data are mean±SD (n=3). Significantly different at *p<0.05 versus non-treated cells. C: The expression of anti-apoptotic proteins B-cell lymphoma 2 (BCL2) and pro-apoptotic proteins BCL2-associated X (BAX) were determined by western blotting. The blots were reprobed with β-actin to confirm equal loading of samples. D: The immunoblot signals were quantified by densitometry and data from independent experiments were normalized and are presented. The data are mean±SD (n=3). Significantly different at *p<0.05 versus non-treated cells.*

caused by several chemotherapeutic drugs occurs through p53-mediated death (20-22). A number of studies have indicated the effect of pro-survival AKT signal on chemotherapeutic resistance in cancer (9, 10, 23, 24). Therefore, we tested whether CIN-RM induced apoptosis of H292 cells *via* p53 and suppressed pro-survival AKT. Cells were treated with CIN-RM (0-60 μM) for 12 h, and subjected to western blot analysis for the detection of p-AKT, total AKT, p53, BCL2, and BAX. Figure 4 shows that treatment of the cells with CIN-RM dramatically suppressed active AKT (AKT phosphorylated at Ser473), while slightly suppressing total AKT. Importantly, CIN-RM increased the p53 level in the cells relative to the untreated control. As a down-stream protein target of p53 pathway,

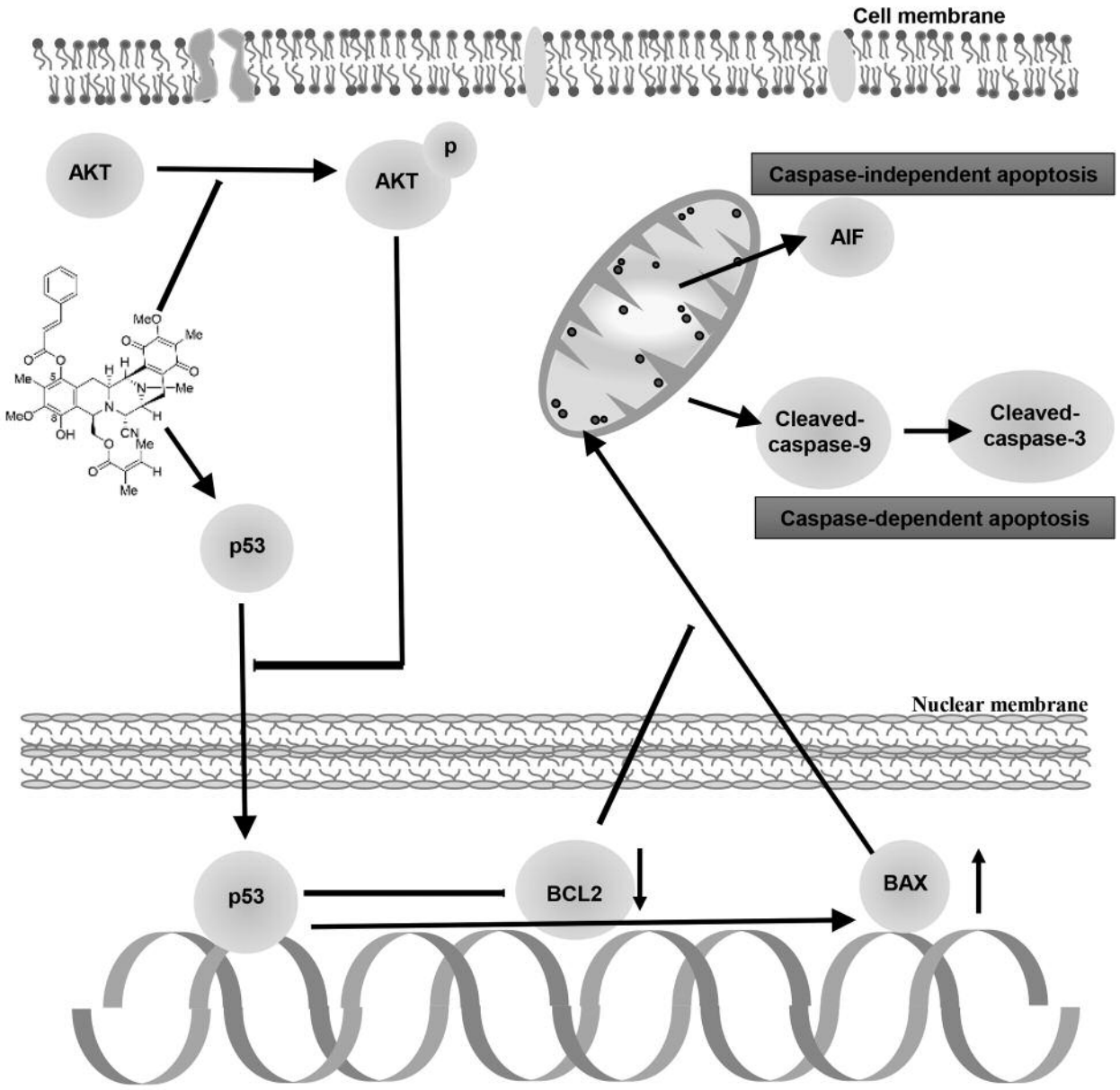

Figure 5. *Schematic overview of apoptosis pathway of 5-O-cinnamoyl ester analog of renieramycin M (CIN-RM). CIN-RM causes apoptosis by reducing expression of pro-survival ATP-dependent tyrosine kinase (AKT) and activating the p53 pathway. The accumulation of p53 suppresses the expression of anti-apoptotic protein B-cell lymphoma 2 (BCL2) while promoting the expression of pro-apoptotic protein BCL2-associated X (BAX). The shift of balance between pro- and anti-apoptotic proteins subsequently triggers the release of apoptosis-inducing factor (AIF), which promotes caspase-independent apoptosis pathway. Moreover, loss of mitochondrial membrane integrity stimulates caspase-dependent apoptosis by activation of caspase-9 and caspase-3.*

expression of BAX was found to subsequently increase, while that of BCL2 was down-regulated (Figure 4C and D). Together, these results indicate that CIN-RM induces apoptosis by activating the p53 pathway as well as suppressing the AKT survival signal.

Discussion

New drugs and novel strategies to overcome cancer with high efficacy are of the greatest interest in cancer-related and pharmacological research fields. In general, most anticancer drugs are used to kill cancer cells through

apoptosis, as it is a mode of cell death with a controlling mechanism (22, 25). Apoptosis is the main mechanism that the human body uses to eliminate unwanted and damaged cells (26, 27). As part of our continuing searching for novel cytotoxic natural products, we isolated and modified a series of renieramycin (RM) alkaloids from blue sponge *Xestospongia* sp. (12, 13, 15, 18). RMs are a group of *bis*tetrahydro-isoquinoline quinone alkaloids possessing potent cytotoxicity against several human cancer cell lines (15-18, 28-29). In addition, RM was shown to have anti-metastasis potential and suppress cancer stem cells in lung cancer (16, 17, 28).

Successful synthesis of 22-*O*-acyl and hydroquinone 5-*O*-acyl ester analogs of RM has led to several more potent compounds for anticancer approaches (15, 18, 19). Interestingly, our previous work of RM derivatives showed that the hydroquinone 5-*O*-acetyl ester analog of RM was the first analog possessing selective apoptotic induction while reduce its necrosis-inducing effect by reducing oxidative stress (30).

As chemotherapeutic resistance in lung cancer has been long recognized as an important obstacle to successful therapy (9, 31), new drugs having high potency as well as the ability to inhibit drug resistance of cancer cells are of great interest in anticancer drug discovery. The AKT signaling pathway has been shown to be a central survival pathway playing a key role in drug resistance in many cancer types, including lung cancer (32, 33). Especially, targeting of the AKT pathway was shown to be a good strategy to boost chemotherapy efficacy (34). Figure 5 indicates that not only does CIN-RM induce apoptosis of lung cancer cells by activation of a p53-dependent mechanism that triggers BCL2 imbalance, the release of AIF, and activation of caspases, but the compound also has the ability to suppress the AKT survival signal.

In conclusion, we have shown that the newly-synthesized CIN-RM has a potent cytotoxic effect against lung cancer cells. The results indicate that the derivative of the lead compound RM possesses a good anticancer activity by inducing apoptosis of lung cancer cells *via* p53-mediated alteration of BCL2 protein expression and suppression of pro-survival AKT signaling.

Conflicts of Interest

The Authors declare that there is no conflict of interest in regard to this research.

Acknowledgements

This work was supported by grants from The Thailand Research Fund through The Royal Golden Jubilee Ph.D. program Grant No. PHD/0063/2557, grant for International Research Integration: Chula Research Scholar, Ratchadaphiseksomphot Endowment Fund grant for Ratchadaphiseksomphot Endowment Fund under Outstanding Research Performance Program.

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Received May 2, 2017
Revised May 22, 2017
Accepted May 24, 2017