Simvastatin and Alendronate Effects against Cholangiocarcinoma Cells

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Buranrat: Effect of Simvastatin and Alendronate against Cancer

New treatments are needed for cholangiocarcinoma, an aggressive cancer with a poor prognosis. Combination of statins and bisphosphonates may be useful in treating cancer through influencing the mevalonate pathway. We studied the synergistic effects of simvastatin and alendronate against cholangiocarcinoma cells, looking first at cytotoxic, apoptotic and anti-migratory effects, then possible mechanisms of action. Individually, both simvastatin and alendronate increased cholangiocarcinoma cell death and the combination was more effective. Additionally, the combination treatment inhibited colony formation, reduced protein-related cell proliferation, NF-κB and cyclin D1, and increased p53 protein-related cell death. Apoptosis was induced by the combination treatment through activation of early apoptotic cells and stimulation of the caspase 3 enzyme. The combination treatment also decreased cancer cell migration by suppressing matrix metalloproteinases, metalloproteinase-2 and metalloproteinase-9 levels. Finally, the combination treatment reduced Rac1 protein expression in the mevalonate pathway. Our study shows that a combination of simvastatin plus alendronate has a direct anticancer and anti-migratory effect on cholangiocarcinoma cells *in vitro*. Mevalonate inhibitors could be used as therapeutic options for cholangiocarcinoma and perhaps, as chemotherapy-sensitizing agents.

Key words: Cholangiocarcinoma, simvastatin, alendronate, mevalonate pathway

Cholangiocarcinoma (CCA) is an aggressive cancer of the bile duct that initiates from the epithelial bile ducts and is a major public health problem in Southeast Asian countries including Thailand. In Thailand, CCA risk factors include *Opisthorchis viverrini* and *Clonorchis sinensis* infections, which promote chronic inflammation and oxidative stress, and lead to an unregulated intracellular signaling pathway[1,2]. Unfortunately, CCA is not usually detected until stage III or IV, when surgical treatment is no longer possible. Chemotherapeutic agents (anticancer drugs) must be used instead and these have low efficacy and high toxicity[3]. Therefore, alternative strategies for CCA treatment are urgently needed for the management of unresectable CCA.

The Mevalonate (MVA) pathway is the key pathway in cholesterol synthesis, and produces various biochemical agents in intracellular processes[4]. The products of the MVA pathway include Rac, Rho, Ras, and Rap, which control protein function in many cellular steps, such as growth, necrosis, apoptotsis, angiogenesis, invasion, and migration. Drugs that repress the MVA pathway could therefore be useful in the treatment of cancer. Statins and Bisphosphonates (BPs), for example, inhibit β-Hydroxy β-Methylglutaryl-CoA (HMG-CoA) reductase and Farnesyl Pyrophosphate Synthase (FPPS), reduce MVA product formation, and lead to an *in vitro* suppression of cancer cell proliferation and migration[5,6].

Statins inhibit the rate-limiting enzyme of the MVA pathway, HMG-CoA reductase, which controls cholesterol synthesis[5], and they are broadly used as lipid-lowering drugs in hyperlipidemia patients, significantly decreasing their risk of Coronary Heart Disease (CHD)[7]. Statins may

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cause decreased levels of MVA and its downstream products, affecting various cell functions including the cellular signaling process, protein synthesis, and cell cycle progression[8]. Several studies have indicated that statins could be beneficial in clinical cancer treatment by regulating tumor initiation and suppressing cancer cell growth invasion and metastasis[9-12], and that statins could reduce the concentration of anticancer drugs needed to treat many cancer cell types.

BPs are approved for treating and preventing osteoporosis and tumor migration to the bones. Many studies have shown that BPs may prevent cancer-associated bone disease. BPs act by repressing the FPPS and MVA enzymes, which can result in changes in Dimethylallyl Pyrophosphate (DMAPP), Isopentenyl Pyrophosphate (IPP) and Farnesyl Pyrophosphate (FPP) products[13,14]. Additionally, BPs affects the downstream production of FPPS and Geranylgeranyl Pyrophosphate Synthase (GGPPS)[15]. BPs have also shown anticancer activities in *in vitro* studies including inhibition of cell growth, proliferation, apoptosis, invasion, and migration[16]. The underlying mechanisms are *via* repression of the MVA pathway although other actions cannot be ruled out.

Given the importance of the MVA pathway in cancer cell processes, the effects of statins and BPs on the MVA pathway, and the growing number of studies demonstrating statin and BP activity against many cancer cell types, it seems plausible that these drugs could have additive or synergistic effects in CCA cancer cells. In the present study, therefore, we investigated the effect of simvastatin and alendronate alone and in combination on CCA cell growth and migration using KKU-100 cells.

MATERIALS AND METHODS

Chemicals and reagents:

All of the cell culture reagents, with the following exceptions, were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA). Simvastatin, alendronate, Radioimmunoprecipitation assay lysis buffer, and sulforhodamine B (SRB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies against NF-kB, p53, cyclin D1, caspase 3, Rac1, RhoA, beta-actin, and anti-rabbit IgG HRP-link antibody were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell lines and culture conditions:

The KKU-100 human CCA cell line was kindly provided by the Department of Pathology at Khon Kaen University Faculty of Medicine. Cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) with antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin) and 10 % Fetal Bovine Serum (FBS).

Cell viability assay:

Cell viability was measured using the SR) assay as described previously[17]. In brief, cells were exposed to simvastatin (0-200 µM) and alendronate (0-1000 µM) for 24-48 h, then fixed and stained with 0.4 % SRB, before addition of 200 µl 10 mM Tris base. The Optical Density (OD) was read at 540 nm using a spectrophotometer. Treatment with a combination of simvastatin and alendronate was also tested using the SRB assay. In these combination treatment experiments, cells were exposed to various concentrations of simvastatin (0-50 cM) with 50 µM alendronate or various concentrations of alendronate (0-250 µM) with 25 µM simvastatin for 24 h, then cytotoxicity was measured by SRB assay as described above.

Colony formation assay

Cancer cell replication was measured using the colony formation assay described previously[17]. In brief, cells were plated overnight and then simvastatin (25-50 µM) and alendronate (25 µM) were added for 24 h. The next day, the cells were transferred to new DMEM medium and allowed to grow for a further 15 d. After this, the cells were fixed with cold absolute methanol, stained with 0.5 % crystal violet, washed, and colony formation was counted.

Wound healing assay:

Cell migration was assessed using the wound healing assay described previously[17]. In brief, cells were seeded overnight and a wound was then made by scratching each monolayer with a 0.2 ml pipette tip. The cell monolayers were treated with 1 µM simvastatin with or without 25 µM alendronate for 48 h, and closure of the distant wounds was captured from 0-48 h by inverted microscopy (10x magnification).

Gelatin zymography analysis:

Metalloproteinases (MMP)-2 and MMP-9 levels were assessed by gelatin zymography analysis as described previously[17]. In brief, cells were seeded overnight, then exposed to 1 µM simvastatin with or without 25 µM alendronate for 24 h, and the medium was collected. Later, protein samples were mixed with 2X non-reducing buffer and the samples (20 µg) were then pipetted onto a 12 % Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel comprising 0.01 % (w/v) gelatin. The gel was incubated with developing buffer at 37° for 8 h and stained with 0.5 % Coomassie Brilliant Blue R-250 for 1 h. After that, the gel was washed several times with destaining buffer. The molecular weights of MMP-2 and MMP-9 were observed to be 72 kDa and 92 kDa.

Matrigel migration assay:

We used the matrigel invasion assay to test cell migration. The cells were plated in the upper Transwell chambers with simvastatin alone (0-25 µM), alendronate alone (0-250 µM), and a combination of simvastatin and alendronate (10 µM simvastatin plus 50 µM alendronate) in serum-free medium. Complete DMEM medium was added to the bottom chamber, and it was then incubated with the tested compounds for 24 h. Cells passing through the membrane were fixed in cold absolute methanol for 30 min at -20°, stained with 0.5 % crystal violet, and then captured with an inverted microscope (100x, CKX53, Olympus Corporation).

Apoptosis by flow cytometry:

Cell apoptosis was assessed by flow cytometry as described previously[18]. In brief, cells were seeded overnight and then exposed to 50 µM simvastatin plus 250 µM alendronate for 24 h. After this, the cells were resuspended in buffer, stained with 5 µl Propidium Iodide (PI) and 5 µl Annexin V-FITC, and allowed to incubate in the dark at room temperature for 15 min before addition of 400 µl of assay buffer. Cancer cell apoptosis was assessed by flow cytometry.

Western blotting:

Protein expression was assessed by western blot as described previously[17]. Cells were seeded overnight and then exposed to 50 µM simvastatin with or without 250 µM alendronate for 24 h prior to collection. Cytosol proteins (20 µg) were loaded onto a 10 % SDS-PAGE gel, then transferred onto Polyvinylidene Fluoride (PVDF), and blocked with 2.5 % albumin. The membrane was treated with primary antibodies for NF-κB, p53, cyclin D1, caspase 3, Rac1, RhoA, and beta-actin (1:2,500) overnight at 4°, and then exposed to secondary antibody (1:5000) for 2 h at room temperature. The intensity of the target band was determined using Western substrate (Bio-Rad Laboratories, USA).

Statistical analysis:

All experimental data were analyzed by one-way Analysis of Variance (ANOVA), followed by Tukey's post-hoc test to compare untreated and treated groups. Half maximal inhibitory concentration (IC_{50}) values were calculated using the Prism 5 program (GraphPad Software, San Diego, CA). All data are expressed as mean±Standard Error of Mean (SEM) and we set the statistically significant difference as less than 0.05.

RESULTS AND DISCUSSION

The effects of the MVA pathway inhibitors, simvastatin and alendronate, on CCA cell viability were assessed using the SRB assay. Cells were exposed to simvastatin alone and alendronate alone for 24-48 h. Simvastatin and alendronate treatment alone decreased the cell viability of KKU-100 cells in a time- and dose-dependent manner (fig. 1A and fig. 1B), with IC_{50} values of 64.18±12.35 µM at 24 h and 15.16±1.51 µM at 48 h for simvastatin, and with IC_{50} values of 999.77±138.26 µM at 24 h and 48.24±9.36 µM at 48 h for alendronate (Table 1).

We also examined the effects of simvastatin and alendronate in combination using the SRB assay, with KKU-100 cells exposed to the combination for 24 h. When various concentrations of simvastatin (0-50 µM) were tested in combination with 50 µM alendronate, the IC_{50} value of simvastatin was reduced from 121.10±0.83 µM to 14.80±1.06 µM. Also, when various concentrations of alendronate (0-250 µM) were tested in combination with 25 µM simvastatin, the IC_{50} value of alendronate was reduced from 229.37±16.09 µM to 85.67±5.81 µM (fig. 1D and Table 1). Clearly therefore, the two MVA pathway inhibitors have synergistic cytotoxic effects against KKU-100 cells.

To examine the effects of simvastatin, alendronate, and a combination of simvastatin and alendronate on CCA cell replication and protein expression, the colony formation assay and western blotting were

used. We found that 25-50 µM simvastatin and 25 µM alendronate reduced the colony formation of cancer cells with approximate percentages of inhibition of 45.83 %, 32.11 %, and 78.68 %, respectively. Alendronate potentiated the inhibition of colony formation with both simvastatin concentrations (25 and 50 µM), and the approximate percentages of inhibition were 18.87 % and 1.47 %, respectively (fig. 2A and fig. 2B). These results show that a combination of simvastatin and alendronate has an antiproliferative effect on KKU-100 cells.

Whether or not the combination of simvastatin and alendronate inhibited KKU-100 proliferation by altering expression of the proteins NK-κB, p53, and cyclin D1 was investigated using western blot analysis. Our results indicate that simvastatin significantly decreased expression of NF-κB and induced wild type p53 (fig. 2C-fig. 2F). These results show that simvastatin in combination with alendronate inhibited cell proliferation and cell regrowth by reducing protein-related cancer cell growth via NF-κB and cyclin D1, and by increasing protein-associated cell death via p53.

The effects of simvastatin alone, alendronate alone, and simvastatin and alendronate in combination on KKU-100 cell apoptosis and protein-related apoptosis were investigated by flow cytometry and western blotting. The results show that 50 µM simvastatin induced early phase apoptosis in KKU-100 cells and 250 µM alendronate slightly increased apoptosis. In combination, simvastatin and alendronate induced apoptosis more than either drug treatment alone (fig. 3A). Based on these results, simvastatin and alendronate have significant effects on CCA cancer cell apoptosis and cell death.

TABLE 1: IC$_{50}$ VALUES OF SIMVASTATIN AND ALENDRONATE, ALONE AND IN COMBINATION, AGAINST CCA CELLS

Treatment groups	Incubation time (h)	IC$_{50}$ µM)
Simvastatin	24	64.2+12.4
	48	15.2+1.5
Alendronate	24	999.8+138.3
	48	48.2+9.4
Simvastatin (0-50 µM)	24	121.1+0.8
Simvastatin+50 µM Alendronate	24	14.8+1.1
Alendronate (0-250 µM)	24	229.4+16.1
Alendronate+25 µM Simvastatin	24	85.7+5.8

Note: Values represent mean±SEM (n=3). Different character indicates significant difference
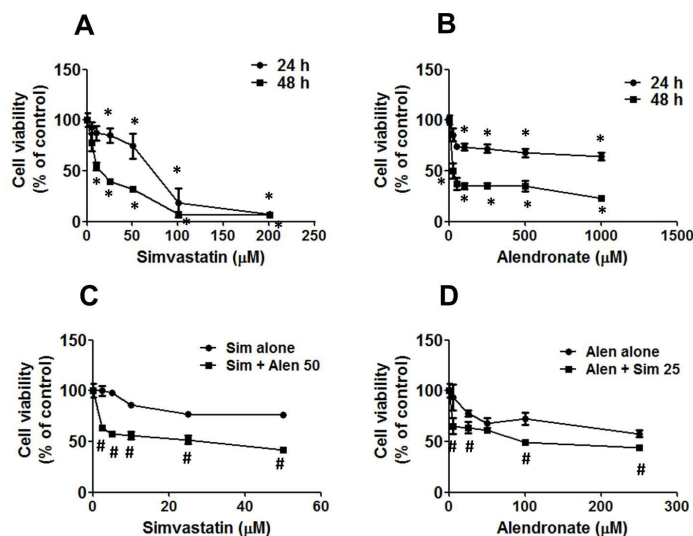
Fig. 1: Effects of simvastatin and alendronate, alone and in combination, on CCA cell proliferation, with cancer cell viability measured using the SRB method; (A,B): KKU-100 cells treated with simvastatin (0-200 µM) or alendronate (0-1000 µM) for 24-48 h; (C): KKU-100 cells treated with 50 µM alendronate plus various doses of simvastatin (0-50 µM) for 24 h; (D): KKU-100 cells treated with 25 µM simvastatin plus various doses of alendronate (0-250 µM) for 24 h. Results are shown as a percentage of control and expressed as mean±SEM values (n=3); *p<0.05 vs. control group of each time point; #p<0.05 vs. simvastatin or alendronate treatment alone; A,B: ( ): 24 h; ( ): 48 h; C: ( ): sim alone; ( ): sim+Alen 50; D: ( ): Alen alone and ( ): Alen+sim 25
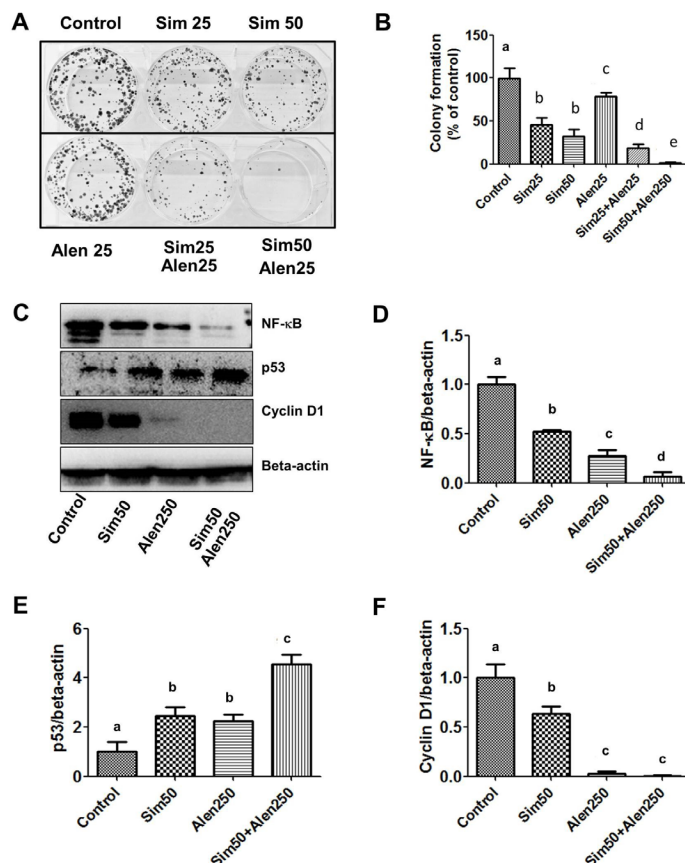
Fig. 2: Effects of simvastatin and alendronate, alone and in combination, on CCA cell growth and protein-related cell proliferation; (A-B): Cells treated with simvastatin alone (25-50 µM), alendronate alone (25 µM), or simvastatin plus alendronate for 24 h, grown for another 15 d, then stained with crystal violet and colony formation counted; (C-F): Cells treated with simvastatin alone (50 µM), alendronate alone (250 µM), or simvastatin plus alendronate for 24 h, with protein expression measured by western blot. All data shown are representative of three independent experiments. Data represent mean±SEM (n=3); different character indicates significant difference; Alen: Alendronate and Sim: simvastatin
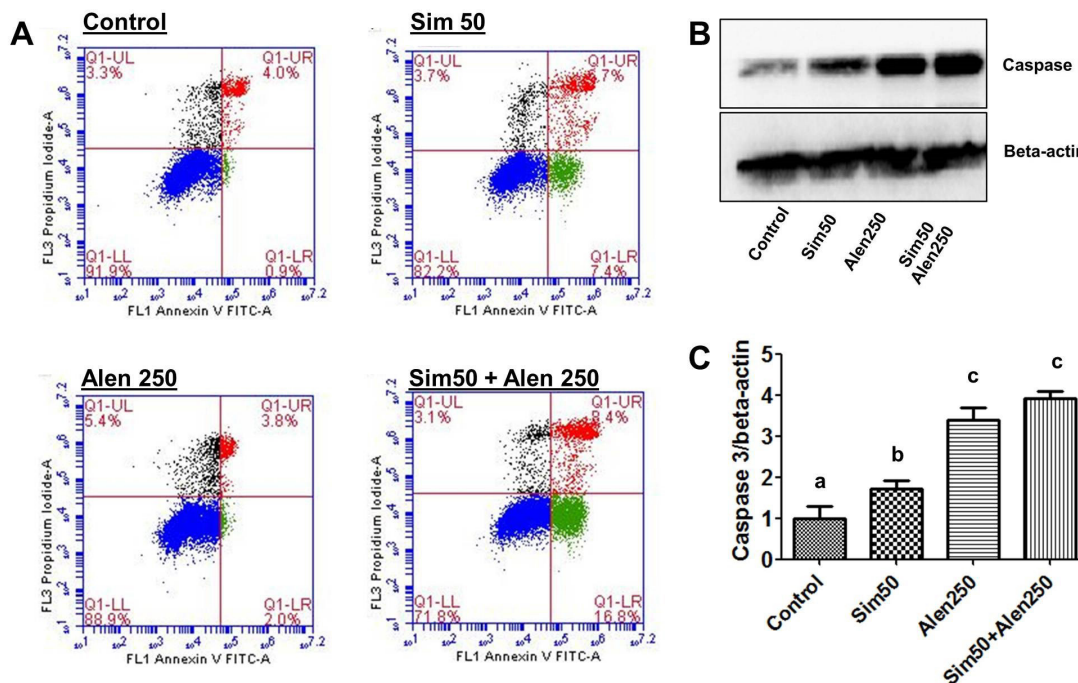

Fig. 3: Effects of simvastatin and alendronate, alone and in combination, on cell apoptosis and apoptosis-associated proteins in CCA cells; (A): Cells treated with simvastatin (50 µM), alendronate (250 µM), or simvastatin plus alendronate for 24 h, then stained with PI and Annexin V FTIC, and cell apoptosis measured by flow cytometry; (B-C): Cells treated with simvastatin alone (50 µM), alendronate alone (250 µM), or simvastatin plus alendronate for 24 h, then protein expression measured by western blot. Bars show mean±SEM (n=3); Different character indicates significant difference; Alen: Alendronate and Sim: Simvastatin

Western blotting was used to gain insight into the molecular mechanism by which the two test drugs induce KKU-100 apoptosis. By comparing flow cytometry and western blotting results, we found that simvastatin treatment alone significantly induced caspase 3 protein expression, but the induction was weaker than with alendronate treatment alone. Simvastatin and alendronate in combination induced higher levels of caspase 3 protein expression than the untreated control group, simvastatin alone, or alendronate alone (fig. 3B and fig. 3C). In conclusion, our results show that simvastatin in combination with alendronate stimulates CCA cell apoptosis more than either drug treatment alone.

The effects of simvastatin, alendronate, and their combination on KKU-100 cell migration were assessed using matrigel migration, wound healing, and gelatin zymography assays. In the first assay, cancer cells were exposed to simvastatin alone (5-25 μM), alendronate alone (50-250 μM), and alendronate supplemented with 10 μM simvastatin, and simvastatin supplemented with 50 μM alendronate for 24 h in matrigel-coated chambers. Our results showed that simvastatin and alendronate significantly decreased KKU-100 cell migration in a dose-dependent manner, and that combination treatment inhibited migration more than the untreated control and simvastatin and alendronate treatment alone (fig. 4A-fig. 4C). In the wound healing assay,

treatment with simvastatin alone and alendronate alone inhibited cancer cell migration (fig. 5A), but combination treatment inhibited CCA migration significantly more.

Next, to investigate the mechanism by which simvastatin and alendronate reduce cell migration, we examined MMP-2 and MMP-9 protein expression using the gelatin zymography assay. Simvastatin treatment and alendronate treatment each exerted an inhibitory effect on the levels of MMP-2 and MMP-9 when compared with the untreated control. In combination, simvastatin and alendronate reduced MMP-2 and MMP-9 protein expression levels further still (fig. 5B). Our results suggest that MMP-2 and MMP-9 levels can be down-regulated in CCA cells by treatment with simvastatin and alendronate.

To explore the mechanism by which simvastatin and alendronate induce cancer cell death, increase cell apoptosis, and inhibit cell migration, we examined Rac1 and RhoA protein expression by western blotting. The effects of the different treatments on Rac1 and RhoA protein expression in the KKU-100 cells are shown in fig. 6A-fig. 6C. Simvastatin did not alter Rac1 expression, but alendronate suppressed Rac1 expression compared to the untreated control and simvastatin treatment. Treatment with simvastatin and alendronate in combination caused an augmented reduction in Rac1 expression.
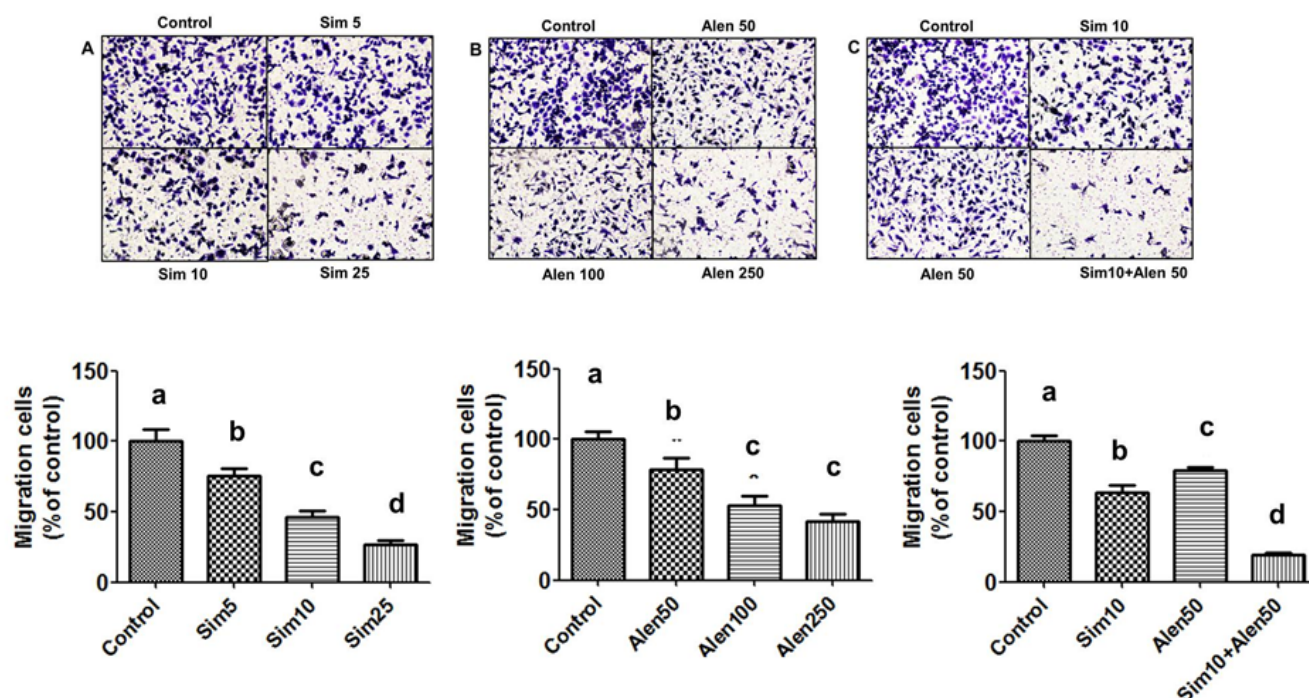

Fig. 4: Effects of simvastatin and alendronate, alone and in combination, on CCA cell migration; (A-C): Cells plated on top of insert-wells, treated with various doses of simvastatin (0-25 μM), alendronate (0-250 μM), or simvastatin plus alendronate for 24 h, and cells passing through the bottom of insert-wells then counted; Data are mean±SEM (n=3); Different character indicates significant difference; Alen: Alendronate and Sim: Simvastatin
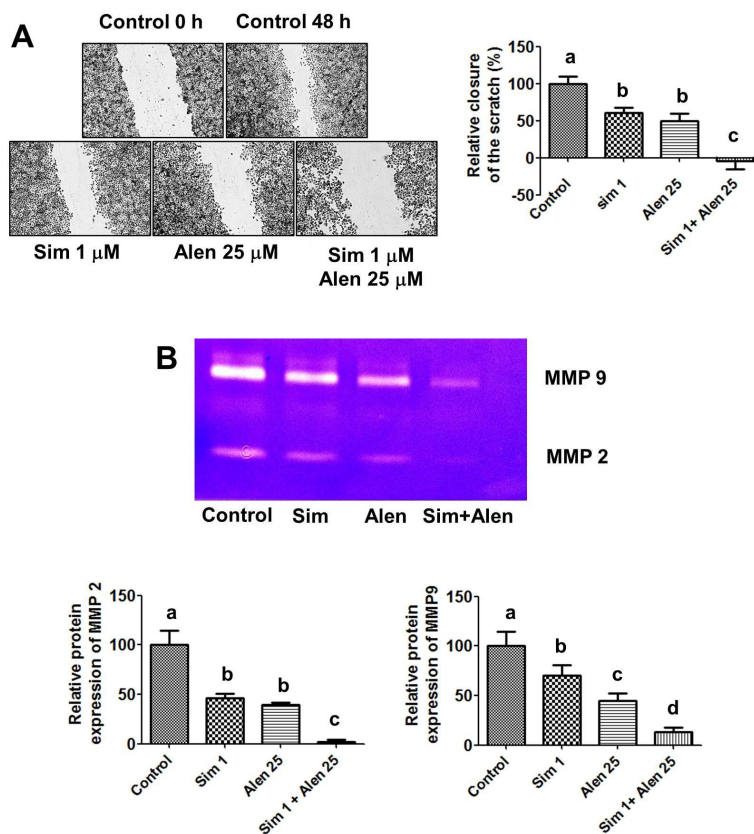
Fig. 5: Effects of simvastatin and alendronate, alone and in combination, on CCA cell migration and protein-related cell migration; (A): Cell monolayer scratched, treated with simvastatin (1 µM), alendronate (25 µM), or simvastatin plus alendronate for 48 h, then wound dimensions measured, and cell migration calculated; (B): Cells treated with simvastatin (1 µM), alendronate (25 µM), or simvastatin plus alendronate for 48 h, then culture medium collected, loaded onto gel with a non-reducing agent, and band density assessed for MMP 2 and MMP 9 levels; data are mean±SEM (n=3); different character indicates significant difference; Alen: Alendronate and Sim: Simvastatin

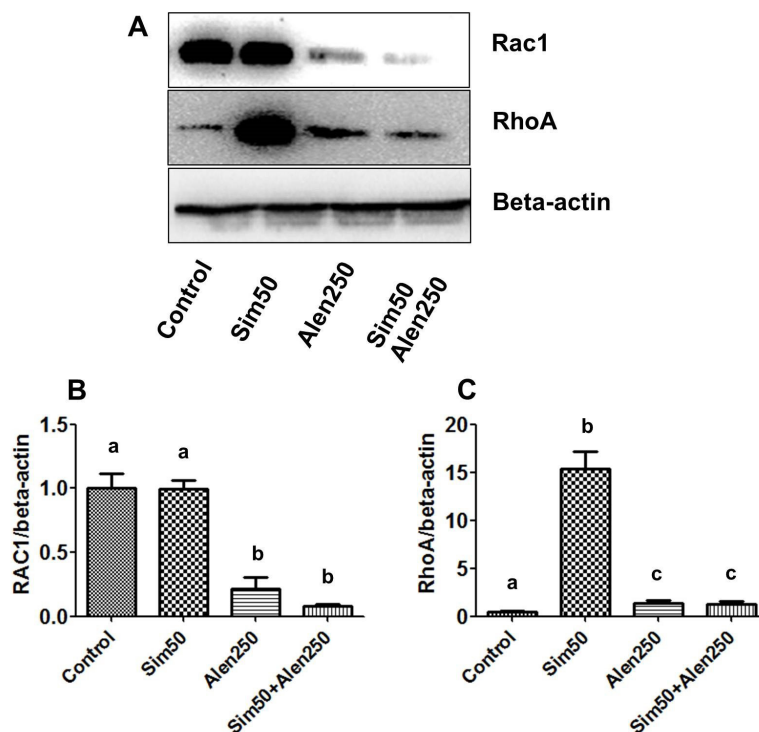**Fig. 6: Effects of simvastatin and alendronate, alone and in combination, on Rac1 and RhoA protein expression; (A-C): Cells treated with simvastatin alone (50 µM), alendronate alone (250 µM), or simvastatin plus alendronate for 24 h with protein expression then measured by western blot; data are mean±SEM (n=3); different character indicates significant difference; Alen: alendronate and Sim: simvastatin**

Whilst simvastatin did not alter Rac1 expression, it did significantly stimulate RhoA protein expression compared to the control, alendronate alone, and combination treatment. Alendronate and the combination treatment increased RhoA levels compared to the control, but not significantly. In conclusion, simvastatin and alendronate modulated Rac1 and RhoA protein expression in the MVA pathway and, although additional studies will be required, this combination could potentially be used to induce cytotoxicity and apoptosis in CCA cells and to inhibit the migration of CCA cells as well.

Simvastatin and alendronate are known to influence cell proliferation and apoptosis in several cancer cell types, perhaps by inhibiting the production of MVA products, such as Rho and Rac. Prior to the current study, however, the effects of simvastatin and alendronate, alone and in combination, on human CCA cells were still unknown. We have shown here that simvastatin and alendronate, in a dose- and time-dependent manner, inhibit CCA cell proliferation, induce cells apoptosis, and suppress the metastatic potential of KKU-100 cells. Moreover, when simvastatin and alendronate were combined, their activity was superior to either drug alone. The combination reduced cancer cell regrowth by reducing expression of NF-κB and cyclin D1, and by increasing expression of the tumor suppressor protein p53. The combination also induced apoptosis by increasing caspase 3 levels, inhibited cancer cell migration by reducing MMP-2 and MMP-9 expression, and modulated the MVA pathway by interfering with Rac1 and RhoA expression. Taken together, these results suggest simvastatin and alendronate could potentially be developed as a therapeutic or preventive treatment for CCA cancer or as an adjunct(s) for potentiating the activity of current anticancer drugs.

The MVA pathway is involved in cholesterol synthesis and protein prenylation, and has been associated with many steps in carcinogenesis, the aggressiveness of tumors, and cancer progression. A few drug classes, such as statins and BPs, suppress the MVA pathway and also act as anticancer agents. We therefore examined the effects of simvastatin and alendronate, alone and in combination, on CCA cells. Both drugs target the HMG-CoA reductase and FPPS enzymes in the MVA pathway and act as cancer suppressors. Our results indicate that both drugs potentiate CCA cancer cell death by reducing NF-κB and cyclin D1 and inducing p53 and caspase 3. Both

p53 and NF-κB are important in many steps in the cellular process[19] and, importantly, both can control cancer cell proliferation and migration. NF-κB is associated with the regulation of cell transformation, proliferation, and tumor development; furthermore, NF-κB activates the transcription of cyclin D1 in the G1 phase of the cell cycle[20], stimulating cancer cell growth. Also, stimulation of the p53 tumor suppressor can lead to DNA damage in cancer cells, resulting in apoptosis and cell cycle arrest[21]. Our results show that simvastatin and alendronate inhibit CCA cell proliferation by inducing the tumor suppressor protein p53 and reducing expression of NF-κB and cyclin D1.

The effects of MVA pathway inhibitors are mostly mediated by apoptosis induction. For example, simvastatin and zoledronic acid suppress drug resistance and additively induce apoptosis[22] in multiple myeloma cells. The aim of many studies is to reduce the concentration of BPs needed to kill or manage cancer cells. In human breast cancer, it was found that zoledronic acid combined with the standard anticancer drug, doxorubicin, could positively induce cancer cell death[23-25]. MVA pathway inhibitors kill cancer cells by reducing protein geranylation more than farnesylation[26], but little is known about the downstream mechanisms. These inhibitors decrease downstream protein and lead to a reduction in Rac, Rho, Ras, and Rab protein expression levels. These effects can suppress cell growth, proliferation, and migration.

The anticancer effects of statins have been demonstrated in both *in vitro* and *in vivo* studies, and against various cancer cell types including gastric, breast, pancreatic, prostate and colon cancer[27]. Our previous report showed that two statins, simvastatin and atorvastatin, strongly suppress CCA cell growth and migration at low concentrations[18]. Work by Wong et al.[6] confirmed that the mechanism of action of statins against cancer cells involves activation of cell death and apoptosis *via* an obstruction of the post-translational modification of various small G proteins (Rac, Rho, and Ras)[6]. Importantly, the Ras protein controls the crucial signaling pathway of growth factor, while Rho and Rac mainly control cell migration, cell adhesion, and the cytoskeleton[28]. Repression of the small G proteins may contribute to MVA inhibitor-induced inhibition of cancer cell growth and migration. MVA inhibitors significantly decrease GPP and FPP production, and inhibit cell

proliferation both *in vitro* and *in vivo*. In the present study, MVA inhibitor-induced repression of cell growth and migration may have been due to altered Rac1 and RhoA protein expression levels, especially Rac1 levels. Our results indicate that MVA inhibitors can modify small G protein, which has a crucial role in the suppression of CCA cancer growth and migration. Another mechanism of action, proposed by Karlic et al.[29] is that simvastatin and ibandronate down regulate energy metabolism by suppressing nicotinamide adenine dinucleotide phosphate, then activate reactive oxygen species, causing cancer cell starvation and death[29]. Statins and BPs may therefore cause cancer cell death *via* multiple mechanisms, but the primary mechanism is likely to be suppression of the MVA pathway.

In an epidemiologic analysis, El-Refai et al.[30] reported that combination of statins with BPs improved overall survival in patients with cancer (hazard ratio 0.85; 95 % confidence interval, 0.80 to 0.91)[30]. Lee et al.[31] also found that patients with advanced non adenocarcinomatous non-small cell lung cancer who received simvastatin (40 mg/d) plus afatinib (40 mg/d) had a longer overall survival than afatinib treatment alone (approximately 10.0 mo *vs.* 7.0 mo), but this difference was not statistically significant (p=0.930)[31]. Simvastatin is usually used at a dose of 40 mg/d to treat hyperlipidemia patients and has low toxicity. These findings suggest that MVA inhibitors, both statins and BPs, could be developed as therapeutic options for CCA cancer and perhaps as sensitizing chemotherapeutic agents.

The MVA inhibitors, simvastatin and alendronate, when used in combination, have a cytotoxic effect against human CCA cells. This activity is partly attributable to a reduction in cancer cell growth, induction of apoptosis, and inhibition of cell migration. The combination also inhibited CCA cancer cell proliferation and regrowth by blocking NF-κB and cyclin D1 and by inducing p53 and caspase 3 protein expression. CCA cell migration was inhibited too, caused by simvastatin and alendronate reducing MMP-2 and MMP-9 levels. The above findings indicate that simvastatin and alendronate have promising activities against CCA cancer cells *via* modulation of Rac1 expression, and may represent a useful new approach for CCA cancer therapy.

Conflict of interest:

The authors declared no conflict of interest.

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