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Original Research Article

Alendronate blocks human cholangiocarcinoma cell proliferation and migration

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Abstract

Purpose: *To explore the effect of alendronate on cell death and migration of cholangiocarcinoma (CCA).*
Methods: *Migration and cell death of CCA cells were determined using sulforhodamine B (SRB), colony formation, wound healing, and gelatin zymography assays. The mechanism of action of alendronate was studied with reverse-transcriptase polymerase reaction (RT-PCR) for gene expression and by Western blotting analysis for protein expression.*
Results: *Alendronate stimulated KKU-100 cell death in dose- and time-dependent manner, with low IC_{50} value, and significantly inhbited colony formation at doses of 5 - 100 µM. Moreover, alendronate at doses of 250 - 1000 µM significantly stimulated CCA apoptosis via reactive oxygen species (ROS) generation, and enhanced caspase 3 activity at a dose of 1000 µM. Moreover, at a dose of 250 µM, it significantly inhibited cell growth through induction of caspase 3 and p53, and reduction of protein expression levels of NF-κB. Furthemore, alendronate altered mevalonate (MVA) pathway via downregulation of Rac1 protein expression. In contrast, it significantly inhibited CCA cell migration, and reduced MMP 2 and MMP 9 levels at doses of 25 - 100 µM.*
Conclusion: *Alendronate may be useful as a novel drug for prevention and chemotherapy of CCA.*

Keywords: *Alendronate, Cholangiocarcinoma, Mevalonate (MVA), Cell death, Matrix metallopeptidase (MMP), Bisphosphonates*

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INTRODUCTION

Bisphosphonates (BPs) are mevalonate (MVA) pathway inhibitors which generally inhibit farnesyl pyrophosphate synthase (FPPS), thereby inhibiting the prenylation of small signaling proteins for example Rab, Ras, and Rho [1]. Many reports have shown that BPs suppress cancer cell growth and stimulate apoptosis in myeloma, breast, pancreas, liver, ovarian, and prostate cancerous cells [2]. Alendronate is a BP which is used for treating osteoporosis as well as cancer. It has been indicated that alendronate and other BPs suppress prostate cancer invasion

[3], decrease human epidermal carcinoma cells proliferation [4], reduce angiogesis [5]. BPs exert their activities on cancer cells by acting on telomerase activity [6], mevalonate (MVA) pathway [7], and by reducing the protein-related proliferation. However, not much is known the effect of alendronate on cholangiocarcinoma (CCA).

CCA is a bile duct cancer which originates from cholangiocyte. Studies have found that CCA-related mortality has increased worldwide [8]. Southeast Asian countries are also involved. Indeed, a study reported that Thailand had the highest incidence of morbidity and mortality from CCA, which is an important health problem in the northeastern part of that country [9]. CCA is correlated with very poor prognosis, and it is refractive to extant to anticancer drugs. Surgical resection is the most effective procedure for prolonging the life of CCA patients, although the overall 3-years survival rates is only 22 % [8]. Therefore, there is need to evolve newer or more effective agents or drugs for treating CCA.

Accordingly, this study was carried out to explore the effect of alendronate on cancer cell growth and migration using the CCA cell line KKU-100. Moreover, the underlying mechanism of alendronate action on the CCA cells death was studied through ROS formation, caspase 3 activity, protein-related cell death, caspase 3, p53, NF-κB as well as MVA expression. Then, the alendronate activities on cell migration assay was explored by wound healing, matrigel, and gelatin zymography.

EXPERIMENTAL

Cell culture and evaluation of cytotoxicity

Cells of the CCA cell line (KKU-100) were cultured in DMEM (10 % FBS, 100 U/mL Pen and 100 mg/mL Step. For cell viability assay, the cells were treated with various doses of 0 - 1000 µM alendronate for 24 - 48 h, and cells death was examined by SRB assay as previously described [10].

Colony formation assay

KKU-100 ells were treated separately with various doses of alendronate (0 - 100 µM) for 24 h, and prior to assay of colony formation as described previously [10].

Wound healing assay

In the wound healing assay, the cells were scratched with 0.2 mL micro pipette tip, after which they were treated with alendronate at doses of 0 – 50 µM for 48 h. Then, wound healing assay was carried out as described previously [10].

Matrigel migration assay

KKU-100 cells were resuspended in serum-free DMEM and seeded into the upper insert wells (Corning), while the bottom chamber contained DMEM complete medium. Then, alendronate was added to the cells at doses of 0 - 100 µM, and the cell was allowed to migrate for 24 h. Therefore, KKU-100 cells were added to absolute methanol, and then exposed to 0.5 % crystal violet for 60 min. The images of the stained cells were captured using inverted microscopy (*10x* magnification) and the cells were counted.

Gelatin zymography assay

In this assay, KKU-100 cell was treated with alendronate (0 - 100 µM) for 48 h, afterwards, the protein expressions of MMP 2 and MMP 9 were examined as described previously [10].

Cell apoptosis assay using flow cytometry

Following incubation with different doses of alendronate (0 - 1000 µM) for 24 h, KKU-100 cells were harvested and then stained with apoptotic assay dye (PI and annexin V-FITC) in dark for 15 min. The stained cell was assessed for degree of apoptosis using flow cytometry.

Determination of reactive oxygen species (ROS)

KKU-100 cells were incubated with alendronate (0 – 1000 µM) and fluorescent probe (25 µM DHE) for 90 min in the dark at 37 °C. Thereafter, ROS production was assessed in line with the procedure outlined previously [11].

Determination of caspase 3 activity

KKU-100 cells were incubated with separately different doses of alendronate (0 – 1000 µM) for 24 h, and further measurement of caspase 3 activity by caspase 3 assay kit according to the manufacturer's instructors [11].

Reverse-transcriptase polymerase chain reaction (RT-PCR)

KKU-100 cells were incubated with 250 µM alendronate for 24 h, after which the mRNA expressions of Rac1 and RhoA were determined using RT-PCR and primer sequences as

described previously [11]. The relative gene expression was measured with comparative Cq analysis ($^{\Delta\Delta}$Cq method).

Western blotting

The cells were exposed to 250 µM alendronate for 24 h and the protein expressions of Rac1, RhoA, caspase 3, p53 and NF-κB were assayed using Western blotting as described previously [12]. The blot was incubated with primary antibody for Rac1, RhoA, caspase 3, p53, NF-κB, beta-actin as an internal control (1:1000 dilution for primary antibodies). The secondary antibody conjugated to horseradish peroxidase was diluted 1:2500.

Statistical analysis

Values are presented as mean ± standard error of the mean (SEM). Data were statistically analysed using Student *t*-test. The statistical analysis was done with SigmaStat software version 3.5 (Systat Software Inc., San Jose, CA, USA). Values of $p<0.05$ were taken as indicative of statistical significance.

RESULTS

Alendronate inhibited CCA cell proliferation and colony formation

Alendronate inhibited KKU-100 cell growth in concentration- and time-dependent manners, relative to the untreated control group (Figure 1 A and 1 B), with IC_{50} value of 736.10 ± 136.44 µM after 24 h, and 360.19 ± 36.52 µM after 48 h. Moreover, alendronate significantly decreased colony formation in a dose-dependent manner, with low IC_{50} value of 11.31 ± 1.62 µM (Figure 1 C to D). Thus, alendronate inhibited colony formation at a lower dose with related to the concentrated which inhibited proliferation.

Alendronate induced CCA cells apoptosis

The cell were exposed to 250 µM of alendronate and subjected to analysis of apoptosis. The results indicated that alendronate induced early and late apoptotic cells, when compared with controls, and also reduced the viability of the cancer cells (Figure 2 A).

Alendronate induced ROS production and increased caspase 3 activity

Alendronate activated ROS formation in a dose-dependent manner, and the activity was significant at the doses range of 250 - 1000 µM (Figure 2 B). Furthermore, alendronate significantly induced caspase 3 activity at a dose of 1000 µM (Figure 2 C). It induced CCA cell death and apoptosis by inducing ROS production and stimulating caspase 3 activity.

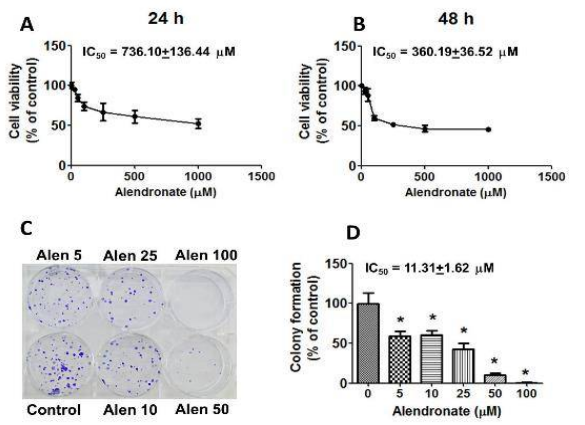

Figure 1: Alendronate effects on cell viability (A-B) and colony formation (C-D) in CCA cells. *p < 0.05 (Alen = alendronate

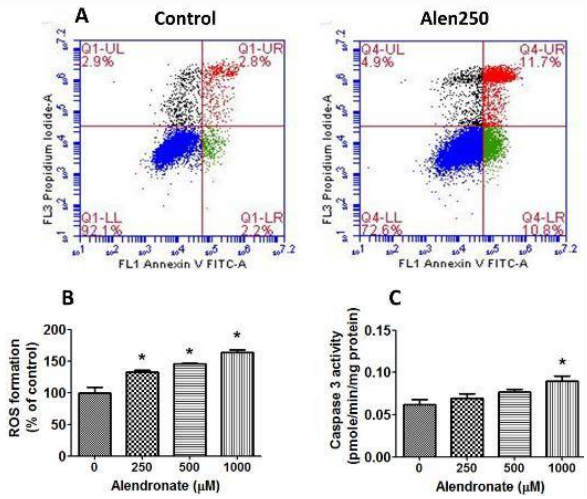

Figure 2: Alendronate effects on cell apoptosis (A), ROS formation (B), and caspase 3 activity (C) in CCA cells. *p < 0.05 (Alen = alendronate)

Alendronate altered mevalonate pathway

The effect of alendronate on MVA products was studied by determining Rac1 and RhoA gene and protein expressions. Alendronate induced Rac1 and RhoA gene expression in CCA cells (Figure 3 A). Furthermore, alendronate downregulated Rac1 protein expression but upregulated RhoA protein expression (Figure 3 B).

Alendronate induced protein-related cells death and inhibited protein-related cells growth

Alendronate effects on protein-related cell proliferation and cell death was studied by using

Western blotting. The results showed that alendronate significantly increased caspase 3 and p53, but decreased NF-κB levels in CCA cells (Figure 3 B). Alendronate activated cancer cell death by increasing enzyme-related cell apoptosis, caspase 3, and tumor suppressor protein, p53; and also induced protein-related cell proliferation, NF-κB.

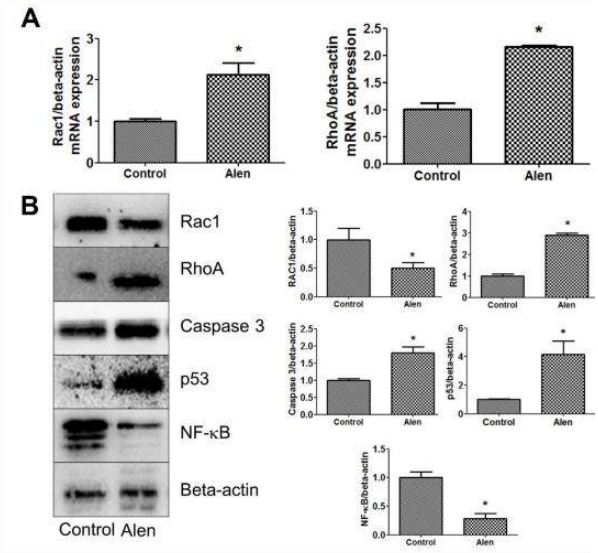

Figure 3: Alendronate effects on Rac1 and RhoA gene expression (A), and protein-related cell death in CCA cells (B). *$p < 0.05$ (Alen = alendronate)

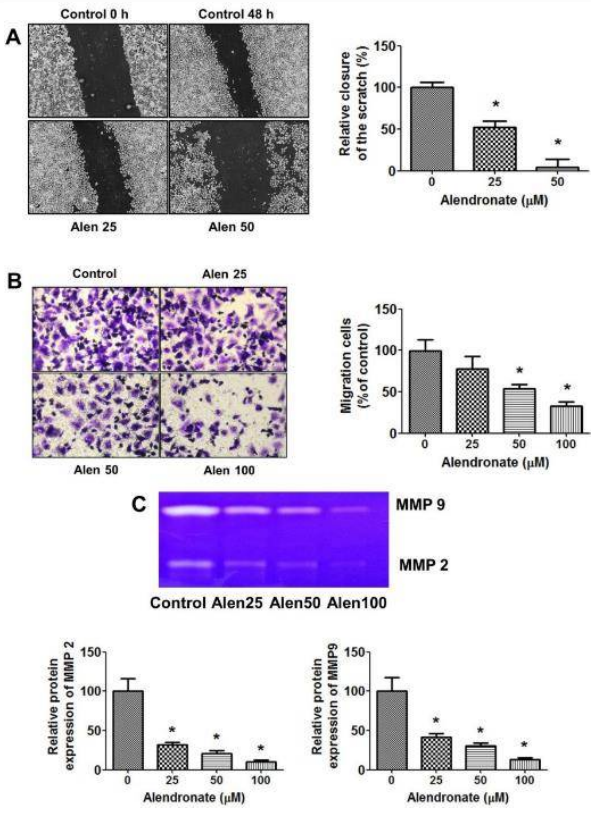

Figure 4: Effect of alendronate on cell migration in CCA cells (A - C); $p < 0.05$ (Alen = alendronate)

Alendronate inhibited cells migration

Alendronate effects on CCA cells migration was determined using wound healing and matrigel migration assay. Alendronate suppressed the closure of the scratch in a dose-dependent manner, and the activity was significant at the dose range of 25-50 µM (Figure 4 A). Moreover, the results from matrigel migration assay showed that alendronate inhibited cell migration significantly at doses of 50 - 100 µM (Figure 4 B). Moreover, alendronate significantly decreased MMP 2 and MMP 9 protein (Figure 4 C) and suppressed the migration of CCA cells by downregulating MMP expression levels.

DISCUSSION

BPs are effective anti-osteoporosis drugs and are also currently used in treating cancer that has metastasized to bone. Presently, research is focus on BPs as possible powerful inhibitors of cancer cells growth, acting either on the suppression of cell growth as inhibitors of migration of cancer cells. However, not much is known the alendronate effect, a BPs, on the migration and proliferation of CCA. Thus, this study was focused on the alendronate potential on CCA cell death and migration.

To evaluate the anticancer potential of alendronate on CCA cells, KKU-100 cells were used. The results showed that alendronate exerted antiproliferative effects, anticolony formation property and antimigratory effects through enhancement of ROS formation, upregulation of protein expression of caspase 3 and p53, downregulation of protein expression of NF-κB. This is the first study to report that alendronate interrupts CCA cell proliferation and migration, and it may provide a basis for developing a new treatment for CCA.

The MVA pathway controls several cellular processes in cancer such as proliferation, apoptosis, migration, and invasion. Therefore, the reduction of MVA products caused repression of cell proliferation and migration. From the results obtained, alendronate modulated Rho GTPase protein through reducing gene and protein expression of Rac1, but not effect on RhoA. The Rac1 gene or protein regulates several signalling pathways such as organisation of cytoskeleton, transcription, and proliferation [13]. The downregulation of Rac1 gene expression enhances cell cycle arrest and induces apoptosis in the human breast cancer cells [14] and in CCA [15].In this study, alendronate facilitated apoptois of CCA cells by

inducing ROS production and activating caspase 3 activity.

It altered protein-related cell death via induction of caspase 3 activity and p53 tumor suppressor gene, and reduction in NF-κB levels. It has been reported that zoledronate mediated augmentation of p53 as well as caspase 3/7 and caspase 9 protein levels in mesothelioma [16]. The Rho GTPases and the NF-κB pathway are critically involved in human diseases and may be potential therapeutic targets [17]. Moreover, zoledronate inhibits proliferation of myeloma cells through suppression of NF-κB/pim-2 [18]. In the present study, alendronate consistently induced CCA cell death and apoptosis by decreasing Rac1, and these results were correlated with the pattern of expression of p53 and NF-κB.

Metastasis is a crucial step in the development of secondary tumors beginning from original tumor. It is a major problem in cancer therapy, including CCA treatment. Animal and clinical researches have revealed that BP interrupt the migration potential of metastatic cells [19]. The antimigrative activities of BPs have been reported in many tumor studies [3,4]. Indeed, the antimigratory effect of alendronate has been demonstrated in prostate and breast cancer cells [3], osteosarcoma [20], and melanoma [21].

In the present investigation, alendronate displayed significant antimigratory potential in CCA cells, and showed promising results in wound healing and matrigel migration assay. These findings are in agreement with those of Molinuevo *et al* who reported that alendronate inhibited cell migration dose-dependently [22] and decreased phosphatase activity in cell-free osteoblastic extracts and osteoblasts. Moreover, studied have shown that low concentration of alendronate inhibited prostate cancer cell migration.

It has also been demonstrated that BPs inhibited cell migration by decreasing MMP 2 and MMP 9 in *in vivo* models of prostate and cervical cancer [23]. Research has also shown that zoledronate and minodronate exert effects on VEGF function and signaling in endometrial cells [23] . Thus, numerous studies have found that BPs decrease the cancer cells migration in several cancer cell types (including CCA cells) with different mechanism.

CONCLUSION

Alendronate exerts anti-cancer effects by activating CCA cell death and cell migration. The results indicate that alendronate is a potentially beneficial treatment for poor-prognosis CCA. However, *in vivo* studies are needed to confirm if indeed alendronate and other BPs would be effective for the management of aggressive and extremely angiogenic CCA.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. BB performed the experiment, analysed the data and then BB, LS, AP, VK read and approved the final manuscript.

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