

Alantolactone suppresses human osteosarcoma through the PI3K/AKT signaling pathway

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Abstract. Osteosarcoma is the most common type of malignant bone cancer and results in cancer-related deaths among adolescents. Alantolactone (ALT) demonstrates antitumor properties in various diseases; however, its potential role in osteosarcoma is relatively unclear. The aim of the present study was to evaluate the effect of ALT on osteosarcoma. ALT significantly decreased the viability of U2OS and HOS osteosarcoma cell lines. Cells flow cytometry assay and Hoechst 33258 staining assay revealed that ALT significantly increased the proportion of apoptotic U2OS cells. In addition, wound healing and Transwell invasion assays demonstrated that the invasion and migration of osteosarcoma were markedly reduced upon ALT treatment. It was hypothesized that the antitumor functions of ALT are mediated through inhibition of the PI3K/AKT signaling pathway. In conclusion, the results of the present study confirmed the inhibition of ALT on osteosarcoma cells via downregulation of PI3K/AKT signaling pathways, suggesting ALT as a potential therapeutic candidate for osteosarcoma.

Introduction

Osteosarcoma is the most common type of malignant bone cancer, which originates from long bones, including the humerus, femur and tibia, and contributes to cancer-related mortality in adolescents (1,2). Although survival rates have improved with the current standard treatment strategies, such as radiotherapy, chemotherapy and surgery, 80% of cases of osteosarcoma still progress to metastasis (3). Therefore, there is an urgent requirement to develop effective and safe compounds, which exert minimal cytotoxicity on healthy tissue that could be potential future therapies for patients with osteosarcoma.

The extracellular matrix (ECM) acts as a support system for normal cells and tissues. In the context of osteosarcoma, which progresses rapidly and can lead to metastasis, aggressive cancer cells can damage ECM components, thus easily invading normal tissues and causing irreversible damage; ECM breakdown is mainly modulated by proteases, specifically matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9 (4-6); activation of MMPs are understood to promote the invasion of osteosarcoma (7,8). Previous studies have reported that the PI3K/AKT signaling pathway is associated with the metastasis of osteosarcoma (9-11); the deregulation of this pathway serves an important role in the tumorigenesis, proliferation, invasion, apoptosis and metastasis of cancer cells (12-16). Thus, the PI3K/AKT signaling pathway may represent a possible target for cancer therapy in osteosarcoma.

Alantolactone (ALT) is a biologically natural compound derived from *Inula helenium* (17). Previous studies have reported that ALT promotes numerous biological effects, including anti-inflammatory and antioxidant functions (18,19). It has also been observed to suppress several types of human cancer, including gastric, pancreatic and breast cancer cells (19-21). It is reported that these anticancer effects of ALT are mediated through the PI3K/AKT signaling pathway (22). Thus, it was hypothesized that ALT may represent a potential agent for the treatment of osteosarcoma. In the present study, the effect of ALT on the apoptosis, proliferation, and invasion of osteosarcoma cells, alongside the underlying mechanism associated with these effects was evaluated.

Materials and methods

Reagents. ALT and Cell Counting Kit (CCK)-8 were obtained from MedChem Express. Primary antibodies against PI3K (4249), phosphorylated (p)-AKT (4060), AKT (9272), cyclin D1 (2978), p27 (3686), Bax (5023), Bcl-2 (15071) and β -actin (3700) were purchased from Cell Signaling Technology, Inc., primary antibodies against cleaved caspase-3 and cleaved caspase-8 were purchased from Abcam, and primary antibodies against MMP-2 and MMP-9 were obtained from ProteinTech Group, Inc. The Annexin V-FITC/propidium iodide (PI) apoptosis detection kit and was purchased from Nanjing KeyGen Biotech Co., Ltd. RPMI-1640 medium and FBS were purchased from Hyclone; GE Healthcare Life Sciences.

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Cell culture and reagents. The human osteosarcoma U2OS and HOS cell lines were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences. U2OS and HOS cells were cultured in RPMI-1640 medium and DMEM (Gibco; Thermo Fisher Scientific, Inc.), respectively, supplemented with 10% FBS and 1% penicillin/streptomycin, and maintained in a humidified atmosphere of 37°C and 5% CO₂.

CCK-8 assay. The U2OS and HOS cell lines were seeded at a density of 8x10³ cells/well in 96-well plates and cells were subsequently exposed to a range of concentrations of ALT (2.5, 5, 10, 20, 40, 80 or 160 μM) for 24 and 48 h at 37°C with 5% CO₂. The CCK-8 assay was used to assess the viability of osteosarcoma cells following drug treatment. A total of 10 μl CCK-8 kit solution was added to each well and the cells were subsequently incubated for a further 4 h at 37°C with 5% CO₂, after which the optical density of the cell lysates was measured at 450 nm. GraphPad Prism version 7.0 software (GraphPad Software, Inc.) was used to calculate the median lethal concentration of ALT (IC₅₀) for osteosarcoma cells.

Colony formation assay. U2OS and HOS cells were collected and seeded in 6-well plates at a density of 1x10³ cells/well. Following cell adherence, the culture medium was replaced with each cell line's respective media containing a range of ALT concentrations (U2OS, 0, 5, 10 or 20 μM; HOS, 0, 15, 30 or 60 μM) and the cells were further incubated for a further 8 days in a humidified cell incubator at 37°C with 5% CO₂. Following incubation, colonies were first fixed with 4% paraformaldehyde for 30 mins at room temperature and then stained with 0.1% crystal violet for 5 min at room temperature. Colonies (>50 cells) were visualized using an optical microscope (magnification, x10).

Hoechst 33258 staining assay. To evaluate the apoptotic rates of ALT-treated U2OS cells, the Hoechst 33258 kit (Beyotime Institute of Biotechnology) was used for nuclear staining. Bright blue nuclear staining indicated nuclear pyknosis, which is a characteristic of apoptotic cells (23). Following treatment with ALT (0, 5, 10 or 20 μM) for 48 h, the U2OS cells were fixed with 4% paraformaldehyde for 30 min at room temperature. The U2OS cells were rinsed with PBS three times both before and after staining with Hoechst 33258 (10 μg/ml; 5 min at room temperature) in the dark. An Eclipse TS100 fluorescence microscope (Nikon Corporation; magnification, x20 and x40) was used to visualize the changes in the nuclear morphology of ALT-treated U2OS cells.

Flow cytometric analysis. A total of 5x10⁵ U2OS cells/well were seeded in 6-well plates and incubated with a range of ALT concentrations (0, 5, 10 or 20 μM) for 48 h at 37°C with 5% CO₂. Following cellular adherence to the plates, the U2OS cells were harvested and rinsed with pre-chilled PBS (4°C). To further evaluate ALT-induced apoptosis of the U2OS cells, an Annexin V-FITC/PI kit was used, according to the manufacturer's protocol. Flow cytometry cell sorting equipment (Navios EX flow cytometer; Beckman Coulter, Inc. and FlowJo v. 10.4; FlowJo LLC) was used to analyze the apoptosis of ALT-treated U2OS cells. The cells stained with

Annexin V (+) were considered early apoptotic and the cells stained with PI (+) were late apoptotic cells.

Wound healing assay. The effect of ALT on the migratory ability of U2OS cells was assessed using a scratch wound healing assay. A total of 5x10⁵ U2OS cells/well were seeded into 6-well plates and cultured to 90% confluence prior to incubation at 37°C with 5% CO₂ with 5% serum and a range of ALT concentrations (0, 5, 10 or 20 μM) for 48 h. The wound scratch was performed by 10 μl pipette tip. The wound area was visualized at the 0, 12, 24 and 48 h time points using an optical microscope (magnification, x10) and the width of the wound was analyzed using ImageJ software (v. d 1.47; National Institutes of Health). The Recovered wound area (%) of each time point (12, 24 and 48 h) was calculated as The wound area of 0 h-the wound area of each time point)/The wound area of 0 h.

Matrigel invasion assay. Matrigel-coated (BD Biosciences) Transwell chambers were used to detect the invasive ability of U2OS cells exposed to 0, 5, 10 or 20 μM ALT. A total of 2x10⁴ U2OS cells/well were plated in the upper chambers of Transwell plates in 100 μl FBS-free RPMI-1640 medium containing the previously indicated concentrations of ALT, 200 μl RPMI-1640 medium containing 20% FBS was placed in the lower chambers. Following incubation for 48 h at 37°C with 5% CO₂, the non-invasive cells remaining in the upper chambers were removed. The invasive cells in the lower chambers were fixed with 4% paraformaldehyde for 30 min and subsequently stained with 0.1% crystal violet for 20 min at room temperature. Stained cells were counted using an optical microscope (magnification, x20).

Western blotting. A total of 5x10⁵ U2OS cells/well were seeded into 6-well plates and incubated with a range of ALT concentrations (0, 5, 10 or 20 μM) for 48 h at 37°C with 5% CO₂. Total protein was extracted from U2OS cells using RIPA lysis buffer containing 1% protease and phosphatase inhibitor (all Beyotime Institute of Biotechnology). Protein samples were maintained on ice for 30 min and subsequently centrifuged (15,000 x g for 10 min at 4°C). Total protein was quantified using a bicinchoninic acid kit (Beyotime Institute of Biotechnology) and the extracted proteins were mixed with loading buffer and boiled at 95°C for 5 min for denaturation. Proteins (40 μg) were separated via SDS-PAGE on a 12% gel. The separated proteins were subsequently transferred onto a 0.22-μm PVDF membrane and blocked for 2 h with 5% non-fat dry milk at room temperature. The membranes were incubated with the following primary antibodies overnight at 4°C: Anti-PI3K, anti-AKT, anti-p-AKT, anti-cyclin D1, anti-p27, anti-Bax, anti-Bcl-2, anti-cleaved caspase-3, anti-cleaved caspase-8, anti-MMP-2, anti-MMP-9 and anti-β-actin, all the primary antibodies were diluted in the primary antibody diluent (Beyotime Institute of Biotechnology) at a concentration of 1%. Membranes were washed three times with TBS-0.1% Tween 20. Following the primary antibody incubation, membranes were incubated with anti-rabbit antibodies (14708, Cell Signaling Technology, Inc.) which were diluted in the secondary antibody diluent (Beyotime Institute of Biotechnology) at a concentration of 0.02%, for 1 h at

room temperature. Protein bands were visualized using the BioSpectrum Imaging system and ImageJ software (v. d 1.47; National Institutes of Health) was used for densitometry.

Statistical analysis. All data are expressed as the mean \pm SD from ≥ 3 independent experimental repeats. Statistical analyses were performed using GraphPad Prism software (version 7.0; GraphPad Software, Inc.). Significant differences between groups were determined by using one-way ANOVA, followed by a Tukey's post hoc test, $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cytotoxicity of ALT in osteosarcoma cell lines. The chemical structure of ALT is presented in Fig. 1A. Cellular viability of U2OS and HOS cells was evaluated in the presence of a range of ALT concentrations for 24 and 48 h using the CCK-8 assay. Concentrations of 20 or 60 μM ALT significantly reduced the viability of U2OS cells and HOS cells respectively, compared with the untreated cells (0 μM ; Fig. 1B). The IC_{50} of ALT in U2OS and HOS cell lines was determined as 29.63 and 67.74 μM at 24 h, and 19.57 and 56.86 μM at 48 h, respectively, for each cell line. Concentrations of 20 or 60 μM ALT presented the half maximal inhibitory concentration, so these concentrations were chosen as the high concentrations in the subsequent experiment.

ALT suppresses the proliferation of osteosarcoma cells. Low concentrations (5 μM) of ALT slightly decreased the number of cells and induced the shrinkage of HOS and U2OS cells compared with untreated cells, whereas higher concentrations (10 and 20 μM) not only decreased the number of osteosarcoma cells, but also made the shrinkage more serious (Fig. 1C). In addition, the colony formation assay demonstrated that ALT markedly reduced the colony-forming ability of U2OS and HOS cells compared with untreated cells (Fig. 1D).

ALT promotes apoptosis of osteosarcoma cells. Therapeutic candidates with antitumor activity often exert cellular cytotoxicity through promoting apoptosis of cancer cells (24,25). As U2OS and HOS cell lines were both collected from young Caucasian female patients with osteosarcoma, this peculiarity also lead to some similarities on tumorigenicity and drug resistance, therefore, subsequent experiments were only performed on U2OS cells. Hoechst 33258 staining, which detects condensed chromatin, demonstrated that the number of apoptotic U2OS cells increased with increasing concentrations of ALT compared with untreated cells (Fig. 2A and B). The rate of apoptosis of U2OS cells following ALT administration was also evaluated using Annexin V-FITC/PI double staining and flow cytometry (Fig. 2C and D). The percentage of apoptotic cells was 6.7 ± 0.12 (5 μM), 13.9 ± 0.36 (10 μM) and 37.4 ± 0.45 (20 μM) compared to 0 μM (5.33 ± 0.20)-for ALT concentrations of 0, 5, 10 and 20 μM respectively (Fig. 2C and D). These data demonstrated that ALT markedly enhanced the apoptosis of osteosarcoma cells in a dose-dependent manner.

ALT inhibits the migratory and invasive ability of osteosarcoma cells. A wound-healing assay was performed to evaluate

the effect of ALT on the migratory ability of U2OS cells. ALT significantly suppressed the migration of the U2OS cells in a dose-dependent manner compared with untreated cells (Fig. 3A and B). Subsequently, a Matrigel assay was performed to assess the effect of ALT on the invasive potential of U2OS cells. The number of invading cells significantly decreased following exposure to ALT for 48 h compared with the untreated cells (Fig. 3C and D). Overall, these results demonstrated that ALT significantly suppressed the invasion and migration of U2OS cells.

Inhibitory mechanisms of ALT in osteosarcoma cells. To evaluate the mechanisms underlying ALT-mediated inhibition of osteosarcoma, western blotting was performed. High concentration of ALT-treated cells demonstrated significantly reduced expression levels of cyclin D1 and increased expression of p27 compared with the untreated cells (Fig. 4A and B). Cyclin D1 and p27 modulate the progression through the G1 and S phases of the cell cycle (26,27), and thus these changes may suggest the reason for inhibition of cell proliferation.

It has previously been reported that ALT promotes apoptosis in osteosarcoma (28-30); therefore, the effect of ALT on the expression levels on Bcl-2, Bax, cleaved caspase-3 and cleaved caspase-8, which all serve important roles in the process of apoptosis was explored. The expression levels of cleaved caspase-3 and cleaved caspase-8 were significantly upregulated in the ALT-treated U2OS cells compared with the untreated cells (Fig. 4C and D). In addition, ALT treatment reduced the expression levels of Bcl-2 and increased the expression levels of Bax compared with untreated cells (Fig. 4C and D).

MMPs are strongly associated with cellular invasion in osteosarcoma (31-33); therefore, the effect of ALT on MMP-2 and MMP-9 was evaluated. The data revealed that ALT decreased the expression of MMP-2 and MMP-9 in a dose-dependent manner compared with untreated cells (Fig. 4E and G).

Previous studies have reported that the PI3K/AKT signaling pathway serves an important role in osteosarcoma (11,34,35); PI3K/AKT modulates the proliferation, invasion, migration and apoptosis of osteosarcoma cell lines (36-39). Thus, the effect of ALT on the PI3K/AKT signaling pathway was investigated to confirm whether it is the mechanism by which ALT affects osteosarcoma cells. ALT demonstrated a significant ability to reduce the activation of p-AKT, which was observed through significantly decreased protein expression levels in U2OS cells compared with untreated cells (Fig. 4F and H) although no effect on the expression of total PI3K was observed. These findings suggested that the cytotoxic effect of ALT is closely associated with regulation of the PI3K/AKT signaling pathway.

Discussion

Osteosarcoma is the most common type of primary bone cancer and affects a large number of adolescents; the incidence of osteosarcoma in the Netherlands was $\sim 0.55/100,000$ (using the European Standardized Rate, ESR) (40). Current standard therapy regimens, such as surgical resection and chemotherapy only delay osteosarcoma progression and are

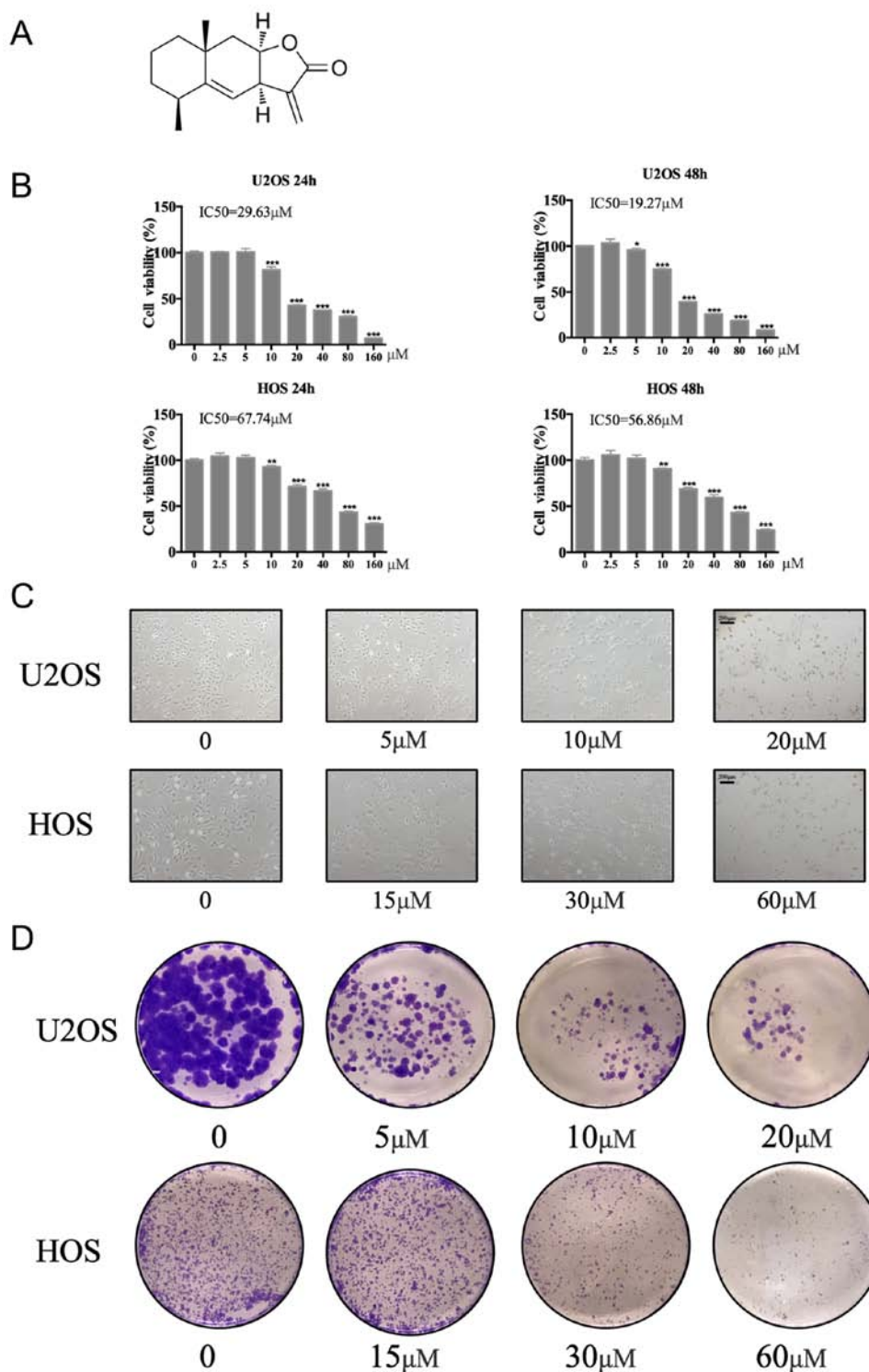


Figure 1. ALT inhibits the proliferation and viability of osteosarcoma cells. (A) Chemical structure of ALT. (B) Cellular viability of U2OS and HOS osteosarcoma cells treated with a range of ALT concentrations for 24 and 48 h was assessed using a CCK-8 assay. Each sample was analyzed in triplicate and normalized to the cell group receiving no ALT treatment. (C) Morphological changes in U2OS and HOS cells treated with a range of ALT concentrations for 48 h. Scale bar, 200 μm . (D) Colony formation assay of U2OS and HOS cells treated with a range of ALT concentrations. Data are presented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. untreated cells. ALT, alantolactone.

unable to prevent tumor metastasis (41). Therefore, patient mortality rates for osteosarcoma remain high, and effective and safe therapeutic agents are urgently required to improve patient outcomes.

ALT is the main biologically active compound derived from *Inula helenium* (42). ALT has been widely researched

as a potential candidate for the treatment of several types of cancer, and previous *in vivo* studies have reported its antiproliferative, anti-metastatic and anti-invasive activity in a variety of cell lines (19,20,42). The present study aimed to evaluate the effect of ALT on osteosarcoma and to determine whether it could be used as a potential future treatment strategy for this

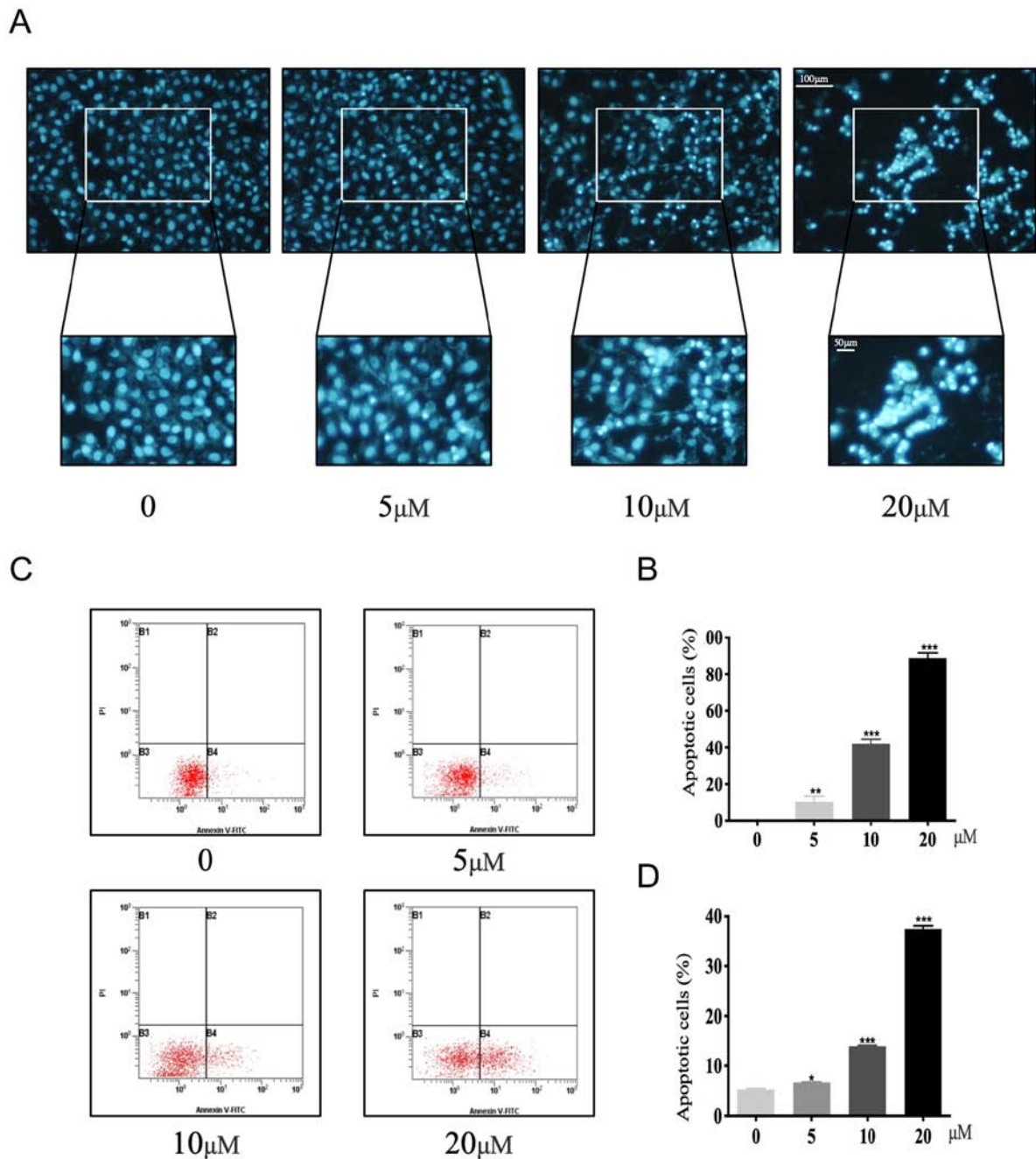


Figure 2. ALT promotes apoptosis of osteosarcoma cells. (A) Hoechst 33258 staining was used to assess the rate of apoptosis in U2OS cells following treatment with a range of ALT concentrations for 48 h. Scale bars, 50 and 100 μ M. (B) Quantification of Hoechst 33258 staining to assess the number of apoptotic cells. The fluorescence intensity was measured by ImageJ. (C) Flow cytometric analysis of the effect of a range of ALT concentrations on the apoptosis of U2OS cells. Annexin V(+)/PI(-) cells were considered early apoptotic cells. (D) Quantitative analysis of the percentage of apoptotic cells from (C). Data are presented as the mean \pm SD. * P <0.05, ** P <0.01 and *** P <0.001. ALT, alantolactone; PI, propidium iodide.

disease. It was demonstrated that ALT inhibited the proliferation of osteosarcoma cell lines in a dose-dependent manner. Previous studies have reported the role of cyclin D1 and p27 in cell proliferation (27,43,44); thus, the effect of ALT on these two proteins was further investigated. The data revealed that ALT decreased the expression of cyclin D1 and increased the expression of p27. In addition, ALT significantly reduced the invasive and migratory ability of osteosarcoma cells, which was attributed to the observed inhibitory effect of ALT over MMP-2 and MMP-9 expression levels. Regarding apoptosis, it was revealed that ALT could promote the apoptosis of

osteosarcoma cells in a dose-dependent manner. The Bcl-2 protein family has previously been shown to regulate apoptosis through the release of pro-apoptotic factors (45,46) and Bax activation increases osteosarcoma cell sensitivity (47). A previous study demonstrated that the downregulation of Bcl-2 and upregulation of BAX significantly promotes cell apoptosis (48). The data from the present study demonstrated that ALT significantly inhibited the expression of Bcl-2 and increased the activation of BAX, thus promoting the apoptosis of osteosarcoma cells. The role of caspase-3 and caspase-8 in apoptosis has also been previously explored (49,50). Western

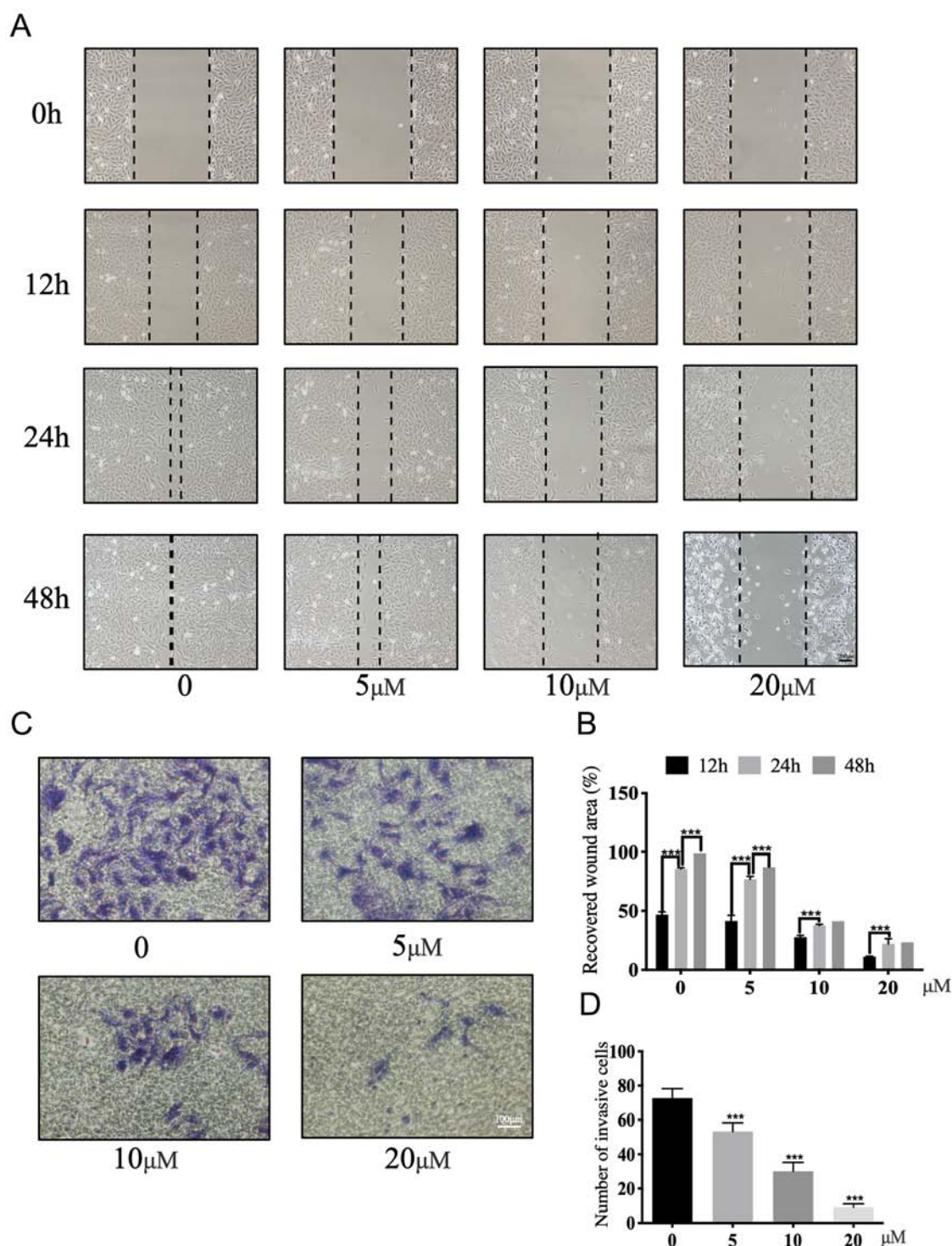


Figure 3. ALT inhibits the migratory and invasive ability of osteosarcoma cells. (A) Migratory ability of U2OS cells treated with a range of ALT concentrations for the indicated times was assessed by the wound healing assay. Scale bar, 200 μm . (B) Quantitative analysis of the wound healing assay from (A). (C) Representative micrographs of the invasive ability of U2OS cells treated with a range of ALT concentrations for 48 h. Scale bar=100 μm . (D) Quantitative analysis of the relative number of invasive cell from (C). Data are presented as the mean \pm SD. *** P <0.001 vs. untreated cells. ALT, alantolactone.

blot analysis was used to evaluate the effect of ALT on these apoptosis-related proteins and revealed that ALT promoted the apoptosis of osteosarcoma cells through modulation of these apoptotic-related factors.

Cyclin D1 and p27 are vital mediators of cell death (43,51,52). Previous studies have reported a crucial role for cyclin D1 and p27 in modulating the G1 and S phase of cells; cell cycle arrest at the G1/S phase

leads to cell apoptosis (53,54). Cyclin D1 is repressed by the p53 protein (55), thus p53 is eventually responsible for growth arrest-induced cell apoptosis. p53 is also able to directly activate Bax to facilitate permeabilization of the mitochondria and it serves a role in the downregulation of Bcl-2 expression, which contributes to DNA damage (56,57). In addition, p53 promotes apoptosis through caspase-3 and -8 (58,59). Thus, the reduced protein expression of cyclin D1 and increased

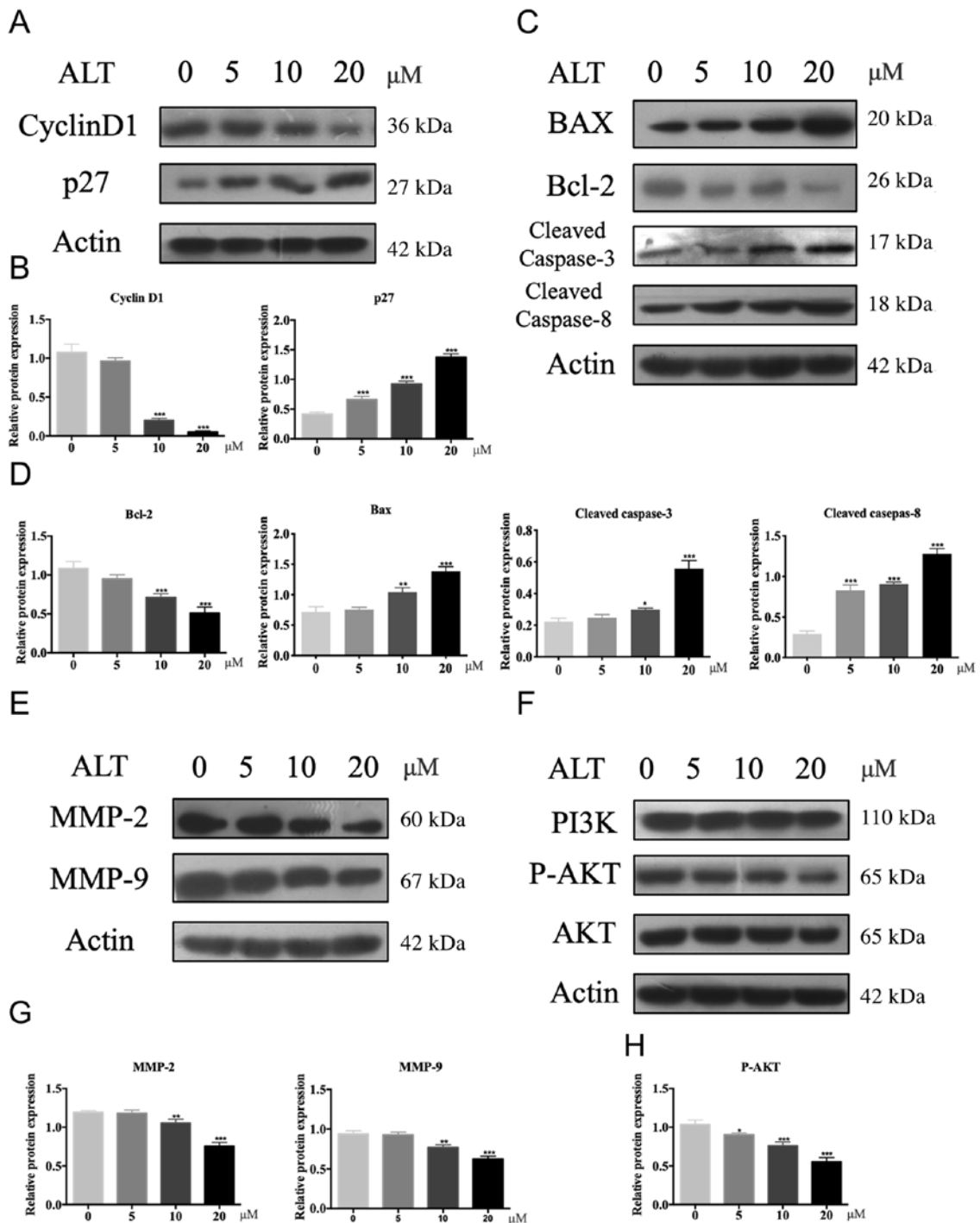


Figure 4. Potential inhibitory mechanism of ALT on osteosarcoma cells. (A-H) Western blotting and semi-quantitative analysis of relative protein expression of (A and B) cyclin D1 and p27. (C and D) Bax, Bcl-2, cleaved caspase-3 and cleaved caspase-8, (E and G) MMP-2 and MMP-9, and (F and H) PI3K, p-AKT and AKT in U2OS cells treated with a range of ALT concentrations. The expression of cyclin D1, p27, BAX, Bcl-2, cleaved caspase-3, cleaved caspase-8, MMP-2 and MMP-9 were relative to β -actin, the expression of p-AKT was relative to AKT. Data are presented as the mean \pm SD of three independent experimental repeats. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. ALT, alantolactone; MMP, matrix metalloproteinase; p, phosphorylated.

expression of p27 caused by ALT provides an explanation for the pro-apoptotic effect of ALT. Future work should aim to explore the effect of ALT on the p53 pathway, which was not undertaken in the current study. The findings from the present study suggested that ALT may suppress the proliferation, invasion, and migration of osteosarcoma cell lines, while promoting their apoptosis. However, the underlying mechanism of action of ALT was unclear.

Accumulating evidence has indicated an important role of the PI3K/AKT signaling pathway in osteosarcoma (11,34,35); PI3K/AKT signaling modulates the proliferation, invasion, migration and apoptosis of osteosarcoma cell lines (36-39,60). Previous studies revealed that alantolactone is a specific inhibitor in several diseases via the PI3K/AKT signaling pathway (7,8). The downregulation of AKT decreases the downstream signaling factors, caspase-8 and caspase-3, which are

responsible for cell apoptosis (61). Protein expression levels of cyclin D1, which is a prominent regulator of cell cycle progression from G1 to S phase (62), were also decreased following AKT downregulation, which has been demonstrated to inhibit cell proliferation (63). Furthermore, invasion-related proteins MMP-2 and MMP-9 are modulated by AKT (64). Other inhibitors of AKT have also demonstrated an ability to suppress osteosarcoma proliferation, invasion and migration (65,66). Consequently, this study explored whether the antitumor activities of ALT were mediated through the PI3K/AKT signaling pathway. The findings indicated that ALT significantly suppressed the expression of AKT, thus, it may act through this mechanism to inhibit osteosarcoma progression.

Nonetheless, the study has several limitations. Firstly, osteosarcoma is composed of several types of cells; therefore, the effect of ALT on other osteosarcoma cell lines should be investigated. Secondly, the antitumor activity of ALT was only investigated *in vitro*, and its *in vivo* protective effect on osteosarcoma remains unclear. In conclusion, the present study demonstrated that the protective effect of ALT on osteosarcoma was mediated through the inhibition of cellular proliferation, migration and invasion, in addition to promoting apoptosis, and that these effects may be explained through the inhibitory effect of ALT on the PI3K/AKT signaling pathway. The present study represents a potential, future therapeutic strategy for osteosarcoma treatment.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YZ and JC conceived the study; YZ and QW conducted the experiments; JH wrote the manuscript and performed statistical analysis; YZ and JC analyzed the results and created the figures. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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