Alpinetin suppresses cell proliferation and metastasis in osteosarcoma by inhibiting PI3K/AKT and ERK pathways

Zhenyu Cao*, Jianwu Ma, Xiaozhong Shen

Department of Orthopedics, Qinghai Provincial People's Hospital, Xining, Qinghai Province, China

*Corresponding Author: Zhenyu Cao, Department of Orthopedics, Qinghai Provincial People's Hospital, No. 2 Gonghe Road, Chengdong District, Xining City, Qinghai Province 810000, China. Email: zhenyu_cao165@163.com

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Abstract

Alpinetin, a natural flavonoid found in medicinal herbs, possesses distinct pharmacological activities, including neuroprotective, antiviral, antibacterial, lung and cardiovascular protective, hepatoprotective, antiinflammatory, and antitumor properties. Here, the role of alpinetin was investigated in osteosarcoma. Osteosarcoma cell lines (143B and U2OS) were treated with 10-, 25-, 50-, 75-, or 100-μM concentration of alpinetin. Cell proliferation was detected by cell counting kit 8 (CCK8) and colony formation assays. Western blotting and flow cytometry were used to investigate cell apoptosis. Cell metastasis was assessed by transwell assay. Administration of alpinetin reduced the viabilities of 143B and U2OS cell lines in a dosage-dependent manner, and decreased the number of colonies of 143B and U2OS cell lines. The apoptosis of 143B and U2OS cell lines was promoted by alpinetin through down-regulation of Bcl-2 and up-regulation of cleaved caspase-3 and Bax. Alpinetin inhibited invasion and migration of 143B and U2OS cell lines through up-regulation of epithelial biomarkers, E-cadherin, and zonula occludens-1 (ZO-1), and down-regulation of mesenchymal biomarkers, Vimentin, and N-cadherin. Alpinetin also reduced the phosphorylation of extracellular signal-regulated kinase (ERK), Ak strain transforming (AKT), and phosphoinositide 3-kinases (PI3K) in 143B and U2OS cell lines. Alpinetin inhibited cell proliferation and metastasis in osteosarcoma through inactivation of PI3K/AKT and ERK pathways, providing potential treatment option for treatment of cancer.

Keywords: alpinetin; proliferation; metastasis; epithelial to mesenchymal transition; osteosarcoma; PI3K/AKT; ERK

Introduction

Osteosarcoma is the most common primary bone tumor observed in adolescents and children (Dengra et al., 2012). Surgery and adjuvant chemotherapy have improved the 5-year overall survival rate of patients with osteosarcoma from 20% to 75% (Abdelgawad et al., 2022). However, development of metastasis, recurrence, and chemoresistance reduces the prognosis of osteosarcoma (He et al., 2014). Therefore, new therapeutic agents to suppress the metastasis of osteosarcoma are urgently required to optimize treatment strategies.

Natural flavonoid-rich herbs, including Urtica dioica (stinging nettle), ameliorated cognitive dysfunction in streptozotocin-induced diabetic mice via reduction of neuroinflammation and hippocampal oxidative stress (Keshvari et al., 2020; Rahmati et al., 2021). Alpinetin, a natural flavonoid found in medicinal herbs, possesses distinct pharmacological activities, including neuroprotective, antiviral, antibacterial, lung and cardiovascular protective, hepatoprotective, antiinflammatory, and anti-tumor properties (Zhao et al., 2022). In tumors, alpinetin promoted cell apoptosis, inhibited cell invasion and metastasis, and induced cell cycle arrest in breast, lung,
and tongue squamous carcinoma, glioma, hepatic, pancreatic, gastric, colon, and cervical cancers (Zhao et al., 2022). Moreover, alpinetin also ameliorated cancer cachexia of Lewis lung carcinoma through activation of peroxisome proliferator-activated receptor-γ (Zhang et al., 2021). The proliferation and invasion of ovarian cancer cells were also repressed by alpinetin (Zhao et al., 2018). However, the anticancer role of alpinetin in osteosarcoma has not been reported for the time being.

Phosphoinositide 3-kinases (PI3K)/Ak strain transforming (AKT) signaling, important for cell survival (Qiu et al., 2021), was abnormally activated in osteosarcoma (Zhang et al., 2015). Activation of PI3K/AKT signaling contributed to cell proliferation, invasion, angiogenesis, metastasis, and chemoresistance of osteosarcoma (Zhang et al., 2015). Inhibition of PI3K/AKT signaling via PI3K inhibitors or natural compounds from plants was used as therapeutic strategies for osteosarcoma (Zhang et al., 2015). Alpinetin has been demonstrated to enhance chemosensitivity of lung cancer cells and suppress progression of tumor through inactivation of PI3K/AKT signaling (Wu et al., 2015). It is thus hypothesized that alpinetin might also suppress osteosarcoma cell proliferation and metastasis through inhibition of PI3K/AKT signaling. Therefore, the effects of alpinetin on cell proliferation, apoptosis, invasion, and migration of osteosarcoma were investigated in this study.

Materials and methods

Cell culture and treatment
Human B lymphoblast (IM-9) and osteosarcoma cell lines (143B and U2OS) were purchased from ATCC (Manassas, VA, USA), and grown in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). Cells were treated with 10-, 25-, 50-, 75-, and 100-μM alpinetin (Sigma-Aldrich) for 24 h, and then subjected to functional assays.

Cell proliferation assays
IM-9 and osteosarcoma cell lines were seeded into 96-well plates, and incubated with different concentrations of alpinetin for 24 h. Cells were then treated with cell counting kit 8 (Beyotime, Beijing, China) for another 2 h. Absorbance at 490 nm was measured by a microplate reader (Thermo Fisher Scientific).

In order to detect cell proliferation, osteosarcoma cells were seeded into 6-well plates, and incubated with different concentrations of alpinetin for 24 h. Cells were then grown in RPMI 1640 medium for 10 days. Cell colonies were fixed in methanol, stained with 0.1% crystal violet (Sigma-Aldrich), and photographed under light microscope (Olympus, Tokyo, Japan).

Cell apoptosis assay
Osteosarcoma cells were performed with trypsin digestion, harvested and resuspended in binding buffer of BD Cytocell™ Plus DNA Reagent Kit (BD Biosciences, San Jose, CA). Cells were stained with propidium oxide and fluorescein isothiocyanate-conjugated annexin V, and analyzed under FACS flow cytometer (Life Technologies, Darmstadt, Germany).

Transwell assays
Osteosarcoma cells in serum-free RPMI 1640 medium were plated into upper Transwell insert chamber (Corning Incorporated, Corning, NY, USA). RPMI 1640 medium with 15% fetal bovine serum was plated into the lower chamber. Invasive cells in the lower chamber were stained with crystal violet and observed under microscope (Olympus) after 24 h.

In order to assess cell invasion, osteosarcoma cells in serum-free medium were also plated into Matrigel-coated upper chambers and subjected to the same protocol.

Western blotting
Osteosarcoma cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime), and the isolated proteins were then separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose membranes, and the membranes were blocked in 5% bovine serum albumin. Membranes were probed with specific antibodies: anti-PCNA and anti-β-actin (1:2,000), anti-cleaved caspase-3 and anti-caspase-3 (1:2,500), anti-Bax and anti-Bcl-2 (1:3,000), anti-Vimentin and anti-N-cadherin (1:3,500), anti-E-cadherin and anti-ZO-1 (1:4,000), anti-p-extracellular signal-regulated kinase (ERK) and anti-ERK (1:4,500), anti-p-AKT and anti-AKT (1:5,000), and anti-p-PI3K and anti-PI3K (1:5,500). The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000). Immunoreactivities were visualized using enhanced chemiluminescence (Sigma-Aldrich), and the blots of proteins were quantified by Image J using β-actin as an internal reference. All the antibodies were obtained from Abcam (Cambridge, MA, USA).

Statistical analysis
All data with at least triple replicates were expressed as mean ± standard error of mean (SEM), and analyzed by Student’s t-test or one-way analysis of variance (ANOVA) with post-hoc analysis using the SPSS software. The normality and homogeneity of data were investigated by
Shapiro–Wilk and Levene’s tests, respectively. \( P < 0.05 \) was considered as statistically significant.

**Results**

**Alpinetin reduced cell proliferation of osteosarcoma**

In order to investigate the cytotoxicity of alpinetin in B lymphoblast, IM-9 cell line was treated with 10-, 25-, 50-, 75-, or 100-μM alpinetin (Figure 1A). Treatment with alpinetin below 50 μM did not affect the viability of IM-9 cell (Figure 1B), and 75- or 100-μM alpinetin reduced no more than 10% of viability of IM-9 cell (Figure 1B). However, treatment with alpinetin decreased the viabilities of osteosarcoma cell lines (143B and U2OS) in a dosage-dependent manner (Figure 1C). Number of colonies in 143B and U2OS cell lines were reduced by alpinetin (Figure 1D) through down-regulation of proliferation-related biomarker, proliferating cell nuclear antigen (PCNA) (Figure 1E), suggesting the antiproliferative effect of alpinetin on osteosarcoma.

**Alpinetin promoted cell apoptosis of osteosarcoma**

In order to investigate the role of alpinetin in cell apoptosis of osteosarcoma, flow cytometry was performed, and the results demonstrated that alpinetin promoted the apoptosis of 143B and U2OS cell lines in a dosage-dependent manner (Figure 2A). The protein expression of pro-survival biomarker, Bcl-2, was down-regulated in 143B and U2OS cell lines by alpinetin (Figure 2B). However, the expression of apoptotic biomarkers, cleaved caspase-3 and Bax, were up-regulated in 143B and U2OS cell lines by alpinetin (Figure 2B), demonstrating the pro-apoptotic effect of alpinetin on osteosarcoma.

**Alpinetin reduced cell metastasis of osteosarcoma**

In order to investigate the role of alpinetin in cell metastasis of osteosarcoma, transwell assays were performed; the results indicated that alpinetin inhibited the migration of 143B and U2OS cell lines (Figure 3A) and decreased the number of invasive cells of 143B and U2OS cell lines in a dosage-dependent manner (Figure 3B), suggesting that the migration and invasion of osteosarcoma cells were...
Alpinetin inhibits osteosarcoma progression

Figure 2. Alpinetin promoted cell apoptosis of osteosarcoma. (A) Treatment with alpinetin promoted the apoptosis of 143B and U2OS cell lines in a dosage-dependent manner, detected by flow cytometry assay. (B) Treatment with alpinetin down-regulated Bcl-2, up-regulated cleaved caspase-3 and Bax of 143B and U2OS cell lines in a dosage-dependent manner. **P < 0.01, ***P < 0.001.

suppressed by alpinetin. The protein expressions of mesenchymal biomarkers, vimentin and N-cadherin, were decreased, while that of epithelial biomarkers, E-cadherin and ZO-1, were increased in 143B and U2OS cell lines by alpinetin in a dosage-dependent manner (Figure 3C), indicating that alpinetin suppressed epithelial to mesenchymal transition in osteosarcoma.

Alpinetin suppressed PI3K/AKT and ERK signaling in osteosarcoma

In order to clarify the mechanism underlying the effects of alpinetin on mediating progression of osteosarcoma, Western blotting was performed. The results demonstrated that the protein expressions of ERK in 143B and U2OS cell lines were not affected by alpinetin (Figure 4), while p-ERK was reduced by alpinetin in a dosage-dependent manner (Figure 4). Moreover, alpinetin down-regulated p-AKT and p-PI3K in 143B and U2OS cell lines (Figure 4), revealing that alpinetin repressed the activation of PI3K/AKT and ERK signaling in osteosarcoma.

Discussion

Naturally, flavonoids in herbs depict beneficial effects on various diseases, and have anticarcinogenic properties through modulation of cellular enzymes (Panche et al., 2016). For example, taxifolin, a plant flavonoid, suppresses cell proliferation and metastasis of osteosarcoma (Chen et al., 2018). The antitumor activity of alpinetin has been widely investigated in various carcinoma cells (Zhao et al., 2022). The present study found that alpinetin enhanced cell apoptosis, reduced cell proliferation, invasion, and migration of osteosarcoma.
Figure 3. Alpinetin reduced cell metastasis of osteosarcoma. (A) Alpinetin inhibited cell migration of 143B and U2OS cell lines in a dosage-dependent manner detected by transwell assay. (B) Alpinetin inhibited cell invasion of 143B and U2OS in a dosage-dependent manner detected by transwell assay. (C) Alpinetin inhibited decreased protein expression of vimentin and N-cadherin, increased E-cadherin and ZO-1 of 143B and U2OS cell lines in a dosage-dependent manner. **P < 0.01, ***P < 0.001.
Alpinetin inhibits osteosarcoma progression

Extracellular signal-regulated kinase signaling is a critical regulator in oncogenic phenotypes of osteosarcoma, including angiogenesis, cell invasion, migration, and proliferation (Chandhanayingyong et al., 2012). Moreover, ERK signaling was also associated with tumor metastasis in osteosarcoma (Yu et al., 2011). Down-regulation of ERK signaling inhibited cell migration in osteosarcoma (Poudel et al., 2014), and ERK targeting therapy established clinical benefits in patients with metastatic osteosarcomas (Chandhanayingyong et al., 2012). Alpinetin reduced the phosphorylation of ERK in chondrocytes (Gao et al., 2020). Here, the protein expression of p-ERK in osteosarcoma cells was down-regulated by alpinetin. The phosphorylation of P13K and AKT was also reduced by alpinetin to inhibit the progression of osteosarcoma through antiproliferative and pro-apoptotic properties.

Metastatic pattern contributed to poor prognosis in patients with osteosarcoma, and inhibition of cell metastasis in osteosarcoma was regarded as a promising strategy for the treatment of osteosarcoma (Sheng et al., 2021). Transition of epithelial to mesenchymal was implicated in the pathogenesis of metastasis in osteosarcoma (Yang et al., 2013). Promotion of epithelial to mesenchymal transition also attributed to osteosarcoma cell metastasis (Zhu et al., 2020). Here, alpinetin suppressed cell migration and invasion in osteosarcoma. Moreover, alpinetin reduced the protein expressions of mesenchymal biomarkers, including vimentin and N-cadherin, while enhanced protein expressions of epithelial biomarkers, including E-cadherin and ZO-1, to suppress transition of epithelial to mesenchymal in osteosarcoma.

Figure 4. Alpinetin suppressed P13K/AKT and ERK signaling in osteosarcoma. Alpinetin down-regulated p-ERK, p-AKT, and p-P13K of 143B and U2OS cell lines in a dosage-dependent manner. *P < 0.05, **P < 0.01, ***P < 0.001.
Competing interests

The authors state that there are no conflicts of interest to disclose.

Author Contributions

Zhenyu Cao designed and carried out the study as well as supervised the data collection. Jianwu Ma analyzed and interpreted the data. Xiaozhong Shen reviewed the draft manuscript and prepared it for publication. All authors read and approved the final manuscript.

References


