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Baicalin inhibits growth and induces apoptosis of human osteosarcoma cells by suppressing the AKT pathway

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Abstract. Osteosarcoma (OS) is one of the most prevalent types of bone malignancies with poor overall prognosis, and is reported mainly in children and adolescents. Therefore, the investigation of novel and efficient treatment strategies for patients with OS is required. Baicalin exhibits potential anticancer effects, including in OS. However, its therapeutic effect against OS and the underlying mechanisms have not been fully evaluated. In the present study, the effect of baicalin on the proliferation and apoptosis of OS cells and its underlying mechanism of AKT pathway activation was explored. Cell confluence and cell number counts revealed suppressed the growth of OS cells that were treated with baicalin. Analysis of cell viability, cell survival and cell cycle, as well as cell apoptosis revealed decreased cell viability and survival, induced cell cycle arrest and apoptosis of treated cells. Western blot analysis demonstrated significantly decreased ratios of phosphorylated-AKT/AKT and Bcl-2/Bax, and decreased protein levels of cyclin D1 and CDK4 in cells treated with baicalin. Thus, the findings from the present study suggest that the suppression of the AKT pathway may be the underlying mechanism of the antitumor effect of baicalin in OS cells.

Introduction

Osteosarcoma (OS), one of the most prevalent types of malignancies of the bone that predominantly affects children and adolescents, was the second leading cause of cancer-associated mortality in this group of individuals in the USA between 1973 and 2004 (1,2). Despite advances in therapeutic strategies (including chemotherapy and surgical resection), the 5-year survival rate of patients with OS who are resistant to treatment or have metastases remains low (3). Moreover, due to poor overall prognosis, >40% of patients develop recurrent or progressive disease following traditional first-line therapy (4). Due to the highly aggressive nature of these tumors, the poor therapeutic outcome and the development of chemoresistance, the exploration of novel and efficient treatment strategies for patients with OS is required (5).

Growing evidence indicates the potential of natural compounds as successful anticancer agents (6). Baicalin (baicalein 7-O-β-D-glucuronide), an important flavonoid, is found in the roots of the Chinese herb Huang Qin (*Scutellaria baicalensis* Georgi) (7). Baicalin exhibits a wide range of pharmacological properties, including against oxidation, tumor, inflammation and proliferation (8-11). Studies on the effect of this compound on various types of cancer cells indicated significantly suppressed tumor growth (12), induction of cell apoptosis and senescence (13-15), as well as inhibition of metastasis (16,17). This is mediated by the suppression of multiple signaling pathways, including ERK, STAT3, β-catenin and p38 mitogen-activated protein kinase (MAPK) (16-19). Treatment of OS cells with baicalin significantly induces apoptosis and inhibits metastasis, through reactive oxygen species-mediated mitochondrial and TGF-β pathways (20,21). However, the role of baicalin in OS and its underlying mechanism has not been fully evaluated.

The AKT pathway, one of the most important intracellular signaling pathways, has been reported to involve a cascade of events that play an essential role in a variety of physiological and pathological processes, including proliferation, migration, cell growth and metabolism (22,23). In most types of cancer in humans, the AKT pathway has been identified as one of the most important oncogenic pathways (24). In OS, the AKT pathway is frequently hyperactivated, playing a critical role in the initiation and development of OS, including tumorigenesis, proliferation, apoptosis and metastasis (25-28). Activation of the AKT pathway contributes to these processes in cancer by mediating the expression of its downstream genes, including cyclin D1, CDK4, anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax (29-32). Due to the essential role of the AKT pathway in OS, the inhibition of the AKT pathway has therapeutic potential in OS (25,26,33-35) and natural compounds

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that target this pathway safely and effectively need to be further investigated.

In the present study, the role of baicalin on U-2 OS cell growth was assessed by cell confluence observation and performing cell number counts. The cell viability, cell survival, cell cycle and cell apoptosis of U-2 OS cells, following baicalin treatment, were further assessed. Moreover, the underlying mechanism of baicalin was explored by investigating the activation of the AKT pathway and its downstream effectors using western blotting.

Materials and methods

Antibodies and chemicals. McCoy's 5A medium, fetal bovine serum (FBS) and the cell cycle determination kit [FxCycle™ Propidium Iodide (PI)/RNase Staining solution; cat. no. F10797] were all purchased from Thermo Fisher Scientific, Inc. The mixture of penicillin and streptomycin was obtained from Hyclone; GE Healthcare Life Sciences. The Annexin V staining kit was provided by Nanjing KeyGen Biotech Co., Ltd. The Cell Counting kit-8 (CCK-8) was provided by Abbkine Scientific Co., Ltd. Baicalin and DMSO were obtained from Beijing Solarbio Science & Technology Co., Ltd. The antibodies against GAPDH (cat. no. 5174), AKT (cat. no. 4691s), phosphorylated (p)-AKT (cat. no. 4060s), cyclin D1 (cat no. 2978s), CDK4 (cat. no. 12790), Bax (cat. no. 5023) and Bcl-2 (cat. no. 15071) were all purchased from Cell Signaling Technology, Inc.

Cell culture and baicalin treatment. U-2 OS cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences. The cells were cultured in McCoy's 5A medium, supplemented with 10% FBS and a mixture of 100 U/ml penicillin and 100 mg/ml streptomycin, in a humidified atmosphere of 37˚C and 5% CO_2. The cells were cultured to 80-90% confluence and were not used after >20 passages. The cells were seeded in multiple plates and treated with various concentrations of baicalin (25, 50 or 100 μM; dissolved in DMSO). The concentrations were selected based on a preliminary study (data not shown). Equal volume of DMSO (≤0.5%) was added to wells as a control treatment.

Cell confluence observation. U-2 OS cells ($0.4x10^5$ cells/well) were seeded on a 6-well plate for 24 h, followed by treatment with 0, 25, 50 or 100 μM baicalin for 24 h. The confluence of cells was observed and images of each well were captured using a phase-contrast inverted light microscope (Leica Microsystems GmbH) at a magnification of x100.

Cell number counts. Following the observation of cell confluence, the cells were trypsinized and diluted with fresh medium. An equal volume of medium containing cells was mixed with 0.2% trypan blue solution (Sigma Aldrich; Merck KGaA) and the cell number was counted using the Countstar Automated Cell Counter (ALIT Life Science Co., Ltd.).

CCK-8 assay. U-2 OS cells ($2x10^3$ cells/well) were seeded on a 96-well plate for 24 h and then treated with various concentrations of baicalin (0, 25, 50 or 100 μM). Following treatment for 24, 48 and 72 h, 10 μl CCK-8 reagent (Abbkine Scientific Co., Ltd.) was added to each well and the cells were incubated at 37˚C for an additional 2 h. The absorbance was measured at 450 nm using an Infinite 200 Pro microplate reader (Tecan Group, Ltd.).

Cell colony-formation analysis. Following the cell number counts for each treatment group, U-2 OS cells treated with or without baicalin were seeded on 12-well plates (500 cells/well), and the medium was changed every 2-3 days. After culture for 8-10 days, the colonies formed were washed with PBS twice and fixed with 4% paraformaldehyde for 15 min at room temperature (RT), followed by 0.01% crystal violet staining for 15 min at RT. Images were captured using an electronic camera (cat. no. DS126201; Canon, Inc.) and the numbers of colonies were recorded. The number of colonies formed was normalized to the control cells.

Cell cycle analysis. U-2 OS cells ($0.4x10^5$ cells/well) were seeded on a 6-well plate, incubated for 24 h, and treated with 0, 25, 50 or 100 μM baicalin for 24 h. The cells were harvested and fixed with 70% ethanol at 4˚C overnight. The fixed cells were washed twice with cold PBS and incubated for 30 min with PI/RNase at RT. The fluorescence signal was detected by the FL2 channel of a flow cytometer and the proportion of DNA at each phase was analyzed using ModFit LT software version 3.0 (Verity Software House, Inc.).

Annexin V staining and cell apoptosis analysis. U-2 OS cells ($0.4x10^5$ cells/well) were seeded on a 6-well plate and incubated for 24 h, after treatment with 0, 25, 50 or 100 μM baicalin for 24 h. The cells were harvested with trypsin without EDTA and washed twice with PBS. The collected cells were incubated with Annexin V-phycoerythrin solution for 15 min at RT. The cells were sorted using a flow cytometer (FACSCalibur™; Becton Dickinson Company). The percentage of cells at the early stage of apoptosis was calculated according to Annexin V-positivity using Cell Quest Pro software (version 6.0; Becton, Dickinson and Company).

Western blotting. U-2 OS cells were cultured in 25-cm^2 flasks at a density of $1.5x10^6$ cells/flask in 5 ml medium for 24 h, and then treated with 0, 25, 50 or 100 μM baicalin for 24 h. After harvesting, the cells were lysed with ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology), containing protease and phosphatase inhibitor cocktails (Roche Diagnostics) for 30 min, and centrifuged at 18,894 x g at 4˚C for 10 min to remove sediment. The concentration of soluble protein was determined by BCA assay (Thermo Fisher Scientific, Inc.). Protein samples (50 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore). The PVDF membranes were blocked with 5% skimmed milk at RT for 1 h. The membranes were then incubated with primary antibodies (1:1,000 dilution) against phosphorylated (p-)AKT, AKT, cyclin D1, CDK4, Bax and Bcl-2 overnight at 4˚C. The blots were then probed with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution) at RT for 2 h, followed by detection using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) with the ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Inc.). The intensities of bands were quantified relative to the intensity of GAPDH bands using the ImageJ software (version 1.46; National Institutes of Health). The levels of target proteins were expressed relative to the levels in untreated cells, defined as 1.00.

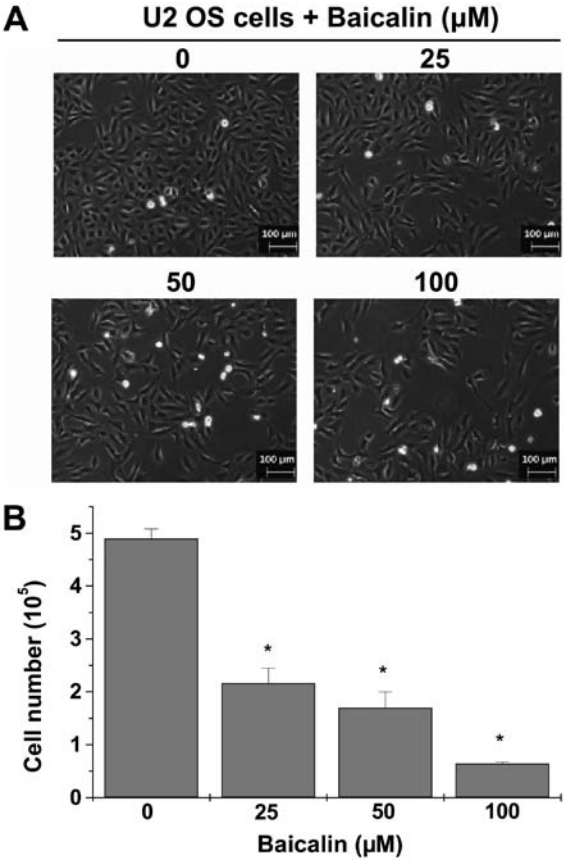

Figure 1. Effect of baicalin treatment on the growth of U-2 OS cells. Cells were treated with 0, 25, 50 or 100 μM baicalin for 24 h. (A) Cell confluence was observed and images were captured using a phase-contrast inverted microscope. (B) Cells were counted using the Countstar Automated Cell Counter, using the trypan blue exclusion principle. Magnification, x100. Data are presented as mean ± SD of n=6 of three independent experiments. *P<0.05 vs. untreated U-2 OS cells.

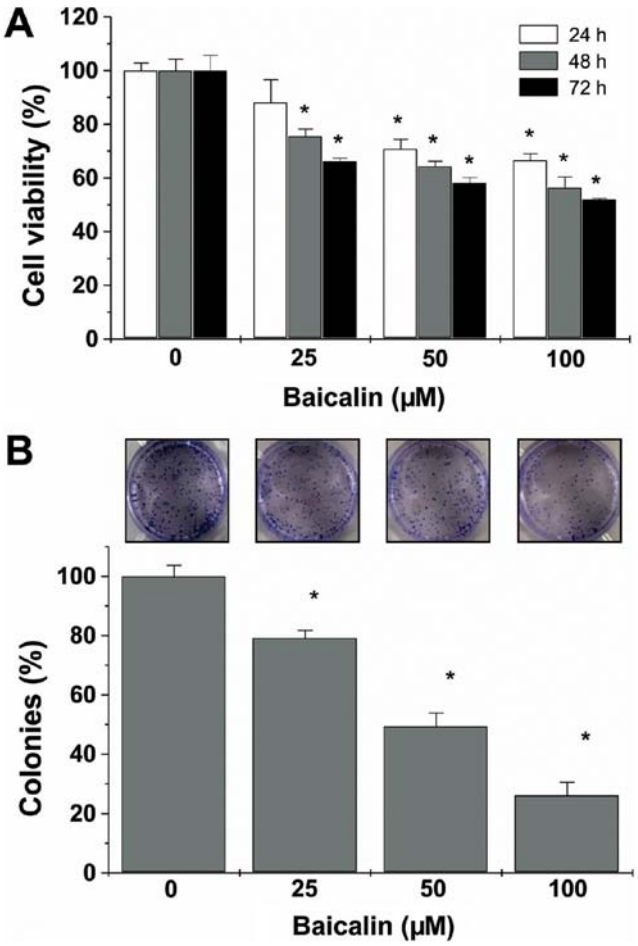

Figure 2. Effect of baicalin treatment on viability and survival of U-2 OS cells. (A) U-2 OS cells were treated with 0, 25, 50 or 100 μM baicalin for 24, 48 or 72 h. Cell Counting Kit-8 was used to measure cell viability relative to the untreated U-2 OS cells. (B) U-2 OS cells were treated with 0, 25, 50 or 100 μM baicalin for 24 h and reseeded in 12-well plates to analyze colony formation. Representative images are shown and the data are normalized to the untreated U-2 OS cells. Data are presented as mean ± SD of three independent experiments. *P<0.05 vs. untreated U-2 OS cells.

Statistical analysis. All statistical analyses were conducted using the SPSS version 20.0 statistical software (IBM Corp.). The data are presented as the mean ± standard deviation of three independent experiments. The significance of the differences between the groups (>3 groups) was tested using ANOVA, followed by the Least Significant Difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Baicalin suppresses the growth of U-2 OS cells. Cell confluence observation by microscopy revealed decreased cell confluence of cultured U-2 OS cells that were treated at various concentrations of baicalin (25, 50 or 100 μM; Fig. 1A). Moreover, cell number counts, using trypan blue staining, demonstrated significantly decreased number of live cells following treatment with 25-100 μM baicalin (P<0.05 vs. untreated U-2 OS cells; Fig. 1B). Thus, baicalin suppresses the growth of U-2 OS cells.

Baicalin decreases cell viability and cell survival of U-2 OS cells. The cell viability of U-2 OS cells was determined using the CCK-8 assay. This demonstrated markedly decreased cell viability of U-2 OS cells that were treated with baicalin (25, 50 or 100 μM) at various time points (24, 48 or 72 h) compared with untreated cells (all P<0.05, except 25 μM baicalin treatment for 24 h; Fig. 2A). The images and calculations of the colonies revealed significantly decreased numbers of colonies at the various concentrations of baicalin (all P<0.05 vs. untreated cells; Fig. 2B).

Baicalin induces the entry of U-2 OS cells into the G_0/G_1 phase. In order to explore the underlying mechanism of the effect of baicalin on cell cycle progression, cell cycle analysis was conducted (Fig. 3). PI staining followed by FACS analysis indicated a significantly increased percentage of U-2 OS cells at the G_0/G_1 phase after 50-μM (36.55±1.16%) and 100-μM (36.37±1.77%) baicalin treatment compared with untreated U-2 OS cells (31.39±1.84%) (both P<0.05). The percentage of U-2 OS cells at the S phase was significantly decreased following treatment with 25 (42.79±1.18%), 50 (43.20±0.42%) and 100 μM (38.24±1.43) baicalin compared with untreated U-2 OS cells (48.03±0.32%) (all P<0.05). In addition, the percentage of cells at the G_2/M phase was increased following treatment with 25

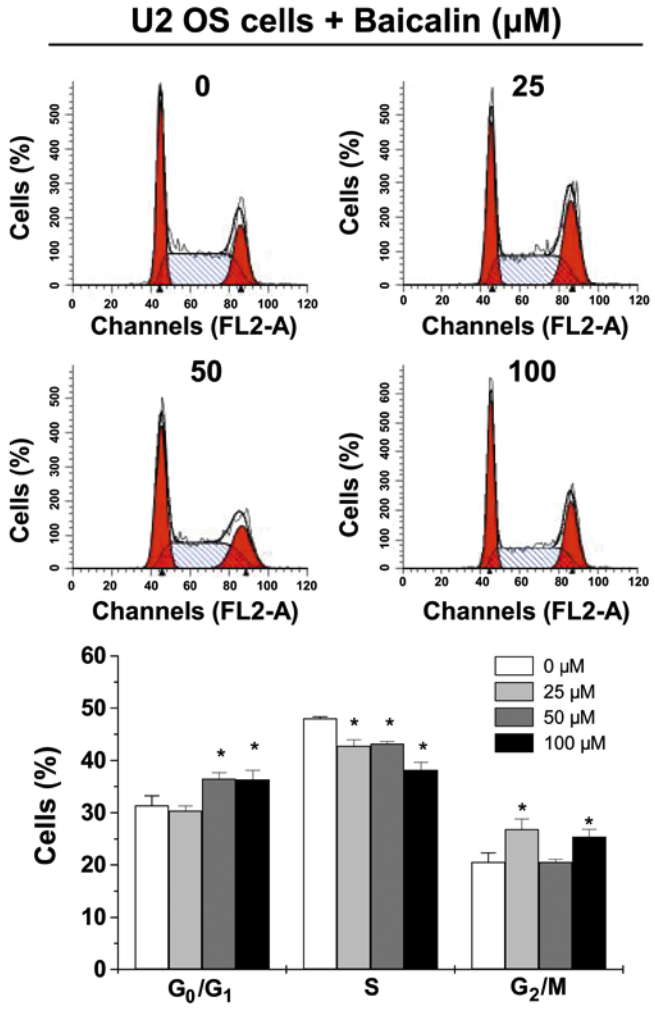

Figure 3. Effect of baicalin treatment on cell cycle progression in U-2 OS cells. U-2 OS cells were treated with 0, 25, 50 or 100 μM baicalin for 24 h. The treated cells were stained with propidium iodide solution in order to analyze cell cycle distribution with flow cytometry. The percentage of cells in the G_0/G_1, S and G_2/M phases was measured. The data are represented as mean ± SD of three independent experiments. *P<0.05 vs. untreated U-2 OS cells; FL2-A, fluorescent light-area.

and 100 μM baicalin (P<0.05), whereas 50 μM baicalin treatment had no effect.

Baicalin induces apoptosis of U-2 OS cells. Annexin V staining followed by FACS analysis revealed a significant shift in the peak of fluorescence to the right and significantly increased percentage of U-2 OS cells that were positively stained with Annexin V following treatment with 25, 50 and 100 μM baicalin (9.03±1.90, 13.28±1.44 and 37.00±1.42%, respectively), compared with untreated cells (all P<0.05; Fig. 4).

Baicalin treatment suppresses the activation of the AKT pathway. The underlying mechanism of the antitumor effect of baicalin was further investigated by western blot analysis (Fig. 5A). As shown in Fig. 5B, treatment with baicalin significantly decreased the levels of p-AKT relative to total AKT (all P<0.05 vs. untreated cells). Determination of the protein levels of its downstream effectors revealed significantly downregulated expression of CDK4 at all tested concentrations, and cyclin D1 at 100 μM only, and decreased Bcl-2/Bax ratio in U-2 OS cells treated with baicalin (all P<0.05 vs. untreated cells; Fig. 5C-E).

Discussion

Treatment of OS by chemotherapy and surgical resection is limited, and patients who are resistant to treatment or have metastasis have poor overall survival (3). Thus, the development of effective therapeutic approaches for OS, with low toxicity and minimal side effects, is required. A number of natural compounds, including kaempferol and oleanolic acid, exhibit potential as anticancer agents with low toxicity (36,37). Baicalin is a naturally bioactive compound extracted from the Chinese herb Huang Qin (*Scutellaria baicalensis* Georgi) (7). Previous studies demonstrated significant effects of baicalin, such as those against oxidation, tumor, inflammation and proliferation (8-11). Moreover, treatment of various cancer cells with baicalin significantly suppresses tumor growth and metastasis, via multiple downstream signaling pathways (13-19). Other studies on OS revealed significant induction of cell apoptosis and inhibition of metastasis following baicalin treatment (20,21). In the present study, the role of baicalin on the growth of OS cells was further explored. This revealed significantly decreased cell confluence and number of OS cells. Moreover, the CCK-8 and colony-formation assays revealed significantly decreased cell viability of OS cells treated with baicalin, suggesting the potential of baicalin as an anticancer therapy for OS. However, the effect of baicalin treatment on tumor growth, metastasis and chemotherapy resistance in OS should be further assessed *in vivo*.

As with most cancer cells, OS cells are characterized by imbalanced cell proliferation and apoptosis. Therefore, the present study determined the progression of the cell cycle in OS cells and found that treatment with baicalin significantly decreased the percentage of cells at the S phase, whereas as an increased percentage at the G_0/G_1 phase was observed. Cyclin D1, together with specific kinases (CDK4 and CDK6), plays an essential role in the regulation of the cell cycle at the G_0/G_1 restriction points. Therefore, western blot analysis of cyclin D1 and CDK4 was performed on U-2 OS cells treated with baicalin, revealing their downregulation. However, other regulators (including p21, p27 and CDK6) that are involved in the control of cell cycle checkpoints at the G_0/G_1 and S phases should be further evaluated in future studies. Moreover, the percentage of cells at the G_2/M phase was increased following treatment with 25 and 100 μM baicalin, whereas 50 μM baicalin had no effect. This observation can be further explored in future studies.

Evading apoptosis is another hallmark of cancer cells, which is mediated by anti-proliferative proteins (such as Bcl-2) and anti-apoptotic proteins (such as Bax). Analysis of the fragmented DNA (a characteristic of late apoptosis) by PI staining revealed a low percentage of cells at the Sub-G1 phase. This was in agreement with the finding of an increased percentage of apoptotic cells by Annexin V staining. However, the role of baicalin in the apoptotic status of cells should be further investigated using double staining of Annexin V-allophycocyanin

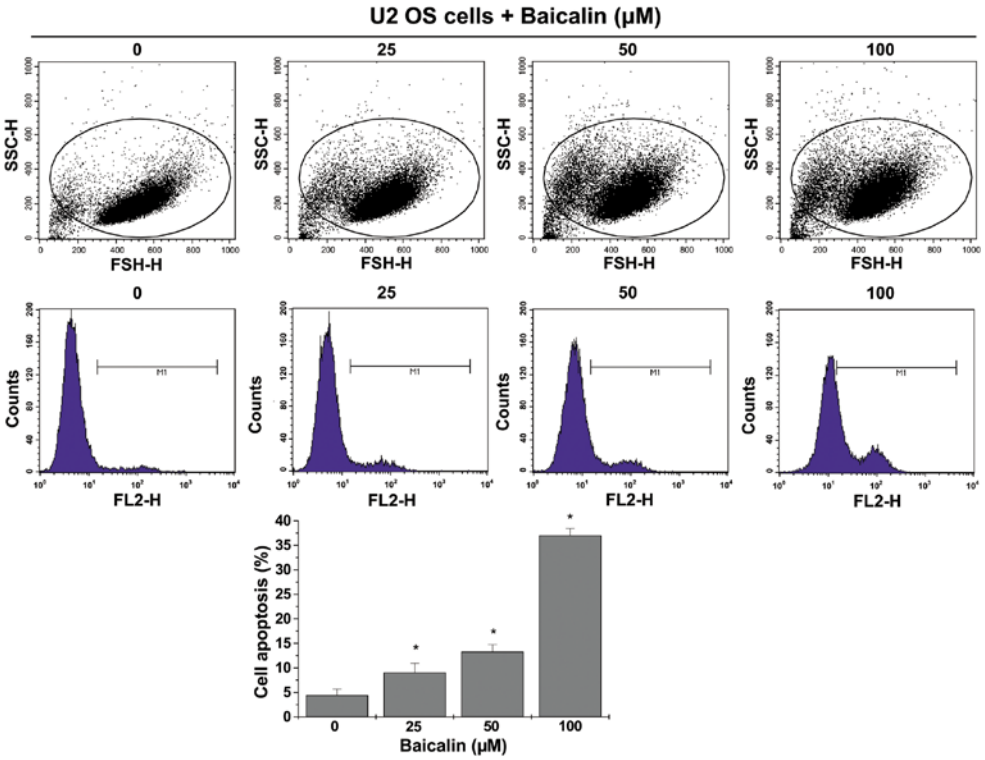

Figure 4. Effect of baicalin treatment on apoptosis of U-2 OS cells. Cells were treated with 0, 25, 50 or 100 μM baicalin for 24 h. The percentage of apoptotic cells was measured using Annexin V-phycoerythrin staining analyzed with flow cytometry. The data are presented as mean ± SD of three independent experiments. *P<0.05 vs. untreated U-2 OS cells. FSH-H, forward angular scattering-height; SSC-H, side scatter-height; FL2-H, fluorescent light-height.

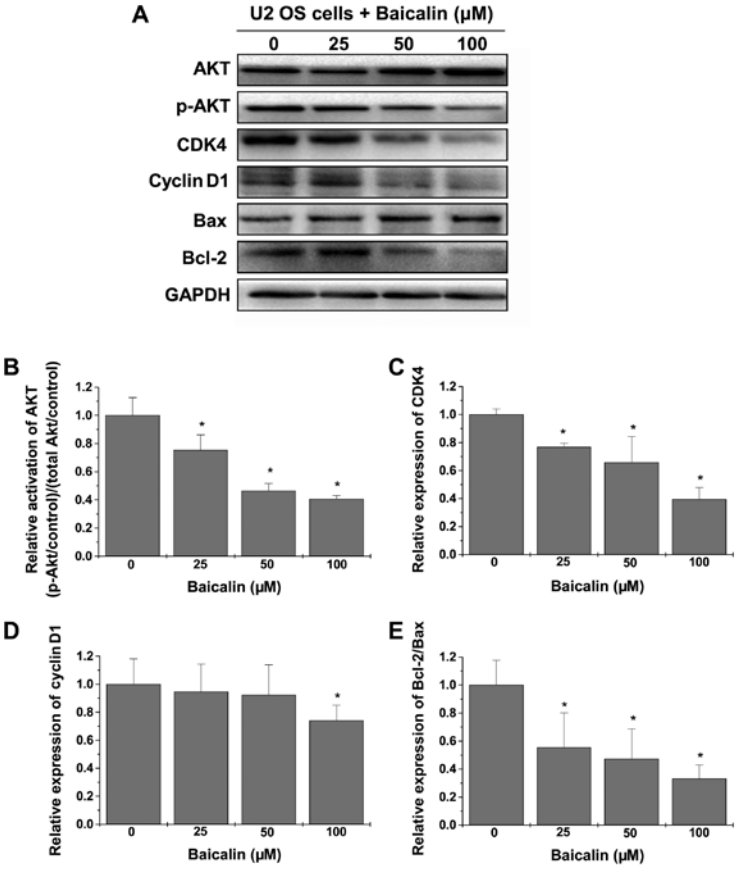

Figure 5. Effect of baicalin treatment on the AKT pathway. U-2 OS cells were treated with 0, 25, 50 or 100 μM baicalin for 24 h. (A) The protein expression of p-AKT, AKT, cyclin D1, CDK4, Bax and Bcl-2 was determined by western blot analysis. GAPDH was used as the internal control. (B-E) The intensities of bands were normalized against those of GAPDH and quantified relative to those of the untreated control cells. The data are presented as mean ± SD of three independent experiments. *P<0.05 vs. untreated U-2 OS cells. p-AKT, phosphorylated AKT.

with PI or 7-aminoactinomycin D. Furthermore, western blot analysis revealed enhanced expression of the anti-apoptotic protein Bax, whereas expression of the anti-apoptotic protein Bcl-2 was decreased, in U-2 OS cells treated with baicalin. These findings thus suggest induced cell cycle arrest and cell apoptosis via the downregulation of cell cycle regulators cyclin D1 and CDK4, and anti-apoptotic protein Bcl-2. However, the underlying mechanism of baicalin needs to be further investigated with omics technologies, including cDNA arrays and sequencing.

Multiple signaling pathways (including ERK, p38 MAPK and STAT3) have been reported to play a critical role in the development and prognosis of OS, which has been reported to be suppressed by baicalin, leading to the inhibition of tumor growth and metastasis (12-19). However, to the best of our knowledge, the regulatory effect of baicalin on the AKT pathway has never been evaluated in OS. In OS, hyperactivation of the AKT pathway is critical in tumorigenesis, proliferation, cell cycle, apoptosis and metastasis, by regulating the expression of its downstream effectors (such as cyclin D1, CDK4, Bcl-2 and Bax) (25-32). Thus, the downregulation of cyclin D1, CDK4, Bcl-2 expression and upregulation of Bax expression observed in OS cells after baicalin treatment prompts further exploration of the role of this compound on the activation of the AKT pathway.

In conclusion, baicalin significantly suppressed the growth of OS cells, inhibited cell viability and survival, and induced cell cycle arrest and apoptosis. Mechanistic studies revealed suppression of the AKT pathway and decreased protein expression of cyclin D1, CDK4 and Bcl-2/Bax ratio as the possible mechanism of the baicalin antitumor effect in OS. The present study provides a basis to further explore the treatment of OS with baicalin in the future, using *in vitro* and *in vivo* studies.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YL and JH conceived and designed the experiments. YL, PC and JW performed the experiments on cells. ZH and YZ conducted the western blot analysis. YL and JW conducted the data analysis. YL and PC produced and revised the manuscript. 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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