


RETRACTED ARTICLE: Cinnamaldehyde Inhibits the Function of Osteosarcoma by Suppressing the Wnt/ β -Catenin and PI3K/Akt Signaling Pathways

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Background: Osteosarcoma (OS) is a primary bone tumor associated with locally aggressive growth and early metastatic potential that typically occurs in children and adolescents. Chinese traditional medicine *Cinnamomum cassia* has been shown to have significant tumor-killing effect, in which cinnamaldehyde (CA) is the main active ingredient.

Purpose: To explore the anticancer effect of CA on the osteosarcoma cells and the possible molecular mechanism.

Methods: Crystal violet assay, MTT assay and colony-forming assay were used to confirm the inhibitory role of CA in the proliferation of 143B and MG63 osteosarcoma cells. Hoechst 33258 staining and flow cytometry were used to observe apoptosis. The migration and invasion role of OS cells were evaluated using transwell assays and wound healing assays. Western blotting was used to analyse the protein expression levels. Nude mice were inoculated with 143B cells to establish an orthotopic OS tumor animal model and to investigate the effect of CA on OS tumors.

Results: According to crystal violet assay, MTT assay and colony-forming assay, CA significantly inhibited cell proliferation. Hoechst 33258 staining and flow cytometry analysis showed that CA-induced apoptosis in a concentration-dependent manner. In addition, transwell assays and wound healing assays showed that CA inhibited the migration and invasion of osteosarcoma cells. In vivo mouse models, CA inhibited the growth of osteosarcoma. The potential mechanisms could be that CA inhibited the transcriptional activity of Wnt/ β -catenin and PI3K/Akt of the osteosarcoma.

Conclusion: CA may inhibit the proliferation, migration, invasion and promote apoptosis of OS cells by inhibiting Wnt/ β -catenin and PI3K/Akt signaling pathways. CA may be a potentially effective anti-tumor drug.

Keywords: osteosarcoma, cinnamaldehyde, anti-tumor, Wnt/ β -catenin, PI3K/Akt

Introduction

Osteosarcoma (OS) is a common skeletal system tumor derived from primordial mesenchymal cells, which occurs in children and adolescents. OS is defined by the presence of malignant mesenchymal cells which produce osteoid.¹ The malignant tumor, which is characterized by local invasion and lung metastasis, leads to metastases or recurrent diseases with a 5-year survival rate of less than 20%. The main treatment methods include neoadjuvant chemotherapy, surgical resection and postoperative chemotherapy at this stage.² Although chemotherapy is an important treatment for osteosarcoma, the toxicity effects of chemotherapeutic agents in the

treatment will increase the pain and cause damage to other physical functions of the patient, such as hematological toxicity, acute liver and renal toxicity, neurocognitive deficits, and cardiomyopathy,³ especially in patients with metastasis. Therefore, the development of auxiliary anti-osteosarcoma natural drugs with high efficiency and low toxicity is necessary.

Cinnamaldehyde (CA) is the main component of the volatile oil of cinnamon, which is an essential medicinal and edible plant. It has various pharmacological effects such as antibacterial, antioxidant, anti-inflammatory, hypoglycemic, and anti-tumor.⁴⁻⁷ In recent years, it has been shown that CA can inhibit the proliferation and induce apoptosis of cancer cells. It can induce apoptosis and reverse epithelial-mesenchymal transition through inhibiting Wnt/ β -catenin pathway in non-small cell lung cancer.⁸ CA was reported to inhibit Colon Cancer cell growth through AP-1 inactivation.⁹ At the same time, CA can induce cell cycle arrest and apoptosis in human oral squamous cell carcinoma Cells.¹⁰ It can also inhibit invasion capacities of human breast cancer cell line by regulating the expression of miR-27a.¹¹ Therefore, the multi-target anticancer activity of CA lays the foundation for research in osteosarcoma.

In this study, we evaluate its anti-tumor effects and relevant molecular mechanisms in OS cell lines. Our results indicated that CA might suppress the growth of osteosarcoma cells in vitro and in vivo through inhibiting Wnt/ β -catenin and PI3K/Akt signaling pathway in OS cells.

Materials and Methods

Materials

Human osteosarcoma cell lines 143B, U2OS, SaoS2, MG63, HEB, HS5 and LO2 were purchased from the American Type Culture Collection (ATCC, USA). Dulbecco's Modified Eagle's Medium (DMEM) were purchased from HyClone (USA). Fetal bovine serum was purchased from Excell bio (China). CA of 20 mg was purchased from Chengdu Herbpurify Co. Ltd (China). MTT reagents were purchased from Sigma, USA, and crystal violet staining solution was purchased from Beijing Solibao Technology Co., Ltd. Transwell was purchased from Corning Corporation, USA. Matrigel was purchased from BD Biosciences, USA. Horseradish peroxidase-labeled goat anti-rabbit and goat anti-mouse IgG (secondary antibody), mouse anti-human β -actin Cloned antibodies, Hoechst 33258 staining solution,

trypsin cell digestion solution (0.25% trypsin), and BCA protein concentration kit were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. Rabbit anti-human Parp, Cleaved Parp, N-cadherin, Cleave-Caspase-3, MMP-9, MMP-7, MMP-2, Caspase3, β -Catenin, Bad, PI3K, cycling D, Phosphorylated AKT (ser473), Phosphorylated AKT (ser308), GSK3 β , Phosphorylated-GSK-3 β (ser9) and C-Myc monoclonal antibodies, and mouse anti-human Bcl-2, BAX, Vimentin, Snail monoclonal antibodies were purchased from Cell Signaling Technology (CST), USA. ChemiDoc MP Imaging System was purchased from Bio-Rad, California, USA. Flow cytometer was purchased from CytoFLEX, Beckman Coulter, Fullerton, CA, USA. Fluorescence microscope was purchased from PHILIPSE Ti, Nikon, Japan.

Cell Culture and Drug Preparations

Human osteosarcoma cell lines 143B, U2OS, SaoS2, MG63, HEB, HS5 and LO2 cultured in 500mL DMEM with 10% fetal bovine serum, 5 mL penicillin-streptomycin solution at 37°C in 5% CO₂. CA of 20 mg was dissolved in 756.7 μ L dimethyl sulfoxide to a final concentration of 200 mM, and then stored at -20°C.

Crystal Violet Assay

OS cell lines were inoculated into a 24-well plate at a density of 5×10^4 /well. After the normal adherent growth of the cells, OS cells were treated with different concentrations of CA. After being treated with CA for 24 hrs, 48 hrs and 72 hrs, cells were stained with crystal violet. Finally, images were obtained with the scanner. After the imaging was completed, the crystal violet in the 24-well plate was fully dissolved with 20% acetic acid solution, and the OD value of each well was detected at the wavelength of 590 nm of the multifunctional enzyme labeling instrument.

MTT Assay

MG63, 143B, HEB, HS5 and LO2 cells in the logarithmic growth phase were firstly seeded into 96-well culture plates at 5×10^3 cells/well and then incubated after treatment with different concentrations of CA for 24 hrs and 48 hrs. MTT 10 μ L/well was added and cultured in an incubator for 4 hrs. The absorbance of each well at a wavelength of 492 nm was measured with a plate reader to reflect the cell proliferation activity.

Colony-Formation Assay

MG63 and 143B cells were seeded in a 6-well plate at a density of 800 cells/well, and cultured in an incubator until the cells completely adhered, and then cultured with low-concentration CA. After 7 days, stained with crystal violet for 10 min. Colonies containing >50 cells by microscopic observation were effective cell colonies, and the number of colonies was counted by Image J software.

Wound Healing Assays

MG63 and 143B cells were inoculated into a 6-well plate and incubated for 24 hrs. After the cells had adhered to the wall, a 200 μ L pipette tip was used to scratch the cell culture plate vertically along the 6-well plate's diameter. Then, the medium was discarded and new medium containing different concentrations of CA added. The scratch area of each group was recorded at different time points (12 hrs, 24 hrs), and the area change of the scratch area was calculated by Image J software.

Cell Migration and Invasion Assays

The matrix collagen solution was diluted with the culture solution at a ratio of 1:5 and spread evenly in the upper cavity of the transwell chamber. In the upper cavity, 2.5×10^5 cells were added to the upper cavity of transwell and different concentrations of CA added to the lower cavity of transwell. After incubation for 24 hrs, the Matrigel film between the upper and lower chambers of the chamber was stained with crystal violet and photographed. The following steps are as described above to reflect the invasion ability. Do not add Matrigel to the upper cavity to determine the ability of cell migration, and the experiments were performed under the same conditions.

Hoechst Apoptosis Staining

MG63 and 143B cells were inoculated on 24 well plates at the density of 5×10^4 cells per hole and cultured until the cells were adherent to the wall. The cells were treated with different concentrations of CA for 24 hrs. Then, the culture medium was sucked out, and 300 μ L Hoechst 33258 dye solution was added to each hole, and shielded from light for 10 min. The size and shape of the nucleus were observed under a fluorescence microscope, and the number of apoptotic cells in each field was counted and analyzed.

Cell Apoptosis Assay

MG63 and 143B cells were inoculated into 6-well plates and treated with different concentrations of CA for 24 hrs. The cells were then collected, washed and resuspended with PBS for 3 times, and finally 500ul of PBS was added to resuspend the cells. The apoptosis rate was detected by flow cytometry according to the process provided by Annexin V-FITC/PI double labeling staining kit.

Western Blot Assay

143B cells and MG63 cells were treated with different concentrations of CA, respectively. After 24 hrs, the cells were lysed, and protein was extracted. The BCA kit was used to detect the protein concentration, and gradient SDS-PAGE separated proteins. The samples were transferred to a PVDF membrane, and 5% skimmed milk powder was blocked for 1 hr. The primary antibody was incubated at 4°C overnight, and the secondary antibody was incubated at 37°C for 1 hr. The protein bands were pictured and analyzed by using the GeneSnap MP Imaging System.

Establishment of Orthotopic OS Tumor Animal Model

Balb/c-nude female mice (3–4 weeks old) weighing from 15 to 20 g were purchased from Beijing HFK BIOSCIENCE Co., Ltd. All animal experiments were approved by IACUC of Animal Protection and Utilization Organization Committee of Chongqing Medical University. And we have obtained the approval number of ethics committee (No.2020–504). After one week of adaptive breeding of mice, 60 μ L of 143B suspension (2×10^7 cells/mL) was injected into the mice's proximal tibia. Then, the rats were treated with different doses of CA (50, 75, 100 mg/kg) or sodium carboxymethyl cellulose (CMC) by gavage every two days. The tumor length and width were measured every 2 days after the 1st week, and the animals were killed 21 days after injection. The formula for calculating the tumor volume was $0.5 \times L \times W^2$ (L is the length of the tumor, and W is the width of the tumor).¹²

Hematoxylin and Eosin Staining and Immunohistochemistry

The tumor tissues were separated, fixed with 4% paraformaldehyde, and embedded in paraffin. Then, the paraffin-embedded specimens were cut into serial sections (4 mm thick) by a microtome. Tumor sections were deparaffinized and stained with hematoxylin and eosin (H&E) for

histological analysis. Tumor sections were immunohistochemically stained with PCNA (1:100), BCL-2 (1:100), Vimentin (1:100), Phosphorylated Akt (ser473)(1:100) and β -Catenin (1:100) antibodies. The image was taken under a light microscope with a magnification of x200 and x400.

Statistical Analysis

Data are presented as the means \pm standard deviation (SD). Statistical analysis was performed by SPSS 19.0 software. All experiments were repeated 3 times. One-way analysis of variance was used for differences between multiple groups, and Tukey's test was used for comparison between groups. $P < 0.05$ was considered statistically significant.

Results

CA Suppressed OS Cells Proliferation

We thought to determine the effect of CA on the proliferation of OS cells. We found by crystal violet staining that CA inhibited the proliferation of OS cells approximately in a dose- and time-dependent (Figure 1A–F, $P < 0.05$). We further confirmed the inhibitory effect of CA on the proliferation of OS cell lines (143B, MG63) using MTT assay and colony-formation assay (Figure 1G–L, $P < 0.05$). After 48h treatment, the half inhibitory concentration (IC_{50}) was MG63 56.68 μ M, 143B 67.95 μ M. We also assessed the toxic effects of CA against normal cells HEB, HS5, LO2. We found that CA could not induce obvious apoptosis in normal cells. The IC_{50} of these cells (HEB 295.1 μ M, HS5 302.9 μ M, LO2 1573 μ M, respectively) was more higher than that of OS cells. In addition, we found that CA inhibited colony-formation ability of OS cells, and reduced the protein level of Proliferating Cell Nuclear Antigen (PCNA), which is a well established indicator of cell proliferation status (Figure 1M–R, $P < 0.05$). Overall, these results indicate that CA may have an inhibitory effect on the proliferation of OS cells, while have low toxicity against human normal cells.

CA Promoted OS Cells Apoptosis

Apoptosis is closely related to tumorigenesis, so we studied the effect of CA on the apoptosis of OS cells. After staining with Hoechst 33258, the morphological changes of cells were observed by the fluorescence microscope. The results indicated that after the CA treatment of OS cell lines, nuclear condensation, fragmentation and chromatin shrinkage increased (Figure 2A and B, $P < 0.05$).

We used AnnexinV-FITC to confirm whether CA-induced apoptosis in OS cell lines. The results showed that CA could increase the early and late apoptosis rate of cells compared to the control group (Figure 2C–F, $P < 0.05$). In order to explore the molecular mechanism of CA-induced apoptosis in human OS cells, we used Western blot to detect apoptosis-related proteins after treatment with CA. The results indicated that the protein expression of Bcl-2 and PARP was down-regulated compared with the control group, while the protein expressions of Bad, cleaved caspase-3, cleaved parp and Bax were significantly up-regulated (Figure 2G–J, $P < 0.05$). In conclusion, CA may induce OS cell apoptosis.

CA-Induced OS Cells Cell Cycle Arrest

To better understand the effect of CA on OS cell lines, we performed a flow cytometry analysis. The results revealed that CA resulted in an increased percentage of arrest at G2/M phase in 143B cells (Figure 3A and B, $P < 0.05$). And the results also revealed that CA resulted in an increased percentage of arrest at G0/G1 phase in MG63 cells (Figure 3C and D, $P < 0.05$). Above results indicated that CA treatment effectively mediated cell cycle arrest in OS cells.

CA Inhibited OS Cells Migration and Invasion

Osteosarcoma is prone to distant metastases. Next, we explored the effect of CA on the migration and invasion of OS cells. We investigated the migration and invasion ability of CA in OS cells by wound healing assay and transwell assay. As shown, transwell migration assay proved that CA suppressed the migration of OS cells compared with control group (Figure 4A–D, $P < 0.05$), and wound healing assay showed similar results (Figure 4I–L, $P < 0.05$). Matrigel transwell assay proved that CA suppressed the invasive potential of OS cells compared with control group (Figure 4E–H, $P < 0.05$). It is well accepted that epithelial–mesenchymal transition (EMT) is essential to tumor invasiveness and migration. Therefore, the expression of EMT-related proteins N-Cadherin, Snail and Vimentin were reduced by the Western blotting (Figure 4M–P, $P < 0.05$). Matrix metalloproteinase (MMP) is an important proteolytic enzyme, which plays a key role in the process of tumor metastasis. CA significantly decreased the expression of MMP-2, MMP-7, and MMP-9. In short, The migration and invasion of OS cells may be inhibited by CA treatment.

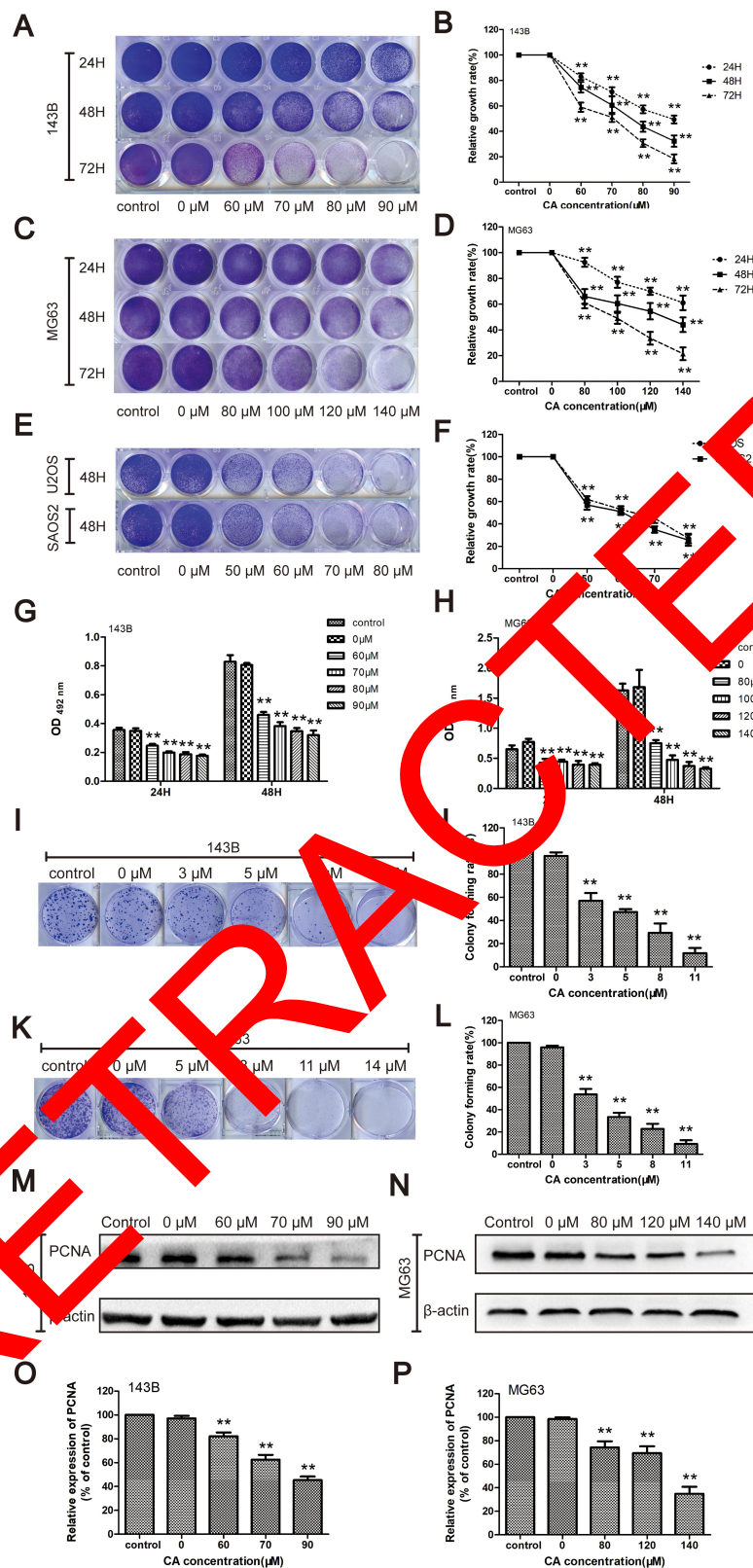


Figure 1 The effect of CA on the proliferation of human OS cells were detected by crystal violet staining (A–F), MTT assay (G–H) and colony-formation assay (I–L). Western blot analysis showed CA down-regulated PCNA (M–P). OS cells were treated with DMSO (as the control group) and 0–140 μ M CA for 24 hrs, 48 hrs and 72 hrs, respectively. CA significantly inhibited the proliferation ability of OS cells (** P <0.01, vs the control group, n =3).

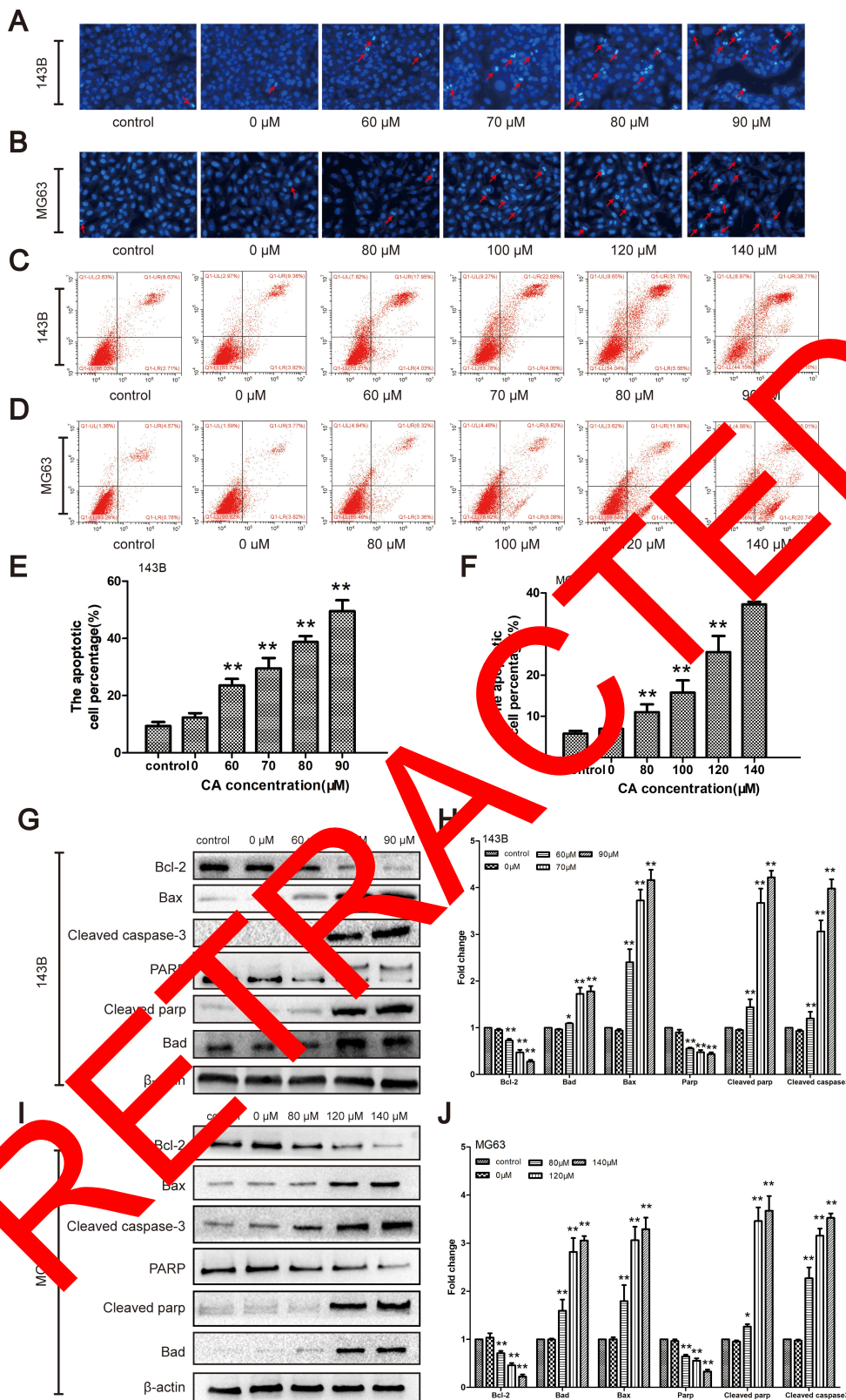


Figure 2 Effect of CA on the apoptosis of human OS cells was detected by Hoechst 33258 staining assay (A and B, ×100), flow cytometry (C–F). Then the expression levels of apoptosis-related proteins Bad, Bax, Bcl-2, Parp, cleaved Parp and cleaved Caspase 3 (c-Caspase 3) were detected by Western blotting (G–J). OS cells were treated with DMSO (as the control group) and 0–140 μM CA, respectively. CA significantly promoted the apoptosis of OS cells (**P*<0.05, ***P*<0.01, vs the control group, n=3).

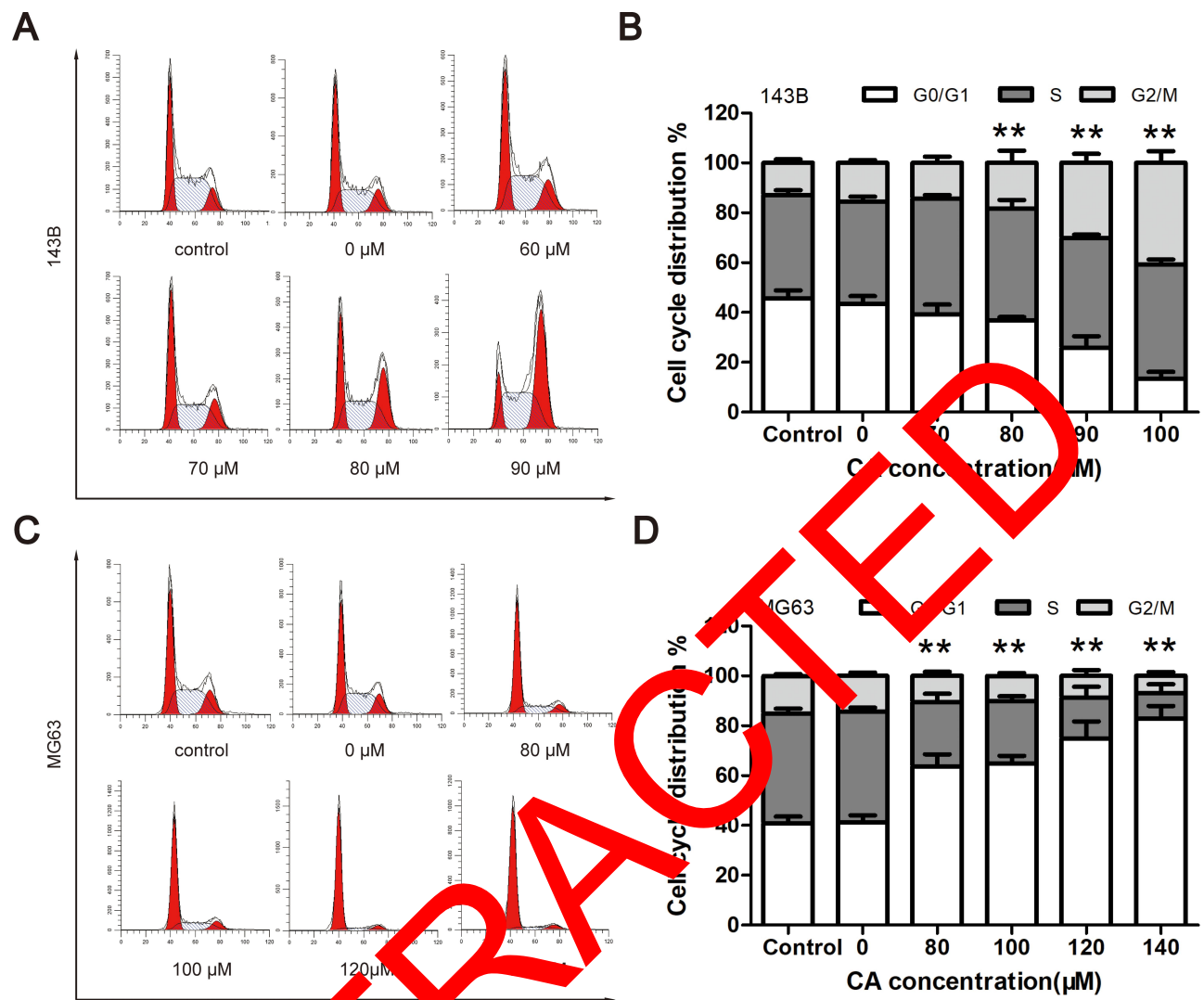


Figure 3 Effect of CA on the cell cycle of human OS cells was detected by flow cytometry assay (A–D). OS cells were treated with DMSO (as the control group) and 0–140 μM CA, respectively. CA-induced OS cells cell cycle arrest (** $P < 0.01$, vs the control group, $n = 3$).

CA Inhibits OS Cells the Wnt/ β -Catenin and PI3K/Akt Signaling Pathway

Wnt signaling pathway plays a vital role in biological development. If there is a mutation in the essential protein in this signaling pathway, it will lead to abnormal signal activation, which may induce cancer.¹³ We performed Western blotting analysis on the components and downstream targets of the Wnt/ β -catenin signaling pathway. The results showed that the expression of β -catenin, a core member of the Wnt signaling pathway, was reduced, and other related molecules, p-GSK-3 β (ser9), cyclin D and C-Myc, were also significantly reduced. At the same time, excessive activation of the PI3K/Akt signaling pathway could promote various cancers. Western blotting analysis showed that PI3K/Akt signaling pathway-related proteins

p-GSK-3 β (ser9), PI3K, p-Akt (ser473) and p-Akt (thr308) expression were reduced (Figure 5A–D, $P < 0.05$). These data indicated that CA might inhibit the growth of OS cells by inhibiting Wnt/ β -catenin and PI3K/Akt signaling pathway.

CA Inhibits Tumor Development in vivo

To further study the effect of CA on tumor growth, we established a tumor model with 143B cells. The results showed that the tumor growth was inhibited with the increase of CA dosage (Figure 6A and B, $P < 0.05$). However, the weight of the mice did not decrease significantly (Figure 6C, $P < 0.05$). The HE staining results of the tumor showed that the nuclear heterogeneity of the control group was apparent, and the high concentration of nuclei

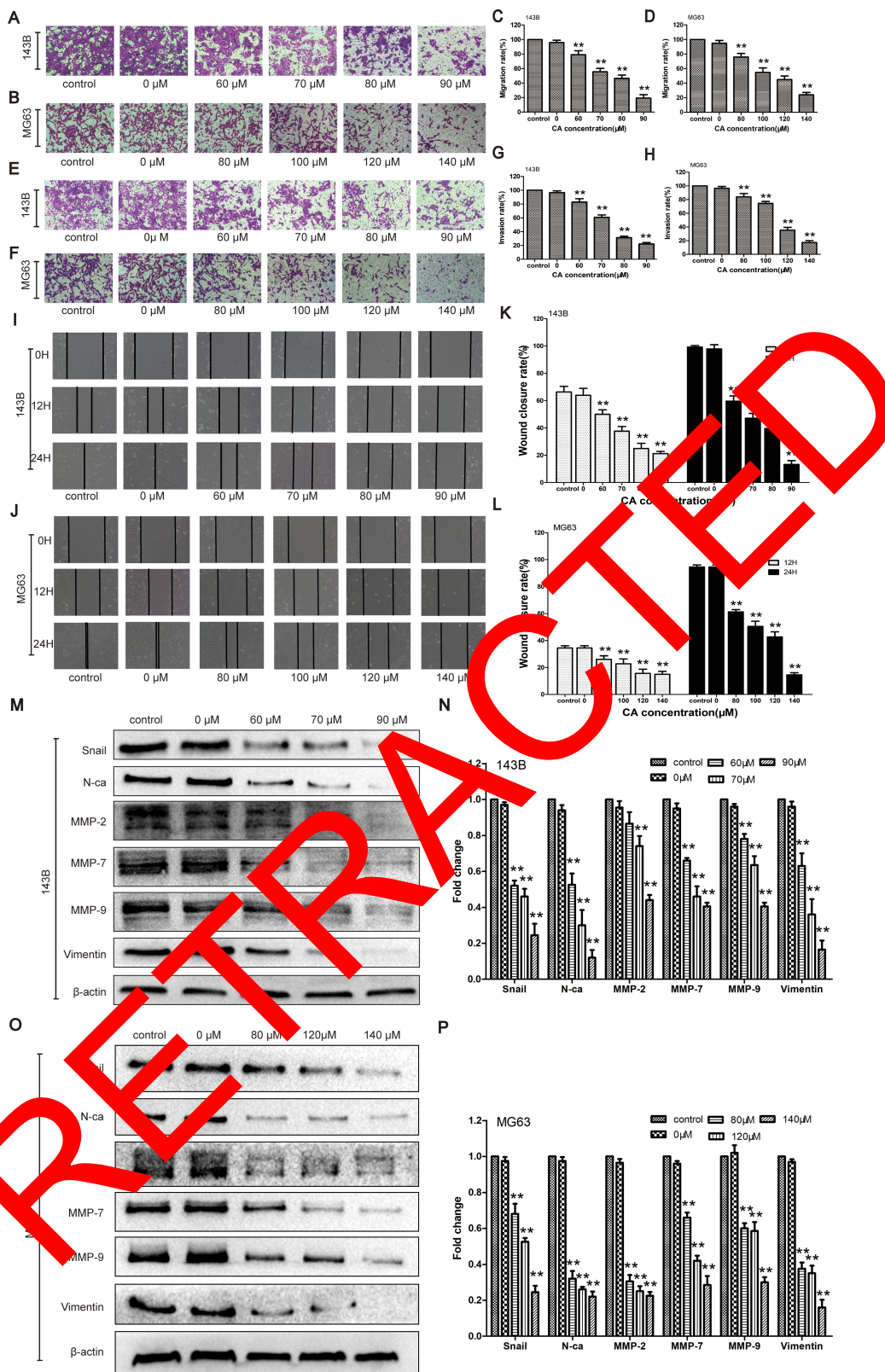


Figure 4 The effects of CA on the migration abilities of human OS cells were detected by transwell assay (A–D, crystal violet staining, ×100) and wound healing test (I–L, ×100), respectively. The effects of CA on the invasive abilities of human OS cells were detected by transwell assay (E–H, crystal violet staining, ×100). Then the expression levels of migration- and invasion-related proteins MMP-2, MMP-7, MMP-9, N-Cadherin, Snail and Vimentin were detected by Western blotting (M–P). OS cells were treated with DMSO (as the control group) and 0–140 μM CA for 12 hrs and 24 hrs, respectively. CA significantly inhibited the migration and invasion of OS cells (**P<0.01, vs the control group, n=3).

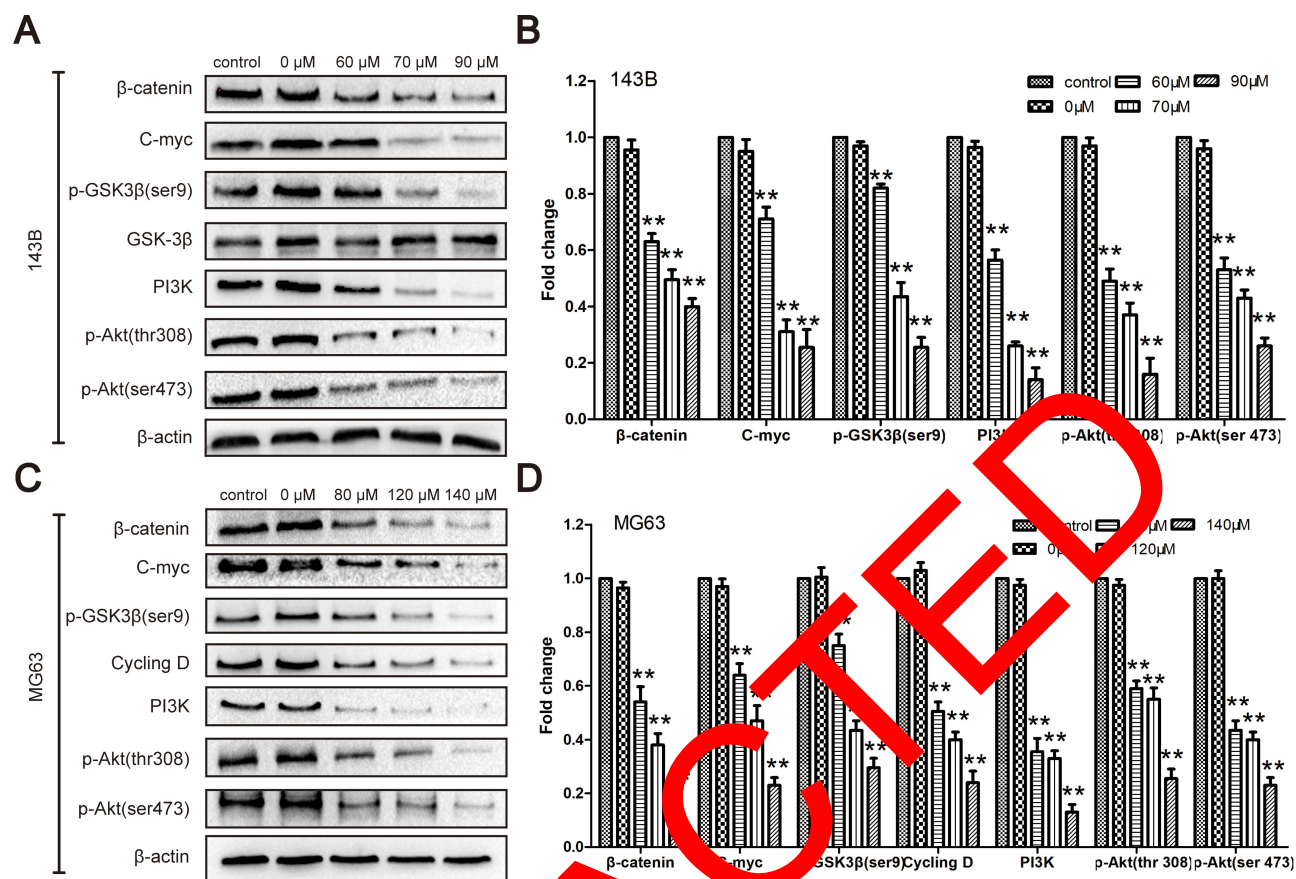


Figure 5 The expression levels of Wnt/ β -catenin and PI3K/Akt signaling pathway-related proteins β -catenin, p-GSK-3 β (ser9), C-Myc, Cyclin D, PI3K, p-Akt (ser473) and p-Akt (thr308) in OS cells treated with CA were detected by Western blotting (A-C). OS cells were treated with DMSO (as the control group) and 0–140 μ M CA, respectively. CA significantly inhibited the activity of Wnt/ β -catenin and PI3K/Akt signaling pathway in OS cells (** $P < 0.01$, vs the control group, $n = 3$).

presented with nuclear shrinkage and nuclear lysis (Figure 6D, $P < 0.05$). Immunohistochemical results revealed that the expression of PCNA, Bcl-2, Vimentin, β -catenin and phosphorylated Akt (ser473) in the high concentration group was reduced compared to the control group (Figure 6E, $P < 0.05$). These results indicated that CA could inhibit the growth of OS in vivo.

Discussion

Osteosarcoma is a highly malignant bone tumor prone to lung metastasis. Over the past 30 years, patients with osteosarcoma have been treated with surgical resection of the affected limb. With the advent of neoadjuvant chemotherapy based on methotrexate and cisplatin, osteosarcoma's 5-year survival rate has gradually increased.¹⁴ Chemotherapy drugs can bring light to the patient while also damaging the body with side effects. Chinese medicine is a treasure trove, and the prevention and treatment of tumors with Chinese medicine has a history of thousands of years. In particular, exploration and research on the prevention and treatment of

osteosarcoma with traditional Chinese medicine have been carried out in recent years.^{12,15,16} CA, the main active ingredient of cinnamon, has been shown to inhibit a variety of tumors, but its underlying mechanism is not fully understood. In this study, we first demonstrated that CA may inhibit the proliferation, promote apoptosis, and inhibit migration and invasion of OS cells through the Wnt/ β -catenin and PI3K/AKT signaling pathways.

The uncontrolled proliferation of tumor cells is an essential factor that causes poor prognosis for patients. We confirmed that CA has a significant inhibitory effect on the proliferation of OS cells in a dose-dependent manner through crystal violet experiments, MTT experiments, and clone formation experiments. Western blot assay results showed that CA effectively reduced PCNA expression, which plays a vital role in the initiation of cell proliferation.¹⁷ The basis of uncontrolled cell proliferation is the disorder of cell cycle regulation. Through flow cytometry analysis, we found that the blockade of OS cells by CA mainly occurred in the G2/M and G0/G1 phase, and the expression of Cyclin D1 and

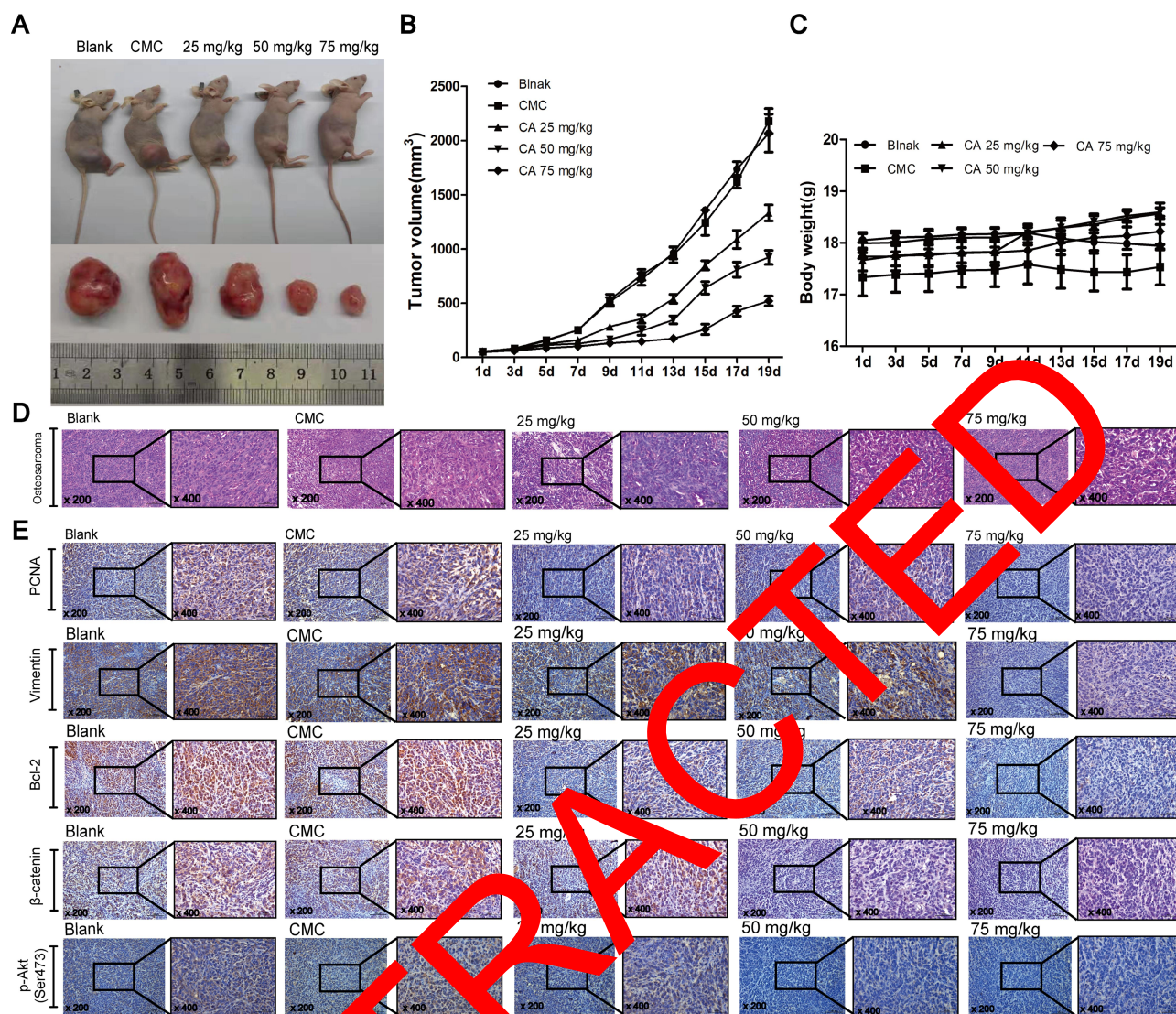


Figure 6 The effect of CA on tumor growth were detected by tumor animal model (A). The effect of CA on tumor volume and mouse weight (B and C). The effect of CA on xenograft tumor were detected by (D). PCNA, Bcl-2, N-Cadherin, β -Catenin and p-Akt (ser308) were detected by immunohistochemistry (E). CA significantly inhibited tumor development in vivo as the CMC group, $n=3$.

cycling B1 reached the peak of G0/G1 and G2/M phases, respectively. The disorder of their expression could lead to uncontrolled cell cycle generation and malignant transformation of cells.¹⁸ However, on the one hand, CA has a strong inhibitory effect on OS cells, and its IC_{50} is estimated to be 56.68 μ M in MG63, and 67.95 μ M in 143B, respectively. On the other hand, the toxicity of CA to HEB, HS5 and LO2 cells is relatively low, with IC_{50} of 295.9 μ M, 302.9 μ M, 1573 μ M, respectively. In vivo experiments showed no significant changes in body weight after ALT treatment. These results suggest that CA may be a relatively safe drug for the treatment of OS.

One crucial step in the development of anticancer drugs is the induction of apoptosis in tumor cells.¹⁹ In

this study, flow cytometric analysis confirmed that CA had a significant increase in the apoptosis rate of OS cells, and Hoechst staining showed that CA induced a change in the nuclear morphology of OS cells to apoptosis. There are two main apoptotic pathways, including the intrinsic/mitochondrial pathway and the death receptor (DR)/extrinsic pathway.²⁰ Poly (ADP-ribose) polymerases (PARPs) and nuclear caspase families are necessary for apoptosis induced by these two pathways.^{21–23} Through the Western blot assay, we found that the protein level of PARP decreased in a dose-dependent manner, while the protein levels of Cleave-PARP and Cleave-Caspase-3 increased. The Bcl-2 family is an essential regulator of apoptotic processes, including BAX, Bad, Bcl-2, etc. Bax

can form pores on the cytoplasmic mitochondria's outer membrane and activate caspase-3 and PARP, leading to the release of cytochrome C into the cytoplasm, and thereby promoting apoptosis.²⁴ Bcl-2 can interact with tBid or Bad on the membrane and change from tail anchoring to multiple transmembrane conformations to effectively inhibit pro-apoptotic proteins.²⁵ Western blot assay results further confirmed the role of Bcl-2 in inhibiting apoptosis and bax in promoting apoptosis. The results of the above experiments proved that CA could affect OS cells through the apoptotic pathway.

Metastasis is the leading cause of death in patients with malignant tumors. Approximately 90% of patients with malignant tumors die from tumor metastases.²⁶ The results of our wound healing assays and transwell assays confirmed that CA could inhibit the migration and invasion of OS cells. Epithelial-mesenchymal transition (EMT) is an essential process in normal embryonic development and a common initiation factor for tumor invasion and metastasis.^{27,28} EMT is a key component that inhibits cell-to-cell connectivity, including Vimentin, N-Cadherin, regulated by the transcription factor Snail. At the same time, matrix metalloproteinases (MMPs) have an auxiliary effect on EMT.^{29,30} The central role of MMPs is to regulate the adhesion of tumor cells to the stroma and activate potentially active proteins to affect tumor invasion and metastasis. At the same time, it also disrupts the dynamic balance of extracellular matrix (ECM) degradation, prompts cancer cells to break through barriers formed by ECM and basement membrane, invades surrounding tissues and metastasizes to distant tissues.³¹ When the expression of N-cadherin and Vimentin is increased, the cell biological characters are changed and the adhesion function is decreased, so that it may easily leave the primary focus and invade or transfer to the surrounding tissues.³² In normal epithelial cells and non-tumor cells, β -catenin is located on the cell membrane. Once EMT occurs, it will transfer to the cytoplasm or nucleus (as activating factor), and promote the expression of EMT-related genes.³³ CA can inhibit the migration and invasion of OS cells, which may be related to blocking EMT process. This hypothesis was confirmed by Western blot assay.

Numerous studies have shown that the abnormal activation of the Wnt signal and the high expression of β -catenin are related to the abnormal histological morphology of osteosarcoma and the abnormal proliferation and differentiation of cells, eventually leading to the occurrence of osteosarcoma.^{34,35} When β -catenin accumulates to a specific concentration in the cytoplasm, it begins to

turn to the nucleus and binds to the nuclear transcription factor TCF/LEF, which leads to the exposure of the promoter factor of the downstream target gene and the activation and expression of the promoter factor, causing the abnormal proliferation and apoptosis resistance of the cells, and promoting the formation of the tumor.³⁶ GSK3 β in the cytoplasm acts as a switch molecule in the β -catenin degradation complex. It reduces the stability of β -catenin and inhibits Wnt signaling pathway by Phosphorylation of β -catenin and ubiquitin ligase β -TrCP-mediated ubiquitin-proteasome pathway.³⁷ Our results reflected that CA inhibited the classic Wnt/ β -catenin signal by down-regulating β -catenin and phosphorylation of GSK- β as well as downstream target proteins C-Myc and MMP7. In addition, Western Blot results showed that CA significantly inhibited PI3K/AKT pathway which has been proved to play a vital role in regulating the proliferation and survival of tumor cells.³⁸ The role of PI3K/Akt in promoting tumorigenesis is not only inhibiting apoptosis but also inducing cell cycle progress.³⁹ Interestingly, studies have reported that activated Akt and GSK3 β can induce the expression of various anti-apoptotic protein genes or inhibit apoptosis through various signal-coupling pathways.³⁷ Akt is an important downstream target in the PI3K signaling pathway. After Akt is activated, it can regulate its activity by phosphorylating a variety of intracellular substrate proteins. GSK3 β is a branch downstream of Akt, and the activity of Akt enhances phosphorylation of GSK3 β , leading to the depression of its activity.⁴⁰ We speculate that CA may inhibit the accumulation of β -catenin and p-Akt by enhancing the activation of GSK3 β , thereby inhibiting the growth of OS. In this study, it was confirmed by Western blot that in addition to down-regulating the phosphorylation level of GSK3 β (Ser9), CA can also down-regulate the expression level of p-Akt protein, inhibit the PI3K/Akt signaling pathway in osteosarcoma cells and activate the pro-apoptotic effect of GSK3 β , which activates the pro-apoptotic effect of Gsk3 β , thereby inhibiting the growth of OS. All in all, CA may inhibit OS through typical Wnt/ β -catenin and PI3K/Akt signaling pathways.

Finally, we further verified the role of CA in vivo by using xenograft models. The results showed that compared with the control group, tumor growth was effectively reduced in all treatment groups of 143B OS xenografts. In addition, CA had no significant effect on the weight of mice. These results indicate that CA may be a relatively effective and secure solution to OS.

Conclusion

Our results indicate that CA can inhibit the growth of OS in vivo and in vitro, and this effect may be related to blocking the Wnt/ β -catenin and PI3K/AKT signaling pathways. These experimental results show that CA is providing a new target drug for the treatment of OS.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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