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ORIGINAL ARTICLE

Oridonin inhibits the proliferation, migration and invasion of human osteosarcoma cells via suppression of matrix metalloproteinase expression and STAT3 signalling pathway

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Summary

Purpose: *Oridonin, a diterpenoid, has been reported to exhibit anticancer activity against a wide range of cancer types.In this study, the effect Oridonin was examined against human osteosarcoma cells.*

Methods: *The human osteosarcoma cells U2OS were treated with various concentrations of Oridonin from 0-200 μM for 24 h. The anti-proliferative effects of Oridonin were measured by cell viability assay. DAPI and annexin V/propidium iodide (PI) assays were employed to examine the induction of apoptosis. Transwell assay was performed to examine the cell migration and invasion. Expression analysis was performed by western blot.*

Results: *Oridonin inhibited the proliferation of U2OScells and exhibited an IC_{50} of 30 μM. The antiproliferative effects were mainly found to be due to induction of apoptosis as indicated by DAPI staining. Moreover, the annexin V/PI staining showed that the percentage of the apoptotic cells increased with increase in the concentration of Oridonin. The induction of apoptosis was also related with upregulation of Bax, Caspase 3 and 9 expression and downregulation of Bcl-2. Oridonin was also found to cause significant decrease in the expression of MMP-2, 3 and 9 concentration-dependently. Transwell assay showed that Oridonin inhibited the migration and invasion of the U2OS cells.*

Conclusion: *It is concluded that Oridonin exhibits significant antiproliferative effects on the osteosarcoma cells and may prove essential in the development of systemic therapy for osteosarcoma.*

Key words: *osteosarcoma, oridonin, apoptosis, migration, invasion*

Introduction

Osteosarcoma is a malignant bone tumor which threatens the life of children and adolescent worldwide. It is characterized as highly aggressive and it has been found that the pulmonary metastases is the major cause for death [1]. Many modern and advanced medications were used to enhance the survival of osteosarcoma patients but the strength of the currently available treatments is very limited because of drug resistance and relapses [2]. Studies have reported that about 30% of osteosarcomas are multidrug-resistant[3]. Many reports established a relationship between the fast bone growth during the development of osteosarcoma andpubescence. It has been shown that 56% of all the osteosarcomas are found around the knees [4].Oridonin, a diterpenoid extracted from the medicinal herb *Rabdosiarubescens*, has been reported to exhibit antiproliferative effects on several types of cancerssuch as

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breast cancer [5,6].However, the anticancer effects of Oridonin have not been examined against osteosarcoma cells. In this work, we aimed to study the suppressive effect of Oridonin on osteosarcoma U2OS cell line *in vitro* and to investigate the mechanism underlying this suppressive effect. Moreover, the effects of oridonin were also examined on the STAT3 signalling pathway.

Methods

Chemicals and reagents

Oridonin was procured from AMSBIO (Cambridge, MA, USA). All other substances used to carry out this work were purchased from Sigma Aldrich Company (Sigma-Aldrich, St. Louis, USA). Stock solution of Oridonin was prepared to the final concentration of 200 µg/ml using the Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Massachusetts, USA) and kept at -20°C in refrigerator. The solution was sterile-filtered and aliquoted before stored for experimental use for further studies.

Cell culture

The osteosarcoma cell line U2OS cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CBTCCCAS, Shanghai, China). U2OS cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) composed of 10% fetal bovine serum (FBS),1 mM L-glutamine, 1% HEPES buffer, 100 µg/ml streptomycin and 100 U/ml penicillin (all obtained from Gibco Laboratories, Maryland, USA) at 37°C and 5% CO_2. The percentage of passage cells were maintained at a confluence of 80-85% and the cell culture medium was changed every 24 h.

Proliferation and colony formation assay

The WST-1 (Roche, Mannheim, Germany) based cytotoxicity assay was carried out to measure the toxicity associated with the treatment of Oridonin. In this experiment, U2OS cells were seeded at a density of 2×10^5 and 3×10^6 respectively, in 100 µl culture medium in 48-well plates and were cultured for one day. Cells were then treated with various Oridonin concentrations and incubated for 48 h. After incubation, WTS-1 reagent (25 µl) was added in both sets and further incubated for the next 2 h. Absorption was then measured at 450 nm with a reference wavelength of 695 nm. The colony formation assay was performed as previously described [9].

Apoptosis assay

The osteosarcoma U2OS cells (0.6×10^6) were grown in 6-well plates and incubated for around 12 h. The U2OS cells were then subjected to Oridonin treatment for 24 h at 37°C. As the cells sloughed off, 25 µl cell culture were put onto glass slides and subjected to staining with DAPI. The slides were covered with coverslip and examined with a fluorescent microscope. Annexin V/PI staining were performed as previously described [10].

Cell migration and invasion assay

The migration and invasion abilities of the Oridonin-treated U2OS cells were examined by transwell chamber assay. In brief, 1×10^4 U2OS cells were seeded in the upper chamber of the transwell(8 µm pore size polycarbonate filters). This was followed by placement of the chambers into 24-well plates and subjected to incubation at 37°C for 48 h. However, in case of invasion assay, the inserts were coated with extracellular matrix gel (50 µl) (ECM, Sigma, USA). Swabbing was performed to remove the non-migrated and non-invaded cells from the upper surface. However, the migrated and invaded cells on the lower surface were subjected to fixation with methanol for about 35 min, and stained with crystal violet (0.5%) for about 50 min, subjected to washing with phosphate buffered saline (PBS) and finally counted under light microscope (5 fields).

Western blotting

The Oridonin-treated U2OS cells were subjected to washing with ice-cold PBS and suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford protein assay. About, 40 µg of protein were loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with tris buffered saline (TBS) and exposed to primary antibody at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualized by enhanced chemiluminescence reagent.

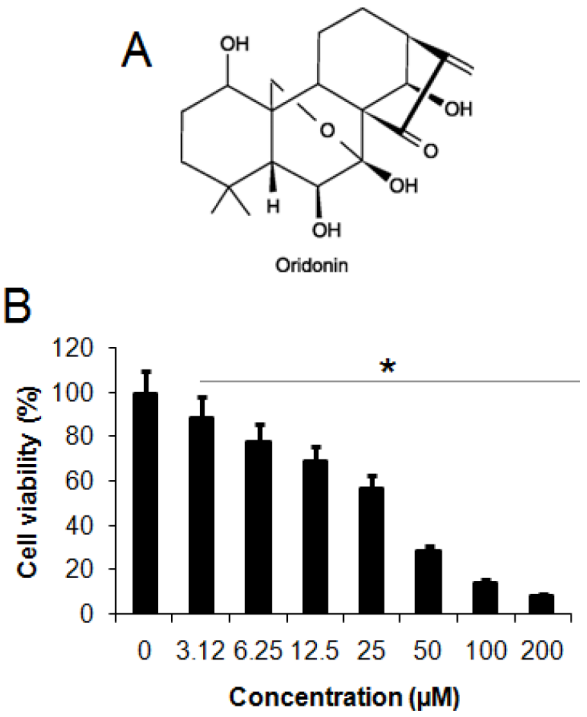

Figure 1. A: Chemical structure of oridonin. **B:** Effect of oridonin on the viability of U2OS cells as determined by WST-1 assay. The values are mean of three experiments ± SD (*p< 0.05).

Statistics

The results from real-time PCR quantification and the enzyme-linked immunosorbent assay were analyzed by using GNU PSPP software (Free Software Foundation, Inc., USA). T-tests were used for unpaired compounds. All the results are shown as mean±standard deviation (SD) and the p value of <0.05 was considered statistically significant.

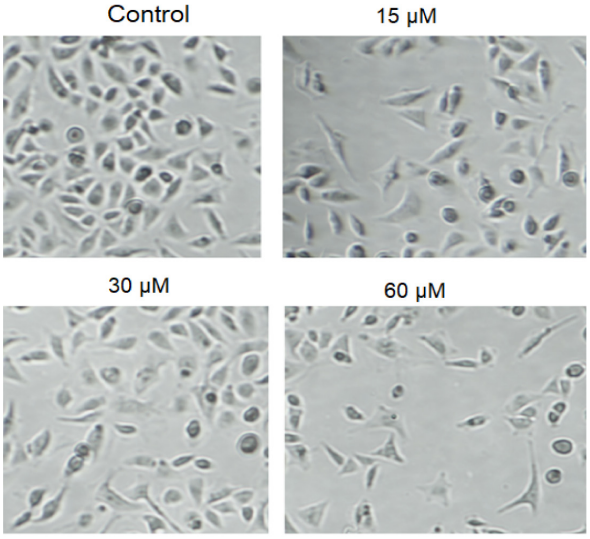

Figure 2. Effect of oridonin on the morphology of the U2OS cells at indicated concentrations. The Figure shows that oridonin causes morphological changes in the U2OS cells in a concentration-dependent manner. The experiments were performed in triplicate.

Results

Oridonin inhibited the proliferation and colony formation of the U2OS cells

The antiproliferative effects of Oridonin (Figure 1A) were assessed on the osteosarcoma cell line U2OS by WST-1 assay. It was found that U2O-Sexerted antiproliferative effects on the U2OS cells and exhibited an IC_{50} of 30μM (Figure 1B). Furthermore, it was found that the anticancer effects of Oridonin on the U2OS osteosarcoma cells were concentration-dependent and Oridonin also caused change in the morphology of the U2OS cells (Figure 2). The results of the colony formation assay showed that Oridonin inhibited the colony development of the U2OS cells concentration-dependently (Figure 3).

Oridonin induced apoptosis in osteosarcoma cells

To ascertain if Oridonin prompts apoptosis in the U2OS cells, DAPI staining was performed which showed remarkable changes in the nuclear morphology and membrane blebbing of the U2OS cells (Figure 4). The percentage of the apoptotic U2OS cells was determined by Annexin V/PI staining which showed that the apoptotic cell percentage increased from 1.14% in the control to 21.94% at 60 μM of Oridonin (p<0.05) (Figure 5). The apoptosis was further confirmed by the increased ex-

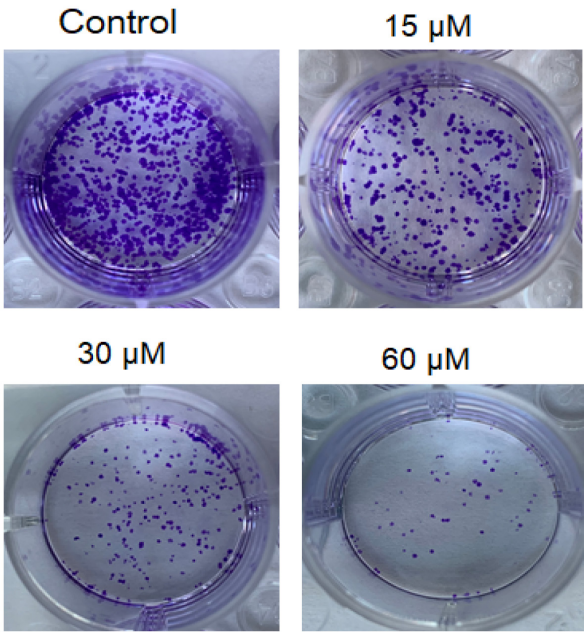

Figure 3. Effect of oridonin on the colony formation of the U2OS cells at indicated concentrations as determined by colony formation assay. The Figure shows that oridonin inhibits the colony formation of the U2OS cells in a concentration-dependent manner. The experiments were performed in triplicate.

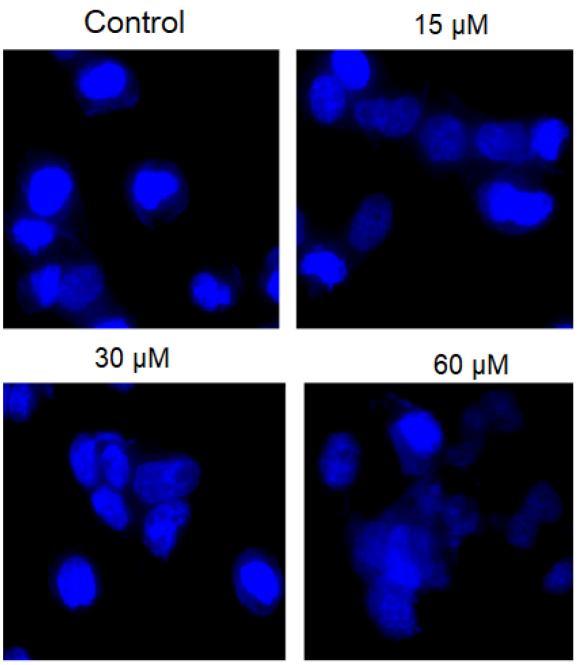

Figure 4. Oridonin induces apoptosis in the U2OS cells as indicated by DAPI staining. The Figure shows that oridonin causes apoptotic cell death in U2OS cells in a concentration-dependent manner. The experiments were performed in triplicate.

pression of Caspase 3, 9 and Bax and decreased expression of the Bcl-2 in U2OS cells (Figure 6).

Oridonin inhibited the migration and invasion of U2OS cells

The effects of Oridonin were also investigated on the migration and invasion of U2OS cells by transwell assay. It was found that Oridonin could inhibit the migration and invasion of the U2OS cells concentration-dependently (Figure 7).

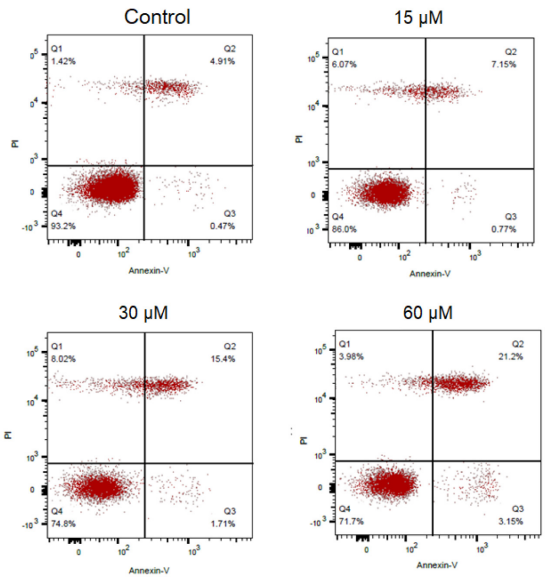

Figure 5. Determination of the percentage of the apoptotic cell populations as determined by Annexin V/PI staining. The Figure shows that oridonin increases the percentage of the apoptotic U2OS cells in a concentration-dependent manner. The experiments were performed in triplicate.

Oridonin inhibited the matrix metalloproteinase (MMP) expression and STAT3 signalling pathway

Next, we sought to know the effects of Oridonin on the expression of MMPs. It was found that Oridonin inhibited the expression of MMP-2, 3 and 9 in a concentration-dependent manner (Figure 8). The effects of Oridonin were also examined on the STAT3 signalling pathway which revealed that Oridonin caused concentration-dependent decline in the phosphorylation of p-STAT3 (Tyr 705) and p-STAT3 (Ser 727) while total STAT3 remained unaltered in U2OS cells (Figure 9).

Discussion

Osteosarcoma causes significant mortality world over and the treatments available are not efficient and are associated with a number of side

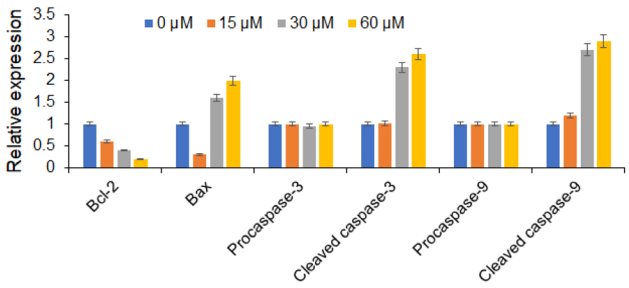

Figure 6. Effect of oridonin on the expression of Bax, Bcl2, Caspase-3 and 9 expression as indicated by western blot analysis. The Figure shows that the expression of Bcl-2 is decreased, the expression Bax is increased and the cleavage of caspase-3 and 9 is also increased upon oridonin treatment. The experiments were performed in triplicate.

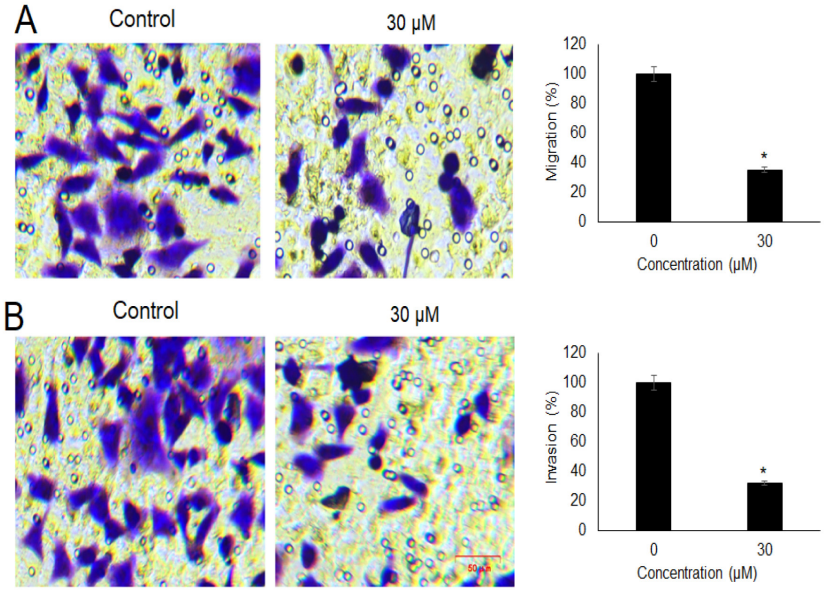

Figure 7. Inhibition of **(A)** cell migration and **(B)** invasion by oridonin at 30 μM concentrations as depicted by transwell assay. The values are mean of three experiments ± SD (*p< 0.05).

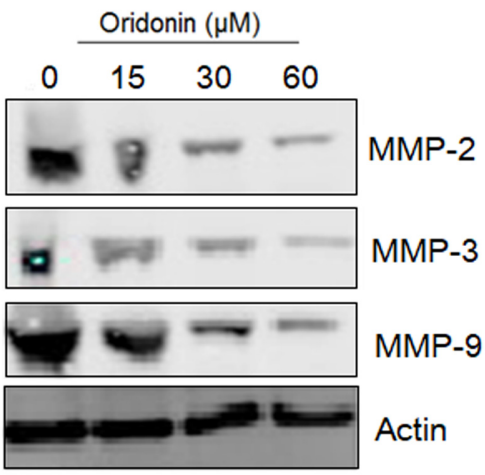

Figure 8. Effect of oridonin on the expression of MMP-2, 3 and 9 at indicated concentrations as depicted by western blot analysis. The Figure shows that the expression of MMP-2, MMP-3 and MMP-9 is decreased upon oridonin treatment in U2OS cells. The experiments were performed in triplicate.

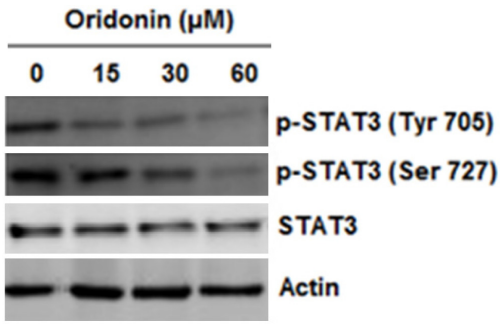

Figure 9. Effect of oridonin on STAT3 pathway at indicated concentrations as depicted by western blot analysis. The Figure shows that the phosphorylation of STAT3 is inhibited upon oridonin treatment. The experiments were performed in triplicate.

effects [11]. There is therefore an urgent need to look for new therapeutic agents that could be utilized to treat osteosarcoma efficiently. Herein, we examined the anticancer effects of Oridonin on the U2OS osteosarcoma cells. The results revealed that Oridonin inhibited the growth of the osteosarcoma cells in a concentration-dependent manner and also halted their ability to develop colonies. Previous studies have shown that Oridonin could inhibit the growth of several types of cancers such as leukemia, pancreatic cancer and gastric cancer [12-14]. Further studies have also shown that Oridonin induces apoptosis in cancer cells. For example, Oridonin has been reported to induce apoptosis in colorectal cancer cells [15]. Therefore, we investigated if Oridonin also induces apoptosis in the U2OS osteosarcoma cells. The results of DAPI and annexin V/PI showed that Oridonin induced apoptosis in the U2OS cells and the percentage of the apoptotic cells increased as the concentration of Oridonin increased. Apoptosis is an important process that helps to kill the harmful and cancer cells and several known anticancer drugs induce apoptosis in the cancer cells [16]. The Oridonin-induced apoptosis was also associated with concomitant increase in the expression of the cleaved caspase-3, 9 and Bax and downregulation of Bcl-2 which are the important markers for apoptosis [17]. Osteosarcoma cells have the capacity to invade neighboring tissues and undergo metastasis [18] and hence we examined the effect of Oridonin on the migration and invasion of the U2OS cells. Interestingly, it was found that Oridonin could suppress the migration and invasion of U2OS cells, concomitant with downregulation of MMP-2, 3 and 9 expression. STAT3 transduction pathway is an important pathway that has been reported to be dysregulated in cancer cells [19] and in this study we found that Oridonin inhibited the phosphorylation of the U2OS osteosarcoma cells.

Conclusion

It is concluded that Oridonin exhibits significant anticancer effects on the osteosarcoma cells via induction of apoptosis. In addition, Oridonin also inhibited the migration and invasion of osteosarcoma cells by modulating the expression of metalloproteinases. Therefore, Oridonin may prove beneficial in the management of osteosarcoma.

Conflict of interests

The authors declare no conflict of interests.

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