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Original Article

The cell death of C6 astrocytoma cells induced by oridonin and its mechanism

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Abstract: Many studies have shown that oridonin, a compound purified from Rabdosia rubescens, was able to suppress proliferation and induce apoptosis in many cell types. In this study, In order to investigate the proliferation suppression and apoptosis-inducing effect of oridonin on Rat C6 astrocytoma cells, we treated C6 cells with different concentrations of oridonin for various time intervals. Oridonin concentration-time viability curve were used to test the effect of oridonin on the C6 cells. The distribution of cell cycle and percentage of apoptosis cells was analyzed by flow cytometry. The protein expression of Bax, Bcl-2, and caspase-3 in the C6 cells was detected by western blot analysis. The results of viability curve demonstrated that oridonin induced suppression of proliferation in a concentration- and time−dependent manner. Hochest 33258 staining and flow cytometry revealed that oridonin induced apoptosis and arrested the entry into G2/M phase of C6 cells. According to the results of Western blot, oridonin down-regulated Bcl-2, up-regulated Bax protein, and activated caspase-3 in the oridonin-treated C6 cells. All together, our results suggested that oridonin can cause the suppression of proliferation in C6 astrocytoma cells and the cell death induced by oridonin might be associated with mitochondria- mediated apoptosis by activating caspase-3.

Keywords: Astrocytoma, C6 astrocytoma cells, oridonin, cell death, apoptosis

Introduction

Astrocytoma, one of the anaplastic gliomas (World Health Organization (WHO) grade III), is a highly aggressive and lethal brain cancer with high morbidity, high mortality and extremely poor prognosis, the median survival of which is generally less than two years despite recent advances in diagnostic and therapeutic approaches [1]. Novel and efficient therapeutic drugs are needed for this deadly disease.

Oridonin, a diterpenoid compound purified from Chinese herb Rabdosia rubescens [2] (molecular structure shown in **Figure 1A**.), was firstly reported for its remarkable anti-proliferative activity in the year of 1976 [3]. Subsequent studies demonstrated remarkable anti-tumor ability of oridonin to suppress the progress of a number of cells from cancers such as primary liver cancer, gastric carcinoma, carcinoma of the esophagus, pancreatic cancer, etc [4-14] . However, the effects of oridonin on astrocytoma cells have not been reported up to now.

C6 astrocytoma cells were produced by Benda *et al.* by repetitively administering N-methylnitrosourea to outbred Wistar rats over a period of approximately 8 mont[15]hs [15]. Comparing the changes in gene expression between the C6 astrocytoma cells and rat stem cell-derived astrocytes, Molecular studies revealed that the changes in gene expression observed in the C6 cell line were the most similar to those reported in human brain tumors [16]. The C6 rat astrocytoma cells have been widely used as experimental model to evaluate the therapeutic efficacy of a variety of drugs [17].

In this study, we investigated the mechanism of oridonin-induced cell death in C6 astrocytoma cells and provided experimental evidence for the potentially application of oridonin on

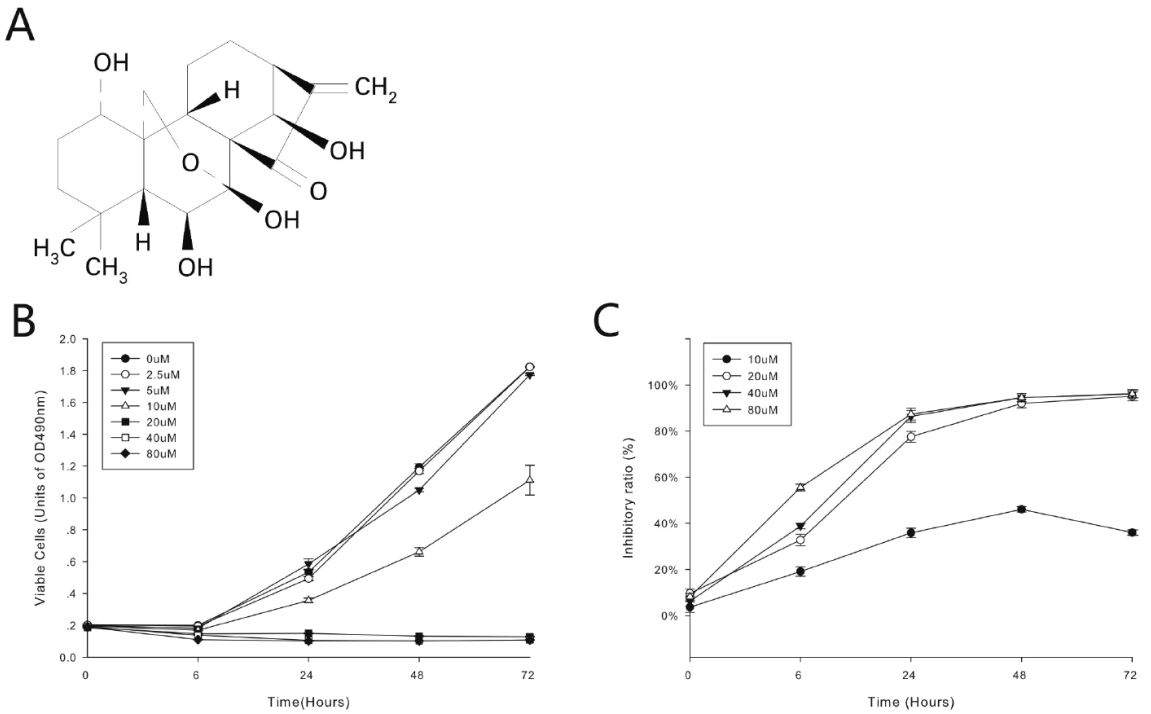

Figure 1. Oridonin inhibits cell proliferation of C6 astrocytoma cells. A. Chemical structure of the diterpenoid oridonin. B. C6 astrocytoma cells were treated with 0, 2.5, 5, 10, 20, 40 and 80 uM oridonin for 0, 6, 24, 48 and 72 hours. Effects of oridonin on cell proliferation were determined by CCK-8 Kit. Error bars represent standard error of the mean (standard deviation divided by the square root of the sample size). C. Time and dose responses of cell death by oridonin treatment. C6 astrocytoma cells were treated with oridonin at a concentration of 10, 20, 40, 80 μmol/L. The inhibitory ratio was calculated according to the following formula:

$$\text{Inhibitory ratio} = \frac{\text{OD}_{490\text{nm}} \text{ value of control group} - \text{OD}_{490\text{nm}} \text{ value of oridonin treatment gorup}}{\text{OD}_{490\text{nm}} \text{ value of control group} - \text{OD}_{490\text{nm}} \text{ value of blank group}} \times 100\%$$

astrocytoma as a new anti-tumor natural medicine.

Materials and methods

Cell lines culture and oridonin dissolution

Rat C6 astrocytoma cells were obtained from American type culture collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing high glucose and pyruvate, with 10% fetal bovine serum (Thermo Scientific, Hyclone, USA) plus antibiotics penicillin and streptomycin (Life Life Technologies, Inc.) Cells were maintained in 100 mm plastic tissue culture dishes at 37°C in a humidified 5% CO2 atmosphere. Confluent cells were harvested by washing in phosphate-buffered saline (PBS) and followed by trypsinization (0.25% in EDTA) for subculture.

Oridonin (purity≧98%) was purchased from Shanghai Standard Biotech Co., Ltd., China. It was dissolved in DMSO at a stock concentration of 100 mmol/L and store at -20°C. The stock solution was further diluted with cell culture medium to yield final oridonin concentrations.

Western blot analysis and antibodies

Cells were washed twice with ice-cold PBS, scraped off the plate, and re-suspended in ice-cold 1×SDS-PAGE lysis buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, and 0.1% bromophenol blue) containing 100 mM DTT. Lysed cells were boiled 5 min before loading for analysis. Protein concentrations in the cleared lysate were quantified using the bicinchoninic acid protein assay (Beyotime, Jiangsu, China), and 30μg protein were loaded on SDS-PAGE gels, and then the proteins were transferred to a nitrocellulose membrane. The membrane

was first rinsed with TBST (20 mmol/L Tris-HCl (pH 7.4), 0.15 mol/L NaCl, and 0.05% Tween 20) and then blocked with 5% (w/v) skim milk in TBST for 1 hour at room temperature. The blocked membrane was subsequently probed overnight at 4°C with 1:200–1:1000 dilutions of first antibodies in blocking buffer. After the membrane had been washed 3 times with TBST, it was incubated for 1 hour at room temperature either with horseradish peroxidase-conjugated antibodies. After the membrane had been washed with TBST, Proteins were detected using enhanced chemiluminescence detection kit (GE Healthcare, Piscataway, NJ, USA). Antibodies employed in this study included anti-bax(1:500, Cell Signaling Technology, Beverly, MA, USA), anti-caspase 3 (1:500, Cell Signaling Technology, Beverly, MA, USA) and anti-β-actin (1:3000, Sigma-Aldrich, St. Louis, MO).

Cell proliferation assay

Cells were seeded into 96-well plates at 3,000 live cells per well and treated with 0, 2.5, 5, 10, 20, 40 and 80 μM oridonin for 3 days. The anti-proliferative effect of oridonin was assessed using Cell Count Kit-8 (Shanghai SunBio Medical Biotechnology Co., Ltd., Shanghai, China).

Flow cytometry

C6 astrocytoma cells treated with oridonin (0, 2.5, 5, 10, 20 and 40 μmol/L) were harvested for flow cytometry analysis on day 3. Cells were fixed in 70% cold ethanol and stained with 0.1 mg/mL propidium iodide (PI) for DNA analysis with FACSCalibur system (Becton–Dickinson, San Jose, CA, USA) and analyzed by modfit LT software (Verity Software House Inc., Topsham, ME, USA).

Hoechst 33258 staining

C6 astrocytoma cells at logarithmic growth were seeded in 96-well plates by density of $1X10^4$/mL. Oridonin treatment group (20 μmol/L) and control group were cultured for 24 hours. Cells were fixed with 3.7% paraformaldelyde for 30 min at room temperature, and then washed and stained with 167μmol/l Hoechst 33258 at 37°C for 30 min. C6 cells were observed under a fluorescence microscope (IX70, Olympus, Tokyo, Japan Nikon, Tokyo, Japan) equipped with a UV filter. The images were recorded on a computer with a digital camera attached to the microscope, and the images were processed by computer. The Hoechst reagent was taken up by the nuclei of the cells, and apoptotic cells exhibited a bright blue fluorescence.

Detection of apoptosis

Apoptosis of C6 astrocytoma cells was analyzed by flow cytometry, which measures cells positively stained with Annexin V (Beyotime, Jiangsu, China) and PI. C6 astrocytoma cells were plated onto a 60-mm dish, trypsin was added to loosen the cells from the plate, and the cells were harvested after the appropriate treatment periods. Briefly, cells were washed twice with ice-cold PBS and precipitated by centrifugation at 500 g for 10 min, and the cell pellets were resuspended in 1× Annexin V binding buffer. To a 100ul aliquot of the cell suspension, 10 ul of PI (50 μg/ml) and then 10ul of Annexin V were added, and the cells were incubated in darkness for 15 min at room temperature. Flow cytometry was performed on FACSCalibur (Becton-Dickinson, San Jose, CA, USA). Data from a total of 10,000 events were analyzed using CellQuest software (Becton-Dickinson Immunocytometry Systems, San Jose, CA). The percentage of Annexin V-positive, and PI-positive cells was calculated to determine cells in the late stage of apoptosis.

Statistical analysis

Graphs were generated by SigmaPlot (SPSS, Chicago, IL, USA) and Microsoft Excel (Excel 2007, Microsoft, Redmont, WA, USA).

Results

Oridonin suppressed C6 astrocytoma cells proliferation

To investigate the possible effect of oridonin on the proliferation of C6 astrocytoma cells, oridonin of various concentrations were used to treat C6 astrocytoma cells. As shown in **Figure 1B**, oridonin could suppress proliferation of C6 astrocytoma cells in a time- and concentration-dependent manner. C6 astrocytoma cells showed sensitivity to the oridonin treatment. The growth of C6 astrocytoma cells was greatly

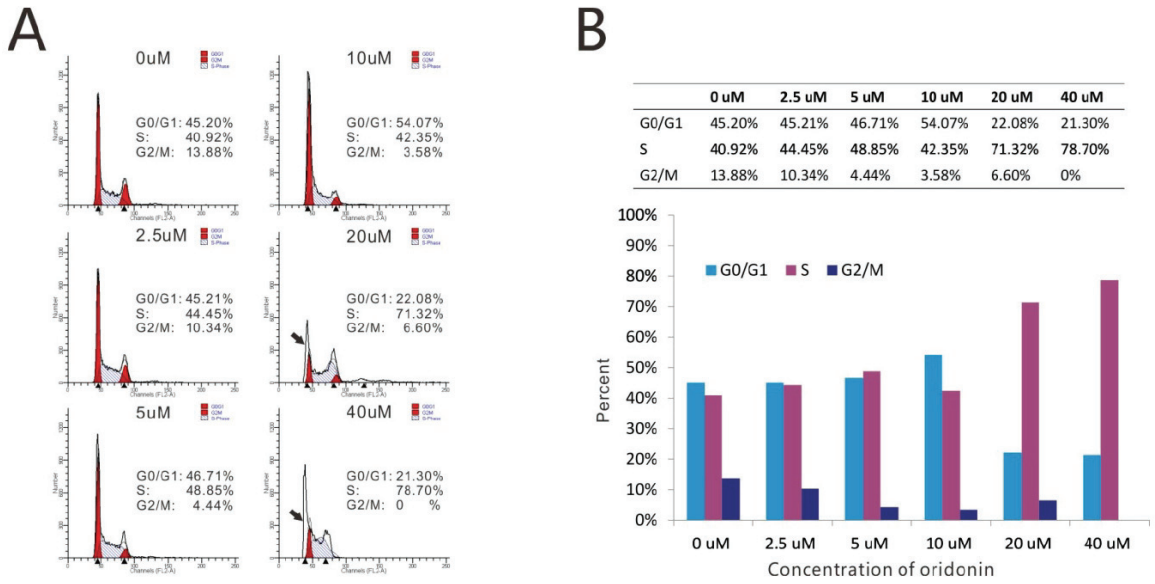

Figure 2. Oridonin induces cell cycle arrest. C6 astrocytoma cells were treated with 0, 2.5, 5, 10, 20 and 40 μM oridonin for 24 hours. Cell cycle distribution was determined by flow cytometry and the representative graphs are shown in A. Sub-diploid population was indicated by black arrow. B. The distribution percentage of G0/G1, S and G2/M phase during the cell cycle corresponding to (A) was shown.

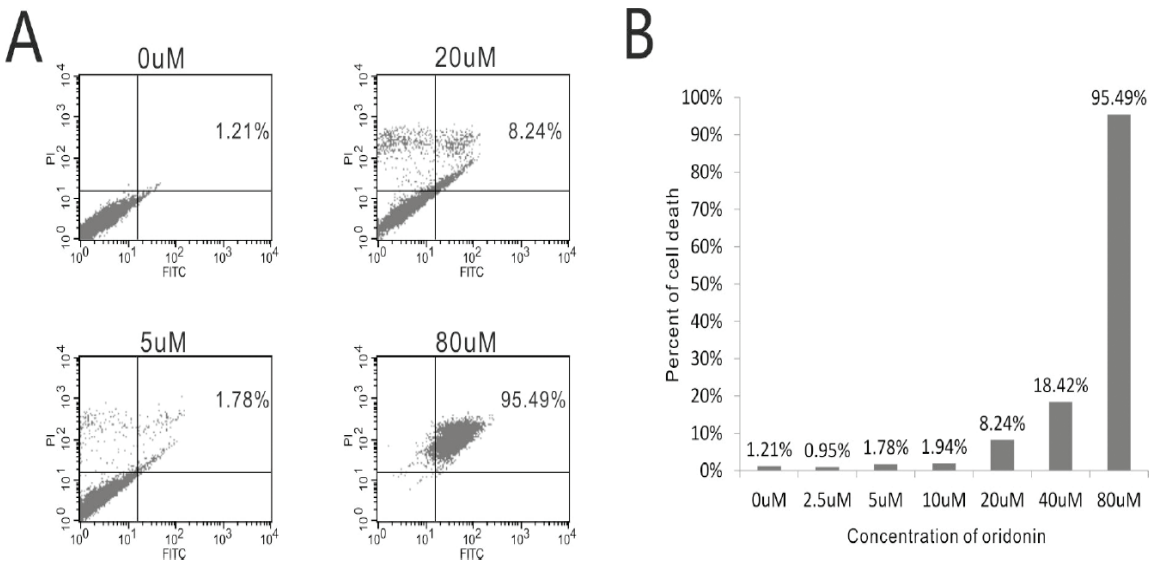

Figure 3. Cell death after oridonin treatment. A. Apoptosis was evaluated after treating C6 astrocytoma cells with 0, 5, 20 and 80 μM oridonin, and staining with Annexin-V at 24 hours. Flow cytometry profile represents Annexin-V-FITC staining in x axis and PI in y axis. The lower left indicates live cells (Annexin V – FITC negative/PI negative); the lower right shows early apoptotic cells (Annexin V – FITC positive/PI negative). The upper left shows damaged cells (Annexin V – FITC negative/PI positive), while the upper right demonstrates necrotic cells and late apoptotic cells (Annexin V – FITC positive/PI positive). The number represents the percentage of necrotic cells and late apoptotic cells in each condition (higher right quadrant). B. The percentage of necrotic cells and late apoptotic C6 astrocytoma cells treated with 0, 2.5, 5, 10, 20, 40 and 80 μM oridonin for 24 hours.

suppressed by oridonin at 20 μM for 24 hours, which lasted to day 3. However, the cells began to show a reduced growth from treatment with 10 μM oridonin after 24 hours. From the concentration of 20 μM, growth of C6 cell lines was completely inhibited after 6 hours.

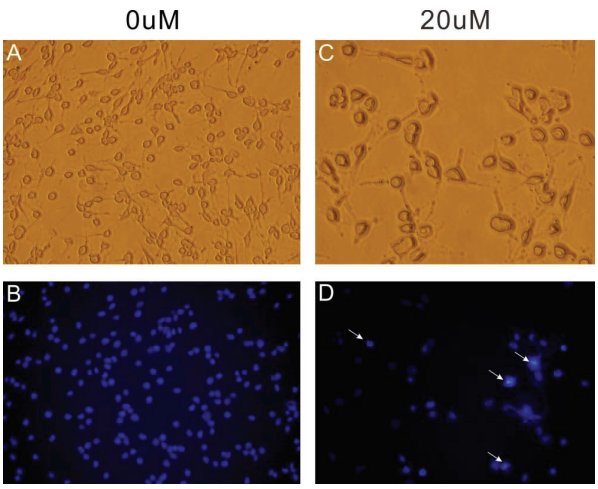

Figure 4. Hochest 33258 staining of C6 astrocytoma cells after treatment with oridonin. C6 astrocytoma cells in the control group (A), (B) and after treatment with 20 uM oridonin (C, D) for 24 hours were shown. Bright-field (A, C) and fluorescence images (B, D) were taken after Hochest 33258 staining. Fragmented nuclei indicated by white arrows. (×200 magnification)

We calculated the inhibitory ratio of oridonin with various concentrations at 0, 6, 24, 48 and 72 hours after treatment (**Figure 1C**). According to these data, oridonin inhibited more than 90% of C6 cells growth at the concentration of 20, 40 and 80 μM after treatment for 48 hours, among which the highest inhibitory ratio, 96.20%, was obtained when 80 μM oridonin treated C6 cells for 72 hours.

Oridonin caused C6 cell cycle arrest

We next examined the effects of oridonin on cell cycle distribution. C6 astrocytoma cells were treated with oridonin at 2.5, 5,10,20,40 μM for 24 hours. 20 and 40 μM oridonin caused obvious S-phase arrest in C6 astrocytoma cells. As shown in **Figure 2A** and **B**, the population of S-phase cells in the groups treated with 20 and 40 μM oridonin increased to 71.32% and 78.70% respectively relative to 40.92% of the control group. Further, the percentage of G0/G1-phase and G2/M-phase of C6 cells treated with oridonin reduced relative to the control group, which implied entries into G0/G1-phase and S-phase were normal and the entry into G2/M-phase from S-phase was arrested. Also, a marked increase of sub-diploid peak was observed (indicated by black arrow), implying the increase of fragments of dead cells. These results suggested that oridonin can arrest the cell cycle of C6 astrocytoma cells accompanied by cell proliferation inhibition.

Oridonin induced apoptosis

The sub-diploid peak indicated the possibilities of existence of apoptotic cells. This apoptosis inducing effect of oridonin was further confirmed using Annexin V-FITC/flow cytometry (**Figure 3A** and **B**). Treatment with 20, 40 and 80 μM oridonin for 24 hours obviously increased the percentage of necrotic cells and late apoptotic cells, especially for the group treated with 80 oridonin in which 95.49% of all cells were identified in the upper right quarter of necrotic cells and late apoptotic cells. Unexpectedly, we didn't detect any obvious change of the percentage of early apoptotic cells.

To characterize the oridonin-induced cell death of C6 astrocytoma cells, we examined the morphologic changes by Hoechst 33258 staining (**Figure 4**). When C6 astrocytoma cells were cultured with 20 μM oridonin for 24 h, apoptotic morphologic changes were observed as compared with the medium control group. In the control group, nuclei of C6 astrocytoma cells were round and homogeneously stained, but the 20 μM oridonin-treated cells showed marked granular apoptotic bodies (indicated by white arrows in **Figure 4D**). According to this characterization of apoptosis, we concluded that oridonin induced the apoptosis of C6 astrocytoma cells.

Oridonin regulated the expressions of Bcl-2, Bax and caspase-3

Accumulating evidence demonstrates that the mitochondria play a critical role in apoptosis [18]: the caspase activation is initiated by mitochondrial damage that leads to cytochrome c–release into cytosol. Cytochrome c, which is normally sequestered between the inner and outer membranes of the mitochondria, then binds and activates Apaf-1. Apaf-1 activates procaspase-9, which in turn cleaves procaspase-3. This pathway is activated by pro-apoptotic family members BAK or BAX and inhibited by Bcl-2 and its anti-apoptotic family members.

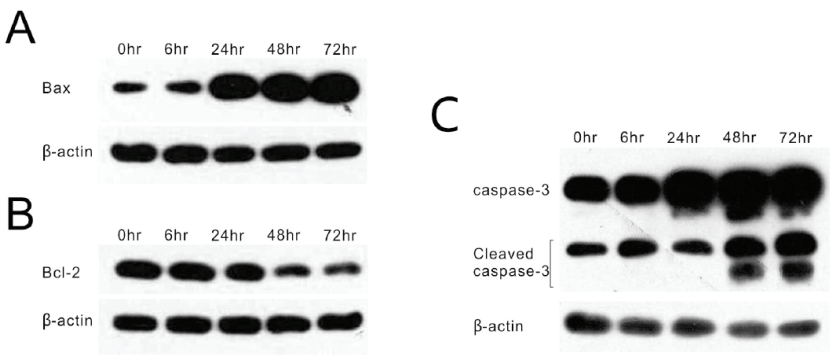

Figure 5. Oridonin regulates the expression of apoptosis-related proteins in vitro. The expressions of protein bax (A), bcl-2 (B) and caspase-3 (C) treated with 20uM oridonin through various time were shown. Three independent experiments were performed and a representative result is shown.

Our results of Western Blot showed that the protein expression level of Bax increased and the protein expression level of bcl-2 decreased as the time of oridonin treatment increased (**Figure 5A** and **5B**). Meanwhile caspase-3 was not only up-regulated but also activated by proteclytic processing of the pro-caspase into smaller subunits after treatment with 20μM oridonin for 48 hours (**Figure 5C**). These results indicated that oridonin-induced apoptosis might be involved in the mitochondria-mediated pathway.

Discussion

The results of cell proliferation assay showed that oridonin inhibited the proliferation of C6 cells in a concentration- and time-dependent manner. There was a significant growth inhibition from the concentration of 20μM oridonin. Based on the result of Hoechst 33258 staining, cell cycle and Annexin V-FITC, we concluded that oridonin caused apoptosis in C6 cells.

Current studies suggest that there are two classic cell apoptosis pathways: mitochondria-mediated apoptosis (the intrinsic pathway) and death receptor-mediated apoptosis (the extrinsic pathway). The intrinsic apoptotic pathway is characterized by permeability of the mitochondria and release of cytochrome c into the cytoplasm; and the extrinsic apoptotic pathway is activated by death receptors on the plasma membrane such as tumor necrosis factor receptor 1 (TNFR1) and Fas/CD95 [19-20]. The expression and activation of caspase-3 is the molecular marker for the cell apoptosis of both pathways [21]. Two factors involve the regulation of mitochondria-mediated apoptosis pathway: B-cell lymphoma-leukemisa-2 gene (Bcl-2) is an inhibitive factor for apoptosis, while Bax is a pro-apoptosis factor [22]. After receiving the apoptosis signals from the upstream, Bax will transfer from cytoplasm to mitochondrial membrane, where it combines with Bcl-2 and forms into dipolymer. The permeability of mitochondrial membrane was changed, and then the pro-apoptosis proteins in the mitochondria such as cytochrome C and apoptosis inducing factor (AIF) were released into the cytoplasm, where they combine with Apaf-1, and activate Caspase-9. Caspase-9 then activates caspase-3 in downstream and finally results in cell apoptosis. Whether the cells can survive or not after receiving apoptosis signals depends on the ratio of Bcl-2/Bax. The cell dies if Bax is dominant, and survives when Bcl-2 is dominant [23].

Our further data indicated that caspase-3 activation was involved in oridonin-induced apoptosis of C6 cells. After 20μM oridonin treatment for 24 hours, the expression of Bcl-2 protein began to decrease, and simultaneously, expression of Bax began to increase. At the same time, caspase-3 activity was increased.

All together, our presented results support that oridonin may induce the apoptosis of C6 cells through the mitochondria pathway, which provides an experimental basis for the animal experiment of clinical application of oridonin.

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