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Oridonin Inhibits Tumor Growth in Glioma by Inducing Cell Cycle Arrest and Apoptosis

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Abstract

Glioma is the most common malignant intracranial tumors. Despite newly developed therapies, these treatments mainly target oncogenic signals, and unfortunately, fail to provide enough survival benefit in both human patients and mouse xenograft models, especially the first-generation therapies. Oridonin is purified from the Chinese herb *Rabdosia rubescens* and considered to exert extensive anti-cancer effects on human tumorigenesis. In this study, we systemically investigated the role of Oridonin in tumor growth and the underlying mechanisms in human glioma. We found that Oridonin inhibited cell proliferations in a dose- and time-dependent manner in both glioma U87 and U251 cells. Moreover, these anti-cancer effects were also confirmed in a mouse model bearing glioma. Furthermore, cell cycle arrest in S phase was observed in Oridonin-mediated growth inhibition by flow cytometry. Cell cycle arrest in S phase led to eventual cell apoptosis, as revealed by Hoechst 33342 staining and annexin V/PI double-staining. The cell apoptosis might be accomplished through a mitochondrial manner. In all, we were the first to our knowledge to report that Oridonin could exert anti-cancer effects on tumor growth in human glioma by inducing cell cycle arrest and eventual cell apoptosis. The identification of Oridonin as a critical mediator of glioma growth may potentiate Oridonin as a novel therapeutic strategies in glioma treatments.

Key words: Oridonin, glioma, growth, cell cycle, apoptosis.

Introduction

Glioma is a brain neoplasm that accounts for 80% of primary tumors in central nervous system (CNS) in the United States (1). Astrocytoma is the largest subset of glioma, comprising of over 50%. The prognosis of glioma is dismal, particularly for patients diagnosed with grade IV astrocytoma (also known as glioblastoma multiforme, GBM). The median survival of patients suffering from GBM is only 14.2 months with the treatments of standard care, which consists of surgical resection, radiotherapy and the alkylating agent temozolomide (TMZ) (2). According to the statistics, 3 out of 100,000 people are suffering from glioma and the 5-year survival rate is approximately 4.7% in the United States. The dismal prognosis of glioma may be due to the inevitability of tumor cell resistance to radiation and chemotherapy (3). Recently, newly developed target therapies are promising and the glioma therapies are also designed which mainly target receptor tyrosine kinases (RTK) signals. Three main classes of drugs have been developed, including vascular endothelial growth factor (VEGF) inhibitors, endothelial growth factor receptor (EGFR) inhibitors, and non-specific tyrosine kinase inhibitors. However, these treatments have fallen short of expectations in mouse models and clinical trials (4-8). These results suggest that exclusively targeting RTK signals may not be so effective to treat glioma. Strategies emanating from other sources need to be tried.

Oridonin is an active component of the Chinese herb *Rabdosia rubescens*. Due to its extensive anti-cancer effects in human cancers, including gallbladder cancer (9), pancreatic cancer (10), lung cancer (11), osteosarcoma (12) and hepatocellular carcinoma (13) et al, Oridonin has been widely studied. Moreover, oridonin also has effects on normal cells. It was reported that Oridonin inhibited hepatic stellate cell proliferation and fibrogeneis by which prevented liver from fibrosis (14). However, researchers have very little knowledge of the role of Oridonin in human glioma. Oridonin has been attached attention because its ability to stimulate cell cycle arrest and apoptosis both *in vitro* and *in vivo*. Various proteins and pathways have been shown to be involved in Oridonin-mediated tumorigenesis, such as nuclear factor-kappa B (NF-κB), cysteine-dependent aspartate-specific proteases (caspase) family, the Bcl-2 family, the mitogen-activated protein kinase (MAPK) family et al (10, 12, 15, 16). However, whether Oridonin could also maintain its cell cycle arrest- and apoptosis-inducing activities remains largely unknown.

In the present study, we aimed to systemically investigate the role of Oridonin in human glioma. The cell proliferation of glioma cell lines treated with different concentrations of Oridonin was initially assessed through the MTT assay and the flat plate-based colony formation assay. Tumor growth was also examined in a

mouse model with intraperitoneal injection of Orodonin (5mg/kg, or 10mg/kg). Cell cycle and cell apoptosis were also included and analyzed in this study.

Materials and methods

Cell lines and cell culture

Human glioma cell lines U87 and U251 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Both cell lines were maintained in Rosewell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY, USA). The culture media were supplemented with 10% fetal bovine serum (FBS, Gibco) and refreshed every two days. Cell culture was maintained in an incubator at 37°C in a humidified atmosphere with 5% CO_2.

Reagents

Oridonin was purchased from Sigma-Aldrich (St. Louis, MO, USA). For *in vitro* studies, oridonin was dissolved in dimethyl sulfoxide (DMSO) to generate a stock solution (0.1 mol/L), which was stored at -20°C. Stock solution was further diluted with culture media to yield the desired oridonin concentrations (2.5, 5, 10, and 20μM/L). For control, cells were also treated with an equal volume of vehicle. For all the cell culture, DMSO concentration was kept lower than 0.1% to avoid any detectable effects on cell growth and cell death.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), annexin V-FITC, Hoechst 33342, and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies against NF-κB, caspase-3, caspase-9, Bax, Bcl-2, PARP-1, cytochrome c, β-actin, and the corresponding secondary antibodies (goat anti-rabbit or goat anti-mouse) were all purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against cyclin A, cyclin B1, and cyclin D1 were commercially purchased from Epitomics (Burlingame, CA, USA).

Cell viability assay

Viability of cells treated with various concentrations of Oridonin was assessed by using the MTT assay. Both U87 cells and U251 cells were seeded into a 96-well plate at a density of 6×10^3 cells/well and cultured in the media containing different concentrations of Oridonin (2.5, 5, 10, and 20μM/L) prior to test. Control cells were treated with DMSO. The culture time was 24, 48, and 72h, respectively. After treatments, a total of 10μl MTT solution (5mg/mL) was added to each well for an incubation of 2-4h under 37°C. At each time point (24, 48, or 72h), the absorbance for each well at 490nm was then measured through a microplate reader (Bio-Tek, USA). Results represented threeindependent experiments.

Colony formation assay

U87 cells and U251 cells were seeded in a 6-well dish in triplicate (500 cells per dish) and allowed to adhere for 12h prior to Oridonin treatments. After adherence, both cell lines were treated with corresponding concentration of Oridonin and incubated at 37°C for 14 days until clones formed. Clones were then washed with PBS twice, fixed with a solution of acetic acid and methanol (1:3) for 15min, and subjected to staining with crystal violet (0.5% crystal violet, 1% paraformaldehyde and 20% methanol in PBS) for 30min. Clone with cell numbers over 50 was considered as a colony. The colonies on each plate were manually counted. Digital images were taken of stained single clones observed under a microscope (Leica, Germany).

Flow cytometry

Cell cycle progression of glioma cells were assessed by flow cytometry. U87 and U251 cells were treated with Oridonin (2.5, 5, 10, and 20μM/L) or vehicle for 48h. After that, cells were washed with cold PBS and fixed with 70% ethanol at 4°C overnight. Cells were then stained with buffer (PBS containing 1 mg/mL PI and 10 mg/mL RNase A; Sigma-Aldrich) at 37°C under darkness for 30 min. Samples were then analyzed with a flow cytometer (BD, San Diego, CA, USA).

Annexin V/PI double staining and Hoechst 33342 morphological staining

Cell apoptosis was analyzed using the annexin V/PI apoptosis kit according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). Briefly, U87 and U251 cells were seeded in a 6-well plate (1×10^6 cells/well) and treated with desired concentrations of Oridonin (according to experimental design) for 48h. The cells were collected after seeding, washed with cold PBS, and were re-suspended in 100μl binding buffer at a concentration of 1×10^6 cells/ml. Thereafter; 5μl of annexin V-FITC together with 5μl of PI working solution (100 μg/ml) were added to the 100μl aliquots of the cell suspension for 15min room temperature incubation. After the incubation under darkness, 400μl of the binding buffer was added to the cell suspension. Samples were then analyzed by flow cytometry. Each sample was tested in duplicate for at least three times.

For nuclei morphological examination, Oridonin-treated and control cells were also performed Hoechst 33342 staining. After 24h cultures, cells from each group were washed with cold PBS twice, followed by fixation in 4% paraformaldehyde for 10min. Thereafter, cells were stained with Hoechst 33342 for 5min and subjected to observation under a fluorescence microscope. Representative images were photographed and apoptotic cells that exhibited condensed, compact and fragmented nuclei were counted and quantified.

Western blot analysis

Total proteins of the sample were extracted 48h after culture. Prior to loading, the concentration of total proteins was determined by using BCA kit (Pierce, Rockford, IL, USA). Equal amounts of proteins were loaded to lanes in a 12% SDS-PAGE gel. Proteins were then transferred to nitrocellulose (NC) membranes (Bio-Rad, Hercules, CA, USA). Thereafter, the membranes were blocked within TBS/0.1% Tween-20 (TBST) containing 5% fat-free milk for 1h, followed by primary antibody incubations overnight at 4°C. After primary antibody incubation, membranes were washed with TBST twice and subjected to incubation with the corresponding secondary antibody at 37°C for 1h. Membranes were then processed for chemiluminescent detection by using an ECL detection kit (Pierce, Rockford, IL, USA). ß-actin was developed for the loading control.

Xenograft model of glioma

Six-week old male athymic nude mice (BALB/$c^{nu/nu}$) (n=18) were used for the current experiment. U87 Cells (1×10^6) were injected intraperitoneally to all the mice. Mice were randomly divided into three groups: vehicle-administrated control group (10% DMSO +90% PBS), 5mg/kg Oridonin-administrated group, and 10 mg/kg Oridonin-administrated group with n=6 for each group. Oridonin was administrated once a day by up to 28 days. Tumor diameters were periodically measured twice a week and tumor volumes (TV) were calculated using the formula as described (17): $TV = (L \times W^2)/2$ L represents the longer dimension, while W represents the short dimension. On day 28, the mice were sacrificed and tumors were dissected, measured and weighed. All efforts were made to minimize the suffering.

Immunohistochemistry (IHC)

For IHC analysis of the tumour tissues from mice, tumour tissues were paraffin-embedded and sliced. Slides were then subjected to antigen retrieval by heating the slides in a microwave at 100°C for 8 min in 0.1mol/L citric acid buffer (PH 6.0), and then incubated with primary antibodies at 4°C overnight. After PBS washes, slides were incubated with corresponding secondary antibodies at room temperature for 1h. Thereafter, the slides were then developed in 0.05% diaminobenzidine (DAB) supplemented with 0.01% hydrogen peroxidase. Representative images were photograghed. For negative controls, specific antibodies were replaced with normal goat serum by co-incubation at 4°C overnight preceding the IHC staining procedure.

Statistical analysis

Data were presented as mean± standard deviation (SD). All data were obtained from at least three independent experiments. Differences between two sample means were analyzed by *Student's* t-test with SPSS v16.0 software. A significance was statistically considered when P value was <0.05.

Results

Oridonin inhibits proliferation of glioma cell lines

To investigate the role of Oridonin in cell proliferation in glioma, U87 cells and U251 cells were cultured and treated with various concentrations of Oridonin (2.5, 5, 10, and 20μM/L). Both cell lines were treated with the desired concentration of oridonin for 24h, 48h, and 72h, respectively. MTT assay was used for determination of cell viability. As shown in Figure 1, both cell lines showed sensitivity to Oridonin. Cell viability was dramatically decreased with the increasing dose of Oridonin. Moreover, under the same concentration of Oridonin, cell proliferation was further inhibited with the prolonged treatment of drug (Figure 1A-1C). At 5μM/L Oridonin, U87 cells were approximately 37% inhibited from proliferations by the time 72h. U251 cells showed slower decreases of cell viability in response to the prolonged treatment of 5μM/L Oridonin (Figure 1D). However, viability of U251 cells was overall lower that the paired U87 cells under four concentrations of Oridonin (Figure 1A-1C). These observations suggest that Oridonin inhibited both cell line proliferations in a dose- and time-dependent manner.

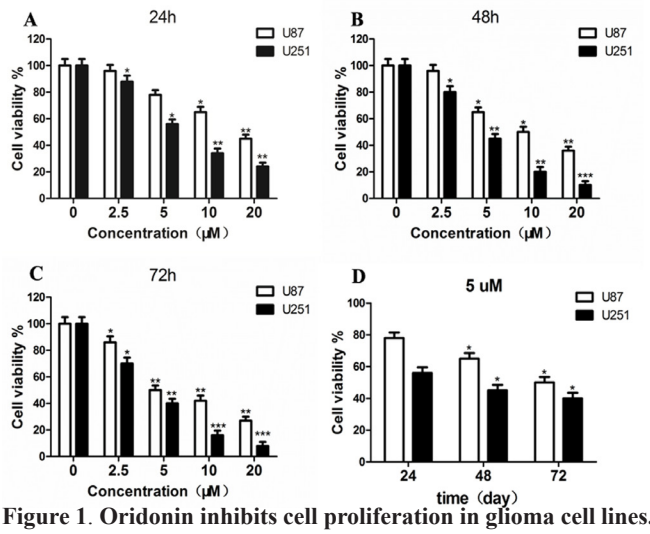

Figure 1. **Oridonin inhibits cell proliferation in glioma cell lines.** **(A, B, C)** U87 cells and U251 cells were cultured and treated with various concentrations of Oridonin (2.5, 5, 10, and 20μM/L). Both cell lines were treated with the desired concentration of oridonin for 24h (A), 48h (B), and 72h (C), respectively. Cell viability was decreased with the raised concentrations and prolonged treatments of Oridonin. (**D**) Under 5μM/L of Oridonin, U87 cells and U251 cells showed increasing sensitivity to Oridonin with the prolonged treatment of drugs (24h, 48h, and 72h). The data were obtained from triplicate independent experiments. *P < 0.05, **P < 0.01, ***P<0.001 *vs*. the control group.

The abilities of glioma cell lines to form colonies under treatments of Oridonin were also assessed by using the flat plate-based colony formation assay (Figure 2A). Colonies were stained by crystal violet. The results showed that Oridonin dose-dependently prevented the presence of colonies in the dishes. Colony counting further showed that U87 cells formed about 270 colonies without any Oridonin treatment. However, only about 40 colonies were formed in the U87 cells under 10μM/L of Oridonin, representing up to 85% inhibition (Figure 2B). Likewise, in U251 cells, colonies were nearly 95% inhibited under 10 μM/L of Oridonin as compared to those without treatments of Oridonin (Figure 2C). Our data suggest that Oridonin may exert a long-term cytotoxic effect to proliferations of glioma cells.

Oridonin induces cell cycle arrest in S phase in glioma cells

To determine whether the proliferation inhibition of Oridonin on glioma cells were mediated by interrupting cell cycle progression, flow cytometry was employed to assess the cell cycle distributions under Oridonin treatments. Oridonin-induced cell cycle changes were shown in Figure 3A (U87 cells) and Figure 3B (U251 cells). In U87 cells, cell proportion in S phase was significantly increased (from 25% to 42%) with a relative deceased cell proportion in G2/M phase, while cell proportion in G1 phase (from 62% to 45%) was dramatically decreased. Consistent with U87 cells, U251 cells were gradually accumulated in S phase (from 20% to 50%), while cells in G1 phase were significantly decreased (from 70% to 42%) with the raised concentration of Oridonin. These data indicate that Oridonin induced cell cycle arrest in S phase. To further confirm these observations, we assessed the expression levels

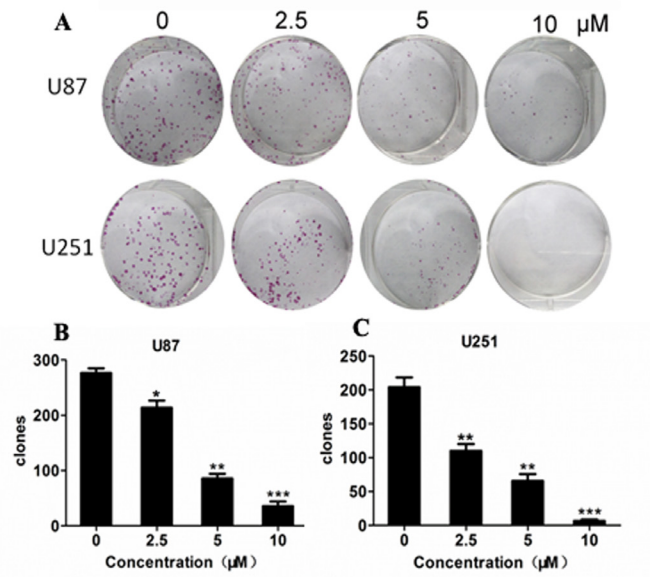

Figure 2. Oridonin inhibited colony formation in glioma cells. (**A**) Crystal violet staining revealed that colonies were dramatically decreased in response to Oridonin treatments in both U87 cells and U251 cells. (**B, C**) statistical analysis showed that U87 cells were approximately 85% inhibited from forming colonies (B), while U251 cells were even up to 95% inhibited (C). *P < 0.05, **P < 0.01, ***P<0.001 *vs*. the control group.

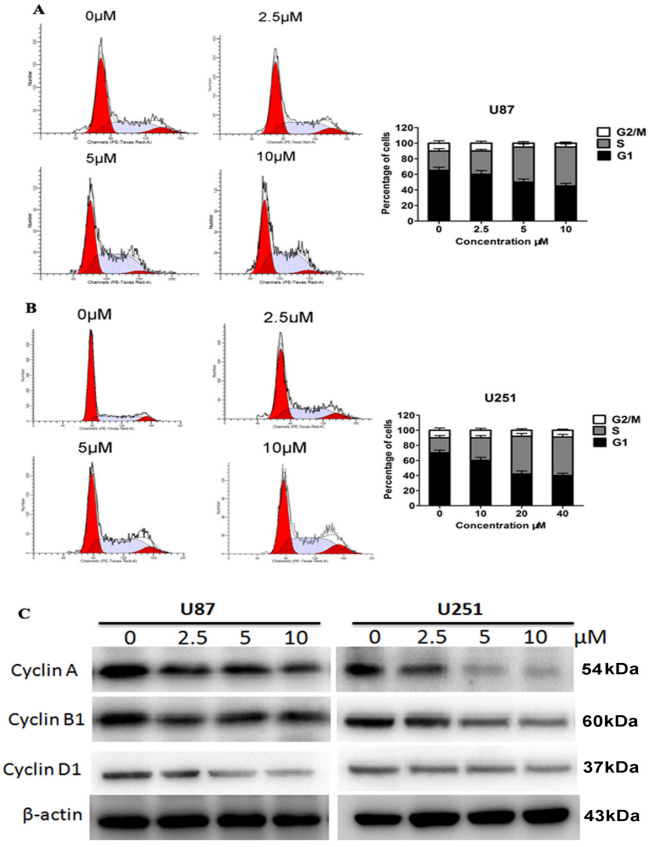

Figure 3. Oridonin induces cell cycle arrest in S phase in glioma cell lines. (**A, B**) flow cytometry was employed to assess the cell cycle distributions under Oridonin treatments. In U87 cells, cell proportion in S phase was significantly increased (from 25% to 42%), while cell proportion in G1 phase (from 62% to 45%) was dramatically decreased. Likewise, U251 cells were gradually accumulated in S phase (from 20% to 50%), while cells in G1 phase were significantly decreased (from 70% to 42%) with the raised concentration of Oridonin. (**C**) Western blot analysis showed that S phase-related Cyclin A1 was decreased after Oridonin treatments. Other cell cycle-related proteins were also correspondingly altered.

of key regulators in cell cycle phases. Consistently, S phase key regulator Cyclin A was decreased in response to Oridonin treatment. Other Cyclins, including G1 phase-related protein Cyclin D1 and G2/M phase-related protein Cyclin B1 were both correspondingly altered under the treatments of Oridonin with both cell lines (Figure 3C).

Oridonin induces cell apoptosis of glioma cells

To further investigate the underlying mechanisms that contribute to Oridonin-mediated growth inhibition, Oridonin-treated U87 cells and U251 cells were subjected to apoptosis assessment by Hoechst 33342 staining and annexin-V/PI double staining. In the morphological Hoechst 33342 staining, both U87 cells and U251 cells exhibited fragmented and condensed nuclei at the presence of Oridonin (Figure 4A, upper panels). Statistical analysis revealed that cell apoptosis induced by Oridonin was dose-dependent. In U87 cells, apoptotic cell proportion raised from 2% in the control cells to 17% under 10μM/L of Oridonin. In U251 cells, apoptotic cells under 2.5μM/L of Oridonin were nearly the same with control cells, but increased dramatically to 17.5%

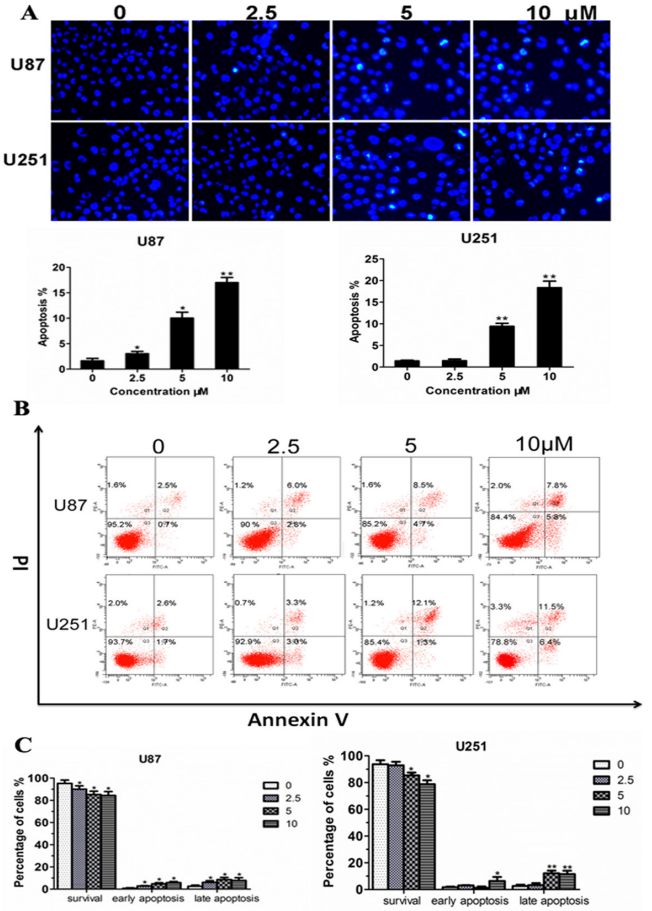

Figure 4. Oridonin induces cell apoptosis in glioma U87 cells and U251 cells. (**A**) Hoechst 33342 staining showed that Oridonin induced fragmented and condensed nucleus in both U87 cells and U251 cells (upper panels). Apoptotic cell proportion in U87 cells raised from 2% in the control to 17% under 10μM/L of Oridonin. In U251 cells, apoptotic cells under 2.5μM/L of Oridonin were nearly the same with control cells, but increased dramatically to 17.5% under 10μM/L of Oridonin (lower panels). (**B, C**) flow cytometry revealed that cell survival rates in both cell lines were dose-dependently decreased, whereas apoptosis were overall increased. *P < 0.05, **P < 0.01 *vs*. the control group.

under 10μM/L of Oridonin (Figure 4A, lower panels).

We further analyzed cell survival, early apoptosis and late apoptosis after treatments of Oridonin to glioma cells (Figure 4B). Early apoptosis is featured with externalization of phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the plasma membrane. The outflows of PS have high affinity for annexin V, making ways for detection of early apoptosis by fluorescent-labeled annexin V. On the contrary, PI could permeate necrotic cells to reveal late stage apoptotic cells. Our results showed that cell survival rates in both cell lines were dose-dependently decreased, whereas apoptosis were overall increased. In U87 cells, cells under early stage of apoptosis were increased with the raised concentration of Oridonin, while cells under late stage of apoptotosis were nearly stable with regard to the three concentrations of Oridonin (2.5, 5, and 10μM/L). In U251 cells, early apoptosis was most prominent when cells were under 10μM/L of Oridonin. And late apoptosis were prominent when cells were treated with 5 and/or 10μM/L of drugs. Taken together, these observations suggest that Oridonin induced significant cell apoptosis in glioma U87 cells and U251 cells.

Apoptosis-related proteins were modulated by Oridonin in glioma cells

It is well-recognized that caspase family, Bcl-2 family, NF-κB and PARP play pivotal roles in the initiation and execution of apoptosis (18-21). We assessed these apoptosis-related proteins expressions with western blot analysis. Our results showed that treatments of Oridonin down-regulated the anti-apoptotic factor Bcl-2 and pro-survival factor NF-κB, whereas up-regulated pro-apoptotic factors cleaved-casepase-3 and cleaved-caspase-9. PARP-1 was also increased in this process. These results confirmed our above conclusion that Oridonin induced cell apoptosis in glioma. Moreover, we further detected that Bax, the pro-apoptotic factor, was up-regulated in mitochondrial and down-regulated in cystol. The

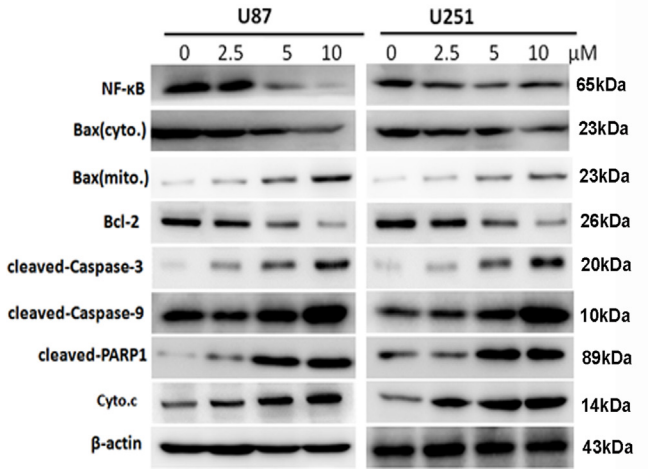

Figure 5. Apoptosis-related proteins were modulated by Oridonin in glioma. treatments of Oridonin down-regulated the anti-apoptotic factor Bcl-2 and pro-survival factor NF-κB, whereas up-regulated pro-apoptotic factors cleaved-casepase-3 and cleaved-caspase-9. PARP-1 was also increased in this process. Moreover, we further detected that Bax mainly re-localized into mitochondrial and cytochrome c in cystol was elevated in response to Oridonin treatments. Mito. is short for mitochondrion, and cyto. is short for cytoplasma. Cyto.c represents cytochrome c.

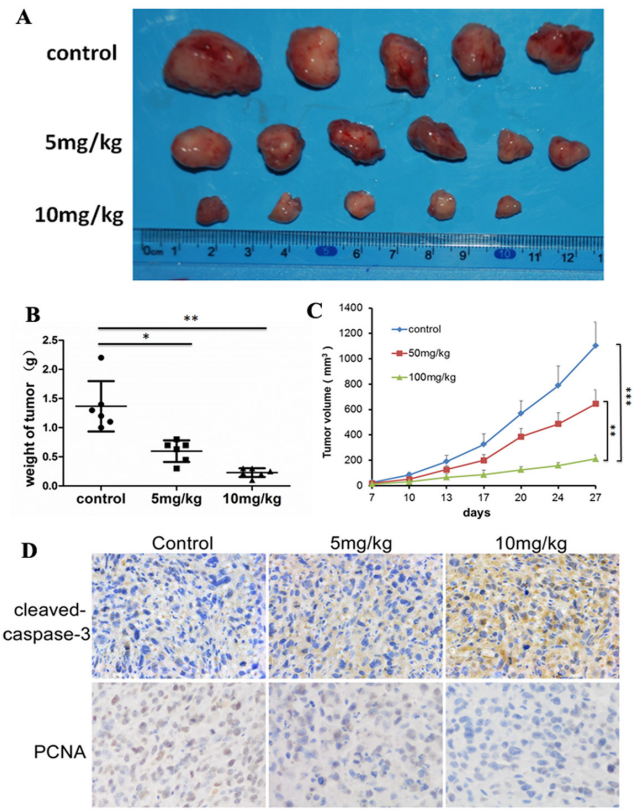

Figure 6. Oridonin inhibits tumor growth in glioma *in vivo*. (**A**) On day 28, tumor volume in Oridonin-administrated groups was significantly smaller than the control group. (**B**) Tumor weights in Oridonin-administrated groups were also prominently decreased as compared with control group. Higher administration of Oridonin had stronger inhibitive effects to tumor volume and tumor weight. (**C**) The periodic monitor of tumor dimensions showed that mice with the Oridonin-administration began to exhibit smaller tumor volume since day 13. On day 27, the mean tumor volume in 5mg/kg Oridonin-administrated mice were approximately 54.5% of that in the control group. And 10mg/kg Oridonin-administrated mice even exhibited 18.2% of that in the control group. (D) IHC analysis showed that cleaved-caspase-3 was elevated in Oridonin-administrated mice groups, whereas the proliferating cell nuclei antigen (PCNA) was decreased. *P < 0.05, **P < 0.01 *vs*. the control group.

re-localization of Bax into mitochondrial is an intrinsic driver of cytochrome c outflow from the mitochondrial to the outer plasma (22, 23). Therefore, we detected cytochrome c in cystol. As expected, cytochrome c in cystol was elevated in response to Oridonin treatments. These data indicated that mitochondrial pathway might be involved in the Oridonin-induced apoptosis.

Oridonin inhibits tumor growth in a mouse xenograft

To determine the effects of Oridonin on tumor growth *in vivo*, we established a mouse model with U87 cells. Mice bearing glioma cells were intraperitonially injected with Oridonin (5mg/kg and 10mg/kg) or vehicle (10% DMSO+90% PBS) each day by up to 28 days. By day 28, a total of 2 mice (one from the control group and the other one from 10mg/kg Oridonin-treated group) were excluded due to death during culture. Tumors were dissected and presented in Figure 6A. On day 28, tumor volume in Oridonin-administrated groups was significantly smaller than the control group. Tumor weights in Oridonin-administrated groups were also prominently decreased as compared with control group (Figure 6B).

Higher administration of Oridonin had stronger inhibitive effects to tumor volume and tumor weight. In addition, the periodic monitor of tumor dimensions showed that mice with the Oridonin-administration began to exhibit smaller tumor volume since day 13. On day 27, the mean tumor volume in 5mg/kg Oridonin-administrated mice were approximately 54.5% of that in the control group. And 10mg/kg Oridonin-administrated mice even exhibited 18.2% of that in the control group (Figure 6C). These results suggest that Oridonin significantly inhibited tumor growth *in vivo*. Furthermore, IHC analysis of the tumor tissues showed that cleaved-caspase-3 was elevated in Oridonin-administrated mice groups, whereas the proliferating cell nuclei antigen (PCNA) was decreased (Figure 6D), indicating the inhibited cell proliferation and eventual apoptosis processes.

Discussion

Glioma is a great threat for human health and accounts for 80% of intracranial tumors (1, 24). Newly developed therapies aim to target oncogenic signals, but unfortunately provide little benefit as regard with glioma patients' clinical treatments. Oridonin is an active diterpenoid compound originally purified from the Chinese herb *Rabdosia rubescens*. This drug has been widely studied due to its extensive pharmacological and physiological functions, such as anti-inflammation and anti-cancer effects (25). Despite the wide reports about the anti-cancer activity of Oridonin in various tumors, whether Oridonin maintains its anti-cancer effects in human glioma remains largely unkown. Based on its extensive roles in human tumorigenesis, Oridonin was focused on in our present study.

In this study, we systemically investigated the growth inhibition role of Oridonin in human glioma. Initially, the *in vitro* MTT assay showed that Oridonin suppressed cell proliferation in a dose- and time-dependent manner in both U87 cells and U251 cells. Both cells were sensitive to Oridonin with a higher sensitivity exhibited to U251 cells. The lethal dose of 50% cells (LD50) in U87 cells was 20μM/L for 24h, or 10μM/L for 48h, or 5μM/L for 72h. In U251 cells, LD50 was 5μM/L for 48h (Figure 1A-1C). Furthermore, we assessed proliferative activities of Oridonin-treated cells with colony formation assay which is closely related to the *in vivo* situation (26). This anchorage-independent assay showed that colony numbers were dramatically decreased with the raised concentrations of Oridonin. To confirm the *in vitro* observations, we established a mouse model bearing human glioma. Mice from each group were administrated Oridonin (5mg/kg, or 10mg/kg) or vehicle (10% DMSO + 90% PBS). Our results showed that both tumor volume and tumor weight in Oridonin-administrated mice were significantly decreased in contrary to the control mice (Figure 6A-6C). Higher dose of Oridonin administration had stronger inhibitive effects on tumor growth *in vivo*. Altogether, the growth inhibition effects of Oridonin in human glioma were concluded through multiple approaches.

In recent years, Oridonin has attracted intense attention for its cytotoxic activity in a wide variety of tumors. These activities were mainly accomplished by inducing cell cycle arrest and apoptosis *in vitro* and *in vivo* (9, 10, 13, 27). In our study, we found that Oridonin inhibited cell proliferation by inducing cell cycle arrest in S phase. In U87 cells, cell proportion in S phase raised significantly from 25% to 42% with cell proportion in G1 phase decreasing from 62% to 45%. Likewise, U251 cells were prominently accumulated in S phase (from 20% to 50%) with a significant drop of cell proportion in G1 phase (from 70% to 42%) (Figure 3A and 3B). The alterations of key cell cycle regulators also verified the above notion (Figure 3C). Furthermore, we found that Oridonin-treated cells exhibited significant fragmented nucleu in morphology and were associated with lower survival rates due to early and late apoptosis (Figure 4). These results indicated that Oridonin might inhibit tumor growth by inducing cell cycle arrest and leading to eventual apoptosis.

Apoptosis is a programmed process with two major pathways contributed: mitochondrial-apoptosome-mediated intrinsic pathway and death receptor-mediated extrinsic pathway (20). In the mitochondrial intrinsic pathway, the pro-survival transcription factor NF-κB plays central role due to its activity to decrease the sensitivity of cells to apoptosis (28). NF-κB inhibits apoptosis by targeting Bcl-2 and regulating other Bcl-2 family members (29). In our study, the down-regulation of NF-κB as well as its target Bcl-2 indicated the activation of mitochondrial-mediated apoptosis. In addition, the mitochondrial-mediated apoptosis is initiated through caspase-9, and subsequent caspase-3, while the death receptor is occurred through caspase-8 (20). Bcl-2 inhibits apoptosis by binding to the outer membrane of the mitochondrion and preventing the release of cytochrome c. Bax antagonizes this process by permeating the mitochondrial membranes and promoting the release of cytochrome c, which leads to the activation of caspase-9 and subsequent caspase-3 (22). Caspase-3 is the key apoptosis executer and cleaved multiple downstream targets, such as PARP-1 (16). Our results showed that cytochrome c and Bax in mitochondrion were increasingly detected with Bcl-2 and Bax in cytoplasma down-regulated in response to the increasing concentrations of Oridonin. Cleaved-PARP-1 was also noted. All these data indicated the activation of mitochondrial-mediated apoptosis. Taken together, our data suggested the involvement of mitochondrial-mediated pathway in Oridonin-induced apoptosis.

In summary, we identified Oridonin as a critical growth inhibitor of glioma cells *in vitro* and *in vivo*. The cytotoxic effect was dose-dependent and was accomplished by inducing S phase arrest and apoptosis. The Oridonin-induced apoptosis might be mediated by mitochondrial pathway. Our data provide evidence for the application of Oridonin as a novel natural anti-cancer therapeutic strategy for the treatment of glioma.

Acknowledgements

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