

Effects of salidroside on glioma formation and growth inhibition together with improvement of tumor microenvironment

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Objective: To test the effects of salidroside on formation and growth of glioma together with tumor microenvironment.

Methods: Salidroside extracted from *Rhodiola rosea* was purified and treated on human glioma cells U251 at the concentration of 20 µg/mL. 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) assay for cytotoxicity and flow cytometry (FCM) for cell cycle analysis were performed. Then for *in vivo* study, xenotransplantation tumor model in nude mice was generated and treated with salidroside at the concentration of 50 mg/kg·d for totally 20 d. Body weight and tumor size were detected every 2 d after the treatment. The levels of 8-isoprostane, superoxide dismutase (SOD) and malondialdehyde (MDA), special markers for oxidative stress, were detected while immunofluorescence staining was performed for astrocyte detection.

Results: For *in vitro* study, salidroside could decrease the viability of human glioma cells U251 and the growth of U251 cells at G₀/G₁ checkpoint during the cell cycle. For *in vivo* study, salidroside could also inhibit the growth of human glioma tissue in nude mice. The body weight of these nude mice treated with salidroside did not decrease as quickly as control group. In the tumor xenotransplantation nude mice model, mice were found of inhibition of oxidative stress by detection of biomarkers. Furthermore, overgrowth of astrocytes due to the stimulation of oxidative stress in the cortex of brain was inhibited after the treatment of salidroside.

Conclusions: Salidroside could inhibit the formation and growth of glioma both *in vivo* and *in vitro* and improve the tumor microenvironment via inhibition of oxidative stress and astrocytes.

Keywords: Glioma; salidroside; cytotoxicity; cell cycle; xenotransplantation; oxidative stress; astrocyte; tumor microenvironment



Submitted Jan 30, 2013. Accepted for publication Mar 04, 2013.

doi: 10.3978/j.issn.1000-9604.2013.10.01

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Introduction

Glioma is a type of tumor mostly in the brain arising from glial cells. It can cause headaches, nausea, vomiting, seizures, and cranial nerve disorders as a result of increased intracranial pressure (1). Gliomas are rarely

curable compared with many other tumors. Glioblastoma multiforme has a worse prognosis with less than a 12-month average survival after diagnosis. Treatment for gliomas is a combined approach of surgery, radiation therapy, and chemotherapy, depending on the tumor location, the cancer

cell type and the grade of malignancy (2). Temozolomide is a chemotherapeutic drug that is able to cross the blood-brain barrier effectively and is currently being used in therapy for high-grade tumors, however, the side effects of temozolomide are serious (3). In our previous research, we found out salidroside could inhibit the growth of human lung adenocarcinoma epithelial cells *in vitro*. Here, we would test the effects of salidroside on the growth of glioma both *in vivo* and *in vitro*.

Salidroside [C₁₄H₂₀O₇, IUPAC: 2-(4-hydroxyphenyl)ethyl-β-D-glucopyranoside], glucoside of tyrosol from *Rhodiola rosea*, is thought to be the most effective compounds responsible for the antidepressant and anxiolytic actions of this plant, even more than rosavin, also found in *Rhodiola rosea* (4). *Rhodiola rosea*, a traditional Chinese herb, belongs to the Crassulaceae family, growing in cold regions and mountains of China. *Rhodiola rosea* has been proved to be effective for neural protection, such as improving mood, alleviating depression, and reducing fatigue (5). It has been proved to be effectively prevented stress-induced changes in appetite, physical activity, weight gain and the estrus cycle. Meanwhile, our former research found out salidroside, extracted from *Rhodiola rosea*, could inhibit the growth of human lung adenocarcinoma epithelial cells. This research mainly focused on the effects of salidroside on the formation and growth of glioma both *in vivo* and *in vitro*. The tumor microenvironment would also be studied.

Materials and methods

Cell culture and drug treatments

Salidroside extracted from *Rhodiola rosea* was purified by high-performance liquid chromatography (HPLC) (ÄKTA, GE Healthcare, USA), then dissolved in 0.9% normal sodium (NS) and stored at 4 °C. Human glioma cells U251 were stored in our lab and propagated at 37 °C with 5% CO₂ in IMDM medium (GIBCO, USA) supplemented with L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% (V/V) fetal bovine serum (FBS) (GIBCO, USA). The salidroside concentration of 20 µg/mL will get a good anti-cancer effect on cells according to our former researches.

3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) assay for cytotoxicity

Viability of U251 cells was determined by MTT (Invitrogen, USA) assay after treatment of salidroside at the

concentration of 20 µg/mL. U251 cells (7×10³/well) were seeded into 96-well plates (Corning, USA). Salidroside was diluted in IMDM/10% FBS. The diluted solution was separately incubated with U251 cells for 24-72 h. At least triplicate measurements were made for each group and time point. At the end of the treatment, 20 µL of 2 mg/mL MTT was added into each well, incubating at 37 °C for 2 h. Then, 100 µL acidic isopropanol was added into each well, and the optical density (OD) at 595 nm was measured using a scanning multiwell spectrophotometer plate reader (Shenzhen Procan Electronic Inc, China).

Cell cycle analysis

U251 cells after treatment of salidroside at the concentration of 20 µg/mL for 24-72 h were seeded in 6-well plates (Corning, USA) at the density of 1×10⁵/mL. Tumor cells were trypsinized, centrifuged (1,000 r/min, 10 min), washed with 1× PBS (pH 7.4), and fixed with cold 70% ethanol/30% phosphate-buffered saline at 4 °C overnight. Cells were digested by 1,000 U RNase A (Invitrogen, USA), and then stained with 1% propidium iodide at 37 °C for 30 min. The DNA profiles were determined within 4 h of staining by flow cytometry (FCM) (EPICS® ALTRA™, Beckman, USA). The results would be divided into G₀/G₁, S, and G₂/M.

Generation of xenotransplantation tumor model and treatment of drug

Human glioma tissue got from clinical operation was cut into 3 mm × 3 mm × 3 mm and then rinsed in DMEM (GIBCO, USA). Male nude mice (2-month old, C57/B6 genetic background) were selected and raised at temperature of 24±2 °C and humidity of (55±15)%. These xenotransplants were subcutaneously injected into the right lower limbs of these mice. After the average tumor size was more than 150 mm³, half of these nude mice were treated with salidroside (intraperitoneal injection) at the concentration of 50 mg/kg·d according to the former research for totally 20 d.

Body weight and tumor size detection

The body weight of these nude mice was weighed every 2 d after the treatment of salidroside. Mice with loss of weight more than 40% would be sacrificed. Tumor size would be determined every 2 d after the treatment of salidroside, together with the body weight detection. Tumor size would be valued as $V = \pi/6 \times a \times b^2$ (a: major axis; b: minor axis).

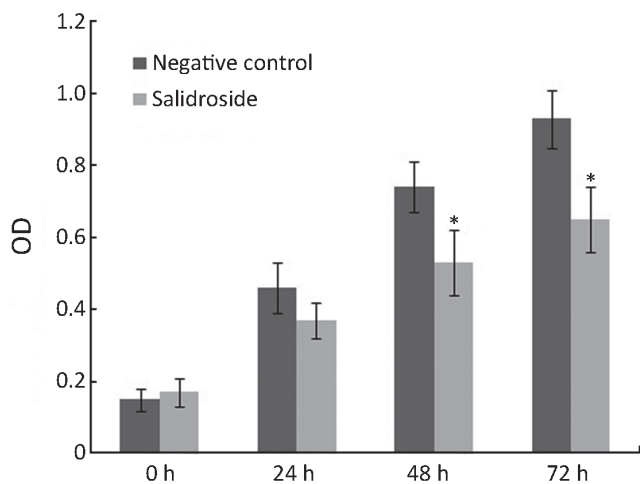


Figure 1 Cell viability test by MTT. *, $P < 0.05$, compared with the negative control group.

Mice with tumor size more than $2,000 \text{ mm}^3$ would be sacrificed. For each group, 10 mice would be used and tested.

Detection of oxidative stress

The cerebral cortex was grinded in tris-buffered saline (TBS) containing AEBSF protease inhibitors (1:1,000), centrifuged at 14,000 r/min for 30 min at 4°C . Supernate was transferred into tubes. The levels of 8-isoprostane, superoxide dismutase (SOD) and malondialdehyde (MDA), special markers for reactive oxygen species (ROS), were determined in the cortex of mice brain in control group and salidroside groups. The isoprostanes were extracted from homogenates of the hemisphere of individual mouse, pulled together and determined using LC/MS/MS method. SOD and MDA extracted from homogenates of the hemisphere were detected using kits (Biobox Company, China). SOD were determined using xanthine oxidase (XO), while thiobarbituric acid (TBA) for MDA.

Immunofluorescence staining detection for astrocytes

Brains of these xenotransplanted nude mice were detected by immunofluorescence staining for the expression of astrocytes. Cryostat mouse brain coronal sections ($16 \mu\text{m}$) were prepared and stained with glial fibrillary acidic protein (GFAP) antibody (Dako, Denmark, 1:1,000) to assess for proliferation of astrocytes, one of the most important glioma cells following protocol. All slides were stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen,

Table 1 Cell cycle analysis by FCM

Time of treatment (h)	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
0 (NC)	59.6±4.0	33.4±3.2	12.9±1.5
12	62.4±2.2*	23.6±1.6	14.0±1.1
24	65.8±3.0*	23.8±1.1	10.4±2.4
36	66.3±1.8**	22.3±2.2	12.3±1.6

*, $P < 0.05$; **, $P < 0.01$, compared with 0 h (negative control).

USA, 1:2,000) for the cell nuclei. Images were taken using confocal microscopy (Zeiss, Germany, 50×).

Data analysis

Each assay was carried out for not less than 3 times. Data were presented as $\bar{x} \pm s$, and Student's *t*-test was used to evaluate the changes between salidroside group and negative control group. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA), and $P < 0.05$ (*) and $P < 0.01$ (**) were considered statistically significant.

Results

Cell viability

As shown in *Figure 1*, salidroside showed significant cytotoxic effect and cell growth inhibition on U251 cells. Compared with control group, the growth and viability of U251 cells treated with salidroside for 24-72 h were significantly inhibited at the concentration of $20 \mu\text{g/mL}$. These results indicated that salidroside had cytotoxicity on human glioma cells U251 for *in vitro* study.

Cell cycle analysis

As shown in *Table 1*, salidroside could inhibit the growth of U251 cells, and induce G₀/G₁ phase arrest. Compared with control group, the percentage of cells at G₀/G₁ phase increased, while the percentage in other phases decreased. The results indicated that salidroside could arrest the cell growth at G₀/G₁ phase.

Body weight and xenotransplanted tumor size of nude mice

As shown in *Figure 2*, the body weight of nude mice was decreased in control group due to the quick growth of xenotransplanted tumor. These nude mice seemed to be weak because of lack of nutrition. However, the body weight

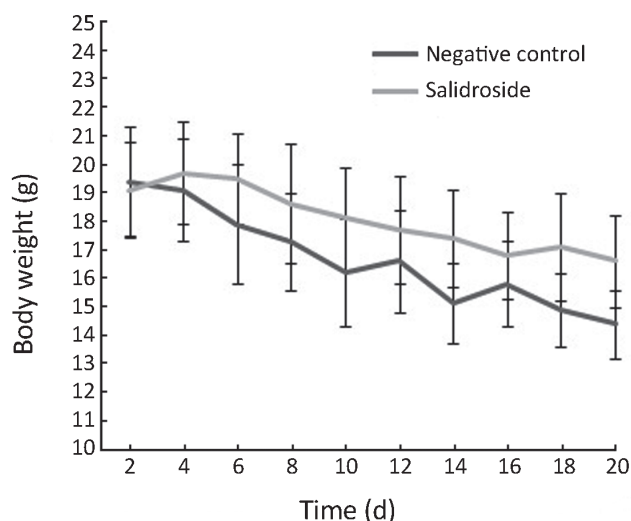


Figure 2 The body weight of nude mice (n=10).

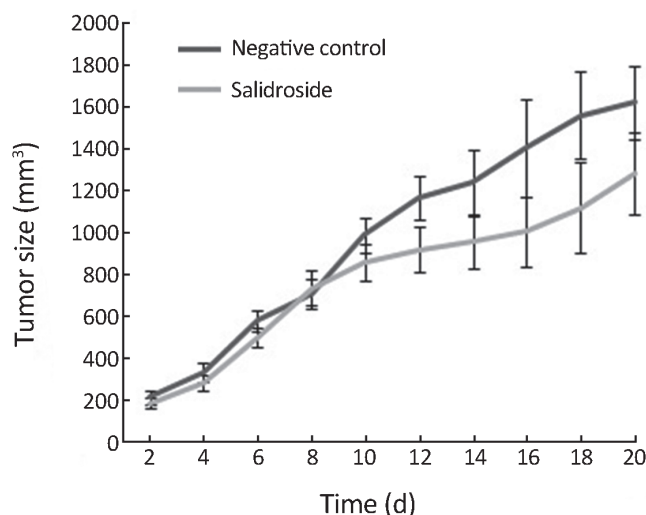


Figure 3 The size of xenotransplanted tumor in negative control group and salidroside group (n=10).

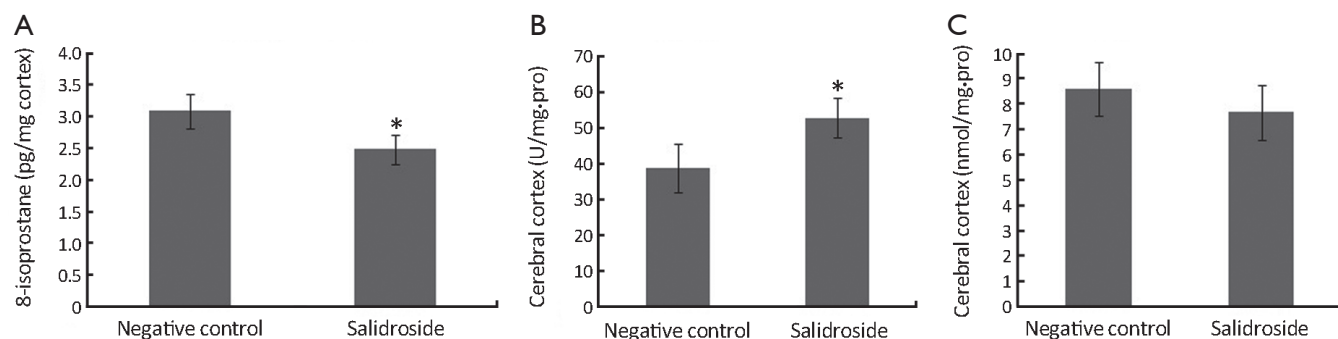


Figure 4 Analysis on the ROS relative enzymes in the cortex of nude mice brain. (A) 8-isoprostane; (B) SOD; (C) MDA. *, P<0.05, compared with the negative control group.

of nude mice was decreased much slower in salidroside group compared with control group due to the slow growth of tumor. Meanwhile, the physical and mental status in salidroside group seemed to be better than control group. As shown in *Figure 3*, the xenotransplanted tumor of nude mice in control group grew quickly without any treatment. The uncontrolled growth of tumors would expend the nutrition and make the mice weak. However, the tumors of nude mice in salidroside group grew much slower than those in control group. This indicated that salidroside could inhibit the growth of human glioma *in vivo*.

Analysis on oxidative stress

As shown in *Figure 4A*, increased concentration of

8-isoprostane was detected in control group since the stimulation of xenotransplants. This increased oxidative stress could in turn make the tumor microenvironment even worse. However, the concentration of 8-isoprostane in salidroside group was significantly lower than that in control group. Decreased SOD was found in salidroside group compared with control (*Figure 4B*) while no obvious change was found for MDA (*Figure 4C*). These results indicated that salidroside could down-regulate the level of oxidative stress *in vivo*.

Formation and growth of astrocytes

The formation and growth of astrocytes could be stimulated by many factors such as tumor, oxidative stress, cytokines,

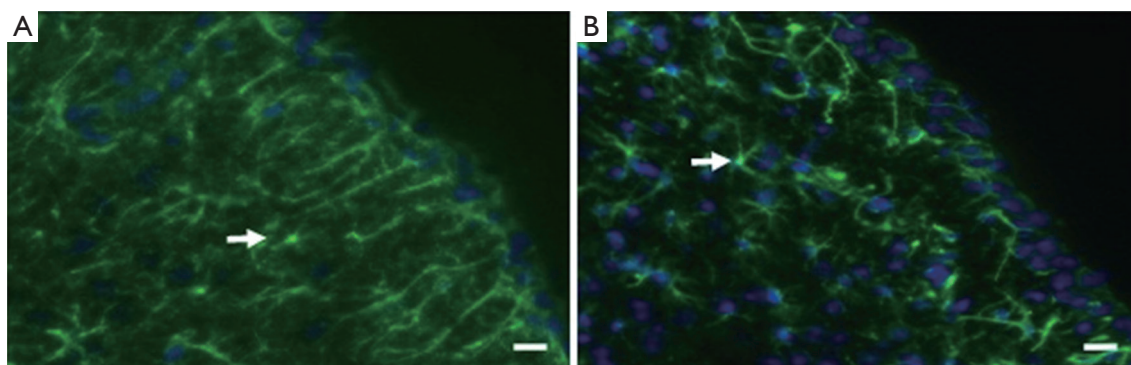


Figure 5 Growth of astrocytes (arrow indicated) near the ventricle of nude mice brains (200×). (A) Overgrowth of astrocytes in negative control group; (B) Inhibition of overgrowth of astrocytes in salidroside group. Bar, 50 μ m.

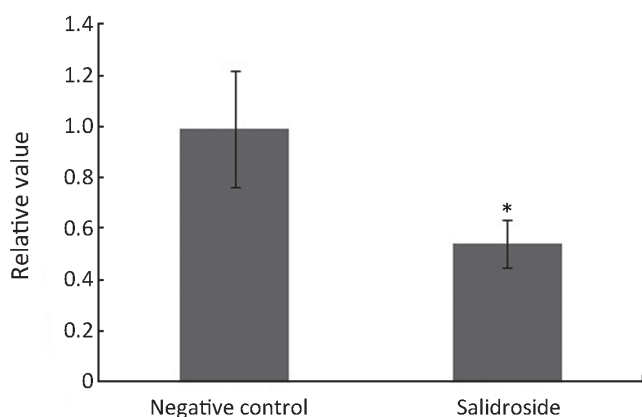


Figure 6 Analysis on formation of astrocytes. *, $P < 0.05$, compared with the negative control group.

and steroid. As shown in *Figure 5*, increased formation and growth of astrocytes were detected in control group. These increased astrocytes could overgrow and then form into tumor if the microenvironment went even worse, which was called astrocytoma. However, the formation and growth of astrocytes were controlled and decreased significantly in salidroside group compared with control group (*Figure 6*). This result indicated that salidroside could regulate the formation and growth of astrocytes, and make them grow normally *in vivo*.

Discussion

Glioma is a type of primary central nervous system (CNS) tumor arising from glial cells, and mostly occurred in the brain. Glioma is the most common and serious form

of brain tumors that affect human adults (6). There are several kinds of gliomas: astrocytomas, ependymomas, and oligodendrogliomas. The advanced astrocytoma is called glioblastoma, accounting for 23% of all primary brain tumors (7). Brain tumors are difficult to treat. One treatment is external beam radiation. Another treatment is surgical removal of the tumor, if possible, followed by chemotherapy. All of these treatments are difficult to go through, and pose risks to the patient. Unfortunately, many gliomas recur even after treatment (8), and finding a useful chemical compound for gliomas treatment is urgent. Salidroside, glucoside of tyrosol extracted from *Rhodiola rosea*, is thought to be the one of the potential compounds for human tumor treatment.

Cytotoxicity analysis is widely used in the pharmaceutical industry for screening compounds. Cytotoxicity can be monitored using MTT assay (9). Salidroside showed cytotoxic effect and cell growth inhibition on U251 cells. The results indicated that the cell cycle of U251 cells would be affected. The cell cycle can be divided in two periods: interphase, during which the cell grows, accumulating nutrients needed for mitosis and duplicating its DNA, and the mitosis phase (M), during which the cell splits itself into two distinct cells (10). Salidroside could arrest the growth of U251 cells at G_0/G_1 phase.

Xenotransplantation of tumor into nude mice, which lack of immunity, is one of the useful and effective methods for valuing the toxicity of compound on formation and growth of tumor (11). The body weight and the size of xenotransplanted tumor are the most important data for detection (12). In this study, the body weight of nude mice was decreased in control group due to the quick growth of xenotransplanted tumor. These nude mice seemed to be

weak because of lack of nutrition. However, the body weight of nude mice decreased much slower in salidroside group due to the slow growth of tumor. Meanwhile, the physical and mental status of mice in salidroside group seemed to be better than those in control group. The xenotransplanted tumor in control group grew quickly without any treatment. The uncontrolled growth of tumors would expend the nutrition and make the mice weak. However, the tumors of nude mice in salidroside group grew much slower than those in control group. This study indicated that salidroside could inhibit the growth of human glioma *in vivo*.

The formation and growth of tumor depend on microenvironment. The metabolic environment of tumor is directly affected by lack of vasculature and subsequent oxygen starvation (13). Tumor microenvironment affects the status and treatment of cancers (14). While the normal cellular microenvironment can inhibit malignant cell growth, the modifications that occur in the tumor microenvironment synergistically support cell proliferation. Tumors shape their microenvironment and support the development of both tumor cells and non-malignant cells (15).

Oxidative stress is one of the most important factors involved in formation and growth of tumor. Oxidative stress means an imbalance between the systemic manifestation of ROS and ability to readily detoxify the reactive intermediates or to repair the resulting damage (16). Oxidative stress can cause disruptions in normal mechanisms of cellular signaling. In humans, oxidative stress is thought to be involved in the development of many diseases or may exacerbate their symptoms including human tumors (17). High level of ROS is often found in cancer cells or tumor tissues. In this research, we found that salidroside could inhibit the level of ROS by detecting several biomarkers of oxidative stress, 8-isoprostane, SOD and MDA. 8-isoprostane, a prostaglandin (PG)-F₂-like compound belonging to the F₂ isoprostane class, is produced *in vivo* by the free radical-catalyzed peroxidation of arachidonic acid. SOD could catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. They are an important antioxidant defense. 8-isoprostane was decreased while SOD increased in salidroside group compared with control group. MDA seems to change little. These results showed salidroside could down-regulate the high and imbalanced condition of oxidative stress.

Astrocytes, star-shaped glial cells in the brain and spinal cord, are the most abundant cells of the human brain (18). They perform many functions, such as biochemical support, provision of nutrients, maintenance of extracellular ion balance, and repairing and scarring of the brain and

spinal cord following traumatic injuries, antioxidant and bioenergetic coupling (19,20). Although these important functions, astrocytoma is primary intracranial tumor derived from astrocytes (21). Overgrowth of astrocytes may promote astrocytoma, which is a dangerous glioma in the brain. In this research, we found that salidroside could inhibit the overgrowth of astrocytes partly due to the oxidative stress, making the formation and growth of astrocytes return to normal.

In general, our research showed salidroside could inhibit the formation and growth of glioma both *in vivo* and *in vitro*, together with improvement of tumor microenvironment, and making oxidative stress and astrocytes return to normal. The conclusions of *in vivo* and *in vitro* study could support each other. Both protection on CNS (22) and inhibition on glioma of salidroside were confirmed. All these suggested that salidroside may be one of the potential compounds responsible for the human tumors, including glioma.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81141080) and Jiangsu Provincial Natural Science Foundation (SBK201340596).

Disclosure: The authors declare no conflict of interest.

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Cite this article as: Zhang Y, Yao Y, Wang H, Guo Y, Zhang H, Chen L. Effects of salidroside on glioma formation and growth inhibition together with improvement of tumor microenvironment. *Chin J Cancer Res* 2013;25(5):520-526. doi: 10.3978/j.issn.1000-9604.2013.10.01