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Original Article

The treatment efficacy of asiaticoside in brain glioma

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Abstract: Brain glioma has a relative higher incidence rate. Current treatments include surgical resection and chemo-/radio-therapy. High recurrent rate and unfavorable prognosis, however, still remained and called for further studies on the proliferation and invasion of glioma cells. As one triterpenes compound, asiaticoside has been shown to inhibit the proliferation of tumors. Its function in glioma, however, still remains unclear. The effects of asiaticoside on the proliferation, cell migration and invasion of rat glioma cell line C6 were tested by MTT, scratch assay and Transwell approach. The C6 cells were inoculated to the caudate nucleus to establish the animal model and different concentrations of asiaticoside were given via intragastric inhalation. Western blotting was also performed to test the expression of cell proliferation and differentiation related proteins including proliferating cell nuclear antigen (PCNA), glial fibrillary acidic protein (GFAP) and CD40. Asiaticoside can inhibit the proliferation, migration and invasion of tumor cells. It can also decrease the expression of PCNA and CD40 in animal models, thus inhibiting tumor cell proliferation, enhancing GFAP expression, and suppressing tumor growth. Asiaticoside can inhibit the growth and proliferation of brain glioma cells, with detailed mechanisms remained to be further elucidated.

Keywords: Asiaticoside, brain glioma, proliferation, migration, invasion

Introduction

As the most common intracranial tumor, brain glioma accounts for about 46% of total brain tumors [1]. The average age of tumor onset is between 45-55 years old. In clinics, most tumors were managed by surgical resection, radio- or chemo-therapy. However, the potent invasiveness and higher recurrence rate of glioma lead to unfavorable prognosis [2]. Therefore, the management of tumor proliferation and invasion is now one research focus currently [3].

As one triterpenes compound, asiaticoside (see **Figure 1** for molecular structure) is extracted from *Centellaasiatica (L.) Urban* [4]. It has multiple drug usages including facilitating healing of wounds, treating scars, anti-tumors, preventing breast gland hypertrophy, protecting gastric mucosa and anti-depression/anxiety [4]. Currently the study about asiaticoside mainly focuses on the wound healing and eliminating scars. The study of its anti-tumor potency has drawn few research interests. It has been reported that asiaticoside can induce the apoptosis of glioma cell line 132N1 (grade II glioma), SW1783 (grade III) and LN18 (grade IV) and inhibit tumor cell proliferation [5]. The role of asiaticoside on the proliferation, migration and invasion of rat glioma cells C6, however, is still unknown.

We thus performed both *in vitro* and *in vivo* studies to elucidate the inhibitory effect of asiaticoside on glioma cells. In cultured rat glioma cells C6, MTT assay was used to determine the effect of asiaticoside on cell proliferation, while the pattern of cell migration and invasion were examined by scratch assay and Transwell method, respectively. Rat glioma model was established upon C6 cell injection. The effect of asiaticoside on tumor size was observed, along with Western blotting assay for expression of proliferating cell nuclear antigen (PCNA), glial fibrillary acidic protein (GFAP) and CD40, all of which are correlated with tumor proliferation, migration and differentiation.

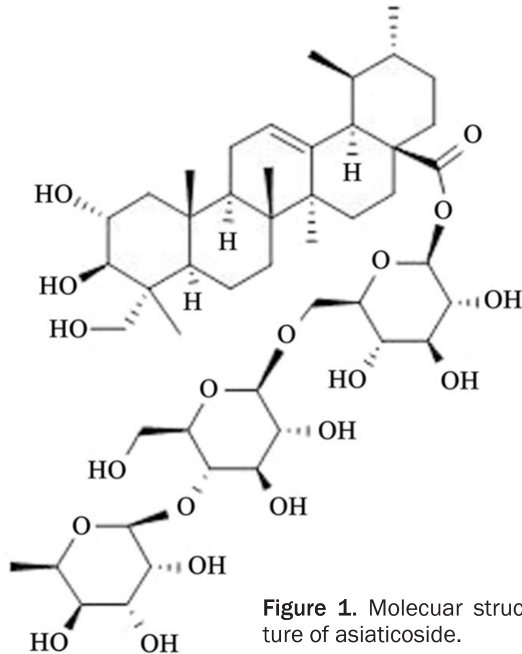

Figure 1. Molecuar structure of asiaticoside.

Materials and methods

Drugs and reagents

Asiaticoside was purchased from Aladdin (China) with >98% purity. MTT reagent was purchased from Sigma (US). DMEM, bovine serum albumin and dual-antibiotics were products of Gibco (US). Transwell chamber was provided by Costar Cambridge (US). Matrigel gel was purchased from BD (US). Rat glioma cell line C6 was obtained from Cell Bank of Chinese Academy of Sciences (China). Rabbit anti-rat PCNA and GFAP monoclonal antibodies were purchased from Abcam (US), while rabbit anti-rat CD40 monoclonal antibody was produced by BioVision (US). Goat anti-rabbit IgG (H+L) was produced by Proteintech (China).

Cell culture

C6 cells were cultivated in DMEM medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified chamber at 37°C with 5% CO_2. Medium was changed every other day.

MTT assay

C6 glioma cells at log-phase were digested in 0.25% trypsin and re-suspended. Cells were seeded into 96-well plate (0.1 mL per well). After attachment of cells, gradient concentrations of asiaticoside (0, 25 μM, 50 μM and 100 μM) were added into each group (N=8). After 24-hour incubation, 10 μL of MTT reagents were added for another 4 hours' incubation until the formation of violet crystal. The supernatants were discarded, with the addition of 0.15 mL reagents for resolving the crystal. After 12 hours, the absorbance value at 490 nm was measured to calculate cell viability.

Scratch assay

C6 glioma cells at log-phase were digested in 0.25% trypsin and re-suspended. Cells were seeded into 6-well plate (1 mL per well). After reaching confluence, parallel lines were drawn by sterilized pipette tips. After PBS washing, cells were incubated with gradient concentrations of asiaticoside (0, 25 μM, 50 μM and 100 μM) for 24 hours. Cell migration was observed under an inverted microscope.

Transwell invasion assay

Transwell chamber was firstly pre-coated with Matrigel on its membrane. The chamber was dried in a 37°C chamber for 30 min. C6 glioma cells at log-phase were digested in 0.25% trypsin and re-suspended. Cells were seeded into 24-well plate containing Transwell chamber (0.2 mL per well). The lower chamber was filled with 0.5 mL medium containing chemotactic factors. Gradient concentrations of asiaticoside (0, 25 μM, 50 μM and 100 μM) were applied to cells for 24 hours. The membrane was cleaned to remove remaining cells. The chamber was fixed and stained in hematoxylin. Under an inverted microscope, the number of perforated cells in each membrane was calculated from three randomly selected fields.

Animal model

A total of 40 SPF-grade SD rats (body weight 160 to 180 g) were provided by Laboratory Animal Institute, Chinese Academy of Science. C6 glioma cells were inoculated as previously documented [6]. In brief, Using stereotaxic apparatus, the rat skull was drilled for inserting microinjection needle and then 25 μL C6 glioma cell suspensions were injected into the caudate nucleus within 10 min. After injection, the needle was remained for 10 min, followed by slow retract. The bone hole was sealed by wax. Then the skin was sutured and prophylactic antibiotics were used.

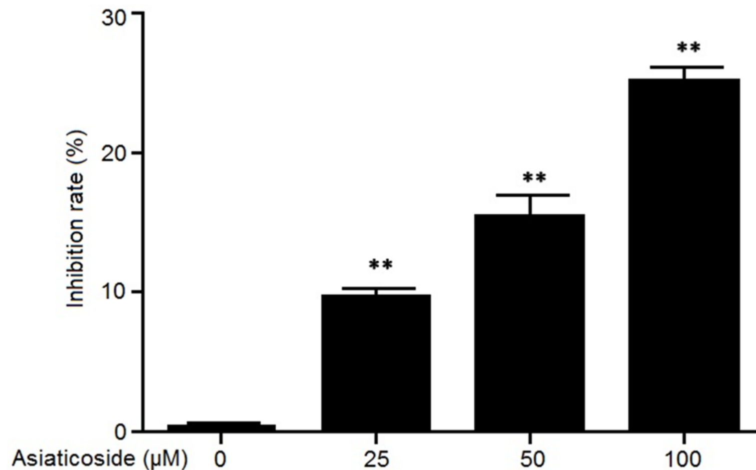

Figure 2. Inhibition of glioma cell proliferation. **P<0.05 compared to control group.

Statistical analysis

All experimental data were collected from triplicate independent studies, and presented as mean ± standard deviation (SD). Student *t*-test was used to compare means between groups. One-way analysis of variance (ANOVA) was employed to compare multiple groups, followed by SNK comparison. A statistical significance was defined when P<0.05.

Animal grouping and treatment

All model animals were randomly divided into 4 groups (N=10 for each): control, low dosage (50 mg/kg asiaticoside), moderate dosage (100 mg/kg asiaticoside) and high dosage (200 mg/kg asiaticoside) group. The drug was applied via gastric intubation at five days after tumor cell inoculation for 15 consecutive days. Animals were then sacrificed.

Determination of tumor inhibition rate

After sacrifice, tumor tissues were removed to measure the tumor size according to previous methods [7]. The tumor inhibition rate was calculated comparing to the control group.

Western blotting

Tumor tissues were lysed in lysis buffer (containing proteinase inhibitor, PMSF and phosphatase inhibitor). After homogenization, the lysate was centrifuged at 14000 g for 10 min. The supernatant was collected and mixed with loading buffer for heating denature. The sample was separated by SDS-PAGE and transferred to PVDF membrane. After blocking in defatted milk powder for 1 hour, primary antibodies against PCNA, GFAP, CD40 or GAPDH were used for overnight incubation. On the next day, the membrane was rinsed in TBS for three times, followed by the addition of secondary antibody and further incubation. ECL reagents were used to develop the membrane.

Results

Glioma cell proliferation was inhibited by asiaticoside

Using MTT to detect the effect of asiaticoside on glioma cell proliferation, we found significantly depressed cell proliferation induced by asiaticoside in a dose-dependent manner (0.68±0.21 for 0 μM, 9.98±1.28 for 25 μM, 16.10±1.96 for 50 μM, 25.60±1.55 for 100 μM) (P<0.05, **Figure 2**).

Asiaticoside inhibited cell migration

The scratch assay revealed inhibitory effect of asiaticoside on glioma cell migration (**Figure 3**).

Asiaticoside depressed cell invasion

Transwell assay showed significantly decreased number of cells that perforated basal membrane. The decrease of invasiveness is of dose-dependent manner (68.6±5.6 for 0 μM, 52.6±3.1 for 25 μM, 28.5±2.6 for 50 μM, 23.1±3.6 for 100 μM) (**Figure 4**).

Asiaticoside suppressed tumor growth

In all groups of rats, solid tumors in the caudal nucleus region were observed. The tumor has irregular round or oval shape, with clear boundary and no capsule (**Table 1**).

PCNA expression

As one indicator for reflecting cell proliferation status, PCNA expression was significantly down-regulated by asiaticoside (0.69±0.04 for 25 μM, 0.53±0.03 for 50 μM, 0.23±0.06 for 100 μM) compared to control (1.02±0.08) (P<0.05, **Figure 5**).

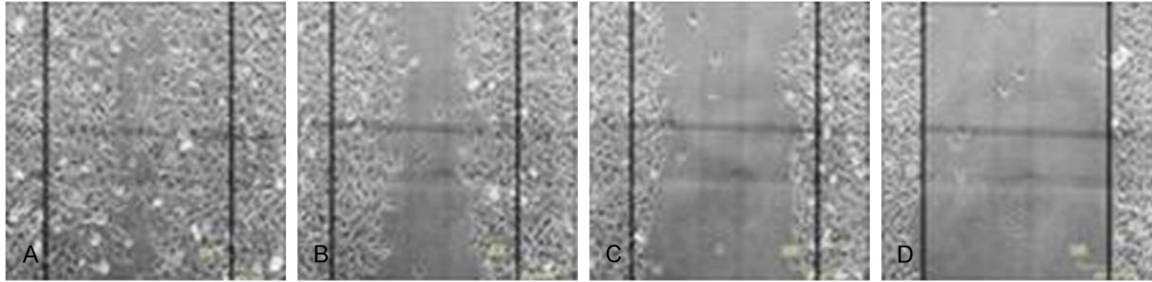

Figure 3. Glioma cell migration. A. Control group; B. Asiaticoside (25 μM); C. Asiaticoside (50 μM); D. Asiaticoside (100 μM).

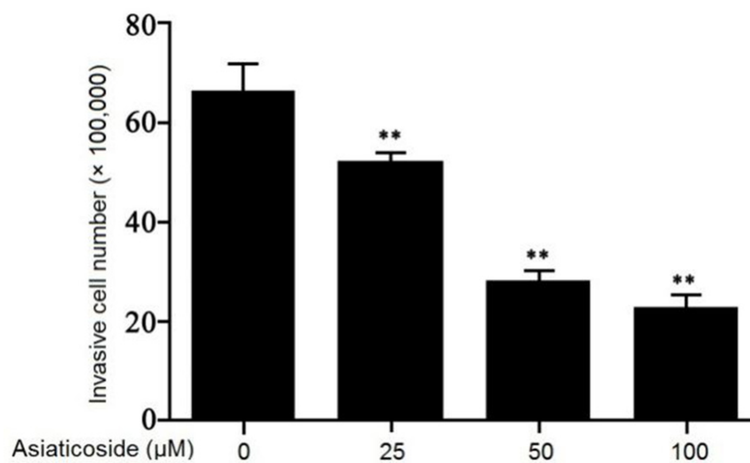

Figure 4. Cell invasiveness. **P<0.05 compared to control group.

Table 1. Asiaticoside and tumor size (N=10)

Group	Tumor size (mm^3)	Inhibition rate (%)
Control	162.1±31.8	-
Asiaticoside (100 mg/kg)	132.3±21.5**	18.38
Asiaticoside (200 mg/kg)	100.3±18.7**	38.12
Asiaticoside (400 mg/kg)	66.5±19.3**	58.98

**P<0.05 compared to control group.

GFAP expression in glioma tissues

Compared to control group, GFAP expression level was up-regulated by asiaticoside in a dose-dependent manner (1.01±0.05 for 0 μM, 1.68±0.13 for 25 μM, 1.99±0.15 for 50 μM, 3.72±1.09 for 100 μM) (**Figure 6**).

CD40 expression in rat glioma tissues

CD40 is expressed in both membrane and cytoplasm of glioma cells. After treated with asiaticoside, the average bind density was lowered (0.49±0.05 for 25 μM, 0.27±0.02 for 50 μM, 0.18±0.04 for 100 μM) compared to control group (1.01±0.05) (P<0.05, **Figure 7**).

Discussion

Brain glioma has a high incidence among all intracranial tumors. It is commonly treated by the combination of surgical and chemo-/radio-therapy [8]. Due to its rapid proliferation and high invasiveness, glioma is predisposed to have recurrence. Therefore, the inhibition of proliferation, migration and invasion of glioma cells is of critical importance. Asiaticoside is one triterpenes compound extracted from *Centellaasiatica (L.) Urban*. Studies have suggested its inhibitory role in tumor proliferation and migration. Its effect on glioma, however, lacked systemic studies.

This study mimicked the inhibitory effect of asiaticoside on brain glioma at both *in vivo* and *in vitro* levels. At cellular level, asiaticoside can inhibit C6 glioma cell proliferation, migration and invasion in a dose-dependent manner. The metastasis of tumor is a complicated process including the detachment of tumor cells from original place toward circulation by penetrating basal tissue and vascular walls [9]. We also employed Transwell chamber to observe the effect of asiaticoside on tumor invasion, which mimicked the *in vivo* tumor metastasis. In animal studies, the size of tumor at caudal nucleus can be inhibited by asiaticoside. The expression of PCNA and CD40 were depressed while

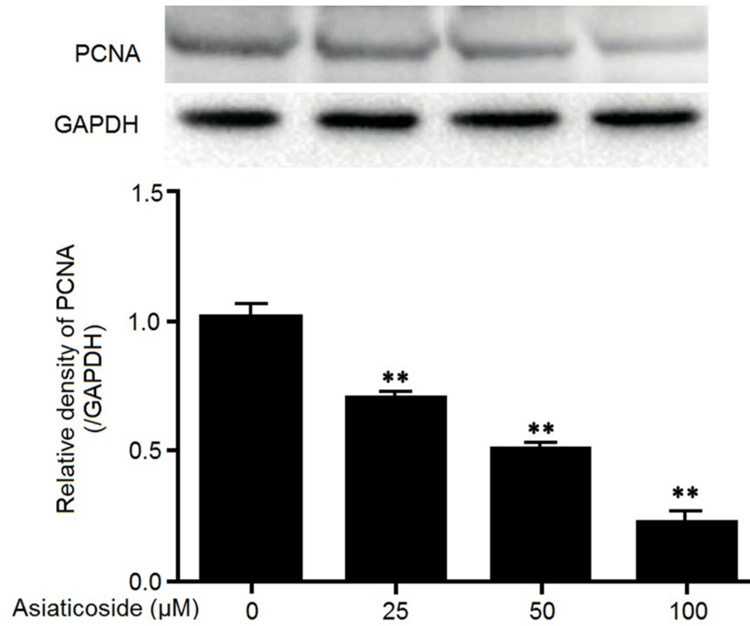

Figure 5. PCNA expression and asiaticoside dosage. **P<0.05 compared to control group.

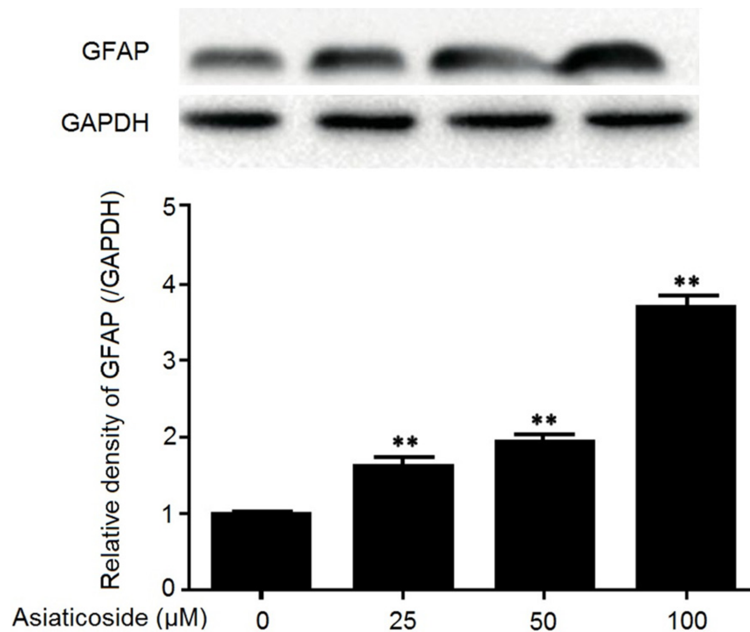

Figure 6. GFAP expression and asiaticoside dosage. **P<0.05 compared to control group.

GFAP expression was enhanced, suggesting satisfactory inhibition on tumor proliferation, angiogenesis and the induction of cell differentiation.

We also investigated the drug effect in model animals. Two weeks after inoculation, further symptoms including congestion peripheral around the eye and even paralysis. All these symptoms mimicked clinical manifestations of glioma.

There have been studies showing the inhibition of tumor growth both *in vitro* and *in vivo* by triterpenes compound extracted from *Centellaasiatica (L.) Urban* while not affecting normal lymphocytes to elongate the survival time of mice by inhibiting DNA synthesis [10]. Other study also showed the significant elevation of intracellular calcium concentration by asiaticoside [11], as well as the lowering of mitochondrial membrane potential to induce cell apoptosis [12]. This study thus examined the expression levels of PCNA, GFAP and CD40 to explore the treatment efficacy of asiaticoside on brain glioma.

PCNA is one effective indicator reflecting cell proliferation status [13] and plays one important role in initiating cell proliferation. The dose-dependent inhibition of PCNA by asiaticoside suggested the inhibition on cell proliferation status. GFAP is one cytoskeleton structural protein that mainly exists in astrocytes [14]. Due to its wide distribution in both cytoplasm and perinuclear regions, GFAP is believed to be one optimal tumor marker for astrocytes [15]. GFAP up-regulation can affect tumor cells to induce cell differentiation [16]. GFAP positive expression commonly reflects the maturation of tumor cells and good differentiation [17]. CD40 signal plays important roles in multiple steps of tumor pathogenesis, in addition to *de novo* angiogenesis [18]. Previous study has shown the inhibition of tumor angiogenesis and metastasis by blocking CD40 signaling

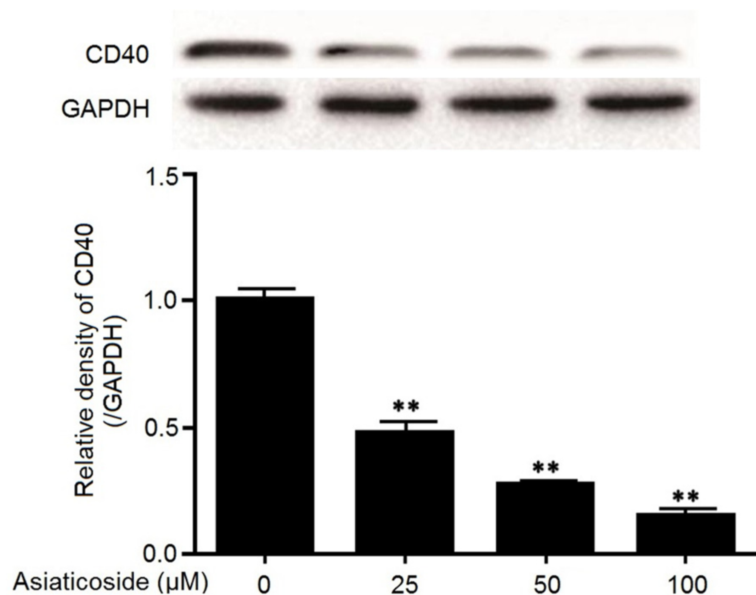

Figure 7. The role of asiaticoside on CD40 expression. **P<0.05 compared to control group.

pathway at the early stage [19]. The specific inhibition of CD40 molecule expression in brain glioma cell U87 can effectively manage the growth and metastasis of tumors [20].

In general, the growth and proliferation of brain glioma is a complicated biological process involving multiple factors. This study demonstrated the inhibition on glioma cell growth by asiaticoside from both *in vitro* and *in vivo* experiments, although detailed mechanism needs further elucidation.

Disclosure of conflict of interest

None.

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