

Baohuoside I via mTOR Apoptotic Signaling to Inhibit Glioma Cell Growth

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Introduction: Baohuoside I, a novel oncotherapeutic agent, has been reported to have anti-cancer effects on a variety of cancers, but its role in glioma and its molecular mechanism are still unclear.

Methods: The proliferation of U251 cells was detected by real-time cellular analysis (RTCA), CCK-8, Ki67 immunofluorescence and colony formation assay. The effect of Baohuoside I on the invasion and migration of U251 cells was measured by transwell and scratch tests. The apoptosis of U251 cells was detected by flow cytometry. The expression level of related protein was detected by western blotting.

Results: Baohuoside I could inhibit the proliferation of human glioma cells and induce apoptosis. Further study showed that the migration and invasion ability of glioma was significantly decreased by Baohuoside I. Western blot revealed the expression of p-AMPK α 1 protein was up-regulated, and the expression of p-mTOR and p-S6K was down-regulated after Baohuoside I treatment. Tumorigenesis in nude mice showed that Baohuoside I had an anti-glioma effect in vivo.

Conclusion: We propose a natural product, which can inhibit the proliferation, invasion and migration of glioma and may be a valuable anti-tumor candidate. The inhibitory effect of Baohuoside I on the glioma is achieved by inducing the apoptosis of the tumor cells, rather than autophagy. In addition, the pathway to induce cell apoptosis of Baohuoside I is to target the mTOR signal.

Keywords: Baohuoside I, mTOR, glioma, apoptosis, proliferation

Introduction

Glioma is a tumor that occurs in the ectodermal tissue of the nerve. In neuroepithelial tissue tumors, the incidence of glioma is about 50%.¹ The high proliferation and invasive behavior of glioma is a difficult treatment problem today, it can effectively “escape” from surgery, radiotherapy, chemotherapy, immunotherapy and other programs, resulting in the ultimate death of patients.² At present, microsurgery can only cut the tumors that are visible to the naked eye, while many “root-like” growths of glioma cells infiltrate into normal brain tissue.³ Chemotherapy toxicity, side effects of radiotherapy and “multi-resistance” cannot be resolved.⁴ Low toxicity, high efficiency and high selective therapy are the most attractive research areas in recent years.

Baohuoside I is used for tonifying the kidney⁵ and strengthening the bone⁶ in Chinese history, and the potential therapeutic effect has been extensively developed in different diseases.⁷ In recent years, Baohuoside I has been proved to be capable of inducing apoptosis of non-small cell lung cancer cells⁶ and inhibiting the growth

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of U266 multiple myeloma through reactive oxygen mitochondrial signaling related reactions,⁸ suggesting that Baohuoside I has potential therapeutic value in the treatment of tumor, but its role in glioma is not clear.

Multiple signal pathways play an important role in the proliferation of glioma cells, such as the JAK2/STAT3, AMPK/mTOR and Nrf2 signaling pathways,⁴ and the high activation of the mTOR signaling pathway is one of the key factors in the occurrence of glioma.⁹ The purpose of this study was to investigate the effects of Baohuoside I on proliferation, migration and apoptosis of glioma cells. The expression level of related proteins was detected by western blotting, including apoptosis-related and JAK2/STAT3 key proteins. This article explores the molecular mechanism of its function and provides new thought for the diagnosis and treatment of glioma.

Materials and Methods

Cell Culture and Reagents

The human glioma cell line (U251) is provided by the Cell Bank of Chinese Academy of Sciences (Shanghai, China). U251 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, California, USA) supplemented with 5% fetal bovine serum (FBS, Invitrogen), 100 µg/mL streptomycin and 100 U/mL penicillin (Invitrogen). The doubling time of U251 cells is about 23 h. The cells were kept at 37 °C and 5% CO₂, passaged every 2–3 days. Baohuoside I was purchased from YuanYe Biotechnology (Shanghai, China) and was dissolved in DMSO as 100 mM. The FITC Annexin V Apoptosis Detection Kit was purchased from BD Pharmingen™ (556547). The Cell Counting Kit-8 (CCK-8) assay was purchased from biosharp (BS350B).

CCK-8 Assay

According to the manufacturer's instructions, the Cell Counting Kit-8 (CCK-8) assay (biosharp, BS350B) was used to determine the viability of U251 cells treated with Baohuoside I (0 µM, 20 µM, 50 µM). After incubation for 24 h, 10 µL reagent was added to the well containing 100 µL of cell suspension (5×10^3 cells), and the absorbance at 450 nm was monitored after incubation for 1 h. The percentage of cell viability was calculated by comparing with the untreated control cells. All of the above tests have been repeated at least three times.

Flow Cytometry Analysis

The U251 cells were treated with Baohuoside I (0 µM, 20 µM, 50 µM) and cultured for 24 hs. Then, cells were collected by centrifugation after trypsinization. In order to analyze the apoptosis, resuspended cells were fully mixed with Annexin V-FITC dissolved in 200 µL binding buffer, incubated at room temperature for 15 min in the dark, then 300 µL binding buffer added and mixed with propidium iodide (PI) for 5 min. Analysis of Annexin V-FITC binding by flow cytometry (Ex = 488 nm; Em = 530 nm, BD FACSVerse, BD Biosciences, USA) was accomplished using the FITC signal detector and PI staining by the phycoerythrin emission signal detector.

Immunofluorescence Staining

U251 cells were cultured on a 6-well plate containing glass slides, treated with Baohuoside I (0 µM, 20 µM, 50 µM) for 24 h, washed in PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) at 4 °C for 30 min. After permeabilization in 0.1% Triton X-100 for 10 min, the sample was washed in PBS and the substrate was blocked with 10% FBS to eliminate non-specific fluorescence. According to previous reports, Ki67 (1:200, Abcam, ab16667) was used as a primary antibody for overnight immunofluorescence staining at 4 °C.¹⁰ After washing in PBS five times, cell preparations and DyLight 594 (Red) labeled secondary antibody (Sigma-Aldrich) were incubated at room temperature for 1 h. After washing in PBS, DAPI was dropped onto the slide and covered with the glass slide. The percentage of Ki67 positive cells was measured by the following formula: (the number of Ki67 positive cells) / (the number of DAPI positive cells).

Real-Time Cellular Analysis (RTCA)

U251 cells were seeded into a cell culture E16-plate (ACEA Biosciences, San Diego, USA) (2×10^4 cells/pores) for proliferation assay, and an unlabeled real-time cell analysis system (RTCA) was used to automatically record the cell growth index of the treatment group and the control group. During cell treatment, the cell growth index was normalized at each time point.

Transwell Assay

Transwell (Costar, New York, NY, USA) assay was utilized to detect the invasion ability of U251 cells in vitro. Cells (2×10^5 cells) in 200 µL serum-free medium with Baohuoside I (0 µM, 20 µM, 50 µM) were inoculated into the upper chamber and coated with Matrigel® (invasion

assay), and the medium containing 10% fetal bovine serum was added to the lower chamber as a chemoattractant. After incubation for 24 h, the cells on the upper surface of the membrane were wiped with Q-TIP, and the invaded cells were fixed with formaldehyde and stained with 0.5% crystal violet (Sigma). Under the microscope, the number of invaded cells was recorded in five randomly selected fields under a microscope.

Colony Formation Assay

U251 cells were inoculated into 12-well plates of 500–800 cells/well. When the cells grew into colonies visible to the naked eye, they were treated with different concentrations of Baohuoside I (0 μ M, 20 μ M, 50 μ M). The colonies were fixed with formaldehyde and stained with crystal violet after 24 h of treatment with Baohuoside I, and the cell colonies were counted.

Migration Assay

The exponential cells were inoculated in a 6-well plate and incubated at 37 °C for 48 h. Then, the culture area was scraped with a crystal pipette tip to make a linear gap in the confluent cell monolayer. The isolated cells were washed off with PBS and then added to the medium containing Baohuoside I (0 μ M, 20 μ M, 50 μ M). Then, we waited for the cells to fill the gap, changed the culture medium every 24 h and used an inverted microscope to capture images of the culture area.

Western Blot Analysis

The U251 cells were seeded in a 6-well plate and treated with 0 μ M, 20 μ M or 50 μ M Baohuoside I for 24 h. Then, the cells were washed with cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer, and quantified with a Bicinchoninic acid protein assay kit (Beyotime Biotechnology). Whole proteins (35 μ g) from each sample were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Solarbio, Beijing, China). After blocking with 5% skim non-fat milk in TBST, membranes were then incubated overnight at 4 °C with the following antibodies: mTOR antibody (ab2732, 1:1000, Abcam), p-mTOR antibody (CST 5536S, 1:1000, Cell Signaling Technology), LC3 antibody (CST 12741, 1:1000, Cell Signaling Technology), p62 antibody (ab155686, 1:1000, Abcam), S6K1 antibody (CST 9202, 1:1000, Cell Signaling Technology), p-S6K1 antibody (CST 9204S, 1:1000, Cell Signaling Technology), AMPK α 1 antibody (CST 2532S, 1:1000, Cell Signaling Technology), p-AMPK α 1 antibody

(CST 2537, 1:1000, Cell Signaling Technology), Bax antibody (50599-2-Ig, 1:1000, Proteintech), caspase 3 antibody (19677-1-AP, 1:1000, Proteintech), caspase 8 antibody (13423-1-AP, 1:1000, Proteintech), Bcl2 antibody (12789-1-AP, 1:1000, Proteintech), JAK2 antibody (AP20700c, 1:1000, ABGENT), p-JAK2 (3771S, 1:1000, Cell Signaling Technology), GAPDH antibody (AP0063, 1:1000, Bioworld Technology). After washing three times, membranes were incubated with secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. After washing another three times with TBST, the protein bands were visualized by chemiluminescence detection on autoradiographic film. The expression of interesting proteins was normalized to GAPDH.

Animal Experiments

For tumorigenesis assays, glioma xenografts were performed in nude mice. U251 (5×10^6 cells) were subcutaneously injected into 6-week-old male BALB/c nude mice (Experimental Animal Centre of Wenzhou Medical University, Wenzhou, China). Then, the nude mice were randomly divided into two groups ($n = 5$). The experimental group received intraperitoneal injection of 35 mg/kg Baohuoside I daily and the control group received the same amount of saline. Tumor volumes were measured every 5 days, and were calculated using the formula: $\text{Volume} = (\text{length} \times \text{width}^2) / 2$. After 30 days, the tumors were removed and analyzed. All animal research consent was approved by the Institutional Review Board of Wenzhou Key Laboratory of Surgery, and the Institutional Animal Care and Use Committee of Wenzhou Medical University, China. The animal experiments were performed on the basis of regulatory institutional guidelines for animal welfare (National Institutes of Health Publications, NIH Publications No. 80-23).

Statistical Analysis

GraphPad Prism software (version 6.02) was used for statistical analysis. A one-way analysis of variance was performed to analyze the data and a p -value of less than 0.05 was considered a statistically significant difference.

Results

Baohuoside I Inhibits Cell Proliferation in Human Glioma

The effects of Baohuoside I on the proliferation of human glioma cells (U251) were detected with CCK8

colorimetric assay, unlabeled real-time cell analysis (RTCA), Ki67 immunofluorescence analysis and colony formation assay. As shown in Figure 1A, significantly decreased proliferation of U251 cells was observed with unlabeled real-time cell analysis after the administration of

both 20 μM and 50 μM Baohuoside I. Likewise, the survival rate of cells treated with different concentrations of Baohuoside I was much lower than that of control cells ($P < 0.05$) (Figure 1B). Ki67, a marker of proliferation,¹¹ immunofluorescence staining (Figure 1C and D) suggested

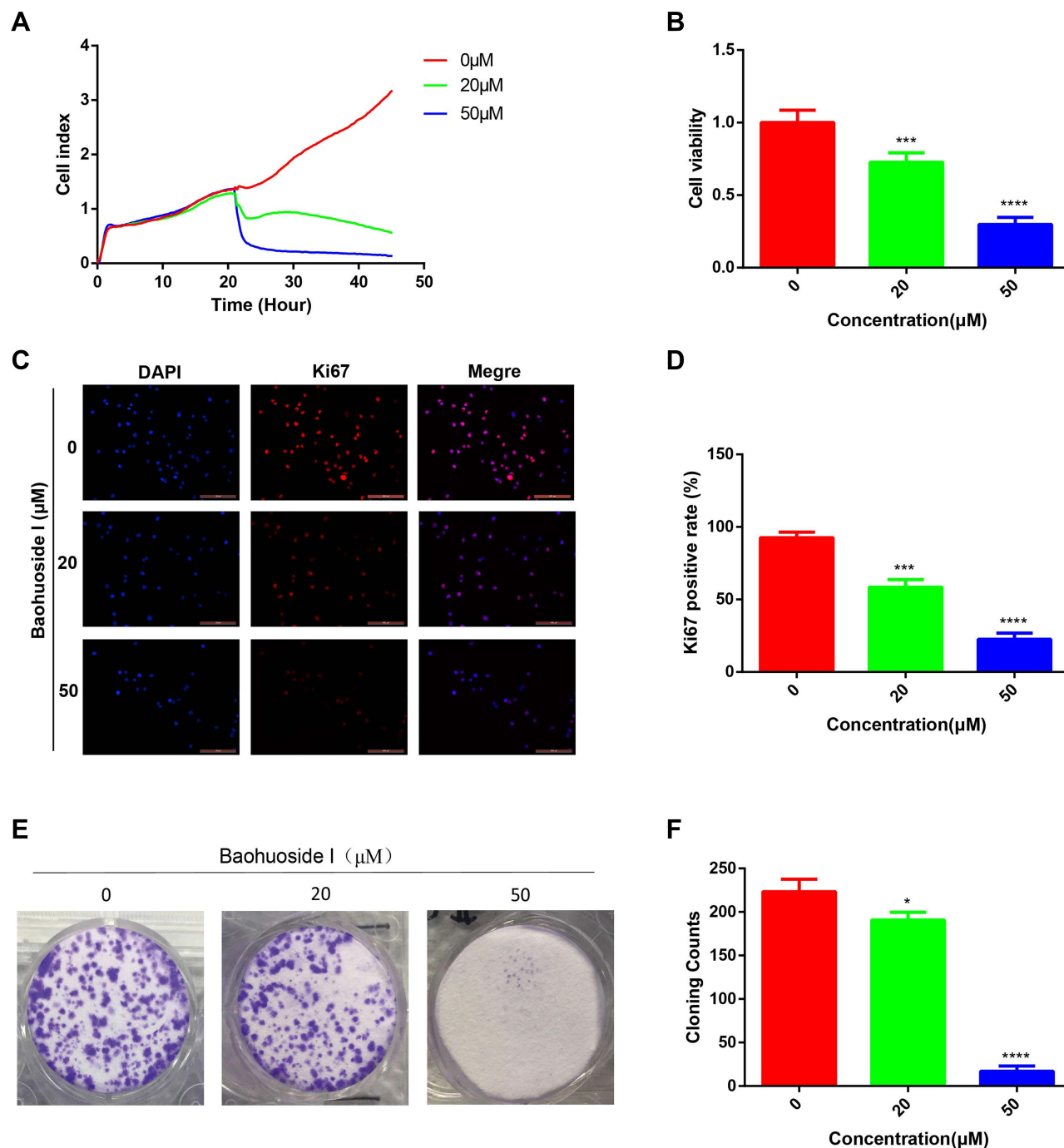


Figure 1 Baohuoside I inhibits cell proliferation of U251. **(A)** RTCA proliferation analysis of U251 cells treated with 0, 20 and 50 μM Baohuoside I. **(B)** CCK-8 assays of U251 cells treated with 0, 20 and 50 μM Baohuoside I for 24 h. **(C)** Ki67 immunofluorescence staining of U251 cells treated with 0, 20 and 50 μM Baohuoside I for 24 h. **(D)** Histogram representing the Ki67 positive rate of U251 cells in each group. **(E)** Colony formation experiment of U251 cells treated with 0.20 and 50 μM Baohuoside I. **(F)** Histogram representing the colony formation assays of U251 cells in each group. All experiments were repeated 3 times and data are presented as mean \pm SD. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, compared with control groups.

that the proliferation of U251 cells decreased significantly after treatment with 20 μM and 50 μM Baohuoside I. In addition, the plate colony formation test was also performed to detect the proliferation of U251 cells (Figure 1E). The number of colonies decreased from 211/well to 17/well after treatment with Baohuoside I (0 μM , 20 μM , 50 μM) (Figure 1F), showing that Baohuoside I could significantly inhibit the proliferation and clonogenicity of U251 cells. All of these results indicate that Baohuoside I treatment inhibits the proliferation of U251 cells by down-regulating Ki67 in a dose-dependent manner.

The Inhibitory Effects of Baohuoside I on Invasion and Migration of Human Glioma

To determine the ability of cell migration after Baohuoside I treatment, we carried out wound healing experiments *in vitro*. As shown in Figure 2A and B, Baohuoside I markedly inhibited the migration of U251 cells compared with the control group. Uniformly, the invasion ability of U251 cells reduced gradually with the treatment of 20 μM or 50

μM Baohuoside I (Figure 2C and D). These results suggest that Baohuoside I treatment inhibits the invasion and migration of U251 cells in a dose-dependent manner.

Baohuoside I Induces Human Glioma Cells Apoptosis Not Autophagy

In order to study the effect of Baohuoside I on apoptosis of the human glioma cell line, the Annexin V-FITC/PI method was carried out to identify U251 cells treated with 0 μM , 20 μM or 50 μM Baohuoside I for 24 h. In response to Baohuoside I, the percentage of apoptosis cells in U251 cells increased from 3.06% to 20.84% compared with the untreated group (Figure 3A and B). Furthermore, the degree of apoptosis of U251 cells was enhanced as Baohuoside I concentrations increased. The results showed that Baohuoside I treatment promoted apoptosis of U251 cells in a dose-dependent manner. In addition, we detected the expression of apoptosis-related key proteins.¹² The expression of apoptosis-promoting protein Bax increased obviously, while the expression of anti-apoptosis protein

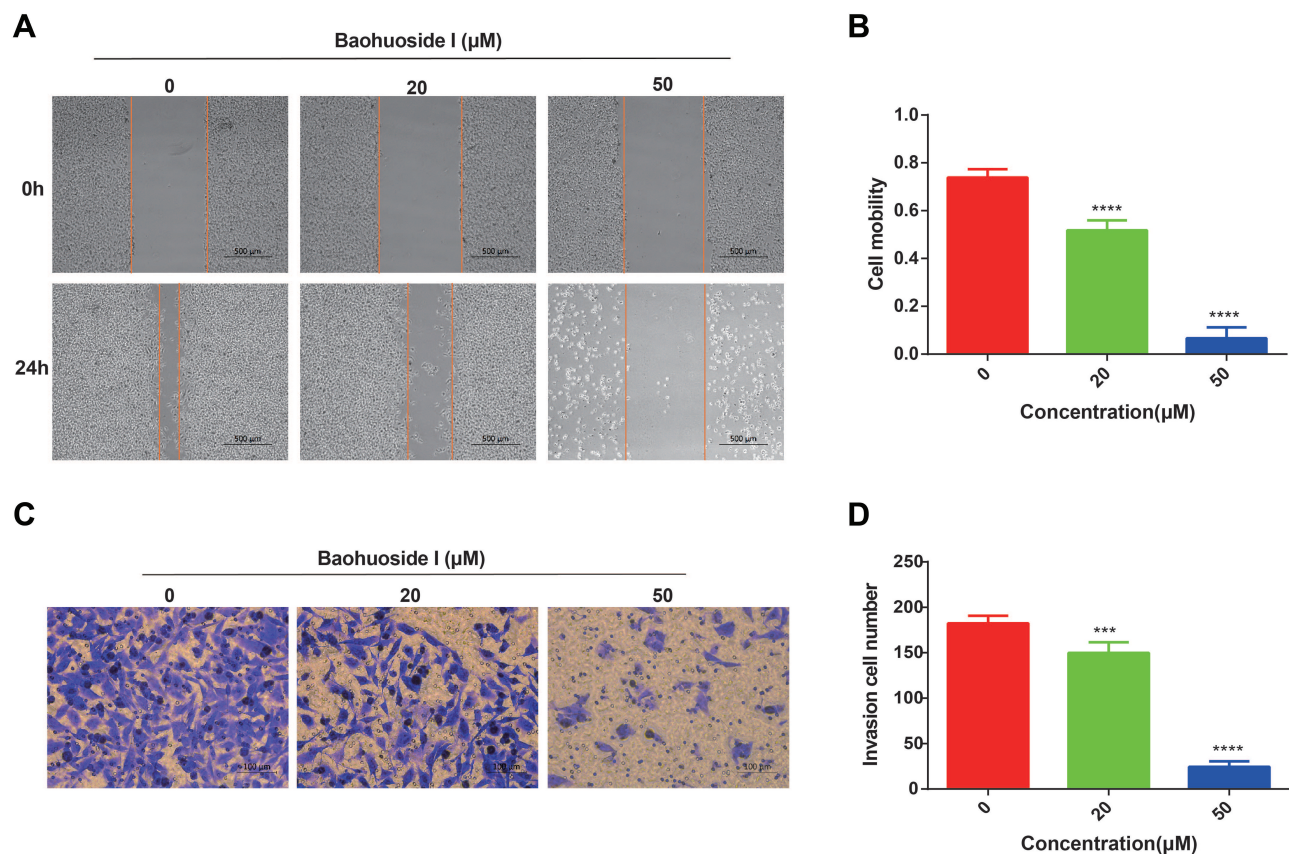


Figure 2 Baohuoside I inhibits the invasion and migration of U251 cells. **(A)** Wound-healing assays of U251 cells treated with Baohuoside I for 24 h. **(B)** Histogram representing the wound-healing assays of U251 cells in each group. **(C)** Transwell assays of U251 cells treated with Baohuoside I for 24 h. **(D)** Histogram representing the Transwell assays of U251 cells in each group. All experiments were repeated 3 times and data are presented as mean \pm SD. *** $p < 0.001$, **** $p < 0.0001$, compared with untreated group.

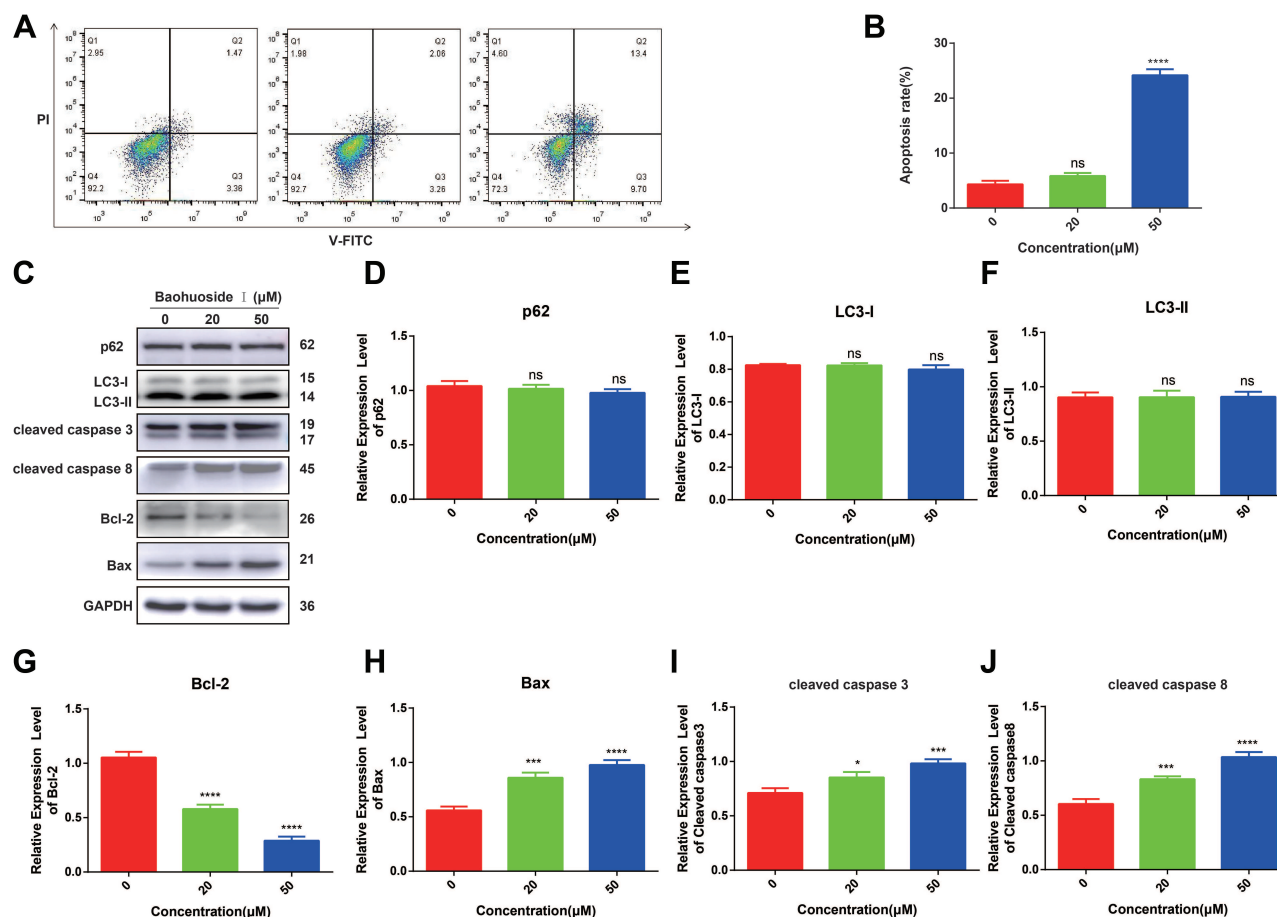


Figure 3 Baohuoside I promotes apoptosis of U251 cells. (A) Flow cytometry analysis of U251 cells after 24 h treated with 0, 20 and 50 μ M Baohuoside I. (B) Histogram of flow analysis in each group. (C) Western blot analysis of apoptosis-related proteins. (D) Histogram representing the relative protein expression level of p62. (E) Histogram representing the relative protein expression level of LC3-I. (F) Histogram representing the relative protein expression level of LC3-II. (G) Histogram representing the relative protein expression level of Bcl-2. (H) Histogram representing the relative protein expression level of Bax. (I) Histogram representing the relative protein expression level of cleaved caspase 3. (J) Histogram representing the relative protein expression level of cleaved caspase 8. All experiments were repeated 3 times and data are presented as mean \pm SD. * p < 0.05, *** p < 0.001, **** p < 0.0001, compared with untreated cells.

Bcl-2 decreased. We also found that the expression of cleaved caspase 3 and cleaved caspase 8 increased significantly, but the changes of LC3 and p62 (Figure 3C–J) were not obvious, which indicated that the autophagy of U251 cells¹³ was not affected by Baohuoside I.

Baohuoside I Inducing Apoptosis by mTOR/S6K1-Caspases/Bcl2/Bax Apoptotic Signaling

In order to explore the molecular mechanism of apoptosis induced by Baohuoside I, we detected the expression of apoptosis-related key proteins by western blot. As shown in Figure 4A, the total mTOR and JAK2 did not change, but the p-mTOR decreased significantly, while the p-JAK2 had no obvious change, indicating that the mTOR signaling pathway may be involved in the apoptosis of U251

cells induced by Baohuoside I (Figure 4A–C). In order to further verify this conjecture, we detected a decrease in the expression of p-S6K1, while an increase in the expression of p-AMPK α 1, but no significant change in total S6K1 and AMPK α 1 (Figure 4D–F). Therefore, we speculate that Baohuo glycoside may induce apoptosis and inhibit the proliferation of U251 cells by mTOR/S6K1-Caspases/Bcl2/Bax apoptotic signaling.

Baohuoside I Inhibits the Growth of Glioma in vivo

To further confirm the potential therapeutic value of Baohuoside I in vivo, we conducted a tumor-forming model of glioma in nude mice. As shown in Figure 5A and B, Baohuoside I treatment obviously suppressed the growth of tumor. Further analysis revealed that Baohuoside I

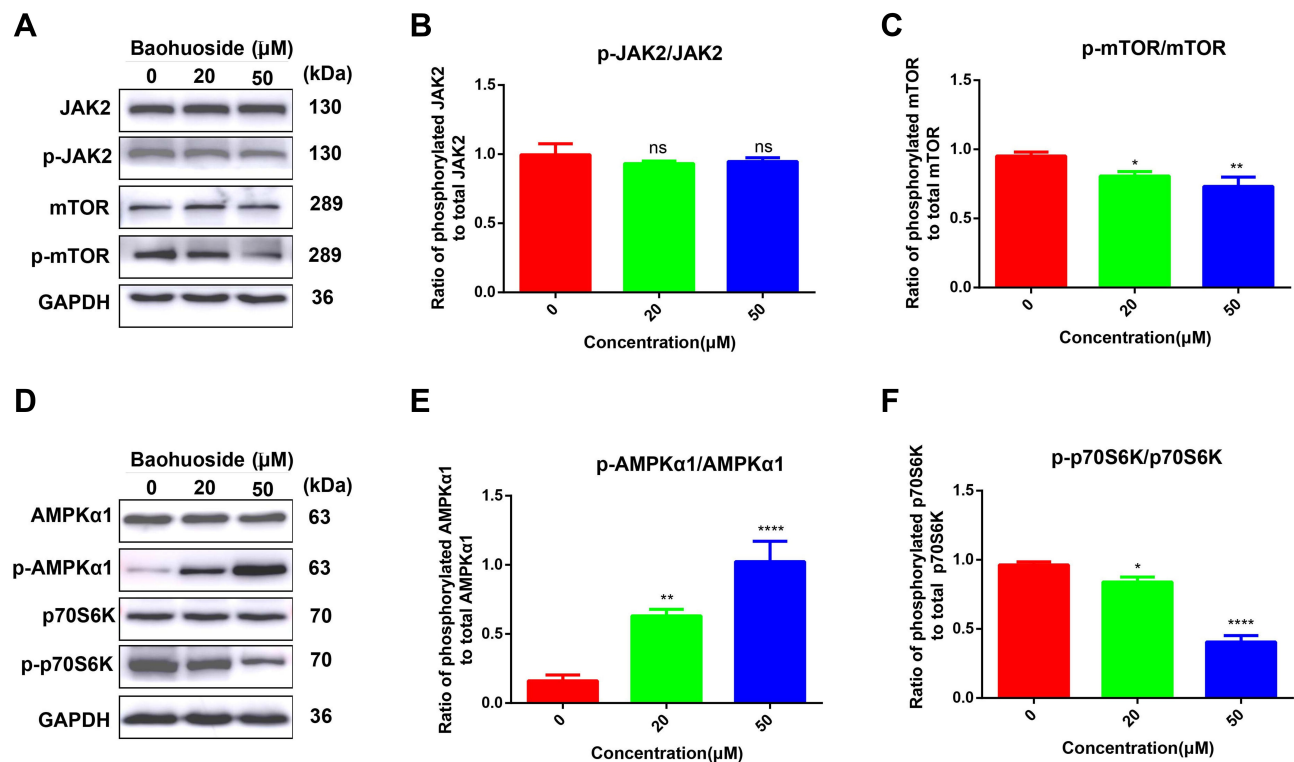


Figure 4 Baohuoside I inhibits the activity of mTOR signaling. (A and D) Western blot analysis of protein expression level in U251 cells treated with 0, 20 and 50 μ M Baohuoside I for 24 h. (B) Histogram representing the ratio of p-JAK2/JAK2. (C) Histogram representing the ratio of p-mTOR/mTOR. (E) Histogram representing the ratio of p-AMPK α 1/AMPK α 1. (F) Histogram representing the ratio of p-p70/p70. All experiments were repeated 3 times and data are presented as mean \pm SD. * p < 0.05, ** p < 0.01, **** p < 0.0001, compared with untreated cells.

obviously decreased the tumor volume (Figure 5C) and weight (Figure 5D) compared to control groups. In addition, as shown in Figure 5E–G, western blot analysis of tumor suggested Baohuoside I treatment increased the expression of p-AMPK α 1 and decreased the expression of p-mTOR, while total AMPK α 1 and mTOR had no obvious changes, which were consistent with the results of experiments in vitro.

Discussion

All present therapies for glioma are traditional treatment including radiation and chemotherapy but ineffective especially for Phase III glioma trials.¹⁴ Recent advances in chemotherapy of glioma suggest some effective drugs such as temozolomide,^{15,16} vincristine and lomustine.¹⁷ However, these drugs are with high side effects that result in nonspecific targeting of cancer cells and normal cells.¹⁸ Temozolomide as an alkylating and methylating agent has been reported to induce central diabetes insipidus.¹⁹ Programmed cell death (PCD) is repressed in cancer cells and agents facilitating PCD are potential specific chemotherapy candidate drugs.²⁰ Apoptosis belonging to type I PCD is important in chemical induced cancer cell death.²¹ Our

results present Baohuoside I as a natural product derived from epimedium that induced glioma cancer cell apoptosis but not autophagy (Figure 3C). The dose-dependent activation of caspase 3, caspase 8 and Bcl2-associated X (Bax) further confirmed the conclusion (Figure 3C).

Janus kinase 2 (JAK2) plays a critical role in tumor apoptosis regulation. The inhibition of JAK2 signaling activates caspase 3 and inhibits Bcl-2 to induce cancer cell apoptosis.²² A recent study further confirms that activation of JAK2 signaling via long noncoding RNA could suppress apoptosis.²³ However, our results showed that the expression of phosphorylated JAK2 was not changed under Baohuoside I treatment (Figure 4A). The data indicate that apoptosis of glioma cancer cells by Baohuoside I was not through JAK2 signaling.

Mammalian target of rapamycin (mTOR) as a core regulatory complex in cell proliferation and development plays a complex role to participate in both autophagy and apoptosis.²⁴ P62 facilitates misfolding protein degradation during the autophagy process.²⁵ Microtubule-associated protein 1A/1B-light chain 3 (LC3) includes two isoforms LC3-I and LC3-II and participates in autolysosome

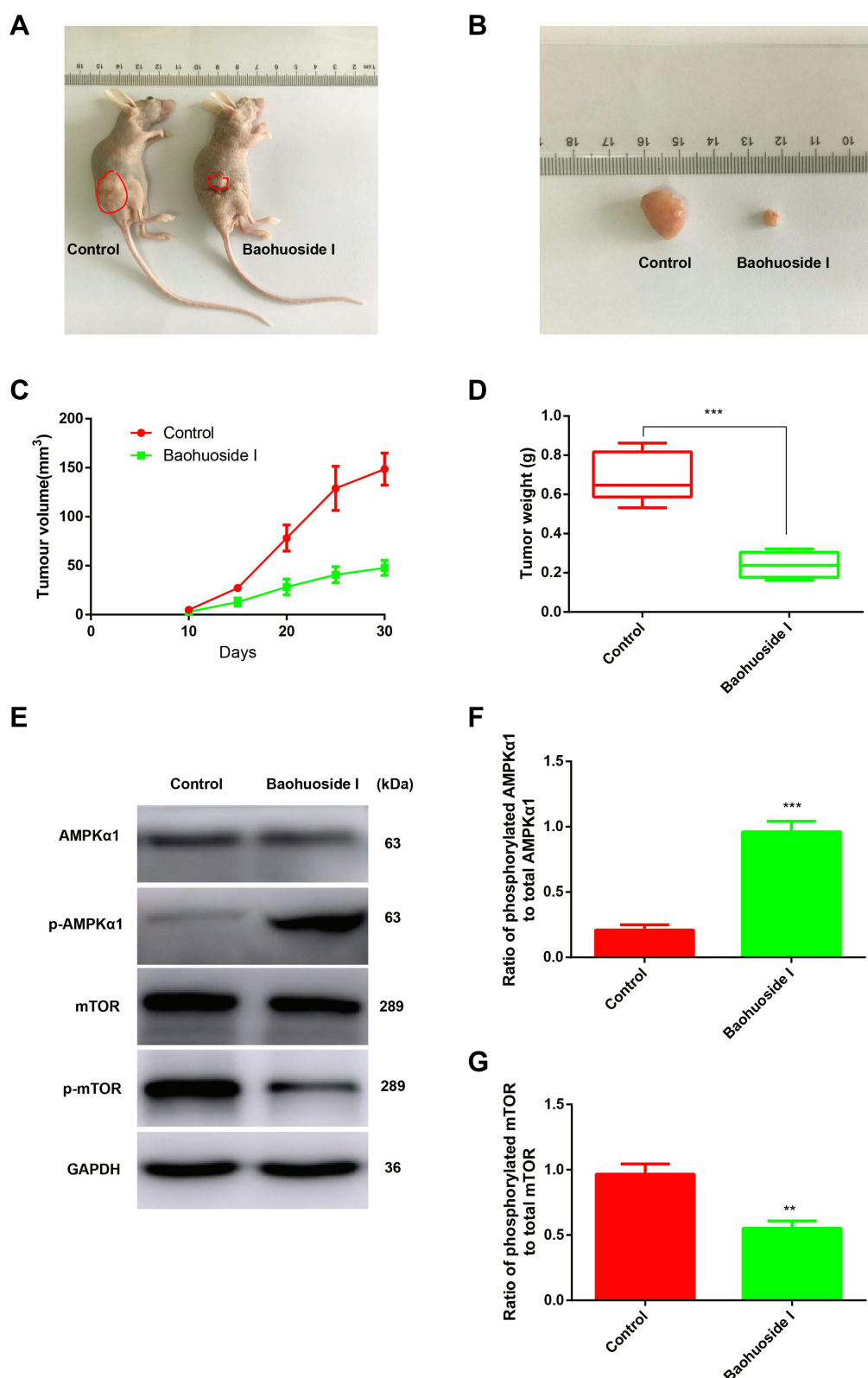


Figure 5 Baohuoside I inhibits the growth of glioma in vivo. (**A** and **B**) U251 cells were subcutaneously injected into nude mice. At the end of the assay, tumors were removed and photographed. (**C**) Tumor volume was detected at various time points. (**D**) Tumor weight was determined after tumors were removed. (**E-G**) Western blot analysis of protein expression. All experiments were repeated 3 times and data were presented as mean \pm SD. ** $P < 0.01$, *** $P < 0.001$, compared with control group.

formation.²⁶ In our results, none of the P62 or LC3 isoforms was induced by Baohuoside I (Figure 3C). The data suggest that the inhibition of glioma proliferation, invasion and migration is not through autophagy. However, Baohuoside I treatment increased the expression of Bax, cleaved caspase 8 and cleaved caspase 3, which suggested intrinsic and extrinsic apoptosis were both involved in apoptosis induced by Baohuoside I (Figure 3C). The role of mTOR in apoptosis has been widely studied. Overexpressed adipocytokine alpha-2-glycoprotein 1 (AZGP1) accelerates mTOR expression that activates caspase 3 to induce apoptosis of gastric carcinoma cells.²⁷ Dual pharmacological inhibition of SGK1 and mTOR induces autophagy-dependent apoptosis in prostate cancer.²⁸ However, the present agents to inhibit mTOR or its partners are highly toxic. Baohuoside I as a natural product is traditionally used to improve sexual function and with low toxicity.²⁹ Our data showed that Baohuoside I specifically activated mTOR in a dose-dependent manner and further suppressed glioma cancer cell proliferation, invasion and migration via apoptosis not autophagy.

In summary, we present a natural product extraction, Baohuoside I, that inhibited glioma proliferation, invasion and migration and may be a valuable candidate drug. The inhibition of glioma by Baohuoside I is through inducing cancer cell apoptosis not autophagy. Moreover, the induction of apoptosis by Baohuoside I is via targeting mTOR signaling not JAK2 signaling.

Data Sharing Statement

Data and materials are available from the authors on reasonable request.

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Disclosure

The authors report no conflicts of interest for this work.

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