

Original Paper

Osthole Exhibits Anti-Cancer Property in Rat Glioma Cells Through Inhibiting PI3K/Akt and MAPK Signaling Pathways

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Key Words

Glioma cell • Proliferation • Apoptosis • Osthole • PI3K/Akt • MAPK

Abstract

Aims: The purpose of this study was to investigate how Osthole affects glioma cell proliferation, apoptosis, invasion and migration. **Methods:** Rat glioma cells were treated with different concentrations of Osthole (0 μ M, 25 μ M, 50 μ M, and 100 μ M). Cell proliferation was assessed by measuring PCNA expression and CCK8 assay at different time points. Apoptosis was evaluated by measuring the expression of pro-apoptotic protein including Bax, Bcl2, PARP, and cleaved Caspase3, and of anti-apoptotic protein Survivin. Cell migration and invasion were assessed using different methods. Signaling pathways such as PI3K/Akt and MAPK, which are involved in the development of glioma cells, were also investigated in this study. **Results:** Treatment with Osthole markedly inhibits glioma cell proliferation, as assessed by western blot with the PCNA antibody. Osthole also induces cell apoptosis by upregulating the expression of pro-apoptotic proteins, and by reducing the expression of anti-apoptotic factors. Moreover, C6 cell migration and invasion were efficiently inhibited in groups treated with Osthole, compared to the control group. Additionally, inhibition of PI3K/Akt and MAPK signaling pathway was also observed in C6 cells treated with Osthole. **Conclusions:** Our findings showed an anti-cancer effect of Osthole on glioma cells, including the proliferation inhibition, apoptosis induction, and migration/invasion inhibition. Further investigation in C6 glioma cells implicated the role of Osthole in essential pathways controlling glioma cell progression. Taken together, our data suggested that Osthole may have a potential application in glioma therapy.

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Introduction

Glioma is one of the most common primary brain tumors, and its standard treatment is thought to be the combination of surgery and chemotherapy. Unfortunately, patients suffered from the most malignant gliomas can only survive for an average of 12 months after diagnosis, due to chemotherapy resistance [1]. The aggressive nature of glioma is attributed to high proliferative capacity and resistance to apoptosis [2]. In spite of enormous efforts towards unveiling the molecular basis of this disease, factors mediating glioma invasion and recurrence are still poorly understood. Therefore, there is an increasingly urgent need to identify critical carcinogenic pathways and to discover new therapeutic targets for glioma.

Osthole, 7-methoxy-8-(3-methyl-2-butenyl) coumarin, is a coumarin derivative clinically ingested as an important ingredient of medicinal plants and herbs [3] in Traditional Chinese Medicine (TCM), exhibiting many pharmacological and biological activities [4, 5]. Osthole has long been used for the treatment of eczema, cutaneous pruritus, trichomonas vaginalis infection, and sexual dysfunction. Recent studies have revealed that Osthole may have anti-inflammatory [6], vasorelaxant [7], and antiallergic [8] effects, as well as the prophylactic effects in hepatitis [9]. In addition, Osthole, as widely reported, exhibits an anti-cancer effect by suppressing cancer cell growth and enhancing apoptosis [10-14]. Given its significant activities in pharmacology as described above, Osthole is becoming a very promising lead compound for drug discovery.

In this study, we found that treatment of C6 glioma cells with Osthole inhibits cell proliferation and enhances apoptotic activity in a concentration-dependent manner. Importantly, Osthole also inhibits cell migration and invasion by downregulating the expression of MMP2 and MMP9. Further explorations suggest the anti-cancer role for Osthole in regulating two signaling pathways PI3K/Akt and MAPK. Together, these findings provide mechanistic insight into how Osthole suppresses glioma cell growth and progression.

Materials and Methods

Cell line and culture

Rat glioma cell line C6, purchased from ATCC (American Type Culture Collection, Manassas, VA, USA), was maintained in high glucose DMEM (Biowest, France) with 10% fetal bovine serum (Biowest, France).

Western blotting

Cells were lysed with lysis buffer (Beyotime, P0013B, China) containing PMSF. Lysates were centrifuged at 12,000 g at 4°C for 10 min, and protein concentration was determined using BCA Protein Assay Kit (Cat. No. 23227, Pierce, USA). Proteins were separated by 15% SDS-PAGE and transferred to PVDF membrane using a standard protocol. The membrane was incubated with primary antibodies and probed with the corresponding secondary antibodies. Enhanced chemiluminescence (Pierce Biotechnology, Rockford, USA) then was used for protein visualization. The following antibodies were used: p-Erk1/2 (#4370, CST, USA), Erk1/2 (#9102, CST, USA), Bcl2(ab7973, USA), Bax (ab7977, USA), PARP (Beyotime, AP102, China), Survivin (Beyotime, AS792, China), PCNA (SC-25280, USA), Akt(#4691, CST, USA), P-Akt-Ser473(#4060, CST, USA), P-Akt-Thr308 (#2965, CST, USA), cleaved Caspase-3 (#9661, CST, USA), and GAPDH (#2118, CST, USA).

Cell Proliferation Assay

Cell proliferation was measured using CCK8 assay (Dojindo, Japan). Briefly, C6 cells were plated at a density of 1×10^4 cells/ml/well in 96-well plates overnight and treated with different concentrations of Osthole (Nanjing Tcm Institute of Materia Medica, China). After 24, 48 or 72 hrs treatment, 10 μ l of CCK8 solution was added to each well and cells were cultured for another 2 hrs at 37°C. Finally, the plates were shaken and the optical density was determined at 450 nm (OD₄₅₀) using ELISA plate reader (SYNERGY4, USA). At least three independent experiments were performed. The inhibition percentage was calculated according to this formula: $(\text{Control}_{\text{OD450}} - \text{experimental group}_{\text{OD450}}) / \text{Control}_{\text{OD450}} \times 100\%$.

Wound closure assay

C6 cells were plated in 6-well plates. When cells reached about 90% confluence, wounds were created with a 200- μ l pipette tip. Cells were rinsed with medium for the removal of floating cells and debris, and then incubated with medium containing different concentrations of Osthole for 24 hrs.

Cell invasion assay

The invasion assay was performed using 24-well transwell inserts with 8 μ m pores (Costar, Cambridge, NY). Each well was coated with ECM gel (E1270, Sigma-Aldrich, Switzerland) to form a continuous thin layer. C6 cells (2×10^5) were cultured with different concentrations of Osthole, and then C6 cells from different groups were counted and plated on the ECM-coated filter with 1% serum, with 10% serum-containing medium in the lower side of the plate. After 24 hrs incubation, cells that had invaded the lower side of the membrane were stained with 4'-6-diamidino-2-phenylindole (DAPI, D9564, Sigma-Aldrich, Switzerland). Stained cells in each well were photographed and counted.

Gelatin zymography analysis

The activities of MMP-2 and MMP-9 in conditional medium from C6 cells were measured using gelatin zymography protease assays (Applygen, Beijing). Confluent cells were incubated in serum-free medium with Osthole (0 μ M, 25 μ M, 50 μ M, and 100 μ M) for 24 hrs. An appropriate volume of conditional medium was collected and subjected to electrophoresis on 8% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin under non-reducing conditions. After electrophoresis, gels were washed with 2.5% Triton X-100 and incubated in a reaction buffer (40 mM Tris-HCl at pH 8.0, 10 mM CaCl_2 , and 0.01% NaN_3) for 12 hrs at 37 °C. The gel was then stained with Coomassie brilliant blue R-250 for 1 hr and washed in 5% methanol and 7% acetic acid to visualize the bands of proteolytic activity.

Statistical analysis

The data were expressed as mean \pm SD. Statistical correlation of data was checked for significance by ANOVA and Student's t test. Differences with $P < 0.05$ were considered significant. These analyses were performed using SPSS 16.0 software.

Results

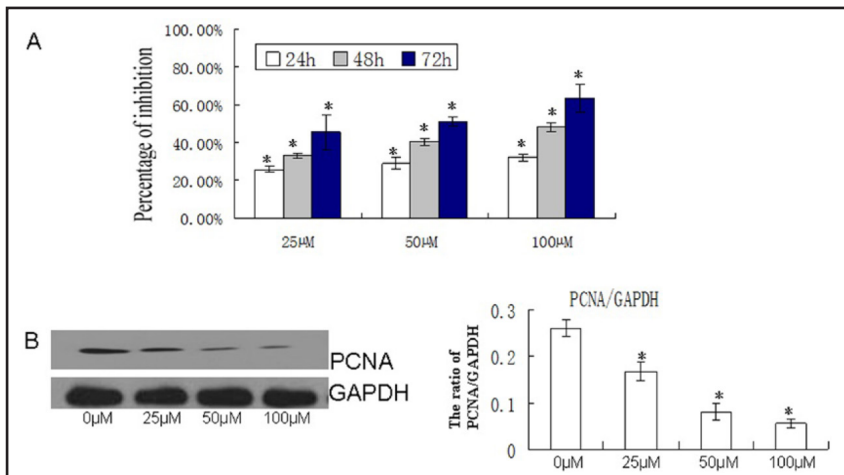
Inhibitory effect of Osthole on C6 cell proliferation

It has been proposed that a high concentration (up to 100 μ M) of Osthole can inhibit proliferation of many tumor cells, which was repeatable in C6 cells in this study. Treatment of C6 cells with Osthole markedly suppressed cell growth in a dose-dependent manner, as assessed using CCK8 method (Fig. 1A). This anti-proliferative role for Osthole in this cell line was further strengthened by the observation that the protein level of PCNA was downregulated in cells treated with Osthole compared to that in control (Fig. 1B). Collectively, these results demonstrated that treatment of C6 cells with Osthole efficiently inhibits cell proliferation.

Treatment of C6 cells with Osthole enhances apoptosis in a dose-dependent manner

C6 cells were passaged and treated with different concentrations of Osthole (0, 25, 50, and 100 μ M) for 24 hrs, and then proteins were extracted and analyzed by Western Blot. As shown in Fig. 2A, the ratio of Bax to Bcl2 in cells treated with Osthole was higher than that in control cells, and the value was improved in a dose-dependent manner. Similarly, we found evidence that Osthole significantly promotes the expression of other pro-apoptotic factors such as PARP (Fig. 2B) and cleaved Caspase3 (Fig. 2C). Conversely, treatment with Osthole resulted in a marked reduction in the expression of Survivin, an anti-apoptotic factor (Fig. 2D). Together, these findings indicate that treatment with Osthole significantly enhances C6 cell apoptosis in a dose-dependent manner.

Fig. 1. Effect of Osthole on the proliferation. (A) C6 cells were treated with increasing concentrations of Osthole (0, 25 μ M, 50 μ M and 100 μ M) for 24h, 48h and 72h. The proliferative inhibition effect of Osthole on C6 cells was assessed by CCK8 kit. * $p < 0.05$ versus control group. (B) C6 cells were treated with



Osthole (0 μ M, 25 μ M, 50 μ M and 100 μ M) for 24hrs. The protein expression level of PCNA was detected by Western blot. The result of one of three separate experiments was shown. Quantification of PCNA protein was normalized to GAPDH using software Image J. Data was expressed as mean \pm SD from three separate experiments. * $p < 0.05$ versus control.

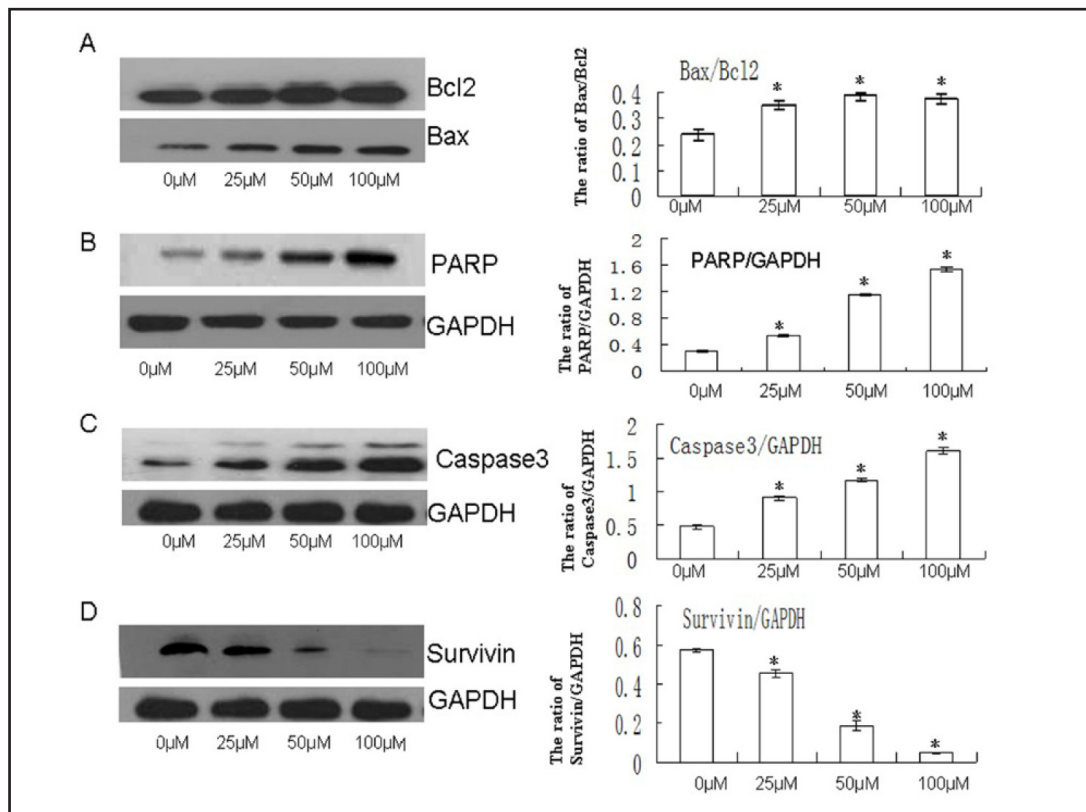
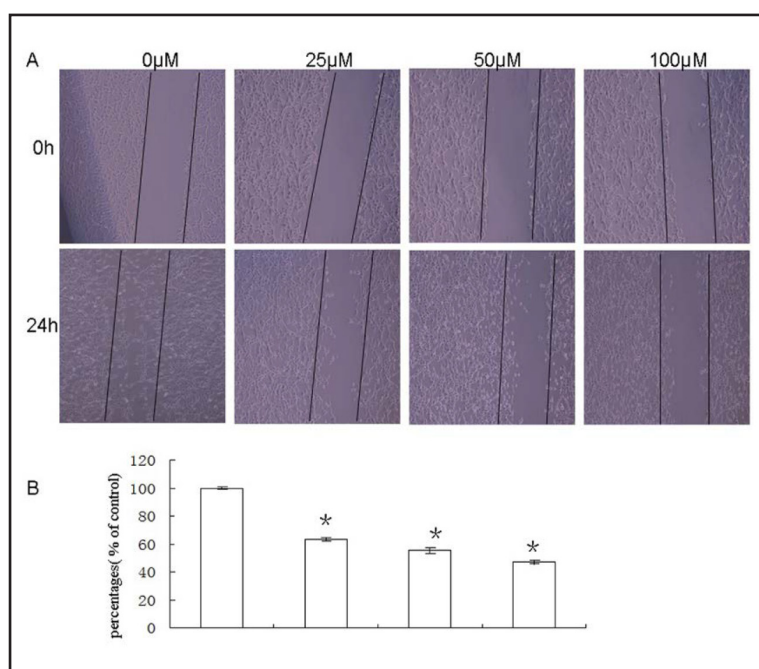


Fig. 2. Effect of Osthole on the expression of apoptotic genes. C6 cells were treated with different concentrations of Osthole (0 μ M, 25 μ M, 50 μ M and 100 μ M) for 24hrs. The protein expression levels of Bcl2, Bax, PARP, Survivin and Caspase3 were assayed by Western blot. The result of one of three separate experiments was shown. Software Image J was used in the Quantification of all proteins. (A) Quantification of Bax protein was normalized to Bcl2. (B) Quantification of PARP protein was normalized to GAPDH. (C) Quantification of Caspase3 protein was normalized to GAPDH. (D) Quantification of Survivin protein was normalized to GAPDH. All data were expressed as mean \pm SD from three separate experiments. * $p < 0.05$ versus control.

Fig. 3. Effect of Osthole on C6 cell migration. A monolayer of confluent C6 cells was scraped with a 200 μ l sterile pipette tip and treated with the indicated concentrations of Osthole (0, 25 μ M, 50 μ M and 100 μ M) (A) Cell migrations of both non-treated and treated were observed by phase-contrast microscopy and photographed at 0h and 24h respectively. Images are representative of three independent experiments. (B) Cells migrating into the wound area were counted between the two lines, migrative cell number compared to control (expressed as percentage of control). * $p < 0.05$ versus control.



Osthole inhibits C6 cell migration

To determine the effect of Osthole on cell migration, scratch wound healing assays using C6 cells were conducted with different concentrations of Osthole for 24 hrs (Fig. 3). When compared with the group untreated, wound closure appeared delayed in groups which were treated with Osthole, as quantified by counting the migrating cells (Fig. 3A). Specifically, the migration percentages in C6 cells were 63.2 ± 1.40 , 55.7 ± 2.08 , 46.8 ± 1.53 compared with control group (Fig. 3B).

Osthole inhibits C6 cell invasion by downregulating the expression of MMP2 and MMP9

To exclude the possibility that reduced proliferation contributes to a reduction in invasive cell number, C6 cells were pre-treated with different concentrations of Osthole and counted. C6 cells were maintained in medium containing different concentrations of serum on the filter and under the filter, respectively, and then they were stained with DAPI after being treated with Osthole for 24 hrs (Fig. 4A). Indeed, DAPI staining indicated that treatment with Osthole concentration-dependently inhibits C6 cell invasion, ranging in invasion percentage from 75.6 ± 2.2 to 68.73 ± 2.5 or 54.3 ± 3.8 (Fig. 4B). Given that MMP-9 and MMP-2 are important extracellular matrix-degrading enzymes, cancer cell migration and invasion are usually assessed by monitoring their activities. In this case, we found that treatment with Osthole results in a reduction in the expression of MMP2 and MMP9 in a concentration-dependent manner (Fig. 4C and 4D). Collectively, these data showed that Osthole inhibits C6 cell invasion through suppressing the expressions of both MMP2 and MMP9. The inhibitory effects on MMP2 expression which Osthole mediated were more evident than that of MMP9. These results suggested that MMP2 was more important than MMP9 in regulating cell invasion after being treated with Osthole.

Treatment of C6 cells with Osthole exhibits inhibitory activities of PI3K/Akt and MAPK signaling pathways

The activities of signaling pathways that involved in the proliferation inhibition, apoptosis and invasion/ migration in glioma cells have been detected. Here, we found that, no changes in total Akt and Erk1/2 protein levels were observed in glioma cell treated with

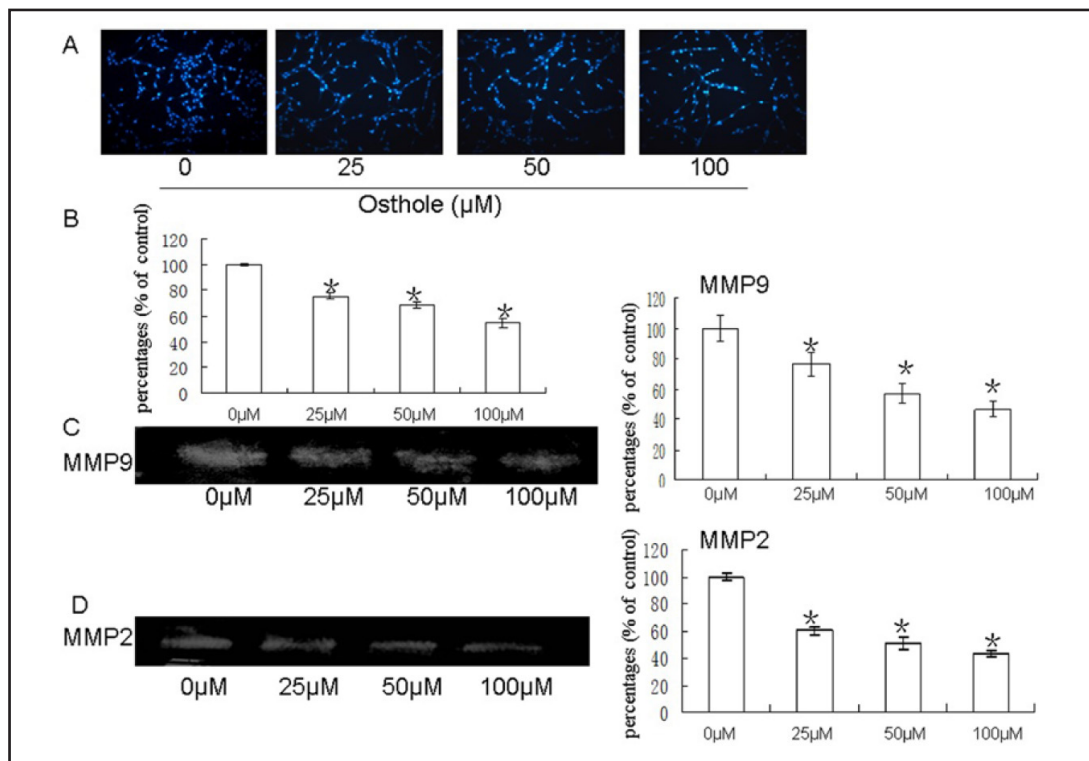
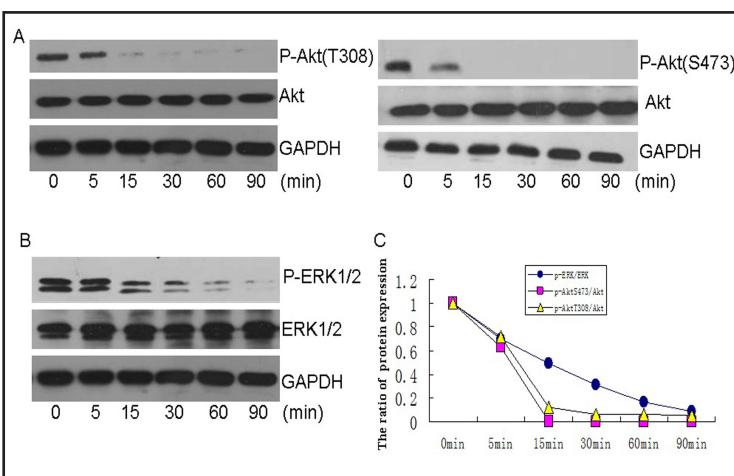


Fig. 4. Effects of Osthole on C6 cell invasion, the expression of MMP2 and MMP9. Concentration-dependent inhibitory effects of Osthole on C6 cell invasion. (A) Photographs (20×) show DAPI-labeled cells invading through a ECM-coated membrane. (B) Bar graphs represented the invasive cells that were treated with various concentrations (0–100 μM) of Osthole for 24 h (expressed as percentage of control) and then subjected to gelatin zymography to analyze the activities of MMP-2 (C) and MMP-9 (D). Data are expressed as mean±SD from three separate experiments. *p < 0.05 versus control.

Fig. 5. The influences of Osthole on the activities of both MAPK and PI3K-Akt signal pathways. C6 glioma cells were treated with 100 μM Osthole for 0–90 min. The phosphorylation levels of Akt (A) and MAPK (B), which represented the activities of PI3K-Akt and MAPK pathway, were analyzed by Western blot. (C) The down-regulated expression level of p-ERK1/2 was dependent on the intervention time of Osthole on C6 cells. The expression level of p-Akt (S473) was inhibited after being treated with Osthole for 5 min and completely repressed from the time point of 15 min. The expression level of p-Akt (T308) was downregulated gradually during the whole process of Osthole treatment.



Osthole, treatment with Osthole significantly inhibits the phosphorylation levels of Akt and Erk1/2 in a time-dependent manner, as assessed by measuring the expression of p-Akt and p-Erk1/2, respectively (Fig. 5A and B). Furthermore, we observed that Osthole can rapidly

abolish the expression of p-Akt within 15 min, whereas the p-Erk1/2 expression failed to be completely diminished within 90 min (Fig. 5C).

Discussion

Amounts of studies showed that Osthole regulates cell apoptosis, proliferation and invasion via multiple signaling pathways in many cancer cells [13, 15, 16]. In addition, it has been reported in recent studies that Osthole has essential roles in brain functions by protecting neurons [17-21], suggesting that Osthole could penetrate the blood-brain barrier for the chemotherapy drugs of brain tumours.

In the present study, the effects of Osthole on glioma cells were investigated. We first evaluated the proliferation of C6 glioma cells between Osthole-treated groups and control group by using different methods. It was reported that Osthole did not display any cell toxicity in normal osteoblasts even at the concentration of 100 μ M [22]. Meanwhile, combined with the concentration of Osthole used in other tumor [12, 23], C6 cells were treated with different concentrations of Osthole (0-100 μ M) for different time points. The effects of proliferation inhibition were more evident in a time and dose dependent manner by CCK8 analysis. Western blot analysis revealed that the treatment of C6 cells with increasing concentrations of Osthole would decrease the protein expression levels of PCNA obviously. The consistent results from these data showed that the growth of C6 cells was inhibited by Osthole.

Apoptosis, an important indicator for anticancer therapy, could also be investigated among Osthole-treated groups compared with control group. Among the Bcl-2 family proteins that regulate apoptosis, Bcl-2 and Bax play opposite roles in apoptosis process. Bcl-2 inhibits apoptosis while Bax promotes apoptosis. The change in the ratios of anti- and pro-apoptotic Bcl-2 family proteins is likely to determine the onset of apoptosis. The results in this study indicated that Osthole treatment increased the protein level of pro-apoptotic protein Bax significantly while also increased the level of Bcl2 slightly, the Bax/Bcl-2 ratio was increased finally. Other apoptotic proteins such as PARP, Caspase3 and Survivin also participate in the process of apoptosis. Caspase-3 was responsible for *in vivo* cleavage of the full-length PARP. Survivin, a member of the inhibitor of apoptosis (IAP) family, functions to negatively regulate apoptosis. The Survivin had been shown to prevent apoptosis by inhibiting the active form of caspase-3[24]. Our findings indicated that the expression levels of Survivin were down-regulated evidently in Osthole-treated groups. Furthermore, cleaved caspase-3 was activated by elevating the expression levels, and the downstream molecule PARP was also cleaved to obtain an 89 kDa fragment. All these results showed that Osthole promoted the obvious apoptosis in C6 cells. These data were consistent with the previous studies which Osthole induced apoptosis in cancer cells from different types of tissue [16, 23].

Except for the excessive proliferation and resistance to apoptosis stimuli, extensive invasion into surrounding normal brain tissue was another major characteristic of glioma. From the results demonstrated above, Osthole had been shown to exert activity against the proliferation and apoptosis. The invasive properties of tumor cells were to secrete and activate proteolytic enzymes such as metalloproteases MMP2 (72-kDa) and MMP9 (92-kDa), which were responsible for degrading extracellular matrix (ECM). These two proteases were expressed endogenously at high levels in glioma, and the elevated expressions and activities have been correlated with high invasiveness and migration [25-28]. In our experiments, we found Osthole exhibited inhibitory effects on both the migration and the invasion of C6 cells via a dose-dependent manner. Furthermore, the protein expression levels of MMP-2 and MMP9 were significantly down-regulated in C6 cells after being treated with Osthole through MMPs zymography assay. These results indicated that Osthole could be further developed as a novel target for control of glioma.

Akt is phosphorylated and subsequently crucial to many divergent physiological functions including cell proliferation, apoptosis, migration, and invasion [29]. In animal models, Akt has been shown to play a crucial role in gliomagenesis. Changes(amplications

or mutations) in receptor tyrosine kinase growth factor Receptors such as EGFR, PDGFR and ERBB2, which result in activation of downstream signaling pathways (for example MAPK, PI3K-Akt) [30, 31]. These two pathways are found in most of World Health Organization grade II, III and IV astrocytomas. The MAPK pathway is also activated in the majority of World Health Organization grade I tumors [32]. Together these data suggest that MAPK signaling is important for glioma development. Activation of this pathway also influenced cell-cycle progression through Cyclin D1 activation and inhibition of apoptosis by regulation of BCL-2-family gene expression and activity [33].

To further reveal the possible biological mechanisms of Osthole's effects underlying apoptosis, proliferation and cell migration/invasion on C6 cells, related pathways were investigated. The activations of MAPK and PI3K-AKT signaling pathways have been reported in glioma [34, 35]. The essential roles of these pathways were in control of the proliferation and apoptosis in cancer cells. To successfully develop new therapeutic approaches for glioma, combinational approaches towards several molecular pathways will likely yield more effective treatments. Inhibition of several oncogenic pathways with their inhibitors respectively could lead to severe cytotoxicity and cell death. The ideal treatment is that a natural drug could aim at several pathways specifically activated in glioma cells.

In our current study, both MAPK and PI3K-AKT signaling pathway were investigated in Osthole-treated C6 cells. Akt is a major downstream target of PI3K. The PI3K/Akt signaling pathway played important roles in regulating the development and progression of various cancers by elevating the phosphorylation level of Akt [36]. C6 cells were treated with Osthole at different time points, and the phosphorylation level of p-Akt (S473 and T308) was reduced evidently while the protein level of total Akt kept the same. These data showed that PI3K-Akt pathway was inhibited efficiently by Osthole.

In addition, the phosphorylation level of p-Erk1/2, a critical downstream molecular of MAPK signal pathway, was declined after C6 cells were incubated with Osthole even for 15 minutes. The activation of MAPK signal pathway was completely blocked at the time point of 90 minutes from the results presented. These results further indicated Osthole exhibited various anticancer effects on C6 glioma cells through inhibiting MAPK signal pathway.

It has been reported that Osthole plays a role of protecting neurons by activating Erk1/2 [20]. The activity inhibition of Erk1/2 in glioma cells by Osthole from our experiment clearly conflicted with this report. The most likely explanation of this apparent difference may be due to different cell types used. Based on these findings, Osthole may be an ideal drug for glioma therapy without destroying neurons in brain.

Conclusions

Osthole inhibited the proliferation, promoted apoptosis and inhibited cell migration/invasion *in vitro* in glioma cells. It was further found that both MAPK and PI3K-Akt pathways, which were endogenously expressed at high level and important for the progression of glioma, were inhibited by Osthole. All our findings implied that Osthole may play an inhibitory role in the progression of glioma and provide an adjuvant therapy to control malignant gliomas.

Abbreviations

CCK8 (Cell Counting Kit); PCNA (Proliferating Cell Nuclear Antigen); EGFR (Epidermal growth factor receptor); PDGFR (Platelet Derived Growth Factor Receptor); MAPK (Mitogen Activated Protein Kinase); MMP2 (Matrix Metalloproteinase 2); MMP9 (Matrix Metalloproteinase 9).

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgements

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