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LABORATORY INVESTIGATION

Phloretin induces cell cycle arrest and apoptosis of human glioblastoma cells through the generation of reactive oxygen species

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Abstract Phloretin, a flavonoid present in various plants, has been reported to exert anticarcinogenic effects. However, the mechanism of its chemo-preventive effect on human glioblastoma cells is not fully understood. This study aimed to investigate the molecular mechanism of phloretin and its associated chemo-preventive effect in human glioblastoma cells. The results indicate that phloretin inhibited cell proliferation by inducing cell cycle arrest at the G0–G1 phase and induced apoptosis of human glioblastoma cells. Phloretin-induced cell cycle arrest was associated with increased expression of p27 and decreased expression of cdk2, cdk4, cdk6, cyclinD and cyclinE. Moreover, the PI3K/AKT/mTOR signaling cascades were suppressed by phloretin in a dose-dependent manner. In addition, phloretin triggered the mitochondrial apoptosis pathway and generated reactive oxygen species (ROS). This was accompanied by the up-regulation of Bax, Bak and c-PARP and the down-regulation of Bcl-2. The antioxidant agents *N*-acetyl-L-cysteine and glutathione weakened the effect of phloretin on glioblastoma cells. In conclusion, these results demonstrate that phloretin exerts potent chemo-preventive activity in human glioblastoma cells through the generation of ROS.

Keywords Phloretin · Cell cycle arrest · Apoptosis · Reactive oxygen species · Glioblastoma

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Introduction

Glioblastoma (GBM) is the most common and aggressive central nervous system tumor. Due to the relentless invasion and growth of this tumor, patients with GBM usually have poor prognosis. The median survival of patients diagnosed with GBM is 12–15 months, and the five-year survival rate is less than 5 % [1]. Current treatments are still limited, and more effective chemotherapeutic drugs for use against malignant glioma remain to be explored. Many studies have focused on the various phytochemicals present in fruits and vegetables as promising chemo-preventive agents [2]. In recent years, flavonoids and their synthetic analogues have been investigated in several cancers, such as skin [3], ovarian [4] and breast [5].

Phloretin [2′, 4′, 6′-trihydroxy-3-(4-hydroxyphenyl)-propiophenone], a naturally occurring flavonoid found mostly in plants of the Rosaceae family, has been shown to exert anti-tumor activity in many studies, mainly through the inhibition of glucose transmembrane transport and protein kinase C activity [6–8]. However, the role of ROS

generation in the anticancer effect of phloretin has not been fully described.

It has been reported previously that the PI3K/Akt signaling pathway is frequently activated in glioblastoma [9]. PTEN (phosphatase and tensin homolog), a major negative regulator of the PI3K/Akt signaling pathway, is a tumor suppressor gene implicated in a wide variety of human cancers, including glioblastoma [10].

In this study, we demonstrate the effect of phloretin on glioblastoma cells and its underlying molecular mechanism in vitro. Our results provide evidence that phloretin induces cell cycle arrest and apoptosis in glioblastoma cells through the accumulation of ROS and activation of the mitochondrial apoptotic pathway.

Materials and methods

Reagents

The U87 and U251 cell lines were obtained from the Cell Culture Centre of the School of Basic Medicine at Peking Union Medical College. DMEM was purchased from Hyclone (USA), and fetal bovine serum (FBS) was purchased from Gibco (USA). Primary astrocyte cultures were prepared from ICR mice as described by Li et al. [11]. and Chen et al. [12]. A Cell Counting Kit-8(CCK8) assay was purchased from Dojindo (Janpan). Phloretin was purchased from Sigma and dissolved in methanol. All antibodies were purchased from Cell Signaling Technology (USA).

Cell proliferation assay and clonogenic assay

The U87 and U251 cell lines were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS). Cells were seeded at 5×10^4 cells/ml with 100 μL of culture medium and treated with the indicated concentration of phloretin. At 12, 24, and 48 h after treatment, 10 μL of CCK-8 solution was added to each well, and the cells were incubated for 1 h. The absorbance at 450 nm was measured using a BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader (USA). To determine the long-term effects, cells were allowed to form colonies for 7 days and were rinsed with fresh medium every 3 days prior to staining with crystal violet (0.4 g/L).

Flow cytometry analysis of cell cycle and apoptosis

For cell cycle analysis, cells (less than 70 % confluent) were trypsinized, collected, centrifuged at 400 × *g* and fixed in cold 70 % ethanol overnight at 4 °C. After washing with PBS, cells were incubated in propidium iodide (PI) for 30 min in the dark. For apoptosis analysis, cells were harvested with trypsin, washed with ice-cold PBS, suspended in 1× binding buffer and incubated with PE and 7-AAD for 15 min. A FACSCalibur flow cytometer (BD, USA) was used to analyze the samples, and data analysis was performed using Modifit LT software.

Assay for caspase-9 activity

This assay was based on spectrophotometric detection of a colored reporter molecule, *p*-nitroaniline (*p*NA), that was linked to the end of the caspase-specific substrate. Cell lysates were incubated with the peptide substrate LEHD-*p*NA (Ac-Leu-Glu-His-Asp-*p* NA) in assay buffer for 4 h at 37 °C. The release of *p*NA was monitored at 405 nm.

ROS measurement

The generation of intracellular ROS was determined using H2DCFDA, which yields fluorescent 2,7-dichlorofluorescein (DCF) when oxidized in the presence of H2O2 and peroxidases. After exposure to different concentrations of phloretin for 24 h, cells were incubated with 10 μM H2DCFDA for 30 min at 37 °C. Cells were harvested with trypsin, and intracellular ROS was detected using a confocal fluorescent microscope (Leica TCS SP5 Confocal Microscope) or using flow cytometry.

Western blot analysis

Protein samples extracted from cells were evenly loaded and separated using 10 % SDS-PAGE electrophoresis and then transferred to nitrocellulose membranes. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce, USA), and signal imaging was performed using a KODAK Image Station 4000.

Statistical analysis

Data were expressed as the mean ± SD. Statistical comparisons of the results were performed using ANOVA and Dunnett's test. Significant differences were accepted as $P < 0.05$.

Results

Phloretin inhibits cell proliferation and colony formation in the U87 and U251 cell lines

We first examined the effect of phloretin on cell proliferation and colony formation. Figure 1b shows that in both cell lines, colony formation was inhibited in a concentration-dependent manner. Phloretin also inhibits cell growth in both a concentration-dependent manner and a time-dependent manner after treatment with various concentrations of phloretin for 12–48 h

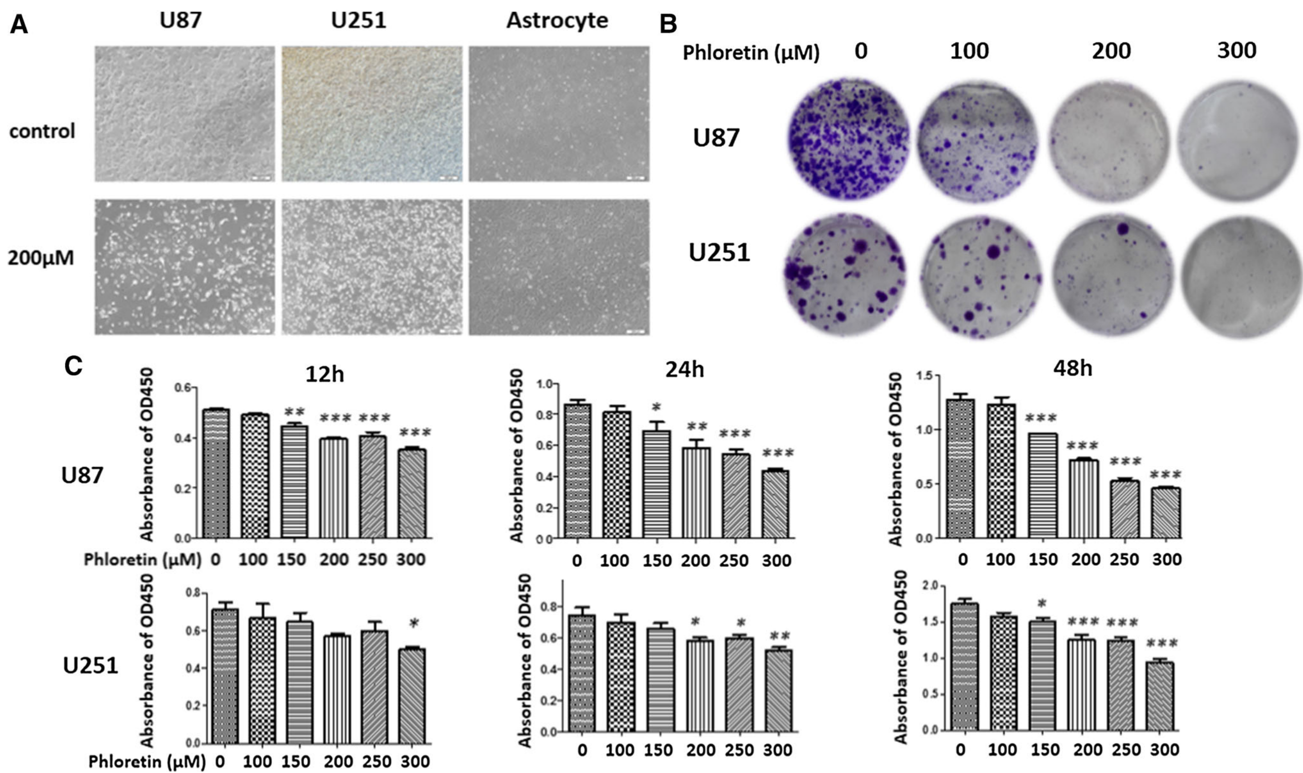


Fig. 1 The effects of phloretin on cell proliferation and colony formation. **a** Phloretin induces morphological change of U87 and U251 but primary astrocytes are unaffected. **b** To determine long-term effects, cells were allowed to form colonies for 7 days. Colony formation of U87 and U251 cell lines were inhibited in a concentration-dependent manner. **c** Inhibition of U87 and U251 cell proliferation by phloretin using the CCK8 assay. The data are shown as the means from three independent experiments. Columns, mean of three experiments; *bars*, SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

(Fig. 1c; Supplementary Tables 1 and 2). Cell viability of primary mouse astrocytes was unaffected by incubating with phloretin at a concentration of 200 μM, which was consistent with several other previous studies (Fig. 1a) [13, 14].

Phloretin induces cell cycle arrest at G0–G1 in U87 cells and regulates the expression of cell cycle-related proteins

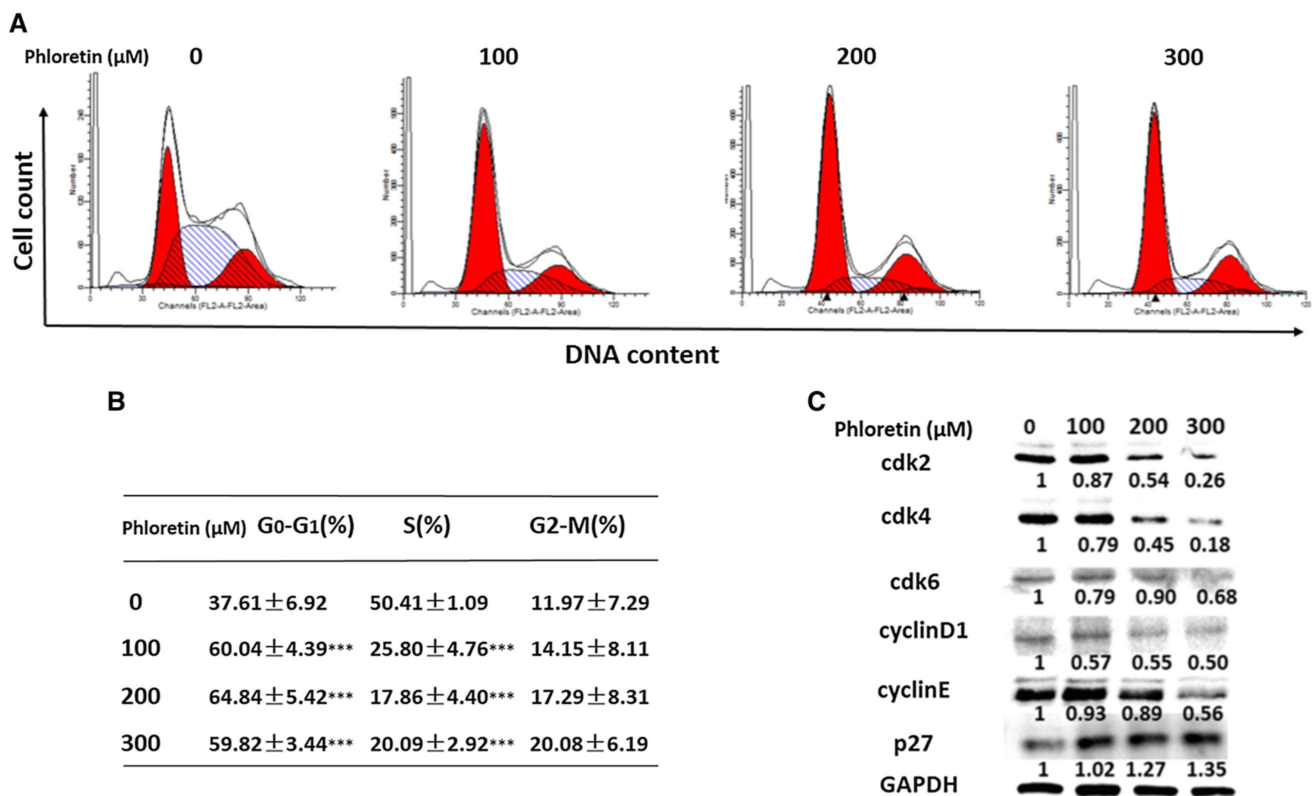
Analysis of cell cycle distribution showed that phloretin-induced cell cycle arrest in U87 cells occurs at the G0–G1 phase. The greatest effect was observed using 200 μM phloretin; approximately 64.8 % of cells were in G0–G1 phase compared to 37.6 % in the control conditions (Fig. 2a, b). Figure 2c shows that phloretin decreased the expression of cyclin D1, cyclin E, CDK2, CDK4 and CDK6 and increased the expression of p27 in a concentration-dependent manner in U87 cells.

Phloretin induced apoptosis and triggered activation of the mitochondrial pathway

Apoptosis analysis showed that phloretin significantly induced apoptosis in U87 cells (38.4 % of cells were stained by Annexin V-PE, and 21.8 % were double stained after treatment with 300 μM for 24 h) (Fig. 3a). The mitochondria of U87 cells appeared to be activated following treatment with phloretin resulting in the activation of caspase-9 (Fig. 3b; Supplementary Table 3), increased expression of pro-apoptotic Bax and Bak and the up-regulation of PARP cleavage. In contrast, the expression of anti-apoptotic Bcl-2 decreased (Fig. 3c). It was suggested that caspase-9 activation and disruption of the mitochondrial membrane were involved in apoptosis. Moreover, it is shown in Fig. 3d and E that phloretin induces ROS accumulation.

Phloretin inhibits PI3K/Akt/mTOR signaling and increases PTEN expression

Following treatment with phloretin, the expression of PTEN, a PI3K/Akt inhibitor, increased, and the expression of p-PI3K, PI3K, p-ERK1/2, total ERK1/2, p-AKT and Akt decreased in a concentration-dependent manner. The expression levels of S6K and p-S6K, both downstream of the PI3K/Akt cascade and substrates of mTOR, were also down-regulated Fig. 4.



Phloretin (μM)	G0-G1(%)	S(%)	G2-M(%)
0	37.61±6.92	50.41±1.09	11.97±7.29
100	60.04±4.39***	25.80±4.76***	14.15±8.11
200	64.84±5.42***	17.86±4.40***	17.29±8.31
300	59.82±3.44***	20.09±2.92***	20.08±6.19

Fig. 2 Phloretin induced G0–G1 phase arrest and affected the expression of proteins involved in cell cycle progression in the U87 cell line. **a** Phloretin-induced cell cycle arrest at the G0–G1 phase. Cells were treated with vehicle or phloretin for 24 h, and cell cycle distribution was assessed using flow cytometry. **b** Statistical analysis of cell cycle distribution from three independent experiments. **c** Regulation of cell cycle protein expression

Antioxidant agents prevent phloretin-induced ROS accumulation, proliferation inhibition and apoptosis

The effects of the antioxidant agents *N*-acetyl-L-cysteine (NAC) and glutathione (GSH) on phloretin-treated cells were investigated. The results demonstrate that pretreatment with NAC or GSH alleviates the accumulation of ROS and prevents the inhibition of proliferation induced by phloretin (Fig. 5a, b; Supplementary Table 4). Moreover, treatment with NAC or GSH also inhibited activation of caspase-9 (Fig. 5c; Supplementary Table 5). These results suggest that ROS generation plays an upstream role in phloretin-mediated proliferation inhibition and mitochondrial apoptosis.

Discussion

This study demonstrates that phloretin-induced cell cycle arrest at G0–G1 and apoptosis in glioblastoma cell lines occurs through the generation of ROS, which has not been previously reported.

It is known that the mitochondrial apoptotic pathway is an important pathway for regulating apoptosis in mammalian cells [15]. ROS have been found to play an important role in stimulating mitotic cell division, inducing cellular senescence and regulating apoptosis. Following treatment with phloretin, we observed a significant increase in the expression of the pro-apoptotic factors Bax and Bak and a decrease in the anti-apoptotic factor Bcl-2, suggesting that changes in the ratio of pro-apoptotic and anti-apoptotic Bcl-2 family proteins might contribute to phloretin-induced apoptosis. Moreover, these changes coincide with the degradation of poly-ADP-ribose polymerase (PARP), a substrate of caspase-3, and the activation of caspase-9. Permeabilization of the outer mitochondrial membrane and subsequent release of cytochrome c could result in the activation of downstream caspases, thus producing excessive ROS [16]. These results indicate that activation of the mitochondrial apoptotic pathway is involved in the apoptosis of glioblastoma cell lines induced by phloretin treatment. The importance of this pathway was further confirmed by treatment with the antioxidant agents, NAC and GSH, which protected cells from ROS generation and inhibited cell proliferation.

A number of studies have indicated that PI3K/Akt/PTEN signaling cascades play an important role in oxidative stress-

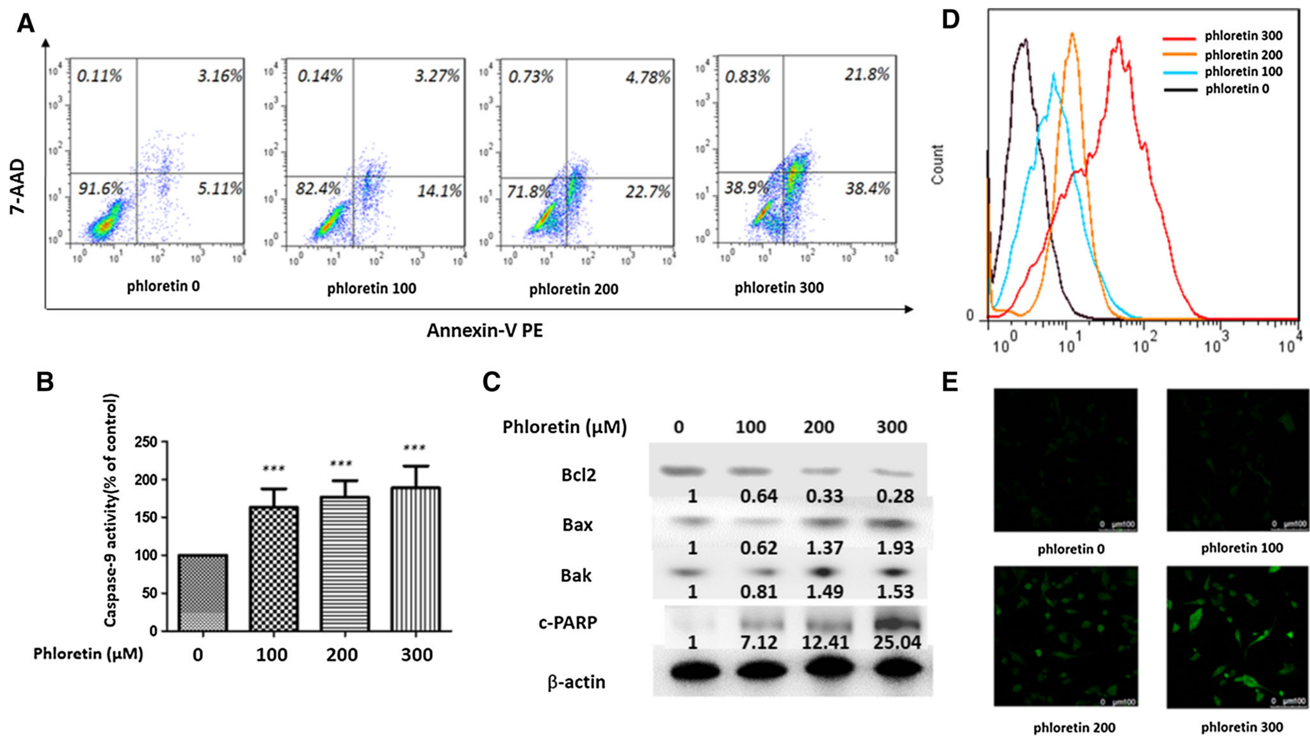


Fig. 3 Phloretin induced apoptosis and generation of ROS in the U87 cell line. **a** Cells were treated for 24 h and analyzed for the apoptotic Annexin-V + population. **b** The activation of caspase-9. **c** The expression of proteins involved in the activation of the mitochondrial pathway is altered following treatment with phloretin. **d** Flow cytometry was used to determine ROS generation after staining with H2DCFDA. **e** The accumulation of ROS was detected by confocal microscopy. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

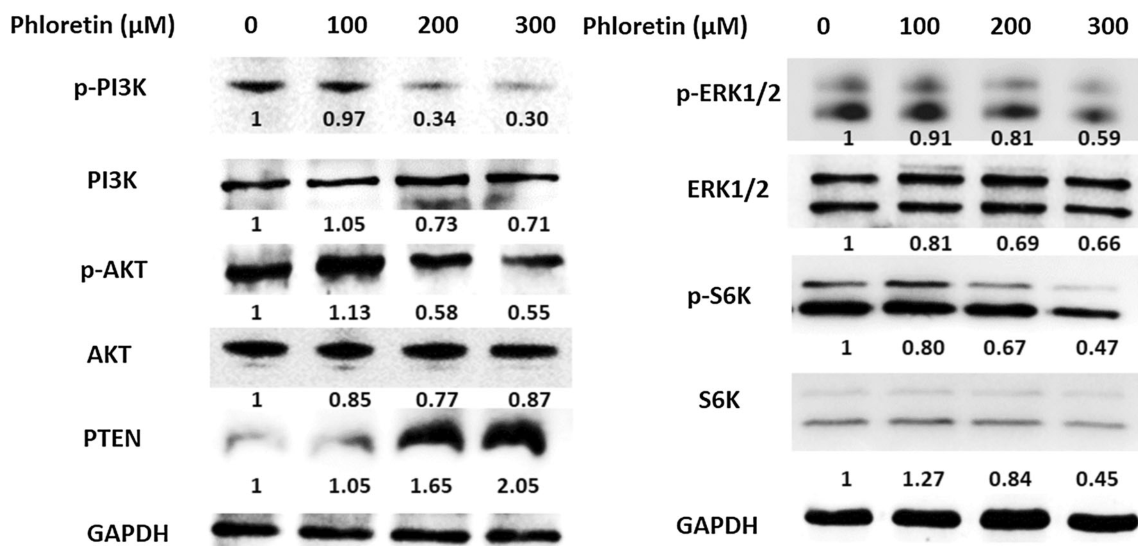


Fig. 4 Phloretin inhibited PI3K/AKT/mTOR signaling and increased PTEN expression; expression of p-ERK1/2 and ERK1/2 were not affected

induced apoptosis in cancer [17, 18]. PTEN is a well-studied tumor suppressor gene implicated in a wide variety of human cancers. Loss of PTEN is a common mechanism for human glioblastoma tumorigenesis. The Akt signaling pathway has been shown to be activated in the majority of primary glioblastoma samples [19], suggesting an important role in the progression of glioblastoma. In this study, we observed up-regulation of PTEN and inhibition of PI3K/AKT activation, which may explain the cell cycle arrest and apoptosis induced by phloretin.

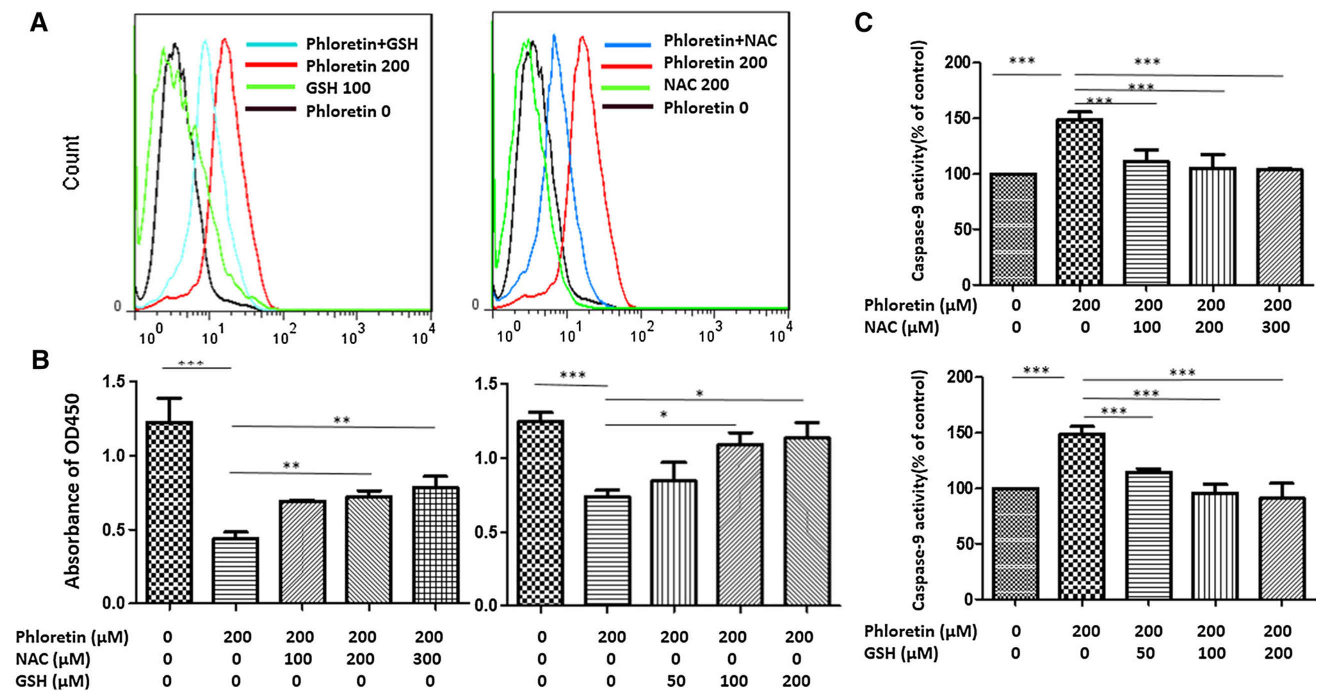


Fig. 5 Antioxidants prevent phloretin-induced ROS accumulation, proliferation and caspase-9 activation. The U87 cell line was pretreated with either NAC or GSH for 1 h prior to phloretin exposure. **a** Flow cytometry analysis for ROS levels after staining with H_2DCFDA. **b** The U87 cell line was assessed using the CCK8 assay at 24 h. **c** Activity of caspase-9 was detected at 24 h. Data are shown as the mean from three independent experiments. Columns, mean of three experiments; *bars*, SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

It has been well established that PTEN induces G1 cell cycle arrest and regulates cell growth in tumor cells by up-regulating the expression of the CDKIs p21 and p27 and down-regulating cyclin and CDKs [20]. Cyclin and cyclin-dependent kinase (CDK) complexes play an important role in regulating the cell cycle. The cyclin Ds, CDK2 and CDK4, are key participants during the transition from G1 to S phase [21]. CDK inhibitors (CDKIs) inhibit the kinase activity of the cyclin-dependent kinase complexes and block cell cycle transitions. CDKIs can be classified into two groups [22]. One group of CDKIs specifically inhibit the cyclin-CDK complexes, including CDK4, CDK6, p16, p15 and p18 [23]. The other group includes p21/Waf1 and p27/Kip1, which can bind to and inactivate cyclin-CDK complexes. These complexes can be composed of various CDKs, including CDK2 and CDK4 [24, 25]. Moreover, our results show that the expression of p27 was up-regulated and the expression of cyclin D1, cyclin E, CDK2, CDK4 and CDK6 were down-regulated following treatment with phloretin.

However, there were some limitations in our study. First, the strong anti-tumorigenic effect of phloretin has not been confirmed in animal models. It is known, however, that dihydrochalcone phloretin can inhibit glucose transmembrane transport (GLUT) [26] and protein kinase C (PKC) [27] and is a naturally occurring non-steroid estrogen [28]. Previous reports suggest that phloretin-induced cancer-specific cytotoxicity is due to GLUT2 inhibition [29] and the inhibition of PKC activity [30]. However, the mechanism of phloretin-induced cytotoxicity in glioblastoma cells has not been previously reported. Further investigation will be necessary to determine the specific molecular target of phloretin in glioblastoma cells.

In conclusion, the results of this study show that phloretin-induced cell cycle arrest and apoptosis of human glioblastoma cell lines occurs through the generation of ROS. We provide evidence that phloretin can induce the up-regulation of PTEN and the inhibition of cyclin-CDK complexes and the PI3K/AKT/mTOR signaling pathway. This suggests that phloretin is a potential anti-tumorigenic agent in glioblastoma, and further studies will be necessary to determine its clinical applications.

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Compliance with ethical standards

Conflict of interest None declared.

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