

Caudatin Inhibits Human Glioma Cells Growth Through Triggering DNA Damage-Mediated Cell Cycle Arrest

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Abstract Caudatin, one of the species of C-21 steroidal glycosides mainly isolated from the root of *Cynanchum bungei Decne.*, exhibits potent anticancer activities. However, the mechanism remains poorly defined. In the present study, the growth inhibitory effect and mechanism of caudatin on human glioma cells were evaluated in vitro. The results revealed that caudatin time- and dose-dependently inhibited U251 and U87 cells growth. Flow cytometry analysis indicated that caudatin-induced growth inhibition against U251 and U87 cells was mainly achieved by the induction of G0/G1 and S-phase cell cycle arrest

through triggering DNA damage, as convinced by the up-regulation of p53, p21, and histone phosphorylation, as well as the down-regulation of cyclin D1. Moreover, caudatin treatment also triggered the activation of ERK and inactivation of AKT pathway. LY294002 (an AKT inhibitor) addition enhanced caudatin-induced AKT inhibition, indicating that caudatin inhibited U251 cells growth in an AKT-dependent manner. Taken together, these results indicate that caudatin may act as a novel cytostatic reagent against human glioma cells through the induction of DNA damage-mediated cell cycle arrest with the involvement of modulating MAPK and AKT pathways.

Xiao-yan Fu and Shuai Zhang are co-first authors.

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Introduction

Malignant glioma represents one of the most common and aggressive primary brain tumors (Su et al. 2006; Chen et al. 2008). Glioma possess high infiltration and invasion capacity into adjacent tissues through migrating along blood vessels, displacing the junction among glial cells and blood vessels, invading, compressing, and destructing the brain parenchyma (Du et al. 2009; Iarosz et al. 2015; Rhee et al. 2009). The survival rate for glioma is extremely low and the mean survival is only 16 months (Ku et al. 2011; Niu et al. 2011). Due to the high invasiveness, high incidence, and resistance toward standard chemotherapy, the cure rate is very low, and new therapeutic strategies are urgently needed (Bourkoula et al. 2014; Liu et al. 2013).

Natural products exhibit potential application in cancer chemotherapy due to their novel pharmacological activities

and less toxicity. *Cynanchum auriculatum*, mainly distributed in China and the other Asia countries, was used as a medicine to nourish the blood and prolong the life in the ancient times (Peng et al. 2008). It has been shown that *C. auriculatum* displayed various pharmacological functions, including immunoenhancement, hepatoprotection, anti-aging, anticancer, and even some nervous system and mental disorders, such as epilepsy, depression, and Meniere's syndrome (Johnson and O'Neill, 2012; Lwin et al. 2013). C-21 steroidal glycoside, a member of Asclepiadaceae families, is thought to be the major active component of *C. auriculatum*. It has been demonstrated that C-21 steroidal glycosides showed multiple pharmacological activities, such as anti-proliferation/invasion of tumor cells, immunoregulation, high efficiency of removing hydroxyl radicals and oxygen free radicals, and protection of liver and nerve cells (Luo et al. 2013). Caudatin, extracted and purified from the root of *Cynanchum bungei* Decne, is a compound of C-21 steroidal glycosides species (chemical structure Fig. 1) (Ma et al. 2011). It is reported that caudatin could induce apoptosis in several cell lines. Besides its prohibitive effect on cancer cell proliferation and invasion, the roles of inhibiting the secretion of HBsAg and HBV DNA replication were also reported (Wang et al. 2012). All the properties make caudatin have powerful chemoprevention and chemotherapy potential against tumor as well as virus infection. However, the underlying mechanisms are not well understood, especially in human brain tumors. Therefore, in the present study, the anticancer activities and mechanism of caudatin on human glioma cells were clarified, and the results revealed that caudatin effectively inhibited human glioma cells growth by triggering DNA-mediated cell cycle arrest with the involvement of modulation of MAPK and AKT pathways.

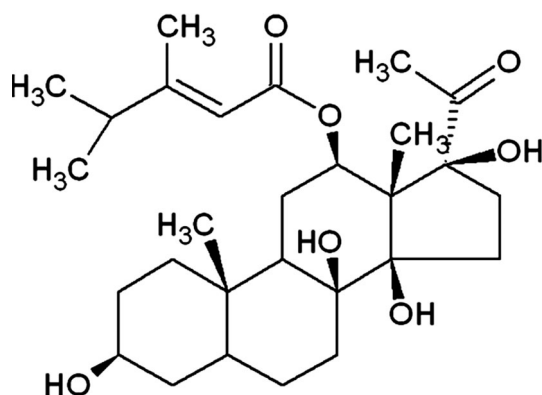


Fig. 1 Chemical structure of caudatin

Materials and Methods

Chemicals

Caudatin, propidium iodide (PI), MTT, and other reagents were all purchased from Sigma. BCA assay kit was bought from CWBIO (Cat CW0014). DMEM and fetal bovine serum (FBS) were bought from Gibco. Parnzyme and penicillin–streptomycin were purchased from Solarbio. All of the solvents related were of high-performance liquid chromatography (HPLC) grade. The water mentioned in this study was provided by a Milli-Q water purification system from Millipore. All antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell Culture

U251 cells (ATCC, USA) were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS and penicillin (100 U/ml) and streptomycin (50 U/ml) at 37 °C under a humidified atmosphere with 5 % CO₂.

MTT Assay

U251 cells (10⁴ cells/well) seeded in 96-well plate were treated with caudatin (0, 25, 50, 100 μM) for 24, 48, and 72 h. After incubation, cell viability was detected by MTT assay. Briefly, MTT could be transformed by succinate dehydrogenase, an important enzyme in cellular metabolism, to a purple formazan crystal in living cells. Therefore, when the cells were treated with caudatin, MTT was added into the wells with 20 μl/well (5 mg/ml) and incubated for another 5 h at 37 °C. Then the medium was replaced by 150 μl/well DMSO. Crystals were dissolved by DMSO and then the intensity of the solvent was measured by microplate reader (Molecular Devices, USA) at 570 nm. The result represents the growth condition of the U251 cells after drug intervention. Cell viability was expressed as the percentage compared to control (as 100 %).

Cell Apoptosis and Cycle Distribution

Cell apoptosis and cell cycle were detected by flow cytometry. Briefly, cells seeded in cell culture dishes (6 cm) were treated with caudatin (0–400 μM) for 48 h. Then the cells were harvested by centrifugation, washed with PBS, fixed with 70 % ethanol at –20 °C overnight, and stained with PI for 2 h in dark. The apoptotic cells show hypodiploid DNA contents (Sub-G1 peak), and therefore the Sub-G1 peak was usually employed to quantify the

apoptotic cells. The cell cycle distribution, G0/G1, S, and G2/M, was analyzed by Modfit LT 4.0 software.

Western Blotting Analysis

Cells were cultured in dishes (10 cm) and treated with caudatin. After treatment, cells were washed by PBS and lysed in lysis buffer in the icy environment. The total cellular protein was extracted and the concentration of the protein was quantified by BCA kit according to the manufacturer's instructions. The total protein was added with samples buffer, boiled for 10 min, and stored at -80°C environment for subsequent SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Briefly, total protein (40 $\mu\text{g}/\text{lane}$) was loaded and separated in 5 % (80 V, 40 min) and 10 % SDS-PAGE (110 V, 70 min), respectively. Then the protein was transferred into polyvinylidene difluoride membrane (PVDF) under 110 V for 90 min. After that, the membranes were blocked with 5 % BSA at room temperature for 2 h. Then, the membranes were incubated with primary antibodies (1:1000) overnight at 4°C and second antibodies (1:2000) for 2 h at room temperature, respectively. At last, the target proteins were scanned with enhanced chemiluminescence reagent (ECL) under an Imaging System (Chemi Doc MP, Bio-Rad). β -actin plays as a positive control.

Results

Caudatin Inhibits Human Glioma Cells Proliferation

Firstly, MTT assay was employed to examine the anti-proliferation activity of caudatin against U251 and U87 cells. As shown in Fig. 2, treatment of cells with caudatin significantly induced cell growth inhibition against U251

and U87 cells in a time- and dose-dependent manner. For instance, cells exposed to 25, 50, and 100 μM of caudatin for 48 h markedly inhibited the U251 cell viability to 78.7, 67.6, and 51.3 %, respectively. And U251 cells treated with 50 μM caudatin for 24, 48, and 72 h showed an obvious decline in cell viability to 80.2, 67.6, and 41.5 %, respectively. This result suggests that caudatin may act as a potential cytostatic agent in hunting human glioma cells growth. Moreover, U251 cells exhibited more sensibility to caudatin than that of U87 cells. Therefore, U251 cells were selected for mechanism study.

Caudatin Triggers Cell Cycle Arrest in Human Glioma Cells

To explore the modes of cell death induced by caudatin, flow cytometry was used to quantify caudatin-induced cell apoptosis and cell cycle distribution. As shown in Fig. 3a, exposure of cells to indicated concentrations of caudatin resulted in significant accumulation of U251 cells in G0/G1 phase, as convinced by the increase of G0/G1 peak, accompanied by the decrease of S and G2/M phases. The statistical analysis of cell cycle distribution exactly confirmed this result (Fig. 3b). However, caudatin inhibited U87 cells growth also by the induction of cell cycle arrest (Fig. S1), but at S-phase arrest, not G0/G1 phase. No significant cell apoptosis in U87 and U251 was observed in 48 h of treatment compared to that of the control group. To evaluate whether cell apoptosis was involved in caudatin-induced cell growth inhibition, the treatment time of caudatin was prolonged to 72 h, and the result indicated that, besides the G0/G1 phase arrest, caudatin also induced obvious cell apoptosis in U251 cells, as convinced by the increase of Sub-G1 peak (Fig. S3).

To evaluate the molecular mechanism involved in the cell growth inhibition induced by caudatin, two regulators of cell cycle, cyclin D1 and cyclin B1, were detected by

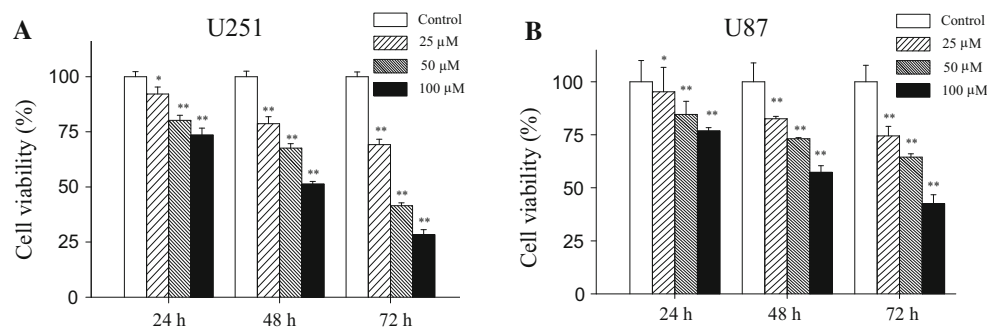


Fig. 2 Caudatin inhibits U251 cells growth. Cytotoxicity of caudatin to U251 (a) and U87 cells (b). Cells seeded in 96-well plate (10,000 cells/well) were treated with 0, 25, 50, or 100 μM caudatin for 24, 48, and 72 h. Cell viability was detected by MTT assay. All data were

expressed as the mean \pm SD of the three independent experiments. Bars with “*” and “***” represent statistical difference at $P < 0.05$ and $P < 0.01$ levels, respectively

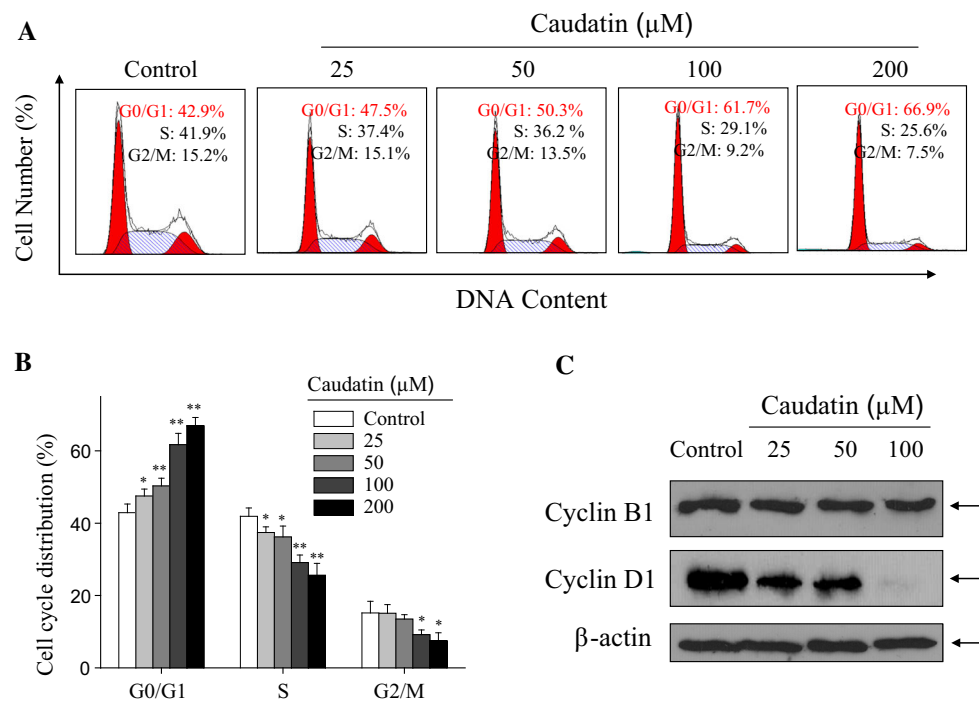


Fig. 3 Caudatin induces G0/G1 phase arrest in U251 cells. **a** Cell cycle distribution. Cells after treatment with caudatin were stained by PI solution, and the cell cycle distribution (G0/G1, S, and G2/M) was analyzed by flow cytometry. **b** Statistical analysis of cell cycle

distribution. **c** Effect of caudatin on cyclin D1. Total protein was extracted from caudatin-treated cells, and the protein expression was detected by western blotting. Bars with “*” and “***” represent statistical difference at $P < 0.05$ and $P < 0.01$ levels, respectively

western blotting method. As shown in Fig. 3c, caudatin significantly decreased the cyclin D1 expression, but had no effect on cyclin B1 expression. Taken together, these results indicate that caudatin inhibited human glioma cells proliferation by the induction of cell cycle arrest.

Caudatin Triggers DNA Damage

Induction of DNA damage by anticancer drugs is an effective way in combating human cancers. Hence, several DNA damage markers were examined. As shown in Fig. 4, caudatin treatment apparently increased the phosphorylated levels of p53, p21, and histone. p53 can be activated in response to DNA damage by affecting several phosphorylated sites, and active p53 can activate p21 and eventually induce apoptosis or/and cell cycle arrest. Therefore, we speculate that the addition of caudatin into U251 cells caused DNA damage and eventually induced G0/G1 cell cycle arrest.

Caudatin Causes Dysfunction of MAPK and AKT Signaling pathways

Both MAPK (including ERK, p38, and JNK) and AKT pathways play important roles in regulating cell proliferation, cell survival, and cell apoptotic signaling. Hence,

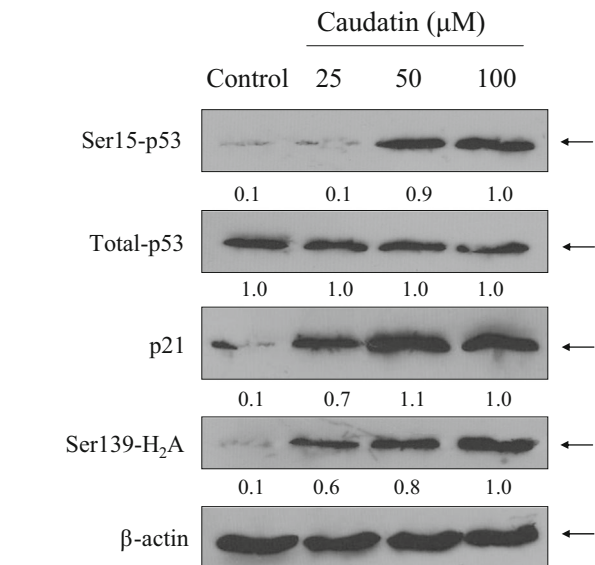


Fig. 4 Caudatin triggers DNA damage. Cells after treatment were collected and lysed, and total protein was used to detect protein expression by western blotting method. The optical density was detected by Quantity-One software to quantify the protein expression. The expression rate (target protein/β-actin) was lined under the bands

we examined whether phosphorylated status of p38, JNK, ERK, and AKT was involved in caudatin-induced cell growth inhibition. The results found that caudatin treatment

dramatically increased ERK expression and decreased AKT expression (Fig. 5), indicating that MAPK and AKT pathways both contributed to caudatin-induced cell growth inhibition. Furthermore, caudatin-induced U87 cells growth inhibition also involved the decline of p-AKT expression (Fig. S2). Moreover, AKT inhibitor (LY294002) pretreatment significantly enhanced caudatin-induced inhibition against AKT expression in U251 cells (Fig. 5c), revealing that caudatin suppressed U251 cells growth in an AKT-dependent manner. No significant changes in expression of p38 and JNK were observed. Besides cell cycle arrest, decreased AKT expression also contributed to caudatin-induced apoptosis (Fig. S4). Taken together, these results above revealed that caudatin inhibited human glioma cells growth mainly by DNA damage-mediated cell cycle arrest with the involvement of modulating MAPK and AKT pathways.

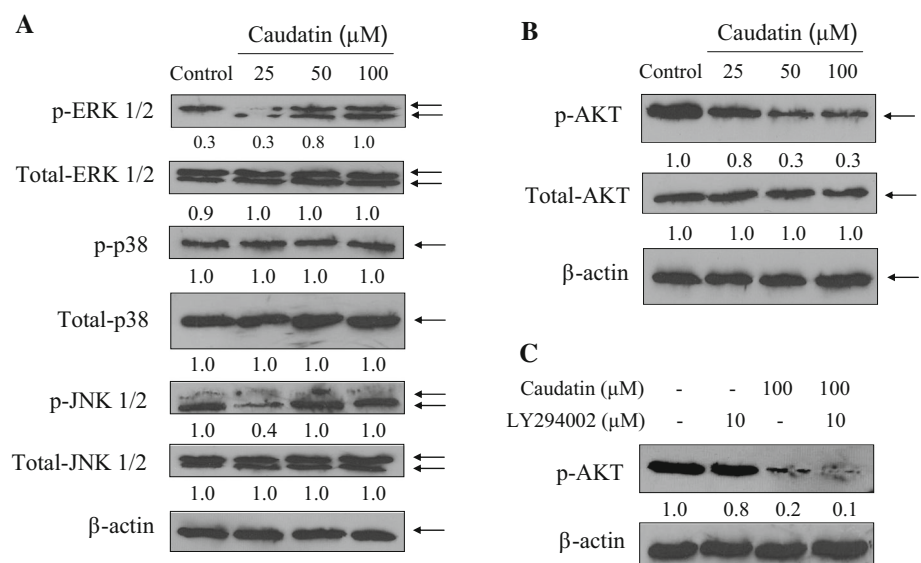
Discussion

Caudatin as a natural product exhibits diverse pharmacological activities (Fei et al. 2012a, b; Li et al. 2013; Ma et al. 2011; Peng et al. 2008). Induction of cell apoptosis or/and cell cycle arrest by natural products are the important ways in hunting cancer cell growth. The cell death process is highly precisely regulated by several signaling pathways which is critical during the development and regulation of cellular homeostasis as well as in the pathogenesis of a variety of diseases including many brain tumors (Charriaut-Marlangue et al. 1998; Czarnota et al. 1999; Jesionek-Kupnicka et al. 1997).

Cell cycle arrest is one of the momentous mechanisms through which many pharmaceutical products exert

antitumor effects (Xiong et al. 2014; Xu and Kim, 2014). Many cytotoxic medicaments and DNA damaging drugs inhibit cell growth by the induction of cell cycle arrest at the G₀/G₁, S, or G₂/M phase (Jin et al. 2014; Liu et al. 2014). As detected by flow cytometry, we observed that the growth inhibition effect of caudatin is highly related to cell cycle arrest of U251 cell lines in the G₀/G₁ phase. G₀/G₁ transition phase is regulated by the controlled expression and activity of various cytokines, including cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs) (Brooks 2005; Warenius et al. 2008). Cyclin and CDK can form a complex to regulate the cell cycle (Chen et al. 2005). Aberrant elevation of cyclins and down-regulation of CKIs have been proven to be poor prognostic features in many cancers (Scott et al. 2004). Numerous studies have demonstrated that p21 played a crucial role in regulating G₁/S transition (Chen et al. 2005). Its overexpression and association with CDKs are implicated in the induction of blockade at a specific stage of the cell cycle. Transcriptional regulation of the p21 gene is controlled by the tumor suppressor protein p53, acting on the p53 responsive element in the distal region of the p21 promoter in response to intracellular signals such as DNA damage (Curro et al. 2014; Liu et al. 2006). Cyclin D1, a regulator of cellular proliferation, is itself regulated by ERK1/2. In the present study, caudatin treatment dose dependently caused a distinct down-regulation in cyclin D1 (a G₀/G₁ phase-regulating protein), whereas the levels of G₂/M phase-regulating cyclin B1 were not changed. Therefore, caudatin-mediated cell cycle arrest at G₀/G₁ phase might be through the inhibition of the formation of CDK/cyclin complexes by the down-regulation of cyclin D1, along with p21 activation in a p53-dependent event in U251 cells.

Fig. 5 Effects of caudatin on MAPK and AKT pathways. Effects of caudatin on MAPK (a) and AKT pathways (b). c Effect of AKT inhibitor (LY294002) on AKT expression in caudatin-treated U251 cells. Cells were pre-treated with LY294002 for 2 h before the caudatin treatment. The protein expression was detected by western blotting and was quantified by Quantity-One software



Progression of cell cycle is governed by the sequential assembly and activation of holoenzyme complexes (Yang et al. 2007). Plenty of evidence indicates that the MAPK and AKT pathways are important mediators of the processes of cell growth and death in response to stressful stimuli, such as UV irradiation, heat, hydrogen peroxide, and DNA damage (Squires et al. 2003; Wang et al. 2010; Ho et al. 2012). Moreover, ERK and JNK have been implicated in p53 phosphorylation that in turn participates in the regulation of p53 activity (Su 2014). In order to testify the apoptotic signaling pathways induced by caudatin, we detect the phosphorylation level of ERK, JNK, and p38 in the caudatin-treated cells. The result suggested that ERK was activated and AKT was inactivated after caudatin treatment in a dose-dependent manner, suggesting that ERK and AKT, but not p38 and JNK, contributed to caudatin-induced G₀/G₁ phase arrest in U251 cells. Furthermore, the activation of p53 and p21 suggested that caudatin-induced G₀/G₁ phase arrest may be mediated by MAPKs through p53-dependent pathways.

In conclusion, our results demonstrated that caudatin inhibited the U251 cell proliferation in a dose-dependent manner by inducing G₀/G₁ arrest through the induction of DNA damage, which was mediated by markedly decreased expression of cyclin D1, as well as the obviously increased expression of p21 and p53 with the involvement of regulating MAPK and AKT pathways. The findings explored that caudatin may act as a beneficial antitumor agent with potential application in chemoprevention and chemotherapy of human glioma.

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Conflict of interest The authors declare that there is no conflict of interest for all the authors.

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