Arctigenin upregulates apoptosis through the AKT/MTOR pathway, inhibiting the proliferation of glioma

ABSTRACT:
Arctigenin (ARG) is a natural lignan compound extracted from arctium lappa and has displayed anticancer functions and effective treatments in a variety of cancers. Studies had shown that Arctigenin (ARG) inhibits tumors through the AKT/MTOR pathway and mediates autophagy. However, the role in glioma cells have not still fully understood. This study was designed to investigate whether Arctigenin (ARG) can mediate AKT/mTOR pathway in glioma to regulate autophagy, and affected glioma cells growth and survival. We found that the dose-dependent downregulation of Arctigenin (ARG), reducing cell proliferation, migration and invasion in two human glioblastoma cell lines (U87, T98G). These phenomena were reversed after the administration of the AKT agonist (SC79). Arctigenin (ARG) also affected other autophagy markers such as p62, LC3B. In addition, the apoptotic molecules cleaved-PARP, caspase-9, and cleaved-caspase3 were also dose-dependently altered.

KEYWORDS: Arctigenin (ARG), AKT/MTOR, autophagy, apoptosis, glioma
1. Arctigenin upregulates apoptosis through the AKT/MTOR pathway, inhibiting the proliferation of glioma

2. Jiang yongan1*, Liu jiayu2*, Hong wangwang1, Fei xiaowei3 and Liu Ru’en2

3. Abstract:

4. Arctigenin (ARG) is a natural lignan compound extracted from arctium lappa and has displayed anticancer functions and effective treatments in a variety of cancers. Studies had shown that Arctigenin (ARG) inhibits tumors through the AKT/MTOR pathway and mediates autophagy. However, the role in glioma cells have not still fully understood. This study was designed to investigate whether Arctigenin (ARG) can mediate AKT/mTOR pathway in glioma to regulate autophagy, and affected glioma cells growth and survival. We found that the dose-dependent downregulation of Arctigenin (ARG), reducing cell proliferation, migration and invasion in two human glioblastoma cell lines (U87, T98G). These phenomena were reversed after the administration of the AKT agonist (SC79). Arctigenin (ARG) also affected other autophagy markers such as p62, LC3B. In addition, the apoptotic molecules cleaved-PARP, caspase-9, and cleaved-caspase3 were also dose-dependently altered.

5. KEYWORDS: Arctigenin (ARG), AKT/MTOR, autophagy, apoptosis, glioma

6. INTRODUCTION:

7. Glioma is the most common central nervous system tumor, and about 50% is glioblastoma. Surgery, radiation therapy, and drug therapy (temozolomide) are routine ways to treat Glioblastoma, but the effect of treatment is not optimistic. The overall survival rate is 12-18 months[1]. Therefore, there is an urgent need to discover new targets or novel drugs for the treatment of glioma. Arctigenin (ARG) is a bioactive lignan extracted from Arctium lappa, and has various biological activities such as anti-cancer[2,3], neuroprotection[4], anti-oxidation effects[5,6], anti-proliferation[7,8], anti-viral[9], and exerting a certain therapeutic effect on the treatment of colorectal cancer, liver cancer, retinoblastoma, prostate cancer. It has been reported that Arctigenin (ARG) can treat colorectal cancer by inducing autophagy[10]. It is also reported that Arctigenin (ARG) inhibited Epithelial mesenchymal transition of Hepatocellular Carcinoma and by suppressing GSK3β-Dependent Wnt/β-Catenin signaling pathway[11]. In addition, it has been reported in the literature that Arctigenin (ARG) can inhibit the proliferation of retinoblastoma Y79 and also promote apoptosis[12]. Although there have been previous articles stating that Arctigenin (ARG) has an inhibitory effect on glioma[13,14], thus deeper mechanisms remains to be discovered. While Autophagy is the process of transporting damaged, denatured or aged proteins and organelles to lysosomes for digestion and degradation, which circulates and degrades intracellular components in response to a lack of nutrients or growth factors to maintain homeostasis. Autophagy has two mechanisms: protective mechanism and death mechanism. The former protects cells from apoptosis by eliminating unfolded proteins, reducing protein load and eliminating damaged organelles (mainly mitochondria), and regulating the activity of caspase8 to regulate cells. Apoptosis, it has been found in the literature that ATG inhibitors can greatly increase caspase8 to cause hepatocyte apoptosis induced by LPS and GalN. The latter is thought to be caused by complete degradation of cellular components due to excessive autophagy, manifested by caspase-8 activation and the formation of apoptotic intracellular death-inducing signaling complex (iDISC), such as knockdown of ATG5, which inhibits ATG7. The initial phase of
phagocytosis to reduce the production of caspase-8 and the occurrence of apoptosis, there are also articles that the degradation of caspase-8 can protect U87 glioma cells from H2O2 injury. However, the AKT/mTOR pathway plays an important role in a variety of cancers, except that it is involved in the differentiation of bone marrow mesenchymal stem cells and various osteoblasts, mainly mediated by autophagy. In this study, we have investigated the effects of Arctigenin (ARG) on the cycle and apoptosis of glioma cells (U87, T98) and further explored whether these effects are due to the AKT/mTOR pathway. In addition, we further determined the important role played by autophagy.

Methods and Materials

Chemicals, Reagents and Antibodies
ARG was purchased from tianjing wanxiang Science and Technology Ltd (Tianjin, China). ARG was dissolved in Dimethyl sulfoxide (DMSO) and diluted to 10 mM with phosphate buffered solution (PBS) and stored at 4°C. Dulbecco’s modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, USA). Antibodies against AKT, P-AKT, AMPK, P-AMPK, mTOR, P-mTOR, p62, LC3B were purchased from Abcam.

Cell Culture
The glioma cell lines (U87, T98) were purchased from Chinese Academy of Medical Sciences (Beijing, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (FBS) and incubated in an incubator containing 5% CO2 at 37 °C.

Cell Viability Assay
2 × 103 cells were uniformly cultured in 96-well plates for 0h, 12h, 24h, 48h, 60h, 72h, and treated with different concentrations of ARG. Add 10μl of CCK8 to each empty and continue to incubate at 37 °C for 30min. The solution was detected by the microplate reader.

Cell monoclonal formation assay
Approximately 5000 cells were cultured in a 10 cm culture dish, and the cells were treated with ARG at a concentration of 100μM, 200μM, 400μM for 15 days. After the treatment, the cells were washed 2-3 times with PBS, fixed with 4% paraformaldehyde for 15 min, and fixed with crystal violet. And calculate the number under the microscope.

Cell Apoptosis Analysis
The mixture was thoroughly mixed in 500 μl of binding buffer, 5 μl of propidium iodide (PI), and 5 μl of FITC-conjugated anti-Annexin V antibody, and the results were measured by Accui C6 flow cytometer (BD, USA) within one hour.

Cell Cycle Analysis
1 × 105 cells (U87, T98) were incubated overnight in 6-well plates, and cells were treated with different concentrations of ARG. The cells were collected, washed with PBS for 2-3 times, resuspended in 70% ethanol for 24 h, and then washed with ethanol in PBS, and added to the mixture with PI/RNase Staining Buffer. Finally, Accui C6 flow cytometer (BD, USA) was used to measure results.

Western Blotting
Cell lysates of glioma cells (U87, T98) were used, and proteins were extracted using Pro-prepTM protein extract (Korea iNtRON Biotechnology). Proteins were separated on different concentrations of sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to
ARG Inhibits The Growth Of Glioblastoma Cells

To investigate whether ARG had an effect on glioma cells, we applied CCK8 assay to detect the cell viability of U87MG and T98G, These Glioma cells were treated with different concentrations of ARG in U87MG and T98G, cell activity decreased from 60% to 40% when the ARG concentration was reduced from 200 μM to 400 μM at 48h (Figure 1a). In the next experiment, we used 100 μM, 200 μM, 400 μM for following experiments, and we functioned the monoclonal antibody conjugated with HRP were incubated for 1 h at room temperature, and the results were obtained with electrochemiluminescence (ECL) (Pierce, Rockford, IL, USA).

Cell was seeding in 96-wells plates and were treated with indicated concentrations of PF for 15 days, and it was fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 1 h. Cells were incubated with 200 μM, 400 μM ARG for 48 hours, and cell proliferation using Edu assay, The nucleus used DAPI (blue), and the proliferated cells used Azide 488 (green).

Cell proliferation, cell cycle and apoptosis effects of ARG on glioma cells (U87MG, T98G), were detected by annexin V-FITC assay and measured within 1hr, The Annexin V-FITC axis represents cells that are early apoptotic, and the PI axis represented cells in the mid-late stage of apoptosis. The cell cycle was determined by PI/rNase staining buffer assay, and the results were obtained with electrophoresis (Figure 2b). Therefore, we confirmed that ARG induced cell cycle arrested and apoptosis.

Statistical Analysis

The experiment was repeated at least three times and using an independent t test to compare between the two groups. One-way analysis of variance was used to compare analysis between multiple groups on SPSS 20.0 software. P < 0.05 was considered statistically significant.
ARG regulated AKT/mTOR and levels of autophagy-associated proteins

Many reports that the AKT/mTOR pathway plays an important role in tumors We therefore considered whether the AKT/mTOR pathway is relevant in the mechanism of ARG treatment of glioblastoma After treatment with ARG at a concentration of 100 μM, 200 μM, 400 μM for 48 h, treated glioma cells (U87MG, T98G) significantly reduced the expression of phosphorylated AKT, mTOR, correspondingly regulated the expression of phosphorylated akt, mTOR, but there were no significant changes in AKT, mTOR, and AMPK of total protein level (Figure 2a). Our study of ARG may inhibit glioma cell proliferation by AKT/mTOR-mediated autophagy pathway Therefore, we further used Western blotting to reveal the expression of important autophagy-associated marker factors (Figure 2a). The cells were treated at a concentration of 100 μM, 200 μM, and 400 μM for 48 h. It is clear that ARG can significantly increase the expression of LC3B II and P62 expression. Therefore, we believe that the reason ARG affects glioma cell proliferation may be due to AKT/mTOR-mediated autophagy.

Fig. 2. ARG regulated AKT/mTOR and levels of autophagy-associated proteins a. Western blotting was used to show AKT, P-AKT, mTOR, P-mTOR, LC3B, P62 expression levels on ARG-treated U87MG, T98G. b. Western blotting was applied to display protein expression levels on ARG-treated U87MG, T98G cell cycle and apoptosis. All experiments were repeated three times, *p<0.05; **p<0.01; ***P<0.001.

SC79 significantly rescued proliferation, cell cycle arrested, apoptosis induced by ARG in glioma cells

It was previously demonstrated that ARG may induce autophagy by AKT/mTOR to inhibit proliferation of glioma cells and affect cell cycle arrested and apoptosis. To further confirm, we used an agonist, SC79, an activator of AKT, to restore the ability of the AKT pathway. We found that glioma cells (U87MG, T98G), were incubated in ARG and SC79 for 48 hours. The proliferative capacity was tested by Edu assay, and it was found that the group treated with ARG and SC79 was more significant than the group treated with ARG alone (Figure 3a). The result indicated ARG significantly restore the proliferation of glioma cells, and we next applied PI/rNase buffer, PI-FITC-annexin to determine their cycle and apoptosis. Interestingly, SC79 not only restores their proliferative capacity, but also repairs cycle arrest and increased apoptosis (Figure 3b). The expression of cell cycle-associated proteins and apoptosis-related proteins was verified by Western blotting (Figure 4b). The results showed that SC79 can reverse the toxic effects of ARG on glioma cells.

Fig. 3: SC79 regulated glioma cell proliferation inhibition, cycle arrested, and autophagy apoptosis treated with ARG. a. After 48 hours of incubation with ARG (400 μM) and SC79 (5 μg/μL), nuclei was stained with DAPI. Edu assay was applied to detection and observation was performed using a confocal microscope. b. After 48 hours, the treated cells were collected and fixed with 70% ethanol for 12 hours, centrifuged and washed twice with PBS, treated with PI/rNase staining buffer assay, and detected by Accuri C6 flow cytometer after 15 minutes. c. After 48 hours, the suspended treated cells were collected, and 20,000 cells were counted. After treatment with PI-FITC-annexin assay, the results were measured with a C6 flow cytometer within 1 hours. All experiments were repeated three times, *p<0.05; **p<0.01; ***P<0.001.

SC79 reversed autophagy of glioma cells induced by ARG through AKT/mTOR pathway

Our study of ARG affects glioma cell proliferation inhibition, cycle arrested, and apoptosis. Therefore, we further used Western blotting to reveal the expression of important autophagy-associated marker factors (Figure 2a). The cells were treated at a concentration of 100 μM, 200 μM, and 400 μM for 48 h. It is clear that ARG can significantly increase the expression of LC3B II and P62 expression. Therefore, we believe that the reason ARG affects glioma cell proliferation may be due to AKT/mTOR-mediated autophagy.

Fig. 2. ARG regulated AKT/mTOR and levels of autophagy-associated proteins a. Western blotting was used to show AKT, P-AKT, mTOR, P-mTOR, LC3B, P62 expression levels on ARG-treated U87MG, T98G. b. Western blotting was applied to display protein expression levels on ARG-treated U87MG, T98G cell cycle and apoptosis. All experiments were repeated three times, *p<0.05; **p<0.01; ***P<0.001.

SC79 significantly rescued proliferation, cell cycle arrested, apoptosis induced by ARG in glioma cells

It was previously demonstrated that ARG may induce autophagy by AKT/mTOR to inhibit proliferation of glioma cells and affect cell cycle arrested and apoptosis. To further confirm, we used an agonist, SC79, an activator of AKT, to restore the ability of the AKT pathway. We found that glioma cells (U87MG, T98G), were incubated in ARG and SC79 for 48 hours. The proliferative capacity was tested by Edu assay, and it was found that the group treated with ARG and SC79 was more significant than the group treated with ARG alone (Figure 3a). The result indicated ARG significantly restore the proliferation of glioma cells, and we next applied PI/rNase buffer, PI-FITC-annexin to determine their cycle and apoptosis. Interestingly, SC79 not only restores their proliferative capacity, but also repairs cycle arrest and increased apoptosis (Figure 3b). The expression of cell cycle-associated proteins and apoptosis-related proteins was verified by Western blotting (Figure 4b). The results showed that SC79 can reverse the toxic effects of ARG on glioma cells.

Fig. 3: SC79 regulated glioma cell proliferation inhibition, cycle arrested, and autophagy apoptosis treated with ARG. a. After 48 hours of incubation with ARG (400 μM) and SC79 (5 μg/μL), nuclei was stained with DAPI. Edu assay was applied to detection and observation was performed using a confocal microscope. b. After 48 hours, the treated cells were collected and fixed with 70% ethanol for 12 hours, centrifuged and washed twice with PBS, treated with PI/rNase staining buffer assay, and detected by Accuri C6 flow cytometer after 15 minutes. c. After 48 hours, the suspended treated cells were collected, and 20,000 cells were counted. After treatment with PI-FITC-annexin assay, the results were measured with a C6 flow cytometer within 1 hours. All experiments were repeated three times, *p<0.05; **p<0.01; ***P<0.001.

SC79 reversed autophagy of glioma cells induced by ARG through AKT/mTOR pathway

Our study of ARG affects glioma cell proliferation inhibition, cycle arrested, and apoptosis. Therefore, we further used Western blotting to reveal the expression of important autophagy-associated marker factors (Figure 2a). The cells were treated at a concentration of 100 μM, 200 μM, and 400 μM for 48 h. It is clear that ARG can significantly increase the expression of LC3B II and P62 expression. Therefore, we believe that the reason ARG affects glioma cell proliferation may be due to AKT/mTOR-mediated autophagy.
As demonstrated by previous experiments, SC79 can reverse the growth inhibition of glioma caused by ARG and can reverse its effect on cycle and apoptosis. To this end, we further study whether SC79 also causes the above phenomenon through the AKT/mTOR pathway. Western blotting technology is applied to next explorations. As shown in the (figure 4 a), after administrated to the AKT agonist SC79, the proteins associated with the AKT/mTOR pathway are altered, and the phosphorylated proteins of AKT and mTOR are quite different from those of ARG alone, such as down-regulating P-AKT. The expression level of AKT, while total AKT did not change significantly, and phosphorylated mTOR also showed the same result. So far, we have further determined the toxic effects of ARG on glioma cells (U87MG, T98G), and this inhibition is mediated by the AKT/mTOR pathway, and SC79 can reverse this result. Interestingly, SC79 also reversed the reduction of autophagy-associated molecules (LC3B II) in glioma cells caused by ARG and reduced the conversion of LC3B I to LC3B II. Therefore, we suspect that the target of ARG inhibition of glioma may be related to autophagy, and further experimental evidence is needed.

**Figure 4:** SC79 regulated autophagy of glioma cells induced by ARG through AKT/mTOR pathway. a. Proteins were separated on different concentrations of SDS-PAGE and incubation with the corresponding monoclonal antibody for more than 18 hours, secondary antibody incubation for less than 2 hours, the results were obtained with ECL, was analyzed with the Bio-Rad gel imaging system. b. The detection method was the same according to the previous Western blot technique, the corresponding primary antibody was used, and the results were observed with the Bio-Rad gel imaging system. All experiments were repeated three times, *p<0.05; **p<0.01; ***P<0.001.

**DISCUSSION**

As the main source of therapeutic drugs, plant extracts are increasingly showing that extracts from plants can greatly prolong the survival of patients and are potential medical treasures in the future. As an extract of Arctium lappa, Arctigenin (ARG) has important potential for anti-cancer, and there are reports that it also has therapeutic effects in the treatment of cancer [10, 15, 16, 6]. However, in our current study, we further determined that Arctigenin (ARG) mediates autophagy via the AKT/mTOR pathway to inhibit glioma proliferation, cell cycle arrested, and apoptosis. Our aim was to investigate the treatment of ARG in Glioblastoma. However, ARG also inhibits the survival of hepatocellular carcinoma, inhibits cell proliferation induced by ROS-mediated mitogen-activated protein kinases [17, 18], and inhibits epithelial-mesenchymal transition in peritoneal mesenchymal cells [19]. ARG-enhanced cisplatin has also been reported, increasing sensitivity to drug resistance in colorectal cancer cells [10]. Therefore, in order to study the mechanism of ARG treatment of glioma, further to determine its potential therapeutic targets. In the present study, ARG dose-dependently inhibited the proliferation of glioma cells, and the monoclonal ability of glioma cells was also inhibited by ARG. Abnormal changes in the activity of the cell cycle and apoptosis-related proteins will result in the proliferation of the cells themselves. Cell cycle regulators are therefore also considered to be very attractive targets for the treatment of tumors. In our study, we revealed that ARG can block cell cycle arrest caused by G1/S arrested. We also detected dose-dependent expression of cytosin CDK4 and Cyclin D1 in U87MG, T98G after treatment with ARG. However, there are articles pointing out that ARG blocks glioma cell cycle in G0 phase [13]. At the same time, apoptosis is also considered to be the mechanism of programmed cell death and is considered to be an important and essential
therapeutic and selective target for the treatment of tumors. ARG has been confirmed by various studies to play a role in inducing apoptosis in different cancers. For example, in hepatocellular carcinoma, colorectal cancer, retinoblastoma, lymphoma, ARG can be triggered by intracellular signals by different pathways, such as apoptosis, such as genotoxic stress, or by external signals, such as binding of ligands to cell surface death receptors. Our research shows that ARG was applied to induce the apoptosis of glioma (U87MG, T98G). And a concentration-dependent transition of early apoptosis to late apoptosis, further depleted expression of related apoptosis proteins caspase-9, cleaved-caspase-3 were increased. After we used the AKT activator SC79, the cell cycle arrested and apoptosis was removed. Autophagy, another mechanism of death, has the basic function of degrading cellular proteins and organelle fragments to recover new nutrients, maintaining cellular homeostasis of normal physiological functions. It has been previously reported in the literature that ARG activation posture enhances the sensitivity of cisplatin to drug-resistant colorectal cancer. The microtubule-associated protein LC3 is an important autophagic regulator, and the conversion of LC3B I to LC3B II is considered to be the mechanism of autophagy. It has cytoprotective and cytotoxic effects. In our study, we determined by Western blotting that ARG dose-dependent increases the expression of LC3B II and P62 in glioma cells. This is a classic manifestation of autophagy. Therefore, ARG is considered as an important regulator of the treatment of glioma and induction of autophagy. However, mTOR is considered as a key protein regulating autophagy, and it has been reported in the literature that autophagy regulates the progression of gastric cancer, pancreatic cancer, and esophageal cancer. The role of AKT in the proliferation of tumors cannot be ignored, by activating mTOR phosphorylation to the occurrence of autophagy. It acts as an intermediate medium between upstream protein kinase B (Akt) and downstream p70S6K and 4EBP1. We used Western blotting to detect AKT, mTOR and phosphorylated forms. ARG alone reduced the level of phosphorylated protein in a dose-dependent manner, whereas after co-treatment with AKT agonist SC79, the phosphorylated form of AKT, mTOR, P70S6K increased. Our studies indicate that ARG leads to activation of autophagy via the AKT/mTOR pathway. This indicates that ARG inhibits glioma cell proliferation by activating autophagy by AKT/mTOR. Although we have perfected the in vitro experiments, we have not been able to determine the role of ARG in the body because we have not performed in vivo experiments, so further proof is needed. In summary, Arctigenin (ARG) inhibits proliferation of glioblastoma, inhibits cell cycle, and increases apoptosis. This mechanism involves AKT/mTOR signaling. Our studies also confirm that Arctigenin (ARG) can be used as a potential promising treatment for glioblastoma.

Conflict of interest: The authors report no conflicts of interest in this work.

Funding: This work was supported by grant from the “National Natural Science Foundation of China” (Grant No. 81573774).

Ethical approval: This research does not include any studies with human participants or animals by any author.

Informed consent: This work received consent from all individuals involved in the research.

References


