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Biological Effects of Artemether in U251 Glioma Cells

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Abstract

Glioma is one of the most common brain tumors, and there is no effective treatment method so far. Thus, it is very important to find a safe and effective agent for Glioma. Artemether (ART) is a derivative of artemisinin, which has been reported as an anticancer agent. To understand the mechanism of ART against cancer, we investigated the impacts of ART on the expression of c-MET, API5, VEGF and Bcl-2 on human glioma cell U251. We treated U251 cells with ART at different concentrations. The inhibition rates of cell growth were tested by using MTT assay. The cell proliferation and invasion were tested with transwell. The cell cycles and apoptosis were tested by using flow cytometry and Western blot, respectively. Finally, the expressions of c-MET, API5, VEGF, Bcl-2 and mTOR proteins were tested by West blotting. Our study demonstrated that the expressions of c-MET, API5, VEGF and Bcl-2 were significantly suppressed by ART compared to the control group ($p < 0.05$). ART can suppress U251 cell proliferation and invasion. The apoptotic bodies were observed after 48 hours ART treatment and cell apoptosis was significantly promoted ($p<0.05$). We concluded that ART can suppress U251 cell proliferation and invasion by downregulating the expression of c-MET, API5, VEGF and Bcl-2. In summary, our study demonstrates ART may be an effective therapeutic agent with multiple targets properties for glioma.

Keywords: Human Glioma Cells; Apoptosis; Invasion; Target Sites; Artemether

Introduction

Gliomas is a tumor that originates from the glial cells of the brain or the spine [1]. Gliomas accounts for about 80 percent of all malignant brain tumors [2]. Glioblastoma is the most aggressive and dominant type of primary brain tumor [3]. After diagnosis, the survival rate of glioblastoma patients is less than two years [4]. Therefore, it remains a priority to seek a therapeutic agent for gliomas.

The C-Met gene, encodes c-MET protein, was first described in 1984 [5]. c-MET proteins possess a tyrosine kinase that is activated by binding to its receptor hepatocyte growth factor (HGF). Then activated c-MET protein leads to the tumor diffusion, proliferation, angiogenesis, metastasis and invasion [6-8]. Studies have confirmed that c-MET is increased in colorectal cancer, thyroid cancer, nasopharyngeal carcinoma, lung cancer, breast cancer and other malignant tumors [9-13]. Apoptosis inhibitor-5

(API5) is encoded by the API5 gene [14] which is increased in cervical cancer [15,16]. Studies have suggested that API5 proteins are potential targets for anti-cancer drug development [14,16].

Vascular endothelial growth factor (VEGF) [17] plays a crucial role in the angiogenesis of tumors [18,19]. It was reported that VEGF was overexpressed in anoxic tumor cells [20]. In addition, B-cell lymphoma 2 (BCL2) gene is an anti-apoptotic factor which is involved in normal B-cell development and differentiation [21,22]. Studies have also reported that BCL 2 protein was overexpressed in many human cancer types, including lymphoma, leukemia and carcinoma [23].

In summary, we proposed that c-MET, API5, VEGF and Bcl-2 were ideal targets for anti-glioma drugs. Artemether (ART) was first isolated from the Artemisia annual L by You-you Tu [24] and has been widely used for the treatment of malaria [25-29]. It was reported that ART and its derivatives have inhibitory activity against breast cancer, leukemia, gastric cancer and endometrial cancer [30-32] and it has been shown that artemether has inhibitory effects on the proliferation of Nb2a cells and glioma C6 cells [33]. Our group has demonstrated that ART has inhibitory effect to the growth and anti-apoptotic effects to C6 cells and CT-26 cells on animal models [34,35].

The success treatment of ART to cerebral malaria proved that it can pass through the blood-brain barrier (BBB) [36-38]. In the present study, we first explored the anti-glioma effects of ART by *in vitro* assay using glioma cells U251. We tested the expression of c-MET, API5, VEGF and Bcl-2 protein levels after ART treatment to understand the molecular mechanism of ART against gliomas.

Materials and Methods

Materials

Human glioma cells (U251) were obtained from ATCC (KCB200965YJ). U251 cells were cultured in DMEM containing 10 % fetal bovine serum (FBS) and were then placed in incubator at 37°C and 5 % CO_2. ART was obtained from Kunming Pharmaceutical Ltd. 2,2′-Bicinchoninic acid (BCA) protein assay kits were purchase from Tiangen Biotech. MTT Cell Proliferation assay kits were purchased from Sigma. DMEM/F12 medium, fetal bovine serum, Opti-MEM medium, phosphate buffer solution (PBS) and 0.25 % trypsin were purchased from Gibco. Rabbit anti-human c- MET, rabbit anti-human API5, rabbit anti-human Bcl-2 and rabbit anti-human VEGF monoclonal antibody were all from Cell Signaling Inc. (USA). Rabbit anti-human GADPH monoclonal antibody and horseradish peroxidase (HRP) conjugated Goat anti- rabbit IgG were from Santa Cruz (USA). SDS-PAGE Gel Preparation Kits were from Shanghai Beyotime Biotechnology. Ampicillin (Amp) was from Shanghai Solarbio Life Science. The Coulter DNA Prep reagents kits were from Beckman Coulter.

Methods

Cell toxicity detection assay

U251 cell growth inhibition and the 50 % inhibitory concentration (IC50) of ART were tested by MTT assay: U251 cells were seeded into 96-well plates at 5×10^3 cells per well. After 24 hours, ART solutions were added to the designated wells to reach at concentrations of 25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL and 800 μg/mL respectively. After treatment with ART, 20 μL of MTT (5 g/L) agent was added to each well and incubated at 37°C for 4 hours in the dark. Then 200 μL dimethyl sulfoxide (DMSO) (Sigma, USA) was added to each well to dissolve the precipitate. Optical density (OD) was measured at the wavelength of 490 nm. Data were presented as the mean ± SD, and each dilution has triplicates. There are least three independent experiments.

The *in vitro* cell invasion assay

Cell invasion was assayed in a 24-well trans-well chamber. Briefly, the cells were plated in 25 cm^2 flasks, maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum, and incubated at 37°C with 5 % CO_2. The cell culture medium was replaced by the Opti-MEM serum-free culture medium for 48 hours after ART treatment, and then cultured for another 12 hours. Afterward, 60 μL of Matrigel diluted with DMEM/F12 was added to the top chamber. After trypsin digestion, cells were seeded into the top chambers at 1×10^5 cells in 200 μL Opti-MEM per well. Subsequently, 600 μL DMEM/F12 containing 10 % FBS was added to the lower chambers and then the Trans-well plate was placed in a CO_2 incubator at 37°C for 24 hours**.** The noninvasive cells were removed with a cotton swab. The cells that migrated through the lower membrane were fixed with methanol and stained with 0.1 % crystal violet. For quantification, cells were counted under a microscope in five predetermined fields. Assays were performed in triplicates at each condition.

The detection of cell apoptosis

The apoptotic rate of U251 cells was detected using flow cytometry (FCM) 48 hours after ART treatment. Briefly, 1×10^6 cells in each treatment group were fixed with 70 % ethanol and placed overnight in an incubator at 4°C. After washing with 1×PBS, the cells were stained for 30 minutes with the Coulter DNA-Prep Reagents Kit (Beckman Coulter). Then, cell cycles and cell apoptosis were measured by Bechman Coulter flow cytometry. Data were calculated with the Wincycle software.

The apoptosis of U251 cells was detected using transmission electron microscopy (TEM) 48 hours after the ART treatment. Cells were fixed with 3.5 % glutaraldehyde solution and 1 % osmic acid solution and then dehydrated by the following steps: 1) 50 % ethanol solution; 2) 70 % ethanol solution; 3) 90 % ethanol solution; 4) a mixture of 90 % ethanol solution and 90 % acetone solution at 1:1; 5) then 90 % acetone solution. Samples were incubated in each of the solutions described from step 1 to 5 for 15 mins; Samples were soaked in 100 % acetone solution for 10 mins for twice. Subsequently, the embedding agents were used embed the sample and thus the semi-thin sections were prepared. In addition, an optical microscope was used to locate and modify the sample block. With a Leica-R type ultrathin sectioning apparatus, sections were obtained for counterstain and then the images were observed and collected by transmission electron microscope.

Western blot

After being treated for 48 hours, cells in each group were collected and washed with 1×PBS and then lysis buffer was added to each cell. Protein concentration was quantified with BCA assay, and then lysis buffer was used to bring to the same concentration. Thirty micrograms of total proteins were separated on a 10 % SDS- PAGE gel and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) at 200 mA for 2 hours. The membranes were blocked for 1 hour at room temperature with 1×TBST buffer containing 5 % skim milk and incubated with the different preliminary antibodies (diluted at 1:1000 with 1×TBST), including rabbit anti-human c-MET, anti-API5, anti-VEGF, anti-Bcl-2, and anti-GAPDH (as an internal reference) and kept at 4°C for overnight, Then membranes were washed for three times at 5 minutes each time with 1×TBST and incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (diluted at a volume ratio of 1:1000 with 1×TBST) for 1 hour at room temperature.

The membranes were then washed three times with 1×TBST. Membranes were detected using chemiluminescence (enhanced chemiluminescence; Amersham Pharmacia Biotech) according to the manufacturer's manual. Each experiment was repeated at least twice with same protocol.

Statistical Analysis

Statistical analysis was performed using Prism 6 (GraphPad Software, La Jolla, USA). Data were presented as mean ± standard error of mean (SEM), and the differences among groups were analyzed by one-way analysis of variance (ANOVA) and followed by using Tukey's multiple comparisons test. The significant level was set at $p< 0.05$.

Results

Our results demonstrate that ART can suppress the growth of U251 glioma cells: The effect of ART on the growth of U251 cells is shown as in Figures 1 and 2. Treatment with ART led to morphologic changes of U251 cells, such as an obvious decrease in adhesion (Figure 1a). Furthermore, ART treatment resulted in the decrease of cell viability with concentration and time-dependent manner and the IC_{50} values is at 400 μg/mL after 72 hours treatment (Figure 1b). Interestingly, we also demonstrated that ART can significantly inhibit cell growth ($P<0.05$) (Figure 2).

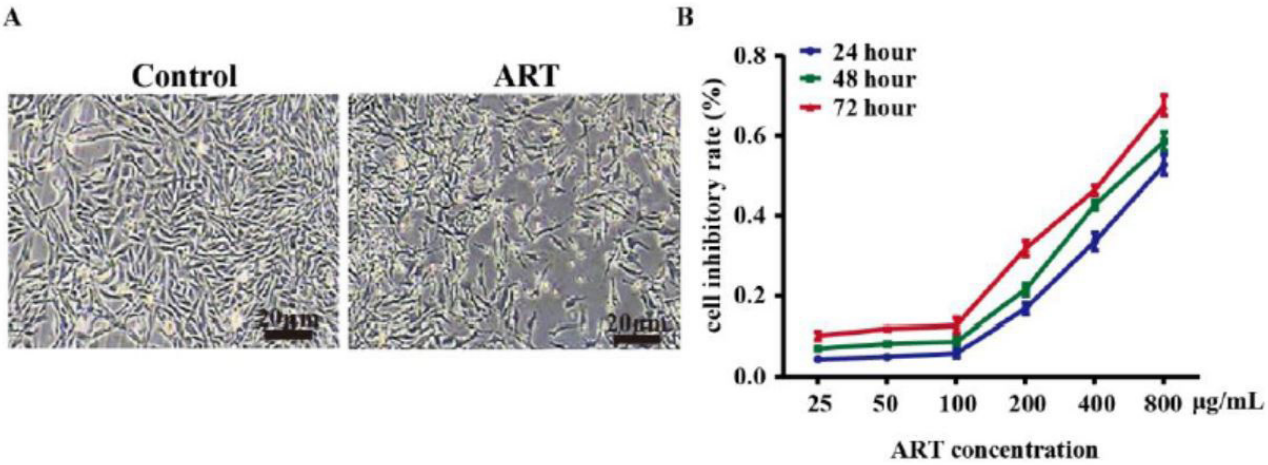

Figure 1: Inhibitory effects of ART on U251 cells. ART could suppress the growth of U251 glioma cells. (A) Morphological changes were observed in ART-treated glioma cells; (B) ART resulted in a comparable dose- and time- dependent decrease in cell viability

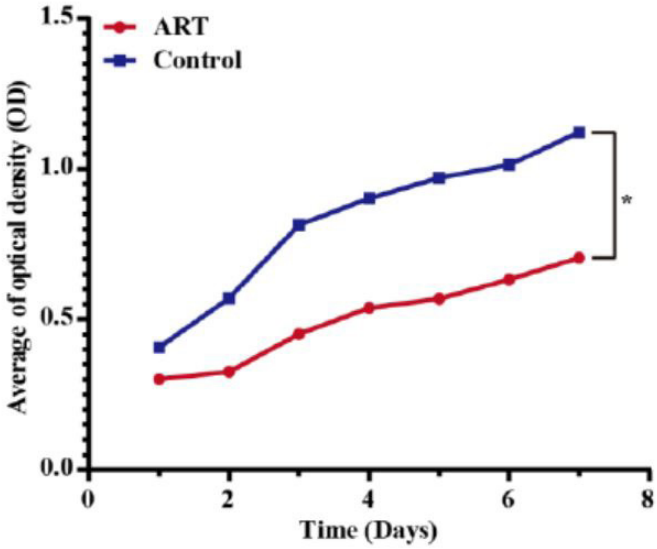

Figure 2: ART inhibits U251 cell proliferation. Cell proliferation was tested by MTT assay. The proliferation rate of U251 cells was inhibited treated with ART at 400ug/ml compared with control group (n = 6, * P < 0.01 compare to control group)

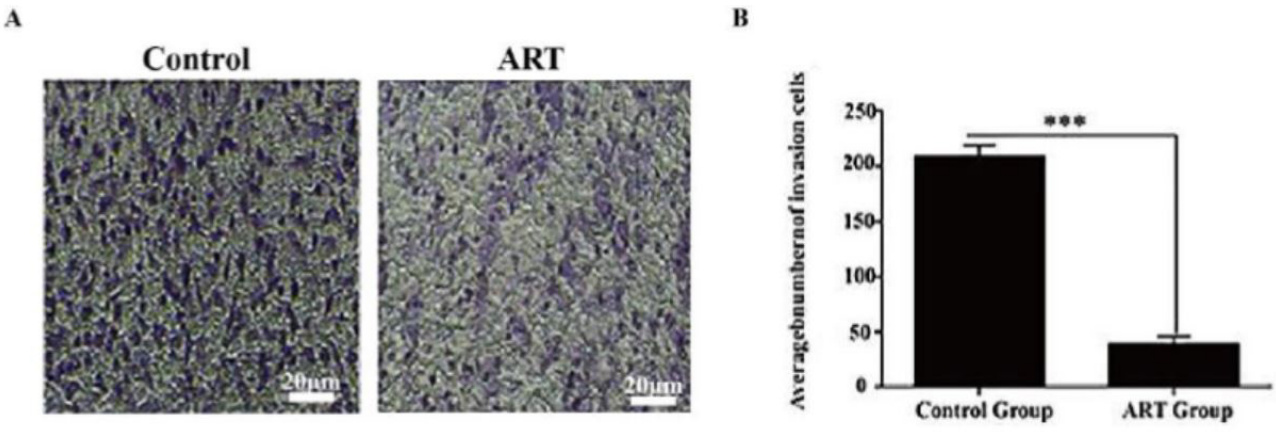

Figure 3: ART inhibits the invasion of U251 glioma cells *in vitro*. (A) The number of invasive cells was determined by the trans-well invasion assays and enumerated under the inverted microscope (100 ×); (B) The average number of invasion cells was calculated from 5 fields under the inverted microscope. (n = 5, *** P < 0.001)

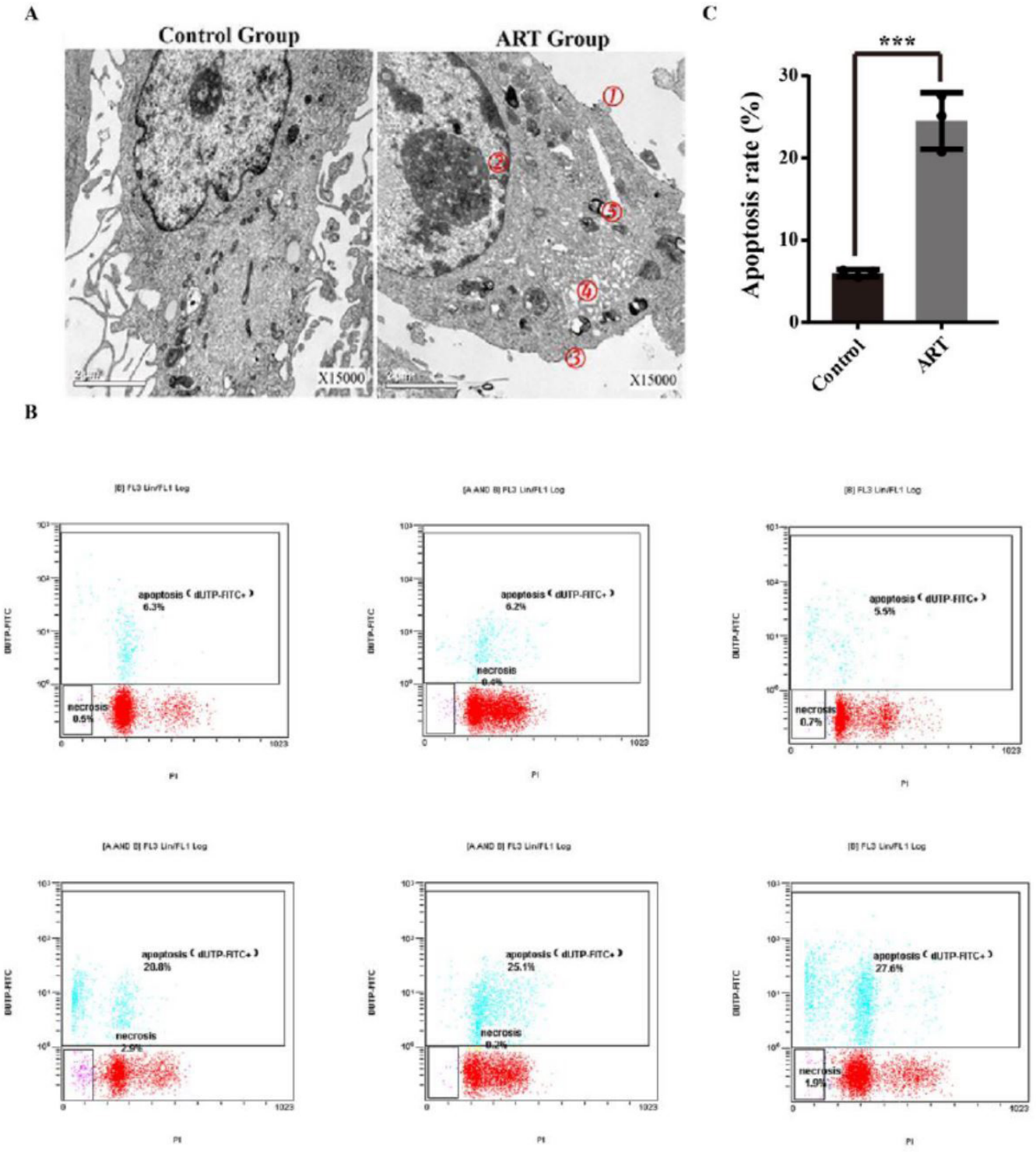

Figure 4: ART induces U251 cell apoptosis. (A)The morphology of apoptotic cells relative to the normal cell morphology at a quantitative level (15000×). Apoptotic cells were analyzed in ART-treated cells after 48 hours drug treatment. These images were recorded by TEM. ① apoptotic body, ② high density chromatin, ③ cell membrane is incomplete, ④ cell vacuolation,⑤ organelles cracking; (B) FCM method was used to analyze the apoptosis rate of cells in each group at a qualitative level; (C) statistical analysis showed there were significantly differences (n=3, P<0.001)

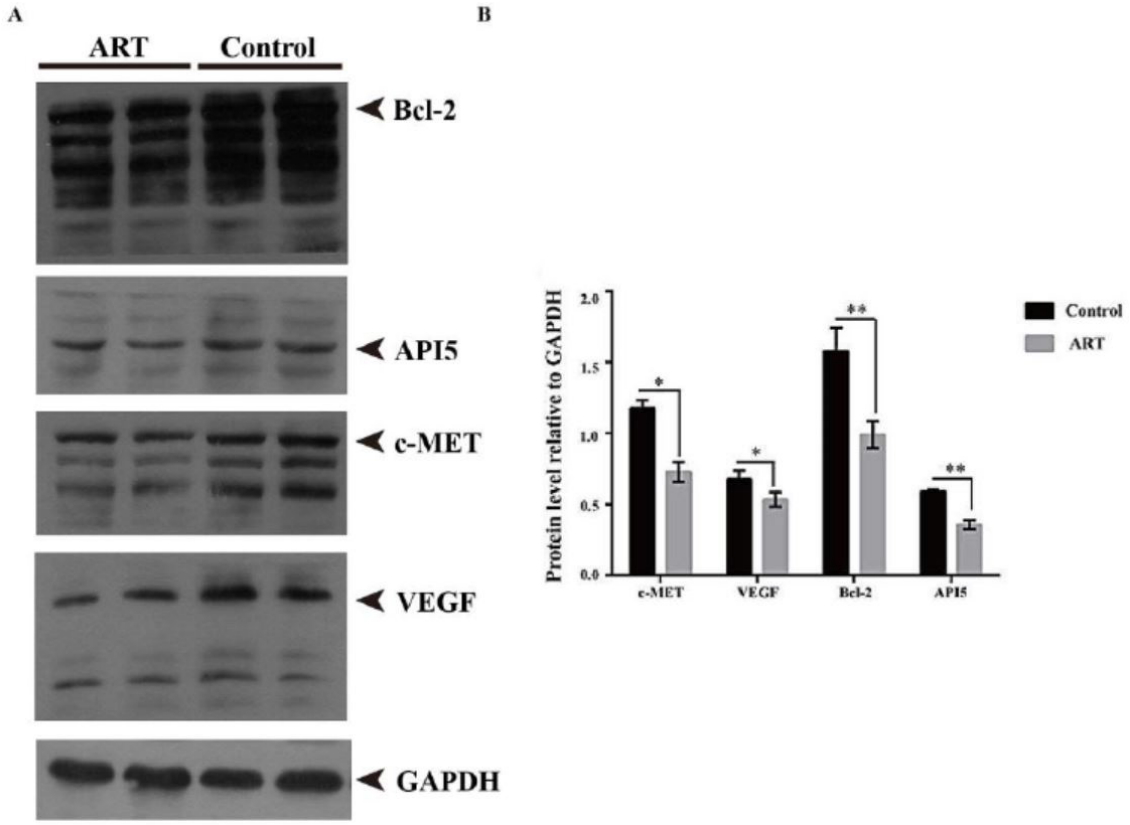

Figure 5: ART downregulates the expression of API5, c-MET, Bcl-2 and VEGF in U251 glioma cells. The expression levels of API5, c-MET, VEGF and Bcl-2 in ART-treated U251 cells were measured by western blot at 48 hours post drug treatment. The GAPDH was also measured as a reference (n = 3, * P＜0.05, and ** P＜0.01)

ART also inhibited the invasion of U251 glioma cells: We used the *in vitro* cell invasion assay to evaluate the effect of ART on the invasion of U251 cell. The result of ART in inhibiting to U251 cells invasion is shown as in Figure 3. We found that there is a significant decrease in invasion in ART-treated cells versus control group ($p<0.05$) ART induced the apoptotic cell death of U251 cell: We used TEM and FCM assays to evaluate the effect of ART on the apoptosis of U251 glioma cells. The features of cell apoptosis were clearly shown by TEM, such as decreased cell volume, cytoplasmic condensation, nuclear pyknosis and the incidence of apoptotic bodies in the ART- treated group (Figure 4A). The FCM assay showed that the total number of apoptotic cells in ART treated U251 cells group is significantly increased after 48 hours. (n=3, $P<0.0001$) (Figure 4B and 4C).

ART downregulated the expression of API5, c-MET, Bcl-2 and VEGF in U251 glioma cells: Computational analysis predicted that API5, c-MET, Bcl-2 and VEGF might potentially be an ART-based therapeutic targets for malignant glioma. U251 glioma cells treated with ART led to a significant decrease in API5, c-MET, Bcl-2 and VEGF protein levels compared with the control group (Figure 5).

Overall, our results confirmed that ART could inhibit proliferation of U251 glioma cell *in vitro.*

Discussion

There is no effective treatment drug for Glioma, so it is pivotal to find an effective therapeutic drug for this devastating disease. In this study, human U251 glioma cells were used as a cell model to test the anti-cancer activity of ART. Our results showed that ART significantly inhibited U251 cell proliferation, cell invasion and lead to cell cycle arrest as well as cell apoptosis. These results suggest that ART has *in vitro* cytotoxic properties. To investigate the molecular mechanism of ART anti-glioma activities, the protein expression of c-MET, API5, VEGF, Bcl-2 and mTOR of U251 glioma cells were detected with or without ART treatment. C-met gene is a member of the receptor tyrosine kinase family [5,39], which could lead to cell proliferation, cytoskeleton reorganization, cell invasion and angiogenesis [40]. Studies showed that c- MET protein was overexpressed in gastric cancer, colorectal cancer, lung cancer, prostate cancer, breast cancer [41-44] and U251 glioma [40]. Our results showed that c- MET level was significantly downregulated by ART. We therefore concluded that ART can suppress U251 cell proliferation,

cytoskeleton reorganization, cell invasion and angiogenesis by decreasing c-MET protein expression.

API5 is an inhibitor of apoptosis, which is highly conserved [41], and the determinant of E2F1-induced apoptosis in Drosophila both *in vivo* and *in vitro* [42]. It was shown that tumor progression was closely linked with overexpressed API5 in patients suffering cervical cancer [16], and API5 induced the decrease of the apoptotic protein Bim. High levels of API5 correlated with resistance to chemoradiation in cervical cancer patients [45]. Importantly, API5 have been overexpressed in glioma [46]. Our results showed that API5 level was significantly reduced under ART treatment.

Angiogenesis is the prerequisite and the basic requirement for tumor growth and metastasis [47]. Many studies confirmed that VEGF is a critical molecule in tumor angiogenesis [48,49]. It was reported that VEGF is overexpressed in U251 glioma cell [50]. Our results showed that VEGF expression was significantly decreased by ART treatment, B-cell lymphoma 2 (BCL2) protein is an anti-apoptotic factor involved in normal B-cell development and differentiation [21,22]. It was reported that Bcl-2 protein was overexpressed in many types of human cancer, such as lymphoma, leukemia and carcinoma [23]. Interestingly, Bcl-2 level increased in glioma [51]. Our results showed that Bcl-2 was significantly decreased after ART treatment. ART may induce U251 cell apoptosis through decreasing Bcl-2 level.

Mammalian target of rapamycin (mTOR) is an untypical serine/threonine kinase (Ser/Thr kinase), and a member of phosphatidylinositol 3 kinase-related kinases [52]. mTOR participates in regulating cell growth, protein synthesis and gene transcription [53]. It was shown tumors such as pancreatic cancer [54], laryngeal carcinoma [55], bladder cancer [56] and glioma [57] could be suppressed by inhibiting mTOR [58]. Our previous results showed that mTOR was significantly decreased by ART treatment. ART may inhibit the gene transcription and protein synthesis necessary for glioma growth through decreasing mTOR protein expression.

Overall, our study showed that ART can inhibit the invasion of U251 glioma cells by decreasing the expression of exogenous c-MET, API5, VEGF, Bcl-2 and mTOR. In addition, previous reports [59] have shown that ART has some clinical advantages, such as fewer side effects and the ability to cross the blood brain barrier (BBB). Furthermore, considering the role of c-MET, API5, VEGF, Bcl-2 and mTOR in cancer, our study implies that ART may be a multi-targeted therapeutic candidate for glioma treatment.

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Statement of Ethics

The study is aimed at research at the cellular level and does not involve animal experiments and human tissue experiments. There is no moral conflict.

disclosure statement

The authors have no conflicts of interest to declare.

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Availability of data and material

All data generated or analyzed during this study are included in this published article.

Author Contributions

Conceived and designed the experiments: Ji-lin Yang, Qi-shun Zhu.

Performed the experiments:

Hong-juan Li performed MTT assay; Xiang Li and Yin Zhang performed cell toxicity detection; Meng-zi Shi performed Western Blot; Da-lun Li and Yun-yi Liu performed in vitro cel

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