Artemisinin reduces cell proliferation and induces apoptosis in neuroblastoma

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Abstract. Artemisinin, a natural product from the Chinese medicinal plant, *Artemisia annua* L., is commonly used in the treatment of malaria, and has recently been reported to have potent anticancer activity in various types of human tumors. Yet, the effect of artemisinin on neuroblastoma is still unclear. In the present study, we aimed to investigate the effects of artemisinin on neuroblastoma cells. We observed that artemisinin significantly inhibited cell growth and proliferation, and caused cell cycle arrest in the G1 phase in neuroblastoma cell lines. Annexin V-FITC/PI staining assay revealed that artemisinin markedly induced apoptosis. Soft agar assays revealed that artemisinin suppressed the ability of clonogenic formation of neuroblastoma cells and a xenograft study in NOD/SCID mice showed that artemisinin inhibited tumor growth and development *in vivo*. Therefore, our results suggest that the Chinese medicine artemisinin could serve as a novel potential therapeutic agent in the treatment of neuroblastoma.

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Introduction

Neuroblastoma is a common childhood malignant tumor of neural crest origin, arising in the paravertebral sympathetic ganglia and the adrenal medulla (1). The clinical characteristics of neuroblastoma include extreme heterogeneity, easy transfer and high malignant potential (2,3). Neuroblastoma accounts for approximately 10% of all pediatric cancers and 15% of cancer-related deaths in children (3-9). Although conventional therapies that are based on surgery, chemotherapy and radiotherapy are available, these approaches have limited efficacy in many cases (8). Therapy failure is generally caused by acquired chemoresistance or high toxicity in neuroblastoma patients. The major challenge in neuroblastoma treatment is the identification and development of new drugs with specific effects (10).

Natural plant compounds represent a large group of untapped potential medicines. Various chemical compounds extracted from natural plants have been reported to have high efficacy and few side-effects (11). Artemisinin (ART), also known as Qinghaosu, was isolated from the Chinese herb *Artemisia annua* L. (Qinghao), a type of wormwood native to Asia. It was discovered in the early 1970s by Tu Youyou (12,13). It has been approved as a potent anti-malarial agent by the Food and Drug Administration (FDA), and artemisinin is commonly used in the clinical management of malaria (14,15). Recently, it was reported that artemisinin or its derivatives have anticancer activity in many types of cancers, such as in multiple tumors (16,17), breast cancer (18), hepatocellular carcinoma (19), leukemia (20), prostate cancer (16) and oral cancer (21). Yet, the role of artemisinin in neuroblastoma has not been well characterized.

In the present study, we investigated the potential role of artemisinin in neuroblastoma cells. We determined that artemisinin induces the inhibition of cell proliferation and induces the apoptosis of the neuroblastoma cells *in vitro*. Moreover, we also demonstrated that artemisinin suppressed clonogenic formation ability and xenograft tumor growth in NOD/SCID mouse models. As a natural product, artemisinin has been confirmed to have no or fewer side effects in the treatment of

malaria. Thus, artemisinin could be used as a novel potential drug for the treatment of neuroblastoma.

Materials and methods

Cell culture. Human neuroblastoma cell lines SK-N-AS, SK-N-DZ and SHEP1 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics penicillin and streptomycin (P/S). BE(2)-C cells were cultured in a 1:1 mixture of DMEM and Ham's nutrient mixture F12 (DMEM/F12), supplemented with 10% FBS and 1% antibiotics (P/S). All four cell lines were purchased from ATCC (Manassas, VA, USA). The growth media, antibiotics and FBS were purchased from Life Technologies. All cells were cultured at 37˚C in a 5% CO_2 humidified incubator.

Cell growth and proliferation assay. Artemisinin was dissolved in DMSO. The cell growth curve was detected using the Cell Counting Kit-8 assay (CCK-8; Beyotime, China). Briefly, approximately 1,000 cells in 200 μl medium were seeded in 96-well plates and incubated with artemisinin at concentrations of 100, 200, 300 and 400 μM; DMSO was used as a control. CCK-8 (20 μl) was added and incubated for 2 h every 2 days, and the absorbance at 450 nm was used to detect metabolically intact cells according to the manufacturer's protocol. Cells were exposed to 300 μM artemisinin or DMSO for 72 h, and cell morphologic examination was carried out using an inverted microscope (TS100, Nikon). Then adherent cells were collected and washed with ice-cold PBS. The sample obtained was analyzed by the TC10™ Automated Cell Counter (Bio-Rad, Hercules, CA, USA).

Immunofluorescence staining. Cells were grown on coverslips and treated with either DMSO or 300 μM artemisinin for 72 h. Cells were washed with PBS, fixed for 20 min in 4% paraformaldehyde (PFA), and permeabilized with 0.3% Triton X-100 for 5 min. The cells were blocked with 10% goat serum in PBS for 1 h, incubated with a primary rat antibody against Ki67 (1:300, 556003; BD) in blocking buffer for 1 h at room temperature, and then incubated with the secondary antibody Alexa Fluor 595 goat anti-mouse IgG (H+L) (1:2,000; A-21422; Invitrogen). DAPI (300 nM) in PBS was used for nuclear staining. Cells were examined using a Nikon microscope (80i) with Image-Pro Plus software for image analysis. Ki67 uptake was calculated in 10 microscopic fields.

Cell cycle assay. Cells were plated in 10-cm plates and treated either with 300 μM artemisinin or DMSO. After 72 h of treatment, cells were fixed with 70% cold ethanol, stained with propidium iodide (PI) and analyzed by flow cytometry (Accuri C6; BD). The data were analyzed with ModfitLT software.

Western blot analysis. Cells treated with artemisinin for 72 h were harvested and suspended in RIPA lysis buffer (Beyotime, China). Protein concentrations were determined with the Enhanced BCA protein assay kit (Beyotime). Sixty micrograms of protein was separated on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), transferred to PVDF membranes, and analysis was performed with the primary

antibodies including anti-cyclinD1 (1:500; Santa Cruz), anti-α-tubulin (1:2,000; Sigma-Aldrich), anti-CDK4 (1:500; Santa Cruz), anti-cyclinE2 (1:500; Santa Cruz), anti-cyclinB1 (1:500; Santa Cruz). Horseradish peroxidase-conjugated goat anti-mouse (1:20,000) and rabbit anti-goat (1:10,000) immunoglobulin G (IgG; KPL, Gaithersburg, MD, USA) were used as secondary antibodies. Proteins were visualized with BeyoECL Plus (Beyotime, China).

Cell death and apoptosis assay. For cell death assay, all cells grown to 60-70% confluency were treated with 300 μM artemisinin for 72 h, and cells were treated with DMSO as control. After treatment, adherent and floating cells were collected by centrifugation, and then the sample obtained was analyzed using the TC10™ Automated Cell Counter. Cell counting was carried out using trypan blue dye (145-0021, Bio-Rad) staining as previously described (22). The apoptotic ratios of the cells were determined with the Annexin V-FITC apoptosis detection kit (Sigma). Briefly, after a 72-h treatment with DMSO or 300 μM artemisinin, the cells were collected and washed twice with cold PBS buffer, resuspended in 100 μl of binding buffer, and incubated with 5 μl of Annexin V conjugated to FITC and 10 μl PI for 15 min at room temperature. Cell were then analyzed by flow cytometry (Accuri C6; BD). The data were analyzed with Flowjo software.

Soft agar clonogenic assay. Five hundred cells were mixed with 0.3% Noble agar in growth medium and plated into 6-well plates containing a solidified bottom layer (0.6% Noble agar in growth medium). After 21 days of cell growth, colonies were stained with 5 mg/ml MTT (Sigma), photographed and recorded.

In vivo tumorigenic assay. BE(2)-C cells were grown to 70-80% confluency and trypsinized. Cells (1×10^6 in 200 μl DMEM) were injected into the flanks of NOD/SCID mice. After one week of tumor growth, the mice were administered intraperitoneal injections of artemisinin at 100 mg/kg (mouse body weight) daily or vehicle control DMSO (1 μl/ml DMEM) (16) for 12 days. Tumor diameter was measured with digital calipers every 3 days, and the tumor volume (V) was calculated by the formula: $V = 1/2$ (length x width^2). After treatment, mice were sacrificed by CO_2, and tumors were observed and weighed. All animal experiments were pre-approved by the Institutional Animal Care and Use Committee of Chongqing University.

Quantification and statistical analysis. Quantitative data are expressed as the mean ± SD. The two-tailed Student's t-test was performed for paired samples. A minimum of three independent experiments was performed. Differences were considered statistically significant at $P<0.05$.

Results

Artemisinin significantly inhibits cell proliferation in neuroblastoma cells. Neuroblastoma cells were treated with various concentrations of artemisinin, from 100 μM to 400 μM, for an indicated period of time. As shown in Fig. 1A, a concentration- and time-dependent response to artemisinin in neuroblastoma
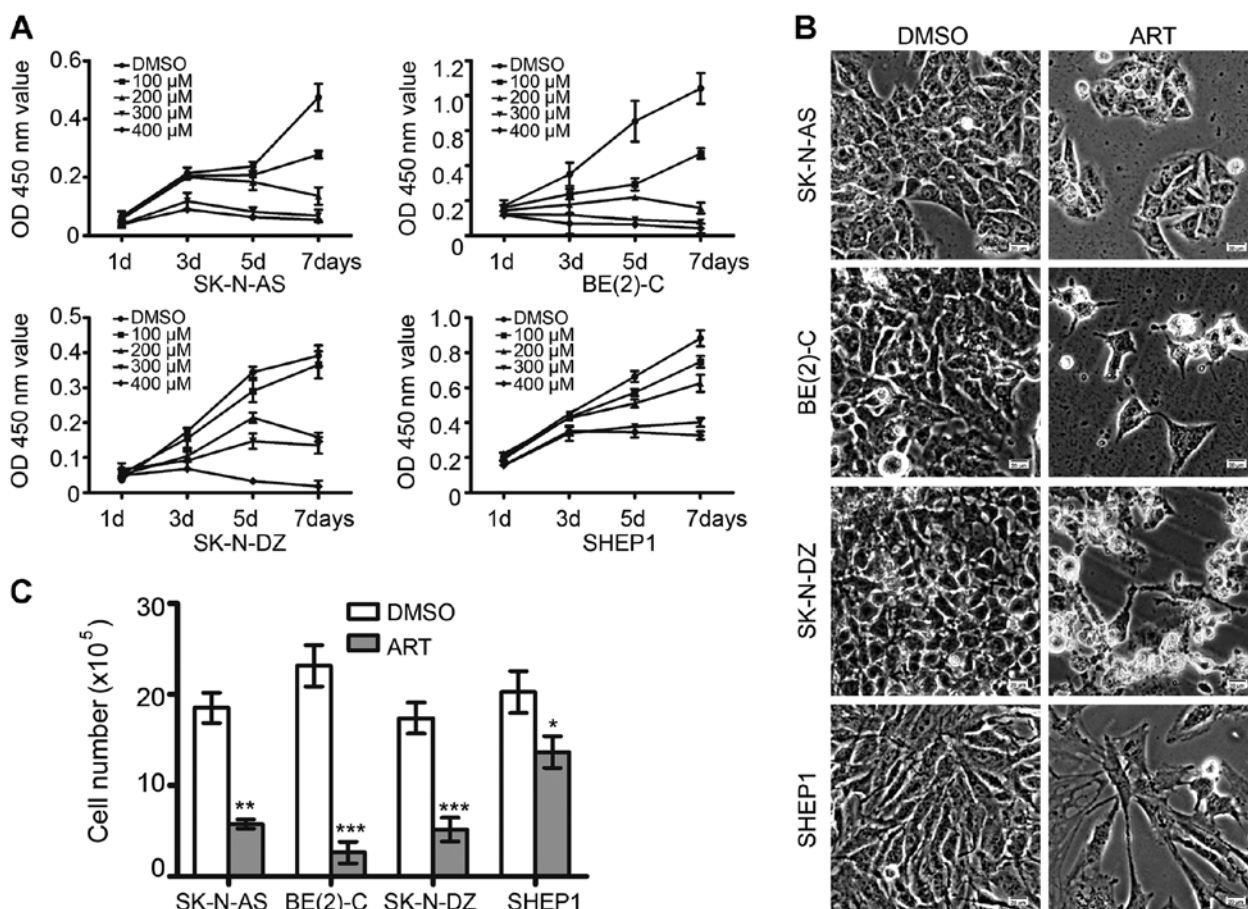
Figure 1. Artemisinin inhibits the cell growth of neuroblastoma cells. (A) SK-N-AS, BE(2)-C, SK-N-DZ and SHEP1 cells were treated with 100, 200, 300 and 400 μM artemisinin; DMSO was used as a control. Cell growth was assessed by the CCK-8 assay every 2 days. (B) Morphologic examination of neuroblastoma cell growth after treated with 300 μM artemisinin or DMSO for 72 h respectively. Scale bar, 20 μm. (C) The cell number of SK-N-AS, BE(2)-C, SK-N-DZ and SHEP1 cells was determined after artemisinin treatment at 300 μM for 72 h; DMSO was used as a control. In A and C, data represent the average ± SD of at least three independent experiments. *P<0.05, **P<0.001, ***P<0.001. ART, artemisinin.

cells was observed. Artemisinin markedly inhibited cell proliferation in the neuroblastoma cells. After 72 h of treatment with 300 μM artemisinin, both morphologic examination and cell counting revealed that artemisinin significantly reduced cell growth in all four neuroblastoma cell lines (Fig. 1B and C). As a proliferation marker, Ki67 staining also confirmed that artemisinin markedly inhibited cell proliferation (Fig. 2A). The histogram statistics of the Ki67-positive rates also demonstrated that artemisinin was an effective agent for inhibiting the proliferation of neuroblastoma cells (Fig. 2B). The percentage of Ki67-positive SK-N-AS cells was reduced from 67.2±2.76 to 25.2±6.11%. The percentage of Ki67-positive BE(2)-C cells was reduced from 72.8±4.94 to 22.7±1.51%. The percentage of Ki67-positive SK-N-DZ cells was reduced from 82.1±4.89 to 41.1±5.08%, and the percentage of Ki67-positive SHEP1 cells was reduced from 57.6±1.88 to 37.9±4.85%.

Artemisinin treatment induces G1 phase cell cycle arrest. Furthermore, to gain insight into artemisinin-induced inhibition of cell proliferation, we examined the cell cycle distribution of the four neuroblastoma cell lines. After artemisinin treatment at 300 μM for 72 h, the proportion of cells in the G1 phase was significantly increased in all four cell lines (Fig. 3A). The cell cycle analysis of artemisinin-treated BE(2)-C cells revealed

a significant increase in the proportion of cells in the G0/G1 phase (54.46±2.14%), compared with the vehicle-treated cells (G0/G1, 37.53±1.82%). Similar results were obtained in the SK-N-AS, SK-N-DZ and SHEP1 cell lines (Fig. 3B). These data demonstrated that artemisinin induced cell cycle arrest in the G1 phase. Western blot analysis also showed that artemisinin led to a marked downregulation of cyclinD1, CDK4 and cyclinE2 (Fig. 3C), which are collectively required for cell cycle progression through the G1 to S phases (23,24). These data suggest that artemisinin inhibits cell proliferation through cell cycle arrest in neuroblastoma cells.

Artemisinin accelerates apoptosis in neuroblastoma cells. To investigate whether artemisinin induces apoptosis in neuroblastoma cells, SK-N-AS, BE(2)-C, SK-N-DZ, and SHEP1 cell lines were incubated with 300 μM artemisinin for 72 h. Trypan blue assay indicated that the cell death was increased after artemisinin incubation when compared with the control (Fig. 4A). Notably, treatment with artemisinin resulted in a higher proportion of cells with positive Annexin V and/or PI staining. Artemisinin markedly induced apoptosis in all four neuroblastoma cell lines (Fig. 4B), which indicated that artemisinin induced cell death through apoptosis in neuroblastoma cells.
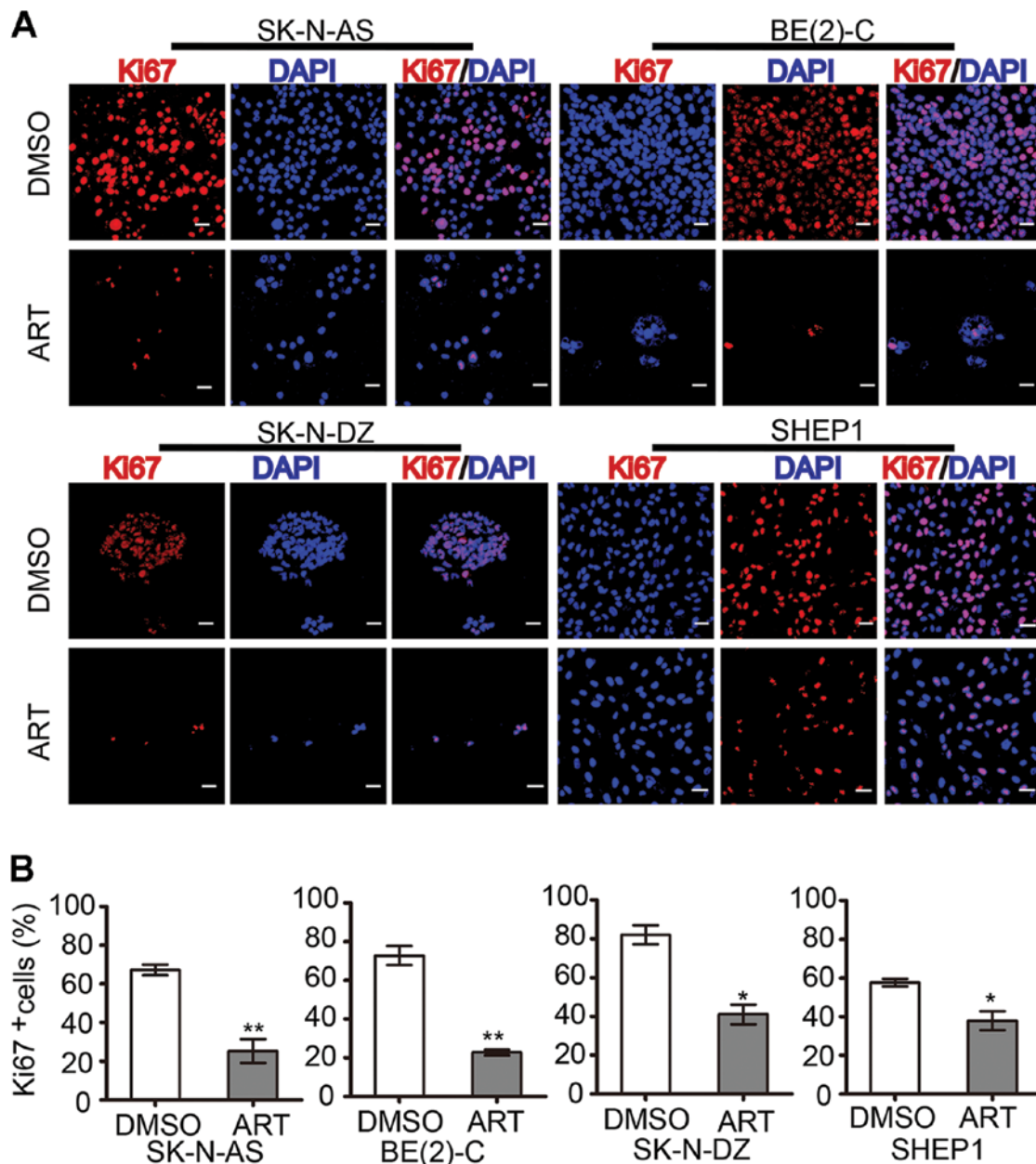
Figure 2. Artemisinin inhibits the cell proliferation of neuroblastoma cells. (A) SK-N-AS, BE(2)-C, SK-N-DZ and SHEP1 cells were grown on coverslips and treated with either DMSO or 300 μM artemisinin for 72 h, respectively. Cells were stained with an antibody against Ki67 (red), and counterstained with DAPI (blue); scale bar, 25 μm. (B) The percentage of Ki67$^+$ cells was determined in a x20 field view under a microscope. Data represent the average ± SD of at least three independent experiments. Statistical analysis was performed using the two-tailed Student's t-test. *P<0.05, **P<0.01. ART, artemisinin.

Artemisinin suppresses the tumorigenicity in neuroblastoma cells. Soft agar is used to test the ability of single cancer cells to proliferate and form colonies, and is also used as a 'human tumor stem-cell assay' (25). In the present study, BE(2)-C cells treated with 300 μM ART were observed to give rise to small and scant colonies in soft agar compared with the cells treated with DMSO (Fig. 5A and B). To determine the effect of artemisinin on tumorigenicity in neuroblastoma cells, we carried out a xenograft study in NOD/SCID mice. BE(2)-C cells were implanted subcutaneously into the flanks of NOD/SCID mice. One week after tumor injection, mice were treated intraperitoneally with either DMSO or artemisinin at 100 mg/kg daily for 12 days. The volume and weight of the xenograft tumors in the artemisinin treatment group

were much smaller and lighter than those in the DMSO group (Fig. 5C and D). These data indicate that artemisinin significantly suppresses the tumorigenicity of neuroblastoma cells.

Discussion

Neuroblastoma is a malignant pediatric tumor with a wide range of stages, requiring a wide range of therapeutic options. However, successful therapeutic options remain limited. Therefore, it is urgently necessary to identify additional chemotherapeutic agents to target this disease. It has been reported that artemisinin has multiple anti-proliferative activity, including cell growth suppression (26,27), apoptosis induction (28),
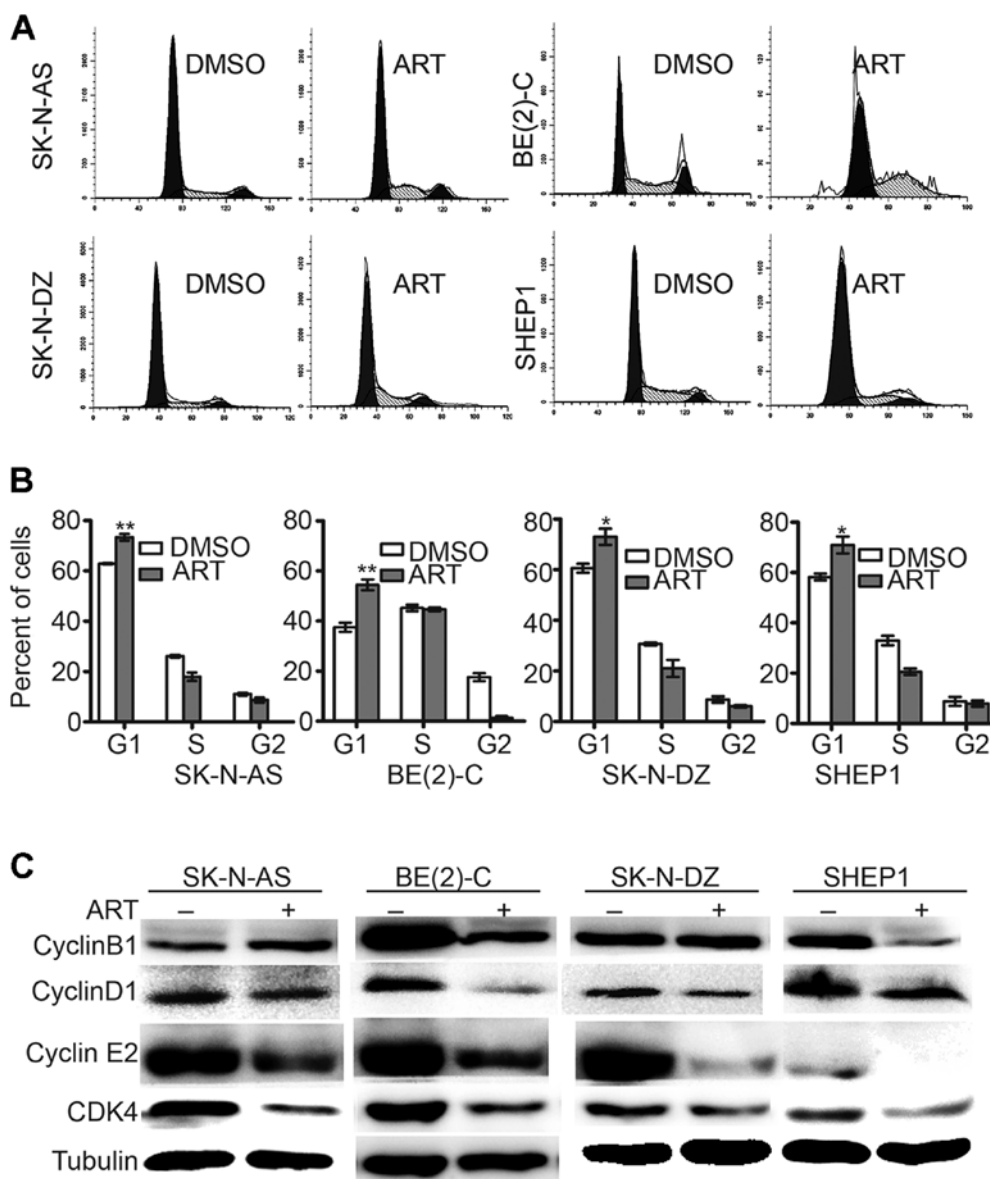
Figure 3. Artemisinin induces G1 phase cell cycle arrest in neuroblastoma cells. (A) SK-N-AS, BE(2)-C, SK-N-DZ and SHEP1 cells were treated with 300 μM artemisinin or DMSO for 72 h. Cells were harvested, fixed with ethanol and stained with propidium iodide, DNA content was determined by flow cytometry. (B) The statistical analysis of the percentage of cells in each cell cycle phase in the four neuroblastoma cell lines is shown. Data represent the average ± SD of at least three independent experiments. Statistical analysis was performed using the two-tailed Student's t-test. *P<0.05, **P<0.01. (C) Western blot analysis of cyclinB1, cyclinD1, cyclinE2 and CDK4 in neuroblastioma cells treated with artemisinin or DMSO for 72 h. α-Tubulin levels are shown as loading control. ART, artemisinin.

angiogenesis inhibition, cell migration disruption (29-31), and modulation of nuclear receptor responsiveness (32,33). Yet, the effect of artemisinin on neuroblastoma remains unclear. The results presented in the present study demonstrated that artemisinin led to significantly decreased cell growth and cell proliferation, and increased apoptosis in neuroblastoma cells. We first demonstrated that artemisinin treatment suppressed the ability of colony formation *in vitro* and tumorigenicity of neuroblastoma cells *in vivo*.

Artemisinin has been suggested to promote cytostasis by G0/G1-phase arrest and to decrease the expression level of cyclinB1, CDK2 and CDC25A in colon cancer (34), and to inhibit the promoter activity of CDK4 in prostate cancer (16). Artemisinin also induced G2/M phase arrest in osteosarcoma cells (35). Our data indicated that artemisinin induced cell

cycle arrest at the G1 phase, together with a decrease in the expression levels of cyclinD1, CDK4 and cyclinE2 in all four neuroblastoma cell lines. cyclinB1 was downregulated only in the BE(2)-C and SHEP1 cells, but no significant difference was noted in the SK-N-AS and SK-N-DZ cells.

Previous studies have shown that artemisinin induced apoptosis in pancreatic tumor cells (36), lung adenocarcinoma cells (37), liver cancer cells (38), and non-small cell lung cancer cells (39). In the present study, we first demonstrated that artemisinin induced the cell death and apoptosis of neuroblastoma cells at a lower dose than that for clinical usage. Next, we investigated the mechanism and the expression of apoptotic relevant proteins.

Collectively, our results revealed that artemisinin inhibited cell proliferation and tumor growth, with cell cycle arrest and
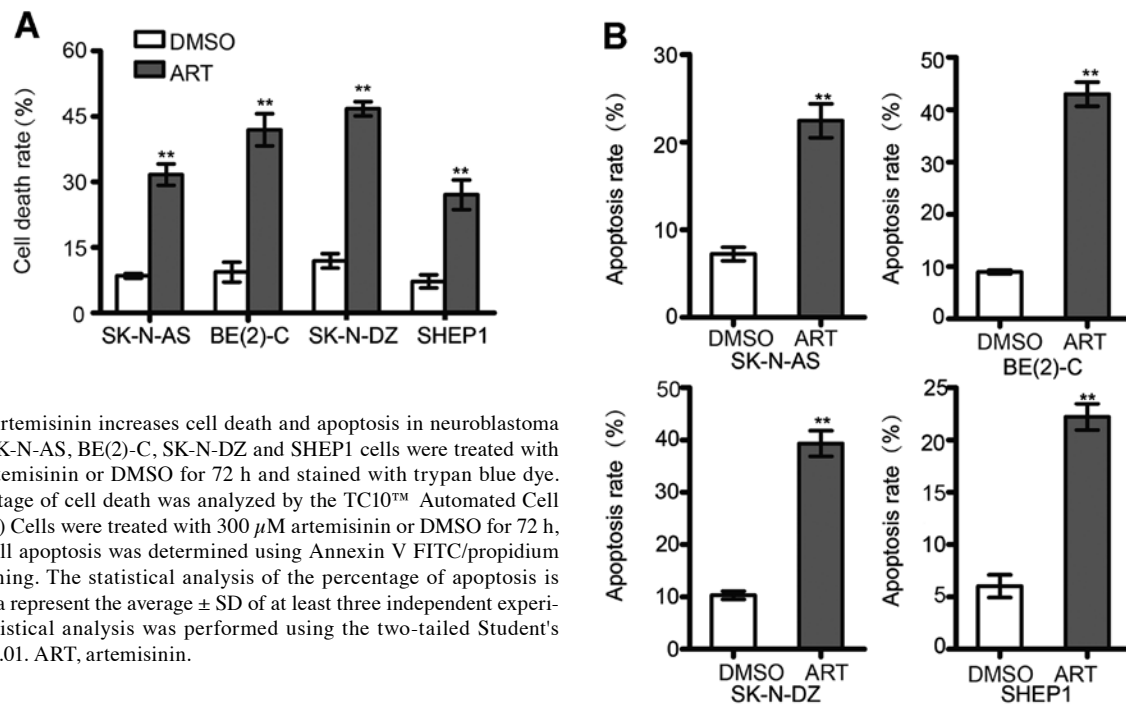
Figure 4. Artemisinin increases cell death and apoptosis in neuroblastoma cells. (A) SK-N-AS, BE(2)-C, SK-N-DZ and SHEP1 cells were treated with 300 μM artemisinin or DMSO for 72 h and stained with trypan blue dye. The percentage of cell death was analyzed by the TC10™ Automated Cell Counter. (B) Cells were treated with 300 μM artemisinin or DMSO for 72 h, and then cell apoptosis was determined using Annexin V FITC/propidium iodide staining. The statistical analysis of the percentage of apoptosis is shown. Data represent the average ± SD of at least three independent experiments. Statistical analysis was performed using the two-tailed Student's t-test. **$P<0.01$. ART, artemisinin.

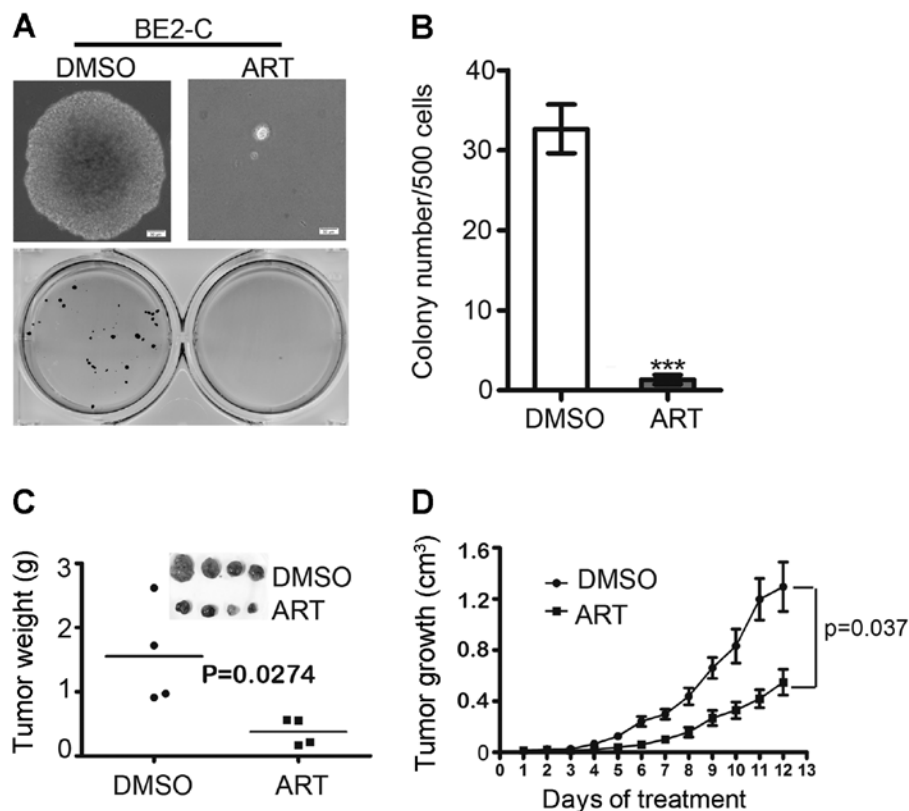Figure 5. Artemisinin suppresses colony formation *in vitro* and tumor growth *in vivo*. (A) BE(2)-C cells were plated at 5x10^2 cells per well in 6-well culture plates and treated with 300 μM artemisinin or DMSO in a soft agar culture system for 21 days. The colonies were then stained with MTT and imaged in order to determine the number of colonies. Scale bar, 50 μm. (B) Colonies >0.5 mm or containing >50 cells were recorded and counted. Data represent the average ± SD of at least three independent experiments. Statistical analysis was performed using the two-tailed Student's t-test. **$P<0.01$. (C) The NOD/SCID mouse xenograft tumors after treatment with DMSO or 100 mg/kg daily artemisinin were photographed and weighed. (D) Xenograft tumor volume was analyzed per group. In C and D, data were analyzed with two-tailed Student's t-test with the P-value as indicated. ART, artemisinin.

apoptosis induction in neuroblastoma cells. Since artemisinin has been used for the treatment of malaria for an extensive

period of time, a large body of data regarding clinical tests and adverse drug reactions in patients are available. Therfore,

artemisinin may serve as a potential new therapeutic agent for the treatment of neuroblastoma.

Acknowledgements

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