Artemisinin reduces cell proliferation and induces apoptosis in neuroblastoma

SHUNQIN ZHU1,2*, WANHONG LIU1,4*, XIAOXUE KE2, JIFU LI2, RENJIAN HU2, HONGJUAN CUI2 and GUANBIN SONG1

1Key Laboratory of Biorheological Science and Technology, Ministry of Education, College of Bioengineering, Chongqing University, Chongqing; 2State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing; 3College of Life Science, Southwest University, Chongqing; 4School of Chemistry and Chemical Engineering, Chongqing University of Science and Technology, Chongqing, P.R. China

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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.

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% CO₂ 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 with either 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 (1x10⁶ in 200 µl DMEM) were injected into the flanks of NOD/SCID mice. After one week of tumor growth, the mice were administered intra-peritoneal 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 width²). After treatment, mice were sacrificed by CO₂, 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
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.
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),
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
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. Therefore,
artemisinin may serve as a potential new therapeutic agent for the treatment of neuroblastoma.

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