Artemisinin inhibits neuroblastoma proliferation through activation of AHP-activated protein kinase (AMPK) signaling

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Recent population studies suggest that the use of artemisinin is associated with reduced incidence and improved prognosis of certain cancers. In the current study, we assessed the effect of artemisinin on neuroblastoma cells using SHSY5Y cells. We found that artemisinin inhibited growth and modulated expression of cell-cycle regulators in these cells. Treatment with artemisinin was also associated with activation of AMP kinase and inhibition of mTORp70S6K/pS6 signaling in SHSY5Y cells. In addition, inhibition of AMPK signaling reversed impact on the anti-proliferative roles of artemisinin. Taken together, these results provide evidence for a mechanism that may contribute to the antineoplastic effects of artemisinin suggested by recent population studies and justify further work to explore its potential roles in neuroblastoma prevention and treatment.

1. Introduction

Neuroblastoma is a tumor originating from nerve tissue, and is the most frequent extracranial solid tumor in children (Pietras 2012; Tonini et al. 2012). A characteristic feature of neuroblastoma is its heterogeneity, ranging from spontaneous regression to fatal outcome. Its prognosis is very variable, with outcome related to age, stage and molecular pathology, which in turn has led to the development of targeted therapies (Pietras 2012). Extensive research projects have been focused on developing new chemotherapies either by exploiting the antitumor activity of novel compounds or by assessing drugs conventionally used in other clinical diseases. Thus, there is always a constant need to develop alternative or synergistic antitumor agents with minimal side-effects. One important strategy to develop effective antitumor agents is to study antitumor agents derived from natural sources. Natural products have been found to be a relevant source of novel and potent bioactive compounds, which have been proven to be effective against a range of diseases with broad antimicrobial activity, and some have also exhibited significant antitumor activity (Ali et al. 2012). One of the promising compounds in artemisinin, a naturally occurring antimalarial with anticancer properties (Ellerth et al. 2001). Artemisinin and its derivatives, which are notably used in malaria therapy (Üzün et al. 2007), have also potent antitumor activity in the nano- to micromolar range in sensitive and drug- or radiation-resistant cell lines (Ellerth et al. 2003). Importantly, artemisinin is one of the very few drugs that have been widely used as antimalarials but has no significant side effects or clinical resistance, although tolerance has been reported (Gordi and Lepist 2004; Reungpatthanaphong and Mankhetkorn 2002).

2. Investigations and results

2.1. Artemisinin treatment inhibited cell growth in a dose-dependent manner

To our knowledge, the effect of artemisinin on neuroblastoma cells remains unexplored. Thus, we selected SHSY5Y cells to investigate whether artemisinin has potential anti-proliferative roles. Cells were treated with artemisinin at several concentrations. After 30 h of treatment, growth was inhibited in a dose-dependent manner in SHSY5Y cells as determined by MTT and BrdU incorporation assays (Fig. 1A-1B). Moreover, these results suggested that the concentration of artemisinin at 20μM was appropriate. Therefore, 20μM of artemisinin was selected for the further analysis of genes expression in SHSY5Y cells.

2.2. Expression of cell-cycle regulators in artemisinin-treated cells

We speculate that growth inhibition in neuroblastoma cells might be caused by cell-cycle arrest following artemisinin treatment. To confirm this hypothesis, we analyzed the expression contents of p21, p27, Cyclin D1 and Cyclin E, which are known as key molecules involved in cell-cycle arrest. As shown in Fig. 2A-2B, expression levels of p21 and p27 were significantly increased in artemisinin cells. Besides, the contents of Cyclin D1 and Cyclin E...
Fig. 1: Artemisinin inhibits cell proliferation in neuroblastoma cells. (A) Cell viability was measured by MTT assays in SHSY5Y cells (A). Cells were treated with various concentrations of artemisinin as indicated. (B) Cell proliferation activity was measured by BrdU incorporation assays in SHSY5Y cells (B).

E were markedly down-regulated in artemisinin-treated cells (Fig. 2A-2B).

2.3. Artemisinin activates AMP kinase activity in neuroblastoma cells

Many studies have indicated that the antiproliferative effects of several compounds or drugs involve the AMP kinase pathway (Kim et al. 2012; Buzzai et al. 2007). Indeed, our western blot analysis indicated that artemisinin stimulated AMPK phosphorylation in SHSY5Y cells (Fig. 3A). Phosphorylated ACC, a downstream target of AMPK, was also enhanced in cells treated with artemisinin (Fig. 3A). Because AMPK activation inhibits energy-consuming pathways and protein synthesis (Gong et al. 2013; Kim et al. 2012). We observed that AMPK activation is associated with a decreased phosphorylation of mTOR and S6 kinase (Fig. 3C).

2.4. Inhibition of AMPK pathway reversed the roles of artemisinin

We next test whether the inhibiting effect of artemisinin on cell proliferation is mediated by AMPK in neuroblastoma cells. As shown in Fig. 4A and 4B, pretreatment with the AMPK inhibitor (Compound C, CC) reverses the inhibitory effect of artemisinin on cell proliferation. Besides, expression levels of cell-cycle regulators were also inhibited by artemisinin in the presence of CC (Fig. 4C). To rule out any possible nonspecific effects of CC, siRNA oligos-mediated knockdown of AMPK 2 subunit was performed (Fig. 4D and 4E). As a result, we also...
observed that artemisinin could not regulate cell proliferation and expression levels of cell-cycle regulators in cells with AMPK 2 subunit depletion (Fig. 4F-4H). Therefore, our results suggest that the antiproliferative effects of artemisinin in neuroblastoma is dependent of AMPK signaling.

3. Discussion

In the present study, we firstly explored the roles of artemisinin and its molecular mechanisms in neuroblastoma cells. Artemisinin was shown to inhibit cell proliferation in SHSY5Y cells as evidenced by MTT and BrdU incorporation assays. Moreover, artemisinin treatment induced p21 and p27 expression while repressed Cyclin D1 and Cyclin E expression while repressed Cyclin D1 and Cyclin E expression and in animal models (Reganti et al. 2009; Alcantara et al. 2013; Gong et al. 2013; Singh et al. 2011). Artemisinin was shown to induce doxorubicin resistance in human colon cancer cells via calcium-dependent activation of HIF-1alpha and P-glycoprotein overexpression (Reganti et al. 2009). In breast cancer cells, artemisinin repressed proliferation and induced a strong G1 cell cycle arrest in MCF-7 cells, an estrogen-responsive human breast cancer cell line that represents an early-stage cancer phenotype, and effectively inhibited the tumor growth of MCF-7 cell-derived tumors from xenografts in vivo. The neuroblastoma cell line SHSY5Y, was purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai), and cultured in Dulbecco modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco, USA).

4. Experimental

4.1. Cell cultures

The neuroblastoma cell line SHSY5Y cells were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai), and cultured in Dulbecco modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco, USA).

4.2. Cell viability and BrdU incorporation assays

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Beyotime, Shanghai). After pretreatment, cells were treated with medium containing different doses of artemisinin (Sigma) and/or different agents as described. MTT assay was performed by incubating the cells with 0.5 mg/mL MTT for 4 hours. The formazan product was dissolved in dimethyl sulfoxide, and absorbance was read at 493 nm. A cell proliferation/immunoassay-linked immunosorbent assay kit (Beyotime, Shanghai) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer’s protocols. All experiments were repeated at least four times.

4.3. RNA isolation and real-time PCR

Total RNAs were isolated from cells by TRIzol reagent (Invitrogen, USA), and reverse transcriptions were performed by Takara RNA PCR kit (Takara, Dalian, China), following the manufacturer’s instructions. In order to determine the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Dalian, China) on an ABI 7900 machine.

4.4. Western blot analysis

Cells after different treatments were lysed with RIPA buffers. An equal amount of protein was subjected to 12% SDS-PAGE, and separated proteins were transferred to nitrocellulose membranes. The membranes were blocked in 5% milk for 2 h at 22–25 °C. The immunoblots were incubated overnight at 4 °C with antibodies. Next day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA) for 2 h at 22–25 °C. The immunoreactive bands were detected with a chemiluminescence substrate kit (ProteinSimple, Santa Clara, CA) under the Fluor Chem FC2 system. Antibodies were purchased from Abcam (anti-β-actin, anti-AMPK, anti-ACC, anti-mTOR and anti-S6K) or Cellsignaling Company (anti-p21, anti-p27, anti-Cyclin D1 and anti-Cyclin E).

4.5. Small interfering RNA (siRNA)

Cells were transfected with siRNA targeting the AMPKβ2 subunit or a negative control (all siRNA oligos from Qiagen, Valencia, CA) using Lipofectamine 3000 (Invitrogen, USA) as described by the manufacturer’s instructions. Cells cultures were incubated for 18 hours with 100 nM siRNA before artemisinin treatment.

4.6. Statistical analysis

Statistical analysis was performed with SPSS version 13.0 software. Numerical data are expressed as mean ± SEM. Statistical significance is shown as *P < .05, **P < .01, or ***P < .001.)

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Fig. 3. Artemisinin induces AMPK activation in neuroblastoma cells. (A) Western blot analysis of phosphorylated AMPK and ACC in SHSY5Y cells (A). Contents of total AMPK, ACC and β-actin were used as loading controls. (B) Western blot analysis of phosphorylated mTOR and S6K in SHSY5Y cells. (B) Contents of total mTOR, S6K and β-actin were used as loading controls.
Fig. 4: The anti-proliferative action of artemisinin is dependent on AMPK signaling activation. (A-B) Cell proliferation activity was measured by MTT or BrdU incorporation assays in SHSY5Y cells. Cells were pretreated with vehicle control (DMSO) or Compound C (CC) for 12 h. (C) mRNA levels of p21, p27, Cyclin D1 and Cyclin E were determined by real-time PCR in SHSY5Y cells. (D-E) Real-time PCR and Western blot analysis of AMPKα2 or scramble siRNA (Ctrl). (F-G) Cell proliferation activity was measured by MTT or BrdU incorporation assays in SHSY5Y cells. Cells were pre-transfected with siRNA oligos against AMPKα2 or scramble siRNA (Ctrl). (H) mRNA levels of p21, p27 and Cyclin D1 were determined by real-time PCR in SHSY5Y cells.

References
ORIGINAL ARTICLES


