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

WEI-QIANG TAN*, GANG CHEN*, BING JIA, MING YE

1. Introduction
Neuroblastoma is a tumor originating from 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 exploring the anticancer ability 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 anticancer drugs with minimal side-effects. One important strategy to develop effective anticancer agents is to study anticancer 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 for cancer prevention and therapy (McLeod 2013; Berkovich et al. 2012). Plant derivatives have been known 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 is artemisinin, a naturally occurring antimalarial with anticancer properties (Efferth et al. 2001). Artemisinin and its derivatives, which are notably used in malaria therapy (Ürüzinger et al. 2007), have also exhibited significant antitumor activity (Ali et al. 2012). Extensive research projects have been focused on developing new chemotherapies either by exploring the anticancer ability 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 anticancer drugs with minimal side-effects. One important strategy to develop effective anticancer agents is to study anticancer 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 for cancer prevention and therapy (McLeod 2013; Berkovich et al. 2012). Plant derivatives have been known to be effective against a range of diseases with broad antimicrobial activity, and some have also exhibited significant antitumor activity (Ali et al. 2012).

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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. Significant antitumor activity of artemisinin and licensed semisynthetic artemisinin derivatives has been documented in vitro and in animal models (Riganti 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 in vivo growth of MCF-7 cell-derived tumors from xenografts in athymic nude mice (Tin et al. 2012). At the molecular level, artemisinin inhibited E2F1 interactions with the endogenous CDK2 and cyclin E promoters (Tin et al. 2012). Moreover, artesunate (ART), a semisynthetic derivative of artemisinin, induces apoptosis in human lung adenocarcinoma cell lines (ASTC-a-1 and A549). ART induces Bak-mediated caspase-independent intrinsic apoptosis in both ASTC-a-1 and A549 cell lines, suggesting a potential therapeutic effect of artemisinin on lung cancer (Zhao et al. 2012). Taken together, artemisinin has been reported by numerous studies to exert anti-cancer effects. At the molecular level, our results demonstrated that artemisinin activated AMPK kinase activation as well as inhibition of mTOR signaling. Interestingly, inhibition of the AMPK pathway using antagonist or siRNA oligos largely abolished the anti-proliferative roles of artemisinin, suggesting that the function of artemisinin was AMPK-dependent. Because of its well-established roles in various aspects of metabolic physiology, AMPK has received great pharmaceutical interest as a well-established target in neuroblastoma cells. Due to its broad therapeutic activity, clinicians should take into account to further investigate the efficacy of artemisinin in patients.

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 mg/ml streptomycin (Gibco, USA).

4.2. Cell viability and BrdU incorporation assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Beyotime, Shanghai). After pre-culture, 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 470 nm. A cell proliferation/inhibitory-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 (LifeTechnologies, 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 SDS-PAGE, and separated proteins were transferred to nitrocellulose membranes. The membranes were blocked in 10% milk for 2 h at 22–25 °C. The immunoblot was 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 (ECL, Amersham, USA). The intensity of bands was measured by Quantity One software. All the primers were designed from Abcam (anti-β-actin, anti-AMPK, anti-ACC, anti-mTOR and anti-S6K). Specific primers were purchased from Bioneer Co. Ltd. (Seoul, South Korea) as described by the manufacturer’s instructions. Cells were incubated for 18 hours with 100 µM siRNA before artemisinin treatment.

4.5. Small interfering RNA (siRNA)

Cells were transfected with siRNA targeting the AMPK or a negative control (all siRNA oligos from QIAGEN, Valencia, CA) using Lipofectamine 2000 (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 were expressed as mean ± SEM. Statistical significance was shown as *P < .05, **P < .01, ***P < .001.
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 pre-treated with vehicle control (DMSO) or Compound C (CC) for 12h. (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 in SHSY5Y cells transfected with siRNA oligos against 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


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