Artesunate inhibits lung cancer cells via regulation of mitochondrial membrane potential and induction of apoptosis

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Abstract. Lung cancer is a common malignant disease with a high incidence rate worldwide, posing a great threat to human health. To date, only a small number of studies have assessed the potential anti-cancer effect of artesunate (Art) and the associated mechanisms in lung cancer. The present study aimed to investigate the inhibitory effects of Art in human lung cancer cells and investigated the underlying molecular mechanisms. The inhibitory effect of Art on the growth of A549 lung cancer cells was detected by the MTT assay, and flow cytometry was utilized to determine cell cycle progression, apoptosis, mitochondrial membrane potential, as well as the expression of Bcl-2 and Bax proteins in A549 cells after Art treatment for 24 h. Art inhibited the growth of A549 cells in a dose-dependent manner, induced cell apoptosis and cell cycle arrest, decreased the expression of Bcl-2 protein and mitochondrial membrane potential, and increased the expression of Bax protein. In conclusion, Art significantly inhibited the growth of lung cancer cells by preventing cell cycle progression. This phenomenon indicated its promising therapeutic potential in the treatment of lung cancer.

Introduction

Lung cancer is a common malignancy and its initiation and progression are complex processes associated with the loss of normal regulatory pathways, including cell proliferation, differentiation and apoptosis (1-4). Current treatment strategies include chemotherapy; however, the efficacy is limited and the side effects of these drugs pose major challenges (5). Therefore, the development of novel lung cancer drugs is imperative. Owing to its long history in tumor treatment, the

search for anti-tumor drugs among Chinese herbal medicines has become a hot topic in cancer research.

Artesunate (Art) is a water-soluble hemisuccinate derivative of dihydroartemisinin and the most widely used member of the family of artemisinin drugs. Artemisinin compounds are widely used for the treatment of severe and complicated malaria in humans. Art is a classic anti-malarial drug for the treatment of severe and drug-resistant malaria (6-10). It also exerts anti-tumor activity (11-17). Several *in vitro* and *in vivo* studies have indicated that the anti-tumor effect of Art is associated with the induction of apoptosis and cell cycle arrest (11-13). It was also demonstrated to inhibit tumor infiltration and metastasis (18,19). However, studies investigating the mechanisms of the anti-tumor effects of Art in lung cancer are limited, and hence, its role in lung cancer therapy remains to be clarified. In the present study, the anti-cancer efficacy of Art in the A549 lung adenocarcinoma cell line was assessed and the underlying molecular mechanisms were investigated.

The balance between apoptosis and cell proliferation is essential for maintaining the function of normal cells and growth of normal tissues (20,21). Disruption of this balance leads to diseases, including tumors, in the body. Cell apoptosis is closely linked to the occurrence of a tumor. At present, apoptosis is the most studied mechanism in anti-cancer therapy. Cellular apoptosis is an automated gene-controlled death program mediated via complex regulatory mechanisms. Bcl-2, Bax and the mitochondrial membrane potential are essential effectors of the intrinsic pathway of apoptosis.

In the present study, it was investigated whether Art is able to induce lung cancer cell apoptosis by regulating the intrinsic apoptosis pathway, thereby providing a novel Art-mediated mechanism for lung cancer treatment.

Materials and methods

Cell line and culture. The human lung cancer cell line A549 was purchased from Procell Life Science & Technology Co., Ltd., and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (both purchased from Gibco; Thermo Fisher Scientific, Inc.), 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Chemicals and reagents. Art was purchased from Guilin Pharmaceutical (Shanghai) Co., Ltd. The Annexin V-FITC/PI

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kit was purchased from Beckman Coulter, Inc., while propidium iodide (PI) was purchased from BD Biosciences.

Cytotoxicity assay. The sensitivity of A549 lung cancer cells to Art was determined using an MTT assay, which is based on the capacity of viable cells to metabolize a yellow tetrazolium salt, MTT, to form purple formazan crystals. These are solubilized in acidified 2-propanol and the absorbance is measured spectrophotometrically at 490 nm. The cells were seeded in 96-well plates at a density of 5×10^4 cells/ml/well. Once cells were attached, serially diluted art solution was added to reach a final concentration of 0, 0.1, 0.5, 1, 5, 10, 50, 100, 200, 400 or 800 $\mu\text{g/ml}$ in a final volume of 200 μl /well. Normal saline (NS) was used for the control group (22). After drug treatment for 24 h, the medium was replaced with an equivalent volume of fresh RPMI 1640 medium containing 0.5 mg/ml MTT, followed by incubation for an additional 4 h. Subsequently, the medium was replaced with 180 μl DMSO, followed by incubation for 10 min at room temperature. The cytotoxic effects of the drug concentrations were determined by measuring the optical density values at 490 nm with a microplate reader. Cell viability was expressed as the relative synthesis of formazan in treated samples compared with the control cells [(treated cells/control cells) $\times 100\%$].

Experimental groups and drug intervention experiments. After the cultured A549 tumor cells were attached, cells were grouped evenly and treated with serial concentrations of artesunate (0, 25, 50 and 100 $\mu\text{g/ml}$) for 24 h. Then, cells were harvested routinely; NS was used as the control. The cell concentration of the suspension was adjusted to $1 \times 10^6/\text{ml}$. Each experiment was performed three times.

Assessment of the cell cycle distribution using flow cytometry (FCM). Single-cell suspension (1 ml containing 1×10^6 cells) was prepared, washed with cold PBS and fixed with 70% ethanol at 4°C for 24 h prior to the addition of 1 ml PI (50 $\mu\text{g/ml}$). After incubation at 4°C for 30 min, FCM (FC-500; Beckman Coulter, Inc.) was performed and MultiCycle AV software (Beckman Coulter, Inc.) was used to analyze the cell cycle. The proliferation status was expressed by the following proliferation index = $(S+G_2/M)/(G_0/G_1+S+G_2/M) \times 100\%$.

Assessment of cell apoptosis of A549 cells using FCM. To detect apoptosis, 1 ml of single-cell suspension was stained with PI (12.5 $\mu\text{g/ml}$) and Annexin V-FITC (0.25 $\mu\text{g/ml}$) and analyzed with an FC500 flow cytometer (Beckman Coulter). Cells with positive staining for Annexin V and negative staining for PI were considered to be in early apoptosis, whereas those that were positive for both Annexin V and PI were considered to be in late apoptosis.

Analysis of mitochondrial membrane potential of A549 cells using FCM. To determine the mitochondrial membrane potential following Art treatment, harvested A549 cells were washed with ice-cold PBS and stained with 1 ml fluorescence reagent containing 10 $\mu\text{g/ml}$ Rhodamine 123. After incubation for 30 min in the dark at 37°C , the stained cells were re-suspended in 1 ml PBS and analyzed using the FC500 flow cytometer.

Figure 1. Cell growth rate of A549 lung cancer cells after treatment with various concentrations of Art. A549 lung cancer cells were treated with various concentrations of Art for 24 h and the cell growth rate was detected by the MTT assay. The cell growth rate was decreased in an Art concentration-dependent manner. Art, artesunate.

Assessment of the expression of Bcl-2 and Bax proteins in A549 cells using FCM. To determine the expression of Bcl-2 and Bax proteins after Art treatment, the harvested cells were fixed overnight in 70% ice-cold ethanol. After washing with ice-cold PBS, the cells were incubated with anti-Bcl-2 (1:100; cat. no. sc-7382; Santa Cruz Biotechnology, Inc.) and anti-Bax antibodies (1:100; cat. no. sc-20067; Santa Cruz Biotechnology, Inc.) for 30 min in the dark at room temperature. Subsequently, the cells were incubated with IgG-FITC antibody (1:100; cat. no. 115-095-003; Jackson ImmunoResearch Laboratories, Inc.) for 30 min in the dark at room temperature. The stained cells were analyzed using the FC500 flow cytometer, with the mean fluorescence intensity representing the expression of Bcl-2 and Bax proteins.

Statistical analysis. Statistical analysis was performed using the SPSS v21 software (IBM Corp.). Values are expressed as the mean $\pm$ standard deviation. Multiple groups were compared using one-way analysis of variance followed by Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Art inhibits A549 cell survival and proliferation. An MTT assay was used to determine the viability of A549 lung adenocarcinoma cells treated with different concentrations of Art at a dose range of 0.1–800 $\mu\text{g/ml}$ for 24 h. The survival of A549 cells was decreased by Art in a dose-dependent manner, with a 50% inhibitory concentration (IC_{50}) value of 52.87 ± 2.36 $\mu\text{g/ml}$ (Fig. 1). Treatment with Art exerted a growth inhibitory effect on A549 cells in a concentration-dependent manner.

In addition, FCM revealed that the proliferation index of A549 cells was significantly lower in the Art-treated groups than that in the control group ($P < 0.01$). The cell proliferation index in the 100 $\mu\text{g/ml}$ group was significantly lower than that in the 25 and 50 $\mu\text{g/ml}$ Art groups ($P < 0.01$). Furthermore, the cell population in G0/G1 phase was significantly higher in

Table I. Cell cycle phase distribution of A549 cells after treatment with various concentrations of Art.

Group	G0/1 (%)	S (%)	G2/M (%)	Proliferation index (%)
Control	59.56±0.11	36.68±1.79	3.76±1.72	40.44±0.11
25 µg/ml Art	61.78±0.19 ^a	32.10±3.31	6.12±3.13	38.22±0.19 ^a
50 µg/ml Art	63.48±0.18 ^a	28.41±1.18 ^a	8.11±1.10	36.52±0.18 ^a
100 µg/ml Art	65.32±0.21 ^a	23.95±1.26 ^a	10.73±1.08 ^a	34.68±0.21 ^a

^aP<0.01 vs. control group. Values are expressed as the mean ± standard deviation (n=3). Multiple groups were compared using one-way analysis of variance followed by Tukey's test. Art, artesunate.

Figure 2. Apoptosis rate of A549 cells after treatment with various concentrations of Art. (A) Representative flow cytometry dot plots with Annexin V-FITC/PI double staining to determine the cell apoptosis rate of A549 cells. (B) Quantitative results for apoptosis of A549 cells calculated as a percentage of the control. The apoptosis rate in the Art-treated groups was significantly higher than that of the control (P<0.01). The apoptosis rate in the 25 and 50 µg/ml Art groups was significantly lower than that of the 100 µg/ml group (P<0.01). *P<0.01 vs. control group; #P<0.01 vs. 100 µg/ml Art group. PI, propidium iodide; Art, artesunate.

the Art-treated groups than in the control and the G0/1 phase population of the cell cycle increased with the concentration of Art (P<0.01; Table I).

Art induces cell apoptosis and modulates the mitochondrial membrane potential. In order to further investigate the mechanism of the decrease in the number of viable A549 cells upon exposure to Art in the MTT assay, Annexin V/PI staining was performed to determine whether apoptosis was induced (Fig. 2). The results indicated that Art triggered apoptosis in a dose-dependent manner within a range of 25-100 µg/ml; furthermore, the cell apoptosis rate in the 100 µg/ml Art group was significantly higher than that in the 25 and 50 µg/ml groups (P<0.01). In addition, the mitochondrial membrane potential was determined in A549 cells using FCM. The mitochondrial membrane potential in the 25, 50 and 100 µg/ml Art groups was significantly lower than that in the control group (P<0.01). Treatment with Art significantly decreased the mitochondrial membrane potential, suggesting that Art triggered apoptosis in A549 cells in a dose-dependent manner (P<0.01; Fig. 3).

Art modulates Bcl-2 and Bax protein expression in A549 cells. Bcl-2 and Bax regulate cell apoptosis via pore formation in mitochondrial complexes, thereby serving as markers

for further analysis of the mechanism of action of Art in A549 cells. FCM revealed significant downregulation of Bcl-2 protein expression compared with the control (P<0.01; Fig. 4A and B). Furthermore, Bax protein expression was significantly upregulated compared with the control following treatment with Art for 24 h (P<0.01; Fig. 4C and D). These expression profiles were in line with the increased apoptotic activity via mitochondrial membrane modulation in A549 lung cancer cells after Art treatment.

Discussion

Lung cancer is one of the most common malignant cancer types worldwide, exhibiting high morbidity and mortality rates (1). Typical treatment regimens include surgery, chemotherapy and radiotherapy (23,24). Chemotherapy is the primary treatment for late-stage, inoperable cases. However, toxic side effects of chemotherapeutic drugs have severely affected the efficacy of such regimens, frequently accounting for treatment failure. Thus, identifying anti-cancer drugs for lung cancer with low toxicity and high efficacy is imperative.

Chinese herbal medicine for tumor treatment has a long history with minimal side effects. Art is a derivative of artemisinin, derived from *Artemisia annua*, and is a classic

Figure 3. Mitochondrial membrane potential following Art treatment was detected by flow cytometry. (A) Histograms for the mitochondrial membrane potential in A549 cells treated with different concentrations of Art for 24 h. (B) Quantified results for the mitochondrial membrane potential calculated as a percentage of the control. The mitochondrial membrane potential of A549 cells in the 100 µg/ml Art group was significantly lower than that in the 25 and 50 µg/ml groups ($P<0.01$). * $P<0.01$ vs. control group; # $P<0.01$ vs. 100 µg/ml Art group. Art, artesunate.

Figure 4. Bcl-2 and Bax protein expression of A549 cells after treatment with Art as detected by FCM. (A) Representative FCM histograms for the detection of Bcl-2 expression in A549 cells treated with Art for 24 h. (B) Quantified levels of Bcl-2 in the different groups calculated as a percentage of the control. Bcl-2 protein expression in A549 cells was significantly lower after Art treatment and the 25 and 50 µg/ml groups exhibited higher expression than the 100 µg/ml group ($P<0.01$). (C) Representative FCM histograms for the detection of Bax expression in A549 cells treated with Art for 24 h. (D) Quantified levels of Bax in the different groups calculated as a percentage of the control. Bax protein expression in A549 cells was significantly higher following Art treatment and the 25 and 50 µg/ml cohorts had lower levels than the 100 µg/ml group ($P<0.05$). Bax protein expression level was calculated as a percentage of the control. * $P<0.01$ vs. control group; # $P<0.01$, ## $P<0.05$ vs. 100 µg/ml Art group. FCM, flow cytometry; Art, artesunate.

anti-malarial drug that was recently demonstrated to have anti-tumor activity and is involved in the regulation of immune function (25-29). Several *in vitro* and *in vivo* studies have indicated that the anti-tumor effect of Art is associated with the

induction of apoptosis and cell cycle arrest. Yang *et al* (30), reported that Art induces mitochondrial apoptosis of retinoblastoma cells via upregulating Kruppel-like factor 6 in *in vivo* and *in vitro* experiments. Wang *et al* (31), indicated

that Art inhibited the proliferation and induced ferroptosis of CA-46 cells *in vivo*. Li *et al* (32), reported that Art may be valuable as a therapeutic agent for osteoarthritis. Chen *et al* (33) suggested that Art promoted type I T-helper cell differentiation from CD4⁺ T cells by downregulating Sirtuin 1 through microRNA-142, thereby enhancing cell apoptosis in ovarian cancer. Chen *et al* (34), demonstrated that Art inhibited β -catenin expression and cell proliferation as well as promoted apoptosis in MG-63 cells, rendering it a promising drug for the clinical treatment of osteosarcoma.

In addition to the anti-tumor effect, Art is also able to reverse the drug resistance of tumors. Nunes *et al* (35), reported that Art disrupts the androgen receptor antagonist-mediated resistance observed in metastatic castration-resistant prostate cancer. Jing *et al* (36), suggested that the combination of Art and sorafenib further improved the apoptosis of hepatocellular carcinoma (HCC) and revealed that Art induces HCC apoptosis via PI3K/AKT/mTOR pathway inhibition, thereby suggesting that the combination of Art and sorafenib is a potential therapeutic regimen for advanced HCC.

Previous studies by our group suggested that Art inhibited the growth of esophageal cancer and gastric cancer cells by inducing apoptosis (22,37,38) and reversed the multidrug resistance of esophageal cancer by modulating the expression of ATP binding cassette G2 (37,39). The present study focused on the inhibitory effects of Art in lung cancer. Compared with previous studies (22,40), the present study investigated the inhibitory effects of Art on lung cancer, including apoptosis. In addition, the present study focused on the molecular mechanisms of apoptosis, which was indicated to be mediated by the endogenous apoptosis pathway centered on the mitochondrial membrane potential; furthermore, Art inhibited the growth of lung cancer cells via induction of cell cycle arrest.

In the present study, the lung cancer cell line A549 was selected to test the potential anti-cancer effects and the tumor-suppressive effects of Art against lung cancer cells *in vitro*. MTT assay suggested that Art inhibited the growth of A549 cells in a dose-dependent manner. The IC₅₀ of Art on the A549 lung cancer cells over 24 h was 52.87 μ g/ml and three concentrations (25, 50 and 100 μ g/ml) were selected for subsequent experiments. However, the molecular mechanisms underlying Art-induced cell death in lung cancer cells remained to be clarified. In the present study, it was determined that Art exerts potent cytotoxic effects on the human lung cancer cell line A549 *in vitro*. The cytotoxicity of Art was mediated by induction of apoptosis and cell cycle arrest, which was further supported by the detection of apoptosis-associated factors.

Malignant tumor cells have strong proliferative abilities, typically with increasing S and G₂/M phase populations during the cell cycle. In the present study, it was determined that the proliferation index of A549 lung cancer cells decreased in a dose-dependent manner following Art treatment, which blocked the cell cycle progression of these cells at the G0/I stage. Thus, these results suggested that Art inhibits the growth of lung cancer cells by inducing cell cycle arrest.

Dysregulation of apoptosis has a critical role in the occurrence and development of tumors and induction of cell apoptosis is vital for drug inhibition in aberrant cell growth. Cellular apoptosis is an automated gene-controlled death mediated via complex regulatory mechanisms. In the present

study, cellular apoptosis was determined by the detection of Annexin V/PI staining by FCM. The results suggested that Art induced apoptosis of lung cancer cells in a dose-dependent manner. The mitochondrial membrane potential, Bcl-2 and Bax are essential effectors of the intrinsic pathway of apoptosis, which were modulated by Art treatment. Since the mitochondrial membrane potential is closely associated with apoptosis, the decrease in the level may cause irreversible cell death. Hence, the content of markers of the intrinsic pathway of apoptosis, namely the Bcl-2 and Bax proteins, which regulate the mitochondrial membrane potential, was determined. The expression of Bcl-2 protein was indicated to be significantly decreased along with the upregulation of Bax following Art administration, which was consistent with decreased mitochondrial membrane potential that results in cell apoptosis.

In conclusion, the present study explored the specific mechanisms of lung cancer cell inhibition by Art. Art induced lung cancer cell apoptosis and cell cycle arrest, reduced the level of Bcl-2 protein and mitochondrial membrane potential and increased the expression of Bax protein. A previous study found that Art was associated with minimal toxic side effects (22), taken together with the present findings, it could be speculated that Art may have potential as a highly effective, non-toxic anti-cancer agent in chemotherapy. The molecular mechanisms of growth inhibition of lung cancer cells by Art are complex, necessitating further studies in the future. In future investigations, a variety of lung cancer cell lines should be used to further verify the growth inhibitory effect of Art on lung cancer cells. In addition, non-cancerous cells should be used to determine the side effects of Art.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YZ performed the experiments and wrote the manuscript. JL performed the experiments and statistical analysis. LL designed the study, performed the experiments and revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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