Original Research Article

Arctigenin-induced reversal of drug resistance in cisplastin-resistant cell line A549/DDP, and the mechanism involved

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Abstract

Purpose: To investigate the drug resistance reversal effect of arctigenin (ARG) on cisplatin-insensitive A549/DDP cancer cells, and to elucidate the underlying mechanism(s).
Methods: Four groups of cells: control, DDP, ARG and ADP were used. The degrees of inhibition of proliferation, drug resistance and apoptotic changes were measured using MTT assay, CCK-8 assay and flow cytometry, respectively. Expressions of PTEN and STAT3 proteins were determined by Western blotting.
Results: At ARG concentration of 5 µmol/L, A549/DDP cells were significantly inhibited (p < 0.05). The combination therapy was more effective in reversing A549/DDP cells resistance than the single therapy. The expression level of PTEN protein increased with increase in ARG concentration, while STAT3 protein expression decreased with increase in ARG concentration. ADP group up-regulated PTEN but decreased STAT3 expression levels.
Conclusion: ARG regulates drug resistance in A549/DDP cells, possibly via a mechanism involving reduction of A549/DDP cell sensitivity to DDP, thereby regulating the stress pathways associated with PTEN and STAT3. The combination of ARG and DDP effectively reduces A549/DDP cells resistance.

Keywords: Arctigenin, Non-small cell lung cancer, Drug resistance

INTRODUCTION

Lung cancer is one of the most harmful malignant tumors in humans. According to statistics, male lung cancer patients number in China ranks first among all cancer patients, and female lung cancer patients number is second only to the number of female breast cancer patients [1]. Non-small cell lung cancer (NSCLC) is seen in 85 % of lung cancer patients [2-4].

The early symptoms of NSCLC are not easily noticeable. Thus, more than 70 % of patients are diagnosed with advanced stage cancer and metastasis, leading to poor prognosis [5,6]. Chemotherapy is the main treatment method for NSCLC. Currently, platinum-containing drugs are the preferred clinical therapy. However, the treatment outcome is not satisfactory due to drug resistance. Moreover, the mechanism associated

with the chemotherapy resistance has not been unraveled. In recent years, bioactive products of plants have been shown to have anticancer effects. The combination of traditional chemotherapeutic drugs and plant bioactive products could result in enhanced anticancer effects, and this has attracted the attention of researchers [7]. It makes sense to find a safe and effective natural anticancer drug in combination with traditional chemotherapy drugs for overcoming chemotherapy resistance and inhibiting tumor growth.

Arctigenin (ARG) is a lignan compound isolated from the dry and mature fruits of second-year herbaceous burdock of Asteraceae. Studies have shown that ARG has anti-tumor, anti-diabetic and other pharmacological effects which could induce cancer cell apoptosis [8,9]. However, there are limited studies on its effect on NSCLC, and the molecular mechanisms involved. The effect of ARG on sensitivity of drug-resistant cancer cells has also not been reported. Hence, the present research was carried out to study the influence of ARG on drug insensitivity, and the effect of combinations with DPP on A549/DDP cells resistance, and the mechanisms involved. This was with a view to providing an experimental basis for the development of newer, highly effective and safer NSCLC treatment regimens.

EXPERIMENTAL

High sugar medium DMEM was obtained from HyClone Fetal bovine serum, phosphate buffered saline (PBS) and 0.25 % Trypsin sin-EDTA were products of Gibco. Cisplatin (freeze-dried powder was procured from Shandong Gilu Pharmaceutical Co. Ltd; ARG and DMSO were bought from Aladdin. Penicillin, streptomycin, tetramethylazolidazole salt (MTT) and RIPA lysate were manufactured by Biyuntian Biological; CCK-8 kit, and Annexin-fitc/PI double dyeing kit were from Kgi Biological. Beta-actin monoclonal antibody was purchased from Bioworld; STAT3 polyclonal antibody was bought from ABclonal, while PTEN polyclonal antibody was product of Cell Signaling Technology.

Cell culture and reagent preparation

The A549/DDP cells were cultured in high-sugar DMEM medium containing 10 % FBS and 1 % DBS, and digested with trypsin (0.25 %). The cells were selected and used at logarithmic stage in this study at temperature of 37 ^{o}C, 95 % humidity and 5 % CO_2. A549 /DDP cells were placed in 6 μmol/L DDP to maintain drug resistance. The reserve solution (2 g/L) was prepared by dissolving 20 mg DDP in 10 ml PBS,

from which the DDP working solution (1 g/L) was prepared. 50 mg ARG was dissolved in 13.4 mL DMSO to prepare 10 mmol/L ARG stock solution.

MTT assay

Four groups of cells were used, based on doses of ARG. Following tryptic digestion, they were counted. They were then inoculated in wells (2 × 10^4/well), and cultured in an incubator at 37 ^{o}C in an atmosphere containing 5 % CO_2. After 12 h, the cells were attached to the wall, and the culture medium was discarded. Serum media containing different concentrations of ARG (1, 5, 10, 20, 50 and 100 μmol/L) were added to the wells, and blank and control well containing only medium were set up simultaneously. The blank well contained only medium, without cells, while the control well had medium and cells, but no drug.

Each group was set in 5 replicate wells. The cells were placed in an incubator at 37 ^{o}C and 5% CO_2 for 24 h, after which 20 μL MTT reagent was added, followed by incubation for 4 h. Thereafter, the formazan formed were solubilized in DMSO, and the absorbance value of each well was read at 490 nm. Three independent replicates were used to calculate the degree of inhibition of cell proliferation.

Evaluation of effect of ARG and DDP on proliferative ability of A549/ DDP cells

Following tryptic digestion, the A549/ DDP cells were suspended and counted. They were then inoculated in well plates (2 × 10^4 /well), and cultured at 37 ^{o}C and 5 % CO_2 overnight. The cells were attached to the wall, and the culture medium was discarded. The corresponding drugs were added along with the configured serum medium. In this experiment, blank wells and control wells were set up as described earlier, with 5 replicate wells per group. The culturing was continued in an incubator at 37 ^{o}C and 5 % CO_2. After 24 h, 10 μL CCK-8 reagent was added, and the culturing was cultured in the incubator for 4 h. Optical density was read at 450 nm in a microplate reader. Three independent replicates were used to calculate the degree of inhibition of cell proliferation.

Flow cytometry

Apoptosis was determined with Annexin v-fitc /PI double staining. Cells in logarithmic growth phase were used. They were inoculated in dishes, and the cell density was 4×10^6 cells/mL. Then, they were inoculated in 6 wells at 100 microns per well. After 24 h, the cells were

divided into control group, DDP group (20 μmol/L), ACR group (5 μmol ARG/L) and ADP group (20 μmol DDP/L + 5 μmol ARG/L). After the cells of each group were cultured for 24 h, the cells were subjected to tryptic digestion using trypsin digestion solution without EDTA, to yield a cell suspension (1×10^6 cells/mL).

After washing twice with PBS, centrifugation was done at 1000 rpm for 5 min, and apoptosis was detected using Annexin V FITC/PI method. In this procedure, Annexin V-FITC and PI with appropriate fluorescence labeling were added to the extracts, followed by mixing and incubation in the dark for 10 min. Following staining, 400 μL staining buffer was added. Finally, flow cytometry was used for detection of apoptotic cells, while ModFit3.0 software was used for analysis.

Western blot analysis

The A549/DDP cells in logarithmic growth phase were inoculated into plates. After adjusting the cell density to 4×10^6 cells/mL, they were seeded (100 μL per well), and cultured for 24 h. Then, the groups were administered their respective drugs. The cells were harvested and dissolved in RIPA buffer for 30 min on ice. The protein content of the cells was determined using BCA assay.

A sample of cellular protein was added to a buffer that was five times the volume of the sample. The samples were boiled for 15 min, and the sample size was adjusted for Western blot. Wet transfer of proteins from 10 % SDS-PAGE to PVDF membrane was done, and the protein was transferred to a blocking solution (5 % BAS) and blocked by shaking at room temperature for 1h. Primary and secondary antibodies were added, and the membrane was washed with PBST and exposed and scanned.

Statistical analysis

Measurement data are expressed as mean ± standard deviation, and were statistically compared with SPSS 17.0 statistical software using *t*-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Inhibition of proliferation of A549/DDP cells by different concentrations of ARG

Figure 1 shows that when ARG concentration was 1 μM, the inhibition of proliferation of A549/DDP cells was not significant $(p > 0.05)$. However, when the concentration of ARG was

higher than or equal to 5 μM, concentration-dependent inhibition of proliferation of A549/DDP cells occurred, relative to control $(p < 0.05)$.
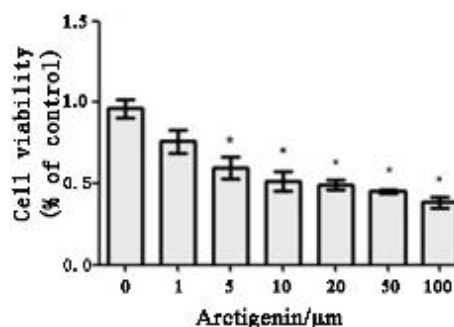
Figure 1: Inhibitory effect of ARG on proliferation of A549/ DDP cells; *p < 0.05, relative to control

Effect of combined administration of ARG and DDP on A549/DDP proliferation

The degree of proliferation was comparable between ADP/DDP cells in DDP and control groups. However, proliferations of A549/DDP cells in ARG group and ADP group were significantly inhibited, when compared with control and DDP group $(p < 0.05)$, and the inhibition was greater in ADP group. This indicated that the combination of ARG and DDP can effectively reverse A549/DDP cells' drug resistance (Figure 2).
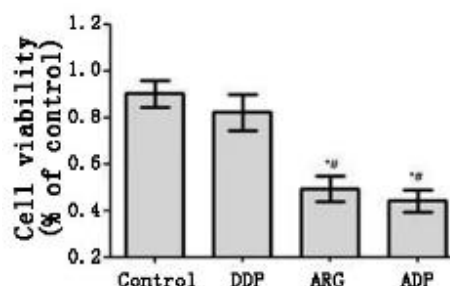
Figure 2: Inhibitory effect of different treatments on the proliferation of A549/DDP cells; *p < 0.05, relative to control; #*p* < 0.05, relative to DDP group

Effect of ARG combined with DDP A549/DDP cells apoptosis

The increasing order of apoptosis in the various groups was: control group (0.185 %) < DDP group (4.64 %) < ACR group (9.91 %) < ADP group (21.6 %). Apoptosis in DDP group, ARG group and ADP group were significantly higher than that in the control group, and the degrees of apoptosis in the ARG and ADP groups were markedly greater than in DDP cells $(p < 0.05)$. These results are presented in Figure 3.
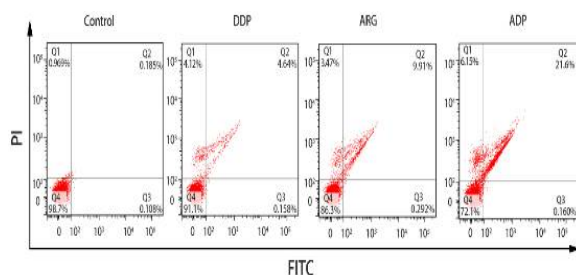
Figure 3: Effect of various treatments on apoptosis of A549/DDP cells. A549 /DDP cells were incubated with 20 μM DDP, 5 μM ARG, and 5 μM ARG for 24 h, and the cells were collected

Effect of different drugs on STAT3 and PTEN protein expression levels

Different concentrations of ARG had different effects on the protein expressions of PTEN and STAT. The protein expression of PTEN in ARG group (5 μM) was greater than those in ARG (1 μM) and ARG groups (10 μM). The protein expression of STAT3 in ARG group (5 μM) was lower than that in ARG group (1 μM) and ARG group (10 μM). Compared with DDP group and ARG group, ADP group had significantly up-regulated expression level of PTEN, and significantly down-regulated STAT3 expression.

Figure 4: Effect of various treatments on protein expressions of PTEN and STAT3

DISCUSSION

Due to smoking, tobacco pollution; NO_2, PM2.5, dust and other environmental pollutants; individual susceptibility and other factors, the occurrence of lung cancer amongst Chinese remains elevated [10,11]. The current treatments for NSCLC include chemotherapy, radiotherapy and surgery. Although surgery is the most effective treatment at present, most NSCLC patients are usually diagnosed with advanced stage or metastasis of cancer cells, thereby making surgery generally unfeasible.

Radiation therapy is also crucial in early and late treatment of lung cancer, but radiation therapy inevitably causes unnecessary damage to normal lung tissues within the radiation field, leading to the generation of radioactive pneumonia, which causes poor prognosis. The current standard chemotherapy regimen for advanced (IIB-IV) NSCLC is platinum-containing drug chemotherapy. However, previous studies found that patients developed drug resistance after 4 - 6 cycles of first-line standard platinum-based chemotherapy, and their condition did not improve subsequently, leading to treatment bottleneck [12,13].

In previous studies, extracts from natural plants were found to improve the anti-cancer effect of platinum-containing drugs during the bottleneck phase of chemotherapy. Therefore, in order to break through the bottleneck in the efficacy of platinum-containing chemotherapy, the combination of cisplatin with natural anticancer drugs has attracted much interest from researchers in recent years. The combination of different anticancer drugs is of great significance for overcoming chemotherapy resistance and improving therapeutic effects in patients.

Arctigenin (ARG) is an important phenyldibenzyl butyllactone compound present in burdock seeds. Signal transduction and transcriptional activation factor-3 (STAT3) are members of the family of transcription factors. Sustained activation of STAT3 triggers multiple oncogenic tyrosine kinase signaling pathways. Not only is STAT-3 related to the proliferation, differentiation and apoptosis of various tumors, it also promotes cancer cell metastasis. At present, STAT3 is considered a new target of tumor therapy. Studies have shown that STAT3 is overexpressed in ovarian and breast cancer cells [14,15].

A tumor suppressor gene, PTEN promotes apoptosis and inhibits tumor development by antagonizing tyrosine kinase activity [16,17]. It has been reported that PTEN is important in the etiology of NSCLC. A study has demonstrated that in the absence of the expression of PTEN, chromosome I stage NSCLC was enhanced [18]. Indeed, it has been shown that patients with high expression of PTEN had longer survival and late disease recurrence and metastasis than patients with low expression of NET, and that there was no correlation with tumor stage, physical condition and weight loss [19].

Thus, PTEN may be an indicator of differentiation, metastasis and prognosis of lung cancer.

There are many studies on the anti-tumor effect of ARG. In the lung cancer clinical treatment, ARG shrinks the tumor and prolongs patient's survival time [20]. It has been found that ARG induces cell cycle arrest and inhibits the growth

of gastric cancer by regulating the cell cycle and the expressions of regulatory proteins [21]. A study has also found that ARG induced cell cycle arrest and apoptosis of bladder cancer T24 cells in a dose-dependent manner [22]. In addition, ARG has been shown to enhance the chemical sensitivity of NSCLC H460 cells to cisplatin [23]. In another study, it was reported that ARG enhanced the sensitivity of tumor cells to DDP by inhibiting signal transduction and transcriptional activation factor STAT3 [24].

Consistent with the above findings, in this study, it was demonstrated that when the concentration of ARG was higher than, or equal to 5 μM, cell proliferation was significantly and dose-dependently inhibited. When combined with ARG and DDP, the degrees of inhibition of proliferation and apoptosis of A549/DDP cells were higher than those in singly treatments. These results suggest that ARG effectively inhibits the proliferation of A549/DDP cells, and the combination of DDP and ARG regulates A549/DDP cells sensitivity. Further studies showed that PTEN protein expression increased, while STAT3 protein expression decreased with increase in ARG level. In the ADP group, PTEN protein expression level was upregulated, while STAT3 protein expression level was decreased. From these results, it can be reasonably expected that ARG may reduce the resistance of A549/DDP cells to DDP by regulating the stress pathways associated with PTEN and STAT3.

CONCLUSION

This study has shown that combining ARG with DDP effectively reduces the resistance of A549/DDP cells, probably via a mechanism linked to reduction of their susceptibility to DDP via regulation of PTEN and STAT3 stress pathways. Thus, ARG is a potential drug for use as a combination therapy for cisplatin-resistant A549 lung cancer.

DECLARATIONS

Acknowledgement

Conflict of interest

No conflict of interest is associated with this study.

Authors' contribution

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Shiying Zheng; Jiaping Li, Xinlu Tao, Zheng Tao, Yan Zhang, Shiying Zheng collected and analyzed the data; Jiaping Li and Xinlu Tao wrote the text. All authors read and approved the manuscript for publication. Jiaping Li and Xinlu Tao contributed equally to this work and should be considered as co-first authors.

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