Cryptotanshinone Reverses Cisplatin Resistance of Human Lung Carcinoma A549 Cells through Down-Regulating Nrf2 Pathway

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Key Words
Lung cancer • Cisplatin • Cryptotanshinone • Chemoresistance • Nrf2

Abstract
Background/Aims: To explore whether Nrf2 was associated with drug-resistance in cisplatin resistant A549 (A549/DDP) cells, and if cryptotanshinone (CTS), one of the bioactive compounds isolated from the roots of Salvia miltiorrhiza Bunge (Danshen), could enhance the sensitivity in A549/DDP cells towards cisplatin. Methods: A549 and A549/DDP cells were subjected to various treatments, and then Sulforhodamine B (SRB) assay, flow cytometry analysis and western immunoblotting analysis were applied to determine IC_{50}, apoptotic status and expressions of Nrf2 and its downstream genes. Results: The endogenous expression levels of Nrf2 as well as its target genes including GCLC, GCLM, HO-1, NQO1 and MRP1 were much higher in A549/DDP cells than those of A549 cells and the susceptibility of A549/DDP cells to cisplatin was partially restored by silencing Nrf2. The combination of CTS and cisplatin led to cell death and apoptosis through sensitizing A549/DDP cells towards cisplatin compared with cisplatin mono-treatment, however, this reversal role could be abolished by Nrf2 knockdown. Specifically, CTS obviously diminished Nrf2 expression, thus contributing to the decrease of Nrf2-target genes expression levels. Meanwhile, we also discovered that CTS triggered several other signals involving in chemoresistance such as MAPKs, Akt and STAT3 pathway. Conclusion: Our data indicated CTS may be developed as a potential sensitizer cooperating with anticancer drugs to combat chemoresistant carcinoma through the inhibition of the Nrf2 pathway.

C. Xia and X. Bai contribute equally to this work.
Introduction

Lung carcinoma is a main cause disturbing public health in the world [1]. Cisplatin belongs to platinum drugs which promotes cancer cell death and thereby is extensively utilized in the treatment of lung carcinoma [2]. But the development of a drug-resistant phenotype remains the primary hindrance towards the platinum-based chemotherapy for lung carcinoma [3]. Finding new drug sensitizers that can overcome drug-resistance and increase the potency of platinum drugs is necessary.

Nuclear factor erythroid-related factor 2 (Nrf2) is a redox-sensitive transcription factor [4]. Hundreds of Nrf2-target genes have been identified including NADP(H): quinone oxidoreductase 1 (NQO1), heme oxygenase (HO-1), and glutamate-cysteine ligase catalytic subunit (GCLC) [5, 6]. Recent studies have shown that Nrf2-mediated cytoprotective genes expression levels were positively correlated to drug resistance such as cisplatin, doxorubicin and etoposide [7, 8]. In lung and breast carcinoma, knockdown of Nrf2 could increase the sensitivity of cancer cells toward chemotherapeutic drugs [9]. So it is urgent to identify novel chemicals that are able to inhibit Nrf2-regulated defensive genes for the adjuvant therapy of chemoresistant lung tumor.

Cryptotanshinone (CTS) is one of the principal active constituents isolated from the roots of the plant Salvia miltiorrhiza Bunge (Danshen) [10]. It has been proposed to be a potent antitumor agent [11, 12]. Chen et al. reported that CTS inhibits lung tumorigenesis and induces apoptosis in cancer cells in vitro and in vivo [13]. Interestingly, we have found a high lung-accumulation phenomenon in the tissue distribution of CTS in rats [14]. However, whether CTS would sensitize chemoresistant cancer cells to chemotherapeutic drugs has not been investigated yet.

Therefore, this study was undertaken to examine whether CTS can suppress Nrf2 and its target genes expression and to restore the susceptibility of cisplatin-resistant A549 cell to cisplatin. Our study revealed that CTS, a natural diterpenoid compound, could reverse chemoresistance by increasing the sensibility in chemoresistant carcinoma towards chemotherapeutic drugs.

Materials and Methods

Reagents

Cryptotanshinone (over 98% purity) was kindly provided by Professor Gu Lianquan (Institute of Pharmacy Synthesis, Sun Yat-sen University). Sulforhodamine B was purchased from Alfa Aesar (Tianjin, China). Cis-diamminedichloroplatinum (II) (cisplatin, DDP) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Monoclonal antibodies against Nrf2, GCLC, Histone H3 were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Monoclonal antibody against NQO1 was purchased from Sigma (St. Louis, Missouri, USA) and other antibodies were obtained from Cell Signaling Technology Inc. (San Francisco, California, USA). All other chemicals and solvents were commercially available and of analytical grade.

Cell culture

A549 and cisplatin-induced multidrug-resistant A549/DDP cells were derived from the Cancer Institute & Hospital, Chinese Academy of Medical Sciences (Beijing, China). Cells were maintained in HAM’S/F-12 (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, New York State, USA), 100 U/mL penicillin and 100 mg/mL streptomycin. Cell lines were incubated at 37 °C in a humidified atmosphere of 5% CO₂.

Sulforhodamine B (SRB) assay

Survival rate of cells or cytotoxicity was measured by SRB assay with the product specification. A549 and A549/DDP cells were plated in 96-well plates (Corning, New York, USA) at a density of 7 × 103 per well. The cells were treated with various concentrations of cisplatin at 37°C in 5% CO₂ for 48 h. Half-maximal (50%) inhibitory concentration (IC₅₀) values were determined by subtracting mean OD value of respective
blank from the mean OD value of experimental set, and the fold of resistance was calculated in accordance with the following formula: Fold of resistance (RF) = IC_{50} (DDP toward A549/DDP) / IC_{50} (DDP toward A549). The reversal index (RI) = IC_{50} (chemotherapeutic drug alone) / IC_{50} (chemotherapeutic drug in the presence of the test drugs).

**Cell cycle analysis**

Cells were fixed in 70% ice-cold ethanol at 4 °C overnight. The fixed cells were washed with cooled PBS and stained with the staining solution containing 100 μg/ml of RNase A and 50 μg/ml of propidium iodide at 37 °C for 30 min and then analyzed for DNA content by flow cytometric analysis (Beckman Coulter EPICS XL, USA), and cell cycle data were determined using the Modfit software (Verity Software House, Topsham, Maine, USA).

**Cell apoptosis analysis**

After treatment of CTS or cisplatin or in combination, cells were detached with EDTA-free trypsin and washed twice with cooled PBS. The cells were then treated with Annexin V-FITC followed by treatment with PI for 15 min at 25 °C in dark place on the basis of the manufacturer’s protocol. Finally, cell apoptosis status was determined by the FACS Calibur analyzer (Beckman Coulter EPICS XL, USA).

**Nrf2 siRNA transient transfection**

Negative control siRNA and siRNAs against hNrf2 (human Nrf2 siRNA) were produced by RiboBio Co. Ltd. (Guangzhou, China). And these sequences for human Nrf2 siRNA were 5'-GAGAAAGAAUUGCCUGUAAdTdT-3'; 3'-dTdTCU -CUUUCUAACGGACAUU-5'. A549/DDP cells were plated into 6-well plate at a density of 1.5×10^5 cells per well. After 24 hours of incubation, negative control siRNA or siRNAs targeting human Nrf2 were transiently transected to A549/DDP cells with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, California, USA) and incubated for 48 h. Then CTS (10 μM) was added to the culture medium for the last 24 h. After that, the cells were harvested for western blot analysis.

**Western blot analysis**

Proteins extracted from cells were prepared with RIPA lysis buffer or Nuclear Extract kit (Active Motif, Carlsbad, New Mexico, USA) following the manufacturer’s specification. Protein concentration was determined by BCA protein assay (Thermo Scientific, Rockford, Illinois, USA). Protein extracts (40 μg) was resuspended and electrophoresed on 10 - 12% sodium dodecyl sulfate polyacrylamide gel and then blotted onto polyvinylidene fluoride membranes (Millipore A) at 230 mA for 1 h. Following blocking with 5% nonfat milk in TBST (TBS-1% Tween 20) for 1h, membranes were immunoblotted with primary antibodies overnight at 4 °C and further incubated with secondary horseradish peroxidase-conjugated anti-rabbit. Finally, protein bands were detected by developing the blots with the enhanced chemiluminescence western blot detection kit (Engreen Biosystem, China).

**Statistical Analysis**

All experiments were performed in triplicate at least. The results of this study are presented as mean ± SD and analyzed by Student’s t test with statistical analysis of data using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Probability values of < 0.05 were considered statistically significant.

**Results**

**Upregulation of Nrf2-dependent defense responses was involved in the chemoresistance of A549/DDP**

To find out the chemoresistance capacity of A549/DDP cells treated with cisplatin, Sulforhodamine B assay was used and RF was calculated. The IC50 of cisplatin in A549 cells was much lower than that in A549/DDP cells (P < 0.05). The fold of resistance (RF) was 8.89 demonstrating A549/DDP cells were cisplatin-resistant. The total or the nuclear Nrf2 protein levels in A549/DDP cells were much higher than those of A549 cells. Moreover, the expression levels of Nrf2-target genes are significant higher as well (Fig. 1).
Xia et al.: Cryptotanshinone Reverses Cisplatin Resistance of Lung Carcinoma

Cellular Physiology and Biochemistry

CTS restored the sensibility of A549/DDP cells towards cisplatin

CTS inhibited the proliferation of A549 and A549/DDP cells in a dose-dependent manner. Co-incubation of A549/DDP cells with various concentrations of CTS for 48 h significantly decreased the IC\textsubscript{50} of cisplatin in A549/DDP cells (Fig. 2A). In addition, treatment of CTS induced an accumulation of cells in the G0/G1 phase fraction with concomitant reduction of cell numbers in S phase (Fig. 2B). We then investigate apoptotic status of A549/DDP cells after 24h of exposure to cisplatin or CTS alone or in combination. The results showed that the apoptotic rate of cells in co-treatment group was much higher than that of mono-treatment. (Fig. 2C, \(P < 0.05\)).

CTS sensitized A549/DDP cells to CDDP by inhibition of Nrf2

The protein expression of Nrf2, p-Nrf2, MRP1, GCLC, GCLM, HO-1 and NQO1 were inhibited by CTS, and this inhibition was dose-dependent as shown in Fig. 3A. Since Keap1 is the major repressor of Nrf2 [4], the effect of CTS on the expression of Keap1 was also investigated. The results showed that the expression of Keap1 was significantly increased by CTS treatment (Fig. 3A). In addition, Nrf2 protein expression began to decline from the 12th hour after CTS treatment (10 μM) (Fig. 3B).

To confirm whether Nrf2 was involved in the chemoresistance of A549/CDDP cells, Nrf2 siRNA was transfected to knock down Nrf2 expression. The reduction of Nrf2 expression was confirmed by Western blot analysis. Silencing Nrf2 rendered A549/DDP cells much more susceptible to cisplatin. The expression levels of Nrf2 and its downstream genes in Nrf2-siNrf2 A549/DDP cells were not significantly changed by CTS (Fig. 4A). Besides, the effect of CTS was decreased in Nrf2- knockdown A549/DDP cells (Fig. 4B), indicating that CTS restored the sensitivity of A549/DDP cells to cisplatin via inhibiting Nrf2.

CTS inhibited MAPKs, AKT and STAT3 pathway.

It is reported that MAPK signaling pathways are involved in chemoresistance and regulating the phosphorylation of Nrf2 [15]. In addition, activating AKT and STAT3 led to
activation of Nrf2 with subsequent induction of defensive genes which protect cells against toxic insults [15]. To determine whether these mechanisms mentioned above were involved in the sensitivity increase of A549/DDP cells to cisplatin by CTS, the effects of CTS on the mitogen-activated protein kinase, Akt and STAT3 pathways were studied. As shown in Fig. 5A, CTS attenuated the expression level of p-P38, p-JNK and p-ERK1/2 levels in a dose-dependent manner. Furthermore, CTS also prevented Akt and STAT3 activation (Fig. 5B).
Discussion

Recently, Nrf2 has been proved to be responsible for the chemoresistant effect against chemotherapy. Higher expression of Nrf2 and its downstream genes has been noted in hepatocellular carcinoma than non-neoplastic liver tissues [16]. Besides, Nrf2 silence rendered some cancer cell lines more sensitive towards chemicals [16]. These studies pointed out that constitutive activation of Nrf2 and Nrf2-regulated genes were responsible for the formation of neoplastic chemoresistance towards anticancer agents.

In the present study, we found that IC\textsubscript{50} of A549 cells to cisplatin was much lower than that of A549/DDP cells indicating that A549/DDP cells had significant cisplatin-resistance. Nrf2 expression level is also lower in A549 cell than that in A549/DDP cells as well as its target genes. Furthermore, transfection of A549/DDP cells with Nrf2 siRNA suppressed Nrf2 signaling pathway leading to the restoration of susceptibility of A549/DDP cells to cisplatin. Defensive genes NQO1, GCLC, HO-1, and drug transporters MRP1 are direct target genes of Nrf2, which play essential roles in drug resistance. Results from our data analysis demonstrated that the constitutive activation of Nrf2 and its downstream genes was at least partially involved in the mechanisms of cisplatin-resistance in A549/DDP cells.

Although Nrf2 has been proposed as an attractive molecular target reversing tumor chemoresistance, few natural compounds have been found as inhibitors of Nrf2 [17-20]. Chrysin, Brusatol and trigonelline were proved to inhibit Nrf2 leading to the restoration of sensibility of cancer cells. Similar to these natural compounds, CTS is a natural product...
Xia et al.: Cryptotanshinone Reverses Cisplatin Resistance of Lung Carcinoma

Cellular Physiology and Biochemistry

with potent anticancer activity [10]. Our results showed that CTS treatment enhanced the susceptibility of A549/DDP cells to cisplatin and blocked Nrf2 signaling pathway. Moreover, Nrf2-deficient A549/DDP cells were much more sensitive to cisplatin than A549/DDP cells, whereas the effects of CTS were diminished in Nrf2 siRNA-transfected A549/DDP cells (Fig. 4B). Therefore our data supported the proposal that the sensibility restoration of A549/DDP induced by CTS was associated with the inhibition of Nrf2 pathways.

It was shown that MAPK, Akt and STAT3 pathways are related to drug-resistance and altering the molecular events of Nrf2 [8]. To further understand the mechanism of sensitization to anticancer drugs by CTS, the effects of CTS on the expression of the MAPKs, Akt and STAT3 were detected and we found that CTS down-regulated the protein levels of...
p-JNK, p-ERK, p-p38, p-Akt and p-STAT3. These findings further indicated that CTS may be useful for targeting Nrf2 in clinical therapy to sensitize tumor to therapeutic drugs.

Keap1 serves as a negative regulator of Nrf2 [21]. Accumulating evidences have shown that mutations of Keap1 exist in some cancers including A549 cells; these mutations enhance Nrf2 activity and are associated with resistance to standard chemotherapy and high mortality of tumor [21]. In present study, we found that the expression of Keap1 was enhanced by CTS, however, the contribution of CTS to Keap1 downregulating Nrf2 demands further study.

In summary, our data support the notion that high expression level of Nrf2 led to the chemoresistance of A549/DDP cells, while CTS suppressed Nrf2 and Nrf2-target genes, which rendered A549/DDP cells more susceptible towards cisplatin and finally facilitated cell death and apoptosis. However, we do not know if CTS have the same effects in animal models as well as in human beings. Therefore more detailed studies are necessary to test whether CTS can act as a potent chemical for adjuvant chemotherapy of tumor.

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Disclosure Statement

The authors have declared no conflict of interest.
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