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Cepharanthine induces apoptosis through reactive oxygen species and mitochondrial dysfunction in human non-small-cell lung cancer cells

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ABSTRACT

Cepharanthine is a medicinal plant-derived natural compound which possesses potent anti-cancer properties. However, there is little report about its effects on lung cancer cells. In this study, we investigated the effects of cepharanthine on the cell viability and apoptosis in human non-small-cell lung cancer H1299 and A549 cells. It was found that cepharanthine inhibited the growth of H1299 and A549 cells in a dose-dependent manner which was associated with the generation of reactive oxygen species(ROS) and the dissipation of mitochondrial membrane potential (Δψm). These effects were markedly abrogated when cells were pretreated with N-acetylcysteine (NAC), a specific ROS inhibitor, indicating that the apoptosis-inducing effect of cepharanthine in lung cancer cells was mediated by ROS. In addition, cepharanthine triggered apoptosis in non-small lung cancer cells via the upregulation of Bax, downregulation of Bcl-2 and significant activation of caspase-3 and PARP. These results provide the rationale for further research and preclinical investigation of cepharanthine's anti-tumor effect against human non-small-cell lung cancer.

1. Introduction

Lung cancer is one of the most common malignant carcinoma in the world, and non-small-cell lung cancer (NSCLC) accounts for about 85% of total lung cancer [1]. Although in the last few decades, prevailing chemotherapy in combination with surgery has resulted in relieving symptoms and improving the life quality of patients suffering from lung cancer, death remains to be the ending for most patients with lung cancer [2,3]. Consequently, exploring more effective therapeutic agents are necessary for developing the treatment of NSCLC.

Reactive oxygen species (ROS), contain oxygen ions and the chemical activity of the peroxidase molecules. ROS generation has important physiological effect in normal cells, whereas it will kill the cell if produced too much [4]. Many studies show that phytochemicals targeting ROS metabolism could kill cancer cells selectively, because the cancer cells showed higher levels of endogenous active oxygen than normal cells, toxic threshold can be easily implemented [5]. The excessive ROS generation can damage DNA in cancer cells, leading to cancer cell death and cell cycle arrest. Apoptosis is a morphologically defined form of programmed cell death [6]. The dissipation of mitochondrial membrane potential (Δψm) is one of the most important mechanisms of apoptosis. As reducing the mitochondrial membrane potential, mitochondrial permeability transition pores (PTP) are opened, leading to cytochrome c and other proapoptotic molecules released, from intermembrane space to cytosol. Finally, caspase-3 and PARP, DNA repair enzymes are activated, resulting in apoptosis [7,8].

Cepharanthine (CEP) is a biscoclaurine alkaloid, extracted from the roots of Stephania cepharantha Hayata, known to have anti-inflammatory and immunomodulatory activities [9,10].In recent decades, many reports reavealed that CEP has anti-tumor activity, anti-tumor invasion and pro-apoptic action in many cancer cells [11–14]. Further studies showed that CEP has anti-tumor action by

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inducing apoptosis and raising ROS generation [15]. However, the effects of cepharanthine on human non-small-cell lung cancer cells are still unknown. The present studies were aimed to exploring the effects of cepharanthine on the proliferation of H1299, A549 cells and to determine the possible mechanism in cepharanthine -induced apoptosis in lung cancer cells with a therapeutic potential.

2. Materials and methods

2.1. Materials

As for cell line selection, we chose A549 and H1299 cells as referencing Dr. Jacques Pouyssegur 's research [16]. Human non-small-cell lung cancer cells H1299 and A549 were purchased from ATCC (Rockvill.Maryland.U.S.A). Cepharanthine which was purchased from the Tongtian company (Shanghai, China), was dissolved in Dimethyl Sulfoxide (DMSO), which was purchased from Shenggong company (Shanghai, China) to make a stock solution. Fatal bovine serum (FBS) was purchased from Gibco. [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (MTT), RPMI 1640 medium and Rh123 mitochondrial specific fluorescent dye were purchased from Sigma. Cell Cycle Analysis Kit and Reactive Oxygen Species Assay Kit were purchased from Beyotime company (Shanghai, China). BCA Protein Assay Kit, Annexin V-FITC Apoptosis Detection Kit were purchased from Keygen company (Nanjing, China). Polyclonal antibodies against β-actin and horseradish peroxidase-conjugated secondary antibodies (goat-anti rabbit, mouse) were purchased from Beyotime company (shanghai, China). Polyclonal antibodies against Bax, Bcl-2, caspase-3, cleaved caspase-3, poly ADP-ribose polymerase (PARP) and horseradish peroxidase-conjugated secondary antibodies (goat-anti rabbit, mouse) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Western Blotting detection kit was purchased from Milipore (Billerica, USA).

2.2. Cell culture

Human non-small cell lung cancer cells H1299 and A549 were maintained in RPMI 1640. Cells were supplemented with 10% FBS at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Cell growth inhibition assay

The effects of cepharanthine on cell viability were evaluated by MTT assay. Briefly, H1299 and A549 cells were treated with cepharanthine in different concentrations, in the presence or absence of NAC. After 24 h, the MTT reagent was added (500 μg/ml) and incubated for additional 4 h. Then 150 μl DMSO was added to dissolve the formazan crystals. The absorbance was read in a microplate reader (Thermo Scientific) at 570 nm.

2.4. Flow cytometric analysis of cell cycle

Cell cycle was detected by flow cytometry using PI cell cycle detection kit. Briefly, the cancer cells were seeded into 6-well plates and H1299 cells were treated with cepharanthine at the concentration of 0 μM, 20 μM and 40 μM, A549 cells were treated with cepharanthine at the concentration of 0 μM, 40 μM and 80 μM, respectively incubated for 24 h. Finally, the sample was incubated with RNase A and propidium iodide (PI) staining solution as the manufacturer described.

2.5. Apoptosis analysis by flow cytometry

The apoptotic rate of cancer cells was detected by flow cytometry using annexin V-FITC/PI staining. The cancer cells were incubated in 6-well plates and H1299 cells were treated with cepharanthine for 24 h at the concentration of 0 μM, 20 μM and 40 μM, A549 cells were treated with 0 μM, 40 μM and 80 μM, respectively. The cells were pretreated with 5 mM NAC for 2 h and H1299 cells added 40 μM cepharanthine, A549 cells were added 80 μM for 24 h in ROS inhibitor group. The cells were collected, washed with phosphate buffered saline (PBS) and resuspended in binding buffer containing Annexinv-FITC and PI according to the manufacturer's instructions. After staining, cells were analyzed by flow cytometry.

2.6. Flow cytometric determination of reactive oxygen species (ROS) in NSCLC

In order to determine the intracellular changes in ROS generation, cancer cells were stained with 2′, 7'-dichlorofluorescein-diacetate (DCFH-DA). Briefly, H1299 cells were treated with 0, 20 and 40 μM, A549 cells were treated with 0, 40 and 80 μM cepharanthine for 24 h. In the ROS inhibitor group, the cells were pretreated with 5 mM NAC for 2 h then H1299 cells were treated with 40 μM cepharanthine while A549 cells with 80 μM for 24 h. After treatment, cells were incubated with 10 μM DCFH-DA in the dark for 30 min. Finally, the cells were analyzed for DCF fluorescence by flow cytometry.

2.7. Flow cytometric determination of mitochondrial membrane potential ($\Delta\Psi m$)

Rhodamine 123 was used to evaluate perturbations in $\Delta\Psi$m in cancer cells by flow cytometry. Briefly, H1299 cells were treated with 0, 20 and 40 μM, A549 cells treated with 0, 40 and 80 μM cepharanthine for 24 h. The cells were pretreated with 5 mM NAC for 2 h then H1299 cells were added 40 μM, A549 cells were added 80 μM cepharanthine for 24 h in the ROS inhibitor group. Then, the cells were incubated with the Rhodamine 123 (10 μM) at 37 °C in the dark for 20 min. After filtration, the samples were analyzed by flow cytometry.

2.8. Western blot analysis of protein expression

To evaluate the effect of cepharanthine, and to explore the mechanism of the apoptotis, the apoptotic related proteins were detected by western blotting. Firstly, H1299 cells were treated with 0 μM, 20 μM and 40 μM, A549 cells were treated with 0 μM, 40 μM and 80 μM of cepharanthine for 24 h. The cell pellets were resuspended in RIPA lysis buffer and ultrasound lysed on ice. After centrifugation for 5 min, the supernatant fluids were collected and the protein content of the supernatant was determined using BCA Protein Assay Kit. The protein samples were frozen at −80 °C. Secondly, the protein lysates were separated by electrophoresis on 10% sodium dodecyl sulphate (SDS) -polyacrylamide gel and transferred to a Polyvinylidene fluoride membrane (Amersham Biosciences, Piscataway, NJ). The membranes were soaked in blocking buffer (5% skimmed milk) for 1 h. To probe for all the proteins, membranes were incubated overnight at 4 °C with relevant antibodies, followed by appropriate horseradish peroxidase (HRP) conjugated secondary antibodies and enhanced chemiluminescence (ECL) detection. We used Gel-Pro Analyzer, which could extract valuable qualitative and quantitative information from electrophoretic gels to document and store our Western blot data.

2.9. Statistical analysis

Data were expressed as mean ± SD. Comparisons were made using a one-way ANOVA followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of cepharanthine on lung cancer cells proliferation

To detect the growth inhibitory of cepharanthine (structure shown in Fig. 1A), H1299 and A549 cells were performed using MTT assay. Our results showed that cepharanthine inhibited the proliferation of H1299 and A549 cells in a dose-dependent manner. The cells were pretreated with 5 mM NAC for 2 h, and the cytotoxic effect on H1299 and A549 cells was significantly reduced in the ROS inhibitor group (Fig. 1B and C).

3.2. Cepharanthine induced cell cycle arrest in lung cancer cells

Cell cycle arrest and apoptosis are two effective mechanisms in the induction of cell death [17]. In order to find out whether cell growth inhibition is due to cell cycle arrest at a specific phase of cell cycle, we anaylzed the effect of cepharanthine on the cell cycle of H1299 and A549 cells by flow cytometry. Our results showed that the percentages of H1299 cells that accumulated in the S phase were 14.2, 24.8 and 33.8%. The percentages of A549 cells in G2/M phase were 26.5, 38.9 and 52.4%. These findings indicated that

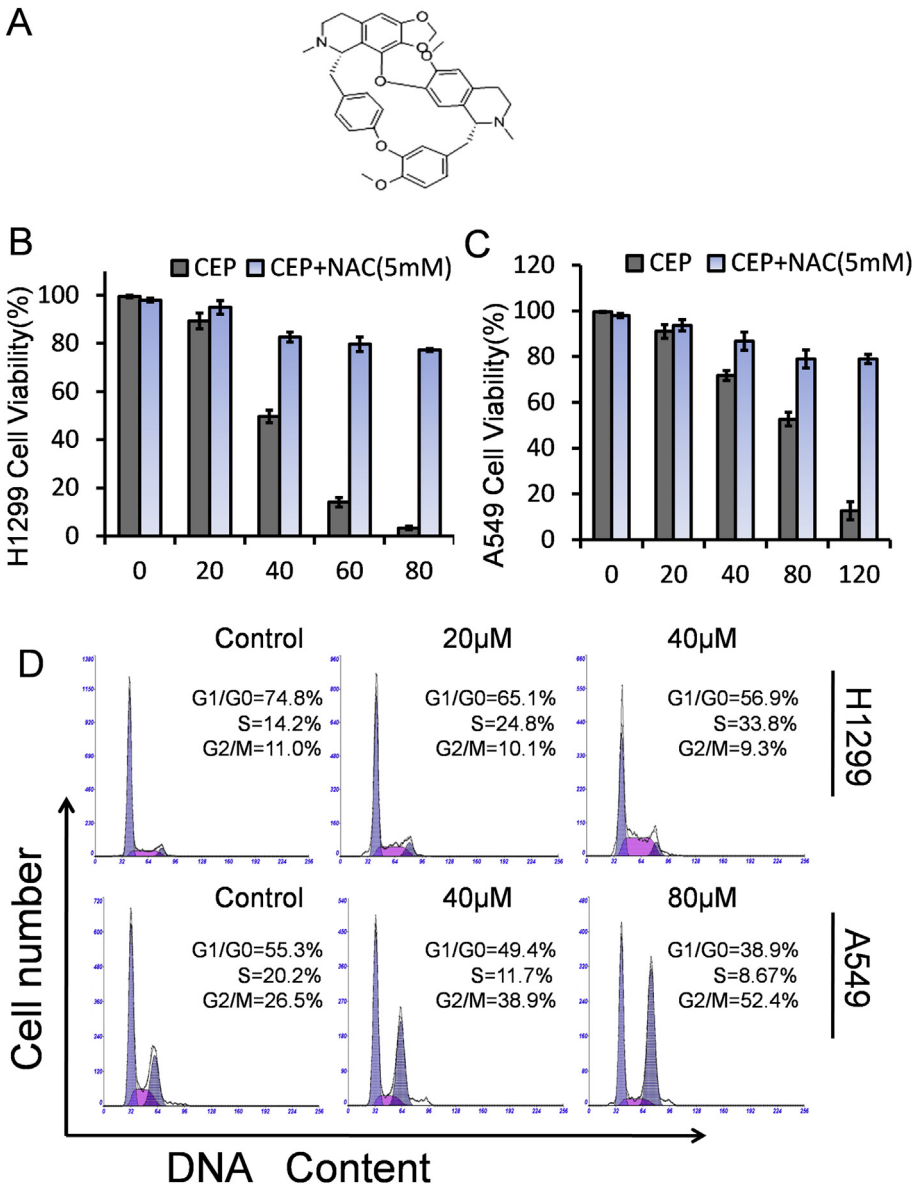

Fig. 1. A. Chemical structures of cepharanthine. B. Cepharanthine induced growth inhibition on H1299 cells. The cells were exposed to the indicated concentrations of cepharanthine for 24 h, in the presence or absence of NAC, and the percentages of viability were measured by MTT assay. Data are expressed as mean ± SD of three independent experiments. Columns not sharing the same superscript letter differ significantly ($P < 0.05$). C. Cepharanthine induced growth inhibition on A549 cells. The cells were exposed to the indicated concentrations of cepharanthine for 24 h, in the presence or absence of NAC, and the percentages of viability were measured by MTT assay. Data were expressed as mean ± SD of three independent experiments. Columns not sharing the same superscript letter differ significantly ($P < 0.05$). D. Effect of cepharanthine on cell cycle distribution. H1299 cells were treated with 0 μM, 20 μM and 40 μM, A549 cells treated with 0 μM, 40 μM and 80 μM of cepharanthine for 24 h and then stained with PI by flow cytometric analysis. Histograms showed the numbers of cells/channel (y-axis) vs. DNA content (x-axis). These values indicated the percentages of cells in the corresponding phases of cell cycle.

cepharanthine arrested the cell cycle of H1299 cells at S phase and A549 cells at G2/M phase in a dose-dependent manner (Fig. 1D).

3.3. Cepharanthine induces apoptosis in H1299 and A549 cells

To further investigate cepharanthine-induced inhibitory effect, H1299 and A549 cells were treated with cepharanthine as described in Materials and Methods section and the percentages of cells undergoing apoptosis/necrosis were determined by flow cytometric analysis after staining with annexin V-FITC and PI. Our results showed that cepharanthine caused a dose-dependent apoptosis. A significant increase was observed in early apoptosis in experimental group compared to control group. Pretreatment with NAC blocked most of the apoptotic effect of cepharanthine (Fig. 2A–C). These results showed that cepharanthine-induced apoptosis was associated with ROS.

3.4. Effects of cepharanthine on ROS generation and mitochondrial membrane potential($\Delta\Psi m$)

According to above findings, we hypothesized that the effect of cepharanthine on cancer cells was associated with ROS. Here, we analyzed the level of ROS generation by flow cytometry. The levels of ROS in H1299 cells treated with 0, 20 and 40 μM cepharanthine for 24 h were 3.69, 45.54 and 95.55% (Fig. 2D). The levels of ROS in A549 cells treated with 0, 40 and 80 μM cepharanthine for 24 h were 5.49, 27.74 and 48.75% (Fig. 3A). Our results demonstrated that cepharanthine significantly increased ROS generation in H1299 and A549 cells in a dose-dependent manner.

Next, we determined the effect of cepharanthine on $\Delta\Psi m$ in cancer cells. As shown in Fig. 3B–D, the levels of $\Delta\Psi m$ in H1299 cells treated with 0, 20 and 40 μM cepharanthine for 24 h were 97.41, 44.34 and 36.81%. And the levels of $\Delta\Psi m$ in A549 cells

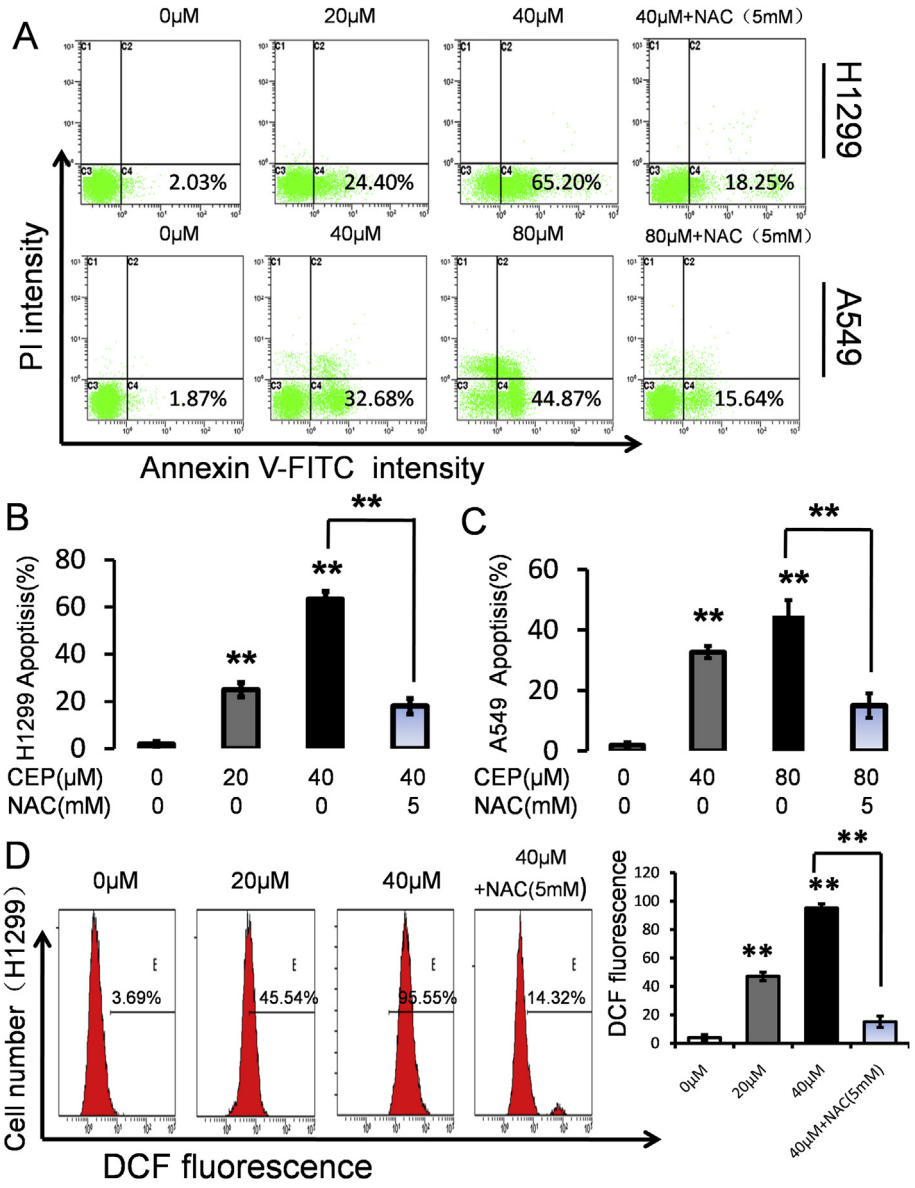

Fig. 2. A. Apoptosis induced by cepharanthine in H1299 and A549 cells. H1299 cells were treated with 0 μM, 20 μM, 40 μM and A549 cells were treated with 0 μM, 40 μM, 80 μM cepharanthine for 24 h. For the caspase and ROS inhibitor analyses, the cells were pretreated with 5 mM NAC for 2 h. Then cells were stained with FITC-conjugated Annexin V and PI for flow cytometric analysis. The flow cytometry profile represents Annexin V-FITC staining in x axis and PI in y axis. (B, C)The number represents the percentages of apoptotic cells in each condition. D. Effect of cepharanthine on the ROS in H1299 cells. The ROS of H1299 cells treated with 20 and 40 μM cepharanthine in the presence or absence of 5 mM NAC for 24 h were analyzed by flow cytometry, respectively. Data are expressed as mean ± SD of three independent experiments. Columns not sharing the same superscript letter differ significantly, $^{**}P < 0.01$, $^{*}P < 0.05$ compared to the control or presence compared to absence of NAC.

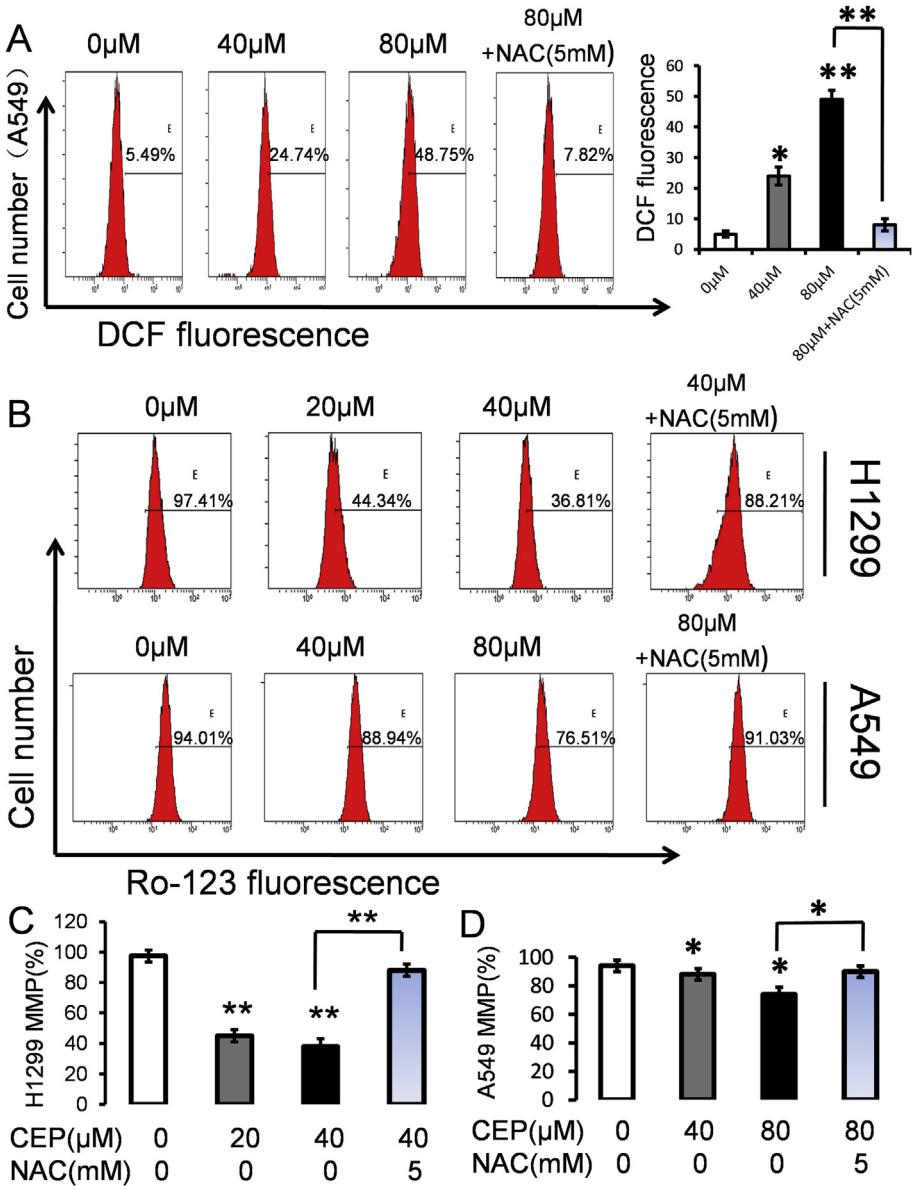

Fig. 3. A. Effect of cepharanthine on the ROS in A549 cells. The ROS of A549 cells treated with 40 and 80 μM cepharanthine in the presence or absence of 5 mM NAC for 24 h were analyzed by flow cytometry, respectively. B. Effect of cepharanthine on the $\Delta\Psi$m in H1299 and A549 cells. The $\Delta\Psi$m of H1299 and A549 cells treated with cepharanthine at different concentrations in the presence or absence of 5 mM NAC for 24 h were analyzed by flow cytometry, respectively. (C, D). The loss of $\Delta\Psi$m in H1299 and A549 cells following cepharanthine treatment in a dose-dependent manner, respectively. The data are expressed as the means ± SEM (n = 3). $^{**}P < 0.01$, $^{*}P < 0.05$ compared to the control or presence compared to absence of NAC.

treated with 0, 40 and 80 μM cepharanthine for 24 h were 94.01, 88.94 and 76.51%. The ROS inhibitor group, the cells were pre-treated with 5 mM NAC almost prevented disruption of mitochondrial membrane potential. These findings indicated that cepharanthine -induced apoptosis was associated with ROS generation and $\Delta\Psi$m disruption.

3.5. *Effects of cepharanthine on the expression of apoptosis regulators*

Our data showed that cepharanthine-induced apoptosis was associated with $\Delta\Psi$m disruption. The Bcl-2 protein family, a large family of apoptosis regulating proteins, modulated the mitochondrial pathway [18]. To further characterize this cell-specific apoptotic effect of cepharanthine in cancer cells, we analyzed the levels of Bcl-2 family proteins in H1299 cells treated with cepharanthine (0, 20 and 40 μM) and A549 cells treated with cepharanthine (0, 40 and 80 μM) for 24 h by Western blot. As a result, the expression of Bax were markedly increased by cepharanthine accompanied with decreased expression of Bcl-2 in a dose-dependent manner (Fig. 4A–C). Next, we examined the effect of cepharanthine on caspase-3 activation by Western blot. The expression of cleaved PARP into 85 kDa fragment, procaspase-3 were decreased and cleaved caspase-3 were increased (Fig. 4A–C).

4. Discussion

In the present study, we investigated the inhibitory effect of cepharanthine on proliferation of non-small lung cancer cells H1299 and A549 *in vitro*. The results showed that cepharanthine reduced the cell viability in a dose-dependent manner. This effect could be blocked by NAC, a ROS inhibitor. These findings indicated

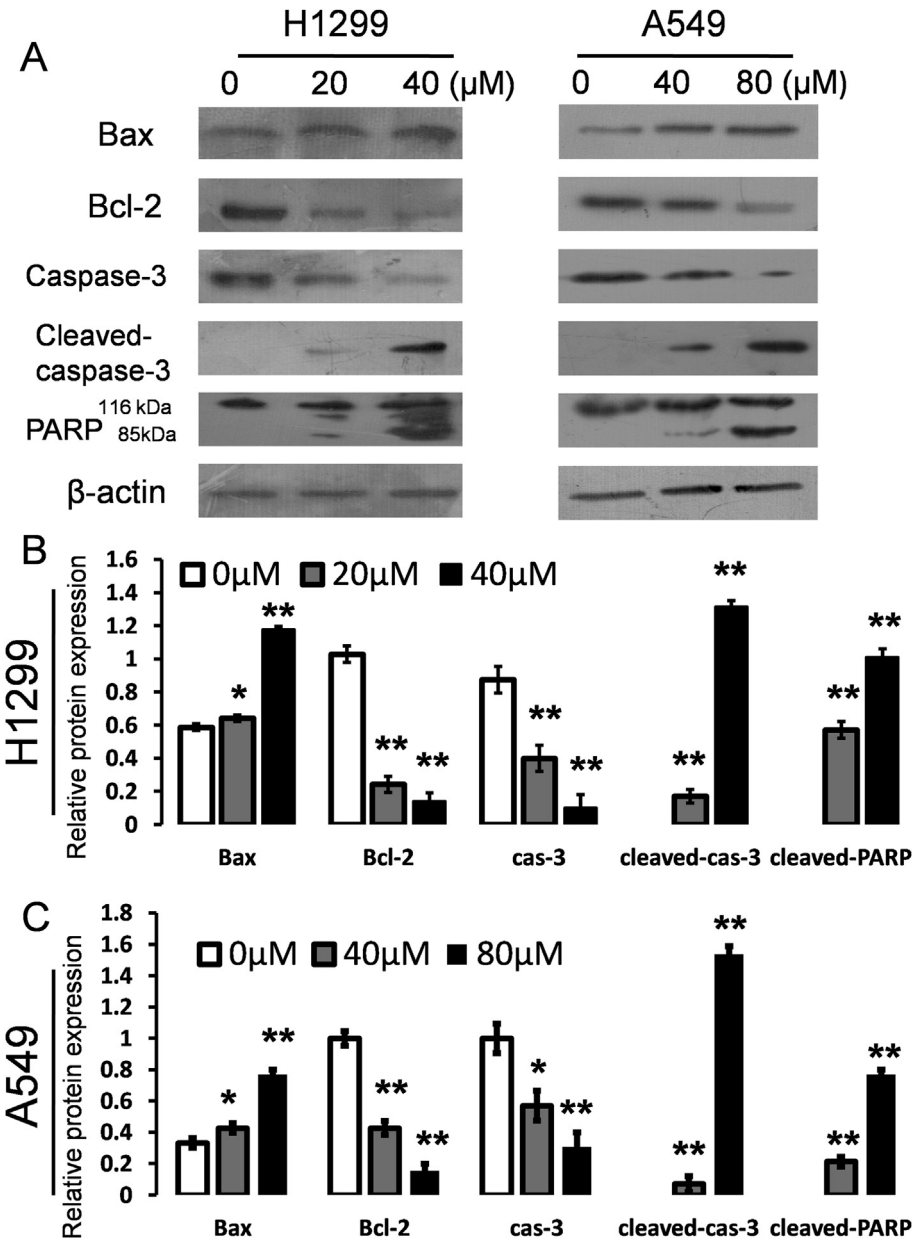

Fig. 4. A.Effect of cepharanthine on the expression of apotosis regulators. H1299 and A549 cells were treated with cepharanthine at different concentrations for 24 h, respectively. The expression of The expression of Bcl-2, Bax, caspase-3 and PARP were determined by Western blot analysis and quantified by Gel-pro analyer software. (B, C). Data are expressed as mean ± SD of three independent experiments. Columns not sharing the same superscript letter differ significantly, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with to the control.

that cepharanthine exerts cytotoxic effect on H1299 and A549 cells through ROS generation.

As known, cell cycle arrest and apoptosis are two mechanisms in the induction of cell death [17]. Recent studies about cell cycle regulation have shown that cell cycle progression is tightly controlled by various checkpoints in normal cells while alterations in the checkpoints of cell cycle progression lead to aberrant cell proliferation and development of cancer [19]. Tumor cells frequently acquiring defects in the checkpoints leads to unrestrained proliferation [20]. Many anticancer drugs induced the cell cycle arrest at a specific checkpoint and thereby induced apoptosis [21,22]. In our study, we showed that cepharanthine induced H1299 cell cycle arrest at S phase and A549 cell cycle arrest at G2/M phase in a dose-dependent manner, suggesting that cell cycle arrest is one of the mechanisms in the growth inhibitory effect of cepharanthine in NSCLC, but the reason of arresting at different phase still remains unclear. A549 cells are defective in LKB1 gene with a nonsense mutation (c.109C > T), but there is a wild-type LKB1 in H1299 cells. Although LKB1 status is different between two cell lines, LKB1 appears to induce cell cycle arrest for G1 but not S or G2/M phases [23], thus the difference at arresting phase by cepharanthine is unlikely to be caused by LKB1. In addition to cell cycle arrest, cepharanthine also exerts its cytotoxic effect via the induction of apoptosis in NSCLC. These data strongly suggested that the cytotoxic effect of cepharanthine in H1299 cells and A549 cells was achieved via induction of apoptosis, which was in agreement with previous studies, the induction of other cancer cells including human cervical adenocarcinoma Hela cell [24], cholangiocarcinoma cell [25], human oral squamous cell [26]and myeloma cells [15]. Pretreatment with NAC could significantly reduce the apoptotic effect of cepharanthine on the H1299 and A549 cells. But there were still about 10% more early apoptosis compared with the control group, indicating that ROS mediated apoptosis is the

main factor,and other pathways also exist, which needs to be further explored in the future.

ROS is known as the mediator of intracellular signal. The excessive generation of ROS can induce oxidative stress, loss of cell function and cell apoptosis [27]. ROS can also participate in the process of lipid peroxidation, mercaptan tissue protein cross-linking, the two processes can be induced by mitochondrial PTP opened [28]. In the present study, we found that cepharanthine significantly increased ROS levels and reduced mitochondrial membrane potential in a dose-dependent manner. These effects would be weakened by pretreatment with NAC, indicating that cepharanthine -induced cell death is associated with ROS level.

The Bcl-2 protein family is a large family of apoptosis regulating proteins that modulate the mitochondrial pathway which includes antiapoptotic proteins and proapoptotic proteins such as Bcl-2 and Bax [17]. To explore the further molecular mechanism of cepharanthine-induced apoptosis on NSCLC, we tested the expression of Bax and Bcl-2 protein in H1299 and A549 cells of each group. The results showed that the expression of Bax gradually increased and Bcl-2 decreased in treatment groups in a dose-dependent manner. Caspase-3 is a frequently activated death protease which cleave PARP, a DNA repair enzyme [8]. In our study, the expression of cleaved PARP into 85 kDa fragment and procaspase-3 were decreased while actived caspase-3 increased. These results clearly demonstrated that the mitochondrial-mediated caspase activation pathway is involved in cepharanthine-mediated apoptosis in NSCLC.

In our study, cepharanthine inhibited the growth and induced apoptosis of A549 cells which were associated with the generation of ROS. The conclusion was similar to that in H1299 cells. This indicated that cepharanthine induced cell death is independent of LKB1 status of NSCLC.

Although the mechanisms of the growth inhibitory effect of cepharanthine in some cancer cells have previously been demonstrated, our studies were the first time to describe the role of ROS in the induction of apoptosis in lung cancer cells. In conclusion, cepharanthine induced apoptosis of H1299 and A549 cells through ROS generation. Cepharanthine-induced apoptosis marked with up-regulation of Bax, down-regulation of Bcl-2 expression, and dissipation of $\Delta\Psi$m, whereas caspase-3 was activated and PARP was cleaved, leading to apoptosis. These findings recommended that cepharanthine may have a potential as a lead compound for future development of anti-lung cancer therapy. For further research, we will explore the mechanism of apoptosis induced by cepharanthine, as well as the effect *in vivo*.

Conflict of interest

None.

Acknowledgments

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