Effects of sodium new houttuynifonate on proliferation, apoptosis and migration of A2780 cells

Linsong Yang1,*, Luyao Wang2

1Biomedicine Laboratory, School of Pharmaceutical Engineering and Life Science, Changzhou University, Changzhou 213164, R.P.China
2Changzhou’s Key Laboratory of Pharmaceutical Manufacture and Quality Control Engineering, Changzhou 213164, R.P.China
*Corresponding author: linsongyang@cczu.edu.cn

Abstract: Objective: To observe the effects of sodium new houttuynifonate on proliferation, apoptosis and migration of human epithelial ovarian cancer A2780 cells, and to investigate the inhibition of invasion and migration of human epithelial ovarian cancer A2780 cells by regulating the expression of apoptosis-related molecules. Methods: blank group and different dose groups (100, 200, 300, 400 and 500 μg·ml−1 of sodium new houttuynifonate) were treated with human ovarian cancer A2780 cells for 48 h, respectively. MTT assay was used to detect cell proliferation and activity, and flow cytometry was used to detect cell proliferation inhibition rate and apoptosis rate. The cell migration was observed and the healing rate and migration rate of scratch were calculated. The mRNA expression of apoptosis-related molecules Bcl-2, Bax, VEGF and NF-κBp65 was detected by real-time PCR. Results: MTT and flow cytometry results showed that compared with blank group, the cell inhibition rate of sodium neohouttuynium was significantly increased (P<0.01), and the total cell apoptosis rate was increased (P<0.05); The results of scratch test showed that compared with blank group, the migration ability of new houttuynia sodium group was weakened, and the number of cell migration was significantly reduced. PCR results showed that compared with the blank group, the mRNA expression of VEGF, Bcl-2 and NF-κBp65 in the group of neohouttuynia sodium decreased, and the expression of Bax increased. Conclusion: sodium new houttuynifonate can inhibit proliferation and promote apoptosis of human ovarian cancer A2780 cells, and may inhibit the migration of A2780 cells by regulating the expression of apoptosis-related molecules.

Keywords: sodium new houttuynifonate, Ovarian cancer A2780, Invasion migration, Proliferation apoptosis

1. Introduction

Ovarian cancer is one of the three most common female genital malignancies. Due to its early symptoms and hidden location, most patients are confirmed to be advanced [1-4]. Advanced ovarian cancer has direct spread, metastasis or abdominal implantation, resulting in a 5-year survival rate of less than 50% [5]. Ovarian cancer is considered to have a good response to chemotherapy, but there are many adverse reactions after chemotherapy, and most patients suffer from intolerance after chemotherapy. Therefore, more effective alternative drugs with less adverse reactions should be explored for these patients [6].

Houttuynia cordata is a medicinal herb included in the Chinese Pharmacopoeia. It comes from the dry aboveground part of the Chameleon plant of the Chameleon family, and also is a plant with both medicinal and food worths. Houttuynia taste hot, slightly cold, go to the lung; It has the effect of clearing heat and detoxifying, eliminating carbuncle and discharging pus, diuresis and drenching. It can be used to treat lung carbuncle and pus, phlegm, heat, asthma, dysentery, heat shower and carbuncle and sore toxin [7]. Houttuynia cordata has a variety of pharmacological activities, such as anti-inflammatory, antibacterial, anti-virus and anti-cancer effects [8]. In this study, the effect of houttuynia sodium on the proliferation and apoptosis of ovarian cancer cell A2780 was studied to deduce the broad-spectrum anticancer effect of houttuynia sodium, and to explore the inhibition of invasion and migration of A2780 cells by regulating the expression of related molecules, in order to provide a basis for further study of the anti-inflammatory mechanism of Houttuynia sodium.
2. Materials and methods

2.1. Cell lines, chemicals and biochemicals

Sodium new houttuynonate (SNH) was purchased from Xi'an Kailai Biological Engineering Co., LTD. (Xi'an, China). We used high performance liquid chromatography to quantitatively analyze SNH with a purity of 98%. A2780 cells were obtained from ATCC. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (USA). Fetal bovine serum (FBS), MTT, penicillin and streptomycin all were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V-FITC/PI apoptosis detection kit, Apoptosis-Hoechst staining kit and DNA ladder extraction kit were from Shanghai Beyotime Biological Technology Co. Ltd. (Shanghai China). The primer of GADPH, Bax, NF-κBp65, VEGF and Bcl-2 were synthesized by ShanghaiSangon Biological Engineering Technology and Service Company. One step RT-PCR kit was bought from Qiagen (Germany). All other reagents were of the highest grade commercially available.

2.2. Cell culture and treatments

A2780 cells were cultured in DMEM complete medium containing 10% fetal bovine serum at 37 ℃ in a 5% CO2 cell incubator. After the cell adherence rate reached 80%, the cells were digested with 0.25% trypsin for subculture. SNH was dissolved in DMEM medium and diluted with DMEM medium containing 2% calf serum to the final concentration of 100, 200, 300, 400 and 500 μg• ml⁻¹. Culture medium containing 10%FBS was used as the solvent control.

2.3. In vitro cytotoxicity was detected by MTT assay

Cells at logarithmic growth stage were placed into 96-well plates at a density of 1 × 10⁵/mL, 0.2 mL per well, and cultured overnight at 37 ℃ and 5% CO2. SNH was treated with different concentrations (0, 100, 200, 300, 500 and 500 μg• ml⁻¹) for 48 h, and the cells were divided into control group and experimental group. After 48 h of culture, 20 μL MTT (5 mg• ml⁻¹) was added to each well, the supernatant was discarded 4 h later, and 100 μL DMSO was added to each well. The absorbance (OD) value at 490 nm was measured by a microplate reader after a mild shock, and the cell proliferation rate was calculated. The experiment was repeated three times for each group, and their average value was taken and the inhibition rate of cell growth and proliferation was analyzed.

2.4. Cell scratch - wound healing assay experiment

A2780 cells were inoculated into 6-well plates at 3×10⁵/mL, and 10%FBS was added. Migration capacity of HepG2 cells. In short, cells were counted and seeded in 6-well plates at a concentration of 3×10⁵ mL⁻¹. When the cells grew to fusion, the cells were damaged with a 200ul sterile pipette tip. The cells were washed lightly with PBS three times, then 2ml DMEM medium containing different concentrations of SNH was added. The cells were incubated for 48 h, and photos were taken at 0, 6, 12, 24, 30, 36 and 48 h, respectively, repeated 3 times. Image J was used to measure the wound gap area, and the data were normalized to the mean value of the control group. When the sample was compared with control cells, if P < 0.01, the difference was considered significant.

2.5. DNA laddering detection

A2780 cells were treated with different concentrations of SNH solution for 48 h, then culture medium and adherent cells were collected. DNA of each group was collected using DNA ladder kit. DNA samples were electrophoresed on 1.2% agarose gel containing 2 uL/50 mL GoodView nucleic acid dye. The gel is examined and photographed by the Ultraviolet Gel Recording System (Bio-RAD, USA).

2.6. Annexin V-FITC/PI staining

A2780 cells (4 × 10⁵ / well) were inoculated in 6-well plates. After overnight cell adherence, cells with different concentrations of SNH were treated for 48 h, then cells with different concentrations of SNH were collected, washed twice with PBS, and 195uL were added into 50,000 cells. Finally, each sample was analyzed by flow cytometry (BD Biosciences). The gated A2780 cells were then mapped as a bidirectional dot plot of Annexin V-FITC and PI to assess the percentage of apoptotic cells. The
apoptotic cells were named Annexin V+/PI- and Annexin V+/PI+ cells. Negative control: untreated cells.

2.7. Real time RT-PCR analysis

A2780 cells were seeded at a density of 2.5×10⁶ cells/well in a six-well plate. After incubation for 24 h at 37°C and 5% CO₂, the experimental group cells were treated with 2mL of 250 μg/ml SNH for 48 h. Meanwhile, 2mL of culture medium was added to the control wells and each group included three replicates. Then, total RNA of A2780 cells was extracted using Trizol reagent and was reverse-transcribed into cDNA with a random hexamer primer using the PrimeScript II 1st strand cDNA Synthesis System, in accordance with the manufacturer’s instructions. 1 μl of the cDNA product was used as the template for RT-PCR. The reaction mixture was composed of Taq DNA Polymerase, dNTP Mix, 10× Taq Buffer, 5× Betaine, 10 pmol of forward and reverse primers, 100 ng of A2780 cDNA and distilled water added to a final volume of 10 μl. The primer sequences and product lengths are listed in Table 1. Thermocycling conditions were 94°C for 2 min (polymerase activation), followed by 32 cycles of 94°C for 10 sec (denaturation), various annealing temperatures for 20 sec, depending on the target gene (55°C for GADPH; 55°C for VEGF; 55°C for Bax; 52°C for Bcl-2 and 61°C for NF-kB p65), and by 72°C for 40 sec for elongation. Normalization of data was performed using the housekeeping gene GADPH as an endogenous control. One real-time PCR run was done in three replicates for each sample.

2.8. Statistical analysis

Data were analyzed using one way ANOVA and T test. *P < 0.05 and **P < 0.01 were considered statistically significant and extremely significant. Data were presented using mean ± standard error.

3. Results

3.1. Cytotoxicity and apoptosis assay

The in vitro cytotoxicity of SNH on A2780 (ovarian cancer) and SKOV-3 (ovarian cancer) cell lines was evaluated by 3-(4, 5-dimethylthiazole-2-yl) -2, 5-diphenyltetrazolium ammonium bromide (MTT) assay. The results showed that SNH had significant cytotoxic effects on A2780 and SKOV-3 (ovarian cancer cells), suggesting that SNH might have a broad spectrum of cytotoxic effects on ovarian cancer cells. SNH showed similar cytotoxicity to A2780 cells and SKOV-3 cells, possibly because both of these cells are human ovarian cancer cells.

![Figure 1: Sodium new houttuynate inhibit the proliferation of A2780 cells](image)

3.2. Effect of SNH on migration of A2780 cell

The effect of SNH on cell migration was studied in an in vitro wound healing model. The wound healing level was measured by the mean reduction of wound edge distance at different time points (0, 6, 12, 24, 30, 36, 48 h) stimulated or not stimulated by SNH (100, 200, 300, 400, 500 μg/ mL). As shown in the figure, compared with the control group, the migration of cells in different concentration groups was inhibited to different degrees. The migration ability of cells in 200 μg/mL and 300 μg/mL groups was decreased more obviously, and the migration ability of cells in 400 μg/mL and 500 μg/mL groups was inhibited more significantly than the control group.
was also decreased, and the number of dead cells increased at this time.

Figure 2: SNH inhibited the migration of A2780 cells during wound healing. Representative images (10x) showed cell migration to the injured area during in vitro scratch healing test. A2780 cells were treated with different concentrations of SNH (0, 100, 200, 300, 400, 500 μg/mL) for 48 h, and photographed at 6, 12, 24, 30, 36, 48 h, respectively. These values are means±SD from three independent experiments. (Independent sample T test, *P < 0.05; ** P < 0.01; *** compared with control group, P < 0.001).

3.3. SNH promoted cell apoptosis of the A2780 cells

The A2780 cells were treated with different concentrations of Sodium houttuylate (0, 100 μg/mL, 200 μg/mL, 300 μg/mL, 400 μg/mL and 500 μg/mL for 48 h by DNA Ladder method. After DNA extraction, 1.2% agarose gel electrophoresis was performed. As shown in Figure 4, sodium neohouttuynium in high concentration group promoted apoptosis of A2780 cells. It can be seen from the figure that, compared with the control group, a small number of DNA Ladder bands appeared in the 100 μg/mL and 200 μg/mL sodium houttuyniate dosage groups. In addition, 300 μg/mL, 400 μg/mL and 500 μg/mL sodium houttuynium groups showed typical DNA Ladder bands, which promoted cell apoptosis.

Figure 3: DNA laddering

3.4. Effects of SNH on apoptosis of A2780 cells

Compared with the control group, some SNH-treated A2780 cells showed signs of apoptosis such as rounding, atrophy, membrane bubble and apoptotic body formation under microscope. To further verify whether SNH induced apoptosis of A2780 cells, cells treated with different concentrations of SNH for 48 h were stained with Annexin V (AV) and propidium iodide (PI). As shown in Figure 4, A2780 cells were treated with different concentrations of sodium neohouttuylin for 48 h, and apoptosis rates of cells
in each group were significantly increased with the increase of concentration, which were 4.35%, 11.31%, 13.82%, 41.30%, 44.41% and 51.32%, respectively. These results suggest that sodium new houttuyfonate can promote the apoptosis of ovarian cancer A2780 cells.

Figure 4: A2780 cells were treated with SNH (0, 100, 200, 300, 400 and 500 mg mL\(^{-1}\)) for 48 h, and apoptosis was measured by flow cytometry after staining with annexin V/PI, (independent-samples t test, **P < 0.01, ***P < 0.001 versus the control group).

3.5. Apoptosis and migration-related mRNA expression

The results showed that SNH induced apoptosis and migration were closely related to mitochondrial pathway and extracellular matrix, respectively. Bcl-2 is one of the most important oncogenes in apoptosis research. Bcl-2 is the main target molecule for the study of molecular mechanism of apoptosis. It regulates the permeability of mitochondrial outer membrane, and most of them are located in the mitochondrial outer membrane or transferred to the mitochondrial outer membrane after being stimulated by signals. In addition, VEGF is also a special gene, and its decline is now considered as a marker of cancer, it plays an important role in the formation of new blood vessels, tumor growth and metastasis of cancer cells.

To investigate the effect of SNH on apoptosis and migration of A2780 cells, we detected the expression of Bcl-2, Bax, NF-κBp65, VEGF and GADPH by RT-PCR. As shown in Figure 7, compared with the untreated group, 300 μg/mL SNH significantly increased the level of Bax. The expression of Bcl-2 decreased with the increase of SNH concentration. The expressions of NF-κBp65 and VEGF in A2780 cells were significantly decreased in SNH treatment group. Studies have shown that THE expression of NF-κB is highly correlated with VEGF. NF-κBp65 and VEGF-C may be involved in the occurrence and development of epithelial ovarian cancer [1]. Therefore, we concluded that the effect of INHIBITION of SNH on VEGF expression may be partially realized by inhibition of NF-κB level in A2780 cells. Our results suggest that SNH promotes apoptosis and metastasis of A2780 cells by increasing the expression of Bax and decreasing the expression of NF-κBp65, VEGF and Bcl-2.

Figure 5: The mRNA expression
4. Discussion

Ovarian cancer may be caused by increased estrogen levels, family inheritance, gene mutations, diet and other factors. The progression of ovarian cancer course is closely related to apoptosis of cancer cells. Commonly used chemotherapy drugs can induce apoptosis of tumor cells through cytotoxic effect, but due to its toxic effect on normal cells, it has obvious side effects. In addition, due to some patients are not sensitive to chemotherapy drugs, often resulting in treatment failure [9], therefore, it is of great significance to find effective drugs to induce apoptosis of ovarian cancer cells. Sodium houttuynia cordata is a bioactive component extracted from Aabaena cordata. It was found that Houttuynia houttuynia had many pharmacological properties such as antibacterial and anti-inflammatory, enhancing resistance and anti-tumor [10]. Other studies have confirmed that flavonoid compounds have anti-bacterial anti-inflammatory, anti-virus, enhance the immune ability of the body and other pharmacological effects, especially the role of flavonoid compounds in promoting tumor cell apoptosis is increasingly concerned [11-13]. In previous studies, Houttuynia cordata may inhibit the proliferation of A549 lung cancer cells by blocking cell cycle and apoptosis [14]. In addition, quercetin, the main flavonoid in Houttuynia cordata, inhibits the growth of human melanoma cells by inducing programmed cell death [15]. In addition, HOUTTuynia cordata inhibited the growth of MDA-MB-468 cells [16] and migration of HMC-1 cells [17]. Our results were consistent with those findings that SNH exhibited similar anti-proliferation and apoptosis effects to polysaccharide and quercetin in Houttuynia cordata.

SNH inhibited proliferation and migration of A2780 cells in apoptotic mode. Some relevant gene levels were also examined to elucidate the underlying mechanism of SNH. Bax and Bcl-2 are two important pro-apoptotic and anti-apoptotic proteins in the Bcl-2 family. The ratio of Bax and Bcl-2 determines whether a cell is apoptotic or survives [18]. It is well known that tumor growth and metastasis require angiogenesis and the participation of VEGF, and NF-KBP65 plays a key regulatory role in immune response, inflammatory response, apoptosis, tumorigenicity and other aspects [19-20]. The results showed that SNH up-regulated Bax level and decreased the expression of Bcl-2, NF-kBp65 and VEGF. Kim et al. [21] has been reported that Houttuynia cordata interferes with the expression of bcl-2 family proteins (Bax, Bcl-2 and Bcl-XL), promotes the activation of HIF-1A-FOXO3 and MEF2A pathways, and induces the apoptosis of human HepG2 hepatoma cells. In addition, houttuynia houttuynia has been reported to inhibit NF-xB activation and is considered as a potential treatment for mastitis. Our results are similar to those of previous studies. SNH may regulate proliferation and apoptosis of A2780 by decreasing THE level of NF-kBp65 and increasing the ratio of Bax to Bcl-2, and may down-regulate cell migration by decreasing the expression of VEGF.

In conclusion, SNH significantly inhibited the proliferation and migration of A2780 cells, and promoted apoptosis by increasing the ratio of apoptotic genes to anti-apoptotic genes. These results suggest that SNH is one of the potential candidates for the treatment of ovarian cancer by inducing apoptosis and reducing migration of ovarian cancer cells.

References


