SUPPLEMENTARY MATERIAL

In vitro anti-tumor activity of the Tanshinone IIA against SKOV3 cells

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To determine the antitumour activity of tanshinone IIA in SKOV3 cells. Results suggested that tanshinone IIA could significantly inhibit (IC₅₀ value = 19.6 μM) the proliferation of SKOV3 cells and induce apoptosis of SKOV3 cells demonstrated by flow cytometry analysis. In addition, tanshinone IIA treatment induced G2/M phase cell cycle arrest in SKOV3 cells. The results of western blotting indicated that tanshinone IIA can suppress the expression of anti-apoptotic protein Bcl-2, increase (0.28 vs 0.62) the expression of pro-apoptotic protein Bax (0.83 vs 0.24) in SKOV3 cells. It can be concluded that the tanshinone IIA may be a possible therapeutic candidate having cytotoxic and anti-tumor potential.

Keywords: Tanshinone IIA, SKOV3, Apoptosis, Cell

1. Experimental

1.1 Materials

Tanshinone IIA (purity 98.6%) was purchased from Shanghai HaoRan Biotech Co., Ltd (Shanghai, China). The dose of tanshinone IIA was determined by our preliminary experiments.

SKOV3 was obtained from the Shanghai Xin Yu Biotech Co., Ltd (Shanghai, China). SKOV3 was maintained in RPMI 1640 (Cambrex Bio Science, Walkersville, MD, USA) with 10% FBS (Hyclone) and 1% penicillin/streptomycin (Hyclone). All cells were grown at 37°C in a humidified 5% CO₂ atmosphere.

1.2 MTT assay

In brief, tumour cells were seeded in 96-well plates at a density of 1000 cells/well in 150 µl culture medium, and after a 24-h culture, the cells were treated with various concentration of tanshinone IIA (0, 10, 20 and 30 µg/µL) for 48 h. Cell proliferation was also verified by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay for SKOV3 cells. Immediately after treatment with tanshinone IIA, the cells were incubated in the dark with MTT dissolved in phosphate buffer saline (PBS) at a final concentration of 50 µg/ml for 2 h. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals which are insoluble in aqueous solutions. The crystals are dissolved in acidified isopropanol. The absorbance of the resulting purple solution is spectrophotometrically measured at a wavelength of 570 nm. The background absorbance was determined at 690 nm and subtract from the 570 nm measurement. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity. Growth inhibition (in %) was expressed as $[100 \times (\text{O.D. control} - \text{O.D. treated})/\text{O.D. control}] \%$.

1.3 Annexin-V/PI apoptosis assay

Cells were seeded in 6-well plates at densities of $1-2 \times 10^5$ cells/well (48 h treatments). Following tanshinone IIA treatment (48 h), cells were collected and washed in ice cold PBS and transferred with medium and detached cells to FACS tubes. Following centrifugation (1,500 rpm; 5 min; 4 °C), supernatants were discarded and 100 µl $1 \times$ annexin-V buffer (10 $\times$; 0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl₂) plus 5 µl annexin-V-FITC (BD Pharmingen) were added to each tube. After gentle vortex, samples were incubated for 15 min at room temperature in the dark. An additional 400 µl annexin-V buffer and 10 µl PI (50 µg/ml in PBS) were added to each sample placed on ice and incubated in the dark at room temperature for 10 min. A Beckman Coulter EPICS-XL flow cytometer was used to analyse the percentage of cells undergoing apoptosis: the sum of early apoptosis (annexin-V-positive cells) and late apoptosis (annexin-V-positive and PI-positive cells).

1.4 Cell cycle analysis

Cells were seeded in 6-well plates at densities of $1-2 \times 10^5$ cells/well (48 h tanshinone IIA treatments). Cells were incubated and allowed to attach overnight before treatment began. Following treatment, detached cells were collected and

attached cells trypsinised, pooled samples were then washed (PBS; 4 °C), cells were centrifuged (1,500 rpm; 5 min; 4 °C) then resuspended in fluorochrome solution (50 µg/ml propidium iodide (PI), 0.1 mg/ml of ribonuclease A, 0.1 % sodium citrate, 0.1 % triton-X-100). Cell cycle analyses were performed using a Beckman Coulter EPICS-XL flow cytometer. Data were evaluated using EXPO32 software.

1.5 Transwell cell invasion and migration assay

SKOV3 cells incubated with various concentrations (0, 10, 20 and 30 µg/µL) of tanshinone IIA for 48 h were removed by trypsinizing, and their in vitro invasion and migration were tested. For invasion assay, SKOV3 cells were trypsinised and resuspended in DMEM containing 0.1% FBS and 2×10^5 cells were added to the upper chamber of each well (6.5 mm in diameter, 8-µm pore size; Corning, NY, USA) coated with 30 mg/cm² Matrigel (ECM gel, Sigma–Aldrich, St. Louis, MO). Medium containing 0.1% FBS and supplemented with HGF (20 ng/ml) was placed in the lower compartment of the chamber. After 48 h at 37 °C, cells on the upper membrane surface were removed by careful wiping with a cotton swab and the filters were fixed by treatment with 95% ethanol for 30 min and stained with 0.2% crystal violet solution for 30 min. The migration assay is the same with invasion assay excepting no matrigel was used and the permeating time for cells was 12 h. Numbers of cells that had migrated and invaded were quantified by counting cell numbers of three random fields.

1.6 Western blot analysis

Cells were harvested and resuspended in lysis buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 4 M urea). Protein samples (50 µg) were each diluted into a 20 µl solution of lysis buffer and 5% 2-mercaptoethanol and heated at 95 °C for 5 min. The protein extracts were separated by 10% SDS–PAGE. The separated proteins were transferred to nitrocellulose membrane and then blocked in a blocking solution (5% dry milk and 0.2% Tween 20 in phosphate buffered saline (PBS)) for 1 h. The membranes were incubated overnight with the primary specific antibodies detecting Bax (1:300), and Bcl-2 (1:200). The membranes were then incubated with HRP-labelled secondary antibodies for 1 h. The proteins were visualised on enhanced chemiluminescence film. Each experiment was repeated three times with consistent results.

Figure S1. Effect of tanshinone IIA on SKOV3 cells growth

**P<0.01, compared with control (0 µg/µL)

Figure S2. Tanshinone IIA induces SKOV3 cells apoptosis

**P<0.01, compared with control (0 µg/µL)

Figure S3. Tanshinone IIA affects SKOV3 cells cycle

*P<0.05, **P<0.01, compared with control (0 µg/µL)

Figure S4. Effect of tanshinone IIA on SKOV3 cells migration and invasion

*P<0.05, **P<0.01, compared with control (0 µg/µL)

A

B

C

Figure S5. Effect of tanshinone IIA on Bax (A), Bcl-2 (B) and Bax/Bcl-2 (C) proteins expression in SKOV3 cells

*P<0.05, **P<0.01, compared with control (0 μg/μL)