Growth inhibition and apoptosis induction by tanshinone I in human colon cancer Colo 205 cells

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Abstract. Tanshinone I (Tan-I) and tanshinone IIA (Tan-IIA) were isolated from Danshen (*Salviae Miltiorrhizae Radix*), a widely prescribed traditional herbal medicine that is used to treat cardiovascular and dysmenorrhea diseases. In our previous study, Tan-IIA was demonstrated to induce apoptosis in human colon cancer Colo 205 cells. However, the effect of Tan-I on human colon cancer cells is not clearly understood yet. In this study, the anti-growth and apoptosis-eliciting effects of Tan-I, as well as its cellular mechanisms of actions, were investigated in Colo 205 human colon cancer cells. Tan-I reduced cell growth in a concentration-dependent manner, inducing apoptosis accompanied by an increase in TUNEL staining and in cells in the sub-G1 fraction. The expression of p53, p21, bax and caspase-3 increased in Tan-I-treated cells. In addition, the cell cycle analysis showed G0/G1 arrest. These findings suggest that Tan-I induces apoptosis in Colo 205 cells through both mitochondrial-mediated intrinsic cell-death pathways and p21-mediated G0/G1 cell cycle arrest. Accordingly, the therapeutic potential of Tan-I for colon cancer deserves further study.

Introduction

Colon cancer is a major health problem in human of developed Western countries. The prognosis of colon cancer is still not satisfactory. The modulation of apoptotic response provided new hope for therapeutic strategies in cancer disease (1,2). Many therapeutic agents were developed to induce the apoptotic process in target cells (3). Many compounds purified from plants have revealed anticancer activity, such as camptothecin (4) and taxol (5) that were able to induce cancer cells to apoptosis. Tan-I and Tan-IIA are derived from the plant of Danshen (*Salviae miltiorrhizae Radix*), the root of the traditional Chinese medicine, *Salvia miltiorrhiza Bunge,* which is widely used for cardiovascular and dysmenorrheal diseases (6). It is known that Tan-IIA exerts its anticancer activity on many human cancer cells (7-13). However, there is no available information to address the effects of Tan-I in human colon cancer cells. In the present study, human colon cancer Colo 205 cells were used to investigate the molecular mechanisms of Tan-I in human colon cancer.

Materials and methods

Chemicals and reagents. Tanshinone I (1,6-Dimethyl-phenanthro[1,2-b]furan-10,11-dione;$C_{18}H_{12}O_3$ (Fig. 1), purity >98%, HPLC) was purchased from Herbasin Co. (Shenyang, China). Aprotinin, antipain, sodium deoxycholate, leupeptin, propidium iodide (PI), sodium orthovanadate, Triton X-100, Tris-HCl, ribonuclease-A and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphate and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Human colon cancer cell line (Colo 205). The human colon cancer cell line (Colo 205: human colon adenocarcinoma) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C in humidified 5% CO_2 and 95% air atmosphere in RPMI-1640 medium (Sigma Chemical Company), contained with 10% heat-inactivated fetal bovine serum, FBS (Gibco BRL), 10 mM HEPES, 10 mM sodium pyruvate (Gibco), 2% penicillin-streptomycin (10,000 U/ml penicillin; 10 mg/ml streptomycin), 1% 10 mM HEPES, 1% 10 mM sodium pyruvate and 1% glutamine. The data presented in this study are from at least 3 independent experiments.

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Cell viability assay. Colo 205 cells were plated in 6-well plate at a density of 2×10^5 cells per well and cultured in a humidified atmosphere with 5% CO_2 at 37˚C for 24 h. Then, Tan-I (1, 2.5, 5 and 10 μg/ml; stock solutions made in DMSO) was applied into the cultured cells for 24, 48, and 72 h; control cells were treated with DMSO vehicle (final concentration <0.5%) in culture medium. The final concentration of DMSO was <0.5% (v/v), which did not affect cell growth. After treating with Tan-I 24, 48, and 72 h, the cells were collected and their viability determined by trypan blue exclusion method.

Cell cycle analysis. The percentage of cells in sub-G1, G0/G1, S and G2/M phases were determined by flow cytometry as previously described (14). Briefly, 1×10^6 cultured Colo 205 cells in 10-cm dishes were treated with different concentrations (0, 1, 2.5, 5 and 10 μg/ml) of Tan-I for 48 and 72 h. The harvested cells were washed twice with PBS, fixed gently with ice-cold 70% ethanol overnight, and then resuspended in PBS containing 40 μg/ml propidium iodide, 0.1 mg/ml RNase (Sigma), and 0.1% Triton X-100. The cells were kept at 37˚C for 30 min before the cell cycle was analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA) using an argon laser at 488 nm. The average of the percentage of each phase in the cell cycle was representative of the 3 independent experiments.

TUNEL assay. Apoptotic cells were identified using a commercially available kit based on the TdT-mediated dUTP nick end-labeling (TUNEL) method in accordance with the manufacturer's instructions (Roche Molecular Biochemicals). Briefly, Colo 205 cells were plated in 6-well plates at a density of 1×10^5 cells per well and cultured for 24 h. Then cells were treated with or without various concentrations (1-10 μg/ml) of Tan-I for 24 h. After treatment, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed with PBS, and permeabilized with 0.1% Triton X-100 for 2 min on ice. Then cells were then washed twice and resuspended in 50 μl TUNEL reaction mixture (5 μl TUNEL enzyme containing terminal deoxynucleotidyltransferase, mixed with 45 μl TUNEL label containing phycoerythrin-dUTP and dNTP nucleotides) for 60 min at 37˚C. Negative controls contained only 50 μl TUNEL label. Finally, the cells were washed 3 times with buffer (PBS, 0.1% NaN$_3$, and 10% autologous serum) and examined by fluorescence microscopy (Nikon, 200X).

DAPI stain. Apoptotic cells were identified by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Briefly, the Colo 205 cells were plated in 6 well plates at a density of 1×10^5 cells/well and grown for 24 h, then they were treated with or without various concentrations of Tan-I at 0, 1, 2.5, 5 and 10 μg/ml for 24 h and were then isolated for DAPI staining. The cells were fixed in 4% paraformaldehyde for 15 min at 15-37˚C then rinsed with PBS, and 0.1% Triton X-100/in PBS was added for 15 min, followed by further rinsing with PBS. DAPI solution (1 μg/ml) was added into cells, which were then incubated for 30 min at 37˚C. The cells were then washed with PBS and examined by fluorescence microscopy (Nikon, 200X).

Molecular structure of Tanshinone I
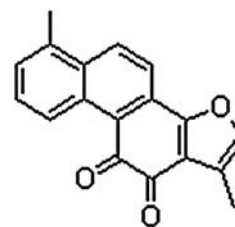
Source: www.chemblink.com/products/568-73-0.htm

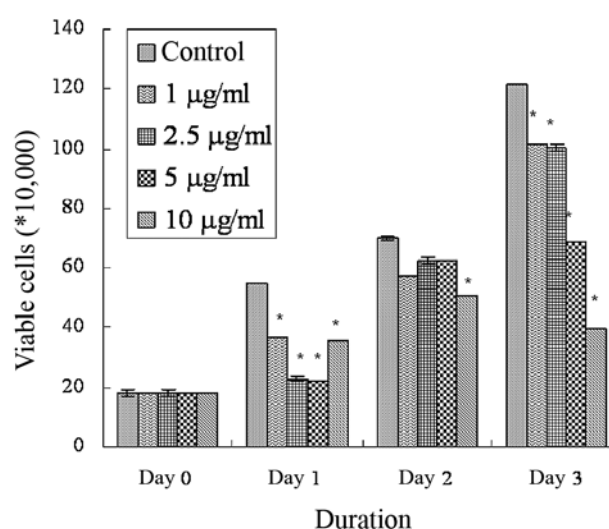Figure 1. Molecular structure of tanshinone I.

Figure 2. The viable cell numbers of Colo 205 cells were treated with various concentrations (control, 1, 2.5, 5 and 10 μg/ml) of Tan-I for 24, 48 and 72 h, then cells were collected by centrifugation and the viable cells were determined by trypan blue exclusion as described in Materials and methods. The viable cell numbers significantly decreased with the increase of the Tan-I concentration. Each point is mean ± S.D. of 3 experiments, *P<0.05.

Protein preparation. For protein extraction, Colo 205 cells (2×10^6 cells) were initially seeded on 10-cm cultured dishes and treated with Tan-I at the concentrations of 0, 2.5, 5, and 10 μg/ml for 12, 24 and 48 h before the cells were harvested by centrifugation. Proteins were extracted as previously described (16). Briefly, cell pellets were resuspended in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium ortho-vanadate, 1 mM sodium fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml antipain) for 30 min at 4˚C. Lysates were immediately centrifuged at 13,000 x g for 20 min at 4˚C and the supernatants were collected, aliquoted (50 μg/tube) and stored at -80˚C until assay. Protein concentrations were measured using the Bradford method (15).

Western blotting. All protein samples were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis
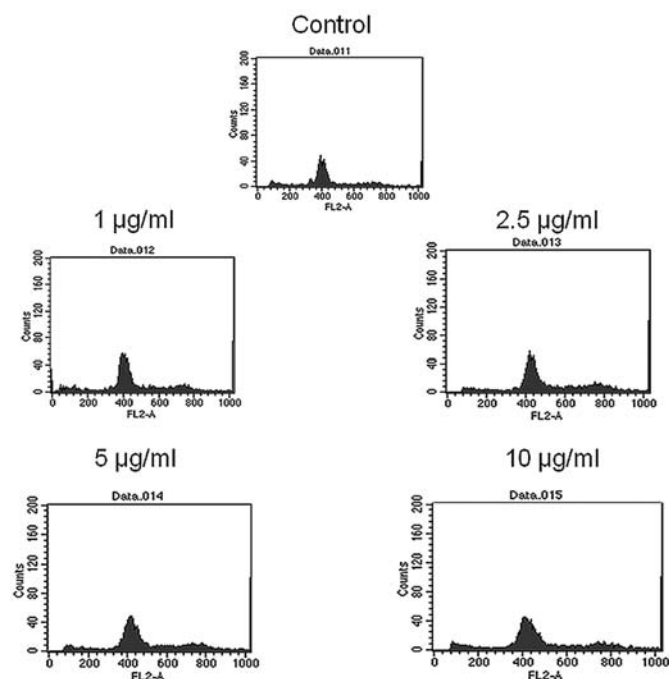
Figure 3. The Colo 205 cells were exposed to various concentrations (0, 1, 2.5, 5 and10 μg/ml) of Tan-I for 48 h, then the cells were harvested and the cell cycle was analyzed by flow cytometry as described in Materials and methods. The G0/G1 phase arrest and sub-G1 phase increased according to the increased concentration of Tan-I.

Figure 4. The Colo 205 cells were exposed to various concentrations (0, 1, 2.5, 5 and10 μg/ml) of Tan-I for 72 h, then the cells were harvested and the cell cycle was analyzed by flow cytometry as described in Materials and methods. The G0/G1 phase arrest and sub-G1 phase increased according to the increased concentration of Tan-I.

(SDS-PAGE) (Bio-Rad Life Science Products, Hercules, CA, USA) as previously described (16). The SDS-separated proteins were equilibrated in transfer buffer (50 mM Tris, pH 9.0, 40 mM glycine, 0.375% SDS, and 20% methanol) and transferred onto an Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA). The membranes were incubated with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 for 1 h. The membranes were then washed and incubated with appropriate dilutions of specific antibodies for p53, p21, bax, caspase-3 and ß-actin (Upstate, Lake Placid, NY, USA) at 4˚C overnight. After incubation with anti-mouse peroxidase-conjugated antibody (Santa Cruz, CA, USA), the immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amerham Pharmacia Biotech) detection kit. ß-actin was used as an internal control in Western blotting. Immunoreactive bands were scanned and analyzed using a digital scanning densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis. Values are presented as mean ± S.D. of control. The Student's t-test was used to analyze the statistical significance between the tanshinone I-treated and control groups. $p < 0.05$ was considered significant.

Results

The cytotoxicity of Tan-I on human colon cancer Colo 205 cells. Colo 205 cells were treated with various concentrations of Tan-I (0, 1, 2.5, 5 and 10 μg/ml) for different durations (24, 48 and 72 h). The results were viewed under contrast-phase microscope (x100). The results showed viable cells
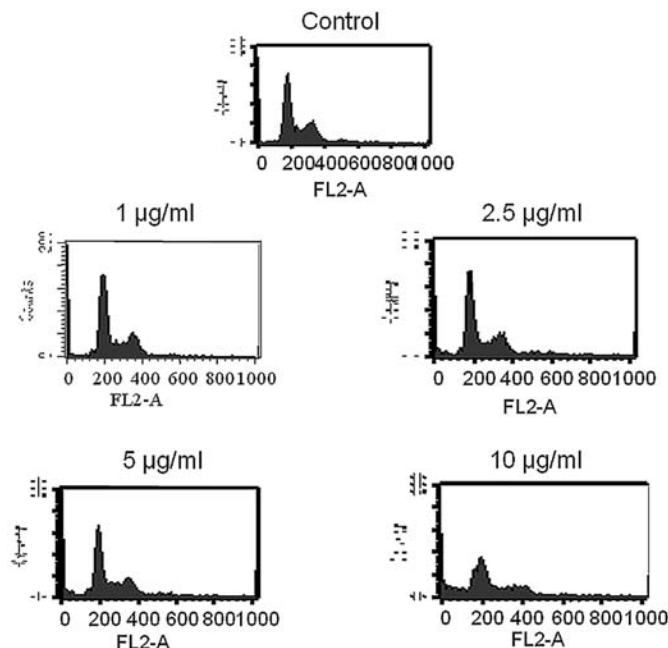
decreased when the concentration of Tan-I increased, then Colo 205 cells were collected by centrifugation and the viable cells were determined by trypan blue exclusion methods. When the cultured cells were exposed to various concentrations of Tan-I (0, 1, 2.5, 5 and 10 μg/ml) for 24 h, the numbers of viable cells were 54.83±1.04, 36.67.0±0.29, 22.92±0.63, 22.17±0.29 and 35.67±1.15 (x10⁴), respectively. After the cells were exposed to Tan-I at 0, 1, 2.5, 5 and 10 μg/ml for 48 h, the numbers of viable cells were 70.0±1, 57.33.7±5.51, 62.33±1.52, 62.67±0.58 and 51.0±1.0 (x10⁴), respectively. After the cells were exposed to Tan-I at 0, 1, 2.5, 5 and 10 μg/ml for 72 h, the numbers of viable cells were 121.33±0.58, 101.67±0.58, 100.33±0.58, 69±2 and 39.67±0.58 (x10⁴), respectively. The results indicated that the viable cell numbers significantly decreased as the Tan I concentration increased. Tanshinone I obviously inhibited Colo 205 cell growth ($p < 0.05$), demonstrating a dose-dependent effect (Fig. 2).

Tan-I induced Colo 205 cell cycle arrest in G0/G1. To analyze the effect of Tan-I on Colo 205 cell growth and apoptosis, the cell cycle distribution was determined by flow cytometry. The fraction of cells in sub-G1 phase increased after the cells were exposed to various concentrations of Tan-I (0, 1, 2.5, 5 and 10 μg/ml) for 48 and 72 h. When Colo 205 cells were treated with Tan-I at 0, 1, 2.5, 5 and 10 μg/ml for 48 h, the percentage of cells in sub-G1 phase increased from 6.44% to 11.4%, 8.58%, 8.86% and 13.1%, respectively (Fig. 3). Treatment with Tan-I at 0, 1, 2.5, 5 and 10 μg/ml for 72 h, increased the proportion of cells in sub-G1 phase from 3.83% to 7.22%, 8.68%, 14.4% and 32.98%, respectively (Fig. 4). Summary results indicate that Tan-I arrests Colo 205 cells in the G0/G1 phase with cell accumulation in the sub-G1 phase.

Control

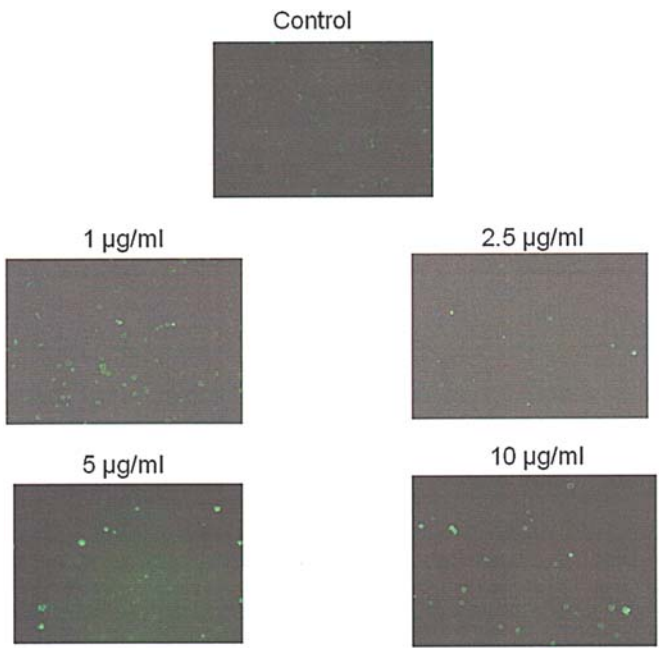1 μg/ml   2.5 μg/ml

5 μg/ml   10 μg/ml

Figure 5. TUNEL assay for the effects of Tan-I on Colo 205 cells apoptosis. The Colo 205 cells were incubated with various concentrations of Tan-I for 24 h and apoptosis was determined by TUNEL assay as described in Materials and methods.

Control

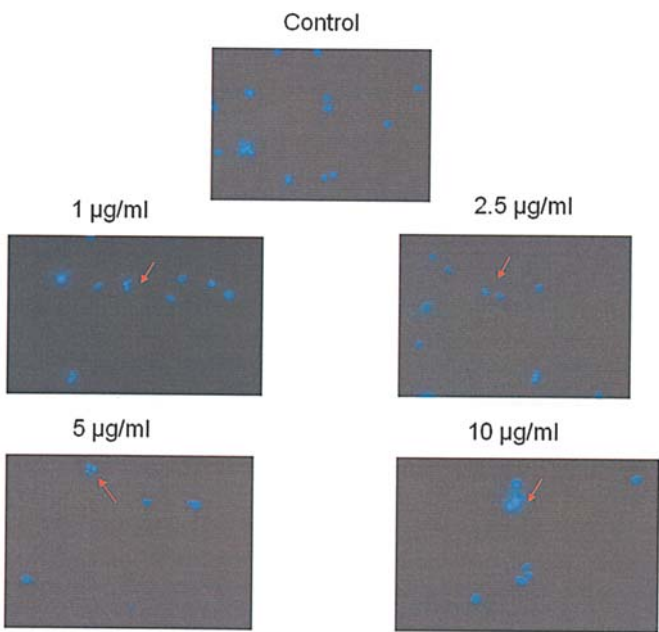1 μg/ml   2.5 μg/ml

5 μg/ml   10 μg/ml

Figure 6. DAPI staining analysis for the effects of Tan-I on Colo 205 cells apoptosis. The Colo 205 cells were incubated with various concentrations of Tan-I for 24 h and apoptosis was determined by DAPI staining as described in Materials and methods.

A

p53

β-actin

B

p21

β-actin

C

bax

β-actin

Figure 7. A, B, and C.

To determine the contribution of apoptosis to Tan-I-induced cytotoxicity, qualitative analyses were performed using TUNEL staining. The number of TUNEL-positive cells significantly increased after Tan-I treatment (1-10 μg/ml for 24 h). The percentage of TUNEL-positive cells by Tan-I treatment at 0, 1, 2.5, 5 and 10 μg/ml was 2.63, 3.99, 18.33,
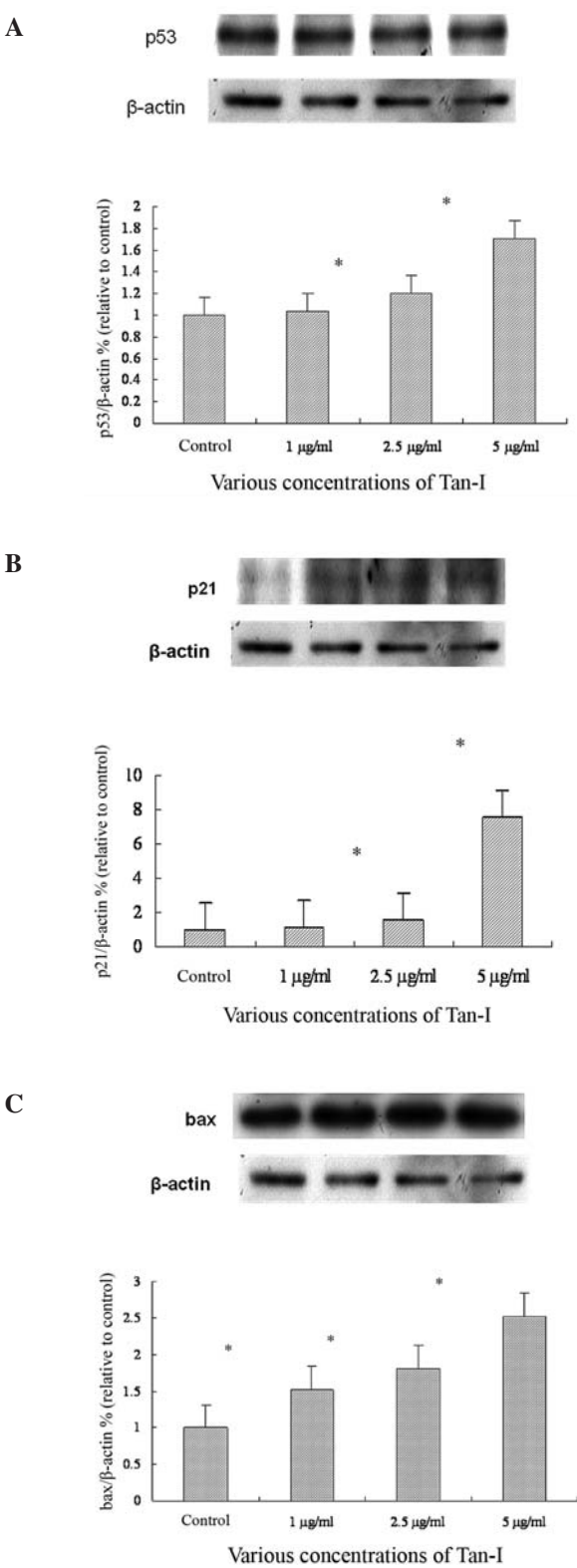
22.36, and 16.33%, respectively. Some cells clearly contained apoptotic bodies, providing further evidence of cell apoptosis (Fig. 5).

To determine the contribution of apoptosis to Tan-I-induced cytotoxicity, qualitative analyses were performed using DAPI staining. Apoptosis was detected (Fig. 6).

The influence of Tan-I on the expression of cell cycle regulator proteins was investigated by Western blot analysis.
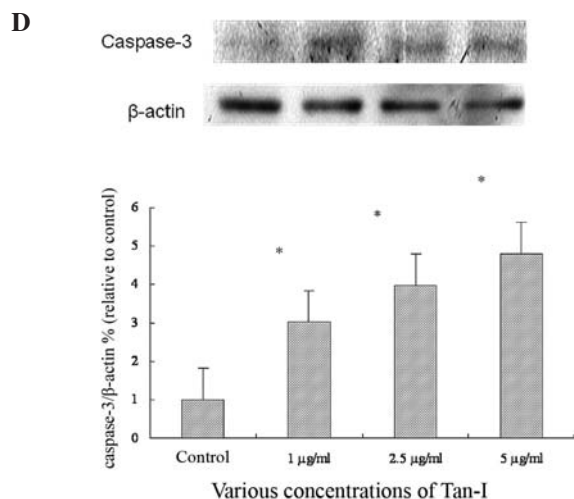
D

Figure 7. Representative Western blot showing changes on the levels of p53 (A), p21 (B), bax (C) and Caspase-3 (D) in Colo 205 cells after exposure to tanshinone I. The Colo 205 cells ($5x10^6$/ml) were treated with various concentrations (0, 1, 2.5 and 5 μg/ml) of Tan-I for 24 h, then cytosolic fraction and total protein were prepared and determined as described in Materials and methods. Evaluation of the levels of p53, p21, bax and caspase-3 expression was carried out by Western blotting as described in Materials and methods.

Figure 9. The proposed anti-proliferation signal pathways for Tan-I in human colon cancer Colo 205 cells.

The expressions of the pro-apoptotic protein p53 and cyclin-dependent kinase inhibitor p21 protein increased significantly after treatment of Tan-I at the concentration of 2.5 and 5 μg/ml for 24 h (Fig. 7A and B). To establish the involvement of a mitochondrial apoptotic event, the protein of bax was measured. When cells were treated with Tan-I at the concentration of 2.5 and 5 μg/ml for 24 h, the expressions of bax and caspase-3 protein increased significantly (Fig. 7C and D). We also characterized the involvement of p53 and p21 in Tan-I effects on cultured colon cancer cells. The protein levels of p53 and p21 were significantly increased after
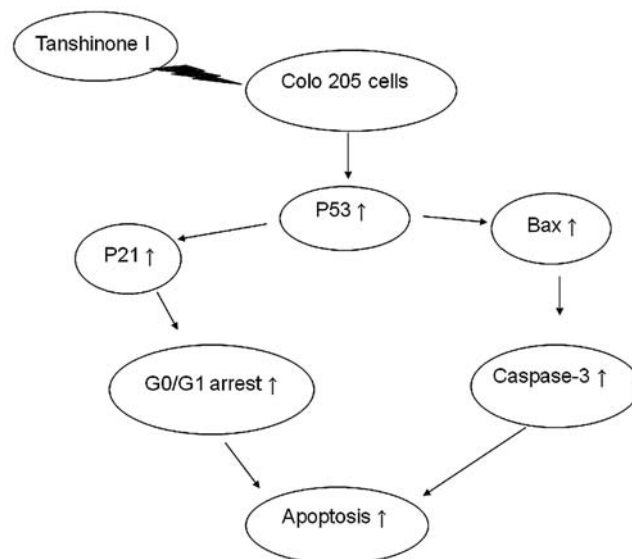
administration of Tan-I (5 and 10 μg/ml) for 24 h (Fig. 8A and B).

Discussion

In this study, the results showed that Tan-I reduced cell growth in a concentration-dependent manner, inducing apoptosis accompanied by an increase in TUNEL staining and in cells in the sub-G1 fraction. This is in agreement with other studies that tanshinone-induced cytotoxicity in Colo 205 and HepG2 carcinoma cell lines (8). It is well documented that p53 is activated and turns on the transcription of one of its downstream genes, *p21 (WAF1, Cip-1)* (17), and have
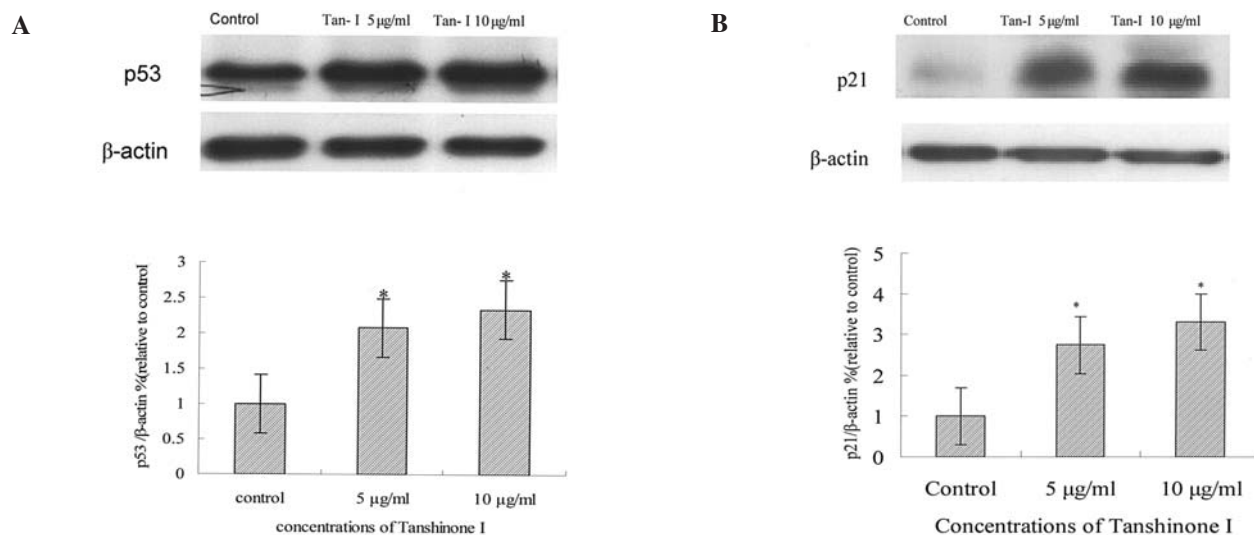
A

B

Figure 8. Representative Western blot showing changes on the levels of p53 (A) and p21 (B) in Colo 205 cells after exposure to Tan-I. The Colo 205 cells ($5x10^6$/ml) were treated with various concentrations (0, 5 and 10 μg/ml) of Tan-I for 24 h, then cytosolic fraction and total protein were prepared and determined as described in Materials and methods. Evaluation of the levels of p53 and p21 expression was carried out by Western blotting as described in Materials and methods.

the potential to induce G1 arrest (18) and apoptosis (19,20). The expressions of p53, p21, bax and caspase-3 increased in Tan-I-treated cells. In addition, the cell cycle analysis showed G0/G1 arrest. These findings suggest that Tan-I induces apoptosis in Colo 205 cells through both mitochondrial-mediated intrinsic cell-death pathways and p21-mediated G0/G1cell cycle arrest (Fig. 9). This is agreement with other reports that Tan-I was able to induce apoptosis of human hepatoma hepG2 cells through up-regulation of the expression of apoptosis-associated gene Bax (21). These observations showed that the therapeutic potential of Tan-I for colon cancer deserves further study.

Acknowledgements

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