

Tanshinone IIA enhances chemosensitivity of colon cancer cells by suppressing nuclear factor- κ B

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Abstract. The aim of the present study was to investigate the effect and molecular mechanism of tanshinone IIA (TSA) on colon cancer cells. Cell viability was determined using Cell Counting kit-8 assay and the results demonstrated that TSA treatment significantly decreased the cell viability of HCT1116 and COLO205 cells in a dose-dependent manner. TSA treatment also sensitized HCT1116 and COLO205 cells to fluorouracil therapy in a concentration-dependent manner. Western blotting was performed in order to investigate the molecular mechanisms of TSA action and determine the level of phosphorylated p65 and nuclear factor- κ B (NF- κ B)-regulated genes, including vascular endothelial growth factor (VEGF), c-Myc, cyclooxygenase-2 (COX-2) and B-cell lymphoma-2 (Bcl-2). The results revealed that TSA treatment greatly decreased the level of phosphorylated p65 in the nucleus, which indicated the inhibition of NF- κ B activation by TSA treatment. TSA also decreased the expression levels of VEGF, c-Myc, COX-2 and Bcl-2. Furthermore, the inhibition of NF- κ B activation with the specific inhibitor, pyrrolidine dithiocarbamate, increased the induction of cell death and chemosensitization effect of TSA in colon cancer cells. In conclusion, these results suggest that TSA induces cell death and chemosensitizes colon cancer cells through the suppression of NF- κ B signaling.

Introduction

Colorectal cancer is the third most common type of human cancer and a major global public health concern (1). The incidence rate of colorectal cancer has been increasing in the Chinese population; in Beijing, the annual incidence of CRC has increased from 16 per 100,000 to 24 per 100,000 in the past decade (2). Chemoprevention, including the use of oxaliplatin, fluorouracil and leucovorin, is considered to be the

most promising strategy, since other therapies fail to control gastrointestinal cancers (3). However, at present, no optimal adjuvant chemotherapy exists; therefore, there is a constant requirement for the development of rationally designed, novel adjuvant therapeutic strategies for the treatment of colon cancer. In the past few decades, traditional Chinese herbal medicines have become a widely accepted treatment option for colorectal cancer and an increasing number of studies have focused on the identification of new bioactive pure compounds and herbs (4,5). Danshen, also known as *Radix Salviae miltiorrhiza*, is widely prescribed in traditional Chinese medicine for the treatment of cardiovascular diseases (6,7). Tanshinone IIA (TSA; C₁₉H₁₈O₃) is extracted from Danshen (8,9) and presents anti-inflammatory (10,11) and antioxidant properties (12,13). Recently, TSA has been proven to also harbor antitumor activities in various human malignant neoplasms (14-17). Su *et al* reported that TSA may inhibit cell growth and induce apoptosis in colon cancer (18); however, the molecular mechanisms of TSA action remain unclear. In the present study, the influence and molecular mechanism of TSA on colon cancer cell growth and chemosensitivity of colon cancer cells to fluorouracil (5-FU) were investigated.

Materials and methods

Cell culture and materials. HCT1116 and COLO205 colon cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL) and 2% penicillin/streptomycin (penicillin, 10,000 U/ml; streptomycin, 10 mg/ml). The cells were placed into tissue culture flasks (75 cm², 250 ml) and grown at 37°C in humidified atmosphere consisting of 5% CO₂ and 95% air. TSA was purchased from Dasherb Corp. (Shenyang, China). Aprotinin and leupeptin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide was purchased from EMD Millipore Corporation (Darmstadt, Germany). The antibodies used in the present study were the following: Mouse monoclonal anti-actin (1:1,000; cat. no. sc-8432; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-vascular endothelial growth factor (VEGF; 1:500; cat. no. ab46154; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-c-Myc (1:1,000; cat. no. 9402S; Cell Signaling

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Technology, Inc., Denvers, MA USA), mouse monoclonal anti-cyclooxygenase-2 (COX2; 1:1,000; cat. no. sc-376861) and rabbit polyclonal anti-B-cell lymphoma-2 (Bcl-2; 1:2,000; cat. no. sc-492) (both from Santa Cruz Biotechnology Inc.).

Cell viability assay. Cell viability was measured using a Cell Counting kit (CCK)-8 kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, cells were seeded in a 94-well plate at a density of 2×10^4 cells per well in 200 μ l culture medium. The following day the cells were incubated with drug for 48 h. At the end of the experiment, 20 μ l CCK-8 solution was added to the cells. The cells were then further incubated at 37°C for 2 h. Subsequently, the optical density (OD) at 450 nm was measured using a VICTOR™ X Multi-Label reader (PerkinElmer, Inc., Waltham, MA, USA) and the percentage of cell viability was calculated as $OD_{\text{drug}}/OD_{\text{control}} \times 100\%$.

Preparation of nuclear extract. Following treatment, the cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS). Next, the cell samples were resuspended in 1 ml PBS and nuclear extracts were prepared on ice as described in a previous study (19). Following centrifugation at 15,000 x g for 10 min at 4°C, the cell pellet was suspended in an ice-cold buffer (consisting of 10 mmol/l HEPES, 1.5 mmol/l $MgCl_2$, 0.5 mmol/l dithiothreitol, 0.2 mmol/l phenylmethylsulfonylfluoride and 0.2 mmol/l KCl), vortexed for 10 sec and centrifuged at 15,000 x g for 5 min at 4°C. Subsequently, the nuclear pellet was washed in 1 ml buffer (consisting of 20 mmol/l HEPES, 25% glycerol, 0.2 mmol/l ethylenediaminetetraacetic acid, 1.5 mmol/l $MgCl_2$ and 0.42 mol/l hypertonic saline), resuspended in 30 ml buffer (20 mmol/l HEPES, 25% glycerol, 0.42 mol/l NaCl, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l EDTA), rotated for 30 min at 4°C and centrifuged at 14,500 x g for 20 min at 4°C. Finally, the supernatants were used as nuclear extracts.

Western blotting. Whole cell lysates or nuclear extracts were separated using 12% SDS-PAGE, as described previously (20). Transfer buffer [25 mM Tris (pH 8.5), 20% methanol and 0.2 M glycine] was used to equilibrate the separated proteins, which were then transferred onto a 0.4- μ m polyvinylidene fluoride membrane (EMD Millipore Corporation, Bedford, MA, USA). The membranes were incubated with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h, followed by washing and then incubation with appropriate dilutions of specific primary antibodies at 4°C overnight. Following incubation with secondary anti-mouse peroxidase-conjugated antibody (dilution, 1:15,000; Sigma-Aldrich), the immune-reactive bands were visualized using an enhanced chemiluminescence detection kit (EMD Millipore Corporation, Darmstadt, Germany). β -actin was used as an internal control for western blotting.

Statistical analysis. The statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and are presented as the mean \pm standard deviation. One-way analysis of variance was performed to compare numeric variables between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

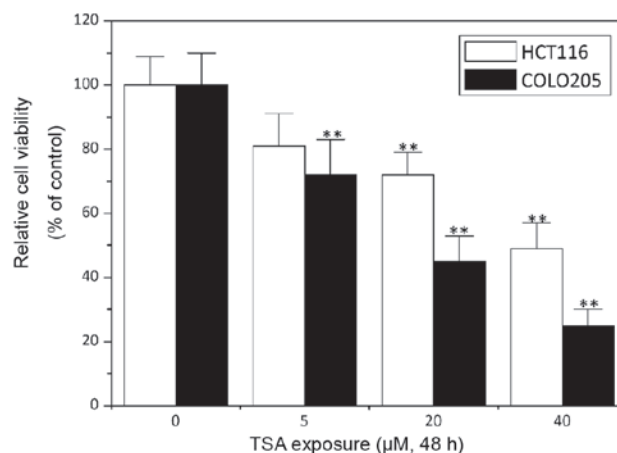


Figure 1. TSA induced cell death in the COLO205 and HCT116 colon cancer cell lines. Cultured cells (2×10^4 cells per well) were treated with different concentrations (5-40 μ M) of TSA for 48 h. Cell viability was determined using Cell Counting kit-8 and presented as a percentage of the control value. Data are presented as the mean \pm standard deviation of five independent experiments. ** $P < 0.05$ vs. the respective control group. TSA, tanshinone IIA.

Results

Effect of TSA on cell viability of colon cancer cells. TSA has been demonstrated to exert an antitumor activity in numerous types of human cancer, including breast, lung, liver, prostate and ovarian cancer and leukemia (15,21-25). The data of the present study showed that the exposure of the HCT116 and COLO205 colon cancer cell lines to TSA greatly decreased their cell viability in a concentration-dependent manner ($P < 0.05$; Fig. 1), which suggested an antitumor effect of TSA in colon cancer.

TSA enhances chemosensitivity of colon cancer cells. Drug resistance is a clinical challenge that can result in the failure of colon cancer therapy (26). In order to examine how TSA influences the sensitivity of colon cancer cells to chemotherapy, 5-FU chemotherapy was combined with TSA treatment. As shown in Fig. 2, 5-FU-induced cell death in HCT116 and COLO205 cells was considerably enhanced by 20 μ M TSA treatment ($P < 0.05$), which suggested that TSA was able to potentiate the chemosensitivity of colon cancer cells.

Involvement of nuclear factor- κ B (NF- κ B) in TSA activity. As shown by the aforementioned data, TSA can decrease cell viability and increase the chemosensitivity of colon cancer cells; however, the molecular mechanisms of TSA action remain to be elucidated. In order to further investigate the mechanisms of TSA action, the influence of TSA on the activation of NF- κ B was determined, since it has been suggested that NF- κ B is involved in the development and progression of various malignant neoplasms (27-33), which made it a target for tumor therapeutics (34,35). The present data revealed that 20 μ M TSA treatment significantly decreased the level of phosphorylation of p65, a subunit of NF- κ B (Fig. 3A), which indicated the inhibitory effect of TSA on NF- κ B activation. In addition, the expression of NF- κ B regulated genes was examined. As illustrated in Fig. 3B, 20 μ M TSA was able to considerably decrease VEGF, c-Myc, COX-2 and Bcl-2 expression.

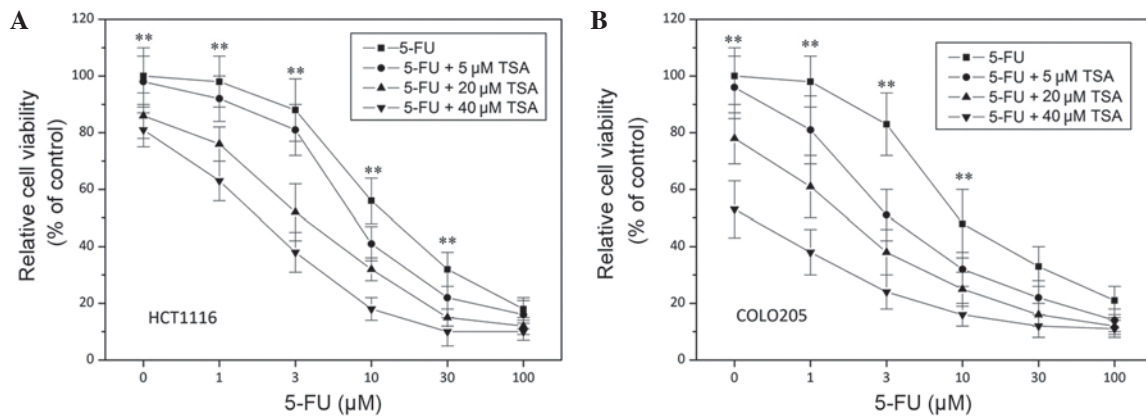


Figure 2. TSA enhances cytotoxicity of 5-FU in (A) HCT1116 and (B) COLO205 colon cancer cell lines. Colon cancer cells were treated with different doses of 5-FU (0-100 μM), with or without different concentrations of TSA (0-40 μM) for 24 h. Cell viability was measured using Cell Counting kit-8 and presented as a percentage of the control values. Data are presented as the mean ± standard deviation of five independent experiments. **P<0.05, 20 μM and 40 μM TSA + 5-FU group vs. 5-FU group. TSA, tanshinone IIA; 5-FU, fluorouracil.

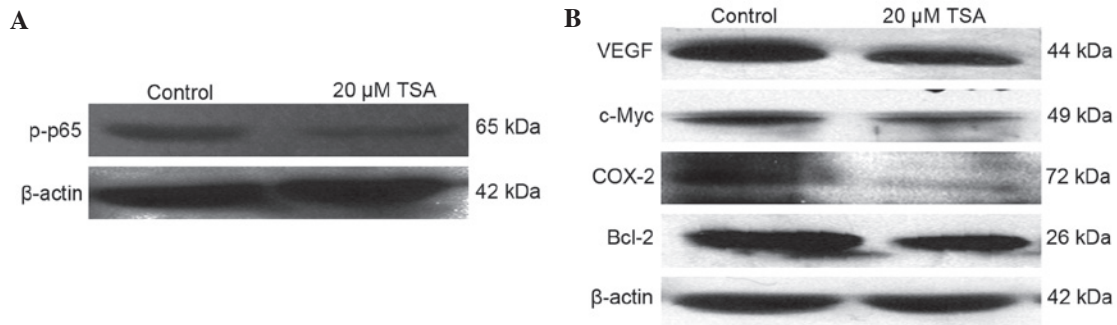


Figure 3. Effect of TSA on the activation of NF-κB and the expression of its downstream gene products. (A) TSA inhibited the phosphorylation of p65, which is a subunit of NF-κB. COLO205 cells were treated with 20 μM TSA for 48 h. Cells were harvested and the expression of phosphorylated-p65 in their nucleus was evaluated using western blotting. (B) TSA downregulated the downstream gene products of NF-κB in COLO205 cells. After 48 h of incubation, the levels of VEGF, c-Myc, COX-2 and Bcl-2 were determined using western blotting. β-actin was used as an internal control. A representative example from three independent experiments is shown. TSA, tanshinone IIA; NF-κB, nuclear factor-κB; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; Bcl-2, B-cell lymphoma-2.

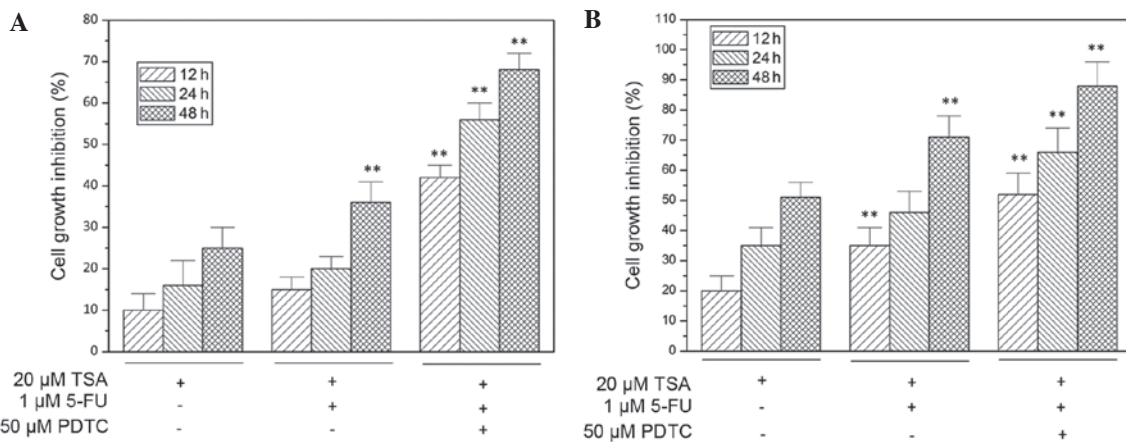


Figure 4. Nuclear factor-κB inhibitor, PDTC enhanced the effect of TSA-induced cell death on the (A) HCT1116 and (B) COLO205 colon cancer cell lines. Cultured cells were seeded in a 96-well plate at the density of 2×10^4 cells per well and treated by 20 μM TSA, with or without 50 μM PDTC. After 12, 24 and 48 h of incubation, cell viability was determined using Cell Counting kit-8. Cell viability was expressed as a percentage of the control value. Data are presented as the mean ± standard deviation of at least three independent experiments. **P<0.05 vs. TSA treatment. TSA, tanshinone IIA; 5-FU, fluorouracil; PDTC, pyrrolidine dithiocarbamate.

NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), enhances TSA activity. As indicated in Fig. 4, the inhibition of NF-κB signaling with specific inhibitor PDTC significantly

potentiated the suppression of TSA-induced cell growth in HCT1116 and COLO205 cells. Furthermore, the chemosensitizing effect of TSA on colon cancer cells was also enhanced

by 50 μ M PDTC treatment ($P < 0.05$), which is illustrated as increased cell growth inhibition in Fig. 4.

Discussion

Due to drug resistance and the toxic effect of current chemotherapy strategies, antitumor drug studies have been attempting to identify natural chemical compounds, and Chinese herbal medicine has been attracting increasing attention (36). TSA has been found to be an effective chemical component extracted from Danshen (*Radix Salviae Miltiorrhizae*) (37), which harbors anti-inflammatory and anti-oxidative activities (10-13). It has also been shown to have a cardioprotective effect by reducing apoptosis (38-40). Recently, an increasing number of studies has focused on the antitumor activity of TSA. For instance, Jung *et al* (41) and Liu *et al* (42) identified that TSA induced apoptosis in leukemic cells *in vitro*, possibly through the JAK/STAT3/5 and SHP1/2 pathways. In addition, Fu *et al* (25) found that TSA blocked the epithelial-mesenchymal transition through the downregulation of hypoxia-inducible factor-1 α , reversing hypoxia-induced chemotherapy resistance in breast cancer cell lines. In addition, increasing evidence indicated that TSA can inhibit tumor cell growth and induce apoptosis (15,17,43); however, despite the fact that in certain type of cancer evidence showed that intrinsic apoptosis pathways were involved (14,44), the underlying mechanisms remain unclear. With regard to colon cancer, in 2008, Su *et al* identified that TSA inhibited cell growth and induced apoptosis in colon cancer cells (18). Shan *et al* (45) also indicated that TSA was able to inhibit colon cancer cell migration and invasion. Furthermore, in 2012 Su *et al* (46) found that TSA potentiated the efficacy of 5-FU in colon cancer cells *in vivo* through the downregulation of P-glycoprotein and LC3-II. The results of the present study confirmed the antitumor activity and chemosensitizing effect of TSA in colon cancer cells and provided new evidence that TSA can sensitize colon cancer cells to 5-FU through the suppression of NF- κ B activation.

NF- κ B is constitutively activated in numerous human cancer types (30-32,35). The suppression of NF- κ B may induce apoptosis and sensitize cancer cells to chemotherapy (33,34,47,48). As an eukaryotic transcription factor, NF- κ B regulates numerous genes that are differentially expressed and implicated in tumorigenesis, including c-Myc, COX-2 and matrix metalloproteinase-9 (49). In colon cancer, NF- κ B activation participates in the promotion and progression of colon cancer (49); therefore, the aim of the present study was to investigate the effect of TSA on NF- κ B activation and on NF- κ B-regulated gene products. The results showed that TSA treatment decreased the level of the phosphorylated p65 in the nucleus of colon cancer cells and the NF- κ B-regulated gene expression levels of VEGF, COX-2, c-Myc and Bcl-2. In addition, the inhibition of NF- κ B with specific inhibitor PDTC may further enhance the induction of cell death and the chemosensitizing effect of TSA in colon cancer cells.

In conclusion, the present study revealed that TSA was able to induce cell death in colon cancer cells and sensitize colon cancer cells to 5-FU therapy by inhibiting NF- κ B activation; therefore, TSA appears to be a good option of adjuvant chemotherapy for colon cancer.

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