

Tanshinone IIA inhibits cell proliferation and tumor growth by downregulating STAT3 in human gastric cancer

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Received November 6, 2017; Accepted February 16, 2018

DOI: 10.3892/etm.2018.6562

Abstract. Gastric cancer is the third leading cause of cancer-associated deaths worldwide. Research into the underlying mechanisms of gastric cancer is essential for the development of novel therapeutic agents to improve the prognoses of patients with gastric cancer. Tanshinone IIA (Tan IIA) is the pure extract of Danshen root (*Salvia miltiorrhiza*) and has been reported to inhibit the proliferation of gastric cancer cells; however, the intrinsic underlying mechanisms remain unclear. The aim of the present study was to investigate whether Tan IIA has a direct anti-cancer effect in gastric cancer cells and determine the underlying mechanisms responsible. The results revealed that Tan IIA effectively inhibits proliferation in three human gastric cancer cell lines (SNU-638, MKN1 and AGS) in a time- and dose-dependent manner. Furthermore, Tan IIA treatment induced an increase in apoptosis, B-cell lymphoma (Bcl-2)-associated protein X expression and cleaved caspase-3 levels, as well as a decrease in Bcl-2 expression. Treatment with Tan IIA inhibited Furthermore, treatment with Tan IIA significantly inhibited the phosphorylation of signal transducer and activator of transcription 3 (STAT3), which may be responsible for the changes in apoptosis gene expression. However, overexpression of STAT3 significantly ameliorated the Tan IIA-induced suppression of cell growth and apoptosis. A nude mouse xenograft model was constructed and the results revealed that intraperitoneal Tan IIA treatment for

28 days significantly inhibited tumor growth and STAT3 activation. The results of the present study suggest that Tan IIA exerts potent anti-cancer activity in gastric cancer cells and this effect is mediated by the downregulation of STAT3 activation.

Introduction

Although the incidence of gastric cancer has decreased in recent years (1,2), it remains the third leading cause of cancer-associated mortality worldwide, with ~1,000,000 new cases diagnosed each year (3,4). Improved techniques for the treatment of gastric cancer have been developed, including novel surgical methods, radiation and chemotherapy protocols; however, the prognoses of patients with advanced gastric cancer remain poor, with a 5-year survival of 5-20% (5,6). As such, identifying efficient chemo-adjuvants from herbal medicines may contribute to improving treatment outcomes for patients with gastric cancer.

Traditional Chinese herbal medicines have been studied as potential candidates for cancer treatment and a number of studies have focused on the identification of new bioactive compounds (7-9). Tanshinone IIA (Tan IIA), a major bioactive compound extracted from the root of *Salvia miltiorrhiza*, has been reported to exhibit a number of pharmacological activities in cardiovascular and cerebrovascular diseases (10-12). A number of studies have reported that Tan IIA serves as an effective adjunctive reagent in different types of cancer, including breast, bladder, colorectal and prostate cancers (13-15). Although a number of studies have indicated that Tan IIA exerts powerful anticancer effects in gastric cancer (16,17), the detailed molecular mechanisms remain to be elucidated.

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family of signal-responsive transcription factors (18). STAT3 has been demonstrated to regulate cell proliferation, survival, angiogenesis and immunosuppression (19). It has previously been reported that STAT3 is constitutively activated in gastric cancer and serves a critical role in tumorigenesis (20,21). The inactive form of STAT3 is activated by cytokines, growth factors and oncogenic proteins via the sequential phosphorylation of tyrosine 705 and serine

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Abbreviations: Tan IIA, Tanshinone IIA; STAT3, signal transducer and activator of transcription 3

Key words: Tanshinone IIA, gastric cancer, signal transducer and activator of transcription 3

727 (22). There is evidence that constitutive STAT3 activation may contribute to the progression of gastric cancer (23,24), and so identifying a novel therapeutic agent that inhibits STAT3 signaling is important for the treatment of human gastric cancer. The aim of the present study was to investigate the effects of Tan IIA on gastric cancer proliferation using SNU-638 cells *in vitro* and *in vivo* and to clarify whether the underlying mechanism is associated with the inhibition of STAT3 activation.

Materials and methods

Cell culture and drug treatment. Human gastric cancer cell lines (SNU-638, MKN1 and AGS) were obtained from the Cell Resource Center (Chinese Academy of Sciences, Shanghai, China). SNU-638 and MKN1 cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and AGS cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Tan IIA was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany; purity, ≥97%) and dissolved in dimethyl sulfoxide (DMSO) to make 2.5 μg/ml stock solutions. Gastric cancer cells were treated with Tan IIA in different times (0, 12, 24, 48 and 72 h) or concentrations (0, 2.5, 5 and 10 μg/ml) at 37°C in humidified air and 5% CO₂ atmosphere.

Cell proliferation assay. A modified MTT assay was used to determine the proliferation of human gastric cancer cells. Briefly, cells were seeded in 96-well plates at a density of 1×10⁴ cells/well and treated with Tan IIA. Following 24 h culture at 37°C, 20 μl MTT dye solution (Sigma-Aldrich; Merck KGaA) was added to each well and incubated for a further 2 h. The medium was subsequently removed and replaced with Solubilization/Stop solution (100 μl/well; Sigma-Aldrich; Merck KGaA). Absorbance was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Apoptosis assay. Apoptosis was determined using an Annexin V-FITC/PI kit (cat. no. BB-4101-3; BestBio company, Shanghai, China) according to the manufacturer's protocol. Following treatment with Tan IIA, SNU-638 cells were harvested and washed with PBS. Cells were subsequently stained with propidium iodide (PI) (20 μg/ml) with or without Annexin V-fluorescein isothiocyanate (FITC) for 30 min in the dark at room temperature. The cells were analyzed using a Beckman Coulter CyAn ADP Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA).

Western blotting. Cells were washed twice with ice-cold PBS and lysed using RIPA lysis buffer (Thermo Fisher Scientific, Inc.) at room temperature for 30 min. Protein concentrations were measured using a BCA Protein Assay Reagent (Pierce; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. An equal amount of protein (40 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were then incubated at

4°C overnight with one of the following primary antibodies: Cleaved caspase-3 (1:1,000; cat. no. 9664), phosphorylated (p)-STAT3 (1:1,000; cat. no. 9145) and STAT3 (1:500; cat. no. 12640) (both from Cell Signaling Technology Inc., Danvers, MA, USA). B-cell lymphoma 2 (Bcl-2; 1:1,000; cat. no. sc-56015) and Bcl-2-associated X protein (Bax; 1:1,000; cat. no. sc-4239; both Santa Cruz Biotechnology Inc., Dallas, TX, USA). Membranes were washed with TBST and incubated with a horseradish peroxidase-conjugated anti-rabbit Immunoglobulin G (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 60 min at room temperature. Blots were developed with enhanced chemiluminescence (cat. no. 34580; Pierce; Thermo Fisher Scientific, Inc.) and detected using the Las 4000 imager (GE Healthcare, Chicago, IL, USA). β-actin (dilution 1:5,000; cat. no. SAB2100037; Sigma-Aldrich; Merck KGaA) was used as an internal control for normalization. The relative density of each band was analyzed using Image J software (version 4.0; National Institutes of Health, Bethesda, MD, USA).

STAT3 reporter plasmid transfection and luciferase assays. SNU-638 cells were transiently co-transfected with 0.4 μg of STAT3 reporter plasmid (pSTAT3-LUC; Sangon Biotech Co., Ltd., Shanghai, China) and 0.4 μg of control plasmid pRL-TK (Promega Corp., Madison, WI, USA) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 24 h following transfection, the luciferase activity of STAT3 was measured using a dual-luciferase reporter assay system (Promega Corp.) according to manufacturer's protocol. Data are expressed as relative fold activation to that of non-stimulated (-) sets.

Xenograft tumor model of human gastric cancer. BALB/c nude mice (male, 6-8 weeks old; weight, 18-24 g) were purchased from the laboratory Animal Center of Southern Medical University (Guangzhou, China). Mice were allowed free access to food and water under standard conditions (temperature, 20-25°C; relative humidity, 50-60%), and exposed to a 12 h light/dark cycle. A total of 24 BALB/c nude mice were randomly assigned to four groups (each, n=6). SNU-638 cells were injected into nude mice subcutaneously. At 14 days following tumor cell injection, each mouse was injected intraperitoneally with Tan IIA (12.5, 25 or 50 mg/kg) 3 times a week for 28 days. The control group received an equal volume of saline. Following 28 days, mice were sacrificed. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Southern Medical University (Guangzhou China).

Statistical analysis. Data are presented as the mean + standard error of the mean. Statistical analysis was performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Statistical comparisons were made using one way analysis of variance followed by Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Tan IIA treatment inhibits the proliferation of human gastric cancer cells. An MTT assay was performed to

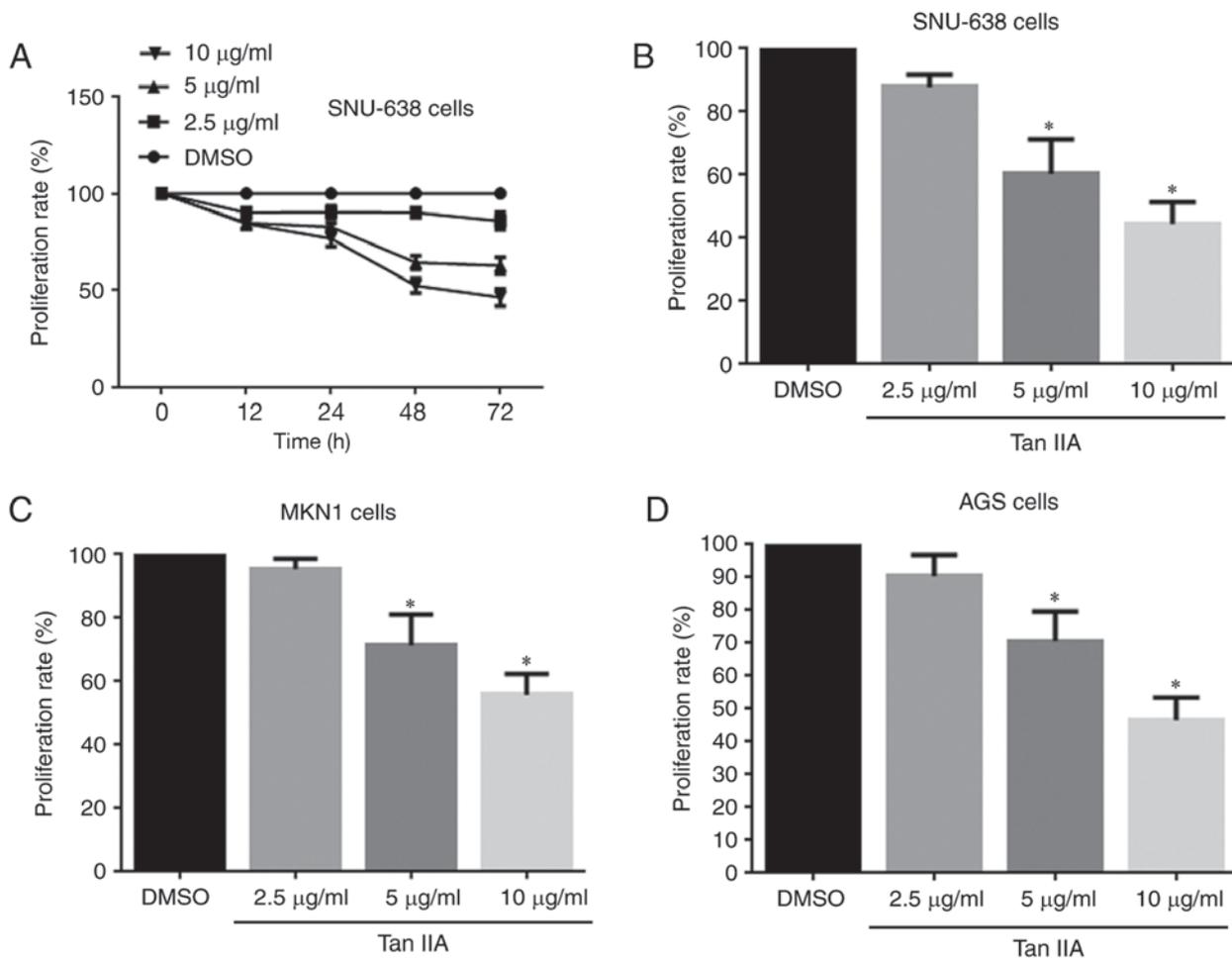


Figure 1. Effects of Tan IIA on human gastric cancer cell proliferation. (A) SNU-638 cells were treated with 2.5, 5 or 10 µg/ml Tan IIA for 12, 24, 48 or 72 h and cell proliferation rate were measured using an MTT assay. (B) SNU-638, (C) MKN1 and (D) HGC27 cells were treated with 2.5, 5 or 10 µg/ml Tan IIA for 48 h and cell proliferation was measured using an MTT assay. *P<0.05 vs. DMSO. Tan IIA, Tanshinone IIA; DMSO, dimethyl sulfoxide.

evaluate the inhibitory effect of Tan IIA on gastric cancer cell proliferation. The results revealed that Tan IIA significantly inhibited the proliferation of SNU-638 cells in a time- and dose-dependent manner (Fig. 1A and B). A dosage of 10 µg/ml Tan IIA was most effective, and so this dosage was used for subsequent experiments. Similar results were observed in MKN1 AGS cells (Fig. 1C and D). These results suggest that Tan IIA inhibits the proliferation of human gastric cancer cells.

Tan IIA treatment induces apoptosis in human gastric cancer cells. The apoptotic effects of Tan IIA were assessed using Annexin V-FITC and PI apoptosis double staining. Tan IIA treatment induced a significant dose-dependent increase in apoptosis (Fig. 2A and B). Furthermore, Tan IIA treatment increased the expression of Bax and cleaved caspase-3, as well as decreasing the expression of Bcl-2 (Fig. 2C). These results suggest that Tan IIA treatment induces gastric cancer cell apoptosis.

Potential role of STAT3 as a target for Tan IIA-induced inhibition of human gastric cancer cell. To examine whether Tan IIA inhibits gastric cancer cell proliferation via the inhibition of STAT3 signaling. Treatment with 5 or 10 µg/ml Tan IIA

significantly inhibited STAT3 phosphorylation at Tyr705 by ~54 and ~77%, respectively (Fig. 3A). Furthermore, Tan IIA treatment significantly reduced STAT3 trans-activity (Fig. 3B). To further confirm whether STAT3 signaling is associated with the anti-proliferative effect of Tan IIA, SNU-638 cells were co-transfected with a STAT3 reporter plasmid (pSTAT3-LUC). The results demonstrated that STAT3 overexpression significantly reversed the Tan IIA-induced inhibition of cell growth and apoptosis, suggesting that STAT3 is required for Tan IIA-induced cell growth inhibition in gastric cancer cells.

Tan IIA suppresses tumor growth in a xenograft model of human gastric cancer. To further examine the anti-tumor efficacy of Tan IIA *in vivo*, a xenograft model of human gastric cancer was constructed using mice. No significant differences in body weight were observed between the treatment groups (Fig. 4A); however, tumor volumes were significantly smaller in the Tan IIA-treated groups (25 and 50 mg/kg) compared with the control after 21 days of treatment (Fig. 4B). In addition, Tan IIA treatment significantly inhibited STAT3 phosphorylation (Fig. 4C). These results suggest that Tan IIA-mediated inhibition of tumor growth occurs via the suppression of STAT3 signaling.

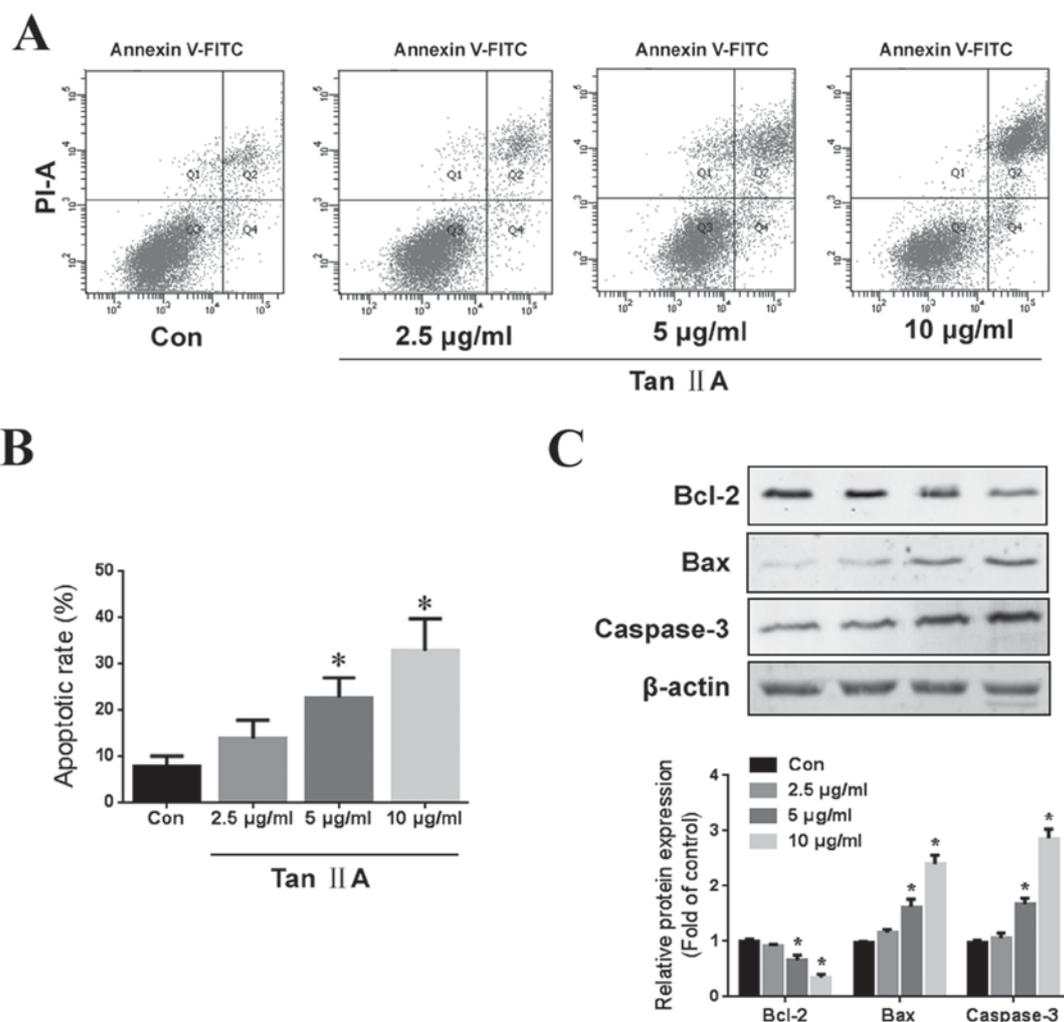


Figure 2. Effects of Tan IIA on apoptosis. SNU-638 cells were incubated with 2.5, 5 or 10 µg/ml Tan IIA for 48 h. (A and B) Annexin V staining and flow cytometry were performed to assess apoptosis and (C) western blotting was performed to measure the expression of apoptosis-associated proteins. *P<0.05 vs. DMSO. Tan IIA, Tanshinone IIA; DMSO, dimethyl sulfoxide; Bcl-2, B-cell lymphoma; Bax, Bcl-2-associated X protein; FITC, fluorescein isothiocyanate.

Discussion

The results of the present study demonstrate that Tan IIA, a bioactive component isolated from traditional Chinese medicine *S. miltiorrhiza*, exerts its anti-cancer effects in 3 human gastric cancer cell lines *in vitro* and a xenograft tumor model *in vivo*. Tan IIA exhibited significantly anti-proliferative and pro-apoptotic effects against gastric cancer, inducing apoptosis and inhibiting STAT3 phosphorylation at Tyr705. The anticancer effect of Tan IIA could be inhibited by co-transfection with STAT3 plasmid to induce STAT3 overexpression. These results suggest that Tan IIA exerts its anti-proliferative effects in gastric cancer cells via downregulating STAT3 activation.

Gastric cancer is the third leading cause of cancer-associated mortality worldwide (25). Although great advances have been made in the diagnosis and treatment of gastric cancer, the outcome for patients with gastric cancer is generally poor (26,27). The side effects associated with chemotherapy reduce patients' quality of life and may also lead to life-threatening complications (28). Identifying effective chemo-preventive agents derived from herbal medicines

are therefore of great importance for improving treatment regimens for gastric cancer (29).

Tan IIA is a herbal medicine extracted from the root or rhizomes of *S. miltiorrhiza* Bunge (30) and has been reported to have potential chemo-preventative effects relevant to various human cancers (31). Xu *et al* (32) reported that Tan IIA reverses the malignant phenotype of SGC7901 gastric cancer cells, which indicates that it may be promising therapeutic agent. The results of the present study indicate that Tan IIA effectively inhibits the proliferation of gastric cancer cells in three cell lines. In addition, a xenograft tumor model was used to demonstrate that Tan IIA reduces the volume of tumors derived from SNU-638 cells. These observations, combined with the effects observed *in vitro*, suggest a direct anticancer effect of Tan IIA.

As a multi-target drug, the molecular targets of Tan IIA include apoptotic-regulating proteins, transcription factors and inflammatory mediators (17,33). In the present study, Tan IIA treatment induced apoptosis, increased the expression of Bax and cleaved caspase-3 and decreased the expression of Bcl-2. In future studies, the effect of Tan IIA on the expression of apoptosis-associated proteins in the xenograft tissues

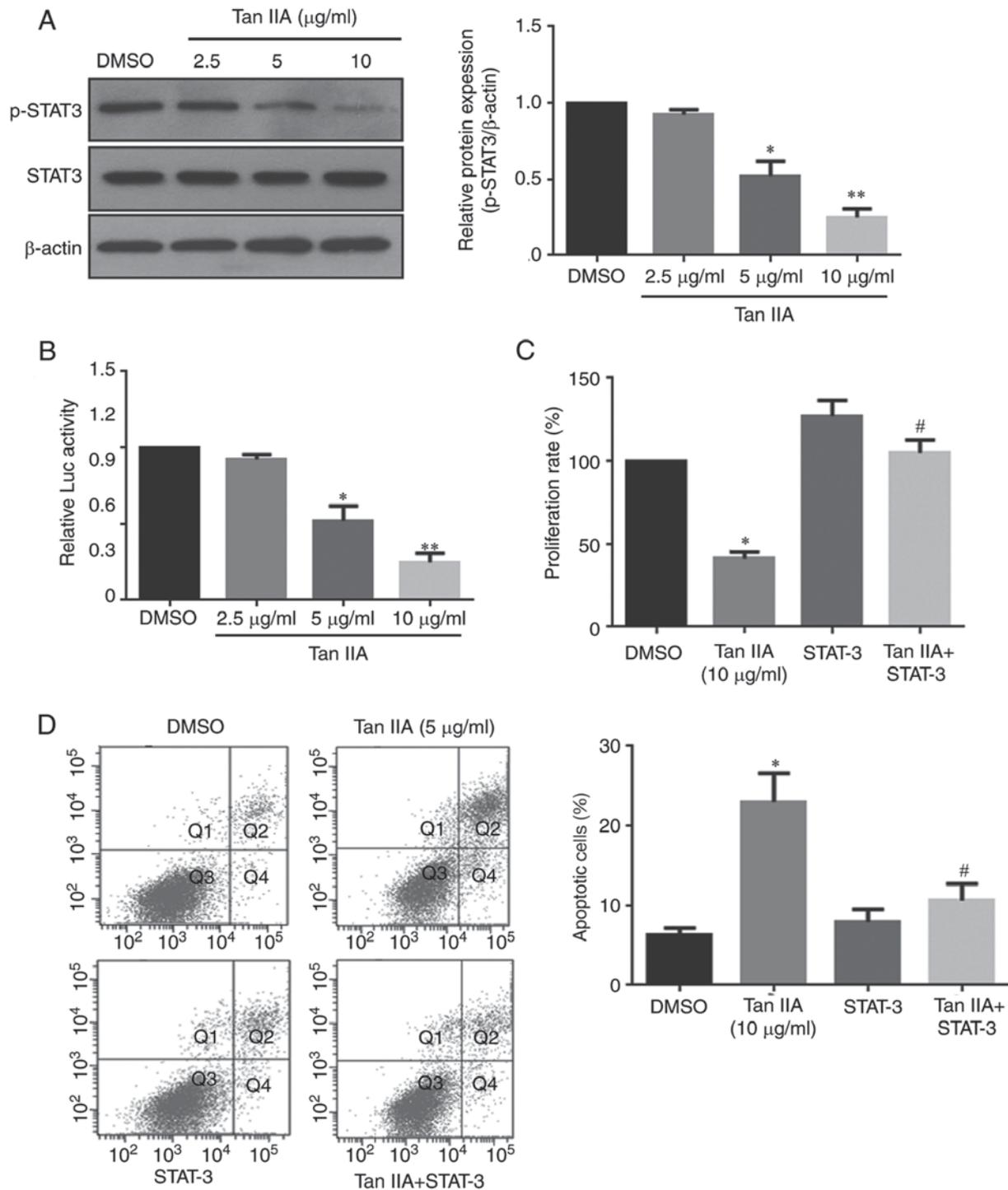


Figure 3. Effect of Tan IIA on STAT3 activity in gastric cancer cells. SNU-638 cells were treated with 2.5, 5 or 10 μg/ml Tan IIA for 48 h. (A) Expression of p-STAT3 and (B) STAT3 activity were measured using western blotting and a dual luciferase reporter assay, respectively. SNU-638 cells were co-transfected with the FLAG-STAT3 plasmid and the control plasmid pRL-TK and treated with 5 μg/ml Tan IIA for 5 h, following which (C) cell proliferation and (D) apoptosis were assessed. *P<0.05, **P<0.01 vs. DMSO and #P<0.05 vs. Tan IIA alone. Tan IIA, Tanshinone IIA; STAT3, signal transducer and activator of transcription 3; p, phosphorylated; DMSO, dimethyl sulfoxide.

of SNU-638 cells should be investigated. It was also demonstrated that Tan IIA significantly prevents the phosphorylation of STAT3. Previous studies have reported that constitutive activation of STAT3 signaling is important for cancer initiation, development and progression (21,34). To further investigate the mechanisms underlying the anticancer effects of Tan IIA in gastric cancer cells, STAT3 phosphorylation was examined.

The results revealed that STAT3 phosphorylation was inhibited by Tan IIA in a dose-dependent manner and similar results were observed in the xenograft model. However, when STAT3 overexpression was induced in SNU-638 cells, Tan IIA failed to inhibited cell proliferation. It has been reported that direct STAT3 suppression induces apoptosis in prostate cancer cells (35). The results of the present study demonstrate that

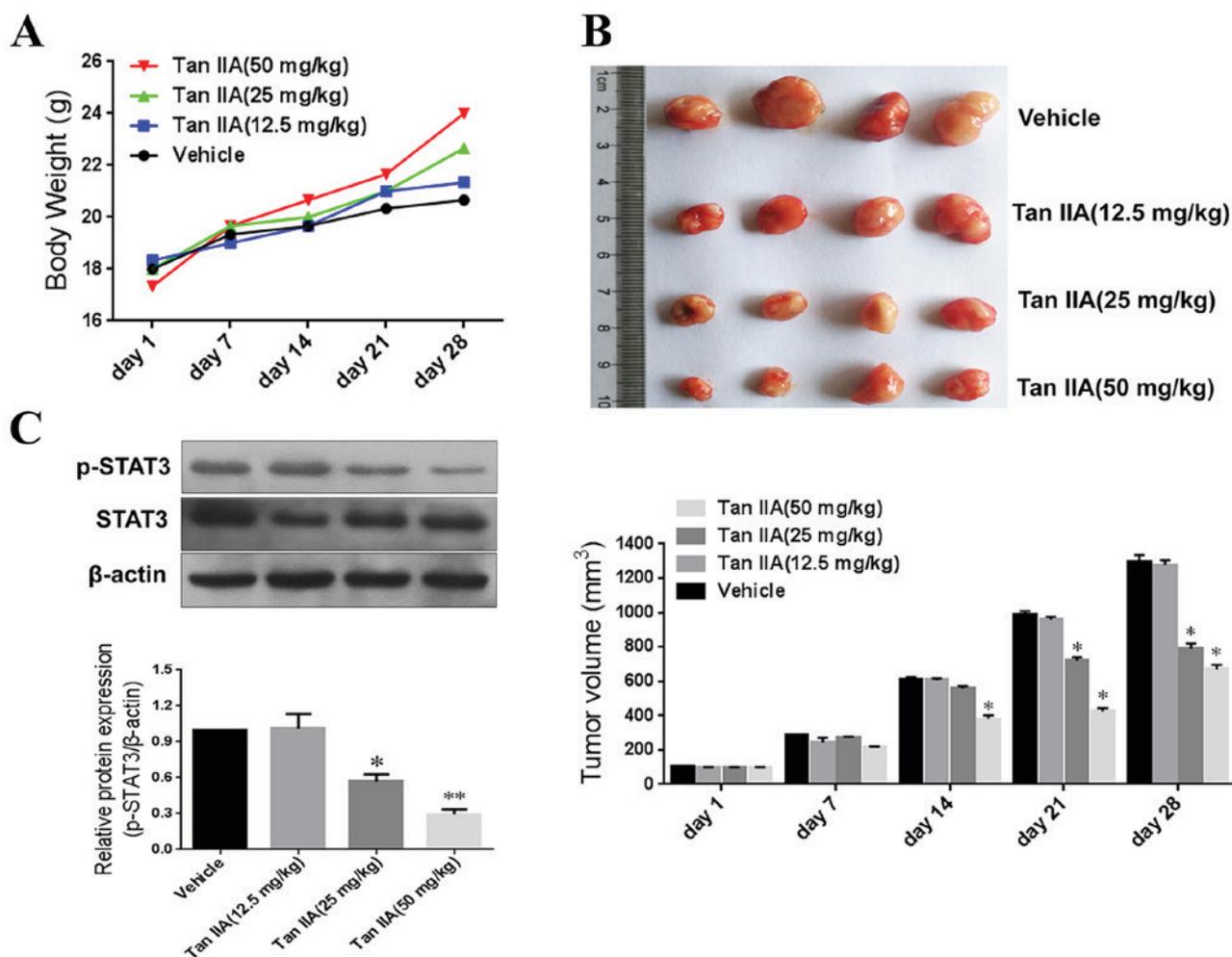


Figure 4. Effects of Tan IIA on gastric tumor growth in a xenograft model of SNU-638 cells. Mice were subcutaneously injected with SNU-638 cells and treated with 12.5, 25 or 50 mg/kg Tan IIA. (A) Body weight and (B) tumor volume were measured over time. (C) Western blotting was performed to measure p-STAT3 expression. * $P < 0.05$, ** $P < 0.01$ vs. vehicle. Tan IIA, Tanshinone IIA; p, phosphorylated; STAT3, signal transducer and activator of transcription 3.

STAT3 overexpression abrogate Tan IIA-induced apoptosis in human cancer SNU-638 cells. However, one limitation of the present study was that normal gastric cells were not included, thus it cannot be determined whether the effects of Tan IIA are cancer-specific.

In summary, the present study demonstrates that Tan IIA exerts significant anticancer effects in human gastric cancer cells and this is mediated at least in part by STAT3 inhibition. However, Tan IIA may also act via other or additional nonspecific mechanisms and this requires further investigation.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YJZ, SG and JF designed the present study. YJZ performed assays, analyzed and interpreted the data and wrote the manuscript. BP, YZ and TC made substantial contributions to the experimental design and revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Southern Medical University Animal Policy and Welfare Committee (Guangzhou, China) and complied with the National Institutes of Health Guidelines (Guide for the Care and Use of Laboratory Animals).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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