Tanshinone IIA induces apoptosis and inhibits the proliferation, migration, and invasion of the osteosarcoma MG-63 cell line *in vitro*

Yi Zhang*, Ren-xiong Wei*, Xiao-bin Zhu*, Lin Cai, Wei Jin and Hao Hu

Tanshinone IIA (Tan IIA) is an active ingredient extracted from the widely used Danshen root (*Salvia miltiorrhiza* Bunge), a traditional Chinese medicine. Recent studies have indicated that Tan IIA may play important roles in anticancer treatment. However, its effects on the most common primary malignant bone tumor, osteosarcoma (OS), are unknown. Here, we report that Tan IIA may be an efficacious anti-OS drug as it could induce cell apoptosis and inhibit proliferation, migration, and invasion *in vitro*. Furthermore, we detected possible molecular mechanisms for Tan IIA activity by examining the levels of Bcl-2, Bax expression, and caspase-3, caspase-8, and caspase-9 activities that regulate apoptosis, matrix metalloproteinase (MMP)-2, and MMP-9 involved in regulating migration and invasion. In this study, we find that Tan IIA inhibits proliferation and induces apoptosis in the human OS cell line MG-63 in a time-dependent and dose-dependent manner. In addition, Tan IIA displays inhibitory activity on OS cell migration and invasion. Mechanistic studies have shown that Tan IIA activity is mediated by caspase activation. Tan IIA was also shown to reduce antiapoptotic Bcl-2, MMP-2, and MMP-9 levels, whereas it increased proapoptotic Bax levels. These data suggest that Tan IIA may be a novel, efficient candidate agent for OS treatment. *Anti-Cancer Drugs* 23:212–219 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Department of Orthopedics, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, People's Republic of China

Correspondence to Lin Cai, PhD, Department of Orthopedics, Zhongnan Hospital of Wuhan University, No.169th Donghu Road, Wuchang District, Wuhan 430071, People's Republic of China
Tel: +86 276 781 3116; fax: +86 276 781 2892;
e-mail: guke3116@yahoo.com.cn

*Yi Zhang, Ren-xiong Wei, Xiao-bin Zhu contributed equally to this study.

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Introduction

Osteosarcoma (OS) is the most common malignant bone tumor, and occurs mainly in adolescents and young adults [1]. Despite significant clinical improvements in the use of combinational chemotherapy and surgery over the past decades, current neoadjuvant chemotherapy outcome for OS remains unsatisfactory in the presence of metastases [2–4]. There is clearly an ongoing need for more effective treatments. In recent years, the preventive use of traditional Chinese medicines in cancer therapy has been widely accepted as a promising option against the development and recurrence of malignant diseases. Increasing evidence has shown that traditional Chinese medicines can yield potential drugs for the prevention or the treatment of various cancers, including OS [5–7].

Danshen (*Salvia miltiorrhiza* Bunge) has been widely used in the practice of traditional Chinese medicine since ancient times to treat various diseases including heart disease, hepatitis, and cerebrovascular diseases, with minimal side effects [8]. Danshen and its derivatives, which are clinically administered to certain cancer patients in China [9], have been proven to be effective as an adjuvant following chemotherapy. It had been shown that tanshinone IIA (Tan IIA, CAS Number: 568-72-9, Fig. 1a), the most abundant and structurally representative ingredient from Danshen, significantly inhibited growth and induced apoptosis in different types of cancers including breast cancer [10,11], hepatocellular carcinoma [12,13], leukemia [9,14,15], and colon cancer [16].

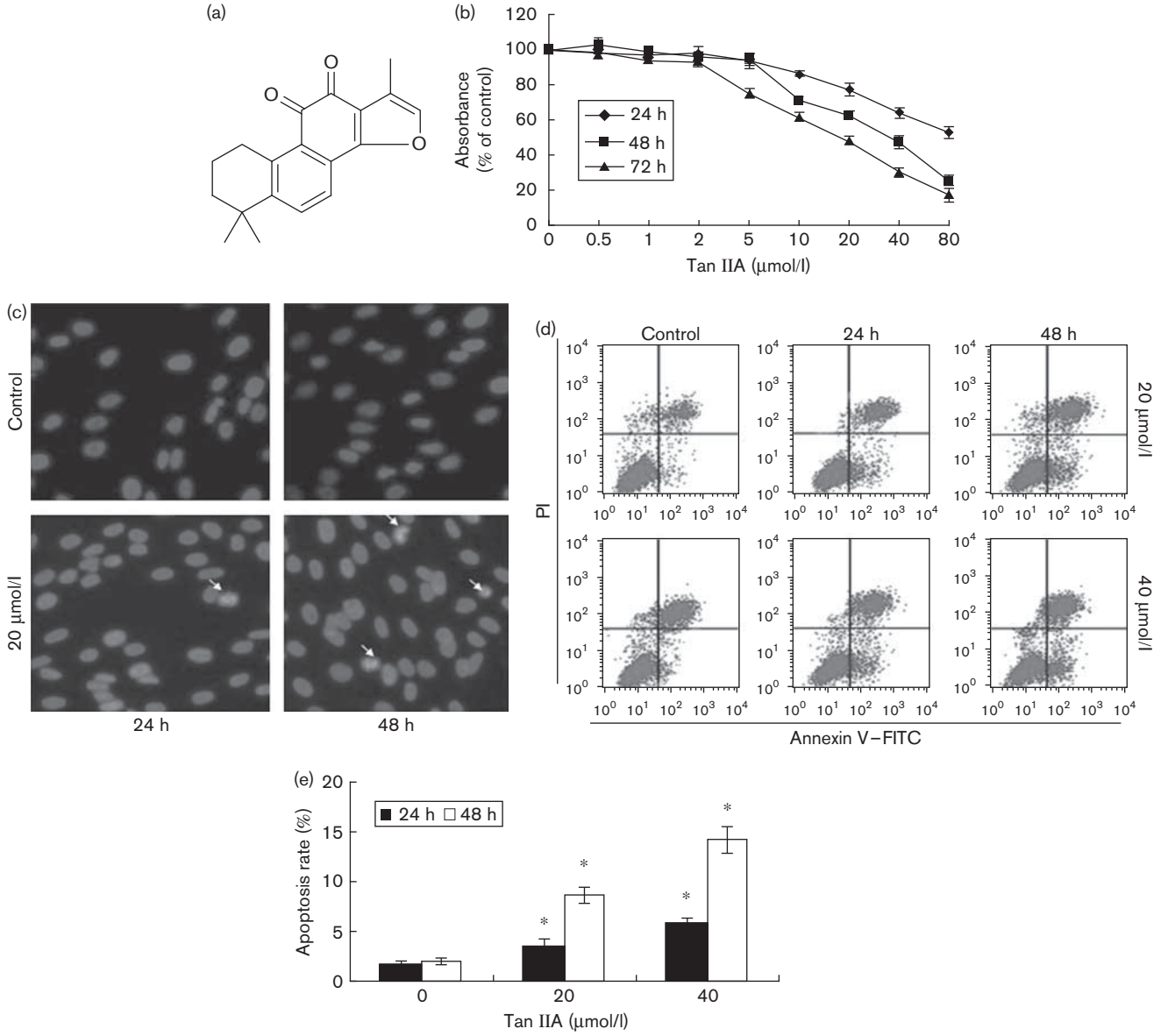
Here, we investigated the effects of Tan IIA on the human OS cell line MG-63 using in-vitro assays for proliferation, apoptosis, migration, and invasion. Possible molecular mechanisms of Tan IIA were detected by examining the levels of matrix metalloproteinase (MMP)-2 and MMP-9, which are involved in regulating migration and invasion. The levels of Bcl-2, Bax, and caspase-3, caspase-8, and caspase-9 activities were shown to mediate apoptosis. Therefore, we infer that Tan IIA may be a potential candidate for novel anti-OS drug development.

Materials and methods

Materials and reagents

Tan IIA was obtained from the Institute of Traditional Chinese Medicine, Zelang Pharmaceutical Co. (Nanjing, China), at 98% purity as verified by high-performance liquid chromatography. Lysis buffer, antibodies against Bcl-2, Bax, MMP-2, MMP-9, and secondary antibodies were purchased from Santa Cruz (Santa Cruz, California,

Fig. 1



Tanshinone IIA (Tan IIA) inhibits MG-63 cell proliferation and induces apoptosis in a time-dependent and dose-dependent manner. (a) Molecular structure of Tan IIA ($C_{19}H_{18}O_3$, molecular weight = 294.3). (b) Tan IIA inhibits MG-63 cell proliferation. Cells were treated with various doses of Tan IIA for 24, 48, or 72 h and cell proliferation was analyzed using CCK-8 cell proliferation assay kits. The results are representative of three independent experiments. (c) Tan IIA treatment induces DNA condensation and fragmentation. White arrows indicate cells with apoptotic bodies (magnification: × 100). Pictures are representative of two independent experiments. (d, e) Apoptotic cells, FITC (+) and propidium iodide (PI) (−), caused by Tan IIA were analyzed using flow cytometry. Data are expressed as mean ± SEM of three independent experiments (*$P < 0.05$ vs. control group).

USA). All other chemicals were purchased from Sigma (St Louis, Missouri, USA) unless otherwise stated.

Cell culture

The human OS MG-63 cell line was obtained from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in MEM/EBSS (Hyclone, Logan, Utah, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin in a humidified incubator with 5% CO_2 at 37°C.

Cell proliferation assays

Cells were seeded in 96-well microplates and allowed to attach overnight. Tan IIA was added at different concentrations at 0 h. Then, cell proliferation was

measured at 24, 48, and 72 h using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. The CCK-8 assay was carried out at 450 nm using a microplate reader (TECAN SPECTRA, Mannedorf, Switzerland), and the relative inhibition rate was calculated from the absorbance (A value) obtained from dose–response data using the following equation [17]:

$$\text{Inhibition ratio}\,(\%) = [(A_{\text{Control}} - A_{\text{Treated}}) / A_{\text{Control}}] \times 100\,\%.$$

IC_{50} was assumed to be the concentration that caused 50% inhibition of cell viability and calculated using the linear regression method.

DNA staining

Cells incubated with or without 20 μmol/l Tan IIA were stained with Hoechst 33258 (Sigma) according to the manufacturer's instructions. The stained cells were examined under a fluorescence microscope (Nikon, Tokyo, Japan) at ×100 magnification.

Cell apoptosis assays

Cell apoptosis was detected using the annexin V—FITC/PI apoptosis detection kit (KeyGen, Nanjing, China). In brief, 1×10^5 treated cells were harvested following trypsinization and centrifugation. Cells were washed with PBS and resuspended in 500 μl binding buffer, to which 5 μl annexin V—FITC was added; after a 5-min incubation, 5 μl propidium iodide was added, followed by a 5-min incubation in the dark. Apoptotic cells were analyzed by flow cytometry (FC500 System; Beckman Coulter, Fullerton, California, USA).

Caspase activity assays

Cytoplasmic protein was extracted from 2×10^6 MG-63 cells treated with 20 or 40 μmol/l Tan IIA for 0, 12, and 24 h. Cells were washed twice with ice-cold PBS and lysed on ice in 50 μl of chilled cell lysis buffer for 10 min. The lysate was centrifuged at 15000*g* for 10 min and the supernatant was retained for further testing. The protein concentration was assessed using a spectrophotometer (NanoDrop Technologies, Montchanin, Delaware, USA), and samples were diluted to 100 μg protein per 50 μl cell lysis buffer for each assay. Next, 5 μl of substrate was added and the samples were incubated at 37°C for 1 h. Caspase-3, caspase-8, and caspase-9 activities were quantified using a microplate reader at a wavelength of 405 nm. The fold increase was calculated as a ratio of the absorbance values from treated and untreated samples. All experiments were performed in triplicate.

Scratch migration assay

For scratch migration assays *in vitro*, 5×10^5 MG-63 cells were seeded onto six-well plates. After a 24-h incubation, the confluent monolayer was scratched with a pipette tip to create a cell-free area and then incubated with 2% serum-supplemented media with or without 40 μmol/l Tan IIA for an additional 24 h. Cell migration was measured by counting cells that migrated from the wound edge into the cell-free area. Cells were photographed using a light microscope equipped with phase-contrast optics.

Cell invasion assay

Cell invasion was determined using modified chambers containing Transwell polycarbonate membranes (Costar, Massachusetts, Cambridge, USA). Briefly, cell culture chambers were coated with 80 μl Matrigel (Sigma, St Louis, Missouri, USA). MG-63 cells were seeded into the upper chamber at 5×10^5 cells per well using an MEM serum-free medium. Meanwhile, 40 μmol/l of Tan IIA was added for the treatment group and untreated MG-63 cells served as the experimental control. After 24 h of incubation, cells inside the chamber were wiped off with a cotton swab. Cells that invaded through the Matrigel and the polycarbonate membrane to the lower surface were stained with 0.2% crystal violet. We randomly selected five fields of vision for each chamber and counted the cell number under a microscope [18].

Quantitative real-time-PCR analysis

For mRNA expression analysis, 5×10^5 MG-63 cells were grown overnight and subsequently treated with various doses of Tan IIA for 24, 48, and 72 h. RNA was isolated using Trizol (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using 1 μg of total RNA and the First-Strand Synthesis Kit (Fermentas, Flamborough, Ontario, Canada) according to the manufacturer's protocol. The reaction was incubated for 60 min at 42°C, and then it was terminated by heating at 70°C for 7 min. The primer sets used for PCR amplification are shown in Table 1.

Table 1 Primer sets used for quantitative real-time–PCR

Gene	Primer sets	OMIM ID
Bcl-2		
Forward	5′-CGCCCTGTGGATGACTGAGTA-3′	151430
Reverse	5′-GGGCCGTACAGTTCCACAAAG-3′	
Bax		
Forward	5′-CCCTTTTGCTTCAGGGTTTCATCCA-3′	600040
Reverse	5′-CTTGAGACACTCGCTCAGCTTCTTG-3′	
MMP-2		
Forward	5′-CTCAGATCCGTGGTGAGATCT-3′	120360
Reverse	5′-CTTTGGTTCTCCAGCTTCAGG-3′	
MMP-9		
Forward	5′-AAGTGGCACCACCACAACAT-3′	120361
Reverse	5′-TTTCCCATCAGCATTGCCGT-3′	
β-actin		
Forward	5′-CTGGAGCATGCCCGTATTTA-3′	102630
Reverse	5′-TTTGGTCTTGCCACTTTT- CC-3′	

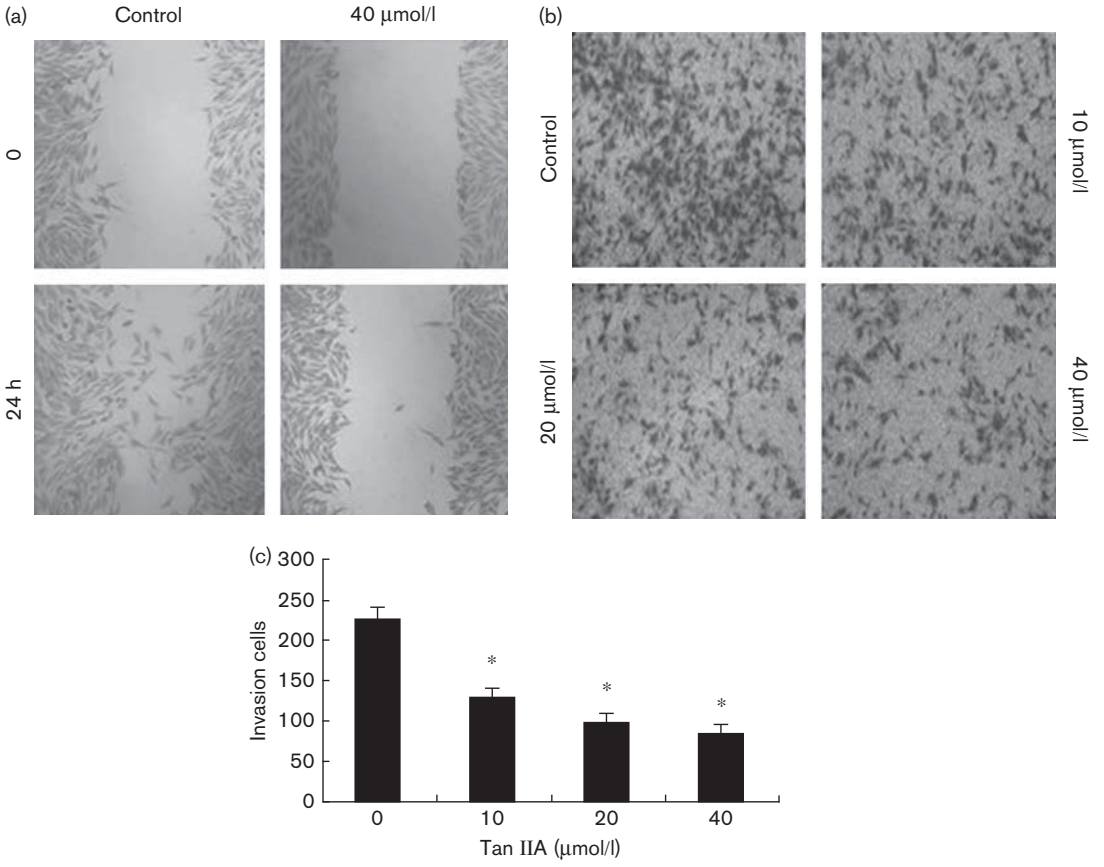
OMIM, Online Mendelian Inheritance in Man; MMP, matrix metalloproteinase.

Quantitative real-time-PCR was performed using the Mx-3000P PCR system (Stratagene, La Jolla, California, USA) and the SYBR Premix Ex Taq real-time PCR kit (TaKaRa, Kyoto, Japan) according to the manufacturer's instructions. Initial denaturation was performed at 95°C for 1 min, followed by 35 cycles of denaturation (20 s, 95°C), annealing at 60°C (for MMP-2), 58°C (for MMP-9, Bcl-2, and Bax), 59°C (for β-actin), and extension (45 s, 72°C); a final extension was performed at 72°C for 10 min. The cycle threshold (CT) value is the fractional cycle number at which fluorescence signals pass the fixed threshold set by the Mx-3000 P PCR System software (Stratagene). The relative expression of mRNA was calculated on the basis of the relative quantification method ($2^{-\Delta\Delta CT}$) and normalized to β-actin [19].

Western blotting

Cells were washed with PBS and lysed in cell lysis buffer. The lysate was centrifuged at 15 000*g* at 4°C for 10 min. The supernatant was reserved and separated on SDS-PAGE, and then transferred onto a nitrocellulose membrane (Millipore, Bedford, Massachusetts, USA) in a standard transfer buffer. After blocking with 5% nonfat milk (blocking solution) at room temperature, the membrane was incubated with primary antibodies diluted in blocking solution overnight at 4°C. The following antibodies and dilutions were used: anti-MMP-2 (1 : 200), anti-MMP-9 (1 : 300), anti-Bcl-2 (1 : 150), anti-Bax (1 : 200), and anti-β-actin (1 : 1000). After washing the blot in TBST three times, secondary antibodies conjugated with HRP were applied for 2 h at room temperature. After extensive washing in TBST, specific immunoreactivity was visualized using enhanced chemiluminescence (ECL) Western Blotting Substrate (Pierce, Rockford, Illinois, USA) and ECL system (Fusion FX7; Vilber Lourmat, Torcy, France). The relative quantitative expression was analyzed on the basis of the intensity ratio of the target protein to that of β-actin. The band intensity was determined using Quantity One Software (Bio-Rad, Hercules, California, USA).

Fig. 2



Tanshinone IIA (Tan IIA) inhibits MG-63 cell migration and invasion. (a) Tan IIA inhibits the migration of MG-63 cells. Cells were treated with 40 μmol/l of Tan IIA. The results are representative of three independent experiments (magnification: ×40). (b, c) Tan IIA inhibits cell migration in a dose-dependent manner. Photos show invasive cells after staining with 0.2% crystal violet under a phase-contrast microscope (magnification: ×40). Data are expressed as mean ± SEM and represents three independent experiments (*$P<0.05$ vs. the control group).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using Statistical Product and Service Solutions (SPSS, Chicago, Illinois, USA) Vision 17.0. Results were considered statistically significant only if the P value was less than 0.05 using a two-tailed paired Student's t-test.

Results

Tanshinone IIA inhibited the proliferation of MG-63 cells

MG-63 cells were treated with various doses of Tan IIA for 24, 48, and 72 h. The results of the CCK-8 cell proliferation assays (Fig. 1b) showed that the IC_{50} dose for Tan IIA was 32.3 μmol/l for the 48-h time point, whereas it was 17.0 μmol/l for the 72-h time point. A statistically significant decrease ($P < 0.05$) in cell proliferation was observed following 10 μmol/l treatment of MG-63 cells for all the time points. At the 48-h time point, we observed that many cells began to detach from the culture surface after treatment with 40 μmol/l or higher Tan IIA. These results suggested that Tan IIA inhibited MG-63 cell proliferation in a time-dependent and dose-dependent manner.

Tanshinone IIA induced apoptosis in MG-63 cells

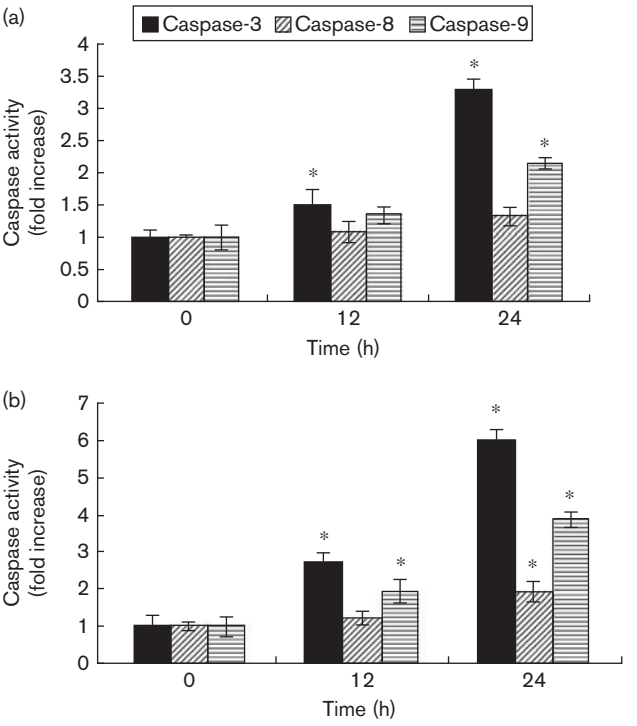
As the effects of Tan IIA on cell proliferation were time and dose dependent, we assayed for the signs of cellular apoptosis following Tan IIA treatment by analyzing its effects on DNA condensation using Hoechst 33258 staining. As shown in Fig. 1c, nuclear chromosomal condensation and fragmentation were detected in MG-63 cells after treatment with 20 μmol/l of Tan IIA for 24 and 48 h.

We then quantified cellular apoptosis following 20 and 40 μmol/l Tan IIA treatment using flow cytometry at 24 and 48 h, respectively (Fig. 1d and e). There were few apoptotic cells when incubated without Tan IIA, but apoptosis increased in a time-dependent and dose-dependent manner in Tan IIA-treated cells. Quantitative analysis revealed a significant increase ($P < 0.05$) in apoptosis following Tan IIA treatment. These results confirmed that Tan IIA inhibited cell proliferation by inducing apoptosis.

Tanshinone IIA inhibited cell migration and invasion *in vitro*

As OS cell migration and invasion were associated with metastatic potential [6], we used a cell scratch migration assay and an invasion assay to corroborate the Tan IIA effects. We observed that there were significantly fewer migrating cells after treatment with Tan IIA than with untreated cells (Fig. 2a). We also observed a statistically significant reduction in invasive properties for Tan IIA-treated MG-63 cells in a dose-dependent manner (Fig. 2b and c). These two observations emphasized the inhibitive role of Tan IIA in MG-63 cell migration and invasion.

Fig. 3



Tanshinone IIA increases caspase activity in a time-dependent and dose-dependent manner. (a, b) shows Tanshinone IIA increases caspase-3, caspase-8, and caspase-9 activities to varying degrees in 12 and 24 h. All data are expressed as mean ± SEM of three independent experiments (*$P < 0.05$ vs. the control group).

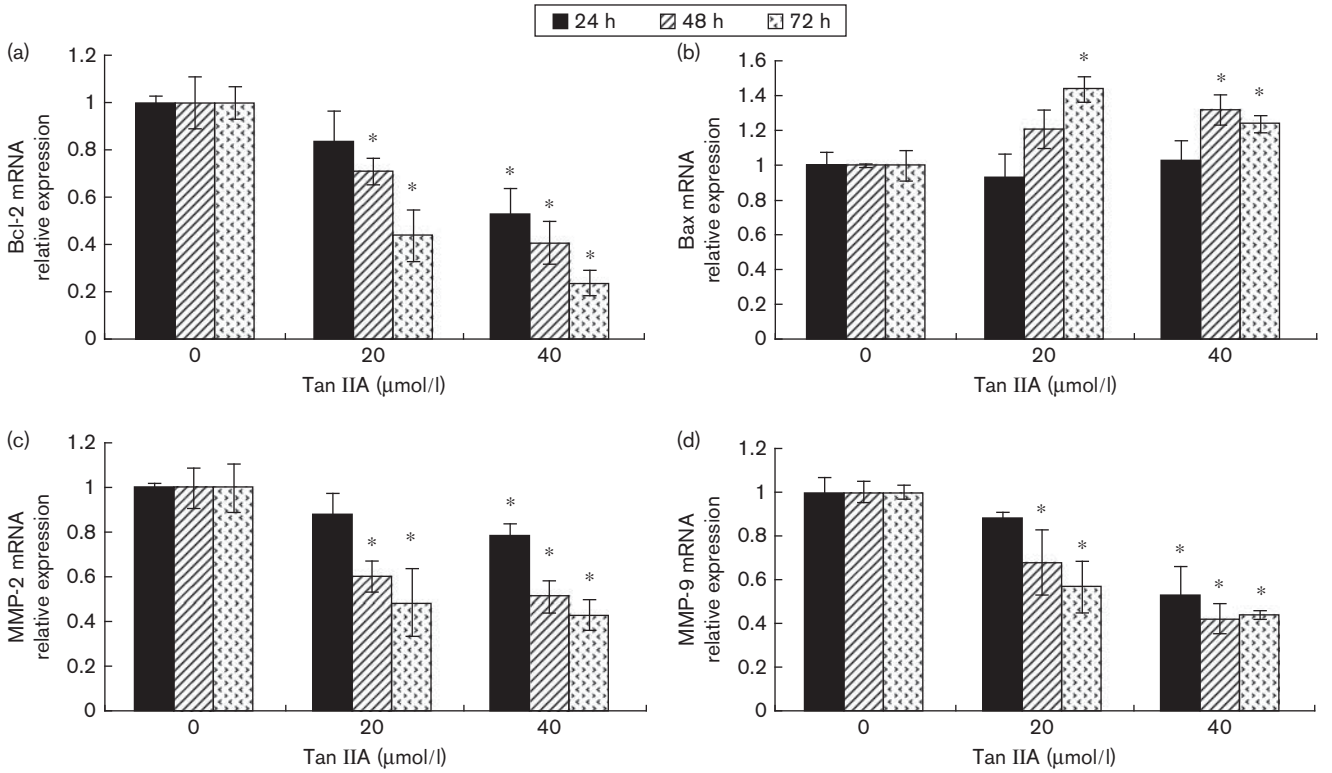
Tan IIA increased caspase activation

We then conducted a mechanistic study to determine how Tan IIA activated apoptosis. Caspase activity assays showed that the activities of caspase-3, caspase-8, and caspase-9 increased after exposure to Tan IIA in a time-dependent manner (Fig. 3a and b). Although the activities of caspase-3 and caspase-9 were significantly increased ($P < 0.05$), only MG-63 cells incubated with 40 μmol/l of Tan IIA for 24 h showed significantly higher ($P < 0.05$) caspase-8 activity than the controls. These findings indicated that Tan IIA induced apoptosis in MG-63 cells by caspase-3, caspase-8, and caspase-9 activations, especially caspase-3 and caspase-9 in 24 h.

Tan IIA altered mRNA and protein expression involved in apoptosis and invasion

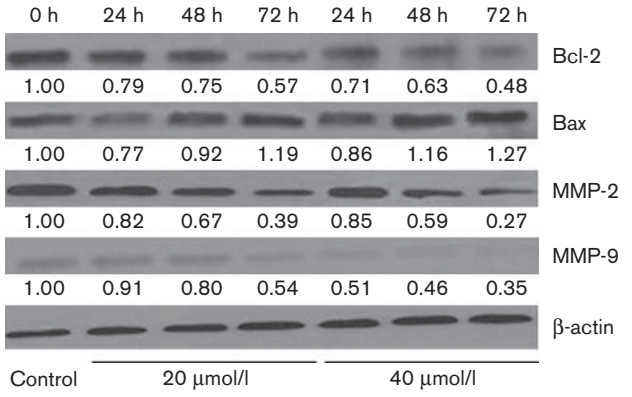
To verify that Tan IIA altered the expression of apoptosis-related proteins in MG-63 cells, we examined the mRNA and protein expressions of apoptotic molecules from the Bcl-2 family using quantitative real-time-PCR and western blot analysis. Figures 4a, b and 5 showed that Tan IIA decreased Bcl-2 expression and increased Bax expression for both mRNAs and proteins in a time-dependent and dose-dependent manner.

Fig. 4



Tanshinone IIA (Tan IIA) increases the expression of mRNAs involved in apoptosis and invasion. (a–d) shows time-dependent and dose-dependent effects on the relative mRNA expression of Bcl-2, Bax, MMP-2, and MMP-9 in MG-63 cells. The relative mRNA expression was calculated as $2^{-\Delta\Delta CT}$ with β-actin as the normalization control. All data are expressed as mean ± SEM of three independent experiments (*$P < 0.05$ vs. the control group). MMP, matrix metalloproteinase.

Fig. 5



Time-dependent and dose-dependent effects of tanshinone IIA on Bcl-2, Bax, MMP-2, and MMP-9 protein levels in MG-63 cells after treatment. Cells were treated with 20 and 40 μmol/l of tanshinone IIA for 24, 48, and 72 h and target proteins were examined by western blot. The relative densitometric values are listed below each row while β-actin was the loading control. MMP, matrix metalloproteinase.

MMPs play a central role in extracellular matrix degradation to promote OS invasiveness and metastasis [20], and this was considered as a possible mechanism of action of Tan IIA. Therefore, we determined the mRNA and protein expression levels of MMP-2 and MMP-9 in cells treated with Tan IIA at different time points using the methods described earlier. Our data revealed that the MMP-2 and MMP-9 levels were markedly decreased in MG-63 cells treated with Tan IIA and the effect was independent of the incubation time or the dosage (Figs 4c and d, and 5).

Discussion

Danshen and its derivatives have been used for centuries without significant adverse effects, and so their active ingredients may be safe candidates for the prevention and/or the treatment of cancer. Previous data demonstrated that Tan IIA could inhibit OS by downregulating prohibitin, an inner membrane-bound chaperone in mitochondrion, as a candidate molecular target, while

concurrently translocating it from the nucleolus to the cytoplasm [21,22]. Recent studies have shown that Tan IIA and its derivatives were protective agents against cardiotoxicity and nephropathy induced by doxorubicin, which is an important drug in neoadjuvant chemotherapy for OS [23,24].

In this study, we found that Tan IIA significantly inhibited MG-63 cell proliferation and induced apoptosis in a time-dependent and dose-dependent manner. CCK-8 assays quantitatively showed that Tan IIA strongly inhibited MG-63 cell proliferation. As shown in Fig. 1b, a significant effect could be observed after 72 h of Tan IIA treatment at a dose of 5 μmol/l, which is quite low. Furthermore, we found that Tan IIA induced apoptosis significantly in MG-63 cells as assessed by DNA staining (Fig. 1c) and flow cytometry analysis (Fig. 1d and e).

To clarify the molecular mechanisms of Tan IIA in apoptosis, we determined the protein levels of the Bcl-2 family including antiapoptotic molecules such as Bcl-2 and proapoptotic molecules such as Bax. We also evaluated caspase-3, caspase-8, and caspase-9 activities because they play important roles in the induction, transduction, and amplification of intracellular apoptotic signals [25]. Our results suggested that Tan IIA may inhibit the growth of human MG-63 cells *in vitro* by inducing Bax activation, while concurrently suppressing Bcl-2 expression.

Caspase activation is central for apoptosis induction, and is considered a reasonable criterion for distinguishing apoptosis from necrosis [26]. Caspase-3, which is the most important factor in apoptosis [27], showed a marked increase following Tan IIA treatment. Caspase-8 and caspase-9, which triggered extrinsic and intrinsic caspase signaling cascades, respectively [27], showed increased activity following Tan IIA treatment to varying degrees. In summary, Tan IIA markedly increased the activities of caspase-3, caspase-8, and caspase-9 in MG-63 cells. This is consistent with the increased rate of apoptosis in a time-dependent and dose-dependent manner. Consequently, we concluded that Tan IIA induces apoptosis in MG-63 cells primarily through the mitochondrial apoptotic pathway rather than the death receptor pathway.

For most patients with OS, lung metastasis is the main cause of death. About 15% of OS patients who present distant metastases at the time of diagnosis (90% lung, 10% others) will die within 5 years. The essential steps for metastases, invasion, and migration require the action of tumor-associated proteases that dissolve the surrounding tumor matrix and basement membrane [6,28]. MMPs play an important role in the degradation of the extracellular matrix, which is a special characteristic of tumor invasion, metastasis in OS. Among previously reported human MMPs, MMP-2 (also known as gelatinase A) and MMP-9 (gelatinase B), are considered particularly good targets for anticancer drugs because they degrade gelatins [29], which are major components of basement membrane.

Our results suggest that Tan IIA partially suppressed the mRNA and protein expression of MMP-2 and MMP-9, inhibited OS cell invasion though Matrigel, and reduced MG-63 migration activity. These results provide new insights into the potential use of Tan IIA in controlling OS invasion and metastasis. We verified that Tan IIA has antiinvasive and antimetastatic activity through the inhibition of MMP-2 and MMP-9 expression in human MG-63 cells in a time-dependent and dose-dependent manner.

In conclusion, the results from our study provided experimental evidence to support Tan IIA as a novel, efficient, and safe candidate agent for the chemoprevention and/or the treatment of OS progression. We showed that this effect is mediated by apoptosis induction and inhibition of their proliferation and metastatic potential.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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