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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Mechanistic studies of the apoptosis induced by the macrocyclic natural product tetrandrine in MGC 803 cells

Sai-Qi Wang[1] · Hong-Lin Hou[1] · Liang-Yu Bie[1] · Cai-Yun Nie[1] · Luo-Nan Wang[2] · Shaobing Gao[3] · Ting-Ting Hu[1] · Xiao-Bing Chen[1]

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Abstract

Tetrandrine (Tet) is a macrocyclic tetrahydroisoquinoline alkaloid isolated from *Stephania tetrandra* S. Moore along with other Chinese and Japanese herbs. It has been used extensively for the treatment of silicosis, autoimmune disorders, inflammatory diseases and cardiovascular diseases. Of late, Tet has garnered increasing attention for its anticancer activity and its efficacy to reverse chemoresistance. In this study, we evaluated the cytotoxicity of Tet on MGC 803 gastric cancer cells using the methyl thiazolyl tetrazolium (MTT) assay. In addition, apoptosis and cell cycle were analyzed through flow cytometry, mitochondrial membrane potential (MMP) was measured using fluorescence microscopy and migration of cells was determined by transwell assay. It was observed that Tet efficiently inhibited the proliferation of MGC 803 cells in a concentration-dependent and time-dependent manner and reduced the number of colonies at low concentrations. It induced apoptosis through mitochondrial dysfunction, which may be related with upregulated Bcl-2-associated X protein (Bax), and downregulated of B cell lymphoma 2 (Bcl-2). On the other hand, Tet blocked the cell cycle at Gap 2 (G2)/mitosis (M) phase, which was associated with the upregulation of $p21^{CIP1/WAF1}$. Furthermore, Tet elevated the intracellular reactive oxygen species (ROS) level and suppressed the migration of MGC 803 cells. Altogether, we demonstrated that Tet inhibited the proliferation and migration of gastric cancer MGC 803 cells and thus might be a potential drug candidate for the treatment of gastric cancer.

Keywords Tetrandrine · Apoptosis · Cell cycle arrest · Migration

These authors contributed equally: Sai-Qi Wang, Hong-Lin Hou.

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✉ Xiao-Bing Chen
2290773710@qq.com

1 Department of Oncology, The Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, 450008 Zhengzhou, Henan, China

2 Department of Oncology, The First Affiliated Hospital of Xinxiang Medical University, 453100 Xinxiang, Henan, China

3 Central Laboratory, The Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, 450008 Zhengzhou, Henan, China

Introduction

Natural products have been recognized as a rich source of new drugs. More than 100 natural products (e.g. Taxol) and their structural analogs, especially the antibiotics, have found applications for the clinical treatment of numerous diseases (Butler et al. 2014; Butler 2005; Yu et al. 2016). Due to their structural diversity, complexity and richness in stereogenic centers, they allow for covering unexplored biologically relevant chemical space (Harvey et al. 2015; Lachance et al. 2012). Of particular interest are macrocyclics with 12-membered or more-membered ring architectures, which possess increased conformational flexibility, stereochemical complexity and favorable pharmacokinetic properties (Driggers et al. 2008). These attractive characteristics render macrocyclic compounds promising as starting points for designing new drugs and also attract attention from organic chemists to synthesize macrocyclic analogs (Marsault and Peterson 2011; Wessjohann et al. 2005). Numerous macrocyclic drugs are currently used

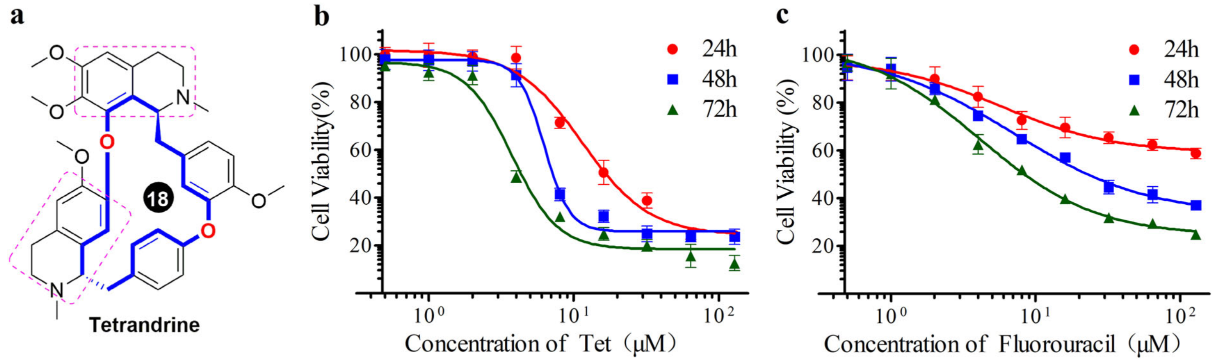

Fig. 1 Tet reduced the viability of MGC 803 cells in a concentration-dependent and time-dependent manner. **a** Chemical structure of tetrandrine (Tet). **b** MGC 803 cells were treated with the specified concentrations of Tet for 24, 48, and 72 h. **c** MGC 803 cells were treated with the specified concentrations of fluorouracil for 24, 48, and 72 h. Cell viability was determined by MTT assay. Data are expressed as means ± SD of three independent experiments performed in triplicate

clinically, for example, vancomycin is widely used for its therapeutic efficacy against the gram-positive bacterial infections (Rybak et al. 2008).

Tetrandrine (Fig. 1a), a macrocyclic tetrahydroisoquinoline alkaloid, is isolated from the root tubers of *Stephania tetrandra* S. Moore. Tet has been traditionally used in China for the clinical treatment of silicosis, autoimmune disorders, inflammatory pulmonary diseases, cardiovascular diseases and hypertension (Liu et al. 2016). The characteristic tetrahydroisoquinoline-incorporated oxygen-tethered 18-membered macrocyclic ring system (highlighted in bold in Fig. 1a) makes Tet an interesting candidate for developing new therapeutic agents. Moreover, earlier investigations established that Tet inhibits proliferation and induces apoptosis of a number of cancer cell lines including human leukemia, prostate cancer (Liu et al. 2015), lung cancer (Lee et al. 2002), breast cancer (Xing et al. 2013), glioma (Wu et al. 2014), and hepatoma (Kuo and Lin 2003), as well as cancer cells isolated from patients (Liu et al. 2008). Additionally, Tet inhibits angiogenesis (Gao et al. 2013) and metastasis, enhances the sensitivity of cancer cells to chemotherapy (Wan et al. 2013; Mei et al. 2015; Chaudhary and Vishwanatha 2014) and radiotherapy (Sun et al. 2007), and reverses multidrug resistance (Sun and Wink 2014; Fu et al. 2002). However, the antitumor activity of Tet on human gastric cancer cells and its potential mechanisms of actions were relatively unexplored. Herein, we investigated the antiproliferative activity of Tet against MGC 803 cells as well as the underlying mechanisms.

Materials and methods

Cell viability assay

The human gastric cancer cell line MGC 803 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were harvested with trypsin, resuspended in the medium and seeded in a 96-well plate. After incubation at 37 °C for 24 h, the medium containing different concentrations of Tet (Meilun Biology Technology, Dalian, China. Purity: HPLC ≥ 98%, supplementary 1–2) was added into the designated wells. Following 72 h of incubation at 37 °C, 20 μL of methyl thiazolyl tetrazolium (MTT) solution (5 mg/mL) was added to each well. After 4 h of incubation, 150 μL of dimethyl sulfoxide (DMSO) was added into each well to dissolve the formazan crystals. Finally, the absorbance was determined at 570 nm using a microplate reader (BioTek Instruments, Inc., USA) and the concentrations required to inhibit 50% of growth (IC_{50}) were calculated by means of the SPSS software 17.0. In this assay, fluorouracil (Fluorouracil Injection, Xudonghaipu pharmaceutical co., LTD, Shanghai, China) was used as the positive control.

Hoechst 33342 staining assay

MGC 803 cells were seeded in a 6-well plate and cultured overnight. After treatment with Tet for 24 h, the medium was discarded and phosphate-buffered saline (PBS) was used to wash each well thrice. Then, the cells were stained by Hoechst 33342 (5 μg/mL; Beyotmie Biotechnology, Haimen, China) with 0.2% Triton X-100 for 20 min at room temperature in the dark. Finally, the images were captured with a Nikon inverted fluorescent microscope (Eclipse Ti-s, Tokyo, Japan).

Colony formation assay

MGC 803 cells were seeded in 6-well plates at a density of 1000 cells per well and the medium containing Tet was refreshed once in every 2 days. After incubation at 37 °C for one week, the plates were washed with PBS, fixed with methanol for 30 min and the cells were stained with 1%

crystal violet for 30 min. Finally, the colonies were enumerated by means of the Image J software.

Apoptosis assay

Cells were plated in a 6-well plate at a density of 2×10^5 cells per well and incubated at 37 °C for 24 h. Different concentrations of Tet were added into each well and incubated for 24 h, following which the cells were washed twice with ice-cold PBS and resuspended in 200 µL of binding buffer containing 0.5 mg/mL fluorescein isothiocyanate (FITC)-Annexin V and 0.5 mg/mL propidium iodide (PI). Subsequently, the cells were incubated for 30 min in the dark and the fluorescence was measured via Flow cytometry (Acurri C6, Becton Dickinson, Franklin Lakes, USA).

Cell cycle assay

After treatment with Tet for 24 h, the cells were fixed and permeabilized with 70% ethanol overnight at 4 °C. Subsequently, the cells were rinsed twice with ice-cold PBS and stained with PI (50 µg/mL) solution containing RNaseA (50 µg/mL) for 30 min in the dark. Finally, the fluorescence was measured by flow cytometry and the data were analyzed with FlowJo software.

Measurement of the mitochondrial membrane potential (MMP)

Cells were seeded into the wells and treated with Tet for 24 h. Then, they were washed with PBS and dyed with the serum-free medium containing 5 µg/mL 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) for 20 min at 37 °C in the dark. After washing thrice, the fluorescence was immediately analyzed using the Nikon fluorescent microscope (Eclipse Ti-s, Tokyo, Japan) (Wang et al. 2017).

Measurement of intracellular ROS

The cell-permeant probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was employed to monitor the levels of ROS. In brief, the cells were seeded in the wells of a 6-well plate at a density of 2×10^5 cells/well and treated with Tet for 12 h. Subsequently, the cells were incubated with 10 µM of DCFH-DA in PBS for 30 min in the dark, washed with PBS and further subjected to flow cytometric analysis.

Transwell assay

Three thousand cells were suspended in 200 µL of medium containing 2% fetal bovine serum (FBS) and diverse concentrations of Tet were added to the upper chambers of the transwell plate, while 400 µL of medium containing 20% FBS was added to the bottom chambers. After incubation at 37 °C for 24 h, the upper chambers were rinsed with PBS before being fixed with 4% paraformaldehyde for 20 min. Subsequently, the cells were stained with Hoechst 33442 (5 µg/mL) for 20 min at room temperature in the dark. After washing with PBS twice, the migrated cells were enumerated by means of the high-content screening system (Thermo Scientific, Waltham, USA).

Western blot analysis

At the end of Tet treatment, the cells were harvested and washed with ice-cold PBS. After draining the PBS, ice-cold lysis buffer with protease inhibitors was added, the cells were agitated for 30 min and then centrifuged at 12,000×*g* for 15 min at 4 °C. Subsequently, the supernatant was aspirated and placed in a fresh tube placed on ice to determine the concentration of protein through the bicinchoninic acid (BCA) assay. Equal amounts of total cell lysates were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose (NC) membranes. Then, the membranes were blocked in 5% milk in Tris-buffered saline with Tween 20 (TBST, 10 mM Tris-HCl, 150 mM NaCl and 0.1% tween 20, pH 7.4) at room temperature for 1 h and incubated with the primary antibody at 4 °C overnight. After that, the membranes were washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Zhongshan Golden Bridge, Beijing, China) at room temperature for 2 h. Finally, the membranes were rinsed and the protein-antibody complex was detected through the enhanced chemiluminescence detection system and the data were accessed using Image J software (Wang et al. 2016).

Statistical analysis

Each assay was performed at least in triplicate. All data are presented as the mean ± standard deviation (SD) and were analyzed using the SPSS 17.0 software.

Statistical significance was evaluated by means of one-way analysis of variance (ANOVA) and the differences were considered to be statistically significant when $P < 0.05$ (Ma et al. 2015; Wang et al. 2016).

Results

Tet inhibited the growth of gastric cancer MGC 803 cells

Initially, the antiproliferative effect of Tet on MGC 803 cells was investigated using the MTT assay. As shown in

Fig. 1b, Tet inhibited the growth of MGC 803 cells in a concentration-dependent and time-dependent manner. After 24, 48, and 72 h of Tet treatment, the IC_{50} values were determined to be 11.44 ± 3.23, 6.24 ± 1.32, and 3.75 ± 1.42 μM, respectively. As the positive control, the IC_{50} values of fluorouracil at 24 h was more than 128 μM, and the IC_{50} value at 48 and 72 h were 29.84 ± 3.09 and 13.17 ± 1.64 μM (Fig.1c). Next, the colony formation assay was carried out to study the effect of Tet on the ability of a single cell to grow into a colony, in which MGC 803 cells were treated with low concentrations (0.25, 0.5, 1.0 μM) that were not cytotoxic. As shown in Fig. 2, after treatment with Tet, the number of the cell colonies decreased sharply.

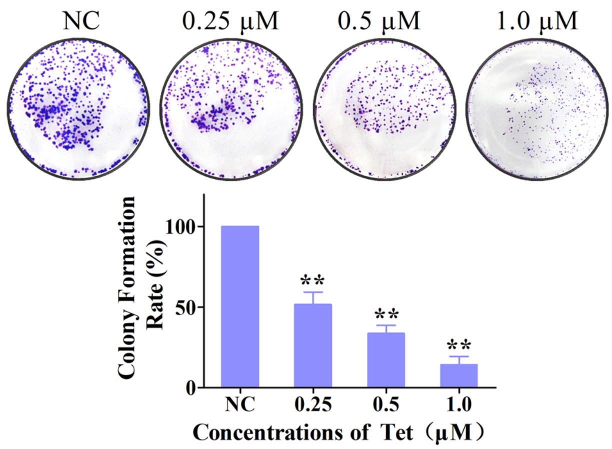

Fig. 2 Tet suppressed the colony-formation of MGC 803 cells at low concentrations. Cells were treated with Tet for 7 days before the colonies were counted. Data are expressed as means ± SD of three independent experiments performed in triplicate. $^{**}P < 0.01$ versus negative control (NC)

Tet triggered apoptosis in MGC 803 cells

Inducing apoptosis is an effective anticancer strategy since uncontrolled proliferation is a hallmark of cancer cells (Hanahan and Weinberg 2011). Consequently, the apoptosis of MGC 803 cells was detected using the Annexin V-FITC/PI double staining through flow cytometry following the treatment with different concentrations of Tet. In Fig. 3a, after incubation for 24 h with 5, 10, and 20 μM of Tet, the early apoptosis rates were about 15.8 ± 3.7, 25.5 ± 2.9, and 44.3 ± 5.4%, respectively ($P < 0.05$). Meanwhile, Tet also resulted in late apoptosis of MGC 803 cells ($P < 0.05$). Typical features such as karyopycnosis are often associated

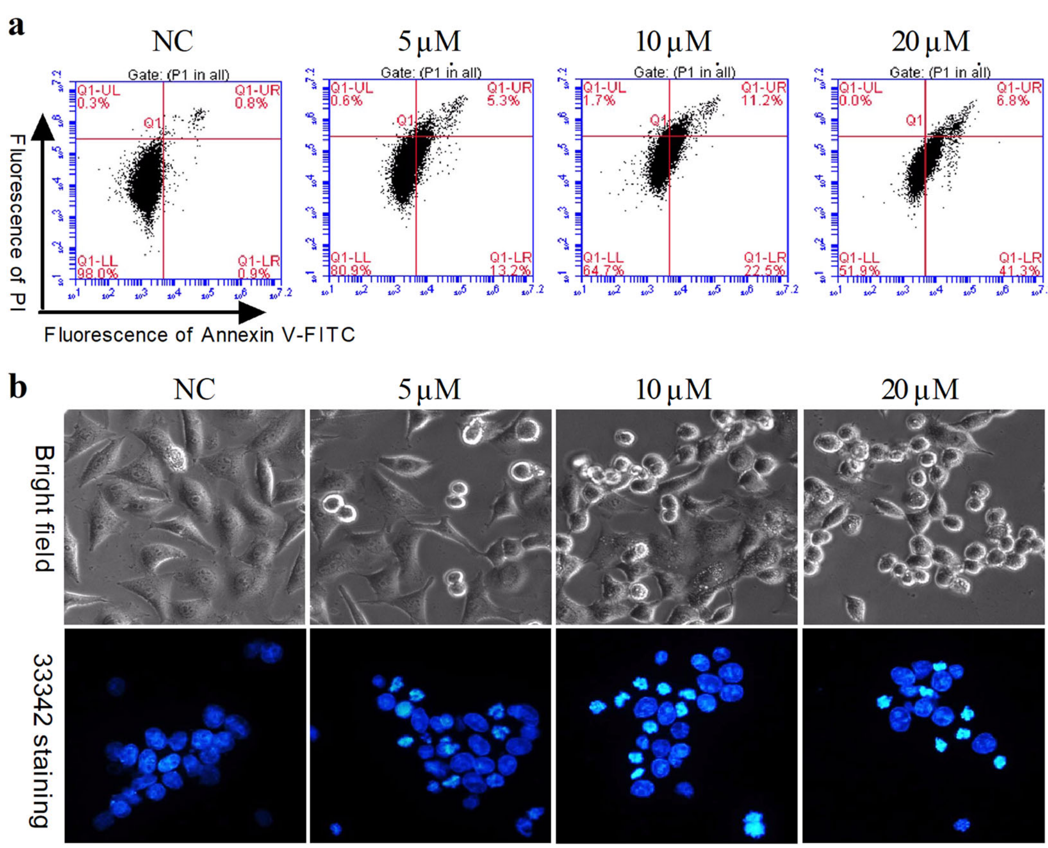

Fig. 3 Tet triggered apoptosis in MGC 803 cells after treatment for 24 h. **a** Cells were stained by Annexin V-FITC/PI and analyzed by flow cytometry. **b** Morphological changes were observed by phase-contrast microscopy and fluorescent microscopy after staining with Hoechst 33342

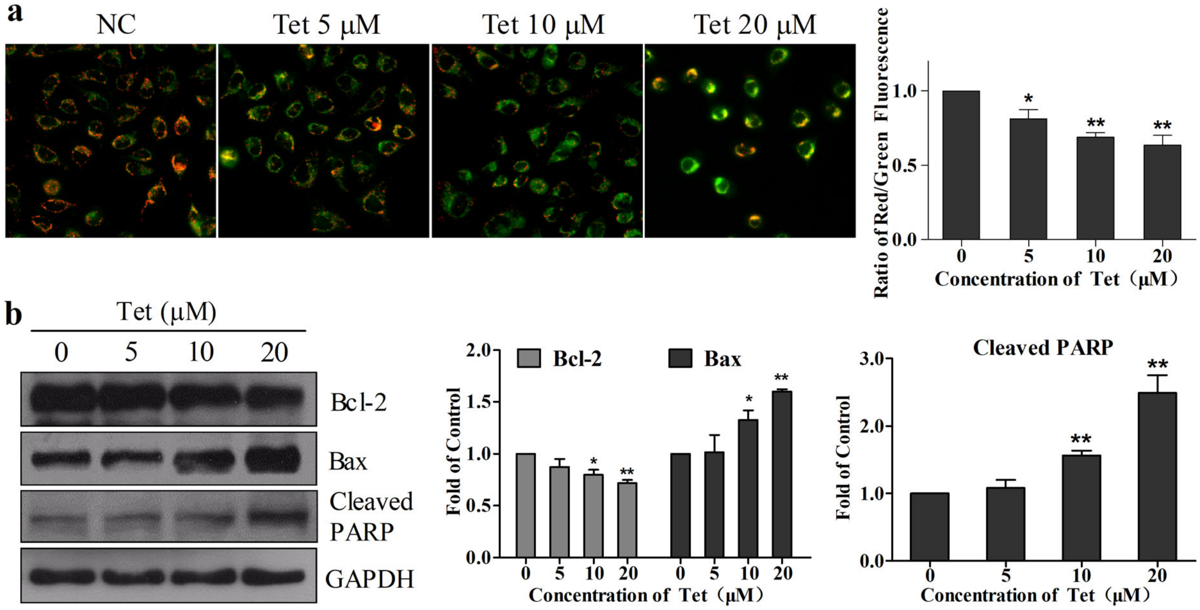

Fig. 4 Tet induced the mitochondrial dysfunction through regulating the Bcl-2 family. **a** Tet induced the loss of mitochondrial membrane potential of MGC 803 cells after 24 h of treatment. **b** Western blot analysis of Bcl-2, Bax and cleaved PARP in MGC 803 cells after treatment with Tet for 24 h. Data are expressed as means ± SD of three independent experiments performed in triplicate. $*P < 0.05$, $**P < 0.01$ versus NC

with apoptosis. After the treatment with Tet, MGC 803 cells became round in shape (Fig. 3b) and their nuclei shrank (bright blue, Fig. 3b).

Tet reduced the mitochondrial membrane potential (MMP) of MGC 803 cells

Apoptosis typically proceeds through the death receptor and the mitochondrial pathways (Qin et al. 2013). In the mitochondrial pathway, the members of the Bcl-2 family play a vital role through their proapoptotic or antiapoptotic activities (Sola et al. 2013; Kroemer et al. 2007; Chao and Korsmeyer 1998; Nunez and Clarke 1994) by maintaining the normal MMP. To explore the underlying mechanisms, JC-1 staining assay was utilized to estimate the MMP. After treatment with Tet, the intensity of red fluorescence (J-aggregates) progressively decreased, whereas the intensity of green fluorescence (monomer) increased correspondingly (Fig. 4a), signifying that Tet induced the loss of MMP. Western blot analysis (Fig. 4b) revealed that Tet upregulated the expression of proapoptotic proteins including Bax, and simultaneously down-regulated the expression of anti-apoptotic protein Bcl-2 in MGC 803 cells. Relative to the negative control, the Bax/Bcl-2 ratio in the treatment group was about 2.1-fold higher, which was ascribed to the increase in mitochondrial permeability. On the other hand, Tet augmented the level of cleaved poly ADP-ribose polymerase (PARP). These results suggested that Tet triggers apoptosis via the mitochondrial pathway by disrupting the balance between the proapoptotic and antiapoptotic members of the Bcl-2 family.

Tet induced cycle arrest at the G2/M phase in MGC 803 cells

Dysfunctional cell cycle progression is another hallmark of cancer and is considered to be a vital target in cancer therapy. To examine the effect of Tet on cell cycle, we analyzed the cell cycle distribution through PI staining after Tet treatment. As presented in Fig. 5a, the percentage of cells in the G2/M phase was 19.91% for the negative control. After 24 h of Tet treatment, the percentages of cells in the G2/M phase were increased in a concentration-dependent manner, accompanying a corresponding decline of cells in the synthesis (S) and Gap 0(G0)/Gap 1(G1) phases. All these results indicated that Tet induced cell cycle arrest in the G2/M phase. Subsequently, Western blot analysis was performed to explore the underlying mechanisms. As seen in Fig. 5b, after treatment with Tet for 24 h, $p21^{CIP1/WAF1}$ was upregulated in a concentration-dependent manner, indicating that Tet may block cell cycle by mediating the expression of the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$.

Tet induced excessive ROS in MGC 803 cells

ROS is considered as a double-edged sword in cancer. On one hand, low levels of ROS facilitate cancer cell survival

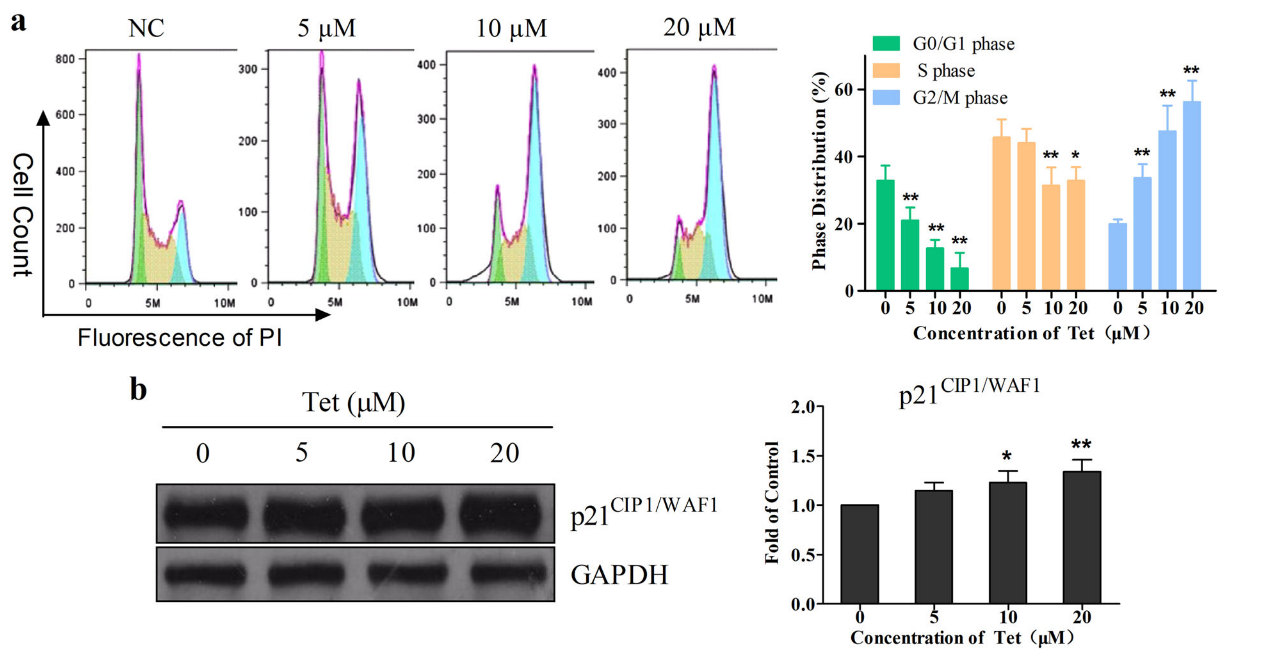

Fig. 5 Tet induced the G2/M phase cell cycle arrest through up-regulating p21$^{CIP1/WAF1}$ in MGC 803 cells. **a** Flow cytometry analysis of MGC 803 cells after treatment with Tet for 24 h. **b** Western blot analysis of p21$^{CIP1/WAF1}$ in MGC 803 cells after treatment with Tet for 24 h. Data are expressed as means ± SD of three independent experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$ versus NC

(Irani et al. 1997), on the other hand, high levels of ROS suppress tumor growth (Ramsey and Sharpless 2006; Takahashi et al. 2006). It is well-known that cancer cells possess higher ROS levels relative to normal cells. Therefore, both ROS-elevating and ROS-eliminating strategies have been developed for the treatment of cancer. Interestingly, Tet has been reported to stimulate ROS generation in several types of cancer cells. Consequently, we investigated whether Tet can elevate ROS levels in the MGC 803 cells. After 12 h of Tet treatment, MGC 803 cells were stained by DCFH-DA. Intracellular ROS level was then analyzed through flow cytometry. As shown in Fig. 6, compared with negative control (1.0%), Tet at 5, 10, 20 μM produced 9.2, 14.6, and 15.9% of right-shift of the green fluorescence (the fluorescence of DCF) respectively, signifying the generation of superfluous ROS in MGC 803 cells.

Tet inhibited the migration of MGC 803 cells

Tumor metastasis, caused by the migration of cancer cells, often results in the failure of cancer therapy and ultimately leads to cancer-related deaths. The transwell assay was employed to assess the impact of Tet on cell migration by means of the high-content screening. It was observed that the number of MGC 803 cells passing through the membrane in the upper chamber gradually decreased in a concentration-dependent manner (Fig. 7), signifying that Tet suppressed the migration of MGC 803 cells.

Discussion

Cancer is a complex disease which is characterized by the abnormal proliferation and differentiation of cells. As the second most common cause of death globally, cancer proves to be a colossal threat to public health and life. Stomach cancer is the fifth most common malignancy after cancers of the lung, breast, colorectum, and prostate and it is the third leading cause of cancer-related mortality in both sexes worldwide.

Over 100 natural products including Taxanes (Sakamoto et al. 2009; Hernandez-Vargas et al. 2007) have been extensively used for the clinical treatment of different types of diseases. Natural products and their derivatives represent over one-third of the new molecular entities approved by the Food and Drug Administration (FDA), and nearly one-half of them are derived from plants. Moreover, in recent years, more and more natural products from food and traditional Chinese medicine have been established as effective anticancer candidates, such as Tetrandrine (Tet) (Qiu et al. 2014), curcumin (Guan et al. 2016; Wu et al. 2016), apigenin (Shukla et al. 2015), and capsaicin (Chen et al. 2016).

Tet, a bisbenzylisoquinoline alkaloid isolated from the traditional Chinese medicine *Stephania tetrandra* S. Moore, inhibits the growth (He et al. 2011) and metastasis (Liu et al. 2015; Xu et al. 2014) of several types of cancer cells. Moreover, Tet reverses multi-drug resistance and is

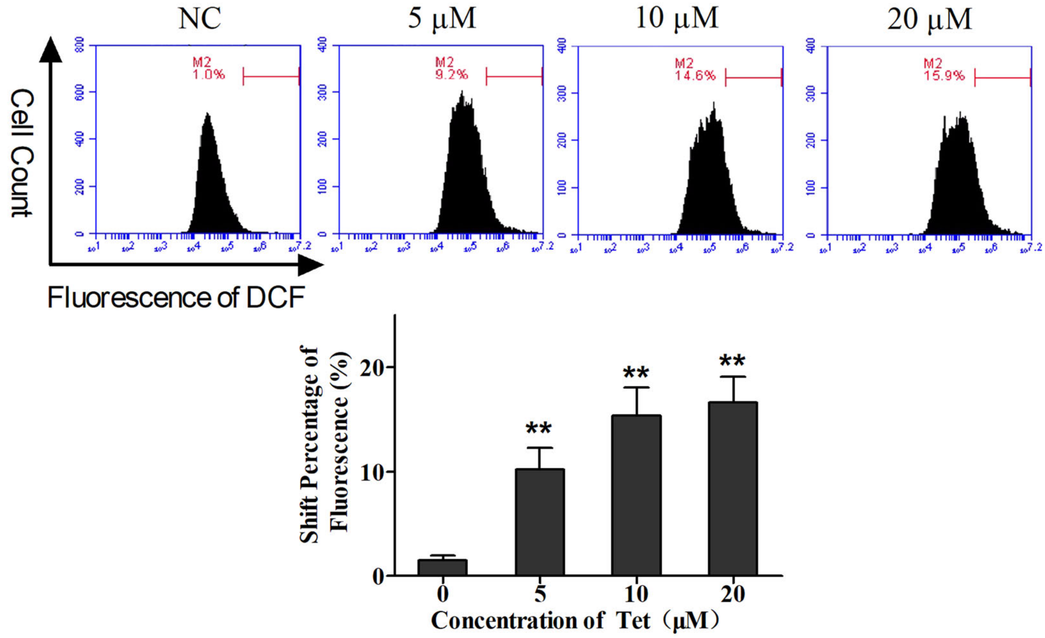

Fig. 6 Tet produced excessive intracellular ROS in MGC 803 cells. The level of intracellular ROS in MGC 803 cells was measured by flow cytometry after treatment with Tet for 12 h. Data are expressed as means ± SD of three independent experiments performed in triplicate. $^{**}P < 0.01$ versus negative control

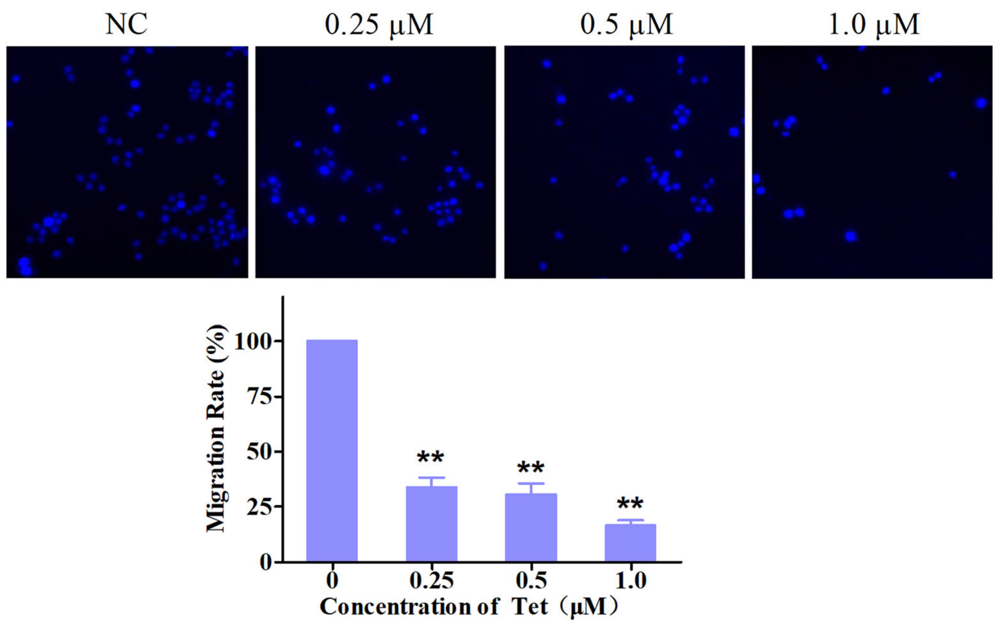

Fig. 7 Tet inhibited the migration of MGC 803 cells. After treatment with Tet for 24 h, the migration rate of MGC 803 cells was detected using the high-content analysis system. Data are expressed as means ± SD of three independent experiments performed in triplicate. $^{**}P < 0.01$ versus NC

considered a potential candidate for cancer chemotherapy (Liu et al. 2016).

In this study, we demonstrated the inhibitory activity of Tet against gastric cancer MGC 803 cells and explored the fundamental mechanisms. Tet inhibited the proliferation and colony formation ability of MGC 803 cells in a concentration-dependent manner. Through up-regulation of the pro-apoptotic member Bax and downregulation of the anti-apoptotic member Bcl-2, Tet treatment led to a decrease in MMP, followed by an increase in the mitochondrial membrane permeability and ultimately resulted in the apoptosis of MGC 803 cells. In addition to the induction of apoptosis, blocking the cell cycle progression is one more effective strategy for the treatment of carcinoma. In this study, we established that Tet induced cell cycle arrest at the G2/M phase through the up-regulation of p21 $^{CIP1/WAF1}$. On the other hand, ROS play dual roles in the progression and inhibition of cancer. The results of the current study suggested that the inhibitory effect of Tet in MGC 803 cells is dependent on the elevated intracellular levels of ROS. Tumor metastasis usually causes failure of treatment and results in mortality, and migration is a fundamental basis of metastasis. In the present study, we found that Tet inhibited the migration of MGC 803 cells in vitro.

Conclusions

In summary, Tet inhibited the proliferation of gastric cancer cells MGC 803 in a ROS-dependent manner and induced apoptosis via the intrinsic apoptotic pathway. In addition, it caused cell cycle arrest at the G2/M phase and inhibited the migration of MGC 803 cells. As a result, Tet may be considered as a potential candidate for the treatment of stomach cancers.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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