Tetrandrine suppresses proliferation, induces apoptosis, and inhibits migration and invasion in human prostate cancer cells

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Tetrandrine (TET), a traditional Chinese medicine, exerts remarkable anticancer activity on various cancer cells. However, little is known about the effect of TET on human prostate cancer cells, and the mechanism of function of TET on prostate cancer has not yet been elucidated. To investigate the effects of TET on the suppression of proliferation, induction of apoptosis, and inhibition of migration and invasion in human prostate cancer cell lines, DU145 and PC-3. Inhibition of growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and clone formation assay, and flow cytometry analysis was performed to detect the induction of apoptosis. Activation of poly (ADP-ribose) polymerase, caspase-3, Akt, phospho-Akt, Bcl-2, and Bax was analyzed by Western blotting. Wound healing assay and transwell migration assay were used to evaluate the effect of TET on migration and invasion of cancer cells. TET inhibited the growth of DU145 and PC-3 cells in a dose- and time-dependent manner. Cell cloning was inhibited in the presence of TET in DU145 and PC-3 cells. TET suppressed the migration of DU145 and PC-3 cells. Transwell invasion assay showed that TET significantly weakened invasion capacity of DU145 and PC-3 cells. TET exhibited strong inhibitory effect on proliferation, migration, and invasion of prostate cancer cells. In addition, TET induced apoptosis in a dose-dependent manner by activating the caspase cascade and inhibiting phosphoinositide 3-kinase-Akt signal pathway. The accumulating evidence suggests that TET could be a potential therapeutic candidate against prostate cancer in a clinical setting.

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INTRODUCTION
Prostate cancer is one of the most common genitourinary malignancies in males. According to the epidemiology statistics of global cancer from World Health Organization (GLOBOCAN 2008), the morbidity from prostate cancer in 2008 was ranked 2nd, accounting for 14% of all cancer types in males.¹ In the past few years, there is a significant upward trend in morbidity and mortality from prostate cancer in China. Its treatment has become a task of priority in modern medicine. At present, several methods are available to treat prostate cancer including endocrine therapy, surgery, radiation therapy, and chemotherapy. Among these methods, radical prostatectomy is still the most effective method for treating localized prostate cancer, but its clinical applications are restricted by tumor grading and staging. Therefore, there is a strong demand to develop new treatment regimens to suppress prostate cancer.

Tetrandrine (TET; International Union of Pure and Applied Chemistry name: 6,6',7,12-tetramethoxy-2,2'-dimethyl-1β-berbaman; molecular structural formula: C₃₅H₄₂N₂O₁₂; molecular weight: 622.74988 g mol⁻¹), a bisbenzylisoquinoline alkaloid, is purified from the roots of Stephania tetrandra (or hang fang ji) (family: Menispermaceae). TET has been used as an effective constituent to treat patients with hypertension, arrhythmia, arthritis, inflammation, even silicosis in traditional Chinese medicine.² There is accumulating evidence suggesting that TET presents anticancer effects against various cancers in vitro and to some extent, in vivo including leukemia,¹ hepatocellular carcinoma,³ gastric cancer,⁴ colon cancer,⁵ lung cancer,⁶ glioma,⁶,¹¹ nasopharyngeal carcinoma,¹² bladder cancer,¹³ and renal cell carcinoma.¹⁴

However, little is known about the effect of TET on human prostate cancer cells. And the mechanism of function of TET on prostate cancer has not yet been elucidated. Hence, this study investigated the effect of TET on the suppression of proliferation, induction of apoptosis, and inhibition of migration and invasion in human prostate cancer cell lines, DU145 and PC-3.

MATERIALS AND METHODS

Cell culture
Human prostate cancer DU145 and PC-3 cell lines were from the American Type Culture Collection (Manassas, VA, USA). Cells were

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cultured in Dulbecco’s Modified Eagle’s medium/1640 supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin, at 37°C, in humidified air containing 5% CO₂.

**Reagents**

Tetrandrine (C₁₅H₁₃N₄O₄) and 3-((4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TET was made into a fine suspension by dissolving the compound in 0.1 mol l⁻¹ HCl at a concentration of 25 mg ml⁻¹, which was diluted to desired concentrations in the medium immediately before each experiment. Antibodies against cleaved caspase-3, poly (ADP-ribose) polymerase (PARP), Akt, phospho-Akt, Bcl-2, Bax and peroxidase-conjugated secondary antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibody against glyceraldehyde-3-phosphate dehydrogenase was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) detection system was obtained from Amersham Life Science, Inc. (Arlington Heights, IL, USA).

**Cell viability assay**

Cell viability was assessed using the MTT assay. DU145 and PC-3 cells were incubated with or without TET for various durations, and incubated with 0.5 mg ml⁻¹ MTT at 37°C for 4 h. After incubation, cells were lysed with dimethyl sulfoxide. The absorbance was determined using a 96-well microplate reader at a wavelength of 490 nm (Bio-Rad, Hercules, CA, USA). The experiments were performed in triplicate.

**Flow cytometry analysis**

DU145 and PC-3 cells were exposed to different doses of TET (HCl, 2.5, 5.0, and 10.0 μmol l⁻¹) for 48 h. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer’s protocol. Apoptotic cells were then analyzed by flow cytometry (BD FACScan Flow Cytometer; BD Biosciences, San Jose, CA, USA). The representative data presented in this study were reproduced in three independent experiments.

**Clone formation assay**

Prostate cancer cell lines (DU145 and PC-3) were seeded onto six-well plates (1000 per well). When cells were adherent, diverse doses of TET or solvent control containing dilute HCl were added to each well. When the cell density in solvent control reached > 50 per cluster, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet (Beyotime, Shanghai, China). After washing with PBS again, cloning of the cells was visible with the naked eye, and cells were counted from five randomly selected fields with microscopy to evaluate the ability of cell migration. The experiments were performed in triplicates.

**Statistical analysis**

All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Statistical differences among the control and various treatment groups were compared using one-way analysis of variance, followed by Dunnett’s t-test for multiple comparisons. Student’s t-test (two-sided) was used for comparisons involving only two groups. P < 0.05 was considered statistically significant.

**RESULTS**

**Tetrandrine inhibits the growth of prostate cancer DU145 and PC-3 cells**

First, the growth inhibitory effects of TET on prostate cancer DU145 and PC-3 cells by the MTT assay and clone formation assay were investigated. As shown in Figure 1, TET inhibited the growth of DU145 and PC-3 cells in a dose- and time-dependent manner. Since TET treatment at 10, 15, 20, and 30 μmol l⁻¹ showed strong inhibitory effects on the proliferation of prostate cancer DU145 and PC-3 cells, TET at 2.5, 5.0, and 10.0 μmol l⁻¹ was chosen as the representative doses for the in vitro treatment in the subsequent studies. Meanwhile, cell cloning was inhibited in the presence of TET in DU145 and PC-3 cells (Figure 2).

**Tetrandrine induces apoptosis in prostate cancer DU145 and PC-3 cells**

Since a significant inhibitory effect of TET on prostate cancer DU145 and PC-3 cells was observed, whether TET could induce apoptosis in prostate cancer cells by annexin V and PI double staining was investigated. The effect of TET on the apoptosis of PC-3 cells, as detected by flow cytometry, is shown in Figure 3. The TET treatments at 2.5, 5, and 10 μmol l⁻¹ for 48 h resulted in 12.01%, 16.56%, and 69.24% of apoptotic cells, respectively, and the baseline apoptosis of the solvent control cells was 5.08% (P < 0.05). Similar effects were observed in DU145 cells (data...
not shown). These results indicated that TET could induce apoptosis in human prostate cancer DU145 and PC-3 cells in a dose-dependent manner. Moreover, as shown in Figure 4, TET could activate the cleavage of caspase-3 (17 kDa and 19 kDa) and PARP (89 kDa) in PC-3 cells in a dose-dependent manner, which indicated that TET triggered caspase cascade. Similar effects were observed in DU145 cells (Figure 4).

The phosphoinositide 3-kinase (PI3K)-Akt signal pathway is a critical pathway that regulates various biological processes including apoptosis, cell cycle, proliferation, etc., Western blotting was used to detect the expression of Akt-related protein, including Akt, phospho-Akt, Bcl-2, and Bax. The results showed that there is a significant concentration-dependent decrease of phosphorylated Akt and Bcl-2; Bax showed the opposite effect (Figure 4).

**Tetrandrine inhibits migration and invasion in prostate cancer DU145 and PC-3 cells**

To evaluate the impact of TET on cell migration, the wound healing assay and transwell migration assay were performed. It was found that TET suppressed the migration of DU145 and PC-3 cells (Figure 5). Consistent with this finding, transwell invasion assay showed that TET significantly weakened invasion capacity of DU145 cells (Figure 6). These results suggested that TET played an important role in inhibiting migration and invasion potential of human prostate cancer cells.

**DISCUSSION**

Tetrandrine has a long history of use in traditional Chinese medicine. There is accumulating evidence that TET partly exhibited antitumor activity against various cancer cells in vitro and vivo. However, whether TET could function on human prostate cancer cells remains under investigation. In this study, the effect of TET on human prostate cancer cells was demonstrated. It was observed that treatment with TET could trigger the inhibition of growth, induction of apoptosis, and inhibition of migration and invasion in DU145 and PC-3 cell lines; in addition, induction of apoptosis may be at least partially associated with the activation of caspase. To date, this is the first report about the effects of TET on human prostate cancer cells.

Tetrandrine elicited a concentration- and time-dependent inhibition of proliferation in DU145 and PC-3 cells. The drug concentration causing 50% inhibition of the desired activity (IC50)
value decreased from 18.87 to 5.84 μmol l⁻¹ for DU145 cells at different incubation times (PC-3 cells: from 6.95 to 1.42 μmol l⁻¹). The low value of IC₅₀ suggested that TET may play an important role as a growth inhibitor in prostate cancer. The availability of TET in vivo remains unknown. Hence, there is an urgent need to establish an animal model to confirm the inhibition of growth of TET on prostate cancer.

In the present study, TET showed a remarkable effect in inducing apoptosis in prostate cancer cells. Compared with DU145, PC-3 cells are more sensitive. As known, apoptosis consists of two diverse pathways: the extrinsic and the intrinsic pathways. Both pathways triggered caspase cascade, converged on caspase-3, and ultimately led to the morphologic characteristics of apoptosis such as DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing. This is the first study that reported that TET could induce activation of caspase-3 and cleavage of PARP on prostate cancer. Akt, as critical kinase, is implicated in cancer cell apoptosis by regulating downstream signal transduction cascade. The downregulation of phospho-Akt and Bcl-2 and the upregulation of Bax suggested that the apoptosis induced by TET on prostate cancer may be at least in part mediated by Akt pathway, although further investigation is required.

Additionally, wound healing assay and transwell assay were used to evaluate the function of TET as a migration or invasion inhibitor. Significant inhibition was observed with the treatment of TET. Thus, molecular mechanisms of TET in regulating the migration and invasion of cancer cells should be focused further.

The present study has several limitations. On the one hand, the lack of animal models is a shortcoming, and the function of TET on animal models should be considered in future studies. On the other hand, the function of TET on prostate cancer cells was alone observed. Whether TET could function on normal cells, or it has cancer specificity has not yet been elucidated. Hence, it is necessary to resolve these questions in further studies.

In summary, this study provided the first evidence that TET inhibited proliferation, migration, and invasion, and presented apoptotic capacity in human prostate cancer DU145 and PC-3 cells. The apoptosis induced by TET might be associated with PI3K-Akt pathway and activation of caspases. These findings suggest that TET may be used as a potential anticancer candidate against prostate cancer in clinical setting in the future.

**AUTHOR CONTRIBUTIONS**

WL and BK participated in the design of the trial, conducted the data acquisition, interpreted and statistically analyzed the data and drafted the manuscript. ZKM and XST designed the study and offered the study materials. CL, MY and JQC conducted the data acquisition, interpreted and statistically analyzed the data. LL and XYW conducted the data acquisition. DLH designed the study, interpreted the data and drafted the manuscript. All authors read and approved the final manuscript.

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