

Original Article

Parthenolide induces apoptosis and inhibits proliferation of human 786-O kidney cancer cells *in vitro*

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Abstract: Background: Clear cell renal carcinoma is one of the most common malignant tumors, with high incidence and mortality rates. Prognosis of RCC is poor, with 5-year survival rates less than 5%. PTL, a naturally occurring sesquiterpene lactone isolated from *Tanacetum parthenium*, can inhibit tumor cell growth and induce tumor cell apoptosis. Methods: MTT and apoptosis assays were performed to evaluate the function of PTL on human 786-O kidney cancer cells. Moreover, Western blotting analysis was performed to detect expression levels of cell apoptosis-related proteins. Results: PTL inhibits cell proliferation and induces apoptosis in human 786-O kidney cancer cells. Western blot analysis indicated a downregulation of phosphorylated survival, in a dose-dependent manner, in 786-O cells with PTL treatment. Regarding gene and protein levels, PTL treatment of 786-O cell-lines resulted in a significant reduction in Bcl-2 expression, but an increase in Bax protein, in a dose-dependent manner. Conclusion: PTL may display powerful activities against kidney cancer. Thus, it should be considered a potential candidate facilitating anticancer treatment.

Keywords: Kidney tumor, parthenolide, apoptosis

Introduction

Kidney cancer is one of the most common malignancies, accounting for 2%-3% of all malignant tumors. More than 80% of kidney cancers are clear cell renal carcinoma [1, 2]. Epidemiological data indicates a decade-long trend of gradually increasing kidney cancer incidence and mortality rates. Regarding the progression of regular physical examinations and imaging technology, asymptomatic renal carcinoma and occasional small kidney cancer detection rates have increased significantly in recent years. However, 20%-30% of patients with kidney cancer are in the late stages upon primary diagnosis with kidney cancer [3, 4]. Advanced kidney cancer is not sensitive to radiotherapy and chemotherapy, resulting in poor prognosis and 5-year survival rates less than 5% [5]. Therefore, exploration of new therapeutic modalities for kidney cancer has become an urgent problem for urologists. Cancer chemoprevention, defined as the use of natural or biological substances to slow or reverse cancer development,

can reduce incidence and mortality rates of malignant tumors. The current study attempted to find a kind of natural plant extract that can be applied for chemoprevention of kidney cancer, aiming to improve prognosis.

Parthenolide (PTL, C₁₅H₂₀O₃, molecular structural formula; see **Figure 1**) is a naturally occurring sesquiterpene lactone isolated from *Tanacetum parthenium* (feverfew). It is used for treatment of fevers, migraines, headaches, rheumatoid arthritis, and skin infections. It has remarkable sterilization, anti-inflammatory, antispasmodic, and pain relieving effects [6-8]. In recent years, one study found that PTL has significant antitumor properties with minimal adverse effects. PTL can inhibit tumor cell growth and induce tumor cell apoptosis in a number of ways, including oxidative stress, mitochondrial dysfunction, and caspase family cascade [9-11]. PTL has been used for several tumors, including glioblastomas and prostate, pancreatic, and colorectal cancer. However, underlying molecular mechanisms of the anti-metastasis

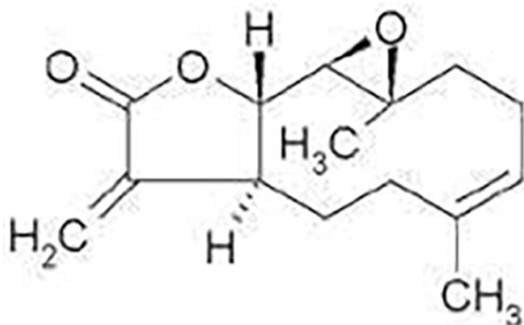


Figure 1. Chemical structure of PTL chemical structure of the sesquiterpene lactone 4 α , 5 β -epoxygermacra-1-(10), 11-(13)-dien-12, 6 α -olide (PTL) isolated from *T. parthenium*.

activity in renal cancer cells have not been elucidated. The current study investigated the effects of PTL on cell viability, proliferation, and apoptosis of human 786-O kidney cancer cells.

Materials and methods

Cells, reagents, and instruments

For this study, 786-O cells were obtained from the Lanzhou Branch of the Chinese Academy of Sciences (Cell source was clear; Cells grew well and had stable character). Dulbecco's modified Eagle's medium (DMEM), endotoxin-free bovine serum albumin (BSA), 0.25% trypsin mixture, fetal bovine serum (FBS), and TRIzol Reagent were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), MTT, and PTL were purchased from Sigma (St. Louis, MO, USA). PTL was dissolved with DMSO to a concentration of 10 mmol/L and stored in the dark at -80°C. A sample of DMSO was stored under the same conditions and used as the control treatment. PrimerScript™ Master Kit was obtained from TaKaRa (Osaka, Japan). SYBR Green PCR Mix was obtained from Toyobo (TaKaRa Biotechnology, Dalian, China). Antibodies against Bax, Bcl-2, and survivin were obtained from CST (Cell Signaling Technology, USA). β -actin monoclonal antibody was obtained from Santa Cruz (Santa Cruz, CA, USA). HRP-conjugated goat anti-rabbit and anti-mouse IgG were obtained from Beyotime Institute of Biotechnology (Shanghai, China).

Cell culturing

For culturing, the 786-O cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and strepto-

mycin (100 mg/mL). Cultured cells were used between passages 5 and 10. All cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Cell treatment

The cells were divided into five groups and treated with parthenolide at various concentrations, including 0, 5, 10, 15, and 20 μ M. After incubation for 24 hours or 48 hours, MTT assays were performed. In the control group, cells were treated with nothing. After 24 and 48 hours of treatment, cell viability was determined using MTT assay. The cells were divided into five groups: 1) Control group, cells treated with nothing; 2) 5 μ M, cells stimulated with 5 μ mol/L of PTL for 24 hours and 48 hours; 3) 10 μ M, cells stimulated with 10 μ mol/L of PTL for 24 hours and 48 hours; 4) 15 μ M, cells stimulated with 15 μ mol/L of PTL for 24 hours and 48 hours; and 5) 20 μ M, cells stimulated with 20 μ mol/L of PTL for 24 hours and 48 hours.

Cell viability assay

Cell viability was determined using MTT assays. Briefly, 786-O (8×10^3 cells/well) and 100 μ L of medium was seeded in 96-well plates. After 24 hours, the medium in each well was replaced with a medium containing different concentrations of parthenolide. The plate was incubated for 24 and 48 hours. Subsequently, 20 μ L of MTT (5 mg/mL) was added into each well. After incubation at 37°C for 4 hours, the supernatant was removed and 200 μ L of DMSO was added to each well. After the precipitate was fully dissolved, absorbance values were measured with the Microplate Reader (Thermo Fisher Scientific, Thermo®RMK3, USA). Data in each treatment group are expressed as percentages of controls.

Apoptosis assay

Cell apoptosis was evaluated using an apoptosis assay with Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA). Briefly, harvested cells were resuspended in 100 μ L of the binding buffer to achieve a concentration of 1×10^6 mL. Next, 5 μ L of Annexin V-FITC and 5 μ L of propidium iodide (PI, 20 μ g/mL) were added. The tubes were incubated for 15 minutes at room temperature in the dark.

Finally, the binding buffer (400 μ L) was added to each reaction tube and the cells were analyzed using flow cytometry. Data were analyzed by software (Becton, Dickinson and Company BD FACS Verse™).

RNA extraction and real-time RT-PCR analysis

The 786-O cells were harvested at indicated time points. Total RNA was extracted from the cells using TRIzol Reagent, according to manufacturer instructions. Single-strand cDNA was synthesized from 2 μ g of total RNA using a reverse-transcription kit (TaKaRa Biotechnology, Dalian, China). Quantitative analyses of Bax and Bcl-2 mRNA were carried out with a real-time system (Stratagene Mx3000P, La Jolla, USA), using the SYBR Green Master Mix real-time RT-PCR kit. Primers for Bcl-2 were forward 5'-TGGCCAGGGTCAGAGTAAA-3' and reverse 5'-TGGCCTCTCTTGCAGGAGTA-3'; for β -actin, forward 5'-GCACCACACCTTCTACAATGAG-3' and reverse 5'-ATAGCACAGCCTGGATAGCAAC-3'; and for Bax, forward 5'-TTGCTTCAGGGTTTCATCCA-3 and reverse 5'-AGACACTCGCTCAGCTTCTTG-3' [12]. Amplification was carried out in a total volume of 20 μ L and cycled 40 times after initial denaturation (95°C for 30 seconds) with the following parameters: 95°C for 5 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. After the completion of cycling, melting curve analysis was performed to establish the specificity of PCR products. Moreover, mRNA levels were obtained from the value of threshold cycle (Ct) for each specific gene and normalized against the Ct of β -actin. Relative changes in gene expression were calculated with the $2^{-\Delta\Delta C_t}$ method [13].

Western blotting analysis

The 786-O cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed for 20 minutes on ice, with a lysis buffer containing 20 mM of Tris-HCl pH 8, 137 mM of NaCl, 10% glycerol, 1% NP-40, 2 mM of EDTA, 5 mM of DL-Dithiothreitol (DTT), and 10 mM of Phenylmethanesulfonyl fluoride (PMSF). Following lysis, the lysates were centrifuged for 4 minutes at 10 800 g. The supernatant was collected in a fresh tube kept on ice. Protein concentrations were determined using a BCA assay. Proteins (40 μ g/lane) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferr-

ed onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Membranes were blocked with 3% fat-free milk in Tri Buffered Saline Tween-20 (TBST) (in mmol/L: Tris-HCl 20, NaCl 150, pH 7.5, 0.1% Tween 20) for 1 hour at 4°C under agitation. They were then washed three times in TBST and incubated with rabbit anti-human Bcl-2 antibody, rabbit anti-human Bax antibody, rabbit anti-human survivin antibody (1:1000 dilution, CST, USA), or β -actin monoclonal antibody (1:1000 dilution, Santa Cruz, CA, USA) for 18 hours at 4°C. After washing three times with TBST, membranes were incubated with horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000, Beyotime Institute of Biotechnology) for 1 hour at room temperature. Proteins were visualized using an enhanced chemiluminescence (ECLTM) Western blotting detection kit (Amersham Pharmacia Biotech). Results were recorded and analyzed using ChemiDoc molecular imaging systems and ImageJ software (ChemiDoc™ XRS+, BIO-RAD).

Statistical analysis

All experiments were repeated three times. Data are expressed as mean \pm SD from a representative experiment. SPSS 16.0 was used for statistical analysis. One-way analysis of variance and Dunnett's post-hoc tests were performed, analyzing the statistical significance of differences in all assays. $P < 0.05$ indicates statistical significance.

Results

PTL inhibits cell proliferation of renal cancer cells

Examining the effects of PTL on proliferation of cells, 786-O cells were exposed to different concentrations of PTL for 24 and 48 hours using MTT assays. Consequently, PTL inhibited proliferation of 786-O cells in a significant dose-dependent manner (* $P < 0.05$, ** $P < 0.01$) (**Figure 2**). The viability of 786-O cells was reduced to 93.21% and 53.61% after treatment with PTL for 24 and 48 hours, respectively. At the same level, with the role of the extension of time, survival rates of 786-O cells were significantly lower.

PTL induces apoptosis in renal cancer cells

Verifying and quantifying apoptotic cells induced by PTL, apoptosis assays were performed

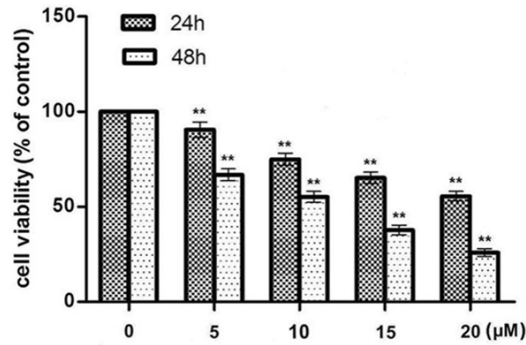


Figure 2. PTL suppressed the viability of 786-O cells. The effects of PTL on cell viability were measured by MTT assay. 786-O cells were treated with PTL for 24 and 48 hours. PTL significantly inhibited cell viability, in a dose-dependent manner. Results are represented as the mean \pm standard deviation (SD) of these experiments and the corresponding standard error. Significant versus control, * $P < 0.05$, ** $P < 0.01$.

to analyze apoptotic rates of PTL-treated cells with Annexin V-conjugated Alexa Fluor 488 and propidium iodide staining. Percentages of early and later apoptotic cells are shown in the lower right (LR) and upper right (UR) quadrant of the histograms, respectively (**Figure 3A-E**). The total percentage of apoptotic cells (UR+LR) increased from 5.7% in non-PTL-treated 786-O cells to 15.1%, 38.3%, 72.1%, and 86.6% in PTL-treated cells (5, 10, 15, and 20 μ M, respectively) after 48 hours (* $P < 0.05$, ** $P < 0.01$, **Figure 4F**). Treatment of 786-O cells with 0, 5, 10, 15, and 20 μ M of PTL for 48 hours induced apoptosis in these cells, in a dose-dependent manner. The significant induction of apoptosis after PTL treatment indicates the anti-cancer effects on 786-O cells.

PTL induces Bax and reduces Bcl-2 mRNA expression in 786-O cells

Determining whether PTL regulates Bax and Bcl-2 expression, 786-O cells were treated with various doses of palmitate (0, 5, 10, 5, and 20 μ M) for 24 and 48 hours. Expression of Bax and Bcl-2 mRNA was analyzed using real-time reverse transcription-polymerase chain reaction (RT-PCR). PTL induced a dose-dependent decrease in Bcl-2 mRNA expression in 786-O cells (* $P < 0.05$, ** $P < 0.01$, **Figure 4A** and **4B**). In contrast, PTL induced a dose-dependent increase in Bax mRNA expression in 786-O cells (* $P < 0.05$, ** $P < 0.01$, **Figure 4C** and **4D**).

Effects of PTL on expression of cell apoptosis-related proteins

Expression of pro-apoptotic protein Bax has been associated with increased apoptosis, while anti-apoptotic protein Bcl-2 has been associated with inhibition of apoptosis in target cells. After treatment with increased concentrations of PTL for 48 hours, expression of Bax and Bcl-2 in 786-O cells was detected. Levels of Bax were positively correlated with apoptosis rates of 786-O cells, while Bcl-2 was just the opposite (**Figure 5A, 5B**). The ratio of Bax/Bcl-2 protein levels is the decisive factor in transmitting the apoptosis signal. Comparing the intensity of their bands, it was found that Bax/Bcl-2 had dose-dependent effects on apoptosis (* $P < 0.05$, ** $P < 0.01$) (**Figure 5C**). With an elevated ratio of Bax/Bcl-2, expression of survivin was decreased (**Figure 5D**).

Discussion

PTL is a naturally occurring sesquiterpene lactone, a plant endemic in Lebanon known in Arabic as “Bahar ghishai” [14]. PTL belongs to the Anthemideae tribe of the Asteraceae (Compositae) family, comprising 25,000 species within three subfamilies and 17 tribes. Many of these are employed in a variety of medicinal applications [15, 16]. In recent years, the PTL molecule and several structurally related sesquiterpene lactone analogs have been extensively studied due to their potent anti-tumor and cytotoxic properties. PTL induces apoptosis in prostate cancer [17], pancreatic cancer [18] and colorectal cancers [19], as well as in Burkitt lymphoma [20] and leukemia cells [21]. However, it is ineffective in normal tissues [22]. PTL exerts antitumor effects in breast and lung cancer cells [23, 24]. Observed differences in the antitumor potential of PTL may be due to different physicochemical properties. Specifically, the lactone skeleton can exert effects via the Michael type addition with sulfhydryl groups of enzymes and other functional proteins (α -methylene- γ -lactones and epoxide structure) [25]. This may interfere with key biological processes in the cells, such as cell signal transduction, line grain with breathing, proliferation, and apoptosis [26]. Considering the apoptosis-promoting nature of PTL, the current study aimed to investigate its potential effects on renal cancer cells.

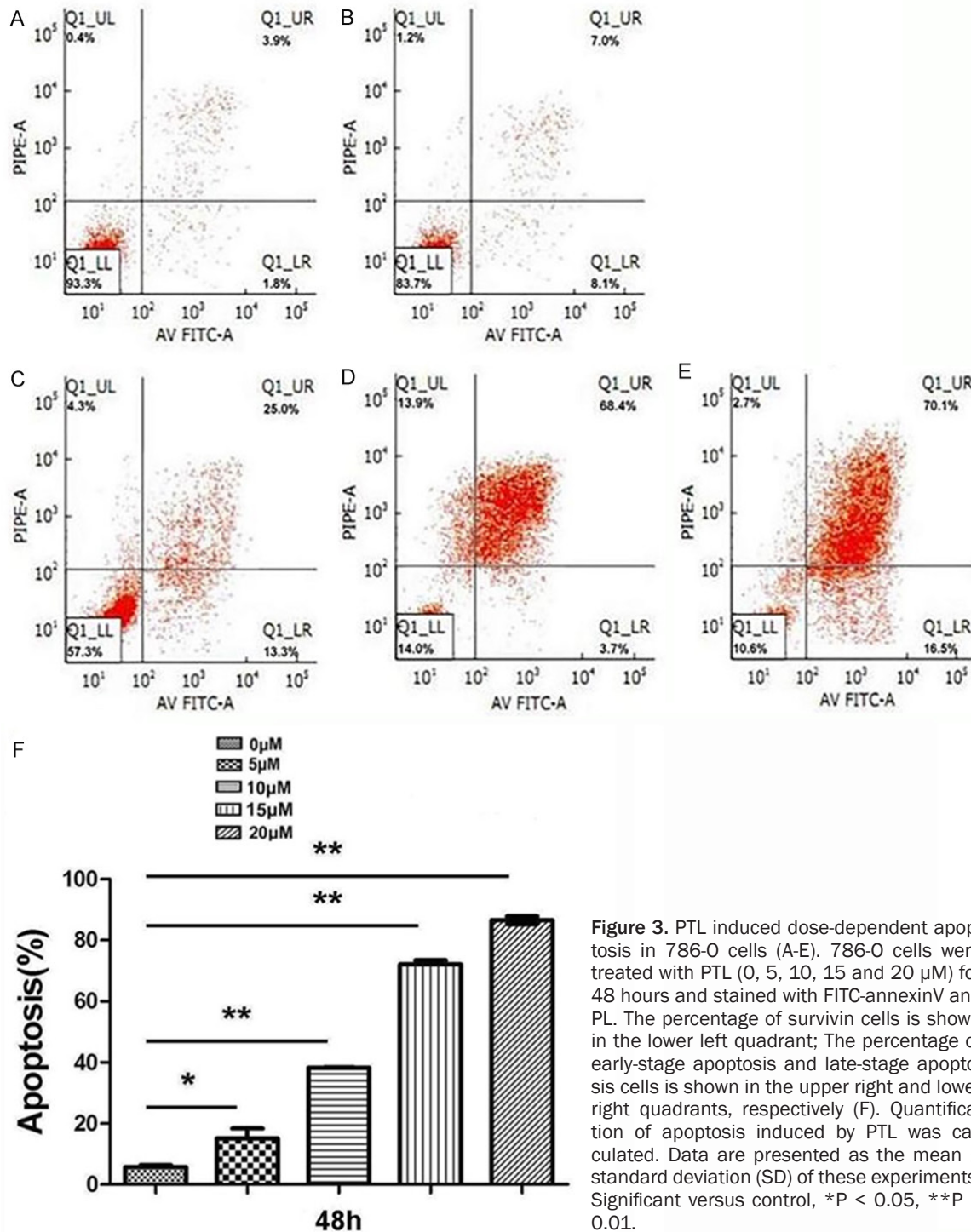


Figure 3. PTL induced dose-dependent apoptosis in 786-O cells (A-E). 786-O cells were treated with PTL (0, 5, 10, 15 and 20 μ M) for 48 hours and stained with FITC-annexinV and PL. The percentage of surviving cells is shown in the lower left quadrant; The percentage of early-stage apoptosis and late-stage apoptosis cells is shown in the upper right and lower right quadrants, respectively (F). Quantification of apoptosis induced by PTL was calculated. Data are presented as the mean \pm standard deviation (SD) of these experiments. Significant versus control, *P < 0.05, **P < 0.01.

Although PTL can promote apoptosis and anti-cancer effects, effects of PTL on 786-O cells have not been reported. The present study found that PTL could potentially inhibit proliferation and promote apoptosis in renal cancer cells *in vitro*. Furthermore, this study examined apoptotic markers after exposure to different

concentrations of PTL. Results indicate that PTL could induce apoptosis, downregulate pro-apoptotic survivin and Bax, and upregulate anti-apoptotic Bcl-2. It has been reported that cell-cycle arrest partly contributes to the growth suppression of PTL in cancer cells. Therefore, further experiments are necessary to deter-

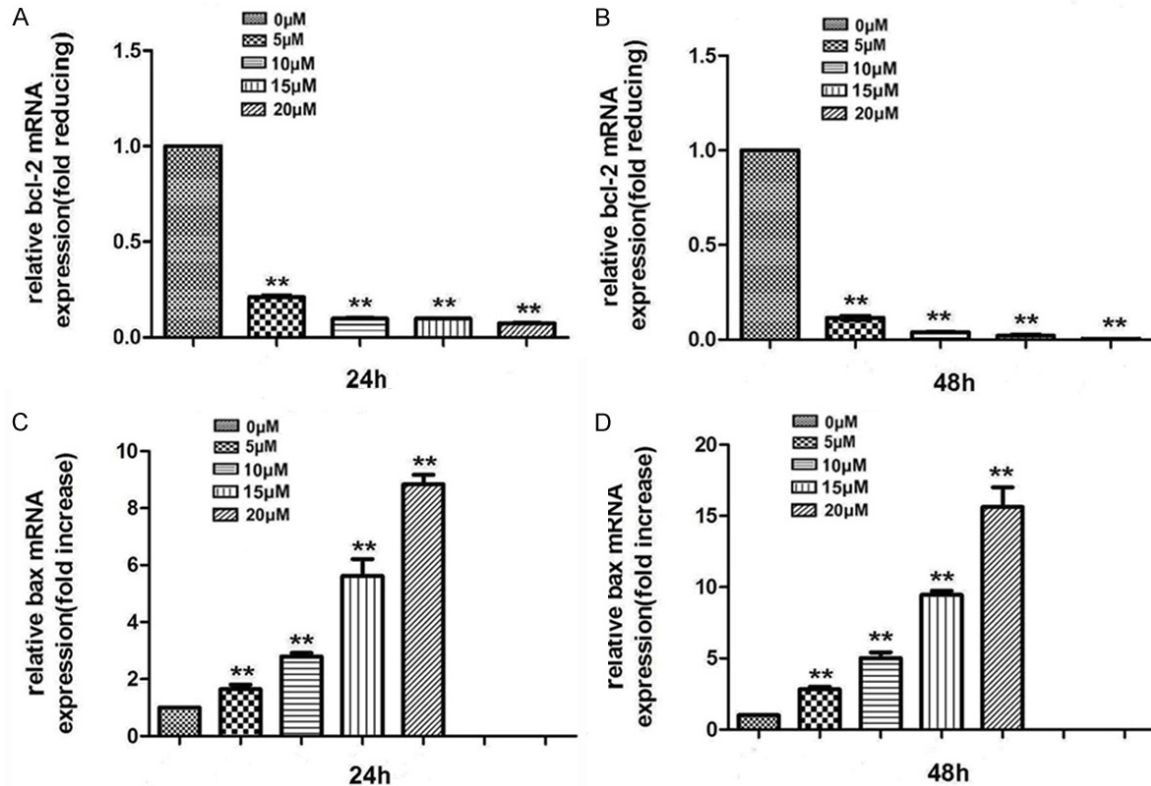


Figure 4. Effects of PTL on Bcl-2 and Bax mRNA expression in human 786-O cells. The cells were treated with PTL (0, 5, 10, 15, and 20 μ M) for 24 and 48 hours. Total RNA was isolated at indicated time points. Bcl-2 RNA expression was analyzed using real-time reverse-transcription polymerase chain reaction (A and B). Bax also used the same method (C and D). Values represent the means \pm standard deviation (SD) of two experiments. Significant versus control, * $P < 0.05$, ** $P < 0.01$.

mine the effects of parthenolide on the cell cycle in 786-O cells.

Molecular mechanisms by which PTL elicits antitumor effects have been a recent focus of investigations. Recent studies have implicated NF- κ B signaling pathways as mediators of PTL-dependent pro-apoptotic effects [17]. It has been reported that PTL could suppress the proliferation of human glioblastoma cancer U87MG and U373 cells and inhibit NF- κ B [27]. PTL promotes apoptosis in neoplastic cells by inducing oxidative stress and inhibiting cancer-promoting transcription factor nuclear factor kappa B (PTL decreases NF- κ B DNA binding by inhibiting I- κ B kinase) [28]. In addition, dysregulation of Raf/MEK/ERK signaling pathways, found in one-third of all kinds of human cancers, alters multiple gene expression, affecting tumor cell differentiation, proliferation, survival, migration, and angiogenesis [29-31]. Due to their powerful potential effects, Raf/MEK/ERK signaling pathways have been a focus of in-

tense investigation for therapeutic targets [32, 33]. Therefore, effects of PTL on these signaling pathways should be examined in future research.

Molecular mechanisms of apoptosis signals pathways have been explored in-depth by many scholars. Survivin, Bcl-2, and Bax are three of the molecular proteins involved in apoptosis. Survivin, a member of the inhibitor of apoptosis protein (IAP) family, can bind the effector cell death proteases caspase-3 and -7 *in vitro* and inhibit caspase activity and cell death in cells exposed to diverse apoptotic stimuli [34]. Bcl-2, an anti-apoptotic membrane-associated protein, exerts its function by binding to Bax and blocking c-Myc-induced apoptosis [35]. Bax, a pro-apoptotic Bcl-2 homologue residing in the cytoplasm or in the cell membrane, promotes apoptosis through interaction with p53/Bcl-2/Bcl-XL/c-Myc [35]. Results of Ku et al. demonstrated that the apoptogenic effects could be due to increased levels of Bax or decreased

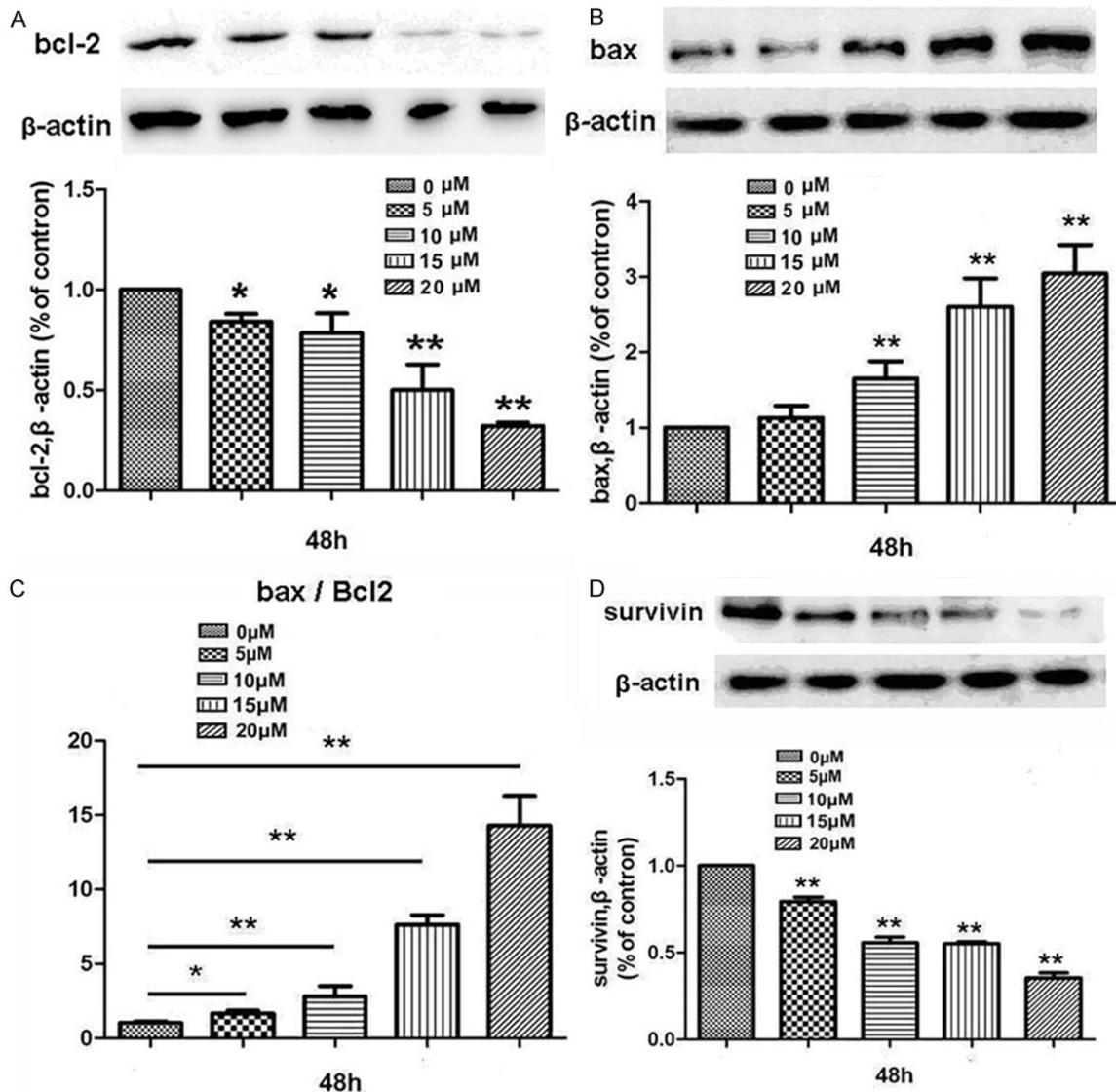


Figure 5. Effects of PTL on protein levels of Bcl-2, Bax, and survivin in 786-O cells (A, B and D). 786-O cells were assayed for Bcl-2, Bax, and survivin by Western blotting analysis with β -actin as a control (C). Bax/Bcl-2 ratios of A498 and 786-O cells. The densitometry value of each band was determined with ImageJ. Values are represented as the means \pm standard deviation (SD) of three experiments. Significant versus control, * $P < 0.05$, ** $P < 0.01$.

Bcl-2 and survivin expression in prostate cancer [36]. The current study found that the ratio of Bax/Bcl-2 was elevated, while expression of survivin was decreased, with an increase of cell apoptosis. This is consistent with previous conclusions.

Accumulating evidence has emphasized the anti-cancer efficacy of PTL against multiple malignant tumors. The current study demonstrated that PTL could effectively inhibit proliferation and induce apoptosis of renal cancer cells *in vitro*. Present results suggest that PTL may be a promising drug for patients with advanced renal cancer.

Conclusion

In summary, current results suggest that parthenolide may possess powerful characteristics against kidney cancer. Therefore, it should be considered a potential candidate for anti-cancer treatment, assuming further positive studies *in vivo* are confirmed.

Disclosure of conflict of interest

None.

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