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Triptolide, a diterpenoid isolated from the Chinese herb *Tripterygium wilfordii*, inhibits the proliferation of human bladder cancer cells by blocking cell cycle progression and inducing apoptosis

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Abstract

The Chinese medicinal herb *Tripterygium wilfordii* Hook and one of its active components triptolide, a diterpenoid triepoxide, have been shown to have anti-inflammatory, anti-fertility and anti-neoplastic activities. Recently, triptolide was shown to have antitumor properties by suppressing the growth and inducing apoptosis of a broad range of human tumor cells. In this study, the antiproliferative activity of triptolide in the human bladder cancer was investigated. The results showed that triptolide inhibited the proliferation of T24 human bladder cancer cells by blocking cell cycle progression in the G0/G1 phase and inducing apoptosis.

Keywords: *Tripterygium wilfordii*, triptolide, bladder cancer, cell cycle, apoptosis

Introduction

Bladder cancer is the second most-common cancer of the genitourinary tract. Currently, there are a wide range of therapeutic modalities available based on the extent of the disease including intravesical chemotherapy, surgery, radiation therapy, and systemic chemotherapy. Unfortunately, patients with advanced bladder cancer face a 5-year survival rate of approximately 20–40% despite the wide range of treatment modalities (Cookson *et al.* 1997; Herr 1997; Sudarshan *et al.* 2005). This is because these classical treatments are hampered by unwanted side effects and, most importantly, by the development of tumor resistance (Igney & Krammer 2002). There is an obvious urgent need for novel and effective therapies against bladder cancer. Apoptosis plays an important role in homeostasis and development of the tissue in organism (Igney & Krammer 2002). Imbalance between cell proliferation and apoptotic cell death will result in serious disease such as cancer. Many studies have demonstrated that cancer treatment by chemotherapy and γ-irradiation kill target cells primarily by the induction of apoptosis (Igney & Krammer 2002; Kasibhatla & Tseng 2003).

Tripterygium wilfordii Hook.f, a member of the Celastraceae family of plants, has been used in Chinese medicine for centuries. Triptolide, a diterpenoid, was first isolated from the plant and structurally characterized in 1972 (Kupchan *et al.* 1972; Carter *et al.* 2006) and has been used for the treatment of a variety of autoimmune diseases and as an

immunosuppressant in patients with organ and tissue transplantations. Triptolide induces apoptosis in a number of cancer cell lines in association with the activation of p53 (Jiang et al. 2001; Kiviharju *et al.* 2002; Wan *et al.* 2006) but triptolide may also induce apoptosis in HL-60, a p53-deficient promyelocytic cell line (Chan *et al.* 2001; Wan *et al.* 2006). A possible route for the p53-independent apoptosis maybe the inhibition of tumor necrosis factor α (TNFα)-mediated activation of NFκB (Lee *et al.* 1999; Wan *et al.* 2006). In this study, we determined the antiproliferative activity of triptolide, and examined its effect on cell cycle distribution and apoptosis in the human bladder cancer cell line, T24.

Materials and Methods

Chemicals and reagents

Fetal bovine serum (FBS), penicillin G, streptomycin, amphotericin B and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO BRL (Gaithersburg, MD). Triptolide was purchased from Calbiochem (La Jolla, CA). Dimethyl sulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO). XTT was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Nucleosome ELISA was purchased from Calbiochem (Cambridge, MA).

Preparation of triptolide

Triptolide was dissolved in dimethyl sulfoxide (DMSO) and stored at -20℃. For all experiments, the final concentrations of the test compound were prepared by diluting the

stock with DMEM. Control cultures received the carrier solvent (0.1% DMSO).

Cell culture

Bladder cancer cell line T24 was obtained from the American Type Cell Culture Collection (Manassas, VA). It was maintained in monolayer culture at 37℃ and 5% CO_2 in DMEM supplemented with 10% FBS, 100 units/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B.

Cell proliferation assay

Inhibition of cell proliferation by triptolide was measured by XTT {sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate} assay. Briefly, cells were plated in 96-well culture plates (1×10^4 cells/well). After 24 h incubation, the cells were treated with triptolide (0.5, 2.5, 5, and 10 μM) for 48 h. Fifty μl of XTT test solution, which was prepared by mixing 5 ml of XTT-labeling reagent with 100 μl of electron coupling reagent, was then added to each well. After 6 h incubation, the absorbance was measured on an ELISA reader (Multiskan EX; Labsystems; Thermo Electron Corporation, Milford, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

Cell cycle analysis

To determine cell cycle distribution, 5×10^5 cells were plated in 60-mm dishes and treated with triptolide (0, 4, and 8 μM) for 24 h. After treatment, the cells were collected by

trypsinization, fixed in 70% ethanol, washed in PBS, re-suspended in 1 ml of PBS containing 1 mg/ml RNase and 50 μg/ml propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by EPICS flow cytometer. The data were analyzed using the Multicycle software (Phoenix Flow Systems, San Diego, CA).

The analysis of apoptosis

Quantitative assessment of apoptosis was analyzed by an Annexin V assay kit (BD Biosciences PharMingen, San Jose, CA). Briefly, cells grown in 10 cm Petri dishes were harvested with trypsin and washed in PBS. Cells were then resuspended in binding buffer (10 mmol/l HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/l $CaCl_2$) and stained with Annexin V-FITC and PI at room temperature for 15 minutes in the dark. Cells were then analyzed in an EPICS flow cytometer (Coulter Electronics) within 1 hour after staining. Data from 10,000 cells were collected for each data file. Apoptotic cells were defined as Annexin V-FITC-positive and PI-negative cells. Our definition of cellular status is as follows: unstained cells were classified as “live”, cells stained for Annexin V only were “early apoptotic”, cells stained for both Annexin V and propidium iodide were “late apoptotic”, and cells stained for propidium iodide only were “dead”. Apoptotic cells were the sum of early and late apoptotic cells (Xu *et al.* 2006; Barton *et al.* 2004; Kuo *et al.* 2006).

Quantitative assessment of oligonucleosomal DNA fragmentation was also assayed using

the Nucleosome ELISA kit. This kit uses a photometric enzyme immunoassay that quantitatively determines the formation of cytoplasmic histone-associated DNA fragments (mono and oligonucleosomes) after apoptotic cell death. For determination of apoptosis by ELISA, cells were treated with vehicle alone (0.1% DMSO) and triptolide (4 and 8 μM) for the indicated time. The induction of apoptosis was evaluated by assessing the enrichment of nucleosome in cytoplasm, and determined exactly as described in the manufacturer's protocol (Kuo *et al.* 2005).

Statistical analysis

Data were expressed as means ± S.D. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p<0.05$) between the means of control and triptolide-treated cells were analyzed by Dunnett's test.

Results and Discussion

Effect of triptolide on T24 cell proliferation

We first tested the antiproliferative effect of triptolide in the bladder cancer cell line, T24. As shown in Fig. 1, the proliferative inhibitory effect of triptolide was observed in a dose-dependent manner. At 48 h, the maximal effect on proliferation inhibition was observed with 10 μM triptolide, which inhibited proliferation in 81.5% of T24 cells. The IC_{50} value was 3.9 μM.

Triptolide-induced cell cycle arrest and apoptosis in T24 cells

The results on the effect of triptolide on cell cycle progression of T24 are shown in Fig. 2. As compared to the control, 4 μM of triptolide increased the population of G0/G1 phase from 35.7% to 47.2%. This effect was enhanced when T24 cells were treated by 8 μM of triptolide (59.6% cell population in G0/G1 phase).

A quantitative evaluation of apoptosis was sought using an Annexin V-FITC dye to detect the translocation of phosphatidylserine from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface). Compared with vehicle-treated cells, 8 μM triptolide induced 30.5% and 42.7% of apoptotic cells in T24 at 24 and 48 h, respectively (Fig. 3A). Additionally, a quantitative evaluation was sought using ELISA to detect histone-associated oligonucleosome DNA fragments. Compared with vehicle-treated cells, 8 μM triptolide induced 6.8 and 9.2-fold of cytoplasmic oligonucleosome in T24 at 24 and 48 h, respectively (Fig. 3B).

Our study has clearly demonstrated that triptolide may be a promising chemopreventive agent for treating bladder cancer.

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Figure legends

Fig. 1. The anti-proliferative effect of triptolide in T24 cells. Adherent cells plated in 96-well plates (10^4 cells/well) were incubated with different concentrations of triptolide at 48 h. Cell proliferation was determined by XTT assay. Results are expressed as percent cell proliferation relative to the proliferation of control. Each value is the mean ± S.D. of three determinations.

Fig. 2. Effects of triptolide on cell cycle distribution in T24 cells. T24 cells following treatment with 0, 4, and 8 μM triptolide for 24 h were fixed and stained with propidium iodide, and cell cycle distribution was then analyzed by flow cytometry. Each value is the mean ± S.D. of three determinations. The asterisk indicates a significant difference between control and triptolide-treated cells as analyzed by Dunnett's test ($P<0.05$).

Fig. 3. Induction of apoptosis in T24 cells by triptolide. Triptolide induced apoptosis in T24 cells determined by Annexin V-FITC/PI dye (A) and Nucleosome ELISA kit (B). For (A) and (B), cells were treated with vehicle and triptolide for indicated times, and then the induction of apoptosis was assessed by Annexin V-FITC/PI dye and Nucleosome ELISA kit. The asterisk indicates a significant difference between control and triptolide-treated cells as analyzed by Dunnett's test ($P<0.05$).

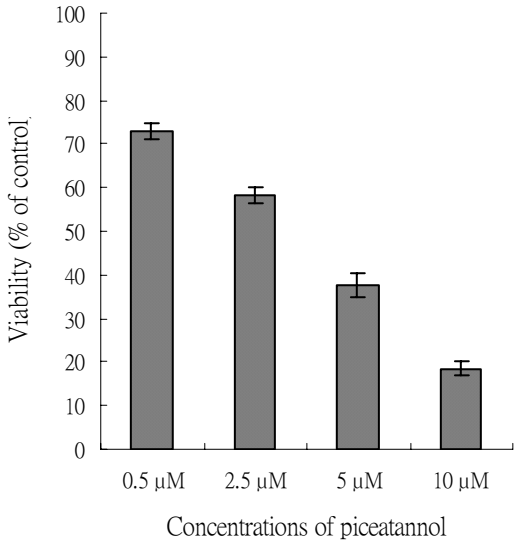
100
90
80
70
60
50
40
30
20
10
0
Viability (% of control)
0.5 μM
2.5 μM
5 μM
10 μM
Concentrations of piceatannol

Fig. 1.

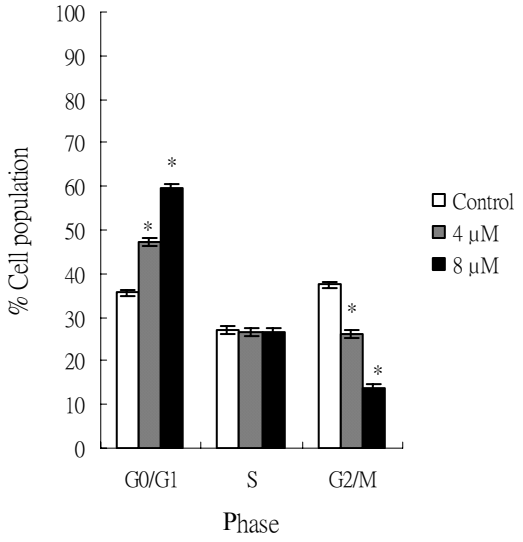
100
90
80
70
60
50
40
30
20
10
0
% Cell population
*
*
*
*
Control
4 μM
8 μM
G0/G1
S
G2/M
Phase

Fig. 2.

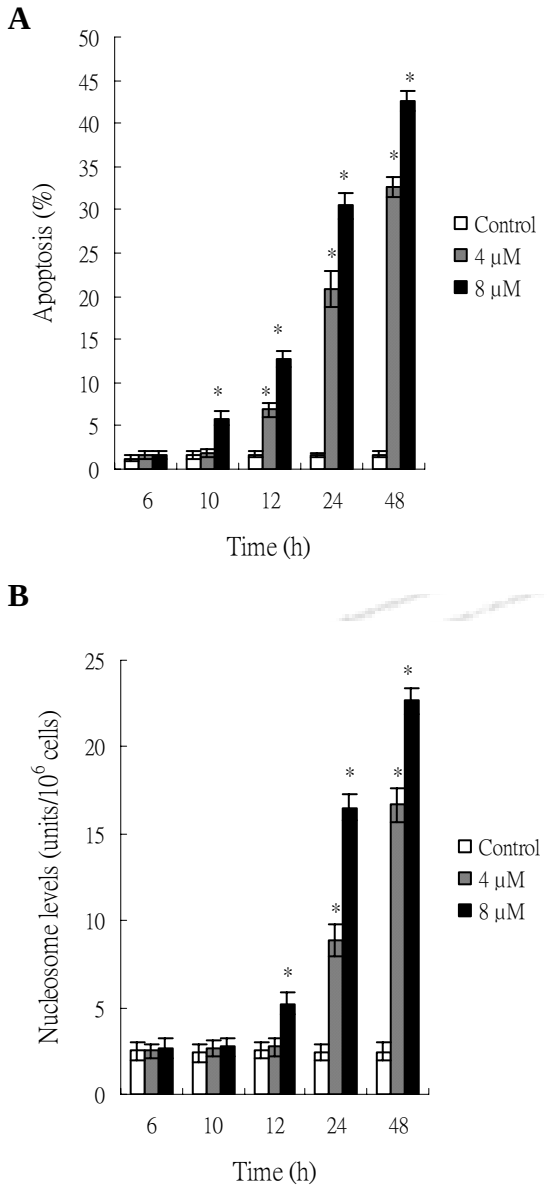
A
Apoptosis (%)
50
45
40
35
30
25
20
15
10
5
0
6
10
12
24
48
Time (h)
Control
4 μM
8 μM
B
Nucleosome levels (units/10^6 cells)
25
20
15
10
5
0
6
10
12
24
48
Time (h)
Control
4 μM
8 μM

Fig. 3.