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**Induction of apoptosis in human breast adenocarcinoma MCF-7 cells by
Triptolide, a diterpenoid isolated from the Chinese herb *Tripterygium
wilfordii***

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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. To identify the anticancer mechanism of triptolide, we assayed its effect on apoptosis, cell cycle distribution. The results showed that triptolide induced apoptosis in MCF-7 cells, without affecting the cell cycle distribution. Our study reports here for the first time that the activity of the apoptotic induction may participate in the anti-proliferative activity of triptolide in MCF-7 cells.

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

Introduction

Worldwide, breast cancer is the most common cancer among women after skin cancer, and is the second leading cause of cancer death (after lung cancer) in women [1,2]. Increasing evidence suggests that breast cancer might result from interactions between genetic elements, various possible environmental factors, and also the difference in ethnicity [1,3]. This pathology is currently controlled through surgery and/or radiotherapy, and is frequently supported by adjuvant chemo- or hormonotherapies. Unfortunately, these classical treatments are hampered by unwanted side effects and, most importantly, the development of tumor resistance. There is no doubt an urgent need for novel and effective therapies against breast cancer [4]. Apoptosis plays an important role in homeostasis and development of the tissue in organism [5]. 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 [5,6].

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 [7,8] 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 [9-11] but triptolide may also induce apoptosis in HL-60, a p53-deficient promyelocytic cell line [11,12]. A possible route for the p53-independent apoptosis maybe the inhibition of tumor necrosis factor α (TNF α)-mediated activation of NF κ B [11,13]. In this study, we determined the antiproliferative activity of triptolide, and examined its effect on cell cycle distribution and apoptosis in the human breast cancer cell line, MCF-7.

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°C. 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

Breast cancer cell line MCF-7 was obtained from the American Type Cell Culture Collection (Manassas, VA). It was maintained in monolayer culture at 37°C and 5% CO₂ 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 (1×10⁴ cells/well). After 24 h incubation, the cells were treated with 0, 0.5, 2.5, 5, and 10 µM triptolide 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, 5×10⁵ cells were plated in 60-mm dishes and treated with triptolide (0, 5, and 10 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).

Measurement of apoptosis by ELISA

The induction of apoptosis by triptolide was 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. MCF-7 cells were treated with 0, 5, and 10 µM triptolide, for 6, 12, 24, and 48 h. The samples of cell lysate were placed in 96 well (1×10^6 per well) microtiter plates. The induction of apoptosis was evaluated by assessing the enrichment of nucleosome in cytoplasm, and determined exactly as described in the manufacturer's protocol.

Statistical analysis

Data were expressed as means \pm 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 MCF-7 cell proliferation

We first tested the anti-proliferative effect of triptolide in the breast cancer cell line,

MCF-7. 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 μ M triptolide, which inhibited proliferation in 73.3% of MCF-7 cells. The IC_{50} value was 5.2 μ M.

Triptolide induced apoptosis in MCF-7 cells, without affecting the cell cycle distribution

To clarify the mechanism of anti-proliferative effect, EPICS flow cytometer and apoptotic ELISA kits were used to analyze cell cycle distribution and apoptosis respectively. In cell cycle distribution, our results did not show any significant change between the control group and the triptolide-treated group for up to 10 μ M at 24 h (Fig. 2). By using Nucleosome ELISA kit, we demonstrated that triptolide induced apoptosis of MCF-7 cells in dose-dependent and time-dependent manners (Fig. 3). In contrast to the controls, when cells were treated with triptolide, the number of cells undergoing apoptosis increased from about 4 fold at 5 μ M triptolide to 8.7 fold at 10 μ M triptolide at 48 h.

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References

- [1] Brinton L, Lacey J, Devesa SS. Epidemiology of Breast Cancer. In: W.L. Donegan and J.S. Spratt, Cancer of the Breast 5 Edition, Philadelphia, WB Saunders, 2002:111-132.
- [2] Fog CK, Christensen IJ, Lykkesfeldt AE. Characterization of a human breast cancer cell line, MCF-7/RU58R-1, resistant to the pure antiestrogen RU 58,668. *Breast Cancer Res Treat* 2005; **91**: 133-144.
- [3] Hsiao WC, Young KC, Lin SL, Lin PW. Estrogen receptor-alpha polymorphism in a Taiwanese clinical breast cancer population: a case-control study. *Breast Cancer Res* 2004; **6**: R180-R186.
- [4] Chopin V, Toillon RA, Jouy N, Le Bourhis X. p21(WAF1/CIP1) is dispensable for G1 arrest, but indispensable for apoptosis induced by sodium butyrate in MCF-7 breast cancer cells. *Oncogene* 2004; **23**: 21-29.
- [5] Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. *Nature reviews. Cancer* 2002; **2**: 277-288.
- [6] Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature* 2001; **411**: 342-348.
- [7] Kupchan SM, Court WA, Dailey RG Jr, Gilmore CJ, Bryan RF. Triptolide and triptolide, novel antileukemic diterpenoid triepoxides from *Tripterygium wilfordii*. *J Am Chem Soc* 1972; **94**: 7194-7195.

- [8] Carter BZ, Mak DH, Schober WD, McQueen T, Harris D, Estrov Z, Evans RL, Andreeff M. Triptolide induces caspase-dependent cell death mediated via the mitochondrial pathway in leukemic cells. *Blood* 2006; **108**: 630-7.
- [9] Jiang XH, Wong BC, Lin MC, Zhu GH, Kung HF, Jiang SH, Yang D, Lam SK. Functional p53 is required for triptolide-induced apoptosis and AP-1 and nuclear factor-kappaB activation in gastric cancer cells. *Oncogene* 2001; **20**: 8009-8018.
- [10] Kiviharju TM, Lecane PS, Sellers RG, Peehl DM. Antiproliferative and proapoptotic activities of triptolide (PG490), a natural product entering clinical trials, on primary cultures of human prostatic epithelial cells. *Clin Cancer Res* 2002; **8**: 2666-2674.
- [11] Wan CK, Wang C, Cheung HY, Yang M, Fong WF. Triptolide induces Bcl-2 cleavage and mitochondria dependent apoptosis in p53-deficient HL-60 cells. *Cancer Lett* 2006; **241**: 31-41.
- [12] Chan EW, Cheng SC, Sin FW, Xie Y. Triptolide induced cytotoxic effects on human promyelocytic leukemia, T cell lymphoma and human hepatocellular carcinoma cell lines. *Toxicol Lett* 2001; **122**: 81-87.
- [13] Lee KY, Chang W, Qiu D, Kao PN, Rosen GD. PG490 (triptolide) cooperates with tumor necrosis factor-alpha to induce apoptosis in tumor cells. *J Biol Chem* 1999; **274**: 13451-5.

Figure legends

Fig. 1. The anti-proliferative effect of triptolide in MCF-7 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 the percent of the cell proliferation of control at 0 h. Standard deviations were less than 10%.

Fig. 2. The effects of triptolide on cell cycle distribution in MCF-7 cells. MCF-7 cells following treatment with 0, 5, and 10 μ M triptolide for 24 h were fixed and stained with propidium iodide, and cell cycle distribution was then analyzed by flow cytometry. The data indicate the percentage of cells in G0/G1, S, and G2/M phases of the cell cycle ($P>0.05$). The results represent the mean values and standard deviations from three individual experiments.

Fig. 3. Induction of apoptosis in MCF-7 cells by triptolide. MCF-7 cells were cultured with 0, 5, and 10 μ M of triptolide for 6, 12, 24 and 48 h. Cells were harvested and lysed with lysis buffer. Cell lysates containing cytoplasmic oligonucleosomes of apoptotic cells were analyzed by means of Nucleosome ELISA. Each value is the mean \pm S.D. of three determinations. The asterisk indicates a significant difference between control and triptolide-treated cells, as analyzed by Dunnett's test ($P<0.05$).

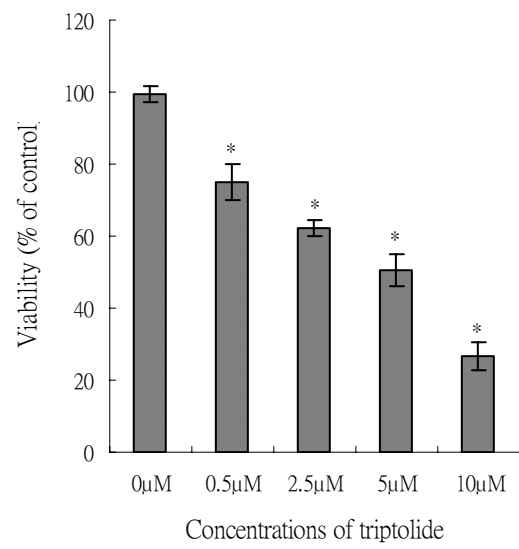


Fig. 1.



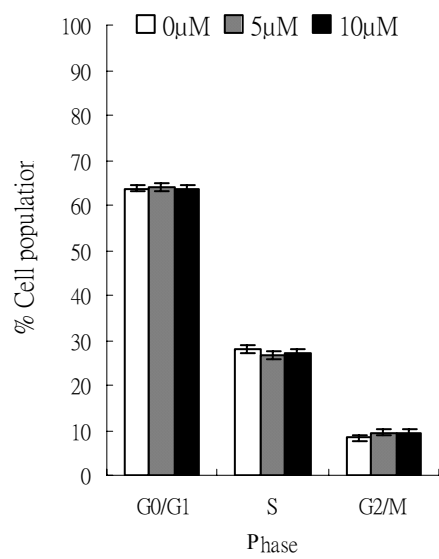


Fig. 2.



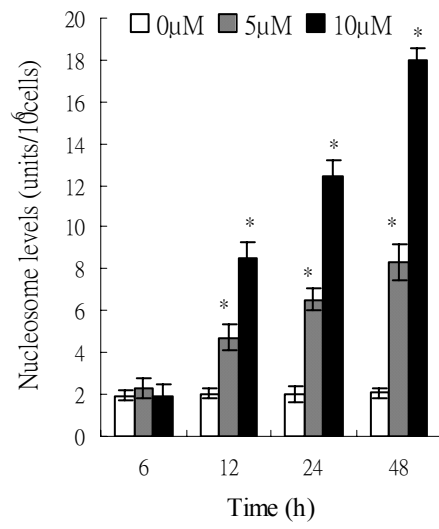


Fig. 3.

