Effects of triptolide from *Tripterygium wilfordii* on ERα and p53 expression in two human breast cancer cell lines

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Abstract

The aim of the study was to discover possible differential cytotoxicity of triptolide towards estrogen-sensitive MCF-7 versus estrogen-insensitive MDA-MB-231 human breast cancer cells. Considering that MCF-7 cells express functional Estrogen receptor α (ERα) and wild-type p53, whereas MDA-MB-231 cells which are ERα-negative express mutant p53, the anti-proliferation effect of triptolide on MCF-7 and MDA-MB-231 cells were examined, the apoptotic effect and cell cycle arrest caused by triptolide were investigated, ERα and p53 expression were also observed in this paper. The results showed that the anti-proliferation effects were induced by triptolide in both cell lines. But the value of IC_{50} in MCF-7 cells for its anti-proliferation effect was about one tenth of that in MDA-MB-231 cells, which indicated that the effect is more potent in MCF-7 cells. Condensed chromatin or fragmented nuclei could be found in MCF-7 cells treated with only 40 nM triptolide but in MDA-MB-231 cells they couldn't be observed until the concentration reached to 400 nM. Triptolide induced significant S cell cycle arrest along with the presence of sub-G0/G1 peak in MDA-MB-231 cells, whereas there was only slightly S cell cycle arrest on cell cycle distribution in MCF-7 cells. The role of p53 in two breast cancer cells was examined, the results showed that the mutant p53 in MDA-MB-231 cells was suppressed and the wild-type p53 in MCF-7 was increased. Moreover, triptolide could down regulate the expression of ERα in MCF-7 cells. The results showed that triptolide is much more sensitive to ERα-positive MCF-7 cells than to ERα-negative MDA-MB-231 cells, and the sensitivity is significantly associated with the ERα and p53 status.

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**Keywords:** Triptolide; *Tripterygium wilfordii*; Estrogen receptor alpha; P53; Human breast cancer cells

Introduction

Triptolide, a tri-epoxide phenanthrene (Fig. 1) and a major component in Chinese herb *Tripterygium wilfordii* Hook F (TWHF), has multiple biological effects. In addition to its potent immunosuppressive and anti-inflammatory activity (Qiu and Kao 2003; Liu et al. 2006), its antineoplastic activity has been a hot topic in recent years. Triptolide induces apoptosis and inhibits the growth and metastasis in many tumors such as prostate cancer, cholangiocarcinoma and multiple myeloma (Yang et al. 2003; Panichakul et al. 2006; Lou et al. 2005; Tong et al. 2007; Zhang et al. 2007). It has been reported that the antitumor effects of triptolide resulted from the decreased expression of cell-cycle promoting
factors such as cyclins (A, B1, and D1) and c-myc in breast tumor MDA-435 cells (Miyata et al. 2005). It has been also reported that triptolide can induce cell arrest in the S phase in human fibrosarcoma HT-1080 cells (Fidler et al. 2003) and prostatic adenocarcinoma cells (Kiviharju et al. 2002). In addition, the antitumor effects induced by triptolide has been reported to be associated with the down regulation of NF-KappaB in human multiple myeloma cells (Lou et al. 2005) and with the involvement of mitochondrial pathway in cytotoxicity induced by triptolide in human normal liver L-02 cells (Yao et al. 2008). Furthermore, the in vitro and in vivo antitumor proliferation caused by triptolide may be associated with apoptosis (Jiang et al. 2001; Chang et al. 2001), but the exact mechanism responsible for the antineoplastic effect of triptolide is not clearly illuminated.

Breast cancer is the main cause of cancer deaths among women (Greenlee et al. 2000). Major advances in breast cancer therapy showed that a number of resistance mechanisms were presented to reduce the effectiveness of chemotherapeutic drugs (Clarke et al. 2005; Evan and Vousden 2001). Therefore, some novel and more effective strategies to prevent and treat breast cancer are still required to be researched. A reasonable treatment plan should be chosen according to the clinical stages, pathological types, tumor positions and the level of estrogen receptors (Wang 1988). The methods of treating the hormone-sensitive breast cancers by inhibiting the estrogen production and its binding to estrogen receptors have been adopted (Jordan and Brodie 2007). Recently, it has been reported that triptolide can inhibit proliferation of MCF-7 human breast cancer cells (Wang et al. 2006). However, the antineoplastic mechanism obtained through comparing the effect of triptolide on different kinds of breast cancers which are greatly impacted by estrogen-sensitivity is not reported yet. It is important to investigate the effect of triptolide dependent on ERα and p53 status.

Materials and Methods

Cell culture and triptolide

MDA-MB-231 cells and MCF-7 cells were purchased from Shanghai Institute of Cell Biology and Biochemistry, Chinese Academy of Sciences, China. Cells were grown on tissue culture flasks in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (Gibco) and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin) at 37 °C in 5% CO2 and 95% air in a humidified incubator. MDA-MB-231 cells and MCF-7 cells designated for experimental usage were grown in culture medium containing serum that had been stripped with Dextran-coated Charcoal (DCC) to remove steroids and other low-molecular weight factors. Triptolide was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (purity >98% (HPLC), Beijing, China).

Cell viability assay

The cells were seeded in 96-well plates at a density of 5×10^4/ml in a humidified incubator with 5% CO2, 37 °C for 2–3 days. The cells were then exposed to 1–1000 nM triptolide for 24, 48 and 72 h, respectively. At the end of treatment period, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (5 mg/ml in PBS) was added to each well. The plates were then incubated for 4 h at 37 °C in the dark. The medium was removed and MTT reduction product (formazan crystals) was dissolved in Dimethyl Sulfoxide (DMSO), and the absorbance at 490 nm was measured with an ELISA reader (Bio-Rad, USA).

Lactic Dehydrogenase (LDH) release assay

LDH release was determined by measuring changes in absorbance at 530 nm due to NADH oxidation with LDH assay kits (Roche Applied Science). The amount of LDH released into the medium under different treatments compared to the total amount of LDH is defined as cytotoxicity, the total amount of LDH consists of the amount derived from cells treated with 2% Triton X-100 and the amount of LDH released from the medium.

Cell cycle analysis

The distribution of cells at specific cell cycle stages was evaluated by Flow Cytometry. 5×10^5 cells/well were cultured on tissue culture plates with triptolide treatment in a humidified incubator with 5% CO2, 37 °C for 48 h, and cells incubated in medium without
triptolide served as controls. After incubation, adherent cells were detached with trypsin (0.5% trypsin and 0.1% EDTA in PBS). The detached and suspended cells were harvested in complete DMEM medium and centrifuged at 500g for 10 min. Pellets were washed with PBS and fixed with ice cold 75% ethanol overnight at 4°C, treated with 100 μg/ml RNase A (Sigma), and subsequently stained with 25 μg/ml propidium iodide (Sigma). The cells were analyzed by BDFACSCanto Flow Cytometer analyzer.

**Hoechst 33258 staining**

The apoptotic effects of triptolide on MCF-7 cells and MDA-MB-231 cells were analyzed by DNA staining. MCF-7 cells and MDA-MB-231 cells were placed on glass cover slips in the wells of a 6-well plate. After 24 h cell culture, they were maintained in the same media lacking phenol red and containing charcoal-stripped FCS for 24 h, and then treated with triptolide for 48 h. The cells were washed by PBS and fixed in 4% paraformaldehyde for 20 min and then stained with Hoechst 33258 (Sigma) at a concentration of 2 mM for 10 min. After being washed with PBS, fluorescent nuclei were observed with fluorescence microscopy (DIAPHOT300, Nikon, Japan). The morphology of the stained nuclei was used to determine apoptosis.

**Western blot analysis**

An equivalent volume of 5 x SDS-sample buffer was added to cell lysates and boiled for 5 min. The supernatants were subjected to electrophoresis on SDS-PAGE gels and transferred to nitrocellulose by using a Bio-Rad apparatus. Membranes were blocked for 1 h at room temperature in PBS containing 5% non-fat dried milk and probed overnight at 4°C with primary antibodies. ERα and p53 detection were performed with mouse primary antibody (ERα, 1:600 (ab2746, abcam); p53, 1:1500 (ab26, abcam), overnight), and a-tubulin antibody and β-actin (1:5000, overnight, 4°C Chemicon, Temecula, CA) were served as internal controls. Protein bands of ERα, p53, a-tubulin and β-actin were visualized using peroxidase-labeled goat anti-mouse secondary antibody (1:1000 dilution, 1 h, room temperature, ab6276, abcam), and the protein expression signals were detected by the enhanced chemiluminescence kit (Rockford, IL, USA.)

**Statistical analysis**

The raw data were analyzed statistically. All the results are expressed as the means ± SD. Significance of difference between groups was evaluated with a one-way analysis of variance (ANOVA) and Dunnett’s t-test.

![Fig. 2. Inhibition of proliferation and induction of cytotoxicity by triptolide. MCF-7 and MDA-MB-231 cells cultured in DMEM supplemented with 10% FBS were incubated with indicated concentrations of triptolide for 24, 48 or 72 h, respectively. The cytotoxic effect was determined by MTT assay (A) and LDH assay (B). Data are expressed as means ± SD (n = 5). Data analysis was performed using Dunnett’s t-test. (**P < 0.05 and ***P < 0.01, compared to control).](image-url)
Results

Inhibiting proliferation and inducing cytotoxicity

The proliferative effect of triptolide on the ERα-positive breast cancer cells line MCF-7 and ERα-negative cells line MDA-MB-231 was examined. Triptolide induced cell death in a concentration and time dependent manner by MTT assay (Fig. 2(A)). The value of IC₅₀ of triptolide in MCF-7 cells was found to be around one tenth of that in MDA-MB-231 cells, which indicated the higher potency of triptolide in MCF-7 cells than in MDA-MB-231 cells. To evaluate cell membrane permeability and integrity, the LDH activity was assayed. As shown in Fig. 2(B), the group exposed to triptolide alone presented a significant increase in LDH when compared to the control.

Morphological observation of nuclear change

Hoechst 33258 staining showed that there were significant morphological changes in the nuclear chromatin of the breast cancer cell lines MCF-7 and MDA-MB-231 (Fig. 3). In the control group, the morphology of nuclei was normal (Figs. 3A and C). However, when cells were treated with 40 nM and 400 nM triptolide corresponding to MCF-7 and MDA-MB-231 for 48 h, condensed chromatin or fragmented nuclei could be found in many treated cells which are the classic characteristics of apoptotic cells (Figs. 3B and D).

Flow Cytometry analysis

Flow Cytometry analysis was performed to provide further evidence that inhibition of the cell cycle by triptolide was responsible for cells anti-proliferation effect. As shown in Fig. 4(A), cell cycle distribution patterns of MCF-7 and MDA-MB-231 cells differed in their response to triptolide treatment. Triptolide induced a significant S cell cycle arrest along with the...

Fig. 3. Fluorescence Photomicrograph of MCF-7 and MDA-MB-231 cells stained with Hoechst 33258. MCF-7 and MDA-MB-231 cells were treated with triptolide for 48 h, and stained with hoechst 33258, then examined by fluorescence microscopy. Nuclear chromatin condensation and presence of granular apoptotic bodies were observed in the cells treated with triptolide (B and D) compared to control (A and C). Magnification is 200 × .
presence of sub-G0/G1 peak in MDA-MB-231, whereas there was only slight S cell cycle arrest on cell cycle distribution in MCF-7 cells, and there was no statistical difference in cell cycle distribution compared to the control (Fig. 4(B)).

**Western blot analysis**

The level of ERα in nucleus of hormone-dependent MCF-7 cells treated with triptolide are shown in Fig. 5, and the levels of p53 in whole-cell lysates of MCF-7 cells and MDA-MB-231 were observed in Fig. 6. Triptolide could up regulate the expression of wild-type p53 and down regulate the expression of ERα in a concentration-dependent manner compared to the control in MCF-7 cells. Triptolide could also down regulate the expression of the mutant p53 in the hormone-independent cells line MDA-MB-231.

**Discussion**

In this paper, we examined the effects of triptolide treatment on two breast cancer cell lines in an attempt to discover differential cytotoxicity towards estrogen receptor positive versus estrogen receptor negative breast cancer. Triptolide could induce cytotoxicity not only in MCF-7 cells but also in MDA-MB-231 cells by MTT and LDH assay. To our surprise, the inhibitory actions reported here for triptolide assay is much more sensitive to ERα-positive MCF-7 cells than to ERα-negative MDA-MB-231 cells. Then, we investigated morphological changes in the nuclear chromatin of the breast
cancer cell lines MCF-7 and MDA-MB-231. Apoptotic features including chromatin condensation was observed in both human breast cancer cell lines, but the concentration used 40 nM and 400 nM respectively corresponding to MCF-7 and MDA-MB-231 for 48 h. The analysis of cell cycle distribution further revealed the difference of treatment between MCF-7 and MDA-MB-231 cells. The MDA-MB-231 cells treated with triptolide could increase the proportion of cells in S phase which has been reported in human fibrosarcoma HT-1080 cells (Fidler et al. 2003), whereas S phase arrest was not obvious in MCF-7 cells treated with triptolide. Therefore, these data suggest that triptolide can alter the cell cycle in a cell-specific manner. Lastly, according to their different characteristics: MCF-7 cells express functional ERα and wild-type p53, whereas MDA-MB-231 cells which are ERα-negative express mutant p53, the expressions of ERα, wild-type p53 and mutant p53 were investigated in this paper. Estrogen is a key regulator for normal growth and differentiation in mammary glands and reproductive organs and the malignant progression of breast cancer (Park and Jordan 2002; Margueron et al. 2004). The biological activities of estrogens are mediated by nuclear estrogen receptors (ERs), which interact directly with estrogen response elements (ERE) in the promoters of target genes and recruit various co-activators to mediate transcriptional regulation (McDonnell and Norris 2002). ERs also regulate transcription through “non-classical” response sites, probably via protein-protein interactions (McDonnell and Norris 2002). The presence of high levels of ERα in benign breast epithelium signifies an increased risk of breast cancer, suggesting a role for ERα in breast cancer initiation, promotion and progression (Ali and Coombes 2000). The ERα is an important target to develop drugs for the treatment and prevention of breast cancer (Jensen and Jordan 2003). The role of ERb in breast cancer growth and development is not as clear as that of ERα (Gustafsson and Warner 2000; Palmieri et al. 2002). The interaction of estrogen with the ER can result in increased proliferation of target cells; accordingly the rationale for therapy is either to block the interaction of estrogen with the ER or to suppress ERα expression. MCF-7 cells express estrogen receptors alpha, and treatment of MCF-7 cells with triptolide could rapidly induce down regulation of ERα in the nucleus. It has been reported that the substances with a phenanthrene backbone such as estrogens and tamoxifen can bind to ERs (Shagufta et al. 2006; Paolo et al. 2006), and triptolide is a triepoxide phenanthrene, thus an involvement of ERs in the action of triptolide, at least in MCF-7 cells, cannot be excluded. Considering that the inhibitory actions of triptolide reported here is more sensitive to ERα-positive MCF-7 cells than to ERα-negative MDA-MB-231 cells, and ERα could be down regulated by triptolide in the MCF-7 cells model, the risk of breast cancer in premenopausal women may be decreased by using triptolide. The tumor suppressor wild-type p53 plays a central role in the protection against DNA damage and physiological stress primarily induced during cell cycle arrest or apoptosis. Mutation of p53, which is the most frequent genetic alteration detected in human cancers, inactivates these growth regulatory functions and causes a loss of tumor suppressor activity (Cadwell and Zambetti 2001). Since MCF-7 expresses a wild p53 gene while MDA-MB-231 expresses a mutated p53 gene, the present results indicate that triptolide could induce apoptosis in human breast cancer cell lines likely via a p53-independent pathway. By dissecting out mechanistic differences between wild-type and mutant P53 activities, it may be possible to develop therapeutics that restore tumor suppressor function of wild-type p53 or selectively inactivate tumor-promoting functions of mutant p53. Triptolide could up regulate the expression of wild-type p53 and down regulate the expression of the mutant p53 in a concentration-dependent manner compared to the control. The experimental results are consistent with the inhibitory effect of triptolide on Glioblastoma Multiforme in vitro (Lin et al. 2007). A number of significant and novel findings in this paper should be further researched in the future.
TWHF has been historically used in traditional Chinese medicine to treat inflammatory and autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus, psoriatic arthritis, and Behcet’s disease (Lipsky and Tao 1997). The therapeutic effects of TWHF are very significant according to many clinical observations and animal experiments (Tao et al. 2002; Cui et al. 2007).

Unfortunately, the good effects of TWHF are always accompanied by adverse events (Arathi et al. 2005), which made the clinical use of TWHF controversial (Lipsky and Tao 1997). However, although the adverse effects were frequent, only a few patients had to discontinue the preparation of TWHF because of them, and all adverse events such as gastrointestinal tract disturbances, loss of appetite, anorexia, vomiting, abdominal pain, diarrhea, esophageal burning, leukopenia, and thrombocytopenia, appeared to be dose related and reversible with cessation of medication (Lipsky and Tao 1997). In an animal experiment, the injection or oral administration of triptolide, which is thought to account for both the effectiveness and toxicity of TWHF, effectively blocks experimental amyloidosis, and all mice tolerated a long-term (105 days) administration of triptolide, no mice died or appeared ill during treatment (Cui et al. 2007).

Therefore, according to the reports above, whether the TWHF can be used clinically or not still remains controversial and need further research. In fact, researchers, doctors and pharmaceutical industries in and abroad, are devoting to study TWHF, trying to explore its good therapeutic effects in clinic and decrease the side effects. We think that, TWHF, with a long history of use in China more than two thousand years (Chen 2001), has good values in both research and development.

In our paper, the proliferative inhibitive effect of triptolide, the main active component of TWHF, on breast cancer cells was studied and differential cytotoxic mechanism of triptolide towards estrogen-sensitive MCF-7 versus estrogen-insensitive MDA-MB-231 human breast cancer cells was discovered. This finding may reveal the characteristics of action of triptolide on breast cancer, and can provide references for triptolide used in breast cancer therapy. The toxicities of triptolide exist now, but it will be solved as technology develops and comprehensive and profound understanding of triptolide. For example, by modifying its structures, developing appropriate dosage form, or combination with other drugs, we may be able to diminish the toxicity of triptolide to minimum, but still maintain good therapeutic effects.

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