RESEARCH ARTICLE

Combined Effects of Curcumin and Triptolide on an Ovarian Cancer Cell Line

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Abstract

Background: As natural medicines in Asia, curcumin and triptolide extracted from different drug plants have proven to possess anticancer potential and widely used for anti-cancer research. The present study attempted to clarify that curcumin and triptolide synergistically suppress ovarian cancer cell growth *in vitro*. Methods: To test synergic effects, cell viability and apoptosis were analyzed after curcumin and triptolide combination treatment on ovarian cancer cell lines. Synergistic effects on apoptosis induction were determined by lactate dehydrogenase (LDH) leakage assay, intracellular reactive oxygen species (ROS) assay, mitochondrial membrane potential (MMP) loss assay and flow cytometry analysis. Critical regulators of cell proliferation and apoptosis related were analyzed by qRT-PCR and Western blotting. Results: We showed that the combination of curcumin and triptolide could synergistically inhibit ovarian cancer cell growth, and induce apoptosis, which is accompanied by HSP27 and HSP70, indicating that HSP27 and HSP70 play the important role in the synergic effect. Conclusions: From the result present here, curcumin and triptolide combination with lower concentration have a synergistic anti-tumor effect on ovarian cancer and which will have a good potential in clinical applications.

Keywords: Curcumin - triptolide - ovarian cancer cell line - cytotoxicity

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Introduction

Ovarian cancer is a malignant neoplasm in female, one of the highest fatality rates of all cancers. Ovarian cancer is usually diagnosed at an advanced stage leading to lack of specific symptoms and absence of reliable screening strategies (Schmitt et al., 2001; Muggia, 2009). The current standard therapy for ovarian cancer is surgical intervention followed by adjuvant carboplatin and taxane-based chemotherapy. Unfortunately, surgical treatment and chemotherapy often achieve poor therapeutic efficacy (Landis et al., 1999; Kaufmann et al., 2000), and the patients' ovary tissue will be damaged at the same time, therefore, new strategies or reagents to tackle this disease are needed.

Natural products play an important role in the area of cancer chemotherapy because of their excellent pharmacological activities and low toxicity. Curcumin, a type of yellow pigment that is extracted from the rhizome of turmeric, has been used for a long time as a food additive and a traditional medicine in Asian (Ono et al., 2013; Rozzo et al., 2013; Qiao et al., 2013; Wei et al., 2013). Triptolide is a diterpenoid triepoxide and the principal active ingredient of Tripterygium wilfordii Hook. f. that also has been used for hundreds of years, which has been used in the treatment of different diseases such as nephritis and rheumatoid arthritis for centuries (Wang et al., 2012; Pacak et al., 2012; Hsu et al.,2013; Tan et al., 2013; Huang et al., 2013). Recently, curcumin (Yu et al., 2011; Li et al., 2013; Zhou et al., 2013; Yin et al., 2013) and triptolide (Kim et al., 2010; Liu et al., 2012; Wen et al., 2012) are able to potently inhibit the growth of human cancer cells in vitro and prevents tumor growth in vivo via inhibiting cell proliferation and inducing apoptosis. However, both of curcumin and triptolide possess side effects in high concentration (Banjerdpongchai et al., 2005; Clawson et al., 2010; Antonoff et al., 2010; Li et al., 2010; Borja-Cacho et al., 2010; Shakibaei et al., 2013; Du et al., 2013; Jiang et al., 2013).

To decrease the side effects and enhance the efficacy of traditional Chinese medicine (TCM) prescriptions, combinations of different TCM herbs is alternative strategy for cancer therapy. The present study was aimed to analysis the combined effect of curcumin and triptolide on ovarian cancer cell lines in vitro.

Materials and Methods

Reagents and cell culture

Curcumin was purchased from the Sigma-Aldrich Trading Co, Ltd (Shanghai, China); it was dissolved in DMSO to obtain 1 mM stock solution and kept at -20 °C until use, and then diluted in medium to different concentrations. Triptolide (purity > 99.0%, Institute of

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Dermatology, Chinese Academy of Medical Sciences, Nanjing, China) was dissolved in dimethyl sulfoxide (DMSO) to obtain 1 μM stock solution and then was added in medium at required concentrations. Methyl–thiazdyldiphenyl–tetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), RNase A and propidium iodide (PI) were obtained from Sigma (St. Louis). Total protein extraction kit P1250 (Applygen Technologies Inc., Beijing, China); BCA protein assay kit (Biosynthesis Biotechnology Co., Ltd., Beijing, China); Primary antibody (Anti-Hsp27, anti-Hsp70 and anti-Hsp90) antibodies and secondary antibody (horseradish peroxidase (HRP)-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit) were from Santa Cruz Biotechnology (CA, USA). TransScript First-Strand cDNA Synthesis SuperMix was purchaseed from TransGen Biotech (Trans, Beijing, China). GeneRuler TM 100 bp DNA Ladder and DreamTaqTM Green PCR Master Mix were purchased from Fermentas Company (Fermentas, Shenzhen, China). Primers were synthetized by Sangon Biotech (Sangon, Shanghai, China).

Human ovarian cancer OVAR3, SKOV3, HO-8910 and A2780 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 μg/ml of streptomycin and 100 U/ml of penicillin at 37 °C in a humidified atmosphere of 5% CO2.

Proliferation analysis

The effect of curcumin on ovarian cancer OVAR3, SKOV3, HO-8910 and A2780 cell proliferation was assessed using MTT. Exponentially growing cells were seeded into 96-well plates using 1×10^4 cells per well for viability measurements and incubated for 24 h. The proliferation inhibitory curcumin (4, 8 and 16 μM) and triptolide (10, 20 and 40 nM) were added to the wells and incubated for varying times at 37 °C. On the day of collection, the cell number was measured at 24, 48 and 72 h using a standard methylthiazolyltetrazolium (MTT)-based assay. One hundred microliters of MTT (working concentration of 1 mg/mL) was added to each well, and the cells were returned to the incubator and incubated at 37 °C for 4 h. After removing the supernatant, 300 μL of DMSO was added to dissolve the formazan crystals, and the optical density was detected at 490 nm using a microplate spectrophotometer (SpectraMax, Molecular Devices, CA, USA). The data represent the mean of three readings, and each dose was tested in triplicate.

Lactate dehydrogenase (LDH) leakage measurement

During damage to cell cytoplasmic membranes, LDH is released from cells and into the surrounding cell culture supernatant. Quantitation of LDH in cell culture supernatant is one method by which investigators analyze cell death levels. After ovarian cancer OVAR3 and SKOV3 cells treated with drugs alone or combination for 24 h. Culture medium was aspirated and centrifuged at 2000 g for 10 min to obtain a cell free supernatant. LDH activity in medium was examined by conversion of lactate to pyruvate using a LDH leakage detection kit (Sigma).

Reactive oxygen species (ROS) assay

ROS generation was assessed using fluorescence dye DCFH-DA. Briefly, OVAR3 and SKOV3 cells (1×10^4 per well) were cultured in 96-well black bottom culture plate, adhere for 24 hours in a CO2 incubator at 37 °C. The cells were then challenged with drugs alone or combination for 24 h. Discard medium and incubate with DCFH-DA (10 μM, Ex/Em = 485 nm/528 nm) for 30 minutes at 37 °C. Aspirate the reaction mixture and replaced by 200 μL of PBS in each well, shaking for 10 minutes at room temperature in the dark. Fluorescence intensity was measured using a Multiwell microplate reader (FLUOstar), and the values were expressed as a percentage of fuorescence intensity.

Mitochondrial membrane potential assay

MitoTracker Red CMXRos (Invitrogen, USA) accumulated in mitochondria during apoptosis; the fluorescence emission will be changed from green to red. Therefore, the apoptosis could be evaluated by the loss of mitochondrial membrane potential. After the drug treatments, cells were harvested, and then centrifuged at 350 × g for 5 min; the cell pellet was resuspended in 0.1 μM final concentration of MitoTracker Red CMXRos (dissolved in DMSO) for 20 min. Washed and resuspended in PBS, fixed with 4% paraformaldehyde. After the final wash with PBS, the microplate were read by a spectrophotometer.

Apoptosis analysis

Approximately 1×10^6 cells were cultured with medium for 24 h. The cells were then treated for a further 48 h with drugs. The foating and adherent cells were collected together for the analysis. Cells were washed with PBS and centrifuged, fixed with 70% (v/v) ice-cold methanol overnight at 4 °C. The fixed cells were collected by centrifugation, washed with PBS, and then resuspended in 100 μl of PBS containing 40 μg/ml RNase A, after being stained 5 μl Annexin V-FITC and 50 μg/ml propidium iodide, cells were placed in dark for 30 min at room temperature. Apoptosis analysis was performed on a FACSCalibur fow cytometer (Becton Dickinson, CA, USA).

RNA isolation and real time RT-PCR analysis

OVAR3 and SKOV3 cells were cultured overnight at 37 °C before treatment. After cells were treated with drugs for 48 h, total RNA was prepared using the TRIzol reagent (Tiangen, Beijing, China), according to the manufacturer's instructions. RNA was reverse transcribed into first-strand cDNA using a kit (Tiangen, Beijing, China) following the manufacturer's procedure. The synthesized cDNA was used as a template for polymerase chain reaction (PCR) amplification. Real-time PCR was performed using a Thermal Cycler Dice Real Time PCR System (Takara, Japan). The primers used for SYBR Green real-time RT-PCR were as follows: for Hsp27, sense primer (CCA GAG CAG AGT CAG CCA GCA T) and antisense primer (CGA AGG TGA CTG GGA TGG TGA); for Hsp70, sense

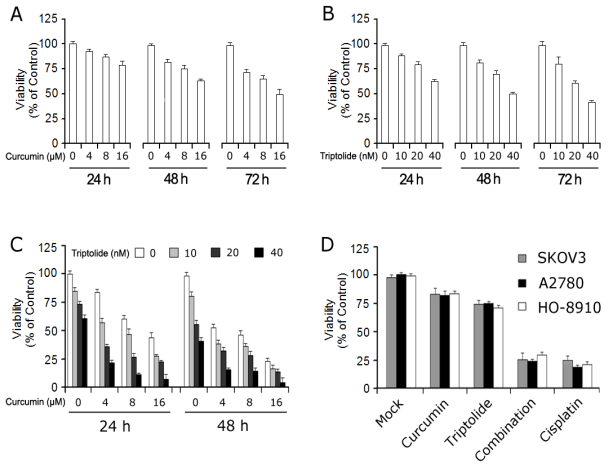

Figure 1. Growth Suppression Induced by Combination of Curcumin and Triptolide. (A) OVAR3 cells were treated by curcumin with various concentrations (4, 8 and 16 μM) for 24 h, 48 h and 72 h. (B) OVAR3 cells were treated by triptolide with various concentrations (10, 20 and 40 nM) for 24 h, 48h and 72 h. (C) The cells were treated with curcumin (4, 8 and 16 μM) and triptolide combination with various concentrations (10, 20 and 40 nM) for 24 h and 48 h. (D) Different ovarian cancer cell lines (SKOV3, A2780 and HO-8910) were used for drug combination (8 μM curcumin plus 20 nM triptolide)

primer (AGA GCC GAG CCG ACA GAG) and antisense primer (CAC CTT GCC GTG TTG GAA); for Hsp90, sense primer (TTA AGG TAC TAC ACA TCT GCC TCT) and antisense primer (TGC TTT CGG AGA CGT TCC ACA A); and for GAPDH, sense primer (AGC CTC AAG ATC ATC AGC AAT G) and antisense primer (ATG GAC TGT GGT CAT GAG TCC TT). A dissociation curve analysis of Hsp27, Hsp70, Hsp90 and GAPDH showed a single peak. The mean Ct of the gene of interest was calculated from triplicate measurements and normalized with the mean Ct of a control gene, GAPDH.

Western blot analysis

Cells were seeded in 6-well plate at a density of 2.5 × 10^5 cells and were then incubated overnight at 37 °C before treatment. After cells were treated with drugs for 48 h, the cells were harvested, washed with ice-cold PBS, suspended in 200 μl of ice-cold solubilizing buffer (300 mM NaCl, 50 mM Tris-HCl (pH 7.6), 0.5% (v/v) Triton X-100, 2 mmol phenylmethanesulfonyl fluoride, 2 μl/ml aprotinin, and 2 μl/ml leupeptin), and incubated at 4 °C for 1 h. The extracts were cleared by centrifugation at 12 000 rpm for 20 min at 4 °C. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Cell lysate were mixed with an equal volume of 5 × SDS sample buffer, boiled for 5 min, and then separated by 10% SDS-PAGE gels and transferred to 0.22 μm polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 0.05 g/ml non-fat dry milk, incubated with primary antibody, including Hsp27 (1:300), Hsp70 (1:800) and Hsp90 (1:800) and GAPDH (1:1000) at 4 °C over night. After washes three times, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, following three times of washing with TBST (Tris-buffered solution, pH 7.6, 0.05% Tween-20), and then developed in ECL-detection reagents, followed by exposure to X-ray film..

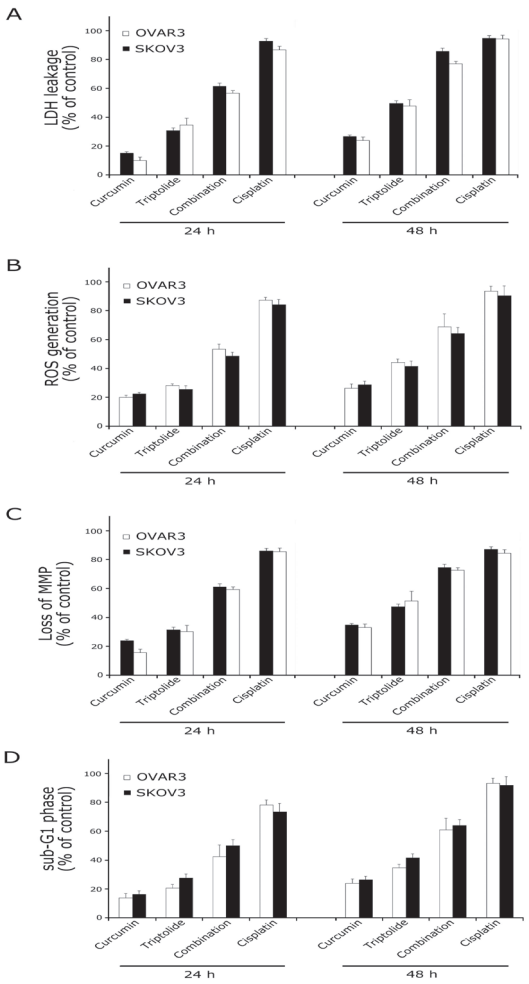

Figure 2. Cell Cytotoxicity of Curcumin and Triptolide Combination on OVAR3 and SKOV3 Cells. (A) Lactate dehydrogenase (LDH) leakage assay for drug combination (8 μM curcumin plus 20 nM triptolide) exposure for 24 h and 48 h. (B) Intracellular reactive oxygen species (ROS) generation assay after drug combination (8 μM curcumin plus 20 nM triptolide) treatment. (C) Mitochondrial membrane potential (MMP) loss assay under 8 μM curcumin plus 20 nM triptolide treatment. (D) The sub-G1 phase cells after drug combination (8 μM curcumin plus 20 nM triptolide)

Statistical analysis

All results are expressed as mean ± standard deviation (SD). Statistical analysis of the difference between treated and untreated groups was performed with Student's t-test. Values of $P < 0.05$ were considered as significant differences.

Results

Curcumin and triptolide synergistically inhibit ovarian cancer cell lines growth

The MTT assay was used to assess the cytotoxicity of different concentrations of curcumin (4, 8 and 16 μM) and triptolide (10, 20 and 40 nM) on OVAR3 cells after 24, 48 and 72h. As seen in Figure 1, the growth of OVAR3 cells was significantly inhibited by curcumin (Figure 1A) and triptolide (Figure 1B) in a dose dependent

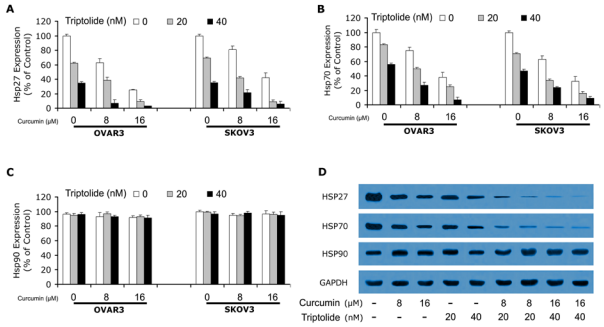

Figure 3. Heat Shock Protein (HSP) Expression after Combination of Curcumin and Triptolide on OVAR3 and SKOV3 Cells. (A) mRNA expression level of Hsp27 detected by qRT-PCR. (B) Hsp70 mRNA expression level analyzed by qRT-PCR. (C) Hsp90 mRNA expression level analysis by qRT-PCR. (D) Western blotting of cell lysates of OVAR3 cells treated with drug used alone or combination (8 μM curcumin plus 20 nM triptolide)

manner. To evaluate the synergistic effect of curcumin and triptolide in combination for subsequent studies, the viability of OVAR3 cells significantly decreased in combination compared with drug used separately (Figure 1C). Moreover, other ovarian cancer SKOV3, A2780 and HO-8910 cells growth also significantly suppressed by curcumin and triptolide in combination (Figure 1D) ($P < 0.05$).

Induction of apoptosis by curcumin and triptolide combination in ovarian cancer cells

To determine whether inhibitory effect of curcumin and triptolide on the cell viability is related to the apoptosis, lactate dehydrogenase (LDH) leakage assay, intracellular reactive oxygen species (ROS) assay, mitochondrial membrane potential (MMP) loss assay and flow cytometry analysis were performed. As is shown in Figure 2, 8 μM curcumin and 20 nM triptolide combination were used to treat OVAR3 and SKOV3 cells. From the LDH leakage assay, as is shown in Figure 2A, the LDH leakage in drug combination is significantly increased compared to drug used separately. ROS was measured as an increase in fluorescence intensity quantification by monitor the enzymatic cleavage of DCFH-DA. In combination exposure, ROS generation is significantly increased at 24 h (Figure 2B). ROS production usually accompanies with MMP loss, after the cells were exposed to the drug treatment for 24 h, MMP loss was significantly increased (Figure 2C). Lastly, apoptosis determination by flow cytometry were used after drug treatment, the number of cells in the sub-G1 phase (Figure 2D) were also significantly increased when compared to drugs used alone ($P < 0.05$).

Hsp27 and Hsp70 down regulated by curcumin and triptolide combination

Among the heat shock proteins, HSP27, HSP70 and HSP90 were important target in anti-tumor therapy. Whether they play a role in apoptosis induced by curcumin and triptolide combination is still unknown. As indicated in Figure 3, the mRNA level of HSP27 (Figure 3A) and HSP70 (Figure 3B) significantly dropped with curcumin and triptolide combination compared with drug used alone, whereas HSP90 mRNA level have no much changes in OVAR3 and SKOV3 cells (Figure 3C). From the Western blot analysis, HSP 27 and HSP70 expression also significantly decreased (Figure 3D) but HSP 90. Therefore, our results indicate that curcumin and triptolide combination induce the decline of HSP27 and HSP70 may be the crucial mechanism underlying the synergic apoptosis on ovarian cancer cells.

Discussion

In women, ovarian cancer is the fifth common gynecological malignancy and one of the leading causes of death. Ovarian cancer is often detected at an advanced stage because of lacking effective strategies for early diagnosis. Cisplatin-based chemotherapy has been used for women to treat ovarian cancer for a long period (Landis et al., 1999; Schmitt et al., 2001; Muggia, 2009). Cisplatin-based chemotherapy is often associated with various side effects. Other drugs such as paclitaxel, docetaxel, vinorelbine, irinotecan and gemcitabine are currently being used in combination with cisplatin to achieve better survival. However, the limited efficacy of cytotoxic chemotherapy remains a key obstacle in the treatment of advanced ovarian cancer (Kaufmann et al., 2000). Therefore, alternative strategy would be one that not only decreases the dose of chemotherapeutics but enhances the sensitivity of cancer cells to chemotherapeutics. Recently, tumor therapy by traditional Chinese herb such as curcumin (Gandhy et al., 2012; Khaw et al., 2013; Blakemore et al., 2013) and triptolide (Phillips et al., 2007; Matsui et al., 2008; Antonoff et al., 2009; Zhu et al., 2012; Dudeja et al., 2013) is becoming more and more attractive (Du et al., 2013). We therefore attempted to examine the effects of curcumin and triptolide on ovarian cancer.

In the present study, we shown that curcumin combined with triptolide acts in synergy to inhibit ovarian cancer cell line growth and induce its apoptosis. Previous studies have shown that HSP27, HSP70 and HSP90 play a key role in carcinogenesis and tumor chemotherapy. Newly reports have shown that these heat shock proteins may be act as a target for anti-tumor therapy [35, 36]. In the present study, we found that HSP27 and HSP70 declined after combination treatment, which means that the synergetic suppression induced by curcumin and triptolide combination was more likely due to the proliferation related pathway triggered by HSP27 and HSP70 but not HSP90.

In summary, the present study shown that growth suppression by curcumin and triptolide combination on ovarian cancer cells resulted from the induction of apoptosis in vitro. Our findings may lay a foundation for ovarian cancer clinical treatment.

Acknowledgements

The author(s) declare that they have no competing interests.

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