Curcuma zedoaria (Berg.) Rosc. essential oil and paclitaxel synergistically enhance the apoptosis of SKOV3 cells

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Abstract. Curcuma zedoaria (Berg.) Rosc. essential oil (CZEO) is the major component of Curcuma zedoaria (Berg.) Rosc., a traditional medicine with antitumor activity. Paclitaxel (PTX) is a first-line chemotherapeutic agent used to treat patients with ovarian cancer. These compounds directly target nuclear DNA, in order to suppress or inhibit tumor cell growth. The present study aimed to determine the synergistic antitumor effects of CZEO and PTX on the SKOV3 human ovarian cancer cell line. SKOV3 cells were treated with CZEO, PTX or a combination of the two and cell viability was detected using cell counting kit-8. In addition, flow cytometry was used to determined cell apoptosis as well as for cell cycle analysis. The morphological changes of apoptosis were assessed using Hoechst 33342 staining and the expression levels of apoptotic pathway proteins, including caspase-3 and poly (ADP-ribose) polymerase (PARP), were quantified using western blot analysis. The cell viability assay indicated that either of these compounds alone or in combination suppressed the growth of SKOV3 cells. Furthermore, flow cytometric analysis indicated that treatment with a combination of CZEO and PTX resulted in increased inhibition of proliferation and induction of apoptosis of SKOV3 cells, as compared with treatment with either of the compounds alone. In addition, the protein expression levels of caspase-3 were increased following treatment with a combination of CZEO and PTX. The results of the present study suggested that CZEO and PTX synergistically enhanced the inhibition of SKOV3 proliferation, and the possible underlying mechanism may be the induction of cell apoptosis and cell cycle arrest. This therefore indicated that PTX supplemented with CZEO may be an effective treatment strategy to decrease the dose and toxicity of PTX.

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Introduction

Paclitaxel (PTX) is a first-line chemotherapeutic agent used to treat patients with ovarian cancer. PTX is a novel microtubule-damaging agent that stabilizes the structure of tubulin, by promoting its polymerization and suppressing its depolymerization (1). Therefore, PTX effectively inhibits cellular mitosis (2). *Curcuma zedoaria* (Berg.) Rosc. is a traditional medicine that is used to treat flatulence, dyspepsia, menstrual disorders, cough and fever (3). Furthermore, previous studies have demonstrated its anticancer action (4-6). A major component of *Curcuma zedoaria* (Berg.) Rosc. is essential oil (3,7-9). PTX and *Curcuma zedoaria* (Berg.) Rosc. essential oil (CZEO) are considered to be potential anticancer drugs. These compounds directly target nuclear DNA, in order to suppress or inhibit tumor cell growth. Furthermore, tumor immunogenicity can be increased by either treatment, resulting in the induction of a stronger cytotoxic response to tumor cells (10).

Apoptosis, also known as programmed cell death, is an active intracellular death program that has a key role in the maintenance of organisms (11). Caspases, a family of cysteine proteases, are the key executors of apoptosis (12). Caspase-3 is situated at pivotal junctions in apoptosis pathways and its activation leads to a series of cellular events (13). Poly adenosine diphosphate-ribose polymerase (PARP), a nuclear protein involved in the DNA damage response, is a well-known substrate for caspase-3 cleavage during apoptosis (14).

In the present study, in order to explore the antitumor effects of the combined treatment of PTX and CZEO, an *in vitro* experiment was conducted using the SKOV3 human ovarian cancer cell line. The effects of the treatment on cell growth were determined, and the underlying mechanisms were investigated.

Materials and methods

Chemicals and reagents. Cell Counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). RPMI-1640 media was purchased from Thermo Fisher Scientific (Beijing, China), and fetal bovine serum (FBS) was obtained from Gibco Life Technologies (Carlsbad, CA, USA). Propidium iodide (PI) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Curcuma zedoaria (Berg.) Rosc. essential oil. Zedoray Turmeric Oil Injection, containing 0.1 g/10 ml CZEO, was purchased from Xuzhou Lai'en Pharmaceutical Co., Ltd (Xuzhou, China). The main components of CZEO are neocurdione, curdione, germacrone, curzerene, furanodiene, γ-elemene and 8,9-dehydro-9-formyl-cycloisolongifolene (15).

Cell lines and cell culture. The SKOV3 human ovarian cancer cells were purchased from the Cell Bank of the Cinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco Life Technologies), at 37°C in a humidified atmosphere containing 5% CO_2.

Cell proliferation assay. The SKOV3 cells were dispersed in culture medium supplemented with 10% FBS and were seeded in a 96-well plate at a density of $2x10^4$ cells/ml. The cells were then treated with PTX (10 nM; Xi'an Sanjiang Bio-Engineering Co. Ltd, Xi'an, China), CZEO (62.5 μg/ml) or a combination of the two for 48 h, following 24 h growth. Cell in the control group were treated with 0.2 ml phosphate-buffered saline (PBS). A total of 10 μl CCK-8 was added to each well, and the cells were then cultured in an incubator for a further 3 h. The optical density (OD) of the cells was measured at 490 nm using a microplate spectrophotometer (Spectramax 190; Molecular Devices Corp., Sunnyvale, CA, USA). Each concentration corresponds to three parallel wells for detection. The cell viability was calculated as follows: Cell viability=$(OD_{treated\ group}-OD_{blank})/(OD_{control}-OD_{blank})$x100. The interaction between PTK and CZEO was analyzed using CalcuSyn 2.0 software (Biosoft, Cambridge, UK), using the Chou and Talalay method (16). The combination index (CI) was determined on the basis of the isobologram analysis: CI<1, synergistic effect; CI=1, additive effect; and CI>1, antagonistic effect.

Hoechst 33342 staining. The SKOV3 cells ($5x10^5$ cells/ml) were seeded in six-well plates, and cultured overnight. Following treatment with PTX (10 nM), CZEO (62.5 μg/ml) or a combination of the two for 24-48 h in a 37°C incubator containing 5% CO_2, the cells were incubated with Hoechst 33342 (5 μl; Sigma-Aldrich) for 30 min. Hoechst 33342-stained cell nuclei were observed using an inverted fluorescence microscope (BX60; Olympus Optical Co., Tokyo, Japan), and images were captured with a confocal microscope (LSM510; Carl Zeiss AG, Oberkochen, Germany).

Detection of apoptosis by flow cytometry. The SKOV3 cells ($5x10^5$ cells/ml) were seeded in six-well plates and cultured for 12 h. Following treatment of the cells with PTX, CZEO, or a combination of the two for 48 h in a 37°C incubator containing 5% CO_2, the cells were collected and washed twice with cold PBS in order to remove the medium. The cells were then resuspended in 100 μl of 1X binding buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), prior to the addition of 5 μl Annexin-V (Invitrogen Life Technologies) and 1 μl propidium iodide (PI; Sigma-Aldrich) and incubation in the dark on ice. Finally, 400 μl of 1X binding buffer was added to the cells and they were analyzed by flow cytometry using a BD FACSAria cell sorter (Becton-Dickinson, San Jose, CA, USA).

Cell cycle analysis by flow cytometry. The SKOV3 cells were seeded in six-well plates and treated as mentioned in the previous section. The cells were then harvested, washed twice with PBS and resuspended in 0.3 ml PBS. RNase A (Roche, Indianapolis, IN, USA) was added, in order to digest the cells for 30 min at 37°C, after which the cells were collected and washed twice with PBS. The reaction was terminated by placing the mixture on ice. PI was added to the cells in the dark, in order to prepare the samples for flow cytometry.

Western blotting of key signal proteins caspase-3 and PARP for apoptosis. The SKOV3 cells ($5x10^5$ cells/ml) were seeded in 60 mm culture dishes in the presence of PTX, CZEO, or a combination of the two for 48 h, and cultured in a 37°C incubator containing 5% CO_2. The cells were collected according to the standard western blotting procedure. The protein samples were used straight after protein concentration determination, or were stored at -20°C until further use.

Protein concentration of the samples was determined using bicinchoninic acid protein reagent .(Pierce Biotechnology, Inc., Rockford, IL, USA). The protein samples were separated by SDS electrophoresis on polyacrylamide gels and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). The membranes were then blocked in 5% bovine serum albumin (Sigma-Aldrich) for 3 h at room temperature, followed by incubation for 2 h at room temperature with primary antibodies (rabbit polyclonal anti-PARP and anti-caspase-3; 1:1,000; Cell Signaling Technology, Inc., Beverly, MA, USA). The membranes were washed (3x10 min) in tris-buffered saline with Tween® (TBST) and were then incubated with a horse-radish peroxidase-conjugated sheep anti-rabbit secondary antibody (1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature, followed by further washing (3x10 min) with TBST. Immunoblot signals were detected by Odyssey Infrared Imaging v1.2 system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis. All of the data are expressed as the mean ± standard error of the mean. Student's t-test was used to determine the statistical significance between two observations and a one-way analysis of variance followed by the Bonferroni test were used for the multiple comparisons. Statistical analyses were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). P≤0.05 was considered to indicate a statistically significant difference.

Results

Treatment with a combination of CZEO and PTX significantly suppresses proliferation of SKOV3 cells. Treatment with CZEO or PTX significantly inhibited the viability of SKOV3 cells in a dose-dependent manner, at concentrations between 2.5 and 40 nM PTX, and between 15.625 and 250 μg/ml CZEO (Fig. 1A). Furthermore, treatment with a combination of CZEO and PTX enhanced the suppressive effect on the SKOV3 cells (Fig. 1B; confidence interval, 0.526 to 0.705).

Treatment with a combination of CZEO and PTX enhances the apoptosis of SKOV3 cells and induces cell cycle arrest at G_2/M phase. Flow cytometry was used to detect apoptosis, following
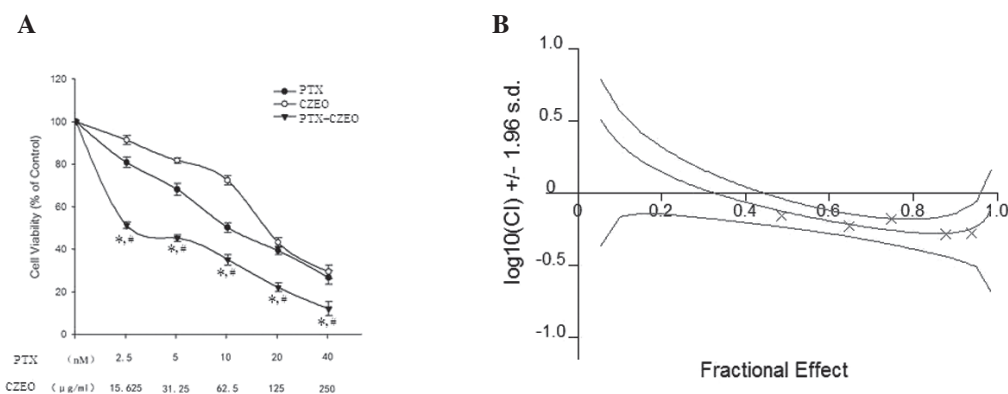
Figure 1. Treatment with a combination of CZEO and PTX significantly inhibited the proliferation of SKOV3 human ovarian cancer cells. (A) Survival rates of SKOV3, as detected using Cell Counting kit-8 (*P<0.05 vs. PTX; #P<0.05 vs. CZEO). (B) Synergistic effects of CZEO and PTX, as analyzed using CalcuSyn software. The middle line represents curve fitting of the CI values as a function of fractional effect and data points are indicated by 'X'. The other two lines represent the 95% confidence interval of the values. CI<1 indicates a synergistic effect. PTX, paclitaxel; CZEO, *Curcuma zedoaria* (Berg.) Rosc. essential oil; CI, combination index.
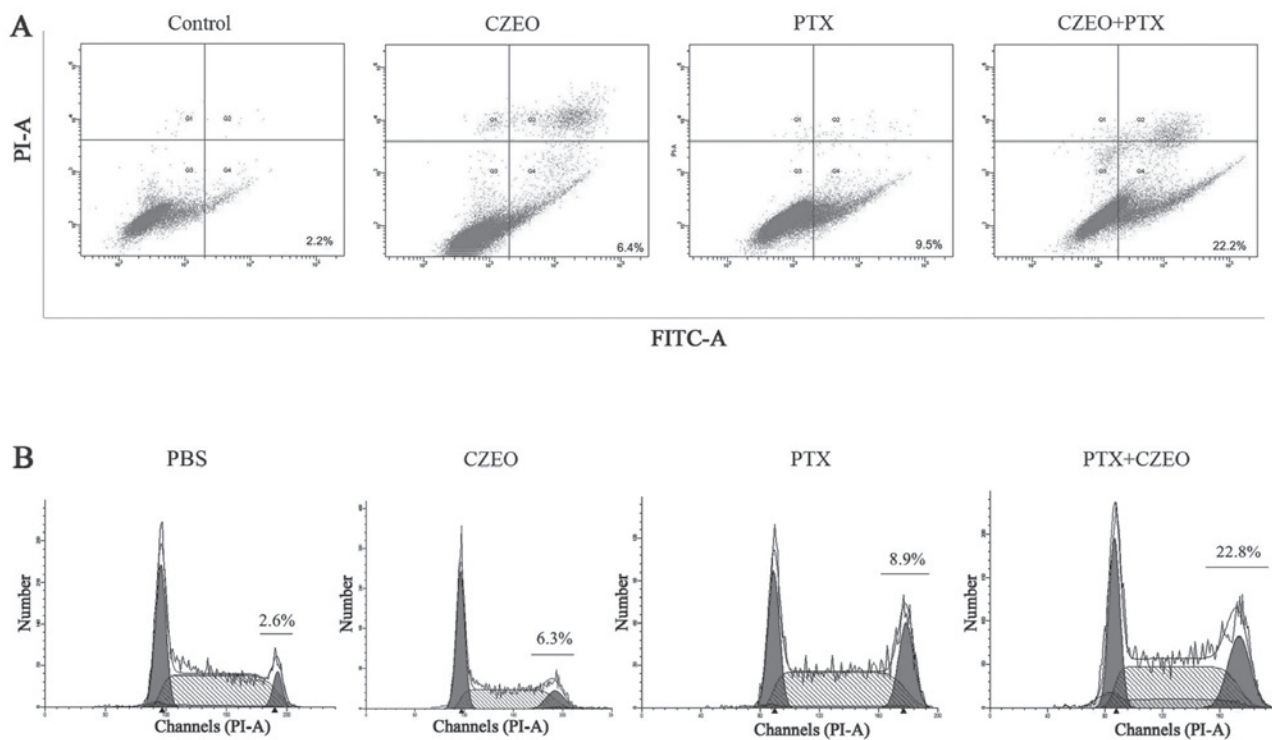

Figure 2. Effect of the combined treatment of PTX and CZEO on cell cycle distribution and apoptosis of SKOV3 human ovarian cancer cells. (A) Apoptosis and (B) cell cycle distribution were detected using flow cytometry. PBS, phosphate-buffered saline; PTX, paclitaxel; CZEO, *Curcuma zedoaria* (Berg.) Rosc. essential oil; FITC, fluorescein isothiocyanate; PI, propidium iodide.

treatment of the SKOV3 cells with CZEO (62.5 μg/ml), PTX (10 nM), or a combination of the two for 48 h. The cells were collected for dual staining using Annexin V/PI and then underwent flow cytometry. The cells stained with Annexin V but not PI were considered to be in the early-apoptotic phase. Treatment with a combination of CZEO and PTX significantly enhanced the rate of apoptosis (Fig. 2A). The rate of apoptosis of the cells treated with CZEO (62.5 μg/ml), PTX (10 nM) and a combination of the two was 6.4%, 9.5% and 22.2%, respectively.

Flow cytometry was used to perform a cell cycle analysis, following treatment of the SKOV3 cells with CZEO (62.5 μg/ml), PTX (10 nM), or a combination of the two for 24 h. The number of cells arrested at G_2/M phase were significantly increased (Fig. 2B), which may be partially attributed to the anti-proliferative effects of CZEO and PTX.

Morphological changes. The SKOV3 cells exhibited morphological changes characteristic of apoptosis following treatment with CZEO (62.5 μg/ml), PTX (10 nM), or a combination of the two. A microscopic observation demonstrated that the cells treated with CZEO (62.5 μg/ml), PTX (10 nM), or a combination of the two for 48 h had significantly reduced growth and exhibited morphological changes characteristic of apoptosis,
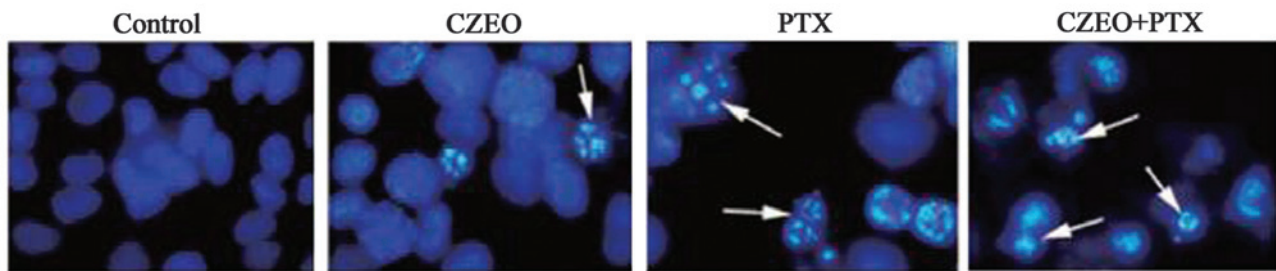
Figure 3. Hoechst 33342 staining was performed to detect the apoptosis of SKOV3 human ovarian cancer cells. Arrows indicate the cells which are immunoreactive for Hoechst 33342 staining (magnification, x1200). PTX, paclitaxel; CZEO, *Curcuma zedoaria* (Berg.) Rosc. essential oil.
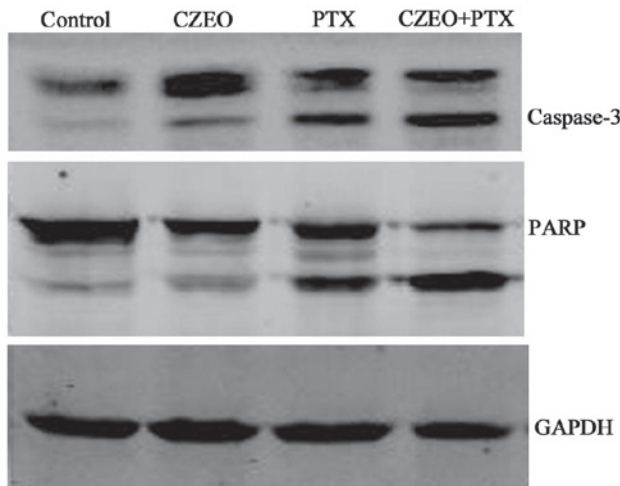

Figure 4. Expression levels of proteins involved in the apoptotic pathway, as detected by western blotting. PARP, poly (ADP-ribose) polymerase; PTX, paclitaxel; CZEO, *Curcuma zedoaria* (Berg.) Rosc. essential oil.

including widespread chromatin condensation, exocytosis, and condensation and fragmentation of nuclei (Fig. 3).

Hoechst 33342 staining was also performed to detect apoptosis following treatment with CZEO (62.5 μg/ml), PTX (10 nM) or a combination of the two for 48 h. The untreated control cells exhibited normal chromatin without condensation or fragmentation, with no bright staining in the nuclei, thus indicating the absence of dying cells. Whereas, the treated cells exhibited significantly condensed and fragmented chromatin, disintegration of nuclei and formation of apoptotic bodies.

Relative expression levels of apoptotic pathway proteins detected by western blotting. Following treatment with CZEO (62.5 μg/ml), PTX (10 nM), or a combination of the two for 24 h, the cells were harvested for western blotting, in order to detect the expression levels of proteins involved in the apoptotic pathway. Treatment with a combination of CZEO and PTX activated caspase-3, to form a cleaved product of the substrate PARP (Fig. 4).

Discussion

Ovarian cancer is one of the three most common malignant cancers that occur within the female reproductive tract, which poses a severe threat to the health of the female population. The incidence of ovarian cancer has increased annually during the past two decades (17). The early stages of ovarian cancer can be effectively treated with chemotherapy; however, ~70% of ovarian cancers are diagnosed at a late stage (18), which severely effects the life quality and mortality rates of patients, since the cancer cells develop resistance to drugs, such as PTX (19). Cyto-reductive surgery is the first-line therapy for patients with all stages of ovarian cancer, which is used in combination with chemotherapy or radiotherapy for patients with late stage or recurrent ovarian cancer, so as to improve their quality of life and chances of survival (20).

PTX is a potent drug of natural origin, which is isolated from the bark of the Pacific yew (*Taxus brevifolia*) (21), and is considered the first-line antitumor drug for ovarian cancer. However, the success of PTX chemotherapy in treating ovarian cancer is limited, due to its extreme toxicity (22,23). Therefore, developing a novel therapeutic strategy with higher therapeutic efficacy and lower toxicity is required.

Previous studies regarding combination chemotherapy have focused on identifying natural compounds that may increase the therapeutic index. Cang *et al* (24) reported that phenethyl isothiocyanate enhanced the apoptosis and α-tubulin hyperacetylation abilities of PTX in MCF7 and MDA-MB-231 breast cancer cell lines. Yang *et al* (25) previously demonstrated that luteolin could enhance PTX-induced apoptosis in MDA-MB-231 human breast cancer cells, by blocking signal transducer and activator of transcription 3. Hossein *et al* (19) showed that PectaSol-C modified citrus pectin could sensitize ovarian cancer cells to PTX by inducing apoptosis, which may lead to an accumulation of cells in the $subG_1$ and G_1 phases, and cleavage of caspase-3 (24-27). Numerous studies have reported that CZEO is a promising antitumor drug, which has a direct cytotoxic effect that inhibits tumor cell growth and proliferation, disrupts nuclear metabolism, inhibits angiogenesis and impairs the membrane potential, all of which can be lethal to cancer cells (3,15,28). To the best of our knowledge, the present study was the first to demonstrate the effect of the combination of CZEO and PTX in suppressing cancer cell growth.

The cell viability assay demonstrated that the tumor survival rate of the cells decreased following treatment with CZEO, in a dose-dependent manner, which is concordant with the findings of Chen *et al* (3) and Chen *et al* (15). Furthermore, the inhibitory effect was increased by treatment with the combination of CZEO and PTX. In addition, the combination also enhanced the rate of apoptosis, which was demonstrated by the observation of morphological changes. These results indicate that it may be possible to reduce the side effects of PTX whilst enhancing its clinical efficacy by using it in combination with CZEO.

PTX can arrest the cell cycle at the G_2/M phase and induce caspase-3 enzymatic activity (29-32). In the present study, treatment with the combination of CZEO and PTX increased the accumulation of cells in the G_2/M phase, and the expression levels of caspase-3. These results indicate that the synergistic antitumor effects of CZEO and PTX are achieved by inducing apoptosis and arresting the cell cycle at G_2/M phase.

In conclusion, the present study demonstrated that CZEO can sensitize ovarian cancer cells to PTX, through inducing apoptosis, which was the result of the accumulation of cells in the G_2/M phase, and cleavage of caspase-3. These results suggest that PTX supplemented with CZEO may be an effective treatment strategy to decrease the dose and toxicity of PTX. Further studies are required to clarify the signaling pathways and key molecules underlying the effects of a combination of CZEO and PTX in human ovarian cancer.

Acknowledgements

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