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ORIGINAL ARTICLE

Ovatodiolide exerts anticancer effects on human cervical cancer cells via mitotic catastrophe, apoptosis and inhibition of NF-kB pathway

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Summary

Purpose: *Being the second most prevalent cancer in females, cervical cancer causes significant mortality across the globe. Owing to the adverse effects and inefficiency of the currently used anticancer drugs, there are increasing efforts for the identification of safer and effective anticancer agents from plants. This study was undertaken to investigate the anticancer effects of Ovatodiolide, a plant-derived macrocyclic diterpenoid, against the human cervical cancer.*

Methods: *The anticancer effects were examined by WST-1 proliferation assay. DAPI and annexin V/propidium iodide (PI) staining were used for apoptosis detection. Flow cytometry was used for cell cycle analysis. Protein expression was used for cell cycle analysis.*

Results: *The results revealed that Ovatodiolide caused inhibition of the viability of all the cervical cancer cells with IC_{50} ranging from to 14 to 56 μM. Ovatodiolide exerted more profound antiproliferative effects on the DoTc2 cells with and IC_{50} of 14 μM. However, minimal cytotoxicity was observed for the normal cervical cells as evidenced from the IC_{50} of 100 μM. Ovatodiolide triggered apoptotic cell death of the DoTc2 cells. The induction of apoptosis was accompanied with increase in Bax and decrease in Bcl-2 expression. Ovatodiolide also caused arrest of the DoTc2 cells at the G2/M phase of the cell cycle, which was also accompanied with suppression of cyclin B1 expression. Investigation of the effects of Ovatodiolide on NF-kB expression revealed that the molecule caused significant decrease in the expression of the NF-kB expression.*

Conclusion: *Taken together, Ovatodiolide may prove a lead molecule for the development of systemic therapy for cervical cancer.*

Key words: *cervical cancer, apoptosis, cell cycle arrest, ovatodiolide*

Introduction

Plants have been used as source of medicines in different systems of traditional medicine since times immemorial [1]. The plants were used as extracts for the treatment of diseases and disorders but in the 19th century plant-derived pure molecules were used first in the treatment of human diseases [2]. Plants produce these molecules (referred to as secondary metabolites) as defense against different environmental stresses [3]. Because of their bioactivities, humans have used them for the treatment of deadly diseases such as cancer [4]. Diterpenoids form a large group of secondary metabolites in plants and have been reported to exhibit anticancer properties [5]. Ovatodiolide is a macrocyclic diterpenoid with significant pharmacological activities [6] and has been shown to inhibit the proliferation of several types of cancer cells. For example, Ovatodiolide has been shown to inhibit the growth and metastasis of the human breast cancer cells [7] and has been reported to trigger apoptotic cell

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death as well as cell cycle arrest of oral cancer cells [8]. In another study, it has been reported to suppress the tumorigenesis of the renal carcinoma [9]. However, there is no study to report the anticancer effects of this molecule on the human cervical cancer cells. Therefore, this study was undertaken to investigate the anticancer effects of Ovatodiolide against different human cervical cancer cells and an attempt was made to elucidate the underlying mechanisms. Cervical cancer imposes huge disease burden for populations across the globe. Approximately 0.26 million cervical cancer deaths and 0.53 million new cases of cervical cancer were reported in 2012 alone [10]. Owning to disproportionate incidence of cervical cancer in low and high-income countries, it is often referred to as the 'disease of disparity' [11]. Cervical cancer represents a major type of cancer [12]. The treatment for cervical cancer involves radical hysterectomy, chemotherapy and/or radiotherapy [13]. Herein, we report that Ovatodiolide inhibits the growth of cervical cancer via induction of apoptosis and cell cycle arrest and may prove a lead molecule for cervical cancer treatment and warrants further investigation.

Methods

Cell culture conditions

The human cervical cancer cell lines DoTc2, SiHa, HeLa, C33A and the normal cell line HCvEpC were obtained from the Cancer Research Institute of Beijing (Beijing, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Massachusetts, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Massachusetts, USA),100 µg/ml streptomycin and 100 U/ml penicillin G (Himedia, Pennsylvania, USA) in an incubator at 37°C with 5% CO_2.

Cell proliferation assay

The proliferation rate of the human cervical cancer cells and normal astrocytes HCvEpC was monitored by WST-1 assay. In brief, cervical cancer cells were cultured in 96-well plates at a density of 2×10^5 cells/well and treated with 0 to 100 µM concentrations of Ovatodiolide for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for 4 h. The absorbance was then measured at 450 nm using a victor 3 microplate reader to determine the proliferation.

Cell cycle analysis

The cultured human cervical cancer DoTc2 cells were firstly treated with varied concentrations of Ovatodiolide for 24 h at 37°C. The cells were then washed with phosphate buffered saline (PBS). Afterwards, the DoTc2cells were stained with propidium iodide (PI) and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Analysis of cell death

The DoTc2 cervical cancer cells were cultured in 24-well plates for 24 h at 37°C. The cells were then collected by centrifugation and washed with PBS. After this, the cells were stained 1.2 mM DAPI or 1 µl of acridine orange (AO)/ethidium bromide (EB) solution for 5 min. The DoTc2 cells were then washed with PBS and observed both by fluorescence and phase contrast microscopy. For annexin V/PI assay, DoTc2 cells (5×10^5 cells per well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC or PI. The percentage of apoptotic DoTc2 cells was determined by flow cytometry.

Table 1. Anticancer effects of Ovadiodine on the cervical cancer and normal cell lines expressed as IC_{50}

S. No	*Cell line*	*IC_{50}*
1	DoTc2	14
2	SiHa	25
3	HeLa	56
4	C33A	25
5	HCvEpC	100

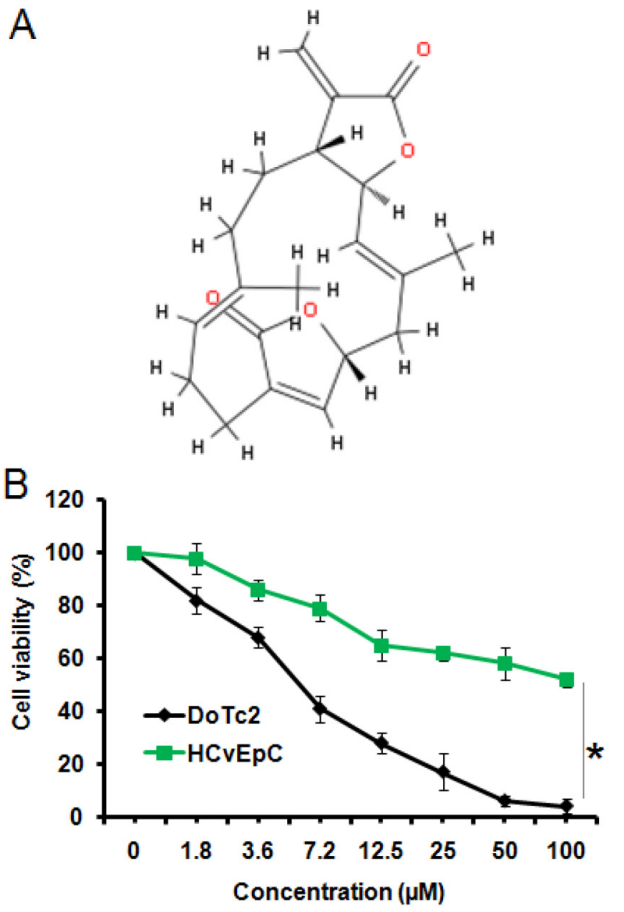

Figure 1. A: Chemical structure of Ovatodiolide. **B:** WST-1 assay showing the effects of Ovatodiolide on the proliferation of DoTc2 and HCvEpC cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Western blot analysis

The DoTc2 cells were then lysed in lysis buffer containing the protease inhibitor. Around 45 µg of proteins from each sample were separated on 10% SDS-PAGE, followed by transferring the gell to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally the protein bands of interest were detected by Odyssey Infrared Imaging System. Actin was used as control for normalization.

Statistics

The experiments were done in triplicate. The values presented are mean of three repeats ± SD. *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant. Student's *t*-test using GraphPad prism 7 software was employed for statistical analyses.

Results

Ovatodiolide exerts growth inhibitory effects in cervical cancer cells

The growth inhibitory effects of Ovatodiolide (Figure 1A) were examined against a panel of four cervical cancer cell lines (CaSki, DoTc2, SiHA, C-33A) and one normal cell line (HCvEpC). The results showed that Ovatodiolide suppressed the proliferation of all the cervical cancer cells with the IC_{50} ranging from 10 to 25 µM (Table 1). Nonetheless, it was observed that Ovatodiolide exerted more significant anticancer effects on the DoTc2 cells with an IC_{50} of 14 µM (Figure 1B). Evaluation of the antiproliferative effects of Ovatodiolide on the normal HCvEpC cells showed that the molecule exerted minimal growth inhibitory effects on these cells (IC_{50} 100 µM). The growth inhibitory effects of Ovatodiolide on the cervical cancer cells showed a dose-dependent pattern.

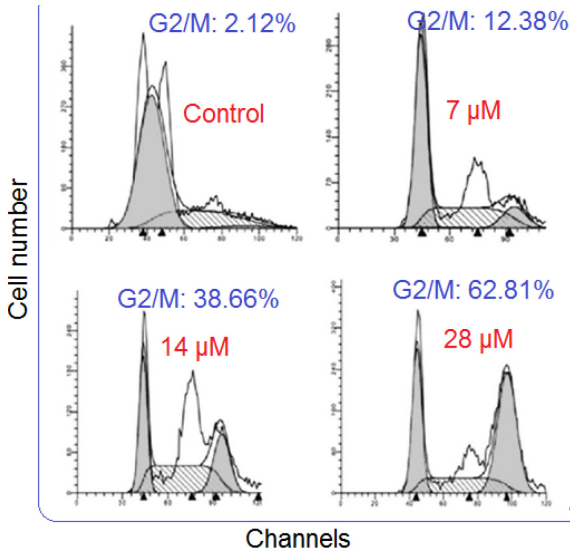

Figure 2. Flow cytometric analysis showing the effects of Ovatodiolide on the cell cycle distribution of the DoTc2 cells. The experiments were performed in triplicate and show that Ovatodiolide induces G2/M cell cycle arrest in these cells.

Ovatodiolide causes G2/M arrest of cervical cancer cells

The DoTc2 cervical cancer cells were treated with various concentrations of Ovatodiolide and the distribution of DoTc2 cells at each phase of the cell cycle was determined by flow cytometry. The results showed that the G2/M phase cells increased remarkably upon Ovatodiolide treatment. The percentage of G2/M phase cells were 2.21, 12.38, 38.66 and 62.81% at 0, 7, 14 and 28 µM concentrations of Ovatodiolide respectively, indicative of G2/M arrest of the DoTc2 cells (Figure 2). Western blot analysis was also performed to examine the effects of Ovatodiolide on the expression of cyclin B1. The results showed that Ovatodiolide inhibited the expression of cyclin B1 in a concentration-dependent manner (Figure 3).

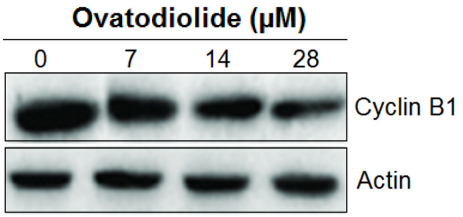

Figure 3. Western blotting showing the effects of Ovatodiolide on the expression of cyclin B1. The experiments were performed in triplicate and show that Ovatodiolide suppresses cyclin B1 dose-dependently.

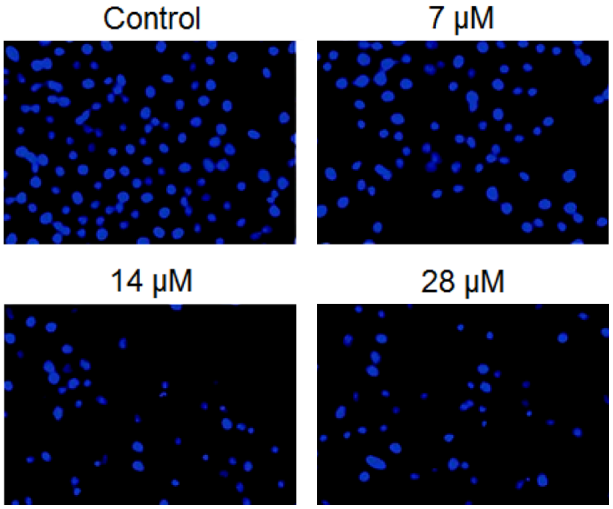

Figure 4. DAPI staining showing the effect of Ovatodiolide on the nuclear morphology of the DoTc2 cells. The Figure shows that Ovatodiolide induces apoptosis of these cells concentration-dependently.

Ovatodiolide causes the apoptotic cell death of DoTc2 cells

To decipher whether Ovatodiolide also causes apoptosis of the DoTc2 cervical cancer cells, DAPI and AO/EB staining assays were performed. Both of the DAPI (Figure 4) and AO/EB staining (Figure 5) showed that caused nuclear fragmentation of the DoTc2 cells in dose-dependent manner, suggestive of apoptosis. The Annexin V/PI staining assay showed that the percentage of apoptotic DoTc2 cells increased with increase in the concentration of Ovatodiolide. The percentage of apoptotic DoTc2 cells was 1.67, 32.24, 50.11 and 64.48% at Ovatodiolide concentrations of 0, 7, 14 and 28 μM (Figure 6). Western blot analysis was performed to determine the effects of Ovatodiolide on the expression of Bax and Bcl-2. The results showed that the expression of Bax increased, while that of bcl-2 decreased in the DoTc2 cells upon Ovatodiolide treatment (Figure 7).

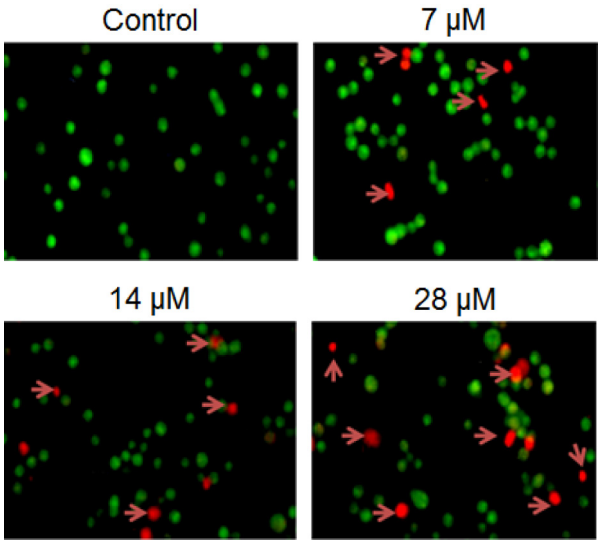

Figure 5. AO/EB staining showing the induction of apoptosis in the Ovatodiolide-treated DoTc2 cells (arrows depict apoptotic cells). The Figure reveals that Ovatodiolide triggers apoptosis of these cells concentration-dependently. The experiments were performed in triplicate.

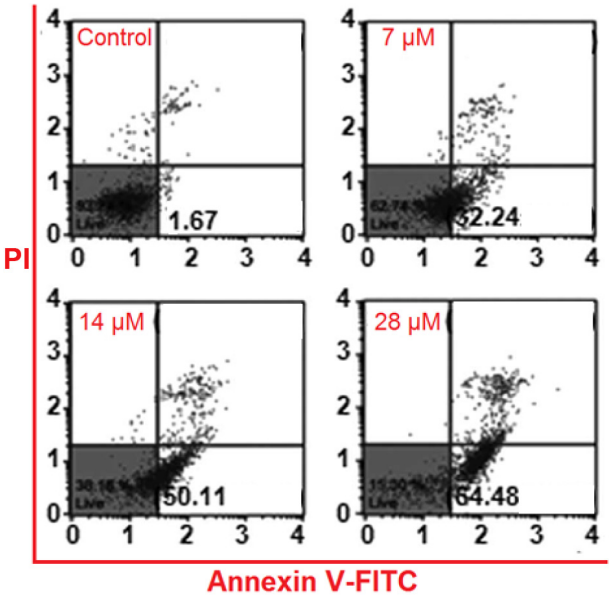

Figure 6. Annexin V/PI staining showing the effects of Ovatodiolide on the percentage of apoptosis in DoTc2 cells. The Figure shows that the percentage of apoptotic DoTc2 cells increased with increasing of Ovadiolide concentration. The experiments were performed in triplicate.

Ovatodiolide inhibits the expression of NF-kB in the DoTc2 cells

The effects of Ovatodiolide were also investigated on the expression of the NF-kB in the DoTc2 cells. The results showed that the expression of NF-kB was significantly and concentration-dependently decreased upon treatment with Ovatodiolide (Figure 8).

Discussion

Because of the drawbacks of cervical cancer chemotherapy, the development of new therapeutic approaches are of utmost priority for researchers across the globe. Huge research efforts are devoted to explore potent treatments and to minimise the

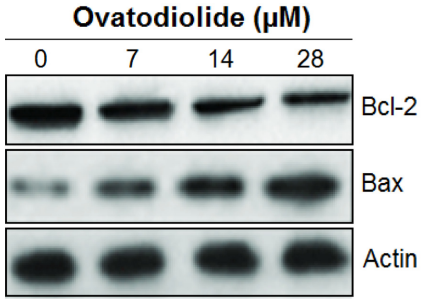

Figure 7. Effect of Ovatodiolide on the expression of Bcl-2 and Bax in DoTc2 cells as depicted by western blot analysis. The experiments were performed in triplicate and show that the expression of Bcl-2 decreases and of Bax increases upon Ovatodiolide treatment.

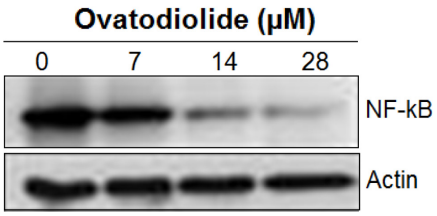

Figure 8. Effect of Ovatodiolide on the expression of NF-kB in DoTc2 cells as depicted by western blot analysis. The experiments were performed in triplicate and show that the expression of NF-kB decreases upon Ovatodiolide treatment.

toxicity of the different drugs [14]. Many of the anticancer drugs exhibit narrow therapeutic window because of their low selectivity against cancer cells. So another goal of the researchers is to develop drugs that selectively target cancer cells or reduce their malignant potential without any effect on normal cells [15]. One of the approaches is to explore the chemical scaffololds isolated from terrestrial plants [16]. Herein, the effects of the diterpenoid Ovatodiolide were examined against a panel of cervical cancer cells. The results showed that Ovatodiolide selectively killed the cervical cancer cells with more profound effects on the DoTc2 cells. The anticancer effects Ovatodiolide have also been reported in previous studies. Ovatodiolide has been shown to suppress the growth of breast cancer and myeloid leukemia cells [17,18]. It has also been reported to halt the proliferation of glioblastoma cells [19]. Previous studies have also shown that Ovatodiolide induces G2/M cell cycle arrest of cancer cells [20]. In another study, Ovatodiolide caused cell cycle arrest of oral cancer cells [8]. Therefore, we also investigated the effects of Ovatodiolide on cell cycle distribution of the DoTc2 cells and found that the molecule caused arrest of the DoTc2 cells at the G2/M checkpoint of the cell cycle which was also accompanied by depletion of cyclin B1 expression. DAPI and AO/EB staining of the Ovatodiolide-treated DoTc2 cervical cancer cells revealed that the molecule caused nuclear fragmentation of the DoTc2 cells, suggestive of apoptosis. Apoptosis was further confirmed by Bax and Bcl-2 expression in the DoTc2 cells. Bax and Bcl-2 are important biomarker proteins of apoptosis [21]. The results showed that Ovatodiolide caused upregulation of Bax and downregulation of Bcl-2. These results are in concordance with previous investigations wherein Ovatodiolide has been reported to trigger apoptosis in nasopharyngeal cancer cells [22]. Finally, Ovatodiolide was also found to decrease the expression of the NF-kB signalling pathway in DoTc2 cervical cancer cells which in agreement with previous investigations wherein Ovatodiolide has been reported to suppress the expression of NF-kB in pancreatic cancer cells [22].

Conclusion

The findings of this study showed that Ovatodiolide exerts growth inhibitory effects in the DoTc2 cells via ROS-mediated apoptosis and cell cycle arrest. Ovatodiolide also suppressed the invasion of the DoTc2 cells via inhibition of NF-kB expression. Taken together, Ovatodiolide may prove a lead molecule for the development of systemic therapy for cervical cancer.

Conflict of interests

The authors declare no conflict of interests.

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