ORIGINAL ARTICLE

Anticancer effects of ovatodiolide on human prostate cancer cells involves cell cycle arrest, apoptosis and blocking of Ras/ Raf/MEK/ERK signaling pathway

Dongsheng Jia^{1*}, Jianbo Zheng^{1*}, Junli Yu², Ning Zhao¹, Shengxing Lu¹, Dongfang Hao¹

¹Department of Urology, Zibo Central Hospital, Zibo, Shandong, 255036 China. ²Zibo Center for Disease Control and Prevention, Zibo, Shandong 255026, China.

in

*These two authors contributed equally to this work.

Summary

Purpose: The current research was set with a goal to characterize the anticancer role of ovatodiolide against human prostate cancer along with the underlying mechanism of its action.

Methods: The proliferation of prostate cancer cells was sessed by using the CCK8 reagent. DAPI and acridine of ange (AO)/ ethidium bromide (EB) staining procedures were employed for the analysis of cell apoptosis. Flow cytometric examination of prostate cancer cells was undertaken for the mitotic cell cycle analysis. The western blotting technique was used for the inference of expression levels of the proteins of interest.



In vitro administration of ovatodiolide led to de-Results: cline of proliferation of prostate cancer cells. The reduction proliferative vates was attributed to the induction of apoptosis of prostate cancer cells and mitotic cell cycle arrest. Furthermore, the anticancer effects of ovatodiolide on pros-

tate cancer cells were exerted through the inhibition of Ras/ Raf/MEK/ERK signaling cascade.

Conclusion: This study established the anticancer role of diterpenoid ovatodiolide in restricting the growth and proliferation of human prostate cancer cells.

Key words: ovatodiolide, prostate cancer, anticancer, apoptosis, cell cycle arrest, flow cytometry

Introduction

Cancer is one of the most deadly human diseases and poses a serious threat to health and well being of humans [1]. Prostate cancer is the most prevalent cancer in males and in North America it ranks 2nd in terms of cancer-specific mortality among the male population [2]. The most common reason for high mortality rates of prostate cancer is its metastatic spread, the mechanics of which are far from being fully understood [3]. The traditional chemo and radiotherapy-based anticancer approaches along with surgical interventions have been seen with limited success because of the chances of recurrence of this disease [4]. Hence,

the researchers are actively engaged in exploring the cellular targets for the management of prostate cancer and to develop more potent anticancer agents against this malignancy. In this regard, the recent research studies have laid lot of focus on the characterization of natural compounds for their anticancer effects against the growth and proliferation of prostate cancer cells [5]. The natural compounds possess health beneficial effects on human body and they act as vital source for the development of effective drug molecules [6]. These compounds possess antioxidant and anticancer properties [7]. Taking these facts into consideration, the

Corresponding author: Dongfang Hao, MD. Department of Urology, Zibo Central Hospital, League West Rd, Zibo, No.54 of Communist Youth, Shandong, China 255036.

Tel and Fax: +86 0533 2360228, Email: hdf1229@126.com Received: 18/01/2020; Accepted: 11/02/2020

present research work was aimed at the exploration of the anticancer effects of a diterpenoid compound, ovatodiolide, against human prostate cancer cells. Ovatodiolide is the principal bioactive component of Anisomeles indica [8]. It has been demonstrated in previous studies that ovatodiolide has anticarcinogenic effects and inhibits the proliferation of human tumor cells [9]. Furthermore, it was shown to induce apoptosis and cell cycle arrest in cancer cell lines [10]. However, the anticancer property of ovatodiolide has not been studied against the human prostate cancer. Against this background the present study was designed to investigate the anticancer effects of ovatodiolide against the human prostate cancer cells and explore the underline mechanism.

Methods

Cell lines and culture

The human prostate cancer cell line PC-3 as well as the normal prostate epithelial cell line PNT1A were acquired from the ATCC collection center, USA. The cell lines were cultured using Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Scientific, Waltham, Mass, USA). The cell lines were maintained in a humidified CO_2 incubator at 37°C with 5% $CO_2/95\%$ air concentration.

Analysis of cell proliferation

The proliferation of prostate cancer cells was determined by cell counting kit-8 (CCK8, Thermo Scientific) following the manufacturer's protocol. The prostate cell lines (both normal and cancerous) were cultured in 96-well plate at 37°C for 48h. The cell cultures were treated with 0, 0, 3.25, 6.5, 9.75, 13, 16,25, 30, 50 or 100µM of ovatodiolide for 24h. Afterwards, exactly 10µl CCK8 reagent was added to each well and incubation of 37°C was continued for another 4h. Finally, a microplate reader was used to record the optical density (OD) measurements at 450nm which were used to calculate the percent cell proliferation using the proliferation rate of cell culture without ovatodiolide treatment as 100%.

DAPI staining

The PC-3 prostate cancer cells were put at the cell density of 10⁶ cells/well in 6-well plates and cultured for 24h at 37°C. The cell cultures were then administered 0, 15, 30 or 45µM ovatodiolide. After 24-h incubation at 37°C, the cells were harvested by centrifugation, phosphate buffered saline (PBS) washed, fixed with 70% ethanol and subsequently stained with 4',6-diamidino-2-phenylindole (DAPI) solution. Then, the cells were then examined under fluorescent microscope.

AO/EB dual staining

The PC-3 cancer cells treated with 0, 15, 30 or $45 \mu M$ ovatodiolide for 24h were centrifuged and the collected cell pellets were washed with PBS and fixed using 70%

ethanol. The cells were then stained with acridine orange and ethidium bromide (AO/EB) dual staining mix and analyzed for morphological examinations under a fluorescent microscope.

Annexin V- FITC/PI fluorescent staining

The annexin V-FITC/PI staining assay was performed to assess the level of cell apoptosis under 0, 15, 30 or 45µM ovatodiolide administration for 24h. The PC-3 cancer cells were fixed using methanol and stained with dual annexin V-FITC/PI staining solution. Then, the cells were examined for nuclear morphology under a fluorescent microscope and the percentage of apoptotic cells was determined.

Flow cytometric examination of the cell cycle

For studying the mitotic cell cycle phase distribution, the PC-3 prostate cancer cells were treated with 0, 15, 30 or 45μ M ovatodiolide for 24h. Centrifugation was used to harvest the treated cells which were then washed with PBS three times, fixed using 4% formaldehyde and stained with PI solution. The cells were then examined using flow cycometer for the analysis of mitosis.

Protein extraction and western blotting

To extract the total proteins the PC-3 cells were lysed with RIPA lysis buffer (Thermo Scientific). The Bradford method was used to determine the total protein concentrations of the cell lysates. Equal protein



Figure 1. Ovatodiolide selectively inhibits the proliferation of prostate cancer cells. **A:** Chemical structure of ovatodiolide. **B:** Assessment of proliferation of PC-2 cancer and PNT1A normal prostate cell lines under ovatodiolide administration. The experiments were performed in triplicate and expressed as mean \pm SD (*p<0.05).

concentrations were loaded on 8% SDS-PAGE gel. The page gel was then blotted to transfer the contents to nylon membranes which were exposed to primary and secondary antibodies designed for respective proteins of interest which were then visualized and their expression levels assessed by the chemiluminescence method.

Statistics

The experiments were performed at least in triplicate. The final values were presented as mean±standard deviation. T-test was performed using the Graphpad Prism 7.0 software and p values ≤ 0.05 were considered as statistically significant.

Results

Ovatodiolide inhibited the cancer cell proliferation in a selective manner

Ovatodiolide is a macrocyclic diterpenoid compound (Figure 1A). To investigate the effects of ovatodiolide on the cell proliferation, the PC-2 cancer and PNT1A normal prostate cell lines were treated for 24h with 0, 3.25, 6.5, 9.75, 13, 16.25, 30, 50 or 100µM ovatodiolide. CCK-8 assay was performed to determine the proliferation rates which were represented in percentages. It was observed that ovatodiolide declined the proliferation of both PC-2 and PNT1A prostate cells (Figure 1B). How ever, the antiproliferative effects were significantly more severe on the PC-2 cancer cells. The TO, of Bax and Bcl-2 protein expression study where the

ovatodiolide for PC-2 cancer cells was 13µM but it was 85µM for PNT1A prostate cells which suggests that ovatodiolide selectively targets the prostate cancer cells and has limited effect against the normal prostate cell.

Ovatodiolide treatment induced apoptosis in prostate cancer cells

The PC-2 cancer cells were treated with 0, 15. 30 and 45µM ovatodiolide to see whether the antiproliferative effects of the molecule against the prostate cancer cells was due to induction of cell apoptosis. The cells were assessed for nuclear morphology by DAPI staining. The observations made indicated that the ovatodiolide treatment led to the nuclear deformation of prostate cancer cells and the effects were more prominent at higher concentrations (Figure 2). Similar observations were noted in the fluorescent microscopic examination of the AO/EB stained cells (Figure 3). The results were further confirmed by the annexin V-FITC/PI dual staining procedure. The percentage of apoptotic cells was seen to increase from 2.3% to 47.5% under 0 and 45µM ovatodiolide administration, respectively (Figure 4A). Taken together, the results were suggestive of the role of ovatodiolide in inncing apoptosis of prostate cancer cells and were further confirmed by the western blot analysis of



Figure 2. DAPI staining showing the nuclear morphological analysis of PC-2 cancer cells treated with ovatodiolide. The Figure shows that ovatodiolide induced apoptosis in PC-2 cells in a concentration-dependent manner (arrows show apoptotic cells). The experiments were performed in triplicate.

Figure 3. AO/EB staining for nuclear morphological analysis of PC-2 cancer cells treated with ovatodiolide. The Figure shows that ovatodiaolide promoted apoptosis in PC-2 cells dose-dependently (arrows depict apoptotic cells). The experiments were performed in triplicate.



Figure 4. Ovatodiolide induces apoptosis in PC-2 cancer cells. **A:** Flow cytometric examination of apoptosis analysis of PC-2 cancer cells treated with ovatodiolide. **B:** Western blotting for Bax and Bcl-2 protein expression analysis of PC-2 cancer cells treated without/with ovatodiolide. The Figure shows that upon treatment with ovatodiolide the expression of Bax increased, while that of Bcl-2 decreased dose-dependently. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

ovatodiolide treatments increased the Bax protein level in a dose-dependent manner (Figure 4B). Bcl-2 protein expression followed the reverse trend.

Ovatodiolide caused G2/M cell cycle arrest of prostate cancer cells

The analysis of prostate cancer cell mitosis treated with 0, 15, 30 and 45µM ovatodiolide for 24h revealed that the molecule treated cells exhibited higher cell percentage at G2 phase and the percentage of G2 phase cells increased in a concentration-dependent manner (Figure 5). The percentage of G2 prostate cancer cells was 67.9 at 45µM ovatodiolide concentration, against only 9.6 in controls.

Anticancer effects of ovatodiolide against the prostate cancer cells were modulated through the inhibition of Ras/Raf/MEK/ERK signaling pathway

The western blotting studies carried out for the expression analysis of Ras, Raf, MEK and ERK proteins revealed that the protein concentration of Ras protein decreased under ovatodiolide treatment. The decrease in the concentrations of phosphorylated Raf, MEK and ERK (p-Raf, p-MEK and p-ERK) was also observed (Figure 6). The concentrations of non-phosphorylated Raf and ERK proteins remained



Figure 5. Flow cytometric examination for mitotic cell cycle phase distribution of PC-2 cancer cells treated without/ with Ovatodiolide (represented in percents). The experiments were performed in triplicate and expressed as mean \pm SD (p<0.05).

unchanged but non-phosphorylated MEK protein concentration increased under the ovatodiolide treatment. The protein concentrations were affected in a dose-dependent manner. The results suggested that the growth inhibitory effects of ovatodiolide against the prostate cancer cells were exerted through the inhibition of Ras/Raf/MEK/ERK pathway.



Figure 6. Western blotting for Raf, Ras, p-Ras, MEK, p-MEK, ERK, p-ERk protein expression analysis of PC-2 cancer cells treated without/with ovatodiolide. The Figure shows that ovatodiolide blocked the Raf/Ras/MEK/ERK pathway dose-dependently.

Discussion

Of the various deadly diseases faced by the mankind, cancer is ranked amongst the most serious health disorder because of the morbidity and mortality it causes annually worldwide N. Prostate cancer is at the top in terms of prevalence rates among the male population and the second most deathly malignancy in males in North America [2]. The mortality associated with the human prostate cancer has been seen to result from the metastasis of this cancer to the neighboring tissues and organs [3]. The currently employed anticancer approaches against the prostate cancer are seen with lesser success rates and are associated with many undesirable side effects [11]. Taking these facts into consideration, the researchers are trying the search for more effective strategies to combat this disorder in a sustainable manner. For this, the natural products are being evaluated for their anticancer role against the

growth and proliferation of human prostate cancer [12-14]. In a similar type of study, we herein deduced the anticancer role of a bio-active substance, ovatodiolide, which is a macrocyclic diterpenoid compound. Ovatodiolide has already been found to be effective against a number of human cancers making the current study more reliable [9,15]. In the current study, the ovatodiolide exhibited selective proliferative inhibition against the human prostate cancer cells and its effects were limited against the normal prostate cells. Such selective inhibitory potential has been noticed for other natural compounds also [16,18]. The inhibitory effects of ovatodiolide on the proliferation of prostate cancer cells were due to induction of apoptosis. This finding is in conformity with previous studies on ovatodiolide [17]. Furthermore, the natural compounds have shown potential to inhibit the cell cycle progression in cancer cells. Our results also support a similar type of anticancer effect of ovatodiolide on the human prostate cancer cells. The ovatodiolide caused the G2/M cell cycle arrest of prostate cancer cells in a dose-dependent manner, which is in accordance with its previous established role [10]. The Ras/Raf/MEK/ERK pathay has been shown to act as a vital target in cancer management and it was stated that molecules may be designed to target this pathway to effectively inhibit the cancer progression [18]. In our study, we concluded that ovatodiolide exerted its anticancer effects on prostate cancer cells by blocking the Ras/ Raf/MEK/ERK signaling pathway which highlights its potential to serve as a vital lead molecule in anticancer drug discovery.

Conclusion

The results of the present research indicate that ovatodiolide is effective in inhibiting the growth and proliferation of prostate cancer cells by modulating the Ras/Raf/MEK/ERK pathway. The effectiveness of ovatodiolide could be enhanced through semi-synthetic chemistry approaches and as such this molecule merits further future research.

Conflict of interests

The authors declare no conflict of interests.

References

- Collins FS, Barker AD. Mapping the cancer genome. Sci Amer 2007;296:50-7.
- 2. Stamey TA, McNeal JE, Yemoto CM, Sigal BM, John-

stone IM. Biological determinants of cancer progression in men with prostate cancer. JAMA 1999;281:1395-400.

- Gervasi LA, Mata J, Easley JD et al. Prognostic significance of lymph nodal metastases in prostate cancer. J Urol 1989;142(2 Part 1):332-6.
- 4. Gelet A, Chapelon JY, Poissonnier L et al. Local recurrence of prostate cancer after external beam radiotherapy: early experience of salvage therapy using high-intensity focused ultrasonography. Urology 2004;63:625-9.
- Naponelli V, Modernelli A, Bettuzzi S, Rizzi F. Roles of autophagy induced by natural compounds in prostate cancer. BioMed Res Int 2015;2015.
- Cragg GM, Newman DJ. Nature: a vital source of leads for anticancer drug development. Phytochem Rev 2009;8:313-31.
- 7. Dai J, Mumper RJ. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 2010;15:7313-52.
- 8. Chen YL, Lan YH, Hsieh PW et al. Bioactive cembrane diterpenoids of Anisomeles indica. J Nat Prod 2008;71:1207-12.
- Hsieh YJ, Tseng SP, Kuo YH et al. Ovatodiolide of anisomeles indica exerts the anticancer potential on pancreatic cancer cell lines through STAT3 and NF-KB regulation. Evidence-Based Complem Altern Med 2016;2016.
- 10. Yu CY, Teng CL, Hung PS et al. Ovatodiolide isolated from Anisomeles indica induces cell cycle G2/M arrest and apoptosis via a ROS-dependent ATM/ATR signaling pathways. Eur J Pharmacol 2018;819:16-29.

- 11. Cairns J. The treatment of diseases and the war against cancer. Sci Amer 1985;253:51-9.
- 12. Samarghandian S, Afshari JT, Davoodi S. Chrysin reduces proliferation and induces apoptosis in the human prostate cancer cell line pc-3. Clinics 2011;66:1073-9.
- Shigemura K, Arbiser JL, Sun SY et al. Honokiol, a natural plant product, inhibits the bone metastatic growth of human prostate cancer cells. Cancer 2007;109:1279-89.
- 14. Saleem M. Lupeol, a novel anti-inflammatory and anticancer dietary triterpene. Cancer Lett 2009;285:109-15.
- Lin KL, Tsai PC, Hsieh CY, Chang LS, Lin SR. Antimetastatic effect and mechanism of ovatodiolide in MDA-MB-231 human breast cancer cells. Chemicobiol Interact 2011;194:148-58.
- Zou J, Lin J, Li C et al. Ursolic Acid in Cancer Treatment and Metastatic Chemoprevention: From Synthesized Derivatives to Nanoformulations in Preclinical Studies. Curr Cancer Drug Targets 2019;19:245-56.
- 17. Rahman MA, Amin AR, Shin DM. Chemopreventive potential of natural compounds in head and neck cancer. Nutrition Cancer 2010;62:973-87.
- Friday BB, Adjei AA. Advances in targeting the Ras/ Raf/MEK/Erk mitogen-activated protein kinase cascade with MEK inhibitors for cancer therapy. Clin Cancer Res 2008;14:342-6.