Tropical Journal of Pharmaceutical Research November 2022; 21 (11): 2353-2358
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i11.12

Original Research Article

Ellipticine induces apoptosis and mitochondrial dysfunction in human endometriosis cell lines by activating MAPK signaling pathway

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Sent for review: 6 August 2022 *Revised accepted: 30 October 2022*

Abstract

Purpose: *To assess the effect of ellipticine (EPT), an alkaloid isolated from the Oleaceae family, on endometriosis, and to identify its possible mechanisms of action.*
Methods: *Human endometriosis-like cell lines exposed to EPT were subjected to bromodeoxyuridine/5-bromo-2´-deoxyuridine and proliferating cell nuclear antigen staining. Flow cytometry and immunoblot analyses were used to assess the effect of EPT on cell apoptosis. Mitochondrial damage was determined by JC-1 staining and immunoblotting. Immunoblot assays were performed to determine the effects of EPT on the MAPK pathway.*
Results: *Ellipticine inhibited the viability of human endometriosis cell lines and stimulated cell apoptosis ($p < 0.01$). It further induced mitochondrial damage in human endometriosis cell lines ($p < 0.01$). Mechanistically, EPT acted on MAPK pathway, and induced apoptosis and mitochondrial dysfunction ($p < 0.01$) in human endometriosis cells.*
Conclusion: *Ellipticine is a potential treatment strategy for the management of endometriosis. However, further exploration of this potential should be explored via in vivo studies.*

Keywords: *Endometriosis, Ellipticine (EPT), Apoptosis, Mitochondrial damage, MAPK pathway*

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INTRODUCTION

Endometriosis is a type of gynecological disorder characterized by the growth of endometrial cells [1]. Abnormal endometrial tissue grows and forms adhesions between the peritoneum and abdominal organs such as those in ovaries [2,3]. As a result, endometriosis causes chronic pelvic and abdominal pain and leads to infertility, with 30 − 50 % of endometriosis cases resulting in infertility. In addition, endometriosis is thought to be a potential cause of malignancies [4]. Previous studies have shown that the promotion of endometriosis in cell lines results in apoptosis and mitochondrial damage, so induction of proliferation alleviates endometriosis. Drugs are used to inhibit periodic endocrine stimulation of the ovaries. Testosterone was initially used, but because of its side effects, and because it was not very effective, the use of testosterone has

been gradually abandoned. Later treatments involved sham pregnancy and sham menopause therapies. However, better drugs are needed to effectively treat endometriosis.

Ellipticine (EPT) has been shown to have anticancer effects in several types of cancer cells [5]. The antitumor effects of EPT are mainly through DNA insertion and inhibition of DNA topoisomerase II activity [6]. Ellipticine can also act through the formation of covalent DNA adducts during peroxidase oxidation. For example, exposure to EPT (1 − 10 μM) induced apoptosis in rL95-2 human endometrial carcinoma cells, trapping the cell cycle at G2/M, promoting the MAPK pathway activation and reactive oxygen species (ROS) accumulation [7]. Ellipticine promotes apoptosis of tumor cells by stimulating DNA damage in lymphomas [8]. It also inhibits proliferation and induces apoptosis of fibroblast synovial cells in rheumatoid arthritis by regulating the STAT3 pathway [9]. It significantly inhibits lipopolysaccharide (LPS)-induced activation of c-fos and c-Jun, inhibits LPS-induced inflammatory response of macrophages, and reduces inflammatory response and mortality in an LPS-induced/endotoxin-induced shock mouse model [10]. However, EPT has rarely been reported in the treatment of endometriosis, and the mechanisms of action are unclear.

In this study, using a series of *in vitro* assays, the effects of EPT on the apoptosis and mitochondrial damage of endometriosis cells were investigated and the possible mechanism was revealed.

EXPERIMENTAL

Cell culture

The VK2/E6E7 and End1/E6E7 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in keratinocyte serum-free medium (Gibco, Waltham, MA, USA) as well as DMEM/F12 medium supplemented with 10 % fetal bovine serum, in a serial culture hood supplied with 5 % CO_2. The cells were stimulated with different doses of EPT for 48 h.

Bromodeoxyuridine/5-bromo-2´-deoxyuridine (BrdU) staining

Cell proliferation was determined using BrdU staining. The cells were plated in a 96-well plate, and treated with EPT for 48 h. After the addition of BrdU, the cells were fixed and treated with anti-BrdU-POD for 90 min. Images of the BrdU-positive cells were captured using a fluorescence microscope.

Immunofluorescence staining

Cells were blocked with 4 % paraformaldehyde in 5 % bovine serum albumin (BSA) in phosphate buffered saline with tween (PBST) and incubated with primary proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After rinsing thrice in PBST, secondary antibodies conjugated with Alexa 488 (Invitrogen, Carlsbad, CA, USA) were added. Then, 4′,6-diamidino-2-phenylindole was used to counterstain the cell nuclei, and images were captured using a fluorescence microscope.

Cell apoptosis

To determine the percentages of apoptotic cells, Annexin V/propidium iodide (PI) apoptosis detection was performed by the kit (Sigma-Aldrich, St. Louis, MO, USA). The cells were digested into single cells and resuspended in reaction buffer containing Annexin V and PI for 5 min at room temperature in the dark. Cell apoptosis was then analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA).

Immunoblot assay

Total proteins were extracted using RIPA buffer (Beyotime Biotechnology, Haimen, China) and the protein concentration was determined using a BCA kit. The proteins were then resolved using SDS-PAGE, followed by electrotransfer to PVDF membranes. The membranes were blocked with 5 % BSA in tris-buffered saline with tween (TBST), followed by incubating with primary antibodies (Abcam, Cambridge, UK) for cleaved caspase 3 (1:1,000), BAX (1:1,000), Bcl-2 (1:1,000), mito-cyt-c (1:1,000), COX IV (1:2,000), cyto-cyt-c (1:1,000), p-JNK (1:1,000), JNK (1:2,000), p-p38 (1:2,000), p38 (1:2,000), GAPDH (1:20,000), and beta-actin (1:20,000) for 2 h at room temperature. Subsequently, the membranes were reacted with secondary antibodies for 2 h, then visualized using an enhanced chemiluminescence (ECL) kit.

JC-1 staining

To analyze mitochondria damage, the cells were plated in 12-well plates and maintained for 24 h for cell adhesion assays. After rinsing, the cells were incubated with 2 μM JC-1 for 15 min at 37 °C. After rinsing thrice in phosphate-buffered saline (PBS) the cells were analyzed using flow cytometry.

ROS assay

The cellular ROS levels after EPT treatment were determined using 2′-7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich). Then, the cells were washed and analyzed using a microplate reader at 350 nm wavelength.

Statistical analysis

Statistical analysis was performed using GraphPad software, version 7.0 (GraphPad, San Diego, CA, USA). All data are expressed as mean ± SEM. Statistical comparisons were conducted using Student's *t*-test, and $p < 0.05$ was considered statistically significant.

RESULTS

Ellipticine inhibited cell proliferation

Cell proliferation in response to increasing doses of EPT (1, 2, 5, and 10 μM) for 48 h in VK2/E6E7 and End1/E6E7 cells was investigated. Ellipticine (5 μM) reduced cell proliferation in both cell types (Figure 1 A). In addition, to confirm the effect of EPT on normal endometrial cells, cell proliferation was determined using PCNA immunofluorescence staining. Overall, only a small percentage of cell proliferation was seen in EPT-treated cells (Figure 1 B).

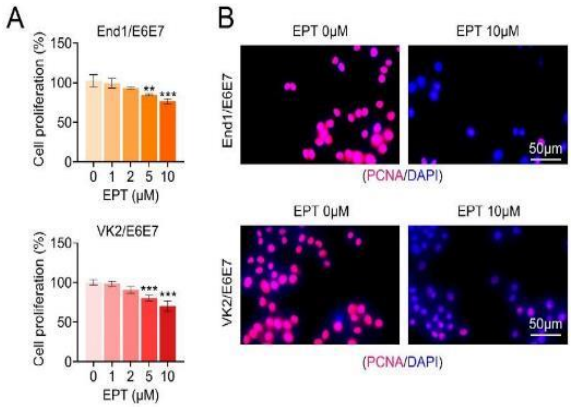

Figure 1: Ellipticine (EPT) inhibited cell proliferation in VK2/E6E7 and End1/E6E7 cells. (A) Cell proliferation of VK2/E6E7 and End1/E6E7 cells in response to increasing doses of EPT. (B) PCNA staining of VK2/E6E7 and End1/E6E7 cells in response to elevated levels of EPT. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs EPT (0 uM)

EPT-induced cell apoptosis

The effect of EPT on cell apoptosis was measured using flow cytometry. Ellipticine (EPT) induced increased cell apoptosis in VK2/E6E7 and End1/E6E7 cells (Figure 2). The expressions of cleaved caspase 3, BAX, and Bcl-2 were also determined. Elevated levels of cleaved caspase 3 and BAX, and decreased levels of Bcl-2 were observed after treatment with EPT of VK2/E6E7 and End1/E6E7 cells (Figure 3). These results confirmed that EPT promoted cell apoptosis in VK2/E6E7 and End1/E6E7 cells.

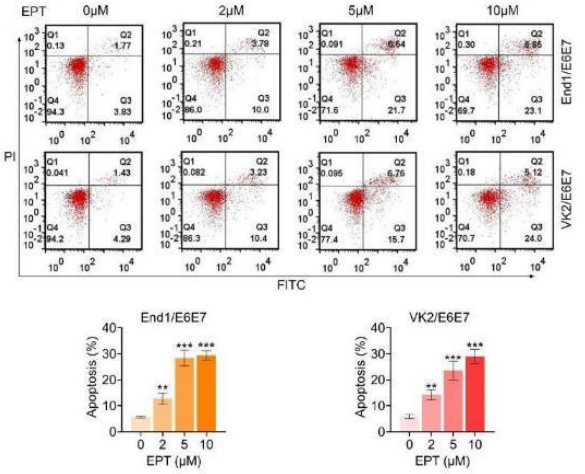

Figure 2: Ellipticine (EPT) induces cell apoptosis in VK2/E6E7 and End1/E6E7 cells. The apoptosis in response to EPT in VK2/E6E7 and End1/E6E7 cells was detected using flow cytometry. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs EPT (0 uM)

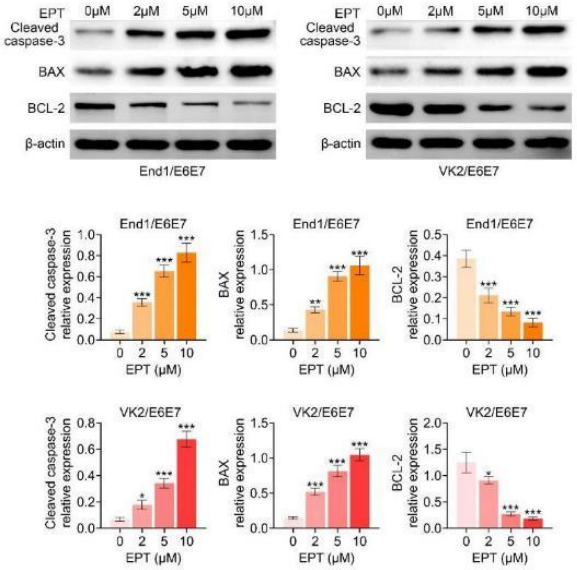

Figure 3: Ellipticine (EPT) affected the expression of cell apoptosis-related proteins in VK2/E6E7 and End1/E6E7 cells. Levels of cleaved caspase 3, BAX, and Bcl-2 in each group were detected. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs EPT (0 uM)

EPT treatment, induced mitochondrial damage

Increased JC-1 monomers and reduced JC-1 aggregates were observed in EPT-treated cells, indicating reduced membrane potential (Figure 4

A). The ROS levels in VK2/E6E7 and End1/E6E7 cells were increased after EPT treatment (Figure 4 B). Moreover, EPT treatment led to reduced levels of mitochondrial-cyto-c and enhanced levels of cytosolic-cyto-c, suggesting impaired mitochondrial membrane integrity in response to EPT treatment (Figures 4 C and D). These results showed that EPT treatment led to mitochondrial damage in VK2/E6E7 and End1/E6E7 cells.

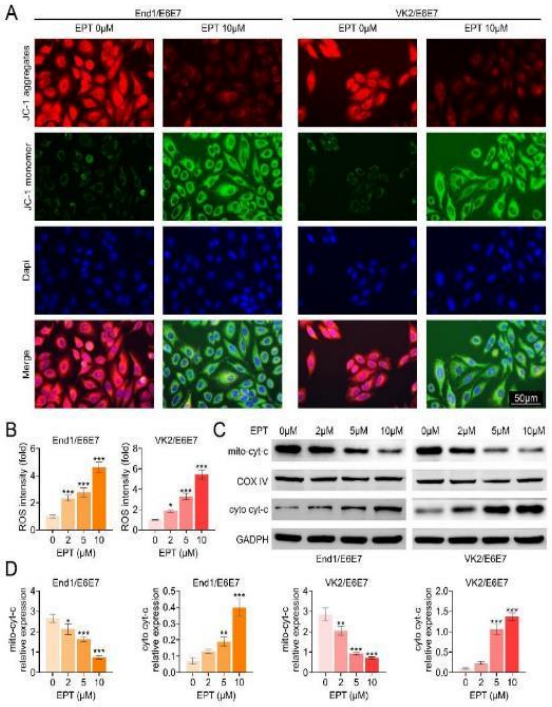

Figure 4: Ellipticine (EPT) led to mitochondria damage in VK2/E6E7 and End1/E6E7 cells. (A). JC-1 staining in VK2/E6E7 and End1/E6E7 cells in response to increasing doses of EPT. (B) Levels of reactive oxygen species in VK2/E6E7 and End1/E6E7 cells in response to increasing doses of EPT. (C, D). The levels of mitochondrial Cyt-c and cytosol Cyt-c in each group were revealed. *P < 0.05; **p < 0.01; ***p < 0.001 vs EPT (0 uM)

Ellipticine promoted apoptosis and mitochondrial damage during endometriosis by activating MAPK pathway

To identify how EPT induced apoptosis and mitochondrial damage during endometriosis, the MAPK pathway was analyzed. The results showed increased levels of p-JNK and p-p38 after EPT treatment of VK2/E6E7 and End1/E6E7 cells (Figure 5). These results suggest that EPT induced apoptosis and mitochondrial damage in endometriosis by activating the MAPK pathway.

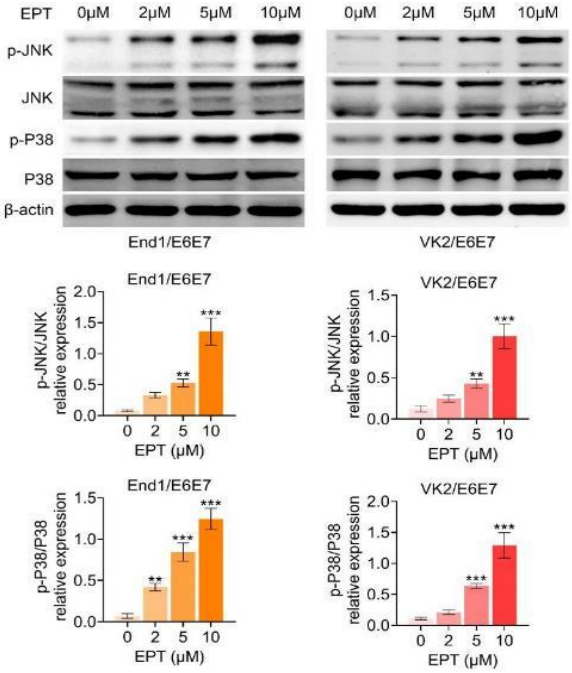

Figure 5: Ellipticine (EPT) promoted apoptosis and mitochondrial damage in endometriosis by activating the MAPK pathway. Immunoblot assays showed the expressions of p-JNK and p-p38 in EPT-treated VK2/E6E7 and End1/E6E7 cells. *P < 0.05; **p < 0.01; ***p < 0.001 vs EPT (0 uM)

DISCUSSION

Endometriosis leads to a series of clinical symptoms, including dysmenorrhea, ovarian cysts, menstrual disorders, and so on [11]. The pathogenesis of endometriosis is still unclear. Some patients may be partially treated with drugs, but some drugs are not effective. The remedial plan for endometriosis differs, depending on the severity of the illness and the patient's age [12]. If the condition is very severe, or if there is severe dysmenorrhea or pelvic examination reveals definite endometriosis nodules, medication or surgery must be used [13], and more effective drugs are needed to treat this disease. Based on the results of the present study, EPT induced apoptosis and mitochondrial damage during endometriosis by activating the MAPK pathway, therefore EPT may be an effective drug to treat this disease.

To confirm the possible effects of EPT on the progression of endometriosis, its effects on VK2/E6E7 and End1/E6E7 endometriosis cells were determined. The results of CCK-8, FCM, MMP, and ROS staining assays showed that EPT suppressed cell viability and induced apoptosis and mitochondrial damage in endometriosis cells. The biological activities of EPT have been widely reported. Ellipticine (EPT) is found naturally in the leaves of *Ochrosia*

elliptica Labill., bark, and leaves of *O. balansae*, *O. moorei*, and in other plants [14]. It could be chemically synthesized and used as a cytotoxic alkaloid with anti-tumor effects. In addition, it inhibits RNA polymerase activity [15] and the activities of acetylcholinesterase and butyrylcholinesterase in humans and experimental animals. Ellipticine (EPT) also has antioxidant activity [16]. Treatment of cells with EPT resulted in the inhibition of cell growth.

Importantly, EPT induced apoptosis of human endometrial cancer cells and arrested the cell cycle via the induction of the MAPK pathway and accumulation of ROS. It further inhibited proliferation and induced apoptosis of fibroblast synovial cells in rheumatoid arthritis by regulating the STAT3 pathway. Similarly, EPT inhibited proliferation and induced apoptosis of endometriosis cells.

The MAPK pathway is an important regulator in several cellular processes, including cell viability and apoptosis. Endometriosis generates a unique microenvironment for the survivability of endometriotic lesions, which include cell proliferation, differentiation, migration, and apoptosis, which are regulated by the MAPK pathway [17]. Several studies reported its possible effects on mitochondrial function. In the present study, EPT induced apoptosis and mitochondrial damage during endometriosis by activating the MAPK pathway, suggesting that this pathway could be a target for endometriosis treatment.

CONCLUSION

Ellipticine (EPT) promotes apoptosis and mitochondrial damage during endometriosis by activating the MAPK pathway. Thus, it is a potential treatment strategy for the management of endometriosis. However, further exploration of this potential should be explored via in vivo studies.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Fenyun Zhang and Qianqian Wang designed and conducted the experiments, analyzed the data, and wrote the manuscript. All authors read and approved the manuscript.

REFERENCES

1. *Allam S, Paris E, Lazcano I, Bitterman P, Basu S, O'Donnell J, Barua A. Detection of cannabinoid receptor expression by endometriotic lesions in women with endometriosis as an alternative to opioid-based pain medication. J Immunol Res 2022; 2022: 4323259.*
2. *Long X, Pei Y, Masokano IB, Zhu X, Lin W, He S, Liu H. A rare case of extrauterine endometrial stromal sarcoma arising from deep pelvic endometriosis: role of multidisciplinary team meeting. Curr Med Imaging 2022.*
3. *Ma W, Shi H, Wei G, Hua M, Yu H. Loureirin B attenuates amiodarone-induced pulmonary fibrosis by suppression of TGFβ1/Smad2/3 pathway. Trop J Pharm Res 2020; 19(7): 1371-1376.*
4. *Peters M, Mikeltadze I, Karro H, Saare M, Estonian Biobank Research T, Salumets A, Magi R, Laisk T. Endometriosis and irritable bowel syndrome: similarities and differences in the spectrum of comorbidities. Hum Reprod 2022.*
5. *Hrabeta J, Groh T, Khalil MA, Poljakova J, Adam V, Kizek R, Uhlik J, Doktorova H, Cerna T, Frei E, et al. Vacuolar-ATPase-mediated intracellular sequestration*

of ellipticine contributes to drug resistance in neuroblastoma cells. Int J Oncol 2015; 47(3): 971-980.

6. *Banerjee A, Sanyal S, Majumder P, Chakraborty P, Jana K, Das C, Dasgupta D. Recognition of chromatin by the plant alkaloid, ellipticine as a dual binder. Biochem Biophys Res Commun 2015; 462(4): 352-357.*
7. *Wang C, Lu H, Liu M, Wang G, Li X, Lu W, Dong W, Wang X, Chen H, Tan C. Effective antibacterial and antihemolysin activities of ellipticine hydrochloride against Streptococcus suis in a mouse model. Appl Environ Microbiol 2021; 87(10).*
8. *Reed L, Indra R, Mrizova I, Moserova M, Schmeiser HH, Wolf CR, Henderson CJ, Stiborova M, Phillips DH, Arlt VM. Application of hepatic cytochrome b5/P450 reductase null (HBRN) mice to study the role of cytochrome b5 in the cytochrome P450-mediated bioactivation of the anticancer drug ellipticine. Toxicol Appl Pharmacol 2019; 366: 64-74.*
9. *Lu H, Liu M, Lu W, Wang C, Wang G, Dong W, Wang X, Chen H, Tan C. Repurposing ellipticine hydrochloride to combat colistin-resistant extraintestinal pathogenic E. coli (ExPEC). Front Microbiol 2020; 11: 806.*
10. *Li X, Ye C, Mulati M, Sun L, Qian F. Ellipticine blocks synergistic effects of IL-17A and TNF-alpha in epithelial cells and alleviates severe acute pancreatitis-associated acute lung injury. Biochem Pharmacol 2020; 177: 113992.*
11. *Bindra V, Sampurna S, Kade S, Mohanty GS, Madhavi N, Swetha P. Primary umbilical endometriosis - case series and review of clinical presentation, diagnosis and management. Int J Surg Case Rep 2022; 94: 107134.*
12. *Darici E, Salama M, Bokor A, Oral E, Dauser B, Hudelist G. Different segmental resection techniques and postoperative complications in patients with colorectal endometriosis: A systematic review. Acta Obstet Gynecol Scand 2022; 101(7): 705-718.*
13. *Hao M, Liu X, Guo SW. Activation of alpha7 nicotinic acetylcholine receptor retards the development of endometriosis. Reprod Biol Endocrinol 2022; 20(1): 85.*
14. *Dan VM, Varghese TS, Viswanathan G, Baby S. Ellipticine, its derivatives: Re-evaluation of clinical suitability with the aid of drug delivery systems. Curr Cancer Drug Targets 2020; 20(1): 33-46.*
15. *Costa de Oliveira R, Soares Pontes G, Kostyuk A, Coutinho Camargo GB, Dhyani A, Shvydenko T, Shvydenko K, Grafov A. Anticancer and immunomodulatory activities of a novel water-soluble derivative of ellipticine. Molecules 2020; 25(9).*
16. *Cerna T, Hrabeta J, Eckschlager T, Frei E, Schmeiser HH, Arlt VM, Stiborova M. The histone deacetylase inhibitor valproic acid exerts a synergistic cytotoxicity with the DNA-damaging drug ellipticine in neuroblastoma Cells. Int J Mol Sci 2018; 19(1).*
17. *Thakur R, Das A, Chakraborty A. The fate of anticancer drug, ellipticine in DPPC and DMPC liposomes upon interaction with HSA: a photophysical approach. J Photochem Photobiol B 2014; 130: 122-131.*