Cancer Letters ■■ (2014) ■■–■■

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Ormeloxifene efficiently inhibits ovarian cancer growth

Diane M. Maher [a,1], Sheema Khan [b,1], Jordan L. Nordquist [a], Mara C. Ebeling [a], Nichole A. Bauer [a], Lucas Kopel [c], Man Mohan Singh [d], Fathi Halaweish [c], Maria C. Bell [a,e], Meena Jaggi [b], Subhash C. Chauhan [b,*]

[a] Cancer Biology Research Center, Sanford Research, Sioux Falls, SD 57104, USA
[b] Department of Pharmaceutical Sciences and Center for Cancer Research, University of Tennessee Health Science Center, Memphis, TN 38163, USA
[c] Department of Chemistry & Biochemistry, South Dakota State University, SD 57007, USA
[d] Saraswati Dental College, Lucknow, Uttar Pradesh, India
[e] Departments of OB/GYN and Basic Biomedical Science Division, Sanford School of Medicine, The University of South Dakota, SD 57105, USA

ARTICLE INFO

Article history:
Received 15 August 2014
Received in revised form 3 October 2014
Accepted 6 October 2014

Keywords:
Ormeloxifene
Novel therapies
Cisplatin resistance
Ovarian cancer
Apoptosis
Xenograft mice

ABSTRACT

Ovarian cancer continues to be a leading cause of cancer related deaths for women. Anticancer agents effective against chemo-resistant cells are greatly needed for ovarian cancer treatment. Repurposing drugs currently in human use is an attractive strategy for developing novel cancer treatments with expedited translation into clinical trials. Therefore, we examined whether ormeloxifene (ORM), a non-steroidal Selective Estrogen Receptor Modulator (SERM) currently used for contraception, is therapeutically effective at inhibiting ovarian cancer growth. We report that ORM treatment inhibits cell growth and induces apoptosis in ovarian cancer cell lines, including cell lines resistant to cisplatin. Furthermore, ORM treatment decreases Akt phosphorylation, increases p53 phosphorylation, and modulates the expression and localization patterns of p27, cyclin E, cyclin D1, and CDK2. In a pre-clinical xenograft mouse ORM treatment significantly reduces tumorigenesis and metastasis. These results indicate that ORM effectively inhibits the growth of cisplatin resistant ovarian cancer cells. ORM is currently in human use and has an established record of patient safety. Our encouraging *in vitro* and pre-clinical *in vivo* findings indicate that ORM is a promising candidate for the treatment of ovarian cancer.

Introduction

Ovarian cancer is the fifth leading cause of cancer mortality in women in the U.S. [1]. Due to the lack of clear symptoms and effective screening mechanisms, 62% of ovarian cancers are diagnosed at the distant stage, which has an average 5 year survival of only 27% [1]. While most ovarian cancer patients will initially respond to chemotherapy, over 66% of patients with advanced stage disease will relapse with disease that becomes resistant to current treatment options [2], highlighting a clear need for additional therapies to treat both initial and relapsed ovarian cancers.

While the discovery and characterization of *de novo* drugs may yield new therapies, there is an appealing option of identifying an effective ovarian cancer therapeutic from a compound that is already in human use, which would dramatically shorten the time and resources required to provide a new treatment option to patients. Ormeloxifene (ORM, also known as Centchroman) is a non-hormonal, non-steroidal oral method of contraception widely used in India [3,4]. In an early report, Misra et al. conducted a trial on advanced breast cancer patients and suggested that ORM may be effective at inhibiting breast cancer [5]. About 38.5% of breast cancer female patients responded to the ormeloxifene therapy and the response to ormeloxifene treatment was more promising for bone, pulmonary, soft tissue, skin, and lymph-node metastases. More recently, ORM has shown anti-cancer effects with *in vitro* models of breast cancer, head and neck cancer, and chronic myeloid leukemia [6–11]. Moreover ORM is reported to have an excellent therapeutic index and is safe for chronic administration [12].

Herein, we have examined the effects of ORM on the growth of cisplatin sensitive (A2780) and cisplatin resistant (A2780-CP and SKOV-3) ovarian cancer cell lines. We show evidence that ORM induces apoptosis and is capable of modulating several proteins involved in cell cycle regulation. ORM efficiently inhibited the growth and spread of ovarian cancer cells in a pre-clinical mouse model of ovarian cancer. Together, these data suggest that ORM may be an effective therapeutic for ovarian cancer and its history of safe human use provides additional evidence for the promising translation of ORM into clinical practice.

* Corresponding author. Tel.: +1 901 448 2175; fax: +1 901 448 1051.
E-mail address: schauha1@uthsc.edu (S.C. Chauhan).
[1] Equal authors.

http://dx.doi.org/10.1016/j.canlet.2014.10.009
0304-3835/

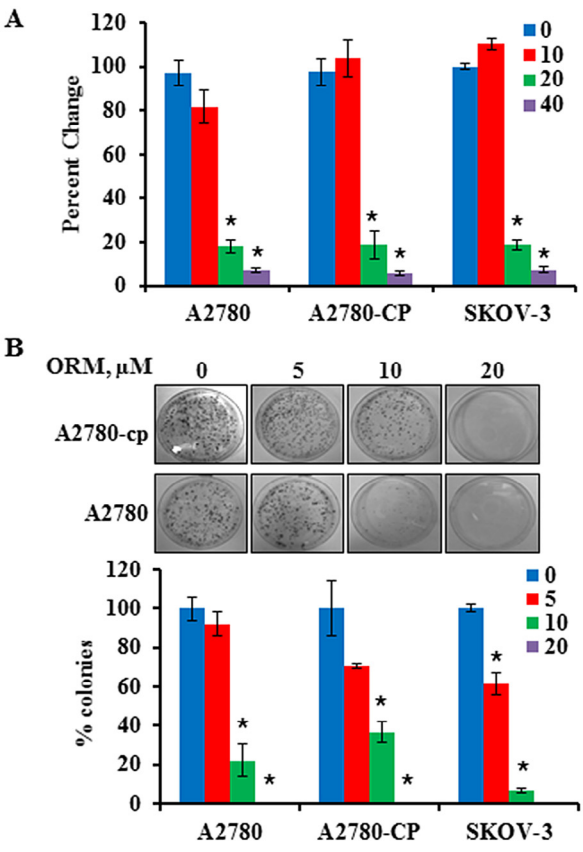

Fig. 1. Ormeloxifene treatment inhibits growth of chemo-sensitive (A2780) and chemo-resistant (A2780-CP and SKOV-3) ovarian cancer cell lines. A) Proliferation was determined with an MTS assay 48 hours after ORM addition and normalized to control wells treated with appropriate amounts of vehicle (ethanol, set at 100%, labeled as 0 ORM). Columns: mean, Bars: SE, n = 3. *Indicates p value: <0.05. B) A colony forming assay was conducted to determine the long term effect of ORM treatment on the clonogenic potential of ovarian cancer cells. Colonies were counted and expressed as a percentage of the number of colonies in the vehicle control (ethanol, set at 100%, labeled as 0 ORM). Columns: mean, Bars: SE, n = 3. *Indicates $p < 0.05$. Representative plates are shown for A2780-CP cells.

Materials and methods

Cell culture, growth conditions, and treatment

The human ovarian carcinoma cell line SKOV-3 was purchased from ATCC and upon receipt cells were expanded and frozen aliquots (passage < 6) were stored in liquid nitrogen. When needed, cells were thawed and grown for less than 6 months. The paired ovarian cancer cell lines, A2780 and A2780-CP cells, were a gift from Dr. Howell (University of CA, San Diego). A2780-CP cells are a cisplatin resistant cell line derived from the parental A2780 cells [13]. SKOV-3, also considered to be cisplatin resistant, was grown in DMEM supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 10 nM non-essential amino acids, 100 nM sodium pyruvate, and 1× antibiotic/antimycotic (Gibco BRL, Grand Island, NY). A2780 and A2780-CP were maintained as monolayer cultures in RPMI-1640 medium (HyClone Laboratories, Inc. Logan, UT) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1× antibiotic/antimycotic (Gibco). All cells were cultured at 37 °C in a humidified atmosphere (5% CO_2). ORM was generously synthesized and provided by FH as described earlier [14]. ORM was solubilized in 100% ethanol and at the time of treatment, ORM was diluted into fresh cell culture media.

Cell proliferation assays

Cells were seeded at 5000 cells per well in 96-well plates and allowed to attach overnight before ORM was added at various concentrations as indicated. Ethanol containing medium served as the vehicle control. The anti-proliferative effect of ORM was determined at 2 days using the CellTiter 96 AQ_{eous} One solution assay (Promega, Madison, WI) as described earlier [15]. The CellTiter reagent was added to each well (20 μl/well) and plates were incubated for 2 hrs at 37 °C. The color intensity was measured at 492 nm using a microplate reader (BioMate 3 UV-Vis spectrophotometer, Thermo Electron Corporation, Waltham, MA). The anti-proliferative effect of each treatment was calculated as a percentage of cell growth with respect to the vehicle control.

Clonogenic assay

For the clonogenic assay, cells were seeded at 500 cells per 100 mm culture dish and allowed to attach overnight. The cells were treated with ORM (or ethanol for the vehicle control) and maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. After 10 days, the dishes were washed twice in PBS, fixed with methanol, stained with hematoxylin (Fisher Scientific, Pittsburgh, PA), washed with water and air dried. Visible colonies (~50 cells) were counted and the percent of colonies was calculated using the number of colonies formed in treatment divided by number of colonies formed in the vehicle control, as described earlier [16].

Cell cycle and TUNEL analysis

Cells (5.0×10^5) were plated in a 100 mm dish and allowed to attach overnight. Cells were then exposed to ORM (10 and 20 μM) for 48 hours. After ORM treatment all cells (including those floating in the media) were collected, fixed with 70% ethanol, stained with Telford Reagent containing propidium iodide, incubated overnight at 4 °C, and analyzed with an Accuri C6 flow cytometer (BD Biosciences). Cells with hypodiploid DNA (content less than G_0/G_1) were deemed apoptotic (sub-G_0/G_1). Alternatively, after ORM treatment, all cells were collected and prepared according to the APO-BrdU™ TUNEL Assay Kit (Invitrogen, Life Technologies) and analyzed with the Accuri C6 flow cytometer.

Mitochondrial membrane potential ($\Delta\Psi m$)

Mitochondrial membrane potential ($\Delta\Psi m$) was measured by uptake of tetramethylrhodamine (TMRE). TMRE is sequestered by active mitochondria and undergoes a dramatic increase in fluorescence intensity. Briefly, cells were treated with ORM (10–20 μM) at three different time points (6, 12, and 18 hours), incubated in 100 nM TMRE for 20 minutes, and fluorescence intensities were measured by flow cytometry. Mean fluorescence values are shown as a percent of the vehicle control (ethanol) values.

Western blotting

Whole cell lysates were prepared as described earlier [17]. Briefly, cells (1×10^6) were allowed to attach overnight and then treated with ORM for the indicated times. After ORM treatment, both adherent and floating cells were collected in 2× SDS lysis buffer (Santa Cruz Biotechnologies), sonicated, and the protein concentration was normalized using SYPRO Orange (Molecular Probes). SDS-PAGE electrophoresis was performed with a 4–20% gradient gel and resolved proteins were transferred onto a PVDF (BioRad) membrane. The following antibodies were used: PARP, Caspase 3, Caspase 9, Rb, phospho-p53 (ser-15), p21, p27, pAKT (ser-473) (each from Cell Signaling Technologies); p53, Cyclin E, and CdK2 (each from Santa Cruz Biotechnologies); and β-actin (Sigma). The primary antibody was detected by a species appropriate HRP-secondary antibody (Promega) followed by incubation with the Lumi-Light detection reagent (Roche).

Confocal microscopy

Cells were plated in 4 well chamber slides at 5×10^4 cells per chamber. Cells were allowed to attach and reach 60% confluency (approximately 24 hr) before being treated with 10 uM ORM for 18 hr. Cells were then processed for confocal microscopy as described earlier [16]. Briefly, following treatment cells were rinsed with 1× HEPES/Hank buffer, fixed and permeabilized with ice cold methanol, washed with 1× PBS and blocked with 10% normal goat serum in PBS. Cells were then incubated with the primary antibody (source listed in prior section) followed by a species specific Alexa Fluor® 488 secondary antibody (Invitrogen). After washing, cells were stained with DAPI and coverslips were mounted in FluoroCare Anti-Fade mounting medium (BioCare Medical). Confocal microscopy was performed with an Olympus Fluoview FV1000 confocal microscope (Olympus Corporation).

In vivo *tumor xenograft model*

Six-week-old female athymic nude (nu/nu) mice (Harlan Laboratories) were injected intraperitoneally with A2780-CP cancer cells (5×10^6 cells in 400 μl PBS). Immediately prior to injection, the cells were mixed with ORM (50 or 100 μg) or vehicle (ethanol). The mice continued to receive the same treatment ORM (50 or 100 μg/mouse) or vehicle (ethanol) via intraperitoneal (*i.p.*) injection once a week for four subsequent weeks. Once per week, the mice were also weighed to help monitor their health. On day 35 post cancer cell injection, mice were euthanized, and tumor burden and metastases were noted. Mouse studies were carried out following procedures approved by the Sanford Research Institutional Animal Care and Use Committee.

Please cite this article in press as: Diane M. Maher, et al., Ormeloxifene efficiently inhibits ovarian cancer growth, Cancer Letters (2014), doi: 10.1016/j.canlet.2014.10.009