Fenretinide: A Novel Treatment for Endometrial Cancer

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Abstract

Resistance to progestin treatment is a major hurdle in the treatment of advanced and reoccurring endometrial cancer. Fenretinide is a synthetic retinoid that has been evaluated in clinical trials as a cancer therapeutic and chemo-preventive agent. Fenretinide has been established to be cytotoxic to many kinds of cancer cells. In the present study, we demonstrate that fenretinide decreased cell viability and induced apoptosis in Ishikawa cells, which are an endometrial cancer cell line, in dose dependent manner in-vitro. This effect was found to be independent of retinoic acid nuclear receptor signaling pathway. Further, we have shown that this induction of apoptosis by fenretinide may be caused by increased retinol uptake via STRA6. Silencing of STRA6 was shown to decrease apoptosis which was inhibited by knockdown of STRA6 expression in Ishikawa cells. Results of an in-vivo study demonstrated that intraperitoneal injections of fenretinide in endometrial cancer tumors (created using Ishikawa cells) in mice inhibited tumor growth effectively. Immunohistochemistry of mice tumors showed a decrease in Ki67 expression and an increase in cleaved caspase-3 staining after fenretinide treatment when compared to vehicle treated mice. Collectively, our results are the first to establish the efficacy of fenretinide as an antitumor agent for endometrial cancer both in-vitro and in-vivo, providing a valuable rationale for initiating more preclinical studies and clinical trials using fenretinide for the treatment of endometrial cancer.

Introduction

Endometrial cancer is the most prevalent gynecologic cancer in the United States, with an estimated 49,560 new cases and 8,190 deaths occurring in 2013 [1]. The probability of a female being diagnosed with this cancer during her lifetime is about one in 38 [2]. The incidence of this reproductive organ malignancy has increased by an average of 1.1% per year from 2004 to 2008 and death rates have also increased by an average of 0.3% per year from 1998 to 2007 [3]. Surgery, chemotherapy and radiation therapy are three main established approaches for the treatment of endometrial carcinoma [4,5]. Surgical removal of the uterus and adjuvant therapy result in a greater than 90% of five year survival rate. Recurrence due to resistance to chemotherapy or radiotherapy presents another major challenge for healthcare professionals. Therefore, it is of utmost important to identify new and effective treatments for endometrial cancers.

Clinically, progestins, including megace (megestrol acetate) have been used to treat endometrial malignancies because of the antagonistic effect of progesterone on estrogen action. Progestins are used as primary agents to treat advanced or recurrent disease, those who are poor candidates for surgery and in younger women who would like to preserve their future fertility. A complete response can be achieved when progestin therapy is used in women with endometrial hyperplasia and well-differentiated endometrial adenocarcinoma; however, the effectiveness of progestin therapy drops with an increase in disease severity. Additionally, recurrences can occur once progesterone therapy is stopped [6,7].

In search of targeted therapeutic agent against endometrial cancer, previous in-vitro studies from our lab demonstrated marked inhibition of proliferation of endometrial cancer Ishikawa cell line by retinoic acid (RA) and the RA agonist AM580 compound [8]. However, their clinical usage has been, thus far, limited by their unfavorable side effects profile [9]. RA and their derivatives (either natural or synthetic compounds) have a recognized role in the regulation of cell growth, differentiation and apoptosis. RA has the potential for the treatment and prevention of cancers [10,11]. Retinol (the dominant form of retinoids in the human body) must be converted into retinoic acid to show its biological activity. The primary source of RA is dietary vitamin A, which is taken up in the intestine and packaged as retinyl esters in the liver. These retinyl esters are secreted into the circulation bound to retinol binding protein (RBP) and subse-
Small interfering RNA (siRNA) knockdown

Materials and Methods

Ishikawa cells were grown to approximately 80% confluency, then transfected with a nontargeting negative control siRNA (Life Technologies, Grand Island, New York). Transfected Ishikawa cells were treated with either DMSO or 6 μM fenretinide (Sigma-Aldrich, St. Louis, MO) solution 60 hours post transfection. Cells were collected after 24 hours of treatment and processed for real-time PCR or Western blotting.

Cell viability assay

 PrestoBlue (Life Technologies) cell viability reagent was used to estimate the cell viability. PrestoBlue is a resazurin-based membrane permeable solution that can be quantitatively measured to determine cell viability [33]. Ishikawa cells were seeded (5x10^3 cells per well) in 96-well plates and cultured for 24 hours at 37°C. The cells were then serum starved for 16-18 hours [34,35]. Cells were then grown in fresh medium and treated with different concentrations of fenretinide and megestrol acetate (Sigma-Aldrich) for 24 h at 37°C. The cytotoxicity of fenretinide and megestrol acetate was measured by adding the PrestoBlue reagent according to the manufacturer’s instructions. The fluorescence signal was measured to determine cell viability on the Synergy HT plate reader from Bio-Tek with the KC4 3.4 software at 530/590 nm.

Western blot analysis

Ishikawa cells were treated with different concentrations of fenretinide and megestrol acetate (Sigma-Aldrich) or vehicle, lysed in RIPA [50 mM Tris, pH 8.0, 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich)]. Clear lysates were obtained after centrifugation at 14,000 rpm for 15 minutes, and the protein concentration was measured using the Micro BCA kit (Thermo Scientific, Rockford, Illinois). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on NOVEX NuPAGE 4-12% Bis-tris gels (Life Technologies) and transferred to polyvinylidene difluoride membranes (Whatman, GE Healthcare Life Sciences, Piscataway, NJ). The membranes were blocked with 5% non-fat dry milk for one hour at room temperature and hybridized with specific primary antibodies for total and cleaved PARP [poly (ADP-ribose) polymerase], Caspase-9 & cleaved Caspase-9 (Cell Signaling Technology, Beverly, MA) overnight at 4°C. The protein bands were visualized using an enhanced chemiluminescence reagent ECL Super Signal West Femto detection kit (Thermo Scientific) after hybridization with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Cell Signaling Technology). The membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) and probed with beta-actin (β-actin) antibody (Sigma-Aldrich) for protein loading control.

Real time PCR

 Total RNA was isolated from Ishikawa cells using Quiaen RNaseasy Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. The purity and concentration of extracted RNA were determined using the ND-1000 Spectrophotometer (NanoDrop). 1 μg of RNA was reverse-transcribed with the Q-scrip cDNA super mix (Quanta Biosciences Inc., Gaithersburg, MD, USA) according to manufacturer’s protocol. Real-time quantitative PCR was performed with the ABI 7900 Sequence Detection and the ABI Power Syber Green gene expression systems (Applied Biosystems, Foster City, CA, USA). The mRNA was quantified using commercially available specific primers to STRA6, CRBP1, CYP26A1, CRABP2, FABP5, RAR-α, RAR-β, and RXR-α (Qiagen) and the housekeeping gene GAPDH (Integrated DNA Technologies, Chicago, IL). The fold change in expression was
calculated using the ΔΔCt method [36] with GAPDH as an internal control. PCRs were performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) for 40 cycles (95°C for 15 s, 60°C for 1 minute) after 10 minutes incubation at 95°C.

**Xenograft mouse model**

Female CD-1 athymic nude mice (4–5 weeks old, Charles River Laboratories International Inc., Wilmington, MA) were maintained under specific pathogen-free conditions based on the guidelines established by the Northwestern University, Institutional Animal Care and Use Committee. Mice were ovariectomized, and an estradiol pellet (1.7 mg/pellet, 60 day release) (Innovative Research of America, Sarasota, Florida) was implanted subcutaneously. Ishikawa human endometrial cancer cell-line cells (2×10⁶/0.1 ml) were suspended in 1:1 PBS/Matrigel (BD Biosciences, San Jose, California) and injected subcutaneously into both right and left flanks of each mouse Tumors were allowed to grow and once the tumors reached 50–150 mm³ (which occurred in about 2 weeks), mice were divided into 4 groups: 1. vehicle, 2. megestrol acetate, 3 fenretinide (Tocris Bioscience, Bristol, UK) and Fenretinide + megestrol acetate. Megestrol acetate pellets (21 mg, 21-day release for 1 mg/day) (Innovative Research of America) were implanted subcutaneously. The mice were given 120 mg/kg of fenretinide at a volume of 10 ml/kg of body weight, 5 times per week for 3 weeks via intraperitoneal injection. Control mice were similarly treated with i.p. injections of 5% ethanol in 0.9% sodium chloride solution containing 1.65 mg/ml of bovine serum albumin. There was no evidence of local or systemic toxicity after i.p. treatment with the dose administered [37] (Fig. S2). Mice were weighed and tumor sizes were measured with calipers twice a week throughout the entire course of treatment. The mice were euthanized using CO₂ asphyxiation and the tumors were resected. Tumor tissues from mice were weighted, then embedded in a paraffin block and subjected to immunohistochemistry or hematoxylin and eosin (H&E) staining. Tumor volumes were calculated using the formula:

$$\text{Tumor volume} = \frac{(a \times a) + b}{2}$$

Where “a” is the shorter of two perpendicular axes [35].

**Figure 1. Effects of Fenretinide on Ishikawa cell viability and apoptosis (A–E).** Ishikawa cells were treated with DMSO (Veh), (A) 2–20µM fenretinide, (B) 0.1–10µM megestrol or (C) a combination of megestrol + fenretinide for 24 h and cell viability was measured using the PrestoBlue reagent. Data are presented as fold change in relative fluorescence units from DMSO treated samples and represent the mean ±SEM of 4–5 independent experiments. An asterisk indicates a significant (*P<0.05) decrease in cell viability in Ishikawa cells treated with fenretinide as compared to the DMSO treated group. (D) Ishikawa cells were treated with DMSO (Veh) or 1–10µM fenretinide which induced apoptosis in a dose dependent manner within 24 h of treatment as demonstrated by increases in cleaved caspase-9 and cleaved-PARP protein levels by western blotting. (E) The treatment of Ishikawa cells with megestrol did not have any effect on these markers of apoptosis. Actin was used as loading control. Blots are representative of 3 independent experiments.
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Immunohistochemistry

Tissues were fixed in formalin, paraffin embedded, and 4 μm tissue sections were cut. After de-paraffinization of tissue sections, heat induced antigen retrieval was performed in a 10 mM sodium citrate buffer (pH 6.0) with 0.05% Tween (Sigma) for 20 min in a pressure cooker. The slides were allowed to cool for 30 minutes at room temperature and were then washed in TBS-T for 5 minutes. The Dako EnVision HRP IHC kit was used (Dako North America, Inc., Carpinteria, CA). Endogenous peroxidase activity was blocked with Hydrogen peroxide (3%) for 10 minutes followed by 2 washes of TBS-T for 10 minutes. For Ki67 immunolabelling, non-specific binding sites were blocked with protein block, and the tissue sections were then incubated with Ki67 primary antibody (Dako) overnight at 4°C in a humidified chamber. Anti-mouse secondary antibody was then applied to the tissue sections for 1 hour at room temperature and then washed in TBS-T twice for 5 minutes. For cleaved caspase-3, non-specific proteins were blocked using 5% normal donkey serum, incubated with cleaved Caspase-3 (Cell Signaling Technology) overnight, then an ABC kit protocol (Vector Laboratories Inc., Burlingame, CA) was followed. DAB solution was used to develop color, and Mayer’s Hematoxylin was used to counterstain the sections. The 28% ammonium hydroxide was used as a bluing reagent. The sections were dehydrated via 2 changes of 95% ethanol, 2 changes of 100% ethanol, and 3 changes of Xylene, and then mounted onto cover slips using Cytoseal XYL mounting media (Richard-Allan Scientific). The slides were visualized using Zeiss upright AXIO TissueFAXS version 3.5.5120.120 scope (TissueGnostics GmbH, Vienna, Austria).

Statistical analysis

All quantitative data are expressed as mean values ± standard error, and significant differences were determined by one-way ANOVA using GraphPad Prism6. A probability value of $P<0.05$ was used as the criterion for statistical significance.

Results

Fenretinide decreases cell viability and increases apoptosis of endometrial cancer Ishikawa cells in-vitro

The treatment of Ishikawa cells with fenretinide significantly inhibited cell viability and increased apoptosis in a dose-dependent manner (Fig.1A & 1D). Fenretinide at 6 to 20 μM caused a decrease of 38% to 99% respectively in cell viability and increase in apoptosis as evident by increases in protein levels of cleaved caspase -9 and cleaved-PARP as compared to DMSO treated group.

![Figure 2. Fenretinide increases retinol uptake genes expression in endometrial cancer Ishikawa cells (A–C).](image-url)
cells. Fenretinide was found to be toxic to the cells at doses higher than 2 μM concentration after 24 hours of treatment (data not shown). In order to determine the effects of megace, Ishikawa cells were treated with megace alone at different concentrations and in combination with fenretinide for 24 hours and cell viability & apoptosis were assessed. Megace did not have any effect on Ishikawa cell viability and apoptosis up to 10 μM alone as well as in combination with 6 μM fenretinide (Fig.1B, 1C & 1E). These results suggest that the fenretinide reduces cell viability and induces apoptosis in Ishikawa cells in-vitro making it a promising candidate to test its efficacy in-vivo.

Fenretinide exerts an increase in retinol uptake via STRA6 and CRBP1

To determine the effect of fenretinide on the retinoic acid signaling pathway in endometrial cancer, real time RT PCR was performed. The mRNA levels of STRA6, CRBP1 and CYP26A1 significantly increased with the fenretinide treatment [Fig.2 (A–C)]. The mRNA of these genes remained unchanged with megace treatment alone or in combination with fenretinide [Fig.3 (A–C)]. However, fenretinide did not have any effect on the mRNA levels of nuclear receptor genes (RAR-α, RAR-β, RXR-α), or the intracytoplasmic carrier proteins CRABP2 and FABP5 (Figure S1).

STRA6 knockdown influences fenretinide mediated apoptosis in endometrial Ishikawa cells

STRA6 is the primary cell surface retinol receptor in the cells responsible for retinol uptake. To establish whether STRA6 is essential for fenretinide induced apoptosis in Ishikawa cells, endogenous STRA6 expression was knocked down using siRNA. As shown in Fig. 4A, we were able to significantly knockdown (76%) the expression of STRA6 in Ishikawa cells. Knockdown of STRA6 abrogates the fenretinide induced apoptosis in Ishikawa cells (Fig. 4B) as shown by diminished levels of cleaved caspase-9 and cleaved PARP when STRA6 is silenced. These results strongly support our hypothesis that STRA6 is crucial for fenretinide induced apoptosis in endometrial Ishikawa cells.
Fenretinide inhibits tumor progression: a mouse xenograft model

Initial in-vitro observations suggested that the anticancer activity of fenretinide may arise from its potential to promote apoptosis in tumor cells. To examine this antitumor activity of fenretinide in-vivo, the mice were engrafted with subcutaneous tumors. Fenretinide treatment significantly suppressed tumor progression in mice as compared to vehicle-treated mice (Figure 5B) based on fold change in tumor size. The decreases in tumor volume were also significant in megace treated as well as megace + fenretinide treated (Figure 5B) as compared to vehicle-treated mice. Moreover, mice seemed to tolerate fenretinide and megace treatments without apparent symptoms of toxicity or serious loss of body weight compared with vehicle-treated group (Figure S2).

Treatment with fenretinide decreases proliferation and increases apoptosis in mice tumors

The effect of fenretinide on cell proliferation and apoptosis was evaluated by immunohistochemistry and H&E staining of Ishikawa endometrial tumor sections after 3 weeks of treatment. (Fig.6). Proliferation of the cells, as measured by Ki67 protein levels, decreased with fenretinide treatment. The levels of cleaved caspase-3 (an apoptosis marker), were negligible in the tumors of vehicle-treated mice, while more staining was evident in the tumors of fenretinide treated mice. Taken together, these data
indicate that Ishikawa tumor development was suppressed by fenretinide through its ability to inhibit cell proliferation and increase in apoptosis of Ishikawa cells both in-vitro & in-vivo.

Discussion

Fenretinide has already been shown to be an effective therapeutic and chemopreventive alternative in breast and ovarian cancers [17]. The present study demonstrates the use of

Figure 5. Effect of fenretinide on endometrial tumor progression in an Ishikawa xenograft mouse model (A–C). (A) Ovariectomized nude CD-1 female mice were injected with Ishikawa cells in both the flanks and estrogen pellets were implanted. Tumors were allowed to grow for 2 weeks. After 2 weeks of tumor establishment, the mice were treated with vehicle (20 mice), meagce (19 mice), fenretinide (21 mice) or meagce + fenretinide (20 mice) and tumors were harvested after 3 weeks of treatment. (B) Images of representative excised tumors from each group. (C) The fold change in tumor volume (from day 1 treatment to day of sacrifice) of vehicle, fenretinide, meagce or both fenretinide + meagce treated mice was plotted after 3 weeks of treatment. Data are shown as means ± min to max of two different experiments. The P values indicate a significant inhibition by fenretinide, meagce or meagce + fenretinide both on tumor growth (*P<0.05). The largest decrease in tumor size was observed in the mice treated with fenretinide alone.

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Figure 6. Inhibition of cell proliferation and increase in apoptosis following fenretinide treatment. Excised tumors were fixed in formalin, paraffin embedded and sectioned. (A) H & E staining and immunohistochemical staining with anti-Ki67 antibody (proliferation marker) and anti-cleaved caspase -3 (apoptosis marker) of whole sections of tumor xenografts. (B) The images are at 100μm.

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fenretinide as a possible anti-tumor agent against endometrial cancer using both in-vitro and in-vivo studies. Fenretinide decreased human endometrial cancer Ishikawa cell viability by inducing apoptosis as demonstrated by an increase in cell death markers (cleaved caspase-9 and cleaved PARP). It also inhibited tumor growth in-vitro, as seen by a decrease in cell proliferation (Ki67) and an increase in apoptosis (Cleaved caspase-3) in mice tumor xenografts. Additionally, we have shown that the antitumor effects of fenretinide are mediated by an increase in retinol uptake, with an increased expression of STRA6 seen in fenretinide treated cells.

Recently, studies by Carrera et al have shown the role of STRA6 in p53 mediated cell death responses in normal and cancer cells, which is similar to our research findings and found to be independent of the downstream activation of RA targets [38]. They demonstrated that transfection with STRA6 induced a substantial amount of apoptosis in RA sensitive ovarian cancer cells (specifically PA-1 and A12847) as well as in RA insensitive HCT116 colon cancer cells. Additionally, the authors found that STRA6 transfection induced both a pro-apoptotic and tumor suppressive affect. These findings, together with the findings of our study, suggest that STRA6 induction may have a potential role in tumor suppression.

Previous literature suggests an interaction in the successful treatment of cancer cells with chemotherapeutic drugs and their potential to trigger apoptotic pathways. It is still not clear whether the biological effects of chemotherapeutic agents are mediated by a direct interaction with nuclear retinoid receptors or via novel nuclear receptor-independent pathways [39]. We found that treatment of Ishikawa cells with fenretinide resulted in cellular apoptosis via induction of Caspase-9, caspase -3 and PARP. The induction of these apoptotic pathways has also been seen in breast cancer [17] and ovarian cancer [40]. Interestingly, a phase III breast cancer prevention trial in premenopausal women has demonstrated that treatment with fenretinide decreased the incidence of second breast malignancies and ovarian cancers [41].

In contrast to our present study, other studies have demonstrated that RA analogues may also act by activating nuclear receptors. Specifically, Ishikawa cells proliferation was decreased and apoptosis was induced via RA signaling involving RAR/RXR activation [8].

Progestins have long been used in reproductive aged women who want to preserve their fertility, as well as in advanced and recurrent endometrial cancer cases which cannot be treated with anticancer agents. However, 30% or more patients with endometrial cancer exhibit resistance to hormonal therapies, limiting the use of progestin therapy [42]. In the present study, megace alone did not induce any apoptosis in our endometrial cancer model, nor did it potentiate the anti-tumor activity of fenretinide in-vitro or in-vivo.

The limitations of the present study are the use of single mouse model and the use of cultured endometrial Ishikawa cells instead of primary endometrial cancer cells. Using primary culture is difficult because of the inability to grow primary cancer cells without stromal factors. Further studies are warranted to establish antitumor capacity of fenretinide in different endometrial cancer cell lines and tumor mouse models.

In conclusion, we have demonstrated that fenretinide decreases cell viability, increases apoptosis, and causes a decrease in tumor size. We believe that fenretinide induced apoptosis because of an increase in retinol uptake, specifically by increasing gene expression of STRA6. We believe that this study is among the first to demonstrate that fenretinide has anti-tumor potential against endometrial cancer. We believe that future preclinical and clinical trials are needed to validate its potential as a new possible therapeutic candidate for the treatment of endometrial cancer.

Supporting Information

Figure S1 Treatment of Ishikawa cells with fenretinide at 0.25–10 μM concentrations for 24 h did not alter the expression of retinoic acid nuclear receptor genes. The data is representative of means ± standard error of three different experiments. (TIF)

Figure S2 Treatments with either fenretinide or megace have no effect on mouse body weights. The body weights were measured from Vehicle treated or drug treated groups of mice twice a week during the whole experiment time. (TIF)

Author Contributions

Conceived and designed the experiments: MEP SEB JJK MD MO. Performed the experiments: NM SM. Analyzed the data: NM SM MEP. Contributed reagents/materials/analysis tools: JCS SEB. Wrote the paper: NM SM MEP.
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