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ORIGINAL ARTICLE

In vitro evaluation of the growth inhibition and apoptosis effect of mifepristone (RU486) in human Ishikawa and HEC1A endometrial cancer cell lines

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Abstract
Purpose To determine the growth inhibitory effects of mifepristone on endometrial cancer cell growth and evaluate its effect on apoptosis using HEC-1-A and Ishikawa human endometrial cancer cell lines.
Methods The human endometrial cancer cell lines, HEC-1-A and Ishikawa, were cultured in vitro. MTT assays were completed in order to estimate the IC_{50} of mifepristone. Both cell lines were then treated with the respective IC_{50} values. Immunohistochemistry assays were performed to investigate the expression of estrogen receptors alpha and beta (ERα/β), progesterone receptor alpha and beta (PR α/β), cyclooxygenase-2 (COX-2), *bax*, p53, and *bcl-2*. Flow cytometry analysis was performed to study cell cycle arrest and apoptosis.
Results The estimated IC_{50} of mifepristone for HEC-1-A and Ishikawa was found to be 16 and 19 μg/ml respectively. At this concentration, there was no change in either ERα/β or PR α/β in Ishikawa. However, PR β expression increased with time of treatment in HEC-1-A. Expression of p53 was increased with duration of treatment in both cell lines. Consequently a decrease in *bcl-2* and an increase in COX-2 expression were seen in HEC-1-A and Ishikawa cells, respectively. Lastly, flow cytometry analysis confirmed an accumulation of cells in G0 phase after 72 h of treatment in both cell lines.
Conclusions Mifepristone demonstrates activity in both HEC-1-A and Ishikawa cells at clinically relevant concentrations based on an oral human dose of about 200 mg/day. While its mechanism of action remains unknown, this data supports an increase in apoptosis that may be due to p53 activation rather than hormone receptor mediation. Additional studies are needed to help further identify mifepristone mechanism of action.

Keywords RU486 · Mifepristone · Progesterone · Endometrial · Cancer · Estrogen receptor

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Introduction

Carcinoma of the uterine corpus, or endometrial carcinoma, is estimated to be the fourth most common cancer among women and the most common gynecologic malignancy with an estimated 39,080 new cases diagnosed and an associated 7,400 deaths for the year 2007 [1]. Frequently women present with early disease because of abnormal vaginal bleeding. Since most treatment interventions are curative, endometrial cancer is associated with an 86% five-year survival rate after surgical and/or radiation treatment [2]. Unfortunately, despite radiation and chemotherapy treatment interventions, the long-term survival of those women with advanced and recurrent endometrial cancer is limited.

In pre-menopausal women under normal physiological conditions, the endometrium will undergo a cyclic pattern of proliferation, differentiation, and breakdown controlled by the steroid hormones, specifically estrogen and progesterone [2]. However, unopposed or increased estrogen exposure is associated with an increase risk for endometrial cancer [3, 4]. The duration of estrogen exposure is increased in women with nulliparity, polycystic ovary syndrome (PCOS), early menarche, late menopause, and specifically obesity [3–5]. Consequently, one therapeutic option for patients with advanced or recurrent disease has been targeted endocrine based treatment with various agents such as anti-estrogens, progesterone agents, gonadotropin-releasing hormone (GnRH) agonists, and most recently, aromatase inhibitors and new generation selective estrogen receptor modulators (SERMs). Historically, response to hormone therapy occurs in about 20% of patients and is associated with multiple side effects [3]. Pre-clinical studies are now being conducted in order to determine the clinical potential of novel agents that modulate progesterone receptors.

Mifepristone, or RU486, is an antiprogestin agent approved by the US Food and Drug Administration (FDA) in September 2000 for the medical termination of intrauterine pregnancy through 49 days pregnancy [6]. While it primarily modulates progesterone activity, since its approval, other physiological effects have been seen with this agent including anti-estrogen and anti-glucocorticoid activity [7, 8]. Accordingly the effect of mifepristone has been examined for other therapeutic uses such as endometriosis and leiomyomata [9–12]. Also, several investigators have shown that mifepristone has direct inhibitory effects on endometrial cell growth, specifically in the EM42, KLE, RL95-2, HEC-1-A, and Ishikawa cell lines, as well as apoptosis-inducing activity [13–17].

Numerous potential therapeutic mechanisms were identified that may contribute to the growth inhibitory activity observed with mifepristone [13–17]. These include estrogen (ER) and progesterone (PR) receptor down-regulation, induction of *bax* and p53 expression, reduction of *bcl-2* expression, and effects on endometrial VEGF production [13–18]. The purpose of this in vitro study was to determine the growth inhibitory effects of mifepristone on endometrial cancer cell growth and evaluate its effect on apoptosis using the two well-differentiated endometrial adenocarcinoma cell lines HEC-1-A and Ishikawa.

Materials and methods

Supplies and buffers

The human endometrial cancer cell line HEC-1-A was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The Ishikawa cancer cell line was generously provided by Dr. Russell Broaddus (Division of Pathology and Laboratory Medicine, The University of Texas M. D. Anderson Cancer Center, Houston, TX). Both cell lines were maintained for less than fifteen passages. Mifepristone, [(3-(4,5-Cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT)] and dimethyl sulfoxide (DMSO), and Tris base were purchased from Sigma–Aldrich Co. (St Louis, MO). Fetal bovine serum (FBS) and trypsid-EDTA were purchased from IBCO Invitrogen Co. (Carlsbad, CA). The methanol, various cell culture flasks, serological pipettes, 96-well plates and other cell culture supplies as needed were obtained from Fisher Scientific (Pittsburgh, PA). BCA Protein kit was purchased from Calbiochem–Novabiochem Co. (San Diego, CA). The primary antibodies, ERα, Erβ, and PR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while COX-2, p53, *bax*, and *bcl-2* were obtained from Calbiochem–Novabiochem Co. (San Diego, CA). All other buffer solutions for immunohistochemistry studies were purchased from Bio-Rad Laboratories (Hercules, CA). ECL Plus Western Blotting Detection Reagents were purchased from Amersham Biosciences (Piscataway, NJ). All buffers for immunohistochemistry studies were prepared as described in previous studies [19].

Cell culture

HEC-1-A is an adenocarcinoma cell line representative of grade II endometrial cancer that was propagated in medium consisting of McCoy's 5A medium with 10% FBS, and the Ishikawa is a well differentiated adenocarcinoma endometrial cancer cell line that was grown in 1:1 mixture DMEM:F12 medium with 10% FBS. All cell lines were grown in 75 cm^2 culture flasks in 5% carbon dioxide (CO_2) in air at 37°C to 90% confluence.

Standard solutions

A 100-mg/ml stock solution of mifepristone was prepared by diluting 100 mg of mifepristone with one milliliter of methanol. A 1:10 dilution was made with methanol for a final concentration of 10-mg/ml. For the MTT assay and immunohistochemistry studies, all additional dilutions were completed with the respective cell culture media for each cell line to achieve a final well concentration range of 5×10^{-8} to 50 μg/ml or the estimated IC_{50} concentration, respectively. The MTT stock solution was prepared by dissolving the MTT into phosphate-buffered saline (PBS) for a final concentration of 3 mg/ml.

Growth inhibition assay (MTT)

The endometrial cancer cells were plated with 15,000 cells per well in 96-well plates and incubated at 37°C for 24 h.

Cells were then treated with mifepristone at a concentration range of 5×10^{-8} to 50 μg/ml. Control wells had media and cancer cells alone. After a 72-h incubation period, 25 μl of MTT solution (3 mg/ml) was added, and cells were incubated for 2 h. Plates were centrifuged, and the supernatant was removed. DMSO of 50 μl was added and absorbance was measured at 562 nm with the FL600 Dual-Band plate reader from BioTek Instruments, Inc. (Winooski, VT). All experiments were completed in quadruplicate.

Immunohistochemistry assays

Protein extracts were prepared by lysing cells on ice in 100–200 μl of NP40 lysis buffer and recovering the supernatant. Each sample protein concentration was determined with the Pierce Micro BCA Protein Assay Kit (Pierce, Rockford, IL), and a standard curve was constructed with known concentrations of BSA.

For immunoblotting, 50 μg of protein was separated by electrophoresis on SDS 10% polyacrylamide and transferred to polyvinylidene difluoride (PVDF) membranes in Tris/glycine buffer using Mini-Protean II Electrophoresis Cell (EMD Biosciences, San Diego, CA) at a current of 30–50 mA respectively. Membranes were then blocked with 5% nonfat dry milk in TBST for 1 h at room temperature. Primary mouse monoclonal antibodies were used as follows: COX2 (1:500 dilution), Bax (1:500 dilution), B-actin (1:10,000 dilution). Primary rabbit monoclonal antibodies were used as follows: p53 (1:1000 dilution), Bcl × 1 (1:1000 dilution), PRα (1:1000 dilution), PRβ (1:1000 dilution), ERα (1:1000 dilution), ERβ (1:1000 dilution). Secondary Goat anti-mouse and anti-rabbit IgG purified antibody were used at dilutions of 1:1000, and 1:2000 respectively. All experiments were completed in quadruplicate.

Flow cytometry

Cellular DNA content was analyzed through the University of Texas M. D. Anderson Cancer Center Flow Cytometry and Image Analysis Core Facility. Treated and untreated HEC-1-A and Ishikawa cells were harvested and fixed in cold 1% PFA in PBS for 30 minutes on ice. Cells were then washed and fixed in ice-cold 70% ethanol. Samples were stored at temperature of 2–8°C until ready for analysis. Cells were then stained with propidium iodide (PI) (final concentration of 10–25 μg/ml containing 5–10 μg/ml of RNAse A) and incubated for 20 min at 37°C. For each cell population, 1×10^6 cells were analyzed by FACS. Flow cytometry results are reported as means, and statistical analysis was performed with paired, two-tailed Student's *t* test. All experiments were completed in quadruplicate.

Results

Growth inhibition assay (MTT)

Growth inhibition assays were completed to determine the growth inhibitory activity of mifepristone on the human endometrial adenocarcinoma cell lines HEC-1-A and Ishikawa. The concentration to achieve 50% growth inhibition (IC_{50}) in HEC-1-A and Ishikawa was estimated to be 16 and 19 μg/ml, respectively (Fig. 1).

Immunohistochemistry assays

The expressions of common targets known to inhibit cell growth were evaluated through immunohistochemistry to identify potential mechanisms of action of mifepristone. Targets evaluated include estrogen receptors alpha and beta (ERα and ERβ), progesterone receptors alpha and beta (PRα and PRβ), p53, bcl-2, COX-2, and *bax* (Fig. 2). There was no change in the expression of ER-α, ER-β or PR-α in HEC-1-A. However, an increase in PR-B expression occurred in HEC-1-A with increasing durations of treatment. Also, there was no expression of COX-2 or *bax*. However, an increase in p53 phosphorylated and a decrease in bcl-2 occurred in HEC-1-A with time of treatment. Conversely, Ishikawa cancer cell lines did not express ERα, bcl-2, or *bax*, and no changes in expression were seen with ERβ or PRα or PRβ. Furthermore, an increase in COX-2 expression was demonstrated in the Ishikawa cells, and similar to HEC-1-A, an increase in p53 phosphorylated expression was shown. A summary of results is shown in Table 1.

Flow cytometry

Flow cytometry analysis was completed to evaluate the effect of mifepristone on the cell cycle progression of

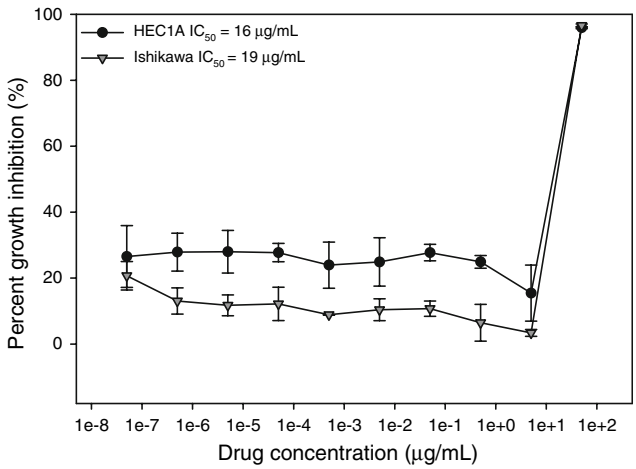

Fig. 1 Summary of mifepristone growth inhibitory activity in selected human endometrial cancer cell lines

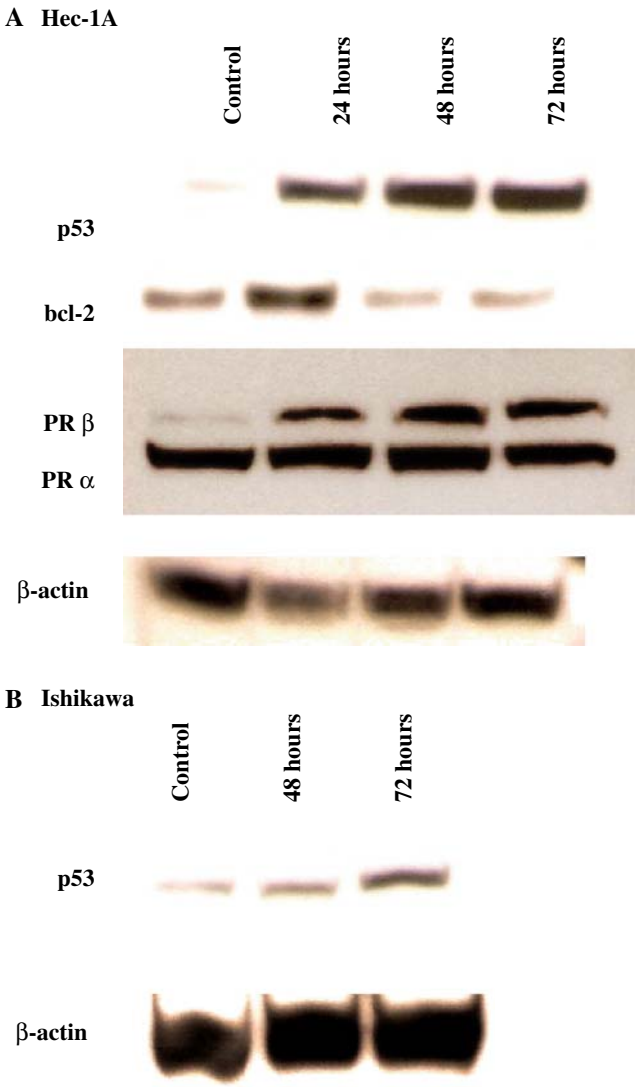

Fig. 2 Summary of mifepristone effects on expression of cellular markers of apoptosis. **a** Result for HEC-1A adenocarcinoma of the endometrium after sampling after 24, 48 and 72-h treatment of mifepristone 16 μg/ml demonstrated increase expression of p53 and decrease expression of bcl-2. **b** Result for Ishikawa endometrial adenocarcinoma sampling after 48 and 72-h treatment of mifepristone 19 μg/ml demonstrated increase expression of p53

HEC-1-A and Ishikawa cancer cells. Exposure of both cell lines to mifepristone at the respective IC_{50} values led to an increase accumulation of cells in the G0 phase with time of treatment when compared to control cells. At 72-h post treatment, the accumulation of HEC-1-A cells in G0 phase doubled (19.76% ± 1.48 vs. 10.72% ± 1.79) while that of Ishikawa increased fourfold (30.37% ± 0.35 vs. 7.17% ± 4.32). Also, the accumulation of HEC-1-A cells in the M phase at 24-h post treatment (20.90% ± 1.93) almost doubled when compared to control cells (11.64% ± 0.6). Comparatively, the accumulation of Ishikawa cells at 72-h post treatment in the G1 phase decreased when compared to control (30.73% ± 0.59 vs. 42.93% ± 0.42,). This decrease also occurred at 72-h post treatment in the M phase when compared to control (12.95% ± 1.45 vs. 27.01% ± 3.4) (Tables 2, 3).

Table 1 Effect of mifepristone on signal transducers

Signal transducers	HEC-1-A	Ishikawa
ER α	No change	No expression
ER B	No change	No change
PR α	No change	No change
PB β	↑ with time of Tx	No change
p53 phosphorylated	↑ with time of Tx	↑ with time of Tx
bcl xl	↓ with time of Tx	No expression
Cox-2	No expression	↑ with time of Tx
Bax	No expression	No expression

Discussion

While initial studies of mifepristone focused on its use for the termination of early pregnancy, it has since been shown to have therapeutic potential for other disease states. For instance, a systematic review of six published trials, involving women with symptomatic uterine leiomyomata, demonstrated a reduction in leiomyoma size and improvement in symptoms [9]. This data was confirmed in a recent randomized, double-blinded, placebo-controlled trial using a dose of mifepristone 5 mg daily [10]. Also, several in vitro studies have demonstrated mifepristone activity in endometriosis, breast cancer, and specifically endometrial cancer [11, 13–17, 20–23]. Schneider et al. [16] studied the effect of mifepristone on three endometrial cancer cell lines, RL95–2, HEC-1-A, and KLE. Investigators found a 50% decrease in growth at a mifepristone dose of 2.5 μg/ml and almost complete growth inhibition at doses greater than or equal to 10 μg/ml. Similarly, another study by Li et al. [15] report activity of mifepristone in the Ishikawa cell line with 50% inhibition occurring at concentrations between 10 and 100 μM. These findings are in agreement with our study and confirm the growth inhibitory effect of mifepristone in both the HEC1-A and Ishikawa cell lines therefore verifying its potential use against endometrial cancer.

Unfortunately, despite the numerous amount of research completed showing mifepristone efficacy, its mechanism of action is still unclear. Initially, the activity of mifepristone activity was thought to be due to mediation of the hormone receptors; however, data suggests that this may not be the only mechanism. Lessey et al. [24] first characterized the functionality of the progesterone receptor in Ishikawa cells in 1996. Authors describe the effect of mifepristone by demonstrating the prevention of PR mRNA and protein level down-regulation caused by progesterone. Accordingly,

Table 2 Accumulation of HEC-1-A cells at various cell cycle phases

HEC-1-A					
	Test 1 (%)	Test 2 (%)	Mean (%)	Standard deviation	*P* value
Percent cell accumulation at G1 phase					
Control	58.7	57.9	58.3	0.5	
24-h treatment	50.2	48.4	49.3	1.2	0.03
72-h treatment	51.7	51.2	51.4	0.4	0.01
Percent cell accumulation at S phase					
Control	18.2	16.2	17.2	1.4	
24-h treatment	18.2	14	16.1	2.9	0.49
72-h treatment	15.1	10.6	12.8	3.2	0.18
Percent cell accumulation at M phase					
Control	11.2	12.1	11.6	0.6	
24-h treatment	19.5	22.3	20.9	1.9	0.06
72-h treatment	11.6	15.5	13.6	2.8	0.43
Percent cell accumulation at G0 phase					
Control	9.5	12	10.7	1.8	
24-h treatment	8.3	10.1	9.2	1.3	0.14
72-h treatment	20.8	18.7	19.8	1.5	0.16

Table 3 Accumulation of Ishikawa cells at various cell cycle phases

Ishikawa					
	Test 1 (%)	Test 2 (%)	Mean (%)	Standard deviation	*P* value
Percent cell accumulation at G1 phase					
Control	42.6	43.2	42.9	0.4	
24-h treatment	40.5	41.3	40.9	0.5	0.02
72-h treatment	31.6	30.7	31.2	0.6	0.04
Percent cell accumulation at S phase					
Control	19.4	19.1	19.2	0.2	
24-h treatment	21.3	20.9	21.1	0.3	0.01
72-h treatment	16.8	14.5	15.6	1.6	0.17
Percent cell accumulation at M phase					
Control	24.6	29.4	27	3.4	
24-h treatment	21.2	23.4	22.3	1.5	0.17
72-h treatment	11.9	14	13	1.5	0.06
Percent cell accumulation at G0 phase					
Control	10.2	4.1	7.2	4.3	
24-h treatment	15.4	13.6	14.5	1.3	0.18
72-h treatment	30.1	30.6	30.4	0.4	0.09

one therapeutic option available to patients with endometrial cancer has included the use of hormonal agents. In this study we found that HEC-1-A expresses ERα, ERβ, and PRα and PRβ; however no change in expression was seen when treated with mifepristone in ERα, ERβ and PR-A. Only the expression of PR-B was increased with time of treatment. Conversely, the Ishikawa cell line seemed to express only ERβ and PRα or PRβ, and no changes were seen with treatment. This lack of observed change may be due to specifics of the PR receptor or inherent differences in the cell lines. In addition, we did not evaluate the presence of inherent "drug resistance".

Mifepristone's antiglucocorticoid activity is well documented in literature and is thought to bind to glucocorticoid receptors with the approximately the same affinity as dexamethasone [25]. However, this may be in opposition to its

anticancer activity. While no previous data has shown an increase in COX-2 expression, there have been reports of reversal of COX-2 inhibition when combined with steroids. Rae et al. demonstrated the direct anti-inflammatory activity of cortisol in the ovarian surface epithelial cells via down-regulation of cytokine-induced COX-2. When these cells were treated with mifepristone, cortisol inhibition of IL1α-stimulated COX-2 mRNA expression was reversed [26]. Similarly, Chiver et al. describe mifepristone inhibition of dexamethasone and budesonide activity in A549 pulmonary cells resulting in the reversal of COX-2 expression inhibition. However, investigators also found an independent inhibitory effect of mifepristone at the level of arachidonic acid release therefore imitating glucocorticoid activity [27]. In our study increased COX-2 expression was observed in the Ishikawa cell line which suggests an increased resistance to apoptosis, however, could not be confirmed since no changes were observed in markers for apoptosis such as bax, or bcl-2 in the Ishikawa cell line.

Several hypotheses now propose additional activity on the expression of several proteins associated with the inhibition of cell growth such as *bax*, *bcl-2*, p53, and COX-2. For instance, while investigating the use of mifepristone on endometriosis, Han et al. report results in the benign endometrial epithelial cell line EM42. Investigators conclude that mifepristone activity is partially due to promotion of cellular apoptosis through increased NF-κB binding resulting in overexpression of *bax* and down-regulation of *bcl-2* [13]. Similarly, Li et al. describe the increase of *bax* mRNA with an associated decrease of *bcl-2* mRNA in Ishikawa cell lines treated with mifepristone at 100 μmol/l. Authors suggest that mifepristone may induce apoptosis via modulation of *bax* and bcl-2 and in turn mediate the FAS/FASLG apoptotic pathway [15]. Unlike previous studies, we did not see either *bax* or *bcl-2* expression in the Ishikawa cell line. Conversely, while *bax* was not expressed in the HEC-1-A cell line, *bcl-2* expression decreased after 72 h of mifepristone treatment. Therefore, the growth inhibitory effect may be due to the decrease in anti-apoptotic *bcl-2* expression. Another study by Kamradt et al. demonstrates the restoration of p53 expression by mifepristone in dexamethasone induced radioresistant cervical carcinoma cells. This corresponded to restoration of radiosensitivity and apoptosis following γ-irradiation [18]. Our study also shows an increase in p53 expression after 72 h of mifepristone exposure further indicating other pathways involved in mifepristone activity. While these mechanisms are possible more research is needed to identify the specific pathways and targets involved due to conflicting reports especially regarding both *bax* and *bcl-2* expression in either cell line.

In order to determine the role of apoptosis in mifepristone activity, flow cytometry analysis was done on both the HEC-1-A and Ishikawa cell lines. Although, significant accumulation of cells at any particular phase was not shown, there was a trend towards accumulation at the G0 phase in the cells treated with mifepristone. Accumulation at this phase indicates an increase in apoptosis when compared to control cells. These results are similar to a study done by Rose et al. on the response of human ovarian carcinoma cell lines to mifepristone. Investigators found antiproliferative effects as well as blockage of cells in the G0/G1 phase resulting in a reduced number of cells in the S phase [28]. However, different results were demonstrated by Li et al. who reported an accumulation of cells in the S phase with a decrease of cells in G2/M phase suggesting selective induction of apoptosis of cells in the S phase [15].

Several studies have been done to demonstrate mifepristone activity, however, its mechanism is still a puzzle. For instance, Jiang et al. conclude that the down-regulation effect of mifepristone on estrogen and progesterone receptors is one of the therapeutic mechanisms of mifepristone on endometriosis [14]. However, another study demonstrates activity of mifepristone on the human endometrial cell line EM42 after loss of its antiprogesterone activity due to reduction of the progesterone-like steroid ring and suggests activity due to antioxidant properties [20]. While many studies indicate modulation of apoptosis-related genes such as *bax* and *bcl-2*, this modulation may be mediated via a variety of transcription factors, including p53, NF-κB, or another unidentified pathway [13, 16, 29]. Other hypotheses based on endometrial and breast cancer in vitro data include induction of apoptosis through caspase or mitogen-activated protein kinase (MAPK) activation [15, 23, 30]. Our study provides further evidence for the possible use of mifepristone in endometrial cancer treatment. The data supports the mechanism of p53 induced apoptosis by showing an increased expression of phosphorylated-p53 in the HEC-1-A cells possibly causing transrepression of the apoptosis-inhibiting gene *bcl-2*. However, despite growth inhibition activity this mechanism was not demonstrated in the Ishikawa cell line. While an increased expression of phosphorylated-p53 was seen, the increased COX-2 expression indicates p53 transactivation of COX-2; therefore suggesting a separate pathway. However, while these mechanisms of action are probable, more studies are needed to identify the specific pathways such as study of NF-KB, MAPK, or VEGF, due to conflicting reports. A phase II clinical trial is underway at MDACC to evaluate the activity of RU486 in PR positive ESS and Endometroid endometrial cancers.

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