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Characterization of Antiestrogenic Activity of the Chinese Herb, *Prunella vulgaris*, Using In Vitro and In Vivo (Mouse Xenograft) Models[1]

Nancy H. Collins,[3,4] Elizabeth C. Lessey,[3,6] Carolyn D. DuSell,[8] Donald P. McDonnell,[8] Lindsay Fowler,[4] Wilder A. Palomino,[7] Maria J. Illera,[9] Xianzhong Yu,[5] Bilan Mo,[4] Angela M. Houwing,[4] and Bruce A. Lessey[2,4]

Department of Obstetrics and Gynecology,[4] University Medical Group, and Developmental Biology Laboratory,[5] Oncology Research Institute, Greenville Hospital System, Greenville, South Carolina 29605
Department of Dermatology,[6] and Laboratory for Reproductive Biology,[7] University of North Carolina, Chapel Hill, North Carolina 27599
Department of Pharmacology and Cancer Biology,[8] Duke University Medical Center, Durham, North Carolina 27710
Departamento de Fisiologia Animal,[9] Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain

ABSTRACT

Prunella vulgaris **(PV), a commonly used Chinese herb, also known as Self-heal, has a wide range of reported medicinal activities. By screening multiple herbs using the endometrial cancer cell line, ECC-1, and an alkaline phosphatase detection assay, we found that PV displayed significant antiestrogenic activity. We investigated the possible usefulness of antiestrogenic activity using both in vitro and in vivo models of endometrial function. Using the well-differentiated, hormone-responsive endometrial cell line, ECC-1, PV extract, at concentrations that were not toxic to the cells, significantly reduced alkaline phosphatase activity and cell proliferation in response to estrogen in a dose-dependent manner. The expression of CYR61, an estrogen-induced protein, was blocked in ECC-1 cells by both the antiestrogen ICI 182 780 and PV extract. Interestingly, PV extract did not appear to directly inhibit estrogen signaling. Rather, we found that its activities were probably related to an ability to function as an aryl hydrocarbon receptor (AHR) agonist in ECC-1 cells. In support of this hypothesis, we noted that PV induced CYP1A1, CYP1B1, and AHR repressor expression in a dose-dependent manner—responses that were blocked by small interfering RNA treatment to reduce AHR and specific AHR antagonists. Ovariectomized immunodeficient RAG-2/gamma(c) knockout mice implanted with human endometrial xenografts developed implants only when treated with estrogen. Mice treated with estrogen and PV tea in their drinking water had fewer and smaller xenograft implants compared with their estrogen-treated counterparts that drank only water ($P < 0.05$). Analysis of the resulting implants by immunohistochemistry demonstrated persistent estrogen receptor (ER), but reduced proliferation and CYR61 expression. Mouse uterine tissue weight in PV-treated mice was not different from controls, and cycle fecundity of intact C57 female mice was unaffected by PV tea treatment. PV, or Self-heal, exhibits significant antiestrogenic properties, both in vitro and in vivo. This activity is likely due to the ability of PV-activated AHR to interfere with estrogen. This herb may be useful as an adjunct for the treatment of estrogen-dependent processes like endometriosis and breast and uterine cancers. Full characterization of this herb will likely provide new insights into the crosstalk between AHR and ESR1, with potential for therapeutic applications in women.**

aryl hydrocarbon receptor, cell lines, chinese herbs, endometriosis, endometrium, estradiol, estradiol receptor, female reproductive tract, herbal therapy implantation,

INTRODUCTION

Estrogens are steroid hormones with a variety of functions in many target tissues. While essential for reproductive function, the effects of estrogen can be detrimental or support abnormal cell growth and/or differentiation. The actions of unopposed estrogen or estrogen in the face of progesterone (P) resistance can promote various gynecologic disorders, including endometriosis, endometrial hyperplasia, and cancer [1–3]. Understanding the role of estrogen and other sex steroids will likely lead to an improved understanding of hormone-dependent diseases and their treatments.

Estrogen replacement therapy has known risks and side effects that may mitigate its usefulness in menopause. Likewise, endogenous estrogen in women of reproductive age may worsen or aggravate preexisting conditions, such as endometriosis or uterine fibroids [4, 5]. Likewise, the use of antiestrogens or medical suppression of estrogen has proven benefit in gynecology [6–9]. However, in some cases, antiestrogens, such as tamoxifen, that benefit target tissues like those of the breast, have unanticipated adverse effects on the endometrium [10, 11]. To this end, pharmaceutical companies have sought to develop selective estrogen receptor modulators (SERMs) that might provide better activity profiles to selectively benefit bone and breast tissues, while not increasing risk of endometrial hyperplasia or cancer and heart disease. Finally, uterine receptivity may be compromised by excess estrogen activity [12–15]. Availability of an effective antiestrogen would likely have utility for the treatment of infertility and such defects in uterine receptivity caused by prolonged or exaggerated estrogen action.

There is increasing interest in herbal and botanical remedies in gynecology [16–19]. For a variety of reasons, many more

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[2]Correspondence: Bruce A. Lessey, Department of Obstetrics and Gynecology, 890 W. Faris Road, Suite 470, Greenville, SC 29605. FAX: 864 455 3095; e-mail: blessey@ghs.org
[3]These authors contributed equally to this work.

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women are actively seeking natural or nontraditional medicinal approaches to treat a wide variety of complaints and conditions [20–22]. Recently, the American College of Obstetrics and Gynecology issued its first *Clinical Update in Women's Health* on the subject of complementary and alternative medicine [23], and the number of publications on gynecologic uses of herbal therapies is rapidly expanding. Over three billion dollars are spent annually by the consumers of complementary and alternative therapies. The use of herbal treatments with estrogenic activities has expanded as well, including primrose oil, black cohosh, and soy protein [24–27]. Many medical practitioners are unaware of the potential drug interactions involving alternative medicines, and many patients do not tell their physicians that they are taking herbal remedies [28]. While some randomized controlled trials involving alternative medicine are occurring [23, 29, 30], there remain many herbal therapies that have not been scientifically studied.

In this study, we investigated the botanical herb *Prunella vulgaris* (PV), a common plant found in Europe and Asia with a long history of use as a remedy for multiple human conditions [31–35]. This herbal remedy contains antioxidant and antimicrobial properties, and has been used to treat dysmenorrhea. During a screening of multiple Chinese herbs, we found that extracts of this plant have antiestrogenic activities, the mechanism(s) of which we probe in this study using both cellular and in vivo models of estrogen action. Based on recent studies suggesting the involvement of the aryl hydrocarbon receptor (AHR) as an antiestrogen in the action of herbs, supplements, and environmental toxins [36, 37], and the link between the AHR agonist dioxin and endometriosis [38], we investigated the potential interactions of PV with this receptor to better understand its mechanism of action.

MATERIALS AND METHODS

Herb Extraction

PV was purchased along with other herbal remedies at a local Chinese medicine distributor in Greenville, South Carolina (Table 1). Initially, all herbs were extracted at room temperature with 100% methanol for 24 h at room temperature. The methanol was filtered and evaporated at 50°C. The residual extract was weighed and reconstituted in 100% ethanol. For animal studies, PV was brewed into a tea using 5 g/250 ml of distilled water.

Cell Proliferation Assay

Using a cell proliferation assay kit (CellTiter96 Aqueous One Solution Cell Proliferation Kit [Promega]), ECC-1 cells were cultured in phenol red-free Dulbecco modified Eagle medium/F12 (DME/F-12) media alone or in the presence of DES (10^{-8} M) or DES plus increasing concentrations of PV extract (wt/vol). Effect of PV extract was also compared to 100-fold excess of the antiestrogen ICI 182 780 (10^{-6} M). Cell number was estimated using a spectrophotometer plate reader at 490 nm using reagents per manufacturer's instructions.

Cell Viability Assay

Well-differentiated endometrial cancer cell line ECC-1 was used for these studies. This cell line was recently characterized by our laboratory [39]. The ECC-1 cells were prepared in 96-well plates with 2×10^4 cells per well in phenol red-free DME/F-12 medium. After 24 h, the medium was switched to the DME/F-12 phenol red-free medium containing the extracts, diethylstilbestrol (DES), or the pure antiestrogen Imperial Chemical Industries ICI 182 780 and was replaced daily and assayed over time. Cell viability was determined using the Cell Titer-Blue TM Cell Viability Assay (Promega, Madison, WI). The medium was changed after 72 h, but only 100 μl was added with 20 μl of reagent provided with the kit and incubated for 2 h. The florescence in the substrate was then observed at 480 nm excitation and 530 nm emission by a fluorescence plate reader (LUMIstar; BMG Labtech, Durham, NC).

Alkaline Phosphatase Assay

To screen for estrogenic or antiestrogenic activity, we used the alkaline phosphatase assay developed by Littlefield and colleagues [40] using ECC-1 cells. Medium was switched to phenol red-free DME/F-12 for 48 h before seeding in 96-well plates (BD Bioscience, Franklin Lakes, NJ). Unless otherwise stated, all reagents were obtained from Sigma (St. Louis, MO). The cells were washed twice with 1× PBS and then trypsinized until cells were dislodged and then resuspended in culture medium. Approximately 2×10^4 cells were added to each well with 200 μl medium and grown for 24 h. The cells then were switched to phenol red-free DME/F-12 medium containing specified concentrations of herbal extracts, DES or DES plus the antiestrogen ICI 182 780 for 72 h. The medium was changed daily. At the end of the treatment, the alkaline phosphatase assay was performed as previously described [40]. Cells were washed twice with 1× PBS and the plates were frozen for 10 min at −80°C and then thawed to lyse the cell membranes. A 20-μl aliquot of cold soluble substrate consisting of Sigma*Fast* p-nitrophenyl phosphate tablets (Sigma) dissolved in deionized H_2O was added to each well on ice. The substrate solution produced a yellow color when alkaline phosphatase was present. After 1–3 h of incubation at room temperature, the absorbance was read in a plate reader at 405 nm (Bio-Rad, Hercules, CA). For comparison purposes, another endometrial cancer cell line (Ishikawa cells) was used.

TABLE 1. Chinese herbs screened for estrogenic or antiestrogenic properties.

Chinese name	Scientific name	Common name
Yizhihuanghua	*Herba solidaginis*	Common goldenrod herb
Bajiaolian	*Dysosma pleianthum*	
Qianliguang	*Herba senecionis scandentis*	Climbing groundsel herb
Liaogewang	*Radix wikstroemiae indicae*	Indian stringbush root
Sankezhen	*Radix berberidis*	Barberry root
Tufuling	*Rhizoma smilacis glabrae*	Glabrous greenbrier rhizome
Tubeimu	*Rhizoma bolbostematis*	
Tuniuxi	*Achyranthes aspera* Linn.	
Daqingye	*Folium isatidis*	Indigowoad leaf
Qianjinteng	*Caulis lonicerae*	Japanese honeysuckle stem
Xiaoji	*Herba cirsii*	Field thistle herb
Mabo	*Lasiosphaera*	Puff-ball
Nyzhenzi	*Fructus ligustri lucidi*	Glossy privet fruit
Mabiancao	*Herba verbenae*	European verbena herb
Maweilian	*Thalictrum glandulosissimum* (Finet et Gagnep.)	Meadowrue root and rhizome
Mujinhua	*Flos hibisci*	Shrubalthea flower
Niubangzi	*Fructus arctii*	Great burdock achene
Niuerdahuang	*Rumex nepalensis* Spreng.	
Shengma	*Rhizoma cimicifugae*	Large trifoliolious bugbane rhizome
Fengweicao	*Pteris multifida*	Chinese brake herb
Xiakucao	*Prunella vulgaris*	Self-heal

TABLE 2. List of antibodies used in the investigation of endometriotic implants.

Name	Catalog no.	Source	Dilution	Host species
ESR1	NCL-ER-6F11	Nova Castra Laboratories, Newcastle, UK	1:250	Mouse
CYR61	H-78	Santa Cruz, Santa Cruz, CA	1:200–500	Rabbit
MKI67	AM410	Biogenex, San Ramon, CA	1:75	Mouse
ACTB	A 5441	Sigma, St. Louis, MO	1:1000	Mouse

Aryl Hydrocarbon Receptor Studies

General. β-Naphthoflavone, DMSO, and real-time quantitative PCR primers were purchased from Sigma. 3-methoxy-4-nitroflavone was a gift from Dr. Stephen Safe (Texas A&M University). Plasmid aryl hydrocarbon receptor response element-thymidine kinase-luc3 (AHRE-TK-luc3) was a gift from Dr. Alvaro Puga (University of Cincinnati).

Real-time PCR. For RNA analysis, ECC-1 cells were seeded in six-well plates in phenol red-free media containing 8% charcoal-stripped serum, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. After 48 h, cells were treated with the appropriate ligand. After 4 h, cells were harvested and total RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad); 1 μg of RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). The Bio-Rad iCycler Realtime PCR System was used to amplify and quantitate levels of target gene cDNA. Quantitative PCR reactions were performed with 1 μl cDNA, 10 μM specific primers, and iQ SYBRGreen supermix (Bio-Rad). Data are normalized to the 36B4 housekeeping gene and presented as fold induction over vehicle. Data are the mean ± SEM from at least two independent experiments.

For small interfering (si) RNA experiments, ECC-1 cells were plated in the presence of 40 nM siAhR or siRNA control (Stealth siRNA; Invitrogen) using DharmaFECT-1 (Dharmacon, Lafayette, CO) as a transfection reagent. After 48 h, cells were treated with the indicated ligand for 4 h, then harvested and assayed for RNA expression, as detailed above.

Transient Transfection

For transient transfections, ECC-1 cells were plated in phenol red-free media containing 8% charcoal-stripped serum, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids in 24-well plates 24 h prior to transfection. Briefly, a DNA-lipofectin mixture (Invitrogen) containing a total of 3 μg of plasmid for each triplicate sample was added to the cells. Each triplicate sample contained 0.1 μg pCMV-βgal (Clontech, Mountainview, CA) and 2.9 μg AHRE-TK-luc3. Ligands were added to the cells 24 h after transfection, and cells were assayed following overnight treatment. Luminescence and β-galactosidase activity were measured on a Fusion luminometer (PerkinElmer, Waltham, MA). Results are expressed as fold activation over vehicle (normalized to β-galactosidase for transfection efficiency) for at least two independent triplicate experiments. Error bars indicate SEM.

Animal Protocols

Using approved Institutional Animal Care and Use Committee protocols at Greenville Hospital System, we purchased 32 RAG-2γ(c) knockout mice (C57BL/6J × C57BL/10SgSnAi[KO]γc[KO]Rag-2; Taconic, Germantown, PA) and then housed them under barrier husbandry conditions. Female RAG mice were ovariectomized using sterile technique and allowed to recover for at least 1 wk prior to further surgery. Additional intact C57 female mice (background strain) were purchased and allowed to mate with male C57 mice, while consuming either H_20 or PV tea for 1 mo prior to mating.

Human Endometrium

All human tissues were obtained after signed informed consent using protocols approved by the Institutional Review Committee at Greenville Hospital System (protocol no. 030607). Endometrial biopsies were obtained from cycling women during laparoscopy using a pipelle device, and the tissue was placed into Hams F12 medium for transport to the laboratory. In the laboratory, small samples (1 mm^3) were dissected and placed into four separate positions in subcutaneous pockets in the abdomen of the ovariectomized, anesthetized RAG mice. The incisions containing the xenograft implants were closed with 5–0 Vicryl interrupted suture (Ethicon, Piscataway, NJ).

Hormone Treatments

Ovariectomized female mice used for the xenograft experiments were implanted with estrogen (E2)-releasing pellets (Innovative Research of America, Sarasota, FL) or E2 plus P pellets. Some mice treated with estrogen received PV tea as their sole source of drinking water. Treatments of xenograft containing mice extended for 4 wk, at which time animals were killed. We harvested uterine tissue and endometriotic xenograft tissue, at which time each was weighed and either snap frozen in liquid nitrogen or placed in 10% buffered formalin for subsequent analysis using immunohistochemistry.

Immunohistochemistry

Sections of endometriotic xenograft tissue were fixed in formalin and paraffin imbedded. Thin sections of representative blocks were immunostained for estrogen receptor-alpha (ESR1), the estrogen-sensitive protein, CYR61, and a marker of cell division, MKI67 (Ki67). Semiquantitative HSCORE was assigned in a blinded manner and compared between groups. The use of HSCORE has previously been validated as a semiquantitative assay for immunohistochemical staining [41]. The antibodies used are listed in Table 2.

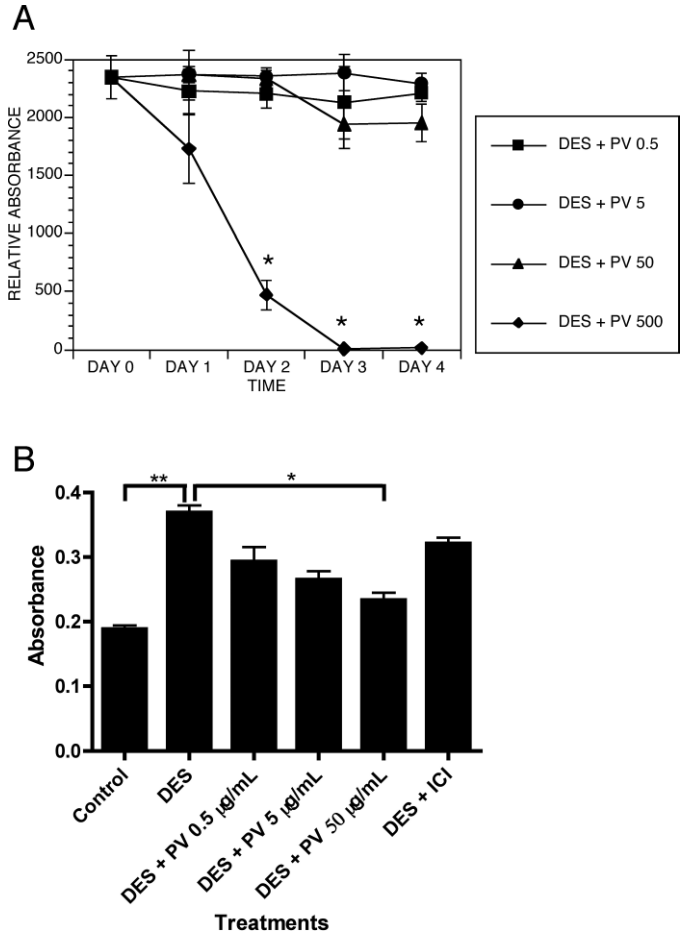

FIG. 1. **A**) Cell viability Assay comparing DES and PV treatments over time (days). At time points 0, 1, 2, 3, and 4 days, cell viability was measured using the Cell Titer-Blue TM Cell Viability Assay. PV only reduced cell viability at the highest dose tested (500 μg/ml). Error bars represent the SEM at each time point for each condition. **B**) Cell proliferation assay was performed using ECC-1 cells treated with or without DES, or with DES plus increasing concentrations of PV up to 50 μg/ml. Error bars represent ± SEM. Significant differences are indicated $^*P < 0.01$; $^{**}P < 0.01$.

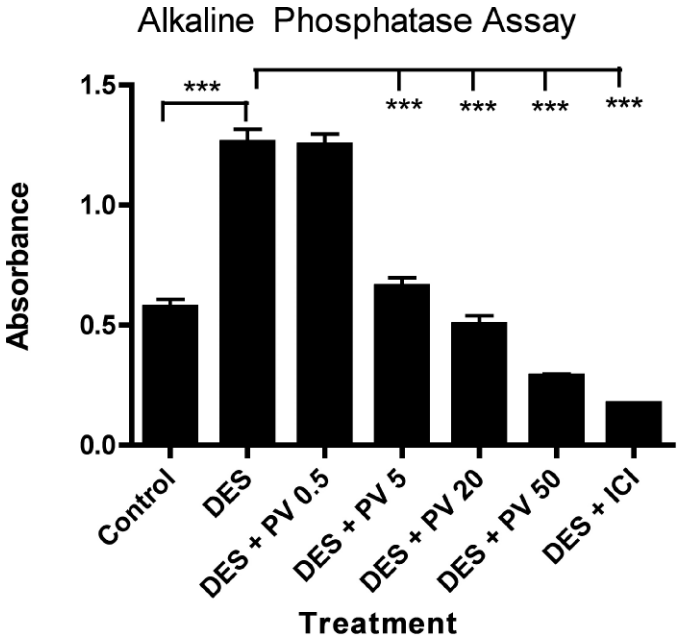

FIG. 2. Alkaline phosphatase assay in ECC-1 cells demonstrates an antiestrogenic activity of the Chinese herbal remedy, PV. Note that at the dose of 50 μg/ml (DES + PV 50), the activity approached that of the antiestrogen ICI 182 780 (ICI). Data represent the mean ± SEM. ***$P <$ 0.001.

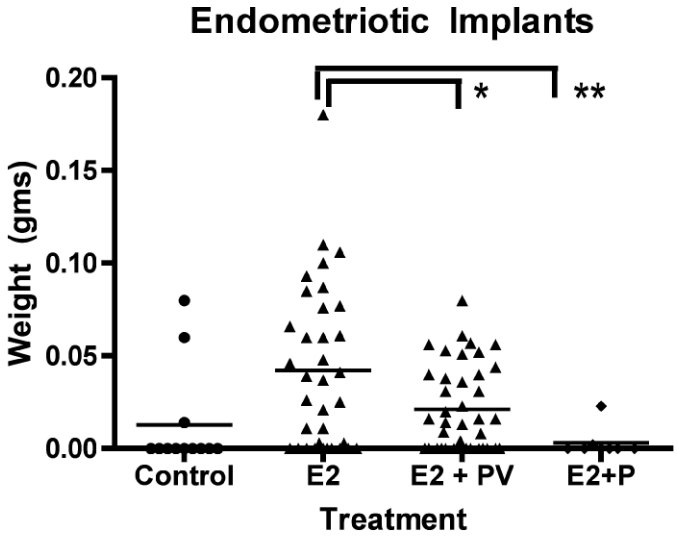

FIG. 4. Summary of the weight in grams of xenograft implants of endometrium placed in the RAG-2γ(c) knockout mice. Cotreatment with estrogen (E2) and progesterone (E2 + P) yielded statistically fewer measurable implants (**$P < 0.01$). PV extract, in the form of tea fed to the mice, reduced the number of implants (*$P < 0.05$), but not to the same degree as estrogen plus progesterone.

Western Blot Analysis

ECC-1 cells were cultured in cell culture dishes (BD Bioscience) to about 80% confluence and treated for various amounts of time, ranging from 6 to 12 h, in treatment medium containing either DES, PV extract, ICI 182 780, or control medium. The cells were washed twice with cold 1× PBS. For each plate, 300 μl of 1× Protease Cocktail Inhibitor (Roche) diluted in RIPA buffer (1% NPH, 0.5% deoxycholate, 0.1% SDS in PBS) was added to each dish and incubated while shaking for 8 min or until cells were visibly dislodged. Cells were then scraped off the dish, removed, and sheared with a 20-gauge needle. After 30 min in an ice bath, the lysate was removed and centrifuged at 14 000 × *g* for 20 min at 4°C. The supernatant was removed and stored at −20°C. Total protein was measured by Bradford assay. Protein concentration was measured using BioProtein Assay reagent and measured with a spectrophotometer (Bio-Rad). Equal amounts of a standard protein concentration or biotinylated protein marker (Cell Signaling, Beverly, MA) with 2× sample buffer LAEMI were then loaded in a 4%–15% ready-made Tris-HCl polyacrymide gel (Bio-Rad). Gels were run at 100 V for approximately 1 h. After removing the gel, the proteins were transferred to a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) for 45 min at 25 V with a semidry transfer cell (Bio-Rad). After blocking for 1 h with blotting-grade nonfat dry milk (Bio-Rad) in a shaking incubator, the membrane was incubated overnight with the primary CYR61 antibody or anti-β-actin in buffer (TBS-T [10% 10× TBS] and 5 ml Tween [BioRad]) at 4°C with continuous shaking. The membrane was then incubated with the matching mouse-HRP-conjugated anti-biotinylated secondary antibody (Cell Signaling) in buffer for 1 h while shaking. After 3 more 15-min TBS-T washes, 3 ml of equal amounts of ECL Western blotting reagent 1 and reagent 2 were added to the membrane for 1 min. The membrane was then developed on a single-coated film (Kodak, Rochester, NY). The protein on the membrane was stripped with stripping buffer consisting of 62.5 mM Tris-HCl, pH 6.8, 2% SDS (Bio-Rad), and 100 mM β-mercaptoethanol (EMD Biosciences, San Diego, CA), and probed for β-actin. β-Actin concentrations were determined by repeating the same method using the anti-β-actin antibody.

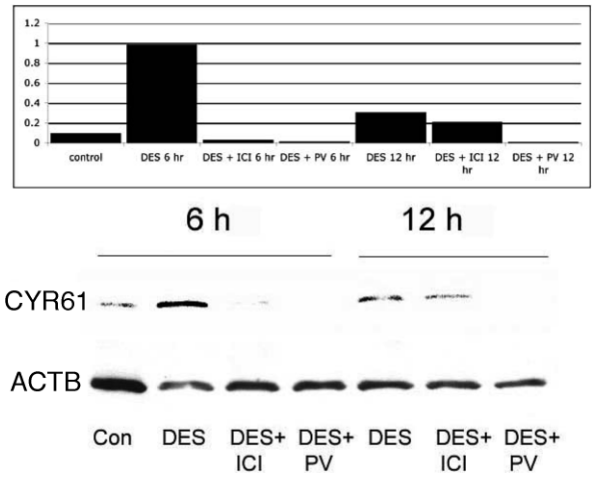

FIG. 3. Western blot showing regulation of CYR61 by estrogen and the inhibition of expression by ICI 182 780 and PV (50 μg/ml) herbal extract at 6 and 12 h. The control for equal loading was β-actin (ACTB), and relative absorbance calculated by the absorbance of CYR61. ACTB is shown in the upper panel.

Statistical Analysis

Comparison between groups was performed using ANOVA with post hoc correction for multiple comparisons using Prism software version 4.0c (Graphpad Software, Inc., San Diego, CA). Two-tailed Student *t*-test was used for other comparisons. A 95% confidence interval was chosen as a determinant for significant differences ($P < 0.05$).

RESULTS

To determine if PV was toxic to the cells, we performed cell viability assays (Fig. 1A). In our study, PV extracts were toxic to cells at only the highest dose (500 μg/ml). The cells were viable following treatment up to 72 h with lower concentrations. At the final time point, none of the cell viability values were different, except at the 500 μg/ml dose when compared with controls ($P < 0.05$). To investigate the effect of PV on cell proliferation, we treated ECC-1 cells with different doses of PV up to 50 μg/ml. As noted in Figure 1B, while DES significantly increased cell proliferation ($P < 0.001$), PV significantly decreased cell proliferation at the highest dose ($P < 0.01$), exceeding the effect of ICI 182 780 on proliferation.

We previously performed a dose-response assay of estrogen treatment using estradiol and DES in ECC-1 and Ishikawa cells

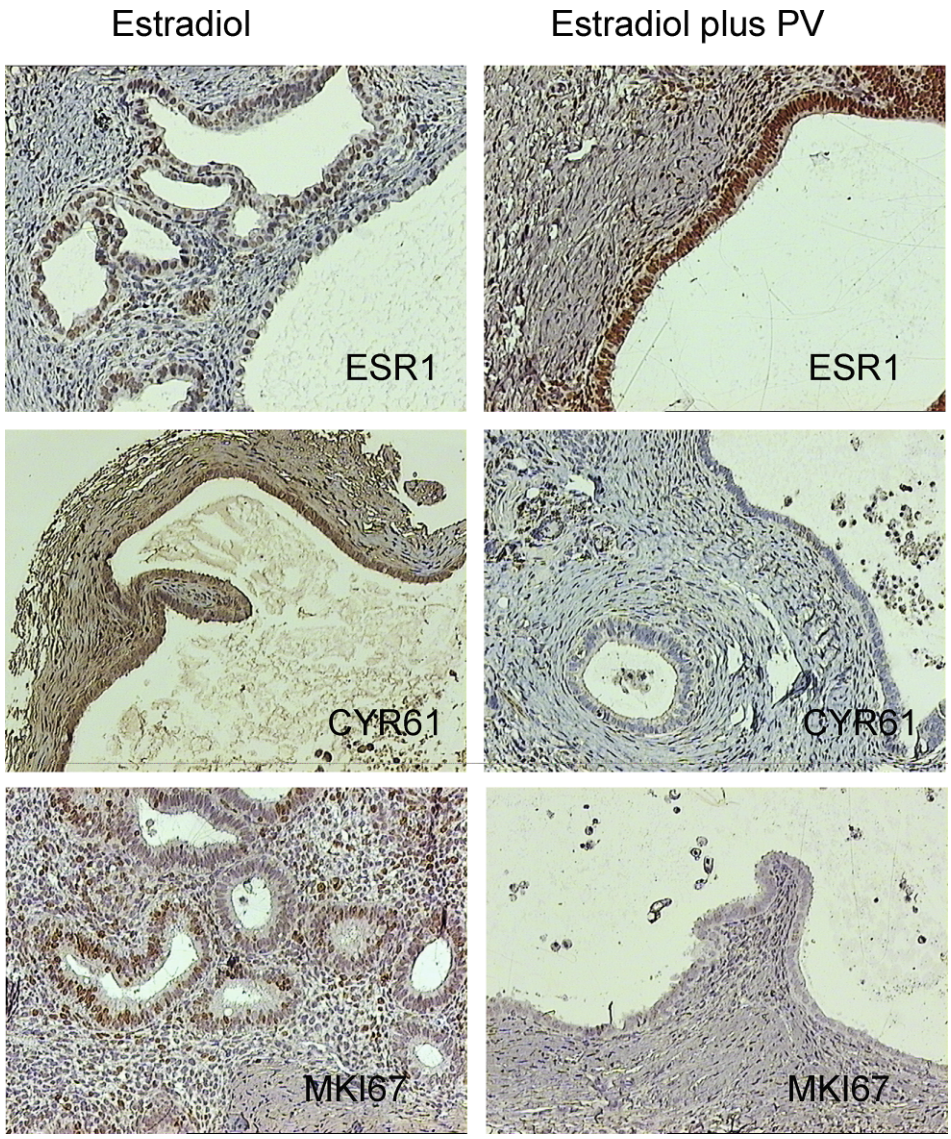

FIG. 5. Immunohistochemical appearance of endometriotic xenografts stained for ESR1, CYR61, and MKI67. While ESR1 was maintained, CYR61 and MKI67 were reduced in many of the implants from mice treated with PV tea. Original magnification ×200.

using the alkaline phosphatase assay (data not shown). Compared to estradiol, DES stimulated the cells to a similar degree and was used because it lacks activity against the AHR and may be more stable than estradiol in cell culture. The ECC-1 cells responded strongly to DES treatment in a dose-responsive manner. Peak activity was reached between 10^{-8} and 10^{-10} M DES, and concentrations above that were toxic to the cells. The Ishikawa cells responded to estrogens as well in the alkaline phosphatase assay, but with a reduced response [36]. Based on these favorable results, ECC-1 cells were chosen for all subsequent experiments. We show that DES significantly increased alkaline phosphatase activity (Fig. 2; $P < 0.001$). Compared with the other herbs tested, PV was uniquely found to exhibit strong antiestrogenic activity with a significant reduction in alkaline phosphatase activity. As shown in Figure 2, PV began to inhibit alkaline phosphatase levels at 5 ng/ml, and continued in a dose-dependent manner (each at $P < 0.001$). At 50 ng/ml, suppression of alkaline phosphatase activity approached that of the synthetic antiestrogen ICI 182 780.

CYR61 is a cystosine-rich secreted protein and a member of the connective tissue growth factor family that has previously been shown to be rapidly induced in endometrial epithelium [14]. To investigate the antiestrogenic effect of PV, ECC-1

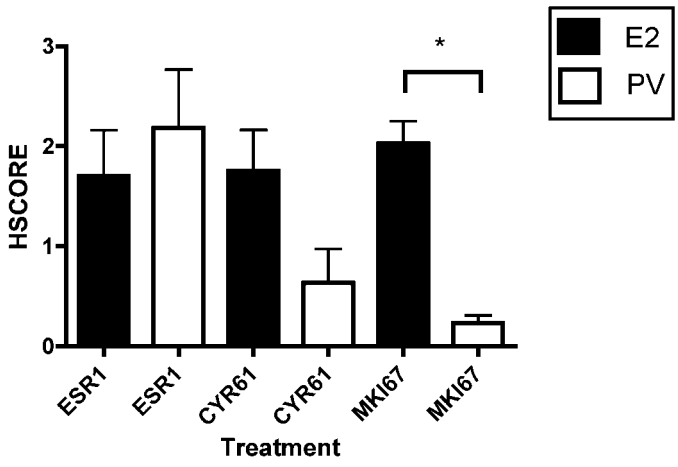

FIG. 6. Summary of immunohistochemical data for ESR1, CYR61, and a marker of cell proliferation (MKI67) in xenografts harvested from mice treated with estradiol (E2) or estradiol and exposed to PV tea in the drinking water. While ESR1 was unchanged, both CYR61 and MKI67 immunostaining appeared to be reduced, with the reduction in MKI67 being statistically significant (*$P < 0.05$). Error bars represent mean ± SEM.

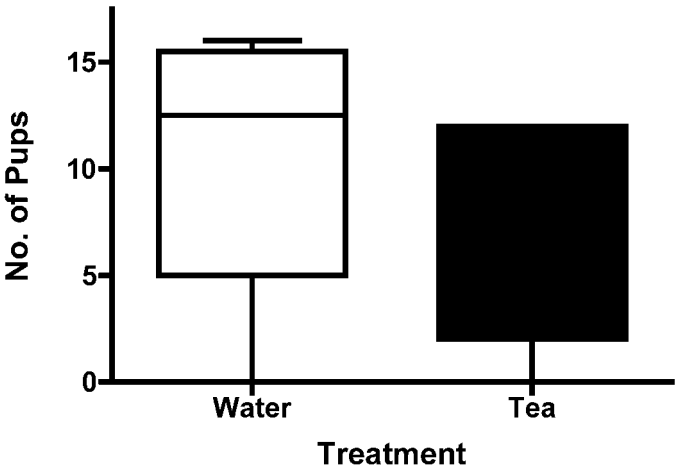

FIG. 7. Box and whisker plot of pregnancy outcome in mice treated with water or PV tea. Pregnancy in C57 mice treated for 2 wk with water (H_2O) or PV (Tea) and then mated with fertile males overnight. The number of pups was not significantly different between groups. In the box and whisker plot, the upper and lower borders of the box represent the 25th and 75th percentile, respectively, and the whiskers, when present, denote the highest and lowest value for each group.

cells were treated for 6 or 12 h with DES in the presence or absence of PV extract (50 μg/ml). Levels of CYR61 were induced by DES, and were highest at 6 h, but maximally reduced by concomitant treatment with either PV extract or ICI 182 780. Both ICI 182 780 and PV effectively reduced CYR61 expression in ECC-1 cells compared with controls (Fig. 3).

To investigate the effect of PV tea in an in vivo model of endometriosis, we used the previously validated RAG-2γ(c) knockout mouse model [42]. As shown in Figure 4, endometrium placed subcutaneously grew under the influence of estradiol pellets to a size that was visible under the skin. Harvested implants provided a histologic model for endometriosis and allowed us to compare the effects of estrogen or estrogen plus PV tea or P. In mice treated for 1 mo with no treatment (control), estradiol pellets alone (E2), E2 plus PV tea, or E2 plus P, there were statistical differences in the weight of implants recovered (Fig. 4). Few implants grew in the absence of estrogen (control). While continuous treatment with E2 plus P resulted in a reduced number of surviving implants compared with E2 ($P < 0.01$). PV tea also reduced the average weight of implants compared with E2 alone ($P < 0.05$). The weight of the uteri recovered from each group of mice was not significantly different between groups (data not shown).

To investigate the biochemical effects of PV tea on endometriotic xenografts, we performed immunohistochemistry for the ESR1, CYR61, and a marker of cell proliferation, MKI67. Representative images are shown in Figure 5 for each marker in implants from estrogen-treated (left column) versus estrogen plus PV-treated (right column) animals. Using the semiquantitative HSCORE assessment of distribution and intensity of immunostaining for each marker protein in multiple implants from each group, we found that ESR1 was not different between groups, while MKI67 was significantly reduced ($P < 0.01$) compared with controls (Fig. 6). CYR61, while lower in the PV-treated mice, did not reach statistical significance ($P = 0.08$).

To test whether PV tea interfered with fertility, PV tea was also fed to intact C57 female mice in a continuous manner. After 2 wk of treatment, females were placed with intact male mice overnight. The number of pups per delivery was similar between PV-treated female mice and water-treated controls (Fig. 7), suggesting that, despite its antiestrogenic activity on xenograft tissues, the herb had little adverse effect on fertility.

There are many possible mechanisms by which a ligand can indirectly regulate ER activity, including crosstalk between the ER and the aryl hydrocarbon receptor (AHR) signaling pathways. We therefore investigated the possibility that a

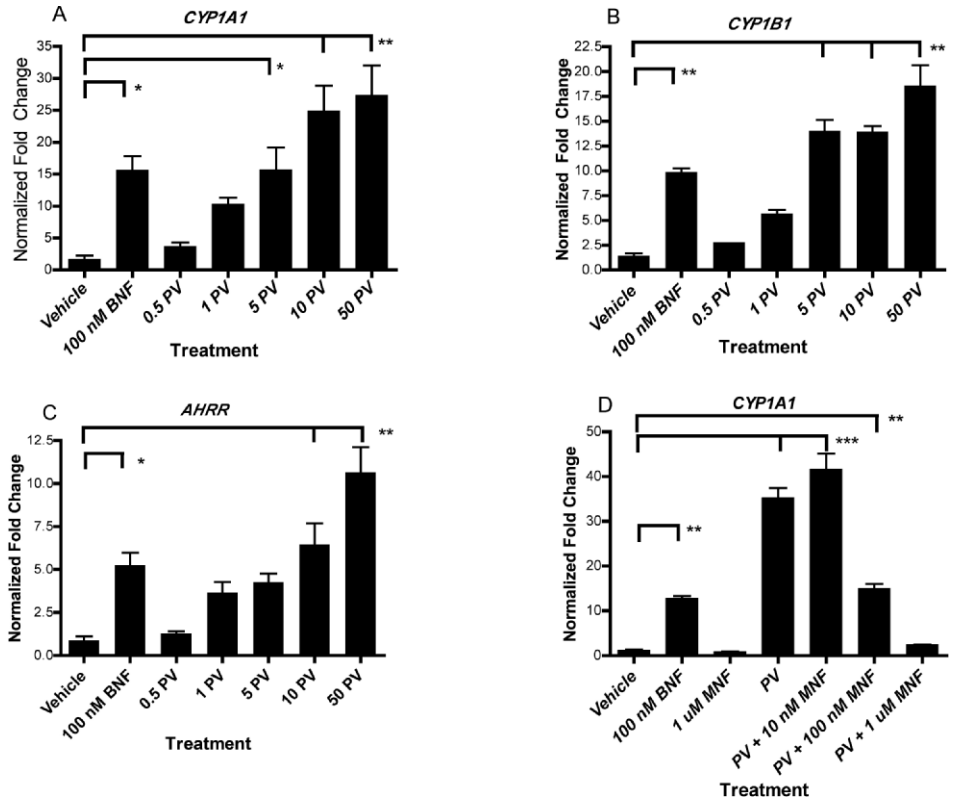

FIG. 8. PV increases AHR target gene expression in a dose-dependent manner (**A**–**C**). Expression of AHR target genes was measured by qRT-PCR in ECC-1 endometrial cells. ECC-1 cells were treated with vehicle, 100 nM BNF, or increasing concentrations of PV for 4 h. Following treatment, cells were harvested, total RNA was isolated, and cDNA was prepared for use as a template for gene expression analysis. All values were normalized to the housekeeping gene, 36B4 (official symbol *RPLP0*). Data are presented as the fold induction over vehicle. Data are the mean ± SEM for three independent experiments. The AHR activity of PV is blocked by an AHR antagonist (**D**). Expression of AHR target genes was measured by qRT-PCR in ECC-1 endometrial cells. ECC-1 cells were treated with vehicle, 100 nM BNF, 50 μg/ml PV with or without the AHR antagonist, MNF, for 4 h. Following treatment, cells were harvested, total RNA was isolated, and cDNA was prepared for use as a template for gene expression analysis. All values were normalized to the housekeeping gene, *ROLP0*. Data are presented as the fold induction over vehicle. Data are the mean ± SEM for two independent experiments. Significant differences are noted as: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

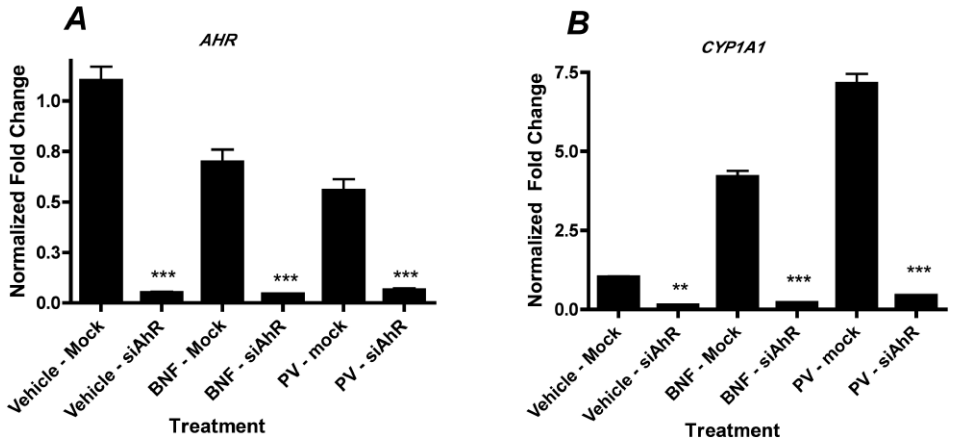

FIG. 9. Induction of CYP1A1 by PV is dependent on AHR expression. The requirement for AHR in PV-mediated induction of *AHR* (**A**) and *CYP1A1* (**B**) was determined in ECC-1 cells. ECC-1 cells were transiently transfected with siRNA to AHR (siAhR) or with siRNA control (mock). After 48 h, cells were treated for 4 h with vehicle, 100 nM BNF, or 50 μg/ml PV. Following treatment, cells were harvested, total RNA was isolated, and cDNA was prepared for use as a template for gene expression analysis. All values were normalized to the housekeeping gene, *RPLP0*. Data are presented as the fold induction over vehicle, and are the mean ± SEM for two independent experiments. Significant differences are as: $^{**}P < 0.01$; $^{***}P < 0.001$.

contributing factor to the antiestrogenic activity of PV is its regulation of AHR. ECC-1 cells have previously been shown to exhibit AHR responsiveness, in addition to estrogen and P responses [43]. Expression of AHR target genes, including *CYP1A1*, *CYP1B1*, and the AHR repressor was measured by quantitative RT-PCR in ECC-1 endometrial cells. ECC-1 cells were treated with vehicle, the AHR agonist, β-napthoflavone (BNF), or increasing concentrations of PV for 4 h. Data (mean ± SEM for three independent experiments) is presented as the fold induction over vehicle and shown in Figure 8, A–C. The stimulatory effect of PV was blocked by the anti-AHR ligand, 3-methoxy-4-nitroflavone (MNF), in a dose-responsive manner (Fig. 8D).

To show that the increased expression of *CYP1A1* by PV was dependent on AHR, we performed knockdown studies using siRNA against AHR. As shown in Figure 9, in the presence of the control vehicle, both BNF and PV stimulated CYP1A1, but this activity was abolished when cells were transfected with AHR siRNA. AHR was not affected by treatment with control vehicle or BNF and PV, but was downregulated by siRNA treatment. Finally, we demonstrated that, like the AHR agonist, BNF, PV activated an AHRE in a dose-dependent manner, and that this action was inhibited by the antagonist MNF (Fig. 10).

DISCUSSION

Herbal medicines are widely used in the United States, with up to one quarter of adults reporting use of such remedies within the past few years [23]. Not surprisingly, the primary physician is often unaware that their patients are using these treatments. The availability and perception that herbal and complementary remedies are safer than prescription medications may have increased the use of these remedies, often leading to self-treatment for a variety of ailments. Failure to recognize side effects of herbal remedies could lead to interactions or unanticipated health consequences. On the other hand, these medicinal agents may have beneficial effects that could be advantageously applied if the mechanisms of action were better understood in the context of Western medicine.

There are several conditions affecting women in which herbal therapies have found a niche. These include menopausal symptoms, menstrual disorders, and treatment for benign conditions, such as endometriosis or uterine fibroids [44, 45]. Many traditional US Food and Drug Administration-approved medications for these conditions have significant side effects or expense [45, 46]. Public concerns about hormone replacement using commercially available estrogens or progestins have also increased after the Women's Health Initiative studies were published [47, 48]. Pre- and postmenopausal women are thus actively seeking natural or nontraditional medical approaches for a wide variety of complaints.

In the present study, we screened 21 herbs using the alkaline phosphatase assay of Littlefield and coworkers [40] to detect either estrogenic or antiestrogenic characteristics. PV was the only herbal extract that had strong biological activity in this assay. In review of the literature, there is one report that supports this finding. Huang and colleagues [49] studied the Chinese herb *Prunella stica* based on its use for the treatment of dysmenorrhea. They showed that this herb blocked the proliferation of Ishikawa cells, but did not interfere with prostaglandin production [49]. Similar to that study, we found that PV extract specifically blocked cell proliferation without affecting cell viability in ECC-1 cells.

The in vivo studies suggest that PV is effective when taken orally, and functions in vivo to block proliferation of ectopic endometrium in response to estrogen. The apparent reduction in CYR61 and MKI67 expression in the endometriotic implants provides evidence that PV interferes with estrogen receptor (ESR1) actions.

The ability of PV to block estrogen action without affecting fecundity in mice suggests that this herb may have advantages

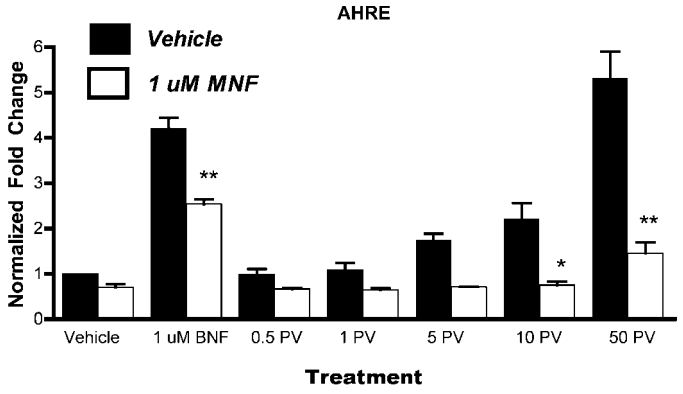

FIG. 10. PV activates an exogenous AHRE in endometrial cells. Transcriptional activity of AHR was examined in the human ECC-1 cell line. Cells were transfected overnight with an AHR-TK-luc3 reporter, then treated overnight with vehicle, 1 μM BNF, or increasing doses of PV with or without 1 μM MNF. After treatment, cells were harvested and assayed for luciferase activity. Luciferase values were normalized to β-galactosidase control. Data are the mean ± SEM for two independent triplicate experiments. Significant differences are noted as follows: $^{*}P < 0.05$; $^{**}P < 0.01$.

for the treatment of women with gynecologic disease. We [12, 13] and others [15, 50–52] have pointed out the relationship between elevated estrogen action and benign endometrial disorders. ESR1 normally declines at the time of implantation, around Cycle Day 19–20 in normal, fertile women [53]. The failure to down-regulate this receptor may be a primary defect leading to altered gene expression patterns seen in endometriosis [1], or the infertility and pregnancy loss in women with polycystic ovary syndrome [12, 14]. Lack of ESR1 down-regulation is a feature of underlying P resistance seen in some women with benign gynecologic disease [1, 2]. Methods to block the inappropriate actions of estrogen during the putative window of implantation could theoretically be helpful and promote improved uterine receptivity toward embryo attachment and implantation. Studies to investigate this possibility are underway.

Understanding the mechanism of the antiestrogenic activity of PV would likely be informative for the design of improved therapies for endometriosis. There is increasing interest in the role of the AHR pathways regulated by dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin [TCDD]) and other environmental toxins in endometriosis. Antagonists of AHR found in food are increasingly being studied [54]. Red clover, thought to have SERM-like activities, is also an AHR ligand [55]. The trihydroxystilbene resveratrol, an active component in red wine, appears to have antagonistic actions via AHR. While many natural flavinoids appear to be AHR antagonists and, therefore, inhibit CYP1A1 expression [56, 57], PV appears to be an AHR agonist with antiestrogenic properties. Like other herbs noted to inhibit estrogen [58, 59], PV uses novel mechanisms to achieve this action. PV appears to activate the AHRE and stimulate AHR target genes in ECC-1 cells. We found little indication that PV had direct effects on ER in endometrial cells.

The mechanism of action of PV to inhibit estrogen responses, potentially through regulation of AHR, remains elusive. AHR, besides regulating target genes, also interacts with both the androgen and estrogen receptors [60], and promotes degradation of these receptors through ubiquitin-mediated mechanisms. Direct action of the AHR to compete with ER activity is another possible explanation for the noted effects of PV on estrogen activity in vivo and in vitro. In addition, evidence points to regulatory elements of AHR as targets for estrogen, further complicating this puzzle [61]. Like resveratrol, PV may also act as a competitive antagonist for TCDD, providing yet another beneficial effect of PV on endometriosis in women who consume this herb. Such a mechanism could account for reported improvements in dysmenorrhea seen in women with endometriosis that take PV.

There remains much to learn about herbal and complementary treatments, especially given the potential for cross-reactivity and unintended consequences when taken with other pharmaceuticals. Unrecognized properties of nontraditional therapies, including their actions as AHR ligands, could provide new opportunities for safe and effective treatment of disorders that have not been adequately addressed by conventional Western medical treatments. The discovery of the antiestrogenic properties of PV provides such an opportunity. This herbal treatment might now be understood at the molecular level as a treatment that blocks estrogen action in endometriosis, and may protect individuals from environmental factors that favor its development. Unlike resveratrol, found in red wine, which cannot as yet be conveniently consumed by women seeking pregnancy, PV may provide a potent alternative, with potential benefits for both pain and infertility. Further investigation and evaluation of the active component(s) of this herb are currently underway.

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