Chronic progesterone antagonist–estradiol therapy suppresses breakthrough bleeding and endometrial proliferation in a menopausal macaque model

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BACKGROUND: Clinicians routinely prescribe progestins along with estrogens during menopausal hormone therapy (HT) to block estrogen-dependent endometrial proliferation. Breakthrough bleeding (BTB) can negate the utility of this treatment. Because progesterin antagonists also inhibit estrogen-dependent endometrial proliferation in women and macaques, we used a menopausal macaque model to determine whether a potent progesterin antagonist (ZK 230 211, Schering AG; ZK) combined with estrogen would provide a novel mode of HT. METHOD: Ovariectomized rhesus macaques were treated for 5 months with either estradiol (E$_2$) alone, E$_2$ + progesterone (two doses) or E$_2$ + ZK (0.01, 0.05 or 0.25 mg/kg). RESULTS: In the E$_2$ + progesterone groups, progesterone suppressed endometrial proliferation and induced a thick decidualized endometrium. In the E$_2$ + ZK 230 211 groups, all doses of ZK blocked endometrial proliferation and induced endometrial atrophy. In all ZK-treated groups, the atrophied endometrium contained some dilated glands lined by an inactive, flattened, non-mitotic epithelium. BTB was much lower in the E$_2$ + ZK groups (17 days of spotting, all groups) than in the E$_2$ and E$_2$ + progesterone groups (155 bleeding days, all groups). ZK suppressed E$_2$ effects in the cervix, but not in the vagina, oviduct or mammary glands. All serum chemistry and lipid profiles were normal. CONCLUSION: The ability of ZK to block estrogen-dependent endometrial proliferation, induce endometrial atrophy and suppress BTB in a menopausal macaque model indicates that progesterin antagonists may provide a novel mode of HT.

Key words: endometrium/estradiol/hormone therapy/progestin antagonist/rhesus macaque

Introduction

For many years, clinicians have prescribed estrogen alone (Ettinger et al., 1993; Ettinger, 1998) or combined estrogen + progestin therapy (Pickar et al., 2001; Speroff, 2002) to control the effects of estrogen deprivation during the menopause. Because excessive endometrial cell proliferation leading to hyperplasia and endometrial cancer can result from estrogen-only therapy, progestins have been administered continuously or sequentially in combination with estrogen to inhibit unwanted endometrial growth (Moyer et al., 1993; Boerrigter et al., 1996). However, chronic estrogen–progestin therapy decidualizes the endometrium and leads to increased breakthrough bleeding (BTB). Also, some women have adverse reactions to the progestin (Curtis et al., 2002). Recently, the Women’s Health Initiative (WHI) study raised concerns regarding the risks associated with typical hormone therapy (HT) (Harman et al., 2004).

Several reports indicate that progestosterone antagonist (PA) therapy can suppress menses in non-human primates (Slayden et al., 2001a) and improve BTB patterns in women treated with Norplant (Glasier et al., 2002; Massai et al., 2004). Therefore, there has been increasing interest in the clinical application of PAs in post-menopausal HT (Cameron et al., 2003) and for the control of BTB bleeding in users of other forms of progestin-only contraception. Other clinical uses for chronic PA therapy include treatment of endometriosis (Grow et al., 1996; Kettel et al., 1996; Spitz and Robbins, 1998), uterine myoma (Eisinger et al., 2003) and meningioma (Lamberts et al., 1992; Carroll et al., 1993) and as an estrogen-free contraceptive (Spitz and Robbins, 1998; Zelinski-Wooten et al., 1998; Bygdeman et al., 1999; Baird, 2000).

Despite the potential of PA therapy, there is concern that chronic long-term PA treatment, by inhibiting the effects of progesterone, could result in unopposed estrogen action leading to endometrial hyperplasia and cancer (Cameron et al., 1996; Newfield et al., 2001). Countering this concern is the growing evidence that these compounds not only block progesterone action but also exert an antiproliferative effect on the endometrium of non-human primates (Wolf et al., 1989;
Slayden et al., 1993; Neulen et al., 1996; Slayden and Brenner, 2003) and women (Cameron et al., 1996). We have examined the endometrial antiproliferative effects of several PAs including mifepristone (RU486) (Slayden et al., 1993; Slayden and Brenner, 1994) ZK 137 316 (Slayden et al., 1998) and ZK 230 211 (Slayden et al., 2001) in rhesus macaques. In short-term 28-day studies, we treated ovariectomized rhesus macaques with estradiol (E2) and ZK 230 211 and showed that estrogen-dependent endometrial proliferation was blocked by ZK 230 211 (Slayden et al., 2001a). ZK 230 211 (Scherer AG, Berlin, Germany) is a PA with high potency, minimal anti-glucocorticoid activity and no PR agonistic activity (Fuhrmann et al., 2000).

Because PAs can inhibit estrogen-dependent endometrial proliferation, induce endometrial atrophy and suppress bleeding, we hypothesized that treatment with E2 + ZK 230 211 could be the basis of a novel form of HT. To explore this in a longer term study, we used the ovariectomized rhesus macaque as a menopausal model and compared the effects of chronic treatment with E2 + progesterone versus E2 + ZK 230 211. In addition, some animals were treated with E2 alone for 5 months to provide baseline data on estrogen-dependent endometrial proliferation.

Methods

Animals and treatments

Animal care was provided by the Division of Animal Resources at the Oregon Regional Primate Research Center (ORPRC), and all procedures were pre-approved by the ONPRC Institutional Animal Care and Use Committee. Twenty-nine adult cycling rhesus macaques were ovariectomized and treated sequentially with E2 and then with E2 + progesterone to induce a pretreatment artificial menstrual cycle (Slayden et al., 1993). To induce these cycles, a 3-cm Silastic capsule (0.34 cm i.d.; 0.64 cm o.d.; Dow Corning, Midland, MI, USA) filled with crystalline E2 was inserted s.c. to induce an artificial proliferative phase. After 14 days of E2 priming, a similar 6-cm Silastic capsule filled with crystalline progesterone was inserted s.c. for an additional 14 days to induce an artificial secretory phase. Removal of the progesterone capsule was followed by reinsertion of the E2 capsule in order to maintain a constant estrogen environment. Treatment groups were summarized in Table I. Treatment with E2, E2 + progesterone [two doses: either a 2-cm progesterone implant (low dose) or a 6-cm progesterone implant (high dose)] or E2 + ZK 230 211 (three doses: 0.01, 0.05 and 0.25 mg/kg) began on day 1 of the second cycle and continued for 150 days. E2 and progesterone-filled capsules were replaced every 30 days. ZK 230 211 was provided by Schering AG and administered by daily, i.m. injection in a non-irritating vehicle that consisted of 37.5% Hanks’ Balanced Salt Solution (Gibco BRL, Grand Island, NY, USA), 37.5% 1,2-propanediol and 25% ethanol (Paper, Shelbyville, KY, USA). Except where indicated, all hormones and other reagents were purchased from the Sigma Chemical Co (St Louis, MO, USA). Serum was obtained from each animal before the initiation of the treatment as well as at monthly intervals to assess hormone release from the implants. Serum was also collected on treatment day 0 and day 150 (necropsy) for analysis of blood lipids and chemistry (see Supplementary tables).

All animals were necropsied and the reproductive tract collected at the end of treatment (150 days). The uterus was separated from the cervix and oviducts. Uterus, oviduct and cervix were weighed, and then the uterus was quartered along the longitudinal axis, and cross-sections (2 mm thick) from two uterine quarters were cut free-hand with a razor blade and prepared for immunocytochemistry (ICC) and morphological study. Endometrial and myometrial weights were obtained from the remaining two quarters after the endometrium was separated from the myometrium with fine scissors and weighed. The oviducts were dissected free from fat and connective tissue and weighed. Samples of fimbriae and ampulla were prepared for morphological study.

Histology and ICC

Samples for morphological study were fixed in 2% glutaraldehyde and 3% paraformaldehyde, embedded in glycol methacrylate (GMA), sectioned (uterus and cervix, 2 μm; oviduct, 1.5 μm) and stained with Gill’s haematoxylin. Samples of fresh tissue for ICC were microwave stabilized (Slayden et al., 1995) in an Amana Radiarrange Touchmatic microwave oven (Amana, IA, USA) for 7 s in 0.5 ml of Hanks’ Balanced Salt Solution (Gibco), then chilled on ice in 10% sucrose dissolved in 0.1 M phosphate-buffered saline (PBS, Sigma), mounted in Tissue Tek II OCT (Miles Inc., Elkhart, IN, USA) and frozen in liquid propane. Cryostat sections (5 μm) were thaw-mounted on Superfrost Plus (Fisher Scientific, Pittsburgh, PA, USA) slides, placed on ice at 5°C and microwave irradiated for 2 s. ICC of estrogen receptor alpha (ERα), progesterone receptor (PR) and Ki-67 was done as previously described (Slayden et al., 1995). ICC for androgen receptor (AR) was conducted similarly with monoclonal anti-human AR antibody F39.4 as previously described (Slayden et al., 2001b; Brenner et al., 2003; Slayden and Brenner, 2004). The microwave-treated sections were lightly fixed (0.2% picric acid and 2% paraformaldehyde in PBS) for 10 min, and the ICC was conducted with monoclonal anti-ERα (1D-5; Biogenex, San Ramon, CA, USA), anti-PR (JZB-39; provided by Geoffre Crop, Carpinteria, CA, USA) and anti-AR F39.4 (Biogenex). In each case, primary antibody was reacted with either biotinylated anti-mouse immunoglobulin G (IgG) (for 1D-5, F39.4 and anti Ki-67) or anti-rat IgG (for JZB-39) secondary antibody and detected with an avidin–biotin peroxidase kit (Vector Laboratories, Burlingame, CA, USA).

Morphometrics

Abundance of mitotic and Ki-67-positive cells in the endometrium were determined by a trained observer who used an ocular micrometer grid to define microscope fields and counted between 1200 and 5000 cells per animal with the aid of a mechanical tabulator. Mitotic index represented the number of mitoses per 1000 epithelial cells. Endometrial stromal cell density values (stromal compaction) were determined with the ImagePro Plus (Media Cybernetics, Inc., Silver Spring, MD, USA) image analysis software package. For this analysis, 10 non-overlapping fields (examined with a ×40 objective) of endometrial stroma in the upper functionalis of each specimen were analysed. The number of stromal cell nuclei per 10 000 μm² provided an index, which reflected the degree to which the endometrial stroma

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>E2 alone (3 cm implant)</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>E2 + low-dose progesterone (2 cm implant)</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>E2 + high-dose progesterone (6 cm implant)</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>E2 + 0.01 ZK 230 211 mg/kg</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>E2 + 0.05 ZK 230 211 mg/kg</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>E2 + 0.25 ZK 230 211 mg/kg</td>
<td>5</td>
</tr>
</tbody>
</table>

E2, estradiol.

*E2 was administered by a 3-cm Silastic capsule implant in all groups.

**ZK 230 211 was injected daily i.m. in all groups.
became expanded (more edematous and lower compaction number) or compacted (less edematous and higher compaction number). ImagePro Plus was also used to measure endometrial thickness (myometrium border–uterine lumen distance) from sections of GMA-embedded samples.

**BTB**

All animals were inspected daily for evidence of bleeding. The perineum was inspected visually, and if blood was observed, it was recorded as frank bleeding. If no blood was evident, the vagina was swabbed with a cotton-tipped swab, and blood on the swab was recorded as spotting. The total number of frank bleeding plus spotting days was recorded. Bleeding of either type typically lasted from 1 to several days, and this time was recorded as the bleeding interval.

**Statistical analysis**

Owing to heterogeneity of variance, data were transformed by (\(\log_{10}\) + 10) before one-way analysis of variance. Differences between means were then analysed by Fisher’s LSD or unpaired Student’s t-tests (Petersen, 1985).

**Results**

**Hormone levels and blood chemistry**

Serum E2 levels (92 ± 9.5 pg E2/ml) were similar among all the groups. Serum progesterone levels were 2.5 ± 0.2 ng/ml (low-dose progesterone) and 7.0 ± 1.9 ng/ml (high-dose progesterone). Blood lipid (see Supplementary table) and serum chemistry values (see Supplementary table) were all within the normal range for rhesus macaques. There were no significant differences among hormonal treatments.

**BTB**

Table II summarizes the incidence of BTB observed as either spotting or frank bleeding throughout the 5-month treatment interval for each group. The five animals treated with E2 alone exhibited both frank bleeding and spotting over a total of 34 days with a bleeding interval around 4.5 days. In the E2 + low-dose progesterone groups, BTB rates were similar to those with E2 alone, but in the E2 + high-dose progesterone group, there was a larger total BTB (63 days) and a longer bleeding interval, although this was entirely accounted for by one animal. In contrast, BTB was minimal in animals treated with ZK 230 211. None of the animals in the 0.01-mg group had any form of BTB, and in the 0.05- and 0.25-mg groups, bleeding intervals were < 2 days and consisted primarily of spotting.

**Assessment of long-term HT on the uterus**

**Effects of E2 alone on the uterus**

Effects on uterine and endometrial weights are presented in Table III to provide baseline comparisons with the other treatment groups. Histological analysis revealed that the endometrium from these animals had a proliferative appearance with primarily tubular glands and abundant mitotic and apoptotic cells in the glandular epithelium (Figures 1a and 2a). The endometrial stroma was moderately compacted, and there was no evidence of hyperplasia, metaplasia or cystic overgrowth. Analysis of mitotic cell counts and Ki-67-positive cells revealed that endometrial cell proliferation was maximal in this group with approximately half of the glandular epithelial cells in the functionalis zone staining positive for Ki-67 (Figure 3a; Table III). Mitotic or Ki-67-positive cells were rare in the basalis zone (Table III).

ICC for ER\(\alpha\), PR and AR is shown in Figure 4. In the animals treated with E2 alone, ER\(\alpha\) and PR staining was strong in both the glands and stroma of the functionalis and basalis zones. AR staining was strong in the endometrial stroma but absent in the glands of both the functionalis and basalis zones (basalis not shown).

**Effects of E2 + progesterone on the uterus**

Treatment with E2 + low progesterone had no significant effect on uterine or endometrial weight compared with E2 alone (Table III). Low progesterone also failed to induce notable endometrial decidualization. The endometrial glands in the E2 + low progesterone-treated animals showed only a moderate degree of glandular sacculation. Treatment with E2 + low progesterone did induce more hypertrophy of the spiral arteries as compared with E2 alone.

E2 + high-dose progesterone significantly increased total uterine weight, endometrial weight and endometrial thickness (\(P < 0.05\)). Histological analysis revealed that the animals in the E2 + high progesterone group had extensively decidualized endometrial stroma (Figure 1c) along with atrophied glands in the functionalis zone (Figure 2c) and excessive deposition of stromal extracellular matrix. Decidualization further resulted in significant stromal expansion compared with E2 alone (\(P < 0.05\)). High-dose progesterone also resulted in excessive hypertrophy of the spiral arteries (see Figure 1c), marked by strikingly thick arterial walls (Figure 2f). Treatment with progesterone at both high and low doses blocked endometrial cell proliferation in the functionalis zone as indicated by the

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>(E_2)</th>
<th>(E_2 + \text{low progesterone})</th>
<th>(E_2 + \text{high progesterone})</th>
<th>(E_2 + 0.01 \text{mg ZK 230 211})</th>
<th>(E_2 + 0.05 \text{ZK 230 211})</th>
<th>(E_2 + 0.25 \text{mg ZK 230 211})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days of spotting</td>
<td>24</td>
<td>19</td>
<td>48</td>
<td>0</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Days of frank menses</td>
<td>10</td>
<td>9</td>
<td>15</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Bleeding interval length (b)</td>
<td>4.5 ± 1.61</td>
<td>4.12 ± 1.58</td>
<td>9 ± 2.61</td>
<td>0</td>
<td>1.8 ± 0.31</td>
<td>1 ± 0</td>
</tr>
</tbody>
</table>

\(E_2\), estradiol.

\(b\)Values represent total days for all animals combined over the 150-day treatment period.

\(b\)Length of each bleeding interval in days (mean ± SE). Includes frank bleeding and spotting days combined.
lack of Ki-67-positive epithelial cells and the absence of mitoses. ERα, PR and AR immunostaining was minimally affected in animals treated with E2 + low progesterone, but staining of all receptors except stromal PR was suppressed by E2 + high progesterone (Figure 4).

**Effects of long-term E<sub>2</sub> + ZK 230 211 on the uterus**

All doses of ZK 230 211 significantly reduced endometrial weight compared to both doses of the E2 + progesterone group and to the group treated with E2 alone (Table III, P < 0.05). Reduction in endometrial mass in the ZK 230 211-treated animals was associated with a dramatic thinning of the endometrium (Figure 1; Table III). There was a dose-dependent degeneration of the glands, especially in the upper regions of the endometrium. At all doses of ZK 230 211, there was increased stromal compaction and formation of dilated glands that were lined by a low cuboidal, non-mitotic epithelium (Figure 2g–i). Analysis of Ki-67 immunostaining (Figure 3) and mitotic cell counts revealed that all doses of ZK 230 211 induced a profound inhibition of glandular cell proliferation. ZK 230 211 at all doses resulted in degeneration of the spiral arteries (Figure 2j–l) with hyalinization of the periarterial stroma. None of these anti-arterial effects were evident in animals treated with E2 alone or E2 + progesterone.

Figure 4j–r shows ICC for ERα, PR and AR in the endometrium of animals treated with E2 + ZK 230 211. Unlike progesterone, ZK 230 211 did not inhibit glandular or stromal ERα or PR expression. Moreover, ZK 230 211 induced a dose-dependent increase in immunostaining of AR in the endometrial glands.

### Oviductal effects

**E2 alone**

As previously found in shorter term studies (Brenner and Slayden, 1994) treatment with E2 alone for 150 days resulted in a fully differentiated oviductal epithelium characterized by a tall columnar epithelium with ~50% ciliated and 50% secretory cells (Figure 5, Table III). There was no evidence of hydrosalpinx in this group.

**E2 + progesterone**

Treatment with E2 + progesterone at both doses significantly reduced oviductal weight (Table III, P < 0.05), ampulla diameter (Figure 5) and cell height (Figure 5b and c) and suppressed differentiation compared with E2 alone. The oviductal epithelium was low cuboidal and was markedly deciliated and non-secretory (Table III). No hydrosalpinx were evident.

**E2 + ZK 230 211**

Unlike the effects of progesterone, treatment with E2 + ZK 230 211 did not suppress oviductal weight or block the ability of E2 to maintain the oviductal epithelium in a fully ciliated secretory state (Figure 5g–i). Rather, there was a trend (non-significant) towards an increase in oviductal wet weight and diameter, accompanied by an increase in the amount of oviductal stroma in the animals treated with E2 + ZK 230 211 compared with those treated with E2 alone or E2 + progesterone. Notably, there were hydrosalpinx in several animals treated with ZK 230 211 (Table III). The hydrosalpinx were usually located on one side (occasionally both sides), were generally located in the isthmus near the uterine end and were generally small.

### Effects on the cervix and vagina

**E2 alone**

In the mid-cervix, after 5 months of continuous treatment with E2 alone, the cervical epithelium showed patches of squamous metaplasia in which regions of columnar, mucous cells were replaced by stratified squamous epithelium (Figure 6a). In the ectocervix, the stratified squamous epithelium was normal in

### Table III. Reproductive tract morphometrics (mean ± SE) in macaques treated with ZK 230 211<sup>1</sup>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>E&lt;sub&gt;2&lt;/sub&gt; (n = 5)</th>
<th>E&lt;sub&gt;2&lt;/sub&gt; + low progesterone (n = 4)</th>
<th>E&lt;sub&gt;2&lt;/sub&gt; + high progesterone (n = 5)</th>
<th>E&lt;sub&gt;2&lt;/sub&gt; + 0.01 mg ZK 230 211 (n = 5)</th>
<th>E&lt;sub&gt;2&lt;/sub&gt; + 0.05 mg ZK 230 211 (n = 5)</th>
<th>E&lt;sub&gt;2&lt;/sub&gt; + 0.25 mg ZK 230 211 (n = 5)</th>
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<tbody>
<tr>
<td>Uterine morphometrics</td>
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<tr>
<td>Uterus weight (g)</td>
<td>5.36 ± 1.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.81 ± 0.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.48 ± 2.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.08 ± 3.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.43 ± 0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.44 ± 0.69&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Endometrial thickness (mm)</td>
<td>0.122 ± 0.169&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.218 ± 0.188&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.710 ± 0.674&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.023 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.013 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.014 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Functionalis Ki-67 (%)</td>
<td>3.5 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.8 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.31 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Functionalis mitotic index&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6.1 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>Basal Ki-67 (%)</td>
<td>0.6 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0 ± 4.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Basal mitotic index&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.88 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>Oviducal morphometrics</td>
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<tr>
<td>Oviduct weight</td>
<td>0.78 (0.12&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>0.32 (0.05&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>0.35 (0.02&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>1.19 (0.71&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>1.22 (0.50&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>0.92 (0.39&lt;sup&gt;c&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Fimbrial ciliation (%)</td>
<td>47.3 ± 8.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7 ± 1.1b</td>
<td>36.1 ± 2.5a</td>
<td>39.0 ± 5.5a</td>
<td>42.3 ± 3.8a</td>
</tr>
<tr>
<td>Frequency of hydrosalpinx&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>4</td>
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<tr>
<td>Cervix morphometrics</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Cervix weight (g)</td>
<td>7.02 (1.06&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>4.77 (0.89&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>6.66 (0.99&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>9.38 (1.54&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>6.38 (0.29&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>5.73 (0.43&lt;sup&gt;c&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

E<sub>2</sub> estradiol; nd, not detected.

<sup>1</sup>Means in each column with different superscripts are statistically different (P < 0.05).

<sup>2</sup>Endometrial weights represent the weight from one-half of the uterus in grams.

<sup>3</sup>Mitotic index represents the number of mitotic cells/1000 cells counted.

<sup>4</sup>Stromal compaction represents the number of stromal nuclei/10 000 μm<sup>2</sup> at x400 magnification.

<sup>5</sup>Value represents the number of animals with at least one hydrosalpinx in each group.
appearance but thickened (Figure 6d). In the rhesus vagina, as previously noted (Otto et al., 2002), the stratified squamous epithelium was thrown up into large spines 3–5 mm in height which covered the vaginal surface except for regions near the introitus and the cervix.

**E₂ + progesterone**

Progesterone (both low and high dose) blocked the appearance of squamous metaplastic foci in the mid-cervix that developed in animals treated with E₂ alone (Figure 6b). High-dose progesterone also blocked the ability of E₂ to support a thick layer of stratified squamous epithelium in the ectocervix (compare Figure 6d–f). In the vagina, progesterone treatment greatly reduced the extent of the vaginal spines in a dose-dependent manner but did not greatly reduce the overall thickness of the stratified squamous epithelium.

**E₂ + ZK 230 211**

At the lowest dose of ZK 230 211, the mid-cervix displayed foci of epithelial metaplasia similar to that seen after E₂ alone (Figure 6f), but at higher doses, ZK 230 211 suppressed the ability of E₂ to induce squamous metaplasia in the mid-cervix (Figure 6g–h). In the ectocervix, E₂ + ZK 230 211 at all doses...
maintained a thick stratified epithelium (Figure 6l–k), and in the vagina, typical spines were evident at all doses, but there was a tendency for the highest dose of ZK 230 211 to produce more ridges and fewer spines. These effects indicate that ZK 230 211 can modify the vaginal effects of E2 somewhat, but the ability of E2 to induce a thick, stratified, squamous epithelium was unaffected.

The mammary gland

E2 alone

After E2 alone, the mammary glands consisted of abundant acini composed of numerous alveoli lined with cuboidal epithelial cells in addition to large, branching ducts containing stratified epithelium. The lobular–alveolar–ductal structures were enveloped by connective and adipose tissue containing an abundant vascular supply and a few nerves (Figure 7a).

E2 + progesterone

The major differences between this group and E2 alone were that alveolar diameters appeared larger and alveoli were packed closer together within a lobule, but these differences were not very great (Figure 7b).

E2 + ZK 230 211

Treatment with all doses of ZK 200 211 resulted in mammary morphology very similar to that of E2 alone. No obvious signs of ductal, alveolar or stromal hyperplasia were evident (Figure 7c).

In sum, there were no notable differences attributable to the different hormone or PA treatments on the mammary glands. The histological structures were identical in all treatment groups (Figure 7a–c), and Ki-67 immunostaining showed no differences between treatments on proliferative rates (Figure 7d–f).

Discussion

Although the literature clearly indicates that chronic unopposed estrogen can lead to endometrial hyperplasia in women, our findings in the current work did not confirm that unopposed E2, at the dose (∼100 pg/ml) and time (5 months) we used, induced hyperplasia in the ovariectomized rhesus macaque. Baskin et al. (2002) reported that endometrial hyperplasia occurred to a variable degree in six ovariectomized rhesus macaques that had received much higher doses of E2 alone, averaging ∼537 pg/ml, for a much longer time (17–22 months). We chose our E2 dose to parallel the physiological levels of E2 that occur during the follicular phase in the rhesus macaque, which, with the exception of the brief pre-ovulatory surge, average ∼80 pg/ml. This dose of E2 was also within the range of doses reported for HT in women (Bennink and Herjan, 2004; Stopinska-Gluszak et al., 2006). Our goal was to use low, physiological doses of E2 in keeping with current clinical trends in which the lowest effective E2 doses are used in contraceptive and other formulations (Boerrigter et al., 1996).

Unopposed E2 did induce squamous metaplasia in the cervix, which confirms older observations that squamous metaplasia is a normal effect of continuous estrogen action in the rhesus monkey cervix (Hisaw and Hisaw, 1961). This metaplasia never becomes anaplastic or dysplastic, disappears when the estrogen treatment is stopped and can be blocked by progesterone treatment (Hisaw and Hisaw, 1961). The data in the current study are the first indication that a PA can block this estrogen-dependent effect in a dose-related manner.

In this study, our working hypothesis was that a potent PA such as ZK 230 211 could be combined with a physiological level of estrogen to provide a novel form of menopausal HT. Estrogen + ZK 230 211 treatment over 5 months in these macaques suppressed endometrial growth and greatly reduced BTB without inducing atrophy in other parts of the reproductive tract such as the oviduct, the cervix or the vagina and without inducing endometrial hyperplasia. Consequently, these data support our suggestion that a PA could be used systemically along with estrogen for menopausal HT.
There were some side effects of the E₂ + ZK 230 211 treatment, namely hydrosalpinges and endometrial glandular cysts that were unexplained, but neither of these changes represented severe pathology. Hydrosalpinges were most common at the higher doses of E₂ + ZK 230 211. Because oviductal fluid normally passes into either the peritoneal cavity or the uterine lumen, any mechanical obstruction to fluid flow will induce hydrosalpinges. Increases either in smooth muscle constrictions at points along the oviductal flexures or in the walls of the intramural region could block oviductal fluid egress. Further studies of fluid flow and smooth muscle contracture during the course of PA treatment would be needed to reveal the mechanism underlying these effects.

Regarding the glandular cysts, there was a tendency for the upper portions of the glands to degenerate and disappear in animals treated with E₂ + ZK 230 211. Consequently, the serum

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**Figure 5.** Photomicrographs showing glycol methacrylate (GMA) sections of the oviduct. With estradiol (E₂) alone, (a) high power shows the epithelium is differentiated into ciliated and secretory cells and (d) low power shows typical oviductal mucosa with extensive folding that partially fills the lumen. With E₂ + low or high progesterone, both doses of progesterone blocked epithelial differentiation (b and e) and reduced the overall diameter of the oviduct (e and f). All doses of ZK 230 211 permitted E₂-driven differentiation of the oviduct into a ciliated and secretory state (g-i), and the overall diameter of the oviducts was similar or slightly greater than that seen after E₂ alone. Bar indicates scale.
transudate pumped across the epithelium of the remaining portions of the glands would lack egress into the uterine lumen, and the resulting pressure could dilate the glandular cavities. The epithelium lining the dilated glands was in a low cuboidal, non-proliferative state typical of other fluid-filled cysts. There was no evidence for excessive mitotic activity, atypia, branching or stromal invasion in the endometria of the ZK 230 211-treated animals. Because hydrosalpinges and glandular cysts were more pronounced at the higher doses of ZK 230 211, lower doses of this very potent PA would likely minimize their occurrence.

A serious disadvantage of combined estrogen + progestin therapy for HT in menopausal women is the increase in BTB that is commonly seen (Anderson, 1992; Critchley, 2003). In our study, animals treated with either E2 alone or E2 + progesterone showed considerable BTB but animals treated with ZK 230 211 showed much less. We reported previously that PA therapy with a related PA, ZK 137 316, can suppress menses in naturally cycling rhesus macaques (Slayden et al., 2001a), and there are several reports that PA treatment can improve BTB in women treated with progestins (Glasier et al., 2002; Massai et al., 2004). Therefore, one of the advantages of E2 + PA treatment for menopausal HT is that the endometrial antiproliferative effect would be accompanied by a reduction in BTB.

Long-term progestin-only contraception also induces endometrial atrophy but is often associated with BTB, which is currently considered due to increased vessel fragility (Hickey and Fraser, 2000) and/or focal expression of stromal matrix metalloproteinases (MMPs) (Marbaix et al., 2000). In contrast, PA therapy suppresses the spiral arteries in macaques and may have other inhibitory effects on endometrial vessels that counteract bleeding tendencies. Therapy that combines progestin-based contraception with intermittent use of a PA can inhibit BTB and improve menstrual cycle control (Gemzell-Danielsson et al., 2002). However, further study of the action of PAs on endometrial vessels is needed to more fully explain the dramatic inhibitory effects of PAs on endometrial bleeding.

The molecular mechanism through which PA compounds like ZK 230 211 specifically block E2-stimulated endometrial growth has not been fully explained. In the current work, as previously noted with other Pas (Slayden et al., 2000), the AR was markedly elevated in the glandular epithelium of the E2 + ZK 230 211-treated animals. Because androgens can inhibit the effects of estrogens in the endometrium, increases in endometrial AR could bind endogenous androgens and mediate the antiproliferative effects of PA. Treatment with the anti-androgen flutamide reversed many of the effects of PA on the endometrium (Slayden and Brenner, 2003), which is strong evidence that AR is involved in the antiproliferative effect. The
nature of the androgenic ligand that occupies AR in the endometrium of PA-treated subjects is unknown. ZK 230 211 does not effectively occupy ER (Fuhrmann et al., 2000), but it binds to AR and has partial agonist activity (Fuhrmann et al., 2000). However, it does not inhibit estrogen effects in the primate oviduct though AR is expressed by the oviduct. Moreover, androgens, administered systemically, can severely inhibit E2-stimulated growth and differentiation in the fallopian tube (Hirst et al., 1992). Therefore, the evidence suggests that local androgens specific to the endometrium are the relevant AR ligands. Androstenedione may be such a ligand, as this androgen, but not others, blocked E2 effects on endometrial cells in vitro (Tuckerman et al., 2000), and its concentration was higher than either testosterone or dihydrotestosterone in human endometrium (Suri, 1997). Little is known about metabolism of androgens in the primate endometrium, but there is evidence that the 17-beta hydroxysteroid dehydrogenases that modulate steroid synthesis are hormonally regulated in the human endometrium (Burton et al., 2003). Additional studies are needed to explore the molecular mechanisms underlying the enhanced synthesis of glandular AR by PA and to understand the role of AR in the endometrial antiproliferative effect.

The macaque is an excellent model in which to pursue such studies because the human and macaque endometria respond so similar to PA treatment. In women, as in macaques, mifepristone reduces endometrial thickness (Baird et al., 2003), suppresses endometrial cell proliferation (Cameron et al., 1996; Baird et al., 2003), reduces endometrial vascular development, increases ER, PR (Cameron et al., 1996) and AR (Slayden et al., 2001b) and increases the occurrence of benign glandular cysts (Baird et al., 2003). Preclinical studies of PA action are best done in macaques.

In these studies, we used a specific, very potent PA as a proof of principle. There are other molecules, in particular, the newer, selective progesterone receptor modulators that may be excellent choices for a similar clinical use. In sum, we showed that a 5-month, long-term treatment with E2 + a potent PA can block endometrial cell proliferation and endometrial growth without inhibiting E2 action in other regions of the reproductive tract. E2 + PA also suppressed the incidence and severity of BTB. This novel approach to menopausal support deserves a careful risk-benefit analysis in the clinic.

### Supplementary material

Supplementary data are available at http://humrep.oxfordjournals.org/.

### References


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Submitted on April 10, 2006; resubmitted on May 17, 2006; accepted on May 26, 2006.