Reversible suppression of menstruation with progesterone antagonists in rhesus macaques

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BACKGROUND: A reliable means of menstrual suppression would greatly improve the quality of life for women. Information is lacking on the direct endometrial effects and appropriate dosages of new antiprogestins that may be useful for this purpose. METHODS: The current work evaluated three different systems in macaque monkeys. First, the range of doses of two relatively new antiprogestins, ZK 137 316 and ZK 230 211, that would block progesterone action directly on the endometrium in artificially cycled, spayed rhesus macaques; second, the direct endometrial effects of ZK 230 211, a type III antiprogestin; and third, investigation of whether endometrial-suppressive doses administered chronically to intact, cycling monkeys could be used for reversible, menstrual suppression. RESULTS: The results in naturally cycling animals showed that ZK 137 316 blocked menstruation in all animals, but doses of 0.05 mg/kg blocked ovulation in 55.5% of animals and doses of 0.1 mg/kg blocked ovulation in 66.6% of the animals. However, all doses of ZK 230 211 that blocked menstruation also blocked ovulation. All progesterone antagonist (PA)-treated animals, regardless of dose, maintained normal follicular phase concentrations of oestradiol and returned to normal menstrual cyclicity within 15–41 days post-treatment. Therefore ZK 137 316, depending on dose, can allow ovulation but block menstruation, while ZK 230 211, a much more potent PA, blocks both ovulation and menstruation at all effective doses. Both PAs block unopposed oestrogenic action on the endometrium through their antiproliferative effects. CONCLUSIONS: Reversible amenorrhoea can be achieved with these two PAs, and they can protect the endometrium from the effects of unopposed oestrogen whether or not ovulation is blocked. Chronic, low dose PA treatment may provide a new option for women who wish to suppress their menstrual periods.

Key words: endometrium/macaque/menstruation/ovulation/progesterone antagonists

Introduction

As has recently been stressed (Coutinho and Segal, 1999; Thomas and Ellertson, 2000), a reliable means of menstrual suppression would greatly improve the quality of life for women. This is particularly so for women in stressful occupations, such as the military, that require strenuous duty, frequent travel and unexpected assignments. The modern woman is accustomed to having control over her reproductive functions, and menstruation is one function that many women would like to control. While administration of oral contraceptives without a pill-free interval can be used for this purpose, not all women respond favourably to synthetic oestrogen-progesterone combinations, and there are some health conditions (e.g. clotting disorders) that prescribe their use. Progesterone antagonist (PA, antiprogestin) therapy can provide an alternative method of menses suppression. The dramatic inhibition of endometrial growth and development that these compounds induce (Slayden et al., 1998) suggests that they may also serve as a treatment for some menstrual disorders, including dysfunctional uterine bleeding, menorrhagia and irregular bleeding. PAs including mifepristone (RU 486), onapristone (ZK 98 299) and others, as well as newly developed progesterone receptor modulators (PRMs), either block or modulate progesterone action by binding to the progesterone receptor (PR), and completely (PAs) or partially (PRMs) inhibiting progesterone-dependent gene expression (Goodman and Hodgen, 1996; Spitz et al., 1996; Elger et al., 2000). In theory, chronic PA therapy would negate any and all of the effects of progestins that lead to unwanted menstrual bleeding.

Experimental menstruation can easily be induced in both women (Critchley et al., 1999) and Old World nonhuman primates (Brenner et al., 1996) by experimental progesterone withdrawal from an oestradiol plus progesterone primed endometrium. Menstruation in rhesus macaques therefore provides an experimental model that can be used to assess the ability of antiprogestins to suppress uterine bleeding.
The ability of antiprogestins to inhibit the menstrual cycle was first described in cynomolgus monkeys. In that study, high doses of RU 486 (5 mg/day) administered on days 10–12 of the menstrual cycle, delayed the mid-cycle LH surge and lengthened the intermenstrual interval from the normal ~30 days, to 61 days, effectively blocking one menstrual cycle (Collins and Hodgen, 1986). Similar results were reported for women treated with RU 486 during the follicular phase, where RU 486 disrupted follicle maturation and delayed progression of the menstrual cycle (Liu et al., 1987). A single high dose of RU 486 administered to women during the midluteal phase of the cycle also suppressed serum LH pulse amplitude and frequency, was luteolytic and induced menstruation (Garzo et al., 1988). The same workers also reported that corpus luteum function after a single dose of RU 486 could be rescued by exogenous HCG, but menstruation was still induced because RU 486 blocked progesterone action in the endometrium.

ZK 137 316 and ZK 230 211 are new-generation PAs with increased potency and reduced antiglucocorticoid activity. ZK 137 316, like RU 486, is a type II antagonist that can under certain circumstances exhibit agonistic activity in vitro and in vivo (Klein-Hitpass et al., 1991; Chwalisz et al., 2000a). ZK 230 211 is a type III antagonist and does not display any PR agonistic activity in vitro or in vivo (Fuhmann et al., 2000). In our previously published studies of ZK compounds we found that 0.03 mg/kg ZK 137 316 would inhibit endometrial development (Slayden et al., 1998) but still allow menstrual and ovarian cyclicity in half of the animals treated while higher doses inhibited menstrual cyclicity (Zelinski-Wooten et al., 1998b). These lower doses of ZK 137 316 were then shown to be contraceptive (Zelinski-Wooten et al., 1998a), but it was unknown if the effects of ZK 137 316 would be acutely reversible once treatment was stopped. In the current study we evaluated higher doses of these PAs to determine whether regimens could be identified that would reliably block menstrual bleeding, whether ovarian function was affected, and whether the effects of such doses would be reversible.

The current work had three aims: first, to discover the range of doses of ZK 137 316 and ZK 230 211 that would block progesterone action directly on the endometrium in artificially cycled, spayed monkeys; second, to document the histological effects of ZK 230 211 on the endometrium; and third, to determine whether such endometrial-suppressive doses administered chronically to intact cycling monkeys would cause reversible, menstrual suppression. Two dose-finding studies were carried out in the spayed animals: (i) menstrual blockade; to determine the dose of PA which, if given chronically for one cycle, would block menstruation on progesterone withdrawal, and (ii) menstrual induction; to determine the dose that would induce menstruation when given acutely at the end of an artificial cycle. The most likely doses were then selected for chronic studies of menstrual suppression in naturally cycling animals. The work was conducted over a three-year period; ZK 230 211 became available for study later in the work. Our overall goal was to obtain preclinical data useful for clinical development of a novel mode of reversible, menstrual suppression for women who may desire such suppression.

Materials and methods

Progestosterone antagonists

ZK 137 316 and ZK 230 211 were provided by Schering AG, Berlin, and administered by i.m. injection in a nonirritating vehicle (HPE) that consisted of 37.5% Hanks Balanced Salt Solution (Gibco BRL; Grand Island, NY, USA), 37.5% 1,2-propanediol, and 25% ethanol (Aaper, Shelbyville, KY, USA). Except where indicated, all hormones and other reagents were purchased from the Sigma Chemical Co (St Louis, MO, USA).

Animal care

Animal care was provided by the Division of Animal Resources at the Oregon Regional Primate Research Center (ORPRC) following the National Institutes of Health guidelines on the care and use of animals. Chronic treatment of cycling macaques with ZK 137 316 or ZK 230 211 had no obvious effects on general animal health. Artificial menstrual cycles were created as previously described (Slayden et al., 1993). Briefly, a 3 cm Silastic capsule (0.3 cm inner diameter; 0.64 cm outer diameter; Dow Corning; Midland, MI, USA) filled with crystalline oestradiol was first inserted s.c. to induce an artificial follicular phase. After 14 days of oestradiol priming, a 6 cm Silastic capsule containing crystalline progesterone was inserted s.c. for an additional 14 days to stimulate an artificial luteal phase. Removal of the progesterone implant on day 28 completed the cycle and induced menstruation. Serum samples were collected during the artificial cycles to confirm normal concentrations of oestradiol and progesterone.

Menstrual blockade of progesterone-withdrawal menses by chronic administration of ZK 137 316 in artificially cycled, ovariectomized macaques

The animals were injected with ZK 137 316 daily in HPE during a complete cycle (oestradiol for 14 days and then oestradiol + progesterone for 14 days). Control animals received vehicle only during the artificial cycle (n = 4). Four doses of ZK 137 316 were tested: 0.01, 0.03, 0.05, and 0.1 mg/kg body weight (n = 3 each). At the end of the cycle, the progesterone implants were removed, and injections of ZK 137 316 were continued for 7 more days. Vaginal swabs were performed daily for 9 days after progesterone implant removal and the incidence of frank menses or minute bleeding was recorded. ZK 230 211 was not available at the time this menstrual blockade study was done. All of the animals tested for menses blockade were allowed to recover for 60 days to clear residual effects of ZK 137 316 and were then returned to the Primate Centre colony.

Menstrual induction in ovariectomized macaques

Menses induction in a progesterone-primed animal is another reliable, non-invasive indicator of antiprogestin action on the endometrium. Beginning on day 28 of an artificial menstrual cycle, the monkeys were injected daily with antiprogestin in HPE vehicle for 7 days while the progesterone implant remained in place. Control animals received vehicle only (n = 4). Five doses of ZK 137 316 were tested: 0.01 (n = 6), 0.03 (n = 6), 0.05 (n = 4), 0.1 (n = 6), and 0.15 (n = 3) mg/kg body weight. Five doses of ZK 230 211 were also tested: 0.005 (n = 2), 0.01 (n = 4), 0.03 (n = 4), 0.05 (n = 2), and 0.1 (n = 2) mg/kg. Vaginal swabs were performed daily for 9 days and the ability of the various doses of antiprogestin to induce frank, overt menses (blood detectable on the external genitalia and the cage floor, confirmed by vaginal swab) was recorded. All of the animals tested for menses induction were allowed to recover for 60 days to clear residual effects of PAs and were then returned to the Primate Centre colony.
**Morphological effects of ZK 230 211 in ovariectomized hormone-treated macaques**

In preliminary work, we found that treatment of artificially cycled, ovariectomized animals for 1 month with 0.1 mg/kg ZK 137 316 produced endometrial suppression essentially equivalent to the suppression we had previously observed in naturally cycling macaques treated with that dose for 5 months (Slayden et al., 1998). Therefore, in this study, we focused attention on dosage effects of ZK 230 211 on histomorphometric analyses. Five groups of ovariectomized macaques were treated as described above to create artificial menstrual cycles. Beginning on day 1 of the second cycle the animals were treated with various doses as shown in Table I. Doses were selected based on findings from the menstrual induction study. Animals treated with oestradiol alone were included to provide a baseline measure of the degree of oestradiol-dependent proliferation.

At the end of treatment, reproductive tracts were collected and the uterus was separated from the cervix and oviducts. The uterus was quartered along the longitudinal axis, and cross-sections (2 mm thick) from two uterine quarters were cut freehand with a razor blade and prepared for immunocytochemistry 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. The oviducts were dissected free from fat and connective tissue and weighed. Samples of fimbriae and ampulla were prepared for morphological study. Details of tissue handling were as follows.

**Histology and immunocytochemistry**

Tissue samples for morphological study were fixed in 2% glutaraldehyde and 3% paraformaldehyde, embedded in glycol methacrylate (GMA), sectioned (uterus, 2 μm; oviduct 1.5 μm) and stained with Gill’s Hematoxylin (Sandow et al. 1979). Samples of fresh tissue for immunocytochemistry were microwave stabilized (Slayden et al., 1995) in an Amana Radarrange Touchmatic microwave oven (Amana, Iowa, USA) for 7 seconds in 0.5 ml Hank’s Balanced Salt Solution (Gibco), then chilled on ice in 10% sucrose dissolved in 0.1 mol/l 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 microwaved for 2 s. Immunocytochemistry of oestrogen receptor alpha (ERα), PR and Ki-67 was carried out as recently described (Slayden et al., 1998). Briefly, the microwave-treated sections were lightly fixed (0.2% picric acid, 2% paraformaldehyde in PBS) for 10 min; and the immunocytochemistry was conducted with monoclonal anti-ERα (1D-5; Biogenex, San Ramon, CA, USA), anti-PR (JZB-39; provided by Geoffrey Greene, University of Chicago) or anti-Ki-67 antigen (Dako Corp., Carpinteria, CA, USA). In each case primary antibody was reacted with either biotinylated anti-mouse IgG (for 1D-5 and anti Ki-67) or anti-rat IgG (for JZB-39) second antibody and detected with an avidin-biotin peroxidase kit (Vector Laboratories, Burlingame, CA, USA).

**Table I. Doses and treatments on day 1 of the second cycle**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Oestradiol alone (n = 4)</td>
<td>Oestradiol for 28 days</td>
</tr>
<tr>
<td>Oestradiol + progesterone (n = 4)</td>
<td>Oestradiol for 14 days then oestradiol plus progesterone for 14 days (one artificial cycle)</td>
</tr>
<tr>
<td>Oestradiol + progesterone + 0.005 mg ZK (n = 3)</td>
<td>One artificial cycle plus 0.005 mg/kg ZK 230 211 daily</td>
</tr>
<tr>
<td>Oestradiol + progesterone + 0.016 mg ZK (n = 3)</td>
<td>One artificial cycle plus 0.016 mg/kg ZK 230 211 daily</td>
</tr>
<tr>
<td>Oestradiol + progesterone + 0.032 mg ZK (n = 3)</td>
<td>One artificial cycle plus 0.032 mg/kg ZK 230 211 daily</td>
</tr>
</tbody>
</table>

**Morphometry**

The abundance of mitotic cells, Ki-67 positive cells, and apoptotic cells in the endometrium was determined by a trained observer who used an ocular micrometer grid to define microscope fields and counted between 1200–5000 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 Optimas 3.0 image analysis software package. For this analysis, 10 non-overlapping fields (examined with a ×25 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 became expanded (more oedematous) or compacted (less oedematous). Apoptotic cell counts were carried out in glycol methacrylate sections. Apoptotic bodies consist of cytoplasmic fragments containing nuclear elements and these can easily be distinguished in such sections. Percentage apoptosis was based on the number of apoptotic bodies per 5000 glandular epithelial cells.

**Naturally cycling macaques: menstrual suppression**

Untreated, adult macaques were first monitored for two complete menstrual cycles to document normal cycle lengths for each animal prior to treatment. Three independent protocols (see below) were conducted in naturally cycling animals. The basic design of these treatment protocols is depicted graphically in Figure 1. After treatments were complete, all of the animals were allowed to rest for a minimum of two normal menstrual cycles and were then returned to the Primate Center colony.

**Protocol 1: Short term (40 day) treatment with ZK 137 316**

Beginning on the day after the onset of menstruation (day 2), adult macaques with normal menstrual cycles were injected daily with ZK 137 316 in HPE for 40 days (see Figure 1). Three dosages were used: HPE vehicle only (control; n = 4), 0.05 mg/kg (n = 9), and 0.1 mg/kg (n = 9). Daily blood samples and vaginal swabs were collected during the treatment period until the animals displayed a menstruation of longer than 2 days. After menstruating, the monkeys were allowed to rest for one menstrual cycle, and then daily blood samples were again collected for one menstrual cycle. All blood samples were analysed for concentrations of oestradiol and progesterone. Lengths of the menstrual cycles, intermensural interval, and serum hormone concentrations were compared between treatment groups.

**Protocol 2: Long term (100 day) treatment with ZK 137 316**

Starting on day 2 of menses, animals with normal menstrual cycles were injected daily for 100 days with ZK 137 316 in HPE. Three dosages (n = 4 each) were used: (i) vehicle only (control), (ii) 0.05 mg/kg ZK 137 316, and (iii) 0.1 mg/kg body weight. Daily vaginal swabs were collected during the entire treatment period. Daily blood samples were collected for analysis of oestradiol and progesterone during the last 30 days of treatment and continued until...
Experimental design

Figure 1. Experimental design for assessment of effects of PAs on menstrual cycles in naturally cycling macaques. The figure shows how intermenstrual interval, recovery period and post-treatment cycles were defined.

Table II. Serum concentrations of oestradiol and progesterone (mean ± SEM) in ovariectomized rhesus macaques treated with Silastic implants of oestradiol, oestradiol + progesterone or oestradiol + progesterone + ZK 230 211 (ZK)

<table>
<thead>
<tr>
<th></th>
<th>Oestradiol alone (n = 5)</th>
<th>Oestradiol + progesterone (n = 5)</th>
<th>Oestradiol + progesterone + 0.005 mg ZK (n = 3)</th>
<th>Oestradiol + progesterone + 0.016 mg ZK (n = 3)</th>
<th>Oestradiol + progesterone + 0.032 mg ZK (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol</td>
<td>80.66 ± 23</td>
<td>67.66 ± 43</td>
<td>74.76 ± 21</td>
<td>62.40 ± 33</td>
<td>73.87 ± 20</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.12 ± 0.07</td>
<td>4.42 ± 1.6</td>
<td>4.99 ± 1.3</td>
<td>4.72 ± 1.1</td>
<td>5.16 ± 1.2</td>
</tr>
</tbody>
</table>

Figure 2. Menstrual blockade: dosage effects of ZK 137 316 in ovariectomized, artificially cycled animals. Values represent mean (± SEM) days of bleeding. Bars with different superscripts are significantly different (P < 0.05).

the monkeys menstruated (2 days). The monkeys were further monitored for menses during the first post-treatment cycle.

Protocol 3: Intermediate (60 day) treatment with ZK 230 211
During protocols 1 and 2 we showed that HPE vehicle injection throughout the entire treatment periods had no effect on hormone concentrations, menstrual cycles or endometrial bleeding patterns. Therefore, in protocol 3, macaques were first injected i.m. daily with HPE alone for one control, pretreatment cycle. Then beginning on the day after menses in the next cycle, the animals received ZK 230 211 in HPE (i.m.) daily for 60 days. Three dosages of ZK 230 211 (at steps of ~3-fold) were compared: (i) 0.005 mg/kg, (ii) 0.016 mg/kg and (iii) 0.05 mg/kg body weight (n = 5 each). Daily vaginal swabs and daily blood samples were collected during the pretreatment cycle, the treatment period (60 days) and the recovery period, until the monkeys menstruated for longer than 2 days. The monkeys were further monitored for menstruation by vaginal swab without blood collection during the first post-treatment cycle. Blood samples were assayed for serum concentrations of oestradiol and progesterone, and once these concentrations were known, samples that flanked the surge (or highest value) of oestradiol were assayed for bioactive LH.

Hormone assays
All blood samples were analysed for concentrations of oestradiol and progesterone by routine radioimmunoassay and LH determinations were made by bioassay (Zelinski-Wooten et al., 1998b). All assays were performed by the ORPRC Hormone Assay Core.

Statistical analysis
Quantitative data were compared by analysis of variance (ANOVA). Significant differences among means were determined by Fisher’s protected least significant difference test (Petersen, 1985). These data included lengths of the pre-treatment menstrual cycles, treatment-induced intermenses interval, length of the recovery period (time to return to menses) after the last injection, post-treatment menstrual cycle length, tissue weights, percentage fimbrial ciliation, mitotic index, percentage Ki-67 positive epithelial cells, apoptotic index and degree of stromal compaction.

Results

Ovariectomized animals

Hormone assays
Table II shows the hormone concentrations produced by Silastic implants of oestradiol and progesterone in ovariectomized macaques treated with ZK 230 211. There was no effect of ZK 230 211 treatment on oestradiol or progesterone concentrations in the ovariectomized animals.

Dose-finding 1. Menstrual blockade induced by daily administration of ZK 137 316.
Figure 2 shows the effects of daily administration of various doses of ZK 137 316 on progesterone withdrawal bleeding in
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Figure 3. Menstrual induction: comparison of dosage effects of ZK 137 316 versus ZK 230 211 in ovarioctomized, artificially-cycled macaques. Values represent mean (± SEM) days bleeding. Bars with different superscripts are significantly different (P < 0.05).

artificially cycled, ovarioctomized animals. As expected, all the control, HPE injected animals menstruated when progesterone was withdrawn at the end of the artificial cycle. Treatment with 0.01 mg/kg ZK 137 316 daily during the artificial cycle did not prevent progesterone withdrawal menses. At the 0.03 mg/kg dose, frank bleeding was detected in one out of three animals; the other two animals showed only minute bleeding. Increasing the treatment dose to ≥0.05 mg/kg blocked all progesterone withdrawal bleeding, indicating that this dose fully blocked progesterone action in the endometrium.

Dose finding 2: Menstrual induction; comparison of ZK 137 316 with ZK 230 211

The ability of short-term administration of various doses of ZK 137 316 and ZK 230 211 to induce menses in oestradiol plus progesterone-primed ovarioctomized animals is shown in Figure 3. Serum concentrations of oestradiol and progesterone produced by Silastic implants in these animals were within the normal range for the luteal phase (mean ± SEM; 89 ± 27 pg oestradiol/ml and 4.39 ± 0.48 ng progesterone/ml).

ZK 137 316:

As Figure 3 shows, the first significant increase in mean days of bleeding was induced by 0.10 mg/kg, and increasing the dose to 0.15 mg/kg induced significantly more days of bleeding. ZK 230 211:

As Figure 3 shows, the first significant increase in mean days of bleeding was induced by 0.03 mg/kg, and increasing the dose to 0.05 mg/kg and 0.1 mg/kg induced significantly more days of bleeding.

Histological effects of ZK 230 211:

Animals treated with oestradiol + progesterone alone displayed an hypertrophied, progestational (secretory) endometrium (Figure 4a) with sacculated glands and expanded stroma in both the functionalis and basalis zones (Figures 4a and e), and well developed spiral arteries (Figure 4i). In the basalis zone, the glandular epithelium was tall, columnar and mitotically active. Under this hormonal condition, the epithelium of the oviductal fimbriae was deciliated and cuboidal (Figure 4m), indicative of the normal suppressive action of progesterone on the oviduct (Brenner and Slayden, 1994). Treatment with ZK 230 211 at 0.005 mg/kg had little or no effect on endometrial differentiation. In contrast, doses of 0.016 and 0.032 mg/kg inhibited progestational differentiation of the endometrium (Figure 4c,d) and blocked the suppressive effects of progesterone on oestradiol action in the oviduct (Figure 4o,p). Compared with vehicle injected controls, the higher doses of ZK 230 211 resulted in an overall thinning of the endometrium (compare Figure 4a–d), which was maximal in the 0.016 mg/kg and 0.032 mg/kg groups. This effect was associated with significantly increased stromal compaction (Table III) and a decrease in number and sacculated of endometrial glands (compare Figure 4 e,f with g,h). These higher doses of ZK 230 211 also caused hyalinization of the walls of the spiral arteries (Figure 4k,l) especially the adventitial layer, indicative of arterial degeneration. At the highest dose, ZK 230 211 also produced some venous dilation (Figure 4d) and the dilated veins also showed increased hyalinization of the perivenous stroma (not shown).

ZK 230 211 at 0.005 mg/kg had no effect on oviductal differentiation or secretion, (Figure 4n), but the 0.016 and 0.032 mg/kg doses resulted in oviducts that were fully differentiated (Figure 4c,d) as indicated by a substantial increase in percentage of ciliation (Table III, P < 0.05); these data indicate that the two higher doses of ZK 230 211 were able to completely block the suppressive effects of progesterone on oestriol-dependent oviductal differentiation.

Immunocytochemical effects of ZK 230 211:

The effect of each dose of ZK 230 211 on endometrial ERα, PR and Ki-67 is presented in Figure 5. In oestradiol + progesterone-treated control animals, oestrogen, progesterone and Ki-67 staining in the functionalis was generally low because of the suppressive action of progesterone (Brenner et al., 1990; Hild-Petito et al., 1992; Okulicz et al., 1993). Treatment with ZK 230 211 at 0.005 mg/kg had no effect but 0.016 and 0.032 mg/kg blocked progesterone suppression and increased ERα (compare Figure 5 a–d) PR (Figure 5 e–h) and Ki-67 (Figure 5 i–l) staining in glands and stroma.

Morphometric indices affected by ZK 230 211:

Table III shows that endometrial thickness and mass were significantly suppressed by ZK 230 211 at doses of 0.016 and 0.032 mg/kg. At these doses, thickness and mass were not only below the amount stimulated by oestradiol + progesterone treatment, but also below the level induced by oestradiol alone. Similarly, ZK 230 211 at doses of 0.016 and 0.032 mg/kg suppressed the mitotic index in the functionalis well below that stimulated by oestradiol alone. These data indicate that ZK 230 211 can induce an endometrial antiproliferative effect, that is, a blockade of oestradiol-dependent mitosis in the functionalis. However, the higher doses of ZK 230 211 did not suppress the Ki-67 index in the functionalis zone.

In the basalis zone of the macaque, mitosis is progesterone-dependent, not oestradiol-dependent (Brenner and Slayden, 1994). Consequently there was considerable Ki-67 and mitotic activity in this zone in the animals treated with oestradiol + progesterone.
progesterone alone. ZK 230 211 treatment with both the 0.016 and 0.032 mg/kg doses dramatically suppressed both the Ki-67 and mitotic indices in the basalis.

In the oestradiol + progesterone treated animals, the apoptotic index was significantly lower than that seen after oestradiol treatment because progesterone treatment tends to suppress endometrial apoptosis. ZK 230 211 at doses $\geq 0.016$ mg/kg, blocked the action of progesterone on apoptosis and raised the level to that seen under oestradiol alone (Table III).

\textbf{Naturally cycling macaques}

Based on the above dosage information, we selected two doses of ZK 137 316 (0.05 and 0.1 mg/kg) and three doses of ZK 230 211 (0.005, 0.016 and 0.05 mg/kg) for study of menstrual suppression in naturally cycling monkeys.

Table IV summarizes the effects of both PAs on (i) treatment-induced intermenstrual intervals, (ii) the lengths of the recovery periods, and (iii) the lengths of the post-treatment cycles.
Figure 5. Colour photomicrographs of endometrium immunostained (brown) for ERα (a–d), progesterone receptor (PR) (e–h) and Ki-67 (i–l). In oestradiol- (E₂) + progesterone- (P) treated animals, ERα (a) and PR (b) staining and the abundance of Ki-67 positive cells was minimal. ZK 230 211 at 0.016 and 0.032 mg/kg resulted in increased intensity of ERα and PR staining and an increase in the abundance of Ki-67 positive cells (arrow). ERα and PR micrographs were photographed at 250X and Ki-67 at ×100 original magnification. S = stroma, Gl = glands.

Table III. Reproductive tract morphometrics (mean ± SEM) in macaques treated with ZK 230 211. Means in each column with different superscripts are statistically different

<table>
<thead>
<tr>
<th>Morphometrics</th>
<th>Oestradiol alone (n = 5)</th>
<th>Oestradiol + progesterone (n = 5)</th>
<th>Oestradiol + progesterone + 0.005 mg ZK (n = 3)</th>
<th>Oestradiol + progesterone + 0.016 mg ZK (n = 3)</th>
<th>Oestradiol + progesterone + 0.032 mg ZK (n = 3)</th>
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<tbody>
<tr>
<td><strong>Uterine morphometrics</strong></td>
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<tr>
<td>Endometrial thickness (mm)</td>
<td>3.4 ± 0.4a</td>
<td>4.5 ± 0.6b</td>
<td>3.01 ± 0.6b</td>
<td>2.2 ± 0.3c</td>
<td>2.0 ± 0.2c</td>
</tr>
<tr>
<td>Endometrial mass (g)</td>
<td>0.35 ± 0.03a</td>
<td>0.41 ± 0.01b</td>
<td>0.29 ± 0.08b</td>
<td>0.08 ± 0.03 c</td>
<td>0.07 ± 0.02c</td>
</tr>
<tr>
<td>Myometrial mass (g)</td>
<td>1.49 ± 0.20a</td>
<td>1.15 ± 0.15a</td>
<td>1.22 ± 0.28c</td>
<td>1.51 ± 0.26a</td>
<td>1.23 ± 0.35a</td>
</tr>
<tr>
<td>Stromal compaction</td>
<td>69.3 ± 10.3a</td>
<td>38.3 ± 9.4a</td>
<td>95.6 ± 8.7b</td>
<td>167.4 ± 17.8c</td>
<td>166.3 ± 8.7c</td>
</tr>
<tr>
<td><strong>Endometrial cell proliferation</strong></td>
<td></td>
<td></td>
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<tr>
<td>Functionalis Ki-67 (%)</td>
<td>39.0 ± 4.0b</td>
<td>1.0 ± 0.0b</td>
<td>2.1 ± 1.1b</td>
<td>28 ± 10.0bc</td>
<td>26.0 ± 4.0c</td>
</tr>
<tr>
<td>Functionalis mitotic index²</td>
<td>18.5 ± 6.0b</td>
<td>nd</td>
<td>5.21 ± 0.7bc</td>
<td>3.23 ± 0.4bc</td>
<td>3.23 ± 0.4bc</td>
</tr>
<tr>
<td>Basalis Ki-67 (%)</td>
<td>3.1 ± 2.0b</td>
<td>39.0 ± 4.0b</td>
<td>19.0 ± 8.0b</td>
<td>3.1 ± 1.0b</td>
<td>1.0 ± 1.0b</td>
</tr>
<tr>
<td>Basalis mitotic index</td>
<td>0.88 ± 0.22a</td>
<td>5.23 ± 1.12b</td>
<td>3.16 ± 2.4b</td>
<td>0.26 ± 0.07c</td>
<td>0.15 ± 0.06c</td>
</tr>
<tr>
<td>Functionalis apoptosis (%)³</td>
<td>3.6 ± 1.03a</td>
<td>0.17 ± 0.26b</td>
<td>0.16 ± 0.37b</td>
<td>3.32 ± 0.88a</td>
<td>3.14 ± 1.24a</td>
</tr>
<tr>
<td><strong>Oviductal morphometrics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oviductal weight (mg)</td>
<td>453 ± 97a</td>
<td>400 ± 32a</td>
<td>433 ± 29a</td>
<td>443 ± 117 xۚ</td>
<td>327 ± 96xۚ</td>
</tr>
<tr>
<td>Fimbrial ciliation (%)</td>
<td>43.3 ± 8.8a</td>
<td>4.7 ± 2.0b</td>
<td>6.1 ± 2.5b</td>
<td>39.0 ± 5.5xۚ</td>
<td>42.3 ± 3.8xۚ</td>
</tr>
</tbody>
</table>

*Endometrial and myometrial weights presented represent the weight from one-half of the uterus.
²Mitotic index represents the number of mitotic cells/1000 cells counted.
³Percentage of apoptotic cells was calculated from counts on 1200–5000 epithelial cells in the functionalis zone.
*Stromal compaction represents the number of stromal nuclei/10 000 µm² at ×400 magnification.
*ₙ = 2.
Normal menstrual cycle

During the 40 day treatment period, the recovery period was 0.1 mg/kg blocked frank menstruation in all of the monkeys (Table IV). Injection with ZK 137 316 (0.05 and 0.1 mg/kg) blocked frank menstruation in all of the monkeys during the menstrual cycle. The area under the curve of progesterone concentrations in control macaques treated with HPE alone for 40 days. Days indicate time from the onset of menses during the menstrual cycle. The area under the curve of progesterone concentrations in control macaques treated with HPE alone for 40 days. Days indicate time from the onset of menses during the menstrual cycle.

The mean (± SEM) inter-menstrual interval in days. Means in the same row with different superscripts are statistically different (P < 0.001).

Recovery period was counted as days from the end of treatment to the next menstruation.

Post-treatment cycle length was calculated as days from the end of treatment to the next menstruation.

Figures 6–11 graphically display the serum oestradiol and progesterone concentrations in control macaques treated with HPE alone for 40 days. Days indicate time from the onset of menses during the menstrual cycle. The area under the curve of progesterone concentrations is dark shaded in this and subsequent graphs. Vertical arrow and M indicates approximate time of menstruation.

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**Table IV. Effect of ZK 137 316 and ZK 230 211 on menstrual cycle lengths in rhesus monkeys**

<table>
<thead>
<tr>
<th>Pretreatment cycle lengths</th>
<th>Treatment-induced inter-menstrual intervals</th>
<th>Recovery Period</th>
<th>Post-treatment cycle lengths</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong> (n = 8)</td>
<td>28.1 ± 1.0a</td>
<td>–</td>
<td>27.7 ± 1.1a</td>
</tr>
<tr>
<td><strong>ZK 137 316 (40 day regimen)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 mg/kg (n = 9)</td>
<td>31.2 ± 1.2a</td>
<td>55.0 ± 5.5b</td>
<td>15 ± 5.5</td>
</tr>
<tr>
<td>0.10 mg/kg (n = 9)</td>
<td>30.1 ± 1.3a</td>
<td>61 ± 2.2b</td>
<td>21 ± 2.2</td>
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<td><strong>ZK 137 316 (100 day regimen)</strong></td>
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<td>0.05 mg/kg (n = 4)</td>
<td>31.6 ± 1.5a</td>
<td>131 ± 10.1b</td>
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<td>0.10 mg/kg (n = 4)</td>
<td>29.6 ± 3.6a</td>
<td>134 ± 8.7b</td>
<td>34 ± 8.7</td>
</tr>
<tr>
<td><strong>ZK 230 211 (60 day regimen)</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0.005 mg/kg (n = 5)</td>
<td>27.4 ± 0.51a</td>
<td>27 ± 0.78a</td>
<td>0</td>
</tr>
<tr>
<td>0.016 mg/kg (n = 5)</td>
<td>27.6 ± 1.0a</td>
<td>101 ± 7.4b</td>
<td>41 ± 7.4</td>
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<tr>
<td>0.050 mg/kg (n = 5)</td>
<td>27.0 ± 2.0b</td>
<td>98 ± 5.9b</td>
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Values represent mean (± SEM) inter-menstrual interval in days. Means in the same row with different superscripts are statistically different (P < 0.001).

Recovery period was counted as days from the end of treatment to the next menstruation.

Post-treatment cycle length was calculated as days from the menstruation at the end of the recovery period to the next menstruation.

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**Treatment period**

**Figure 6.** Mean (± SEM) oestradiol and progesterone concentrations in control macaques treated with HPE alone for 40 days. Days indicate time from the onset of menses during the menstrual cycle. The area under the curve of progesterone concentrations is dark shaded in this and subsequent graphs. Vertical arrow and M indicates approximate time of menstruation.

**Figure 6.** Mean (± SEM) oestradiol and progesterone concentrations in control macaques treated with HPE alone for 40 days. Days indicate time from the onset of menses during the menstrual cycle. The area under the curve of progesterone concentrations is dark shaded in this and subsequent graphs. Vertical arrow and M indicates approximate time of menstruation.

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Values represent mean (± SEM) inter-menstrual interval in days. Means in the same row with different superscripts are statistically different (P < 0.001).

Recovery period was counted as days from the end of treatment to the next menstruation.

Post-treatment cycle length was calculated as days from the menstruation at the end of the recovery period to the next menstruation.

---

All treated monkeys showed normal non-surge concentrations of oestradiol (~30–100 pg/ml). When treatment ended (at 100 days) most of the animals in both groups developed an oestradiol surge, ovulated, expressed a normal luteal phase and menstruated when progesterone declined. These data indicate that the 100 day treatment was fully reversible.
Intermediate term (60 day) suppression of menses with ZK 230 211:

Treatment with 0.005 mg/kg ZK 230 211 had no effect on ovulation or menstruation, but the 0.016 and 0.05 mg/kg doses suppressed all ovulation and menstruation, significantly extending the intermenstrual interval to ~100 days ($P < 0.01$; Table IV).

The recovery period was around 40 days (see Table IV). Both 0.016 mg and 0.05 mg/kg doses also increased the length of the first post-treatment menstrual cycle to ~50 days (Table IV, $P < 0.05$); menstrual cycles thereafter were of normal length.

In this protocol we bled the animals daily throughout the entire study period until the first menstruation occurred. Macaques treated with 0.005 mg/kg had normal oestradiol surges and luteal phase progesterone concentrations. The 0.016 mg/kg dose resulted in follicular phase oestradiol surges of normal amplitude, but normal luteal phase concentrations of progesterone did not develop (Figure 10, and see Figure 11). Monkeys treated with 0.05 mg/kg failed to develop either a normal oestradiol surge or normal luteal phase concentrations of progesterone (Figures 10 and 11). All ZK 230 211 treated monkeys showed normal non-surge concentrations of oestradiol (~30–100 pg/ml). Approximately 10 days after treatment ended, there was a rise in serum progesterone and a normal length luteal phase for both higher dose groups. Menses occurred following progesterone decline at the end of this luteal phase (Figure 10).

To determine whether ZK 230 211 could suppress LH, serum samples flanking the highest concentrations of oestradiol in each group were re-assayed for LH by bioassay (bLH, Figure 11). Samples from the pretreatment period, the recovery period, and those treated with 0.005 mg ZK 230 211 all showed rises of bLH associated with peak concentrations of oestradiol. However, bLH concentrations remained low in
Menses suppression with progesterone antagonists

Discussion

Dose finding studies

The menstrual blockade and menstrual induction studies in spayed macaques provided information on doses that directly suppress the endometrium, and these were useful in selecting doses for trial in naturally cycling animals. In addition, they provided new information on the relative potency of these two PAs in macaques. For example, in the menstrual induction study, the first significant increase in days of bleeding induced by ZK 137 316 required 0.10 mg/kg but only 0.03 mg/kg of ZK 230 211. This suggests that ZK 230 211 is ~3-fold more potent than ZK 137 316. Additionally, a dose of 0.15 mg/kg of ZK 137 316 was required to induce ~2 days of frank menstrual bleeding while only 0.03 mg/kg of ZK 230 211 was needed to induce the same 2 days of bleeding. This is an ~5-fold difference. On this basis, ZK 230 211 is somewhere around 3–5 times more potent than ZK 137 316, depending on the criterion used.

Histological and morphometric effects

ZK 230 211 dramatically suppressed the endometrium in oestradiol plus progesterone-primed animals at 0.032 mg/kg over a 1 month treatment period. This dose is around 3-fold lower than the 0.1mg/kg dose of ZK 137 316 that blocked the endometrium in naturally cycling monkeys over a 5 month period (Slayden et al., 1998). As stated in Materials and methods, we also found that 0.1 mg/kg of ZK 137 316 severely suppressed the endometrium within a 1 month treatment period (data not shown).

The specific histological effects induced by ZK 230 211 included overall shrinkage, stromal compaction, glandular

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Figure 8. Oestradiol and progesterone concentrations (mean ± SEM) during the second post-treatment menstrual cycle after a 40 day treatment with ZK 137 316. All of the animals expressed normal patterns of oestradiol and progesterone and normal menses, indicating no residual effects of ZK 137 316.

Figure 9. Oestradiol and progesterone concentrations (mean ± SEM) during the last 30 days of treatment in macaques treated with vehicle (A), 0.05 mg/kg ZK 137 316 (B), and 0.1 mg/kg ZK 137 316 (C) for 100 days. Days represent time from the beginning of treatment.
atrophy and hyalinization of the spiral artery walls. In addition, the suppressive effects of progesterone on endometrial ERα and PR were blocked, as were the antagonistic effects of progesterone on oestradiol action in the oviduct. The data in Table III indicate that ZK 230 211 suppressed endometrial mass, endometrial thickness and the functionalis mitotic index significantly below the level in animals treated with oestradiol alone, which is a clear antiproliferative effect. Because ZK 230 211 treatment elevated both ERα and Ki-67 concentrations, the findings suggest that oestradiol acts through ERα (directly or indirectly) to stimulate cells to produce Ki-67 and enter the cell cycle, but some unknown aspect of ZK 230 211 action blocks the cells from entering mitosis. The exact mechanism underlying this mitotic suppressive effect remains to be discovered.

In addition, ZK 230 211 elevated apoptotic counts in the functionalis to the level seen during oestradiol treatment alone (Table III). Because ZK 230 211 treatment suppressed the mitotic rate but raised the apoptotic index, cell death would greatly overtake cell birth in the functionalis zone, and over time this difference would contribute to the decrease in endometrial cell mass and thickness induced by these doses.

In the basalis, which grows under progesterone influence in macaques, the blockade of progesterone action by ZK 230 211 would block the growth of this zone as well. Suppression of growth of both zones contributes to the overall shrinkage of the endometrium. Clearly, moderate doses of ZK 230 211 can block the effects of both oestradiol and progesterone on endometrial growth and development. This is the first report to show that low doses of ZK 230 211 can induce these effects directly on the endometrium and oviduct within 28 days in ovariectomized, artificially cycled macaques.

Studies in naturally cycling animals

ZK 137 316:
The combined results of the 40 and 100 day studies with ZK 137 316 suggest that treatment with 0.05 mg/kg, long or short term, is near but generally below the threshold dose that blocks ovulation, while the 0.1 mg/kg dose generally blocked ovulation. There was no obvious residual effect of antiprogestin treatment and effects appeared completely reversible, as post-treatment cycles were normal in all respects. The two treatment periods, one for 40 and one for 100 days, were designed to mimic those cases where women might want to block either just one or several menstrual periods. During the 40 day study ovulation was blocked in 5/9 animals at 0.05 mg/kg and 6/9 animals at 0.1 mg/kg. In those animals in which ovulation occurred, the luteal phase length, the serum concentrations of progesterone and oestradiol, and the time of decline of progesterone were normal, but there was no frank menstrual bleeding. Undoubtedly this was due to the combined antagonistic effects of ZK 316 on both oestradiol-dependent growth, (Wolf et al., 1989; Slayden et al., 1994, 1998) and progesterone-dependent endometrial progestational development (Brenner and Slayden, 1994).

In the 40 day study, the ovulatory animals showed minute bleeding, detectable only by vaginal swab, around the time of progesterone decline. These positive vaginal swabs detected bleeding that was far less in quantity than the so-called spotting or breakthrough bleeding which occurs in women on continuous progestin treatment (e.g. Norplant, or Depo Provera). However, in the 100 day study, animals that ovulated were completely amenorrheic with no minute bleeding. Apparently such minute bleeding can disappear with continued treatment.

ZK 230 211:
At 0.005 mg/kg, ZK 230 211 failed to suppress either menstruation or ovulation, but the higher doses of 0.016 and 0.05 mg/kg completely suppressed both ovulation and menstruation. The suppression of the LH surges induced by these doses clearly indicates that this compound had central inhibitory effects. We previously noted that the antiovulatory effects of ZK 137 316 were accompanied by suppression of LH surges, implicating the hypothalamic-pituitary axis as the site of the antiovulatory action for both these antiprogestins (Zelinski-Wooten et al., 1998b). Of great interest were the animals treated with the 0.016 mg/kg dose that showed oestradiol surges but no LH surges (Figure 11), which suggests that ZK 230 211 has separate, dose related antagonistic effects.

In the basalis, which grows under progesterone influence in macaques, the blockade of progesterone action by ZK 230 211 would block the growth of this zone as well. Suppression of growth of both zones contributes to the overall shrinkage of the endometrium. Clearly, moderate doses of ZK 230 211 can block the effects of both oestradiol and progesterone on endometrial growth and development. This is the first report to show that low doses of ZK 230 211 can induce these effects directly on the endometrium and oviduct within 28 days in ovariectomized, artificially cycled macaques.
Menses suppression with progesterone antagonists

**Conclusions**

The anti-ovulatory and anti-endometrial effects of ZK 137 316, a Type II PA, were somewhat dissociated, that is, menstruation could be inhibited in many of the animals without inhibiting ovulation. On the other hand, ZK 230 211, a Type III PA, blocked ovulation at all doses that blocked menstruation. Moreover, other data indicate that ZK 230 211 is a pure PA, while ZK 137 316 has some agonist action (Chwalisz et al., 2000b). These results suggest that pure PAs are most suitable for therapeutic approaches which require a consistent inhibition of ovulation, whereas compounds exhibiting some agonistic activity, such as ZK 137 316, and particularly the PRMs, appear to be more endometrium-selective.

Of great importance, however, circulating oestradiol concentrations were never suppressed below normal, follicular phase concentrations by either PA at any dose. Therefore, if women used these compounds to block menses, and if ovulation were also blocked, it is unlikely there would be any associated symptoms of oestrogen deprivation, such as hot flushes, vaginal atrophy or loss of bone density, though we did not address these issues in this study. Moreover, the antiovulatory effects of PAs would be accompanied by secretion of naturally

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**Figure 11.** Concentrations (mean ± SEM) of bioactive LH (bLH) in animals treated with ZK 230 211. Samples were first analysed for oestradiol. In cases where a clear oestradiol surge occurred, as in panels A and B, samples flanking the surge were reassayed for bLH. When no surge was evident, as in panel C, samples flanking the highest value of oestradiol were reassayed for bLH.

at the ovarian and pituitary levels. These divergent effects deserve additional attention in a separate research study.
occurring oestrogens, an important difference from the current mode of menstrual inhibition, namely blockade of ovulation by combined synthetic oestrogens and progestins in a continuous contraceptive tablet regimen. Synthetic progestins are usually administered to prevent unopposed actions of synthetic oestrogens on the endometrium, but PA therapy, through its endometrial antiproliferative effects, may obviate this need.

Chronic low dose PA therapy is therefore a powerful method to reversibly suppress menstrual bleeding, and it can act in several ways depending on dose and PA type: (i) it can allow ovulation, but block the effects of both oestradiol and progesterone on endometrial growth and development, suppress menstruation upon progesterone withdrawal and induce a state of amenorrhea, (ii) it can inhibit ovulation so that progesterone concentrations remain low, resulting in amenorrhea, (iii) it can allow normal follicular phase concentrations of oestradiol so that the effects of oestrogen on bone and other peripheral target tissues should be normal and (iv) it can block any unopposed oestrogenic effects in the endometrium through the endometrial antiproliferative effect. The potential also exists for suppression of various sorts of abnormal uterine bleeding, though long term studies in women are needed to validate these various possibilities.

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References

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