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Differential Endometrial Responses of Primates vs Rodents: Screening for Antiproliferative Effects of Antiprogestins

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DIFFERENTIAL ENDOMETRIAL RESPONSES OF PRIMATES VS RODENTS: SCREENING FOR ANTIPROLIFERATIVE EFFECTS OF ANTIPROGESTINS

by

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ABSTRACT

DIFFERENTIAL ENDOMETRIAL RESPONSES OF PRIMATES VS RODENTS: SCREENING FOR ANTIPROLIFERATIVE EFFECTS OF ANTIPROGESTINS.

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Eastern Virginia Medical School
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The antiprogestin, mifepristone, has previously been shown to noncompetitively inhibit estrogen-induced endometrial proliferation in nonhuman primates (van Uem et al., 1989; Wolf et al., 1989b; Neulen et al., 1990; Neulen et al., 1996). For both economical and ethical reasons, we are encouraged to identify comparative laboratory rodent models which can substitute the need to use primate models. In the following study, we compared capabilities of the rat uterine weight bioassay versus a primate uterine bioassay, to identify the noncompetitive antiestrogenic/antiproliferative effects of mifepristone.

Long-term ovariectomized monkeys were exposed to exogenous 17β-estradiol (E2) and mifepristone in doses and regimes already demonstrated to curtail endometrial growth (Wolf et al., 1989b). Results show that mifepristone decreased endometrial proliferation in a dose-dependent manner, and this decrease occurred in the presence of physiologic serum E2 levels.

In the rat model, ovariectomized immature (day 20) and adult Sprague-Dawley rats were pretreated with E2 for 3 days, followed by E2 plus mifepristone (various doses) for 3 additional days. E2 replacement was either given as 0.5 μg/100 g body weight (in oil, sc) or as a 0.5 mg sc pellet. Mifepristone did not induce a decrease in uterine wet or blotted weights in immature or adult rats receiving E2 replacement as 0.5 μg/100 g body weight ($P>0.05$). This lack of effect was not due to insufficient E2 stimulation as histological evaluation of the endometrium showed increased numbers of mitotic figures in all treatment groups and serum E2 levels were in the diestrous-proestrous range. In contrast, mifepristone did inhibit an increase in uterine wet weight of adult rats ($P<0.05$)

and immature rats ($P<0.01$) receiving E2 replacement as 0.5 mg E2 pellet. Uterine blotted weights were also effected by treatment with mifepristone in the immature rats ($P<0.05$). Serum E2 levels attained in rats given the 0.5 mg E2 pellet were pharmacological (>800 pg/ml), illustrating a relationship between E2 levels and capability of mifepristone to affect rat uterine weight.

Based on the results summarized here, we do not recommend using the rat uterine weight bioassay as a substitute model for screening antiprogestins for noncompetitive antiestrogenic/antiproliferative effects on primate uterine endometrium.

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To the EVMS animal husbandry staff for giving my animals such wonderful care, your help was appreciated. To my neighbor, Roger Counce, Sr., whom looked forward to this day with as much eagerness as myself, but is not here to share the joy. To all my friends in Norfolk, who I offer my bitter-sweet good-byes, Knox, Estella, Leah, Radek, Diane, Renie, Susan, Suzanne, Michelle, Jim, Christine, Euni, Gustavo, and of course, all the friends and acquaintances who are unnamed but still dearly missed.

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INTRODUCTION

History

The drug RU38486 (17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4, 9-dien-3-one; Roussel-Uclaf), also called mifepristone, was developed in 1980 as a potential antiglucocorticoid. Because previous company data showed some binding cross-reactivity between the glucocorticoid receptor (GR) and the progesterone receptor (PR), Roussel-Uclaf conducted receptor binding tests to identify possible receptor cross-reactivity. The receptor binding tests revealed that mifepristone, in addition to recognizing the glucocorticoid receptor, showed significant binding affinity for the progesterone receptor; and to a much lesser extent, the androgen receptor (Baulieu, 1989). This cross-reactivity is not surprising as subsequent work has shown a 90% amino acid sequence identity in the GR and PR DNA-binding regions and a 55% amino acid sequence identity in the COOH-terminal region (Evans, 1988).

As the capability of a synthetic steroid compound to bind a steroid receptor can not accurately predict its *in vivo* agonistic or antagonistic effects (Baulieu, 1989), studies evaluating the antiprogestational effects of RU486 were necessary. The secretion of progesterone by the corpus luteum is well known to be necessary for the maintenance of pregnancy in women (Csapo et al., 1972; Jones, 1976), as luteectomy followed by progesterone replacement therapy prevents pregnancy loss (Csapo et al., 1973). Philibert and others (1982) evaluated the use of mifepristone to block progesterone action and induce termination of pregnancy in rats. Building from the success of these experiments, the capability of mifepristone to block progesterone action and induce abortion in women was also investigated. Principally due to mifepristone's capability to induce early pregnancy termination in women, the steroid quickly became labeled by anti-abortion groups as the abortion-pill.

The following dissertation text style is modeled after Molecular Reproduction and Development.

Multiple uses for mifepristone have been proposed and identified, including, ovulation inhibition (Danforth et al., 1989; Wolf et al., 1989a), treatment for breast cancer (McDonnell and Goldman, 1994), pregnancy termination (Ulmann, 1987; Ji, 1988), contragestation (Glasier et al., 1992; Webb et al., 1992), treatment of leiomyomas (Murphy et al., 1993), treatment of progesterone receptor containing meningiomas (Grunberg, 1994), and treatment of endometriosis (Kettel et al., 1991; Grow et al., 1996; Kettel et al., 1996). Yet, it is the highly controversial abortive properties of the drug that have garnered so much attention of the press and public and made RU486 recognized.

Generalized Description of Gene Transcription

Figure 1A diagrams a very simplified representation of a steroid-inducible eukaryotic gene. In general, the transcription of mRNA from such a gene requires RNA polymerase II and accessory proteins which recognize the promoter sequence. These accessory proteins are termed transcription factors, which are proteins needed for the initiation of transcription but are not themselves a component of RNA polymerase II. The promoter is a region of DNA that contains binding sites for the transcription factors. Most eukaryotic promoters contain the consensus sequence TATA approximately 25 base pairs upstream of the transcription start site. In addition, the gene sequence contains a hormone response element (HRE). The HRE is a DNA binding site that induces transcription when activated by its respective ligand-receptor complex, and prevents transcription in the nonactivated state; thereby regulating hormone-induced transcription. Steroid receptors are proteins that can be viewed as ligand-dependent transcription factors (Kraus et al., 1995). It is generally accepted that unliganded steroid receptors reside within the nucleus and are believed to be loosely associated with DNA (Mester and Baulieu, 1995; Catt, 1996). Following activation by a ligand, the receptors undergo tight DNA binding to the hormone response element (HRE) and initiate gene activation.

Figure 1B illustrates a possible model for activation of an estrogen-inducible gene. Upon entering the cell, estrogen binds to its receptor (ER) and promotes tight association of the ligand-receptor complex to the estrogen response element (ERE). Binding of the

activated estrogen-receptor complex to the ERE initiates formation of the transcription complex and the binding of RNA polymerase II. It is thought that binding of the estrogen-receptor complex to the ERE likely induces a change in the three dimensional structure of the gene, bringing the transcription complex into close physical contact with the estrogen-receptor complex (Nardulli et al., 1993). Interaction between these two elements activates RNA polymerase II and initiates mRNA transcription. Translation of the mRNA leads to the synthesis of proteins with various functions, among them, regulation of cell growth and mitogenesis.

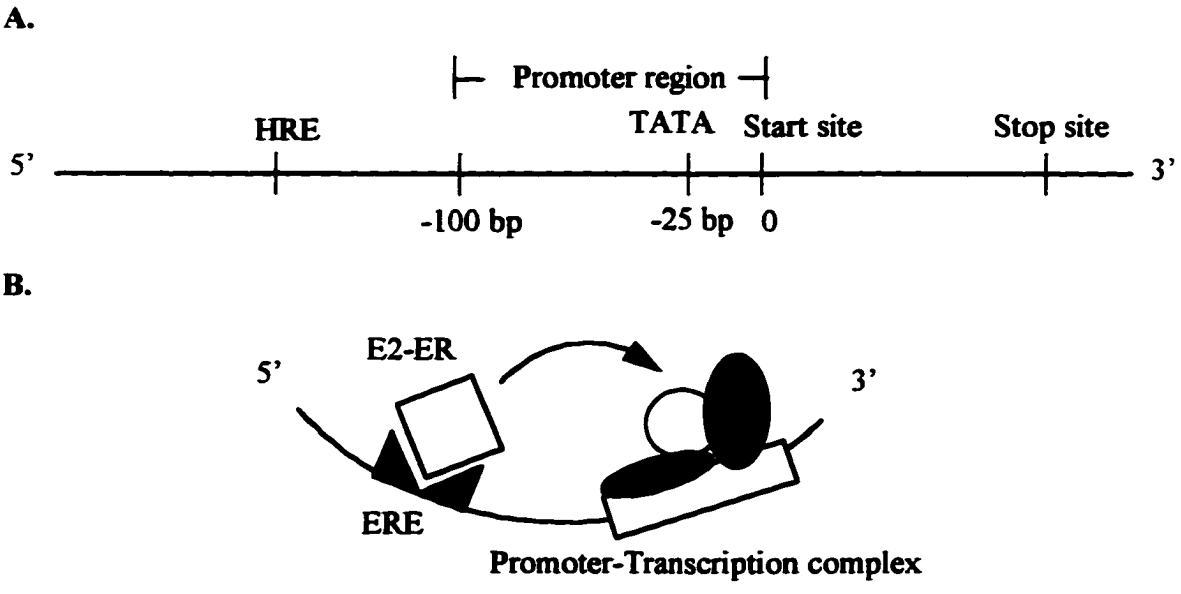

Fig. 1. Conceptualized view of steroid-inducible gene activation. (A) Illustrates binding regions for the promoter complex and hormone receptor complex. (B) Binding of the activated estrogen receptor complex to the ERE induces a 3-D change in the gene and brings the promoter complex into close physical contact with the receptor, initiating mRNA transcription. Model adapted from Kraus and others (1995).

Cell Cycle

The progression of a cell through the cell cycle, is in part, regulated by the activity of a special class of protein kinases called cyclin-dependent kinases. In turn, activation of the cyclin-dependent kinases are positively regulated by association with a catalytic subunit called cyclin. Numerous cyclins have been identified and classified according to their conserved amino acid sequence motifs, and apparent function (Geum et al., 1997). Cyclin-dependent kinase D1 has been identified in steroid regulation of cell growth and appears to be necessary for progesterone-dependent mammary development, as cyclin D1-deficient mice do not develop lobular mammary alveoli during pregnancy (Musgrove et al., 1997). Cyclin-dependent inhibitor, p21, is known to inhibit cyclin-dependent kinase activity. In the G_1 phase of the cell cycle (Figure 2), D-type cyclins are involved in activating the powerful growth-inhibitory protein retinoblastoma tumor suppresser protein (pRB). When pRB is hypophosphorylated it actively blocks cell mitosis by sequestering transcription factors. Cyclin-dependent kinase D1 is involved in the hyperphosphorylation of pRB, which then releases the transcription factors and allows the progression of cell mitotis (Weinberg, 1996).

Musgrove and others (1997) have recently presented data describing mifepristone-induced cell cycle inhibition in T-47D breast cancer cells. T-47D cells are progesterone responsive cancer cells and are frequently used by investigators to study the *in vitro* effects of progestins. In these experiments a 3- to 4-fold increase in p21 levels was observed in the cells 12-24h after treatment with mifepristone. The increase in p21 levels was associated with a decrease in cyclin-D1 kinase activity and an increase in the ratio of hypophosphorylated pRB:hyperphosphorylated pRB.

These data would strongly indicate that mitotic arrest of T-47D cells (and possibly other types of progesterone responsive cells) by mifepristone is due to an upregulation of p21, decreased activity of cyclin-D1 kinase, and inactivation of pRB. These actions most likely occur during G_1 phase, at which point in time proteins required for mitosis are synthesized. This would suggest that the lack of necessary cell-division proteins acts to direct cells into a long-term, or permanent, G_0 phase (Cameron et al., 1996).

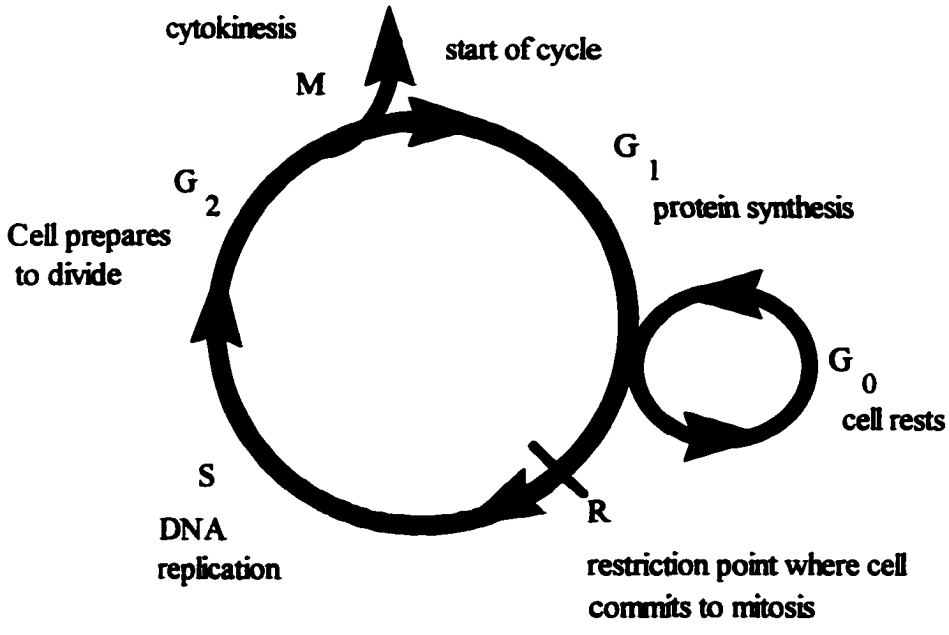

Fig. 2. Stages of the cell cycle. Mifepristone-induced inhibition of cell mitosis may occur during the G_1 phase by altering the synthesis and activity of cell-division proteins. Model adapted from Weinberg (1996).

Repression of Estrogen-Induced *In Vitro* Transcription by Mifepristone

Four models describing the repression of transcription have been proposed by Levine and Manley (1989) and Kraus and others (1995), these being, direct repression, competitive repression, squelching, and quenching. In direct repression a repressor protein binds to the DNA at a site distant to the promoter region and sterically interferes with formation of the transcription complex. Competitive repression occurs when the repressor protein binds to a DNA site overlapping or shared by the transcription complex, and prevents its ability to bind to the DNA. In comparison, squelching is observed when the over expression of a transcription factor sequesters other needed factors and prevents them from forming the transcription complex. Lastly, quenching occurs when a repressor and activator bind distinct DNA sites, yet interaction between the two prevents the activator from making proper contact with the transcription complex.

McDonnell and Goldman (1994) have proposed that mifepristone induces antiestrogenic activities through the progesterone receptor A isoform; and not B isoform. In their studies, monkey kidney CV-1 fibroblasts were transiently transfected with a mouse mammary tumor virus-estrogen response element-luciferase reporter. The transfected cells also included expression vectors for human progesterone receptor A or B (PRA and PRB), and an expression vector for hER. Results showed that luciferase activity was decreased to approximately 60% of control levels in cells transfected with the PRA. This decrease in luciferase activity was completely absent in cells transfected with the PRB; indicating a partial estrogen receptor antagonism in cells coexpressing PRA. These authors favored the mechanism of squelching to describe the noncompetitive inhibition of hER by hPRA.

In a similar set of experiments by Kraus and others (1995), rat uterine cells were transiently transfected with Rous sarcoma virus promoter-driven rat estrogen receptor vector (pRSV-rER), pRSV-human progesterone receptor A (-hPRA), pRSV-hPRB, hormone response elements ERE and PRE, and a chloramphenicol acetyltransferase reporter gene. Results from these experiments showed that cells treated with mifepristone had an approximately 85% decrease in CAT activity, regardless of which hPR isoform they contained. These data would indicate that in rat uterine cells, both PRA or PRB can act as an inhibitor of ER-mediated transcriptional activity. These authors supported the model of quenching to explain their data on transcriptional repression of the ER by activated PR.

Antiestrogenic Effects of Mifepristone in Primate Endometrium

Mifepristone was first reported to inhibit estrogen-induced endometrial proliferation in the non-human primate by van Uem and others (1989). van Uem and others measured a decreased endometrial thickness in intact monkeys treated with mifepristone. As mifepristone has been reported to not bind the ER (Moguilewsky and Philibert, 1985), these authors concluded that the antiestrogenic effect of the antiprogestin was very likely the result of noncompetitive actions. This experiment by van Uem and others was quickly followed by an additional study from Wolf and others (1989b), who

showed that mifepristone induced a decrease in endometrial thickness and reduced the mitotic index in the endometrium of ovariectomized estrogen-supplemented monkeys. The paradoxical noncompetitive antiestrogenic effect of mifepristone in nonhuman primates has been supported by Slayden and Brenner (1994). These authors found that mifepristone decreased uterine wet weight and increased amount of DNA per mg uterine tissue (DNA μg/mg tissue wet weight). Surprisingly, these antiestrogenic effects were only noted in the endometrium and not the oviduct, thus showing tissue selectivity.

The mitogenic capacities of estrogen on uterine tissue are well known. As estrogen induces transcription of cyclin-D1 kinase mRNA during mouse uterine cell proliferation (Geum et al., 1997), it is possible that cyclin-D1, p21, or pRB proteins are effected during the inhibition of estrogen-induced endometrial proliferation by mifepristone in nonhuman primates. The atrophic appearance of mifepristone-treated nonhuman primate endometrium is not due to decreased estrogen receptor concentration (Neulen et al., 1990; Slayden and Brenner, 1994; Neulen et al., 1996).

Purpose

Since the late 1930s it has been established that the proestrus surge of estrogen in rats stimulates uterine fluid retention (Astwood, 1939). Release of this uterine fluid is accomplished by actions of progesterone in relaxing the cervix and allowing it to escape via the vagina (Armstrong, 1968). Building upon this known effect of estrogen, the standard accepted animal model assay used to assess the estrogenic or antiestrogenic capability of new compounds is the *in vivo* immature rat uterine weight assay (Kirkland et al., 1979). In this assay, ovariectomized rats are given estradiol replacement to stimulate water retention and endometrial proliferation. A positive estrogenic response can be determined by an increase in uterine wet weight and uterine blotted weight, respectively. The simultaneous administration of estradiol and an antiestrogenic compound should both decrease water retention and uterine blotted weight, in comparison to the estradiol-treated control. In regards to estrogen controlled growth, no model has been proven to be more effective than the rat uterine bioassay. Accordingly, here we have compared the rodent model with a primate model for evaluation of antiproliferative actions of mifepristone.

Our laboratory has a continuing interest in examining the antiestrogenic effects of mifepristone and other antiprogestins. The use of antiprogestins may be beneficial in controlling breakthrough bleeding during progestin-only contraception (Williams et al., 1997) and treatment of endometriosis (Grow et al., 1996; Kettel et al., 1996). While evaluating the antiestrogenic effects of mifepristone using the rat uterine weight bioassay, results from our preliminary experiments indicated that the bioassay might fail to identify certain compounds capable of suppressing endometrial proliferation in nonhuman primates, and as such, may not be suited for conventional high-throughput screening of compounds. High throughput screening can be loosely defined as the testing of a large number of compounds for detection of a limited number of specific endpoints. Based upon whether these endpoints are observed, each compound is then accepted or eliminated from further testing. Validity of this testing procedure is dependent on the chosen endpoints truly reflecting the compounds' desired capability. The negative results in our preliminary studies are indirectly supported by two earlier studies. An acute study by Okulicz (1987) indicated that ovariectomized rats retained E2-induced uterine fluid during treatment with mifepristone. In addition, Szabo and others (1996) reported that proestrus rats given mifepristone showed uterine imbibition [uterine ballooning]. Both of these studies suggested that administration of mifepristone would not affect the estrogen-induced accumulation in uterine fluid. As these two studies and our preliminary rat experiments failed to show noncompetitive antiestrogenic/antiproliferative effects of mifepristone, we chose to replicate the study of Kraus and Katzenellenbogen (1993) which reported antiproliferative effects of mifepristone in immature rats. Replication of this study was necessary to show that differences their and our study were due to experimental methods (i.e., method of E2 replacement). In addition, we conducted an experiment using cynomolgus monkeys to reassert that mifepristone does cause antiproliferative effects on primate endometrium. Therefore, the goal of this study was to determine if a difference exists between the rat uterine weight bioassay and a nonhuman primate uterine assay in their capability to identify antiestrogenic effects of mifepristone . Those interested in the screening of antiprogestin candidate compounds may benefit from our experiences and observations reported herein.

MATERIALS AND METHODS

Reagents

17β-estradiol and progesterone were purchased from Sigma Chemical Comp., (St. Louis, MO). 17β-estradiol pellets were purchased from Innovative Research of America (Cat. #E121, Sarasota, FL). The mifepristone used in these experiments was purchased from China and its bioavailability and potency are equivalent to RU486.

Animals and Treatments

All Sprague Dawley rats used in these experiments were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The animals were maintained in a temperature controlled room (24-27°C) under a 14h-10h, light-dark cycle, and given food and water *ad libitum*. In the experiments using adult animals, females were ovariectomized when approximately 250 g of weight and allowed a minimum of 10 days to recover before starting hormone treatment. In experiments using immature animals, females were ovariectomized on day 16 of age and allowed to recover for 4 days before starting hormone treatment. The animals were randomly assigned to a treatment group following ovariectomy (5 per group). Hormone treatments for animals of both ages were given for 6 days, 3 days of 17β-estradiol pretreatment (either 17β-estradiol in oil or as a pellet), followed by 3 days of 17β-estradiol plus mifepristone. 17β-estradiol was given as a daily single sc injection (0.5 μg/100 g body weight (BW), in corn oil) or as a single sc 0.5 mg pellet implant. Mifepristone was dissolved in corn oil and administered as a daily single sc injection at the doses indicated in figures or tables. Vehicle control animals received the appropriate volume of corn oil. Cardiac blood samples were collected under ketamine-rompun induced anesthesia from a group of similarly treated animals on days 1, 3, 5, and 7 for determination of estradiol levels.

Fifteen long-term ovariectomized cynomolgus monkeys (*Macaca fascicularis*), weighing approximately 2-4 kg, were maintained in a temperature controlled room (22°C) with a 12h light-dark cycle, and given food and water *ad libitum*. The animals were

assigned to one of five treatment groups (3 per group), as follows: **1**, vehicle control, 3 cm placebo containing silastic capsule, implanted sc, plus, daily sc injections of corn oil; **2**, 17β-estradiol (E2) control, 3 cm estradiol containing silastic capsule (~150 mg E2), implanted sc, plus, daily sc injection of corn oil; **3**, E2 capsule plus mifepristone (0.01 mg/kg/day, sc, in oil); **4**, E2 capsule plus mifepristone (0.1 mg/kg/day, sc, in oil); **5**, E2 capsule plus mifepristone (1.0 mg/kg/day, sc, in oil). The estradiol capsule was implanted on day 1 and left in place for 21 days. Starting on day 1, animals were treated with vehicle or mifepristone for 20 days. Femoral blood samples (3.5 cc) were collected under ketamine-induced anesthesia on days 1, 5, 9, 13, 17, and 21 for determination of estradiol levels.

Uterine Tissue Collection and Histology

One hour prior to tissue collection, all rats received a 0.1 ml sc injection of corn oil containing 5 μg E2 and 2 mg progesterone to activate the steroid receptors and induce nuclear translocation (Kraus and Katzenellenbogen, 1993). Rats were euthanized by CO_2 asphyxiation and the entire reproductive tract removed as one piece from the cervix to the distal ends of the uterine horns. The tissue was stripped of fat and quickly weighed to obtain the uterine wet weight, distal ends of the uterine horns were cut and the fluid gently expelled, and the tissue was weighed again to obtain the uterine blotted weight. After weighing, the uterine tissue was placed into 10% buffered formalin and processed for hemotoxylin and eosin (H&E) staining.

Histology was performed on two cross-sections from each rat. Each cross-section was morphologically examined for the number of mitotic figures in the endometrium and glands. Mitotic activity was determined by counting the number of cells showing metaphase chromosomes or a condensed nucleus with clear cytoplasm. Each cross-section was also examined for the number of cells showing nuclear fragmentation.

Uterine biopsies were obtained from each monkey on day 21 of the experiment. Full thickness endometrial biopsies were placed in 10% buffered formalin and processed for H&E staining. Histological analysis of the endometrium was performed on a single section by the criteria of Noyes and others (1950). Each section was morphologically

examined for the number of mitotic figures in the endometrium, glands, and stroma. Mitotic activity was determined by counting the number of cells showing metaphase chromosomes or a condensed nucleus with clear cytoplasm. Mean endometrial thickness for each animal was obtained by averaging at least four separate determinations using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) on a Olympus BX50 microscope.

Radioimmunoassay

Monkey serum levels of 17β-estradiol were measured by radioimmunoassay (RIA) using a commercially available kit from ICN Biomedicals, Inc., (Costa Mesa, CA), and quality control standards of known potency from BioRad (Hercules, CA). All serum samples were measured in a single assay. The intraassay coefficient of variance for the BioRad control of similar potency as the unknowns was 9%. Our labs historical interassay coefficient of variance for this assay is <10%. The assay sensitivity for 17β-estradiol was 10 pg/ml.

Rat serum levels of 17β-estradiol were measured by Covance Laboratories Inc., (Vienna, VA), using an in-house assay with rabbit antiserum, HWA E2. Cross-reactivity of this antiserum is 8.48% for 17β-dihydroequilenin and negligible for all other tested steroids. The reported intraassay coefficient of variance and interassay coefficient of variance from Covance are 10.76% and 12% respectively. The assay sensitivity for 17β-estradiol was 10 pg/ml.

Preliminary Rat Experiments and Post-Ovariectomy Recovery Period

In our initial experiments (estrogen dose response curve and Preliminary Experiments 1-4), adult Sprague Dawley rats, were purchased, cared for, and treated by identical conditions as previously described; with the following exceptions. Both the estrogen dose response experiment and Preliminary Experiment 1 used adult rats that had a post ovariectomy recovery period of ≥4 days. The method of E2 replacement and/or length of E2 treatment changed between experiments and is described with the associated Figure and Tables.

In the rat experiments presented herein, it is acknowledged that the post-ovariectomy recovery period, was a variable we did not totally control. Two experiments with adult rats used a 4 day recovery period (approximately 1 estrous cycle). The recovery period was increased to 10 days (2 estrous cycles) in all subsequent adult studies to facilitate the clearance of all circulating ovarian steroids and to allow uterine atrophy prior to experiment initiation. In the Latin Square design experiments that repeated a published treatment regime (Kraus and Katzenellenbogen, 1993), we maintained our established 10 day surgery recovery period for adult rats, but observed the 4 day recovery period for immature rats used by these authors.

For the following reasons, it is our belief that this variable did not play a role in determining experimental outcome: 1) Immature rats were ovariectomized at 16 days of age prior to the initiation of significant endogenous ovarian steroid production; 2) Serum E2 levels were barely detectable in adult rats sampled 10 days after surgery; and 3) Vehicle control tissue in all experiments consistently showed the lack of exposure to E2.

In addition, it is acknowledged that the length of time from ovariectomy to initiation of an experimental treatment is not consistent between monkeys and rats. Long-term ovariectomized monkeys (versus short-term) were used in these experiments for reasons of availability, because steroid hormone levels were barely detectable, and because maximal uterine atrophy had been established.

Statistical Analysis

Number of mitotic cells, cells showing nuclear fragmentation, uterine wet weight, and uterine blotted weight, were statistically compared by analysis of variance using Instat GraphPAD Software, version 1.11a (1990). Significant differences between group means were determined by Fisher's least significant difference test, with values being considered significant at $P \leq 0.05$.

Ethical Treatment of Animals

All animals were housed in facilities approved by the American Association for the Accreditation of Laboratory Animal Care. These studies were approved by the Animal Care and Use Committee of Eastern Virginia Medical School and carried out according to NIH guidelines and USDA regulations.

RESULTS

Monkey Endocrine Analysis

As expected, mifepristone was highly proficient in the inhibition of estrogen-driven mitogenesis of primate endometrial tissue. These data confirm our prior published results (van Uem et al., 1989; Wolf et al., 1989b; Neulen et al., 1990). All animals used in this study were long-term ovariectomized (≥ 9 months). In order to stimulate growth of the endometrium, monkeys received estrogen replacement therapy given as a 3 cm E2 containing silastic capsule, sc, for 21 days. The mean serum E2 levels attained in each treatment group are shown in Figure 3. All treatment groups receiving an E2 capsule maintained serum E2 levels in the physiological range. The higher serum E2 values in the 0.1 mg/kg mifepristone group were likely due to random variation in implant steroid releasing kinetics.

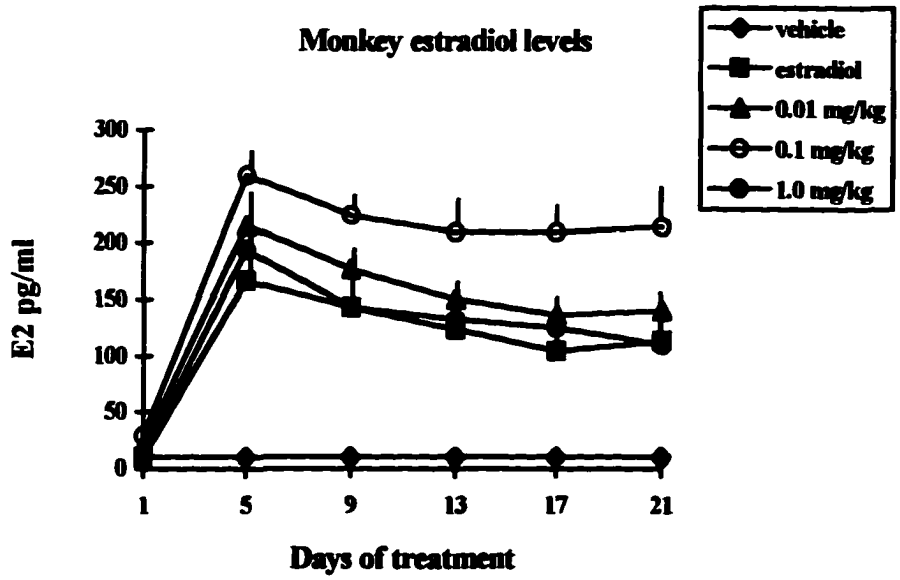

Fig. 3. Serum estradiol levels in monkeys given a 3 cm E2-containing silastic capsule and treated with mifepristone. All points represent the mean + SEM of three determinations, except for E2 control (day 1) and 1.0 mg mifepristone (day 21) which represent two determinations + range. All treatment groups given the E2 capsule maintained a physiological level of E2 throughout the experiment.

TABLE 1. Histological Classification of the Cynomolgus Endometrium Developed Under Different Steroid Treatments.

Group (n=3)	Thickness (mm ± SEM)	Glandular morphology	Glandular mitotic activity[1]	Stromal mitotic activity[2]	Histological classification
Vehicle	0.92 ± 0.1^{a}	small rounded	0/10	0/10	atrophic
E2 control	2.83 ± 0.3^{b}	dilated tubular	19/10	0.3/10	mid proliferative
E2 plus mifepristone (0.01 mg/kg)	2.11 ± 0.2^{b}	tubular	10/10	0.3/10	mid proliferative
E2 plus mifepristone (0.1 mg/kg)	1.64 ± 0.1^{ab}	small rounded	3/10	0/10	weak proliferative
E2 plus mifepristone (1.0 mg/kg)	0.67 ± 0.1^{a}	small rounded	1.7/10	0/10	very weak proliferative

Animals were treated with either a 3 cm E2-containing silastic implant or a vehicle-containing implant for 21 days. Daily injections of mifepristone (at doses indicated) began on day 1 and were continued for an additional 19 days. Uterine biopsies were obtained on day 21.

[1] Glandular mitotic activity = average number of mitotic cells/10 glands.

[2] Stromal mitotic activity = average number of mitotic cells/10 fields @ 400X.

Means with different superscripts are significantly different ($P<0.05$).

Antiestrogenic Effects of Mifepristone on Monkey Endometrium

Histological results showing the effect of mifepristone on monkey endometrium are illustrated in Table 1 and Figure 4. In the vehicle control group, the lack of ovarian steroids was associated with endometrium showing very densely positioned nuclei in the stroma and being completely void of glandular and stromal mitotic activity (Fig. 4A). With the addition of E2 to the experimental model (E2 control group), a marked induction of endometrial growth was observed. The endometrial classification for this group of monkeys was mid-proliferative, as shown by appearance of dilated tubular-shaped glands, stromal edema, and presence of glandular mitotic activity (Fig. 4B). The mean endometrial thickness in the E2 control group was significantly different from the vehicle control group ($P<0.01$). In relationship to the E2 control group, treatment with E2 plus 0.01 mg/kg mifepristone did not inhibit E2-induced endometrial growth ($P>0.05$) nor affect general endometrium morphology (Fig. 4C). Increasing the dose of mifepristone to 0.1 mg/kg, reduced the level of glandular mitotic activity, produced an endometrium with small rounded glands, and further reduced the endometrial thickness (Fig. 4D). The endometrial thickness of the 0.1 mg/kg mifepristone group was not different from either the vehicle control or E2 control groups ($P>0.05$), indicating the appearance of antiestrogenic effects. Animals treated with 1.0 mg/kg mifepristone showed a further reduction in glandular mitotic activity and a compact stromal morphology closely resembling that in the vehicle control group. As shown in Figure 4E the glands were few, small and rounded. The endometrial thickness attained in the 1.0 mg/kg mifepristone group was not different from the vehicle control group ($P>0.05$), and was different from the E2 control group ($P<0.001$). Histological classification of the 1.0 mg/kg mifepristone group was weak proliferative and not atrophic, as in the vehicle control group. This difference in classification indicates that mifepristone can suppress growth of the endometrium to a level equal to the vehicle control, yet at the same time does not completely inhibit all estrogenic activity.

Fig. 4. Comparison of endometrial appearance from cynomolgus monkeys treated with E2 plus mifepristone. Paraffin embedded tissue was sectioned at 5μm, stained with H&E and photographed at (X40). Tissue from the vehicle control group (panel A) shows a dense, compact stromal cell density with no observable edema. In contrast, tissue from the E2 control group (panel B) is characterized by increased endometrial thickness, elongated dilated glands, and stromal edema. (*) Approximate border of the endometrium and myometrium.

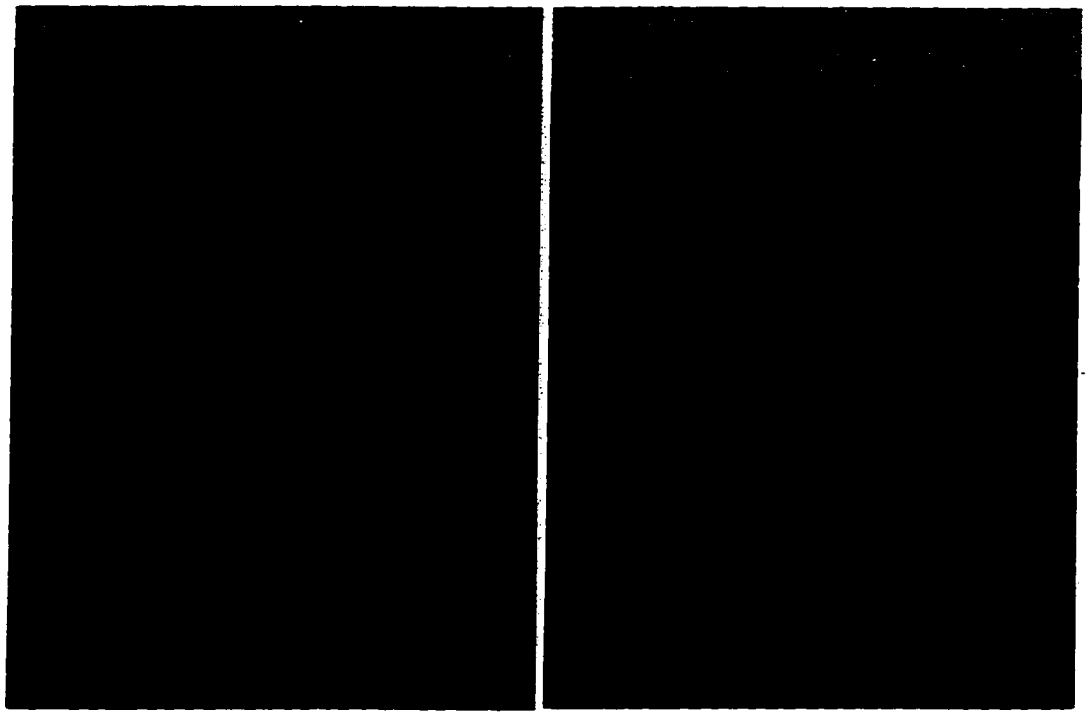

Fig. 4. (continued). Photographs of monkey endometrial tissue. Tissue from the E2 + 0.01 mg/kg mifepristone treatment group (panel C) shows tubular glandular morphology and stromal edema. Endometrium thickness in this group did not differ from the E2 control group ($P>0.05$). Panel D shows endometrial tissue from the E2 + 0.1 mg/kg mifepristone treatment group. tissue from this group was characterized by small round glands, decreased stromal edema, and decreased endometrial thickness. Endometrial thickness of this group did not differ from the vehicle control group or E2 control group ($P>0.05$). (*) Approximate border of the endometrium and myometrium.

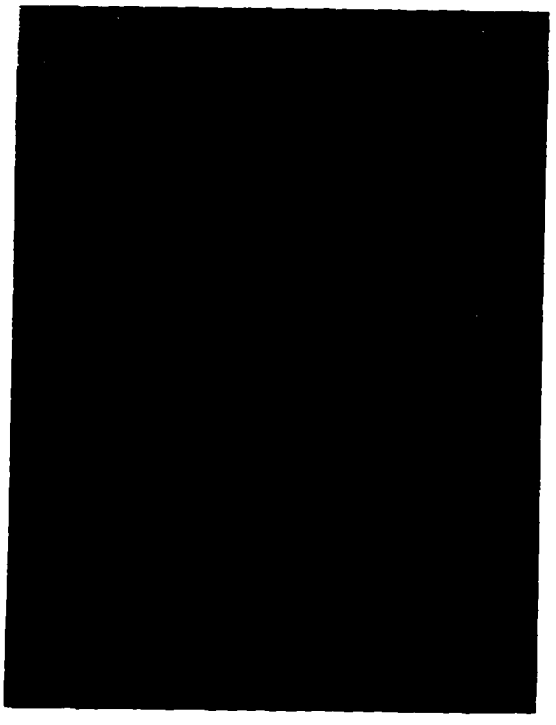

Fig. 4. (continued). Photograph shows endometrial tissue from the E2 + 1.0 mg/kg mifepristone treatment group (panel E). The endometrium in this group was marked by an almost complete lack of glands, stromal compaction, and an endometrial thickness not different from the vehicle control group ($P>0.05$). (*) Approximate border of the endometrium and myometrium.

Rat Preliminary Experiments

A series of experiments using the rodent model were conducted to determine optimal doses of E2 and mifepristone required to affect uterine imbibition and uterine blotted weight. As our overall study design included establishment of a rodent model to replace the primate uterine model, our initial studies used adult rats as a comparative physiological model for adult monkeys.

Figure 5 illustrates the increase in uterine wet weight in response to various doses of E2. The ideal E2 dose desired would be a value approximating the midpoint of the uterine response curve. As shown in Figure 5, the E2 dose most closely meeting this criteria was 6 μg E2/100 g BW; consequently, this dose was then selected to be used in subsequent experiments.

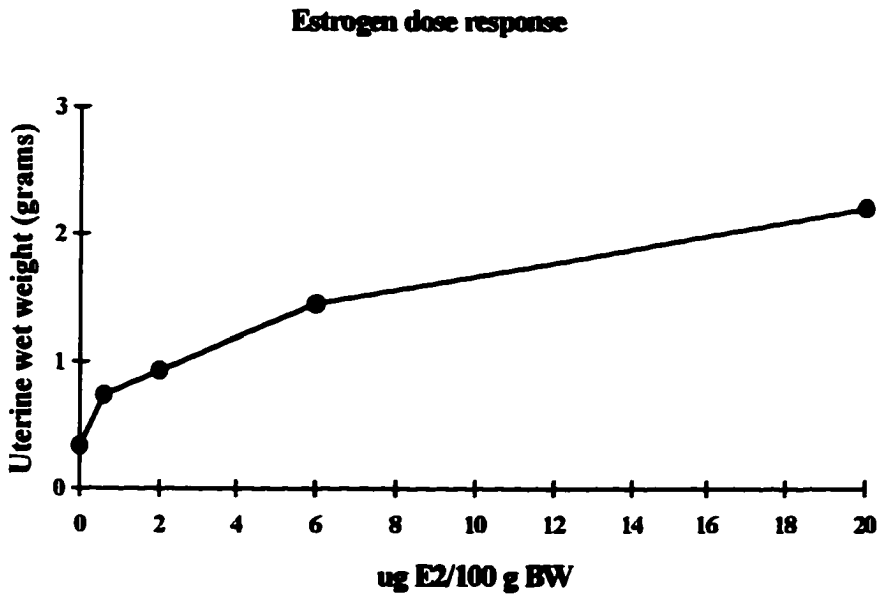

Fig. 5. Estrogen dose response curve. Ovariectomized adult rats were treated once daily for 3 days with various doses of E2 (in oil, sc). Uterine wet weight values were determined on the morning of day 4. Animals in this experiment were ovariectomized ≥4 days prior to steroid treatment.

TABLE 2. Preliminary Experiment 1. Uterine Weights of Ovariectomized Adult Rats[1] Treated for 3 Days with 6μg E2/100 g BW plus Mifepristone.

	wet weight (g)	blotted weight (g)
Treatment	mean ± SEM	mean ± SEM
Vehicle control	0.307 ± 0.015[a]	0.293 ± 0.014[a]
E2 control	1.627 ± 0.131[b]	0.547 ± 0.014[b]
E2 + 0.005 mg mifepristone	1.597 ± 0.144[b]	0.565 ± 0.021[b]
E2 + 0.05 mg mifepristone	1.646 ± 0.196[b]	0.588 ± 0.068[b]
E2 + 0.5 mg mifepristone	1.206 ± 0.138[b]	0.548 ± 0.031[b]

[1]Rats were given a ≥4 day post-ovariectomy recovery period before starting steroid treatment.

Treatment means with a different superscript are significantly different ($P<0.05$).

TABLE 3. Preliminary Experiment 2. Uterine Weights of Ovariectomized Adult Rats[1] Treated for 3 Days with 6μg E2/100 g BW plus Mifepristone.

	wet weight (g)	blotted weight (g)
Treatment	mean ± SEM	mean ± SEM
Vehicle control	0.216 ± 0.007[a]	0.211 ± 0.007[a]
E2 control	1.340 ± 0.171[b]	0.497 ± 0.034[b]
E2 + 0.5 mg mifepristone	1.535 ± 0.184[b]	0.486 ± 0.028[b]
E2 + 1.5 mg mifepristone	1.121 ± 0.108[b]	0.460 ± 0.011[b]
E2 + 5.0 mg mifepristone	1.203 ± 0.155[b]	0.459 ± 0.015[b]

[1]Rats were given a ≥10 day post-ovariectomy recovery period before starting steroid treatment.

Treatment means with a different superscript are significantly different (P<0.05).

Previous studies already mentioned that used the monkey model had indicated that mifepristone inhibited endometrial proliferation. In an effort to maintain a similar mifepristone dose (mg/kg) in the rat model, we began these studies examining the effect of 0.005 mg, 0.05 mg, and 0.5 mg mifepristone, total dose per animal (doses approximate 0.015 mg/kg, 0.15 mg/kg, and 1.5 mg/kg). In addition, the rat uterine weight bioassay conventionally uses a 3 day steroid treatment period and we followed this accepted protocol.

Data from Preliminary Experiment 1 did not identify antiestrogenic effects of mifepristone in adult rats, as determined by a decrease in uterine imbibition or uterine blotted weight ($P>0.05$, Table 2). Consequently, in our following experiment we chose to increase the mifepristone total dose per animal to 0.5 mg, 1.5 mg, and 5.0 mg. Data from this experiment (Preliminary Experiment 2) show that even these high levels of antiprogestin did not inhibit the increase in uterine wet and blotted weights induced by 6 μg E2/100 g BW ($P>0.05$, Table 3). Concern was expressed that the dose of 6 μg E2/100 g BW may establish serum E2 levels exceeding the physiologic range, and therefore, possibly over-ride the noncompetitive antiestrogenic effects of mifepristone. The dose 0.5 μg E2/100 g BW was selected from the literature (L'Horset et al., 1993) as sufficient to mimic E2 peak levels during proestrus. Uterine weights of adult rats treated with 0.5 μg E2/100 g BW and mifepristone are shown in Table 4 (Preliminary Experiment 3). These data show that all treatment groups receiving mifepristone were not significantly different from the E2 control group ($P>0.05$).

It had been reported that ovariectomized rats not pretreated with E2 failed to respond to progestin treatment (Kraus and Katzenellenbogen, 1993; L'Horset et al., 1993). The lack of response is thought to be due to decreased expression of PR. As our previous three experiments failed to identify antiestrogenic effects of mifepristone in the ovariectomized rat, it was possible that the lack of response was due to very low uterine PR expression. To determine if E2 pretreatment was necessary in our rodent model, we treated rats with 0.5 μg E2/100 g BW for 3 days, followed by the same dose of E2 plus mifepristone for 3 consecutive days (Preliminary Experiment 4). Mifepristone did not demonstrate any antiestrogenic affects under these conditions. Thus, results of this

experiment show that E2 pretreatment did not affect the capacity of mifepristone to cause antiestrogenic effects in adult rats ($P>0.05$, Table 5).

Kraus and Katzenellenbogen (1993) have reported antiestrogenic effects of mifepristone in ovariectomized immature rats as determined by a decrease in uterine imbibition. Since the methodology used by these authors differed considerably from our own, we believed it necessary to conduct a comparative study. A Latin Square 2x2 study was designed to test adult rats versus immature rats given E2 replacement as either 0.5 μg E2/100 g BW or 0.5 mg E2 pellet.

Although the treatment regime modeled after that described by Kraus and Katzenellenbogen (1993), i.e., Preliminary Experiment 4, did not identify noncompetitive antiestrogenic effects of mifepristone, we empirically chose to continue this treatment regime. The variable, post-ovariectomy recovery period, was held at 10 days for adult rats to facilitate ovarian steroid clearance and uterine atrophy. To replicate the experiment described by Kraus and Katzenellenbogen (1993), we were obligated to observe the 4 day surgery recovery period for immature rats. In addition, we continued to use the E2 dose of 0.5 μg E2/100 g BW to maintain E2 stimulation within the physiologic range.

TABLE 4. Preliminary Experiment 3. Uterine Weights of Ovariectomized Adult Rats[1] Treated for 3 Days with Physiologic Levels of E2 Replacement (0.5 μg E2/100 g BW) plus Mifepristone.

	wet weight (g)	blotted weight (g)
Treatment	mean ± SEM	mean ± SEM
Vehicle control	0.204 ± 0.018[a]	0.201 ± 0.017[a]
E2 control	1.179 ± 0.073[b]	0.442 ± 0.039[b]
E2 + 0.1 mg mifepristone	1.080 ± 0.144[b]	0.445 ± 0.029[b]
E2 + 0.5 mg mifepristone	1.203 ± 0.170[b]	0.452 ± 0.032[b]
E2 + 1.5 mg mifepristone	1.114 ± 0.110[b]	0.466 ± 0.032[b]
E2 + 5.0 mg mifepristone	1.192 ± 0.096[b]	0.484 ± 0.032[b]

[1]Rats were given a ≥10 day post-ovariectomy recovery period before starting steroid treatment.

Treatment groups with a different superscript are significantly different ($P<0.05$).

TABLE 5. Preliminary Experiment 4. Effect of E2 Pretreatment on Uterine Weights of Ovariectomized Adult Rats[1] Treated for 3 Days with 0.5 μg E2/100 g BW plus Mifepristone.

	wet weight (g)	blotted weight
Treatment	mean ± SEM	mean ± SEM
Vehicle control	0.195 ± 0.004[a]	0.192 ± 0.003[a]
E2 control	0.841 ± 0.216[a]	0.518 ± 0.031[b]
E2 + 0.1 mg mifepristone	1.088 ± 0.252[a]	0.531 ± 0.040[b]
E2 + 0.5 mg mifepristone	1.006 ± 0.188[a]	0.484 ± 0.023[b]
E2 + 1.5 mg mifepristone	0.881 ± 0.179[a]	0.493 ± 0.012[b]
E2 + 5.0 mg mifepristone	1.168 ± 0.109[a]	0.517 ± 0.014[b]

Animals were pretreated with E2 at 0.5 μg E2/100 g BW alone for 3 days, followed by an additional 3 days of E2 plus mifepristone or E2 alone. Treatment groups with a different superscript are significantly different ($P<0.05$).

[1]Rats were given a ≥10 day post-ovariectomy recovery period before starting steroid treatment.

Rat Endocrine Analysis

In order to establish the approximate serum E2 levels attained by the two different E2 delivery methods, we treated a representative group of animals and collected cardiac blood samples on days 1 (pretreatment control), 3, 5, and 7. The mean serum E2 values are shown in Figure 6. Administration of 0.5 μg E2/100 g BW to both immature and adult rats maintained daily average serum E2 levels in the range of 10-35 pg/ml. These values correspond to physiologic levels attained in diestrus and proestrus. In comparison, animals given the 0.5 mg E2 pellet attained extraordinary supraphysiologic serum levels of E2. These values ranged from 80- to 600-fold greater than the levels reached by treatment with 0.5 μg E2/100 g BW. The higher E2 levels measured in the immature rats versus adult rats are possibly due to the difference in volume of distribution and/or slower hepatic metabolism.

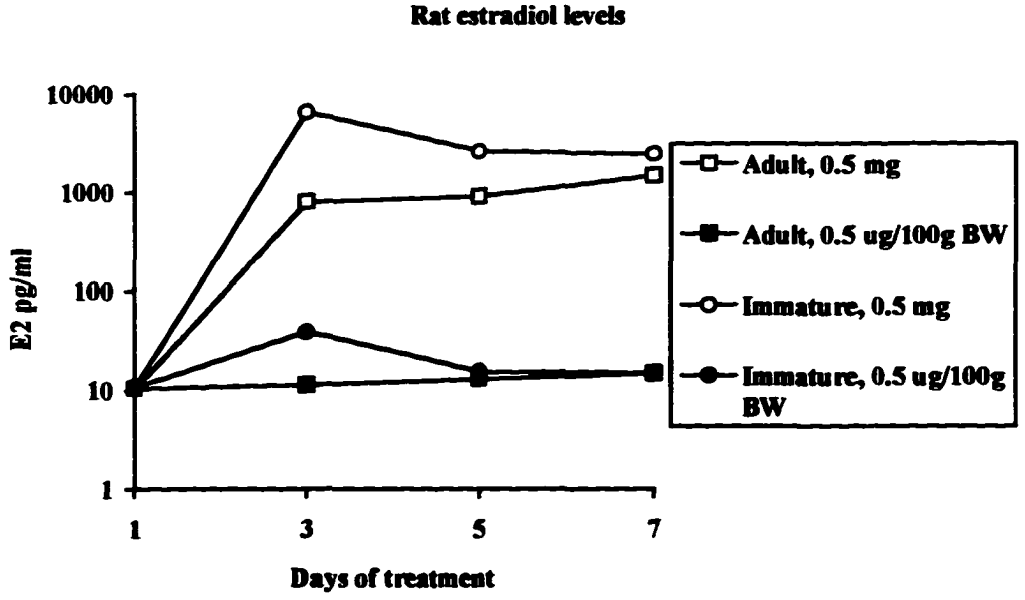

Fig. 6. Serum estradiol levels in adult and immature rats. Animals were either implanted on day 1 with a 0.5 mg E2 pellet, sc, or treated daily with 0.5 μg E2/100 g BW for 6 days. Serum samples were collected by cardiac puncture on the days indicated. Animals receiving the 0.5 mg pellet attained E2 levels 80- to 600-fold greater than animals given 0.5 μg E2/100 g BW. All points represent the mean + SEM of three determinations, except immature day 1 (control) which represents mean + range of two determinations.

Antiestrogenic Effects of Mifepristone on the Estradiol-Primed Rat Uterus

In contrast to the monkey data, the rat uteri did not manifest the capacity of mifepristone to curtail endometrial growth, even at doses 16-fold greater (mg/kg) than that used in the monkey. Interestingly, mifepristone did appear to impair water retention in certain experiments, even while the rat uterine weight bioassay proved inappropriate for the demonstration of the antiproliferative actions of this antiprogestin.

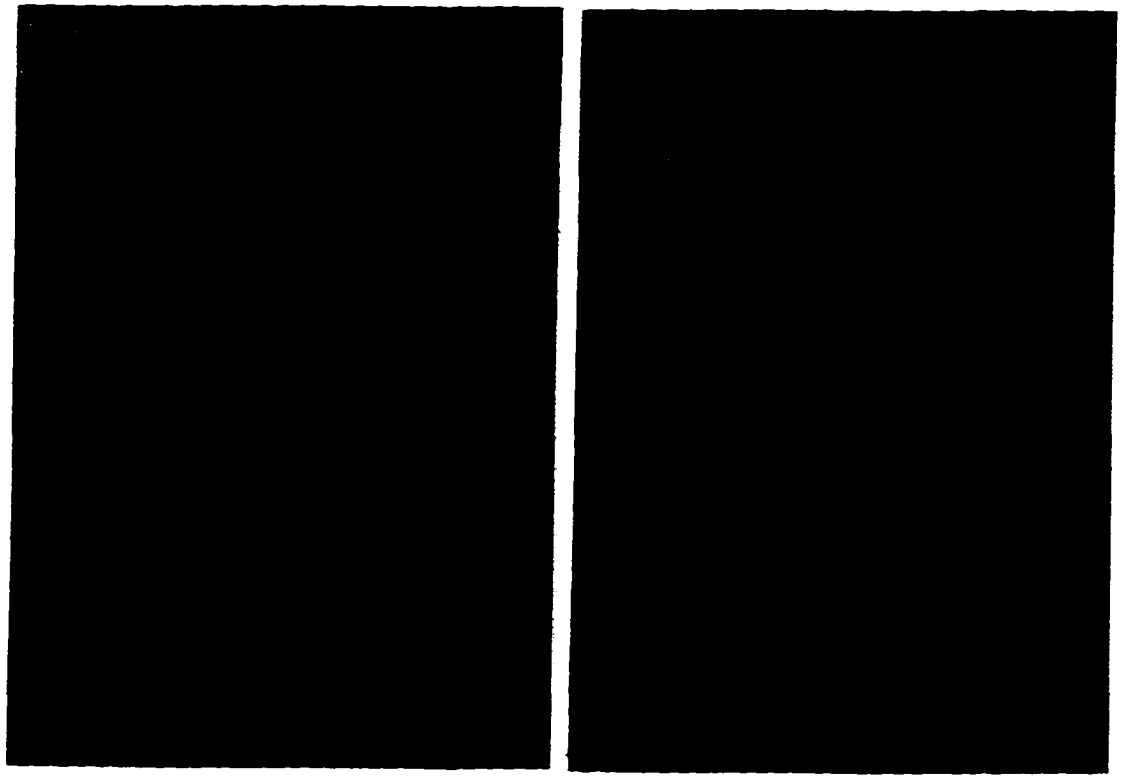

Fig. 7. Effect of 0.5 μg E2/100 g body weight on adult rat uterine tissue. (A) Control tissue, (B) E2 treated tissue. Note uterine ballooning and increased tissue growth induced by the treatment with estrogen.

In the first experiment, the antiestrogenic effect of mifepristone was assessed in adult animals treated with 0.5 μg E2/100 g BW/day. The uteri of animals from the vehicle control group were small, flaccid, and contained a negligible weight of fluid (Fig. 7A). In contrast, the uteri of animals from the E2 control group were large and turgid (Fig. 7B). The E2 control group had a mean fluid volume of 0.3 ml and showed a 3.6-fold increase in wet weight and a ~2-fold increase in blotted weight compared to that observed in the vehicle control group. These results clearly illustrate that this amount of E2 does have stimulatory effects on rat uterine tissue.

The uterine wet and blotted weights of adult rats treated with 0.5 μg E2/100 g BW are shown in Figure 8. In this experiment, uterine weights of all treatment groups receiving mifepristone did not differ from their respective E2 control ($P>0.05$; Fig. 8A and B).

Similar results were obtained in the second experiment which assessed the antiestrogenic effects of mifepristone in immature animals treated with 0.5 μg E2/100 g BW/day. In this experiment, uteri of animals from the E2 control group showed a 4-fold increase in both uterine wet weight ($P<0.05$; Fig. 9A) and blotted weight ($P<0.05$; Fig. 9B) in comparison to the vehicle control group. As in the adult rat experiment, the immature rat uterine wet and blotted weights from all mifepristone-treated groups did not differ from their respective E2 control group ($P>0.05$). Taken together, data from these two experiments indicate that mifepristone does not affect the change in uterine wet weight or uterine blotted weight induced by 0.5 μg E2/100 g BW.

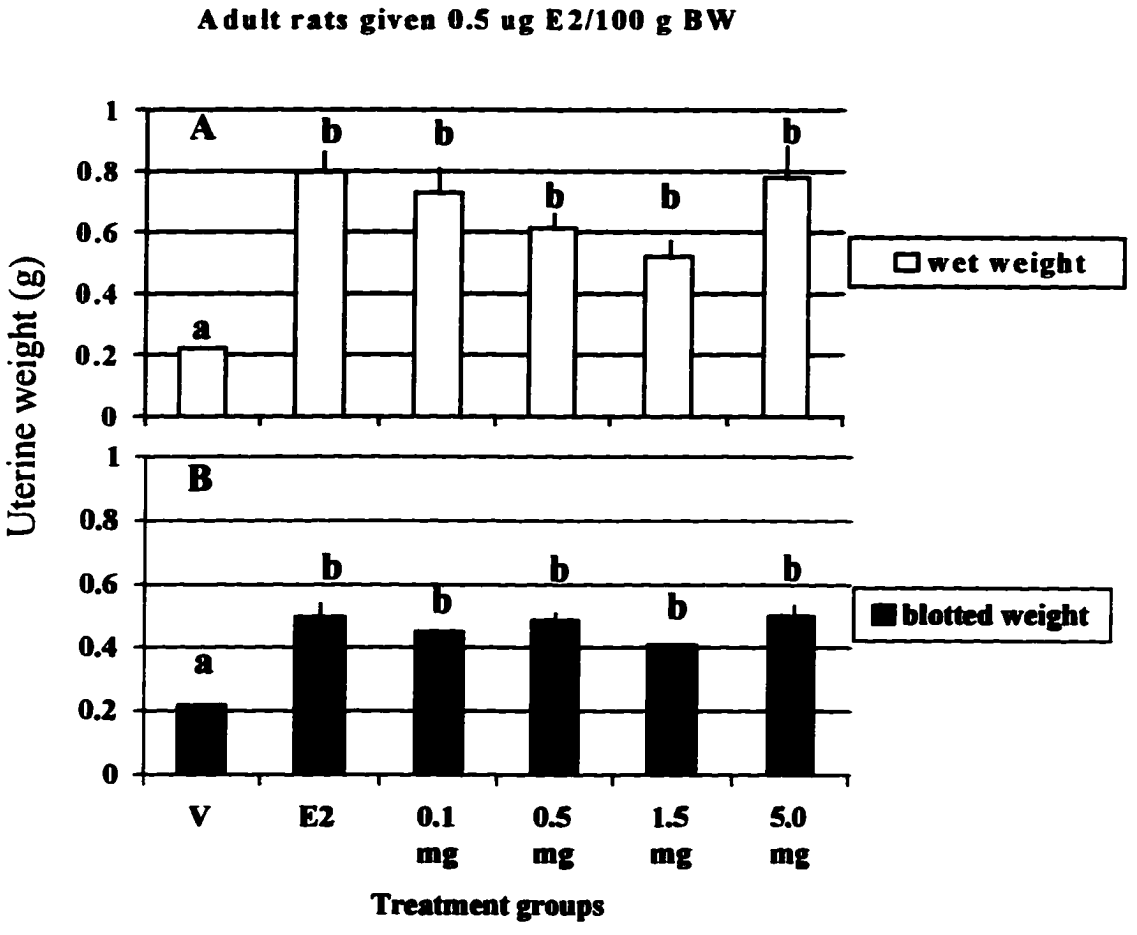

Fig. 8. Uterine weight values attained in adult rats given 0.5 μg E2/100 g BW/day and treated with mifepristone. Treatment groups are: vehicle control (V); estradiol control (E2); E2 + 0.1 mg mifepristone; E2 + 0.5 mg mifepristone; E2 + 1.5 mg mifepristone; and E2 + 5.0 mg mifepristone. Each column represents the mean + SEM for five determinations. Columns with a different superscript are significantly different ($P<0.05$). Uterine wet and blotted weights in the mifepristone treated groups were not significantly different from their respective E2 control group. Columns without bars indicate that the SEM is within the column.

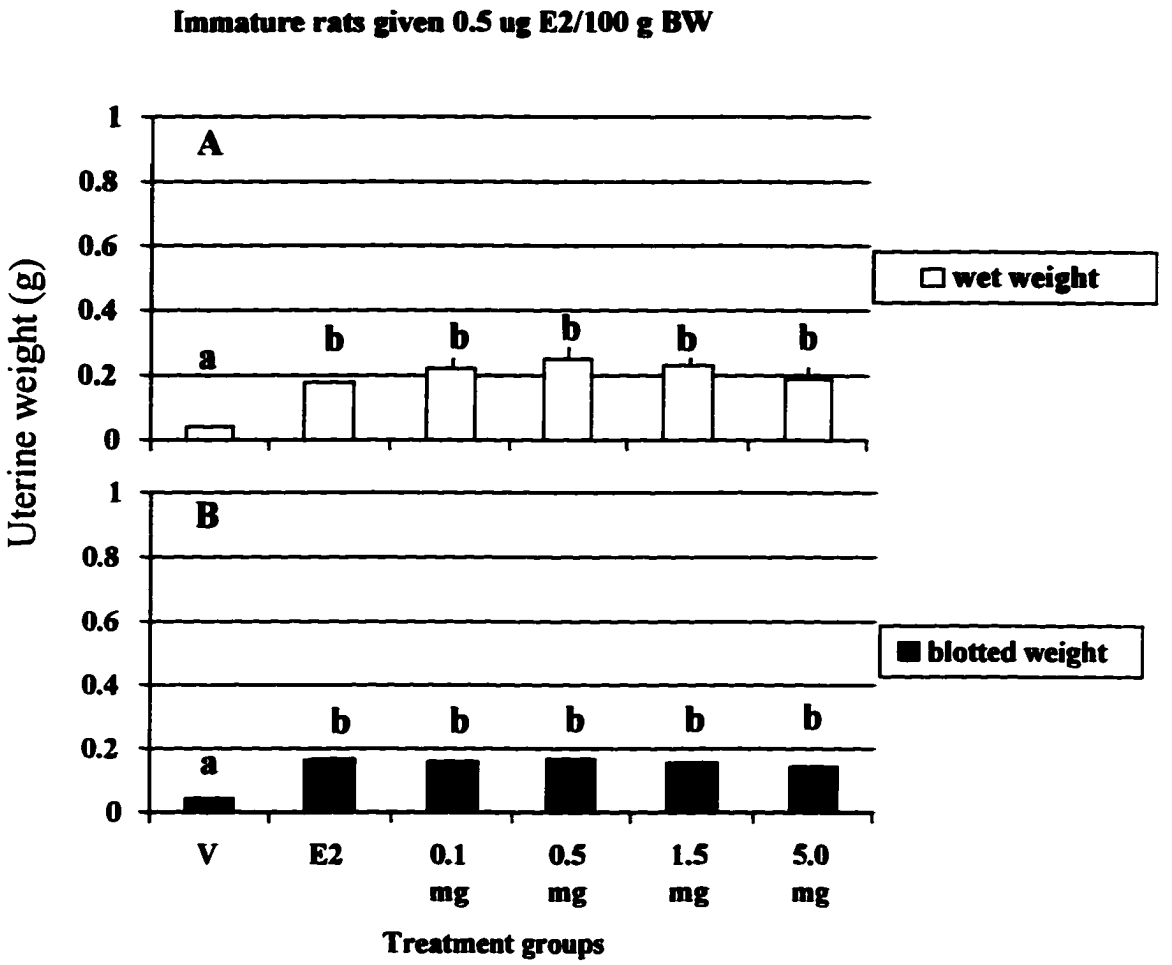

Fig. 9. Uterine weight values attained in immature rats given 0.5 μg E2/100 g BW/day and treated with mifepristone. Treatment groups are: vehicle control (V); estradiol control (E2); E2 + 0.1 mg mifepristone; E2 + 0.5 mg mifepristone; E2 + 1.5 mg mifepristone; and E2 + 5.0 mg mifepristone. Each column represents the mean + SEM for five determinations. Columns with a different superscript are significantly different ($P<0.05$). Animals treated with mifepristone did not show a decrease in uterine wet or blotted weights in comparison to their respective E2 control group. Columns without bars indicate that the SEM is within the column.

Previous work by Kraus and Katzenellenbogen (1993) reported antiestrogenic effects of 1.5 mg mifepristone in immature rats treated with a 0.5 mg E2 pellet. As our previous experiments failed to detect any antiestrogenic effect of mifepristone in rats treated with 0.5 μg E2/100 g BW, we were interested in comparing our experimental model to that of Kraus and Katzenellenbogen.

In this experiment adult animals treated with a 0.5 mg E2 pellet showed a tremendous increase in uterine wet weight (2.21 g) in comparison to the vehicle control (0.2 g). This increase in uterine wet weight was inhibited by treatment with mifepristone at all doses ($P<0.05$; Fig. 10A), with there being no statistically significant difference between the lowest and highest dose groups. The uterine blotted weight of the 0.1 mg mifepristone and 0.5 mg mifepristone groups were different from the E2 control group ($P<0.05$; Fig. 10B), however, this significance was marginal since the alpha probability was between 0.04 and 0.05. Blotted uterine weights of the 1.5 mg and 5.0 mg mifepristone groups were not significantly different from the E2 control ($P>0.05$).

When immature animals were treated with the 0.5 mg E2 pellet, an ~15-fold increase in uterine wet weight and 5-fold increase in uterine blotted weight were observed in comparison to the vehicle control, (0.62 g vs 0.04 g) and (0.2 g vs 0.04 g) respectively. Similar to that shown in the adult animals, all doses of mifepristone were able to inhibit the E2-induced increase in uterine wet weight ($P<0.01$; Fig. 11A). In addition, mifepristone decreased uterine blotted weight by approximately 15% in all treatment groups ($P<0.05$; Fig. 11B). These data are in agreement with Kraus and Katzenellenbogen (1993), who reported a 17% decrease in uterine wet weight of animals treated with 0.5 mg E2 pellet and 1.5 mg mifepristone.

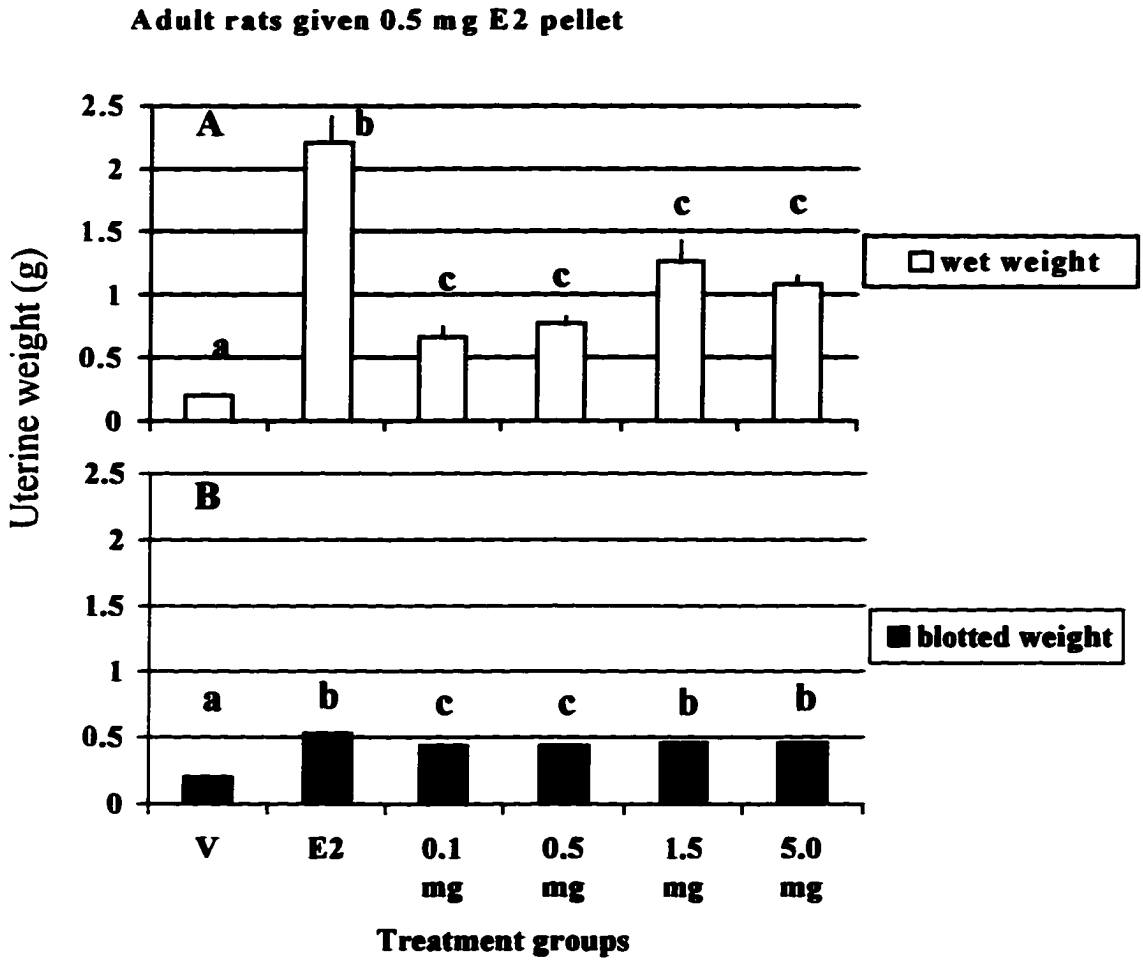

Fig. 10. Uterine weights attained in adult rats given a 0.5 mg E2 pellet and treated with mifepristone. Treatment groups are: vehicle control (V); estradiol control (E2); E2 + 0.1 mg mifepristone; E2 + 0.5 mg mifepristone; E2 + 1.5 mg mifepristone; and E2 + 5.0 mg mifepristone. Each column represents the mean + SEM for five determinations. Columns with a different superscript are significantly different ($P<0.05$). E2 control animals showed a dramatic increase in uterine wet weight. This increase was effectively inhibited by treatment with mifepristone at all four doses. Columns without bars indicate that the SEM is within the column. *Note change in scale.

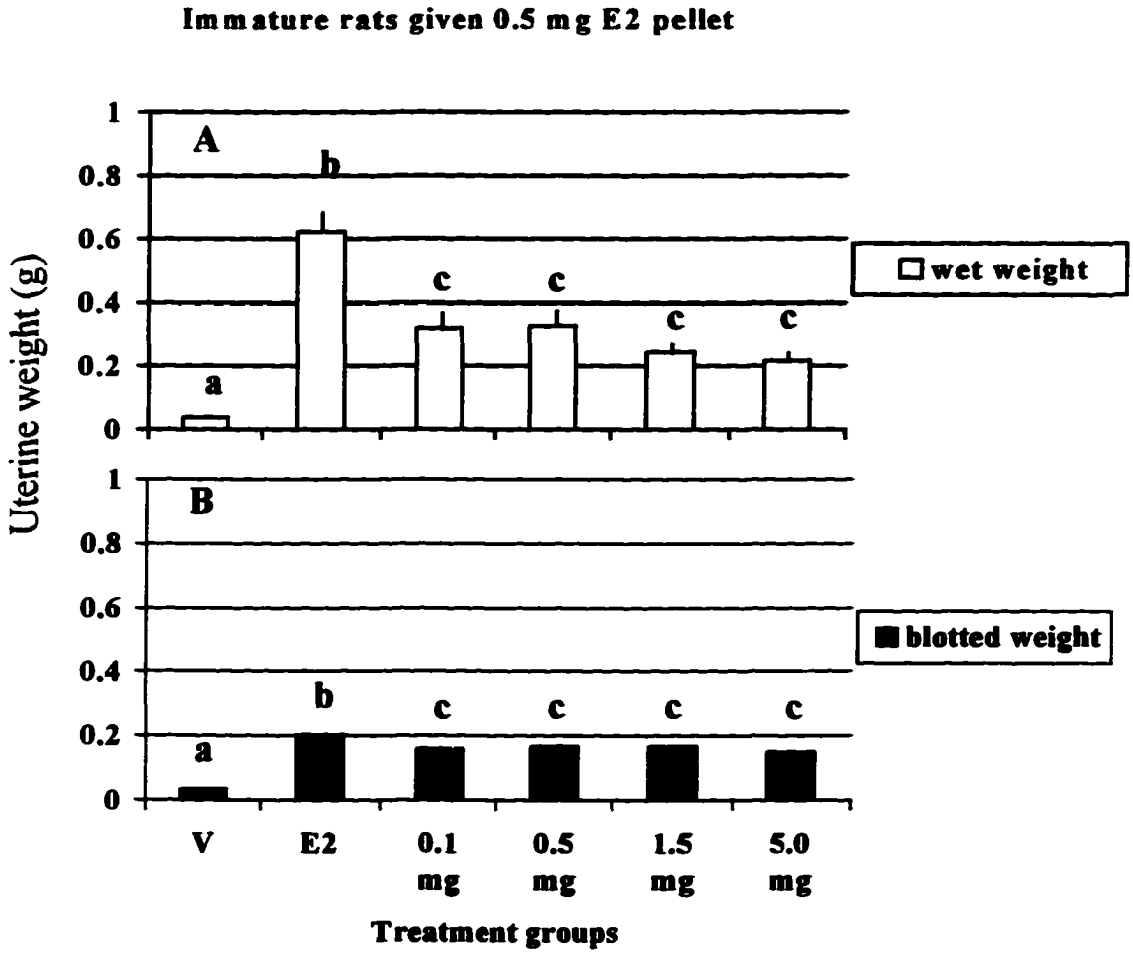

Fig. 11. Uterine weight attained in immature rats given a 0.5 mg E2 pellet and treated with mifepristone. Treatment groups are: vehicle control (V); estradiol control (E2); E2 + 0.1 mg mifepristone; E2 + 0.5 mg mifepristone; E2 + 1.5 mg mifepristone; and E2 + 5.0 mg mifepristone. Each column represents the mean + SEM for five determinations. Columns with a different superscript are significantly different ($P<0.05$). The E2 treated group showed a 15-fold increase in uterine weight in comparison to the vehicle control group. This estrogen-induced weight increase was inhibited by treatment with mifepristone. Columns without bars indicate that the SEM is within the column.

Histological Analysis

To help understand the effect of mifepristone on rat endometrial morphology, uteri from the four rat experiments were stained with H&E and histologically examined for number of mitotic figures and cells with nuclear fragmentation. A comparison of these parameters are shown in Tables 6 and 7. These data show that within each experiment, the number of mitotic cells and cells with a fragmented nucleus in the E2 control group were not significantly different from the mifepristone-treated groups ($P>0.05$). As such, the difference in uterine blotted weight observed in those experiments using the 0.5 mg E2 pellet is not due to a mifepristone-induced decrease in mitotic activity or increase in cell death. It is interesting to note that in animals given 0.5 μg E2/100 g BW/day, the number of mitotic cells and cells showing nuclear fragmentation were greater in relationship to their age-matched counter parts given a 0.5 mg E2 pellet.

TABLE 6. Number of Mitotic Figures[1] (Mean ± SEM) Measured in Ovx, Adult and Immature Rats, Treated with 17β-Estradiol and Mifepristone.

Experimental model	Vehicle control	Estradiol control (E2)	E2 + 0.1 mg mifepristone	E2 + 0.5 mg mifepristone	E2 + 1.5 mg mifepristone	E2 + 5.0 mg mifepristone
Adult rats 0.5 μg E2/100 g	2 ± 1	44 ± 8	38 ± 4	34 ± 7	36 ± 4	36 ± 5
Adult rats 0.5 mg E2 pellet	2 ± 0.5	6 ± 1	9 ± 1.5	8 ± 1	9 ± 1	7 ± 1
Immature rats 0.5 μg E2/100 g	0.2 ± 1	18 ± 5	15 ± 4	14 ± 2	17 ± 3	26 ± 5
Immature rats 0.5 mg E2 pellet	0 ± 0	3 ± 0.5	4 ± 0.5	3 ± 0.4	3 ± 0.5	4 ± 1

Animals were pretreated with E2 at (0.5 μg/100 g BW) or (0.5 mg pellet) alone for 3 days, followed by an additional 3 days of E2 plus mifepristone or E2 alone. Mifepristone treated groups were not significantly different from their respective E2 control group ($P > 0.05$).

[1] Mean number of cells/tissue cross-section/animal.

TABLE 7. Number of Cells with Nuclear Fragmentation[1] (Mean ± SEM) Measured in Ovx, Adult and Immature Rats, Treated with 17β-Estradiol and Mifepristone.

Experimental model	Vehicle control	Estradiol control (E2)	E2 + 0.1 mg mifepristone	E2 + 0.5 mg mifepristone	E2 + 1.5 mg mifepristone	E2 + 5.0 mg mifepristone
Adult rats 0.5 μg E2/100 g	0.1 ± 0.1	13 ± 9	21 ± 15	31 ± 21	27 ± 8	45 ± 35
Adult rats 0.5 mg E2 pellet	0.2 ± 0.2	1 ± 0.5	3 ± 1	2 ± 1	1.5 ± 0.5	1.5 ± 0.5
Immature rats 0.5 μg E2/100 g	0 ± 0	10 ± 3	8 ± 4	4 ± 1	10 ± 3	11 ± 3
Immature rats 0.5 mg E2 pellet	0 ± 0	0.5 ± 0.4	0.5 ± 0.3	0.4 ± 0.2	0.5 ± 0.3	0 ± 0

Animals were pretreated with E2 at (0.5 μg/100 g BW) or (0.5 mg pellet) alone for 3 days, followed by an additional 3 days of E2 plus mifepristone or E2 alone. Mifepristone treated groups were not significantly different from their respective E2 control group ($P > 0.05$).

[1] Mean number of cells/tissue cross-section/animal.

DISCUSSION

Before our laboratory's initial reports in 1989, there was no appreciation of a class of antiprogestin compounds that conferred antiestrogenic/antiproliferative effects upon uterine tissue. Accordingly, in the absence of binding to the ER, antiestrogenic effects of mifepristone were simply not anticipated. Although the primate is an extremely useful animal model for comparative physiological studies in humans, considerable financial and ethical issues remain a constant companion when conducting primate research. As such, the application of other animal models to replace the primate is important.

The immature rat uterus is frequently used by corporate scientists as a target organ for high-throughput screening of estrogenic compounds. Two easily observable classic responses in the rat uterus to exogenous estrogen include increased cell proliferation and water retention. Utilization of the rat model greatly decreases the economical and ethical burdens surrounding the use of primates in research. Therefore, it is prudent for investigators to use this less controversial animal model when possible.

The experiments reported herein, have examined the ability of the rat uterine weight assay to detect previously established noncompetitive antiestrogenic effects of mifepristone on estrogen-induced endometrial proliferation. Our findings suggest that the rat uterine weight bioassay is not an appropriate laboratory model suitable for screening antiprogestins for medical indications in which women's tissues grow in response to estrogen.

Results from our nonhuman primate experiment have clearly confirmed the noncompetitive antiestrogenic effect of mifepristone on endometrial proliferation. These data by themselves although not novel, do confirm the antiestrogenic nature of mifepristone and allow a direct comparison to the rat model. The present experiment and those of others (van Uem et al., 1989; Wolf et al., 1989b; Neulen et al., 1990; Slayden and Brenner, 1994) all show that mifepristone reliably inhibits E2-induced endometrial proliferation in ovariectomized monkeys in a dose dependent manner. Stromal compaction was not statistically evident in this study at the two lowest mifepristone doses (0.01 and 0.1 mg/kg). However, the 0.1 mg/kg dose appeared to be a partially effective dose as the

endometrial thickness did not differ from either the vehicle control or E2 control groups. The 1.0 mg/kg mifepristone dose did not induce development of an atrophic endometrium as shown in the vehicle control group. This limited ability of mifepristone to completely suppress estrogenic effects may increase its applicability to treat various diseases.

During this experiment, all monkeys were observed daily for the presence of menstrual bleeding. Throughout the entire 21 day study period, all animals remained amenorrheic (data not shown).

Our preliminary rat experiments that used adult animals and various treatment regimes did not identify noncompetitive antiestrogenic effects of mifepristone. As our nonhuman primate data clearly shows the noncompetitive antiestrogenic capacity of mifepristone, validity of the rat uterine weight assay was brought into question. To correct for the possible lack of, or paucity of progesterone receptor and ineffectiveness of mifepristone in the rat model, the total treatment time was lengthened to 6 days; 3 days of E2 pretreatment, and 3 days of E2 plus mifepristone treatment. Indeed, E2 pretreatment may be necessary to upregulate the progesterone receptor in ovariectomized rats before effects of progestins can be assessed (Katzenellenbogen, 1980; L'Horset et al., 1993).

The mean serum E2 levels in rats given 0.5 μg E2/100 g BW were in the normal physiologic range of diestrus and proestrus. In comparison, serum E2 levels established in rats given the 0.5 mg E2 pellet were pharmacologic. Animals treated with the E2 pellet maintained a daily mean serum E2 level ranging from 80- to over 600-fold greater than rats given 0.5 μg E2/100 g BW. These values are much higher than the 40 pg/ml average for the proestrus estradiol surge in rats (Smith et al., 1975). The lower level of serum E2 established by 0.5 μg E2/100 g BW did not stimulate as dramatic an increase in uterine wet weight in the E2 control animals as did treatment with the 0.5 mg E2 pellet. In addition, rats given 0.5 μg E2/100 g BW did not show a decrease in uterine wet weight when treated with mifepristone. At this time the association between serum E2 levels and the capability of mifepristone to affect uterine wet weight is not fully understood.

It has been reported by Kraus and Katzenellenbogen (1993) that ovariectomized immature rats treated with a 0.5 mg E2 pellet show a down-regulation in uterine ER expression. After 2 days of treatment, uterine tissue from rats receiving the E2 pellet

expressed only 15% of control ER levels, and this level remained steady throughout the remaining 6 day treatment period. Levels of uterine PR (B isoform) were also measured in these same animals. A 7-fold increase in PR levels was attained after 2 days of treatment and a 10-fold increase was attained by end of the 6 day treatment period. The regulation of steroid receptors in ovariectomized rats given various levels of E2-supplementation has also been reported by Manni and others (1981). These authors report that rats given from 0 to 50 μg E2/day for 5 days show a dose-related decrease in total level of uterine estrogen receptors. Although uterine ER and PR expression were not measured in our experiments, it is likely our animals given the 0.5 mg E2 pellet would have shown a similar regulation of these steroid receptors. The possible increased number of ERs in our animals given 0.5 μg E2/100 g BW is supported by the observation of a greater number of mitotic cells in these animals; which is a classic estrogen-driven response. The increased mitotic index in these animals occurred despite having much lower serum E2 levels in comparison to animals given the 0.5 mg E2 pellet. These data would then suggest that the decreased numbers of mitotic cells observed in the E2 pellet-treated animals could be due to down-regulation of the ER. If these assumptions are valid, it appears that the decrease in uterine wet weight caused by mifepristone in our experiments required the presence of a low level of ER expression, a high level of PR expression, and a high serum E2 level. Slayden and Brenner (1994) have proposed that increased levels of PR expression is important for the ability of mifepristone to have antiestrogenic effects in the rhesus monkey. Up-regulation of the PR may provide a mechanism by which squelching or quenching of estrogen gene activity could occur. Therefore, it is possible that the ratio of PR:ER coupled with high serum E2 levels are key factors that may regulate the ability of mifepristone to have antiestrogenic effects in the rat.

Mifepristone decreased the uterine blotted weight of rats in both experiments that used the 0.5 mg E2 pellet. Although statistically significant, the absolute weight differences between treatment groups within these two experiments is very small, and as such, emphasis should not be strongly placed upon the difference. When comparing age-matched rat experiments, it is evident that E2 replacement given as 0.5 μg /100 g BW versus 0.5 mg pellet, led to approximate equal uterine blotted weight values; yet at the

same time, those animals treated with 0.5 μg E2/100 g BW had greatly increased numbers of mitotic cells and cells showing nuclear fragmentation. The disparity of these data indicate that the observed decrease in uterine wet weight in animals given a E2 pellet was more likely due to a decrease in uterine fluid retention, and not in addition, the apparent inhibition of cell growth. Kraus and Katzenellenbogen (1993) also observed a decrease in uterine wet weight in mifepristone treated rats. These authors interpreted their results as an indication of the ability of mifepristone to inhibit E2-induced increase in uterine growth; however, this assessment was made in the absence of histological evaluation. Although our data do indicate a difference in mitotic and fragmenting cell numbers between experiments, this activity was not associated with a change in uterine weight.

It has been proposed that mifepristone elicits its antiestrogenic effects via the PR A isoform (McDonnell and Goldman, 1994). The absence of observed antiestrogenic effects by mifepristone in these experiments is not due to the lack of PRA in the rat uterus, as Ilenchuk and Walters (1987) have measured a 3:1 ratio of PRA:PRB in rat uteri. Second, the only species identified to date that do not recognize mifepristone are the chicken, hamster, and tammar wallaby (Benhamou et al., 1992; Lim-Tio et al., 1996). Lastly, it has been reported that mifepristone does have antiprogestin effects in the rat, as shown by impairment of implantation and embryo development (Psychoyos and Prapas, 1987; Roblero and Croxatto, 1991), and pregnancy termination (Chang et al., 1993).

Other mechanisms have been proposed to describe the antiestrogenic effects of mifepristone. Two of these mechanisms include, inhibition via PRA and PRB (Kraus et al., 1995), or by an over expression of the ER (Neulen et al., 1996). It is apparent that the observed inhibition is not due to a down-regulation of uterine ER, as both Slayden and Brenner (1994) and Neulen and others (1996) have measured an increased expression of ER in mifepristone treated animals. The mechanism(s) by which mifepristone induces its antiproliferative effects on the nonhuman primate endometrium remains unknown.

CONCLUSIONS

In the absence of both physiological and pharmacological serum estradiol levels, failure of the rat uterine weight assay to confirm the nonhuman primate data indicating curtailment of endometrial proliferation, illustrates an extreme limitation and brings in to question the utility of using the rat uterine weight bioassay to screen lead antiprogestins compounds for potential noncompetitive antiestrogenic activity. Although we only analyzed the single antiprogestin mifepristone, it seems likely that other antiprogestins possess antiproliferative effects on primate endometrium. Our data indicate that the antimitogenic capability of these compounds would likely not be identified using the rat uterine weight bioassay. In summary, the rat uterine weight bioassay, in contrast to the primate uterine assay, is not a valid *in vivo* model for detecting, or for quantifying the noncompetitive antiestrogenic/antiproliferative effects of mifepristone.

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