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Modulation of oestrogenic effects by progesterone antagonists in the rat uterus

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TABLE OF CONTENTS

Antiprogestins can modulate oestrogenic effects in various oestrogen-dependent tissues, dependent on species, tissue, dose and duration of treatment. Enhanced oestrogenic responses to mifepristone and onapristone occur *in vitro* and *in vivo*. However, the antiprogestins mifepristone, onapristone, and ZK 137 316 can block the ability of oestradiol to increase endometrial growth in non-human primates. Our purposes were firstly, to decide whether mifepristone and onapristone had direct oestrogenic activity *in vitro* and in the uterus of spayed and immature rats, and secondly, to discover whether antiprogestins exhibit inhibitory effects on oestrogen action in the uterus in spayed, oestrogen-substituted rats. In transactivation assays, mifepristone induced oestrogenic response, whereas onapristone had only marginal effects on reporter gene transcription. In immature rats, onapristone and mifepristone markedly increased uterine weights, and onapristone, but not mifepristone significantly enhanced endometrial luminal epithelial height, a sensitive oestrogen parameter. Conversely, in spayed and adrenalectomized rats, neither onapristone nor mifepristone changed uterine weights or endometrial morphology, indicating that their effects in immature rats were indirect. In spayed, oestrogen-substituted rats, antiprogestins did not block oestradiol-stimulated endometrial growth and luminal and glandular epithelium were stimulated more after antiprogestin plus oestrogen, than after oestradiol alone. All compounds induced compaction of the uterine stroma. In spayed rats, onapristone and some other 13α-configured (type 1) antagonists (ZK 135 569, ZK 131 535) reduced oestradiol-stimulated myometrial proliferation and induced an overall uterine weight reduction in animals treated with oestrogen and antiprogestins, in comparison with oestradiol-treated controls. 13β- configured (type II) antagonists, including mifepristone, lilopristone and ZK 112 993, were not effective. In the uteri of spayed rats, onapristone was also found to enhance the oestradiol-stimulatory effect on expression of the oestrogen-dependent proto-oncogene, *c-fos*. In conclusion, antiprogestins do not inhibit, but rather enhance, oestrogen-induced uterine glandular and luminal epithelium in spayed rats, contrary to their effects in primates. The rat model is unsuitable to study endometrial antiproliferative effects of antiprogestins in primate uteri.

Key words: oestrogen action/progesterone antagonists/proliferation/uterus

Introduction

11β-aryl-substituted steroidal progesterone antagonists (antiprogestins), e.g. mifepristone (RU 486), onapristone (ZK 98 299), lilopristone (ZK 98 734), ZK 112 993, and other structurally-related compounds bind with high-affinity to progesterone receptors (PR) and block progestagenic effects both *in vitro* and *in vivo* (Neef *et al*., 1984; Phillibert *et al*., 1985). Mifepristone and onapristone are the most widely-studied antiprogestins. Both also bind to glucocorticoid (GR) and androgen (AR) receptors and exhibit

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antiglucocorticoid and anti-androgenic activity *in vitro* (U.Fuhrmann, unpublished results) and *in vivo*; onapristone being less antiglucocorticoid than mifepristone. These two antiprogestins show a very weak binding to human and rat oestrogen receptors (ER) (Chwalisz *et al*., 1995).

Overall control of growth and functions in the reproductive tract is regulated by oestrogen and progesterone. In the uterus, oestrogens stimulate endometrial epithelial proliferation, control many metabolic events, and are necessary for normal uterine growth. Progesterone generally inhibits oestrogen-dependent uterine epithelial proliferation and involves endometrial differentiation to the secretory type. Specific mechanisms proposed for the antiproliferative action of progesterone include: (i) a down-regulation of ER in target tissues (Hsueh *et al*., 1975; Katzenellenbogen 1980); (ii) uterine enzyme induction catalysing oestradiol conversion to less active metabolites (Tseng and Gurpide, 1975); (iii) a decrease in oestrogen-induced specific protein expression (Bhakoo *et al*., 1977); and (iv) an inhibition of oestrogen-induced proto-oncogenes (Kirkland *et al*., 1992; Fuhrmann and Stöckemann, 1993).

Antiprogestins and oestrogen responses in the uterus

Progesterone is a major sex steroid controlling oestrogen action in reproductive tracts and other oestrogen-dependent tissues. Therefore, it is not surprising that antiprogestins also interfere with various oestrogenic responses. The major concern of chronic antiprogestins administration in women is endometrial hyperplasia due to unopposed oestrogen effects. Paradoxically, antiprogestins, including mifepristone (Wolf *et al*., 1989; Slayden *et al*., 1993; Slayden and Brenner, 1994; Heikinheimo *et al*., 1996), onapristone (Chwalisz *et al*., 1994) and the new antiprogestin ZK 137 316 (Slayden *et al*., 1997), inhibit endometrial proliferation in both ovariectomized and intact monkeys. Antiprogestins administered chronically at relatively low doses inhibit mitotic activity in endometrial epithelium and induce a dose-dependent stromal compaction in spayed and intact monkeys at high oestradiol concentrations. Similar endometrial antiproliferative effects arise in ovariectomized rabbits where onapristone selectively inhibits oestrogen-induced endometrial gland formation (Chwalisz *et al*., 1991). Recently, Gemzell-Danielsson *et al*. (1998) found that low-dose mifepristone (0.5 mg daily for 3 months) delayed endometrial maturation and significantly reduced the glandular diameter in premenopausal women. These effects were accompanied by a reduction in endometrial glycodelin expression and *Dolichus biflorus* agglutinin (DBA)–lectin binding. Stromal compaction and an absence of mitoses arose with 2 mg mifepristone daily for 30 days (Cameron *et al*., 1996). In monkeys and rabbits antiproliferative effects were endometrium-specific and oestrogenic effects in the oviduct and vagina were not inhibited by antiprogestins (Chwalisz *et al*, 1991; Slayden and Brenner, 1994). The endometrial antiproliferative effects of antiprogestins in the primate endometrium is a most important property of antiprogestins offering a unique opportunity to selectively inhibit oestrogenic effects in the uterus without affecting oestrogenic response in other tissues. The mechanism underlying this endometrial antiproliferative effect is still unknown, yet it differs from the inhibitory effect of a progesterone on the endometrium.

However, uterine oestrogenic responses occur in monkeys and women after treatment with chronic, high-dose antiprogestins. Cystic endometrial hyperplasia occurred after chronic, high dose oral onapristone (50 mg/kg, Schering toxicological study, unpublished data) of intact cynomolgus monkeys. Atypical cystic changes also arose after chronic treatment of endometriotic women with a relatively high dose (50 mg daily) of mifepristone (Murphy *et al*., 1995). Recently, Croxatto *et al*. (1998) reported the occurrence of endometrial gland dilation in 34% of women treated with 1 mg/day mifepristone for 150 days. The significance of this finding is unclear, since no signs of endometrial hyperplasia were found in this study. A similar dilation of endometrial glands was frequently observed in monkeys after chronic treatment with different antiprogestins (K.Chwalisz, unpublished data). Interestingly, this effect could be observed even within an atrophic endometrium accompanied by a drastic reduction of mitotic activity in the glandular epithelium strongly suggesting that antiprogestin-induced gland dilation is due to altered glandular fluid outflow rather than to endometrial hyperplasia in non-human primates. Moreover, there are experimental studies in rats and mice which suggest that both mifepristone (Dibbs *et al*., 1995) and onapristone (Bigsby and Young, 1994) may exhibit some oestrogenic-like activities by directly interacting with ER.

These conflicting results indicate that the precise mechanism of the divergent antiprogestin effects on the endometrium are still poorly understood. These studies also show that the modulatory impact of oestrogen by antiprogestins is quite complex, since it may depend on species, tissue, antiprogestin dose and type, and duration of treatment. We describe the modulatory effect of various antiprogestins in the non-pregnant rat uterus, since rats are widely used to study oestrogenic and anti-oestrogenic activities. We specifically address the question of whether antiprogestins exert oestrogenic or antiproliferative (anti-oestrogenic) effects in the uteri of castrated rats, and whether type I (onapristone-type) and type II (mifepristone-type) antiprogestins act differently in the rat uterus. The

oestrogen-like effects of antiprogestins are described using in-vitro and in-vivo models. In addition, the modulatory effects of antiprogestins on various parameters of oestrogen action, including *c-fos* expression, uterine growth, morphology, and morphometry are discussed. Experiments were performed on spayed and immature rats, both commonly used to study oestrogenic effects. We also wished to find out whether rat models are of use in predicting antiproliferative endometrial effects of antiprogestins in primates. We also examined onapristone and mifepristone effects on uterine ER protein concentrations in ovariectomized rats, in the presence and absence of oestradiol.

Immature and adult Wistar rats (Schering, Berlin, Germany) were kept in Makrolon cages (type III) in an air-conditioned room at a temperature of 22 ± 2°C and relative humidity of 50 ± 5%, under a regime of 14 h light: 10 h dark cycle (light 6:30–20:30). The animals had free access to the standard pellet diet Altromin® (Altromin Ltd, Lage, Germany) and to tap water containing 0.9% saline.

Figure 1 shows the progesterone antagonists used in this study. The 13β-methyl-substituted (type II) antiprogestins used were: mifepristone (RU 486; ZK 95 890: 11β-[4-(dimethylamino)-phenyl]-17β-hydroxy-17β-(prop-1-ynyl)estra-4, 9-dien-3-one); lilopristone (ZK 98 734; 11β-[4-(dimethylamino)-phenyl]-17β-hydroxy-17α-(3-hydroxyprop-1-ynyl)estra-4, 9-dien-3-one). The 13α-methyl-substituted (type I) antiprogestins used were: onapristine (ZK98 299; 11β-[4-(dimethylamino)-phenyl]-17α-hydroxy-17β-(3-hydroxypropyl)-11β-13α-estra-4, 9-dien-3-one); ZK 131 535 (17α-hydroxy-17β-(3-hydroxypropl) (11β-[4-1-methylethenyl)phenyl]-13α-estra-4, 9-dien-3-one); and ZK 135 695 (11β-[4-(3-furanyl)phenyl]-17α-hydroxy-17β (3-hydroxypropyl)-13α-estra-4, 9-dien-3-one). All compounds were from Schering AG. For oral administration, the antiprogestins were formulated in 0.5 ml Myrj® 53 (ICI, Essen, Germany) saline (85 mg Myrj® 53 in 100 ml 0.9% saline). For s.c. administration, the compounds were formulated in 0.2 ml benzylbenzoate + castor oil (1:4 v/v). 17β-oestradiol and the pure anti-oestrogen ICI 182 780 (ZK 156 901) were formulated in 0.2 ml benzyl benzoate plus castor oil (1:4 v/v).

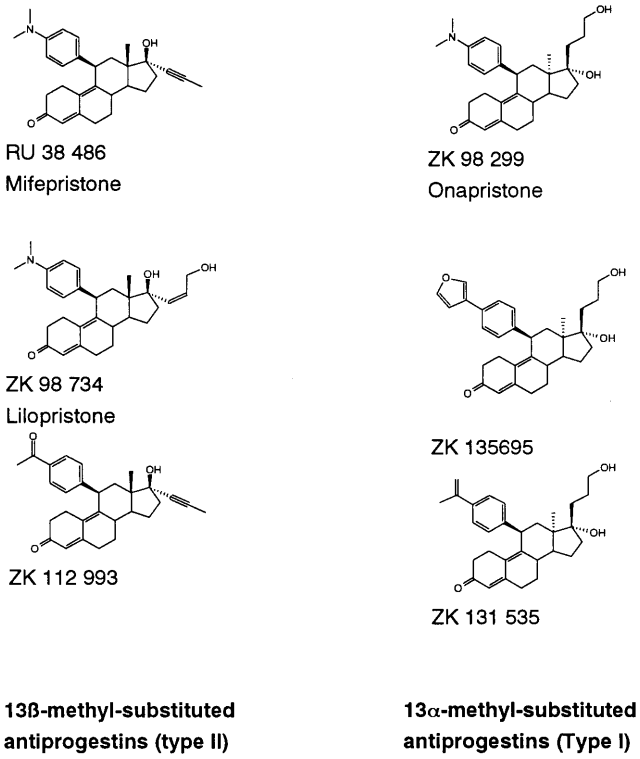

Figure 1. Structure of 13α- (type I) and 13β-methyl substituted (type II) antiprogestins used in the experiments.

Oestrogenic effects of anti-progestins *in vitro* and *in vivo*

Oestrogen-like activities of onapristone and mifepristone

Effects of onapristone and mifepristone on the oestrogen-responsive reporter gene VITtk-LUC in MVLN cells expressing human endogenous ER

MVLN cells (MCF-7 cells stably transfected with Vitellogenin-Luciferase-reporter gene and Neomycin resistance gene) were cultured in Dulbecco's modified Eagle's medium (DMEM) without Phenol Red, supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, penicillin, and streptomycin. To study hormonal effects, MVLN cells were trypsinized, pooled and replated onto 96-well dishes at a density of 1.2×10^4 cells/well. Cells were cultured in medium supplemented with 3% charcoal-stripped FCS in the presence of 10^{-9} M of the anti-oestrogen ICI 182 780 to reduce high background and the appropriate compound. Cells cultured in 1% ethanol were used as negative controls for reporter gene induction. Transactivation assays were carried out at least three times. The Luc Assay was performed using the Promega kit.

Oestrogenic-like effects of onapristone and mifepristone in immature rats

Rats aged 21 days (body weight ~50 g) were randomly allocated to nine groups (n = 5/group) and treated s.c. for 3 consecutive days as follows: group 1, vehicle; group 2, oestradiol 0.1 μg/rat; group 3, onapristone 500 μg/rat; group 4: ICI 182 780 (ZK 156 901, 500 μg/rat); group 5, oestradiol plus ICI 182 780 (0.1 μg/rat and 500 μg/rat respectively); group 6, onapristone plus ICI 182 780 (500 μg/rat each); group 7, onapristone plus oestradiol (500 μg/rat and 0.1 μg/rat respectively); group 8,

mifepristone (RU 486) at 500 μg/rate. Oestradiol doses induced a submaximal (50–60%) stimulation of uterine growth. During autopsy, the uteri were carefully excised from surrounding tissue, weighed and placed in Bouin's solution for histology, morphometric analysis, and proliferating cell nuclear antigen (PCNA) staining.

Effects of onapristone and mifepristone on selected parameters of oestrogen action in ovariectomized and adrenalectomized adult rats

Adult female rats (n = 40; body weight 170–180 g) were ovariectomized and adrenalectomized under ether anaesthesia. An additional group of 10 rats remained intact until the start of experiment (group 1, intact controls). At 12 days after surgery the ovariectomized and adrenalectomized animals were randomly allocated to four groups and treated s.c. for 15 consecutive days with either vehicle (group 2, ovariectomized plus adrenalectomized controls), oestradiol (0.3 μg/rat; group 3), onapristone, 10 mg/rat (group 4), and mifepristone 10 mg/rat (group 5). During autopsy, which took place 1 day after cessation of treatment, the uteri and vaginae were carefully excised from the surrounding tissue, weighed and placed in Bouin's solution for histological and morphometric analysis.

Modulation of oestrogenic effects by onapristone and mifepristone

Effects of onapristone on the oestrogen-induced expression of c-fos in the rat uterus

Adult female Wistar rats (225–250 g body weight) were ovariectomized and randomly allocated to seven experimental groups (six rats per group). At 10 days after ovariectomy the animals were treated s.c. as follows: group 1, vehicle, 0.2 ml; group 2, oestradiol, 3 μg/rat; group 3, oestradiol plus progesterone, 3 μg/rat and 3 mg/rat respectively; group 4, oestradiol plus onapristone, 3 μg/rat and 10 mg/rat respectively; group 5, oestradiol plus onapristone plus progesterone, 3 μg/rat, 10 mg/rat, and 3 mg/rat respectively; group 6, progesterone, 3 mg/rat, group 7, onapristone, 10 mg/rat. At 2 h after the treatment the animals were decapitated and the uteri were removed for RNA preparation.

Effects of onapristone and mifepristone on oestrogen receptor (ER) protein in the uterus of ovariectomized rats

With the exception of group 1 (intact controls, n = 10), all other rats were ovariectomized under ether anaesthesia 14 days prior to the experiment, randomly allocated to nine groups and treated s.c. for 3 days as follows: group 2, vehicle (n = 25); group 3, oestradiol (0.3 μg/rat/day) plus vehicle (n = 5); group 4, onapristone (10 mg/rat/day) plus vehicle (n = 15); group 5, mifepristone (10 mg/rat/day) plus vehicle (n = 15); group 6, oestradiol plus onapristone (1 mg/rat, n = 10); group 7, oestradiol plus onapristone (3 mg/rat/day; n = 10); group 8, oestradiol plus onapristone (10 mg/rat/day; n = 10); group 9, oestradiol plus mifepristone (10 mg/rat/day; n = 15). The animals were killed 24 h after the last treatment and the whole uteri were removed for ER measurements.

Effects of various type I and type II antiprogestins on selected parameters of oestrogenic action in ovariectomized, oestradiol-substituted rats

Adult Wistar rats were ovariectomized under ether anaesthesia at least 11 days before the experiment. The rats were then randomly allocated to treatment and control groups and treated for 3 consecutive days with a substitution dose of oestradiol (0.3 μg/rat s.c.) in combination with oral treatment of various antiprogestins including onapristone, mifepristone (RU 486), lilopristone (ZK 95 734) and ZK 122 993 (1, 3, and 10 mg/rat each). During autopsy, which was performed ~24 h after the last treatment, the uterine wet weights were measured and the uteri were fixed in Bouin's solution for histological and morphometric analysis. In a separate experiment the effects of two additional 13α-configurated compounds (ZK 131 535, ZK 135 695) were studied. (see Figure 7 for details).

Molecular and morphometric analyses

RNA preparation and Northern blot analysis

Total RNA was prepared according to Maniatis *et al*. (1982). Briefly, RNA was extracted from the uteri by immediate homogenization in a buffer containing 4 M guanidinium thiocyanate using a Polytron homogenizer (Kinematic AG, Littau, Switzerland). Uteri from each group were pooled for the preparation of each RNA sample. RNA was purified by ultracentrifugation through 5.7 M CsCl dissolved in SET buffer (10 mM Tris–HCl, pH 7.4) containing 5 mM EDTA, and 0.1% sodium dodecyl sulphate) and precipitated with ethanol. Poly $(A)^+$ RNA was separated from total RNA by affinity chromatography on oligo(dT)-cellulose columns (Pharmacia, Freiburg, Germany). Northern blot analysis was carried out according to Maniatis *et al*. (1982), applying 5 μg poly$(A)^+$ RNA per lane. Blots were hybridized with [^{32}P]-labelled cDNA probes for c-*fos* (Dianova, Hamburg, Germany). To assure that a constant amount of RNA was loaded, the blots were re-hybridized with the cDNA probe for 1A (subunit of cytochrome C

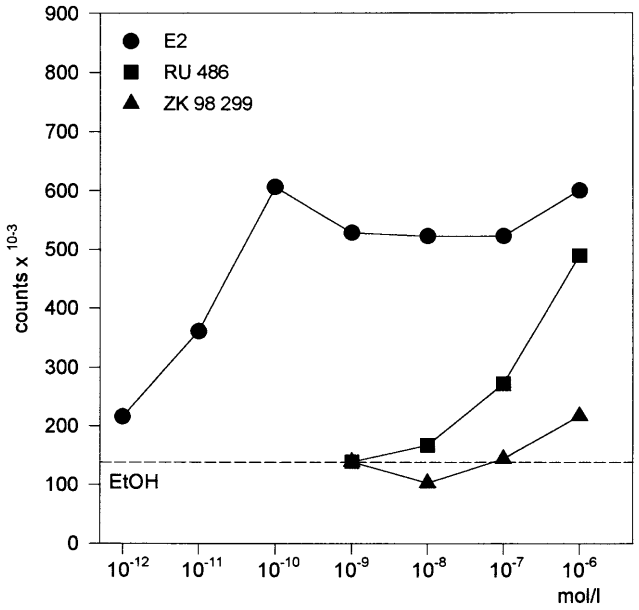

Figure 2. Effects of onapristone and mifepristone (RU 486) in the transactivation assay. MVLN cells expressing endogenous human ER and stably transfected with VITtk-LUC were cultured in the absence (EtOH control) and presence of increasing amounts of oestradiol (E2), mifepristone (RU 486) and onapristone (ZK 98 299). Note the weak oestrogenic activity of mifepristone.

oxidase; M.Lessl, unpublished data) a ubiquitous mRNA in the rat uterus whose expression is not regulated by steroid hormones.

Assay for total (cytosolic and nuclear) ER

After excision, rat uteri were deep frozen in liquid nitrogen and stored at –80°C until use. Receptor determination was performed essentially as described earlier (Chwalisz *et al*., 1991). Briefly, 1 g frozen tissue was broken up in a microdismembrator (Braun, Melsungen, Germany). The powder was suspended (5 ml/g) in homogenization buffer (10 mM Tris–HCl, pH 7.4, 1.5 mM EDTA, 0.4 mM KCl, 5 mM $NaMoO_4$, 10% glycerol) and was homogenized with a Polytron homogenizer. The homogenate was incubated at 0°C for 60 min. During incubation the slurry was gently stirred. After that the homogenate was centrifuged for 90 min at 100 000 *g*. Aliquots of the supernatant were diluted to a protein concentration of 1 mg/ml and were analysed by an ER enzyme immunoassay kit from Abbott Laboratories, (Chicago, IL, USA) in accordance with the manufacturer's instructions.

Immunohistochemistry

Tissue sections were prepared from Bouin-fixed paraffin-embedded uterine samples. Proliferation was studied by using a monoclonal antibody against PCNA and by applying the avidin–biotin–peroxidase (ABC) technique. After deparaffinization and washing in methanol, the slides were incubated in 3% hydrogen peroxide and diluted in methanol for 15 min. Non-specific binding of avidin/biotin reagents was prevented by using a blocking kit (Vector Laboratories, Burlingame, CA, USA) for 20 min. Thereafter the slides were incubated with the specific mouse monoclonal antibody against PCNA (DAKO–PCNA; PC Dako, Glostrup, Denmark), diluted 1:100, for 60 min at room temperature. The sections were then incubated with a biotinylated sheep anti-mouse immunoglobulin (Ig)G (RPN 1061; Amersham Life Science, Braunschweig, Germany, diluted 1:300) for 60 min at room temperature, and followed by incubation with avidin DH–biotinylated horseradish peroxidase H complex (Vectastain Elite ABC Kit; Vector Laboratories) for 60 min at room temperature in accordance with the manufacturer's instructions. Finally, the sections were developed in a substrate solution of 0.05% diaminobenzidine tetrachloride and 0.01% hydrogen peroxide. Slides were then washed in tap water, dehydrated in ethanol, cleared in xylene and mounted in DPX. Control sections were prepared by substituting the primary antibody with unspecific mouse IgG.

Morphometric evaluation

Measurements of the luminal epithelial height and luminal perimeter were performed on haematoxylin/eosin-stained paraffin sections, which were sectioned vertically to the longitudinal axis using Axioplan II microscope (Carl Zeiss GmbH, Jena, Germany) and Vidas 2.0 (Kontron Electronics, Eching, Germany) software. A double-sided *t*-test ($\alpha = 0.5$) was used for the statistical comparison of the treatment and the corresponding control groups with respect to various parameters.

Oestrogen-like activities of onapristone and mifepristone

Effects of onapristone and mifepristone in transactivation assays in vitro

To determine the oestrogenic activity of mifepristone and onapristone, MVLN cells expressing endogenous ER and stably transfected with VITtk-LUC were used. ER-mediated activity was investigated by treating the cells

with increasing amounts of mifepristone and onapristone as illustrated in Figure 2. For a positive control of ER-meditated reporter gene induction, cells were treated with oestradiol. Almost no induction of reporter gene expression was observed after onapristone administration up to a concentration of 10^{-6} M. However, a slight increase in reporter gene expression occurred after 10^{-7} M mifepristone with a pronounced effect at 10^{-6} M.

Oestrogen-like effects of onapristone and mifepristone in immature rats

This experiment was performed to determine whether the antiprogestins onapristone and mifepristone exhibit any oestrogenic-like activity in immature rats. Both onapristone (group 3) and mifepristone (group 8), significantly ($P < 0.05$) increased uterine wet weights and reached ~50% of the oestrogen stimulation (group 2) (Figure 3, lower panel) in immature rats. However, substantial differences in the effects of onapristone (group 3) and mifepristone (group 8) were observed on uterine epithelial height which is a very sensitive parameter of oestrogen action in the rodent uterus. Onapristone, but not mifepristone, led to a marked increase in epithelial height exceeding even the oestradiol effects by >50%. The stimulatory effects of onapristone on epithelial height was completely blocked by the pure anti-oestrogen ICI 182 780 (group 6).

Table I shows the results of the semiquantitative evaluation of the proliferation marker PCNA. The staining for PCNA was most intensive after oestradiol treatment and was evident in all uterine compartments (luminal and glandular epithelium, endometrial stroma and myometrium). Interestingly, compared with the vehicle control group (group 1), onapristone (group 3) moderately increased PCNA staining in the glandular epithelium, endometrial stroma and the myometrium, but not in the luminal epithelium. The stimulatory effects of mifepristone (group 7) on PCNA staining were less pronounced and were restricted to the endometrial stroma and the myometrium.

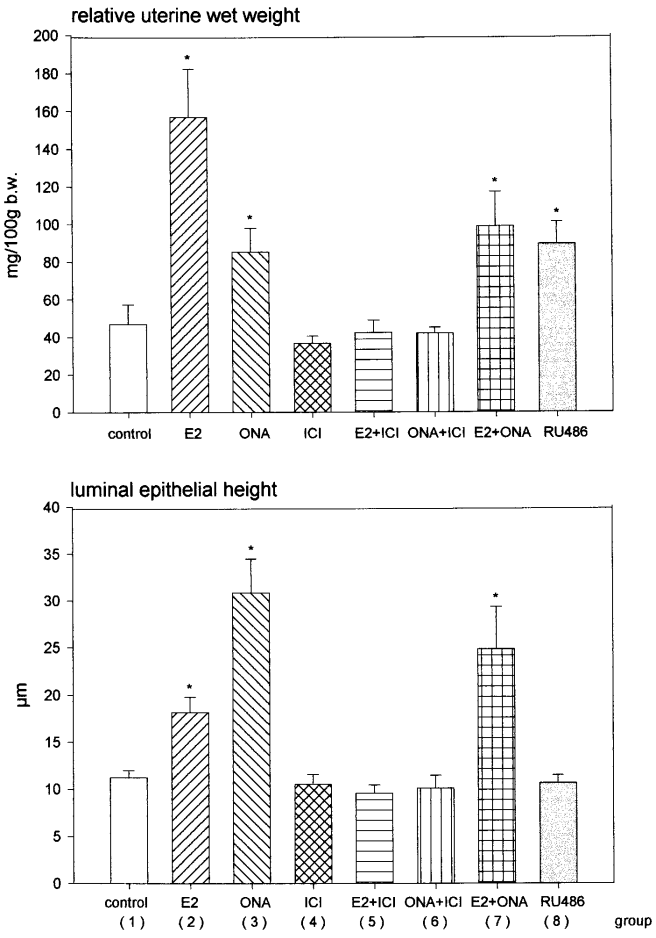

Figure 3. Effects of onapristone and mifepristone on uterine wet weights (upper panel) and luminal epithelial height lower panel in immature rats. 21 day old rats were treated s.c. for 3 consecutive days with either the vehicle (group 1); 0.1 µg/rat oestradiol (E2, group 2); 500 µg/rat onapristone (group 3); 500 µg/rat ICI 182 780 (anti-oestrogen, group 4); oestradiol plus ICI 182 780 (500 µg/rat each, group 5); onapristone plus ICI 182 780 (same doses; group 6); oestradiol plus onapristone (group 7, same doses); and mifepristone (RU 486; 500 µg/rat, group 8). Note a significant ($P < 0.05$) increase in uterine weights after both onapristone (group 3, upper panel) and mifepristone (group 8, upper panel) treatment, and a marked luminal epithelial hypertrophy after oestradiol (group 2), onapristone alone (group 3, lower panel), and onapristone plus oestradiol treatment (group 7, lower panel) compared with vehicle-treated control rats (group 1).

Effects of onapristone and mifepristone on selected parameters of oestrogen action in ovariectomized and adrenalectomized adult rats

The aim of this study was to determine whether the antiprogestins onapristone and mifepristone exhibit any oestrogen-like activities in ovariectomized and adrenalectomized rats, i.e. in the total absence of oestrogen and progesterone secretion. In contrast to immature rats, neither onapristone (group 4) nor mifepristone (group 5) increased the uterine wet weights or luminal epithelial height (Figure 4) after s.c. treatment for 15 days, when compared with the vehicle control group (group 2, ovariectomized and adrenalectomized rats). There was also no stimulatory effect on vaginal weights (Figure 4, lower panel). The morphological evaluation did not reveal any oestrogenic effect in the uterus or vagina after either antiprogestin (data not shown).

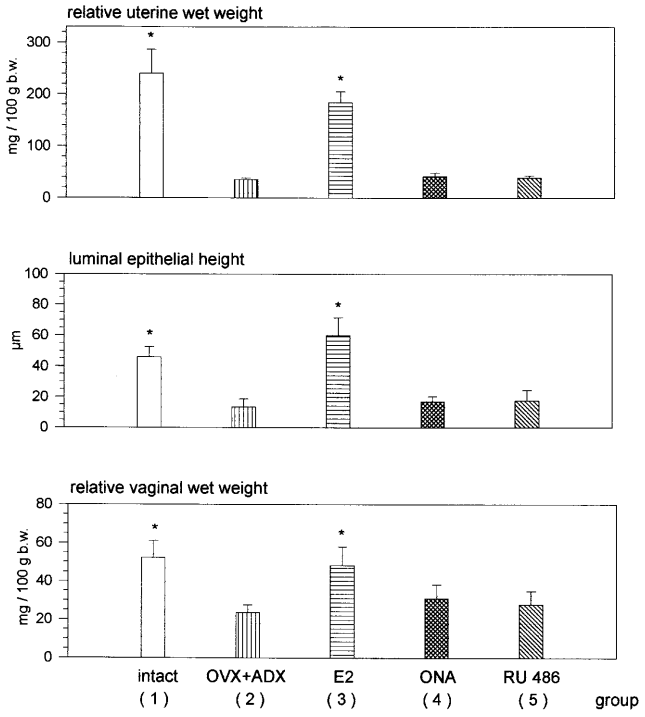

Figure 4. Effects of onapristone (ONA) and mifepristone (RU) on uterine wet weights (upper panel), luminal epithelial height (middle panel) and vaginal weights (lower panel) in ovariectomized and adrenalectomized adult rats. The animals were treated s.c. for 15 consecutive days with either the vehicle (OVX+ADX; group 2), oestradiol (0.3 µg/rat; group 3), onapristone, (10 mg/rat, group 4), or mifepristone (10 mg/rat, group 5). The intact animals (intact, group 1) were treated with the vehicle). The effects of oestradiol treatment (group 3) differed significantly ($P < 0.05$) from the ovariectomized and adrenalectomized control group (group 2) and were similar to those seen in intact controls. Note that neither parameter of oestrogen action was significantly ($P < 0.05$) influenced by the antiprogestins onapristone and mifepristone. *Values are significantly different ($P < 0.05$) from ovariectomized and adrenalectomized controls (group 2).

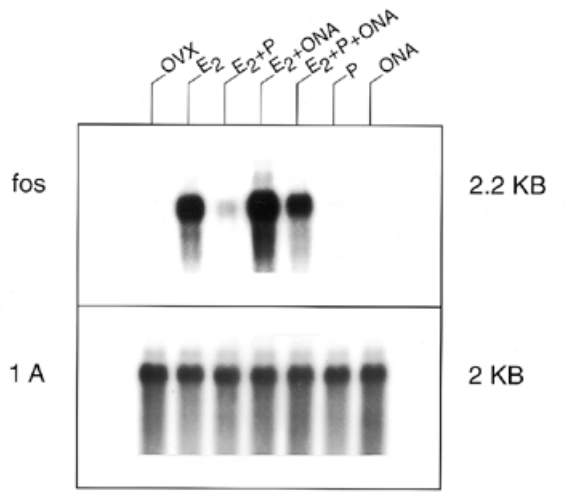

Figure 5. Effects of onapristone (ONA) and progesterone (P) on the oestrogen-induced expression of *c-fos* in the rat uterus 2 h after treatments. Adult female ovariectomized rats were treated s.c. with the vehicle (OVX), oestradiol (E_2, 3 µg), 3 µg oestradiol plus 3 mg progesterone (E_2+P), 3 µg oestradiol plus 10 mg onapristone (E_2+ONA); 3 µg oestradiol plus 10 mg onapristone plus 3 mg progesterone (E_2+P+ONA); 3 mg progesterone (P), and 10 mg onapristone (ONA). Note the inhibition of oestradiol-induced *c-fos* expression by progesterone (E_2+P) and a strong expression after a combined oestradiol plus onapristone treatment (E_2+ONA). Note also lack of *c-fos* expression after onapristone alone.

Modulation of oestrogenic effects by onapristone and mifepristone

Effects of onapristone on the oestrogen-induced expression of c-fos in the rat uterus

The aim of this ex-vivo experiment was to determine whether the antiprogestin onapristone modulates the oestrogen-dependent *c-fos* expression in the presence and absence of progesterone treatment. The design of this experiment was established in a pilot study in which a single injection of oestradiol resulted in the expected transient increase in *c-fos* peaking 2 h after injection (data not shown). Therefore, this particular time period was selected for the present experiment. Oestradiol treatment exerted a pronounced stimulatory effect on *c-fos* expression, which was substantially inhibited by progesterone and slightly enhanced by onapristone (Figure 5). Neither progesterone nor onapristone showed any stimulatory effects on *c-fos* expression when administered alone. However, the inhibitory effect of progesterone was competely blocked by onapristone.

Effects of onapristone and mifepristone on ER protein in the uterus of ovariectomized rats

This experiment was performed to determine whether onapristone and mifepristone modulate the synthesis of ER in spayed rats. The results of this experiment are presented in Figure 6. Relatively high ER levels were measured in ovariectomized animals (group 2). Oestrogen treatment reduced ER synthesis by ~50% (group 3). Surprisingly, onapristone (group 4), but not mifepristone (group 5) treatment alone (10 mg/rat/day each) increased ER protein levels markedly. The effect of mifepristone alone (group 5) was comparable to that of oestradiol (group 3). A combined oestradiol plus onapristone (groups 6–8) or mifepristone treatment (group 9) increased ER concentrations beyond oestradiol treatment level alone (group 3). The effects of onapristone were dose-dependent.

Table I. Proliferating cell nuclear antigen (PCNA) staining in the uterus of immature rats treated s.c. with oestradiol, onapristone (ONA), mifepristone (RU 486) and onapristone plus the pure anti-oestrogen ICI 182 780 (ICI)

Treatment	Vehicle	Oestradiol	ONA	ICI	Oestradiol + ICI	ONA + ICI	RU 486
Luminal epithelium	+	+++	+	–	–	–	+
Glandular epithelium	+	+++	++	–	–	–	+
Stroma	+	+++	++	–/+	+	+	+/++
Myometrium	+	+++	++	–/+	+	+	+/++

– = no staining; + = low staining; ++ = medium staining; +++ = strong staining.

Effects of various type I and type II antiprogestins on selected parameters of oestrogenic action in ovariectomized, oestradiol-substituted rats

The aim of these experiments was to determine whether antiprogestins (type I or type II) exert any antiproliferative effects in spayed rats. The results of this experiment demonstrate the anti-uterotrophic effect of type I antiprogestins (onapristone ZK 131 35, ZK 135 695) on oestrogen-induced uterine growth after s.c. administration for 3 days (Figure 7, lower panel). A similar effect was seen after oral administration of onapristone (Figure 7, upper panel). In contrast, neither type II antagonist tested had any anti-uterotrophic effects after oral (Figure 7, upper panel) or s.c. administration (data not shown). The anti-uterotrophic effects of onapristone were dose-dependent. The morphometric analysis indicates that the anti-uterotrophic effect of onapristone was not accompanied by an inhibition of luminal epithelial height. Neither onapristone nor mifepristone influenced the oestradiol-induced increase in luminal epithelial height (Figure 8, upper panel). Interestingly, the luminal epithelial perimeter was dose-dependently reduced by onapristone treatment indicating that the amount of luminal epithelial cells decreased after a combined onapristone plus oestradiol treatment (Figure 8, lower panel).

The results of the morphological evaluation (Figure 9) of onapristone and mifepristone were consistent with the morphometric analysis. There was no evidence of any inhibitory effects on the luminal and glandular epithelium after treatment with oestradiol plus antiprogestin (in either group). Conversely, there appeared to be a greater stimulation of both the luminal and glandular epithelium in animals treated with oestradiol plus an antiprogestin than in the oestrogen-treated controls. The morphological appearance of the epithelium (luminal and glandular) of rats treated with oestradiol plus onapristone was similar to that of the oestradiol-treated controls. In addition, an enlarged fluid-filled uterine lumen was observed in animals treated with mifepristone, lilopristone and ZK 112 993 in combination with oestradiol. This effect did not occur after onapristone plus oestradiol treatment (Figure 9C,E). Pronounced subnuclear vacuolization was observed after a combined treatement with mifepristone and oestradiol (Figure 9E,F). A similar, but less pronounced, effect could be seen after ZK 112 993 plus oestradiol and lilopristone plus oestradiol, but not after treatment with type I antiprogestins and oestradiol. However, endometrial stroma was higly condensed in animals treated with oestradiol plus antiprogestins (all compounds) compared wiht the oestradiol-only group. The effect of antiprogestins on stromal compaction was dose-dependent. There was also a marked decrease in myometrial growth after onapristone (and other type I antiprogestins) plus oestradiol, which suggests that the anti-uterotrophic effects of type I antiprogestins was mainly the result of their myometrial impact.

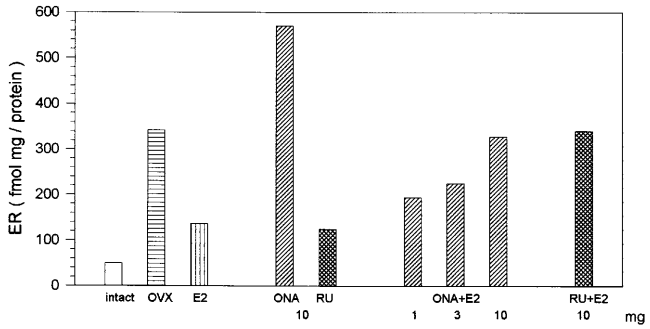

Figure 6. Effects of onapristone (ONA) and mifepristone (RU) on the total oestrogen receptor (ER) protein concentrations in the uterus of ovariectomized, oestradiol (E2)-substituted (0.3 μg/rat/day) rats. The ER protein was measured in uterine homogenates using an enzyme immunoassay kit (Abbott). The uteri of each group were pooled prior to the homogenization procedure. Note that onapristone markedly increased ER concentrations when administered alone, and dose-dependently increased ER concentrations in combination with oestradiol.

Oestrogen-like properties of antiprogestins

In the first part of the study we raised the question whether the antiprogestins onapristone and mifepristone induce any oestrogen-like activities *in vitro* and *in vivo*. In the transactivation assay carried out in MVLN cells expressing

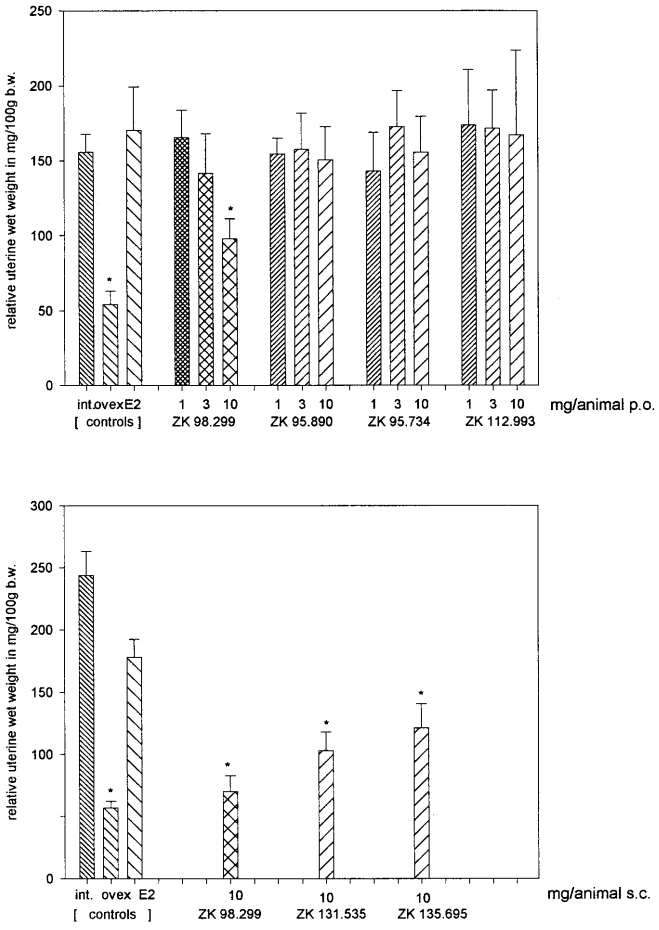

Figure 7. Anti-uterotrophic effects of the 13α-methyl substituted antiprogestins, onapristone, ZK 131 535 and ZK 135 695 in ovariectomized, oestradiol-substituted rats. Upper panel shows dose-dependent anti-uterotrophic effect of onapristone (ZK 98 299) and lack of effect of the 13β-methyl substituted antiprogestins, mifepristone, lilopristone and ZK 112 993 after oral treatment for 3 consecutive days. The lower panel shows the anti-uterotrophic effects of two additional 13α-configured antiprogestins, ZK 131 535 and ZK 135 695, compared with onapristone (ZK 98 299) after s.c. administration with 10 mg/rat for 3 consecutive days. *The uterine weight of animals treated with onapristone (both application routes), ZK 131 535 and ZK 135 695 was significantly lower ($P < 0.05$) than that of the oestradiol (E2, 0.3 μg/rat)-treated animals.

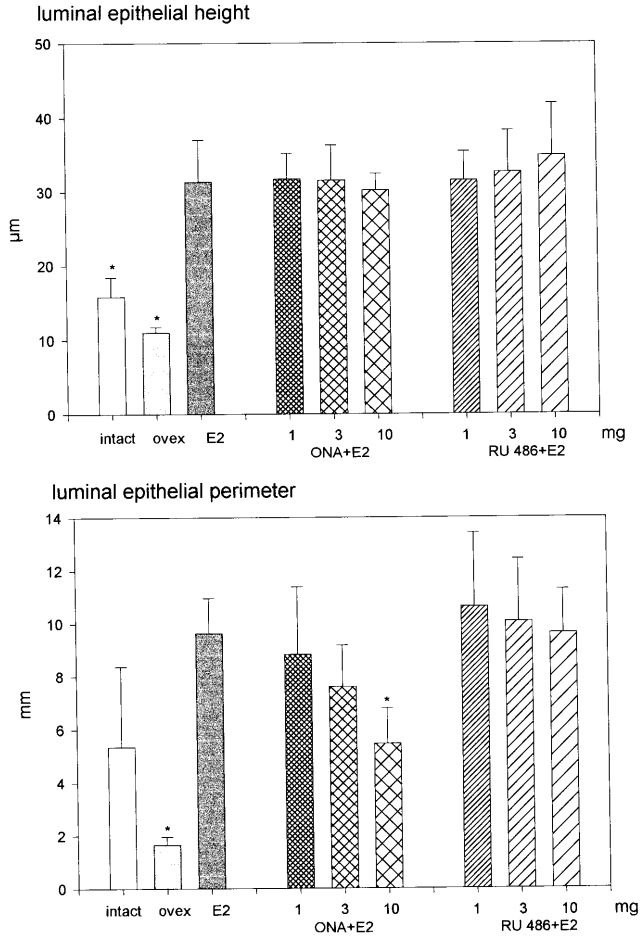

Figure 8. The effects of onapristone (ZK 98 299) and mifepristone (ZK 95 890) on luminal epithelial height (upper panel) and luminal epithelial perimeter (lower panel) in ovariectomized, oestradiol-substituted rats. Both onapristone and mifepristone were administered orally. *Note a dose-dependent decrease in luminal epithelial perimeter after onapristone treatment ($P < 0.05$).

the endogenous ER and stably transfected with Vit-TK-LUC, which is a very sensitive model for detecting ER-mediated gene expression, mifepristone showed a weak oestrogen activity (at 10^{-6} to10^{-7} M), whereas onapristone exerted virtually no activity (Figure 2) which is consistent with the results obtained in HeLa cells transiently transfected with the human oestrogen receptor and a *Vit-TK-CAT* reporter gene (Michna *et al.*, 1996).

However, in immature rats both antiprogestins possessed a weak oestrogen-like activity in terms of uterine growth stimulation (~50% of oestradiol response, Figure 3), and PCNA staining, a parameter of cell proliferation (Kelman 1997) (Table I). Surprisingly, onapristone, but not mifepristone markedly increased the luminal epithelial height, which is a very sensitive parameter of oestrogen action in rats. The stimulatory effects of onapristone on uterine wet weights, luminal epithelium hypertrophy and PCNA staining could be completely blocked by additional anti-oestrogen treatment indicating that these effects were ER-mediated. Similar stimulatory effects of onapristone were previously described in 21 day old immature rats (Bigsby *et al.*, 1994). A single injection of onapristone, but not mifepristone and lilopristone increased luminal epithelial cell height and stimulated the uterine epithelial DNA synthesis. These effects were blocked by tamoxifen.

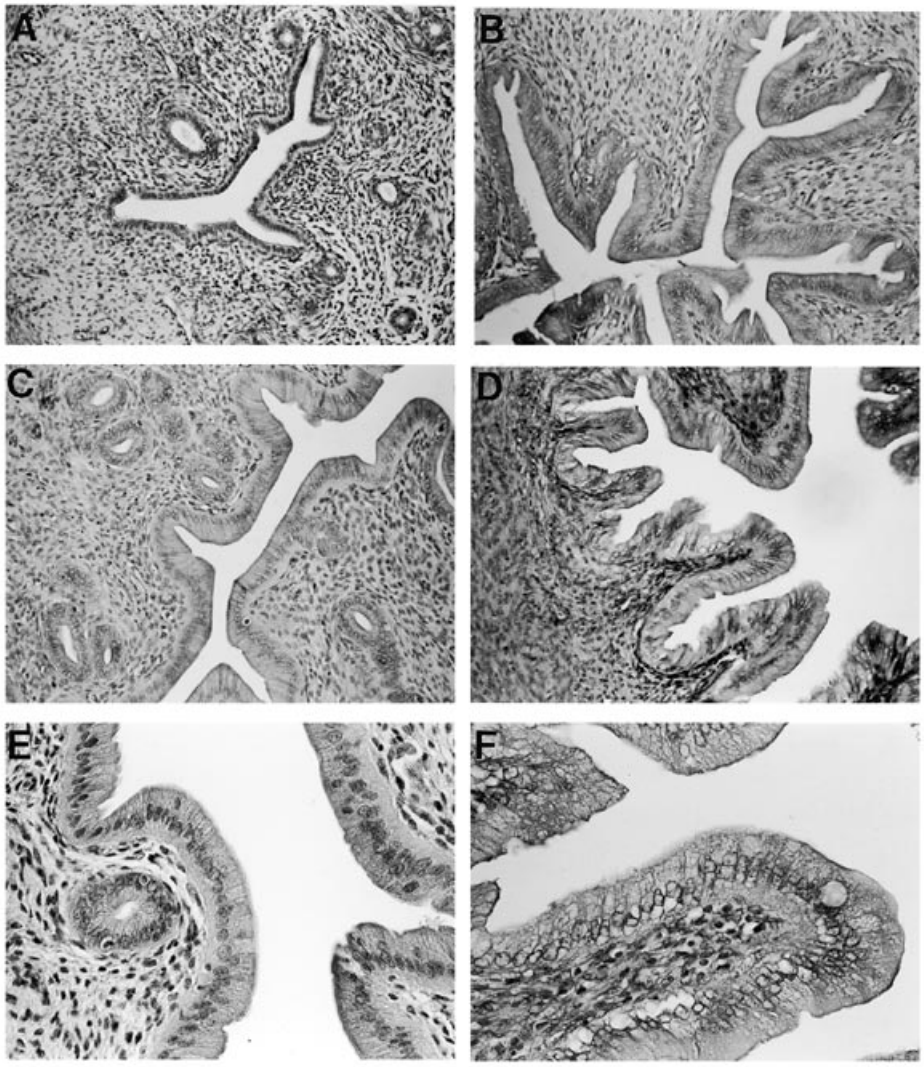

Figure 9. Photomicrographs of paraffin sections through the uterus of ovariectomized rats treated with the vehicle (**A**), oestradiol (0.3 μg/rat) alone (**B**), onapristone plus oestradiol (**C**, **E**), and mifepristone plus oestradiol (**D** and **F**), (10 mg/rat s.c. each). Oestradiol treatment led to typical stimulation of luminal and glandular epithelium growth (**B**) compared with the vehicle-treated, castrated animals (**A**). The columnar epithelium is subtended by a vascular, sparsely cellular and oedematous stroma (**B**). Note a pronounced stromal compaction and highly stimulated luminal and glandular epithelium after onapristone (**C**, **E**) and mifepristone (**D**, **F**). Note also the presence of subnuclear vacuolization and degenerative changes in the luminal epithelium after mifepristone plus oestradiol treatment (D, F). Magnification: haematoxylin–eosin (**A**, **B**, **C**, **D**) ×200 and (**E**, **F**) ×400.

Based on experiments performed in immature rats one could in fact conclude that onapristone shows some oestrogenic activity *in vivo*. However, in contrast to the oestrogenic effects observed in immature rats, there was no evidence for any oestrogen-like activity either after 3 days (data not shown) or 15 days s.c. treatment (Figure 4) in ovariectomized and adrenalectomized adult rats, i.e. in the total absence of sex steroid secretion. Neither compound significantly increased uterine or vaginal weights or induced luminal epithelial hypertrophy. There was also no evidence of any oestrogenic effect of onapristone and mifepristone in uterine morphology in this experiment. The contrasting results obtained in immature intact rats versus ovariectomized adult rats raises the question as to the relevance of studies performed in immature rats. It is likely that onapristone directly or indirectly (e.g. via prolactin or gonadotrophin increase) stimulated ovarian oestrogen secretion which in turn was responsible for enhanced uterine growth and luminal epithelial hypertrophy. The precise mechanism underlying the oestrogenic-like effects of onapristone in immature rats and its different mode of action in this model compared

with the type II antiprogestin mifepristone remains to be evaluated. In particular, further studies of the ovarian effects on type I antiprogestins in young rats are needed.

Understanding antiprogestin effects on oestrogenic responses

In the second part of the study we evaluated the modulatory effects of different antiprogestins on various oestrogenic responses in the rat uterus. The transcription of the proto-oncogenes, including *c-fos* and *c-jun*, are some of the most rapid responses yet seen upon stimulation by a number of growth factors, hormones, neurotransmitters and other factors. In the uterus, *c-fos* is considered to be an 'early response' oestrogen-dependent gene which functions in signal transduction pathways by secondarily regulating cell proliferation (Ransone and Verma, 1990; Angel and Karin, 1991). In the human endometrium, *c-fos* and *c-jun* are strongly expressed during the proliferative and early secretory phase of the menstrual cycle, but are down-regulated during pregnancy (Salmi *et al*., 1996). There was a temporal association between immunoreactive ERs and the expression of *c-fos* and *c-jun* mRNA which suggests that the activation of both proto-oncogenes is ER-mediated in the human endometrium. Our study demonstrates that onapristone treatment did not inhibit the oestrogen-dependent *c-fos* expression. On the contrary, those animals treated with oestradiol plus onapristone appeared to have an increased *c-fos* expression when compared with oestradiol alone (Figure 5). Moreover, onapristone did not exert any effect on the oestrogen-dependent uterine *c-fos* expression when administered alone in ovariectomized rats. Therefore, it does not posses any oestrogenic activity in this model. In addition, progesterone treatment clearly down-regulated *c-fos* expression in the uterus of ovariectomized rats which is consistent with results previously reported (Kirkland *et al*., 1992; Fuhrmann and Stöckemann, 1993). Hence, onapristone showed no 'anti-oestrogenic' effects on uterine expression in the 'early response', oestrogen-dependent gene *c-fos* in rats. In contrast, onapristone even increased oestrogen-dependent *c-fos* expression in the rat uterus.

We also wanted to know whether antiprogestins exhibit an antiproliferative effect in the endometrium of spayed, oestradiol-treated rats as described in non-human primates (Wolf *et al*., 1989, Slayden *et al*., 1993; Slayden and Brenner, 1994) and rabbits (Chwalisz *et al*., 1991). Surprisingly, neither antiprogestin tested inhibited the oestrogen-induced endometrial epithelial growth in ovariectomized, oestrogen-treated rats. In contrast to the effects seen in monkeys and rabbits, the uterine luminal and glandular epithelium was even more stimulated (hypertrophic) in rats treated with oestradiol plus antiprogestin than in those treated with oestradiol alone (Figure 9). In addition, accumulation of uterine fluid was observed after treatment with type II antiprogestins; the effects of mifepristone being most pronounced. These effects appear to be partly correlated with a recently described reproductive phenotype of the progesterone receptor null mutant mouse after oestrogen treatment (Lydon *et al*., 1996). Overall, these results provide further in-vivo evidence for the pivotal role of progesterone and PR in controlling uterine epithelium growth in rodents. Interestingly, an antiproliferative effect of the antiprogestins onapristone and ZK 136 799 on the ectopic endometrium was observed in rats with surgically-induced endometriosis (Stöckemann *et al.*, 1995), indicating that the mechanism of antiprogestin action may differ in the ectopic and eutopic endometrium.

The morphological appearance of epithelial cells is, however, dependent on the antiprogestin type. Type II antiprogestins, including mifepristone, ZK 112 993 and lilopristone, induced subnuclear vacuolization of endometrial epithelial cells, with the effects of mifepristone being most pronounced. In spayed, oestrogen-substituted rats, a comparable effect was exerted by progesterone and synthetic progestins (unpublished data). Type I antiprogestins (onapristone, ZK 135 695, ZK 131 535) had no such effect on the glandular and luminal epithelium. It is likely that the subnuclear vacuolization induced by mifepristone and other type II antagonists may represent their progesterone–agonistic activity in this model.

All antiprogestins exert a dose-dependent substantial stromal compaction in the presence of oestradiol in ovariectomized rats. Thus, there was no major difference between the rat and primate uterus in terms of the stromal effects. In fact, this effect was independent of the type of compound used, since it was seen in all antiprogestins tested. It is well established that the proliferation of endometrial stroma is progesterone-dependent in rats. In spayed, adrenalectomized rats oestradiol treatment alone stimulates mitosis in the luminal and glandular epithelium but not in the stroma. Additional treatment with progesterone, suppressed epithelial mitosis and instead caused a mitotic response to oestradiol in the stroma (Clark, 1971). A dramatic proliferation and differentiation of endometrial stroma in response to progesterone and other pregnancy-related factors occurs during the decidual reaction. In fact, progesterone is essential for stromal cell proliferation induced by a panel of growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor I (IGF-I) (Piva

et al., 1996). Stromal compaction also occurred in monkeys (Ishwad *et al*., 1993; Brenner and Slayden, 1994; Slayden *et al*., 1997) and in humans (Cameron *et al*., 1996) after chronic low-dose antiprogestin treatment. Therefore, stromal compaction observed in spayed rats after antiprogestin plus oestradiol treatment may be indicative of an antiprogestagenic effect. Interestingly, this effect can be observed in ovariectomized and adrenalectomized rats, i.e. in the absence of progesterone. This is further evidence for the role of the unliganded progesterone receptor (PR) in controlling various physiological functions, including cell proliferation and differentiation as proposed previously in the rabbit model (Chwalisz *et al*., 1991).

Another interesting finding of this study was the observation that type I antiprogestins (onapristone, ZK 131 535 and ZK 135 695) exhibit an anti-uterotrophic effect in ovariectomized, oestradiol-substituted rats. A dose-dependent reduction of oestrogen-induced uterine growth was mainly due to the inhibition of myometrial growth and stromal compaction, since epithelial growth was not inhibited. The anti-uterotrophic effects of type I antagonists could also be observed in ovariectomized and adrenalectomized rats, i.e. in the total absence of endogenous progesterone (data not shown). Moreover, the anti-androgenic partial activity did not promote the anti-uterotropic activity of onapristone, since this effect could not be blocked by the androgen testosterone propionate (K.Chwalisz, unpublished data). In addition, mifepristone which also possesses anti-androgenic properties did not exert inhibitory effects on oestrogen-induced uterine growth (Figure 8, upper panel). The anti-uterotrophic effect of type I antiprogestins is another example of the different activity *in vivo* compared with type II antiprogestins. The exact mechanism of this effect remains to be established.

We also measured total ER concentrations in the uterine rat homogenates treated with either onapristone or mifepristone with and without oestradiol (Figure 6). Oestradiol treatment reduced ER concentrations below the levels of ovariectomized controls. Unexpectedly onapristone, but not mifepristone, substantially elevated uterine ER concentrations in those animals not treated with oestradiol, the exact mechanism of this interesting observation being unclear at present. However, in the presence of oestradiol both antagonists dose-dependently increased ER concentrations in ovariectomized, oestradiol-substituted rats beyond the level of the oestradiol-treated controls. Interestingly, the up-regulation of ER concentrations by the antiprogestins was observed in the absence of ovarian progesterone secretion. A similar up-regulation of ER by onapristone was also seen in ovariectomized and adrenalectomized rats, again in the total absence of progesterone (data not shown). We previously described a similar effect in ovariectomized rabbits and suggested that the unliganded PR must exert an inhibitory effect on ER expression in the rabbit uterus (Chwalisz *et al*., 1991). This inhibitory effect is abolished after binding with an antiprogestin resulting in an increase in ER synthesis. In this case we discovered a similar effect in the rat uterus. The up-regulation of ER concentrations by both mifepristone and onapristone was also observed in the endometrium of ovariectomized, oestrogen-treated monkeys (Neulen *et al*., 1990; Hodgen *et al*., 1994). Thus, the inhibition of ER expression, and perhaps some other ER-dependent genes, by unliganded PR may be a general phenomenon occurring in the uterus and possibly in other oestrogen- and progesterone-dependent tissues. Such a mechanism should be taken into consideration when describing the paradoxical effects of antiprogestins.

Conclusions

Overall, the results of this study showed that the type I antiprogestin onapristone differs from the type II antagonist mifepristone in a number of end-points. Firstly, onapristone, but not mifepristone exerted glandular epithelial hypertrophy in immature rats, perhaps due to ovarian stimulation. Secondly, onapristone and some other type I antiprogestins showed an anti-uterotrophic effect in adult, spayed rats. Thirdly, onapristone, but not mifepristone, increased uterine ER synthesis in the absence of oestradiol. It is likely that these differences are due to the different molecular action sof these compounds, although different pharmocokinetic properties may also play a role. The 13α-methyl-substituted (type I) antiprogestins, e.g. onapristone, ZK 135 695 and ZK 131 535 are believed to differ in their molecular action mechanism from the 13β-methyl-substituted (type II) antiprogestins, e.g. mifepristone, lilopristone and ZK 112 993. The type II antagonists promote PR binding to the DNA. On the other hand, the 13α-configured compounds have been shown to impair the binding of PR-complexes to the progesterone responsive element in the promoter of a PR-regulated gene, as evidenced by gel-retardation assays (Klein-Hitpass *et al*., 1991; Horwitz, 1992; Gronemeyer *et al*., 1992; U.Fuhrmann, unpublished data). This effect was recently corroborated by our laboratories in COS-1 cells being transiently transfected with human PR_B (Chwalisz *et al*., 1995). However, recent in-vivo DNA footprinting studies carried out in T47D cells show that under these conditions both types of antiprogestins prevent hPR binding to DNA, questioning to some extent the initial classification of

antiprogestins based on gel-retardation assay (Truss *et al*., 1994). Nevertheless, there are clear differences between type I and type II antiprogestins in terms of receptor phosphorylation and in their response to the protein kinase A signalling pathway, such as cAMP. The type II antagonist mifepristone (but not type I onapristone), stimulates PR phosphorylation to a level comparable with progesterone, and can function as a PR agonist in the presence of cAMP (reviewed by Fuhrmann *et al*., 1998). Therefore, it was suggested that type II antiprogestins, depending on the tissue, may act as mixed agonist/antagonists *in vivo*. In fact, ZK 112 993 displays weak progestagenic effects in the uterus of ovariectomized rabbits (unpublished data). Similarly, agonistic properties of mifepristone were described in the primate endometrium (Koering *et al*., 1986). On the other hand the type I antiprogestins may not express any agonistic activities and may be regarded as 'pure' progesterone receptor antagonists (Horwitz, 1992).

The most important finding of this study is the observation that neither antiprogestin tested induced any antiproliferative effects in the uterine epithelium in ovariectomized, oestradiol-treated rats. Conversely, there was a greater stimulation of the endometrial glandular and luminal epithelium in animals treated with oestradiol plus an antiprogestin than in those treated with oestradiol alone. This stimulatory effect, which was dose-dependent and occurred in all antiprogestins tested, may be the result of an enhanced oestrogen effect on the rat endometrium. The underlying mechanism may involve the unliganded PR, which 'brakes' or opposes the action of oestradiol even in the absence of progesterone, an effect that is blocked when antiprogestins occupy PR. This indicates that the antiprogestin effects on the non-pregnant uterus are species-dependent, since in macaques antiprogestins clearly inhibit oestrogen-dependent endometrial growth, mainly affecting the glandular and luminal epithelium (Wolf *et al*., 1989; Slayden *et al*., 1997).

Accordingly, the question arises as to why antiprogestin effects on the endometrium differ so profoundly in non-human primates versus rats. It is well known that there is a fundamental difference in the endometrial physiology, particularly with regard to progesterone action, between rodents and primates. In rodents, the ovarian cycle is very short (4 days) and there is no endometrial sloughing (menstruation) after progesterone withdrawal. Menstrual bleeding, a phenomenon which exclusively occurs in primates, involves progesterone withdrawal-induced vaso-constriction of the spiral arteries. Interestingly, in rhesus monkeys the profound antiproliferative endometrial effect of 5 months of continuous treatment with the antiprogestin ZK 137 316 was accompanied by endometrial arterial atrophy. It therefore appears likely that in monkeys inhibition of endometrial growth by antiprogestins is the result of a reduced blood supply to the endometrium either due to the atrophy of spiral arteries and/or the inhibition of endometrial angiogenesis, e.g. via suppression of endometrial vascular endothelial growth factor (VEGF) production (Slayden *et al*., 1997; Greb *et al*., 1997). However, these effects might not occur in rats.

It has been suggested that antiprogestins can be employed to treat sex-steroid hormone-dependent disorders, including endometriosis and uterine fibroids, as well as for contraception and hormone replacement therapy. In each such therapeutic application, chronic antiprogestin use is anticipated. The antiproliferative effects of antiprogestins, as described in primates, may effectively counter the possibility of 'unopposed' oestrogen action in the primate endometrium and reduce the risk of endometrial stimulation during chronic antiprogestin administration. Unfortunately, the rat model, which is commonly used to study oestrogenic effects in the uterus, is not suitable for the study of these relationships. Investigating the mechanisms underlying the endometrial antiproliferative effects of antiprogestins, particularly their molecular aspects, will require further experimental and clinical research in both non-human primates and women.

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