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Further investigation of the antifertility effects in the rat of the antiprogestational steroid, RMI 12,936, and related compounds

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Summary. The antifertility activities of RMI 12,936 and 7α-methyltestosterone may be reversed by progesterone implants in ovariectomized, but not in intact, rats on Day 8 of pregnancy. The presence of the ovary for 48 h after administration of RMI 12,936 is necessary for irreversible antifertility activity to be developed, and it is concluded that this activity is due to a metabolite other than 7α-methyltestosterone. 7α-Methylandrostenedione was significantly less potent than RMI 12,936 in antifertility tests, while the uterotrophic activity of RMI 12,936 was not inhibited by aromatization inhibitors, suggesting that the active metabolite is not produced by dehydrogenation or aromatization of 7α-methyltestosterone.

Introduction

Investigations of the antifertility effects of 17β-hydroxy-7α-methylandrost-5-en-3-one (RMI 12,936) in the pregnant rat have shown that implantation may be prevented by acceleration of egg transport, fetal resorption may be induced or the animals may abort depending upon the time of administration (Kendle, 1975). Administration on Day 8 of pregnancy causes fetal resorption, induces ovarian hypertrophy and a reduction in peripheral progesterone level. The hypertrophy also occurs in non-pregnant rats with functional corpora lutea, such as pseudopregnant or gonadotrophin-treated animals, and similar results following hysterectomy led to the conclusion that the effect was probably a direct one on the ovary and was not due to any factor of uterine origin (Kendle, 1976). Further investigations gave rise to the hypothesis that the compound inhibits progesterone synthesis in the ovary by acting as a competitive substrate for Δ^5 3-ketosteroid isomerase. Enzymic isomerization of RMI 12,936 results in the production of 7α-methyltestosterone by ovarian homogenate *in vitro* (Kendle, 1976; Hardy, Kendle, Lawrie & Omand, 1977), and 7α-methyltestosterone may also be identified in ovarian homogenates after administration of RMI 12,936 *in vivo* (R. B. Taylor & K. E. Kendle, unpublished observations). The antifertility effects of 7α-methyltestosterone were not associated with significant reduction in plasma progesterone, suggesting that this compound, or some other metabolite, may inhibit progesterone utilization (Hardy *et al.*, 1977).

The present experiments were therefore undertaken to determine whether (1) the effects of RMI 12,936 could be attributed solely to inhibition of progesterone synthesis, (2) 7α-methyltestosterone might act by a different mechanism, and (3) any evidence of further metabolism of 7α-methyltestosterone could be obtained.

Materials and General Methods

The general methods were as described previously (Kendle, 1975, 1976). Rats of the Sprague–Dawley strain were used throughout. Steroids were suspended in an aqueous vehicle containing 0·25% sodium carboxymethylcellulose (w/v) and 1% Tween 80 (w/v) and administered subcutaneously in a dose volume of 0·5 ml except when otherwise stated. Progesterone implants weighing approximately 25 mg were formed by cold compression of pure progesterone. 7α-Methyltestosterone was prepared from

RMI 12,936 as described previously (Kendle, 1976) and 7α-methylandrostenedione was prepared from 7α-methyltestosterone by oxidation with sodium dichromate/acetic acid (Belgian patent 610,385; May 16, 1962). The melting point, ultraviolet and nuclear magnetic resonance spectra of the product were used to confirm its identity as 7α-methylandrostenedione.

The aromatization inhibitors androst-4-ene-3,6,17-trione and androsta-1,4,6-triene-3,17-dione were purchased (Steraloids, U.K. Ltd, Radcliffe Road, Croydon).

All surgical procedures were performed under light ether anaesthesia. Preliminary studies showed that subcutaneous implantation of progesterone on a single occasion at the time of ovariectomy was sufficient to maintain pregnancy provided that 2 implants and some form of oestrogen were given. The slight oestrogenicity of RMI 12,936 or 7α-methyltestosterone was sufficient to provide the oestrogen in animals receiving these substances, but it was necessary to inject control animals with an equivalent amount (0·2μg) of ethinyloestradiol (Sigma London Chemical Co. Ltd, Kingston-upon-Thames, Surrey).

Detailed Methods and Results

Maintenance of pregnancy by progesterone implants

Six groups, each of 10 pregnant rats, were given the treatments shown in Table 1 on Day 8 of pregnancy and killed on Day 15. The results show that progesterone implants, capable of maintaining pregnancy in the ovariectomized, oestrogen-treated rats, also maintained pregnancy in those treated with RMI 12,936 or 7α-methyltestosterone and ovariectomized. Neither of the last two treatments was able to maintain pregnancy if the ovaries were still present.

In a second experiment, groups of rats were given 2 mg RMI 12,936 subcutaneously on Day 8 of pregnancy. Ovariectomy and progesterone implantation was then carried out at various times as shown in Table 2. The results show that pregnancy was maintained when ovariectomy was carried out up to 24 h after dosing, that significantly fewer animals were pregnant when ovariectomy was carried out

Table 1. Maintenance of pregnancy in rats (10/group) given progesterone implants (2× 25 mg, s.c.) on Day 8 and treated in various ways

Treatment on Day 8	Ovariectomy	No. pregnant on Day 15
Vehicle only	No	10
Ethinyloestradiol, 0·2 μg	Yes	7
RMI 12,936, 2 mg	No	0
RMI 12,936, 2 mg	Yes	9
7α-Methyltestosterone, 2 mg	No	0
7α-Methyltestosterone, 2 mg	Yes	10

Table 2. Effect on maintenance of pregnancy in rats of ovariectomy and progesterone (2 × 25 mg implants, s.c.) at various times after administration of RMI 12,936 (2 mg/rat) on Day 8 of pregnancy

Time of treatment after RMI 12,936 (h)	No. pregnant/ no. tested	Difference from first treatment period*
1–3	10/10	—
6–12	9/10	N.S.
24	8/10	N.S.
48	5/16	$P = 0{\cdot}001$
72	0/7	$P = 0{\cdot}0001$
96	0/6	$P = 0{\cdot}0002$

* Fisher Exact Test; N.S. = not significant.

48 h after dosing, and that pregnancy was not maintained when longer periods elapsed between dosing and ovariectomy.

In a further experiment, animals were given 2 mg RMI 12,936 on Day 9. When ovariectomy and progesterone implantation was carried out 24 or 48 h later, 12 of 16 rats and 1 of 7 rats respectively were pregnant at autopsy on Day 15.

Antifertility activity of 7α-methylandrostenedione

Various doses of RMI 12,936 or 7α-methylandrostenedione were given to groups of 10 rats on Day 8 of pregnancy as shown in Table 3. There were no significant deviations from linearity or parallelism and the potency ratio was 0·24 with fiducial limits ($P = 0·05$) of 0·21 to 0·60.

Uterotrophic activity of RMI 12,936 given with aromatization inhibitors

Investigation of uterotrophic activity was carried out with groups of 5 immature rats as described previously (Kendle, 1976). The treatments are shown in Table 4 and the results indicate that neither inhibitor significantly reduced the uterotrophic response to RMI 12,936.

Table 3. Antifertility activities in rats (10/group) of various doses of RMI 12,936 and 7α- methylandrostenedione after subcutaneous administration on Day 8 of pregnancy

Compound	Dose (mg/rat)	No. not pregnant	Median effective dose (mg)*
RMI 12,936	0·25	0	
	0·5	4	0·33
	1·0	10	
7α-Methylandrostenedione	0·5	0	
	1·0	4	
	2·0	6	1·41
	4·0	10	

* Calculated after logit transformation of the quantal response.

Table 4. Uterotrophic effects of RMI 12,936 in the presence of aromatization inhibitors in immature rats (5/group)

Experiment	Treatment (dose/day)†	Mean (± s.e.m.) uterine wt (mg)‡
1	Vehicle only	32·1 ± 3·8
	RMI 12,936 (0·2 mg)	117·3 ± 3·9*
	RMI 12,936 (0·2 mg) + androst-4-ene-3,6,17-trione (2 mg)	108·3 ± 4·6*
2	Vehicle only	36·2 ± 3·0
	RMI 12,936 (0·2 mg)	123·5 ± 4·2**
	RMI 12,936 (0·2 mg) + androsta-1,4,6-triene-3,17-dione (5 mg)	114·0 ± 5·7**

* Not significantly different, $P > 0·05$, Student's t test.
** Not significantly different, $P > 0·05$, Student's t test.
† For 3 days.
‡ 24 h after the last dose.

Discussion

The hypothesis that the sole antifertility effect of RMI 12,936 is due to inhibition of progesterone synthesis has been investigated and the results show that pregnancy is maintained by progesterone implants in rats given RMI 12,936 only if the ovaries are removed. It may therefore be concluded that the antifertility activity of the compound itself is due to inhibition of progesterone synthesis because it is ineffective with exogenous progesterone. If the compound was capable of inhibiting progesterone utilization it should be effective against exogenous and endogenous progesterone, as is the case with the competitive antagonist 13-ethyl-17α-ethynyl-17-hydroxy-gona-4,9,11,trien-3-one (R2323) (Sakiz, Azadian-Boulanger & Raynaud, 1974). Because administration of RMI 12,936 prevents maintenance of pregnancy by exogenous progesterone in animals with intact ovaries, it is presumably metabolized in the ovary to one or more substances which have different mechanisms of antifertility activity, such as inhibition of progesterone utilization.

It is possible that RMI 12,936, or a metabolite produced elsewhere, e.g. liver or adrenal gland, acts on the ovary to induce synthesis or release of a factor such as prostaglandin or relaxin which causes fetal resorption. However the present work was undertaken as an investigation of ovarian metabolism because studies with ovarian homogenate indicated that RMI 12,936 is metabolized in the ovary to 7α-methyltestosterone. If this metabolite was a competitive antagonist of progesterone it should be capable of inhibiting maintenance of pregnancy by exogenous progesterone. The present results, however, show that this is not the case and that, like RMI 12,936, 7α-methyltestosterone will prevent maintenance of pregnancy by progesterone in intact but not in ovariectomized rats. It must therefore be concluded that 7α-methyltestosterone undergoes further metabolic change in the ovary and that the unchanged compound either has no antifertility activity or, like RMI 12,936, acts by inhibiting progesterone synthesis. Previous work (Kendle, 1976) has shown that 7α-methyltestosterone, unlike RMI 12,936, does not cause significant reduction of systemic progesterone levels 24 h after administration. It is, however, possible that significant inhibition does occur but does so at some different time after administration.

If the termination of pregnancy in intact, progesterone-treated animals is due to the effects of some ovarian metabolite of RMI 12,936, there should be a period during which the ovaries are necessary before 'progesterone resistant' antifertility is developed. After administration of RMI 12,936 on Day 8, significantly fewer pregnancies could be maintained by exogenous progesterone if ovariectomy was carried out after an interval of 48 h or more. This finding indicates either that effective concentrations of the active metabolite are produced only after 48 h or that the metabolite is produced earlier and the physiological events occurring on Day 10 of pregnancy make this the time of greatest sensitivity. The results of the experiment in which RMI 12,936 was given on Day 9 with ovariectomy 24 or 48 h later show that the presence of the ovary for 48 h after dosing is necessary rather than there being any special sensitivity associated with Day 10.

These experiments also show that pregnancy is maintained when ovariectomy and progesterone administration are delayed until Day 9 following administration of RMI 12,936 on Day 8; exogenous progesterone is therefore being given at a time when previous work (Kendle, 1976) has shown endogenous progesterone to be less than 50% of control levels, and is too late to prevent this fall. The observed inhibition of progesterone synthesis cannot be sufficient to produce antifertility effects and may be unrelated to the antifertility effect observed when RMI 12,936 is given to the intact pregnant animal.

If 7α-methyltestosterone is metabolized along pathways similar to those of testosterone it may be anticipated that it would be dehydrogenated to 7α-methylandrostenedione, aromatized to 7α-methyloestradiol or be subject to both reactions. Since the metabolic production of 7α-methylandrostenedione and any further metabolites of it is limited by the amount of RMI 12,936 given, the antifertility of RMI 12,936 can only be due to production of 7α-methylandrostenedione if the latter is as or more potent than RMI 12,936. However, this is not so because 7α-methylandrostenedione is significantly less potent ($P = 0{\cdot}05$) than RMI 12,936. It may be concluded, therefore, that the active substance is not produced by this metabolic pathway, provided that both substances have similar pharmacokinetic characteristics, such as absorption from subcutaneous aqueous suspension. Analysis

of the particle size distribution of the two suspensions used showed that they were very similar but no data are available on other physicochemical parameters which would validate the assumption of pharmacokinetic similarity. The logarithmic partition coefficients of testosterone and androstenedione are 0·32 and 0·97 respectively in a *N*-heptane/water system (Leo, Hansch & Elkins, 1971) and these steroids may be separated by a high-performance liquid-chromatography system which is unable to resolve 7α-methyltestosterone and 7α-methylandrostenedione. A smaller difference in partition coefficients of the methylated compounds may be anticipated therefore, but investigation of relative blood levels of these substances following subcutaneous administration is necessary when suitable techniques have been developed.

The enzyme system responsible for the aromatization of androstenedione to oestrone may be competitively inhibited by androst-4-ene-3,6,17-trione and androsta-1,17-dione (Schwarzel, Kruggel & Brodie, 1973). These inhibitors are effective *in vivo* in an inhibitor:substrate ratio of 6:1 (Christensen & Clemens, 1975). If the antifertility or slight oestrogenic activity of RMI 12,936 or 7α-methyltestosterone was due to the metabolic production of 7α-methyloestrone and 7α-methyloestradiol, these activities should have been prevented by simultaneous administration of the inhibitors. Only the uterotrophic response was investigated because limited supplies of the inhibitors were available, but the failure of the inhibitors to prevent the response suggests that it is not due to metabolic aromatization. The only alternative explanation would be that 7α-methyl substrates have a higher affinity for the aromatization enzymes than the natural substrates or the inhibitors. This possibility requires further investigation in a system similar to that used by Schwarzel *et al.* (1973).

It is shown that the antifertility activity of RMI 12,936 is independent of its activity in inhibiting progesterone synthesis but may be due to the production of a metabolite by the ovary some 48 h after administration. Evidence is obtained that the active metabolite is probably not produced by the metabolic dehydrogenation or aromatization of 7α-methyltestosterone. Further work is needed to elucidate the identity of the metabolite and to investigate the possibility that a non-ovarian metabolite acts via the ovary as a target organ.

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