OVULATION INDUCTION AND ANTIFERTILITY EFFECTS OF AN LH-RH ANALOGUE (AY-25,205) IN CYCLIC RATS

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Summary. Pro-oestrous rats, treated with fluphenazine dihydrochloride to block ovulation, were used to compare the ovulation-inducing activity of synthetic LH-RH with one of its analogues, [D-Ala⁶, des-Gly-NH₂¹⁰]-LH-RH ethylamide (AY-25,205). The lowest dose of LH-RH which produced a significant response was 125 ng compared with 2.9 ng AY-25,205. Statistical analysis of the data showed that AY-25,205 was approximately thirty-six times more potent than LH-RH. Treatment with AY-25,205 also induced partial ovulation in metoestrous rats (80 or 160 ng/rat) and apparently normal ovulation in dioestrous rats (10 to 160 ng/rat). The compound (160 ng) failed to induce further ovulation in oestrous rats which ovulated during the previous night. Advancement of premature ovulation in dioestrous rats with AY-25,205 prevented mating behaviour and pregnancy during the treatment cycle. The anti-fertility effect of the compound disappeared during the following cycle.

INTRODUCTION

Since the isolation, purification, characterization and synthesis of porcine LH- and FSH-releasing hormone (Matsuo, Arimura, Nair & Schally, 1971; Schally, Kastin & Arimura, 1971), many investigators (see White, Hedlund, Rippel, Arnold & Flouret, 1973) have successfully synthesized this decapeptide. Potent analogues of this decapeptide have also been synthesized and their potencies compared to that of synthetic LH-RH with respect to hypophysial LH- and FSH-release (Coy & co-authors, 1974; Kastin & co-authors, 1974; Arimura, Vilchez-Martinez & Schally, 1974a; Arimura, Vilchez-Martinez, Coy, Coy, Hirotsu & Schally, 1974b) and ovulation-inducing activity in rats (Fujino & co-authors, 1974a, b). A synthetic LH-RH and a few of its analogues have also been synthesized in our laboratories (Immer, Nelson, Revesz, Sestanjan & Götz, 1974).

The purpose of this communication is to report the comparative capacity of synthetic LH-RH and one of its analogues (AY-25,205) to induce ovulation in pro-oestrous rats treated with fluphenazine dihydrochloride (FD). In this report, the results of induction of premature ovulation in metoestrous and dioestrous rats and the antifertility effects of AY-25,205 in female rats are also described.

MATERIALS AND METHODS

Experiments were conducted on adult (225 ± 25 g) 4-day cyclic Sprague-Dawley rats obtained from the Canadian Breeding Laboratories, St Constant, Quebec. The rats were maintained in an air-conditioned room (24 to 25°C; 45 to 50% humidity) and exposed to equal periods of light and darkness. Standard Purina Chow and water were freely available. At least six or more animals were used in each group.

For blocking ovulation, FD was administered subcutaneously (500 μ g/0.2 ml of 0.9% saline) between 14.15 and 14.30 hours on the day of pro-oestrus. For inducing ovulation in FD-treated rats, different single doses of the following two synthetic decapeptides were injected in 0.2 ml of 0.9% saline intravenously between 15.00 and 15.30 hours on the same day: (1) synthetic LH-RH: H-Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂, (2) LH-RH analogue (AY-25,205): H-Pyr-His-Trp-Ser-Tyr-[D-Ala]-Leu-Arg-Pro-NH-CH₂-CH₃. Control rats received the vehicle only.

For experiments on the induction of ovulation in untreated rats, 160 ng AY-25,205/rat was injected intramuscularly between 15.00 and 15.30 hours on the appropriate day of the cycle so that all stages were represented. Similarly, different doses of AY-25,205 were administered intramuscularly to metoestrous and dioestrous rats in order to establish the minimum effective dose (MED) for premature induction of ovulation. Evidence for ovulation was obtained by counting microscopically freshly shed ova from the oviduct the morning after administration of the decapeptides, according to the method described by Banik & Herr (1969).

For exploring the antifertility and post-antifertility effects, different doses of AY-25,205 were administered intramuscularly between 15.00 and 15.30 hours to dioestrous rats. Each treated and control rat was allowed to cohabit with a fertile male for 3 consecutive days and nights. Females were inspected on the morning after pairing for a mucus plug or spermatozoa in the vaginal smear, and the number of uterine implantations were counted 9 days after coitus or the last day of cohabitation.

Statistical analysis of the number of rats which ovulated, mated and became pregnant was made using the fourfold contingency test tables of Mainland & Murray (1952); the average number of ova or implantations in each group was compared with those of the appropriate control and other groups by using Student's *t* test.

RESULTS

Induction of ovulation in pro-oestrous rats treated with FD

It is evident from the results in Table 1 that FD blocked ovulation in 19/20 rats, and increasing doses of LH-RH and AY-25,205 induced ovulation in rats treated with FD. Treatment with LH-RH (125 ng or more) induced ovulation in a significant number of rats, when compared to control rats injected with FD and saline, whereas only 2.9 ng or more of AY-25,205 was needed for a comparable effect (Table 1). The relative potency of LH-RH and AY-25,205 was determined by probit analysis (Finney, 1947) and the latter compound was

Table 1. Induction of ovulation with a synthetic LH-RH or AY-25,205 in pro-oestrous rats treated with fluphenazine dihydrochloride

<i>Treatment</i>	<i>No. of rats ovulating</i>	<i>No. of ova (Mean $\pm$ S.E.)*</i>
LH-RH (ng/rat)		
Saline (control)	10/10**	11.5 $\pm$ 0.5
FD+saline (control)		
16.22	1/10	9.0
31.25	0/10	0
62.5	1/10	15.0
125.0	2/10	9.0
187.5	8/10**	7.5 $\pm$ 1.1
250.0	9/10**	8.2 $\pm$ 0.8
	10/10**	10.1 $\pm$ 1.2
AY-25,205 (ng/rat)		
Saline (control)	10/10**	11.5 $\pm$ 0.5
FD+saline (control)		
0.97	0/10	0
1.95	1/10	10.0
2.9	2/10	3.5
3.9	7/10**	11.0 $\pm$ 0.7
7.7	9/10**	11.4 $\pm$ 1.0
	10/10**	10.6 $\pm$ 1.2

FD, fluphenazine dihydrochloride.

* Calculated from the rats which ovulated. Statistical comparisons were made between different groups when feasible.

** $P < 0.01$ versus FD+saline control group.

approximately 36.33 ± 6.8 (S.E.) times more potent than the former; moreover, when the log-dose of each compound was plotted against the proportion of rats ovulating, two parallel lines were obtained (Text-fig. 1).

TEXT-FIG. 1. The relative potency of AY-25,205 compared to that of LH-RH in pro-oestrous rats treated with fluphenazine dihydrochloride.

Induction of ovulation with AY-25,205 administered on different days of the cycle

A single dose of AY-25,205 was unable to induce further ovulation in oestrous rats which ovulated during the previous night, but this compound was effective in inducing premature ovulation in metoestrous and dioestrous rats (Table 2).

Table 2. Attempted induction of ovulation with AY-25,205 (160 ng/rat) in oestrous, metoestrous, dioestrous and pro-oestrous rats

Stage of cycle	Treatment	No. of rats ovulating†	No. of ova (Mean $\pm$ S.E.)‡
Oestrus	Saline (control)	0/6	0
	AY-25,205	0/7	0
Metoestrus	Saline (control)	0/6	0
	AY-25,205	7/10**	2.3 $\pm$ 0.5
Dioestrus	Saline (control)	0/12	0
	AY-25,205	10/10**	10.1 $\pm$ 1.0**
Pro-oestrus	Saline (control)	6/6	11.5 $\pm$ 1.5**
	AY-25,205	8/8	13.4 $\pm$ 0.5**

** $P < 0.01$.

† Versus saline control of respective group.

‡ Versus AY-25,205 metoestrous group.

The mean number of ova in the rats treated during metoestrus was lower than that in the dioestrous and pro-oestrous rats ($P < 0.01$). All pro-oestrous animals treated with saline or with AY-25,205 ovulated.

Induction of premature ovulation in metoestrous and dioestrous rats with an increasing dose of AY-25,205

Premature ovulation was induced by a single dose (80 ng/rat) of AY-25,205 in 6/10 metoestrous rats, though the mean number of ova detected was lower than in pro-oestrous rats treated with saline (Table 3). In dioestrous rats, a dose of ≥ 10 ng AY-25,205 induced ovulation in all but one rat. None of the dioestrous control rats or those receiving the lowest dose of AY-25,205 ovulated.

Table 3. The induction of premature ovulation in metoestrous and dioestrous rats with a single increasing dose of AY-25,205

Treatment	Metoestrous rats		Dioestrous rats	
	No. ovulating	No. of ova (Mean $\pm$ S.E.)*	No. ovulating	No. of ova (Mean $\pm$ S.E.)*
Saline (control)	0/6	0	0/10	0
AY-25,205 (ng/rat)				
2.5	0/10	0	0/10	0
10	0/10	0	10/10**	7.3 $\pm$ 0.8
20	0/10	0	9/10**	10.3 $\pm$ 0.8
40	0/10	0	10/10**	11.0 $\pm$ 1.4
80	6/10**	4.7 $\pm$ 1.0	10/10**	9.0 $\pm$ 1.2

* See Table 1 for statistical treatments.

** $P < 0.01$ versus saline control.

Antifertility and post-antifertility effects of AY-25,205 in female rats

The results presented in Table 4 show that ≥ 10 ng AY-25,205 administered on the day of dioestrus interfered with the mating behaviour during the ensuing pro-oestrus and oestrus. No such significant antifertility effect was seen in the control rats or in those treated with 2.5 ng AY-25,205. Except in these two groups, there was a change in the vaginal smear cytology during the treatment cycle; the vaginal smears from rats receiving the higher doses of AY-25,205 did not contain the expected predominance of squamous cells on the morning of oestrus. During the ensuing cycle, however, most of the rats treated with AY-25,205 and all of the remaining controls mated with fertile males during the night of pro-oestrus and became pregnant with a normal number of implantations (Table 4). The pro-oestrous type of vaginal cytology was found 1 day earlier than usual.

Table 4. Antifertility and post-antifertility effects of AY-25,205 in female rats

Treatment	Antifertility effect			Post-antifertility effect		
	No. of rats mated	No. of rats pregnant	Implantations (Mean $\pm$ S.E.)†	No. of rats mated	No. of rats pregnant	Implantations (Mean $\pm$ S.E.)‡
Saline (control)	8/10	8/8	11.1 $\pm$ 0.4	2/2	2/2	10.5 $\pm$ 0.1
AY-25,205 (ng/rat)						
2.5	7/10	5/7	12.0 $\pm$ 0.1	2/3	2/2	13.0
10	1/10**	1/1	4.0	9/9	9/9	10.6 $\pm$ 0.4
20	4/10*	2/4	3.5	6/6	6/6	10.8 $\pm$ 0.6
40	0/10**	0/0	0	10/10	10/10	10.2 $\pm$ 0.4
80	0/10**	0/0	0	10/10	10/10	10.2 $\pm$ 1.0

* $P < 0.05$ versus saline control group; ** $P < 0.01$ versus saline control group.

† Calculated from the rats which mated. Statistical comparisons were made between different groups when feasible.

‡ Remaining rats which mated with fertile males within two subsequent cycles.

DISCUSSION

These experiments demonstrated that LH-RH and an analogue, AY-25,205, were effective in inducing ovulation in pro-oestrous rats treated with FD. Under these conditions, AY-25,205 was approximately thirty-six times more potent than LH-RH in inducing ovulation. In a recent study (U. K. Banik, M. A. Hirsch and M. L. Givner, unpublished data), we were unable to induce ovulation in metoestrous and dioestrous rats with a single intravenous dose of 500 ng LH-RH. It appears, therefore, that AY-25,205 is much more potent than LH-RH in inducing ovulation in metoestrous and dioestrous rats. Fujino *et al.* (1974a) also observed that a synthetic preparation of an LH-RH analogue with an identical chemical structure to AY-25,205 was about fifty to eighty times more potent in inducing ovulation in dioestrous rats. In the present study, the MED₁₀₀ of AY-25,205 administered intramuscularly was found to be approximately 10 ng/dioestrous rat. Fujino *et al.* (1974b) calculated the MED₅₀ of the same LH-RH analogue to be between 2.0 and 5.1 ng/100 g body weight.

Recently, Coy *et al.* (1974) and Arimura *et al.* (1974b) have indicated that a synthetic compound, [D-Ala⁶, des-Gly-NH₂¹⁰]-LH-RH ethylamide, chemically

identical to AY-25,205, was many times more active than synthetic LH-RH in releasing hypophysial LH and FSH in ovariectomized rats treated with oestrogen and progesterone and also in immature male rats. The greater potency of AY-25,205 compared to LH-RH in inducing ovulation in pro-oestrous rats treated with FD and in normal dioestrous rats was, therefore, to be expected.

In laboratory animals (see Labhsetwar, 1973; Schwartz, 1974) as well as in women (Taymor, Berger, Thomson & Karam, 1972), an interplay between FSH and LH is essential for ovulation. The advancement of ovulation with the appropriate dose of AY-25,205 in all dioestrous and even in some metoestrous rats in the present study, suggests that this compound is capable of releasing both FSH and LH for inducing final follicular growth and ovulation. By contrast, much higher doses (500 to 1000 ng/rat) of synthetic LH-RH have been shown to be incapable of inducing satisfactory ovulation in metoestrous and early dioestrous rats (Morishita & co-authors, 1974; U. K. Banik, M. A. Hirsch and M. L. Givner, unpublished data). These findings suggest that, unlike AY-25,205, synthetic LH-RH could not induce final maturation and rupture of nearly matured follicles, perhaps due to inadequate release of both FSH and LH.

In 4-day cyclic rats, the peak serum LH levels occur between 15.00 and 17.30 hours on the day of pro-oestrus (see Neguin, Talley, Mann & Schwartz, 1974). Although the serum LH levels in FD-treated rats and in those treated with FD and synthetic peptides were not measured in the present study, it was presumed that the endogenous release of LH was blocked due to injection of FD before the critical period, and that the synthetic LH-RH and its analogue induced ovulation through the endogenous release of LH and perhaps also FSH.

In an earlier study (Humphrey & co-authors, 1973), synthetic LH-RH, given intramuscularly in μg quantities or orally in mg amounts, induced ovulation in pro-oestrous rats treated with chlorpromazine. The lowest intramuscular dose of LH-RH inducing 100% ovulation (MED_{100}) in pro-oestrous rats treated with chlorpromazine was 1 μg (3.7 $\mu\text{g}/\text{kg}$ body wt). In the present study the lowest intravenous doses of synthetic LH-RH and AY-25,205 per rat for MED_{100} were 250 and 7.7 ng, respectively.

The antifertility effect with a single dose of AY-25,205 administered 1 day before the expected day of pro-oestrus was due to premature induction of ovulation (Tables 2, 3 and 4), and perhaps a change in ovarian steroidogenesis. This was evident from the disturbance in the vaginal cytology. On the expected day of pro-oestrus, despite showing predominantly vaginal epithelial cells, very few animals mated with fertile males. This was probably due to induction of premature rupture of follicles at 'a wrong time' thus causing a quick change from oestrogen-producing granulosa cells to progesterone-producing luteal cells (Short, 1964). In a separate study (results not included in this report), we have observed that, by repeated medication of rats with AY-25,205, 1 day before the expected day of pro-oestrus, the antifertility effect could be demonstrated for a longer period of time without inducing anoestrus. In most of the animals, however, the cycle became 1 day shorter (U. K. Banik and M. L. Givner, unpublished data).

Our preliminary studies demonstrate that, on a molar basis, AY-25,205 is not only more potent than LH-RH in inducing ovulation in rats but also possesses a different biological profile on the pituitary-ovarian system which may be useful for correcting infertility, controlling fertility and regulating the menstrual cycle in women and promoting fertility in farm animals.

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