Contraception by induction of luteinized unruptured follicles with short-acting low molecular weight FSH receptor agonists in female animal models

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Abstract

During recent decades minor innovative drugs have been developed for the female contraceptive market and they all contain steroidal progestagens (and estrogens) that act centrally and have side effects that can be attributed to this central action. In this study, we present an innovative tissue-specific approach for female contraception by low molecular weight (LMW) FSH receptor (FSHR) agonists, which interact with the FSHR that is dominantly expressed in the granulosa cells. The oral administration of LMW FSHR agonists with a short circulation time, induced formation of luteinized unruptured follicles (LUFs) from the Graafian follicles, thereby preventing the release of the oocyte. The short-acting LMW FSHR compounds were fully agonistic to FSHR ($EC_{50} = 4\text{--}5\text{ nM}$). In an isolated mouse follicle culture, a short incubation period (2 h) resulted in inhibition of follicular rupture, where continuous incubation induced follicle growth.

Pharmacokinetics after oral administration showed a surge-like exposure in rats and monkeys. Oral administration of short-acting LMW FSHR agonists inhibited ovulation at 10 mg/kg in rats and guinea pigs by generating LUFs without affecting cyclicity. Also, inhibition of follicular rupture was shown to be reversible within one cycle. Finally, LUFs were induced without affecting the hormonal cyclicity in cynomolgus monkeys, a mono-ovulatory species. In healthy women LUF formation occurs naturally, with a LUF acting as corpus luteum that produces enough progesterone to ensure normal menstrual cyclicity. Together with the presented data this indicates that the innovative approach with short-acting LMW FSHR agonists could lead to oral contraception for females at the ovarian level.

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Introduction

Since approval of the first contraceptives in the sixties, steroidal contraceptives have been widely used to control female fertility. The combined estrogen and progestagen contraceptives act to suppress the ovulation indirectly due to a blockade in GnRH secretion from the hypothalamus and thus FSH and LH secretion from the pituitary while they also thin the endometrial lining. Progestagen-only contraceptives thicken the cervical mucus and prevent entry of sperm and fertilization. Although the effectiveness of hormonal contraceptives is very high (>99%), side effects such as headaches, weight gain, breakthrough bleedings, depression, and increased risk of venous thromboembolism account for ~30% of new hormonal contraceptive users who discontinue treatment in the first year of use. Moreover, around 25% of all pregnancies worldwide are unintended (Nass & Strauss 2004) and evidence shows that a wider range of available contraception options increases usage and compliance. Therefore, there is

a need for new contraceptives with similar efficacy and fewer side effects.

Our strategy to identify a novel female contraceptive was similar to that of Chengalvala *et al.* (2006). Key criteria for a novel contraception approach are: a) selective expression of the molecular target in the gonads, b) inhibition of fertility function, and c) reversibility of fertility function.

The molecular target of interest is the FSH receptor (FSHR), which is selectively expressed in ovarian tissue, mainly in granulosa cells, and plays a pivotal role in normal follicle development. The endogenous ligand FSH induces follicular maturation and concomitant aromatase expression leading to estradiol production that is required for ovulation and preparation of the uterus for implantation in combination with progesterone.

The first attempts to use FSH preparations for contraception were based on deglycosylation of the high molecular weight protein. Deglycosylated FSH had a 50-fold larger binding affinity to the FSHR than

wild-type FSH but was incapable of stimulating either cAMP production or steroidogenesis (Keene *et al.* 1994). Unfortunately, due to the short half-life of the deglycosylated molecule there was no *in vivo* follow up.

The first report of a bioactive low molecular weight (LMW) gonadotropin describes an FSHR antagonist as a potential compound for female contraception (Arey *et al.* 2002). More investigators have reported small molecule modulators of the FSHR including diketopiperazines (Guo *et al.* 2004a, 2004b), thienopyrimidines (Hanssen & Timmers 2003, van Straten *et al.* 2005), dihydropyrimidines (Grima Poveda *et al.* 2006), thiazolidinones (Pelletier *et al.* 2005, Wrobel *et al.* 2006, Yanofsky *et al.* 2006), and others (for a review see van Straten & Timmers (2009)), all having high affinity for FSHR. Oral bioavailability or oral efficacy for any of these LMW FSHR modulators, however, has not been reported.

Obviously, follicle (over) stimulation in the mouse, rat, and monkey can only be achieved when sufficiently high plasma concentrations of the FSHR agonist are retained for a prolonged period of time. This pharmacokinetic/pharmacodynamic (PK/PD) relationship of long exposure above a threshold is in line with the long half-life of FSH itself (Zelevnik 2004). We found that oral short-acting LMW FSHR agonists with low time of maximum concentration (T_{max}) and short half-life ($T_{1/2}$) values and high maximum concentration (C_{max}) levels in plasma (surge-like) inhibit follicular rupture in rats by generating luteinized unruptured follicles (LUFs) without affecting cyclicity. In LUFs, the oocyte remains within the follicle, while the normal processes of luteinization and subsequent progesterone production are maintained. By definition, absence of the oocyte in the fallopian tube prevents the chance of fertilization and pregnancy. Since formation of LUFs occurs in normal cycling women in 5–10% of the cycles (Kerin *et al.* 1983, Killick & Elstein 1987) and increases after treatment of infertile women undergoing first line treatment with clomiphene citrate (Qublan *et al.* 2006), a contraceptive approach based on induction of LUFs without affecting cyclicity is a very promising concept.

We found that in rats a short, but high, FSHR agonist plasma level (surge) induces physiological changes in antral follicles (FSH dependent) leading to LUF formation and inhibition of follicular rupture without affecting the smaller growing follicles, still being FSH independent, and steroid hormone production. In this study, we report the use of short-acting LMW FSHR agonists that reversibly inhibit follicular rupture in multiple species. The use of these compounds in women might result in a novel contraceptive approach with a possibly very benign side effect profile due to the maintenance of physiological ovarian processes.

Results

In vitro pharmacological profile

The LMW FSHR agonists used for LUF induction were profiled *in vitro* on the human G protein-coupled receptors (GPCRs) FSHR and LHR and in addition on the FSHR of rat (rFSHR), mouse (mFSHR), and cynomolgus (cFSHR).

Both compounds, LMW FSHR agonist nr. 1 and nr. 2, are very potent on the human FSHR (hFSHR) with nanomolar activity (EC_{50} values) of 4 and 5 nM respectively. On the rFSHR and cFSHR the activity was comparable, although the activity for only LMW FSHR agonist nr. 2 was approximately ten times less on the mFSHR (48 nM) with a low efficacy of 58%. The activity of the protein reference compound rec-FSH is in the picomolar range for all species. For the human LH receptor (hLHR) the activity was about 100 times less than for the FSHR (543 nM for LMW FSHR agonist nr. 1 and 218 nM for nr. 2). The activities on the LHR for the rat, mouse, and cynomolgus were in the micromolar range or higher, indicating a selectivity of three orders of magnitude for FSHR over LHR for both compounds. No activity was found at the human TSH receptor (Table 1). As control, we also tested the action of the three main metabolites of FSHR agonist nr. 1 but no activity on hFSHR, rFSHR, and cFSHR, hLHR or human TSHR could be found.

Table 1 EC_{50} values (in M) of low molecular weight (LMW) FSH receptor (FSHR) agonists nr. 1 and nr. 2, and rec-FSH in Chinese hamster ovary (CHO) cells stably expressing the human (h), rat (r), mouse (m), or cynomolgus (c) FSHR, LHR, or TSHR.

	LMW FSHR agonist nr. 1	Efficacy (%)	LMW FSHR agonist nr. 2	Efficacy (%)	Rec-FSH	Efficacy (%)
CHO.hFSHR ago	4 nM ($n=11$)	93	5 nM ($n=6$)	93	12 pM ($n>10$)	100
CHO.rFSHR ago	1 nM ($n=4$)	113	6 nM ($n=1$)		3 pM ($n=2$)	100
CHO.mFSHR ago	8 nM ($n=2$)	101	48 nM ($n=2$)	58	9 pM ($n=2$)	100
CHO.cFSHR ago	1 nM ($n=3$)	82	4 nM ($n=3$)	83	35 pM ($n=2$)	100
CHO.hLHR ago	543 nM ($n=8$)	114	218 nM ($n=7$)	108	NA	
CHO.rLHR ago	2 μ M ($n=3$)	62	> 3.16 μ M	0	NA	
CHO.mLHR ago	> 3.16 μ M ($n=3$)	0	1 μ M	48	NA	
CHO.cLHR ago	2 μ M ($n=3$)	108	1 μ M	100	NA	
CHO.hTSHR ago	> 10 μ M ($n=5$)	21	> 10 μ M	0	NA	

No antagonistic activities were reported for these compounds. Efficacy is relative to the maximal effect of the natural hormone, FSH, LH, or TSH. NA, no activity.

Figure 1 Ovulation (follicular rupture) of isolated mouse follicles after 2 h of treatment per indicated culture day with LMW FSHR agonist nr. 1 (10 µM, 4–7 follicles/group). All follicles were incubated with continuous rec-FSH (75 mIU/ml, days 1–3) and received ovulation induction on day 4 (hCG 5 IU/ml). Control group was without 2 h intermittent incubation. Arrow indicates inhibition of follicular rupture of follicles incubated for 2 h at day 3.

Ex vivo ovulation inhibition mouse model

When LMW FSHR agonist nr. 1 was tested as an FSHR agonist by adding the compound continuously to the culture media from days 1 to 3 it showed stimulation of follicle growth at 1 µM, comparable to rec-FSH induced follicle growth, without any adverse effects (data not shown).

However, when LMW FSHR agonist nr. 1 was incubated in shorter periods very different results were noted. First, the crucial timing of administration was explored by varying the incubation day. Therefore, LMW FSH-agonist nr. 1 was incubated only for 2 h at days 1, or 2, or 3. Follicle growth by continuous rec-FSH (75 mIU/ml) combined with either day LMW FSHR agonist was not affected (no FSHR antagonist effect). However, when ovulation was induced by hCG (5 IU/ml) the next day, all the follicles treated with LMW FSHR agonist on day 3 of culture were unable to rupture (Fig. 1), whereas the follicles treated on days 1 and 2 did rupture.

In the next experiment the duration of compound administration on day 3 was further explored. The hypothesis that a short exposure to FSHR agonist can induce LUF formation was tested by short incubations of 1, 2, or 4 h. In the control group 92% of the follicles ruptured. After 1 h of incubation with LMW FSHR agonist nr. 1 the ovulation rate declined to 60% and after 2 or 4 h the ovulation rate dropped to 0% (Table 2). In all tested conditions for LMW FSHR agonist nr. 1 the follicle growth was not disturbed. The LMW FSHR agonist nr. 2 showed also inhibition of follicular rupture

at 2, 4, or 6 h with, respectively, 60, 0, and 20% ovulation remaining. However, this was accompanied by significantly diminished follicle growth after 6 h incubation and premature ovulation of follicles leading to a decline in the number of follicles (from 14 to 5) that could be used for ovulation induction (Table 2).

PKs in female Wistar rats and female cynomolgus monkeys

In the rat, LMW FSHR agonist nr. 1 showed a rapid absorption with a very short T_{max} of 0.5 h and a high C_{max} of 2.6 µM after oral administration of 10 µmol/kg. The compound showed a short half-life after i.v. (3.0 h) as well as p.o. (3.4 h) administration. This short half-life is caused by very high clearance (4.1 l/h per kg) in combination with a moderate volume of distribution (2.5 l/kg). The oral bioavailability was very good with 95%, which is surprising in view of the high clearance. The LMW FSHR agonist nr. 2 showed a similar short-acting profile with a T_{max} of 0.7 h, a C_{max} of 4.0 µM, and an oral bioavailability of 100%. This compound showed moderate clearance and a low volume of distribution, resulting in an even shorter half-life after i.v. (0.8 h) as well as p.o. (1.5 h) administration. These compounds perfectly mimic short surge-like profiles in the rat (Fig. 2). PK parameters of multiple experiments with the LMW FSHR agonists in the rat are presented in Table 3.

Both compounds were also tested in monkeys of the same colony as used for the ovulation inhibition experiments. In the monkey, the T_{max} values for agonist nr. 1 and nr. 2 were short, 1.0 and 0.8 h respectively. However, the C_{max} values were lower than in the rat. LMW FSHR agonist nr. 1 exhibited a C_{max} of 0.7 µM, whereas LMW FSHR agonist nr. 2 showed a C_{max} of 0.2 µM. Both compounds showed short terminal

Table 2 Follicular growth and rupture (ovulation) of isolated mouse follicles after short incubation with FSH and low molecular weight (LMW) FSH receptor (FSHR) agonists nr. 1 and nr. 2 on day 3 of culture.

Test condition	Follicle growth from days 3 to 4 in µm (mean ± s.d. (n))	Ovulation % (n/n)
Control group (rec-FSH 75 mIU/ml, continuously)	72 ± 20 (26)	92 (21/23)
LMW FSHR ago nr. 1 10 µM: 1 h	80 ± 14 (11)	60 (6/10)
LMW FSHR ago nr. 1, 2 h	78 ± 30 (27)	0 (0/23)
LMW FSHR ago nr. 1, 4 h	82 ± 24 (10)	0 (0/9)
LMW FSHR ago nr. 2, 10 µM: 2 h	69 ± 20 (10)	60 (6/10)
LMW FSHR ago nr. 2, 4 h	70 ± 36 (11)	0 (0/3)
LMW FSHR ago nr. 2, 6 h	45 ± 22 (14)*	20 (1/5)
Rec-FSH 750 mIU/ml, 2 h	62 ± 20 (15)	0 (0/11)

Data of two separate experiments are merged. The number of follicles used for ovulation is lower than used for follicle growth determination due to exclusion of follicles (reasons: too small for ovulation induction (<350 µm), premature ovulation or damaged follicle). * $P < 0.05$ compared with control group.

Figure 2 Single-dose pharmacokinetics of LMW FSHR agonist nr. 1 (A) and nr. 2. (B) in the rat. Straight line represents i.v. administration (4 µmol/kg) and dotted line represents orally administered single dose of 10 µmol/kg.

half-lives after i.v. administration as a result of moderate to high clearance and a moderate volume of distribution. However, after p.o. administration the terminal half-life was surprisingly long for agonist nr. 1 (12.5 h) and short for agonist nr. 2 (1.0 h). Oral bioavailability was moderate for LMW FSHR agonist nr. 1 with 29% and poor for LMW FSHR agonist nr. 2 with only 3% (Table 3).

Ovulation inhibition in cyclic Orga rats

Dose response study

When LMW FSHR agonist nr. 1 was tested in an ovulation inhibition model in rats, the active dose where 100% inhibition of follicular rupture was achieved was determined to be 10 mg/kg p.o. after 3 days of oral treatment (twice-daily, treatment on met-, di-, and pro-estrus of cycle; Fig. 3A). The ovaries did not show increase in weight suggesting that there was no LMW FSHR agonist induced follicle growth at doses up to 100 mg/kg. However, at 10 mg/kg p.o., the ovary weight was significantly increased (90 ± 2 mg compared with placebo 80 ± 2 mg, Fig. 3C). For the LMW FSHR agonist nr. 2 the active dose was also determined to be 10 mg/kg after oral administration (Fig. 3B). This compound did show minor significant ovarian weight changes in the used administration scheme at 2.5 mg/kg (91 ± 4 mg) and 10 mg/kg (90 ± 4 mg), and a significant increase in ovarian weight suggestive for follicle growth comparable to rec-FSH stimulation (100 ± 3 mg) at 20 mg/kg (100 ± 5 mg) (Fig. 3D).

In ovarian tissue of ovulation-inhibited animals, LUFs were detected after histological analysis (Fig. 4). The

oocytes were still present in the large antral follicles that should have ovulated. Part of the ovulation process such as cumulus expansion and oocyte maturation (germinal vesicle breakdown (GVBD)) seemed to have been completed, however, the final follicle rupture did not take place. The follicles showed signs of luteinization of the granulosa cells and the intrusion of small blood vessels which are normally only seen in the theca layer of the follicles.

Timing of administration

To explore the most critical time of administration in the 4-day cycle of the rat, the moment of (twice-daily) administration was varied from day 1 (met-estrus) to day 2 (di-estrus) or to day 3 (pro-estrus, day before ovulation), as shown in Fig. 5. In this experiment it was demonstrated that administration of the LMW FSHR agonist (nr. 1) on di-estrus was the most effective day to obtain 100% inhibition of follicular rupture (Fig. 5A). Treatment on day 1 (met-estrus) showed no effect at all and treatment on day 3 (pro-estrus), which is just before ovulation resulted in partial inhibition of follicular rupture. When the ovarian weights were determined, no differences between treatment groups and placebo were observed (Fig. 5B). However, when progesterone was determined in the sera at autopsy day (estrus, day 4) it became clear that progesterone concentrations were enormously elevated in the day 2 (di-estrus) group compared with the placebo group that normally ovulated, indicative for premature luteinization in these animals (Fig. 5C). LMW FSHR agonist nr. 2 was not tested in this experimental setting.

Table 3 Pharmacokinetic parameters of low molecular weight (LMW) FSH receptor (FSHR) agonist nr. 1 and nr. 2 in rat and monkey.

	LMW FSHR agonist nr. 1	LMW FSHR agonist nr. 2
	Vehicle: 10% Cremophor in water	Vehicle: 0.5% gelatin/5% mannitol in water
Rat (4 µmol/kg i.v., 10 µmol/kg p.o.)		
CL (l/h per kg)	4.1 ± 0.3	0.6 ± 0.1
Vss (l/kg)	2.5 ± 0.1	0.4 ± 0.17
Terminal half-life i.v. (h)	3.0 ± 0.3	0.8 ± 0.2
Terminal half-life p.o. (h)	3.4 ± 0.3	1.5 ± 0.4
C_{max} (µM)	2.6 ± 0.8	4.0 ± 1.3
T_{max} (h)	0.5 ± 0.0	0.7 ± 0.2
Oral bioavailability (%)	95 ± 52	100 ± 0
Monkey (2 µmol/kg i.v., 5 µmol/kg p.o.)		
CL (l/h per kg)	1.6 ± 0.3	0.6 ± 0.0
Vss (l/kg)	0.9 ± 0.1	0.6 ± 0.0
Terminal half-life i.v. (h)	0.6 ± 0.0	1.3 ± 0.2
Terminal half-life p.o. (h)	12.5 ± 2.8	1.0 ± 0.4
C_{max} (µM)	0.7 ± 0.3	0.2 ± 0.0
T_{max} (h)	1.0 ± 0.0	0.8 ± 0.1
Oral bioavailability (%)	29 ± 13	3 ± 1

Data presented as mean $\pm$ s.d., $n=3$.

Figure 3 Effect of dose range of LMW FSHR agonists nr. 1 and 2 in cyclic rats on ovulation inhibition (A and B) and on ovarian weight (C and D). LMW FSHR agonist nr. 1 tested from 1 to 100 mg/kg p.o. (A and C). LMW FSHR agonist nr. 2 tested from 0.5 to 20 mg/kg p.o. (B and D). Summary of multiple experiments (* $P < 0.05$, compared with control, one-way ANOVA, and the multiple range test by Fisher's LSD).

Reversibility of ovulation inhibition in rats after prolonged treatment

To determine the effect of LMW FSHR agonist nr. 1 on growing follicles, rats were treated for one, two, or three consecutive cycles. In rat the complete folliculogenesis from primordial to antral follicle takes place in three waves during three 4-day cycles. We demonstrated that inhibition of follicular rupture is complete in all three treatment cycles and recovered to normal ovulation rates within <5 days after stopping the treatment (10 mg/kg p.o.: Fig. 6A). Furthermore, the ovarian weights (Fig. 6B) differ in treatment cycles 1 and 2 but not after three treatment cycles, compared with the accompanying placebo group, but were not elevated due to follicular growth (as expected with FSH) as concluded from the histology data. Histological analysis showed that part of the ovulation process such as cumulus expansion and oocyte maturation (GVBD) seemed to have been completed; however, the final follicle rupture did not take place. Histological evaluation also demonstrated that the ovaries from animals treated for more than one cycle showed young LUFs (antrum still present) as well as old LUFs (comparable to corpora lutea, no antrum) shown in Fig. 7.

The cycles of all animals as determined by taking vaginal swabs remained regular. Estradiol and progesterone concentrations showed no significant changes compared with control animals after 1, 2, or 3 cycles of treatment (data not shown).

Ovulation inhibition in cyclic guinea pigs

No LUFs were found in the placebo-treated animals ($n = 3$). However, premature luteinization of antral follicles was found in all the LMW FSHR agonist-treated animals ($n = 3$ /group) leading to LUFs.

Ovulation inhibition in cyclic *Macaca fascicularis*

Cyclic animals were treated with LMW FSHR agonist nr. 1 from cycle days 5 to 14 in the follicular phase. Two animals were dosed 10 mg/kg p.o., but this dose appeared to be too low, according the measured C_{max} to cause any effect on ovulation. A higher dose tested (50 mg/kg p.o.) in four cycles resulted in 50% ovulation inhibition by follicle growth disruption without LUF formation, one animal showed premature ovulation and the last animal did not respond at all. Therefore, this compound was not further tested in monkeys.

LMW FSHR agonist nr. 2 was tested in three dosages (10, 20, and 50 mg/kg p.o.) in cyclic monkeys from cycle days 5 to 14 in the follicular phase. Again the low doses (10 and 20 mg/kg p.o.) showed no significant effect on ovulation. The higher dose of 50 mg/kg p.o. was tested in ten animals and resulted in four animals with premature ovulation (ovulation was scored 2–3 days earlier than in the control cycle, Fig. 8A) and two animals showed ovulation inhibition by disturbing the follicle growth during treatment (Fig. 8B). After discontinuation of the treatment (on day 14) a new follicle started to grow, ovulated and completed the cycle with a delayed menstrual bleeding.

Figure 4 Luteinized unruptured follicle in ovary of rat treated with LMW FSHR agonist nr. 1 (10 mg/kg p.o.). Red circle indicates the retained oocyte. Cumulus expansion and oocyte maturation (GVBD) seemed to have been completed; however, the final follicle rupture did not take place. Obj. 10×.

Figure 5 Effect of placebo (1 ml/kg p.o., 10% Cremophor in water) or LMW FSHR agonist nr. 1 (10 mg/kg p.o., twice per day) administered at three different cycle days in 4-day cyclic female rats on ovulation (follicular rupture) (A), ovarian weight (B), and progesterone serum level at day 4 (C) ($n=5/\text{group}$, $*P<0.05$, compared with control, one-way ANOVA, and the multiple range test by Fisher's LSD). Arrow indicates no ovulation at all.

The remaining four animals showed a large dominant follicle with a gradual or no decrease in size around the time of expected follicular rupture together with blood vessel formation indicative for LUF formation as determined by ultrasound scanning (Fig. 8C). These large follicles did not rupture at all and started to produce progesterone earlier.

Two of these four animals were selected for unilateral ovariectomy (with the accompanying fallopian tube), and the ovaries were dissected and processed for histology to validate the existence of a LUF structure. In these ovaries of both monkeys the dominant follicle (early corpus luteum) comprising the former antrum and the layer of luteinizing granulosa cells was distinctly present. The retained oocyte was surrounded by luteinizing granulosa cells of the follicle but not by cumulus cells and showed oocyte maturation (GVBD)

indicating two of the three ovulation processes had taken place including formation of newly formed blood vessels for luteinization. The detection of the oocyte demonstrated the occurrence of LUFs (Figs 9 and 10). This strongly indicates that probably all four animals showed LUFs after oral treatment with LMW FSHR agonist nr. 2 at a dose of 50 mg/kg.

All treated animals remained cyclic after treatment, but inhibition of follicular rupture resulted in a prolonged treatment cycle of 42 ± 3 days where a LUF cycle resulted in a shortened cycle of $\sim 23 \pm 1$ days instead of the average cycle length of 30 ± 2 days. Data are summarized in Table 4.

Discussion

In the search for orally active LMW FSHR agonists for follicle stimulation we identified compounds that were very active *in vitro* but inactive *in vivo*, although the oral availability was high ($>20\%$). We discovered that the duration of stimulation was important for the *in vivo*

Figure 6 Effect of LMW FSHR agonist nr. 1 (10 mg/kg p.o., twice per day) or placebo (10% Cremophor in water) administered during one, two, or three adjacent cycles in 4-day cyclic female rats on ovulation (follicular rupture) (A) and ovarian weight (B) ($n=5/\text{group}$, $*P<0.05$, compared with control, one-way ANOVA, and the multiple range test by Fisher's LSD; //, not significant).

Figure 7 Photographs of LUFs from the multiple cycle experiment with LMW FSHR agonist nr. 1 (10 mg/kg p.o., twice per day) in the rat. The upper panel shows a LUF scored as young. The bottom panel shows an older LUF that is completely filled with luteinized granulosa cells. Red circle indicates the retained oocyte in the luteinized unruptured follicle. Cumulus cells have disappeared and oocyte maturation (GVBD) has taken place. Obj. 10 $\times$.

activity of these compounds. When administered subcutaneously they behaved as LMW FSHR agonists by stimulation of antral follicle growth. However, our primary goal at that time was to identify orally active compounds and therefore we studied more frequent oral dosing in attempt to achieve follicle stimulation. By serendipity, we discovered that the PK profile of these compounds did not induce follicle growth but premature luteinization, leading to the formation of LUFs in the treated rats. In a LUF, the oocyte is not released but luteinization and progesterone production is not disturbed so normal cyclicity is remained. Hence, the use of this phenomenon could be a promising new method of contraception.

In this study, we describe the exploration of this new contraceptive method of LUF induction by two LMW FSHR agonists from different chemotypes, which are orally bioavailable, with a short surge-like PK profile and therefore not suitable as follicle stimulants in assisted reproductive techniques.

The advantage of LMW FSHR agonists for contraceptive purposes is that they selectively bind to the FSHR that is exclusively expressed in the ovaries. Because the FSHR is dominantly expressed in granulosa cells of antral follicles and needed for the shift from pre-antral to antral follicle formation, the compounds will probably not interfere with primary or secondary follicles where the expression is much lower and will probably also therefore not interfere with the recruitment of the follicle pool and the ovarian reserve. In initial studies in the rat

there was no increase in the number of follicles initiated to grow; however, further studies are required to confirm this and that there are no other effects on early follicle growth.

First, we demonstrated *in vitro* at the receptor level that the compounds used were highly selective FSHR agonists with very little LHR agonism and did not show any FSHR and LHR antagonism. Also, the main metabolites were not antagonistic to the hFSHR, rFSHR, mFSHR, and cFSHR.

The PK data in rat and monkey showed that both LMW FSHR agonists used in these studies are orally bioavailable, having a relatively short exposure (only several hours) with a very steep increase in plasma concentrations (high C_{max} within 1–3 h) leading to a surge-like PK profile of the compounds. This surge-like way of exposure of the LMW FSHR agonists is probably the cause of a totally different effect on the growing follicles in the ovary than continuous FSH exposure.

Figure 8 Examples of a premature ovulation cycle (A), an ovulation inhibited cycle (B), and a LUF cycle (C) in the cynomolgus monkey. Animals were treated with LMW FSHR agonist nr. 2 (50 mg/kg p.o., once-daily from days 5 to 14 of the follicular phase). OVX, day of ovariectomy. Black arrow indicates sharp decrease in follicle size indicative of ovulation.

Figure 9 Bouin-fixed sections of the ovary and fallopian tube of the first of the two monkeys (LMW FSHR agonist nr. 2, 50 mg/kg p.o.) fulfilling the criteria for LUF formation. (A) Overview of a section in the center of the sample showing the dominant follicle (early corpus luteum) comprising the former antrum and the layer of luteinizing granulosa cells. (B) Section at the periphery of the sample showing the retained oocyte surrounded by luteinizing granulosa cells and newly formed blood vessels, giving evidence of a LUF. (C) Details of the retained oocyte, in a PAS-stained section (left) and the adjacent HE-stained section (right) showing the diameter of about 140 µm and the slightly degenerated nucleus. (A) Obj. 5×. (B) Obj. 10×. (C) Obj. 40×.

In an isolated mouse follicle culture, we confirmed that a limited incubation time of the compound is very important for its contraceptive effect. Continuous incubation showed pure FSHR agonistic effects like follicle growth comparable with rec-FSH. However, short incubation with high concentrations of the LMW FSHR agonists, instead of causing follicle growth, induced a premature suboptimal ovulation process, without interfering with the follicle growth (only LMW FSHR agonist nr. 1).

These *in vitro* data strengthen the hypothesis that a peak level of FSH during a short period disrupts the growing antral follicle by inducing a suboptimal ovulation process that does not result in the release of the oocyte.

In the rat, our most prominent explorative PD ovulation model, where we found by serendipity the first LUF induction by LMW FSHR agonists, we confirmed inhibition of follicular rupture and demonstrated a clear dose response of the LMW FSHR agonists

with 100% effectivity from 10 mg/kg on, without any exaggerated follicle growth at doses up to 100 mg/kg.

Timing of administration turned out to be very important when explored in the 4-day cyclic rats. Only at one specific day (di-estrus) the LMW FSHR agonists showed 100% inhibition of follicular rupture whereas treatment 1 day earlier had no effect at all and 1 day later (pro-estrus, day before ovulation) showed only partial effects. In later studies we were also able to reproducibly induce ovulation inhibition by LUF formation by one single administration at di-estrus (data not shown).

Together, with the specific timing needed in the isolated mouse follicles it is obvious that a specific stage of follicle development is susceptible for LUF formation by LMW FSHR agonists and that exposure during other periods seems to have no (or partial) effect at all. This specific stage is reasonably the period with the highest FSHR expression of the granulosa cells.

The possibility that the compounds are active via the LHR seems to be unlikely. For LH activity in rats a surge of more than 3 h is needed and the exposure of the LMW FSHR agonists above in the micromolar is very short.

Figure 10 Bouin-fixed and periodic acid-Schiff-stained sections of the ovary and fallopian tube of the second of the two monkeys (LMW FSHR agonist nr. 2, 50 mg/kg p.o.) fulfilling the criteria for LUF formation. (A) Overview of a section in the center of the sample showing the dominant follicle (early corpus luteum) comprising the former antrum and the layer of luteinizing granulosa cells. (B) Section at 4 mm distance of A, showing the retained oocyte surrounded by luteinizing granulosa cells, giving evidence of a LUF. The oocyte appears to be slightly shrunken, attributable to the processing. The latter was confirmed by the likewise shrunken oocyte in the pre-antral follicle (see B left and C, at a distance of 50 µm of B. (A) Obj. 5×. (B) Obj. 5× (left) and 20× (right). (C) Obj. 40×.

Table 4 Results of treatment with the low molecular weight (LMW) FSH receptor (FSHR) agonists in cyclic monkey.

LMW FSHR	Dose (mg/kg)	Effect on follicle	Effect on menstrual cycle	C _{max}	LUF formation	Comment
Agonist nr. 1	10	NC	NC	0.2	Inactive	Dose too low
	10	NC	NC	0.3	Inactive	Dose too low
	50	NC	NC	0.5	Inactive	Exposure too low
	50	Premature ovulation	Shortened	4.0	Inactive	
	50	Growth disrupted	Prolonged	6.5	Inactive	No ovulation, no LUF
	50	Growth disrupted	Prolonged	0.8	Inactive	No ovulation, no LUF
Agonist nr. 2	10	NC	NC	0.3	Inactive	Dose too low
	10	NC	NC	0.8	Inactive	Dose too low
	20	NC	NC	0.5	Inactive	Dose too low
	20	NC	NC	0.8	Inactive	Dose too low
	50	Premature ovulation	Shortened	2.7	Inactive	High activity too early?
	50	Premature ovulation	Shortened	7.3	Inactive	High activity too early?
	50	Premature ovulation	Shortened	6.4	Inactive	High activity too early?
	50	Premature ovulation	Shortened	11.2	Inactive	High activity too early?
	50	Growth disrupted	Prolonged	12.1	Inactive	No ovulation, no LUF
	50	Growth disrupted	Prolonged	2.3	Inactive	No ovulation, no LUF
	50	Luteinized	Shortened	4.8	Active	LUF
	50	Luteinized	Shortened	5.6	Active	LUF
	50	Luteinized	ND	13.2	Active	Confirmed LUF
	50	Luteinized	ND	5.9	Active	Confirmed LUF

NC, no changes; Growth disrupted, follicle stops growing and disappears. Premature ovulation (2–3 days earlier). Prolonged means cycle > 33 days; shortened, cycle < 27 days. ND, not determined due to ovariectomy before menstrual bleeding.

Besides, LHR is expressed after induction by FSH. Furthermore, we performed similar studies with LH or LHR agonists and this resulted in less reproducible LUF formation than with LMW FSHR agonists and was always accompanied with exaggerated ovarian weight gain. Finally, in the mouse follicle culture, in which species no LH activity was proven, we were able to inhibit the follicular rupture with 100%.

Although some LH activity cannot be ruled out completely it seems very unlikely that this is accountable for the inhibition of follicular rupture.

Reversibility of ovulation is very important for a new female contraceptive method (Chengalvala *et al.* 2006). In the rat we tested reversibility by administering the compound during the entire follicle development period (three complete cycles) and looked for spontaneous ovulation recovery after discontinuation of the treatment. It was very promising to see that already within 4–5 days all animals ovulated with a relevant number of oocytes, implying that reversibility seems to be no issue. This was confirmed by the histological analysis of the ovaries of these animals. Newly formed LUFs were found after one or more treatments, always in numbers that resemble the number of follicles expected to ovulate under normal circumstances (10–15 oocytes/animal), and older LUFs resembling old corpora lutea from the previous cycle, indicating that a LUF is comparable with a corpus luteum with an entrapped oocyte. The number of smaller antral follicles did not increase compared with control animals. These data indicate that only larger (antral) follicles, expressing the FSHR, seem to be affected by the treatment. Because fertility returned almost immediately after treatment for three cycles,

we can conclude that the smaller antral follicles are not affected and remain healthy and can continue to grow and ovulate.

To exclude rat specificity, ovulation inhibition with LUF formation by LMW FSHR agonists was also explored in another rodent species, the guinea pig. LUF induction in the guinea pig, by hCG administration on day 14 of the cycle, has already been proven to induce LUFs (Westfahl 1993). Our experiments clearly demonstrated LUF formation after treatment with the LMW FSHR agonists in this species, excluding a rat-specific phenomenon.

Since the inhibitory effects of follicular rupture have only been studied in multi-follicular rodent species such as rat and guinea pig, it was of primary importance to show that LUF formation and inhibition of follicular rupture can also be accomplished in mono-ovulatory species, such as primates. Therefore, a cynomolgus monkey PK/PD study was performed with the LMW FSHR agonists and inhibition of follicular rupture was confirmed by ultrasonography and histology of the ovaries.

Although we treated the monkeys during the entire follicular phase to ensure adequate timing of treatment we were unable to gain reproducible LUF formation in all animals treated. This failure cannot be totally accounted for by insufficient exposure concentrations that were for the higher doses as expected (daily treatment still results in peak-like surges since the half-life is very short). In some monkeys, the follicles seemed to be more sensitive to the LMW FSHR agonist, since the follicles were already inhibited in their development before they were ready to ovulate. Other monkeys,

however, showed premature ovulation. However, in 40% of the treatments we were able to induce LUFs, and this was confirmed by histological evaluation. This is the first report of LUF confirmation other than by ultrasound imaging only. Due to this large variation in response in the monkey, the current lack of knowledge about receptor profiles and mode of action and the lack of a reliable serum biomarker, it was decided not to proceed with this contraceptive approach.

The differences in results between the rodent test species on the one hand (mouse, rat, and guinea pig) and the mono-ovulatory species (cynomolgus monkey) on the other hand are difficult to explain. In the rodents we were able to induce inhibition of follicular rupture in 100% of the cases if we treat at the right time of the follicular development. We were unable to correlate this sensitive timepoint in the development of the follicles of rodents to a similar sensitive timepoint in the monkey. Due to several limitations we only tested between days 5 and 14 in the monkey (last part of follicle development before time of expected ovulation). Perhaps also the duration of the follicle development (days in rodents and 3 months in the monkey) is important in this matter.

LUFs are also found in normal cyclic women and this has been attributed to a suboptimal LH surge around the time of ovulation (Hamilton *et al.* 1985). In house, in rats we also experienced the fact that a suboptimal ovulation stimulus leads to anovulation due to LUF formation. However, the timing of such a suboptimal LH surge just before ovulation is very difficult. It might be hypothesized that the effect of the LMW FSHR agonists is simply a mimic in the rise of cAMP in granulosa cells that is seen following the LH surge. It is known that FSHR activates cumulus expansion *in vitro* in a similar manner as the LH surge acts *in vivo* and it is well accepted that the action of FSH is achieved through secondary messengers such as cAMP (Sirard *et al.* 2007). The most optimal timing in the rat is one full day before the actual LH surge. At that time the large antral follicles are the most susceptible to FSH. It seems that the surge of LMW FSHR agonists mimics an LH-like activity by cAMP increase leading to suboptimal ovulation, resulting in premature luteinization and the retainment of the oocytes in the formed corpus luteum. Indeed, several investigators found a strong positive correlation between premature luteinization and high exogenous FSH doses administered in humans, suggesting that progesterone elevation in the follicular phase is not related to serum LH concentrations but may reflect the mature granulosa cell response to high FSH exposure (Ubaldi *et al.* 1996, Filicori *et al.* 2002, Bosch *et al.* 2003).

In conclusion, potent LMW FSHR agonists, which are orally active and short-acting due to their surge-like PK profile, have a specific inhibitory effect of follicular rupture on a large antral follicles of cyclic animals. This eventually leads to the formation of LUFs that act as

corpora lutea without releasing the oocyte and therefore this novel approach presents a unique way of reversible female contraception without the use of steroidal hormones.

However, more studies are required to elucidate the mechanism and ensure the safety behind this innovative approach.

Materials and Methods

Animals

All animal procedures were in accordance with the Dutch animal welfare law and received permission of the Animal Ethics Committee, MRL, Oss (The Netherlands).

Immature female mice (B6CBAF1/Crl) for follicle culture studies were obtained from Charles River (Koln, Germany). Mature female Wistar rats (used for PK studies), mature female Orga rats with a regular 4-day estrus cycle (used for ovulation inhibition studies) and female guinea pigs (HsdPoc:DH) aged 9- to 14-weeks were obtained from Hsd/Cpb Harlan (Horst, The Netherlands) and kept in our animal facility for at least 1 week before the experiments. The rats were housed in polypropylene type L cages (4–5/cage), with a red shelter as cage enrichment. Guinea pigs were housed in polypropylene cages with shelter (4–5/cage). All animals had free access to standard pellets of dry food and bottled water and were kept in a 12 h light:12 h darkness schedule (lights on at 0600 h).

Female cynomolgus monkeys (*M. fascicularis*) weighing 3.5–6 kg with a regular menstrual cycle were used. Monkeys were group-housed with a 12 h light:12 h darkness cycle and a four times daily feeding schedule with water *ad libitum*. The monkeys have been trained to undergo different handlings with minimal stress. This includes procedures such as blood sampling, treatment with compounds, and taking vaginal swabs. The menstrual cycle was monitored by taking daily vaginal swabs using cotton tipped applicator to detect vaginal bleeding. The first day of menstruation was considered as day 1 of the cycle.

Drugs and reagents

General chemicals of analytical grade were obtained from Sigma–Aldrich. Two LMW FSHR agonists (nr. 1 and nr. 2) of different chemical classes were synthesized in-house by the Department of Medicinal Chemistry at MRL (Oss, The Netherlands). Urinary human chorionic gonadotropin (Pregnyl) used as an ovulation inducer in rats and Recombinant FSH (Puregon) were obtained from NV Organon (Oss, The Netherlands).

Progesterone and estradiol serum concentrations were determined at 37 °C on a Hitachi Modular P-800 biochemical analyzer supplied with ion selective electrodes, i.e. according to International Federation of Clinical Chemistry (IFCC) directives to define the phase of the menstrual cycle and to visualize the effect of the treatment on the menstrual cycle (e.g. ovulation inhibition effect, no progesterone). LH serum concentrations were determined by the LH IFMA as described earlier (van Casteren *et al.* 2000).

In vitro pharmacological profile

In vitro bioassays for FSHR, LHR, and TSHR

The FSHR agonistic activity was tested in a Chinese hamster ovary (CHO) cell line that stably expresses hFSHR as well as a cyclic-AMP-response-element-inducible luciferase gene. In similar assays the activity was tested on other GPCRs: the human LH and TSH receptor and the FSH and LHR of rat, mouse, and cynomolgus monkey.

CHO cells were grown in DMEM/F12 medium containing 5% bovine calf serum and supplemented with penicillin G (100 units/ml) and streptomycin sulfate (0.1 mg/ml), together with or without geneticin or hygromycin (Invitrogen) when appropriate. Cells were cultured in humidified atmosphere in 5% CO₂ at 37 °C.

For the measurement of luciferase activity, wells from a 384-well plate received 10 µl of DMEM/F12 medium supplemented with bovine insulin (1 µg/ml), penicillin G (100 U/ml), streptomycin sulfate (100 µg/ml) (assay medium) with or without FSH, and 10 µl of assay medium containing 0.3% DMSO with or without LMW FSHR agonist. Then, 10 µl of CHO cells stably expressing hFSHR, rFSHR, mFSHR, cFSHR, hLHR, rLHR, mFSHR, or cLHR (7500 cells) were added per well. After incubation for 4 h in 5% CO₂ at 37 °C, 15 µl of SteadyLite (Perkin Elmer, Groningen, The Netherlands) were added and luciferase activity was measured in an Envision after lysis of the cells for 1 h at room temperature. Data points of concentration-effect curves were fitted using GraphPad Prism 4.03 (GraphPad Software, La Jolla, CA, USA).

Ex vivo ovulation inhibition mouse model

Cultured mouse follicles were used to induce ovulation *in vitro* as described previously (Rose *et al.* 1999). Briefly, ovaries from 3 to 5 immature mice (F1: B6BCA; 21–23 days of age) were used per experiment. Pre-antral follicles with a diameter of 170–200 µm were mechanically isolated. Isolated follicles were incubated in a humidified incubator gassed with 5% CO₂ in air at 37 °C and individually cultured in Millicell-CM culture plate inserts with 250 µl medium supplemented with 5% immature mouse serum. Recombinant human FSH 75 mIU/ml (7.5 ng/ml) was present to induce follicular growth. Culture medium was exchanged every other day. The diameter of the follicles was measured using ×100 magnification and a calibrated micrometer. At the end of the culture period (4 days), follicles which reached the pre-ovulatory stage (>400 µm) were used for ovulation induction. Follicles were induced to ovulate with 5 IU/ml hCG. After 15 h of incubation, follicles were visually examined for ovulation: follicular rupture, oocyte extrusion, and oocyte maturation (as determined by GVBD). Culture medium was collected 24 h after hCG addition to measure progesterone production. LMW FSHR agonist incubation was varied and indicated per experiment in the Results section.

PKs in female Wistar rats and female cynomolgus monkeys (M. fascicularis)

PK studies were performed in female cannulated rats ($n=3$ /dose) or cyclic female cynomolgus monkeys

($n=3$ /dose). Blood samples were withdrawn from the cephalic vein (0.25 ml (rat) or 0.6 ml blood (cynomolgus) per withdrawal) with a 23G needle (Terumo, Leuven, Belgium) attached to a 2 ml syringe. Blood was sampled at regular time intervals during a 24 h period and allowed to clot overnight at 4 °C to obtain serum after centrifugation (2000 *g*, 15 min). Serum concentrations of compound were measured by LC–MS/MS. PK parameters were calculated after i.v. (5 nmol/kg single dose) and oral (10 nmol/kg single dose) administration in 10% Cremophore in water. Based on serum concentrations the following parameters were calculated: area under the curve, clearance (CL), elimination half-life ($t_{1/2}$), time (T_{max}), and level (C_{max}) of maximum concentration and oral bioavailability (program WinNonlin, Pharsight, with the non-compartmental model).

Ovulation inhibition in cyclic rats

The effect of LMW FSHR agonists on ovulation inhibition or LUF induction was investigated in regular 4-day cycling Orga rats ($n=6$ /dose) (Orga is a substrain of Wistar). For this, vaginal smears were prepared daily in the morning and evaluated microscopically by determining the percentage of cornified and nucleated epithelial cells and leukocytes. Rats with at least two consecutive 4-day estrus cycles were treated once or twice a day (between 0800 and 0900 h and between 1600 and 1700 h) for 1–3 consecutive days depending on the aim of the experiment: A) dose response curves were determined after 3 days of treatment, B) in the timing of administration experiment animals were treated on one specific day of the cycle, and C) in this reversibility experiment animals were treated for one, two, or three cycles (3, 7, or 11 days). Treatment started on the day after full vaginal epithelial cell cornification (met-estrus, day 1) and the rats were weighed just before the first administration (starting body weight). Autopsy was performed in the morning of day 4 (estrus) of the experiment. The rats were anesthetized by inhalation of a mixture of O₂/medical air 1:2 (O₂ 0.2 l/min and medical air 0.4 l/min) and isoflurane 3%. The anesthetized rats were killed by exsanguination via the abdominal aorta. The genital tract was dissected free and the oviducts were removed and placed in saline (NaCl 0.9%). The oviducts were gently pressed between two glass plates and the number of ovulated oocytes was counted microscopically. Blood was allowed to clot for 24 h at 4 °C and serum was stored for analysis of biomarkers. The ovaries were dissected, weighed, and preserved in a fixative solution to perform histopathology.

Ovulation inhibition in cyclic guinea pigs

To exclude that the contraceptive approach with LMW FSHR agonists is only applicable to rats, a small comparable study in guinea pigs was performed. The effect of LMW FSHR agonists on inhibition of follicular rupture was investigated in regular 16-day cycling animals. Cyclicity was scored by visual scoring of the opening of the vagina, with day 1 as the first day of vaginal opening. Female cyclic guinea pigs were treated daily for 3 days (days 10–12) of the cycle with placebo (1 ml/kg p.o., Cremophor 10% in water, $n=3$) or LMW FSHR agonist

(nr. 1 10 mg/kg p.o., $n=3$ /group). Autopsy was performed at days 12, 14, and 16 for histological analysis of the ovaries.

The cycle days 10–12 in the guinea pig can be compared with the di-estrus phase in the rat, where at that moment a number of antral or Graafian follicles are present.

Ovulation inhibition in cyclic cynomolgus monkeys

The effect of short-acting LMW FSHR agonists on inhibition of follicular rupture and LUF formation was also evaluated in a mono-ovulatory species, the cynomolgus monkey (*M. fascicularis*).

First, a control cycle was fully documented (estradiol and progesterone concentrations, follicle size, menstrual bleeding pattern, etc.). This cycle was used for normal reference values and was followed by treatment cycle(s) where the monkeys were treated once-daily in the morning in the follicular phase from cycle day 5 till 14. The monkeys were fed several hours after administration of the compound.

Blood samples were taken twice-weekly (on Monday and Thursday) from early follicular phase until the end of the luteal phase. Blood samples were withdrawn from the cephalic vein (0.6 ml blood per withdrawal) with a 23G needle (Terumo) attached to a 2 ml syringe. Blood samples were allowed to clot for at least 2 h at room temperature and serum was prepared by centrifugation of the clotted blood (Heraeus Multifuge 1 L-R, 10 000 g for 15 min at 4 °C) and stored at -20 °C until further analysis.

Ultrasound scan images were prepared with the ALOKA Prosound Alpha 7 Ultrasound scanner with a 7–13 Mhz Linear array transabdominal transducer, type UST-5412. Animals were sedated with ketamine/xylazine for 30–45 min to facilitate scanning and after scanning anesthesia were antagonized by Antisedan to speed up recovery. Rupture of the follicle can be measured by the decline in follicle diameter (Van Diepen *et al.* 2011).

In case of LUF formation, the dominant follicle does not rupture and the granulosa cells are starting already to luteinize. Therefore, the absence of a sharp decrease of the follicle size around the expected time of ovulation, measured by daily abdominal ultrasound scanning (US), is indicative for rupture failure. In addition, both blood vessel formations as visualized by color flow Doppler and increasing progesterone concentrations are indicative for luteinization.

To confirm LUF formation, histopathology was performed on the ovaries of monkeys that fulfilled the above two criteria for LUF formation, namely a gradual instead of sharp decrease in follicular size as measured by ultrasound scan and the onset of luteinization as suggested by the development of blood vessels around the dominant follicle and an elevated progesterone level in serum (>1 nmol/l for more than 1 day).

Additional daily blood sampling during the treatment cycle was performed for the measurement of biomarkers (LH and FSH). For measuring snapshot PK of the compound, blood samples were collected at 1, 3, 6, and 24 h on days 1 and 8 of treatment.

In total 13 animals were used and seven animals were treated twice.

Histopathological examination of the ovaries

Rats and guinea pigs

At autopsy, anesthetized rats and guinea pigs were killed by exsanguination via the abdominal aorta. The ovaries were dissected free of adjacent fat and other surrounding tissues and preserved in 4% buffered formaldehyde. All samples were dehydrated and embedded in paraffin wax. Sections (3–4 μ m thick) made from these blocks were stained with hematoxylin and eosin (HE) staining method and examined histopathologically for the entrapped oocyte in a luteinized follicle.

Cynomolgus monkeys

At ovariectomy on day 17 (i.e. 3 days after the expected ovulation date), the monkeys were anesthetized with domitor (medetomidine) and ketamine. Unilaterally, the ovary was removed, together with the accompanying fallopian tube and fixed in Bouin's fixative for 24 h. Before dissection, at both ends of the fallopian tube ligatures were made to prevent any possible escape of the oocyte during removal of the tissues. All samples were dehydrated and embedded in paraffin wax. Serial sections (3–4 μ m thick) were prepared from these blocks. With an interval of 25 μ m two serial slides was stained, one with the HE staining method and the other one with Periodic Acid Schiff reagents (PAS) as described (Bancroft & Stevens 1996) and examined histopathologically. The PAS method was used to obtain conspicuous staining of the zona pellucida of the oocyte.

Statistical analysis

Data are presented as mean $\pm$ s.e.m. All statistical analyses were performed on absolute values. Group sizes were determined by a power of 80% and $\alpha=0.05$. Differences between groups were tested by one-way ANOVA and the Multiple Range Test by Fisher's least significant difference. Significance was assumed at $P<0.05$. The tests were performed by the software Statgraphics Plus for Windows version 2.1.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Arey BJ, Deecker DC, Shen ES, Stevis PE, Meade EH Jr, Wrobel J, Frail DE & Lopez FJ 2002 Identification and characterization of a selective, nonpeptide follicle-stimulating hormone receptor antagonist. *Endocrinology* **143** 3822–3829. (doi:10.1210/en.2002-220372)
- Bancroft JD & Stevens A (Eds) 1996 *Theory and Practice of Histological Techniques*, 4th edn. Edinburgh: Churchill Livingstone Ltd.
- Bosch E, Valencia I, Escudero E, Crespo J, Simon C, Remohi J & Pellicer A 2003 Premature luteinization during gonadotropin-releasing hormone antagonist cycles and its relationship with *in vitro* fertilization outcome. *Fertility and Sterility* **80** 1444–1449. (doi:10.1016/j.fertnstert.2003.07.002)
- van Casteren JJ, Schoonen WG & Kloosterboer HJ 2000 Development of time-resolved immunofluorometric assays for rat follicle-stimulating hormone and luteinizing hormone and application on sera of cycling rats. *Biology of Reproduction* **62** 886–894. (doi:10.1095/biolreprod62.4.886)
- Chengvalva MV, Meade EH Jr, Cottom JE, Hoffman WH, Shanno LK, Wu MM, Kopf GS & Shen ES 2006 Regulation of female fertility and identification of future contraceptive targets. *Current Pharmaceutical Design* **12** 3915–3928. (doi:10.2174/138161206778559632)
- Filicori M, Cognigni GE, Pocognoli P, Tabarelli C, Spettoli D, Taraborrelli S & Ciampaglia W 2002 Modulation of folliculogenesis and steroidogenesis in women by graded menotropin administration. *Human Reproduction* **17** 2009–2015. (doi:10.1093/humrep/17.8.2009)
- Grima Poveda PM, Karstens WFJ & Timmers CM 2006 4-Phenyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline derivatives as medicaments for the treatment of infertility. 1. WO 2006117368 A1.
- Guo T, Adang AE, Dolle RE, Dong G, Fitzpatrick D, Geng P, Ho KK, Kultgen SG, Liu R, McDonald E *et al.* 2004a Small molecule biaryl FSH receptor agonists. Part 1: Lead discovery via encoded combinatorial synthesis. *Bioorganic & Medicinal Chemistry Letters* **14** 1713–1716. (doi:10.1016/j.bmcl.2004.01.042)
- Guo T, Adang AE, Dong G, Fitzpatrick D, Geng P, Ho KK, Jibilian CH, Kultgen SG, Liu R, McDonald E *et al.* 2004b Small molecule biaryl FSH receptor agonists. Part 2: Lead optimization via parallel synthesis. *Bioorganic & Medicinal Chemistry Letters* **14** 1717–1720. (doi:10.1016/j.bmcl.2004.01.043)
- Hamilton CJ, Wetzels LC, Evers JL, Hoogland HJ, Muijtjens A & de Haan J 1985 Follicle growth curves and hormonal patterns in patients with the luteinized unruptured follicle syndrome. *Fertility and Sterility* **43** 541–548.
- Hanssen RGJM & Timmers CM 2003 Preparation of thienopyrimidines with combined LH and FSH agonistic activity. Patent publication number WO 2003020726. Patent number EP 1427733.
- Keene JL, Nishimori K, Galway AB, Matzuk MM, Hsueh AJW & Boime I 1994 Recombinant deglycosylated human FSH is an antagonist of human FSH action in cultured rat granulosa cells. *Endocrine Journal* **2** 175–180.
- Kerin JF, Kirby C, Morris D, McEvoy M, Ward B & Cox LW 1983 Incidence of the luteinized unruptured follicle phenomenon in cycling women. *Fertility and Sterility* **40** 620–626.
- Killick S & Elstein M 1987 Pharmacologic production of luteinized unruptured follicles by prostaglandin synthetase inhibitors. *Fertility and Sterility* **47** 773–777.
- Nass SJ & Strauss JF III 2004 Strategies to facilitate the development of new contraceptives. *Nature Reviews. Drug Discovery* **3** 885–890. (doi:10.1038/nrd1526)
- Pelletier JC, Rogers J, Wrobel J, Perez MC & Shen ES 2005 Preparation of highly substituted gamma-lactam follicle stimulating hormone receptor agonists. *Bioorganic & Medicinal Chemistry* **13** 5986–5995. (doi:10.1016/j.bmc.2005.07.025)
- Qublan H, Amarín Z, Nawasreh M, Diab F, Malkawi S, Al-Ahmad N & Balawneh M 2006 Luteinized unruptured follicle syndrome: incidence and recurrence rate in infertile women with unexplained infertility undergoing intrauterine insemination. *Human Reproduction* **21** 2110–2113. (doi:10.1093/humrep/del113)
- Rose UM, Hanssen RG & Kloosterboer HJ 1999 Development and characterization of an *in vitro* ovulation model using mouse ovarian follicles. *Biology of Reproduction* **61** 503–511. (doi:10.1095/biolreprod61.2.503)
- Sirard MA, Desrosier S & Assidi M 2007 *In vivo* and *in vitro* effects of FSH on oocyte maturation and developmental competence. *Theriogenology* **68** (Supplement 1) S71–S76. (doi:10.1016/j.theriogenology.2007.05.053)
- van Straten N & Timmers C 2009 Non-peptide ligands for the gonadotropin receptors. *Annual Reports in Medicinal Chemistry* **44** 171–188. (doi:10.1016/S0065-7743(09)04408-X)
- van Straten NC, van Berkel TH, Demont DR, Karstens WJ, Merckx R, Oosterom J, Schulz J, van Someren RG, Timmers CM & van Zandvoort PM 2005 Identification of substituted 6-amino-4-phenyltetrahydroquinoline derivatives: potent antagonists for the follicle-stimulating hormone receptor. *Journal of Medicinal Chemistry* **48** 1697–1700. (doi:10.1021/jm049676l)
- Ubaldi F, Camus M, Smits J, Bennink HC, Van SA & Devroey P 1996 Premature luteinization in *in vitro* fertilization cycles using gonadotropin-releasing hormone agonist (GnRH-a) and recombinant follicle-stimulating hormone (FSH) and GnRH-a and urinary FSH. *Fertility and Sterility* **66** 275–280.
- Van Diepen HA, Pansier J, Oude Wesselink P, Van Drie A, Van Duin M & Mulders S 2011 Non-invasive translational Cynomolgus model for studying folliculogenesis and ovulation using color Doppler ultrasonography. *Journal of Medical Primatology* [in press]. (doi:10.1111/j.1600-0684.2011.00514.x)
- Westfahl PK 1993 Comparison of luteinized unruptured follicles and corpora lutea: steroid hormone production and response to luteolytic and luteotropic agents. *Biology of Reproduction* **48** 807–814. (doi:10.1095/biolreprod48.4.807)
- Wrobel J, Jetter J, Kao W, Rogers J, Di L, Chi J, Perez MC, Chen GC & Shen ES 2006 5-Alkylated thiazolidinones as follicle-stimulating hormone (FSH) receptor agonists. *Bioorganic & Medicinal Chemistry* **14** 5729–5741. (doi:10.1016/j.bmc.2006.04.012)
- Yanofsky SD, Shen ES, Holden F, Whitehorn E, Aguilar B, Tate E, Holmes CP, Scheuerman R, MacLean D, Wu MM *et al.* 2006 Allosteric activation of the follicle-stimulating hormone (FSH) receptor by selective, nonpeptide agonists. *Journal of Biological Chemistry* **281** 13226–13233. (doi:10.1074/jbc.M600601200)
- Zeleznik AJ 2004 The physiology of follicle selection. *Reproductive Biology and Endocrinology* **2** 31. (doi:10.1186/1477-7827-2-31)

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