Evolution of Design and Achievement of Inhibitors of the Luteinizing Hormone-Releasing Hormone as Inhibitors of Ovulation

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Luteinizing Hormone–Releasing Hormone

Detailed structure-activity studies on inhibitors of the luteinizing hormone releasing hormone (LH–RH) have been described. The most potent ovulation inhibitors have substitutions in positions 1, 2, 3, and 6. Currently four basic structural requirements for potent antiovulatory activity are: a D-aromatic amino acid, such as D-Trp or D-Phe, in position 6; a D-Phe residue in position 2; substitutions in positions 1 and 3.

For inhibitors based on substitutions in positions 2, 3, and 6, the substitution of a Pro, N–Me–Leu or D-Trp residue in position 3 is equally acceptable, and gives analogues which inhibit ovulation at 750 μg/rat. For inhibitors based on substitutions in positions 1, 2, 3, and 6, D-Trp appears necessary in position 3 in order for ovulation to be inhibited at 200 μg/rat.

Many analogues based on the [residue^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH–RH sequence are known which inhibit ovulation at 200 μg/rat. These include those analogues having D-<Glu, Ac–Pro, N–Ac–Hyp and N–Ac–Thr in position 1. The choice between L- or D-residues in this position is structure dependent (Ac-L-Pro > Ac-D-Pro, D-<Glu >L-<Glu, *etc.*). In addition, a "protected" N-terminal residue having some polar character appears to be important. Substitution of the dipeptide residue, <Glu–Pro–, into position 1 has produced a new category of potent ovulation inhibitors based on linear peptides longer than decapeptides. Continued studies on other analogues in this later class could provide more potent inhibitors by (1) utilizing new binding sites on or in the vicinity of the LH–RH receptor(s); (2) altering transportation properties; (3) producing "pro-drugs".

The substitution of N–Me–Leu into position 7 was not advantageous, presumably because of the presence of bulky D-aromatic amino acids in position 6. Nonapeptide ethylamide analogues also had very low antiovulatory potencies. The analogue [chlorambucil^1, Leu^2, Leu^3, D-Ala^6]-LH–RH acted as an agonist, but also inhibited in a modified assay *in vitro*.

Comparative assays measuring the inhibition of LH–RH, and inhibition of ovulation have emphasized other factors of importance to inhibitor design. Although all ovulation inhibitors active at 750 or 200 μg/rat strongly inhibited *in vivo*, at a ratio of analogue to LH–RH of 166:1, other analogues of comparable *in vitro* potency have displayed a range of antiovulatory activities. Similar discrepancies have been observed in the results of *in vivo* LH–RH inhibition assays. The most potent ovulation inhibitors always inhibited LH–RH at 333:1 in adult male chimpanzees, and at 100:1 in adult male rats. The dissociation of the results of the LH–RH and antiovulatory assays have been rationalized in two cases. The Cpc-analogues were active in inhibiting LH–RH in rats and in chimpanzees when given i.v., but were inactive in rats when given s.c. which is the mode of administration in the antiovulatory assay. The results for inhibition of LH–RH *in vivo* paralleled the results for inhibition of ovulation, and raised a question as to differences in absorption of peptides though the lipid layers of subcutaneous tissue. The reduced *in vivo* activities of the L-Trp^3 analogues in both the LH–RH and antiovulatory assays suggest an increase in enzymatic inactivation for these compounds.

[D-Phe^2, Pro^3, D-Phe^6]-LH–RH can inhibit endogenous LH–RH in the Rhesus monkey and inhibit ovulation. Infusion of [D-Phe^2, Pro^3, D-Trp^6]-LH–RH at 375 μg/day for 4 days from a s.c. implanted minipump completely inhibited ovulation in cycling female rats and decreased serum LH levels in castrated rats. In contrast with LH–RH or des-Gly^{10}-[D-Ala^6]-LH–RH ethylamide the Pro^3 analogue did not block uterine implantation sites of mated rats, indicating a difference in the mechanism of contraception for LH–RH agonists and inhibitors.

Introduction

Knowledge of the sequence of the luteinizing hormone-releasing hormone (LH–RH), <Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 [1–3], opened a new field in peptide research of medicinal importance. LH–RH, formed in the hypothalamus, releases the luteinizing hormone (LH), in the pituitary. Also, LH–RH can release the follicle stimulating hormone (FSH). Both LH and FSH

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are required for ovulation and pregnancy. LH–RH and analogues which are more potent and stable *in vivo* would be expected to be useful for stimulating ovulation. Inhibitors of LH–RH would be expected to be useful for contraception.

Reviews on structure-activity studies and on clinical evaluation have appeared [4–6]. We have written this review as a critique of eight years of our research on the design and evolution of inhibitors of ovulation of ever increasing potency. This critique allows the activities of many analogues, *in vitro* and *in vivo*, to be compared by the same assays. Also, some very recent new data are included.

For the *in vitro* assays, only the results for inhibition of LH release are given because, with few exceptions, the inhibition of FSH release approximately parallels the inhibitions of LH release. However, for the more active inhibitors, the inhibition of LH release appears to be slightly more facile than the inhibition of FSH release.

Several discoveries, which now have historical importance, are particularly worthy of emphasis, because they provided the basis for most current studies. Five such events are as follows.

(1) The replacement of Gly^6 in LH–RH by D-amino acids [7], especially D-aromatic amino acids [8, 9], resulted in agonists of enhanced activity. This modification has also been found to be desirable in the design of inhibitors.

(2) The replacement of $-Pro^9-Gly^{10}-NH_2$ by, for example, $-Pro^9$–NHET [10] led to agonists of enhanced potency. Although the incorporation of this C-terminal modification into inhibitor sequences has led to inhibitors in which the *in vitro* potency was retained, reduced, or enhanced, the antiovulation potency was significantly reduced, in general.

(3) The replacement of Leu^7 by N–Me–Leu in certain agonist sequences has led to retained or enhanced activity [11]. In the design of ovulation inhibitors, this modification has not been useful, probably because D-Phe or D-Trp, rather than D-Ala was in position 6.

(4) The replacement of His^2 or Trp^3 [12, 13] has given inhibitors *in vitro*. Later work has shown that for potent ovulation inhibitors, substitutions at positions 2 and 3 and incorporation of a D-Phe^2 residue are essential.

(5) The suitable replacement of $<Glu^1$ has given highly potent inhibitors, but in agonist sequences the $<Glu^1$ residue appears to be important for activity.

Currently, the most active agonists of LH–RH have substitutions at position 6 and the C-terminus. The most active ovulation inhibitors have substitutions at positions 1, 2, 3 and 6 and are based on the sequence [$Residue^1$, D-Phe^2, D-Trp^3, D-Trp^6] –LH–RH.

In early studies on the design of inhibitors of LH–RH, it was assumed that a potent inhibitor *in vitro* would be an inhibitor of ovulation. The demonstration that [D-Phe^2, D-Ala^6]-LH–RH inhibited spontaneous ovulation in rats at 12 or 24 mg/kg (six divided injections, s.c.), depressed the pre-ovulatory LH–FSH surge and inhibited pregnancy in successfully inseminated recipients, bore out this assumption [14].

Results and Discussions

Inhibitors having L-amino acids in positions 2 and 3 and alkyl amino acids in position 6 (Table I)

The decapeptide [Leu^2, Leu^3]-LH–RH (**1**) inhibited the action of LH–RH *in vitro*, with an analogue to LH–RH ratio of 300,000:1. Although this analogue was of very low potency and was inactive *in vivo*, it was the challenging basis which led to the current substitutions in both positions 2 and 3.

The replacement of Leu^3 in [Leu^2, Leu^3]-LH–RH by Ser (**2**) and Asn (**3**) decreased activity and the replacement of Leu^2 by Thr (**4**) led to the retention of inhibitory activity *in vitro*.

The analogue [Leu^2, Leu^3, D-Ala^6]-LH–RH (**5**) was up to ten-fold more potent than analogue 1 and demonstrated the desirability of substituting a D-amino acid in position 6 for an antagonist. Structural variation at position 2 gave an order of potency of Phe^2 (**7**) > Leu^2 (**5**), Val^2 (**6**), which demonstrated the desirability of substituting an aromatic amino acid into position 2. The replacement of Leu^3 in analogue **5** by Ala (**10**) or Val (**11**) decreased potency. The D-Leu^6 analogues **8** and **9** were of similar potencies to that of analogue **5**.

The weak antiovulatory activity at 3 mg observed for analogues **5** and **8** reflects their weak *in vitro* activity (10 μg).

Inhibitors having substitutions in positions 2, 3, 6 and 10

Table II shows the effect of modifying the C-terminus of 2,3,6-trisubstituted sequences. Most of this work was completed before the synthesis of the more potent D-Phe[2] analogues (section 7), and are based on the des-Gly[10]-[Leu[2], Leu[3], D-Ala[6]]-LH–RH ethylamide sequence. The order of *in vitro* potencies for 2-substitution was Trp (**24**), Phe (**25**), Leu (**17**) (1 μg) >Nva (**19**), Nle (**20**), Val (**21**), Ile (**22**) (10 μg) > Gly (**18**), D-Ala (**23**), Tyr (**26**) (100 μg). The results indicated a requirement for a

Table I. Inhibitors having L-amino acids in positions 2 and 3 and alkyl amino acids in position 6.

No.	LH–RH Analogue Position 2	3	6	Inhibition, *in vitro*[a] LH–RH	Analogue dosage (μg/ml) 0.1	1	10	100	Antiovulatory activity Dose, sc mg/rat	Response[b]	Reference
1	Leu	Leu	(Gly)	200 ± 49	177 ± 32 (ns)	207 ± 57 (ns)	238 ± 55 (ns)	83 ± 20 (0.05)	–	–	13
2	Leu	Ser	(Gly)	106 ± 12	88 ± 27 (ns)	140 ± 27 (ns)	88 ± 38 (ns)	90 ± 19 (ns)	–	–	15
3	Leu	Asn	(Gly)	257 ± 56	275 ± 54 (ns)	269 ± 76 (ns)	213 ± 33 (ns)	185 ± 13 (ns)	–	–	15
4	Thr	Leu	(Gly)	408 ± 59	–	–	180 ± 70 (~0.02)	180 ± 37 (< 0.01)	–	–	15
5	Leu	Leu	D-Ala	552 ± 58	–	460 ± 59 (ns)	3 ± 67 (< 0.001)	–	6[c]	3/5	16
6	Val	Leu	D-Ala	327 ± 74	–	246 ± 22 (ns)	28 ± 11 (< 0.001)	–	–	–	16
7	Phe	Leu	D-Ala	145 ± 14	85 ± 20 (< 0.05)	2 ± 16 (< 0.001)	−19 ± 12 (< 0.001)	7 ± 17 (< 0.001)	–	–	–
8	Val	Leu	D-Leu	408 ± 59	–		62 ± 15 (< 0.001)[d]	56 ± 12 (< 0.001)	6[c]	3/5	–
9	Thr	Leu	D-Leu	408 ± 59	315 ± 46 (ns)	191 ± 50 (< 0.02)	36 ± 4 (< 0.001)	36 ± 7 (< 0.001)	3	4/4	–
10	Leu	Ala	D-Ala	332 ± 76	–	230 ± 28 (ns)	149 ± 32 (0.05)	115 ± 19 (0.02)	–	–	15
11	Leu	Val	D-Ala	262 ± 65	–	–	150 ± 43 (ns)	13 ± 20 (< 0.01)	–	–	16
12	Val	Val	D-Ala	282 ± 51	–	–	313 ± 57 (ns)	35 ± 11 (< 0.001)	–	–	16

[a] Values represent ΔLH ng/ml medium ± SEM (p value), 0.3 ng/ml LH–RH. [b] Response is No. of rats ovulating/No. of rats treated. [c] Peptide administered in two equally divided injections. [d] ns at 1 μg/ml.

Table II. Inhibitors having substitutions in positions 2, 3, 6 and 10.

No.	LH–RH Analogue Position 2	3	6	10	Inhibition, *in vitro*[a] LH–RH	Analogue dosage (μg/ml) 0.1	1	10	100	Antiovulatory Activity Dose, sc mg/rat	Response[b]	Reference
13	Leu	Gly	D-Ala	EA	257 ± 56	207 ± 34 (ns)	270 ± 89 (ns)	245 ± 59 (ns)	169 ± 14 (ns)	–	–	17
14	Leu	Abu	D-Ala	EA	111 ± 14	–	157 ± 18 (ns)	150 ± 13 (ns)	45 ± 20 (< 0.05)	–	–	17
15	Leu	Nva	D-Ala	EA	227 ± 42	213 ± 45 (ns)	93 ± 34 (0.02)	45 ± 14 (< 0.001)	–	6[c]	4/5	17
16	Leu	Nle	D-Ala	EA	111 ± 14	–	70 ± 14 (0.05)	28 ± 13 (0.001)	12 ± 9 (< 0.001)	–	–	17
17	Leu	Leu	D-Ala	EA	219 ± 43	166 ± 98 (ns)	60 ± 14 (< 0.01)	45 ± 20 (< 0.01)	55 ± 48 (0.02)	3	6/6	17
18	Gly	Leu	D-Ala	EA	383 ± 65	–	238 ± 37 (ns)	214 ± 18 (< 0.05)	69 ± 12 (< 0.001)	–	–	–
19	Nva	Leu	D-Ala	EA	150 ± 28	–	148 ± 36 (ns)	15 ± 30 (< 0.01)	20 ± 9 (0.001)	–	–	–
20	Nle	Leu	D-Ala	EA	150 ± 28	–	174 ± 24 (ns)	61 ± 36 (ns)	7 ± 5 (< 0.001)	–	–	–
21	Val	Leu	D-Ala	EA	257 ± 56	–	272 ± 75 (ns)	63 ± 21 (< 0.01)	–	–	–	17
22	Ile	Leu	D-Ala	EA	219 ± 43	–	186 ± 37 (ns)	−53 ± 40 (< 0.001)	25 ± 25 (< 0.01)	–	–	17
23	D-Ala	Leu	D-Ala	EA	383 ± 65	–	191 ± 38 (< 0.05)	276 ± 38 (ns)	80 ± 20 (0.001)	–	–	–
24	Trp	Leu	D-Ala	EA	235 ± 33*	193 ± 47 (ns)	73 ± 17 (< 0.01)	36 ± 10 (< 0.01)	55 ± 18 (< 0.01)	0.75 1.5	3/5 1/6	17
25	Phe	Leu	D-Ala	EA	207 ± 16	130 ± 19 (< 0.01)	77 ± 20 (< 0.001)	47 ± 22 (< 0.001)	87 + 30 (< 0.01)	3	5/6	17
26	Tyr	Leu	D-Ala	EA	241 ± 16	–	265 ± 39 (ns)	145 ± 26 (< 0.01)	11 ± 7 (< 0.001)	–	–	–
27	Phe	Leu	D-Ala	D-Ala	190 ± 23	199 ± 26 (ns)	58 ± 14 (< 0.001)	28 ± 14 (< 0.001)	32 ± 16 (< 0.001)	–	–	–
28	Abu	Ala	D-Ala	EA	282 ± 89	–	157 ± 22 (ns)	56 ± 31 (< 0.05)	62 ± 16 (< 0.05)	–	–	15
29	Ile	Ala	D-Ala	EA	293 ± 68	–	173 ± 50 (ns)	103 ± 25 (0.02)	49 ± 11 (< 0.01)	–	–	15
30	Phe	Nva	D-Ala	EA	275 ± 57*	221 ± 78 (ns)	32 ± 27 (< 0.01)	−7 ± 18 (< 0.001)	−20 ± 15 (< 0.001)	3	3/6	17
31	D-Phe	Pro	D-Phe	EA	295 ± 53*	252 ± 46 (ns)	37 ± 32 (< 0.01)	−43 ± 11 (< 0.001)	−113 ± 24 (< 0.001)	0.375 0.75	6/6 5/5	18

[a] Values represent ΔLH ng/ml medium ± SEM (p value), 0.3 ng/ml LH–RH except where marked * when 0.6 ng/ml was used. [b] Response is No. of rats ovulating/No. of rats treated. [c] Peptide administered in six equally divided injections.

large, hydrophobic side-chain in position 2 for more potent activity.

Similar investigation of substitutions in positions 3 resulted in the order Leu (**17**), Nle (**16**), Nva (**15**) > Abu (**14**) > Gly (**13**).

The incorporation of the C-terminal modification into the antiovulatory sequence, [D-Phe^2, Pro^3, D-Phe^6]-LH–RH, resulted in a decrease in the *in vitro* activity and no antiovulatory activity at 750 μg/rat (analogue **31**). A similar result has been observed for the incorporation of this modification in the inhibitor [D-Phe^2, D-Ala^6]-LH–RH [19].

Therefore, in contrast with studies on LH–RH agonists, the ethylamide modification has not been desirable in the design of inhibitors.

Table III. Inhibitors based on the sequence [D-Phe^2, amino acid^3, D-Trp^6]-LH–RH.

No.	Amino acid^3	Inhibition, *in vitro*[a] LH–RH	Analogue dosage (μg/ml) 0.1	1	10	100	Antiovulatory activity Dose, sc mg/rat	Response[b]	Reference
12	Ala	329 ± 36	163 ± 23 (< 0.01)	39 ± 25 (< 0.001)	42 ± 23 (< 0.001)	29 ± 6 (< 0.001)	0.75	5/5	–
13	Nva	440 ± 24	162 ± 19 (< 0.001)	83 ± 13 (< 0.001)	48 ± 26 (< 0.001)	128 ± 12 (< 0.001)[c]	0.75	5/5	–
14	Nle	207 ± 34	82 ± 27 (< 0.02)	19 ± 6 (< 0.001)	19 ± 10 (< 0.001)	85 ± 23 (< 0.02)	0.75	5/6	–
							1.5	1/5	
15	Met	408 ± 27	77 ± 12 (< 0.001)	52 ± 6 (< 0.001)	17 ± 4 (< 0.001)	35 ± 12 (< 0.001)	0.75	4/5	20
16	Val	207 ± 34	44 ± 5 (< 0.001)	−32 ± 24 (< 0.001)	17 ± 8 (< 0.001)	13 ± 5 (< 0.001)	0.75	5/6	20
							1.5	2/5	
17	Ile	440 ± 24	143 ± 24 (< 0.001)	21 ± 14 (< 0.001)	51 ± 17 (< 0.001)	132 ± 12 (< 0.001)	0.75	5/5	20
18	Leu	275 ± 57	1 ± 37 (< 0.01)	26 ± 20 (< 0.01)	32 ± 15 (< 0.01)	124 ± 13 (< 0.05)[c]	0.3	7/7	21
							0.75	6/13	
							1.5	0/11	
							3	0/6	
19	Pro	220 ± 35	61 ± 11 (< 0.01)[d]	15 ± 10 (< 0.001)	2 ± 15 (< 0.001)	11 ± 3 (< 0.001)	0.375	4/9	21
							0.75	0/11	
10	D-Phe	228 ± 30	97 ± 19 (~0.01)	39 ± 20 (< 0.001)	30 ± 30 (< 0.001)	12 ± 13 (< 0.001)	0.75	3/5	20

[a] Values represent Δ LH ng/ml medium ± SEM (p value), 0.6 ng/ml LH–RH. [b] Response is No. of rats ovulating/No. of rats treated. [c] Agonist activity detected at this dosage. [d] Inhibited ($p < 0.001$) at 0.03 μg/ml.

Table IV. Inhibitors based on the sequence [D-Phe^2, amino acid^3, D-Phe^6]-LH–RH.

No.	Amino acid^3	Inhibition, *in vitro*[a] LH–RH	Analogue dosage (μg/ml) 0.1	1	10	100	Antiovulatory activity Dose, sc mg/rat	Response[b]	Reference
41	Leu	197 ± 17	159 ± 23 (ns)	70 ± 9 (< 0.001)	42 ± 14 (< 0.001)	25 ± 7 (< 0.001)	0.375	5/5	21
							0.75	0/10	
42	Nva	478 ± 15	258 ± 50 (~0.001)	137 ± 36 (< 0.001)	107 ± 35 (< 0.001)	44 ± 27 (< 0.001)	0.75	3/5	–
43	Pro	313 ± 62	46 ± 2 (~0.001)[c]	17 ± 4 (< 0.001)	19 ± 6 (< 0.001)	30 ± 9 (0.001)	0.375	4/5	20
							0.75	0/5	
							0.75[d]	2/5	
44	Me–Leu	193 ± 18	83 ± 12 (< 0.001)	64 ± 15 (< 0.001)	63 ± 14 (< 0.001)	103 ± 8 (< 0.001)	0.375	4/6	–
							0.75	0/6	
45	Me–Abu	293 ± 27	109 ± 22 (< 0.001)	8 ± 4 (< 0.001)	16 ± 7 (< 0.001)	6 ± 6 (< 0.001)	0.75	4/4	–
46	Hyp	266 ± 76	179 ± 40 (ns)	23 ± 3 (< 0.01)	15 ± 5 (< 0.01)	5 ± 2 (< 0.01)	0.75	5/7	20
47	Sar	290 ± 48	339 ± 36 (ns)	127 ± 24 (~0.01)	22 ± 3 (< 0.001)	21 ± 8 (< 0.001)	0.75	3/6	20
48	Thr	193 ± 18	159 ± 11 (ns)	97 ± 29 (< 0.02)	18 ± 5 (0.001)	42 ± 4 (< 0.001)	0.75	4/4	20
49	His	520 ± 104	577 ± 67 (ns)	140 ± 23 (< 0.01)	105 ± 29 (< 0.01)	83 ± 5 (< 0.01)	0.75	4/4	–
50	Tyr	478 ± 15	426 ± 18 (0.05)	195 ± 66 (~0.001)	71 ± 33 (< 0.001)	4 ± 4 (< 0.001)	0.75	2/5	–
51	Arg	313 ± 62	276 ± 65 (ns)	51 ± 7 (< 0.01)	34 ± 12 (~0.001)	21 ± 4 (< 0.001)	0.75	3/5	20
52	Glu	442 ± 6	442 ± 11 (ns)	431 ± 15 (ns)	310 ± 34 (< 0.01)	−8 ± 19 (< 0.001)	0.75	5/5	–

[a] Values represent Δ LH ng/ml medium ± SEM (p value), 0.6 ng/ml LH–RH. [b] Response is No. of rats ovulating/No. of rats treated. [c] ns at 0.03 μg/ml. [d] Administered in propylene glycol.

Table V. Other 2,3,6-tri-substituted inhibitors.

No.	LH–RH Analogue Position 2	3	6	7	Inhibition, *in vitro*[a] LH–RH	Analogue dosage (μg/ml) 0.1	1	10	100	Antiovulatory Activity Dose, sc mg/rat	Response[b]	Reference
53	D-Trp	Pro	D-Phe	(Leu)	243 ± 26	272 ± 64 (ns)[c]	48 ± 9 (< 0.001)	29 ± 18 (< 0.001)	62 ± 11 (< 0.001)	0.75	5/5	22
54	D-His	Pro	D-Phe	(Leu)	227 ± 46	221 ± 50 (ns)	346 ± 90 (ns)	115 ± 10 (< 0.05)	6 ± 2 (~0.001)	0.75	5/5	22
55	D-Phg	Pro	D-Phe	(Leu)	243 ± 30	256 ± 14 (ns)	181 ± 42 (ns)	28 ± 19 (< 0.001)	5 ± 6 (< 0.001)	0.75	4/4	22
56	Me-D-Phe	Pro	D-Phe	(Leu)	195 ± 20	135 ± 23 (ns)	18 ± 9 (< 0.001)	−12 ± 8 (< 0.001)	10 ± 2 (< 0.001)	0.75	4/4	–
57	Phe	Pro	D-Phe	(Leu)	295 ± 53	313 ± 38 (ns)	100 ± 33 (~0.01)	11 ± 25 (< 0.001)	−13 ± 19 (< 0.001)	0.75	5/5	22
58	D-Trp	Leu	D-Trp	(Leu)	165 ± 9	220 ± 59 (ns)[c]	83 ± 18 (< 0.01)	129 ± 31 (ns)	272 ± 38 (0.02)[d]	0.75	5/5	–
59	D-Phg	Leu	D-Phe	(Leu)	239 ± 30	156 ± 11 (~0.02)	59 ± 8 (< 0.001)	31 ± 3 (< 0.001)	−3 ± 9 (< 0.001)	0.75	5/5	–
60	D-Phe	Leu	D-Phg	(Leu)	243 ± 45	297 ± 58 (ns)	125 ± 18 (< 0.05)	26 ± 7 (< 0.001)	27 ± 6 (< 0.001)	0.75	5/5	–
61	Phe	D-Leu	D-Phe	(Leu)	471 ± 78	213 ± 33 (~0.01)	67 ± 11 (< 0.001)	50 ± 9 (< 0.001)	98 ± 12 (< 0.001)	0.75	5/5	–
62	Me-D-Phe	D-Trp	D-Phe	(Leu)	269 ± 20	124 ± 41 (< 0.05)	2 ± 2 (< 0.001)	6 ± 2 (< 0.001)	42 ± 6 (< 0.001)	0.75	4/4	–
63	D-Phe	Leu	D-Trp	Me-Leu	408 ± 27	281 ± 26 (~0.01)	280 ± 34 (< 0.02)	196 ± 19 (< 0.001)	20 ± 7 (< 0.001)	0.75	4/5	–
										3[e]	3/4	
										6[e]	2/4	
64	D-Phe	Leu	D-Phe	Me-Leu	385 ± 59	134 ± 29 (< 0.01)	−15 ± 6 (< 0.001)	6 ± 4 (< 0.001)	48 ± 10 (< 0.001)	0.75	5/5	–
65	D-Phe	Nva	D-Trp	Me-Leu	291 ± 43*	245 ± 46 (ns)	80 ± 17 (< 0.001)	63 ± 20 (< 0.001)	349 ± 45 (ns)[d]	1	4/5	–
										6[e]	4/4	

[a] Values represent Δ LH ng/ml medium ± SEM (p value), 0.6 ng LH–RH except where marked by * when 0.3 ng was used. [b] Response is No. of rats ovulating/No. of rats treated. [c] ns at 0.03 μg/ml. [d] Agonist activity detected at this dosage. [e] Administered in two equally divided injections.

Table VI. *In vitro* reversibility of LH–RH inhibitors[a].

Peptide	Dose Peptide (I_3) (μg)	LH–RH (I_3–I_6) (ng)	Δ LH ± SEM (ng ml) I_3	I_4	I_5	I_6
–	–	0.6	248 ± 59 –	236 ± 28 –	265 ± 32 –	278 ± 23 –
[D-Phe^2, Pro^3, D-Trp^6]-LH–RH (39)	1	0.6	49 ± 13 0.01	132 ± 17 0.01	222 ± 18 ns	282 ± 19 ns
[D-Phe^2, Pro^3, D-Phe^6]-LH–RH (43)	1	0.6	41 ± 12 < 0.01	152 ± 30 ns	280 ± 25 ns	349 ± 11 0.0
	10	0.6	31 ± 11 < 0.01	54 ± 35 < 0.01	97 ± 31 < 0.01	207 ± 37 ns
	100	0.6	58 ± 12 0.01	103 ± 31 0.01	113 ± 35 < 0.01	201 ± 39 ns
[D-Phe^2, Met^3, D-Trp^6]-LH–RH (35)	1	0.6	41 ± 10 < 0.01	90 ± 26 < 0.01	121 ± 18 < 0.01	222 ± 35 ns
[D-Phe^2, Val^3, D-Trp^6]-LH–RH (36)	1	0.6	70 ± 11 ~0.01	175 ± 34 ns	219 ± 42 ns	268 ± 24 ns
[D-Phe^2, Nle^3, D-Trp^6]-LH–RH (34)	1	0.6	58 ± 1 < 0.01	92 ± 13 < 0.001	191 ± 6 0.05	249 ± 23 ns

[a] The analogue + LH–RH were added during incubation period I_3 and LH–RH was added alone during the next consecutive hourly incubation periods I_4, I_5, and I_6.

Table VII. Inhibitors based on the sequence [Residue[1], D-Phe^2, Pro^3, D-Phe^6]-LH–RH.

No.	Residue[1]	LH–RH	Inhibition, *in vitro*[a] Analogue Dosage (μg/ml) 0.03	0.1	1	10	100	Antiovulatory activity Dose, sc mg/rat	Response[b]	Reference
66	Cpc	145 ± 12	–	19 ± 6 (< 0.001)	11 ± 3 (< 0.001)	21 ± 8 (< 0.001)	75 ± 25 (< 0.05)	0.75	5/5	18
67	Chc	457 ± 26	–	172 ± 24 (< 0.001)	22 ± 19 (< 0.001)	6 ± 18 (< 0.001)	–	0.75	3/4	22
68	Bz	408 ± 87	–	292 ± 31 (ns)	106 ± 40 (~0.01)	56 ± 15 (< 0.01)	68 ± 21 (< 0.001)	0.75	5/5	22
69	Ac	442 ± 6	–	56 ± 20 (< 0.001)	16 ± 12 (< 0.001)	12 ± 6 (< 0.001)	13 ± 10 (< 0.001)	0.75	6/6	22
70	Ac–Met	151 ± 32	–	437 ± 157 (ns)	190 ± 17 (ns)	43 ± 20 (< 0.05)	10 ± 10 (< 0.01)	0.75	5/5	22
71	Pro	645 ± 12	–	641 ± 4 (ns)	628 ± 20 (ns)	–	67 ± 27 (< 0.001)	0.75	5/5	22
72	Hyp	269 ± 20	–	127 ± 18 (< 0.001)	112 ± 27 (< 0.01)	32 ± 9 (< 0.001)	−11 ± 14 (< 0.001)	0.75	4/4	22
73	Glu	192 ± 20	–	123 ± 23 (~0.05)	89 ± 11 (~0.001)	6 ± 8 (< 0.001)	12 ± 4 (< 0.001)	0.75	5/5	18
74	Kic	1014 ± 166	600 ± 90 (~0.05)[c]	216 ± 48 (< 0.001)	−22 ± 25 (< 0.001)	33 ± 14 (< 0.001)	–	0.75	5/5	–
75	H	321 ± 56	–	84 ± 12 (< 0.01)	14 ± 3 (< 0.001)	8 ± 2 (< 0.001)	16 ± 3 (< 0.001)	0.75	4/5	22
76	D-<Glu	451 ± 15*	197 ± 42 (< 0.001)	144 ± 10 (< 0.001)	42 ± 6 (< 0.001)	48 ± 15 (< 0.001)	43 ± 18 (< 0.001)	0.2	5/5	22
								0.75	5/5	
								1.0	6/6	

[a] Values represent Δ LH ng/ml medium ± SEM (p value), 0.6 ng LH–RH except where marked by * when 0.47 ng LH–RH used. [b] Response is No. of rats ovulating/No. of rats treated. [c] ns at 0.01 μg/ml.

Inhibitors having D-aromatic amino acids in positions 2 and 6 and substitution in position 3

The incorporation of D-Phe into position 2 and either D-Trp or D-Phe into position 6 has been found to significantly enhance inhibitory potency and has led to analogues which completely inhibit ovulation at 750 μg/rat and which inhibit the action of 0.6 ng LH–RH *in vitro*, at 0.1 μg (analogue to LH–RH ratio 166:1).

Substitution into position 3 of the sequence [D-Phe^2, amino acid^3, D-Trp^6]-LH–RH gave the order of antiovulatory activity at 750 μg/rat for 3-substitution as Pro (**39**), D-Trp (100%) > Leu (**38**), D-Phe (**40**) (partial) > Ala (**32**), Nva (**33**), Nle (**34**), Val (**36**), Ile (**37**), Met (**35**) (Table III).

The order of antiovulatory potency at 750 μg/rat for analogues having the sequence [D-Phe^2, amino acid^3, D-Phe^6]-LH–RH was Pro (**43**), Leu (**41**), N–Me–Leu (**44**) (100%) > Nva (**42**), Hyp (**46**), Sar (**47**), Tyr (**50**), Arg (**51**) (partial) > N–Me–Abu (**45**), Thr (**48**), His (**49**), Glu (**52**) (Table IV).

For the Pro^3-analogues **39** and **43**, the substitution of D-Trp or D-Phe into position 6 gave equipotent antiovulatory activities. The best ovulation inhibitors inhibited at 750 μg/rat and had Pro, N–Me–Leu or D-Trp in position 3.

It appears that D-Phe in position 2 of [D-Phe^2, Pro^3, D-Phe^6]-LH–RH (**43**) is essential for potent antiovulatory activity and *in vitro* activity (Table V). The order of *in vitro* potency when D-Phe^2 in **43** was substituted by other aromatic amino acids was D-Phe (**43**) (0.1 μg) > D-Trp (**53**) (1 μg) > D-Phg (**55**), L-Phe (**57**) (10 μg) > D-His (**54**) (100 μg). The analogues **53** to **57** were inactive at 750 μg/rat in the antiovulation assay. Therefore, a monocyclic, non-polar aromatic side chain, in the D-configuration and spaced by at least one CH_2 group from the α-carbon appears to be necessary, although it has not been definitely established that aromaticity is essential. An unsubstituted α-NH in position 2 is also important because the N–Me–D-Phe^2 analogues **56** and **62**, and **76** (see later) were significantly less active than the corresponding D-Phe^2 analogues.

The variation of position 6 in the sequence [D-Phe^2, Leu^3, D-amino acid^6]-LH–RH gave the order of potency *in vitro*, as D-Trp (**38**) > D-Phe (**41**) > D-Phg (**60**). In the corresponding Pro^3 series the D-Trp^6 (**39**) and D-Phe^6 (**43**) analogues were comparable *in vitro*. Displacement of the D-Phe

Table VIII. Other inhibitors with variations in positions 1, 2, 3 and 6.

No.	LH–RH Analogue Position 1	2	3	6	Inhibition, *in vitro*[a] LH–RH	Analogue Dosage (μg/ml) 0.03	0.1	1	10	100	Antiovulatory activity Dose, sc mg/rat	Response[b]	Reference
77	Cpc	Me-D-Phe	Pro	D-Phe	321 ± 56	–	284 ± 39 (ns)	209 ± 37 (ns)	170 ± 37 (~0.05)	110 ± 15 (< 0.01)	0.75	5/5	–
78	D-<Glu	D-Phe	D-Trp	D-Phe	451 ± 15*	88 ± 17 (< 0.001)	63 ± 17 (< 0.001)	13 ± 6 (< 0.001)	18 ± 7 (< 0.001)	43 ± 14 (< 0.001)	0.1 0.2 0.35	2/9 0/7 1/10	22
79	D-<Glu	D-Phe	(Trp)	D-Phe	622 ± 59	507 ± 52 (0.05)	273 ± 14 (< 0.001)	31 ± 18 (< 0.001)	80 ± 26 (< 0.001)	104 ± 16 (< 0.001)[c]	0.75	6/7	22
80	D-<Glu	D-Phe	Me-Leu	D-Phe	537 ± 87	228 ± 27 (< 0.01)	15 ± 40 (< 0.001)	23 ± 6 (< 0.001)	36 ± 17 (< 0.001)	36 ± 90 (< 0.01)	0.2 0.35 0.75	5/5 6/6 5/5	22
81	D-<Glu	D-Phe	Me-Phe	D-Phe	599 ± 14	–	433 ± 60 (< 0.05)	91 ± 24 (< 0.001)	47 ± 29 (< 0.001)	–	0.75	5/5	22
82	D-<Glu	D-Phe	Pro	D-Trp	622 ± 59	469 ± 94 (ns)	600 ± 38 (ns)	176 ± 23 (< 0.001)	42 ± 20 (< 0.001)	−2 ± 11 (< 0.001)	0.75	5/5	– –
83	Cpc	D-Phe	Pro	D-Trp	322 ± 65	−11 ± 27 (< 0.001)	2 ± 30 (< 0.001)	−24 ± 9 (< 0.001)	34 ± 21 (~0.001)	18 ± 7 (< 0.001)	0.75 1.5	6/6 3/3	–
84	Chc	D-Phe	Pro	D-Trp	1110 ± 108	384 ± 28 (< 0.001)	160 ± 37 (< 0.001)	2 ± 8 (< 0.001)	125 ± 52 (< 0.001)	74 ± 58 (< 0.001)	0.75	6/6	–
85	D-<Glu	D-Phe	(Trp)	D-Trp	143 ± 24	83 ± 29 (< 0.001)	19 ± 10 (< 0.001)	9 ± 7 (< 0.001)	−10 ± 8 (< 0.001)	6 ± 16 (< 0.001)	0.75	4/5	–

[a] Values represent Δ LH ng/ml medium ± SEM (p value), 0.6 ng LH–RH except where indicated by * when 0.47 ng LH–RH was used. [b] Response is No. of rats ovulating/No. of rats treated. [c] Agonist activity detected at this dosage.

from position 6 to position 5 (**112**) greatly reduced activity. The results of the N–Me–Leu[7] analogues 63 to 65) indicate that this modification was not beneficial in these examples.

The data in Table VI show that these analogues inhibit reversibly *in vitro* with the ease of reversal being structure dependent.

Inhibitors based on changes in positions 1, 2, 3 and 6

The analogues of Tables VII to IX show the effect of structural modification of the <Glu residue in position 1 for 1,2,3,6-tetra-substituted analogues.

The Cpc analogue, [Cpc[1], D-Phe[2], Pro[3], D-Phe[6]]-LH–RH (**66**), was as active *in vitro* as the corresponding <Glu analogue **43**. Therefore, the <Glu[1] residue is not necessary for potent inhibitory activity at least at the pituitary level. Surprisingly, the Cpc analogue did not inhibit ovulation at 750 μg/rat under our standard assay conditions. The related analogues having acetyl in position 1 (**69**) and the shortened des[1]-sequence **75** significantly inhibited *in vitro* at 0.1 μg. The corresponding Chc (**67**), Bz (**68**), Ac–Met (**70**), Pro (**71**), Hyp (**72**), Glu (**73**), Kic (**74**) and D-<Glu (**76**) analogues were less active. None of these Pro[3] analogues inhibited ovulation at 750 μg/rat.

The replacement of <Glu[1] in [D-Phe[2], Pro[3], D-Trp[6]]-LH–RH (**39**) by D-<Glu (82), Cpc (**83**) and Chc (**84**) has given analogous results.

The announcement that [D-<Glu[1], D-Phe[2], D-Trp[3], D-Trp[6]]-LH–RH had enhanced antiovulatory activity [23] provided a key to rationalizing these results by comparing the activities of a series of analogues based on the sequence [D-<Glu[1], D-Phe[2], amino acid[3], D-Phe[6]]-LH–RH with substitution in position 3 (Tables VII and VIII). The order of *in vitro* potencies was D-Trp (**78**) (0.03 μg) > N–Me–Leu (**80**) (0.1 μg) > Pro (**76**), N–Me–Phe (**81**),

Table IX. Inhibitors based on the sequence [Residue[1], D-Phe[2], D-Trp[3], D-Trp[6]]-LH–RH.

No.	Residue[1]	Inhibition, *in vitro*[a] LH–RH	Analogue Dosage (μg/ml) 0.01	0.03	0.1	1	10	Antiovulatory activity Dose, sc mg/rat	Response[b]	Reference
86	D-<Glu	511 ± 85	204 ± 6 (<0.01)[c]	59 ± 3 (<0.001)	–	–	–	0.2	0/7	24
								0.75	0/5	
87	Ac–Pro	1003 ± 178	–	2 ± 2 (<0.001)	−12 ± 10 (<0.001)	1 ± 11 (<0.001)	–	0.1	4/5	24
								0.2	0/4	
								0.75	0/6	
88	Ac-D-Pro	1003 ± 178	–	41 ± 31 (<0.001)	4 ± 10 (<0.001)	20 ± 13 (<0.001)	–	0.2	4/4	24
								0.75	0/4	
89	Ac–Hyp	414 ± 94	177 ± 32 (<0.05)	54 ± 18 (<0.01)	81 ± 20 (<0.01)	27 ± 18 (~0.001)	17 ± 15 (~0.001)	0.1	2/5	–
								0.2	0/6	
90	Ac–Sar	273 ± 27	–	–	24 ± 12 (<0.001)	−5 ± 6 (<0.001)	32 ± 9 (<0.001)	0.2	6/6	–
								0.75	5/6	
91	Pro	341 ± 22	130 ± 21 (<0.001)	147 ± 21 (<0.001)	49 ± 7 (<0.001)	3 ± 7 (<0.001)	26 ± 8 (<0.001)	0.2	2/5	–
								0.75	0/5	
92	Sar	273 ± 27	–	–	58 ± 12 (<0.001)	21 ± 7 (<0.001)	23 ± 9 (<0.001)	0.2	5/6	–
								0.75	0/6	
93	Kic	1014 ± 166	279 ± 48 (~0.001)	311 ± 51 (<0.01)	99 ± 24 (<0.001)	−15 ± 20 (<0.001)	−12 ± 18 (<0.001)	0.2	4/4	24
								0.75	0/4	
94	Cpc	1003 ± 178	230 ± 25 (<0.01)[d]	105 ± 15 (0.001)	53 ± 21 (<0.001)	8 ± 8 (<0.001)	–	0.2	7/7	24
								0.75	4/4	
95	Ac	463 ± 36	121 ± 23 (<0.001)	34 ± 5 (<0.001)	15 ± 2 (<0.001)	–	–	0.2	6/6	–
								0.75	1/4	
96	H	463 ± 36	294 ± 22 (<0.01)	109 ± 27 (<0.001)	3 ± 7 (<0.001)	–	–	0.2	6/6	–
								0.75	5/5	
97	N–Ac–Thr	562 ± 158	–	60 ± 12 (0.01)	5 ± 37 (<0.01)	33 ± 24 (<0.01)		0.05	4/9	
								0.1	2/9	–
								0.2	1/7	
98	N^{α}–Ac–Trp	562 ± 150	212 ± 110 (ns)	94 ± 57 (<0.02)	14 ± 23 (<0.01)	20 ± 4 (<0.01)		0.2	5/6	
								0.75	4/6	–
								1.5	2/4	

[a] Values represent Δ LH ng/ml medium ± SEM (p value), 0.6 ng LH–RH; [b] response is No. of rats ovulating/No. of rats treated; [c] Δ LH at 0.003 μg/ml was 256 ± 48 (~0.02); [d] ns at 0.003 μg/ml.

Trp (79) (1 to 10 μg). Although the D-Trp[3] analogue 78 completely inhibited ovulation at 200 and 350 μg/rat, the other analogues were inactive at 750 μg/rat. Similar results were obtained with the corresponding D-Trp[6] analogues having D-Trp (86), Pro (82) or Trp (85) in position 3 (Tables VIII and IX).

Although the antiovulatory activities of 2,3,6-trisubstituted LH–RH sequences having D-Trp, Pro or N–Me–Leu in position 3 were comparable (Section 7), the substitution of <Glu[1] by D-<Glu in these sequences resulted in enhancement of potency for the D-Trp[3] analogue and a reduction of potency for the Pro[3] and N–Me–Leu[3] analogues. It is possible that the Pro and N–Me–Leu, but not the D-Trp, in position 3 exists in a *trans-cis* equilibrium, and that the substitution of D-<Glu adversely affects this equilibrium by altering binding capability and/or transportation. Although such an explanation could also be applied, at least in part, to analogues 66 to 75, other factors, such as enzymatic and transportation effects, also need to be considered.

The analogues 86 to 98 in Table IX show the effect of varying position 1 in the sequence [residue[1], D-Phe[2], D-Trp[3], D-Trp[6]]-LH–RH. The most active inhibitors in this group are characterized by inhibiting *in vitro* by greater than 50% at 0.03 μg or less and completely inhibiting ovulation at 200 μg/rat.

The order of potency in the antiovulatory assay was D-<Glu (86), Ac–Pro (87), N–Ac–Hyp (89), N–Ac–Thr (97) (100% at 200 μg/rat) > Ac-D-Pro (88), Pro (91), Sar (92), Kic (93), Ac (95) (100% at 750 μg/rat) > Ac–Sar (90), Cpc (94), H (96), N^{α}–Ac–Trp (98).

These results lead to the following conclusions concerning the nature of residue[1] for highest activity: (1) position 1 can equally well accommodate residues of the L- and D-configuration (contrast the corresponding Pro[3] series); (2) the configuration of the most potent optical isomer depends on the residue substituted (*e. g.* D-<Glu > L-<Glu, Ac–Pro > Ac-D-Pro); (3) some polar character is required; (4) an N-protected residue appears important (Ac–Pro > Pro, Ac > H). It is also interesting to note that the shortened analogues 75 and 96 and their N^{α}-acetylated derivatives, 69 and 95, significantly inhibited at 0.1 μg, *in vitro*, as did [D-Phe[2], Pro[3], D-Phe[6]]-LH–RH, but that only 95 inhibited ovulation at 750 μg/rat.

Inhibitors based on linear sequences longer than decapeptides

One aspect of our structure-activity studies on the Ac–Pro[1] analogue (87) involved replacing the CH_3CO-group, which can be regarded as des-amino-Gly, by other amino acid residues. That is, peptide fragments were substituted into position 1 in the [residue[1], D-Phe[2], D-Trp[3], D-Trp[6]]-LH–RH sequence (Table X).

The order of antiovulatory potency for some undecapeptide analogues in which residue 1 was varied was <Glu–Pro (99) (100%, 200 μg) >

Table X. Inhibitors based on linear sequences longer than decapeptides: [Residue[1], D-Phe[2], D-Trp[3], D-Trp[6]]-LH–RH.

No.	Residue[1]	Inhibition, *in vitro*[a] LH–RH	Analogue Dosage (μg/ml) 0.01	0.03	0.1	1	10	Antiovulatory activity Dose, sc mg/rat	Response[b]
99	(<Glu–Pro)	341 ± 22	51 ± 3 (<0.001)	76—12 (<0.001)	13 ± 6 (<0.001)	14 ± 7 (<0.001)	1 ± 4 (<0.001)	0.1	2/5
								0.2	0/4
100	(<Glu–Gly)	414 ± 94	111 ± 28 (0.01)	60 ± 28 (<0.01)	98 ± 19 (<0.01)	60 ± 22 (<0.01)	–	0.2	3/3
								0.75	1/6
101	(Gly–Pro)	242 ± 15	55 ± 14 (<0.001)	54 ± 24 (<0.001)	6 ± 13 (<0.001)	−31 ± 29 (<0.001)	45 ± 17 (<0.001)	0.2	5/5
								0.75	4/5
101a	(<Glu–Asn)	–	–	–	–	–	–	0.2	3/4
101b	Ac–(Pro–Pro)	422 ± 39	–	97 ± 24 (<0.001)	7 ± 9 (<0.001)	3 ± 7 (<0.001)	–	0.2	0/5
101c	Ac–(Gln–Pro)	369 ± 26	327 ± 24 (ns)	–	263 ± 23 (<0.02)	50 ± 11 (<0.001)	–	0.2	5/5
101d	(D-<Glu–Pro)	422 ± 39	–	119 ± 37 (<0.001)	15 ± 6 (<0.001)	2 ± 7 (<0.001)	–	0.2	5/9
101e	(<Glu–Gln–Pro)	369 ± 26	295 ± 9 (<0.05)	–	198 ± 21 (<0.001)	133 ± 12 (<0.001)	–	0.2	7/7

[a] Values represent Δ LH ng/ml medium ± SEM (p value), 0.6 ng LH–RH. [b] Response is No. of rats ovulating/No. of rats treated.

< Glu–Gly (**100**) (100%, 750 μg) > Gly–Pro (**101**). The high in *vitro* potency of the (Gly–Pro)-analogue **101**, in contrast with its lack of *in vivo* activity, may be due to enzymatic inactivation *in vivo*.

Therefore, in contrast to agonist sequences, position 1 in inhibitors can accomodate at least dipeptide fragments and linear peptides, longer than a decapeptide, now constitute a new class of potent ovulation inhibitors. The presence of the rigid ring of Pro and a "protected" N-terminus may be important.

Miscellaneous analogues

These analogues, shown in Table XI, exhibited low inhibition activities and/or agonist properties. The analogue **110** significantly released LH and FSH at 100 μg at a ratio of LH:FSH greather than that induced by LH–RH.

Irreversible inhibition

The incorporation into peptide sequences of chemically reactive groups capable of reacting with moieties on or in the vicinity of the LH–RH receptor(s), represents an alternative design. One analogue, [chlorambucil^1, Leu^2, Leu^3, D-Ala^6]-LH–RH (**113**), has been shown to inhibit the action of LH–RH, in a modified *in vitro* assay in which the pituitaries were pre-incubated with the analogue (3 to 5 μg) prior to adding LH–RH (0.3 ng) (Table XII). This protocol was necessary, because the analogue unexpectedly acted as an agonist at dosages of 1 to 100 μg in contrast to [Leu^2, Leu^3, D-Ala^6]-LH–RH. Multiple treatments were more effective than a single incubation. Unlike [Leu^2, Leu^3, D-Ala^6]-LH–RH, the chlorambucil^1-analogue **113** irreversibly inhibited *in vitro*. The chlorambucil^1-analogue did not release TSH or GH indicating that its activities could be specific at the receptor site for LH–RH [28].

On the correlation of LH–RH inhibition assays and antiovulatory assays

In general, only a partial correlation exists between the results of *in vitro* and antiovulation assays. All analogues which inhibit ovulation at 750 μg/rat or less strongly inhibit *in vitro* at an analogue to LH–RH ratio of 166:1. However, many exceptions are now evident. Not all analogues active at 166:1 or less *in vitro* inhibit ovulation at 750 μg/rat or at substantially increased dosages.

Comparative studies have given the following results [29] (Table XIII).

(1) Inhibitors having comparable potency *in vitro* can display a range of antiovulatory activities. For example, Ac-[Pro^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH–RH and [Cpc^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH–RH were essentially equipotent at 0.03 μg *in vitro*, but the Ac–Pro analogue inhibited ovulation at 200 μg/rat whilst the Cpc analogue was inactive at 750 μg/rat.

Table XI. Miscellaneous analogues.

No.	Analogue	Inhibition, *in vitro*[a] LH–RH	Analogue Dosage (μg/ml) 0.1	1	10	100	Antiovulatory activity Dose, sc mg/rat	Response[b]	Reference
102	LH–RH–OH	516 ± 89	–	583 ± 53 (ns)[c]	[c]	–	–		26
103	[Tyr^3, Trp^5]-LH–RH–OH	635 ± 25	–	–	603 ± 50 (ns)[c]	[c]	–		26
104	[Gly^{1a}]-LH–RH–OH	590 ± 27	–	–	559 ± 24 (ns)	[c]	–		26
105	[Gly^{2a}]-LH–RH–OH	398 ± 53	–	–	478 ± 53 (ns)	[c]	–		26
106	Thr–Pro–Arg–Lys–OH	150 ± 7	–	117 ± 37 (ns)	91 ± 12 (< 0.01)	176 ± 19 (ns)	0.6[d]	4/5	27
107	LH–RH(1–6)-Thr–Pro–Arg–Lys–OH	146 ± 32	308 ± 82 (ns)	307 ± 90 (ns)	303 ± 67 (ns)	616 ± 15 (< 0.001)[c]	6[d]	6/6	–
108	[Ile^2]-LH–RH	590 ± 27	–	572 ± 31 (ns)[c]	–	–	–		13
109	[Tyr^3, Trp^5]-LH–RH	635 ± 25	–	–	–	671 ± 23 (ns)	–		13
110	[D-Phe^2, Ala^4, D-Phe^6]-LH–RH	146 ± 32	79 ± 31 (ns)	17 ± 19 (< 0.01)	−50 ± 37 (< 0.01)	507 ± 54 (< 0.001)[c]	6[d]	3/6	–
111	[D-Phe^2, Phe^5, D-Phe^6]-LH–RH	165 ± 9*	165 ± 34 (ns)	75 ± 21 (< 0.01)	118 ± 36 (ns)	189 ± 50 (< 0.001)[c]	0.75	5/5	–
112	[D-Phe^2, Pro^3. D-Phe^5]-LH–RH	192 ± 20	302 ± 28 (< 0.01)	279 ± 51 (ns)	161 ± 18 (ns)	20 ± 6 (< 0.001)	0.75	6/6	–

[a] Values represent Δ LH ng/ml medium ± SEM (p value), 0.3 ng LH–RH except where indicated by * when 0.6 ng LH–RH was used. [b] Response is No. of rats ovulating/No. of rats treated. [c] Agonist activity detected at this dosage. [d] Administered in six equally divided injections.

Table XII. Agonist and inhibition activities, of [Chl[1], Leu[2], Leu[3], D-Ala[6]]-LH–RH.

Additions to medium Analogue (μg/ml) P_1	P_2	I_3	LH–RH (ng/ml) I_5 and I_6	Δ LH ng/ml medium ± SEM (p value)
–*			–	48 ± 21
0.1			–	102 ± 19 (ns)
1			–	320 ± 58 (0.001)
10			–	486 ± 58 (< 0.001)
100			–	> 652 (< 0.001)
–			0.3	307 ± 46
1			0.3	279 ± 80 (ns)
3			0.3	120 ± 37 (0.01)
10			0.3	38 ± 17 (< 0.001)
–			0.3	276 ± 37
1	1		0.3	178 ± 26 (< 0.05)
3	3		0.3	93 ± 17 (0.001)
10	10		0.3	30 ± 23 (< 0.001)
1	1	1	0.3	105 ± 50 (0.02)

* Propylene glycol control.

Antiovulatory activity: 1.5 × 2 mg/rat, 5/6 rats ovulated.

(2) The above two analogues were essentially equipotent in inhibiting LH–RH in adult male chimpanzees at a ratio of 333:1 and in adult male rats at 100:1, in contrast with their relative antiovulatory activities. However, at a ratio, of 30:1 in rats, only the Ac–Pro[1] analogue inhibited.

(3) The analogues [D-Phe[2], Pro[3], D-Trp[6]]-LH–RH, [Cpc[1], D-Phe[2], Pro[3], D-Trp[6]]-LH–RH, Ac-[Pro[1], D-Phe[2], D-Trp[3], D-Trp[6]]-LH–RH, Ac-[Hyp[1], D-Phe[2], D-Trp[3], D-Trp[6]]-LH–RH, and [(<Glu–Pro)[1], D-Phe[2], D-Trp[3], D-Trp[6]]-LH–RH inhibited LH–RH in adult male rats at a dosage ratio of 30:1 but the antiovulatory activities were 750 μg, inactive at 750 μg, 200 μg, 200 μg and 200 μg, respectively.

(4) Ovulation inhibitors, active at 200 μg/rat, also inhibited LH–RH in adult male chimpanzees at a ratio of analogue to LH–RH of 333:1.

Since the Cpc-analogues inhibit LH–RH *in vitro* and *in vivo*, their lack of activity in the antiovulation assay cannot readily be explained in terms of enzymatic inactivation or differences in metabolism.

Table XIII. Comparative assays of LH–RH inhibitors for inhibition of LH–RH and inhibition of ovulation.

No.	LH–RH analogue	Inhibition of LH–RH *in vitro*[b] Control	Assay	Adult male rats (iv)[d] Control	Assay	Adult male rats (sc)[f] Dose (mg)	Assay	Adult male chimpanzees (iv)[g] 0 min	+ 15 min	+ 30 min	Antiovulatory activity Dosage, sc mg/rat	Response[b]
39	D-Phe[2], Pro[3], D-Trp[6]	220 ± 35	61 ± 11 (< 0.01)	11.8 ± 0.6	4.0 ± 0 (< 0.001)	–	–	6.4 ± 0.8	20.0 ± 2.9 (ns)	16.6 ± 1.4 (ns)	0.375 0.75	4/9 0/11
94	Cpc[1], D-Phe[2], D-Trp[3], D-Trp[6]	1003 ± 178	53 ± 21 (< 0.001)	9.0 ± 0.6	8.8 ± 1.0 (ns)[e]	0.75	7.4 ± 0.92 (ns)	3.8 ± 2.4	7.5 ± 1.6 (< 0.001)	7.0 ± 2.1 (< 0.01)	0.2 0.75	7/7 4/4
83	Cpc[1], D-Phe[2], Pro[3], D-Trp[6]	322 ± 65	2 ± 30 (< 0.001)	11.8 ± 0.6	7.4 ± 1.5 (~0.02)	0.75	6.2 ± 0.86 (ns)	3.8 ± 1.6	11.7 ± 1.9 (< 0.01)	9.5 ± 1.3 (< 0.01)	0.75 1.5	6/6 3/3
87	Ac-Pro[1], D-Phe[2], D-Trp[3], D-Trp[6]	1003 ± 178	–12 ± 10 (< 0.001)	9.0 ± 0.6	3.2 ± 0.5 (< 0.001)	0.3	1.93 ± 0.6 (< 0.01)	1.5 ± 0.5	7.5 ± 0.9 (< 0.001)	5.0 ± 1.1 (< 0.001)	0.1 0.2	2/3 0/12
89	Ac-Hyp[1], D-Phe[2], D-Trp[3], D-Trp[6]	414 ± 94	81 ± 20 (< 0.01)	10.7 ± 0.8	1.3 ± 0.3 (< 0.001)	–		–	–	–	0.1 0.2	2/5 0/6
99	(<Glu–Pro)[1], D-Phe[2], D-Trp[3], D-Trp[6]	341 ± 22	13 ± 6 (< 0.001)	10.7 ± 0.8	2.0 ± 0.3 (< 0.001)	0.2	4.12 ± 0.67 (< 0.02)	3.3 ± 0.5	5.8 ± 1.7 (< 0.001)	2.3 ± 1.9 (< 0.001)	0.1 0.2	3/8 0/4
85	D-<Glu[1], D-Phe[2], (Trp[3]), D-Trp[6]	511 ± 85	19 ± 10 (< 0.001)[c]	11.8 ± 1.0	8.8 ± 1.0 (ns)*	–	–	–	–		0.75	4/5

[a] Values represent Δ LH ng/ml medium ± SEM (p value). Saline controls were performed. [b] Control was 0.6 ng/ml LH–RH and Assay was 0.6 ng/ml LH–RH + 0.1 μg/ml analogue (dosage ratio 166:1). [c] Inhibited ($p < 0.001$) at 0.03 μg/ml. [d] Control was 0.1 μg LH–RH (iv) and Assay was 0.1 μg LH–RH + 3 μg analogue (iv) (dosage ratio 30:1) except where marked by * when the ratio was 100:1, LH after + 15 min. [e] Inhibited ($p < 0.01$) at dosage ratio of 100:1. [f] Analogue administered sc 2 h before 0.1 μg LH–RH (iv). Δ LH measured 15 min after administration of LH–RH. Control was 0.1 μg LH–RH producing Δ LH of 10 ± 1.84 ng/ml and Assay was 0.1 μg LH–RH (iv) + analogue (sc). [g] Control was 3 μg LH–RH (iv) giving Δ LH of 7.7 ± 1.1 (0 min), 22.4 ± 2.7 (+ 15 min) and 18.4 ± 2.9 (+ 30 min). Assay was 3 μg LH–RH + 1000 μg analogue (iv) (dosage ratio 333:1). [h] Analogues administered sc in corn oil on noon of proestrus. Response is No. of rats ovulating/No. of rats treated.

However, the peptides were administered i.v. in the above *in vivo* LH–RH assays and s.c. in the anti-ovulation assay. This difference raised a question on the absorption of the peptides through the lipid layers of subcutaneous tissue, *i.e.* altered membrane transportation properties. The Cpc residue is much less polar than, for example, <Glu or Ac–Pro residues. Support for this notion has been obtained by measuring the inhibition of LH–RH, given i.v., in adult male rats, when the peptides are administered s.c. In this case, Ac-[Pro^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH–RH and [(< Glu–Pro)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH–RH effectively inhibited the action of 0.1 μg LH–RH at dosages of 300 μg and 200 μg, respectively, whilst [Cpc^1, D-Phe^2, Pro^3, D-Trp^6]-LH–RH and [Cpc^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH–RH were ineffective at 750 μg. For this particular assay the results of the LH–RH *in vivo* inhibition assay paralleled the antiovulatory results.

Dissociated activities have also been observed for analogues **78** and **86** having L-Trp in position 3. Although [D-<Glu^1, D-Phe^2, Trp^3, D-Trp^6]-LH–RH (**86**) strongly inhibited *in vitro* at 0.03 and 0.1 μg, it did not inhibit ovulation at 750 μg/rat. In contrast with the Cpc-analogues, analogue **86** did not inhibit LH–RH in adult male rats at a ratio of 100:1, suggesting that enzymatic inactivation may be important. Similarly, analogues [D-Phe^2, D-Trp^6]-LH–RH and [D-Phe^2, Pro^3, D-Trp^6]-LH–RH (**39**) had comparable activities *in vitro*, but only analogue **39** inhibited ovulation at 750 μg/rat. Surprisingly, analogue **78** had significant agonist activity at 100 μg *in vitro*, which presumably masked its inhibitory activity. The results on these L-Trp^3 analogues, *i.e.*, inhibitors based on changes in positions 2 and 6, or in positions 1, 2, and 6, emphasize the importance of designing inhibitors having suitable substitution in position 3.

A priori, it seems reasonable to conclude that LH–RH inhibitors inhibit ovulation in rats by acting on the pituitary and inhibiting the LH–FSH preovulatory surge on proestrus. The analogues are most effective when administered near the LH–FSH surge and the most active ovulation inhibitors very effectively inhibit both the LH and FSH responses of LH–RH *in vitro* and *in vivo*, although the reverse is not always true.

Small hypothalamic hypophysiotropic peptides act at multiple anatomic sites and exhibit multiple functional activities. Although there is indirect evidence that LH–RH inhibitors could act at additional sites, for example, the ovary, direct evidence is not yet available.

Inhibition of ovulation in rhesus monkeys

One of our analogues, [D-Phe^2, Pro^3, D-Phe^6]-LH–RH (**43**), was synthesized in sufficient quantity (3 g) for evaluation in the Rhesus monkey (Macaca Mulatta). Preliminary data indicate that treatment with 300 mg of **43**, in six divided injections administered over a 40 h time span (injection, s.c., every 8 h of 50 mg dispersed in 1 ml corn oil) did inhibit the action of endogenous LH–RH during the spontaneous menstrual cycle. The absence of clear sites of follicular rupture in two of the three treated animals also strongly suggested that ovulation did not occur. More definitive results will presumably be achieved when more potent inhibitors, such as [(<Glu–Pro)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH–RH, are evaluated.

Inhibition by a minipump (Alza Corp.)

The infusion of [D-Phe^2, Pro^3, D-Trp^6]-LH–RH in propylene glycol at a rate of 375 μg/day for 4 days from a s.c. implanted minipump completely inhibited ovulation in cycling female rats and decreased serum LH levels in castrated male rats. The corresponding Leu^3 analogue was not effective. The infusion of LH–RH and the super agonist des-Gly^{10}-[D-Ala^6]-LH–RH ethylamide at 375 and 6 μg/day, respectively, for 4 days completely blocked uterine implantation sites of mated rats. In contrast, the Pro^3 and Leu^3 inhibitors did not block the uterine implantation sites indicating a difference in mechanism of contraception for agonists and inhibitors of LHRH [30].

Perspectives

With the currently available analogues, the proposal that LH–RH inhibitors can act ac ovulation inhibitors has been proven at least in rats and monkeys.

In the design of these inhibitors, it has been found that certain structure-activity relationships for LH–RH agonists can be carried over the design of inhibitors. The most potent ovulation inhibitors have substitutions in positions 1, 2, 3 and 6, *i.e.* in four out of ten positions. Structural modification

in, for example, positions 1, 2 and 3, could influence the conformation of the molecule to such an extent that the optimum substitution for other parts of the molecule could be different for agonists and inhibitors. This would necessitate the synthesis of LH–RH analogues with five or more changes in the molecule.

The discovery of the highly potent undecapeptide analogue, [(<Glu–Pro)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH–RH, has opened a new category of peptides longer than decapeptides. Analogues in this category could also be designed which (1) have different degress of lipophilicity (*cf.* the high antiovulatory activity of the N–Ac–Thr-analogue 97), or (2) "prodrug" characteristics.

With the ever growing synthesis of specially designed sequences and the expansion of primate studies, the design of inhibitors or LH–RH as ovulation inhibitors is one of great promise.

Experimental

Synthesis of analogues of LH–RH

Amino acid derivatives. – Intermediates were purchased from Peninsula Laboratories, San Carlos, California 94070, which markets products made by the Protein Research Foundation, Japan.

Protecting groups

N^a-protection. – The butyloxycarbonyl group (Boc-) was used for all amino acid derivatives with the exception of Arg, when the more soluble Aoc-protected derivative was used. The <Glu- residue was incorporated as the more soluble Z–<Glu–OH derivative.

Side chain protection. – The following protecting groups were used: Tos for His and Arg; BZl for Ser, Thr and Gly; *o*-Br–Z for Zyr; *o*-Cl–Z for Lys.

Active ester derivatives. – Gln and Asn residues were incorporated as their *p*-nitrophenyl esters.

Resins. – As most of these LH–RH analogues are peptide amides, the benzhydrylamine hydrochloride resin (1% cross-linked) as market by Beckman Instruments, Palo Alto, California 94304, was used. When analogues with other C-terminals are desired, the Merrifield chloromethylated resin was used. The PAM-resin which has a more acid stable peptide to resin covalent bond attachment has also been used.

Solid phase synthesis

Attachment of first amino acid to the resin. – The benzhydrylamine resin hydrochloride was neutralized with 25% triethylamine (redistilled from NaOH pellets and ninhydrin) in methylene chloride. The first amino acid derivative was attached by the DCC method until the ninhydrin color test was negative.

The Merrifield resin was stirred overnight with an equivalent amount of the lithium salt of the protected amino acid in 6–8 ml DMF per gram resin at 50 °C.

Deprotection. – The protecting group of the α-nitrogen (Boc, Aoc) was removed by stirring the protected peptide-resin with 50% (w/v) trifluoroacetic acid (TFA) in methylene chloride containing 0.1% (w/v) indole for 30 min after the resin had been prewashed with this reagent.

Neutralization. – The trifluoroacetate salt of the peptide-resin from the deprotection step was neutralized with 10% (v/v) triethylamine (redistilled from ninhydrin and NaOH pellets) in methylene chloride for 10 min after 2 prewashes with the neutralizing reagent.

Coupling. – In all dicyclohexylcarbodiimide (DCC)-mediated coupling reactions, 2- to 3-equivalents of a solution of 10% (v/v) DCC (redistilled) in methylene chloride was used. Generally, a double coupling procedure was performed to insure complete coupling of amino functions on the resin. A 2- to 3-fold excess of the amino acid derivative was used.

Monitoring coupling reactions. – The ninhydrin color test of Kaiser *et al.* was used, in duplicate, and with a blank reference for comparison of color.

Avoiding incorrect sequences. – The free residual amino groups (after several incomplete couplings) were acylated with 3% (w/v) nitrophthalic anhydride in pyridine.

Automated peptide synthesis. – All reactions were under an atmosphere of pre-purified grade nitrogen. Liquids were removed from the teflon reaction vessel containing the resin (1 to 10 g) by means of a positive pressure of N_2, through the syntered disc at the base of the reaction vessel. An adjustable drain time dial controls the time of operation. Five mixing timers allows the instrument to select suitable times for the washing, deprotection, neutralization, and coupling steps. The amino acid delivery control sets the time necessary for delivery of the CH_2Cl_2, CH_2Cl_2–DMF, or DMF solutions of the amino acid derivatives. Reagents and wash solvents were contained in reservoirs and metered by N_2-pressure to two metering columns. Metering column B was for TFA and one CH_2Cl_2 wash reservoir. All other reagents and solvents were metered into metering column A. The volumes of these solutions were controlled by photo-electric sensors.

Programs for synthesis. – The peptide synthesizer operates, when in the "Automated Mode", by reading instructions from a punched mylar tape loop. A loop may contain either complete programs for commonly used coupling procedures or the single steps like deprotection, neutralization, various

couplings, and washing, as different programs. The latter loop allows a combination of various steps, individually.

1. Deprotection step; CH_2Cl_2 (2 washes); 50% (w/v) TFA-CH_2Cl_2 (1 prewash); 50% (w/v) TFA-CH_2Cl_2 (deprotection); and CH_2Cl_2 (2 washes).

2. Neutralization step: CH_2Cl_2 (2 washes); 10% (v/v) Et_3N–CH_2Cl_2 (2 prewashes); 10% (v/v) Et_3N–CH_2Cl_2 (neutralization); and CH_2Cl_2 (2 washes).

3. DCC-mediated coupling step: CH_2Cl_2 (2 washes); amino acid derivative (addition); 10% (v/v) DCC-CH_2Cl_2 (addition and coupling); CH_2Cl_2 (rinse and hold, coupling); and CH_2Cl_2 (2 washes).

4. Active-ester-mediated coupling step: CH_2Cl_2 (2 washes); DMF (2 washes); active ester (5–10-fold excess) (addition); CH_2Cl_2 (rinse and hold, coupling); and CH_2Cl_2 (2 washes).

5. Acetylation step: CH_2Cl_2 (2 washes); acylation reagent (addition); CH_2Cl_2 (rinse and hold, acylation); and CH_2Cl_2 (2 washes).

6. Washing step (#1): CH_2Cl_2 (2 washes); isopropanol (2 washes); and CH_2Cl_2 (2 washes).

7. Washing step (#2): CH_2Cl_2 (2 washes); isopropanol (2 washes); DMF (3 washes); and CH_2Cl_2 (3 washes).

Anhydrous HF reactions. – Reactions were conducted in a Toho Kasei HF line. The HF was distilled from an on-line HF cylinder into a cooled (CO_2/acetone) reservoir containing anhydrous CoF_3. Stirring this mixture for 45 to 60 min at room temperature removed traces of moisture from the liquid HF. The anhydrous HF was then distilled into a cooled (CO_2/acetone) reaction vessel containing the protected peptide resin (or a protected peptide) and 10–25% anisole (redistilled). Cleavage of the peptide from the resin and simultaneous deblocking of side-chain protecting groups occurred when the peptide resin (1–9 g) was stirred with the HF/anisole mixture for 1 h at 0 °C. The HF was then removed rapidly (aspirator) and the residue dried *in vacuo* over NaOH pellets. The mixture of peptide and resin was washed thoroughly with ethyl acetate to remove anisole products and then the free peptide was extracted with AcOH and AcOH–H_2O mixtures. After lyophilization, fluoride ion was exchanged for acetate on Dowex AG–X1 resin.

Purification of peptides. – Two different methods for purification were used. The first one consisted of several column chromatographic steps including ion exchange chromatography, gel filtration and partition chromatography. Separation was monitored either by UV absorption measurements at 254 or 280 nm, or chlorine-tolidine color spot tests after TLC on silica gel plates. Fraction cuts for pooling were made after the TLC spot pattern and were based on purity rather than yield. This purification sequence has been established over many years.

The second method includes High Pressure Liquid Chromatography on a Waters C_{18} preparative column with a capacity of more than one gram of material. Divisibility of the mixture was checked first on an analytical μ-Bondapak C_{18} reversed phase column. Separation on both the analytical and the preparative column was monitored by UV absorption at 210, 254, or 280 nm.

Determination of purity and characterization. – The following procedures were used: thin layer chromatography; thin layer electrophoresis; amino acid analysis; optical rotation; high pressure liquid chromatography.

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