Relative Potencies of Antagonists of the Luteinizing Hormone Releasing Hormone with Lys^8 and Arg^8 and Substitutions in Positions 3, 5, 6, 7 and 8

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Luteinizing Hormone Releasing Hormone, Ovulation, Peptide, Antagonist, Histamine Release

Antagonists of the luteinizing hormone releasing hormone (LHRH) of increased potency is a goal for control of ovulation. In the design and synthesis of 26 decapeptides, emphasis was given to analogs with Lys^8 and Arg^8 and with various substitutions in positions 3, 5, 6, 7 and 8. Two antagonists, [N–Ac–D-2-Nal^1,D-pClPhe^2,D-3-Pal^3,Ser^4,Tyr^5,D-Arg^6,Leu^7,Lys^8,Pro^9,D-Ala^{10}]–NH_2 and [N–Ac–D-2-Nal^1,D-pClPhe^2,D-3-Pal^3,Ser^4,Arg^5,D-3-Pal^6,Leu^7,Arg^8,Pro^9,D-Ala^{10}]–NH_2 showed 80–85% antiovulatory activity (AOA) at 0.25 μg in the rat. The latter antagonist showed 60% AOA at 0.125 μg. Of four pairs of analogs with Arg^8 and Lys^8, respectively, two pairs favored Lys^8 over Arg^8 for potency. One pair showed negligible difference and another pair favored Arg^8 over Lys^8. There is specificity of substitution for potency. In other antagonists, D-3-Pal^3, Tyr^5 or Phe^5, D-Arg^6 and Leu^7 or Nle^7 or Val^7 and Arg^8 were variously effective substitutions for increase of potency and reduction of histamine release.

Introduction

Karten and Rivier [1] published in 1986 a comprehensive report of GnRH analog design and summarized structure function studies toward the development of agonists and antagonists by many investigators. They emphasized rationale and perspective, and included a section on peptide-induced histamine release of timely importance.

Schmidt *et al.* [2] reported the pivotal observation that an antagonist, [Ac–D-2-Nal^1,4FD–Phe^2,D-Trp^3,D-Arg^6]GnRH, caused in rats a transient edema of the face and extremities when the peptide was subcutaneously administered. On the basis of this and subsequent observations, it was concluded [1] that those antagonists of LHRH which were the most potent in causing histamine release had a structural combination of a basic D-amino acid in position 6 and Arg^8 and a grouping of hydrophobic aromatic amino acids in the three positions of the N-terminus.

Abbreviations: D-3-Pal, D-3-pyridylalanine; D-pClPhe, D-p-chlorophenylalanine; D-2-Nal, D-2-naphthylalanine; Cit, Citrulline; 6-Qal, 6-quinolylalanine; NicLys, N$^\varepsilon$-nicotinoyllysine; α-MeArg, α-methylarginine; AOA, antiovuktory activity; LHRH, luteinizing hormone releasing hormone; GnRH, gonadotropin releasing hormone; Boc, N-testbutoxycarbonyl.

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At this stage of the multidisciplinary and multiple group search for safe and effective antagonists of LHRH to control ovulation, it became compelling to design antagonists which not only would be adequately potent but cause negligible release of histamine which perhaps could be no greater than that of the clinically important superagonists of LHRH.

The release of histamine is not restricted to these antagonists of LHRH since somatostatin analogs caused histamine secretion [3], and neuropeptides caused histamine release and vascular changes [4], and compound 48/80 and substance P induced release of histamine and serotonin [5], and gastrin induced histamine release from human cutaneous mast cells [6].

Subsequent to the realization that designs of antagonists of LHRH must maintain potency of AOA and minimize histamine release, progress has been reported. Prominent in design change has been deletion of D-Arg in position 6 and transfer of Arg in the L-form to position 5. Roeske *et al.* [7] synthesized [Ac–D-2-Nal^1,4ClD-Phe^2,D-Trp^3,Arg^5,D-Trp^6,D-Ala^{10}]GnRH and found that it was about one-tenth as potent as the D-Arg^6 antagonist in causing histamine release.

Folkers *et al.* [8] synthesized the Arg^5,D-3-Pal^6 antagonist, [N–Ac–D-2-Nal^1,D-pClPhe^2,D-3-Pal^3,Ser^4,Arg^5,D-3-Pal^6,Leu^7,Arg^8,Pro^9,D-Ala^{10}]–NH_2, which caused 60% inhibition of ovulation in the rat at

125 ng and appeared to be the most potent antagonist yet described. The strategy of the design was replacement of D-Arg^6 with D-3-Pal^6 and of Tyr^5 with Arg^5. They also observed that histamine release was less for the D-3-Pal^6 peptides of three pairs of analogs.

Millar [9] observed the stimulation of gonadotropin release by a non-GnRH peptide sequence of the GnRH precursor. Sundaram *et al.* [10] recorded species differences in the sensitivity to the antitesticular effects of [Ac−D-2-Nal^1,4FD−Phe^2,D-Trp^3,D-Arg^6]LHRH. Folkers *et al.* [8] cited six relevant accounts of other investigators who had given primary emphasis to potency of AOA rather than to potency and histamine release.

Herein, we describe the design, synthesis and bioassay for potency of AOA and histamine release in rats of 26 peptides with particular emphasis of substitutions in positions 6 and 8 to replace D-Arg^6 with D-His^6, D-3-Pal^6, D-2-Nal^6 and Arg^8 with Lys^8, Leu^8, Cit^8 and other substitutions.

Experimental

Materials

The purchase of the amino acid intermediates, the protective groups of the alpha-amino functions, the source and nature of the resin, solvents, and other chemicals were described [8], but with modifications as follows.

Boc ε-Nicotinoyl-lysine (NicLys), Boc-N-α-methyl-arginine (α-MeArg), and 6-Quinolylalanine (6-Qal) were synthesized. L-Citrulline was purchased from Aldrich and Boc protection of the alpha-amino group was added before coupling.

Synthesis

The 26 peptides of Table I were synthesized as described [8].

Purification and purity

All of the peptides were first purified by chromatography over silica gel, and finally by HPLC using the solvent systems which were described [8]. Single peaks were observed on analytical μ-Bondpak C_{18} columns (3.9 mm × 30 cm) when phosphate-acetonitrile buffer systems were used at the various concentrations of acetonitrile according to Table II. The chromatographic data on these peptides are in Table II. Table III contains the amino acid analytical data, which were obtained as described [8].

Biological assays

The peptides were bioassayed to determine their activities to inhibit ovulation in rats as described [8], and the data are in Table I. Hook *et al.* [11] described a system to assay analogs for histamine release, which we have used to provide the data in Table I. The system utilizes mast cells from adult male Sprague-Dawley rats. A concentration of 575 μg/ml of LHRH in this system released 50% of the total histamine.

Results and Discussion

Activities for inhibition of ovulation in rats

Table I contains the data on the antiovulatory activities and the activities for the release of histamine by the 26 peptides. Only two of the Peptides showed no antiovulatory activity (AOA) at a dosage of 1 μg. It was policy not to test the peptides above this level. Frequently, these peptides would not be tested at a level above 0.5 μg in recognition of the goal which was to seek ever increasing potency. In general, the release of histamine was tested at a dosage of 10 μg unless a peptide was of special interest in which case the dosage was reduced to 0.01 μg.

All of these peptides have the substitutions of [N−Ac−D-2-Nal^1,D-pClPhe^2,( )^3,Ser^4,( )^5,( )^6,( )^7,( )^8,Pro^9,D-Ala^{10}]−NH_2.

Two objectives on sequence-activity were emphasized in the design of these 26 peptides which were to compare analogs with Lys^8 and Arg^8 and to compare analogs with substitutions in positions 3, 5, 6, 7 and 8.

Comparison of analogs with Lys^8 and Arg^8

Antagonists 1−8 constitute four pairs of antagonists, each with Arg^8 and Lys^8 (*i.e.*, 1 and 2, 3 and 4, etc.). For the pair 1 and 2 and the pair 3 and 4, the Lys^8 analogs were more potent than the Arg^8 analogs (80% *vs.* 57%/0.25 μg and 87% *vs.* 25%/0.5 μg, respectively). For the 5 and 6, the potencies of the Lys^8 and Arg^8 analogs were similar (90% *vs.* 85%/1.0 μg), but for the pair 7 and 8, the Lys^8 analog was 1/2 as active as the Arg^8 analog (50% *vs.* 100%/0.5 μg). The first three pairs were identical except for Lys^8 and Arg^8, but each of the three pairs are different in position 6 which makes the comparisons more meaningful. In the fourth pair, Arg was in position 5 in contrast to Tyr^5 in the first three pairs.

Table I. Antagonists of LHRH based on [N−Ac−D-2-Nal[1],D-pClPhe[2],( )[3],Ser[4],( )[5],( )[6],( )[7],( )[8],Pro[9],D-Ala[10]−NH_2]−LHRH.

	( )[3]	( )[5]	( )[6]	( )[7]	( )[8]	% AOA/μg 0.125	0.25	0.5	1	Wheal area [mm/μg] 10	1	0.1	0.01
Comparison of Analogs with Lys[8] and Arg[8]													
1	D-3-Pal	Tyr	D-Arg	Leu	Arg*	–	57	100	–	184 ± 35	–	–	–
2	D-3-Pal	Tyr	D-Arg	Leu	Lys	–	80	100	–	158	123	105	51
3	D-3-Pal	Tyr	D-His	Leu	Arg	–	–	25	100	119	–	–	–
4	D-3-Pal	Tyr	D-His	Leu	Lys	–	–	87	100	96 ± 10	–	–	–
5	D-3-Pal	Tyr	D-3-Pal	Leu	Arg**	–	–	–	85	134	–	–	–
6	D-3-Pal	Tyr	D-3-Pal	Leu	Lys	–	–	–	90	120	–	–	–
7	D-3-Pal	Arg	D-3-Pal	Leu	Arg**	60	85	100	100	120	–	–	–
8	D-3-Pal	Arg	D-3-Pal	Leu	Lys	–	–	50	–	154	–	–	–
Comparison of Analogs with Substitutions in Positions 3, 5, 6, 7 and 8													
9	D-3-Pal	Ile	D-Arg	Leu	Arg	10	100	100	100	189	–	–	–
10	D-3-Pal	3-Pal	D-Arg	Leu	Leu	–	–	–	70	154	128	92	68
11	D-3-Pal	3-Pal	D-2-Nal	Leu	Leu	–	–	0	–	–	–	–	–
12	D-3-Pal	3-Pal	D-3-Pal	Leu	Cit	–	–	0	–	101 ± 6.1	–	–	–
13	D-3-Pal	3-Pal	D-3-Pal	Leu	NicLys	–	–	0	–	99 ± 10.3	–	–	–
14	D-3-Pal	3-Pal	D-3-Pal	Leu	His	–	–	0	–	144 ± 10.6	–	–	–
15	D-3-Pal	Tyr	D-Arg	Leu	D(L)αMeArg	–	–	0	–	–	–	–	–
16	D-3-Pal	Tyr	D-Arg	Leu	D-Trp	–	–	–	0	–	–	–	–
17	D-3-Pal	Tyr	D-Arg	Leu	3-Pal	–	–	0	20	–	–	–	–
18	D-3-Pal	Tyr	D-Arg	Leu	Trp	–	–	10	10	–	–	–	–
19	D-3-Pal	Tyr	D-Arg	Nle	Arg	–	60	–	–	–	–	–	–
20	D-Phe	Phe	D-Arg	Val	Arg	–	40	100	–	–	–	–	–
21	D-Phe	Tyr	D-Arg	Phe	Arg	–	30	–	–	–	–	–	–
22	D-3-Pal	Tyr	D-Arg	Lys	Arg	–	–	8	–	–	–	–	–
23	D-Phe	Tyr	D-Arg	6-Qal	Arg	–	0	–	–	–	–	–	–
24	D-3-Pal	3-Pal	D-Arg	Ile	Arg	–	27	–	–	–	–	–	–
25	D-3-Pal	Arg	D-3-Pal	Cit	Arg	–	–	0	–	95 ± 0	–	–	–
26	D-Arg	Tyr	D-Arg	Leu	3-Pal	–	–	0	0	–	–	–	–

* Ref. 12.
** Ref. 8.

The most potent antagonist of Table I was peptide 7 with Arg^5 and Arg^8 which showed 60% AOA at 0.125 μg [8]. However, peptides 2 and 7 had similiar potencies (80 and 85%) at 0.25 μg. Again, a given substitution may be superior in one pair of analogs but not in another pair with only a single additional change.

Comparisons of analogs with substitutions in positions 3, 5, 6, 7 and 8

Of the eighteen analogs (No. 9−26) with the substitutions in positions 3, 5, 6, 7 and 8, the analog number 9 with Ile^5, D-Arg^6, Arg^8 was the most potent showing 100% AOA at 0.25 μg and 10% AOA at 0.125 μg. In this analog, the replacement of Ile^5 with 3-Pal^5 and Arg^8 with Leu^8 greatly reduced activity to 70%/1.0 μg.

The introduction of D-2-Nal^6 and D-3-Pal^6 in place of D-Arg^6 and the introduction of Cit^8, $NicLys^8$ and His^8 gave inactive peptides at 0.5 μg. The introduction of Tyr^5 for Ile^5 and D(L)N-α-$MeArg^8$ in place of Arg^8 was also detrimental.

The maintenance of D-3-Pal^3 or D-Phe^3 and various introductions of Tyr^5 and Phe^5 and 3-Pal^5 all with D-Arg^6 but variously with Nle^7, Val^7, Phe^7 and Ile^7 gave antagonists showing 27−60% AOA at 0.25 μg, and which were relatively successful sequence changes.

For analogs 12 and 25, the change from Leu^7, Cit^8 in analog 12 to Cit^7, Arg^8 in analog 25 did not bestow activity.

The exchange of D-3-Pal, Arg^8 in peptide 1 for D-Arg^3, 3-Pal^8 significantly reduced activity to 0%/1.0 μg.

In summary, substitutions with D-3-Pal^3 and Tyr^5 or Phe^5 with D-Arg^6 and Nle^7 or Val^7 with Arg^8 were

Table II. Chromatographic data on LHRH antagonists.

Analog No.[a]	Linear gradient in % change of CH_3CN	HPLC Retention time [min]	TLC R_{f_1}	R_{f_2}	R_{f_3}	R_{f_4}	R_{f_5}	R_{f_6}	R_{f_7}	R_{f_8}	R_{f_9}
2	30 to 60 in 20 min*	10.4	0.11	0.15	0.24	0.20	0.28	–	–	–	–
3	32 to 64 in 15 min	6.0	0.15	0.18	0.49	0.18	–	0.75	–	–	–
4	16 to 64 in 15 min	9.1	–	0.16	0.47	0.19	–	–	0.56	0.61	–
6	24 to 60 in 15 min*	8.6	0.16	0.17	0.25	0.21	0.29	–	–	–	–
8	16 to 64 in 15 min	3.0	–	0.10	–	0.25	–	–	0.59	0.19	–
9	32 to 80 in 15 min	7.1	–	0.24	–	0.19	–	–	0.58	0.76	0.15
10	32 to 80 in 15 min	2.7	–	0.26	–	0.13	–	–	0.83	0.81	0.33
11	24 to 80 in 15 min	8.2	–	–	–	0.24	–	–	0.68	0.66	–
12	24 to 64 in 15 min	5.4	–	0.06	0.45	0.16	–	–	0.51	0.58	–
13	32 to 80 in 15 min	4.1	–	0.14	–	0.28	–	–	0.65	0.61	0.27
14	32 to 80 in 15 min	3.3	–	0.08	–	0.19	–	–	0.58	0.56	0.20
15	–	–	–	–	–	0.21	–	–	–	–	–
16	20 to 50 in 20 min	13.5	0.29	0.15	0.45	0.30	–	0.76	–	–	–
17	32 to 80 in 20 min**	11.2	–	–	–	–	–	–	–	–	–
18	32 to 80 in 20 min**	15.0	–	–	–	–	–	–	–	–	–
19	30 to 60 in 25 min*	20.8	0.25	0.08	0.37	0.21	–	0.67	–	–	–
20	32 to 80 in 15 min	8.5	0.32	0.26	0.41	0.25	–	0.75	–	–	–
21	32 to 80 in 15 min	8.0	0.35	0.29	0.43	0.24	0.38	0.76	–	–	–
22	20 to 50 in 20 min	8.9	–	–	–	0.00	0.40	–	–	–	0.01
23	32 to 80 in 15 min	7.6	0.14	0.22	0.31	0.27	0.35	–	–	–	–
24	32 to 64 in 15 min	6.0	0.13	0.04	0.47	0.15	–	0.71	–	–	–
25	16 to 64 in 15 min	7.7	–	0.01	0.39	0.14	–	–	0.43	0.10	–
26	32 to 80 in 20 min**	10.1	–	–	–	–	–	–	–	–	–

HPLC Solvent System:
Buffer A = 0.01 M KH_2PO_4, pH = 3; buffer B = 20% A in CH_3CN, eluted in linear gradient at various percentages of CH_3CN in 15 to 25 min as listed at a flow rate of 2 ml per minute.
* Buffer A = 0.1 M NH_4OAc, pH = 5, buffer B = 20% A in CH_3CN.
** Buffer A = 0.05 M NH_4OAc, pH = 5, buffer B = 20% A in CH_3CN.
[a] Analog No. 1 (Ref. 12), analog No. 5 and 7 (Ref. 8).

TLC Solvents:
R_{f_1} = *n*BuOH:EtOAc:HOAc:H_2O = 15:5:1:3.
R_{f_2} = *n*BuOAc:*n*BuOH:HOAc:H_2O = 2:8:2:3.
R_{f_3} = *n*BuOH:HOAc:H_2O = 4:1:2.
R_{f_4} = *n*BuOH:HOAc:H_2O = 4:1:5 (upper).
R_{f5} = *n*BuOAc:*n*BuOH:HOAc:H_2O:pyridine = 2:8:2:2:1.
R_{f_6} = *n*BuOH:pyridine:HOAc:H_2O = 5:3.3:1:4.
R_{f_7} = *n*BuOH:pyridine:HOAc:H_2O = 4:1:1:2.
R_{f_8} = EtOAc:*n*BuOH:HOAc:H_2O = 1:1:1:1.
R_{f_9} = EtOAc:pyridine:HOAc:H_2O = 20:5:3:3.

relatively good substitutions for the potency of AOA.

Activity for release of histamine

It is understood that the bioassays for AOA are presumably more quantitative than are the assays for the release of histamine by the method used. Of the four pairs of antagonists with Arg^8 and Lys^8, three of the four pairs with Lys^8 were possibly less active for release of histamine than those with Arg^8, but the reverse was probably true for the fourth pair (peptides 7 and 8).

The two peptides (No. 2 and 7) showing 80 and 85% AOA, respectively, at 0.25 µg were less active for histamine release (158 and 120 mm) than was analog 9 which showed 100% AOA/0.25 µg and a wheal area of 189 mm.

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Table III. Amino acid analytical data for LHRH antagonists.

Analog No.*	Amino acid Ser	Tyr	Arg	Leu	Pro	Ala	Lys	His	Ile	Phe	2-Nal	pClPhe	3-Pal	Others
2	1.05	1.02	1.01	0.98	0.92	1.08	0.94	–	–	–	+	+	+	
3	0.88	0.92	1.07	1.01	1.02	1.06	–	1.05	–	–	+	+	+	
4	0.96	0.91	–	1.04	0.99	1.06	1.03	1.01	–	–	+	+	+	
6	0.89	0.95	–	0.99	0.98	0.99	0.99	–	–	–	+	+	++	
8	0.89	–	1.03	0.99	0.99	0.96	1.04	–	–	–	+	+	2.10	
9	1.06	–	1.63	1.06	1.15	1.09	–	–	0.82	–	+	+	+	
10	0.94	–	1.08	2.09	0.88	1.02	–	–	–	–	+	+	++	
11	0.93	–	2.10	0.96	1.01	0.89	–	–	–	–	++	+	1.11	
12	0.82	–	0.96	1.01	1.22	1.00	–	–	–	–	+	+	++	Cit(+)
13	0.83	–	1.01	1.03	1.10	0.97	1.06	–	–	–	+	+	++	
14	0.88	–	1.05	1.05	0.99	1.00	–	1.03	–	–	+	+	++	
15	1.00	0.96	0.99	0.99	0.97	1.10	–	–	–	–	+	+	+	α-MeArg(+)
16	0.90	1.05	0.94	0.89	1.02	1.02	–	–	–	–	+	+	+	Trp(+)
17	0.95	1.07	1.04	0.94	1.02	1.07	–	–	–	–	+	+	++	
18	0.92	1.04	1.01	0.97	1.01	1.04	–	–	–	–	+	+	+	Trp(+)
19	0.82	0.93	2.05	–	1.05	0.92	–	–	–	–	+	+	+	Nle(+)
20	1.06	–	1.87	–	1.22	1.08	–	–	–	2.03	+	+	–	Val(0.75)
21	1.10	0.94	1.93	–	1.03	1.11	–	–	–	1.92	+	+	–	
22	0.90	0.97	1.97	–	1.09	1.09	0.97	–	–	–	+	+	+	
23	0.95	0.98	2.06	–	0.95	1.01	–	–	–	1.05	+	+	–	6-Qal(+)
24	0.97	–	1.99	–	1.04	1.07	–	–	0.93	–	+	+	++	
25	0.82	–	1.96	–	1.27	0.96	–	–	–	–	+	+	++	Cit(+)
26	0.91	1.03	1.97	0.91	1.02	1.15	–	–	–	–	+	+	+	

* Analog 1 is found in Ref. 12 and analogs 5 and 7 are recorded in Ref. 8.

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