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Effective antagonists of luteinizing hormone releasing hormone modified at position one

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Summary. The amino acid, D-2-naphthylalanine, has been used by many investigators as a substituent for position one of antagonists of LHRH. We have newly designed substituents for position one in which the carboxy groups of 2-naphthoic acid, 3-quinoline- and 2-quinoxaline-carboxylic acids are linked to the five amino acids, DAla, DThr, DNVal, DSer, and Gly. The substituents in positions 2–10 were DpClPhe[2], DPal[3], Ser[4], PicLys[5], DPicLys[6], Leu[7], ILys[8], Pro[9], $DAlaNH_2$[10].

Remarkably, DThr, acylated on the amino group by 3-quinolinecarboxylic acid or by 3-quinoxalinecarboxylic acid, and introduced into position one of a relatively potent antagonist, gave a new class of antagonists of LHRH, which released as little histamine as yet recorded, and yet possessed reasonable antiovulatory activity and greatly improved solubility.

These structure-activity results advance the basic knowledge of understanding the structural features of such decapeptides which cause antiovulatory activity and histamine release.

Keywords: Amino acids – LHRH antagonists – Antiovulatory activity – Histamine release – Solid phase peptide synthesis

Abbreviations: ILys – N^{ε}-isopropyllysine; 1-Nal – 3-(1-naphthyl)alanine; 2-Nal – 3-(2-naphthyl)alanine; Nap – 2-naphthoic acids; NicLys – N^{ε}-nicotinoyllysine; Pal – 3-(3-pyridyl)alanine; pClPhe – 3-(4-chlorophenyl)alanine; PicLys – N^{ε}-picolinoyllysine; c-PzACAla – cis-3-(4-pyrazinylcarbonylaminocyclohexyl) alanine; 3-Qal – 3-(3-quinolyl)alanine; Qui – 3-quinolinecarboxylic acid; Qux – 2-quinoxalinecarboxylic acid

Introduction

The hypothalamic hormone LHRH, which releases the gonadotropins LH and FSH, is either directly or indirectly involved in normal fertility in animals. Synthetic antagonists of this hormone are a basis for an approach to contraception.

The activity of "superagonists", which desensitize the pituitary LHRH receptors as contraceptive agents, has recently been demonstrated in humans. The "superagonists" are also utilized for the management of steroid dependent carcinomas (prostate and breast), and treatment of precocious puberty and of endometriosis.

Although a few thousand antagonists have already been internationally synthesized, the use of such antagonists in humans is still limited, because of the lack of sufficiently potent and safe peptides.

Early investigation of antagonists centered on position 2, when it was shown that [desHis^2]LHRH was an antagonist (Vale et al., 1972). In position 1, DpGlu (Rivier et al., 1978) and Pro (Humphries et al., 1978) were tried. Then the first aromatic D-amino acids were reported in position one (Channabasavaiah et al., 1979). A very important improvement came with the successful incorporation of D-2-Nal in this position (Horvath et al., 1982). Surprisingly, the other isomer, D-1-Nal had no activity at the same dose level. Since 1982, acetylated D-2-Nal has been the dominant amino acid in position one. An appropriate combination of size, aromaticity and lypophilicity of this molecule has been very unique and difficult to substitute. Similar in conformation, but more hydrophilic, D-3-Qal introduced at position one (Ljungqvist et al., 1991) was less effective than D-2-Nal in most peptides, but the peptides were very potent when c-PzACAla^5 was also present (Janecka et al., 1993).

Recently, a tricyclic aromatic residue was introduced into position one. D-3-(2-dibenzofuranyl)alanine was introduced in only one analog, and was slightly less potent than D-2-Nal (Ljungqvist et al., 1993).

Though advantageous for potency, the presence of lypophilic residues at the N-terminus appears to cause both histamine release and insufficient solubility of such peptides for significant formulation as drugs.

The new antagonists described herein with more hydrophilic substituents in the N-terminus have good solubility and plausible potency. Of greatest importance is the finding that certain substituents in position one very greatly reduce the release of histamine.

Experimental

Synthesis

The analogs were synthesized by solid phase either manually or by a Beckman 990 Peptide Synthesizer by the described protocols (Janecka et al., 1991) on a benzhydrylamine resin (BHA), and by using the tert-butyloxycarbonyl (Boc) group for N-amino protection.

Purification was achieved by gel filtration on Sephadex G-25 with 6% aqueous acetic acid as the eluent, followed by chromatography on Sephadex LH-20, 1–3 times with the solvent system, water: n-butanol:acetic acid:methanol 90:10:10:8. The purity was checked by TLC, amino acid analysis and HPLC. The peptides gave single spots on TLC in four different solvent systems (Table 1).

Table 1. Chromatographic data of LHRH antagonists

Analog #[a]	TLC[b] R_fA	R_fB	R_fC	R_fD	HPLC[c] R_t [min]	gradient
1	0.18	0.65	0.90	0.73	6.6	30–80
2	0.38	0.59	0.82	0.67	3.3	0–80
3	0.12	0.57	0.79	0.66	18.0	30–80
4	0.40	0.65	0.84	0.71	3.2	0–80
5	0.17	0.61	0.89	0.73	17.5	30–80
6	0.29	0.61	0.82	0.66	17.3	0–80
7	0.23	0.63	0.91	0.74	18.5	0–80
8	0.37	0.40	0.65	0.40	17.8	0–80
9	0.09	0.64	0.87	0.74	17.5	30–80
10	0.44	0.60	0.78	0.63	18.5	0–80
11	0.40	0.60	0.76	0.60	4.1	0–80
12	0.26	0.55	0.84	0.59	13.3	30–80
13	0.28	0.59	0.80	0.60	5.1	0–80
14	0.25	0.51	0.77	0.53	17.9	0–80
15	0.40	0.46	0.72	0.50	4.7	0–80
16	0.32	0.57	0.79	0.60	19.4	0–80
17	0.28	0.56	0.79	0.66	17.2	0–80

[a] For sequence see Table 2 and 3
[b] *TLC* Solvent Systems: *A* n-BuOH:py:HOAc:H_2O = 4:1:1:2; *B* n-BuOH:HOAc:H_2O = 4:1:2; *C* n-BuOH:py:HOAc:H_2O = 30:10:3:12; *D* EtOAc:py:HOAc:H_2O = 5:5:1:3
[c] *HPLC* conditions are described in Methods

Amino acid analyses were carried out on a Beckman 118CL Amino Acid Analyzer after hydrolysis in constant boiling HCl for 24 h. The unnatural amino acids were qualitatively determined with the exception of Pal which was quantified. The results were in agreement with theory within the limits of experimental error. The purity was further checked by HPLC using a Waters Instrument with a 660 solvent programmer and a Vydac C_{18} column. The flow rate was 1.5 ml/min and the absorbance was recorded at 210 nm; buffer A was 0.01M KH_2PO_4 adjusted to pH3 with H_3PO_4; buffer B was 80% acetonitrile, 20% A. A linear gradient of 30–80% or 0–80% B in 20 min was used to elute the peptides. Retention times are in Table 1.

Bioassay

The AOA was determined in rats as reported (Humphries et al., 1979). The *in vitro* histamine release test in rat mast cells was performed, as described (Hook et al., 1985), and the results are reported as ED_{50} values which is the concentration in μg/mL that releases 50% of the total releasable histamine.

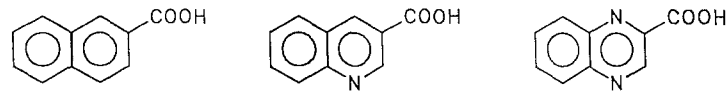
Results and discussion

NAcD-2-Nal has been the most common residue for position one of LHRH antagonists. It has an aromatic, bicyclic nucleus, which is quite unlike the monocyclic, nonaromatic pGlu that occupies position one of LHRH.

It was questioned whether this aromatic nucleus, which has been effective for the antagonistic potency, has to be in the side chain of the amino acid in

position one (as it is in Nal) or whether it can be attached to an aliphatic amino acid in position one by acylation with the use of an appropriate carboxylic acid.

Three carboxylic acids were selected for acylation: 2-naphthoic acid Nap(I), 3-quinolinecarboxylic acid Qui(II), and 2-quinoxalinecarboxylic acid Qux(III).



I II III

Nine analogs were synthesized. The potent antagonist used for comparison was: NAcD-2-Nal-DpClPhe-DPal-Ser-PicLys-DpicLys-Leu-ILys-Pro-DAlaNH$_2$ (PC), and changes were made only in position one. The analogs are in Table 2.

The AOA's were determined at the concentration of 0.5 μ g/rat. The analogs 1–3 have DAla[1]. Analog 1, acylated with Nap, showed 17% AOA while analogs 2 and 3 acylated with Qui and Qux, respectively, were inactive at this dose level.

The next choice for position one was DThr[1] to have a more hydrophilic N-terminal. Analog 4 with NapDThr[1] had only 20% AOA, but analogs 5 and 6 with QuiDThr[1] and QuxDThr[1] both had 60% AOA at 0.5 μg. For analog 5, total inhibition of ovulation occurred at 2.5 μg/rat. Analogs 5 and 6 were much more hydrophilic at the N-terminal than the analogs with NAcD-2-Nal[1], because of both the hydroxyl group of Thr and the nitrogen atom (or atoms) in the nucleus.

In accordance with experience, the analogs with more hydrophilic N-terminals should release less histamine in the *in vitro* assay. The ED_{50} for histamine release of analogs 5 and 6 was 307 μg/mL and 315 μg/mL, respectively, to compare with 93 for PC. The solubility of these two analogs is significantly better than the parent compound with NAcD-2-Nal[1].

Table 2. Biological activities of LHRH analogs in the antiovulatory assay (*AOA*) and in the assay for histamine release (*HRA*)
General Sequence: X-DpClPhe-DPal-Ser-PicLys-DPicLys-Leu-ILys-Pro-DAlaNH$_2$

Analog #	X	AOA (rats ovulating/total rats, %) dose in μg 0.5		1.0		2.5		HRA $ED_{50} \pm$ SEM (μg/mL)
PC	NAcD-2-Nal	6/10	40	0/6	100			93 ± 11
1	NapDAla	5/6	17					
2	QuiDAla	6/6	0					
3	QuxDAla	6/6	0					
4	NapDThr	4/5	20					
5	QuiDThr	4/10	60	1/5	80	0/5	100	307 ± 0
6	QuxDThr	4/10	60			0/5	100	315 ± 15
7	QuiDNVal	4/5	20					
8	QuiDSer	4/5	20					
9	QuiGly	5/5	0					

Table 3. Antiovulatory activities of LHRH analogs with acylated DThr in position one

Analog #	Sequence 1	2	3	4	5	6	7	8	9	10	AOA (rats ovulating/total rats, %) 0.5 μg	
10	NapDThr	DpClPhe	DPal	Ser	PicLys	DPicLys	Leu	Arg	Pro	$DAlaNH_2$	1/6	14
11	QuiDThr	DpClPhe	DPal	Ser	PicLys	DPicLys	Leu	Arg	Pro	$DAlaNH_2$	6/8	25
12	QuiDThr	DpClPhe	DPal	Ser	PicLys	DPicLys	Abu	Arg	Pro	$DAlaNH_2$	3/6	50
13	QuiDThr	DpClPhe	DPal	Ser	NicLys	DNicLys	Leu	ILys	Pro	$DAlaNH_2$	6/6	0
14	QuiDThr	DpClPhe	DPal	Ser	PicLys	D(PicSar)Lys	Leu	ILys	Pro	$DAlaNH_2$	4/5	20
15	QuxDThr	DpClPhe	DPal	Ser	PicLys	D(PicSar)Lys	Leu	ILys	Pro	$DAlaNH_2$	3/5	40
16	QuiDThr	DpClPhe	DPal	Ser	cPzACAla	DPicLys	Leu	Arg	Pro	$DAlaNH_2$	4/6	33
17	QuiDThr	DpClPhe	DPal	Ser	cPzACAla	DPicLys	Leu	ILys	Pro	$DAlaNH_2$	4/5	20

Analogs 7,8 and 9 in Table 2 were not potent. Analog 7 with QuiDNVal[1] was an example of a larger residue than in DAla and had 20% AOA while analog 9 with QuiGly was inactive at 0.5 μg. Analog 8 with QuiDSer[1] showed 20% AOA though it contained a hydroxyl group.

The eight analogs, 10–17 in Table 3, were all designed with acylated DThr in position 1 and some changes in other positions. Substituting ILys[8] for Arg[8] (analogs 10–12) resulted in a decrease of potency, but Abu[7] instead of Leu[7] was advantageous. Analogs 13–17 are congeners of prior analogs in which position 1 was substituted by QuiDThr[1] or QuxDThr[1], but decreases in potency were observed.

In this research to achieve clinically promising antagonists of LHRH, two biological activities of the analogs are dominant. They are antiovulation and histamine release. The desirable antagonist should have maximal antiovulatory activity and minimal histamine release. The structural features which most significantly cause these two biological activities are not yet fully elucidated. Our finding that a bicyclic and aromatic carboxylic acid, Qui (II) or Qux (III), used in acylation with DThr as the substituent in position one very greatly reduces the histamine release, as evidenced by an ED_{50} of ca. 300 μg/mL, is basic for future designs of antagonists.

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