

Highly potent antagonists of luteinizing hormone-releasing hormone free of edematogenic effects

(inhibitory luteinizing hormone-releasing hormone analogs/histamine release/anaphylactic reaction)

S. BAJUSZ*, M. KOVACS*, M. GAZDAG*, L. BOKSER*, T. KARASHIMA*, V. J. CSERNUS*, T. JANAKY*, J. GUOTH*, AND A. V. SCHALLY*†

†Endocrine, Polypeptide and Cancer Institute, Veterans Administration Medical Center and *Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70146

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ABSTRACT To eliminate the undesirable edematogenic effect of the luteinizing hormone-releasing hormone (LH-RH) antagonists containing basic D amino acids at position 6, exemplified by [Ac-D-Phe(pCl)^{1,2}, D-Trp³, D-Arg⁶, D-Ala¹⁰]LH-RH [Phe(pCl) indicates 4-chlorophenylalanine], analogs with D-ureidoalkyl amino acids such as D-citrulline (D-Cit) or D-homocitrulline (D-Hci) at position 6 were synthesized and tested in several systems *in vitro* and *in vivo*. HPLC analysis revealed that the overall hydrophobicity of the D-Cit/D-Hci⁶ analogs was similar to that of the basic D-Arg⁶ antagonists. *In vitro*, most of the analogs completely inhibited LH-RH-mediated luteinizing hormone release in perfused rat pituitary cell systems at an antagonist to LH-RH molar ratio of 5:1. *In vivo*, the most active peptides, [Ac-D-Nal(2)¹, D-Phe(pCl)², D-Trp³, D-Cit⁶, D-Ala¹⁰]LH-RH [Nal(2) indicates 3-(2-naphthyl)alanine] and its D-Hci⁶ analog, caused 100% inhibition of ovulation in cycling rats in doses of 3 μg and suppressed the luteinizing hormone level in ovariectomized female rats for 47 hr when administered at doses of 25 μg. Characteristically, these peptides did not exert any edematogenic effects even at 1.5 mg/kg. These properties of the D-Cit/D-Hci⁶ antagonists may make them useful clinically.

Since the isolation and structural elucidation of hypothalamic luteinizing hormone-releasing hormone (LH-RH) more than 2000 analogs have been synthesized, in view of their expected medical applications (1). Chronic administration of potent LH-RH agonists leads to the inhibition of pituitary and gonadal functions (2-6). While repeated administration of LH-RH agonists is required to lower the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and sex steroids, similar effects can be obtained with single administration of LH-RH antagonists (7). Competitive antagonists of LH-RH were developed by multiple modification of the parent molecule, <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂ (8-14) (<Glu, pyroglutamic acid). Agonist activity is reduced by substitution of aromatic D amino acids at positions 2 and 3, and receptor affinity is retained when there are replacements at residues 1 and 6, and in some analogs also in position 10. LH-RH antagonists are frequently characterized by the nature of the residue in position 6. Analogues with hydrophobic D residues, such as D-Trp, are referred to as hydrophobic antagonists, and those having a hydrophilic basic residue, such as D-Arg, are known as hydrophilic antagonists. Another type of antagonist, developed by exchanging Arg⁵, D-Tyr⁶ for Tyr⁵, D-Arg⁶ in hydrophilic antagonists, may be referred to as transposition hydrophilic antagonists. However, the most potent D-Arg⁶-containing hydrophilic antagonists, such as [Ac-D-Nal(2)¹, D-Phe(pF)², D-

Trp³, D-Arg⁶]LH-RH [I; Nal(2), 3-(2-naphthyl)alanine; Phe(pF), 4-fluorophenylalanine] and [Ac-D-Phe(pCl)^{1,2}, D-Trp³, D-Arg⁶, D-Ala¹⁰]LH-RH (II), produce transient edema of the face and extremities when administered subcutaneously (s.c.) to rats at doses of 1.25-1.5 mg/kg (15, 16). In addition, these analogs cause a dose-related whealing response (17), increase histamine levels in rats (16), and elicit histamine release from rat mast cells (18). The anaphylactoid reactions to these antagonists are not invariably associated with their edematogenic potencies (16). A highly active transposition antagonist with Arg⁵, [Ac-D-Nal(2)¹, D-Phe(pCl)², D-Pal(3)³, Arg⁵, D-Abu(AA)⁶, D-Ala¹⁰]LH-RH (III; Pal(3), 3-(3-pyridyl)alanine; D-Abu(AA), 4-(p-methoxybenzoyl)-D-2-aminobutyric acid], was reported to be 1/10th as potent as I in releasing histamine and showed no edematogenic effect in the rat at the doses tested (0.5-1.0 mg/kg) (14). Antagonist II, which induced edema at the challenge dose of 1.5 mg/kg, was also about 1/10th as potent in histamine release assays as the other hydrophilic antagonists studied (16).

In contrast to hydrophilic antagonists, hydrophobic antagonists having a neutral aromatic D amino acid at position 6, for instance [Ac-Δ³-Pro¹, D-Phe(pF)², D-Trp^{3,6}]LH-RH (IV), did not exhibit edematogenic effects at a dose of 1.25 or 5.0 mg/kg (19) and were about 1/200th as potent as I in triggering the release of histamine (18). Thus, it seems likely that a structural combination of the basic D-Arg⁶ residue and a cluster of three aromatic D amino acids at the N terminus confers the anaphylactoid side reactions on LH-RH antagonists (19). Consequently, it could be conjectured that the introduction of a neutral hydrophilic residue into position 6 might lead to highly potent antagonists that are devoid of edematogenic effects. This paper reports the synthesis and biological evaluation of such a class of LH-RH antagonists containing D-citrulline (D-Cit) or D-homocitrulline (D-Hci)—that is, the neutral ureido analogs of D-arginine and D-homoarginine (D-Har), respectively, at position 6.

MATERIALS AND METHODS

Synthesis. LH-RH antagonists with D-Cit⁶ and D-Hci⁶ residues were prepared from their respective D-Orn⁶ and D-Lys⁶ analogs through carbamylation with potassium cyanate in dimethylformamide or aqueous dimethylformamide. The N^ω-ethylureido derivatives were similarly obtained by

Abbreviations: LH-RH, luteinizing hormone-releasing hormone (luteinizing hormone); LH, luteinizing hormone (lutropin); FSH, follicle-stimulating hormone (follicle-stimulating hormone); Cit, citrulline (2-amino-5-ureidopentanoic acid); Cit(Et), N^ω-ethylcitrulline; Har, homoarginine (2-amino-6-guanidinoheptanoic acid); Har(Et), N^ω, N^{ω'}-diethylhomoarginine; Hci, homocitrulline (2-amino-6-ureidohexanoic acid); Hci(Et), N^ω-ethylhomocitrulline; Nal(2), 3-(2-naphthyl)alanine; Orn, ornithine (2,5-diaminopentanoic acid); Pal(3), 3-(3-pyridyl)alanine; Phe(pCl), 4-chlorophenylalanine; Phe(pF), 4-fluorophenylalanine; D-Abu(AA), 4-(p-methoxybenzoyl)-D-2-aminobutyric acid (AA, anisic acid).

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using *N*-ethylisocyanate in place of potassium cyanate. The D-Orn⁶- and D-Lys⁶-containing intermediate peptides were assembled in a Beckman model 990 automatic synthesizer using standard solid-phase procedures (20). Benzhydrylamine resin (0.5–1.0 mmol/g) was used as starting material. Amino acids were coupled as their *N*^α-*t*-butoxycarbonyl (Boc) derivatives, and side-chain functional groups of the *N*^α-Boc-amino acids were protected as follows: serine, *O*-benzyl; tyrosine, *O*-2,6-dichlorobenzyl; D-ornithine, *N*^δ-benzyloxycarbonyl; D-lysine, *N*^ε-2-chlorobenzoyloxycarbonyl; arginine, *N*^G-*p*-toluenesulfonyl. The coupling reaction was achieved with a 3-fold excess of Boc-amino acid and *N,N'*-diisopropylcarbodiimide. With some amino acids, the preformed *N*-hydroxybenzotriazole ester procedure was used as follows: Equivalent amounts of components were allowed to react in 10–50% (vol/vol) dimethylformamide/dichloromethane in mmol/ml concentration at 0°C for 20 min, then added to the peptide-resin and rinsed with dichloromethane. After a coupling time of 2 hr, the completeness of acylation was monitored by the Kaiser test (21). Acetylation (after incomplete coupling and at the N terminus of decapeptides) was performed with an excess (30-fold) of acetic anhydride in dichloromethane. Intermediate deblocking was achieved with 50% trifluoroacetic acid in dichloromethane followed by neutralization with 10% (vol/vol) triethylamine in dichloromethane. Final deprotection was performed with liquid HF in the presence of 10% *m*-cresol at 0°C, using a cleavage time of 45 min.

Purification. Crude peptides were purified by using a Beckman Prep-350 preparative HPLC system. Gradient elution was used. Solvent A consisted of 0.1% aqueous trifluoroacetic acid and solvent B was 0.1% trifluoroacetic acid in 70% (vol/vol) aqueous acetonitrile.

Analytical HPLC. The HPLC analysis of crude and purified peptides was carried out with a Hewlett-Packard model 1090 liquid chromatograph. The peptides were chromatographed on a 4.6 × 250 mm W-Porex 5-μm C₁₈ column (Phenomenex, Rancho Palos Verdes, CA) at a flow rate of 1.2 ml/min, using two solvent systems.

Amino Acid Analysis. Amino acid analyses were performed in a Beckman 6300 amino acid analyzer, on samples that were hydrolyzed at 110°C for 20 hr in evacuated sealed tubes containing 4 M methanesulfonic acid and 0.2% 3-(2-aminoethyl)indole.

Detection of LH-RH Antagonist Activity *in Vitro*. LH-RH antagonist activity of the peptides was assayed by using a superfused rat pituitary cell system (22). Each peptide was perfused through the cells for 9 min (3-ml perfusate) at 3–100 nM. Immediately after that, a mixture containing the same concentration of the peptide and 1 nM LH-RH was administered for 3 min. This was followed by four consecutive infusions of 1 nM LH-RH for 3 min (1 ml) at 30-min intervals (30, 60, 90, and 120 min). LH content of the 1-ml fractions collected was determined by radioimmunoassay (RIA).

***In Vivo* Antiovolatory Assay.** This assay was carried out in 4-day-cycling rats as described by Corbin and Beattie (23).

Anaphylactoid Activities. Assays for the edematogenic activity *in vivo*, cutaneous anaphylactoid reaction *in vivo*, and histamine-releasing potency *in vitro* were carried out essentially as described by Morgan *et al.* (16).

Edematogenic Effect. Six groups of 6–10 rats were injected s.c. once a day on 2 consecutive days with the antagonists (1.5 mg/kg dissolved in 0.2 ml of sterile deionized water). Control rats were injected with diluent. The rats were observed during 8 hr on each day. The reactions of rats were classified as follows: no apparent reaction (NR), partial responders (PR; edema of nasal and paranasal area), and full responders (FR; facial edema with edematous extremities).

Cutaneous Anaphylactoid Reactions. Rats were anesthetized with pentobarbital (6 mg/100 g of body weight). One

milliliter of 0.5% Evans blue dye was injected into the jugular vein. The antagonists were diluted with saline to concentrations of 0.5, 1, 5, and 10 μg/ml. A standard volume (0.05 ml) of these solutions was injected intradermally. Each rat was injected with a negative control (0.05 ml of saline). Rats were decapitated 20 min after the injection and the reactions were read by measuring the blue lesions in reflected skin.

Histamine Release *in Vitro*. Rat peritoneal exudate cells were harvested and histamine release from mast cells was triggered by antagonists as described (16). Histamine levels were determined with a histamine RIA kit (NMS Pharmaceuticals, Newport Beach, CA). The concentration of each antagonist that released 50% of total mast cell histamine (HRD₅₀) was determined.

RESULTS

A series of LH-RH decapeptides related to hydrophilic antagonists [Ac-D-Phe(*p*Cl)^{1,2},D-Trp³,D-Arg⁶,D-Ala¹⁰]LH-RH (II) (8) and [Ac-D-Nal(2)¹,D-Phe(*p*Cl)²,D-Trp³,D-Har(Et₂)⁶,D-Ala¹⁰]LH-RH (V) (11), but containing D-ureidoalkylamino acid substitutions at position 6, as shown in general formula A₁, were synthesized and tested in various systems *in vitro* and *in vivo*.

A, [Ac-R¹,D-Phe(*p*Cl)²,D-Trp³,R⁶,D-Ala¹⁰]LH-RH.

In A₁, R¹ = D-Phe(*p*Cl) or D-Nal(2) and
R⁶ = D-Cit, D-Hci, D-Cit(Et), or D-Hci(Et);

in A₂, R¹ = D-Phe(*p*Cl) or D-Nal(2) and
R⁶ = D-Orn or D-Lys.

The peptides A₁, referred to as ureido antagonists, were prepared by carbamoylation with potassium cyanate and *N*-ethylisocyanate, respectively, of the corresponding D-Orn⁶- and D-Lys⁶-containing analogs having the general formula A₂. The precursor peptides, A₂, were made by standard solid-phase methods (20). After purification by preparative HPLC, the purity of isolated peptides was calculated to be greater than 95% on the basis of absorbance data obtained on HPLC in two systems. Amino acid analyses of the pure products showed the expected amino acid compositions.

The effects of D-ureidoalkylamino acid residues on overall hydrophobicity or hydrophilicity of a peptide as compared to the related basic amino acids, such as D-Orn, D-Lys, and D-Arg, were estimated by HPLC. Compounds A₁, their D-Orn⁶/D-Lys⁶ analogs, compounds A₂, and the D-Arg⁶-containing antagonist II and its D-Nal(2) analog (VI) were analyzed by HPLC under isocratic conditions using 0.03 M ammonium acetate in 40% (vol/vol) aqueous acetonitrile as eluent at pH 7.2 (24, 25). The capacity factor *k'* was used as a measure of the overall hydrophobicity of the analogs. The analogs are shown in Table 1 in order of increasing *k'* within the two series of peptides containing D-Phe(*p*Cl)¹ and D-Nal(2)¹, respectively.

The peptides were tested for their ability to inhibit LH-RH-mediated LH release from perfused rat pituitary cells *in vitro*. Every antagonist tested at 10 and 100 nM completely blocked LH-RH response. Every peptide also caused a significant decrease in response to LH-RH even at 3 nM (Table 2). The duration of the inhibitory effect of the antagonists after four consecutive exposures to LH-RH at 30-min intervals was variable. Antagonist activity of some analogs decreased rapidly. However, other analogs, when tested at 3 nM, showed the strongest blocking activity 60 or even 120 min after their infusion was stopped (Table 2).

Table 1. Order of hydrophobicity of [Ac-R¹,D-Phe(pCl)²,D-Trp³,R⁶,D-Ala¹⁰]LH-RH analogs as measured by their *k'* values

R ¹	<i>k'</i> for given R ⁶						
	D-Cit	D-Hci	D-Lys	D-Orn	D-Arg	D-Cit(Et)	D-Hci(Et)
D-Phe(pCl)	3.01	3.18	3.23	3.75	4.18	4.66	5.13
D-Nal(2)	3.96	4.24	4.92	5.11	5.43	6.11	6.76

Capacity factor *k'* = (retention volume – void volume)/void volume in HPLC.

In vivo antioviulatory activity of peptides 1–8 as compared to their D-Arg⁶-containing parent compound, II, was determined by injecting the peptides s.c. in aqueous solution into rats at noon on the day of proestrus. Table 3 shows the results as a percentage of the inhibition of ovulation and as the ratio of rats that did not ovulate to the total number of rats treated. Parent compound II and its D-Hci⁶ analog (3) were about equipotent, while the D-Cit⁶ congener (1) showed somewhat less potency. Introduction of D-Nal(2) into position 1 resulted in analogs with enhanced activities (2 and 4). However, analogs 5–8 with D-Cit(Et)⁶ or D-Hci(Et)⁶ showed lower potencies than the D-Cit⁶/D-Hci⁶-containing peptides or were inactive at 3 μg per rat.

To assess the duration of inhibitory action of the D-Cit/D-Hci⁶ analogs on LH secretion, as compared to the antagonist with D-Arg⁶, analogs 1–4 and II were injected s.c. in 40% (vol/vol) propylene glycol in saline to ovariectomized female rats and blood samples were drawn at various time intervals. Fig. 1 shows that antagonists II and 1 induced a significant (*P* < 0.01) inhibition of LH secretions for 23 hr at doses of 100 μg. The suppression after the high dose of antagonist II was also significant at 30 hr. Analogs 2–4 suppressed LH for 47 hr at both doses (*P* < 0.05).

Anaphylactoid activities of the D-Cit/D-Hci⁶ antagonists 1–4 and two reference compounds—the D-Arg⁶ antagonist II and the Arg⁵ analog of 4, 4a—as measured in three assays, are shown in Table 4. In accord with previous findings (16), the D-Arg⁶ antagonist II proved to be highly edematogenic at a dose of 1.5 mg/kg. Seven of 10 rats reacted 60–120 min after injection on day 1, and all 10 rats showed edema on day 2. Edema formation started 45 min after the injection. The peak reaction occurred between 60 and 90 min, was present during the 8 hr of observation, but subsided completely 24 hr after the injection. No edematous reactions could be observed with the D-Cit/D-Hci⁶ antagonists. Only antagonist 3 produced a very light edema in 1 of 9 rats, 3 hr after injection on day 2. Light edema of the face could also be observed in 2 of 6 rats treated with 4a. In the cutaneous anaphylaxis assay, all antagonists elicited skin reactions in a dose-dependent fashion, and all analogs induced histamine release

from rat peritoneal mast cells, with HRD₅₀ values of 1.0–3.5 μg/ml.

DISCUSSION

Structure–activity studies on LH-RH antagonists (19) indicated that substitution of a hydrophilic basic D amino acid residue at position 6 paired with a hydrophobic N terminus produces simultaneously high antagonistic potency and edematogenic activity in LH-RH analogs such as [Ac-D-Phe(pCl)^{1,2},D-Trp³,D-Arg⁶,D-Ala¹⁰]LH-RH (II) (9) and its D-Nal(2)¹ congener (VI) (26). In a search for LH-RH antagonists without undesirable edematogenic effect, we synthesized the analogs of II and VI containing D-ureidoalkyl amino acids at position 6. The D-ureidoalkyl amino acids, such as D-Cit and D-Hci, can be regarded as the neutral isomers of D-Arg and D-Har, respectively, that form hydrogen bonds through their —NH—CO—NH₂ (ureido) group. HPLC analysis of the peptides and their analogs with a basic residue at position 6 revealed that the order of overall hydrophobicity, as measured by the corresponding capacity factor (*k'*) (Table 1), was D-Cit < D-Hci < D-Lys < D-Orn < D-Arg < D-Cit(Et) < D-Hci(Et) within this homologous family of [Ac-R¹,D-Phe(pCl)²,D-Trp³,R⁶,D-Ala¹⁰]LH-RH analogs. It is evident from these data that the neutral D-Cit and D-Hci, because of their hydrogen-bond-forming ability, convey at least as much hydrophilicity to a peptide as the basic residues D-Arg, D-Lys, and D-Orn. However, D-Cit(Et) and D-Hci(Et) substitutions increase the overall hydrophobicity compared to the replacements with basic residues.

The intensity and duration of the LH-RH antagonistic activity of the analogs were examined in the superfusion assay. The intensity of the instant antagonistic activity was determined by exposure to 1 nM LH-RH for 3 min in the presence of the analog after incubation with it for 9 min. In every case a complete inhibition of response to LH-RH was found at 10 and 100 nM. Antagonists II, 2, 3, 5, and 6 also canceled LH-RH responses at 5 nM (Table 2).

The dynamics of the antagonistic activity were analyzed by exposing cells preincubated with the antagonists to 1 nM

Table 2. LH-RH-inhibiting activities of [Ac-R¹,D-Phe(pCl)²,D-Trp³,R⁶,D-Ala¹⁰]LH-RH analogs in perfused rat pituitary cell systems at various ratios of antagonist to LH-RH

No.	Peptide		% inhibition of LH response for given antagonist to LH-RH molar ratio						
	R ¹	R ⁶	3:1				5:1		10:1
			0 min	30 min	60 min	90 min	120 min	0 min	0 min
II	D-Phe(pCl)	D-Arg	71	81	69	—	55	100	100
1	D-Phe(pCl)	D-Cit	84	65	47	—	—	90	100
2	D-Nal(2)	D-Cit	0	40	45	—	40	100	100
3	D-Phe(pCl)	D-Hci	75	85	95	—	—	100	100
4	D-Nal(2)	D-Hci	39	77	70	70	—	70	100
5	D-Phe(pCl)	D-Cit(Et)	66	62	37	—	—	100	100
6	D-Nal(2)	D-Cit(Et)	52	46	10	—	—	100	100
7	D-Phe(pCl)	D-Hci(Et)	72	61	44	—	—	90	100
8	D-Nal(2)	D-Hci(Et)	33	58	62	—	65	80	100

The cells were first exposed to the analogs at 3, 5, 10, or 100 nM for 9 min. At that time 1 nM LH-RH was also given for 3 min together with the analogs (0-min response). LH-RH was also administered 30, 60, 90, and 120 min later. At 100 nM all antagonists gave 100% inhibition (data not shown).

Table 3. Antiovolutory activities of [Ac-R¹,D-Phe(pCl)²,D-Trp³,R⁶,D-Ala¹⁰]LH-RH analogs in rats

No.	Peptide		Dose, μ g	No. rats ovulating/ no. tested	% blockade of ovulation
	R ¹	R ⁶			
II	D-Phe(pCl)	D-Arg	3	1/8*	87.5*
1	D-Phe(pCl)	D-Cit	3	5/17	70
			1	6/12	50
2	D-Nal(2)	D-Cit	3	0/7	100
			1	3/7	57
3	D-Phe(pCl)	D-Hci	3	2/13	85
			1	3/11	73
4	D-Nal(2)	D-Hci	3	0/11	100
			1.5	3/8	62.5
5	D-Phe(pCl)	D-Cit(Et)	3	8/8	0
6	D-Nal(2)	D-Cit(Et)	3	4/7	43
7	D-Phe(pCl)	D-Hci(Et)	3	5/7	29
8	D-Nal(2)	D-Hci(Et)	3	8/8	0
Control			—	—	0

*Results reported previously for antagonist II indicate 2 of 9 rats ovulating (78% blockade of ovulation) at 3 μ g (9).

LH-RH, four times for 3 min at 30-min intervals. The time needed for the recovery of the normal LH-RH response varied for different peptides. At the highest doses (10 and 100 nM) only the duration of the inhibitory effect could be determined. However, at the 3 nM dose, more subtle differences between the analogs could be detected. Some peptides showed the highest inhibitory activity at 0 min (Table 2) and the normal LH-RH response continued to recover after that. Other analogs, II, 2, 3, 4, and 8, first exhibited only a moderate antagonistic activity and showed the strongest inhibitory effect 30, 60, or even 90 min after the preincubation had been stopped. These findings suggest that the initial association rates for LH-RH and some antagonists are different. In a 3-min reaction, LH-RH may bind to its receptor much faster than some antagonists. Binding of antagonists to sites that have relatively high affinity (or avidity) for these hydrophobic compounds but that cannot mediate gonadotropin release also takes place. In time, the dormant portion of antagonists residing at binding sites not concerned with the release of LH can migrate to the receptor site. This event results in a prolonged blockade of action of LH-RH and an apparent increase of antagonist potency. These results relate to the early events of receptor-peptide interaction and are consistent with reports by Perrin *et al.* (27).

Data in Table 3 show that replacement of the basic D-Arg⁶ residue of II with the neutral D-Cit and D-Hci leading to

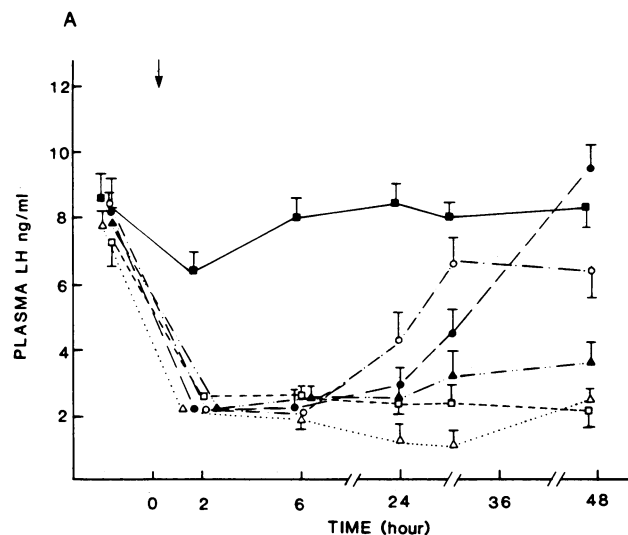


FIG. 1. Plasma LH concentrations in ovariectomized female rats after s.c. injection (arrow) of LH-RH antagonists in doses of 100 μ g. The numbering of the analogs is the same as in Table 3. ■, Saline; ●, II; ○, 1; △, 2; ▲, 3; □, 4. Vertical lines indicate the SEM.

analogs 1 and 3, respectively, produces the retention of potent inhibiting activity in the antiovolutory assay. D-Nal(2)¹, a substitution known to improve potency of D-Arg⁶ (26) and D-Har(Et)₂⁶ (28) antagonists, was introduced in 1 and 3. The high activity of analogs 2 and 4 thus obtained indicates that D-Nal(2)¹ and D-Cit/D-Hci⁶ substitutions confer desirable characteristics on this type of analog. D-Cit(Et) and D-Hci(Et) substitutions, however, resulted in much less potent compounds (5–8). Interestingly, no such difference could be observed among the basic analogs; that is, the D-Arg⁶-containing antagonist II and its N^ω-alkylated homolog, V, with D-Har(Et)₂⁶, were found to be equipotent when compared on the basis of ED₅₀ values (1.7 μ g) determined with 40% propylene glycol/saline as vehicle (28).

The results obtained in the LH suppression assay (Fig. 1) reveal that D-Cit/D-Hci⁶ analogs, like their parent structure II, are long-acting antagonists. A comparison of the duration of action of analogs 1, 2, and II suggests that D-Cit⁶ may be similar to or somewhat less effective than D-Arg⁶ when paired with D-Phe(pCl)¹. Only a combination of D-Cit⁶ and D-Nal(2)¹ substitutions can produce a peptide, 2, that causes LH suppression at 24 hr. However, D-Hci⁶ appears to contribute to the prolonged action of both the D-Phe(pCl)¹ and D-Nal(2)¹ peptides (analogs 3 and 4).

Table 4. Anaphylactoid activities of LH-RH antagonists [Ac-R¹,D-Phe(pCl)²,D-Trp³,R⁶,D-Ala¹⁰]LH-RH and one of their Arg⁵ analogs

No.	Peptide		Edematous response* at 1.5 mg/kg, no. rats						Cutaneous anaphylaxis, diameter of skin lesion, mm, at given dose				HRD ₅₀ , [†] μ g/ml
			Day 1			Day 2							
			NR	PR	FR	NR	PR	FR	10 μ g/ml	5 μ g/ml	1 μ g/ml	0.5 μ g/ml	
II	D-Phe(pCl)	D-Arg	3	7	0	0	0	10	13.7 ± 0.7	12.8 ± 1.2	8.9 ± 0.3	7.2 ± 0.4	1.0
1	D-Phe(pCl)	D-Cit	9	0	0	9	0	0	9.5 ± 0.2	8.4 ± 0.3	5.0 ± 1.2	0.2 ± 0.03	2.5
2	D-Nal(2)	D-Cit	8	0	0	8	0	0	10.8 ± 0.5	9.7 ± 0.3	5.1 ± 0.6	2.3 ± 0.7	3.5
3	D-Phe(pCl)	D-Hci	9	0	0	8	1 [‡]	0	9.3 ± 0.3	6.5 ± 0.4	4.2 ± 1.0	2.0 ± 0.4	2.5
4	D-Nal(2)	D-Hci	6	0	0	6	0	0	7.3 ± 0.5	6.0 ± 0.8	4.0 ± 0.3	1.7 ± 0.2	2.0
4a [§]	D-Nal(2)	D-Hci	6	0	0	4	2 [‡]	0	7.9 ± 1.3	6.2 ± 0.9	4.2 ± 0.8	2.1 ± 0.3	ND
Control			6	0	0	6	0	0					

*Classification of edematous response: NR, nonresponders (no apparent reaction); PR, partial responders (edema of nasal and paranasal area); FR, full responders (facial edema with edematous extremities).

[†]HRD₅₀ = concentration of the LH-RH antagonist required to induce release of 50% of rat peritoneal mast cell histamine *in vitro*. ND, not determined.

[‡]Very light edema in the face.

[§]Arg⁵ analog of 4.

Studies on the anaphylactoid activity of the antagonists 1–4 (Table 4) revealed that D-Cit/D-Hci⁶ substitutions in the highly edematogenic D-Arg⁶ antagonists II and VI could drastically reduce (as in analog 3) or abolish (as in antagonists 1, 2, and 4) this undesirable side reaction of the parent molecules, even when tested at a dose of 1.5 mg/kg. The fact that analog 3 produced light edema in 1 of 9 rats indicates that this toxic side effect (at least to a slight degree) could also be conveyed by a nonbasic hydrophilic residue at position 6, such as D-Hci⁶, in a suitable structural combination—e.g., when paired with D-Phe(pCl)¹. Another combination, such as D-Nal(2)¹, D-Hci⁶, afforded peptide 4, which did not exert an edematogenic effect. However, introduction of Arg⁵ into analog 4 led to compound 4a, which induced edema in 2 of 6 rats when tested at 1.5 mg/kg, suggesting that a basic hydrophilic residue, such as Arg, can confer this effect on an otherwise nontoxic molecule in the 5th position as well. Although analogs 1, 2, and 4 were absolutely free from the edematogenic effect, all four D-Cit/D-Hci⁶ antagonists showed some anaphylactoid activity in the two other assays. Thus, they induced skin lesions following intradermal injection, although less intense than analog II when compared at 1- and 0.5- μ g doses. They also elicited histamine release but with intensities 1/2 to 1/3.5 the intensity of II, which is a relatively weak histamine releaser, about 1/10 as strong as the related edematogenic antagonists I (14) and VI (16). These data provide further evidence that the activity of antagonists in the last two tests cannot predict their anaphylactoid activity in general. The most relevant of these side effects, edema, should be determined directly and at the doses of 1.25–1.5 mg/kg as suggested previously (15, 16).

Studies on the hydrophilic LH-RH antagonists presented here show that D-Cit/D-Hci⁶ substitutions in the highly active D-Arg⁶ antagonists produced analogs, such as [Ac-D-Nal(2)¹, D-Phe(pCl)², D-Trp³, D-Cit⁶, D-Ala¹⁰]LH-RH (2) and its D-Hci⁶ congener (4), that are devoid of the highly undesirable edematogenic effect, possess high and prolonged activity of the parent compounds, and are easily obtainable from the corresponding D-Orn/D-Lys peptides. These properties of the D-Cit/D-Hci⁶ antagonists may make them useful clinically.

In view of favorable clinical results with LH-RH agonists in the treatment of various tumors, the development of LH-RH antagonists is indicated (6, 7, 29, 30). The advantage of the antagonists is based on the fact that they inhibit LH and FSH and, thus, sex steroids from the start of administration (30). The use of antagonists would avoid the transient stimulation that occurs initially in response to LH-RH agonists and prevent the temporary clinical “flare-up” of the disease. LH-RH antagonists could be useful for contraception, the treatment of hormone-dependent tumors, and prevention of gonadal damage caused by radiation and chemotherapy (7, 30).

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