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GONADOTROPIN RELEASING HORMONE ANTAGONISTS: NOVEL STRUCTURES INCORPORATING N^{ω}-CYANO MODIFIED GUANIDINE MOIETIES

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A series of GnRH antagonists with substitutions at positions 1, 2, 3, 5 and 6 that included the recently reported homoArg-N^{ω}-cyano-$N^{\omega'}$-alkyl- or Lysine-N^{ε}-5'-(3-amino-1*H*-1,2,4-triazole) [Lys(atz)] amino acid derivatives was synthesized, characterized and tested for antiovulatory and anaphylactoid activities and binding affinity. Overall, these analogs were found to be considerably more soluble at neutral pH than their homologs Nal-Glu or Antide. The decapeptides with these substitutions in positions 5 and/or 6 retained high in vivo potency while those with similar substitutions at positions 1, 2 and 3 were significantly less potent than Nal-Glu or Antide. Of the 16 new analogs reported here, Azaline (Ac-DNal[1],DCpa[2],DPal[3], Lys[5](atz),DLys[6](atz), ILys[8],DAla[10]]-GnRH) showed the most promising physico-chemical and biological properties [Lys(atz)=N^{ε}-5'-(3-amino-1*H*-1,2,4-triazole) lysine]. Azaline is readily soluble in dilute buffers at pH 7.0, completely inhibits ovulation at 2.0 to 3.0 μg per rat, is equipotent to GnRH in releasing histamine in the rat and has a weaker anaphylactoid response in the rat than other analogs such as Nal-Glu or even Antide.

Since the discovery of mammalian gonadotropin releasing hormone (GnRH, <EHWSYGLRPG-amide)(1,2) approximately three to four thousand analogs have been synthesized in a wide ranging search for potent and long acting agonists and antagonists for potential use as therapeutic agents for endocrine diseases as well as for nonsteroidal contraception

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Abbreviations:

IUPAC Rules are used for nomenclature except for the following: Ac=Acetate; bCN=N^{ω}-cyano-$N^{\omega'}$-butyl; Cpa=4-Chloro-phenylalanine; DGlu[6](AA)=D-2-amino-5-oxo-5-(4-methoxyphenyl)pentanoic acid; Har=homoarginine; ILys=N^{ε}-Isopropyl lysine; Lys(atz)=N^{ε}-5'-(3-amino-1*H*-1,2,4-triazole) lysine; Lys(Nic)=N^{ε}Nicotinoyl Lysine; 2mpCN=N^{ω}-cyano-$N^{\omega'}$-2-methylpyridyl; Nal=3-(2-naphthyl)-alanine; Pal=3-(3-pyridyl)-alanine; TFA=trifluoroacetate

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(3,4). The rationale that led to the discovery of these different analogs has been previously described (5-8). By controlling the secretion of the gonadotropins, GnRH plays a major role in initiating reproductive events suggesting that inhibition of GnRH action at the level of the pituitary could alter reproductive processes. The validity of this hypothesis has recently been demonstrated in humans with the use of synthetic superagonists that desensitize the pituitary GnRH receptors, and to a much lesser extent with the use of competitive antagonists of GnRH.

The *in vitro* potency in the agonist series was improved by increasing the hydrophobic character and conformational stability of GnRH by introduction of D-aromatic residues in position 6 (such as DTrp, DNal and others). These analogs were found to exhibit prolonged duration of action *in vivo* (9). Accordingly, the first generation of potent antagonists with modifications at positions 1, 2, and 3 contained similar substitutions in position 6. Later, Nekola et al. (10) found that substitution of basic amino acids such as D-Arg in position 6 significantly increased potency of GnRH antagonists containing a hydrophobic aromatic N-terminus. This led to the development of analogs typified by the "Nal-Arg" analog, [Ac-DNal^1, DFpa^2,DTrp^3,DArg^6]-GnRH, which gave 100% inhibition of ovulation in rats on the day of proesterus at a dose of 1.0 μg (11). However, it was found that subcutaneous (sc) injection of "Nal-Arg" and structurally related antagonists produced transient edema of the face and extremities in rats (12) and a systemic effect in some humans at the highest doses tested (20 mg per individual) (13). Subsequently, the "Nal-Arg" antagonist was withdrawn from clinical investigation due to its histamine related side effects. This undesired histamine release was traced to a combination of two structural elements: the presence of hydrophobic residues at the N-terminus and of basic residues at positions 6 and 8 (14). A mechanism of action for this effect has recently been proposed by Mousli et al. (15).

While the utilization of the superagonists for the management of steroid dependent carcinomas (prostate and breast), treatment of precocious puberty, in vitro fertilization and endometriosis is well documented, the use of antagonists in humans is still limited because of a lack of sufficiently potent and safe compounds. Despite the fact that extensive clinical investigations have been carried out with the "Nal-Glu" antagonist, [Ac-DNal^1,DCpa^2,DPal^3,Arg^5,DGlu^6(AA), DAla^{10}]-GnRH (6), we recognized the need for more potent antagonists with even safer therapeutic indices (antiovulatory versus histamine releasing potencies or anaphylactoid activity). Recent work in this field therefore has focused on devising GnRH antagonists with minimal histamine releasing activity and high inhibitory potency *in vivo* (6,7). One recent approach to solving this problem is

best exemplified by the work of Ljungquist et al. (7) who introduced into the basic structure of an antagonist (Ac-DNal[1],DCpa[2],DPal[3],DAla[10]-amide), two N^{ε} nicotinoyl-lysines at positions 5 (L-configuration) and 6 (D-configuration) and an N^{ε} isopropyl lysine in position 8 to yield Antide. Antide showed very little activity in the *in vitro* histamine release assay (ED_{50} >300 μg/mL), little anaphylactoid activity in the rat (Table 1) while retaining favorable potency in the AOA and unexpected, but not always reproducible, duration of action at high doses. Unfortunately, this peptide is insufficiently soluble in aqueous buffers at pHs higher than 4.0 to 5.0 thus complicating significantly its formulation. New concepts in analog design therefore had to be developed and implemented.

In this report we describe a general approach which led to the synthesis of Azaline [Ac-DNal[1],DCpa[2],DPal[3],Lys[5](atz),DLys[6](atz),ILys[8], DAla[10]]-GnRH (Compound 6 in Table 1) and congeners. Success in attenuating the basicity of various arginine residues of active analogs was achieved by the introduction of an electronegative N^{ω}-substituent on the guanidino function (16-19). This allowed arious arginine residues to be replaced with less basic N^{ω}-cyano-$N^{\omega'}$-alkyl or aryl homoarginine and arginine and N^{ε} Isopropyl or N^{ω}-triazolyl lysine residues in active analogs (See Table 1). These novel analogs were synthesized using solid phase techniques employing a modification of the N^{ω}-NH_2 of lysine and ornithine residues in otherwise protected, resin bound peptides (16,17). These peptides were purified using preparative HPLC (20), fully characterized chemically and tested in a rat for antiovulatory (AOA) (21) and anaphylactoid activities and for binding affinity to rat pituitary cell membranes (22) (see Table 1).

Methods (See Table 1)

Analogs were synthesized by solid phase either manually or on a Beckman 990 peptide synthesizer with use of previously described protocols on a methylbenzhydryl amine (MBHA) resin using the tert-butyloxycarbonyl (Boc) group for N^{α} amino protection (6). The distal amino of the lysine residues to be modified was protected as the 9H-fluorenylmethoxycarbonyl (Fmoc) derivative. The fully assembled peptide resin was treated with freshly distilled 20% piperidine in DMF (mixed at the time of deblocking) to remove the Fmoc group. Following the procedure of Webb and Labaw (19) for the preparation of cyanoguanidines, diphenyl cyanocarbonimidate (PCI) in DMF was added, at room temperature, to the selectively deprotected peptide resin. After completion of the PCI coupling (negative ninhydrin test) an amine, RNH_2, was added. The peptides were cleaved from the resin with HF and purified using two reverse phase HPLC systems (20). Retention times using isocratic conditions and specific rotations are given in Table 1. Amino acid analyses, including quantitation of Cpa and Nal, were consistent with expected results. The new amino acids were not characterized nor were they detected by standard amino acid analysis in our standard system. Calculated values for protonated molecular ions were in agreement with those obtained using FAB mass spectrometry. FT-IR of selected analogs were also consistent (16). In order to determine the relative hydrophobicity of selected GnRH antagonists at pH 7.0, peptides

(5μg/μl) dissolved in TEAP pH 7.0/CH_3CN (76%/24%) were applied to an analytical (25 x 0.45 cm) Vydac C_{18} column. All peptides were soluble in the above buffer except for Antide (Cpd 2) which was loaded in an acidified (H_3PO_4) buffer. A gradient was run between 30% and 48% CH_3CN in 30 min. at a flow rate of 1.5 mL/min. UV detection was 0.1 AUFS at 210 nm. Under these conditions, the agonists [DTrp^6,Pro^9-NHEt]GnRH and [DHis(imBzl)^6,Pro^9-NHEt]GnRH had retention times of 7.55 and 7.85 min. respectively.

Bioassays (See Table 1)

In binding studies, the K_D for the potent agonist [DAla^6,NMeLeu^7,Pro^9-NHEt]-GnRH (taken as standard) was determined from a Scatchard analysis to be approximately 0.3 nM. All the other K_D values were calculated from the potencies of the analogs (relative to the standard) determined from displacement data (22). The AOA was carried out as described by Corbin and Beattie (21); cycling rats (250-300 g at the time of the assay) were injected subcutaneously with the peptides dissolved in saline or other appropriate solvents (200 μL) at noon on proestrus. Results are expressed in terms of the dosage in micrograms/rat (rats ovulating/total number of treated rats). In the anaphylactoid assay, an acute change in vascular permeability due to a localized injection is determined by measuring the staining of the surrounding connective tissue following the intravenous administration of Evan's blue. Evan's blue is a dye which binds to serum proteins allowing the escape of serum proteins into the interstitium to be observed. For the purpose of this assay, male Spague-Dawley rats (200-250 g) are injected intravenously with Evan's blue (1 mL of a 0.5% solution). Dilutions of peptides are injected interdermally through shaved section of each animal's back. Four sites representing vehicle and three peptide dilutions are injected in each animal. Fifteen minutes after intradermal injection, animals are sacrificed and the dorsal skin reflected. The area of the wheal formed at each injection site is measured. Regression analysis is used to estimate the dose of the peptide required to produce an 11 x 11 mm wheal.

Results and Discussion

On the basis of results indicating that the Arg^5 substitution was compatible with retention of considerable potency in the AOA, we synthesized a series of GnRH analogs including the "Nal-Glu" antagonist (Cpd 1) presently under clinical investigation in humans. This analog however still suffers from some residual histamine releasing activity, with an *in vitro* histamine release ED_{50} of 1.6 μg/mL compared to >300 μg/mL for Antide (Cpd 2) or ca 150 μg/mL for GnRH itself (14) and an anaphylactoid ED μg/std wheal of 2.02 (see Table 1). Using these two compounds as leads for the development of more potent analogs, Cpds 3-6 were prepared using the rationale presented in the introduction. Of these three analogs, Azaline (Cpd 6), has an intermediate affinity (between that of Antide and Nal-Glu) for the GnRH receptor and is about half as potent as Antide in the AOA [inhibition of ovulation in the rat by 90% at 2μg with an ED_{50} comparable to that of GnRH in the in vitro histamine release assay (data not shown) and an anaphylactoid ED μg/std wheal of 16.03 (see Table 1)]. Because its potency in releasing histamine or in the anaphylactoid test is minimal, and because of its greatly improved solubility in aqueous media at neutral pH, Azaline is a good candidate for further studies. For the purpose of comparing the relative hydrophobicity of different peptides at neutral pHs using HPLC, an order of elution on an acetronitrile gradient using a

Table 1. $[\alpha]_D$, HPLC behavior, affinity to pituitary cell membranes, antiovulatory potency and anaphylactoid activity of GnRH antagonists

Compounds	$[\alpha]_D$[a]	HPLC[b] RT (%CH_3CN)	K_D, (nM)[c]	AOA[d]	Anaphylactoid activity[e]
1. [Ac-DNal^1,DCpa^2,DPal^3,Arg^5,DGlu^6(AA),DAla^{10}]-GnRH "Nal-Glu"	-29°	4.17 (36.0)	0.67 (0.43-1.0)	0.5 (8/16), 1.5 (0/10)	2.02
2. [,Lys(Nic)^5,DLys(Nic)^6,ILys^8,DAla^{10}]-GnRH "Antide"	-31°	3.50 (29.0)	0.35 (0.25-0.47)	1.0 (2/10)	9.75
3. [,Har^5(bCN),DHar^6(bCN),ILys^8,DAla^{10}]-GnRH	-27°	4.11 (37.2)	1.3 (0.9-1.9)	0.5 (6/6),1.0 (4/14)	nd
4. [,Arg^5(bCN),DArg^6(bCN),ILys^8,DAla^{10}]-GnRH	-38°	5.11 (39.0)	2.1 (1.2-3.5)	1.0 (5/8), 2.5 (0/5)	nd
5. [,Arg^5,DHar(bCN)^6,DAla^{10}]-GnRH	-24°	3.85 (36.6)	0.92 (0.64-1.3)	1.0 (4/15)	nd
6. [,Lys^5(atz),DLys^6(atz),ILys^8,DAla^{10}]-GnRH "Azaline"	-34°	3.72 (27.0)	0.48 (0.38-0.57)	2.0 (1/10)	16.03
7. [,Lys(Nic)^5,DLys(Nic)^6,Har(bCN)^8,DAla^{10}]-GnRH	-31°	3.77 (36.0)	nd	5.0 (14/20)	nd
8. [,Lys(Nic)^5,DLys(Nic)^6,Lys(atz)^8]-GnRH	-37°	5.11 (34.8)	nd	5.0 (4/6), 10 (4/5)	nd
9. [,Lys(Nic)^5,DLys(atz)^6,ILys^8]-GnRH	-37°	3.76 (29.4)	nd	1.0(4/6), 2.5 (0/11)	nd
10. [,Lys(atz)^5,DLys(Nic)^6,ILys^8,DAla^{10}]-GnRH	-32°	4.15 (29.4)	nd	1.0(7/7), 2.0(0/5)	nd
11. [,DHar(bCN)^3,Har(bCN)^5,DHar(bCN)^6,ILys^8,DAla^{10}]-GnRH	-31°	4.04 (45.6)	10.8(6.7-17.2)	10 (4/6)	nd
12. [,DHar(bCN)^3,Arg^5,DNal^6,DAla^{10}]-GnRH	-36°	4.57 (47.0)	6.7 (4.1-11.2)	5.0 (4/5)	nd
13. [,DLys(atz)^3,Arg^5,DNal^6,DAla^{10}]-GnRH	-37°	4.39 (39.6)	4.3 (2.7-6.7)	25 (2/8)	nd
14. [,DHar(2mpCN)^3,Arg^5,DNal^6,DAla^{10}]-GnRH	nd	5.16 (39.0)	5.1 (3.2-8.0)	5.0 (4/6)	nd
15. [,DLys(atz)^3,Lys(Nic)^5,DLys(Nic)^6,ILys^8,DAla^{10}]-GnRH	-32°	4.34 (28.8)	nd	5.0 (6/6)	nd
16. [,DHar(bCN)^2,DTrp^3,DArg^6,DAla^{10}]-GnRH	-35°	4.16 (33.6)	99 (63-153)	25 (6/6)	nd
17. [,DHar(2mpCN)^2,DTrp^3,DArg^6,DAla^{10}]-GnRH	-26°	3.66 (28.8)	117 (74-184)	25 (6/6)	nd
18. [Ac-DHar(bCN)^1,DCpa^2,DTrp^3,DArg^6,DAla^{10}]-GnRH	-13°	4.00 (32.4)	nd	25 (4/4), 100(3/4)	nd

- nd: not determined.

a c=1 (weight of lyophilized peptide in 50% HOAc/H_2O).

b Peptides (10 μg/10 μL) dissolved in 0.1% TFA were applied to a Vydac C_{18} column (5 μm, 300Å pore size; 4.5 x 250 mm) under isocratic conditions, 0.1% TFA/H_2O with %CH3CN shown, at a flow rate of 2.0 mL/min. UV detection was 0.1 AUFS at 210 nm.

c Dissociation constant: see ref 22.

d AOA=antiovulatory assay: dosage in micrograms/rat (rats ovulating/total number of rats treated: weight of rat was 250-300 g).

e Anaphylactoid effective dose (μg/std wheal). Std wheal is 11 x 11 mm.

triethylammonium phosphate pH 7.0 buffer was obtained: Azaline {RT=9.32 min} was followed by Antide {RT=17.1 min}and Nal-Glu {RT=22.8 min}. Cpds 7-10 are variations on Antide whereby either the nicotinoyllysines or isopropyl lysine are substituted one at a time by homo-arginine residues or Lys(atz). The idea was to confer solubility to Antide (or hydrophobicity to Azaline) without compromising its potency *in vivo* and low histamine release potency. Except for Cpds 9 and 10, the other two analogs are significantly less potent in the AOA. This again points to the rigorous steric and charge requirements at position 8 which have been observed by many investigators (5). Because some hydrophilicity/aromaticity at position 3 was not necessarily detrimental to potency in the AOA (DPal is more hydrophilic than DTrp which was often found in early generations of potent GnRH antagonists), we investigated the effect of substituting DPal in position 3 by $N^{\omega},N^{\omega'}$ substituted cyanoguanidino DHar. Compounds 11-15 were synthesized and found to be significantly less potent in both assays suggesting that the GnRH receptor is indeed extremely sensitive to modifications in this region of the molecule. We also investigated the effect of substituting DCpa at position 2 (Cpds 16, 17) and DNal at position 1 (Cpd 18) by more hydrophilic residues; once more, the impotency of most of these analogs at doses five to ten time those at which closely related congeners showed significant activity, indicate that the substitutions selected in the past could not be further improved on within this series of analogs.

In summary, we have successfully demonstrated the applicability of a general synthetic method for modifying ω-amino-containing residues on a resin-bound peptide. A relatively high level of GnRH antagonistic activity was retained and, as hypothesized, the histamine releasing potency of some potent (in the AOA) analogs was reduced upon introduction of these less basic residues. The flexibility of this method, combined with its procedural simplicity and good yields enhances its applicability to peptide synthesis for the study of structure activity relationships and to begin to assess the role of basic residues in other bioactive peptides.

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