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The structural features of effective antagonists of the luteinizing hormone releasing hormone

Review Article

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Summary. The structure-activity data of 6 years on 395 analogs of the luteinizing hormone releasing hormone (LHRH) have been studied to determine effective substituents for the ten positions for maximal antiovulatory activity and minimal histamine release. The numbers of substituents studied in the ten positions are as follows: $(41)^1$-$(12)^2$-$(12)^3$-$(5)^4$-$(47)^5$-$(52)^6$-$(16)^7$-$(18)^8$-$(4)^9$-$(8)^{10}$. In position 1, DNal and DQal were effective with the former being more frequently the better substituent. DpClPhe was uniquely effective in position 2. Positions 3 and 4 are very sensitive to change. D3Pal in position 3 and Ser in position 4 of LHRH were in the best antagonists. PicLys and cPzACAla were the most successful residues in position 5 with cPzACAla being the better substituent. Position 6 was the most flexible and many substituents were effective; particularly DPicLys. Leu^7 was most often present in the best antagonists. In position 8, Arg was effective for both antiovulatory activity and histamine release; ILys was effective for potency and lesser histamine release. Pro^9 of LHRH was retained. $DAlaNH_2{}^{10}$ was in the best antagonists.

Keywords: Amino acids – LHRH antagonists – Histamine release – Structure-activity relationship

Abbreviations: AABLys N^{ε}-(4-acetylaminobenzoyl)lysine; AALys N^{ε}-anisinoyllysine; AAPhe 3-(4-acetylaminophenyl)lysine; Abu 2-aminobutyric acid; ACLys N^{ε}-(6-aminocaproyl)lysine; ACyh 1-aminocyclohexanecarboxylic acid; ACyp 1-aminocyclopentanecarboxylic acid; Aile alloisoleucine; AnGlu 4-(4-methoxyphenylcarbamoyl)-2-aminobutyric acid; 2ANic 2-aminonicotinic acid; 6ANic 6-aminonicotinic acid; APic 6-aminopicolinic acid; APh 4-aminobenzoic acid; APhe 4-aminophynylalanine; APz 3-amino-2-pyrazinecarboxylic acid; Aze

azetidine-2-carboxylic acid; Bim 5-benzimidazolecarboxylic acid; BzLys N^{ε}-benzoyllysine; Cit citrulline; Cl_2Phe 3-(3,4-dichlorphenyl)alanine; cPzACAla cis-3-(4-pyrazinylcarbonylaminocyclohexyl)alnine; cPmACAla cis-3-[4-(4-pyrimidylcarbonyl)aminocyclohexyl]alanine; Dbf 3-(2-dibenzofuranyl)alanine; DMGLys N^{ε}-(N,N-dimethylglycyl)lysine; Dpo N^{δ}-(4,6-dimethyl-2-pyrimidyl)-ornithine; F_2Ala 3,3-difluoroalanine; hNal 4-(2-naphthyl)-2-aminobutyric acid; HOBLys N^{ε}-(4-hydroxybenzoyl)lysine; hpClPhe 4-(4-chlorophenyl)-2-aminobutyric acid; Hse homoserine, 2-amino-4-hydroxybutanoic acid; ICapLys N^{ε}-(6-isopropylaminocaproyl)lysine; ILys N^{ε}-isopropyllysine; Ind indoline-2-carboxylic acid; INicLys N^{ε}-isonicotinoyllysine; IOrn N^{δ}-isopropylornithine; Me_3Arg N^G,N^G,N^G-trimethylarginine; Me_2Lys N^{ε},N^{ε}-dimethyllysine; MNal 3-[(6-methyl)-2-naphtyl]alanine; MNicLys N^{ε}-(6-methylpicolinoyl)lysine; MPicLys N^{ε}-(6-methylpicolinoyl)lysine; MOB 4-methoxybenzoyl; MpClPhe N-methyl-3-(4-chlorphenyl)lysine; MPZGlu glutamic acid, γ-4-methylpiperazine; Nal 3-(2-naphthyl)alanine; Nap 2-naphthoic acid; NicLys N^{ε}-nicotinoyllysine; NO_2B 4-nitrobenzoyl; NO_2Phe 3-(4-nitrophenyl)alanine; oClPhe 3-(2-chlorphenyl)alanine; Opt O-phenyl-tyrosine; Pal 3-(3-pyridyl)alanine; 2Pal 3-(2-pyridyl)alanine; 2PALys N^{ε}-(3-pyridylacetyl)lysine; pCapLys N^{ε}-(6-picolinoylaminocaproyl)lysine; pClPhe 3-(4-chlorophenyl)alanine; pFPhe 3-(4-fluorophenyl)-alanine; Pic picolinic acid; PicLys N^{ε}-picolinoyllysine; Pip piperidine-2-carboxylic acid; PmcLys N^{ε}-(4-pyrimidylcarbonyl)lysine; Ptf 3-(4-trifluromethyl phenyl)alanine; Pz pyrazinecarboxylic acid; PzAla 3-pyrazinylalanine; PzAPhe 3-(4-pyrazinylcarbonylaminophenyl)alanine; Qal 3-(3-quinolyl)alanine; QndLys N^{ε}-quinaldoyllysine; Qui 3-quinolinecarboxylic acid; Qux 2-quinoxalinecarboxylic acid; Tic 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TinGly 2-thienylglycine; tNACAla trans-3-(4-nicotinoylaminocyclohexyl)-alanine; tPACAla trans-3-(4-picolinoylaminocyclohexyl)alanine.

Introduction

Since the isolation, identification and synthesis of the luteinizing hormone releasing hormone (LHRH), in 1971, by the groups of Schally and of Guillemin (Matsuo et al., 1971a,b; Burgus et al., 1972) the research on agonists and antagonists of LHRH has experienced an incredible growth rate.

1 2 3 4 5 6 7 8 9 10

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2

Structure of LHRH

Initially, the synthetic effort was directed toward finding potent agonists which could be used as pro-fertility agents, and toward finding potent antagonists for use in contraception. It soon became apparent that the agonists which stimulated the release of gonadotropins when given acutely proved to be potent inhibitors of gonadotropin secretion when administered chronically. This finding has led to the use of potent agonists, e.g. zoladex, nafarelin, buseralin, and others in the treatment of hormone-dependent tumors.

The rationale for the development of the antagonists was derived from the recognition that a competitive antagonist of LHRH has the potential of being a nonsteroidal contraceptive agent (Schally et al., 1980). A specific physiological action was expected of the antagonists (Corbin et al., 1975) which were intended primarily for female contraception (Schally et al., 1980). These peptides were expected to be free of the toxicities which are associated with either of the components of the estrogen-progestagen combination pill.

The initial enthusiasm was, however, dimmed by the fact that the early antagonists were found to cause histamine mediated side effects, both *in vivo* and *in vitro* (Morgan et al., 1986). Current research, therefore, is focused on designing LHRH antagonists with minimized histamine releasing activity and maximized inhibitory activity, *in vivo*, (Rivier et al., 1991; Theobald et al., 1991; Janecka et al., 1991a,b; Ljungqvist et al., 1991).

Although about 3-4 thousand analogs of LHRH have been synthesized, the research for more potent and long-acting antagonists for use as therapeutic agents for endocrine diseases and for nonsteroidal contraception is still in progress.

Background for design of antagonists

According to classical theory, an effective antagonist should firmly occupy the receptor without triggering the biological response. In the case of LHRH, an agonist induces conformational changes in the receptor, which then, through secondary mechanisms, cause altered cell function and gonadotropin release. A receptor occupied by an antagonist does not adopt conformational changes.

Today, it is uncertain as to which amino acids of LHRH are functional for binding and which amino acids trigger the biological response. There is the possibility that some amino acids may possess both types of function.

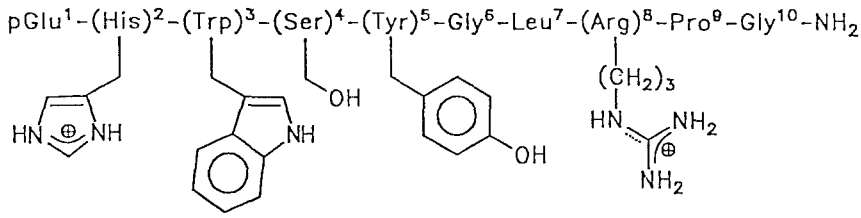
According to recent research, however, it is believed that the N-terminal is more critical than the C-terminal for effector function (Nikolics et al., 1988). This belief is based on the fact that modification of Trp^3 of LHRH caused complete loss of LH-releasing activity while [Orn^8]-LHRH is an analog with full agonist activity, but with reduced potency. Also, the N-terminal is more conserved among species than the C-terminal. Despite differences in the C-terminal, avian and teleost LHRH's show low, but full LH-releasing activities across species (Millar et al., 1983; Miyamoto et al., 1984). It seems that the N-terminal is conserved for the stimulation of LH release, and that the C-terminal contributes to species specific binding.

Chemical studies on the LHRH receptor indicate that at least two carboxyl groups may participate in the binding of the hormone (Hazum, 1987). One of these groups most probably reacts with the protonated guanidino function of Arg^8, and the other group probably reacts with the protonated basic imidazole nucleus of His^2. It is, however, conceivable that both carboxyl groups could bind to the positively charged guanidino group of Arg^8. One group could bind as a salt, and the other group through hydrogen bonding. If such binding existed, His^2 would likely be involved in other interactions.

Other amino acids of the receptor for LHRH that have been implicated in the interaction with the hormone are tyrosine and tryptophane (Keinan et al., 1985). These two amino acids could participate in aromatic stacking or in charge-transfer interactions. The indole nucleus of tryptophane is electron rich, and could strongly bind to electron poor aromatic nuclei or to positively charged species like the protonated imidazole nucleus of histidine. The phenolic OH of tyrosine could also be involved in hydrogen bonding.

The bioactive conformation of LHRH, although not established, has frequently been postulated to involve a Type II β-turn around residues 5–8 (Momany, 1976; Kopple, 1981; Karten et al., 1986) which could place the N- and C-terminals in close proximity at the receptor. The mid-portion of LHRH would, according to this concept, not bind to the receptor, but would be important for conformation, and could bind to membrane structures in the vicinity of the receptor.

The sequence of LHRH with the side chains most likely to be involved in receptor interactions may be depicted as follows:



His^2 Charge-transfer, ionic, aromatic stacking; *Trp*^{3}: Aromatic stacking, charge-transfer, hydrophobic interactions; *Ser*^{4} Hydrogen bonding; *Tyr*^{5} Aromatic stacking, charge transfer, hydrogen bonding; *Arg*^{8} Ionic interactions.

Influence of variation in sequence on the positional structure-activity relationships

In classical medicinal chemistry, one is generally concerned with structure-activity relationships of a molecule as a single unit. In peptide chemistry, and in the decapeptide chemistry of LHRH, one is seemingly concerned with ten molecules (ten residues) bonded together in contrast to a molecule as a single unit. Research on the syntheses of antagonists of LHRH has revealed that if substituent A is exchanged for substituent B in one of the ten positions, activity may increase or decrease, but this increase or decrease may not hold true if a change is made in the substituents in one of the other nine positions. In other words, the variables in the structure-activity relationships of such a decapeptide are considerably greater than are the variables in classical medicinal chemistry. Such variables also influence the anaphylactoid activity, or the histamine release, since it was observed (Phillips et al., 1987) that an antagonist without Arg may elicit some degree of anaphylactoid activity, and the structural features responsible for this activity could not be discerned.

Extensive and diversified research on the antagonists of LHRH, which encompassed these variables, and positive feedback from the multitudinous data

can facilitate progress and success. Even so, the decision making on priorities for syntheses of new analogs are very critical. Plans to synthesize a very large number of antagonists rather than a modest or a small number of antagonists is the best policy to overcome the difficulties of the molecular variations and influences, because sooner or later, in the acquisition of a large number of antagonists, a breakthrough in activity will be achieved, either by design or by "serendipity."

A summary of the best antagonists

During 1986 to 1992, 395 analogs of LHRH have been designed and synthesized in this Institute for Biomedical Research toward more potent antagonists. Only 25/395 have potencies of 50% or higher at 0.25 μg, and they are summarized in Table 1.

All ten positions of LHRH have been substituted by numerous natural, but mostly by unnatural amino acids, in order to discover structure-activity relationships and toward increases in potency and specificity of action.

Compilation of substituents

A compilation from position 1 to 10 of the different amino acids tried in each of these positions is as follows.

Position 1

NAcDNal, NAcDQal, NAcDpClPhe, DAlaDNal, NAcDOpt, NAcDCl_2Phe, Butyryl-DNal, NAcDDbf, DpGlu, NAcDPal, QuxDAla, DTyrDNal, NAc-DhNal, QuiDAla, NO_2BPro, NAcDNO_2Phe, NapDAla, MOBPro, TyrDNal, BimDAla, NAcDTic, NAcDMNal, QuiGly, NAcDTrp, NAcDPtf, QuiDNval, NAcDPhe, NAcDHis, QucDThr, DNal, Pic, NapDThr, desAcNHNal, DTyr-DQal, NapDpClPhe, DPalDNal, NAcInd, QuxDThr, QuiDSer, Qui, QuiDVal.

Position 2

DpClPhe, DPtf, DpFPhe, DCl_2Phe, DMpClPhe, DPal, DPhe, DhpClPhe, DHis, DNO_2Phe, DoClPhe, DAAPhe.

Position 3

DPal, DNO_2Phe, DHis, DhNal, DPzAla, DNal, DTrp, DNicLys, DTinGly, DPal, DpClPhe, D2Pal.

Position 4

Ser, Hse, Tyr, Abu, Thr.

Table 1. LHRH antagonists with 100% AOA at 0.5 μg and/or 50% or more AOA at 0.25 μg/rat

Position										AOA* (%)		HRA**
1	2	3	4	5	6	7	8	9	10	.25 μg	.5 μg	$ED_{50} \pm SEM$ μg/mL
NAcDNal	DpClPhe	DPal	Ser	PicLys	cDPzACAla	Leu	ILys	Pro	$DAlaNH_2$	73	100	28 ± 7.5
NAcDNal	DpClPhe	DPal	Ser	PicLys	DPicLys	Leu	IOrn	Pro	$DAlaNH_2$	50	90	42 ± 3.1
NAcDNal	DpClPhe	DPal	Ser	cPzACAla	DPicLys	Leu	ILys	Pro	$DAlaNH_2$	67	90	48 ± 4.8
NAcDNal	DpClPhe	DPal	Ser	PicLys	cDPzACAla	Leu	IOrn	Pro	$DAlaNH_2$	50	100	15 ± 1.8
NAcDNal	DpClPhe	DPal	Ser	PicLys	cDPzACAla	Val	ILys	Pro	$DAlaNH_2$	73	100	84 ± 5.6
NAcDNal	DpClPhe	DPal	Ser	cPzACAla	cDPzACAla	Leu	ILys	Pro	$DAlaNH_2$	57	100	33 ± 1.3
NAcDNal	DpClPhe	DPal	Ser	PicLys	DPicLys	Abu	Arg	Pro	$DAlaNH_2$	50	88	27 ± 3.3
NAcDNal	DpClPhe	DPal	Ser	Ile	DArg	Leu	Arg	Pro	$SarNH_2$	70		0.72 ± 0
NAcDNal	DpClPhe	DPal	Ser	cPzACAla	DPicLys	Leu	ILys	Pro	$SarNH_2$	62	100	28 ± 6.4
NAcDQal	DpClPhe	DPal	Ser	cPzACAla	DPicLys	Leu	ILys	Pro	$DAlaNH_2$	55	100	117 ± 17
NAcDNal	DpClPhe	DPal	Ser	cPzACAla	DPicLys	Leu	Me_3Arg	Pro	$DAlaNH_2$	67	89	38 ± 2
NAcDNal	DpClPhe	DPal	Ser	cPzACAla	DPicLys	Leu	ILys	Pro	$DAlaNH_2$	63		
NAcDNal	DpClPhe	DPal	Ser	PicLys	D(DSer)Lys	Leu	ILys	Pro	$DAlaNH_2$	69	95	18 ± 6.4
NAcDNal	DPtf	DPal	Ser	PicLys	DPicLys	Leu	ILys	Pro	$DAlaNH_2$	50	86	
NAcDNal	DpClPhe	DPal	Ser	Pal	DPal	Abu	Arg	Pro	$DAlaNH_2$	50		9.8 ± 0.1
NAcDQal	DpClPhe	DPal	Ser	cPzACAla	DPicLys	Leu	Arg	Pro	$DAlaNH_2$	89	100	30.8 ± 0.6
NAcDQal	DpClPhe	DPal	Ser	cPzACAla	DPicLys	Leu	Arg	Aze	$DAlaNH_2$	60		28.4 ± 0.2
NAcDQal	DPtf	DPal	Ser	cPzACAla	DPicLys	Leu	Arg	Pro	$DAlaNH_2$	100	100	40 ± 0
NAcDQal	DpClPhe	DPal	Ser	cPzACAla	DPzcLys	Leu	Arg	Pro	$DAlaNH_2$	50		
NAcDQal	DpClPhe	DPal	Ser	cPzACAla	DNicLys	Leu	Arg	Pro	$DAlaNH_2$	67		5.1 ± 2.2
NAcDQal	DpClPhe	DPal	Ser	cPzACAla	DTrp	Leu	Arg	Pro	$DAlaNH_2$	50		
NAcDQal	DpFPhe	DPal	Ser	cPzACAla	DPicLys	Leu	Arg	Pro	$DAlaNH_2$	60		
NAcDNal	DpClPhe	DPal	Ser	cPzACAla	DNicLys	Leu	ILys	Pro	$DAlaNH_2$	67	100	104 ± 2
NAcDNal	DpClPhe	DPal	Ser	PicLys	(PicSar)DLys	Leu	ILys	Pro	$DAlaNH_2$	50	100	34 ± 0.3
NAcDNal	DpClPhe	DPal	Ser	PicLys	D(6ANic)Lys	Leu	ILys	Pro	$DAlaNH_2$	50	83	96 ± 0

* Antiovulatory activity, in corn oil. ** Histamine-releasing activity.

Position 5

cPzACAla, PicLys, Pal, Ile, Me_3Arg, PmcLys, NicLys, HOBLys, Tyr, Cit, INicLys, 2PALys, NicOrn, Me_3hArg, Arg, PzAla, MNicLys, (DSer)Lys, Dpo, His, DMGLys, hCit, PzcLys, QalLys, MPicLys, PzAPhe, (PzGly)Lys, ProLys, PicLys, (PicGly)Lys, ACyhLys, PalLys, PyrLys, (PicSer)Lys, (PicACyh)Lys, APhLys, AsnLys, (ACyp)Lys, pGluLys, (PicPal)Lys, HisLys, SarLys, CitLys, (PicSar)Lys, (PzSar)Lys, (AcSar)Lys, NMeTyr.

Position 6

D(DSer)Lys, DNicLys, DPicLys, DPzcLys, cDPzACAla, DPzAla, DArg, DPal, DTrp, D(AcDSer)Lys, DAABLys, DILys, DACLys, DINicLys, DhCit, DBzLys, D(Sar)Lys, DDpo, D(DAla)Lys, DMNicLys, DEt_2hArg, D(Ser)Lys, DAnGlu, D(DThr)Lys, DMPZGlu, D(Pip)Lys, DMe_2Lys, DNal, tDNACAla, DQal, cDNACAla, D(DPro)Lys, DQndLys, D(DPal)Lys, tDPACAla, D(DSer)Orn, tDPzACAla, cDPACAla, D(Qux)Lys, DMPicLys, D(Pro)Lys, DPmcLys, DTyr, cDPmACAla, DSer, DPCapLys, D(PicSar)Lys, D(6ANic)Lys, D(AA)Lys, D(APz)Lys, D(2ANic)Lys, D(APic)Lys.

Position 7

Leu, Abu, Val, Ser, Aile, Phe, Nle, Ile, Nval, DLeu, Ala, hpClPhe, NMeLeu, Trp, ACyp, Thr.

Position 8

ILys, IOrn, Arg, Me_3hArg, Me_3Arg, Lys, NicLys, Pal, CypLys, Cit, Dpo, Arg-(NO_2), ICapLys, His, DILys, SarLys, (DSer)Lys, isoBuLys.

Position 9

Pro, Aze, Pip, βAla.

Position 10

$DAlaNH_2$, DAlaOH, $SarNH_2$, DF_2AlaNH_2, $DAbuNH_2$, $DSerNH_2$, desGly-NHEt, $GlyNH_2$.

Discussion of the best substituents

Of the very large number of substituents used in every position of LHRH, only a few were found to provide the most potent antagonists, and they are listed in Table 1. The best substituents for each of the ten positions are shown in Table 2.

A study of the data in Tables 1 and 2 have allowed the following conclusions:

Position 1

Only two amino acids were useful: DNal and DQal. DNal was effective in twice as many peptides as DQal. The large number of amino acids (41) tried in this

Table 2. The best substituents for each of the ten positions of LHRH present in the most potent antagonists

Position	Substituent	Occurrence in the 25 best antagonists	Per cent occurrence in the 25 best antagonists	Number of substituents tried in this position
1	DNal	17	68	41
	DQal	8	32	
2	DpClPhe	22	88	12
	DPtf	2	8	
	DpFPhe	1	4	
3	DPal	25	100	12
4	Ser	25	100	5
5	PicLys	9	36	
	cPzACAla	14	56	47
	Ile	1	4	
	Pal	1	4	
6	cDPzACAla	4	16	
	DPicLys	12	48	
	DArg	1	4	
	DPzAla	1	4	52
	D(DSer)Lys	1	4	
	DPal	1	4	
	DNicLys	2	8	
	DTrp	1	4	
	(PicSar)DLys	1	4	
	(6ANic)DLys	1	4	
7	Leu	22	88	
	Val	1	4	16
	Abu	2	8	
8	ILys	12	48	
	IOrn	2	8	
	Arg	10	40	18
	Me_3hArg	1	4	
9	Pro	24	96	4
	Aze	1	4	
10	DAlaNH$_2$	24	96	
	SarNH$_2$	1	4	8

position indicates that the better substituents will likely be bicyclic and aromatic. It is structurally significant that the monocyclic nucleus of pGlu of LHRH is replaced by the sterically larger bicyclic nucleus of Nal and Qal, and of the D-configuration.

Position 2

DpClPhe has been the best amino acid, and only exceptionally can it be replaced by the very similar, in the steric shape and properties, DpFPhe or DPtf.

Position 3 and 4

Both positions are very sensitive to any change. In position 3, DPal was the only residue that was present in all of the best antagonists. Even the very similar

D2Pal caused a partial loss of activity. In position 4, Ser, which occurs naturally in LHRH was exclusively present in all of the potent peptides. Not one of five different substitutents which were tried was effective.

Position 5

In almost all of the best analogs, PicLys or cPzACAla were present in position 5 in a 36% to 56% ratio, respectively, although up to 47 different residues were substituted. Analogs with cPzACAla[5] were the most potent antagonists.

Position 6

This position has been the most flexible to changes with up to ten different amino acids out of 52 being present in the 25 best antagonists. Among the successful substitutents, one-half were acylated D-lysines, with DPicLys being present in 48% of the potent antagonists.

Position 7

Although Leu was the best residue for position 7 in 88% of the most potent analogs, the similar amino acids such as Val, Abu, Ile, Nval, etc., slightly improved the potency or decreased histamine release, in some cases. Changes to other structurally different amino acids have always been detrimental in position 7.

Position 8

This position is very important for both potency and histamine release. ILys and Arg are present in almost the same number of the best peptides, and one is not decisively the better substituent. Generally, Arg seems to contribute more to potency, but unfortunately also to histamine release. ILys may be the best choice for potency and safety. The other two substituents that were successful in position 8 are closely related; they are IOrn and Me_3hArg.

Position 9

Natural Pro in position 9 has been seldom substituted. Only three other amino acids were tried, including Aze, which is the four-member homolog of Pro. It was introduced with some success, but was not better than Pro.

Position 10

DAlaNH_2 was present in 96% of the best antagonists. The only other successful substituent was SarNH_2.

A summary of the best substituents for the 10 positions of LHRH is as follows:

Position 1 – DNal, DQal
Position 2 – DpClPhe
Position 3 – DPal
Position 4 – Ser
Position 5 – cPzACAla, PicLys
Position 6 – DPicLys was most often used. Many substitutents are effective.
Position 7 – Leu
Position 8 – ILys, Arg
Position 9 – Pro
Position 10 – $DAlaNH_2$

General observations

It seems that the substitutions in positions 2, 3, 4, 7, 9, and 10 have the highest efficacy by potency, at least at this stage of knowledge, and their replacement is not yet likely to be successful. These substituents are common for almost all of the LHRH antagonists which are now being internationally synthesized.

The study of structure-activity relationships for LHRH antagonists has mostly been concentrated on the remaining four positions, that is 1, 5, 6, and 8.

Specific structure-activity relationships for positions 1, 5, 6, and 8

Position 1

Position 1 was, in most cases, occupied by DNal since this residue was first introduced (Horvath et al., 1982). This substitution is very interesting when considering the large difference in structure between Nal and the natural substitutent pGlu. It has been suggested that the N-terminal of LHRH is associated with the effector mechanisms and C-terminal with binding (Nikolics et al., 1988). It is thus possible that the effectiveness of DNal is due to the very fact that it is so dissimilar to pGlu. It may not bind to the same function of the receptor as pGlu; it may not bind to the receptor at all. It may exert its effect by shielding parts of the N-terminal from interactions with the receptor, e.g., interactions involving hydrogen bonding.

Table 3 illustrates the structure-activity studies for position 1 in the sequence: X-DpClPhe-DPal-Ser-PicLys-DPicLys-Leu-ILys-Pro-$DAlaNH_2$. Among the 21 residues tried in position 1 in this sequence, DNal was definitely the best.

The more hydrophilic DQal (Ljungqvist et al., 1991) can produce very potent analogs but, though it is difficult to explain, only in combination with $cPzACAla^5$. It may be that the high hydrophilicity provided by the DQal moiety needs to be balanced by high lipophilicity somewhere in the sequence. This high lipophilicity may be provided by the cPzACAla residue in position 5.

The peptides in Table 4 have DQal in position 1, but different residues in position 5, 6 and 8. They are divided into groups which have different substituents only in position 5. In all groups, except for one, the analog with $cPzACAla^5$ was the most potent.

Table 3. The comparison of AOA's for the peptides with different substituents in position 1 of the general sequence: NAc( )[1]-DpClPhe-DPal-Ser-PicLys-DPicLys-Leu-ILys-Pro-DAlaNH$_2$

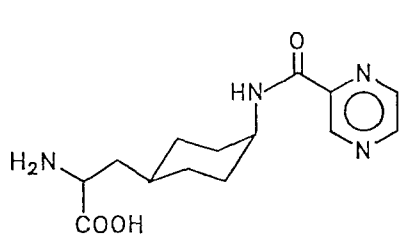
#	Position 1	AOA (%) 0.5 μg/rat	Reference
1	DNal	100	Ljungqvist et al., 1987
2	DQal	60	Janecka et al., 1993a
3	DMNal	56	Janecka et al., 1991c
4	DPtf	20	Janecka et al., 1991c
5	NO_2BPro	0	unpublished data
6	MOBPro	0	unpublished data
7	DCl_2Phe	38	unpublished data
8	QuxDAla	0	Janecka et al., 1993b
9	QuiDAla	0	Janecka et al., 1993b
10	NapDAla	17	Janecka et al., 1993b
11	BimDAla	0	Janecka et al., 1993b
12	QuiGly	0	Janecka et al., 1993b
13	QuiDNval	20	Janecka et al., 1993b
14	QuiDThr	60	Janecka et al., 1993b
15	NapDTrp	0	Janecka et al., 1993b
16	NapDpClPhe	0	Janecka et al., 1993b
17	NapDThr	20	Janecka et al., 1993b
18	QuxDThr	60	Janecka et al., 1993b
19	Qui	0	Janecka et al., 1993b
20	QuiDSer	20	Janecka et al., 1993b
21	QuiDVal	0	Janecka et al., 1993b

Table 4. Comparison of the AOA data for analogs with DQal[1] of general sequence: NAcDQal-DpClPhe-DPal-Ser-( )[5]-( )[6]-Leu-( )[8]-Pro-DAlaNH$_2$

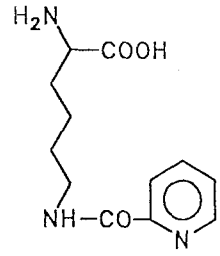
	Position			AOA (%) dose in μg/rat		
#	5	6	8	0.25	0.5	Reference
1	cPzACAla	DPicLys	ILys	55	100	Ljungqvist et al., 1991
2	PicLys	DPicLys	ILys		60	Janecka et al., 1993a
3	(PicSar)Lys	DPicLys	ILys		20	Janecka et al., 1993c
4	cPzACAla	DPicLys	Arg	89	100	Janecka et al., 1991a
5	PicLys	DPicLys	Arg	0	33	Janecka et al., 1991b
6	His	DPicLys	Arg		20	unpublished data
7	PzcLys	DPicLys	Arg		17	unpublished data
8	Arg	DPicLys	Arg		0	unpublished data
9	PzAPhe	DPicLys	Arg	33		Janecka et al., 1991b
10	Cit	DPicLys	Arg	29		Janecka et al., 1991b
11	cPzACAla	DPicLys	His	33	45	Janecka et al., 1993a
12	PicLys	DPicLys	His		0	Janecka et al., 1993a
13	cPzACAla	DNicLys	ILys	25		unpublished data
14	NicLys	DNicLys	ILys	0	0	unpublished data
15	cPzACAla	D(PicSar)Lys	ILys		18	Janecka et al., 1993c
16	Tyr	D(PicSar)Lys	ILys		17	Janecka et al., 1993c
17	PicLys	D(PicSar)Lys	ILys		43	Janecka et al., 1993c
18	cPzACAla	D(PicSar)Lys	Arg		80	unpublished data
19	PicLys	D(PicSar)Lys	Arg		57	Janecka et al , 1993c

Position 5

In position 5, our two best substituents were cPzACAla and PicLys. They are difficult to compare, because cPzACAla is the most effective with DQal[1]. PicLys is more effective in combination with DNal[1].



cPzACAla



PicLys

The data in Table 5 shows that both DNal[1], PicLys[5] or DQal[1], cPzACAla[5] antagonists can be equally potent in some sequences and it has been unpredictable which combination of the two moieties would be more effective in another sequence.

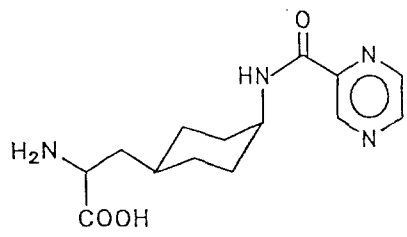
Position 6

Position 6 has been the most flexible to changes. Ten residues out of fifty two which were tried in this position were present in the best antagonists. DPicLys was used the most frequently, which is why this amino acid is present in 48% of the best compounds (Table 1). However, other residues can be equally or even more effective.

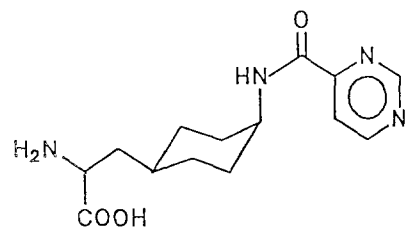
Table 6 presents structure-activity relationships for the sequence NAcDNal-DpClPhe-DPal-Ser-PicLys-( )[6]-Leu-ILys-Pro-DAlaNH$_2$. The best residues for position 6 in this sequence were DPicLys, cDPzACAla, D(PicSar)Lys, and DSarAPhe.

The comparison of data in Table 6 clearly shows that very small structural changes can affect activity. Peptides 6 and 7 both have in position 6, 3-(4-aminocyclohexyl)alanine acylated with pyrazinecarboxylic acid (Pz) or 4-pyrimidinecarboxylic acid (Pm), respectively.

A different position of only one nitrogen atom in the aromatic ring can be responsible for a loss of AOA from 100% to only 9%.



cPzACAla



cPmACAla

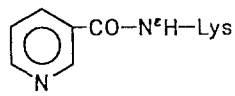
Table 5. Comparison of AOA's for analogs with DQal[1] and cPzACAla[5] or DNal[1] and PicLys[5] of the general sequence NAc( )[1]-( )[2]-DPal-Ser-( )[5]-( )[6]-Leu-( )[8]-Pro-DAlaNH$_2$

	Position					AOA (%) dose in μg/rat		
#	1	2	5	6	8	0.25	0.5	Reference
1	NAcDNal	DpClPhe	PicLys	DPicLys	ILys	40	100	Ljungqvist et al., 1987
2	NAcDQal	DpClPhe	cPzACAla	DPicLys	ILys	55	100	Ljungqvist et al., 1991
3	NAcDNal	DpClPhe	PicLys	DPicLys	Arg		0	Janecka et al., 1993a
4	NAcDQal	DpClPhe	cPzACAla	DPicLys	Arg	89	100	Janecka et al., 1993a
5	NAcDNal	DpClPhe	PicLys	D(DSer)Lys	ILys	69	95	Ljungqvist et al., 1991
6	NAcDQal	DpClPhe	cPzACAla	D(DSer)Lys	ILys		14	unpublished data
7	NAcDNal	DPtf	PicLys	DPicLys	ILys	50	86	unpublished data
8	NAcDQal	DPtf	cPzACAla	DPicLys	ILys	20		Janecka 1991b
9	NAcDNal	DpClPhe	PicLys	D(PicSar)Lys	ILys	50	100	Janecka et al., 1993c
10	NAcDQal	DpClPhe	cPzACAla	D(PicSar)Lys	ILys		18	Janecka et al., 1993c
11	NAcDNal	DpClPhe	PicLys	D(PicSar)Lys	Arg	50	100	Janecka et al., 1993c
12	NAcDQal	DpClPhe	cPzACAla	D(PicSar)Lys	Arg		80	Janecka et al., 1993c

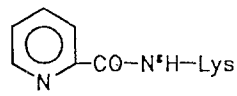
Table 6. Comparison of AOA data for analogs with different substituents in position 6. General Sequence: NAcDNal-DpClPhe-DPal-Ser-PicLys-( )^6-Leu-ILys-Pro-DAlaNH$_2$

#	Position 6	AOA (%) dose in μg/rat 0.25	0.5	Reference
1	DNicLys		64	Ljungqvist et al., 1987
2	DPicLys	40	100	Ljungqvist et al., 1987
3	tDPACAla		50	Ljungqvist et al., 1990
4	tDPzACAla		44	Ljungqvist et al., 1990
5	cDPACAla		54	Ljungqvist et al., 1990
6	cDPzACAla	73	100	Ljungqvist et al., 1990
7	cDPmACAla		9	Ljungqvist et al., 1990
8	DPzAla	9		unpublished data
9	DAABLys		25	Ljungqvist et al., 1991
10	D(DSer)Lys	69	95	unpublished data
11	D(AcDSer)Lys	0	0	Suzuki et al., 1992
12	D(DPro)Lys		56	Suzuki et al., 1992
13	D(DPal)Lys		57	unpublished data
14	D(Qui)Lys		25	unpublished data
15	D(Qux)Lys		10	Suzuki et al., 1992
16	D(Sar)Lys	33	86	Janecka et al., 1993c
17	D(PicSar)Lys	50	100	Janecka et al., 1993c
18	DSarAPhe	29	100	Janecka et al., 1993c
19	D(PicSar)APhe		0	Janecka et al., 1993d
20	D(6ANic)Lys	50	83	Janecka et al., 1993d
21	D(APz)Lys		77	Janecka et al., 1993d
22	D(2ANic)Lys		0	Janecka et al., 1993d
23	D(APic)Lys		33	Janecka et al., 1993d

As was also observed before (Folkers et al., 1991), substitution of DPicLys (peptide 2) for DNicLys (peptide 1) caused an increase in potency from 64% to 100%, although the structural difference is minor.



NicLys



PicLys

A complete loss of activity was also observed when D(Sar)APhe (peptide 18, 100% AOA) was changed for D(PicSar)APhe (peptide 19, 0% AOA). However, the same picolinic acid residue was advantageous when connected to D(Sar)Lys (peptide 16), resulting in the more potent peptide 17 with D(PicSar)Lys.

Position 8

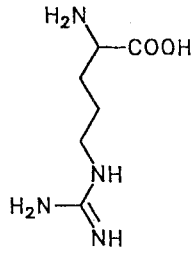
Arg, naturally occurring in position 8 of LHRH, has been used with success in antagonists over many years until it was realized that basic residues in position

Table 7. The comparison of AOA and HRA data of LHRH antagonists with Arg^8 or $ILys^8$. General sequence: NAc( )[1]-DpClPhe-DPal-Ser-( )[5]-( )[6]-( )[7]-( )[8]-Pro-DAlaNH$_2$

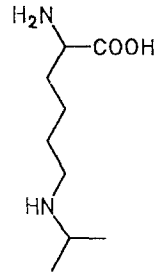
#	Positions					AOA (%) dose in μg/rat		HRA $ED_{50} \pm SEM$ (μg/mL)	Reference
	1	5	6	7	8	0.25	0.5		
1	DQal	cPzACAla	DPicLys	Leu	ILys	55	100	171 ± 17	Ljungqvist, 1991
2	DQal	cPzACAla	DPicLys	Leu	Arg	89	100	30.8 ± 0.54	Janecka, 1991a
3	DNal	cPzACAla	DPicLys	Leu	ILys	64	90	49 ± 4.9	Janecka, 1993a
4	DNal	cPzACAla	DPicLys	Leu	Arg		75	5.3 ± 0	Janecka, 1991a
5	DQal	cPzACAla	DPicLys	Abu	ILys	11			Janecka, 1991a
6	DQal	cPzACAla	DPicLys	Abu	Arg	29		40 ± 5.4	Janecka, 1991a
7	DQal	cPzACAla	DPzcLys	Leu	ILys	17			unpublished data
8	DQal	cPzACAla	DPzcLys	Leu	Arg	50			unpublished data
9	DQal	cPzACAla	DNicLys	Leu	ILys	17			unpublished data
10	DQal	cPzACAla	DNicLys	Leu	Arg	67			unpublished data
11	DNal	(PicSer)Lys	DPicLys	Leu	ILys		75	45 ± 8	Janecka, 1993c
12	DNal	(PicSer)Lys	DPicLys	Leu	Arg		67	3.6 ± 0.1	Janecka, 1993c
13	DNal	PicLys	D(PicSar)Lys	Leu	ILys	50	100	34 ± 2	Janecka, 1993c
14	DNal	PicLys	D(PicSar)Lys	Leu	Arg	50	100	10.5 ± 0	Janecka, 1993c
15	DNal	PicLys	D(6ANic)Lys	Leu	ILys	62	83	96 ± 0	Janecka, 1993d
16	DNal	PicLys	D(6ANic)Lys	Leu	Arg	67	83	7.7 ± 3	Janecka, 1993d
17	DNal	PicLys	D(2ANic)Lys	Leu	ILys		0		Janecka, 1993d
18	DNal	PicLys	D(2ANic)Lys	Leu	Arg		0		Janecka, 1993d
19	DNal	PicLys	D(APz)Lys	Leu	ILys		77	100 ± 0	Janecka, 1993d
20	DNal	PicLys	D(APz)Lys	Leu	Arg		0	8.2 ± 0	Janecka, 1993d
21	DNal	(6ANic)Lys	D(6ANic)Lys	Leu	ILys		0		Janecka, 1993d
22	DNal	(6ANic)Lys	D(6ANic)Lys	Leu	Arg		0		Janecka, 1993d
23	DNal	(2ANic)Lys	D(2ANic)Lys	Leu	ILys		0		Janecka, 1993d
24	DNal	(2ANic)Lys	D(2ANic)Lys	Leu	Arg		0		Janecka, 1993d
25	DNal	(APz)Lys	D(APz)Lys	Leu	ILys		14		Janecka, 1993d
26	DNal	(APz)Lys	D(APz)Lys	Leu	Arg		33		Janecka, 1993d

6 and 8, together with an hydrophobic N-terminal, are responsible for the undesired histamine release (Schmidt et al., 1984; Hahn et al., 1985; Mousli et al., 1990). Then, Arg was successfully replaced by ILys (Hocart et al., 1987), which is much less basic. Higher ED_{50} values for histamine release were observed for antagonists with $ILys^8$ in comparison with Arg^8 (Table 7). The comparison of AOA values for antagonists with Arg or ILys in position 8, as made in Table 7, does not determine which of these two residues is the more effective. The data of Table 7 indicate that, in some cases, Arg is superior to ILys, but in other cases, ILys is better, and sometimes they are equipotent.

It is questioned why the very basic arginine residue, which is supposed to bind to the carboxylic groups of the receptor through ionic interaction, can be replaced by the much less basic isopropyllysine, which cannot interact in the same way with the receptor, but still be effective. Perhaps binding is more steric than ionic.



Arg



ILys

The similar steric shape of these two molecules may be partly responsible for the successful substitution.

Histamine release

The discovery of the edematogenic and anaphylactoid properties of some LHRH antagonists necessitated further structural changes if these LHRH antagonists were to receive serious consideration as potential contraceptive agents.

The release of histamine by LHRH agonists and antagonists, as well as by other peptides, appears to be a function of specific structural parameters which are independent of other inherent biological activities. It should, therefore, be possible to incorporate structural elements which would retain the gonadotropin-suppressive properties of the most potent LHRH antagonists, and yet would greatly reduce the histamine releasing potency of these analogs.

As is now known, the structural characteristics of the most potent LHRH analogs in triggering histamine release *in vitro* involve a combination of a strongly basic D-amino acid side chain (Arg or Lys) at position 6 in close proximity to Arg^8, and a cluster of hydrophobic aromatic amino acids at the N-terminal (Schmidt et al., 1984). A great reduction of histamine releasing activity was achieved in Antide (Ljungqvist et al., 1987), by substituting Arg^6 by acylated lysine and Arg^8 by alkylated lysine.

The ED_{50} value of Antide was >300 μg/mL. It is difficult to explain which structural features or their combination is responsible for this high ED_{50} value,

because some other antagonists with the same substitutions in positions 6 and 8 have lower ED_{50} values (Table 8). Also, analogs with DPicLys^6 instead of DNicLys^6 released more histamine (Table 8). However, as shown in Table 7, ILys^8 is generally superior to Arg^8 in terms of histamine-dependent side effects.

A reduction of histamine releasing potency by introducing more hydrophilic N-terminals was observed when DQal was substituted for DNal^1. Even better results in terms of safety were obtained with the very hydrophilic DThr^1 acylated by quinoline-, or by quinoxalinecarboxylic acids (Table 9).

The analogs with the highest ED_{50} values (>300 ug/mL) are summarized in Table 10. It is difficult to identify the structural features responsible for these

Table 8. ED_{50} data for histamine release for LHRH antagonists with acylated Lys in position 6 and alkylated Lys in position 8. General sequence: NAcDNal-DpClPhe-DPal-( )^4-( )^5-( )^6-( )^7-ILys-Pro-DAlaNH$_2$

#	Position 4	5	6	7	HRA $ED_{50} \pm$ SEM (μg/mL)	Reference
1	Ser	NicLys	DNicLys	Leu	300 ± 0	Ljungqvist et al., 1988
2	Ser	Ile	DNicLys	Leu	324 ± 20	Ljungqvist et al., 1988
3	Ser	Arg	DNicLys	Leu	4.3 ± 0.52	Ljungqvist et al., 1988
4	Ser	Pal	DNicLys	Leu	43 ± 13	Ljungqvist et al., 1988
5	Ser	DMGLys	DNicLys	Leu	24 ± 0.4	Ljungqvist et al., 1988
6	Ser	PicLys	DNicLys	Leu	39 ± 1.0	Ljungqvist et al., 1988
7	Ser	2PALys	DNicLys	Leu	260 ± 0	Ljungqvist et al., 1988
8	Ser	cPzACAla	DNicLys	Leu	104 ± 0.4	Ljungqvist et al., 1988
9	Thr	NicLys	DNicLys	Leu	100 ± 0	Ljungqvist et al., 1988
10	Ser	NicLys	DNicLys	Thr	298 ± 3	Ljungqvist et al., 1988
11	Ser	PicLys	DPicLys	Leu	93 ± 11	Ljungqvist et al., 1987
12	Ser	NicLys	DPicLys	Leu	60 ± 1.4	Ljungqvist et al., 1987
13	Ser	cPzACAla	DPicLys	Leu	49 ± 4.8	Ljungqvist et al., 1990
14	Ser	SerLys	DPicLys	Leu	22 ± 4.7	Ljungqvist et al., 1990
15	Thr	PicLys	DPicLys	Leu	32.5 ± 2	Ljungqvist et al., 1990
16	Ser	PicLys	DPicLys	Thr	30.5 ± 2	Ljungqvist et al., 1990

Table 9. The comparison of ED_{50} values for histamine release for LHRH antagonists with DNal and more hydrophilic residues in position 1. General sequence: ( )^1-DpClPhe-DPal-Ser-( )^5-DPicLys-Leu-( )^8-Pro-DAlaNH$_2$

#	1	Position 5	6	8	HRA $ED_{50} \pm$ SEM (μg/mL)	Reference
1	NAcDNal	PicLys	DPicLys	ILys	93 ± 11	Ljungqvist et al., 1987
2	NAcDQal	PicLys	DPicLys	ILys	100 ± 0	Janecka et al., 1993a
3	QuiDThr	PicLys	DPicLys	ILys	307 ± 0	Janecka et al., 1993b
4	QuxDThr	PicLys	DPicLys	ILys	315 ± 15	Janecka et al., 1993b
5	NAcDNal	cPzACAla	DPicLys	ILys	49 ± 4.8	Ljungqvist et al., 1990
6	NAcDQal	cPzACAla	DPicLys	ILys	171 ± 17	Ljungqvist et al., 1991
7	NAcDNal	cPzACAla	DPicLys	Arg	5.5 ± 0.8	Ljungqvist et al., 1991
8	NAcDQal	cPzACAla	DPicLys	Arg	30.8 ± 0.6	Janecka et al., 1991a

Table 10. LHRH antagonists with ED_{50} value for histamine release of 300 μg/mL or more. General structure: ( )[1]-( )[2]-DPal-Ser-( )[5]-( )[6]-( )[7]-( )[8]-Pro-DAlaNH$_2$

#	Position 1	2	5	6	7	8	HRA $ED_{50} \pm$ SEM μg/mL	AOA (%) dose in μg/rat 0.5	1.0
1	NAcDNal	DpClPhe	NicLys	DNicLys	Leu	ILys	>300	36	100
2	NAcDNal	DpClPhe	NicLys	DNicLys	Leu	Dpo	>300		18
3	NAcDNal	DpClPhe	Ile	DNicLys	Leu	ILys	324 ± 20		82
4	NAcDNal	DpClPhe	Tyr	DNicLys	Leu	IOrn	>300		54
5	NAcDNal	DpClPhe	NicLys	DBzLys	Leu	ILys	>300		50
6	NAcDNal	DpClPhe	MNicLys	DMNicLys	Leu	ILys	>300	56	100
7	NAcDNal	DpClPhe	NicLys	DAnGlu	Leu	ILys	>300		67
8	NAcDNal	DpClPhe	NicLys	DNicLys	Thr	ILys	298 ± 3	0	
9	NAcDNal	DpClPhe	PicLys	DPicLys	Leu	ICapLys	>300	84	
10	NAcDQal	DpClPhe	cPzACAla	DPicLys	Leu	His	300 ± 0	45	
11	NAcDQal	DpClPhe	cPzACAla	DPicLys	Thr	ILys	310 ± 0	0	
12	NAcDQal	DpFPhe	cPzACAla	DPicLys	Leu	ILys	300 ± 0	50	
13	NAcDQal	DpClPhe	PzcOrn	DPicLys	Leu	ILys	300 ± 0	50	
14	NAcDCl$_2$Phe	DpClPhe	Tyr	DNicLys	Leu	ILys	311 ± 6.5	0	89
15	QuiDThr	DpClPhe	PicLys	DPicLys	Leu	ILys	307 ± 9	60	
16	QuxDThr	DpClPhe	PicLys	DPicLys	Leu	ILys	315 ± 15	60	

high values. Unfortunately none of the antagonists from Table 10 has 100% AOA at 0.5 ug.

The search for an antagonist which is both safe and potent goes on.

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