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SUPERIORITY OF AN ANTAGONIST OF THE LUTEINIZING HORMONE RELEASING HORMONE WITH EMPHASIS ON ARGININE IN POSITION 8, NAMED ARGTIDE

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SUMMARY: In the research for more potent antagonists of the luteinizing hormone releasing hormone (LHRH), 13 new peptides with emphasis on arginine in position 8 were designed, synthesized and tested for anti-ovulatory activity (AOA). Two very potent analogs were achieved. N-Ac-D-3-Qal, D-pClPhe, D-3-Pal, Ser, c-PzACAla, D-PicLys, Leu, Arg, Pro, D-AlaNH$_2$ showed 63% AOA at 0.125 µg and 89% at 0.25 µg, and an ED_{50} of 30.8 ± 0.59 and presently may be the most promising antagonist reported. It is named Argtide. N-Ac-D-3-Qal,D-pClPhe, D-3-Pal,Ser, c-PzACAla, D-PicLys, Val, Arg, Pro, D-AlaNH$_2$ showed 18% AOA at 0.125 µg. Arg^8 in antagonists may be significant for receptor binding.

Effective antagonists of the luteinizing hormone-releasing hormone (pGlu,His,Trp,Ser,Tyr,Gly,Leu,Arg,Pro,Gly-NH$_2$) have been objectives of international research. This field has been aptly reviewed by Karten et al. (1).

Lunenfeld and Insler (2) summarized four different rationales for potential therapy of clinical modalities. They are: (1) to suppress steroid -dependent mechanisms of malignancies and endometriosis; (2) to inhibit precocious puberty, etc.; (3) to control gonadotropin secretion in ovulation, etc.; (4) to exploit other effects depending upon future proof of applicability. The existing wide-scale clinical use of LHRH agonists is a background for potential uses of antagonists, but the lower activities of antagonists, by one-thousandth that of agonists means that per unit dose the potency of the presently known antagonists need to be increased up to ten- fold for promising clinical use.

Abbreviations for the unnatural Amino Acids:

c-PzACAla	-	cis-3-(4-pyrazinylcarbonylaminocyclohexyl)alanine			
iLys	-	Nε-isopropyllysine	pClPhe	-	3-(4-chlorophenyl)alanine
2-Nal	-	3-(2-naphthyl)alanine	PicLys	-	Nε-picolinoyllysine
NicLys	-	Nε-nicotinoyllysine	PzLys	-	Nε-pyrazinylcarbonyllysine
3-Pal	-	3-(3-pyridyl)alanine	3-Qal	-	3-(3-quinolyl)alanine

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In the early years, antagonists such as [D-Phe^2,Pro^3,DTrp^6] LHRH (3) showed AOA_{100} at 750 μg/rat. Introduction of basic D-amino acids in position 6 resulted in a significant increase of antiovulatory activity. Antagonists like [N-Ac-D-2-Nal^1;D-pClPhe^2,D-Trp^3,D-Arg^6, D-Ala^{10}]LHRH by Horvath et al. (4) and [N-Ac-D-2-Nal^1, D-pClPhe^2, D-3-Pal^3, D-Arg^6, Trp^7, D-Ala^{10}] LHRH by Folkers et al. (5) were examples of antagonists with AOA of 100% at ca. 0.5 μg/rat.

However, the most potent D-Arg^6-containing antagonists produced edema in the face and extremities (6,7) and a dose-related wheal response (8). These undesired effects apparently caused by release of histamine from mast cells (9) have been ascribed to the presence of strongly basic residues in position 6 and 8, e.g., D-Arg^6, Arg^8, and a cluster of hydrophobic amino acids at the N-terminal (10, 11).

With new emphasis on decreasing the histamine releasing activity, structural modification of antagonists was focused primarily on reducing basicity in positions 6 and 8. This goal was achieved by different approaches. Folkers et al. (12) introduced D-3-Pal^6 instead of DArg^6 and obtained the relatively potent antagonist [N-Ac-D-2-Nal^1, DpClPhe^2, D-3-Pal^3,Arg^5,D-3-Pal^6, DAla^{10}]LHRH, but the histamine release was undesirable. Supression of histamine release occurred by introduction of D-ureidoalkyl amino acids such as D-citrulline or D-homocitrulline at position 6 by Bajusz et al (13), but the most active antagonist of their series, [N-Ac-D-2-Nal^1, D-pClPhe^2, D-Trp^3, D-Cit^6, D-Ala^{10}]LHRH, caused 100% AOA in doses as high as 3 μg/rat.

Combining aspects of safety and AOA was achieved by Ljungqvist et al. (14) by a new class of antagonists which featured acylated Lys residues in positions 5 and 6 in combination with alkylated Lys in position 8. The prominent example of this class of antagonists is Antide, [N-Ac-D-2-Nal^1, D-pClPhe^2, D-3-Pal^3,NicLys^5,D-NicLys^6,ILys^8,D-Ala^{10}]LHRH which completely inhibited ovulation at 1 μg/rat and had an ED_{50} for histamine release > 300 μg/ml. Antagonists with AOA superior to Antide with acylated aminocyclohexylalanines and acylated lysines in position 5 and 6 were next reported; Ljungqvist et al. (15).

We describe the design, synthesis and bioassay data on new antagonists of LHRH, toward the goal of a ten-fold increase in potency. Since there are no established guidelines for design for a high probability of increased potency, many alternatives of design are required in the hope that greatly increased potency will be unpredictably achieved. Two of 13 antagonists have activities at dosages of 0.125 μg. One of these antagonists may be comparable to the most potent antagonists reported in the literature to date. This antagonist, named Argtide, is [N-Ac-D-3-Qal^1, D-pClPhe^2,

D-3-Pal^3, c-PzACAla^5, D-PicLys^6, D-Ala^{10}]-LHRH and it showed 63% AOA at 0.125 μg and 89% AOA at 0.25 μg.

MATERIALS AND METHODS

Materials: The natural amino acids were obtained from Peninsula Laboratories, San Carlos, CA. BOC-Abu was purchased from Sigma Chemical Co., St. Louis, MO. BOC-D-2-Nal, BOC-D-3-Qal, BOC-D-pClPhe, BOC-D- and L-3-Pal were provided by Dr. Narashima Rao of the Southwest Foundation for Biomedical Research, San Antonio, TX. α-BOC-cis-L-aminocyclohexylalanine, from Dr. Narashima Rao, was converted to the corresponding BOC-cis-L-PzACAla derivative by acylation with the p-nitrophenylester (16) of pyrazinecarboxylic acid from the Aldrich Chemical Co., Milwaukee, WI, in DMF. BOC-D- and L-PicLys and BOC-PzcLys were similarly prepared from BOC-D- and L-Lys and picolinic acid (17) or, pyrazine carboxylic acid p-nitrophenylester in DMF. The benzhydrylamine hydrochloride resin was purchased from Advanced Chem Tech, Louisville, KY. The dicyclohexylcarbodiimide was from Sigma and was not distilled before use. The dichloromethane was distilled from sodium carbonate. All other solvents and reagents were of reagent grade.

Synthesis: The peptides were synthesized by the manual solid phase method as described (5). The peptides were cleaved from the resin with the concomitant removal of the protecting groups by treatment with doubly distilled HF(1) at 0°C for 1 h in the presence of about 10% p-cresol. The HF was then evaporated in vacuo. The residue was extracted 3 times with ether to remove non-peptidic material. The crude peptides were extracted with 20-50% aq. acetic acid and the extracts were lyophilized.

Purification and Characterization: Purification was achieved by gel filtration on Sephadex G-25 with 20% 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).

Amino acid analyses were carried out on a Beckman 118CL Amino Acid Analyzer after hydrolysis in constant boiling HCl for 24 h using standard procedures (5). The unnatural amino acids were qualitatively determined with the exception of 3-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. Different gradients of increasing concentration of acetonitrile in 0.01 M KH_2PO_4, adjusted to pH3 with H_3PO_4, were employed. All peptides were estimated to be 97-99% pure in this system.

Biological Assays: The AOA was determined in rats as reported (18). The in vitro histamine release test in rat mast cells was performed, as described (9, 19), and the results are reported as ED_{50} values which is the concentration in μg/ml that releases 50% of the total releasable histamine.

The data from the determinations of AOA and ED_{50} are in Table I.

RESULTS AND DISCUSSION

Based on the importance of the antagonist-receptor structural relationship, Arg^8 has been introduced in 13 new analogs towards achieving an increase in the AOA potency. Changes were made in positions 1, 5, 6 and

Table 1. Data for LHRH antagonists of the general sequence: N-Ac-( )[1],D-pClPhe[2],D-3-Pal[3],Ser[4], ( )[5],( )[6],( )[7],( )[8], Pro[9],D-Ala-NH_2[10]

#	1	5	6	7	8	%AOA/μg dosage 0.125	0.25	0.5	TLC Data Rf^1	Rf^2	Rf^3	Rf^4
1[a]	D-2-Nal	Tyr	D-NicLys	Leu	Arg			60				
2	"	"	D-PicLys	"	"		0		0.37	0.67	0.90	0.75
3	"	PicLys	D-Tyr	"	"		0		0.37	0.42	0.70	0.57
4	"	Tyr	D-Trp	Val	"			44	0.30	0.61	0.88	0.63
5	"	c-PzACAla	"	Leu	"		17		0.41	0.56	0.83	0.72
6	"	"	D-Tyr	"	"			50	0.37	0.58	0.83	0.73
7	"	"	D-Qal	Val	"			50	0.18	0.56	0.87	0.78
8	"	PicLys	"	"	"			25	0.43	0.54	0.96	0.56
9[b]	"	c-PzACAla	D-PicLys	Leu	ILys		64	90				
10[c]	"	"	"	Val	"		12					
11	"	"	"	Leu	Arg			75	0.38	0.63	0.80	0.72
12	"	"	"	Val	"		20	100	0.16	0.58	0.87	0.77
13	"	PzcLys	"	"	"		0		0.37	0.59	0.88	0.64
14[de]	D-3-Qal	c-PzACAla	"	Leu	ILys	17	55	100				
15[f]	"	"	"	"	Arg	63	89		0.31	0.63	0.70	0.58
16	"	"	"	Val	"	18			0.30	0.51	0.94	0.61
17	"	PzcLys	"	"	"		17		0.29	0.46	0.71	0.59

a) From reference 14
b) " " 15, 20
c) " " 15
d) " " 21
e) ED_{50}, 171 ± 17 μg/ml
f) ED_{50}, 30.8 ± 0.59 μg/ml

Solvent Systems for TLC:
1. n-BuOH : HOAc : H_2O : 4:1:2
2. n-BuOH : py : HOAc: H_2O : 30:10:3:12
3. EtOAc : py : HOAc: H_2O : 5:5:1:3
4. n-BuOH : py : HOAc: H_2O : 4:1:1:2

7 toward an increase in water solubility and a decrease in histamine release.

In position 1, D-3-Qal was used in 3 analogs. In position 5, c-PzACAla was used in 7 analogs since c-PzACAla seems to contribute strongly to AOA (15, 20). In position 6, D-PicLys, D-3-Qal, D-Trp, and D-Tyr were used as promising substituents. Leu in position 7 was frequently substituted by Val.

Based on analog 1 (12) containing Arg^8, antagonists 2 - 8 were synthesized. Analog 2 contained D-PicLys instead of D-NicLys, and its AOA was 0%/0.25 μg. It was not tested at higher dose levels. In analog 3, amino acids in positions 5 and 6 were exchanged, but again, 0% AOA/0.25 μg was obtained. Analog 4 contained $D\text{-}Trp^6$, a substituent often used in this position in former years, but the activity of analog 4 was slightly lower than the AOA of the parent compound (peptide 1); 44% vs 60% at 0.5 μg.

Analogs 5 and 6 have large, rigid c-PzACAla in position 5. This amino acid was very promising in other peptides (15, 20). The activity of analog 5 with $c\text{-}PzACAla^5$,$D\text{-}Trp^6$ was 17%/0.25 μg, while $c\text{-}PzACAla^5$ in combination with $D\text{-}Tyr^6$ (analog 6) showed only 50% AOA/0.5 μg.

Analogs 7 and 8 have hydrophilic D-3-Qal in position 6, which is also weakly basic. Basic residues in position 6 were considered to increase

potency. However, analogs 7 and 8 had only 50% and 25% activity at 0.5 μg, respectively.

The next analog chosen as a parent compound was peptide 9 (15, 20) [N-Ac-D-2-Nal^1,D-pClPhe^2,D-3-Pal^3, c-PzACAla^5,D-PicLys^6,ILys^8,D-Ala^{10}] LHRH with AOA of 90% at 0.5 μg and 64% at 0.25 μg. Its congener with Val^7 (peptide 10) was less potent and had 12% AOA at 0.25 μg.

Peptides 11 and 12 are analogs of peptides 9 and 10, respectively, with Arg in position 8 instead of ILys, as the only difference. The activity of peptide 11 with Leu^7 decreased from 90 to 75% at 0.5 μg while the activity of peptide 12 with Val^7 increased from 12 to 20% at 0.25 μg. As observed many times before, the same change in two different analogs might cause opposite changes in AOA. The changes here are small, however, and may not be significant.

Substitution of PzcLys^5 instead of c-PzACAla^5 was not successful in analog 13. These two amino acids contain the same pyrazine ring, but its distance from the α-carbon atom in c-PzACAla is longer by one more CH_2 group than for PzcLys, and also the substituent with the cyclohexyl nucleus is more rigid than the substituent with the straight chain, PzcLys. Analog 13 with PzcLys^5 showed 0% activity at 0.25 μg in comparison with 20% for its c-PzACAla^5 congener (peptide 12).

Peptide 14 [N-Ac-D-3-Qal^1, D-pClPhe^2, D-3-Pal^3, c-PzACAla^5,D-PicLys^6, ILys^8, D-Ala^{10}]LHRH (21) is the best LHRH antagonist reported so far if considering both AOA and histamine release as well as water solubility. The Arg congener of this peptide (peptide 15) shows even higher AOA, 89% at 0.25 μg and 63% at 0.125 μg and maybe the most potent analog ever reported. It was named Argtide. Substitution at Leu^7 by Val^7 decreased the activity sharply to 18% at 0.125 μg which is in contrast with peptides 11 and 12.

PzcLys in position 5 instead of c-PzACAla again resulted in the less active analog 17 with 17% AOA at 0.25 μg.

The histamine release was tested only for Argtide (peptide 15). The ED_{50} value was 30.8 ± 0.59 μg/ml, which seems quite promising, considering that both Arg and c-PzACAla are present in the sequence.

Both Antide and Argtide, described herein, have five D-amino acids. It seems likely that Argtide would have a long duration of action comparable to that of Antide. Aubert et al. (22) considered that the long-lasting effects of Antide are not due to an unusual receptor-affinity of Antide and that other effects need to be elucidated. Gordon et al. (23) observed that low daily doses or slow-release implants of Antide may provide desirable therapy. Didolkar et al. (24) observed that a sustained release formulation of Antide may provide the desirable physiological responses. Presumably, these observations are at least qualitatively applicable to Argtide.

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REFERENCES

1. Karten, M.J. and Rivier, J.E. (1986) Endocrine Rev. 7, 44-66.
2. The Current Status of GnRH Analogues, Lunenfeld, B. and Insler, V., Eds., (1991) Parthenon Publishing Group, Park Ridge, NJ, p. 13-14
3. Humphries, J., Wan, Y.P., Folkers, K. and Bowers, C.Y. (1976) Biochem. Biophys. Res. Comm. (1976) 72, 939-944.
4. Horvath, A., Coy, D.H., Nekola, M.V., Coy, E.J., Shally, A.V. and Teplan, I. (1982) Peptides 3, 969-973.
5. Folkers, K., Bowers, C.Y., Shieh, H.-M., Liu, Y.-Z., Xiao, S.-B., Tang, P.-F.L., and Chu, J.-Y. (1984) Biochem. Biophys. Res. Commun. 123, 1221-1226.
6. Schmidt, F., Sundaram, K., Thau, R.B., and Bardin, C.W. (1984) Contraception 29, 283-280.
7. Morgan, J.E., O'Neil, C.E.,Coy, D.H., Hocart, S.J., and Nekola, M.V. (1986) Int. Arch. Allergy Appt. Immunol. 80, 70-74.
8. Hahn, D.W., McGuire, J.L., Vale, W.W., and Rivier, J. (1985) Life Sci. 37, 505-509.
9. Hook, W.A., Karten, M., and Siraganian, R.P. (1985) Fed. Proc. 44, 1323.
10. Nekola, M.V., O'Neil, C., Morgan, J., and Coy, D. (1984) Clin. Res. 32 865-869.
11. Roeske, R.W., Chaturvedi, N.C., Hrinyo-Pavlina, T., and Kowalczuk, M., (1987) LHRH and its Analogs, Vickery, B.H. and Nestor, J.J., Jr., eds., MTP Press, Lancaster, U.K., pp. 17-24.
12. Folkers, K., Bowers, C.Y., Shaobo, X., Tang, P.-F.L., and Kubota, M. (1986) Biochem. Biophys. Res. Comm. 737, 709-715.
13. Bajusz, S., Kovacs, M., Gazdag, M., Bokser, L., Karashima, T., Csernus, V.J., Janaky, T., Groth, J., and Schally, A.V.(1988) Proc. Natl. Acad. Sci. USA 85, 1637-1641.
14. Ljungqvist, A., Feng, D.-M., Tang, P.-F.L., Kubota, M., Okamoto, T., Zhang Y., Bowers, C.Y., Hook W.A., and Folkers, K. (1987) Biochem. Biophys.Res. Comm. 148, 849-856.
15. Ljungqvist, A., Feng, D.-M., Bowers, C.Y., Hook, W.A., and Folkers, K. (1990) Tetrahedron 46, 3297-3304.
16. Folkers, K., Ljungqvist, A., Feng, D.-M., Bowers, C.Y., Tang, P.-F.L., and Kubota, M. (June 19, 1990) U.S. Pat. 4,935,491.
17. Ljungqvist, A. and Folkers, K. (1988) Acta Chem. Scand. 842, 408-410.
18. Humphries, J., Wan, Y.P., Folkers, K., and Bowers, C.Y. (1987) J. Med. Chem. 21, 120-123.
19. Karten, M.J., Hook, W.A., Siraganian, R.P., Coy, D.H., Folkers, K., Rivier, J.E., and Roeske, R.W. (1987) LHRH and its Analogs, Vickery, B.H., and Nestor, J.J., Jr., eds. pp. 179-180.
20. Ljungqvist, A., Feng, D.-M., Hook, W.A., Shen, Z.-I., Bowers, C.Y., and Folkers, K. (1988) Proc. Natl. Acad. Sci. USA 85, 8236-8240.
21. Ljungqvist, A., Feng, D.-M., Bowers, C.Y., Hook, W.A., and Folkers, K., Z. Naturforsch. B., In Press.
22. Aubert, M.L., Li, S.L., Gruaz, N.M., Eshkol, A., and Sizonenko, P.C. (1991) Abs. 1688, 73, Am. Endocr. Soc. Meeting, June 19-22, Washington, D.C.
23. Gordon, K., Williams, R.F., Danforth, D.R., and Hodgen, G.D. (1991) Abs. 1212, 73, Am. Endocr. Soc. Meeting, June 19-22, Washington, D.C.
24. Didolkar, A.K. and Sundaram, K. (1991) Abs. 1704, 73, Am. Endocr. Soc. Meeting, June 19-22, Washington, D.C.