PRESENCE OF PROLINE IN POSITION 3 FOR POTENT INHIBITION OF THE ACTIVITY OF THE LUTEINIZING HORMONE RELEASING HORMONE AND OF OVULATION *

by

John Humphries, Yieh-Ping Wan, and Karl Folkers

Institute for Biomedical Research
The University of Texas at Austin
Austin, Texas 78712

and

Cyril Y. Bowers

Tulane University School of Medicine
New Orleans, Louisiana 70112

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SUMMARY

[D-Phe^2,Pro^3,D-Trp^6]-LHRH completely inhibited the release of LH and FSH by LHRH from isolated rat pituitaries at a ratio of analog:LHRH of 50:1. Complete inhibition also resulted from [D-Phe^2,Leu^3,D-Trp^6]-LHRH and [D-Phe^2,Leu^3, D-Phe^6]-LHRH, but at higher ratios. These analogs had no agonist activity. The Pro^3-analog completely inhibited ovulation in rats at a single sc injection of 750 μg, and 375 μg gave 50% inhibition. The Leu^3-analogs gave complete inhibition of ovulation at 750 μg with D-Phe^6, and at 1.5 mg with D-Trp^6. The substitution of proline in position 3 of an LHRH inhibitory sequence effectively increased both the in vitro potency, and the antiovulatory potency in rats.

Humphries et al. (1) in 1974 emphasized dual substitutions of His^2-Trp^3 in the luteinizing hormone releasing hormone (LHRH <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), toward achieving inhibitors of the release of LH and FSH by LHRH. The exemplary [Leu^2,Leu^3]-LHRH did not release either LH or FSH, and unequivocally inhibited the LHRH-induced release of LH and FSH, but the ratio of inhibitor:LHRH was high, or 300,000 fold. However, a guideline emerged, which is still acceptable, from these studies that an effective inhibitor should probably not exhibit even weak agonist activity, and at a high dose level.

Continuing to focus upon positions 2 and 3, and including the introduction of a D-amino acid in position 6, as based upon analogs of Monahan et al. (2), Wan et al. (3) made a number of additional dual substitutions for His^2-Trp^3 and with D-Ala in position 6 to constitute a triple change, and observed ratios of inhibition of 30,000:1 for certain analogs of LHRH. It was again observed

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that the effective analogs of Wan et al. (3), for example, [Leu^2,Leu^3,D-Ala^6]-LHRH, were also devoid of agonist activity. Humphries et al. (4) observed that the substitution of threonine for His in position 2 in addition to changes in position 3 were beneficial as exemplified by [Thr^2,Leu^3]-LHRH which inhibited the release of LH at a ratio of 30,000:1. The decapeptides, [Leu^2,Leu^3,D-Ala^6, des-Gly^{10}]-LHRH ethylamide, and [Leu^2,Nva^3,D-Ala^6,des-Gly^{10}]-LHRH ethylamide, were found to inhibit, in vitro, with a ratio of 3,000:1 (5). The substitution of a D-amino acid, such as D-Phe for His in position 2, was reported by Corbin and Beattie (6). The substitution of a D-aromatic amino acid, such as D-Trp, in position 6 of LHRH was found by Rivier et al. (7) and Coy et al. (8) to greatly enhance the agonist activity of LHRH.

We have continued emphasis upon His-Trp in positions 2 and 3 and have included D-amino acids in positions 2 and 6, and found that inhibitory activity has been substantially increased, and particularly when proline was substituted for Trp in position 3. Notably, [D-Phe^2,Pro^3,D-Trp^6]-LHRH inhibits the release of LH by LHRH at the low ratio of 50:1 for inhibitor:LHRH. These increases of inhibition potency from ratios of 300,000:1 to 50:1 have been based upon in vitro assays.

We are continuing to find (5) that antagonistic activities, in vitro, using isolated rat pituitaries do not necessarily parallel anti-ovulatory activity, in rats.

We have found that this potent in vitro inhibitor, [D-Phe^2,Pro^3,D-Trp^6]-LHRH, is also potent for the complete inhibition of ovulation in rats as described herein.

SYNTHESIS

Three analogs of LHRH, [D-Phe^2,Leu^3,D-Trp^6]-LHRH, [D-Phe^2,Leu^3,D-Phe^6]-LHRH, and [D-Phe^2,Pro^3,D-Trp^6]-LHRH, have been synthesized by the solid phase method (9) on a benzhydrylamine resin support. The Beckman Model 990 automatic peptide synthesizer was used. The coupling procedure was carried out essentially as described (4). The cleavage and deprotection steps were also carried out essentially as described (4).

[D-Phe^2,Pro^3,D-Trp^6]-LHRH was purified by gel filtration over Sephadex G15 with 33% aqueous acetic acid followed by ion exchange chromatography on a column of CM-Sephadex with a continuous linear gradient of NH_4OAc buffers (1mM pH 4.5, to 125 mM). The major peak was located at 256 nm. The peptide solution of the major peak was lyophilized to give a product which was homogeneous in three TLC systems. The R_f values were 0.70, 0.81, and 0.76, in the systems 1-BuOH, AcOH, EtOAc, H_2O (1:1:1:1), 2-propanol, 1 N AcOH (2:1), and 0.1% AcOH, 1-BuOH, pyridine (11:5:3), upper phase, respectively.

TABLE 1. ASSAYS, IN VITRO, FOR ANTAGONIST AND AGONIST ACTIVITY

Analog	Dose		LH			FSH		
	ng/ml of medium		Δ ng/ml	SEM	p value	Δ ng/ml	SEM	p value
	Peptide	LHRH	of medium	(±)		of medium	(±)	
[D-Phe^2,Pro^3,D-Trp^6]-LHRH	-	-	-15	3	-	296	106	-
	30	-	21	6	<0.001	206	272	ns
	100	-	-5	3	<0.05	201	181	ns
	1,000	-	4	2	<0.001	109	188	ns
	10,000	-	10	9	<0.05	42	92	ns
	100,000	-	9	3	<0.001	175	181	ns
	-	0.6	220	35	-	6617	601	-
	30	0.6	26	7	<0.001	-161	334	<0.001
	100	0.6	61	11	<0.01	1270	248	<0.001
	1,000	0.6	15	10	<0.001	135	167	<0.001
	10,000	0.6	2	15	<0.001	-328	239	<0.001
	100,000	0.6	11	3	<0.001	-243	183	<0.001
[D-Phe^2,Leu^3,D-Phe^6]-LHRH	-	-	17	8	-	577	270	-
	100,000	-	19	6	ns	316	366	ns
	-	0.6	197	17	-	7389	423	-
	100	0.6	159	23	ns	5733	276	<0.01
	1000	0.6	70	9	<0.001	5339	853	ca.0.001
	10,000	0.6	42	14	<0.001	-86	445	<0.001
[D-Phe^2,Leu^3,D-Trp^6]-LHRH	-	-	-48	38	-	-1079	331	-
	100,000	-	47	2	<0.05	-131	371	ns
	-	0.6	275	57	-	6712	658	-
	100	0.6	1	37	<0.01	919	548	<0.001
	1,000	0.6	26	20	<0.01	-628	330	<0.001
	10,000	0.6	32	15	<0.01	-2017	305	<0.001

Amino acid analysis after hydrolysis in 6 N HCl at 110° for 18 hours was determined on a Beckman Model 119 amino acid analyzer to be Glu 1.02, Phe 1.0, Pro 2 x 1.18, Ser 0.84, Tyr 1.08 Leu 0.94, Arg 1.08, Gly 1.02.

The other decapeptide analogs were purified over columns of Sephadex LH-20 with 1-BuOH, H_2O, (6:100). Their homogeneity was similarly demonstrated by TLC and by amino acid analysis.

BIOLOGICAL ASSAYS

In vitro assays. - The LH and FSH agonist and antagonist data were obtained from studies using pituitaries of 20-day-old female Sprague-Dawley rats, essentially as described (4). LHRH was added at I_5 and I_6. When LHRH and the analog were added together, the analog was always added to the incubation medium 5 min before LHRH. The values were calculated in terms of ng of the following standards: LH-LER-1240-2 (0.60 NIH-FSH-SI units/mg).

Antiovulation assays. - The antiovulatory data were determined by counting the number of ova. The analog, in 0.3 ml corn oil, was administered as a single subcutaneous injection to cycling female Sprague-Dawley rats (Charles River) between 12 and 12:30 p.m. on proestrus. The control rats received 0.3 ml of the vehicle. On estrus, the ova were counted as the index of the antiovulatory activity.

RESULTS AND DISCUSSION

The data from the tests for agonist and antagonist activities, in vitro, are in Table 1. The decapeptide, [D-Phe^2,Pro^3,D-Trp^6]-LHRH, is the most active in vitro inhibitor. It completely inhibits the release of LH and FSH by 0.6 ng LHRH at all of the dosages tested from 100 μg to 30 ng. The ratio of peptide inhibitor to LHRH for complete inhibition is 50:1. This extremely low ratio of inhibition reflects the encouraging advances made in the design of LHRH inhibitors by several groups of investigators. Our early data on [Leu^2,Leu^3]-LHRH showed inhibition at a ratio of 300,000:1 and this analog was was about one-third as potent as the pioneer [des-His^2]-LHRH (10) under our assay conditions.

The in vitro agonist test results of Table 1 show that [D-Phe^2,Pro^3, D-Trp^6]-LHRH is not effective in causing the release of LH and FSH at high dosages up to 100 μg. This lack of agonist activity of an inhibitor of LHRH, especially at high dosages, is still considered as one criterion which is important for an effective antiovulation agent, in vitro.

The antiovulatory activity of the LHRH inhibitors is given in Table 2. A single sc injection of 750 μg of [D-Phe^2,Pro^3,D-Trp^6]-LHRH at noon on proestrus, produced a 100% inhibition of ovulation in the 11 rats tested. A single sc injection of 375 μg of the [Pro^3]-analog inhibited ovulation by

TABLE 2. ANTIOVULATORY ACTIVITY OF THE ANALOGS

Analog	Total dose (sc) mg	No. of rats	No. of ova	SEM (±)	% Inhibition of ovulation
[D-Phe^2,Pro^3,D-Trp^6]-LHRH	-	11	12.4	1	-
	0.375	9	5	3	50
	0.750	11	0	0	100
[D-Phe^2,Leu^3,D-Phe^6]-LHRH	-	7	13.7	0.15	-
	0.375	5	11.0	2.8	0
	0.750	10	1.1	1.1	100
	1.5	5	0	0	100
[D-Phe^2,Leu^3,D-Trp^6]-LHRH	-	26	12.8	0.4	-
	0.750	13	5.5	0.68	50
	1.5	11	0.6	0.6	100
	3.0	6	0	0	100

50%. This high potency of inhibition of ovulation is greater, under our assay conditions, than that observed for [D-Phe^2,D-Ala^6]-LHRH (6), which only inhibited ovulation by 100% at dosage of 1.5 mg (5).

The structurally related analogs, [D-Phe^2,Leu^3,D-Trp^6]-LHRH and [D-Phe^2, Leu^3,D-Phe^6]-LHRH, produced 100% inhibition of ovulation at dosages of 1.5 mg and 750 μg, respectively. The substitution of proline in positions 3 is beneficial. When leucine is in the 3 position, D-phenylalanine was more effective than D-tryptophan in position 6.

These results again support our approach of making dual substitutions in the biologically important 2 and 3 positions of LHRH. The inclusion of other structural substitutions, such as in position 6, may further enhance potency and biological stability.

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REFERENCES

1. Humphries, J., Fisher, G., Wan, Y.P., Folkers, K., and Bowers, C.Y., J. Med. Chem. 17, 569 (1974).
2. Monahan, M.W., Amoss, M.S., Anderson, H.A., and Vale, W., Biochemistry, 12, 4216 (1973).
3. Wan, Y.P., Humphries, J., Fisher, G., Folkers, K., and Bowers, C.Y., J. Med. Chem. 19, 199 (1976).

4. Humphries, J., Wan, Y.P., Folkers, K, and Bowers, C.Y., J. Med. Chem. submitted.
5. Unpublished data.
6. Corbin, A., and Beattie, C.W., Endocrine Res. Commun., 2, 1 (1975).
7. Rivier, J., Ling, N., Monahan, M., Rivier, C., Brown, M., and Vale, W. in "Peptides: Chemistry, Structure and Biology", Proceedings, Fourth American Peptide Symposium, New York, 1975, Eds. Walter, R., and Meienhofer, J., Ann Arbor Science, page 863.
8. Coy, D.H., Labrie, F., Savary, M., Coy, E.J., and Schally, A.V., Biochem. Biophys. Res. Commun., 67, 576 (1975).
9. Merrifield, R.B., J. Amer. Chem. Soc., 85, 2149 (1963).
10. Vale, W., Grant, G., Rivier, J., Monahan, M., Amoss, M., Blackwell, R., Burgus, R., and Guillemin, R., Science, 176, 933 (1972).