Vol. 86, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1979 Pages 843-848

A NEW CATEGORY OF OVULATION INHIBITORS

LINEAR LH-RH ANALOGUES HAVING MORE THAN TEN RESIDUES

by

Tadeusz Wasiak**, John Humphries, 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

Received December 26,1978

SUMMARY

A linear analogue of the luteinizing hormone-releasing hormone, longer than a decapeptide, is described for the first time, which is equivalent in potency to the best known inhibitors of ovulation, and which constitutes an important new lead to the design of inhibitors of even greater potency. At a dosage of 200 μg/rat, the undecapeptide [(<Glu-Pro)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH caused 100% inhibition of ovulation. The related analogues, [(<Glu-Gly)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH and [(Gly-Pro)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH, were less active, in vivo. All of these undecapeptides inhibited the action of 0.6 ng/ml of LH-RH by greater than 50% at the very low level of 10 ng/ml.

INTRODUCTION

The design of ovulation inhibitors based on modification in positions 2,3, and 6 has followed from some of our earlier work (1,4). The most potent inhibitors were [D-Phe^2, Pro^3, D-Trp^6]-LH-RH (3), [D-Phe^2, Pro^3, D-Phe^6]-LH-RH (4), [D-Phe^2, N-Me-Leu^3, D-Phe^6]-LH-RH (5), and [D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH (6), which inhibited ovulation completely at 750 μg/rat.

In the investigation of new probes that could generate important leads to inhibitors with enhanced activities, we have synthesized various tetra-substituted LH-RH analogues; in particular, we have emphasized the importance of position 1 (8-10).

Peptide Hormone 135.

**Present address: Institute of Physiology and Biochemistry, Medical School, Łódź, Poland.

0006-291X/79/030843-06$01.00/0

Copyright © 1979 by Academic Press, Inc.
All rights of reproduction in any form reserved.

The antiovulatory potency of 1,2,3,6-tetra-substituted LH-RH analogues was found to be dependant on the nature of the residues in positions 1 and 3 (10). The substitution of D-<Glu (9-11) and Ac-Pro (9) into position 1 of sequences containing D-Trp in position 3 has led to equipotent inhibitors with increased antiovulatory potency, in which ovulation was inhibited at 200μg/rat. In contrast, all substitutions into position 1 of sequences having Pro or N-Me-Leu in position 3 have given analogues with decreased activity (10).

Our discovery of the highly active Ac-[Pro^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH (9), which is the most potent ovulation inhibitor yet reported having an L-amino acid at position 1, has established that potent ovulation inhibitors could be designed having either L- or D-amino acid residues in position 1.

As a consequence of structure-activity studies on the Ac-Pro^1 analogue, we now describe the activities of three undecapeptide analogues of LH-RH. The observation that one of these, [(<Glu-Pro)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH, completely inhibits ovulation at 200μg/rat, provides the first example that potent ovulation inhibitors can be designed which have linear sequences longer than ten amino acid residues.

EXPERIMENTAL

The peptides were synthesized by solid-phase procedures in a Beckman model 990 peptide synthesizer, followed by treatment with HF, as described (10). The (<Glu-Pro)^1 and (<Glu-Gly)^1 analogues were purified over columns of Sephadex G-25 with 20% AcOH, followed by partition chromatography over Sephadex G-25 with the system 1-BuOH, 2 N AcOH (1:1). The (Gly-Pro)^1 analogue was purified over Sephadex G-25 with 20% AcOH, followed by chromatography over Sephadex LH-20 with 1-BuOH, AcOH, H_2O (1:10:90). The peptides were homogeneous by TLC on silica Gel with the systems (v/v) R_f^1 1-BuOH, AcOH, EtOAc, H_2O (1:1:1:1); R_f^2 2-propanol, 1 N AcOH (2:1); R_f^3 EtOH, H_2O (7:3); and R_f^4 1-BuOH, pyridine, AcOH, H_2O (30:20:6:24). The presence of Trp was established but not quantitated.

[(<Glu-Pro)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH: $[\alpha]_D^{20}$ -54.04° (c 0.997, MeOH); R_f^1 0.78, R_f^2 0.78, R_f^3 0.67, R_f^4 0.63; Amino acid analysis gave Glu 1.08, Pro 2 x 1.1, Phe 0.99, Ser 1.02, Tyr 0.80, Leu 0.82, Arg 1.06, Gly 1.00.

[(<Glu-Gly)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH: $[\alpha]_D^{20}$ -41.95 (c 1.013, MeOH); R_f^1 0.67, R_f^2 0.78, R_f^3 0.69, R_f^4 0.65; Amino acid analysis gave Glu 0.92, Gly 2 x 0.97, Phe 1.00, Ser 1.09, Tyr 1.00, Leu 0.82, Arg 1.1, Pro 1.1.

[(Gly-Pro)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH: $[\alpha]_D$ -51.52 (c 1.068, MeOH); R_f^1 0.75, R_f^2 0.72, R_f^3 0.48, R_f^4 0.62; Amino acid analysis gave Gly 2 x , Pro 2 x0.96, Phe 1.09, Ser 0.9, Tyr 1.14, Leu 0.97, Arg 0.99.

The procedures for measuring in vitro activities and antiovulatory potencies have been described (4).

RESULTS AND DISCUSSION

The results of the in vitro assays are in Table 1. All of the analogues inhibited the release of LH from 0.6 ng/ml of LH-RH by significantly greater than 50%, at a dosage of 10 ng/ml. The release of FSH by LH-RH appeared to be less effectively inhibited.

Table 1. ASSAYS, IN VITRO, FOR LH-RH ANTAGONIST AND AGONIST ACTIVITY OF THE UNDECAPEPTIDES*

Analogue	Dose		LH			FSH		
	ng/ml of Peptide	medium LH-RH	Δng/ml of medium	SEM (±)	p value	Δng/ml of medium	SEM (±)	p value
[(<Glu-Pro)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH	-	0.6	341	22	-	5036	634	-
	10	0.6	51	3	<0.001	2030	295	<0.01
	30	0.6	76	12	<0.001	2226	382	<0.01
	100	0.6	13	6	<0.001	418	71	<0.001
	-	-	21	10	-	76	54	-
	10,000	-	8	5	ns	-23	116	ns
[(<Glu-Gly)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH	-	0.6	414	94	-	3412	508	-
	10	0.6	111	28	0.01	1897	355	<0.05
	30	0.6	60	28	<0.01	1023	180	<0.01
	100	0.6	98	19	<0.01	575	130	<0.001
	-	-	-47	22	-	108	89	-
	1,000	-	19	15	<0.05	125	130	ns
[(Gly-Pro)^1, D-Phe^2, D-Trp^3, D-Trp^6]-LH-RH	-	0.6	242	15	-	3692	479	-
	10	0.6	55	14	<0.001	2166	347	<0.05
	30	0.6	54	24	<0.001	1924	448	<0.05
	100	0.6	6	13	<0.001	7	416	<0.001
	-	-	-10	9	-	-299	276	-
	1,000	-	-34	23	ns	-128	251	ns
	10,000	-	59	12	<0.001	1181	93	<0.001

* For brevity, not all dosages have been reported.

Table 2. ANTIOVULATORY ACTIVITY OF THE UNDECAPEPTIDES

Analogue	Dose µg/rat sc	No. of rats	No. of rats ovulated	No. of ova per ovulating rat	SEM (±)	% Inhibition of ovulation
[(<Glu-Pro)^1, D-Phe^2,	-	4	4	11.25	0.25	0
D-Trp^3, D-Trp^6]-LH-RH	200	4	0	0	0	100
	100	5	2	5.2	3.2	60
[(<Glu-Gly)^1, D-Phe^2,	-	5	5	11.6	1.5	0
D-Trp^3, D-Trp^6]-LH-RH	200	3	3	11.0	1.2	0
	-	4	4	11.3	0.25	0
	750	6	1	2.0	2.0	83
[(Gly-Pro)^1, D-Phe^2	-	5	5	11.6	1.5	0
D-Trp^3, D-Trp^6]-LH-RH	200	5	5	12.4	1.4	0
	-	4	4	11.3	0.25	0
	750	5	4	10.4	2.6	20

The rat antiovulatory data are in Table 2. The (<Glu-Pro)[1] analogue was the most potent inhibitor and completely suppressed ovulation at a dosage of 200 μg/rat. A 60% inhibition of ovulation was observed at 100 μg/rat. The (< Glu-Gly)[1] analogue was completely effective at 750 μg/rat, and the (Gly-Pro)[1] analogue was considerably less potent.

Since the antiovulatory potency of the (<Glu-Pro)[1] analogue is essentially equivalent to that of the corresponding Ac-Pro[1] analogue, it is now apparent that position 1, in the [Residue[1], D-Phe[2], D-Trp[3], D-Trp[6]]-LH-RH sequence, can probably accomodate innovated substitutions and still maintain high potency.

The observation that the (<Glu-Gly)[1] analogue was less potent than the (<Glu-Pro)[1] analogue may indicate that a more rigid residue is required in that position.

The (Gly-Pro)[1] analogue is analogous to the Ac-Pro[1] analogue, in that a NH_2CH_2CO-residue occupies the position of the CH_3CO-group. This change did not appear to be beneficial for antiovulatory activity, although this analogue did strongly inhibit in the *in vitro* assay. The lower *in vivo* potency may be a consequence of the presence of a protonated α-amino function, and/or to increased enzymatic degradation due to the "unprotected" α-amino function on the Gly residue. Currently, all of the most potent ovulation inhibitors have a "protected" amino-terminal, e.g. L- or D- <Glu, and CH_3CO-, which in peptide nomenclature, can be regarded as des-amino-Gly.

This work demonstrates, for the first time, that a new category of potent ovulation inhibitors can be designed which are longer than decapeptides.

ACKNOWLEDGMENTS

Appreciation is expressed to Dr. Marvin Karten and for the support of Contract NO1-HD-8-2820 of the National Institutes of Health, and for Public Health Service Research Grant No. CA-14200-05 from the National Cancer Institute, and for a grant from the Robert A. Welch Foundation. The RIA reagents for FSH were distributed by NIAMD, NIH. We are grateful to Drs. G. Niswender, L.E. Reichert and A. Parlow for their RIA preparations and procedures.

REFERENCES

1. J. Humphries, G. Fisher, Y.P. Wan, K. Folkers, and C.Y. Bowers, J. Med. Chem., 17, 569 (1974).
2. Y.P. Wan, J. Humphries, G. Fisher, K. Folkers, and C.Y. Bowers, J. Med. Chem., 19, 199 (1976).
3. J. Humphries, Y.P. Wan, K. Folkers, and C.Y. Bowers, Biochem. Biophys. Res. Commun., 72, 939 (1976).
4. J. Humphries, Y.P. Wan, K. Folkers, and C.Y. Bowers, J. Med. Chem., 21, 120 (1978).
5. K. Folkers, unpublished data.

6. D.H. Coy, J.A. Vilchez-Martinez, and A.V. Schally in "Peptides 1976", A. Loffet, Ed., Editions de l'Universite de Bruxelles, Brussels, 1977, p. 660.
7. J. Humphries, Y.P. Wan, K. Folkers, and C.Y. Bowers, J. Med. Chem., 20, 1674 (1977).
8. J. Humphries, Y.P. Wan, K. Folkers, and C.Y. Bowers, Biochem. Biophys. Res. Commun., 78, 506 (1977).
9. J. Humphries, T. Wasiak, Y.P. Wan, K. Folkers, and C.Y. Bowers, Biochem. Biophys. Res. Commun., 85, 709 (1978).
10. J. Humphries, Y.P. Wan, T. Wasiak, K. Folkers, and C.Y. Bowers, J. Med. Chem., submitted.
11. J. Rivier and W. Vale, Salk Institute, La Jolla, California.