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LONG-TERM INHIBITION OF OVULATION BY A GnRH-ANTAGONIST AT LOW DOSE LEVEL

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SUMMARY: Ac-D-Trp[1,3], D-Cpa[2], D-Lys[6], D-Ala[10]-GnRH[**] has been prepared by solid phase synthesis. The peptide was found to completely inhibit ovulation when administered on proestrus day in a dose of 1.5 µg/rat, s.c. The peptide completely inhibited ovulation for a period corresponding to three to four cycles when administered daily in a dose of 5 µg/rat, s.c. and caused 70% inhibition of ovulation in a dose of 3 µg/rat.

Synthetic analogues of gonadotropin releasing hormone (GnRH) are potential contraceptives by inhibiting the secretion of LH and FSH from the pituitary (reviewed in 1,2). This is achieved either by pituitary desensitization by "superagonistic" GnRH analogues (3) or by competitive inhibition by "antagonistic" GnRH analogues (4). Major progress has been made in the development of competitive inhibitors of GnRH, which are able to block ovulation. Inhibition of ovulation is generally tested in a single experiment when the peptides are given in proestrus and the number of ova is evaluated on the next day (5).

The present GnRH antagonists have reached a very high level of effectiveness and therefore it has become necessary to conduct long-term experiments with these. This paper describes the preparation of a highly active competitive inhibitor of GnRH and its effectiveness in both single and long-term application.

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** Cpa refers to 4-chloro-phenylalanine.

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MATERIALS AND METHODS

1. Synthesis of Ac-D-Trp^{1,3}, D-Cpa^{2}, D-Lys^{6}, D-Ala^{10}-GnRH

The peptide was synthesized by solid phase synthesis on 975 mg benzhydrylamine resin (1.05 meq/g; CKB-Wolfen, GDR) using Nα-butyl-oxycarbonyl amino acids. For side chain protection, the following groups were applied: Arg:tosyl, Lys:benzyloxycarbonyl, Tyr:benzyl, Ser:benzyl. The amino acids (3 equiv) were coupled for 4 hours in the presence of N,N'-diisopropylcarbodiimide (3 equiv). Boc-D-Ala and Boc-Pro were coupled for 15 hours. Coupling efficiency was tested by the ninhydrin test of Kaiser et al (6,7). Repeated coupling was needed in the case of arginine only. N-terminal acetylation was carried out with acetic anhydride in methylene chloride in the presence of imidazole.

The peptide-resin (2.725 g, weight increase: 93% of the theoretical maximum) was suspended in 5 ml anisole in the presence of 200 mg dithiothreitol. 45 ml liquid HF was distilled onto the material and the mixture was stirred for 1 hour at 0°C. After removal of HF, the residue was powdered with ether and the peptide was dissolved in 33% acetic acid.

The peptide was purified on a Sephadex G-50 column (1.5mx2.5cm) eluting with 33% acetic acid. The main peak containing ninhydrin and Erlich positive material was lyophylized to give 970 mg crude decapeptide. 500 mg of this material was further purified on a silica gel column (1.2mx2cm) eluting with n-butanol - acetic acid - water 6:1:1 (v/v) mixture. The main peak after lyophylization resulted 137 mg peptide. This was found to be homogeneous by tlc in n-butanol - acetic acid - water 6:1:1, Rf=0.11; ethyl acetate - pyridine -acetic acid - water 30:20:6:11, Rf=0.75; n-butanol - acetic acid - water - ethyl acetate 1:1:1:1, Rf=0.67 and isopropanol - 1M acetic acid 2:1, Rf=0.66. The material was homogeneous by reverse phase HPLC on a Shandon Hypersil-ODS, 5μ, 25x0.5 cm column, retention time in 67% methanol - 33% 0.05M ammonium acetate, pH 4.2 at a flow rate of 1 ml/min was 5.6 min and in 20% isopropanol - 0.1% trifluoroacetic acid was 4.3 min. $[\alpha]_D = -24.4°$ and $[\alpha]_{436} = -51.4°$ (c=0.5; 1M acetic acid).

Amino acid analysis after methanesulfonic acid hydrolysis: Trp 1.83, Ser 0.92, Tyr 0.98, Lys 1.03, Leu 1.00, Arg 0.98, Pro 0.94, Ala 1.05. 4-chlorophenylalanine was not determined. Peptide content was 78%.

2. Assay of short-term inhibition of ovulation.

The peptide was dissolved in 40% propylene glycol - 60% 0.9% NaCl solution at a concentration of 20 μg/ml. Aliquots of this solution containing 5, 3, 1.5 and 0.75 μg peptide were injected subcutaneously into female Wistar R-Amsterdam (200-250 g) rats at noon of proestrus day. The animals were housed under constant light (5:00-19:00) and dark (19:00-5:00) conditions. Only those rats that previously showed three consecutive regular 4-day cycles as tested from vaginal smears were used for the experiments. The rats were ovariectomized one day after the injection and their oviducts prepared and tested for ova present. Results are expressed in terms of rats ovulating/total number of rats injected.

3. Assay of long-term inhibition of ovulation

Rats showing two consecutive 4-day cycles were used for these experiments. The animals were injected with 5 or 3 μg peptide subcutaneously daily for 11 to 15 days. The treatment started either on proestrus or estrus day at noon. Cycles were followed by testing daily vaginal smears. After one delayed cycle (6-7 days) the animals were unilaterally ovariectomized and their oviducts examined for the presence of ova. Treatment was continued and the other oviduct was prepared and tested on day 11 to 15 of treatment.

Table 1

Peptide dose, µg/rat	Number of rats ovulating/ total number of rats
5	0/4
3	0/10
1.5	0/10
1.0	3/10
0.75	5/10

Short-term inhibition of ovulation by Ac-D-Trp^{1,3}, D-Cpa^{2}, D-Lys^{6}, D-Ala^{10}-GnRH in cycling rats. The peptide was administered by subcutaneous injection at 12:00 noon on proestrus day as described under Materials and Methods.

RESULTS

Ac-D-Trp^{1,3}, D-Cpa^{2}, D-Lys^{6}, D-Ala^{10}-GnRH was prepared by solid phase peptide synthesis. After purification the peptide was found to be homogeneous by different criteria of purity and characterized.

The peptide was found to completely inhibit ovulation in a single 5, 3 and 1.5 µg injection given at noon on proestrus day as shown in Table 1. Lower doses did not cause complete inhibition of ovulation.

This inhibitory decapeptide was found to be a very effective inhibitor of ovulation when applied daily for a period corresponding to three to four consecutive cycles. In one series of experiments involving 10 rats, the peptide was administered in a daily s.c. dose of 5 µg/rat and treatment was started on proestrus day. No ovulation occured in any of the treated rats during the treatment period of 11 to 15 days. Individual cycle patterns of the animals, however, showed variations. 7 animals remained in oestrus phase for 2 to 3 days, 2 animals for 1 day. 1 animal remained in oestrus from the second day of treatment. Oviducts were examined in the first diestrus phase following oestrus, however, no ova were detected. 1 animal showed constant oestrus during the whole treatment period and did not ovulate. This experiment using 5µg daily dose of the antagonist was repeated with another 10 rats starting the treatment in oestrus phase. The results confirmed complete inhibition of ovulation during the entire treatment period.

In a lower daily dose, namely 3 µg/rat, 7 of 10 treated animals did not ovulate during the 12 to 15-day treatment period. In this experiment, treatment was started in oestrus phase. Following regular diestrus I and II and proestrus, 7 animals had a 2-day oestrus phase, while 3 had a 3-day oestrus phase. This was followed by dioestrus on the 6th or 7th day of treatment. In this phase unilateral ova counting revealed 3 ovulations. Daily injections were continued and after a 2 to 5-day diestrus followed by proestrus, oestrus lasted for 2 to 3 days in 6 animals and remained constant in 4 animals for the rest of the treatment period. In those animals that showed diestrus, no ova could be detected in their oviducts.

After termination of the treatment with 5 µg antagonist, vaginal smears of four rats were examined on consecutive days. In all 4 animals, normal, regular 4-day cycles returned after a 3-day oestrus phase.

DISCUSSION

Antagonistic analogues of GnRH are able to inhibit ovulation by blocking the action of the endogenous releasing hormone (1,2). When GnRH antagonists are given in a single injection to rats in proestrus, ovulation is inhibited on the following day. In such tests, todays most effective GnRH antagonists cause complete inhibition of ovulation in a low dose range, namely 0.7 to 3 µg/rat (8 - 10). This range is close to wide practical application, therefore long-term testing of such peptides provides vital informations. Earlier experiments of long-term application showed that a potent antagonist of GnRH was able to permanently inhibit ovulation in a very high daily dose only (500 µg/rat) although the peptide inhibited ovulation in the single-dose test in 5 µg/rat (11-13). In the present experiments we have found Ac-D-Trp^{1,3}, D-Cpa^{2}, D-Lys^{6}, D-Ala^{10}-GnRH to be fully inhibitory over a period corresponding to three to four cycles in a dose only slightly higher than the single inhibitory dose (5 µg vs 1.5 µg). This result may be very promising for the development of further GnRH antagonists.

Another similar GnRH antagonist has been reptorted to be able to inhibit ovulation by oral administration although in high amounts (14). Both the orally active analogue and the one reported here contain a basic amino acid in position 6. This seems to be a very effective substitution for enhancing the inhibitory activity of these peptides.

Finally, it is very important to point out that the long-term application had a reversible inhibitory effect on ovulation. Ovulation returned to normal very rapidly after termination of the treatment. Both the low dose of the inhibitory peptide required for long-term inhibition of ovulation and the reversible effect on reproductive function provide strong support that GnRH antagonists may become powerful alternatives of contraception in long-term application.

REFERENCES

1. Schally, A.V., Coy, D.H. and Arimura, A. (1980) Int. J. Gynecol. Obstet. 18, 318 - 324
2. Vale, W., Rivier, C. and Brown, M. (1977) Ann. Rev. Physiol. 39, 473 - 492
3. Keri, G., Nikolics, K., Teplan, I. and Molnar, J. (1983) Mol. Cell. Endocrinol. 30, 109 - 120
4. Vale, W., Grant, G., Rivier, J., Monahan, M., Amoss, M., Blackwell, R., Burgus, R. and Guillemin, R. (1972) Science 176, 933 - 935
5. de la Cruz, A., Coy, D.H., Vilchez-Martinez, J.A. and Schally, A.V. (1976) Science 191, 195 - 196
6. Kaiser, E., Colescott, R.L., Bossinger, C.D. and Cook, P.J. (1970) Anal. Biochem. 34, 595 - 598
7. Kaiser, E., Bossinger, C.D., Colescott, R.L. and Olsen, D.B. (1980) Anal. Chim. Acta 118, 149 - 151
8. Coy, D.H., Horvath, A., Nekola, M.V., Coy, E.J., Erchegyi, J. and Schally, A.V. (1982) Endocrinology 110, 1445 - 1447
9. Folkers, K., Bowers, C.Y., Kubiak, T and Stepinski, J. (1983) Biochem. Biophys. Res. Commun. 111, 1089 - 1095
10. Mezo, I., Seprodi, J., Erchegyi, J., Horvath, A., Nikolics, K., Teplan, I., Vigh, S., Kovacs, M., Flerko, B., Pedroza, E., Nekola, M. Coy, D.H. and Schally, A.V. (1983) Acta Chim. Budapest, in press
11. Rivier, C., Rivier, J. and Vale, W.W. (1981) in: Zatuchni, G.I., Shelton, J.D. and Schiarra, J.J., eds. Harper&Row, Philadelphia, pp. 140 - 150
12. Rivier, C., Rivier, J. and Vale, W. (1981) Endocrinology 108, 1998 - 2001
13. Rivier, C. and Vale, W. (1983) Biol. Reprod. 24, 1061 - 1067
14. Nekola, M.V., Horvath, A., Ge, J.J., Coy, D.H. and Schally, A.V. (1982) Science 218, 160 - 162