

New Placental Gonadotropin Releasing Hormone Receptor Antagonist

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Abstract: We isolate for the first time in Literature a new placental gonadotropin releasing hormone receptor antagonist in trying to test the effectiveness of this newly discovered compound we compared with other known GnRH antagonists Antagonand Cotrotide. We found that the potency of placental GnRH Antagonists is more than other compounds with a minimal side effect so a new placental GnRH Antagonists has been born [Ali Farid Mohamed Ali, Anas El-Attar and Laila Ali. **New placental gonadotropin releasing hormone receptor antagonist.** *J Am Sci* 2013;9(12):251-254]. (ISSN: 1545-1003). <http://www.americanscience.org>. 31

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1. Introduction

We demonstrated for the first time⁽¹⁾ in the Literature that GnRHmessenger and peptide in the placental suggesting that trophoblast are the source of GnRH. Also we demonstrate a wide heterogeneity for the GnRH-R-gene in human placenta, which may be attributed to alternative splicing and autocrine/paracrine role of GnRH in human Placenta.

We discovered recently⁽¹⁾ a new placental gonodotropin releasing hormone receptor antagonist, for the first time in Literature, the aim of this work is to review the pharmacology and the preclinical studies and compare between placental gonadotropin releasing hormone receptor antagonist and other GnRHantagonis (Antagon, and Cetrotide).

2. Material and Methods

Ten placentas from the first trimester, 10 placentas from the second trimester, and 10 placentafrom the third trimester were enrolled in the study. We used *in situ* polymerase chain reaction (PCR) alone or in combination with immunocytochemistry; placental section (74 m) or single trophoblasts in monlayer cultures of up to 5 days were created with proteinase K following reverse transcription (RT) with GnRH and GnRH-R-specific oligoprimers. Polymerase chain reaction was performed using primers with exon- exon overlaps to exclude nonspecific DNA amplification; detection of the 380 bpamlicon was accomplished by nested PCR which was performed with digoxigenin-labeled dUTP and NBT IBCIP for substrate visualization. The GnRH peptide was detected in a sandwich antibody assay. GnRH-R-C DNA was detected by RT-PCR. After Assay a control plasmid-coded cDNA to confirm the integrity of the PCR products of expected lengths, we optimized the experimental condition for

GnRH-R RT-PCR. Under these conditions, we obtained PCR products from placental total RNA extracts, which represented almost the complete GnRH-R open reading frame. However, several other products could also be observed in the same reaction; some were longer, others were smaller.

Animal Assays

All rats were housed; temperature was kept at 25⁰C with 12 hours light: 12 hours dark cycle (lights on at 06.30) standard food and water were available. Monkey studies were performed in the primate.

Primary rat pituitary cell culture studies⁽²⁾

Hemisected rat pituitaries from immature male rats were enzymatically dispersed using Collagenase followed by viokase. All tissue culture supplies were purchased from GIBCO BRL unless otherwise specified. Aliquots of a single cell suspension in DMEM/F12 medium supplemented with 8% fetal bovine serum 0.52 µg/ml transferrin. 0.5 ng/ml parathyroid hormone 1 ng/ml basic fibroblast growth factor, 0.29 mg/ml glutamine, 5 µg/ml insulin and penicillin streptomycin and nystatin were placed in a 24-well tissue culture plate and placed in a 5% CO₂ incubator at 37°C for 3 days. On the fourth day, the wells were washed with and reincubated in the nutrient medium. At this time the cultures were treated with 0.1 nmol/l lupron alone or in combination with various concentrations. After 4 hours at 37°C, the media were removed and stored at - 20° until assayed for gonadotropin release by radioimmunoassay (RIA). Gonadotropin levels in tissue culture media were determined by standard RIA techniques.

Rat anti-ovulatory activity⁽²⁾

Female rats (180-200g), with predictable 4-day estrous cycles were injected subcutaneously with a GnRH antagonist between 13.00 and 14.00 hours on

the day of proestrus. The vehicle was 10 nmol/1 Hc1 in normal saline. The animals were sacrificed the following morning and the presence or absence of ova in the oviduct was determined by microscopic inspection. The animals were scored as ovulating if one or more ova were present in the oviduct. ED50 was calculated using the SAS probit analysis program.

Overiectomized rat studies ⁽³⁾

Female rats (180-200g) were ovariectomized using Metofane. After 2-3 weeks, one jugular vein was cannulated. The following day, the GnRH antagonists dissolved in either 5% mannitol or acidified saline were injected subcutaneously. Blood samples were then collected through the cannulae. Approximately 400 µl of plasma was obtained from each sampling and the red blood cells, resuspended in sterile saline, were replaced through the cannula into each rat. This blood collection procedure was followed for studies lasting less than 15 days. For the 15-30 day time course studies, jugular vein cannulations were not performed, but instead 1.0 ml or less of blood was collected by heart puncture after Metofane anesthesia. All blood samples were then centrifuged 1500 r.p.m for 15 min at room temperature, and the resulting supernatant (plasma fraction) was stored at -20°C until assayed by RIA for gonadotropin levels. Gonadotropin levels in plasma samples were determined by standard.

Castrate monkey Studies ⁽⁴⁾

In Overiectomized monkeys a pretreatment femoral blood sample was collected on day 0 followed by daily blood collections for 4 consecutive days. All blood collections were made under ketamine-induced anesthesia. The GnRH antagonist was administered subcutaneously on day 0, following the zero-time blood collection. All serum samples were analyzed for LH by RIA. The treatment groups were: Placental GnRH Antagonist 1.5, 5.0, 15 and 45 µh/kg; Antagon 5.0 and 45 µh/kg; cetrorelix 5.0 and 45 µh/kg.

Studies in intact rats ⁽⁴⁾

Intact male rats were injected subcutaneously for 15 days with the indicated GnRH antagonist at the following doses: 8, 18, 60, 180 and 512 µh/kg per day. The vehicle was 5% mannitol in water. On the day following the last injection, animals were sacrificed and the testes, ventral prostate and seminal vesicles were extracted, trimmed free of any surrounding fatty tissue and weighted. Intact rats treated with vehicle served as intact controls. Rats castrated on the first day of treatment served as castrate controls.

Subacute studies in cycling monkeys

Normally cycling female monkeys were utilized for these studies. On menstrual cycle days 20-22 in

the pretreatment cycle, an evaluation of serum progesterone concentrations was undertaken. If the serum progesterone concentration was greater than 1.0 ng/ml, the monkey was assigned to a treatment group at the start of the next menstrual cycle. The four treatment groups with placental GnRH Antagonist were 5.0, 15, 25 and 40 µh/kg day., Placental GnRH Antagonist was administered daily as a subcutaneous injection from cycle days 2 to 15. Femoral blood samples were collected daily from cycle days 2 to 16 in all treatment groups and were collected on alternation days from days 18 to 42 from monkeys in the two higher dosage groups. Estradiol concentrations were analyzed in all serum samples using a commercial RIA kit.

Coetaneous and pulmonary anaphylactoid activity: ⁽⁴⁾

Male guinea pigs (500-590g) were anesthetized with urethane (2.0g/kg intraperitoneally) and placed in a whole-body plethysmograph. Transpleural pressure was measured. Tidal volume, Blood pressure was also monitored. The guinea pigs were pretreated with succinylcholine spontaneous respiration. After a 5 min stabilization period one of the GnRH antagonists was administered in a volume of 0.1 ml intravenously over 30s. Percentage change from baseline was monitored over 30 min for all parameters and recorded at 1, 3, 5 and 30 min after administration.

Male rats were injected intravenously with 1.0 ml of 0.5% solution of Evan's blue dye. Various concentrations of GnRH antagonists in acidified saline as well as vehicle control were injected intradermally into a shaved section on the back of the animal. Five injections at separate sites were made into each animal, and were used for determining a dose – response relationship. At 15 min after the intradermal the animals were sacrificed and the area of each wheal (as indicated by the intradermal presence of the Evan's blue dye) was measured as the product of the longest perpendiculars.

3. Results

Inhibition of LH release in rat pituitary cell culture

The GnRH Agonist Luporn (0.1 nmol/l) induced a sixfold increase in LH secretion and a four- to fivefold increase in FSH secretion, over a 4-hour incubation period in static cultures. This stimulation was inhibited completely, in a dose-dependent fashion, by co-incubation with any of three different GnRH antagonists. All compounds showing an ED₅₀ of approximately 0.69 nmol/l.

Inhibition of ovulation in proestrus rats

Subcutaneous administration of a GnRH antagonist to female rats on the afternoon of proestrus

was effective in reducing the percentage of rats ovulating (n=10/ group), in a dose-dependent manner. In this model, the dose-response curves were parallel, but placental GnRH antagonist showed the greatest potency in anti-ovulatory activity. The ED₅₀ was 5.2 µg/kg for Placental GnRH Antagonist.

Inhibition of LH levels after a single injection to ovariectomized rats and monkeys

The potency of placental GnRH Antagonist in suppressing gonadotropins in the castrated female rat was examined via single subcutaneous administration in 1 5% mannitol vehicle. Placental GnRH Antagonist at 1 µg/kg was indistinguishable from vehicle. At 3 µg/kg LH suppression of about 50% was evident within 2 hours, and a nadir (>90%) was reached by 6 hours; recovery was complete within 24 hours. Higher doses induced even more rapid suppression of LH₂, with near-maximal inhibition in evidence by 2 hours after injection. Speed of recovery proceeded inversely with dose, with a return to baseline occurring between 30 and 48 hours at 10 and 30 µg/kg, respectively. To explore the limits of duration of this inhibitory action on gonadotropins, higher doses of placental GnRH antagonist were examined in experiments that were extended in time. At 200 µg/kg, gonadotropins returned to baseline at approximately 3 days, whereas at 2000 µg/kg both LH and FSC remained maximally suppressed for at least 15 days following administration of azaline B. Thereafter recovery proceeded gradually and was complete by 30-35.

In ovariectomized monkeys, a single subcutaneous administration of Placental GnRH Antagonist in 5% mannitol at a dose of 1.5 µg/kg had no effect on LH levels, whereas doses of 5, 15 and 45 µg/kg induced maximal (80-90%) LH suppression between 6 and 24 hours. The duration of this suppression was again dose-dependent at 5 µg/kg, return toward baseline was evident by day 2 and complete by day 3, at 15 µg/kg, complete suppression was sustained on day 2 but by day 3 LH had returned to baseline levels. At the 45 µg/kg dose, significant (> 70%) suppression was still in effect 4 days after administration.

Potency relative to other GnRH antagonists during and after 2 weeks of daily administration of intact rats

The effect of daily administration of GnRH antagonists over an extended (2-weeks) treatment period was evaluated in intact male rats; change in testicular, ventral prostate, and seminal vesicles weight after 14 days of dosing was compared with that observed following surgical castration. While decreases of approximately 30 and 50% were seen with Antagon at the highest dose tested (570 µg/kg per day), castrate values were not achieved with

either of these two compounds. Cetrorelix was ineffective at 63 µg/kg per day, but induced castrate organ weights at a dose of 190 µg/kg per day. Placental GnRH Antagonist demonstrated the greatest potency in this model, with about 30% suppression following a dose of 20 µg/kg per day and near-maximal (about 90%) suppression at 56 µg/kg per day; castrate values were seen at 190 µg/kg per day.

Inhibition and recovery of estradiol levels during and after 2 weeks of daily administration to cycling monkeys.

To determine its potency in suppressing estradiol secretion, placental GnRH Antagonist was administered subcutaneously to cycling monkeys via daily injection for 2 weeks (cycle days 2 to 15). At 5 µg/kg per day, suppression of estradiol was inconsistent, with mean levels varying between approximately 20 and 50 pg/ml. At doses of 15, 25 and 40 µg/kg per day, mean estradiol levels remained in the castrate range, below 20 pg/ml, for the duration of treatment. Following cessation of antagonist administration, estradiol recovery began within 1 to 5 days, even at the highest dose, with ovulation occurring in all monkeys by cycle day 30-35, i.e. within 15-20 days of discontinuing azaline B.

Assessment of anaphylactoid potential

The potential of placental GnRH Antagonist and other GnRH antagonists to induce anaphylaxis was assessed using whole-body plethysmography to measure changes in lung compliance and airway resistance following intravenous administration of 10 mg of drug to guinea pigs. At this dose of Antagon a significant 80% diminution in lung compliance was seen; this was accompanied by a reciprocal increase in airway resistance. Virtually no changes in these parameters were encountered with similar injections of 10 mg of either Antagon or Placental GnRH Antagonist.

Further analysis of comparative histaminic potential was undertaken via the intradermal injection of 1, 3, 6 or 10 µg/kg of different antagonists to male rats. In this model, dose-related increases in wheal response were observed with Antagon and Cetrorelix, in that order of potency. Placental GnRH Antagonist at all doses tested was ineffective in eliciting any wheal response greater than control

4. Discussion:

The above studies evaluated the activity of placental GnRH Antagonist, alone and in comparison to several other GnRH antagonists now in development(5), in pituitary cell culture and in both intact and castrated animal models. The gonadotroph remains the principal target tissue for the action of

GnRH antagonists(6,7), which renders suppression of gonadotropins in pituitary cultures and in the castrate model a critical measure of the activity of these compounds.

In cell culture, Antagon, and placental GnRH Antagonist exhibited similar potency, though in the anti-ovulatory assay in rats these same antagonists were clearly separable, with Placental GnRH Antagonist(8) demonstrating greater potency. In Castrated rats and monkeys, the minimally effective dose of Placental GnRH Antagonist in suppressing LH below castrate levels was between 2 and 5 µg/kg, Increasing the dose of Placental GnRH Antagonist beyond this did not further enhance the degree of suppression achieved, but did extend(9).

The minimal dose of Placental GnRH Antagonist required to suppress ovarian estradiol secretion reliably in the cycling monkey appears to lie between 5 and 15 µg/kg per day. Even at higher doses (40 µg/kg per day), however ovarian follicular activity quickly resumed after cessation of treatment, with ovulation occurring with approximately 2 weeks, Reversibility of suppression after chronic treatment thus appears assured, as one would wish for a therapy which induces a medical castration.

A critical obstacle to the successful development of GnRH antagonists as useful therapeutic agents has been the propensity of this class of peptides to induce histamine release. Anaphylaxis is the result of multiple cascading immunological and inflammatory. In the forgoing studies, we have demonstrated that placental GnRH Antagonist, of all compounds tested, remains the most selective for the GnRH receptor, in exhibiting the least potential for inducing anaphylactoid responses in these in thses animal models. The preclinical studies presented herein have elucidated the doses of Placental GnRH Antagonist that produce gonadotropin suppression and castration in rats and monkeys , its high relative potency and prolonged duration of action with respect to other antagonists in these animal models, and the minimal anaphylactoid potential of placental GnRH Antagonist compared to other antagonists. The foregoing studies would lead one of predict that Placental GnRH Antagonist will have a wide therapeutic index relative to other GnRH antagonists(10).

Conclusion:

A new placental GnRH Antagonist has been born it's effectiveness is more than Antagon, Cetrotide (and with minimal side effect).

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