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In vivo characterization of a novel GnRH (gonadotropin-releasing hormone) antagonist, LXT-101, in normal male rats

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Abstract

LXT-101 is a newly developed GnRH (gonadotropin-releasing hormone) analogue. In this study, the *in vivo* pharmacological profile in intact male rats and binding characters of LXT-101 were illustrated, and regulation of mRNA of hormone receptors related to the pituitary–gonadal axis during and after administration was observed to reveal its molecular mechanism of potent effect and reversibility. After single subcutaneous injections, LXT-101 produced a dose- and time-dependent suppression of serum testosterone level. Multiple administrations and osmotic pump implantation revealed that the time of onset and dose needed to maintain the effect of chemical castration decreased as the frequency of injection increased and gave direct proof that depot formulation could significantly improve the duration of antagonist delivery and pharmacological activities compared to the injectable formulation. And LXT-101 showed excellent character of regulating the pituitary–gonadal axis quickly and reversibly. Competitive binding assay showed that LXT-101 could specifically bind a pituitary GnRH receptor with high affinity. These results indicated that LXT-101 is fit for sustained-release formulation and it might possibly be developed as an ideal candidate for treating sex hormone-sensitive tumors and other disorders.

Keywords: GnRH analog; GnRH antagonist; Chemical castration; Serum testosterone; Competitive binding; mRNA expression

1. Introduction

Since the clinical availability of the first GnRH agonist, no significant improvement has been made in the field of medical castration [1–3]. A potential problem with the GnRH agonist therapy is the transitory testosterone flare that begins approximately 2 or 3 days after the initial injection and lasts through approximately the first week of therapy [4,5] and that causes severe side-effects such as bone pain, bladder outlet obstruction, cord compression and cardiovascular effects [6]. With the development of GnRH antagonists, which have no agonist properties, the question of the frequency and severity of GnRH agonist-induced flares has renewed relevance. The third generation of GnRH antagonists is characterized by substituting the appropriate combination of amino acids at positions 5, 6, and 8 thus reducing anaphylactic reactions caused by histamine release. These antagonists inhibit the reproductive system through competition with endogenous GnRH for the receptor and, in view of their rapid effects and low anaphylactic reactions, are being increasingly used for clinical trial [7]. But to date, only a few GnRH antagonists as cetrorelix, abarelix and ganirelix are available on the market [8–10]. Cetrorelix and ganirelix are used in assisted reproduction and puberty prematurity, and abarelix was developed for treating prostate cancer [11]. However, the development of GnRH antagonist lags behind that of the agonists, therefore, there is still more requirement in developing potent GnRH antagonists for the treatment of variable hormone-related diseases [12].

Recently, a new GnRH analog named LXT-101 is developed by our institute. Although this compound shares a similar structure with GnRH antagonists, its binding property to the pituitary GnRH receptors and the *in vivo* pharmacological profile of LXT-

Abbreviations: GnRH, Gonadotropin-releasing hormone; GnRH-R, Gonadotropin-releasing hormone receptor; LH, luteinizing hormone; AR, androgen receptor; P, pituitary; H, hypothalamus; T, testis; Pr, prostate; BS, Abovine serum albumin.

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101 on the sex hormone need to be investigated, which might be most important in choosing the appropriate therapeutic formation. Previously several groups have reported that GnRH analogs are suitable to be developed as depot formation for the treatment of hormone-sensitive diseases, yet most of their conclusion is based on the clinical hint and reasoning that multiple doses are better than single-shot and little direct experimental argument is given [13–15]. In addition, the molecular regulation of GnRH antagonist on the regulation of the gonadal axis besides pituitary is still unclear, although some groups tried to explain the mechanism of GnRH antagonist on the pituitary–gonadal axis on the hormone secretion level [16]. Our experiments are aimed to solve these problems and give clear descriptions of the new GnRH antagonist LXT-101.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (200–220 g) were purchased from Vital River Experimental animals Technique Ltd. (Beijing, China) and kept for at least 5 days before initiating experiments. Food and water were supplied *ad libitum*. All studies were carried out in accordance with the Declaration of Helsinki.

2.2. Drugs

LXT-101 [Ac-D-Nal-D-Phe(4-Cl)-D-Pal-Ser-Mop-D-Pal-Leu-Arg-Pro-D-Ala·NH_2] was synthesized by our institute. It has a good solubility and solutions were prepared in 5% aqueous mannitol. Dilutions were made for each dose level to give rats an injection volume of 0.1 ml/0.1 kg weight. The GnRH antagonists cetrorelix and abarelix were kindly provided by Dr. Guangcheng Jiang (Ferring Research Institute Inc., San Diego, CA 92121). The [^{125}I, D-Trp^6]GnRH was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA 02118).

2.3. Drug administration in rats

LXT-101 were given either by injecting subcutaneously (S.C.) in the dorsal region or by releasing from osmotic pump implanted under dorsal skin. Blood samples (500 μl) were collected by puncture of the ophthalmic venous plexus with micro-hematocrit tubes in Eppendorf tubes, left at 4 °C for 4 h and then centrifuged at 3000 rpm for 10 min then stored at −20 °C until hormone determination. After the experiment, rats were decapitated and the hypothalamus, anterior pituitaries, testis, and prostates were rapidly removed and frozen in liquid nitrogen for mRNA studies.

Before the osmotic pump transplantation, rats were anaesthetized with ketamine hydrochloride (i.p., 0.05 g/kg). The ALZET® osmotic pump (ALZET, model 2001, 2 ml reservoir, 1 ml/h delivery, Durect Corporation, USA) filled with different concentrations of LXT-101 under sterile condition was implanted under the dorsal skin according to the manufacturer's protocol, resulting in releasing LXT-101 at 12.5, 25, and 50 μg/day slowly and regularly for 7 days. The control group was treated with sham operation. Blood samples were drawn and treated as above.

2.4. Hormone determination

Total serum testosterone levels are determined by specific immunochemiluminescence assay using the Access Testosterone Immunoassay Kit (Beckmann-Coulter Inc., CA). The Access Testosterone assay is a paramagnetic particle, a chemiluminescent immunoassay and also a competitive binding immunoenzymatic assay. A sample is added to a reaction vessel along with Sample Treatment Solution, mouse monoclonal anti-testosterone antibody, testosterone alkaline phosphatase conjugate, and paramagnetic particles coated with goat anti-mouse polyclonal antibody. Testosterone in the sample is released from the carrier proteins by the sample treatment solution and competes with the anti-testosterone monoclonal antibody. The resulting antigen–antibody complexes are then bound to the solid phase by the capture antibody. After incubation in a reaction vessel, separation in a magnetic field and washing remove materials not bound to the solid phase. A chemiluminescent substrate, Lumi-Phos 530, is added to the reaction vessel and light generated by the reaction is measured with a luminometer. The light production is determined from a stored, multi-point calibration curve. The lower limit of detection and the highest calibrator value are 0.1 ng/ml and 16 ng/ml respectively. The imprecision is $\leq$20% at 0.5 ng/ml and $<$10% at 2–10 ng/ml of testosterone.

2.5. Membrane preparation and receptor binding assay

Pituitary membrane fractions were prepared as described before [17]. Receptor binding of GnRH was performed as reported [18] using a sensitive *in vitro* ligand competition assay based on binding of radiolabeled [D-Trp^6]GnRH to rat anterior pituitary membrane homogenates. Briefly, membrane homogenates containing 20 μg of protein were incubated in triplicate with 60,000–80,000 cpm ($\approx$0.2 nM) [^{125}I, D-Trp^6]GnRH as radioligand and with increasing concentrations of nonradioactive peptides in a total volume of 150 μl of binding buffer. And unspecific binding was controlled with adding 1 μM of GnRH. At the end of the incubations, 125-μl aliquots of suspension were transferred onto the top of 1 ml of ice-cold binding buffer containing 1.5% (wt/vol) BSA in polypropylene microcentrifuge tubes (Axygen, preincubated with binding buffer of 1.5% BSA overnight and dried to reduce absorption). The tubes were centrifuged at 12,000 ×*g* for 3 min at 4 °C. Supernatants were aspirated, and the bottoms of the tubes containing the pellet were cut off and assayed in a γ counter (Micromedic Systems, Beijing, China). Protein concentration was determined by the Bradford method.

2.6. RNA preparation and real-time quantitative reverse-transcript PCR

The isolation of total RNA from individual organs of animals in the experiment with an intermittent injection was carried out with the TRIZOL® Total RNA Isolation Kit (Invitrogen, USA) according to the manufacturer's instructions. RNA quantity was evaluated by photometric concentration measurements