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Evaluation of Orntide Microspheres in a Rat Animal Model and Correlation to In Vitro Release Profiles

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ABSTRACT Orntide acetate, a novel luteinizing hormone-releasing hormone (LHRH) antagonist, was prepared and evaluated in vivo in 30-day and 120-day sustained delivery formulations using a rat animal model. Orntide poly(d,l- lactide-co-glycolide) (PLGA) and poly(d,l- lactide) (PLA) microspheres were prepared by a dispersion method and administered subcutaneously in a liquid vehicle to rats at 2.2 mg Orntide/kg of body weight (30-day forms) or 8.8 mg Orntide/kg (120-day forms). Serum levels of Orntide and testosterone were monitored by radioimmunoassays, and a dose-response study at 4 doses (3, 2.25, 1.5, and 1.75 mg Orntide/kg) was conducted to determine the effective dose of Orntide. Microspheres with diameters between 3.9 and 14 μ were prepared. The onset and duration of testosterone suppression varied for different microsphere formulations and were influenced both by polymer properties and by microsphere characteristics. Microspheres prepared with 50:50 and 75:25 copolymers effectively sustained peptide release for 14 to 28 days, whereas an 85:15 copolymer and the PLA microspheres extended the pharmacological response for more than 120 days. Increase in drug load generally accelerated peptide release from the microspheres, resulting in higher initial serum levels of Orntide and shorter duration of the release. In general, apparent release was faster in vivo than under in vitro conditions. Orntide microspheres effectively suppressed testosterone in rats, providing rapid onset of release and extended periods of chemical castration. Testosterone suppression occurred immediately after microsphere administration without the initial elevation seen with LHRH superagonists.

Key Words: LHRH antagonist; Orntide acetate; Peptide controlled delivery; PLGA microspheres; Prostate cancer.

INTRODUCTION

Since the discovery and structural elucidation of luteinizing hormone–releasing hormone (LHRH), numerous analogues have been synthesized and studied for potential application in gynecology and oncology as antigonadotropic agents for ovarian and testicular suppression [1-4]. Leuprolide acetate, developed and marketed by TAP Pharmaceuticals, Inc. (Deerfield, Ill.) as Lupron Depot®, is one of the most widely used LHRH superagonists in the treatment of sex hormone-dependent tumors, such as prostatic carcinoma in men [5-7]. During continuous administration, Leuprolide initially stimulates production and release of testosterone, but the prolonged exposure to an LHRH superagonist eventually causes down-regulation of LHRH receptors and inhibition of luteinizing hormone (LH) release, which finally leads to chemical castration [8,9]. Due to this initial testosterone elevation, which may last in humans from 5 to 8 days, approximately 11% of all treated patients experience painful and potentially dangerous flare-ups of disease [10-14].

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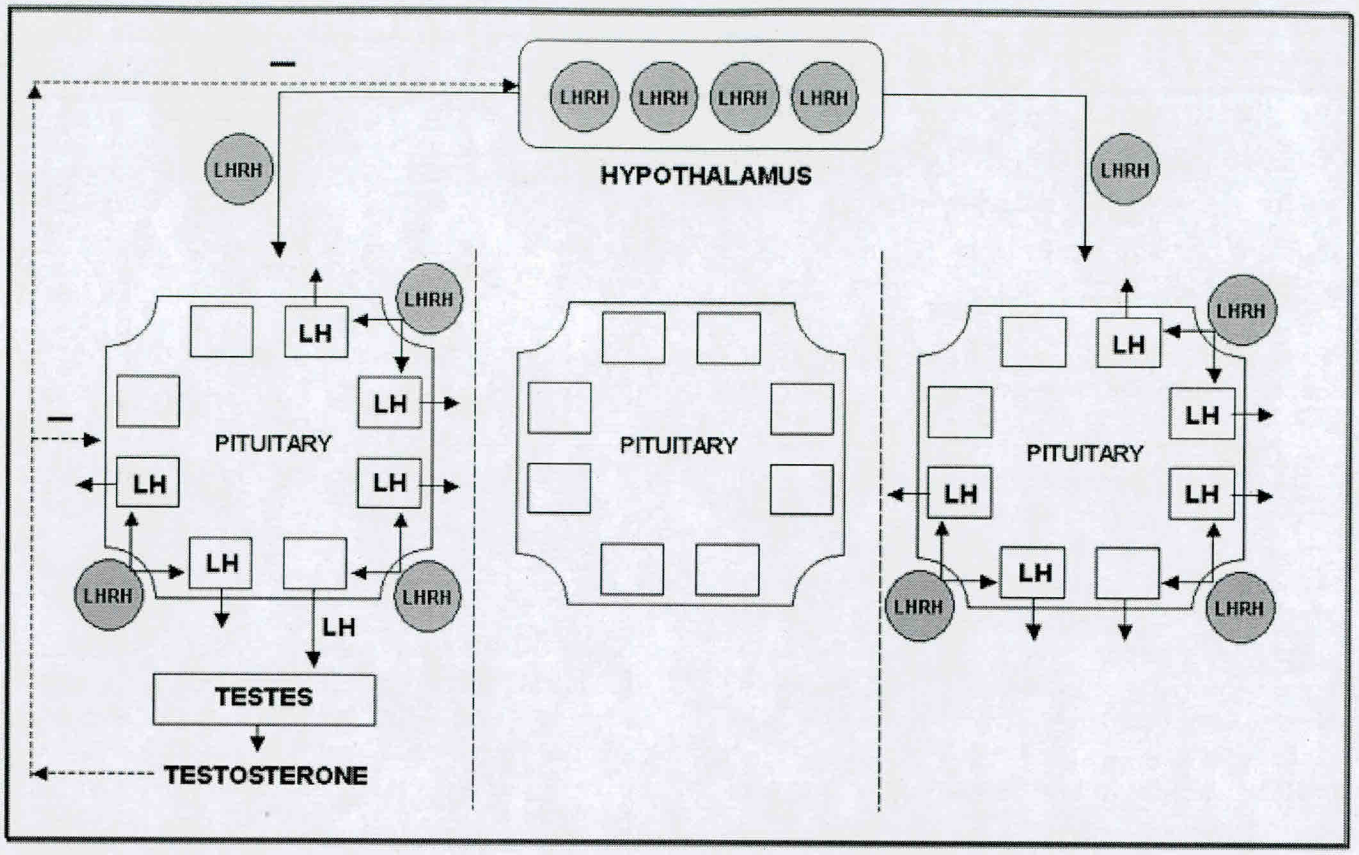
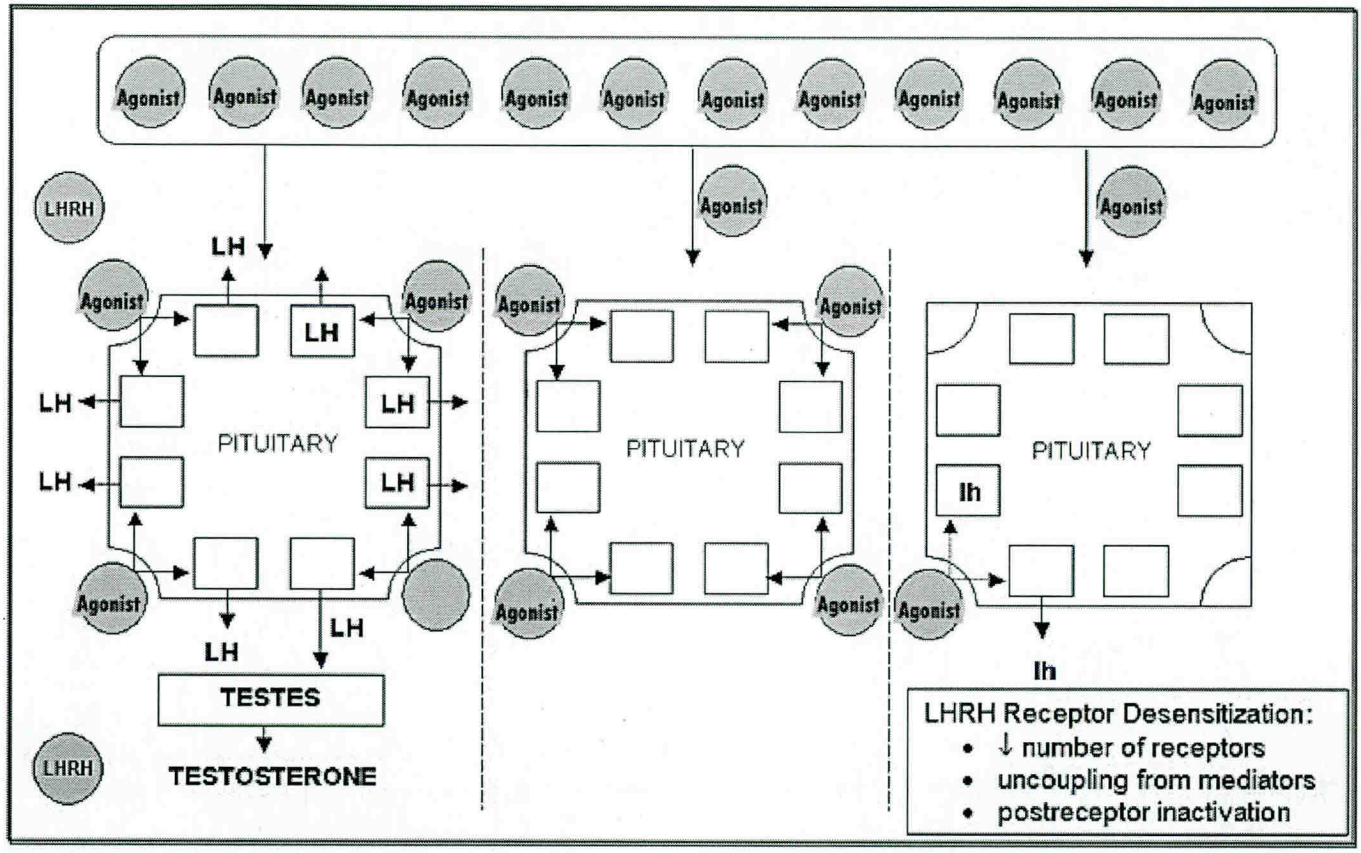


Scheme 1. Normal physiology of the pituitary-gonadal axis.

Scheme 1 illustrates normal physiology of the hypothalamo-pituitary-gonadal axis. LHRH, a peptide hormone synthesized in the arcuate nucleus of the hypothalamus, is released in response to low testosterone levels and is transported to the pituitary gland, via capillaries known as the hypothalamic-pituitary portal system, where it binds to the surface receptors of gonadotropin-producing cells, or gonadotropes. Once the LHRH receptors are stimulated, the gonadotropes synthesize and secrete gonadotropins by exocytosis of storage granules containing LH and follicle-stimulating hormone (FSH). Both hormones are glycoproteins that consist of €α and ß subunits. FSH stimulates the development of the seminiferous tubules and maintains spermatogenesis, and LH stimulates the Leydig cells in the testes to produce and secrete testosterone. Under normal physiologic conditions, LHRH is rapidly removed from the site by enzymes that cleave the glycine-leucine linkage between positions 6 and 7. In this way the gonadotropins are released in a regular pulsatile fashion, which is necessary to maintain normal functions of the pituitary gonadotropes and gonads [2].

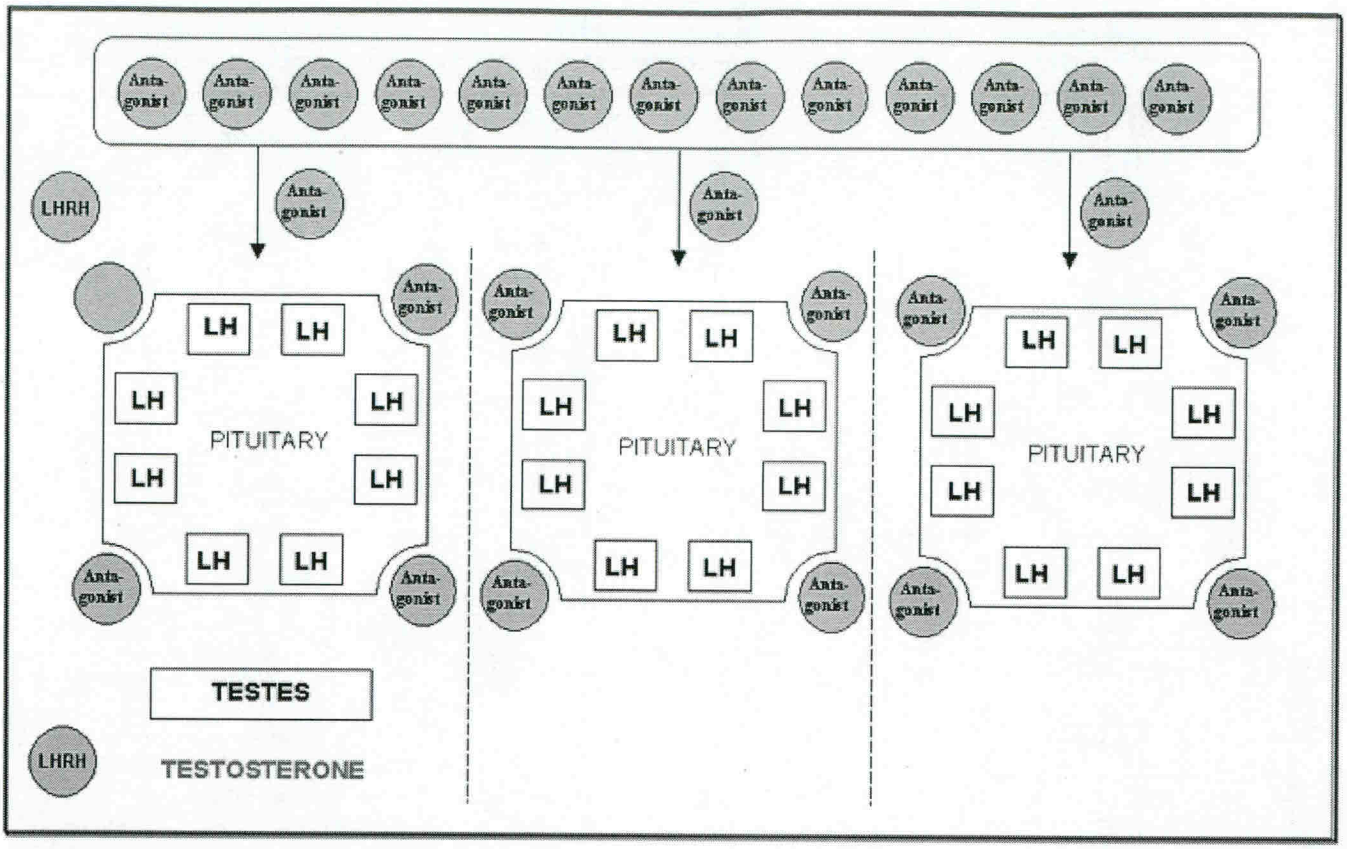
Scheme 2 shows the effect of continuous administration of an LHRH superagonist on the pituitary-testicular axis. Initially, a transient phase of testosterone elevation caused by stimulation of the pituitary LHRH receptors and increased release of LH is observed. Soon after, however, the gonadotrophic receptors become desensitized and unresponsive to further stimulation. The receptor down-regulation process includes reduction in the number of surface receptors, uncoupling of the receptors from intracellular mediators, and postreceptor inactivation [2,15-17]. Finally, instead of producing active LH, gonadotrophs start to produce and release an inactive form of LH (shown in **Scheme 2** as "lh") that is unable to stimulate production of testosterone in testes.



Scheme 2. The effect of continuous exposure to a LHRH superagonist on the pituitary-gonadal axis physiology.

It has been reported that immediate and complete testosterone suppression may be achieved with LHRH antagonists [2]. These compounds show a much higher affinity to the pituitary LHRH binding sites than does native LHRH; they also compete with endogenous LHRH at the LHRH receptor sites. Blockade of the pituitary LHRH receptors prevents stimulatory effects of native LHRH and results in rapid inhibition of the pituitary- gonadal axis; thus, testosterone suppression is immediate without the characteristic stimulatory phase for LHRH superagonists (**Scheme 3**). In addition, it has been reported that LHRH antagonists directly inhibit growth of numerous tumors, including mammary, pancreatic, and prostatic tumors, by binding to receptors localized on tumor cells [18-21].

More than 3000 LHRH analogues have been synthesized and studied [2]. Whereas the LHRH agonists have already found clinical application in the therapy of gonadal steroid–associated diseases, the LHRH antagonists have not reached this stage. Animal studies with early LHRH antagonists revealed that, due to high histamine release from mast cells, these compounds caused severe adverse effects, including anaphylactoid reactions, hypotension, skin lesions, and formation of edema [22,23]. During the past 2 decades, developers of safer LHRH antagonists focused on increasing antigonadotropic potency and minimizing histamine-releasing properties. As a result of enormous efforts by several research groups, a series of modern, safer, and more potent LHRH antagonists has been synthesized [24,25]. Orntide acetate, synthesized by Cyril Y. Bowers, Karl Folkers, and Anna Janecka at Tulane School of Medicine, New Orleans, LA, is one of the most promising LHRH antagonists developed to date (**Table 1**).



Scheme 3. The effect of continuous exposure to a LHRH antagonist on the pituitary-gonadal axis physiology.

Table 1. Leading LHRH antagonists (Modified from: Cyril Y. Bowers, Karl Folkers, and Anna Janecka, United States Patent #US5480969, 1996)

LHRH Antagonist	Antiovulatory Activity (%/μg)					Histamine Release ED_{50} (μg/mL)
	0.125	0.25	0.5	1.0	1.5	
Nal-Arg (Rivier et al., 1984)			50			0.17
Nal-Glu (Rivier et al., 1986)			50		100	1.6
Antide (Ljungqvist et al., 1987)			36	100		>300
Argtide (Janecka et al., 1991)	63	89				31
SB-75 (Bajusz et al., 1988)					75	1.5
RS-26306 (Nestor et al., 1985)		50				13
Orntide (Bowers et al., 1996)		25	100			100

Note: The most preferred LHRH analogues of the table have high antiovulatory activity and also a high effective dose level for histamine (ED_{50} = dose releasing 50% of total histamine from mast cells; for native LHRH ED_{50}=170 μg/mL).

It combines both high antigonadotropic potency and safety. At present, this analogue is undergoing preclinical evaluation in animals and soon will enter the first phase of clinical trials. Therefore, sustained-release formulations of Orntide acetate are urgently needed to make the clinical use more feasible. Such preparations may, in addition, help to reduce the therapeutic dose required for continuous suppression of pituitary and gonadal functions and may cause fewer adverse effects.

To enable further studies and clinical trials with Orntide, this analogue must be formulated as a therapeutic system that provides the highest efficiency and safety of the treatment and preferably good patient compliance. Therefore, the purpose of this work was to prepare controlled-release microparticulate Orntide formulations using biodegradable low-molecular-weight polylactides, poly(d,l- lactide) (PLA) and poly(d,l- lactide-co-glycolide) (PLGA), and to evaluate these formulations for their efficacy in suppressing testosterone in a rat animal model.

MATERIALS AND METHODS

Materials

Orntide acetate ([NacDNal1 DpClPhe 2 D3Pal 3 PicLys 5 D(6Anic)Orn 6 Ilys 8 DAla 10] - LHRH) was supplied by California Peptide Research, Inc (Napa, CA). Orntide 30-day and 120-day PLGA and PLA microspheres were developed in collaboration with Oakwood Laboratories, LLC (Oakwood, OH) [19]. Three 50:50 PLGA copolymers (MW 10 777–31 281) with different degrees of hydrophilicity, 75:25 PLGA (MW 11 161), and a PLA homopolymer (MW 9489) were obtained from Boehringer Ingelheim, Inc (Ingelheim, Germany), and 85:15 PLGA (MW 17 903) was obtained from Birmingham Polymers, Inc (Birmingham, AL). The hydrophilic resomers (H) had free carboxyl end groups, whereas the hydrophobic ones were capped with long-chain alkyl alcohols. Polyvinyl alcohol (PVA) (average MW, 30 000-70 000) was obtained from Sigma Chemical Co (St Louis, MO). The solvents and other excipients were analytical grade and were purchased from commercial sources; dialysis tubes (Tube-O-Dialyzer®) were from Research Products International Corp (Mount Prospect, IL), and a Spectra/Por® CE dialysis membrane (molecular weight cut-off, or MWCO, 300 000 Da) was from Spectrum Medical Industries, Inc (Houston, TX). Injections of free Orntide acetate were prepared by dissolving the peptide in water to reach the desired concentration. Male Sprague Dawley rats weighing approximately 300 g were purchased from Harlan (Indianapolis, IN). The studies were conducted at the University of Kentucky College of Pharmacy Animal Research Facility.

Methods

High-Performance Liquid Chromatography (HPLC) Method for Peptide Assay

The peptide was analyzed by reverse-phase HPLC (Bondclone 10 C18 column, 150 x 3.90 mm) using an elution phase of 34% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid in water. Ultraviolet detection was at 215 nm [26].

Preparation of Orntide Microspheres

Orntide microsphere batches (0.9-2.7 g) were prepared by a dispersion method, followed by solvent extraction and evaporation [26]. A solution of peptide in methanol was combined with a solution of PLGA or PLA in methylene chloride and stirred until clear. The solution was then slowly injected into a 1 L reactor with baffles (Ace Glass Inc, Vineland, NJ) containing the continuous phase (CP; 0.35% [wt/vol] solution of PVA; pH, 7.2) and stirred at 5500 rpm with a Silverson™

L4R homogenizer (Silverson Machines Ltd, Waterside, England). The temperature of the reactor was maintained initially at 25°C for 30 minutes and then at 40°C for 60 minutes. Once microspheres were formed and hardened, the contents of the reactor were transferred to a filtration apparatus equipped with a 0.8 µ membrane filter (Gelman Sciences, Ann Arbor, MI) and the recovered product was washed with water and dried under reduced pressure for 48 hours at room temperature.

Microsphere Characterization

Peptide content in microspheres was determined by HPLC after dissolving the microspheres in chloroform and extracting the peptide with a 0.1 mol/L acetate buffer (AB) with a pH of 4.0. Total product yield was assessed gravimetrically on the basis of polymer/drug recovery. Microsphere bulk density was determined with the tapping method using approximately 100 taps and a 10 mL graduated glass test tube. Particle size distribution was determined using a laser diffraction technique (Malvern 2600c Particle Sizer, Malvern, UK).

In Vitro Release Study

A previously described dialysis technique was used to determine the in vitro release from Orntide microspheres [27]. Approximately 20 mg of the microspheres were quantitatively transferred to a 7 mL dialysis tube (Tube-O-Dilalyzer; MWCO, 300 000 Da) containing 5.0 mL of AB, which in turn was placed in a 50 mL tube containing 40 mL of the same release medium. The contents of the larger tube were continuously stirred with a magnetic stirrer. At various times, 1.0 mL of the supernatant was removed from the

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