Effect of the Concurrent LHRH Antagonist Administration with a LHRH Superagonist in Rats

Janusz W. Kostanski,[1] Bhas A. Dani,[1] Bruce Schrier,[2] and Patrick P. DeLuca[1,3]

Received August 13, 1999; accepted December 30, 1999

Purpose. The purpose of this study was to investigate the effect of a novel LHRH antagonist, Orntide acetate, on the initial testosterone elevation in rats during treatment with a LHRH superagonist, Leuprolide acetate.

Methods. Thirteen groups of a rat animal model were administered either liquid Orntide or Orntide PLGA microspheres before or simultaneously with Leuprolide injections. Serum levels of testosterone were monitored during the time course of the study using a radioimmunoassay method.

Results. Administration of a single daily dose of liquid Orntide resulted in testosterone suppression within 6 h to levels below 0.5 ng/ml (castration level). However, combined administration of liquid Orntide and liquid Leuprolide did not have a significant effect on the initial testosterone elevation in studied rats. Similarly, there was no effect when liquid Orntide was co-administered with Leuprolide microspheres. Administration of Orntide microspheres 48 h before Leuprolide microspheres suppressed testosterone levels below the castration level within 24 h, however, did not prevent a rise in testosterone serum concentration upon administration of Leuprolide microspheres. Also, a second testosterone peak was observed between days 3 and 15 in the animals which were simultaneously treated with Orntide microspheres and Leuprolide microspheres.

Conclusions. Orntide acetate was found to be an effective LHRH antagonist with a rapid onset of pharmacological action and a short biological half-life. Administration of a single dose of liquid Orntide or Orntide microspheres, resulted in rapid testosterone suppression without an initial elevation, as seen with LHRH superagonists. However, combined administration of Orntide and Leuprolide did not have an effect on the initial testosterone elevation in rats.

KEY WORDS: LHRH antagonist; orntide acetate; LHRH superagonist; leuprolide acetate; prostate cancer flare up; PLGA microspheres.

INTRODUCTION

Hormonal therapy of sex hormone-dependent diseases, such as prostate cancer, became an important and effective treatment option over the past decade (1–5). Better understanding of human endocrinology, progress in peptide chemistry, and development of novel and sophisticated drug delivery systems contributed to the evolution of this group of antigonadotropic agents.

The idea of treatment of prostate cancer with Luteinizing Hormone-Releasing Hormone (LHRH) analogues is based on the observation made by Huggins et al., in the early 1940s, that growth of prostate cancer was under the trophic influence of male hormones, such as testosterone and dihydrotestosterone (6). Indeed, administration of exogenous androgens causes acceleration of growth of tumor cells, whereas suppression of testosterone to castration levels in males causes regression of tumor growth (7).

Continuous administration of potent LHRH superagonists, such as Leuprolide acetate, causes an initial elevation of testosterone level followed by subsequent testosterone suppression. This transient stimulatory phase may persist for up to one week in rats and as long as four weeks in humans before chemical castration is achieved (8,9). During this time an accelerated tumor growth may be observed causing painful flare of the disease and profound worsening of the quality of life (10–14).

Initial testosterone elevation is typical to chronic treatment with LHRH agonists and is due to the initial stimulation of pituitary LHRH receptors and increased release of Luteinizing Hormone (LH), which in turn stimulates testicular steroidogenesis and release of gonadotropins. After the initial stimulation by a LHRH agonist, pituitary gonadotrophic receptors become desensitized and unresponsive to further stimulation. This leads to an inhibition of production and release of active LH from the pituitary gland followed by a subsequent testosterone suppression (8). In contrast, LHRH antagonists inhibit gonadotropin release without an initial stimulatory phase (15).

Currently, very limited data are available regarding the impact of concurrent LHRH antagonist administration on the initial gonadotropin elevation during LHRH agonist treatment. The purpose of this study was to evaluate potential use of a new LHRH antagonist, Orntide acetate, in free form and formulated as slow-releasing biodegradable microspheres, to decrease or eliminate the initial testosterone elevation and associated side effects observed during treatment with Leuprolide. Also, the effect of continuous delivery of Orntide on testosterone suppression and on the initial testosterone elevation was studied using biodegradable microspheres containing encapsulated Orntide.

MATERIALS AND METHODS

Materials

Leuprolide acetate ([DLeu^{6}Pro^{9}Des-Gly^{10}]-LHRH Ethyl Amide) was purchased from Bachem, Inc. (Torrance, CA). Orntide acetate ([NacDNal^{1}DpClPhe^{2}D3Pal^{3}PicLys^{5}D(6Anic)Orn^{6}Ilys^{8}Dala^{10}]-LHRH) was supplied by California Peptide Research, Inc. (Napa, CA). Liquid injections of Leuprolide acetate and Orntide acetate were prepared by dissolving peptides in purified water at desired concentrations. Poly(d,l-lactide-co-glycolide) (PLGA, M_w 26,878) was obtained from Boehringer Ingelheim, Inc., Germany, and used for microsphere

ABBREVIATIONS. NacDNal, N-acetyl-3-(12-naphtyl) alanine; DpClPhe, 4-(4-chlorophenyl)-2-amino-butyric acid; Pal, 3-(3-pyridyl) alanine; PicLys, N$^{\varepsilon}$-picolinoyllysine; D(6Anic)Orn, 6-aminonicotinyl ornithine; IprLys, N$^{\varepsilon}$-isopropyllysine; LHRH, luteinizing hormone—releasing hormone; LH, luteinizing hormone; LL, liquid Leuprolide; LO, liquid Orntide; PLGA, poly(d,l-lactide-co-glycolide); MS, microspheres; LMS, Leuprolide microspheres; OMS, Orntide microspheres.

[1] Faculty of Pharmaceutical Sciences, University of Kentucky College of Pharmacy, Lexington, Kentucky 40536.
[2] Oakwood Laboratories, LLC, Oakwood, Ohio 44146.
[3] To whom correspondence should be addressed: (e-mail: ppdelu1@pop.uky.edu)

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preparation. The solvents and other excipients used were analytical grade and were purchased from commercial sources.

Microsphere Preparation

PLGA microspheres containing Leuprolide acetate or Orntide acetate were prepared by a dispersion method followed by solvent extraction / evaporation (16). Briefly, solution of peptide in methanol was combined with a solution of poly(d,l-lactide-co-glycolide) in methylene chloride and stirred using a magnetic stirrer for approximately 10 min. The clear solution was then slowly injected into a reactor containing the continuous phase (0.35% (w/v) solution of polyvinyl alcohol) and stirred at 3500 rpm with a Silverson L4R homogenizer. The temperature of the reactor was maintained initially at 25°C for 30 min. and after that at 40°C for 60 min. using a circulating water bath. Once microspheres were formed and hardened the contents of the reactor were passed through a 0.8 μ membrane filter (Gelman Sciences, Ann Arbor, MI) and the recovered microspheres were washed with water and dried under reduced pressure for 48 hours at room temperature.

Design of Animal Studies

Combination of Liquid Orntide and Liquid Leuprolide

Five groups of animals (n = 4 for each group) were used in this study. Groups I and II were injected with LO or LL, respectively, and were considered as study controls. Animals in Group III were simultaneously administered with a daily dose (0.1 mg/kg) of liquid Orntide (LO) and liquid Leuprolide (LL) at the beginning of the study. Injections of LL were repeated at 3, 6, 24, 48, and 96 hours. Group IV was injected with LO at a dose 3 times higher than Group III (0.3 mg/kg). The dosing regimen of LL was the same as for Group III. Animals in Group V received a single daily dose of LO 6 hours prior to the first LL administration. Blood samples were taken at 0, 3, 6, 24, 48, and 96 hours, right before injections of the drugs. In Group V an additional blood sample was taken at −6 hours right before LO injection.

Combination of Liquid Orntide and Leuprolide Microspheres

Five groups of animals (n = 4 for each group) were used in this study. Group VI was administered with PLGA microspheres containing a 30-day dose of Leuprolide acetate (3 mg/kg/30d) and was treated as a control. Animals in Group VII were injected with a single daily dose of LO 6 hours prior to the administration of Leuprolide microspheres (LMS). Injections of LO and LMS were given to rats at the same time at the beginning of the study in Group VIII. Group IX was injected simultaneously with a dose of LO 10 times higher than the daily dose (1 mg/kg) and a 30-day dose of LMS. Two injections of LO were given to each rat in Group X at 0 and 24 hours and a single injection of LMS was given at 0 h. Blood levels of testosterone were monitored in all animals for 7 days.

Combination of Orntide Microspheres and Leuprolide Microspheres

Three groups of animals (n = 4 for each group) were used in this study. Groups XI and XII were injected with a 30-day dose of Orntide microspheres (OMS) or Leuprolide microspheres (LMS), respectively, and were considered as study controls. Animals in Group XIII were injected with the same dose of OMS two days prior to the administration of LMS.

In Vivo Testosterone Suppression

Male Sprague Dawley rats weighing approximately 300 g were used to evaluate testosterone suppression. All formulations were injected subcutaneously at the back of the neck. Blood samples were collected from the tail vein at specific time points. The samples were centrifuged in Microtainer tubes obtained from Becton Dickinson and Co., NJ, and serum was collected. Serum samples were frozen and stored at −20°C until analysis. Serum testosterone was assayed using Active™ Testosterone RIA DSL-4000 kits purchased from Diagnostic Systems Laboratories, Inc., Webster, TX. The lower limit of detection for this assay was 0.08 ng/ml and the intra- and interassay coefficients of variation were 10 and 9%, respectively. The cross-reactivity of the testosterone antiserum was less than 6%.

Statistical Evaluation of Data

Data are presented as means ± standard deviation. For values below the assay detection limit the detection limit was used for calculations. To evaluate the effect of LHRH antagonist on the initial testosterone elevation during treatment with LHRH agonist, the areas under testosterone curves were calculated between 0 and 48 h (with exception of Group I where 0–6 h time interval was considered) using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The results were analyzed by one-way analysis of variance at α-level of 0.05.

Results

Combination of Liquid Orntide and Liquid Leuprolide

As expected, multiple injections of liquid Leuprolide resulted in an initial elevation of testosterone from 1.8 ng/ml to 17.1 ng/ml in 3 hours after the first injection (Figure 1). This transient higher level of testosterone was observed for at least 24 hours. At 48 hours testosterone was suppressed to levels close to castration (0.84 ng/ml). However, at 96 hours testosterone level started to rise suggesting that during the 48-hour interval between the fifth and the sixth Leuprolide injections most of the drug was metabolized and removed from the body.

Injection of a single daily dose (100 μg/kg) of liquid Orntide resulted in immediate suppression of testosterone (Fig. 1). The initial mean level of testosterone in rats (4.1 ng/ml) decreased to 1.1 ng/ml after 3 hours and at 6 hours was below castration level (0.3 ng/ml). However, after 24 hours testosterone levels returned to initial values suggesting that Orntide was rapidly removed from the system.

Simultaneous administration of LO and LL resulted in a behavior similar to that in which no Orntide was administered (Fig. 2 Groups III and IV). Both groups showed an initial elevation in testosterone levels. Also, no effect of Orntide dose on the initial increase in testosterone level was observed.

In the case where LO was administered 6 hours prior to LL (Fig. 2, Group V), testosterone levels were suppressed within 6 hours to 0.4 ng/ml but upon administration of LL, the levels

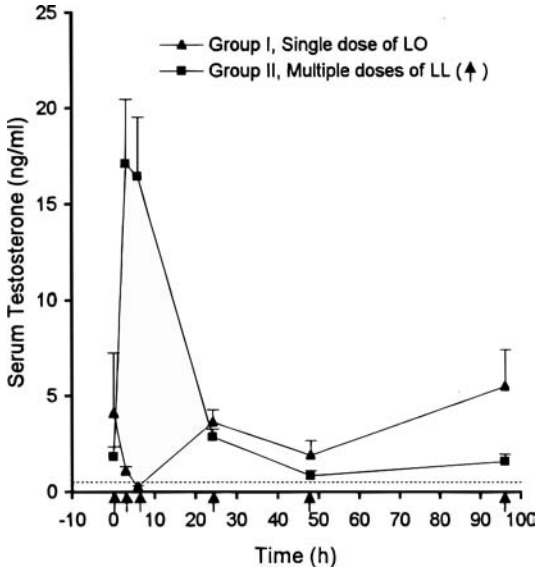

Fig. 1. Testosterone levels after administration of Liquid Orntide (LO, Group I), and liquid Leuprolide (LL, Group II) in rats. Broken line at 0.5 ng/mL designates castration level.

elevated to above 16 ng/ml and subsequent behavior was the same as in the previous two groups.

These studies indicate that while liquid Orntide effectively suppresses testosterone without an initial elevation it does not prevent the elevation upon concurrent or subsequent administration of liquid Leuprolide. The possible explanation will be discussed in the following section.

Combination of Liquid Orntide and Leuprolide Microspheres

Administration of a 30-day dose of Leuprolide microspheres resulted in the expected behavior shown with Group VI in Fig. 3. Testosterone levels peaked to above 21 ng/ml in 3 hours, then started to gradually decrease to levels below 2 ng/ml after 1 week. Administration of LO 6 hours prior to Leuprolide microspheres (LMS) decreased testosterone levels to below castration. However, upon administration of LMS the testosterone levels elevated again to above 15 ng/ml (Fig. 3, Group VII).

Simultaneous administration of LO and LMS (Group VIII) did not prevent the initial peak of testosterone. Also similar behavior was observed with Group X where two injections of LO were given (Fig. 3). Different behavior was observed when a high dose of LO (1 mg/kg) was simultaneously administered with LMS (Group IX, Fig. 3). After the initial peak testosterone level decreased to baseline after 24 hours. However, no further decrease was observed but the testosterone level started to rise and reached 7.6 ng/ml at 168 hours.

These studies reveal that simultaneous administration of liquid Orntide and Leuprolide microspheres dose not eliminate the initial testosterone peak. Also, a 10-fold increase in the LO dose or administration of LO 6 hours prior to the application of LMS does not have an effect on the initial testosterone elevation due to Leuprolide.

Combination of Orntide Microspheres and Leuprolide Microspheres

Administration of a 30-day dose of Orntide acetate (3 mg/kg/30d) incorporated into PLGA microspheres resulted in immediate testosterone suppression and castration was achieved within one day. Low levels of testosterone (below 0.1 ng/ml) were maintained for 28 days (Fig. 4, Group XI). In contrast to Orntide microspheres, Leuprolide microspheres resulted in elevated levels of testosterone before testosterone suppression was observed (Fig. 4, Group XII).

In Group XIII, where OMS were injected 48 hours prior to the injection of LMS, testosterone level initially decreased to below 0.5 ng/ml (Fig. 5). Injection of LMS two days later resulted in a typical testosterone peak as seen in Group XII, where no Orntide was present in the system. However, starting on day 2, testosterone level started to increase and reached the baseline level on day 8. After that testosterone level started to decrease reaching castration level on day 15 and remained below 0.5 ng/ml for the remainder of the study.

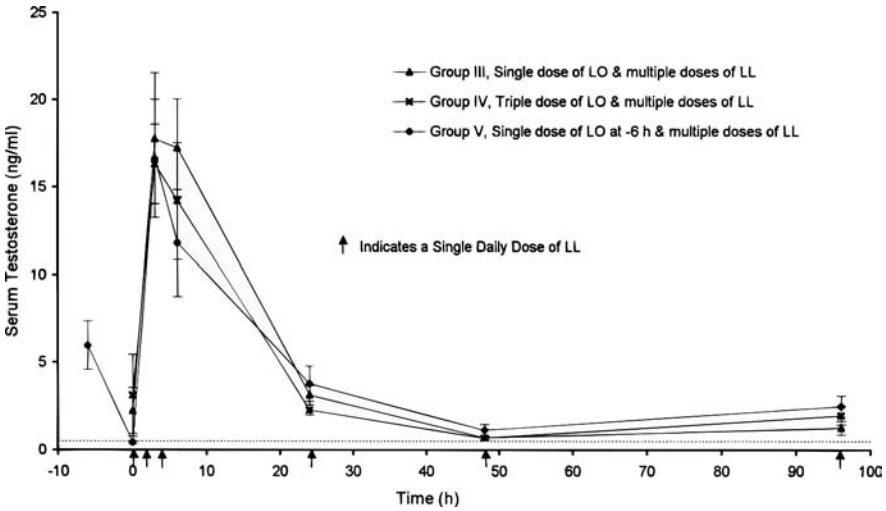

Fig. 2. Testosterone levels after administration of three different combinations of liquid Orntide (LO) and liquid Leuprolide (LL) in rats. Broken line at 0.5 ng/mL designates castration level.

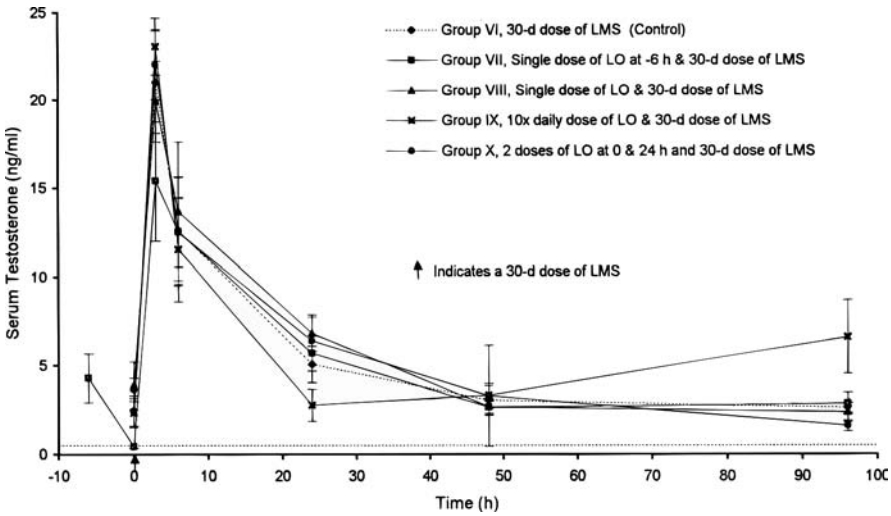

Fig. 3. Testosterone levels after administration of four different combinations of liquid Orntide (LO) and Leuprolide microspheres (LMS) in rats. Broken line at 0.5 ng/mL designates castration level.

These results show that continuous administration of Orntide produces an instant and profound suppression of testosterone. However, earlier administration of Orntide microspheres does not prevent LHRH receptors stimulation upon injection of Leuprolide microspheres. The second elevation in testosterone levels seen in Group XIII may be caused by a competition between Orntide and Leuprolide for the LHRH receptors and by temporary protection of these receptors by Orntide from a desensitizing effect of Leuprolide.

DISCUSSION

The animal data show substantial initial increase in testosterone levels in rats after administration of Leuprolide acetate. Testosterone peaks were observed in animals that received either subcutaneous injections of liquid Leuprolide or slowly releasing microspheres containing a 30-day dose of Leuprolide (Figs. 1, 3, and 4). In both cases the initial transient testosterone elevation was followed by testosterone suppression.

Injection of a potent LHRH superagonist with much higher affinity for LHRH receptors than that of native LHRH causes immediate release of all LH stored in gonadotrophs and stimulates the cells to intensified production and release of new LH. This results in a surge of LH that in turn stimulates testicular production and release of testosterone. If a sufficient blood level of Leuprolide is maintained for several days or weeks (daily injections of LL or application of LMS) then testosterone suppression is observed. This is caused by desensitization of pituitary LHRH receptors due to prolonged and continuous exposure to LHRH superagonist. Under normal conditions native LHRH is released from the hypothalamus in a pulsatile manner thus giving gonadotrophs enough time to recover and prepare for the next pulse of LHRH. However, if superagonist

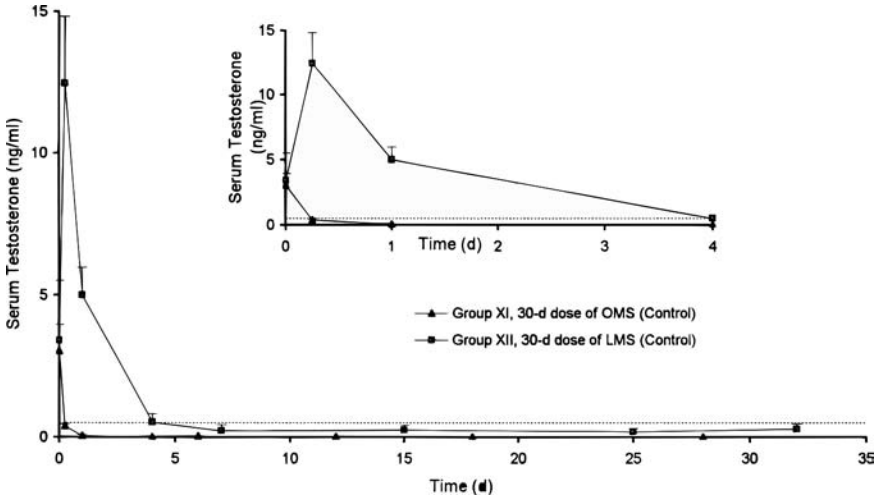

Fig. 4. Testosterone levels after administration of Orntide microspheres (OMS) and Leuprolide microspheres (LMS) in rats. The insert captures the first four days to better illustrate the testosterone rise with Leuprolide. Broken line at 0.5 ng/mL designates castration level.

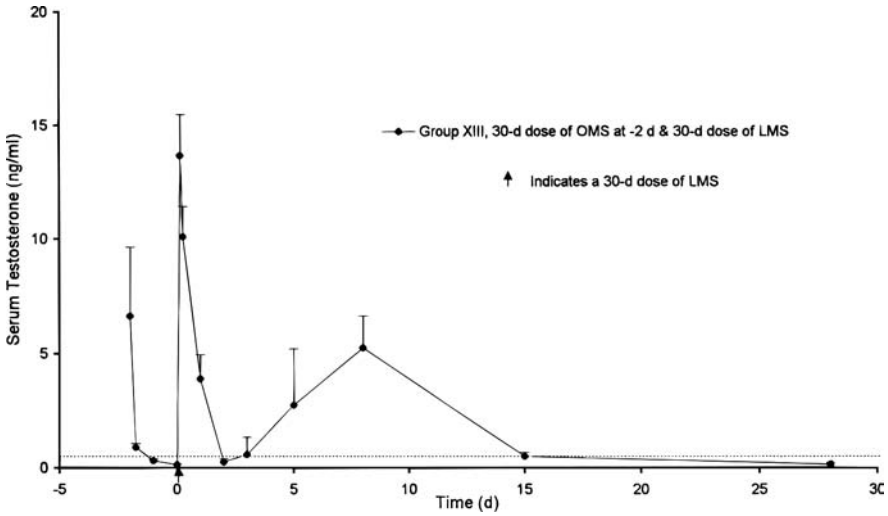

Fig. 5. Testosterone levels after administration of a combination of Orntide microspheres (OMS) and Leuprolide microspheres (LMS) in rats. Broken line at 0.5 ng/mL designates castration level.

occupies LHRH receptor sites in the pituitary for an extended period then a series of events leading to receptor desensitization is observed including reduction in the number of surface receptors, uncoupling of the receptors from intracellular mediators, and postreceptor inactivation (15,17–19). Finally, instead of active LH, gonadotrophs start to produce and release inactive form of LH unable to stimulate production of testosterone in testes.

Injection of Orntide acetate caused immediate testosterone suppression in rats (Fig. 1). As shown in *Table I*, the initial area under the testosterone curve was significantly smaller for Group I than for Group II. This behavior was expected since LHRH antagonists do not stimulate gonadotrophic receptors but simply block them and prevent activation by native LHRH (15).

Combined administration of liquid Leuprolide and liquid Orntide did not have an effect on the initial testosterone elevation (Fig. 2, Table I). This suggests that Leuprolide acetate may have higher affinity to the LHRH receptors and may compete with Orntide for the binding sites. Administration of a single daily dose of Orntide acetate caused, as expected, immediate decrease of testosterone level (Group V). However, administration of LL 6 hours later resulted in testosterone elevation. This suggests that Leuprolide may be able to displace Orntide from the receptor sites. Furthermore, these results indicate that binding of Orntide to the LHRH receptors does not cause desensitization of gonadotrophs and upon stimulation by LHRH agonist, gonadotrophs start to function normally.

A similar scenario was observed when injections of LO were combined with application of LMS. Again, both simultaneous injections of LO and LMS and administration of LO 6 hours before LMS did not eliminate the initial testosterone elevation (Fig. 3) and there was no significant difference in the AUCs for Groups VII–X when compared to Group VI (Table I).

An interesting response was observed when a combination of Orntide MS and Leuprolide MS was used. As shown in Fig. 5, injection of OMS resulted in complete castration within 2 days to levels below 0.5 ng/ml. Administration of LMS 48 hours later resulted in the elevation of the testosterone level and although the increase was not as high as in the control Group XII the difference was not statistically significant (Table I). This is consistent with the response observed in the previous groups. However, in the previous cases, only liquid Orntide injections were used with combination of either multiple injections of LL or single injection of LMS. In contrast to that, administration of OMS into animals in Group XIII assured that Orntide was continuously present in the system. In this case longer competition between Orntide and Leuprolide for the receptor sites resulted in a second testosterone peak between days 3 and 15. This suggests that by binding to LHRH receptors Orntide not only causes their blockage but also protects them

Table I. Response Areas Under Testosterone Curves During 0–48 h of Administration of Leuprolide In Rats

Group	Dosing	Mean AUC (ng d/mL)
I	Single LO injection at 0 h (Control 1)	10 ± 5[a,b]
II	Multiple LL injections (Control 2)	297 ± 42
III	Single LO inj. at 0 h and multiple LL inj.	313 ± 37
IV	Triple LO inj. at 0 h and multiple LL inj.	260 ± 36
V	Single LO inj. at −6 h and multiple LL inj.	268 ± 37
VI	Single inj. of a 30-d dose of LMS (Control 3)	344 ± 36
VII	Single LO inj. at −6 h & 300-d dose of LMS at 0 h	330 ± 61
VIII	Single LO inj. at 0 h & 30-d dose of LMS at 0 h	385 ± 61
IX	Ten times daily inj. of LO at 0 h & 30-d dose of LMS at 0 h	291 ± 46
X	Two inj. of LO at 0 and 24 h & 30-d dose of LMS at 0 h	375 ± 32
XI	30-d dose of OMS (Control 4)	0.7 ± 0.2[c]
XII	30-d dose of LMS (Control 5)	347 ± 38
XIII	Inj. of OMS at −2 d & LMS at 0 d	247 ± 33

[a] AUC calculated for 0–6 h time interval.
[b] $P < 0.01$ vs. Group II.
[c] $P < 0.01$ vs. Group XII.

from desensitization by Leuprolide and thus a complete castration of animals in Group XIII could not be achieved during the initial 15 days. After that testosterone levels remained below the castration level indicating that the release of active LH from the pituitary gland was inhibited due to a complete downregulation of the LHRH receptors and exhaustion of stored active LH in gonadotrophs.

Our results suggest that Orntide acetate does not produce desensitization of gonadotrophic LHRH receptors in rats at the doses used in our studies. This finding is consistent with results obtained previously by other researchers (17), however desensitization of gonadotrophic LHRH receptors chronically exposed to higher doses of LHRH antagonists has been reported in the literature (18,19). These reports may indicate that desensitization of gonadotrophic LHRH receptors is dependent upon the dose and the exposure time to a LHRH antagonist.

It is also clear that concurrent administration of liquid Orntide with Leuprolide microspheres does not eliminate or significantly reduce the initial testosterone peak and therefore probably has no therapeutic advantage over the Leuprolide monotherapy. However, as shown in Table IV, the area under the testosterone curve after administration of liquid Orntide (Group I) or Orntide microspheres (Group XI) was overwhelmingly smaller when compared with the area under the testosterone curve produced by Leuprolide microspheres (Groups VI and XII). These data suggest that Orntide acetate is an effective LHRH antagonist and that it may be a good candidate for further development as an antigonadotropic agent for the treatment of sex hormone-dependent diseases where an immediate and profound testosterone suppression would be desired.

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