# In-vitro/in-vivo studies of the biodegradable poly-(*d,l*-lactide-co-glycolide) microspheres of a novel luteinizing hormone-releasing hormone antagonist for prostate cancer treatment

Lina Du[a,b], Xingguo Mei[b], Chenyun Wang[c], Xin Li[c], Fucheng Zhang[d] and Yiguang Jin[a]

**The introduction of luteinizing hormone-releasing hormone (LHRH) analogs and their antagonists is revolutionizing the treatment of prostate cancer. In this study, poly(*d,l*-lactide-*co*-glycolide) (PLGA) microspheres containing a highly potent LHRH antagonist (LXT-101) of interest in the indication of prostate cancer were evaluated on release mechanisms *in vitro* and biological performance *in vivo*. LXT-101 microspheres were prepared by the water/oil/water double emulsion method and the solid/oil/oil method. The results showed that the mechanism of LXT-101 releasing from PLGA 14 000 microspheres was the cooperation of drug diffusion and polymer degradation. This clarified the relationship between the microsphere characterization and hormone level *in vivo*. The larger microspheres (33 μm) could inhibit the testosterone level to castration for a longer time (35 days) than the smaller microspheres (15 μm, 14 days). The formulation containing the hydrophilic additive (polyethylene glycol 6000) could suppress the testosterone level to castration for a longer time (>35 days) than the formulation without polyethylene glycol (14 days). The appearance of testis, vesicular seminalis, and prostates changed after treatment. The weights of sexual organs decreased significantly. The in-vivo release of the LXT-101 PLGA 14 000 microspheres curve showed that in-vivo release started immediately after day 1 (22.7%) and was rapid during the first 5 days (40.2% release). The LXT-101 microspheres could be a promising drug delivery system candidate to treat sex hormone-dependent tumors and other related disorders. *Anti-Cancer Drugs* 22:262–272 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

**Anti-Cancer Drugs** 2011, **22**:262–272

**Keywords: anticancer, luteinizing hormone-releasing hormone antagonist, microspheres, poly(*d,l*-lactide-*co*-glycolide), testosterone**

[a]Department of Pharmaceutical Chemistry, Beijing Institute of Radiation Medicine, [b]Beijing Institute of Pharmacology and Toxicology, [c]General Hospital of PLA and [d]General Hospital of Airforce, Beijing, China

Correspondence to Yiguang Jin, PhD, Department of Pharmaceutical Chemistry, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, China
Tel: +86 10 88215159; fax: +86 10 68214653;
e-mail: jinyg@nic.bmi.ac.cn

**Received** 5 October 2010 **Revised form accepted** 7 November 2010

## Introduction

In the 1980s, luteinizing hormone-releasing hormone (LHRH) agonists were introduced, and revolutionized the treatment of advanced prostate cancer. The two most common drugs among LHRH agonists are leuprolide and goserelin. A potential problem with the LHRH 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, eventually causing severe side effects such as bone pain, urinary obstruction, cord compression, and cardiovascular effects. This testosterone surge can result in a transient increase in prostate cancer growth, known as the flare phenomenon [1].

In comparison with LHRH agonists, LHRH antagonists offer a more advantageous option. The major advantage of LHRH antagonists such as cetrorelix and abarelix over agonists is that they avoid the flare phenomenon [2]. Moreover, other potential advantages include the fact that there is no need for combination therapy with an antiandrogen, simplification of management by removing the need to educate patients about antiandrogen use, and more targeted therapy (with a direct effect on tumor cells). To date, however, only a few LHRH antagonists such as cetrorelix [3–5], abarelix [6–8], and ganirelix [9,10] are available on market. Cetrorelix and ganirelix are used in assisted reproduction and premature puberty, and abarelix is used for treating prostate cancer. The development of LHRH antagonists lags behind that of the agonists. Therefore, there are still more requirements for the development of potent LHRH antagonists for the treatment of variable hormone-related diseases. The third generation of LHRH 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 LHRH for the receptor. In addition, their rapid action and low anaphylactic reactions have led to an increasing need in clinical applications.

A decapeptide of 1412 Da, LXT-101 (Ac-D-Nal[1]-D-Cpa[2]-D-Phe[3]-Ser[4]-Arg[5]-D- Pal[6]-Leu[7]-Arg[8]-Pro[9]-D-Ala[10]-$NH_2$), as the third generation of LHRH antagonist, was

0959-4973 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e3283425c2a

developed in our laboratory. In comparison with other antagonists, LXT-101 shows a lower potential to release histamine, which hampers the use of the first generation of LHRH antagonists. However, because of its short plasma half-life (approximately 4 h), LXT-101 has to be administered parenterally at least once a day, and this is undesirable for long-term treatment [11]. Moreover, LXT-101 as a novel peptide passes poorly through biological barriers because of their poor diffusivity and low partition coefficient [12]. As a result, an effective and suitable sustained-release system of LXT-101 was a key issue for a promising new antiprostate cancer drug. Peptide encapsulation within biodegradable polymer microspheres offers important opportunities for the protection and sustained release of these labile molecules [13–16]. Aliphatic polyesters such as poly(lactide) (PLA), poly(glycolide) (PGA), and especially copolymers of lactide and glycolide-poly (poly(*d,l*-lactide-*co*-glycolide) (PLGA) probably constitute the most commonly used family of bioerodible polymers for microspheres at present [17–20]. The advantages of the microsphere delivery system based on PLGA are its long duration of action, the ease of its administration through regular intramuscular or subcutaneous injections, and its biodegradability. Moreover, the biodegradable microspheres could provide a relatively constant rate of drug release, thus improving the efficacy and reducing the side effects. In addition, the microspheres of LHRH agonists are already in the market (e.g. Lupron, Decapeptyl), whereas LHRH antagonist microspheres have not reached this stage [21–23]. Recently, however, the microgranule of LHRH antagonist teverelix was investigated in a dog model for efficacy in terms of testosterone suppression and provided an encouraging 80-day chemical castration [24]. On the basis of the above analysis, the development of sustained-release biodegradable microsphere injections for peptide delivery will help LXT-101 to enhance patient compliance and convenience. As an effective antiprostate cancer sustained release system, it should release sufficient drugs to inhibit the testosterone level to castration and release a certain amount of drugs continuously for inhibition. However, because there are more synthesized amino acids in the structure, it is much less soluble. This is disadvantageous for the microsphere preparation.

This study provides a feasible method for the PLGA microsphere preparation and documents the release mechanisms *in vitro* and biological performance (including the testosterone level *in vivo*, pharmacokinetics, histological alterations, appearances, and weight changes in sexual organs) of LXT-101 microspheres *in vivo*. The factors mentioned above may be important for the therapeutic effect of LXT-101 microspheres.

## Materials and methods

### Materials

LXT-101 was synthesized in our laboratory and confirmed in structure. PLGA [molecular weight (MW), 14 and 36 kDa] was supplied by the Institute of Chemistry, Nankai University, China. Polyvinyl alcohol (PVA, 88% hydrolyzed, MW = 130 kDa), and all salts (NaCl, $KH_2PO_4$, $K_2HPO_4$) were purchased from Beijing Chemical Reagent Co. (Beijing, China). All other chemicals were obtained commercially as analytical grade reagents.

### Animals

Male Sprague–Dawley rats (0.20–0.25 kg) from Laboratory Animal Center of Beijing Institute of Pharmacology and Toxicology were 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.

### Preparation of LXT-101 microspheres

PLGA-microspheres of LXT-101 were obtained by the double-emulsion solvent evaporation (water/oil/water, W/O/W) method in the presence of PVA as described earlier [25], or by the solid/oil/oil (S/O/O) method. The details of the process are as follows.

#### *Water/oil/water method*

In brief, LXT-101 PLGA-microspheres were produced by homogenization with a tissue mixer (Model 985370; Cole Parmer Instrument Co., Vernon Hills, Illinois, USA) [25]. The W/O emulsion formed was then slowly injected into an aqueous PVA solution. The resultant W/O/W emulsion was transferred into another PVA solution containing NaCl. After removing organic solvents, the hardened microspheres were centrifuged and collected. The microspheres were screened using a 200-mesh sieve before size measurement, the in-vitro release experiment, and in-vivo administration to animals.

#### *Solid/oil/oil method*

The drug powder was first micronized. The micronization of LXT-101 using amphiphilic polymers was conducted as described earlier with modifications. In brief, LXT-101 was added into a glass test tube with polyethylene glycol (PEG) 20 000 solution. The mixtures were frozen on a precooled shelf at −50°C in a freeze-dryer (a LGJ-18 freeze-dry system; Beijing Sihuan Scientific Instrument Works, Beijing, China) and then dried at −20°C for 3 h and at 20°C for 12 h in turn. The lyophilized white solid was first dispersed in methylene chloride to dissolve PEG 20 000 and then centrifuged to remove the amphiphilic polymer. The obtained lyophilized product was dispersed in acetonitrile to obtain LXT-101 suspension. PLGA was added to the suspension to form an organic phase. The suspension was added into 20 ml cottonseed oil containing Span 85 (Fisher, Waltham, Massachusetts, USA) and homogenized with a propeller stirrer. Petroleum ether (30 ml, boiling point 60–90°C) was added into the cottonseed oil to extract acetonitrile. The hardened microspheres were collected through centrifuging and

washed with an excess amount of petroleum ether and finally freeze-dried. The microspheres were screened in the same manner as the W/O/W microspheres.

### Particle size

The freeze-dried microspheres were suspended in water containing 0.02% (w/v) Tween 20 (Sigma, St Louis, Missouri, USA), and bath-sonicated for 3 min to disperse the microspheres before measurement. The volume mean diameter $V_{md}$ of microspheres was investigated on a laser light scattering instrument (LS800 Particle Size Analyzer, Omec Instruments, Zhuhai, China).

### Surface morphology

A layer of freeze-dried microsphere powder without screening was adhered on copper nets using adhesive tapes. A thin layer of gold powder was vacuum-coated on the nets, which were then observed on a scanning electron microscope (Model JSM-5600LV; JEOL, Tokyo, Japan) at 15 kV.

### Determination of peptide content

LXT-101 was determined by high-performance liquid chromatography (HPLC) (Hitachi LC equipment, column Kromasil 100 Å, 250 × 4.6 mm; Dikma Technologies, China; mobile phase: acetonitrile:water 48 : 52, 0.1% (v/v) trifluoroacetic acid, 0.2% (v/v) triethylamine) and particle size measurements were made by laser light scattering (a LS 800 Particle size analyzer; Omec Instruments, Zhuhai, China). Surface morphologies were examined by a scanning electron microscope (Model JSM-5600LV; JEOL).

### In-vitro release of LXT-101 from microspheres

The phosphate buffer solution (10 mmol/l, 2 ml, pH 7.4), containing 0.02% Tween 80 and 0.02% Tween 20 (Amresco, Solon, Ohio, USA), was added into a 5-ml tube, and then the drug-loaded microspheres (10 mg) were added into the tube and suspended thoroughly. The tube was placed in a 50°C water bath and shaken at 160 rpm speed horizontally. At predetermined time intervals, the tube was centrifuged at 3500 rpm for 10 min, a 2-ml supernatant was withdrawn for determination, and the equal volume of fresh phosphate buffer was re-added. The released LXT-101 was assayed by the spectrophotometry on a fluorescence spectrometer (F4500; Hitachi, Japan) at excitation wavelength, $E_x$ 280 nm and emission wavelength, $E_m$ 335 nm. The tests were carried out in triplicate.

### Detection of hormone level of animals

Male Sprague–Dawley rats (0.20–0.25 kg, $n = 5$) were used to evaluate the in-vivo performance of LXT-101 microspheres prepared by the W/O/W and S/O/O techniques. The microspheres were subcutaneously injected with different doses of LXT-101 at the left legs of rats after reconstitution with a suitable medium (0.5% carboxymethylcellulose and 5% mannitol, w/v). The rats administered with the equal volume of blank reconstituted medium were used as controls. The blood sample (0.5 ml) was collected by puncture of the ophthalmic venous plexus with the microhematocrit tubes in Eppendorf tubes at predetermined time intervals. The samples were kept at 4°C for 4 h followed by centrifuge at 3000 rpm for 10 min, and then stored at −20°C until hormone determination.

The total serum testosterone levels were measured with the specific immunochemiluminescence assay with the Access Testosterone Immunoassay Kit (Beckmann-Coulter Inc., California, USA). The access testosterone assay is a paramagnetic particle, a chemiluminescent immunoassay, and also a competitive binding immunoenzymatic assay. A sample was added to a reaction vessel along with the sample treatment solution, the mouse monoclonal antitestosterone antibody, the testosterone alkaline phosphatase conjugate, and the paramagnetic particles coated with the goat antimouse polyclonal antibody. Testosterone in the sample was released from the carrier proteins by the sample treatment solution and competed with the antitestosterone monoclonal antibody. The resulting antigen–antibody complexes were then bound to the solid phase by the capture antibody. After incubation in a reaction vessel, separation in a magnetic field and washing removed materials not binding to the solid phase. A chemiluminescent substrate, Lumi-Phos 530, was added to the reaction vessel, and light generated from the reaction was measured with a luminometer. The light production was determined from a stored, multipoint calibration curve. The lower limit of detection and the highest calibrator value were 0.1 and 16 ng/ml, respectively. The intra-assay and interassay coefficients of variation were 10 and 9%, respectively.

### Histological assessment, appearance, and weight changes in sexual organs

The W/O/W microspheres were subcutaneously injected into the back of the rat with a dose of 15 mg LXT-101/kg. After 4 months administration, the sexual organs of ethically killed rats were isolated, including testis, prostate glands, and seminal vesicles. Testes were rinsed with a saline solution, fixed with 10% neutral carbonate buffered formaldehyde, embedded in paraffin using an embedding center, and cut into slices. The slices were stained with hematoxylin and eosin and observed under a light microscope (CK30/CK40 Olympus, Japan). The three kinds of glands were weighed. The rats administered with the equal volume of the blank reconstituted medium were used as controls. Results for serum concentrations of testosterone (ng/ml) and the weight of both sex organs and body (g) were the means ± SEM.

### Pharmacokinetic investigation

Eighteen male Sprague–Dawley rats (160–200 g) were used with a dose of 7.5 mg LXT-101/kg. The microspheres

were suspended in 0.9% saline solution (0.5 ml) containing 2% (w/w) sodium carboxymethylcellulose and 0.2% (w/w) Tween 20. The suspension was injected into the quadriceps muscles of rats using a 23-gauge needle. The residual amount of microspheres in the syringe and needle was measured and subtracted from the injection dose to obtain the actual dose of injection. The actual dose of microspheres was 7.02 mg LXT-101/kg.

Microspheres tend to agglomerate and form bolus after intramuscular injection, so that the drug in-vivo release profile may be obtained by determining the residual LXT-101. The rats were ethically killed at 1, 3, 5, 10, 21, and 28 days after injection, and the epidermis was split carefully. The microsphere bolus and its surrounding muscles were cut down and sheared into small pieces that were mixed with 2.5 ml acetonitrile/water (9:1, v/v) to homogenate for 10 min. The formed homogenate was transferred into a 10-ml volumetric flask, and diluted with $KH_2PO_4$ solution (0.184 mol/l, containing 0.02% Tween 20) to obtain the suspension. The suspension was centrifuged at 3000 rpm for 10 min, and the supernatant was filtered with a 0.22-μm filter. The filtrate (20 μl) was injected into HPLC. The tests were carried out in triplicate. The limit of quantity of the method was 1.002 μg/ml, the average interday and intraday precision was 5.5%, and the average extraction recovery was higher than 85% for the method.

### Statistical analysis

Statistically significant difference between individual groups was determined using a one-way analysis of variance with a Student's *t*-test. All testing was done using the SPSS 16.0 (SPSS Inc., Somers, New York, USA).

## Results and discussion

### Release mechanism of LXT-101 from microspheres

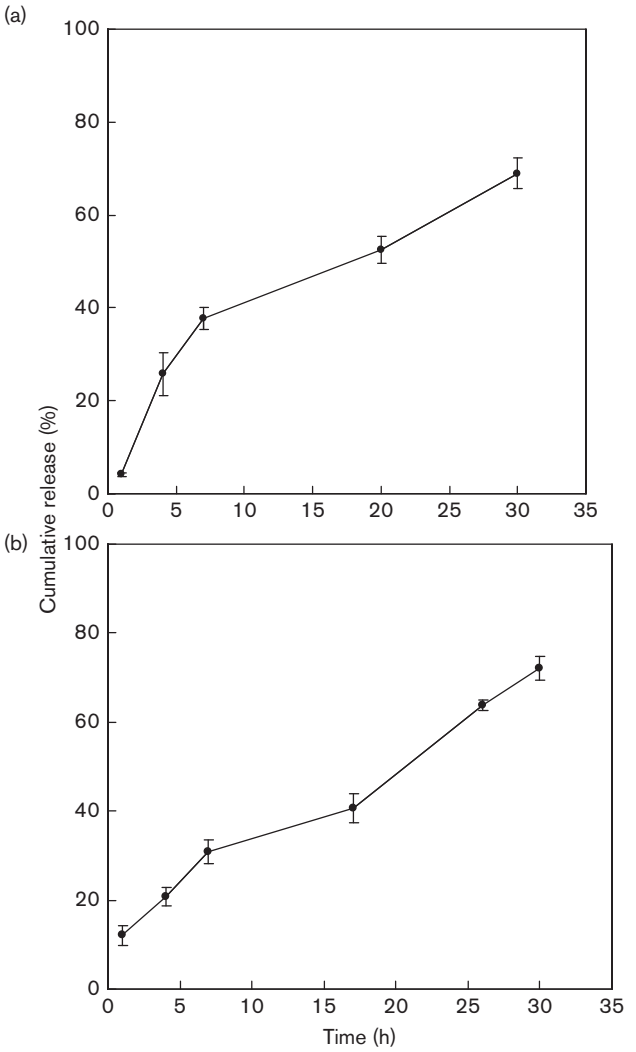
The burst drug release was 30–50% for most of the microspheres prepared by the emulsion methods [26,27]. From the in-vitro profiles of LXT-101 microspheres (Fig. 1), it was noted that the burst release was low. The probable reason may be most of drugs distributing in the inner water phase at the initial process, and water could migrate to the outer surface when drying.

We also used some release models to describe the in-vitro release behavior, including zero order, first order, Higuchi equation, and the Ritger–Peppas exponent equation. The drug release mechanism could be deduced based on the equations, possibly involving diffusion and degradation, or the mixed mechanism of two factors. The model equations were as follows:

Zero-order model

$$y = k_{ro}t \tag{1}$$

**Fig. 1**




In-vitro release profiles of luteinizing hormone-releasing hormone antagonist, LXT-101, from (a) the poly(*d,l*-lactide-*CO*-glycolide) (PLGA) 36 000 microspheres and (b) the PLGA 14 000 microspheres, $n=3$.

In the equation above, $y$ was the cumulative release percentage, $k_{ro}$ zero release rate constant, and $t$ time.

First-order model

$$\log(y_\infty - y) = \frac{-k_{r1}t}{2.303} + \log M \tag{2}$$

In the equation above, $y_\infty$ was the maximum cumulative release percentage, $k_{r1}$ first release rate constant, $t$ time, and $M$ a constant.

Higuchi-order model

$$Q = k_H t^{1/2} \tag{3}$$

In the equation above, $Q$ was the cumulative release percentage, $k_H$ Higuchi release rate constant, and $t$ time.

Ritger–Peppas exponent model

$$F(t) = kt^n \quad (4)$$

In the equation above, $F(t)$ was the cumulative release percentage at a certain time point, $k$ a constant, $t$ time, and $n$ the diffusion index.

The W/O/W microspheres were prepared from two different PLGAs (MW 14, 36 kDa), and the in-vitro release profiles were consistent with the Higuchi equation (Tables 1 and 2), indicating the diffusion mechanism of microspheres. Meanwhile, the different values of $n$ in the Ritger–Peppas exponent model represented the different release mechanisms (Table 3). $n = 0.43$ stands for Fick's diffusion and $n = 0.85$ stands for the dissolution controlling mechanism. The value of $n$ between 0.43 and 0.85 showed that the release mechanism was the cooperation of Fick's diffusion and dissolution mechanism. In this study, the representative parameter $n$ was between 0.43 and 0.85, suggesting that the mixed mechanism occurred, involving the cooperation of drug diffusion and polymer degradation.

The drug release mechanism of biodegradable microspheres may be classified into two types: diffusion-controlling release and degradation-controlling release.

**Table 1 The models describing the in-vitro release profiles of the drug-loaded poly(*d,l*-lactide-*CO*-glycolide) 36 000 microspheres**

| Models | Model equation | Correlation coefficient ($R^2$) |
|---|---|---|
| Zero-order model | $Y = 1.9203t + 14.012$ | 0.8910 |
| First-order model | $Y = -0.0347t - 0.1154$ | 0.9585 |
| Higuchi-order model | $Y = 13.36t^{1/2} - 3.8453$ | 0.9646 |
| Ritger–Peppas exponent model | $Y = 5.8393t^{0.7874}$ | 0.9076 |

**Table 2 The models describing the in-vitro release profiles of the drug-loaded poly(*d,l*-lactide-*CO*-glycolide) 14 000 microspheres**

| Fitting model | Model equation | Correlation coefficient ($R^2$) |
|---|---|---|
| Zero-order model | $Y = 2.0124t + 13.003$ | 0.9749 |
| First-order model | $Y = -0.0391t - 0.0704$ | 0.9649 |
| Higuchi-order model | $Y = 13.589t^{1/2} - 4.5674$ | 0.9794 |
| Ritger–Peppas exponent model | $Y = 11.15t^{0.5158}$ | 0.9737 |

**Table 3 The diffusion index (*n*) and release mechanism from the various controlled release systems**

| Diffusion exponent ($n$) | | | |
|---|---|---|---|
| Film | Cylinder | Sphere | Mechanism |
| 0.50 | 0.45 | 0.43 | Fick's |
| 0.5–1.0 | 0.45–0.89 | 0.43–0.85 | Non-Fick's |
| 1.0 | 0.89 | 0.85 | Zero-order (Case-II transport) |

In the diffusion-controlling release, the release rate was slow at first. However, the corrosion of biodegradable polymers would lead to increasingly fast release rates. In most cases, drug release from the microspheres was the cooperation of the two mechanisms mentioned earlier. In fact, drug distribution in microspheres may determine the release behavior. Generally, the drug absorbing or attaching to the microsphere surface tends to 'burst release'. The sequentially formed channels might help the medium enter the microsphere interior phase. Moreover, the interior porosity structure of microspheres provides many tunnels for drug diffusion and medium ingression. Thus, the swelling and biodegradation rate of polymers was improved. Therefore, the release mechanism of LXT-101 from PLGA microspheres should be the cooperation of drug diffusion and polymer degradation. PLGA of higher molecular weight would be degraded more slowly than that of lower molecular weight. The microsphere structure prepared with the former could be more compact, preventing water entry. Therefore, the release of LXT-101 from the PLGA 36 000 microspheres was slower than that from the PLGA 14 000 microspheres. A similar result was also reported elsewhere [28]. PLGA microspheres are degraded through the hydrolysis of ester bonds of the polymer by means of so-called zipping and unzipping processes. The product of low molecular weight liberated from the polymer may further accelerate microspheres degradation. PLGA of high molecular weight is highly crystallized, and has a highly rigid hydration structure on the microspheres surface, so that the degradation rate is reduced [29].

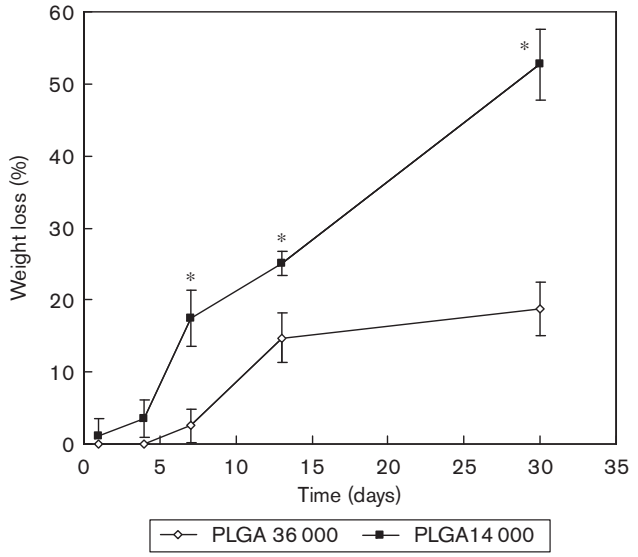
The PLGA 14 000 microspheres showed a significant weight loss in day 7 (Fig. 2). In addition, the percentage of weight loss further increased with time, indicating that the drug release mainly depended on diffusion before day 7. After 7 days, diffusion and degradation cooperated with the erosion and degradation of PLGA 14 000.

### Effect of LXT-101 microspheres on the in-vivo hormone level

Androgen as a steroid hormone is secreted by the interstitial cells of the testis. Testosterone is the major natural androgen, promoting the development of male animals' accessory sex organs and facilitating the formation of the secondary sexual characteristics. Hormone-dependent prostate cancer is an androgen-sensitive cancer. There is a definite cause and effect correlation between androgen and prostate cancer. The incidence of prostate cancer in populations with a higher androgen levels is much higher than that of populations with lower androgen levels. We selected the in-vivo testosterone level as an assessment factor to evaluate the pharmacodynamic action of LXT-101 microspheres.

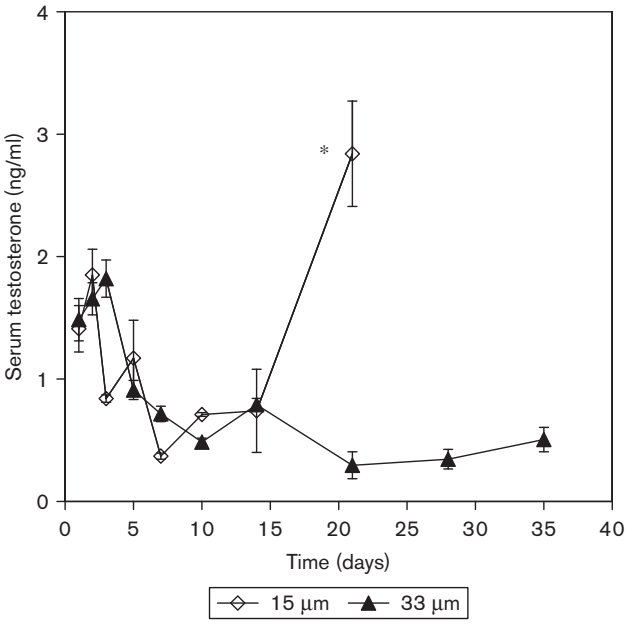
LXT-101 decreased the sex hormone level immediately without inducing hormone flare-up after a single subcutaneous injection of LXT-101 microspheres. A low dose

**Fig. 2**




Weight loss of luteinizing hormone-releasing hormone antagonist, LXT-101, microspheres during incubation in phosphate-buffered solutions (50 ml, pH 7.4), $n=3$. *$P<0.01$.

**Fig. 3**




The effects of microsphere size on the serum testosterone level of rats, $n=3$. *$P<0.01$.

of 3 mg/kg also led to a dramatic fall in testosterone to castration levels for 14 days. Therefore, LXT-101 is a super LHRH antagonist, and has the capacity to regulate the pituitary–gonadal axis quickly and reversibly. It is shown again that LHRH antagonists have advantages compared with the agonists [30]. The following sections discuss the effects of different factors on the microspheres.

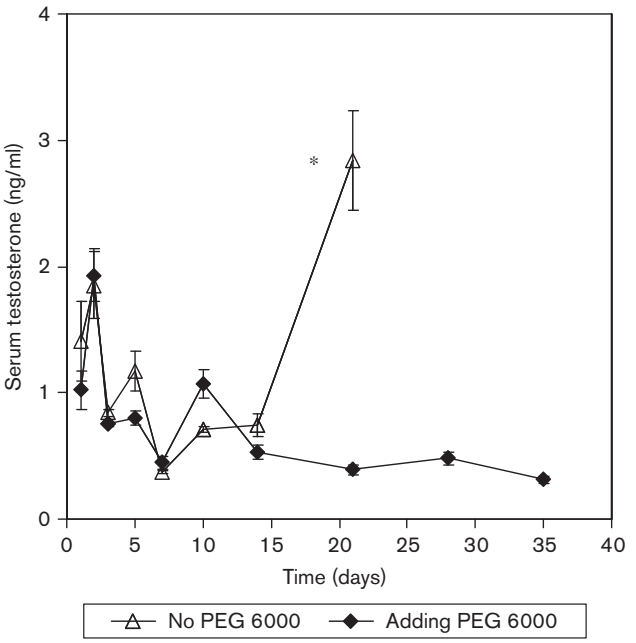
### *Effect of the size of LXT-101 microspheres on the in-vivo hormone level*

The large microspheres (33 μm) inhibited the testosterone level to castration for a long time (35 days). However, the small microspheres (15 μm) prepared with the same formulation and technique inhibited the testosterone level to castration for only approximately 14 days (Fig. 3). It was deduced that the drug release rate increased for the small microspheres because the surface area/volume ratio of the smaller microspheres was relatively higher than that of the larger microspheres. Therefore, the release of drug could not be maintained for a long time for the small microspheres.

### *Effect of the addition of hydrophilic excipient-polyethylene glycol 6000 on the in-vivo hormone level*

The microspheres containing the hydrophilic additive-PEG 6000 suppressed the testosterone level to castration for a long time (> 35 days). The microspheres without PEG 6000 only maintained castration for approximately 14 days (Fig. 4). The hydrophilic PEG 6000 could precipitate the hydrophobic LXT-101 and make its crystalline density high in the microspheres, decreasing the release rate of LXT-101 and favoring sustained release.

**Fig. 4**

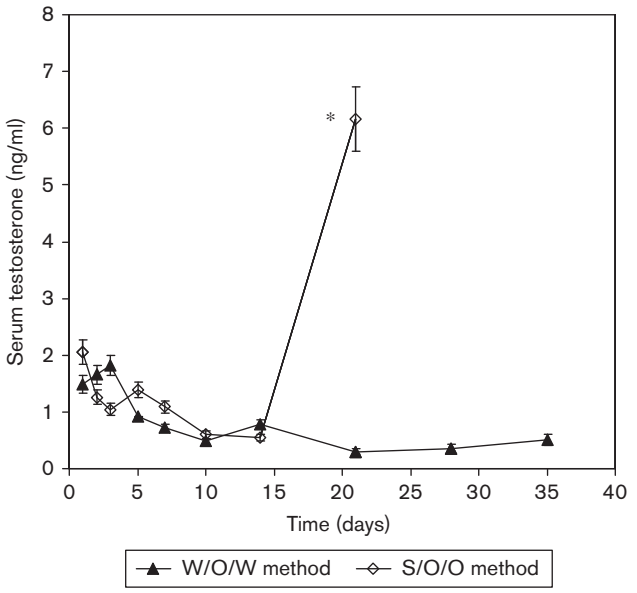



The effects of hydrophilic additives polyethylene glycol (PEG) 6000 on the serum testosterone of rats, $n=3$. *$P<0.01$.

### *Effect of various preparation methods on the in-vivo hormone level*

The W/O/W microspheres continuously suppressed the testosterone level to castration for more than 35 days. However, the testosterone-suppressing effect of S/O/O microspheres merely maintained for approximately 14 days (Fig. 5). Therefore, the preparation methods had a great effect on the release behavior of LXT-101 from the microspheres. For the W/O/W microspheres, a clear homogeneous solution of polymers and drugs was used, and the drugs tended to homogeneously distribute in the PLGA matrix. However, for the S/O/O microspheres, parts of the LXT-101 particles could be absorbed on the surface of microspheres, although most of them could be encapsulated within the microspheres. This led to an initial burst release of LXT-101 from the S/O/O microspheres, and the remaining LXT-101 were not enough to suppress the testosterone level for the same long period of time as the W/O/W microspheres. Furthermore, the effect of preparation methods was also seen from the surface morphologies of microspheres. The W/O/W microspheres showed a smooth surface, whereas the S/O/O microspheres had a coarse surface because of the preparation process (Fig. 6). The drug may be tightly encapsulated in the inner space of W/O/W microspheres, resulting in the well sustained release of drug. However, the drug could release rapidly from the S/O/O microspheres because the possible formed holes on the surface provided a route of drug diffusion.

**Fig. 5**

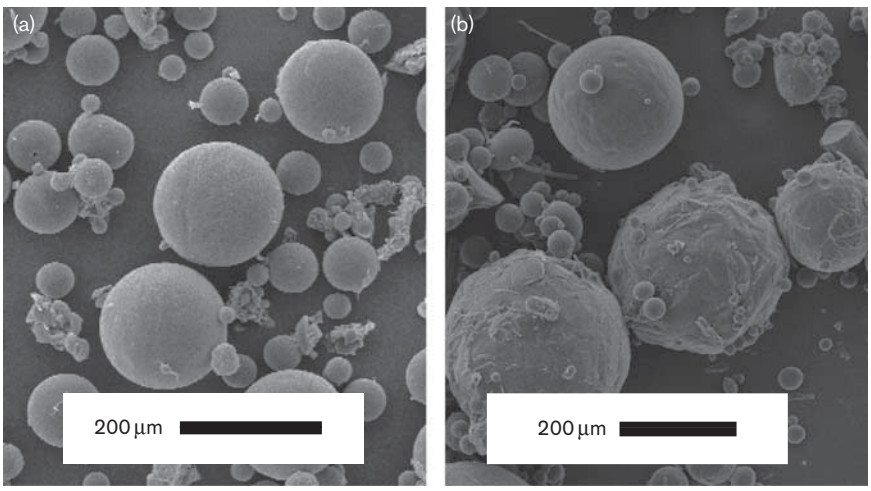



The effects of different preparation methods on the serum testosterone of rats, $n=3$. *$P<0.01$. S/O/O, solid/oil/oil; W/O/W, water/oil/water.

### Therapeutic action of LXT-101 microspheres based on histological assessment, appearance, and weight changes in sexual organs

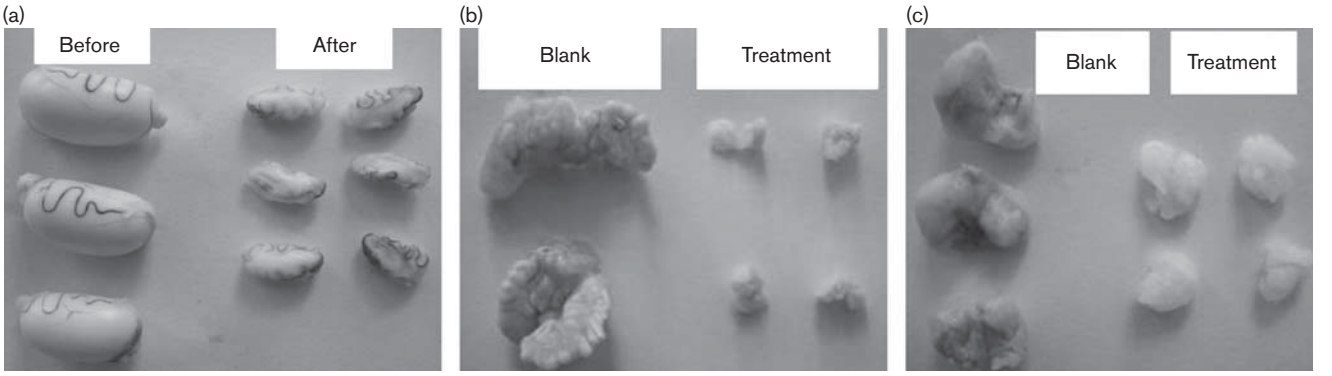
The appearance of three sexual organs, the testis, vesicular seminalis, and prostates, showed obvious changes after treatment using LXT-101 microspheres (Fig. 7). After administration for 4 months, all of three main sexual organs had shrunk. However, the global appearance did not change significantly. The testes of blank control rats were smooth ellipses, and there was a piece of transparent compact connective tissue encapsulating the testis, that is, tunica albuginea testis. The gubernaculums testis near the caudal end was distinct and clear. After treatment for 4 months, the rat testis was wrinkled and

**Fig. 6**




Scanning electron microscopic images of (a) the water/oil/water microspheres and (b) the solid/oil/oil microspheres.

**Fig. 7**




Morphologies of sexual organs of rats, including (a) testes; (b) vesicula seminalis; (c) prostates.

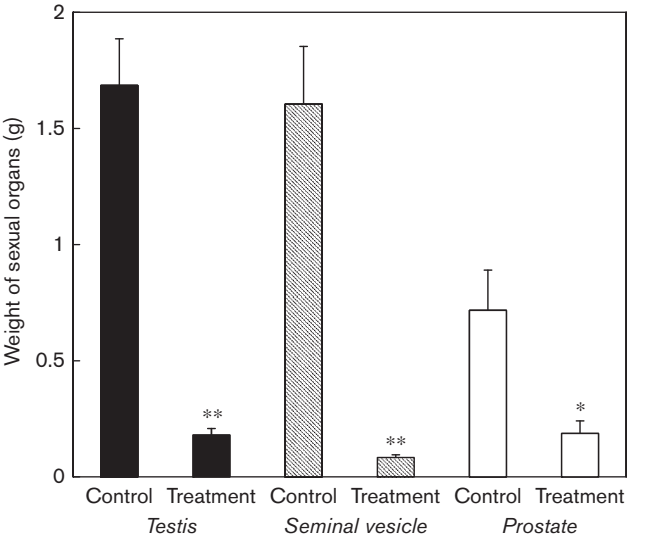
atrophied. Tunica albuginea testis was no longer significant. The results showed that LXT-101 microspheres suppressed the testosterone level continuously and exert the pharmacodynamic action by regulating the pituitary–gonadal axis quickly and efficiently. However, it was not clear that the phenomenon of shrinking was reversible.

Moreover, the testis, seminal vesicle, and prostate weights of treatment groups decreased evidently compared with the blank control. It has been shown that LXT-101 sustained-release microspheres can treat prostate cancer by suppressing the testosterone level to castration (Fig. 8). It is expected that tumor volume could also decrease faster with the testosterone level decreasing.

The rat testis sections showed slight atrophy of seminiferous tubules and tubular degeneration after treatment. However, many tubules still showed normal appearances (Fig. 9a and b). The vesicular seminalis was composed of mucous membrane, muscle layer, and outer membrane. The normal muscle layer was thin and well distributed (Fig. 9c). However, after treatment the muscle layer of vesicular seminalis was thickened, and there were few infiltrations of inflammatory cells (Fig. 9d). This indicated that some chronic inflammation stimulations occurred. Compared with the normal prostate, the cellular nucleus of the prostate of treatment groups was clear and distinct. Connective tissue and fibrous tissue proliferated. Some inflammation cells in the interstitium appeared, most of which were lymphocytes (Fig. 9e and f).

The above-mentioned results showed that LXT-101 released from the beginning and continued for a long time. Therefore, the testosterone level maintained the level of castration for a long time, and the main sex organs were degenerated. The testis weights of rats decreased significantly compared with the blank control after 4 months. It was found that testes were the primary organs to excrete testosterone. The significant decrease in testis weights must lead to a decrease in castration level. The testosterone level reversed to normal after 42 days in this study. However, it was not clear whether the main sex organs would return to the normal state after degeneration.

**Fig. 8**

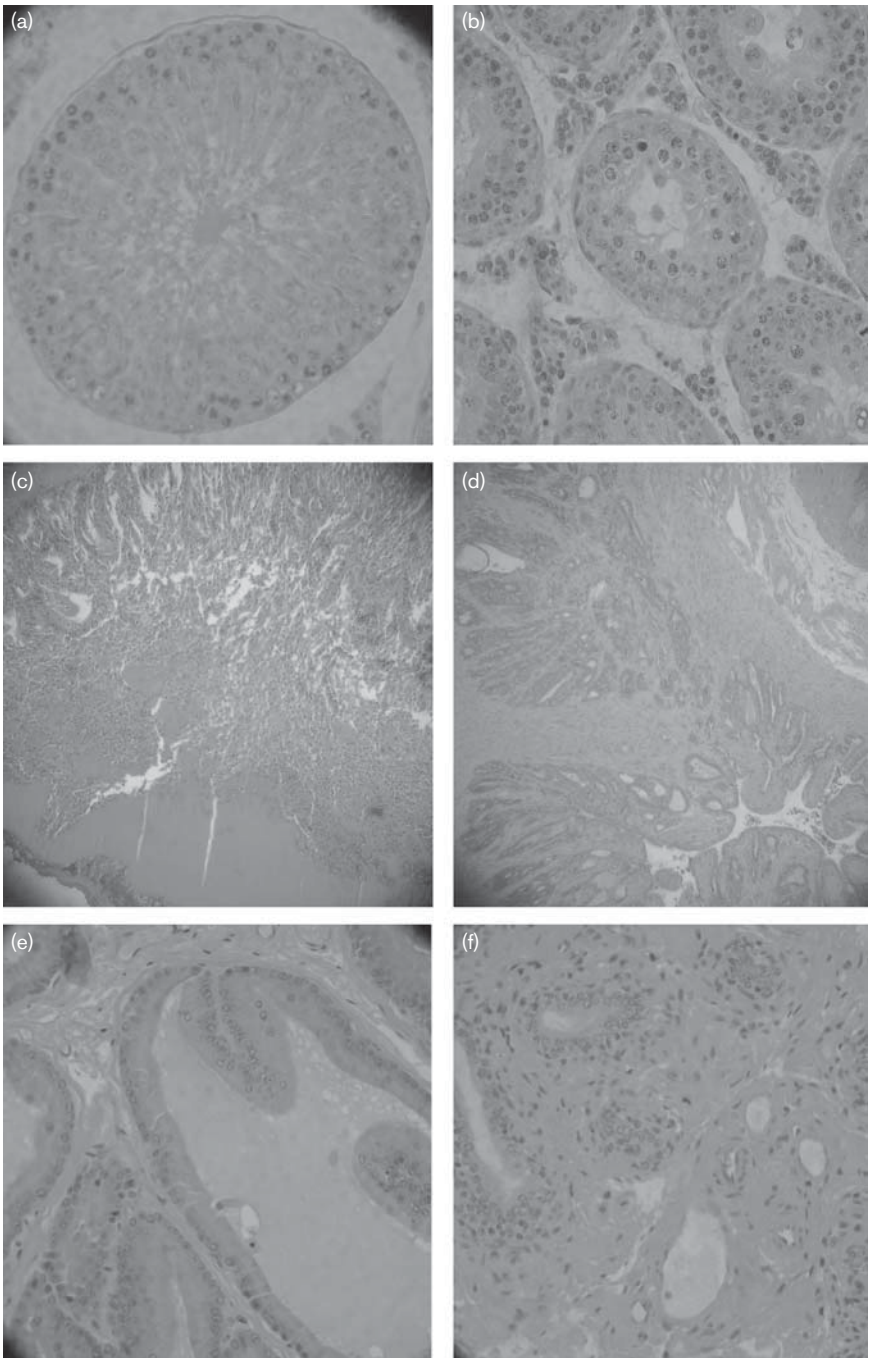



Weight changes in sexual organs of rats after subcutaneous administration. $N=6$. $^{*}P<0.05$. $^{**}P<0.01$.

### LXT-101 release kinetics *in vivo*

LXT-101 is a sensitive hormone, meaning that a very small amount of the drug in circulation can perform action *in vivo*. However, it is hard to determine when there is too little of the drug in circulation, although a sensitive liquid chromatography–mass spectrometry method of LXT-101 in serum is being developed. Therefore, an alternative method of measurement was developed in our laboratory, which had been shown to be sufficient for the in-vivo release kinetic study of LXT-101. Using this method, the

**Fig. 9**

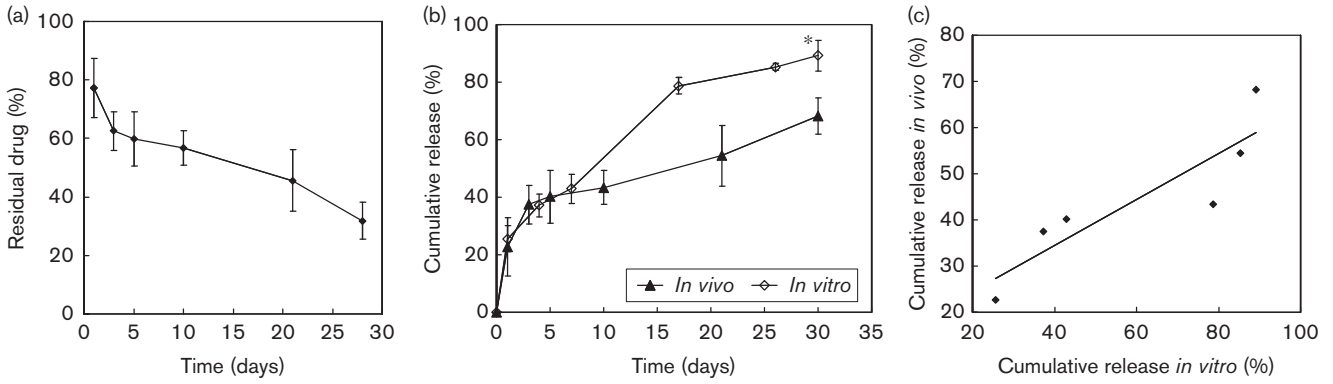



Histopathological appearances of testes, vesicula seminalis, and prostates after hematoxylin and eosin staining. (a) Normal testes ( × 400); (b) testis after treatment (× 400); (c) normal vesicular seminalis (× 400); (d) vesicular seminalis after treatment (× 400); (e) normal prostates ( × 400); (f) prostates after treatment (× 400).

residual drug *in situ* was isolated and measured using HPLC. A rapid initial release appeared within 3 days (37.4%), and the release profiles were then steady with an only 28% release ratio from day 5 to day 30 (1.12% per day), and reached 68.2% after day 30 (Fig. 10a and b). The phenomenon of initial burst release and continual controlled release is the advantage of the biodegradable PLGA microspheres [31].

The correlation analysis of in-vitro and in-vivo releases of the PLGA 14 000 microspheres indicates that the in-vitro release is much higher than that of *in vivo* (Fig. 10c). The

**Fig. 10**




In-vivo release of the luteinizing hormone-releasing hormone antagonist, LXT-101, poly(*d,l*-lactide-*CO*-glycolide) 14 000 microspheres in rats. $N=6$. (a) Residual drug percentage *in vivo*; (b) drug release percentage *in vivo* and *in vitro*; (c) correlation of in-vitro release with in-vivo release. $*P<0.01$.

in-vivo release was rapid with 22.7% at day 1, 40.2% within day 5, and 50% within day 15, which was similar to the in-vitro release behavior with 37.2% within day 4 and 50% within day 9. After the initial release field, the in-vivo and in-vitro release rates were significantly different during the last 25 days in the 30-day duration.

A study using the electron paramagnetic resonance method showed the rapid formation of an acidic microenvironment during the inner phase of biodegradable microspheres [32]. However, the buffered environments were supplied whether *in vitro* or *in vivo* so that the pH would be not the key to determining PLGA degradation and drug release. Another study showed that plasma protein adsorption could accelerate drug release from microspheres because of hydrophobic interactions [33,34]. This was not consistent with our study results. In fact, unlike the microspheres in circulation, the surroundings of microspheres had few proteins after subcutaneous administration. Furthermore, the narrow space in the injection site led to slow diffusion of degradation products of polymers and slow drug release. More importantly, after administration for several days, a significant connective tissue was found to envelop the microsphere bolus. Therefore, the in-vivo drug release was much slower than that of *in vitro* in the latter period.

The latter higher release *in vitro* resulted in low in-vivo–in-vitro correlation. The key reason may be higher peptide dissolution in the buffered solutions than in tissues. A recently published study showed that LXT-101 can form gels in 0.9% saline solution [35]. The released LXT-101 *in vivo* can form gels in the injection sites because of hard diffusion, leading to slow dissolution.

LXT-101 can penetrate biomembranes into circulation despite poor diffusivity and low partition coefficient, based on the results of this study and those of another [36]. It was also shown that a very small amount of LXT-101 can produce sufficient pharmacological action, although the released peptides could be readily destroyed by enzymes *in situ*. Therefore, the pharmacological action might be produced after injecting the aqueous suspension of pure LXT-101. However, the action might be kept for a very short time because total drugs would be quickly degraded. However, LXT-101 is released from the microspheres in a sustained mode for a very long time and enters into circulation to perform its action. Therefore, the release mode of LXT-101 determines the duration of pharmacological action.

### Conclusion

The LXT-101 PLGA microspheres not only immediately suppressed gonadotropins, but also made the hormone reversible after discontinuation of treatment. More importantly, the action can last for at least 1 month with just one injection. It was confirmed that treatment efficiency was related to the characteristics of drug-loaded microspheres, such as size, excipients, and preparation techniques. The inhibition of LXT-101 on the pituitary–gonadal axis was reversible, and thus it might also be suitable for the treatment of endometriosis, leiomyomas, and benign prostatic hyperplasia in addition to prostate cancer. It is believed that LXT-101 is a promising LHRH antagonist and could give much benefit to patients.

## Acknowledgements

The authors thank Professor Keliang Liu, Dr Jiankun Qie, Xiaoli Chi, Aiying Kong, and Junping Cheng for their valuable assistance during drug synthesis and analysis.

## References

1 Weckermann D, Harzmann R. Hormone therapy in prostate cancer: LHRH antagonists versus LHRH analogues. *Eur Urol* 2004; **46**:279–284.
2 Stricker HJ. Luteinizing hormone-releasing hormone antagonists in prostate cancer. *Urology* 2001; **58**:24–27.

3 Lizio R, Klenner T, Borchard G, Romeis P, Sarlikiotis AW, Reissmannb T, *et al.* Systemic delivery of the GnRH antagonist cetrorelix by intratracheal instillation in anesthetized rats. *Eur J Pharm Sci* 2000; **2000**:253–258.
4 Erb K, Klipping C, Duijkers I, Pechstein B, Schueler A, Hermann R. Pharmacodynamic effects and plasma pharmacokinetics of single doses of cetrorelix acetate in healthy premenopausal women. *Fertil Steril* 2001; **75**:316–323.
5 Niwa M, Enomotoa K, Yamashita K. Measurement of the novel decapeptide cetrorelix in human plasma and urine by liquid chromatography–electrospray ionization mass spectrometry. *J Chromatogr B* 1999; **729**:245–253.
6 Beer TM, Garzotto M, Eilers KM, Lemmon D, Wersinger EM. Targeting FSH in androgen-independent prostate cancer: abarelix for prostate cancer progressing after orchiectomy. *Urology* 2004; **63**:342–347.
7 McLeod D, Zinner N, Tomera K, Gleason D, Fotheringham N, Campion M, *et al.* A phase 3, multicenter, open-label, randomized study of abarelix versus leuprolide acetate in men with prostate cancer. *Urology* 2001; **58**:756–761.
8 Koch M, Steidle C, Brosman S, Centeno A, Gaylis F, Campion M, *et al.* An open-label study of abarelix in men with symptomatic prostate cancer at risk of treatment with LHRH agonists. *Urology* 2003; **62**:877–882.
9 Gustofson RL, Segars JH, Larsen FW. Ganirelix acetate causes a rapid reduction in estradiol levels without adversely affecting oocyte maturation in women pretreated with leuprolide acetate who are at risk of ovarian hyperstimulation syndrome. *Hum Reprod* 2006; **21**:2830–2837.
10 Martínez F, Clua E, Parera N, Rodríguez I, Boada M, Coroleu B. Prospective, randomized, comparative study of leuprorelin + human menopausal gonadotropins versus ganirelix + recombinant follicle-stimulating hormone in oocyte donors and pregnancy rates among the corresponding recipients. *Gynecol Endocrinol* 2008; **24**:188–193.
11 Xiao S. LHRH-antagonists. WO1992008733. 1992
12 Lee VHL. Enzymatic barriers to peptide and protein absorption. *CRC Crit Rev Ther Drug Carrier Syst* 1988; **5**:69–97.
13 Langer R. New methods of drug delivery. *Science* 1990; **249**:1527–1533.
14 Ubaidulla U, Khar RK, Ahmad FJ, Sultana Y, Panda AK. Development and characterization of chitosan succinate microspheres for the improved oral bioavailability of insulin. *J Pharm Sci* 2007; **96**:3010–3023.
15 Boonsongrit Y, Abe H, Sato K, Naito M, Yoshimura M, Ichikawa H, *et al.* Controlled release of bovine serum albumin from hydroxyapatite microspheres for protein delivery system. *Mater Sci Engineering B* 2008; **148**:162–165.
16 Sun Y, Wang B, Wang H, Jiang J. Controllable preparation of magnetic polymer microspheres with different morphologies by miniemulsion polymerization. *J Colloid Interface Sci* 2007; **308**:332–336.
17 Ito F, Fujimori H, Honnami H, Kawakami H, Kanamura K, Makino K. Effect of polyethylene glycol on preparation of rifampicin-loaded PLGA microspheres with membrane emulsification technique. *Colloids Surf B Biointerfaces* 2008; **66**:65–70.
18 Brandhonneur N, Chevanne F, Vié V, Frisch B, Primault R, Potier M-FL, *et al.* Specific and non-specific phagocytosis of ligand-grafted PLGA microspheres by macrophages. *Eur J Pharm Sci* 2009; **36**:474–485.
19 Ribeiro-Costa RM, Cunha MRD, Gongora-Rubio MR, Michaluart-Júnior P, Ré MI. Preparation of protein-loaded-PLGA microspheres by an emulsion/solvent evaporation process employing LTCC micromixers. *Powder Tech* 2009; **190**:107–111.
20 Bae SE, Son JS, Park K, Han DK. Fabrication of covered porous PLGA microspheres using hydrogen peroxide for controlled drug delivery and regenerative medicine. *J Controlled Release* 2009; **133**:37–43.
21 Couvreur P, Puisieux F. Nano- and microparticles for the delivery of polypeptides and proteins. *Adv Drug Delivery Rev* 1993; **10**:141–162.
22 Shive M, Anderson J. Biodegradation and biocompatibility of PLA and PLGA microspheres. *Adv Drug Delivery Rev* 1997; **28**:5–24.
23 Okada H. One- and three-month release injectable microspheres of the LHRH superagonist leuprorelin acetate. *Adv Drug Delivery Rev* 1997; **28**:43–70.
24 Schwach G, Oudry N, Giliberto J-P, Broqua P, Lück M, Lindner H, *et al.* Biodegradable PLGA microparticles for sustained release of a new GnRH antagonist: part II in vivo performance. *Eur J Pharm Biopharm* 2004; **57**:441–446.
25 Du L, Cheng J, Chi Q, Qie J, Liu Y, Mei X. Biodegradable PLGA microspheres as a sustained release system for a new luteinizing hormone-releasing hormone (LHRH) antagonist. *Chem Pharm Bull* 2006; **54**:1259–1265.
26 Leo E, Scatturin A, Vighi E, Dalpiaz A. Polymeric nanoparticles as drug controlled release systems: a new formulation strategy for drugs with small or large molecular weight. *J Nanosci Nanotechnol* 2006; **6**:3070–3079.
27 Danhier F, Lecouturier N, Vroman B, Jérôme C, Marchand-Brynaert J, Feron O, *et al.* Paclitaxel-loaded PEGylated PLGA-based nanoparticles: in vitro and in vivo evaluation. *J Control Release* 2009; **133**:11–17.
28 Ravivarapua HB, Burtonb K, DeLucac PP. Polymer and microsphere blending to alter the release of a peptide from PLGA microspheres. *Eur J Pharm Biopharm* 2000; **50**:263–270.
29 Makino K, Arakawa M, Kondo T. Preparation and in vitro degradation properties of polylactide microcapsules. *Chem Pharm Bull* 1985; **33**: 1195–1201.
30 Malmusi S, La Marca A, Giulini S, Xella S, Tagliasacchi D, Marsella T, *et al.* Comparison of a gonadotropin-releasing hormone (GnRH) antagonist and GnRH agonist flare-up regimen in poor responders undergoing ovarian stimulation. *Fertil Steril* 2005; **84**:402–406.
31 Csernus V, Szende B, Schally A. Release of peptides from sustained delivery (microcapsules and microparticles) in vivo. A histological and immunohistochemical study. *Int J Peptide Protein Res* 1990; **35**:557–565.
32 Mäder K, Bittner B, Li Y, Wohlauf W, Kissel T. Monitoring microviscosity and microacidity of the albumin microenvironment inside degrading microparticles from poly(lactide-co-glycolide) (PLG) or ABA-triblock polymers containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethyleneoxide) B blocks. *Pharm Res* 1998; **15**:787–793.
33 Makino K, Ohshima H, Kondo T. Effects of plasma proteins on degradation properties of poly(l-lactide) microcapsules. *Pharm Res* 1987; **4**:62–65.
34 Makino K, Ohshima H, Kondo T. Potential distribution across a plasma protein-coated poly(l-lactide) microcapsule surface. *J Microencapsul* 1990; **7**:199–208.
35 Chi X, Zhou W, Cheng J, Zhang Y, Liu K. In vivo characterization of a novel GnRH (gonadotropin-releasing hormone) antagonist, LXT-101, in normal male rats. *Reg Peptides* 2006; **136**:122–129.
36 Kong A, Zhang Z, Qiao J, Zhang F, Zhou W, Liang K, *et al.* Determination of decapeptide LXT-101 in plasma by HPLC-MS/MS and its pharmacokinetics in beagle dogs. *Acta Pharm Sin* 2008; **43**:946–950.