Bioorganic & Medicinal Chemistry xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

PEGylated leuprolide with improved pharmacokinetic properties

Mian Fu[a], Xiaomei Zhuang[b], Tianhong Zhang[b], Ying Guan[a,*], Qingbin Meng[b,*], Yongjun Zhang[a,*]

[a] Key Laboratory of Functional Polymer Materials and State Key Laboratory of Medicinal Chemical Biology, Institute of Polymer Chemistry, College of Chemistry, Nankai University, Tianjin 300071, China

[b] State Key Laboratory of Toxicology and Medical Countermeasures, Beijing Institute of Pharmacology and Toxicology, 27 Taiping Road, Beijing 100850, China

ARTICLE INFO

Keywords:
Leuprolide
PEGylation
Pharmacokinetics

ABSTRACT

Leuprolide, a gonadotropin-releasing hormone (GnRH) agonist widely used in androgen deprivation therapy for the treatment of advanced prostate cancer, suffers from a short circulating half-life like other peptide therapeutics. As an attempt to improve its pharmacokinetic properties, two PEGylated leuprolides with different molecular weight were synthesized utilizing *N*-hydroxysuccinimidyl (NHS) conjugation chemistry. The reaction conditions, including reaction temperature, reaction time and feed ratio of the reactants, were optimized to obtain a higher yield. Reverse-phase high performance liquid chromatography (RP-HPLC) characterization indicates a high purity of the resulting conjugates. Matrix-assisted laser desorption mass spectrometry (MALDI-MS) characterization suggests a 1:1 PEGylation. ^{1}H NMR study reveals that the reaction occurs on the imidazolyl group on the histidine residue and the conjugates are stable in pH7.4 aqueous solutions. The in vitro bioactivity of the conjugates was evaluated using both hormone-sensitive and hormone-insensitive cell lines. It was found that the PEGylated peptides can still counteract the stimulatory action of androgens and the mitogenic action of epidermal growth factor on cell proliferation. The in vivo bioactivity of the conjugates was also tested. Like the unmodified peptide, administration of the conjugates to male rats leads to an initial testosterone surge, followed by a suppression of testosterone secretion. Pharmacokinetics of the drugs after i.v. and s.c. administrations were determined. In both cases, a prolonged circulating half-life, an increased AUC, and a decreased Cl_F were observed for the PEGylated drugs.

1. Introduction

Prostate cancer is one of the most common types of cancer in men and also a major cause of cancer death.[1] As testosterone promotes the growth of prostate cancer cells, a reduced testosterone level could slow cancer growth or shrink it temporarily, therefore androgen deprivation therapy (ADT) has become a standard-of-care treatment for advanced prostate cancer.[1] Besides surgical castration, a more common type of ADT is to use gonadotropin-releasing hormone (GnRH) agonists to suppress the testosterone level.[1]

GnRH is secreted by hypothalamic neurons in a pulsatile fashion.[2,3] It stimulates the secretion of gonadotropin in the pituitary,[2] and in turn the secretion of testosterone in the testes.[4] GnRH agonists have a high affinity for the GnRH receptors in the pituitary. Long-term exposure to a GnRH agonist results in down-regulation of GnRH receptors, and therefore reduced synthesis of gonadotropin in the pituitary and testosterone in the testes.[4] A number of GnRH agonists have been developed for androgen deprivation therapy. Among them, leuprolide acetate, a nonapeptide analogue of the natural GnRH, was introduced for prostate cancer treatment in 1985 and now widely used for the treatment of prostate cancer.[3,5–7] It was also used to treat many other sex hormone-related diseases, including endometriosis, precocious puberty, and uterine fibroids.[3]

Like other peptide therapeutics, leuprolide has a short circulating plasma half-life because of its poor chemical and physical stability. To address this problem, various drug carriers, particularly biodegradable poly(lactic-*co*-glycolic acid) (PLGA) microspheres,[1,8–10] were designed to encapsulate the drug and to achieve its sustained release. Recently Kim et al developed a new sustained-release injection formulation using liquid crystal-forming materials as drug carrier.[11] In another approach, the peptide was linked covalently to polymeric micelles via hydrolysable ester linkages. The drug was administrated via intravenous injection and the circulation half-life was significantly extended.[12] Sustained release of leuprolide was also achieved using an implantable device in which the diffusion of the drug is controlled by physico-electrostatic confinement of the nanochannels.[13]

PEGylation is another popular method to extend the circulating half-life of a peptide/protein therapeutic. Conjugation a peptide/

* Corresponding authors.
E-mail addresses: yingguan@nankai.edu.cn (Y. Guan), nankaimqb@sina.com (Q. Meng), yongjunzhang@nankai.edu.cn (Y. Zhang).

https://doi.org/10.1016/j.bmc.2020.115306
Received 17 November 2019; Received in revised form 26 December 2019; Accepted 2 January 2020

protein therapeutic with polyethylene glycol (PEG) reduces its renal clearance, protects it from proteolytic degradation, and thus increases its stability and prolongs its circulating half-life. In addition, PEGylation could also reduce its immunogenicity and antigenicity.[14–17] Improved therapeutic efficacy and efficiency have been demonstrated from various PEGylated peptide/protein therapeutics including interferon,[18,19] human growth hormone,[20] insulin,[21] and salmon calcitonin.[22,23]

Although PEGylation has been well-established as a technology to improve therapeutic efficacy and efficiency of peptide/protein therapeutics,[24–27] it is surprising that, to the best of our knowledge, no study has been reported about the PEGylation of leuprolide. Here to improve the pharmacokinetic properties of leuprolide, two PEGylated leuprolides were synthesized and characterized. In vitro and in vivo tests demonstrated that the peptide remains its bioactivity after PEGylation. Meanwhile its pharmacokinetic properties were improved successfully.

2. Results and discussion

2.1. Synthesis of PEGylated leuprolides

A variety of activated PEGs were developed to attach PEG chain to protein or peptide.[15,17] Here *N*-hydroxylsuccinimide (NHS) functionalized polyethylene glycol, mPEG-SPA, was selected to synthesize PEGylated leuprolides (Fig. 1). To identify an optimized reaction temperature, the reaction between mPEG2K-SPA and leuprolide was carried out at 4, 25 and 37 °C, respectively. The reaction mixture was then analyzed by RP-HPLC. As shown in Fig. 2A, the retention time of the unmodified peptide is ~19.1 min. After reaction with mPEG2K-SPA, a new species, i.e., the resulting conjugate PEG2K-LEU, appears at a retention time of ~20.2 min. When the reaction temperature increases from 4 °C to 25 and 37 °C, the peak ratio of PEG2K-LEU to the unreacted leuprolide increases from 0.34 at 4 °C to 0.47 at 25 °C and further to 0.60 at 37 °C. The result suggests that a higher temperature is more favorable for the reaction, therefore a reaction temperature of 37 °C was chosen in the following studies. To study the influence of reaction time, the reaction time was varied from 1 to 24 h, and the results were shown in Fig. 2B. At a reaction time of 1 h, the peak ratio of PEG2K-LEU to unreacted leuprolide is 0.43. Extending reaction time to 4 h increases the ratio to ~0.60. The value keeps almost constant when further prolonging the reaction time to 12 and 24 h. The result suggests the reaction stopped at a reaction time of 4 h, possibly due to the consumption of mPEG-SPA as a result of the concomitant hydrolysis. So a reaction time of 4 h was chosen in the following studies. To study the influence of the feed molar ratio of mPEG2K-SPA to leuprolide, a feed molar ratio of 1:1, 2:1, 4:1, 6:1, and 8:1 was tested (Fig. 2C). The peak ratio of PEG2K-LEU to unreacted leuprolide was determined to be ~0.60, ~0.55, ~0.48, and ~0.42, respectively. The result suggests that a higher feed molar ratio of mPEG2K-SPA to leuprolide is not favorable for the reaction, probably because a high concentration of mPEG2K-SPA leads to an increased viscosity, which is unfavorable for the reaction. Therefore a feed molar ratio of 1:1 was selected for further studies.

Using the conditions identified above, leuprolide was reacted with two mPEG-SPAs, i.e., mPEG2K-SPA and mPEG5K-SPA, which has a MW of 2000 and 5000 respectively. The products were purified by ion-exchange chromatography, the most commonly used technique for purification of PEGylated proteins.[28] The final yield is ~31.3% for PEG2K-LEU and ~35.8% for PEG5K-LEU.

To assess the purity of the conjugates, they were first examined by reverse-phase (RP) HPLC. As shown in Fig. 3, only a single peak was detected in the chromatogram of both PEG2K-LEU and PEG5K-LEU, indicating both conjugates have a high purity (> 95%). It is noteworthy that the retention time of the two conjugates is almost identical.

The molecular weight and molecular weight distribution of the conjugates were then determined by MALDI-TOF analysis. As shown in Fig. 4, like other PEGylated peptides, the mass spectra of the PEG-leuprolide conjugates are composed of a series of peaks equally spaced 44 ± 1 Da apart,[27] because the parent PEG polymers are disperse and the molecular weight of the repeating unit, ethylene glycol, is 44. The mass of the most abundant peak is 3235.6 *m/z* for PEG2K-LEU and 6144.2 *m/z* for PEG5K-LEU. Since the molecular weight of leuprolide is 1209.5 Da[12] and that of the two PEGs is ~2000 and ~5000 Da, the result clearly indicates that each leuprolide molecule was conjugated with only one PEG chain.[24]

When utilizing the NHS conjugation chemistry for pegylation, the target site can be any nucleophilic sites on the peptide. The common target sites, i.e., α-amino group of the *N*-terminal residue and the ϵ-amino groups of lysine residues, are absence in the leuprolide structure. However the leuprolide structure still has many nucleophilic sites, including the hydroxyl group on serine residue, the phenolic hydroxyl group on tyrosine residue, and the imidazolyl group on histidine residue, which could be possible pegylation site.[29,30] Which site will be the pegylation site depends on many factors, such as peptide conformation, solvent accessibility, local electronic and pKa effects determining the relative reactivity of the potential pegylation sites.[29] To identify the pegylation site, ^{1}H NMR spectra of leuprolide and the two conjugates were measured. As shown in Fig. 5A, the peak at 8.23 ppm in the spectra of LEU was assigned to *Ha* on the imidazole ring. In PEG2K-LEU, this peak was divided into 2 peaks and shifted to 8.05 and 7.98 ppm. Division and upfield shift of the peak (to 7.82 and 7.76 ppm) was also observed when conjugated with PEG5K. This observation indicates that pegylation occurs on the imidazolyl group on the histidine residue site (Fig. 1).[29] Similar upfield shift of the imidazolyl proton was previously observed by Wyss et al.[29] when pegylating interferon alpha-2b with succinimidyl carbonate PEG. The division of the peak suggests PEGylation may occur at the N1 or N2 position on imidazole ring. (Fig. 1) From the intensity of the two peaks, the ratio of the two positional isomers is about 58:42 for PEG2K-LEU and about 63:37 for PEG5K-LEU. It is interesting that a larger shift was found for PEG5K-LEU. We speculate the phenomenon could be explained as follows. The PEG chain can bind with leuprolide via the formation of intramolecular hydrogen bond between the PEG chain and the phenol hydroxyl group on leuprolide. This will increase the influence of the PEG chain on the chemical shift of the imidazolyl proton. PEG5K-LEU has a longer PEG chain, therefore a larger possibility for the formation of the intramolecular hydrogen bond. As a result, a larger shift of the imidazolyl proton was found for PEG5K-LEU.

It was previously reported that pegylated peptide using imidazole as pegylation site may depegylate because the PEG-imidazole bond is labile.[29] To investigate the stability of the two conjugates, the change in their NMR spectra with time was followed. To mimic physiological conditions, buffered D_2O with a pH of 7.4 was used as solvent. As shown in Fig. 5B and C, no detectable change was found for either PEG2K-LEU nor PEG5K-LEU. Particularly no new peak appeared at 8.23 ppm, suggesting no nonpegylated leuprolide formed. In other words, no depegylation occurred. The stability of the two conjugates at pH3.0 and pH5.0 were also studied. Again no depegylation was found (Fig. S1). Therefore the PEG-imidazole bond is quite stable in the PEG-LEU conjugates synthesized here.

2.2. In vitro bioactivity evaluation

Conjugation with a PEG chain likely leads to a reduction or even loss of the bioactivity of a peptide therapeutic, because the large PEG chain will interfere with the interaction between the peptide therapeutic and its binding site.[31] To check the effect of PEGylation on the bioactivity of leuprolide, the two conjugates were tested using both in vitro and in vivo models.

First the conjugates were tested using in vitro cell models. Previous studies revealed that, although leuprolide is ineffective in regulating the growth of LNCaP cells, a hormone-sensitive cell line, when used alone, it counteracts the stimulatory action of androgens on the

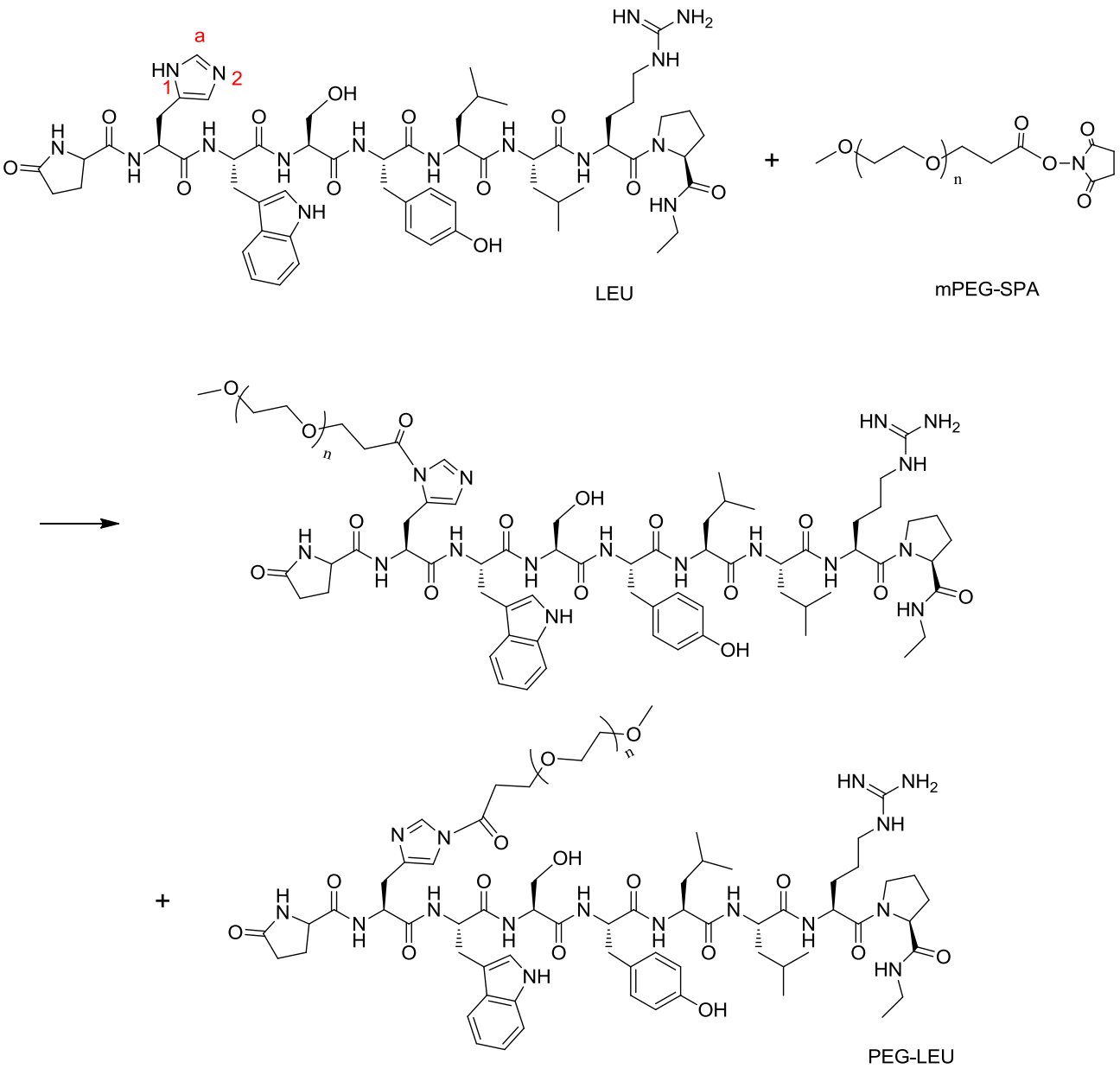

Fig. 1. Synthesis of PEGylated leuprolide by coupling of PEG to the imidazolyl group on leuprolide using mPEG-SPA.

proliferation of the cells.[32] To examine if the PEG-leuprolide conjugates remains to be bioactive, LNCaP cells were treated with dihydrotestosterone (DHT), DHT and unmodified leuprolide, and DHT and PEG-leuprolide conjugates. As shown in Fig. 6, an enhanced growth of LNCaP cells was observed after the cells were treated with 10^{-8} M DHT for 2, 4, and 6 days, confirming that the growth of the cells can be stimulated by androgens. When treated simultaneously with DHT and unmodified leuprolide, however, the cell growth become slower, suggesting the androgen-stimulated cell proliferation was reduced.[32] Similar results were observed when the cells were simultaneously treated with DHT and PEG-leuprolide conjugates (PEG2K-LEU or PEG5K-LEU). The results indicate that the ability of leuprolide to counteract the stimulatory action of androgens on the proliferation of LNCaP cells is remained after PEGylation.

Similarly it was found that leuprolide does not affect the growth of PC-3 cells, a hormone-insensitive cell line, when used alone. However it counteracts the mitogenic action of epidermal growth factor (EGF) on them.[32] Fig. 7 examined the effect of EGF alone, EGF and unmodified leuprolide, EGF and PEG-leuprolide conjugates on the growth of PC-3 cells. A significantly enhanced growth was observed after the cells were stimulated with EGF for 4 days because of the mitogenic action of the growth factor, however, the effect of EGF was reduced when the cells were treated simultaneously with either unmodified or PEG-modified leuprolide. The results again suggest leuprolide remains to be active after PEGylation. It is interesting that from Figs. 6 and 7, after PEGylation, the ability of leuprolide to counteract the stimulatory action of androgens on LNCaP cells and EGF on PC-3 cells is somewhat enhanced, not reduced.

2.3. *In vivo bioactivity evaluation*

The in vivo bioactivity of the conjugates was then checked using male SD rats. A major physiological function of leuprolide is to suppress the serum testosterone level in male animals. Acting as an agonist of the pituitary GnRH receptor in the hypothalamo-pituitary-gonadal axis, leuprolide disrupts the maintenance of the normal hypothalamo-pituitary-gonadal axis and desensitizes the GnRH receptor. Therefore the secretion of luteinizing hormone and follicle-stimulating hormone will

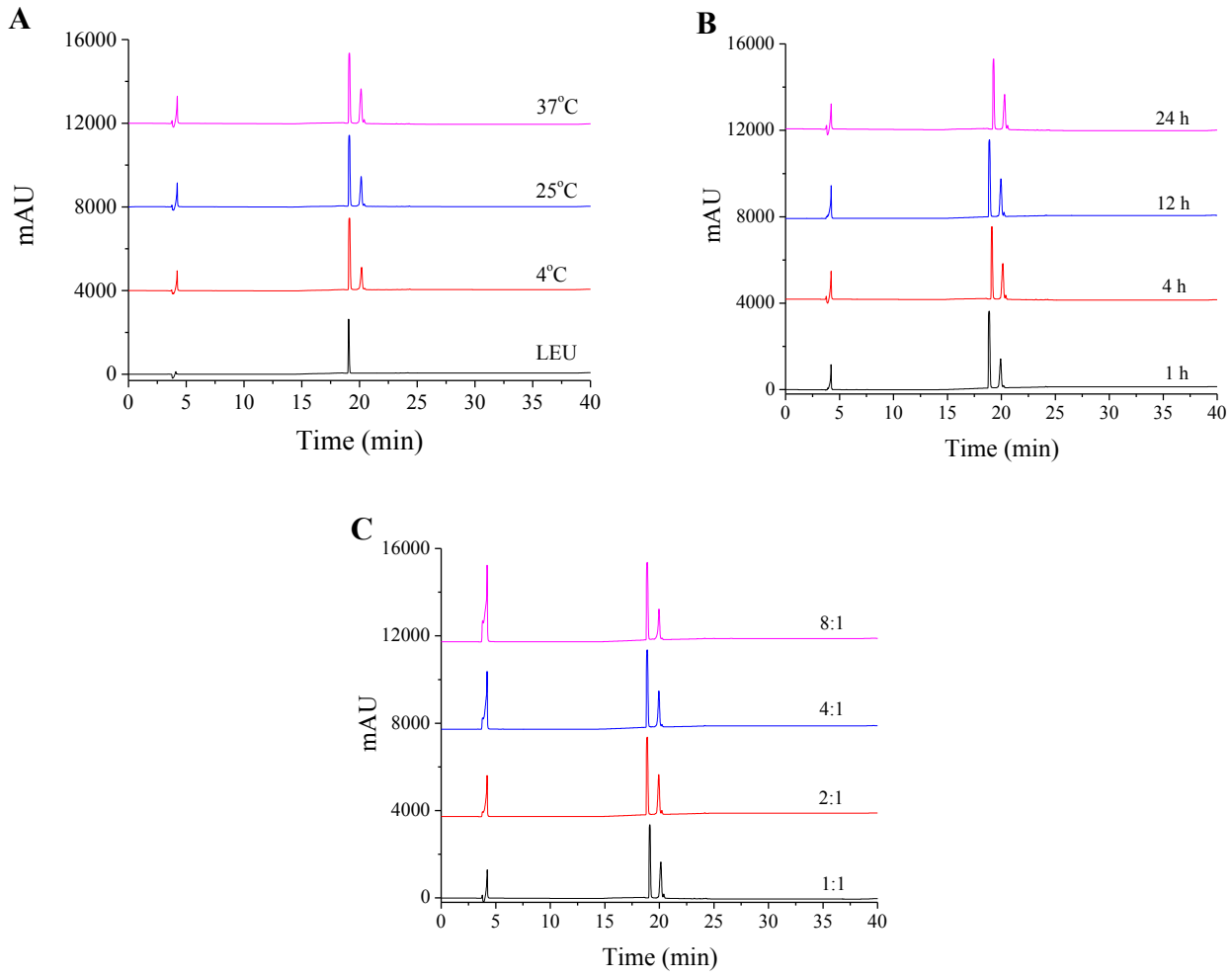

Fig. 2. RP-HPLC analysis of the reaction mixture of mPEG2K-SPA and leuprolide after reaction under various conditions. Analysis was performed on a RP-C4 column (Phenomenex Jupiter 5 μm, 4.6 × 250 mm, 300 Å) using water/acetonitrile/TFA as eluent at a flow rate of 1 mL/min. The peak was monitored at λ = 214 nm. The reaction mixture was diluted 10 times before analysis. For clarity the chromatogram were shifted along the vertical axis. (A) [mPEG2K-SPA] = [leuprolide] = 0.002 mol/L. Reaction time = 4 h. (B) [mPEG2K-SPA] = [leuprolide] = 0.002 mol/L. T = 37 °C. (C) [leuprolide] = 0.002 mol/L. Molar ratios of mPEG2K-SPA to leuprolide was labelled. T = 37 °C. Reaction time = 4 h.

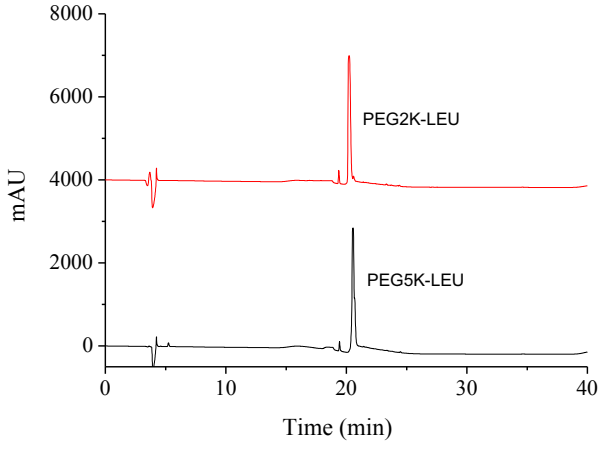

Fig. 3. RP HPLC chromatogram of PEG2K-LEU and PEG5K-LEU conjugates. Analysis was performed on a RP-C4 column (Phenomenex, Jupiter 5 μm, 4.6 × 250 mm, 300 Å) column using water/acetonitrile/TFA as eluent at a flow rate of 1 mL/min. The peak was monitored at λ = 214 nm. For clarity the chromatogram were shifted along the vertical axis.

be down-regulated, and the levels of estradiol and testosterone in the blood will be lowered.[7] The testosterone-suppressive effect of leuprolide has been widely demonstrated in previous studies.[2,33,34] To check if the PEGylated leuprolides remain to be active as GnRH agonist, they were daily administrated via subcutaneous injection to male SD rats for 14 days. For comparison, another group of rats were treated with the same dose of unmodified leuprolide. Rats without treatment were used as control.

Fig. 8A shows the change in serum testosterone level after the first injection of leuprolide. Upon leuprolide administration, serum testosterone level of the rats increases sharply. It is known that GnRH, when occurs within a narrow range of pulse frequencies and amplitudes, stimulates pituitary to synthesize and secret the gonadotropins, and subsequently controls gonadal function.[2] As a synthetic nonapeptide analog of GnRH, the administration of leuprolide will stimulate the secretion of gonadotropin, resulting in an increased testosterone level.[4] The initial testosterone surge after leuprolide administration has been widely observed in both animals and human.[4,12] From Fig. 8A, the administration of same dose of PEGylated leuprolides also results in an initial testosterone surge. In addition, the extent of the testosterone surge is almost the same for all three peptides, either PEGylated or not.

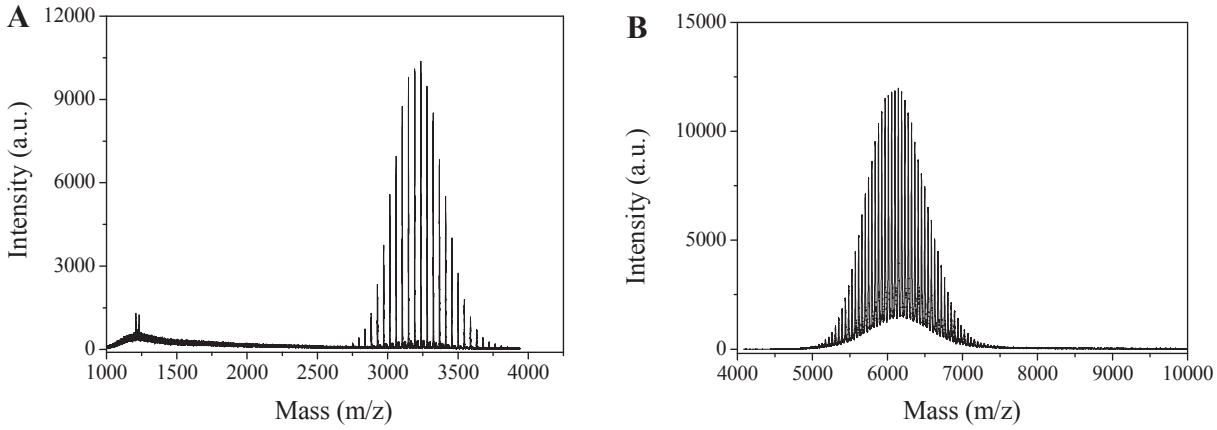

Fig. 4. MALDI-TOF mass spectra of (A) PEG2K-LEU and (B) PEG5K-LEU conjugates. The molecular weight of un-modified leuprolide is 1209.6 Da. The most probable mass are 3235.6 and 6144.2 Da for PEG2K-LEU and PEG5K-LEU, respectively.

These results demonstrate that the PEGylated leuprolides can still act as GnRH analog to stimulate the secretion of gonadotropins.

Fig. 8A also shows that, after the brief surge, the testosterone level decreases and becomes to be close to the normal level 24 h post administration. As Fig. 8B revealed, continuous daily administration of leuprolide results in a testosterone level lower than that of the control group, in which the rats did not receive leuprolide. As previous studies revealed, continuous exposure to GnRH analogs, such as leuprolide, leads to desensitization of pituitary. Therefore the gonadotropin secretion is suppressed, which in turn leads to the suppression of testosterone secretion.[2–4,7] For all three peptides studied, a similar testosterone-suppressive behavior was found, suggesting PEGylated leuprolides remain its ability to down-regulate the pituitary receptors for GnRH. Like the unmodified peptide, for both PEGylated leuprolides, a testosterone level lower than 0.5 ng/mL, the target level for androgen deprivation therapy of advanced prostate cancer, was achieved.[1,11]

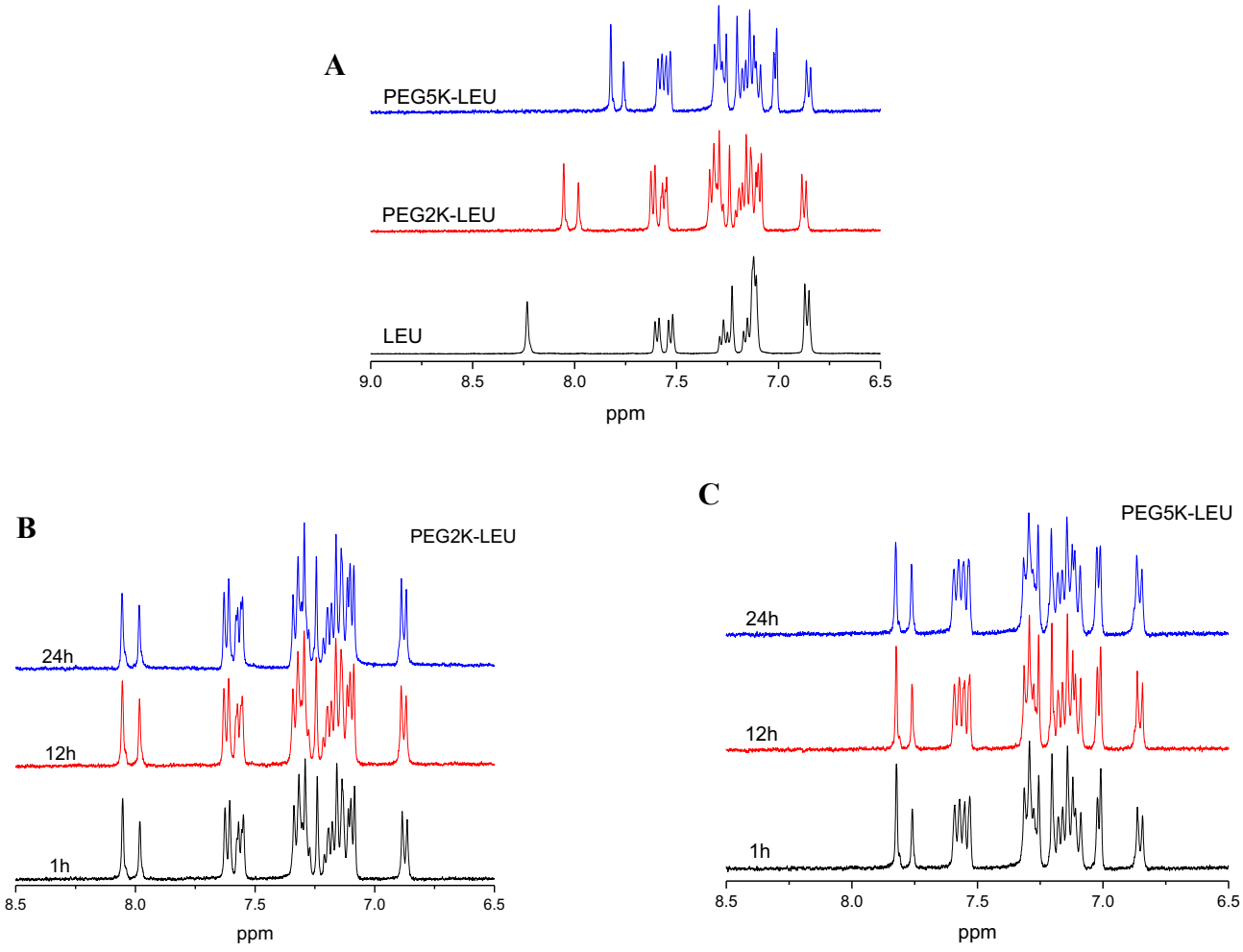

Fig. 5. (A) Aromatic region of the ^{1}H NMR spectra of leuprolide, PEG2K-LEU and PEG5K-LEU. (B, C) Aromatic region of the ^{1}H NMR spectra of PEG2K-LEU (B) and PEG5K-LEU (B) after being aged at 37 °C for 1 h, 12 h and 24 h. The samples were dissolved in phosphate buffered D_2O (pH7.4).

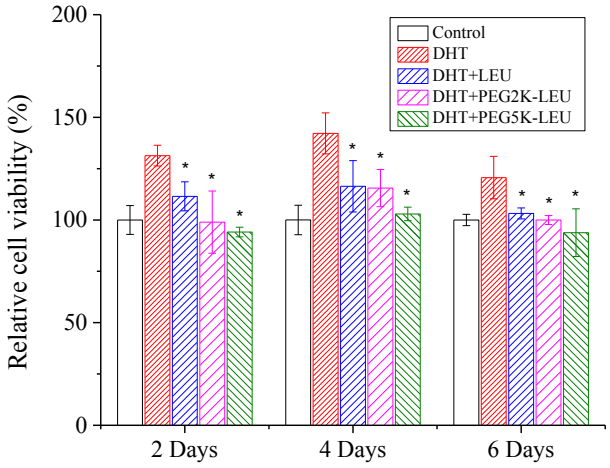

Fig. 6. Growth of LNCaP cells in the presence of DHT, DHT and leuprolide, DHT and PEG2K-LEU, and DHT and PEG5K-LEU. DHT concentration is 10^{-8} M. The concentrations of leuprolide or PEG-modified leuprolides are all 10^{-6} M. The cells were cultured in medium supplemented with 5% CH-FBS. Cell viability was determined by MTT assay at 490 nm. Columns represent the average of 3 different experiments. Bars represent SD. * $p < 0.05$ vs DHT-treated cells.

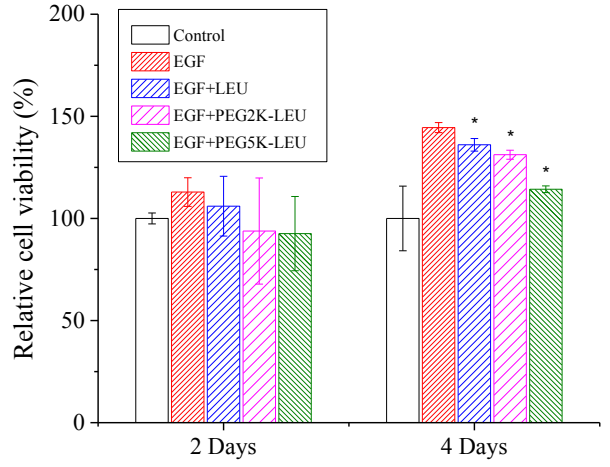

Fig. 7. Growth of PC-3 cells in the presence of EGF, EGF and leuprolide, EGF and PEG2K-LEU, and EGF and PEG5K-LEU. EGF concentration is 10^{-8} M. The concentrations of leuprolide or PEG-modified leuprolides are all 10^{-5} M. The cells were cultured in medium supplemented with 5% CH-FBS. Cell viability was determined by MTT assay at 490 nm. Columns represent the average of 3 different experiments. Bars represent SD. * $p < 0.05$ vs EGF-treated cells.

After 14 day treatment, the weight of the reproductive organs of the rats were examined. As shown in Fig. 9, compared to the untreated control group, a significant lowered weight of the reproductive organs was found for the groups treated with both unmodified and PEGylated leuprolides. The treatment with unmodified leuprolide leads to a decrease of ~43%, ~33%, and ~49% in the weight of testis, prostate and seminal vesicle, respectively. Similar suppression of the growth of reproductive organs was previously reported for male rats after continuous administration of leuprolide, which was correlated with the suppressed testosterone level in the animals.[33,35] Similar therapeutic effect was observed after treatment with the same dose of PEGylated leuprolides. As an example, the weight of testis of the rats received unmodified leuprolide, PEG2K-LEU and PEG5K-LEU decreases to be ~57%, ~58%, and ~52% of the untreated group. These results again indicate that the PEGylated leuprolides remain active as GnRH analog in vivo.

2.4. Pharmacokinetics

To study the effect of PEGylation on the pharmacokinetics of leuprolide, a single dose of the unmodified and PEGylated leuprolides was administrated via intravenous (i.v.) or subcutaneous (s.c.) route to male SD rats. The blood samples were then collected at predetermined time intervals, and the serum drug concentrations were determined. The pharmacokinetic properties were evaluated by noncompartmental analysis of the drug concentration–time curves.

From Fig. 10, after i.v. injection, the unmodified drug was rapidly eliminated from the circulation. The serum level was detectable only for 4 h post-administration. Similar results were previously reported by other researchers.[12] The half-life ($t_{1/2}$) was calculated to be only 0.43 h (Table 1). Like other peptides, leuprolide is also susceptible to proteolysis and can be filtrated through the glomeruli of kidney,[36] therefore it can be cleared from systemic circulation rapidly following parenteral administration.[12] Like the unmodified drug, the PEGylated drugs were also eliminated from the circulation quickly, but at a relatively slower rate. (Fig. 10) PEG2K-LEU was still detectable at 6 h post-administration, and its half-life was extended to be 0.53 h (Table 1). An even greater improvement was observed for PEG5K-LEU. At 10 h post-administration it was still detectable. Its half-life was calculated to be 1.28 h, representing a 2-fold increase compared to the unmodified drug (Table 1). By conjugation with a PEG chain, the molecular weight and hydrodynamic volume of the peptide increases, which makes the renal clearance more difficult. In addition, the attached PEG chain can protect the peptide from proteolytic degradation. Therefore the circulation time of the peptide is prolonged.[31] It is understandable that a longer PEG chain will retard renal clearance and protect the peptide from proteolytic degradation more effectively, therefore PEG5K-LEU exhibits

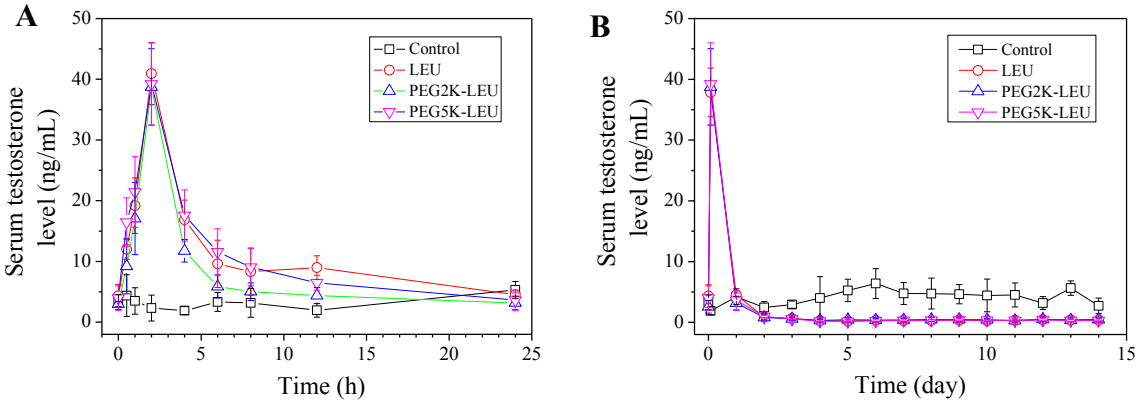

Fig. 8. Serum testosterone levels in male SD rats which were daily administrated via subcutaneous injection with leuprolide, PEG2K-LEU, or PEG5K-LEU at a dose of 0.1 mg/kg in terms of leuprolide. (A) in 24 h period after the first administration. (B) in 14 days period. Data are expressed as the mean ± SD (n = 5).

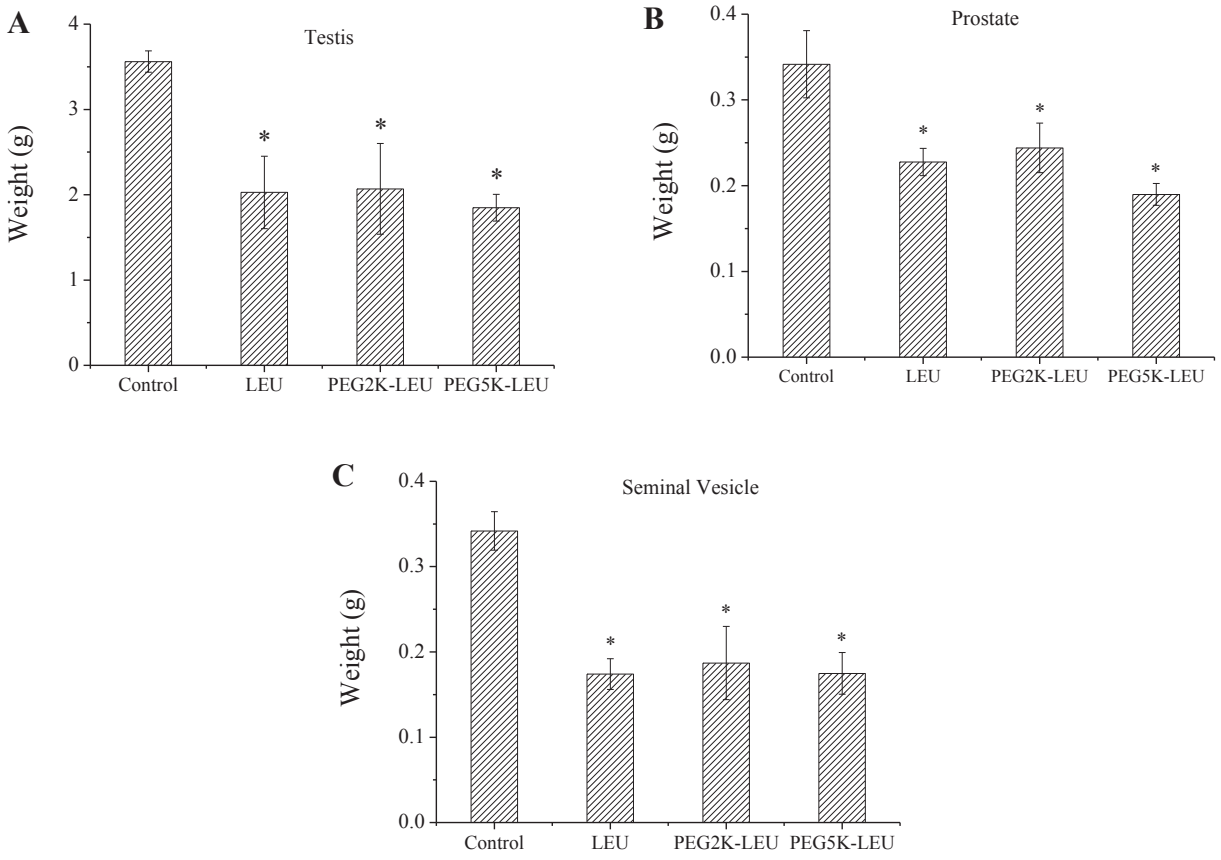

Fig. 9. Weight of genital organs, (A) testis, (B) prostate and (C) seminal vesicle, in male SD rats after daily s.c. injection of leuprolide, PEG2K-LEU, or PEG5K-LEU for 14 days (Dose = 0.1 mg/kg/day, in terms of leuprolide). Data are expressed as the mean ± SD (n = 5). * $p < 0.05$ vs control.

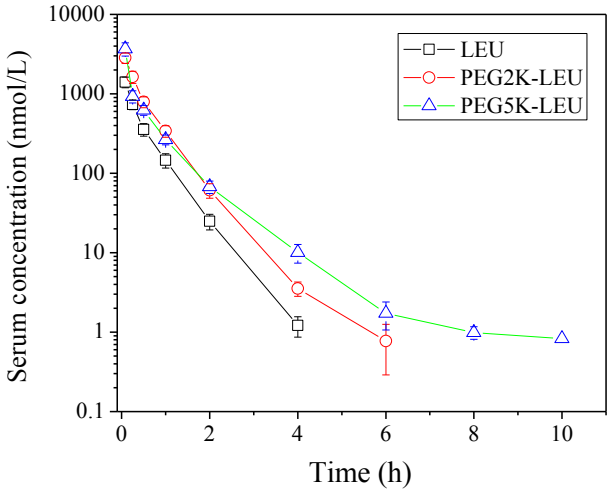

Fig. 10. Serum concentrations of the drug (unmodified leuprolide, PEG2K-LEU, or PEG5K-LEU) after a single i.v. administration in rats. Data are expressed as the mean ± SD (n = 5).

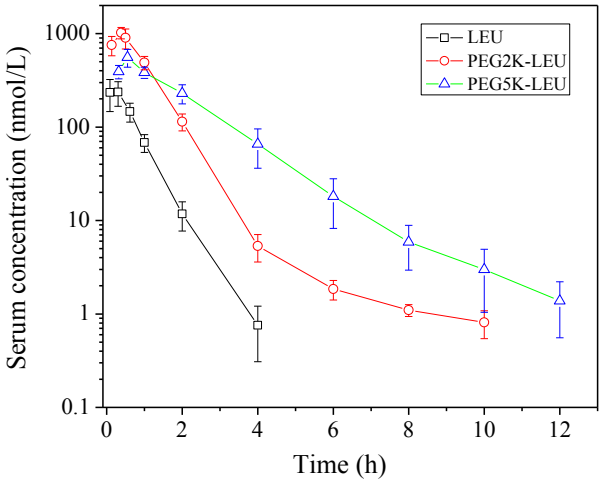

Fig. 11. Serum concentration of the drug (unmodified leuprolide, PEG2K-LEU, or PEG5K-LEU) after single s.c. administration in rats. Data are expressed as the mean ± SD (n = 5).

Table 1
PK parameters of leuprolide and PEG-modified leuprolides administrated by i.v. injection.

Parameters	leuprolide	PEG2K-LEU	PEG5K-LEU
$t_{1/2}$ (h)	0.43 ± 0.025	0.53 ± 0.032	1.28 ± 0.64
AUC_{last} (h*nmol/L)	708 ± 68.5	1503.99 ± 124.34	1521.94 ± 128.84
Cl_F (L/h/kg)	1.14 ± 0.20	0.53 ± 0.09	0.52 ± 0.09
V_Z (L/kg)	0.72 ± 0.14	0.41 ± 0.09	1.0 ± 0.42

an even longer half-life than PEG2K-LEU. In line with the extended circulation time, compared to the unmodified peptide, the AUC of the two PEGlyated peptides increased by 1 fold, meanwhile the Cl_F is lowered by 1 fold (Table 1).

More significant changes were found when the drugs were administrated via the subcutaneous (s.c.) route. In this case, the serum drug concentration first increases fast with time, reaches a peak value and then decays quickly. As shown in Fig. 11 and Table 2, PEGylation delays the time to reach the maximum concentration (T_{max}). For

Table 2
PK parameters of the pristine and PEGylated leuprolide after s.c. administration.

Parameters	leuprolide	PEG2K-LEU	PEG5K-LEU
$t_{1/2}$ (h)	0.43 ± 0.06	0.59 ± 0.023	1.51 ± 0.127
C_{max} (nmol/L)	248.74 ± 69.35	985.79 ± 104.99	575.38 ± 75.11
T_{max} (h)	0.23 ± 0.13	0.43 ± 0.06	0.55 ± 0.00
AUC_{last} (h*nmol/L)	213.71 ± 52.10	1123.45 ± 146.62	1172.66 ± 85.80
Cl_F (L/h/kg)	3.87 ± 1.1	0.74 ± 0.20	0.67 ± 0.05

unmodified drug, its serum concentration peaked 0.23 h after the subcutaneous injection, while peak concentration was observed at 0.43 h and 0.55 h after the injection of PEG2K-LEU and PEG5K-LEU, respectively. Following s.c. injection, peptide molecules diffuse into blood capillaries or lymphatic vessels and thus enter into systemic circulation.[36] It will become more difficult for the PEGylated peptide to diffuse into blood capillaries or lymphatic vessels because of their increased size. Therefore their absorption becomes slower. However, the maximum serum drug concentration (C_{max}) observed is much higher for the PEGylated drugs (C_{max} is 248.74, 985.79 and 575.38 nmol/L for unmodified leuprolide, PEG2K-LEU, and PEG5K-LEU, respectively), suggesting less drug is eliminated during the process, which should be attributed to the increased stability of the PEGylated ones. For the same reason, the half life is also extended from 0.43 h for unmodified leuprolide to 0.59 h for PEG2K-LEU and 1.51 h for PEG5K-LEU. Thanks to the prolonged circulation time and higher serum concentration, AUC of PEG2K-LEU and PEG5K-LEU is about 4 fold higher than that of the unmodified peptide, and the Cl_F is only 1/5 of the unmodified peptide (Table 2). It is noteworthy that although an improved pharmacokinetics was found for the conjugates, their influence on testosterone level and weight of genital organs is similar to the unconjugated peptide, possibly because of the saturation and down-regulation of the pituitary GnRH receptors by the analogue under the experimental conditions.[11,12]

3. Conclusions

In conclusion, two PEGylated leuprolide, PEG2K-LEU and PEG5K-LEU, were synthesized by reaction with succinimidyl derivative of PEG propionic acid, PEG-SPA. The reaction conditions were optimized to obtain a higher yield of conjugates. The products have a high purity as demonstrated by RP-HPLC characterization. The molecular weights of the conjugates were determined by MALDI-MS, which is the sum of the molecular weight of leuprolide and the corresponding PEG chain, suggesting a 1:1 PEGylation. The pegylation site was identified to be the imidazolyl group on the histidine residue by ^{1}H NMR. Both conjugates are stable in pH 7.4 aqueous solutions. In vitro and in vivo bioactivity evaluation suggest the conjugates remain to be active. Like the unmodified leuprolide, the PEGylated peptides can counteract the stimulatory action of androgens on the proliferation of LNCaP cells and the mitogenic action of epidermal growth factor on the proliferation of PC-3 cells. More importantly, they can also significantly suppress the serum level of testosterone in mal SD rates, just like the unmodified drug. As expected, the pharmacokinetic properties of the PEGylated drugs were improved compared to that of the unmodified one. Both conjugates display a prolonged circulating half-life and a slower clearance. Particularly when administrated via s.c. injection, AUC of both conjugates is increased by about 4 folds. The pharmacokinetic properties of the drug could be improved further by zero-order release. For this purpose, the PEGylated drugs synthesized here will be incorporated into dynamic layer-by-layer films with tannic acid via hydrogen bonding.[23,37–39] This work is now actively ongoing.

4. Materials and methods

4.1. Materials

Leuprolide and Alarelin were purchased from Zhejiang Peptites Biotech company (Hangzhou, China). Methoxypoly(ethylene glycol)-succinimidyl propionate (mPEG-SPA) with a Mw of 2000 and 5000 (mPEG2K-SPA and mPEG5K-SPA) was purchased from Chemgen Pharma (Beijing, China). Epidermal growth factor (EGF) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Dihydrotestosterone (DHT) was purchased from Meilun Bio (Dalian, China).

4.2. Synthesis of PEGylated leuprolides

The PEGylated leuprolide was synthesized via a reaction between leuprolide and mPEG-SPA. In a typical example, 63.5 mg of leuprolide acetate (~0.05 mmol) was first dissolved in 15 mL of deionized water, to which 100 mg of PEG2K-SPA (~0.05 mmol) was added. The mixture was stirred at 37 °C for 4 h. Subsequently the reaction mixture was purified on a SP Sepharose preparative ion-exchange column equilibrated with 5 mM pH 6.5 PBS at a flow rate of 1.5 mL/min. The bound protein was eluted using a NaCl gradient from 0 to 0.2 M and UV detection was carried out at 220 nm. The major peak was collected and dialyzed against deionized water. It was then lyophilized and stored at −20 °C prior to use. The product was labelled as PEG2K-LEU. The reaction between leuprolide and PEG5K-SPA was carried out in the same way. The resulting conjugate was labelled as PEG5K-LEU.

4.3. Characterization of PEGylated leuprolides

Reverse-phase HPLC (RP-HPLC) was performed on a Shimadzu LC-20AT reverse-phase HPLC system equipped with Phenomenex Jupiter RP-C4 column (5 μm, 4.6 × 250 mm, 300 Å) and a UV detector operated at 214 nm to assess the purity of the conjugates. Gradient elution was performed at a flow rate of 1.0 mL/min with a mobile phase A (0.1% trifluoroacetic acid (TFA) in water) and a mobile phase B (0.1% TFA in 90% acetotitrile/10% water). A gradient program was set as follows: 10% solvent B for 5 min, 85% solvent B over 10 min, 85% solvent B for 15 min, 10% solvent B over 4 min, 10% solvent B for 6 min.

To measure molecular weights of the PEGylated peptides, MALDI-TOF MS analyses were performed on a Bruker Autoflex III TOF mass spectrometer. α-Cyano-4-hydroxycinnamic acid was used as the matrix for all samples. ^{1}H NMR spectra were measured on a Varian UNITY-plus 400 NMR spectrometer using phosphate buffered D_2O (pH7.4) as solvent.

4.4. In vitro bioactivity evaluation

The hormone-sensitive prostate cancer cell lines LNCaP and hormone-insensitive PC-3 cell lines were obtained from China Infrastructure of Cell Line Resources (Beijing, China). LNCaP cells were grown in RPMI-1640 medium (HyClone, USA) supplemented with penicillin (100 IU/ml), streptomycin (100 IU/ml) and 10% fetal bovine serum (FBS; BI, Israel). PC-3 cells were cultured with F12K medium (Meilunbio, Dalian, China) supplemented with penicillin (100 IU/ml), streptomycin (100 IU/ml) and 10% fetal bovine serum. The cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

To study the additives on the cell proliferation, the cells were seeded in 24-well microplates (Corning Costar, USA) at 20,000 cells/well and cultured using standard culture medium. Forty-eight hours later the medium was renewed with fresh RPMI-1640 or F12K supplemented with 5% charcoal-treated FBS. Leuprolide or PEG-leuprolide conjugates were added daily to the medium at the concentration of 10^{-6} M in LNCaP cell and 10^{-5} M in PC-3 cell. DHT or EGF was added every 48 h at the concentration of 10^{-8} M separately. In all the experiments, the medium was renewed every two days. At predetermined time intervals,

the cell viability was assessed by MTT assay by adding 100 μL of MTT solution (5 mg/ml in PBS) into the medium. After additional 4 h incubation, the medium was removed and 1 mL of DMSO was added. The cell density in each well was then determined using a Tecan Spark microplate reader (Switzerland) at 490 nm.

4.5. In vivo bioactivity evaluation

Healthy male Sprague Dawley (SD) rats weighing ~300 g were obtained from Spfanimals, China. All the animals were cared in accordance with the national regulations and approved by the local ethical committee for animal experimentation. Twenty rats were randomly divided into four groups and subcutaneously administrated daily with PBS solution, leuprolide, PEG2K-LEU, or PEG5K-LEU at a dose of 0.10 mg/kg in terms of leuprolide. Blood samples were drawn from rats orbit at 0, 0.5, 1, 2, 4, 6, 8 and 12 h during the first 12 h after subcutaneous administration and then once a day at 9 a.m. in the morning for 14 days. The samples were centrifuged at 3000 rpm/min for 10 min. The serums were collected and stored at −20 °C before analysis. The serum testosterone levels were determined by radioimmunoassay using a commercially available kit (Beijing North Institute of Biotechnology, China) according to the manufacturer's instructions. After the rats were sacrificed, the weight of the organs, including testis, prostate and seminal vesicle, were measured.

4.6. Pharmacokinetic studies

To study the pharmacokinetics of the drugs, thirty SD rats were divided into six groups. The first three groups were intravenously administered with leuprolide, PEG2K-LEU or PEG5K-LEU at a dose of 1.0 mg/kg in terms of leuprolide. The other 3 groups were subcutaneously administrated with the drugs at the same dose. Blood samples were collected from rats orbit at predetermined time intervals. The samples were centrifuged at 3000 rpm/min for 10 min. The serum were collected and stored at −10 °C before analysis.

The concentration of leuprolide in the serum samples was analyzed by validated ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method, as described previously.[12,40–43] Briefly, 5 μL of internal standard solution (alarelin, 50.0 ng/mL) and 150 μL acetonitrile were added to 50 μL of serum. After being vigorously vortexed for 2 min and centrifuged at 12,000 rpm/min for 10 min at 4 °C, the upper supernatant was transferred to a clean tube and evaporated to dryness at 40 °C. Then the mixture was dissolved in 100 μL 0.1 M NaOH solution and incubated for 30 min at 37 ± 0.5 °C on an orbital shaker to break the ester bond and release leuprolide. Acetic acid was used to adjust pH of the samples. After being centrifuged at 12,000 rpm/min for 10 min at 4 °C, the supernatant (5 μL) was injected into the LC-MS/MS system. The chromatographic separation was achieved on Kromasil C-18 column (1.8 μm, 2.1 × 50 mm) using gradient elution at a flow rate of 0.5 mL/min. The mobile phase consisted of 1% acetic acid in water (A) and 1% acetic acid in acetonitrile (B). The gradient program was set as follows: from 10% to 90% solvent B over 2.5 min, 90% solvent B for 0.5 min, 10% solvent B over 0.1 min. The mass spectrometer was operated in the positive ion mode using an electrospray ionization (ESI) source. Quantification was performed using multiple reaction monitoring (MRM) of the transitions m/z 605.5 → m/z 249.05 for leuprolide and m/z 584.5 → m/z 249.15 for alarelin (IS).

4.7. Statistical analysis

Statistical analysis was performed on OriginPro 8.5. The one way analysis of variance (ANOVA) or student's t test were used to access statistical significance between different groups separately. $P < 0.05$ was considered to be statistically significant. The pharmacokinetic parameters of leuprolide and PEG-leuprolide were calculated by non-compartmental analysis of the serum drug concentration versus time curves using WinNonlin software (version 6.3).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank financial support for this work from the National Natural Science Foundation of China (Grant Nos: 51625302, 51873091, and 81573354) and the National Key Research and Development Program of China (2017YFC1103501).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2020.115306.

References

1. Crawford ED, Moul JW, Sartor O, Shore ND. *Expert Opin Drug Metab Toxicol.* 2015;11:1465–1474.
2. Weckermann D, Harzmann, Hormone R. *Eur Urol.* 2004;46:279–284.
3. Wilson AC, Vadakkadath Meethal S, Bowen RL, Atwood CS. *Expert Opin Inv Drug.* 2007;16:1851–1863.
4. Teutonico D, Montanari S, Ponchel G. *Expert Opin Drug Del.* 2012;9:343–354.
5. Trachtenberg JJ. *Urology.* 1983;129:1149–1151.
6. Sennello LT, Finley RA, Chu S, et al. *J Pharm Sci.* 1986;75:158–160.
7. Lee D, Kim S, Choi G, Lee Y, Cho H. *Molecules.* 2018:23.
8. Sophocleous AM, Desai KH, Mazzara JM, et al. *J Control Release.* 2013;172:662–670.
9. Okada H, Heya T, Igari Y, Ogawa Y, Toguchi H, Shimamoto T. *Int J Pharm.* 1989;54:231–239.
10. Periti P, Mazzei T, Mini E. *Clin Pharmacokinet.* 2002;41:485–504.
11. Ki M, Lim J, Ko J, et al. *J Control Release.* 2014;185:62–70.
12. Hu Q, van Gaal EVB, Brundel P, et al. *J Control Release.* 2015;205:98–108.
13. Ferrati S, Fine D, You J, et al. *J Control Release.* 2013;172:1011–1019.
14. Veronese FM. *Biomaterials.* 2001;22:405–417.
15. Zalipsky S. Chemistry of polyethylene glycol conjugates with biologically active molecules. *Adv Drug Deliver Rev.* 1995;16:157–182.
16. Veronese FM, Monfardini C, Caliceti P, Schiavon O, Scrawen MD, Beer D. *J Control Release.* 1996;40:199–209.
17. Roberts MJ, Bentley MD, Harris JM. *Adv Drug Deliver Rev.* 2012;64:116–127.
18. Monkarsh SP, Ma Y, Aglione A, et al. *Anal Biochem.* 1997;247:434–440.
19. Glue P, Fang JWS, Rouzier-Panis R, et al. *Clin Pharmacol Ther.* 2000;68:556–567.
20. Clark R, Olson K, Fuh G, et al. *J Biol Chem.* 1996;271:21969–21977.
21. Hinds K, Koh JJ, Joss L, Liu F, Baudys M, Kim SW. *Bioconjug Chem.* 2000;11:195–201.
22. Lee KC, Tak KK, Park MO, et al. *Pharm Dev Technol.* 1999;4:269–275.
23. Zhao Y, Xu X, Wen N, et al. *Sci Rep.* 2017;7:5524.
24. Chang L, Lee H, Chung M, Yang VC. *Bioconjug Chem.* 2005;16:147–155.
25. Han J, Fei Y, Zhou F, et al. *Brit. J. Pharmacol.* 2018;175:544–557.
26. Hamley IW. *Biomacromolecules.* 2014;15:1543–1559.
27. Park EJ, Na DH. *Anal Chem.* 2016;88:10848–10853.
28. Janson J. *Protein purification: principles, high resolution methods, and applications.* John Wiley & Sons; 2011.
29. Wang Y, Youngster S, Bausch J, Zhang R, McNemar C, Wyss DF. *Biochemistry.* 2000;39:10634–10640.
30. Wang Y, Youngster S, Grace M, Bausch J, Bordens R, Wyss DF. *Adv Drug Deliv Rev.* 2002;54:547–570.
31. Fishburn CS. *J Pharm Sci.* 2008;97:4167–4183.
32. Sica G, Iacopino F, Settesoldi D, Zelano G. *Eur Urol.* 1999;35(suppl 1):2–8.
33. Ogawa Y, Okada H, Heya T, Shimamoto T. *J Pharm Pharmacol.* 1989;41:439–444.
34. Chodak GW. *Rev Urol.* 2004;6(Suppl 8):S18–S23.
35. Okada H, Doken Y, Ogawa Y, Toguchi H. *Pharm Res.* 1994;11:1199–1203.
36. Di L. *AAPS J.* 2014;17:134–143.
37. Zhao Y, Yuan Q, Li C, Guan Y, Zhang Y. *Biomacromolecules.* 2015;16:2032–2039.
38. Zhao Y, Gu J, Jia S, Guan Y, Zhang Y. *Soft Matter.* 2016;12:1085–1092.
39. Wen N, Dong Y, Song R, et al. *Biomacromolecules.* 2018;19:1918–1925.
40. Zhang S, Han J, Leng G, et al. *J Chromatogr B.* 2014;965:183–189.
41. Gómez-Canela C, Cortés-Francisco N, Ventura F, Caixach J, Lacorte S. *J Chromatogr A.* 2013;1276:78–94.
42. Zhan Y, Chen X, Zhao X, Zhong D. *J Chromatogr B.* 2009;877:3194–3200.
43. Lee D, Heo S, Kim S, Lee Y, Cho HJ. *Pharm. Investig.* 2017;47:531–540.