Engineering a protease-based and site-specific PEGylation-based strategy for the controlled release of exenatide

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Using the commercially available antidiabetic drug exenatide (exendin-4) as a model peptide, we designed a novel exenatide derivative, termed LEX-1, comprising a 12-mer albumin-binding peptide, a protease-sensitive linker and a native exenatide. In addition, site-specific PEGylation was performed using LEX-1 as a lead sequence to generate four exenatide derivatives (LEX-2 to LEX-5). Moreover, we determined the optimal molecular weight of maleimide-derivatized PEG for the site-specific PEGylation of LEX-1 by an in vitro stability assay and an in vivo hypoglycemic efficacy test. As a result, LEX-3 (PEG10 kDa) exerted enhanced proteolytic stability, rational release rate of free exenatide and the best glucose-stabilizing capability compared with others. In addition, the prolonged hypoglycemic effects of LEX-1 and LEX-3 were demonstrated in type 2 diabetic mice by multiple OGTTs and a hypoglycemic duration test. Furthermore, a pharmacokinetic test was conducted using Sprague Dawley (SD) rats: LEX-3 (PEG10 kDa) showed the best circulating t_{1/2} of ~119.7 h for exenatide release from LEX-3, suggesting that LEX-3 has the potential to be developed into a once-weekly antidiabetic agent. The consecutive 8 week treatment of both LEX-1 and LEX-3 exhibited enhanced beneficial efficacies on body weight gain, cumulative food intake, % fat and hemoglobin A1c (HbA1c) reduction compared with exenatide treatment. Meanwhile, the chronic administration of LEX-1 and LEX-3 also effectively improved the blood biochemical indexes. Our results indicate the enhanced antidiabetic effects of LEX-1 and LEX-3, and our strategy of PEGylation and albumin conjugation can be applied to other bioactive agents.

Introduction

Numerous therapeutic peptides have already been investigated for medical applications. It is well-known that the efficacy of a therapeutic agent is mainly dependent on its in vivo lifespan. However, the efficacies of pharmaceutical peptides are limited due to their rapid clearance in circulation, resulting in poor druggability. To date, mechanisms including glomerular filtration in the kidney and proteolysis by proteases are the reasons for the short t_{1/2} of peptide drugs. The increase in the molecular size of protein drugs reduces the renal clearance and further extends the in vivo exposure of peptide therapies. As a result, extensive research has been carried out to prolong the in vivo lifespan of peptide drugs. Among these methods, a certain domain of polypeptides, which can bind to a plasma protein, such as human serum albumin (HSA), or suitable site-specific PEGylation has shown potential to prolong the short in vivo t_{1/2} of the therapeutic agents.

HSA, as one of the most useful in vivo molecule transporters, exerts a maximum t_{1/2} of ~19 days. Peptides or proteins that bind tightly with HSA exert longer lifespans compared with the free ones. Similarly, a site-specific PEGylation technology, covalent attachment of PEG to targeted peptides or has been applied for drug modification to make the existing biopharmaceuticals clinically more effective than before. The active sites of modified peptides or proteins for enzymes or antibodies are protected by PEG conjugation, but the size of the PEG chain and the modified sites are closely related to the biological activity of the peptides or proteins. For example, the application of random PEGylation may result in an exponential decrease in biological activity because the attachment of PEGs may reject the binding of the target molecules.

According to a previously reported protease-based strategy, we used exendin-4, a clinically approved therapeutic drug for type 2 diabetes with a short elimination t_{1/2} of 3–4 h, as a domain of bioactive peptides to design new agents. We selected the albumin-binding domain (LPHSRHALSLPP), which possesses high binding affinity (K\text{d} = 1.67 \mu M) for HSA, to construct an albumin-binding fusion peptide. Previous research has proved that thrombin is specific for the sequence Phe (F)-
Asn (N)-Pro (P)-Arg (R) or Lys (K)-Xxx (X)-Zxx (Z), while dipeptidyl peptidase-4 (DPP4) is specific for an N-terminal dipeptide with Pro (P) or Ala (A). The optimized protease-cleavable linker sequence was determined as FNPKCP, which is scissile to protease hydrolysis by human thrombin and DPP4, and generated a novel fusion exendin-4 derivative, termed LEX-1. Furthermore, we used LEX-1 as an active intermediary agent for site-specific PEGylation (5, 10, 20, and 30 kDa) at the 17th position of cysteine to generate LEX-2 to LEX-5 for the delayed release rate of exendin-4. After entering into the blood circulation, the newly designed exendin-4 derivatives can be protected from elimination by glomerulus and from proteinase due to the dual protection activities of albumin binding and PEGylation. When the derivative was dissociated from HSA, the linker was consequently digested by proteinase including thrombin and DPP4, and thereby the native exendin-4 was slowly released into the circulation at a proper rate controlled by PEGylation.

In the present study, we determined the suitable molecular weight of PEG by evaluating the hypoglycemic effects of LEX-2 to LEX-5 in fasted C57BL/6J mice. The antidiabetic efficacies and pharmacokinetic characteristics of LEX-1 and its PEGylated analog were further assessed using db/db mice and SD rats, respectively.

Experimental

Materials and animals
Exendin-4 and LEX-1 were prepared by a previously reported method of solid-phase synthesis.26,27 PEGylated derivatives were prepared by site-specific conjugation of LEX-1 with a free thiol group with maleimide-derivatized PEG. Peptides were evaluated by mass spectrometry and reversed-phase high-performance liquid chromatography (RP-HPLC) with a purity of more than 95%. Six to eight-week-old male C57BL/6J mice, db/db mice and SD rats were allowed to access water freely and feed separately in accordance with their different sources and different experimental purposes. All animal experimental procedures were approved by the Harbin Medical University Animal Ethics Committee (approval code: HMAE192013) and performed according to the experimental regulations.

Preparation and characterizations of LEX peptides
Maleimide-derivatized PEG (1 mM) in 1 mL of 0.1% DMSO at 4 °C for 24 h. The final mixture was purified by RP-HPLC using a SHIMADZU C18 column (10 × 250 mm, 5 μm), and LEX-2 to LEX-5 were collected. Separately, LEX peptides were detected by high-performance liquid chromatography. The accurate molecular weights of LEX peptides were identified using an Autoflex III smartbeam-MALDI-TOF/MS. The characterization of the fusion peptides bound to HSA were performed by the surface plasmon resonance (SPR) using a BIAcore 8K system (G& Electronics, USA) according to the manufacturer’s instructions.

Plasma stability tests
Compared with the native exendin-4, in vitro stabilities of five exendin-4 analogues in a rat kidney homogenate and plasma were researched. At 37 °C, six kinds of samples (native exendin-4, LEX-1 and LEX-2 to -5) were incubated in the plasma and supernatant of the homogenized rat kidney at a final concentration of 1 mM. Sex replicates were carried out and the collected samples were frozen in liquid nitrogen. Subsequently, the collected samples were measured using the commercially available exendin-4 ELISA kits brought from Millipore (Merck, Germany).

Protease cleavage test
The controlled release of exendin-4 from LEX-1 to LEX-5 was analysed in a PBS buffer (pH 7.4) containing thrombin at a concentration of 0.4 U mL⁻¹. The reaction mixture was incubated at 37 °C for 104 h shielded from light and was taken out at 0, 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 88, 96 and 104 hours, respectively. Finally, the ELISA and LC-MS/MS methods were used to analyze the released exendin-4, respectively.

Multiple OGTTs in fasted db/db mice
In this study, modified OGTTs were conducted in male C57BL/6J mice and db/db mice under overnight (18 h) fasting conditions. The animals were allocated based on body weight and initial BGLs. A single subcutaneous injection of exendin-4 and LEX-1 to LEX-5 (25 nmol kg⁻¹) was given to the male C57BL/6J mice 0.5 h before the glucose administration at a dose of 2.0 g kg⁻¹ body-weight. Meanwhile, the glucose values of blood samples were detected pre-dose and at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3 h after each administration of D-glucose using a hand-held glucometer. The AUC was calculated from 0 to 3 h, 72 to 75 h and 144 to 147 h, respectively, to assess the in vivo hypoglycemic efficacies.

Hypoglycemic efficacies in non-fasted db/db mice
The animals received a single s.c. administration of saline, exendin-4, LEX-1 and LEX-3 (25 and 250 nmol kg⁻¹ body wt). The samples were measured at various time-points (0, 0.5, 1, 2, 4, 8, 12, 16, 24, 32, 48, 72, 96, 120 and 144 h). The glucose-lowering effects within the treatment period (0–144 h) were also assessed by calculating the area under the curve (AUC) of different groups.

Pharmacokinetics tests
The samples were collected after a single subcutaneous injection of exendin-4 (25 nmol kg⁻¹), LEX-1 (25 and 250 nmol kg⁻¹) and LEX-3 (25 and 250 nmol kg⁻¹) at various time-points (0, 0.5, 1, 2, 3, 4, 6, 12, 24, 48, 72, 96, 120 and 144 h). The free exendin-4 concentration of the collected samples was detected to be within the range of 0.01–3.20 μg mL⁻¹ using commercial exendin-4 ELISA kits as mentioned above. The intact LEX-1 or LEX-3 concentration was measured by the established LC-MS/MS method according to the previous reports.

Chronic studies
Male type 2 diabetic mice were grouped according to the average body weight and were treated with exendin-4 (250 nmol kg⁻¹), LEX-1 (25 and 250 nmol kg⁻¹) and LEX-3 (25 and 250 nmol kg⁻¹) for 8 consecutive weeks. The cumulative food intake and body
weight were measured once every four days, and % of body mass was detected after the treatment cycle. After 8 week administration, OGTT was performed after a week wash-out period. The measurement of % HbA1c values and blood biochemical parameter levels was performed using an automatic biochemical analyzer.

**Statistical analysis**

All data are expressed as means ± SD. The differences in all parameters were tested using one-way ANOVA. The $P$ values lower than 0.05 were considered significant.

**Results**

**Characterization of exendin-4 derivatives**

LEX-1 was prepared by a previously reported method of solid-phase synthesis, and optimized site-specific PEGylation was employed to obtain LEX-2 (5 kDa), LEX-3 (10 kDa), LEX-4 (20 kDa) and LEX-5 (30 kDa).20,21 The elution and purity profiles of LEX peptides were identified by RP-HPLC, and the results indicated that the elution time of exendin-4 was 15.4 min, LEX-1 was 16.4 min, LEX-2 was 16 min, LEX-3 was 15.7 min, LEX-4 was 14.2 min and LEX-5 was 13.2 min. In addition, the purity of all the peptides was over 99% (Fig. 2A). The MALDI-TOF/MS was further performed to identify exendin-4 and its derivatives. By further peptide mapping, the calculated molecular weights of the five molecules well match the theoretical values.

Binding constants of LEX peptides for HSA including the association rate constant ($k_a, M^{-1} s^{-1}$), dissociation rate constant ($k_d, s^{-1}$) and overall affinity constant ($K_D, M$) are listed in Table 1. The results indicated that LEX-3 ($0.22 \times 10^{-6} M$) associated with HSA with higher affinity than LEX-1 ($1.33 \times 10^{-6} M$), LEX-2 ($3.16 \times 10^{-6} M$), LEX-4 ($4.31 \times 10^{-6} M$) or LEX-5 ($3.12 \times 10^{-6} M$).

**Fig. 1** Strategy for multistage sustained release of bioactive peptides via protease cleavage and site-specific PEGylation.

**Fig. 2** Characterizations of exenatide derivatives. (A) HPLC chromatograms and (B) mass spectra of exendin-4, and LEX peptides.
Table 1 Binding affinities of LEX peptides for HSA

| Molecule | $k_a$ (M\(^{-1}\) s\(^{-1}\)) | $k_d$ (s\(^{-1}\)) | $K_D$ (M) |
|----------|----------------|----------------|----------|
| LEX-1    | $1.38 \times 10^4$ | $1.83 \times 10^{-2}$ | $1.33 \times 10^{-6}$ |
| LEX-2    | $1.14 \times 10^4$ | $3.60 \times 10^{-2}$ | $3.16 \times 10^{-6}$ |
| LEX-3    | $1.28 \times 10^4$ | $0.28 \times 10^{-2}$ | $0.22 \times 10^{-6}$ |
| LEX-4    | $1.24 \times 10^4$ | $5.33 \times 10^{-2}$ | $4.31 \times 10^{-6}$ |
| LEX-5    | $2.01 \times 10^4$ | $6.27 \times 10^{-2}$ | $3.12 \times 10^{-6}$ |

Proteolytic stabilities of LEX peptides

The susceptibility of peptides or proteins to enzymatic digestion is one of the barriers to obtain prolonged bioactivities.\(^{26}\) The stability tests were performed with the rat kidney homogenate and plasma to evaluate the proteolytic stabilities of LEX-1 to LEX-5. As demonstrated in Fig. 3, a rapid decrease in the concentration of exendin-4 was shown with a $t_{1/2}$ of 31.2 ± 5.9 min in the rat kidney homogenate. In contrast, LEX-1 to LEX-5 exert significantly prolonged half-lives compared with the native exendin-4 (56.5 ± 12.3 min, 92.2 ± 13.4 min, 123.1 ± 21.5 min, 152.9 ± 21.3 min and 169.4 ± 22.0 min vs. 31.2 ± 5.9 min, respectively). Similarly, the $t_{1/2}$ of LEX-1 to LEX-5 was also notably prolonged compared with exendin-4 in rat plasma (6.8 ± 0.8 h, 7.5 ± 1.7 h, 17.0 ± 2.3 h, 17.1 ± 2.0 h and 34.8 ± 4.2 h vs. 3.2 ± 0.5 h), suggesting that the strategy of albumin binding and PEGylation in LEX-1 strikingly improved the in vitro proteolytic stability of the native exendin-4.

Evaluation of the controlled release of exendin-4

As shown in Fig. 4, the concentration of exendin-4 transiently released from LEX peptides via the plasma TBN was detected. The concentration of released exendin-4 varies as a function of time during protease digestion and was monitored by an ELISA method. As a result, LEX-1 exerts a very fast release of exendin-4 due to thrombin digestion when they enter the blood circulation. However, the concentration of the intact LEX-1 was also rapidly decreased and became nearly exhausted after 48 h. PEGylation of LEX-1 effectively slows digestion effects of blood proteases and the peak concentration of free exendin-4 in LEX-2 to LEX-5 groups, especially for LEX-4 and LEX-5, all lower than that of LEX-1. Moreover, the duration of controlled release of exendin-4 in PEGylated LEX-1 was significantly prolonged. However, the concentration of transient exendin-4 in both the LEX-4 and LEX-5 groups was obviously lower than those of LEX-1 to LEX-3 probably due to the larger molecular weight of PEG. Furthermore, a very low concentration of free exendin-4 may not exert effective hypoglycemic actions. Therefore, we need to perform in vivo experiments to screen the suitable molecule for further preclinical studies.

Glucose-stabilizing capabilities of LEX peptides

In order to confirm the suitable molecule weight of PEG, we further identified the glucose-stabilizing capabilities of LEX-1 to LEX-5 by modified multiple oral glucose tolerance tests (OGTTs) in normal male C57BL/6j mice. As evidenced in Fig. 5, the blood glucose levels (BGLs) of the placebo-treated group promptly reached the peak at 15 min and then slowly dropped to baseline (approximately 5 mmol L\(^{-1}\)) after 2 h, while the native exendin-4, LEX-1 and LEX-2 to LEX-5 resulted in considerable hypoglycemic effects in the first OGTT. Remarkably, the glucose-lowering effects of LEX-4 and LEX-5 were worse than those of other conjugates probably due to the increased steric hindrance caused by the larger molecular weight of PEG, which affected the proper release of exendin-4. Interestingly, better glucose-
stabilizing capability of LEX-3 was observed during the third OGTT, suggesting longer hypoglycemic duration and effects than other conjugates. Therefore, LEX-3 was selected to evaluate further in vivo bioactivities.

**Hypoglycemic duration tests of LEX-1 and LEX-3**

To further evaluate the antihyperglycemic efficacies of LEX-1 and LEX-3, hypoglycemic duration tests were investigated in non-fasting diabetic mice. The BGLs of exendin-4 (250 nmol kg\(^{-1}\)), LEX-1 (25 and 250 nmol kg\(^{-1}\)) and LEX-3 (25 and 250 nmol kg\(^{-1}\)) treated groups all rapidly decreased to the hypoglycemic state (< 8.35 mmol L\(^{-1}\)) in 4 h and the time required to rebound to initial level were nearly 48, 72, 120, 120 and 144 h, respectively, while the placebo-treated group exhibited a hyperglycemic state (>20 mmol L\(^{-1}\)) during the entire experiment (0–144 h) (Fig. 5A). Furthermore, LEX-3 at 250 nmol L\(^{-1}\) remarkably decreased the area under the curve (AUC) to 72.5% for a treatment period of 0–144 h compared with the placebo-treated group, while for exendin-4- and LEX-1-treated groups it was only 18.5% and 58.0% at the same dose, indicating that the sustained release effects from protease cleavage and site-specific PEGylation could notably prolong the hypoglycemic duration of LEX-3 (Fig. 5B). In short, LEX-3 showed enhanced hypoglycemic efficacy compared with LEX-1 or exendin-4.

**Glucose-stabilizing capability tests of LEX-1 and LEX-3**

We further assessed in vivo glucoregulatory activities of LEX-1 and LEX-3 using multiple OGTTs in overnight fasted diabetic mice. These mice were subcutaneously injected with the doses of exenatide (250 nmol kg\(^{-1}\)), LEX-1 (25 and 250 nmol kg\(^{-1}\)) and LEX-3 (25 and 250 nmol kg\(^{-1}\)) at 0.5 h before first treatment of D-glucose. As illustrated in Fig. 7A, the glucose level of placebo-treated groups rapidly showed a peak at half an hour after each round of administration of glucose and then slowly returned to the baseline level within 3 h. As shown in Fig. 7A, the injection of exendin-4, LEX-1 and LEX-3 all obviously reduced the BGL compared with the control during the first period of multiple OGTTs (P < 0.01). Nevertheless, exendin-4 was completely ineffective after 10 h, while the treatment of LEX-1 and LEX-3 resulted in significant glucose-lowering effects during the experimental period (0–144 h) at the same dose. In particular, a single s.c. administration of LEX-3 exerts higher AUC-lowering percentage than LEX-1 in the third

![Image](image_url)
period of multiple OGTTs (24.0% vs. 18.3%, versus placebo-treated groups) (Fig. 7B–D). The above-mentioned results suggested that glucose-stabilizing capabilities of LEX-1 and LEX-3 at 250 nmol kg\(^{-1}\) could sustain over 74 h and 144 h, respectively.

In vivo pharmacokinetics of LEX-1 and LEX-3

Albumin-binding and PEGylation significantly improved the pharmacokinetic properties of modified exendin-4 derivatives in Sprague Dawley (SD) rats. As we expected, the plasma concentration of exendin-4 peaked within an hour, and then quickly returned to the original blood glucose level at 12 h post-injection with a calculated \(t_{1/2}\) of 4.4 ± 0.9 h, whereas the \(t_{1/2}\) of intact LEX-1 or LEX-3 were 78.4 ± 11.5 h or 119.7 ± 15.0 h, and that of released exendin-4 were 67.4 ± 9.8 h or 109.6 ± 10.4 h, respectively, at a dose of 250 nmol kg\(^{-1}\) (Fig. 8). From the combined results of the hypoglycemic duration test and multiple OGTTs, it was found that LEX-3 has the potential to be developed as a once-weekly antidiabetic agent.

Chronic studies

As LEX-1 and LEX-3 exert remarkable in vivo efficacies and metabolic characters in acute pharmacodynamic and pharmacokinetic studies, we further performed chronic studies to evaluate their ulteriorly therapeutic potential. The diabetic mice subcutaneously received LEX-1 and LEX-3 at doses of 25 and 250 nmol kg\(^{-1}\) twice-weekly for 8 consecutive weeks using exendin-4 as the positive control. As shown in Fig. 9A and B, cumulative food intake and body weight gain significantly decreased in
both LEX-1- and LEX-3 (25 and 250 nmol kg\(^{-1}\) body wt)-treated mice but continuously increased for the placebo-treated ones. Moreover, LEX-3 treatment exerts more significant suppression effects than exendin-4 and LEX-1 at the same dose (\(P < 0.05\)). Similarly, long-term treatment of LEX-1 and LEX-3 at 250 nmol kg\(^{-1}\) resulted in a reduction in fat (% of body mass) for 41.1% and 56.0%, respectively, while it was only 10.6% after exendin-4 treatment compared with the placebo-treated ones (Fig. 9C).

To determine whether long-term administration of LEX peptides effectively improved glucose metabolism in diabetic mice, we conducted an OGTT at weeks 8 and 9 (after a week wash-out period). It was found that 8 week treatment of LEX-3 exerted significantly improved glucose-stabilizing effects in db/db mice compared with LEX-1- or exendin-4-treated ones (Fig. 9D). Interestingly, the results graphed in Fig. 9E revealed that the LEX-3-treated group exhibited a significantly higher AUC-lowering effect than the exendin-4- and LEX-1-treated group (44.3% vs. 26.7% and 37.3%, \(P < 0.02\) and \(P < 0.05\), respectively, compared with the placebo-treated group). The following % HbA1c values, which represent the cumulative blood glucose concentrations for more than 3 months,\(^{27}\) were further detected, and the results are shown in Fig. 9F. LEX-3 treatment significantly improved the reduction of % HbA1c values compared with exendin-4 or LEX-1 treatment, indicating

![Fig. 9](image_url)  
**Fig. 9** Chronic in vivo studies of LEX-1 and LEX-3 in diabetic mice. Efficacies of chronic treatment of LEX peptides on (A) food intake, (B) body weight gain, (C) % fat, (D) time–BGLs curve, (E) OGTT AUC and (F) % HbA1c of db/db mice. All results are shown as the means ± SD (\(n = 8\)). ***\(P < 0.001\), **\(P < 0.02\), *\(P < 0.05\).

![Fig. 10](image_url)  
**Fig. 10** Efficacies of 8 consecutive weeks of administration of LEX peptides on the blood biochemical indexes in diabetic mice. All results are expressed as means ± SD (\(n = 8\)). ***\(P < 0.001\), **\(P < 0.02\), *\(P < 0.05\).
that PEG conjugation notably enhanced the in vivo stability and induced better potential therapeutic utilities.

After the 8 week experimental period, the mice were killed and the blood biochemical indexes were measured (Fig. 10). A significant decline in values of either total cholesterol (TC) or triglyceride (TG) was observed in LEX-1- and LEX-3- (250 nmol kg⁻¹) treated groups. Herein, the activities of ALT and AST were determined to assess the hepatocyte damage. As we expected, treatment with both LEX-1 and LEX-3 at a dose of 250 nmol kg⁻¹ resulted in a remarkable reduction compared with placebo treatment ($P < 0.05$). Furthermore, long-term treatment with LEX-3 at a dose of 250 nmol kg⁻¹ decreased the LDL/HDL ratio up to 30.9%, while exendin-4 and LEX-1 exert a reduction of only 20.9% and 25.4% at the same doses, respectively.

**Discussion**

Therapeutic effects of polypeptide drugs need frequent injection to sustain the effective duration due to rapid renal filtration or proteolysis. Hence, many strategies have been applied to achieve administration efficacies of peptide drugs in vivo.²⁻⁻²⁻⁻⁻⁻²⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓
found that LEX-3 exerts a significantly higher AUC-lowering percentage than exendin-4 or LEX-1 at the same dose ($P < 0.02$ and 0.05) (Fig. 9D–E). % HbA1c, a well-known indirect indicator of cumulative blood glucose concentrations, was a more sensitive index of glycemic control than glucose concentration.\textsuperscript{a} As presented in Fig. 9F, an obvious reduction in the % HbA1c value was observed in LEX-3-treated groups, which was significantly lower than those of exendin-4 and LEX-1-treated ones (both $P < 0.05$). Furthermore, chronic treatment with LEX peptides significantly improved the blood biochemical indexes in diabetic mice (Fig. 10).

**Conclusion**

A novel anti-diabetic drug, LEX-1, has been designed by the fusion of an albumin-binding domain and a thrombin-cleavable linker to a native exendin-4. Furthermore, the site-specific PE\textsubscript{G}ylation (10 kDa) of LEX-1 generated a long-acting exendin-4 derivative, termed LEX-3. Considering that exenatide (exendin-4) is a FDA-approved anti-diabetic drug, LEX-1 and LEX-3, which could stably release the native exendin-4, hold potential to be developed as promising once-weekly anti-diabetic agents. This multistage sustained-release strategy could also be applied for other peptides with short lifespans.

**Author contributions**

Conceptualization, Mei Dong; Data curation, Aihong Zhang; Methodology, Aihong Zhang and Yin Li; Supervision, Aihong Zhang, and Yin Li; Validation, Mei Dong; Writing-original draft, Aihong Zhang; Writing-review & editing, Yin Li.

**Conflicts of interest**

Authors declare no conflict of interest.

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**Notes and references**

1 J. S. Ryu, A. Y. Cho, W. S. Sang and H. Min, *Methods Mol. Biol.*, 2014, 1088, 35–50.
2 Y. Shechter, M. Mironchik, S. Rubinraut, A. Saul, H. Tsubery and M. Fridkin, *Bioconjugate Chem.*, 2005, 16, 913–920.
3 D. P. McGregor, *Curr. Opin. Pharmacol.*, 2008, 8, 616–619.
4 L. Benzi, P. Cecchetti, A. Ciccarone, A. Pilo, G. Di Cianni and R. Navalesi, *Diabetes*, 1994, 43, 297–304.
5 D. B. M. Fep and D. H. Z. Fep, *J. Clin. Pharmacol.*, 2013, 52, 548–625.
6 J. Lin and A. Lu, *Pharmacol. Rev.*, 1997, 49, 403–449.
7 D. Kumar Malik, S. Baboota, A. Ahuja, S. Hasan and J. Ali, *Curr. Drug Delivery*, 2007, 4, 141–151.
8 R. Pawar, A. Ben-Ari and A. J. Domb, *Expert Opin. Biol. Ther.*, 2004, 4, 1203–1212.
9 H. Chen, W. Guohao, L. Xixin, J. Orit, D. Kiesewetter, L. Yi, M. Ying, Z. Xianzhong, W. Hua and Z. Lei, *Theranostics*, 2016, 6, 243–253.
10 F. M. Veronese and G. Pasut, *Drug Discovery Today*, 2005, 10, 1451–1458.
11 S. Mitragotri, P. A. Burke and R. Langer, *Nat. Rev. Drug Discovery*, 2014, 13, 655–672.
12 D. Sleep, J. Cameron and L. R. Evans, *Biochim. Biophys. Acta*, 2013, 1830, 5526–5534.
13 N. Gong, A.-N. Ma, L.-J. Zhang, X.-S. Luo, Y.-H. Zhang, M. Xu and Y.-X. Wang, *Br. J. Pharmacol.*, 2011, 163, 399–412.
14 Y. Chi, H. Zhang, J. Zhou, W. Huang and S. Ni, *Lett. Org. Chem.*, 2008, 5, 399–402.
15 C. Yushi, Z. Huibin, H. Wenlong and Z. Jinpei, *Bioorg. Med. Chem.*, 2008, 16, 7607–7614.
16 J. Han, L. Sun, X. Huang, Z. Li and W. Huang, *Br. J. Pharmacol.*, 2014, 171, 5252–5264.
17 M. C. Guillin, A. Bezeaud, M. C. Bouton and M. Jandrot-Perrus, *Thromb. Haemostasis*, 1995, 74, 129–133.
18 K. Bohensztajn, M. P. Peppelenbosch and C. A. Spek, *Trends Mol. Med.*, 2008, 14, 429–440.
19 T. J. Wang, J. Li, L. J. Liang, M. Zhong, X. H. Chen and B. I. Kai shun, *Chin. J. Pharm. Anal.*, 2007.
20 A. Zorzi, S. J. Middendorp, J. Wilbs, K. Dyle and C. Heinis, *Nat. Commun.*, 2017, 8, 16092.
21 R. Malka, D. M. Nathan and J. M. Higgins, *Sci. Transl. Med.*, 2016, 8, 359ra130.
22 J. Lee, C. Lee, I. Kim, H. R. Moon, T. H. Kim, K. T. Oh, E. S. Lee, K. C. Lee and Y. S. Yoon, *Int. J. Pharm.*, 2012, 424, 50–57.
23 L. Diao and B. Meibohm, *Clin. Pharmacokinet.*, 2013, 52, 855–868.
24 A. P. Chapman, *Adv. Drug Delivery Rev.*, 2002, 54, 531–545.
25 S. Choi, M. Baudys and S. Kim, *Pharm. Res.*, 2004, 21, 827–831.
26 J. Buse, R. Henry, J. Han, D. Kim, M. Fineman and A. Baron, *Diabetes Care*, 2004, 27, 2628–2635.
27 T. H. Kim, H. H. Jiang, S. Lee, Y. S. Yoon, C. W. Park, Y. Byun, X. Chen and K. C. Lee, *Bioconjugate Chem.*, 2011, 22, 625–632.
28 A. K. Saleh and M. A. A. Moussa, *Clin. Chem.*, 1985, 31, 1872–1876.
29 R. Webster, V. Elliott, B. Park, D. Walker, M. Hankin and P. Taupin, *PEGylated Protein Drugs: Basic Science and Clinical Applications*, 2009, pp. 127–146, DOI: 10.1007/978-3-7643-8679-5_8.