Biochemical Pharmacology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Site-specific fatty chain-modified exenatide analogs with balanced glucoregulatory activity and prolonged in vivo activity

Lidan Sun [a,1], Xun Huang [b,1], Jing Han [a,c], Xingguang Cai [a], Yuxuan Dai [a], Yingying Chu [a], Chuandong Wang [d], Wenlong Huang [a,*], Hai Qian [a,*]

[a] Jiangsu Key Laboratory of Drug Discovery for Metabolic Diseases and State Key Laboratory of Natural Medicines, Center of Drug Discovery, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China
[b] Division of Antitumor Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200090, China
[c] School of Chemistry and Chemical Engineering, Jiangsu Normal University, Xuzhou 221116, China
[d] Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Jiao Tong University School of Medicine and Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

ARTICLE INFO

Article history:
Received 1 March 2016
Accepted 27 April 2016
Available online xxxx

Keywords:
Ex-4
Cysteine modified
Fatty chain
Protracted antidiabetic effects

ABSTRACT

The therapeutic utility of exenatide (Ex-4) is limited due to short plasma half-life of 2.4 h and thus numerous approaches have been used to obtain a longer action time. However, such strategies often attend to one thing and lose another. The study aimed to identify a candidate with balanced glucoregulatory activity and prolonged in vivo activity. A series of fatty chain conjugates of Ex-4 were designed and synthesized. First, thirteen cysteine modified peptides (**1**–**13**) were prepared. Peptides **1**, **10**, and **13** showed improved glucagon-like peptide-1 (GLP-1) receptor activate potency and were thus selected for second step modifications to yield conjugates **I-1**–**I-9**. All conjugates retained significant GLP-1 receptor activate potency and more importantly exerted enhanced albumin-binding properties and in vitro plasma stability. The protracted antidiabetic effects of the most stable **I-3** were further confirmed by both multiple intraperitoneal glucose tolerance test and hypoglycemic efficacies test in vivo. Furthermore, once daily injection of **I-3** to streptozotocin (STZ) induced diabetic mice achieved long-term beneficial effects on hemoglobin A1C (HbA1C) lowering and glucose tolerance. Once daily injection of **I-3** to diet induced obesity (DIO) mice also achieved favorable effects on food intake, body weight, and blood chemistry. Our results suggested that **I-3** was a promising agent deserving further investigation to treat obesity patients with diabetes.

1. Introduction

Exendin-4, a 39-amino acid polypeptide, is a natural dipeptidyl peptidase-IV (DPP IV) resistant GLP-1 receptor agonist which shares 82% homology with human GLP-1 in N-terminal segment [1,2]. As the first clinically available incretin mimetic, Ex-4 has attracted much interest in terms of the treatment of type 2 diabetes due to beneficial anti-diabetic effects, such as suppressing glucagon secretion, enhancing glucose-dependent insulin secretion, reducing energy intake and gastric mobility, and increasing β-cell mass [3–5]. However, the therapeutic utility of Ex-4 is limited due to short plasma half-life of 2.4 h and thus, the high doses must be administered and frequent injections required which is inconvenient for patients and compliance is low [6].

Abbreviations: Ex-4, exendin-4; T2DM, type 2 diabetes mellitus; GLP-1, glucagon-like peptide-1; DPP IV, dipeptidyl peptidase IV; STZ, streptozotocin; DIO, diet induced obesity; PEG, polyethylene glycol; HSA, human serum albumin; UPLC–MS, ultra performance liquid chromatography–mass spectrometry; RP-HPLC, reversed phase high-performance liquid chromatography; cAMP, cyclic adenosine monophosphate; OGTT, oral glucose tolerance test; AUC, area under the curve; IPGTT, intraperitoneal glucose tolerance test; HFD, high fat diet; LFD, low fat diet; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; HDL, low-density lipoprotein; TFA, trifluoroacetic acid; FBS, fetal bovine serum; BSA, bovine serum albumin; FACS, flow cytometric analysis; HbA1C, hemoglobin A1C.

* Corresponding authors.
E-mail addresses: ydhuangwenlong@126.com (W. Huang), qianhai24@163.com (H. Qian).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bcp.2016.04.016
0006-2952/

To address this issue, numerous approaches, such as the developments of long-acting incretin mimetics or sustained delivery systems have been used to obtain a longer action time [7]. To date, developments described concern three distinct strategies, i.e., increasing molecular size via chemical conjugation to reduce renal elimination [8–10], coupling to biodegradable polymer microspheres confers protracted release from the subcutaneous tissue [11,12] and the introduction of functional moieties that interact physically with biological molecules, like serum albumin [13–15]. In particular, our group has previously investigated the use of GLP-1 bio-conjugated with fatty acids, coumarin and dicoumarin for long-acting delivery [16–18] and found site-specific cysteine-substitution offered an effective and convenient strategy to choose peptides with desired characteristics to make further modifications.

In this study, we firstly introduced cysteine step by step to Ex-4 to identify modified peptides with favorable potency. Considering the unique physicochemical properties of fatty chain-modified conjugates, such as the self-aggregating behavior in aqueous media and effective physical interactions with serum albumin [19], the selected sequences were then covalent coupled to various fatty chains, hypothesizing that the site-specific covalent coupling of Ex-4 to fatty chains might offer a practical strategy of developing long-acting Ex-4 derivatives which could be applied independently or in combination with other sustained release strategies. Moreover, the structural, biological, and physicochemical characteristics of all synthesized Ex-4 conjugates were explored and antidiabetic characteristics of compound **I-3** were investigated to identify its long-acting antidiabetic properties. Furthermore, the long-term beneficial effects of compound **I-3** in streptozotocin induced diabetic model and diet induced obesity mice were evaluated.

2. Experimental section

2.1. Materials and animals

Fmoc-protected amino acids, Fmoc Rink Amide-MBHA resin, liraglutide and Ex-4 were purchased from GL biochem (Shanghai, China). Acetonitrile and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany). cAMP dynamic kit was purchased from Cisbio (Bedford, MA, USA). HbA1c kit was purchased from Glycosal (Deeside, UK). Serum leptin and adiponectin levels using EZML-82K and EZMADP-60K ELISA kits supplied by Linco Research (St. Charles, MO, USA), respectively. Serum TG, TC, HDL, LDL, ALT and AST were assayed on a by Beckman Coulter Chemistry Analyzer (Beckman Coulter, CA, USA). Unless indicated, all other reagents were purchased from Sigma (Saint Louis, MO, USA). Peptides were synthesized in a Discover focused single mode microwave synthesis system (CEM, NC, USA) using microwave irradiation procedures at 2450 MHz and the mass of obtained peptides and target conjugates were confirmed by Waters ACQUITY UPLC Systems (Waters, Milford, MA, USA). Sprague–Dawley rats (SD rats, male, 200–250 g) and Kunming mice (male, 10 weeks old) were purchased from the comparative medical center of Yangzhou University (Jiangsu, China). C57BL/6J mice (male, 6–8 weeks old) were obtained from Jiesijie Laboratory Animal (Shanghai, China). Animals were housed in groups of three (rat) and six (mice) in cages under controlled temperature (22 ± 2 °C) and relative air humidity (set point 50%) with a 12 h light:12 h dark cycle. Tap water and standard laboratory chow were provided ad libitum throughout the study. C57BL/6J mice were fed with a HFD (D12492; 60% fat, 20% protein and 20% carbohydrate; 5.24 kcal/g) or a LDF (D12450B; 10% fat, 20% protein and 70% carbohydrate; 3.85 kcal/g) and watered ad libitum. Both diets were supplied by Research Diets (New Brunswick, NJ, USA). All animal experimental protocols were approved by an ethical committee at China Pharmaceutical University and conducted according to the Laboratory Animal Management Regulations in China and adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (revised 2011). The experiments were conducted in such a way that the number of animals used and their suffering was minimized. Prior to the blood sampling, animals were anesthetized with diethyl ether.

2.2. General synthetic route and HPLC purification of peptides ***1–13***

According to the standard solid-phase peptide synthesis protocol, thirteen cysteine altered peptides (**1–13**) were synthesized [20,21] and further identified by electrospray mass spectrometry after HPLC purification [22,23]. To be brief, Fmoc Rink Amide-MBHA resin underwent repeated procedures of deprotection and coupling with relevant Fmoc-protected amino acids and then the final peptide was cleaved from the resulting resin by reagent K (TFA/thioanisole/water/phenol/EDT, 82.5:5:5:5:2.5) for 1.5 h at room temperature. The crude peptides were purified on Shimadzu preparative RP-HPLC with the following condition: Shimadzu C18 reversed-phase column (5 μm, 340 mm × 28 mm), a linear gradient of mobile phase 30–75% B in 30 min at a flow rate of 6.0 mL/min (mobile phase A: water containing 0.1% TFA, mobile phase B: acetonitrile containing 0.1% TFA).

2.3. General synthetic route of fatty chain modified conjugates ***I-1–I-9***

Three fatty chain-maleimide (1-octyl-1H-pyrrole-2, 5-dione, 1-dodecyl-1H-pyrrole-2,5-dione, and 1-hexadecyl-1H-pyrrole-2,5-dione) were synthesized as previously reported [18]. Cysteine altered peptide (5 μmol) and fatty chain-maleimide (12 μmol) were reacted in 10 mL of 0.05 mol/L sodium phosphate buffer (pH 7.0) at 20 °C under N_2 till UPLC showed completion. The analytical condition was as follows: Acquity UPLC HSS T3 column (1.8 μm, 2.1 mm × 100 mm, Waters); a linear gradient of mobile phase 5–95% B (mobile phase A: water with 0.2% formic acid, mobile phase B: acetonitrile with 0.2% formic acid) in 3.5 min at a flow rate of 0.3 mL/min with ultraviolet (UV) detection at 214 nm. The crude conjugate was purified on Shimadzu preparative RP-HPLC.

2.4. GLP-1 receptor activation assay

The GLP-1 receptor activation assay was conducted as previously reported [18]. Briefly, HEK293 cells over expressing human GLP-1 receptor stably were constructed and used to assess the potency of peptides and conjugates toward the GLP-1 receptor [24,25]. Cells were grown in Dulbecco's modified Eagle's medium-31053 (Invitrogen, Carlsbad, CA, USA) supplemented with 0.5% FBS, 2 mmol/L L-glutamine (Sangon Biotech, Shanghai, China), 20 mmol/L HEPES (Sigma, Saint Louis, MO, USA), 50 units/ml penicillin, and 50 μg/ml streptomycin (Gibco, Grand Island, NY, USA) at 37 °C in 5% CO_2. Cells were plated in 96-well half area, solid black microplates 2 h before the test started. Meanwhile, test articles were solubilized in DMSO and further diluted in medium containing 0.1% BSA fraction V (Genview Scientific, Florida, USA). The resulting solution was added to cells and incubated for 20 min, then assayed for cAMP using the cAMP dynamic 2 kit with homogenous time-resolved fluorescence technology (Cisbio, Bedford, MA, USA) using an Envision 2104 Multilabel Reader according to the manufacturer's instructions. The potency of the conjugates (EC_{50} values) was calculated by sigmoidal curve fitting using GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA).

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2.5. Plasma stability test

Plasma were obtained from adult male Sprague–Dawley (SD) rats and compounds were then incubated with plasma over 72 h using a modification of a previously described method [16]. Samples from each time points underwent solid phase extraction and the resulting extract was analyzed by LC-MS/MS system to assess the profile of plasma degradation [26]. In vitro stability test, the initial concentration in rat plasma of fatty chain modified conjugates **I-1**–**I-9** and the positive controls (Ex-4 and liraglutide) was 1000 ng/mL at 37 °C. At 0, 1, 2, 4, 6, 8, 12, 24, 36, 48 and 72 h time points, 100 μL mixture was aliquoted and extracted on an Oasis HLB 96-well plate (Waters, Milford, MA, USA) and then analyzed by the LC-MS/MS system. The signal of test articles were detected by multiple reaction monitoring with the use of electrospray ionization mass spectrometry on a Sciex API-4000 and Turbo Ionspray (Applied Biosystems, Foster City, CA, USA). The condition of reverse phase liquid chromatographic separation was as aforementioned.

2.6. Albumin binding

The albumin binding properties of the fatty chain-modified conjugates **I-1**–**I-9** were investigated in an albumin-binding assay with the use of albumin-conjugated Sepharose resin according to the reported literatures [18,27]. Firstly, albumin-conjugated resin was prepared by mixing NHS-activated Sepharose 4 fast flow (5 mL, wet resin volume, Amersham Bioscience, Uppsala, Sweden) and human serum albumin (HSA; 33 mg in 10 mM PBS, pH 7.4) to react at room temperature by gentle shaking for 4.5 h. Then, the albumin-conjugated resin was recovered by centrifugation (1000 rpm, 5 min). Meanwhile, the resin was inactivated by hydrolysis of the NHS-active ester to give an albumin-free control resin. The HSA content of the wet resin was 6–7 mg/mL. Conjugates **I-1**–**I-9**, Ex-4 or liraglutide (100 μg/mL in PBS, 50 μL) were mixed with HSA resin or albumin-free resin and incubated for 3 h at room temperature (25 °C). The supernatant was separated by centrifugation (1000 rpm, 10 min) to determine the unbound peptide contents using the aforementioned method by LC-MS/MS system.

2.7. Cell viability assay

Cell viability assay was conducted as previously reported [28,29]. Rat pancreatic INS-1 cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) with 11.2 mM glucose supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM N-2-hydroxyethylpiperazine-N-ethane-sulfonic acid, 50 μM β-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere (5% CO_2). INS-1 cells were seeded in 96-well plates (~5000 cells/well) for 24 h, then cells were treated with glucolipotoxicity media consisting of RPMI media made to 25 mM glucose and 0.4 mM oleate (oleic acid-albumin from bovine serum, Sigma, Saint Louis, MO, USA), or treated with H_2O_2 (50 μM) to induce oxidative stress, or treated with 1 μM streptozotocin (STZ, β-cell-specific DNA-damaging agent) to induce apoptosis followed by the addition of Ex-4 (10 nM), liraglutide (10 nM) or **I-3** (1, 10 or 100 nM). After incubation for 24 h, cell viability was measured by adding 200 g/ml 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Dingguo, Beijing, China) and incubated for 3 h at 37 °C. The reaction was stopped and the purple formazan precipitate formed was dissolved in dimethyl sulfoxide and the color intensity was measured at 550 nm with a multiwall spectrophotometer (Thermo Labsystems, Waltham, MA, USA).

2.8. Detection of apoptotic cells

In flow cytometric analysis (FACS), INS-1 cell were seeded in 96-well plates (~5000 cells/well) for 24 h, then treated with glucolipotoxicity media, H_2O_2 (50 μM) or 1 μM STZ followed by the adding of Ex-4 (10 nM), liraglutide (10 nM) or **I-3** (10 nM) according to the previously reported methods [28,29]. After incubation for 24 h, cells were collected by trypsinization, washed with cold phosphate-buffered saline and incubated with Annexin V (Sigma, Saint Louis, MO, USA) for 15 min, then stained with propidium iodide (Sigma, Saint Louis, MO, USA). Cells in early apoptosis were Annexin V positive and PI negative. The analysis was performed with a FAC Scan flow cytometer using the CellQuest software (BD Biosciences, San Jose, CA, USA).

2.9. Glucoregulatory and insulin secretion assay

The glucoregulatory and insulin secretion assays were carried out in accordance with a previous method [16,18]. Briefly, overnight fasted (12 h) SD rats (n = 6/group, 200–250 g) were orally administrated with **I-3**, Ex-4 and liraglutide (25 nmol/kg) with saline used as negative control. Male SD rats were administered saline, Ex-4, liraglutide or **I-3** half hour prior to oral glucose load (10 g/kg) (the time point was set as 0 min). At −30, 0, 15, 30, 45, 60, 90, 120 and 180 min, blood sample was collected from the cut tip of the tail vein to measure the blood glucose levels using a blood glucose monitor. Meanwhile, blood samples (0.1 ml) were collected in EDTA-containing microcentrifuge tubes from the lateral tail vein at the same aforementioned time point. Plasma samples were then obtained by centrifugation (1000 rpm, 15 min) and assayed for insulin levels using a Rat Insulin ELISA kit (Millipore, Billerica, MA, USA).

2.10. Multiple intraperitoneal glucose tolerance test

Multiple intraperitoneal glucose tolerance test (IPGTT) was carried out on C57BL/6 mice to assess the ability of **I-3** to reduce glucose levels for longer time using a modification of a previously described method [16]. Briefly, the fasted overnight (12 h) male C57BL/6 mice (n = 6/group, 6 weeks) were first intraperitoneally administrated with saline, Ex-4, liraglutide, or **I-3** (25 nmol/kg). Half an hour later, they were intraperitoneally challenged with glucose (2.0 g/kg) (0 h) at the interval of 6 h to simulate the condition of three meals a day. The blood glucose levels were monitored at 0, 0.25, 0.5, 1, 2, 3 h using a blood glucose monitor. The following glucose load time points were at 6 and 12 h after the first IPGTT.

2.11. Hypoglycemic efficacies test

Hypoglycemic efficacies of **I-3** were evaluated in STZ-induced diabetic mice using a modification of a previously described method [13,30]. Kunming mice received a single intraperitoneal injection of saline, Ex-4, liraglutide, **I-3** (25 nmol/kg) with free access to food and water. At 0, 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 30 and 36 h, the second drop of blood was drawn from a tail vein to determine the blood glucose levels by blood glucose monitor. Moreover, we also calculated the hypoglycemic durations with the blood glucose level <8.35 nmol/L (150 mg/dL).

2.12. Chronic in vivo studies

2.12.1. Chronic in vivo studies on STZ-induced diabetic mice

Male KM mice, 6–8 weeks old, weighing 18–22 g, were injected with STZ intraperitoneally (40 mg/kg/day) for 5 consecutive days after fasted overnight. Five days after STZ injection, blood glucose was measured to validate diabetic hyperglycemia. Mice with

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fasting blood glucose level 11.1 mM or higher were assigned to groups with matched body weight and were injected intraperitoneally with 25 nmol/kg Ex-4, liraglutide, or **I-3** once daily for 3 weeks. On days 0, 5, 10, 15, and 20, overnight fasting blood samples were obtained from the tail vein to measure blood glucose levels. At the end of the study, mice were sacrificed and blood samples were collected to determine the levels of HbA1c by Beckman Coulter Chemistry Analyzer (Tokyo, Japan).

2.12.2. Chronic in vivo studies on DIO mice

C57BL/6 mice (6 weeks) were maintained on prescribed HFD for 12 weeks. They were injected intraperitoneally once daily with 25 nmol/kg Ex-4, liraglutide or **I-3**, respectively with saline as control after randomly assigned to treatment groups ($n = 6$) with matched body weight. C57BL/6 mice fed with LFD ($n = 6$/group) were used to index responses to normal values. Water consumption, food intake, and body weight were measured daily. At the end of the study, blood samples were collected and sera separated subsequently for further analyses.

2.13. Data analysis and statistical assessment

Data were analyzed using Prism version 5 software (GraphPad, San Diego, CA, USA). General effects were tested using a 1-way ANOVA with Tukey's multiple-comparison post hoc test. Data throughout are stated as mean ± SD. $p < 0.05$ considered significant.

3. Results

3.1. Synthesis and characterization of the cysteine altered Ex-4 analogs

According to the standard solid-phase peptide synthesis protocol, thirteen cysteine altered Ex-4 analogs were prepared and further identified by Waters ACQUITY UPLC–MS Systems after HPLC purification (Table 1). Furthermore, the potency of all cysteine altered peptides was determined using HEK293 cells over expressing human GLP-1 receptor stably. As shown in Table 1, cysteine altered peptides showed high potency in activating GLP-1 receptor. In particular, compared with Ex-4, peptides **10** and **13** exhibited favorably improved capacity and thus were chosen to further conjugate with fatty chain to develop long-acting Ex-4 conjugates. Moreover, as the substituted sites of **10** and **13** located in the end and central of C-terminal of Ex-4, **2** was also selected to further conjugate with fatty chain to determine whether different conjugation sites affected the activity and long-term characteristics.

3.2. Synthesis and characterization of the fatty chain-modified conjugates ***I-1*–*I-9***

As shown in Table 1, the selected cysteine altered peptides (**2**, **10** and **13**) were reacted with different lengths of fatty chain-maleimide to give nine fatty chain-modified conjugates (**I-1**–**I-9**) (Fig. 1). The crude conjugates were then purified by preparative RP-HPLC and further identified by Waters ACQUITY UPLC–MS

Table 1
Characterization of the cysteine altered Ex-4 peptides and their potency on the cloned human GLP-1 receptor activation.

Peptide	Retention time [a] (min)	Molecular mass		EC_{50} [b], (pM)
		Calculated	Found	
Ex-4-NH_2				1.873 ± 0.912
Cys_{11}-Ex-4-NH_2 (**1**)	0.67	$[M+3H]^{3+}$ 1402.3 $[M+4H]^{4+}$ 1051.9	$[M+3H]^{3+}$ 1402.5 $[M+4H]^{4+}$ 1051.4	45.660 ± 1.420*
Cys_{12}-Ex-4-NH_2 (**2**)	0.49	$[M+3H]^{3+}$ 1388.2 $[M+4H]^{4+}$ 1041.4	$[M+3H]^{3+}$ 1388.2 $[M+4H]^{4+}$ 1041.7	0.407 ± 0.035*
Cys_{14}-Ex-4-NH_2 (**3**)	0.51	$[M+3H]^{3+}$ 1387.5 $[M+4H]^{4+}$ 1040.9	$[M+3H]^{3+}$ 1387.1 $[M+4H]^{4+}$ 1040.5	1.889 ± 0.791
Cys_{15}-Ex-4-NH_2 (**4**)	0.44	$[M+3H]^{3+}$ 1388.2 $[M+4H]^{4+}$ 1041.4	$[M+3H]^{3+}$ 1388.8 $[M+4H]^{4+}$ 1041.4	0.405 ± 0.141*
Cys_{16}-Ex-4-NH_2 (**5**)	0.48	$[M+3H]^{3+}$ 1388.2 $[M+4H]^{4+}$ 1041.4	$[M+3H]^{3+}$ 1387.9 $[M+4H]^{4+}$ 1041.0	0.671 ± 0.432
Cys_{17}-Ex-4-NH_2 (**6**)	0.51	$[M+3H]^{3+}$ 1388.2 $[M+4H]^{4+}$ 1041.4	$[M+3H]^{3+}$ 1387.9 $[M+4H]^{4+}$ 1041.0	0.349 ± 0.171*
Cys_{18}-Ex-4-NH_2 (**7**)	0.62	$[M+3H]^{3+}$ 1407.6 $[M+4H]^{4+}$ 1055.9	$[M+3H]^{3+}$ 1407.6 $[M+4H]^{4+}$ 1056.0	1.220 ± 0.450
Cys_{21}-Ex-4-NH_2 (**8**)	0.61	$[M+3H]^{3+}$ 1393.5 $[M+4H]^{4+}$ 1045.4	$[M+3H]^{3+}$ 1393.9 $[M+4H]^{4+}$ 1045.3	2.105 ± 0.514
Cys_{24}-Ex-4-NH_2 (**9**)	0.44	$[M+3H]^{3+}$ 1388.2 $[M+4H]^{4+}$ 1041.4	$[M+3H]^{3+}$ 1388.4 $[M+4H]^{4+}$ 1041.7	0.262 ± 0.195*
Cys_{25}-Ex-4-NH_2 (**10**)	0.44	$[M+3H]^{3+}$ 1369.2 $[M+4H]^{4+}$ 1027.1	$[M+3H]^{3+}$ 1370.0 $[M+4H]^{4+}$ 1127.7	0.081 ± 0.025*
Cys_{27}-Ex-4-NH_2 (**11**)	0.44	$[M+3H]^{3+}$ 1388.5 $[M+4H]^{4+}$ 1041.7	$[M+3H]^{3+}$ 1388.5 $[M+4H]^{4+}$ 1041.6	148.5 ± 12.5*
Cys_{29}-Ex-4-NH_2 (**12**)	0.57	$[M+3H]^{3+}$ 1412.2 $[M+4H]^{4+}$ 1059.4	$[M+3H]^{3+}$ 1412.4 $[M+4H]^{4+}$ 1059.6	0.499 ± 0.146
Cys_{40}-Ex-4-NH_2 (**13**)	0.66	$[M+3H]^{3+}$ 1430.9 $[M+4H]^{4+}$ 1073.4	$[M+3H]^{3+}$ 1430.5 $[M+4H]^{4+}$ 1073.7	0.093 ± 0.023*

[a] UPLC conditions: 5–95% acetonitrile (mobile phase A: water with 0.2% formic acid, mobile phase B: acetonitrile with 0.2% formic acid) in 3.5 min at a flow rate of 0.3 mL/min with ultraviolet (UV) detection at 214 nm with the use of Acquity UPLC HSS T3 column (1.8 μm, 2.1 mm × 100 mm, Waters).
[b] The receptor potency data are expressed as mean ± SD. All experiments were performed in triplicate and repeated three times ($n = 3$).
* $p < 0.05$, compared with Ex-4.

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Fig. 1. Structure of fatty chain-modified Ex-4 conjugates.

Systems (Table 2). Furthermore, the same receptor activation experiment was conducted to evaluate the potency of **I-1**–**I-9**. As shown in Table 2, slight reduction in the activation potency was observed due to the fatty chain modifications. Notably, the N-terminal modified conjugates (**I-1**, **I-2**, and **I-3**) showed comparable potency to that of the C-terminal modified conjugates (**I-4**–**I-9**), indicating that cysteine-specific conjugations to the N-terminal did not affect the receptor activation potency of GLP-1 for our conjugates. Furthermore, the shorter fatty chain exhibited better receptor activation potency than the longer fatty chain, suggesting that the receptor activation potency was closely related to the length of the fatty chain (Table 2).

3.3. Plasma stability

As the fatty chain-modified conjugates preserved most of their receptor activation capacity, the plasma stability of **I-1**–**I-9** were then performed to evaluate their in vitro stability and compared with Ex-4 and liraglutide. As shown in Fig. 2, Ex-4 had an approximate half-life of 3.6 h in rat plasma at 37 °C, the long-acting GLP-1 agonist liraglutide had a nearly 4-fold longer half-life ($t_{1/2}$ = 15.4 h). As expected, most fatty chain-modified conjugates were more stable than Ex-4 and the longest one was **I-3** ($t_{1/2}$ = 35.6 h). Importantly, plasma half-life values of **I-1**–**I-9** were closely related to the length of the fatty chains.

3.4. Albumin binding

The increased metabolic stability observed above might be attributed to the increased albumin binding affinities of our conjugates. To further prove the hypothesis, albumin binding tests were performed using compounds **I-1**–**I-9**, Ex-4 and liraglutide. As illustrated in Fig. 2 and 86.7 ± 2.5% of liraglutide and 21.0 ± 3.6% of Ex-4 were found to associate with albumin resin respectively under physiological conditions (in PBS pH 7.4). Most fatty chain-modified conjugates showed greatly increased albumin binding ability than Ex-4. Interestingly, the binding ability correlated well with stability. The relationship of in vitro stability and albumin binding ability of conjugates **I-1**–**I-9** revealed in our study was consistent with previous successful reports regarding the protracted in vivo circulation half-life of fatty acid-modified insulin or GLP-1 analogs [18,31]. In this study, **I-3** not only retained the receptor activation capacity, but also possessed the longest plasma half-life and the highest albumin binding ability, all of which were important for the achievement of long-acting in vivo activity. Therefore, the in vivo biological activity of **I-3** was evaluated in the following glucoregulatory and insulin secretion assay.

3.5. INS-1 cells protective effects against cytotoxicity induced by glucolipotoxicity, H_2O_2, or STZ

INS-1 cells were exposed to glucolipotoxicity media (25 mM glucose plus 0.3 m Moleate), 50 μM H_2O_2 or 1 μM STZ. **I-3** was added simultaneously and incubated for 24 h to determine the protective effect of **I-3** on cell viability and apoptosis. Compared with saline-treated controls, **I-3** at 1–100 nM significantly retained cell viability against glucolipotoxicity media, 50 μM H_2O_2 or 1 μM STZ. After 24 h incubation with glucolipotoxicity media, cell viability of control INS-1 cells was significantly decreased by ~25%, while only ~20% in 1 nM and ~10% in ⩾10 nM **I-3** treated INS-1 cells ($p < 0.05$ for 10 Nm and 100 nM, compared with saline) (Fig. 3A). For oxidative stress, cell viability of control INS-1 cells was significantly decreased by ~25% after 24 h incubation with 50 μM H_2O_2, while only ~15% in 1 nM ($p < 0.05$ for 1 nM) and ~10% in ⩾10 nM **I-3** treated INS-1 cells ($p < 0.05$, compared with saline) (Fig. 3B). After 24 h incubation with 1 μM STZ, cell viability

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Table 2
Characterization of the fatty chain-modified Ex-4 derivatives and their potency on the cloned human GLP-1 receptor activation.

Peptide	Retention time[a] (min)	Molecular mass		EC_{50}[b], (pM)
		Calculated	Found	
Ex-4				1.873 ± 0.912
I-1	2.51	$[M+3H]^{3+}$ 1457.9 $[M+4H]^{4+}$ 1093.7	$[M+3H]^{3+}$ 1457.7 $[M+4H]^{4+}$ 1093.2	5.103 ± 1.32*
I-2	2.46	$[M+3H]^{3+}$ 1476.6 $[M+4H]^{4+}$ 1107.7	$[M+3H]^{3+}$ 1476.8 $[M+4H]^{4+}$ 1107.9	14.56 ± 1.45*
I-3	3.00	$[M+3H]^{3+}$ 1495.5 $[M+4H]^{4+}$ 1121.9	$[M+3H]^{3+}$ 1495.2 $[M+4H]^{4+}$ 1121.9	38.47 ± 9.86*
I-4	2.28	$[M+3H]^{3+}$ 1438.9 $[M+4H]^{4+}$ 1079.4	$[M+3H]^{3+}$ 1439.1 $[M+4H]^{4+}$ 1079.7	10.76 ± 2.16*
I-5	2.34	$[M+3H]^{3+}$ 1457.6 $[M+4H]^{4+}$ 1093.1	$[M+3H]^{3+}$ 1457.9 $[M+4H]^{4+}$ 1093.2	30.81 ± 7.56*
I-6	2.43	$[M+3H]^{3+}$ 1476.5 $[M+4H]^{4+}$ 1107.6	$[M+3H]^{3+}$ 1476.6 $[M+4H]^{4+}$ 1107.6	44.19 ± 8.99*
I-7	2.38	$[M+3H]^{3+}$ 1500.6 $[M+4H]^{4+}$ 1125.7	$[M+3H]^{3+}$ 1500.6 $[M+4H]^{4+}$ 1125.4	7.25 ± 1.25*
I-8	2.50	$[M+3H]^{3+}$ 1519.3 $[M+4H]^{4+}$ 1139.7	$[M+3H]^{3+}$ 1519.8 $[M+4H]^{4+}$ 1139.5	14.09 ± 2.45*
I-9	2.60	$[M+3H]^{3+}$ 1538.6 $[M+4H]^{4+}$ 1153.9	$[M+3H]^{3+}$ 1538.7 $[M+4H]^{4+}$ 1153.3	36.85 ± 3.49*

[a] UPLC conditions: 5–95% acetonitrile (mobile phase A: water with 0.2% formic acid, mobile phase B: acetonitrile with 0.2% formic acid) in 3.5 min at a flow rate of 0.3 mL/min with ultraviolet (UV) detection at 214 nm with the use of Acquity UPLC HSS T3 column (1.8 μm, 2.1 mm × 100 mm, Waters).
[b] The receptor potency data are given as mean ± SD. All experiments were performed in triplicate and repeated three times ($n = 3$).
* $p < 0.05$, compared with Ex-4.

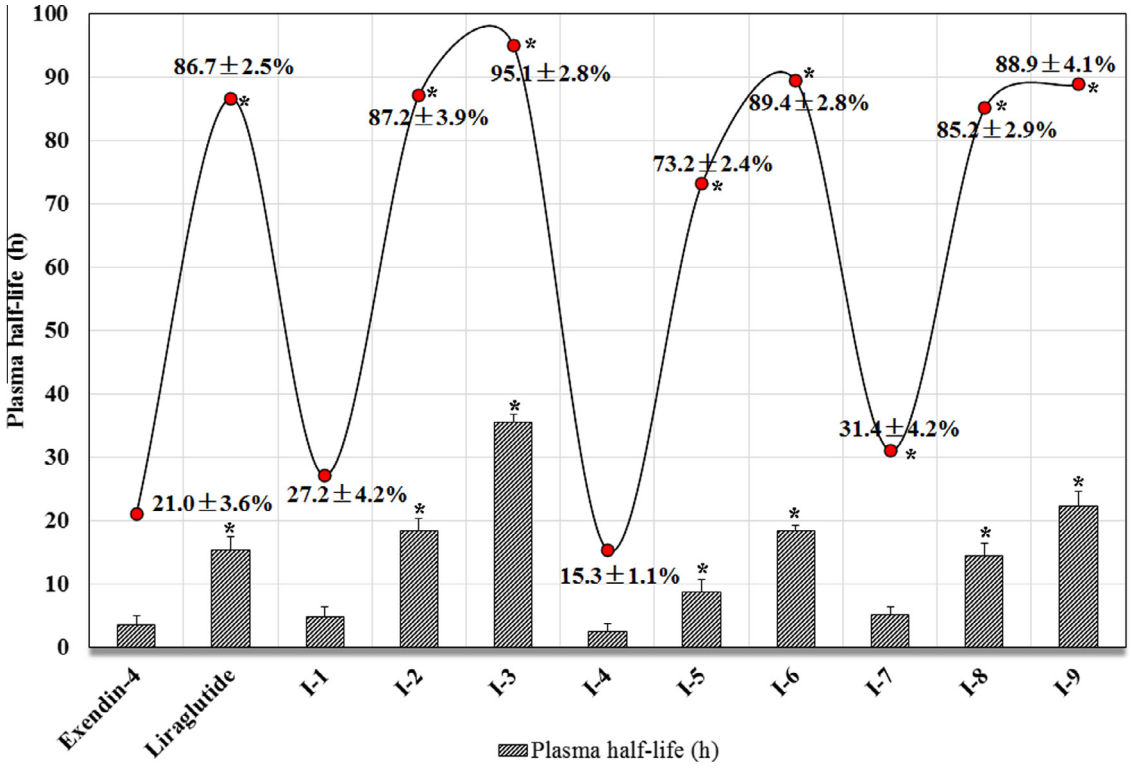

Fig. 2. The plasma stability and albumin binding affinity of the fatty chain-modified Ex-4 conjugates, Ex-4 and liraglutide.

of control INS-1 cells was significantly decreased by ~45%, while only ~30% in 1 nM and ~20% in ⩾10 nM **I-3** treated INS-1 cells ($p < 0.05$, compared with saline) (Fig. 3C). Flow cytometric analysis also showed similar results, 24 h treatment with glucolipotoxicity media, 50 μM H_2O_2 or 1 μM STZ caused a drastic increase of apoptosis in control INS-1 cells, however, less apoptosis was found in Ex-4, liraglutide and **I-3** treated INS-1 cells (Fig. 4A–C), suggesting that **I-3** could protect β-cell viability and inhibited apoptosis

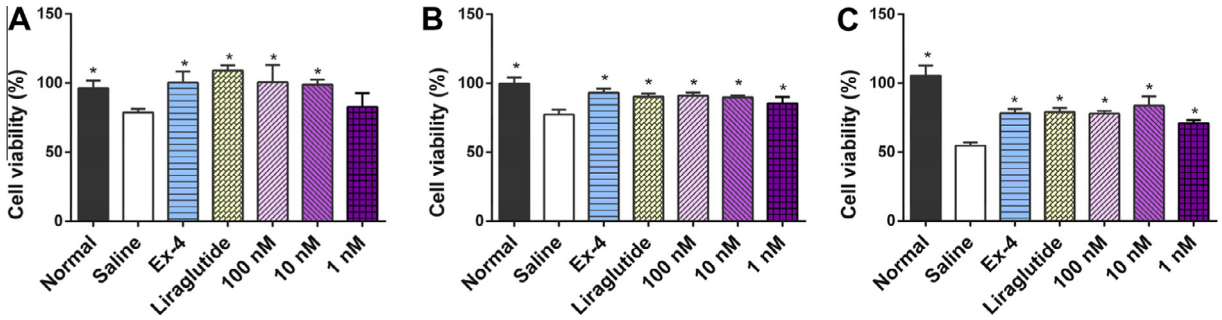

Fig. 3. I-3 attenuates glucolipotoxic and oxidative stress and enhances viability and survival of INS-1 cells.

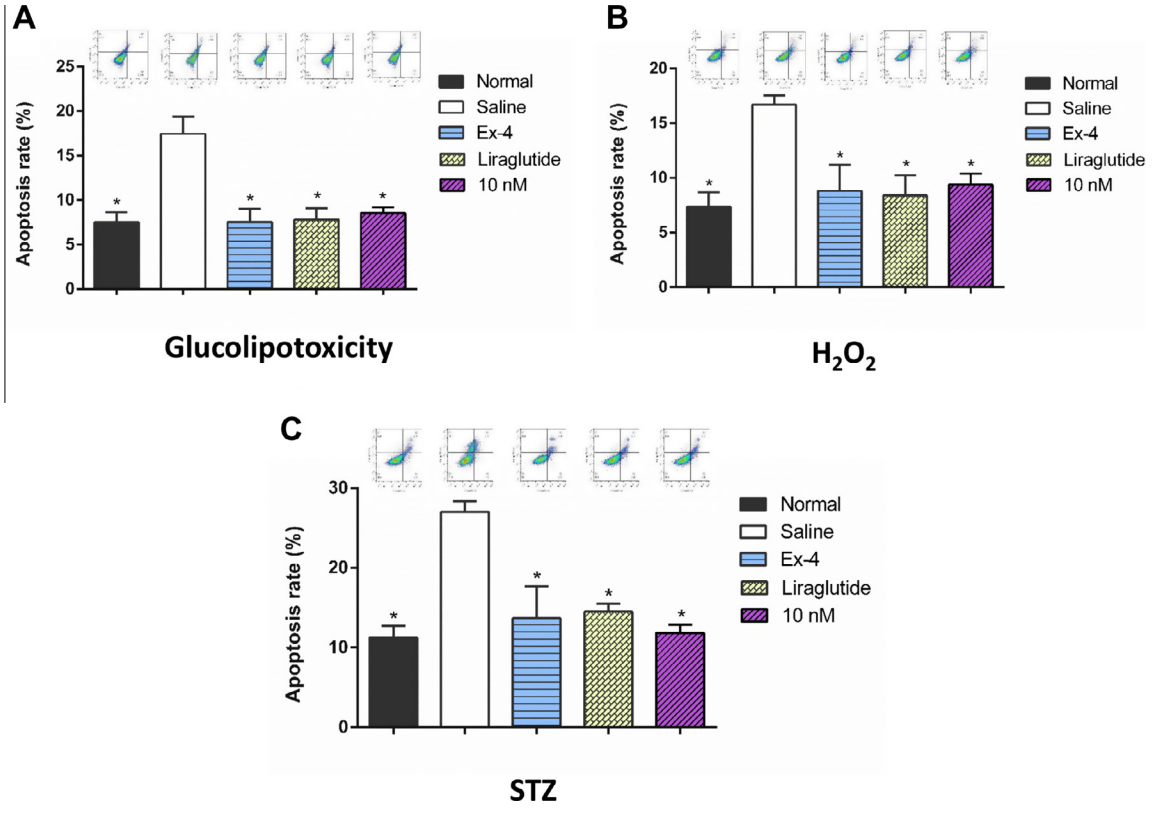

Fig. 4. I-3 decreased cell apoptosis in the presence glucolipotoxic, oxidative stress and STZ.

against glucolipotoxicity, oxidative stress and DNA damage-mediated stress.

3.6. Glucoregulatory and insulin secretion assay

The in vivo glucoregulatory and insulinotropic activities of **I-3** was first examined in SD rats by oral glucose tolerance test (OGTT). After the intraperitoneal administration of **I-3**, liraglutide or Ex-4 (25 nmol/kg,) at −30 min and a glucose challenge of 10 g/kg at 0 min, the mean blood glucose levels at 15 min, 30 min, 45 min, 60 min, 90 min, 120 min and 180 min were measured and the glucose tolerance patterns exhibited a significant increase (Fig. 5A). After a glucose challenge, the mean blood glucose level in the saline treated group rapidly increased to 13.8 ± 1.31 mmol/L at 30 min while in those receiving liraglutide, Ex-4 and **I-3** dramatically the level reduced to ~7.2, ~7.6, and ~7.8 mmol/L at the same time point, respectively. Moreover, the glucose AUC of liraglutide, Ex-4 and **I-3** was significantly lower than that of the saline treated group (Fig. 5B, $p < 0.05$). Meanwhile, the decreases in plasma glucose were accompanied by the increases in plasma insulin concentration which was consistent with the GLP-1-dependent mechanism (Fig. 5C). Similar time courses for plasma insulin concentrations in **I-3**, liraglutide or Ex-4 treated group were observed and all plasma insulin concentrations from 15 min to 60 min time points were significantly greater than those of the control group. In particular, **I-3** showed comparable promoting insulin secretion ability than liraglutide and Ex-4 ($p < 0.05$). The insulinotropic activities and glucose-lowering abilities of **I-3** were comparable with those of liraglutide and Ex-4 (Fig. 5D).

3.7. Multiple intraperitoneal glucose tolerance test

Based on the remarkable in vitro stability of **I-3**, the abilities of **I-3** to reduce glucose levels for longer time were assessed in DIO mice through a modified multiple intraperitoneal glucose tolerance test to simulate the human dietary habits of three meals a

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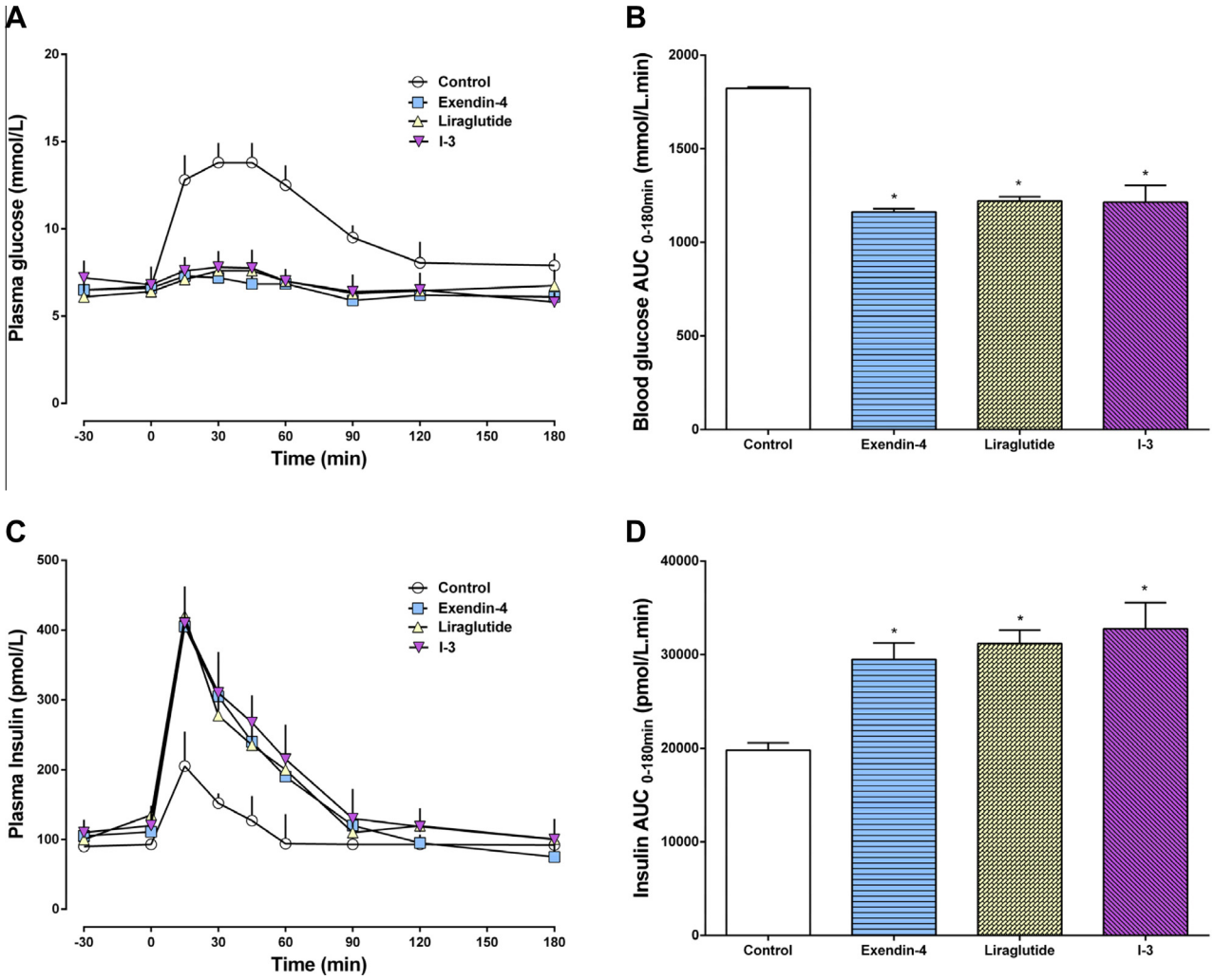

Fig. 5. In vivo biological activity tests of **I-3**.

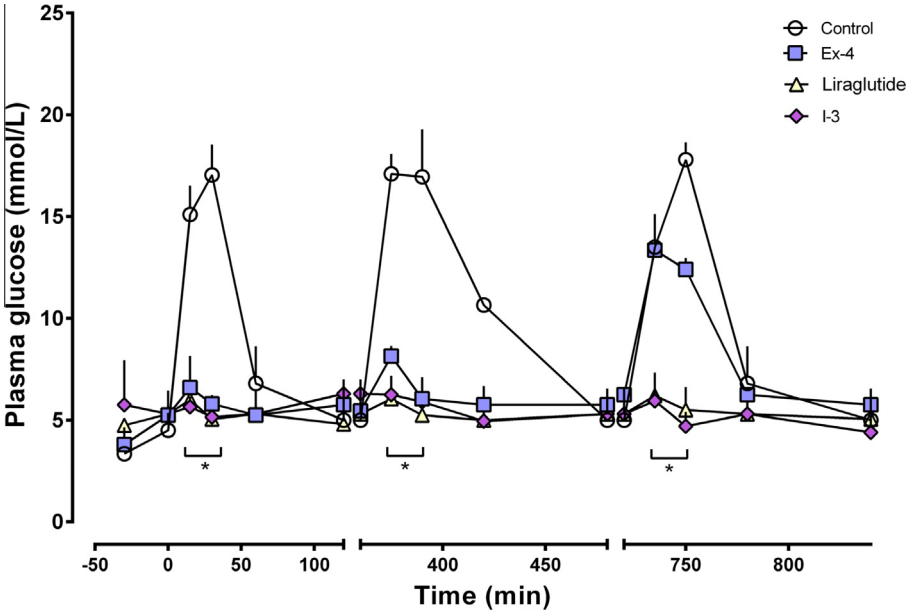

Fig. 6. Long time glucose-lowering effect of **I-3** was studied using multiple IPGTT in C57BL/6 mice.

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day. 2 g/kg glucose was loaded 0.5 h after intraperitoneal injection of Ex-4, liraglutide, **I-3** (25 nmol/kg) and control (saline). The second and third glucose loads were conducted at time points 6 and 12 h, respectively. As shown in Fig. 6, the blood glucose levels in the saline treated group rapidly increased over 15 mmol/L 0.5 h after the load of glucose while those of Ex-4, liraglutide, **I-3** treated group were below 10 mmol/L. Furthermore, the glucose-lowering ability of Ex-4 maintained for 0–6 h but decreased dramatically after 6 h and was almost ineffective after 12 h. During the whole experiment period (0–15 h), liraglutide and **I-3** showed comparable glucose lowering ability. These results indicated that a single dose of **I-3** (25 nmol/kg) could maintain glucoregulatory abilities over 15 h.

3.8. Hypoglycemic duration test

In the present study, the hypoglycemic effects of **I-3** were assessed at two doses (25 and 150 nmol/kg, i.p.) in STZ-induced diabetic mice. Normal blood glucose level was set below 8.35 mmol/L. Euglycemic durations under this value were calculated and viewed as a practical indication for antidiabetic treatment [32]. As illustrated in Fig. 7, saline treated control mice exhibited a hyperglycemic state (average > 20 mmol/L) while those injected with Ex-4 and liraglutide (25 nmol/kg) rapidly maintained normal blood glucose for nearly 4.8 h and 17.7 h, respectively. The time required to rebound to a glucose level of 8.35 mmol/L was ~22.6 h in **I-3** treated mice which was much longer than in Ex-4 treated mice and better than in liraglutide treated mice at a dose of 25 nmol/kg (Fig. 7A). At a dose of 150 nmol/kg, the $AUC_{0-36\ h}$ values of **I-3** were 2.49 times lower than those of Ex-4 and 1.50 times greater than those of liraglutide (Fig. 7B). In particular, the $t_{\sim 8.35}$ mmol/L value in mice that received **I-3** at the highest dose (150 nmol/kg) was 33.1 h.

3.9. Chronic in vivo studies on STZ-induced diabetic mice

STZ-induced diabetic mice received chronic administration of **I-3** to probe in vivo activity and potential therapeutic utility. Ex-4, liraglutide and **I-3** (25 nmol/kg) were intraperitoneally injected (once daily) with saline used as control. Fasting blood glucose level in diabetic mice prior to treatment were 17.5 ± 1.9 mmol/L and worsened somewhat to 20.5 ± 1.3 mmol/L

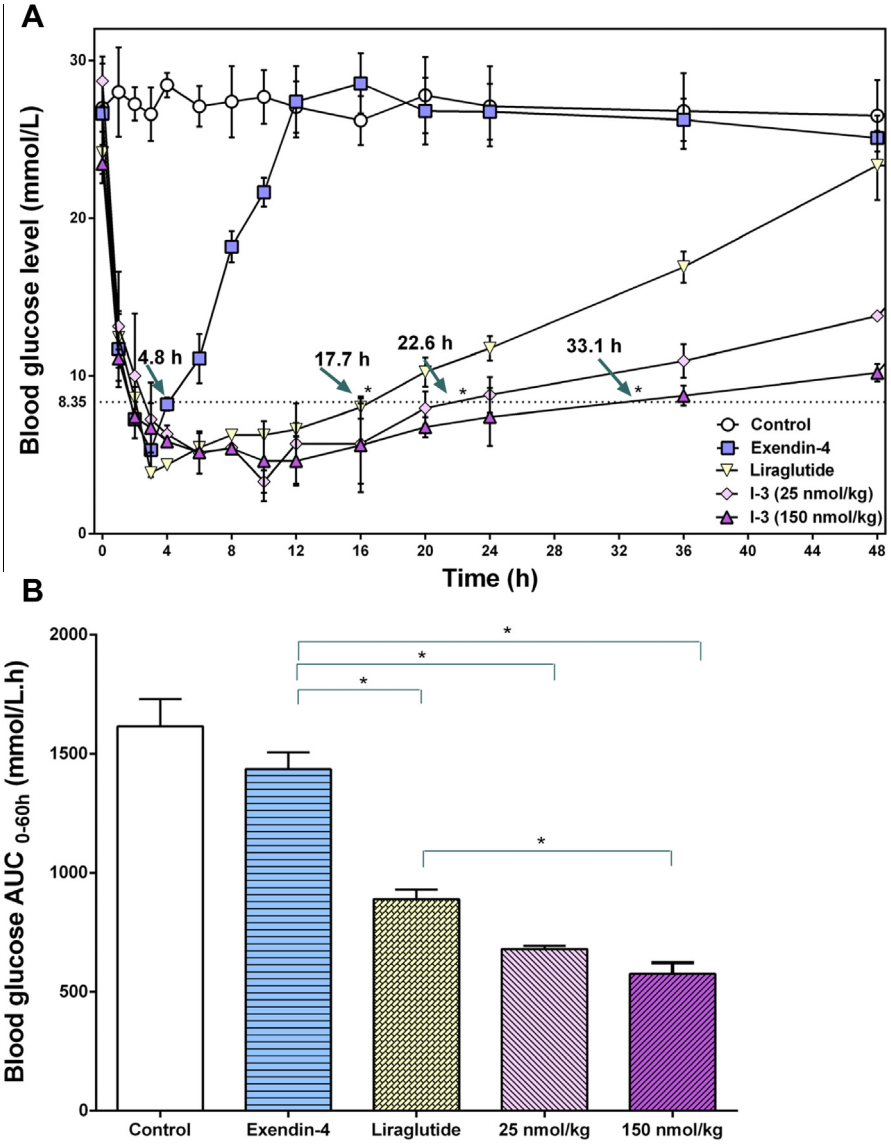

Fig. 7. Glucose-lowering and stabilizing effect of Ex-4, liraglutide and **I-3** as determine by hypoglycemic duration test in nonfasted diabetic mice.

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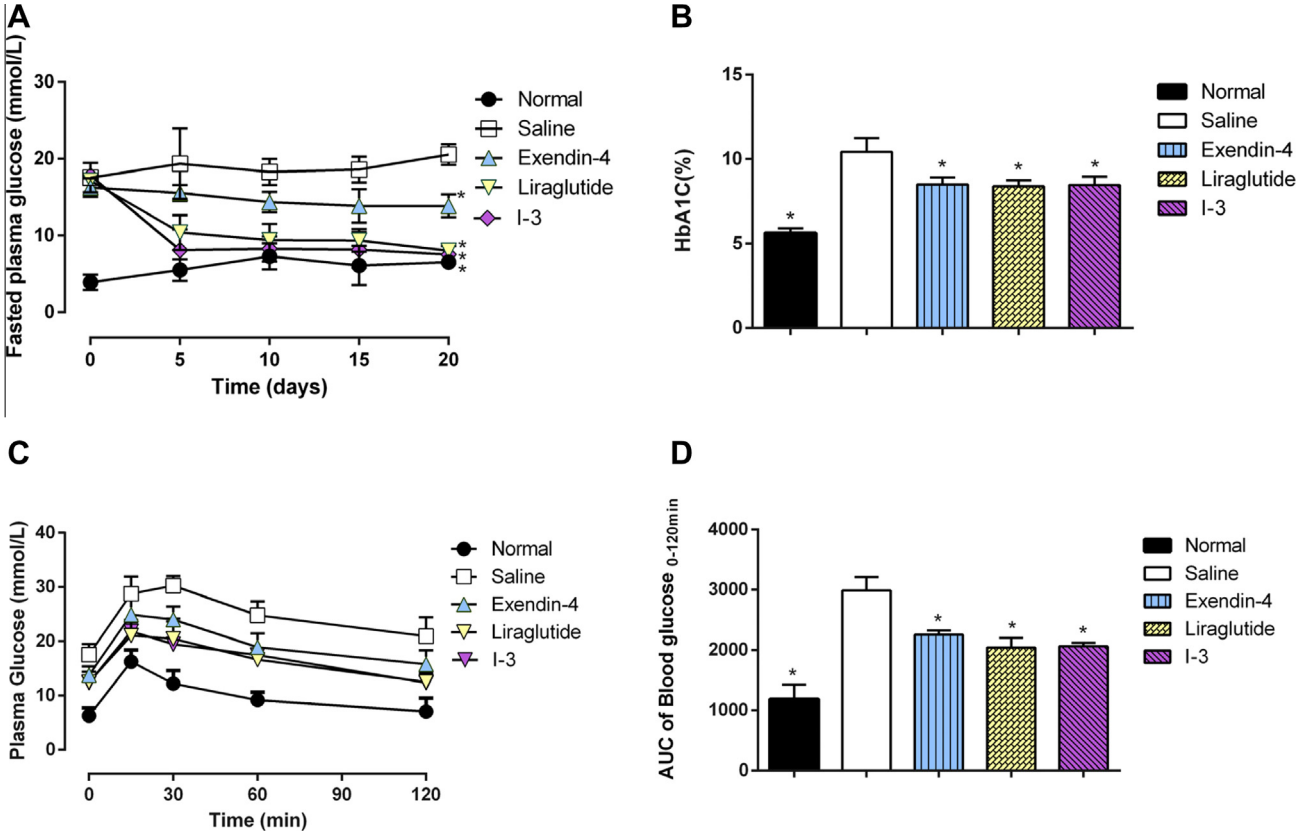

Fig. 8. The effects of chronically administered **I-3**, Ex-4 and liraglutide in diabetic mice.

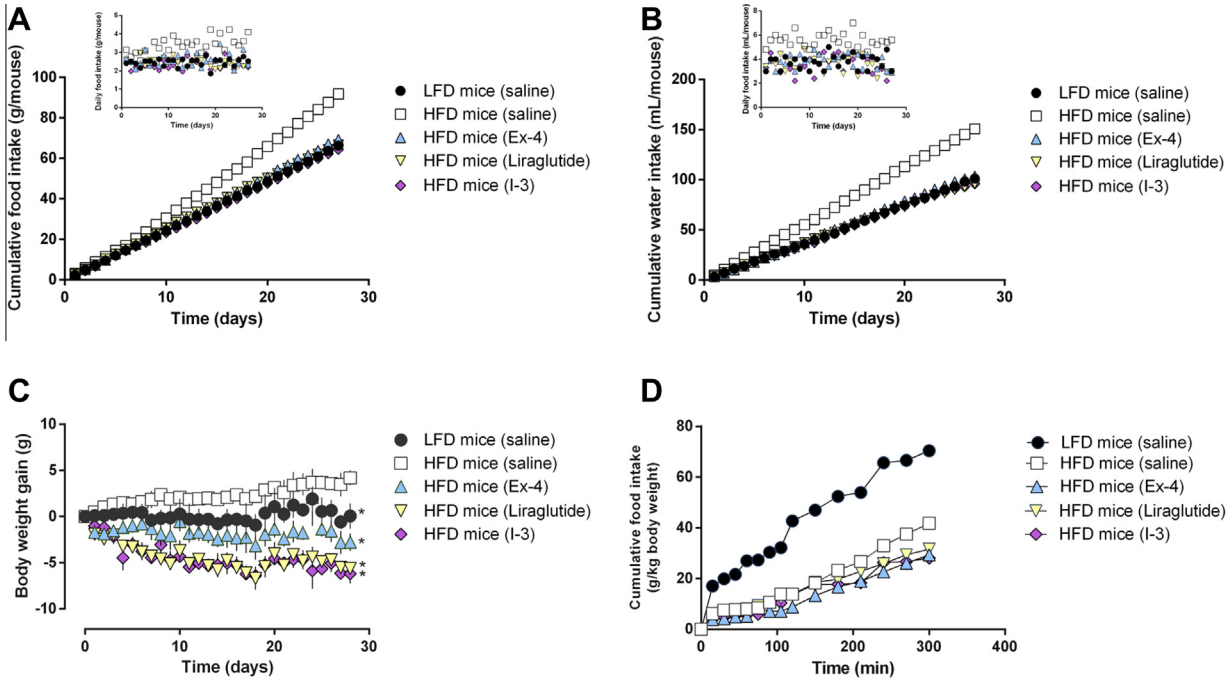

Fig. 9. Effects of **I-3** on food and water intake.

after 21 days. In contrast with saline treated controls, **I-3** treatment lowered fasting glucose toward the 6.7–8.3 mmol/L levels (Fig. 8A). HbA1c was resulted from non-enzymatic irreversible glycation and thus regarded as an indirect indicator of cumulative blood glucose concentrations which was a more sensitive index of glycemic control than glucose concentration [33]. As shown in Fig. 8B, HbA1c was initially well matched in all diabetic groups which was significantly higher than in nondiabetic littermates. During the 21-day treatment, diabetic mice daily injected **I-3** exhibited a gradual reduction while HbA1c in control group continued to be

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high. The HbA1c reduction caused by the administration of **I-3** is indicative of promising role of **I-3** in clinical studies.

To determine whether long-term **I-3** treatment improved glucose tolerance, IPGTT test was performed before and after treatment (day 0 and day 24). At beginning of treatment, no difference was observed between mice in every group. However, at the end the treatment, **I-3**, Ex-4 and liraglutide treated mice showed rapid blood glucose reductions and returned to base line at 120 min after glucose administration, whereas saline treated mice showed a typical diabetic pattern of slow reduction in the hyperglycemic state at 120 min (Fig. 8C). The blood glucose AUC of **I-3** was significantly lower than that of the saline treated diabetic mice ($p < 0.05$) (Fig. 8D).

3.10. Chronic in vivo studies on DIO mice

During the 4-week treatment course, **I-3** inhibited cumulative food and water intake by up to 18% and 25%, respectively (Fig. 9A and B). Furthermore, nearly the same amount of water as LFD mice was taken by **I-3**-treated mice. To further confirm the appetite suppression effect, an acute in vivo study was conducted and **I-3** inhibited food intake by up to 32% at 6 h after administration (Fig. 9D). Body weight gain of the control HFD mice continually increased over the 4-week treatment period by ≈5 g whereas it was suppressed after administration of either **I-3**, Ex-4 or liraglutide (Fig. 9C).

Two important adipocytokines secreted from white adipose tissue, the serum levels of leptin and adiponectin, were also altered after **I-3** treatment. Circulating leptin concentration in DIO mice was significantly higher than that in LFD controls ($p < 0.05$). In contrast, **I-3** administration significantly ($p < 0.05$) reduced serum leptin level to a near normal value of 2.3 ng/ml (Fig. 10F). Fig. 10G shows that, prolonged exposure to HFD induced nearly 39% lower serum adiponectin level in DIO than in lean mice ($p < 0.05$), whereas this level was remarkably increased ($p < 0.05$) by **I-3**. Recent evidence suggests that overweight represents important risk factors for steatohepatitis [34]. In the present study, hepatocyte damage was assessed by examining serum enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Our data (Fig. 10D) showed that **I-3** treatment resulted in a reduction in ALT ($p < 0.05$) and AST ($p < 0.05$) than that in obese. Furthermore, same reduction trend was also observed in serum total cholesterol (TC) (Fig. 10A) and triglyceride (TG) (Fig. 10B) ($p < 0.05$ and $p < 0.05$, respectively, compared to HFD mice). In addition, the LDL/HDL ratio "good" (HDL) and "bad" (LDL) cholesterol values were calculated to analyze the magnitude of increases in the two lipoproteins among different treatment groups. As shown in Fig. 10C, long-term exposure to HFD increased the LDL/HDL ratio by 20% while **I-3** treatment resulted in a reduction trend though not significant following administration.

4. Discussion

We designed and prepared a series of novel fatty chain modified Ex-4 analogs to search for long-acting Ex-4 conjugates with retained efficacy. The initial GLP-1 receptor activation experiments provided direct evidence of the relevant structure–activity relationships. As illustrated in Table 2, the receptor activation potentials of compounds **I-1**–**I-9** depended on the length of the fatty chain of fatty chain-maleimide and were independent of the conjugated position of cysteine residues (Cys_{12}, Cys_{25}, and Cys_{40}). The following in vitro stability test and albumin binding test revealed that the albumin binding ability and plasma stability of all conjugates **I-1**–**I-9** were improved to different degrees. Importantly, the plasma stabilities of test compounds correlated well with their albumin binding abilities. **I-3** exhibited the best albumin binding ability and the longest plasma half-life and was further evaluated for in vivo insulinotropic activities and glucose-lowering abilities. It was observed that **I-3** showed comparable protection effects against glucolipotoxic, oxidative stress and STZ (Figs. 3 and 4), insulinotropic activities and glucose-lowering abilities with those of liraglutide and Ex-4 (Fig. 5). Importantly, the in vivo biological action of **I-3** was shown to be exerted via the glucose-dependent action mode. This offered the advantage of increased drug safety compared with agents that increased insulin secretion via glucose-independent mechanisms (e.g., sulfonylureas), suggesting that **I-3** can be used clinically without the risk of hypoglycemia. The in vivo long-acting glucose-lowering profiles of **I-3** were then evaluated. The long-acting characteristic of **I-3** was significantly more improved than Ex-4, probably due to its reduced enzyme metabolism and renal clearance resulting from the strong albumin binding ability. The absorption of **I-3** into systemic circulation was delayed probably due to the formation of self-assembled colloidal structures in aqueous environment [31,35]. The interesting findings of this study concerned albumin binding by **I-3** and its long-acting profiles were consistent with previous successful reports regarding the protracted in vivo circulation half-life values of fatty acid-modified GLP-1 analogs, attributed to their physical interactions with serum albumin. The long-acting glucose-lowering effects of **I-3** were further confirmed by the hypoglycemic duration

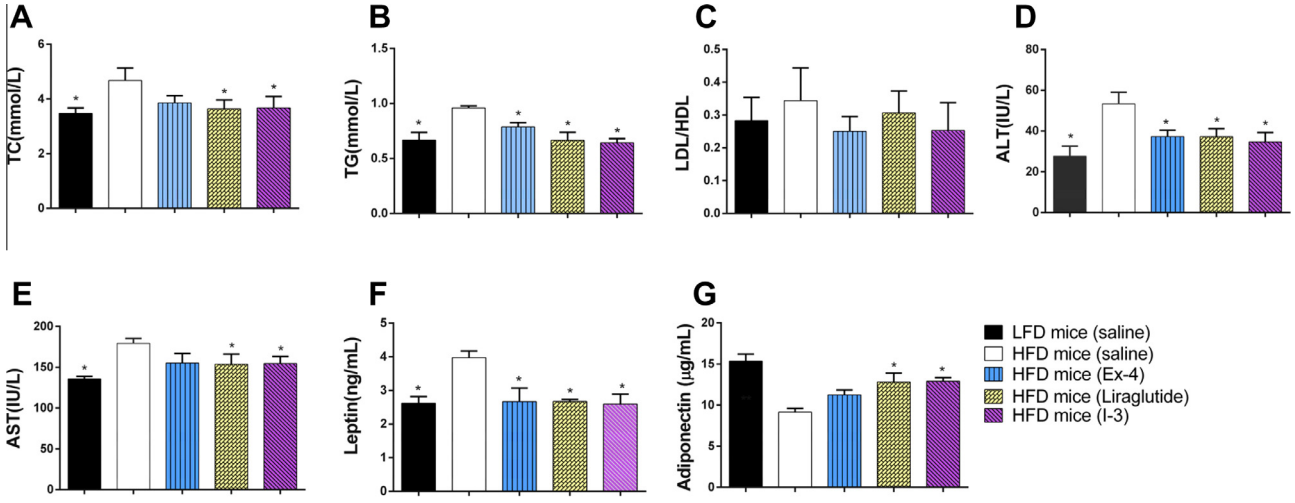

Fig. 10. Effects of **I-3** on serum biomarkers examined at the end of the 4-week treatment.

test in STZ-induced diabetic mice. The hypoglycemic durations of **I-3** in diabetic mice were much greater than those of Ex-4 and better than those of liraglutide, regardless of the dose administered. Furthermore, once daily injection of **I-3** to STZ-induced diabetic mice achieved long-term beneficial effects on HbA1c lowering and glucose tolerance. The enhanced glucose tolerance of **I-3** treated mice presumably resulted from the protracted biological activity (β cell neogenesis and/or proliferation) [36]. The results of chronic administration of **I-3** to HFD mice were also consistent with the chronic effects of peripherally administered GLP-1 receptor agonists including appetite suppression and weight loss reduction [37].

In summary, the present study demonstrates that the substitution of GLP-1 residues by cysteine and further fatty chain modification offers a useful approach to the development of long-acting incretin-based antidiabetics. This study also shows that **I-3**, which has well preserved biological activity, long-acting antidiabetic characteristics, and long-term beneficial effects, is a promising agent deserving further investigation to treat obesity patients with diabetes.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No. 81172932 and 81273376), the Natural Science Foundation of Jiangsu Province (No. BK2012356) and the Project Program of State Key Laboratory of Natural Medicines, China Pharmaceutical University (No. JKGZ201103 and SKLNMZZ201212).

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Please cite this article in press as: L. Sun et al., Site-specific fatty chain-modified exenatide analogs with balanced glucoregulatory activity and prolonged in vivo activity, Biochem. Pharmacol. (2016), http://dx.doi.org/10.1016/j.bcp.2016.04.016