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# Novel lipid side chain modified exenatide analogs emerged prolonged glucoregulatory activity and potential body weight management properties

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ABSTRACT

Exenatide is known as the first marketed GLP-1 agonist for antidiabetic treatment, but it need twice injection a day because of its fast clearance. This work aims to prolong the half-life of exenatide by modified with novel lipid chain. Four optimized exenatide analogs named as Cys12-Exenatide (1–39)-$NH_2$, Cys40-Exenatide (1–39)-$NH_2$, Cys12-Tyr22-Gln24-Glu28-Arg35-Exenatide (1–39)-$NH_2$ and Tyr22-Gln24-Glu28-Arg35-Cys40-Exenatide (1–39)-$NH_2$ were selected and applied for conjugation. Then a series of evaluations including GLP-1R activation assay were conducted, conjugation **C2** was selected for further investigation. Glucoregulatory and insulin secretion assay and hypoglycemic duration test were accessed and showed that **C2** was capable of comparable insulinotropic activities and glucose-lowering abilities with those of liraglutide and exenatide. Cell protective effects in INS-1 cells confirmed that **C2** had relatively protection effects. Meanwhile, once daily injection of **C2** to STZ-induced diabetic mice achieved long-term beneficial effects on glucose tolerance, body weight and blood chemistry. Acute feeding studies were evaluated in DIO mice. These results suggested that **C2** is a promising agent for further investigation of its potential to treat diabetes patients with obese.

## 1. Introduction

Glucagon-like peptide-1 (GLP-1) has received much attention, because of its unique mechanisms of glucose-dependent potentiating insulin secretion, and other properties, like inhibiting glucagon release, delaying gastric emptying, and reducing appetite.[1] However, endogenous GLP-1 has a short half-life of 1–2 min due to dipeptidyl peptidase-IV (DPP-IV) cleavage and renal filtration.[2]

Exenatide is a 39-amino acid peptide isolated from the venom of the Gila monster lizard *Heloderma suspectum*,[3] which is known as the first marketed incretin mimetic and the first GLP-1 receptor agonist approved by the FDA in 2005. Exenatide is not a substrate for DPP-IV because of the introduction of a Gly residue at position 2. Additionally, exenatide features a C-terminal extension (Cex) that enhances chemical stability by a compact tertiary fold (termed as Trp cage) that inhibits peptidase activity and improved physicochemical properties.[4,5] However, exenatide still can be eliminated quickly by kidney filtration, which exhibits a relatively long terminal half-life of ~2.4 h after intravenous injection. Exenatide is well known to have relatively potent weight lowering efficacy in rodents, as well as a modest weight lowering effect in humans.[6]

Chemical conjugation approach is proved to be a feasible strategy to afford GLP-1 based therapeutics, like PEGylation, albumin conjugation and lipidation. PEGylation has offered to prolong plasma half-life and

*Abbreviations:* AUC, area under the curve; cAMP, cyclic adenosine monophosphate; DIO, diet induced obese; DPP-IV, dipeptidyl peptidase-IV; $EC_{50}$, half maximally at an effective concentration; Ex-4, exenatide; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; HFD, high fat diet; HPLC, high-performance liquid chromatography; i.p, intraperitoneal administration; LC–MS, liquid chromatography–tandem mass spectrometry; LFD, low fat diet; NA, not active; NT, not test; TFA, trifluoroacetic acid

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improve stability in vivo. PEGylation of Exenatide results in an increase in molecule size, which slows down renal ultrafiltration and, as a result, can prolong the residence time of exenatide in the circulation.[7] CJC-1134-PC, developed by ConjuChem, is a chemical bioconjugate of exenatide and albumin, in which Lys is inserted into position 40 of exenatide, and a chemical linker bearing a terminal maleimide is attached for chemical conjugation to a single Cys residue on albumin. CJC-1134-PC has a half-life of approximately 8 days, making it suitable for once-weekly dosing.[8] However, PEGylation results in accumulation in cells (vacuolation) because of non-biodegradable of PEG moiety,[9] meanwhile nausea and vomiting was observed in CJC-1134-PC clinical trial, and no recent development has been reported. Lipid modification as applied in insulin degludec, a long-acting insulin analog, which is composed with hexadecanedioic acid via a γ-L-glutamic acid linker to Lys in B chain. Lipophilic substituent promotes the formation of a multimolecular self-association complex at the injection site that slows diffusion kinetics into circulation. The hexadecanedioic acid moiety also confers reversible binding to circulating albumin to slow renal clearance and extend circulating half-life.[10]

In this work, we report our efforts to identify four novel lipid-modified exenatide analogs **C1–C4** (Fig. 1). It is reported that the number of free carboxyl groups in the lipid chain affects the binding efficient.[11] When the lipid chain contains two free carboxyl groups, glycemia stabilization is more extended than one. We designed a novel lipidized chain, and introduced into four selected exenatide analogs, which exhibits exceptional glucoregulatory activities named as Cys12-Exenatide (1–39)-$NH_2$, Cys40-Exenatide (1–39)-$NH_2$, Cys12-Tyr22-Gln24-Glu28-Arg35-Exenatide (1–39)-$NH_2$ and Tyr22-Gln24-Glu28-Arg35-Cys40-Exenatide (1–39)-$NH_2$.[12,13] The thiol group of Cys in selected analogs was attached to maleimide modified lipid chain. Afterwards, relevant pharmacological assessments were evaluated of conjugates. Compounds **C2** were identified as long-acting and highly biologically active exenatide analog, and its antidiabetic properties and further evaluations were also investigated.

## 2. Methods and materials

### 2.1. Materials and animals

#### 2.1.1. Chemical materials

Fmoc-protected amino acids, Fmoc Rink Amide-MBHA resin, Liraglutide and Exenatide 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 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).

#### 2.1.2. Animals

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 DIO (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 *1–4*

Four optimized polypeptide chains were synthesized by the method of the standard solid-phase peptide synthesis protocol, Cys12-Exenatide (1–39)-$NH_2$ (**1**), Cys40-Exenatide (1–39)-$NH_2$ (**2**), Cys12-Tyr22-Gln24-Glu28-Arg35-Exenatide (1–39)-$NH_2$ (**3**) and Tyr22-Gln24-Glu28-Arg35-Cys40-Exenatide (1–39)-$NH_2$ (**4**). Then the peptides were purified on preparative RP-HPLC and were identified by electrospray mass spectrometry. Generally speaking, 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).

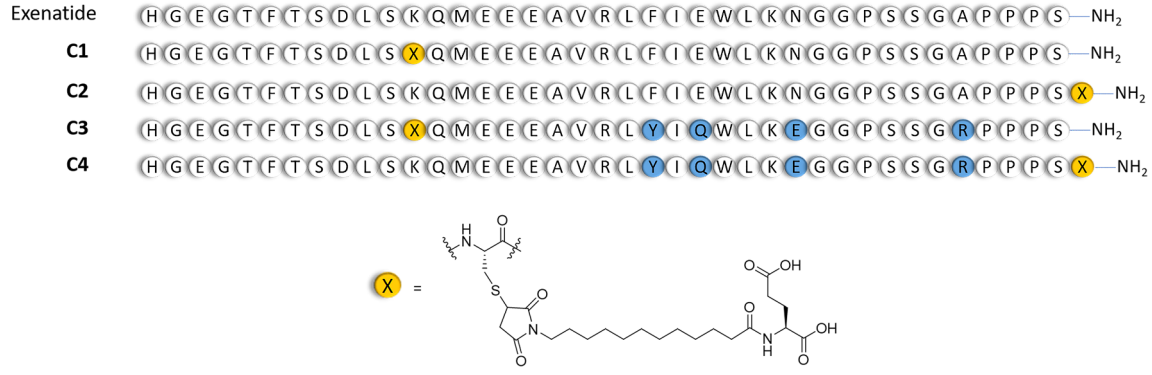
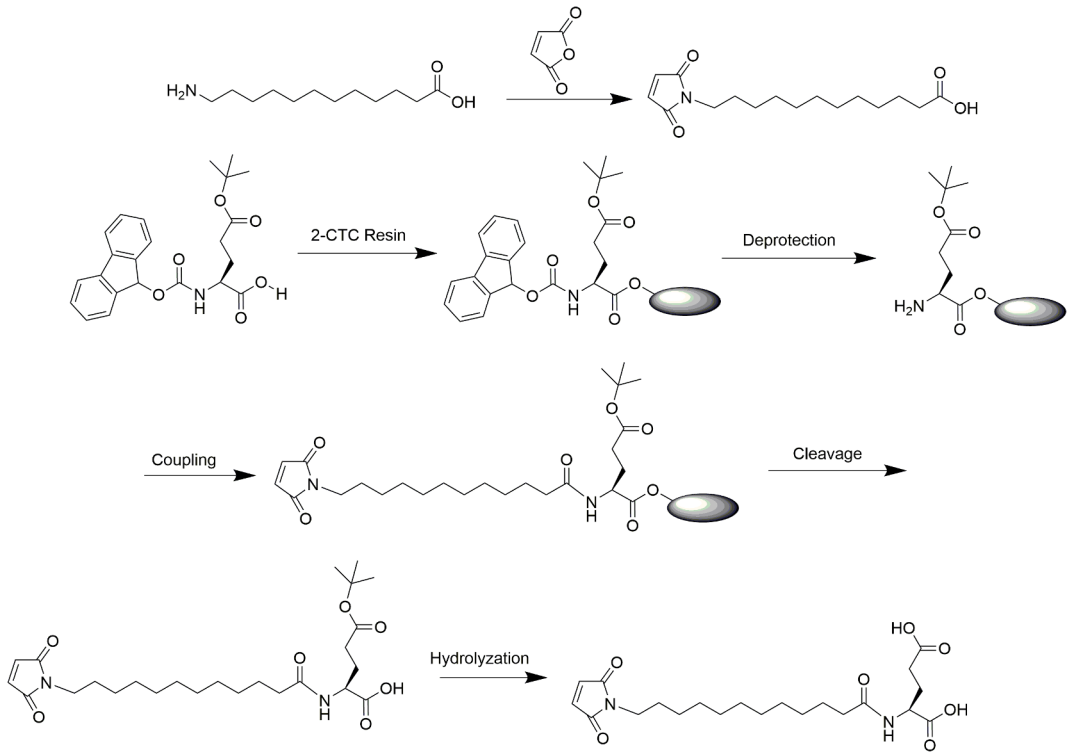



**Fig. 1.** Structure of four lipid chain-modified exenatide conjugates.




**Scheme 1.** Synthetic route of lipid chain-maleimide.

### 2.3. General synthetic route of lipid side chain-maleimide

#### 2.3.1. Synthetic route of 12-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) dodecanoic acid

As shown in Scheme 1, 12-aminododecanoic acid (10 mmol, 2151.9 mg) was dissolved in a three-necked flask with acetic acid, and maleic anhydride (12 mmol, 980.6 mg) was dissolved in acetic acid, and then slowly dropped to three-necked flask. The mixture was heated to reflux for 6 h at 120 °C. Purification was performed by column chromatography (ethyl acetate/petroleum ether) to yield white powdery solid (1.73 g, 81.9%).

#### 2.3.2. Synthetic route of (12-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) dodecanoyl)-L-glutamic acid

As shown in Scheme 1, 2-CTC resin (0.2 mmol, 363.6 mg, degree of substitution 0.55 mmol/g) was swelled by DCM (dichloromethane) for 30 min in a polypeptide reaction tube. Then Fmoc-Glu (tBu)-OH (0.6 mmol, 255.3 mg) and DIPEA (1.6 mmol, 264 μL) which dissolved in 7 mL of DCM was added and reacted at room temperature, with nitrogen bubbling for 4 h. Methanol/DIPEA = 9:1 was applied for capping for 30 min, Before deprotection step, resin was washed with DMF and DCM. Then 12-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl) dodecanoic acid (0.6 mmol, 882 mg), EDC (0.9 mmol, 690 mg), NHS (0.9 mmol, 414 mg) which dissolved in 7 mL of DCM was added and reacted at room temperature with nitrogen bubbling. The resin was cleaved by 0.5% TFA solution in DCM for 30 min, and the filtrate was collected and evaporated.

1 mL of TFA was slowly added dropwise to the filtrate which dissolved in DCM, and reacted for 3 h. 5 mL of 1 M aqueous NaOH solution was added, and a white solid was precipitated and centrifuged, and the precipitate was collected to obtain a white powdery product (80.0 mg, 94.2%).

### 2.4. General synthetic route of conjugates (C1–C4)

The conjugates were synthesized as previously reported.[14] Cysteine altered peptide (5 μmol) reacted with lipid chain-maleimide (12 μmol) in 10 mL of 0.05 mol/L sodium phosphate buffer(pH 7.0) at 20 °C under $N_2$ till UPLC displayed the end of reaction. 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. The condition for purification: Shimadzu C18 reversed-phase column (5 mm, 340 mm × 28 mm), a linear gradient of mobile phase 40–70% B (mobile phase A: water with 0.1% TFA, mobile phase B: acetonitrile with 0.1% TFA) in 30 min at a flow rate of 6.0 mL/min, and ultraviolet (UV) detection at 214 nm. The molecular mass of the purified conjugates was confirmed by LC–MS.

### 2.5. GLP-1 receptor activation assay

The GLP-1 receptor activation assay was conducted as previously reported.[15] To put it simply, our group constructed HEK293 cells over expressing human GLP-1 receptor stably, which was used to assess the potency of peptides and conjugates toward the GLP-1 receptor.[16] Cells were grown in Dulbecco's modified Eagle'smedium-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 7.0 (GraphPad, San Diego, CA, USA).

### 2.6. Plasma stability test

With a modification of a previously described method, Plasma was obtained from adult male Sprague-Dawley (SD) rats and compounds were then incubated with plasma over 72 h. 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. In vitro stability test, the initial concentration in rat plasma of lipid chain modified conjugates **C1**–**C4** and the positive controls (Exenatide 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 was 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.

### 2.7. Hypoglycemic efficacies test

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. Hypoglycemic efficacies of **C2** were evaluated in STZ-induced diabetic mice using a modification of a previously described method.[14] Kunming mice received a single intraperitoneal injection of saline, exenatide, liraglutide, **C2** (25 nmol/kg) with free access to food and water. At 0, 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 30, 36 and 48 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).

### 2.8. Cell viability assay

Cell viability assay was conducted as previously reported.[15] 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-hydroxyethylpi perazine-*N*-ethane-sulfonic acid, 50 μM b-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, b-cell-specific DNA-damaging agent) to induce apoptosis followed by the addition of exenatide (10 nM), liraglutide (10 nM) or **C2** (10 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 570 nm with a multiwall spectrophotometer (Thermo Lab systems, Waltham, MA, USA).

### 2.9. Glucoregulatory and insulin secretion assay

The glucoregulatory and insulin secretion assays were carried out in accordance with a previous method.[14] Briefly, overnight fasted (12 h) SD rats (n = 6/group, 200–250 g) were intraperitoneal injected with **C2**, exenatide and liraglutide (25 nmol/kg) half hour before oral glucose loaded (10 g/kg) (the time point was set as 0 min) with saline used as negative control. 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 with 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. 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 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 exenatide, liraglutide, or **C2** once daily for 3 weeks. Water consumption, food intake, and body weight were measured daily. 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 ALT, AST and HbA1c by Beckman Coulter Chemistry Analyzer (Tokyo, Japan), while liver and pancreas for further analyses.

### 2.11. Acute feeding studies and IPGTT on DIO mice

C57BL/6 mice (6 weeks) were maintained on prescribed DIO for 12 weeks before 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. After fasted for 12 h, they were injected intraperitoneally with 25 nmol/kg exenatide, liraglutide or **C2**, respectively with saline as control, the remaining food and water were measured in 0, 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min.

IPGTT were performed after fasted for 12 h, they were injected intraperitoneally with 25 nmol/kg exenatide, liraglutide or **C2**, respectively with saline as control. After 30 min, 18 mmol/kg of glucose was injected and glycemia was measured. The time of glucose injection was set at 0 min. The blood sugar levels were measured at 15 min, 30 min, 60 min, 120 min and 180 min, respectively.

### 2.12. Data analysis and statistical assessment

Data were analyzed using Prism version 7 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 lipid exenatide analogs and GLP-1 receptor activation assay

Four exenatide analogs (Cys12-Exenatide (1–39)-$NH_2$, Cys40-Exenatide (1–39)-$NH_2$, Cys12-Tyr22-Gln24-Glu28-Arg35-Exenatide (1–39)-$NH_2$ and Tyr22-Gln24-Glu28-Arg35-Cys40-Exenatide (1–39)-$NH_2$) were synthesized followed by standard solid-phase peptide synthesis (SPPS) method. Lipid chain was prepared by 2-CTC resin (Scheme 1) and reacted with the exenatide analogs by cysteine-maleimide in sodium phosphate buffer to obtain 4 lipid chain modified-peptide conjugates. Waters ACQUITY UPLC–MS Systems was applied for identification (Table 1) and HPLC for purification. The activation assay was determined using HEK293 cells over expressing human GLP-1 receptor stably. As shown in Table 1, all four conjugates showed pretty high potency in activating GLP-1 receptor compared to liraglutide, although slight reduction compared to exenatide. The assay results indicated that the four exenatide analogs modified with lipid chain considerably activated GLP-1 receptor.

**Table 1**
Characterization of Exenatide analogs and their potency on GLP-1 receptor activation.

| Name | $EC_{50}$[a] (pM) | Retention time[b] (min) | Molecular mass | Molecular mass calculated | | Molecular mass found | |
|---|---|---|---|---|---|---|---|
| | | | | $[M+3H]^{3+}$ | $[M+4H]^{4+}$ | $[M+3H]^{3+}$ | $[M+4H]^{4+}$ |
| Exenatide | 5.8 ± 0.6 | N/A | N/A | N/A | N/A | N/A | N/A |
| Liraglutide | 65.7 ± 0.8 | N/A | N/A | N/A | N/A | N/A | N/A |
| **C1** | 21.0 ± 0.7$^{**\#\#}$ | 2.56 | 4586.1 | 1529.7 | 1147.5 | 1529.6 | 1148.3 |
| **C2** | 16.9 ± 1.8$^{**\#\#}$ | 2.67 | 4714.3 | 1572.4 | 1179.6 | 1572.5 | 1180.0 |
| **C3** | 18.2 ± 0.1$^{**\#\#}$ | 2.56 | 4701.2 | 1568.1 | 1176.3 | 1568.8 | 1175.6 |
| **C4** | 20.2 ± 1.7$^{**\#\#}$ | 2.58 | 4829.4 | 1610.8 | 1208.4 | 1611.8 | 1208.0 |

$^{*}P < 0.05$, $^{**}P < 0.01$ vs Exenatide, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs Liraglutide.
[a] Results are expressed as mean ± SD. All experiments were performed in triplicate and repeated three times (n = 3).
[b] HPLC conditions: 10–90% acetonitrile in formic acid aqueous (0.1%) 7 min, flow rate of 0.3 mL/min (CSH, 1.7 μm, 2.1 × 50 mm, Waters).

### 3.2. Plasma incubation assay

When incubated with plasma, exenatide analogs can be degraded by DPP-IV and other proteases. Therefore, plasma incubation assay was conducted to explore the plasma stability of exenatide analogs by rat plasma. UPLC–MS/MS was applied to measure the concentration of compounds at different intervals, then the plasma half-life was calculated. As illustrated in Fig. 2, the plasma half-life of most lipid chain modified conjugates were prolonged compared with exenatide and liraglutide. More specifically, the plasma half-life of exenatide is 4.34 h, liraglutide is 13.93 h, and the plasma half-life of conjugate **C2** is up to 39.03 h. Consequently, the in vivo biological activity of **C2** was performed in the subsequent assay.

### 3.3. Hypoglycemic duration test

STZ-induced diabetic mice were selected for assessing long-acting hypoglycemic levels of conjugates **C2**. The postprandial blood glucose of STZ-induced diabetic mice is generally above 16.7 mmol/L, and glycemia will decrease to normal level after intraperitoneal injection of exenatide, liraglutide or **C2** (25 nmol/kg). As compounds were gradually cleared, the glycemia slowly returned to hyperglycemic state. In this paper, duration for glycemia under 8.35 mmol/L (150 mg/dL) was calculated and viewed as indication for glucoregulatory level. As exhibited in Fig. 3, hypoglycemic state (< 8.35 mmol/L) observed in the exenatide-treated, liraglutide-treated and **C2**-treated groups were about 2.8 h, 13.1 and 29.4 h. It is shown that the stable glycemia levels of the preferred compounds **C2** were significantly better than exenatide and liraglutide ($p < 0.01$).

### 3.4. Glucoregulatory and insulin secretion assay

Glucose dependent insulin secretion levels were accessed, which representing exenatide analogs stimulated physiological GLP-1R expression properties. In this paper, SD rats were injected intraperitoneally with normal saline, exenatide, liraglutide, **C2** (25 nmol/kg). After 30 min, oral glucose tolerance test (OGTT) was performed to evaluate plasma insulin levels and glycemia changes. As shown in Fig. 4A, B, after injection of glucose, the insulin levels in the saline group increased slightly (~203 pmol/L), while the insulin secretion in the administration group increased significantly. After 15 min, the insulin secretion of exenatide, liraglutide and **C2** was ~523 pmol/L, ~411 pmol/L and ~439 pmol/L, respectively. The time courses for

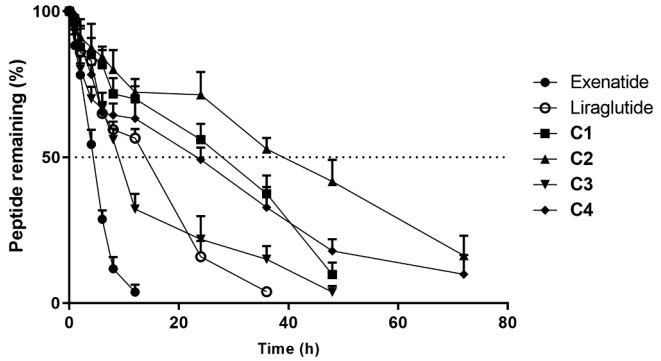



**Fig. 2.** Degradation of the lipid chain-modified conjugates by rat plasma over 72 h.

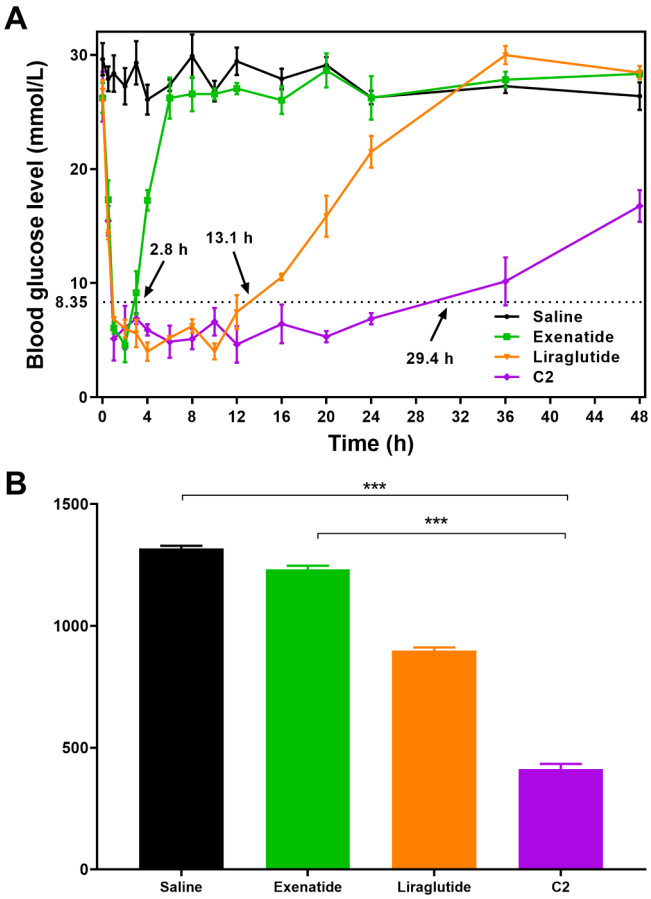



**Fig. 3.** Glucose-lowering and stabilizing effect of exenatide, liraglutide and **C2** as determined by hypoglycemic duration test in nonfasted STZ-induced diabetic mice. (A) Time-course average blood glucose levels of diabetic mice after an i.p. injection of exenatide, liraglutide or **C2** (25 nmol/kg). Times depict hypoglycemic duration rebound to 8.35 mmol/L. (B) Hypoglycemic effects of exenatide, liraglutide and **C2** based on the calculated glucose $AUC_{0-48h}$ values. n = 6 per group. Data are given as mean ± SD. *p < 0.05, **p < 0.01 compared with saline (control).

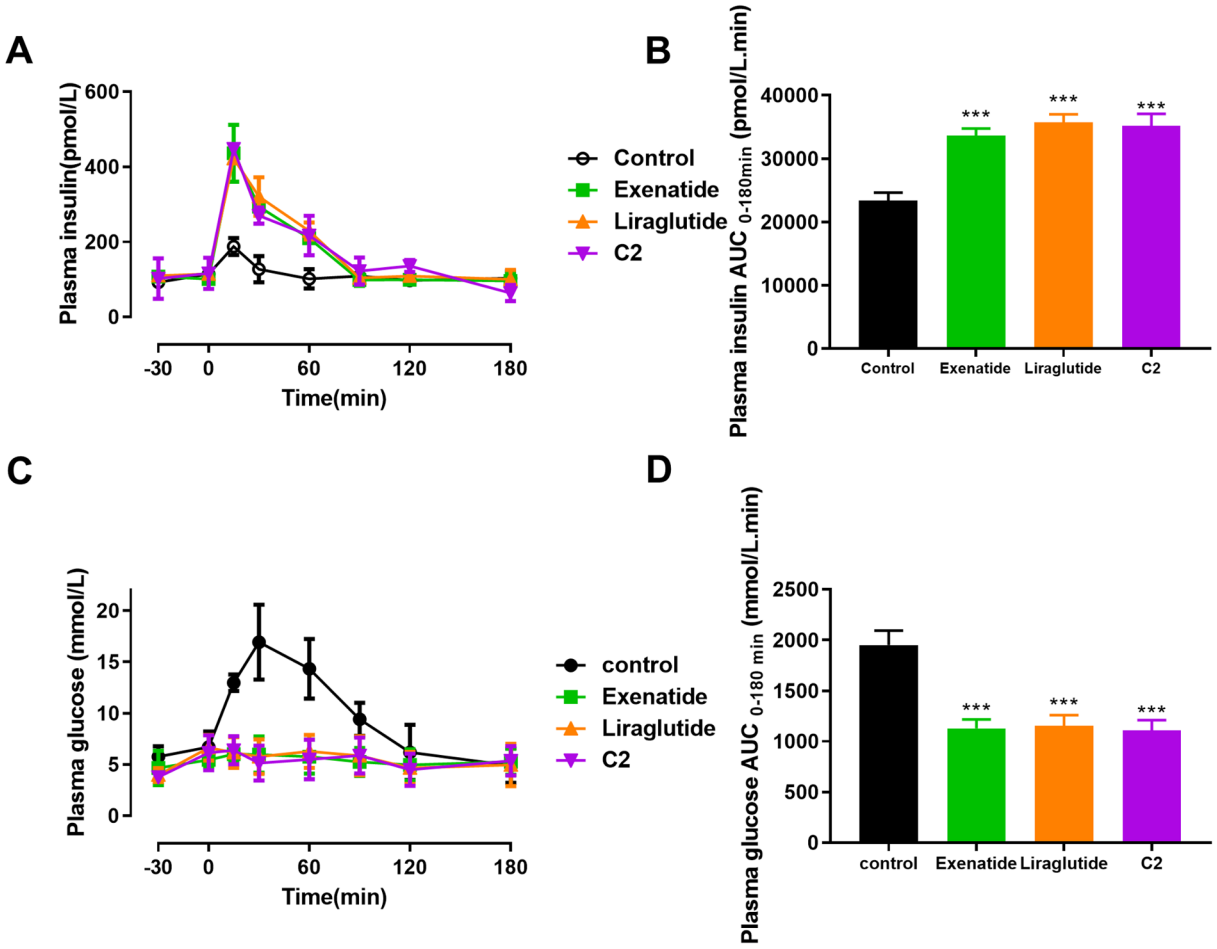



**Fig. 4.** In vivo biological activity tests of **C2**. Liraglutide, exenatide and **C2** (25 nmol/kg) were i.p. injected into SD rats; glucose was taken orally (10 g/kg). (A) Insulinotropic activities and calculated insulin $AUC_{0–180min}$ (B) of liraglutide, exenatide and **C2**. (C) The glucose-lowering effects and calculated plasma glucose $AUC_{0-180min}$ (D) of liraglutide, exenatide and **C2**. n = 6 per group. Data are given as mean ± SD. *p < 0.05, **p < 0.01 compared with saline (control).

plasma insulin concentrations in exenatide, liraglutide and **C2** observed was similar in where all plasma insulin concentration from 15 to 60 min were notably greater than those of the control group. The blood glucose in the administration group tended to be stable, while the blood glucose in the saline group increased significantly and returned to normal after 120 min (Fig. 4C, D). It is shown that glucose-lowering and insulintropic abilities of **C2** were comparable with those of exenatide and liraglutide.

### *3.5. INS-1 cells protective effects against cytotoxicity induced by $H_2O_2$ or STZ*

To evaluate the protective activity of **C2**, apoptosis induced by STZ and H2O2 in INS-1 cells was applied. Compared to saline control, exenatide, liraglutide and **C2** at 10 nM incubated cells were espoused to 1 μM STZ or 50 μM $H_2O_2$. As illustrated in Fig. 5, after 24 h, cell viability of saline INS-1 cells was significantly reduced, while almost no reduce in 10 nM **C2** treated INS-1 cells. After 24 h incubation with

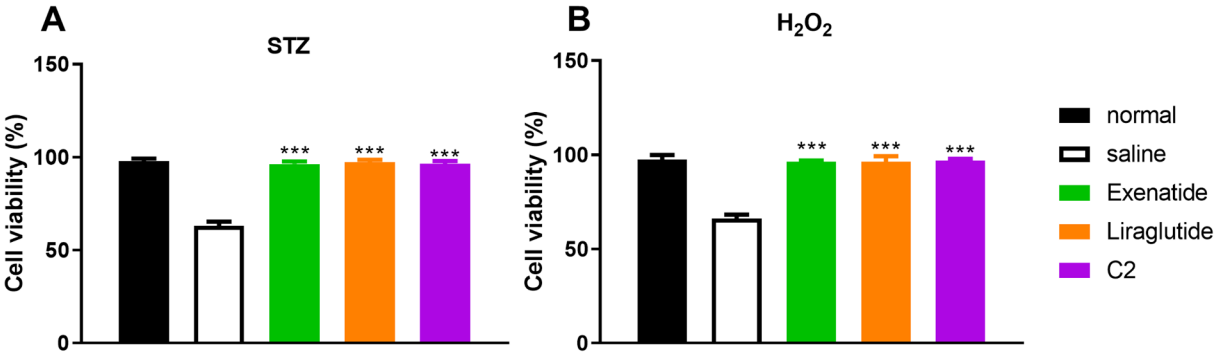



**Fig. 5. C2** enhances viability and survival of INS-1 cells. (A) **C2** preserved cell viability in response to STZ. (B) **C2** preserved viability and survival of INS-1 cells against oxidative stress. All experiments were performed in triplicate and repeated three times (n = 3). Data are given as mean ± SD. *p < 0.05, compared with saline.

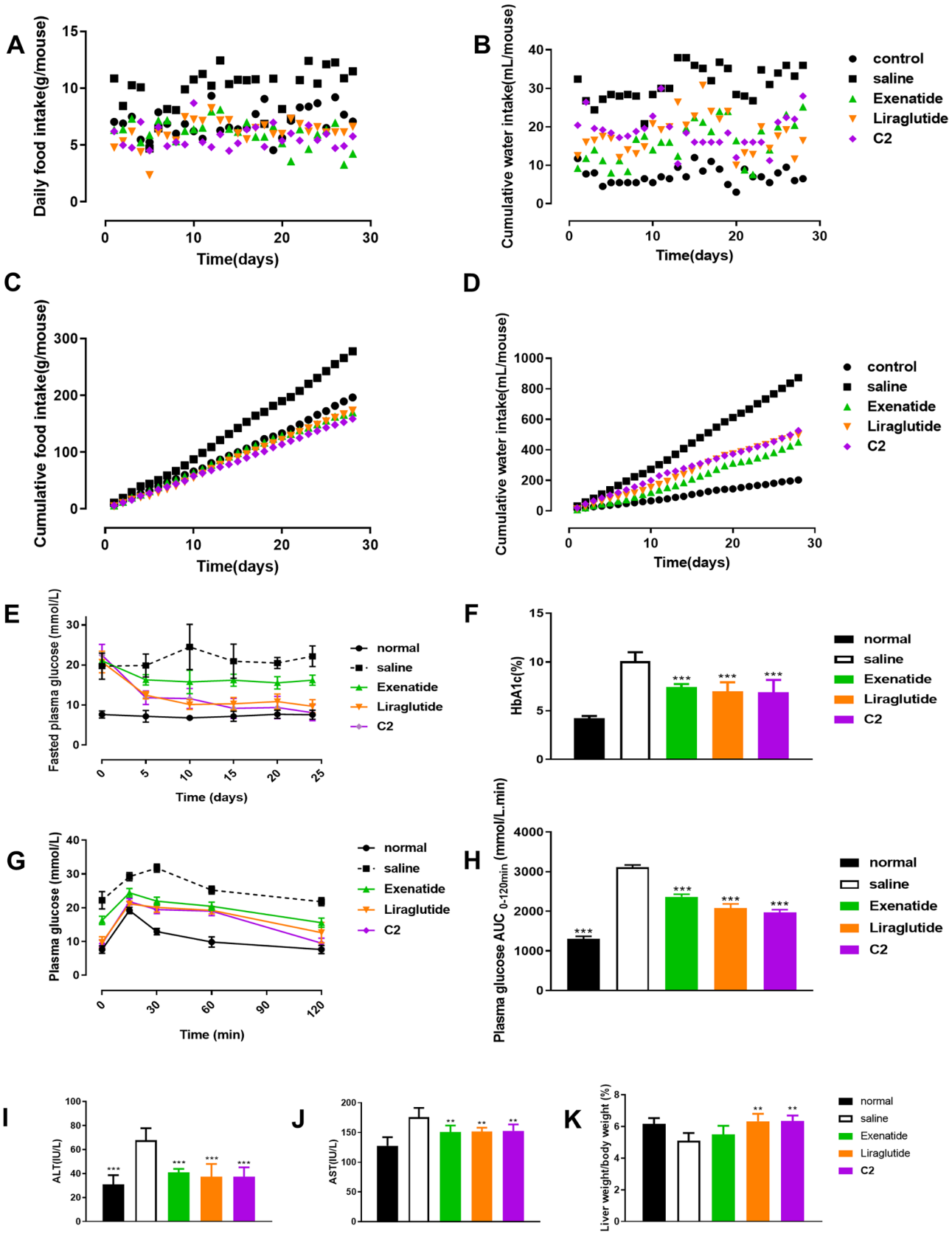



**Fig. 6.** The effects of chronically administered **C2**, exenatide and liraglutide in diabetic mice. Time course for chronic effect of **C2** administered for 3-weeks in STZ-induced diabetic mice on daily food and water intake. Time course for chronic effect of **C2** administered for 4 weeks in STZ-induced diabetic mice on daily food (A) and water (B) intake and cumulative food (C) and water (D) intake. (E) Fasted plasma glucose measured every five days. (F) $HbA_{1C}$ measured at day 24. (G) IPGTT test results in mice at day 24. (H) AUC of IPGTT test results in mice at day 24. (I) ALT. (J) AST. (K) Regeneration ratio of liver corrected by body weight. ALT, alanine aminotransferase; AST, aspartate aminotransferase. n = 6 per group. Data are given as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 compared with saline.

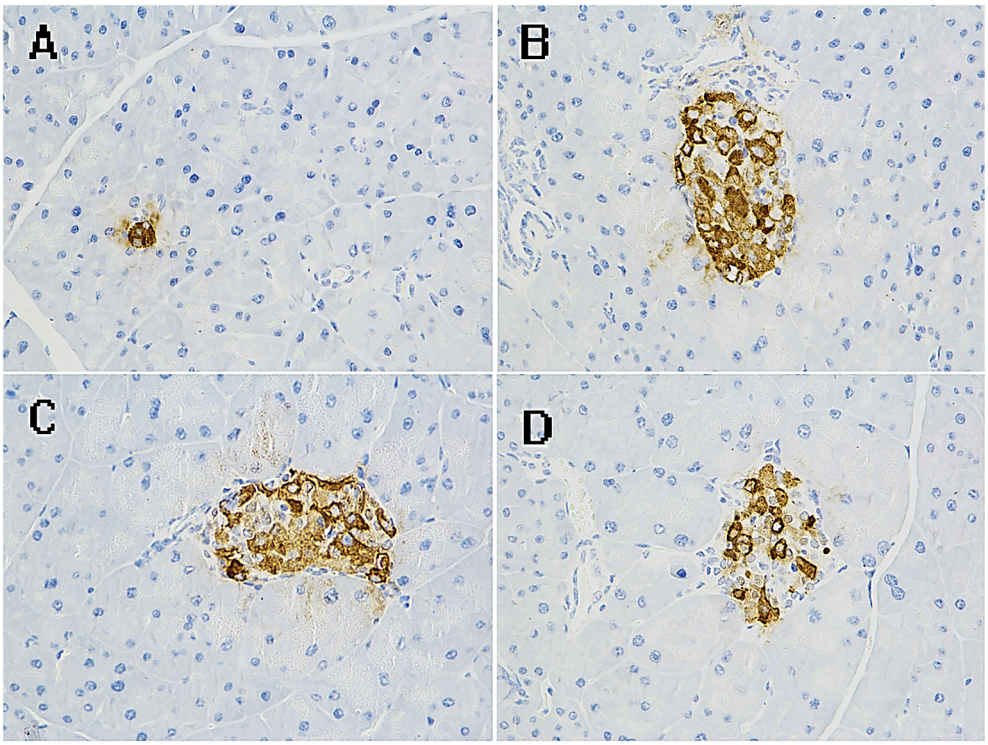



**Fig. 7.** Representative images of pancreatic insulin-positive β cells after 3-weeks treatment of Liraglutide, Exenatide, **C2** (25 nmol/kg).

50 μM $H_2O_2$, similar results were observed, indicating that **C2** could protect cell viability and inhibit apoptosis against STZ and $H_2O_2$.

### 3.6. Chronic in vivo studies on STZ-induced diabetic mice

STZ-induced diabetic mice were daily injected with saline, exenatide, liraglutide or **C2** (25 nmol/kg) for 21 days for evaluating the long-term therapeutic effect. Food intake and water intake during the treatment were measured every day, and cumulative food intake and water intake with time was calculated to evaluate the appetite suppressing effect of conjugates **C2** (Fig. 6A, B). Compared with normal mice, food intake and water intake of diabetic mice in the saline group increased. Meanwhile, all administered groups were significantly lower than those of the saline group. Markedly, cumulative food intake and water intake of the **C2**-treated group was 77.52 g and 251.2 g respectively lower than those of the saline group, which indicating that **C2** had a significant appetite suppressing effect.

Fasting blood glucose level of diabetic mice in administration group gradually tended to normal level after 21 days treatment. Notably, **C2**-treated group lowered fasting glucose toward 8.1 ± 1.8 mmol/L, while the fasting blood glucose of diabetic mice in the saline group was above 20 mmol/L (Fig. 6C). HbA1c reflects the level of glycemia to which the cell has been exposed during its life-cycle, which was regarded as indicator of cumulative blood glucose concentrations.[17] HbA1c of the diabetic mice was significantly higher than that of the normal mice, while the HbA1c of administration groups were decreased, which was significantly lower than that of the saline group (Fig. 6D, $p < 0.01$).

IPGTT test was performed after 21 days treatment. As illustrated in Fig. 6E, F, saline treated diabetic mice had very poor glucose tolerance levels and the peak blood glucose level was above 30 mmol/L. The glucose tolerance levels of administration groups were improved. The AUC of blood glucose **C2** treated was considerably lower than that of the saline treated diabetic mice ($p < 0.05$).

Liver lesion was known to raise in diabetic mice, which can be evaluated by detecting the content of alanine aminotransferase, aspartate aminotransferase, and the proportion of liver weight to body weight. On day 24, animals were sacrificed for collecting serum to measure ALT and AST levels and calculating the percentage of liver weight to total body weight. As shown in Fig. 6G, H, I, the ALT and AST levels were significantly lower in the administration group than in the saline group ($p < 0.01$). In addition, the liver weight/body weight ratio of the saline group mice was significantly decreased, indicating that the liver injury in administration groups was significantly improved. It is indicated that **C2** had protective effects on liver damage in diabetic mice.

At the end of the long-term treatment experiment (24 days), the pancreas tissues of mice were gathered and sliced, and the insulin-positive cells were observed by immunohistochemical method. As shown in Fig. 7, the number of islet positive cells in diabetic mice was significantly reduced and the number of insulin-positive cells was significantly increased in exenatide, liraglutide and C2 treated groups. It is shown that **C2** has excellent islet cell protection effect.

### 3.7. Acute feeding studies and IPGTT on DIO mice

GLP-1R agonists have the effect of delaying gastric emptying and appetite suppression. In this paper, diet-induced obesity (DIO) mice were used for body weight experiments. After 12 h of fasting, injected intraperitoneally with normal saline, exenatide, liraglutide or preferred compound **C2** (25 nmol/kg), the interval food intake of the mice after injection was recorded (Fig. 8A). As the experiment progressed, the food intake of the mice gradually increased, and the food intake of the DIO mice in the administered groups were significantly less than those in the saline group and the control group. Notably, **C2** exhibited a comparable appetite suppression level to liraglutide.

IPGTT was also examined to evaluate the hypoglycemic level of **C2** at DIO mice. As illustrated in Fig. 8B, C, after 15 min of intraperitoneal injection of glucose, the blood glucose of the control group and the saline group increased rapidly, peaking at nearly 20 mmol/L, while glycemia was stable in administration groups. The AUC of blood glucose **C2**-treated was considerably lower than that of the saline treated DIO mice ($p < 0.05$).

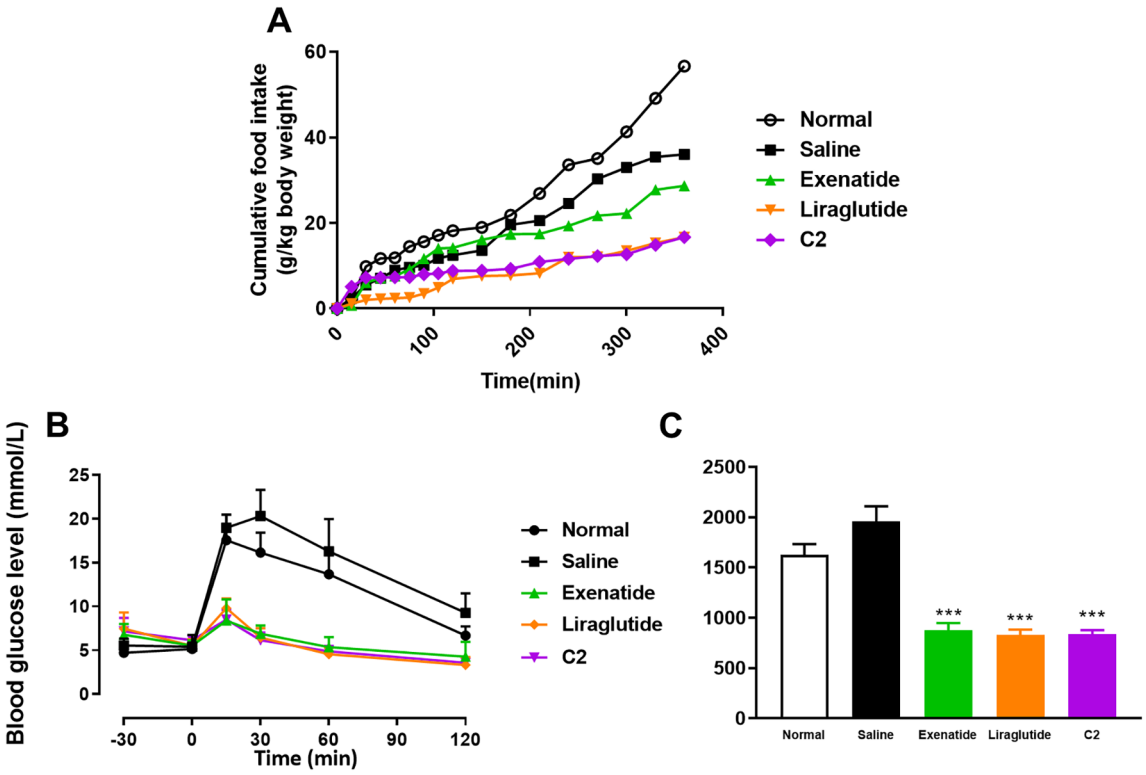



**Fig. 8.** (A) Time course of acute effect on food intake in fasted mice with **C2**. (B) IPGTT test results in DIO mice. (C) AUC of IPGTT test results in DIO mice. n = 6 per group. Data are given as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 compared with saline (control).

## 4. Discuss and conclusion

In this paper, we proposed a novel lipid chain that conjugated to optimized exenatide analogs. Four modified exenatide analogs were synthesized and characterized to prolong short half-life. For proving the relevant structure-activity relationships, GLP-1 receptor activation assay was firstly evaluated. As shown in Table 1, the receptor activation of compounds **C1–C4** retained relatively high potency compared to exenatide. The following in vitro stability test revealed that the plasma stability ability of all conjugates **C1–C4** were improved (Fig. 2). Notably, **C2** exhibited the best plasma half-life, and **C2** was further corroborated for in vivo glucose-lowering abilities in STZ-induced diabetic mice by calculating the hypoglycemic duration (Fig. 3). The hypoglycemic durations of **C2** in diabetic mice were much longer than those of exenatide and comparable with those of liraglutide. The long-acting characteristic of **C2** was notably more improved than exenatide, most likely due to its reduced renal clearance resulting from the strong albumin binding ability and enzyme resistance. It is interesting to find that concerned albumin binding by **C2** and its long-acting profiles were harmonious with previous successful reports regarding the protracted in vivo circulation half-life values of fatty acid chain-modified GLP-1 analogs, attributed to their physical interactions with serum albumin. What's more, **C2** exhibited comparable insulinotropic activities and glucose-lowering abilities with those of liraglutide and exenatide (Fig. 4), and protection effects against oxidative stress and DNA damage mediated stress (Fig. 5). Like GLP-1 biological action mode, the in vivo glucose management action of **C2** was observed to be exerted by glucose-dependent mode. Which means, such mechanism offered the advantage of increased drug safety so that **C2** can be used clinically without hypoglycemia risk. Furthermore, once daily administration of **C2** to STZ-induced diabetic mice achieved long-term beneficial effects on HbA1c lowering and glucose tolerance. The enhanced glucose tolerance of **C2** treated mice probably resulted from the prolonged biological activity (β cell neogenesis and/or proliferation). The results of liver and pancreas proved that **C2** has a protective effect on liver and pancreas. The results of acute feeding studies of **C2** treated DIO mice revealed its potential body weight management.

In summary, this present study demonstrates that lipid chain modified strategy offers a practical approach to the development of long-acting exenatide-based antidiabetics. This study also shows that **C2**, preserved biological activity, long-acting antidiabetic characteristics, and long-term beneficial effects, is a promising biological agent deserving further investigation to treat obesity patients with diabetes.

## 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.

## Acknowledgement

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