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# Novel fatty acid chain modified GLP-1 derivatives with prolonged in vivo glucose-lowering ability and balanced glucoregulatory activity

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ABSTRACT

Glucagon-like peptide-1 is a potent hypoglycemic hormone with beneficial properties for the treatment of diabetes. However, its half-life is short because the rapid metabolic degradation. This study aims to prolong the half-life of glucagon-like peptide-1 through conjugation with the fatty acid side chain which helps the conjugates to interact with the albumin. Firstly, we chose two optimized polypeptide chains which have tremendous hypoglycemic effect named $Cys_{17}$-$Gly_8$-GLP-1(7-36)-$NH_2$ and $Cys_{37}$-$Gly_8$-GLP-1(7-37)-$NH_2$, and various fatty acid chains were modified. All conjugates preserved relatively strong GLP-1R activation and **I-6** behaved best in glucose-lowering ability. The prolonged antidiabetic effects of **I-6** were further confirmed by hypoglycemic efficacy test in vivo. Meanwhile, once daily injection of **I-6** to diabetic mice achieved long-term beneficial effects on glucose tolerance, body weight and blood chemistry. It is concluded that **I-6** is a promising agent for further investigation of its potential to treat obese patients with diabetes.



## 1. Introduction

Glucagon-like peptide-1 (GLP-1) is a potent hypoglycemic hormone, emphasized a great opportunity for its potential for the treatment of diabetes.[1] GLP-1 exhibits stimulation of pancreatic insulin secretion in glucose-dependent way.[2] Thus, the risk of hypoglycemia is reduced.[3] A short half-life of endogenous GLP-1 limits its clinical application, which accounts for the rapid metabolic degradation and elimination by kidney filtration.[4]

Up to now, several distinct strategies have been described.[5] Methods have been developed to reduce renal elimination through enlarging the molecular size, such as, PEG or ELP conjugates. Lee et al. linked two Ex-4 molecules together by using a commercially available bimaleimide amine to which a PEG chain had already been conjugated so as to prolong pharmacokinetic duration by increasing the hydrodynamic radius.[6] Although the Ex-4 polymer showed a moderate improvement in binding affinity for GLP-1 receptor over parent Ex-4, PEGylation reduced its affinity. In expectation, the conjugate possessed a far more better pharmacokinetic profile than parent Ex-4 itself. Luginbuhl. et al. fused GLP-1 to an elastin-like polypeptide biopolymer and injected it as a subcutaneous injection depot which had improved in vivo half-life and controlled release features.[7] It is reported that the depot formed after a single injection of GLP-1-ELP conjugate results in zero-order release kinetics. However, PEG is non-biodegradable and some PEGylated proteins have been associated with generation of anti-PEG antibodies and cellular toxicity.[8] Meanwhile, the industrialization of ELPylated peptide also takes a long time. Thus, the GLP-1 receptor agonist field still has a need for new sustained-release therapies that can achieve safety hypoglycemic action and convenience preparation of production. Such a formulation would be likely to reduce gastrointestinal side effects, improve patient comfort, and further dissociate compliance from therapeutic outcome.

Towards this goal, considering the physicochemical characteristics of fatty acid chain modified conjugates, like interacting with serum albumin.[9] Our group has investigated the hypoglycemic effect of many GLP-1 conjugates and supplied a series of site-specific cysteine substitution polypeptide chains which have tremendous hypoglycemic effects and created an effective and

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convenient strategy to screen desired peptides for further investigation.[5,10]

In this study, we firstly chose two optimized polypeptide chains which have tremendous hypoglycemic effects named as $Cys_{17}$-$Gly_8$-GLP-1(7-36)-$NH_2$**(1)** and $Cys_{37}$-$Gly_8$-GLP-1(7-37)-$NH_2$**(2)**.[10] In the previous work, these two polypeptide chains which acted excellently in antidiabetic characteristics had superior competency to activate GLP-1 receptor.[11] Through the thiol group with high chemo selectivity, maleimide modified fatty acid chain was then attached to the cysteine. A series of fatty acid chain modified GLP-1 conjugates were designed (Fig. 1), afterwards their structural, biological, and biochemical analysis were explored. The optimized compound **I-6** possessed the most long acting antidiabetic properties. Further investigation revealed that **I-6** could also promote INS-1 cell viability (Fig. 5). Compounds **I-6** was identified as long-acting and highly biologically active GLP-1 derivative, Long-term beneficial effects in diabetic mice (Fig. 6) and diet induced mice (Fig. 8) were also evaluated.

## 2. Results

### 2.1. Synthesis and characterization of the fatty acid chain-modified GLP-1 analogs

Applying standard solid-phase peptide synthesis protocol with N-Fmoc/tBu chemistry and microwave irradiation, two cysteine altered peptides $Cys_{17}$-$Gly_8$-GLP-1(7-36)-$NH_2$**(1)**, $Cys_{37}$-$Gly_8$-GLP-1(7-36)-$NH_2$**(2)** were synthesized. Three different fatty acid chains with maleimide were prepared and reacted with the cysteine altered peptides to obtain 6 fatty acid chain-modified conjugates (**I-1** ∼ **I-6**). These conjugates (Fig. 1) were purified and identified by preparative PR-HPLC and Waters ACQUITY UPLC–MS System. Using HEK293 cells over expressing human GLP-1R, we determined the potency of all 6 conjugates. As the data shown in Table 1, all conjugates preserved relatively strong receptor activation while slightly reduction was observed. Furthermore, the longer fatty acid chain exhibited weaker receptor activation than the shorter fatty acid chain, indicating that the length of the fatty acid chain had a bearing on the GLP-1 receptor activation. The results suggested that the conjugates of cysteine-substituted peptides and fatty acid chain were strong GLP-1R agonists.

### 2.2. Albumin binding

The expected long-acting effects may be attributed to the remarked albumin binding affinities of the fatty acid chain. Thus, the albumin binding assay was achieved using conjugates **I-1** ∼ **I-6**, Ex-4 and liraglutide. As shown in Fig. 2, 21.0 ± 3.6% of Ex-4 and 86.7 ± 2.5% of liraglutide were found to associate with albumin resin respectively under physiological conditions (in PBS pH 7.4). The majority fatty acid chain-modified conjugates presented considerably increased albumin binding ability than Ex-4.

### 2.3. Plasma stability

The in vitro stability of the 6 conjugates **(I-1** ∼ **I-6)**, compared with Ex-4 and liraglutide, was performed in the plasma stability assay. As illustrated in Fig. 2, the half-life of Ex-4 was approximate 3.6 h while that of long-acting agonist liraglutide was 15.4 h at 37 °C in rat plasma, nearly 4-fold longer. As expected, most fatty acid chain modified conjugates were more stable than Ex-4. What's more, the longer fatty acid chain exhibited better in long-acting effects than the shorter.

The albumin binding ability and in vitro stability of conjugates **I-1** ∼ **I-6** were correlated well each other. In this study, fatty acid chain-modified conjugate **I-6** showed noticeably highest plasma stability and albumin binding ability, which was very important for the accomplishment of long-acting in vivo activity. Consequently, the in vivo biological activity of **I-6** was performed in the subsequent assay.

### 2.4. Glucoregulatory and insulin secretion assay

The in vivo antidiabetic activity of fatty acid chain-modified conjugate **I-6** was examined by oral glucose tolerance test (OGTT) in SD rats. As illustrated in Fig. 3, after the intraperitoneal administration of **I-6,** the mean blood glucose level rapidly increased to 13.8 ± 1.31 mmol/L in the saline treated group while that in the Ex-4, liraglutide and **I-6** treated group reduced to ∼7.6, ∼7.2 and 6.7 mmol/L at 30 min, respectively (Fig. 3A). Meanwhile, the glucose AUC of Ex-4, liraglutide and **I-6** was considerably lower than that of the saline treated group (Fig. 3B). In this assay, the decrease in plasma glucose was associated with the increase in plasma insulin concentration which was coherent with the GLP-1-dependent mechanism (Fig. 3C). The time courses for plasma insulin concentrations in Ex-4, liraglutide and **I-6** observed was similar in where all plasma insulin concentration from 15 to 60 min were notably greater than those of the control group. Especially, **I-6** behaved comparable insulin secretion promoting ability than Ex-4 and liraglutide ($p < 0.05$). The glucose-lowering abilities and insulin tropic activities of **I-6** were comparable with those of Ex-4 and liraglutide.

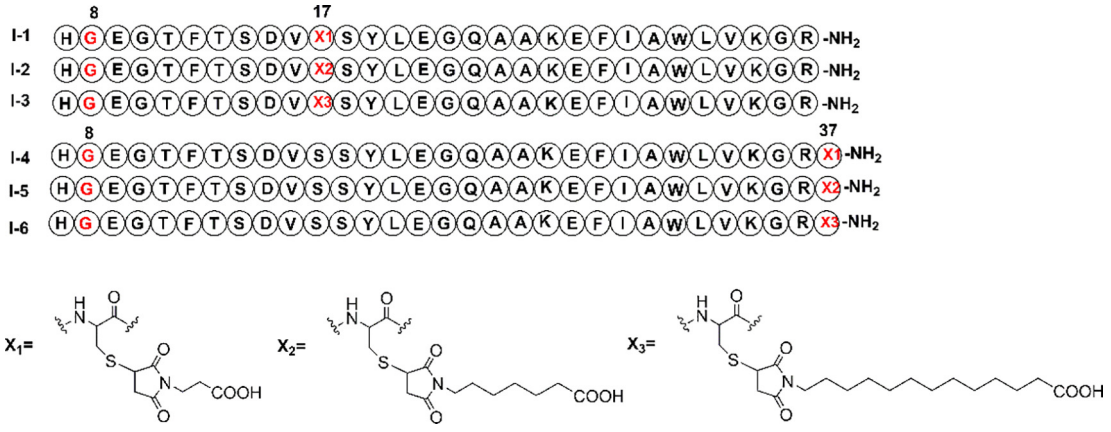


**Fig. 1.** Structure of compounds **I-1** ∼ **I-6.**

**Table 1**
MS data of conjugates.

| Conjugates | $EC_{50}$(pM)[a] | Purity | Formula | Molecular mass | Molecular mass calculated | | Molecular mass found | |
|---|---|---|---|---|---|---|---|---|
| | | | | | $[M + 3H]^{3+}$ | $[M + 4H]^{4+}$ | $[M + 3H]^{3+}$ | $[M + 4H]^{4+}$ |
| $Gly_8$-GLP-1(7-36)-$NH_2$ | 5.6 ± 0.9 | N/A | N/A | N/A | N/A | | N/A | |
| Ex-4 | 1.8 ± 0.8 | N/A | N/A | N/A | N/A | | N/A | |
| I-1 | 5.6 ± 1.1* | 95.7% | $C_{155}H_{231}N_{41}O_{48}S$ | 3468.8 | 1157.3 | 868.2 | 1157.6 | 868.9 |
| I-2 | 14.3 ± 2.0* | 95.8% | $C_{158}H_{237}N_{41}O_{48}S$ | 3510.9 | 1171.3 | 878.7 | 1171.1 | 878.7 |
| I-3 | 26.3 ± 2.5* | 97.2% | $C_{164}H_{249}N_{41}O_{48}S$ | 3595.1 | 1199.3 | 899.7 | 1199.3 | 899.2 |
| I-4 | 6.1 ± 1.9* | 96.3% | $C_{158}H_{236}N_{42}O_{50}S$ | 3555.9 | 1186.3 | 890.0 | 1186.8 | 890.5 |
| I-5 | 9.3 ± 1.1* | 96.8% | $C_{161}H_{242}N_{42}O_{50}S$ | 3598.0 | 1200.3 | 900.5 | 1200.9 | 900.4 |
| I-6 | 19.8 ± 2.1* | 95.1% | $C_{167}H_{254}N_{42}O_{50}S$ | 3682.2 | 1228.4 | 921.6 | 1228.8 | 921.3 |

[a] 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.

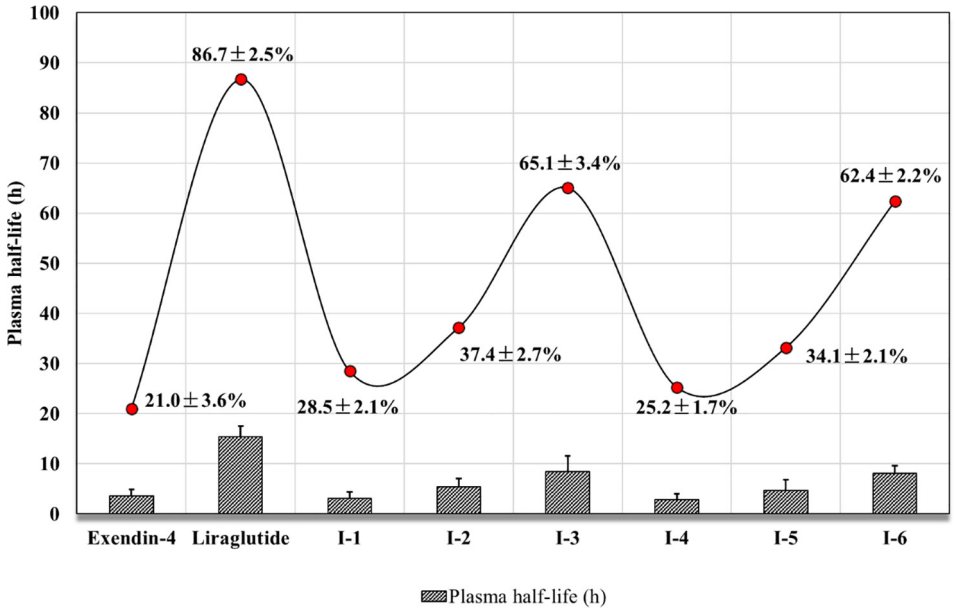


**Fig. 2.** The plasma stability and albumin binding affinity of the fatty chain-modified GLP-1 conjugates, Ex-4 and liraglutide.

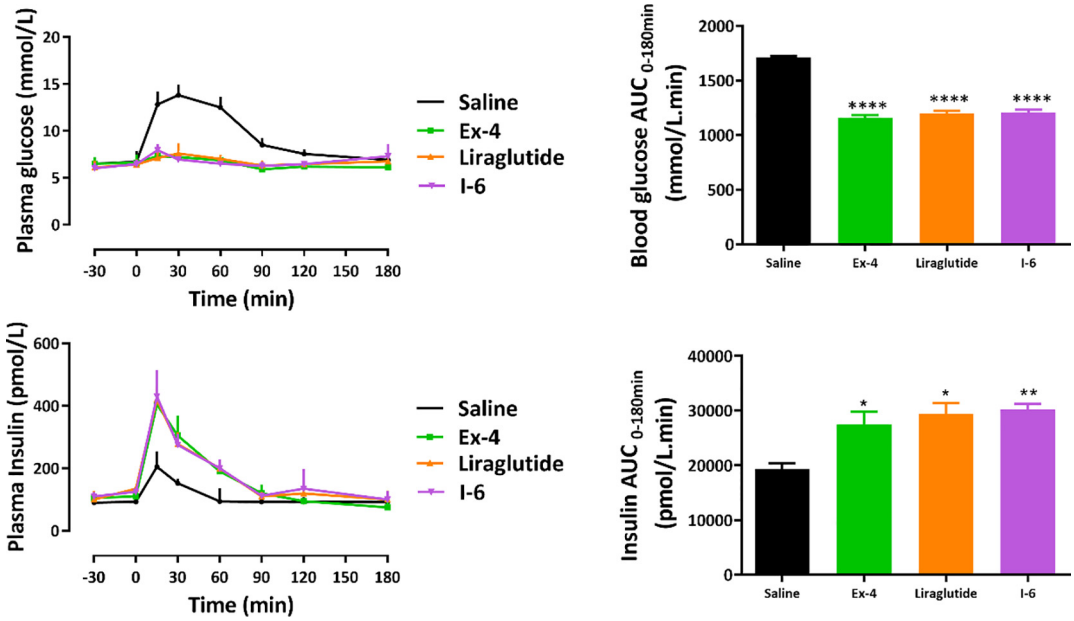


**Fig. 3.** In vivo biological activity tests of **I-6**. Liraglutide, Ex-4 and **I-6** (25 nmol/kg) were i.p. injected into SD rats; glucose was taken orally (10 g/kg). (A) The glucose-lowering effects and calculated plasma glucose $AUC_{0-180min}$ (B) of liraglutide, Ex-4 and **I-6**. (C) Insulinotropic activities and calculated insulin $AUC_{0-180min}$ (D) of liraglutide, Ex-4 and **I-6**. n = 6 per group. Data are given as mean ± SD. *$p < 0.05$, **$p < 0.01$ compared with saline (control).

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### 2.5. Hypoglycemic duration test

In the present study, normal blood glucose level was set as below 8.35 mmol/L, and englycemic durations below this magnitude were calculated and viewed as a practical indication for antidiabetic treatment.[10] The hypoglycemic effects of compound **I-6** were performed at two does (25 and 150 nmol/kg, i.p.) in STZ-induced diabetic mice. As shown in Fig. 4A, a hyperglycemic state (average > 20 mmol/L) was observed in saline treated control mice but a normal blood glucose level was maintained in those treated with Ex-4 and liraglutide for nearly 4.8 h and 17.7 h, respectively. At the dose of 25 nmol/kg, the target durations in **I-6** treated mice (~9.7 h) were much longer than Ex-4 and were shorter than that in liraglutide (Fig. 4A). When the dose was raise to 150 nmol/kg, the $AUC_{0-36h}$ values of I-6 were much lower than those of saline or Ex-4 treated mice (Fig. 4B). In this assay, the time required to below a glucose level of 8.35 mmol/L was 11.5 h.

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

To determine the protective effect of **I-6** on cell viability and apoptosis, Compared with saline controls, **I-6** 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 1 μM STZ, cell viability of control INS-1 cells was significantly decreased by ~45%, while only ~28.8% in 1 nM and ~21.9% in ≥10 nM **I-6** treated INS-1 cells ($p < 0.05$ for 10 nM and 100 nM, compared with saline) (Fig. 5A–C). After 24 h incubation with glucolipotoxicity media or 50 μM $H_2O_2$, similar results were observed. Flow cytometric analysis was also performed, 24 h treatment with glucolipotoxicity media, 50 μM $H_2O_2$ or 1 μM STZ caused a drastic increase of apoptosis in INS-1 cells at saline-treated control group, however, less apoptosis was found in Ex-4, liraglutide and **I-6** treated INS-1 cells (Fig. 5D), indicating that **I-6** could protect β-cell viability and inhibit apoptosis against glucolipotoxicity, oxidative stress and DNA damage mediated stress.

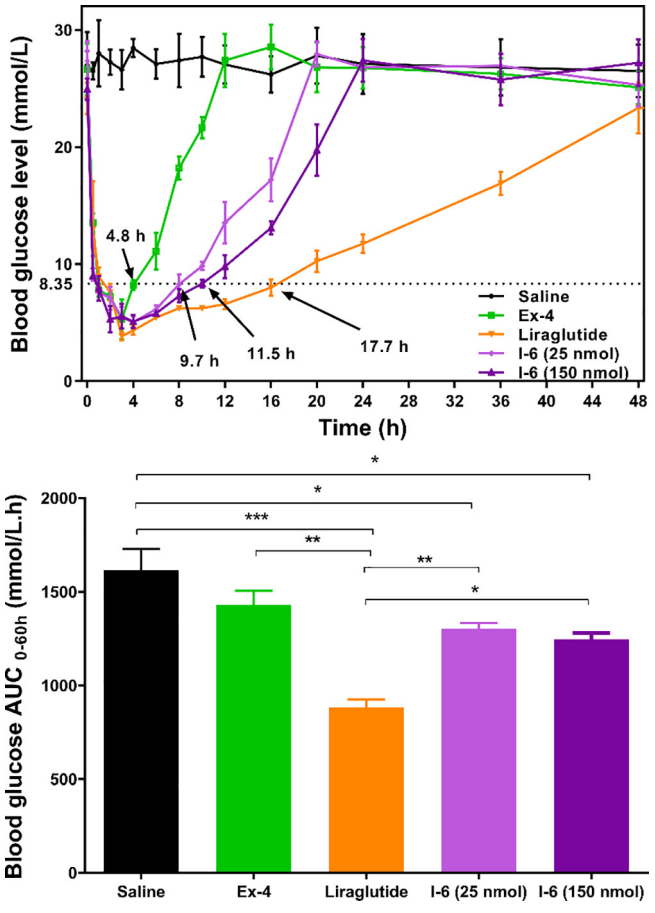



**Fig. 4.** Glucose-lowering and stabilizing effect of Ex-4, liraglutide and **I-6** as determine by hypoglycemic duration test in nonfasted diabetic mice. (A) Time-course average blood glucose levels of diabetic mice after an i.p. injection of Ex-4, liraglutide or **I-6** (25 nmol/kg or 150 nmol/kg). Times depict hypoglycemic duration rebound to 8.35 mmol/L. (B) Hypoglycemic effects of Ex-4, liraglutide and **I-6** based on the calculated glucose $AUC_{0-60h}$ values. n = 6 per group. Data are given as mean ± SD. *$p < 0.05$, **$p < 0.01$ compared with saline (control).

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

In vivo activity and potential therapeutic utility was probed STZ-induced diabetic mice received chronic administration of **I-6**. Ex-4 and liraglutide (25 nmol/kg) were injected (once daily) and saline was used as control. Fasting blood glucose level in diabetic mice prior to treatment was 17.5 ± 1.9 mmol/L and in saline treated group it was worsened somewhat to 20.5 ± 1.3 mmol/L after 21 days. In contrast with saline treated control, administration of **I-6** lowered fasting glucose toward the 10.7 ~ 11.3 mmol/L levels (Fig. 6**A**). HbA1c was regarded as an indirect pointer of cumulative blood glucose concentrations which was a more responsive indicator of glycemic control than glucose concentration and resulted from non-enzymatic irreversible glycation.[12] Initially HbA1c was well matched in all diabetic groups which was considerably higher than in nondiabetic littermates. As shown in Fig. 6B, after a 21-day treatment, HbA1c in diabetic mice daily injected **I-6** revealed a gradual reduction while that in control group stayed to be high. The medication of **I-6** caused the HbA1c reduction, which was indicative of promising role of **I-6** in clinical studies.

IPGTT test was performed before and after treatment (day 0 and day 24), to determine if long-term treatment of **I-6** improved glucose tolerance or not. At the beginning of the treatment, no difference was observed between each group. However, at the end of the treatment, Ex-4, liraglutide and **I-6** treated mice exhibited rapid blood glucose reductions and the blood glucose of those returned to base line at 120 min after injection of glucose, whereas saline treated mice manifested a typical diabetic outline of slow reduction on the hyperglycemic state after 120 min of administration (Fig. 6C). The AUC of blood glucose **I-6** treated was considerably lower than that of the saline treated diabetic mice ($p < 0.05$) (Fig. 6D).

To estimate the protective and rehabilitation of **I-6**, pancreas of mice was gathered, followed with fixation, section and HE stain. As shown in Fig. 7**A**, the edge of islet β cells was such rough and the nucleus was diminished along with the β cells narrowed. As stated in Fig. 7B, islet area in the saline treat mice was significant smaller than that in the normal mice while the islet area in the Ex-4, liraglutide and **I-6** treated mice was raised notably after 21 days treatment.

### 2.8. Chronic in vivo studies on DIO mice

As shown in Fig. 8A, after the treatment in STZ-induced diabetic mice, body-weight change was noticed while the body weight of **I-6** treated group was lower than that in saline treated group. As shown in Fig. 8A, during the 4-week treatment progression, **I-6** inhibited cumulative water and food intake by up to 33.5% and 24.9%, respectively. Meanwhile, nearly the same amount of water was taken by **I-6** treated mice as lean mice. To further verify the appetite suppression effect, an in vivo acute study was performed and **I-6** inhibited food intake by up to 34.5% at 6 h after administration. Body weight gain of the saline treated DIO mice continually

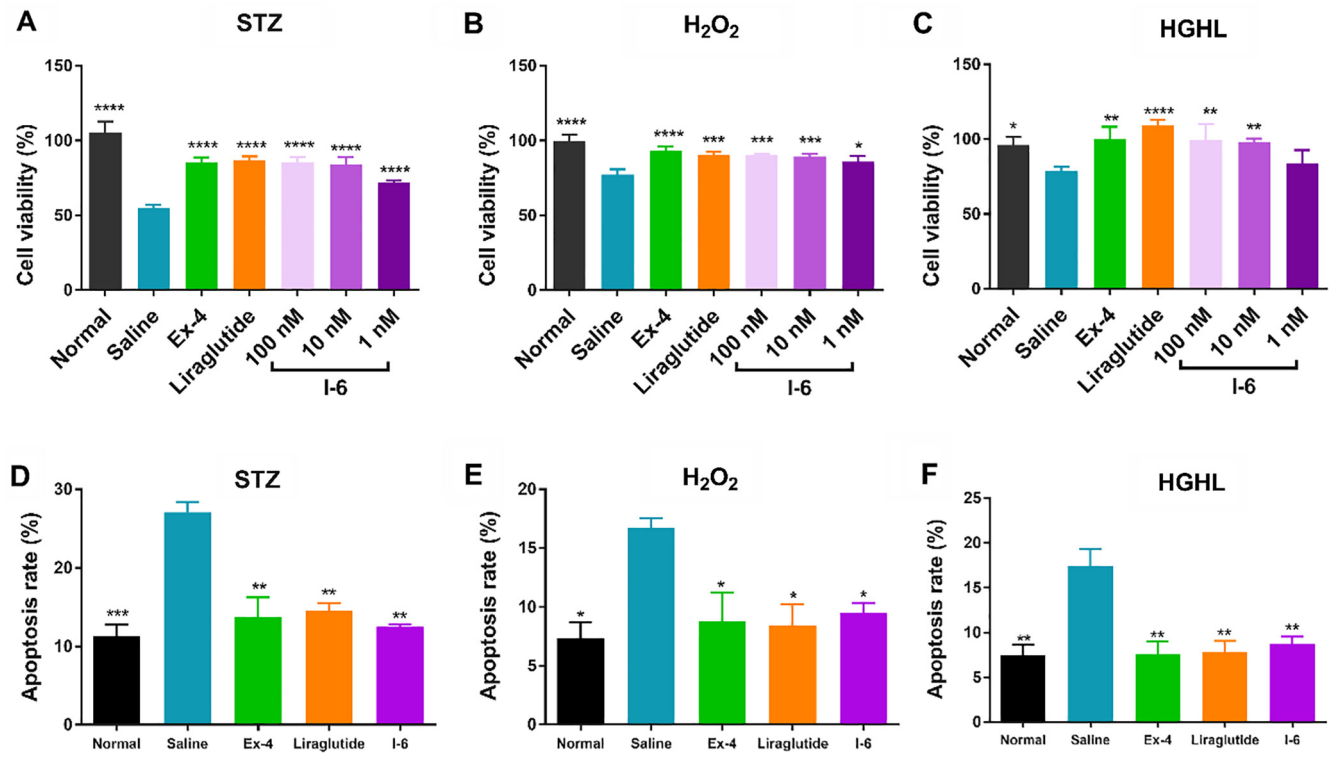



**Fig. 5.** **I-6** attenuates glucolipotoxic and oxidative stress and enhances viability and survival of INS-1 cells. **I-6** preserved cell viability in response to STZ (A). **I-6** preserved viability and survival of INS-1 cells against oxidative stress (B). **I-6** preserved viability and survival of INS-1 cells against glucolipotoxic stress (C). **I-6** decreased cell apoptosis in response to STZ (D), oxidative stress (E) and glucolipotoxic stress (F). All experiments were performed in triplicate and repeated three times (n = 3). Data are given as mean ± SD. * $p < 0.05$, compared with saline.

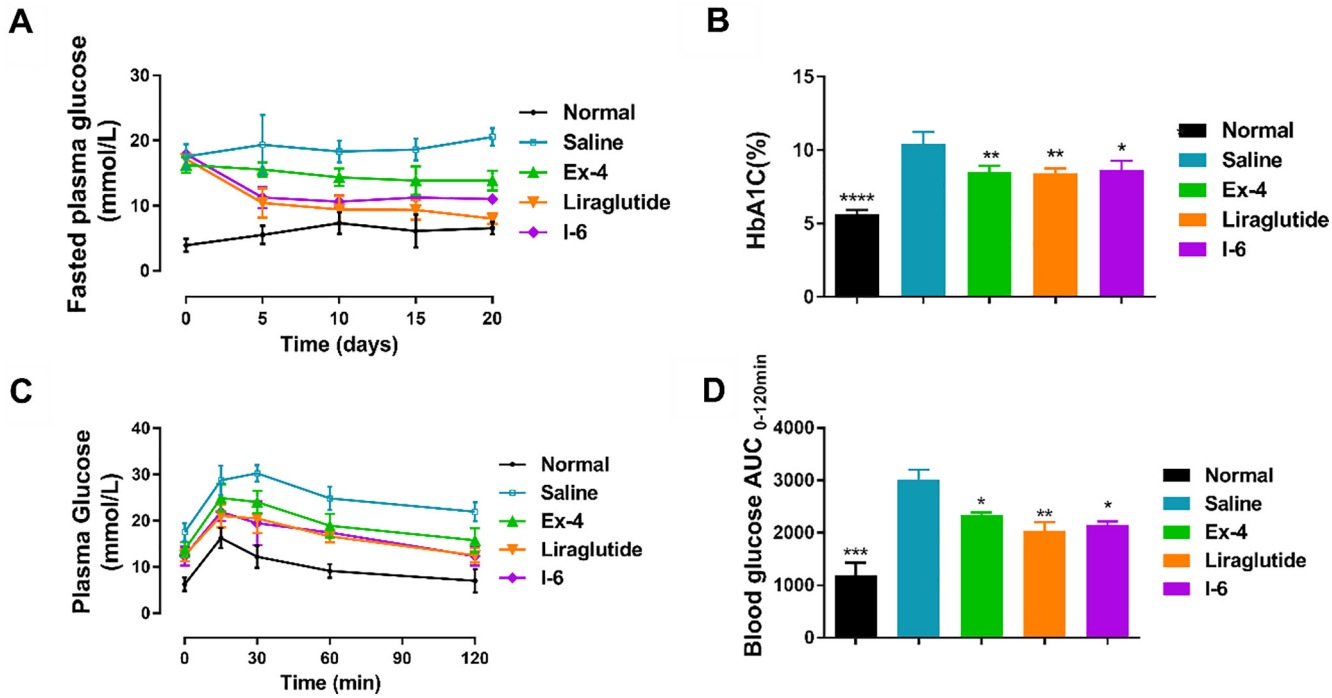



**Fig. 6.** The effects of chronically administered **I-6**, Ex-4 and liraglutide in diabetic mice. (A) Fasted plasma glucose measured every five days. (B) HbA1C measured at day 24. (C) IPGTT test results in mice at day 24. (D) AUC of IPGTT test results in mice at day 24. n = 6 per group. Data are given as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with saline (control).

increased over the 4-week treatment progression by ~5 g whereas it was suppressed after administration of either Ex-4, liraglutide or **I-6**.

The serum levels of leptin and adiponectin were also amended after **I-6** treatment which were important adipocytokines secreted from white adipose tissue. As shown in Fig. 8B, leptin

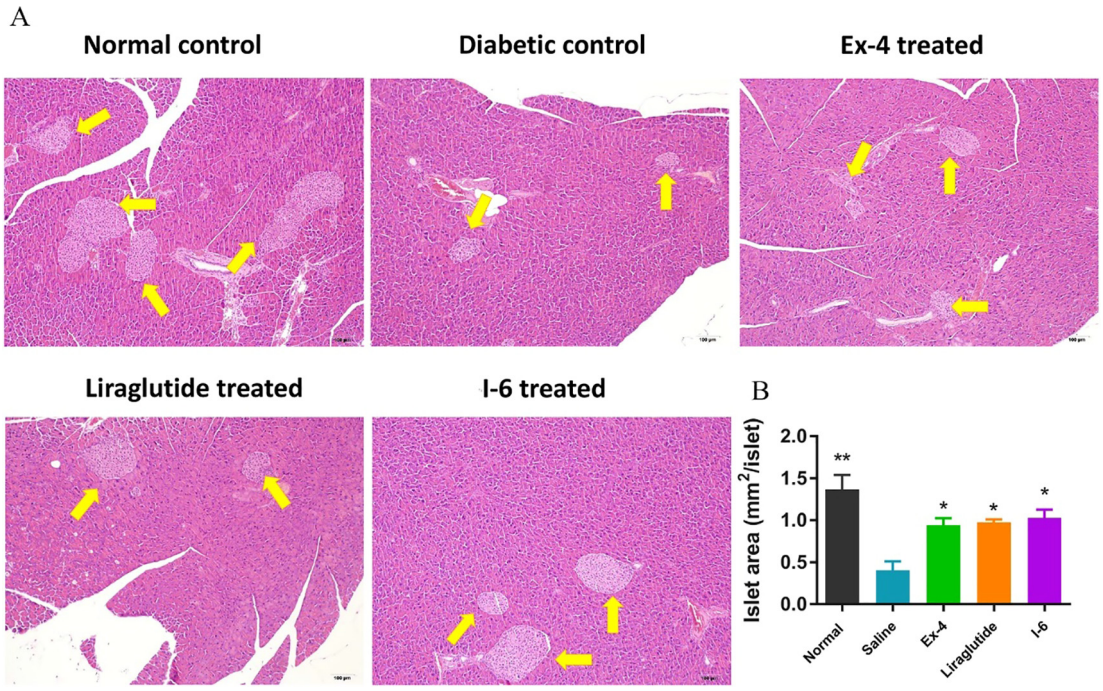



**Fig. 7.** Effects of **I-6** on islet size. Histological analysis was performed on the islet tissue from saline treated, normal mice, diabetic mice, Ex-4, liraglutide and **I-6** treated diabetic mice, respectively (A). Sections (5 μm) were stained with H&E and the area of each islet. Islet areas were assessed by manually drawing around the islet perimeter with the measuring tool contained in the software Image-Pro Plus 6.0. n = 6 per group (B). *$p < 0.05$, **$p < 0.01$ compared with saline (control).

concentration circulating in DIO mice was notably higher than that in lean mice controls ($p < 0.05$). Meanwhile, **I-6** administration notably ($p < 0.05$) reduced leptin level circulating to a near normal value of 2.5 ng/ml. Nearly 39% serum adiponectin level in DIO was reduced after prolonged exposure to high diet induced compare with that in lean mice ($p < 0.05$), and this level was significantly increased ($p < 0.05$) by **I-6**. Recent evidence shows that overweight represents high risk factors for steatohepatitis.[13] In present study, hepatocyte damage was also assessed by examining serum enzyme activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The result showed that **I-6** treatment resulted in a reduction in ALT ($p < 0.05$) and AST ($p < 0.05$) than that in saline treated LFD mice. Moreover, similar reduction trend was also detected in serum total cholesterol (TC) and triglyceride (TG) ($p < 0.05$ and $p < 0.05$, respectively, compared to DIO mice). In addition, the ratio between "good" (HDL) and "bad" (LDL) cholesterol values, LDL/HDL, were considered to analyze the degree of increases in the two lipoproteins in different groups. As shown in Fig. 8G, prolonged exposure to HFD increased this ratio by 20% while **I-6** treated DIO mice lead to a reduction trend but not significant following administration. Furthermore, the brown adipocytes and the white adipocytes were gathered after treatment. As shown in Fig. 9, the white or brown adipocytes cells in **I-6** treated DIO mice was significantly smaller than that in DIO mice. **I-6** could reduce body weight in obese mice.

## 3. Discussion

In this study, a series of novel fatty chain modified GLP-1 analogs were designed and prepared to search for long-acting GLP-1 conjugates with retained efficacy. The initial GLP-1 receptor activation assay granted direct evidence of the relevant structure-activity relationships. As stated in Table 1, the receptor activation of compounds **I-1** ~ **I-6** depended on the length of the fatty acid chain of fatty acid chain-maleimide and were independent of the position of cysteine residues (Cys17 and Cys37). The following in vitro albumin binding test and stability test revealed that the plasma stability and albumin binding ability of all conjugates **I-1** ~ **I-6** were improved to different degrees. Notably, the plasma stabilities of the test conjugates associated well with their albumin binding abilities. **I-6** exhibited the longest plasma half-life and the highest albumin binding ability, **I-6** was further estimated for in vivo glucose-lowering abilities and insulinotropic activities. It was shown that **I-6** showed comparable protection effects against glucolipotoxicity, oxidative stress and DNA damage mediated stress (Figs. 3 and 4), insulinotropic activities and glucose-lowering abilities with those of liraglutide and Ex-4 (Fig. 5). Vitally, the in vivo biological action of **I-6** was observed to be exerted via the glucose-dependent action mode. This action offered the advantage of increased drug safety compared with drugs that increased insulin secretion via glucose-independent mechanisms (e.g., sulfonylureas), indicating that **I-6** can be used clinically without the risk of hypoglycemia. Then it was evaluated that the in vivo long-acting glucose-lowering profiles of **I-6**. What's more, the long-acting characteristic of **I-6** was notably more improved than Ex-4, most likely due to its reduced renal clearance resulting from the strong albumin binding ability and enzyme metabolism. The absorption of **I-6** into systemic circulation was delayed possibly due to the formation of self-assembled colloidal structures in aqueous environment.[14] It is interesting to find of this study that concerned albumin binding by **I-6** 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. The long-acting glucose-lowering effects of **I-6** were further corroborated by the hypoglycemic duration test in STZ-induced diabetic mice. The hypoglycemic durations of **I-6** in diabetic mice were much longer than those of Ex-4 and comparable with those of liraglutide, regardless of the dose administered. Furthermore, once daily administration of **I-6** to STZ-induced diabetic mice achieved long-term beneficial effects on HbA1c lowering and glucose toler-

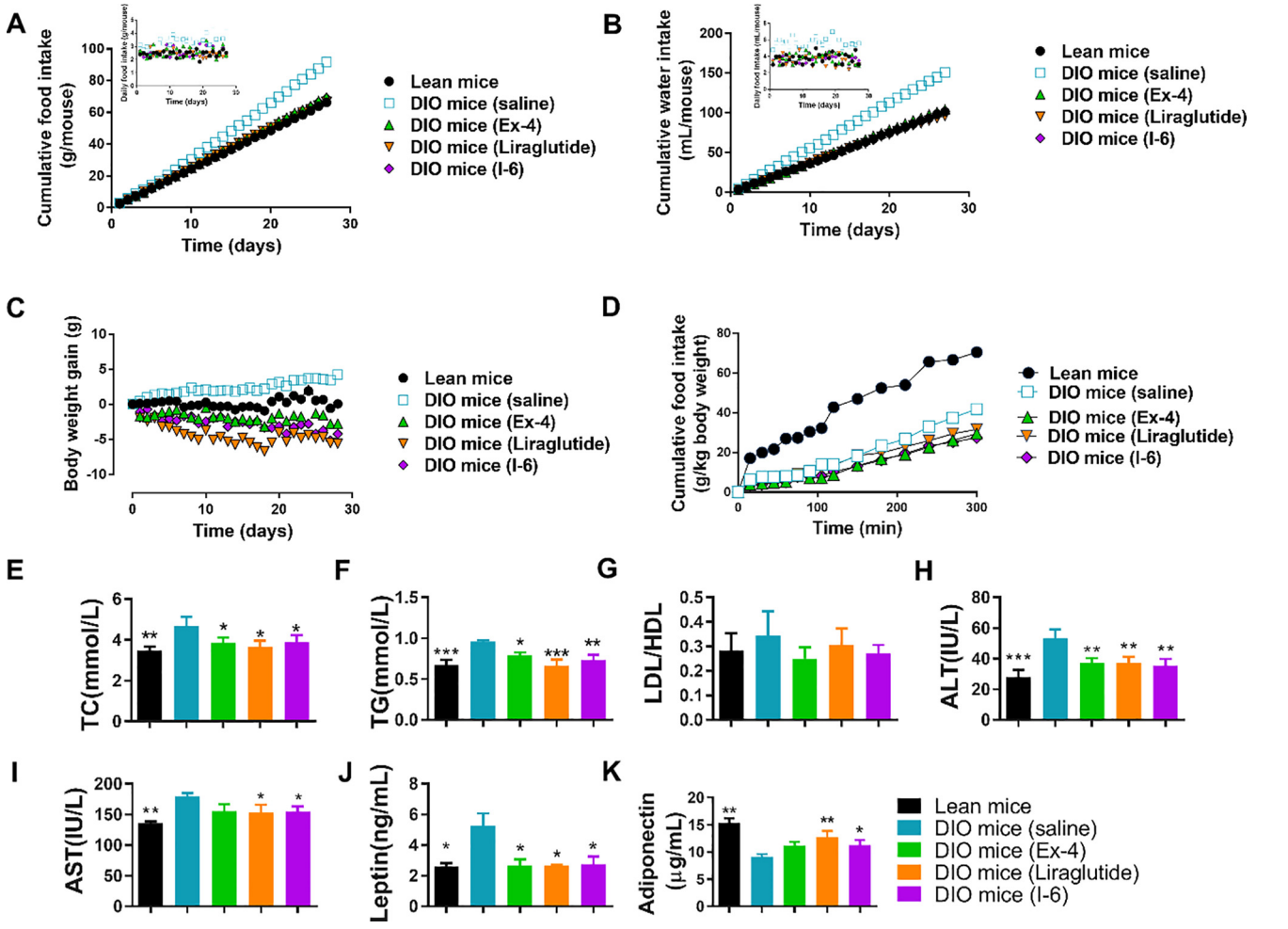



**Fig. 8.** Effects of **I-6** on food and water intake. Time course for chronic effect of **I-6** administered for 4 weeks in DIO mice on cumulative food intake. Time course for chronic effect of **I-6** administered for 4 weeks in DIO mice on cumulative food (A) and water (B) intake. Time course of **I-6** effect on changes in body weight. Time course of acute effect on food intake in fasted mice with intraperitoneal doses of **I-6**. Effects of **I-6** on serum biomarkers examined at the end of the 4-week treatment. Serum TC (E), TG (F), HDL/LDL (G), ALT (H), AST (I), leptin (J) and adiponectin (K) levels were assayed on a by Beckman Coulter Chemistry Analyzer. TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; 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 HDF (saline).

ance. The enhanced glucose tolerance of **I-6** treated mice probably resulted from the prolonged biological activity (β cell neogenesis and/or proliferation).[15] Including appetite suppression and weight loss reduction, the results of chronic administration of **I-6** to DIO mice were also consistent with the chronic effects of administered GLP-1 receptor agonists.

In summary, this present study demonstrates that the cysteine substitution of GLP-1 residues and further fatty acid chain modification offers a useful scientific approach to the development of long-acting incretin-based antidiabetics. This study also shows that **I-6**, preserved biological activity, long-acting antidiabetic characteristics, and long-term beneficial effects well, is a promising biological agent deserving further investigation to treat obesity patients with diabetes.

## 4. Experimental section

### 4.1. 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 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

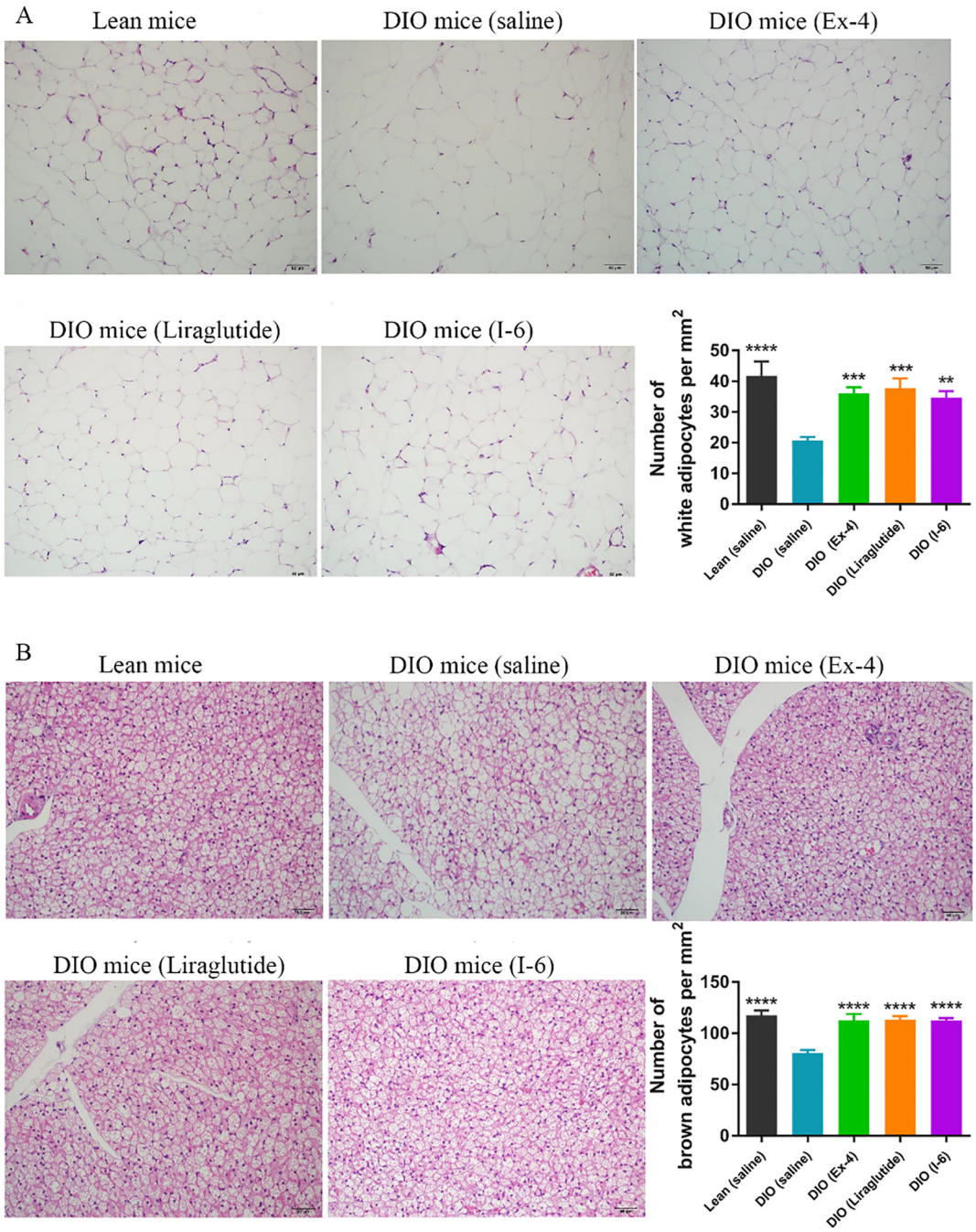
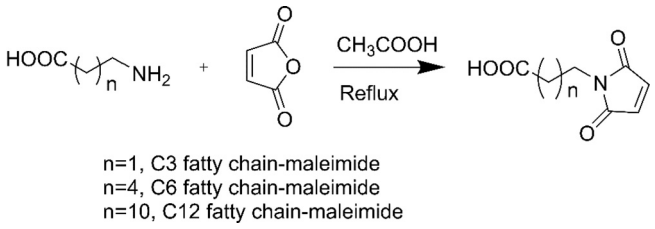



**Fig. 9.** Effects of **I-6** on white or brown adipocytes cells.

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.

### 4.2. General synthetic route and HPLC purification of peptides 1 and 2

Two optimized polypeptide chains were synthesized by the method of the standard solid-phase peptide synthesis protocol, $Cys_{17}$-$Gly_8$-GLP-1(7-36)-$NH_2$ **(1)** and $Cys_{37}$-$Gly_8$-GLP-1(7-37)-$NH_2$ **(2)**. Then the peptide was purified on preparative RP-HPLC and was identified by electrospray mass spectrometry.[11] 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).




n=1, C3 fatty chain-maleimide
n=4, C6 fatty chain-maleimide
n=10, C12 fatty chain-maleimide

**Scheme 1.** Synthetic route of fatty acid chain-maleimide.

### 4.3. General synthetic route of fatty acid chain-maleimide

As shown in Scheme 1, three fatty acid chain maleimide (3-(2, 5-Dioxo-2H-pyrrol-1(5H)-yl)propanoic acid, 6-(2,5-Dioxo-2H-pyrrol-1(5H)-yl)hexanoic acid, 12-(2,5-Dioxo-2H-pyrrol-1(5H)-yl)dodecanoic acid were synthesized as previously reported.[10]

### 4.4. General synthetic route of conjugates (I-1, I-2, I-3, I-4, I-5, I-6)

The conjugates were synthesized as previously reported. 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.

### 4.5. GLP-1 receptor activation assay

The GLP-1 receptor activation assay was conducted as previously reported.[16] 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.[17] 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 (EC50 values) was calculated by sigmoidal curve fitting using GraphPad Prism version 7.0 (GraphPad, San Diego, CA, USA).

### 4.6. Plasma stability test

With a modification of a previously described method, Plasma were obtained from adult male Sprague–Dawley (SD) rats (Certificate No.: 0011253) 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 fatty chain modified conjugates **I-1** ~ **I-6** 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 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.

### 4.7. Albumin binding

The albumin binding properties of the fatty acid chain modified conjugates were investigated in an albumin-binding assay with the use of albumin-conjugated Sepharose resin according to the reported literatures.[10] 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 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.

### 4.8. Cell viability assay

Cell viability assay was conducted as previously reported.[16] 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 lM 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 570 nm with a multiwall spectrophotometer (Thermo Labsystems, Waltham, MA, USA).

### 4.9. Detection of apoptotic cells

In flow cytometry analysis, INS-1 cell were seeded in 96-well plates (~5000 cells/well) for 24 h, then treated with glucolipotoxicity media, $H_2O_2$ (50 M) or 1 μM STZ followed by the adding of Ex-4 (10 nM), liraglutide (10 nM) or **I-6** (10 nM) according to the previously reported methods. After incubation for 24 h, cells were collected by trypsinization, washed with cold PBS and incubated with Annexin V-FITC (Sigma, Saint Louis, MO, USA) for 15 min, then stained with propidium iodide (PI, Sigma, Saint Louis, MO, USA). Cells in early apoptosis were Annexin V-FITC positive and PI nega-

tive. The percentage of apoptotic cells was analyzed by the flow cytometry (Beckman Coulter Accuri C6).

### 4.10. Glucoregulatory and insulin secretion assay

The glucoregulatory and insulin secretion assays were carried out in accordance with a previous method.[10] Briefly, overnight fasted (12 h) SD rats (n = 6/group, 200–250 g) were orally administrated with **I-6**, 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).

### 4.11. Multiple intraperitoneal glucose tolerance test

Multiple intraperitoneal glucose tolerance test (IPGTT) was carried out on C57BL/6 mice to assess the ability of **I-6** to reduce glucose levels for longer time using a modification of a previously described method.[10] 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-6** (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.

### 4.12. Hypoglycemic efficacies test

Hypoglycemic efficacies of **I-6** were evaluated in STZ-induced diabetic mice using a modification of a previously described method.[10] Kunming mice received a single intraperitoneal injection of saline, Ex-4, liraglutide, **I-6** (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).

### 4.13. Chronic in vivo studies

#### 4.13.1. 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 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 **2c** 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).

#### 4.13.2. Chronic in vivo studies on DIO mice

C57BL/6 mice (6 weeks) were maintained on prescribed DIO for 12 weeks. They were injected intraperitoneally once daily with 25 nmol/kg Ex-4, liraglutide or **I-6**, 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.

### 4.14. 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.

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Notes

The authors declare no conflict of interest to disclose.

## A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2018.04.022.

## Reference

1. Ali S, Davies MJ, Brady EM, et al. Guidelines for managing diabetes in Ramadan. *Diabetic Med*. 2016;33:1315–1329.
2. Bagger JI, Knop FK, Holst JJ, Vilsboll T. Glucagon antagonism as a potential therapeutic target in type 2 diabetes. *Diabetes Obes Metab*. 2011;13:965–971.
3. Manandhar B, Ahn JM. Glucagon-like Peptide-1 (GLP-1) analogs: recent advances, new possibilities, and therapeutic implications. *J Med Chem*. 2015;58:1020–1037.
4. Emery EC, Diakogiannaki E, Gentry C, et al. Stimulation of GLP-1 Secretion Downstream of the Ligand-Gated Ion Channel TRPA1. *Diabetes*. 2015;64:1202–1210.
5. Sun L, Huang X, Han J, et al. Site-specific fatty chain-modified exenatide analogs with balanced glucoregulatory activity and prolonged in vivo activity. *Biochem Pharmacol*. 2016;110:80–91.
6. Kim TH, Jiang HH, Lee S, et al. Mono-PEGylated dimeric exendin-4 as high receptor binding and long-acting conjugates for type 2 anti-diabetes therapeutics. *Bioconjugate Chem*. 2011;22:625–632.
7. Luginbuhl KM, Schaal JL, Umstead B, et al. One-week glucose control via zero-order release kinetics from an injectable depot of glucagon-like peptide-1 fused to a thermosensitive biopolymer. *Nat. Biomed. Eng.*. 2017;1(6).
8. Gaberc-Porekar V, Zore I, Podobnik B, Menart V. Obstacles and pitfalls in the PEGylation of therapeutic proteins. *Curr Opin Drug Discov Devel*. 2008;11:242.
9. Jonassen I, Havelund S, Hoegjensen T, Steensgaard DB, Wahlund P, Ribel U. Design of the novel protraction mechanism of insulin degludec, an ultra-long-acting basal insulin. *Pharm Res*. 2012;29:2104–2114.
10. Han J, Sun L, Chu Y, et al. Design, synthesis, and biological activity of novel dicoumarol glucagon-like peptide 1 conjugates. *J Med Chem*. 2013;56:9955–9968.
11. Han J, Huang X, Sun L, Li Z, Qian H, Huang W. Novel fatty chain-modified glucagon-like peptide-1 conjugates with enhanced stability and prolonged in vivo activity. *Biochem Pharmacol*. 2013;86:297–308.
12. Young AA, Gedulin BR, Bhavsar S, et al. Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (Macaca mulatta). *Diabetes*. 1999;48:1026–1034.
13. Olleros ML, Martin ML, Vesin D, et al. Fat diet and alcohol-induced steatohepatitis after LPS challenge in mice: role of bioactive TNF and Th1 type cytokines. *Cytokine*. 2008;44:118–125.
14. Kim K, Kwon S, Park JH, et al. Physicochemical characterizations of self-assembled nanoparticles of glycol chitosan-deoxycholic acid conjugates. *Biomacromolecules*. 2005;6:1154–1158.

15. Rolin B, Larsen MO, Gotfredsen CF, et al. The long-acting GLP-1 derivative NN2211 ameliorates glycemia and increases β-cell mass in diabetic mice. *Am J Physiol-Endoc M*. 2002;283(4).
16. Han J, Sun L, Huang X, et al. Novel coumarin modified GLP-1 derivatives with enhanced plasma stability and prolonged in vivo glucose-lowering ability. *Brit J Pharmacol*. 2014;171:5252–5264.
17. Sun L, Wang C, Dai Y, et al. Coumaglutide, a novel long-acting GLP-1 analog, inhibits β-cell apoptosis in vitro and invokes sustained glycemic control in vivo. *Eur J Pharmacol*. 2015;767:211–219.