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PII: S0223-5234(17)30568-8

DOI: 10.1016/j.ejmech.2017.07.046

Reference: EJMECH 9609

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 27 April 2017

Revised Date: 19 July 2017

Accepted Date: 22 July 2017

Please cite this article as: J. Zhou, X. Cai, X. Huang, Y. Dai, L. Sun, B. Zhang, B. Yang, H. Lin, W. Huang, H. Qian, A novel glucagon-like peptide-1/glucagon receptor dual agonist exhibits weight-lowering and diabetes-protective effects, *European Journal of Medicinal Chemistry* (2017), doi: 10.1016/j.ejmech.2017.07.046.

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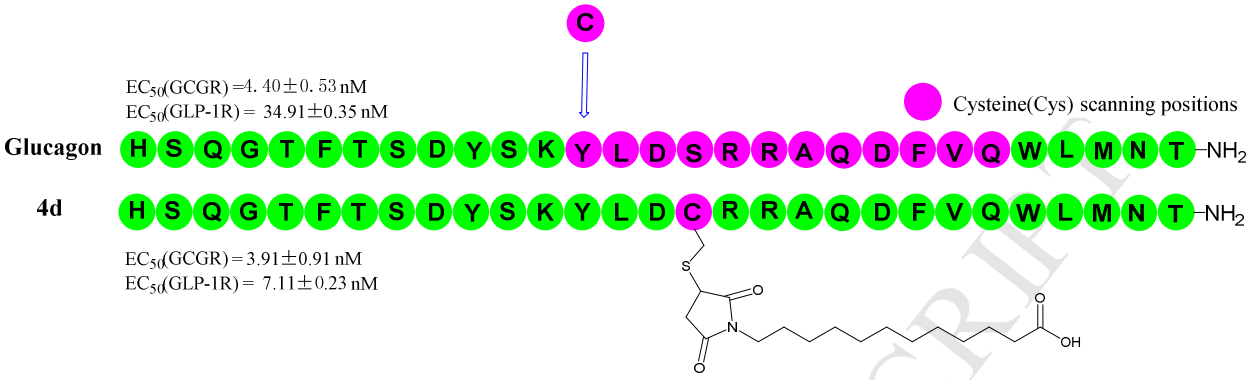
Graphical Abstract



Highlights

- Twenty-four glucagon-like peptide-1/glucagon receptor dual agonists were designed and synthesized.
- Compound 4d behaves well in lowering body weight and maintain energy expenditure in DIO mice.
- 4d showed notable diabetes-protective effects in type 2 diabetic mice.
- No hyperglycaemia effect were observed in the OGTT of 4d.

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ABSTRACT

Glucagon has plenty of effects via a specific glucagon receptor(GCGR) like elevating the blood glucose, improving fatty acids metabolism, energy expenditure and increasing lipolysis in adipose tissue. The most important role of glucagon is to regulate the blood glucose, but the emergent possibilities of hyperglycaemia is exist. Glucagon could also slightly activate glucagon-like peptide-1 receptor(GLP-1R), which lead to blood glucose lowering effect. This study aims to erase the likelihood of hyperglycaemia and to remain the inherent catabolic effects through improving GLP-1R activation and deteriorating GCGR activation so as to lower the bodyweight and show diabetes-protective effects. Firstly, twelve cysteine modified GLP-1/GCGR dual agonists were synthesized (**1**-**12**). Then, the GLP-1R/GCGR mediated activation and biological activity in normal ICR mice were comprehensively performed. Compounds substituted by cysteine at positions 22, 23 and 25 in glucagon were observed to be better regulators of the body weight and blood glucose. To prolong the half-lives of derivatives, various fatty side chain maleimides were modified to optimal glucagon analogues. Laurate maleimide conjugate **4d** was the most potent. Administration of 1000 nmol/kg **4d** once every two days for a month normalized adiposity and glucose tolerance in diet-induced obese (DIO) mice. Improvements in plasma metabolic parameters including insulin, leptin, and adiponectin were observed. These studies suggest that compound **4d** behaves well in lowering body weight and maintaining energy expenditure without a chance of hyperglycaemia, **4d** has strong clinical potential as an efficient GLP-1/GCGR agonist in the prevention and treatment of obesity and dyslipidemia.

Keywords: Glucagon, GLP-1/GCGR agonist, weight-lowering, diabetes-protective

1. Introduction

The global number of patients with diabetes was projected to increase to 366 million by 2030, while more than 80% of patients with type 2 diabetes are obese [1]. Glucagon-like peptide-1 (GLP-1) is a potent hypoglycemic hormone, emphasized a great opportunity for the treatment of diabetes **(Figure 1)** [2]. GLP-1 has a lot of benefits in a glucose-dependent manner like promoting insulin secretion, reducing blood glucose and delaying gastric emptying via a glucagon-like peptide-1 receptor (GLP-1R) [3, 4]. However, studies have found that it is difficult to further improve hypoglycemic activity if GLP-1R is activated merely when a certain point reached, meanwhile, nausea, vomiting and other adverse reactions may be caused [5]. Derived from processing of proglucagon like GLP-1, glucagon is a 29-amino acid pancreatic hormone secreted in the islets alpha cells [6]. Via a specific glucagon receptor(GCGR), glucagon could raise the blood glucose to be the emergency treatment of severe hypoglycaemia, but emergent possibilities of hyperglycaemia is exist [7]. Meanwhile, glucagon has versatile effects on hepatic glucose metabolism, renal, glucagon affects the cardiovascular, pulmonary, gastrointestinal systems vascular and gastrointestinal smooth muscle [8], like increasing lipolysis in adipose tissue, and glucagon acts as a regulation of insulin to raise the blood glucose [9].

Oxyntomodulin (OXM), a 37-amino acid hormones containing the entire 29-amino acid sequence of glucagon followed by other eight amino acid carboxy-terminal extension **(Figure 1)**, has been known as a GLP-1R/ GCGR dual agonist that reduces the body weight, lowers

the lipid and so on [10]. OXM could slightly activate GLP-1R which lead to lower blood glucose [11]. But the likelihood of hyperglycaemia is not completely erased [12]. Meanwhile, just like the endogenous GLP-1, the half-life of OXM or glucagon is very short, which accounts for the rapid metabolic degradation and elimination by kidney filtration [5, 13]. Aim to improve the GLP-1 activation, we modified the structure of glucagon, to deteriorate GCGR activation and combine good GCGR and GLP-1R activation in a single peptide so as to the activation of GCGR will induce catabolic effects which favor these on weight loss products [14], while GLP-1R activation will modulate glucose homeostasis [15], along with eliminating the possibility of hyperglycaemia. The raised hypoglycemic property of GLP-1R activation would minimize the potential diabetogenic risk of excessive GCGR activation, proper GCGR activation would help promote the lipolytic and thermogenic properties [16-18].

For another, the N-terminal sequence of native glucagon is highly conserved, and positions 8, 9, 16 and 18 are noteworthy in maintaining glucagon activity [18, 20, 21]. It also achieves a degree of selectivity from its C-terminal residues, and a significant contribution is made by the C-terminal acid [18]. In preliminary study, we have studied that the fatty acid side chain helps the molecules to interact with the albumin so that the half-time of GLP-1 could be prolonged to ~18 h or even longer [19]. And our group contrived a method to modify the peptide rapidly and quantitatively through cysteine [22-24], maleimide modified fatty acid chain was attached to the cysteine through the thiol group with high chemoselectivity to prolong the half-lives of peptide analogues. Therefore, in the present study, the middle section of glucagon was modified and substituted by cysteine at positions 19-30 to afford

twelve glucagon peptide analogues **(Figure 1)**. A key question is, how the sensitivity of glucagon single amino site on receptor activation and how to find more balanced GLP1-R/GCGR agonist, and even further provide guidance for further GLP-1R/GCGR agonist design. Consequently, through cysteine scanning, **1-12** was synthesized. The GLP-1R/GCGR mediated activation and biological activity in normal ICR mice were comprehensively performed to trace the better regulators of blood glucose and body weight. GLP-1 exists as 30 amino acids, C-terminally amidated peptide (GLP-1(7-36)-NH_2), or as the glycine-extended form, GLP-1(7-37). The former is the predominant form in human plasma being[25-27]. In this study, all of peptides are C-terminally amidated peptides to activate GLP-1R more effectively. To prolong the half-lives of peptide analogues, various fatty acid chains with maleimide were added to optimal cysteine modified glucagon analogues to synthesize long-acting compounds. The GLP-1R/GCGR activation and biological activity were also explored. The most potent conjugates with superior hypoglycemic activity and long term effects were identified. In addition, the novel compounds could be used to control blood glucose and reduced body weight in diet induced obese (DIO) mice. Simultaneously, the beneficial influence to liver morphology and overall energy balance were also observed.

Put Figure 1 here.

Put Figure 2 here.

2. Results

2.1. Receptor activation by GLP-1/GCGR dual agonists

Cyclic adenosine monophosphate (cAMP) is the primary effector of GLP-1R induced glucose-dependent secretion of insulin or GCGR induced glycogenosis and gluconeogenesis. Each peptide was tested for its ability to stimulate cAMP release via GLP-1R and GCGR in HEK293 over expressing human GLP-1R or GCGR **(Table 1)**. Native glucagon activated GCGR half maximally at an effective concentration (EC_{50}) of 0.97 ± 0.15 nM and activated GLP-1R with an EC_{50} of 7.70 ±0.29 nM. OXM had weak activation both on GCGR and GLP-1R. Most of the glucagon analogues were observed to slightly diminish activation on GCGR and to improve that on GLP-1R compare to glucagon. Peptide **5** and **7** showed comparable activation on GCGR with 2 or 4-fold greater than that of native glucagon respectively, suggesting that these positions could be modified to further improve GLP-1R potency while remaining GCGR activation. In particular, compound **4** showed a remarkable 8.17-fold increase in GLP-1R potency and slightly reduced potency at GCGR compared with glucagon, cysteine substitution of glutamate could improve GLP-1R receptor activation potency while maintaining GCGR potency. However, compounds **1**, **6**, **8**, **10** and **11** substituted by cysteine at positions 19, 24, 26, 28 and 29 respectively, had lower activation on GLP-1R relative to glucagon, especially compounds **10, 11**. Compounds **10** and **11** almost lost activation on GLP-1R, indicating that these sites may be sensitive to GLP-1R and not suitable for replacement. And compound **10** lost its activity on both GLP-1R and GCGR, suggesting that the original residue was important for maintaining GLP-1R and GCGR activation.

Put Table 1 here

2.2. Oral glucose tolerance test (OGTT) measurement of GLP-1/GCGR dual agonists in normal ICR mice

In vivo test, glucose tolerance test was firstly performed to evaluate peptides response to glucose. Before the OGTT, the Dose of dual agonists were explored between 100, 500, 1000 and 1500 nM/kg, while 1000 nM/kg (3.5 mg/kg) preformed best. GLP-1 is not included in the assay cause its short half-life. As shown in **Figure 3**, blood glucose levels of **4**, **5**, **7** treated mice were significantly lower than those of 0.9% saline-treated control mice at 30 and 60 min after glucose administration. It is stated clearly that glucagon analogues **4, 5** and **7** could maintain the blood glucose level in a proper range.

Put Figure 3 here.

Put Figure 3A .csv here

2.3. Acute food intake in ICR mice

Slimming drugs mainly reduce weight through appetite suppression and energy metabolism promotion. The anorexia effect of the GLP-1/GCGR dual agonists was monitored in normal

fasted ICR mice and compared with glucagon. As shown in **Figure 4**, most of the peptides reduced food intake in the first hour after administration compared with 0.9% saline control. Glucagon almost lost its anorectic effect at 2h. And **4**, **5**, **7** treated mice had a greater anorectic effect than other peptides for up to 8h. All the peptides had no significant difference of cumulative food intake for up to 24h (data not shown).

Put Figure 4 here.

2.4. Chronic effect on body weight in ICR mice of every other day dosing by 1-12

To further evaluate the peptides effect on body weight, i.p administration of normal saline, glucagon or **1-12** at 1000 nmol/kg(~3.5 mg/kg) every other day in normal ICR mice for two weeks **(Figure 5A)**. The dosage of 1000 nmol/kg in vivo test was chosen according to the OGTT test former**.** The weight of all the mice increased after treatment because the mice were in growth period which is hard to avoid. Among them, mice treated **4, 5 or 7** had lower weight gain than normal saline after two weeks (3.17±0.10g, 2.56±0.02g, 3.37±0.18g and 4.90±0.26g for **4, 5, 7** and normal saline, respectively) ($P < 0.01$), indicating these compounds may have good weight control potency. As shown in **Figure 5B**, the dynamic body weight change of the excellent compounds **4**, **5 and 7**. The body weight of **5** treated mice increased slowly versus saline-treated animals. Since the day 6, the **5** treated mice weight barely increased and remained stable.

All the experiments above indicating that **4**, **5** and **7** were the most potent **GLP-1/GCGR dual agonists**. Furthermore, to prolong the life-lives of peptide analogues, various fatty acid chains with maleimide were modified through cysteine residue to afford novel compounds.

Put Figure 5 here.

Put Figure 5B .csv here

2.5. Glucagon and GLP-1 receptor-activation by short fatty chain glucagon-related conjugates

Each conjugate was also tested for its ability to stimulate cAMP release via GCGR and GLP-1R in HEK293 cells over expressing human GLP-1R or GCGR. Most of the glucagon-related conjugates maintained high GCGR/GLP-1R activation efficacy in this assay except for **5c(Table 2)**. The receptor activation potency of **4c**, **5c** and **7c** conjugates were markedly less potent than **4a**, **5a**, **7a**, **4b**, **5b** and **7b**, indicating that the longer fat chain may lead to weaker receptor activation. The reason may be that their solubility was reduced and in turn the receptor activation potency was weaken. In order to improve the receptor activation, hydrophilic carboxyl group was introduced to the alkyl chain. The cAMP release results showed the fatty acid glucagon-related conjugates were more potent than fat chain conjugates

as expected. In particular, **4d** showed a remarkable 7.93-fold increase in GLP-1R potency and a little GCGR potency loss compared with glucagon.

Put Table 2 here

2.6. Oral glucose tolerance test (OGTT) in ICR mice

OGTT was performed to evaluate the ability to regulate blood glucose **(Figure 6)**, which is the premise and guarantee for the further research. Compared with control (saline), **4d**, **5a** and **7d** exhibited moderate hypoglycemic effects. Their capability to maintain normal glucose levels over longer durations was further explored using a multiple oral glucose tolerance test in ICR mice. As shown in **Figure 6**, **4d** exhibited somewhat blood glucose lowering activity for the significant decrease in the area under curve. So did **5a** and **7d**, data is not shown here.

Put Figure 6 here.

2.7. Energy balance physiology measurements in DIO mice

Considered its preliminary pharmacodynamics, **4d** and **7d** were chosen to further evaluate energy metabolism in DIO mice model. 1000 nmol/kg(3.8 mg/kg) of peptides **4d** and **7d** were intraperitoneal injected daily for five days **(Figure 7)**. In this experiment, daily injection was chosen in view of the drug life-time. The injections decreased body weight of DIO mice by

1.45±0.2 g for **4d** compared to control increased by 1.75±0.12 g (P<0.01) **(Figure 7D)**. Energy expenditure, however, was increased with **4d** (19.83±3.67 kcal/(kg.h^{-1})) and **7d** (17.46±3.90 kcal/(kg.h^{-1})) compared to saline(15.47±2.22 kcal/(kg.h^{-1}); P <0.01, and the respiratory quotient tended to be decreased **(Figure 7B, C)** (0.807±0.069, 0.820±0.06 and 0.832±0.05 for **4d, 7d** and control(saline), respectively), which indicates that increased thermogenesis and altered nutrient partitioning may explain the overall negative energy balance. Increased energy expenditure was not associated with a change in spontaneous physical activity–induced thermogenesis since locomotor activity did not differ between treatment groups and controls **(Figure 7A)**. Automated online monitoring of acute feeding of food intake did not reveal any differences in food intake **(Figure 7E)**.

Put Figure 7 here.

2.8. One-month therapy in DIO mice

In a follow-up experiment, we tested the weight loss effect of chronic treatment of **4d** or **7d**. Considering the mice tolerance, we chose every other day injection of **4d** and **7d** for one month. The injections decreased body weight of DIO mice by 3.59±0.15 g for **4d** compared with control increased by 1.66±0.32g (P<0.01) **(Figure 8A)**. **7d** treated mice also decreased body weight of 0.19±0.36g. And long-term effects of these conjugates on food intake were not statistically significant either (data not shown). OGTT performed on day 0 and 30 revealed that glucose tolerance was significantly and comparably improved in both treatment

groups **(Figure 9)**, especially **4d**. Furthermore, basal blood glucose levels were normalized by chronic treatment with either peptide **(Figure 9C, t = -30, 0min)**. The body weight changes were associated with the white adipose tissue(WAT) cell in the same area, as shown in **Figure 8B**, the number of WAT cell per mm^2 were increased because the reduction of the cell size.

Put Figure 8 here.

Put Figure 9 here.

2.9. Chronic treatment improves metabolism

Several other metabolic parameters in plasma were also improved by chronic treatment with the peptides **(Table 3)**. After treatment, the serum levels of leptin and adiponectin, two important adipocytokines secreted from white adipose tissue, were also altered. Increases in adiponectin and leptin correlated with the decreased adiposity observed at the end of the study in each treatment group. So does decrease in insulin levels. Reduced cholesterol and decreased hepatic lipid droplets size **(Figure 10)** relative to control(saline) were also noted, especially for animals treated with **4d**. But these peptides had no significant effect on triglycerides.

Put Table 3 here.

Put Figure 10 here.

3. Conclusions

In the present study, with the specific aims of erasing the likelihood of hyperglycaemia and to remain the inherent catabolic effects through improving GLP-1R activation and deteriorating GCGR activation, optimizing GCGR/GLP-1R activation to develop long-acting therapeutic agents for diet induced obesity, dyslipidemia and diabetes mellitus. We firstly designed and prepared a series of novel cysteine replaced glucagon analogues. The initial GLP-1R/GCGR activation experiments provide direct evidence of the relevant structure-function relationship of glucagon. Compounds **3**, **4**, **5**, **7** and **12** exhibited increased GLP-1R activation and preserved GCGR activation well relative to glucagon, which shows these changes are beneficial. Two positions essential for GLP-1 recognition were found in this study, compounds **10**, **11** almost had little activation at GLP-1R, indicating that these residues are sensitive to GLP-1R. Because Phe^{28} is critical residue for receptor binding and activation on GLP-1R[28]. And compound **10** not only lost its potency at GLP-1R but GCGR, suggesting that this position is important for maintain GLP-1R and GCGR activation. The peptide in vivo results including OGTT and acute food intake and chronic study correlated well with peptides' EC_{50} and rate of GLP-1R/GCGR activation.

Then, various fatty side chain maleimides were added to optimal **4**, **5**, **7** to prolong peptides lifetime by increased interaction with serum albumin[29, 30]. Although the negligible reductions were observed in receptor activation, **4d** and **7d** still showed well-preserved

weight-lowering and lipid-regulation effects. Then one-week therapy (the energy balance physiology measurements) and one-month therapy (chronic treatment) in DIO mice further confirmed that **4d** had a significant effect in weight loss and energy expenditure increase. Chronic treatment of **4d** also lowered body adiposity and hepatic lipid droplets in DIO mice. As expect, the hyperglycemia-promoting effect of **4d** is not observed in either one-week or the chronic study, supporting the hypothesis that the increased GLP-1R activation of these molecules opposes and potentially neutralizes GCGR-mediated diabetogenic effects. These preclinical studies indicate that when full GLP-1R activation is augmented with an appropriate degree of GCGR activation, weight reduction can be substantially enhanced without any overt adverse effects. The weight reduction is directly related to the two receptors activation. But how much relative receptor activity is optimal for clinical benefit remains inaccurate and more study is needed.

In summary, this study shows that compound **4d** has strong clinical potential as an efficient GLP-1/GCGR agonist exhibits weight-lowering, lipid-regulation and diabetes-protective effects and plays an important role in the prevention and therapy of diet induced obesity and dyslipidemia. The present study also demonstrates that GCGR and GLP-1R are potential targets for drugs lowering body weight and protecting diabetics. Modification of glucagon offers a useful approach to balance GCGR/GLP-1R activation and to develop therapeutic agents for weight-lowering and diabetes-protective.

4. Materials and Methods

4.1. Materials and Animals

Reagents and materials were purchased from the following companies: Fmoc Rink amide-MBHA resin and Fmoc-protected amino acids, GL Biochem (Shanghai, China); HPLC grade acetonitrile and methanol, Merck (Darmstadt, Germany); cAMP dynamic kit, Cisbio (Perkin-Elmer); All other reagents, unless otherwise indicated, were obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA) and used as received. Microwave irradiation procedures were performed in a discover focused single mode microwave synthesis system (CEM, NC, USA), which produced continuous irradiation at 2450 MHz HPLC analysis and purification were performed on a Shimadzu 2010C HPLC system and a Shimadzu LC-10 preparative RP-HPLC system, respectively. ESI mass spectra were obtained with a Waters ACQUITY UPLC system (Milford, MA, USA). ICR mice (male, 6 weeks old) and C57BL/6 mice (male, 6weeks old) were purchased from the Comparative Medical Center of Yangzhou University (Jiangsu, China). Animals were housed in groups of six in cages under controlled temperature (22 ± 2 ℃) 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. 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. Synthesis and HPLC Purification of Compounds 1-12.

Peptides **1−12** were prepared and purified using previously described methodology[31, 32]. Fmoc Rink amide-MBHA resin (0.015mmol) was deprotected with 20% piperidine in 5 mL of DMF for 4 min under microwave irradiation (microwave power of 10W). A mixed solution of 0.045mmol of first amino acid, 0.045mmol of HBTU, 0.045mmol of HOBt, and 0.090mmol of DIPEA dissolved in 4 mL of DMF was added. The mixture was bubbled with N_2 for 10 min under microwave irradiation (10W) and washed with DMF. Deprotection and coupling were repeated with the relevant Fmoc-protected amino acids, and peptides were cleaved with 7 mL of 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 using a Shimadzu preparative RP-HPLC. Samples were injected into a Shimadzu C18 reverse phase column (5μm, 340 mm × 28 mm) and purified using a linear gradient from 30% to 75% phase B (acetonitrile and 0.1% TFA) over 30 min (phase A (water, 0.1% TFA)) at a flow rate of 5.0 mL/min with UV detection at 214 nm. Compounds were obtained with approximately 97% purity, as estimated by HPLC analysis. The molecular mass of the purified peptides was confirmed by LC−MS. 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.

4.3. General Procedure I for Preparation of Compounds 13-16

Different fatty chain and 1.2mol of maleic anhydride in 20 mL of acetic acid were stirred at 120°C for 6h **(Scheme.1)**. The reaction mixture was based with saturated $NaHCO_3$ after

cooling to room temperature and little yellow solid gained. The solid was filtered and washed with little water. **13-16** were prepared and purified using previously described methodology[33].

Put Scheme 1 here.

1-Hexyl-1*H*-pyrrole-2,5-dione (C6 fatty chain-maleimide) (**13**)

^{1}H NMR (DMSO-d_6, 300 mHz): δ 6.97 (s, 2H,COC<u>H</u>=C<u>H</u>CO), 3.39 (t, 2H, J = 7.1 Hz, NC<u>H</u>$_2$), 1.49–1.45 (m, 2H,NCH$_2$C<u>H</u>$_2$(CH$_2$)$_3$CH$_3$), 1.23 (s, 6H, NCH$_2$CH$_2$(C<u>H</u>$_2$)$_3$CH$_3$), 0.90 (t, 3H, J = 6.3 Hz, NCH$_2$CH$_2$(CH$_2$)$_3$C<u>H</u>$_3$); MS(ESI, m/z): 181.3 [M+H]$^+$.

1-Dodecyl-1*H*-pyrrole-2,5-dione (C12 fatty chain-maleimide) (**14**)

^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.01 (s, 2H, COC<u>H</u>=C<u>H</u>CO), 3.39 (t, 2H, J = 7.1 Hz, NC<u>H</u>$_2$), 1.49–1.45 (m, 2H, NCH$_2$C<u>H</u>$_2$(CH$_2$)$_9$CH$_3$), 1.23 (s, 18H, NCH$_2$CH$_2$(C<u>H</u>$_2$)$_9$CH$_3$), 0.85 (t, 3H, J = 6.3 Hz, NCH$_2$CH$_2$(CH$_2$)$_9$C<u>H</u>$_3$); MS(ESI, m/z): 265.8 [M+H]$^+$.

1-Hexadecyl-1*H*-pyrrole-2,5-dione (C16 fatty chain-maleimide) **(15)**

^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.01 (s, 2H, COC<u>H</u>=C<u>H</u>CO), 3.38 (t, 2H, J = 6.2 Hz, NC<u>H</u>$_2$), 1.49–1.45 (m, 2H, NCH$_2$C<u>H</u>$_2$(CH$_2$)$_{13}$CH$_3$), 1.23 (s, 26H, NCH$_2$CH$_2$(C<u>H</u>$_2$)$_{13}$CH$_3$), 0.85 (t, 3H, J = 6.4Hz, NCH$_2$CH$_2$(CH$_2$)13C<u>H</u>$_3$); MS(ESI, m/z): 322.0 [M+H]$^+$.

12-(2,5-Dioxo-2,5-dihydro-1*H*-pyrrol-1-yl) dodecanoic acid (C12 fatty chain acid-maleimide) (**16**)

^{1}H NMR (DMSO-d_6, 300 MHz): δ 11.00 (s, H, COOH), 7.01 (s, 2H, COCH=CHCO), 3.39 (t, 2H, J = 7.1 Hz, NCH_2), 1.49–1.45 (m, 2H, $NCH_2CH_2(CH_2)_9CH_3$), 1.23 (s, 18H, $NCH_2CH_2(CH_2)_9CH_3$); MS(ESI, m/z): 295.3 $[M - H]^-$.

4.4. General Synthetic Route of fatty side chain of GLP-1 conjugates (4, 5, 7-a, b, c, d). Cysteine modified peptides (**4**, **5 and 7**, 5 μmol) were conjugated with fatty side maleimides (**13-16**, 12 μmol) in 5 mL of 0.05 M sodium phosphate buffer, pH 7.0, as previous reported **(Scheme.2)**[24]. The structure of conjugates are shown in **Figure 2**. The reaction mixture was stirred at 20 °C under N_2 for 1.5 h until HPLC confirmed completion. Crude conjugates were purified on a Shimadzu preparative RP-HPLC system using a Shimadzu C18 reverse phase column (5μm, 340mm × 28mm) equilibrated in phase A (water, 0.1% TFA) and eluted with a linear gradient of 35−85% phase B (acetonitrile, 0.1%TFA) over 30min at a flow rate of 5.0mL/min with UV detection at 214 nm. The purity of each synthetic peptide was above 95%. 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.

Put Scheme 2 here.

4.5. GCGR and GLP-1R mediated cAMP release

a) Construction of GLP-1 receptor retroviral expression plasmid: Preparation of a large number of retroviral vector pBABE-puro plasmid with puromycin resistance and pMSCV-sulfuric acid GLP-1 receptor plasmid containing GLP-1 receptor full-length cDNA sequence. The primers of GLP-1 receptor gene were synthesized by Shanghai Shenggong Biology Co. Ltd. and the ends of the receptor gene were digested with EcoRI and NcoI respectively. The target gene sized 1.7 kb was amplified by PCR while the template was the pMSCV- sulfuric acid GLP-1 receptor plasmid preservative. The PCR product was digested with the restriction endonuclease EcoRI and NcoI, and then the TaKaRa DNA Ligation Kit (TaKaRa Biotechnology Co. Ltd. Dalian) was used to connect the PCR target gene and the pBABE-puro empty vector for the product named pBABE-HPA. The recombinant was transformed into Escherichia coli DH5α competent cells and cultured in LB solid medium containing Ampicillin and selected for positive clones of Ampicillin. Transfection can be identified by PCR and restriction endonuclease digestion and sequencing.

b) Preparation of retrovirus: 24 h before transfection, Phoenix 293 cell plate culture, about 70% -80% fusion when the plasmid transfection. Trypsin digests Phoenix 293 cells and seeded into 6-well plates in 2 x 105 cells / well and incubated to about 70-80% when cultured to about 1 mL of serum-free antibiotic Opti-MEM medium, 2 μl of plasmid DNA and 5 μL of Lipofectamin 2000 Reagent were diluted with 130 μL serum-free antibiotic Opti-MEM medium, respectively, standing for 5 min and mixed. The mixed solution was added to a 6-well cell culture plate. 4-6 h after the orifice plate to replace the complete medium to continue training for 48 h. After the virus was produced in Phoenix 293 cells, the virus

supernatant was collected by centrifugation and protected from light at 4 ° C after filtration using a 0.45 μm pore size filter.

c) The establishment of stable expression of cell lines: normal HEK293 cells in the six-well plate, 2×10^5 cells each hole, cultured 24h, followed by adding 2 mL each virus supernatant and Polybrene (final concentration of 6 μg/mL). Cultured in the 32□ incubator after 30min centrifugation with 1000 rpm at room temperature, 5 h later the medium was replaced to normal, 37□ culture 48 h. In order to obtain a stable virus-infected cell line, 0.45 μg / mL of puromycin was added to the platelet for one week.

d) Human embryonic kidney (HEK293) cells transfected with either the human GLP-1R or the human GCGR, were used to assess effects on cAMP production[14, 34]. 2 h before the test started, cells were plated in 96-well half area, solid black microplates. The conjugates were solubilized in DMSO and further diluted in medium containing 0.1% BSA fraction V (Genview Scientific, Florida, USA). The maximum final DMSO concentration in the assay is 0.1%. Add the resulting solution to cells and incubate for 20 min, then assayed for cAMP using the kit with homogenous time-resolved fluorescence technology (Cisbio, Bedford, MA, USA) tested by 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 5.0 (GraphPad, San Diego, CA, USA).

4.6. Glucose tolerance test in normal ICR mice

ICR mice were acclimatized to laboratory conditions for at least one week and handled daily before the first study, during which time they received two injections of saline to minimize

stress on the study days. For the determination of glucose tolerance, the mice (8weeks, male) were subjected to 12h of fasting. At 0.5h prior to the oral glucose forced loaded (2g/kg glucose in 0.9% w/v saline), mice were administered glucagon, glucagon analogues or glucagon-related conjugates (1000 nmol/kg) intraperitoneally (0 min). The blood glucose levels were measured at the tip of tail by a handheld glucometer (Sannuo Changsha, Changsha, China) before -30min and at 0, 15, 30, 60, 90 and 120min after injection.

4.7. Acute feeding studies in normal ICR mice

For each study, animals (9 weeks, male) were fasted 8h preceding the injection. All peptides were dissolved in 0.9% saline, and 0.9% saline was administered as a control injection. After intraperitoneal injection (0 min), animals were returned to their home cages containing a preweighed amount of food that was reweighed at 0.5, 1, 2, 4, 8, and 24h after injection.

4.8. Chronic food intake and body weight change study in normal ICR mice.

Mice (10 weeks, male) were grouped to treatment groups based on their body weight, randomly. The mice were administrated glucagon, control (0.9% saline) or glucagon analogues **1-12** intraperitoneally at 1000 nmol/kg every other day for 14 days. Body weight and food consumption were also measured every day.

4.9. Energy balance physiology measurements.

Energy intake and expenditure, as well as home-cage activity, were assessed by a combined indirect calorimetry system (TSE Systems). O_2 consumption and CO_2 production were measured every 30 min for a total of 120h (including 24 h of adaptation) to determine the

respiratory quotient and energy expenditure. Food intake was determined continuously for 120h at the same time as the indirect calorimetry assessments by integration of scales into the sealed cage environment. Home-cage locomotor activity was determined using a multidimensional infrared light beam system with beams scanning the bottom and top levels of the cage, and activity being expressed as beam breaks.

4.10. Chronic study in DIO mice

C57Bl/6 mice were fed on either low fat diet (D12491: 20% kcal from fat; Research Diets) or high fat diet (D12492: 60% kcal from fat; Research Diets) in a 12h light/12h dark cycle for 12 weeks. The average weight of lean mice was 28±1.2g. Thirty DIO (20 weeks, male) mice (body weight ~38g, diet D12492) were infused intraperitoneally with **4d** (3.79mg/kg, 10 mL/kg), **7d** (3.80mg/kg, 10 mL/kg), glucagon (3.48mg/kg, 10 mL/kg), OXM (4.45mg/kg, 10 mL/kg) or 0.9% saline (10 mL/kg) every other day at 9 o'clock in the morning for 30 days. Body weight, food consumption and water intake were also measured every day.

4.11. Biochemical analyses

At the end of the study, blood samples were collected via arteria carotis under anaesthesia by sodium pentobarbital and sera separated subsequently for further analyses. Insulin, leptin and adiponectin were measured by ELISA (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Plasma triglyceride and total cholesterol were determined using an Olympus AU400e Bioanalyzer (Mishima Olympus, Shizooka-Ken, Japan).

4.12. Histopathological analysis of the liver and white adipose tissue (WAT) of DIO mice

Liver and white adipose tissue were isolated immediately after sacrifice, washed with ice cold saline and fixed in 10% (v/v) formalin overnight. After dehydration, sections were embedded in paraffin, and 4 mm sections were cut and stained with H&E for histopathological assessment. white adipose tissue cell number was estimated by counting focal fat on five sections of each white adipose tissue, each spaced 245 mm (35 sections) a part (mean ± SD).

4.13. Statistical analysis

All data are presented as mean ± SD. Comparisons among groups were made using ANOVA or unpaired student's t-test, as appropriate. $P<0.05$ was regarded as statistically significant. $P<0.01$ was regarded as extremely significant difference.

Funding Sources

This study was supported by grants from the National Natural Science Foundation of China (81673299 and 81370878) and Innovation Project Program of Hua Hai Pharmaceutical (HH13B003).

Notes

The authors declare no competing financial interest.

Abbreviations

AUC, area under the curve; cAMP, cyclic adenosine monophosphate; DIO, diet induced obese; $EC_{50,}$ half maximally at an effective concentration; GCG, glucagon; GCGR, glucagon receptor; 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; OGTT, oral glucose tolerance test; OXM, oxyntomodulin; TFA, trifluoroacetic acid; WAT, white adipose tissue.

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Figure legend

Scheme 1. General synthetic route of fatty chain-maleimide

Scheme 2. General synthetic route of fatty chain-modified conjugates.

Figure 1. Structures of glucagon, GLP-1, OXM and glucagon analogues, the green parts show the same amino acid sequence, the violet show the different parts.

Figure 2. Structures of fatty chain-glucagon-related conjugates

Figure 3. Oral glucose tolerance in ICR mice, i.p. administration of 4, 5, 7 and glucagon at 1000 nmol/kg, respectively.

Figure 4. Acute effect on cumulative food intake for 8 h after an overnight fast in ICR mice, i.p administration of glucagon or **1~12** at 1000 nmol/kg (n= 6-8 per group).

Figure 5. Effects on ICR mice of once other day for two weeks, i.p. administration of glucagon or glucagon analogues at 1000 nmol/kg: (A) Cumulative changes in body weight in ICR mice for two weeks (n=6-8 per group). (B) Dynamic body weight change of preferred peptides **4**, **5**, **7** in ICR mice for two weeks. *p<0.05, **p<0.01 and***p<0.001 compared with respective saline control; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared with glucagon.

Figure 6. OGTT in ICR mice, the blood glucose(A) and the area under curve of 0-120 min(B), i.p administration of saline, OXM and **4d,** respectively.

Figure 7. One-week treatment of diet-induced obese mice with OXM, **4d** and **7d** (A-E): (A) Locomotor activity. (B) Energy expenditure. (C) Respiratory quotient. (D) Dynamic body weight change. (E) Cumulative food intake. (n=6-8 per group)

Figure 8. Dynamic body weight change of DIO mice for one month.

Figure 9. One-month treatment of DIO mice with glucagon and **4d** and **7d** (A-D): (A) OGTT at day 0. (B)AUC of OGTT at day 0. (C) OGTT at day 30. (D)AUC of OGTT at day 30.

Figure 10. Chronic effect on liver lipid droplets of DIO mice (A–D) (Lipid droplets are marked with blue circle): (A) **4d**. (B) **7d.** (C) glucagon. (D) control(saline).

Table 1 GCGR and GLP-1R activation of **1~12**

Peptide	mGLP1R EC_{50} (nM)	mGCGR EC_{50} (nM)	mGLP1R/ mGCGR	Molecular	Mass found	Molecular	Mass Calculated
				$[M+3H]^{3+}$	$[M+4H]^{4+}$	$[M+3H]^{3+}$	$[M+4H]^{4+}$
GLP-1	0.07 ±0.01	NA	NA	NT	NT	NT	NT
glucagon	7.70 ±0.29	0.97 ±0.15	7.93	1161.5	871.4	1161.9	871.7
glucagon-NH_2	3.21 ±0.37	1.68 ±0.51	1.91	1161.2	871.1	1161.6	871.5
OXM	12.17 ±0.47	1.23 ±0.58	9.89	1483.3	1112.8	1483.3	1112.1
1	10.31 ±0.97	1.41 ±0.37	7.33	1141.0	855.9	1141.6	856.4
2	5.86 ±0.22	3.88 ±0.32	1.51	1158.0	868.6	1158.2	868.9
3	1.40 ±0.79**	0.46 ±0.57	3.02	1157.5	868.3	1157.2	868.6
4	0.94 ±0.09***	1.41 ±0.35	**0.67**	1166.2	875.0	1166.2	874.9
5	1.84 ±0.45**	1.43 ±0.31	**1.29**	1143.8	858.3	1143.2	857.6
6	16.16 ±0.67	3.36 ±0.05	4.89	1144.6	857.6	1143.2	857.6
7	1.72 ±0.08**	6.99 ±0.55**	**0.24**	1172.1	879.0	1172.3	879.5
8	31.93 ±0.59	5.30 ±0.84	NA	1153.2	865.2	1153.2	865.2
9	6.14 ±0.58	1.27 ±0.54	4.82	1157.3	867.6	1157.6	868.5
10	NA	122.57 ±0.89	6.03	1146.41	860.3	1146.9	860.4
11	NA	0.75 ±0.02	NA	1162.6	872.2	1162.9	872.4
12	3.58 ±0.24	0.90 ±0.17	3.96	1153.0	865.1	1153.3	865.2

EC_{50} values represent peptide concentrations at which half-maximum activation is occurring at the GCGR and GLP-1R. All the peptides were conducted three times. Selectivity ratio = [EC_{50}(GLP-1R)] / [EC_{50}(GCGR)].

Table 2 GCGR and GLP-1R mediated cAMP release by short alkyl chain glucagon-related conjugates

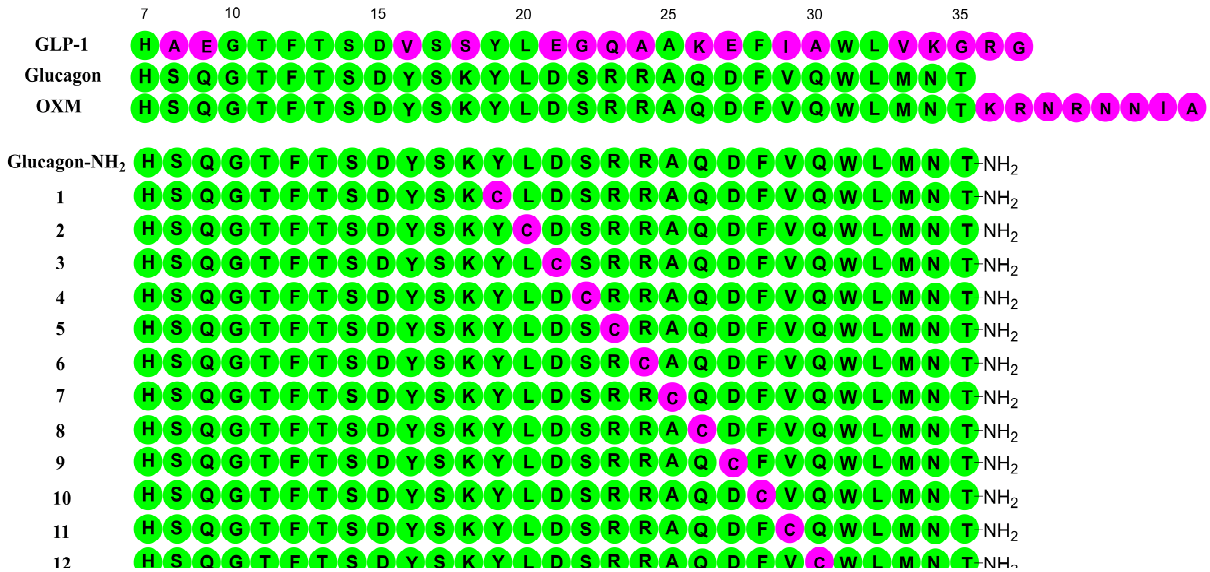
Peptide	mGLP1R EC_{50} (nM)	mGCGR EC_{50} (nM)	mGLP1R/ mGCGR	Molecular	Mass found	Molecular	Mass Calculated
				$[M+3H]^{3+}$	$[M+4H]^{4+}$	$[M+3H]^{3+}$	$[M+4H]^{4+}$
GLP-1	0.07 ±0.01	NA	NT	NT	NT	NT	NT
glucagon	7.70 ±0.29	0.97 ±0.15	7.93	1161.5	871.4	1161.9	871.7
glucagon-NH_2	3.21 ±0.37	1.68 ±0.51	1.91	1161.2	871.1	1161.6	871.5
OXM	12.17 ±0.47	1.23 ±0.58	9.89	1483.3	1112.8	1483.3	1112.1
4a	3.30 ±0.43	0.86 ±0.14	3.85	1227.0	920.6	1226.6	920.2
4b	4.54 ±0.56	2.40 ±0.38	1.89	1254.7	941.3	1254.6	941.2
4c	88.23 ±0.19	4.49 ±0.29	19.6	1273.7	955.6	1273.4	955.3
4d	0.86 ±0.08**	1.57 ±0.02	0.55	1264.2	948.9	1264.7	948.7
5a	1.60 ±0.64*	1.29 ±0.12	1.24	1204.1	903.4	1203.6	902.9
5b	2.00 ±0.62	2.99 ±0.74	0.67	1232.1	924.1	1231.6	924.0
5c	51.72 ±0.60	14.60 ±0.52	3.54	1250.7	938.1	1250.4	938.0
5d	3.00 ±0.02	2.84 ±0.54	1.05	1241.2	931.4	1241.6	931.5
7a	8.96 ±0.33	2.81 ±0.23	3.19	1232.4	924.9	1232.6	924.7
7b	29.40 ±0.54	4.78 ±0.78	6.15	1260.7	945.6	1260.7	945.8
7c	112.03 ±0.02	18.64 ±0.79	6.01	1279.1	959.6	1279.3	959.7
7d	3.01 ±0.31	4.37 ±0.04	0.69	1270.9	953.1	1270.6	953.2

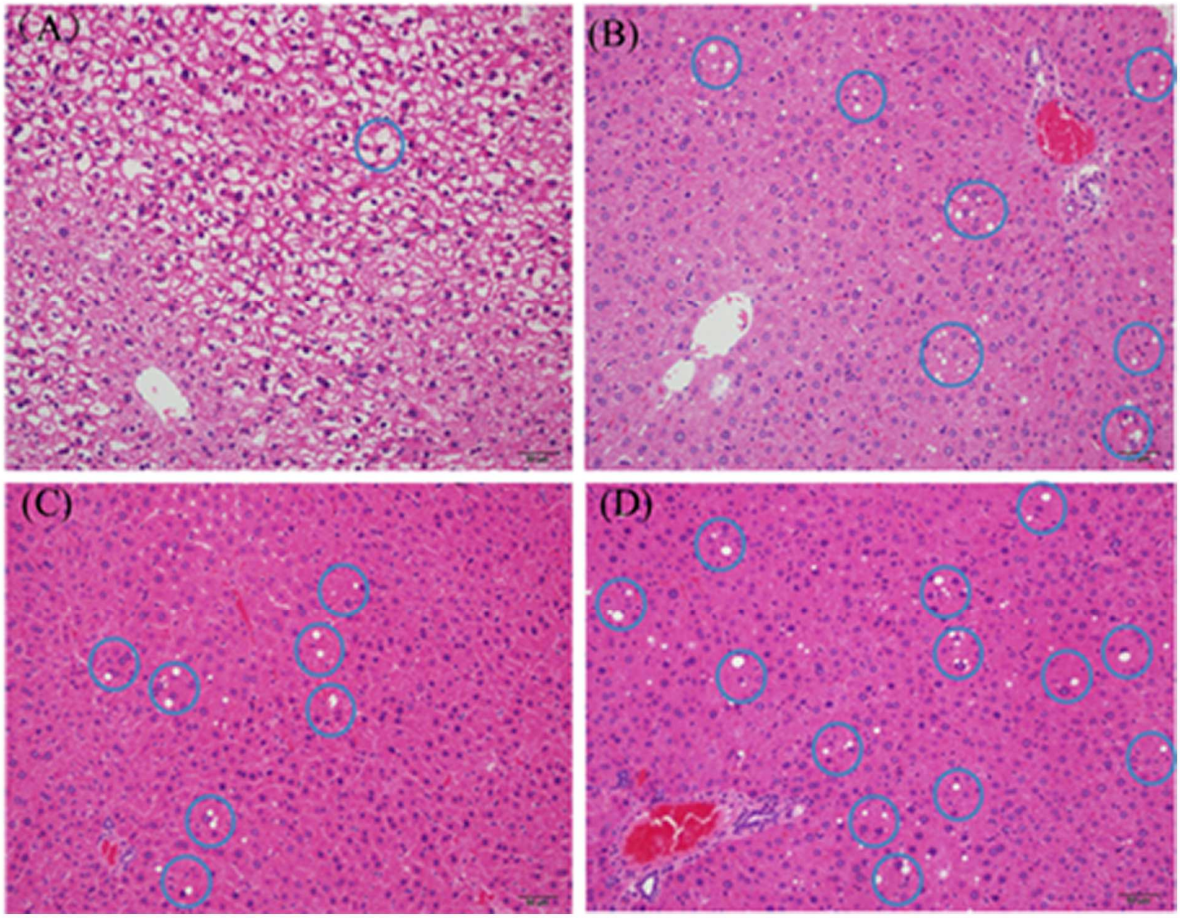
EC_{50} values represent peptide concentrations at which half-maximum activation is occurring at GCGR and GLP-1R. All the peptides were conducted three times. Selectivity ratio = [EC50(GLP-1R)] / [EC50(GCGR)].

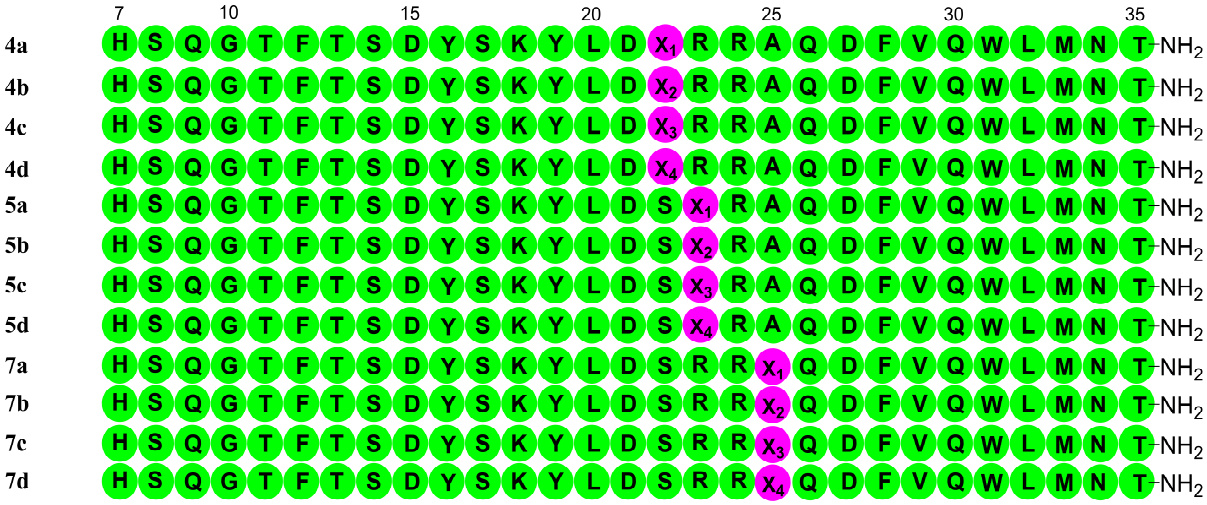
Table 3 Chronic treatment with fatty chain glucagon-related conjugates **4d, 7d,** OXM and glucagon in DIO mice: plasma determinations at the end of the chronic study(n=6-8 per group).

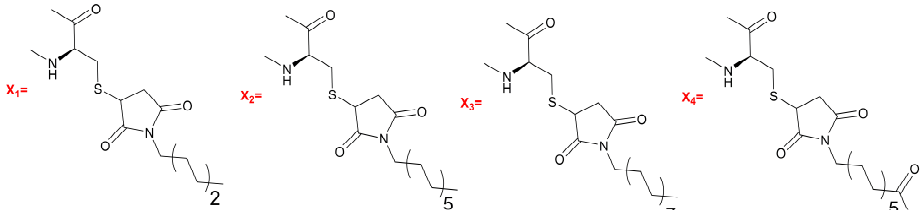
plasma parameters	Insulin (ng/ml)	Leptin (ng/ml)	Adiponectin(ug/ml)	Cholesterol (mmol/l)	Triglycerides(mmol/l)
Lean mice	8.02±0.23	4.98±0.25	25.12±0.37	1.80±0.22	1.05±0.12
DIO mice-saline	13.33±0.45	1.9±0.07	8.9±0.38	4.33±0.19	0.84±0.12
DIO mice-OXM	11.2±0.33	3.79±0.26**	17.4±0.57***	4.18±0.34	1.07±0.35
DIO mice-glucagon	8.41±0.26***	1.79±0.24	12.2±1.3**	5.08±0.57	1.19±0.29
DIO mice-4d	7.94±0.13***	5.36±0.23***	26.3 ±1.1***	2.58±0.15**	0.89±0.12
DIO mice-7d	11.50±0.56	3.36±0.25**	23.2±0.9***	3.42±0.80	0.96±0.10

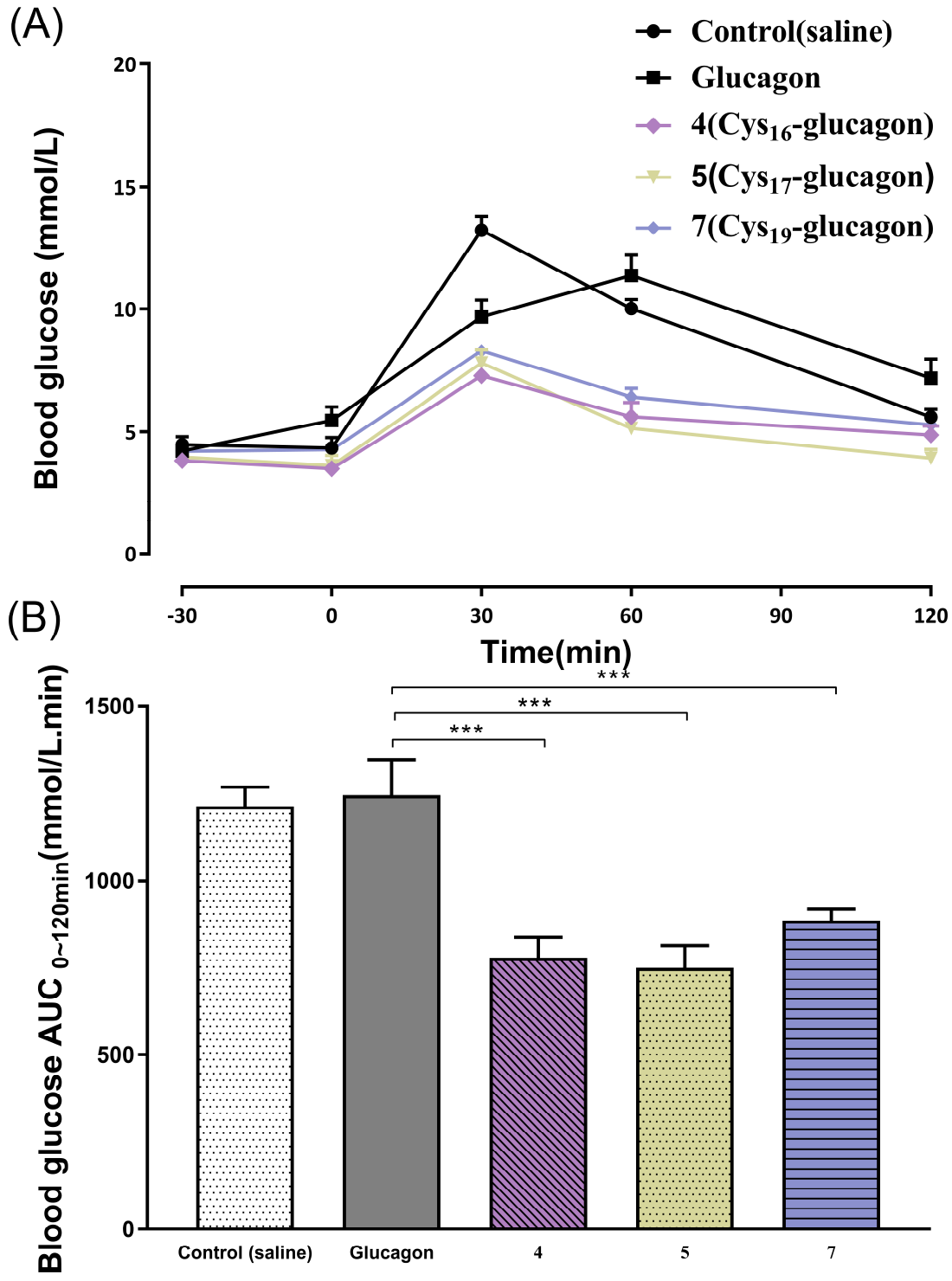
Data are means ± SD. n=6, *P < 0.05, **P < 0.01, *** P < 0.001 **4d**, **7d**, GLP-1, glucagon vs. saline.

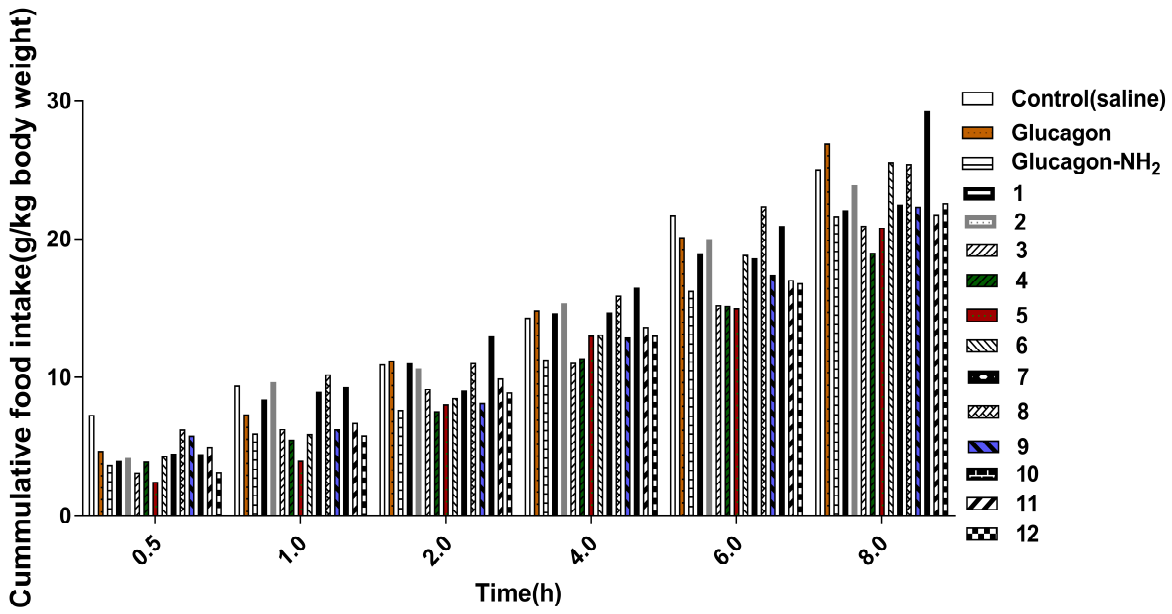


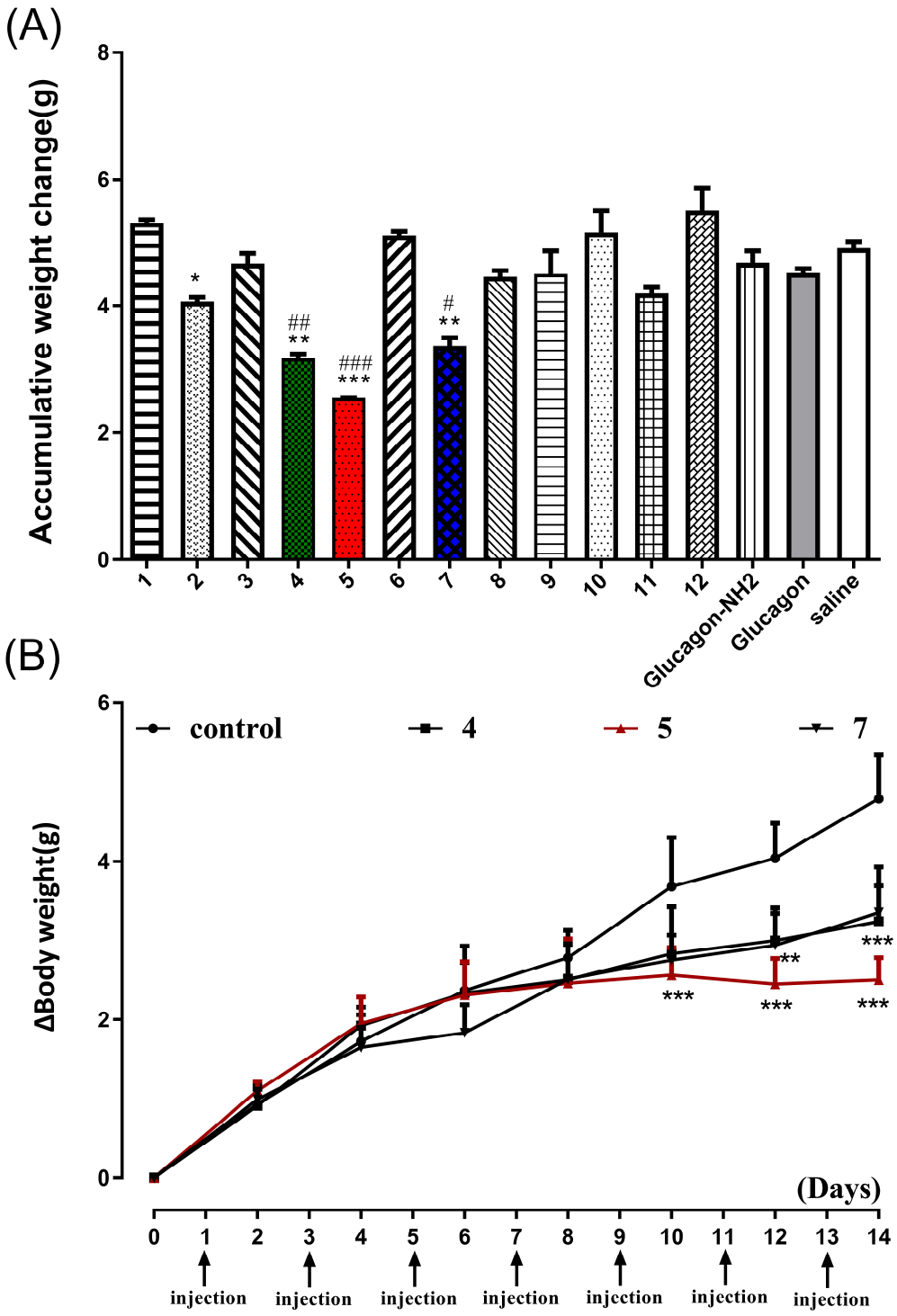


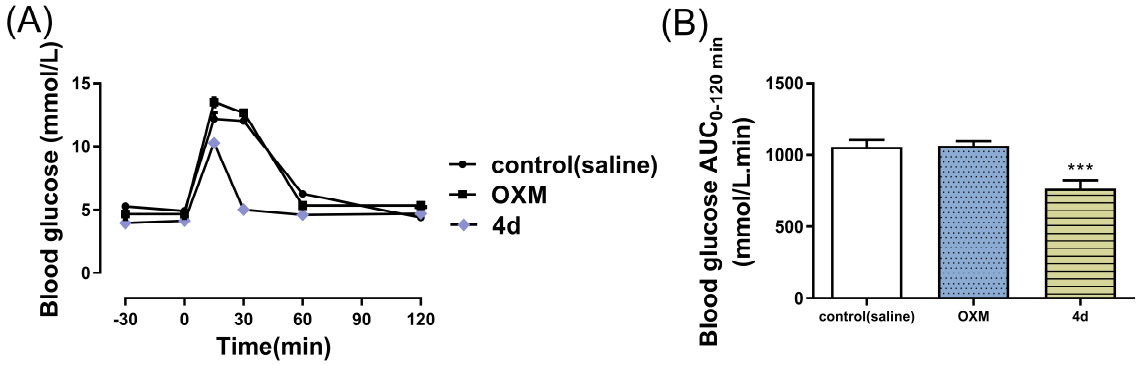


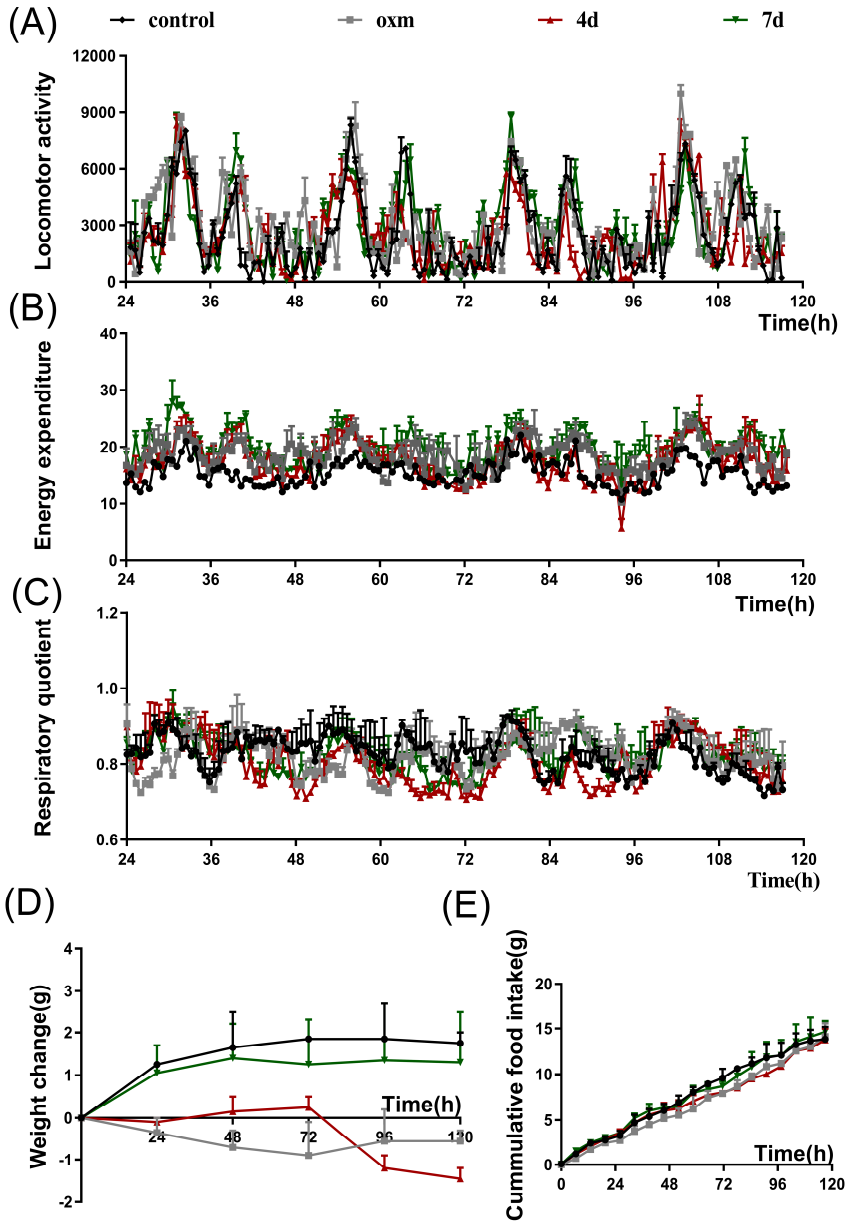


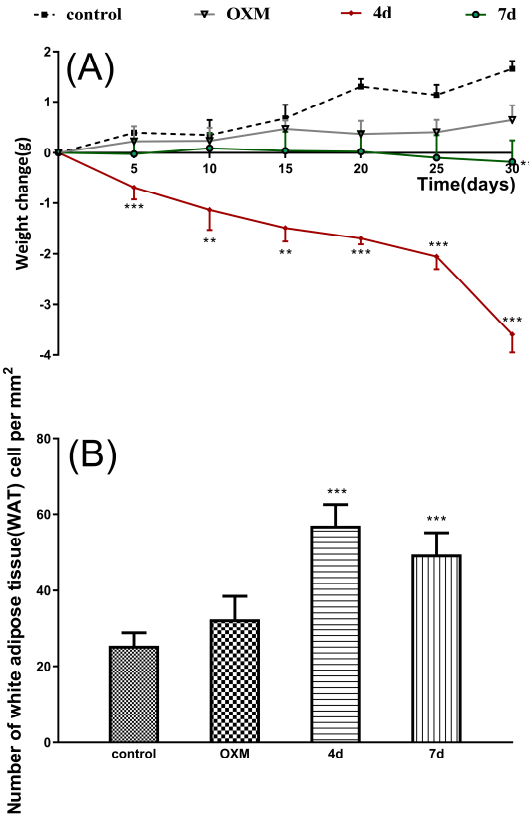

(A)
Control(saline)
Glucagon
4(Cys16-glucagon)
5(Cys17-glucagon)
7(Cys19-glucagon)
Blood glucose (mmol/L)
20
15
10
5
0
-30
0
30
60
90
120
Time(min)
(B)
Blood glucose AUC 0~120min(mmol/L.min)
1500
1000
500
0
***
***
***
Control (saline)
Glucagon
4
5
7

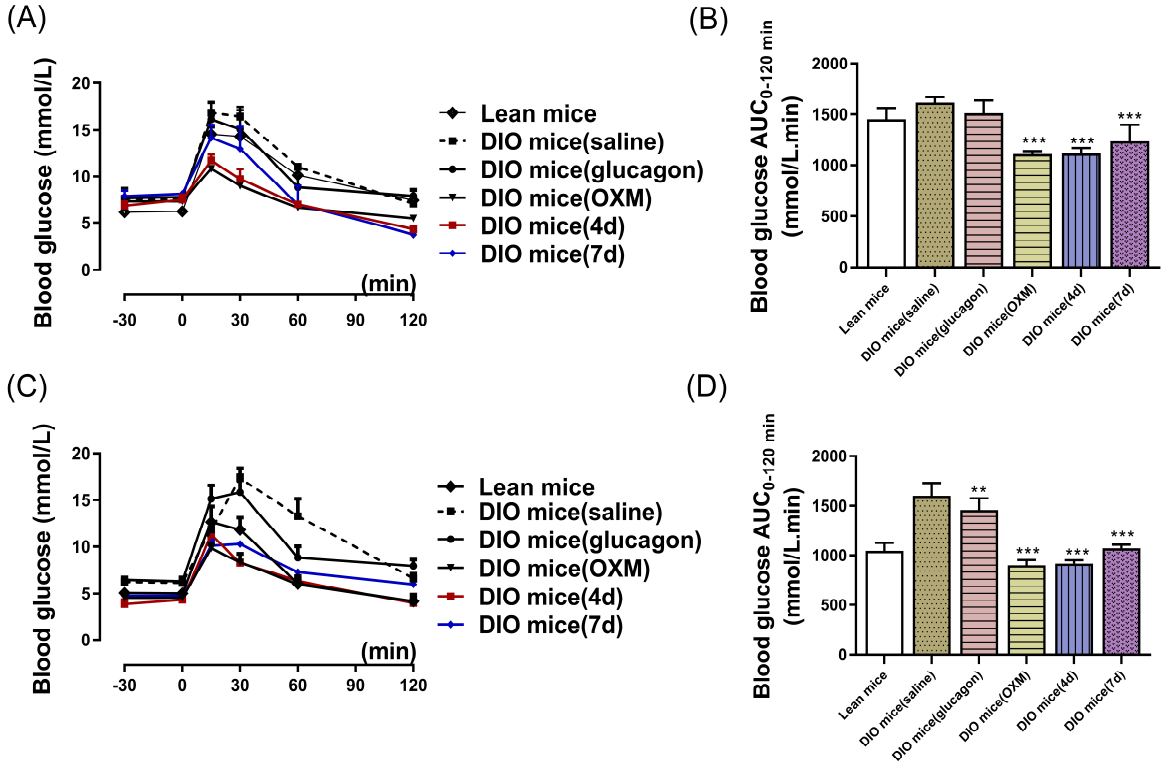


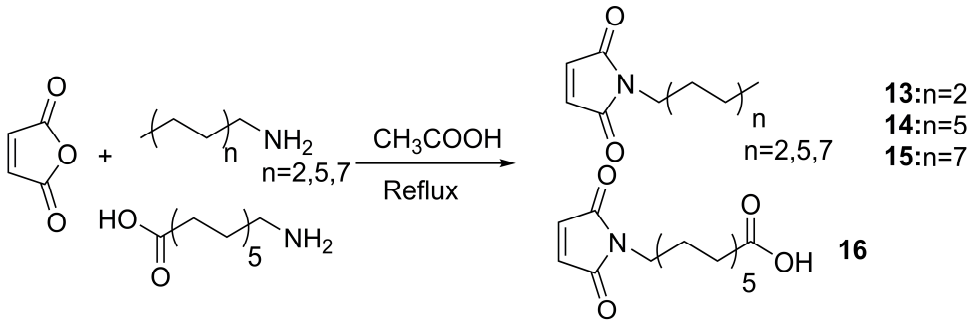

(A)
Accumulative weight change(g)
8
6
4
2
0
*
##
**
###
***
#
**
1
2
3
4
5
6
7
8
9
10
11
12
Glucagon-NH2
Glucagon
saline
(B)
ΔBody weight(g)
control
4
5
7
6
4
2
0
***
**
***
***
***
(Days)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
injection
injection
injection
injection
injection
injection
injection

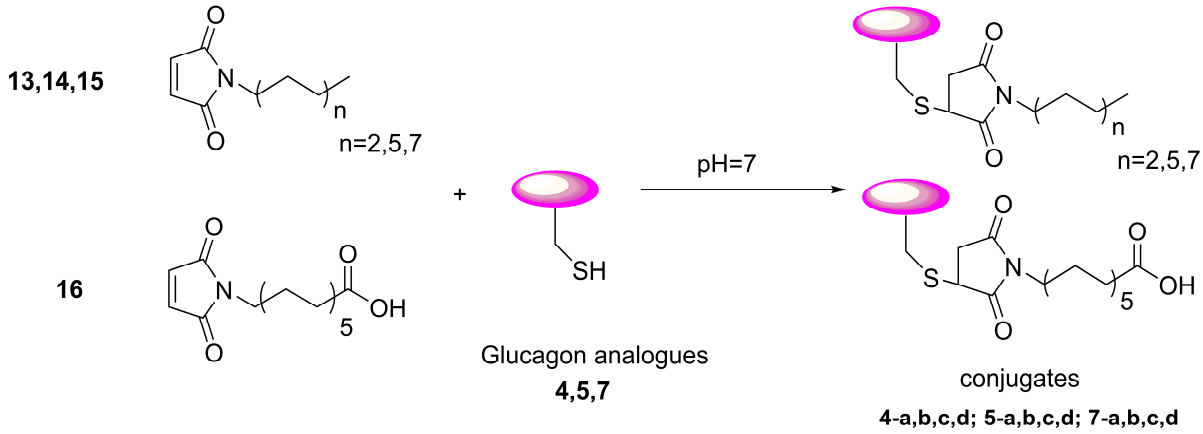



(A)
control
oxm
4d
7d
Locomotor activity
12000
9000
6000
3000
0
24
36
48
60
72
84
96
108
120
Time(h)
(B)
Energy expenditure
40
30
20
10
0
Time(h)
(C)
Respiratory quotient
1.2
1.0
0.8
0.6
Time(h)
(D)
Weight change(g)
4
3
2
1
0
-1
-2
Time(h)
24
48
72
96
120
(E)
Cummulative food intake(g)
20
15
10
5
0
Time(h)









Highlights

- Twenty-four glucagon-like peptide-1/glucagon receptor dual agonists were designed and synthesized.
- Compound 4d behaves well in lowering body weight and maintain energy expenditure in DIO mice.
- 4d showed notable diabetes-protective effects in type 2 diabetic mice.
- No hyperglycaemia effect were observed in the OGTT of 4d.