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Novel glucagon- and OXM-based peptides acting through glucagon and GLP-1 receptors with body weight reduction and anti-diabetic properties

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ABSTRACT

Oxyntomodulin (OXM) is an endogenous gastrointestinal hormone, which activates both the Glucagon-like peptide-1 receptor (GLP-1R) and the glucagon receptor (GCGR). However, OXM has shortcomings including poor GLP-1R agonism to control glycemia, short half-life and others. Inspired from the sequence relationship between OXM and glucagon, in this study, we introduced different C-terminus residues of GLP-1, exenatide and OXM to glucagon to get a series of hybrid peptides with enhanced GLP-1R activation. The formed glucagon-exenatide hybrid peptide shows higher GLP-1R activation properties than OXM. Then the peptides based on the glucagon-exenatide hybrid peptide were coupled with fatty acid side chains to prolong their half-lives. As a result, the most potent compound **16a** could stimulate insulin secretion and maintain blood glucose in normal level for ~42.6 h in diabetic mice. **16a** exhibited reduced HbA1c level in diabetic mice, lowered body weight significantly in obesity mice on chronic treatment assay. **16a**, combined efficient GCGR/GLP-1R activity, is potential as novel treatment for obesity and diabetes. This finding provides new insights into balancing GLP-1/GCGR potency of glucagon-exenatide hybrid peptide and is helpful for discovery of novel anti-diabetic and bodyweight-reducing drugs.

1. Introduction

Obesity is highly associated with type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) [1] and all-cause mortality in people with body mass index (BMI) ≥ 35 kg/m^2 compared to those with normal body weight [2]. Glucagon like peptide-1(GLP-1, Peptide **1**) analog is one of the few medications which are available for good management of obesity and related comorbidities, like diabetes [3]. GLP-1 as the mostly studied incretin, clinically, is a gut hormone that enhances the secretion of insulin after nutrient ingestion. The functions of GLP-1 include delaying gastric emptying, stimulating insulin secretion and mediating satiety in central nervous system (CNS). These actions are beneficial to individuals with obesity and type 2 diabetes [4]. However, its function in reducing body weight is not prominent.

Exenatide (Peptide **2**), known as the first approved GLP-1R agonist, is a synthetic version of exendin-4 which was found in the saliva of the Gila monster. It has only 53% amino acid sequence homology with human GLP-1 [5]. In addition to the amino acid differences throughout the sequence compared to human GLP-1, exendin-4 contains a C-terminal extension (Cex) sequence that greatly improves its biophysical and metabolic stability compared to human GLP-1 [6,7]. The effect of exenatide was tested in DMGO trials [8–10]. Which showed that exenatide could reduce the HbA1c by approximately 1.0–1.2% and decrease the fasting plasma glucose by about 1.0–1.4 mmol/L compared

Abbreviations: AUC, area under the curve; cAMP, cyclic adenosine monophosphate; DIO, diet induced obese; EC50, half maximally at an effective concentration; Ex-4, exenatide; 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; OXM, oxyntomodulin; TFA, trifluoroacetic acid; WAT, white adipose tissue

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to placebo [8]. Glucagon (Peptide **3**) is a well-established insulin counter hormone, which is processed from proglucagon, the same biosynthetic precursor as GLP-1 and Oxyntomodulin (OXM, Peptide **4**) [11]. Recent reports demonstrated that glucagon has beneficial effects on increasing energy expenditure. Additionally, glucagon has both catabolic and thermogenic actions [12–14].

Diseases like obesity and diabetes are concurrent and heterogeneous etiology. Improved efficacy in the treatment of such diseases could be achieved by targeting multiple biochemical pathways within a single therapy [15–17]. However, physical combinations of independent medications normally would complicate the processes of drug development and clinical practice. Therefore, a single molecule with appropriate balanced activities at multiple receptors is the best option [18]. OXM, for instance, binds to and fully activates both the GLP-1 receptor (GLP-1R) and the glucagon receptor (GCGR) in vitro with similar potencies [19]. Interestingly, OXM is composed of the entire 29 amino acid sequence of glucagon, along with an octapeptide extension to the C-terminus. In a one-month study, an average weight loss of 2.3 kg on patients with overweight and obesity was observed after three times daily preprandial subcutaneous administration of OXM, while an average weight loss of 0.5 kg was observed in the placebo group [20].

However, OXM is not the most potent agonist on GLP-1R or GCGR, and OXM could slightly reduce the blood glucose level [21]. Similar to GLP-1, endogenous OXM is quickly degraded (about 12 min) *in vivo* due to *N*-terminal degradation by DPP-IV [22]. Currently, a few candidates are in preclinical or clinical research development. MEDI0382 is in phase II clinical development at MedImmune. It was accepted by once-daily injection. MEDI0382 consist of 30 amino acids based on modified OXM, with gamma-glutamate and palmitic acid coupled at lysine residue [23]. Phase II results showed marked reductions in fasting plasma glucose and postprandial glucose from baseline with no increase in hypoglycemia. Body weight was significantly reduced after injected by MEDI0382, with a weight loss of −3.8 kg from baseline compared to −1.7 kg with placebo [24]. Evers *et al.* designed a series of dual agonists based on exenatide to adjust the selectivity ratio of GLP-1/glucagon. Anti-obese activity was studied on the selected peptide as well as Liraglutide which were dosed at 50 μg/kg twice daily over 33 days. A significant decrease of body weight by 29.1% was observed [25].

In this paper, we proposed a novel approach to overcome the disadvantages of OXM including weak activation of GLP-1 and short half-life. Inspired from the sequence connection between OXM and glucagon, C-terminus residues of GLP-1, Exenatide and OXM were introduced to the glucagon C-terminus. Further modification and fatty acid side chain conjugation were also performed to prolong the half-life of the selected hybrid peptide. In our previous work, it was proved that the fatty acid side chain attached at position 16 had a positive effect on stabilizing plasma glucose and lowering body weight [26]. In this study, the activation of GLP-1R/GCGR and intra-peritoneal glucose tolerance test was performed and the influences on body weight changes were observed. Since fatty liver diseases may be associated with diabetes, biomarkers of liver diseases like ALT and AST were detected. Compound **16a** was identified as the most potent GLP-1/GCGR agonist which could stabilize plasma glucose level, lower body weight of diet-induced-obesity (DIO) mice and have long-term beneficial effects on streptozotocin (STZ)-induced diabetic mice.

2. Results

2.1. Design of C-terminus hybrid peptides and receptors activation assay

According to the relationship between OXM and glucagon, we hypothesized that introducing different C-terminus extensions to glucagon may result in peptides with different agonism ratio on GLP-1R and GCGR. cAMP was chosen to prove the activation of the two receptors by our compounds (Fig. 1, Table 1). Native OXM activated GCGR at an effective concentration (EC_{50}) of 6.1 ± 0.2 pM and GLP-1R at an EC_{50} of 15.7 ± 0.2 pM. Compared to OXM, most of the OXM analogues had improved potency on GLP-1R and maintained activity on GCGR, indicating that the C-terminus modification method was feasible. The octapeptide of OXM was substituted by part of its sequences, GLP-1 and Exenatide to afford compounds **5**, **6** and **7** respectively. Truncated peptide **5** showed 1.86-fold weaker GCGR agonism than native OXM. Compound **7** showed a slight increase in GLP-1R potency and little reduced potency at GCGR compared to OXM. Substitution of three amino acid residues of glucagon C-terminus to exenatide residue led to compound **8**. It exhibited great GLP-1R activation and moderate GCGR potency, which was nearly comparable to native OXM. This indicates that the C-terminus of glucagon could be rationally modified to further improve the relative ratio of GLP-1R/GCGR co-agonism, although a degree of conservation for both receptors was observed [27]. Compound **10** showed a 2.06-fold increase in GLP-1R potency compared with OXM.

2.2. Preliminary activity assessment of hybrid peptides in Kunming mice

The activity of the hybrid peptides on feeding was monitored in normal fasted Kunming mice. As shown in Fig. 2**A**, most of the peptides could reduce food intake in the first hour after administration compared with vehicle. Peptides **6**, **7**, **10** had a greater anorectic effect than other peptides. Among all peptides, no significant difference in cumulative food intake over up to 24 h was observed (data not shown), which indicates that the duration of peptides was less than 24 h. To further evaluate chronic lowering effects of body weight, administration of hybrid peptides at 25 nmol/kg once per day to Kunming mice for two weeks was conducted (Fig. 2**B**). After two weeks treatment of **7**, **9** and **10**, the average body weight gain was lower than that in the vehicle treatment group ($P < 0.05$), indicating good weight control potency of these analogues. Particularly, the least body weight increase was observed in mice groups treated with **7** and **10** versus vehicle-treated mice ($P < 0.01$, 2.36 ± 0.23 g for **10**, 2.66 ± 0.32 g for **7**, 3.88 ± 0.16 g for vehicle (saline), respectively).

To evaluate whether the administration of hybrid peptides could improve systemic response to glucose, IPGTT was performed to Kunming mice. Most OXM analogues exhibited good hypoglycemic effects. Especially, after **7** and **10** were administrated, plasma glucose levels decreased significantly ($p < 0.001$) at 15, 30 and 60 min (Fig. 2**C**).

2.3. Energy balance and physiological measurements in DIO mice

Peptides **7** and **10** were administered at 25 nmol/kg intraperitoneally once daily for five days (Fig. 3). The injections of **10** led to body weight decrease in DIO mice by 1.3 ± 0.2 g, while increased body weight of 1.75 ± 0.13 g was shown in the vehicle group ($P < 0.01$, $n = 5$ per group) (Fig. 3**A**). For **7** and **10**, energy expenditure was also increased compared to vehicle (18.44 ± 3.46 kcal/($kg.h^{-1}$) and16.20 ± 3.03 kcal/($kg.h^{-1}$) compared to 15.47 ± 2.22 kcal/($kg.h^{-1}$), $P < 0.05$). They had no significant difference with OXM (17.5 ± 3.67 kcal/($kg.h^{-1}$)), and the respiratory quotient tended to decrease slightly (Fig. 3**B**, **C**). However, the 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 (Fig. 3**D**), which suggested that **7** and **10** reduced weight through appetite suppression and energy expenditure.

2.4. One-month therapy on DIO mice by 7 and 10

In the follow-up experiment, we tested the effect of intraperitoneal injections of **7** or **10** (25 nmol/ kg) every other day for one month. The injections decreased the body weight of DIO mice by 0.78 ± 0.22 g for **7** and 1.49 ± 0.36 g for **10**, respectively. In contrast, in the vehicle

Fig. 1. Structures of compounds.

group, the body weight of DIO mice was increased by 1.66 ± 0.32 g ($P < 0.01$) (Fig. 4**A**). Decreased white adipose content was observed in **7**- and **10**-treated DIO mice. The white adipose tissue (WAT) cell number in the unit area was associated with body weight changes. The more cells counted; the smaller white adipose cells are. The results are as follows:34.4 ± 3.36 for **7**, 64.4 ± 4.23 for **10**, 33.6 ± 5.96 for OXM, and 25.4 ± 3.44 for controls, $P < 0.001$ (Table 2).

The IPGTT performed on day 1 and 30 revealed that the glucose tolerance ability was significantly improved in both groups (Fig. 4**B**, **C**). Furthermore, basal blood glucose level was normalized by chronic treatment with **10**, and this result was comparable with that using **OXM** (Fig. 4**D**).

Several other metabolic parameters in plasma were also improved by chronic treatment with the peptides (Table 2). Increases in adiponectin and leptin as well as decreases in insulin levels correlated with decreased adiposity were observed at the end of the study in each treatment group. Reduced cholesterol and decreased hepatic lipid droplets relative to vehicle treatment group were also noted, especially for mice treated with **10**. Nevertheless, these peptides had no significant effects on triglycerides levels.

2.5. Optimization of long-acting effect of hybrid peptides

As demonstrated above, compound **10** displayed favorable GLP-1/GCGR activation, and *in vivo* study showed that compound **10** could improve glucose tolerance and decrease body weight in Kunming mice. Compound **10** also increased energy expenditure and decreased the respiratory quotient. In the long-period therapy on DIO mice, compound **10** lowered the body weight significantly, improved glucose tolerance and several other metabolic parameters in plasma. Therefore, compound **10** shows good prospects as exenatide C-terminal sequence hybrid peptide. Doyle *et al.* investigated the *in vivo* biological activity of exenatide (1–30) and also found that exenatide (1–30), the C terminal-truncated exendin peptide, had a reduced affinity for GLP-1R and a lessened ability to increase intracellular cAMP in insulinoma cells [28].

To prolong the acting effect of glucagon-exenatide hybrid peptides, we tried to raise the homologies compared with Exenatide by changing a series of amino acids in the middle of the peptides (Fig. 5). Ser in position 2 was substituted with Gly to prevent rapid inactivation by the metabolic enzymes like DPP-IV [29]. Meanwhile, in compound **11–16**, amino acid residues located at 14, 15, 17, 19, 21 and 24 were substituted respectively by the residues of Exenatide in the same location. Position 16 was replaced by Cys for coupling hybrid peptides with fatty chains in the next step to yield compound **11a-16a**. As shown in Table 3, most hybrid peptides preserved their GLP-1R and GCGR activation ability, only compound **11** had a 19.06-fold decrease in GLP-1R activation and 12.46-fold decrease in GCGR activation compared with OXM. Compounds **13** to **16** showed good GLP-1R activation ability. It was demonstrated that the C-terminus sequences of Exenatide had positive effect on raising GLP-1R activation in hybrid peptides without resulting in drastic reduction in GCGR activation. It was reported that the fatty acid chain would interrupt the interaction force between OXM/GLP-1 analogues and GLP-1R/GCGR in many studies [30,31]. However, in this work, we found a well-marked elevation on GLP-1/GCGR activation after fatty acid was introduced in each hybrid peptide. The activation on GLP-1R of compound **13a** had a 3.65-fold increase compared with that of compound **13**, while that of compound **16a** had a 10.95-fold increase compared with that of compound **16**. The most potent agonist on GLP-1/GCGR in this group was compound **16a** that could activate GLP-1R with an EC_{50} of 0.20 ± 0.5 pM and activate GCGR with an EC_{50} of 2.70 ± 0.4 pM. Therefore, the potential of compound **16a** for further research is optimistic.

2.6. Preliminary activity assessment and hypoglycemic test

Traditional IPGTT has a limitation that it could not evaluate the long-acting plasma glucose lowering effect. Therefore, we ameliorate the procedure by injecting the compound intraperitoneally in Kunming

Table 1
EC_{50} of GLP-1, Exenatide, Glucagon, OXM and OXM analogues.

Peptides	mGLP1R (pM)	mGCGR (pM)	Peptides	mGLP1R (pM)	mGCGR (pM)
1(GLP-1)	4.3 ± 0.4	> 1000	**6**	7.8 ± 0.2** ##	8.4 ± 0.2**
2(Exenatide)	5.8 ± 0.6	> 1000	**7**	4.2 ± 0.2** ##	7.2 ± 0.5
3(Glucagon)	34.9 ± 0.4	1.1 ± 0.5	**8**	7.4 ± 0.6** ##	8.8 ± 0.5**
4(OXM)	15.7 ± 0.2	6.1 ± 0.2	**9**	12.6 ± 0.7** ##	24.1 ± 0.5**
5	15.8 ± 0.2##	11.4 ± 0.3**	**10**	1.9 ± 0.1** ##	14.4 ± 0.9**

Results are expressed as mean ± SD.
*$P < 0.05$.
#$P < 0.05$.
** $P < 0.01$ vs OXM.
$P < 0.01$ vs Exenatide.

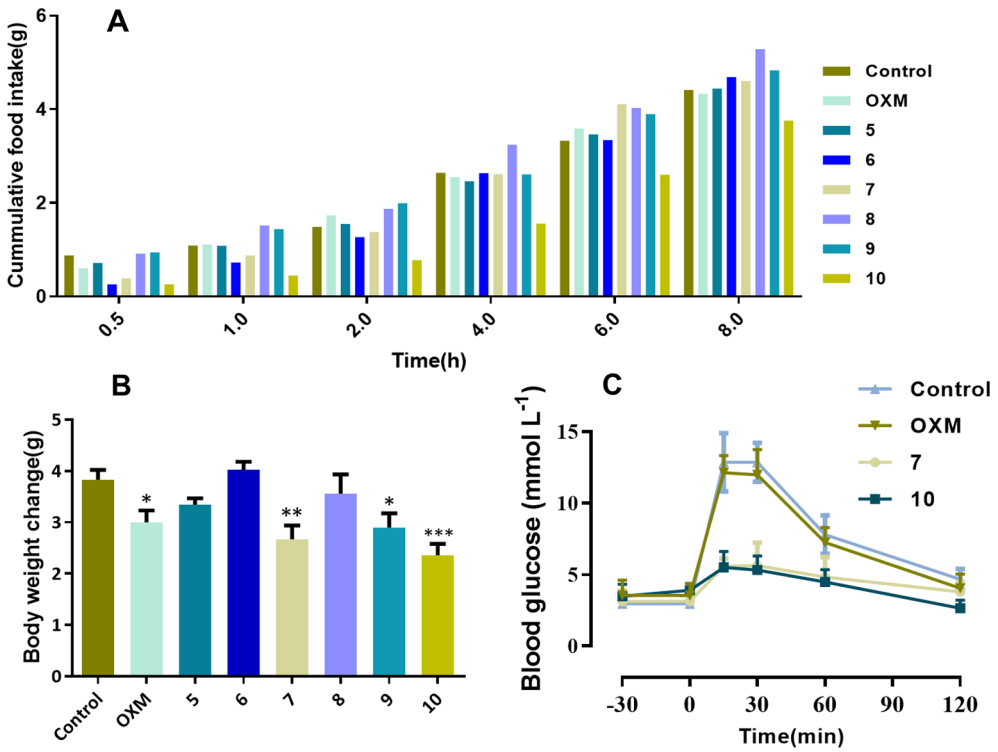

Fig. 2. (A) Cumulative food intake(g) in normal fasted Kunming mice for 8 h with 25 nmol/kg hybrid peptides (B) Body weight change with 25 nmol/kg once per day to Kunming mice for two weeks (C) Glucose-lowering effect of control (saline), OXM, **7** and **10** using IPGTT in Kunming mice.

mice and loading the glucose 24 h later. After the glucose was injected, the plasma glucose was measured at 0, 15, 30, 60 and 120 min. As shown in Fig. 6**A**, the activation abilities of compound **13a, 15a** and **16a** were superior in IGPTT while the others including Exenatide and Liraglutide almost lost their activation. Afterwards, compound **11a-16a**, OXM and saline were administrated once a day for 2 weeks (Fig. 6**B**), and compound **16a** exhibited good slimming effect, thus it was chosen for further studies.

Plasma and compounds were incubated over 96 h for evaluating in vitro stability of long-acting compounds **11a-16a**. As illustrated in Fig. 6**C**, the in vitro half-life of most compounds was prolonged compared with exenatide and liraglutide. More specifically, the plasma half-life of compound **16a** is up to 71.79 h.

The hypoglycemic effects of compound **16a** were assessed via intraperitoneal injection in STZ-induced diabetic mice. Normal blood glucose level was set as no more than 8.35 mmol/L (150 mg/dL). Euglycemic durations under this value were calculated and considered as a practical indication for antidiabetic treatment [32]. As shown in Fig. 6**D-E**, saline-treated control mice exhibited a hyperglycemic state (average $>$ 25 mmol/L) while those injected with Exenatide and Liraglutide (25 nmol/kg) rapidly maintained normal blood glucose level for nearly 3.8 h and 12.4 h, respectively. At a dose of 25 nmol/kg, the time required to rebound to a glucose level of 8.35 mmol/L was ~42.6 h in **16a**-treated mice which was much longer than that in Exenatide-treated mice and better than that in Liraglutide-treated mice. The $AUC_{0–60h}$ values of **16a** were 2.63 times lower than those of Exenatide and 1.93 times lower than those of Liraglutide.

The *in vivo* glucoregulatory and insulin tropic activities of **16a** were examined in SD rats by IPGTT. After intraperitoneal injection of **16a**, Liraglutide or Exenatide at -30 min, a glucose challenge of 10 g/kg was loaded at 0 min, then the mean blood glucose levels at 15, 30, 45, 60, 90, 120 and 180 min were measured. A significant increase in the glucose tolerance patterns was observed. As shown in Fig. 7, after a glucose challenge, the mean blood glucose level in the saline-treated group rapidly increased to 16.94 $\pm$ 3.6 mmol/L at 30 min, while in those injected with Liraglutide, Exenatide and **16a**, the level dramatically reduced to ~7.0, ~6.4, and ~6.9 mmol/L at the same time point, respectively. Moreover, the glucose AUC of Liraglutide, Exenatide and **16a** were significantly lower than that of the saline-treated group. The decreases in plasma glucose came with the increases in plasma insulin concentration which was consistent with the GLP-1-dependent mechanism. Similar time courses for plasma insulin concentrations in **16a-**, Liraglutide- or Exenatide-treated group were observed and all plasma insulin concentrations from 15 to 60 min time points were significantly greater than those of the control group. In particular, **16a** showed comparable promoting insulin secretion ability than Liraglutide and Exenatide ($p < 0.05$). The insulin tropic activities and glucose-lowering abilities of **16a** were also comparable with those of Liraglutide and Exenatide.

Glucose-lowering and stabilizing effect of Liraglutide, Exenatide and **16a** as determined by hypoglycemic duration test in nonfasted diabetic mice. (D) Time-course average blood glucose levels of diabetic mice after an i.p. injection of Liraglutide, Exenatide or **16a** (25 nmol/kg). Times depict hypoglycemic duration rebound to 8.35 mmol/L. (E) Hypoglycemic effects of Liraglutide, Exenatide and **16a** based on the calculated glucose $AUC_{0\text{-}60h}$ values.

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

To prove *in vivo* activity and potential therapeutic efficiency towards diabetes, compound **16a**, Liraglutide, Exenatide and saline were chronically administrated to STZ-induced diabetic mice every day. As shown in Fig. 8**A**, before the treatment, fasting blood glucose level in diabetic mice was ~20.0 mmol/L. After 21 days, it increased to ~22.0 mmol/L in saline-treated group, while treatment by compound **16a** lowered the fasting blood glucose toward the normal levels. Furthermore, HbA1c was a more sensitive index of diabetes control than blood glucose level. HbA1c was a more sensitive index of diabetes control than blood glucose level. HbA1c was generated from non-enzymatic irreversible glycation and thus it could be regarded as an

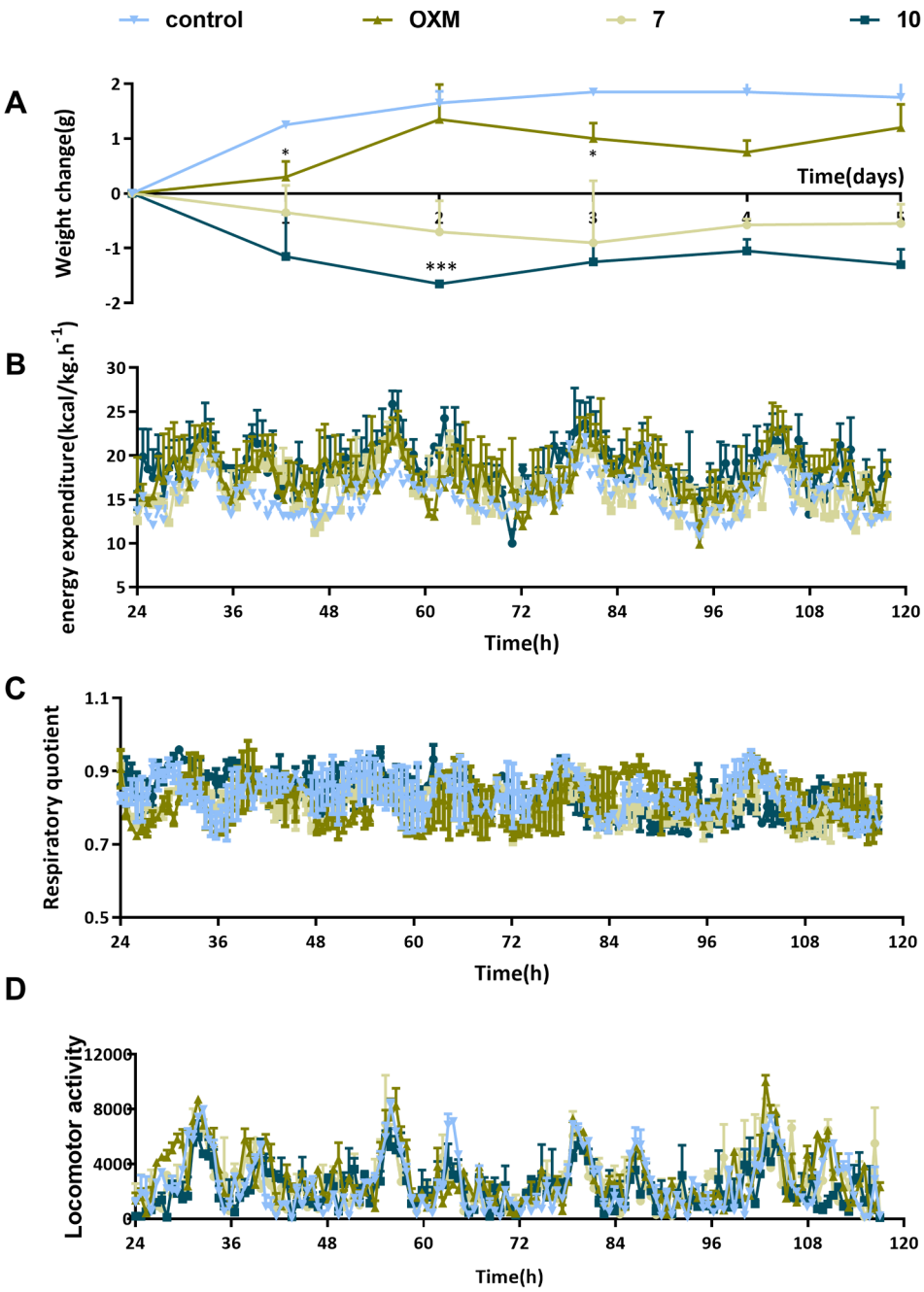

Fig. 3. One-week treatment of diet-induced obese mice with OXM, **7** and **10**: (A) dynamic body weight change, (B) energy expenditure, (C) respiratory quotient, (D) locomotor activity.

indirect indicator of cumulative blood glucose concentrations[42]. As shown in Fig. 8**B**, initially, HbA1c was well matched in all diabetic groups. During the 21-day treatment, diabetic mice daily injected with **16a** exhibited a marked reduction on HbA1c, while it was high in the control group. The HbA1c reduction caused by the administration of **16a** is indicative of the promising role of **16a** in clinical studies. To determine whether long-term treatment by **16a** could improve glucose tolerance or not, IPGTT test was performed before and after treatment (day 0 and day 24). At the beginning of treatment, few differences were observed between mice in each group. However, at the end the treatment, **16a-**, Liraglutide- and Exenatide-treated mice showed rapid blood glucose reductions, and it returned to baseline at 120 min after glucose administration, whereas saline-treated mice showed a typical diabetic pattern of slow reduction in the hyperglycemic state at 120 min (Fig. 8**C**). The blood glucose AUC of **16a** was significantly lower than that of the saline-treated diabetic mice ($p < 0.05$) (Fig. 8**D**).

Compound **16a** improved liver regeneration in STZ-induced diabetic mice, and regeneration ratio of liver corrected by body weight was analyzed at the end of the 3-week treatment of saline, Liraglutide, Exenatide or **16a**. Saline-treated diabetic mice had a low ratio of liver weight/body weight compared to the normal mice (Fig. 8**E**). Compound **16a**-treated mice had a significant increase in liver weight/body weight ratio. Meanwhile, serum ALT and AST were assayed on a Beckman Coulter Chemistry Analyzer. As shown in Fig. 8**F-G**, ALT and AST increased in saline-treated mice, while treatment by compound **16a** reduced both ALT and AST levels. To evaluate the effect of compound **16a** on the morphology of pancreatic islets in STZ-induced diabetic mice. The number of insulin-positive beta cells were reduced in saline-treated

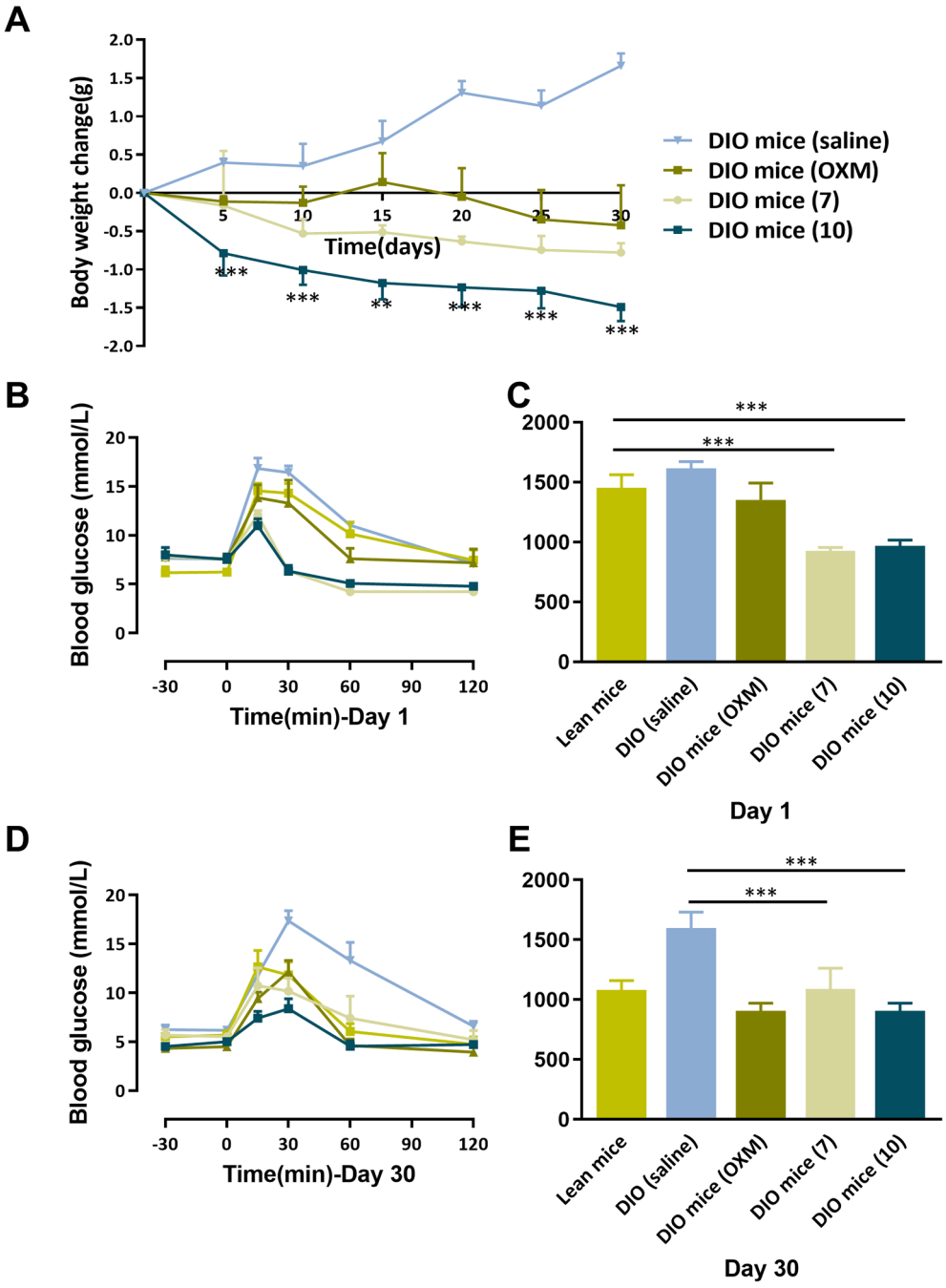

Fig. 4. (A) Body weight change of DIO mice for one month. (B-E) One-month treatment of DIO mice with OXM, **7** and **10**: (B) IPGTT results in mice at day 1. (C) IPGTT results in mice at day 30. (D) AUC of IPGTT results at day 1. (E) AUC of IPGTT results at day 30.

Table 2
Metabolic parameters in plasma at the end of the chronic study.

Compound	White adipose cell numbe	Insulin(ng/ml)	Leptin(ng/ml)	Adiponectin(ug/ml)	Cholesterol(mmol/l)	Triglycerides(mmol/l)
Control	25.44 ± 3.44	13.33 ± 0.45	1.9 ± 0.07	0.89 ± 0.38	4.33 ± 0.19	0.84 ± 0.12
OXM	33.60 ± 5.90	8.96 ± 0.13*	1.64 ± 0.007	1.15 ± 0.05*	3.82 ± 0.56	1.04 ± 0.21
7	34.40 ± 3.36	10.34 ± 0.23*	3.26 ± 0.36*	1.60 ± 0.11**	3.06 ± 0.05*	0.81 ± 0.23
10	64.40 ± 4.63***	9.40 ± 0.35*	2.66 ± 0.39*	1.94 ± 0.09***	3.78 ± 0.30*	0.81 ± 0.14

Results are expressed as mean ± SD. Significantly different from vehicle.
* P < 0.05.
** P < 0.01.
*** P < 0.001.

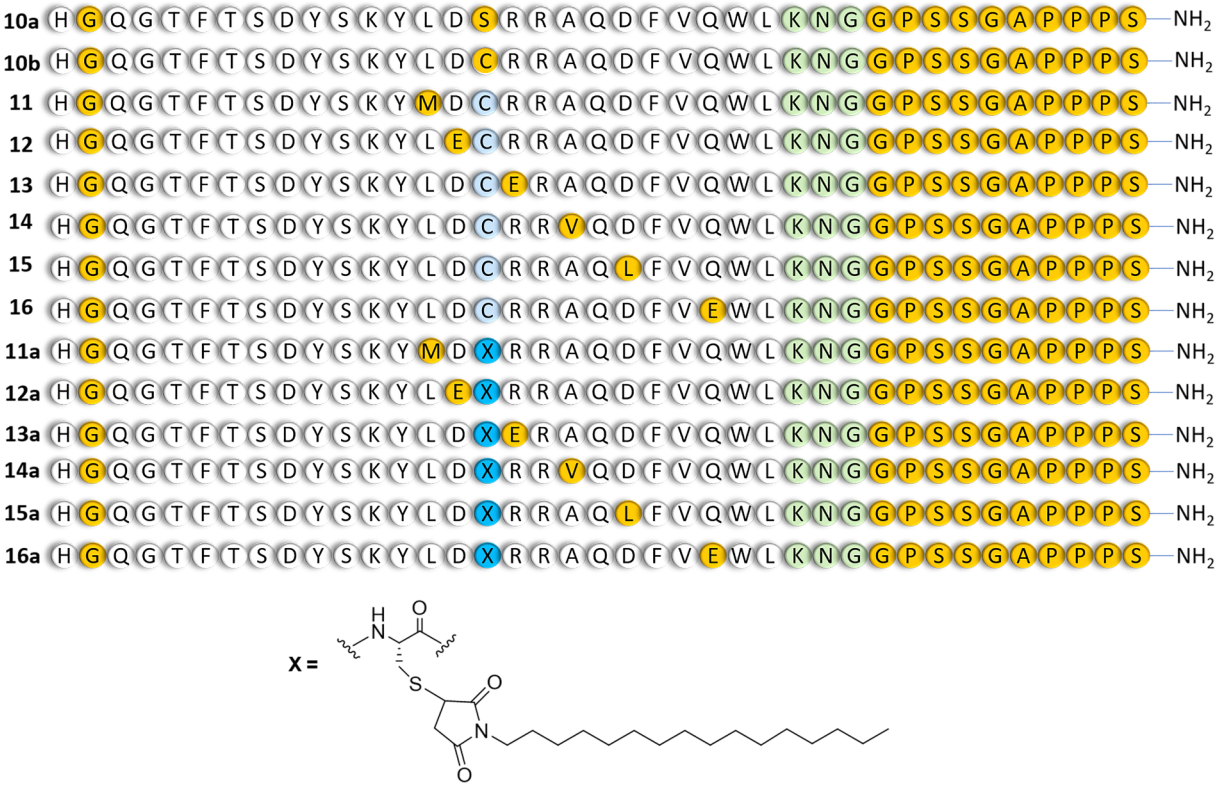

Fig. 5. Structures of compounds.

mice compared with that in normal mice. As shown in Fig. 8**H**, **16a**-treated mice had a significant increase in the number of insulin-positive beta cells than saline-treated mice.

2.8. Chronic in vivo studies on DIO mice

During the 4-week treatment course, saline-treated mice did not inhibit cumulative food and water intake (Fig. 9**A-B**), while compound **16a** reduced cumulative food and water intake by up to 49.6% and 42.1%, respectively. Noticeably, after 4-week treatment (Fig. 9**C**), compound **16a** lowered body weight of DIO mice significantly (~4.3 g). To further confirm the appetite suppression effect, an acute *in vivo* study was conducted (Fig. 9**D**), and compound **16a** could inhibit food intake by up to 70.2% at 6 h after administration. In addition, in the saline-treated DIO mice, total cholesterol (TC) and triglyceride (TG) level were higher than those in the lean mice. However, compound **16a** reduced TC and TG to a normal level after treatment. Two important adipocytokines secreted from white adipose tissue, the serum levels of leptin and adiponectin, were also altered after treatment by **16a**. Circulating leptin concentration in DIO mice was significantly higher than that in LFD controls ($p < 0.05$). In contrast, administration of **16a** significantly ($p < 0.05$) reduced serum leptin level to a near normal value of 2.3 ± 0.2 ng/ml. As shown in Fig. 9**I**, compound **16a**-treated mice had a significant increase in the number of insulin-positive beta cells than saline-treated mice.

3. Conclusions

In summary, six hybrid peptides based on glucagon were designed and synthesized. The glucagon-exenatide hybrid peptide, compound **10** had better receptor activation and glucose tolerance abilities. The intraperitoneal injections of **10** every other day for one month decreased the body weight of DIO mice significantly. A series of fatty acid attached compounds were designed and synthesized to prolong the half-life of compound **10**. A well-marked elevation was found on GLP-1/GCGR activation after fatty acid was introduced. In hypoglycemic test, the most potent GLP-1/GCGR agonism, compound **16a**, maintained normal blood glucose level for nearly 42.6 h in the STZ-induced

Table 3
EC_{50} of **OXM** analogues.

peptides	mGLP1R (pM)	mGCGR (pM)	peptides	mGLP1R (pM)	mGCGR (pM)
10a	6.9 ± 0.7**	16.5 ± 0.6**	**10b**	5.4 ± 0.6**	12.3 ± 1.1**
11	133.2 ± 1.5** ##	102.5 ± 1.2**	**11a**	52.2 ± 0.8** ##	27.4 ± 0.6
12	12.4 ± 1.1** ##	7.6 ± 1.7	**12a**	3.5 ± 0.8**	1.6 ± 0.3**
13	15.4 ± 0.6##	22.5 ± 1.7**	**13a**	2.3 ± 0.5** ##	128.0 ± 1.9**
14	19.5 ± 1.5** ##	62.5 ± 1.2**	**14a**	11.4 ± 0.9** ##	19.3 ± 1.8**
15	6.9 ± 0.9**	22.1 ± 0.4**	**15a**	2.5 ± 0.4** ##	3.4 ± 0.6
16	3.8 ± 0.4**	13.6 ± 1.3**	**16a**	0.3 ± 0.1** ##	2.5 ± 0.3*

Results are expressed as mean ± SD.
# $P < 0.05$.
* $P < 0.05$.
** $P < 0.01$ vs OXM.
$P < 0.01$ vs Exenatide.

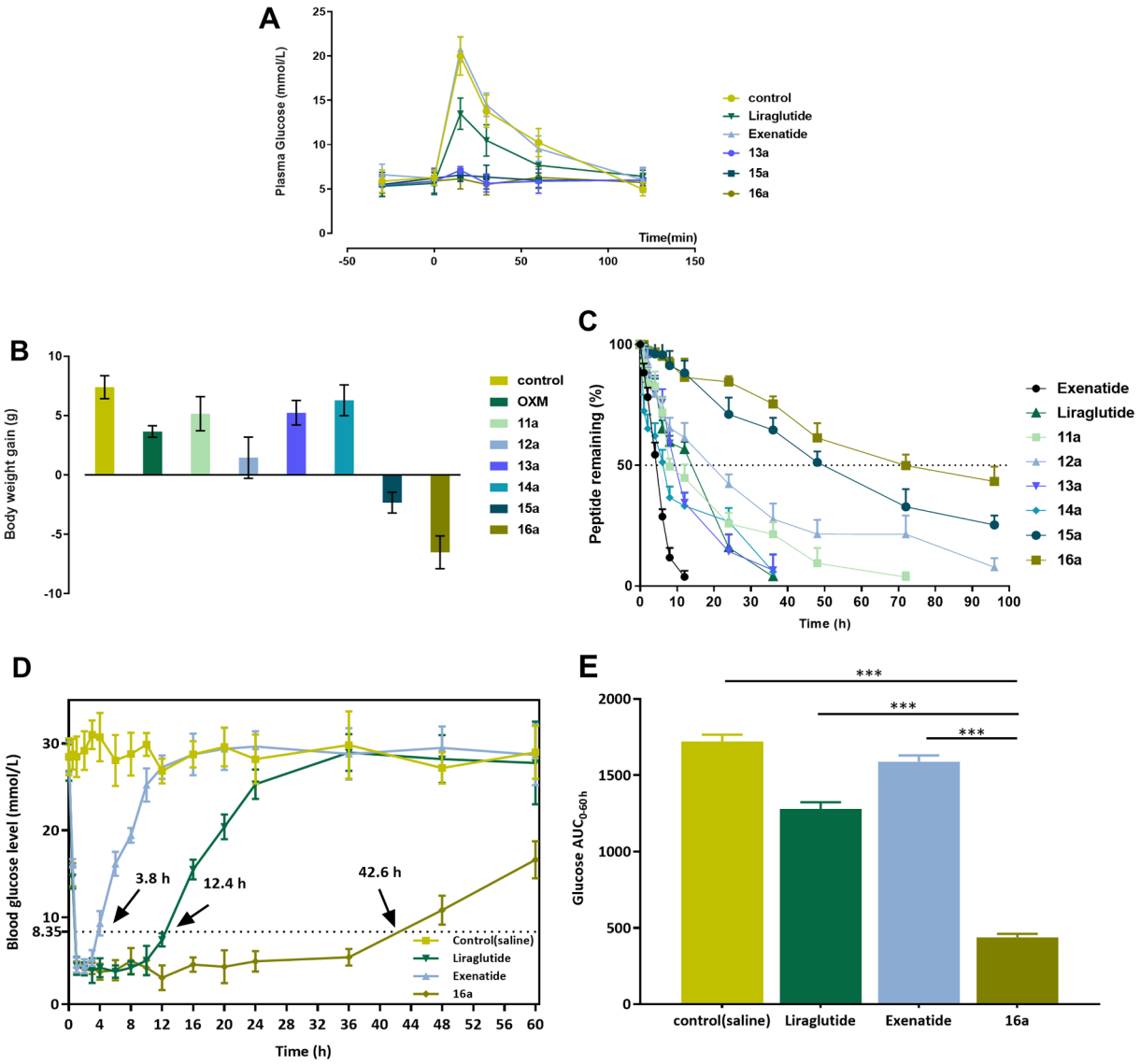

Fig. 6. (A) IPGTT result of **13a, 15a** and **16a**. (B) Body weight change after 2 weeks of treatment by **11a-16a**. (C) Plasma half-life of compounds.

diabetic mice. Moreover, compound **16a** showed comparable promoting insulin secretion ability to Exenatide and Liraglutide. The insulin tropic activities and glucose-lowering abilities of **16a** were comparable with those of Liraglutide and Exenatide. During the 21-days treatment, the HbA1c in the diabetic mice daily injected with **16a** exhibited a marked reduction, while it continued to be high in the control group. Furthermore, compound **16a** improved liver regeneration in the STZ-induced diabetic mice. In the 4-week treatment on DIO mice, compound **16a** reduced cumulative food and water intake and lowered body weight of DIO mice significantly. Compound **16a** with good receptor activation ability, plasma glucose regulator properties, body weight lowering effect, long-term beneficial effects and most importantly, long-acting antidiabetic properties, could be potentially useful for the development of OXM analogues.

4. Materials and methods

4.1. Chemistry

Fmoc Rink amide-MBHA resin, Fmoc-protected amino acids, Liraglutide, Exenatide, and Gly8-GLP-1(7–36)-NH_2 were purchased fromGL Biochem (Shanghai, China); HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany); cAMP dynamic kit was purchased from Cisbio (Bedford, MA); rat insulin ELISA kit was purchased from Millipore (Billerica, MA); HbA1c kit was purchased from Glycosal (Deeside, UK). 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), which generated continuous irradiation at 2450 mHz. HPLC purification were performed on a Shimadzu LC-8A preparative RP-HPLC system. Purity verification and ESI mass spectra were obtained on a Waters ACQUITY UPLC system (Milford, MA). Sprague–Dawley (SD) rats (male, 200–250 g) were purchased from the Comparative Medical Center of Yangzhou University (Jiangsu, China). Kunming mice, ob/ob mice and wild-type nondiabetic C57BL/6 littermate (male and female, 6–8 weeks old) were obtained from the Model Animal Research Center of Nanjing University (Jiangsu, 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. High fat diet (D12492; 60% fat, 20% protein and 20% carbohydrate; 5.24 kcal/g) and low fat diet (D12450B; 10% fat, 20% protein

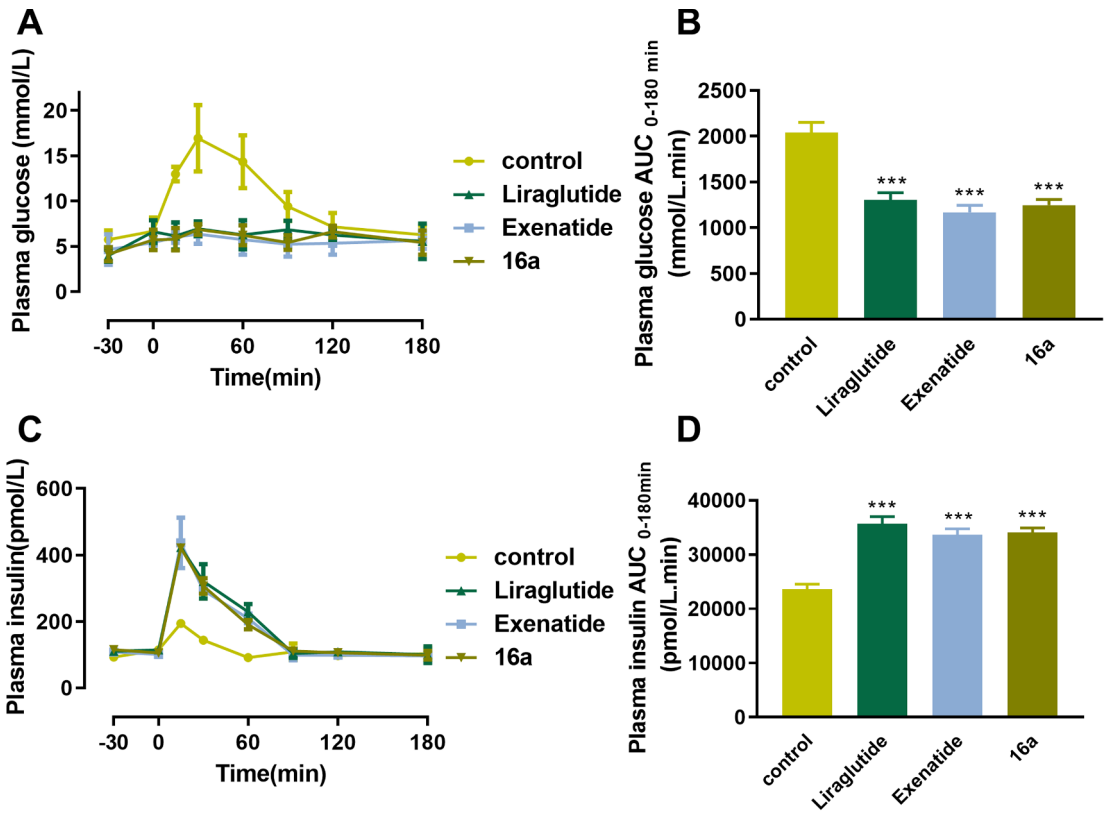

Fig. 7. *In vivo* biological activity tests of **16a**, Liraglutide and Exenatide (25 nmol/kg) i.p. injected into SD rats; glucose was taken orally (10 g/kg). (A) Insulinotropic activities and calculated insulin $AUC_{0-180min}$ (B) of Liraglutide, Exenatide and **16a**. (C) The glucose-lowering effects and calculated plasma glucose $AUC_{0-180min}$ (D) of Liraglutide, Exenatide and **16a**.

and 70% carbohydrate; 3.85 kcal/g) were supplied by Research Diets (New Brunswick, NJ, USA). All animal experimental protocols adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85–23, revised 1986).

4.1.1. General synthetic route and HPLC purification of peptides

Peptides were prepared using methodology called solid phase peptide synthesis (SPPS). Briefly, Fmoc Rink amide-MBHA resin (0.01 mmol) was deprotected with 25% piperidine in 5 mL of DMF for 4 min under microwave irradiation (10 W). A mixed solution of 0.025 mmol of first amino acid, 0.030 mmol of HBTU, 0.030 mmol of HOBt, and 0.050 mmol of DIPEA dissolved in 2.5 mL of DMF was added after washing three times with DMF. The mixture was bubbled with N_2 for 10 min under microwave irradiation (31 W) and washed three times 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 4 h at room temperature. Then, peptides were precipitated by addition of 50 mL of cold ethyl ether. After centrifugation, the crude peptides were purified using a Shimadzu preparative RP-HPLC: samples were injected into a Shimadzu C18 reverse phase column (5 μm, 340 mm × 28 mm) equilibrated in water with 0.1% TFA and purified using a linear gradient from 30% to 75% acetonitrile with 0.1% TFA over 30 min at a flow rate of 6.0 mL/min with UV detection at 214 nm. The molecular mass of the purified peptides was confirmed by LC–MS.

4.1.2. General synthetic route of fatty chain-maleimide

Hexadecan-1-amine (482.6 mg, 2 mmol) and furan-2, 5-dione (235.5 mg, 2.4 mmol) were dissolved in acetic acid, stirred at 120 °C for 8 h. The reaction was cooled to room temperature, and solvent was removed by reduced pressure condense. The crude solid was purified by column chromatography over silica gel using light petroleum/ethyl acetate (1:3, v/v) to give the final compound (579.6 mg, yield 90.0%), m.p. 103–105 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): d 7.01 (s, 2H, $COCH_5CHCO$), 3.38 (t, 2H, J = 6.2 Hz, NCH_2), 1.49–1.45 (m, 2H, $NCH_2CH_2(CH_2)_{13}CH_3$), 1.23 (s, 26H, $NCH_2CH_2(CH_2)_{13}CH_3$), 0.85 (t, 3H, J = 6.4 Hz, $NCH_2CH_2(CH_2)_{13}CH_3$); ESI-MS: *m/z* Calcd for $C_{20}H_{35}NO_2$ $[M + H]^+$ 322.0 found.

4.1.3. General synthetic route of conjugates

Cysteine altered peptide (5 mmol) was conjugated with fatty chain-maleimide (12 mmol) in 5 mL of 0.05 mol/L sodium phosphate buffer (pH 7.0). The reaction mixture was stirred at 20 °C under N_2 for 1.5 h until UPLC showed completion. The analytical condition was as follows: C18 column (5 mm, 150 mm × 4.6 mm); a linear gradient of mobile phase 20–80% B in 20 min (mobile phase A: water with 0.1% TFA and mobile phase B: acetonitrile with 0.1% TFA) at a flow rate of 1 mL/min, and ultraviolet (UV) detection at 214 nm was utilized. The crude conjugate was purified on Shimadzu preparative RP-HPLC: Shimadzu C18 reversed-phase column (5 mm, 340 mm × 28 mm), a linear gradient of mobile phase 40–90% 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 UPLC–MS.

4.2. Pharmacology

4.2.1. GLP-1 and glucagon receptor activation assay

(a) *Construction of GLP-1 receptor retroviral expression plasmid.* A large number of retroviral vector pBABE-puro plasmid with puromycin resistance and pMSCV-sulfuric acid GLP-1 receptor plasmid containing GLP-1 receptor with full-length cDNA sequence were prepared. The primers of GLP-1 receptor gene were synthesized by Shanghai Shenggong Biology Co. Ltd., and the ends of the receptor gene were

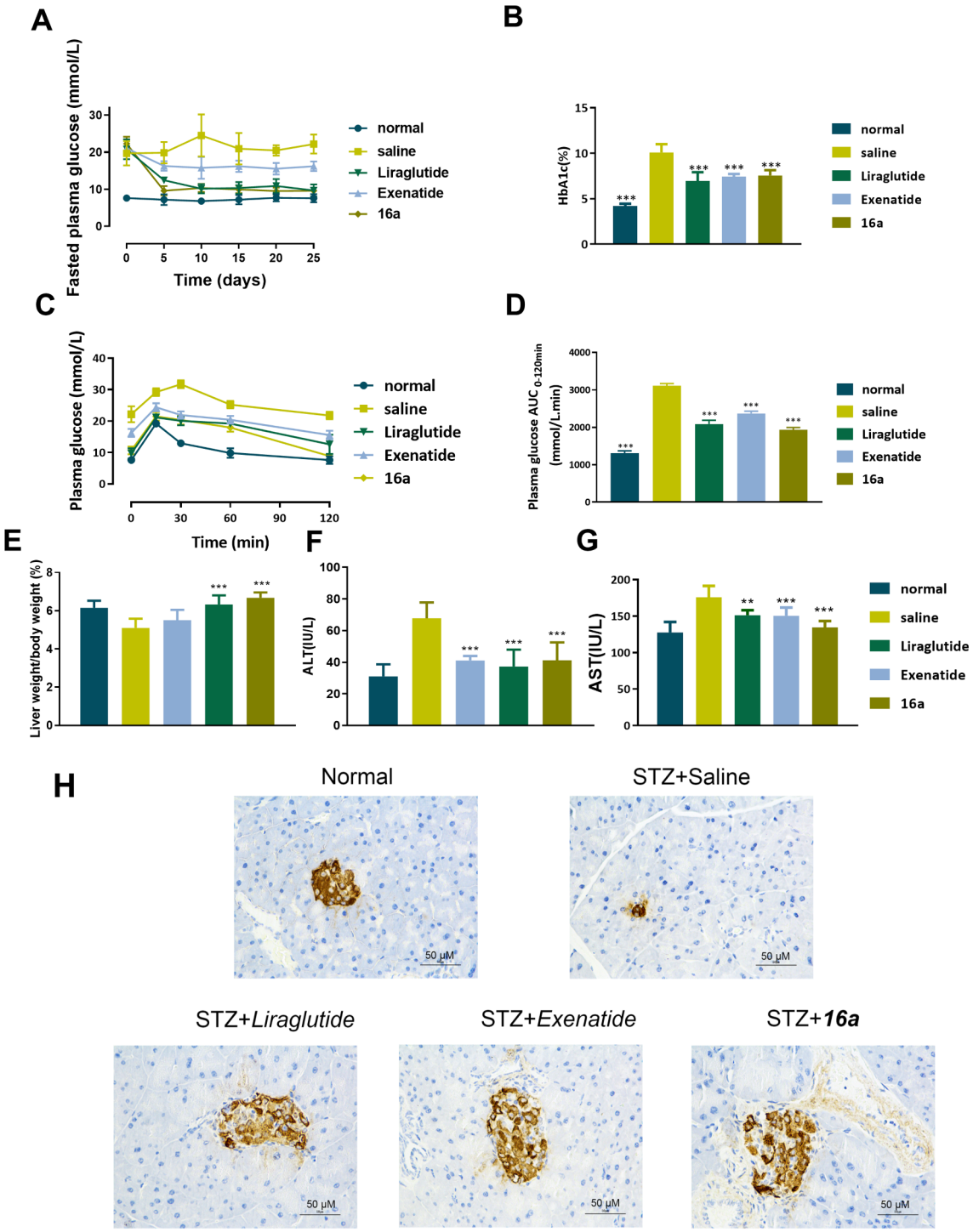

Fig. 8. The effects of chronically administered **16a**, Liraglutide and Exenatide in diabetic mice. (A) Fasted plasma glucose measured every five days. (B) HbA1C measured at day 24. (C) IPGTT results in mice at day 24. (D) AUC of IPGTT test results in mice at day 24. (E) Regeneration ratio of liver corrected by body weight was analyzed at the end of the 4-week treatment of saline, Liraglutide, Exenatide or **16a**. Effects of **16a** on serum biomarkers examined at the end of the 4-week treatment. (H) Representative images of pancreatic insulin-positive β cells after treatment.

digested with EcoRI and NcoI respectively. The target gene sized 1.7 kb was amplified by PCR, and 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, cultured in LB solid medium containing Ampicillin and selected for positive clones of Ampicillin. Transfection could be identified by PCR, restriction endonuclease digestion and sequencing.

(b) *Preparation of retrovirus*. 24 h before transfection, Phoenix 293 cells were cultured in plate. Trypsin digests Phoenix 293 cells and seeded into 6-well plates in 2 × 10^5 cells/well, incubated to about 70–80% when cultured to about 1 mL of serum-free antibiotic Opti-MEM medium. Plasmid DNA (2 μL) and 5 μL of Lipofectamin 2000

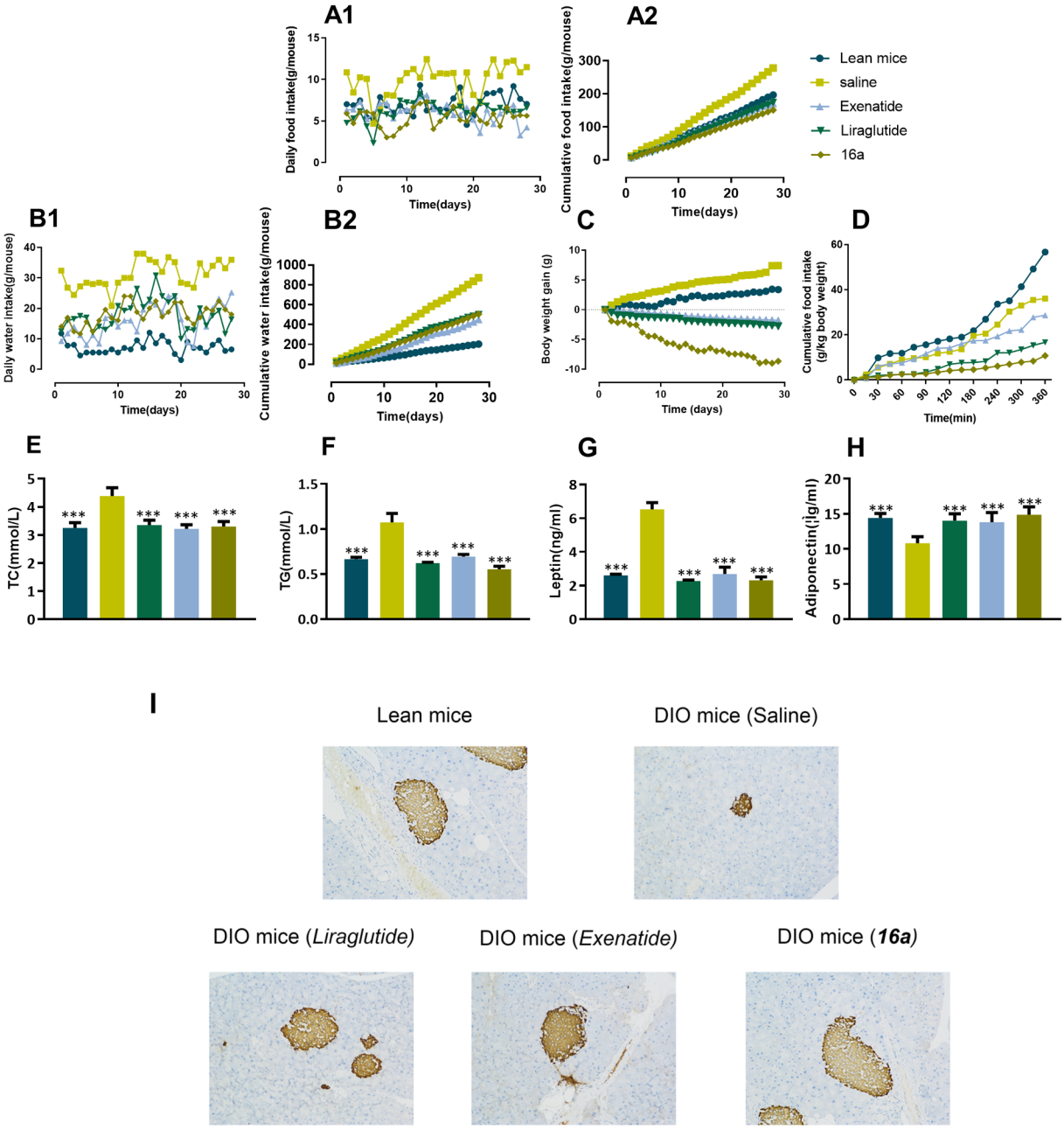

Fig. 9. Effects of **16a** in DIO mice. (A) Time course for chronic effect of **16a** administered for 4 weeks in DIO mice on cumulative food intake. (B) Time course for chronic effect of **16a** administered for 4 weeks in DIO mice on cumulative water intake. (C) Time course of **16a** effect on changes in body weight. (D) Time course of acute effect on food intake in fasted mice with intraperitoneal doses of **16a**. Effects of **16a** TC (E), TG (F), leptin (G), adipocytokines (H) examined at the end of the 4-week treatment. (H) Representative images of pancreatic insulin-positive β cells after treatment.

Reagent were diluted with 130 μL of serum-free antibiotic Opti-MEM medium, respectively, standing for 5 min before mixing. The mixed solution was added to a 6-well cell culture plate. The complete medium was replaced by the orifice place after 4–6 h 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) *Establishment of stable expression of cell lines*. Normal HEK293 cells in the six-well plate, 2×10^5 cells each hole, cultured 24 h, was added with 2 mL of each virus supernatant and polybrene (final concentration of 6 μg/mL). The cells were cultured in the incubator (32 °C) after 30 min of centrifugation with 1000 rpm at room temperature. Five hours later, the medium was replaced to normal, which was cultured at 37 °C for48 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. Two hours before the test started, cells were plated in 96-well half area with 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 was 0.1%. The resulting solution was added to cells which was incubated for 20 min. It was 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).

4.2.2. Plasma stability test

Plasma were obtained from adult male Sprague-Dawley (SD) rats and compounds were then incubated with plasma over 96 h. Samples from each time points underwent solid-phase extraction and the resulting extract was analyzed by LC-MS/MS to assess the profile of plasma degradation. In the in vitro stability test, the initial concentration in rat plasma of lipid chain modified conjugates **11a-16a**, and the positive controls (Exenatide and liraglutide) were 1000 ng/mL at 37 °C. At 0, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h time points, 100 μL of mixture was aliquoted, extracted on an Oasis HLB 96-well plate (Waters, Milford, MA, USA) and analyzed by LC-MS/MS. 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.

4.2.3. Glucose tolerance test in normal Kunming mice

Male Kunming mice (8 weeks, male, 6–8 per group) were acclimatized to laboratory conditions for at least one week. For the determination of glucose tolerance, the mice were subjected to 12 h fasting. At 0.5 h prior to the oral glucose loading by intubation (18 mmol/kg glucose in 0.9% w/v saline), mice were administered with **OXM** or **OXM** analogues (25 nmol/kg in 0.9% w/v saline) intraperitoneally. Tail blood glucose levels were measured by using the tail cut method and blood glucose strips (San Nuo Changsha, Changsha, China) at-30, 0, 15, 30, 60, 90 and 120 min.

4.2.4. Acute feeding study and body weight change in normal ICR mice

For acute feeding study, mice (9 weeks, male, 6–8 per group) were fasted for 8 h preceding the injection. All peptides were dissolved in 0.9% saline. After intraperitoneal injection of **OXM** analogues (25 nmol/kg) and 0.9% saline as control at 9 o'clock AM, animals were returned to their home cages containing a preweighed amount of food that was reweighed at 0.5, 1, 2, 4, 8, and 24 h after injection.

For body weight change study, mice (10 weeks, male, 6–8 per group) were assigned to treatment groups based on their body weight. The mice were fasted for 12 h preceding the injection. All peptides were dissolved in 0.9% saline. **OXM** analogues (25 nmol/kg) and 0.9% saline as control were intraperitoneally injected once daily at 9 o'clock AM for two weeks. Body weight and food consumption were measured every day at the same time.

4.2.5. Energy balance physiology measurements

C57Bl/6 mice (8 weeks) were singly housed and fed on either standard chow (D12491: 20% kcal from fat; Research Diets) or high fat diet (D12492: 60% kcal from fat; Research Diets) in a 12 h light/12 h dark cycle for 14 weeks with free access to food and water. Mice (22 weeks) were randomized and distributed to test groups according to body weight and body composition. Energy intake and expenditure, as well as home-cage activity, were assessed by using a combined indirect calorimetry system (TSE Systems) in DIO mice (22 weeks, male, 6 per group, body weight ~ 40 g). O_2 consumption and CO_2 production were measured every 39 min for a total of 120 h (including 24 h of adaptation) to determine the respiratory quotient and energy expenditure. Food intake was determined continuously for 120 h at the same time. Home-cage locomotor activity was determined using a multi-dimensional infrared light beam system with beams scanning the bottom and top levels of the cage and activity being expressed as beam breaks

4.2.6. Chronic treatment on DIO mice by 7 and 10

DIO mice (23 weeks, male, 6 per group, body weight ~40 g, diet D12492) were administered intraperitoneally with **7** (25 nmol/kg), **10** (25 nmol/kg), control (**OXM** (25 nmol/kg), **Exenatide** (25 nmol/kg) or 0.9% saline as single injection every other day at 9 o'clock in the morning for 30 days. Body weight, food consumption and water intake were measured every day.

4.2.7. Hypoglycemic efficacies test

Hypoglycemic efficacies of compounds were evaluated on the STZ-induced diabetic mice using a modified previously described method [30]. Kunming mice received a single intraperitoneal injection of compound at 25 nmol/kg body weight intraperitoneally with free access to food and water. At 0, 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 30, 36, 48, 60 h, the second drop of blood was drawn from a tail vein to determine the blood glucose levels by blood glucose monitor. Moreover, the hypoglycemic durations were also calculated with the blood glucose level $<$ 8.35 nmol/L (150 mg/dL).

4.2.8. Glucoregulatory and insulin secretion assay

The glucoregulatory and insulin secretion assays were carried out in accordance with a previous method. Briefly, overnight fasted (12 h) SD rats (n = 6/group, 200–250 g) were orally administrated with **16a**, **Exenatide** and **Liraglutide** (25 nmol/kg) with saline used as negative control. Male SD rats were administered saline, **Exenatide**, **Liraglutide** or **16a** half hour prior to loading of oral glucose (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 level 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).

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

Male Kunming 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 of 11.1 mM or higher were assigned to groups with matched body weight and were injected intraperitoneally with 25 nmol/kg **Exenatide**, **Liraglutide**, or **16a** 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). 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.

4.2.10. 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 of **Exenatide**, **Liraglutide** or **16a**, 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 via arteria carotis under anaesthesia by sodium pentobarbital and sera separated subsequently for further analyses.

4.2.11. Data analysis and statistical assessment

Data were analyzed using Prism version 5 software (GraphPad, San Diego, CA, USA). General effects were tested using a 1-way ANOVA

with Tukey's multiple-comparison post hoc test. Data throughout are stated as mean ± SD. $p < 0.05$ was considered as significant.

Declaration of Competing Interest

The authors declare no competing financial interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103538.

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