Life Sciences 260 (2020) 118339

Contents lists available at ScienceDirect

Life Sciences

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

Rational design of a GLP-1/GIP/Gcg receptor triagonist to correct hyperglycemia, obesity and diabetic nephropathy in rodent animals

Jie Cui[a,b], Anquan Shang[c], Weiwei Wang[d], Wei Chen[a,*]

[a] Center for Laboratory Medicine, the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, PR China
[b] Center for Laboratory Medicine, General Hospital of Ningxia Medical University, Yinchuan 750004, PR China
[c] Department of Laboratory Medicine, Tongji Hospital of Tongji University, Shanghai 200065, PR China
[d] Department of Pathology, Yancheng Sixth People's Hospital of Southeast University Group, Yancheng 224001, PR China

ARTICLE INFO

Keywords:
GLP-1
GIP
Glucagon
Triagonist
Diabetes
Obesity
Diabetic nephropathy

ABSTRACT

Aims: To design and screen a potent GLP-1/GIP/Gcg receptors triagonist with therapeutic potential in rodent animals with diabetes and obesity.

Main methods: First, we obtained a 12-mer dual GIP/Gcg receptor agonist from a large combinatorial peptide library *via* high-throughput screening technique and then fused to the Exendin (9–39) to generate a potent GLP-1/GIP/Gcg triagonist. Further site fatty chain modification was performed to improve the druggability *via* enhancing *in vivo* stability and cyclic half-life. *In vitro* signaling and functional assays in cell lines expressing each receptor and *in vivo* efficacy evaluation in rodent model animals with hyperglycemia and obesity were all carefully performed.

Key findings: We screened and obtained a potent GLP-1/GIP/Gcg triagonist, termed XFL0, which promotes *in vitro* GLP-1, GIP, Gcg receptor activation comparable to native GLP-1, GIP and glucagon, respectively. Site-specific fatty acid modification significantly enhanced plasma stability of XFL0 and exhibited no obvious impact on receptor activation. The selected XFL0 conjugates termed XFL6, showed glucose-dependent insulin secretion and improved glucose tolerance by acting on all GLP-1, GIP and Gcg receptors in gene-deficient mice of which the effects were all significantly greater than any single receptor agonist. After chronic treatment in rodent animals with diabetes and obesity, XFL6 potently decreased body weight and food intake, ameliorated the hyperglycemia and hemoglobin A1c levels as well as the lipid metabolism and diabetic nephropathy related disorders.

Significance: XFL6, as a novel GLP-1/GIP/Gcg receptor triagonist, held potential to deliver outstanding improvement in correcting hyperglycemia, obesity and diabetic nephropathy.

1. Introduction

Type 2 diabetes mellitus (T2DM) is an endocrine and metabolic disease characterized by hyperglycemia and continuously deteriorating diabetic complications [1,2]. The etiology of type 2 diabetes mainly includes tissue resistance to insulin, decreased insulin sensitivity and relative deficiency of insulin secretion by pancreatic β-cells [3,4]. Not only that, obesity is also one of the important risk factors affecting the occurrence and development of diabetes and its complications [5,6]. Therefore, anti-diabetic therapy accompanied by weight loss has become an effective strategy for the treatment of T2DM [6].

Glucagon-like peptide-1 (GLP-1), as a kind of incretins secreted from intestinal L-cell, could regulate blood glucose levels in T2DM patients by increasing insulin secretion and inhibiting glucagon release [3,7,8]. Therefore, GLP-1 receptor agonists (GLP-1RAs) are well-established therapy in the treatment of type 2 diabetes [9]. The marketed GLP-1RA, including Liraglutide and Semaglutide, have shown good hypoglycemic effect in clinical practice without the risk of hypoglycemia [10,11]. However, long-term use of GLP-1RA often leads to worse tolerability and insufficient efficacy in T2DM patients with obesity or other broader demand [12,13]. In general, such single-target drugs failed to meet expectations, especially for body weight control and treatment of diabetes-related complications [13].

Glucose-dependent insulinotropic polypeptide (GIP) and glucagon (Gcg), as two important growth factors and play important roles in energy metabolism [7,14]. GIP, as a polypeptide hormone secreted by intestinal K cells in response to the food intake, can promote the insulin secretion to maintain glucose homeostasis and promote the

* Corresponding author.
E-mail address: chenwei6311@163.com (W. Chen).

https://doi.org/10.1016/j.lfs.2020.118339
Received 1 August 2020; Received in revised form 16 August 2020; Accepted 20 August 2020
Available online 22 August 2020

decomposition and deposition of white adipose tissues [15,16]. Similarly, glucagon is secreted by islet α cells and could stimulate energy expenditure, lower lipid, inhibit gastric emptying and appetite *in vivo*, thereby reducing body weight [17]. Currently, a novel therapy based on peptide agonist combining the activity of GLP-1, GIP and glucagon holds capability of superior therapeutic application prospect [12]. Finan et al. developed a triple agonist (GLP1/GIP/glucagon) for the treatment of metabolic disorders, which effectively reduced body weight and blood glucose levels in diabetic mice to a greater extent than each monomeric agent [18]. Such strategy of multi-target treatment was proved to more effective than that of single-target therapy in treating T2DM and obesity.

Phage display technology is a high-throughput method which can be applied to screen drugs with high affinity and selectivity as well as to identify target-specific peptides and proteins against various disease-related targets [19]. As previously reported [20], we used the F linear 12-mer peptide library, which displayed as N-terminal coat fusion protein on the surface of filamentous phage M13, to screen the target peptide. In present study, we used this phage display peptide library to screen the dual agonist with the high affinity for both GIPR and GcgR. The selected dual receptor agonists were further fused to the exendin (9–39) to generated novel triagonists (Scheme 1). The three receptors activation of selected candidate was assessed in cell-based assay *in vitro*. Furthermore, the anti-diabetes and anti-obesity potential of the side chain modified triagonist were assessed through acute and chronic *in vivo* study in rodent animals.

2. Materials and methods

2.1. Materials

Ph.D.-12 phage library (New England Biolabs, USA) was applied for screening the dual GIPR/GcgR agonist sequence. The 12-mer and fused peptides were prepared by Chinapeptides Co. (Shanghai, China) by using traditional method of solid phase synthesis with the purify over 98%. HEK293 cells over-expressing the human GLP-1R, GIPR or GcgR were bought from Procell Life Science&Technology Co., Ltd. (Shanghai, China). Other common reagents were obtained from ThermoFisher Scientific or Sigma (USA).

2.2. Animals

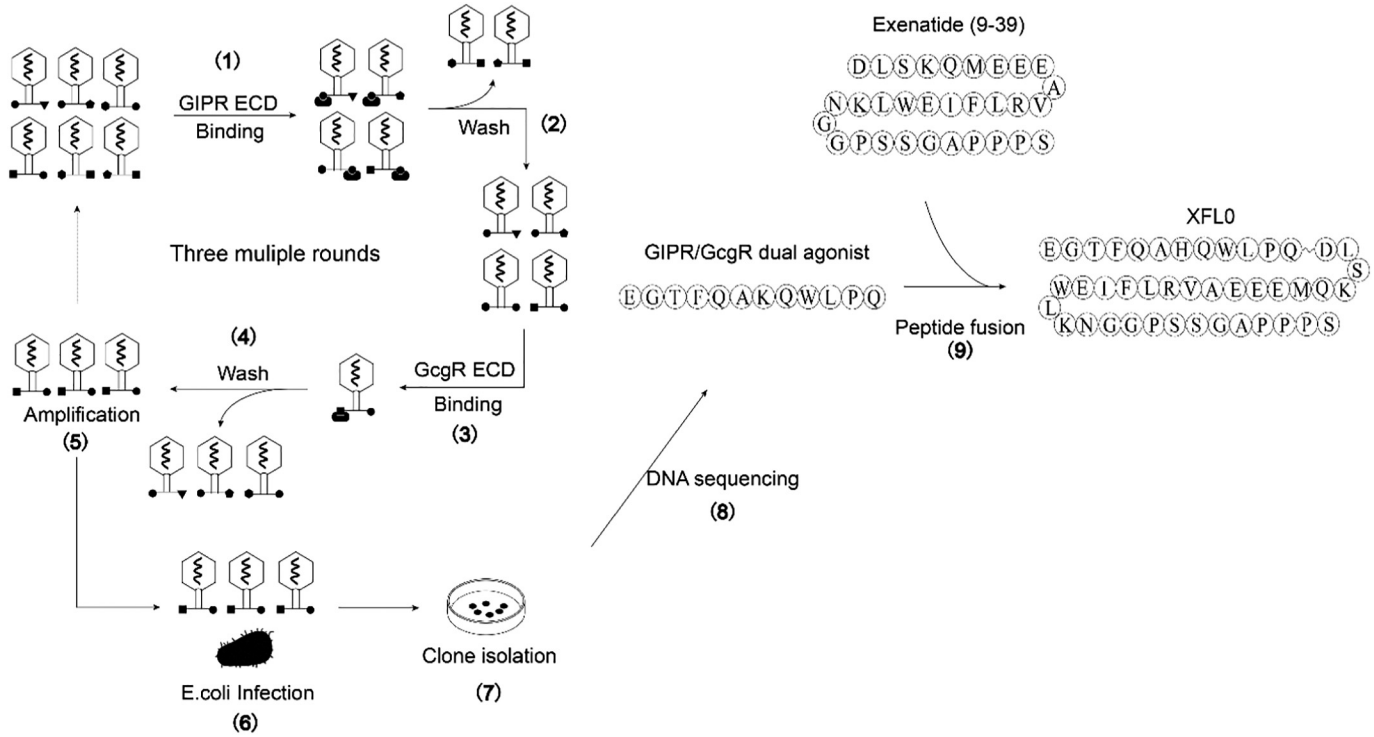
All animals used in this study were acquired from Biofavor Biotechnology Co., Ltd. (Wuhan, China) and housed in 6/cage under the constant temperature (25 ± 2 °C) and humidity (60 ± 5%) with a 12-h light/dark cycle environment, and fed with normal-chow diet and normal drinking water. The animal experiments were approved by Xi'an Jiaotong University and performed in consistent with the approved guidelines.

2.3. Phage display

The Ph.D.™-12 phage library was applied to screen the dual GIPR/GcgR agonists with high affinity to both extracellular domain (ECD) of GIPR and GcgR. In brief, the ECD of GIPR and GcgR (Shanghai Sangon Biotech Co., Ltd., China) were coated in 96-well plates, respectively, and further blocked with 1% bovine serum albumin (ThermoFisher Scientific, USA) for 1 h. The 12-mer peptide library was also pre-incubated in 1% bovine serum albumin for 15 min, and then sequentially added to ECD proteins-coated well for the three rounds screening. The selected phage was amplified with *E. coli* ER 2738 (Shanghai Qincheng Biotechnology Co., Ltd., China) and the total DNA was isolated by using the sequencing kit (Shanghai Sangon Biotech Co., Ltd., China) according to the manufacturer's protocol. Peptides were prepared by standard method of solid-phase synthesis and the crude peptides were purified by RP-HPLC. Molecular weights of purified peptides were further detected *via* LC-MS (Bruker, Germany).

2.4. Surface plasmon resonance (SPR) measurement

We used SPR method to determine the binding affinities of XFL peptides for the ECD of GLP-1R, GIPR and GcgR. Briefly, 30 μg/mL



Scheme 1. Schematic selection and design of novel triagonists for GLP-1R, GIPR and GcgR.

ligand solutions were immobilized onto the CM5 sensor surface to attain response value of 300 RU. The XFL peptides solutions (dissolved in HBS-EP buffer) were injected over CM5 sensor surface at 30 μL/min flow rate for 60 s of association and 60 s of dissociation. The detailed kinetics of the association and dissociation were calculated by BIA evaluation 3.0 software.

2.5. In vitro GLP-1R, GIPR and GcgR activation assay

The ability of the triagonist to activate the target receptor was detected by a cell-based luciferase reporter assay that indirectly measures cAMP production. HEK293 cells over-expressing the human GLP-1R, GIPR or GcgR were incubated with serial dilutions of triagonist in 96-well plate for 5 h at the condition of 37 °C, 5% CO_2 and 90% humidity. As a contrast, GLP-1, GIP and Gcg were also incubated with the HEK293 cells. At the end of the incubation, luciferase substrates were added to 96-well plate and the data were collected by using microplate reader (Beckman Coulter, USA).

2.6. Oral glucose tolerance test

In oral glucose tolerance test (OGTT), 6- to 8-week-old male C57BL/6J (wild-type, WT) mice and gene-deficient mice were divided based on average body weight and fasting blood glucose level (n = 6/group). After 12 h fasting, mice subcutaneously received single dose of XFL6 (10, 30 and 90 nmol/kg) half an hour before oral glucose administration (2 g/kg body weight). The blood samples were collected at 0, 15, 30, 45, 60 and 120 min after the glucose challenge, and the blood glucose levels (BGLs) were measured by using a glucometer (Johnson& Johnson, USA). Plasma insulin levels were measured detected *via* the insulin ELISA kit (ThermoFisher Scientific, USA). Multiple OGTTs were performed in db/db mice through the above method carrying out at time points of 0, 72 and 144 h after drug administration.

2.7. Pharmacokinetic test

In pharmacokinetic test, age-matched 6- to 8-week-old male Sprague Dawley (SD) rats were divided based on body weight and received a single subcutaneously administration of XFL6 (10, 30 and 90 nmol/kg). The blood samples were collected at 0, 4, 8, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h, and the plasma concentration of XFL6 were further detected by previously established LC-MS/MS method.

2.8. Chronic study

Chronic study, age-matched 6- to 8-week-old male DIO mice and db/db mice were divided based on body weight and free-eating blood glucose, respectively. All animals subcutaneously received XFL6 (10, 30 and 90 nmol/kg) and the positive control Semaglutide (90 nmol/kg) once every three days for consecutive 8 weeks. During the chronic treatments, food intakes and body weight were measured weekly. OGTT was conducted before and after the long-term treatment. The Hemoglobin A1c (HbA1c) value was detected by the commercial HbA1c ELISA kit (Merck, USA). At the end of the 8-week treatment, db/db mice were sacrificed and the blood samples were collected to detect the serum lipid profiles including triglyceride (TG), total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C) and high density lipoprotein- cholesterol (HDL-C) *via* commercial enzymatic assay kits (all from Merck, USA). The expression levels of peroxisome proliferator-activated receptor γ (PPARγ) mRNA, IL-6 and TNF-α, and the insulin and HOMA-IR values were detected using commercial ELISA kits (Millipore, USA). At the end of the treatment cycle, pancreatic tissues were isolated and fixed in 10% formalin for 8 h. Then the tissues were prepared and detected according to the previously reported standard histopathological procedures [21].

2.9. Data analysis

Statistical significance was assessed by one-way ANOVA using Graphpad Prism 6 software (GraphPad Prism, USA). Data are presented as means ± SD, and the *P* value lower than 0.05 was defined as statistically significant.

3. Results

3.1. Screening, design and characterization of novel long-lasting triple agonists

Here, the phage-displayed 12-mer peptides library was used to discovery the dual agonist that was able to bind to GIPR and GcgR with the high affinity. As showed in scheme 1, we sequentially probed the peptides library with ECD of GIPR and GcgR and finally identified a 12-mer peptide. Moreover, this screened peptide was further fused to Exendin (9–39) to generate a potent triagonist of GLP-1R/GIPR/GcgR (termed XFL0). In order to enhance the predictable short half-life of XFL0, three lysine sites at position at 7, 16 and 31 were modified with different lengths of fatty chain maleimide (C12, C14 and C16) to generate nine XFL0 conjugates, termed XFL1-XFL9 (Fig. 1). All nine XFL0 conjugates as well as XFL0 were synthesized by the strategy of standard fmoc solid phase synthesis method with the purities all > 98%. The actual molecular weights are consistent with its theoretical molecular weights (data not shown).

Subsequently, the binding affinities of XFL0–9 for ECD of GLP-1R, GIPR and GcgR were detected by SPR measurement and the results were shown in Table 1. Fatty chain modification on lys [7] and lys [31] led to decreased affinity for three kind of receptor. Nevertheless, C16 fatty chain modification on lys [16] of XFL0, exhibited the enhanced binding affinity for each corresponding receptor compared to GLP-1, GIP and GcgR, respectively. Hence, *in vitro* cell-based assay of GLP-1R, GIPR and GcgR activation by XFL peptides were further investigated in following study.

3.2. In vitro receptor activation activity of novel triagonists

The HEK293 cells stably expressing the human GLP-1R, GIPR or GcgR were used to confirm the activation of XFL peptides on target receptor. As shown in Table 2, XFL6 exhibited more balanced capabilities to activate the human GLP-1R (0.12 nM), GIPR (0.33 nM) and GcgR (1.01 nM) compared with other XFL peptides. Moreover, above results suggesting that the C16 fatty chain modification on lys [14] of XFL0 could retain most receptor activation potency of XFL0. Hence, XFL6 was selected as candidate molecule to perform the following *in vivo* study.

3.3. Glucose tolerance and insulin secretion test in gene-deficient mice

To investigate biological activities of different moiety of XFL6, we subsequently examined the effect of XFL6 treatment on glucose tolerance and the insulin secretion in GLP-1R$^{-/-}$, GIPR$^{-/-}$ and GcgR$^{-/-}$ mice, respectively. As shown in Fig. 2, XFL6 improved the glucose tolerance and insulin secretion in wild-type mice, but not obvious in GLP-1R$^{-/-}$ mice, indicating that the activation of GLP-1R is essential for rapid balance of blood glucose level. Actually, a single dose treatment of XFL6 even worsened the glucose tolerance in GLP-1R$^{-/-}$ mice suggesting that XFL6 increased blood glucose level without GLP-1 activity to enhance and neutralize the activity of Gcg or GIP moiety, respectively. Moreover, we also found that GcgR deficiency lead to the enhanced glucose lowering effect and insulin secretion of XFL6 treatment compared with of those of WT mice. Together, these results indicate that XFL6 can induce glucose-dependent insulin secretion *in vivo* through GLP-1R, and the activation of glucagon did not cause an obvious elevation in blood glucose.

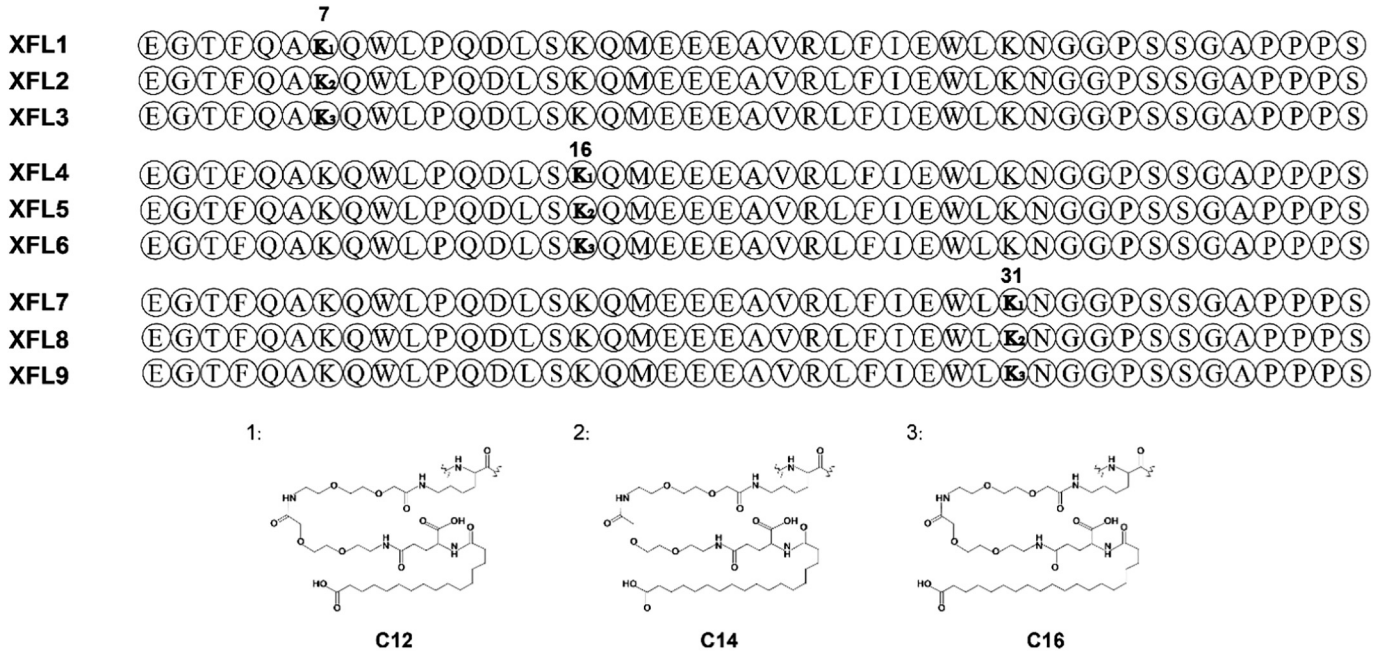


Fig. 1. Structure of fatty chain-modified nine novel triple agonists.

Table 1
The binding affinities of XFL peptides for ECD of GLP-1R, GIPR and GcgR.

Peptides	K_D (M)		
	GLP-1R ECD	GIPR ECD	GcgR ECD
GLP-1	2.52×10^{-7}	4.89×10^{3}	8.42×10^{3}
GIP	6.42×10^{2}	3.46×10^{-8}	1.53×10^{2}
Gcg	5.03×10^{-3}	3.74×10^{3}	2.08×10^{-7}
XFL0	2.43×10^{-7}	5.63×10^{-7}	1.35×10^{-6}
XFL1	7.68×10^{-7}	5.24×10^{-7}	9.66×10^{-7}
XFL2	5.34×10^{-6}	6.33×10^{-7}	7.14×10^{-6}
XFL3	1.35×10^{-6}	1.75×10^{-7}	2.46×10^{-6}
XFL4	3.59×10^{-7}	8.78×10^{-8}	7.88×10^{-6}
XFL5	6.34×10^{-7}	1.37×10^{-7}	7.02×10^{-7}
XFL6	3.60×10^{-7}	8.12×10^{-8}	1.74×10^{-7}
XFL7	5.64×10^{-7}	5.23×10^{-7}	6.11×10^{-6}
XFL8	5.36×10^{-7}	5.34×10^{-7}	6.78×10^{-6}
XFL9	8.63×10^{-6}	4.05×10^{-7}	3.98×10^{-7}

Table 2
In vitro receptor activation profiles of XFL peptides for GLP-1R, GIPR and GcgR.

Peptides	EC_{50} (nM)		
	GLP-1R	GIPR	GcgR
GLP-1	0.11	1163.78	2169.35
GIP	1429.10	1.87	369.57
Gcg	10.91	1291.33	0.58
XFL0	0.16	0.46	0.91
XFL1	0.37	0.73	2.79
XFL2	0.13	2.43	2.67
XFL3	0.26	0.71	0.99
XFL4	0.19	1.47	1.65
XFL5	1.42	0.53	2.34
XFL6	0.12	0.33	1.01
XFL7	0.32	1.99	0.68
XFL8	0.35	1.80	1.52
XFL9	0.49	3.75	1.37

3.4. Multiple oral glucose tolerance tests in db/db mice

To further investigate the *in vivo* hyperglycemic duration of XFL6, we performed the multiple OGTTs in db/db mice using Semaglutide as the positive control. After first oral glucose administration, blood glucose levels of XFL6 and Semaglutide treated group exhibited a significant decrease within ~1 h compared to the saline treated ones. In addition, the $AUC_{0\text{-}2h,\ 72\text{-}74h\ and144\text{-}146h}$ values of XFL6 treatment all revealed that XFL6 exerts its hypoglycemic effect in a dose-dependent manner within a range of 10–90 nmol/kg. Interestingly, the hypoglycemic effect of XFL6 at 90 nmol/kg was slightly better than that of Semaglutide at the same dose in first OGTT, and similar trends were also shown in the second and third OGTT, suggesting that XFL6 held similar hyperglycemic intensity and duration as Semaglutide, one of the best GLP-1RA-based hypoglycemic drug (Fig. 3).

3.5. Pharmacokinetic test in SD rats

The serum concentration-time curves of XFL6 at three doses (10, 30 and 90 nmol/kg) were shown in Fig. 4. The drug concentration of XFL6 at all three doses rapidly reached peak 8 h after single subcutaneous injection and then slowly decrease to the baseline within 96 h for 10 nmol/kg, 144 h for 30 nmol/kg, and 90 nmol/kg for 168 h, respectively. The detailed pharmacokinetic parameters were summarized in Table 3. After single subcutaneous administration of 90 nmol/kg XFL6, the $AUC_{0\text{-}t}$ was calculated to be 10.75 ± 0.90 ng/mL.h × 10 [4], which obviously higher than that of Semaglutide at same dose (6.94 ± 0.51 ng/mL.h × 10 [4]). Similarly, XFL6 also exhibited a significant longer $t_{1/2}$ than Semaglutide (75.81 ± 3.58 h *vs.* 56.74 ± 3.06 h, $P < 0.01$). All these results clearly indicated that XFL6 can maintain higher serum concentration than Semaglutide at same time and effective drug concentrations for longer duration at the same dose of 90 nmol/kg.

3.6. Chronic study in DIO mice

Based on the encouraging *in vitro* and acute *in vivo* anti-diabetic efficiencies, 8-week chronic treatment of XFL6 at two doses of 30 and 90 nmol/kg were performed in DIO mice to evaluate chronic anti-

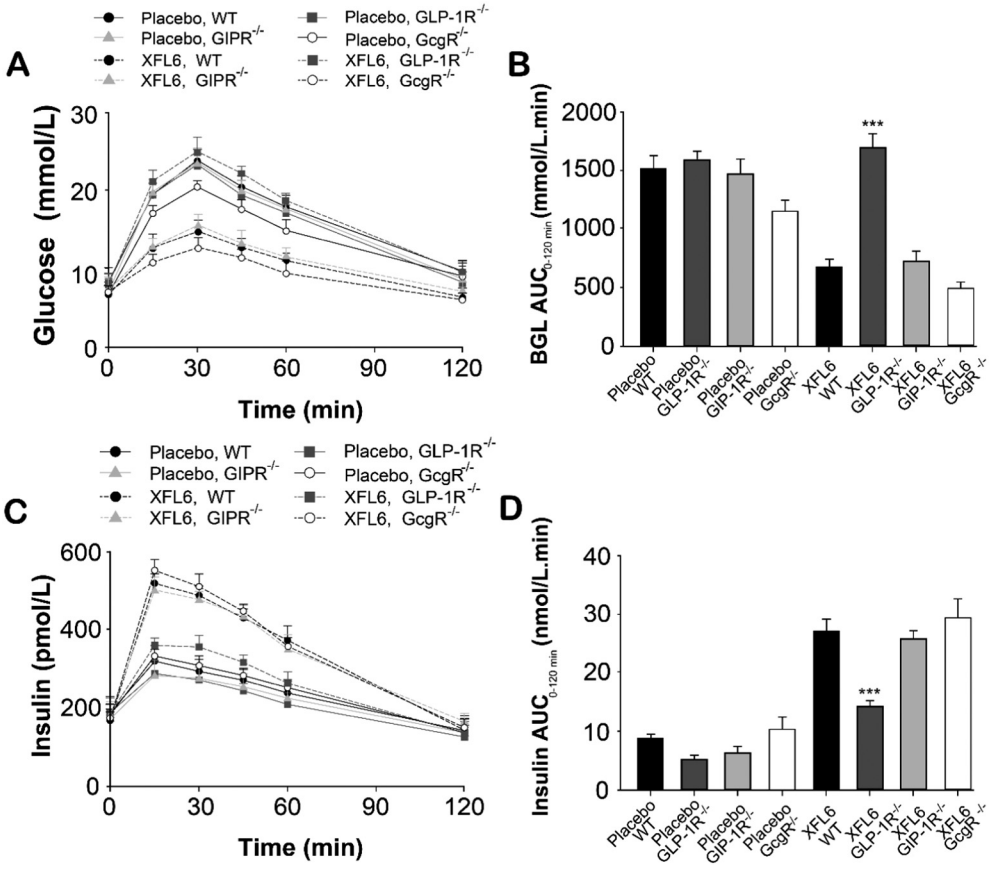


Fig. 2. Oral glucose tolerance test of XFL6 in GLP-1R$^{-/-}$, GIPR$^{-/-}$ and GcgR$^{-/-}$ mice. (A) Blood glucose levels, (B) area under the curve of blood glucose, (C) plasma insulin level and (D) area under the curve of plasma insulin during the oral glucose tolerance test. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* XFL6 treated WT mice. All values are presented as mean ± SD (n = 6).

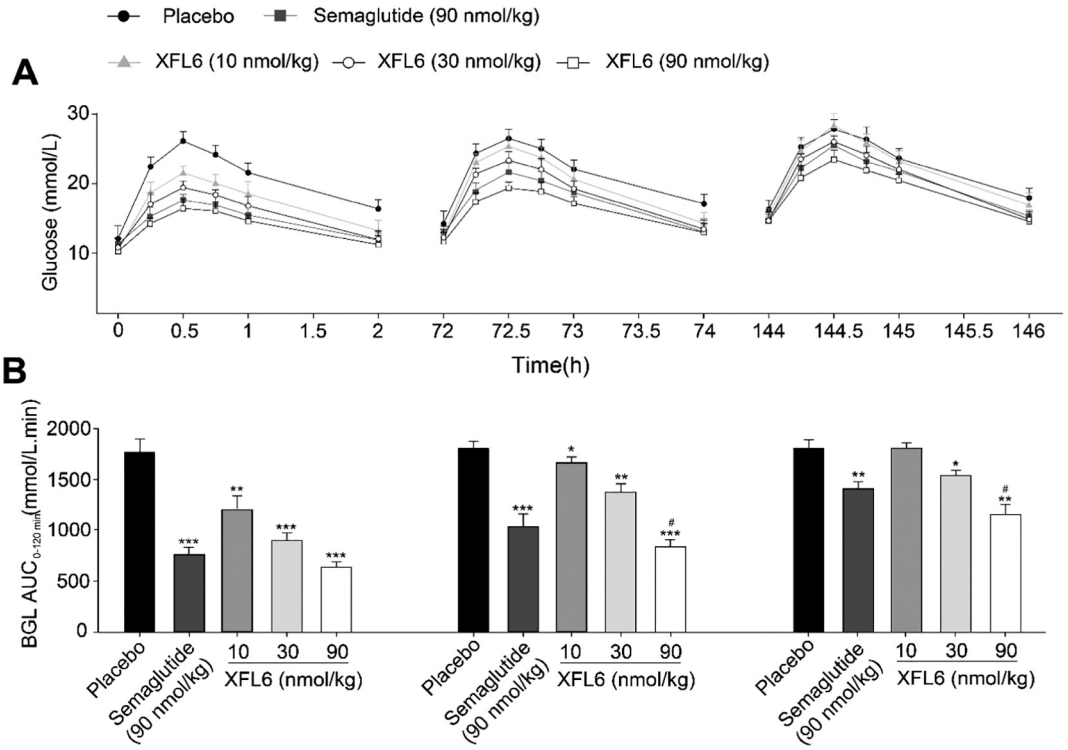


Fig. 3. Multiple oral glucose tolerance tests in db/db mice. (A) Blood glucose levels and (B) area under the curve of blood glucose during the multiple oral glucose tolerance tests. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* saline treated group; $^{\#}P < 0.05$ *vs.* Semaglutide treated group. All values are presented as mean ± SD (n = 6).

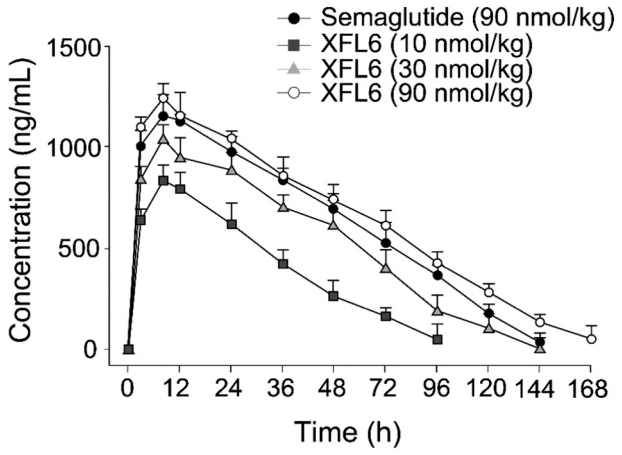


Fig. 4. Pharmacokinetic test of XFL6 at three doses in SD rats. All values are presented as mean ± SD (n = 6).

obesity effects using Semaglutide (90 nmol/kg) and saline as positive and negative control, respectively. As shown in Fig. 5A, those DIO mice received two doses of XFL6 both exhibited more significant decrease in food intake compared with those of saline or Semaglutide treated ones. Meanwhile, body weights were also reduced in the both two XFL6 treated groups at the end of chronic treatment period compared with saline treated group, and also significantly lower than that of Semaglutide treated ones at same dose ($P < 0.05$) (Fig. 5B). Moreover, as the results shown in Fig. 5C, OGTT was performed before and after the 8-week treatment. Substantial decreasing of OGTT $AUC_{0–120\ min}$ values were observed in both two XFL6 treated mice, whereas not notably changes in saline treated ones. In addition, treatment of two doses of XFL6 at 30 and 90 nmol/kg reduced the %HbA1c, representing the 3-month average of plasma glucose concentration, for 0.79% and 1.17%, respectively, which were both significantly better than that of saline-treated ones (Fig. 5D). At the end of chronic treatment, DIO mice were sacrificed and the blood samples were collected to detect the serum lipid profiles. As showed in Table 4, the serum levels of TC, TG and LDL-C were all obviously decreased in XFL16 treated groups at both two doses ($P < 0.05$), accompanied by significantly increased serum level of HDL-C. Interestingly, XFL6 exhibited a obviously better therapeutic potential in improving lipid metabolism of DIO mice compared with Semaglutide at same dose (90 nmol/kg).

The expression level of PPARγ, as an important factor which regulates bioactivities of insulin and leptin in white adipose tissue (WAT). The expression of PPARγ mRNA in XFL6 treated groups were both obviously increased compared with saline treated DIO mice as well as Semaglutide group (Fig. 6A). According to the previously reported results, the expansion of WAT was correlated with the formation and occurrence of insulin resistance inducing by the accumulation of pro-inflammatory macrophage in adipose tissues. We further assessed the effect of chronic treatment of XFL6 on the levels of TNF-α and IL-6 in DIO mice. As the results presented in Fig. 6B–C and Table 5, 8-week treatment of XFL at both two doses obviously down-regulated the levels of IL-6 and TNF-α as well as the fasting blood insulin levels and HOMA-IR values compared with those of the saline-treated group.

3.7. Chronic study in db/db mice

We also explored the anti-diabetic effects of XFL6 at two doses (30 and 90 nmol/kg) in db/db mice for consecutive 8 weeks also using Semaglutide and saline as positive and negative control, respectively. As shown in Fig. 7A and B, the food intake and body weight of saline treated db/db mice maintained a continuous increase trend. In contrast, XFL6 could decrease the food intake and body weight gain in db/db mice which were more significant than those of Semaglutide at the same dose ($P < 0.05$). Furthermore, the OGTT $AUC_{0–120min}$ values were reduced for 49.6% and 30.2% in XFL6- and Semaglutide-treated db/db mice at the dose of 90 nmol/kg, respectively. Moreover, long-term treatment of XFL6 at the doses of 30 and 90 nmol/kg exhibited decrease HbA1c% by 1.01% and 1.28%, respectively, compared with that before treatment, which is significantly higher than Semaglutide treated group (1.14%). Above results demonstrated that XFL6, as a triple agonist, possesses a higher therapeutic potential than single-receptor agonist for treating diabetes. Furthermore, the histological analysis of pancreas by using H&E staining method indicated that chronic treatment of XFL6 in db/db mice preserved proper islet number and area which were significantly better than those of the saline treated db/db mice (Fig. 7E–G). In addition, we further investigated the effects of long-term administration of XFL6 at different doses on renal function-related parameters in db/db mice. As the results showed in the Table 6, serum levels of the β2-MG, Crea and UA in negative control group were all obviously higher than those of normal mice as well as two XFL6 groups (30 and 90 nmol/kg, both $p < 0.01$). Moreover, BUN level in placebo-treated db/db mice was significantly lower than that of the mice in normal group or XFL6 treated groups ($p < 0.05$). Above results demonstrated that chronic treatment of XFL6 at both two doses could significantly improve the body weight control and glucose metabolism as well as diabetic nephropathy of the db/db mice.

4. Discussion

Diabetes mellitus is a slow progressive disease with increasing blood glucose leading to complications in various parts of the body and raising the risk of premature death [22]. The main cause of type 2 diabetes mellitus is insulin resistance which induces the uneffective usage of insulin and finally results in hyperglycemia as well as complications [23,24]. Therefore, improvements on insulin resistance and rational blood glucose control as well as efficient body weight control are critical to control the progression of type 2 diabetes [13,25].

GLP-1 receptor agonists are a class of drugs that can reduce body weight at the same time without causing hypoglycemia on the basis of effective control of blood glucose balance [3,6,26]. In addition, GLP-1 can also act on relevant receptors in the hypothalamus to produce satiety and loss of appetite [27]. Due to its unique advantages and mechanism of action, incretin-based agonists have become a hot spot for the study of diabetic therapeutics [28,29]. However, recent reports found that hypoglycemic activity and body weight loss are difficult to achieved if only GLP-1 receptor (GLP-1R) is activated, meanwhile, some other adverse reactions, including nausea and vomiting, may also be induced [30].

Similar to the GLP-1, glucagon and GIP are also important pancreatic hormones which were secreted in the islet alpha cells and small

Table 3
Pharmacokinetic parameters of XFL6 in SD rats. *$P < 0.05$, **$P < 0.01$ *vs.* Semaglutide treated group. All values are presented as mean ± SD (n = 6).

Parameters	Semaglutide (90 nmol/kg)	XFL6 (nmol/kg)		
		10	30	90
$AUC_{0\text{-}t}$ (ng/mL.h × 10^4)	8.34 ± 0.51	3.89 ± 0.23**	7.78 ± 0.84*	10.75 ± 0.90*
C_{max} (ng/mL)	1182.8 ± 78.3	810.5 ± 92.3**	1096.3 ± 132.2*	1265.9 ± 82.4*
$T_{1/2}$ (h)	64.74 ± 3.06	41.35 ± 1.42*	58.23 ± 2.17*	75.81 ± 3.58**

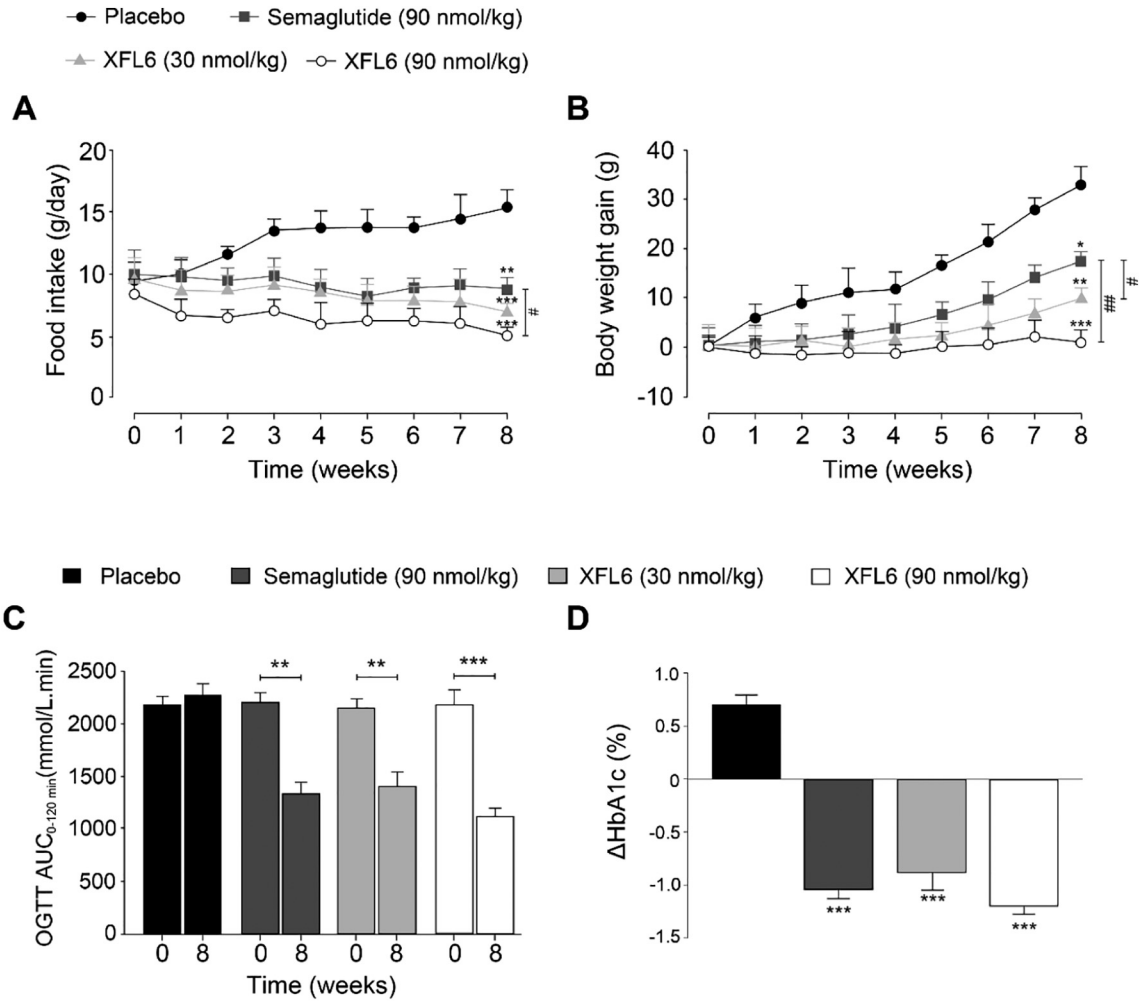


Fig. 5. Chronic study of XFL6 in DIO mice. The changes of (A) cumulative food intake, (B) body weight, (C) OGTT AUC value and (D) △HbA1c (%) values in DIO mice after 8-week XFL6 treatment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* saline treated group. All values are presented as mean ± SD (n = 6).

Table 4
The serum lipid profiles of DIO mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* saline treated group; $^{\#}P < 0.05$ *vs.* Semaglutide treated group. All values are presented as mean ± SD (n = 6).

Parameters	Normal	Saline	Semaglutide (90 nmol/kg)	XFL6 (nmol/kg)		
				10	30	90
TG (mmol/L)	0.78 ± 0.13	1.24 ± 0.44	0.93 ± 0.25**	1.18 ± 0.14	1.05 ± 0.35*	0.82 ± 0.18**
TC (mmol/L)	3.28 ± 0.25	5.47 ± 0.72	4.39 ± 0.36*	4.98 ± 0.56	4.47 ± 0.37*	3.63 ± 0.66**/#
LDL-C (mmol/L)	0.66 ± 0.11	1.77 ± 0.14	1.23 ± 0.16**	1.46 ± 0.12*	1.39 ± 0.14**	1.02 ± 0.09***/#
HDL-C (mmol/L)	3.34 ± 0.27	2.28 ± 0.23	2.67 ± 0.22*	2.39 ± 0.42	2.58 ± 0.35	2.76 ± 0.38*

intestinal mucosa K cells, respectively [31]. Glucagon effectively raises blood glucose level to be the emergency treatment of severe hypoglycaemia [32]. The main physiological effects of glucagon are to inhibit insulin secretion and stimulate hepatic gluconeogenesis to increase blood glucose levels, and also to affect the body's metabolic and thermogenic processes [33]. The catabolic effects of glucagon and increased body heat production are beneficial in obese patients, but treatment of obesity with GcgR agonists may lead to abnormally hyperglycemia [18]. Therefore, the key problem to be solved in the application of GcgR agonists in obesity treatment is to make full use of the decomposition promoting metabolism produced by GcgR activation and increase the body's thermogenic effect while weakening or eliminating its blood glucose increasing effect. Therefore, fusion of GLP-1R agonists with GcgR agonists or in combination are ideal choice.

GIP, as an incretin, is secreted in response to food intake and subsequently raised blood glucose [7]. Under normal physiological condition, postprandial blood level of GIP is almost 4-fold more than GLP-1 [31]. Moreover, GIP exerts most insulinotropic effects and some other additional function which is distinct from GLP-1 [7]. Furthermore, GIP is both insulinotropic and glucagonotropic in a glucose-dependent model to stimulate the secretion of glucagon under hyperglycemia [7]. Historically, clinical applications of GIP were severely limited due to the functions of GIP were blunted in patients with diabetes which possibly because of that high blood circulating glucose down-regulate the GIPR [34]. However, the resistance of GIP could be mostly improved by lowering the circulating glucose level which paves the application direction of GIP combine or fuse with the glucose-lowering agent, like GLP-1.

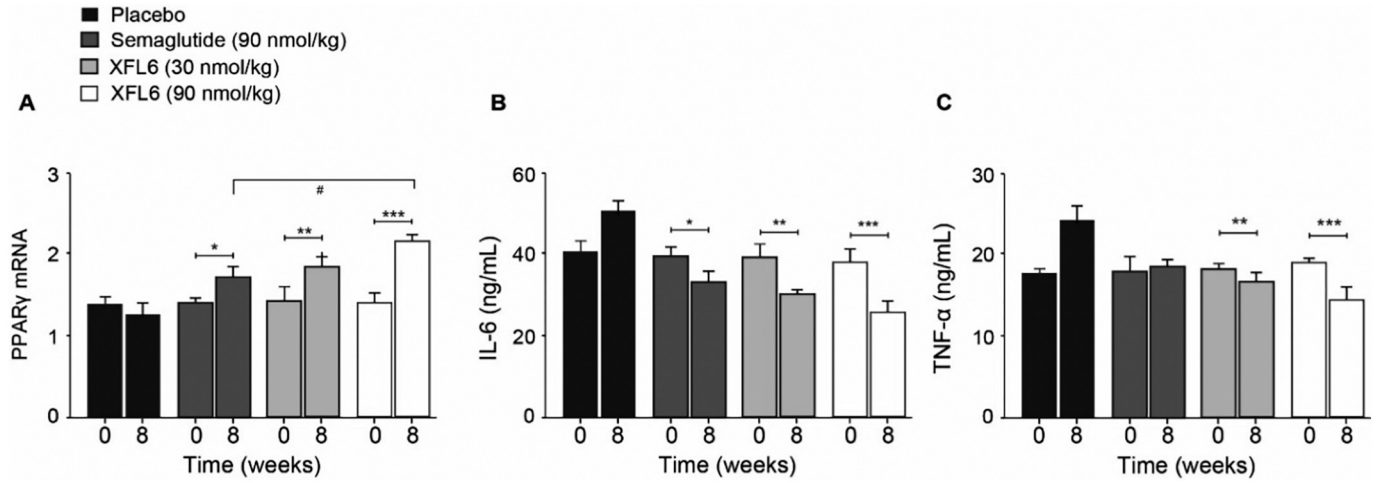


Fig. 6. Effects of chronic XFL6 treatment on adipose tissues in DIO mice. The expression level of (A) PPARγ mRNA, (B) IL-6, (C) TNF-α in male DIO mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* saline treated group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* Semaglutide treated group. All values are presented as mean ± SD (n = 6).

Herein, we attempt to determine whether newly screened and designed monomeric peptide with agonism at all three receptors for GLP-1, GIP and glucagon is effective in improving hyperglycemia, insulin resistance and dyslipidemia in rodent model animals. Firstly, we used the phage-displayed 12-mer peptide library to discovery a sequence targeting both GIPR and GcgR, as dual receptors agonists with the high affinity (Scheme 1). In order to obtain a potential triagonist, selected 12-mer sequence was fused to exendin (9–39), a GLP-1R antagonist, and generate a fusion peptide (named XFL0) with balanced activation on all three receptors. Considering that the predictable short *in vivo* $t_{1/2}$ of XFL0, different lengths of fatty chain maleimide (C12, C14 and C16) were further modified at the three lysine sites in XFL0 to generate nine derivatives, termed XFL1-XFL9 (Fig. 1). In addition, these conjugates were prepared by chemically synthesize according to the standard fmoc solid phase synthesis method with high purities (> 98%). Moreover, the binding affinities of these derivatives for three receptors were detected and results indicated that fatty chain modification on lys [7] and lys [31] decreased affinity for all three receptors, while, the C16 fatty chain on lys [16] enhanced the binding affinity (Table 1). According to our speculation, the increased HSA binding stability of XFL6 observed above might be attributed to the most suitable steric hindrance brought from the modification at the sixteenth Lysine acylation site. Further *in vitro* cell-based assay of GLP-1R, GIPR and GcgR activation by XFL peptides were investigated in HEK293 cells over-expressing the human GLP-1R, GIPR or GcgR. Balanced activation on three receptors is critical for a rationally designed triagonist due to their functional complementarity. As the data listed in Table 2, XFL6 exhibited more balanced potential to activate all three receptors compared with others. Combined with the SPR results, C16 fatty chain modification on lys [16] of XFL0 retain most receptor activation potency. Hence, XFL6 was selected as candidate molecule to perform the following *in vivo* study.

In order to clarify the biological function of different moieties, acute treatment of XFL6 was performed in in GLP-1R$^{-/-}$, GIPR$^{-/-}$ and GcgR$^{-/-}$ mice, respectively, to assess its effects on glucose tolerance and insulin secretion. As the results showed in the Fig. 2, XFL6 exhibited little improvement on the glucose tolerance and insulin secretion in GLP-1R$^{-/-}$ mice compared with that in wild type, indicating that the activation of GLP-1R is essential for rapid balance of blood glucose level. Moreover, deletion of GIPR did not exhibited obvious influence on glucose tolerance or insulin secretion, while the in GcgR$^{-/-}$ mice, the glucose tolerance and insulin secretion were both improved due to the activity of Gcg was blocked. In general, XFL6 induced glucose-dependent insulin secretion and improve the glucose tolerance mainly *via* activating GLP-1R which plays core role in realizing the multiple-function.

Further hyperglycemic duration test and multiple OGTTs were performed in db/db mice to assess the anti-diabetes potential of XFL6 using Semaglutide, one of the present best GLP-1RA, as the positive control. The BGLs of XFL6 treated mice exhibited a significant decrease within 1 h after first oral glucose administration. Interestingly, hypoglycemic effects of XFL6 at high dose of 90 nmol/kg was slightly better than Semaglutide at all three OGTTs. In addition, the $AUC_{0-2\ h,\ 72-74\ h\ and\ 144-146\ h}$ values of XFL6 groups exhibited in in a dose-dependent manner. Above results suggested that XFL6 held better hyperglycemic intensity and duration than Semaglutide, one of the best GLP-1RA-based hypoglycemic drug. In order to explain the pharmacodynamic data, the pharmacokinetic test of XFL6 at three doses of 10, 30 and 90 nmol/kg were performed. As serum concentration-time curves shown in Fig. 4, XFL6 exhibited a significant longer $t_{1/2}$ than Semaglutide (75.81 ± 3.58 h *vs.* 56.74 ± 3.06 h, $P < 0.01$). All these results clearly indicated that XFL6 can maintain higher serum concentration than Semaglutide at same time and effective drug concentrations for longer duration at the same dose of 90 nmol/kg.

Based on outstanding acute anti-diabetic efficacies, we further conducted the long-term of XFL6 at two doses of 30 and 90 nmol/kg in DIO mice and db/db mice to evaluate chronic anti-obesity and anti-

Table 5
Effects of XFL6 on insulin levels and HOMA-IR values of DIO mice.

Group	Insulin level (μU/ml)		HOMA-IR	
	0 week	8 weeks	0 week	8 weeks
Normal	24.2 ± 2.7	34.2 ± 5.2	15.2 ± 2.5	10.1 ± 1.9
Saline	86.1 ± 12.2	291.8 ± 43.1	56.3 ± 8.2	125.2 ± 14.1
Semaglutide (90 nmol/kg)	92.2 ± 10.1	97.2 ± 10.1***	64.2 ± 7.1	39.2 ± 9.2***
XFL6 (30 nmol/kg)	88.2 ± 9.2	82.2 ± 9.2***	57.3 ± 6.1	33.1 ± 8.7***
XFL6 (90 nmol/kg)	94.2 ± 8.1	54.2 ± 8.1***	61.6 ± 6.2	20.3 ± 4.6***

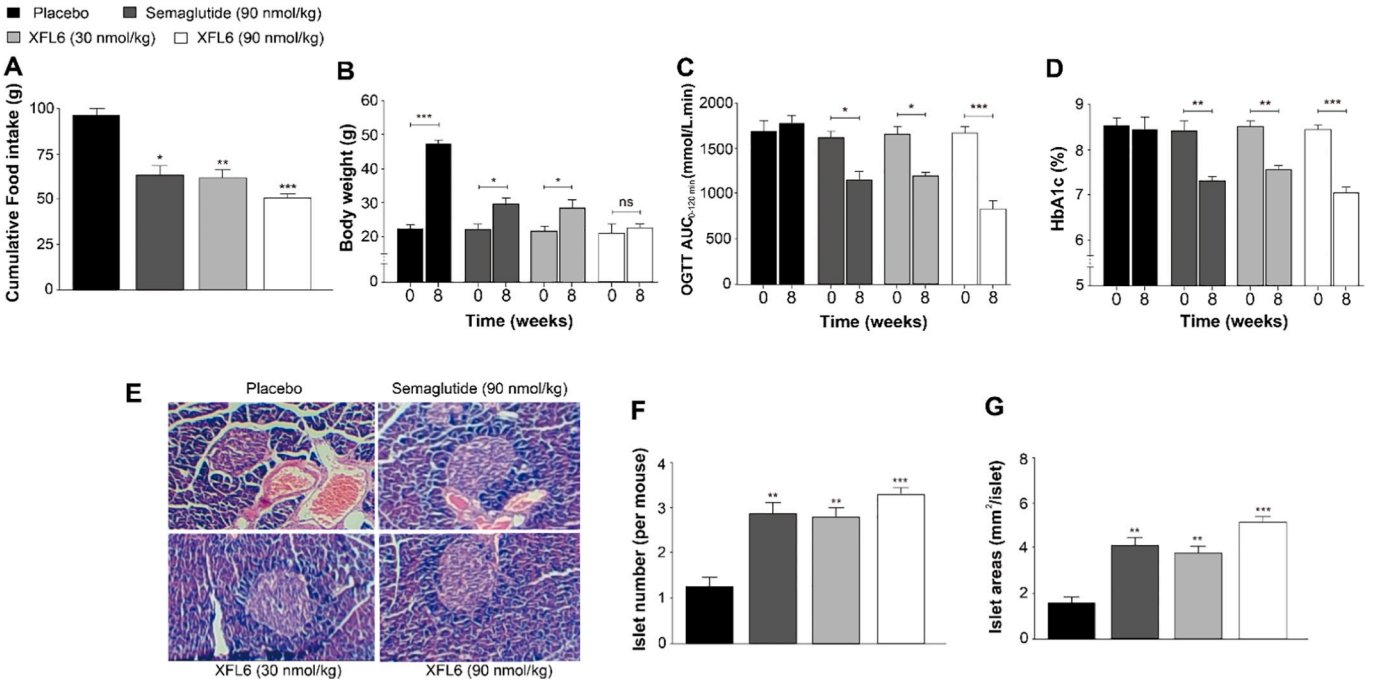


Fig. 7. Chronic study in db/db mice. The change trends of (A) food intake and (B) body weight gain, (C) OGTT AUC and (D) HbA1c (%), (E) Representative images and (F) number and (G) area of pancreatic islet in db/db mice after 8-week XFL6 treatment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* saline treated group; $^{\#}P < 0.05$ *vs.* Semaglutide treated group. All values are presented as mean ± SD (n = 6).

Table 6
Effects of XFL6 on the renal function-related parameters in db/db mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* Placebo group. Results were showed as means ± SD (n = 6 each group).

Characters	Normal mice	db/db mice			
		placebo	Semaglutide (90 nmol/kg)	XFL6 (30 nmol/kg)	XFL6 (90 nmol/kg)
Crea (mmol/L)	54.89 ± 11.52	96.77 ± 15.83	69.48 ± 12.14***	72.64 ± 8.21**	63.51 ± 10.09***
BUN (mmol/L)	9.93 ± 1.27	5.59 ± 1.85	7.51 ± 1.48**	7.03 ± 2.54**	9.71 ± 1.74***
UA (mmol/L)	67.52 ± 5.99	164.78 ± 13.11	94.74 ± 10.56**	114.26 ± 27.58**	86.47 ± 11.81***
β2 -MG (ng/mL)	1.40 ± 0.35	5.64 ± 1.75	2.17 ± 0.81***	2.64 ± 0.75**	1.52 ± 0.54***

diabetes effects. Chronic treatment of XFL6 at two doses both exhibited more significant decrease in food intake and body weight gain compared with those of saline or Semaglutide treated ones. Based on our finding, we speculate that XF6-induced weight loss is likely due to thermogenic properties of glucagon enhance the energy metabolism *in vivo*. Similar improvements were also showed in the glucose tolerance and %HbA1c. At the end of chronic treatment, the serum levels of TC, TG and LDL-C were all obviously decreased in XFL6 treated groups at both two doses ($P < 0.05$), accompanied by significantly increased serum level of HDL-C. Furthermore, 8-week treatment of XFL6 at both two doses obviously down-regulated the levels of IL-6 and TNF-α as well as the fasting blood insulin levels and HOMA-IR values compared with those of the saline-treated group indicating the effectively improved insulin resistance of DIO mice. The food intake, body weight and glucose tolerance as well as %HbA1c were all significantly improved after long-term treatment of XFL6 which were better than those of Semaglutide at same dose. Furthermore, H&E staining result indicated that chronic treatment of XFL6 significantly improved the pancreas morphology of db/db mice. Moreover, the results showed in Table 6 revealed that chronic XFL6 treatment significantly increased the renal function parameters of db/db mice, including the levels of creatinine, uric acid, and β2-microglobulin, which suggesting that the XFL6 could effectively improve renal function in db/db mice. Together, these results indicated the 8-week treatment of XFL6 obviously exhibited efficiencies on body weight and food intake reduction as well as glucose/lipid metabolism by improving the morphology and function of pancreas as well as insulin resistance in the DIO and db/db mice.

In brief, our data demonstrated that the sequence fusion and fatty acid chain modification brought newly designed triagonist, XFL6, balanced potencies for GLP-1R/GIPR/GcgR and prolonged anti-diabetes/obesity efficacies which may provide a novel therapy for treating diabetes and obesity.

CRediT authorship contribution statement

Wei Chen: Conceptualization, Supervision; Jie Cui: Conceptualization, Data curation, Data analysis, Writing-original draft, Writing-review & editing; Anquan Shang: Methodology, Validation; Weiwei Wang: Methodology, Writing-review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

References

[1] A. Tielmans, M. Laloi-Michelin, M. Coupaye, M. Virally, T. Meas, P.J. Guillausseau, Drug treatment of type 2 diabetes, Presse Med. 36 (2 Pt 2) (2007) 269.
[2] W.T. Friedewald, Effects of intensive glucose lowering in type 2 diabetes, 358 (24) (2008) 2545.
[3] D.K. Arulmozhi, B. Portha, GLP-1 based therapy for type 2 diabetes, Eur. J. Pharm.

Sci. 28 (1–2) (2006) 96–108.
[4] J.J. Holst, Treatment of Type 2 diabetes mellitus based on glucagon-like peptide-1, Expert Opin. Investig. Drugs 8 (9) (1999) 1409–1415.
[5] N. Ullah, K. Hafeez, S. Farooq, A. Batool, N. Aslam, M. Hussain, et al., Anti-diabetes and anti-obesity: a meta-analysis of different compounds, Asian Pacific J. Trop. Dis. 6 (9) (2016) 749–756.
[6] K.M. Heppner, P.-T. Diego, GLP-1 based therapeutics: simultaneously combating T2DM and obesity, Front. Neurosci. 9 (2015).
[7] L. Laurie, Baggio, J. Daniel, et al., Biology of incretins: GLP-1 and GIP, Gastroenterology. 132 (6) (2007) 2131–2157.
[8] Y. Fan, L. Dong, W. Ma, GLP-1 biology and GLP-1 based antidiabetic therapy, J. Chin. Pharm. Sci. 22 (1) (2013).
[9] A. Bo, GLP-1 receptor agonists in the treatment of Type 2 diabetes, Diabetes Management 3 (5) (2013) 401–413.
[10] T. Vilsbøll, M. Zdravkovic, T. Le-Thi, T. Krarup, O. Schmitz, J.P. Courrèges, et al., Liraglutide, a long-acting human glucagon-like peptide-1 analog, given as monotherapy significantly improves glycemic control and lowers body weight without risk of hypoglycemia in patients with type 2 diabetes, Diabetes Care 30 (6) (2007) 1608–1610 Epub 2007/03/21 https://doi.org/10.2337/dc06-2593 (PubMed PMID: 17372153).
[11] J. Lau, P. Bloch, L. Schäffer, I. Pettersson, T. Kruse, Discovery of the once-weekly glucagon-like peptide-1 (GLP-1) analogue semaglutide, J. Med. Chem. 58 (18) (2015) 7370.
[12] V.A. Gault, V.K. Bhat, N. Irwin, P.R. Flatt, A novel GLP-1/glucagon hybrid peptide with triple-acting agonist activity at GIP, GLP-1 and glucagon receptors and therapeutic potential in high-fat fed mice, J. Biol. Chem. 288 (49) (2013) 35581–35591.
[13] S.J. Henderson, A. Konkar, D.C. Hornigold, J.L. Trevaskis, R. Jackson, M. Fritsch Fredin, et al., Robust anti-obesity and metabolic effects of a dual GLP-1/glucagon receptor peptide agonist in rodents and non-human primates, Diabetes Obes. Metab. 18 (12) (2016) 1176–1190 Epub 2016/07/06 https://doi.org/10.1111/dom.12735 (PubMed PMID: 27377054; PubMed Central PMCID: PMCPMC5129521).
[14] V. Patel, A. Joharapurkar, S. Kshirsagar, B. Sutariya, M. Patel, H. Patel, et al., Central administration of coagonist of GLP-1 and glucagon receptors improves dyslipidemia, Biomed. Pharmacother. 98 (2018) 364–371.
[15] M.L. Hartman, A.J. Sanyal, R. Loomba, J.M. Wilson, A. Haupt, Effects of novel dual GIP and GLP-1 receptor agonist tirzepatide on biomarkers of nonalcoholic steatohepatitis in patients with type 2 diabetes, Diabetes Care 43 (6) (2020) dc191892.
[16] V.K. Bhat, B.D. Kerr, P.R. Flatt, V.A. Gault, A novel GIP-oxyntomodulin hybrid peptide acting through GIP, glucagon and GLP-1 receptors exhibits weight reducing and anti-diabetic properties, Biochem. Pharmacol. 85 (11) (2013) 1655–1662.
[17] A. Evers, T. Haack, M. Lorenz, M. Bossart, R. Elvert, B. Henkel, et al., Design of novel exendin-based dual glucagon like peptide 1 (GLP-1)/glucagon receptor agonists, J. Med. Chem. 60 (10) (2017) 4293–4303.
[18] B. Finan, B. Yang, N. Ottaway, D. Smiley, T. Ma, C. Clemmensen, et al., A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents, Nat. Med. 21 (1) (2014) 27–36, https://doi.org/10.1038/nm.3761.
[19] H.M.E. Azzazy, W.E. Highsmith, Phage display technology: clinical applications and recent innovations, Clin. Biochem. 35 (6) (2002) 425–445.
[20] X. Zhong, Z. Chen, Q. Chen, W. Zhao, Z. Chen, Novel site-specific fatty chain-modified GLP-1 receptor agonist with potent antidiabetic effects, Molecules 24 (4) (2019) 779–789.
[21] H. Zhang, E. Sturchler, J. Zhu, A. Nieto, P.A. Cistrone, J. Xie, et al., Autocrine selection of a GLP-1R G-protein biased agonist with potent antidiabetic effects, Nat. Commun. 6 (2015) 8918 Epub 2015/12/02 https://doi.org/10.1038/ncomms9918 (PubMed PMID: 26621478; PubMed Central PMCID: PMCPMC4686834).
[22] Association AD, Diagnosis and classification of diabetes mellitus, Diabetes Care 33 (1) (2010) S62 Suppl.
[23] G.I. Shulman, Cellular mechanisms of insulin resistance, J. Clin. Invest. 106 (2) (2000) 171–176, https://doi.org/10.1172/JCI10583 (PubMed PMID: 10903330).
[24] A. Penfornis, S. Borot, D. Raccah, Therapeutic approach of type 2 diabetes mellitus with GLP-1 based therapies, Diabetes Metab. 34 Suppl 2 (2) (2008) S78–S90.
[25] S.A. Sadry, D.J. Drucker, Emerging combinatorial hormone therapies for the treatment of obesity and T2DM, Nat. Rev. Endocrinol. 9 (7) (2013) 425–433.
[26] G.A. Hitman, R.D. Leslie, R.I.G. Holt, GLP-1-based diabetes therapies; trial by media, Diabet. Med. 30 (10) (2013) 1147.
[27] J.P. Chanoine, K.J. Mackelvie, S.I. Barr, A.C.K. Wong, D.H. Elahi, GLP-1 and appetite responses to a meal in lean and overweight adolescents following exercise, Obesity 16 (1) (2012) 202–204.
[28] L.L. Nielsen, Incretin mimetics and DPP-IV inhibitors for the treatment of type 2 diabetes, J. Am. Board Fam. Med. 10 (10) (2005) 703–710.
[29] A. Wick, K. Newlin, Incretin-based therapies: therapeutic rationale and pharmacological promise for type 2 diabetes, J. Am. Acad. Nurse Pract. 21 (s1) (2009) 623–630.
[30] A. Consoli, G. Formoso, Potential side effects to GLP-1 agonists: understanding their safety and tolerability, Expert Opin. Drug Saf. 14 (2) (2015) 207–218.
[31] P.L. Brubaker, D.J. Drucker, Structure-function of the glucagon receptor family of G protein-coupled receptors: the glucagon, GIP, GLP-1, and GLP-2 receptors, Receptors Channels 8 (3–4) (2002) 179–188 (Epub 2003/01/18. PubMed PMID: 12529935).
[32] E. Ralf, B. Martin, A.W. Herling, W. Tilo, B. Zhang, K. Aimo, et al., Team players or opponents: coadministration of selective glucagon and GLP-1 receptor agonists in obese diabetic monkeys, Endocrinology 159 (8) (2018) 3105–3119.
[33] K.W. Sloop, M.D. Michael, J.S. Moyers, Glucagon as a target for the treatment of Type 2 diabetes, Expert Opin. Ther. Targets 9 (3) (2005) 593.
[34] R. Mentlein, Mechanisms underlying the rapid degradation and elimination of the incretin hormones GLP-1 and GIP, 23 (4) (2009) 443–452.