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ORIGINAL ARTICLE

DR10601, a novel recombinant long-acting dual glucagon-like peptide-1 and glucagon receptor agonist for the treatment of obesity and type 2 diabetes mellitus

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Abstract

Purpose Both glucagon-like peptide-1 (GLP-1) and glucagon (GCG) belong to the incretin family. This study aimed to investigate the pharmacokinetics and pharmacodynamics of DR10601, a fully recombinant hybrid peptide with dual GLP-1/GCG receptor agonistic activity.

Methods The agonistic ability of DR10601 was indirectly assessed by inducing cAMP accumulation in Chinese hamster ovary cells transfected with GLP-1R or GCGR in vitro. Following s.c. administration, the plasma pharmacokinetics of DR10601 were analysed in male Sprague-Dawley rats. The antiobesity effects and improved glycaemic control of DR10601 in vivo were evaluated by administering DR10601 to high-fat DIO mice and ICR mice as a single dose or repeated s.c. doses once every 4 days for 24 days.

Results DR10601 exhibits dual agonistic activity on GLP-1 and glucagon receptors. The plasma half-life of DR10601 in Sprague-Dawley rats following s.c. administration was 51.9 ± 12.2 h. In an IPGTT, a single s.c. dose of DR10601 (30 nmol/kg) produced similar glycaemic control effects and a longer duration of action compared to dulaglutide (10 nmol/kg). Compared with that achieved with liraglutide (40 nmol/kg) s.c. administered daily, DR10601 administered s.c. once every 4 days at 90 nmol/kg exerted a nearly equivalent effect on food intake and significantly reduced the body weights of high-fat DIO mice at 24 days.

Conclusions Repeated administration of DR1060 provides potent and sustained glycemic control and body weight loss effect in high-fat DIO mice. DR10601 is a promising long-acting agent deserving further investigation for the treatment of type 2 diabetes and obesity.

Keywords Body weight · Glucagon-like peptide-1 · Obesity · Type 2 diabetes

Abbreviations

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
DIO	Diet-induced obese
FDA	Food and Drug Administration
GCGR	Glucagon receptor
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLP1R	GLP-1 receptor
HRP	Horseradish peroxidase
OXY	Oxyntomodulin
PYY	Peptide YY
TMB	3,3′5,5′-Tetramethylbenzidine
SD rats	Sprague-Dawley rats
CHO cells	Chinese hamster ovary cells

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Introduction

Diabetes is a metabolic disorder that has become an important epidemic disease. Epidemiological survey results indicate that obesity is an important risk factor for the development of diabetes and that 90% of patients with type 2 diabetes are overweight or obese [1]. Evidence has shown that body weight loss is beneficial to the effects of antidiabetic drugs in treating type 2 diabetes or even reversing type 2 diabetes progression.

GLP-1 is an incretin that is released into the blood by enteroendocrine cells of the gut within minutes after a meal. GLP-1 has been shown to exert a number of beneficial effects on diabetes, including potentiating insulin release, delaying gastric emptying and inducing satiety, and plays an important role in nutrient intake and the regulation of energy metabolism [2].

Currently, six GLP-1RAs are approved by the FDA for treating type 2 diabetes. Randomized controlled trials have demonstrated that all GLP-1RAs improve glycaemic control, reduce body weight, at the expenses of increased risk of adverse gastrointestinal symptoms compared with a placebo. In addition, liraglutide is approved as an injectable weight loss drug by the FDA for obesity treatment [3]. However, long-term glucose control by GLP-1RAs is still less than perfect, and reductions in adiposity remain far below that desired by patients and physicians [4].

Current research results indicate that GLP-1RAs combined with other agonists or unimolecular dual/multiple incretin receptor agonists, such as dual GLP-1R/GCGR agonists [5], dual GLP-1R/GIPR agonists [6], and GLP-1R/GCGR/GIPR triagonists at different phases of clinical trials [7], show synergistic benefits on glycaemic control and weight loss compared to those achieved with GLP-1RA monoagonists. For example, the dual GLP-1R/GCGR agonist MEDI0382 exhibits superior weight reduction and a similar glucose-lowering ability compared to liraglutide in DIO mice [8].

Native incretin is less stable in plasma due to fast renal clearance and degradation by proteases; thus, to meet clinical requirements for drugs, existing GLP-1 agonists usually function via mutating enzyme-sensitive sites to improve enzyme stability and modifying GLP-1 polypeptides with fatty acids or polyethylene glycol (PEG) to reduce renal clearance and prolong half-life. Compared to native incretin, the current GLP-1RA used in the clinic typically has multiple mutation sites and even unnatural amino acid residues. The results of clinical GLP-1RA application indicate that a lower homology between GLP-1 and the endogenous incretin peptide sequence is correlated with a greater risk of forming corresponding antidrug antibodies, which may be detrimental to the long-term clinical application of such medicines [9]. To overcome the deficiencies of existing GLP-1RAs, DR10601 was designed to be fully recombinantly expressed as a unimolecular polypeptide with dual GCGR and GLP1R agonistic activities (Fig. 1). In this study, we aimed to evaluate the pharmacokinetics and pharmacodynamics of DR10601 for the treatment of type 2 diabetes and obesity using a rodent model.

Methods

In vitro glucagon receptor and GLP-1 receptor activation potency test

The ability of DR10601 to activate GLP-1R and GCGR was indirectly tested by measuring the cAMP accumulation in a cell-based luciferase reporter gene assay. CHO cells (Thermo Fisher Scientific (China) Co., Ltd, Shanghai, China) cultured on six-well plates were cotransfected with cDNA for each individual receptor (Zeocin selection) and a luciferase reporter gene construct fused to a cAMP response element (hygromycin B selection) [10, 11]. In total, 6.0×10^4 cotransfected CHO cells were plated in 96-well cell plates and incubated overnight in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS and 1 μg/ml puromycin. Subsequently, serial dilutions of DR10601 (in 0.5% BSA) were added to the cells in the 96-well plates, which were incubated for 6 h at 37 °C and 5% CO_2 in a humidified environment. Following incubation, the luciferase activity of the CHO cells was measured by using a Bright-Glo™ Luciferase Assay System purchased from Promega Biotech Co., Ltd. (Beijing, China) according to the manufacturer's instructions.

HSQGT FTSDY SKYLD SQAAQ DFVQW LMNGG PSSGA PPPSG	
GGGSG GGGSG GGGSA Fc	DR10601
HSQGT FTSDY SKYLD SRRAQ DFVQW LMNT	Glucagon
HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPPS	Exenatide
HGEGT FTSDV SSYLE EQAAK EFIAW LVKGG GGGGG SGGGG	
SGGGG SAFc	Dulaglutide
HAEGT FTSDV SSYLE GQAAK EFIAW LVKGR	GLP-1 (7-37)

Fig. 1 Amino acid sequence of DR10601. Sequences of the related peptides dulaglutide, glucagon, GLP-1 (amino acids 7–37) and exenatide are also shown. Differences in amino acids from native glucagon are denoted in red. Fc represents the IgG4 hinge-Fc fragment. In addition, N297 of Fc was mutated to alanine to eliminate glycosylation

Animal studies

The mice, obtained from the animal experiment center of the Zhejiang Academy of Medical Sciences, were housed with a 12 h light/12 h dark cycle at standard temperature and humidity conditions and had ad libitum access to food and water unless noted otherwise. All experiments were performed according to the guidelines of the regulation for the administration of affairs concerning experimental animals of P.R. China. The mouse experiments were approved by the Zhejiang Academy of Medical Sciences Animal Ethics Committee (no. 201786-89).

Pharmacokinetics assay in Sprague-Dawley rats

Adult male SD rats ($n = 6$/group) were injected s.c. with 16.3 nmol/kg DR10601, and blood samples were collected at 3, 8, 24, 36, 48, 72, 96, 120, 144 and 168 h after administration. The serum concentrations of DR10601 in the rats were measured by the ELISA. Briefly, a 96-well plate was coated with a rabbit anti-glucagon monoclonal antibody (JF0960, HuaAn Biotechnology Co., Ltd, Hangzhou, China) overnight at 4 °C in PBS. After incubation and washing, 100 µL samples were added to the wells and incubated for 2 h at 37 °C. A biotin-labelled mouse anti-human IgG4 Fc antibody (9200-08, Southern Biotech, Birmingham, AL, USA) and HRP-labelled streptavidin were used to detect DR10601; TMB was used to develop the ELISA. All experiments were repeated three times.

Glucose-lowering effects of a single DR10601 administration in ICR mice

Normal male ICR mice (6–8 weeks old) were randomly assigned into 5 groups (body weight, 27–29 g; $n = 10$/group), including a vehicle group, a dulaglutide group (10 nmol/kg) and 3 dose groups of DR10601 (10, 30, 90 nmol/kg).

The mice were fasted for 12 h after receiving a s.c. injection of vehicle (50 mM phosphate buffer, pH 7.5) once daily for 3 days, and then each group of animals was injected s.c. with the respective doses of DR10601, vehicle, and dulaglutide. After 2 h, the mice were intraperitoneally injected with glucose (2 g/kg). Blood samples were collected from the tail vein at 0 (prior to glucose administration), 30, 60 and 120 min post-glucose challenge. Then, the mice were given free access food and water. To evaluate the duration of reduced blood glucose levels induced by DR10601, the mice were intraperitoneally injected with glucose (2 g/kg) after an overnight fast on the 3rd, 5th, 6th and 7th days following DR10601 treatment. Blood samples were collected from the tail vein at 0 and 30 min after the glucose challenge.

To assess the antihypoglycaemic properties of DR10601, 6- to 8-week-old normal male ICR mice were randomly assigned into 6 groups (body weight, 27–29 g; $n = 8$/group). After being fasted for 4 h, the mice were s.c. injected with 0.4 IU/kg insulin to induce hypoglycaemia. The mice were administered s.c. injections of vehicle (PBS buffer, 10 µl/kg), glucagon (15 nmol/kg), DR10601 (40 nmol/kg), DR10601 (40 nmol/kg) + exendin (9-39) (1 µmol/kg), dulaglutide (5 nmol/kg) + exendin (9-39) (1 µmol/kg), or dulaglutide (5 nmol/kg) + exendin (9-39) (1 µmol/kg) + glucagon (15 nmol/kg) at 45 min after the insulin injection. Blood was collected from a tail snip, and glucose was measured at 0, 15, 30, 45, 60, 75, 90, 105, 120, 150 and 180 min post-insulin treatment.

Effects of repeated DR10601 administration on the body weights and metabolism of DIO mice

Six-week-old male C57BL/6 mice were fed a high-fat diet (HFD; 60% kcal from fat, Rodent Diet D12492; Research Diets, Inc., New Brunswick, NJ, USA) ad libitum for 11 weeks. The mice were screened for a body weight of approximately 40 g, and the eligible mice were randomly assigned into four groups ($n = 8$/group): C (vehicle group), DR30 (DR10601 30 nmol/kg test group), DR90 (DR10601 90 nmol/kg test group), and L (liraglutide group).

The mice were s.c. injected with saline (PBS, pH 7.5) for 3 days prior to the start of the study. Mice in the C and L groups were s.c. injected daily with saline or liraglutide (40 nmol/kg). Mice in the DR30 and DR90 groups were s.c. injected with DR10601 (at doses of 30 and 90 nmol/kg, respectively) once every 4 days; on the days without DR10601 injection, mice in the DR groups received the same daily doses of saline as those in the C group. All groups of DIO mice were treated for 24 days and then maintained on a HFD for ten additional days.

On day 21, an IPGTT was performed. The mice were given an intraperitoneal injection of glucose (2 g/kg) after being fasted for 6 h. Mice in the C group were injected with saline, while those in the L group were administered liraglutide (40 nmol/kg) 1 h prior to the glucose challenge, and mice in the DR groups received DR10601 (30 or 90 nmol/kg) 12 h prior to glucose challenge ($t = 0$ min). Blood glucose levels were measured at −45, 0 (immediately prior to glucose challenge), 30, 60, 90, 120 and 180 min post-glucose challenge.

On day 24, blood samples were taken from the tail veins of mice fasted for 4 h for further biochemical analyses. Throughout the duration of the study, the food consumption and body weights of DIO mice were recorded daily in the evening before drug administration. On the 35th day, all the mice were killed; blood samples were collected for further analysis, and the livers were weighed.

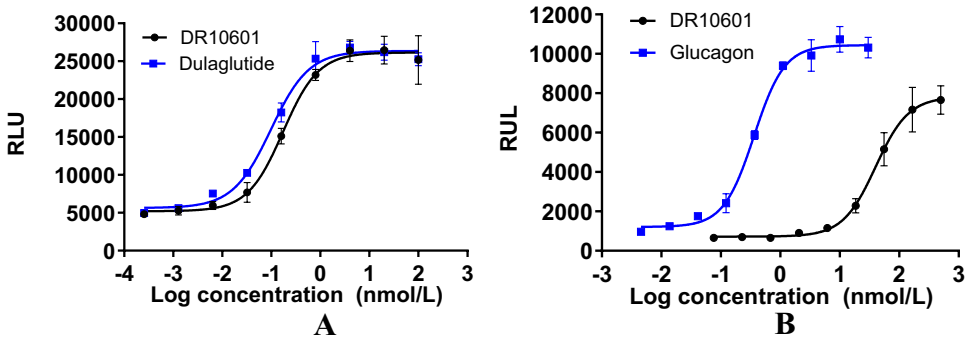

Fig. 2 Potency of DR10601 in transfected receptor systems. Representative concentration–response curves of DR10601, dulaglutide and glucagon (GCG) in cAMP accumulation assays of CHO cell lines expressing human GLP-1 receptors (**a**) or human GCG receptors (**b**), both of which were cultured in the presence of 0.1% BSA. Values are presented as the mean (±SD) from duplicate analyses fitted with a 4-parameter logistic fit to determine the EC_{50} value. The data shown are representative of $n \geq 3$ experiments

Table 1 Pharmacokinetic parameters in SD rats (mean ± standard deviation)

	C_{max} (pmol/ml)	T_{max} (h)	$AUC_{0-\infty}$ (pmol /h/ml)	*T*1/2 (h)	CL/F (ml/h/kg)	Vss/F (ml/kg)
Rat	84.5 ± 14.62	32.0± 9.8	7572.5± 1317.8	51.9± 12.2	2.2 ± 0.4	166.3± 54.3

Cmax the maximal observed plasma concentration; T_{max} the time of the maximal observed plasma concentration; $AUC_{0-\infty}$ the area under the plasma concentration curve from zero to infinity; *T1/2* the elimination half-life; *CL/F* clearance as a function of bioavailability; *Vss/F* the volume of distribution at steady state as a function of bioavailability

Statistical analysis

Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA) followed by the paired Student's *t* test or unpaired Student's *t* test. All results are presented as the mean ± SD. Differences with *p* values less than 0.05 were considered statistically significant and are identified with an asterisk.

Results

DR10601 is a dual GLP-1 and glucagon receptor agonist in vitro

As shown in Fig. 2, DR10601 stimulated cAMP accumulation in CHO cells in a concentration-dependent manner. The potency (EC50) value of DR10601 as measured by cAMP generation was 183.7±33.04 pmol/L for GLP-1R, which is approximately 5% of the potency (9.92 ± 4.47 pmol/L) of dulaglutide. The potency (EC50) value of DR10601 for GCGR was 37.39 ± 3.95 nmol/L, which is approximately 1% of the potency (0.40 ± 0.06 nmol/L) of native glucagon.

DR10601 has an extended half-life in vivo

The pharmacokinetics of DR10601 were assessed in rats, and the resulting pharmacokinetic curves were fit to a noncompartmental pharmacokinetic model. The pharmacokinetic parameters for DR10601 in plasma are presented in Table 1; the terminal half-life of DR10601 in rats was found to be 51.9 ± 12.2 h (Fig. 3).

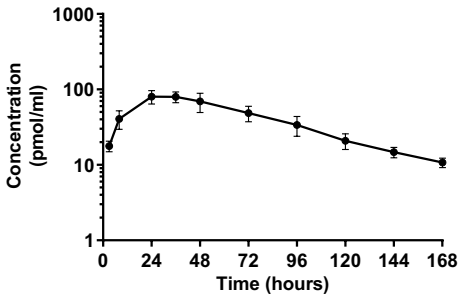

Fig. 3 Pharmacokinetics of DR10601 in SD rats. Blood samples were collected at the indicated time points after a single s.c. injection of DR10601 (16.3 nmol/kg) in SD male rats ($n = 6$). The concentration of DR10601 was analysed by a sandwich ELISA using an anti-human Fc antibody and a glucagon-specific antibody. PK parameters were calculated with PKSolver using the noncompartmental method

A single dose of DR10601 significantly lowers blood glucose in ICR mice

Following a single s.c. injection of vehicle, DR10601 or dulaglutide, 10 nmol/kg dulaglutide and 10, 30, and 90 nmol/kg DR10601 significantly lowered blood glucose

levels ($p < 0.001$) in ICR mice at all time points compared with that achieved with vehicle (Fig. 4; $p < 0.001$). Moreover, as shown in Fig. 4c, the DR10601 has a longer duration of action in decrease glucose excursion in ICR mice than dulaglutide (7 days vs 5 days).

To evaluate the agonistic activation of GCGR by DR10601 in vivo, the acute effect of DR10601 alone or in combination with the selective GLP-1R antagonist exendin (9-39) on glycaemic control was assessed in insulin-induced hypoglycaemic male ICR mice. At 75 min after insulin administration, treatment with DR10601 alone resulted in further lowering of blood glucose levels further than that achieved with the vehicle (4.87 ± 1.07 mmol/L vs 7.20 ± 0.87 mmol/L) in ICR mice (Fig. 5). Treatment with glucagon at a 15 nmol/kg dose robustly increased blood glucose levels compared with that achieved with vehicle treatment (10.27 ± 0.82 mmol/L vs 7.20 ± 0.87 mmol/L). In contrast, coadministration of the GLP-1R antagonist exendin (9-39) (1 μmol/kg) reversed the blood glucose-lowering effects of DR10601, robustly elevating the blood glucose to levels that were approximately equal to those achieved with glucagon treatment (by 10.45 ± 0.31 mmol/L vs 10.27 ± 0.82 mmol/L). These results indicated that DR10601 has both GCGR agonistic activity and GLP-1R agonistic activity.

Interestingly, the blood glucose levels following the coadministration of both dulaglutide and exendin (9-39) were significantly higher than those following vehicle treatment (9.62 ± 0.79 mmol/L vs 7.20 ± 0.87 mmol/L) and were almost equal to those after glucagon treatment (9.62 ± 0.79 mmol/L vs 10.27 ± 0.82 mmol/L). The dulaglutide and exendin (9-39) and glucagon-administered groups showed the highest blood glucose levels among all the treatment groups analysed (13.23 ± 1.13 mmol/L).

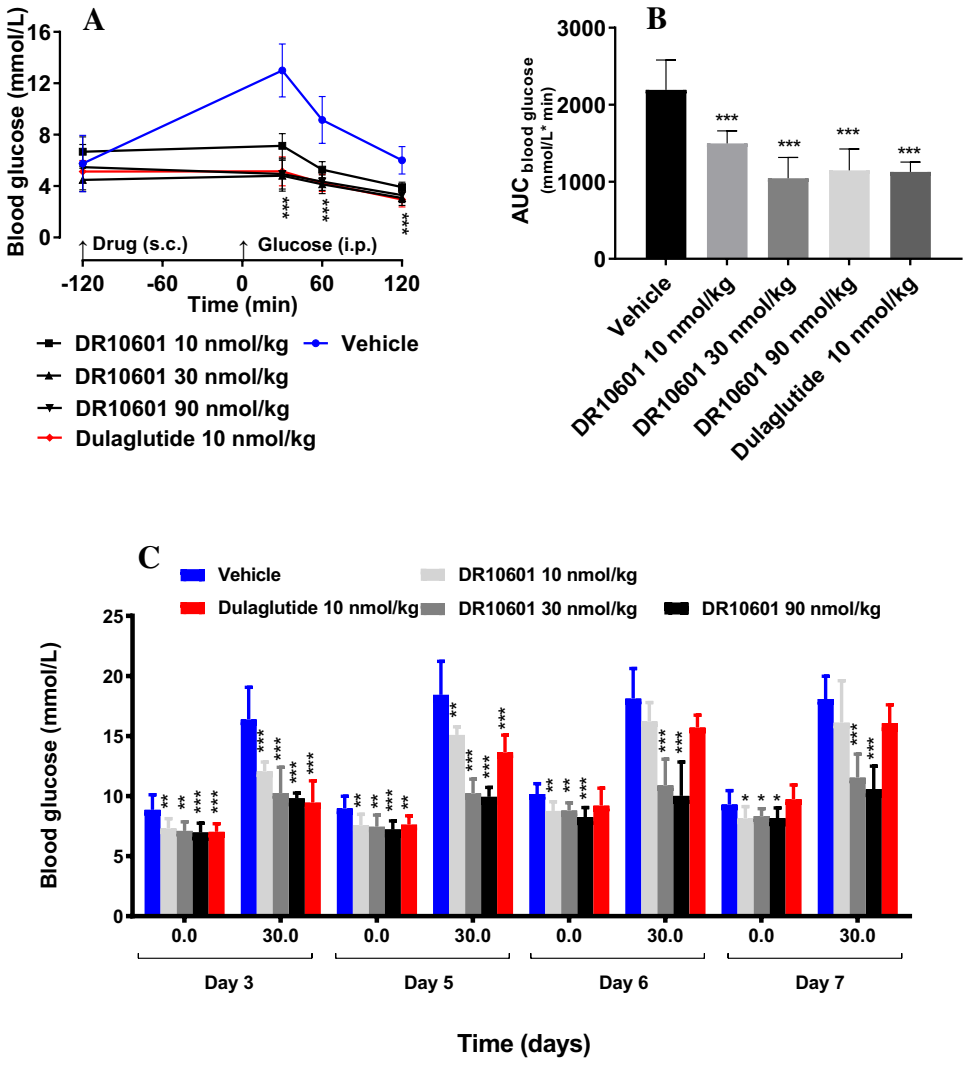

Fig. 4 Effects of single doses of DR10601 and dulaglutide on glucose tolerance in normal ICR mice. Glucose levels of mice at 0, 30, 60 and 120 min post-glucose challenge (**a**) and overall area under the curve (AUC) values for blood glucose on the first day (**b**). The blood glucose levels of ICR mice at 0 and 30 min post-glucose challenge on the third, fifth, sixth and seventh days following treatment (**c**). Mice fasted overnight were administered a single s.c. dose of DR10601 (10, 30, or 90 nmol/kg) or dulaglutide (10 nmol/kg) prior to the first day. On the following days, mice were intraperitoneally injected with glucose (2 g/kg) after overnight fasting. Blood samples were collected at the indicated time points. Time 0 was immediately prior to glucose challenge. The values represent the mean ± S.D. n = 10 mice/group. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ compared to vehicle

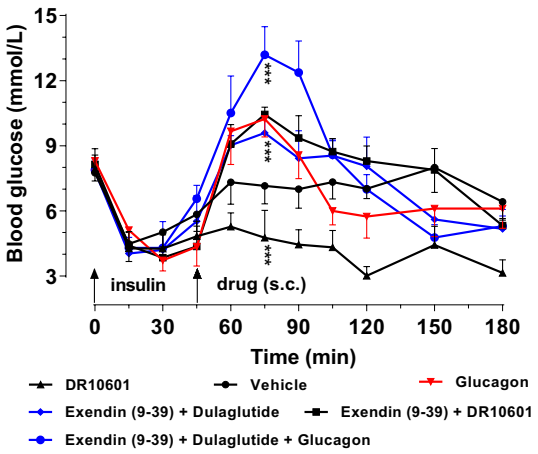

Fig. 5 Effect of exendin (9-39) and DR10601 coadministration on blood glucose control in insulin-induced hypoglycaemic mice. The ICR mice ($n = 10$/group) were injected s.c. with insulin (0.4 IU/kg) at $t = 0$ and with vehicle (PBS buffer, 10 µl/kg), glucagon (15 nmol/kg), DR10601 (40 nmol/kg), DR10601 (40 nmol/kg) + exendin (9-39) (1 µmol/kg), dulaglutide (5 nmol/kg) + exendin (9-39) (1 µmol/kg), or dulaglutide (5 nmol/kg) + exendin (9-39) (1 µmol/kg)+ glucagon (15 nmol/kg) s.c. at $t = 45$ min. Blood samples were collected from the tail vein at 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 min post-insulin treatment. Mice were fasted 4 h prior to the study and for the duration of the study. The values represent the mean ± S.D. $n = 10$ mice/group. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to vehicle)

Repeated administration of DR10601 reduced body weight and increased glucose tolerance in DIO mice

Figure 6a shows that the body weights of the vehicle-treated control mice did not increase significantly over the treatment period. Both repeated s.c. administration of DR10601 once every 4 days and a once-daily s.c. administration of liraglutide in DIO mice significantly ($p < 0.05$) reduced the body weights of DIO mice over the 24-day study period. The mean body weight of vehicle-treated animals increased by 0.89 ± 3.82% compared to the starting body weight over the course of the 24-day study, whereas the mean body weights of mice treated with 30 nmol/kg DR10601, 90 nmol/kg DR10601 and 40 nmol/kg liraglutide were reduced by 10.19 ± 4.59%, 26.5 ± 3.59% and 16.39 ± 5.04%, respectively, compared to that at the start of the study.

Because appetite reduction is expected to be the major mechanism by which these agents reduce body weight [12], we tested the effect of DR10601 on food intake in DIO mice over a period of 34 days.

The cumulative total food intake of DIO mice receiving repeated treatment with 30 nmol/kg DR10601 was slightly lower and not significantly different than that of the vehicle-treated mice (64.5 ± 3.59 vs 69.4 ± 4.33 g), but was significantly higher than that of mice treated with 40 nmol/kg liraglutide (64.5 ± 3.59 vs 49.7 ± 4.01 g, $p < 0.01$) by the 24th day of treatment (Fig. 6b). However, compared with that achieved with vehicle treatment, repeated treatment with 90 nmol/kg DR10601 led to a significant reduction in the cumulative total food intake of DIO mice (50.4 ± 1.65 vs 69.4 ± 4.33 g, $p < 0.01$), which was approximately equal to that of mice treated with 40 nmol/kg liraglutide (50.4 ± 1.65 vs 49.7 ± 4.01 g) at 24 days. These results indicated that 90 nmol/kg DR10601 had a nearly equal inhibitory effect on the food intake of DIO mice and a better weight-reducing effect compared with that achieved with 40 nmol/kg liraglutide (Fig. 6B).

To assess the metabolic impact of DR10601, an IPGTT was performed on day 21 (Fig. 6c). Glucose tolerance was significantly improved ($p < 0.001$) in all treatment groups, with glucose areas under the curve (AUCs) of 2359, 1537, 1557 and 1425 mmol/L min for the vehicle, 30 nmol/kg DR10601, 90 nmol/kg DR10601 and 40 nmol/kg liraglutide groups, respectively. Furthermore, blood glucose levels measured prior to the glucose challenge ($t = 0$ min) were also significantly reduced in all treatment groups compared with those in the vehicle group.

Discussion

Gut-derived hormones include PYY, GLP-1, GIP and oxyntomodulin, and bariatric surgery significantly elevates circulating gut hormone levels and is proven to affect acute remission of type 2 diabetes [13]. Moreover, research results indicate that gut hormones are secreted by enteroendocrine cells following a meal and have a combinatory effect of promoting insulin secretion, glycaemic control and appetite regulation. Thus, gut hormones are ideal antidiabetic and antiobesity targets.

Currently, six monotherapy GLP-1RAs are approved by the FDA for treating type 2 diabetes. However, a variety of enterogenous hormones directly and indirectly participate in the regulation of blood glucose in the body. GIP and GLP-1 are known to indirectly downregulate blood glucose levels by promoting insulin secretion, and glucagon can directly increase blood glucose levels and antagonize the blood glucose-reducing effects of both GIP and GLP-1. Because the plasma concentration of GLP-1RA is much higher than the levels of endogenous hormones after administration, the balance of various gut hormone levels in the body is disturbed. Although GLP-1RAs can exert a glycaemic control effect, a slight risk of adverse reactions, such as nausea, vomiting and hypoglycaemia, is inevitable. In contrast, a combination of several enteric hormones can closely simulate the changes in endogenous hormone concentrations, which is beneficial to glycaemic control and weight loss and reduces the occurrence of adverse reactions. However, because the combination of

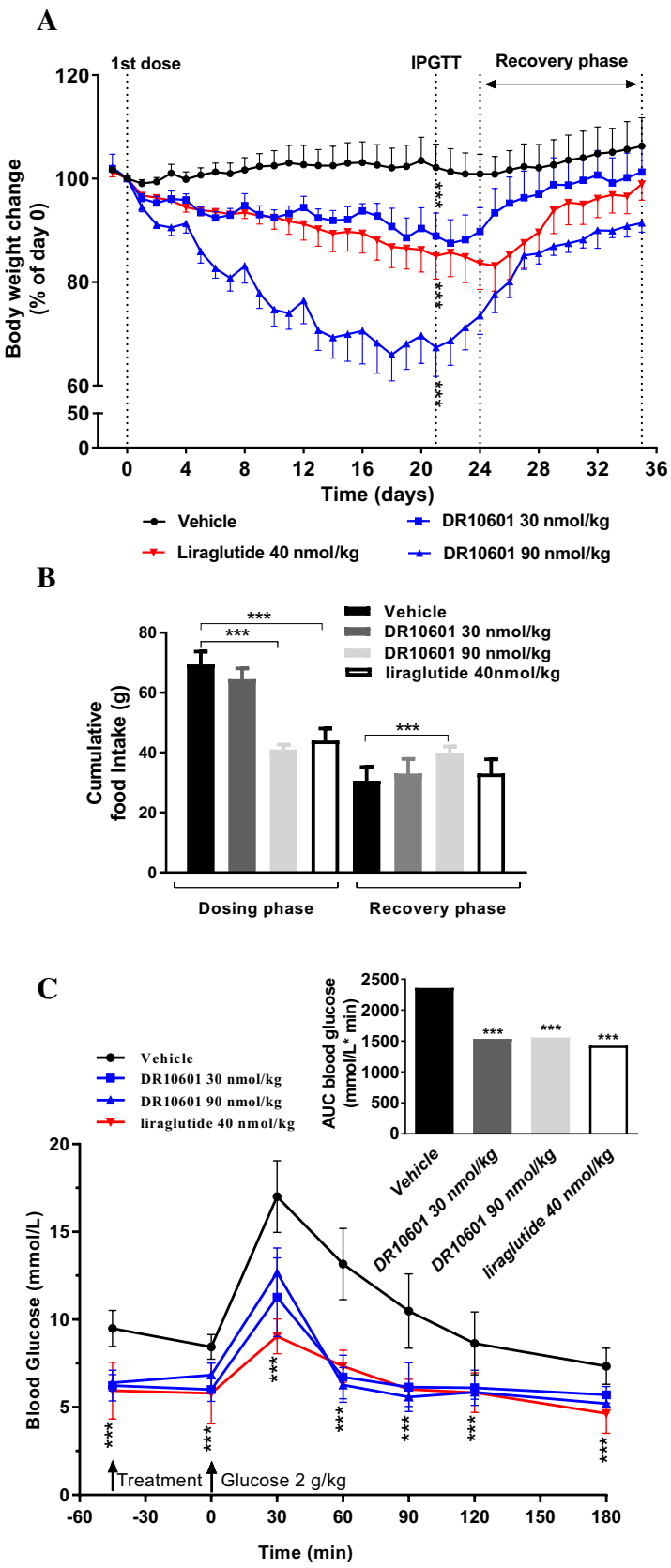

Fig. 6 Effects of 24 days of repeated-dose treatment with DR10601 on body weight (**a**), cumulative food intake (**b1** and **b2**) and glucose tolerance (**c**) in male DIO mice. Male DIO mice ($n = 8$/group) were treated with either a daily s.c. administration of vehicle or liraglutide (40 nmol/kg) or a s.c. administration of DR10601 (30 and 90 nmol/kg) once every 4 days. Glucose tolerance was measured by the i.p. administration of glucose (2 g/kg) on day 21. Time 0 was immediately prior to glucose challenge. The insets show the overall area under the curve (AUC) values for blood glucose (**c**). The stippled lines indicate the start of dosing, IPGTT, and recovery phase in **a** and **b1**, respectively. The values represent the mean ± SD; $n =$ 8 mice/group. *$p < 0.05$; **$p <$ 0.01; ***$p < 0.001$ compared to vehicle

two simple gut hormone drugs increases the difficulty of quality control and approval of the formulation, the use of single molecules with dual or triple agonistic activity is preferred for new antiobesity and antidiabetic drugs.

Obesity and overweightness are multifactorial problems caused by unbalanced energy intake and expenditure. Currently, reducing energy intake and increasing energy expenditure are the pillars of treatment. Glucagon, a hormone that counter-regulates insulin, can antagonize insulin- or GLP-1-induced hypoglycaemia in a glucose-dependent manner, restore blood glucose levels to normal and increase energy expenditure [14]. Moreover, compared with selective GLP-1RAs at equimolar doses, OXY, the only endogenous dual GLP-1R/GCGR agonist to date, exhibits superior weight loss effects, antihyperglycaemic effects and lipid-lowering activity in obese mice and humans [15, 16]; However, the short plasma half-life of OXY (approximately 10 min) prevents its direct use in clinical practice [17]. In addition, MEDI0382, a dual GLP-1R/GCGR agonist produced by Millennium, exhibits superior weight loss effects and a comparable glucose-lowering ability to liraglutide [8]. The above facts indicate that glucagon is suitable in combination with GLP-1 in antiobesity and antidiabetic drugs.

Furthermore, to meet the needs of clinical application, existing GLP-1RAs typically use multiple amino acid mutations, even introducing nonnatural amino acids to increase resistance to protease degradation and modifying fatty acid or PEG molecules to reduce the renal clearance rate. However, these measures reduce the homology of GLP-1RAs with endogenous GLP-1 and increase the risk of antidrug antibody development.

Therefore, to address the problems mentioned above, DR10601 was designed as a single molecule with molecular modification of the glucagon amino acid sequence using as few natural amino acid residues as possible to increase resistance to proteases while endowing DR10601 with GLP-1R/GCGR dual agonistic activity (Fig. 1). Previous studies indicated that glucagon possesses poor solubility in aqueous buffers at physiological pH values [18] and has lower stability in plasma due to renal clearance and degradation by proteases, such as DPP4 and endopeptidase. The degradation of glucagon mainly occurs at the penultimate (P_1) position [19] as well as between Arg17 and Arg18 [20]. In addition, analysis of the structure–activity relationship of both GLP-1 and glucagon upon receptor binding showed that the middle sequence has an important influence on the selectivity of incretin for its corresponding receptor [5, 21]. OXY consists of the whole glucagon sequence with an octapeptide at its C-terminal end. Interestingly, glucagon has only GCGR agonistic activity, while OXY has GLP-1R/GCGR dual agonistic activity. These facts indicate that the intermediate and C-terminal regions have important effects on the receptor selectivity of glucagon.

In addition, the COOH-terminal extension (CEX) of exenatide can provide additional metabolic stability and enhance the binding affinity for GLP1R when added to GLP-1 [22, 23]. To increase the agonistic activity of DR10601 on GLP-1R and improve resistance to enzyme degradation, the amino acid residues Arg17 and Arg18 in the glucagon sequence were replaced with Gln17 and Ala18 derived from GLP-1. Furthermore, Try29 at the C-terminus of glucagon was replaced with the CEX sequence. Reducing the renal clearance of DR10601 can prolong its plasma half-life. The Fc fragment of human IgG4 was fused to the C-terminus of DR10601 through the GS linker to further extend the plasma half-life of DR10601. SDS-PAGE analysis indicated that DR10601 is recombinantly expressed as a dimer (Supplemental, Figure S1A), and mass spectrometry revealed that the MW of the DR10601 dimer is 61.513 kDa (Supplemental, Figure S1B). The rat plasma stability test showed that DR10601 is more resistant to protease degradation than native glucagon (Supplemental, Figure S2). Table 1 shows that the terminal half-life of DR10601 in rats was 51.9 ± 12.2 h, while the half-life of dulaglutide in rats was shown to be 38.2 ± 2 h [24]. These results indicated that DR10601 is suitable for once-weekly dosing to treat type 2 diabetes and that using only a few natural amino acid mutations can increase the enzymatic resistance.

Following the s.c. administration of a single dose of DR10601 or dulaglutide to ICR mice, 10, 30, and 90 nmol/kg DR10601 and 10 nmol/kg dulaglutide significantly reduced blood glucose levels compared with those in the vehicle group. The blood glucose levels in the DR10601 (30 and 90 nmol/kg) groups were not significantly different from those in the 10 nmol/kg dulaglutide group (Fig. 4). The mechanisms underlying the dose-independent improvement in blood glucose by DR10601 may involve the antagonistic effects of GLP-1 and glucagon on blood glucose regulation. The DR10601-induced decrease in fasting plasma glucose levels lasted up to 7 days (Fig. 4c) and was longer than that of dulaglutide, which lasted for only 5 days. These results are consistent with the results of DR10601 observed in rat pharmacokinetic tests.

The agonistic action of DR10601 on GCGR was further verified by coadministration of both DR10601 and the GLP-1R selective antagonist exendin (9-39) in insulin-induced hypoglycaemic male ICR mice (Fig. 5). Coadministration with exendin (9-39) (1 μmol/kg) reversed the blood glucose-lowering effects of DR10601 and robustly elevated blood glucose levels, which were approximately equal to those in the glucagon group and significantly higher than those in the vehicle group. These results showed that exendin (9-39) (1 μmol/kg) completely antagonized the GLP-1 activating activity of DR10601, resulting in DR10601 showing only glucagon activity, which led to increased blood glucose levels.

The blood glucose levels in the dulaglutide and exendin (9-39) coadministration group were slightly lower than those in the DR10601 and exendin (9-39) coadministration group and were also higher than those in the vehicle group. These results showed that exendin (9-39) (1 μmol/kg) not only completely antagonized the GLP-1 activating activity of dulaglutide, but also antagonized the activity of some endogenous GLP-1, resulting in elevated blood glucose levels. The second reason why the blood glucose level in the dulaglutide and exendin (9-39) coadministration group was lower than that in the DR10601 and exendin (9-39) coadministration group may be that DR10601 has a potency for GLP-1R that is approximately 5% of that of dulaglutide, which is consistent with the in vitro activity analysis of DR10601. In addition, compared with the other treatment groups, the dulaglutide and exendin (9-39) and glucagon coadministration group showed the highest blood glucose level, probably due to the superposition of glucagon and exendin (9-39) on endogenous GLP-1 antagonism.

Figure 6a shows that DR10601 treatment induced weight loss in DIO mice in a dose-dependent manner compared to that achieved with the vehicle over the course of the 24-day study.

The mean body weight loss induced by 30 nmol/kg DR10601 was less than that caused by 40 nmol/kg liraglutide. The cumulative total food intake in the 30 nmol/kg DR10601 group was significantly greater than that in the 40 nmol/kg liraglutide group and was approximately equal to that in the vehicle group. These results show that 30 nmol/kg DR10601 has a smaller effect on feed intake than 40 nmol/kg liraglutide but has similar effects on weight loss. Therefore, DR10601 is thought to have a lower inhibitory effect on appetite than liraglutide. Compared with 40 nmol/kg liraglutide, 90 nmol/kg DR10601 has almost equal effects on feed intake in DIO mice, but induced more weight loss. The underlying mechanism may be that DR10601 has glucagon activity and thus increases energy expenditure.

To assess the metabolic impact of DR10601, an IPGTT was performed on day 21 (Fig. 6c). Glucose tolerance was significantly improved in all treatment groups compared with that in the vehicle group. No significant difference was found in blood glucose levels between the liraglutide group and the groups treated with different doses of DR10601, suggesting that DR10601 is less likely to induce the risk of hypoglycaemia in future clinical applications than other agents currently in use.

The liver is a central organ in carbohydrate metabolism, and hepatocytes are one of the main tissues expressing GCGRs. In accordance with previous reports, DR10601 treatment decreased AST and ALT and triglyceride levels in serum and reduced serum cholesterol, mainly due to a reduction in LDL cholesterol (Supplemental, Figure S3). Furthermore, 90 nmol/kg DR10601 reduced the liver weight more than liraglutide (Supplemental, Figure S4), potentially because glucagon can reduce the accumulation of liver lipids [25, 26].

In summary, repeated administration of DR10601 results in excellent glycaemic control and profound weight loss in DIO mice. DR10601 has equal weight loss efficacy and a reduced inhibitory effect on appetite compared with liraglutide and a longer duration of action than dulaglutide.

Author contributions WW designed DR10601 and wrote the manuscript. WW, WD and YH conceived and designed the study. XW, JD, GY and ZZ constructed the expression vector and performed the protein expression experiment. ZY and YC separated and purified the target protein. XW, YF and JF were responsible for the relevant experimental analyses. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interests The authors have no conflicts of interest to declare.

Ethical approval The experimental animals used in this paper were purchased from the animal experiment center of the Zhejiang Academy of Medical Sciences, All experiments were performed according to the guidelines of the administration for the administration of affairs concerning experimental animals of PR China. The mouse experiments were Approved by the Zhejiang Academy of Medical Sciences Animal Ethics Committee (no. 201786-89).

Informed consent For this type of study, formal consent is not required.

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