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Glutazumab, a novel long-lasting GLP-1/anti-GLP-1R antibody fusion protein, exerts anti-diabetic effects through targeting dual receptor binding sites

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ABSTRACT

Aims: Glucagon like-peptide-1 (GLP-1)-based drugs have been proposed as mono- or combined therapy for type 2 diabetes mellitus. Thus we characterized a novel antibody fusion protein engineered by linking the human GLP-1 derivative to a humanized GLP-1 receptor (GLP-1R) antibody *via* a peptide linker.
Materials and methods: Glutazumab was characterized by receptor binding and reporter activation assays, and its specificity was investigated with the aid of the cognate receptor antagonist exendin (9-39) and antibody Ab1. Pharmacokinetics was evaluated in Sprague-Dawley (SD) rats and cynomolgus monkeys, and pharmacodynamics was assessed in normal ICR and spontaneous type 2 diabetic KKAy mice. Hypoglycemic effects were evaluated after acute administration and glucose metabolism and β-cell function were assessed with repeated administrations. Dulaglutide was a positive control in all experiments.
Results: Glutazumab significantly bound and activated GLP-1R, but the receptor antagonist exendin (9-39) did not inhibit the activation except when combined with Ab1. Single injection of glutazumab reduced the blood glucose in ICR mice and KKAy mice, and the half-lives in SD rats and cynomolgus monkeys were 18 h and 33.6 h. Repeated injections of glutazumab controlled glycemic fluctuations and improved β-cell function in KKAy mice.
Conclusions: As a novel GLP-1R agonist, glutazumab may be a potential treatment for T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease characterized by insulin resistance and progressive β-cell failure. To compensate insulin resistance, β cells must secrete increasingly more insulin to maintain glucose homeostasis and this causes metabolic stress and deterioration, loss of glycemic control and the onset of T2DM. Therefore, restoration of β-cell function is crucial to the treatment of T2DM [1]. Obesity is a major risk factor for onset of T2DM, while hypoglycemia is a common adverse effect of T2DM treatment with insulin and sulphonylureas and has important consequences on cognitive function [2,3], so T2DM drugs that inhibit weight gain and promote stable blood glucose are ideal.

Glucagon-like peptide-1 (GLP-1) is an important endogenous incretin for glucose metabolism and a novel treatment for T2DM [4]. GLP-1 binds to GLP-1R to protect β cells from progressive failure by decreasing apoptosis and promoting proliferation, simultaneously enhancing insulin secretion upon glucose stimulation and suppressing appetite and gastric emptying without hypoglycemia or weight gain [5,6]. But natural GLP-1 is of little clinical utility because of rapid inactivation by dipeptidyl peptidase-IV (DPP-IV) and natural endogenous peptidase, as well as glomerular filtration. Therefore, it is essential to extend the half-life of GLP-1 for the development of GLP-1-based therapeutics. Five GLP-1 analogs in the US and six in Europe have been approved [7]. Fusing GLP-1 to a large “carrier” moiety is an effective method to extend half-life and dosing interval. Albiglutide is a DPP-IV resistant GLP-1 dimer fused to human albumin with a half-life of four to seven days, and dulaglutide is a DPP-IV resistant GLP-1 analog fused to a modified immunoglobulin G (IgG4) Fc fragment. Both drugs are injected once weekly [8,9].

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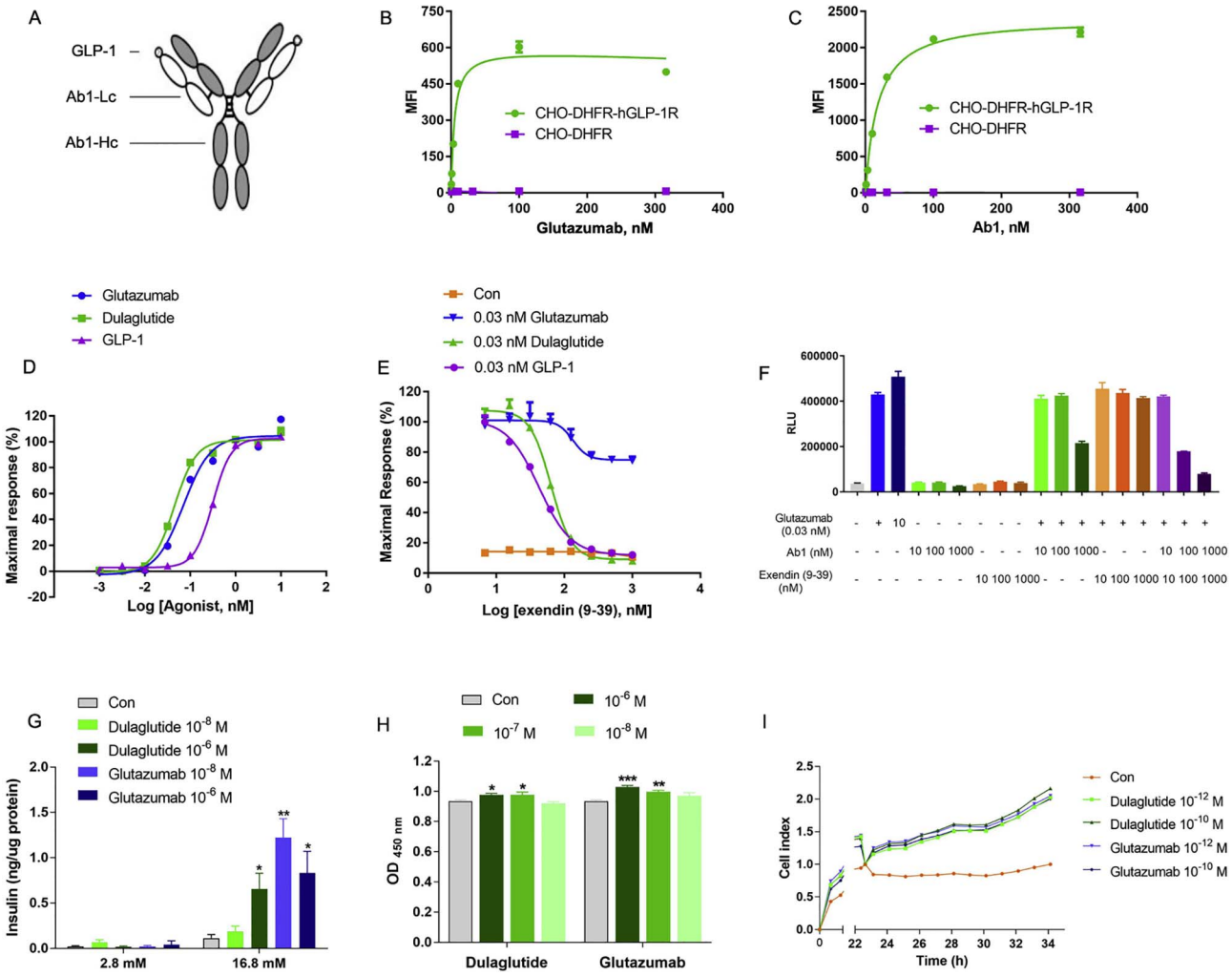

Fig. 1. Glutazumab enhanced glucose-stimulated insulin secretion and β-cell survival by specifically binding and activating GLP-1 receptor. A) Schematic diagram of glutazumab, B) Binding of glutazumab to human GLP-1 receptor (hGLP-1R), C) Binding of Ab1 to hGLP-1R, D) Luciferase activity induced by glutazumab, dulaglutide and natural GLP-1, E) Inhibition of the luciferase activity by exendin (9-39), an antagonist of GLP-1R, F) Inhibition of the luciferase activity by exendin (9-39), Ab1 or the combination of exendin (9-39) and Ab1, G) Insulin secretion induced by glutazumab and dulaglutide with 2.8 and 16.8 mM glucose, H) NIT-1 cell viability induced by glutazumab and dulaglutide, I) Real-time NIT-1 cell growth . All data were expressed as means ± SEM, n = 3. For G and H, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* Con.

Here we describe glutazumab, a novel GLP-1R agonist that is constructed by fusing a DPP-IV-resistant GLP-1 (7-35) fragment to the light chains of humanized GLP-1R antibody (IgG2) via a 23-amino acid peptide linker. Glutazumab possesses pharmacological effects of the natural GLP-1 and the structural attributes of a highly specific antibody (Fig. 1A), and its pharmacology and pharmacokinetics were fully characterized.

2. Materials and methods

2.1. Materials

Geneticin, sodium azide, pentobarbital sodium, exendin (9-39), penicillin, streptomycin, streptavidin, bovine serum albumin (BSA) and HRP conjugated goat anti-human IgG Fc were obtained from Sigma-Aldrich (St. Louis, MO, USA). DMEM/F12, fetal bovine serum (FBS) and dialyzed FBS were purchased from Gibco (Rockville, MD, USA). CHO-DHFR and NIT-1 cells were obtained from Cell Resource Center of Shanghai Institute for Biological Sciences (Shanghai, China) and ATCC (Manassas, VA, USA) respectively. The steady-Glo luciferase assay system and pEXP-TF and pGL3 vectors were obtained from Promega (Madison, WI, USA). The DyLight 488 NHS ester was purchased from Thermo Fisher Scientific (Waltham, MA, USA). BCA assay kit was obtained from Applygen Technologies (Beijing, China). GLP-1 (7-37) and dulaglutide were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA) and Eli Lilly (Indianapolis, IN, USA) respectively. DPP-IV inhibitor was obtained from Millipore (Darmstadt, Germany). Acetaminophen was purchased from National Institutes for Food and Drug Control (Beijing, China). The biotinylated antibody recognizing the N-terminus of GLP-1 was supplied by Gmax Biopharm (Hangzhou, China). CCK-8 assay reagent was purchased from Dojindo Laboratory (Kumamoto, Japan). Insulin (mouse) ultrasensitive ELISA kit was bought from Alpco (Salem, NH, USA). HbA1c assay kit was bought from Homa Biological (Beijing, China). Glucose assay kit was obtained from Biosino Bio-Technology & Science Inc. (Beijing, China). Fluorescence-activated cell sorting (FACS) analysis was performed on Guava of Merck KGaA (Darmstadt, Germany). The iCELLigence Real Time Cell Analyser system and the 8-well E-plates were purchased from ACEA Biosciences Inc. (Hangzhou, China). Glucose, saline and reagents for preparing KRH and PBS buffers were obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China).

2.2. Construction of plasmids and stable cell lines

The human GLP-1 receptor (hGLP-1R) cDNA was amplified and cloned into pEXP-TF vector, and the reporter plasmid was constructed

by cloning three copies of multiple response elements (5′-ATGCTAAA GGACGGTCACATTGCA-3′), one copy of cAMP response element (5′-CGTCATACTGTGACGTC-3′) and the luciferase reporter gene into pGL3 vector. The two plasmids containing either hGLP-1R gene (3 μg) or the reporter gene (1 μg) were co-transfected into 5×10^5 CHO-DHFR cells. Selection medium, DMEM/F12 containing 300 μg/ml geneticin and 10% dialyzed fetal bovine serum (FBS) minus hypoxanthine and thymidine, was added 48 h later and replaced with fresh medium every 3 days until colonies were formed. Single colonies were chosen and expanded. A stable cell line expressing hGLP-1R was confirmed by the reporter assay with GLP-1 stimulation.

2.3. Preparation and generation of GLP-1R mAbs and glutazumab

The preparation and screening of anti-hGLP-1R monoclonal antibodies (mAbs) using hybridoma technology were conducted as previously described [10]. The humanization was carried out via complementarity-depending region (CDR) grafting as described previously [11]. The heavy chain and light chain variable sequences were independently aligned with the human germline database of NCBI IgBLAST and the optimal germline sequences were selected as possible templates. A total of 23 mouse monoclonal antibodies with high affinity toward hGLP-1R were obtained and the best antibody was humanized and referred as Ab1. Ab1 was used to construct glutazumab (Fig. 1A). Both Ab1 and glutazumab were of human IgG2 isotype.

2.4. Receptor binding assay

Glutazumab or Ab1 were labeled with DyLight 488 NHS ester according to the manufacturer's instructions. Glutazumab or Ab1 (0.0316–316 nM) were incubated with the cells stably expressing hGLP-1R or the parental cells for 30 min at 4 °C in PBS containing 2% FBS and 0.02% sodium azide. Cell surface binding was quantified by FACS analysis. Data analysis was performed using Guava InCyte Software 2.2.

2.5. Receptor activation and specificity assay

The cells stably expressing hGLP-1R were seeded in 96-well cell culture plates (35,000 cells/well) and cultured overnight at 37 °C. For the assay, GLP-1 (7-37), dulaglutide and glutazumab were added at a final concentration of 0.003–31.6 nM. After incubation for 6 h, cells were lysed and luciferase activity was measured using a Steady-Glo luciferase assay system.

To evaluate the specificity of GLP-1R activation, 0.03 nM GLP-1 (7-37), dulaglutide and glutazumab were separately co-incubated with GLP-1R antagonist exendin (9-39) (from 6.8 to 1000 nM) in cells expressing hGLP-1R. Luciferase activities were measured and IC_{50} values were calculated. To assess the contribution of the Ab1 moiety to GLP-1R binding and activation, Ab1 and exendin (9-39) (10, 100 and 1000 nM) were incubated separately or together glutazumab (0.03 nM) in cells expressing hGLP-1R. Luciferase activities were measured and data were compared between groups.

2.6. Glucose-stimulated insulin secretion in primary islets

Normal male ICR mice (20–22 g) were fasted for 4 h with water *ad libitum* and anesthetized with pentobarbital sodium (80 mg/kg) *via* intraperitoneal (ip) injection. Islets were isolated as previously reported [12]. All islets were incubated in KRH solution containing 0.1% BSA and 2.8 mM glucose at 37 °C, 5.0% CO_2 for 1 h. Then the islets were transferred to Eppendorf tubes and incubated in the buffer for 2 h in the presence of 2.8 or 16.8 mM glucose and glutazumab or dulaglutide (10^{-8} and 10^{-6} M). Insulin in the supernatant was measured with ELISA kit and total proteins were quantified with BCA assay kit.

2.7. Cell proliferation assay

NIT-1 cells (18,000 cells/well) were seeded in 96-well plates and cultured for 24 h in DMEM/F12 containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin with the addition of PBS, glutazumab or dulaglutide. Then 5 μL of CCK-8 assay reagent was added and incubated for 4 h. OD was read at 490 nm. In another experiment, NIT-1 cells were seeded in 8-well E-plates (30,000 cells/well) and cultured as above to monitor growth using an iCELLigence Real Time Cell Analyser system according to the manufacturer's instructions.

2.8. Animals

Male ICR mice (20–22 g; Beijing Vital River Laboratory Animal Technology Co., Ltd., China), adult SD rats (200–220 g; Fengtai Experimental Animal Center, Academy of Military Medical Sciences, Beijing, China) and cynomolgus monkeys (2–5 kg; Guangxi Guidong Quadrumana Development Experimental Co. Ltd., Wuzhou, China) were kept in a temperature- and humidity-controlled environment with a 12 h light/dark cycle. These animals were fed with food and water *ad libitum*. Female spontaneous diabetic KKAy mice (35–40 g; HFK Bioscience Co., Ltd., Beijing, China) were housed and fed a high-fat diet consisting of 78.8% basic feed, 1% cholesterol, 10% yolk powder, 10% lard and 0.2% bile salt (HFK bioscience Co. Ltd., Beijing, China) with free access to water until hyperglycemia and obesity were confirmed. All the animals were handled according to guidelines (GB14925-2001 and MOST 2006a) established by China.

2.9. Pharmacokinetics of glutazumab in SD rats and cynomolgus monkeys

Adult rats (N = 8/group, M4 + F4) received a subcutaneous (sc) injection of glutazumab (4 mg/kg, sc) or Ab1, and blood was collected at 0 (pre-administration), 2, 4, 8, 12, 24 h and 2, 4, 6, 8, 10, 12, 18 and 28 d after administration *via* tail tip. Blood samples were stabilized with 10 μL DPP-IV inhibitor/mL, and immuno-reactive glutazumab was measured by ELISA with biotinylated antibody recognizing the N-terminus of GLP-1 and HRP conjugated goat anti-human IgG Fc. The ELISA plates were coated with streptavidin. Ab1 was quantified as well. The experiment was also carried out in cynomolgus monkeys (N = 8/group, M4 + F4) and glutazumab or Ab1 was given at 2 mg/kg.

2.10. Single injection of glutazumab in normal ICR and spontaneous diabetic KKAy mice

2.10.1. Effects of glutazumab on blood glucose after oral glucose loading in ICR mice

Male ICR mice were randomized into five groups (N = 10/group): vehicle group (Nor), glutazumab (2.5, 5.0 and 10 mg/kg) and dulaglutide (1.0 mg/kg) groups. All mice were fasted overnight with free access to water before collecting blood from tail tips to measure fasting glucose (0 min). Then saline, glutazumab and dulaglutide were administrated *via* sc injection, followed by oral glucose loading (2 g/kg). Blood glucose was measured at 30, 60 and 120 min after glucose administration and area under curve of blood glucose-time (AUC) was calculated. In addition, all mice were given oral glucose gavage after a 5 h fasting with water *ad libitum* in the next 6 days, and blood glucose before glucose gavage (0 min) and after 30 min was measured.

2.10.2. Effects of glutazumab on non-fasting blood glucose, food intake and body weight in ICR mice

Another batch of male ICR mice were grouped, fasted and treated as above. Quantitative feed was given to each group immediately after sc injection of glutazumab, dulaglutide or saline (sc), then blood glucose, surplus feed and body weight were monitored 5 h later and at 9:00 am for the next 4 days. Food intake for each group was calculated.

2.10.3. Effects of glutazumab on gastric emptying in ICR mice

Male ICR mice were grouped and fasted as above and followed by collection of blood from tail tips (0 min) and by sc injection of saline, glutazumab (2.5, 5.0 and 10 mg/kg) and dulaglutide (1.0 mg/kg). Acetaminophen (500 mg/kg) was orally administrated 24 h after injection, and 30 and 60 min later blood samples were collected from tail tips. Blood acetaminophen was measured using a diazo reaction assay and the AUC was calculated [13].

2.10.4. Effects of glutazumab on non-fasting blood glucose in diabetic KKAy mice

Based on non-fasting and fasting blood glucose, fasting blood triglyceride and total cholesterol and body weight, female KKAy mice were randomized to five groups (N = 10/group): saline (Con), glutazumab (2.5, 5.0 and 10 mg/kg) and dulaglutide (1.0 mg/kg) groups. All mice were simultaneously fed immediately after treatment and non-fasting blood glucose, surplus diet and body weight were measured 4 h later and at 9:00 am for the next 3 days. Food intake and water consumption for each group were calculated.

2.11. Repeated injections of glutazumab in diabetic KKAy mice

2.11.1. Dosing regimen

Five groups of female KKAy mice were dosed with saline, glutazumab (2.5, 5.0 and 10 mg/kg) or dulaglutide (1.0 mg/kg) *via* sc injection for every 3 days, and then every 2 days after the 8th injection, and finally once daily from the 10th injection until the end of the experiment. The change of injection frequency was based on variations in non-fasting blood glucose (NFBG) at 72 h after each treatment. Body weight and food and water intake were monitored during the experiment.

2.11.2. NFBG and HbA1c assays

All mice were housed with food and water *ad libitum*. NFBG was measured from tail tips 72, 48 or 24 h after each treatment. HbA1c was measured 37 days after the treatment.

2.11.3. FBG, OGTT, FBI and GSI assays

All mice were fasted for 4 h with free access to water before experiments. Fasting blood glucose (FBG) and oral glucose tolerance test (OGTT) were evaluated once weekly at 48 h after the 3rd and 5th injections, 24 h after the 9th injection and immediately after the 15th and 21st injections. After a 4 h fasting, FBG (0 min) was assayed, then an oral glucose gavage was given (2 g/kg). 30, 60 and 120 min later, blood glucose was measured and AUC was calculated. Fasting blood insulin (FBI) was quantified 15 days after treatment, and glucose stimulated insulin (GSI) was measured 30 min after oral glucose loading (2 g/kg) 37 days after treatment.

2.11.4. Hyperglycemic clamp test

The hyperglycemic clamp test was used to evaluate β-cell function 41 days after treatment (N = 3–4/group). All mice were fasted overnight with water *ad libitum* and anesthetized with pentobarbital sodium (80 mg/kg) *via* ip injection. Then the hyperglycemic clamp was applied [14], and glucose infusion was adjusted to create a steady state with hyperglycemia at 14 ± 0.5 mM. At the end of clamp test, the glucose infusion rate (GIR) was calculated according to the formula of [glucose infusion at steady state (μl/min) × glucose (mg/μl)/weight (kg)].

2.12. Statistical analysis

EC_{50} values were determined from curves plotting maximal response *versus* agonist concentrations by GraphPad Prism 7.01 (GraphPad Software, San Diego, USA) and expressed as means ± SEM with three identical experiments. All other data are expressed as means ± SEM., unless otherwise stated. Data were analyzed using a one-way ANOVA with a Bonferroni correction and a Student *t*-test ($p < 0.05$ as statistically significant).

3. Results

3.1. Binding and activation of GLP-1R by glutazumab

Glutazumab is shown in Fig. 1A. Ab1 and glutazumab specifically bound to hGLP-1R of the recombinant cell line, and the Kd data of glutazumab and Ab1 were 5.09 and 18.59 nM, respectively (Fig. 1B-C). Both glutazumab and dulaglutide dose-dependently elicited luciferase responses through GLP-1R activation with potencies ~7 times greater than GLP-1 (7-37) (Fig. 1D).

3.2. Specificity and targeting of GLP-1R activation by glutazumab

Exendin (9-39) significantly inhibited luciferase responses induced by dulaglutide and GLP-1 (7-37) but did not inhibit that induced by glutazumab. Thus, binding and activation of GLP-1R by glutazumab was not solely mediated by its GLP-1 moiety (Fig. 1E). The GLP-1R antibody Ab1, is the other moiety of glutazumab, had no inhibitory effect on glutazumab-induced luciferase responses at 10 and 100 nM, while it significantly inhibited luciferase response at 1000 nM ($p < 0.001$). The Ab1 moiety may contribute to the binding and activation of GLP-1R by glutazumab (Fig. 1F). Combination of 100 nM exendin (9-39) and Ab1 significantly inhibited glutazumab-induced luciferase responses ($p < 0.001$), while exendin (9-39) or Ab1 alone did not (Fig. 1F). This further confirmed that both Ab1 and the GLP-1 fragment in glutazumab contributed to the binding and activation of GLP-1R.

3.3. Insulin secretion and β-cell proliferation induced by glutazumab

16.8 mM glucose stimulated much more insulin secretion (about 5.5 times, $p = 0.051$) from primary islets than 2.8 mM glucose. Glutazumab (10^{-6} and 10^{-8} M) significantly stimulated insulin secretion from primary mouse islets in the presence of 16.8 mM glucose but not in the presence of 2.8 mM glucose, suggesting glucose dependence for stimulating insulin secretion (Fig. 1G). CCK-8 assay data showed that glutazumab (10^{-6} and 10^{-7} M) increased NIT-1 cell viability ($p < 0.01$; Fig. 1H). Glutazumab (10^{-12} and 10^{-10} M) also increased NIT-1 cell indices that reflected cell numbers but with reverse concentration-effect relationship (Fig. 1I).

3.4. Pharmacokinetics of glutazumab in SD rats and cynomolgus monkeys after a single injection

The pharmacokinetics of glutazumab and Ab1 in rats and cynomolgus monkeys was summarized (Table 1 and Fig. 2). The half-lives of glutazumab and Ab1 after a single dose of 4 mg/kg were approximately 18.0 h and 254.8 h in SD rats, respectively, while the half-lives for a single dose of 2 mg/kg were approximately 33.6 h and 152.4 h in cynomolgus monkeys, respectively.

3.5. Single injection of glutazumab reduced blood glucose, body weight, diet intake and gastric emptying in normal ICR and diabetic KKAy mice

In the oral glucose tolerance test, glutazumab dose-dependently decreased blood glucose and AUC 30 min after the injection in ICR mice (Fig. 3A), and the inhibitory effect on 30 min blood glucose lasted over 6 days (Fig. 3B). Glutazumab also reduced fasting blood glucose (0 min) before oral glucose loading over 4 days in ICR mice (Fig. 3B). Single dose of glutazumab also decreased non-fasting blood glucose of ICR mice for at least 3 days (Fig. 3C) and reduced food intake (Fig. 3D, 3.19, 2.62 and 2.39 g/mouse for glutazumab group *versus* 4.24 g/mouse for Nor group on day 2; 4.56, 3.42 and 3.59 g/mouse for glutazumab group

Table 1
Pharmacokinetic parameters of glutazumab and Ab1 in SD rats and cynomolgus monkeys.

	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (μg/mL)	AUC_{0-t} (μg·h/mL)	CL/F (ml/h/kg)	Vd/F (ml/kg)
SD rats						
Glutazumab	18.0 ± 0.7	24	2.5 ± 0.4	110.0 ± 16.0	44.1 ± 8.3	1156.7 ± 228.4
Ab1	254.8 ± 30.1	96–144	61.0 ± 2.0	23216.7 ± 683.0	0.1 ± 0.0	25.3 ± 1.8
Cynomolgus monkeys						
Glutazumab	33.6 ± 1.2	9.7 ± 0.8	17.9 ± 0.8	896.2 ± 36.4	3.3 ± 1.0	102.8
Ab1	152.4 ± 15.3	22.3 ± 4.5	24.5 ± 1.1	5480.6 ± 450.2	0.4 ± 0.0	74.7 ± 4.1

The concentrations of glutazumab in the blood were determined with a sandwich ELISA recognizing the N-terminus of GLP-1. Ab1 was quantified as well. All data were expressed as means ± SEM; T_{max} of SD rats and Vd/F of monkeys were means only. C_{max}: maximal observed plasma concentration, T_{max}: time of maximal observed plasma concentration, AUC_{0-t}: area under the plasma concentration curve from zero to the last chosen time point, $T_{1/2}$: elimination half-time, CL/F: clearance as a function of bioavailability, Vd/F: volume of distribution as a function of bioavailability.

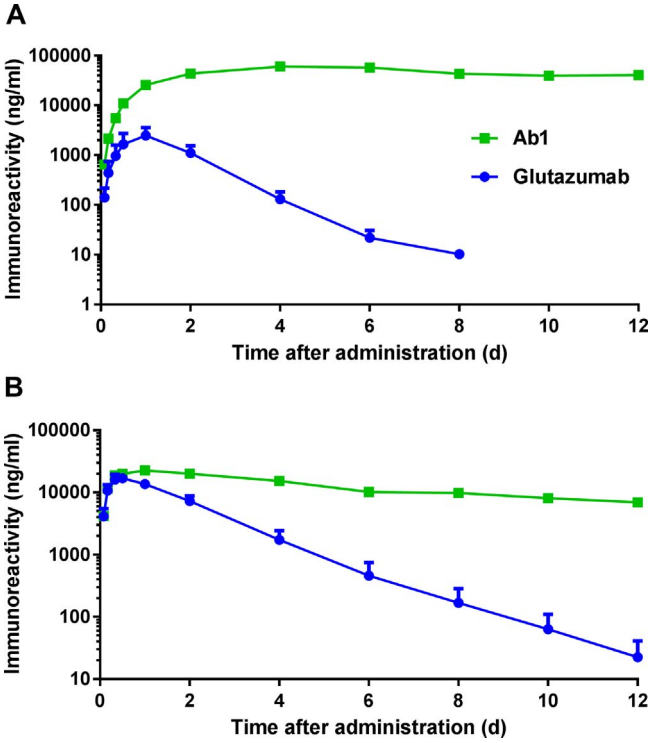

Fig. 2. Pharmacokinetics of glutazumab and Ab1 in SD rats (A) and cynomolgus monkeys (B). Glutazumab and Ab1 were administered as a single subcutaneous dose of 4 mg/kg in SD rats and 2 mg/kg in cynomolgus monkeys, and glutazumab in the blood was determined by sandwich ELISA recognizing the N-terminus of GLP-1. Ab1 was quantified by ELISA as well. All data were expressed as means ± SEM, n = 8.

versus 5.29 g/mouse for Nor group on day 3) and body weight for 2 days (Fig. 3E). For 24 h, glutazumab significantly reduced blood acetaminophen and AUC, suggesting inhibition of gastric emptying (Fig. 3F). Single injection of glutazumab in diabetic KKAy mice induced long-lasting hypoglycemic effects and reduced food intake and water consumption, but had no significant effect on body weight (Fig. 3G–J).

3.6. Repeated injections of glutazumab reduced blood glucose, body weight, food intake and water consumption in diabetic KKAy mice

Repeated injections of glutazumab significantly decreased non-fasting blood glucose in the initial stage, and the hypoglycemic effect gradually was attenuated with time, but immediately recovered when the dosing frequency was changed to once daily (Fig. 4A). Repeated injections of glutazumab also significantly lowered fasting blood glucose in a dose-dependent manner, especially by the end of treatment (Fig. 4B), and reduced HbA1c by 0.18%, 0.90% ($p < 0.05$) and 0.77% ($p < 0.05$) at 2.5, 5.0 and 10 mg/kg doses, respectively (Fig. 4C). This suggested excellent glycemic control. Repeated injections of glutazumab also significantly decreased the body weight of KKAy mouse with time (Fig. 4D), and probably reduced food intake and water consumption (Fig. 4E and F).

3.7. Repeated injections of glutazumab improved the impaired glucose tolerance, insulin secretion and β-cell function in diabetic KKAy mice

Repeated injections of glutazumab for 9 and 37 days significantly reduced the blood glucose and AUC after oral glucose loading (2 g/kg) (Fig. 5A and B). Glutazumab increased fasting blood insulin after 15 days of treatment (Fig. 5C), and enhanced glucose-stimulated insulin secretion after 37 days of treatment (Fig. 5D). Glucose infusion rate (GIR) in the steady state of hyperglycemic clamp test was significantly decreased in diabetic KKAy mice, and repeated injections of glutazumab at 2.5, 5.0 and 10 mg/kg doses significantly increased GIR by 44.1%, 132.3% and 126.5% (Fig. 5E).

4. Discussion

GLP-1R agonists have been used for diabetic therapy for glycemic control and improved β-cell function with few side effects [15]. Here we describe a novel GLP-1 receptor agonist, glutazumab, constructed by linking a human GLP-1 fragment to a humanized GLP-1R antibody for longer half-life and receptor targeting *via* antibody-receptor binding. Glutazumab is different from dulaglutide, an approved GLP-1/IgG4 Fc fusion protein [9], and Ab1, the GLP-1R antibody moiety, may decrease the off-target effects by receptor targeting. Receptor binding and activation assays confirmed that glutazumab specifically bound to GLP-1R and activated reporter gene expression with the same potency as dulaglutide. However, the activation of GLP-1R by glutazumab was more robust than dulaglutide, since the specific GLP-1R antagonist, exendin (9-39), suppressed the activity induced by dulaglutide but not that induced by glutazumab. Furthermore, Ab1 and exendin (9-39) significantly inhibited reporter gene expression in comparison to Ab1 or exendin (9-39) alone. This suggested that the GLP-1R antibody moiety may increase the binding robustness and functional activity of glutazumab and may reduce off-target effect *in vivo*.

The protection of β-cell survival and enhancement of glucose-stimulated insulin secretion are two important characteristics of GLP-1 [16]. In this study, the mouse primary islets were isolated to evaluate insulin secretion, and a mouse pancreatic β cell line, NIT-1, was used to study cell viability and growth [17]. Data show that glutazumab preserved characteristics of natural GLP-1, stimulating insulin secretion and enhancing β-cell proliferation.

Pharmacokinetic and pharmacodynamic data show that glutazumab was long-acting and the experimental differences in ICR and KKAy mice were likely due to different glucose metabolism of normal and diabetic states, especially different β-cell sensitivity to glucose stimulation. GLP-1-based drugs enhance insulin secretion in a glucose-dependent manner, so glucose is key in assessing efficacy and duration of action.

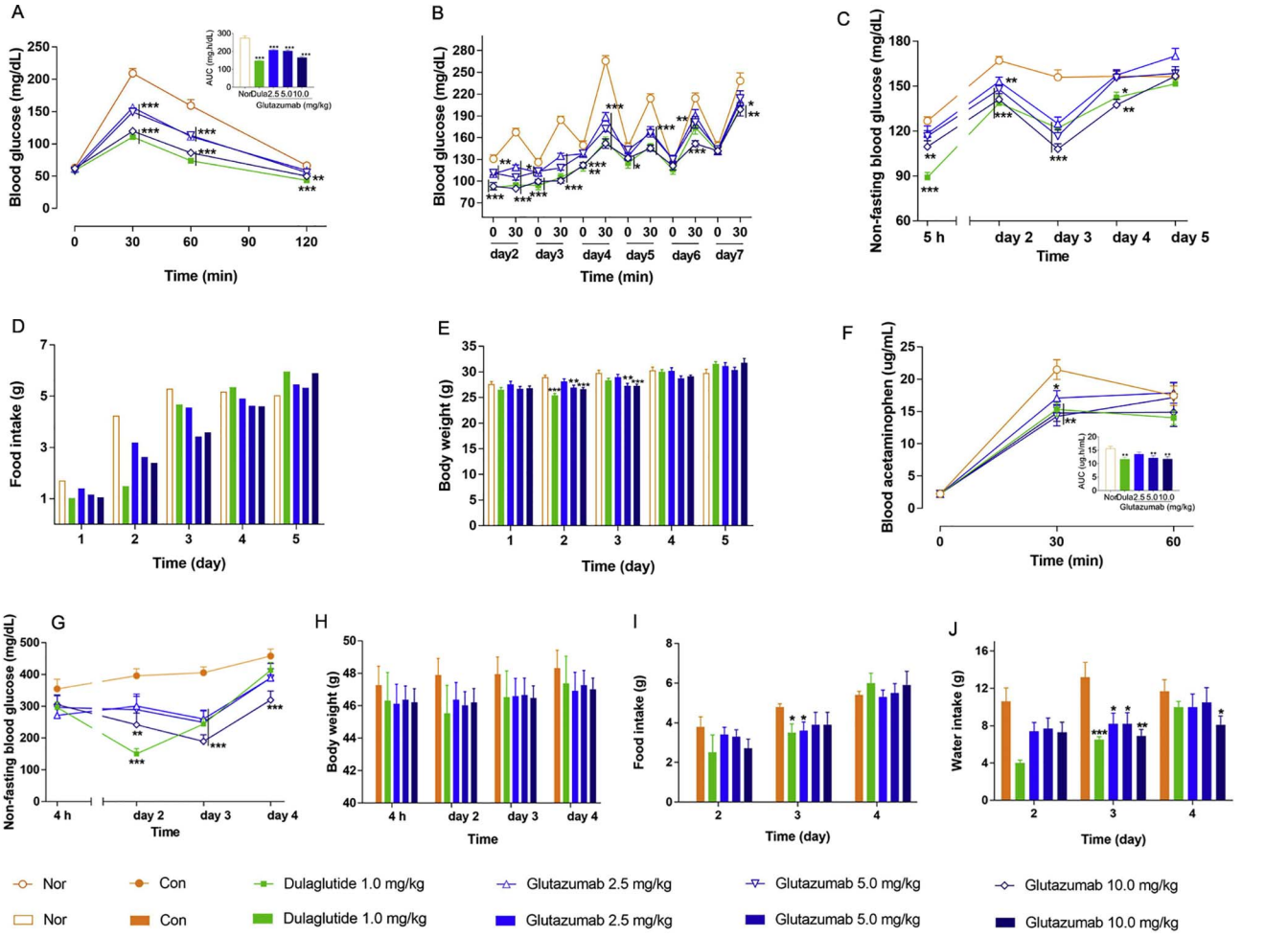

Fig. 3. Effects of glutazumab on blood glucose, gastric emptying, food intake, water consumption and body weight after a single injection in normal ICR and diabetic KKAy mice. A) Blood glucose-time curve and AUC measured after oral glucose loading 30 min following injection in ICR mice, B) Blood glucose at 30 min after oral glucose loading from d2 to d7 in ICR mice, C) Non-fasting blood glucose in ICR mice, D) Food intake of ICR mice, E) Body weight of ICR mice, F) Blood acetaminophen-time curve and AUC measured 24 h after glutazumab injection in ICR mice, G) Non-fasting blood glucose of diabetic KKAy mice, H) Body weight of KKAy mice, I) Food intake of KKAy mice, J) Water consumption of KKAy mice. All data are expressed as means ± SEM; For D, the data are means only, n = 10. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* Nor in ICR mice and Con in KKAy mice.

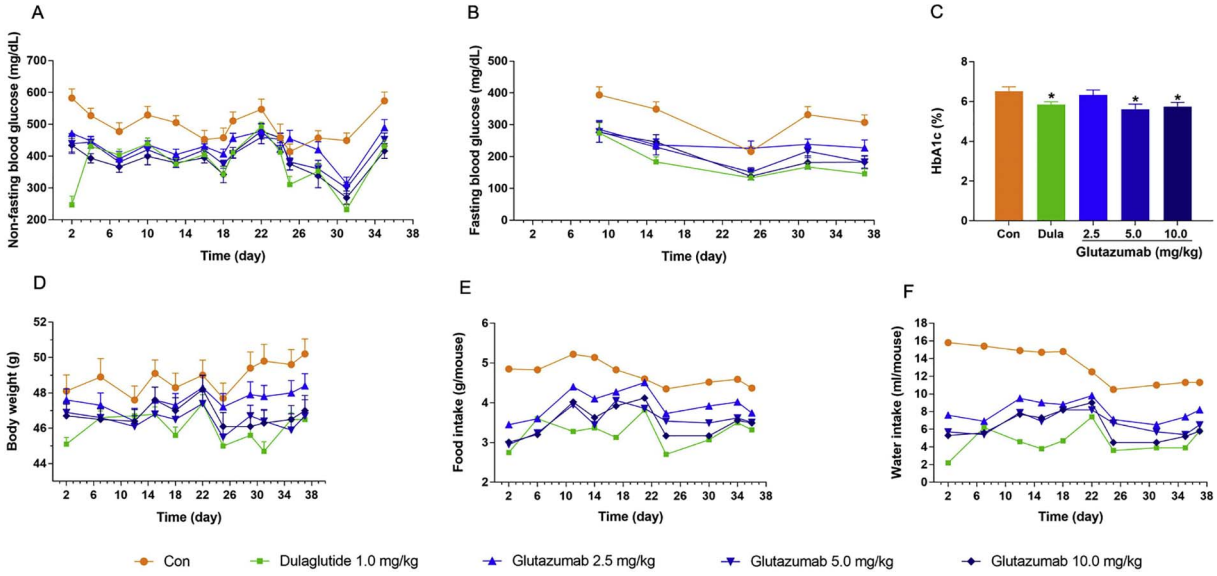

Fig. 4. Effects of glutazumab on blood glucose, body weight, food intake and water consumption after repeated injections in diabetic KKAy mice. A) Non-fasting blood glucose, B) Fasting blood glucose, C) Blood HbA1c, D) Body weight, E) Food intake, F) Water consumption. All data are expressed as means ± SEM; For E and F, the data are means only, n = 11, $^{*}p < 0.05$ *vs* Con.

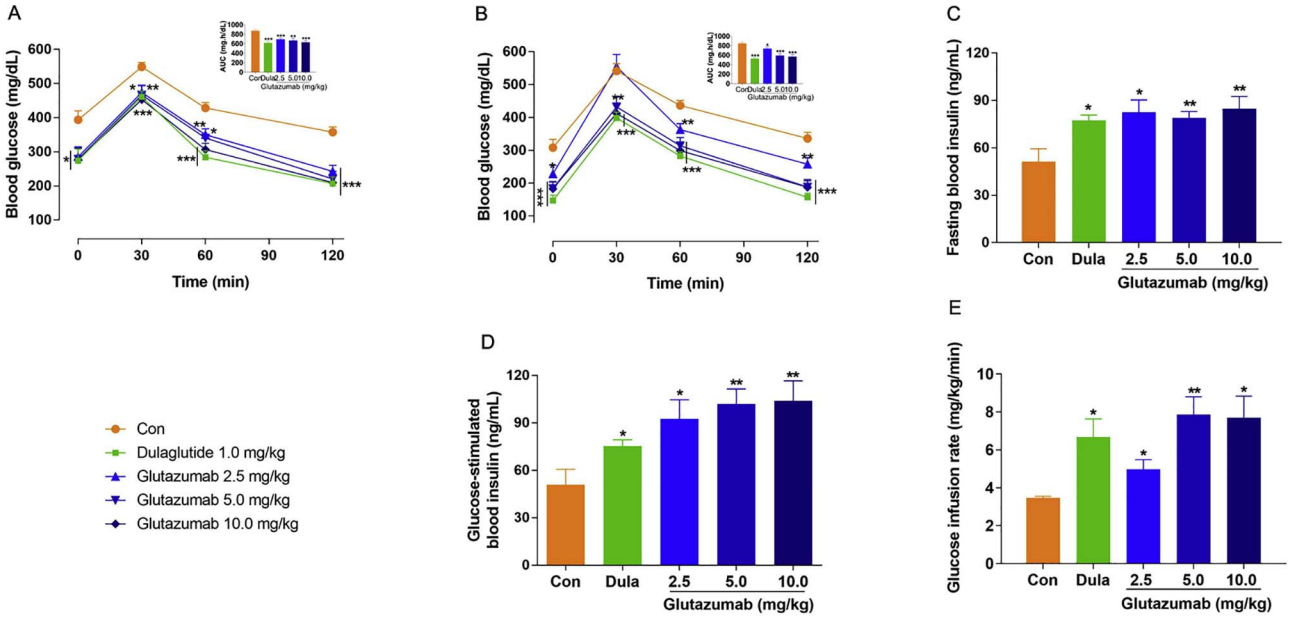

Fig. 5. Effects of glutazumab on β-cell function after repeated injections in diabetic KKAy mice. A) Blood glucose-time curve and AUC for oral glucose tolerance test 9 d after treatment, B) Blood glucose-time curve and AUC for the oral glucose tolerance test 37 d after treatment, C) Fasting blood insulin 15 d after treatment, D) Glucose-stimulated insulin secretion 37 d after treatment, E) GIR in hyperglycemic clamp test. All data are expressed as means ± SEM, n = 11 for A-D, 3-4 for E, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* Con.

GLP-1 was reported to inhibit gastric emptying and to reduce food intake, water consumption and body weight [18]. Delayed gastric emptying also reduced post-prandial blood glucose by delaying entry of nutrients into the circulation and subsequently decreasing post-prandial insulin [19]. O'Donovan's group reported that post-prandial glucose excursion was not determined by total glucose delivered, but by the rate of glucose delivery into the duodenum [20]. In the current study, the effect of glutazumab on gastric emptying was evaluated by measuring blood acetaminophen after gavage (500 mg/kg) [13]. Data show that glutazumab had the characteristics of the natural GLP-1 and inhibited gastric emptying, and the inhibitory effect was long-lasting. Furthermore, glutazumab might also decrease the body weight, food intake or/and water consumption in normal ICR and diabetic KKAy mice, likely due to delayed gastric emptying and increased satiety *via* an indirect vagal and gastric sensory mechanism, or due to a direct effect on the brain [21].

To evaluate the effects of glutazumab on glycemic control and β-cell function, spontaneous diabetic KKAy mice were given glutazumab every three days, then every two days and eventually once daily. To ensure efficacy, the dosing interval was shortened when the effects on glycemic control started to disappear. Reduced efficacy may be due to production of anti-drug antibodies arising from species differences between mice and humans [22]. Data show that repeated injections of glutazumab suppressed glycemic fluctuations, improved the impaired oral glucose tolerance and increased insulin secretion. However, the fasting blood insulin was similar to the glucose-stimulated insulin. We thought this may be related to the hyperinsulinemia of KKAy mice and the time of 30 min after glucose stimulation, likely in the second phase of insulin secretion. Besides, the hyperglycemic clamp test was used to evaluate β-cell function [23]. Glutazumab (2.5–10 mg/kg) significantly improved β-cell function, attenuated glycemic fluctuations and slowed down disease progression.

In summary, glutazumab is a novel and unique G-protein-coupled receptor (GPCR)-targeted antibody fusion protein. Glutazumab can consistently improve β-cell function and reduce glycemic fluctuations, and it may serve as a new treatment for T2DM.

Acknowledgements

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None.

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