

Original Article

A noncytolytic antibody-like extendin-4-IgG4 fusion protein as a long-acting potential anti-diabetic agent

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Abstract: Background: GLP-1 and its analogs have a variety of anti-diabetic effects. However, short half-life and rapid degraded by DPP-IV limits the therapeutic potential of the native GLP-1. So, many DPP-IV-resistant and long-acting GLP-1 analogs were developed. In this study, an antibody-like extendin-4-IgG4 fusion protein was developed. Methods: The $\gamma 4$ constant region contains two amino acid substitutions relative to native $\gamma 4$ (S228P and L235E) lead to affinity for Fc γ RI to be low and stability of the IgG4 molecular. The fusion protein was expressed in CHO cells and assembled into an immunoglobulin-like structure with molecular weight of approximately 130 kDa. Results: The Extendin-4-IgG4 fusion protein was found to affinity bind GLP-1R in vitro. In vivo when compared the potency and duration of glucose-lowering effects in diabetic (db/db) mice at the same dose, exendin-4 resulted in a glucose-lowering effect that persisted only for 6 hours, but the extendin-4-IgG4 fusion protein for more than 168 hours. Injecting subcutaneously with a high dose of the fusion protein led normal BALB/c mice to the lower blood glucose level but did not cause serious hypoglycemia. Especially, the half-life time of the fusion protein in cynomolgus monkeys was about 180 hours, almost the longest half-life time among the developed GPL-1 analogues, which suggested a longer half-life time in human. Conclusions: The intact antibody-like fusion protein has more advantages than the Fc fusion protein including the intent of prolonging the half-life. These results also suggested the fusion protein was a safe and long-acting potential anti-diabetic agent.

Keywords: Extendin-4-IgG4 fusion protein, anti-diabetic agent, long-acting, GLP-1 analog, antibody-like

Introduction

Diabetes is a metabolic disorder characterized by chronic hyperglycemia. On May 16, 2012, a World Health Statistics report was released in Geneva indicating that one-tenth of the adult population worldwide suffers from diabetes (<http://www.cmt.com.cn/>). Diabetes is usually classified as type 1 or type 2. Type 2 diabetes, also termed non-insulin-dependent mellitus diabetes, accounts for about 90% of the total number of diabetic patients. The treatment for type 2 diabetes is aimed at improving insulin resistance and protecting pancreatic islet beta cell function. In addition to insulin, commonly used drugs in the clinic include biguanides, sulfonylurea, glinides, thiazolidinediones, and alpha-glucosidase inhibitors. These drugs have good efficacy in lowering blood glucose levels,

but the most significant problems are that these drugs lack specificity in reducing the function of alpha and beta cells and have significant adverse affects. Approximately 25% of patients with type 2 diabetes switch from oral hypoglycemic agents to insulin or combination insulin therapy. However, the occurrence of insulin resistance desensitizes patients to insulin. Increased doses of insulin and its long-term use may produce hyperinsulinemia, which increases the risk of cardiovascular diseases [1-5].

Incretins have attracted widespread attention because of their unique role in the treatment of type 2 diabetes. These agents include glucagon-like peptide-1 (GLP-1) and glucose-dependent insulin releasing polypeptide (GIP), among which GLP-1 plays a more important role. There

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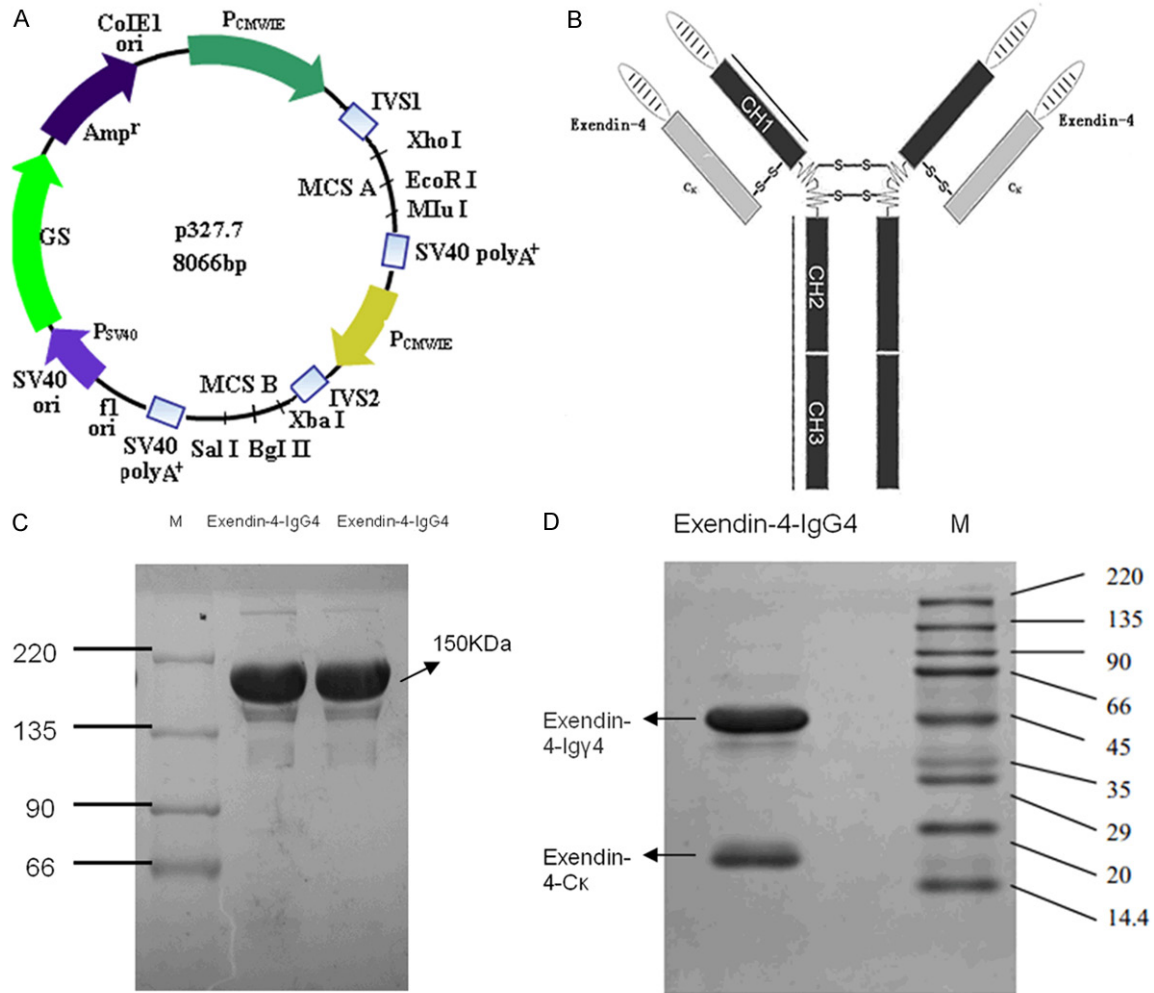


Figure 1. Construction of Exendin-4-IgG4 fusion protein. A. Schematic diagram of the Exendin-4-IgG4 fusion protein expression vector. B. Schematic diagram of the Exendin-4-IgG4 fusion protein. The amino acid sequence of Exendin-4 including the linker is as follows: HGEFTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSGGGGSGGGGGSSGGGS. The $\gamma 4$ constant region contains two amino acid substitutions relative to native $\gamma 4$ (S228P and L235E). C. Non-reducing SDS-PAGE electrophoresis of proteins purified supernatant. D. Reducing SDS-PAGE electrophoresis of purified proteins. Exendin-4-IgG4, Exendin-4-IgG4 fusion protein; M, molecular weight protein markers. The position of the band corresponding to Exendin-4-IgG4 and Exendin-4-Ck was shown (arrow).

are two biologically active forms of GLP-1, GLP-1 (7-37) and GLP-1 (7-36) amide, which differ in sequence in only one amino acid [6]. GLP-1 receptor is widely expressed in various types of cells, including the beta cells of the pancreas, gastric pits, the small intestine, the heart and lung, and the central nervous system. Glucose and fatty acid can stimulate the secretion of GLP-1, which interacts with a specific GLP-1 receptor to exert its effect. Soon after release into the blood, GLP-1 is degraded by dipeptidyl peptidase-IV (DPP-IV) and cleared by the liver and kidney, with a half life of only 1-2 min, therefore limiting the clinical applica-

tion of native GLP-1 [7]. Exendin-4 is a GLP-1 analogue, which is almost identical to GLP-1 in biological effects. Exendin-4 contains 39 amino acids, with a relative molecular mass of 4186.57 [8]. The amino acid sequence of Exendin-4 has 53% similarity with human GLP-1. Both Exendin-4 and GLP-1 bind to the GLP-1 receptor [9]. Exendin-4 can induce beta cell regeneration, promote beta cell proliferation [10-14], and inhibit beta cell apoptosis [15-23], thereby improving the function and increasing the quality of beta islet cells [24, 25]. After binding to the GLP-1 receptor, Exendin-4 increases the intracellular cAMP concentration,

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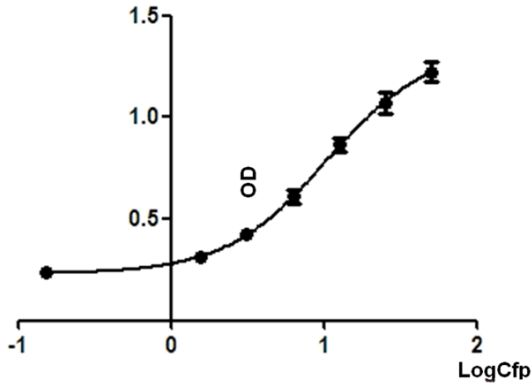


Figure 2. Binding activity of Exendin-4-IgG4 fusion protein with recombinant human glucagon-like peptide 1 receptor (GLP-1R). LogCfp was the logarithmic concentration values of Exendin-4-IgG4 fusion protein, OD was the optical density measured. The 50% effective dose value of Exendin-4-IgG4 fusion protein in vitro binding with recombinant human GLP-1R was determined from this dose-response curve.

promoting insulin secretion from pancreatic beta cells [26]. Exendin-4 can reduce the glucagon level [27] and inhibit glycogenolysis and gluconeogenesis, thus significantly reducing blood glucose levels [28]. Unlike other hypoglycemic agents, Exendin-4 stimulates insulin secretion only under the conditions of normal or high blood glucose, but is non-functional in the case of low blood glucose, which reduces the associated risk of hypoglycemia in patients [29]. Therefore, Exendin-4 is more effective than the existing drugs in restoring the ratio of insulin and glucagon in the portal vein. In patients with type 2 diabetes, Exendin-4 can retard gastric emptying, which may occur directly through the regulation of gastrointestinal motility via GLP-1 receptors on the central nervous system, or indirectly through the delay of gastric emptying via the afferent pathway of the vagus nerve [30]. As a new anti-diabetes agent, Exendin-4 (Exenatide, approved by USFDA, 2005, trade name Byetta), jointly developed by Eli Lilly and Company and Amylin Pharmaceuticals, is a fully chemically synthesized therapeutic drug for type 2 diabetes. The half-life of Exenatide is only 2.4 hours and need to be injected twice a day for the treatment of type 2 diabetes. Currently available GLP-1 analogs were also Liraglutide (approved by EU, Japan, 2009, USFDA, 2010) and Exenatide LAR (approved by USFDA, 2011). Now many GLP-1 analogs were developing, for example Taspoglutide, Lixisenatide, Albiglutide, Du-

glutide (LY2189265), etc. But almost of them were the half-life of no more than 7 days [31].

The aim of this study was tried to create an efficacious, safe and the longest acting potential anti-diabetic agent, like IgG4. Firstly, the Exendin-4-encoding gene was linked to the human antibody heavy-chain constant region ($\gamma 4$) and a light chain constant region C κ encoding gene by the $(G_4S)_3$ linker respectively. Then the expression vector was transfected into CHO cells. Finally, a molecular weight of approximately 130 kDa IgG4-like structure fusion protein was assembled in CHO cell and then excreted into the culture medium, which we refer to as the "Exendin-4-IgG4 fusion protein". Our results suggest that this fusion protein can be easily purified and has improved efficacy over Exendin-4 in terms of the longevity of its effects.

Material and methods

Construction of an expression vector encoding

The gene encoding Exendin-4-Ig $\gamma 4$ and Exendin-4-C κ were synthesized by Shanghai General Biological Engineering Co., Ltd. (Shanghai, China) and cloned into a double expression cassettes glutamine synthetase (GS) expression p327.7 vector by Xho I-EcoR I and Xba I-Sal I (**Figure 1A**) respectively. Exendin-4 was linked to Ig $\gamma 4$ and C κ by the $(G_4S)_3$ linker respectively. The $\gamma 4$ constant region contains two amino acid substitutions relative to native $\gamma 4$. Substitution of glutamic acid for leucine at residue 235 in the CH2 region reduces residual Fc γ RI binding, and substitution of proline for serine at residue 228 in the hinge region, stabilizes disulfide bond interactions between the heavy chains of the mAb [32].

Expression and purification

The stable cell lines were developed by Edmonds MC's method [33]. Briefly, CHO cells were adapted and cultured adherently in CD OptiCHO medium (Invitrogen) supplemented with 10% fetal bovine serum and 4 mM glutamine. The cells were then planted into a T75 flask. After cultured for 24 hours, cells were transfected using Lipofectin 2000 reagent according the manufacturer's instructions (Invitrogen). Forty eight hours after transfected cells were trypsinized and seeded into 96-well

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Table 1. The binding activity of Exendin-4-IgG4 fusion protein at different concentrations (mean \pm SD)

C _{fp} (mg/ml)	50	25	12.5	6.25	3.13	1.56	0.78
OD	1.223 \pm 0.065	1.072 \pm 0.076	0.865 \pm 0.045	0.609 \pm 0.046	0.428 \pm 0.026	0.311 \pm 0.034	0.237 \pm 0.036

C_{fp} indicates concentration of Exendin-4-IgG4 fusion protein.

plates at cell density 1.0×10^4 cells/well. Meanwhile, the medium was replaced with CD OptiCHO selection medium containing 50 μ M L-Methionine sulfoximine (Sigma, USA) but without glutamine. Wells containing single colonies were expanded from the 96-well plates into 24-well plates and then subsequently into 6-well plates. The higher producing transfectants were expanded into T75 flasks in final serum free selection medium and then suspension adapted into 500-mL vented shake flasks. Supernatants were collected from the 24-well plates, 6-well well plates, T75 flasks, 250-mL and 500-mL vented shake flask for initial titer ranking by monoclonal enzyme-linked immunosorbent assay (ELISA) or Protein A HPLC.

The serum-free culture supernatants were harvested. Firstly, a HiTrap MabSelect Sure column (GE Healthcare Life Sciences, USA) was equilibrated with PBS, pH 7.4 solution by adding 10 column-bed volumes. The culture supernatants were filtered using a 0.45 μ m membrane and then loaded onto the column. The column was washed with an additional 5-10 column-bed volumes of PBS solution (pH 7.4), and then proteins were eluted in 100 mM citric acid buffer solution (pH 3.6). Then multistep purification process has been developed to optimize the level of purity and pathogen safety. Chromatography resins and filters used are SP Sepharose FF®, Q Sepharose FF®, and ultrafiltration. The denaturing and non-denaturing SDS-PAGE electrophoresis of purified fusion proteins were performed according to the conventional method.

In vitro characterization

To evaluate the binding activity of Exendin-4-IgG4 fusion protein with GLP-1R, a sandwich enzyme-linked immunosorbent assay (ELISA) was used. The extracellular domains of recombinant human GLP-1 receptors (MyBioSource) were coated onto 96-well EIA/RIA plates to capture Exendin-4-IgG4 fusion protein. Exendin-4-IgG4 fusion proteins diluted into 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 μ g/ml, each concentration was repeatedly measured for 3 times.

Then HRP-labeled goat anti-human IgG (Beijing Zhongshan Biotech Co., Ltd., China) was added. 50% effective dose of Exendin-4-IgG4 fusion protein was calculated using a four-parameter algorithm with Graphad Prism software.

In vivo studies in mice

The Jiangsu Institutional Animal Care and Use Committee approved this research (Approval Number: ACU13-627). For all in vivo studies, the treatments of animals conformed to national guidelines for ethical animal of China. In our studies, all animals only were collected a small amount of blood samples without suffering anesthesia, operation and euthanasia.

All animals were raised in stainless steel cages in the common room (db/db mice in Suzhou Airmite Experimental Animal Center, BALB/c mice in Yangzhou University Medical Academy, and cynomolgus monkeys in JOINN Laboratories, China) feeding fodders twice a day in the morning and afternoon. The cynomolgus monkeys were fed fruits and vegetables once a day. Animals free drank tap water in Chinese National City Drinking Water Sanitation Standard. Animal room temperature was set at 16 to 26°C, humidity 40% to 70%, 12 hours of light and shade alternate. The cages were cleaned once a day and the floor and walls of the room were disinfected with 0.1% Benzalkonium bromide disinfectant or 0.5% of Sodium hypochlorite disinfectant once a week. After the study, all animals were still raised in the provide institutions and not put to death.

To compare effects of Exendin-4-IgG4 fusion protein with Exendin-4, twelve six-week-old male db/db mice (Suzhou Airmite Experimental Animal Center, China) were randomly divided into two groups (n = 6) and acclimatized for one week. The basal levels of blood glucose were measured after 3 h fasting using a Accu-Chek Performa Nano Blood glucose meter (Roche Excellence Tiny). The mice were injected subcutaneously with Exenatide/Byetta (Baxter Pharmaceutical Solutions LLC, USA) 8 nmol/Kg, or Exendin-4-IgG4 fusion protein 8 nmol/Kg. At 1,

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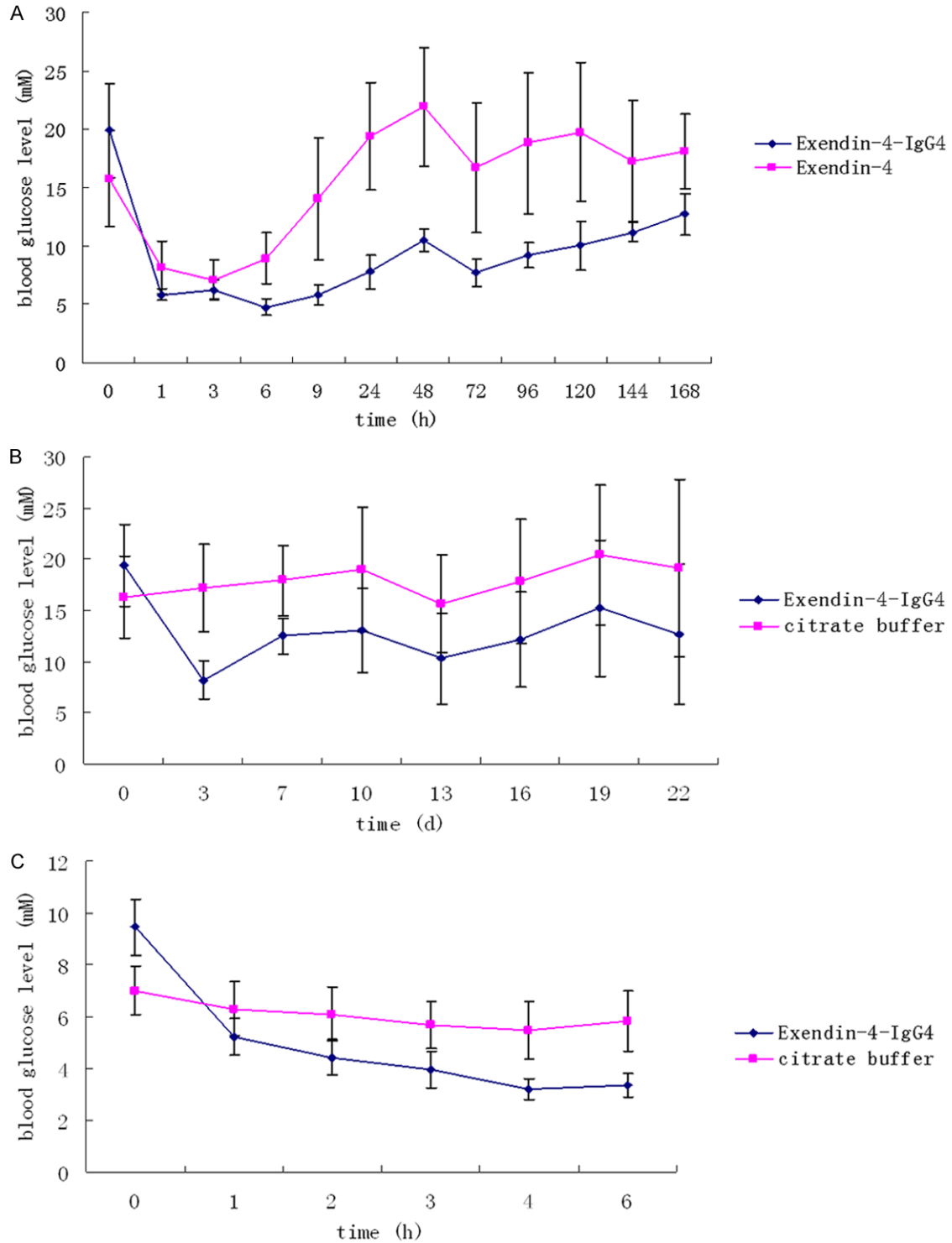


Figure 3. Extended effects of Exendin-4-IgG4 fusion protein in lowering blood glucose levels in normal BALB/c mice and in db/db diabetic mice. Purified Exendin-4-IgG4 fusion protein was injected into db/db diabetic mice (A) or normal BALB/c mice (C), and blood glucose levels were examined over a time course following the injection. These results suggest that is equally effective in reducing blood glucose levels as Exendin-4 shortly after injection, and that the blood-glucose-reducing effect for the fusion protein also has more long-lived effects than Exendin-4. (B) Exendin-4-IgG4 fusion protein administrated once weekly for more than 21 days can still remain the significant blood-glucose-reducing effect.

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Table 2. The blood glucose levels of db/db diabetic mice at per time point after administration (mM) (mean \pm SD)

Time (h)	0	1	3	6	9	24	48	72	96	120	144	168	H	P
Exendin-4-IgG4	19.9 \pm 4.0	5.82 \pm 0.51	6.23 \pm 0.79	4.75 \pm 0.71	5.8 \pm 0.86	7.77 \pm 1.40	10.47 \pm 0.97	7.67 \pm 1.18	9.23 \pm 1.07	10.02 \pm 2.07	11.17 \pm 0.83	12.7 \pm 1.81	61.207	0.0000
Exendin-4	15.8 \pm 4.14	8.17 \pm 2.26	7.07 \pm 1.74	8.90 \pm 2.20	14.05 \pm 5.23	19.4 \pm 4.61	21.95 \pm 5.09	16.73 \pm 5.58	18.82 \pm 6.05	19.75 \pm 5.94	17.3 \pm 5.18	18.12 \pm 3.25	43.371	0.0000

Kruskal-Wallis rank test for comparison among groups.

Table 3. The long-term effects of Exendin-4-IgG4 fusion protein on db/db diabetic mice with once-weekly dosing (mM) (mean \pm SD)

Time (d)	0	3	7	10	13	16	19	22
Exendin-4-IgG4	19.4 \pm 4.02	8.2 \pm 1.9	12.5 \pm 1.73	13.03 \pm 4.11	10.32 \pm 4.47	12.15 \pm 4.6	15.2 \pm 6.69	12.68 \pm 6.85
citrate buffer	16.3 \pm 3.97	17.2 \pm 4.3	17.92 \pm 3.45	18.98 \pm 6.08	15.65 \pm 4.78	17.8 \pm 6.07	20.43 \pm 6.89	19.18 \pm 8.65

Table 4. The blood glucose levels of normal BALB/c mice at per time point after administration (mM) (mean \pm SD)

Time (h)	0	1	2	3	4	6	F	P
Exendin-4-IgG4	9.45 \pm 1.08	5.23 \pm 0.71*	4.43 \pm 0.70*	3.95 \pm 0.71*	3.18 \pm 0.39*, ^Δ	3.33 \pm 0.46*, ^Δ	64.821	0.0000
citrate buffer	7.0 \pm 0.94	6.3 \pm 1.03	6.1 \pm 1.03	5.67 \pm 0.90	5.47 \pm 1.10	5.82 \pm 1.18	0.1690	0.1674

*P \leq 0.01, compared with Time 0 (fasted blood glucose level); ^ΔP \leq 0.05, compared with Time 3 (blood glucose level at 3 h after administration).

Table 5. The plasma concentrations of Exendin-4-IgG4 fusion protein in cynomolgus monkeys after a single SC administration (mean \pm SD)

Time (h)	0.5	1	6	24	48	72	120	168	240	336	408	504	576	672
C _p (ng/ml)	261.20 \pm 73.85	709.44 \pm 26.25	3425.37 \pm 493.11	4958.23 \pm 1693.81	3302.04 \pm 1587.19	2077.52 \pm 360.93	1400.86 \pm 195.94	1219.82 \pm 85.82	1250.01 \pm 114.35	1076.54 \pm 131.10	270.82 \pm 15.89	274.87 \pm 1.63	262.68 \pm 10.10	255.48 \pm 14.38

C_p indicates plasma concentration of Exendin-4-IgG4 fusion protein.

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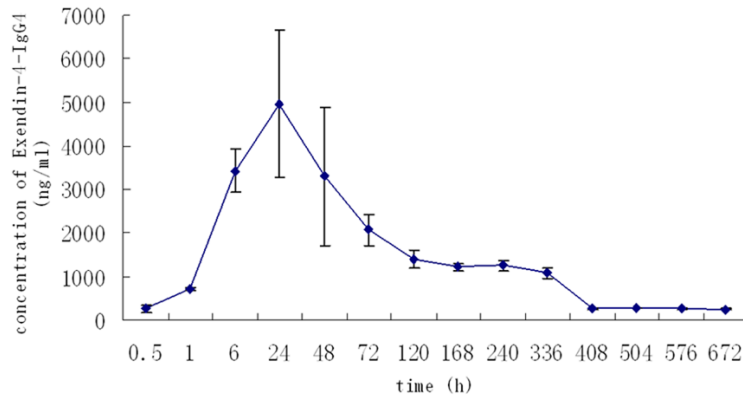


Figure 4. Pharmacokinetics of Exendin-4-IgG4 fusion protein in cynomolgus monkeys. The plasma concentrations of Exendin-4-IgG4 fusion protein in cynomolgus monkeys after a single SC administration at the dose of 0.72 mg/kg.

3, 6, 9, 24, 48, 72, 96, 120, 144 and 168 hours after injection, blood samples were taken from mouse tails and the blood glucose levels were measured. Mice were fasted 9 h after injection but had free access to water.

To examine the long-term effects of Exendin-4-IgG4 fusion protein in controlling the blood glucose levels, twelve six-week-old male db/db mice were randomly divided into two groups ($n = 6$) and acclimatized for one week. The mice were injected subcutaneously with Exendin-4-IgG4 fusion protein 8 nmol/Kg or equal volume of citrate buffer once weekly for 4 times. Fasted blood glucose was measured at 0 (pre-administration), 3, 7, 10, 13, 16, 19 and 22 day as above.

Twelve six-week-old male BALB/c mice (Yangzhou University Medical Academy, China) were randomly divided into experimental and control groups ($n = 6$) and acclimatized for one week. Basic blood glucose levels were measured after 3 h fasting. Exendin-4-IgG4 fusion protein was injected subcutaneously at a high dose of 80 nmol/Kg in 100 μ L citrate buffer, the controls were injected with equal volume of citrate buffer. At 1 h, 2 h, 3 h, 4 h, 6 h after injection, blood samples were taken from mouse tails to measure the blood glucose levels. Mice were fasted throughout the experiment, but had free access to water.

Pharmacokinetics in cynomolgus monkeys

Adult male cynomolgus monkeys (JOINN Laboratories, China) ($n = 3$ /group) received Exendin-4-IgG4 fusion protein at a single sub-

cutaneous (SC) dose of 0.72 mg/0.2 ml/kg, and 0.5 ml blood were collected in the cold tubes containing heparin (31 IU) at 0 (pre-administration), 0.5, 1, 6, 24, 48, 72, 120, 168, 240, 336, 408, 504, 576 and 672 hours after administration. Plasma samples were separated within 2 hours by centrifugation at 2,400 g for 10 min at 4°C, and Exendin-4-IgG4 fusion protein concentration was determined using Exendin-4 Fluorescent EIA Kit (Phoenix Pharmaceuticals, Inc., USA). The

standard curves were prepared for Exendin-4-IgG4 fusion protein in monkey plasma and the concentrations of Exendin-4-IgG4 fusion protein were detected according to the kit instructions. The pharmacokinetic parameters, $t_{1/2}$, T_{max} , C_{max} , $AUC_{(0-t)}$, AUC_{inf} , V_d , Cl and MRT , were calculated using a non-compartment model with WinNonlin V6.2 software. During the experiment, the cynomolgus monkeys were observed mortality, morbidity, respiration, secretion, feces, nausea, vomit, diet and drinking twice a day (morning and afternoon).

Statistical analysis

Homogeneity test of variance was carried out, and then two-way ANOVA was used for blood glucose levels of mice, Kruskal-Wallis rank test for comparing multiple samples with the unequal variances otherwise noted below the table. Mean value, standard deviation and coefficient of variation were calculated by using the Microsoft Office EXCEL. All statistical tests were two tailed with $P \leq 0.05$ set as significant. SAS software (SAS institute Inc., Cary, North Carolina) was used for statistical analysis.

Results

Expression and purification

In 3 weeks, colonies appeared then chose 168 single colonies from the 96-well plates into 24-well plates.

Step by step, the highest-producing colony was chosen among the 168 single colonies. Once a high-producer was identified, fed-batch studies

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Table 6. Pharmacokinetic parameters of Exendin-4-IgG4 fusion protein in cynomolgus monkeys (mean \pm SD)

Monkeys	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (μ g/ml)	AUC_{last} (h mg/ml)	AUC_{inf} (h mg/ml)	Vd (ml/kg)	Cl (ml/h/kg)	MRT (h)
1	184.24	24	6.77	0.78	0.85	225.11	0.85	168.29
2	169.39	24	4.69	0.72	0.78	225.07	0.92	186.88
3	169.78	6	3.89	0.64	0.71	249.01	1.02	190.75
Mean	174.47	18.00	5.12	0.72	0.78	233.07	0.93	181.97
SD	8.46	10.39	1.49	0.07	0.07	13.81	0.09	12.01
CV	4.85	57.74	29.10	9.49	9.10	5.93	9.17	6.60

Pharmacokinetic parameters were determined from the mean plasma concentration data from three animals per time point. C_{max} indicates maximal observed plasma concentration; T_{max} indicates time of maximal observed plasma concentration; AUC_{last} indicates area under the plasma concentration curve from zero to last observed time; AUC_{inf} indicates area under the plasma concentration curve from zero to infinity; $t_{1/2}$ indicates elimination half-life; Cl indicates clearance as a function of bioavailability; Vd indicates apparent volume of distribution at steady state as a function of bioavailability; MRT indicates mean residence time.

were carried out in 7.5-L bioreactors to demonstrate if the high growth and titers obtained in batch cultures led to further improved productivity with nutrient feeding and scale up. Specific productivities could reach 350 mg/L.

Like IgG, Exendin-4-IgG4 and the Exendin-4-Ck were shown to become assembled via disulfide linkage to compose an (Exendin-4-IgG4)₂ (Exendin-4-Ck)₄ fusion protein with tetramer structure (**Figure 1B**) and a molecular weight of 130 kDa. Non-reducing SDS-PAGE electrophoresis and reducing SDS-PAGE electrophoresis of the purified Exendin-4-IgG4 fusion protein were performed to verify expression and to determine the apparent molecular weight of the expressed protein. The Exendin-4-IgG4 fusion protein was approximately 150 kDa as determined by non-reducing SDS-PAGE electrophoresis (**Figure 1C**). The reducing SDS-PAGE electrophoresis showed a 50 kDa band indicative of the expression of Exendin-4-IgG4 and a 15 kDa band indicative of Exendin-4-Ck (**Figure 1D**), suggesting the formation of a higher level structure of Exendin-4-IgG4 fusion under native conditions, consistent with theoretical predictions.

In vitro characterization

We used a sandwich enzyme-linked immunosorbent assay to evaluate the binding activity of Exendin-4-IgG4 fusion protein with GLP-1R. The fusion protein can bind with the extracellular domain of recombinant human GLP-1R, the binding activity was increased with the increasing Exendin-4-IgG4 fusion proteins with a 50%

effective dose of 10.23 μ g/ml (**Figure 2; Table 1**).

In vivo studies in mice

In order to examine the effects of the Exendin-4-IgG4 fusion protein and to compare its effect with equimolar Exendin-4, glucose levels were measured following injection into db/db diabetic mice (8 nmol/Kg). Exendin-4 and Exendin-4-IgG4 fusion protein each showed a significant effect in reducing blood glucose levels in db/db diabetic mice 1-3 hours after injection ($F = 100.182$, $P < 0.001$). However, the effects of the two forms of the proteins differed at later time points. The average blood glucose level reached a lowest value of 7.1 mmol/L at 3 h after administration with Exendin-4, then gradually rose to even higher than the basal levels after 9 hours and had a rebound peak level between 24 h and 48 h, and the effect of Exendin-4 in lowering blood glucose levels disappeared. In contrast, injection with Exendin-4-IgG4 fusion protein cause a significantly decreased the blood glucose level at 1 h that reached the lowest level of 4.75 mmol/L at 6 h and then was stably maintained under 13.0 mmol/L throughout the 168 hours time course after injection (**Figure 3A; Table 2**). If once-weekly dosing of db/db diabetic mice with 8 nmol/Kg Exendin-4-IgG4 fusion protein for more than 3 weeks resulted in consistently lowered plasma glucose over the 3-week period compared to that of controls ($F = 10.811$, $P < 0.01$) (**Figure 3B; Table 3**). These results suggest that the Exendin-4-IgG4 fusion protein is equally effective in reducing blood glucose lev-

els as Exedin-4, and that the blood-glucose-reducing effect for the fusion protein also has longer acting than Exendin-4.

In order to further study the effects and safety of a high dose of Exendin-4-IgG4 fusion protein in normal mice, BALB/c mice were injected with a higher dose of Exendin-4-IgG4 fusion protein (80 nmol/Kg). The blood glucose levels in the control group (injected with citrate buffer solution) remained stable ($P = 0.167$), fluctuating between 5.5-7.0 mmol/L; However, at one hour after injection with a high dose of Exendin-4-IgG4 fusion protein, the blood glucose levels decreased significantly ($F = 29.033$, $P < 0.001$) (**Figure 3C**; **Table 4**). At 4 hours after injection, the blood glucose level dropped to 3.2 mmol/L and then maintained a steady state ($P > 0.05$). No serious hypoglycemia was observed. These results show that the Exendin-4-IgG4 fusion protein is very safe and does not induce serious hypoglycemia in high subcutaneous doses.

Pharmacokinetics in cynomolgus monkeys

After a SC dose of 0.72 mg/kg Exendin-4-IgG4 fusion protein, the plasma concentrations of Exendin-4-IgG4 fusion protein reached the peak value between 6 h and 24 h and disappeared slow (**Figure 4**; **Table 5**). The pharmacokinetic profile of Exendin-4-IgG4 fusion protein in cynomolgus monkeys was summarized in **Table 6**. The average values of $t_{1/2}$, T_{max} , C_{max} and AUC_{last} were 174.47 ± 8.46 h, 18.00 ± 10.39 h, 5.12 ± 1.49 $\mu\text{g/ml}$ and 0.78 ± 0.07 h mg/ml respectively. The half-life of Exendin-4-IgG4 fusion protein after a single dose of 0.72 mg/kg was more than 174 hours in three monkeys (**Table 6**). Throughout the experiment, the monkeys showed no abnormalities in mental state and behavioral activities, no monkey died during the experiments, and Exendin-4-IgG4 fusion protein was well tolerated.

Discussion

The anti-diabetes drug Exendin-4 (Exenatide, trade name Byetta) is a new class of injectable type 2 diabetes drug, which was jointly developed by Eli Lilly and Company and Amylin Pharmaceuticals. It was approved by the US Food and Drug Administration (FDA) in 2005. On January 27, 2012, the FDA approved a sus-

tained-release dosage form of Exenatide, which was the first once-a-week therapeutic drug in the treatment of type 2 diabetes [34]. Dulaglutide (LY2189265) a GPL-1 analogue covalently linked to a constant fragment (Fc) of a human immunoglobulin class 4 (IgG4) has almost completed the clinical trials [35]. Others GPL-1 analogue were developing including Lixisenatide, Albiglutide as well as CJC-1134-PC. CJC-1134-PC was a recombinant human serum albumin-exendin-4 conjugated protein [36]. In this study, we describe the engineering of Exendin-4-IgG4 fusion protein, a recombinant fusion protein linking the Exendin-4-encoding gene and the genes encoding the human antibody heavy-chain constant region ($\gamma 4$) and a light chain constant region C κ respectively. There are four Exendin-4 functional domains in the Exendin-4-IgG4 fusion protein. Exendin-4-IgG4 fusion protein bound to the recombinant human GLP-1R in vitro, and reduced blood glucose levels in vivo. Given that the half-life of IgG4 immunoglobulin is up to 21 days. This might explain a 174-hour half-life in monkeys and the prolonged effect in reducing blood glucose levels in db/db diabetic mice of the Exendin-4-IgG4 fusion protein as compared to non-fused Exendin-4. The fusion protein is expressed in the CHO cell system, which may have an advantage over prokaryotic systems that often do not facilitate the proper folding and modification of eukaryotic proteins. Mass production of Exendin-4-IgG4 fusion protein may be possible through large-scale mammalian cell expression technology, which could effectively reduce the production costs, lower the price to an acceptable level by patients, and provide a treatment of equal or greater effectiveness.

Human IgG1 Fc has been widely used as a bioconjugate, but exhibits shortcomings, such as antibody- and complement-mediated cytotoxicity, when applied to agonistic proteins. The actions of GLP-1 are mediated through the GLP-1 receptor (GLP-1R), which is a seven-transmembrane G protein-coupled receptor (GPCR) expressing on pancreatic β cells. It is important to assure noncytolytic in the treatment of type 2 diabetes. Here, we constructed a non-immunogenic, noncytolytic and flexible IgG4-like fusion protein. The rationale for choosing IgG4 among the four subclasses of IgG in the present study is that IgG4 has the

weakest binding affinity to C1q, so that activation of classical pathway of complement and complement-dependent cytotoxicity (CDC effects) are negligible [37]. Human IgG4 binds Fc γ RI (high-affinity Fc receptor) with a 10-fold lower affinity than IgG1 [38] and does not bind to monocytes/macrophages, which results in that IgG4 does not mediate antibody-dependent cellular cytotoxicity (ADCC effect) [32]. However, the disadvantage of IgG4 is that the dimers that form are not entirely stable and have some ability to bind to Fc γ R on effector cells (T lymphocytes, neutrophils and monocytes). S228P and L235E mutations were introduced to the amino acid residues in IgG4 hinge and CH2 constant regions (The Fc amino acid residue is numbered according to the Kabat database), and effectively overcome these drawbacks of the IgG4 molecule. Leu248 was mutated to Glu in the IgG4 version to yield a mAb with a predicted affinity for Fc γ RI to be 1000-fold lower than the IgG1 mAb. Further, Ser241 was replaced by Pro to enhance stability of the IgG4 heavy chain dimer. Reddy MP and Parekh BS had proved this structure was lack of binding to complement component C1q, and inability to mediate complement-dependent cytotoxicity [32, 39]. Exendin-4-IgG4 fusion protein is a noncytolytic protein to avoid damages to pancreatic β cells.

The interaction between therapeutic antibody and neonatal Fc receptor (FcRn), which is one of the critical factors in determining the circulating antibody and albumin half-life [40]. FcRn regulates pH dependent on intracellular trafficking of immunoglobulin G (IgG) and albumin, resulting in enhanced serum persistence and transcellular permeability of these proteins compared to other proteins of similar size. Based on the functional roles of FcRn in regulating serum persistence and transcellular permeability, protein engineers have sought to exploit this receptor as a means of enhancing the absorption, distribution, metabolism and excretion of IgG-based therapeutics. Nearly 350 IgG-based therapeutics are approved for clinical use or are under development for many diseases lacking adequate treatment options. These include molecularly engineered biologicals comprising the IgG Fc-domain fused to various effector molecules (so-called Fc-fusion proteins) that confer the advantages of IgG, including binding to the neonatal Fc receptor (FcRn) to facilitate in vivo stability, and the therapeutic benefits of the specific effector func-

tions [41]. As to GLP-1analogs, at least two types of Fc-fusion proteins were developed. One is Fc of IgG4 fusion protein (Dulaglutide) [35], the other one is Fc of IgG2 fusion protein [42]. To reduce interactions of GLP-Fc with high-affinity Fc receptors, Dulaglutide was also introduced mutations F234A and L235A as we did. But the half-life of Dulaglutide in cynomolgus monkeys is 51.6 ± 3.2 hours and much less than the 174.47 ± 8.46 -hour half-life of Exendin-4-IgG4 fusion protein, the half-life of Dulaglutide in human is 90 h [31], so we suggested Exendin-4-IgG4 fusion protein is maybe longer half-life time than 180 h in human being. Obviously, the Exendin-4-IgG4 fusion protein in the half-life is far longer than Dulaglutide, which is due to the size of whole immunoglobulin G more than the fragment of Fc. Although the Fc domains are used with the intent of prolonging the half-lives of proteins, the half-lives tend not to be fully prolonged to the level of IgG [43].

No experimental monkey or mouse was observed the abnormalities in mental state and behavioral activities. All of these results indicated the potential efficacious, safety and well tolerability of Exendin-4-IgG4 fusion protein. Together, the long-acting Exendin-4-IgG4 fusion protein retains native GLP-1 activities and thus may serve as a potent GLP-1 receptor agonist.

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Disclosure of conflict of interest

None.

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