Pharmacological Research 122 (2017) 130–139

Contents lists available at ScienceDirect

Pharmacological Research

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

Perspective

BPI-3016, a novel long-acting hGLP-1 analogue for the treatment of Type 2 diabetes mellitus

Lieming Ding[a], Sisi Lu[a], Yanping Wang[b], Haibo Chen[a], Wei Long[c], Cunbo Ma[b], Erlong He[a], Dan Yan[a], Fenlai Tan[a,*]

[a] Department of Preclinical Research, Betta Pharmaceuticals Co., Ltd, Beijing, China
[b] Department of Preclinical Research, Beijing Jacobio Pharma Co., Ltd, Beijing, China
[c] Department of Immunology, Peking University School of Basic Medical Science, Beijing, China

ARTICLE INFO

Article history:
Received 23 February 2017
Received in revised form 10 May 2017
Accepted 11 May 2017
Available online 13 June 2017

Chemical compounds studied in this article:
NN9535 (PubChem CID: 56843331)
Liraglutide (PubChem CID: 16134956)
Exenatide LAR (PubChem CID: 45588096)

Keywords:
BPI-3016
Type 2 diabetes
Long-acting GLP-1 analogues

ABSTRACT

Glucagon-like peptide-1 (GLP-1) analogues have been commonly used as add-on medications for patients with Type 2 diabetes mellitus (T2DM). Currently, the development of long-acting GLP-1 analogues which allow the freedom and flexibility of once-weekly injections while maintaining their potency for a relatively long period has become the mainstream. Here, we successfully developed a long-acting human GLP-1(7–37) analogue (BPI-3016) with significantly extended half-life and increased resistance to dipeptidyl peptidase IV (DPP-IV) cleavage by structural modifications of human GLP-1. *In vitro* activity of BPI-3016 including GLP-1 receptor affinity and stimulation of cyclic adenosine monophosphate (cAMP) production was measured. *In vivo* activity of BPI-3016 such as its effects on glycemic control, β-cell mass and body weight was evaluated in *ob/ob* mice, *db/db* mice, and spontaneous diabetic cynomolgus monkeys. The results indicated that BPI-3016 preserved receptor affinity to GLP receptors, and was capable of stimulating cAMP production. In *in vivo* pharmacokinetic study, the half-life of BPI-3016 was more than 95 h after single dosing in diabetic cynomolgus monkeys. Also, BPI-3016 reduced fasting and postprandial plasma glucose levels for up to a week after a single dose; It reduced body mass index (BMI), body fat, improved glucose tolerance and showed insulinotropic effects after once-weekly injection for 7 weeks. In conclusion, BPI-3016 retains the effects of GLP-1 with significantly prolonged half-life, making it a promising therapy for type 2 diabetes with once-weekly treatment in the clinic.

1. Introduction

Progressive β-cell dysfunction and insulin resistance represent major pathological events evolved during the development of hyperglycemia in type 2 diabetes mellitus (T2DM) [1,2]. Multiple therapeutic agents such as some oral medicines and/or exogenous insulin targeting on restoring β-cell function, likely through reducing β-cell workload or inducing β-cell rest, have been developed in recent decades. However, many conventional treatments for T2DM cannot significantly improve β-cell function which also undesirably bring some side effects such as weight gain, increased hypoglycemia risk and peripheral hyperinsulinemia [3–5]. Thus, a lot of recent studies were stimulated to develop novel drugs which could remarkably improve β-cell function and meantime avoid the commonly-seen side effects. The incretin drugs GLP-1 analogues which are used to reduce the level of blood glucose mainly *via* suppressing glucagon secretion and increasing insulin secretion, become ideal candidates for T2DM therapy because they are usually well-tolerated without causing severe adverse effects. They can reduce weight by slowing gastric emptying and reducing appetite, and typically have a low risk of hypoglycemia [6,7].

GLP-1 is produced in enteroendocrine cells in the distal small bowel and colon [8]. Its plasma level rapidly increases in response to food intake to reduce circulating glucose through promoting glucose concentration-dependent insulin secretion and suppressing glucagon secretion [9]. Bioactive GLP-1 generated from GLP-1(1–37) exists as two equipotent circulating molecular forms, GLP-1(7–37) and GLP-1(7–36) amide, in which GLP-1(7–36) amide represents the majority of circulating active GLP-1 in human plasma [10]. The plasma half-life of native GLP-1 is very short due to rapid degradation by DPP-IV and neutral endopeptidase 24.11(NEP) [11,12]. Therefore, engineering GLP-1 analogues which retain the biological activity of native GLP-1 with stronger resistance to cleavage of the enzymes became one promising strategy. Current FDA-approved GLP-1 analogues include two once or twice-daily administration ones (Exenatide and Liraglutide) and three

* Corresponding author.
E-mail address: fenglaitan789@gmail.com (F. Tan).

http://dx.doi.org/10.1016/j.phrs.2017.05.007
1043-6618/

once-weekly administration ones (Albiglutide, Dulaglutide, and Exenatide LAR). Once-weekly drugs can be injected at any time of the day, avoiding food-drug interaction commonly observed in their oral formulations, which reduce the number of injections and potentially improve patient compliance. To improve the safety and efficacy of GLP-1 analogues usage, substantial effort has been put into developing once-weekly forms of GLP-1 analogues.

The amide bond (—NHCO—) of Ala^8 at N-terminal in GLP-1(7–37) or GLP-1(7–36) is very susceptive to DPP-IV cleavage [13]. Theoretically, modifications on N-terminal of GLP-1 can lead to resistance to DPP-IV inhibition, thereby prolonging half-life and efficiency. As reported in our previous research study, we engineered a novel GLP-1(7–36) analogue BPI-3006 by structurally substituting the-NHCO-of Ala^8 of native GLP-1(7–36) with a connected unit containing -CF_3- [14]. Similarly, here we develop a GLP-1(7–37) analogue BPI-3016 with the same structural substitution of N-terminal and other modifications including amino acid replacement at position 34 (lysine to arginine) and acylation of the lysine in position 26 with a C-18 fatty acid chain. The conjugation of long fatty acids to peptides overcomes the degradation of DPP-IV and prevents native GLP-1 from renal filtration through facilitating binding to plasma protein, such as albumin, and minimizing elimination by the kidney [15]. The significantly extended half-life of native GLP-1 makes it possible for once-weekly administration for type 2 diabetes. In this study, we evaluated the *in vitro* biological activities of BPI-3016 by assessing its affinity to GLP-1 receptors, stimulation of cAMP production and resistance to DPP-IV degradation. Furthermore, we described the *in vivo* activities of BPI-3016 on plasma glucose level, insulin secretion, body weight, glucose tolerance, and pancreatic β-cell functions in three typical diabetic animal models including *ob/ob*, *db/db* mice and spontaneous diabetic cynomolgus monkeys.

2. Materials and methods

2.1. Peptides

The GLP-1, BPI-3016, and NN9535 were synthesized, purified and characterized by Betta Pharmaceuticals Co., Ltd (China) (peptide sequence of BPI-3016 seen in Fig. S1). GLP-1 used for GLP-1 receptor binding experiment was obtained from HD Biosciences Co., Ltd (Shanghai, China). The GLP-1 analogue Liraglutide used for *in vitro* study was provided by Betta. Liraglutide (Victoza) and Exenatide LAR (Bydureon) used in animal studies were purchased from Novo Nordisk and AstraZeneca, respectively. For all experiments, BPI-3016 was dissolved in 0.01 M Na_2HPO_4 buffer (pH = 8 ~ 9). For *in vivo* studies, BPI-3016 was diluted by saline or 0.01 M Na_2HPO_4 buffer (pH = 7.96).

2.2. Cell culture and cell transfection

HEK293 cells (ATCC) were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% (v/v) FBS, 100 units/mL of penicillin and 100 μg/mL streptomycin at 37 °C, 5% (v/v) CO_2 humidified environment. To transfect HEK293 cells with the human GLP-1, Lipofectamine™ 2000 agent was used according to the manufacturer's protocol after the cells were seed at about 70% confluency.

2.3. Measurement of binding affinity to GLP-1 receptor by radioactive ligand binding assay

A competitive radioactive ligand binding assay was applied to obtain BPI-3016 inhibitory constant Ki, a parameter to reflect binding affinity to the GLP-1 receptor. HEK293 cells transfected with human GLP-1 receptor were homogenated for 10 s in 15 mL ice-cold binding buffer (50 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl). The cell homogenate was centrifuged at 18000*g* for 20 min. The precipitate contained cell membrane was resuspended with 1 mL ice-cold binding buffer. Peptide stock solutions (1 mM) were serially diluted by binding buffer containing 0.1% BSA (pH = 7.4). 15 μL of peptides solution (300 pM–10 μM, 10×), 15 μL of ^{125}I-GLP-1(2.5 nM, 10×) (Perkin Elmer) and 120 μL cell membrane homogenate were incubated for 1 h in 96-well plates at room temperature. In addition, 15 μL binding buffer or GLP-1 (1 μM) were incubated with ^{125}I-GLP-1 and homogenate of cell membrane in 96-well plate as total binding or non-specific binding controls. After incubation, the reaction mixture was filtrated into a 96-well filter plate (Perkin Elmer) by cell harvester (FilterMate Universal Harvester, PerkinElmer). The plates were washed with HEPES buffer for 3 times and dried at 37 °C overnight. The radioactivity (corrected counts per minute, CCPM) in each well was subsequently analyzed by a microplate scintillation counter (1450 MicroBeta TriLux, PerkinElmer). The inhibitory receptor-binding rate of ^{125}I-GLP-1 was calculated according to below formula:

Inhibitory binding rate (%) = $(CCPM_{compound} - CCPM_{non\text{-}specificcontrol}) \times 100/(CCPM_{totalbindingcontrol} - CCPM_{non\text{-}specificcontrol})$.

Data were analyzed by Graph Pad Prism 5.0, and the value of Ki was calculated with IC_{50}.

2.4. Determination of BPI-3016-induced cAMP production

DPBS buffer [supplemented with 0.1% (w/v) BSA and 0.5 mM IBMX] was used to dilute GLP-1 and other peptide stock solutions into 200 nM. Three-fold serial dilution was performed to obtain GLP-1 and BPI-3016 solutions at different concentrations. The resuscitated HEK 293/GLP1R/CRE/LUCIFERASE cells (HD Biosciences Co., Ltd.) were collected by centrifugation at 900*g* for 5 min and resuspended in DPBS buffer. Then the cells were diluted to a density of 1.6×10^5/mL and transferred (5 μL) to the 384-well plates. Peptides (5 μL) with various concentrations (0.042 pM–200 nM, 2×) were subsequently added to the cells. After 30 min incubation at 37 °C, cAMP was measured using cAMP Dynamic 2 Kit (Cisbio bioassays) according to protocol instructions. HTRF signal was read at 665 nm and 615 nm on EnVision plate reader (PerkinElmer). The readout counts were converted to cAMP concentrations according to the cAMP standard curve. Data were analyzed by Graph Pad Prism 5.0.

2.5. Degradation of BPI-3016 by DPP-IV

BPI-3016, Liraglutide and NN9535 (50 μM) were incubated with DPP-IV purified from pig kidney (Sigma-Aldrich) (100 U/L) in Tris–HCl (pH = 8.0) for 0, 1, 8, 24, 32 and 48 h in water bath at 37 °C. To compare the resistance to DPP-IV among the above GLP-1 analogues and native GLP-1, GLP-1 (50 μM) was incubated with DPP-IV for 0, 0.25, 0.5, 0.75 and 1 h. All the above enzymatic reactions were terminated by adding trifluoroacetic acid (TFA, 10%). Samples for 0, 1 or 48 h without DPP-IV were used as controls. The above processed samples were stored at −20 °C for future evaluation of parental peptide by a Kromasil 300-5 C4 column (Akzo Nobel). The absorbance was monitored at 214 nm using a photodiode array (PDA) UV detector (Agilent Technologies) and the peak area was analyzed. The results were expressed as relative content of parental peptide.

2.6. Animals

To determine the pharmacodynamic effects of BPI-3016 *in vivo*, 60 *ob/ob* (B6.Cg-Lep^{ob}/J, 30–45 g) and 12 wild-type mice (15–25 g) with half males and half females were obtained from Shanghai Institute of Materia Medica; 32 male *db/db* (BKS.Cg-

Dock7$^{m+/+}$ Lepr^{db}/JNju) and 8 m/m mice aged 6–8 weeks, were purchased from Model Animal Research Center of Nanjing University. Mice were housed as one per cage and maintained in specific pathogen free (SPF)-class experimental animal room (23.0 ± 1.0 °C, 50–70% relative humidity, 12 h light/dark cycle). *ob/ob* mice were fed with high-fat chow, all other types of mice were fed with standard chow. All mice were fed with sterilized water *ad libitum*. 38 male spontaneous diabetic cynomolgus monkeys (fasting glucose: 94–300 mg/dL, glycated hemoglobin(HbA1c) ≥ 4%, $K_{Glucose}$ < 2%) and 6 normal cynomolgus monkeys with weights around 7–15 kg and age of 13–23 years old were purchased from Kunming Yaling Biological Technology Co., Ltd and individually housed in a controlled environment(18–29 °C, 30–70% relative humidity, 12 h light/dark cycle). Diabetic and healthy monkeys were fed with a fixed amount of standard diet. All animal studies were conducted according to the standards for laboratory animals established by the People's Republic of China (GB14925-2001). And the study on cynomolgus monkeys was approved by the Institutional Animal Care and Use Committee of Kunming Biomed International (KBI) (Protocol No.KBI K001115002-01,01).

2.7. Pharmacokinetics in spontaneous diabetic cynomolgus monkeys

Spontaneous diabetic cynomolgus monkeys received a single subcutaneous dose of 0.2 mg/kg BPI-3016, then blood samples collected at −2 (pre-administration), 2, 8, 22, 46, 94, 118, 142, and 166 h after administration were stored at −80 °C for analysis. The plasma concentration of BPI-3016 at each time point was measured by the electrospray ionization LC–MS/MS system (LC-30AD, Shimadzu; AB 6500, AB Sciex). This chromatographic separation system consisted of a guard column (C18, Phenomenex), an analytical column (XB-C18, Phenomenex), and a gradient mobile phase of 5 mM ammonium acetate (phase A) and acetonitrile (phase B). All the analytical data were processed by the Analyst software (version 1.6, AB Sciex). The pharmacokinetic parameters of BPI-3016 were obtained from non-compartmental analysis with Phoenix (version 1.3, Pharsight).

2.8. Blood glucose and insulin level measurement

7 week-old *ob/ob* mice were randomized into 5 groups (n = 12/group), and the 12 wild-type mice were treated as normal control group. To specifically demonstrate the short term effect of BPI-3016, random glucose levels were monitored 0, 2, 4, 6, 10, 24, 30, 34 and 48 h after subcutaneous administration with saline (vehicle control), BPI-3016 (0.5, 1, 2 mg/kg), or Liraglutide. For evaluation of the long-term effect of BPI-3016, saline and BPI-3016 were administered subcutaneously every other day over 31 days (administration at Day 21 was skipped) respectively, and Liraglutide (0.1 mg/kg, positive control) was administered twice daily in the same period (without drug withdrawal). Random blood glucose levels of *ob/ob* and wild-type mice were determined by ACCU-CHEK® Performa glucose meter (Roche Diagnostics). Fasting serum insulin levels of *ob/ob* mice were determined by enzyme-linked immunosorbent assay (ELISA) kit (Crystal Chem) on Day 30.

db/db mice were randomized into four groups (n = 8): saline (vehicle control), low-dose and high-dose BPI-3016, and NN9535 were treated accordingly. Animals received subcutaneous injection of vehicle (normal saline), BPI-3016 (0.2, 0.5 mg/kg) or NN9535 (0.2 mg/kg) every other day over a 8-week period. And 8 m/m mice treated with saline were used as normal control. In this experiment, the fasting blood glucose levels in each group were measured by ACCU-CHEK® Integra glucose meter (Roche Diagnostics).

38 spontaneous diabetic cynomolgus monkeys were randomly divided into 5 groups according to body weight, fasting blood glucose, HbA1c levels and $K_{Glucose}$ (Table. S1): the diabetic control group (n = 8) was treated with phosphate buffer, the positive control group (n = 8) received 0.15 mg/kg Exenatide LAR, and the other three groups received BPI-3016 at 0.1 mg/kg (n = 6), 0.2 mg/kg (n = 6) and 0.4 mg/kg (n = 10). Additionally, 6 obese monkeys at the same age with normal blood glucose were selected as normal obese control group and treated with the vehicle. All the animals received once-weekly subcutaneous administrations for 7 times. Fasting and post-prandial plasma glucose levels of diabetic and normal cynomolgus monkeys were monitored before 7th administration by Roche cobas c311 automatic biochemical analyzer.

2.9. Measurement of HbA1c level

In the 8-week study of *db/db* mice, HbA1c was measured by NycoCard Reader II HbA1c detector (Axis-Shield) at Day 0 and the 8th week. And the measurements of HbA1c level of diabetic cynomolgus monkeys were performed before and after 6 times dosing, whose blood samples were collected for HbA1c measurements using the Roche C311 biochemical analyzer.

2.10. Glucose tolerance in spontaneous diabetic cynomolgus monkeys

Pre-dosing and post-dosing Intravenous Glucose Tolerance Test (IVGTT) procedures were conducted in the study of cynomolgus monkeys. And the post-dosing IVGTT was performed at 22 h after the 7th treatment. Animals were anesthetized after fasted for 16 h. Blood samples were collected for basal plasma glucose measurement. Then 50% glucose solutions were injected intravenously according to body weight (0.5 g glucose/kg body weight) of monkeys. Blood samples were collected at 1, 3, 5, 10, 20, 40, and 60 min upon completion of the intravenous glucose load to measure the level of plasma glucose and insulin. Glucose tolerance was evaluated by calculating the rate of glucose disappearance ($K_{Glucose}$) during 10–40 min after an exogenous glucose load:

$$K_{Glucose} = (Glu_{10\,min} - Glu_{40\,min})/30 * Glu_{10\,min}.$$

2.11. BMI and body fat measurement in spontaneous diabetic cynomolgus monkeys

Body Mass Index (BMI, kg/m^2) calculated by dividing the body weight with the square of the height was assessed before and after the 7-time dosing period for each animal. Whole body scans were conducted by dual energy x-ray absorptiometry (DEXA) twice, before and after 7-time dosing, using the Hologic Discovery QDR Series (Bedford, MA) Densitometer to measure body fat. The animals were sedated with Zoletil (3 mg/kg, IM) and then securely positioned within the scanning region on the DEXA bed to perform scanning. The total and regional body compositions of monkeys were analyzed by Hologic Body Composition Software according to established guidelines.

2.12. Histological assessment of β-cell mass

9 male *ob/ob* mice from vehicle control, BPI-3016 and Liraglutide group were sacrificed to obtain their pancreas which were weighed, fixed in the 10% formaldehyde and embedded in paraffin. The pancreas was sliced into 3 μm-thick section from the head to tail. Four representative sections (each 1st specimen of consecutive 35 sections) for each pancreas were used for islet insulin staining. The sections were deparaffinized by xylene and rehydrated and

then blocked with normal goat serum (5%, v/v) for 45 min. Guinea pig polyclonal anti-insulin antibody (Abcam; 1:100) and rabbit polyclonal anti-guinea pig IgG antibody labeled by horseradish peroxidase (Abcam; 1:150) were used as the primary and secondary antibody, subsequently. Then, the sections were washed by Tris-buffered saline(containing 0.1% Tween-20) and covered by diaminobenzidine substrate solution for 15 min. Then the sections were washed again and counterstained with hematoxylin. The sections were observed with a NIKON microscope and analyzed by Image J software (National Institutes of Health). β-cell mass was calculated by percentage of β-cell area in the pancreas and the weight of the pancreas before fixation, according to the following formula:

$$\beta\text{-cell mass (mg)} = [\text{area immunostained/cross-sectional area of total section} * 100\%] \times \text{weight of pancreas (g)} * 1000$$

$$\text{weight percentage of } \beta\text{-cellmass} = \beta\text{-cellmass (mg)}/[1000 * \text{body weight (g)}] * 100\%.$$

2.13. Statistical analyses

All data were expressed as mean ± SEM. The data obtained from *ob/ob* mice studies were analyzed by Student-*t*-test. For *db/db* mice experiments, data were analyzed by one-way ANOVA (Dunnett's test) and independent-*t*-test with SPSS Statistics 19.0 (IBM). In monkey experiments, the data were statistically analyzed by one-way ANOVA (LSD-*t*-test), independent-*t*-test and Kruskal-Wallis test were also used if necessary by using statistical software SAS 9.3.

3. Results

3.1. In vitro *pharmacological profile of BPI-3016*

To evaluate the biological activities of BPI-3016, the binding affinity of BPI-3016 to the GLP-1 receptor was firstly measured.

Table 1
Resistance of peptides to the degradation by DPP IV *in vitro*.

Peptides	Relative content of parental peptide (%)						
	0 h	0.5 h	1 h	8 h	24 h	32 h	48 h
GLP-1	100	25.82	5.22	[a]	[a]	[a]	[a]
BPI-3016	100	[a]	102.33	101.92	98.19	98.83	98.97
Liraglutide	100	[a]	97.42	89.09	69.28	58.22	46.65
NN9535	100	[a]	91.91	94.43	90.49	89.76	89.80

50 μM GLP-1, BPI-3016, Liraglutide and NN9535 were incubated with DPP-IV(100 U/L) purified from pig kidney in 37 °C for different intervals. Then reactions were terminated by adding 10% TFA. Absorbance for each peptide was monitored at 214 nm using an HPLC system (containing PDA UV detector) through analyzing peaks in the chromatograms. The relative content of parental peptide was expressed in percentile by comparing to the peak area at 0 h.

[a] Represents the samples were not assayed considering the necessity.

The results indicated that BPI-3016 had a lower binding affinity (Ki = 19.4 nM) to GLP-1 receptors compared to GLP-1 (Ki = 1.2 nM) (Fig. 1A). Besides, BPI-3016 showed dose-dependent stimulatory effect on cAMP production with an EC_{50} at 1.80 nM, and EC_{50} for native GLP-1 was 0.10 nM (Fig. 1B). In addition, BPI-3016 showed a better resistance to DPP-IV *in vitro* with about 98.97% parental peptide left after 48 h incubation compared to that of 46.65% for Liraglutide and 89.80% for NN9535, while GLP-1 was rapidly degraded by DPP-IV with only 5.22% parental peptide left after 1 h incubation (Table 1). In the same reaction system without DPP-IV, BPI-3016, Liraglutide, NN9535, and the GLP-1 parental peptide was 96.87%, 87.26%, 100.90% and 97.99%, respectively, suggesting an *in vitro* stability of BPI-3016 (Table 2).

3.2. BPI-3016 exerts long-acting glucose-regulatory effect, increases β-cell mass and promotes insulin secretion in ob/ob mice

The random blood glucose levels in *ob/ob* mice treated with vehicle, BPI-3016 (0.5, 1, 2 mg/kg) and Liraglutide were measured to assess the glucose-regulatory effect of BPI-3016 over a 48-h period. The results indicated 36.2%, 41% and 49.2% reductions in random glucose levels after single-dose treatment of 0.5, 1 and 2 mg/kg BPI-3016 compared to vehicle controls within 34 h ($P < 0.001$). Remarkably, 48 h after administration, the random blood glucose levels of *ob/ob* mice showed 19.8% and 22.2% reduction after 1 and

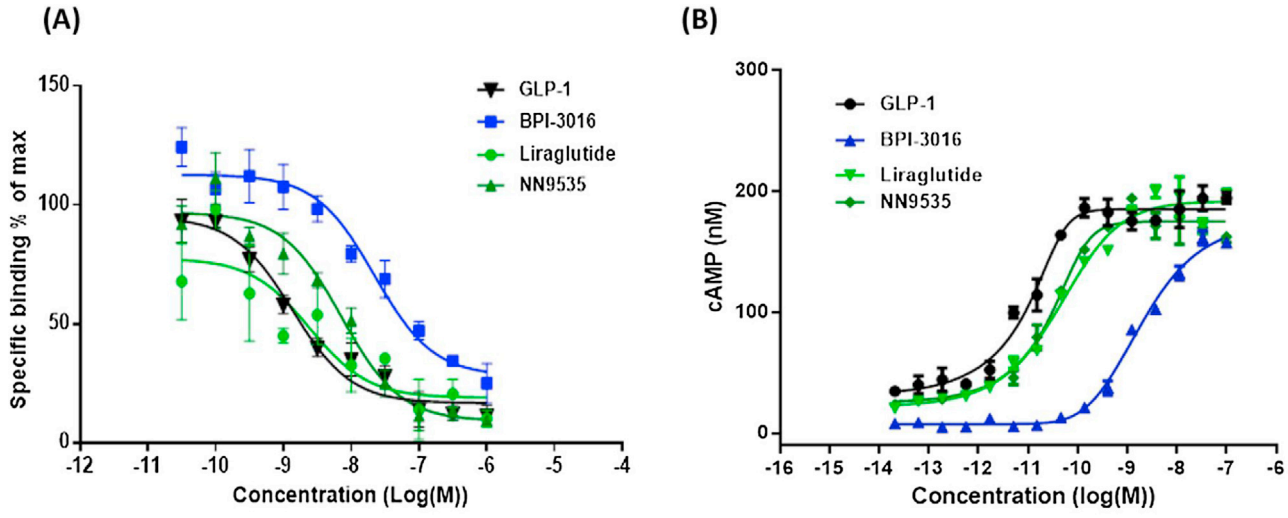

Fig. 1. *In vitro* pharmacological profile of BPI-3016.
A) The binding affinity of unlabelled GLP-1, BPI-3016, Liraglutide and NN9535 (30 pM–1 μM) with increasing concentrations which was demonstrated by displacing binding of the radiolabeled ligand ^{125}I GLP-1 (0.25 nM). B) Intracellular cAMP responses in HEK293 cells to stimulation by increasing concentrations of GLP-1, BPI-3016, Liraglutide and NN9535 (0.021 pM–100 nM). Data are presented as means ± SEM, n = 2.

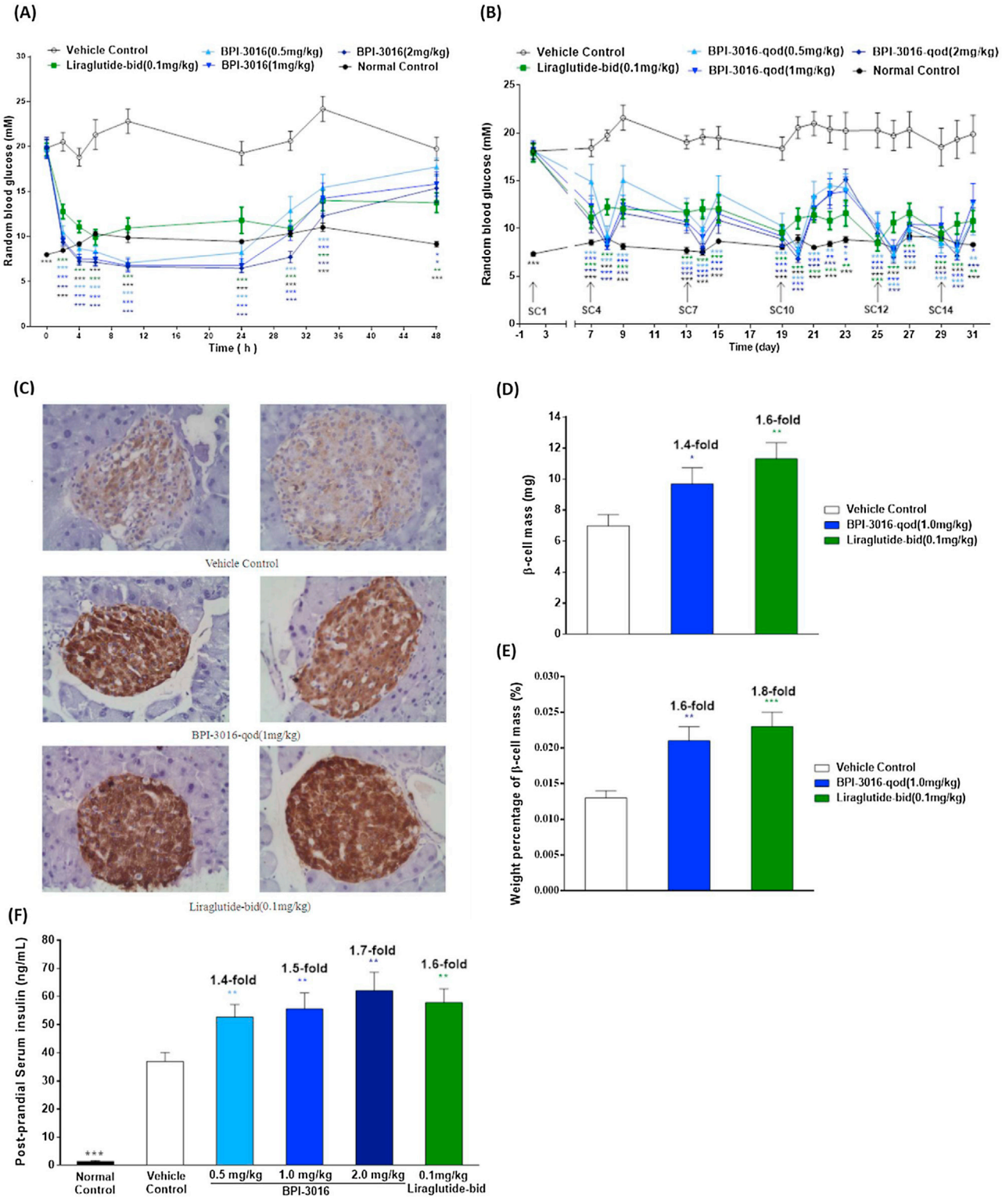

Fig. 2. BPI-3016 exerts long-acting glucose-regulatory effect, increases β-cell mass and promotes post-prandial insulin secretion in *ob/ob* mice.
A) A single dose of BPI-3016 significantly reduces random blood glucose level in *ob/ob* mice: single dose s.c. administration of 0.5, 1, 2 mg/kg BPI-3016 showed significant reductions in random blood glucose levels at 2, 4, 6, 10, 24, 30, and 34 h after administration (***$P < 0.001$, 0.5, 1, 2 mg/kg BPI-3016 groups compared with vehicle control group) (n = 12/group). B) 14 times s.c. administration of 0.5, 1, 2 mg/kg BPI-3016 significantly lowered random blood glucose level in *ob/ob* mice (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; 0.5, 1, 2 mg/kg BPI-3016 groups compared with vehicle control group) (n = 12/group). C) Pancreatic sections from 31-day long-term injections of 1 mg/kg BPI-3016 and Liraglutide in *ob/ob* mice were observed under Nikon microscope (×40) to analyze the characteristics of Insulin-positive β-cells. D) β-cell mass in BPI-3016 (1 mg/kg) group was significantly increased (*$P < 0.05$, compared with vehicle control group) (n = 3/group). E) The weight percentage of β-cell mass in the medium-dose BPI-3016 group also significantly increased compared to vehicle control group (**$P < 0.01$, compared with vehicle control group) (n = 3/group). F) Single dose of 0.5, 1, 2 mg/kg BPI-3016 significantly increased post-prandial insulin secretion in *ob/ob* mice (**$P < 0.01$, 0.5, 1, 2 mg/kg BPI-3016 groups compared with vehicle control group) (n = 12/group). Serum insulin level was determined by ELISA kit at 2 h after administration. Data are presented as means ± SEM.

Table 2
In vitro stability of peptides without DPP IV treatment.

Peptides	Relative content of parental peptide (%)		
	0 h	1 h	48 h
GLP-1	100	97.99	–
BPI-3016	100	–	96.87
Liraglutide	100	–	87.26
NN9535	100	–	100.90

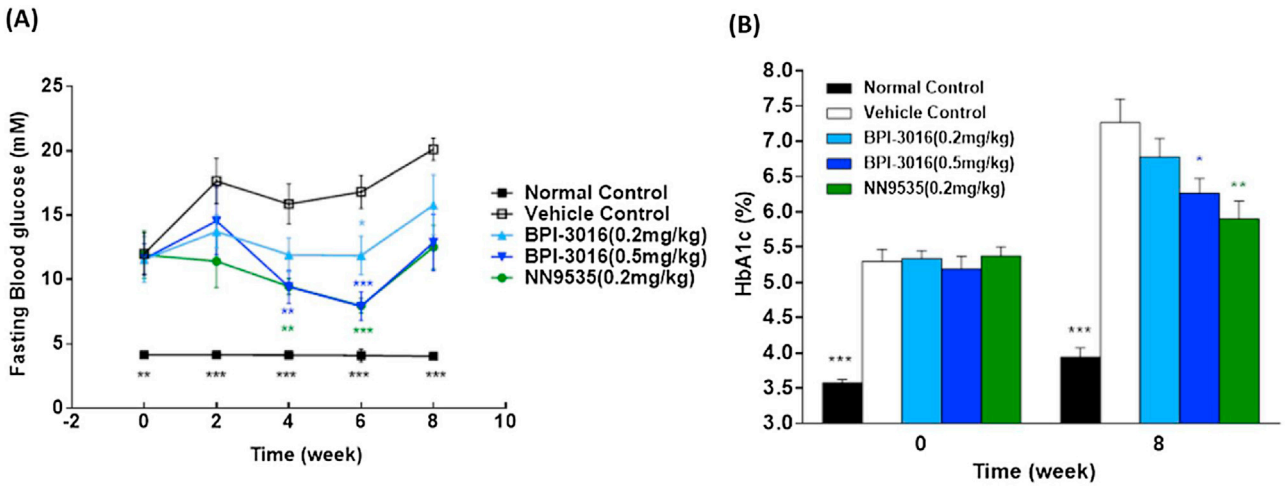

Fig. 3. Chronic treatment of BPI-3016 improves glycemic control in *db/db* mice.
Fasting blood glucose levels in *db/db* mice were measured on 0, 2nd, 4th, 6th and 8th week. HbA1c levels were detected on 0 and 8th week. A) Effect of BPI-3016 on fasting blood glucose: 6-week treatment with 0.2 and 0.5 mg/kg BPI-3016 (s.c.) significantly lowered the fasting blood glucose levels (*$P<0.05$, ***$P<0.001$; compared with vehicle control group) (n = 8/group). A 4-week treatment with 0.5 mg/kg BPI-3016 (s.c.) showed the similar effect (**$P<0.01$, compared with vehicle control group) (n = 8/group). B) Effect of BPI-3016 on HbA1c levels. Chronic injection of 0.5 mg/kg BPI-3016 resulted in a significant reduction in HbA1c after 8-week treatment (*$P<0.05$, compared with vehicle control group) (n = 8/group). Data are presented as means ± SEM.

2 mg/kg BPI-3016 administration ($P<0.05$), suggesting BPI-3016 significantly lowered blood glucose levels (Fig. 2A, Tables S2 and S3).

The changes in random blood glucose levels of *ob/ob* mice were observed over a 31-day period with BPI-3016 administration given once every twice days to indicate its long-term effect. Compared to vehicle control group, random blood glucose levels were significantly decreased in BPI-3016 groups ($P<0.05$, $P<0.01$, $P<0.001$) after 4th dosing. To specifically evaluate the efficacy of BPI-3016 in reducing glucose levels after long-term administration, the levels of random blood glucose in all groups were measured before and after injection at 10th dosing (24, 48, 72 and 96 h). The results showed that 0.5, 1 and 2 mg/kg BPI-3016 still significantly reduced glucose levels even at 96 h after administration ($P<0.05$, $P<0.01$, $P<0.001$). Besides, compared to single administration of BPI-3016, chronic BPI-3016 treatment with multiple injections induced more significant reduction in random glucose levels at 48 h after the initial administration (0.5 mg/kg: 10.2% *vs* 36.2%; 1 mg/kg: 19.8% *vs* 42.3%; 2 mg/kg: 22.2% *vs* 42.3%), suggesting continuous alternate-day subcutaneous injection with 0.5, 1 and 2 mg/kg BPI-3016 for 14 times could significantly reduce the level of random blood glucose and their effects are long-acting (Fig. 2B, Tables S4 and S5).

To investigate whether the injection of 1 mg/kg BPI-3016 every other day for 31 days could increase the β-cell mass of *ob/ob* mice, the insulin-positive β-cells in pancreatic sections were analyzed under the Nikon microscope. We found the β-cell mass (Fig. 2C and D) and its weight percentage (Fig. 2E) in *ob/ob* mice were significantly increased compared to vehicle controls (9.70 mg *vs* 6.98 mg, $P<0.05$; 0.021% *vs* 0.013%, $P<0.01$) after 31-day BPI-3016 treatment. As positive control group, Liraglutide also significantly increased β-cell mass (11.32 mg *vs* 6.98 mg; $P<0.05$) and the weight percentage (0.023% *vs* 0.013%; $P<0.001$).

To determine the effect of BPI-3016 on insulin secretion, postprandial serum insulin levels in *ob/ob* mice were measured after the treatments (Fig. 2F). In 0.5, 1 and 2 mg/kg BPI-3016 groups, serum insulin level reached to 52.68, 55.60 and 62.11 ng/mL respectively, which were significantly higher than vehicle control group (36.93 ng/mL) ($P<0.01$). Besides, serum insulin level in Liraglutide group (57.85 ng/mL) was also significantly increased ($P<0.01$).

3.3. Chronic treatment of BPI-3016 improves glycemic control in db/db mice

To determine the chronic effect of treatments of BPI-3016 on glycemic control in *db/db* mice, the levels of fasting plasma glucose and HbA1c among vehicle control, low-dose BPI-3016 (0.2 mg/kg), high-dose BPI-3016 (0.5 mg/kg) and NN9535 (0.2 mg/kg) group were compared. The results demonstrated that 0.2 mg/kg BPI-3016 (16.81 mM *vs.* 11.86 mM, $P<0.05$) consistently reduced fasting blood glucose levels in *db/db* mice after a 6-week administration (Fig. 3A, Table S6) compared to vehicle controls. The effect of high-dose BPI-3016 on reducing fasting blood glucose in high-dose group was more significant: a significant reduction in the level of fasting blood glucose appeared after 4 weeks administration (11.91 mM *vs.* 9.43 mM; $P<0.01$), and the reduction maintained after 6 weeks administration (16.81 mM *vs.* 7.90 mM; $P<0.001$). Similarly, we found NN9535 could remarkably reduce fasting blood glucose level. As indicated in Fig. 3B, the levels of HbA1c in the high-dose BPI-3016 group (7.26% *vs.* 6.26%; $P<0.05$) and NN9535 group ($P<0.01$) were significantly decreased after 8 weeks treatment, suggesting a strong ability of BPI-3016 in glycemic control.

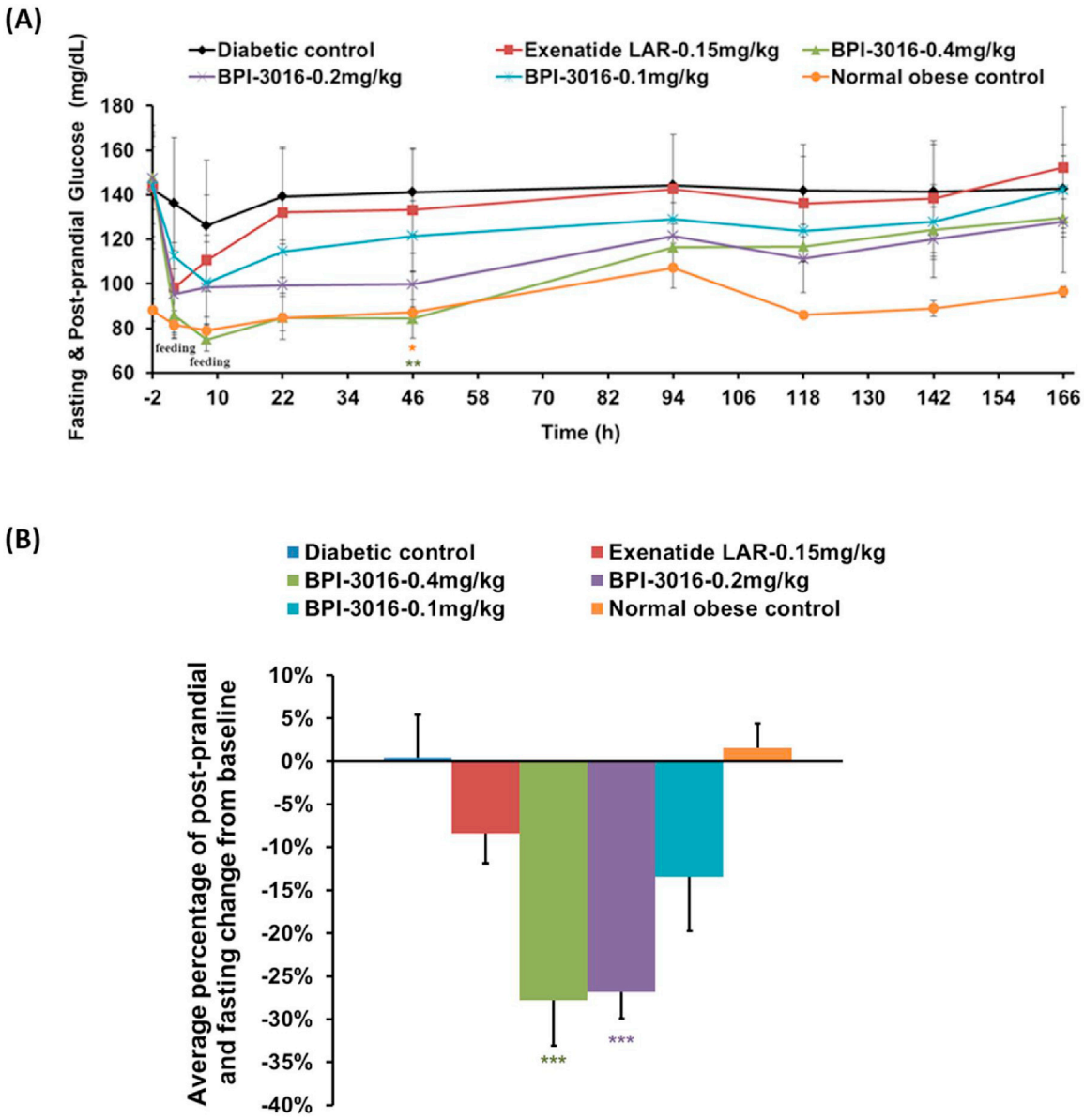

Fig. 4. Effect of single administration of BPI-3016 on plasma glucose in spontaneous diabetic monkeys.
Post-prandial glucose levels were measured at 2, 8 h and fasting glucose levels were measured at 0, 22, 46, 94, 118, 142, 166 h after administration. Percentage of plasma glucose change at each time points after administration was calculated with individual fasting glucose level before 1st administration. And the average percentage of post-prandial and fasting glucose change from baseline in each group were calculated too. A) Post-prandial and fasting glucose levels. Single subcutaneous injection of 0.4 mg/kg BPI-3016 significantly lowered the fasting glucose level at 46 h after administration (**$P < 0.01$, compared with diabetic control group; data were analyzed by one-way ANOVA followed by LSD-*t*-test) (n = 6–10/group). B) The average percentage of post-prandial and fasting glucose change from baseline. Single subcutaneous injection of 0.2, 0.4 mg/kg BPI-3016 lowered the mean percentage of plasma glucose change within 166 h (***$P < 0.001$, compared with diabetic control group; data were analyzed by one-way ANOVA followed by LSD-*t*-test) (n = 6–10/group). Values are shown as mean ± SEM.

3.4. A single administration of BPI-3016 exerts glucose-lowering effect in spontaneous diabetic cynomolgus monkeys

The pharmacokinetic properties of BPI-3016 in spontaneous diabetic cynomolgus monkeys were investigated. The half-life of BPI-3016 in diabetic cynomolgus monkey models was 95 h (Table 3). Post-prandial and fasting glucose were monitored before the single administration of BPI-3016 or Exenatide LAR and at 2, 8, 22, 46, 94, 118, 142 and 166 h after feeding (Fig. 4A). To exclude individual variations among the monkeys, the mean percentage of glucose change from individual baseline glucose level was calculated for each spontaneous diabetic cynomolgus monkey (Fig. 4B). The result showed that the single subcutaneous injection of 0.4 mg/kg BPI-3016 significantly lowered the fasting glucose level at 46 h after administration ($P < 0.01$). Besides, the average change percentage of plasma glucose levels from baseline over 166 h was significantly decreased in 0.2 and 0.4 mg/kg BPI-3016 groups (percentage change: −27%, −28% *vs.* 0%, $P < 0.001$), while no significant decrease in Exenatide LAR group was detected. In summary, 0.2 and 0.4 mg/kg BPI-3016 treatment had an obvious hypoglycemic effect and their efficacy in reducing glucose level was superior to Exenatide LAR.

3.5. Chronic treatment of BPI-3016 alleviates diabetic symptoms in spontaneous diabetic cynomolgus monkeys

To evaluate the chronic effect of BPI-3016, fasting glucose levels, HbA1c levels, and glucose tolerance were measured in diabetic

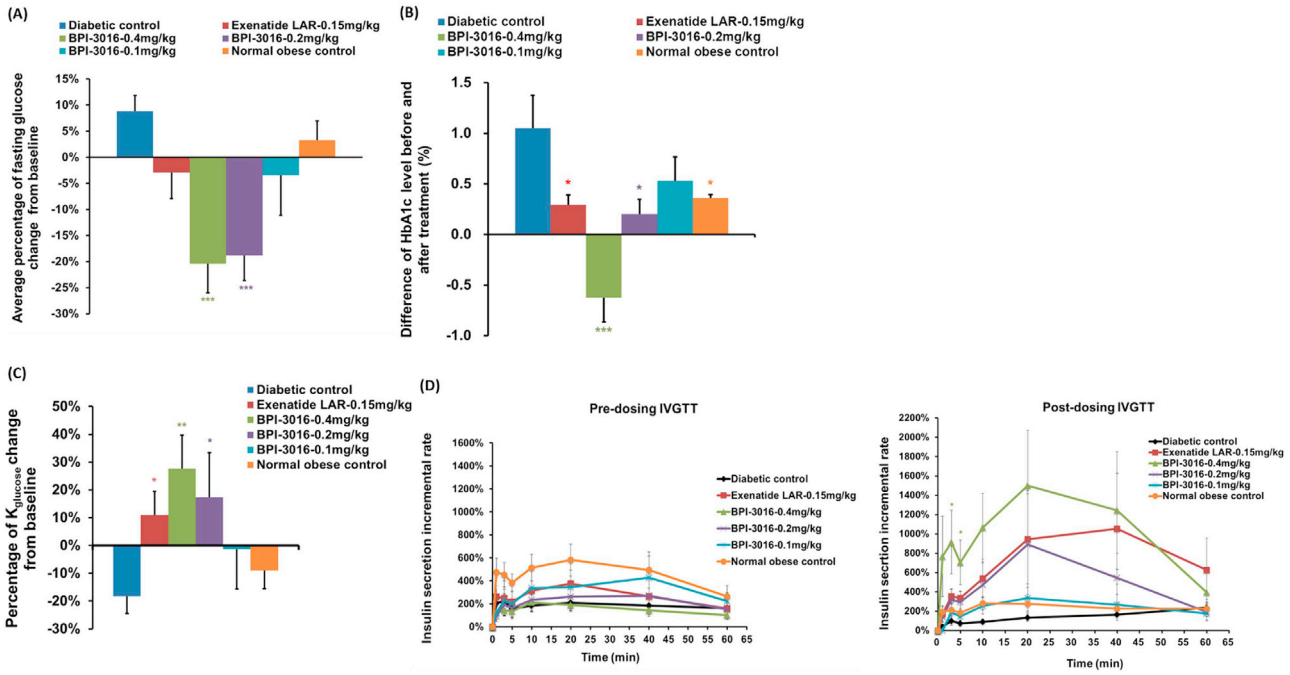

Fig. 5. Chronic treatment of BPI-3016 alleviates diabetic symptoms in spontaneous diabetic cynomolgus monkeys.
A) The average percentage of fasting glucose change from baseline (fasting glucose level before 1st dosing). Once-weekly subcutaneous injection of 0.2, 0.4 mg/kg BPI-3016 for 6 times lowered the mean percentage of blood glucose change (***$P<0.001$, compared with diabetic control group; data were analyzed by one-way ANOVA followed by LSD-*t*-test) (n = 6–10/group). B) Effect of BPI-3016 on HbA1c levels: changes of the HbA1c level before and after 6-time dosing were calculated by following formula: $HbA1c_{change}(\%) = HbA1c_{6\text{-}week}(\%) - HbA1c_{0\text{-}week}(\%)$. 0.2 and 0.4 mg/kg BPI-3016 significantly reduced HbA1c levels (*$P<0.05$, ***$P<0.001$, compared with diabetic control group) (n = 6–10/group; data were analyzed by one-way ANOVA followed LSD-*t*-test). **C**) The change in $K_{Glucose}$ before and after treatment. After 7 times of dosing, 0.4 mg/kg BPI-3016 significantly increased the percentage of $K_{Glucose}$ change from baseline ($K_{glucose}$ value of pre-dosing IVGTT test), compared to the diabetic control (**$P<0.01$, data were analyzed by independent *t*-test). Increases in the $K_{Glucose}$ change percentage were also observed for the 0.2 mg/kg BPI-3016 and Exenatide LAR-treated groups (*$P<0.05$, compared with diabetic control group; data were analyzed by independent *t*-test) (n = 6–10/group). D) Pre-dosing and post-dosing IVGTT test on plasma insulin secretion. For post-dosing IVGTT, the increments in insulin secretion at 3 and 5 min after glucose-load significantly increased (*$P<0.05$, compared with diabetic control group; data were analyzed by Kruskal-Wallis test) (n = 6–10/group). Data are presented as means ± SEM.

Table 3
Pharmacokinetic parameters in spontaneous diabetic cynomolgus monkeys.

Dose (mg/kg)	T_{max} (h)	C_{max} (ng/mL)	$AUC_{0\text{-}t}$ (ng h/mL)	$AUC_{0\text{-}\infty}$ (ng h/mL)	$T_{1/2}$ (h)
0.2	9 ± 3	3055 ± 310	274218 ± 20534	384170 ± 34667	95 ± 20

Plasma concentrations of BPI-3016 (0.2 mg/kg) in diabetic cynomoglus monkeys were determined by an electrospray ionization LC–MS/MS system. The following pharmacokinetic parameters were measured on 6 animals per time point, values are shown as mean ± SEM. T_{max} indicates the time of maximal observed plasma concentration; C_{max} indicates maximal observed plasma concentration; $AUC_{0\text{-}t}$ indicates area under the plasma concentration curve from zero to last time point; $AUC_{0\text{-}\infty}$ indicates area under the plasma concentration curve from zero to infinity; $T_{1/2}$ indicates half-life.

controls, BPI-3016 groups (0.1, 0.2, 0.4 mg/kg), Exenatide LAR group and normal controls. The results indicated that the level of fasting glucose in the normal obese control group remained stable at a relatively low level compared to the diabetic control group, the difference of fasting glucose level between the two groups was gradually expanded over time. With the increase in times of administration, the hypoglycemic effect of once-weekly injection of BPI-3016 especially with the dose of 0.2 mg/kg and 0.4 mg/kg was extended in a long term (Fig. S3). Mean percentage of changes in fasting glucose from baseline over the 6-week period was further calculated (Fig. 5A). The result showed that the mean percentage of changes in fasting glucose level in the low, medium and high-dose BPI-3016 groups was −3%, −19% and −20% respectively, statistically significant differences were observed in medium and high-dose BPI-3016 group ($P<0.001$) compared to diabetic controls (9%), reflecting the potent hypoglycemic effect of BPI-3016. The low-dose BPI-3016 (−3%) and Exenatide LAR group didn't show any statistical difference in the mean percentage of changes in fasting glucose level. The results above suggested that once-weekly administration with BPI-3016 for 6 times had chronic dose-dependent efficacy in lowering fasting glucose levels on diabetic obese monkeys, and 0.2, 0.4 mg/kg BPI-3016 showed more significant glucose-regulatory effect than the Exenatide LAR.

The alteration of the HbA1c level before and after treatments was analyzed to demonstrate the action of BPI-3016 in reducing HbA1c level (Fig. 5B). The result indicated that after 6 times weekly administration, the high-dose BPI-3016 group showed a significant decrease in HbA1c level compared to diabetic control (−0.62% *vs.* 1.05%, $P<0.001$). Slight increases in the HbA1c level were observed in the medium-dose BPI-3016 group (0.20%) and Exenatide LAR group (0.29%) ($P<0.05$).

Two IVGTT tests were performed on the monkeys prior to 1st and after 7th dosing (Fig. S3) to evaluate alterations in glucose tolerance, which was indicated by $K_{Glucose}$. After 7-time weekly of dosing, the high-dose BPI-3016 group showed a significant increase in the percentage of changes in $K_{Glucose}$, compared to the diabetic control (27.6% *vs.* −18.2%, $P<0.01$). Significant increases in the changes of $K_{Glucose}$ were also identified in the 0.2 mg/kg BPI-3016 (17.4%) and Exenatide LAR-treated (11.0%) groups ($P<0.05$) (Fig. 5C). Since exogenous glucose load led to insulin secretions in the IVGTT tests, insulin secretion incremental rates (increased level of insulin normalized with baseline level) were compared among

different groups (Fig. 5D). The normal control monkeys showed the highest glucose-induced insulin secretion compared to other diabetic groups during pre-treatment IVGTT test. In post-treatment IVGTT test, dose-dependent increments in insulin secretion were observed in BPI-3016 group treated with the dose of 0.1, 0.2, 0.4 mg/kg and Exenatide LAR group. And there were statistical differences between high-dose BPI-3016 group and diabetic control group at 3 min, 5 min after glucose loading ($P < 0.05$).

3.6. Chronic treatment of BPI-3016 reduces BMI and body fat in diabetic cynomolgus monkeys

BMI data were collected before and after 7-time treatment (Fig. 6A). BMI in diabetes control group arose from 54.9 to 56.2 kg/m^2. In contrast, treatment with BPI-3016 at 0.4 mg/kg significantly reduced BMI after treatment (55.8–48.5 kg/m^2, $P < 0.001$). Slight BMI reductions were observed in the middle dose group (55.7–53.0 kg/m^2) and low dose group (57.5–56.3 kg/m^2), suggesting dose-dependent effects of BPI-3016 on body weight. BMI reduction was also observed in the Exenatide LAR-treated group. Body composition measurement by DEXA scanning was performed before and after weekly-dosing at 7th week. The percentage of changes in body fat among BPI-3016 groups and Exenatide LAR group after 7 times of once-weekly dosing was measured (Fig. 6B). The result showed that 0.4 mg/kg BPI-3016 significantly lowered body fat percentage in the obese diabetic monkeys in high dose group (−18.6% *vs.* 0.2%, $P < 0.001$), compared to the diabetic control group. No remarkable significance was observed in low-dose, medium-dose BPI-3016 group and Exenatide LAR group. The above results suggested that 7-time weekly treatment with BPI-3016 efficiently suppressed obesity and improved symptoms of metabolic syndrome in spontaneous diabetic cynomolgus monkeys.

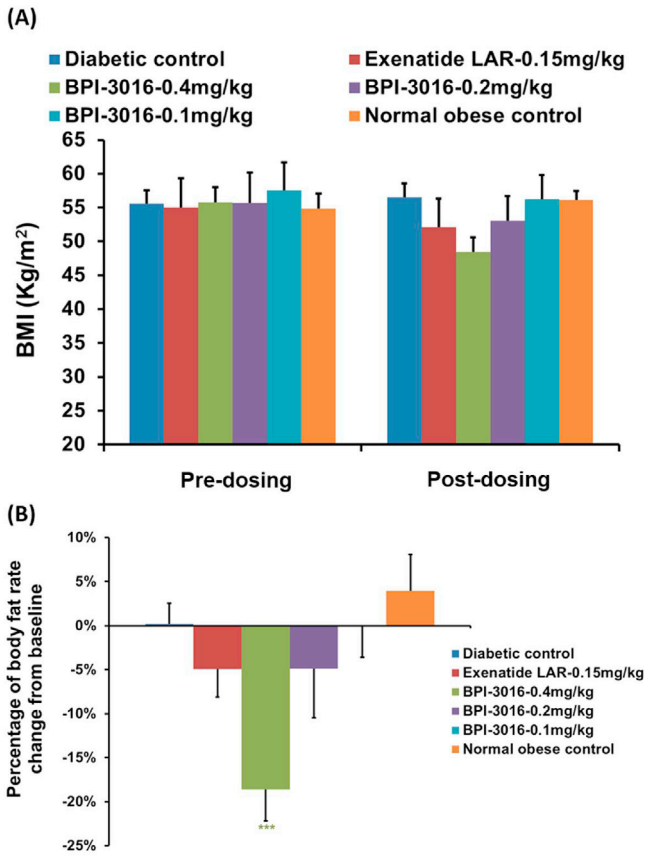

Fig. 6. Chronic treatment of BPI-3016 reduces BMI and body fat in diabetic cynomolgus monkeys.
A) BMI was measured before and after the 7th dosing(n = 6–10/group). Indexes in BPI-3016 treated groups tended to decrease. B) The alteration of body fat after the 7th dosing. Percentage of body fat rate change was calculated with individual body fat rate before 1st administration. Treatment with 0.4 mg/kg BPI-3016 significantly lowered body fat in the obese diabetic monkeys (***$P < 0.001$, compared with diabetic control group; data were analyzed by one-way ANOVA followed by LSD-*t*-test) (n = 6–10/group). Data are presented as means ± SEM.

4. Discussion

In this study, we designed a GLP-1(7–37) analogue BPI-3016 by substituting the N-terminal penultimate amide bond with a —CH(CF_3)NH—, replacing an amino acid and linking a long fatty acid chain on human GLP-1(7–37). BPI-3016 exhibited similar biological activities to native GLP-1 and showed strong resistance to DPP-IV after the structural modification. Specifically, BPI-3016 maintained stability after 48 h incubation with DPP-IV, showed affinity to the GLP-1 receptor, dose-dependently stimulated intracellular cAMP production *in vitro*. We also demonstrated pharmacological characteristics of BPI-3016 in animal models of T2DM. For instance, structural modifications on GLP-1 significantly prolonged the half-life (≥95 h in spontaneous diabetic cynomolgus monkeys) *in vivo*. Single-dose administration of BPI-3016 reduced the level of random blood glucose over a period of 34–48 h and increased post-prandial insulin secretion in *ob/ob* mice. And a single dose of BPI-3016 had obvious hypoglycemic effect in diabetic monkeys. Also, chronic treatment of BPI-3016 exerted a long-acting effect on reducing the level of plasma glucose and HbA1c in *ob/ob* mice, *db/db* mice, and diabetic cynomolgus monkeys. The hypoglycemic effect of BPI-3016 was maintained over 166 h in cynomolgus monkeys after repeated once-weekly dosing (data not shown). Moreover, chronic treatment with BPI-3016 increased the β-cell mass of *ob/ob* mice, improved glucose tolerance and reduced BMI and body fat in diabetic monkeys.

The *in vivo* potency of BPI-3016 in improving symptoms of diabetic animals has promised it a great potential as a once-weekly drug for treating type 2 diabetes. Our records for observing immunogenicity responses after BPI-3016 treatment showed that after multiple-dose administrations, no anti-drug antibody was detected in cynomolgus monkeys (Figs. S4 and S5). Also, no severe adverse event was observed in the non-human animal safety evaluation test (Table S7). These findings indicate the potential advantages of chemical structural modification in BPI-3016: including smaller molecular weight, less injection site pain or skin nodules and fewer chances of generating anti-drug antibody in response to injection compounds with the huge molecular weight *in vivo*.

In conclusion, BPI-3016 with structural modifications based on native GLP-1 has significantly prolonged half-life both *in vitro* and *in vivo*, and meantime maintains GLP-1 receptor affinity and activity of stimulating intracellular generation of cAMP. BPI-3016 also significantly reduces blood glucose levels, decreases HbA1c levels and improves glycemic control, insulin resistance and β-cell function, and shows a long-acting therapeutical effect in spontaneous T2DM animal models. Thus, BPI-3016 has a potential acting as a long-acting GLP-1 analogue for type 2 diabetes treatment in the clinic.

Acknowledgment

This work was supported by National Science and Technology Major Project for Significant New Drugs Development of the Ministry of Science and Technology of China, Grant No. 2013ZX09103001-011.

References

[1] B. Gallwitz, The fate of Beta-cells in type 2 diabetes and the possible role of pharmacological interventions, Rev. Diabet. Stud.: RDS 3 (4) (2006) 208–216.
[2] C. Weyer, C. Bogardus, D.M. Mott, R.E. Pratley, The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus, J. Clin. Invest. 104 (6) (1999) 787–794.
[3] UK Prospective Diabetes Study (UKPDS) Group, Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34), Lancet 352 (9131) (1998) 854–865.
[4] S.E. Kahn, S.M. Haffner, M.A. Heise, W.H. Herman, R.R. Holman, N.P. Jones, B.G. Kravitz, J.M. Lachin, M.C. O'Neill, B. Zinman, et al., Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy, N. Engl. J. Med. 355 (23) (2006) 2427–2443.
[5] D.M. Nathan, J.B. Buse, M.B. Davidson, E. Ferrannini, R.R. Holman, R. Sherwin, B. Zinman, American Diabetes A, European Association for Study of D, Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes, Diabetes Care 32 (1) (2009) 193–203.
[6] D.J. Drucker, M.A. Nauck, The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes, Lancet 368 (9548) (2006) 1696–1705.
[7] M. Lind, J. Jendle, O. Torffvit, I. Lager, Glucagon-like peptide 1 (GLP-1) analogue combined with insulin reduces HbA1c and weight with low risk of hypoglycemia and high treatment satisfaction, Prim. Care Diabetes 6 (1) (2012) 41–46.
[8] R. Eissele, R. Goke, S. Willemer, H.P. Harthus, H. Vermeer, R. Arnold, B. Goke, Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man, Eur. J. Clin. Invest. 22 (4) (1992) 283–291.
[9] D.J. Drucker, The biology of incretin hormones, Cell Metab. 3 (3) (2006) 153–165.
[10] C. Orskov, L. Rabenhoj, A. Wettergren, H. Kofod, J.J. Holst, Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans, Diabetes 43 (4) (1994) 535–539.
[11] R. Mentlein, B. Gallwitz, W.E. Schmidt, Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7–36)amide, peptide histidine methionine and is responsible for their degradation in human serum, Eur. J. Biochem./FEBS 214 (3) (1993) 829–835.
[12] K. Hupe-Sodmann, G.P. McGregor, R. Bridenbaugh, R. Goke, B. Goke, H. Thole, B. Zimmermann, K. Voigt, Characterisation of the processing by human neutral endopeptidase 24.11 of GLP-1(7–36) amide and comparison of the substrate specificity of the enzyme for other glucagon-like peptides, Regul. Pept. 58 (3) (1995) 149–156.
[13] C.F. Deacon, What do we know about the secretion and degradation of incretin hormones? Regul. Pept. 128 (2) (2005) 117–124.
[14] C. Li, Y. Huan, N. Shen, L. Ji, S. Sun, S. Liu, Q. Liu, L. Gao, F. Tan, Y. Wang, et al., A novel GLP-1 analog, BPI3006, with potent DPP IV resistance and good glucoregulatory effect, Biochem. Biophys. Res. Commun. 400 (4) (2010) 563–568.
[15] B.D. Green, V.A. Gault, M.H. Mooney, N. Irwin, P. Harriott, B. Greer, C.J. Bailey, F.P. O'Harte, P.R. Flatt, Degradation, receptor binding, insulin secreting and antihyperglycaemic actions of palmitate-derivatised native and Ala8-substituted GLP-1 analogues, Biol. Chem. 385 (2) (2004) 169–177.