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Albumin-binding domain extends half-life of glucagon-like peptide-1

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) is considered to be a promising peptide for the treatment of type 2 diabetes mellitus (T2DM). However, the extremely short half-life of GLP-1 limits its clinical application. Albumin-binding domain (ABD) with high affinity for human serum albumin (HSA) has been used widely for half-life extension of therapeutic peptides and proteins. In the present study, novel GLP-1 receptor agonists were designed by genetic fusion of GLP-1 to three kinds of ABDs with different affinities for HSA: GA3, ABD035 and ABDCon. The bioactivities and half-lives of ABD-fusion GLP-1 proteins with different types and lengths of linkers were investigated *in vitro* and *in vivo*. The results demonstrated that ABD-fusion GLP-1 proteins could bind to HSA with high affinity. The blood glucose-lowering effect of GLP-1 was significantly improved and sustained by fusion to ABD. Meanwhile, the fusion proteins significantly inhibited food intake, which was beneficial for T2DM and obesity treatment. The half-life of GLP-1 was substantially extended by virtue of ABD. The *in vivo* results also showed that a longer linker inserted between GLP-1 and ABD resulted in a higher blood glucose-lowering effect. The fusion proteins generated by fusion of GLP-1 to GA3, ABD035 and ABDCon exhibited similar bioactivities and pharmacokinetics *in vivo*. These findings demonstrate that ABD-fusion GLP-1 proteins retain the bioactivities of natural GLP-1 and can be further developed for T2DM treatment and weight loss. It also indicates that the ABD-fusion strategy can be generally applicable to any peptide or protein, to improve pharmacodynamic and pharmacokinetic properties.

1. Introduction

According to investigations, 80%–90% of patients with Type 2 diabetes mellitus (T2DM) are coupled with obesity (Evers et al., 2018). Therefore, an agent with the properties of promoting weight loss and lowering blood glucose is an ideal treatment route for T2DM. Glucagon-like peptide-1 (GLP-1) is considered to be a promising peptide for T2DM treatment due to its multi-functionality through GLP-1 receptor (GLP-1R) binding, with effects such as glucose-dependent insulin secretion, inhibition of gastric emptying and decreasing appetite (Cho et al., 2012; Baggio and Drucker, 2007). However, the half-life of GLP-1 in circulation is very short, typically about 1–2 min, due to rapid inactivation by dipeptidyl peptidase 4 (DPP-4) and clearance by the kidneys (Graaf et al., 2016; Ren et al., 2019), thus limiting its therapeutic utility.

To improve their pharmacological properties, GLP-1R agonists have been extensively developed through chemical or genetic modifications. To date, seven GLP-1 agonists have been approved for the treatment of T2DM by the FDA or EMA, including the twice-daily taken exenatide, once-daily taken lixisenatide and liraglutide, once-weekly taken exenatide (extended release), albiglutide, dulaglutide and semaglutide (Madsbad, 2016; Sharma et al., 2018; Pratley et al., 2014). These GLP-1 agonists are developed through three

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key half-life extension strategies: (i) sequence modification; (ii) fusion to a large protein to increase the hydrodynamic volume; and (iii) exploitation of the recycling mechanism of neonatal Fc receptor (FcRn) by binding to the human serum albumin (HSA) or IgG-Fc (Kontermann, 2016; Strohl, 2015).

Albumin-binding domain (ABD) with high HSA-binding affinity is also used widely for half-life extension through non-covalent binding to HSA. The naturally occurring ABD is a small, three-helical protein domain (Nilvebrant and Hober, 2013). G148-ABD3 (GA3), expressed by streptococcal strain G148, is one of the best characterized domains and has been developed widely to extend the half-lives of therapeutic peptides or proteins (Nilvebrant and Hober, 2013; Stork et al., 2007; Gapizov et al., 2019).

Inspired by the promising application of GA3 in half-life extension, many variants have been designed to further improve the pharmacodynamic and pharmacokinetic profiles of fusion partners. ABD035, selected by phage display technology, showed an apparent affinity of 50-50 fM for HSA, corresponding to several orders of magnitude of improvement compared with wild-type GA3 (1.2 nM) (Jonsson et al., 2008). ABDCon, with an affinity of 75 pM for HSA, is a variant designed by a consensus sequence design method (Jacobs et al., 2015). It was noteworthy that both of the variants retained high affinity for albumin from other species, such as mouse, rat and cynomolgus, facilitating preclinical, toxicology and pharmacology experiments (Jacobs et al., 2015; Li et al., 2016). ABD is therefore a suitable scaffold for half-life extension of peptides and proteins.

Here, we designed a series of chimeric constructs by genetic fusion of GLP-1 to GA3, ABD035 and ABDCon, respectively, through a linker. The bioactivities and half-lives of fusion proteins were then measured *in vivo*. We expected that the ABDs could improve the pharmacodynamic and pharmacokinetic properties of GLP-1 *in vivo*, resulting in a promising agent for lowering blood glucose.

2. Materials and methods

2.1. Protein construction

The DNA sequences containing the modified GLP-1 fused to the N-terminus of ABDs (GA3, ABD035 and ABDCon) via different types of linkers were synthesized by the company GENEWIZ (Suzhou, China). The modified GLP-1 moiety consisted of 31 amino acids from human GLP-1 (7–37) with a substitution of Gly for Ala at position 8 to prevent degradation by DPP-4. A His-tag was placed at the N-terminus of the sequence and a TEV protease cleavage site was inserted between the His-tag and GLP-1, facilitating removal of the His-tag to obtain the free N-terminus of GLP-1. The GLP-1 was separated from the ABD by different types of linkers, such as a flexible linker composed of Gly and Ser or a rigid linker containing Glu and Ala. The linkers were extended by the addition of amino acids, such as GSGGGS (denoted as L1), GS $(GGGGS)_2$ (L2), GS $(GGGGS)_3$ (L3). L0 signifies that no linker existed between GLP-1 and ABD. The gene sequence was then digested by *Nco*I and *Eco*RI restriction endonucleases (Thermo, USA) and cloned into a pET-28a (+) plasmid.

2.2. Protein expression and purification

The expression and purification of the fusion proteins were conducted as previously described by Tan et al. (2017) with modifications. All plasmids containing fusion proteins were transformed into *E. coli* BL21 (DE3). One colony was picked randomly and cultured in LB medium with 60 μg/ml kanamycin overnight at 37 °C. The overnight cultures were grown in LB medium at 37 °C, diluted 50-fold, and grown until the OD_{600} reached 0.4–0.6. IPTG was then added with a final concentration of 0.5 mM and bacteria were cultured for an additional 4 h at 25 °C. The cells were harvested by centrifugation at 5500*g* for 20 min at 4 °C, then resuspended in lysis buffer (25 mM Tris/HCl, 300 mM NaCl, 10 mM imidazole, pH 8.0), after which the cells were lysed, followed by centrifugation at 26,000*g* for 40 min.

The supernatants were then applied to a Ni^{2+}-NTA column (GE, USA) pre-equilibrated with lysis buffer. The column was washed with wash buffer (25 mM Tris/HCl, 300 mM NaCl, 30 mM imidazole, pH 8.0), after which the bound proteins were eluted with elution buffer (25 mM Tris/HCl, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted fractions were dialyzed against 25 mM Tris/HCl, pH 8.0.

The fusion proteins were subjected to His-tag removal by adding 1/50 TEV protease and 1 mM dithiothreitol (DTT). The reaction was carried out at room temperature for 2 h and the solution was then dialyzed overnight against 25 mM Tris/HCl, pH 8.0. The solution was passed through the Ni^{2+}-NTA column again, and the flow-through fractions from the column were collected and pooled. The untagged protein was further purified by a Q ion exchange column (GE, HiTrap Q HP) pre-equilibrated with 25 mM Tris/HCl, pH 8.0. The proteins eluted from the Q column were analyzed by 15% SDS-PAGE (EpiZyme, Shanghai, China).

2.3. Binding assay for HSA

The ability of a fusion protein to bind to HSA was measured by two methods: size-exclusion chromatography and enzyme-linked immunosorbent assay (ELISA). The binding of a fusion protein with HSA (Sigma-Aldrich, Shanghai, China) was qualitatively assessed by mixing the proteins in a 1.2:1 ratio in accordance with McMahon et al. (2018) with some modifications. The mixture was incubated at room temperature for 1 h, then separated by a Superdex 200 10/300 gel filtration column (GE, USA) in PBS buffer (pH 7.4). The fractions were analyzed by 12% SDS-PAGE (EpiZyme, Shanghai, China).

The binding of ABD-fusion GLP-1 protein to HSA was also determined by ELISA as described by Li et al. (2016) with some modifications. HSA was diluted to 200 μg/ml using the carbonate-bicarbonate buffer (pH 9.6). The HSA (100 μl/well) was immobilized in a 96-well plate at 4 °C overnight. The coated plated was washed three times with PBST (PBS + 0.05% Tween 20), then blocked with 5% BSA (Sigma-Aldrich, Shanghai, China) overnight. After washing, 100 μl of serial concentrations of ABD-fusion GLP-1 proteins prepared in PBS (pH 7.4) were added to the wells followed by incubation at room temperature for 2 h. Unbound proteins were removed with three PBST washes. The GLP-1 in fusion proteins were detected by rabbit monoclonal human GLP-1 antibody (MAB12492, R&D systems, USA) with a dilution of 1:6000 by PBST. After washing, the HRP-conjugated goat anti-rabbit IgG (CW0103S, CWBIO, Beijing, China) with 1:8000 dilution in PBST was added to each well. After another round of washing, TMB substrate (HE101-01, TransGen Biotech, Beijing, China) was applied to detect the bound HRP. The reaction was stopped by the addition of 1 M HCl (100 μl/well) followed by measurement of the absorbance at 450 nm using a SpectraMax M2 micro-plate reader (Molecular Devices, USA).

2.4. Animals

The male C57BL/6 mice (20–25 g) and female BALB/c mice (20–25 g) were all obtained from the Academy of Military Medical Sciences (Beijing, China). The animals were housed under a 12 h light-dark cycle and the temperature of the environment was maintained at 24 ± 2 ˚C with free access to food and water, except where noted. All experiments were carried out following the guideline principles for the care and use of laboratory animals approved by the Animal Care Committee of China and were approved by the Experiment Animal Center of Academy of Military Medical Sciences (SCXK-2019-0013).

2.5. Feeding studies and non-fasting blood glucose measurements

The male C57BL/6 mice were fasted overnight (14–16 h), and then weighed. The mice were randomly grouped according to body weight (n = 5–6 per group). A single bolus of 30 nmol/kg of fusion proteins was administered subcutaneously. Mice in the control group were given the same volume of PBS. The mice were then placed into individual cages containing the pre-weighed rodent chow with free access to water. The food was measured at the time preset. Food intake was calculated by the food decrease (g)/body weight (g) and the non-fasting blood glucose was measured using a OneTouch UltraEasy glucometer (Johnson & Johnson, USA) at different time-points after administration (Su et al., 2020).

2.6. Oral glucose tolerance test (OGTT)

In order to verify whether the fusion proteins improved the glucose tolerance of mice, OGTTs were performed as described by Finan et al. (2015). Briefly, the male C57BL/6 mice were grouped according to body weight (n = 5–6 per group), followed by fasting overnight (14–16 h). Fusion proteins were injected subcutaneously at a dose of 15 nmol/kg 30 min before the oral glucose challenge (2 g glucose per kg body weight). Mice in the control group received the same volume of PBS. The liraglutide (Novo Nordisk, Denmark) was injected at the same dose as a positive control. The blood glucose levels were measured by a OneTouch UltraEasy glucometer at −30, 0, 15, 30, 60, 90 and 120 min after the glucose load.

2.7. Pharmacokinetics in mice

To determine the pharmacokinetic profiles of fusion proteins, FITC (Sigma-Aldrich, Shanghai, China) was chemically conjugated to the amine terminal and primary amines in fusion proteins. The process of fusion protein labeling was conducted following the manufacturer's instructions. Briefly, 50 μl FITC (1 mg/ml in DMSO) was added into 1 ml protein solution (2 mg/ml) in sodium carbonate-bicarbonate, pH 9.0. The mixture was incubated in the dark for 8 h at 4 ˚C. The reaction was stopped by the addition of NH_4Cl with a final concentration of 50 mM followed by incubation for 2 h at 4 ˚C. Glycerol with a final concentration of 5% was added. The unbound FITC was then separated by a G-25 desalting column (GE, USA) pre-equilibrated with PBS. The fusion proteins conjugated with FITC were stored in PBS in a light-proof container at 4 ˚C. Protein concentration was measured by BCA protein assay kit (CWBIO, Beijing, China).

The determination of pharmacokinetics was performed as described by Fan et al. (2018) and Lee et al. (2017) with some modifications. In brief, FITC labeled fusion proteins (50 μg/mouse) were subcutaneously injected into female BALB/c mice (n = 3 per group); then, at different time-points, about 20 μl of blood was collected from the tail vein to an ice-cold Ep tube containing EDTA-Na_2 for anticoagulation. The plasma was separated by centrifugation at 1500*g* for 15 min at 4 ˚C. The plasma was then diluted with PBS, followed by analysis on a black 96-well microplate at a volume of 100 μl. The fluorescence was determined by a SpectraMax M2 micro-plate reader using excitation at 485 nm and emission at 535 nm. Quantified plasma concentrations of fusion proteins were calculated according to the standard curves. Non-compartmental analysis of the final data was performed using the PK Solver Excel Add-in program (Zhang et al., 2010) to calculate the half-life (Jacobs et al., 2015).

2.8. Statistical analysis

All data are presented as mean ± S.E.M, graphed and analyzed using the GraphPad Prism 6.01 (GraphPad software, San Diego, CA, USA). Statistical analyses were performed using the one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison analysis. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Design and production of ABD-fusion GLP-1 proteins

To improve the pharmacokinetic and pharmacodynamic properties of GLP-1, three kinds of ABDs were genetically fused to the C-terminus of GLP-1 molecules, through a linker with different types. An N-terminal His-tag was used for purification and a TEV protease recognition site was inserted between the His-tag and GLP-1 to remove the His-tag (Fig. 1A). The ABD035 and ABDcon are variants derived from GA3 with improved affinity for HSA (Fig. 1B). For steric separation of GLP-1 and ABD, in order to avoid misfolding and bioactivity loss, we investigated several linkers with different conformations and lengths (Fig. 1C). The GS-linkers are relatively flexible, facilitating free movement and interaction with the receptor, whereas the EA-linker is rigid, forming an α-helix in favor of separating domains more effectively (Chen et al., 2013). The lengths of linkers were prolonged by addition of amino acids, from 0 to 17. The ABD-fusion GLP-1 proteins were designated as GLP-GS/EA-ABD-GS/EA-Lx, where x refers to the number as described in Fig. 1B. For example, GLP-GA3-GS-L3 indicates that GLP-1 was genetically fused to GA3 via a GS-linker with 17 amino acids. A modeling simulation of GLP-GA3-GS-L3 generated by the SynLinker system (Liu et al., 2015) demonstrated that the GLP-1 and GA3 were sterically separated, and therefore did not influence binding to the GLP-1 receptor (GLP-1R) and HSA (Fig. 1D).

Genes encoding ABD-fusion GLP-1 proteins were synthesized and cloned into pET-28a (+) vectors followed by expression in *E. coli*. All the fusion proteins were expressed in the soluble fraction, and purified by Ni^{2+}-NTA resin, after which the fusion proteins were subjected to His-tag cleavage by TEV protease to obtain the free N-terminal of GLP-1 for GLP-1R binding. The SDS-PAGE analysis of the purified fusion proteins revealed single bands corresponding to expected molecular weight, showing that the proteins were high purity (Fig. 1E).

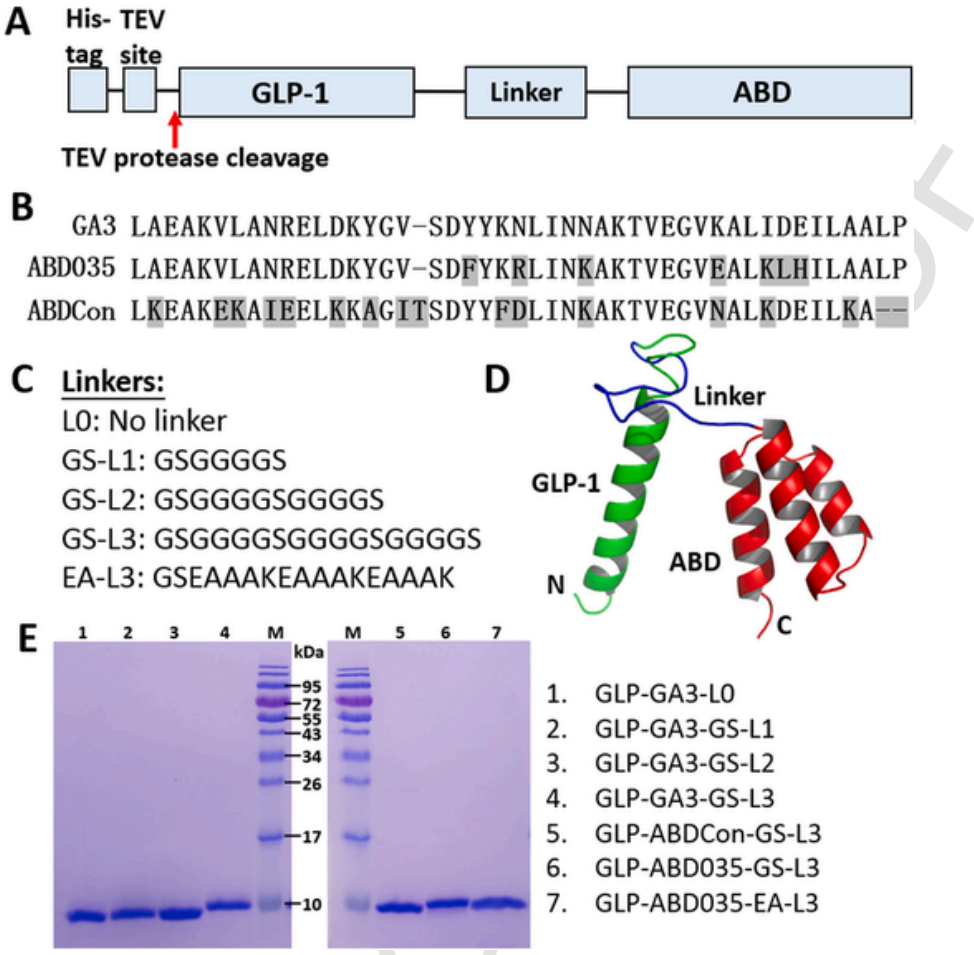

Fig. 1. Design and production of ABD-fusion GLP-1 proteins. (A) Schematic representation of GLP-1 genetically fused to ABD via a linker. (B) Sequence alignment of GA3, ABD035 and ABDCon. The amino acids different from GA3 are shown in gray. (C) The designed linkers between GLP-1 and ABD. (D) Modeling simulation of GLP-GA3-GS-L3. The model was generated by SynLinker system using structures of GLP-1 (based on PDB 5VAI) and GA3 (based on PDB 1GJT) through a linker, and then visualized by PyMOL. (E) SDS-PAGE analysis of purified ABD-fusion GLP-1 proteins.

3.2. Binding assay for HSA

To confirm that the affinity of ABD for HSA was not affected by the GLP-1, both ELISA and gel filtration were carried out. First, a 96-well plate was coated with HSA with immobilized concentration. Increasing concentrations of fusion proteins were then added and an antibody against GLP-1 was used to detect the bound fusion proteins. As shown in Fig. 2, ABD-fusion GLP-1 proteins all bound to HSA with high affinity. It was interesting that the apparent affinities for HSA, indicated by concentrations for half maximal binding, were very similar, in the range of 2.2–5.0 nM. There was no significant interference in binding affinities of linkers, regardless of lengths and types, for HSA. This may be because GLP-1 has a small molecular size, resulting in a smaller steric hindrance. Based on the ELISA method, we also found that the fusion proteins containing GA3, ABD035 and ABDCon showed no significant difference in apparent affinity for HSA ($P > 0.50$). However, the signals in the wells of GLP-ABD035-GS-L3 were 28.6%

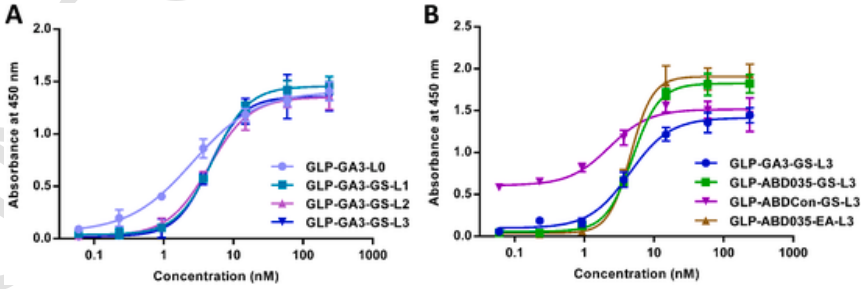

Fig. 2. Affinity determination of ABD-fusion GLP-1 proteins for HSA by ELISA method. Microtiter plates were coated with HSA and bound fusion proteins were detected by antibody against GLP-1. (A) Binding of GLP-GA3 fusion proteins with different lengths of linkers to HSA pre-coated in the 96-well plate. (B) Binding of ABD-fusion GLP-1 proteins containing GA3, ABD035 and ABDCon with GS- or EA-linker to HSA.

and 20.0% greater than GLP-GA3-GS-L3 and GLP-ABDCon-GS-L3, respectively, and were comparable to GLP-ABD035-EA-L3 (Fig. 2B).

Next, to further verify the HSA binding properties, fusion proteins were incubated with HSA at room temperature for 1 h, followed by analysis using size-exclusion chromatography. As shown in Fig. 3, an obvious earlier peak was eluted, corresponding to a larger molecular weight than individual proteins, indicating that the ABD-fusion GLP-1 protein bound to HSA. SDS-PAGE analysis also demonstrated that peak 1 was composed of a fusion protein and HSA. This suggests that the ABD-fusion GLP-1 proteins, containing GA3, ABD035 and ABDCon, all retained the capacity to bind to HSA in solution in a non-covalent manner.

3.3. Non-fasting blood glucose and food intake measurements

To confirm that the ABD-fusion GLP-1 proteins retained the bioactivities of natural GLP-1 *in vivo*, the non-fasting glucose levels of the mice were measured after one single injection of fusion proteins at a dose of 30 nmol/kg. As shown in Fig. 4, the fusion proteins exerted all bioactivities of natural GLP-1, significantly reduced blood glucose levels and inhibited food intake. It was noteworthy that the fusion proteins containing a longer linker resulted in a higher blood glucose-lowering effect (Fig. 4A, $P < 0.05$). A single dose of GLP-GA3-GS-L3 significantly decreased non-fasting blood glucose levels for at least 8 h ($P < 0.001$). The GLP-GA3-L0 construct showed the weakest potency in glucose reduction (Fig. 4A, $P < 0.05$). The calculated glucose area under curve (AUC) for GLP-GA3-GS-L3 was significantly lower than that of GLP-GA3-L0 ($P < 0.05$). The native GLP-1 was found to have no obvious blood glucose-lowering effect (Fig. 4B), probably because of its extremely short half-life.

We then compared the blood glucose-lowering effect of GLP-GA3-GS-L3 with GLP-ABD035-GS-L3 and GLP-ABDCon-GS-L3. All three of the fusion proteins significantly decreased the non-fasting blood glucose levels for at least 8 h ($P < 0.05$). The GLP-ABD035-GS-L3 was the most potent in blood glucose lowering (Fig. 4C, $P < 0.001$). The effect of GLP-ABD035-EA-L3, containing a rigid EA-linker, on lowering blood glucose was comparable to GLP-ABD035-GS-L3, with no statistical difference ($P = 0.32$). The blood glucose-lowering effect was further confirmed in diabetic mice, and the results demonstrated that GLP-ABD035-GS-L3 significantly decreased glucose levels in an STZ-induced diabetic model (Fig. S1, $P < 0.05$). The anorectic effect of GLP-1 was also retained by the fusion proteins, as shown in Fig. 4D. All fusion proteins significantly reduced food intake in *ad libitum*-fed mice for at least 10 h ($P < 0.05$). At 2–4 h, the food intake was the lowest, which corresponded to the lowest blood glucose levels. This indicates that the anorectic effect also plays an important role in blood glucose control. There was no significant difference between fusion protein groups ($P > 0.05$).

3.4. Oral glucose tolerance test (OGTT)

To further evaluate the suppressive effect of fusion proteins on blood glucose, the OGTTs were performed on fasted mice. The results showed that the GLP-GA3-GS-L3, GLP-ABD035-GS-L3 and GLP-ABDCon-GS-L3 at a dose of 15 nmol/kg significantly reduced blood glucose levels immediately after the oral glucose challenge (2 g/kg, $P < 0.001$). GLP-ABD035-GS-L3 resulted in lower blood glucose levels at 15 min and 30 min after the oral glucose challenge. However, no statistically significant difference in glucose levels was found between the three fusion protein groups (Fig. 5A) and the calculated AUCs from 0

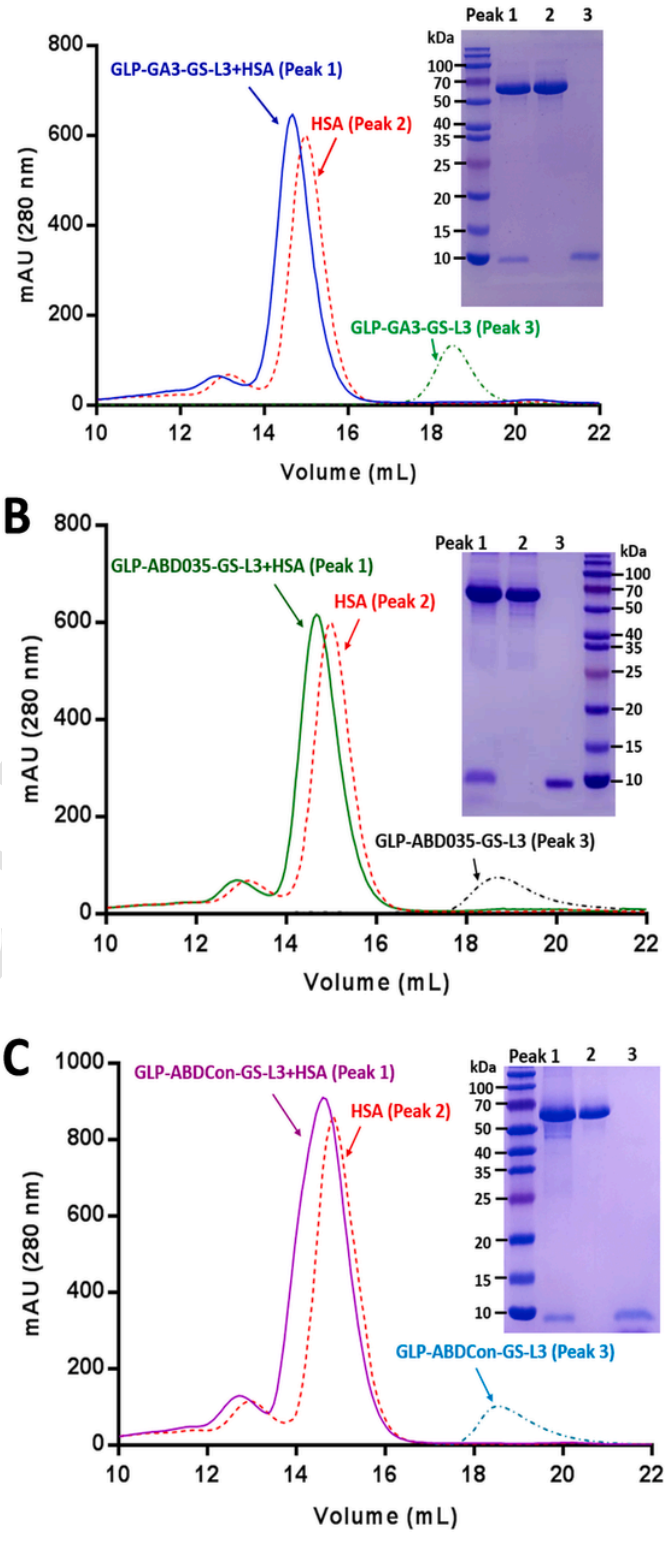

Fig. 3. Binding of ABD-fusion GLP-1 proteins to HSA analyzed by size-exclusion chromatography and SDS-PAGE. GLP-GA3-GS-L3 (A), GLP-ABD035-GS-L3 (B) and GLP-ABDCon-GS-L3 (C) were incubated with HSA at 1.2:1 ratio at room temperature for 1 h, and then analyzed by size-exclusion chromatography. Samples from peak 1 were analyzed by 12% SDS-PAGE.

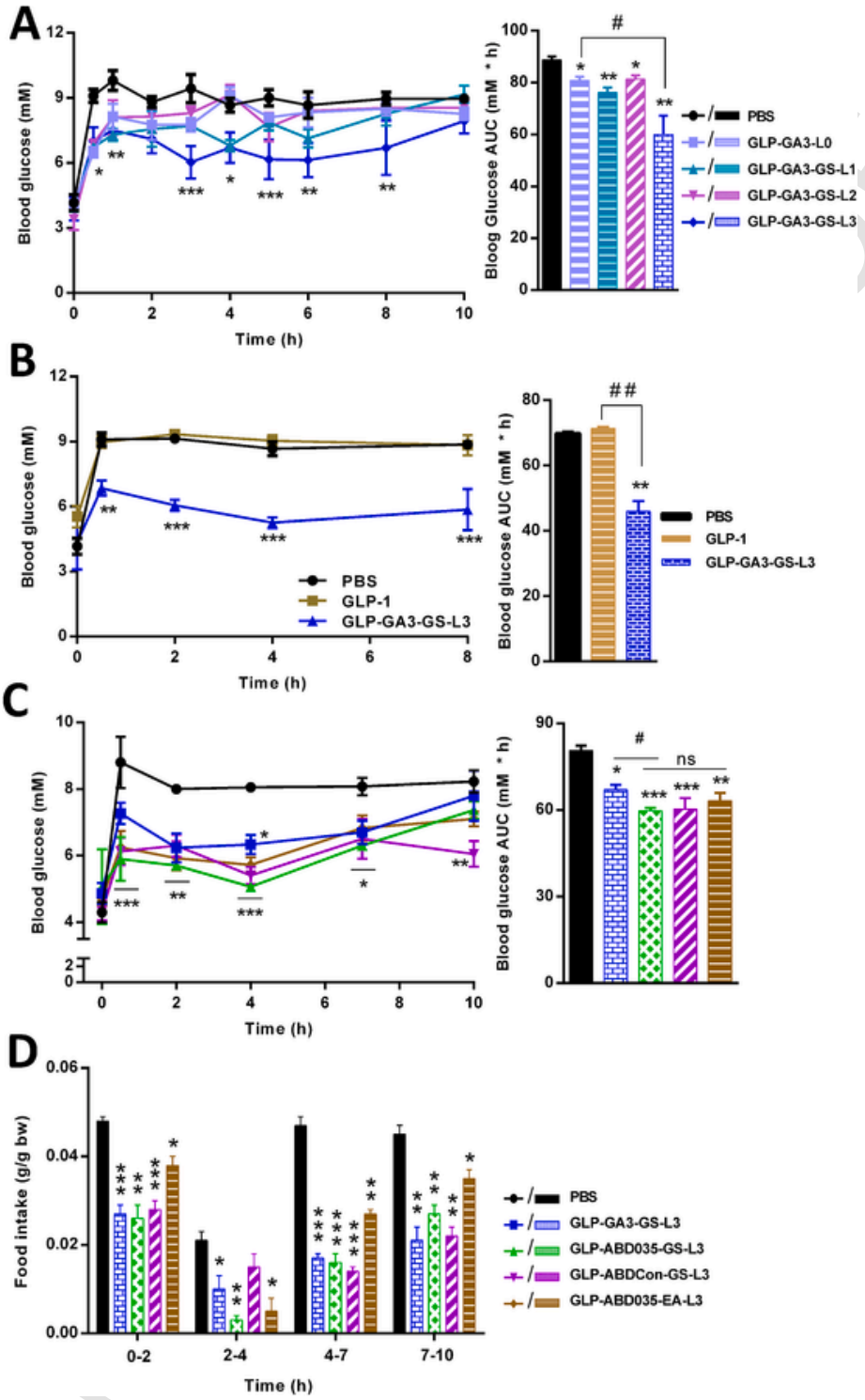

Fig. 4. Effects of ABD-fusion GLP-1 proteins on non-fasting blood glucose and food intake. The mice were fasted overnight (for 14–16 h) followed by subcutaneous injection of fusion proteins at a dose of 30 nmol/kg. After administration, blood glucose levels were measured by a glucometer at different time points, and food intake was also determined. (A) Blood glucose levels and area under curve (AUC) in mice treated with GLP-GA3 fusion proteins with different lengths of linkers. (B) Blood glucose levels and AUC treated with GLP-GA3-GS-L3 and GLP-1. (C and D) Blood glucose levels, AUC (C) and food intake (D) after one single injection of GLP-GA3-GS-L3, GLP-ABD035-GS-L3, GLP-ABDCon-GS-L3 and GLP-ABD035-EA-L3. Data are presented as mean ± S.E.M, n = 5–6 mice for the blood glucose study. *P < 0.05, **P < 0.01, ***P < 0.001 for test groups versus PBS group. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 for GLP-GA3-GS-L3 group versus other test groups. The "ns" represents no significance.

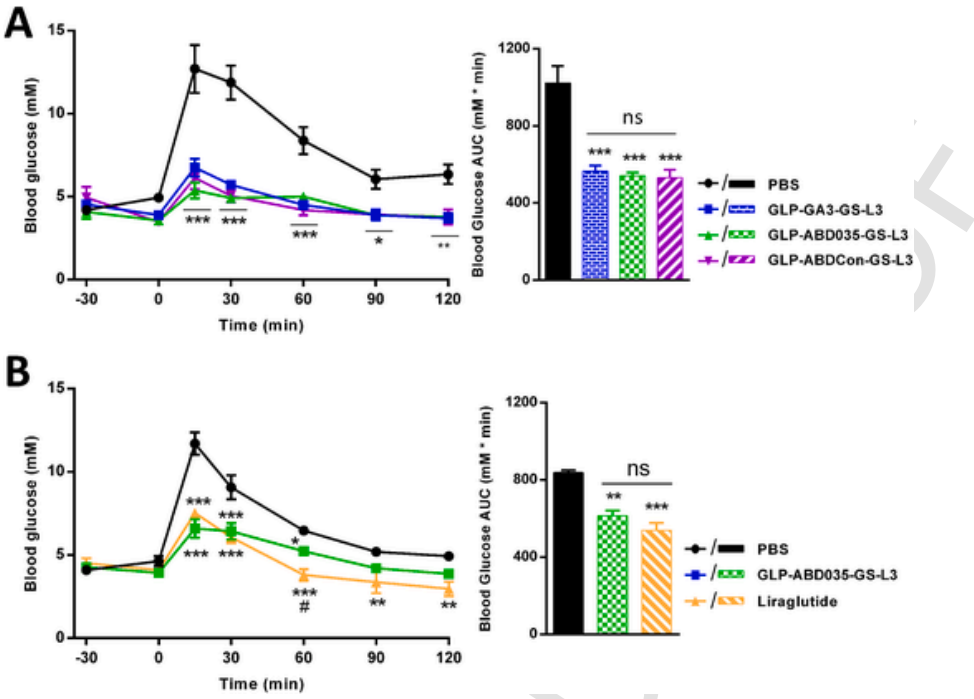

Fig. 5. Acute effects on oral glucose tolerance in mice treated with ABD-fusion GLP-1 proteins or liraglutide. The mice were fasted overnight (14–16 h) followed by subcutaneous injection of fusion proteins at a dose of 15 nmol/kg 30 min before oral glucose challenge (2 g glucose per kg body weight). (A) Blood glucose and AUC during glucose challenge in mice after injection of 15 nmol/kg of GLP-GA3-GS-L3, GLP-ABD035-GS-L3 and GLP-ABDCon-GS-L3. (B) Blood glucose and AUC during glucose challenge in mice after injection of GLP-ABD035-GS-L3 and liraglutide at a dose of 15 nmol/kg. Data are presented as mean ± S.E.M, n = 5–6 mice for the blood glucose study. *P < 0.05, **P < 0.01, ***P < 0.001 for test groups versus PBS group. #P < 0.05 for liraglutide group versus GLP-ABD035-GS-L3 group. The "ns" represents no significance.

to 120 min were not significant different between the three groups (P > 0.62). This indicates that GLP-GA3-GS-L3, GLP-ABD035-GS-L3 and GLP-ABDCon-GS-L3 have a similar bioactivity in the reduction of blood glucose.

We next compared the acute glycemic effects of GLP-ABD035-GS-L3 with liraglutide, approved by the FDA for the treatment of T2DM. Both GLP-ABD035-GS-L3 and liraglutide significantly inhibited the acute, transient hyperglycemic effect after the glucose challenge. At 15 and 30 min, the blood glucose-lowering effect of GLP-ABD035-GS-L3 was comparable to liraglutide (P = 0.21 and P = 0.78, respectively). However, from 60 min to 120 min, GLP-ABD035-GS-L3 exhibited less potency than liraglutide (P < 0.05). The calculated AUC from 0 to 120 for liraglutide was less than for GLP-ABD035-GS-L3, though without a statistical difference (Fig. 5B, p = 0.20).

3.5. Pharmacokinetic studies

To determine the pharmacokinetic profiles of GLP-GA3-GS-L3, GLP-ABD035-GS-L3 and GLP-ABDCon-GS-L3, FITC was used to label fusion proteins. The half-lives of fusion proteins were analyzed by the fluorescence intensity measurements of the plasma samples obtained after a single subcutaneous injection of FITC-labeled proteins into mice. The results showed that the calculated half-lives of GLP-GA3-GS-L3, GLP-ABD035-GS-L3 and GLP-ABDCon-GS-L3 were 36.3 ± 7.8 h, 31.3 ± 1.0 h and 38.3 ± 2.7 h, respectively (Fig. 6). That was substantially longer than the half-life of natural GLP-1, which is only 1–2 min *in vivo*. There was no statistically significant difference observed amongst the three fusion proteins (P > 0.23). This indicates that the ABD fused to GLP-1 played a pivotal role in half-life extension.

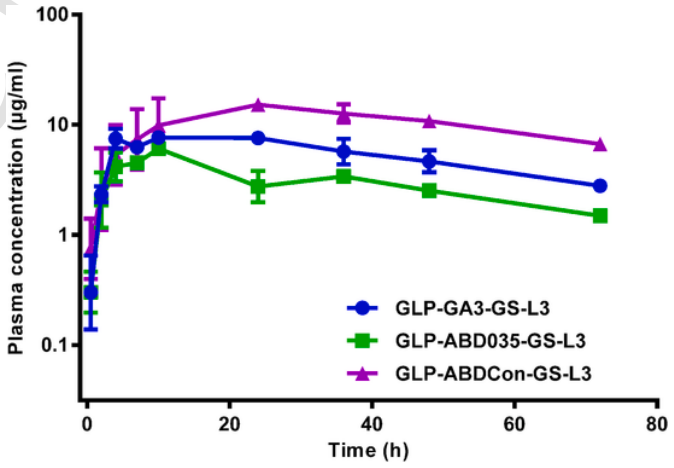

Fig. 6. Pharmacokinetic profiles of ABD-fusion GLP-1 proteins in mice. The GLP-GA3-GS-L3, GLP-ABD035-GS-L3 and GLP-ABDCon-GS-L3 labeled with FITC were subcutaneously injected into female BALB/c mice at a dose of 50 μg/mouse (n = 3 per group). Plasma was then separated by centrifugation after collection from the tail vein. The plasma was diluted with PBS followed by analysis on a black 96-well microplate at a volume of 100 μl.

4. Discussion

GLP-1 has the ability to influence glycemic control and body weight regulation. Meanwhile, recent studies have revealed that GLP-1 has a beneficial effect on inflammatory responses and cardiovascular (CV) outcomes in diabetic patients (Insuela and Carvalho, 2017; Knudsen and Lau, 2019). In addition, persistent hyperglycemia can lead to β-cell dam-

age and loss of insulin secretion ability. Previous studies have proven that GLP-1 analogs can increase β-cell proliferation, restoring the function of the damaged islets and maintaining insulin secretion ability. Therefore, injection of a GLP-1 analog has long-term benefits for T2DM patients. Consequently, GLP-1-based therapies have become increasingly attractive for the treatment of T2DM (Underwood et al., 2010). However, their extremely short half-life prevents therapeutic utilization. Many half-life extension strategies have been employed to modify GLP-1 and exenatide, a GLP-1 analog isolated from the saliva of the Gila monster, either by chemical conjugation or genetic fusion, such as PEGylation, lipidation, albumin-binding, Fc-fusion, XTEN-fusion and antibody-fusion (Gao et al., 2012; Kontermann, 2016; Li et al., 2018; Schellenberger et al., 2009; Strohl, 2015).

ABD, with its association with the FcRn-mediated recycling system, is studied extensively as a means to prolong the half-life of a therapeutic moiety by genetic fusion. A crystal structure of ABD in complex with HSA revealed that the binding site is located on the exterior of domain II of albumin, without interfering with albumin-FcRn binding (Lejon et al., 2004). Besides half-life extension, ABD-fusion has several advantages for production. Recombinant ABD-fusion proteins can be expressed in an *E. coli* system with low cost and the ABD-fusion method facilitates manufacturing, since it yields homogeneous products. Furthermore, the process of recombinant protein purification is relatively simple, avoiding the additional steps of conjugation and subsequent downstream purification (Tan et al., 2018). These features render ABD a promising alternative approach to extending the half-lives of therapeutic peptides or proteins.

Here, we designed a novel long-acting GLP-1 agonist by genetically fusing GLP-1 to the N-terminus of ABD. In order to obtain a fusion protein with improved bioactivity, we first investigated different types and lengths of linkers between GLP-1 and GA3. The flexible GS-linker was the most widely used linker, providing a certain degree of movement and separation of functional domains. This is exemplified by dulaglutide, which is a GLP-1R agonist approved for the treatment of T2DM, produced by the fusion of a modified GLP-1 to IgG-Fc through a $(GGGGS)_3$ linker (Scheen, 2016). The rigid EA-linker, forming an α-helix, can separate functional domains more effectively (Amet et al., 2009; Chen et al., 2013). By adjusting the number of amino acids, the length of a linker can be optimized to achieve a balance between mobility and the appropriate distance between functional domains. To fully clarify the effect of the linker on bioactivities, we designed a series of linkers with numbers of amino acids from 0 to 17, denoted as L0, L1, L2 and L3, respectively. The HSA binding determined by ELISA showed that there was no significant difference in the affinity of GLP-GA3 with different types and lengths of linkers for HSA. That might be because GLP-1 is a 31-amino-acid peptide, which is relatively small, and it therefore does not interfere with the interaction between GA3 and HSA. In addition, GLP-1 fused to the N-terminus of GA3, located in helix 1, might have little impact on helix 2 and helix 3, which are responsible for HSA binding (Lejon et al., 2004). However, the *in vivo* bioactivity of GLP-1 in the GLP-GA3 fusion protein was affected greatly by the length of linker. The longer linker resulted in higher bioactivity *in vivo*. The EA-linker and GS-linker with the same number of residues had no significant difference in blood glucose-lowering effect. This indicates that the effector molecule should be efficiently separated spatially from ABD bound to HSA. As described in previous studies, the flexible GS-linker with 15 or 17 residues has been used widely to separate the ABD and fusion partner, retaining the bioactivities of human necrosis factor-related apoptosis-inducing ligand (hTRAIL) (Li et al., 2016), αvβ3-integrin-binding protein (JCL) (Gapizov et al., 2019) and immunotoxin (Guo et al., 2016; Wei et al., 2018).

Previous research has demonstrated that the half-life of an ABD-fusion protein is closely related to its affinity for HSA. Higher affinity tends to translate into longer half-life. Jacobs et al. (2015) demonstrated that the higher binding affinity of ABDCon variants for MSA within a span of 3–1600 nM led to a longer half-life of fusion partners in mice. The half-life of JCL-L_{14}-ABD with a higher affinity for HSA was 10 times longer than ABD-L_{15}-JCL in mice (Gapizov et al., 2019). The half-life of recombinant immunotoxin (RIT) was prolonged to 194 min in mice for LMB-164 with a higher affinity of 0.1 nM for MSA. However, LMB-172 and LMB-170 with weaker affinities of 0.4 nM and 2.15 nM for MSA had half-lives of 144 min and 101 min. This clearly demonstrates that the affinity contributes to half-life (Wei et al., 2018). Based on the above results, three ABDs, GA3, ABD035 and ABDCon were chosen as carriers for GLP-1. The affinity of GA3, ABD035 and ABDCon for HSA is 1.2 nM, 500 fM and 75 pM, respectively (Jacobs et al., 2015; Jonsson et al., 2008). We expected to discover whether a higher affinity for HSA represented a higher bioactivity and longer half-life *in vivo*.

The pharmacokinetic results in mice showed no significant difference in the half-lives of GLP-GA3-GS-L3, GLP-ABD035-GS-L3 and GLP-ABDCon-GS-L3, in the range of 31.3–38.3 h. The half-life was in accordance with that of MSA in mice, 35–39 h (Wei et al., 2018). This indicates that ABD-fusion GLP-1 proteins bound to MSA with high affinity in blood and recycled with MSA. It was worth noticing that the half-lives of three fusion proteins were very similar, even though the affinity of ABD035 and ABDCon for MSA and HSA was much higher than that of GA3 (Jacobs et al., 2015; Jonsson et al., 2008). This might due to the high concentration of albumin in blood (30–50 mg/ml) (Kratz, 2008), resulting in a larger excess of albumin compared to the ABD-fusion GLP-1 proteins. Furthermore, the affinities of the three ABDs were still high enough for albumin, even the GA3, leading almost all fusion proteins to bind to albumin and resulting in similar half-lives. This was, to some extent, in agreement with the conclusion from Hopp et al. who indicated that the half-life-extending property of ABD in mice was only weakly influenced by its affinity for MSA (Hopp et al., 2010). Our data indicated that a significant difference in serum exposure would require a much lower affinity of ABD variant for albumin. Tencon25-ABDCon5, with an affinity of 1650 nM for albumin, possessed a two-fold shorter half-life than Tencon25-ABDCon, with an affinity of 1.86 nM (Jacobs et al., 2015).

The pharmacodynamic properties of GLP-1 were significantly improved by fusion to ABD, with sustained bioactivity of blood glucose-lowering and anorectic action *in vivo*. GLP-GA3-GS-L3, GLP-ABD035-GS-L3 and GLP-ABDCon-GS-L3 all controlled non-fasting blood glucose levels for at least 8 h after a single injection. The resulting AUC for GLP-ABD035-GS-L3 was significantly lower than for GLP-GA3-GS-L3, however, comparable to GLP-ABDCon-GS-L3. In OGTTs, GLP-ABD035-GS-L3 exhibited a similar glucose-lowering effect to GLP-GA3-GS-L3 and GLP-ABDCon-GS-L3, indicating that ABD-fusion significantly improved the glucose tolerance. The difference in bioactivity might due to the relatively long measurement time in the non-fasting glucose test. The observed significant

blood glucose-lowering effect mainly resulted from the increased secretion of insulin, as described by previous research showing that the hypoglycemic effect was accompanied by insulin increasing (Kim et al., 2010; Sun et al., 2019). We further determined the bioactivity of GLP-ABD035-GS-L3 compared with liraglutide, approved by the FDA for the treatment of T2DM. The results showed that GLP-ABD035-GS-L3 exhibited less potency than liraglutide from 60 to 120 min, though with the similar AUCs. The data indicates that ABD-fusion GLP-1 proteins are a potential T2DM treatment.

Anorectic action was one advantage of a GLP-1R agonist for the treatment of T2DM, leading to food intake inhibition and weight loss (Heppner and Perez-Tilve, 2015). In two 56-week clinical trials, liraglutide, at a dose of 3 mg, resulted in 5%–10% weight reduction in overweight and obese patients, compared to a placebo with only around 2% reduction (Davies et al., 2015; Pi-Sunyer et al., 2015). Based on the results, liraglutide was approved for treatment of T2DM at a dose of 1.2 mg or 1.8 mg per day, and approved for weight management at a dose of 3.0 mg per day in combination with diet and exercise interventions (O'Neil et al., 2018). Our results found that the ABD-fusion GLP-1 proteins all significantly inhibited food intake for 10 h. That might be positive for the blood glucose-lowering and weight loss strategies.

In view of the significant hypoglycemic activity and food intake inhibitory effect, combined with the improved pharmacokinetic properties, ABD-fusion GLP-1 proteins may have great potential in the development of anti-diabetic and anti-obesity drugs for the future.

However, further research should be performed to increase the application potential of these fusion proteins. Although the short-term control of blood glucose was confirmed, it is necessary to test the long-term effects of ABD-fusion GLP-1 proteins on blood glucose levels to provide a better reference for future applications. In addition, ABD-fusion GLP-1 proteins could be a source of new, long-acting anti-diabetic and anti-obesity drug, therefore, the toxicity of the fusion protein, including any immunogenicity and tissue toxicity, should be evaluated systematically. Furthermore, the administration route, stability, solubility and formulation are important factors for the future application and should be carefully considered. Finally, the application of the ABD-fusion strategy for use in prolonging the half-life of other pharmaceutic peptides should be investigated in future.

5. Conclusions

We have developed a novel GLP-1R agonist by genetic fusion of a modified GLP-1 to ABD. The designed ABD-fusion GLP-1 protein could be expressed in *E. coli* and purified simply, and could bind to HSA with high affinity. The ABD-fusion GLP-1 protein retained the bioactivity of native GLP-1, significantly lowered blood glucose levels and inhibited food intake. The pharmacokinetic properties of the fusion protein were also significantly improved in mice. The ABD-fusion GLP-1 protein may be promising as an agent for anti-diabetic and anti-obesity treatment. In addition, our data indicates that ABD-fusion can be applied as a universal strategy for improve the pharmacodynamic and pharmacokinetic profiles of therapeutic peptides and proteins.

6. Availability of data

The raw/processed data required to reproduce these findings cannot be shared temporally at this time, as the data also forms part of an ongoing study.

CRediT authorship contribution statement

Huanbo Tan: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Wencheng Su:** Methodology, Investigation, Formal analysis. **Wenyu Zhang:** Methodology, Investigation. **Jie Zhang:** Methodology, Investigation. **Michael Sattler:** Writing - review & editing. **Peijian Zou:** Conceptualization,, Supervision, Writing - review & editing.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

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