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Generation of novel long-acting GLP-1R agonists using DARPins as a scaffold

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) has been considered to be a promising peptide for treatment of type 2 diabetes mellitus (T2DM). However, the extremely short half-life (minutes) of native GLP-1 limits its clinical application potential. Here, we designed two GLP-1 analogues by genetic fusion of GLP-1 to one or two tandem human serum albumin-binding designed ankyrin repeat proteins (DARPins), denoted as GLP-DARPin or GLP-2DARPin. The two DARPin-fusion GLP-1 proteins were expressed in *E. coli* and purified, followed by measurements of their bioactivities and half-lives in mice. The results revealed that the half-life of GLP-2DARPin, binding two HSA molecules, was approximately 3-fold longer than GLP-DARPin (52.3 h versus 18.0 h). In contrast, the bioactivity results demonstrated that the blood glucose-lowering effect of GLP-DARPin was more potent than that of GLP-2DARPin. The oral glucose tolerance tests indicated that blood glucose levels were significantly reduced for at least 48 h by GLP-DARPin, but were reduced for only 24 h by GLP-2DARPin. Injected once every two days, GLP-DARPin substantially reduced blood glucose levels in streptozotocin (STZ)-induced diabetic mice to the same levels as normal mice. During the treatment course, GLP-DARPin significantly reduced the food intake and body weight of diabetic mice up to approximately 17% compared with the control group. A histological analysis revealed that GLP-DARPin alleviated islet loss in diabetic mice. These findings suggest that long-acting GLP-DARPin holds great potential for further development into drugs for the treatment of T2DM and obesity. Meanwhile, our data indicate that albumin-binding DARPins can be used as a universal scaffold to improve the pharmacokinetic profiles and pharmacological activities of therapeutic peptides and proteins.

1. Introduction

Diabetes is increasing globally, and has become one of the leading causes of high mortality and morbidity rates (Tan et al., 2019). Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance and progressive β-cell failure, eventually leading to hyperglycemia (Saisho, 2015). According to investigations, 85% of patients with T2DM are overweight or obese (Evers et al., 2018). Weight loss can lead to a reduced need for anti-hyperglycemic drugs in T2DM patients with obesity (Yang et al., 2020). Therefore, it is of critical importance to develop drugs or drug combinations that promote weight loss and lower of blood glucose for the T2DM patients (Gilroy et al., 2020).

Glucagon like peptide-1 (GLP-1) is a naturally occurring incretin hormone, secreted from intestinal L-cells in response to nutrient digestion (Baggio and Drucker, 2007). GLP-1 can maintain glucose homeostasis through binding to the GLP-1 receptor (GLP-1R). GLP-1 stimulates glucose-dependent insulin secretion, β-cell proliferation and survival, and inhibits glucagon secretion. Meanwhile, GLP-1 also plays a significant role in delaying gastric emptying, decreasing appetite and reducing food intake, which results in weight loss (Cho et al., 2012; Wang et al., 2020). Moreover, recent studies have demonstrated that GLP-1 also has beneficial physiological effects on the cardiovascular and neurological systems (Reed et al., 2020). The versatility of GLP-1 has sparked considerable interest in the application of GLP-1 as a drug for treatment of T2DM and obesity. However, one major limitation of native GLP-1 hampers its application in the clinic. The half-life of GLP-1 in

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circulation is very short, typically about 1–2 min, attributed to both rapid cleavage by ubiquitous dipeptidyl peptidase 4 (DPP-4) and clearance by the kidney (Deacon et al., 1998; Kieffer et al., 1995). Consequently, GLP-1 needs to be injected repeatedly or infused continuously to maintain therapeutic levels, resulting in significant inconvenience (Baggio et al., 2008).

The great promise of GLP-1 has fostered ongoing efforts to develop long-acting GLP-1R agonists, and has achieved great success. To date, several GLP-1R agonists have been approved by the FDA or EMA, including twice-daily exenatide (Byetta), once-daily lixisenatide (Lyxumia) and liraglutide (Victoza), once-weekly exenatide-LAR (Bydureon), albiglutide (Tanzeum), dulaglutide (Trulicity) and semaglutide (Ozempic) (Eissa et al., 2021; Reed et al., 2020). These long-acting GLP-1R agonists exploit several strategies to overcome the two main factors which lead to a short half-life of GLP-1. Firstly, the susceptible amino acid in GLP-1 has been modified into a mutant that can resist DPP-4 degradation (albiglutide, dulaglutide and semaglutide). Secondly, the sequences of Exenatide, lixisenatide and exenatide-LAR are derived from exendin-4, found in the saliva of the Gila monster, sharing 53% homology with human GLP-1 and resistant to DPP-4 (He et al., 2019). Additionally, the half-life of GLP-1 can be substantially prolonged by virtue of the recycling mechanism of the neonatal Fc receptor (FcRn) through binding to IgG-Fc or human serum albumin (HSA). This binding leads to an increased hydrodynamic volume, resulting in slowed renal clearance (liraglutide, albiglutide, dulaglutide and semaglutide) (Kontermann, 2016; Strohl, 2015).

HSA exhibits an average half-life of 19 days in circulation, and has been used widely as a versatile carrier for improving pharmacokinetic properties of therapeutic peptides or proteins (Kratz, 2008; Yang et al., 2016). Genetic fusion or non-covalent binding of GLP-1 to HSA has proven to be an effective strategy for half-life extension which has brought three products to the market. Albiglutide consists of two tandem DPP-4 resistant GLP-1 molecules genetically fused to HSA (Tomkin, 2009). Both liraglutide and semaglutide extend half-lives by conjugating a fatty acid chain non-covalent associate with HSA (Bech et al., 2018; Ribel et al., 2002).

Furthermore, besides fatty acid chains, albumin-binding moieties (Jonsson et al., 2008; McMahon et al., 2018; O'Connor-Semmes et al., 2014; Steiner et al., 2017) with high affinity for HSA are also reported to be used as a carrier for half-life extension through non-covalent binding to HSA. DARPins are derived from natural ankyrin repeat proteins, a class of binding proteins with high abundance in the human genome (Stumpp et al., 2008). Ankyrins are characterized by their high specificity and high affinity to a large variety of different proteins, and are composed of up to 29 tandem copies of a 33-amino-acid repeat (Caputi and Navarra, 2020). DARPins, engineered by a consensus design approach based on the natural ankyrins, have only two to four 33-residue modules flanked by the first N-capping repeat and the last C-capping repeat (Binz et al., 2003; Caputi and Navarra, 2020). DARPins can be developed to bind with high specificity to a series of proteins, such as membrane proteins, kinases, proteases, cytokines and growth factors, based on ribosome display, phage display and yeast-surface display technologies (Stumpp et al., 2008; Stumpp et al., 2020). DARPins exhibit many advantages, such as high thermal and thermodynamic stability, high storage stability, high production in *E. coli* and no proteolytic digestion in human serum *in vitro*. Therefore, DARPins can be envisioned as an alternative to antibody-based scaffolds (Caputi and Navarra, 2020). To date, several candidate drugs based on DARPins have been tested in clinical trials. MP0250 is a tri-specific DARPin drug, consisting of one DARPin domain binding to vascular endothelial growth factor (VEGF), one DARPin domain binding to hepatocyte growth factor (HGF) and two DARPin domains that target HSA for half-life extension (Binz et al., 2017). The half-life of MP0250 in human was about 2 weeks. MP0250 is now in phase 2 clinical trials (Stumpp et al., 2020). This suggests that DARPins with a high affinity for HSA can be used as a universal carrier to prolong the half-lives of their fusion partners.

Here, we designed two novel GLP-1R agonists by genetic fusion of one GLP-1 molecule with substitution of Gly for Ala to prevent degradation from DPP-4 to one or two DARPins, which bind to HSA with a high affinity. The pharmacokinetic and pharmacodynamic profiles of DARPin-fusion GLP-1 proteins are significantly improved. DARPin-fusion GLP-1 proteins exhibit a promising potential for the treatment of T2DM and obesity.

2. Materials and methods

2.1. Protein construction

DARPins with a high affinity for HSA were derived from MP0250 (Steiner et al., 2017). The GLP-1 molecule consisted of 31 amino acids from human GLP-1(7–37) with a Gly instead of Ala at position 8 to prevent degradation from DPP-4. The GLP-1R agonist, a GLP-DARPin fusion protein, was constructed by the genetic fusion of modified GLP-1 to the N-terminal of DARPins through a flexible linker, GS $(GGGGS)_3$, denoted as GLP-DARPin. Similarly, one modified GLP-1 molecule was genetically fused to the N-terminal of two tandem DARPins assembled via a Pro-Thr-rich polypeptide generated GLP-2DARPin fusion protein, resulting in GLP-2DARPin. A His_6-tag was added to the N-terminus of the above fusion proteins for affinity chromatography and a TEV protease cleavage site was inserted between the His_6-tag and GLP-1 for acquisition of a free N-terminus of GLP-1. The DNA sequences encoding GLP-DARPin and GLP-2DARPin fusion proteins were optimized according to the codon usage of *E. coli* and synthesized by the company GENEWIZ (Suzhou, China). The genes were then digested by the restriction endonucleases of *Nco* I and *EcoR* I (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 according to the protocol described by Su et al. (Su et al., 2020) with some modifications. The plasmids encoding fusion proteins were transformed into chemically competent *E. coli* BL21(DE3) cells. One colony was picked randomly from the overnight plate and cultured in LB medium with 60 μg/mL kanamycin overnight at 37 °C. The overnight cultures were added into fresh LB medium at a ratio of 1:100, and grown until the OD_{600} reached 0.4–0.6 at 37 °C. Then, IPTG was added with a final concentration of 0.1 mM and bacteria were cultured at 25 °C. After 16 h, the cultures were centrifuged at 5500 g for 20 min at 4 °C, then resuspended in the lysis buffer (25 mM Tris/HCl, 300 mM NaCl, 10 mM imidazole, pH 8.0), and lysed by high-pressure homogenizer (ATS, Taizhou, China) for three cycles with a pressure of 600–800 bar, followed by centrifugation at 26,000*g* for 30 min.

The supernatants were added to a Ni^{2+}-NTA column (GE, USA) pre-equilibrated with 5 column volumes (CVs) of the lysis buffer. The column was washed with 5 CVs of the wash buffer (25 mM Tris/HCl, 300 mM NaCl, 30 mM imidazole, pH 8.0). The bound fusion proteins were eluted with the elution buffer (25 mM Tris/HCl, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted fractions were pooled and dialyzed against 25 mM Tris/HCl, pH 8.0.

The N-terminal His_6-tag of fusion proteins were removed by adding 1/50 TEV protease of the purified proteins and 1 mM dithiothreitol (DTT). After reaction for two hours at room temperature, the mixture was dialyzed against 25 mM Tris/HCl, pH 8.0, overnight. Then the mixture was applied to a Ni^{2+}-NTA column again, and the flow-through fractions from the column were collected and pooled. The untagged fusion proteins were further purified by a Q ion exchange column (GE, HiTrap Q HP) pre-equilibrated in 25 mM Tris/HCl, pH 8.0. The fusion proteins eluted from the Q column were analyzed by 15% SDS-PAGE (EpiZyme, Shanghai, China).

2.3. Binding assay for HSA

Enzyme-linked immunosorbent assay (ELISA) and size-exclusion chromatography (SEC) were applied to determine whether a DARPin-fusion protein had the ability to bind to HSA. The ELISA method was conducted as described by Tan et al. (Tan et al., 2021) and Li et al. (Li et al., 2016) with some modifications. HSA (Sigma-Aldrich, Shanghai, China), diluted to 200 μg/mL using a carbonate-bicarbonate buffer (pH 9.6), was immobilized in a 96-well plate (100 μL/well) at 4 °C overnight. The coated plate was then washed three times using PBST (PBS + 0.05% Tween 20), then blocked with 5% BSA (Sigma-Aldrich, Shanghai, China) overnight. After washing three times with PBST, a series of concentrations of fusion proteins prepared in PBS (pH 7.4, 100 μL) were added to the wells, and then incubated at room temperature for 2 h. Unbound proteins were removed with three PBST washes and the rabbit monoclonal human GLP-1 antibody (MAB12492, R&D systems, USA) with a dilution of 1:6000 by PBST was added to detect the GLP-1 in the fusion proteins. Washing was repeated three times and the HRP-conjugated goat anti-rabbit IgG (ProteinFind, HS101-01, TransGen Biotech, Beijing, China) with 1:5000 dilution in PBST was added to each well. After washing, the bound HRP was detected by addition of TMB substrate (HE101-01, TransGen Biotech, Beijing, China). The reaction was stopped by adding 100 μL of 1 M HCl, followed by measurement on a SpectraMax M2 micro-plate reader (Molecular Devices, USA) at 450 nm.

The SEC method was also applied to qualitatively determine the affinity of fusion proteins for HSA according to the description by McMahon et al. (McMahon et al., 2018), with some modifications. The DARPin-fusion proteins were mixed with HSA in a 1.2: 1 ratio in PBS (pH 7.4), followed by incubation at room temperature for 1 h. The mixture was then separated by a Superdex 200 10/300 gel filtration column (GE, USA) in PBS buffer (pH 7.4).

2.4. Animals

Male C57BL/6 mice (20–25 g) were purchased from the Academy of Military Medical Sciences (Beijing, China). The mice were housed in a temperature-controlled room maintained at 24 ± 2 °C. The room was under a 12 h/ 12 h light/ dark cycle. The mice were allowed to eat food and drink water freely, except where noted. Mice were group-housed and allowed a one-week acclimation before the study. All injections and measurements were carried out during the light cycle. Studies *in vivo* were performed following the guideline principles for the care and use of laboratory animals with the approval of the Animal Care Committee of China and were approved by the Experimental Animal Center of the Academy of Military Medical Sciences (SCXK-2020–0015).

2.5. Pharmacokinetics determination in mice

For quantifying the pharmacokinetic properties of fusion proteins, FITC (Sigma-Aldrich, Shanghai, China) was used to label the DARPin-fusion proteins, following the manufacturer's instructions and as described by Tan et al. (Tan et al., 2021) Briefly, 50 μL FITC at a concentration of 1 mg/mL dissolved in DMSO was mixed with 1 mL protein solution (2 mg/mL) prepared in sodium carbonate-bicarbonate, pH 9.0, followed by incubation in the dark for 8 h at 4 °C. Then, NH_4Cl with a final concentration of 50 mM was added into the mixture to stop the reaction. Five percent glycerol was also added. The FITC-labelled fusion proteins were separated from unbound FITC by a G-25 desalting column (GE, USA) pre-equilibrated with PBS (pH 7.4). The FITC-conjugated fusion proteins were stored in a black Ep tube at 4 °C. Protein concentration was measured by BCA protein assay kit (CWBIO, Beijing, China).

To determine the pharmacokinetics of fusion proteins, the FITC-conjugated fusion proteins (50 μg/mouse) were administered subcutaneously into normal male C57BL/6 mice (n = 3 per group). At 0.5, 2, 4, 7, 10, 24, 36, 48 and 72 h after injection, 20 μL of blood was collected from the tail vein into an ice-cold Ep tube and mixed with EDTA-Na_2 for anticoagulation. The plasma, separated by centrifugation at 1500 g for 15 min at 4 °C, was diluted with PBS. The fluorescence detection of diluted plasma (100 μL) was performed on a black 96-well microplate using a SpectraMax M2 micro-plate reader at excitation and emission wavelengths of 485 and 535 nm. The quantified concentration of FITC-labelled fusion proteins in plasma was calculated from a standard curve. Non-compartmental analysis of the final data was performed by the PK Solver Excel Add-in program (Zhang et al., 2010) to calculate the half-life (Jacobs et al., 2015).

2.6. Non-fasting blood glucose test

Blood glucose-lowering effects of fusion proteins were tested in mice. Male C57BL/6 mice were randomly divided into three groups according to blood glucose levels and body weight (n = 4 per group). The mice were fasted overnight (14–16 h). The fusion proteins were subcutaneously injected into mice as a single dose of 30 nmol/kg. The mice in control group received the same volume of PBS. The mice were then given free diet and water during the test. The non-fasting blood glucose of mouse in each group was measured using a OneTouch UltraEasy glucometer (Johnson & Johnson, USA) at pre-set time-points after administration.

2.7. Oral glucose tolerance test (OGTT)

To evaluate whether the fusion proteins retained the ability to suppress the acute increase of blood glucose, OGTTs were conducted 24 h after a single subcutaneous injection, as described by Su et al. (Su et al., 2020), with some modifications. The male C57BL/6 mice were divided into four groups according to blood glucose levels and body weight (n = 4 per group). The fusion proteins were administered subcutaneously at a dose of 30 nmol/kg. Liraglutide, developed by Novo Nordisk (Denmark), was also injected at the same dose as a positive control. The same volume of PBS was given to the mice in the control group. Twenty-four hours after administration, the mice fasted overnight (14–16 h) were orally administered 2 g of glucose per kg body weight. Their blood glucose levels were measured by a OneTouch UltraEasy glucometer at 0, 15, 30, 60, 120 min after the glucose load.

To examine whether the fusion proteins sustained the blood-lowering effect of GLP-1, the OGTTs were also carried out 48 h and 72 h after a single dose of fusion proteins (90 nmol/kg). The procedures of OGTTs were performed as described above.

2.8. Repeated dose study in STZ-induced diabetic mice

To confirm that the fusion proteins had the ability to reduce the blood glucose levels in diabetic mice, streptozotocin (STZ) was used to induce diabetes in C57BL/6 mice. The STZ solution (0.2 mL in citrate buffer, pH 4.5) at a dose of 45 mg/kg was injected intraperitoneally into fasted (14–16 h) C57BL/6 mice for 5 consecutive days. After another 7 days with free access to food and water, non-fasting blood glucose levels were measured. The mice with non-fasting morning blood glucose levels over 11.1 mM were regarded as diabetic model mice. The diabetic mice were randomly divided into 4 groups (n = 6 per group). GLP-DARPin (90 nmol/kg) and liraglutide (10 nmol/kg) were subcutaneously injected into diabetic mice every 2 days for three weeks. Normal mice were used as a control and were given the same volume of PBS. The mice were provided access to pre-weighed food and water. Non-fasting blood glucose levels, food intake and body weight were measured in the morning before each injection. The last injection was performed on day 21, followed by measurements for another 6 days. Blood glucose was measured with a handheld glucometer as described above.

2.9. Histopathology analysis of pancreases of STZ-induced diabetic mice

Pancreases (n = 3) were rapidly removed from mice in each group on

day 27 after measuring glucose levels and body weight, washed with ice-cold PBS, and then fixed with 10% (v/v) formalin. The fixed pancreases were embedded in paraffin after dehydration in alcohol. The tissues were sectioned into 5 μm thick sections and stained with hematoxylin and eosin (H&E). The stained tissue sections were examined using a microscope (Leica, DM2000 LED).

2.10. Statistical analysis

Data were analyzed and graphed using GraphPad Prism 8 (GraphPad software, San Diego, CA, USA). All data are presented as mean ± S.E.M. Curve fitting was performed using a four-parameter logistic equation to calculate the EC_{50}. Statistical analyses were performed using the one-way or two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison analysis. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Construction and purification of DARPin-fusion GLP-1 proteins

DARPins with properties of high stability and high affinity for HSA can be applied as a scaffold to prolong the half-life of a fusion partner. To improve the pharmacokinetic and pharmacological activity of GLP-1, one or two DARPin domains were genetically fused to the C-terminal of GLP-1 through a flexible GS-linker with a length of 17 amino acids, referred to as GLP-DARPin or GLP-2DARPin, respectively (Fig. 1A). For purification, a His_6-tag was fused to the N-terminal of sequence. A TEV protease recognition site was inserted between the His_6-tag and GLP-1 to release the GLP-1 with a free N-terminus, which was essential for bioactivity. GLP-2DARPin was constructed by fusion of a second DARPin domain to the C-terminal of GLP-DARPin via a PT-rich linker, which was more rigid than the GS-linker, in order to separate the domains more effectively (Steiner et al., 2017).

The structures of the GLP-DARPin and GLP-2DARPin were built by modelling using Modeller program (version 9.21), based on the crystal structures of GLP-1 (5VAI) and DARPins (PDB: 4GRG) as templates (Fig. 1B). The models of fusion proteins demonstrated that GLP-1 and the DARPin domain were sterically separated, leading to no interference with the binding affinity for GLP-1R and HSA.

The recombinant fusion proteins were expressed in *E. coli* BL21(DE3) and both fusion proteins were highly soluble. After purifying the fusion proteins with a Ni^{2+}-NTA column, they were cleaved by TEV protease to free the N-terminal of GLP-1 for GLP-1R binding. The untagged fusion proteins were further purified by a second Ni^{2+}-NTA column and a Q ion exchange column. The eluted proteins were analyzed by 15% SDS-PAGE, exhibiting two bands on SDS-PAGE (Fig. 1c). We further analyzed the fusion proteins by Q ion exchange and size-exclusion chromatography and found that they eluted as monomeric peaks without aggregation or multimerization (data not shown). This indicated that the additional band might be ascribed to incomplete unfolding of DARPin domains on SDS-PAGE due to their high thermodynamic stability. That was consistent with the results of previous studies on anti-EGFR E01 DARPin and DARPin variants, where more than one band was observed when analyzed by SDS-PAGE (Balakrishnan et al., 2019; Binz et al., 2003).

3.2. Binding assay to HSA

The affinity of the DARPin domain for HSA has an important impact on the pharmacokinetic profiles of fusion proteins. To determine the binding affinity of DARPin-fusion GLP-1 fusion proteins for HSA, ELISA and analytical size-exclusion chromatography (SEC) were conducted. In the ELISA test, increasing concentrations of fusion proteins were added into a 96-well plate pre-coated with HSA, followed by detection of the bound fusion proteins using monoclonal antibodies against GLP-1. The results showed that GLP-2DARPin had a much higher affinity for HSA compared to GLP-DARPin. The concentration for 50% of maximal effect (EC_{50}) of GLP-2DARPin was 0.51 ± 0.04 nM, which was 18 times lower than the concentration for GLP-DARPin, 9.06 ± 0.15 nM (Fig. 2A). This suggested that both of the two DARPin domains in the GLP-2DARPin protein could bind to HSA effectively. Furthermore, the SEC method was also used to analyze the binding properties of DARPin-fusion GLP-1 proteins to HSA. The chromatography showed that an obvious earlier peak appeared after incubation of fusion proteins with HSA. The peak, corresponding to the GLP-2DARPin-HSA complex (12.20 mL), was much earlier than that of GLP-DARPin-HSA (14.12 mL, Fig. 2B, C). The apparent molecular weight of the GLP-DARPin-HSA complex, calculated from the standard curve, was 102.3 kDa, which was slightly larger than its actual 83.9 kDa, possibly because of the non-spherical structure of the complex. This indicated that GLP-DARPin bound to one HSA molecule. The calculated apparent molecular weight of GLP-2DARPin-HSA complex was 277.3 kDa, suggesting that one GLP-2DARPin bound to two HSA molecules, resulting in an actual weight of 165.2 kDa (Fig. S1). The results of ELISA and SEC demonstrated that the fusion proteins, GLP-DARPin and GLP-2DARin, retained the ability to bind to HSA with

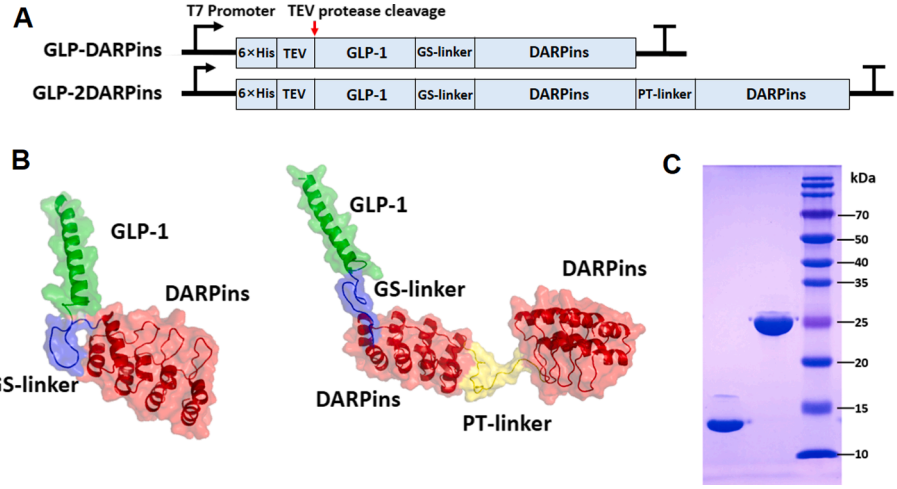

Fig. 1. Design and SDS-PAGE analysis of DARPin-fusion GLP-1 proteins. (A) Schematic representation of expression plasmids of GLP-DARPin and GLP-2DARPin. (B) The structures of the GLP-DARPin and GLP-2DARPin modelled by Modeller program (version 9.21) based on the crystal structures of GLP-1 (5VAI) and DARPins (PDB: 4GRG) as templates. (C) 15% SDS-PAGE analysis of purified GLP-DARPin and GLP-2DARPin.

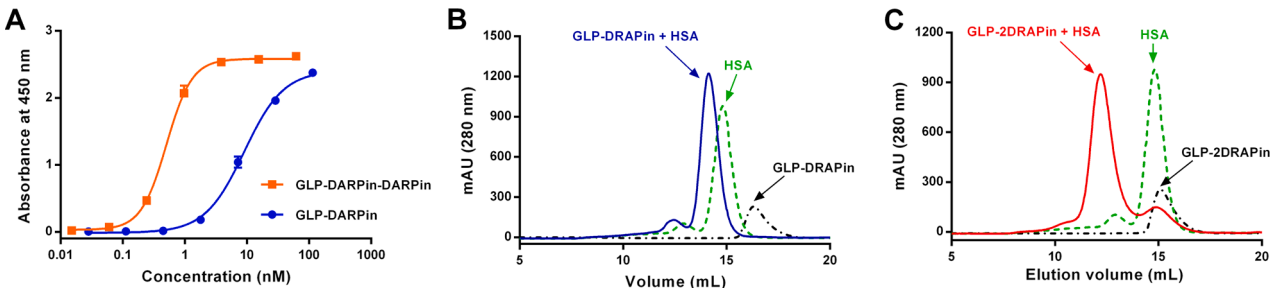

Fig. 2. Binding of GLP-DARPin and GLP-2DARPin proteins to HSA analyzed by ELISA and SEC methods. (A) Affinity determination of GLP-DARPin and GLP-2DARPin for HSA by ELISA. A series of concentrations of GLP-DARPin or GLP-2DARPin were incubated with HSA pre-coated on a microtiter plate. The bound fusion proteins were detected by antibody against GLP-1. (B and C) Binding of GLP-DARPin (B) and GLP-2DARPin (C) to HSA were analyzed by SEC. DARPin-fusion GLP-1 proteins were incubated with HSA at a ratio of 1.2: 1 at room temperature for 1 h followed by SEC analysis using a Superdex 200 column (GE, 10/300 GL).

high affinity in a non-covalent manner.

Additionally, the bioactivities of GLP-DARPin and GLP-2DARPin were evaluated *in vitro*, based on the accumulation of cyclic adenosine monophosphate (cAMP) in cells expressing GLP-1R. The results showed that GLP-DARPin had a similar potency to native GLP-1, due to the small molecular weight of the DARPin domain (only 13 kDa). However, a slightly reduced efficacy was observed for GLP-2DARPin, perhaps because of the presence of two DARPin domains (Fig. S2).

3.3. Determination of half-life

To confirm whether the DARPins with a high affinity for HSA had the capacity to prolong the half-life of a fusion partner, FITC was applied to label the fusion protein for half-life determination. The FITC-labelled DARPin-fusion GLP-1 proteins were subcutaneously injected into mice, followed by analysis of fluorescence intensity in plasma samples. The calculated half-life of FITC-labelled GLP-2DARPin was 52.3 ± 4.6 h, which was approximately 3-fold longer than that of GLP-DARPin, 18.0 ± 2.8 h (Fig. 3). The half-lives of both fusion proteins were significantly longer than the half-life of native GLP-1, which is only 1–2 min *in vivo* (Kim et al., 2010). These results demonstrated that DARPin, bound to HSA, could extend the half-life of a payload, and it is noteworthy that a higher affinity for HSA resulted in longer retention time in circulation.

3.4. DARPin-fusion GLP-1 proteins decreased blood glucose in mice

To examine the glucose-lowering effect of GLP-DARPin and GLP-2DARPin, the fusion proteins were subcutaneously injected into C57BL/6 mice, followed by measurement of glucose levels at different time points. The non-fasting blood glucose levels of mice were detected following a single administration of GLP-DARPin or GLP-2DARPin at a dose of 30 nmol/kg. The results demonstrated that both of the fusion proteins retained the bioactivity of natural GLP-1, markedly reducing blood glucose levels for at least 24 h (Fig. 4A). The duration of this blood glucose-lowering effect was considerably longer than for modified GLP-1 with an A8G mutation, which only exhibited 0.5 h even at a dose of 50 nmol/kg in mice (Fremaux et al., 2019). It is worth noting that the GLP-DARPin group had a significantly lower blood glucose level at 2 h compared with the GLP-DARPin group (Fig. 4A). However, the calculated glucose area under curve (AUC) for GLP-DARPin was not significantly different from that of GLP-2DARPin (Fig. 4A).

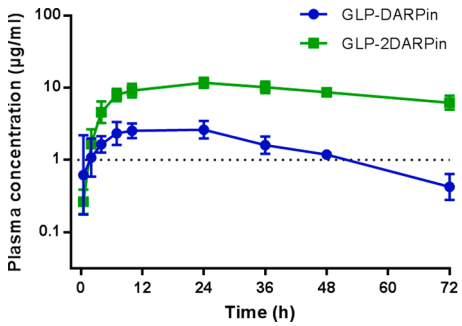

Fig. 3. Half-life determination of GLP-DARPin and GLP-2DARPin. The DARPin-fusion GLP-1 proteins labelled by FITC were administered subcutaneously into male C57BL/6 mice at a dose of 50 μg/mouse (n = 3 per group). The plasma obtained from the tail vein at 0.5, 2, 4, 7, 10, 24, 36, 48 and 72 h after injection was diluted with PBS followed by analysis using a black 96-well microplate at excitation and emission wavelengths 485 and 535 nm.

To more closely compare the hypoglycemic activities of GLP-DARPin and GLP-2DARPin, oral glucose tolerance tests (OGTTs) were conducted 24 h after subcutaneous injection at a dose of 30 nmol/kg in fasted mice. Moreover, to further confirm whether the DARPin domain could extend the blood glucose-lowering effect of GLP-1, liraglutide, an FDA-approved drug developed by Novo Nordisk for T2DM treatment with once-daily administration, was administered as a positive control. After 24 h, the results demonstrated that GLP-DARPin still reduced the blood glucose significantly at 120 min, resulting in the lowest AUC value when compared with GLP-2DARPin and liraglutide. Liraglutide only lowered the blood glucose levels at 15 and 30 min, leading to a comparable AUC value to PBS. The blood glucose-lowering effect of GLP-DARPin was more potent than GLP-2DARPin at 120 min. The calculated AUC value, from 0 to 120 min, of GLP-2DARPin was lower than that of liraglutide, though the difference was not statistically significant (Fig. 4B).

To further clarify how long the GLP-DARPin could reduce blood glucose levels *in vivo*, OGTTs were performed 48 h and 72 h after subcutaneous administration at a dose of 90 nmol/kg. After 48 h, GLP-DARPin still retained the ability to inhibit acute hyperglycemic effects, reducing blood glucose significantly at 15 and 30 min, resulting in a much lower AUC value compared with PBS (Fig. 4C). After 72 h, GLP-DARPin led to a lower blood glucose level from 15 to 120 min, though the difference was not statistically significant (Fig. 4D).

3.5. GLP-DARPin reduced blood glucose of STZ-induced diabetic mice

To further verify whether GLP-DARPin held potential for the treatment of T2DM in clinic, diabetic model mice were induced by STZ. After induction, a mouse with a non-fasting blood glucose level higher than 11.1 mM was considered to be diabetic. GLP-DARPin was subcutaneously injected into diabetic mice once every two days at a dose of 90 nmol/kg. Liraglutide was used as a control. Blood glucose levels, food intake and body weight were measured for 27 days. The results demonstrated that the non-fasting blood glucose levels of diabetic mice were 20 ~ 30 mM, and 6 ~ 8 mM for normal mice (Fig. 5A). After injection of GLP-DARPin, the non-fasting blood glucose of diabetic mice gradually decreased to the normal levels at day 5. The GLP-DARPin maintained the blood glucose of diabetic mice at a level comparable to normal mice for at least 18 days. At day 21, the last injection was conducted. The blood glucose levels gradually increased from day 25 to

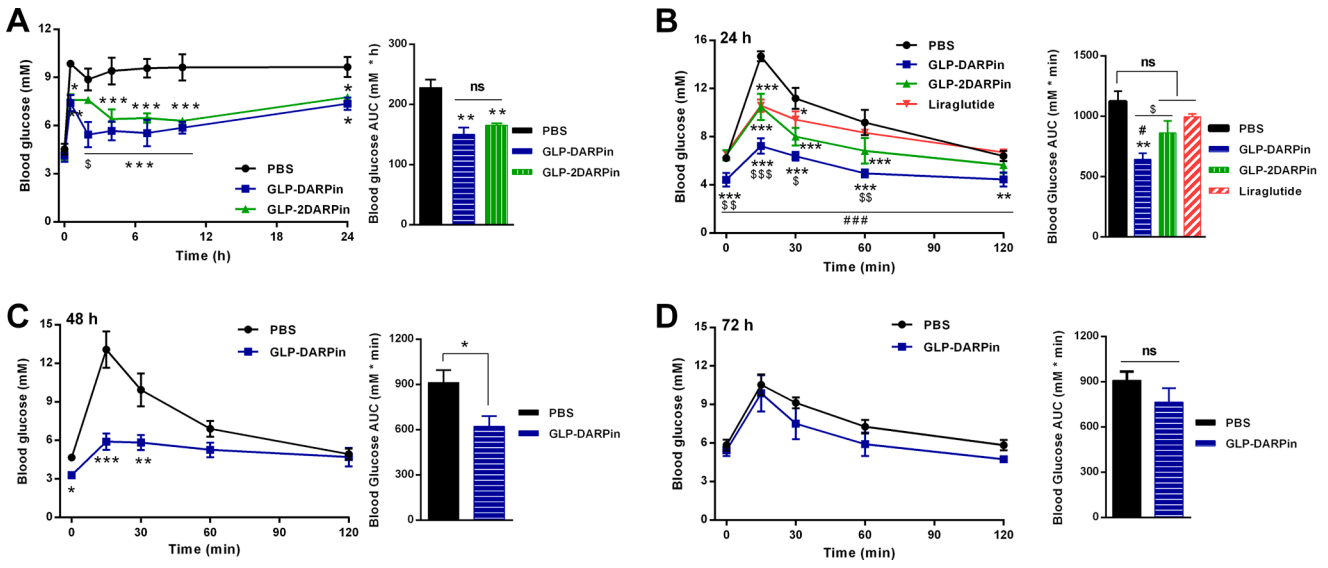

Fig. 4. Pharmacological activities of GLP-DARPin and GLP-2DARPin in normal C57BL/6 mice. (A) Blood glucose levels and area under curve (AUC) after one single subcutaneous injection of GLP-DARPin and GLP-2DARPin proteins at a dose of 30 nmol/kg. (B-D) Blood Glucose levels and AUC during OGTTs performed 24 h (B), 48 h(C) and 72 h (D) after one single injection in mice. The mice, fasted overnight, were subcutaneously injected with GLP-DARPin, GLP-2DARPin or liraglutide at a dose of 30 nmol/kg. Data are presented as mean ± SEM, n = 4 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for test groups versus PBS group. $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ for DARPin group versus liraglutide group. $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ for GLP-DARPin group versus GLP-2DARPin group. The "ns" represents no significance.

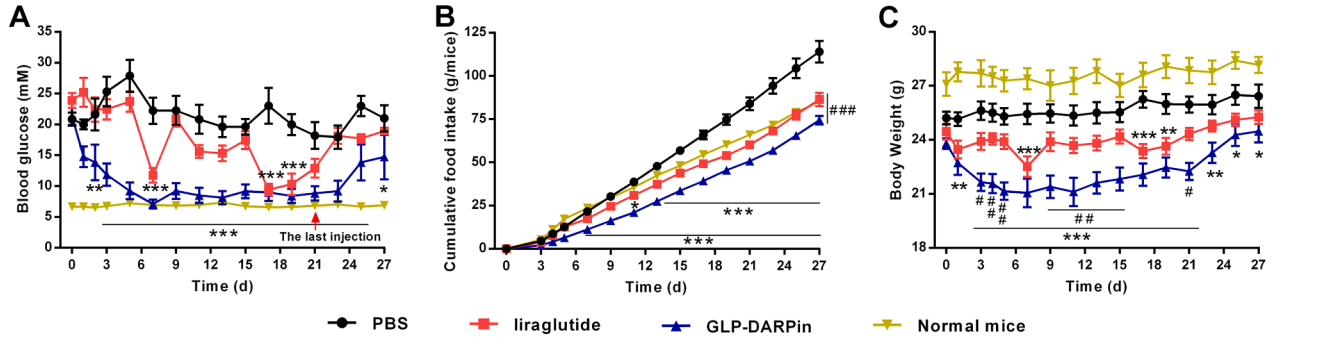

Fig. 5. Long-term effects of GLP-DARPin on STZ-induced diabetic mice. The non-fasting blood glucose level (A), cumulative food intake (B) and body weight changes (C) of STZ-induced diabetic mice during the chronic repeated injections of GLP-DARPin and liraglutide. The diabetic C57BL/6 mice were induced by STZ. The GLP-DARPin at a dose of 90 nmol/kg and liraglutide at a dose of 10 nmol/kg were subcutaneously injected into mice every 2 days. The blood glucose levels, food intake and body weight of diabetic mice were then measured. The last injection was carried out on day 21. Normal mice were used as a control. Data are presented as mean ± SEM, n = 6 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for test groups versus PBS group. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ for GLP-DARPin group versus liraglutide group. The "ns" represents no significance.

day 27, though they remained much lower than in the PBS group. Liraglutide significantly decreased the blood glucose of diabetic mice only at day 7, day 17 and day 19. Liraglutide could not maintain the blood glucose of diabetic mice at a stable and lower level with an injection frequency of every two days (Fig. 5A).

Cumulative food intake was measured to verify the food intake inhibition effect of GLP-DARPin. The results showed that both GLP-DARPin and liraglutide considerably inhibited food intake. The cumulative food intakes of the GLP-DARPin and liraglutide groups were much lower than that of the PBS group from day 7 and day 11, respectively. GLP-DARPin led to a much lower cumulative food intake compared with liraglutide from day 9 to day 27. After the last injection at day 21, the GLP-DARPin and liraglutide groups still exhibited a reduced food intake. At the end of the study, the cumulative food intakes of the PBS, GLP-DARPin, liraglutide and normal groups were 114.0 ± 6.2, 74.4 ± 2.5, 86.4 ± 3.8 and 85.1 ± 2.2 g/mouse, respectively (Fig. 5B).

To test whether the decreased food intake could result in lower body weight, the body weights of diabetic mice in different groups were also measured. Both GLP-DARPin and liraglutide could substantially lower the body weights of diabetic mice. The body weights of the GLP-DARPin group began to decline gradually at day 1 after injection. The lowest average body weight was found at day 7 in the GLP-DARPin group, at 21.1 ± 0.8 g/mouse, which was a 16.9% reduction compared with the PBS group, and a 10.5% weight loss compared with the liraglutide treatment. The body weight in the GLP-DARPin group was much lower than in the liraglutide group from day 3 to day 21. As with the blood glucose levels, GLP-DARPin could control the body weight at a lower level throughout the treatment course. However, liraglutide significantly reduced body weight at day 7, day 17 and day 19. After the last injection, body weights began to increase in both the GLP-DARPin and liraglutide groups (Fig. 5C).

3.6. Histopathology analysis of pancreas in STZ-induced diabetic mice

To elucidate the protective effect of GLP-DARPin fusion proteins on islet cells, H&E staining of pancreases in STZ-induced diabetic mice was

conducted after the treatment. The results demonstrated that the islets in normal mice were regular, and had the largest β-cell area (Fig. 6A). STZ-induced diabetic mice exhibited decreased area of pancreatic islets, and showed significant damage and shrinkage (Fig. 6B). Both liraglutide and GLP-DARPin could significantly improve the islet area and pancreas architecture. The islet area in the GLP-DARPin group was much larger than in the liraglutide group (Fig. 6C, D). This indicated that the GLP-DARPin fusion protein could protect islets from damage and increase the β-cell count.

4. Discussion

Albumin-binding moiety has been applied extensively to improve the pharmacodynamics and pharmacokinetic profiles of therapeutic peptides or proteins by means of the FcRn-mediated recycling system through genetic fusion or non-covalent association to albumin. Examples of such therapeutics include streptococcal protein G-derived albumin-binding domain (ABD) (Li et al., 2016), serum albumin-binding domain antibodies (AlbudAbs) (Walker et al., 2010), fatty acid moiety (Lau et al., 2015), nanobodies (Steeland et al., 2016), and DARPins (Stumpp et al., 2008). DARPins are a class of binding proteins with the potential to bind any given target protein with high affinity and specificity by selection (Stumpp et al., 2008). Albumin-binding DARPins can be targeted to HSA with a high affinity. Genetically fusing non-HSA-binding DARPins or other therapeutic proteins to albumin-binding DARPins can significantly prolong the half-lives of the therapeutic protein, as exemplified by MP0250 (Steiner et al., 2017).

DARPins exhibit many advantages that render them an ideal scaffold for protein drug development. DARPins show high thermal stability with midpoints of denaturation between 66 °C and 95 °C (Binz et al., 2003; Stumpp et al., 2008). Formulation experiments revealed the high solubility of DARPins in solution, up to 100 mg/mL. No proteolytic digestion was found when incubated with human serum (*ex vivo*), demonstrating high stability for potential use as a drug. DARPins can be expressed at high levels in soluble form in *E. coli*, up to 15 g/L in fed-batch high-cell-density fermentation (Binz et al., 2017). In term of safety, DARPins are predicted to be non-immunogenic based on their chemical characterization (Caputi and Navarra, 2020). Furthermore, the albumin-binding DARPin domain showed nanomolar affinities for serum albumin of human, cynomolgus monkey, mouse and rat regardless of whether pH was 7.4 or 6.0. This broad species selectivity makes it possible to extrapolate half-life data from mice to human (Steiner et al., 2017). These properties make albumin-binding DARPins a potential alternative scaffold to extend the half-lives of therapeutic peptides or proteins.

Here, we designed two novel GLP-1R agonists based on GLP-1 and DARPins: the GLP-DARPin and GLP-2DARPin fusion proteins. Comparison with Fc-fusion or albumin-fusion strategies for half-life extension, DARPin fusion may have several advantages in production cost and safety. Both recombinant DARPin-fusion GLP-1 proteins were highly expressed in *E. coli*, yielding homogeneous products. Furthermore, fusion proteins could be easily purified using Ni^{2+}-NTA and Q ion exchange columns at low cost, avoiding the chemical conjugation process and subsequent downstream purification. The production costs of DARPin-fusion GLP-1 proteins were lower compared with dulaglutide and albiglutide, both of which must be produced by mammalian or yeast cells with long fermentation periods and high costs. Additionally, DARPin-fusion GLP-1 proteins contain no Fc-fragment, thereby avoiding antibody-mediated cytotoxicity and complement-mediated cytotoxicity (Shilova and Deyev, 2019).

The half-life of GLP-2DARPin was 3 times longer than GLP-DARPin in mice, which corresponded to a higher affinity of GLP-2DARPin for HSA, 18-fold higher than that of GLP-DARPin. The results indicated that a higher affinity for HSA could translate into a longer half-life. This was in accordance with previous results from other albumin-binding domains of streptococcal protein G (ABD) and variants. The half-life of Tencon25-ABDCon, with an affinity of 1.86 nM for murine albumin, was approximately 2-fold longer than Tencon25-ABDCon5 with 887-fold lower affinity (60.4 h versus 34.2 h in mice). The Tencon25 with no ABDCon has a half-life of just 0.67 h in mice (Jacobs et al., 2015). The half-life of a recombinant immunotoxin (RIT) in LMB-164 fusion protein was extended to 194 min; an approximately 2-fold longer half-life than in LMB-170 fusion protein (101 min), which can be attributed to a 21.5-fold higher affinity for murine albumin (Wei et al., 2018).

However, a previous study also showed that Tencon25-ABDCon3 and Tencon25-ABDCon5, with HSA binding affinities of 103 nM and 20.6 nM, respectively, possessed a comparable half-life in mice (45.8 h versus 46.8 h) (Jacobs et al., 2015). ScDb-ABD_2, with 4-fold higher affinity for murine albumin, exhibited comparable half-life to ScDb-ABD: 37.9 h and 36.4 h, respectively (Hopp et al., 2010). Our previous study also showed that the similar apparent affinities of ABD-fusion constructs for HSA resulted in comparable half-lives (Tan et al., 2021). DARPin construct-1, with 2.5-time higher affinity for albumin, showed a similar

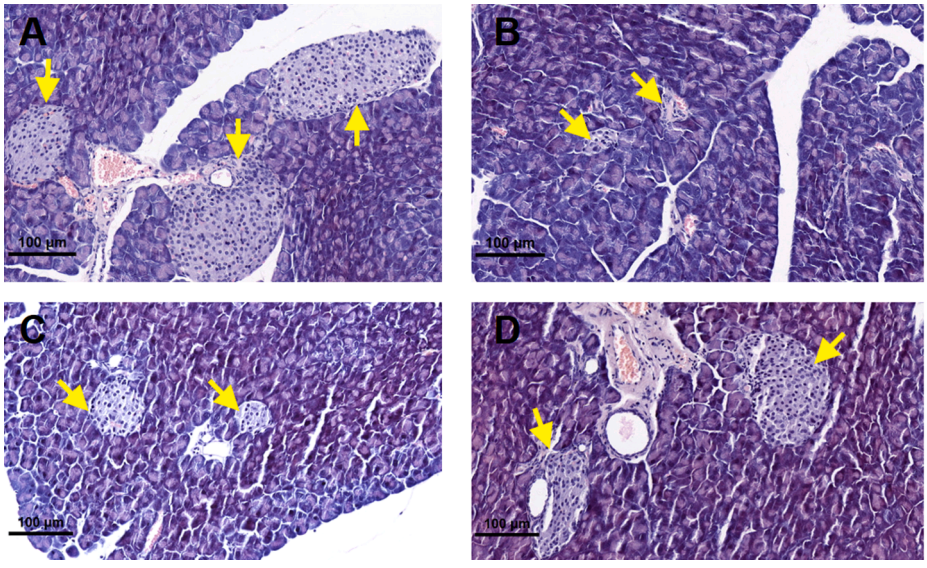

Fig. 6. H&E staining of pancreatic tissues in STZ-induced diabetic mice. Representative islets of normal mice (A) and PBS (B), liraglutide (C) and GLP-DARPin (D) treated mice. At the end of the chronic treatment of STZ-induced diabetic mice, the pancreatic tissues were prepared and stained with H&E. The stained tissue sections were visualized using a microscope with 100 × magnification. The arrows in the figures shows the islets β-cells.

half-life in mice to DARPin construct-3 (Steiner et al., 2017). These results suggested that a significantly higher affinity for albumin may be needed to obtain substantially increased half-lives of therapeutic proteins with albumin-binding moieties. An affinity increased by<5-fold may not be sufficient to lead to further half-life extension. Nevertheless, it is noteworthy that, in comparison with constructs lacking an albumin-binding domain, even a low affinity for albumin still contributes to a significant extension of half-life (Hopp et al., 2010).

The long half-lives of GLP-DARPin and GLP-2DARPin can be attributed in part to the FcRn-mediated recycling mechanism, made possible by HSA binding, and partly to the increased hydrodynamic size of fusion proteins due to HSA association, which causes slowed renal clearance. The SEC results demonstrated that one GLP-2DARPin molecule, when bound to two HSA, had a considerably increased hydrodynamic size. Additionally, two DARPin domains may protect fusion proteins from degradation in the endosome during the process of FcRn-mediated recycling, providing an alternative escape mechanism via interaction with the second albumin (Steiner et al., 2017). Further studies are needed to elucidate the mechanism by which half-life is extended through the second albumin-binding DARPin moiety. However, in terms of activity, the increased molecular size might exhibit greater steric hindrance, resulting in a lower affinity for the GLP-1 receptor and thereby leading to a reduced glucose-lowering effect *in vivo*. GLP-DARPin significantly improved glucose tolerance for at least 48 h. GLP-2DARPin, on the other hand, exhibited this improvement for just 24 h. This indicated that the DARPin moiety with a greater affinity for albumin needed to be further screened to pursue higher bioactivity and a longer half-life. GLP-DARPin was more potent than liraglutide, an FDA-approved GLP-1R agonist used for T2DM treatment. The duration of the glucose-lowering effects of DARPin-fusion GLP-1 proteins were much longer than those of GLP-1 (8G) with no DARPin moiety, which lasted only 0.5 h (Fremaux et al., 2019).

In the chronic treatment of STZ-induced diabetic mice, subcutaneous administration of GLP-DARPin once every two days could reverse hyperglycemia and reduce non-fasting blood glucose to the levels of normal mice. Over the course of the entire treatment, GLP-DARPin retained a continuous and strong hypoglycemic effect, indicating no immunogenicity and generating no neutralizing antibodies. Repeated injection of glutazumab, a long-acting GLP-1/anti-GLP-1R antibody, resulted in a shortening of dosing interval from once every three days to once daily. This may be due to the production of anti-drug antibodies leading to a reduced efficacy (Li et al., 2018). In diabetic mice, the GLP-DARPin fusion protein also retained the actions of natural GLP-1, stimulating β-cell proliferation and protecting islets from damage, thereby contributing to an improvement of islet area, which is highly beneficial for T2DM patients. These results were in line with previously reported chronic GLP-1R agonist treatments, which suggested that the agonists alleviated islet loss, increased β-cell mass, inhibited β-cell apoptosis and improved β-cell function in diabetic mice (Kim et al., 2010; Li et al., 2018; Reed et al., 2020; Sun et al., 2015).

Besides the blood glucose-lowering effect, GLP-1 also exerts anorectic action by activation of central nervous system (CNS) centers or vagal afferent pathways through binding to GLP-1R (Baggio et al., 2008). GLP-DARPin exerted a strong inhibitory effect on food intake, resulting in the lowest cumulative food intake compared with liraglutide and PBS treatment in diabetic mice. The body weights of diabetic mice treated with GLP-DARPin also decreased the most during treatment, leading to approximately 16.9% and 10.5% greater weight loss compared with PBS and liraglutide treatment, respectively. A drug that simultaneously has blood glucose-lowering and weight loss effects would be highly attractive and beneficial to patients, considering that more than 80% of T2DM patients are overweight or obese (Lorenz et al., 2013). In a 56-week trial, liraglutide, also approved for weight management by the FDA, at a dose of 3 mg led to 5%-10% reduction in body weight (Pi-Sunyer et al., 2015). GLP-DARPin, therefore, has great potential for the clinical treatment of T2DM and obesity simultaneously.

Recent studies have revealed that GLP-1 also has other highly beneficial effects on the cardiovascular and neurological systems and on inflammatory responses in diabetic patients (Knudsen and Lau, 2019; Reed et al., 2020). Therefore, GLP-1R agonists have the potential to treat cardiovascular disease and dementia. Moreover, in a phase 2 clinical trial, a GLP-1R agonist, semaglutide, showed significant improvements in non-alcoholic steatohepatitis (NASH) and played a role in preventing disease progression, possibly by improving glycemic control, promoting weight loss and reducing alanine aminotransferase (ALT) levels (Newsome et al., 2021; Reed et al., 2020). GLP-1 also likely plays an important role in maintaining kidney, neuronal and liver homeostasis during healthy conditions (Reed et al., 2020). GLP-1R agonists could therefore simultaneously improve several complications in the treatment of T2DM and obesity.

However, further studies should be conducted to increase the potential for the clinical application of fusion proteins. With respect to the half-life *in vivo*, the sandwich ELISA method may need to be developed further in order to obtain more accurate half-life data for fusion proteins. Moreover, an albumin-binding DARPin domain exhibiting higher affinity for HSA must be screened to obtain improved bioactivity and half-life for the fusion partner. Additionally, in further studies, semaglutide administered once-weekly is more suitable as a reference agent due to the long half-lives of fusion proteins.

5. Conclusion

In summary, we designed a novel, long-acting GLP-1R agonist, GLP-DARPin, by genetic fusion of GLP-1 to DARPins with a high affinity for HSA. The recombinant GLP-DARPin could be highly expressed in *E. coli* and easily purified at low cost. The half-life of GLP-1 was considerably prolonged by the fused DARPin moiety. GLP-DARPin retained the bioactivities of naturally occurring GLP-1 and activated a broad spectrum of GLP-1R-dependent actions, including significantly lowering blood glucose levels, inhibiting food intake and reducing body weight in diabetic mice. These results suggest that the long-acting GLP-DARPin may have great potential in clinical applications for the treatment of T2DM and obesity. It is worth noting that the HSA-binding DARPin domain can be applied as a universal scaffold to effectively generate long-acting protein drugs.

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:** Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2021.121043.

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