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Research article

Exploring a novel long-acting glucagon-like peptide-1 receptor agonist built on the albumin-binding domain and XTEN scaffolds

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ABSTRACT

In recent years, glucagon-like peptide-1 (GLP-1) has demonstrated considerable potential in the treatment of type 2 diabetes (T2D) and obesity. However, the half-life of naturally occurring GLP-1 is quite short *in vivo*. Two common strategies employed for half-life extension are the use of the Albumin-binding domain (ABD) and XTEN polypeptide, which operate through different mechanisms. In this study, we designed an innovative GLP-1 receptor agonist with an extended duration of action. This new construct incorporated an albumin binding domain (ABD) and an XTEN sequence (either XTEN144 or XTEN288) as carriers. We referred to these fusion proteins as GLP-ABD-XTEN144 and GLP-ABD-XTEN288. In an *E. coli* system, the said constructs were efficaciously produced in substantial quantity. It was observed from *in vitro* studies that the fusion protein GLP-ABD-XTEN144 demonstrated a five times stronger affinity towards human serum albumin (HSA), boasting a binding affinity (K_d) of 5.50 nM. This was in contrast to GLP-ABD-XTEN288, whose K_d value was registered at 27.78 nM. Moreover, GLP-ABD-XTEN144 presented a half-life of 12.9 h in mice, thus exceeding the corresponding value for GLP-ABD-XTEN288, 7.32 h in mice. Both these fusion proteins significantly mitigated non-fasting blood sugar levels and overall food consumption for 48 h subsequent to a one-time injection in mice. Notably, GLP-ABD-XTEN144 exhibited more pronounced hypoglycemic activity and food inhibitory effects than GLP-ABD-XTEN288. The designed GLP-ABD-XTEN144 fusion protein shows promising prospects for clinical application in T2D treatment. Our findings also suggest that ABD and XTEN polypeptides synergistically contribute to half-life extension, further enhancing the pharmacokinetic characteristics of a payload.

1. Introduction

The International Diabetes Federation (IDF) reports that approximately one in every ten individuals globally now suffers from diabetes, impacting a staggering 537 million people worldwide [1]. Type 2 Diabetes (T2D), one of the most perilous chronic metabolic diseases globally, poses a significant threat to human health. It wreaks cumulative havoc on the cardiovascular, nervous, visual, and hepatic systems [2]. Primarily, insulin resistance and eventual insufficient insulin secretion by pancreatic beta cells lead to T2D [3].

The peptide hormone known as Glucagon-like Peptide-1 (GLP-1) is acclaimed for its beneficial effects on metabolic disorders. It

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promotes insulin production in response to glucose, moderates after-meal blood sugar levels, and decreases levels of plasma HbA1c, all while helping to suppress appetite [4,5]. Nonetheless, its application in clinical therapy is constrained due to the rapid breakdown by the enzyme dipeptidyl peptidase 4 (DPP-4) and its small molecular size, which consequently gives the natural GLP-1 a brief *in vivo* lifespan of merely 1–2 min [6,7].

In the last two decades, the Food and Drug Administration (FDA) has sanctioned multiple extended-release medications to tackle T2D. These sustained-release GLP-1 receptor (GLP-1R) agonists utilize three distinct approaches to prolong the half-life of GLP-1: fatty acid side-chain conjugation (e.g., liraglutide and semaglutide), amino acid mutation to avert DPP-4 inactivation (e.g., A8G or A8Aib mutation), and genetic fusion to human serum albumin (HSA) or IgG-Fc [7,8]. Such modifications enhance the structural stability of GLP-1, augment the molecular size, slow down kidney clearance, and facilitate binding to the neonatal Fc receptor (FcRn) in the bloodstream, thus extending the half-life of GLP-1 through FcRn-mediated recycling [9].

The Albumin-binding domain (ABD), a peptide group comprised of 46 amino acids, is seen on the surface of select streptococcal strains. These peptides have the ability to attach to HSA with remarkable affinity at the nanomolar scale, and this interaction allows them to prolong their half-life [10]. Consequently, peptides or proteins can extend their stability by genetically merging with ABD [11,12]. The half-life of several molecules, such as G-CSF [12], interferon-α [13], bispecific single-chain diabody (ScDb) [14], and human fibronectin type III domain [15], has been significantly improved by fusing to ABD.

XTEN is a class of polypeptides composed of six chemically stable amino acids: A, E, G, T, S, and P [16]. This polypeptide has been engineered to prolong the stability of peptides or proteins via genetic merging or chemical bonding [17,18]. Modifying the length of XTEN from 144 to 864 amino acids generates molecules with a broad spectrum of half-lives [18]. The fusion of XTEN288 with glucagon significantly increases the half-life of glucagon in monkeys, changing it from 10 min to 9 h [19]. Moreover, using XTEN864 as a carrier enables exendin-4 to achieve a half-life of 139 h in humans, far exceeding its native form [16].

In our research, we developed two unique GLP-1R stimulants by combining GLP-1 with sequential ABD and XTEN peptides of different sizes. We hypothesized that ABD and XTEN would synergistically extend the half-life of GLP-1. We expressed and purified the fusion proteins from an *E. coli* system, then evaluated the stability and bioactivities of the GLP-ABD-XTEN fusion proteins *in vivo*.

2. Materials and methods

2.1. Fusion proteins design

To construct a long-acting GLP-1 agonist fusion protein, the GLP-1 molecule was linked to the N-terminus of ABD via a $(GGGGS)_3$ linker (GLP-ABD). Subsequently, ABD was connected to the N-terminus of the XTEN polypeptide (consisting of either 144 or 288 amino acids) through a $(GGGGS)_3$ linker. The resulting fusion proteins were labeled GLP-ABD-XTEN144 and GLP-ABD-XTEN288. For the purpose of purification, a His-tag was affixed to the N-terminus of GLP-1. A TEV recognition site was integrated in the space between the His-tag and GLP-1, which made the separation of GLP-1 easier. The TEV-GLP-1 sequence was as follows: ENLYFQH-GEGTFTSDVSSYLEGQAAKEFIAWLVKGR (the TEV cleavage site is indicated by underline). The DNA strands that encode these combined proteins were adapted specifically for use in an *E. coli* system, and were synthesized by GENEWIZ based in Suzhou, China. These sequences were inserted into the pET-28a(+) plasmid via the *Eco*R I and *Xho*I restriction sites. Moreover, for the assessment of pharmacokinetics, fusion proteins featuring a C-terminal His-tag were constructed. The TEV protease was unable to remove the C-terminal His-tag.

2.2. Protein expression and purification

The plasmids were transferred into the *E. coli* BL21(DE3) strain. A single clone was randomly selected after 24 h and cultivated in LB medium, augmented with 50 μg/mL kanamycin, at 37 °C overnight. A 50 mL sample of the cultures was inoculated into 5 L of fresh LB medium and left to grow until the OD_{600} reached between 0.6 and 0.8. The isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM) was added to induce protein expression, and the bacteria were cultured for an additional 12 h at 25 °C.

The cells underwent a centrifugal process at 5000 rpm, in a 4 °C environment for a quarter of an hour, before being reconstituted in a lysis buffer solution made up of 20 mM Tris/HCl, 100 mM NaCl, and 20 mM imidazole at a pH of 8.0. An ultrasonic homogenizer (Scientz, China) was used to break down the cells, and then, to eliminate any sediment, a second centrifugation at 15,000 rpm, lasting half an hour at 4 °C was carried out. The resulting supernatant was introduced onto a Ni^{2+}-NTA affinity chromatography column (5 mL) from GE Healthcare (USA), which had been previously balanced with the lysis buffer. The column underwent a cleansing process with 10 column volumes of a washing buffer, comprising 20 mM Tris/HCl, 100 mM NaCl, and 40 mM imidazole, with a pH value of 8.0. Finally, fusion proteins were extracted using an elution buffer that contained 20 mM Tris/HCl, 100 mM NaCl, and 250 mM imidazole, also at a pH of 8.0.

The eluted proteins were subsequently dialyzed against 20 mM Tris/HCl, 1 mM EDTA, pH 8.0. To remove the His-tag, 25 mL of the fusion proteins were digested at room temperature for 2 h by 1 mg TEV protease with the addition of 1 mM DTT. The protein solution was further purified through a Ni^{2+}-NTA column. Proteins without the His-tag were collected and further dialyzed in phosphate-buffered saline (PBS). The purity of the final proteins was analyzed using 12.5% SDS-PAGE, and the protein concentration was examined using a BCA assay kit (CWBIO, China).

2.3. Removal and quantification of endotoxin

Endotoxins were eliminated from the purified fusion proteins utilizing a ToxinEraser™ Endotoxin Removal Kit (Genscript, Nanjing, China). The levels of endotoxin were quantified with the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript). The resultant fusion proteins employed in animal experiments contained endotoxin levels below 5 EU/mg of protein.

2.4. Binding affinities of fusion proteins for HSA in vitro

An ELISA was used to determine the binding affinities of the fusion proteins to HSA, following the methodology described by Tan et al. [11]. An immobilized 96-well plate was prepared by incubating 10 μg of HSA in 100 mM carbonate buffer (pH 9.6) overnight at 4 °C. After three washes with PBST (PBS + 0.05% Tween 20), the plate was blocked with 5% BSA (Solarbio, Beijing, China). A series of GLP-ABD, GLP-ABD-XTEN144 or GLP-ABD-XTEN288 fusion proteins at different concentrations, from 0.032 to 500 nM, were subsequently introduced into the wells and left to incubate for 2 h in a room temperature setting. The GLP-1 present in these combined proteins was identified by employing a mouse anti-GLP-1 antibody (ab23472, Abcam, UK) as well as a HRP-conjugated goat anti-mouse IgG (CW0202S, CWBIO, China). After completing a series of three rinses, a TMB substrate (Solarbio, China) was employed to pinpoint the HRP that had successfully bonded. The reaction was concluded by adding 1 M HCl, followed by a measurement of the absorbance at 450 nm using a SpectraMax M2 microplate reader (Molecular Devices, USA).

2.5. Pharmacokinetic studies in mice

Sixty-two male C57BL/6 mice, aged eight weeks and weighing approximately 20 g, were maintained under a 12-h light/dark cycle at 25 °C with free access to food and water. These mice were given a week to acclimatize before being subjected to experiments. All procedures performed in the studies adhered to the principles set by China's Animal Care Committee, and received prior approval (No. SYXK 2022-0135) from the Laboratory Animal Center at Ningbo University.

In the pharmacokinetic research, two sets of mice were allocated: one to the GLP-ABD-XTEN144 group (comprising 3 mice) and the other to the GLP-ABD-XTEN288 group (also with 3 mice). GLP-ABD-XTEN144 and GLP-ABD-XTEN288 fusion proteins, dissolved in PBS, with a C-terminal His-tag were administered subcutaneously to the mice at a single dose of 5 nmol/mouse. At 1, 3, 7, 12, 18, 24, 30, 36 h, approximately 30 μL of blood was drawn from the tail vein and centrifuged at 4000 g for 10 min at 4 °C. The plasma was then diluted with PBS. The plasma concentration of the fusion protein was ascertained using the ELISA method, as previously detailed. In summary, immobilized anti-His-tag antibodies (ab18184, Abcam, UK) were used to capture the fusion proteins in plasma. The identification of the attached fusion proteins was accomplished using a monoclonal anti-GLP-1 antibody from a rabbit (ab111125, Abcam, UK) alongside an HRP-linked goat anti-rabbit IgG (CW0103S, CWBIO, China). A standard curve method was employed to quantify the fusion proteins. The half-life and the pharmacokinetic parameters of the fusion proteins were ascertained through Microsoft Excel's PK Solver application [20,21].

2.6. Hypoglycemic activities of fusion proteins in vivo

C57BL/6 mice were randomly assigned into eight groups (4 mice per group). The evaluation of the blood glucose reducing potential of fusion proteins was carried out, with minor adaptations, based on the method outlined in a previous study [22]. Before undergoing the procedure, all the mice were subject to an overnight fast. We performed oral glucose tolerance tests (OGTTs) to ascertain if the fusion proteins could prevent abrupt surges in blood glucose levels. C57BL/6 mice were subcutaneously administered GLP-ABD-XTEN144 or GLP-ABD-XTEN288 fusion proteins at a single dose of 3, 10, or 30 nmol/kg, 30 min prior to an oral glucose dose of 2 g/kg. Mice assigned to the PBS group (control) received injections of an equivalent volume of PBS (0.1 mL), which was followed by an oral glucose dose of 2 g/kg. Subsequently, blood glucose levels were measured by a glucometer (Sanocare, China) at −30, 0, 15, 30, 60, and 120 min intervals.

In order to delve deeper into the longevity of the decrease in blood glucose levels triggered by the fusion proteins, new experiments were performed and the mice allocated to GLP-ABD-XTEN144 group or GLP-ABD-XTEN288 group (4 mice per group) were subcutaneously administered the corresponding fusion proteins at a single dose of 30 nmol/kg. The mice in PBS group (control) received injections of a consistent volume of PBS (0.1 mL). Non-fasting blood glucose measurements were taken from the tail vein at 0, 1, 3, 8, 12, 24 and 48 h using a glucometer (Sanocare). Food intake was also monitored. To determine if the GLP-ABD-XTEN144 and GLP-ABD-XTEN288 fusion proteins retained their ability to lower blood glucose levels after 48 h, an additional set of experiments was conducted. The mice were divided into three groups: GLP-ABD-XTEN144 group, GLP-ABD-XTEN288 group and PBS group (4 mice per group). OGTT experiments were performed 48 h following a single injection of 30 nmol/kg. Blood glucose levels were measured by a glucometer (Sanocare) at −30, 0, 15, 30, 60, and 120 min intervals. At time point zero (0 min), the mice were orally received 2 g of glucose per kg body weight. As for the PBS group (control), the mice received an injection of 0.1 mL PBS.

2.7. Statistical analysis

For all analyses, each value is expressed as the mean ± S.E.M. GraphPad Prism 8.01 (GraphPad Software, USA) was used for analysis, and multiple comparisons were conducted using one-way or two-way analysis of variance (ANOVA), followed by Tukey's test. Statistical significance was determined when the p value was <0.05. The binding affinity (K_d, the concentration for 50% of

maximal effect), was calculated by fitting the obtained absorbance curve into a four-parameter non-linear model.

3. Results

3.1. Design and purification of GLP-ABD-XTEN fusion proteins

In order to extend the half-life of GLP-1 *in vivo*, tandem ABD and XTEN polypeptides were used as carriers to enhance its pharmacodynamic and pharmacokinetic properties. ABD enhances the lifespan of GLP-1 by non-covalently associating with albumin in the blood, whereas XTEN amplifies its hydrodynamic radius and offers spatial protection. As shown in Fig. 1A, GLP-1 was fused to the N-terminus of ABD-XTEN to ensure the N-terminus of GLP-1 was free after digestion by TEV protease. A flexible $(GGGGS)_3$ linker was added between GLP-1 and ABD, and ABD and XTEN. This flexible linker could assist the domains in the fusion proteins to fold correctly and allow each domain to independently bind to its receptor.

The fusion proteins were successfully expressed in the *E. coli* BL21(DE3) strain under the induction conditions of 25 °C and 0.1 mM IPTG. After purification using a Ni^{2+}-NTA column and His-tag removal by TEV protease digestion, GLP-ABD-XTEN144 and GLP-ABD-XTEN288 fusion proteins were obtained, having molecular weights of approximately 23 kDa and 37 kDa, respectively. Following an additional Ni^{2+}-NTA purification step to remove uncut fusion proteins and His-tag peptide, the final yield of GLP-ABD-XTEN144 and GLP-ABD-XTEN288 fusion proteins reached 70 mg/L and 42 mg/L, respectively. SDS-PAGE analysis displayed a single band corresponding to the expected molecular weight, with a target protein purity exceeding 95% (Fig. 1B).

3.2. Binding affinity for HSA in vitro

The ELISA technique was utilized to evaluate the binding capacities of the GLP-ABD-XTEN fusion proteins to HSA, employing an anti-GLP-1 antibody to measure the amount of attached fusion proteins. As depicted in Fig. 2, the K_d value of GLP-ABD-XTEN144 and GLP-ABD-XTEN288 were 5.50 ± 0.51 nM and 27.78 ± 5.70 nM, respectively. The affinity of the fusion protein containing XTEN288 for HSA was lower about fivefold compared to the fusion protein containing XTEN144. However, the K_d value for GLP-ABD was 2.27 ± 0.38 nM, which is significantly lower than the fusion proteins containing an XTEN unstructured polypeptide. This suggests that the XTEN polymer might introduce steric hindrance that affects the binding of the ABD domain in the fusion protein to HSA. Furthermore, the lengthier the XTEN polypeptide, the more significant the steric hindrance.

3.3. Half-lives of GLP-ABD-XTEN fusion proteins in mice

The GLP-ABD-XTEN fusion proteins' pharmacokinetic attributes were assessed in a study involving C57BL/6 mice. The fusion proteins with a C-terminal His-tag were given to the mice in a one-time subcutaneous injection at a dose of 5 nmol. An ELISA assay, utilizing an anti-His-tag antibody for capture and an anti-GLP-1 antibody for detection, was employed to determine the levels of these

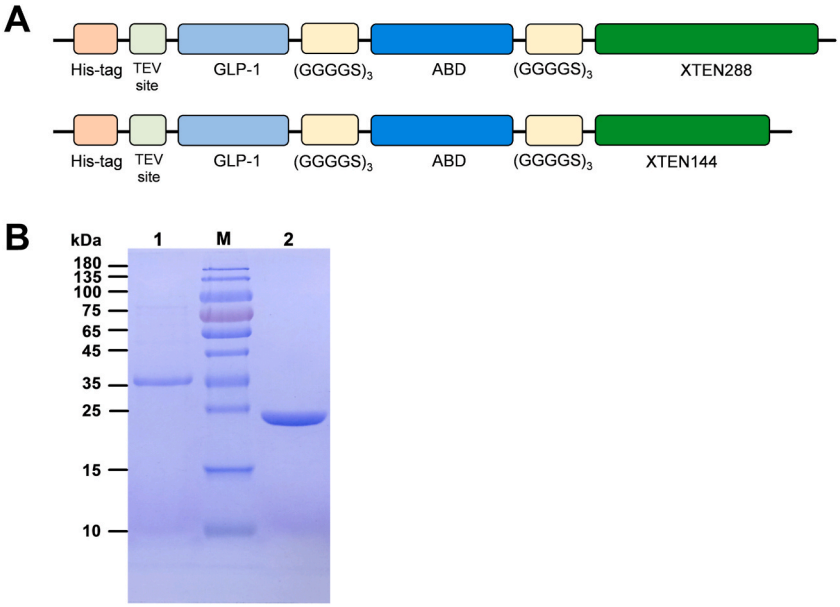

Fig. 1. Construction and Purification GLP-ABD-XTEN fusion proteins. (A) Schematic illustration of the fusion of GLP-1 to the N-terminus of ABD-XTEN carrier via a flexible linker. (B) SDS-PAGE analysis of purified GLP-ABD-XTEN fusion proteins. His-Tag was removed by TEV protease. M, prestained protein marker; Lane 1, GLP-ABD-XTEN288; Lane 2, GLP-ABD-XTEN144.

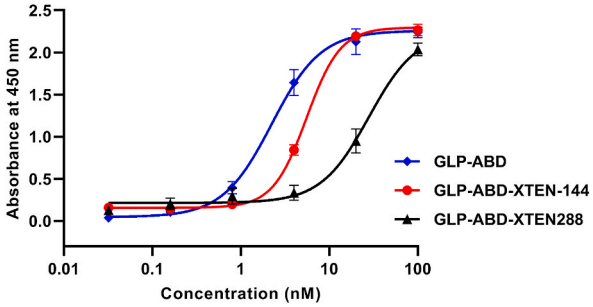

Fig. 2. ELISA analysis the binding affinity of purified GLP-ABD-XTEN fusion proteins to human serum albumin (HSA). HSA were immobilized onto a 96-well plate. Subsequently, varying concentrations of either GLP-ABD-XTEN144 or GLP-ABD-XTEN288 fusion proteins were introduced into these wells. Post-incubation, the attached fusion proteins were identified using a mouse anti-GLP-1 antibody followed by a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG.

fusion proteins in the bloodstream. The calculated half-life of GLP-ABD-XTEN144 was 12.9 ± 2.7 h, which was significantly longer than that of GLP-ABD-XTEN288 (7.32 ± 0.9 h) in mice. Both GLP-ABD-XTEN144 and GLP-ABD-XTEN288 groups achieved their T_{max} at 7 h (Fig. 3). The peak concentration (C_{max}) for GLP-ABD-XTEN144 was found to be 930.3 ± 146.1 ng/mL, which is marginally lower compared to GLP-ABD-XTEN288's C_{max} of 1208.1 ± 297.3 ng/mL. However, this difference in C_{max} was not statistically significant. Regarding the area under the curve (AUC(0-∞)), GLP-ABD-XTEN144 registered 20983.3 ± 3374.3 h × ng/mL, which is somewhat higher than GLP-ABD-XTEN288's 18652.5 ± 4269.7 h × ng/mL, but again, the difference was not significant. Notably, the clearance rate for GLP-ABD-XTEN144, at 165.7 ± 31.8 mL/h/kg, was considerably slower than GLP-ABD-XTEN288's rate of 285.0 ± 68.6 mL/h/kg. This resulted in a longer half-life for GLP-ABD-XTEN144 in comparison to GLP-ABD-XTEN288. The shorter half-life of the fusion protein containing XTEN288 might be due to the larger steric hindrance that affects the binding of ABD to HSA. Both fusion proteins had a significantly longer half-life than that of the native GLP-1 peptide (1–2 min) [23]. Thus, it can be concluded that the tandem ABD-XTEN carrier can significantly extend the half-life of GLP-1 in circulation.

3.4. Effect of GLP-ABD-XTEN fusion proteins on blood glucose in mice

To ascertain whether the GLP-ABD-XTEN fusion proteins maintain the biological functionality of native GLP-1, the hypoglycemic effects of these proteins were analyzed in mice. OGTTs were performed to establish if these fusion proteins had the capacity to mitigate sudden glucose spikes in a living organism. In the group receiving PBS, a significant increase in blood glucose levels was noted within 30 min following the administration of glucose at a dose of 2 g/kg. However, both GLP-ABD-XTEN288 and GLP-ABD-XTEN144 could dose-dependently inhibit this increase and drastically lower the blood glucose levels within 120 min, compared to the control group (Fig. 4A and B). At a dose of 30 nmol/kg, both fusion proteins resulted in the most significant reduction in blood glucose levels and the minimal AUC values.

To investigate how long the GLP-ABD-XTEN fusion proteins could maintain low blood glucose levels *in vivo*, the non-fasting glucose levels were measured after a single subcutaneous injection of the fusion proteins at a dose of 30 nmol/kg. The findings indicated a significant decrease in non-fasting blood glucose levels within 48 h post-injection with either GLP-ABD-XTEN288 or GLP-ABD-XTEN144, as depicted in Fig. 5A. GLP-ABD-XTEN144 led to lower non-fasting blood glucose levels (4–5 mM) than GLP-ABD-XTEN288. Furthermore, the hypoglycemic action of GLP-ABD-XTEN144 lasted for at least 48 h, a longer duration than that of GLP-ABD-XTEN288. The calculated AUC values of non-fasting blood glucose also indicated that GLP-ABD-XTEN144 had better glucose-

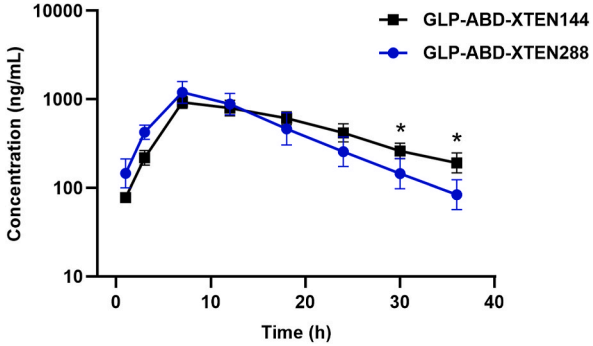

Fig. 3. The plasma concentrations of GLP-ABD-XTEN fusion proteins. The fusion proteins were subcutaneously injected into C57BL/6 mice at a dose of 5 nmol/mice. The plasma concentrations of GLP-ABD-XTEN fusion proteins at different time points were determined by an ELISA method. *$p < 0.05$ for GLP-ABD-XTEN144 group versus GLP-ABD-XTEN288 group.

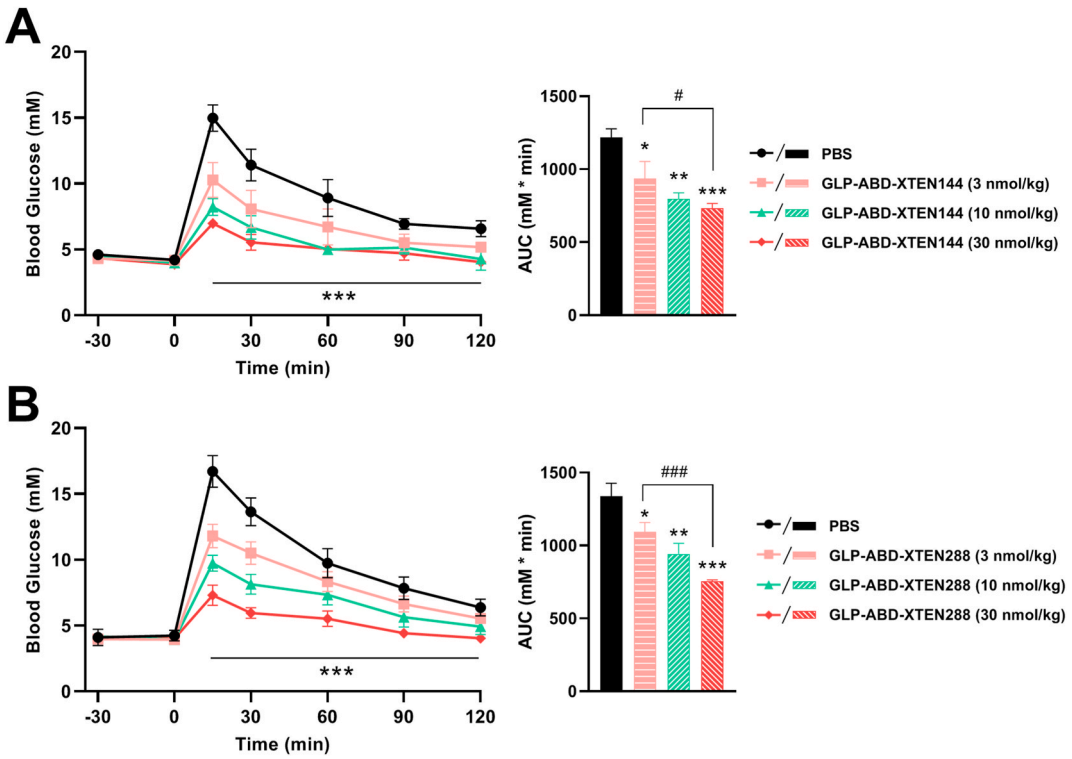

Fig. 4. Blood glucose levels in OGTT after subcutaneous injection of GLP-ABD-XTEN fusion proteins in C57BL/6 mice. GLP-ABD-XTEN144 (A) and GLP-ABD-XTEN288 (B) fusion proteins were subcutaneously injected into C57BL/6 mice at a single dose of 3, 10 or 30 nmol/kg. Blood glucose and calculated AUC values were determined after orally administering 2 g/kg glucose. Results are represented by the mean ± SD, n = 4 mice for each group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ for GLP-ABD-XTEN fusion protein groups versus PBS group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ for 3 nmol/kg group versus 30 nmol/kg group in both treatments. OGTT, oral glucose tolerance test.

lowering efficacy than GLP-ABD-XTEN288 (Fig. 5A). Similarly, GLP-ABD-XTEN144 significantly inhibited the cumulative food intake within 48 h, more so than GLP-ABD-XTEN288 (Fig. 5B).

At 48 h, an OGTT experiment was conducted to determine if the remaining fusion proteins could still inhibit the rapid increase of blood glucose levels. Both GLP-ABD-XTEN144 and GLP-ABD-XTEN288 fusion proteins were still able to significantly reduce blood glucose levels within 120 min. The calculated AUC values demonstrated that GLP-ABD-XTEN144 was more effective in lowering blood glucose levels than GLP-ABD-XTEN288 (Fig. 5C). The findings suggest that the GLP-ABD-XTEN fusion proteins can maintain glucose homeostasis *in vivo*.

4. Discussion

Despite the short half-life of GLP-1, which limits its direct clinical use, its significant potential in the treatment of T2D has prompted institutions and pharmaceutical companies to develop novel GLP-1 receptor (GLP-1R) agonists with improved pharmacokinetic profiles. Over the past two decades, several GLP-1 receptor agonists have been approved to treat T2D and obesity, including dulaglutide and semaglutide. The drug injection schedule has progressed from twice a day (for exenatide) to once a week (for dulaglutide and semaglutide) [4]. The association with HSA through genetic fusion, chemical conjugation, or non-covalent binding is a commonly employed strategy [24,25]. Lipidation, or modification with fatty acid side chains, is another method that can significantly prolong the half-lives of peptide or protein drugs by association with HSA in circulation, as seen with the once-daily liraglutide and once-weekly semaglutide [8].

Alternative strategies for half-life extension frequently utilize ABD, nanobodies [26], and albumin-binding domain antibodies (AlbudAbs) [27] that target HSA. Using phage display, ABD mutants with significantly improved affinity for HSA can be identified, such as ABD035, which has an affinity for HSA of 50 fM [28]. The half-life of Tumor Necrosis Factor-related Apoptosis-Inducing Ligand (TRAIL) was extended more than 40-fold by fusion to ABD035 [29]. Research suggests that, within a certain range, the higher the affinity between ABD and HSA, the longer the *in vivo* half-life of the ABD-fusion protein [20]. Through the process of genetic fusion with ABDCon, which possesses a 1.86 nM affinity for mouse albumin, the half-life of Tencon25 in mice was significantly increased from 40 min to 60.4 h. On the contrary, when Tencon25 was combined with ABDCon9 — characterized by a much lower affinity for mouse albumin at 550 nM — its half-life was considerably shorter, clocking in at only 34 h [20].

Unstructured XTEN polypeptides slow kidney clearance and increase the half-life of fusion proteins by significantly enhancing their

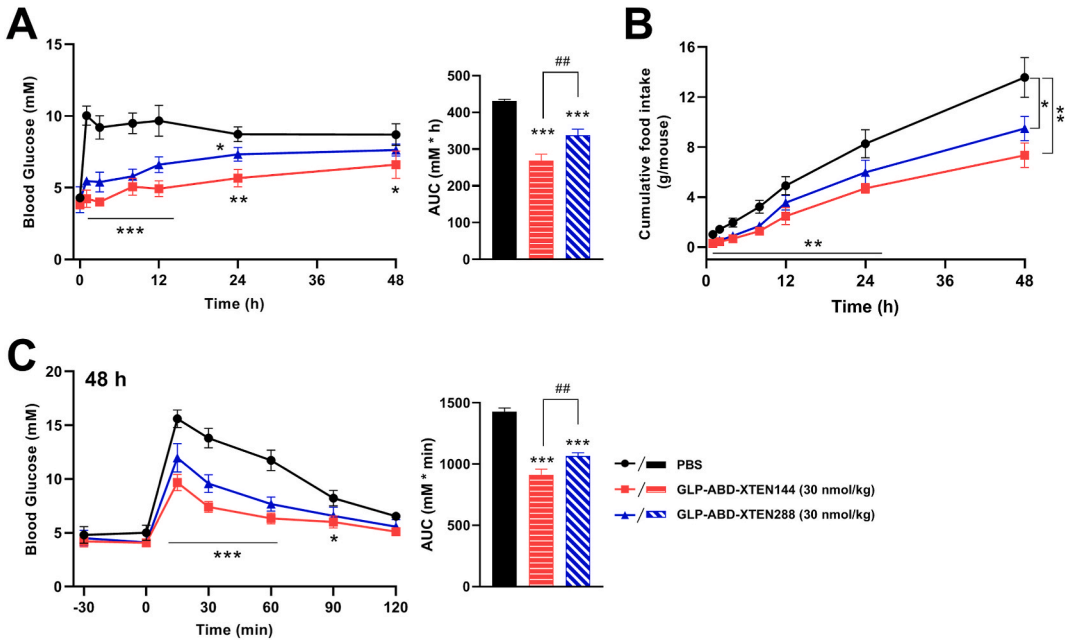

Fig. 5. Beneficial effects of GLP-ABD-XTEN fusion proteins after one single subcutaneous injection. The mice were injected subcutaneously at a single dose of 30 nmol/kg of GLP-ABD-XTEN fusion proteins. The non-fasting blood glucose levels and calculated AUC values (A), cumulative food intake (B) and OGTT performed 48 h after injection were measured. Values are expressed in the manner of mean ± SD, n = 4 mice for each group. *p < 0.05, **p < 0.01 and ***p < 0.001 for GLP-ABD-XTEN fusion protein groups versus PBS group. # #p < 0.01 for GLP-ABD-XTEN144 group versus GLP-ABD-XTEN288 group.

hydrodynamic volume [30]. For instance, the volume of an XTEN polypeptide comprising 576 amino acids, weighing 53 kDa, was equivalent to the 670 kDa thyroglobulin, based on size-exclusion chromatography (SEC) method [18]. Generally, the half-life of the fusion partner is extended with an increasing number of amino acids in the XTEN polypeptide, due to the larger molecular size. The XTEN polypeptide has been used to extend the half-life of exenatide, Glucagon-Like Peptide-2 (GLP-2), growth hormone (GH), factor VIII, factor IX, and glucagon [16,18].

Recognizing the potential of ABD and XTEN polypeptides for extending half-life, we hypothesized that combining these two domains could have a synergistic effect and further increase the half-life of the payload fused to them. Therefore, we developed GLP1R agonists, GLP-ABD-XTEN144 and GLP-ABD-XTEN288, by genetically fusing GLP-1 to the tandem ABD and XTEN polypeptide. The design of the three components of the fusion protein was influenced by several considerations: first, GLP-1 must have an exposed N-terminus to bind to receptors via a TEV protease digestion; second, the ABD domain can form a non-covalent bond with albumin *in vivo*, creating a larger molecular complex, slowing renal clearance, and enabling FcRn-mediated recycling; third, the XTEN component has distinct biochemical qualities for drug development, such as high thermal stability, solubility, and ease of production in *E. coli*; and finally, the flexible linker $(GGGGS)_3$ provided necessary spatial distance between each domain, allowing for correct folding during expression.

The fusion proteins GLP-ABD-XTEN144 and GLP-ABD-XTEN288 were effectively produced in high quantities in *E. coli*. The subsequent purification procedures were simple and efficient, achieving more than 95% purity of these fusion proteins using techniques such as affinity chromatography and ion exchange chromatography. The production of these fusion proteins was simpler and more high-yielding than some existing market drugs, such as dulaglutide and semaglutide, which are produced through mammalian cells or chemical synthesis with high costs and longer time requirements [22]. Therefore, the use of an *E. coli* expression system with lower cost will likely have greater market competitiveness.

To better understand the binding affinity of GLP-ABD-XTEN fusion proteins for HSA, we employed a sandwich ELISA method. GLP-ABD-XTEN288 showed a K_d value of 27.78 nM, which was higher than that of GLP-ABD-XTEN144, with a K_d value of only 5.50 nM. GLP-ABD without any XTEN polypeptide had the highest affinity for HSA, with a K_d value of 2.27 nM. This indicates that due to their lack of secondary structure, a certain length of XTEN (XTEN144 or XTEN288 polymer) impacts the binding between HSA and ABD because of steric hindrance. This is in line with a previous report in which VRS-317, constructed by the genetic fusion of XTEN1 (83.6 kDa) and XTEN2 (13.3 kDa) to the N-terminus and C-terminus of GH, showed a 12-fold reduction in *in vitro* potency compared with recombinant human GH [31]. However, the *in vivo* activity of VRS-317 was significantly enhanced, suggesting that the loss of *in vitro* activity can be offset by significantly increased tissue and organ exposure *in vivo* [31].

The half-lives of GLP-ABD-XTEN144 and GLP-ABD-XTEN288 were influenced by both ABD and XTEN polypeptides. The half-life of GLP-ABD-XTEN144 was extended to over 12.9 h in mice, almost twice that of GLP-ABD-XTEN288. This is due to GLP-ABD-XTEN144 having a 5-fold higher affinity than GLP-ABD-XTEN288. Despite GLP-ABD-XTEN288's larger molecular size, it had a shorter half-life

than GLP-ABD-XTEN144, suggesting that the ABD component played a larger role in half-life extension for these two constructs. Another possible explanation could be that the XTEN polypeptides with 144 or 288 residues were too short to create a significant steric hindrance, slowing renal clearance. The use of XTEN864 notably extended the half-life of the fusion partner, much longer than XTEN288 or XTEN144 [18]. Therefore, to further extend the half-life of GLP-ABD, XTEN576 or XTEN864 could be employed and evaluated.

In order to evaluate the *in vivo* efficacy of GLP-1R agonists, we conducted tests to measure the blood glucose levels following the application of the fusion proteins. The findings revealed that both GLP-ABD-XTEN144 and GLP-ABD-XTEN288 maintained the biological activities of the innate GLP-1. In the context of OGTTs, both fusion proteins consistently reduced the sudden surge in blood glucose in a dose-dependent manner. A single dose of the fusion protein was able to keep non-fasting blood glucose levels reduced for a minimum duration of 48 h. This is a significant enhancement when compared to GLP-ABD devoid of the XTEN component, which could only sustain this effect for 8 h [11]. It implies that the XTEN polypeptide is vital in extending the half-life of GLP-1. Additionally, the fusion proteins considerably diminished the overall food consumption in mice. This suggests that ABD and XTEN polypeptides effectively improved the pharmacodynamic properties by increasing GLP-1 exposure *in vivo*. In terms of activity, GLP-ABD-XTEN144's activity was higher than that of GLP-ABD-XTEN288, possibly due to the larger steric hindrance caused by XTEN288 interfering with GLP-1 receptor binding. This finding aligns with the *in vitro* affinity of fusion proteins for HSA. These results suggest that although XTEN reduced the *in vitro* binding activity of GLP-ABD, the significantly extended half-life could compensate for this decrease [31]. The experimental results from this study showed that combining two half-life extension strategies could have a synergistic effect, improving both payload activity and half-life. This combined strategy is also employed in the design of BIVV001 (efanesoctocog alfa), which uses both Fc fusion and XTEN polypeptide for half-life extension of Factor VIII (FVIII). The injection frequency of BIVV001 was reduced from three times per week to once per week [32].

Several limitations within this study warrant addressing in subsequent research. The potential safety implications associated with administrating protein drugs via injection are notably concerning. Post-injection, the possible induction of inflammatory factors like TNF-α and IL-6 by E. *coli*-derived endotoxins requires meticulous evaluation and exploration. Moreover, it's crucial to acknowledge that this study did not include a comparative analysis concerning the bioactivity of the fusion proteins relative to other FDA-approved GLP-1R agonists, such as dulaglutide or semaglutide.

Additionally, there are several opportunities for further improvement in this study to enhance the pharmacological profiles of GLP-1. First, longer XTEN peptides such as XTEN864 should be tested to further reduce the kidney clearance rate. Second, an ABD with higher affinity for HSA may further increase the half-life of the payload. Third, linkers of different lengths or rigid linkers could be used to further reduce steric hindrance between domains, enabling each component to bind to its corresponding receptor more effectively. Fourthly, to ascertain the pharmacokinetic attributes of the fusion proteins with greater precision, the development of a highly sensitive ELISA method is essential, one that can detect concentrations as low as 0.1 ng/mL or less.

5. Conclusion

In the current research, we developed unique, enduring GLP-1 agonists, integrating both ABD and XTEN polypeptide dual-domain carriers, attached to the C-terminal of GLP-1. These hybrid proteins were effectively produced in the *E. coli* system with an elevated yield and simple purification steps. The binding capacity of GLP-ABD-XTEN288 for HSA was considerably less than that of GLP-ABD-XTEN144. Nevertheless, both GLP-ABD-XTEN144 and GLP-ABD-XTEN288 fusion proteins exhibited substantially prolonged half-lives *in vivo*. The cooperative action of ABD and XTEN polypeptide was instrumental in extending the half-life of GLP-1. Both fusion proteins effectively mitigated blood glucose levels and curbed food consumption in mice. GLP-ABD-XTEN144 was found to be more potent in preserving blood glucose balance compared to GLP-ABD-XTEN288. Therefore, GLP-ABD-XTEN144 shows considerable promise for potential clinical use in managing T2D. Moreover, the utility of combining two distinct strategies for enhancing half-life has been shown to be a viable approach to optimizing the pharmacodynamic characteristics of a payload.

CRediT authorship contribution statement

Yan Zhou: Writing – original draft, Methodology, Formal analysis. **Jianhui Li:** Methodology, Investigation, Formal analysis. **Guosheng Gao:** Methodology, Formal analysis. **Yafeng Li:** Methodology, Investigation. **Changzhen Zhang:** Writing – review & editing, Supervision, Resources, Conceptualization.

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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References

[1] K. Ogurtsova, L. Guariguata, N.C. Barengo, P.L. Ruiz, J.W. Sacre, S. Karuranga, H. Sun, E.J. Boyko, D.J. Magliano, IDF diabetes Atlas: global estimates of undiagnosed diabetes in adults for 2021, Diabetes Res. Clin. Pract. 183 (2022) 109118, https://doi.org/10.1016/j.diabres.2021.109118.

[2] S. Demir, P.P. Nawroth, S. Herzig, B. Ekim Ustunel, Emerging targets in type 2 diabetes and diabetic complications, Adv. Sci. 8 (2021) e2100275, https://doi.org/10.1002/advs.202100275.

[3] D.H. van Raalte, C.B. Verchere, Glucagon-like peptide-1 receptor agonists: Beta-cell protection or exhaustion?, 27, 2016, pp. 442–445, https://doi.org/10.1016/j.tem.2016.04.009.

[4] J. Reed, S. Bain, V. Kanamarlapudi, Recent advances in understanding the role of glucagon-like peptide 1, F1000Res 9 (2020) 239, https://doi.org/10.12688/f1000research.20602.1.

[5] G. Cantini, E. Mannucci, M. Luconi, Perspectives in GLP-1 research: new targets, new receptors, Trends Endocrinol. Metabol. 27 (2016) 427–438, https://doi.org/10.1016/j.tem.2016.03.017.

[6] L. Ren, Q. Cui, W. Liu, L. Wang, Y. Liao, Y. Feng, W. Sun, Y. Yang, Z. Zhang, T. Jin, G.J. Prud'homme, L. Zhang, Y. Li, Y. Leng, Q. Wang, Novel GLP-1 analog supaglutide stimulates insulin secretion in mouse and human islet beta-cells and improves glucose homeostasis in diabetic mice, Front. Physiol. 10 (2019) 930, https://doi.org/10.3389/fphys.2019.00930.

[7] M. Yu, M.M. Benjamin, S. Srinivasan, E.E. Morin, E.I. Shishatskaya, S.P. Schwendeman, A. Schwendeman, Battle of GLP-1 delivery technologies, Adv. Drug Deliv. Rev. 130 (2018) 113–130, https://doi.org/10.1016/j.addr.2018.07.009.

[8] H. Tan, W. Su, W. Zhang, P. Wang, M. Sattler, P. Zou, Recent advances in half-life extension strategies for therapeutic peptides and proteins, Curr. Pharmaceut. Des. 24 (2018) 4932–4946, https://doi.org/10.2174/1381612825666190206105232.

[9] J.T. Sockolosky, F.C. Szoka, The neonatal Fc receptor, FcRn, as a target for drug delivery and therapy, Adv. Drug Deliv. Rev. 91 (2015) 109–124, https://doi.org/10.1016/j.addr.2015.02.005.

[10] J. Nilvebrant, S. Hober, The albumin-binding domain as a scaffold for protein engineering, Comput. Struct. Biotechnol. J. 6 (2013) 1–8, https://doi.org/10.5936/csbj.201303009.

[11] H. Tan, W. Su, W. Zhang, J. Zhang, M. Sattler, P. Zou, Albumin-binding domain extends half-life of glucagon-like peptide-1, Eur. J. Pharmacol. 890 (2021) 173650, https://doi.org/10.1016/j.ejphar.2020.173650.

[12] F.Y. Nikravesh, S. Shirkhani, E. Bayat, Y. Talebkhan, E. Mirabzadeh, M. Sabzalinejad, H.A.M. Aliabadi, L. Nematollahi, Y.H. Ardakani, S. Sardari, Extension of human GCSF serum half-life by the fusion of albumin binding domain, Sci. Rep. 12 (2022) 667, https://doi.org/10.1038/s41598-021-04560-6.

[13] J. Guo, J. Sun, X. Liu, Z. Wang, W. Gao, Head-to-tail macrocyclization of albumin-binding domain fused interferon alpha improves the stability, activity, tumor penetration, and pharmacology, Biomaterials 250 (2020) 120073, https://doi.org/10.1016/j.biomaterials.2020.120073.

[14] J. Hopp, N. Hornig, K.A. Zettlitz, A. Schwarz, N. Fuss, D. Muller, R.E. Kontermann, The effects of affinity and valency of an albumin-binding domain (ABD) on the half-life of a single-chain diabody-ABD fusion protein, Protein Eng. Des. Sel. 23 (2010) 827–834, https://doi.org/10.1093/protein/gzq058.

[15] S.S. Gapizov, L.E. Petrovskaya, L.N. Shingarova, E.A. Kryukova, E.F. Boldyreva, E.P. Lukashev, S.A. Yakimov, E.V. Svirshchevskaya, D.A. Dolgikh, M. P. Kirpichnikov, Fusion with an albumin-binding domain improves pharmacokinetics of an αvβ3-integrin binding fibronectin scaffold protein, Biotechnol. Appl. Biochem. 66 (2019) 617–625, https://doi.org/10.1002/bab.1762.

[16] V. Schellenberger, C.W. Wang, N.C. Geething, B.J. Spink, A. Campbell, W. To, M.D. Scholle, Y. Yin, Y. Yao, O. Bogin, J.L. Cleland, J. Silverman, W.P. Stemmer, A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner, Nat. Biotechnol. 27 (2009) 1186–1190, https://doi.org/10.1038/nbt.1588.

[17] V.N. Podust, B.C. Sim, D. Kothari, L. Henthorn, C. Gu, C.W. Wang, B. McLaughlin, V. Schellenberger, Extension of in vivo half-life of biologically active peptides via chemical conjugation to XTEN protein polymer, Protein Eng. Des. Sel. 26 (2013) 743–753, https://doi.org/10.1093/protein/gzt048.

[18] V.N. Podust, S. Balan, B.C. Sim, M.P. Coyle, U. Ernst, R.T. Peters, V. Schellenberger, Extension of in vivo half-life of biologically active molecules by XTEN protein polymers, J. Contr. Release 240 (2016) 52–66, https://doi.org/10.1016/j.jconrel.2015.10.038.

[19] N.C. Geething, W. To, B.J. Spink, M.D. Scholle, C.W. Wang, Y. Yin, Y. Yao, V. Schellenberger, J.L. Cleland, W.P. Stemmer, J. Silverman, Gcg-Xten, An improved glucagon capable of preventing hypoglycemia without increasing baseline blood glucose, PLoS One 5 (2010) e10175, https://doi.org/10.1371/journal.pone.0010175.

[20] S.A. Jacobs, A.C. Gibbs, M. Conk, F. Yi, D. Maguire, C. Kane, K.T. O'Neil, Fusion to a highly stable consensus albumin binding domain allows for tunable pharmacokinetics, Protein Eng. Des. Sel. 28 (2015) 385–393, https://doi.org/10.1093/protein/gzv040.

[21] Y. Zhang, M.R. Huo, J.P. Zhou, S.F. Xie, PKSolver: an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel, Comput, Methods Programs Biomed. 99 (2010) 306–314, https://doi.org/10.1016/j.cmpb.2010.01.007.

[22] H. Tan, W. Su, W. Zhang, J. Zhang, M. Sattler, P. Zou, Generation of novel long-acting GLP-1R agonists using DARPins as a scaffold, Int. J. Pharm. 607 (2021) 121043, https://doi.org/10.1016/j.ijpharm.2021.121043.

[23] C. Graaf, D. Donnelly, D. Wootten, J. Lau, P.M. Sexton, L.J. Miller, J.M. Ahn, J. Liao, M.M. Fletcher, D. Yang, A.J. Brown, C. Zhou, J. Deng, M.W. Wang, Glucagon-like peptide-1 and its class B G protein-coupled receptors: a long march to therapeutic successes, Pharmacol. Rev. 68 (2016) 954–1013, https://doi.org/10.1124/pr.115.011395.

[24] R. Zaman, R.A. Islam, N. Ibnat, I. Othman, A. Zaini, C.Y. Lee, E.H. Chowdhury, Current strategies in extending half-lives of therapeutic proteins, J. Contr. Release 301 (2019) 176–189, https://doi.org/10.1016/j.jconrel.2019.02.016.

[25] R.E. Kontermann, Half-life extended biotherapeutics, Expet Opin. Biol. Ther. 16 (2016) 903–915, https://doi.org/10.1517/14712598.2016.1165661.

[26] P. Bannas, J. Hambach, F. Koch-Nolte, Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics, Front. Immunol. 8 (2017) 1603, https://doi.org/10.3389/fimmu.2017.01603.

[27] A. Walker, G. Dunlevy, D. Rycroft, P. Topley, L.J. Holt, T. Herbert, M. Davies, F. Cook, S. Holmes, L. Jespers, C. Herring, Anti-serum albumin domain antibodies in the development of highly potent, efficacious and long-acting interferon, Protein Eng. Des. Sel. 23 (2010) 271–278, https://doi.org/10.1093/protein/gzp091.

[28] A. Jonsson, J. Dogan, N. Herne, L. Abrahmsen, P.A. Nygren, Engineering of a femtomolar affinity binding protein to human serum albumin, Protein Eng. Des. Sel. 21 (2008) 515–527, https://doi.org/10.1093/protein/gzn028.

[29] R. Li, H. Yang, D. Jia, Q. Nie, H. Cai, Q. Fan, L. Wan, L. Li, X. Lu, Fusion to an albumin-binding domain with a high affinity for albumin extends the circulatory half-life and enhances the in vivo antitumor effects of human TRAIL, J. Contr. Release 228 (2016) 96–106, https://doi.org/10.1016/j.jconrel.2016.03.004.

[30] M.F. Lee, C.L. Poh, Strategies to improve the physicochemical properties of peptide-based drugs, Pharm. Res. (N. Y.) 40 (2023) 617–632, https://doi.org/10.1007/s11095-023-03486-0.

[31] J.L. Cleland, N.C. Geething, J.A. Moore, B.C. Rogers, B.J. Spink, C.W. Wang, S.E. Alters, W.P. Stemmer, V. Schellenberger, A novel long-acting human growth hormone fusion protein (VRS-317): enhanced in vivo potency and half-life, J. Pharm. Sci. 101 (2012) 2744–2754, https://doi.org/10.1002/jps.23229.

[32] T. Lissitchkov, A. Willemze, S. Katragadda, K. Rice, S. Poloskey, C. Benson, Efanesoctocog alfa for hemophilia A: results from a phase 1 repeat-dose study, Blood Adv. 6 (2022) 1089–1094, https://doi.org/10.1182/bloodadvances.2021006119.