Journal of Controlled Release 256 (2017) 114–120

Contents lists available at ScienceDirect

Journal of Controlled Release

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

An approach for half-life extension and activity preservation of an anti-diabetic peptide drug based on genetic fusion with an albumin-binding aptide

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ARTICLE INFO

Keywords:
Aptides
Exenatide
Human serum albumin
Fusion peptide
GLP-1
Type 2 diabetes

ABSTRACT

Although the peptide, exenatide, has been widely used as a drug for the treatment of type 2 diabetes, its short plasma half-life requires frequent subcutaneous injection, resulting in poor patient compliance in addition to side effects such as infection at the sites of injection. Here, we report a novel long-acting fusion peptide comprising exenatide and a human serum albumin (HSA)-binding aptide. A phage display screen of a library of aptides, yielded an HSA-specific aptide (APT_{HSA}) that bound HSA with a K_d of 188 nM. The recombinant fusion peptide comprising exenatide and APT_{HSA} (exenatide-APT_{HSA}) was expressed in *Escherichia coli* and purified by affinity and size-exclusion chromatography. The resulting exenatide-APT_{HSA} fusion peptide showed glucose-induced insulin secretion activity similar to that of native exenatide when tested *in vitro* using the INS-1 cell line. A pharmacokinetic analysis of exenatide-APT_{HSA} after subcutaneous administration revealed a 4-fold longer plasma half-life (1.3 *vs.* 0.35 h) compared with exenatide. Furthermore, exenatide-APT_{HSA} showed significantly improved anti-hyperglycemic effects in oral glucose tolerance tests and enhanced hypoglycemic effects compared with exenatide in a *db/db* type 2 diabetes mouse model. These results suggest that the exenatide-APT_{HSA} fusion peptide could be used as a potential anti-diabetic agent for the treatment of type 2 diabetes.

1. Introduction

Patients with type 2 diabetes have greatly impaired incretin-mediated insulin secretion, mainly owing to decreased secretion of glucagon-like peptide-1 (GLP-1) [1]. The 39-amino acid peptide exenatide, a synthetic version of the hormone exendin-4 found in Gila monster, is a GLP-1 mimetic peptide that has been widely used as an adjunctive therapy to improve glycemic control in diabetes mellitus type 2 patients [2–5]. However, exenatide exhibits a short plasma half-life of ~1.5–4 h in humans [6] owing to its size, which is far smaller than the cut off for renal excretion, and thus necessitates twice daily subcutaneous injection. In addition to causing side effects, such as infection at the sites of injection, the requirement for frequent injections results in poor patient compliance [7,8], a limitation that recent efforts have sought to address.

Among the technological solutions that have been investigated for limiting renal clearance of exenatide is fusion of the peptide to large artificial or natural macromolecules, such as polyethylene glycol, XTEN polypeptide, and human serum albumin (HSA) [9–19]. Although such technological approaches have increased the plasma half-life of exenatide up to ~12–38 h, the biological activity of the drug in these conjugates is dramatically reduced (up to 98%) compared to pristine exenatide, limiting further development [20–22]. This significant loss of activity in exenatide fusion conjugates may result from blockage of the peptide's receptor binding site by the larger molecular fusion partners. As alternative approaches, technologies based on albumin-binding peptides (ABP) or albumin-binding domains (ABD) that allow a reversible, non-covalent association with the abundant serum protein have emerged [23,24]. Such approaches rely on the fact that albumin binds to neonatal Fc receptor (FcRn) in an acidic endosome and is recycled back into the blood circulation at physiological pH, resulting in long circulation half-life in bloodstream [25]. Although such approaches result in a diminished loss of exenatide activity compared with fusions with larger molecular scaffolds, such as XTEN and HSA, concerns remain regarding the potential immunogenicity of ABD and the low-affinity of HSA for ABP. This experience with different fusion partners suggests that a new affinity molecule with a smaller size than ABD and higher affinity than ABP would be a suitable candidate fusion

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http://dx.doi.org/10.1016/j.jconrel.2017.04.036
Received 14 October 2016; Received in revised form 19 April 2017; Accepted 25 April 2017
Available online 27 April 2017

partner for short-acting peptide drugs like exenatide.

Aptides, developed by our group, are a novel class of structure-constrained peptides containing a randomizable binding region and a constant β-hairpin scaffold. By combining this genetic variability with *in vitro* directed evolution strategies, high-specificity and -affinity peptide binders for various biological targets can be identified from screens of aptide libraries [26–30]. Here, we report a new fusion peptide between exenatide and an HSA-binding aptide (ATP_{HSA}), designated exenatide-ATP_{HSA}. ATP_{HSA} was initially identified by phage display screening, its affinity and specificity for HSA was characterized, and the pharmacokinetics and biological activity of the resulting exenatide-ATP_{HSA} fusion conjugate were examined *in vitro* and *in vivo*. These analyses revealed that exenatide-ATP_{HSA} exhibits an extended half-life while retaining antidiabetic biological activity.

2. Materials and methods

2.1. Materials

Exenatide (*N′*-HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-*C′*) was synthesized by AnyGen Co. (Gwangju, South Korea). All oligonucleotides for libraries and cloning were purchased from Genotech Inc. (Daejeon, South Korea). Female C57BL/6 *db/db* mice (6 wk. old) were supplied by the Korean Research Institute of Bioscience and Biotechnology (Daejeon, South Korea) and maintained under pathogen-free conditions in the animal facility at Korea Advanced Institute of Science and Technology (KAIST). Animal experiments were approved by the KAIST Animal Care and Use Committee. Exenatide enzyme-linked immunosorbent assay (ELISA) kits were purchased from Phoenix pharmaceuticals, Inc. (Burlingame, CA, USA) and insulin ELISA kits were purchased from Mercodia (Uppsala, Sweden). The glucose-sensitive pancreatic cell line, INS-1, was cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. A One-Touch blood glucose meter (CodeFree) was purchased from SD Biosensor, Inc. (Suwon, South Korea).

2.2. Phage display screening for HSA-binding aptides

An HSA-specific aptide (ATP_{HSA}) was identified using a phage display-based screening method, as described previously [26]. Briefly, HSA was immobilized on 96-well plates (Corning, Steuben, NY, USA) overnight at 4 °C. After incubating phage library pools on HSA-immobilized plates for 1 h at 25 °C, unbound phages were removed by washing with phosphate-buffered saline (PBS) containing 0.5% Tween-20 (PBS-T), and bound phages were eluted with 0.2 M glycine buffer (pH 2.2), followed by immediate neutralization with Tris-HCl (pH 8.8). The eluted phages were amplified in log-phase cultures of *Escherichia coli* ER (New England Biolabs, Ipswich, MA, USA). This biopanning process was reiterated several times using the amplified phages until enrichment of specific phages was achieved. After biopanning, phage ELISAs were carried out. Bound phages were incubated with horseradish peroxidase (HRP)-conjugated anti-M13 antibody (GE Healthcare, Little Chalfont, UK) and then detected by incubation with the chromogenic substrate tetramethylbenzidine (TMB) with subsequent monitoring of absorbance at 450 nm. The sequence of ATP_{HSA} identified by these screens was *N′*-HAHFNFGSWTWENGKWTWKGIWLPAR-*C′*, where the underlined sequence denotes the constant β-hairpin scaffold portion of the aptide structure.

2.3. Specificity assessment of APT_{HSA}

To investigate the specificity of APT_{HSA}, we carried out phage ELISA experiments using HSA and phages displaying APT_{HSA}; negative control protein targets included streptavidin (New England Biolabs, Ipswich, MA, USA), tumor necrosis factor alpha (TNFα; R & D Systems, Minneapolis, USA), CD7-Trx, and visfatin (Sigma, St. Louis, MO, USA). All proteins (10 μg/mL in PBS) were immobilized on 96-well plates (Corning, Steuben, NY, USA) by incubating overnight at 4 °C. After blocking with 2% skim milk, phages displaying APT_{HSA} (1×10^8 PFU) were incubated with HSA or control protein for 1 h at 25 °C. Then, each plate was washed five times with PBS-T and incubated with HRP-conjugated anti-M13 antibody (GE Healthcare, Little Chalfont, UK, cat. no. 27-9420-01). The results were visualized by incubating with the TMB substrate (BD Biosciences, Franklin Lakes, NJ, USA) and measuring absorbance at 450 nm.

2.4. Measurement of APT_{HSA} affinity

Affinity of peptides was measured using a BIAcore X instrument (GE Healthcare, Little Chalfont, UK) as described by the manufacturer. Briefly, HSA protein was immobilized on a CM5 chip (GE Healthcare, Little Chalfont, UK). Different concentrations of APT_{HSA} in running buffer (PBS, pH 7.4) were then injected and allowed to interact with HSA at a flow rate of 30 μL/min. All kinetic data for interactions were analyzed using BIAevaluation 3.1 software.

2.5. Preparation of an exenatide-APT_{HSA} fusion peptide

Exenatide-APT_{HSA} was prepared using recombinant DNA technology. A fusion gene for exenatide-APT_{HSA} was generated by concatenating the oligonucleotides encoding exenatide, a linker (GSEGSEGEGGSEGSEGEG), and APT_{HSA}. The gene was amplified by polymerase chain reaction (PCR) using primers obtained from Genotech Inc. (Daejeon, South Korea). PCR conditions were 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. The amplified gene was cloned into the prokaryotic expression vector pET32a (Novagen, Darmstadt, Germany), and the resulting exenatide-APT_{HSA} expression plasmids were transformed into *E. coli* strain BL21 (DE3) (Stratagene, La Jolla, CA, USA). After induction with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO, USA) for 20 h at 18 °C, the exenatide-APT_{HSA} fusion conjugate was purified using Ni-NTA affinity resin (ELPIS biotech, Daejeon, South Korea) following by gel filtration using a Superdex 75 column (Amersham Pharmacia, Little Chalfont, UK) pre-equilibrated with PBS (pH 7.4). The Trx-fusion tag was cleaved by incubating the fusion peptide with enterokinase for 16 h at 20 °C, after which exenatide-APT_{HSA} was purified by gel filtration using a Superdex 75 column (Amersham Pharmacia, Little Chalfont, UK) pre-equilibrated with PBS (pH 7.4). Endotoxins were removed using a high capacity endotoxin removal spin column (Pierce, Waltham, MA, USA).

2.6. *In vitro* biological activity of exenatide-APT_{HSA}

The *in vitro* biological activity of exenatide-APT_{HSA} was determined by measuring insulin secretion by the glucose sensitive pancreatic-cell line, INS-1, after treatment with the fusion peptide. The cells were maintained in complete RPMI-1640 medium (11.1 mM glucose) containing 10% (v/v) FBS, 50 μM 2-mercaptoethanol, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin and 100 μg/mL streptomycin, and incubated at 37 °C in a humidified 5% CO_2 atmosphere. INS-1 cells were incubated in 500 μL of Kreps–Ringer–HEPES (KRH) buffer (11.1 mM glucose) containing exenatide or exenatide-APT_{HSA} (0.1–100 nM) for 1 h, and the levels of insulin released were measured using an insulin ELISA kit.

2.7. *In vivo* pharmacokinetics of exenatide-APT_{HSA}

The pharmacokinetic profiles of exenatide and exenatide-APT_{HSA} that were mixed with HSA (600 μM) through incubation with HSA for 1 h were assessed following subcutaneous (s.c.) administration in ICR mice ($n = 3$) at a dose of 25 nmol/kg. Blood samples were obtained from the retro-orbital sinus and centrifuged at 700 × *g* and 4 °C for