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Pluronic modified leptin with increased systemic circulation, brain uptake and efficacy for treatment of obesity☆

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ABSTRACT

Modification of hydrophilic proteins with amphiphilic block copolymers capable of crossing cell membranes is a new strategy to improve protein delivery to the brain. Leptin, a candidate for the treatment of epidemic obesity, has failed in part because of impairment in its transport across the blood–brain barrier (BBB) that develops with obesity. We posit that modification of leptin with poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly (ethylene oxide), Pluronic P85 (P85) might permit this protein to penetrate the BBB independently of its transporter, thereby overcoming peripheral leptin resistance. Here we report that peripherally administered leptin–P85 conjugates exhibit biological activity by reducing food intake in mouse models of obesity (ob/ob, and diet-induced obese mouse). We further generated two new leptin–P85 conjugates: one, Lep(ss)–P85(L), containing one P85 chain and another, Lep(ss)–P85(H), containing multiple P85 chains. We report data on their purification, analytical characterization, peripheral and brain pharmacokinetics (PK). Lep(ss)–P85(L) crosses the BBB using the leptin transporter, and exhibits improved peripheral PK along with increased accumulation in the brain compared to unmodified leptin. Lep(ss)–P85(H) also has improved peripheral PK but in a striking difference to the first conjugate penetrates the BBB independently of the leptin transporter via a non-saturable mechanism. The results demonstrate that leptin analogs can be developed through chemical modification of the native leptin with P85 to overcome leptin resistance at the level of the BBB, thus improving the potential for the treatment of obesity.

1. Introduction

Leptin is a 16 kDa regulatory protein secreted by fat cells and acting within the brain to control appetite and thermogenesis [1–3]. It crosses the blood–brain barrier (BBB) by a specific, saturable transport system [4]. A decade ago, recombinant leptin was tested unsuccessfully as a therapy for reduction of body weight in obese patients. We now appreciate that the early attempts of leptin monotherapy failed because of leptin resistance manifested in the obese patients. Animal and human studies show that leptin resistance can occur at three levels: reduced transport at the BBB, impaired leptin receptor function in the hypothalamus, and altered response of anorectic/orexigenic downstream neuronal circuitries [5–7]. A great deal of effort has been made to address leptin receptor resistance. For example, a combination of amylin with leptin led to profoundly synergistic reductions in food intake and body weight in the obese patients and diet-induced obese (DIO) mice [8,9]. By activating amylin receptor within the hindbrain area postrema and subsequent polysynaptic connection to the hypothalamus, amylin sensitizes the ventromedial hypothalamus, resulting in the augmentation of leptin-induced phosphorylated signal transducer and activator of transcription (STAT) 3 activation [8]. Leptin resistance at the level of the BBB has also been demonstrated. Indeed, an impaired BBB transport is important in the maintenance and probably in the progression of obesity [7,10–12]. In particular, leptin transporter defects predominate over brain receptor defects early on in outbred models of rodent DIO [7]. Modeling based on cerebrospinal fluid (CSF) and serum levels of leptin indicates that in advanced obesity in humans (leptin levels of about 40 ng/mL), transporter defects account for about 2/3 of resistance to peripheral leptin [13]. Thus, just as in the rodent and canine models [10,14,

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15], it is expected that moderately obese humans with poor response to peripherally administered leptin [16] would still respond better to a leptin that could reach brain receptors.

Several strategies have been explored to improve leptin action by way of peripheral administration route. Modification of leptin with polyethylene glycol (PEG) extended leptin serum circulation [17]. However, subcutaneous administration of PEGylated leptin failed in clinical trials as a weight control drug for obesity treatment [18,19]. It is known that PEGylation of proteins sometimes increases and sometimes decreases the permeability of those proteins at the BBB [20–22], but in a specific study a PEGylated leptin was completely unable to cross the BBB [23]. Similarly, a genetically engineered Fc–leptin fusion protein had no anti-obesity effect on DIO mice, although it increased serum half-life of leptin and induced more weight loss than leptin in ob/ob mice [24]. To target brain, leptin was also modified with trans-activating transcriptional activator (TAT), a cell-penetrating peptide that is widely explored to facilitate cellular uptake of various molecular cargos. After intravenous (i.v.) injection, the modified leptin was detected in a greater amount than leptin in brain hypothalamus and showed greater response than leptin in inducing weight loss in high-fat diet Balb/c mice [25,26]. The most recent attempt in leptin development is a leptin peptide mimics that carries a carbohydrate moiety aiming for active transport across the BBB. Following multiple daily intraperitoneal (i.p.) (0.5 mg/kg/day for 11 days) or intranasal (0.1 mg/kg/day for 10 days) injections, this leptin peptide controlled body weight gain in DIO mice [27]. The transport rate at the BBB is a key factor underlying the ability of any substance to enter the brain and efforts have been made to improve leptin transporter activity at the BBB. For example, leptin co-administration with epinephrine showed a greater uptake by mouse brain [28]. Altogether, these studies suggest that overcoming transporter resistance at the BBB and increasing delivery of leptin to the brain could significantly improve leptin therapeutic efficacy and increase weight loss. Among the aforementioned delivery strategies, some (e.g. leptin peptide mimics) might move to clinical tests, while others (e.g. TAT modification) might encounter safety concerns. However, the problem remains unresolved. Thus developing a safe, efficacious and translatable approach to overcome peripheral resistance to leptin could address an unmet medical need and result in a therapeutic product for the treatment of obesity.

Toward this goal we proposed to modify leptin with pluronic block copolymers, aiming to improve the peripheral pharmacokinetics (PK) of leptin and its brain uptake. “Pluronics” or “poloxamers” are amphiphilic triblock copolymes consisting of (poly(ethylene oxide)-*b*-poly (propylene oxide)-*b*-poly(ethylene oxide) (PEO–PPO–PEO, same as poly(polyethylene glycol)-*b*-poly(propylene glycol)-*b*-poly(polyethylene glycol) or PEG–PPG–PEG). We reported on the first generation of the modified product, Lep(ss)–P85, showing an elongated serum half-life time, higher stability in blood and brain, and rapid transport rate across the BBB in a non-saturable and leptin transporter independent manner [29]. The *in vivo* biological activity of this Lep(ss)–P85 was shown by an acute reduction in food intake in normal body weight mice [29]. In an extension of this approach, we have continuously produced leptin–pluronic conjugates with the goal to improve their purity, PK profile, and efficacy. Herein, we report the new data obtained during this optimization process that involved conjugate production, analytical characterization, peripheral and brain PK analysis and efficacy evaluation in models of obese mice.

2. Materials and methods

2.1. Materials

Mouse recombinant leptin (Lep) and a chimera leptin receptor (ObR-Fc) were purchased from R&D Systems (Minneapolis, MN). 4-Methoxyltrityl chloride (MTr-Cl), 1,1′-carbonyldiimidazole (CDI), 1,2-ethylenediamine (EDA), ninhydrin, L-glutathione (reduced), ethylenediaminetetraacetic acid (EDTA), sinapinic acid, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), iodoacetamide (IAA), triethylamine, anhydrous acetonitrile, anhydrous pyridine, methanol, dichloromethane, toluene, acetone, ethanol, isopropanol, dimethylformamide (DMF), PEG-SOD1 (S9549), human male AB serum and silica gel (288616, 70–270 mesh, 60 Å) were purchased from Sigma-Aldrich Co. (St-Louis, MO). Pluronic P85 (P85) (lot no. WPOP-587A, average M.W. 4600) was kindly provided by BASF Corp. (Parispany, NJ). Dithiobis(succinimidyl propionate) (DSP), disuccinimidyl propionate (DSS), dithiothreitol (DTT) and bovine serum albumin (BSA) were from Thermo Fisher Scientific (Rockford, IL). Carboxymethyl dextran chip (CM5), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), ethanolamine-HCl, HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4), protein G, Sephadex LH-20 gel and Illustra NAP-10 or 25 columns were from GE Healthcare (Piscataway, NJ). Amicon Ultra 0.5 mL centrifugal filters (10 kDa MWCO) and Amicon Ultra centrifugal filter units Ultra-15 (MWCO 10 kDa) were from Sigma-Aldrich Co. (St-Louis, MO). FLOAT-A-LYZER G2 (8–10 kDa MWCO) was from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Flexible thin-layer chromatography (TLC) plates were from Whatman Ltd (Mobile, AL).

2.2. Synthesis, purification and analysis

2.2.1. Synthesis of leptin–P85 conjugates

The synthesis was carried out as previously reported [30]. Briefly, P85 was reacted with MTr-Cl (1:1 molar ratio) and purified on Silicagel column (3 × 40 cm) with stepwise elution in dichloromethane containing 2%, 5% and 10% methanol. The resulting mono-MTr-P85 (80% wt. yield) was activated by excess of CDI, mixed with EDA and finally treated with TFA to remove MTr protection. The resulting monoamine P85 was further purified using gel-permeation chromatography on Sephadex LH-20 column (2.5 × 30 cm) in methanol. The amino groups were assayed qualitatively by TLC and quantitatively by ninhydrin assay. To produce leptin–P85 conjugates, the obtained monoamine P85 was activated by excess of DSP linker followed by elution in Illustra NAP-25 columns in 20% aqueous ethanol to remove excess of unreacted linker. Fractions containing activated P85 copolymers were immediately mixed with leptin (molar ratio of leptin to P85 1:10) in 20% aqueous ethanol supplemented with sodium borate buffer (0.1 M, pH 8.0). After incubation overnight at 4 °C, the reaction mixture was precipitated in cold acetone to remove excess of free P85 copolymers, as described previously [30]. The obtained precipitates were then desalted, sterilized and stored for animal feeding study or further purified as described below.

2.2.2. Purification of leptin–P85 conjugates

The above obtained conjugates (Lep(ss)–P85) were subjected to size exclusion chromatography (SEC) on TSKgel G2000SW column (7.8 mm × 30 cm, Tosoh Bioscience LLC, Grove City, OH) using a Shimadzu HPLC system with a multiple-wavelength UV-detector (Shimadzu Scientific Instruments, Columbia, MD) and eluted in 0.1 M Na_3PO_4/0.2 M NaCl (pH 7.4) containing 10% methanol at a flow rate of 1 mL/min. Protein fractions were collected, desalted using Amicon Ultra centrifuge filter unit and analyzed by electrophoresis and mass spectra. Fractions eluted at 8.8 min (designated as Lep(ss)–P85(H)) and 9.5 min (designated as Lep(ss)–P85(L)) were further sterilized and stored for animal pharmacokinetic study.

2.2.3. MALDI-TOF spectra

Mass values of leptin–P85 conjugates were determined by matrix-assisted laser desorption/ionization time of fly (MALDI-TOF) spectroscopy in 4800 MALDI TOF/TOF™ analyzer (Applied Biosystems/MDS SCIEX) at a laser power of 3000 V and in a positive reflector mode. Solution containing saturated sinapinic acid in 50% acetonitrile with 0.1% TFA was used as matrix for sample preparation. Briefly, 0.5 μL of sinapinic acid solution was coated on the plate followed by 1)