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PASylation of Murine Leptin Leads to Extended Plasma Half-Life and Enhanced *in Vivo* Efficacy

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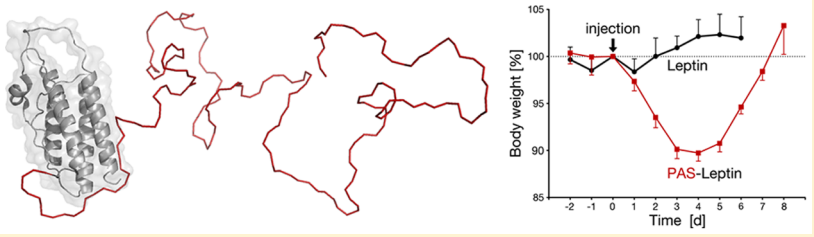

ABSTRACT: Leptin plays a central role in the control of energy homeostasis and appetite and, thus, has attracted attention for therapeutic approaches in spite of its limited pharmacological activity owing to the very short circulation in the body. To improve drug delivery and prolong plasma half-life, we have fused murine leptin with Pro/Ala/Ser (PAS) polypeptides of up to 600 residues, which adopt random coil conformation with expanded hydrodynamic volume in solution and, consequently, retard kidney filtration in a similar manner as polyethylene glycol (PEG). Relative to unmodified leptin, size exclusion chromatography and dynamic light scattering revealed an approximately 21-fold increase in apparent size and a much larger molecular diameter of around 18 nm for PAS(600)-leptin. High receptor-binding activity for all PASylated leptin versions was confirmed in BIAcore measurements and cell-based dual-luciferase assays. Pharmacokinetic studies in mice revealed a much extended plasma half-life after ip injection, from 26 min for the unmodified leptin to 19.6 h for the PAS(600) fusion. *In vivo* activity was investigated after single ip injection of equimolar doses of each leptin version. Strongly increased and prolonged hypothalamic STAT3 phosphorylation was detected for PAS(600)-leptin. Also, a reduction in daily food intake by up to 60% as well as loss in body weight of >10% lasting for >5 days was observed, whereas unmodified leptin was merely effective for 1 day. Notably, application of a PASylated superactive mouse leptin antagonist (SMLA) led to the opposite effects. Thus, PASylated leptin not only provides a promising reagent to study its physiological role *in vivo* but also may offer a superior drug candidate for clinical therapy.

KEYWORDS: *adipokine, drug development, kidney, obesity, PEGylation, pharmacokinetics, satiety hormone, therapeutic protein*

1. INTRODUCTION

Leptin is primarily secreted from white adipose tissue and plays a key role in energy balance regulation.[1] The plasma concentrations of this adipokine communicate the state of energy storage to the brain, as well as some other organs, thus controlling food intake and energy expenditure.[2,3] Leptin can enter the central nervous system by a saturable transport mechanism across the blood−brain barrier.[4] Subsequently, binding to the long leptin receptor isoform—also referred to as ObR, CD295, or LepRb—triggers intracellular JAK/STAT signaling.[5,6] Leptin receptor activation causes a reciprocal regulation of anorexigenic and orexigenic neuron populations in several nuclei of the hypothalamus.[7] The significance of leptin for the control of energy homeostasis is impressively demonstrated by rare, naturally occurring loss-of-function mutations in the leptin gene.[8,9] In both humans and rodents, congenital leptin deficiency results in hyperphagia and morbid obesity and is accompanied by hyperinsulinemia and impaired fertility.[10−12] Indeed, constant infusions or frequent injections of recombinant leptin ameliorate the disease phenotype of individuals with functional leptin deficiencies.[13−16]

However, originally developed by Amgen up to clinical phase II trials, the expectations of using leptin as a drug for obesity treatment remained unmet,[9,17,18] mainly due to its low potency and short circulating half-life. In addition, common obesity is associated with high leptin levels and leptin resistance, which is considered responsible for only minor energy balance responses to recombinant leptin administration.[19] This resistance is due to (i) impaired transport of leptin across the blood−brain barrier and (ii) cellular resistance through enhanced activity of negative feedback loops in neurons.[20] Furthermore, apart from

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diet-induced obesity, leptin receptor mutations, photoperiod, and also pregnancy can alter leptin responsiveness.[21−23]

Beyond the treatment of obesity, leptin exerts positive effects on glycemic control, insulin sensitivity, and plasma triglycerides, thus raising prospects for therapy of type 1 and type 2 diabetes[24,25] as well as other hypoleptinemic, pathophysiological conditions such as hypothalamic amenorrhea[26] and, in particular, lipodystrophy.[27] In 2013 Metreleptin, a recombinant analogue of human leptin developed by Amylin/Bristol-Myers Squibb (BMS), received market approval for the treatment of rare forms of lipodistrophy in Japan.[28] Furthermore, this drug is subject to ongoing clinical trials and is under review by the US Food and Drug Administration (FDA) for various indications related to obesity and diabetes.

Mature murine leptin (UniProt ID P41160), which shares 85% amino acid sequence identity with the human orthologue (UniProt ID P41159), is a non-glycosylated 16 kDa cytokine of 146 amino acids. Its three-dimensional structure comprises a four-helix bundle with an up−up−down−down topology, carrying one structural disulfide bond that is essential for biological activity.[29] Leptin belongs to the long-chain helical cytokine family[30] for which a receptor activation mechanism is proposed that involves a quaternary signaling complex of two leptin proteins and two LepRb chains.[31] Three major interaction sites were identified on the surface of the leptin molecule: while site II is predominantly responsible for high affinity receptor binding,[32,33] site III—which is only found in cytokines of the "long" class—plays a role in the activation of the leptin receptor.[34] Recently, a superactive mouse leptin antagonist (SMLA) was developed by combining a set of mutations in these two sites:[34] the side chain replacement D23L close to site II reduces electrostatic repulsion and, thus, increases affinity to the leptin receptor, whereas the substitutions L39A, D40A, and F41A in site III prevent its activation.

In spite of the high pharmaceutical potential of leptin, its therapeutic efficacy is hampered by the small molecular size as well as low solubility.[29] In fact, leptin is rapidly eliminated by kidney filtration,[35] with a reported very short terminal half-life of about 50 min in mice and a similarly short circulation in monkeys and humans.[36] Indeed, there is general expectation that leptin variants with prolonged circulation should not only reduce the required frequency of dosing but also exert enhanced pharmacological properties and therapeutic performance.[37]

A common strategy to improve pharmacokinetic (PK) properties of small proteins involves increasing their hydrodynamic molecular volume beyond the pore size of the glomerular basal membrane that is responsible for renal filtration. Chemical conjugation with polyethylene glycol (PEG) is a widely employed approach to extend the plasma half-life of biologics.[38,39] So far, PEGylation has been used for more than ten approved biopharmaceuticals and was even applied to enhance the bioactivity of leptin—as well as its antagonistic variants—in preclinical[34,40,41] and clinical[42] studies. However, PEGylation in general has several drawbacks such as the necessity to identify a permissive coupling position in the biologically active protein, high cost for pharmaceutical grade activated PEG, and additional efforts for chemical conjugation and purification of the PEGylated protein. Furthermore, during long-term treatment the poor biodegradability can lead to kidney vacuolization and tissue accumulation of the synthetic polymer, in particular in the choroid plexus epithelial cells of the brain, and there is an increasing number of reports on the emergence of neutralizing antibodies against PEGylated biologics.[43−46]

To circumvent these caveats, "PASylation" was recently developed as a biological alternative to PEGylation.[47] PASylation involves the genetic fusion of a therapeutic protein (or peptide) with a conformationally disordered polypeptide of defined sequence comprising the small amino acids Pro, Ala, and/or Ser. This approach offers a superior way to attach a solvated molecular random chain with large hydrodynamic volume to a biologically active compound, showing biophysical properties very similar to those of PEG.[48] PASylation has so far been used to extend the plasma half-life of various biologics such as antibody fragments, growth hormones, and interferons both with regard to therapeutic applications and for *in vivo* imaging.[47,49,50] Here, we describe the design and biochemical analysis of PASylated murine leptin versions as well as their functional characterization in mice, demonstrating substantially prolonged circulation and much enhanced suppressive effects on food intake *in vivo*.

2. EXPERIMENTAL SECTION

2.1. Construction of Expression Plasmids. The structural gene for murine leptin (UniProt ID P41160) was initially amplified from mouse white adipose cDNA and inserted into a cloning vector. The coding region for the mature polypeptide was reamplified using the primers 5′-CAC CAT GGC GCC AGC TCT TCT GCC GTG CCT ATC CAG AAA GTC C-3′ and 5′-CCC CTC AAG CTT AGC ATT CAG GGC TAA CAT CC-3′ (*Kas*I, *Sap*I, and *Hin*dIII recognition sites underlined). The resulting PCR product was cleaved with *Kas*I and *Hin*dIII restriction endonucleases and finally cloned on the *Escherichia coli* expression vector pASK75-His_6-hGH.[47] This vector already encoded the OmpA signal peptide for secretion into the bacterial periplasm as well as an N-terminal His_6-tag for affinity purification (Figure 1).

Leptin with this N-terminal tag (Ala-Ser-His_6-Gly-Ala-Ser_3-Ala; 14 residues), including the linker for subsequent insertion of the PAS gene cassette, is here referred to as the unmodified recombinant protein and has a total mass of 17.4 kDa. The corresponding expression plasmid for SMLA, which differs from murine leptin by the four amino acid replacements D23L, L39A, D40A, and F41A,[34] was generated using QuikChange (Agilent Technologies, Waldbronn, Germany) site-directed mutagenesis with appropriate primers. One to three PAS#1.2(200) gene cassettes[47] were inserted into the leptin expression plasmid via the singular *Sap*I site between the His_6-tag and the coding region for the mature part of the protein.

2.2. Protein Production and Purification. For protein production, *E. coli* KS272[51] was co-transformed with the appropriate leptin expression plasmid and the helper plasmid pTUM4,[52] followed by recombinant gene expression as previously described.[47] Briefly, bacteria were cultivated in an 8 L benchtop fermenter at 25 °C with a synthetic glucose mineral salt medium supplemented with 100 mg/L ampicillin, 30 mg/L chloramphenicol, and 1 g/L L-proline. Gene expression was induced by addition of 500 μg/L anhydrotetracycline (Acros Organics, Geel, Belgium) when the culture reached OD_{550} = 20, followed by further incubation for up to 2.5 h. Immediately thereafter, the cells were harvested by centrifugation, and a periplasmic extract was prepared by resuspending the cells in ice-cold 500 mM sucrose, 15 mM EDTA, 100 mM Na-borate pH 8.0 supplemented with 250 μg/

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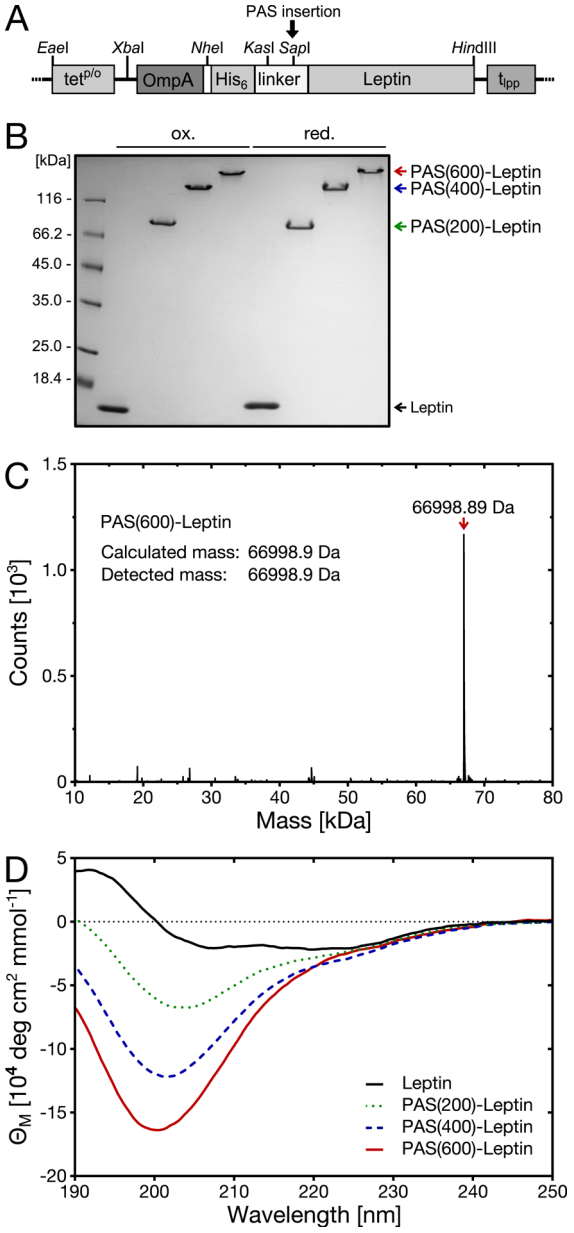

Figure 1. Preparation of recombinant murine leptin and its PASylated fusion proteins. (A) Expression plasmids are based on the generic pASK75 vector comprising the chemically inducible $tet^{p/o}$ promoter, an OmpA signal sequence for periplasmic secretion, a His_6-tag for affinity purification, a short linker allowing insertion of the PAS gene cassette, and the coding region for mature murine leptin. The preparation of PASylated leptin versions from *E. coli* in a functional monomeric form was confirmed by SDS−PAGE (B) and ESI-TOF mass spectrometry (C). (D) The correct fold of the leptin moiety and the secondary structure of the attached PAS polypeptide were investigated by CD spectroscopy.

mL lysozyme, and 1 mM dithiodipyridin (Sigma-Aldrich, Taufkirchen, Germany).

After dialysis, the recombinant protein was purified from the periplasmic extract, using a Ni^{2+}-charged 25 mL HisTrap HP IMAC column (GE Healthcare, Uppsala, Sweden). For further purification, anion exchange chromatography was performed on a Resource Q column (GE Healthcare) using 20 mM Tris/HCl pH 8.5 as running buffer and elution with an NaCl concentration gradient. Finally, the monomeric protein was isolated by size exclusion chromatography (SEC) on a Superdex 75 or 200 pg HiLoad 26/60 column (GE Healthcare) in phosphate-buffered saline (PBS; 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4). Purified proteins were concentrated by ultrafiltration using Amicon Ultra centrifugal filter units (10,000 MWCO; Millipore, Billerica, MA). Finally, endotoxin content was quantified using an Endosafe-PTS system (Charles River Laboratories, Wilmington, MA).

SDS−PAGE was performed using a high molarity Tris buffer system[53] followed by Coomassie brilliant blue staining. Protein concentrations were determined according to the absorption at 280 nm using calculated extinction coefficients[54] of 2680 $M^{-1}\cdot cm^{-1}$ for both recombinant leptin and SMLA as well as their PAS fusion proteins. Electrospray ionization mass spectra (ESI-MS) were recorded on maXis Q-TOF (Bruker Daltonics, Bremen, Germany) or 62100 time-of-flight LC/MS (Agilent, Santa Clare, CA) mass spectrometers.

2.3. Analytical Size Exclusion Chromatography. Analytical SEC was performed on a 24 mL bed size Superdex 200 HR 10/300 GL column (GE Healthcare) at a flow rate of 0.5 $mL\cdot min^{-1}$ using an ÄKTA Purifier 10 system (GE Healthcare) with PBS as running buffer. To this end, the purified proteins (250 μL) were applied and respective elution volumes were measured. The apparent molecular masses were estimated by interpolation from a calibration line (R^2 = 0.99) (Figure 2A) obtained with the reference proteins thyroglobulin, apoferritin, β-amylase, albumin, ovalbumin, carbonic anhydrase, cytochrome C, and aprotinin (all from Sigma-Aldrich).

2.4. Dynamic Light Scattering (DLS). DLS measurements were performed in PBS at 20 °C using a Zetasizer nano S photometer (Malvern Instruments, Malvern, U.K.) equipped with a 3 mm path length quartz cuvette (Hellma, Müllheim, Germany) using the standard operating procedure "Protein at 20 °C". Depicted data represent the averaged mean intensity from the volume particle size distribution of nine measurements for each sample, which all met the internal quality criteria. Conversion of hydrodynamic radii to apparent molecular masses and *vice versa* was performed using the "Hydrodynamic Radius Estimate" function provided by the Zetasizer software.

2.5. Circular Dichroism (CD) Spectroscopy. Protein secondary structure was analyzed using a J-810 spectropolarimeter (Jasco, Easton, MD) equipped with a 0.1 mm path length quartz cuvette (Hellma). Spectra were recorded at room temperature from 190 to 250 nm by accumulating 16 runs (bandwidth 1 nm, scan speed 100 $nm\cdot min^{-1}$, response 4 s) using 6.7−9.5 μM protein solutions in 50 mM K_2SO_4, 20 mM K-P_i pH 7.5. After correction for buffer blanks, spectra were smoothed using the instrument software (Spectra viewer with J-800 Control Drive v. 1.24.00). After that, the molar ellipticity Θ_M was calculated according to the equation $\Theta_M = \Theta_{obs}/(c\cdot d)$, wherein Θ_{obs} denotes the measured ellipticity, c the protein concentration [mol/L], and d the path length of the quartz cuvette [cm]. Finally, the molar ellipticity Θ_M was plotted against the wavelength. Secondary structure content was estimated with the instrument software.

2.6. Surface Plasmon Resonance (SPR) Spectroscopy. Real-time affinity measurements were performed on a BIAcore 2000 instrument (BIAcore, Uppsala, Sweden). To measure the biochemical activity of leptin as well as its PASylated versions, a mouse leptin receptor Fc chimera (R&D Systems, Minneapolis, MN) was immobilized in 10 mM Na-acetate pH 5.5 at a concentration of 5 μg/mL on a CMD 2D sensorchip (Xantec,

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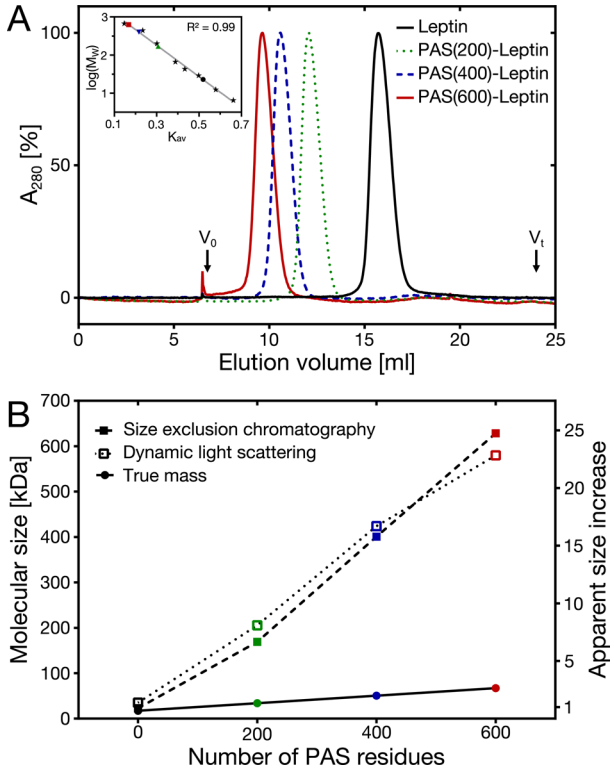

Figure 2. Size measurements for PASylated leptin versions. The effect of the genetic fusion of leptin with the conformationally disordered PAS polypeptide was investigated by analytical size exclusion chromatography (SEC) and dynamic light scattering (DLS). (A) SEC in the presence of phosphate-buffered saline (PBS) resulted in a single peak with decreasing elution volume for PAS fusions with increasing number of amino acid residues. Comparison with a half-logarithmic calibration curve (inset) led to the apparent molecular masses listed in Table 1. (B) Absolute and relative molecular sizes determined by both SEC and DLS measurements in the presence of PBS vs the length of the PAS tag.

Düsseldorf, Germany) using an amine coupling kit (GE Healthcare), resulting in a surface density of approximately 630 resonance units (ΔRU). The leptin versions were injected in appropriate concentration series (1−64 nM) using PBS supplemented with 0.05% (v/v) Tween 20 (PBS/T) as running buffer. Complex formation was observed at a continuous flow rate of 25 μL·min^{-1}, and the kinetic parameters were determined by data fitting to a Langmuir binding model for bimolecular complex formation using BIAevaluation software. Standard errors (SE) were calculated with the help of the software as previously described.[55] The sensorgrams were corrected by double subtraction of the corresponding signals measured for the in-line control blank channel and an averaged baseline determined from several buffer blank injections.[56] Chip regeneration between measurement cycles was achieved by applying a pulse of 10 mM glycine/HCl pH 2.2.

2.7. Cell Culture Experiments. HEK293 cells were transiently co-transfected with three different plasmids encoding the murine leptin receptor as well as reporter genes: (a) LepRb pcDNA3.1/Zeo (long form of murine leptin receptor; kindly provided by Dr. C. Bjorbaek); (b) pAH32 (STAT3-responsive *Photinus* luciferase reporter gene; kindly provided by Dr. C. Bjorbaek); (c) phRG-b (constitutive *Renilla* luciferase expression vector; Promega, Mannheim, Germany). Initially, HEK293 cells were cultured in DMEM (Sigma-Aldrich) containing 10% (v/v) FBS (Biochrom, Berlin, Germany) and 200 U/mL penicillin (1600 U/mg; Carl Roth, Karlsruhe, Germany). One day prior to transfection, cells from one 10 cm dish (Sarstedt, Nümbrecht, Germany) were split 1:5 and seeded onto new 10 cm dishes using 10 mL of medium. For calcium phosphate transfection, 5 μg of each plasmid DNA was mixed with 62 μL of 2.5 M $CaCl_2$ and adjusted to a volume of 500 μL with nuclease-free water, then mixed with an equal volume of 2×HBS (42 mM HEPES/NaOH, 2 mM Na_2HPO_4, 300 mM NaCl, 10 mM KCl, 10 mM Dextrose, pH 7.1) under vortexing and added to the cells of one dish. Twenty-four hours after transfection, cells were transferred from the 10 cm dish to a poly-D-lysine coated 48-well culture plate. 48 h post transfection, cells were incubated for 18 h with the different leptin versions, which were added to the culture medium at varying concentrations (0.05−500 nM). After that, cells were washed with PBS and treated with passive lysis buffer (Promega) for 20 min at room temperature.

The total cell lysates were combined with dual luciferase assay reagents (Promega), and bioluminescence was quantified according to the manufacturer's instructions in a Sirius luminometer (Berthold Technologies, Bad Wildbad, Germany). At least four independent measurements, each in duplicate, were performed for each leptin version. Induced *Photinus* luciferase activities were normalized to those of the constitutively expressed *Renilla* enzyme. Average of the normalized luminescence intensities (I) was plotted against the applied sample concentration (C) and fitted according to a one-site competition sigmoidal dose−response curve using Prism4 software (GraphPad Software, La Jolla, CA) to yield the half-maximally effective concentration (EC_{50}) and the respective standard error (SE):

$$I = I_{min} + \frac{I_{max} - I_{min}}{1 + 10^{\log(EC_{50}/C)}}$$

2.8. Animal Studies. All animal experiments were performed with permission from the district government of Upper Bavaria (permission number 55.2.1.54-2532-183-11). Mice were housed in a specific-pathogen free animal facility under controlled conditions of relative humidity (55%), temperature (22 °C), and light/dark cycles (12 h/12 h).

For pharmacokinetic (PK) studies, 8−10 week old male or female C57BL/6J mice with an average body weight of 24.7 or 19.1 g, respectively, were injected intraperitoneally (ip) with purified solutions of leptin or its PASylated versions in PBS (<10 endotoxin units (EU) per injection). All mice per group ($N = 3 \times 3$ for each test protein) received a dose of 287 nmol·kg^{-1} body weight (bw), typically 150 μL of an appropriately concentrated protein solution. Blood samples were collected from three groups (I, II, III), each with three animals, as follows: (group I) 15 min, 2 h, 6 h, 24 h; (group II) 30 min, 3 h, 36 h, 48 h; (group III) 1 h, 4 h, 8 h, 12 h. From each blood sample, the plasma was prepared by centrifugation at 14,000 rpm for 20 min at 4 °C and stored at −20 °C. To determine the PK of each test protein, the plasma concentration values, as determined from ELISA measurements (see below), were plotted against time post injection and numerically fitted using WinNonlin v. 6.1 software (Pharsight, St. Louis, MO), assuming a one-compartment model (corresponding to the Bateman function). Data are reported with standard errors (SE).

Pharmacodynamic (PD) studies were performed with 10 week old male C57BL/6J mice with an average initial body

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weight of 22.9 g (N = 8). Continuous monitoring of body weight and food consumption was enabled by placing mice in LabMaster phenotyping devices (TSE systems, Bad Homburg, Germany). Therefore, mice were housed individually in type III cages (820 cm^2) with food and water *ad libitum*. Baseline values for body weight and food intake were determined after repeated ip injections of vehicle (PBS), 1 h before lights off, on 3 consecutive days prior to a single application of leptin (287 $nmol \cdot kg^{-1}$ bw), SLMA, or their PASylated versions on "day 0". Mice received daily vehicle injections until the end of the experiment to expose them to the same handling procedure during the entire feeding study. Food intake and body weight trajectories were statistically analyzed by repeated measures two-way ANOVA, with time and leptin version as independent variables, and subsequent Holm−Sidak post hoc comparison using SigmaPlot v. 12.5 software (Systat, Erkrath, Germany). $P < 0.05$ was considered statistically significant. Error bars indicate standard deviation (SD). Area under the curve (AUC) was calculated to integrate amplitude and duration of the effect of leptin administration on body weight into one value.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA). Leptin and its PASylated versions in the plasma samples from the mouse PK study were quantified with a Leptin Mouse ELISA Kit (Abcam, Cambridge, U.K.) using a microtiter plate coated with an anti-leptin antibody in combination with a biotinylated second anti-leptin antibody. To this end, the plasma samples were applied to the microtiter plate in dilution series in the buffer supplied, supplemented with up to 0.5% (v/v) mouse plasma from untreated animals (to maintain constant concentration of sample matrix), and incubated for 2 h. The wells were then washed five times with the buffer and incubated for 2 h with 50 μL of the biotinylated anti-leptin antibody. After washing five times, 50 μL of streptavidin horseradish peroxidase conjugate was added and incubated for 30 min. The wells were washed five times, and then 50 μL of the supplied chromogenic substrate was added and incubated for about 20 min. After addition of 50 μL of stop solution the absorption was measured at 450 nm using a SpectraMax 250 microtiter plate reader (Molecular Devices, Sunnyvale, CA). Concentrations of the test proteins in the plasma samples were quantified by comparison with standard curves determined in parallel under the same conditions for dilution series of the corresponding purified recombinant protein, indicating negligible background levels (as possibly arising from endogenous leptin).

2.10. Quantification of STAT3 Phosphorylation via Western Blotting. Mice from the PK study were sacrificed at the end of the experiment, and the hypothalami were prepared. To quantify both total STAT3 and phosphorylated STAT3 levels, ventral hypothalami were minced in lysis buffer (30 mM Tris/HCl pH 8.0, 2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 1% (w/v) DTT) supplemented with each 1% (w/v) protease and phosphatase inhibitor cocktails (#P8340, #P5726; Sigma-Aldrich), sonicated for 1 min and centrifuged at 16,000*g*, 4 °C for 15 min. Total protein concentrations were first quantified via Bradford assay (Roti-Quant; Carl Roth). Samples were adjusted to 65 μg of protein each and separated by SDS−PAGE under reducing conditions, electroblotted onto a nitrocellulose membrane (LI-COR Biosciences, Lincoln, NE), and then incubated with antibodies against total STAT3 and phospho-Tyr^{705}-STAT3, respectively (#9139, #9145; Cell Signaling, Cambridge, U.K.). Bands were stained with secondary antibodies conjugated to an infrared dye using the LI-COR Odyssey Infrared Imaging device. Densitometric analysis of Western blot signals was conducted with the Odyssey software v. 3.0 (LI-COR). Western blot data were statistically analyzed by two-way ANOVA with time and leptin version as independent variables and subsequent Holm−Sidak post hoc comparison using SigmaPlot. $P < 0.05$ was considered statistically significant. Error bars indicate standard deviation (SD).

3. RESULTS

3.1. Preparation of Recombinant Leptin Versions with Extended Plasma Half-Life Using PASylation. For bacterial biosynthesis of murine leptin and its PASylated versions, a series of plasmids based on the generic expression vector pASK75[57] were constructed (Figure 1). These encode leptin, preceded by a bacterial signal peptide, together with an N- or C-terminal His_6-tag as well as a short linker comprising a unique *Sap*I recognition site. This restriction site served for subsequent insertion of one or multiple PAS#1.2(200) gene cassettes—an improved version of the previously described PAS#1(200) cassette, coding for the same amino acid sequence—in the proper reading frame according to a published cloning strategy.[47]

Leptin fusion proteins, including the version without a PAS tag (the so-called unmodified recombinant protein), were initially produced by periplasmic secretion (to ensure formation of the structural disulfide bridge in an oxidizing milieu) in an *E. coli* K12 strain at the shake flask scale and purified via IMAC and SEC. Notably, in the case of C-terminally PASylated leptin versions a disulfide-linked dimer was predominantly formed, whereas the leptin versions with N-terminal PASylation yielded mainly monomeric protein. In fact, the single intrachain disulfide bridge of leptin connecting Cys^{96} with the last residue in the polypeptide chain, Cys^{146}, is known to be important for folding, stability, and biological activity.[29,58,59] Notably, a leptin dimer linked by a nonphysiological intermolecular disulfide bond was already described before.[29,58]

Generally, fusion of the PAS moiety to the N-terminus of leptin appeared preferable as this region is solvent exposed, not involved in receptor activation,[31,33,60] and more flexible according to the published crystal structure.[29] Indeed, N-terminal modification of leptin was previously employed for preparation of an Ig Fc fusion protein[37] and also for chemical PEGylation.[34] Consequently, our final design of the PASylated leptin fusion proteins investigated in this study comprised an N-terminal PAS polypeptide of varying length (preceded by the His_6-tag) and the mature murine adipokine (Figure 1A).

Preparative production of these leptin versions was performed via fermentation of *E. coli*, followed by extraction of the periplasmic proteins. Subsequent high quality purification was accomplished by His_6-tag affinity purification (IMAC), anion exchange chromatography (AEX), and a final size exclusion chromatography (SEC). AEX was essential for the depletion of certain proteolytic degradation products, apparently comprising the His_6-tag, the intact PAS polypeptide, and three, six, or 18 N-terminal residues of leptin. Isolation of the homogeneous full-length monomeric PASylated leptin—and separation from a minor disulfide-linked dimer species—were eventually achieved by SEC (Figure 1B). Finally, uniform protein composition was confirmed by ESI-TOF mass spectrometry (Figure 1C).

The unmodified recombinant leptin showed a size of approximately 17 kDa in SDS−PAGE, as expected (see

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Table 1. Molecular Parameters of PASylated Leptin Versions

	leptin	PAS(200)-leptin	PAS(400)-leptin	PAS(600)-leptin
		Molecular Size		
no. of residues	160	360	560	760
theor mass [Da]	17443.8	33962.2	50480.6	66998.9
mass measd by DLS [kDa]	35.2	205.6	424.0	579.8
mass measd by SEC [kDa]	22.9	169.0	400.9	628.4
		Receptor Binding		
K_D measd by SPR [nM]	0.6 ± 0.001	2.0 ± 0.005	3.3 ± 0.010	4.2 ± 0.013
k_{on} measd by SPR [$M^{-1}\cdot s^{-1}$]	$9.2 \times 10^5 \pm 1.1 \times 10^3$	$2.4 \times 10^5 \pm 3.9 \times 10^2$	$1.7 \times 10^5 \pm 4.4 \times 10^2$	$1.4 \times 10^5 \pm 3.5 \times 10^2$
k_{off} measd by SPR [s^{-1}]	$5.5 \times 10^{-4} \pm 6.4 \times 10^{-7}$	$4.9 \times 10^{-4} \pm 7.7 \times 10^{-7}$	$5.7 \times 10^{-4} \pm 9.4 \times 10^{-7}$	$5.8 \times 10^{-4} \pm 9.1 \times 10^{-7}$
EC_{50} measd in cell culture [nM]	0.8 ± 1.3	3.2 ± 1.3	5.7 ± 1.3	6.1 ± 1.2
		Pharmacokinetics		
plasma half-life $\tau^{\beta}_{1/2}$ [h]	0.43 ± 0.08 ♀	3.3 ± 0.4 ♀	7.0 ± 0.3 ♀	19.6 ± 2.4 ♀
	0.66 ± 0.03 ♂			19.6 ± 3.5 ♂
AUC [h·μM]	3.9 ± 0.7 ♀	5.4 ± 0.4 ♀	38.8 ± 2.4 ♀	218.8 ± 19.8 ♀
	2.4 ± 0.1 ♂			252.5 ± 30.3 ♂
CL [$mL\cdot h^{-1}\cdot kg^{-1}$]	72.4 ± 12.9 ♀	53.1 ± 3.8 ♀	7.4 ± 0.5 ♀	1.3 ± 0.1 ♀
	120.7 ± 2.5 ♂			1.1 ± 0.1 ♂
		Pharmacodynamics		
AUC STAT3 phosphorylation [h][a]	9.0 ♂	35.5 ♂	58.9 ♂	114.6 ♂
AUC body weight change [%·d][a]	−1.6 ♂	−10.1 ♂	−32.0 ♂	−45.0 ♂

[a]Area under the curve (AUC) is stated relative to the baseline.

Experimental Section), while exhibiting higher electrophoretic mobility in the oxidized state due to the disulfide cross-link, in line with earlier reports.[61] In contrast, the PASylated proteins exhibited strongly altered electrophoretic mobility and migrated significantly more slowly than predicted from their calculated masses (Figure 1B), with the following apparent sizes: ∼75 kDa for PAS(200)-leptin (calculated mass: 44.0 kDa), ∼180 kDa for PAS(400)-leptin (50.5 kDa), and ∼300 kDa for PAS(600)-leptin (67.0 kDa). A similar behavior was previously observed for both PASylated interferon and growth hormone and was explained by the weak interaction between the hydrophilic PAS polymer and SDS.[47]

3.2. Biophysical and Functional Characterization. Correct folding of the leptin versions was investigated using circular dichroism (CD) spectroscopy (Figure 1D). The spectrum for the unmodified recombinant protein showed an ellipticity maximum at 194 nm as well as the characteristic double dip minimum for α-helical proteins in the region of 210−220 nm,[62] very similar to the published far-UV CD spectrum of recombinant human leptin.[61] The secondary structure content was estimated as 54−56% α-helical. Notably, the spectra of the PASylated leptin versions showed an additional negative band at approximately 200 nm. This minimum became more pronounced with increasing length of the fused PAS polypeptide, thus indicating random coil conformation of the PAS moiety, in full agreement with previous observations.[47]

The effect of PASylation on the molecular size of leptin in physiological solution was studied by analytical SEC (Figure 2). Both the unmodified recombinant leptin and its N-terminally PASylated versions showed a single peak, confirming the monodisperse nature of each protein preparation. Interpolation of the SEC elution volume of the unmodified leptin versus a set of protein standards led to an apparent molecular size of 22.9 kDa, which is just slightly above the theoretical mass of 17.4 kDa. However, all PASylated versions showed much larger apparent molecular sizes of 169 kDa for PAS(200), 401 kDa for PAS(400), and 628 kDa for PAS(600) (Table 1). Compared with the much smaller actual molecular masses, this phenomenon clearly reveals the dramatically enlarged hydrodynamic volume of each fusion protein owing to the expanded random coil conformation of the PAS tag, correlating with its length. In fact, the PAS(600)-leptin version exhibited an almost 10-fold increased apparent size of 628 kDa compared with its calculated mass of 67 kDa.

A similar effect was observed in dynamic light scattering (DLS) experiments (Table 1; Figure 2B). In this case, the following molecular sizes were determined: 35 kDa for the unmodified leptin, 206 kDa for its PAS(200) fusion, 424 kDa for PAS(400)-leptin, and 580 kDa for PAS(600)-leptin. These values are in remarkable agreement with the SEC data (Figure 2B), hence confirming the anticipated biophysical effect on leptin of the expanded PAS random chain polymer[47] with two independent methods. Accordingly, PAS(600)-leptin showed a 3.7-fold increased diameter (18.4 vs 5.0 nm) and a 21-fold larger apparent molecular volume (604 vs 29 kDa) compared with the unmodified leptin (based on the averaged values from SEC and DLS).

Binding activity of the PASylated leptin versions to the leptin receptor was first investigated in real-time surface plasmon resonance (SPR) spectroscopy (Figure 3) using a LepRb-Fc fusion protein. The unmodified recombinant leptin showed a dissociation constant (K_D) of 0.6 nM, nicely matching the previously published value of 0.5 nM.[63] In comparison, the PASylated versions showed a slightly decreased receptor affinity, which was already apparent for the initial fusion with the PAS(200) tag while longer PAS lengths only caused minor additional effects (Table 1). Eventually, the PAS(600)-leptin fusion protein had a K_D value of 4.2 nM. This was mainly due to a decrease in the association rate (k_{on}) whereas the dissociation rate (k_{off}) of the PASylated leptin versions was mostly unaffected (Figure 3B).

The potency of PASylated leptin to effect intracellular LepRb signaling was assessed with HEK293 cells transiently expressing the long form of the murine leptin receptor, together with a STAT3-inducible *Photinus* luciferase (including constitutively

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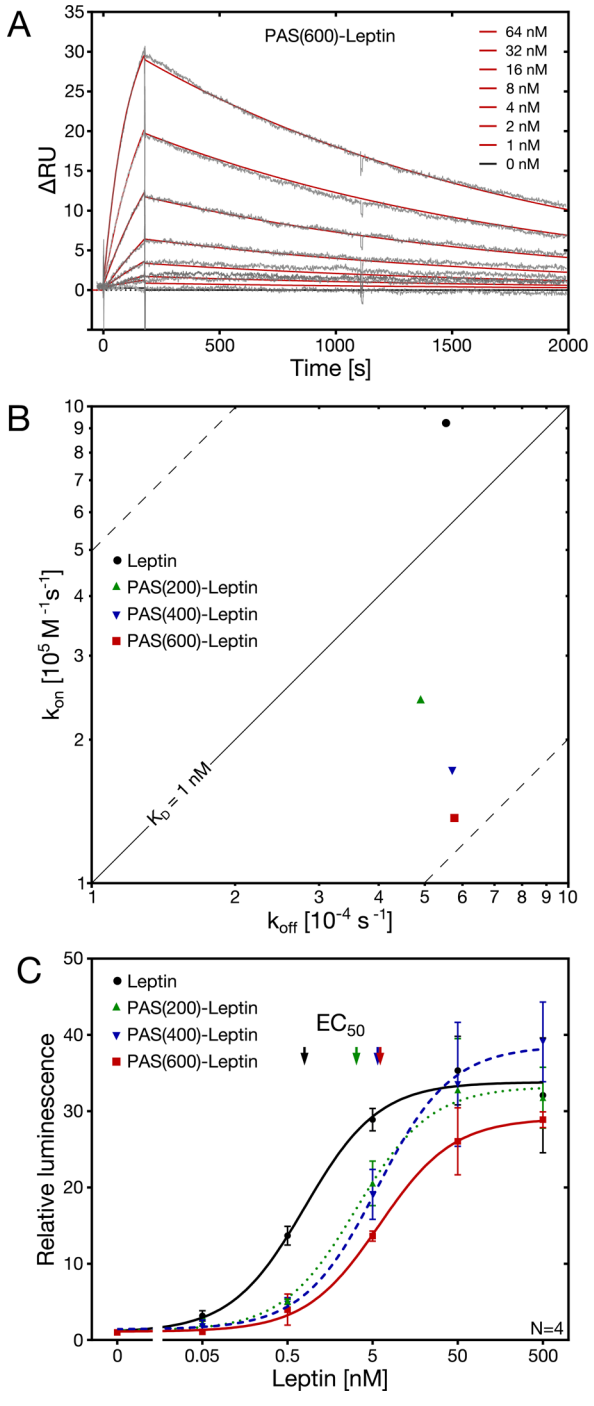

Figure 3. Investigation of receptor binding activity for PASylated leptin by real-time SPR analysis and in a cell culture reporter assay. (A) Dilution series of leptin versions from 64 to 1 nM were applied in PBS/T to a sensor chip charged with a murine LepRb-Fc fusion protein. Biacore sensorgrams were fitted to a 1:1 Langmuir binding model, and resulting dissociation constants and kinetic parameters are listed in Table 1. (B) Visualization of the parameters from plot A in a k_{on}/k_{off} plot. (C) Leptin versions were applied to HEK cells transiently co-transfected with an LepRb-expression vector and a STAT3-responsive *Photinus* luciferase gene. The leptin versions were applied in a dilution series in duplicate, and after incubation for 18 h luciferase activity was assayed (N = 4). Luminescence values (normalized against constitutively coexpressed *Renilla* luciferase) for each test protein were fitted to a sigmoidal dose−response curve; resulting EC_{50} values are listed in Table 1.

coexpressed *Renilla* luciferase for signal normalization). Like the unmodified leptin, all three PASylated versions were fully capable of triggering leptin receptor signaling as indicated by essentially unchanged signal amplitudes (Figure 3C). However, PASylation slightly raised the EC_{50} values for receptor activation, indicating a 7-fold decrease in apparent receptor affinity for the PAS(600)-leptin fusion protein, very similar to the effect observed before in the SPR measurements (Table 1).

3.3. Pharmacokinetic (PK) and Pharmacodynamic (PD) Analysis of PASylated Leptin. The plasma half-life of PASylated leptin was investigated in both female and male C57BL/6J mice (N = 9) after ip administration of 287 nmol·kg^{-1} bw purified protein by taking blood samples at time points from 15 min to 48 h post injection (p.i.) (Figure 4). The time-dependent concentration of each test protein was measured by a leptin-specific sandwich ELISA. For both the unmodified protein and the PASylated leptin versions the plasma concentration profile exhibited the expected pattern for ip administration: a biphasic curve with an initial distribution phase followed by a terminal elimination phase.[64]

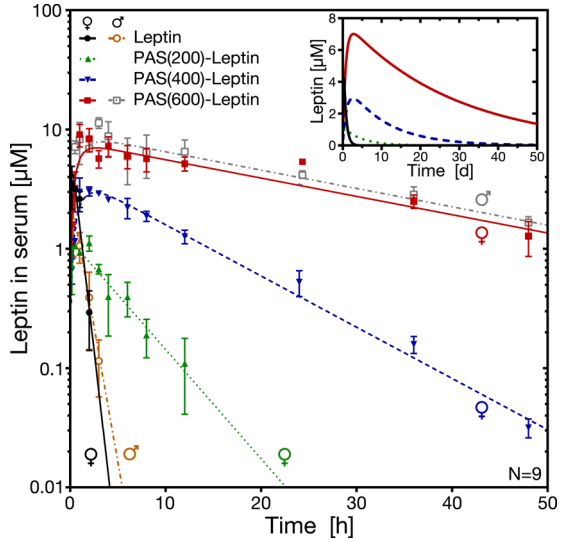

Figure 4. PK of recombinant murine leptin and its PASylated versions in mouse plasma. C57BL6/J mice (N = 9) were injected ip with a protein dose of 287 nmol·kg^{-1} bw. The leptin concentration in plasma was quantified in a sandwich ELISA with antibodies directed against the adipokine, using appropriate calibration curves, and data were plotted in a semilogarithmic fashion against the time of sampling post injection. The PK profiles show distinct resorption and elimination phases; deduced terminal half-lives, clearance, and AUC parameters are listed in Table 1. Plot of the same data in a linear fashion (inset) illustrates the strongly increased AUC, indicating high bioavailability of PASylated leptins.

Curve fitting with WinNonlin software revealed a rather short terminal plasma half-life ($\tau^{\beta}_{1/2}$) of 26 ± 5 min for the unmodified leptin, which was considerably prolonged by PASylation from 3.3 ± 0.4 h for PAS(200)-leptin over 7.4 ± 0.5 h for PAS(400)-leptin to 19.6 ± 2.4 h for PAS(600)-leptin (in the female mice). Thus, fusion with a PAS(600) polypeptide led to extension of the elimination half-life of leptin by a factor of 46. The strong effect of PASylation on leptin PK was reflected by the values obtained for clearance (CL), with a decrease from 72 mL·h^{-1}·kg^{-1} for the unmodified leptin to 1.3 mL·h^{-1}·kg^{-1} for the PAS(600) fusion protein. The strongly retarded kidney filtration led to an almost 60-fold

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increase in the area under the curve (AUC), from 3.9 to 218 h·μM. Interestingly, the PK parameters determined for male mice (N = 9) were highly similar (see Table 1).

At the end of the PK study, female mice were killed and phosphorylation of the STAT3 signal transducer, which represents a major intracellular signaling event of leptin,[5,6] was investigated by quantitative Western blot analysis of hypothalamic protein extracts (Figure 5). Twelve hours after

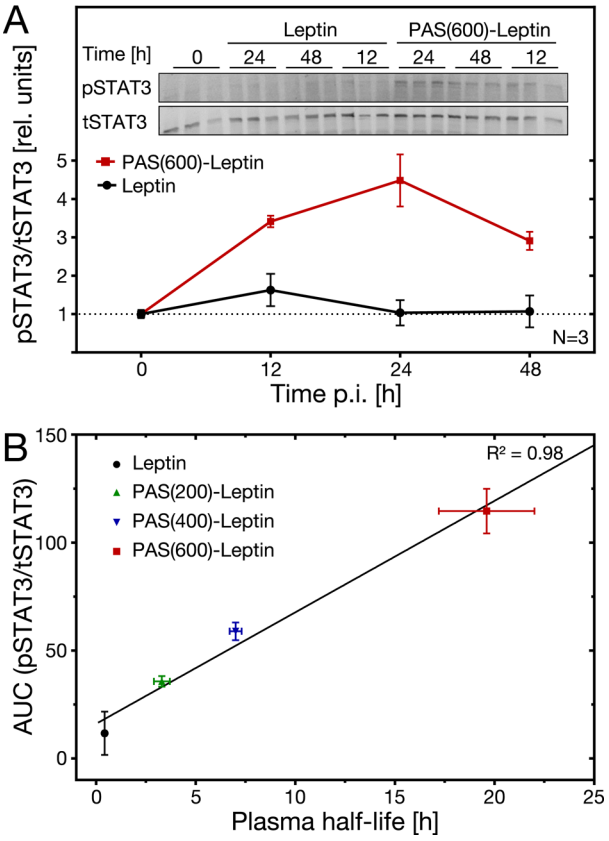

Figure 5. Hypothalamic receptor signaling of PASylated murine leptin versions. The PD of unmodified recombinant leptin and its PASylated versions was investigated in lean C57BL/6J mice by quantifying STAT3 phosphorylation in the hypothalamus after ip injection of 287 nmol·kg^{-1} bw for each test protein. (A) Downstream signaling in neurons of the hypothalamus was examined by immunostaining of phosphorylated (pSTAT3) and total STAT3 (tSTAT3) on a Western blot. For determination of basal STAT3 phosphorylation, a group of mice that had only received PBS injections was included (samples from these mice are labeled on the Western blot as "0"). The bands were quantified by densitometry (N = 3), and the change in the pSTAT3/tSTAT3 ratio relative to the mock samples is shown in the lower panel. (B) The area under the curve from the test proteins shown in panel A as well as some other PASylated leptin versions (see Table 1) was plotted against the corresponding terminal plasma half-life (N = 3), revealing a linear correlation.

injection, mice treated with the unmodified leptin showed an up to 1.5-fold increase in phosphorylation of STAT3 and returned to baseline values at 24 and 48 h. While this initial signal was hardly statistically significant, it reflects the decay of the early phosphorylation effect of the native adipokine, which is known to reach its maximum already after around 1 h.[65] In contrast, mice treated with PAS(600)-leptin showed a much later maximum, at around 24 h p.i., with significantly augmented and sustained STAT3 phosphorylation (Figure 5A). In fact, the relative pSTAT3 level was ~3.5-fold and ~4.5-fold increased (over baseline) at 12 and 24 h, respectively (p < 0.001). The prolonged response to the PASylated protein was particularly evident as even 48 h p.i. STAT3 phosphorylation was still ~3-fold enhanced (p < 0.001). PAS(200)-leptin and PAS(400)-leptin caused intermediate responses (see Table 1). Notably, the length-dependent effects of PASylation were characterized by a strong correlation between PK and PD (Figure 5B), suggesting that longer PAS tags may even lead to a superior response.

The systemic effects of single ip injections of the different PASylated leptin versions (287 nmol·kg^{-1} bw) on daily food consumption and body weight progression were assessed in another cohort of male C57BL/6J mice (N = 8), also in comparison with the leptin antagonist SMLA[34] and its PAS(600) fusion (Figure 6). The strongest reduction in food

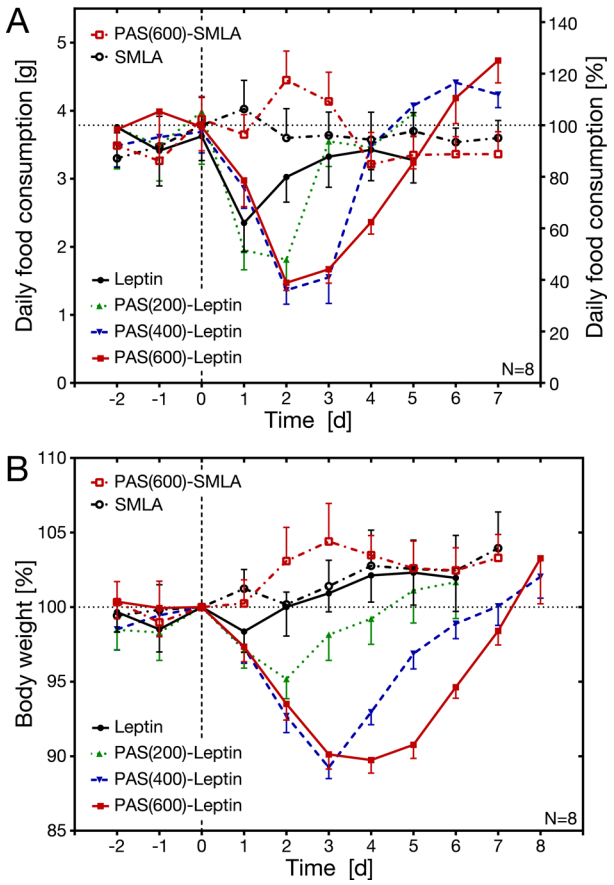

Figure 6. Physiological effects of PASylated murine leptin versions. The PD of unmodified recombinant leptin, as well as the superactive mouse leptin antagonist (SMLA), and its PASylated versions was investigated in mice with regard to daily food consumption (A) and body weight change (B) using a phenotyping device (N = 8). Lean C57BL/6J mice were injected with a single ip dose of 287 nmol·kg^{-1} bw test protein on day 0.

intake was observed after administration of PAS(600)-leptin (Figure 6A), with a maximal decrease by 60% on day 2 and day 3 (p < 0.001), whereas the unmodified leptin only caused a modest decrease by 35% on day 1 (p < 0.001). In the case of PAS(600)-leptin, even on day 4 p.i. food intake was still reduced by 35% compared to the preinjection level (p < 0.001), thus demonstrating its sustained efficacy. The development of body weight showed the following pattern (Figure 6B): a 10%

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loss of body weight was observed 3 days after treatment with PAS(600)-leptin and lasted until day 5 ($p < 0.001$), whereas merely a very modest body weight loss of maximally 2% was observed for the unmodified leptin at the end of day 1 ($p < 0.05$). The PAS(200) and PAS(400) fusions caused intermediate effects (cf. Figure 6), resulting in statistically significant body weight differences compared with the unmodified leptin at least from day 2 to 4 ($p < 0.001$).

Interestingly, administration of SMLA and PAS(600)-SMLA led to the opposite effects on both food intake and body weight progression. Unmodified SMLA caused a weak elevation of mean food consumption and body weight on day 1 after injection (Figure 6A,B) without statistical significance ($p = 0.864$ and $p = 0.116$, respectively). On the other hand, PASylation of SMLA boosted its known orexigenic activity as demonstrated by a significantly increased food intake on day 2 and a 4% increase in body weight 3 days p.i. ($p < 0.05$ and $p < 0.001$, respectively).

The cumulated PD effects monitored over the duration of the experiment were further compared using the area under the curve (AUC) for the relative body weight change (Table 1). As a result, the integrated loss of body weight was 28-fold stronger for PAS(600)-leptin than for the unmodified recombinant protein. Moreover, the time span for which the body weight significantly dropped below baseline after the single drug injection was extended from 1 day for the unmodified leptin to 6 days for its fusion with the PAS(600) polypeptide, in line with the successful extension of the plasma half-life of this PASylated adipokine.

4. DISCUSSION

Upon its discovery in 1994,[1] leptin gained immediate attention as a potential therapeutic agent for treatment of human metabolic diseases.[17] However, its poor solubility and, importantly, the very short circulation half-life affected clinical drug development, necessitating frequent administration in high doses to achieve at least modest pharmacological effects and, thus, leading to injection site events. PASylation, which can be accomplished by the convenient fusion of PAS gene cassettes of tunable length with the open reading frame of a therapeutic protein or peptide, may offer a promising opportunity to convert leptin into a viable biotherapeutic with prolonged and enhanced action.

Indeed, attachment of 600 PAS residues to the N-terminus of murine leptin led to a 46-fold increase in terminal plasma half-life after ip injection into mice and to a 56-fold increase in the pharmacokinetic AUC. These effects are comparable to those described before for other PASylated therapeutic proteins of similar size.[47] The secretory production in *E. coli* and subsequent purification of homogeneous PASylated leptin in a functional state was achieved for all investigated versions, independent of the length of the fused PAS polypeptide, i.e., 200, 400, or 600 residues. It seems feasible that even longer PAS-leptin fusion proteins may be prepared if considered useful for therapeutic application.

The strong increase in molecular size conferred to leptin by fusion with the PAS moiety led to an expanded hydrodynamic volume and lowered the filtration coefficient in the kidney, which is the main organ for elimination of non-glycosylated small to midsize proteins from blood.[66] Native leptin has a mass of 16.0 kDa, equivalent to a globular diameter ⌀ = 3.9 nm, which is in agreement with the crystal structure of this protein, revealing main dimensions of 2.0 × 2.5 × 4.5 nm^3, that is ⌀ = 3.5 nm on average.[29] Renal filtration is mainly determined by the pore size of the glomerular basal membrane, which is around 4 nm,[67] such that proteins with masses below 70 kDa (⌀ = 7.3 nm) are increasingly eliminated from the blood plasma, approaching a sieving coefficient of 1 for ≤5 kDa. Proteins with larger molecular size progressively resist glomerular filtration and, consequently, show longer plasma half-life.[48]

The present data reveal an essentially linear increase in apparent molecular size with growing length of the PAS polypeptide for the investigated PAS-leptin fusion proteins based on experimental evidence from two independent methods, SEC and DLS, which is also in line with published data for other PASylated proteins.[47] Comparison of the SEC measurements with literature data[40] for a PEGylated leptin antagonist (having essentially the same molecular size) indicates that attachment of a PAS(200) polypeptide exerts an equivalent apparent size effect as a single 20 kDa PEG chain (169 [206 from DLS] vs 220 kDa) while PAS(400)-leptin shows a similar size as the conjugate with a single 40 kDa or a branched 2 × 20 kDa PEG (401 vs 400 kDa for both PEG versions). Notably, PAS(600)-leptin shows a larger hydrodynamic volume than any of the PEGylated leptin antagonists that were previously investigated by gel filtration.

Retention of high receptor-binding activity of a modified pharmaceutically active protein is a prerequisite for therapeutic development. Binding of the unmodified recombinant leptin to its receptor was investigated using real-time SPR spectroscopy (BIAcore), resulting in a K_D value of 0.6 nM, which is very close to the previously published number of 0.5 nM for a similar recombinant version of murine leptin.[63] SPR analysis also allowed determination of association and dissociation kinetics. Interestingly, the association rate constant (k_{on}) of the PASylated leptin versions was slightly reduced compared to the unmodified protein while the dissociation rate (k_{off}) was almost unchanged for all measured versions. This observation indicates that PASylation slightly slows target binding, possibly due to retarded diffusion through the hydrogel matrix of the sensorchip or to sterical hindrance, as also described for PEGylated proteins before,[68] but does not affect the kinetic stability of the cytokine−receptor complex.

In addition, leptin receptor-binding activity was investigated in cells co-transfected with the LepRb cDNA in conjunction with luciferase reporter genes. The half-maximally effective concentration found for the unmodified murine leptin with the luciferase assay (EC_{50} = 0.8 nM) was slightly higher than the value determined for human leptin and its cognate receptor (EC_{50} = 0.15 nM) in a comparable assay,[69] which is also in agreement with the 2-fold stronger binding of human leptin to its receptor as measured by SPR spectrometry.[63] Fusion of murine leptin to PAS polypeptides of various lengths caused some reduction in affinity, which was 7-fold for the PAS(600)-leptin according to both the cell-based luciferase assay and SPR analysis.

The major loss was caused by the initial PASylation with 200 residues whereas subsequent extension of the PAS tag only had minor effects, suggesting that modifications to the N-terminus of the cytokine can generally interfere with receptor binding. Similar observations were made before with PASylated human IFNα2b[47] and also in a study on the comparison of different linkers for N-terminal PEGylation of human growth hormone.[70] However, even stronger negative effects on receptor-binding activity were described for other approaches

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to prolong the plasma half-life of leptin. For example, a 6- to 26-fold[40] loss in affinity was determined after chemical PEGylation of leptin while a 2- to 4-fold affinity reduction was observed after genetic conjugation (again at the N-terminus) with an Ig Fc fragment,[37] despite the gain in avidity due to the bivalent nature of this type of fusion protein. In comparison, PASylation of leptin maintains a relatively high receptor-binding activity.

The effect of PASylation on the PK profile of leptin was investigated in mice after ip injection of the different versions. The terminal plasma half-life of the unmodified recombinant leptin was determined as 26−40 min, which is expected for a protein hormone of this size and is in agreement with literature values.[36,71] Compared with that, PASylation led to a marked increase in plasma half-life, up to 19.6 h for a PAS tag of 600 residues, i.e., a factor 46, thus demonstrating the strong effect of the expanded molecular volume on kidney clearance (cf. Table 1). For the Fc-leptin fusion protein already mentioned above a shorter half-life of 8.8 h after iv injection in mice was reported.[37] In comparison, chemical coupling of two 20 kDa PEG chains (to the N-terminus and to a specifically introduced Cys side chain) resulted in half-lives of approximately 19 h (iv) and 16 h (sc) in mice[72] whereas a leptin conjugate with 40 kDa branched PEG showed a 35-fold increased half-life.[40] Our PAS(600)-leptin fusion reveals at least the same improved PK behavior while retaining high receptor affinity and avoiding PEG vacuole formation.[72] Taken together, due to the longer plasma half-life the AUC of PASylated leptin is up to 56-fold increased, which leads to a much higher tissue exposure to the drug and clearly overcompensates the small reduction in binding activity compared with the unmodified protein.

This was finally demonstrated in a PD study in mice. Pharmacological efficacy of the PASylated leptin versions was first investigated for lean C57BL/6J mice by quantifying the level of STAT3 phosphorylation in the hypothalamus after single ip injection. In this experiment, a drastically increased effect was observed for the PASylated leptins. With growing length of the PAS tag the point of highest STAT3 activation was shifted from a known value of around 1 h for native leptin[65] to 24 h for the PAS(600) fusion protein. Both the PAS(400) fusion and the PAS(600) fusion led to significantly enhanced STAT3 phosphorylation even after 48 h while, in the case of the unmodified recombinant leptin, baseline level was reached already at 24 h. These observations not only demonstrate a prolonged and generally enhanced action, which can be explained by the longer plasma half-life and much higher AUC of the circulating drug (cf. Table 1), but also indicate that PASylation does not impair activation of leptin receptor signaling in the hypothalamus. Whether PASylated leptin penetrates the blood−brain barrier as intact fusion protein or may become degraded during transcytosis, releasing shorter fragments comprising intact leptin that effect STAT phosphorylation, remains to be clarified in future studies.

A similarly boosted PD effect was seen on reduction of food consumption and body weight loss in another mouse experiment after single ip injection of the PASylated leptin versions. With increasing length of the PAS tag, both the reduction in daily food intake and the duration of this response were considerably augmented in comparison with the unmodified adipokine, reaching a maximum of 60% less food consumption and a detectable pharmacological effect up to 5 days for PAS(600)-leptin. As expected, the antagonist SMLA showed the opposite effects, characterized by increased food intake and, consequently, gain in body weight, which were both clearly enhanced again for the PASylated compound.

The latter finding confirms the crucial role of the protein component for the pharmacological activity of the investigated PASylated leptin versions and rules out the possibility that the PAS moiety itself may have caused reduced food intake of treated animals. In the case of SMLA, the absolute size of both (reverse) effects was less drastic, which can be explained by the different mode of action: SLMA not only blocks binding to the neuronal leptin receptor in a competitive manner but also inhibits the import of endogenous leptin into the hypothalamus by competing with its receptor-mediated transport across the blood−brain barrier.[40,73]

Suppression of leptin signaling by means of specific and highly potent leptin receptor antagonists such as SMLA[34] might be a useful research approach for identifying further relevant leptin-dependent physiological pathways. Moreover, leptin antagonistic compounds constitute putative drug candidates themselves. In fact, beside its beneficial metabolic activities, leptin can mediate undesired pathophysiological effects like elevated immune responses in autoimmune disorders, tumor growth, cardiovascular dysfunction, and osteoarthritis. Therefore, antagonistic inhibition of leptin signaling may offer a potential therapeutic strategy in these indications.[74]

Generally, engineered variants of leptin with enhanced agonistic or antagonistic activity are interesting from both a scientific and a therapeutic perspective. It is noteworthy that the PASylated versions of both types of proteins developed in this study show not only prolonged but also stronger pharmacological effects in mice, which makes them valuable reagents to study the physiological role of leptin *in vivo* as well as its precise mechanism of receptor signaling in the central nervous system. Furthermore, PAS sequences are not immunogenic as shown in several previous studies[47,49] and also for leptin (after multiple repeated administration) in a mouse disease model for genetic leptin deficiency ($Lep^{ob/ob}$) which will be published elsewhere. Based on such future studies, a PASylated human leptin may turn out as a promising drug candidate for clinical therapy.

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Notes

The authors declare the following competing financial interest(s): A.S. is a managing director and A.S. and M.S. are shareholders of XL-protein GmbH.

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REFERENCES

(1) Zhang, Y.; Proenca, R.; Maffei, M.; Barone, M.; Leopold, L.; Friedman, J. M. Positional cloning of the mouse obese gene and its human homologue. *Nature* **1994**, *372*, 425−432.

(2) Maffei, M.; Halaas, J.; Ravussin, E.; Pratley, R. E.; Lee, G. H.; Zhang, Y.; Fei, H.; Kim, S.; Lallone, R.; Ranganathan, S.; et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat. Med.* **1995**, *1*, 1155−1161.

(3) Friedman, J. M. Leptin at 14 y of age: an ongoing story. *Am. J. Clin. Nutr.* **2009**, *89*, 973S−979S.

(4) Banks, W. A.; Kastin, A. J.; Huang, W.; Jaspan, J. B.; Maness, L. M. Leptin enters the brain by a saturable system independent of insulin. *Peptides* **1996**, *17*, 305−311.

(5) Vaisse, C.; Halaas, J. L.; Horvath, C. M.; Darnell, J. E., Jr.; Stoffel, M.; Friedman, J. M. Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nat. Genet.* **1996**, *14*, 95−97.

(6) Ghilardi, N.; Skoda, R. C. The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. *Mol. Endocrinol.* **1997**, *11*, 393−399.

(7) Varela, L.; Horvath, T. L. Leptin and insulin pathways in POMC and AgRP neurons that modulate energy balance and glucose homeostasis. *EMBO Rep.* **2012**, *13*, 1079−1086.

(8) Farooqi, I. S.; O'Rahilly, S. Leptin: a pivotal regulator of human energy homeostasis. *Am. J. Clin. Nutr.* **2009**, *89*, 980S−984S.

(9) Paz-Filho, G.; Wong, M. L.; Licinio, J. Ten years of leptin replacement therapy. *Obes. Rev.* **2011**, *12*, e315−e323.

(10) Ingalls, A. M.; Dickie, M. M.; Snell, G. D. Obese, a new mutation in the house mouse. *J. Hered.* **1950**, *41*, 317−318.

(11) Montague, C. T.; Farooqi, I. S.; Whitehead, J. P.; Soos, M. A.; Rau, H.; Wareham, N. J.; Sewter, C. P.; Digby, J. E.; Mohammed, S. N.; Hurst, J. A.; Cheetham, C. H.; Earley, A. R.; Barnett, A. H.; Prins, J. B.; O'Rahilly, S. Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* **1997**, *387*, 903−908.

(12) D'Souza, A. M.; Asadi, A.; Johnson, J. D.; Covey, S. D.; Kieffer, T. J. Leptin deficiency in rats results in hyperinsulinemia and impaired glucose homeostasis. *Endocrinology* **2014**, *155*, 1268−1279.

(13) Halaas, J. L.; Gajiwala, K. S.; Maffei, M.; Cohen, S. L.; Chait, B. T.; Rabinowitz, D.; Lallone, R. L.; Burley, S. K.; Friedman, J. M. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* **1995**, *269*, 543−546.

(14) Farooqi, I. S.; Jebb, S. A.; Langmack, G.; Lawrence, E.; Cheetham, C. H.; Prentice, A. M.; Hughes, I. A.; McCamish, M. A.; O'Rahilly, S. Effects of recombinant leptin therapy in a child with congenital leptin deficiency. *N. Engl. J. Med.* **1999**, *341*, 879−884.

(15) Licinio, J.; Caglayan, S.; Ozata, M.; Yildiz, B. O.; de Miranda, P. B.; O'Kirwan, F.; Whitby, R.; Liang, L.; Cohen, P.; Bhasin, S.; Krauss, R. M.; Veldhuis, J. D.; Wagner, A. J.; DePaoli, A. M.; McCann, S. M.; Wong, M. L. Phenotypic effects of leptin replacement on morbid obesity, diabetes mellitus, hypogonadism, and behavior in leptin-deficient adults. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4531−4536.

(16) Wabitsch, M.; Funcke, J. B.; Lennerz, B.; Kuhnle-Krahl, U.; Lahr, G.; Debatin, K. M.; Vatter, P.; Gierschik, P.; Moepps, B.; Fischer-Posovszky, P. Biologically inactive leptin and early-onset extreme obesity. *N. Engl. J. Med.* **2015**, *372*, 48−54.

(17) Friedman, J. M.; Halaas, J. L. Leptin and the regulation of body weight in mammals. *Nature* **1998**, *395*, 763−770.

(18) Ravussin, E.; Smith, S. R.; Mitchell, J. A.; Shringarpure, R.; Shan, K.; Maier, H.; Koda, J. E.; Weyer, C. Enhanced weight loss with pramlintide/metreleptin: an integrated neurohormonal approach to obesity pharmacotherapy. *Obesity (Silver Spring)* **2009**, *17*, 1736−1743.

(19) Heymsfield, S. B.; Greenberg, A. S.; Fujioka, K.; Dixon, R. M.; Kushner, R.; Hunt, T.; Lubina, J. A.; Patane, J.; Self, B.; Hunt, P.; McCamish, M. Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *J. Am. Med. Assoc.* **1999**, *282*, 1568−1575.

(20) Münzberg, H. Leptin-signaling pathways and leptin resistance. *Forum Nutr.* **2010**, *63*, 123−132.

(21) Osborn, O.; Sanchez-Alavez, M.; Brownell, S. E.; Ross, B.; Klaus, J.; Dubins, J.; Beutler, B.; Conti, B.; Bartfai, T. Metabolic characterization of a mouse deficient in all known leptin receptor isoforms. *Cell. Mol. Neurobiol.* **2010**, *30*, 23−33.

(22) Tups, A.; Ellis, C.; Moar, K. M.; Logie, T. J.; Adam, C. L.; Mercer, J. G.; Klingenspor, M. Photoperiodic regulation of leptin sensitivity in the Siberian hamster, *Phodopus sungorus*, is reflected in arcuate nucleus SOCS-3 (suppressor of cytokine signaling) gene expression. *Endocrinology* **2004**, *145*, 1185−1193.

(23) Grattan, D. R.; Ladyman, S. R.; Augustine, R. A. Hormonal induction of leptin resistance during pregnancy. *Physiol. Behav.* **2007**, *91*, 366−374.

(24) Wang, M. Y.; Chen, L.; Clark, G. O.; Lee, Y.; Stevens, R. D.; Ilkayeva, O. R.; Wenner, B. R.; Bain, J. R.; Charron, M. J.; Newgard, C. B.; Unger, R. H. Leptin therapy in insulin-deficient type I diabetes. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 4813−4819.

(25) Coppari, R.; Bjorbaek, C. Leptin revisited: its mechanism of action and potential for treating diabetes. *Nat. Rev. Drug Discovery* **2012**, *11*, 692−708.

(26) Chou, S. H.; Chamberland, J. P.; Liu, X.; Matarese, G.; Gao, C.; Stefanakis, R.; Brinkoetter, M. T.; Gong, H.; Arampatzi, K.; Mantzoros, C. S. Leptin is an effective treatment for hypothalamic amenorrhea. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 6585−6590.

(27) Oral, E. A.; Simha, V.; Ruiz, E.; Andewelt, A.; Premkumar, A.; Snell, P.; Wagner, A. J.; DePaoli, A. M.; Reitman, M. L.; Taylor, S. I.; Gorden, P.; Garg, A. Leptin-replacement therapy for lipodystrophy. *N. Engl. J. Med.* **2002**, *346*, 570−578.

(28) Chou, K.; Perry, C. M. Metreleptin: first global approval. *Drugs* **2013**, *73*, 989−997.

(29) Zhang, F.; Basinski, M. B.; Beals, J. M.; Briggs, S. L.; Churgay, L. M.; Clawson, D. K.; DiMarchi, R. D.; Furman, T. C.; Hale, J. E.; Hsiung, H. M.; Schoner, B. E.; Smith, D. P.; Zhang, X. Y.; Wery, J. P.; Schevitz, R. W. Crystal structure of the obese protein leptin-E100. *Nature* **1997**, *387*, 206−209.

(30) Bravo, J.; Heath, J. K. Receptor recognition by gp130 cytokines. *EMBO J.* **2000**, *19*, 2399−2411.

(31) Mancour, L. V.; Daghestani, H. N.; Dutta, S.; Westfield, G. H.; Schilling, J.; Oleskie, A. N.; Herbstman, J. F.; Chou, S. Z.; Skiniotis, G. Ligand-induced architecture of the leptin receptor signaling complex. *Mol. Cell* **2012**, *48*, 655−661.

(32) Fong, T. M.; Huang, R. R.; Tota, M. R.; Mao, C.; Smith, T.; Varnerin, J.; Karpitskiy, V. V.; Krause, J. E.; Van der Ploeg, L. H. Localization of leptin binding domain in the leptin receptor. *Mol. Pharmacol.* **1998**, *53*, 234−240.

(33) Peelman, F.; Van Beneden, K.; Zabeau, L.; Iserentant, H.; Ulrichts, P.; Defeau, D.; Verhee, A.; Catteeuw, D.; Elewaut, D.; Tavernier, J. Mapping of the leptin binding sites and design of a leptin antagonist. *J. Biol. Chem.* **2004**, *279*, 41038−41046.

(34) Shpilman, M.; Niv-Spector, L.; Katz, M.; Varol, C.; Solomon, G.; Ayalon-Soffer, M.; Boder, E.; Halpern, Z.; Elinav, E.; Gertler, A. Development and characterization of high affinity leptins and leptin antagonists. *J. Biol. Chem.* **2011**, *286*, 4429−4442.

(35) Cumin, F.; Baum, H. P.; de Gasparo, M.; Levens, N. Removal of endogenous leptin from the circulation by the kidney. *Int. J. Obes.* **1997**, *21*, 495−504.

(36) Ahren, B.; Baldwin, R. M.; Havel, P. J. Pharmacokinetics of human leptin in mice and rhesus monkeys. *Int. J. Obes.* **2000**, *24*, 1579−1585.

(37) Lo, K. M.; Zhang, J.; Sun, Y.; Morelli, B.; Lan, Y.; Lauder, S.; Brunkhorst, B.; Webster, G.; Hallakou-Bozec, S.; Doare, L.; Gillies, S. D. Engineering a pharmacologically superior form of leptin for the treatment of obesity. *Protein Eng., Des. Sel.* **2005**, *18*, 1−10.

(38) Jevsevar, S.; Kunstelj, M.; Porekar, V. G. PEGylation of therapeutic proteins. *Biotechnol. J.* **2010**, *5*, 113−128.

DOI: 10.1021/mp5007147
Mol. Pharmaceutics 2015, 12, 1431−1442

(39) Pasut, G.; Veronese, F. M. State of the art in PEGylation: the great versatility achieved after forty years of research. *J. Controlled Release* **2012**, *161*, 461−472.
(40) Elinav, E.; Niv-Spector, L.; Katz, M.; Price, T. O.; Ali, M.; Yacobovitz, M.; Solomon, G.; Reicher, S.; Lynch, J. L.; Halpern, Z.; Banks, W. A.; Gertler, A. Pegylated leptin antagonist is a potent orexigenic agent: preparation and mechanism of activity. *Endocrinology* **2009**, *150*, 3083−3091.
(41) Müller, T. D.; Sullivan, L. M.; Habegger, K.; Yi, C. X.; Kabra, D.; Grant, E.; Ottaway, N.; Krishna, R.; Holland, J.; Hembree, J.; Perez-Tilve, D.; Pfluger, P. T.; DeGuzman, M. J.; Siladi, M. E.; Kraynov, V. S.; Axelrod, D. W.; DiMarchi, R.; Pinkstaff, J. K.; Tschöp, M. H. Restoration of leptin responsiveness in diet-induced obese mice using an optimized leptin analog in combination with exendin-4 or FGF21. *J. Pept. Sci.* **2012**, *18*, 383−393.
(42) Hukshorn, C. J.; Westerterp-Plantenga, M. S.; Saris, W. H. Pegylated human recombinant leptin (PEG-OB) causes additional weight loss in severely energy-restricted, overweight men. *Am. J. Clin. Nutr.* **2003**, *77*, 771−776.
(43) Gaberc-Porekar, V.; Zore, I.; Podobnik, B.; Menart, V. Obstacles and pitfalls in the PEGylation of therapeutic proteins. *Curr. Opin. Drug Discovery Dev.* **2008**, *11*, 242−250.
(44) Knop, K.; Hoogenboom, R.; Fischer, D.; Schubert, U. S. Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. *Angew. Chem., Int. Ed.* **2010**, *49*, 6288−6308.
(45) EMA. CHMP Safety Working Party's response to the PDCO regarding the use of PEGylated drug products in the paediatric population. *EMA/CHMP/SWP/647258/2012*.
(46) Verhoef, J. J.; Carpenter, J. F.; Anchordoquy, T. J.; Schellekens, H. Potential induction of anti-PEG antibodies and complement activation toward PEGylated therapeutics. *Drug Discovery Today* **2014**, *19*, 1945−1952.
(47) Schlapschy, M.; Binder, U.; Börger, C.; Theobald, I.; Wachinger, K.; Kisling, S.; Haller, D.; Skerra, A. PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins. *Protein Eng., Des. Sel.* **2013**, *26*, 489−501.
(48) Binder, U.; Skerra, A. Half-life extension of therapeutic proteins via genetic fusion to recombinant PEG mimetics. In *Therapeutic Proteins—Strategies to Modulate Their Plasma Half-lives*; Kontermann, R., Ed.; Wiley-VCH: Weinheim, 2012; pp 63−80.
(49) Harari, D.; Kuhn, N.; Abramovich, R.; Sasson, K.; Zozulya, A. L.; Smith, P.; Schlapschy, M.; Aharoni, R.; Köster, M.; Eilam, R.; Skerra, A.; Schreiber, G. Enhanced *in vivo* efficacy of a type I interferon superagonist with extended plasma half-life in a mouse model of multiple sclerosis. *J. Biol. Chem.* **2014**, *289*, 29014−29029.
(50) Mendler, C. T.; Friedrich, L.; Laitinen, I.; Schlapschy, M.; Schwaiger, M.; Wester, H.-J.; Skerra, A. High contrast tumor imaging with radio-labeled antibody Fab fragments tailored for optimized pharmacokinetics via PASylation. *mAbs* **2015**, *7*, 96−109.
(51) Meerman, H. J.; Georgiou, G. Construction and characterization of a set of *E. coli* strains deficient in all known loci affecting the proteolytic stability of secreted recombinant proteins. *Nat. Biotechnol.* **1994**, *12*, 1107−1110.
(52) Schlapschy, M.; Grimm, S.; Skerra, A. A system for concomitant overexpression of four periplasmic folding catalysts to improve secretory protein production in *Escherichia coli*. *Protein Eng., Des. Sel.* **2006**, *19*, 385−390.
(53) Fling, S. P.; Gregerson, D. S. Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. *Anal. Biochem.* **1986**, *155*, 83−88.
(54) Gill, S. C.; von Hippel, P. H. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **1989**, *182*, 319−326.
(55) Schönfeld, D.; Matschiner, G.; Chatwell, L.; Trentmann, S.; Gille, H.; Hülsmeyer, M.; Brown, N.; Kaye, P. M.; Schlehuber, S.; Hohlbaum, A. M.; Skerra, A. An engineered lipocalin specific for CTLA-4 reveals a combining site with structural and conformational features similar to antibodies. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 8198−8203.
(56) Myszka, D. G. Improving biosensor analysis. *J. Mol. Recognit.* **1999**, *12*, 279−284.
(57) Skerra, A. Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in *Escherichia coli*. *Gene* **1994**, *151*, 131−135.
(58) Rock, F. L.; Altmann, S. W.; van Heek, M.; Kastelein, R. A.; Bazan, J. F. The leptin haemopoietic cytokine fold is stabilized by an intrachain disulfide bond. *Horm. Metab. Res.* **1996**, *28*, 649−652.
(59) Boute, N.; Zilberfarb, V.; Camoin, L.; Bonnafous, S.; Le Marchand-Brustel, Y.; Issad, T. The formation of an intrachain disulfide bond in the leptin protein is necessary for efficient leptin secretion. *Biochimie* **2004**, *86*, 351−356.
(60) Peelman, F.; Iserentant, H.; De Smet, A. S.; Vandekerckhove, J.; Zabeau, L.; Tavernier, J. Mapping of binding site III in the leptin receptor and modeling of a hexameric leptin·leptin receptor complex. *J. Biol. Chem.* **2006**, *281*, 15496−15504.
(61) Churgay, L. M.; Kovacevic, S.; Tinsley, F. C.; Kussow, C. M.; Millican, R. L.; Miller, J. R.; Hale, J. E. Purification and characterization of secreted human leptin produced in baculovirus-infected insect cells. *Gene* **1997**, *190*, 131−137.
(62) Sreerama, N.; Venyaminov, S. Y.; Woody, R. W. Estimation of the number of α-helical and β-strand segments in proteins using circular dichroism spectroscopy. *Protein Sci.* **1999**, *8*, 370−380.
(63) Mistrík, P.; Moreau, F.; Allen, J. M. BiaCore analysis of leptin-leptin receptor interaction: evidence for 1:1 stoichiometry. *Anal. Biochem.* **2004**, *327*, 271−277.
(64) Meibohm, B. *Pharmacokinetics and Pharmacodynamics of Biotech Drugs: Principles and Case Studies in Drug Development*; Wiley-VCH: Weinheim, 2006.
(65) Mori, H.; Hanada, R.; Hanada, T.; Aki, D.; Mashima, R.; Nishinakamura, H.; Torisu, T.; Chien, K. R.; Yasukawa, H.; Yoshimura, A. Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. *Nat. Med.* **2004**, *10*, 739−743.
(66) Maack, T.; Johnson, V.; Kau, S. T.; Figueiredo, J.; Sigulem, D. Renal filtration, transport, and metabolism of low-molecular-weight proteins: a review. *Kidney Int.* **1979**, *16*, 251−270.
(67) Haraldsson, B.; Sörensson, J. Why do we not all have proteinuria? An update of our current understanding of the glomerular barrier. *News Physiol. Sci.* **2004**, *19*, 7−10.
(68) Kubetzko, S.; Sarkar, C. A.; Plückthun, A. Protein PEGylation decreases observed target association rates via a dual blocking mechanism. *Mol. Pharmacol.* **2005**, *68*, 1439−1454.
(69) Rosenblum, C. I.; Vongs, A.; Tota, M. R.; Varnerin, J. P.; Frazier, E.; Cully, D. F.; Morsy, M. A.; Van der Ploeg, L. H. A rapid, quantitative functional assay for measuring leptin. *Mol. Cell. Endocrinol.* **1998**, *143*, 117−123.
(70) Wu, L.; Ji, S.; Shen, L.; Hu, T. Phenyl amide linker improves the pharmacokinetics and pharmacodynamics of N-terminally mono-PEGylated human growth hormone. *Mol. Pharmaceutics* **2014**, *11*, 3080−3089.
(71) Klein, S.; Coppack, S. W.; Mohamed-Ali, V.; Landt, M. Adipose tissue leptin production and plasma leptin kinetics in humans. *Diabetes* **1996**, *45*, 984−987.
(72) Gegg, C.; Kinstler, O. Site-directed dual pegylation of proteins for improved bioactivity and biocompatibility. US Patent No. US6420339 B1, 2002.
(73) Banks, W. A.; Gertler, A.; Solomon, G.; Niv-Spector, L.; Shpilman, M.; Yi, X.; Batrakova, E.; Vinogradov, S.; Kabanov, A. V. Principles of strategic drug delivery to the brain (SDDB): development of anorectic and orexigenic analogs of leptin. *Physiol. Behav.* **2011**, *105*, 145−149.
(74) Gertler, A.; Solomon, G. Leptin-activity blockers: development and potential use in experimental biology and medicine. *Can. J. Physiol. Pharmacol.* **2013**, *91*, 873−882.

DOI: 10.1021/mp5007147
Mol. Pharmaceutics 2015, 12, 1431−1442