Pegylated Human Leptin D23L Mutant—Preparation and Biological Activity *In Vitro* and *In Vivo* in Male *ob/ob* Mice

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Recombinant monomeric human leptin (hLEP) and its D23L mutant were prepared in *Escherichia coli* and pegylated at their *N*-terminus using 20-kDa methoxy pegylated (PEG)–propionylaldehyde. As determined by both SDS-PAGE and size-exclusion chromatography, the pegylated proteins consisted of >90% monopegylated and <10% double-pegylated species. Circular dichroism spectra showed that their secondary structure, characteristic of all four α-helix bundle cytokines, was not affected by either the D23L mutation or pegylation. Because of the D23L mutation, affinity for hLEP receptor increased 25- and 40-fold for the pegylated and nonpegylated mutant, respectively. However, whereas the proliferation-promoting activity *in vitro* of nonmutated and mutated nonpegylated hLEP was identical, that of the respective pegylated mutant was approximately sixfold higher compared with the pegylated nonmutated hLEP. This difference was also seen *in vivo*. Both pegylated hLEPs at all doses significantly decreased body weight and food consumption, as compared with the vehicle-treated control. Once-daily administration of pegylated hLEP D23L at doses of 0.1, 0.3, and 1 mg/kg for 14 consecutive days in ob/ob mice resulted in significantly decreased body weight and food consumption as compared with respective pegylated hLEP–treated animals, with the biggest difference observed at 0.1 mg/kg. Repeated administration of either pegylated hLEP D23L or pegylated hLEP significantly decreased blood glucose levels compared with the control before glucose challenge and after oral glucose tolerance test, but with no difference between the two treatments. The pegylated hLEP D23L mutant seems to be a more potent reagent suitable for *in vivo* studies than the pegylated nonmutated hLEP. **(*Endocrinology* 160: 891–898, 2019)**

Human leptin (hLEP) is a pleiotropic hormone that acts centrally and peripherally (1–4). It participates in a variety of biological processes, including energy metabolism, reproduction, and modulation of the immune response (5, 6). Though a three-dimensional hLEP structure was reported 14 years ago (7), no crystallized complex between hLEP and hLEP receptor has yet been reported, to our knowledge. Lack of such a structure hampers valid structural interpretations of the presently reported D23L mutation or other D23 mutations. Our interpretation, therefore, is based on theoretical complex models and small-angle X-ray scattering data proposed in the last few years by Tavernier's laboratory (8–12).

To search for high-affinity mutants of hLEP antagonist, (L39A/D40A/F41A mutant), we used random mutagenesis of hLEP antagonists, followed by selection of mutants with higher affinity toward biotinylated hLEP receptor extracellular domain by yeast-surface display. We discovered that replacing residue D23 with a non-negatively charged amino acid leads to dramatically enhanced affinity of hLEP for its soluble receptor (13). Rational mutagenesis of D23 with several hydrophobic

ISSN Online 1945-7170

Received 5 July 2018. Accepted 20 February 2019.
First Published Online 25 February 2019

Abbreviations: AUC, area under the curve; CD, circular dichroism; hLBD, human leptin–binding domain; hLEP, human leptin; mPEG propionyl-ALD 20 kDa, 20-kDa methoxy pegylated propionylaldehyde; OGTT, oral glucose tolerance test; PBST, PBS containing 0.05% w/v Tween 20; PEG, pegylated; PEG-hLEP, pegylated human leptin; SEC, size-exclusion chromatography.

amino acids revealed the D23L substitution to be most effective. D23 is conserved in all mammalian leptins; thus, we documented that such mutations lead to increased affinity not only in mouse and human leptins (13) but also in ovine (14) and rat (15) leptins or leptin antagonists. In 2011, we had already observed that the increased affinity of mouse and human hLEPs modified at position D23 is not accompanied by an increase in agonistic activity in Ba/F3 cells stably transfected with hLEP receptor 13.

In the present work, we checked the activity of the hLEP D23L mutant *in vivo*. Because pegylating mouse or human LEP antagonists is preferable for studying their effects in animals (13, 15, 16), to achieve a more suitable reagent for *in vivo* studies, we prepared the monopegylated analog of the hLEP D23L mutant and studied its action *in vitro* and *in vivo*. Monopegylation aimed at the *N*-terminus, using 20-kDa methoxy PEG-propionylaldehyde (mPEG-propionyl-ALD 20 kDa), was preferred, because according to our previous experience, random pegylation, also leads to pegylation of ε amino groups with other reagents, resulting in lower biological activity.

Materials and Methods

Materials

Recombinant human leptin–binding domain (hLBD) was prepared in our laboratory as described previously (17); hLEP and its D23L mutant were also prepared as described previously (13, 18). Gibco RPMI-1640 medium and Gibco DMEM were purchased from Invitrogen (Carlsbad, CA); fetal bovine serum and penicillin-streptomycin solution were obtained from Biological Industries (Beit Haemek, Israel); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue) and puromycin were purchased from Sigma-Aldrich (St. Louis, MO); Superdex 200 HR 10/30 column and Q-Sepharose were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden); antibiotic–antimycotic solution (5×10^4 U/mL penicillin, 50 mg/mL streptomycin, and 0.125 mg/mL fungisone), NaCl, and Tris base were purchased from Bio-Laboratory (Jerusalem, Israel). Bacto-tryptone, Bacto-yeast extract, glycerol, EDTA, HCl, Triton X-100, and urea were obtained from ENCO Diagnostics (Petah-Tikva, Israel), and molecular markers for SDS-PAGE were purchased from Bio-Rad (Hercules, CA). mPEG-propionyl-ALD 20 kDa was purchased from Jenkem Technology (Allen, TX). Peroxidase-conjugated streptavidin was from Jackson ImmunoResearch (West Grove, PA), and 3,3′,5,5′-tetramethylbenzidine was from DakoCytomation (Copenhagen, Denmark). All other reagents were of analytical grade.

Pegylation of hLEP and hLEP D23L mutant

mPEG-propionyl-ALD 20 kDa was used for pegylation under conditions that favor pegylation of the *N*-terminal amino group. hLEP (384 mg) was dissolved in 200 mL of 0.1 M Na-acetate buffer (pH 5) and centrifuged at 35,000*g* for 10 minutes to remove the insoluble material. Then 0.02 M $NaBH_3CN$ (4.32 mL) was added and the dissolved protein was conjugated with 1.62 g of mPEG-propionyl-ALD 20 kDa dissolved in 16.2 mL of 1 mM HCl. After 20 hours of stirring at 4°C, 310 μL of acetic acid (17 M) was added. The solution was stirred for a few seconds, diluted with ~2 L double-distilled H_2O and applied at maximal flow rate (*i.e.*, 400 to 500 mL/h) to an SP-Sepharose column (30-mL bead volume), pre-equilibrated with 10 mM Na-acetate (pH 4). The column was then washed with 400 mL of 10 mM Na-acetate (pH 4), and the pegylated protein was eluted in 10 mM Na-acetate (pH 5), containing 100 and 150 mM NaCl. Fractions containing the monopegylated protein, as determined by gel filtration on an analytical Superdex 200 HR 10/30 column, were pooled, dialyzed against $NaHCO_3$ to ensure a 2:1 protein-to-salt ratio, and lyophilized. Protein concentrations were determined by absorbance at 280 nm using an extinction coefficient of 0.87 for a 0.1% (w/v) solution of pegylated protein. This value applies to the protein part of the pegylated product.

Determination of purity and monomer content

SDS-PAGE was carried out according to Laemmli (19) in a 12% polyacrylamide gel under reducing conditions. The gel was stained with Coomassie Brilliant Blue R. Size-exclusion chromatography (SEC) was performed on a Superdex 200 HR 10/30 column with 0.2-mL aliquots using TN buffer (*i.e.*, 25 mM Tris-HCl, 300 mM NaCl, at pH 8), developed at 0.7 mL/min.

Determination of circular dichroism spectra and secondary structure

The circular dichroism (CD) spectra in the wavelength range of 200 to 240 nm were measured with a Jasco J-810 Spectropolarimeter (Tokyo, Japan) using a 0.020-cm rectangular QS Hellma cuvette with a spectral resolution of 1 nm and signal-to-noise ratio of ~1% at 210 to 220 nm. Solutions were prepared by dissolving the lyophilized samples in 50 mM phosphate buffer (pH 7.6), followed by centrifugation (10,000*g* for 15 minutes). A protein concentration of ~40 to 80 μM was determined spectrophotometrically at 280 nm using light-scattering correction and an extinction coefficient of 0.87 = 1 mg/mL.

The proteins' secondary structure was determined by applying the procedure and computer program CONTIN developed by Provencher and Glöckner (20), where α-helices, β-sheets, and β-turns are presented as a percentage of amino acids involved in these ordered forms. The constraint that the sum of all elements of the secondary structure in a protein must equal unity produces a remainder, which may be interpreted as the content of random coil (21). In the present study, for calculations by the CONTIN program, a set of standard CD spectra of 21 proteins was used (22).

Binding assay

Biotinylated hLEP served as the ligand in all competitive-binding experiments and the respective hLEP muteins were competitors. hLBD was used as the receptor source. Polystyrene 96-well microtiter plates were coated overnight at 4°C with 100 μL of 40 pM hLBD in PBS (pH 7.4). Wells were then washed once with PBS containing 0.05% w/v Tween 20 (PBST) and blocked with PBS containing 3% (w/v) skim milk for 2

hours at room temperature. All additional incubations were carried out at room temperature.

Wells were washed once again with PBST and incubated with different concentrations of unlabeled hLEP (50 μL per well) for 30 minutes; then, 50 μL of 62.5 pM biotinylated human leptin was added to each well for another 2 hours. The wells then were washed three times with PBST and incubated with 1:15,000 streptavidin–horseradish peroxidase in PBST for 1 hour. Wells were washed three times with PBST and the reaction was quantified at 450 nm by ELISA Micro-Plate Reader ELx808 (Bio-Tek Instrument, Winooski, VT) using 3,3′,5,5′-tetramethylbenzidine according to the manufacturer's instructions.

Ba/F3 proliferation assay

The proliferation rate of hLEP-sensitive Ba/F3 cells stably transfected with the long form of hLEP receptor was used to estimate the agonistic activity of pegylated hLEP (PEG-hLEP), nonpegylated hLEP and the corresponding hLEP D23L mutants. The average absorbance in wells without hLEP (negative control) was used as a blank value and subtracted from other absorbance values to yield the corrected absorbance values. The dose-response curves were drawn using the Prisma (version 4.0) nonlinear regression sigmoidal one-site program and the EC_{50} values were calculated. Note that all mammalian leptins are capable of activating hLEP receptor to an almost identical degree (18).

Stability studies

On day 0, the lyophilized proteins were dissolved in double-distilled H_2O at 2 mg/mL or 4 mg/mL, filter sterilized, and stored at 4°C. The proteins were tested immediately after solubilization by SEC analysis. An aliquot of the proteins was subjected to two cycles of freezing and thawing and tested by SEC and binding assay, respectively compared with freshly dissolved pegylated and nonpegylated hLEP or hLEP D23L. In addition, the samples were tested by Ba/F3 bioassay using freshly prepared nonpegylated hLEP as a control. The rest of the material was frozen, thawed, and kept at 4°C for 4 weeks. After weeks 1, 2, 3, and 4, the samples were tested by SEC and by binding assay. After week 4, an additional Ba/F3 bioassay was also carried out. SEC was performed on a Superdex 200 analytical column in TN buffer pH 8, at 0.7 mL/min. The fraction concentrated to 2 mg/mL was diluted 1:8 and the fraction concentrated to 4 mg/mL was diluted 1:16 with TN buffer immediately before analysis.

In vivo experiment

Male ob/ob mice were obtained from Harlan Laboratories Israel (Rehovot, Israel). The health status of the animals used in this study was examined on arrival and only animals in good health were used for the study after 5 days of acclimation. During acclimation and throughout the entire study, animals were housed in groups of six per filtered polypropylene cage. Animals were provided food *ad libitum* and had free access to drinking water, which was supplied to each cage via polyethylene bottles with stainless-steel sipper tubes. There was a total of 42 animals.

During the acclimation period, animals were randomly assigned to the various test groups according to their body weight, such that the mean body weight in each group was similar; the average was ~45 g ± 15% at study initiation. The test item (PEG-hLEP D23L), reference compound (PEG-hLEP), or control vehicle (hereafter, control) was administered once daily in the morning by IP injection for 14 consecutive days. The daily dose was 1.0, 0.3, or 0.1 mg/kg body weight, the volume dosage was adjusted according to determined body weights, and all formulations were administered at a constant dose volume of 5 mL/kg. Measurements of estimated food consumption per cage were initially carried out during the acclimation period (before the first dosing session) and twice weekly thereafter. Body weight was determined once a day. At the end of the dosing period, all animals underwent a 14-day recovery period and at the scheduled termination of the study, the animals were euthanized by CO_2 asphyxiation.

For glucose monitoring, all animals were fasted overnight before oral administration of glucose (2 g/kg) on day 14. Glucose challenge by oral glucose tolerance test (OGTT) was performed 15 minutes after the last dosing session on day 14. Glucose baseline levels were evaluated using a glucometer immediately before dosing. Then, blood glucose levels were determined immediately before glucose challenge (time 0), and 15, 30, 60, and 120 minutes after challenge for all groups. The joint ethics committee of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare. The Hebrew University is an Association for Assessment and Accreditation of Laboratory Animal Care International accredited institute.

Determination of *in vivo* half-life

Male C57BL/6J WT mice (22 g; 5 weeks old) were purchased from Harlan Laboratories. The mice were acclimatized for 1 week and fed a normal chow diet. They were housed three per cage in a temperature- (21° to 22°C) and light- (lights on from 8 AM to 6 PM) controlled room. Food (Harlan Laboratories) and water were provided *ad libitum*. At 0 hour, each mouse received subcutaneous injections of 20 μg (protein) in 100 μL of saline of either PEG-hLEP or PEG-hLEP D23L. Blood was collected from the tail, allowed to clot in tubes chilled on ice for 30 minutes, and centrifuged, and the serum was removed and stored frozen at −80°C for analyses. hLEP was assayed in duplicate using the 108879 Abcam (Cambridge, United Kingdom) hLEP ELISA kit (23) according to the manufacturer's instructions. According to the information provided by the producer, the sensitivity of this ELISA toward mouse leptin is <1%. Because the sensitivity of PEG-LEP and PEG-LEP D23L was lower than leptin, standard curves for each of them were prepared and used for calculation.

Results

Preparation and characterization of PEG-hLEP D23L and PEG-hLEP

The nonpegylated D23L mutant of hLEP was prepared according to the protocol used previously for superactive mouse LEP antagonists (13). Briefly, the following steps were performed after refolding: (i) collecting the flow-through fraction from the Q-Sepharose column developed at pH 9 with 10 mM Tris-HCl containing 150 mM NaCl, (ii) concentrating this fraction by ultrafiltration, and (iii) isolating the monomer by

preparative SEC. Nonpegylated hLEP was prepared as described previously (18). The pegylation of both preparations was carried out according to the protocol described in "Materials and Methods." The first batch of PEG-hLEP D23L mutant consisted of 136 mg of protein and the second of 168 mg. The two batches were combined, dialyzed against $NaHCO_3$, concentrated to 1.63 mg/mL, and lyophilized. Similarly, the PEG-hLEP was dialyzed, concentrated to 1.72 mg/mL, and lyophilized.

The SDS-PAGE profile of each protein after lyophilization is shown in Fig. 1A, and consists of a main band at ~50 kDa (monomer) and a very weak band at ~100 kDa (dimer). The electrophoretic mobility of PEG-hLEP was considerably reduced because of PEG's large hydrodynamic volume, resulting in a higher molecular mass compared with that calculated and confirmed by mass spectroscopy analyses (*i.e.*, ~36 kDa for monopegylated hLEP or hLEP D23L; data not shown). SEC analysis (Fig. 1B and 1C) confirmed these results; the main peak of monopegylated hLEP corresponded to a molecular mass of ~220 kDa, owing to its hydrodynamic volume. The pegylation site (*N*-terminus) was confirmed by sequencing the 5 *N*-terminal amino acids, which were detected only in the non–*N*-terminal-pegylated and not in the *N*-terminal-pegylated proteins (data not shown).

The binding activities of hLEP, hLEP D23L, and their respective pegylated analogs are presented in Fig. 2A and 2B, showing that the affinity of the nonpegylated hLEP D23L as calculated by EC_{50} was 38.2-fold higher than wild-type hLEP. A similar increase in affinity (25.5-fold) was also found comparing pegylated D23L hLEP with pegylated wild-type hLEP. Despite these higher affinities, agonistic proliferation-promoting activities of the nonpegylated hLEP and hLEP D23L in Ba/F3 cells stably transfected with hLEP receptor were identical (Fig. 2C). In contrast, the activity of PEG-hLEP D23L was 5.3-fold higher than that of PEG-hLEP (Fig. 2D). This result was unexpected; thus, we performed six additional experiments comparing the activities of hLEP and hLEP D23L, and eight experiments comparing the activities of PEG-hLEP and PEG-hLEP D23L. The (mean ± SEM) comparative activities of the D23L mutants were 1.07- ± 0.15-fold higher for hLEP D23L but 6.35- ± 0.84-fold higher for PEG-hLEP D23L than their respective nonmutated counterparts. To check whether this difference originated from structural changes imposed by pegylation of hLEP D23L, we performed CD analyses of all four proteins. As shown in Fig. 3 and Table 1, neither mutation nor pegylation changed the secondary structure.

Stability studies revealed that sterile PEG-hLEP and PEG-hLEP D23L, frozen, thawed, and then kept at 4°C for 4 weeks at 2 or 4 mg/mL, retained their full biological activity, as evidenced by binding assay, SEC, and growth-promoting activity in hLEP-responsive Ba/F3 cells (not shown).

In vivo experiment

No animals died during the dosing or recovery periods, and no abnormal clinical signs were observed in any of them throughout the study period. The mean group body weight gain for PEG-hLEP– and PEG-hLEP D23L–treated animals was significantly lower ($P < 0.01$) than that of the control vehicle–treated animals at all doses tested from day 1 of the study until termination of the dosing phase on day 14 (Fig. 4A). The mean group body weight loss of PEG-hLEP D23L–treated animals at all three doses was significantly lower than that of the PEG-hLEP–treated animals at the same dose levels. The biggest difference ($P < 0.01$) was in animals treated with 0.1 mg/kg (Fig. 4A and 4B). In fact, the overall weight loss (mean ± SEM) in animals treated with 0.3 mg/kg

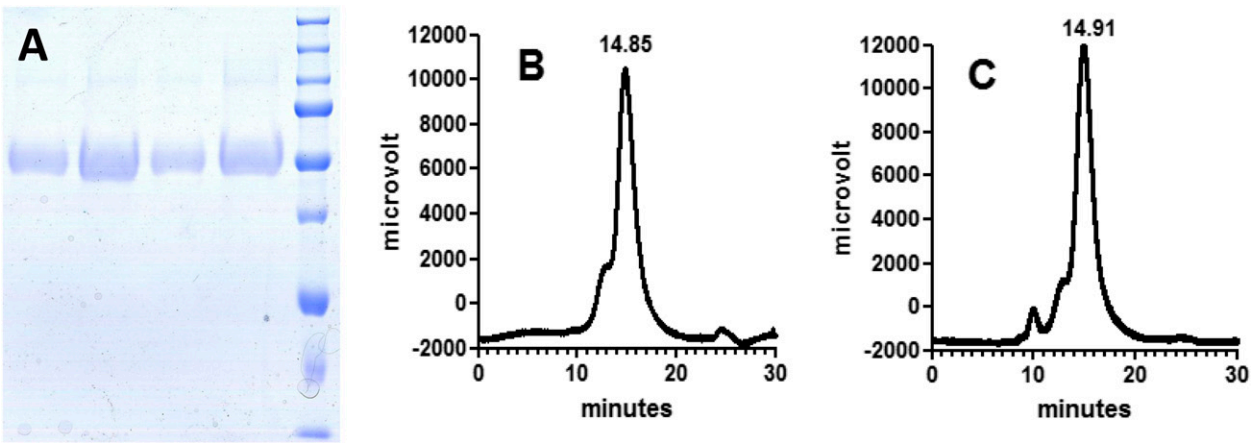

Figure 1. (A) SDS-PAGE (12%) of lyophilized PEG-hLEP (from left to right: lane 1, 2.5 μg; lane 2, 5.0 μg); PEG-hLEP D23L mutant (lane 3, 2.5 μg; lane 4, 5.0 μg); and lane 5, markers from top to bottom: 250, 150, 100, 75, 50, 37, 25, 15, and 10 kDa. SEC (on an analytical Superdex 200 column) analysis of lyophilized (B) PEG-hLEP and (C) PEG-hLEP D23L.

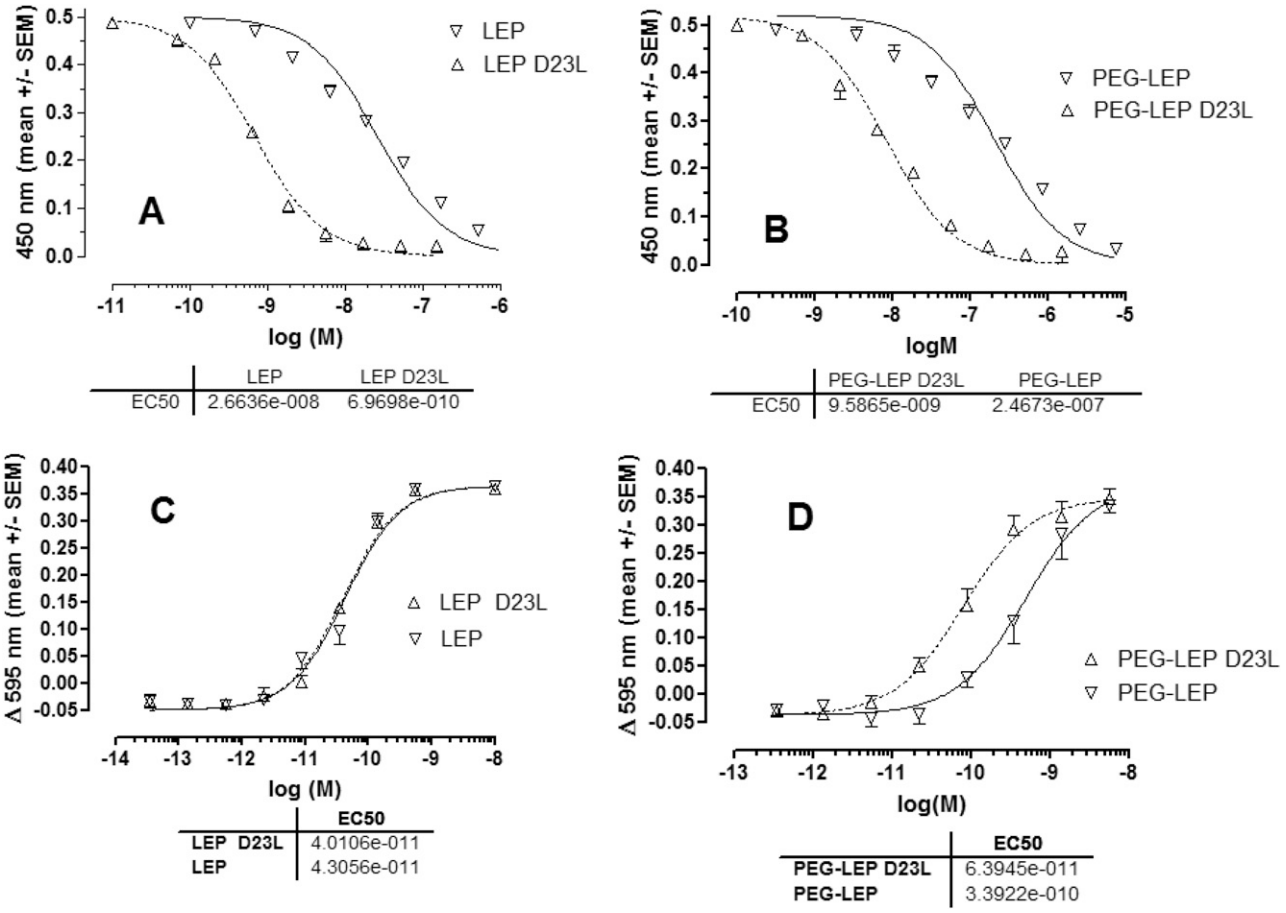

Figure 2. Comparison of (A, B) binding properties and (C, D) biological activity in Ba/F3 cells of (A) hLEP and (C) hLEP D23L mutant and (B) PEG-hLEP and (D) PEG-hLEP D23L.

(−14.55 ± 0.29 g) was not significantly different from that for animals treated with 1.0 mg/kg PEG-hLEP (−15.50 ± 0.68 g). The mean food consumption of PEG-hLEP D23L–treated animals at all doses tested was reduced compared with PEG-hLEP–treated animals over the course of the study. The lowest dose of PEG-hLEP D23L (0.1 mg/kg) showed the greatest relative reduction in food consumption compared with PEG-hLEP at a similar dose level (Fig. 4C).

PEG-hLEP D23L and PEG-hLEP were also evaluated for *in vivo* efficacy at regulating glucose homeostasis using an OGTT on dosing day 14 (Fig. 4D). Blood glucose levels of overnight-fasted ob/ob mice rose quickly after oral administration of glucose challenge (2 g/kg).

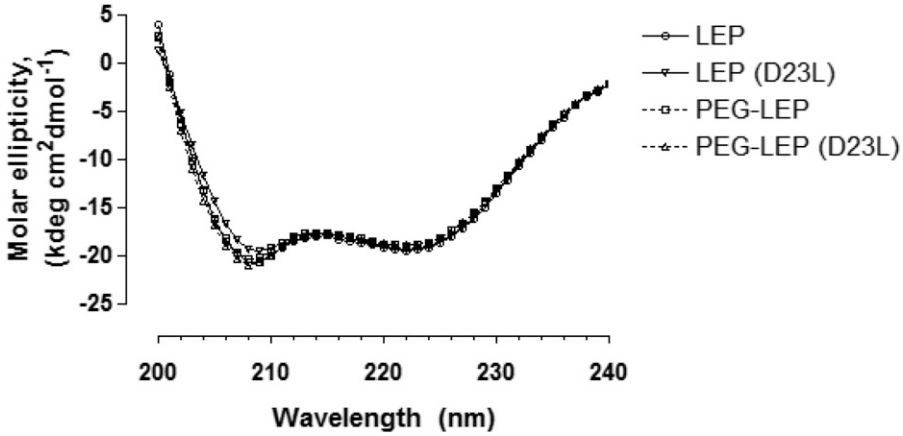

Figure 3. Secondary structure of hLEP, hLEP D23L mutant, PEG-hLEP, and PEG-hLEP D23L. The CD spectra were collected over 200 to 240 nm at 25°C. Lyophilized proteins were dissolved in water, dialyzed against 50 mM phosphate buffer (pH 7.6) for 20 hours and adjusted to 50 μM.

Table 1. Secondary Structure of Nonpegylated hLEP, PEG-hLEP, and Their D23L Mutants at pH 7.6

Secondary Structure	hLEP	hLEP D23L	PEG-hLEP	PEG-hLEP D23L
α-Helix	56 ± 0.3	58 ± 0.5	55 ± 0.8	54 ± 0.8
β-Strand	1 ± 0.0	1 ± 0.8	1 ± 0.8	2 ± 1.0
β-Turn	14 ± 0.6	15 ± 0.7	14 ± 0.5	17 ± 0.6
Remainder	29 ± 1.0	26 ± 0.9	30 ± 1.2	27 ± 1.0

Data are reported as mean percent ± SD. Errors arose only from uncertainty in the fitting of the experimental CD spectrum with the set of standard protein CD spectra in the CONTIN program. Errors in the CD measurements and in the protein concentration determination are not included.

However, this increase was significantly reduced in PEG-hLEP D23L– and PEG-hLEP–treated animals at all doses tested. This decrease in glucose levels was significant ($P < 0.01$) at all time points tested after glucose challenge (*i.e.*, 15, 30, 60, and 120 minutes postglucose challenge), as compared with the vehicle-treated group. Except for animals dosed with PEG-hLEP at 0.1 mg/kg once daily for 14 days, all PEG-hLEP D23L– and PEG-hLEP–treated animals had significantly decreased blood glucose levels ($P < 0.01$) 15 minutes before glucose challenge (not shown), meaning that repeated administration of both test items for 14 consecutive days significantly decreased high glucose levels exhibited by ob/ob mice treated with vehicle. Furthermore, PEG-hLEP D23L–treated (dose of 0.1 mg/kg) animals had significantly reduced blood glucose levels ($P < 0.01$) at 0 minutes before OGTT compared with PEG-hLEP D23L– and PEG-hLEP–treated animals at a similar dose level, suggesting that repeated administration of PEG-hLEP D23L at 0.1 mg/kg improved efficacy compared with the PEG-hLEP treatment. However testing the area under the curve (AUC) using 50 mg/dL as a base revealed no differences between the corresponding doses of PEG-hLEP D23L and PEG-hLEP. The respective AUC values (in arbitrary units) were 31,140 for the control and for PEG-hLEP D23L and PEG-hLEP, respectively: 12,495 and 13,635 at 1 mg/kg; 10,403 and 11,573 at 0.3 mg/kg; and 15,158 and 15,833 at 0.1 mg/kg.

Determination of the *in vivo* half-life of pegylated hLEP and pegylated hLEP D23L

To test whether the difference between PEG-hLEP and PEG-hLEP D23L in *in vivo* experiments could originate from a different half-life in circulation, three mice per treatment received subcutaneous injections (20 μg per mouse) of either PEG-hLEP and PEG-hLEP D23L, and the blood levels of each was determined using hLEP ELISA non–cross-reactive with the endogenous mouse LEP (Fig. 5). The removal of PEG-hLEP D23L was

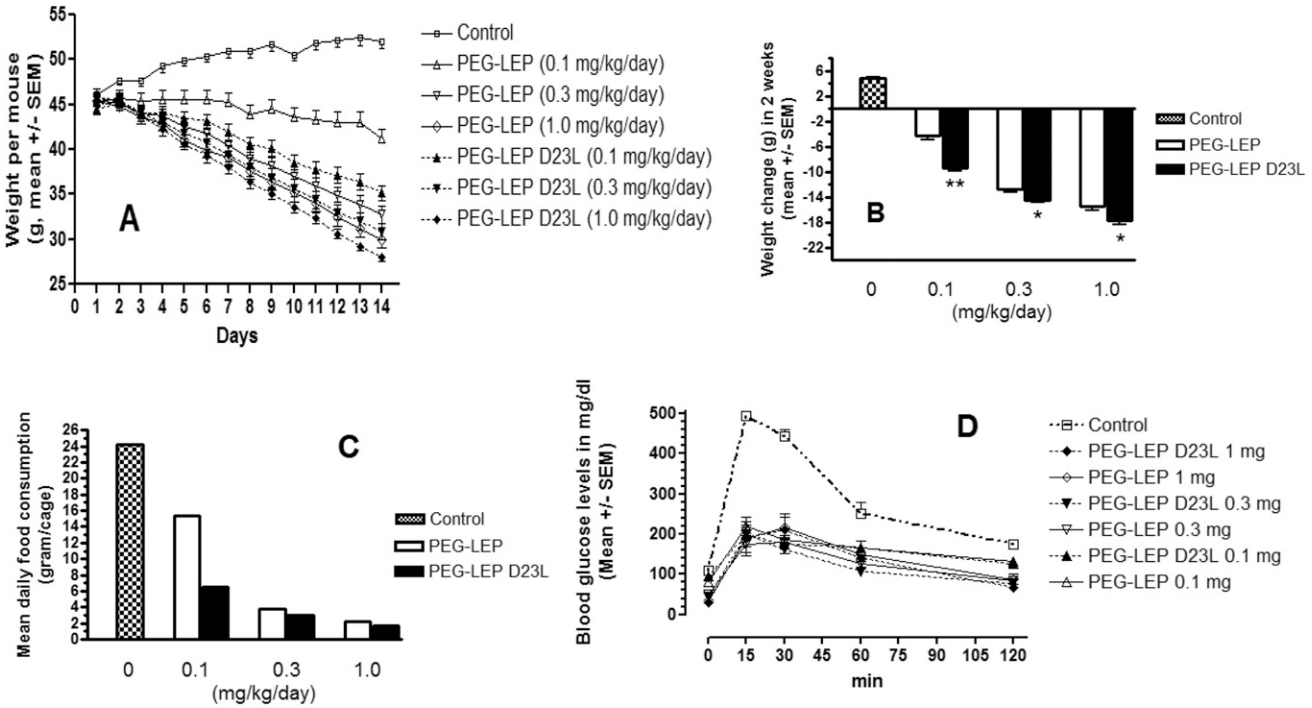

Figure 4. Measures (mean ± SEM) of weight, changes in group body weight, daily food consumption, and effect on blood glucose levels after IP administration of PEG-hLEP, PEG-hLEP D23L, or control once daily for 14 consecutive days. (A) Weight of male ob/ob mice at doses of 0.1, 0.3, or 1.0 mg/kg, or control. (B) Changes in group body weights. *$P < 0.05$, **$P < 0.01$ for comparison between two groups given the same injection. (C) Daily food consumption per group (n = 6 male ob/ob mice). (D) Effect on blood glucose levels (mg/dL) in OGTT tests. In all experiments, there were six mice per group.

slightly faster than PEG-hLEP, but the difference was not significant and resembled the results published previously for PEG-hLEP (14). The respective AUC values of 4798 and 5042 were also similar.

Discussion

The lack of a crystallographic structure for the hLEP: hLEP receptor complex prevents providing a structural explanation for the dramatically enhanced affinity of hLEP for its soluble receptor when residue D23 is replaced with a nonnegatively charged amino acid. Cytokine receptor homology domain has been suggested as the main high-affinity binding site (8). Using a molecular modeling and mutagenesis approach, Tavernier's group showed that D9, T12, K15, T16, and R20 located on helix A, and Q75, N82, D85, and L86 located on helix C (8) have the same orientation and are most likely involved in the interaction with cytokine receptor homology domain of the receptor. The D23 residue is also located on helix A and likely faces the same direction. The suggestion that D23 faces residues V537 and F540 in the hLEP receptor (J. Tavernier, personal communication) could explain increased hydrophobic interaction is why its mutation to hydrophobic residues, and particularly to leucine, elevates the affinity.

Although a reduction in *in vitro* activity is routinely noted after pegylation, we (13, 16, 24–26) and others (27) have shown the significantly improved half-life compensates for this effect, resulting in a net enhancement of biological activity, as shown with pegylated human growth hormone (27), pegylated hLEP antagonists (13, 16), and more recently, pegylated chicken prolactin (26). However, mutagenesis of agonists that leads to increased affinity toward the respective receptor does not necessarily lead to increased activity, as exemplified with human growth hormone (13, 28), and as shown in the present work. Therefore the approximately sixfold increase in PEG-hLEP D23L activity *in vitro* and the approximately two-- to threefold increased activity *in vivo*, although we found no difference between hLEP D23L and human HLEP *in vitro*, was quite unexpected. Pegylation did not change the secondary structure of the mutated or nonmutated hLEP, and their half-lives in circulation were not different; therefore, no explanation can be proposed at present. We previously observed that pegylated hLEP conjugated to Alexa Fluor680 rapidly penetrates the blood–brain barrier in mice (unpublished data). However because pegylation of hLEP D23L increased not only its *in vivo* activity but its *in vitro* activity even more, we speculate that this increase is not related to brain penetration but rather to activation of hLEP receptor.

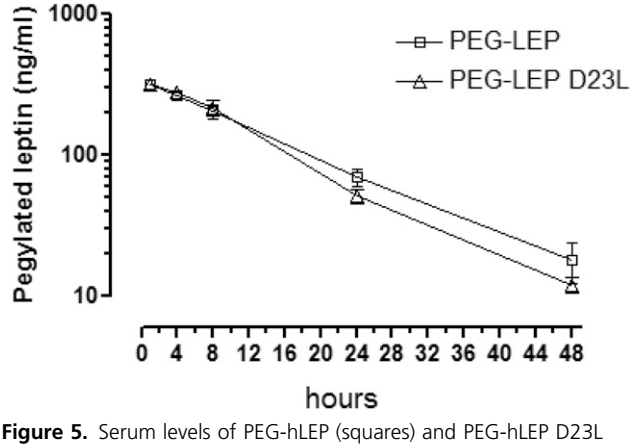

Figure 5. Serum levels of PEG-hLEP (squares) and PEG-hLEP D23L (triangles) in male mice after a single subcutaneous injection of 20 μg of protein per mouse. Serum levels were measured by ELISA. Data are reported as mean ± SEM of three mice.

Acknowledgments

***Financial Support*:** This research was supported by Israel Science Foundation Grant 521/07 (to A.G.).

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Disclosure Summary: The authors have nothing to disclose.

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