NRC Research Press

INVITED REVIEW

Leptin-activity blockers: development and potential use in experimental biology and medicine[1]

Arieh Gertler and Gili Solomon

Abstract: The first adipokine, leptin, discovered almost 20 years ago, is secreted into circulation mainly from adipose tissue and acts both centrally and peripherally. Leptin regulates energy metabolism, reproductive function, bone metabolism, and immune response. However in some physiological or pathological situations such as enhancement of undesired immune responses in autoimmune diseases, tumorigenesis, elevated blood pressure, and certain cardiovascular pathologies, leptin activity may be harmful. In this review we screen different approaches to blocking leptin action, in vitro and in vivo. The recent development of superactive leptin muteins exhibiting antagonistic properties, and other leptin-action-blocking peptides, proteins, monoclonal antibodies, and nanobodies, opens new perspectives for their use in research, and eventually, therapy for cachexia, autoimmune disease, cancer, and other pathologies.

Key words: leptin, antagonist, inflammatory disease, anti-autoimmune diseases, cancer, uremic cachexia, blood pressure, metabolic syndrome, T2DM model.

Résumé : La leptine, première adipokine découverte il y a presque 20 ans, est principalement sécrétée dans la circulation par le tissu adipeux et elle agit de façon centrale et périphérique. La leptine régule le métabolisme énergétique, la fonction reproductrice, le métabolisme osseux et la réponse immunitaire. Toutefois, dans certaines situations physiologiques ou pathologiques comme l'accroissement de réponses immunes indésirables dans les maladies auto-immunes, la tumorigenèse, l'hypertension et certaines pathologies cardiovasculaires, l'activité de la leptine peut être néfaste. Dans cet article de revue, nous passons au crible différentes approches de blocage de l'action de la leptine *in vitro* et *in vivo*. Le développement récent de mutéines de leptine très actives montrant des propriétés antogonistes, ainsi que d'autres peptides, anticorps et nanocorps monoclonaux bloquant l'action de la leptine, ouvre ainsi de nouvelles perspectives à leur utilisation en recherche et, éventuellement, dans la thérapie de la cachexie, des maladies auto-immunes, du cancer et autres pathologies. [Traduit par la Rédaction]

Mots-clés : leptine, antagoniste, maladie inflammatoire, maladies auto-immunes, cancer, cachexie urémique, pression sanguine, syndrome métabolique, modèle de DT2.

Introduction

Although leptin (LEP) and leptin receptor (LEPR) were cloned almost 20 years ago (Tartaglia et al. 1995; Zhang et al. 1994) and LEP's 3D structure was resolved a few years later (Zhang et al. 1997), the 3D structures of LEPR and the LEPR:LEP complex have not yet been elucidated. The extracellular domain (ECD) of LEPR (Fig. 1) consists of several subdomains (from the N terminus): cytokine homology region 1 (CHR 1), an immunoglobulin-like domain (IGD), CHR 2, and two consecutive fibronectin type III (FNIII) domains. ~~Fong and co-workers~~ Fong et al. 1998) localized the leptin-binding domain (LBD) to the membrane-proximal CHR 2 (~210 aa in length) in the LEPR-ECD. This domain was subcloned in our lab from human (Sandowski et al. 2002) and chicken LEPRs (Niv-Spector, Raver et al. 2005*b*) and expressed as a recombinant protein showing a 1:1 molar interaction with LEP. In 2003, Tavernier and his group (Zabeau et al. 2003, 2004) suggested that leptin binding to its receptor resembles the interaction between interleukin 6 (IL6) and its receptor (Chow et al. 2001; Boulanger et al. 2003; Muller-Newen 2003), and they formulated the existence of the putative leptin site III as a major site responsible for the formation of active 2:2 or 4:2 LEPR:LEP complex. The IGD located between the distal and proximal CHR domains was clearly documented as essential for productive dimerization (or even tetramerization) of the LEPR, as its removal attenuated activation, but not binding, of the ligand (Zabeau et al. 2004). A schematic illustration of a 2:2 LEPR:LEP complex, showing interaction of each LEP molecule with the CHR 2 of one receptor (through binding site I/II) and with the IGD of the second receptor (through binding site III), is presented in Fig. 1. This model was further substantiated by the extensive mutagenesis of mouse and human LEPs, which led to the identification of aa S120 and T121, located in the N-terminal part of helix D, as contributors to site III (Peelman et al. 2004). Mutation of these amino acids to A led to the formation of LEPR antagonist that binds to LEPR but is unable to activate it (see further on).

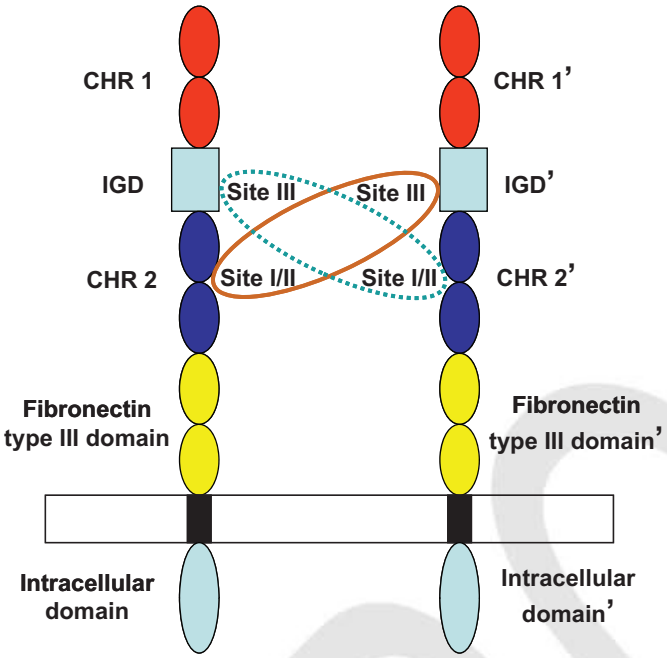
Because LEP is a pleiotropic hormone secreted mainly by adipose tissue, but also by placenta, gut, and other tissues, studying its action by classical replacement therapy is not feasible. LEP action can be blocked by (*i*) direct blocking of the LEPRs that are responsible for transferring LEP through the blood–brain barrier, for its action in the hypothalamus, or for its peripheral action, by LEP muteins acting as antagonists, by LEP fragments acting as low-molecular-weight LEPR antagonists, or by LEPR antibodies (Abs); (*ii*) neutralizing circulating LEP by LEP-binding proteins or specific Abs. Such reagents may therefore serve as novel and potent research tools for studying LEP-related metabolic processes in vitro and in vivo, and may eventually be developed into therapeutic modalities aimed at blocking harmful LEP actions. Undesired

Received 8 January 2013. Accepted 15 April 2013.

A. Gertler and G. Solomon. The Institute of Biochemistry, Food Science, and Nutrition, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel.
Corresponding author: Arieh Gertler (e-mail: arieh.gertler@mail.huji.ac.il).

[1]*This Invited Review is one of a selection of papers published in the special issue on Adipobiology.*

Fig. 1. Schematic illustration of the interaction of 2 leptin molecules with the extracellular domains of 2 leptin receptors. The first leptin molecule (oval with solid line; brown, on the Web site only) interacts through its binding site I/II with cytokine homology region 2 (CHR 2) of the left receptor, and through its binding site III with the immunoglobulin-like domain (IGD') of the second receptor. In parallel, the second leptin molecule (oval with broken line; green, on the Web site only) interacts through its binding site I/II with the CHR 2' of the right receptor, and through its binding site III with the IGD of the left receptor. There is no known interaction between the 2 leptins. Mutation L39A/D40A/F41A abolishes leptin's interaction with IGD, preventing receptor activation, but not its binding to CHR 2, and thus acts as an antagonist. Mutation D23L dramatically increases the affinity of leptin (or leptin antagonist) to CHR 2.



LEP actions may cause several different pathologies such as extensive immune responses in autoimmune diseases, tumorigenesis, elevated blood pressure, certain cardiovascular pathologies, and cachexia. In this review, we survey the different approaches that have been explored in an effort to develop such reagents. A different approach that warrants mention but is beyond the scope of this review, is to silence or modify LEP expression in different tissues by small interfering RNA or hormonal treatments.

LEP muteins acting as LEPR antagonists

R128Q mutants

The first LEPR antagonist was reported in 1998, when an R128Q mutant of human LEP was prepared (Verploegen et al. 1997). This LEP mutein (a protein arising as a result of a mutation) acted in the presence of anti-LEP monoclonal (m) Ab as an in-vivo antagonist in C57BL/6J and *ob*/*ob* (obese) mice; it lacked biological activity in vitro in BAF/3 cells transfected with a chimeric receptor composed of an extracellular transmembrane domain of the murine LEPR fused to the intracellular domain of the human βc receptor, a common subunit present in several cytokine receptors. As the VVALSRLQ sequence (amino acids 123–130) is conserved in both mammalian and nonmammalian LEPs (Table 1), we questioned whether the R128Q mutation plays the same role in all LEPs or is restricted to human LEP only. To answer this, we prepared recombinant R128Q muteins of ovine and chicken LEP, and compared their activity to similarly prepared human LEP and human R128Q mutein (Raver et al. 2002). The R128Q mutation did not change the binding properties to BAF/3 cells stably transfected with the long form of human LEPR compared with nonmutated human, ovine, and chicken LEPs, but the biological activity tested in a proliferation assay in the same cells was drastically reduced: the human R128Q mutein lost its activity and even became a weak antagonist, whereas the activities of the ovine and chicken muteins were reduced 25- and 80-fold, respectively, without exhibiting any antagonistic activity (Raver et al. 2002).

S120A/T121A mutants

Another LEPR antagonist was reported in 2004 by Tavernier's group (Peelman et al. 2004). This antagonist was designed to prevent the interaction of LEP bound to receptor 1 through binding site I/II, to the IGD domain of receptor 2 through its binding site III, as depicted schematically in Fig. 1. As claimed by the authors, who based their argument on a carefully designed 3-D model of the LEPR:LEP complex, amino acids S120 and T121 (for their location in the different LEPs see Table 1) contribute to this interaction, and their mutagenesis to A converts both human and murine (LEPs to antagonists (Peelman et al. 2004). Two variants of this antagonist were prepared: the mouse hemagglutinin (HA)-tagged S120A/T121A mutant, and an analogous human nontagged mutant. Both antagonists inhibited mouse LEPR signaling in HEK293T cells co-transfected with mouse or human LEPR and luciferase reporter plasmid in a dose-dependent manner. However, human LEP S120A/T121A was a less potent antagonist for mouse LEPR signaling. The human S120A/T121A mutant also exhibited an in-vivo effect. After 17 to 21 days of intraperitoneal (i.p.) injections in DBA/1 mice in the presence of mAb 2A5 (which prolongs the half life of the antagonists in the circulation), the antagonist caused a statistically significant weight gain of ~15%. To the best of our knowledge, no additional results for in-vivo experiments using this antagonist have been reported.

L39A/D40A/F41A and L39A/D40A/F41A/I42A: development and production

Another approach to developing alternative LEPR antagonists was taken by our group. Using sensitive bidimensional hydrophobic cluster analysis (Callebaut et al. 1997), and comparing the known structures of IL6 receptor complexes [viral (v) IL6/gp130] and IL6/IL6Rα/gp130 complex (Chow et al. 2001; Boulanger et al. 2003), in which site III of IL6 was first identified], we identified aa L39/D40/F41/I42 in the A–B loop of LEP as responsible for the interaction with LEPR that leads to the functional dimerization or tetramerization required for receptor activation. To verify this hypothesis and to test its generality, we prepared and purified to homogeneity several ovine and human recombinant LEP A muteins in the A–B loop (L39A/D40A, L39A/D40A/F41A or L39A/D40A/F41A/I41A) and documented their activity as potent competitive LEPR antagonists (Niv-Spector et al. 2005*a*). To verify the preservation and importance of this sequence for activation of LEPRs (for sequence comparison see Table 1), we also prepared the corresponding muteins of mouse and rat LEP and documented their similar antagonistic activity (Salomon et al. 2006). In a subsequent work, we increased the half-life of the leptin antagonist by pegylation, resulting in significant extension of its bioavailability while retaining antagonistic activity (Elinav et al. 2009*b*). In view of the potential pharmaceutical uses of LEP antagonists, the general question of how the biopotency of recombinant proteins can be enhanced in vivo needs to be explored. One possible approach is to increase the antagonist's affinity for the receptor by increasing k_{on}, or mainly by decreasing k_{off}, and thus prolonging receptor occupation. Theoretical thermodynamic considerations show that if antagonists and agonists exhibit the same affinity, at a 100-fold molar excess of antagonist, 99% of all occupied receptors will be occupied by antagonists. A 100-fold increase in antagonist affinity will lead to similar results at an approximate 1:1 molar antagonist-to-agonist ratio. Pegylation of such muteins combines

rich2/jpp-cjpp/jpp-cjpp/jpp99912/jpp0125d12z | xppws | S=3 | 7/17/13 | 3:33 | 4/Color Fig: F1 | Art: cjpp-2013-0012 | Input-1st disk, 2nd ??
Pagination not final/Pagination non finale

Table 1. Compilation of most of the known mammalian and non-mammalian leptin sequences (the areas whose mutations may lead to creation of leptin antagonists and D23 in mammalian leptins whose mutation to non-negatively charged leads to increased affinity are shaded.

```
                    Helix  A
              --[=======================]----------------------
Human          VPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILT
Sheep          VPIRKVQDDTKTLIKTIVTRINDISHTQSVSSKQRVTGLDFIPGLHPLLS
Pig            VPIWRVQDDTKTLIKTIVTRISDISHMQSVSSKQRVTGLDISHMQSVSSK
Mouse          VPIQKVQDDTKTLIKTLVTRINDISHTQSVSAKQRVTGLDFIPGLHPILS
Rat            VPIHKVQDDTKTLIKTIVTRINDISHTQSVSARQRVTGLDFIPGLHPILS
Horse          VPIRKVQDDTKTLIKTIVTRINDISHMQSVSSKQRVTGLDFIPGLHPVLS
Dog            VPIRKVQDDTKTLIKTIVARINDISHTQSVSSKQRVAGLDFIPGLQPVLS
Chicken        VPCQIFQDDTKTLIKTIVTRINDISHT-SVSAKQRVTGLDFIPGLHPILS
Frog       RAIKADRVKNDAKLLASTLITRIQEHPIQFLFPSNLKISGLDFIPDEQLLES
Salamander   IMVDQLRMDAKNLTLTIMARLQEHPSQFLLPMNLKVSGLDFIPGEQSLES
Pufferfish   LPGALDAMDVEKMKSKVTWKAQGLVARIDKHFPDR--GLRFDTDKVEGST

              Helix  B                    Helix C
             [================]--[======================]-----
Human       LSKMDQTLAVYQQILTSMPSQNVIQISNDLENLRDLLHVLAFSKSCHLPW
Sheep       LSKMDQTLAIYQQILASLPSRNVIQISNDLENLRDLLHLLAASKSCPLPQ
Pig         LSKMDQTLAIYQQILTSLPSRNVIQISNDLENLRDLLHLLASSKSCPLPQ
Mouse       LSKMDQTLAVYQQVLTSLPSQNVLQIANDLENLRDLLHLLAFSKSCSLPQ
Rat         LSKMDQTLAVYQQILTSLPSQNVLQIAHDLENLRDLLHLLAFSKSCSLPQ
Horse       LSKMDQTLAIYQQILTSLPSRNVIQISNDLENLRDLLHLLASSKSCPLPQ
Dog         LSRMDQTLAIYQQILNSLHSRNVVQISNDLENLRDLLHLLASSKSCPLPR
Chicken     LSKMDQTLAVYQQVLTSLPSQNVLQIANDLENLRDLLHLLAFSKSCSLPQ
Frog        LEHMDETLEVFQKILSSLPMENVDQMLSDMENLRSLLQSLSTIMGCTARK
Salamander DDSVDETLEIFHAILSSLHMDNMEQILSDIENLRRLLHALSSLLGCNAQK
Pufferfish --SVVASLESYNNLISDRF-GGVSQIKTEISSLAGYLNHWRE-GNC---Q

                   Helix  E           Helix  D
             -----[=========]--[======================]---
Human       ASGLETLDSLGGVLEASGYSTEVVALSRLQGSLQDMLWQLDLSPGC
Sheep       VRALESLESLGVVLEASLYSTEVVALSRLQGSLQDMLRQLDLSPGC
Pig         ARALETLESLGGVLEASLYSTEVVALSRLQGALQDMLRQLDLSPGC
Mouse       TSGLQKPESLDGVLEASLYSTEVVALSRLQGSLQDILQQVDLSPGC
Rat         TRGLQKPESLDGVLEASLYSTEVVALSRLQGSLQDILQQLDVSPEC
Horse       ARGLETLASLGGVLEASLYSTEVVALSRLQGSLQGMLQQLDLSPGC
Dog         ARGLETFESLGGVLEASLYSTEVVALNRLQAALQDMLRRLDLSPGC
Chicken     TSGLQKPESLDGVLEASLYSTEVVALSRLQGSLQDILQQLDISPEC
Frog        HSQCDTQVNLTEEYAKAPYTTEKVALDRLQKSLHSIVKHLDHITDC
Salamander SVHPDTLGNLTEEYAKSPFTTEKVALDRFQKNLHSIVKHDEHTLSC
Pufferfish ----EQQPKVWPRRNIFNHTVSLEALMRVREFLKLLQKNVDLLERC
```

Note: The sequences were compiled from the NCBI data bank as follows: human NP_000221, sheep Q28603, pig NP_999005, mouse NP_032519, rat NP_037208, horse AAR88257; and from our unpublished results as follows: chicken AAC60368, dog NP_001003070, frog AAX77665, salamander AAY68394, pufferfish NP_001027897.

Table 2. Effect of PEG-MLA and PEG-SMLA on weight gain in male mice determined 1 and 2 weeks after initiation of the injections.

	Weight gain	
Dose ($mg \cdot kg^{-1} \cdot d^{-1}$)	1 week (%)	2 weeks (%)
Control		
—	0.5±0.1	0.9±0.6
PEG-MLA		
0.72	7.8±0.8a	11.3±1.3a
2.2	10.1±0.9b	14.7±1.5ab
6.7	12.0±1.3b	16.6±1.8b
20.0	18.7±2.0c	22.8±1.5c
PEG-SMLA		
0.72	12.7±2.1b	22.0±2.1c
2.2	19.6±1.7c	30.2±2.3d
6.7	27.3±2.1d	42.3±1.8e
20.0	29.0±2.6d	44.7±3.4e

Note: Each material was injected daily at a dose of 20.0, 6.7, 2.2, and 0.72 mg·(kg body mass)$^{-1}$, $n = 8$ mice per dose. The results are presented as percent of body mass change (mean ± SEM). The initial body weight was 20 to 22 g. PEG-MLA, mono-pegylated mouse LEP antagonist; PEG-SMLA, mono-pegylated superactive mouse antagonist. Values in a column that are followed by the same letters do not differ statistically ($p < 0.05$). The values for the control were not included in the statistical analysis.

both approaches, resulting in a potent and effective long-acting competitive antagonist in vivo. To explore this approach, we employed random mutagenesis of mouse LEP followed by selection of high-affinity mutants by yeast-surface display, and discovered that replacing residue D23 with a non-negatively charged amino acid leads to dramatically enhanced affinity of LEP for LEPR. Rational mutagenesis of D23 revealed the D23L substitution to be the most effective. Coupling the D23 mutation (for sequence homology of this amino acid see Table 1) with A mutagenesis of the 3 amino acids previously reported to convert LEP into an antagonist (L39A/D40A/F41A) (Niv-Spector et al. 2005*a*; Salomon et al. 2006) resulted in potent antagonistic activity (Shpilman et al. 2011). Those novel superactive mouse and human LEP antagonists (D23L/L39A/D40A/F41A) termed, respectively, SMLA and SHLA, exhibited over 60-fold increased binding to LEPR, and 14-fold higher antagonistic activity in vitro relative to the L39A/D40A/F41A mutants. To prolong and enhance their in-vivo activity, SMLA and SHLA were monopegylated at the N terminus. Administration of the pegylated (PEG) SMLA to mice resulted in a remarkably rapid, significant and reversible 27-fold more potent increase in body weight as compared with PEG-MLA (Table 2). To test whether the D23L mutation confers increased affinity in other species' LEP, we recently prepared D23L/L39A/D40A/F41A muteins of ovine (Niv-Spector et al. 2012) and rat (G. Solomon and A. Gertler, unpublished results) LEPs and found that they also act as potent LEPR antagonists.

L39A/D40A/F41A and L39A/D40A/F41A/I42A mutants: use for research and therapy

We and others have explored several ways of employing the LEPR antagonists developed in our laboratory for use in research and for eventual therapeutic purposes. The main use of the antagonists as research reagents is to verify the involvement of LEP activity in a particular pathway of interest. Thus, for example, Tam et al. (2012) showed that appetite and weight reduction by cannaboid receptor inverse agonist is mediated by resensitizing mice with diet-induced obesity (DIO) to endogenous LEP by

Table 3. Weight gain and biochemical and hormonal parameters in 8-week-old female mice (after 4 weeks of treatment) and in 16-week-old female mice (after 12 weeks of treatment) subcutaneously injected with PEG-SMLA at 10 mg·(kg body mass)$^{-1}$·d^{-1}, every other day.

	After 4 weeks		After 12 weeks	
Parameter tested	Control	PEG-SMLA	Control	PEG-SMLA
Weight gain	4.30±0.02	14.03±0.03***	7.82±0.70	20.42±0.58***
Glucose ($mg \cdot dL^{-1}$)	188±15	237±21	131±8	183±10*
Cholesterol ($mg \cdot dL^{-1}$)	95.8±8.1	147.6±14.9**	105.4±5.5	153.0±3.7**
Triglycerides ($mg \cdot dL^{-1}$)	97.0±11.9	153.5±15.3*	92.5±6.1	121.7±9.5**
Insulin ($ng \cdot mL^{-1}$)	0.80±0.13	4.11±0.70***	0.31±0.05	1.84±0.30***
HOMA-IR (arbit. units)	57.2±9.4	380.1±90.0***	17.7±4.1	139.5±27.8***

Note: Values are the mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for mice treated with PEG-SMLA (PEG-SMLA, mono-pegylated superactive mouse antagonist) compared with the control mice.

reversing the hyperleptinemia; this is achieved by decreasing LEP expression and secretion by adipocytes, and increasing LEP clearance via the kidney. Such an effect could be blocked by using PEG-SMLA, confirming the role of LEP. Karmazyn and co-workers (Majumdar et al. 2009) investigated the role of LEP and its interaction with endothelin 1 (ET-1) in FN synthesis and cardiomyocyte hypertrophy using human umbilical vein endothelial cells (HUVECs). These cells were examined for FN production, and the neonatal rat cardiomyocytes for hypertrophy, following incubation with glucose, ET-1, LEP, and specific blockers. Glucose caused increased FN mRNA and protein expression in HUVECs, and increased cardiomyocyte hypertrophy along with upregulation of ET-1 mRNA, and LEP mRNA and protein. Glucose-mimetic effects were seen with LEP, and LEPR antagonist normalized these abnormalities.

Our group has recently shown that LEPR antagonist can be used for the creation of a rapid and reversible mouse model of metabolic syndrome and type 2 diabetes mellitus (T2DM) (Solomon et al. 2012). PEG-SMLA has strong orexigenic properties, and when given to mice every 24 to 48 h leads to remarkable weight gain. To date, over 15 experiments have been performed over the course of 2 to 12 weeks to test the effects of PEG-SMLA on weight gain in mice, resulting in a uniform 25% to 45% weight gain in 14 to 21 days. Representative data from these studies, including a long-term experiment in female mice starting at the age of 4 weeks and ending at the age of 4 months, are presented in Table 3. The results clearly show that weight gain is accompanied by elevated glucose, cholesterol, and triglyceride levels, and an even more dramatic increase in insulin levels and insulin resistance (HOMA-IR). Interestingly, no hepatic damage, which would be evidenced by increased levels of liver enzymes, was observed, even after 12 weeks of treatment, although histologically the livers of PEG-SMLA-treated animals seemed fatty. PEG-SMLA treatment did not affect other blood parameters, such as albumin, globulin, creatinine, urea, calcium, potassium, phosphorus, or bilirubin. An additional experiment carried out to test the effects of PEG-SMLA showed abnormal glucose tolerance (by oral glucose tolerance test) after 3 weeks of treatment (Solomon et al. 2012). This change, along with others such as weight gain, increased fat content, hyperinsulinemia, and hypertriglyceridemia, were fully reversible with cessation of PEG-SMLA injections, disappearing within 10–14 days (Elinav et al. 2009*b*; Levi et al. 2011; Solomon et al. 2012). These authors also attenuated endogenous LEP signaling in normal mice with PEG-MLA to determine the contribution of LEP signaling to the regulation of glucose homeostasis. The antagonist

was either injected or continuously administered via osmotic minipumps for several days, and various metabolic parameters were assessed. PEG-MLA-treated mice showed increased fasting and glucose-stimulated plasma insulin levels, decreased whole-body insulin sensitivity, elevated hepatic glucose production, and impaired insulin-mediated suppression of hepatic glucose production. Moreover, PEG-MLA treatment resulted in increased food intake and increased respiratory quotient without significantly altering energy expenditure or body composition, as assessed by the lean-to-lipid ratio. They concluded that alterations in insulin sensitivity occur before changes in the lean-to-lipid ratio and energy expenditure during the acute disruption of endogenous LEP signaling.

Our long-term experiment also allowed us to test the effect of PEG-SMLA treatment on the expression of a large number of genes in white adipose tissue (WAT), liver, brain, and muscle after 4 and 12 weeks of treatment. The most remarkable change was the respective 6.4- and 15.3-fold increase in leptin expression in WAT, paralleled by corresponding 4.8- and 1.1-fold decreases in the expression of LEPR, while the expression of insulin receptor did not change. Interestingly, genes indicating inflammation, such as *IL6*, *inhibitor of nuclear factor kappa-β kinase*, and even *tumor necrosis factor-α* (TNF-α), were hardly changed. The latter did show 4.8-fold higher expression in WAT, but only after 12 weeks, indicating that in contrast to DIO, leptin antagonist-induced obesity leads to only weak inflammation. Interestingly, cluster of differentiation 14 (CD14) was down regulated in the liver by 1.6- and 2.5-fold after 4 and 12 weeks, respectively. CD14 is an important regulatory factor in the expression of the inflammatory response to lipopolysaccharide (LPS) and enhances the effect of LPS considerably in Kupffer cells (Imajo et al. 2012). Upregulation of CD14 in Kupffer cells and hyper-reactivity against low doses of LPS were observed in high-fat (HF) diet-induced steatosis in mice and in contrast, *ob*/*ob* and *db*/*db* (diabetic) mice show decreased hepatic CD14 expression, resulting in a decreased responsiveness to LPS (Imajo et al. 2012). Those results further indicated that DIO and PEG-SMLA-induced obesity are different. Expression of peroxisome proliferator-activator receptor gamma (PPARγ), which regulates fatty acid storage and glucose metabolism, was 4.8- and 1.9-fold higher in the WAT of PEG-SMLA-treated mice but unchanged in the liver. Genes activated by PPARγ stimulate lipid uptake and adipogenesis by fat cells. Sterol coenzyme A desaturase (SCD1), which is an iron-containing enzyme that catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids, was also elevated in both WAT (1.3- and 2.3-fold) and liver (3.1- and 4.8-fold) after 4 and 12 weeks of treatment, respectively. Lipoprotein lipase was correspondingly elevated by 2.1- and 3.3-fold in WAT, but decreased by 3.5- and 1.4-fold in the liver. Another gene of interest is *sirtuin 1* (SIRT1), which is known to be downregulated in cells that have high insulin resistance; inducing its expression increases insulin sensitivity. We found statistically borderline down-regulation of SIRT1 in both the liver and muscles, particularly after 3 months of PEG-SMLA treatment. Liver expression of fibroblast growth factor 21 (FGF21), which stimulates glucose uptake in adipocytes, was not changed after 1 month of PEG-SMLA treatment, but after 3 months it was reduced 5.5-fold, possibly reflecting down-regulation of its known positive regulator, SIRT1. Surprisingly, liver genes related to increased gluconeogenesis, and mostly observed in metabolic syndrome and T2DM, such as *PPARγ co-activator 1*, *phosphoenolpyruvatecarboxy kinase* (PEPCK), *glucose 6-phospatase*, and *fructose bi-phosphatase 1*, were not changed or even decreased. Only minor changes in gene expression in the muscle were observed. LEPR, pyruvate dehydrogenase kinase, glucose transporter 1, PPARδ, and SIRT1 expression were almost unchanged after 4 weeks of treatments, but reduced by 1.5-, 1.5-, 1.4-, 1.4, and 3.4-fold, respectively, after 12 weeks. In the brain, the most significant changes after 12 weeks were moderate decreases in the expression of the anorexigenic apolipoprotein E and pro-opiomelanocortin by 1.3- and 1.4-fold, respectively, while the expression of orexigenic neuropeptide Y was 1.6-fold higher after 4 weeks, but did not change later on.

Another way to use LEPR antagonists for research is to explore the putative role of LEP in bone metabolism. This was first demonstrated in *ob*/*ob* or *db*/*db* mice, which lack LEP or its receptor (Ducy et al. 2000). Both mutant mice showed increased bone formation, leading to high bone mass. This phenotype was dominant, independent of the presence of fat, and specific for the absence of LEP signaling. As most of the experiments in mice were carried out in genetically modified animals, we decided to use the aforedescribed 3 month experiment (see Table 3) to test the effect of blocking LEP on several bone parameters. LV3 and LV4 and tibias were removed after 1 and 3 months of treatment and analyzed for different bone parameters, such as trabecular morphometric analysis of LV3 and LV4, cortical morphometric analysis of tibia, and the biomechanical 3-point bending test. The results indicated a significantly higher (27%–30%) bone volume fraction and 20%–22% increased trabecular thickness in LV3 and LV4 of PEG-SMLA-treated (compared with the control) mice. Cortical morphometric analysis of the tibia from the PEG-SMLA group showed significantly increased cross-sectional (14%–16%) and medullary (19%–22%) area, and a 42% to 46% increase in mean polar moment of inertia with no changes in cortical thickness or (bone mass density (BMD). A mechanical assay that measures the ultimate load required for fracture, the stiffness, and the fracture yield showed significantly higher (15%–60%) values in the PEG-SMLA-treated mice. Interestingly, all of these differences appeared after only 1 month of treatment of 4-week-old mice, and remained for the whole period of the experiment. In addition, dynamic histomorphometric analysis was performed: bones were labeled by injecting calcein on day 20 of the experiment, and by injecting alizarin complexone on day 26. The mean distance/time interval between labels indicated that bone growth in mice injected with PEG-SMLA is (mean ± SEM, $n = 5$) 0.84 ± 0.05 compared with 0.63 ± 0.05 in the control mice ($p < 0.05$). All of those results favored the notion that blocking LEPR enhances bone formation.

We also used LEPR antagonist to study the effect of early postnatal LEP blockage on long-term LEP resistance and susceptibility to diet-induced obesity in rats. We showed that blockage of LEP action during the critical period of early life in rodents has long-term consequences by altering the capacity to respond to LEP during adulthood, thus predisposing the animals to obesity. We concluded that the physiological importance of the postnatal LEP surge lies in its being required for optimal onset of metabolic regulation, at least in rodents, and in its implication in the prevention of unfavorable developmental programming (Attig et al. 2008). Similar results in rats were also reported recently by Beltrand et al. (2012) who concluded that neonatal LEPR antagonist treatment exert effects on growth and metabolism in adulthood but is dependent upon interactions between maternal and post-weaning nutrition.

Rat LEPR antagonist L39A/D40A/F41A was extensively studied by Scarpace's group (Scarpace and Zhang 2009). Use of this antagonist revealed that the normalization of caloric intake and the thermic effect of food after HF feeding are LEP-dependent. Rats were then fed a HF diet (60% kcal as fat) or chow and simultaneously infused with antagonist (25 $\mu g \cdot d^{-1}$ into the lateral ventricle) for 7 days, and compared with vehicle-infused chow-fed rats. Daily caloric intake of both HF groups peaked on day 2. HF feeding elevated caloric intake, which was nearly normalized by day 7, whereas in the presence of the antagonist, caloric intake remained elevated. Moreover, the HF-mediated increase in UCP1 in brown adipose tissue (BAT) was prevented by the antagonist. These results demonstrated that LEP is essential for the homeostatic restoration of caloric intake after HF feeding and that this LEPR antagonist blocks central LEP signaling and LEP-mediated UCP1 elevation (Zhang et al. 2007). The same group also used LEPR

antagonists to document uncoupling between LEPR signal transducer and activator of transcription 3 (STAT-3) signaling and metabolic responses in rats with central LEP resistance. Such resistance was induced in lean rats by intracerebroventricular injection of a recombinant adeno-associated viral (rAAV) vector encoding LEP. Following development of LEP resistance, on day 153, these rats and the control rats were centrally infused with either vehicle or a rat LEPR antagonist for 14 days. Food intake, body weight, adiposity, and UCP1 expression increased with antagonist infusion in the controls, but increased only marginally in the LEP-resistant rats (Zhang et al. 2007).The same group also showed that hypothalamic overexpression of LEP elevates blood pressure by 18 mm Hg (1 mm Hg = 133.322 Pa), but a 14 day central infusion of the LEPR antagonist reverses LEP-induced hypertension. In contrast, a HF diet increased blood pressure (by approximately 8–9 mm Hg) and tyrosine hydroxylase activity (by 76%) in superior cervical ganglia, compared with chow feeding. Infusion of LEPR antagonist accelerated weight gain, food intake, and adiposity in rats fed the HF diet compared with the chow-fed rats, and tyrosine hydroxylase activity was also reversed in the superior cervical ganglia, thus it can be concluded that central overexpression of LEP-induced hypertension can be reversed by a LEPR antagonist. In contrast, this LEPR antagonist did not reverse the HF-diet-induced elevation in blood pressure, even though there was apparent blockage of other LEP-mediated metabolic and sympatho-excitatory responses (Tumer et al. 2007).

The potential use of our LEPR antagonists for therapeutic purposes was explored by us and others in mouse models of autoimmune disease and cachexia. As a role for LEP had been suggested in the pathogenesis of intestinal autoinflammation, we first investigated the effect of LEPR antagonists in a mouse model of inflammatory bowel disease (IBD). IBD is a chronic inflammatory disorder affecting 0.3% of the Western world's population; its pathogenesis is thought to result from loss of tolerance of the intestinal immune system in the presence of constant antigenic stimuli mediated by resident microflora. LEP's central role as a mediator of colonic autoinflammation has been suggested in both animal models and human studies. LEP-deficient *ob*/*ob* mice are resistant to acute and chronic experimental colitis (Siegmund et al. 2002, 2004). In those studies, LEP was suggested to enhance proinflammatory cytokine secretion, including that of interferon gamma (IFN-γ), TNF-α, IL18, IL1β, IL6, and IL10, and to inhibit inflammation-associated apoptosis of colonic mononuclear cells. Inflamed colonic epithelial cells were also found to express and release LEP apically into the intestinal lumen. Intrarectal administration of LEP induced activation of NF-κB and epithelial wall damage associated with neutrophil infiltration (Sitaraman et al. 2004). In human IBD patients, LEP levels have been suggested to correlate with the severity of the disease (Ballinger et al. 1998; Karmiris et al. 2005, 2007). To study the effect of LEPR antagonists on an experimental mouse model of IBD, we used the dextran sodium sulfate (DSS)-axoxymethane-induced model of IBD. DSS (2%) in the drinking water induced severe colitis, manifested as significant colitis on day 6 and maximal scores at later stages. Administration of PEG-SMLA induced a very significant survival advantage over wild-type mice. Colitis at an early time point (day 6) was significantly milder in PEG-SMLA-treated mice than in the vehicle- and PEG-MLA-treated groups. At later time points, however, colonoscopy scoring (grading the most severe inflammatory foci in each mouse) was similar in all groups. In severe colitis, differences in severity were minimal. Administration of PEG-SMLA without DSS induced significant weight gain in mice, with no apparent adverse general or colonic effects (E. Elinav and A. Gertler, unpublished data). In another study, the effect of LEPR antagonist in a mouse model of liver fibrosis was determined in vivo, using thioacetamide (TAA)-induced fibrosis. To induce liver cirrhosis, mice were given an i.p. injection of 200 mg·(kg body mass)$^{-1}$ TAA twice a week for 12 weeks. Administration of LEPR antagonist induced significant amelioration in this model of chronic liver inflammation and fibrosis (Elinav et al. 2009*a*).

We also found that the superactive LEPR antagonist induces protection from innate inflammation by inhibiting infiltration of mononuclear phagocytes. Superactive pegylated LEPR antagonist or pegylated LEPR agonist was administered to female C57BL mice for 4 days. This was followed by induction of innate hepatitis via administration of LPS and D-GalNac, a known model for the induction of innate hepatitis through infiltration and TNF-α secretion by mononuclear phagocytes. Administration of PEG-MLA resulted in significantly enhanced mortality, compared with vehicle-treated mice. In contrast, administration of PEG-SMLA resulted in significant protection, manifested as improved survival. In testing the effect of LEPR antagonism on the infiltrating macrophages, PEG-SMLA's protective effect was accompanied by a dramatic reduction in the inflammation-induced population of liver-infiltrating macrophages, compared with vehicle-treated mice. A reverse phenotype was noted in mice treated with PEG-MLA, whereby liver macrophage infiltration was enhanced, compared with vehicle-treated mice (E. Elinav and A. Gertler, unpublished results). Taken together, these results demonstrated a significant protective effect of the superactive LEPR antagonist in innate immune-mediated inflammation, via the inhibition of mononuclear macrophage infiltration into the inflamed organ.

However, the most impressive use of LEPR antagonists was observed in the treatment of experimental chronic kidney disease (CKD)-associated cachexia (Cheung et al. 2011). The negative role of LEP in uremic patients has been reviewed (Teta 2012). LEP has been defined as a true uremic toxin, and reducing its levels in uremic patients, particularly those suffering from uremia-related cachexia, may have beneficial effects. This suggestion is based on the finding that plasma LEP is associated with reduced energy intake and protein-wasting in uremic patients (Mak et al. 2006). It was also shown that elevated circulating levels of cytokines such as LEP might be an important cause of CKD-associated cachexia (Cheung et al. 2005). Experimental CKD was induced by 5/6 nephrectomy in 8-week-old c57BL/6J male mice. CKD and sham (S) mice received either PEG-SMLA (7 mg·kg^{-1}, i.p.) or vehicle (V) once a day for 28 days. All mice were fed ad libitum during this period. Metabolic rate was measured by Oxymax, body composition by Echo-MRI, and muscle function by Rotarod activity and grip strength. The results clearly showed that PEG-SMLA treatment reverses anorexia in CKD. The food intake of the CKD/PEG-SMLA mice was significantly increased compared with CKD/V mice (3.7 ± 0.0 vs. 3.1 ± 0.1 g·mouse^{-1}·d^{-1}; $p < 0.001$). CKD/PEG-SMLA mice also gained more weight than CKD/V mice (15.1 ± 0.4% vs. 3.0 ± 0.3%; $p < 0.001$). CKD/PEG-SMLA mice gained fat mass (gain of 0.4 ± 0.1 g) and lean mass (gain of 0.2 ± 0.1 g), while CKD/V mice continued to lose fat mass (loss of 0.2 ± 0.05 g) and lean mass (loss of 1.3 ± 0.1 g; $p < 0.001$). Basal metabolic rate was increased in CKD/V mice (3845 ± 62 mg·kg^{-1}·h^{-1}) compared with S/V mice (3369 ± 43 mg·mL^{-1}·h^{-1}; $p < 0.001$), and was normalized in CKD/PEG-SMLA mice (3246 ± 79 mg·mL^{-1}·h^{-1}). Rotarod activity and grip strength were lower in CKD/V mice (130.3 ± 5.8 s and 1149.6 ± 49.2/100 g, respectively; $p < 0.001$) compared with S/V mice (187.0 ± 12.0 s and 1643.3 ± 28.2/100 g, respectively), and were normalized in CKD/PEG-SMLA mice (175.8 ± 3.8 s, 1568.2 ± 46.0·(100 g)$^{-1}$, respectively). Furthermore, the expression of PAX 3, a member of the paired box (PAX) family of transcription factors that has been shown to contribute to early striated muscle development, was reduced >2-fold in CKD/V mice, but almost normalized in CKD/PEG-SMLA mice. In contrast, expression of 2 other proteins, atrogin-1 (which is an E3 ubiquitin ligase that mediates proteolysis events in response to a variety of catabolic states in muscle), and RING-finger protein-1 (MuRF1; a ubiquitin ligase that has been proposed to trigger muscle protein degradation during pathophysiological muscle wasting), was increased by >2-fold in CKD/V mice, but almost normalized in CKD/PEG-SMLA mice. In addition, the expression

of proinflammatory cytokines (IL1α, IL1β, IL6, and TNF-α) in the liver of CKD/V mice was increased from 2- to 4-fold compared with S/V mice, but almost normalized in CKD/PEG-SMLA mice (M. Ayalon-Soffer, personal communication). In conclusion, PEG-SMLA, a peripheral LEPR antagonist, reverses anorexia, ameliorates lean body mass loss, and normalizes muscle function in a mouse model of CKD-associated cachexia (Cheung et al. 2011). These results imply that LEP blocking may be a suitable strategy for the treatment of CKD patients.

LEP fragments acting as low-molecular-weight LEPR antagonists

LPA-2 and LP-1 peptides

Gonzalez and Leavis (2003) and Gonzalez et al. (2006) were the first to report that LEP activity can be antagonized by peptides derived from the LEP sequence. They prepared 26- and 32-aa-long peptides termed, respectively, LPA-2 and LP-1, corresponding to amino acids 70–95 and 3–34 of human LEP. They showed that at high doses, these peptides inhibit LEP action in vitro and in vivo. These inhibitory effects included attenuation of LEP-dependent increases in the level of β3-integrin, IL1, leukemia inhibitory factor, and their corresponding receptors in mouse endometrial cells (Styer et al. 2008), and reduction in the number of implantation sites and uterine horns in implanted embryos (Gonzalez et al. 2006). The same group also reported that LPA-2 attenuates LEP-induced growth of mouse mammary tumor (MT) cells (Styer et al. 2008). They stated that this effect was more pronounced in vivo than in vitro. The mechanism of action of these peptides has yet to be clarified, as their direct interaction with LEPR has not yet been proven, and the binding experiments (see Fig. 1 in Gonzalez and Leavis 2003) show a nontypical dose–response curve extending over 8 orders of magnitude. The impact of blocking LEPR signaling in the mouse endometrium was assessed in subsequent work. Intrauterine injection of either LEP peptide antagonists or LEPR Ab on day 3 of pregnancy impaired mouse implantation in comparison with the intrauterine injection of scrambled peptides (LPA-Sc) or species-matched IgGs. A significant reduction in the number of implantation sites and uterine horns with implanted embryos was found after intrauterine injection of LPA-1 and LPA-2. The respective scrambled peptides were used as controls (Ramos et al. 2005). The same group also studied the importance of LEP signaling in experimental MTs in mice. Pretreatment of syngeneic mice with LPrA2 (formerly LPA-2) prior to inoculation with mouse 4T1 mammary cancer cells delayed the development and slowed the growth of MTs (up to 90%) compared with the controls. The in-vivo effects obtained by using pegylated LPrA2 were even more pronounced. Serum levels of vascular endothelial growth factor (VEGF) and VEGF/VEGF-R2 expression in MTs were significantly lower in mice treated with LPrA2. Interestingly, LPrA2-induced effects were more pronounced in vivo than in vitro, suggesting paracrine actions in stromal, endothelial, and (or) inflammatory cells that may impact the growth of MTs (Gonzalez et al. 2006). Additional studies carried out by the same group explored the effect of disruption of in-vivo LEP signaling on the establishment and (or) maintenance of an endometriosis-like lesion in a syngeneic immunocompetent mouse model of endometriosis. Findings showed that disruption of LEP signaling by i.p. injection of the pegylated LEP peptide receptor antagonist LPrA impaired the establishment of endometriosis-like lesions, and resulted in a reduction of viable organized glandular epithelium VEGF A expression and mitotic activity. PEG-LPrA treatment resulted in a significant reduction in microvascular density in endometriosis-like lesions after continuous and acute courses, confirming that LEP signaling is a necessary component in lesion proliferation, early vascular recruitment, and maintenance of neo-angiogenesis in a murine model of endometriosis (Styer et al. 2008). More recently, it has also been shown that phosphorylation of VEGF-R2, p38(MAPK), and Akt, as well as COX-2 induction in cells challenged with LEP, are blocked by LPrA2. It was also demonstrated that HUVECs that express LEPRs LPrA2 (30 μmol/l) inhibit the LEP-promoted phosphorylation of VEGF-R2, while VEGF-induced receptor phosphorylation is unaffected. In cells treated with LPrA2, LEP did not promote Stat-3 phosphorylation, while VEGF-induced Stat-3 phosphorylation was still evident. Similarly, exposure to LPrA2 reduced LEP-stimulated COX-2 induction without affecting the response to VEGF. Those authors concluded that leptin-mediated VEGF-R2 phosphorylation and its downstream effects require binding to LEPR (Garonna et al. 2011).

Antagonistic peptides analogous to site III of LEP

A different approach was explored by the group of Surmacz and Otvos (for their most recent review see (Otvos and Surmacz 2011). Those researchers first developed a modified glycopeptide (corresponding to amino acids 119–130 of LEP) that exhibited agonistic activity (Otvos et al. 2008) and then, using non-natural amino acids, modified its structure to generate LEPR antagonists (Otvos et al. 2011*b*). The 3 most potent peptides were termed as follows: (*i*) Aca1 (H-Thr-Glu-Nva-Val-Ala-Leu-SerVALSR-Aca-NH_2); (*ii*) Allo-Aca (H-alloThr-Glu-Nva-Val-Ala-Leu-Ser-Arg-Aca-NH_2); and (*iii*) D-Ser (H-(D)Ser-Glu-Nva-Val-Ala-Leu-Ser(N-Me)-Arg-βAla-NH_2). The authors reported that Allo-aca reduces LEP-dependent growth and signaling in hormone-positive and -negative breast cancer cell lines with IC_{50} values of 50 to 200 pmol·L^{-1} (Otvos et al. 2011*a*, 2011*b*). In immunocompromised mice, Allo-aca suppressed the growth of established hormone-sensitive orthotopic human breast cancer xenografts by 45%–51% when administered either i.p. or subcutaneously (s.c.) for 38 days at a daily dose as low as 0.1 mg·kg^{-1}·d^{-1} (Otvos et al. 2011*a*). The LEPR antagonist peptides penetrated the blood–brain barrier, probably via short-form LEPR-mediated transport at the blood–brain barrier, and interfered with CNS functions (Otvos et al. 2011*a*, 2011*b*). The peptides were distributed to the brain, and when added to normally growing C57BL/6 mice i.p., s.c., or orally, the lead antagonist accelerated normal weight increase without producing any toxic effects. Weight gain increases could not be observed after 10–12 days of treatment, indicating that the mice had become resistant to the CNS activity of the LEP antagonists. However, in normally growing rats, intranasal administration at 0.1 mg·kg^{-1}·d^{-1} for 20 d resulted in a net total body weight gain of 2% with no signs of resistance induction. It should be noted, however, that the half-life of those antagonists, where determined, was quite short, 5–10 min at most (Otvos et al. 2011*b*). The same group also explored the role of LEP and LEPR antagonism in vitro and in vivo in 2 preclinical models of rheumatoid arthritis: K/BxN, and adjuvant-induced arthritis (Otvos et al. 2011*c*). Allo-aca peptide treatment (0.01 to 1 mg·kg^{-1}·d^{-1}) partially attenuated the severity of the disease in a dose-dependent manner, as measured by the number of arthritic joints, but only the highest dose showed borderline statistical significance ($p > 0.1$). Using another parameter (percentage of total paw swelling), Allo-aca peptide (0.1 mg·kg^{-1}·d^{-1}) very significantly ameliorated ($p > 0.01$) paw swelling. However, a similar effect was obtained by injecting LEP, thus questioning whether LEPR blockage is responsible for that effect. In the K/BxN model, LEP injection (0.05 mg·kg^{-1}·d^{-1}) increased the arthritic score, and this effect could be attenuated by co-injection of the Allo-aca peptide (0.1 mg·kg^{-1}·d^{-1}). Furthermore, the same group was the first to demonstrate that an Aca-1 peptide inhibits proangiogenic and growth effects of LEP in glioblastoma endothelial cells, and that the pharmacological potential of this compound might be enhanced by combining it with drugs targeting the VEGF pathway (Ferla et al. 2011). The second-generation LEPR antagonist, Allo-aca, has shown similar activity in several endothelial cell models (E. Surmacz, personal communication).

LEPR Abs acting as LEPR antagonists

Nanobodies (variable domains of the *Camelidae* family heavy-chain antibodies)

An alternative approach was undertaken by Tavernier's group who targeted the LEPR by generating LEPR-specific nanobodies (Zabeau et al. 2012). They created a large number of nanobodies and selected them by measuring their interaction with various domains of the extracellular part of LEPR (see Fig. 1). Nanobodies against CHR 1 did not block LEP binding or signaling, whereas those raised against CHR 2 competed with both. In contrast, nanobodies against the IGD and FNIII domain blocked the signaling but not the binding. Based on those experiments, 3 neutralizing nanobodies were selected: (*i*) 2.17 (against CHR 2, interfering with LEP binding), (*ii*) 4.10 (against IGD), and (*iii*) 4.11 (against FNIII). To prolong their in-vivo half-life in the circulation, those nanobodies were genetically fused to monoclonal-albumin nanobody (m-Alb) using a flexible G-S linker, without compromising the latter's ability to interact with albumin. Subsequently, the respective recombinant proteins (termed 2.17-mAlb, 4.10-mAlb, and 4.11-mAlb) were produced in HEK293 cells and purified to homogeneity. All 3 nanobodies interfered with LEP-induced JAK2 phosphorylation and STAT3 activation, and were tested in vivo in C57BL/6 mice. The strongest effect in the in-vivo experiment in mice, using body weight gain as a readout, was of 4.10-mAlb, while injections of 2.17-mAlb had intermediate effects, and 4.11-mAlb had no effect at all. As the effects on body weight could also be explained by differences in the half lives of the 3 nanobodies in the circulation, the authors measured serum concentrations of the mAlb-fused nanobodies after 1 week of treatment, and showed that the level of 4.10-mAlb was ~2-fold higher than that of 2.17-mAlb, and ~f4-fold higher than that of 4.11-mAlb. In an additional in-vivo experiment, they showed that a 14 d treatment with 4.10-mAlb increases food intake by 40%, doubles the weight of the fat pad, and elevates the blood insulin levels by 77%. In conclusion, Tavernier et al. documented that nanobodies targeting different domains of the LEPR interfere with its activation via different mechanisms: nanobodies against CHR 2 block LEP binding, whereas nanobodies against the IGD and FNIII domains block receptor activation (the latter only in vitro) without affecting LEP binding.

Monoclonal Abs against LEPR

Ross and Strasburger's groups developed mAbs against human LEPR, and verified their antagonistic activity using a LEP-signaling bioassay (Fazeli et al. 2006). The most promising mAb was 9F8, which showed dose-dependent antagonist activity in the LEP bioassay. Specificity of the mAb for the LEPR was confirmed using a plate-binding assay. The Fab fragment of 9F8 was cloned, and recombinant 9F8 Fab (rFab) was purified from the periplasmic fraction of *E. coli* using a C-terminal His tag. Purified 9F8 rFab bound to human LEPR and exhibited LEP-antagonist activity. In-vitro studies demonstrated that the 9F8 mAb inhibits LEP-induced TNF-α production in human monocytes, and that anti-CD3 mAb induces proliferation of human T cells in PBMC culture (Fazeli et al. 2006). The same group had previously confirmed that the anti-LEPR Ab 9F8 binds specifically to HEK293 cells transfected with human LEPR (Zarkesh-Esfahani et al. 2001). Most recently, those researchers crystallized the Fab fragment of 9F8, both in its uncomplexed state and bound to the LBD of human LEPR, and described the structure of the LBD-9F8 Fab complex and the conformational changes in 9F8 associated with LEP binding. A single copy of the LBD-9F8 Fab complex showed that the latter component binds to the N-terminal subdomain of the LBD. The IC_{50} values for LEP and 9F8 mAb binding to LEPR were comparable (0.76 and 1.0 nmol·L^{-1}, respectively). Using a gel-filtration experiment in which complex formation of 9F8 rFab (or LEP) to LBD that corresponds to CHR 2, it was documented that LEP and 9F8 Fab cannot create complexes simultaneously. A molecular model of the putative LEP–LBD complex revealed that 9F8 Fab blocks LEP binding through only a small (10%) overlap in their binding sites, and that LEP binding is likely to involve an induced fit mechanism (Carpenter et al. 2012).

Another group (Purdham et al. 2008) used anti-LEPR Ab produced by Alpha Diagnostic International (San Antonio, Texas, USA) to demonstrate that it mitigates hypertrophy and hemodynamic dysfunction in the post-infarcted rat heart; LEP has been shown to exert direct hypertrophic effects on cultured cardiomyocytes, although its role as an endogenous contributor to post-infarction remodeling and heart failure has not been determined. They further investigated the effect of LEPR blockade in vivo on hemodynamic function and cardiac hypertrophy following coronary artery ligation (CAL). CAL produced myocardial hypertrophy, which was most pronounced 28 days post-infarction, as demonstrated by increases in both left ventricular mass : body mass ratio and atrial natriuretic peptide gene expression. Both phenomena were abrogated by LEPR antagonism. LEPR blockade also significantly improved left ventricular systolic function, attenuated the increase in left ventricular end-diastolic pressure, and reduced the expression of genes associated with ECM remodeling 28 days after CAL. They concluded that the LEPR-neutralizing Ab's ability to improve cardiac function indicates that endogenous LEP contributes to cardiac hypertrophy following CAL.

LBD and LEP-neutralizing Abs

Matarese and co-workers investigated the mechanisms by which in-vivo abrogation of LEP effects protects SJL/J mice from proteolipid protein (PLP) peptide (139–151)-induced autoimmune encephalomyelitis, termed experimental autoimmune encephalomyelitis (EAE). Blockade of LEP with anti-LEP Abs or with a soluble mouse LEPR chimera (LEPR:Fc from R&D Systems), either before or after onset of EAE, improved clinical score, slowed disease progression, reduced disease relapses, inhibited PLP(139–151)-specific T-cell proliferation, and switched cytokine secretion to a Th2/regulatory profile. This was also confirmed by the induction of forkheadbox p3 (FOXP3) expression in CD4 T cells in LEP-neutralized mice. Importantly, anti-LEP treatment induced a failure to down-modulate the cyclin-dependent kinase inhibitor p27 (p27) in autoreactive CD4 T cells. These effects were associated with increased tyrosine phosphorylation of both extracellular-related kinases 1 and 2 (ERK1/2) and STAT6. In summary, blocking LEP led to significant clinical improvement and delayed disease progression during the subsequent 40 days of observation, indicating that LEP blockade inhibited both development and progression of EAE (De Rosa et al. 2006). In another study, the same group reported that LEP can act as a negative signal for the proliferation of human naturally occurring $FOXP3^+CD4^+CD25^+$ regulatory T (T_{reg}) cells. Freshly isolated T_{reg} cells produced LEP and expressed high amounts of LEPR. In-vitro neutralization with anti-LEP mAb during anti-CD3 and anti-CD28 stimulation resulted in T_{reg} cell proliferation, which was IL2-dependent. T_{reg} cells that proliferated in the presence of LEP mAb showed increased expression of Foxp3 and remained suppressive. The phenomena appeared secondary to LEP signaling via ObR and, importantly, LEP neutralization reversed the anergic state of the T_{reg} cells, as indicated by down-modulation of p27 (p27kip1) and phosphorylation of ERK1/2. Together with the finding of enhanced proliferation of T_{reg} cells observed in LEP- and LEPR-deficient mice, these results suggest a potential for therapeutic intervention by LEP blocking (De Rosa et al. 2007). More recently, Matarese's group has also reported the development of another anti-LEP mAb (971212) capable of in-vitro blocking of LEP's effects (Moon et al. 2011).They also recently studied the LEP-induced activation of mTOR, which in turn controls LEP production and signaling and causes a defined cellular, biochemical, and transcriptional signature that determines the outcome of $CD4^+CD252$ FOXP32 T cells' (Teffs') re-

sponses, both in vitro and in vivo. The blockade of LEP/LEPR signaling induced by anti-LEP mAb impaired mTOR activity, which inhibited the proliferation of Teffs in vivo (Procaccini et al. 2012).

Conclusions

The studies reviewed in this paper clearly indicate that developing different means of blocking LEP activity is a timely pursuit, as some of the pleiotropic activities of LEP are undesirable. Several groups have tried different ways of addressing this problem. Future research is needed to determine which of the proposed means will give the optimal result. The main questions that have yet to be answered are as follows: (*i*) Which is preferable? Blocking of circulating LEP or blocking of membrane-embedded LEPR? (*ii*) Are small peptides or large proteins such as mAbs, nanobodies, or LEP mutants exhibiting antagonistic activity better blockers in vivo? (*iii*) How should the half-life of proteins in the circulation be prolonged? By pegylation, preparation of proteins fused to albumin or albumin-binding peptides, or PASylation? PASylation is the genetic fusion of a therapeutic protein with a conformationally disordered polypeptide of defined sequence comprising the aa P, A, and S. This technology provides a superior way to attach a solvated random molecular chain with large hydrodynamic volume to a biologically active protein. Owing to this size effect, the typically rapid clearance of a biopharmaceutical via kidney filtration can be retarded by a factor of 10 to 100, depending on the length of the PAS tag. PAS sequences are highly soluble, although uncharged and biochemically inert. They offer efficient recombinant protein production in *E. coli* as well as in eukaryotic host cells, without the need for chemical coupling procedures. They are nontoxic, nonimmunogenic, and stable against plasma proteases, while being biodegradable. Another as yet unexplored issue in the study of LEP-blocking agents is targeting to specific tissues or cells. This can be achieved by fusion of cytokines to Ab or Ab fragments that enable targeted delivery, and should, therefore, improve efficacy and pharmacokinetics. A recent review provides a comprehensive summary of the developments in the field of targeted cytokine delivery by genetic engineering of Ab–cytokine fusion proteins (Kontermann 2012). In conclusion, different approaches for blocking leptin action were reviewed. Some of those reagents such as (D23L/L39A/D40A/F41A) antagonists and antagonistic peptides analogous to site III of LEP, underwent pre-clinical trials and should be tested in Phase I experiments in the near future.

References

Attig, L., Solomon, G., Ferezou, J., Abdennebi-Najar, L., Taouis, M., Gertler, A., et al. 2008. Early postnatal leptin blockage leads to a long-term leptin resistance and susceptibility to diet-induced obesity in rats. Int. J. Obes. (Lond.), **32**(7): 1153–1160. doi:10.1038/ijo.2008.39. PMID:18379577.

Ballinger, A., Kelly, P., Hallyburton, E., Besser, R., and Farthing, M. 1998. Plasma leptin in chronic inflammatory bowel disease and HIV: implications for the pathogenesis of anorexia and weight loss. Clin. Sci. (Lond.), **94**(5): 479–483. PMID:9682669.

Beltrand, J., Sloboda, D.L., Connor, K.L., Truong, M., and Vickers, M.H. 2012. The effect of neonatal leptin antagonism in male rat offspring is dependent upon the interaction bewteen prior maternal nutrition status and post-weaning diet. J. Nutr. Metab. 2012. doi:10.1155/2012/296935. PMID:22548153.

Boulanger, M.J., Chow, D.C., Brevnova, E.E., and Garcia, K.C. 2003. Hexameric structure and assembly of the interleukin-6/IL-6 alpha-receptor/gp130 complex. Science, **300**(5628): 2101–2104. doi:10.1126/science.1083901. PMID:12829785.

Callebaut, I., Labesse, G., Durand, P., Poupon, A., Canard, L., Chomilier, J., et al. 1997. Deciphering protein sequence information through hydrophobic cluster analysis (HCA): current status and perspectives. Cell. Mol. Life Sci. **53**(8): 621–645. doi:10.1007/s000180050082. PMID:9351466.

Carpenter, B., Hemsworth, G.R., Wu, Z., Maamra, M., Strasburger, C.J., Ross, R.J., et al. 2012. Structure of the human obesity receptor leptin-binding domain reveals the mechanism of leptin antagonism by a monoclonal antibody. Structure, **20**(3): 487–497. doi:10.1016/j.str.2012.01.019. PMID:22405007.

Cheung, W., Yu, P.X., Little, B.M., Cone, R.D., Marks, D.L., and Mak, R.H. 2005. Role of leptin and melanocortin signaling in uremia-associated cachexia. J. Clin. Invest. **115**(6): 1659–1665. doi:10.1172/JCI22521. PMID:15931394.

Cheung, W., Ayalon-Soffer, M., and Mak, R.H. 2011. Peripheral administration of the leptin antagonist BL-5040 ameliorates cachexia and normalizes muscle function in mice with chronic kidney disease. J. Cachexia Sarcopenia Muscle, **2**(3): 209–261. doi:10.1007/s13539-011-0045-3. PMID:22476871.

Chow, D., He, X., Snow, A.L., Rose-John, S., and Garcia, K.C. 2001 Structure of an extracellular gp130 cytokine receptor signaling complex. Science, **291**(5511): 2150–2155. doi:10.1126/science.1058308. PMID:11251120.

De Rosa, V., Procaccini, C., La Cava, A., Chieffi, P., Nicoletti, G.F., Fontana, S., et al. 2006. Leptin neutralization interferes with pathogenic T cell autoreactivity in autoimmune encephalomyelitis. J. Clin. Invest. **116**(2): 447–455. doi:10.1172/JCI26523. PMID:16410832.

De Rosa, V., Procaccini, C., Cali, G., Pirozzi, G., Fontana, S., Zappacosta, S., et al. 2007. A key role of leptin in the control of regulatory T cell proliferation. Immunity, **26**(2): 241–255. doi:10.1016/j.immuni.2007.01.011. PMID:17307705.

Ducy, P., Amling, M., Takeda, S., Priemel, M., Schilling, A.F., Beil, F.T., et al. 2000. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell, **100**(2): 197–207. doi:10.1016/S0092-8674(00)81558-5. PMID:10660043.

Elinav, E., Ali, M., Bruck, R., Brazowski, E., Phillips, A., Shapira, Y., et al. 2009*a*. Competitive inhibition of leptin signaling results in amelioration of liver fibrosis through modulation of stellate cell function. Hepatology, **49**(1): 278–286. doi:10.1002/hep.22584. PMID:19065677.

Elinav, E., Niv-Spector, L., Katz, M., Price, T.O., Ali, M., Yacobovitz, M., et al. 2009*b*. Pegylated leptin antagonist is a potent orexigenic agent: preparation and mechanism of activity. Endocrinology, **150**(7): 3083–3091. doi:10.1210/en.2008-1706. PMID:19342450.

Fazeli, M., Zarkesh-Esfahani, H., Wu, Z., Maamra, M., Bidlingmaier, M., Pockley, A.G., et al. 2006. Identification of a monoclonal antibody against the leptin receptor that acts as an antagonist and blocks human monocyte and T cell activation. J. Immunol. Methods, **312**(1–2): 190–200. doi:10.1016/j.jim.2006.03.011. PMID:16690078.

Ferla, R., Bonomi, M., Otvos, L., Jr., and Surmacz, E. 2011. Glioblastoma-derived leptin induces tube formation and growth of endothelial cells: comparison with VEGF effects. BMC Cancer, **11**: 303. doi:10.1186/1471-2407-11-303. PMID:21771332.

Fong, T.M., Huang, R.R., Tota, M.R., Mao, C., Smith, T., Varnerin, J., et al. 1998. Localization of leptin binding domain in the leptin receptor. Mol. Pharmacol. **53**(2): 234–240. PMID:9463481.

Garonna, E., Botham, K.M., Birdsey, G.M., Randi, A.M., Gonzalez-Perez, R.R., and Wheeler-Jones, C.P. 2011. Vascular endothelial growth factor receptor-2 couples cyclo-oxygenase-2 with pro-angiogenic actions of leptin on human endothelial cells. PLoS One, **6**(4): e18823. doi:10.1371/journal.pone.0018823. PMID:21533119.

Gonzalez, R.R., and Leavis, P.C. 2003. A peptide derived from the human leptin molecule is a potent inhibitor of the leptin receptor function in rabbit endometrial cells. Endocrine, **21**(2): 185–195. doi:10.1385/ENDO:21:2:185. PMID:12897384.

Gonzalez, R.R., Cherfils, S., Escobar, M., Yoo, J.H., Carino, C., Styer, A.K., et al. 2006. Leptin signaling promotes the growth of mammary tumors and increases the expression of vascular endothelial growth factor (VEGF) and its receptor type two (VEGF-R2). J. Biol. Chem. **281**(36): 26320–26328. doi:10.1074/jbc.M601991200. PMID:16825198.

Imajo, K., Fujita, K., Yoneda, M., Nozaki, Y., Ogawa, Y., Shinohara, Y., et al. 2012. Hyperresponsivity to low-dose endotoxin during progression to nonalcoholic steatohepatitis is regulated by leptin-mediated signaling. Cell Metab. **16**(1): 44–54. doi:10.1016/j.cmet.2012.05.012. PMID:22768838.

Karmiris, K., Koutroubakis, I.E., and Kouroumalis, E.A. 2005. The emerging role of adipocytokines as inflammatory mediators in inflammatory bowel disease. Inflamm. Bowel Dis. **11**(9): 847–855. doi:10.1097/01.mib.0000178915.54264.8f. PMID:16116320.

Karmiris, K., Koutroubakis, I.E., Xidakis, C., Polychronaki, M., and Kouroumalis, E.A. 2007. The effect of infliximab on circulating levels of leptin, adiponectin and resistin in patients with inflammatory bowel disease. Eur. J. Gastroenterol. Hepatol. **19**(9): 789–794. doi:10.1097/MEG.0b013e3282202bca. PMID:17700265.

Kontermann, R.E. 2012. Antibody-cytokine fusion proteins. Arch. Biochem. Biophys. **526**(2): 194–205. doi:10.1016/j.abb.2012.03.001. PMID:22445675.

Levi, J., Gray, S.L., Speck, M., Huynh, F.K., Babich, S.L., Gibson, W.T., et al. 2011. Acute disruption of leptin signaling in vivo leads to increased insulin levels and insulin resistance. Endocrinology, **152**(9): 3385–3395. doi:10.1210/en.2011-0185. PMID:21750049.

Majumdar, P., Chen, S., George, B., Sen, S., Karmazyn, M., and Chakrabarti, S. 2009. Leptin and endothelin-1 mediated increased extracellular matrix protein production and cardiomyocyte hypertrophy in diabetic heart disease. Diabetes Metab. Res. Rev. **25**(5): 452–463. doi:10.1002/dmrr.964. PMID:19391127.

Mak, R.H., Cheung, W., Cone, R.D., and Marks, D.L. 2006. Leptin and inflammation-associated cachexia in chronic kidney disease. Kidney Int. **69**(5): 794–797. doi:10.1038/sj.ki.5000182. PMID:16518340.

Moon, H.S., Matarese, G., Brennan, A.M., Chamberland, J.P., Liu, X., Fiorenza, C.G., et al. 2011. Efficacy of metreleptin in obese patients with type 2 diabetes: cellular and molecular pathways underlying leptin tolerance. Diabetes, **60**(6): 1647–1656. doi:10.2337/db10-1791. PMID:21617185.

Muller-Newen, G. 2003. The cytokine receptor gp130: faithfully promiscuous. Sci. STKE, **2003**(201): PE40. doi:10.1126/stke.2003.201.pe40. PMID:14506288.

Niv-Spector, L., Gonen-Berger, D., Gourdou, I., Biener, E., Gussakovsky, E.E., Benomar, Y., et al. 2005*a*. Identification of the hydrophobic strand in the A-B loop of leptin as major binding site III: implications for large-scale preparation of potent recombinant human and ovine leptin antagonists. Biochem. J. **391**(Pt. 2): 221–230. PMID:15952938.

Niv-Spector, L., Raver, N., Friedman-Einat, M., Grosclaude, J., Gussakovsky, E.E., Livnah, O., et al. 2005*b*. Mapping leptin-interacting sites in recombinant leptin-binding domain (LBD) subcloned from chicken leptin receptor. Biochem. J. **390**(Pt. 2): 475–484. PMID:15842201.

Niv-Spector, L., Shpilman, M., Boisclair, Y., and Gertler, A. 2012. Large-scale preparation and characterization of non-pegylated and pegylated superactive ovine leptin antagonist. Protein Expr. Purif. **81**(2): 186–192. doi:10.1016/j.pep.2011.10.003. PMID:22040607.

Otvos, L., Jr., and Surmacz, E. 2011. Targeting the leptin receptor: a potential new mode of treatment for breast cancer. Expert Rev. Anticancer Ther. **11**(8), 1147–1150. doi:10.1586/era.11.109. PMID:21916566.

Otvos, L., Jr., Kovalszky, I., Riolfi, M., Ferla, R., Olah, J., Sztodola, A., et al. 2011. Efficacy of a leptin receptor antagonist peptide in a mouse model of triple-negative breast cancer. Eur. J. Cancer, **47**(10): 1578–1584. doi:10.1016/j.ejca.2011.01.018. PMID:21353530.

Otvos, L., Jr., Kovalszky, I., Scolaro, L., Sztodola, A., Olah, J., Cassone, M., et al. 2011. Peptide-based leptin receptor antagonists for cancer treatment and appetite regulation. Biopolymers, **96**(2): 117–125. doi:10.1002/bip.21377. PMID:20564005.

Otvos, L., Jr., Shao, W.H., Vanniasinghe, A.S., Amon, M.A., Holub, M.C., Kovalszky, I., et al. 2011. Toward understanding the role of leptin and leptin receptor antagonism in preclinical models of rheumatoid arthritis. Peptides, **32**(8): 1567–1574. doi:10.1016/j.peptides.2011.06.015. PMID:21723351.

Otvos, L., Jr., Terrasi, M., Cascio, S., Cassone, M., Abbadessa, G., De Pascali, F., et al. 2008. Development of a pharmacologically improved peptide agonist of the leptin receptor. Biochim. Biophys. Acta, **1783**(10): 1745–1754. doi:10.1016/j.bbamcr.2008.05.007. PMID:18555805.

Peelman, F., Van Beneden, K., Zabeau, L., Iserentant, H., Ulrichts, P., Defeau, D., et al. 2004. Mapping of the leptin binding sites and design of a leptin antagonist. J. Biol. Chem. **279**(39): 41038–41046. doi:10.1074/jbc.M404962200. PMID:15213225.

Procaccini, C., De Rosa, V., Galgani, M., Carbone, F., Cassano, S., Greco, D., et al. 2012. Leptin-induced mTOR activation defines a specific molecular and transcriptional signature controlling CD4+ effector T cell responses. J. Immunol. **189**(6): 2941–2953. doi:10.4049/jimmunol.1200935. PMID:22904304.

Purdham, D.M., Rajapurohitam, V., Zeidan, A., Huang, C., Gross, G.J., and Karmazyn, M. 2008. A neutralizing leptin receptor antibody mitigates hypertrophy and hemodynamic dysfunction in the postinfarcted rat heart. Am. J. Physiol. Heart Circ. Physiol. **295**(1): H441–H446. doi:10.1152/ajpheart.91537.2007. PMID:18469142.

Ramos, M.P., Rueda, B.R., Leavis, P.C., and Gonzalez, R.R. 2005. Leptin serves as an upstream activator of an obligatory signaling cascade in the embryo-implantation process. Endocrinology, **146**(2): 694–701. doi:10.1210/en.2004-1186. PMID:15539553.

Raver, N., Vardy, E., Livnah, O., Devos, R., and Gertler, A. 2002. Comparison of R128Q mutations in human, ovine, and chicken leptins. Gen. Comp. Endocrinol. **126**(1): 52–58. doi:10.1006/gcen.2001.7766. PMID:11944966.

Salomon, G., Niv-Spector, L., Gussakovsky, E.E., and Gertler, A. 2006. Large-scale preparation of biologically active mouse and rat leptins and their L39A/D40A/F41A muteins which act as potent antagonists. Protein Expr. Purif. **47**(1), 128–136. doi:10.1016/j.pep.2005.09.016. PMID:16289983.

Sandowski, Y., Raver, N., Gussakovsky, E.E., Shochat, S., Dym, O., Livnah, O., et al. 2002. Subcloning, expression, purification, and characterization of recombinant human leptin-binding domain. J. Biol. Chem. **277**(48): 46304–46309. doi:10.1074/jbc.M207556200. PMID:12226096.

Scarpace, P.J., and Zhang, Y. 2009. Leptin resistance: a prediposing factor for diet-induced obesity. Am. J. Physiol. Regul. Integr. Comp. Physiol. **296**(3): R493–R500. doi:10.1152/ajpregu.90669.2008. PMID:19091915.

Shpilman, M., Niv-Spector, L., Katz, M., Varol, C., Solomon, G., Ayalon-Soffer, M., et al. 2011. Development and characterization of high affinity leptins and leptin antagonists. J. Biol. Chem. **286**(6): 4429–4442. doi:10.1074/jbc.M110.196402. PMID:21119198.

Siegmund, B., Lehr, H.A., and Fantuzzi, G. 2002. Leptin: a pivotal mediator of intestinal inflammation in mice. Gastroenterology, **122**(7): 2011–2025. doi:10.1053/gast.2002.33631. PMID:12055606.

Siegmund, B., Sennello, J.A., Jones-Carson, J., Gamboni-Robertson, F., Lehr, H.A., Batra, A., et al. 2004. Leptin receptor expression on T lymphocytes modulates chronic intestinal inflammation in mice. Gut, **53**(7): 965–972. doi:10.1136/gut.2003.027136. PMID:15194645.

Sitaraman, S., Liu, X., Charrier, L., Gu, L.H., Ziegler, T.R., Gewirtz, A., et al. 2004. Colonic leptin: source of a novel proinflammatory cytokine involved in IBD. FASEB J. **18**(6): 696–698. doi:10.1096/fj.03-0422fje. PMID:14977884.

Solomon, G., Monsonego-Ornan, E., Chapnik, N., Smith, E., Froy, O., D'Alessio, D., et al. 2012. New fast and reversible leptin antagonist-induced mice model of metabolic syndrome and T2DM. Paper presented at the ECE/ICE 2012, Florence, Italy.

Styer, A.K., Sullivan, B.T., Puder, M., Arsenault, D., Petrozza, J.C., Serikawa, T., et al. 2008. Ablation of leptin signaling disrupts the establishment, development, and maintenance of endometriosis-like lesions in a murine model. Endocrinology, **149**(2): 506–514. doi:10.1210/en.2007-1225. PMID:17962343.

Tam, J., Cinar, R., Liu, J., Godlewski, G., Wesley, D., Jourdan, T., et al. 2012. Peripheral cannabinoid-1 receptor inverse agonism reduces obesity by reversing leptin resistance. Cell Metab. **16**(2): 167–179. doi:10.1016/j.cmet.2012.07.002. PMID:22841573.

Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., et al. 1995. Identification and expression cloning of a leptin receptor, OB-R. Cell, **83**(7): 1263–1271. doi:10.1016/0092-8674(95)90151-5. PMID:8548812.

Teta, D. 2012. Adipokines as uremic toxins. J. Ren. Nutr. **22**(1): 81–85. doi:10.1053/j.jrn.2011.10.029. PMID:22200420.

Tumer, N., Erdos, B., Matheny, M., Cudykier, I., and Scarpace, P.J. 2007. Leptin antagonist reverses hypertension caused by leptin overexpression, but fails to normalize obesity-related hypertension. J. Hypertens. **25**(12): 2471–2478. doi:10.1097/HJH.0b013e3282e9a9fd. PMID:17984669.

Verploegen, S.A., Plaetinck, G., Devos, R., Van der Heyden, J., and Guisez, Y. 1997. A human leptin mutant induces weight gain in normal mice. FEBS Lett. **405**(2): 237–240. doi:10.1016/S0014-5793(97)00192-0. PMID:9089297.

Zabeau, L., Lavens, D., Peelman, F., Eyckerman, S., Vandekerckhove, J., and Tavernier, J. 2003. The ins and outs of leptin receptor activation. FEBS Lett. **546**(1): 45–50. doi:10.1016/S0014-5793(03)00440-X. PMID:12829235.

Zabeau, L., Defeau, D., Van der Heyden, J., Iserentant, H., Vandekerckhove, J., and Tavernier, J. 2004. Functional analysis of leptin receptor activation using a Janus kinase/signal transducer and activator of transcription complementation assay. Mol. Endocrinol. **18**(1): 150–161. doi:10.1210/me.2003-0078. PMID:14525952.

Zabeau, L., Verhee, A., Catteeuw, D., Faes, L., Seeuws, S., Decruy, T., et al. 2012. Selection of non-competitive leptin antagonists using a random nanobody-based approach. Biochem. J. **441**(1): 425–434. doi:10.1042/BJ20110438. PMID:21851341.

Zarkesh-Esfahani, H., Pockley, G., Metcalfe, R.A., Bidlingmaier, M., Wu, Z., Ajami, A., et al. 2001. High-dose leptin activates human leukocytes via receptor expression on monocytes. J. Immunol. **167**(8): 4593–4599. PMID:11591788.

Zhang, F., Basinski, M.B., Beals, J.M., Briggs, S.L., Churgay, L.M., Clawson, D.K., et al. 1997. Crystal structure of the obese protein leptin-E100. Nature, **387**(6629): 206–209. doi:10.1038/387206a0. PMID:9144295.

Zhang, J., Matheny, M.K., Tumer, N., Mitchell, M.K., and Scarpace, P.J. 2007. Leptin antagonist reveals that the normalization of caloric intake and the thermic effect of food after high-fat feeding are leptin dependent. Am. J. Physiol. Regul. Integr. Comp. Physiol. **292**(2): R868–R874. doi:10.1152/ajpregu.00213.2006. PMID:17023670.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature, **372**(6505): 425–432. doi:10.1038/372425a0. PMID:7984236.