Novel Superactive Leptin Antagonists and their Potential Therapeutic Applications

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Abstract: Random mutagenesis of mouse leptin antagonist (L39A/D40A/F41) followed by selection of high-affinity mutants by yeast-surface display indicated that replacing residue D23 with a non-negatively charged amino acid (most specifically with Leu) leads to dramatically enhanced affinity of leptin toward LEPR leading to development of superactive mouse, human, ovine and rat leptin antagonists (D23L/L39A/D40A/F41A). Superactive leptin antagonist mutants of mouse, human, rat or ovine leptins were developed in our laboratory, expressed in E. coli, refolded and purified to homogeneity as monomeric proteins. Pegylation of leptin antagonists resulted in potent and effective long-acting reagents suitable for in vivo studies or therapies. In the present review we explain the mechanism of leptin inhibition and summarize the possible use of leptin antagonists as possible leptin blockers in various human pathologies such as anti-inflammatory and anti-autoimmune diseases, uremic cachexia, and cancer. We also suggest the use of leptin antagonists as research reagents for creation of a novel, fast and reversible model of T2DM in mice.

Keywords: Leptin, antagonist, inflammatory disease, anti-autoimmune diseases, cancer, uremic cachexia, T2DM model.

DEVELOPMENT OF LEPTIN ANTAGONISTS

Though leptin and leptin receptor (LEPR) were cloned almost 20 years ago [1, 2] and leptin’s 3D structure has been resolved [3], the mechanism of leptin:LEPR interaction and activation has not yet been elucidated. Nevertheless, several putative mutants have been proposed [4]. The extracellular domain (ECD) of LEPR, as depicted in (Fig. 1), consists of several subdomains termed (from the N-terminus): cytokine homology region 1 (CHR 1), followed by an immunoglobulin-like domain (IGD), then by another CHR 2 and finally by two consecutive F3 domains. Fong and co-workers [5] localized the leptin-binding domain to the membrane-proximal CHR 2 (~200 amino acids) in the LEPR-ECD. This domain from human [6] and chicken LEPRs [7] was subcloned in Gertler’s laboratory and expressed as a recombinant protein which showed a 1:1 molar interaction with leptin. A decade ago, Tavernier and his group [8] revealed that leptin binding to its receptor resembles the interaction between interleukin 6 (IL6) and IL6 receptor [9-11], and they formulated the existence of the putative leptin site III as a major site responsible for the formation of active 2:2 or 2:4 leptin:LEPR complex. The IGD located between the distal and proximal CK-F3 domains was clearly documented to be essential for productive dimerization (or even tetramerization) of the LEPR, as its removal attenuated activation, but not binding, of the ligand [8]. Schematic illustration of 2:2 leptin:LEPR complex showing interaction of two leptin molecules, each with the CHR 1 of one receptor (through binding site 1/2) and with the IGD of the second receptor (through binding site 3) is shown in (Fig. 1). This model was further substantiated by the extensive mutagenesis of mouse and human leptins which led to the identification of Ser 120 and Thr 121, located in the N-terminal part of helix D, as contributors to site III [12]. Mutation of these amino acids to Ala led to the formation of leptin antagonist. In addition to the N-terminal part of helix D, we found that other parts of the leptin molecule also contribute to the interaction between leptin and LEPR’s IGD. Carefully considering the known structures of IL6-receptor complexes [viral (v) IL6/gp130] [9] and IL6/IL6Rα/gp130 complex [10] in which site III was first identified, we examined the interface between the tips of the vIL6 bundle, comprising an A-B loop and the beginning of helix D, and the edge of one of the IGD β-sheets, involving strands F and G. We further analyzed the corresponding A-B loop in leptin and though its sequence differed greatly in length and in composition compared to IL6, we were able to identify, thanks to the use of the sensitive bidimensional Hydrophobic Cluster Analysis [13], a common short β-strand which interacts in the vIL6/gp130 complex (the only complex for which an ordered structure has been observed in this region) with a short β-strand in the receptor IGD. This strand was located before the first strand of the IGD core and is predicted to also be present in LEPR IGD. To verify this hypothesis and to test its generality, we prepared and purified to homogeneity several ovine and human recombinant leptin Ala mutants in the A-B loop (L39A/D40A, L39A/D40A/F41A or L39A/D40A/F41A/F41A) and documented their activity as potent competitive LEPR antagonists [14]. To verify the preservation and importance of this sequence for activation of LEPRs, we also prepared the corresponding mutants of mouse and rat leptin and documented their antagonistic activity [15]. In subsequent work, we increased the half life of the leptin antagonist by pegylation, resulting in significant extension of its bioavailability while retaining antagonistic activity [16]. In mice, administration of the pegylated antagonist produced rapid and dramatic weight gain, due to enhanced appetite and increased food consumption. The resulting fat was confined to the mesenteric region with no accumulation in the liver. Remarkably, weight changes were reversible upon cessation of leptin-antagonist treatment. The mechanism of severe central leptin deficiency was found to involve inhibition of leptin transport across the blood-brain barrier, as well as accumulation of pegylated antagonist within the central nervous system and direct inhibition of LEPRs in the hypothalamic region.

In view of the potential pharmaceutical uses of leptin 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
Thus recognition and mutagenesis of D23 enabled construction of novel compounds that induce potent and reversible central and peripheral leptin deficiency. In addition to enhancing our understanding of leptin interactions with its receptor, these antagonists have enabled in-vivo study of leptin’s role in metabolic and immune processes, and hold potential for future therapeutic use in disease pathologies involving leptin.

**LEPTIN ANTAGONISTS AS POTENTIAL ANTI-INFLAMMATORY AND ANTI-AUTOIMMUNE DISEASE TREATMENTS**

For years, white adipose tissue has been regarded as an inert organ, with the exclusive function of long-term energy storage. With the cloning of leptin in 1994, that notion has drastically changed [1]. In subsequent years, a plethora of tissue-specific and nonspecific adipose-secreted factors were discovered and characterized and found to play fundamental roles in multiple aspects of metabolic processes, including energy balance, and in distant organs. Moreover, white adipose tissue has been found to dynamically integrate metabolic and immune signals through complex interactions between adipocytes and a complex network of innate and adaptive immune cells, including neutrophils, and mononuclear T and B cell subsets. Through this complex interaction it was suggested that immune components fundamentally affect metabolic processes such as insulin sensitivity, and conversely, adipocyte-secreted factors affect the innate and adaptive immune response through modulation of tissue-resident cells of hematopoietic origin [19, 20].

Leptin, has been most widely studied for its effects on the immune response. Leptin secretion is inducible upon signaling from inflammatory mediators such as tumor necrosis factor alpha (TNF-α), IL1, and IL6. Indeed leptin levels have been shown to increase during infection and chronic inflammation [21]. Like leptin itself, LEPRs resemble cytokine receptors [22] and the long functional isoform of LEPR is found on T-cell subsets, monocytes [23], natural-killer (NK) lymphocytes [24], dendritic cells [25], hepatic stellate cells [26] and a variety of bone marrow progenitor cells. Functionally, leptin signaling promotes macrophage proliferation, phagocytosis and cytokine secretion [27]. NK cell development, activation and survival [28], MAPK-mediated neutrophil chemotaxis [29], T-cell proliferation and IL2 secretion, especially when co-administered with classical T-cell mitogens [30]. Leptin has also been suggested to suppress regulatory T-cell proliferation and inflammation suppressive functions, further contributing to a general pro-inflammatory effect [31].

In vivo, leptin-deficient ob/ob mice are extremely vulnerable to the development of systemic infection and lipopolysaccharide (LPS)-induced organ damage, and resistant to several Th1 cells-mediated immune disorders, including experimental allergic encephalomyelitis, concanavalin A hepatitis, experimental arthritis, and autoimmune nephritis [32-34]. Leptin replenishment reverses these disorders [35]. These mice feature reduced cell density in their bone marrow and thymus, highlighting the importance of leptin signaling in the development of various subsets of the hematopoietic system. Similarly, humans with inherited or starvation-induced leptin deficiency feature thymic atrophy and severe functional alterations in the innate and adaptive immune response, rendering them susceptible to bacterial infection [36, 37].

In humans, the causative correlation between leptin levels and the propensity and severity of autoimmune disease remains controversial. Perhaps the strongest association between leptin levels and autoimmunity has been found in multiple sclerosis (MS), a T-cell-mediated autoimmune disorder of the central nervous system; ob/ob mice are protected from EAE, the most common small animal model of MS, possibly through leptin-deficiency-induced expansion of nTregs cells [38, 39]. In humans, leptin levels have been suggested to correlate with disease activity and to decrease following successful treatment with interferon beta (INF-β) [40]. In other systemic autoimmune disorders, such as rheumatoid arthritis and systemic lupus erythematosus, leptin levels have been suggested in some, but not all, reports to be elevated independently of gender or
weight and to possibly correlate with disease severity [41-46]. Interestingly, one study suggested that acute fasting promotes improvement in multiple immune parameters in rheumatoid arthritis patients, an effect that may be linked to the reduced leptin levels in these patients [47]. Similarly, leptin was suggested to contribute to the pathogenesis of diabetes mellitus in mice, while a mutation in the LEPR of mice with a NOD background ameliorated diabetes severity in these mice [48, 49]. Yet another example of leptin’s effect on the immune response was recently demonstrated by our groups [50], whereby administration of competitive leptin antagonists induced significant amelioration in a model of chronic liver inflammation and fibrosis.

A role for leptin was suggested in the pathogenesis of intestinal autoinflammation. Inflammatory bowel disease (IBD) is a chronic inflammatory disorder affecting 0.3% of the Western 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. In recent years, mounting evidence has suggested that IBD pathogenesis is closely related to a combination of abnormal challenges by normal gut microflora, coupled with inherent or acquired aberrations in the intestinal innate immune response. Leptin’s central role as a mediator of colonic autoinflammation has been suggested in both animal models and human studies. Leptin-deficient ob/ob mice are resistant to acute and chronic experimental colitis [51, 52]. In those studies, leptin was suggested to augment pro-inflammatory cytokine secretion, including IFN-γ, TNF-α, IL18, IL1b, IL6 and IL10, and to inhibit inflammation-associated apoptosis of colonic mononuclear cells. Inflamed colonic epithelial cells were also found to express and release leptin apically into the intestinal lumen. Intrarectal administration of leptin induced activation of NF-kB and epithelial wall damage associated with neutrophil infiltration [53]. The source of local and/or systemic leptin has not been elucidated, but it has been suggested not to be the lamina propria T cells themselves. Similarly, the leptin-responsive cells have not yet been identified. Colateral evidence for a possible pro-inflammatory interaction between leptin and lamina propria dendritic cells (lpDCs) comes from studies showing that LEPRb is expressed on immature and mature human DCs, and that leptin induces STAT3 and NF-kB activation, production of IL1, IL6, IL12, TNF-α and MIP-1α, an anti-apoptotic effect through Bcl-2 and Bcl-XL, downregulation of IL10, polarization into Th-1, and increased CD40 expression [54, 55]. In human IBD patients, leptin levels have been suggested to correlate with the severity of the disease [56-58].

Taken together, a large body of evidence suggests an association between leptin levels and responsiveness in terms of propensity for autoimmunity, autoinflammation and infection. While much of the current data, particularly in humans, is observational and associative, they suggest that leptin may play direct or indirect roles in the modulation and regulation of innate and adaptive immune responses and that excessive leptin signaling might result in a deleterious tendency toward autoinflammation in susceptible populations. As such, inhibition of leptin signaling, as has been recently suggested by Gertler's and Elinav's groups [16, 17, 50], may offer a unique therapeutic modality targeting leptin signaling in the auto-inflammatory setup.

LEPTIN ANTAGONISTS AS ANTI-CANCER AGENTS

The epidemiological evidence linking obesity and breast, cervical, colon, rectal, esophageal, gall bladder, kidney, liver, ovarian, pancreatic, stomach and uterine cancers is well-established [59-64]; the high level of leptin in obese subjects thus raises the notion of a possible link between leptin and cancer. Leptin is an anti-angiogenic molecule in many cell types and its possible role in obesity-linked cancers originates from its known pro-angiogenic, pro-inflammatory and mitogenic activities. Furthermore, leptin was defined as a growth factor not only because of its proliferative activity but also due to its effect on cell motility and migration [65]. Leptin is overexpressed in at least 80% of cases of human skin melanoma [66], breast [67], ovarian [68] and prostate [69] cancers. In most of those cases, LEPR is overexpressed as well [70]. Interestingly, cancer stem cells, and particularly breast cancer stem cells, also overexpress LEPR, thus sensitizing those cells to leptin action [71]. In one such cell type, termed triple-negative breast cancer cell, leptin attenuated the inhibitory effect of cisplatin on cell proliferation and viability [72].

Leptin-induced modulation of colorectal cancer (CRC) has been reported in several in-vitro and in-vivo studies (for most recent report see ref. [79]). Leptin's stimulation of proliferation and inhibition of apoptosis have been shown in several human epithelial colon cancer cells, such as HT-29, CACO-2, DLD-1, SW480, HCT116, LS174-T and Love [73-75]. However in the in vivo role of leptin in CRC remains unclear. In spontaneous CRC models, such as in ApoMin/+ mice, leptin did not increase and even decreased intestinal tumorigenesis in ApoMin/+ mice, a mutation that predisposes the animal to tumor development in the intestine and colon [74, 76]. In contrast, inflammation-induced CRC induced by Dextran Sodium Sulphate - aoxyxymethane was attenuated in ob/ob and db/db mice as compared to wild-type mice [77]. Note that leptin's effect may be indirect as leptin is known to induce inflammatory cytokines in colonic tissue that are implicated in colon carcinogenesis, such as IL6, IL1β and CXC [78]. Likewise the impact of leptin on colon cancer in humans remains unclear. While some reports show increased leptin expression with progressing tumorigenesis, other have failed to confirm such observations [79]. However, it should be noted that serum leptin levels may not be indicative of local leptin levels. Putatively, blocking localized leptin secreted by leptin antagonists endoscopically applied to colon cancer cells may be considered as a possible future treatment option.

The role of leptin in hepatocellular carcinoma (HCC), the third leading cause of cancer death in the world, is also not clear. Patients with cirrhosis resulting from hepatitis B or C infection were found to exhibit increased leptin levels which were suggested to represent a negative prognostic factor [80], but again, studies have reported contradictory findings [81]. Another major factor leading to HCC is non-alcoholic fatty liver disease (NAFLD) ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). In an animal model of NAFLD, leptin was suggested to contribute to insulin resistance and steatosis, and leptin injections increased the expression of procollagen-I, TGF-β1 and smooth muscle actin and led to increased liver fibrosis [82]. In another study without leptin signaling, neither fibrosis nor HCC developed in the rat NASH model [83]. However, exogenous leptin significantly decreased tumor size and increased survival rate in an HCC mouse model [84]. In human studies, leptin levels were significantly higher in NASH patients and correlated with severity of hepatic steatosis [85], but in another study no differences were found [86]. Furthermore, high leptin expression correlated with better survival in HCC patients [87].

More recently, attention has been given to the role of leptin in glioblastoma and is reviewed elsewhere (see ref. [65]). Though extensive in-vitro studies are still lacking and the information linking leptin to glioblastoma stems mainly from studies with glioma cell line C6 [88, 89], the authors strongly suggest leptin's role not only in cell proliferation and inhibition of apoptosis but also in leptin-induced cell migration [90]. Moreover, leptin's effect on VEGF levels may also play an important role in leptin promotion of glioblastoma and other cancers [91-94].

One of the most interesting findings connecting leptin to sensitivity to cancer in mouse models of melanoma and colon cancer, related to environmental enrichment, showed that mice kept in an enriched environment express higher levels of brain-derived nerve factor (BDNF), which in turn activates sympathetic nerve fiber innervation of white adipose tissue, resulting in decreased
leptin secretion, cancer inhibition and remission [95]. Thus, although the role of leptin in tumor promotion is controversial and may depend on tumor type, at least in those cases in which leptin plays a negative role, leptin antagonists may be potentially used as both important research tools and potential therapeutic modalities.

LEPTIN ANTAGONISTS IN UREMIC CACHEXIA

Most recently, the negative role of leptin in uremic patients has been reviewed [96]. Leptin has been defined as a true uremic toxin, and reducing leptin levels in uremic patients, particularly those suffering from uremia-related cachexia, may have potential beneficial effects. This suggestion is based on the finding that plasma leptin is associated with reduced energy intake and protein-wasting in uremic patients [97]. In a murine animal model of uremic cachexia, application of pegylated superactive leptin antagonist prevented weight gain, protein and fat losses and normalized muscle function (Michal Ayalon-Soffer and Robert Mak, personal communication). Use of leptin antagonists in the treatment of uremic patients seems at present as a most feasible therapeutic application.

LEPTIN ANTAGONISTS IN OTHER PATHOLOGIES

The role of leptin signaling in myocardial hypertrophy, heart diseases associated with metabolic syndrome, endothelial dysfunction, arterial hypertension are reviewed in depth in the present issue. In all of these conditions, leptin’s negative effects can be potentially antagonized by leptin antagonists.

LEPTIN ANTAGONIST-INDUCED RAPID AND REVERSIBLE MOUSE MODEL OF T2DM

Obesity and its major consequence, type II diabetes mellitus (T2DM), has become an epidemic in Western society. T2DM accounts for 95% of the diabetes worldwide. One limitation to the development of new T2DM treatments has been a lack of effective animal models to use in research. There are no rodent models that recapitulate the pancreatic β-cell lesions of humans with T2DM. Moreover, animal models of obesity require either overfeeding, which is expensive and takes weeks to months to establish, or specific genetic mutations that cause lifelong metabolic dysfunction. Thus the ability to rapidly induce obesity in healthy rat and mouse strains would constitute a major advance in diabetes and obesity research leading to the development of novel therapies. In its monopegylated form, the compound PEG-SMLA has strong orexigenic properties, and when given to mice every 24 to 48 h leads to remarkable weight gain. So far, 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, achieving a uniform 25 to 45% weight gain in 14 to 21 days. Some representative data from these studies are presented in Table 1 and 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 was observed, even after 12 weeks of treatment, although morphologically the livers of PEG-SMLA-treated animals seem fatty. PEG-SMLA treatment did not affect other blood parameters such as albumin, globulin, creatinine, urea, calcium, potassium, phosphorus or bilirubin (not shown). An additional experiment was carried out to test the effects of PEG-SMLA, and showed abnormal glucose tolerance (by oral glucose tolerance test) after 3 weeks of treatment. 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 to 14 days.

Determining insulin and glucose levels, and HOMA scores can indicate whether mice are normal (euglycemic with normal insulin levels), insulin-resistant, or have developed T2DM according to the following criteria:

Normal: Glucose and insulin are less than 2.5 SD above control average
Insulin-resistant: Insulin more than 2.5 SD above control average but glucose less than

Table 1. Weight gain and selected biochemical and hormonal parameters in 8-week-old male and 4-week-old female mice after long-term 4 or 12 weeks IP administration of PEG-SMLA at 12 (male) or 5 (female) mg/kg daily.

<table>
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<tr>
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<th>Males 1</th>
<th>Females 2</th>
<th>after 4 weeks</th>
<th>after 12 weeks</th>
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<tr>
<td></td>
<td>Control PEG-SMLA</td>
<td>Control PEG-SMLA</td>
<td>Control PEG-SMLA</td>
<td>Control PEG-SMLA</td>
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<tr>
<td>Weight gain (%)</td>
<td>15 ± 0.3</td>
<td>65 ± 1.2**</td>
<td>20 ± 1</td>
<td>69 ± 7***</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>97 ± 6</td>
<td>151 ± 8**</td>
<td>178 ± 15</td>
<td>246 ± 21*</td>
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<tr>
<td>Chol (mg/dl)</td>
<td>133 ± 4</td>
<td>173 ± 10**</td>
<td>96 ± 8</td>
<td>147 ± 9**</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>63 ± 3</td>
<td>86 ± 4**</td>
<td>63 ± 3</td>
<td>87 ± 4*</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>Not tested</td>
<td>0.80 ± 0.1</td>
<td>4.1 ± 0.7**</td>
<td>0.31 ± 0.07</td>
</tr>
<tr>
<td>HOMA (arbit U)</td>
<td>Not tested</td>
<td>57 ± 9</td>
<td>380 ± 80***</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>SGOT (IU/ml)</td>
<td>150 ± 19</td>
<td>165 ± 20</td>
<td>120 ± 23</td>
<td>249 ± 54</td>
</tr>
<tr>
<td>SGPT (IU/ml)</td>
<td>158 ± 37</td>
<td>136 ± 16</td>
<td>74 ± 41</td>
<td>203 ± 67</td>
</tr>
<tr>
<td>AP (IU/ml)</td>
<td>122 ± 10</td>
<td>136 ± 18</td>
<td>192 ± 6</td>
<td>166 ± 19</td>
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1Initial weight 20.8 g at 8 weeks of age.
2Initial weight 11.8 g at 4 weeks of age.

Values are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; male: 4 h post-fasting, females: not fasted.
2.5 SD above control average

**T2DM:** Both insulin and glucose more than 2.5 SD above control average.

Using these criteria, we have found in various experiments that 36 to 100% of PEG-SMLA-treated animals develop insulin resistance or T2DM in a time-dependent manner within 3 to 6 weeks of PEG-SMLA treatment. In view of these results, we conclude that PEG-SMLA treatment leads to the appearance of metabolic syndrome and even to a reversible T2DM phenotype.

In conclusion, administration of PEG-SMLA or even PEGMLA to mice causes reversible obesity, hyperglycemia and hyperinsulinemia. These findings raise questions as to whether islet β-cells are specifically affected by a LEPR blockade preventing their full compensation for insulin resistance. A second issue raised by these findings is the role of hepatic glucose production and insulin sensitivity in the rapid development of hyperglycemia. Hypothalamic LEPRs have been implicated in the regulation of hepatic glucose production, and direct effects of leptin on hepatocytes have also been demonstrated. The ability to rapidly and directly affect systemic and CNS leptin signaling with high potency LEPR antagonists provides a powerful means of addressing the breadth of leptin action.

One-month exposure to PEG-SMLA altered the expression of key regulatory genes in adipose tissue, muscle and brain. These findings indicate that leptin antagonism induces systemic dysmetabolism in a rapid and practical manner, and provides a valuable tool for research in obesity and diabetes.

**CONFLICT OF INTEREST**

Both authors are employed as consultants to BiolineRx, a R&D company exploring the therapeutic potential of leptin antagonists.

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