

## RESEARCH PAPER

# Anti-obesity efficacy of LH-21, a cannabinoid CB<sub>1</sub> receptor antagonist with poor brain penetration, in diet-induced obese rats

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### Keywords

cannabinoids; diet-induced obesity; rat; adipose tissue; appetite; PPARs; adiponectin; leptin; SCD-1

### Received

13 April 2011

### Revised

12 September 2011

### Accepted

14 September 2011

## BACKGROUND AND PURPOSE

Peripheral blockade of cannabinoid CB<sub>1</sub> receptors has been proposed as a safe and effective therapy against obesity, putatively devoid of the adverse psychiatric side effects of centrally acting CB<sub>1</sub> receptor antagonists. In this study we analysed the effects of LH-21, a peripherally acting neutral cannabinoid receptor antagonist with poor brain penetration, in an animal model of diet-induced obesity.

## EXPERIMENTAL APPROACH

To induce obesity, male Wistar rats were fed a high-fat diet (HFD; 60 kcal% fat) whereas controls received a standard diet (SD; 10 kcal% fat). Following 10 weeks of feeding, animals received a daily i.p. injection of vehicle or 3 mg·kg<sup>-1</sup> LH-21 for 10 days. Plasma and liver samples were used for biochemical analyses whereas visceral fat-pad samples were analysed for lipid metabolism gene expression using real-time RT-PCR. In addition, the potential of LH-21 to interact with hepatic cytochrome P450 isoforms and cardiac human *Ether-à-go-go* Related Gene (hERG) channels was evaluated.

## KEY RESULTS

LH-21 reduced feeding and body weight gain in HFD-fed animals compared with the control group fed SD. In adipose tissue, this effect was associated with decreased gene expression of: (i) leptin; (ii) lipogenic enzymes, including SCD-1; (iii) CB<sub>1</sub> receptors; and (iv) both PPAR $\alpha$  and PPAR $\gamma$ . Although there were no significant differences in plasma parameters between HFD- and SD-fed rats, LH-21 did not seem to induce hepatic, cardiac or renal toxicity.

## CONCLUSIONS AND IMPLICATIONS

These results support the hypothesis that treatment with the peripherally neutral acting CB<sub>1</sub> receptor antagonist, LH-21, may promote weight loss through modulation of visceral adipose tissue.

## Abbreviations

CYP, cytochrome P450; FAME, fatty acid methyl esters; hERG, *Ether-à-go-go* Related Gene; HFD, high fat diet; LH-21, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-hexyl-1H-1,2,4-triazole; SD, standard diet

## Introduction

Although the incidence of obesity and related metabolic disorders continues to increase in modern society, there are no pharmacological therapies for its treatment. Thus, development of safe and effective therapies against obesity represents a priority for both researchers and health systems.

Among the new targets for pharmaceutical development of anti-obesity drugs, the endocannabinoid system remains a focus of attention. A growing body of evidence has clearly demonstrated that endocannabinoids and their receptors (mainly cannabinoid CB<sub>1</sub> receptor) play a fundamental role in appetite regulation and energy homeostasis. Obesity and its complications appear to be associated with a dysregulated and hyperactive endocannabinoid system in humans and rodents displaying an increase in circulating endocannabinoid levels and altered expression of the components of this system in several organs such as adipose tissue, liver and pancreas. In fact, increased concentrations of endocannabinoids might overstimulate CB<sub>1</sub> receptors in a pathophysiological manner contributing to obesity (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Di Marzo, 2008; Engeli, 2008; Matias *et al.*, 2008; Starowicz *et al.*, 2008).

Consequently several cannabinoid CB<sub>1</sub> receptor antagonists have been developed as anti-obesity drugs. Synthetic and plant-derived cannabinoid CB<sub>1</sub> receptor blockers have been reported to suppress food intake, whereas chronic treatments lead to weight loss and improved cardiometabolic risk profile in rodents including both genetic and dietary models of obesity (Hildebrandt *et al.*, 2003; Ravinet Trillou *et al.*, 2003; Vickers *et al.*, 2003; Thornton-Jones *et al.*, 2006; Gary-Bobo *et al.*, 2007; Riedel *et al.*, 2009; Tam *et al.*, 2010). One of these antagonists, rimonabant (also referred as SR141716A), reached the market after successful clinical trials revealing both, metabolic benefits and body weight reduction in overweight and obese subjects (Van Gaal *et al.*, 2005; Pi-Sunyer *et al.*, 2006). However, due to the appearance of psychiatric adverse effects, derived from central cannabinoid CB<sub>1</sub> receptor blockade and its inverse agonistic activity, the drug was withdrawn from the market (Christensen *et al.*, 2007; Christophoulou and Kiortsis, 2011).

A growing number of new CB<sub>1</sub> blockers with limited brain penetration and/or neutral antagonism activity are being synthesized and characterized to prevent adverse effects observed with rimonabant while maintaining anti-obesity properties.

Molecules acting as neutral CB<sub>1</sub> antagonists include AM4113 and VCHSR1, and both have been evaluated in food intake behaviour. For example, VCHSR1 is a rimonabant-derived compound with lower affinity for CB<sub>1</sub> that inhibits milk ingestion and growth in newborn mice (Fride *et al.*, 2007). AM4113 has been more extensively studied; it is a pyrazole congener of AM251 that also acts as a neutral CB<sub>1</sub> antagonist and reduces feeding in rats on high fat, high carbohydrate and lab chow diets (Chambers *et al.*, 2007; Sink *et al.*, 2008). Another recent study has reported that rats treated with AM4113, *i.p.* or *p.o.*, display a transient reduction in food intake, which results in a long-term reduction in body weight gain (Cluny *et al.*, 2011).

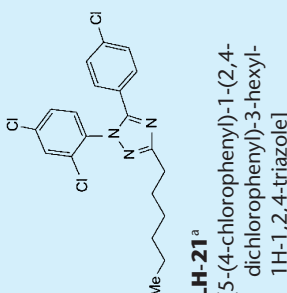
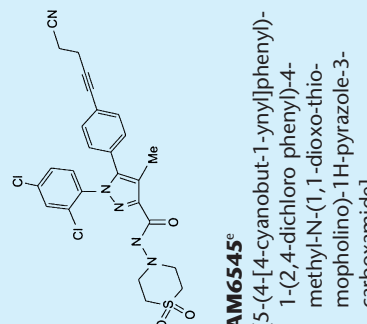
Because it was demonstrated that peripheral cannabinoid CB<sub>1</sub> receptor blockade is sufficient to suppress appetite

(Gomez *et al.*, 2002), increase energy expenditure and reduce lipogenesis on both liver and adipose tissue (Cota *et al.*, 2003; Osei-Hyiaman *et al.*, 2005), the use of cannabinoid receptor antagonists with limited penetration into the brain is a matter of active research. Thus, several CB<sub>1</sub> receptor antagonists with restricted brain penetrability have been developed and evaluated in obesity. For example, non-brain-penetrant CB<sub>1</sub> antagonists JD-2114 and JD-5006 have been shown to reduce body weight gain and improve metabolic parameters in obese mice maintained on a high-fat diet (HFD; McElroy *et al.*, 2008). URB447, a mixed CB<sub>1</sub> antagonist/CB<sub>2</sub> agonist with reduced brain penetration reduces feeding and body weight gain in mice (LoVerme *et al.*, 2009). Another interesting compound is AM6545, a neutral CB<sub>1</sub> antagonist with relatively poor penetrability into the brain, as shown in Table 1 (Tam *et al.*, 2010). This drug reduces food intake in animals fed high-fat and high-carbohydrate diets, but it is less efficacious at reducing lab chow intake (Randall *et al.*, 2010). AM6545 improves the metabolic profile of mice with genetic or diet-induced obesity in a food intake-independent manner (Tam *et al.*, 2010). Recently, it has been reported that AM6545 reduces food intake and body weight gain in rodents including CB<sub>1</sub> gene-deficient mice, but not in CB<sub>1</sub>/CB<sub>2</sub> double knockout mice (Cluny *et al.*, 2010).

Following the research of this new range of compounds based on peripheral CB<sub>1</sub> blockade, the present study has focused on LH-21, a triazole-derived compound with anorectic properties, which was synthesized and identified initially as a neutral CB<sub>1</sub> receptor antagonist with a paradoxical low affinity for CB<sub>1</sub> receptors (Jagerovic *et al.*, 2004) (Table 1), although recently it has been considered a weak CB<sub>1</sub> inverse agonist by Chen and colleagues (Chen *et al.*, 2008). Diverse studies in rodents have demonstrated that this anorectic drug shows a different pharmacological profile in comparison with rimonabant (Pavon *et al.*, 2006; 2008). These studies reported that acute administration of LH-21 reduces feeding behaviour in rats, whereas subchronic administration reduces food intake and body weight gain in genetically obese Zucker rats (*fa/fa*, missense mutation in the leptin receptor), albeit with no metabolic benefits (Pavon *et al.*, 2008). Both, *in vivo* and *in vitro* assays have demonstrated that LH-21 has low permeability through the blood-brain barrier. The poor penetration of LH-21 predicted by *in vitro* permeability assay into the brain was confirmed *in vivo* by the absence of effects on anxiety-like behaviours, motor stereotypies and ethanol self-administration (Pavon *et al.*, 2006). Indeed, systemic administration (*i.p.*) of LH-21 was unable to antagonize the motor inhibition induced by central administration of the CB<sub>1</sub> receptor agonist CP55940 in Wistar rats (Pavon *et al.*, 2006). These studies suggest that its effects on feeding behaviour and body weight are mainly mediated by peripheral targets, *i.e.* cannabinoid CB<sub>1</sub> receptors located in metabolically relevant organs, such as adipose tissue or the liver. Although a recent report (Chen *et al.*, 2008) suggested that LH-21 penetrates freely into the brain of Sprague-Dawley rats, this was not sufficiently demonstrated as: (i) the study lacks positive and negative control drugs for brain penetration in their analysis; (ii) the study used an *i.v.* administration route instead of *p.o.* or *i.p.* route, skipping the liver influence; and (iii) they do not report *in vivo* behavioural data. Moreover, their data on efficacy on feeding inhibition showed effective

Table 1

Structures and pharmacological characteristics of LH-21 and AM6545

Characteristics					Peripheral administration (i.p.) Effects on feeding behaviour (animal model and effective dose)
Chemical structures	Activity at CB <sub>1</sub>	CB <sub>1</sub> IC <sub>50</sub>	CB <sub>2</sub> IC <sub>50</sub>	K <sub>i</sub>	Behavioural central effects
 <p><b>LH-21</b><sup>a</sup> [5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-hexyl-1H-1,2,4-triazole]</p>	Neutral antagonist <sup>a, b</sup> –Moderate affinity <sup>a</sup> –High efficacy <sup>a</sup>	77 nM <sup>b</sup>	>1000 nM <sup>b</sup>	748 nM <sup>a</sup>	<p><b>Poor</b></p> <p>–(PAMPA)<sup>c</sup></p> <p>–i.p.</p> <p>Behavioural studies in Wistar rats</p> <p>–Non-stereotypies in rats<sup>c</sup></p> <p>–Non-motor actions in rats<sup>c</sup></p> <p>–Failed to reverse motor depression induced by CB<sub>1</sub> agonists in rats (i.c.v.)<sup>c</sup></p> <p>–Non-anxiety behaviours in rats<sup>c</sup></p> <p>–Failed to decrease ethanol self-administration in rats<sup>c</sup></p> <p>Reduced food intake<sup>b, c</sup></p> <p>Reduced body weight gain<sup>b, c</sup></p> <p>–Wistar rats (0.3 and 3 mg·kg<sup>-1</sup>)</p> <p>–Zucker rats (3 mg·kg<sup>-1</sup>)</p>
 <p><b>AM6545</b><sup>e</sup> [5-(4-[4-cyanobut-1-ynyl]phenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-(1,1-dioxo-thiomopholino)-1H-pyrazole-3-carboxamide]</p>	Weak inverse agonist <sup>d</sup> –Low affinity <sup>d</sup> –High efficacy <sup>d</sup>	690 nM <sup>d</sup> 631 nM <sup>d</sup>	1330 nM <sup>d</sup>	High –i.v. Mass Spec. in Sprague-Dawley rats (in vivo) <sup>d</sup>	<p>Reduced food intake<sup>d</sup></p> <p>Reduced body weight gain<sup>d</sup></p> <p>–C57BL6 mice (60 mg·kg<sup>-1</sup>)</p> <p>–CB<sub>1</sub>-null mice (60 mg·kg<sup>-1</sup>)</p> <p>Reduced food intake<sup>e, f</sup></p> <p>Reduced body weight gain<sup>e, f</sup></p> <p>–C57BL6 mice (10 mg·kg<sup>-1</sup>)</p> <p>–Wistar rats (10 mg·kg<sup>-1</sup>)</p> <p>–Non-motor actions in mice<sup>e</sup></p> <p>–Failed to reverse motor depression induced by CB<sub>1</sub> agonists (i.c.v.) in mice<sup>e</sup></p> <p>–Non-anxiety like behaviours in mice<sup>e</sup></p> <p>–Failed to produce conditioned gaping or conditioned taste avoidance in rats<sup>f</sup></p>
	Neutral antagonist <sup>e</sup> –High affinity <sup>e</sup> –High efficacy <sup>e</sup>	1.7 nM <sup>f</sup>	523 nM <sup>f</sup>	3.3 nM <sup>e</sup>	<p><b>Markedly poor</b></p> <p>–i.p./po.</p> <p>Mass Spec. in C57BL/6j mice (in vivo)<sup>f</sup></p> <p>–i.p.</p> <p>Behavioural studies in C57BL/6j mice<sup>e</sup></p> <p>–Non-motor actions in mice<sup>e</sup></p> <p>–Failed to reverse motor depression induced by CB<sub>1</sub> agonists (i.c.v.) in mice<sup>e</sup></p> <p>–Non-anxiety like behaviours in mice<sup>e</sup></p> <p>–Failed to produce conditioned gaping or conditioned taste avoidance in rats<sup>f</sup></p>

<sup>a</sup>Jagerovic et al., 2004; <sup>b</sup>Pavon et al., 2008; <sup>c</sup>Chen et al., 2008; <sup>d</sup>Tam et al., 2010; <sup>e</sup>Cluny et al., 2010. <sup>f</sup>K<sub>i</sub>, inhibition constant (binding affinity); BBB, blood–brain barrier; PAMPA, parallel artificial membrane permeation assay.

doses of 60 mg·kg<sup>-1</sup>, whereas we found that the drug was effective at doses three times lower than this in mice and 20 times lower in rats.

In the present study we investigated the toxicity and effects of a 10 day treatment with LH-21 in a diet-induced obesity model, and compared its effects in rats fed a HFD compared with a standard/low-fat diet (SD). We have focused this study on visceral white adipose tissue and liver because both organs have a critical role in energy homeostasis and obesity/overweight leads to a dysfunction caused by excessive fat accumulation. Our results indicate that: (i) LH-21 reduces feeding and body weight gain in HFD-induced obese rats by modulating lipogenic pathways in the adipose tissue; and (ii) this drug has a safe profile, confirming the therapeutic utility of peripheral blockade of cannabinoid receptors in obesity.

## Methods

### Animals

Feeding studies and experiments related to diet-induced obesity were performed on 10–12 week-old male Wistar rats (Charles Rivers, Barcelona, Spain) weighing 200–250 g. Animals were housed in pairs under a 12 h light/dark cycle (lights off 20 h 00 min) in a room with temperature and humidity control. Unless otherwise indicated, water and rat chow pellets were available *ad libitum* throughout the course of these studies. Additional feeding studies were performed on adult male mice weighing 25–30 g. Both wild-type (129S1/SvImJ, stock #002448) and PPAR $\alpha$ -null (129S4/SvJae-Ppara<sup>tm1Gonz/J</sup>, stock #003580) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained as an inbred colony of mice (Suardiaz *et al.*, 2007). All animal care and experimental procedures were conducted in accordance with the European Community Directive 86/609 regulating animal research.

### Drugs

LH-21 was synthesized in our laboratory as previously described (Hernandez-Folgado *et al.*, 2008). LH-21 was dissolved in vehicle (10% Tocrisolve™ 100, Tocris Bioscience, Bristol, UK, diluted in saline). The following doses of LH-21 were used: (i) 1, 3, 10 and 30 mg·kg<sup>-1</sup> for acute studies in rats deprived of food (fasted rats); (ii) 3 mg·kg<sup>-1</sup> for a 10 day administration in diet-induced obese rats; and (iii) 20 mg·kg<sup>-1</sup> for acute studies using mice deprived of food (fasted mice). Drugs were injected *i.p.* at the beginning of light cycle (09 h–10 h) at a dose volume of 1 mL·kg<sup>-1</sup> body weight in rats or 10 mL·kg<sup>-1</sup> body weight in mice.

The drug/molecular target nomenclature used in this study conforms with the BJP's *Guide to Receptors and Channels* (Alexander *et al.*, 2011).

### Cytochrome P450 (CYP) fluorometric inhibition assay

CYP inhibition assay was used to study if LH-21 could produce adverse drug reactions or toxicity, and performed in collaboration with the Fundación Medina (Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, Granada, Spain). The fluorometric assays repre-

sent the most common approach used to test compounds, as CYP inhibitors, in early drug discovery. They are high-throughput systems based on metabolism of substrates into highly fluorescent products. LH-21 and control inhibitors (ketoconazole, sulphafenazole and quinidine) were serially diluted by using a dilution factor of 2:1 to provide different concentrations. Dimethyl sulphoxide (DMSO; 0.35%) and acetonitrile (0.65%) were used as organic solvents with a maximum dose in the assay established as 105  $\mu$ M. Both fluorescence interference and quenching interference were determined for each compound in triplicate. Therefore, IC<sub>50</sub> values were obtained in fluorometric CYP inhibition assays with three different CYPs: CYP3A4; CYP2C9; and CYP2D6. LH-21 was categorized as moderate or weak inhibitor when compared with its respective control.

### Human Ether-à-go-go Related Gene (hERG) channel assay using a cell fluorescence functional assay

Testing the interaction of drugs with the hERG potassium channels in heterologous expression systems is recommended in order to identify drugs, such as LH-21, that may have the potential to cause cardiotoxicity. This study was also performed in collaboration with the above mentioned institution.

The FluxOR™ Potassium Ion Channel Assay is based on the activation of a fluorescent dye using thallium influx as a surrogate indicator of K<sup>+</sup> channel activity. HEK-293 (human embryonic kidney) cells expressing hERG K<sup>+</sup> channels were seeded into poly-D-lysine-coated 96-wells plates. The FluxOR™ potassium channel assay was performed as outlined in the product information sheet (available from Invitrogen Co., Carlsbad, CA, USA), and measured at room temperature via FLIPR Tetra System (Molecular Devices Inc., Sunnyvale, CA, USA). After 24 h of incubation, the plates were washed with assay buffer of the following composition (mM): 165 NaCl, 4.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES and 10 glucose, pH 7.4. Next, loading buffer containing the FluxOR™ dye mix was added into each well and the cells were incubated for 1 h at room temperature (23–25°C). They were washed once in assay buffer and then incubated with the same buffer containing LH-21 and control inhibitors (amiodarone, bepridil, haloperidol and terfenadin) at a dilution of 1:200, on the EP3 pipetting platform. Subsequently, compounds and LH-21 were measured in triplicate in each of three independent plates seeded with hERG-expressing HEK-293 cells, using 12 point curves (1:2 dilutions, and 150  $\mu$ M as maximum concentration). Astemizol (1  $\mu$ M) was added to each well as positive control whereas 0.5% DMSO was used as negative control. Next, plates containing both control inhibitors and LH-21 were allowed to equilibrate for 30 min at room temperature. Finally, stimulation buffer (Ti<sub>2</sub>SO<sub>4</sub> + K<sub>2</sub>SO<sub>4</sub>) was added to the plates via FLIPR Tetra System for kinetic analysis during 120 s, and IC<sub>50</sub> values were obtained.

### Acute feeding studies in LH-21-treated rodents

The acute effects of LH-21 on feeding behaviour were analysed in adult male Wistar rats ( $n = 6–8$  per dose group), and both wild-type and PPAR $\alpha$ -null adult male mice ( $n = 10–11$  per group).



For acute feeding studies, animals were deprived of food for 24 h but had free access to water (Gomez *et al.*, 2002; Pavon *et al.*, 2006). Before the beginning of this study, LH-21 at different doses was administered i.p. for 15 min before access to food and water. Animals were then returned to their individual home cages without any bedding material. Subsequently, a measured amount of food and water were placed into the cages ( $t = 0$  h). For Wistar rats, food pellets and food spillage were then weighed at time intervals of 30, 60, 120 and 240 min, and 24 h. Although a similar protocol was used for wild-type and PPAR $\alpha$ -null mice, pellets were weighed at 15, 30, 60, 120 and 240 min. The cumulative food intake ( $\text{g}\cdot\text{kg}^{-1}$  body weight) was calculated from these data.

### Diet-induced obesity models

To induce obesity, Wistar rats ( $n = 16$  per diet group) were fed an HFD of  $5.24 \text{ kcal}\cdot\text{g}^{-1}$  energy density (D12492; 60 kcal% fat, 20 kcal% protein, 20 kcal% carbohydrate), whereas controls received a standard diet (SD) containing  $3.85 \text{ kcal}\cdot\text{g}^{-1}$  (D12450B; 10 kcal% fat, 20 kcal% protein, 70 kcal% carbohydrate) (Research Diets, Inc., New Brunswick, NJ, USA) for 10 weeks. HFD contained fat constituted by soybean oil (9.26 kcal% of total fat content) and lard (90.74 kcal% of total fat content; cholesterol  $300.8 \text{ mg}\cdot\text{kg}^{-1}$  lard) (formulated by E.A. Ulman, PhD, Research Diets, Inc., 26 August 1998 and 11 March 1999). Food intake ( $\text{kcal}\cdot\text{kg}^{-1}$  body weight) and body weight (g) were measured daily for 10 weeks.

### A 10 day study of feeding behaviour in rats treated with LH-21

Following 10 weeks of feeding, animals received a daily i.p. injection of vehicle or LH-21 ( $3 \text{ mg}\cdot\text{kg}^{-1}$ ) for 10 consecutive days. Eight rats per group were used. Food intake ( $\text{kcal}\cdot\text{kg}^{-1}$  body weight) and body weight (g) were measured daily.

### Sample collection

The animals were killed 2 h after the last dose. LH-21-treated and control animals were anaesthetized (sodium pentobarbital,  $50 \text{ mg}\cdot\text{kg}^{-1}$ , i.p.) and blood samples were collected from the retro-orbital plexus. Blood was centrifuged ( $2100\times g$  C9 for 8 min,  $4^\circ\text{C}$ ) and the plasma kept for further analysis. Visceral white adipose tissue and liver samples were then removed, snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until analyses.

### RNA isolation and RT-PCR analysis

Total RNA was extracted from 100 mg frozen visceral adipose tissue and liver samples using Trizol Reagent (Gibco BRL Technologies, Baltimore, MD, USA), and reverse-transcribed with the Transcriptor Reverse Transcriptase kit (Transcriptor RT; Roche Diagnostics GmbH, Mannheim, Germany). Primer sequences were designed by our laboratory and synthesized by Sigma-Proligo (Paris, France). PCR amplification was performed using primer sets outlined in Table 2. All primer sequences were determined through established GenBank sequences. The cDNA obtained from each sample was used as a template for *q*PCR using both the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) and the iCycler iQReal-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Samples omitting reverse transcriptase were

included as negative controls in each set of reactions. The rat SP-1 and Rpl19 genes were amplified as controls and their expression was used to normalize gene expression levels.

### Liver triglyceride extraction and fatty acid analyses

Total lipids were extracted from frozen liver samples as described previously (Bligh and Dyer, 1959), and the liver fat content was expressed as a percentage of tissue weight. The lipid extracts were separated by TLC using hexane-diethylether-acetic acid (80:20:1, v/v/v) as the solvent system. After separation, the lipid spots corresponding to triglycerides were scraped from silica gel plates (Merck KGaA, Darmstadt, Germany), and fatty acid methyl esters (FAME) extracted with methanol benzene (4:1, v/v) for 1 h at  $90^\circ\text{C}$ . FAME were then analysed by gas-liquid chromatography, and individual fatty acids were identified with the following synthetic standards purchased from Supelco Analytical (Sigma Aldrich Co, St. Louis, MO, USA): lauric (12:0); myristic (14:0); palmitic (16:0); stearic (18:0); arachidic (20:0); palmitoleic (16:1); oleic (18:1); vaccenic (18:1); eicosenoic (20:1); linoleic (18:2);  $\gamma$ -linolenic (18:3);  $\alpha$ -linolenic (18:3); eicosadienoic (20:2); dihomogamma-linolenic (20:3); arachidonic (20:4); eicosapentaenoic (20:5); adrenic (22:4); docosapentaenoic (22:5); and docosahexaenoic acid (22:6). Fatty acids were quantified by area normalization and each fatty acid was expressed as a percentage of total fatty acids. They were finally grouped into saturated (SFA), mono-unsaturated (MUFA) or polyunsaturated (PUFA) fatty acids.

### Biochemical assays

The following plasma metabolites were measured in plasma obtained from the retro-orbital plexus of LH-21-treated and control animals: urea, creatinine, uric acid, cholesterol, HDL-cholesterol, glutamate-pyruvate transaminase (GPT), glutamate-oxaloacetate transaminase (GOT), and  $\gamma$ -glutamyl transpeptidase (GGT). They were analysed in the Hematology Service at the Carlos Haya Hospital (Málaga, Spain) using commercial kits according to manufacturer's instructions and a Hitachi 737 Automatic Analyser (Hitachi Ltd, Tokyo, Japan).

### Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA) by one-way or two-way ANOVA followed by Bonferroni's *post-hoc* test as appropriate. The results are expressed as the mean  $\pm$  SEM of 6–8 rats and 10–11 mice in each group. A *P*-value of less than 0.05 was considered significant.

## Results

### LH-21 does not inhibit P450 activity

The CYP family of enzymes is primarily responsible for drug metabolism. Drug-induced changes in CYP activity are a major source of adverse drug reactions, because CYP inhibition may affect the metabolism and clearance of many therapeutic drugs. Indeed, the inhibition of CYP enzymes may

Table 2

Primers sequences used for RT-PCR

Gene description	GenBank® accession	Oligosense (5'→3') Oligoantisense (5'→3')	Product size (bp)
<b>Ppara</b> (PPAR $\alpha$ )	NM_013196.1	5'TGCTGTCCTCCTTGATGAAC 5'GCTTGAGCACGTGCACAATC	270
<b>Acox1</b> (acyl-CoA oxidase 1)	NM_017340.1	5'CACCTTCGAGGGAGAGAACA 5'CGCACCTGGTCGTAGATTTT	75
<b>Pparg</b> (PPAR $\gamma$ )	NM_013124.1	5'GACCACTCCCATTCTTTGA 5'CGCACTTTGGTATTCTTGGAG	153
<b>Fasn</b> (fatty acid synthase)	NM_017332.1	5'AGTTTCCGTGAGTCCATCCT 5'TCAGGTTTCAGCCCCATAGA	182
<b>Scd1</b> (stearoyl-CoA desaturase 1)	NM_139192.1	5'GAAGCGAGCAACCGACAG 5'GGTGGTCGTGTAGGAAGTGG	71
<b>Cnr1</b> (CB <sub>1</sub> )	NM_012784.3	5'ACAGCCAGCATGCACAGGGC 5'CGGCCGACGTGTCTGTGGAC	94
<b>Cnr2</b> (CB <sub>2</sub> )	NM_020543.4	5'AGGATAAGCAGGAGTTGGGAGGAGA 5'TGAATCTGCCAGAGACAGCATGGG	80
<b>Gpr55</b> (GPCR 55)	XM_576605.2	5'AAAACCTTTGGGATCTGCTG 5'TAGATGGGGATGCTTCCAAC	62
<b>Adipoq</b> (adiponectin hormone)	NM_144744.2	5'AGGGATTACTGCAACCGAAG 5'TCCTGTCCATTCCAGCATCTC	211
<b>Lep</b> (leptin hormone)	NM_013076.1	5'AGGAAAATGTGCTGGAGACC 5'ATACCGACTGCGTGTGTGAA	160
<b>Sp1</b> (Sp1 transcription factor)	NM_012655.2	5'GCTATAGCAAACACCCAGGT 5'GATCAGGGCTGTCTCTCCTT	115
<b>Rpl19</b> (ribosomal protein L19)	NM_031103.1	5'TGCCGGAAGAACACCTTG 5'GCAGGATCCTCATCCTTCG	121

Table 3

Interaction of LH21 with hepatic cytochrome P450 isoforms

Cytochrome P450 fluorometric inhibition assay							
CYP subtype	Control Inhibitors*			LH-21 Inhibition IC <sub>50</sub> (μM)	Lower 95% CL	Upper 95% CL	Inhibitor category
	Inhibition IC <sub>50</sub> (μM)	Lower 95% CL	Upper 95% CL				
CYP3A4	0.052 <sup>A</sup>	0.041 <sup>A</sup>	0.066 <sup>A</sup>	1.62	1.46	1.79	Moderate
CYP2C9	0.23 <sup>B</sup>	0.18 <sup>B</sup>	0.29 <sup>B</sup>	8.14	6.84	9.70	Moderate
CYP2D6	0.018 <sup>C</sup>	0.015 <sup>C</sup>	0.021 <sup>C</sup>	>105	–	–	Weak

\*Control Inhibitors in each CYP inhibition assay: (<sup>A</sup>) Ketoconazole; (<sup>B</sup>) Sulphafenazole; (<sup>C</sup>) Quinidine. CL, confidence level.

produce elevated blood concentrations of drugs resulting in adverse drug effects and toxicity.

As shown in Table 3, LH-21 did not display fluorescent or quenching interference. LH-21 exhibited much higher IC<sub>50</sub> values than the control inhibitors of CYP isoenzymes (CYP3A4, CYP2C9 and CYP2D6). LH-21 can thus be considered to be a moderate/weak CYP inhibitor. Consequently, LH-21 is not expected to induce drug–drug interactions that could lead to adverse reactions or toxicity.

### LH-21 produces no cardiotoxic effects

Drug-induced sudden cardiac death is associated with a prolongation of the QT interval in the electrocardiogram. When the QT interval is prolonged, there is an increased risk of ventricular tachyarrhythmia due to an excessive time between depolarization and repolarization. Inhibition of the delayed rectifier K<sup>+</sup> current (I<sub>Kr</sub>) is the most common mechanism responsible for drug-induced prolongation of QT

interval in humans and such inhibition is conducted by hERG potassium channels.

In our studies with HEK-293 cells expressing hERG, LH-21 displayed no activity in this assay. Thus, it exhibits a high IC<sub>50</sub> value (>150 μM) as compared with standard control inhibitors (IC<sub>50</sub> values for amiodarone, 1.7 μM; bepridil, 2.2 μM; haloperidol, 1.9 μM; terfenadine, 1.0 μM). Thus, LH-21 is a drug with no inhibitory activity on hERG and can be considered a safe drug devoid of cardiotoxicity.

### LH-21 causes a dose-dependent decrease in food intake in fasted Wistar rats

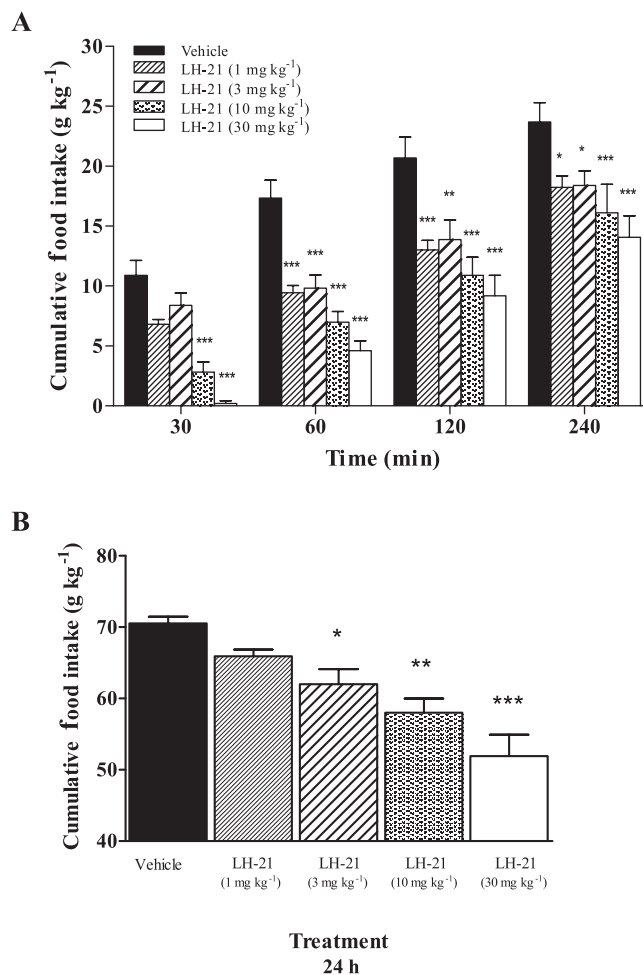
In order to select an effective anorectic dose of LH-21 for subsequent sub-chronic studies, we performed an acute study on feeding behaviour in 24 h-fasted Wistar rats. We evaluated the effect of different doses of LH-21 on feeding at different times during a period of 240 min (Figure 1A), and 24 h after the injection of the drug (Figure 1B). Two-way ANOVA analysis of acute effects revealed the different doses of LH-21 that produced a significant effect on cumulative food intake ( $F_{4,108} = 39.86$ ;  $P < 0.0001$ ) with no interaction with time ( $F_{12,108} = 0.57$ ; n.s.). As expected, the time factor reflected the increase in food intake ( $F_{3,108} = 78.13$ ;  $P < 0.0001$ ). Bonferroni's *post-hoc* test indicated that LH-21 produced a dose-dependent inhibitory effect on feeding, resulting in a significant decrease with all doses tested: 1 mg·kg<sup>-1</sup> ( $P < 0.001$  at 60 and 120 min;  $P < 0.05$  at 240 min), 3 mg·kg<sup>-1</sup> ( $P < 0.001$  at 60 min;  $P < 0.01$  at 120;  $P < 0.05$  at 240 min), 10 mg·kg<sup>-1</sup> ( $P < 0.001$  at 30, 60, 120 and 240 min) and 30 mg·kg<sup>-1</sup> of LH-21 ( $P < 0.001$  at 30, 60, 120 and 240 min), when compared with the vehicle-treated group (Figure 1A). At 240 min all tested doses (1, 3, 10 and 30 mg·kg<sup>-1</sup>, i.p.) exhibited a significant reduction of cumulative food intake (relative percentages over vehicle-treated rats of 77, 78, 68 and 59%, respectively).

Twenty-four hours after the injection of LH-21, the cumulative food intake was evaluated again and a one-way ANOVA showed that the different doses had a marked effect on feeding ( $F_{4,27} = 12.33$ ;  $P < 0.0001$ ). Significant dose-dependent reductions in feeding were observed with LH-21 at doses of 3 mg·kg<sup>-1</sup> ( $P < 0.05$ ), 10 mg·kg<sup>-1</sup> ( $P < 0.001$ ) and 30 mg·kg<sup>-1</sup> ( $P < 0.001$ ) when compared with the vehicle-treated group (Figure 1B). Relative percentages of cumulative food intake were approximately 93, 88, 82 and 74%, with respect to vehicle-treated rats after administration of LH-21, 1, 3, 10 and 30 mg·kg<sup>-1</sup> i.p., respectively.

Overall, the results indicate that LH-21 is a potent hypophagic drug capable of maintaining its anorectic properties for at least 24 h. In accord with previous studies in rats (Pavon *et al.*, 2006; 2008 2008), 3 mg·kg<sup>-1</sup> of LH-21 was an effective dose to perform a 10 day treatment in diet-induced obese rats.

### LH-21 inhibits body weight gain and reduces the food intake in diet-induced obese rats

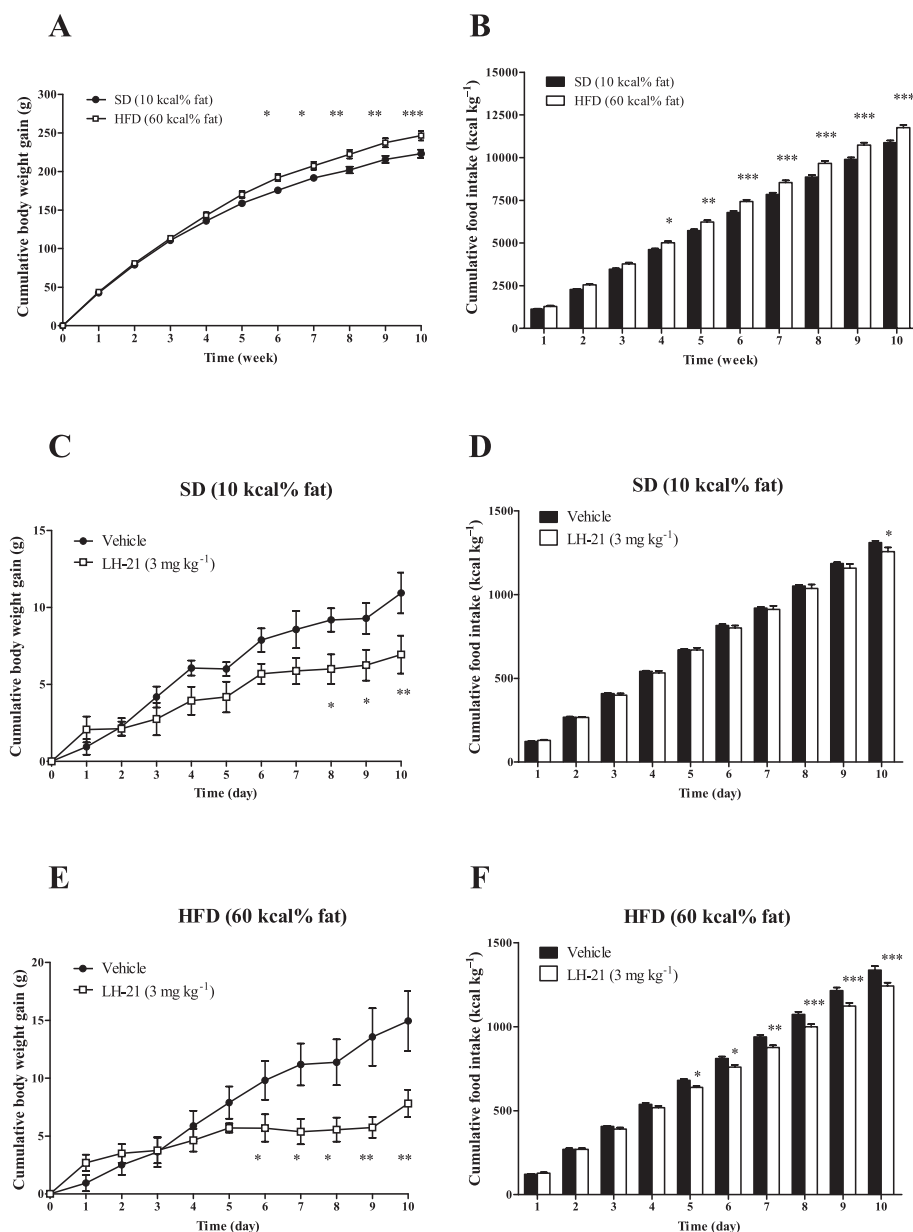
To further explore the anti-obesity properties of LH-21 previously studied on naïve rats and genetically obese rats (*fa/fa*, missense mutation in the leptin receptor), we generated another animal model of obesity, induced by exposure to HFD for 10 weeks. As shown in Figure 2A, both diets had a



**Figure 1**

Effects of acute administration of LH-21 on cumulative food intake in male Wistar rats. Time course of the dose-response effect of LH-21 (1, 3, 10 and 30 mg·kg<sup>-1</sup>, i.p.) at 30, 60, 120 and 240 min (A), and 24 h (B) on cumulative food intake (g·kg<sup>-1</sup> body weight). Columns are means ± SEM ( $n = 8$  animals per group). Data were analysed by one- or two-way ANOVA (treatment and time) and Bonferroni's *post-hoc* test. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote significant differences compared with the vehicle-treated group.

significant effect on cumulative body weight gain of Wistar rats (two-way ANOVA,  $F_{1,330} = 43.77$ ;  $P < 0.0001$ ). This analysis also revealed a significant interaction between diet and time ( $F_{10,330} = 2.553$ ;  $P = 0.0056$ ), because the differences in weights due to diet were greater with time. The HFD produced a significant enhancement in weight gain when compared with the SD, that started on the 6th week of exposure ( $P < 0.05$ , 6th and 7th week;  $P < 0.01$ , 8th and 9th week;  $P < 0.001$ , 10th week). At the beginning of the treatment with LH-21 (10<sup>th</sup> week), HFD-fed rats displayed an increase of 10% in cumulative body weight gain compared with SD-fed rats. With respect to food intake, the statistical analysis revealed differences in feeding between both diets (diet effect,  $F_{1,300} = 151.1$ ;  $P < 0.0001$ ) and a significant interaction between diet and time of exposure ( $F_{9,300} = 43.77$ ;  $P = 0.0007$ ) (Figure 2B). HFD-exposure resulted in a marked and significant increase



**Figure 2**

Effects on body weight and food intake in male Wistar rats fed SD or HFD, and following a 10 day treatment with LH-21. Cumulative body weight gain (A) and cumulative food intake (B) were evaluated weekly for 10 week exposure to SD or HFD. Points and columns are means  $\pm$  SEM ( $n = 16$  animals per group). Data were analysed by two-way ANOVA (diet and time) and Bonferroni's *post-hoc* test. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote significant differences compared with the SD-fed group. Cumulative body weight gain (C, E) and cumulative food intake (D, F) in SD and HFD-fed animals, respectively, were evaluated after a 10 day exposure to vehicle or LH-21 (3 mg·kg<sup>-1</sup>, daily, i.p.). Points and columns are means  $\pm$  SEM ( $n = 8$  animals per group). Data were analysed by two-way ANOVA (diet and treatment) and Bonferroni's *post-hoc*. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote significant differences compared with the vehicle-treated group.

in cumulative food intake that started on the 4th week ( $P < 0.05$ , 4th week;  $P < 0.01$ , 5th week;  $P < 0.001$ , 6th to 10th week) as compared with SD-fed rats. Significant time effects were detected in weight gain and feeding using ANOVA (data not shown).

After a significant segregation of both diet groups were observed, we examined the effect of prolonged LH-21 treatment on body weight and feeding for 10 days (3 mg·kg<sup>-1</sup>,

once daily, i.p.). In rats fed SD, LH-21 reduced the body weight gain (two-way ANOVA analysis,  $F_{1,154} = 24.82$ ;  $P < 0.0001$ ) and only produced a modest effect on cumulative food intake ( $F_{1,140} = 5.185$ ;  $P < 0.05$ ) when compared with the vehicle-treated group (Figure 2C and D). However, LH-21 produced a significant inhibition of body weight gain ( $F_{1,154} = 25.06$ ;  $P < 0.0001$ ) and a marked reduction of feeding ( $F_{1,140} = 61.43$ ;  $P < 0.0001$ ) at all time points analysed in the HFD-fed



Table 4

Hepatic lipid content of diet-induced obese male Wistar rats treated with LH-21

Hepatic lipid levels	Treatment and diet		HFD	
	SD Vehicle	LH-21 (3 mg·kg <sup>-1</sup> )	Vehicle	LH-21 (3 mg·kg <sup>-1</sup> )
Total fat content (% tissue)	4.08 ± 0.34	4.39 ± 0.36	8.21 ± 1.03***	7.91 ± 0.71**
Triglycerides (% tissue)	0.86 ± 0.09	0.88 ± 0.10	2.913 ± 0.42***	2.74 ± 0.31***
SFA (% total fatty acids)	41.96 ± 2.89	45.02 ± 1.84	33.56 ± 1.86*	30.78 ± 1.07**
MUFA (% total fatty acids)	38.68 ± 1.11	41.45 ± 1.54	32.96 ± 0.96*	34.67 ± 1.20
PUFA (% total fatty acids)	19.36 ± 2.21	13.53 ± 1.47	33.48 ± 1.26***	34.55 ± 1.18***
SFA/(MUFA + PUFA)	0.75 ± 0.09	0.83 ± 0.07	0.51 ± 0.04*	0.45 ± 0.02**

Wistar rats fed standard (SD: 10 kcal% fat) or high fat diet (HFD: 60 kcal% fat) for 10 weeks, then treated with vehicle or LH-21 (3 mg·kg<sup>-1</sup>, i.p.) daily for 10 days. Lipid levels in liver were analysed 2 h after the last injection as described in the Methods section. Data were analysed by two-way ANOVA (diet and treatment) for each parameter (see details in the Results section). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote significant differences compared with vehicle-treated SD group (Bonferroni's *post-hoc* test). Values are expressed as mean ± SEM ( $n = 8$  animals per group).

rats that started on the 5<sup>th</sup> day of treatment (Figure 2E and F). This higher efficacy detected with LH-21 on reducing food intake in HFD was statistically confirmed when we analysed the differences on feeding in each diet the last day (10th day) after a continuous treatment. Therefore, an additional two-way ANOVA showed significant effects caused by treatment ( $F_{1,28} = 12.80$ ;  $P = 0.0013$ ) as expected, but the post-test revealed a significant reduction of cumulative food intake only in HFD-fed rats ( $P < 0.01$ ) with no effects on SD when compared with vehicle-treated rats.

These data indicate that LH-21 is able to maintain its anti-obesity properties in diet-induced obesity at doses that do not target brain CB<sub>1</sub> receptors, as was reported in leptin signalling-deficient animals (Pavon *et al.*, 2006; 2008).

### LH-21 does not affect fat composition and content in liver

As the liver is an essential organ involved in lipid metabolism, we investigated the lipid content and its fatty acid composition to identify any effects of the diet and/or LH-21 treatment. As shown in Table 4, two-way ANOVA revealed that LH-21 had no effect on the lipid variables analysed (total fat, triglycerides and fatty acid composition) in both diet-groups. However, important changes were found between SD and HFD groups regardless of whether animals received LH-21 or not. HFD induced a twofold increase in total fat content in liver as compared with SD rats ( $F_{1,28} = 32.40$ ;  $P < 0.0001$ ); a marked increase in triglycerides (threefold) was observed in HFD rats ( $F_{1,28} = 52.75$ ;  $P < 0.0001$ ). Additionally, liver fatty acids of triglycerides were identified and quantified by chromatography. The distribution of the most abundant fatty acids in HFD rats were different from those found in vehicle-treated SD group, with the exception of oleic acid. LH-21 did not appear to have any effect on the fatty acid distribution. The relative percentage of palmitic, oleic and linoleic acid represents about 85% of total for each group, and were as follows: [palmitic acid (16:0): vehicle-treated SD 36.88 ± 2.51%, LH-21-treated SD 39.86 ± 1.70%, vehicle-treated HFD

28.86 ± 1.58% ( $P < 0.05$ ), LH-21-treated HFD 26.20 ± 1.03% ( $P < 0.001$ ); oleic acid (18:1): vehicle-treated SD 30.43 ± 0.66%, LH-21-treated SD 32.89 ± 1.02%, vehicle-treated HFD 29.84 ± 0.83%, LH-21-treated HFD 31.34 ± 0.96%; linoleic acid (18:2): vehicle-treated SD 16.64 ± 1.68%, LH-21-treated SD 12.03 ± 1.38%, vehicle-treated HFD 25.34 ± 0.81% ( $P < 0.001$ ), LH-21-treated HFD 26.62 ± 0.82% ( $P < 0.001$ )] (see Supporting Information Table S1).

Data were also analysed by a two-way ANOVA (diet and treatment) after being grouped as SFA, MUFA and PUFA. The type of diet had a significant effect on each type of fatty acid analysed in both treatment-subgroups (vehicle and LH-21) [SFA: ( $F_{1,28} = 31.41$ ;  $P < 0.0001$ ); MUFA ( $F_{1,28} = 26.22$ ;  $P < 0.0001$ ); and PUFA ( $F_{1,28} = 123.13$ ;  $P < 0.0001$ )], and only PUFA showed an interaction between treatment and diet ( $F_{1,28} = 4.77$ ;  $P = 0.0379$ ). To calculate the SFAs/unsaturated fatty acids ratio we used the formula SFA/(MUFA + PUFA). Two-way ANOVA revealed a significant effect of the HFD on this lipid ratio ( $F_{1,28} = 27.25$ ;  $P < 0.0001$ ). Within-group differences were obtained by Bonferroni's *post hoc* test (see Table 4).

### Effects of LH-21 on blood biochemical parameters

In order to investigate the effect of LH-21 on blood biochemistry indices of toxicity, we analysed the following plasma values: urea, creatinine and uric acid, primary nitrogenous waste products of protein, which are excreted by the kidneys; GPT, GOT and GGt, transaminases whose activities are related to liver toxicity; and finally cholesterol and HDL-C.

Two-way ANOVA revealed that LH-21 had minimal effects on biochemical metabolic parameters related to toxicity in plasma (Table 5). Indeed, LH-21 induced only a significant decrease in the creatinine level ( $F_{1,28} = 42.67$ ;  $P < 0.0001$ ), which showed no interaction with diet. However, diet produced significant effects on both urea and uric acid levels in plasma. Thus, HFD reduced urea ( $F_{1,28} = 13.76$ ;  $P = 0.0009$ ) and uric acid levels ( $F_{1,28} = 4.26$ ;  $P = 0.0484$ ) as compared with the

Table 5

Metabolic biochemical parameters in plasma of diet-induced obese male Wistar rats treated with LH-21

Plasma biochemical parameter	Treatment and Diet SD		HFD	
	Vehicle	LH-21 (3 mg kg <sup>-1</sup> )	Vehicle	LH-21 (3 mg kg <sup>-1</sup> )
Urea (mg L <sup>-1</sup> )	3.95 ± 0.18	3.68 ± 0.14	3.24 ± 0.18	3.28 ± 0.09
Creatinine (mg mL <sup>-1</sup> )	82.4 ± 2.5	64.9 ± 3.9	86.3 ± 2.1	59.8 ± 4.5
Uric acid (mg mL <sup>-1</sup> )	57.3 ± 17.1	90.0 ± 28.5	42.5 ± 7.1	34.3 ± 3.3
Cholesterol (mg L <sup>-1</sup> )	9.20 ± 0.74	7.36 ± 0.84	8.16 ± 0.87	7.19 ± 0.58
HDL-C (mg L <sup>-1</sup> )	2.74 ± 0.12	2.34 ± 0.16	2.49 ± 0.27	2.36 ± 0.11
GPT (U L <sup>-1</sup> )	41.13 ± 3.89	52.25 ± 8.38	48.13 ± 4.75	50.25 ± 6.93
GOT (U L <sup>-1</sup> )	149.88 ± 41.66	119.50 ± 14.78	112.38 ± 20.62	114.25 ± 10.79
GGT (U L <sup>-1</sup> )	6.13 ± 0.74	4.50 ± 0.76	5.13 ± 0.87	6.25 ± 0.56

Wistar rats fed standard (SD: 10 kcal% fat) or high-fat diet (HFD: 60 kcal% fat) for 10 weeks, then treated with vehicle or LH-21 (3 mg·kg<sup>-1</sup>, i.p.) daily for 10 days. Plasma parameters were analysed 2 h after the last injection as described in the Methods section. Data were analysed by two-way ANOVA (diet and treatment) for each parameter (see details in the Results section).

Values are expressed as mean ± SEM ( $n = 8$  animals per group).

GGT,  $\gamma$ -glutamyl transpeptidase; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; HDL-C, high-density lipoprotein cholesterol.

SD-fed group. Neither diet nor LH-21 treatment-dependent effects were observed on the other parameters (cholesterol, HDL-C, GPT, GOT and GGT).

### Effects of LH-21 on lipid metabolism-related gene expression in adipose tissue

The expression of the mRNA of both, enzymes and receptors related to lipid metabolism, was analysed by RT-PCR in adipose tissue of treated animals. We selected genes implicated in pathways for lipolysis [PPAR $\alpha$  and acyl-CoA oxidase (ACOX)] or lipogenesis [PPAR $\gamma$ , fatty acid synthase (FAS) and SCD-1]. Overall, LH-21 modulated both pathways as can be seen in Figure 3.

Although diet did not affect the mRNA expression of PPAR $\alpha$  (Ppara) (Figure 3A) LH-21 treatment had a strong effect on this nuclear receptor ( $F_{1,27} = 15.08$ ;  $P = 0.0006$ ) inhibiting significantly its expression in both SD-fed ( $P < 0.01$ ) and HFD-fed ( $P < 0.05$ ) rats, when compared with vehicle groups. In contrast, ACOX expression (Acox1) was affected by both, diet and treatment. Thus, HFD induced a significant increase in the gene expression of ACOX ( $P < 0.001$ ) in vehicle-treated animals when compared with SD-fed animals. However, LH-21 prevented selectively this increase only in the HFD group ( $P < 0.01$ ). A two-way ANOVA showed that both treatment ( $F_{1,27} = 5.73$ ;  $P = 0.0238$ ) and diet ( $F_{1,27} = 16.76$ ;  $P = 0.0003$ ) had significant effects on this oxidative enzyme. Additionally, a significant interaction between diet and treatment was also detected ( $F_{1,27} = 11.31$ ;  $P = 0.0023$ ) confirming a selective effect of LH-21 on ACOX mRNA in HFD-fed rats.

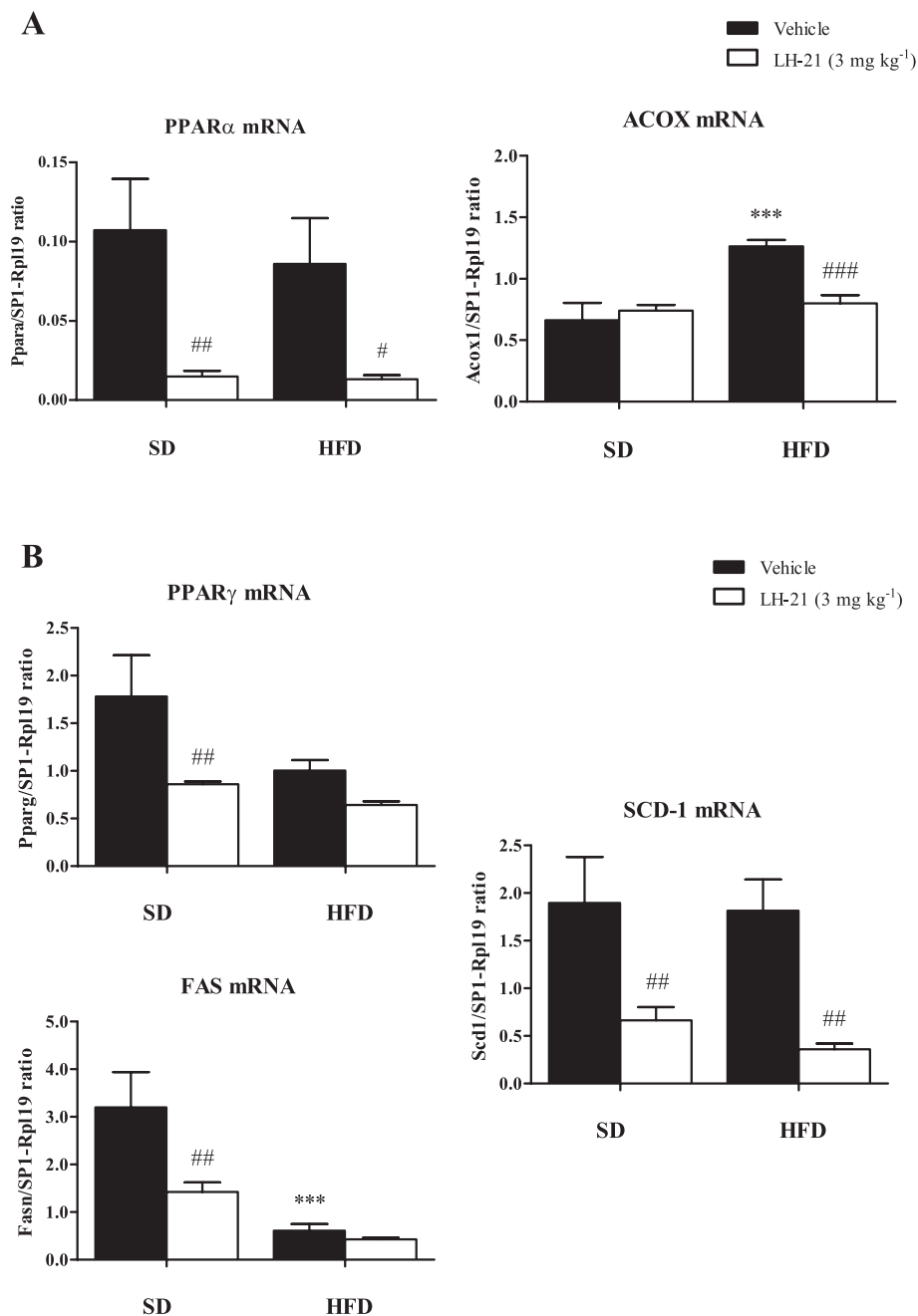
Regarding lipogenic genes, effects due to diet and treatment are depicted in Figure 3B. Gene expression of PPAR $\gamma$  (Pparg) was significantly affected by both diet and treatment in an independent way. In particular, diet induced significant effects on this transcriptional factor ( $F_{1,26} = 5.62$ ;  $P = 0.0254$ ,

where HFD reduced its expression in the adipose tissue. LH-21 reduced PPAR $\gamma$  expression in both diet groups ( $F_{1,26} = 9.38$ ;  $P = 0.0051$ ) with a higher effect in SD-fed rats ( $P < 0.01$ ).

Fatty acid synthase (FAS) is an enzyme that plays a key role in fatty acid synthesis. A two-way ANOVA revealed that diet and treatment induced significant changes in the expression of FAS (Fasn) mRNA, and both factors displayed a significant interaction ( $F_{1,26} = 4.72$ ;  $P = 0.0391$ ). Thus, diet had a very strong effect on FAS ( $F_{1,26} = 24.00$ ;  $P < 0.0001$ ); HFD-fed rats had a significant decrease in FAS when compared with vehicle-treated animals ( $P < 0.001$ ). LH-21 also decreased significantly FAS expression ( $F_{1,26} = 7.08$ ;  $P = 0.0132$ ), but this effect was only observed in the SD-fed group because of the strong inhibition of HFD on FAS expression ( $P < 0.01$ ). The lipogenic enzyme SCD-1 is associated with increased fat accumulation and mono-unsaturation of SFAs. The gene expression of SCD-1 (Scd1) was only affected by LH-21 ( $F_{1,26} = 22.46$ ;  $P < 0.0001$ ) with no interaction between diet and treatment; LH-21 produced a strong reduction in SCD-1 in both SD-fed ( $P < 0.01$ ) and HFD-fed ( $P < 0.01$ ) animals.

### Effects of LH-21 on gene expression of cannabinoid receptors and GPR55 in adipose tissue

Classical CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors and the GPR55 orphan receptor were also analysed in adipose tissue. GPR55 was included as it was originally reported to be a putative cannabinoid receptor (Baker *et al.*, 2006), although conflicting pharmacological data do not further support this classification because this receptor displays great affinity for cannabinoid antagonists but not for endocannabinoids. These receptors were determined by RT-PCR in adipose tissue (Figure 4). Gene expression of CB<sub>1</sub> receptor (Cnr1) was significantly modified by diet and treatment as revealed

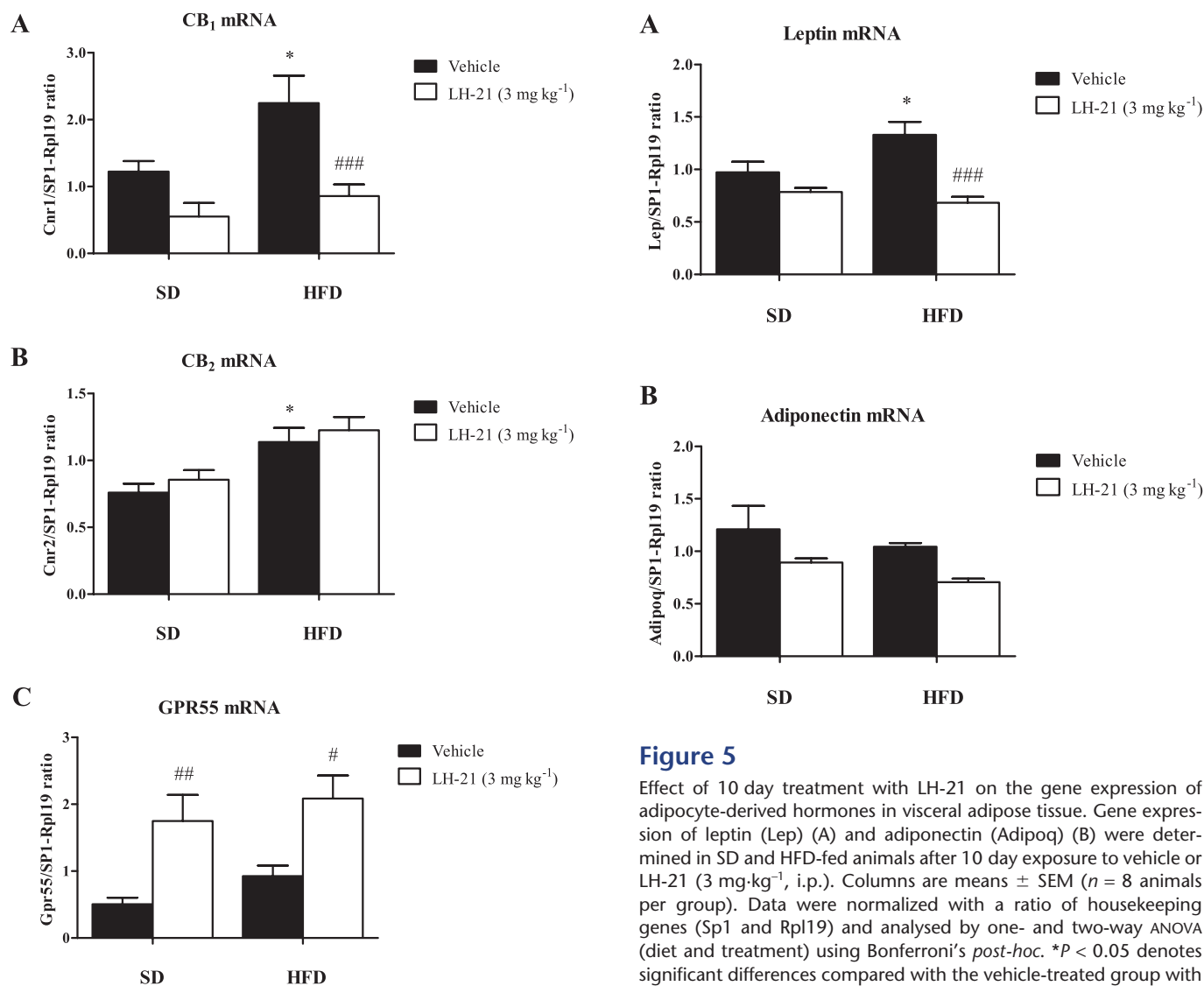


**Figure 3**

Effect of 10 day treatment with LH-21 on the gene expression of lipid metabolism-related proteins in visceral adipose tissue. Gene expression of PPAR $\alpha$  and ACOX (Acox1) were determined as lipolytic molecules (A); and gene expression of PPAR $\gamma$ , FAS (Fasn) and SCD-1 (Scd1) were determined as lipogenic molecules (B) in SD and HFD-fed animals after 10 day exposure to vehicle or LH-21 (3 mg·kg<sup>-1</sup>, i.p.). Columns are means  $\pm$  SEM ( $n = 8$  animals per group). Data were normalized with a ratio of housekeeping genes (Sp1 and Rpl19) and analysed by two-way ANOVA (diet and treatment) using Bonferroni's *post-hoc*. \*\*\* $P < 0.001$  denotes significant differences compared with the vehicle-treated group with SD; # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  denote significant differences compared with the corresponding vehicle-treated group.

by two-way ANOVA with no interaction between these factors. Diet had a significant influence on CB<sub>1</sub> mRNA ( $F_{1,26} = 7.28$ ;  $P = 0.0121$ ) and the *post-hoc* test confirmed this effect. Thus, vehicle-treated HFD-fed group showed a significant increase (~2-fold) of CB<sub>1</sub> as compared with vehicle-treated SD group ( $P < 0.05$ ). Interestingly, LH-21 also exerted

a significant effect on CB<sub>1</sub> ( $F_{1,26} = 17.57$ ;  $P = 0.0003$ ). In fact, LH-21 markedly decreased the gene expression of CB<sub>1</sub> in both diet groups, suppressing the diet-induced increase by 50% ( $P < 0.01$ ) in HFD-fed rats (Figure 4A). CB<sub>2</sub> receptor expression (Cnr2) was found to be elevated in rats fed a HFD ( $F_{1,27} = 18.72$ ;  $P = 0.0002$ ) when compared with the

**Figure 4**

Effect of 10 day treatment with LH-21 on the gene expression of cannabinoid receptors in visceral adipose tissue. Gene expression of CB<sub>1</sub> (Cnr1) (A), CB<sub>2</sub> (Cnr2) (B), and GPR55 (C) were determined in SD and HFD-fed animals after 10 day exposure to vehicle or LH-21 (3 mg·kg<sup>-1</sup>, i.p.). Columns are means ± SEM (*n* = 8 animals per group). Data were normalized with a ratio of housekeeping genes (Sp1 and Rpl19) and analysed by two-way ANOVA (diet and treatment) using Bonferroni's *post-hoc*. \**P* < 0.05 denotes significant differences compared with the vehicle-treated group with SD; #*P* < 0.05, ##*P* < 0.01 and ###*P* < 0.001 denote significant differences compared with the corresponding vehicle-treated group.

respective SD-fed groups. However, LH-21 had no effect on CB<sub>2</sub> receptor expression, independently of diet, as shown in Figure 4B.

The mRNA expression of GPR55 (Gpr55) receptor was only affected by LH-21 ( $F_{1,27} = 17.80$ ;  $P = 0.0002$ ), which exerted a robust and significant increase in GPR55 mRNA in both the SD and HFD groups (~three- and ~twofold, respectively) when compared with vehicle-treated rats ( $P < 0.01$  and  $P < 0.05$ ) (Figure 4C).

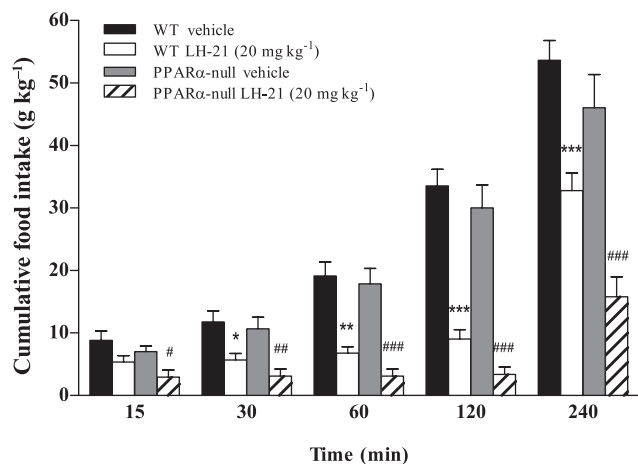
**Figure 5**

Effect of 10 day treatment with LH-21 on the gene expression of adipocyte-derived hormones in visceral adipose tissue. Gene expression of leptin (Lep) (A) and adiponectin (Adipoq) (B) were determined in SD and HFD-fed animals after 10 day exposure to vehicle or LH-21 (3 mg·kg<sup>-1</sup>, i.p.). Columns are means ± SEM (*n* = 8 animals per group). Data were normalized with a ratio of housekeeping genes (Sp1 and Rpl19) and analysed by one- and two-way ANOVA (diet and treatment) using Bonferroni's *post-hoc*. \**P* < 0.05 denotes significant differences compared with the vehicle-treated group with SD; ###*P* < 0.001 denotes significant differences compared with the corresponding vehicle-treated group.

### Effects of LH-21 on gene expression of adipose-derived hormones

Both, leptin and adiponectin (Adipoq) are peptide hormones released from adipose tissue that play a key role in regulating energy intake and energy expenditure. With regard to leptin, we observed a clear diet × treatment interaction ( $F_{1,24} = 7.04$ ;  $P = 0.0139$ ). Thus, HFD increased leptin (Lep) expression in the adipose tissue ( $F_{1,24} = 23.09$ ;  $P < 0.0001$ ) and LH-21 significantly decreased leptin expression ( $P < 0.001$ ) in HFD-fed animals. In the SD group, LH-21 showed a similar profile but this decrease in leptin expression was not significant (Figure 5A).

On the other hand, diet had no effects on Adipoq mRNA, although LH-21 significantly decreased its expression, as revealed by two-way ANOVA ( $F_{1,26} = 8.96$ ;  $P < 0.0060$ ) (Figure 5B).



**Figure 6**

Effects of acute administration of LH-21 on cumulative food intake in wild-type and PPAR $\alpha$ -null mice. Time course of the effect of LH-21 (20 mg·kg<sup>-1</sup>, i.p.) on cumulative food intake (g·kg<sup>-1</sup> body weight). Columns are means  $\pm$  SEM ( $n = 9$  animals per group). Data were analysed by two-way ANOVA (treatment and time) and Bonferroni's post-hoc. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  denote significant differences compared with the wild-type vehicle-treated group; # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  denote significant differences compared with the PPAR $\alpha$ -null vehicle-treated group.

### LH-21 causes a decrease in food intake in both PPAR $\alpha$ -null and wild-type mice

As LH-21 was able to suppress PPAR $\alpha$  mRNA expression (Ppara) dramatically in adipose tissue of both diet groups (Figure 3A), we investigated if it could influence feeding through the well-described actions of PPAR $\alpha$  on feeding behaviour (Fu *et al.*, 2003; Rodriguez de Fonseca *et al.*, 2001). To this end, the actions of LH-21 were evaluated in 24 h-fasted PPAR $\alpha$ -null mice and wild-type control mice. We tested a single effective dose of LH-21 (20 mg·kg<sup>-1</sup>, i.p.) on feeding for 240 min, measuring cumulative food intake at different times (Figure 6). We detected no differences between both genotypes. Thus, statistical analysis revealed that neither LH-21 treatment ( $F_{3,195} = 75.05$ ;  $P < 0.0001$ ) nor time intervals ( $F_{4,195} = 114.41$ ;  $P < 0.0001$ ) were different between the genotypes, on which we observed a clear reduction of feeding induced by LH-21 treatment that was sustained over time. LH-21-treated mice displayed a significant decrease in cumulative food intake at 15 min ( $P < 0.05$  in PPAR $\alpha$ -null mice), 30 min ( $P < 0.05$  in wild-type mice;  $P < 0.01$  in PPAR $\alpha$ -null mice), 60 min ( $P < 0.01$  in wild-type mice;  $P < 0.001$  in PPAR $\alpha$ -null mice), 120 min ( $P < 0.001$  in wild-type mice;  $P < 0.001$  in PPAR $\alpha$ -null mice) and 240 min ( $P < 0.001$  in wild-type mice;  $P < 0.001$  in PPAR $\alpha$ -null mice) when compared with their respective vehicle-treated mice (Figure 6). At 120 min the anorectic effect on feeding behaviour between mice exposed to LH-21 and vehicle reached the highest difference as indicated by the relative percentages of cumulative food intake for each genotype compared to vehicle-treated mice: in wild-type was approximately 27% whereas in PPAR $\alpha$ -null mice was 12%. Food intake after 120 min was practically

the same as that during the first 15 min in both genotypes. However, at 240 min these feeding values were increased to 61% and 35% in wild-type and PPAR $\alpha$ -null mice, respectively. Therefore, a single dose of 20 mg·kg<sup>-1</sup> i.p. of LH-21 in mice was enough to induce a significant and sustained decrease in food intake for at least 4 h and the absence of PPAR $\alpha$  had no effect on this anorectic activity.

## Discussion and conclusions

Three main important findings on the pharmacological properties of LH-21 arose from the present study. Firstly, this cannabinoid CB<sub>1</sub> receptor antagonist displays a safe pharmacological profile, as: (i) it does not interact with the hERG cardiac potassium channels; (ii) it has no or moderate effects on the activity of different isoforms of hepatic cytochrome P450; and (iii) it does not induce liver or kidney toxicity after short-term repeated administration. Secondly, LH-21 maintains its anorectic activity in a model of diet-induced obesity, as previously reported in either food-deprived Wistar rats or obese Zucker rats (Pavon *et al.*, 2006; 2008), and is more effective at reducing food intake and body weight gain in animals fed a HFD as compared with an SD. Thirdly, these effects were associated with changes in mRNA expression of genes related to lipogenesis and leptin in the adipose tissue, whereas we detected no changes in metabolic abnormalities in liver or plasma due to diet in contrast to other CB<sub>1</sub> blockers.

In accordance with previous observations, a 10 week exposure to HFD caused a higher increase in body weight gain and food intake in comparison with SD-fed animals (Crespillo *et al.*, 2010). A 10 day treatment with LH-21 produced a significant reduction in cumulative food intake and body weight gain in both diets. These results show that the changes observed on body weight in LH-21-treated rats were more evident than changes in food intake. Indeed, several studies have reported that body weight reductions observed in diet-induced obese mice treated with CB<sub>1</sub> antagonists, such as rimonabant and AM6545, have been related to food intake-independent of effects on energy expenditure (Kunos and Tam, 2011; Ravinet Trillou *et al.*, 2003; Tam *et al.*, 2010). LH-21 appeared to be more efficacious as an anti-obesity agent in rats fed HFD than SD. This increased sensitivity of HFD-obese animals has been described previously (Matias *et al.*, 2008; Starowicz *et al.*, 2008; Izzo *et al.*, 2009; 2010) and can be explained by a higher sensitivity to the effects of CB<sub>1</sub> antagonists due to the dysregulation and overactivation of the endocannabinoid system. Increased concentrations of endocannabinoids in peripheral tissues, for example, increased 2-AG levels in adipose tissue (Matias *et al.*, 2006) and increased anandamide levels in liver (Osei-Hyiaman *et al.*, 2005), have been reported after exposure to HFDs in obese rodents. Additionally, studies with AM251 have demonstrated that its anorectic action is enhanced in animals fed a HFD (Judge *et al.*, 2009). Also, AM6545, a non-brain penetrant neutral CB<sub>1</sub> antagonist that reduces caloric intake of high fat and high carbohydrate diets, is less effective at reducing lab chow intake (Randall *et al.*, 2010). Further, classical studies with rimonabant show that it is more effective in



obese rats than in lean controls (Colombo *et al.*, 1998; Bensaid *et al.*, 2003; Ravinet Trillou *et al.*, 2003; Vickers *et al.*, 2003).

Overall, these observations support the idea that CB<sub>1</sub> blockers such as LH-21 display a strong ability to reduce weight gain via CB<sub>1</sub> receptor blockade with a greater potency in obese animals exposed to highly caloric diets. The effects of global cannabinoid CB<sub>1</sub> receptor blockade on weight reduction are the combination of central and peripheral mechanisms. Although central actions include a decrease in caloric intake via interference with the hypothalamic and limbic mechanisms controlling appetite, peripheral mechanisms include an increase in energy expenditure involving tissues such as adipose tissue and muscle. The fact that LH-21 has poor penetration into the CNS indicates the importance of peripheral CB<sub>1</sub> receptor blockade to produce weight reduction without interfering with neurobehavioural control of appetite, as has been observed with other non-brain penetrant CB<sub>1</sub> antagonists (Tam *et al.*, 2010). Thus, our effects of LH-21 on appetite and weight gain were probably mediated through its interaction with CB<sub>1</sub> receptors located peripherally in gut sensory terminals that control satiety (Gomez *et al.*, 2002), and also with cannabinoid receptors located in metabolically relevant tissues (Cota *et al.*, 2003; Tam *et al.*, 2010).

Obesity can lead to some forms of hepatic steatosis, mainly due to excessive fat accumulation in the liver. Here, we showed diet-induced alterations in the level and composition of lipids in rat liver; HFD-fed animals displayed an increase in liver fat content and triglycerides compared with SD-fed rats. Significant changes in fatty acid composition of hepatic triglycerides were also observed. The reduction in SFA observed in HFD-fed rats was basically produced by a decrease in palmitic acid levels. This observation might be related to inhibition of lipogenesis detected in these animals, as palmitic acid is the first product of fatty acid synthesis. HFD-fed rats also exhibited an elevation in PUFA, mainly due to the increase in essential fatty acids (linoleic and arachidonic acids); these changes could be caused by the specific composition of the fat-enriched diet, which contained soya oil. Systemic administration of LH-21 did not modify the total percentage of liver fat content or fatty acid composition in any of the groups, it did not reduce the elevated fat content produced by HFD. Again, these results accord with previous observations demonstrating that LH-21 does not reduce liver fat stores in leptin signalling-deficient obese rats (Pavon *et al.*, 2008). This absence of metabolic benefits of LH-21 contrasts with the hepatic fat reduction induced by CB<sub>1</sub> receptor inverse agonists, rimonabant and AM251 (Osei-Hyiaman *et al.*, 2005), and peripheral CB<sub>1</sub> receptor neutral antagonists such as AM6545 (Tam *et al.*, 2010). The fact that these compounds can improve or not the obesity-associated hepatic steatosis might suggest a different profile due to several factors. They include: (i) the inverse agonism/antagonism activity at cannabinoid CB<sub>1</sub> receptors; (ii) penetrability into the brain; (iii) bioavailability to act at CB<sub>1</sub> receptors according to their chemical properties and vehicle used; and (iv) the existence of other non-cannabinoid targets that could counteract or interfere in the metabolic effects promoted with CB<sub>1</sub> blockers in liver. Indeed, pyrazole-derived compounds structurally related to rimonabant have been found to be potent

activators of nuclear receptors in the nanomolar range, specifically PPAR $\alpha$  (Alvarado *et al.*, 2008).

In addition to the liver, adipose tissue has a key role in the peripheral energy homeostasis, as obesity is also associated with dysfunctional adipose tissue. Weight reduction improves the function of adipose tissue, probably by reducing fat mass. In our study, we observed that chronic exposure to a fat-enriched diet induced important changes at the transcriptional level of several enzymes and hormones involved in lipid metabolism and energy in adipocytes. We grouped our data into lipolytic or lipogenic genes to evaluate the net effects caused by diet and/or treatment. A 10 day treatment with LH-21 had an important effect on the gene expression of receptors (PPARs) and enzymes involved in lipid metabolism. LH-21 inhibited mRNA levels of PPAR $\alpha$  in both diet groups in a similar way, as well as abolishing the elevation in ACOX mRNA expression observed in the HFD-fed animals. It is important to note that ACOX expression is PPAR $\alpha$ -dependent. Interestingly, LH-21 exerted an important inhibitory effect on the pro-lipogenic PPAR $\gamma$  receptor and on each lipogenic enzyme analysed, as we observed a reduced mRNA expression of FAS and SCD-1. These data accord with results from previous studies conducted by Matias and colleagues, who showed that the cannabinoid CB<sub>1</sub> receptor agonist HU-210 increases PPAR $\gamma$  expression in mouse adipocytes (Matias *et al.*, 2006). Similar effects on lipogenic enzymes have been described after treatment with rimonabant and AM6545 in fat enriched diet-induced obese mice with a decrease in SCD-1 and FAS expression in visceral adipose tissue (Jourdan *et al.*, 2010; Tam *et al.*, 2010). Gene expression of SCD-1 was similarly reduced in both diet groups, which might implicate a decrease in fat accumulation and mono-unsaturation of SFAs in adipose tissue in LH-21-treated animals. The suppressed transcription of the lipogenic genes may be related to the enhancement of PUFA observed in these animals (Mater *et al.*, 1998). Additionally, this strong inhibitory effect of LH-21 on lipogenesis signalling could be due to compensatory actions of the suppressor effects on lipolytic genes described previously, for example PPAR $\alpha$ . Adipose tissue could be improving its functionality in order to recover an energy balance status by potentiating a down-regulation of the lipogenic pathway, reducing lipid accumulation in the adipocyte, and consequently decreasing lipolysis. Similar observations have been described in obese humans with a decreased expression of lipogenic gene in adipose tissue (Diraison *et al.*, 2002).

As LH-21 is a CB<sub>1</sub> receptor antagonist, we evaluated the expression of both CB<sub>1</sub> and CB<sub>2</sub> receptors in adipose tissue, which controls adipogenesis and lipogenesis in adipocytes (Bellocchio *et al.*, 2008; Vettor and Pagano, 2009). Our data support the presence of cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors in the visceral adipose tissue (Roche *et al.*, 2006). Indeed, HFD produced significant alterations in the expression of both CB<sub>1</sub> and CB<sub>2</sub> receptors; an increase in their mRNA levels was associated with the diet-induced lipogenesis. As mentioned previously, the endocannabinoid system is dysregulated in obesity (Engeli, 2008) and genetically obese rats show an overexpression of CB<sub>1</sub> receptors in adipose tissue compared with lean rats (Bensaid *et al.*, 2003). LH-21 had selective and important effects on these cannabinoid receptors; it reduced the gene expression of CB<sub>1</sub> in the HFD group to values of

SD-fed animals, preventing the increase in CB<sub>1</sub> induced by the HFD. However, LH-21 decreased the expression of CB<sub>1</sub> receptors in both diet groups. Hence, the CB<sub>1</sub> blockade under a 10 day regimen may induce a down-regulation of such receptor signalling in adipose tissue. As some authors have suggested a direct adipogenic role of CB<sub>1</sub> activation (Matias *et al.*, 2006), the CB<sub>1</sub> receptor blockade may be a mechanism to promote oxidative pathways or/and to block synthetic pathways, which is consistent with effects on gene expression of proteins related to lipid metabolism in adipose tissue. In contrast, the expression of CB<sub>2</sub> receptors was unchanged by LH-21 and LH-21 did not affect the increased expression of CB<sub>2</sub> mRNA produced by the HFD. Our results confirm a selective effect of LH-21 on CB<sub>1</sub> versus CB<sub>2</sub> receptors as described previously (Jagerovic *et al.*, 2004; Chen *et al.*, 2008; Pavon *et al.*, 2008). However, recently very high doses of LH-21 have been shown to have effects in CB<sub>1</sub> knockout mice, suggesting alternative targets for this compound (Chen *et al.*, 2008). In this regard, AM6545, a potent neutral CB<sub>1</sub> antagonist with reduced brain penetration, inhibits food intake in CB<sub>1</sub> receptor gene-deficient mice, but not in CB<sub>1</sub>/CB<sub>2</sub> receptor double knockout mice, also indicating the existence of other alternative pathways to inhibit appetite. Two of these pathways may be either the PPAR $\alpha$  receptors or the orphan receptor GPR55. We observed that the mRNA expression of PPAR $\alpha$  in adipose tissue was reduced after 10 day exposure with LH-21, suggesting a down-regulation of this nuclear receptor. As other pyrazole-derived drugs structurally related to LH-21 and other CB<sub>1</sub> antagonists were found to activate PPAR $\alpha$  receptors (Alvarado *et al.*, 2008), we tested whether the hypophagia induced by LH-21 *in vivo* was through a PPAR $\alpha$ -mediated pathway by using PPAR $\alpha$ -null mice. LH-21 (20 mg·kg<sup>-1</sup>, i.p.) potently reduced food intake in both the PPAR $\alpha$ -null mice and wild-type animals, which suggests that the suppressive effect on feeding produced by LH-21 was via PPAR $\alpha$ -independent mechanism. However, we cannot exclude long-term metabolic effects of LH-21 though interactions with PPAR $\alpha$  receptors. LH-21 treatment clearly increased the expression of the orphan receptor GPR55, suggesting a potential interaction of this drug with GPR55-regulatory mechanisms. This receptor is strongly activated by rimonabant and AM251 (Henstridge *et al.*, 2009; 2010; Kapur *et al.*, 2009), therefore, the interaction of LH-21 with GPR55 will be addressed in future studies to elucidate its role.

Adipose tissue is also an endocrine organ. Leptin and Adipoq are the major adipocyte-derived hormones and both are involved in the regulation of energy homeostasis, glucose and lipid metabolism (Zhang *et al.*, 1994; Scherer *et al.*, 1995; Hu *et al.*, 1996). Leptin is known to regulate appetite and body weight by decreasing caloric intake and increase peripheral energy expenditure (Friedman, 1998; Elmquist *et al.*, 1999), although its functionality is lost in obese rodents that display leptin resistance as a consequence of the constantly elevated circulating levels of the hormone. In our study, leptin was the main adipocyte-derived hormone that was affected by both diet and LH-21 treatment. Leptin expression was up-regulated in HFD-fed rats, perhaps indicating the onset of HFD-induced leptin resistance, and LH-21 significantly reduced leptin transcription in both the HFD and SD groups. A decrease in leptin is related to weight reduction and it may indicate a recovery of leptin function. LH-21, however,

failed to up-regulate Adipoq, producing only a moderate decrease in its expression. Although it has been reported that Adipoq mRNA levels in adipose tissue are lower in obese than in lean humans and rodents (Arita *et al.*, 1999; Weyer *et al.*, 2001; Milan *et al.*, 2002), we observed no changes in Adipoq mRNA levels with diet. Nonetheless, the effects of CB<sub>1</sub> antagonists on Adipoq levels are contradictory. Some studies have reported an increase in the expression of Adipoq in the circulation and in adipose tissue of obese Zucker rats and diet-induced obese rats treated with rimonabant (Bensaid *et al.*, 2003; Thornton-Jones *et al.*, 2006). Also, rimonabant has been shown to stimulate Adipoq expression and secretion *in vitro* by murine adipocytes (Matias *et al.*, 2006; Perwitz *et al.*, 2006), and AM6545 was reported to increase serum Adipoq levels in obese mice (Tam *et al.*, 2010). In contrast, other studies have found no differences on Adipoq expression after CB<sub>1</sub> activation in mature human adipocytes (Pagano *et al.*, 2007).

In summary, our results demonstrate the anti-obesity effect of the neutral CB<sub>1</sub> receptor antagonist LH-21 in a non-genetic model of obesity. LH-21 produced hypophagia and inhibited weight gain in diet-induced obese rats. In contrast, continuous treatment with this antagonist was unable to counteract some conditions associated with obesity, such as hepatic steatosis. LH-21 affected lipid metabolism through a general inhibitory effect on gene expression in the adipocyte, mainly lipogenic proteins and leptin. These observations suggest that visceral adipose tissue is the main peripheral target of LH-21 responsible for its weight reduction effect.

## Acknowledgements

This study was supported by grants to F.R.F. from the European Union's 7th Framework Programme (Health-F2-2008-223713, *Reprobesity*); the following grants from the Spanish Ministry of Science and Innovation: Grants SAF 2010-20521; SAF 2009-12422-C02-02; National Institute of Health 'Carlos III' (PI07/0880), UE-ERDF, Red de Trastornos Adictivos (RD06/0001 and RETICS RD06/001/0014), and CIBER OBN; grants from the Consejería de Economía, Innovación y Ciencia de la Junta de Andalucía, UE/ERDF(CTS433, CVI1038 and 740486); and a grant from the Consejería de Salud de la Junta de Andalucía (PI0232/2008), Spain. L.H.F. is recipient of a post-doctoral contract (JAEDoc\_07\_00204) from CSIC. MMG is supported by the Research Stabilization Program of the Instituto de Salud Carlos III (CES 10/004). FJBS holds Miguel Servet research contracts CD07/00283 and JS holds a Sara Borrell postdoctoral contract CD08/00203, all from the National Institute of Health 'Carlos III', Madrid, Spain.

## Conflict of interest

The authors state no conflicts of interest.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1** Fatty acid composition (%) of hepatic triglycerides of diet-induced obese male Wistar rats treated with LH-21

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