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Chronic 5-HT_6 receptor modulation by E-6837 induces hypophagia and sustained weight loss in diet-induced obese rats

[1]Angels Fisas, [1]Xavier Codony, [1]Gonzalo Romero, [1]Alberto Dordal, [2]Jesus Giraldo, [1]Ramon Mercé, [1]Jörg Holenz, [3]David Heal, [1]Helmut Buschmann & *,[1]Petrus Johan Pauwels

[1]Laboratorios Dr. Esteve S.A., Av. Mare de Déu de Montserrat 221, 08041 Barcelona, Spain; [2]Grup Biomatemàtic de Recerca, Institut de Neurociències and Unitat de Bioestadística, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain and [3]RenaSci Consultancy Ltd, Biocity, Nottingham, NG1 1GF

1 E-6837 is a novel, selective and high-affinity 5-HT_6 receptor ligand (pK_i: 9.13) which *in vitro* demonstrates partial agonism at a presumably silent rat 5-HT_6 receptor and full agonism at a constitutively active human 5-HT_6 receptor by monitoring the cAMP signaling pathway.

2 The effects of chronic treatment with E-6837 were determined in diet-induced obese (DIO)-rats on changes in body weight, food and water intake, plasma indices of comorbid risk factors, and weight regain on compound withdrawal. The centrally acting antiobesity drug, sibutramine, was used as the reference comparator.

3 Sustained body weight loss and decreased cumulative food intake of DIO-rats was observed with E-6837 (30 mg kg^{-1}, p.o., twice a day) during the 4-week treatment period. The onset of the E-6837 effect on body weight was slower than that of sibutramine (5 mg kg^{-1}, p.o.), while its maximal effect was greater, that is −15.7 versus −11.0%.

4 E-6837-induced weight loss was exclusively mediated by a decrease (31.7%) in fat mass, with a concomitant reduction (49.6%) in plasma leptin. Reduced obesity was also reflected in improved glycemic control.

5 Although weight regain occurred after withdrawal from either compound, the body weights after E-6837 (−6.6%) remained lower than after sibutramine (−3.8%) indicating that the greater efficacy of the former did not result in profound rebound hyperphagia/weight gain.

6 These results show that the 5-HT_6 receptor partial agonist, E-6837, is a promising new approach to the management of obesity with the potential to produce greater sustained weight loss than sibutramine.
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Abbreviations: α-MSH, α-melanocyte-stimulating hormone; 5-HT, 5-hydroxytryptamine; AM-251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; CB1, cannabinoid-1 receptor; CNS, central nervous sytem; DIO, diet-induced obese; DMSO, dimethylsulphoxide; E-6837, 5-chloro-*N*-(3-(2-(dimethylamino)ethyl)-1*H*-indol-5-yl)naphthalene-2-sulphonamide; GABA, γ-aminobutyric acid; HEK, human embryonic kidney; HPMC, hydroxyl-propyl-methyl-cellulose; HTRF, homogeneous time resolved fluorescence; LSD, lysergic acid diethylamide; mCPP, 1-(m-chlorophenyl)piperazine; NEFAs, non-esterified fatty acids; Ro 04-6790, *N*-(2,6-bis(methylamino)pyrimidin-4-yl)-4-aminobenzenesulphonamide; SB-271046, 5-chloro-*N*-(4-methoxy-3-(piperazin-1-yl)phenyl)-3-methylbenzo[b]thiophene-2-sulphonamide hydrochloride; SB-357134, *N*-(2,5-dibromo-3-fluorophenyl)-4-methoxy-3-(piperazin-1-yl)benzenesulphonamide; SB-399885, *N*-(3,5-dichloro-2-methoxyphenyl)-4-methoxy-3-(piperazin-1-yl)benzenesulphonamide

Introduction

Rising levels of obesity across the Western world and now in many developing countries is associated with numerous health complications, which range from nonfatal debilitating conditions like osteoarthritis to life-threatening chronic diseases such as Type 2 diabetes, cerebro- and cardiovascular disease and certain cancers (Bays, 2004). In addition, the psychological consequences of obesity can range from lowered self-esteem to clinical depression (Friedman *et al*., 2005; Wardle & Cooke, 2005). Currently, there are two broad categories of medication approved for the long-term management of obesity, the peripherally acting nonselective lipase inhibitor, orlistat, (Xenical®), which prevents the pancreatic absorption of lipids and the centrally acting monoamine reuptake inhibitor, sibutramine, which reduces food consumption by enhancing satiety (Yanovski & Yanovski, 2002). Sibutramine is primarily a 5-hydroxytryptamine (5-HT)/noradrenaline reuptake inhibitor, but it has also small effects on dopamine reuptake inhibition; current data suggest that it does not have abuse potential (Schuh *et al*., 2000; Arfken *et al*., 2003). Although increased basal energy expenditure also contributes to the weight-loss in rats induced by sibutramine (Connoley *et al*., 1999; Skill *et al*., 2000), the evidence that this mechanism contributes to weight loss in humans is equivocal (Hanssen

*Author for correspondence; E-mail: ppauwels@esteve.es

et al., 1999; Starling *et al*., 2001). Patients administered sibutramine can experience increases in blood pressure and heart-rate, and as a consequence, sibutramine's use is contra-indicated in patients with uncontrolled hypertension, coronary heart disease, cardiac dysrhythmias, congestive heart failure, or stroke (Bays & Stein, 2003).

Pre-eminent amongst the different approaches for novel antiobesity drugs, are the cannabinoid-1 receptor (CB1) antagonists, with several compounds currently undergoing clinical trials in the U.S.A. and Europe. In animal models, CB1 receptor blockade produces a lean phenotype, with resistance to diet-induced obesity (DIO) and associated dyslipidaemia (Hildebrandt *et al*., 2003; Ravinet-Trillou *et al*., 2003; 2004). The CB1 antagonist, rimonabant, reverses the rodent DIO phenotype through the regulation of lipolysis and energy balance (Jbilo *et al*., 2005). In clinical trials, results from the Rimonabant In Obesity (RIO) – Europe, RIO – North America and the RIO – Lipids indicate that modulating the activity of the endocannabinoid system by blocking CB1 receptors holds therapeutic promise for clinically significant weight loss in obesity with concomittent improvements in associated risk factors (Van Gaal *et al*., 2005; Després *et al*., 2005; Pi-Sunyer *et al*., 2006).

The 5-HT$_6$ receptor is a promising new central nervous system (CNS) target that may have a role to play in obesity (Vickers & Dourish, 2004). Early studies indicated that chronic administration of 5-HT$_6$ mRNA antisense oligonucleotides produced a significant reduction in food intake and body weight in rats (Bentley *et al*., 1997). 5-HT$_6$ receptor knockout mice were also resistant to weight gain when exposed to a high-fat diet (Caldirola, 2003). *In vivo* studies have demonstrated a role for selective 5-HT$_6$ receptor antagonists in the regulation of feeding. Ro 04-6790 (30 mg kg^{-1} i.p.) administered to rats over 3 days induced a reduction in body weight (Woolley *et al*., 2001) and other studies have revealed a dose-related reduction in food consumption following acute injection of Ro 04-6790 ($ID_{50} = 18.6$ mg kg^{-1} i.p.) or SB-271046 ($ID_{50} = 14.5$ mg kg^{-1} i.p.) in rats accustomed to a fixed daily feeding regimen (Bentley *et al*., 1999; Woolley *et al*., 2004). Recently, Pérez-Garcia & Meneses (2005) reported that two other 5-HT$_6$ receptor antagonists, SB-357134 and SB-399885, also suppressed food intake in food-deprived animals. Drugs targeted at 5-HT$_6$ receptors may have relatively few directly peripheral side-effects because the receptor is distributed almost exclusively within the CNS (Hirst *et al*., 2003). To date, there are no published studies describing the effect of chronic administration of 5-HT$_6$ receptor ligands on body weight and its comorbidities in a rodent model that closely mimics human obesity. In view of this, we chose to assess the effect of a novel 5-HT$_6$ receptor ligand E-6837, a 5-sulphonamide tryptamine derivative: 5-chloro-*N*-(3-(2-(dimethylamino) ethyl)-1*H*-indol-5-yl)naphthalene-2-sulfonamide (Holenz *et al*., 2005), in obese rats. It has previously been reported that when mature, female rats given access to a highly palatable, calorie-dense simplified cafeteria diet, they develop obesity with insulin resistance and other metabolic disturbances (Dickinson *et al*., 1998; Heal & Jagger, 2005). The effect of chronic E-6837 treatment was investigated in these dietary-induced obese DIO-rats to determine its effects on body weight, food and water intake and weight loss outcomes, including plasma lipids and indices of glycemic control and adiposity. Sibutramine was chosen as a positive control in the present studies since it is efficacious in reducing body weight in both this DIO rat model (Heal & Jagger, 2005) and in obese patients (Ryan *et al*., 1995). The results suggest that the 5-HT$_6$ receptor is an interesting central target for the development of novel antiobesity drugs. Furthermore, 5-HT$_6$ receptor modulation by the partial agonist, E-6837, indicates that such type of compound is likely to have beneficial effects in the treatment of clinical obesity in terms both of reducing adiposity and of improving glycemic control.

Methods

Binding assays

Receptor binding to human 5-HT$_6$ receptor was performed using transfected human embryo kidney (HEK)-293 membranes (35 μg protein/assay) from Perkin-Elmer (Boston, MA, U.S.A.) using [^{3}H]-lysergic acid diethylamide (LSD, 2.7 nM). Nonspecific binding was determined with 5 μM methiothepin. Competition binding data were analyzed using a Ligand program. Experiments determined E-6837's affinity values for various receptors, uptake sites, ion channels and its ability to inhibit the enzyme, acetylcholinesterase. For the sake of brevity, details of these assays are not provided here. The neurotransmitter receptors being studied were: serotonergic 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_3$, 5-HT$_4$, 5-HT$_7$; adenosine A_1, A_{2A}, A_{2B}; adrenergic α_1, α_{1A}, α_{1B}, α_{1D}, α_2, α_{2A}, α_{2B}, β_1, β_2; bradykinin B_1, B_2; dopaminergic D_1, D_2, D_{2L}, D_3, $D_{4.2}$; GABA-A agonist site, GABA-A benzodiazepine, GABA-B; glutamate kainate, NMDA, NMDA glycine, NMDA phencyclidine; histaminergic H_1, H_2, H_3; imidazoline I_2; muscarinic nonselective, M_1, M_2, M_3; opiate δ, κ, μ; purinergic P_{2X}, P_{2Y}; sigma σ_1, σ_2; tachykinin NK_1; nicotinic acetylcholine and phorbol ester. Also studied were receptors for peptides (endothelin ETA, ETB; interleukin 1L-1α; leukotrien B_4, D_4; platelet activating factor), growth factor (epidermal growth factor), steroid receptors (estrogen ERα, testosterone, glucocorticoid), uptake sites (serotonin, dopamine, noradrenaline, GABA) and ion channels (calcium, potassium, sodium).

cAMP assay

cAMP measurements using HEK-293F cells that stably expressed either a rat 5-HT$_6$ (Ruat *et al*., 1993) or human 5-HT$_6$ receptor (Kohen *et al*., 1996) were performed using homogeneous time-resolved fluorescence (HTRF). Cells in suspension were added to a 96-well culture plate (20,000/well) in incubation buffer composed of Ham's F12 supplemented with 1 mM 3-isobutyl-1-methyl-xanthine and 20 μM pargyline and preincubated for 10 min at room temperature with either vehicle (DMSO) or compound. Thereupon, 10 μl of either vehicle or 5-HT was added for 30 min incubation at 37°C. Agonism was expressed as E_{max} (in percentage of maximal stimulation as obtained with 5-HT) and pEC_{50} values. Antagonism was determined against 0.5 μM 5-HT and expressed as I_{max} (in percentage of maximal antagonism as obtained with SB-271047, Bromidge *et al*., 1999) and pIC_{50} values. Nonlinear regression analyses, using XLfit (IDBS) and GraphPad Prism Version4 programs, were performed to estimate pEC_{50} and pIC_{50} values.

Acute effect of E-6837 on food intake and activity

Sprague–Dawley rats (Charles River Laboratories, Wilmington, U.S.A.) weighing approximately 190 g at the time of arrival were housed three per cage for 1 week, then transferred to individual cages mounted with feeders containing powdered chow and single-housed for the remainder of the study. Rats were handled during the single housing period to accustom them to the injection procedure. Rats were housed under a 12/12 light/day cycle lights on at 0300 h and in temperature and humidity controlled rooms. At 2 weeks after arrival rats were transferred to Mani Feedwin cages and randomized into weight-matched groups (eight per group). Rats were subjected to four injections, each separated at least 3 days. E-6837 was dissolved in 0.5% HPMC and administered by gavage at 5 and 30 mg kg^{-1}. For 2 days prior to transfer to the Mani Feedwin cages, in addition to the daily handling procedure rats were gavaged daily with vehicle. Compound was administered prior to light out (1430–1500 h). Food intake (digital balance) and locomotor behavior (registered as consecutive beam brakes) was monitored online every fifth minute for 60 h.

Animals for the DIO rat studies

Female Wistar rats (250–300 g, Charles River, Margate, Kent, U.K.) were housed in pairs in polypropylene cages with solid floors and sawdust bedding at a temperature of $21 \pm 4^{\circ}$C and $55 \pm 20\%$ humidity. Animals were maintained on a reverse phase light–dark cycle (lights off for 8 h from 1000 to 1800 h) during which time the room was illuminated by red light. Animals were given free access to tap water and powdered VRF1 diet supplemented with 20% refined lard (supplied by Special Diet Services Dietex International Ltd, Witham, Essex, U.K., 20.8 kJ g^{-1}), ground chocolate (Cadbury's Dairy Milk chocolate, 23.4 kJ g^{-1}) and ground peanuts (Big D salted, roasted peanuts, 30.3 kJ g^{-1}). The three different diets were contained in separate glass feeding jars with aluminium lids (Solmedia Laboratory Suppliers, Romford, Essex, U.K.). Each lid had a 3–4 cm hole cut in it to allow access to food. Animals were housed in pairs for 12 weeks. At 2 weeks before the start of the baseline readings, animals were housed individually in polypropylene cages with wire grid floors to enable the food intake of each rat to be recorded. Polypropylene trays with cage pads were placed beneath each cage to detect food spillage.

Experimental procedures for the DIO rat studies

At the start of the first DIO study, animals were weighed (to the nearest 0.1 g. using an electronic top-pan balance) and allocated into three weight-matched treatment groups, each containing 10 animals. Following a 7 day baseline run-in period, during which time all animals were dosed orally once a day with vehicle (0.5% HPMC), rats were dosed for 28 days with vehicle at 0 and 6 h or test compound either E-6837 (30 mg kg^{-1}, p.o., twice a day at 0 and 6 h) or sibutramine (5 mg kg^{-1}, p.o. at 0 h/vehicle p.o. at 6 h). The first compound dose was given at the onset of the 8 h dark period (0 h). This strategy was taken to maximize the impact of any inhibitory effect of the compounds on food intake as rats are predominantly nocturnal feeders. After 6 h, animals were dosed again with either vehicle or compound as described above. Rats, feeding jars and water bottles were weighed (to the nearest 0.1 g) every day at 0 h. At each reading, the tray below each cage was examined for spilt food, which was returned to the appropriate jar before it was weighed. Spillage of food from the feeding jars was normally negligible. Approximately 1 h after the initial compound administration and at the 6 h reading, the animals were examined and any overt behavior was recorded. Variations in body weight and energy levels of the different types of food were accounted for expressing the food intake results in terms of kJ kg^{-1} rat weight. Water intake results were expressed in g kg^{-1}. A glucose tolerance test (D-glucose, 800 mg kg^{-1}, s.c.) was performed at the end of the study upon overnight fasting and blood samples were taken from tail vein at 30, 60 and 120 min into heparinized tubes to measure various metabolic parameters. Plasma was separated by centrifugation and frozen until required for assay of insulin and glucose. Glucose levels were determined using a colorimetric assay (Sigma-Aldrich Co., U.K.) and insulin levels were determined using an ELISA assay (Mercodia, Sweden). Plasma levels of leptin, adiponectin, total cholesterol, triacylglycerol, glycerol, nonesterified fatty acids (NEFAs), glucose and insulin were determined before glucose challenge using commercially available kits and reagents. After the final blood sample was taken, animals were killed by CO_2 to minimize fluid loss. Carcasses were weighed, frozen and stored at -75°C until water levels, body fat and protein of carcasses were determined using standard chemical analysis for determination of changes.

A second DIO rat experiment was carried out which followed the same protocol as first experiment up to Day 28; compound treatment was ceased and the food and water intakes and body weights of the rats were monitored on a daily basis for a further 42 days. Both experiments yielded similar compound-induced effects during the treatment phase.

For conciseness, only results for daily body weight, food and water intake measurements from this second DIO rat study are presented in the manuscript figures. This experiment was chosen because it also contains information about off-dose, as well as on-dose, effects of E-6837 and sibutramine. Weight loss data from the first DIO rat experiment reported in Figure 5 demonstrate the outcome was very similar to that reported for the second DIO study.

Conditioned taste aversion and kaolin consumption

Sprague–Dawley rats (Charles River Laboratories) aged at 6 weeks at the time of the intervention (approximately 190 g) were used. All rats were daily handled and acclimatized to the gavage procedure three times prior to the start experiment. For conditioned taste aversion, food and water were removed in the morning. At 4 h before lights out, the rats were introduced to bottled water with a distinct flavor (0.1% saccharin flavored) with high palatability. At 1 h before lights out, the saccharin bottles were removed and the rats received by gavage either vehicle (0.5% HPMC), E-6837 (5–60 mg kg^{-1}) or LiCl solution (0.15 M, i.p.) as a positive control followed by food and water *ad lib*. At 72 and 96 h after the conditioning, rats were offered a choice between tap water and the palatable saccharin solution. Water/saccharin intake was measured during the following 12-h period both at 72–84 and at 96–108 h. The ratio between saccharin and total liquid intake (water + saccharin intake) was calculated.

Table 1 *In vitro* functional activity of E-6837 as compared with 5-HT and SB-271046 at a presumably silent rat 5-HT$_6$ and constitutively active human 5-HT$_6$ receptors stably expressed in HEK-293F cells, and corresponding p*Ki* values

	cAMP formation						*5-HT$_6$ binding*
	Rat 5-HT$_6$		*Rat 5-HT$_6$*		*Human 5-HT$_6$*		
	Antagonism (I_{max}, %)	*pIC$_{50}$*	*Agonism (E_{max}, %)*	*pEC$_{50}$*	*Agonism (E_{max}, %)*	*pEC$_{50}$*	*pKi*
5-HT	—	—	100	8.57±0.13	100	9.00±0.09	6.96±0.18
E-6837	41±3	8.37±0.09	67±4	9.19±0.11	96±4	9.53±0.09	9.13±0.17
SB-271046	100	7.88±0.05	−3±0.1	—	−39±2	8.32±0.12	8.68±0.09

Basal and 5-HT (1 μM) cAMP values were 1.0±0.1 and 12.6±0.4 pmol × 10^6 cells for HEK-293F/rat 5-HT$_6$ (expression level: 0.40±0.01 pmol mg protein^{-1}) and 3.7±0.2 and 20.6±0.8 pmol × 10^6 cells for HEK-293F/human 5-HT$_6$ cell line (expression level: 4.30±0.04 pmol mg protein^{-1}), respectively. Antagonism was performed against 0.5 μM 5-HT. I_{max} values are expressed as percentage of antagonism as obtained by 1 μM SB-271046. E_{max} values are expressed as percentage of agonism as obtained with 1 μM 5-HT. Values correspond to mean±s.e.m. of at least six independent experiments, each one performed in duplicate.

For kaolin consumption, 9 days before starting the study animals were situated in single cages. Rats were daily offered a fresh block of kaolin clay to decrease the novelty of the block. On the morning of the test day, animals were given clean cages with minimal bedding and subsequently gavaged according to the above mentioned groups. After the gavage/injection animals were offered a novel kaolin block and food and water. Kaolin intake was measured every hour for a 4-h period. At the end of the period food and water intake was measured.

Statistical analyses

Data during both treatment and withdrawal periods were analyzed by a two-way (treatment, time) analysis of variance (ANOVA) with time as a repeated measure. A covariate was included to adjust for differences between treatment groups at baseline. In case of body weight, the covariate was the individual Day 1 values (body weight measured just before dosing started); in case of water and food intake, the covariate was the averages of the baseline phase (days −6 to 0). Experimental points were expressed as mean±s.e.m. Comparisons between average treatment profiles were performed by defining appropriate tests for contrasts within the ANOVA analysis. *Post hoc* Tukey's test was used for the analysis of differences between treatments at the end of the selected time intervals. A value of $P<0.05$ was considered significant. Data analysis was carried out with the SAS/STAT® release 9.1 statistical package (SAS Institute Inc., Cary, NC, U.S.A.).

Materials

Molecular biology reagents were purchased either from Invitrogen (Frederick, MD, U.S.A.), Qiagen (Germantown, MD, U.S.A.) or Roche (Penzberg, Germany). Cell culture media and reagents were purchased from Gibco (Paisley, U.K.). HTRF cAMP kit was obtained from CisBio (Bagnols, France). [^{3}H]-LSD was purchased from NEN (Boston, MA, U.S.A.). 5-Hydroxytryptamine, 3-isobutyl-1-methyl-xanthine, pargyline and DMSO were obtained from Sigma (Poole, U.K.). Methiothepin was from Tocris (Bristol, U.K.). Sibutramine was provided by RenaSci Consultancy Ltd. SB-271046 was prepared *intramuros*. E-6837 is described in WO 2003/042175 A1 (Merce-Vidal *et al*., 2003). Compounds were dissolved in 0.5% HPMC and administered using dose volume in the range 1–5 ml kg^{-1}. Compound doses were expressed as free base.

Results

In vitro *activity of E-6837 as compared to 5-HT and SB-271046*

E-6837 displayed selective and high-affinity binding (pKi: 9.13±0.17) for the recombinant human 5-HT$_6$ receptor. Some affinity was also measured at about 150-fold or higher concentrations for human 5-HT$_{2B}$ receptor (pKi: 6.95), rat dopamine transporter (pKi: 6.90), human 5-HT$_{1A}$ receptor (pKi: 6.80±0.03), human 5-HT$_{2A}$ receptor (pKi: 6.80), human α_{2A}-adrenergic receptor (pKi: 6.70±0.07), human 5-HT$_7$ receptor (pIC$_{50}$: 6.51±0.01), rat α_1-adrenoceptor (pKi: 6.20) and rat muscarinic receptor (pIC$_{50}$: 6.00). No significant binding of E-6837 could be detected at 10 μM for a panel of 61 proteins, including receptors for neurotransmitters, peptides, growth factor and steroids, uptake sites and ion channels, and the compound exerted no acetylcholinesterase inhibitory activity. Determination of functional activity at a presumably silent rat 5-HT$_6$ receptor stably expressed in HEK-293F cells indicated that E-6837 is a potent partial agonist (Table 1). Further analysis at a constitutively active human 5-HT$_6$ receptor in HEK-293F cells demonstrated E-6837 is a potent and efficacious agonist, with E_{max} and pEC$_{50}$ values similar to 5-HT (Table 1).

Acute effect of E-6837 on food intake and activity in normal rats

Administration of E-6837 at 30 mg kg^{-1} p.o. to male Sprague–Dawley rats reduced food intake transiently. The onset of food intake suppression started approximately 2 h into the nightly feeding session, but only lasted for approximately 6 h. The dose of 5 mg kg^{-1} p.o. was without effect on food intake. No effects on locomotor activity were observed with either dose. A more thorough examination of adverse effects was performed in a series of conditioned taste aversion and kaolin consumption experiments. E-6837 did not elicit conditioned taste aversion up to the dose of 60 mg kg^{-1} p.o. Treatment with 60 mg kg^{-1} of E-6837 resulted in a saccharin preference ratio of 77.0±10.7% (72–84 h) similar to vehicle treatment (76.7±10.9%), while LiCl (0.15 M, i.p.) attained a ratio of 30.4±10.5% ($P<0.005$ *versus* vehicle, Anova factoral, Fischer's *post hoc*). In the kaolin intake experiment, E-6837 had no effect on kaolin consumption at either of the doses tested. Treatment with LiCl (0.15 M, i.p.) resulted in a robust induction of kaolin consumption compared to vehicle

treatment (2 h: 0.4±0.0 g *versus* 0.0±0.0 g; 4 h: 0.6±0.1 g *versus* 0.0±0.0 g ($P<0.001$ *versus* vehicle, Anova factoral, Fischer's *post hoc*)). Therefore, we selected the dose of 30 mg kg^{-1} p.o. for E-6837, but a twice a day dosing regime was employed for the repeated-dose DIO rat studies because E-6837 had a relatively short duration of effect on food intake.

In vivo *activity of E-6837 compared with sibutramine in DIO rats*

Chronic administration of either E-6837 (30 mg kg^{-1}, twice a day, p.o.) or sibutramine (5 mg kg^{-1}, p.o. once daily + vehicle p.o. once daily) to female DIO Wistar rats induced a significant body weight loss *versus* vehicle-treated animals during the 4-week treatment period (Figure 1a). Body weight curves intersected on Day 14, with E-6837-treated rats continuing to lose more weight than the sibutramine group before the plateau was reached. Analysis of body weight at the end of treatment period (Day 28) demonstrated a superior (Tukey's test, $P<0.05$) weight loss by E-6837 (−15.7 %) than sibutramine (−11.0%). Analysis of body weight loss by week of treatment indicated E-6837 caused continued weight loss for 3 weeks, whereas the effect of sibutramine was present only during the first week of treatment (Figure 1b). Moreover, sibutramine-treated DIO rats began to demonstrate weight gain from week 1 onwards like vehicle-treated animals. Following cessation of compound treatment, DIO rats having received either E-6837 or sibutramine showed transient rebound hyperphagia Days 29–52 (see Figure 2) with parallel increase in body weights for both groups. The mean body weights of the sibutramine-treated rats were not significantly different from those of the controls from Day 44 onwards; however, those of the E-6837 group remained significantly ($P<0.05$) below control values for the duration of the withdrawal phase (Figure 1a). On Day 71, the mean difference in body weight compared with controls was −6.6% ($P<0.05$) for E-6837 and −3.8% (NS) for sibutramine. In contrast to sibutramine, analysis of the efficacy of E-6837 on the sum of its treatment and withdrawal periods also indicated a significant effect on loss in body weight (Figure 1c).

Figure 2a illustrates the effects of E-6837 and sibutramine on daily food intake. Both compounds produced an overall attenuation of daily food intake during the treatment period. The effect of E-6837 was significant during Weeks 1–3 of treatment, whereas that of sibutramine was only significant during Week 1 (Figure 2b). Daily food intake was slightly increased upon withdrawal of both E-6837 and sibutramine but returned to the vehicle level after 4 and 3 weeks of withdrawal, respectively (Figure 2c). A preferential effect of E-6837 was observed on attenuation of chocolate intake, while sibutramine appeared more effective on high-fat chow intake (Figure 3).

Analysis of the water intake of the vehicle- and compound-treated groups of DIO rats revealed some differences between E-6837 and sibutramine. Consistent with rats being predominantly prandial drinkers, a large reduction (−42%, $P<0.01$) of water intake was observed on Day 1 with sibutramine when food intake was markedly suppressed. Thereafter, water intake returned to control values with sporadic increases above this level during the treatment phase (Figure 4). Consistent with hyperphagia during the early withdrawal phase water intake was similarly greater than control values during this period (Figure 4). E-6837, which decreased food intake for longer than sibutramine, also tended to decrease water over the first 3 weeks, thereafter water consumption remained at control levels during the treatment phase. Unlike the sibutramine-treated rats, those withdrawn from E-6837 showed no marked increase in water intake during the period of hyperphagia off-dose (Figure 4).

As reported in Figure 5, the percentage reductions in body weight at Day 28 evoked by E-6837 (30 mg kg^{-1}, p.o. twice daily) and sibutramine (5 mg kg^{-1}, p.o. once daily + vehicle p.o. once daily) in the first rat DIO experiment 13.6% ($P<0.001$) and 10.0% ($P<0.001$), respectively, are similar to those reported for the second DIO rat experiment, that is E-6837 = 15.7% ($P<0.001$) and sibutramine = 11.0% ($P<0.001$) (Figure 1a).

Body composition analysis of the carcasses (Figure 5) showed that the weight reductions produced by either E-6837 or sibutramine were mediated exclusively by a reduction in fat mass with no significant decreases in water, protein or ash content. In general, no overt behavioral effects were observed in animals given E-6837 that could have influenced energy output. Body temperature was measured 2 and 6 h following compound administration on Days 1 and 28. The doses of E-6837 and sibutramine did not significantly alter rectal temperature on either the first or final day of treatment. When rats were given a D-glucose (800 mg kg^{-1}, s.c.) challenge test after overnight starvation on Day 30, the excursions in plasma glucose and insulin were decreased in E-6837-treated DIO rats relative to vehicle; the decrease in insulin fell short of statistical significance ($P=0.002$ and 0.06 for glucose and insulin, respectively; Figure 6a and b). For sibutramine-treated DIO rats, the glucose excursion was unaltered relative to vehicle treatment, whereas a similar, nonsignificant decrease to that observed with E-6837 treatment was measured for plasma insulin. Measurements of glucose and insulin concentrations in plasma samples taken from fed rats (Table 2) indicated that in agreement with the results obtained in fasted rats (Figure 6), the vehicle-treated rats were probably insulin-resistant, but not diabetic. Chronic treatment with E-6837 produced nonsignificant falls in both glycemic indices (Table 2). Sibutramine also reduced glucose concentration in the DIO rats (Table 2).

Consistent with the reduction of fat mass, levels of plasma leptin were decreased by 50% ($P<0.05$) with E-6837 and by 28% (NS) with sibutramine, but adiponectin levels remained unchanged (Table 2). In terms of plasma lipid profiles, the weight loss induced by either compound had no significant influence on plasma concentrations of cholesterol, triacylglycerol or glycerol (Table 2). NEFAs were unchanged after E-6837 treatment, but were significantly elevated by sibutramine (27%, $P<0.05$) (Table 2).

Discussion

The effects of chronic treatment with a novel 5-HT$_6$ receptor ligand, E-6837 (Holenz *et al*., 2005), on body weight, food and water intake and obesity-related risk factors were determined in DIO rats. In addition to characterizing E-6837 effects on food and water intake and body weight during treatment, these parameters were also monitored for a 6-week period after compound withdrawal. Sibutramine was used as a reference comparator in these studies.

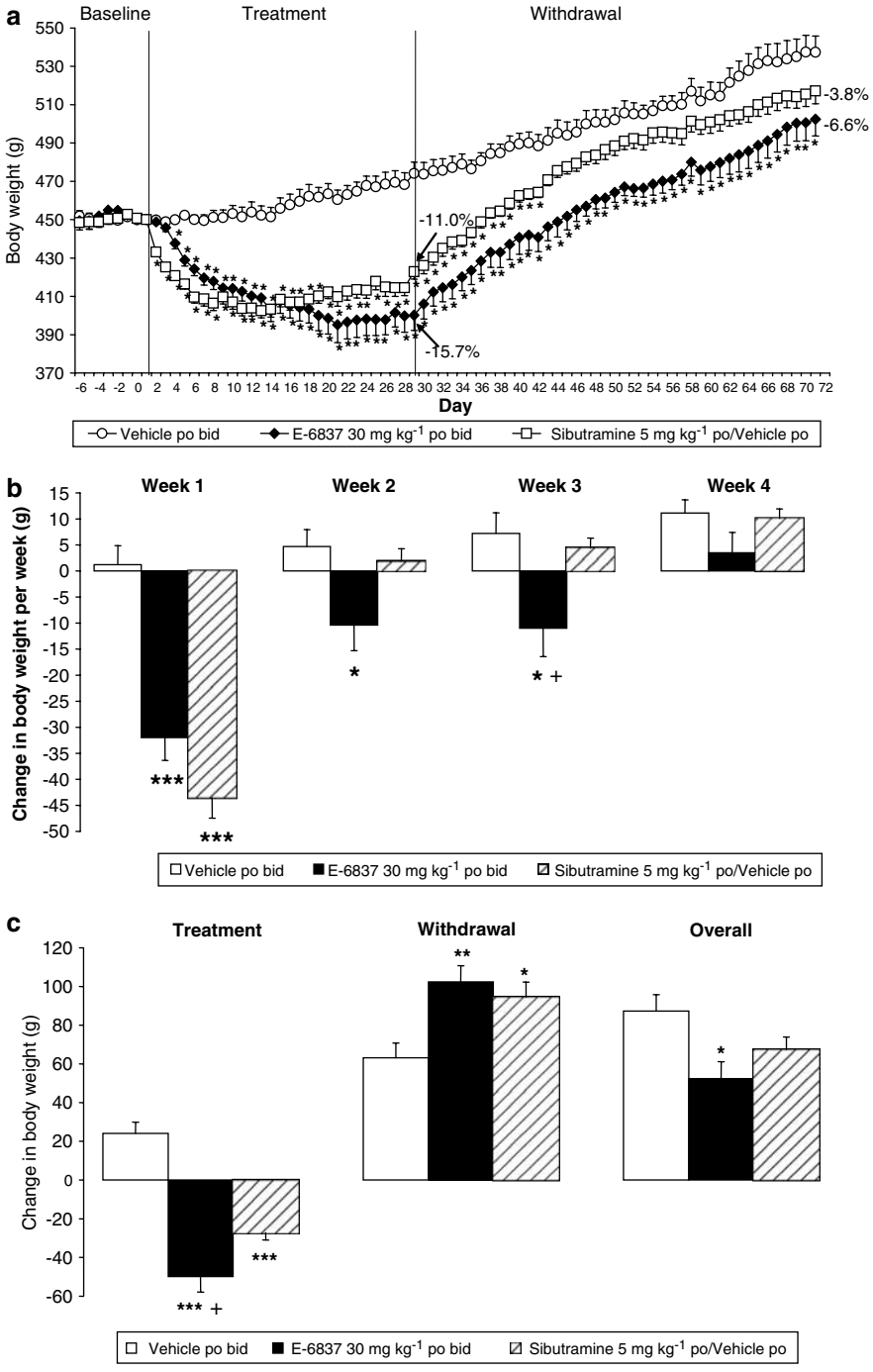

Figure 1 Effects of E-6837 and sibutramine on body weight in DIO female Wistar rats during treatment and withdrawal in comparison with vehicle. (a) Subsequent to a baseline period of 7 days (Days −6 to 0), rats were treated with E-6837 (30 mg kg^{-1}, twice a day, p.o.) and sibutramine (5 mg kg^{-1} day^{-1}, p.o.) for 4 weeks (Days 1–28) and monitored for another 6 weeks (Days 29–70) upon withdrawal. Treatment and withdrawal periods range between Days 2 and 29 and between Days 30 and 71, respectively, thereby representing body weight for a particular day as the effect of the compound dose administered the day before. *P*-values for mean comparisons between compounds along treatment and withdrawal periods: treatment period: vehicle *versus* E-6837: $P<0.0001$, vehicle *versus* sibutramine: $P<0.0001$, E-6837 *versus* sibutramine: $P=0.80$; withdrawal period: vehicle *versus* E-6837: $P<0.0001$, vehicle *versus* sibutramine: $P=0.01$, E-6837 *versus* sibutramine: $P=0.01$. $^*P<0.05$ denotes significant differences from the control group in Dunnett's test within ANOVA single point calculations (one independent test for each time value). As a result of the known multiple testing problem they do not represent true *P*-values; instead, they were included for illustrative purposes to visualize the time-intervals registering the most important differences. (b) Body weight changes per week during treatment period. Significant differences (Tukey's test: *, relative to vehicle; $^+$, between E-6837 and sibutramine) at the end of each week are indicated: $(^*,^+)P<0.05$, $^{***}P<0.001$. (c) Body weight change during treatment, during withdrawal and following both treatment and withdrawal (overall) periods. Significant differences (Tukey's test: *, relative to vehicle; $^+$, between E-6837 and sibutramine) at the end of each period and at the end of the whole study are indicated: $(^*,^+)P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

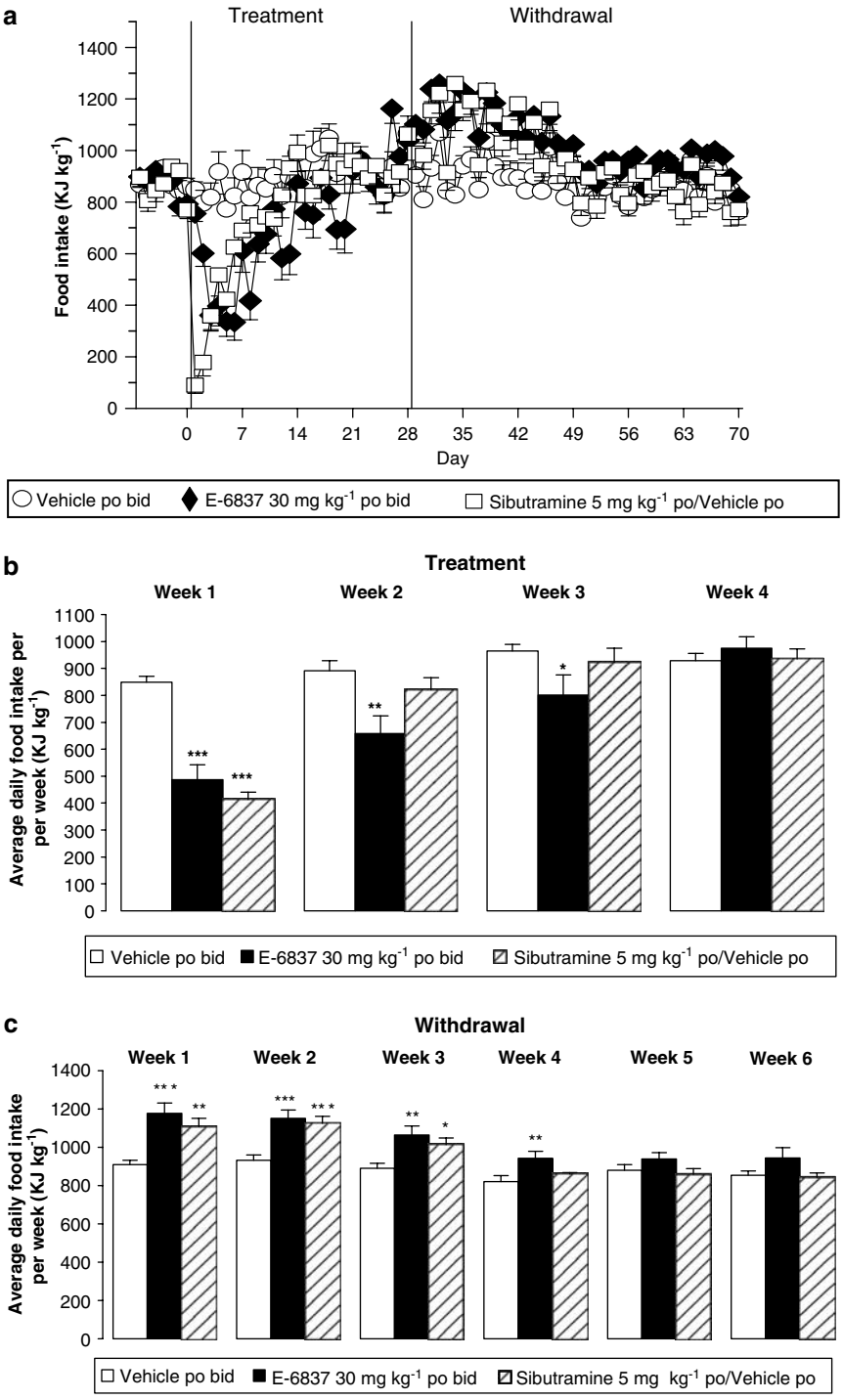

Figure 2 Effects of E-6837 and sibutramine on daily food intake in female DIO Wistar rats during treatment and withdrawal in comparison with vehicle. (a) Rats were treated with E-6837 (30 mg kg^{-1}, twice a day, p.o.) and sibutramine (5 mg kg^{-1} day^{-1}, p.o.) for 4 weeks and followed for 6 weeks upon withdrawal. *P*-values for mean comparisons between compounds along treatment and withdrawal periods. Treatment period: vehicle *versus* E-6837: $P=0.001$, vehicle *versus* sibutramine: $P=0.02$, E-6837 *versus* sibutramine: $P=0.39$; withdrawal period: vehicle *versus* E-6837: $P<0.0001$, vehicle *versus* sibutramine: $P=0.01$, E-6837 *versus* sibutramine: $P=0.04$. (b) Food intake changes per week during the treatment period. (c) Food intake changes per week during the withdrawal period. Significant differences relative to vehicle, at the end of each weak are indicated: $*P<0.05$, $**P<0.01$, $***P<0.001$.

E-6837 is a selective 5-HT$_6$ receptor ligand that showed potent partial to full agonism *in vitro*. As E-6837 is a tryptamine derivative, it is not surprising to find agonist activity as it probably binds to the 5-HT$_6$ receptor close to the 5-HT-binding site. In contrast, the nontryptamine-related 5-HT$_6$ ligand, SB-271046, behaved as an inverse agonist/antagonist at this receptor. Partial agonism for E-6837 is the most probable action at most 5-HT$_6$ receptor systems, *in vivo*,

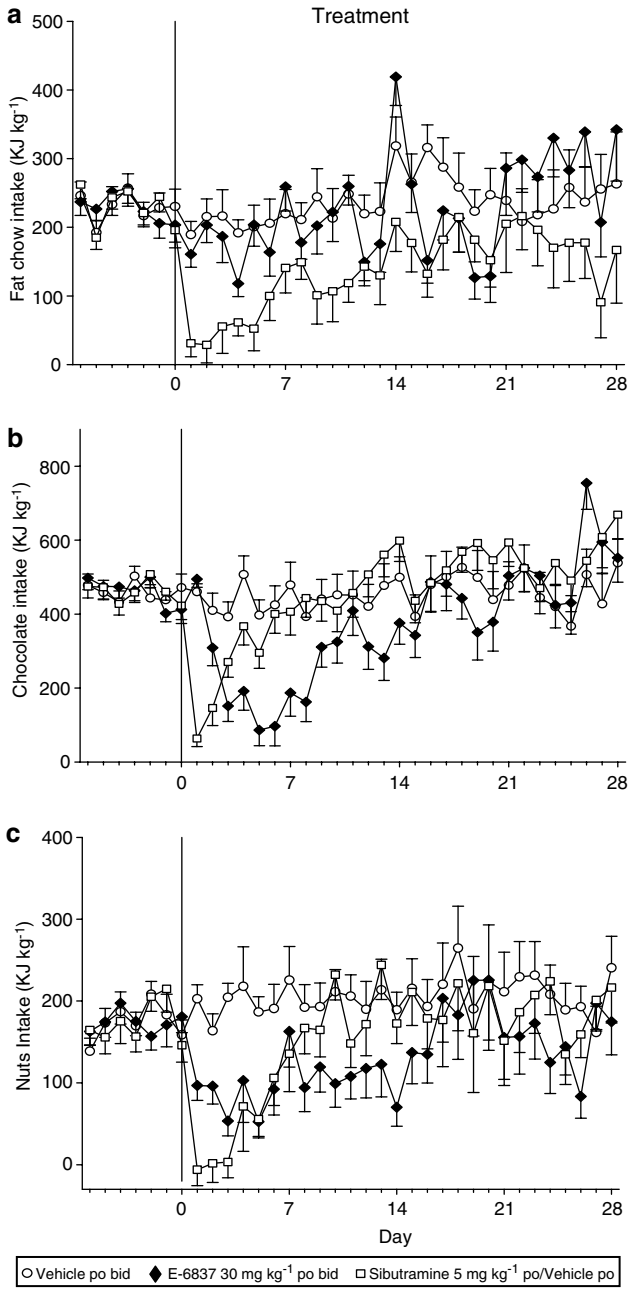

Figure 3 Effects of E-6837 and sibutramine on daily fat chow, chocolate and nuts intake in female DIO Wistar rats during treatment in comparison with vehicle. Rats were treated with E-6837 (30 mg kg^{-1}, twice a day, p.o.) and sibutramine (5 mg kg^{-1} day^{-1}, p.o.) for 4 weeks. *P*-values for mean comparisons between compounds along treatment period. (a) Daily fat chow intake: vehicle *versus* E-6837: $P=0.79$, vehicle *versus* sibutramine: $P=0.006$, E-6837 *versus* sibutramine: $P=0.01$; (b) Daily chocolate intake: vehicle *versus* E-6837: $P=0.08$, vehicle *versus* sibutramine: $P=0.87$, E-6837 *versus* sibutramine: $P=0.06$; (c) Daily nuts intake: vehicle *versus* E-6837: $P=0.02$, vehicle *versus* sibutramine: $P=0.10$, E-6837 *versus* sibutramine: $P=0.51$.

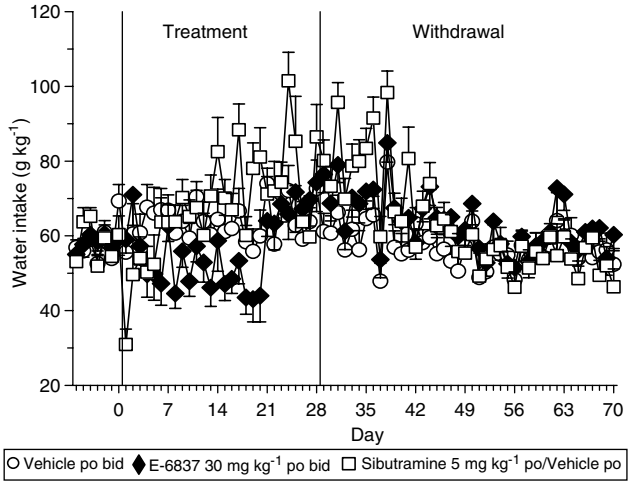

Figure 4 Effects of E-6837 and sibutramine on daily water intake in female DIO Wistar rats during treatment and withdrawal in comparison with vehicle. Rats were treated with E-6837 (30 mg kg^{-1}, twice a day, p.o.) and sibutramine (5 mg kg^{-1} day^{-1}, p.o.) for 4 weeks and followed for 6 weeks upon withdrawal. *P*-values for mean comparisons between compounds along treatment and withdrawal periods. Treatment period: vehicle *versus* E-6837: $P=0.10$, vehicle *versus* sibutramine: $P=0.34$, E-6837 *versus* sibutramine: $P=0.02$; withdrawal period: vehicle *versus* E-6837: $P=0.14$, vehicle *versus* sibutramine: $P=0.19$, E-6837 *versus* sibutramine: $P=0.94$.

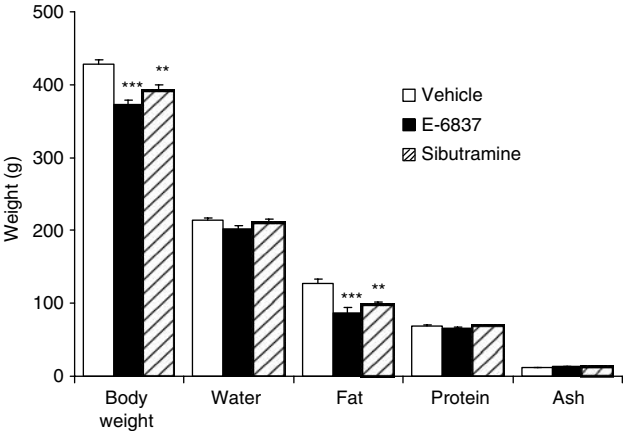

Figure 5 Effect of chronic treatment of female DIO Wistar rats with E-6837 or sibutramine on body composition determined by chemical analyses. Rats were treated with E-6837 (30 mg kg^{-1}, twice a day, p.o.) and sibutramine (5 mg kg^{-1} day^{-1}, p.o.) for 4 weeks. Results (weight per rat (g)) are expressed as treatment group means (adjusted for differences in body weight of the groups at baseline (Day 1)) and s.e.m. (calculated from the residuals of the statistical means). Significant differences from vehicle controls by Tukey's test are denoted by $^{**}P<0.01$ and $^{***}P<0.001$.

although the whole spectrum of activities from agonist to antagonist could be predicted for a partial agonist depending on intrinsic level of activity of the receptor system. Thus, antagonism by E-6837 will be seen in poorly coupled 5-HT$_6$ receptor systems, and while agonism by E-6837 will predominate in efficiently coupled 5-HT$_6$ receptor systems. Although a direct action of E-6837 on 5-HT$_6$ receptors is more probable at this stage, it is not possible to discount the possibility that E-6837 exerts its effect at the 5-HT$_6$ receptor not by an antagonist action *per se,* but by desensitizing the 5-HT$_6$ receptor as a result of prolonged agonist activation. Activation of the 5-HT$_6$ receptor in recombinant HEK-293 cells using 100 μM 5-HT evokes receptor desensitization, causing a decrease in the efficacy of receptor/adenylyl cyclase coupling

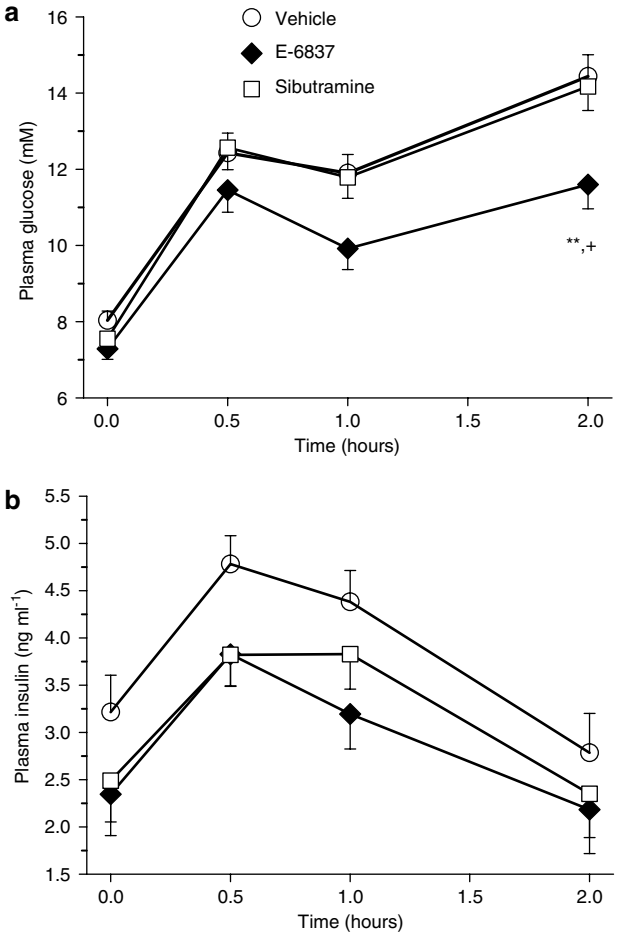

Figure 6 Effects of E-6837 and sibutramine on plasma glucose and insulin levels in female DIO Wistar rats after a glucose challenge test. Rats were treated with E-6837 (30 mg kg^{-1}, twice a day, p.o.) and sibutramine (5 mg kg^{-1} day^{-1}, p.o.) for 4 weeks. Following overnight starvation at the end of the treatment period all groups of rats were challenged with 800 mg kg^{-1}, s.c. glucose load. (a) Plasma glucose concentrations; P values for mean comparisons between compounds along treatment period: vehicle *versus* E-6837: $P=0.002$, vehicle *versus* sibutramine: $P=0.70$, E-6837 *versus* sibutramine: $P=0.007$. (b) Plasma insulin concentrations; P values for mean comparisons between compounds along treatment period: vehicle *versus* E-6837: $P=0.06$, vehicle *versus* sibutramine: $P=0.15$, E-6837 *versus* sibutramine: $P=0.63$. Significant differences from Tukey's test are denoted by ** (between compound and vehicle) and by $^+$ (between E-6837 and sibutramine), $^{**}P<0.01$, $(^+)P<0.05$.

(Max *et al*., 1995). In contrast to this reported desensitization *in vitro*, it appears that *in vivo* the 5-HT$_6$ receptor is not rapidly desensitized. Thus, Schechter *et al*. (2004) have reported that chronic administration (14 days) of the 5-HT$_6$ receptor agonist, WAY-466 (10 mg kg^{-1}, s.c.) produced a significant elevation in cortical GABA levels (845%), an effect that was greater than that induced by acute treatment (360%).

During the 4-week treatment period, E-6837 produced sustained weight loss in DIO-rats and even at the end of the 6-week withdrawal period their weights were still significantly lower than those of the vehicle-treated controls indicating no rapid weight regain after cessation of treatment. The onset of the effect of E-6837 on body weight loss was slower than sibutramine, while its maximum was greater (−15.7 *versus* −11.0%). As the latter is a clinically effective drug that has been approved for the long-term treatment of obesity (Ryan *et al*., 1995), and compound-induced weight-loss in the DIO rat is a predictor of efficacy in the clinic (Heal & Jagger, 2005), the results suggest that E-6837 is a promising clinical candidate for the treatment of obesity. This view is further supported by the findings that the weight loss evoked by E-6837 is solely due to a reduction of fat-mass, with no losses in either protein or water. Moreover, the decrease in adiposity resulted in a significant improvement in glycemic control.

The dynamics of the effects of E-6837 on food intake are different from those observed with sibutramine (this study, Jackson *et al*., 2004; 2005) and other direct or indirect monoamine agonists, for example amphetamine (Cawthorne, 1981), fenfluramine (Stallone & Levitsky, 1994) and mCPP (Vickers *et al*., 2003). The present findings with sibutramine differ somewhat from a previous report by Brown *et al*. (2001) where sibutramine (3 mg kg^{-1} day^{-1}, p.o.) for 3 weeks decreased food intake throughout in DIO rats; however, it should be noted that not only was the dose different but the model used was not a cafeteria DIO model. A transient decrease of food intake was also reported with the CB1 antagonists, rimonabant and AM-251, due to tolerance, notwithstanding both compounds demonstrated a sustained effect on body weight loss in DIO mice (Hildebrandt *et al*., 2003; Ravinet-Trillou *et al*., 2003; Jackson *et al*., 2005). Such findings suggest that although a decrease in food intake may be the main cause for initially reducing body weight for both sibutramine and the CB1 antagonists, other mechanisms are involved in their long-lasting antiobesity effects. One

Table 2 Effect of chronic administration of E-6837 (30 mg kg^{-1}, twice a day, p.o.) or sibutramine (5 mg kg^{-1} day^{-1}, p.o.) to DIO rats on various plasma indices of obesity and its comorbidities

	Adipose signals		*Plasma lipids*				*Glycaemic control*	
Treatment	*Leptin* (ng ml^{-1})	*Adiponectin* (μg ml^{-1})	*Cholesterol* (mg dl^{-1})	*Triglycerides* (mM)	*Glycerol* (mM)	*NEFAs* (mM)	*Glucose* (mM)	*Insulin* (ng ml^{-1})
Vehicle	166±19	14±1	108±7	0.59±0.10	0.50±0.04	0.93±0.07	9.1±0.4	5.90±0.66
E-6837 (30 mg kg^{-1} p.o. bid)	83±19*	12±1	98±7	0.72±0.11	0.43±0.04	0.98±0.07	8.6±0.4	4.28±0.66
Sibutramine (5 mg kg^{-1} p.o./ vehicle p.o.)	119±20	14±1	98±7	0.64±0.12	0.54±0.04	1.18±0.07*	7.9±0.4	5.32±0.70

Results (plasma levels) are expressed as treatment group means (adjusted for bleeding order (animal number), differences between runs and differences between the groups in body weight at baseline (Day 1)) and s.e.m. (calculated from the residuals of the statistical model). Significant differences from Tukey's test are denoted by $^*P<0.05$ between compound and vehicle. Group sizes: vehicle, $n=10$; E-6837, $n=10$; sibutramine, $n=9$.

additional mechanism is likely to be activation of metabolic processes, such as increases of both fatty acid oxidation and energy expenditure (Connoley *et al*., 1999; Skill *et al*., 2000; Ravinet-Trillou *et al*., 2003; Jbilo *et al*., 2005; Liu *et al*., 2005). This mechanism is unlikely to account for the effects of E-6837 because daily water intake was not increased in contrast to results with sibutramine (this study; Jackson *et al*., 2004; 2005). This increase in drinking may be related to the increase in thermogenesis with sibutramine administration in animals (Connoley *et al*., 1999; Skill *et al*., 2000). Hence, moderate, prolonged decreases in food intake appear to be the principal cause for reducing body weight by the interaction of E-6837 with the 5-HT$_6$ receptor. As sibutramine enhances basal metabolic rate by selectively increasing central sympathetic drive to brown adipose tissue (BAT) and this mechanism is of no relevance to juvenile or adult humans, the data suggest that E-6837 may evoke greater reductions in body weight as a result of its more prolonged action to reduce food intake. However, some caution should be exercised because although core body temperature was measured, more sophisticated studies on metabolic rate with E-6837 treatment have not yet been performed.

In terms of alterations in plasma indices of comorbid risk factors, reduced adiposity evoked by E-6837 was accompanied by a highly significant reduction of plasma leptin, which is known to correlate with fat mass (Maffei *et al*., 1995). Although the sibutramine-induced fall in plasma leptin did not reach statistical significance in this study, Brown *et al*. (2001) and Jackson *et al*. (2005) previously reported significant reductions in this hormone along with sibutramine-induced weight loss. No improvements in plasma lipid profiles, for example cholesterol, triacylglycerol, glycerol, NEFAs, were observed with E-6837/sibutramine (with exception of NEFAs) which contrasts with reports of lower concentrations in obese, diabetic, ob/ob mice after treatment with sibutramine (Day & Bailey, 1998). While no definitive explanation can be offered for this difference, the metabolic disturbances present in the ob/ob mouse are much more profound than in the DIO rat, and as a consequence, perhaps more susceptible to improvement by antiobesity drugs.

Hyperinsulinemia, hyperglycemia and insulin resistance are frequently associated with human obesity (Kahn & Flier, 2000). The weight-loss observed after treatment with E-6837 improved the outcome of a glucose tolerance test with a reduced plasma glucose excursion and a trend for reduced plasma insulin levels. These data are probably of biological significance indicating an improvement in insulin sensitivity, and hence, glycemic control.

The mechanisms by which 5-HT$_6$ receptors may modulate food intake are under investigation. The localization of 5-HT$_6$ receptors in the brain may shed further light on these mechanisms. One brain region specifically linked to the regulation of food intake and energy expenditure is the hypothalamus (see Horvath, 2005) and it has been reported to contain significant amounts of 5-HT$_6$ receptor mRNA; in particular, in the arcuate nucleus and detectable 5-HT$_6$ receptor-like immunoreactivity (Hamon *et al*., 1999). A potential interaction between the 5-HT$_6$ receptor and GABAergic interneurones in the hypothalamus, as suggested for several brain regions, may make synaptic contact with pro-opiomelanocortin containing neurones in the arcuate nucleus and inhibit release of anorectic neuropeptide α-melanocyte-stimulating hormone (α-MSH) (Woolley *et al*., 2004). Food consumption in rats given the combination GABA$_A$ agonist, muscimol, with the 5-HT$_6$ receptor ligand, Ro 04-6790, was reduced as compared to that seen following treatment with muscimol alone; a finding that is consistent with GABAergic neurones also being involved in the hypophagic effect of 5-HT$_6$ ligands in rats (Woolley *et al*., 2004). It is, therefore, possible that 5-HT$_6$ receptor ligands, which either block or desensitize the serotonergic receptors could reduce GABA and increase α-MSH release, thereby suppressing food intake.

In summary, the 5-HT$_6$ receptor partial agonist E-6837 appears to offer a promising new CNS-mediated strategy for the management of obesity. E-6837 induces hypophagia and profound and sustained weight loss in DIO rats. These changes are accompanied by a preferential loss of fat mass, decreased plasma leptin and improved glycemic control. On the basis of comparison with sibutramine, it is possible that E-6837 will result in greater sustained weight loss than sibutramine.

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