

## RESEARCH PAPER

# Increase in weight induced by muraglitazar, a dual PPAR $\alpha$ / $\gamma$ agonist, in *db/db* mice: adipogenesis/or oedema?

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**Background and purpose:** Muraglitazar, a dual PPAR $\alpha$ / $\gamma$  agonist, caused a robust increase in body weight in *db/db* mice. The purpose of the study was to see if this increase in weight was due to oedema and/or adipogenesis.

**Experimental approach:** The affinity of muraglitazar at PPAR $\alpha$ / $\gamma$  receptors was characterized using transactivation assays. Pre-adipocyte differentiation, expression of genes for adipogenesis (aP2), fatty acid oxidation (ACO) and sodium reabsorption (ENaC $\gamma$  and Na $^{+}$ , K $^{+}$ -ATPase); haemodilution parameters and serum electrolytes were measured to delineate the role of muraglitazar in causing weight gain *vis a vis* rosiglitazone.

**Key Results:** Treatment with muraglitazar (10 mg kg $^{-1}$ ) for 14 days significantly reduced plasma glucose and triglycerides. Reduction in plasma glucose was significantly greater than after similar treatment with rosiglitazone (10 mg kg $^{-1}$ ). A marked increase in weight was also observed with muraglitazar that was significantly greater than with rosiglitazone. Muraglitazar increased aP2 mRNA and caused adipocyte differentiation in 3T3-L1 cells similar to rosiglitazone. It also caused a marked increase in ACO mRNA in the liver of the treated mice. Expression of mRNA for ENaC $\gamma$  and Na $^{+}$ , K $^{+}$ -ATPase in kidneys was up-regulated after either treatment. Increased serum electrolytes and decreased RBC count, haemoglobin and haematocrit were observed with both muraglitazar and rosiglitazone.

**Conclusions and implications:** Although muraglitazar has a better glucose lowering profile, it also has a greater potential for weight gain than rosiglitazone. In conclusion, muraglitazar causes both robust adipogenesis and oedema in a 14-day treatment of *db/db* mice as observed in humans.

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**Keywords:** PPAR dual agonist; antidiabetic; hypolipidaemic; weight gain; adipogenesis; oedema; *db/db* mice

**Abbreviations:** ACO, acyl CoA oxidase; CPT-1, carnitine palmitoyl-transferase-1; ENaC, epithelial sodium channel; PPAR, peroxisome proliferator-activated receptors

## Introduction

Diabetes mellitus is a metabolic disease characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action or both. Over the last decade, hyperglycaemia has been shown to be only one component of a series of anomalies afflicting patients with type II diabetes mellitus (T2DM). Concurrent maladies including insulin resistance, obesity, hypertension and dyslipidaemia define diabetes as a metabolic syndrome.

The peroxisome proliferator-activated receptor (PPAR) family stands out as a well-validated target in search for new and improved treatment options for diabetic patients. PPARs are members of the nuclear hormone superfamily of ligand-activated transcription factors and include three distinct PPAR subtypes that are products of different genes and are commonly designated as PPAR $\alpha$ ,  $\gamma$  and  $\delta$  (Desvergne and Wahli, 1999). The knowledge that PPAR $\alpha$  and PPAR $\gamma$  are the molecular targets for the actions of fibrates and thiazolidinediones, respectively, has led to a renaissance in nuclear receptor research to develop drugs for diabetes and associated dyslipidaemia. Specific PPAR $\gamma$  agonists such as rosiglitazone and pioglitazone are effective compounds but have an unattractive side effect profile that includes weight gain, oedema, neutropenia and haemodilution (Mudaliar *et al.*, 2003). Clinical experience shows that

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thiazolidinediones induce oedema in 10–15% of patients, sometimes predisposing them to reversible congestive heart failure, requiring discontinuation of therapy (Nesto *et al.*, 2003). Activation of PPAR $\gamma$ , expressed in adipose tissue, lower intestine and immune system, regulates glucose and lipid homeostasis and triggers insulin sensitization in addition to robust pre-adipocyte differentiation leading to weight gain (Desvergne and Wahli, 1999). PPAR $\alpha$ , predominantly expressed in the liver, specifically regulates the transcription of genes involved in hepatic fatty acid uptake and oxidation, mainly acyl CoA synthetase, acyl CoA oxidase (ACO) and carnitine palmitoyl-transferase-1 (CPT-1) (Desvergne and Wahli, 1999) and thus, may have a positive effect on body weight through increased catabolism of fat in addition to its lipid-lowering effects (Staels and Fruchart, 2005). PPAR $\alpha$ -/- mice show a phenotype with monogenic, late-onset, spontaneous obesity with a stable caloric intake, implicating the PPAR $\alpha$  in resistance to obesity (Costet *et al.*, 1998).

Given the importance of simultaneously controlling glucose homeostasis, insulin sensitization and lipid metabolism in T2DM, activation of both PPAR  $\alpha$  and  $\gamma$  subtypes is beneficial (Staels and Fruchart, 2005). This had led to the development of a number of dual  $\alpha/\gamma$  agonists such as muraglitazar, tesaglitazar and several others, which were shelved subsequently (Hegarty *et al.*, 2004; Barlocco, 2005; Oakes *et al.*, 2005). This is because although dual PPAR $\alpha/\gamma$  activators seem to have a favourable pharmacodynamic–pharmacokinetic profile, several serious concerns have arisen in preclinical and clinical studies. Ragaglitazar has been discontinued owing to bladder tumours in rodents and KRP-297 followed a similar fate (Egerod *et al.*, 2005). Thus, application of dual PPAR agonists requires further scrutiny. Muraglitazar, a dual PPAR agonist, with its high efficacy and no tumorigenic potential could not be developed further owing to safety concerns of oedema and induction of congestive cardiac failure in susceptible patients (FDA advisory committee transcript on muraglitazar, 2005; FDA statistical review, 2005; BMJ-News, 2006) and tesaglitazar owing to anticipated nephrotoxicity (Astra-Zeneca press release, 2006).

PPAR $\gamma$  activators are known to cause both oedematous weight gain and adipogenesis (Mudaliar *et al.*, 2003; Cock *et al.*, 2004). Recent reports have implicated the activation of PPAR  $\gamma$  receptors in oedema owing to their presence in the distal nephron epithelium, in the renal medullary ducts (Guan *et al.*, 2005; Zhang *et al.*, 2005). Oedema is postulated to be caused by stimulation of the epithelial sodium channel (ENaC) and Na<sup>+</sup>, K<sup>+</sup>-ATPase system in the collecting ducts as observed in rodents (Guan *et al.*, 2005; Zhang *et al.*, 2005). Specific deletion of PPAR $\gamma$  in the collecting ducts has been observed to block thiazolidinedione-induced fluid retention (Guan *et al.*, 2005; Zhang *et al.*, 2005).

In earlier studies with diet-induced obese C57BL/6 mice and *db/db* mice, muraglitazar showed either no weight gain (Hariharan *et al.*, 2002) or a nonsignificant trend towards a weight gain (Harrity *et al.*, 2006). As we observed a robust increase in body weight with muraglitazar treatment in *db/db* mice, our major aim was to examine if this increase in weight was oedematous and/or due to increased adipogenesis.

## Methods

### *In vitro studies*

**PPAR transactivation assays.** HEK-293 cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco modified Eagle's medium (DMEM) containing 10% charcoal stripped serum. The cells were plated in six-well plates and transiently transfected with vectors containing full-length hPPAR $\gamma$ , hPPAR $\alpha$  or hPPAR $\delta$  and Rat-PPRE-pTAL-Luc plasmids (Chakrabarti *et al.*, 2004). pAdvantage vector was used as a luciferase enzyme translation enhancer and pRL-cytomegalovirus vector as a transfection efficiency control. Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used for the transfections. The plasmid constructs were prepared in-house. After 24 h, the cells were harvested and plated into 96-well plates at a density of  $3 \times 10^4$  cells per well and allowed to attach for 1 h. The cells were then incubated with test compounds for 24 h. Dimethylsulphoxide (DMSO) (0.1%) was used as a vehicle control. After 24 h, cells were lysed and assayed for luciferase activity using the Steady-Glo luciferase system according to the manufacturer's instructions (Promega, Madison, WI, USA). The activity of the compounds as PPAR agonists was related to the transcription of the reporter (Luciferase) gene. Plates were read in a Wallac scintillation counter.

**Mouse pre-adipocyte differentiation assay.** Compounds were assayed for their ability to induce PPAR $\gamma$ -mediated differentiation of mouse 3T3L-1 pre-adipocytes (Shibata *et al.*, 1999). 3T3-L1 pre-adipocytes (ATCC) were seeded at  $5 \times 10^4$  cells ml<sup>-1</sup> per well in a 24-well collagen-coated plate. Two days post-confluent cells were induced for 48 h with a mixture of 1  $\mu$ M dexamethasone, 10  $\mu$ g ml<sup>-1</sup> insulin and 0.5 mM isobutylmethylxanthine (IBMX) in addition to different concentrations of the test compound. After 48 h, the media was replaced with DMEM supplemented with 10% fetal bovine serum (FBS) and 10  $\mu$ g ml<sup>-1</sup> insulin along with different concentrations of test compound. This treatment continued for 3 days, after which the cells were re-fed every 48 h with DMEM supplemented with 10% FBS. At the end of the incubation, wells were washed with phosphate-buffered saline (PBS) and lysed by 100  $\mu$ l 1% (v/v) NP40 in PBS. Cell lysates were used to quantitate triglycerides and protein using GPO reagent kit (Pointe Scientific Inc., Canton, MI, USA) and BCA method (Sigma, St Louis, MO, USA) respectively. Triglyceride content was expressed as mM. The activity of the reference PPAR $\gamma$  agonist, rosiglitazone was defined to be 100% in this assay.

### *Gene expression studies*

**In vitro gene expression studies.** To delineate the role of muraglitazar in adipocyte differentiation and subsequent adipogenesis, expression of aP2 was measured in 3T3-L1 cells. For this, 3T3-L1 cells were seeded ( $5 \times 10^4$  cells/well) in 24-well tissue culture plates and grown to confluence in DMEM with 100 U ml<sup>-1</sup> penicillin, 100  $\mu$ g ml<sup>-1</sup> streptomycin and 1 mM sodium pyruvate, supplemented with 10% fetal calf serum. Cells were grown at 37°C in 5% CO<sub>2</sub> in a humidified chamber and re-fed fresh media every 2 days. The

PPAR ligands were dissolved in DMSO and diluted with DMEM that contained 10% fetal bovine serum, 1  $\mu$ M dexamethasone, and 150 nM insulin. The final media concentration of DMSO was 0.1% in the dilutions. Cells treated with 0.1% DMSO in media served as vehicle controls. The cells were incubated with PPAR ligands for 5 days and were re-fed with fresh dilutions on the third day of the treatment period, except where noted. Each compound concentration was tested in duplicate or triplicate wells.

**In vivo gene expression studies.** Administration of PPAR $\alpha$  agonists increase the expression of peroxisomal (ACO) and mitochondrial  $\beta$ -oxidation enzymes (carnitine acetyl-transferase, CPT-1) which facilitate fatty acid uptake and catabolism. In this study, *db/db* mice were treated either with rosiglitazone or muraglitazar at 10 mg kg<sup>-1</sup> for 14 days and liver tissue was harvested on day 14 and snap frozen for analysing the ACO expression. To explore the role of muraglitazar in renal electrolyte homeostasis, the mRNA expression of selected genes in the kidney were determined using real-time-polymerase chain reaction (RT-PCR). To measure expression of the mRNA for ENaC $\gamma$  and Na<sup>+</sup>, K<sup>+</sup>-ATPase- $\alpha$ , *db/db* mice were treated with rosiglitazone (10 mg kg<sup>-1</sup>) or muraglitazar at 10 mg kg<sup>-1</sup> for 14 days and whole kidneys were harvested and snap frozen.

**Total RNA extraction and RT-PCR.** Total RNA from 3T3-L1 cells (for ap2 mRNA expression), mice liver (ACO mRNA expression) and kidney (for ENaC $\gamma$  and Na<sup>+</sup>-K<sup>+</sup>-ATPase- $\alpha$  mRNA expression) were isolated from frozen kidney tissues of treated mice using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified using RNeasy Mini kits (Qiagen, Valencia, CA, USA). Total RNA (1–2  $\mu$ g) was reverse-transcribed in a 50  $\mu$ l reaction using Taqman reverse transcription reagents (ABI). RT-PCR multiplex reaction was performed using gene specific Taqman primers and probes and analysed using ABI 7900 HT Sequence Detection System. A quantitative mRNA expression level of ap2, ACO, ENaC $\gamma$  subunit (ENaC $\gamma$ ) and Na<sup>+</sup>-K<sup>+</sup>-ATPase- $\alpha$  was determined. Data were normalized to the expression of 18s endogenous control mRNA and presented as fold of vehicle control. mRNA expression data were analysed using the relative quantitation (RQ) study SDS software (Applied Biosystems, CA, USA).

#### *In vivo studies*

C57BL/KsJ-*db/db* mice (10–12 weeks old, either sex, from Jackson Laboratory, Bar Harbor, ME, USA) were from the animal facility of Ranbaxy Laboratories Ltd, Gurgaon, India. Animals were maintained on a 12-h day/night schedule with *ad libitum* access to standard mouse diet (Harlan Teklad, Oxon, UK) and water. All animal experiments were conducted according to the Guidelines of Experimental Animal Care issued by the Committee for Purpose of Control & Supervision of Experiments on Animals (CPCSEA). At the end of the acclimatization period, the mice were bled under light ether anaesthesia by a retro-orbital puncture and plasma samples were analysed for glucose levels. Animals

were grouped on the basis of random plasma glucose values so that the average glucose level of one group was not significantly different from that of the other.

**Drug treatment and blood sampling.** Drugs were administered orally by gavage, once a day at a volume of 10 ml kg<sup>-1</sup> of body weight for 14 days starting from day 1. Blood sampling was performed retro-orbitally under the fed condition on the 14th day 1 h after dosing and plasma was separated to measure the plasma glucose and triglyceride levels. The animals were grouped as follows: Group 1: control; Group 2: rosiglitazone (10 mg kg<sup>-1</sup>); Group 3: muraglitazar (10 mg kg<sup>-1</sup>); Group 4: fenofibrate (100 mg kg<sup>-1</sup>). Another group of animals were treated with increasing doses (0.3, 1, 3 and 10 mg kg<sup>-1</sup>) of muraglitazar for 14 days and glucose and triglyceride levels were measured.

**Plasma levels of glucose and triglycerides in *db/db* mice.** Plasma samples were analysed for biochemical parameters using commercial diagnostic kits for triglyceride and glucose procured from Ranbaxy Diagnostics. Quality control samples (for determination of inter and intra-assay variability) were co-analysed.

**Liver and body weight changes after drug treatment in *db/db* mice.** The animals were weighed weekly to monitor any change in body weight. Increase in the body weight after 14 days treatment with various treatments and muraglitazar (at 0.3, 1, 3 and 10 mg kg<sup>-1</sup>) was determined by subtracting the weight of each mouse on day 1 from its weight on day 14. On day 15, the liver was weighed and the change in weight was expressed as a percentage change with respect to the body weight.

**Oedema parameters in treated *db/db* mice.** To study the role of muraglitazar on renal electrolyte homeostasis, plasma electrolyte concentrations were measured in plasma. The blood collected was also analysed for haemodilution markers such as RBC count, haemoglobin and haematocrit.

#### *Statistical analysis*

Nonlinear regression analysis was used to determine the EC<sub>50</sub> of the agonists. Efficacy was calculated as % maximum of a standard response. Rosiglitazone was used as standard for hPPAR $\gamma$ . Data are presented as mean  $\pm$  s.e.m. Calculations of the % reduction in plasma triglyceride and fed glucose levels were performed using MS Excel and Graph Pad Prism software (version 4.02). Difference between groups was analysed by performing one-way analysis of variance followed by Dunnett's or Newman Keul's multiple comparison test for various parameters of vehicle- and drug-treated groups.

#### *Chemicals*

Muraglitazar was synthesized in the Department of Medicinal Chemistry, New Drug Discovery Research, Ranbaxy Laboratories Ltd. Rosiglitazone was procured from Sun

Pharmaceuticals Ltd (Mumbai, India) and fenofibrate from Sigma (St Louis, MO, USA). Muraglitazar and rosiglitazone were freshly suspended in 0.25% carboxy methylcellulose just before use. All other chemicals used in the study were of analytical grade.

## Results

### Transactivation assays

Muraglitazar, synthesized in our chemical division, showed a significant activation of both hPPAR $\alpha$  and hPPAR $\gamma$  in an *in vitro* transactivation assay. Potent dose-dependent agonistic activity of both PPAR $\alpha$  and PPAR $\gamma$  was demonstrated in transiently transfected HEK-293 cells with EC<sub>50</sub> values of 0.28 and 0.16  $\mu$ M, respectively (Table 1). The potency of muraglitazar as prepared in our lab was comparable to the reported values of muraglitazar (Barlocco, 2005) with EC<sub>50</sub> values of 0.24 and 0.12  $\mu$ M for PPAR $\alpha$  and PPAR $\gamma$ , respectively. No significant PPAR $\delta$  activity was observed at >30  $\mu$ M (data not shown). Rosiglitazone was used as a primary standard for PPAR $\gamma$  activity. Muraglitazar shows functional activity comparable to that of rosiglitazone at PPAR $\gamma$ ; rosiglitazone has negligible potency at PPAR $\alpha$ .

### Differentiation of mouse 3T3L-1 pre-adipocytes

In a pre-adipocyte differentiation assay, which measures the extent of predominantly PPAR $\gamma$ -mediated differentiation of pre-adipocytes into triglyceride loaded adipocytes, muraglitazar-induced dose-dependent differentiation at potency similar to rosiglitazone. The EC<sub>50</sub> values for rosiglitazone and muraglitazar in adipogenesis assays were  $94 \pm 58.1$  and  $131 \pm 46.8$  nM, respectively, which were not significantly different from each other and showed potent activation of PPAR $\gamma$ .

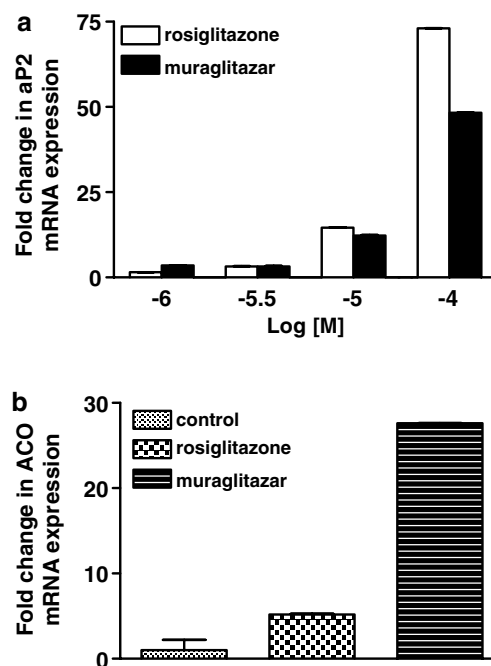
### Gene expression studies

aP2 mRNA expression data were analysed using the relative quantitation (RQ) study SDS software (Applied Biosystems). At 100  $\mu$ M concentration, aP2 mRNA expression by rosiglitazone was higher than muraglitazar (Figure 1a). However, at lower concentrations, both had similar levels of expression. These findings are suggestive of a similar mode and extent of lipid sequestration into fat cells by both the drugs and support the observed weight gain in *in vivo* experiments. Induction of ACO mRNA was observed with muraglitazar suggesting its role in fatty acid uptake and mitochondrial oxidation, whereas rosiglitazone was devoid of this effect. ACO mRNA expression was 4.3-fold higher in livers of muraglitazar-treated *db/db* mice as compared to rosiglitazone treatment at the same dose (Figure 1b). To determine if muraglitazar induced any fluid retention/oedema, the mRNA expression levels of ENaC $\gamma$  and Na<sup>+</sup>, K<sup>+</sup>-ATPase- $\alpha$  were studied by RT-PCR in tissues from *db/db* mice treated with muraglitazar for 14 days. The gene expression results showed that there is an increasing trend in the mRNA expression level of ENaC $\gamma$  and Na<sup>+</sup>, K<sup>+</sup>-ATPase- $\alpha$  (data not shown) in both muraglitazar and rosiglitazone-treated

**Table 1** *In vitro* transactivation activity profile of rosiglitazone and muraglitazar

Compound	Transactivation EC <sub>50</sub> ( $\mu$ M)		
	hPPAR $\gamma$	hPPAR $\alpha$	hPPAR $\delta$
Rosiglitazone (BRL 49653)	$0.056 \pm 0.017$	> 30	> 30
Muraglitazar (BMS 298585)	$0.16 \pm 0.04$	$0.28 \pm 0.03$	> 30

EC<sub>50</sub> values for PPAR $\alpha$ ,  $\gamma$  or  $\delta$  agonist activity were calculated as the concentration of the test ligand ( $\mu$ M) required for the half-maximal fold induction of luciferase activity. Data shown are means  $\pm$  s.e.m. from three experiments for each treatment.



**Figure 1** (a) Relative quantitation of aP2 mRNA expression by rosiglitazone and muraglitazar as determined by semiquantitative RT-PCR. Confluent 3T3-L1 cells were incubated for 5 days with various concentrations of PPAR- $\gamma$  ligands, rosiglitazone, muraglitazar or 0.1% DMSO (as vehicle control) and RNA was isolated as described under 'Methods'. aP2 mRNA levels were normalized by endogenous 18S control. Data shown are means  $\pm$  s.d. of duplicate determinations. A representative bar graph is shown. In (b) mRNA expression of ACO after 14 days treatment with muraglitazar (10 mg kg<sup>-1</sup>) and rosiglitazone (10 mg kg<sup>-1</sup>); *n* = 2.

groups as compared to vehicle-treated *db/db* mice. However, the mRNA expression level of ENaC $\gamma$  was significantly elevated (4.4-fold) in mice treated with muraglitazar compared to control *db/db* mice (Figure 2).

### Glucose and triglyceride lowering activity

Muraglitazar showed a dose-dependent reduction in random glucose levels and triglycerides in *db/db* mice treated for 14 days (Figure 3a and c). Rosiglitazone and muraglitazar at 10 mg kg<sup>-1</sup> showed significant reduction in plasma glucose levels under fed conditions in comparison with vehicle control after 14 days of treatment. The glucose-lowering activity was significantly more in the muraglitazar-treated

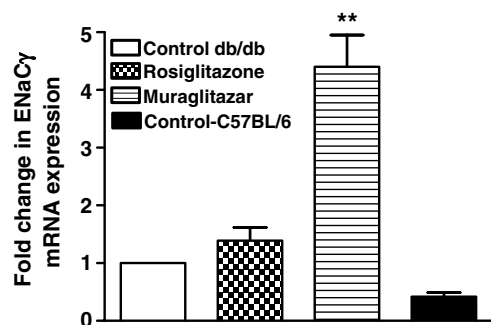
group compared to rosiglitazone-treated group (Figure 3b). Rosiglitazone and muraglitazar at  $10 \text{ mg kg}^{-1}$  also showed significant reduction in plasma triglycerides in comparison with vehicle control after 14 days of treatment (Figure 3d).

#### Effect on body weight

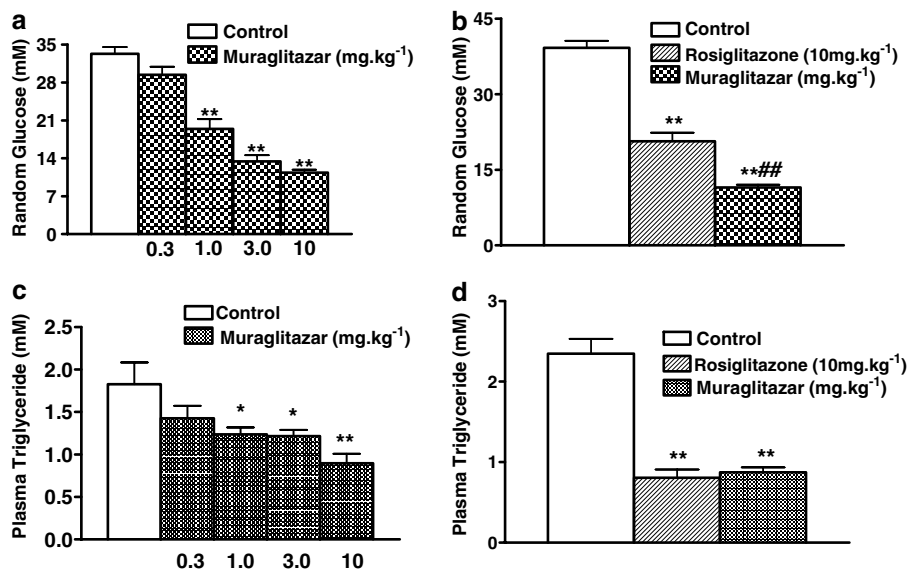
There was dose-dependent increase in body weight gain in grams (g) in muraglitazar (0.3, 1, 3 and  $10 \text{ mg kg}^{-1}$ )-treated groups in comparison with vehicle control group (Figure 4a). Also a significant increase in body weight gain was observed in rosiglitazone group. The effect, however, was more prominent in the muraglitazar-treated group and was significantly greater than in rosiglitazone (Figure 4b).

#### Change in liver weight owing to PPAR $\alpha$ -induced activity

Muraglitazar ( $10 \text{ mg kg}^{-1}$ ) did not show any increase in liver weight after a 14-day treatment (Figure 5). Fenofibrate ( $100 \text{ mg kg}^{-1}$ ) induced a significant increase in the liver

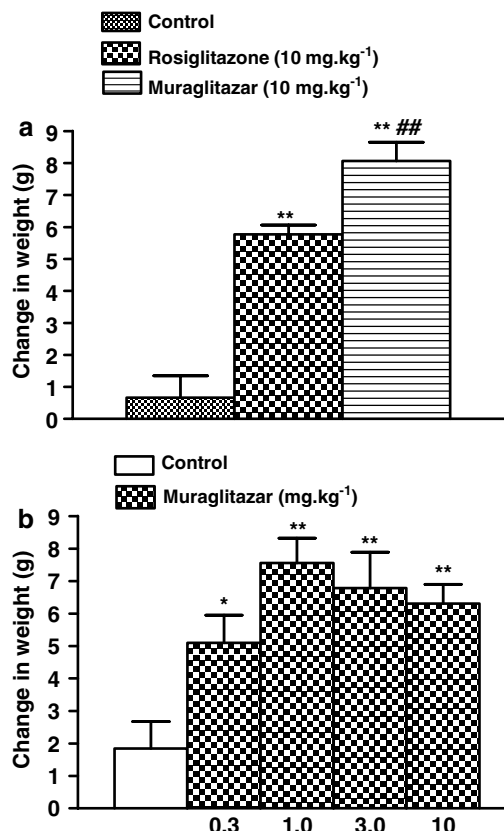


**Figure 2** mRNA expression of ENaC $\gamma$  in *db/db* mice and C57BL/6 mice after 14 days treatment with muraglitazar ( $10 \text{ mg kg}^{-1}$ ) and rosiglitazone ( $10 \text{ mg kg}^{-1}$ ). \*\* $P < 0.01$  vs control;  $n = 4$ .

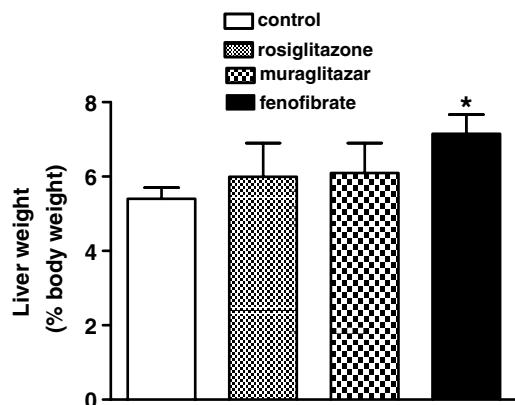


**Figure 3** Dose-dependent effect of muraglitazar on random glucose (a) and triglyceride levels (c). Effect of rosiglitazone and muraglitazar on random glucose (b) and triglyceride levels (d) after 14 days treatment in *db/db* mice. \* $P < 0.05$ , \*\* $P < 0.01$  vs control; ### $P < 0.01$  vs rosiglitazone group;  $n = 12$  per group.

weight as was expected owing to its PPAR $\alpha$  activity (Lee *et al.*, 1995; Figure 5). Rosiglitazone serving as a negative control showed no increase in liver weight (Figure 5).



**Figure 4** Weight gain in *db/db* mice. (a) Effect of rosiglitazone ( $10 \text{ mg kg}^{-1}$ ) and muraglitazar ( $10 \text{ mg kg}^{-1}$ ) on body weight change after a 14-day treatment. (b) Dose-response for muraglitazar on body weight change in *db/db* mice after a 14-day treatment. \* $P < 0.5$ ; \*\* $P < 0.01$  vs control; ### $P < 0.01$  vs rosiglitazone group;  $n = 8$  per group.



**Figure 5** Effect of rosiglitazone ( $10 \text{ mg kg}^{-1}$ ), muraglitazar ( $10 \text{ mg kg}^{-1}$ ) and fenofibrate ( $100 \text{ mg kg}^{-1}$ ) on liver weight (shown as % body weight) after 14-day treatment in *db/db* mice. \* $P < 0.05$  vs control;  $n = 6$  per group.

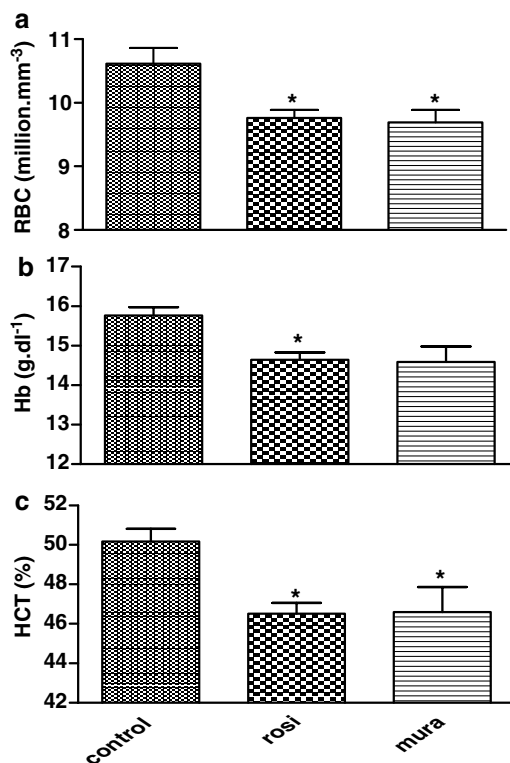
#### Effect of muraglitazar and rosiglitazone on oedema parameters

An increase in sodium and chloride concentrations (control values:  $147 \pm 0.8$  and  $100 \pm 0.8 \text{ mmol l}^{-1}$ , respectively) was observed in plasma of mice treated with both muraglitazar ( $150 \pm 0.5 \text{ mmol l}^{-1}$ ,  $P < 0.05$  vs control for  $\text{Na}^+$  and  $102 \pm 0.5 \text{ mmol l}^{-1}$ ,  $P < 0.05$  vs control for  $\text{Cl}^-$ ) and rosiglitazone ( $150 \pm 0.5 \text{ mmol l}^{-1}$ ,  $P < 0.05$  vs control for  $\text{Na}^+$  and  $102.5 \pm 0.6 \text{ mmol l}^{-1}$ ,  $P < 0.05$  for  $\text{Cl}^-$ ). As sodium retention is always followed by an increase in water reabsorption via collecting ducts, a haemodilution is expected. Muraglitazar ( $10 \text{ mg kg}^{-1}$ ) showed a marked decrease in RBC count, haemoglobin and haematocrit in treated *db/db* mice (Figure 6a–c). Similar changes were seen with  $10 \text{ mg kg}^{-1}$  rosiglitazone (Figure 6–c).

## Discussion

Muraglitazar, with a 25-fold greater affinity at  $\text{PPAR}\alpha$  receptors than fenofibrate ( $\text{EC}_{50} 10 \mu\text{M}$ ), or WY 14643 ( $\text{EC}_{50} 8.1 \mu\text{M}$ ), was expected to cause no weight gain in rodents. Potent  $\text{PPAR}\alpha$  agonist activity is normally associated with, and has been shown to cause, a lipid-lowering effect as well as body weight reduction in the hamster model of dyslipidaemia (Glaxo Wellcome group, 1997; Minnich *et al.*, 2001) and in rodents (Chaput *et al.*, 2000; Guerre-Millo *et al.*, 2000). In a recent study in *ob/ob* mice, fenofibrate prevented the rosiglitazone-induced weight gain (Carmona *et al.*, 2005) in addition to correction of their dyslipidaemic profile. Fenofibrate and its combination groups showed a decrease in the adipose tissue mass and an increase in hepatic fatty acid oxidation (Carmona *et al.*, 2005) validating the role of  $\text{PPAR}\alpha$  in clinical settings where the clinical efficacy of fibrates is accompanied with 'on treatment' weight loss in humans.

It thus seems clear that  $\text{PPAR}\alpha/\text{PPAR}\gamma$  dual agonists with a higher propensity towards  $\text{PPAR}\alpha$  activation should ideally show a weight decreasing, or a weight neutralizing, tendency to counteract  $\text{PPAR}\gamma$  activity. However, in our study, a marked increase in body weight was observed with muraglitazar that was significantly greater than the weight gain



**Figure 6** Haemodilution parameters after 14 days treatment with muraglitazar ( $10 \text{ mg kg}^{-1}$ ) and rosiglitazone ( $10 \text{ mg kg}^{-1}$ ). Mean ( $\pm \text{s.e.m.}$ ) values for RBC count (a), haemoglobin (Hb, b) and % haematocrit (%HCT, c) are shown. \* $P < 0.05$  vs control;  $n = 8$  per group.

observed with rosiglitazone after a 14-day treatment in *db/db* mice. A similar weight gain has also been reported with tesaglitazar in Zucker *fa/fa* rats treated for 21 days (Hegarty *et al.*, 2004; Oakes *et al.*, 2005), and this has been attributed to its  $\text{PPAR}\gamma$  agonist activity. In the present study, in mouse pre-adipocyte differentiation assay, both rosiglitazone and muraglitazar were observed to have similar  $\text{EC}_{50}$  values indicating that muraglitazar through its  $\text{PPAR}\gamma$  activation has a similar propensity as rosiglitazone in causing adipogenesis. Moreover, both rosiglitazone and muraglitazar had similar levels of the adipocyte gene, aP2, mRNA expressed in 3T3 L1 cells suggesting a similar mode and extent of lipid sequestration into fat cells by both compounds (Bernlohr *et al.*, 1985; Kletzien *et al.*, 1992; Gregoire *et al.*, 1998). In earlier reports, rosiglitazone had shown a potential for adipogenesis by activating aP2 mRNA in high-fat-fed rats (Chakrabarti *et al.*, 2004). The increased weight gaining potential of muraglitazar in our study was accompanied by a better glucose-lowering ability than rosiglitazone bringing to mind the adage, 'the paradox of  $\text{PPAR}\gamma$ : The greater the weight gain, the better the drug works'. The triglyceride-lowering efficacy of muraglitazar, however, was similar to that of rosiglitazone.

The robust weight gain and glucose lowering by muraglitazar in *db/db* mice demonstrated its activation of  $\text{PPAR}\gamma$ , a trend similar to the rosiglitazone group of animals, whereas the fenofibrate-treated group with only activation of  $\text{PPAR}\alpha$  did not show such changes. Fenofibrate caused a significant

increase in liver weight owing to its PPAR $\alpha$  activation; however, no such increase was observed with muraglitazar implying that somehow the PPAR $\alpha$  agonist activity of muraglitazar does not translate into increased liver weight. This is an obvious offset of direct PPAR $\alpha$  activation as has been observed with fenofibrate and tesaglitazar (Hegarty *et al.*, 2004; Oakes *et al.*, 2005; Harrity *et al.*, 2006). PPAR $\alpha$  activators have been found to significantly increase the expression of hepatic genes such as ACO in Zucker rats and *db/db* mice (Schoonjans *et al.*, 1996). Muraglitazar has been previously shown to increase ACO mRNA in the liver of *db/db* mice and has shown weight neutrality in DIO C57BL/6J and *db/db* mice (Hariharan *et al.*, 2002). However, in our study, in spite of a significant increase in ACO mRNA in muraglitazar-treated *db/db* livers, no weight neutrality or weight loss was observed showing that a high agonistic potency at PPAR $\alpha$  receptors is not enough to neutralize the weight gain caused by PPAR $\gamma$  activation. This is supported by observations with DRF 2655, which has a PPAR $\alpha$  activity 10-fold weaker than muraglitazar, is a potent anti-obesity and lipid-lowering and glucose-lowering molecule, perhaps, because of a 10-fold lesser activity at PPAR $\gamma$  receptors than rosiglitazone (Vikramadithyan *et al.*, 2003). Based on our observations with muraglitazar, we hypothesize that the increased body weight gain observed could be due to the utilization and siphoning of the fatty acids generated as a result of the PPAR $\alpha$ -mediated effect, towards PPAR $\gamma$  mediated lipogenesis. Pre-clinical data are now being corroborated with clinical studies. Muraglitazar has shown oedema and weight gain in patients undergoing phase II clinical trials and has presented problems of precipitating congestive cardiac failure in susceptible patients (DeFronzo *et al.*, 2005).

The weight gaining tendency of muraglitazar is postulated to be due to either enhanced adipogenesis or to water retention, shown as increased plasma or extracellular volume (Harrity *et al.*, 2006). Normally, thiazolidinediones are associated with dose-related fluid retention, believed to be a re-feeding oedema owing to enhanced insulin action significantly compounded by direct PPAR $\gamma$  activation leading to increased sodium reabsorption in the distal renal tubule (Chen *et al.*, 2005). Data from the muraglitazar trial show that muraglitazar also shares these presumed PPAR $\gamma$ -mediated effects (Belder, 2005). Patients with peripheral and pulmonary oedema associated with thiazolidinedione therapy fail to respond to thiazide and loop diuretics. Clinical improvement ensues only after discontinuation of thiazolidinedione therapy (Mudaliar *et al.*, 2003). A recent report, however, observes complete inhibition of the early weight gain in C57BL/6J mice, induced by pioglitazone, on pretreatment with amiloride, a K<sup>+</sup>-sparing diuretic which is a selective inhibitor of ENaC (Guan *et al.*, 2005). These results point towards a requirement for ENaC-mediated Na<sup>+</sup> absorption in the collecting duct in mediating pioglitazone-associated fluid accumulation (Guan *et al.*, 2005). Selective genetic deletion of *Pparg* gene from the renal collecting ducts using *Pparg*<sup>flox/flox</sup> mice prevented weight gain and thus confirmed the PPAR $\gamma$ -ENaC-dependent mechanism of thiazolidinedione-induced oedema (Zhang *et al.*, 2005).

In the present study, marked haemodilution was observed after muraglitazar treatment for 14 days. A significant

decrease in RBC count, haemoglobin and haematocrit was also observed, indicating an increase in plasma volume. As water reabsorption is secondary to increase in sodium retention, we measured plasma electrolytes wherein a significant increase was observed in plasma Na<sup>+</sup> and Cl<sup>+</sup> ions. Na<sup>+</sup>-reabsorption is enhanced by upregulation of expression of ENaC and Na<sup>+</sup>, K<sup>+</sup>-ATPase. We observed a significant increase in mRNA expression of ENaC $\gamma$  and a trend towards increase in Na<sup>+</sup>, K<sup>+</sup>-ATPase in whole kidneys of *db/db* mice treated with muraglitazar. A similar trend was also observed with rosiglitazone. This indicated that oedema was also seen with treatment with muraglitazar as is reported with rosiglitazone.

To summarize, it is evident that molecules can be ascribed as PPAR $\alpha$  or PPAR $\gamma$  agonists by receptor binding, co-activation studies and gene expression analyses (Oakes *et al.*, 2005). However, on translating these activities *in vivo*, it is difficult to ascribe metabolic effects to distinct activation of PPAR $\alpha$  vs PPAR $\gamma$ , as the patterns of effect resulting from stimulation of selective agonists show considerable overlap. It can be inferred from our observations that the PPAR $\alpha$  activity of muraglitazar does not translate effectively so as to counteract the PPAR $\gamma$ -induced weight gain in rodents. This study clearly indicates that muraglitazar causes both significant oedema and adipogenesis with a 14-day treatment in *db/db* mice, as it does in humans.

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## Conflicts of interest

The authors state no conflict of interest.

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