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ScienceDirect
European Journal of Pharmacology 584 (2008) 192–201
www.elsevier.com/locate/ejphar

A novel selective peroxisome proliferator-activator receptor-γ modulator—SPPARγM5 improves insulin sensitivity with diminished adverse cardiovascular effects

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Received 22 May 2007; received in revised form 28 November 2007; accepted 16 December 2007
Available online 14 February 2008

Abstract

The use of the thiazolidinedione insulin sensitizers rosiglitazone and pioglitazone for the treatment of type 2 diabetes mellitus in recent years has proven to be effective in helping patients resume normal glycemic control. However, their use is often associated with undesirable side effects including peripheral edema, congestive heart failure and weight gain. Here, we report the identification and characterization of a novel selective PPARγ modulator, SPPARγM5 ((2S)-2-(2-chloro-5-{[3-(4-chlorophenoxy)-2-methyl-6-(trifluoromethoxy)-1*H*-indol-1-yl]methyl} phenoxy) propionic acid), which has notable insulin sensitizing properties and a superior tolerability profile to that of rosiglitazone. SPPARγM5 is a potent ligand of human PPARγ with high selectivity versus PPARα or PPARδ in receptor competitive binding assays. In cell-based transcriptional activation assays, SPPARγM5 was a potent partial agonist of human PPARγ in comparison to the PPARγ full agonist rosiglitazone. Compared to rosiglitazone or the PPARγ full agonist COOH (2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid), SPPARγM5 induced an attenuated PPARγ-regulated gene expression profile in fully differentiated 3T3-L1 adipocytes and white adipose tissue of chronically treated *db/db* mice. SPPARγM5 treatment also reduced the insulin resistance index by homeostasis model assessment (HOMA), suggesting an improvement in insulin resistance in these *db/db* mice. Treatment of obese Zucker rats with either rosiglitazone or SPPARγM5 resulted in an improvement in selected parameters that serve as surrogate indicators of insulin resistance and hyperlipidemia. However, unlike rosiglitazone, SPPARγM5 did not cause significant fluid retention or cardiac hypertrophy in these rats. Thus, compounds such as SPPARγM5 may offer beneficial effects on glycemic control with significantly attenuated adverse effects.

Keywords: Selective PPARγ modulators; Insulin sensitizer; Adverse cardiovascular effects

1. Introduction

The anti-diabetic thiazolidinediones are high-affinity ligands and full agonists for peroxisome proliferator-activated receptorγ (PPARγ), a member of the nuclear receptor superfamily. Two isoforms of the peroxisome proliferator-activated receptor$_\gamma$ (PPARγ1 and PPARγ2) have been identified. PPARγ1 has been shown to be expressed in many tissues including muscle and liver at low levels, while PPARγ2 is expressed most abundantly in adipose tissue (Vidal-Puig et al., 1996). The thiazolidinediones are insulin sensitizers which enhance the effects of endogenous insulin as a result of the cumulative effects of altering the expression of genes involved in nutrient metabolism, adipocyte differentiation,

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doi:10.1016/j.ejphar.2007.12.036

insulin signal transduction and inflammation. Despite the beneficial effects of the thiazolidinediones rosiglitazone and pioglitazone in treating non-insulin dependent diabetes mellitus patients, use of these drugs has been limited because it has been associated with, peripheral edema, weight gain and anemia due to increased plasma volume (Kaplan et al., 2001; King and Levi, 2004; Lebovitz and Banerji, 2001; Niemeyer and Janney, 2002; Werner and Travaglini, 2001). Similar side effects, including hemodilution, fluid retention, weight gain and cardiac hypertrophy, also have been observed following chronic thiazolidinediones and non-thiazolidinedione PPARγ full agonist treatment in preclinical species (Actos®, 2003; Aleo et al., 2003; Avandia®, 2003; Carley et al., 2004; Pickavance et al., 1999; Rezulin®, 1997). The fluid retention that may accompany thiazolidinedione use is of concern because it may lead to or exacerbate congestive heart failure in non-insulin dependent diabetes mellitus patients, who are at increased risk for cardiovascular disease or may have preexisting cardiac disease (Nesto et al., 2004).

In light of the frequently observed side effects associated with thiazolidinediones, there is a need to develop PPARγ ligands that can effectively mitigate insulin resistance with improved tolerability. Recently, a novel class of PPARγ effectors classified as selective PPARγ modulators has been described (Berger et al., 2003; Burgermeister et al., 2006; Minoura et al., 2004; Misra et al., 2003; Mukherjee et al., 2000; Oberfield et al., 1999; Rocchi et al., 2001). In comparison with full agonists, these novel selective PPARγ modulators induce unique alterations in receptor conformation stability that can lead to distinctive gene expression profiles, reduced adipogenic effects, and potentially improved *in vivo* biological activities.

This manuscript documents a novel PPARγ ligand, SPPARγM5, which has notable insulin sensitizing properties and a unique cardiovascular tolerability profile, including diminished effects on fluid retention and cardiac hypertrophy in obese Zucker rats. Consistent with its categorization as a selective PPARγ modulator, SPPARγM5 binds the receptor with high affinity, acts as a partial agonist in a cell-based PPARγ transcriptional activity assay, and generates an attenuated gene signature relative to two structurally distinct PPARγ full agonists in both cultured 3T3-L1 adipocytes and the epididymal white adipose tissue of diabetic *db/db* mice.

2. Materials and methods

2.1. Materials

SPPARγM5 ((2S)-2-(2-chloro-5-{[3-(4-chlorophenoxy)-2-methyl-6-(trifluoromethoxy)-1*H*-indol-1-yl]methyl}phenoxy) propionic acid), rosiglitazone (5-(4-{2-[methyl(pyridin-2-yl) amino] ethoxy}benzyl)-1,2-thiazolidine-2,4-dione) and the non-thiazolidinedione PPARγ agonist, COOH (2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid (Carley et al., 2004; Laplante et al., 2006), nTZD3 (3-(4-(3-phenyl-7-propyl-benzisoxazole-6-yl)oxy)butyloxy-phenylactic acid) and nTZD4 (3-chloro-4(3-(3-trifluoromethyl-7-propyl-benzisoxazole-6-yl) oxypropyl)thiophenylactic acid) (Berger et al., 2003) were used in these studies.

2.2. Preparation of recombinant PPARs and binding assay

Recombinant PPARs were prepared and the receptor binding assays were performed as previously described (Berger et al., 2003). Briefly, the full length human cDNAs for each was subcloned into pGEX-KT expression vectors (Pharmacia, Piscataway, NJ, USA), followed by production of purified recombinant proteins in *E. coli*. Using the purified GST-hPPAR receptors, Scintillation Proximity Assay-based receptor competitive binding assays were performed in Packard OptiPlate-96 well polystyrene microplates (Packard BioScience, Meriden, CT, USA) using [$^3H_2$]nTZD3 for PPARγ and PPARα and [$^3H_2$] nTZD4 for PPARδ. K_is were calculated by the equation of Cheng and Prusoff (1973). The four-parameter logistic equation used is shown below:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10(\text{LogEC}_{50} - X)^{*}\text{Hillslope}}.$$

Bottom is the Y value at the bottom plateau, while Top is the Y value at the top plateau. LogEC_{50} is the X value when the response is halfway between Bottom and Top. HillSlope describes the steepness of the curve (GraphPad Prism version 4.00 for Windows, GraphPad Software, Inc., San Diego, CA, USA).

2.3. Cell-based transcriptional activity assays

COS-1 cells were cultured and transactivation assays were performed as previously described (Berger et al., 1999). Briefly, cells were transfected with a pcDNA3-hPPAR/GAL4 expression vector, pUAS(5X)-tk-luc reporter vector and pCMV-lacz as an internal control for transactivation efficiency using Lipofectamine (Invitrogen, Carlsburg, CA, USA). After a 48 h exposure to compounds, cell lysates were produced, and luciferase and β-galactosidase activity in cell extracts were determined. Inflection points (EC_{50}) of normalized luciferase activity were calculated by the four-parameter logistic equation described in Section 2.2.

2.4. Gene signature profiling in 3T3-L1 cells

3T3-L1 cells were grown to confluence in medium A (Dulbecco's modified Eagle's medium with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) at 37 °C in 5% CO_2 and induced to differentiate as previously described (Zhang et al., 1996). Briefly, differentiation was induced by incubating the cells with medium A supplemented with methylisobutylxanthine (IBMX), dexamethasone, and insulin for 2 days (days 1 to 2), followed by another 2-day incubation with medium A supplemented with insulin (days 3 to 4). The cells were further incubated in medium A for an additional 4 days to complete the adipocyte conversion (days 5 to 8). At day 8 following the initiation of differentiation, cells were incubated in medium A +/− compounds for 24 h at saturating concentrations: vehicle, rosiglitazone (10,000 nM), COOH (10,000 nM), or SPPARγM5 (3000 nM). Following treatment, total RNA was prepared from the adipocytes using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy kits (Qiagen, Valencia, CA,

USA) according to the manufacturers' instructions and RNA concentration was estimated from absorbance at 260 nm. Microarray processing was performed as previously described (Hughes et al., 2001). Briefly, labeled cRNA was hybridized for 48 h onto Agilent 60mer 2-color spotted microarrays. Individual treatment samples (including individual vehicle treatment samples) were hybridized against a vehicle treatment pool. LogRatio and *P* values were generated by averaging replicates (2 replicates per treatment) and the AHEM algorithm using the Rosetta Resolver v4.0 system (Rosetta Inpharmatics LLC, a wholly owned subsidiary of Merck & Co., Inc., Seattle, WA, USA). LogRatio values represent the difference in regulation of the compound treated samples versus the vehicle-treated sample where a positive value signifies up-regulation following compound treatment and vice versa. A gene signature score (termed activity score) indicative of overall adipocyte gene regulation was generated for each compound using a subset of 3336 reporter genes and the chi-square fitting approach (Tan Y, Muise E, Thompson J, and Berger J, manuscript in preparation). This subset of reporter genes is composed of the intersection of the rosiglitazone and COOH signature reporter genes with $P<0.01$. The *P* value (*P*) was computed by a more conservative error model than the standard error weighted average approach in order to obtain more reliable gene signatures with a lower false positive rate. This *P* value is computed from the complementary error function of the absolute value of LogRatio/LogRatioError, where LogRatioError takes the maximum of the within group variance, derived from all treatment groups that share a common vehicle control pool, and the variance derived from the standard error weighted average. A clustergram was generated for the 3336 reporter genes using the k-means cluster algorithm in the Rosetta Resolver v4.0 system.

2.5. Gene signature profiling in db/db mice

Ten week old male *db/db* mice (Charles River Laboratories, Wilmington, MA, USA) were housed 8 animals per cage and were provided food (Purina LabDiet 5001, St. Louis, MO, USA) and water *ad libitum*. Animals were housed in a temperature-, humidity-, and light-controlled room (21–23 °C, 47–65%, 12–12 h light–dark cycle). For gene expression profiling, mice ($n=3$ per treatment group) were dosed once daily in the morning by oral gavage with vehicle (0.25% methylcellulose), rosiglitazone (30 mg/kg), COOH (30 mg/kg), or SPPARγM5 (50 mg/kg), for 8 days. Fasting tail nick blood samples were collected one day before the start of dosing and one day after 7th dose and plasma concentrations of glucose (Sigma glucose Trinder assay kit, St. Louis, MO, USA) and insulin (rodent insulin RIA from American Laboratory Products Company, Windham, NH, USA) were determined. Fasting plasma concentrations of glucose and insulin from vehicle, rosiglitazone, and SPPARγM5 groups were used to calculate an insulin resistance index by a homeostasis model assessment (HOMA) as insulin ($\mu U\ ml^{-1}$) × glucose (mM)/22.5 (Matthews et al., 1985; Pickavance et al., 1999). Animals were euthanized by CO_2 asphyxiation 6 h after dosing on the 8th day. The epididymal white adipose tissues were excised and flash-frozen in liquid nitrogen. Total RNA was isolated from frozen tissues after homogenizing in UltraSpec RNA reagent (Biotecx Laboratories, Inc. Houston, TX) with a PT10/35 Polytron (Kinematica AG, Clifton, NJ, USA) and processed as described above. Microarray processing was performed and LogRatio values, activity scores, and a clustergram were generated as described above using a subset of 1733 reporter genes which represents the intersection of rosiglitazone and COOH signature reporter genes with $P<0.01$, minus those regulated in the vehicle treatment (52 reporter genes).

2.6. Effect of SPPARγM5 on insulin sensitization, fluid retention and cardiac hypertrophy in obese Zucker rats

2.6.1. Animals and treatments

Nine to ten week old male Zucker *fa/fa* rats (Charles River Laboratories, Wilmington, MA, USA) were housed 2 animals per cage and were provided food (Harlan Teklad Diet #7012, Madison, WI, USA) and water *ad libitum*. Animals were housed in a temperature-, humidity-, and light-controlled room (21–23 °C, 47–65%, 12–12 h light–dark cycle, $n=8$/group). Following 7 days of acclimation, rats were treated once daily for 7 days by oral gavage with vehicle (0.25% methylcellulose, 10 ml/kg), rosiglitazone (1, 10, and 100 mg/kg), or SPPARγM5 (2, 20, 50, and 100 mg/kg).

2.6.2. Evaluation of insulin sensitization

Tail nick blood samples were collected one day before the start of dosing and one day after 7th dose and plasma glucose (Sigma glucose Trinder assay kit, St. Louis, MO, USA), insulin (rat insulin RIA from American Laboratory Products Company, Windham, NH, USA), free fatty acids (FFA) and triglyceride (both from Roche Diagnostics, Basel, Switzerland) concentrations were determined.

2.6.3. Bioelectrical impedance analysis for determination of extracellular fluid volume

Twenty-four hours following the final dose, rats were anesthetized with ketamine (85 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). Animals were positioned on a non-conductive surface in dorso-lateral recumbency and *extracellular fluid* volume was determined by bioelectrical impedance analysis, following procedures described by the manufacturer (Hydra ECF/ICF Impedance Analyzer Model 4, Xitron, San Diego, CA, USA) and by B. H. Cornish et al. (1996) and Rutter et al. (1998). A tetrapolar impedance monitor was used to measure impedance, and hence the total body water, over a frequency range of 5 kHz to 1 MHz. Source electrodes (1 cm × 26G stainless steel needles) were inserted 5 mm subcutaneously. Detector electrodes were inserted along the midline at the anterior point of the sternum and the anterior point of the penis. The distance between electrodes was measured and included for data modeling.

2.6.4. Plasma volume measurement

After determination of *extracellular fluid* volume, plasma volume was measured in anesthetized animals using a dye dilution technique following methods described by Belcher and

Harriss (1957) with minor modifications. Evans blue dye solution (25 mg/ml in physiological saline) was filtered through a 0.22 μm filter prior to injection into a femoral vein. Twenty minutes after injection, a heparinized blood sample (2 ml) was withdrawn from the descending aorta. Plasma was separated by centrifugation of the blood at 1100 ×*g* for 15 min.; samples were kept at −80 °C until assayed. Absorbance of the thawed plasma was read at 620 nm, and plasma Evans blue dye concentrations were calculated according to a standard curve generated by a serial dilution of the 25 mg/ml Evans blue dye-saline solution. Plasma volume was calculated by using the dilution factors of Evans blue as shown below.

$$\text{Plasma volume} = \frac{[\text{dye}] \text{ injected} \times \text{volume of dye injected}}{[\text{dye}] \text{ in plasma}}.$$

2.6.5. Hematocrit and heart weight measurements

EDTA blood samples collected from the descending aorta were analyzed for % HCT using a Bayer ADVIA-120 hematology analyzer (Bayer Diagnostics, Tarrytown, NY, USA). Animals were then euthanized by pneumothorax and exsanguination and the heart was excised, blotted, and weighed.

2.6.6. Plasma drug level measurements

All plasma samples collected from the descending aorta were precipitated with acetonitrile and subsequently subject to LC/MS/MS for rosiglitazone and SPPARγM5 measurements.

2.7. Animal care and handling

All animal experiments and euthanasia protocols were conducted in strict accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*. Animal experiment protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories. The laboratory animal facilities of Merck Research Laboratories are certified by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.8. Statistical analysis

Data were expressed in one of the two ways as noted in legends or footnotes: mean±S.E.M. or mean±S.D. Dunnett's *t* test was performed to determine if there were significant differences between the vehicle group and each incremental dosage group of a compound for the following parameters: plasma glucose, triglyceride, free fatty acids and insulin concentration, plasma volume, extracellular fluid volume and hematocrit. A P value <0.05 was considered statistically significant.

3. Results

3.1. In vitro ligand binding and transactivation activities

SPPARγM5 is a potent ligand of human PPARγ demonstrating a Ki=0.2 nM in a competitive binding assay; rosiglitazone bound the receptor with a Ki=131 nM under the same assay conditions (Fig. 1A). Neither compound binds significantly to human PPARα or PPARδ at concentrations up to 4.5 μM (data not shown). In cell-based transcriptional activation assays using a non-adipocyte cell line and a chimeric receptor, SPPARγM5 was a potent (EC_{50}=0.7 nM) partial agonist of human PPARγ, attaining a maximal activation of 11% in comparison to the PPARγ full agonist rosiglitazone which activated the receptor with an EC_{50}=33 nM under the same assay conditions (Fig. 1B). Neither of the compounds activated human PPARα or PPARδ at concentrations up to 3 μM in accordance with our binding data (data not shown). COOH is, as previously reported (Carley et al., 2004), a non- thiazolidinedione selective full agonist that binds human PPARγ with a Ki=46 nM and activates the receptor with an EC_{50}=180 nM.

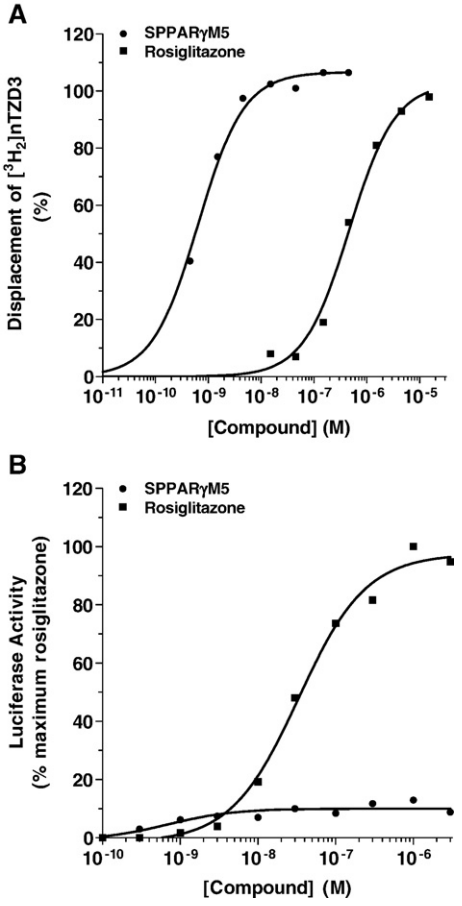

Fig. 1. SPPARγM5 is a potent ligand and partial agonist of hPPARγ. (A) Competition binding curves generated by incubation of [$^3H_2$]nTZD3 with GST-hPPARγ. The displacement of radioligand after incubation in the presence of the indicated concentration of SPPARγM5 or rosiglitazone for ~16 h is plotted. Similar results were obtained in at least two independent experiments performed in duplicate. (B) Activation of hPPARγ by SPPARγM5 or rosiglitazone. COS-1 cells were transiently co-transfected with pSG5-hPPARγ/GAL4 and both pUAS (5X)-tk-luciferase and pCMV-*lacz* then incubated with the indicated concentrations of SPPARγM5 or rosiglitazone for 48 h. Cell lysates were produced, and luciferase and β-galactosidase activity in cell extracts was determined. Normalized luciferase activity is plotted. Similar results were obtained in at least two independent experiments performed in duplicate.

Table 1
PPARγ gene expression activity score computed from the expression of the 3336 or 1733 reporter probes, for 3T3-L1 adipocytes or *db/db* adipose, respectively, that represent the PPARγ full agonist signature, with rosiglitazone set to 1 as reference

Source of RNA	PPARγ activity score
3T3-L1 Adipocytes, 3336 reporter genes	
Rosiglitazone (10,000 nM)	1.00±0.02 [a]
COOH (10,000 nM)	0.97±0.03
SPPARγM5 (3000 nM)	0.43±0.01
db/db Mouse epididymal white adipose tissue, 1733 reporter genes	
Rosiglitazone (30 mg/kg)	1.00±0.09
COOH (30 mg/kg)	1.06±0.13
SPPARγM5 (50 mg/kg)	0.41±0.04

This score provides a single relative measure of the magnitude of their expression and therefore of PPARγ activation relative to rosiglitazone. Each 3T3-L1 data point represents an error weighted average of 2 biological replicates while each *db/db* data point was generated with 3 biological replicates.
[a] All values are expressed as mean±S.D.

3.2. Gene signature profiling in 3T3-L1 adipocytes

Gene expression studies in fully differentiated 3T3-L1 adipocytes treated with saturating concentrations of SPPARγM5, rosiglitazone, or COOH for 24 h were performed using Agilent 25K spotted microarrays. Analysis of data from these microarrays identified a set of 3336 reporter genes that compose the intersection of rosiglitazone and COOH signature reporter genes with $P<0.01$. Due to the significant structural differences between these two PPARγ ligands, we believe that this intersection represents a "true" PPARγ target gene signature (both directly and indirectly regulated genes) in the adipocytes. An activity score was then computed by chi-square fitting of the expression of these 3336 reporter genes in order to provide a single relative measure of the magnitude of their expression and therefore of PPARγ activation. While the activity scores for the PPARγ full agonists were virtually indistinguishable (1.00±0.02 and 0.97±0.03 for rosiglitazone and COOH, respectively), the activity score for SPPARγM5 was 0.43±0.01 due to its attenuated gene expression profile (Table 1). This attenuation can be visualized by a k-means cluster comparing the LogRatio values of these 3336 reporter genes between rosiglitazone, COOH, and SPPARγM5 (Fig. 2A).

3.3. SPPARγM5 has an attenuated white adipose tissue gene expression profile but maintains insulin sensitizing activity in db/db mice

Diabetic *db/db* mice were treated with SPPARγM5, rosiglitazone or COOH for 8 days. Gene expression studies on epididymal white adipose tissue removed from the mice at the end of this treatment regimen were performed using Agilent 25K spotted microarrays (with $n=3$ biological replicates per treatment). Analysis of microarray data identified a set of 1733 reporter genes that compose the intersection of rosiglitazone and COOH signature reporter genes with $P<0.01$. Activity scores were computed and, similar to 3T3-L1 adipocytes, they were virtually indistinguishable for the PPARγ full agonists (1.00 and 1.06 for rosiglitazone and COOH, respectively) while that for SPPARγM5 was 0.41 due to its attenuated gene expression profile (Table 1). This attenuation can be visualized by a k-means cluster comparing the LogRatio values of these 1733 reporter genes between rosiglitazone, COOH, and SPPARγM5 (Fig. 2B).

In addition to determining the effects of the PPARγ ligands on adipose tissue gene expression, their anti-diabetic activities were also examined. Homeostasis model assessments of fasting plasma glucose and insulin concentrations both before and after 7 days of treatment of the *db/db* mice showed that both SPPARγM5 and

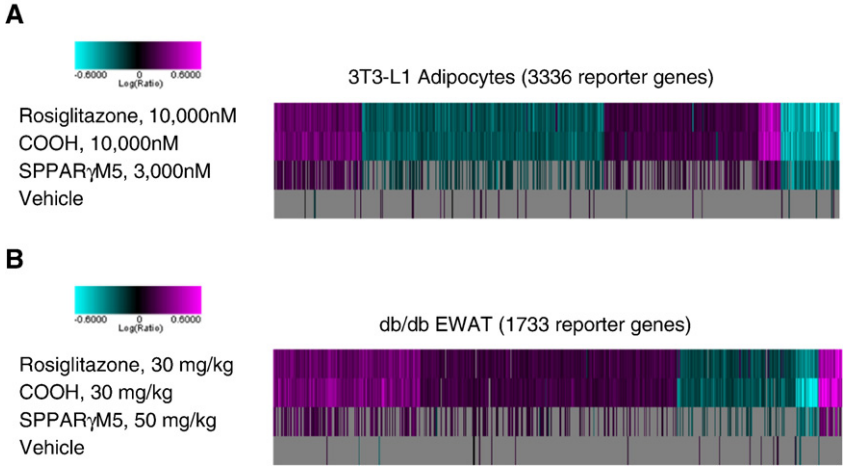

Fig. 2. SPPARγM5 treatment shows attenuated gene expression in both (A) 3T3-L1 adipocytes and (B) epididymal white adipose tissue from *db/db* mice compared to rosiglitazone and COOH. (A) Fully differentiated 3T3-L1 adipocytes were treated with saturating concentrations of compounds as indicated for 24 h (with $n=2$ biological replicates per treatment) and purified mRNA was hybridized to Agilent 25K spotted microarrays as described in Materials and methods. The set of 3336 reporter genes that compose the intersection of rosiglitazone and COOH signature reporter genes with $P<0.01$ are plotted in the clustergram. Displayed are the LogRatio values in the cyan-magenta color range (+/−0.6 LogRatio=+/−4 fold regulation) for each reporter data point. Data points with $P>0.05$ are shaded gray. (B) *db/db* mice were treated with SPPARγM5, rosiglitazone, or COOH at the indicated efficacy doses by oral gavage once per day for 8 days (with $n=3$ biological replicates per treatment). The mRNA from the epididymal white adipose tissues was hybridized to microarrays as described above. The set of 1733 reporter genes that compose the intersection of rosiglitazone and COOH signature reporter genes with $P<0.01$ are plotted in the clustergram. Plot details are as in (A).

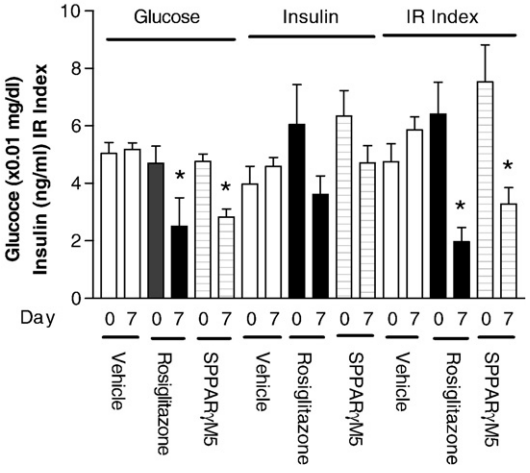

Fig. 3. SPPARγM5 was efficacious in lowering plasma glucose concentration and insulin resistance (IR) Index. Animals ($n=3$ per group) were dosed once daily in the morning by oral gavage with vehicle (0.25% methylcellulose), rosiglitazone (30 mg/kg body weight) or SPPARγM5 (50 mg/kg body weight), for 7 days. Fasting tail nick blood samples were collected one day before the start of dosing and one day after 7th dose for plasma glucose and insulin assays and for the calculation of insulin resistance (IR) index by HOMA. * denotes significant difference at $P<0.05$ between either compound group versus vehicle group on changes in plasma glucose concentration and IR index. All data are expressed as mean ± S.E.M.

rosiglitazone were effective in lowering their fasting plasma glucose concentration and insulin resistance index in comparison with vehicle treatment (Fig. 3). The changes in plasma glucose concentrations over 7 days were +9, −63 ($P<0.05$), and −39% ($P<0.05$) for vehicle, rosiglitazone and SPPARγM5, respectively. The changes in plasma insulin concentrations were −12, −46, and −37% without reaching statistically significant difference. Insulin resistance indices determined by HOMA were −5, −80 ($P<0.05$), and −61% ($P<0.05$) for vehicle, rosiglitazone, and SPPARγM5, respectively.

3.4. Treatment with SPPARγM5 enhances insulin sensitivity in obese Zucker rats

Treatment of obese Zucker rats with either rosiglitazone or SPPARγM5 resulted in an improvement in selected parameters indicative of insulin resistance and hyperlipidemia (Table 2). Plasma insulin levels were significantly lowered by rosiglitazone in a dose-dependent manner while SPPARγM5 significantly lowered insulin by 56% at the lowest dose of 2 mg/kg with further reductions at higher doses. Plasma triglyceride levels were significantly lowered by rosiglitazone by 56% at the 1 mg/kg dose with the greatest correction at 10 mg/kg (−86%) while SPPARγM5 elicited a dose-related lowering of triglyceride with a 44% reduction at the lowest dose of 2 mg/kg and further reductions at higher doses. Plasma free fatty acids levels were significantly lowered to a similar degree in a dose-dependent manner by both compounds.

3.5. Fluid retention, hemodilution and cardiac hypertrophy are attenuated in obese Zucker rats treated with SPPARγM5

Although SPPARγM5 displayed similar efficacy to rosiglitazone in promoting insulin sensitivity, it did not cause significant fluid retention or cardiac hypertrophy (Table 2). Rosiglitazone (10 and 100 mg/kg) caused significant increases in plasma volume (30 and 56%, respectively), while SPPARγM5 treatment resulted in non-significant 13–17% increases in plasma volume at all dose levels. Extracellular fluid volume was increased by rosiglitazone treatment in a dose-dependent manner with a significant increase (23%) at 100 mg/kg; no significant increase in extracellular fluid volume was observed with SPPARγM5. Treatment with rosiglitazone caused a dose-related decrease (3–17%) in hematocrit, while SPPARγM5 had less of an effect in reducing hematocrit, with a significant decrease (11%) at 50 mg/kg only (Table 2). Heart weight was significantly increased by rosiglitazone treatment in a dose-dependent manner, while no significant cardiomegaly was noted with SPPARγM5. Both the SPPARγM5- and rosiglitazone-treated cohorts had normal weight gain, comparable to vehicle-treated animals.

Rosiglitazone treatment at 1, 10, 100 mg/kg body weight for 7 days resulted in systemic plasma exposures of 28, 325, and 3,079 μM h, respectively, while SPPARγM5 treatment at 2, 20, 50, and 100 mg/kg resulted in plasma exposures of 8, 104, 261, and 686 μM h, respectively (Fig. 4A and B). Such treatments

Table 2
Effects of SPPARγM5 and rosiglitazone on changes in plasma volume, heart weight, hematocrit, and extracellular fluid volume as well as plasma insulin, triglyceride, and free fatty acids in male Zucker *fa/fa* rats

Parameter	PV/BW%	ECF	HW/BW	HCT	Insulin	TG	FFA
Vehicle	2.52±0.13	71.9±3.1	2.15±0.04	43.3±0.7	31.8±4.2	467±61	437±57
Rosiglitazone							
1 mg/kg[b]	2.69±0.10	76.1±1.9	2.15±0.03	41.9±0.6	18.3±4.2[a]	204±36[a]	254±33[a]
10 mg/kg	3.16±0.14[a]	79.4±2.4	2.32±0.03	39.3±0.9[a]	10.7±1.8[a]	66±6[a]	90±13[a]
100 mg/kg	3.89±0.49[a]	88.6±3.3[a]	2.55±0.09[a]	35.9±0.9[a]	4.4±0.4[a]	86±8[a]	42±6[a]
SPPARγM5							
2 mg/kg	2.85±0.10	77.1±1.2	2.22±0.05	41.6±0.9	13.9±2.5[a]	260±34[a]	218±30[a]
20 mg/kg	2.93±0.17	78.3±2.0	2.21±0.03	40.0±1.0	11.0±2.2[a]	183±14[a]	155±29[a]
50 mg/kg	2.91±0.20	78.8±3.8	2.17±0.06	38.7±1.1[a]	12.0±1.6[a]	212±9[a]	133±20[a]
100 mg/kg	3.05±0.51	76.3±2.0	2.21±0.04	41.6±1.4	12.6±1.2[a]	123±20[a]	109±21[a]

PV/BW% = plasma volume (ml/100 g body weight), ECF = extracellular fluid volume (ml), HW/BW = heart weight (g/kg body weight), HCT = hematocrit (%), TG = triglyceride (mg/dl), and FFA = free fatty acids (mM). [a] denotes significant difference between vehicle and individual treatment groups for each parameter at $P<0.05$; all values are expressed as mean ± S.E.M. from 8 animals per dosage group. [b] All treatment solutions were administered once daily through oral gavage for 7 days.

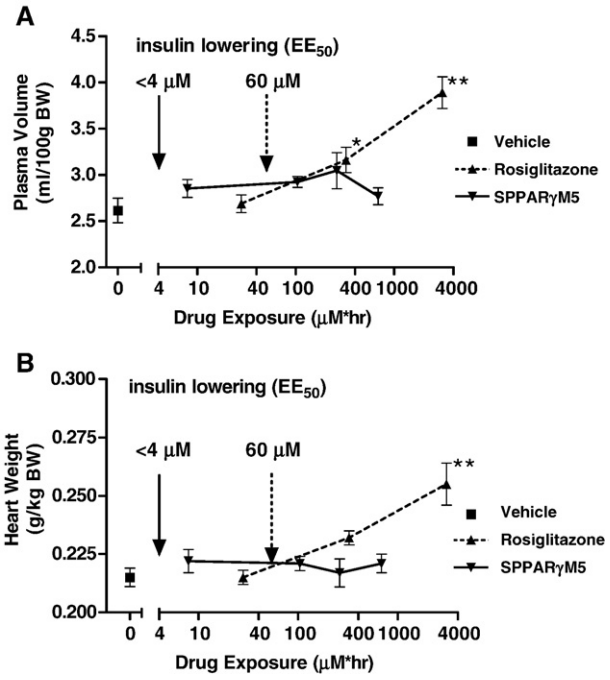

Fig. 4. Rosiglitazone treatment but not SPPARγM5 induced adverse cardiovascular effects in Zucker *fa/fa* rats ($n=8$ per group). Animals ($n=8$ per group) were treated daily for 7 days with vehicle, rosiglitazone (1, 10, and 100 mg/kg) or SPPARγM5 (2, 20, 50, and 100 mg/kg). Circulating drug and insulin levels, plasma volume (A) and heart weight (B) were then determined as described in the Materials and methods section. The drug exposure levels that caused a 50% reduction in plasma insulin lowering (EE_{50}) for rosiglitazone and SPPARγM5 were 60 μM h and <4 μM h, respectively. * and ** denote significant difference from vehicle at $P<0.05$ and $P<0.01$, respectively. All data are expressed as mean±S.E.M.

resulted in reductions in insulin levels, indicative of increased insulin sensitivity. The efficacy exposure at which a 50% decrease in insulin levels was achieved (EE_{50} was 60 μM*h and <4 μM*h for rosiglitazone and SPPARγM5, respectively. Rosiglitazone treatment produced clear dosage-responsive increases in plasma volume and heart weight. In contrast, SPPARγM5 produced no dosage-responsive increases in plasma volume or heart weight, but provided a similar degree of insulin lowering efficacy to rosiglitazone. Thus, at the dose range evaluated, SPPARγM5 has an effective insulin lowering activity and a superior tolerability profile to rosiglitazone in these obese, hyperinsulinemic Zucker *fa/fa* rats.

4. Discussion

The use of the thiazolidinedione insulin sensitizers rosiglitazone and pioglitazone for the treatment of non-insulin dependent diabetes mellitus in recent years has proven to be effective in improving insulin resistance and helping patients resume normal glycemic control. However, their use often is associated with undesirable side effects such as plasma volume expansion, edema, congestive heart failure and weight gain which has limited their clinical utility. We now describe a novel non-thiazolidinedione selective PPARγ modulator, SPPARγM5, which demonstrated an effective insulin sensitizing activity with attenuated adverse cardiovascular side effects in Zucker fatty rat.

Like a number of previously described thiazolidinedione and non-thiazolidinedione PPARγ full agonists as well as the novel selective PPARγ modulators GW0072 (Oberfield et al., 1999), LG100641 (Mukherjee et al., 2000), nTZDpa (Berger et al., 2003), FK614 (Minoura et al., 2004) and PA-082 (Burgermeister et al., 2006), SPPARγM5 is a potent specific ligand of PPARγ. Additionally, as was the case with these other selective modulators, SPPARγM5 was shown to be a PPARγ partial agonist in cell-based transcriptional activity assays. When small molecular activators bind to PPARγ, the conformation of the receptor is altered such that transcriptional co-repressors are released and a cohort of co-activators is recruited to the PPARγ-RXR heterodimer complex (Olefsky and Saltiel, 2000). A subset of these co-activators possess histone acetylase activity capable of causing chromatin remodeling while others appear to serve to link the nuclear receptors to the cellular transcriptional machinery. Together, these cofactors facilitate the activation of gene transcription. Subtle differences in full agonist versus selective PPARγ modulator-induced changes in the conformation of PPARγ can lead to differences in cofactor recruitment and transcriptional regulation (Oberfield et al., 1999; Mukherjee et al., 2000; Rocchi et al., 2001; Berger et al., 2003; Burgermeister et al., 2006). Consonant with those observations, we report here that in comparison with two PPARγ full agonists, SPPARγM5 has an attenuated PPARγ-regulated gene signature in both fully differentiated 3T3-L1 adipocytes and the epididymal white adipose tissue of *db/db* mice. The potent binding affinity, reduced transcriptional activity, and the attenuated gene expression profile of SPPARγM5 lead us to conclude that it is a novel selective PPARγ modulator.

Despite its diminished gene signature, SPPARγM5 was shown, like rosiglitazone, to diminish fasting hyperglycemia and improve insulin resistance in *db/db* mice as determined by the HOMA developed by Matthews et al. (1985). In that publication, the HOMA estimate of insulin resistance was shown to correlate well with estimates obtained by use of the euglycemic clamp ($Rs=0.88$, $P<0.0001$) and the fasting insulin concentration ($Rs=0.81$, $P<0.001$). In additional studies performed in the hyperinsulinemic, hyperlipidemic Zucker *fa/fa* rats, SPPARγM5 demonstrated insulin sensitizing and hypolipidemic activities similar to those of rosiglitazone. However, this selective modulator did not promote plasma volume expansion or cause cardiac hypertrophy as the full agonist did. Thus, these results support the proposition that separation of PPARγ-mediated anti-diabetic activities from well established adverse effects can be obtained by appropriate ligand modulation of the receptor. Since the major pharmacological activities of PPARγ ligands are thought to be mediated by alterations in gene expression, the attenuated gene signature of SPPARγM5 may provide the basis, at least in part, for its diminished adverse effects.

SPPARγM5 activates many similar sets of genes relative to PPAR full agonists. These gene expression changes in turn regulate anti-diabetic actions in multiple insulin-sensitive tissues through their effects in adipose tissue as well as skeletal muscle and liver, the two primary tissues that are responsible for insulin-mediated glucose disposal and metabolism (Berger et al., 2005).

In addition to its anti-diabetic effects, both SPPARγM5 and rosiglitazone reduced plasma triglyceride and free fatty acid levels in Zucker *fa/fa* rats (Table 2). The hypolipidemic effects of SPPARγM5 and rosiglitazone may have been the result of enhanced adipocyte insulin signaling, lipid uptake and anabolic lipid metabolism, attenuated lipolysis and free fatty acid release due to altered expression of related genes.

Fluid retention, manifest as weight gain and peripheral and pulmonary edema, has emerged as the most common undesirable side effect associated with the use of rosiglitazone and pioglitazone in non-insulin-dependent diabetes mellitus patients. Importantly, such edema can lead to or exacerbate congestive heart failure. Thus, the United States Food and Drug Administration has recently required a "black box" warning for both thiazolidinediones stating that their use can lead to congestive heart failure. The mechanism of this fluid retention is at present unknown and multiple factors/pathways could be involved. In obese Zucker rats, we observed a significant dose-dependent increase in plasma volume and extracellular fluid volume and a dose-dependent decrease in hematocrit following 7-day rosiglitazone treatment, whereas no significant changes were noted with SPPARγM5 treatment. Rosiglitazone-induced plasma volume increase and hematocrit decrease could be the results of its direct effects on Na^+ and water retention at the inner medullary collecting ducts (Guan et al., 1997; Yang et al., 1999; Chen et al., 2005; Zhang et al., 2005). Targeted proteomics profiling in kidney homogenates demonstrated that rosiglitazone-induced Na^+ and water retention is associated with a fall in blood pressure and a rise in renal abundance of αNa-K-ATPase, ENaC, the aquaporins 2 and 3 (likely due to direct PPARγ effects at the inner medullary collecting ducts), and NKCC2, NaPi-2, and NHE3 (likely through indirect PPARγ influence on renal hemodynamics) in conscious rats (Song et al., 2004). A recent study showed that rosiglitazone was ineffective in increasing plasma volume or decreasing hematocrit in collecting duct-specific PPARγ gene knock out mice, suggesting that PPARγ is a positive regulator of sodium transport process in the distal nephron which likely underlies thiazolidinedione-induced fluid retention (Zhang et al., 2005). Furthermore, Guan et al. (2005) demonstrated that deletion of the PPARγ gene prevented the stimulation of amiloride-sensitive Na^+ flux by pioglitazone, thereby supporting the proposition that PPARγ directly stimulates ENaCγ subunit transcription and amiloride-sensitive Na^+ absorption. It is yet to be determined if SPPARγM5 treatment will demonstrate an attenuated effect upon pertinent factors/pathways involved in the renal Na^+ and water retention processes.

In this study, we demonstrate that treatment with rosiglitazone, but not SPPARγM5, led to the development of cardiac hypertrophy in obese Zucker rats. The cardiac hypertrophy response to rosiglitazone in rats was not unexpected, as the regulatory filings for rosiglitzone, pioglitazone, and troglitazone, as well as a number of literature reports, indicate that treatment with thiazolidinedione and non-thiazolidinedione PPARγ full agonists cause this effect in pre-clinical species (Carley et al., 2004; Rezulin® package insert, 1997; Actos® package insert, 2003; Avandia® package insert, 2003). There is no data at the present time indicating that thiazolidinediones cause cardiac hypertrophy in humans. Nevertheless, it is still encouraging to observe that specific PPARγ modulator insulin sensitizers do not induce this malady in rodents. Given the increasing use and development of PPARγ effectors as therapeutic agents for metabolic disease, more information about the cardiac effects of PPARγ activation is needed.

While the mechanism(s) mediating PPARγ agonist-induced cardiac hypertrophy is not yet known, it is likely to be an indirect effect resulting from increased pre-load due to plasma volume expansion. This hypothesis is consistent with published data showing that the expression and activity of PPARγ is low or absent in rodent heart and isolated cardiac myocytes (Escher et al., 2001; Gilde et al., 2003) as well as that demonstrating rosiglitazone can induce cardiac hypertrophy in cardiomyocyte-specific PPARγ null mice (Duan et al., 2005). Therefore, we speculate that in contrast to PPARγ full agonists SPPARγM5 does not promote cardiac hypertrophy because it does not induce plasma volume expansion. In a 14-day study in normal male Sprague Dawley rats, both SPPARγM5 (100 mg/kg) and rosiglitazone (150 mg/kg) treatments were well-tolerated and neither caused any abnormalities in liver function enzymes (data not shown). Nevertheless, only rosiglitazone treatment caused cardiac hypertrophy in these Sprague Dawley rats.

In conclusion, we have demonstrated that SPPARγM5 is a novel selective PPARγ modulator that has a notable insulin sensitizing activity with attenuated adverse cardiovascular side effects in a pre-clinical model of insulin resistance. Clinical development of such ligands should prove useful in furthering our understanding of the mechanisms mediating the differential effects of full agonists and selective PPARγ modulators on gene expression, plasma volume expansion, hematocrit, extracellular fluid volume and cardiac hypertrophy observed in pre-clinical species. Furthermore, by successfully developing selective PPARγ modulators that provide anti-hyperglycemic efficacy without the associated undesirable cardiovascular side effects such as edema, a major step forward will be taken in the treatment of non-insulin dependent diabetes mellitus patients.

Acknowledgements

The authors wish to thank Ms. Neelam Sharma and Mr. John Menke for performing the SPA assays, Ms. Bahanu Habulihaz and Dr. Lawrence Colwell for the plasma drug level measurements, Ms. Marie Thompson for the isolation of RNA for microarray processing, the Rosetta Gene Expression Laboratory for the microarray processing, Mr. Charlie Berman, Mr. Eric Rickes, and Mr. Roger Meurer for the animal studies, and Drs. Gerry Hickey, Mike Forrest, Euan Macintyre, Bei Zhang, David Moller, James Heck, and Alan Sachs for their guidance and support for the studies.

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