LT175 Is a Novel PPARα/γ Ligand with Potent Insulin-sensitizing Effects and Reduced Adipogenic Properties*  

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**The abbreviations used are: T2D, type 2 diabetes mellitus; PPAR, peroxisome proliferator-activated receptor; hPPAR, human PPAR; mPPAR, murine PPAR; TZD, thiazolidinedione; PPRE, peroxisome proliferator-activated receptor response element; Luc, luciferase; WAT, white adipose tissue; qPCR, quantitative PCR; CREB, cAMP response element-binding protein; AZ, AZ12063233; HFD, high-fat diet.

Background: PPARs are attractive targets of antidiabetic agents. However, PPAR ligands show side effects that hinder their clinical use.  

Results: LT175 improves insulin sensitivity and reduces body weight via selective gene activation in adipose tissue.  

Conclusion: LT175 shows an improved pharmacological profile linked to characteristic binding and differential coregulator recruitment.  

Significance: LT175 may be a scaffold molecule to design a safer generation of PPAR ligands.

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors regulating lipid and glucose metabolism. Ongoing drug discovery programs aim to develop dual PPARα/γ agonists devoid of the side effects of the marketed antidiabetic agents thiazolidinediones and the dual agonists glitazars. Recently, we described a new dual PPARα/γ ligand, LT175, with a partial agonist profile against PPARγ and interacting with a newly identified region of the PPARγ-ligand binding domain (1). Here we show that LT175 differentially activated PPARγ target genes involved in fatty acid esterification and storage in 3T3-L1-derived adipocytes. This resulted in a less severe lipid accumulation compared with that triggered by rosiglitazone, suggesting that LT175 may have a lower adipogenic activity. Consistent with this hypothesis, in vivo administration of LT175 to mice fed a high-fat diet decreased body weight, adipocyte size, and white adipose tissue mass, as assessed by magnetic resonance imaging. Furthermore, LT175 significantly reduced plasma glucose, insulin, non-esterified fatty acids, triglycerides, and cholesterol and increased circulating adiponectin and fibroblast growth factor 21 levels. Oral glucose and insulin tolerance tests showed that the compound improves glucose homeostasis and insulin sensitivity. Moreover, we demonstrate that the peculiar interaction of LT175 with PPARγ affected the recruitment of the coregulators cyclic-AMP response element-binding protein-binding protein and nuclear corepressor 1 (NCoR1), fundamentals for the PPARγ-mediated adipogenic program. In conclusion, our results describe a new PPAR ligand, modulating lipid and glucose metabolism with reduced adipogenic activity, that may be used as a model for a series of novel molecules with an improved pharmacological profile for the treatment of dyslipidemia and type 2 diabetes.

Type 2 diabetes mellitus (T2D)5 strikes nearly 300 million people worldwide (2) and represents a major cause of morbidity and mortality. This chronic metabolic disorder results in a combination of dyslipidemia, glucose intolerance, and inflammation. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that play a pivotal role in the regulation of glucose homeostasis and lipid metabolism, and for this reason they have been considered suitable targets for the treatment of metabolic disorders. In the last decade, thiazolidinediones (TZDs), agonists of the PPARγ subtype, have been widely prescribed for the treatment of T2D. However, recent studies have demonstrated that the use of these compounds is associated with unwanted effects, such as weight gain, fluid retention (3), increased incidence of cardiovascular events (4), and bone fractures (5, 6). As a direct consequence, the European Association for the Study of Diabetes no longer recommends rosiglitazone for the treatment of T2D (7). Different strategies have been conceived to obtain compounds with good therapeutic potential, trying to avoid the known side effects. New classes of ligands, PPARα/γ dual agonists (glita-
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zonars) and PPARpan agonists, represented two promising solutions, but they failed to avoid the known TZD-induced side effects, presenting themselves with adverse toxicity profiles (8, 9). On the other hand, recent studies demonstrated that modulation of PPARγ activity by selective PPAR modulators might uncouple the insulin-sensitizing effects from adverse effects. In fact, full and partial activation as well as antagonism of PPARγ can all improve insulin sensitivity (10, 11). Interestingly, several groups demonstrated that posttranslational modifications of PPARγ induced by different ligands affect the transcriptional effects on certain target genes (12–14). In this regard, recent observations indicate that peculiar ligand-receptor interactions result in a different structural conformation of the complex that gives rise to different PPAR transcriptional signatures and potentially uncouples the adverse effects of the ligand from therapeutic effects. In previous works published by our group, we showed that the specific interaction of different ligands with PPARγ leads to selective recruitment of PPAR coregulators (15). We also synthesized a new compound called LT175 that interacts with PPARγ in a newly identified hydrophobic region called “diphenyl pocket” (1). Here we report how the interaction of LT175 with PPARγ influences the activation of the receptor and the expression of PPARγ target genes, leading to an improved therapeutic profile compared with rosiglitazone. LT175 shows a strong antidiabetic and insulin-sensitizing effect in a mouse model of diet-induced obesity and insulin resistance. Lastly, this compound avoids weight gain, one of the most important adverse effects induced by TZDs. We show that this feature is likely due to the reduced capability of LT175 to induce the expression of genes involved in fatty acid esterification and storage in adipose tissue.

EXPERIMENTAL PROCEDURES

Cell Cultures—Adipogenesis assays were performed as described previously (16). Briefly, 3T3-L1 and C3H10T1/2 cells (ATCC) were cultured in DMEM supplemented by 10% fetal calf serum and 1% pen/strep. Differentiation was induced in DMEM supplemented with 10% fetal calf serum and 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and 10 µg/ml insulin/ml. After 48 h, the medium was changed with the addition of insulin and ligands. For the adipogenesis assay, 2 days after reaching confluence, cells were differentiated in the presence of PPAR ligands at the indicated concentration and 5 µg insulin/ml. The medium was replenished with ligands three times every other day. Lipid accumulation was determined by staining cellular lipids with Oil Red O. Pictures were taken with an Axiovert 200 microscope (Zeiss) at X20 magnification. The quantification of the intracellular dye was performed after isopropanol extraction and spectrophotometric reading at 490 nm.

Animal Studies—Six-week-old C57Bl/6 male mice (at least 6 animals/group) (Charles River Laboratories, Calco, Italy) were fed a high-fat diet containing 45% fat as the calorie source for 16 weeks. PPAR ligands were administered once a day for 2 weeks by oral gavage in 0.5% hypromellose (Sigma-Aldrich). LT175 was administered at 100 mg/kg/day. Fenofibrate (100 mg/kg/day), rosiglitazone (10 mg/kg/day), and AZ12063233 (5 mg/kg/day) were used as reference compounds for PPARα, γ, and α/γ activation, respectively. The optimal dose of LT175 was determined in a pilot experiment by using as a readout the expression of two PPARγ target genes, Glut4 and Adipoq. All animal studies were approved by the local ethical committee and by the Italian Ministry of Health and followed Italian and European Community legislation.

Luciferase Enzymatic Assay in Tissues—PPRE-Luc transgenic mice were obtained as described previously (17). All experiments were carried on with 10- to 14-week-old male PPRE-Luc transgenic mice. Mice were kept under a 12-h light/dark regimen and were treated once a day with PPAR ligands by oral gavage. After 3 days, mice were euthanized, and tissue extracts were prepared by homogenization in 200 µl of 100 mM KPO4 lysis buffer (pH 7.8) (1 mM dithiothreitol, 4 mM EGTA, 4 mM EDTA, and 0.7 mM phenylmethylsulfon fluoride), three cycles of freezing and thawing, and 30 min of Minifuge centrifugation (Eppendorf, Hamburg, Germany) at maximum speed. Supernatants containing luciferase were collected, and protein concentrations were determined by Bradford assay. Luciferase enzymatic activity in tissue extracts was measured by a commercial kit (Luciferase assay system, Promega), according to the instructions of the supplier. The light intensity was measured with a luminometer (Lumat LB 9501/16, Berthold) in 10-s time periods and expressed as relative light units per microgram of protein.

Oral Glucose Tolerance Test and Insulin Tolerance Test—For the oral glucose tolerance test, mice were fasted overnight and administered 2 g glucose/kg body weight per os. Blood was taken from the tail at 15-min intervals, and glucose concentration was determined with a OneTouch Ultra glucometer (LifeScan, Milan, Italy). For the insulin tolerance test, mice were fasted for 6 h and were administered 0.6 IU/kg body weight of insulin (Eli Lilly, Firenze, Italy) with an intraperitoneal injection. Blood samples were taken from the tail at 15-min intervals, and glucose concentration was determined with a glucometer.

In Vivo Magnetic Resonance Imaging—Monitoring of total body fat distribution was performed by in vivo magnetic resonance imaging as described previously (18, 19). Briefly, mice were anesthetized with 1% isoflurane and were analyzed in a 4.7 T Avance II MRI scanner (Bruker Corp., Karlsruhe, Germany). 16 axial, 1-mm-thick, T1 weighted slices were placed in the abdominal region spanning from the kidneys to the bladder (included). To maximize consistency between different animals, the slice immediately frontal with respect to the ilium bone was chosen for visceral fat estimation and was computed in a dark regimen and was treated once a day with PPAR ligands by oral gavage. After 3 days, mice were euthanized, and tissue extracts were prepared by homogenization in 200 µl of 100 mM KPO4 lysis buffer (pH 7.8) (1 mM dithiothreitol, 4 mM EGTA, 4 mM EDTA, and 0.7 mM phenylmethylsulfon fluoride), three cycles of freezing and thawing, and 30 min of Minifuge centrifugation (Eppendorf, Hamburg, Germany) at maximum speed. Supernatants containing luciferase were collected, and protein concentrations were determined by Bradford assay. Luciferase enzymatic activity in tissue extracts was measured by a commercial kit (Luciferase assay system, Promega), according to the instructions of the supplier. The light intensity was measured with a luminometer (Lumat LB 9501/16, Berthold) in 10-s time periods and expressed as relative light units per microgram of protein.

Histology—At sacrifice, WAT was embedded in OCT (Sakura, Torrance, CA) and frozen on dry ice. Sections of 12-µm thickness were made and submitted to hematoxylin-eosin staining. Cell size was measured using ImageJ software.

Plasma Biochemistry—At sacrifice, blood was taken for the determination of biochemical parameters and circulating hormones. After centrifugation at 8000 rpm and 4 °C for 5 min, plasma was collected. Total plasma cholesterol, triglycerides, nonesterified fatty acids, and glucose were measured with standard commercial kits (for cholesterol, Horiba ABX, Rome, Italy).
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Italy; for triglycerides and glucose, Sentinel Diagnostics, Milan, Italy; for nonesterified fatty acids, Wako, Neuss, Germany) following the instructions of the manufacturer. Cholesterol distribution in lipoprotein fractions was determined by FPLC. Insulin, adiponectin, and fibroblast growth factor 21 (FGF21) levels were determined with an ELISA kit (insulin, Mercodia, Uppsala, Sweden; adiponectin, Genway, San Diego, CA; FGF21, Biovendor, Germany).

**Cholesterol and Triglyceride Levels in the Liver—**100 mg of tissue were homogenized with TissueLyser (Qiagen GmbH, Hilden, Germany). 50 µg of 5a-cholestane was added as an internal standard. 20 ml of chloroform-methanol (2:1, v/v) was added to the samples. After overnight incubation at 4 °C, the aqueous phase was removed, and the organic phase was filtered and dried by nitrogen flushing. Samples were dissolved in 1 ml of hexane and split into two aliquots. The first aliquot was dried by nitrogen flushing and dissolved in 100 µl of 5% Triton X-100 in chloroform and then dried again and dissolved in 100 µl of 0.9% NaCl in H2O. Triglycerides were then measured with a commercial kit (Sentinel Diagnostic). The second aliquot followed a saponification for determination of total cholesterol levels, adding 2 ml of 1 M NaOH in 90% EtOH and incubating samples for 1 h at 60 °C under nitrogen. After diluting samples with 1 ml of H2O, 10 ml of petroleum ether was added, and the solution was shaken vigorously. The organic residues were dissolved in 50 µl of Bis(trimethylsilyl)trifluoroacetamide-pyridine (4:1, v/v), incubated for 1 h at 60 °C, and then 1 µl was injected for cholesterol analysis in GC-MS.

**RNA Extraction and Gene Expression Analysis—**Total RNA was extracted from 3T3-L1 and C3H10T1/2 adipocytes with TRIzol (Sigma), followed by purification on a Nucleospin RNA II column (Macherey Nagel, Germany) according to the instructions of the manufacturer. Mouse tissues were weighted and snap-frozen in liquid nitrogen for gene expression analysis. Total RNA was extracted with TRIzol followed by purification on a Nucleospin RNA II column. RNAsptidic cartridge (Qiagen) were used to test RNA samples. Purified total RNA samples were quantified with Nanodrop (Thermo Scientific, Wilmington, DE). 1 µg of total RNA was used to quantitate mRNA levels of PPAR target genes by real-time qPCR using iScriptTM One Step RT-PCR for Probes (Bio-Rad) in a CFX 384 thermal cycler (Bio-Rad). 36B4 was used as housekeeping gene for data normalization.

**Fluorescence Resonance Energy Transfer—**Fluorescence resonance energy transfer assays to evaluate peptide recruitment were performed in 384-well plates in a final volume of 10 µl as described previously (15, 20). Briefly, a mixture of 8 ng of human PPARγ-ligand-binding domain, 0.8 ng of europium-labeled anti-His antibody (PerkinElmer Life Sciences), and 86 ng of allopochycyanin-labeled streptavidin (PerkinElmer Life Sciences) in a FRET buffer containing 50 mM Tris (pH 7.5), 50 mM KCl, 1 mM DTT, and 0.1% free fatty acid BSA was prepared. The tested ligands were added to the mixture at a concentration ensuring the saturation of the receptor. The biotinylated peptides (PRIMM, Milan, Italy) were added in 12-point dose response curves starting at 9 µM as the highest concentration. The reactions were equilibrated for 1 h at room temperature and then measured in an Envision multiplate reader (PerkinElmer Life Sciences) using 340 nm as excitation and 615 and 665 nm as emission wavelengths. The ratio between 665 (allophycocyanin signal) and 615 (europium signal) was used to evaluate the peptide recruitment on the receptor. The peptide sequences used were Biotin-CPSSHSLTER-HILHRLQEGSPS-COOH (NR box 2) for SRC-1 spanning amino acids 676–700 (reference no. NP_671766), Biotin-DGTPPPQAEPEPSLKLKLLAPANT-COOH (NR box 1) for PGC-1α spanning amino acids 130–154 (reference no. NP_037393), Biotin-LENNIKQAANSSLLHLKKSQ-TIP-COOH (NR box 4) spanning amino acids 366–390, Biotin-VPSPQDFSKNGLSRLLRQRNQQDSYL-COOH (NR box 7) spanning amino acids 805–831 for RIP140 (reference no. NP_003480), Biotin-SGNLVPDASKHQLSELLRGGSGS-COOH (NR box 1) spanning amino acids 56–80 for CREB-binding protein (CBP) (reference no. NP_004371), Biotin-GSTH-GTSLKKEHKILHRLQDSSSPVD-COOH (NR box 2) for TIF2 spanning amino acids 676–702 (reference no. NP_006531), and Biotin-SFADPASNLGEDIR-KALMGSGFD-COOH (NR box 1) for N-CoR1 spanning amino acids 2253–2277 (reference no. NP_006302).

**Statistical Analyses—**Statistical analyses were performed via one-way analysis of variance with Dunnet or Bonferroni post-test analysis for multiple group comparisons using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Differences with p values of less than 0.05 were considered statistically significant.

**RESULTS**

**Low Adipogenic Activity of LT175—**Previously, we reported the activity and the x-ray crystal structures of two enantiomeric forms of an analog of clofibric acid. In particular, the S-enantiomer called LT175 (Fig. 1A) has been shown to be a dual PPARα/PPARγ ligand with a partial agonism profile toward PPARγ (EC50 of hPPARγ, 0.22 µM; EC50 of mPPARγ, 0.26 µM; EC50 of hPPARγ, 0.48 µM) because of the peculiar position adopted by the molecule in the PPARγ ligand-binding pocket (1). To determine whether the particular interaction between LT175 and PPARγ ligand-binding domain could affect its adipogenic activity, 3T3-L1 fibroblasts were induced to differentiate to adipocytes with medium containing both LT175 and insulin. Rosiglitazone (EC50 of hPPARγ, 0.04 µM) or the dual PPARα/γ agonist AZ12063233 (hereafter referred to as AZ) (EC50 of hPPARα, 0.22 µM; EC50 of mPPARγ, 0.011 µM; EC50 of hPPARγ, 0.15 µM) were used as reference compounds. LT175 activated the PPARγ-dependent program of adipocyte differentiation, as shown by increased expression of genes encoding adiponectin (Adipoq), the insulin-sensitive glucose transporter Glut4, and fatty acid binding protein 4 (Fabp4) at least 10 times compared with treatment with insulin alone. The increased expression of these genes was lower compared with rosiglitazone and AZ, although it was strongly significant (Fig. 1B). Nevertheless, cells differentiated in the presence of LT175 accumulated much less lipids than those treated with rosiglitazone or AZ (Fig. 1, C and D), further indicating that LT175 features lower adipogenic activity than a full PPARγ agonist. Besides stimulating the expression of typical adipocyte mark-
ers, such as Fabp4, fully activated PPARγ can induce genes involved in lipid uptake and storage, thus favoring fat retention in adipocytes. Accordingly, the decreased lipid accumulation observed in LT175-treated cells could rely on differential induction of such PPARγ targets by the ligand. To test this hypothesis, we evaluated the expression of the class B scavenger receptor Cd36, which mediates fatty acid uptake into adipose tissue, and of phosphoenolpyruvate carboxykinase 1 (Pck1) and glycerol kinase, both involved in glycerol 3-phosphate formation required for triglyceride synthesis and storage as lipid droplets. Interestingly, these genes were significantly less induced by LT175 than by rosiglitazone or AZ (Fig. 1B). Similar results were obtained in the murine C3H/10T1/2 fibroblasts, which can also be differentiated to adipocyte-like cells (Fig. 1, E and F). Taken together, these observations suggest that dampened induction of genes involved in lipid uptake and storage by LT175 underlies the reduced lipid accumulation observed in LT175-treated cells and explain its low adipogenic activity.

**Metabolic Effects and Antidiabetic Activity of LT175 in Diet-induced Insulin-resistant Mice**—The adipogenic activity of PPARγ agonists is responsible for one of the important side effects of TZDs, contributing to weight gain associated with the use of this class of drugs (21). The low adipogenic potential of LT175 in vitro prompted us to investigate its metabolic effects in vivo. First, the bioavailability of the compound in different target organs was assessed by treating PPRE-Luc reporter mice bearing the luciferase reporter gene under the control of a PPRE (17, 22). LT175 was administered orally at 100 mg/kg/day for 3 days. As expected, LT175 was able to activate PPAR-dependent transcription in liver and in WAT (Fig. 2, A and B), indicating that the compound can reach these tissues. Subsequently, the optimal dose of LT175 was determined in a pilot experiment, which indicated that, when administered at 100 mg/kg/day, the effect of LT175 on the expression of two PPARγ target genes, Glut4 and Adipoq, was comparable with that observed with 10 mg/kg/day of rosiglitazone (data not shown). Next, we evalu-
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FIGURE 2. LT175 is bioavailable in liver and white adipose tissue. PPRE-Luc mice were treated with LT175 once a day. After 3 days, mice were sacrificed 6 h after treatment, and liver (A) and epididymal adipose tissue (B) were collected to measure PPAR-regulated luciferase enzymatic activity. Ctrl, control mice; LT175, 100 mg/kg/day LT175. RLU, relative light units. *, p < 0.05 versus control. Data are mean ± S.E. (n = 4).

To assess glucose homeostasis and insulin sensitivity in HFD-fed mice treated with LT175, we performed glucose tolerance and insulin tolerance tests. As shown in Fig. 4, treated with LT175 showed lower insulin and higher adiponectin (Fig. 4G) levels, further indicating that this molecule is highly effective in improving glucose homeostasis.

LT175 Reduces Visceral White Adipose Tissue Mass—The marked decrease of body weight observed in mice treated with LT175 and AZ was associated with a strong reduction of WAT. Subcutaneous and visceral adipose tissues have different metabolic properties, the latter being more related to the development of insulin resistance and other complications of obesity (24). To assess which WAT depot was chiefly affected by the treatment, we performed an MRI analysis of mouse abdomens, and we found that LT175 significantly reduced only visceral WAT, whereas no significant differences were observed in the subcutaneous WAT fraction (Fig. 5). A smaller decrease in visceral WAT was observed in mice treated with the other compound activating both PPARα and PPARγ, AZ (Fig. 5). This effect was confirmed by the weighing of visceral fat content at the end of treatment (Fig. 5A). Moreover, histological sections of visceral WAT showed that the adipocytes of mice treated with LT175 were smaller, a condition that can preserve adipocyte life (Fig. 5, E and F) (25). No differences were observed in macrophage infiltration in the presence of the tested ligands (data not shown). The strong effect of LT175 on the visceral adipose depot prompted us to evaluate its transcriptional effects in mature adipocytes. As expected, in epididymal WAT from mice treated with LT175, the expression of several PPARγ target genes, such as Adipoq, Glut4, and Fabp4, was induced significantly compared with control mice (Fig. 6A). Conversely, consistent with the results observed in 3T3-L1 and C3H10T1/2 fibroblasts induced to differentiate to adipocytes (Fig. 1), mRNA levels of both Cd36 and Pck1 were not affected by LT175 as opposed to rosiglitazone, which significantly increased the expression of these two key genes for fatty acid uptake and esterification. To corroborate the lack of induction of Cd36 and Pck1 by LT175 in mature adipocytes, 3T3-L1 cells were first differentiated with a differentiation mixture containing insulin, dexamethasone, and isobutylmethylxanthine and then exposed either to rosiglitazone or LT175. In terminally differentiated adipocytes, none of the tested PPARγ ligands were able to further stimulate the expression of Glut4 and Adipoq, whereas only rosiglitazone induced Fabp4 (Fig. 6B). Furthermore, rosiglitazone significantly enhanced the expression of Pck1 and Cd36, whereas LT175 was completely ineffective on these genes (Fig. 6B). Collectively, these observations suggest that the decreased lipid accumulation observed in adipocytes of WAT upon treatment with LT175 may be due to the differential regulation of these genes, which are required for fatty acid esterification and storage in adipose tissue in both differentiating and terminally differentiated adipocytes.

Hepatic Effects of LT175—Because LT175 is a dual PPARα/PPARγ ligand, at the systemic level, its metabolic effects are also determined by its activity in organs expressing high levels of PPARα, such as the liver. In mice treated with LT175, in analogy with fenofibrate and AZ, the hepatic mass was augmented significantly compared with controls (Fig. 7A), possibly because of peroxisome proliferation elicited by PPARα agonists, as typically observed in rodents (26). Furthermore, different from fenofibrate and AZ, the effect of LT175 on hepatic triglyceride content was modest (Fig. 7B), and no effect was...
observed on cholesterol accumulation (Fig. 7C). In the liver, PPARα mainly regulates genes involved in fatty acid β-oxidation and ketone bodies. Accordingly, we found that the treatment with all PPARα agonists induced the expression of acyl-CoA oxidase 1 (Acox1), acyl-CoA dehydrogenase medium chain (Acadm), acyl-CoA dehydrogenase long chain (Acadl), HMG-CoA synthase 2 (Hmgcs2), and Fgf21 (Fig. 7D), indicating that LT175 activates the genetic program of hepatic fatty acid β-oxidation and ketone body synthesis.

**Effects of LT175 on Kidney and Heart**—Together with weight gain, the treatment with TZDs has been shown to induce fluid retention in the kidney (3, 27) and cardiovascular events, most likely due to pressure overload (28, 29). Our data demonstrate that, in kidney medullae, LT175, as opposed to rosiglitazone and AZ, does not induce the expression of Scnn1g (Fig. 8A), the gene encoding the γ subunit of the renal sodium transporter whose induction may result in fluid retention. No differences in the other genes analyzed in the kidney were noticed. In parallel, we measured heart weight, which was not altered significantly by the ligands (Fig. 8B). The analysis of the cardiac gene expression profile showed that both rosiglitazone and LT175 enhance the expression of myosin heavy chain (Mhc), ATPase, Na⁺/K⁺ transporting, α 2 polypeptide (Atp1a2) and ATPase, Ca²⁺ transporting, cardiac muscle, and slow twitch 2 (Atp2a2), which can oppose pressure overload (although the statistical significance was not reached by rosiglitazone for the latter) (Fig. 8C). In contrast, none of the ligands induced the heart failure marker gene natriuretic peptide type B (Bnp). LT175 also showed a favorable profile concerning the genes related to cardiac fatty acid metabolism, enhancing the expression of Pgc1α and Acadl...
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LT175 Induces Differential Coregulator Recruitment on PPARγ—Next, we aimed to investigate the behavior of PPARγ in the presence of different ligands to gain insights into the molecular mechanisms underlying the different biological effects of LT175 compared with other ligands. The biological activity of a given nuclear receptor can be influenced significantly by coregulator recruitment, yielding a differential modulation of target gene expression. To investigate whether the unique interaction mode with PPARγ influences the recruitment of coregulators by the ligand-activated receptor, FRET assays were set at saturating concentrations of the ligand and increasing amounts of a panel of biotinylated peptides corresponding to known coregulators of PPARγ. Interestingly, LT175 impaired the recruitment of the CBP coactivator peptide on PPARγ (Fig. 9D), which was recruited to PPARγ in the presence of the full agonist rosiglitazone (Fig. 9B) and the dual
PPARγ agonist AZ (Fig. 9C). As opposed to rosiglitazone and AZ, LT175 failed to completely release the NCoR1 corepressor peptide from PPARγ (Fig. 9D), confirming the partial agonist behavior observed in previous transcriptional assays (1). Thus, the unusual mode of interaction of LT175 with PPARγ determines the differential recruitment of coregulators compared with the PPARγ full agonist rosiglitazone and the dual PPARα/γ agonist AZ (Fig. 9). We conclude that the differential recruitment of coregulators confers the distinctive gene activation profile induced by LT175 that is responsible for the peculiar in vitro and in vivo pharmacological properties of this ligand.

DISCUSSION

PPAR ligands have shown great promise for therapeutic interventions in metabolic disorders such as T2D. However, the prescription of PPARγ ligands as insulin sensitizers in the clinical setting has been disappointing because of serious adverse effects reported in a significant portion of patients treated with these drugs (32–34). To avoid the side effects of drugs targeting PPARs, several groups have been deploying their efforts to design new generations of ligands (12, 13). In this frame, the understanding of the structure-activity relationship of nuclear receptor-ligand complexes is fundamental to give rise to a safer generation of compounds for the treatment of T2D and metabolic disorders that uncouple the therapeutic effects from the side effects observed with TZDs. The simultaneous activation of PPARα that triggers triglyceride catabolism and fatty acid oxidation may offset adipogenesis resulting from PPARγ activation, one of the major side effects of TZDs. Previously, we described a dual PPARα/γ agonist, LT175, featuring a peculiar interaction with the PPARγ ligand binding domain (1). In this work, we explored the biological effects of LT175 in cell cultures and in a mouse model of diet-induced insulin resistance. We hypothesized that this peculiar interaction could elicit a different transcriptional signature, leading to a safer therapeutic profile than that of rosiglitazone in this mouse model. Here we provide a proof of principle where, by targeting a specific pocket of the ligand binding domain of PPARγ, previously named diphenyl pocket (1), it is possible to obtain a ligand with

FIGURE 5. LT175 decreases body weight and visceral fat content in high-fat diet-fed mice. A, visceral fat of mice was weighed at sacrifice. HFD, high-fat diet-fed mice; Rosi, 10 mg/kg/day rosiglitazone; Feno, 100 mg/kg/day fenofibrate; AZ, 5 mg/kg/day AZ12063233; LT175, 100 mg/kg/day LT175. B, visceral fat area of mice after 2 weeks of treatment was quantified by MRI of total body fat. C, subcutaneous fat area of mice after 2 weeks of treatment was quantified by MRI of total body fat. D, representative image of MRI performed in mice treated with the indicated ligands. Chow, chow diet-fed mice. E, hematoxylin-eosin stain of WAT sections from high-fat diet-fed mice. *, p < 0.05 versus HFD; **, p < 0.01 versus HFD; ***, p < 0.001 versus HFD; §§§, p < 0.001 versus Rosi; ###, p < 0.001 versus AZ. Data are expressed as the mean ± S.E. (n = 8–12).
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FIGURE 6. LT175 elicits no changes in the expression of genes related to lipid uptake and storage in white adipose tissue and mature 3T3-L1 adipocytes. A, C57Bl/6J mice were treated for 3 days with PPAR ligands. Total RNA from epididymal white adipose tissue was extracted, and the expression of Adipoq, Glut4, Fabp4, Pck1, and Cd36 was measured by real-time qPCR. Ctrl, mice treated with vehicle; Rosi, 10 mg/kg/d rosiglitazone; LT175, 100 mg/kg/d LT175. **, p < 0.01; ***, p < 0.001 versus Ctrl. Data are mean ± S.E. (n = 8). B, 3T3-L1 mouse fibroblasts were differentiated by I/D/I and then treated with the PPAR ligands. Total RNA was extracted, and the expression of Adipoq, Glut4, Fabp4, Pck1, and Cd36 was measured by real-time qPCR. Results are expressed as the mean ± S.E. of triplicate samples. Ctrl, control undifferentiated cells; I/D/I, cells differentiated to adipocytes with 10 μg insulin/ml, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine; Rosi, cells differentiated to adipocytes with I/D/I and then treated with 5 μM rosiglitazone; LT175, cells differentiated to adipocytes with I/D/I and then treated with 25 μM LT175. ***, p < 0.001 versus I/D/I; §, p < 0.001 versus Rosi.

remarkable pharmacological features devoid of important side effects that hindered the clinical application of TZDs and other PPAR ligands. Our results indicate that the reduced adipogenic effect of LT175 may be a consequence of the differential expression of genes encoding Cd36, Pck1, and glycerol kinase involved in lipid uptake and storage in adipocytes compared with rosiglitazone and the other dual PPARα/γ agonist, AZ, tested in this study. Moreover, the FRET assay showed that LT175 induced a conformational change, allowing the recruitment of the corepressor peptide NCoR1, a typical behavior observed with other partial agonists of PPARγ (15). Conversely, as opposed to rosiglitazone and AZ, LT175 did not induce the recruitment of the coactivator CBP on PPARγ. Notably, because CBP has been shown to be required for PPARγ-mediated adipogenesis (35, 36), the lack of CBP recruitment to PPARγ may explain why both 3T3-L1 and C3H10T1/2 differentiating adipocytes treated with LT175 show less lipid accumulation than those treated with rosiglitazone and AZ and the reduced lipid accumulation in WAT of mice administered with LT175. This unique feature of this new ligand may represent an advantage in that it may reduce one of the known side effects that have been associated with the clinical use of TZDs and other PPARγ full agonists as insulin sensitizers in patients with T2D. Thus, we suggest that LT175 minimally activates the adipogenic program, a condition that prevents the excessive expansion of visceral fat but, at the same time, determines very low ectopic deposition of lipids in the liver, as opposed to other ligands tested in this study.

The magnitude of PPARγ target gene induction (Glut4, Adipoq, and Fabp4) by LT175 was clearly lower than that with rosiglitazone or AZ in 3T3-L1 adipocytes and in visceral fat. Nonetheless, this compound was able to achieve a potent insulin-sensitizing effect, suggesting that full activation of PPARγ may not be strictly required to attain the antidiabetic effects. This observation is consistent with other findings showing that PPARγ ligands with reduced efficacy or even non-agonists (13) are sufficient to obtain beneficial effects. At the same time, the limited PPARγ activation may offset the adverse effects of TZDs.

Previous studies showed that TZDs affect the dynamics of white adipose tissue maintenance by acting upon the adipocyte progenitor population in the fat depot (37). In this regard, we found that the effects of LT175 were evident both in differentiating 3T3-L1 and in mature 3T3-L1 adipocytes. Because TZDs exert their insulin-sensitizing effect by enhancing lipid deposition in adipose tissue and inducing weight gain in vivo, it was surprising to find that mice treated with LT175, as opposed to other PPAR ligands described previously, showed both improved insulin sensitivity and decreased body weight by as much as 11% after only a treatment of 15 days. Body weight reduction can be explained with the 65% decrease of visceral WAT mass in mice treated with LT175, consistent with the results obtained in cultured adipocytes. Moreover, because the frequency of adipocyte death is positively correlated with increased adipocyte size (25), LT175, by avoiding the expression of the genes involved in lipid uptake and storage, reduces adipocyte size in WAT. This is particularly important because of the association of low-grade inflammation with the expansion of visceral adipose tissue that contributes to the onset of insulin resistance (24, 38). On the basis of these observations, we speculate that the strong insulin-sensitizing effect of LT175 may be explained, at least in part, by its effects in the visceral fat. Reduced body weight and fat mass observed in diet-induced insulin-resistant mice treated with LT175 could also be ascribed to PPARα activation in the liver that contributes to fat burning via FGF21-promoting lipolysis in adipose tissue and hepatic fatty acid β oxidation and ketone bodies synthesis (39–42). Of note, although the dual PPARα/γ ligand AZ was not able to reduce lipid accumulation in cultured adipocytes, in vivo it showed a significant reduction of visceral fat. In analogy with LT175, this could also be related to the PPARα-mediated induction of hepatic FGF21. Furthermore, as opposed to all ligands tested in this study, LT175 caused minimal triglyceride accumulation in the liver. In this regard, Lin et al. (43) have shown recently that the beneficial effects of FGF21 on hepatic triglyceride accumulation are mediated by adiponectin. Coincidently, in mice treated with LT175, we observed the highest levels of circulating adiponectin and of FGF21. Therefore, we infer...
that this could explain the lower accumulation of hepatic triglycerides in mice on LT175. Remarkably, although the ligands used in this study decreased the levels of plasma glucose, triglycerides, and nonesterified fatty acids, LT175 was the only compound able to reduce plasma cholesterol levels. This effect is due to the known repression of apolipoprotein A-I gene transcription already reported in rodents treated with agonists of PPARα, which leads to decreased HDL cholesterol as opposed to humans (23).

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**FIGURE 7. LT175 induces the PPARα-dependent fatty acid oxidation program in the liver.** A, liver weight. At sacrifice, the liver was collected and weighed. Data are expressed as the ratio between liver and total body weight. HFD, high-fat diet-fed mice; Rosi, 10 mg/kg/day rosiglitazone; Feno, 100 mg/kg/day fenofibrate; AZ, 5 mg/kg/day AZ12063233; LT175, 100 mg/kg/day LT175. ***, p < 0.001 versus HFD.** B, liver triglyceride levels expressed as micrograms of triglycerides per milligram of tissue. ***, p < 0.001 versus HFD; §§, p < 0.05 versus Rosi; §§§, p < 0.01 versus AZ. C, liver cholesterol levels expressed as micrograms of cholesterol per milligram of tissue. ***, p < 0.001 versus HFD. Data are mean ± S.E. (n = 8). D, C57Bl/6J mice were treated for 3 days with the indicated PPAR ligands. Total RNA from the liver was extracted, and the expression of Acox1, Acad1, HMGC2, and Fgf21 was measured by real-time qPCR. Ctrl, mice treated with vehicle. Data are mean ± S.E. (**, p < 0.01 versus control.

**FIGURE 8. LT175 induced effects on tissue functionality and fluid retention in kidney and heart.** A, C57Bl/6J mice were treated for 3 days with PPAR ligands. Total RNA from kidney was extracted, and the expression of Scnn1a, Scnn1g, Aqp2, and Aqp3 was measured by real-time qPCR. Ctrl, mice treated with vehicle; Rosi, 10 mg/kg/day rosiglitazone; Feno, 100 mg/kg/day fenofibrate; AZ, 5 mg/kg/day AZ12063233; LT175, 100 mg/kg/day LT175. **, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control.
Oral glucose tolerance tests and insulin tolerance tests showed the insulin sensitizing effect of LT175, which was confirmed by reduced plasma levels of insulin and increased circulating adiponectin and FGF21. The circulating adiponectin levels were significantly higher than expected from gene expression in cell culture and WAT. This is most likely a direct consequence of the action of circulating FGF21 in WAT (43), which contributes to enhance adiponectin levels and to improve the insulin-sensitizing effects. Consequently, we infer that LT175, by increasing circulating FGF21, promotes fatty acid oxidation in the liver as a consequence of enhanced adiponectin secretion from adipose tissue (44). Together, these results demonstrate that LT175 is a very effective insulin sensitizer that displays differential effects compared with TZDs. Further studies are warranted to investigate the global effects of LT175 to define the safety profile of this molecule. In this regard, it is worth mentioning that LT175 induced a differential gene expression profile not only in WAT but also in other tissues that are affected by the typical side effects induced by TZDs. Although the reported data regarding the role of PPARγ in fluid retention are controversial, it is clear that the enhancement of sodium and fluid reabsorption contributes to TZD-induced edema formation (3, 27, 45). In kidney medullae, we found that LT175, in contrast with rosiglitazone and AZ, did not induce the expression of Scnn1g, one of the subunits of the epithelial sodium channel ENaC contributing to fluid retention in mice treated with TZDs (3, 27). Even though the effect of LT175 on Scnn1g expression may partially contribute to explain the lack of body weight gain with this ligand, it is clear that the major effects of LT175 on body weight can be explained with reduced adipogenesis. Concerning the cardiac effect of LT175, the length of the treatment with PPAR ligands in our experimental conditions is not sufficient to induce the development of myocardial dysfunction (41, 46). Therefore, in future studies, it would be interesting to investigate the cardiac effects of LT175 in a model of chronic exposure to this ligand.

In conclusion, in this work we show that LT175 is a peculiar dual PPARα/γ agonist with insulin-sensitizing properties, inducing differential expression of genes involved in lipid uptake and storage in adipocytes, thus avoiding weight gain. At the same time, this ligand, by activating PPARα in the liver, triggers triglyceride and fatty acid catabolism and provides additional beneficial effects because of lipid clearing. These features make LT175 a promising scaffold molecule to design new ligands with better metabolic profiles on both glucose and lipid parameters and with a therapeutic value to reduce cardiovascular risk and improve glycemic control in patients with T2D and metabolic syndrome.

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