T2384, a Novel Antidiabetic Agent with Unique Peroxisome Proliferator-activated Receptor γ Binding Properties

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The nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) plays central roles in adipogenesis and glucose homeostasis and is the molecular target for the thiazolidinedione (TZD) class of antidiabetic drugs. Activation of PPARγ by TZDs improves insulin sensitivity; however, this is accompanied by the induction of several undesirable side effects. We have identified a novel synthetic PPARγ ligand, T2384, to explore the biological activities associated with occupying different regions of the receptor ligand-binding pocket. X-ray crystallography studies revealed that T2384 can adopt two distinct binding modes, which we have termed “U” and “S”, interacting with the ligand-binding pocket of PPARγ primarily via hydrophobic contacts that are distinct from full agonists. The different binding modes occupied by T2384 induced distinct patterns of coregulatory protein interaction with PPARγ in vitro and displayed unique receptor function in cell-based activity assays. We speculate that these unique biochemical and cellular activities may be responsible for the novel in vivo profile observed in animals treated systemically with T2384. When administered to diabetic KKAy mice, T2384 rapidly improved insulin sensitivity in the absence of weight gain, hemodilution, and anemia characteristics of treatment with rosiglitazone (a TZD). Moreover, upon coadministration with rosiglitazone, T2384 was able to antagonize the side effects induced by rosiglitazone treatment alone while retaining robust effects on glucose disposal. These results are consistent with the hypothesis that interactions between ligands and specific regions of the receptor ligand-binding pocket might selectively trigger a subset of receptor-mediated biological responses leading to the improvement of insulin sensitivity, without eliciting less desirable responses associated with full activation of the receptor. We suggest that T2384 may represent a prototype for a novel class of PPARγ ligand and, furthermore, that molecules sharing some of these properties would be useful for treatment of type 2 diabetes.

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Peroxisome proliferator-activated receptor γ (PPARγ (NR1C3)) is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors (1, 2). PPARγ, together with PPARG (NR1C1) and PPARδ (NR1C2), form a subfamily of “lipid sensing” receptors that regulate important aspects of lipid and glucose homeostasis (3, 4). At least two PPARγ isoforms exist, γ1 and γ2, resulting from transcription from two different promoters upstream of the PPARγ gene (5). PPARγ1 is expressed broadly in many tissues, whereas PPARγ2, which possesses an additional 30 amino acids at its N terminus, is expressed predominantly in adipose tissue. Both “gain of function” and “loss of function” studies have firmly established PPARγ as a master regulator of adipocyte differentiation and a link with the diseased state in type 2 diabetes (3). The important roles PPARγ plays in lipid metabolism, inflammatory cytokine production, and macrophage function may also have direct impacts on dyslipidemia, atherosclerosis, and cardiovascular diseases (6).

The first insight into the link between PPARγ and diabetes came from the discovery of PPARγ as the biologic target for the thiazolidinedione class of antidiabetic drugs (TZDs) (7, 8). Two such drugs, rosiglitazone (Avandia™; GlaxoSmithKline) and pioglitazone (Actos™; Takeda Pharmaceuticals), have been prescribed either as monotherapy or in combination with sulfonylureas, metformin, or insulin to achieve glycemic control in diabetic patients. Interestingly, although TZDs lower glucose and improve lipid and adipocytokine profiles in type 2 diabetics, as full agonists they also stimulate adipocyte differentiation in vitro and result in weight gain in vivo, which normally aggravates the diabetic state. Additional undesirable side effects associated with TZD treatment include edema/hemodilution, cardiomyopathy, and anemia (9). These side effects most likely result directly from activation of the receptor, because other PPARγ agonists with unrelated chemical structures show similar profiles (10).

Paradoxically, the reduction of PPARγ activity also results in improvement in insulin sensitivity. Human genetic studies have identified genetic variants in PPARγ associated with altered risks to development of type 2 diabetes. For example, one common polymorphism, Pro12Ala, correlates both with a reduction in the risk of type 2 diabetes and with a reduction in body
mass index (11, 12). The in vitro characterization of this polymorphism suggests that the Ala allele causes a partial loss of PPARγ function as a result of decreased DNA binding affinity and transcription activity (13, 14). In rodents, the reduction of PPARγ expression in PPARγ<sup>−/−</sup> heterozygotes protects these animals from high fat diet- or aging-induced adipocyte hypertrophy, obesity, and insulin resistance. The heterozygous animals also show reduction in factors associated with insulin resistance, including free fatty acids and tumor necrosis factor α, an up-regulation of leptin and adiponectin, and a significantly increased rate of fatty acid β-oxidation. This latter finding may explain the decrease in triglyceride content of white adipose tissue, skeletal muscle, and liver seen in these animals (15). These results, in addition to the analysis of other PPARγ polymorphisms (11, 16), suggest that the receptor-mediated side effects associated with TZD treatment could potentially be decoupled from the insulin sensitization effect. In theory, a PPARγ ligand that could modulate receptor activity and mimic the effects observed with a P12A allele or PPARγ heterozygotes might abolish or at least reduce the unwanted side effects associated with full activation of the receptor while still triggering the physiologic responses that allow proper glycemic control.

The PPARγ ligand-binding site is a large Y-shaped cavity with a total volume of ~1,300 Å<sup>3</sup>, as seen in the apo-PPARγ crystal structure (17). Rosiglitazone binds in this pocket in a “U-shaped” conformation, wrapping around helix 3 with its central benzene ring directly behind helix 3 and the TZD head group extending toward the AF2 helix to form a direct H-bond with the hydroxyl moiety of the Tyr<sup>473</sup> side chain. This interaction is critically important for stabilizing the AF2 helix in a conformation that interacts with coactivator proteins. Because this large binding pocket underlies the surface important for receptor interactions with coactivator and corepressor proteins (17, 18), we reason that ligands bind to different regions of this large pocket, may differentially affect receptor-coregulatory protein interactions, and selectively modulate aspects of receptor activity.

Here we describe a novel, potent, and selective PPARγ ligand, T2384, which may represent this new class of molecule. Using a variety of biochemical and cell-based assays, we demonstrate that T2384 binds to PPARγ in multiple conformations, with potentially distinct biological activities in vitro. Moreover, we suggest that the unique in vitro properties of the ligand we have described might be responsible for this distinctive in vivo pharmacology. In rodent models of diabetes, T2384 effectively lowered plasma glucose and insulin levels without the induction of body weight gain and anemia.

**EXPERIMENTAL PROCEDURES**

**In Vitro Reagents and Assay Protocols**—Plasmids used, homogeneous time-resolved fluorescence (HTRF) assay, ligand-binding assay, and transient transfection assay were all described previously (19). Briefly for HTRF assays, the reaction conditions were as follows: a 100-μl reaction volume contains 50 mM Tris, pH 7.9, 50 mM KCl, 1 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 800 ng/ml anti-GST-(Eu)K antibody (PerkinElmer), 1 ng/ml GST-PPARγ, 1.5 μg/ml streptavidin conjugated with allophycocyanin (Streptavidin-APC, PerkinElmer), 200 nM biotin-peptide, 5 μl of compound in Me<sub>2</sub>SO as indicated in figure legends. GST-PPARγ/anti-GST-(Eu)K and biotin-peptide/streptavidin were pair-wise preincubated separately in 20 μl each for 1 h at room temperature before being combined with the remaining components for additional 1 h at room temperature. The reactions were set up in 96-well plates (black polystyrene, Whatman Inc.), and fluorescence was measured on LJM Analyst (LJM Biosystems). The data were expressed as the ratio, multiplied by a factor of 1000, of the emission intensity at 665 nm to that at the 620 nm.

Mutagenesis was performed using a QuickChange site-directed mutagenesis kit from Stratagene. All of the data in the manuscript are representative of at least two to three independent experiments, and the results are presented as the means of duplicate or triplicate determinations.

**Adipocyte Differentiation Assays**—Isolated human preadipocytes were cultured and induced to differentiate as described by the manufacturer’s (ZenBio, NC) protocol, except either rosiglitazone or T2384 was used in place of the PPARγ agonist in the supplied differentiation medium. The lipid content of the cells was measured with Nile Red staining (Molecular Probes #N1142).

**Protein Preparation**—Human PPARγ LBD (residues 206–478) was PCR-cloned into a pET-30 vector (Novagen, WI) with an N-terminal His<sub>6</sub> tag, and human RXRα LBD (residues 221–462) was in pET-15b vector (Novagen, WI) with an N-terminal His<sub>6</sub> tag as well. Both proteins were expressed in *Escherichia coli* BL21(DE3) cells (Invitrogen) by growing in LB medium. The proteins were purified by a Ni<sup>2+</sup>-nitrilotriacetic acid-agarose column (Qiagen) and an anion exchange column of Mono-Q (Pharmacia) and then further purified by a gel filtration column of Superdex 75 (Pharmacia). For the heterodimer complex of PPARγ LBD and RXRα LBD, the two proteins were mixed and purified by a cation exchange column of Source 30S (Pharmacia) to remove the excess RXRα. The purified PPARγ LBD protein was concentrated to 1 mg/ml in 20 mM Tris-HCl, pH 7.9, 100 mM NaCl, 2 mM EDTA, and 5 mM dithiothreitol before mixing with 5-fold excess of T2384. The protein-ligand mixture was further concentrated to 5–7 mg/ml for crystallization. The purified heterodimer complex of PPARγ LBD and RXRα LBD was then concentrated to 1 mg/ml before mixing with 5-fold excess of T2384 as well as 9-cis-retinoic acid and 2–3-fold excess of a coactivator peptide containing the LXXLL motif derived from helical domain one (TSHKLVQLTTL) of SRC-1. The mixture was then concentrated to 5–7 mg/ml for crystallization.

**Crystallization**—Cocrystals of wild-type PPARγ LBD with T2384 were grown at 20 °C by either hanging drop or sitting drop with 2.5 μl of the protein solution and 2.5 μl of the well solution containing 22–26% (w/v) polyethylene glycol 4000, 0.1 M Hapes, pH 7.5, and 0.2 M sodium acetate. Cocrystals of two mutant PPARγ LBDs with T2384 were obtained with the heterodimer of PPARγ/RXRα LBDs in the presence of 9-cis-retinoic acid and the coactivator peptide in 0.1 M Tris, pH 9.0, 25–27% polyethylene glycol 2000 monomethyl ether, 0.8 M sodium formate. The crystals were transferred into a well solution that contained an additional 15–20% (w/v) of ethylene glycol and then flash frozen in liquid nitrogen.
**PPARγ Modulator**

Data Collection, Structure Determination, and Refinement—
X-ray diffraction data sets were collected on synchrotron radiation beam lines 5.0.1 and 5.0.2 at the Advanced Light Source (Berkeley, CA). The data were integrated using either DENZO/SCALEPACK (20) or MOSFLM (21). The PPARγ crystals grew in space group C2 with unit cell dimensions \(a = 92, b = 63, c = 119 \text{ Å}, \beta = 105^\circ\). The PPARγ/RXRα heterodimer crystals also grew in space group C2 with unit cells of \(a = 180, b = 54, c = 67 \text{ Å}, \beta = 107^\circ\).

| Crystal | Wild type | G284I | L228W/A292W/L333W |
|---------|-----------|-------|-------------------|
| Wavelength (Å) | 1.0 | 1.0 | 1.0 |
| Space group | C2 | C2 | C2 |
| Cell constants (Å) | | | |
| \(a\) | 91.6 | 180.0 | 180.4 |
| \(b\) | 62.4 | 54.0 | 53.5 |
| \(c\) | 118.9 | 67.1 | 66.9 |
| \(\beta\) | 102.6 | 107.7 | 107.3 |
| Resolution (Å) | 2.5 | 2.4 | 2.3 |
| Unique reflections | 22667 | 24345 | 27310 |
| Total reflections | 214064 | 89826 | 100297 |
| Completeness (%) | 94.9 (88.4) | 100.0 (100.0) | 100.0 (99.7) |
| Sym (%) | 4.0 (53.9) | 5.8 (37.5) | 4.6 (19.4) |
| \(R_{	ext{free}}\) (%) | 9.9 | 7.9 | 9.5 |
| \(R_{	ext{cryst}}\) (%) | 35.9 | 37.5 | 37.5 |
| Refinement | | | |
| \(R_{	ext{cryst}}\) (%) | 22.6 | 26.2 | 24.2 |
| \(R_{	ext{free}}\) (%) | 27.3 | 35.4 | 29.4 |
| Rmsd bond lengths | 0.01 | 0.02 | 0.02 |
| Rmsd bond angles | 1.16 | 2.24 | 2.44 |
| Total non-hydrogen atoms | 9951 | 4901 | 4165 |

**TABLE 1**

Statistics of crystallographic data and refinement

Rmsd is the root-mean-square deviation from ideal geometry. The numbers in parentheses are for the highest resolution shell. \(R_{	ext{cryst}} = \sum |F_o| - |F_c| / \sum |F_o|\) and \(R = \sum |F_o| - |F_c| / \sum |F_o|\), where \(F_o\) and \(F_c\) are the observed and calculated structure factors, respectively. \(R_{	ext{free}}\) was calculated from a randomly chosen 10% of reflections excluded from the refinement, and \(R_{	ext{cryst}}\) was calculated from the remaining 90% of reflections.

The cocrystal structures of PPARγ LBD with T2384 were solved by molecular replacement using previously published PPARγ coordinates as a search model. The Protein Data Bank code of 2LBD was used for the PPARγ LBD cocrystal structure, and Protein Data Bank code of 1FM9 was used for the heterodimer cocrystal structures. Manual model building was carried out in Quanta (Accelrys), and refinement was carried out with both CNSX (22) and REFMAC5 in CCP4 (23). The x-ray data collection and refinement statistics are shown in Table 1 (Table 1). Note that the overall heterodimer structures we obtained for the two mutants are similar to the one reported previously (24), especially the RXRα part that contains 9-cis retinoic acid in the ligand-binding site and the coactivator peptide.

**In Vivo Profiling PPARγ Ligands—**KKA\text{y} male mice (5–7 weeks of age; Harlan) were prescreened for body weight and a range of clinical parameters (glucose, insulin, leptin, nonsterified free fatty acids, triglycerides, total cholesterol, and high density lipoprotein cholesterol). All blood sampling was performed from the retro-orbital plexus under ether anesthesia. The mice were singly housed and randomized into groups of \(n = 8\)/treatment group. Compounds were dissolved in ethanol and added to powdered diet (Purina 5053) and after drying overnight to allow for removal of ethanol, delivered to mice in preweighed food jars (day 1). On day 4 of the study, body weight change and food intake were determined, and blood was sampled and assayed for glucose and insulin. EDTA-treated blood samples were submitted for hematology and serum samples for clinical chemistry (Quality Clinical Labs, Mountain View, CA).

**RESULTS**

**T2384 Partially Activates PPARγ and Antagonizes Rosiglitazone**

Promoted Adipocyte Differentiation—
T2384 is chemically distinct from the thiazolidinedione class of PPARγ agonist (Fig. 1A) but binds to the receptor with an affinity similar to that of rosiglitazone with a \(K_i\) of 200 nM (Fig. 1B) and shows specificity toward PPARγ (data not shown). Despite a similar affinity for PPARγ, these two ligands show distinct profiles in cell-based assays for receptor activation. Cell-based reporter gene assays in which HEK293 cells were transiently transfected with an expression construct containing the PPARγ LBD fused to the Gal4-DNA-binding domain, together with a luciferase reporter gene under the transcriptional control of the Gal4 upstream activating sequence (Gal4-UAS), were used to measure ligand-dependent effects on transcription mediated by PPARγ. In these cotransfection assays, rosiglitazone activated transcription by 12-fold,
whereas in this same context, T2384 only partially activated the receptor (~3-fold) with an EC\textsubscript{50} value of 0.56 μM. Moreover, T2384 inhibited PPAR\textgamma transactivation in the presence of rosiglitazone (Fig. 1C), as expected for a partial agonist. Similar results have also been observed using a full-length receptor together with a luciferase reporter gene under the control of a DR1 sequence (data not shown).

PPAR\textgamma agonists are known to promote the conversion of a variety of preadipocyte cell lines and primary cells into mature adipocytes. Therefore, we compared the ability of rosiglitazone and T2384 to stimulate triglyceride accumulation in preadipocytes (Fig. 1D). Incubation of isolated human preadipocytes with rosiglitazone resulted in their efficient conversion to adipocytes, as indicated by dramatically increased lipid accumulation in these cells. In contrast, T2384 treatment stimulated very little lipid accumulation in these cells (Fig. 1D). T2384 was able to efficiently antagonize the ability of rosiglitazone to convert the preadipocytes into adipocytes (Fig. 1D), consistent with its effects in the reporter gene assay (Fig. 1C).

**T2384 Can Adopt Multiple Conformations in the Ligand-binding Pocket of PPAR\textgamma**—Binding of ligands to PPAR\textgamma triggers conformational changes that promote interactions between the receptor and an assortment of coregulatory proteins. These transcriptional regulatory proteins, including both coactivators and corepressors, mediate contact between the PPAR\textgamma-RXR heterodimer, chromatin, and the basal transcriptional machinery to promote activation or repression of target gene expression.

To study T2384-bound PPAR\textgamma conformations and their effects on receptor and transcriptional regulatory protein interactions, a homogeneous HTRF based assay was developed (19) using a panel of peptides derived from these regulatory proteins. Rosiglitazone showed an interaction profile characteristic of full agonists in these assays (Fig. 2). In contrast, T2384 induced a profile that was distinct both from rosiglitazone and a previously described PPAR\textgamma antagonist T0070907 (data not shown and Ref. 19). For example, when a peptide derived from coactivator protein DRIP205 was used, T2384 showed a partial agonist profile at concentrations less than 0.1 μM and an antagonist profile at higher concentrations (Fig. 2A). This biphasic profile is also observed when a peptide from the NCoR corepressor protein was used. The first partial agonist phase, where the NCoR peptide is displaced from the receptor, occurred at concentrations lower than 0.1 μM, and the second antagonist phase, where NCoR peptide is recruited to the receptor, occurred at higher concentrations (Fig. 2B). One possible explanation for these intriguing observations is that T2384 is structurally flexible, adopting multiple conformations with different intrinsic affinities to the ligand-binding pocket of PPAR\textgamma. We speculate that these different conformations of T2384 and their unique interactions with the receptor may be responsible for the different activities seen.

More direct evidence for multiple binding conformations came from x-ray crystallographic analysis of T2384-bound PPAR\textgamma crystals. We solved the cocystal structure of T2384 with the PPAR\textgamma ligand-binding domain to a resolution of 2.5 Å. Strikingly, the structure shows two crystallographically independent monomers, each with T2384 bound in a different conformation (Fig. 3, A and B). One monomer of PPAR\textgamma has one T2384 molecule in the ligand-binding pocket with T2384 wrapping around Cys\textsuperscript{285} from helix 3 in a U-shaped conformation (Fig. 3C), with B and C rings bind in a similar area as rosiglitazone (17). In this conformation, the A ring occupies the space between helix 7 and helix 3, which rosiglitazone does not exploit, and makes aromatic stacking interactions with Phe\textsuperscript{365}. Hydrogen bonds are formed between Lys\textsuperscript{367} in helix 7 and one of the sulfonamide oxygen atoms of T2384, whereas the B ring is sandwiched between Cys\textsuperscript{285} of helix 3 and Leu\textsuperscript{330} of helix 5. The region between helix 3 and the β-sheet forms a pocket into which the C ring binds, and this region is defined as the U pocket. The high affinity binding between T2384 and PPAR\textgamma results from the single hydrogen bond and numerous hydrophobic interactions between the receptor and the bound ligand. Unlike full agonists, such as the TZDs or tyrosine analogs (17, 24), T2384 shows no direct hydrogen bond interactions with the AF2 helix. Because stabilization of the AF2 helix in the activated conformation is a key characteristic of full agonism, the lack of direct interaction between this domain of the receptor and T2384 may explain the partial agonist/modulator activities of T2384 observed in vitro.

The crystal structure of the second PPAR\textgamma monomer revealed the unexpected finding that two molecules of T2384 bound simultaneously to the same ligand-binding pocket. The first molecule (shown in color light blue in Fig. 3, A, B, and D)
These crystallographic studies provide direct evidence that T2384 can bind to PPARγ in multiple conformations. Based on these observations, we speculate that these different conformations may exist in equilibrium in solution. To directly test this hypothesis, we generated mutations in different regions of the ligand-binding pocket to selectively affect the binding of T2384 conformers to the receptor. Coregulator recruitment was monitored to assess the functional consequences of these directed changes. To this end, two mutant PPARγ receptors were generated and subsequently expressed and purified from bacteria. Specific substitutions were selected based on identification of key contacts between T2384 in its various conformations and the ligand-binding domain of PPARγ.

The first mutant protein harbors three amino acid changes located in the S pocket, L228W, A292W, and L333W, and was designed to disrupt interactions between the C-ring of the T2384 S conformer and the receptor. The second PPARγ mutant contains a G284I substitution within the U pocket. Notably, both the mutant and wild-type proteins prepared in this manner behaved similarly during purification as well as crystallographic studies, suggesting that neither the structural integrity nor the biochemical properties of the mutants were globally affected by the amino acid substitutions.

The effects of these mutations on ligand binding were examined using both x-ray crystallography and coregulator recruitment assays. Notably, the profiles for both rosiglitazone-induced recruitment of the DRIP205 coactivator and displacement of NCoR corepressor were indistinguishable for the wild-type receptor and the L228W/A292W/L333W S pocket triple mutant (Fig. 4, A and C, respectively). In contrast, the characteristically biphasic association of NCoR in response to T2384 is markedly altered in the mutant receptor (Fig. 4D). In particular, the second phase of the response associated with NCoR recruitment at high concentrations of T2384 was completely eliminated in assays with the S pocket mutant, resulting in a dose-response profile reminiscent of rosiglitazone. Remarkably, crystallography revealed that the L228W/A292W/L333W mutations effectively prevented access of T2384 to the S pocket and allowed the intrinsically flexible ligand to bind PPARγ exclusively in the U pocket.
suggest that T2384 can adopt multiple binding conformations in the PPARγ LBD in solution and, furthermore, that these distinct binding modes lead to different biological activities.

**T2384 Improves Plasma Glucose and Insulin Levels and Blocks the Weight Gain and Anemia Caused by Rosiglitazone in KKAY Mice**—Because we speculated that different regions in the large PPARγ ligand-binding pocket may differentially affect its function, the in vivo effects of T2384 were evaluated in the KKAY mouse diabetes model to understand the functional consequences of its distinctive molecular pharmacology. Compounds were administered in the food for 4 days before the animals were sacrificed for terminal analysis. The food consumption was not significantly affected by either the addition of T2384 or rosiglitazone (data not shown). Compared with the vehicle-treated group, rosiglitazone (3 mg/kg) effectively reduced the fasting plasma glucose levels (Fig. 5A) and plasma insulin levels (Fig. 5B), indicating overall improvement in insulin sensitivity. Similarly, T2384 treatment improved insulin sensitivity with an efficacy comparable with that of rosiglitazone, lowering plasma levels of insulin and glucose in a dose-dependent fashion (30 and 100 mg/kg; Fig. 5, A and B).

![Figure 4](image-url)

**Figure 4. Mutational analysis to distinguish multiple binding modes for T2384.** A, mutations L228W/L292W/L333W do not affect rosiglitazone-induced recruitment of DRIP205 peptide to PPARγ in an HTRF assay. B–D, mutations L228W/L292W/L333W removed the second phase of the biphasic response curve induced by T2384 binding to PPARγ in an HTRF assay using NCoR peptide (D) and blocked the binding of T2384 into the S-pocket (B) but did not affect rosiglitazone induced displacement of NCoR peptide from the receptor (C). E–G, mutations G284I removed the first phase of the biphasic response curve induced by T2384 binding to PPARγ in an HTRF assay using NCoR peptide (G) and blocked the binding of T2384 into the U-pocket (E); in addition, it right-shifted the dose-response curve for rosiglitazone induced displacement of NCoR peptide from receptor (F). The blue mesh shown in B and E is the 2Fo − Fc electron density map (contoured at 1 σ) for the ligand and those highlighted residues. B and E show the structures of T2384-bound mutant proteins.

conformation. Taken together, these data suggest that the S pocket, a novel region in the ligand-binding domain of PPARγ defined initially by its interactions with T2384 may play an important role in mediating ligand-dependent conformational changes that facilitate recruitment of coregulator proteins, such as NCoR.

The converse was observed for the U pocket mutant G284I. When T2384 was crystallized with this mutant protein, the G284I substitution prevented binding of T2384 in the U conformation, whereas the S conformer was still observed to occupy the pocket (Fig. 4E). In HTRF assays, the U pocket mutation reduced the potency of rosiglitazone-mediated NCoR displacement curve by ~25-fold (Fig. 4F), suggesting that rosiglitazone is conformationally stable and, unlike T2384, may not be able to adopt alternative binding modes. T2384, on the other hand, still binds to the mutant receptor but only in the S conformation (Fig. 4E). Consequently, the biphasic profile was disrupted, and only the second phase, associated with occupancy of the S pocket of the LBD and antagonism of receptor activation, was observed (Fig. 4G). These results strongly suggest that T2384 can adopt multiple binding conformations in the PPARγ LBD in solution and, furthermore, that these distinct binding modes lead to different biological activities.

In contrast, the PPARγ-mediated side effects elicited by these two ligands were demonstrably different in this model. As shown in Fig. 5, rosiglitazone treatment resulted in an increased weight gain over the vehicle group, as well as a significant reduction in the red blood cell count at the end of the 4 day treatment. In contrast, treatment with T2384 at either dose did not increase body weight over the vehicle-treated animals, and in fact, the average body weight in the high dose groups was reduced compared with that of the control groups. Neither dose of T2384 caused anemia, as evidenced by red blood cell count that was unaffected by treatment (Fig. 5D). Importantly, when T2384 and rosiglitazone were coadministered, T2384 dampens the side effects of rosiglitazone, antagonizing both the body weight gain and red blood cell count reduction induced by rosiglitazone treatment (Fig. 5, C and D), without interfering with PPARγ-dependent effects on insulin sensitivity (Fig. 5, A and B). These striking results indicate that T2384 has retained
PPARγ Modulator

FIGURE 5. T2384 improves plasma glucose and insulin levels in KKAy obesity/diabetes mode without causing weight gain and anemia. Effects of T2384 and rosiglitazone on various parameters in KKAy mice after 4 days treatment. A, plasma glucose; B, insulin levels; C, body weight; D, red blood cell counts. Compounds were supplied in food as described under “Experimental Procedures,” and the doses are as indicated.

DISCUSSION

Nuclear receptors, including PPARγ, comprise a large family of ligand-dependent transcription factors that regulate the expression of target genes. The ability of these receptors to promote or suppress transcription of responsive genes is mediated by interaction with ligand and the assembly of multiprotein complexes of coregulatory factors. Upon binding of ligand, receptor conformation is altered and stabilized, and in the case of agonists, a binding cleft is exposed that allows the recruitment of transcriptional coactivators that potentiate the activation of target genes.

PPARγ displays several properties that may allow the receptor to adopt multiple activated states. First, structural studies have revealed that the ligand-binding domain of PPARγ contains a large, Y-shaped binding pocket that is almost twice the volume compared with that of other nuclear receptors. Agonists, such as rosiglitazone, bind in a U-shaped conformation within the binding site while occupying about 40% of the pocket volume. Direct interactions between the thiazolidinedione head group of rosiglitazone and the AF2 helix of PPARγ lock the receptor in an activated conformation to which coactivators can bind. In addition, because of its large volume, the PPARγ ligand-binding domain appears to be intrinsically mobile in solution and may be capable of assuming a continuum of conformations. Finally, experimental evidence suggests that different surfaces of PPARγ may interact with different coregulators. A variety of chemically different ligands have been shown to interact with the PPARγ ligand-binding domain and, depending on the molecular structure, induce distinct conformational changes in the receptor, resulting in differential interactions with coregulatory factors. Moreover, the ability to recruit coregulatory proteins to the receptor in a combinatorial fashion provides a mechanism by which the binding of ligand is transduced into a specific biological action. In this way, different ligands for the same nuclear receptor can elicit different biological responses.

In this report, we take advantage of the unique properties of a conformationally flexible PPARγ ligand, T2384, to further explore the relationship between biological activity and occupation of different regions of the binding pocket. Analysis of x-ray diffraction data from crystals of a ternary complex including the PPARγ LBD and ligand suggests that T2384 can bind to the receptor in two different conformations, which we have designated as the U and S binding modes. In the U mode, T2384 adopts a conformation similar to that reported for tyrosine analogs that fills the lower arm of the Y-shaped binding pocket as defined in these previous reports. However, in contrast to rosiglitazone, in which a network of hydrogen bonds forms directly between the thiazolidinedione heterocycle and the AF-2 helix to stabilize the activated conformation of the receptor, the interactions between T2384 and the receptor appear to be primarily hydrophobic in nature and do not include direct contacts with key residues in the AF-2 domain that are characteristic of agonists. X-ray crystallography has also revealed that T2384 can adopt an alternative conformation, the S binding mode, in which the benzothiazole group occupies the upper arm of the binding pocket.

Notably, these binding modes appear to have distinct effects on PPARγ in biochemical assays of receptor activation. In particular, the recruitment of sensor peptides derived from representative coregulatory factors exhibited a biphasic response as a function of T2384 concentration. At low concentrations of ligand, T2384 behaves as a partial agonist of PPARγ in these assays. Interactions between T2384 and PPARγ triggered the partial recruitment of the coactivator protein DRIP205-derived peptide, and displacement of corepressor protein NCoR-derived peptide from the receptor. In contrast, the second phase of T2384 activity is observed at higher ligand concentrations and is characterized by the release of DRIP205 from, and recruitment of NCoR to, the receptor. Despite exhibiting an affinity for the receptor that is nearly identical to that of rosiglitazone, interactions between T2384 and PPARγ result in a distinct, concentration-dependent profile for recruitment of coregulators.

We wondered whether this biphasic response might be caused by the two different binding sites with distinct binding affinities, corresponding to the U and S pockets of the PPARγ LBD described in the crystal structures. To test this hypothesis, we introduced mutations into the binding domain that selectively preclude the binding of ligand to either of the two arms of the binding pocket. Using this molecular genetic approach, we demonstrate that mutations in the ligand-binding domain that block occupancy of the S pocket and force T2384 to bind...
exclusively in the U conformation (Fig. 4, B–D) resulted in the disappearance of the second, lower affinity portion of the biphasic response curve. In the context of the mutant receptor, T2384 behaves as a conventional PPARγ partial agonist. However, when we introduced mutations in the LBD that blocked occupancy of the U pocket, T2384 binds only in the S conformation, and consequently, only the lower affinity, antagonist phase of the response curve was triggered upon binding to PPARγ (Fig. 4, E–G). These observations support the idea that the flexible scaffold of T2384 allows interaction with PPARγ in both S and U conformations. Moreover, by constraining the conformation of bound T2384 using mutational substitutions, we provide evidence that occupation of these two pockets can confer distinct effects on receptor activity.

This unique feature of T2384 has not been observed with other PPARγ ligands and suggests that T2384 may have the ability to modulate the receptor activity and function under different conditions or in different tissues, potentially resulting in a novel and more attractive in vivo profile. Indeed, in the KKAy mouse diabetes model, T2384 treatment lowered plasma glucose and insulin levels in a dose-dependent manner, and the magnitude of these effects was similar to those seen with rosiglitazone (Fig. 5, A and B). However, unlike rosiglitazone, T2384 treatment did not increase body weight and in fact, at the higher dose, T2384 actually induced a significant weight loss in the treatment group while maintaining the same food intake levels (Fig. 5C). Unlike rosiglitazone, however, T2384 treatment did not cause anemia (Fig. 5D). These effects are presumably receptor-mediated because coadministration of T2384 with rosiglitazone prevented both the body weight gain and anemia seen with rosiglitazone treatment alone, and therefore, T2384 acted as an antagonist in this context with respect to the receptor function. Overall, the data suggest that T2384 represents a novel class of PPARγ ligand that can restore insulin sensitivity without inducing the well known TZD-related side effects of weight gain and anemia.

These results suggest that, mechanistically, T2384 may generate a metabolic state that is more similar to that of the heterozygous PPARγ animals than to that of rosiglitazone. Instead of redistributing fatty acids from peripheral tissues to newly formed, more insulin-sensitive small fat cells, as has been suggested for rosiglitazone (4), T2384 may reduce fat accumulation and increase insulin sensitivity directly, either through improved adipocytokine production or fatty acid oxidation, or both. More detailed studies are needed to address these mechanistic aspects. In addition, whether T2384 may affect N-terminal modifications on PPARγ (26–28) and its stability could also be explored in the future.

Genetic evidence suggests that the insulin sensitization effects and the undesirable side effects induced by TZD treatment could potentially be decoupled (16). It appears that there may be an optimal PPARγ receptor activity level that would result in increased insulin sensitivity without undesirable effects, as seen in the PPARγ<sup>+/−</sup> heterozygous animals and the human genetic variations of P12A. However, to achieve this optimal receptor activity level pharmacologically might be a major challenge, because too much activity could result in side effects, and too little could result in insulin resistance, as seen in humans with certain dominant negative mutations of the receptor (P461L and V290M, 29) and also in PPARγ knock-out animals (3). A few other compounds, in addition to T2384, seem to have achieved this correct balance of receptor activity; for example, SR-202 (30), Fmoc (31), nTZDpa (32), and GW0072 (33) have all been described to induce insulin sensitivity without TZD-associated weight gain in animal models. It is interesting to note that from mass spectrometry studies, it was suggested that two molecules of Fmoc bind to PPARγ (31), and in the cases of nTZDpa and GW0072, x-ray crystallographic studies have shown that both of these molecules also occupy the S pocket (33). These results suggest an interesting possibility that the S pocket occupancy in the absence of direct interaction with AF2 helix might favor a correct balance of coactivator and corepressor binding and therefore achieve the optimal PPARγ activity that would result in the more desirable in vivo profile of improved insulin sensitivity without the undesirable side effects. The functional significance of the S pocket binding as well as the possibility of a structural-based drug design to identify novel PPARγ ligands will be the subject of future studies.

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