**Current Topics**

**Recent Progress in the Structural Understanding of Peroxisome Proliferator-Activated Receptor (PPAR)–Ligand Interaction**

Akihiro Honda,a Shotaro Kamata,a Chihiro Satta,a Yui Machida,a Kie Uchii,a Kazuki Terasawa,a Ayane Nemoto,a Takuji Oyama,b and Isao Ishii*a

*aDepartment of Health Chemistry, Showa Pharmaceutical University; Machida, Tokyo 194–8543, Japan; and bFaculty of Life and Environmental Sciences, University of Yamanashi; Kofu, Yamanashi 400–8510, Japan.

Received March 17, 2021; accepted June 11, 2021

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptor-type transcription factors that consist of three subtypes (α, γ, and β/δ) with distinct functions and PPAR dual/pan agonists are expected to be the next generation of drugs for metabolic diseases. Saroglitazar is the first clinically approved PPARα/γ dual agonist for treatment of diabetic dyslipidemia and is currently in clinical trials to treat non-alcoholic fatty liver disease (NAFLD); however, the structural information of its interaction with PPARα/γ remains unknown. We recently revealed the high-resolution co-crystal structure of saroglitazar and the PPARα-ligand binding domain (LBD) through X-ray crystallography, and in this study, we report the structure of saroglitazar and the PPARγ-LBD. Saroglitazar was located at the center of “Y”-shaped PPARα-ligand-binding pocket (LBP), just as it was in the respective region of PPARα-LBP. Its carboxylic acid was attached to four amino acids (Ser289/His323/His449/Thr473), which contributes to the stabilization of Activating Function-2 helix 12, and its phenylpyrrole moiety was rotated 121.8 degrees in PPARγ-LBD to interact with Phe264. PPARδ-LBD has the consensus four amino acids (Thr253/His287/His413/Tyr437) towards the carboxylic acids of its ligands, but it seems to lack sufficient space to accept saroglitazar because of the steric hindrance between the Trp228 or Arg248 residue of PPARδ-LBD and its methylthiophenyl moiety. Accordingly, in a coactivator recruitment assay, saroglitazar activated PPARα-LBD and PPARγ-LBD but not PPARδ-LBD, whereas glycine substitution of either Thr228, Arg248, or both of PPARδ-LBD conferred saroglitazar concentration-dependent activation. Our findings may be valuable in the molecular design of PPARα/γ dual or PPARα/γ/δ pan agonists.

**Key words** saroglitazar; peroxisome proliferator-activated receptor; dual agonist; X-ray crystallography; non-alcoholic fatty liver disease; steric hindrance

**INTRODUCTION**

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptor-type transcription factors that consist of three subtypes: PPARα, PPARγ, and PPAR(β/δ). These three subtypes have distinct tissue distributions, physiological roles, and ligand specificity.1,2 PPARα is predominantly expressed in the liver, kidney, heart, and muscle, and it controls the liver/skeletal muscle lipid metabolism and glucose homeostasis by regulating the expression of a variety of genes that are involved in general metabolism.2 PPARγ is most highly expressed in white/brown adipose tissues and regulates lipid uptake, adipogenesis, and insulin sensitivity.3 Meanwhile, PPARδ is ubiquitously expressed and has some physiological functions related to those of PPARα, such as energy dissipation (e.g., fatty acid β-oxidation), and those of PPARγ, such as insulin sensitivity enhancement.4 It is on account of these characteristics that PPARs have been used as therapeutic targets against various metabolic diseases.4 Fibrates such as fenofibrate and bezafibrate, which are synthetic PPARα agonists, are widely used to treat hypertriglyceridemia, whereas “thiazolidinediones (glitazones)” such as rosiglitazone and pioglitazone, which are synthetic PPARγ agonists, are anti-diabetic drugs with potent insulin-sensitizing effects that confer long-term glycemic control. PPARδ agonists are not yet clinically available, but it is expected that they will be used in the treatment of metabolic/cardiovascular diseases.5

Human PPARα/γ/δ–ligand binding pockets (LBPs) share 62–71% amino acid sequence identity and form similar “Y”-shape structures, enclosed by α-helices and β-sheets,3 that consist of the Center and Arm I–III regions (the nomenclature of LBD differs depending on the researcher; here we use our nomenclature5). The PPARα/γ-LBD pockets are significantly larger than the PPARδ-LBD pocket because of the narrowing of the pocket adjacent to the Activating Function-2 (AF-2) helix 126); the data describe the 1400 Å³, 1440 Å³, and 1300 Å³ LBP cavities for PPARα, PPARγ, and PPARδ, respectively.5 Although nearly 80% of the 34 residues defining LBP are conserved across the three subtypes, the slight differences between them could alter the ligand specificity.3 Several PPAR subtype-specific as well as dual/pan agonists have been developed so far for clinical purposes.3 Among them, PPAR
dual/pan agonists are expected to treat metabolic diseases including non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). NAFLD comprises a continuum of liver conditions, which vary in severity of injury and resulting fibrosis that extends it to its most advanced stage, NASH. In the United States, the number of patients with NAFLD is expected to increase from 83.1 million (approx. 25% of the population) in 2015 to 100.9 million in 2030. This rise will be accompanied by substantial increases in the number of patients with NASH, cirrhosis, hepatocellular carcinoma, and end-stage liver disease requiring liver transplantation, which may impose a large burden on society. Although many anti-NASH drug candidates have been developed, none of them have yet been approved. Among PPAR-targeted drugs, saroglitazar (α/γ dual agonist, Lipaglyn, ZYHI) and lanifibranor (α/β pan agonist, IVA-337) are currently in clinical trials aimed at studying their application for NAFLD and NASH. Saroglitazar is the first glitazar that has been granted marketing authorization in India for the treatment of diabetic dyslipidemia. It acts as a predominant PPARα agonist with moderate PPARγ agonist activity and improves lipid and glycemic parameters in patients with type 2 diabetes mellitus (T2DM) and NAFLD.

Although the pharmacological properties of saroglitazar have been well established (predominantly through research studies carried out in India), the structural information on its interactions with PPARs was lacking until our recent observations, which revealed, through X-ray crystallography, a high-resolution (2.03 Å) structure of human PPARα/γ-LBD and saroglitazar co-crystals. This study provides a novel structure of human PPARγ-LBD and saroglitazar co-crystals, which enables the comparative analysis of how saroglitazar binds or not to each PPAR subtype.

MATERIALS AND METHODS

PPARα/γ/δ-LBD Expression and Purification Human PPARα-LBD (amino acids 200–468), PPARγ-LBD (amino acids 203–477 in isofrom 1), and PPARδ-LBD (amino acids 170–441) were expressed as amino-terminal histidine (His)-tagged proteins from a pET28a vector (Novagen) in Rosetta (DE3) pLysS competent cells (Novagen) and purified using three-step chromatography as described in human PPARα-LBD preparations. Transformed cells were cultured in an LB medium (with 15 μg/mL kanamycin and 34 μg/mL chloramphenicol) at 30 °C, and 50 mL of overnight culture was seeded in 1 L of a TB medium (with 15 μg/mL kanamycin), and 50 mL of overnight culture was added to 1 L of a TB medium (with 15 μg/mL kanamycin) at 30 °C, and 50 mL of overnight culture was cultured at 30 °C for 1.5 h and then at 15 °C for 2 h. Protein overexpression was induced by adding 0.5 mM isopropyl β-D-thiogalactopyranoside, which was later cultured at 15 °C for 48 h. The cells were harvested and resuspended in 40 mL of buffer A (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM Tris 2-carboxyethylphosphine (TCEP)-HCl, and 10% glycerol) in the case of PPARα/γ or buffer A’ (20 mM Tris–HCl [pH 8.0], 500 mM ammonium acetate, 1 mM TCEP-HCl, and 10% glycerol) for PPARδ; both buffers contained a complete ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor (Sigma-Aldrich, St. Louis, MO, U.S.A.). The cells were then lysed by sonication five times, for 2 min each time, using a UD-201 sonicator (Tomy, Tokyo, Japan) at an output of 8; they were clarified using centrifugation at 12000 × g at 4 °C for 20 min (these conditions were used throughout the study unless otherwise noted); then, polyethyleneimine, with a final concentration of 0.15% (v/v), was added to the supernatant to remove nucleic acids. After centrifugation, 35 mL of the supernatant was mixed with 20 g of ammonium sulfate at 4 °C for 30 min using gentle rotation. After centrifugation, the pellet was resuspended in 30 mL of buffer B (for PPARα/γ) or B’ (for PPARδ); these buffers were based on buffer A and A’ with 10 mM imidazole added. The suspension was loaded on a cobalt-based immobilized metal affinity column (TALON Metal Affinity Resin, Clontech), equilibrated with buffer B (or B’) and eluted with a linear gradient of 10–100 mM imidazole. The PPARα/γ/δ-LBD-containing elutes were incubated with 33 U/mL thrombin protease (Nacalai Tesque, Kyoto, Japan) to cleave the His tag and, at the same time, dialyzed against buffer A overnight at 4 °C using a Slide-A-Lyzer G2 dialysis cassette (20 kDa cutoff, Thermo Fisher Scientific, Waltham, MA, U.S.A.). The sample was later dialyzed against buffer C, which was buffer A minus 150 mM NaCl (or buffer A minus 0.5 M ammonium acetate for PPARδ), at 4 °C for 3 h. The sample was then loaded onto a HiTrap Q anion-exchange column (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.), equilibrated with buffer C, and eluted with a linear gradient of 0–150 mM NaCl (or 0–0.5 M ammonium acetate for PPARδ). The elutes were loaded onto a HiLoad 16/600 Superdex 75 pg gel-filtration column (GE Healthcare), which had been equilibrated with buffer A (or A’), and further eluted with buffer A (or A’) Site-directed mutagenesis to create glycine substitution mutants of human PPARδ-LBD has been performed using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa Bio, Shiga, Japan).

PPAR Activation (PGC1α Coactivator Recruitment) Assay The activation status of each PPARα/γ/δ subtype can be determined using a time-resolved fluorescence resonance energy transfer (TR-FRET) assay, which is used to detect physical interactions between His-tagged human PPARα/γ/δ-LBD proteins and biotin-labeled PGC1α coactivator peptides (biotin-EAAEPSSLKKLLAPANTQ synthesized by GenScript) using a LANCE Ultra TR-FRET assay (PerkinElmer, Inc., Waltham, MA, U.S.A.). The 9.5 μL aliquot of PPARα/γ/δ-LBD (200 nM in buffer D for PPARα/γ-LBD or 400 nM in buffer E for PPARδ-LBD), 0.5 μL of a 100× ligand solution (in dimethyl sulfoxide (DMSO)), and 5 μL of biotin-PGC1α peptide (4 μM) were mixed in one well of a Corning 384 well low-volume, white-round-bottom, polystyrene non-binding surface microplate (buffer D comprised 10 mM N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid (HEPES)–NaOH [pH 7.4], 150 mM NaCl, 0.005% Tween 20, 0.1% fatty acid-free bovine serum albumin; buffer E: 50 mM HEPES–NaOH [pH 7.4], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% fatty acid-free bovine serum albumin). Then, 5 μL of 4 nM Eu-W1024-labeled anti-6×His antibody (PerkinElmer, Inc.)/80 nM ULight-Streptavidin (PerkinElmer, Inc.) was added to each well, and the microplate was incubated in the dark for 2 h at room temperature. FRET signals were detected at one excitation filter (340/12) and at two emission filters (615/12 and 665/12) using a Varioskan Flash double monochromator microplate reader (Thermo Fisher Scientific). The parameters for the measurements at 615 and 665 nm were an integration time of 200 μs and a delay time of 100 μs. The 665 nm emissions were due to ULight-FRET, and the 615 nm emissions were due to
Eu-W1024. The 665/615 ratio was calculated and normalized to the negative control reaction using 1% DMSO. The nonlinear fitting and calculation of half maximal effective concentration (EC$_{50}$) were performed using Prism 5 software (GraphPad, San Diego, U.S.A.). Saroglitazar ([S]-2-ethoxy-3-(4-(2-(2-methyl-5-(4-(methylthio)phenyl)-1H-pyrrol-1-yl)ethoxy)phenyl)propanoic acid), GW7647, and fenofibric acid were purchased from ChemScene (Monmouth Junction, NJ, U.S.A.), Cayman Chemical, and FUJIFILM Wako (Osaka, Japan), respectively. GW1929 and rosiglitazone were purchased from Sigma-Aldrich.

Co-crystallization of PPARγ-LBD and Saroglitazar

Co-crystallization was performed in hanging-drop mixtures of 0.5 µL PPARγ-LBD (20 mg/mL in buffer A), 0.5 µL ligand (2 mM in buffer A), and 1 µL reservoir solution (0.1 M Tris (pH 8.5), 1.2 M trisodium citrate, and 0.1 M magnesium chloride) at 20°C for several weeks. The obtained crystals were briefly soaked in a cryoprotection buffer (reservoir solution plus 30% glycerol); afterwards, these were flash-cooled in a stream of liquid nitrogen until the X-ray crystallography was conducted.

**Table 1. Data Collection and Refinement Statistics (Molecular Replacement)**

| Protein                      | Human PPARγ-LBD | Human PPARγ-LBD |
|------------------------------|-----------------|-----------------|
| Ligand                      | Saroglitazar    | Saroglitazar    |
| PDB ID                      | 7E0A            | not deposited   |
| Wavelength (Å)              | 1.00000         | 1.80000         |

**Data collection**

|                          | P4$_1$2$_2$2   | P4$_1$2$_2$2   |
|--------------------------|---------------|---------------|
| Space group              |               |               |
| Cell dimensions           |               |               |
| a, b, c (Å)              | 65.80, 65.80, 156.76 | 65.80, 65.80, 156.73 |
| a, β, γ (°)              | 90.00, 90.00, 90.00 | 90.00, 90.00, 90.00 |
| Resolution (Å)           | 46.53–1.77 (1.81–1.77) | 46.53–2.28 (2.36–2.28) |
| R$_{merge}$              | 0.062 (0.308)  | 0.137 (0.387)  |
| R$_{free}$               | 0.019 (0.109)  | 0.028 (0.078)  |
| CC$_{1/2}$               | 0.999 (0.969)  | 0.998 (0.990)  |
| I/σ                       | 23.8 (6.2)     | 19.4 (8.9)     |
| Completeness (%)         | 99.9 (99.1)    | 100.0 (100.0)  |
| Redundancy               | 10.8 (8.7)     | 25.0 (25.3)    |

**Refinement**

|                          |               |               |
| Resolution (Å)           | 32.198–1.771  |               |
| No. reflections          | 34250         |               |
| R$_{merge}$/R$_{free}$    | 0.1956/0.2205 |               |
| No. of atoms             |               |               |
| Protein                  | 2195          | 400000         |
| Ligand                   | 31            |                |
| Water                    | 87            |                |
| B-factors                | 28.04         |                |
| Protein                  | 28.00         |                |
| Ligand                   | 26.53         |                |
| Water                    | 29.68         |                |
| Ramachandran plot (%)    |               |                |
| Favored                  | 98.48         |                |
| Allowed                  | 1.14          |                |
| Outliers                 | 0.38          |                |
| R.M.S. deviations        |               |                |
| Bond lengths (Å)         | 0.009         |                |
| Bond angles (°)          | 1.01          |                |

Values in parentheses are for the highest-resolution shell.
The structure was performed using iterative cycles of model adjustment in two programs: COOT and PHENIX. Feature Enhanced Maps (FEMs) were calculated using PHENIX. The structures were constructed using PyMOL programs. All collection data and refinement statistics are included in Table 1. The human PPARγ-LBD–saroglitazar structure was deposited in the PDB (ID: 7E0A).

RESULTS

Saroglitazar Activates PPARα/γ but Not PPARδ

Reinaud et al. proposed an activation model of the ligand-dependent transcription factors (including PPARs). In this model, which has been widely accepted, the AF-2 helix 12 approaches the LBD in response to ligand binding, and the resulting active form of the receptor can bind with a coactivator (peptide), which in turn stabilizes its active form. Therefore, we evaluated PGC1α coactivator recruitment activities toward the three PPARα/γ/δ-LBD subtypes using a TR-FRET assay as measures of ligand-dependent activation of PPARs. Fenofibric acid, the pharmacological active metabolite of triglyceride-lowering fenofibrate, activated PPARα with an EC50 of 8.93 µM and PPARγ with that of over 30 µM (Fig. 1A). GW7647, the PPARα-selective agonist with approx. 200-fold selectivity over PPARγ/δ, displayed PPARα activation with an EC50 of 51.8 nM, which was 3–3.5 orders lower than those for PPARγ/δ (Fig. 1B). Rosiglitazone, a glitazone-type antidiabetic drug, activated PPARγ but not PPARα/δ (Fig. 1C).

Fig. 1. Differential Activation of PPARα/γ/δ Subtypes by Fenofibric Acid, GW7647, Rosiglitazone, GW1929, and Saroglitazar

The activation status of human PPAR subtypes (α, γ, and δ) by fenofibric acid (A), GW7647 (B; a PPARα-selective agonist), rosiglitazone (C), GW1929 (D; a PPARγ-selective agonist), and saroglitazar (E; a PPARα/γ dual agonist) was examined using a TR-FRET-based PGC1α coactivator recruitment assay. The activity was expressed as fold induction of the control (no agonist) level. Data are means ± standard errors of 3–4 experiments with duplicate samples. The calculated EC50 values are presented.
Fig. 2. Structure of PPARγ-LBD–Saroglitazar Co-crystals

(A, C) Overall crystal structure (left) and its magnified view (right) of human PPARγ-LBD–saroglitazar complex (A) and PPARα-LBD–saroglitazar complex (C). The electron density is illustrated in the mesh by Feature Enhanced Maps (FEMs) contoured at 1.0σ. PDB identities and resolutions are labeled, and water molecules are presented as cyan spheres. (B, D) Magnified views of saroglitazar in PPARγ-LBD (B) and PPARα-LBD (D) are superimposed with sulfur signals located using anomalous difference Fourier maps, conducted with a 1.8 Å X-ray. The map is illustrated in the mesh, and water molecules are presented as cyan spheres. (E) Superimposed pictures of PPARγ-LBD–saroglitazar (cyan) and PPARα-LBD–saroglitazar (pink) complex structures. The phenylpyrrole moieties (surrounded by dotted lines) of saroglitazar are rotated 121.8 degrees in PPARγ-LBD. The PPARα-LBD–saroglitazar structures (Figs. 2C, D) were reprinted with permission from Kamata et al.3) (Color figure can be accessed in the online version.)
Fig. 3. Structural Basis for Saroglitazar as a PPARα/γ Dual Agonist

(A) Ligand binding cavities composed of Arm I–III and Center regions (in meshes) of PPARα-LBD (pink), PPARδ-LBD (green), and PPARγ-LBD (cyan) and their ligands (saroglitazar for α and γ, and GW0742 for δ). The originals and their rotated views illustrate their location within the Arm II and Center regions. (B) Possible saroglitazar interactions (speculated from the merged four images) with the surrounding amino acid residues of PPARδ-LBD through its carboxylic acid and methylthiophenyl moieties. Both PPARα-LBD (PDB ID: 6LXC from our previous study) and PPARγ-LBD (PDB ID: 7E0A from this study) have enough space to accept saroglitazar; however, it is difficult to similarly locate saroglitazar in the two typical types of PPARδ-LBD that have altered Trp228 locations (PDB ID: 3TKM, the complex with GW0742, and 3GWX, the complex with EPA) because of the steric hindrance between Trp228 (or Arg248) and its methylthiophenyl moiety. The calculated shortest distances (Å) between the methylthiophenyl moiety and Trp228 (or Arg248) of PPARδ-LBD are indicated. (Color figure can be accessed in the online version.)
GW1929, a non-glitazone-type PPARγ-selective agonist, exhibited PPARγ activation with an EC50 of 32.2 nM, which was approximately three orders lower than that of PPARδ; it did not activate PPARα (Fig. 1D). Saroglitazar activated both PPARα/γ but not PPARδ like fenofibric acid; however, it activated PPARγ at the lower concentrations and PPARα up to the higher levels, which is the opposite of fenofibric acid (Fig. 1E).

Overall Structure of Human PPARγ-LBD–Saroglitazar Complex

Although there has been limited structural information for the human PPARα-LBD–ligand compared with that for PPARγ-LBD (with only 21 PDB records for PPARα in contrast to 224 for PPARγ as of September 2020), we recently supplemented 34 novel high-resolution PPARα-LBD structures of complexes with 17 ligands including saroglitazar using X-ray crystallography with various crystallization techniques such as co-crystallization, cross-seeding, soaking, delipidation, and coactivator peptide supplementation. To obtain human PPARγ-LBD–saroglitazar co-crystals, we screened various co-crystallization buffer conditions that were based on previous studies that had reported high-resolution structures of PPARγ-LBD–ligand co-crystals formed in sodium citrate-based buffers. We investigated various sodium citrate, pH, and metal salt concentrations and finally obtained co-crystals that had a good appearance in the buffer made up of 0.1 M Tris–HCl (pH 8.5), 1.2 M trisodium citrate, and 0.1 M magnesium chloride. Crystallography of the co-crystals using a 1.0 Å X-ray gave a high-resolution structure (1.77 Å; Table 1) as a single unit complex of PPARα-LBD and saroglitazar, which forms the AF-2 helix, a hallmark of the active PPAR conformations (Fig. 2A). Saroglitazar includes a single sulfur atom in its molecule, and anomalous difference Fourier maps, which were obtained using a 1.8 Å X-ray (Table 1), detected sulfur signals from saroglitazar and its surrounding methionine/cysteine (Cys285, Met348, and Met364) in the proximal positions that conventional 1.0 Å X-ray crystallography was deployed (Fig. 2B). When compared with the human PPARα-LBD–saroglitazar structures that we had previously obtained (Figs. 2C, D), the phenylpyrrole moiety was rotated 121.8 degrees in PPARγ-LBD from that in PPARα-LBD (Fig. 2E).

Structural Basis for Saroglitazar as a PPARα/γ but Not PPARδ Agonist

To understand the PPAR subtype specificity of saroglitazar, the obtained structures were compared with the reported structure of human PPARδ-LBD and its ligand GW0742 (PDB ID: 3TKM). Saroglitazar is located at the Center and Arm II regions of PPARα-LBD and PPARγ-LBD (Fig. 3A). Both saroglitazar and

Fig. 4. Activation of PPARδ Mutants (W228G, R248G, and W228G/R248G) by Saroglitazar

The activation status of human (wild-type: WT) PPARδ and its three mutants (W228G, R248G, and W228G/R248G) by saroglitazar was examined using a TR-FRET-based PGC1α coactivator recruitment assay. The activity was expressed as fold induction of the control (no agonist) level. Data are means ± standard errors of 4 experiments with duplicate samples.

Fig. 5. Merged Pictures of Three PPARα/γ Dual Agonists in PPARα-LBD and PPARγ-LBD

The co-crystal structures of PPARα-LBD (left) and PPARγ-LBD (right) bound with the three PPARα/γ dual agonists, saroglitazar (pink), tesaglitazar (blue), and aleglitazar (green), are presented. The shortest distances (Å) between these molecules and Cys275 in PPARα-LBD or Gly284 in PPARγ-LBD (both as the counterpart of Arg248 in PPARδ-LBD) are indicated in the same color. The structures were obtained from the PDB (IDs are indicated). All three PPARα/γ dual agonists are located at similar positions of PPARα-LBD and PPARγ-LBD by avoiding the steric hindrance with side residues of these amino acids. (Color figure can be accessed in the online version.)
GW0742 have a single carboxylic acid in their molecules; this is firmly attached to the surrounding consensus four amino acids (Ser289/Tyr314/His440/Tyr464 for PPARα; Thr253/His287/His413/Tyr437 for PPARγ; and Ser289/His323/His449/Tyr473 for PPARγ, Fig. 3B) to stabilize the AF-2 helix 12. The relatively large Arm II domains of PPARα-LBD and PPARγ-LBD would be able to accept the methylthiophenyl moiety of saroglitazar; however, this might not be possible with the relatively small Arm II domains of PPARδ-LBD due to the steric hindrance between that moiety and the Trp228 or Arg248 in PPARδ-LBD (only 1.7 Å from Trp228 in case of the same saroglitazar location within PPARα-LBD and 2.1/2.2 Å from Arg248 in case of the same saroglitazar location within PPARγ-LBD; Fig. 3B). Wu et al. reported that the PPARδ-LBD helices 2–3 region containing Trp228 is flexible and is capable of interacting with various ligands by changing its conformation although the interaction between Trp228 and Arg248 could stabilize the helices 2–3.21,23 When we check the location of Trp228 side residues in all 44 PPARδ-LBD structures deposited in the PDB, Trp228 side residues were located in the positions quite proximal (almost identical) to that of PDB: 3TKM in 14 structures, and in the another positions quite proximal (almost identical) to that of PDB: 5U3Y in 20 structures; Arg248 positions are almost identical (Fig. 3B). The steric hindrance seems to exist even if Trp228 or Arg248 is in the position of PDB: 5U3Y (1.2 and 2.2/2.3 Å respectively; Fig. 3B). In accordance with this, glycine substitution of either Trp228, Arg248, or both in PPARδ-LBD conferred saroglitazar concentration-dependent activation in the TR-FRET assay (Fig. 4). Saroglitazar was located at a similar position to those of the other PPARα/γ dual agonists (aleglitazar and tesaglitazar) in both PPARα-LBD and PPARγ-LBD by avoiding the steric hindrance against Cys275 in PPARα-LBD or Gly284 in PPARγ-LBD (the counterpart of Arg248 in PPARδ-LBD) (Fig. 5).

**DISCUSSION**

The molecular design of PPARα/γ dual agonists is of major medical interest, as these drugs may combine the benefits of both fibrate- and glitazone-class drugs within a single molecule, and they are expected to be free of fibrate-related serious side effects, such as rhabdomyolysis, and glitazone-related adverse effects, including edema, bone fracture, and heart failure. To date, several PPARα/γ dual agonists have progressed to late-phase clinical trials, including muraglitazar, tesaglitazar, aleglitazar, and MK0767. All of these drugs normalized glucose/lipid abnormalities in patients with T2DM when used as a monotherapy or combined therapy with other glucose-lowering drugs, but most of them had serious safety concerns. Similarly, navelgitazar, ONO-5129, and DSP8658 (other PPARα/γ dual agonists) also suffered similar setbacks early in the clinical pipeline and are no longer in development.

It is within this context that saroglitazar has been developed and clinically approved in India for the treatment of diabetic dyslipidemia. A recent meta-analysis has indicated that saroglitazar produces significantly lower triglycerides and fasting glucose when compared with those in controls who received placebo/diabetes medications but not in those who received anti-lipid medications (statins/fibrates). It also indicated that no significant changes can be observed in blood hemoglobin A1c (HbA1c), total/low density lipoprotein (LDL)/high density lipoprotein (HDL) cholesterol, and apolipoprotein-B. With saroglitazar, there were no serious safety concerns, such as edema, carcinogenic potential, and cardiovascular side effects, except for a significant increase in serum creatinine. Another recent study noted that saroglitazar treatment effectively improved glycemic control and lipid parameters over a 56-week period in patients with T2DM who received background metformin therapy. Saroglitazar was “Non Inferior” in terms of its improved HbA1c levels, as well as body weight gains as a side effect, compared with pioglitazone. These data suggest that saroglitazar reduces blood triglycerides in a similar manner to fibrates and that it has the potential to treat diabetic dyslipidemia, like pioglitazone, without serious side effects.

Some clinical and observational studies have suggested that saroglitazar could improve the parameters of NAFLD/NASH. Saroglitazar (4 mg once daily) reduced serum triglyceride and improved liver stiffness and other glycemic/lipid parameters in patients with T2DM and NAFLD. In a diet-induced NASH model in mice, saroglitazar (4 mg/kg/d, oral gavage) improved hepatic steatosis, lobular inflammation, hepatocellular ballooning, and the fibrosis stage, which resulted in the resolution of NASH. In the U.S.A., saroglitazar applications are currently in a Phase 3 clinical trial for patients with NAFLD/NASH (in combination with vitamin E supplementation and lifestyle modification), two Phase 2 trials for liver transplant recipients with NAFLD and NAFLD in women with polycystic ovarian syndrome, and two Phase 1 trials (ClinicalTrials.gov: https://clinicaltrials.gov/ct2/home). The present study, along with our recent study, has provided the structural basis for such saroglitazar as a PPARα/γ dual agonist.

In contrast to GW7647/rosiglitazone/GW1929, and similar to fenofibric acid, saroglitazar activated both PPARα/γ but not PPARδ (Fig. 1). The EC_{50} value of saroglitazar for PPARγ (1.48 µM) was slightly lower than that for PPARα (5.66 µM), which was the opposite of fenofibric acid, and was much higher than that of rosiglitazone for PPARγ (168 nM). The EC_{50} value of saroglitazar for PPARα (5.66 µM) was equivalent to that of fenofibric acid (8.93 µM). Saroglitazar was located at the Center and Arm II regions of PPARγ-LBD (Figs. 2A, 3A), as it had been in the respective regions of PPARα-LBD (Figs. 2C, 3A), with its carboxylic acid firmly attached to the surrounding consensus four amino acids (Ser289/His323/His449/Tyr473) and its methylthiophenyl moiety attached to Phe264 (PDB ID: 7E0A; Fig. 3B). Such placement could not happen in PPARδ-LBD from the steric hindrance between that moiety and Trp228 or Arg248 irrespective of flexible locations of Trp228 as observed in other PPARδ-LBD structures (Fig. 3B).

The co-crystal structures of human PPARα/γ-LBD with other PPARα/γ agonists, namely aleglitazar and tesaglitazar, have been reported, which are quite similar to the PPARα/γ-LBD–saroglitazar structures in this study (Fig. 5). To avoid the serious side effects of PPARγ full agonism, PPARα partial agonists or even antagonists have been developed. All such partial agonists/antagonists have been designed in such a way that they do not associate with the consensus Ser289/His323/His449/Tyr473 residues, which bind to the ligand’s carboxylic acid; they have reduced PPARγ transcripational activity while retaining the insulin-sensitizing effects. Many anti-diabetic PPARγ ligands have been dem-
onstrated to have a distinct function that is independent of classical transcriptional agonism; some ligands block the obesity-linked phosphorylation of the Ser273 residue of PPARγ located in Arm II that faces the outside) by cyclin-dependent kinase 5 (Cdk5). Notably, SR1664 lacks PPARγ transcriptional agonism but blocks Cdk5-mediated PPARγ phosphorylation in cultured adipocytes and insulin-resistant mice, and it has potent insulin-sensitizing effects without serious side effects. Furthermore, the alanine mutation of PPARγ Ser273 in mice offers protection against insulin resistance in obesity and other serious side effects. Although only the positive side of saroglitazar has been emphasized in published literature so far, there is substantial PPARγ transactivation activity with saroglitazar, and therefore, saroglitazar should be used with caution.

In conclusion, this study has revealed a high-resolution structure of PPARα-LBD and saroglitazar co-crystals compared with the PPARα-LBD–saroglitazar structure that our recent study reported. Our findings elucidate a molecular basis for its selectivity toward the three PPAR subtypes and might contribute to the molecular design of PPAR dual/pan agonists (e.g., through modification of the methylthiophenyl moiety of saroglitazar).

Acknowledgments S.K. and I.I. acknowledge funding from Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (Grant numbers: 19K16359 [S.K.] and 16H05107 [I.I.]), the Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research [BINDS]) from AMED (Grant No. JP19am010107I; Support No. 1407 [S.K. and I.I.]), and a Grant-in-Aid for Young Scientists of Showa Pharmaceutical University [S.K.]. T.O. acknowledges funding from a Grant-in-Aid for Scientific Research from JSPS (Grant number: 18K06081) and the Adaptable and Seamless Technology Transfer Program through Target-driven R&D (A-STEP) from the Japan Science and Technology Agency (Grant No. JPMJTM19AT). This work was performed under the approval of the Photon Factory Program Advisory Committee (Proposal No. 2018G658). A.H. is supported by a scholarship from the Tokyo Biochemical Research Foundation.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Zoete V, Grosdidier A, Michielin O. Peroxisome proliferator-activated receptor structures: ligand specificity, molecular switch and interactions with regulators. Biochim. Biophys. Acta, 1771, 915–925 (2007).
2) Lefebvre P, Chinetti G, Fruchart JC, Staels B. Sorting out the roles of PPARα in energy metabolism and vascular homeostasis. J. Clin. Invest., 116, 571–580 (2006).
3) Ahmadian M, Suh JM, Han N, Liddle C, Atkins AR, Downes M, Evans RM. PPARα signaling and metabolism: the good, the bad and the future. Nat. Med., 19, 557–566 (2013).
4) Dubois V, Eckhoute J, Lefebvre P, Staels B. Distinct but complementary contributions of PPAR isoforms to energy homeostasis. J. Clin. Invest., 127, 1202–1214 (2012).
5) Kamata S, Oyama T, Saito K, Honda A, Yamamoto Y, Suda K, Ishikawa R, Itoh T, Watanabe Y, Shibata T, Uchida K, Suematsu M, Ishi I. PPARα ligand-binding domain structures with endogenous fatty acids and fibrates. iScience, 23, 101727 (2020).
6) Xu HE, Lambert MH, Montana VG, Plunket KD, Moore LB, Collins JL, Oplinger JA, Kliewer SA, Gaume RT Jr, McKee DD, Moore JT, Willson TM. Structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors. Proc. Natl. Acad. Sci. U.S.A., 98, 13919–13924 (2001).
7) Cheng HS, Tan WR, Low ZS, Marvalin CM, Lee JYH, Tan NS. Exploration and development of PPAR modulators in health and disease: an update of clinical evidence. Int. J. Mol. Sci., 20, 5055 (2019).
8) Friedman SL, Neuschwander-Tetri BA, Pinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Nat. Med., 24, 908–922 (2018).
9) Agrawal R. The first approved agent in the Glitazar’s Class. Saroglitazar. Curr. Drug Targets, 15, 151–155 (2014).
10) Jain RH, Kansagra K, Jain MR, Patel H. Pharmacokinetics, safety, and tolerability of saroglitazar (ZYHI), a predominantly PPARδ agonist with moderate PPARγ agonist activity in healthy human subjects. Clin. Drug Investig., 33, 809–816 (2013).
11) Mitra A. An observational study of reduction in glycemic parameters and liver stiffness by saroglitazar 4mg in patients with type 2 diabetes mellitus and nonalcoholic fatty liver disease. Curr. Rev., 12, e9965 (2020).
12) Roy S. Clinical case series of decrease in shear wave elastography values in ten diabetic dyslipidemia patients having NAFLD with saroglitazar 4mg: an Indian experience. Case Rep. Med., 2020, 4287075 (2020).
13) Kaul U, Parmar D, Manjunath K, Shah M, Parmar K, Patil KP, Jaisswal A. New dual peroxisome proliferator activated receptor agonist-Saroglitazar in diabetic dyslipidemia and non-alcoholic fatty liver disease: integrated analysis of the real world evidence. Cardiovasc. Diabetol., 18, 80 (2019).
14) Kamata S, Oyama T, Ishi I. Preparation of co-crystals of human PPARα-LBD and ligand for high-resolution X-ray crystallography. STAR Protoc., 2, 100364 (2021).
15) Liu Q, Dahmene T, Zhang Z, Assur Z, Brasch J, Shapiro L, Mancia F, Hendrickson WA. Structures from anomalous diffraction of native biological macromolecules. Science, 336, 1033–1037 (2012).
16) Kabsch W. XDS. Acta Crystallogr. D Biol. Crystallogr., 66, 125–132 (2010).
17) Evans PR, Murshudov GN. How good are my data and what is the resolution? Acta Crystallogr. D Biol. Crystallogr., 69, 1204–1214 (2013).
18) McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. J. Appl. Crystallogr., 46, 658–674 (2013).
19) Famsley J, Cowtan K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr., 66, 2126–2132 (2020).
20) Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr., 66, 213–221 (2010).
21) Renaud JP, Rochel N, Low ZS, Marvalin C, Lee JYH, Chambon P, Gronemeyer H, Moras D. Crystal structure of the RAR-γ ligand-binding domain bound to all-trans retinoic acid. Nature, 378, 681–689 (1995).
22) Brown PJ, Stewart LW, Hurley KP, Lewis MC, Winegar DA, Wilson JT. Wilkison WO, Ittoop OR, Willson TM. Identification of a sub-type selective human PPARα agonist through parallel-array synthesis. Bioorg. Med. Chem. Lett., 11, 1225–1227 (2001).
23) Brown KK, Henke BR, Blanchard SG, et al. A novel N-aryl tyrosine activator of peroxisome proliferator-activated receptor-gamma
reverses the diabetic phenotype of the Zucker diabetic fatty rat. Diabetes, 48, 1415–1424 (1999).

24) Jang JY, Bae H, Lee YJ, Choi YI, Kim HJ, Park SB, Suh SW, Kim SW, Han BW. Structural basis for the enhanced anti-diabetic efficacy of lobeglitazone on PPARγ. Sci. Rep., 8, 31 (2018).

25) Jiang H, Zhou XE, Shi J, Zhou Z, Gao C, Zhang X, Sun Y, Suino-Powell K, Ma L, Gao H, Yu X, Li J, Li J, Melcher K, Xu HE, Yi W. Identification and structural insight of an effective PPARγ modulator with improved therapeutic index for anti-diabetic drug discovery. Chem. Sci., 11, 2260–2268 (2020).

26) Batista FA, Trivella DB, Bernardes A, Gratieri J, Oliveira PS, Figueira AC, Webb P, Polikarpov I. Structural insights into human peroxisome proliferator activated receptor delta (PPAR-delta) selective ligand binding. PLOS ONE, 7, e33643 (2012).

27) Wu CC, Baiga TJ, Downes M, La Clair JJ, Atkins AR, Richard SB, Fan W, Stockley-Noel TX, Bowman ME, Noel JP, Evans RM. Structural basis for specific ligand of the peroxisome proliferator-activated receptor δ. Proc. Natl. Acad. Sci. U.S.A., 114, E2563–E2570 (2017).

28) Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ, Stertnbach DD, Lehmann JM, Wisely GB, Willson TM, Kliwer SA, Milburn MV. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol. Cell, 3, 397–405 (1999).

29) Bénardeau A, Benz J, Binggeli A, Blum D, Boehringer M, Grether U, Hilpert H, Kuhn B, Märki HP, Meyer M, Puntener K, Raab S, Schlatter D, Mohr P. Aleglitazar, a new, potent, and balanced dual PPARα/γ agonist for the treatment of type II diabetes. Bioorg. Med. Chem. Lett., 19, 2468–2473 (2009).

30) Cronet P, Petersen JF, Folmer R, Blomberg N, Sjoblom K, Karlsson U, Lindstedt EL, Bamberg K. Structure of the PPARα and -γ ligand binding domain in complex with AZ 242; ligand selectivity and agonist activation in the PPAR family. Structure, 9, 699–706 (2001).

31) Willson TM, Brown PJ, Stertnbach DD, Henke BR. The PPARs: from orphan receptors to drug discovery. J. Med. Chem., 43, 527–550 (2000).

32) Dutta D, Bhattacharya S, Sarana V, Aggarwal S, Singla R, Khandelwal D, Sharma M. Efficacy and safety of saroglitazar in managing hypertriglyceridemia in type-2 diabetes: A meta-analysis. Diabetes Metab. Syndr., 14, 1759–1768 (2020).

33) Krishnappa M, Patil K, Parmar K, Trivedi P, Mody N, Shah C, Fald U, Maroo S, Parmar D. Effect of saroglitazar 2 mg and 4 mg on glycemic control, lipid profile and cardiovascular disease risk in patients with type 2 diabetes mellitus: A 36-week, randomized, double blind, phase 3 study (PRESS XII study). Cardiovasc. Diabetol., 19, 93 (2020).

34) Kumar DP, Caffrey R, Marineaux J, Santhekadur PK, Bhat M, Alonso C, Koduru SV, Philip B, Jain MR, Giri SR, Bedossa P, Sanyal AJ. The PPAR α/γ agonist saroglitazar improves insulin resistance and steatohepatitis in a diet induced animal model of nonalcoholic fatty liver disease. Sci. Rep., 10, 9350 (2020).

35) Kröker AJ, Bruning JB. Review of the structural and dynamic mechanisms of PPARα partial agonism. PPAR Res., 2015, 816856 (2015).

36) Choi JH, Banks AS, Estall JL, Kajimura S, Boström P, Laznik D, Ruas JL, Chalmers MJ, Kamenecka TM, Blüher M, Griffin PR, Spiegelman BM. Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARγ by Cdk5. Nature, 466, 451–456 (2010).

37) Choi JH, Banks AS, Kamenecka TM, Busby SA, Chalmers MJ, Kumar N, Kuruvilla DS, Shin Y, He Y, Bruning JB, Marciano DP, Cameron MD, Laznik D, Jurczak MJ, Schütz SC, Vidovic D, Shalman GI, Spiegelman BM, Griffin PR. Antidiabetic actions of a non-agonist PPARγ ligand blocking Cdk5-mediated phosphorylation. Nature, 477, 477–481 (2011).

38) Hall JA, Ramachandran D, Roh HC, et al. Obesity-linked PPARγ S273 phosphorylation promotes insulin resistance through growth differentiation factor 3. Cell Metab., 32, 665–675.e6 (2020).