Please cite as:
Jademilson Celestino os Santos, Amanda Bernardes, Letizia Giampietro, Alessandra Ammazzalorso, Barbara De Filippis, Rosa Amoroso, Igor Polikarpov (2015), Different binding and recognition modes of GL479, a dual agonist of Peroxisome Proliferator-Activated Receptor α/γ, Journal of Structural Biology 191 (2015) 332–340
doi: http://dx.doi.org/10.1016/j.jsb.2015.07.006

Different binding and recognition modes of GL479, a dual agonist of Peroxisome Proliferator-Activated Receptor α/γ

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a b s t r a c t

Peroxisome Proliferator-Activated Receptors (PPARs) are ligand-dependent transcription factors that control various functions in human organism, including the control of glucose and lipid metabolism. PPARγ is a target of TZD agonists, clinically used to improve insulin sensitivity whereas fibrates, PPARα ligands, lower serum triglyceride levels. We report here the structural studies of GL479, a synthetic dual PPARα/γ agonist, designed by a combination of clofibric acid skeleton and a phenyldiazenyl moiety, as bioisosteric replacement of stilbene group, in complex with both PPARα and PPARγ receptors. GL479 was previously reported as a partial agonist of PPARγ and a full agonist of PPARα with high affinity for both PPARs. Our structural studies reveal different binding modes of GL479 to PPARα and PPARγ, which may explain the distinct activation behaviors observed for each receptor. In both cases the ligand interacts with a Tyr located at helix 12 (H12), resulting in the receptor active conformation. In the complex with PPARα, GL479 occupies the same region of the ligand-binding pocket (LBP) observed for other full agonists, whereas GL479 bound to PPARγ displays a new binding mode. Our results indicate a novel region of PPARs LBP that may be explored for the design of partial agonists as well dual PPARα/γ agonists that combine, simultaneously, the therapeutic effects of the treatment of insulin resistance and dyslipidemia.

1. Introduction

The lifestyle of the 21st century, characterized by people sedentary routine and diets rich in fat and carbohydrates, has contributed to an increase in the occurrence of metabolic syndromes, including type 2 diabetes mellitus, dyslipidemia, obesity and other cardiovascular diseases (Eckel et al., 2005). Several of the metabolic pathways involved in these disorders are regulated by Peroxisome Proliferator-Activated Receptors (PPARs), which are members of the superfamily of the nuclear receptors and function as transcription factors activated by several synthetic and natural ligands (Forman et al., 1997). These receptors form heterodimers with the Retinoid X Receptor (RXR) and the heterodimerization is essential for the recruitment of coregulators and consequent initiation of transcription (Auwerx, 1999; Berger and Moller, 2002; Michalik et al., 2006; Rastinejad et al., 2015). There are three PPAR isotypes, encoded by distinct genes and designated as PPARα, PPARβ/δ and PPARγ. PPARα is expressed predominantly in metabolically active tissues, including liver, adipose tissue, kidney and heart. PPARβ/δ expression occurs in a wide variety of tissues and cells, with relatively higher levels in adipose tissue, brain, liver and skin. PPARγ expression is abundant in fatty tissues, liver and heart (Auwerx, 1999; Auwerx et al., 2003; Kota et al., 2005).

Among the three isotypes, PPARα and PPARγ have been the most widely studied, as they have important roles in regulating glucose, lipids and cholesterol metabolism as well as in the fatty acid b-oxidation and homeostasis. It makes these receptors important pharmacological targets of the drugs used for treatment of type 2 diabetes mellitus (T2DM) and dyslipidemia (Harmon et al., 2011; Mandard et al., 2004). The PPARα is a molecular target for fibrates, a class of hypolipidemic drugs used in the treatment of

Abbreviations: PPAR, Peroxisome Proliferator-Activated Receptor; LBD, ligand binding domain; DMSO, dimethyl sulfoxide; GL479, (*E*)-2-methyl-2-(4-(2-(4-(phenyldiazenyl)phenoxy)ethyl)phenoxy)propanoic acid; DTT, dithiothreitol; IPTG, Isopropyl b-D-1-thiogalactopyranoside; HDL, high density lipoprotein; LBP, ligand binding pocket.

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dyslipidemia in humans. PPARα activation by fibrates decreases triglyceride levels, while increasing HDL cholesterol via metabolism control of fatty acids and lipoproteins. Clinical studies have also demonstrated that fibrates reduce the incidence of atherosclerosis and cardiovascular events. Additionally, activation of PPARα produces an increased sensitivity to insulin and glucose tolerance in patients with T2DM. Fibrates are well tolerated by most patients, however some adverse effects have been reported, mostly gastrointestinal, but also anxiety, headache, dizziness, sleep disorder, rash and hives. Because fibrate metabolism and excretion are mainly performed by the kidneys, patients with kidney problems are advised not to use them (Barter and Rye, 2008). PPARγ is the molecular targets of several marketed drugs, mostly the thiazolidinediones (TZDs) that were developed mainly to have high affinity and full agonism towards this receptor subtype (Henke et al., 1998). These pharmaceutical molecules increase insulin sensitivity and are used clinically for the treatment of T2DM, which is associated with various metabolic disorders including obesity, hypertension and dyslipidemia. Albeit their antidiabetic effect, the TZDs present risks to the patients (Levinet al., 2015); troglitazone was withdrawn from the market for causing hepatotoxicity, while rosiglitazone has a restricted use because of the greater risk of death from cardiovascular events. Currently, only pioglitazone is in unrestricted use, however many adverse effects are attributed to the continued use of pioglitazone, such as increased incidence of bone fractures distal, fluid retention,weight gain and increased occurrence of heart failure (Inzucchiet al., 2015; Nathan et al., 2009). The undesirable side effects attributed to the TZDs appear to be linked to the full activation (fullagonists) of gene expression in diverse tissues, which is related to anon-specificity of this class of ligands (Liu et al., 2015). Consistent with this notion, some of the non-TZD ligands, that are also full agonists with antidiabetic activity, exhibited similar side effects (Liu et al., 2015). Therefore, a promising approach for the development of PPARγ agonists, with an acceptable safety profile, is the search for agonists that partially modulate PPARγ target genes (Argmann et al., 2005; Cock et al., 2004; Liu et al., 2015). Despite weak receptor activation, partial PPARγ agonists may have a higher selectivity and fewer side effects (Choi et al., 2010). Structurally, full agonists generally make interactions with residues of H12, whereas partial agonists stabilize other regions of the ligand binding pocket (LBP), without direct contact with the H12 (Bruning et al., 2007).

A challenge to activate both PPARα and PPARγ with a single drug, thus simultaneously normalizing glucose and lipids levels, has led to intensive research efforts. Several PPARα/γ dual agonists, such as muraglitazar, ragaglitazar, tesaglitazar, and aleglitazar, have been synthesized and tested in clinical phase 2 or 3 (Fiévet et al., 2006). Most of these tests have been canceled because of the pronounced side effects of the tested dual agonists. This situation might arise from the fact that all cited glitazars have significantly higher affinity to PPARγ than to PPARα and some of them can be considered as pure PPARγ agonists. When fenofibric acid and rosiglitazone were used as controls of PPARα and PPARγ activity, respectively, some glitazars (muraglitazar and farglitazar, for example) were significantly more potent to PPARγ than a rosiglitazone, when administered in clinically prescribed doses (Fiévet et al., 2006). Nevertheless, better dual agonists, developed to increase insulin sensitivity and concurrently prevent dia- betic cardiovascular complications, still offer a very attractive therapeutic option, particularly if the compounds are able to combine an intermediate to higher PPARα affinity with a selective PPARγ-modulating capacity (Fiévet et al., 2006). Aiming to contribute to better understanding of a structural basis of full and partial activation of PPARs, here we present the structures of the ligand binding domain (LBD) of PPARα and PPARγ complexed with GL479, a PPARα/γ dual agonist (Fig. 1A). This compound was synthesized by Giampietro and colleagues in the search for novel PPAR ligands based on a combination of two key pharma- cophores: the clofibric acid skeleton and natural products as stilbene, chalcone, and their bioisosters (Giampietro et al., 2012). The aim of this project was to improve the pharmacological activ- ity of classical fibrates by introducing the antioxidant, antilipi- demic and antiplatelet properties of natural scaffolds. The introduction of a diazenyl function spaced by a three atom linker from clofibric acid resulted in GL479, a good PPAR α/γ agonist (PPARa EC_{50} = 0.6 μM, PPARc EC_{50} = 1.4 μM). GL479 was also able to influence the gene expression of CPT1A, an enzyme involved in lipid metabolism in liver, related with long-chain fatty acid transport into hepatocyte mitochondria (Giampietro et al., 2012). Our present crystallographic studies revealed that GL479 interacts with ligand binding pocket (LBP) of both PPARα and PPARγ but displays different binding modes. Considering the fact that GL479 acts as a full agonist of PPARα and partial agonist of PPARγ, we advocate that the observed differences in the binding modes are directly related to the efficacy of the PPARs activation by the ligand. Additionally, our structural analysis offers clues for amelioration of the ligands design for pharmaceutical applications, aimed to simultaneously activate more than one PPAR isoform.

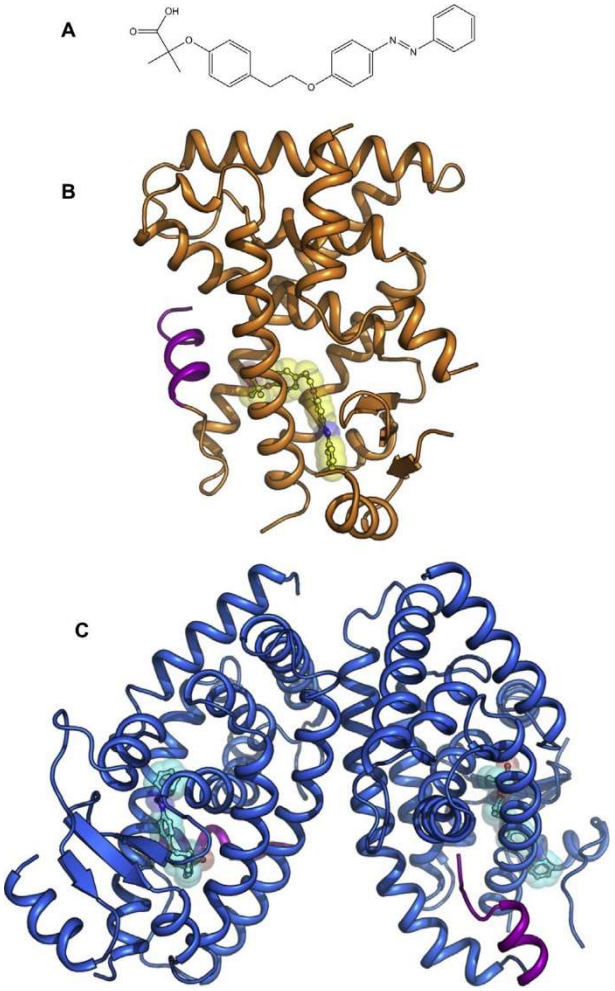

Fig. 1. Structures of PPARα and PPARγ LBDs bound to GL479. (A) Chemical structure of GL479 dual PPARα/γ agonist. (B) An overall schematic representation of the PPARα-LBD structure. The ligand GL479 is shown as a ball and stick model in yellow. (C) The PPARγ-LBD crystallized as an asymmetric unit containing active and inactive forms of the receptor. In both models the H12 is shown in purple.

2. Materials and methods

2.1. Protein expression and purification

The recombinant LBD of PPARα (residues 195–468) and PPARγ (residues 193–441) were expressed and purified as previously described (Bernardes et al., 2013; Puhl et al., 2012). Briefly, PPARα-LBD and PPARγ-LBD were cloned into pET28a (+) plasmid and introduced in competent cells of BL21 DE3 *Escherichia coli* strain. The transformed cells were cultured at 37 °C until $OD_{600\ nm}$ reached 0.6. Protein expression was induced with 1 mM IPTG for 16 h at 18 °C. Cells were collected by centrifugation and disrupted by sonication. Following centrifugation to remove cell debris, purification of the His-tagged protein was conducted by metal affinity chromatography using a $NiSO_4$-loaded HiTrap™5 mL column High Performance (GE Healthcare) pre-equilibrated with 10 mM imidazole, 20 mM Tris–HCl pH 7.5, 50 mM NaCl, 5% glycerol and 1 mm DTT. Clarified cell lysate were applied to the column followed by extensive washing with the same buffer, and subsequently the protein elution with a 10–500 mM gradient of imidazole. Each eluted sample was submitted to another step of protein purification using a Superdex 75 16/60 column (GE Healthcare) coupled to an AKTA Purifier (Amersham Bioscience) and equilibrated with the buffer 20 mM Tris–HCl pH 8.0, 1 mM DTT, 150 mM NaCl and 5% glycerol, at a continuous flow of1 mL min^{-1}. The His-tag was removed using thrombin proteolysis. The protein purity was monitored by SDS–PAGE stained with Coomasie Blue. Finally, complexes between PPARα/PPARγ and GL479 were obtained by incubation of receptors at 15/10 mg mL^{-1} with 3-fold/5-fold of GL479 excess, respectively.

2.2. Crystallization, data collection and structure determination

The crystals of both complexes were obtained using hanging drop vapor diffusion method, at 18 °C, mixing in the drops equal volumes of protein and reservoir solution (1 μL of each). For PPARα-GL479 complex crystallization, the reservoir solution was composed by 27% PEG 20,000 and 0.1 M Tris–HCl pH 7.0, whereas the crystals of PPARγ–GL479 complex grew using the reservoir solution consisting of 0.8 M sodium citrate dehydrate and 0.1 M Hepes at pH 8.0. The crystals were cryoprotected by addition of 15% (v/v) of ethylene glycol in the same reservoir solution and flash cooled by immersion in liquid nitrogen prior to X-ray data collection. Diffraction data were collected at the MX-2 beamline, at LNLS (Brazilian Synchrotron Light Laboratory; Campinas, Brazil) and processed with XDS (Kabsch, 2010) and SCALA (Evans, 2011). The structures were determined by molecular replacement using PHASER (McCoy et al., 2007) and PDB entries 2P54 and 3SZ1 for PPARα and PPARγ, respectively.

2.3. Refinement and structural analyses

Manual adjustments of the model to electron density were carried out using COOT (Emsley and Cowtan, 2004) and the structure refinement was performed with PHENIX (Adams et al., 2002). The geometry of the final models were checked with MOLPROBITY (Chen et al., 2010) and the interatomic contacts between protein–ligand were visualized by LIGPLOT program (Wallace et al., 1995). The protein alignment and analysis were carried with Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC) and Chimera (Pettersen et al., 2004).

3. Results

3.1. Overall description of the X-ray structures of PPARα and γ in complex with GL479

Molecular structure of GL479 contains an acidic head group, an aromatic center and a hydrophobic tail connected by a three atoms linker (Fig. 1A). To understand the structural basis of PPARα/γ activation by this dual agonist and to gain further insights into its binding mode in each receptor, we co-crystallized GL479 with the human PPARα and PPARγ ligand binding domains and determined the structures of both receptors bound to GL479. The

Table 1
Statistics of crystallographic data and refinement.

	PPARα:GL479	PPARγ:GL479
Data collection		
Wavelength Å/Beamline	1.459/LNLS-MX2	1.459/LNLS-MX2
Space group	$P4_12_12$	$C2$
Unit-cell parameters (A, °)	64.53, 64.53, 124.4	88.49, 64.25, 118.78 90.0, 102.6, 90.0
Resolution range (Å)	44.78–2.30 (2.39–2.30)	37.85–1.77 (1.87–1.77)
Mean $I/\sigma(I)$	17.5 (3.0)	13.2 (2.0)
Multiplicity	5.3 (5.0)	3.2 (3.1)
Completeness	99.8 (98.7)	98.8 (97.9)
R_{merge} [a] (%)	6.1 (49.8)	5.7 (62.5)
No. of molecules in asymmetric unit	1	2
No. of reflections	12,240 (1230)	62,608 (8968)
Refinement		
Resolution range (Å)	42.84–2.30	36.25–1.77
R_{factor}/R_{free}	21.88/26.64	16.94/20.82
Average B factor		
Protein	55.70	34.20
Ligand	60.7	43.34
RMSD		
Bond lengths (Å)	0.009	0.019
Bond angles (°)	1.10	1.75
Ramachandran plot (%)		
Most favoured regions	98.35	99.02
Disallowed regions	0	0

Statistics for the highest-resolution shell are shown in parentheses.
[a] $R_{merge} = \Sigma|I_j - \langle I\rangle|/\Sigma\langle I_j\rangle$, where I_j is the observed intensity of an individual reflection and $\langle I\rangle$ is the average intensity of that reflection.

complexed PPARα:GL479 and PPARγ:GL479 structures were solved to a resolution of 2.30 and 1.77 Å, respectively. The structure of the PPARα:GL479 complex (Fig. 1B) belongs to the tetragonal space group $P4_12_12$, and contains a monomer (encompassing the amino acid residues from Lys204 to Met467) in the asymmetric unit. This corresponds to a V_M of 16.7 Å^3/Da and a calculated solvent content of 41.17%. In addition to the protein monomer, the final model consists of one GL479 and 52 water molecules. The statistics of data collection, refinement and quality parameters of the final model are summarized in Table 1. The Ramachandran plot analysis showed that 98.35% of the residues are in the most favored and none in the disallowed regions.

The structure of the PPARγ:GL479 complex was solved in the monoclinic space group *C*2. Similar to the previously determined structures, the final model consists of two monomers in the asymmetric unit (encompassing the amino acid residues from Ala206 to Tyr477). The monomer designated as chain A represents the ''active'' conformation with displaced H12 (closed), and chain B in inactive conformation (H12 open) (Fig. 1C). The conformations adopted for both chains are almost identical, except for the position of H12. The inactive conformation found in the chain B is caused by the crystal contacts with the chain A which prevent H12 to adopt the active conformation (Nolte et al., 1998). Furthermore, the final model contains two molecules of GL479,one in each chain, and 493 water molecules. The LBD structurehas an overall fold practically identical to other of previously pub- lished PPAR LBDs complexes in the agonist-type conformation.

The structure is mainly formed by helices organized in a three-layered sandwich (13 α-helices and 3-stranded antiparallel β-sheet and unlike the other nuclear receptors, PPAR LBD containsan extra helix located between the first β-strand and H3 designatedas H2'). The structures of PPARα-LBD and ΠΠΙΑΡχ–LBD determined here are highly similar with an RMS (Root Mean Square) distance of 0.97 Å calculated for 242 common Ca positions of the chain A.
Some regions showed a weak electron density, because of their high dynamic and disorder, and therefore were omitted from the final model. For PPARα, disordered regions correspond to residues 230–237 and 255–266, whereas in the PPARγ structure the region between residues 262–275 is disordered. The later region of the PPARχ and the correspondent region of PPARα consist of the

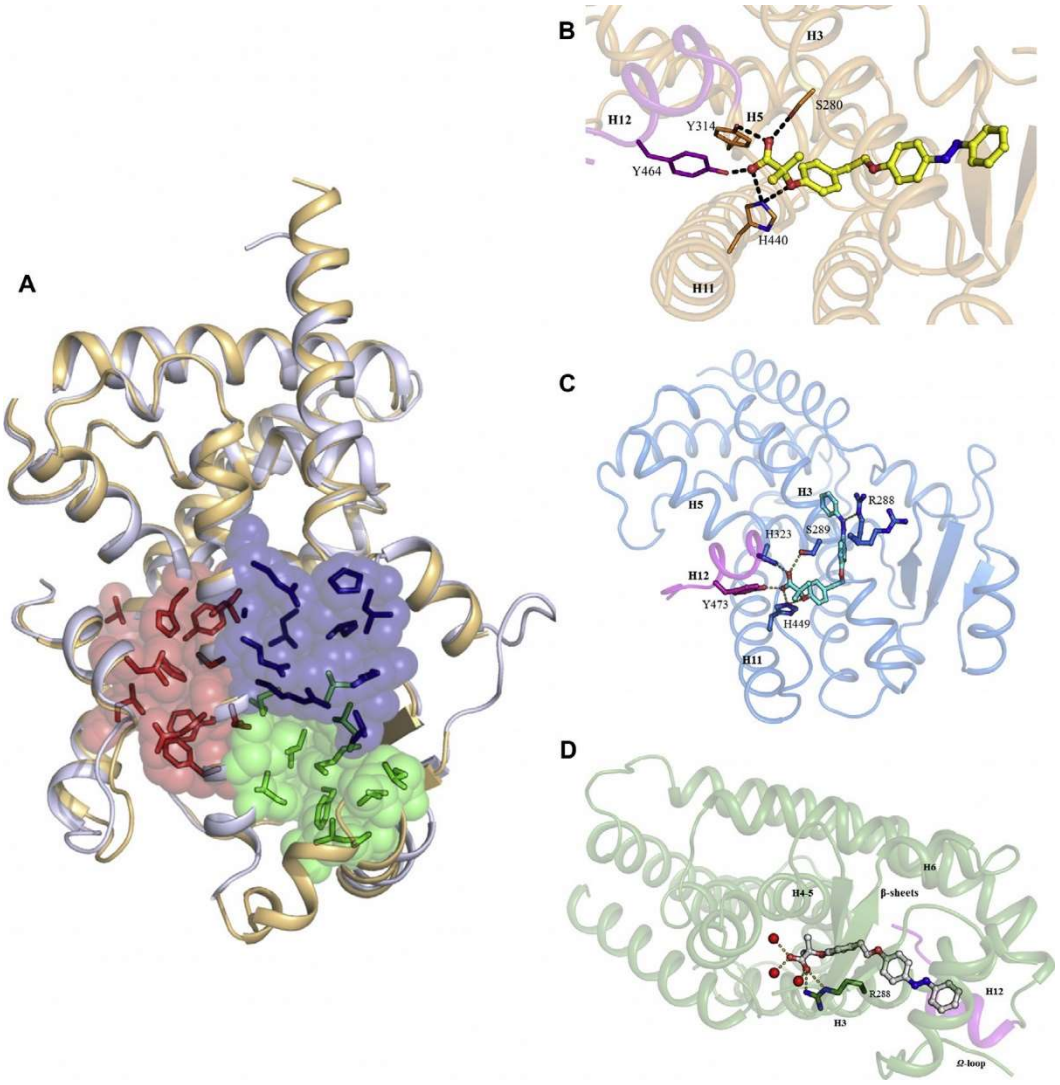

Fig. 2. Structural characterization of GL479. (A) Overall structure of LBDs PPARα (light orange) and PPARγ (blue white) displaying the three arms of the LBP: the arm I (red), the arm II (blue) and the arm III (green). (B) Details of interactions between PPARα and GL479. The GL479 head carboxylic group makes interactions with the polar side chains, including Y464, which results in the PPARα LBD canonical active conformations of helix 12. (C) Active monomer and (D) inactive B-subunit of PPARγ complexed with GL479. The polar amino acid residues that interact with the ligands are labeled and shown as sticks. H12 is painted in purple.

Fig. 3. Overlap of GL479 in complexes with PPARα and PPARγ. GL479 occupied the ligand-binding pocket of PPARα (red) and PPARγ (gray) in two distinct conformations. The acid group adopted essentially the same configuration in both conformations. However, its hydrophobic tail was in two completely configurations. In the first binding mode, exemplified by PPARα structure, the hydrophobic tail bends upward into the upper arm of the *Y*-shaped pocket (arm II). In the second binding mode (inside of PPARγ LBP), the hydrophobic tail of GL479 bends downward into the arm III at the bottom of the LBP. Ligands are shown as sticks and balls.

Ω-loop, located between H2′ and H3. The Ω-loop is highly flexible in nuclear receptors and a number of studies suggest that thisregion is responsible for the entrance of ligands inside the PPAR LBP (Liberato et al., 2012; Lu et al., 2006; Puhl et al., 2012; Zoete et al., 2007) and is highly mobile (Kuwabara et al., 2012; Nolteet al., 1998; Pochetti et al., 2007).

3.2. Different binding modes of GL479 for PPARα and PPARγ - both dependent of H12

The ligand molecules were not included in the protein model until the final stages of refinement. In order to reduce the effects of model bias, the omit maps of the ligand pockets were calculated for both structures and they revealed clear electronic densities for the bound ligands, thus enabling the unambiguously positioning of them into the LBP of each receptor (Supplementary material).

The PPARs have Y-shaped ligand-binding cavity, which extends from the C-terminal helix to the β-sheet, divided into three arms that display significant homology (60–70%) between the subtypes (Xu et al., 2001). The left arm of the Y (arm I) is composed of a mix of hydrophobic and polar residues, including two residues from the AF-2 helix, whereas, the right and lower arms (arms II and III, respectively) are mainly composed by hydrophobic residues(Fig. 2A).

GL479 occupies about 30% of the total volume of the PPARα and PPARγ *Y*-shaped ligand-binding cavity, which extends from the C-terminal helix to the β-sheet, lying between helices H3 and H6.
In both PPAR subtypes structures, GL479 is stabilized through a combination of hydrogen bonds and hydrophobic interactions. The region occupied by GL479 inside of the PPARα LBP fits *U*-shaped conformation and adjoin the H2′, H3, H7, H10/11, H12, and b-strand 2 and 3, so occupying the region of arms I and III. Such ligand arrangement inside of protein pocket allows the formation of four hydrogen bonds of its carboxylic group withthe residues S280 (H3), Y314 (H5), H440 (H11) and Y464 (H12), including one bond with the first phenoxy group S280 (H3). In addition to the hydrogen bonds, many hydrophobic interactions are formed with ligand tail assisting in the stabilization of the GL479. These contacts are formed by the residues L247, F273,C276, Q277, T279, F318, L321, V332, I354, M355 and L460. In addition, there are several polar interactions between the GL479 and PPARα-LBD which are shown in Fig. 2B

Strikingly, for PPARγ complex, the ligand adopted a conformation and occupation very different from that observed for PPARα and for the most full agonists in a *U*-shaped conformation (Bruning et al., 2007; Nolte et al., 1998). Similarly to PPARα:GL479, PPARγ:GL479 complex (chain A), also presents the typical pattern of hydrogen bonds cluster, located in the arm I of its LBP, involving the residues S289 (H3), H323 (H6), H449 (H11) and Y473 (H12) (Fig. 2C). The conservation of the network of hydrogen bonds and H12 direct stabilization suggests that it should be critical for GL479-mediated activation of both receptors.
However, the set of hydrophobic interactions made by the nonpolar ligand tail differs in each context. The most residues that form the nonpolar contacts with GL479 occupy the arm II of PPARγ LBP, encompassing F226, R288, A292, M329, L330 and M364. This forces the ligand to adopt an *S*-shaped conformation (Li et al., 2008). Additionally, the diazene group of the ligand tail forms a weak hydrogen bond with R288, also located in the ligand pocket arm II (Fig. 2C). This interaction appears to be essential to the partial agonism of GL479 and it has been poorly explored in the discovery of new partial agonists. In other PPARχ structures, where a hydrogen bond between ligand and R288 is also found, this correlates with a low receptor activation elicited by the ligands (Casimiro-Garcia et al., 2013; Connors et al., 2009). However, contrasting with PPARγ:GL479 complex, these others ligands do not present direct interaction with the receptor H12. Many regions of the PPARs structures already have been associated with the partial activation, but the importance of the interaction between the ligand and R288 and its effect on partial agonism has not been widely recognized as so far. Nevertheless, when this interaction is observed, the partial agonism is predominant, suggesting that this interaction could be explored further in the search for new partial PPARγ agonists.

In the PPARγ LBD chain B, the ligand is also held in place by several interactions. There are five hydrogen bonds, two are between the polar head of the ligand and R288 side chain and the other three are formed with waters molecules. In addition, the ligand is also stabilized by the hydrophobic contacts (Fig. 2D). However, since this chain is forced into inactive conformation by the crystal packaging, physiological relevance of these interactions is not clear.

Comparisons between PPARα/γ:GL479 complexes showed that despite the dissimilarities, both structures include a hydrogen bond between the residues located in the AF2-helix, whichcompels it to adopt the active conformation, i.e., the ‘‘closed state’’. The divergence of GL479 occupancy in each receptor could be better visualized in the Fig. 3.

3.3. PPAR LBP occupancy versus full/partial agonism

The two X-ray complex structures nicely allow to rationalizethe functional patterns observed in the transactivation assays, describing GL479 as a full agonist for PPARα and as a partial agonist for PPARγ. Based on previous studies, it has been suggested that PPAR full agonists generally have direct interactions with residues of helix 12, primarily through the formation of a hydrogen bond between the carboxylic group of ligands and tyrosine residues located in the AF-2 region. Differently, partial agonists

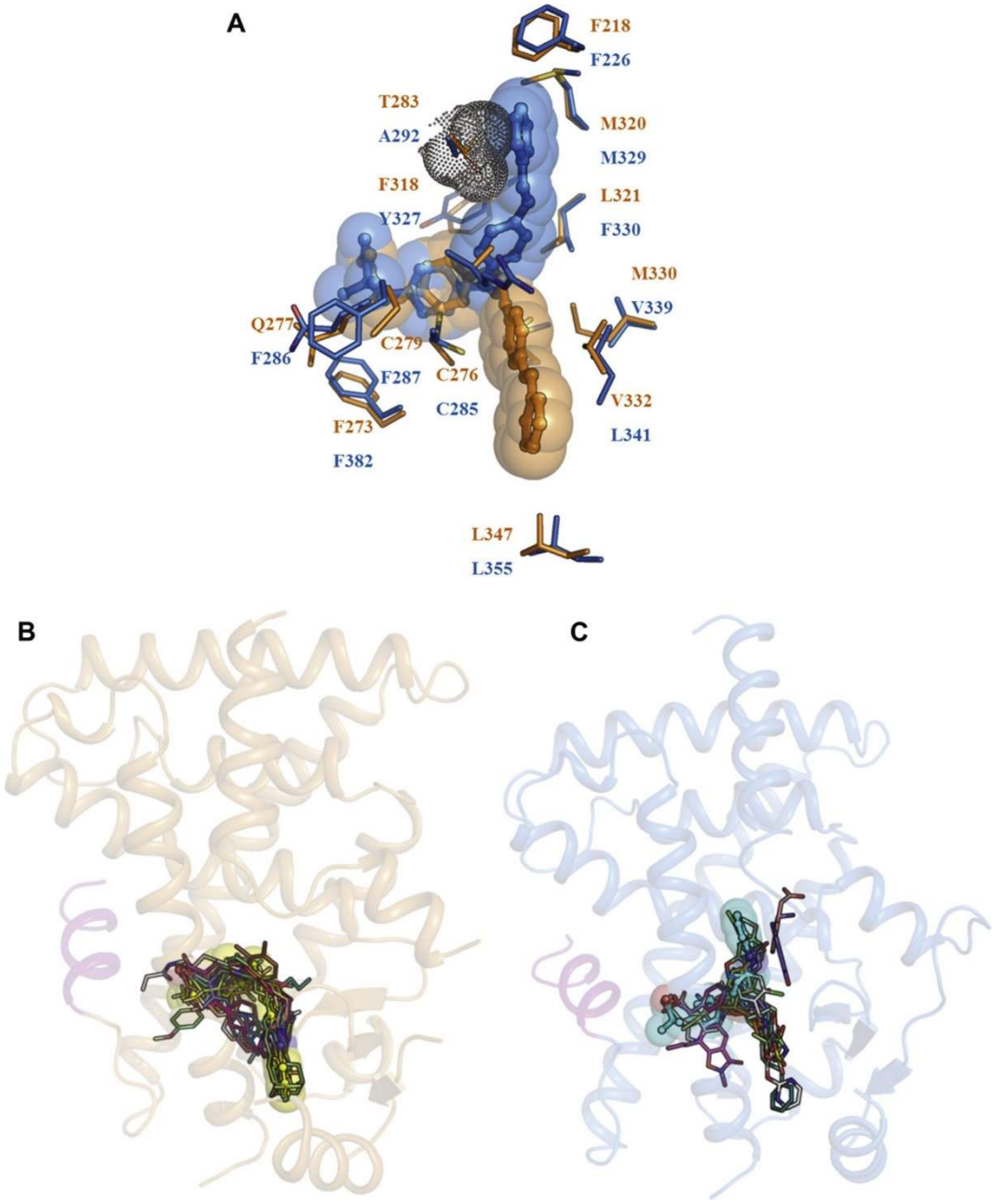

Fig. 4. Structural comparisons between the crystallographic models of PPARα and PPARγ LBD. (A) Superposition of the GL479 ligand bound PPARα (orange) and PPARγ (blue). Only residues that are involved in nonpolar interactions are shown. The position of PPARγ A292 is replaced by PPARα T273, and the later bigger side chain (dots) impairs the accommodation of GL479 rings in the same region of LBP. (B) All the reported crystal structures of PPARα:agonists complex with the ligands in the *U*-shaped inside of receptor pocket which coincide with GL479 (stick and ball yellow) occupation. (C) Some PPARγ agonists were observed occupying S-pocket in agreement with GL479 position (stick ball cian).

stabilize other regions of the LBP, without direct contact with the helix 12 (Bruning et al., 2007). The variations observed in the receptor apo, full-agonist-, and partial-agonist-bound activities leads to the premise that it is controlled by ligand binding modes and mainly by H12 dynamics. The studies with fluorescence labeled H12 (Kallenberger et al., 2003) demonstrated that the degree of H12 helix stabilization is proportional to the degree of agonism and transcriptional output for full agonists.

To comprehend potential molecular determinants of differentiated binding mode and an activation pattern of two subtypes, we carried out a detailed comparison of both three-dimensional structures by their superposition and alignment of their backbones (R.M.S. of 0.814). Among residues involved in the ligand tail stabilization only the position of PPARα T273 is replaced by PPARγ A292. The side chain of PPARα T273 occupies more space and impairs the accommodation of GL479 rings in the region described for PPARγ complex formation (Fig. 4A). This substitutioncould also be the reason why it has not been yet reported X-ray structures with ligand binding in the region of arm II. PPARα agonists always bind in the ligand binding pocket in an U-conformation as can be observed in Fig. 4B. On the contrary, PPARγ agonists were observed occupying S-pocket (Fig. 4C), and this binding mode might play an important role in mediating ligand-dependent conformational changes that facilitate recruitment of coregulator proteins, such as NCoR (Li et al., 2008), which are consistent with lower transcriptional activity and partial

agonism pattern observed for PPARγ. However, the preference of GL479 for PPARγ arm II may be due to hydrogen bond formation between side chain of PPARγ R288 and nitrogen atoms of ligand tail in addition to the increased number of apolar interactions.There are some residues substitutions in PPARγ as compared to PPARα receptor that are involved in the hydrophobic interactions with the ligand (PPARα M330/PPARγ V339 and PPARα C275/PPARγ G284). Thus, the same interactions cannot be formedin PPARγ LBP arm III.

GL479 and two other compounds, all synthesized and described by Giampietro et al. (2012), share the same functional features, i.e. full agonist for the PPARα agonist and partial agonist for PPARγ. The unique difference among these compounds structure is the length of the linker between the aromatic lipophilic tail and the center (one, two or three carbons). Although only the studies with GL479 (linker with two carbons) have resulted in the crystal structures of complexes, we believe that this minor variation on the link size is not enough to change the current position of the ligand in each receptor structure, since the addition or deletion of this carbon atoms would not cause steric hindrance with residues of the PPARs active sites.

B-factors analysis of our structures showed that GL479 stabilizes differently several regions of the PPARα and PPARγ structures. The GL479 produces a stronger stabilization of the loop that comprises H11 and H12 of PPARγ (Fig. 5A), while the region close to Χ-loop is better stabilized in PPARα complex (Fig. 5B). This greater stability of the region adjacent to O-loop for PPARα receptor might cause conformational changes that produce a decrease in the helix 12 mobility leading to stabilization of its the interactions with coactivators (Bernardes et al., 2013).

In fact, these data agree with the hypothesis that the direct interaction with H12 is not the only mediator of transcriptional responses (Bruning et al., 2007), but additional stabilization of certain regions of receptor structure provided by interaction with ligand hydrophobic tail could indirectly influence the H12 dynamic. Furthermore, a polar interaction with γR288 may be also related with partial agonism profile of ligands. In other words, the transcriptional activity observed for a ligand is dictated by its receptor binding mode, and it is not only restricted to the presence or absence of direct interactions with H12. PPARs ligand-binding domains have large volumes and display high intrinsic mobility, which allow them to assume a continuum of conformations, adopting multiple activated states and facilitating interactions with different coregulators (Li et al., 2008; Xu et al., 2002).

We propose that the active conformation of both PPAR subtypes is accessible due to hydrogen bonds network, but the ligand configuration adopted for PPARγ receptor is not sufficient to stabilize the protein structure like in a *U*-shaped full agonists mode. Consequently, the coactivator recruitment becomes weaker, which restricts the receptor activity. Nevertheless, since the ligand fills in the arm III of the ligand-binding pocket, this shifts the equilibrium toward the active configuration of the receptor via indirect AF-2 stabilization.

4. Discussion

The important role of PPARs in metabolic diseases such as T2DM and dyslipidemia has been well established by numerous functional studies (Berger and Moller, 2002; Forman et al., 1995; Kota et al., 2005; Lehmann et al., 1995; Nolte et al., 1998). It is also known that the TZDs drugs rosiglitazone and pioglitazone improve insulin resistance by PPARγ activation, and that PPARα activation by fibrates induces a decrease in circulating lipid levels. Despite the fact that many dual PPAR agonists been synthesized, a drug that could have both therapeutic effects is not available yet because of serious side-effects already observed in both preclinical and clinical studies. However, balanced dual PPARα/γ agonists could potentially benefit patients with diabetes and metabolic disorder (Cavender and Lincoff, 2010). Thus, it is of considerable importance to identify, describe and understand the action mechanism of novel dual PPAR-α/γ agonists. To date the mode of receptor binding has been structurally characterized for only a few dual PPARα/γ agonists (Bénardeau et al., 2009; Cronet et al., 2001; Grether et al., 2009; Xu et al., 2001), and this information about ligands occupation in the binding pocket is crucially important. In all cases described, the ligand adopts the identical active conformation, occupying the arm I and III of the ligand binding pocket and has a similar mode of binding for both PPARs (Fig. 6). The GL479 in complex with the PPARα stabilizes the region occupied by others full agonists such as AZ242 (Cronet et al., 2001), APHM13 (Kuwabara et al., 2012) and oxybenzylglycine derivatives(Li et al., 2010) mainly decreasing mobility of the helices H3, H5, H7, H11 and H12 of the LBD. In these complexes, the acidic head of the ligand buried the arm I forms hydrogen bonds network with the polar residues S280, Y314, Y464 and H440, whereas the hydrophobic part of the ligand occupies the arm II and is stabilized mainly by extensive van der Waals interactions. It is assumed that

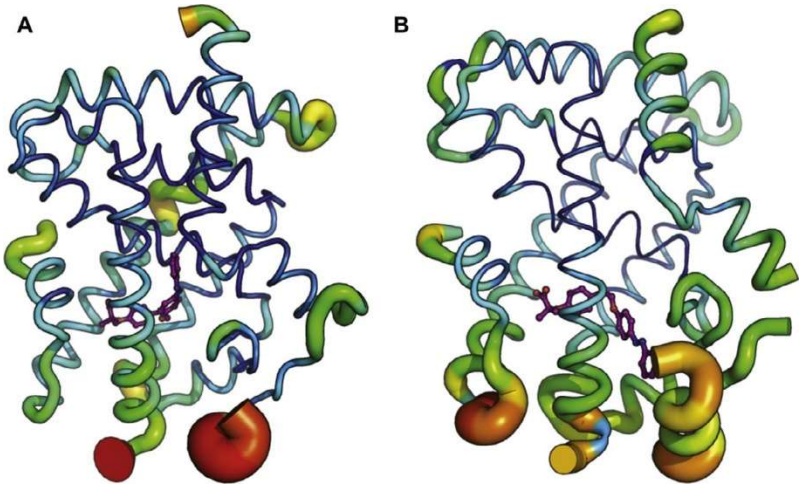

Fig. 5. GL479-induced stabilization of different regions of PPARα and γ. The structures are colored and represented according to their *B*-factors. (A) PPARγ-LBD and (B) PPARα-LBD.

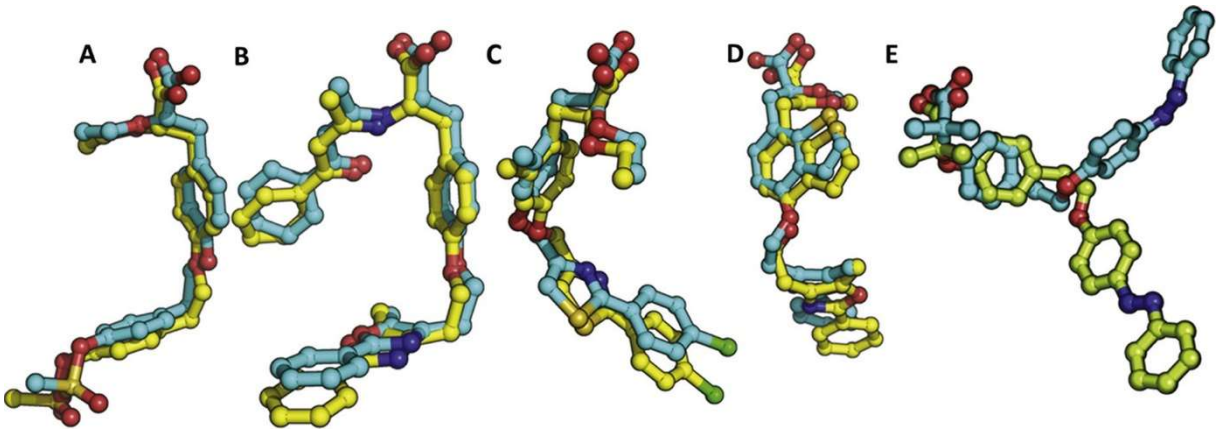

Fig. 6. Superimposed PPARα and PPARγ active monomers crystallized with different dual agonists. Only GL479 (*E*) displayed a divergent binding mode for α and γ subtypes.

the full agonist activity is associated that the later mode of binding observed in all known structures of the PPARα. As a general rule the potent PPARα ligands which have high affinity to the receptor make direct interactions with H12 (Grether et al., 2009; Kuwabara et al., 2012; Li et al., 2010; Oon Han et al., 2007). Surprisingly, the GL479 binds to PPARγ in a singular mode of binding, different from all currently available structures of the receptor:ligand complexes. As a rule, the PPARs partial agonists do not interact with the tyrosine residue of H12 in active conformation (Bruning et al., 2007; Xu et al., 2001). Nevertheless, in our structure we can clearly observe the interaction between GL479 and Tyr473 (H12) of PPARγ. For the first time, we found a partial agonist interacting H12. Another important aspect of this complex is that the GL479 adopts a particular conformation in the active site, extending through the arms I and arm II of LBP, differently of the occupation described for full agonists. This mode of ligand binding has not been described to date for any available PPARs complexes and might indicate that simultaneous occupation of arms I and III ensures the most active conformation of the receptor.

We have provided a detailed structural analysis of a PPARα/γ dual agonist mode of binding to the receptors. Two particular structural features distinguish the binding of this amphipathic ligand to PPARα/γ subtypes. First, the carboxylic acid head makes an intricate series of hydrogen bonds with the receptor, whichare the chiefly responsible for ligand:protein interactions and for

inducing the active conformation of H12. However, the receptor activation pattern is mediated by the ligand tail conformation, demonstrating that the stabilization of specific regions of the LBDs could ensure the mode of receptor activation.

Importantly, the ligands with attenuated PPARγ activation may be promising, since the clinical use of PPARγ agonists has been complicated by weight gain, fluid retention and arising in cardiovascular events that is often encountered (Calkin and Thomas, 2008). On the other hand, full agonism of PPARα has been largely well tolerated. If novel functional studies confirm the dual PPARα/γ agonist nature of GL479 and the theoretical benefit of this ligand class, GL479 may become a promising alternative of the therapy currently employed in the treatment of dyslipidemia and dysglycemia in patients with type 2 diabetes.

Author contributions

JCS, AB, LG, AA BF designed experiments; JCS performed the experiments; JCS and AB analyzed data and wrote the manuscript; RA and IP conceived and supervised the study and made manuscript revisions.

Acknowledgments

This work is supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) via grants 2009/14333-6 (JCS), 2007/58443-4 (AB) and 2008/56255-9 (IP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (IP) and Italian MIUR (RA). We are also grateful to the Brazilian National Synchrotron Light Laboratory (LNLS, Campinas, Brazil) for the use of the MX-2 beam line.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsb.2015.07.006.

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