Novel Glitazones with Diverse Peroxisome Proliferator Activated Receptor Modulatory Potential

Thaggikuppe Krishnamurthy Praveena, Moola Joghee Nanjan Chandrasekarb, Moola Joghee Nanjanb*

a TIFAC CORE HD, JSS College of Pharmacy, Off campus JSS University, Ootacamund-643 001, Tamilnadu, India.
b Department of Pharmaceutical Chemistry, JSS College of Pharmacy, Off campus JSS University, Ootacamund-643 001, Tamilnadu, India.
*bCorresponding author

Dr. Moola Jogee Nanjan, Director, TIFAC CORE HD, JSS College of Pharmacy, Post box No. 20, Rocklands, Ootacamund-643 001, Tamilnadu, India. Telefax: +91 423 2447135, E-mail address: mjinanjan@gmail.com

Running title: Glitazones with PPAR Modulatory Potential
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Abstract

The clinically used glitazones for the management of type 2 diabetes mellitus are full agonists of peroxisome proliferator activated receptors (PPAR)-γ and potent insulin sensitizers. Unfortunately, these molecules suffered with various side effects which resulted in their restriction/ban. Various new strategies were, therefore, developed to overcome the existing problems with the full agonists. Among them the development of partial and dual PPAR agonists are some of the important strategies adopted to improve the adverse profiles of the full agonists. In the present study, 11 novel (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione derivatives were designed, synthesized and evaluated for their possible partial and dual agonistic activities using in silico and in vitro methods. The in silico docking and in vitro PPAR-α & γ competitive binding studies confirm the dual binding potential of these molecules. The post docking analysis reveal that the molecules, 4a, 4b, 4c, 4d, 4e, 4h and 4j, exhibit binding modes similar to a full agonist and molecules, 4f, 4g, 4i and 4k, show binding modes similar to a partial agonist of PPAR-γ receptors. On PPAR-α receptors, all the molecules except, 4a and 4g, show binding modes similar to a full agonist, The molecules, 4a and 4g, however, show binding modes similar to that of a partial agonist. Further, in the in vitro 3T3L1 preadipocytes assay, all the molecules promote the adipocyte differentiation confirming their PPAR-γ agonistic activity. These molecules, therefore, show potential to overcome the problems of the clinically used glitazones in the management of type 2 diabetes mellitus.

Key words: Dual PPAR agonists, Glitazones, PPAR-α, PPAR-γ, PPAR-δ, Type 2 diabetes mellitus, Thiazolidines-2,4-diones.
1. Introduction

Peroxisome proliferator activated receptors (PPARs) are transcription factors that can be turned on or off by binding to small lipophilic compounds. These receptors are reported to act by coordinating the activities of multiple pathways involved in metabolism instead of acting through one major target like one enzyme or one pathway [1]. This unique property of PPARs has attracted the attention of researchers because of the possibility that complex metabolic disorders such as type 2 diabetes mellitus (T2DM), which often requires a combination of symptomatic therapies, can be treated effectively by the use of a single agent which can act by modulating PPARs. PPARs are, therefore, one of the most promising druggable targets in the management of metabolic syndrome.

Thiazolidinediones (TZDs) or glitazones are an important class of drugs which act by increasing the transactivation activity of PPARs, as a result of which, they reduce hepatic glucose production, increase peripheral utilization of glucose and promote lipid metabolism [2]. These actions, therefore, ensure reduced preload and after load on β-cells and lipid homeostasis. In addition, unlike sulfonylureas, these agents are devoid of mechanism based hypoglycemic side effects. There is, therefore, an excellent rationale for the use of TZDs in the management of T2DM. Unfortunately, the clinically used TZDs (troglitazone, pioglitazone and rosiglitazone) suffered with some serious side effects like idiosyncratic hepatotoxicity, fluid retention, weight gain, etc., as a result of which troglitazone and rosiglitazone were banned and the pioglitazone label was updated for the risk of bladder cancer [3-7].

In recent years, however, new approaches have been made to address the problems associated with current TZDs. These include the development PPAR-α/γ dual agonists, PPAR-δ/γ dual agonists, PPAR-pan agonists, selective PPAR-γ modulators or partial agonists [8,9]. The dual agonists and the pan agonist molecules have been claimed to achieve a broad spectrum of metabolic effects by improving insulin resistance, hyperglycemia (PPAR-γ activation) and atherosclerotic dyslipidemia (PPAR-α or δ activation). They are most beneficial in those T2DM patients coexisting with dyslipidemia, which is most common in patients with T2DM. In addition, PPAR-α /δ promotes lipid oxidation and decreases adiposity and, therefore, neutralizes the PPAR-γ mediated weight gain and thus provide better therapeutic benefits with minimization of the adverse effects associated with the PPAR-γ activation [10-12]. PPAR partial agonists or selective PPAR modulators (SPPARMs), similar to selective estrogen receptor modulators (SERMs), thus emerged as a new strategy to tackle the existing problems of PPAR full agonists. These molecules provide a target oriented therapeutic profile by maintaining the desired therapeutic benefits and at the same time have minimal adverse effects due to their inability to fully activate the receptor as that of a full agonist [13-15]. SPPARMs are reported to achieve these effects by selectively recruiting the coactivators to PPAR receptors and thus selectively activating the genes responsible for insulin sensitization, adipogenesis, fluid retention and bone remodeling [16].

Based on the protein-ligand interaction studies the topological features of typical PPARs are defined. These features include an acidic head group, two linker groups, an aromatic centre (spacer ring) and a cyclic tail (Fig. 1) [17]. The acidic head group is reported to form H-bond interactions with the Arm-I residues and the cyclic tail is reported to interact with the Arm-II of of the ligand binding domain (LBD) of PPAR [18]. The modification carried out on the 5 regions has been reported to result in a number of molecules with varied degrees of activities [19].
In the present study, an effort has been made by us to design and synthesize glitazones with 3 carbon chain length of the linker between cyclic tail and aromatic centre instead of 2 carbon chain length as normally observed with typical full agonists. As compared to a full agonist the free rotation of carbon bonds of the linker connecting the acidic head and the aromatic centre is thus restricted by retaining the double bond between the linker carbon and the thiazolidine head. In addition, we have employed various phenolic derivatives as part of cyclic tail instead of substituted oxazoles and pyridyl derivatives as observed in the case of typical full agonists. Based on these modifications to a typical full agonist structure, we have designed hundred and twelve (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidin-2,4-dione derivatives (Fig. 2) and docked them against PPAR-α and γ LBD. Based on the glide scores obtained and synthetic feasibility, a total of 11 novel (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidin-2,4-dione derivatives were synthesized, characterized and evaluated for their possible modulatory potential as partial and dual agonists using in silico and in vitro methods.

![Simplified topological representation of typical PPAR agonist and structures of some the clinically used glitazones](image)

**Fig. (1).** Simplified topological representation of typical PPAR agonist and structures of some the clinically used glitazones

![The general structure of (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidin-2,4-dione derivatives.](image)

**Fig. (2).** The general structure of (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidin-2,4-dione derivatives.

2. Materials and methods
2.1. General

All the glassware’s were oven dried prior to use. All the chemicals used were obtained from Sigma Aldrich Co., Ltd., (St Louis, MO). All the solvents used in the synthesis were obtained from SD fine chemicals Ltd., Mumbai and distilled prior to use as per the standard methods. Thin layer chromatography was performed using precoated TLC plates (silica gel 60 F254, Merck Ltd., Germany). The PPAR-α/γ assay kits were obtained from Invitrogen Cooperation (Carlsbad, CA).

2.2. Docking studies

Docking studies were carried out on PPAR-α and γ receptors separately using Glide, version 5.7, Schrödinger Suite 2011, LLC, New York, 2011, on a Maestro graphical user interface.

2.2.1. Ligand structure preparation

The structures of all the molecules were drawn with ChemBioDraw Ultra (Version 12, PerkinElmer Inc.). These structures were then subjected to ligand preparation process using LigPrep module of Glide. They were converted from 2D to 3D structures by including stereochemical, ionization and tautomeric variations, as well as energy minimization and optimized for their geometry, desalted, and corrected for their chiralites and missing hydrogen atoms. The charged groups were neutralized. The ionization and tautomeric states were generated between a pH of 6.8 to 7.2 using Epik module. The energy minimization was carried out by using Optimized Potentials for Liquid Simulations-2005 (OPLS-2005) force field.

2.2.2. Protein structures preparation

The X-ray crystal structure of the human apo-PPAR-γ LBD in complexation with Rosiglitazone (PDB ID: 2PRG) at 2.2Å resolution and human apo PPAR-α LBD in complexation with Aleglitazar (PDB ID: 3G8I) at 2.2Å resolution, obtained from the RCSB Protein Data Bank (PDB), were used separately in order to model the protein structures in this study. In general the protein structures were refined for their bond orders, formal charges and missing hydrogen atoms, topologies, incomplete and missing residues and terminal amide groups. The water molecules beyond 5Å of the hetero atom were removed. The possible ionization states were generated for the heteroatom present in the protein structure and the most stable state was chosen. The hydrogen bonds were assigned and orientations of the retained water molecules were corrected. Finally, a restrained minimization of the protein structure was carried out using OPLS 2005 force field to reorient side-chain hydroxyl groups and alleviate potential steric clashes.

2.2.3. Receptor grid generation

The prepared proteins were used for the receptor grid construction. The various potential energies of the binding site of the protein were calculated based on the grid with a box size of 17Å. The search space in the binding site of the protein was defined by another box with 12Å. In the receptor grid of the PPAR-γ protein, the flexibility was assigned to the hydroxyl groups of the serine 289, 332, 342 and 355, tyrosine 327 and 473 and threonine 297
residues, whereas in PPAR-α receptor grid the flexibility was assigned to the hydroxyl groups of the serine 280, 322, 323 and 346, tyrosine 214, 314, 334 and 464, and threonine 253, 279, 283, 340 and 438 residues.

2.2.4. Validation of the docking programme

Extra precision Glide docking procedure was validated by removing the cocrystallized ligand from the binding site of the respective proteins and redocking them with their respective binding sites. The Root Mean Square Deviation (RMSD) between the predicted conformation and the observed X-ray crystallographic conformation were used for analyzing the results.

2.2.5. Glide Ligand docking

The ligand docking was carried out using the previously prepared receptor grid and the ligand molecules. The favorable interactions between ligand molecules and the receptor were scored using the Glide ligand docking program. All the docking calculations were performed using the Extra Precision (XP) mode and the force field employed was OPLS-2005. The above docking process was run in a flexible docking mode which automatically generates conformations for each input ligand.

2.3. Synthesis of glitazones (Scheme 1)

2.3.1. Synthesis of 3-phenoxypropan-1-ol derivatives (2a-k)

To a stirred mixture of phenol derivative (1a-k) (1.0g, 1.0m/e) and 3-bromopropan-1-ol (1.2m/e) in dry acetonitrile (20ml), dry potassium carbonate (5.0m/e) was added and the reaction mixture was refluxed for 12h at 70˚C under nitrogen environment [20]. The progress of the reaction was monitored by TLC (n-hexane-ethyl acetate). After the completion of the reaction, the mixture was filtered and the solvent was evaporated in a rotary evaporator to obtain the crude product. The crude product was then purified by flash column using n-hexane and ethyl acetate.

2.3.2. Synthesis of 4-(3-phenoxypropoxy)benzaldehyde derivatives (3a-k)

To a stirred mixture of 3-phenoxypropan-1-ol derivative (2a-k) (1.0g, 1.0m/e), p-hydroxybenzaldehyde (1.0m/e), triphenylphosphine (PPh₃) (1.5m/e) in dry THF (10ml) at 0˚C under nitrogen environment, diisopropyl azodicarboxylate (DIAD) (1.5m/e) was added and the mixture was stirred for 12h at room temperature [21]. The progress of the reaction was monitored by TLC (n-hexane-ethyl acetate). After the completion of the reaction the solvent was evaporated under vacuum and the crude product obtained was purified by flash column using n-hexane-ethyl acetate.

2.3.3. Synthesis of (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidin-2,4-dione derivatives (4a-k)

To a stirred mixture of 4-(3-phenoxypropoxy)benzaldehyde derivative (3a-k) (0.5g, 1.0m/e), thiazolidine-2,4-dione (1.0m/e), molecular sieves (1.0g) in dry toluene (20ml), piperidine (0.025m/e) and glacial acetic acid (0.025m/e) were added. The mixture was refluxed for 4 h at 100˚C in nitrogen atmosphere [22]. The completion of the reaction was confirmed by TLC (n-hexane-ethyl acetate). The precipitated product was dried under vacuum to remove the solvent. To this, brine solution was added and extracted with ethyl acetate in a separating funnel. The ethyl acetate layer was separated and dried over anhydrous sodium sulphate and evaporated when a crude product was obtained.
The crude product was purified by flash column (n-hexane-ethyl acetate as solvent) to obtain the final compound. The structures of the final compounds obtained were confirmed by IR, NMR and Mass spectral data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substitution</th>
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<tbody>
<tr>
<td>4a</td>
<td>H H H H H</td>
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<tr>
<td>4b</td>
<td>H H H C,H</td>
</tr>
<tr>
<td>4c</td>
<td>H H Br H H</td>
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<tr>
<td>4d</td>
<td>C,H H H H H</td>
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<tr>
<td>4e</td>
<td>H H C,H H H</td>
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<td>4f</td>
<td>H NO, Cl H H</td>
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<tr>
<td>4g</td>
<td>C,H H H H</td>
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<tr>
<td>4h</td>
<td>H F H F H</td>
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<td>4i</td>
<td>Br H F H H</td>
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<td>4j</td>
<td>F H Br H H</td>
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<tr>
<td>4k</td>
<td>H Br H F H</td>
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Scheme 1. Reagents and conditions: (i) 1a-k (1.0g, 1.0m/e), 3-bromopropan-1-ol (1.2m/e), K₂CO₃ (5.0m/e), acetonitrile (20ml), 70°C, 12h; (ii) 2a-k (1.0g, 1.0m/e), p-hydroxybenzaldehyde (1.0m/e), triphenylphosphine (1.5m/e), diisopropyl azodicarboxylate (1.5m/e), THF (20ml) 0°C, 12h; (iii) 3a-k (0.5g, 1.0m/e), thiazolidine-2,4-dione (1.0m/e), acetic acid (0.025m/e), piperidine (0.025m/e), toluene (10ml), 100°C, 4h.

2.4. In vitro PPAR competitive binding assay

PPAR-γ and PPAR-α competitive binding assays were carried out using time-resolved fluorescence resonance energy transfer (TR-FRET) PPAR-α and γ Competitive Binding Assay kits (Invitrogen Corporation, Carlsbad, CA). The test and the standard (20µl) were serially diluted (1:1) with the assay buffer to obtain 8 different concentrations in a 96 well plate. Fluromone™ Pan-PPAR Green (10µl) was added to all the wells and the assay was initiated by adding PPAR-γ-LBD/Tb-anti-GST Ab (10µl, for PPAR-γ assay) and PPAR-α-LBD/Tb-anti-GST Ab (10µl, for PPAR-α assay) to all the wells except blank wells. The plate was incubated at room temperature in dark for 3h. After incubation the fluorescent emission signal of each well was recorded at 495nm and 520nm using Synergy HT Multidetection Microplate reader. The TR-FRET ratio was calculated by dividing the emission signals at 520nm and 495nm. The percentage inhibition for each concentration of the test was calculated by using the average control ratio. The IC₅₀ values were then determined from the straight-line equation obtained after plotting % inhibition vs concentration.

2.5. Adipogenesis assay in 3T3-L1 preadipocyte

2.5.1. Preparation of the samples and culture media

The test compounds and the standard, rosiglitazone, were prepared in the assay media using 5% DMSO as solubilizer. Concentrations of 500, 250, 125, 62.5 and 31.25µg/ml were used for the cytotoxicity study and a concentration of 10µM was used for the main assay. Differentiation media was prepared in the Dulbecco’s Modified
Eagle’s Medium (DMEM) by adding 10µg/ml insulin, 0.5mM dexamethasone, 0.8mM isobutylmethyl xanthine and 10% fetal bovine serum (FBS). Progression media was prepared in DMEM by adding 10µg/ml insulin and 10% FBS. Maintenance media was prepared by adding 10% FBS to DMEM.

2.5.2. Cytotoxicity assay

The cytotoxicity of the compounds was assessed in the 3T3-L1 cell lines before carrying out the adipogenesis assay. Preconfluent 3T3-L1 preadipocytes (100µl) were seeded in 96 well plates at a density of 10,000 cells/ml. The maintenance media with or without the various concentrations of test compounds (100µl) were added in triplicates and the plates incubated at 37°C for 48h. After 48h the viability of the cells were assayed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The CTC50 was then estimated from the absorbance readings.

2.5.3. Adipogenesis assay

Mouse 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% bovine calf serum and antibiotics (100U/ml penicillin and 100µg/ml streptomycin) at 37°C under a humidified 5% CO2 atmosphere. Cells at 2 days after reaching confluency (defined as day 0) were cultured in differentiation medium. After 2 days, the culture medium for 3T3-L1 preadipocyte differentiation was changed to progression media. After 2 more days the medium was replaced with maintenance media. The adipocytes were used at 6 days after the initiation of differentiation. In adipogenesis studies, 3T3-L1 preadipocytes, cultured in maintenance media (day 0), were treated with progression media with or without test compounds/rosiglitazone (10µM) in triplicates for 9 days. Fresh progression medium with or without the test compounds/rosiglitazone was replenished every 3 days. After differentiation, the cells were fixed with 10% formal buffered saline for 1 h at room temperature, washed three times with PBS and stained with filtered Oil Red O (0.5% in 60% isopropanol) for 1h. After three washes with distilled water, the cells were photographed under a microscope. The oil Red O was extracted using isopropanol and the absorbance was measured at a wavelength of 520nm using Bio-Rad spectrophotometer to estimate its content [23].

3. Results

3.1. Docking studies

The accuracy of the docking procedure was determined by finding how closely the lowest energy pose (binding conformation) of the cocrystallized ligand predicted by the object scoring function, Glide score (Gscore), resembles an experimental binding mode as determined by X-ray crystallography. The validation results of Extra Precision Glide docking procedure for both PPAR-α and γ are given in Fig. 3. The results reveal a very good agreement between the localization of ligand upon docking and the crystal structure. The RMSD between the predicted conformation and the observed X-ray crystallographic conformation of the ligands, rosiglitazone and aleglitazar, are found to be 0.4930Å and 0.1735Å, respectively. The parameters set for Glide docking are, therefore, reasonable to reproduce the X-ray structure. The docking results for the synthesized molecules are shown in Fig. 4 & 5 and Table 1. The results reveal a good glide score (> 8) for all the molecules. Most of these molecules received lipophilic, hydrophobic enclosure, hydrophobically packed H-bond, hydrophobically packed correlated H-bond, hydrogen bonding, electrostatic, site map and low molecular weight rewards. In addition, all the molecules received rotatable bond penalties.
3.2. Synthesis of the (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidin-2,4-dione derivatives

3.2.1 (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione (4a)

Yield: 0.59 g; mp 140-142°C; IR (KBr, V cm⁻¹): 3504, 3039, 2934, 2878, 1717, 1736, 1683, 1654, 1598, 1244, 1056, 754, 691; ¹H NMR (400 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 12.07 (s, 1H), 7.7 (s, 1H), 7.69-7.25 (m, 4H), 6.99-6.89 (m, 5H), 4.21-4.14 (m, 4H), 2.262 (s, 2H); ¹³C NMR (100 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 168.10, 167.66, 160.25, 158.39, 132.03, 131.76, 129.17, 125.59, 120.47, 114.92, 114.15, 64.38; 63.89, 28.76; HRMS (ESI) m/z calcd. for C₁₉H₁₇NO₄SNa (M+Na)+ 378.0076, found 378.0779.

3.2.2 (5Z)-5-[4-[3-(naphthalen-1-yloxy)propoxy]benzylidene]-1,3-thiazolidine-2,4-dione (4b)

Yield: 0.41 g; mp 175-177°C; IR (KBr, V cm⁻¹): 3401, 3143, 3036, 2936, 1735, 1686, 1593, 1257, 1179, 1019, 767, 689; ¹H NMR (400 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 8.22 (d, J= 8.0, 1H), 7.85-7.71 (m, 1H), 7.68 (s, 1H), 7.49-7.34 (m, 6H), 7.03 (d, J= 8.8, 2H), 6.87 (d, J=7.2, 1H), 4.35-4.31 (m, 4H), 2.45-2.39 (m, 2H); ¹³C NMR (100 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 168.16, 167.70, 160.28, 154.10, 134.15, 132.09, 131.80, 127.21, 126.16, 125.71, 125.24, 124.94, 121.57, 120.48, 120.03, 114.98, 104.54, 64.85, 64.19, 28.90; HRMS (ESI) m/z calcd. for C₂₃H₁₉NO₄SNa (M+Na)+ 428.0932, found 428.0938.

3.2.3. (5Z)-5-[4-[3-(4-bromophenoxy)propoxy]benzylidene]-1,3-thiazolidine-2,4-dione (4c)

Yield: 0.50 g; mp 180-182°C; IR (KBr, V cm⁻¹): 3376, 3136, 3023, 2901, 1725, 1701, 1587, 1367, 1241, 1048, 812, 689; ¹H NMR (400 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 12.12 (s, 1H), 7.71 (S, 1h), 7.45 (d, J=8.8, 2H), 7.36 (d, J=8.2, 2H) 7.01 (d, J=8.8, 2H) 6.81 (d, J=8.8, 2H), 4.22 (t, J=11.6, 2H), 4.14 (t, J=12.0, 2H), 2.31-2.25 (m, 2H); ¹³C NMR (100 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 168.02, 167.58, 160.13, 157.57, 131.95, 131.84, 131.71, 125.59, 120.41, 116.06, 114.89, 112.23, 64.20, 64.09, 28.60; HRMS (ESI) m/z calcd. for C₁₉H₁₆BrNO₄SNa (M+Na)+ 455.9881, found 455.9879.

3.2.4. (5Z)-5-[4-[3-(2-propylphenoxy)propoxy]benzylidene]-1,3-thiazolidine-2,4-dione (4d)

Yield: 0.51 g; mp 153-155°C; IR (KBr, V cm⁻¹): 3371, 3158, 3033, 2956, 2929, 1744, 1684, 1589, 1509, 1266, 1244, 1049, 831, 753, 633, 604; ¹H NMR (400 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 8.73 (s, 1H), 7.82 (s, 1H), 7.45 (d, J=8.8, 2H), 7.17-7.12 (m, 2H), 7.00 (d, J=8.8, 2H), 6.90-6.84 (m, 2H), 4.26 (t, J=12.0, 2H), 4.16 (t, J=11.6, 2H), 2.58 (t, J=6.0, 2H), 2.34-2.28 (m, 2H), 1.63-1.56 (m, 2H), 0.92 (t, J=14.8, 3H); ¹³C NMR (100 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 167.44, 166.95, 160.99, 156.46, 134.42, 132.36, 131.10, 129.94, 126.79, 125.52, 120.48, 119.11, 115.23, 111.06, 64.85, 63.87, 32.29, 29.20, 23.03, 14.09.; HRMS (ESI) m/z calcd. for C₂₂H₂₃NO₄SNa (M+Na)+ 420.1245, found 420.1241.

3.2.5. (5Z)-5-[4-[3-(4-propylphenoxy)propoxy]benzylidene]-1,3-thiazolidine-2,4-dione (4e)

Yield: 0.53 g; mp 145-147°C; IR (KBr, V cm⁻¹): 3216, 3034, 2953, 1750, 1715, 1677, 1601, 1510, 1252, 1061, 824, 691; ¹H NMR (400 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 8.76 (s, 1H), 7.82 (s, 1H), 7.45 (d, J=8.8, 2H), 7.10-6.99
(m, 4H), 6.84 (d, J=8.4, 2H), 4.23-4.14 (m, 4H), 2.55-2.51 (m, 2H), 2.28 (t, J=11.6, 2H), 1.64-1.56 (m, 2H), 0.93 (t, J=14.4, 3H); 13C NMR (100 MHz, CDCl3 + 0.1 ml of DMSO-d6): δ 167.19, 166.61, 160.99, 156.71, 135.05, 134.34, 132.31, 129.30, 125.51, 119.08, 115.27, 114.21, 64.79, 64.05, 37.07, 29.14, 24.69, 13.69; HRMS (ESI) m/z calcd. for C22H23NO4SNa (M+Na)+ 420.1245, found 420.1240.

3.2.6. (5Z)-5-[4-[3-(4-chloro-3-nitrophenoxy)propoxy]benzylidene]-1,3-thiazolidine-2,4-dione (4f)

Yield: 0.23g; mp 163-165°C; IR (KBr, V cm-1): 3252, 3113, 2995, 1743, 1694, 1592, 1257, 1054, 823, 687; 1H NMR (400 MHz, CDCl3 + 0.1 ml of DMSO-d6): δ 11.86 (s, 1H), 7.73(s, 1H), 7.46-7.42 (m,4H), 7.11-7.09 (m, 1H), 7.00 (d, J=3.6, 2H), 4.24-4.21 (m, 4H), 2.35-2.29 (m, 2H); 13C NMR (100 MHz, CDCl3 + 0.1 ml of DMSO-d6): δ 173.08, 172.45, 168.24, 167.79, 160.14, 157.53, 156.48, 147.95, 132.25, 132.16, 131.68, 125.97, 120.72, 120.03, 117.85, 115.00, 110.93, 69.06, 65.14, 64.00, 35.58, 28.61, 21.87; HRMS (ESI) m/z calcd. for C19H15ClN2O6SNa (M+Na)+ 457.0237, found 457.0239.

3.2.7. (5Z)-5-[4-[3-(2,6-diisopropylphenoxo)propoxy]benzylidene]-1,3-thiazolidine-2,4-dione (4g)

Yield: 0.43; mp 146-148°C; IR (KBr, V cm-1): 3352, 3142, 2962, 1696, 1589, 1257, 1056, 820, 687; 1H NMR (400 MHz, CDCl3 + 0.1 ml of DMSO-d6): δ 7.84 (s, 1H), 7.48 (d, J=8.4, 2H), 7.10-7.03 (m, 5H), 4.34 (t, J=5.6, 2H), 3.94 (t, J=11.6, 2H), 3.27-3.22 (m, 2H), 2.31-2.28 (m, 2H), 1.28-1.16 (m, 12H); 13C NMR (100 MHz, CDCl3 + 0.1 ml of DMSO-d6): δ 167.78, 167.40, 161.05, 152.84, 141.69, 134.43, 132.40, 125.57, 124.60, 123.96, 119.24, 115.22, 70.08, 64.31, 29.89, 26.46, 26.33, 23.98, 22.03, 21.94; HRMS (ESI) m/z calcd. for C22H23NO4SNa (M+Na)+ 462.1715, found 462.1713.

3.2.8. (5Z)-5-[4-[3-(3,5-difluorophenoxo)propoxy]benzylidene]-1,3-thiazolidine-2,4-dione (4h)

Yield: 0.54; mp 168-170°C; IR (KBr, V cm-1): 3453, 3116, 3014, 2787, 1744, 1694, 1592, 1265, 1055, 822, 687; 1H NMR (400 MHz, CDCl3 + 0.1 ml of DMSO-d6): δ 12.09 (s, 1H), 7.72 (s, 1H), 7.45 (d, J=4.4, 2H), 7.00 (d, J=8.4, 2H), 6.47-6.39 (m, 3H), 4.23-4.14 (m, 4H), 2.32-2.26 (m, 2H); 13C NMR (100 MHz, CDCl3 + 0.1 ml of DMSO-d6): δ 167.98, 167.55, 164.47, 162.02, 161.87, 160.43, 160.06, 131.89, 131.70, 125.64, 120.45, 114.89, 98.15, 98.07, 97.94, 97.87, 64.59, 64.05, 28.41; HRMS (ESI) m/z calcd. for C19H15F2NO4SNa (M+Na)+ 414.0588, found 414.0587.

3.2.9. (5Z)-5-[4-[3-(2-bromo-4-fluorophenoxo)propoxy]benzylidene]-1,3-thiazolidine-2,4-dione (4i)

Yield: 0.50g; mp 173-175°C; IR (KBr, V cm-1): 3434, 3211, 3042, 2922, 1733, 1691, 1595, 1510, 1492, 1263, 1182, 1057, 1018, 801, 610; 1H NMR (400 MHz, CDCl3 + 0.1 ml of DMSO-d6): δ 12.03 (s, 1H), 7.72 (s, 1H), 7.51-7.43 (m, 2H), 7.29-7.26 (m, 1H), 7.03-6.97 (m, 3H), 6.92-6.89 (m, 1H), 4.29 (t, J=1.2, 2H), 4.19 (t, J=11.6, 2H), 2.35-2.29 (m, 2H); HRMS (ESI) m/z calcd. for C19H15BrFNO4SNa (M+Na)+ 473.9787, found 473.9785.

3.2.10. (5Z)-5-[4-[3-(4-bromo-2-fluorophenoxo)propoxy]benzylidene]-1,3-thiazolidine-2,4-dione (4j)
Yield: 0.45 g; mp 154.156°C; IR (KBr, ν cm⁻¹): 3439, 3115, 3020, 2795, 1727, 1687, 1589, 1510, 1244, 1262, 1054, 824, 691; ¹H NMR (400 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 12.03 (s, 1H), 7.72 (s, 1H), 7.44 (d, J=8.8, 2H), 7.33-7.17 (m, 2H), 6.99 (d, J=8.4, 2H), 6.89-6.85 (m, 1H), 4.24-4.19 (m, 4H), 2.34-2.28 (m, 2H); ¹³C NMR (100 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 168.33, 167.84, 160.31, 153.61, 151.12, 146.17, 146.06, 132.36, 131.94, 127.15, 125.93, 120.63, 119.43, 116.05, 115.06, 112.02, 65.78, 64.21, 28.89; HRMS m/z calcd. for C₁₉H₁₅BrFNO₄S Na (M+Na)⁺ + 473.9787, found 473.9786.

3.2.11. (5Z)-5-{4-[3-(3-bromo-5-fluorophenoxy)propoxy]benzyli-dene}-1,3-thiazolidine-2,4-dione (4k)

Yield: 0.43 g; mp 192-194°C; IR (KBr, ν cm⁻¹): 3352, 3120, 3010, 2967, 2778, 1743, 1693, 1592, 1511, 1435, 1264, 1051, 821, 686; ¹H NMR (400 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 9.87 (s, 1H), 7.83 (d, J=7.2, 1H), 7.70 (s, 1H), 7.45 (d, J=7.2, 1H), 7.05-7.00 (m, 2H), 6.88-6.82 (m, 2H), 6.63-6.61 (m, 1H), 4.25-4.16 (m, 4H), 2.31-2.28 (m, 2H); ¹³C NMR (100 MHz, CDCl₃ + 0.1 ml of DMSO-d₆): δ 190.25, 167.87, 167.46, 164.01, 163.32, 161.54, 160.22, 160.10, 159.96, 131.73, 131.63, 131.49, 129.48, 125.53, 122.18, 122.06, 120.36, 114.82, 114.42, 113.62, 111.01, 110.76, 101.26, 64.54, 64.59, 64.12, 63.93, 28.30; HRMS m/z calcd. for C₁₉H₁₅BrFNO₄S Na (M+Na)⁺ + 473.9787, found 473.9786.

3.3. In vitro PPAR competitive binding assay

The results of PPAR receptors binding studies are shown in Table 2. The data reveal a moderate to good binding activity for all the synthesized compounds. The synthesized molecules show comparable binding as that of the standards, rosiglitazone and benzafibrate.

3.4. Adipogenesis assay in 3T3-L1 preadipocyte

The CTC₅₀ values for the synthesized compounds, 4a-k, on 3T3-L1 preadipocyte cells are given in Table 3. The data reveal that the CTC₅₀ values for all the compounds are above 300 µg/ml, indicating a very low cytotoxic potential of these compounds. Based on these results a noncytotoxic concentration of 10 µM was chosen for adipogenesis assay. In the adipogenesis assay all the compounds tested showed moderate to good differentiation effects on 3T3-L1 preadipocytes, as evident from the increase in the basal levels of lipid accumulation (Fig. 6). The quantification of the Oil Red-O in the cells was done to estimate the amount of lipid accumulation. Compound 4b shows the highest concentration of Oil Red-O comparable to the standard, rosiglitazone (Fig. 7). Based on these results it may be concluded that all synthesized compounds promote adipocyte differentiation through activation of PPAR-γ.

4. Discussion

The structural features of LBDs of all the 3 isotypes of PPARs are reported to be common and also resemble the other nuclear receptors. The LBD is folded into a single domain with 13 helices (H1 to H12) and 4 stranded β-sheets (S1 to S4) (Fig. 8a) [8, 18, 24]. The ligand binding site is reported to be a large Y shaped cavity (volume 1300 to 1400 Å³) (Fig. 8b). This cavity is enclosed by helices H2’, H3, H4, H5, H7, H10/11, H2 and β-strands S3 and S4. The C-terminal helix H12 is positioned closer to the LBD and it is known as AF-2 and reported to play a very important role in the receptor activation. The surface of the Y shaped cavity is reported to extend from the surface of
the protein and branches into two arms (each 12Å in length) (Fig. 8b). Arm-I extends towards the AF-2 (H12 helix) and is found to be substantially polar. The 4 polar residues of Arm-I is reported to be highly conserved in each isotypes [Ser280, Tyr314, His440 and Tyr464 in PPAR-α; Ser289, His323, His449 and Tyr473 in PPAR-γ; Thr289, His323, His449 and Tyr473 in PPAR-δ]. These residues are reported to take part in the hydrogen bonding interactions with the natural ligands (carboxylic acid group of fatty acids and eicosanoids) and with synthetic ligands (TZD head of rosiglitazone, carboxylic acid group of AZ242) [25-27]. Arm-II is situated in between H3 and β-sheet and it is found to be hydrophobic. The solvent accessible part of the entrance is composed of polar residues (Pro227, Arg288, Glu295 and Glu343 for PPAR-γ) (Fig. 8c) and the interior is found to be highly hydrophobic, which may explain the hydrophobic nature of natural ligands for these receptors. The flexible nature of the Ω loop between H2 and H3 facilitates the entry of large ligands without affecting the overall structure of LBD [8, 18, 24].

The acidic head group of the ligand has been reported to form up to 4-hydrogen bonding interactions with the Arm-I of the LBD [25-27]. For a full agonist, the acidic head group should form hydrogen bonding interactions with the conserved residues of the Arm-I. These residues in PPAR-γ include Ser 289 (H3), His323 (H5), His449 (H11) and Tyr473 (H12 or AF-2). Among these the hydrogen bonding with the Tyr473 residue is reported to play a vital role in the stabilization of AF-2 helix which is essential for the recruitment of coactivators necessary for transcriptional activation. The ligands which fail to form hydrogen bonding with the Arm-I residues, especially with Tyr473, would fail in stabilizing the AF-2 helix and results in the destabilization of the LBD. These agents, therefore, are reported to act as either partial agonists or as antagonists [17,19, 24]. Recent finding of the ligand receptor interaction studies have also shown that effective PPAR-γ agonists should show a low transactivation activity (a minimal binding at Arm-I) but a high binding affinity (through hydrophobic interactions at the Arm-II and at the entrance, and hydrogen bond at the entrance with Ser342) to inhibit phosphorylation at Ser273. Inhibition of Ser273 phosphorylation is reported to prevent the unregulated expression of some of the genes including adipin and adiponectin [15]. The spacer ring, linker and the tailpiece portions of the ligand are reported to interact with the hydrophobic portion of the LBD (Arm-II and entrance). In addition the part of the tailpiece is reported to be solvent exposed and, therefore, polar and other diverse substitutions on this portion of the ligand are well tolerated [17]. Although PPARs are highly homologous, the LBD exhibits specific differences that convey isotype selectivity. In case of PPAR-δ the binding site near AF-2 is narrower than the other two isotypes. In addition, in AF-2 region the hydrogen bonding to His 323 of PPAR-γ is found to be less sterically demanding when compared to the corresponding Tyr314 residue in PPAR-α [8].

In the present study, the post docking analysis of the synthesized molecules, 4a, 4b, 4c, 4d, 4e, 4h and 4j, show binding modes similar to a full agonist by forming H-bond interactions with the conserved residues of Arm-I of PPAR-γ LBD with their acidic head. The tail portions of these molecules occupy the hydrophobic region of Arm-II (Fig. 4 & 5, Table 4). The molecules, 4f, 4g, 4i and 4k, show binding modes similar to that of an partial agonist by failing to form H-bond interaction with the Tyr 473 residue of the Arm-I of PPAR-γ LBD (Fig. 4 & 5, Table 4). On the other hand, with PPAR-α LBD, all the molecules except, 4a and 4g show binding modes similar to a full agonist by forming H-bonds with the conserved residues of Arm-I and their tail portions occupy the hydrophobic region of
Arm-II (Fig. 4 & 5, Table 4). In case of molecules 4a and 4g, the polar acidic head of molecules occupy hydrophobic Arm-II and their non polar tails occupy the polar Arm-I region and thus fail to form H-bonds with the polar residues of Arm-I which are important for transcriptional activation of the receptor. In addition, these two molecules form H-bonds with the Ala-333 residue of the entrance. These molecules, therefore, show binding modes similar to that of a partial agonist (Fig. 4 & 5, Table 4). The results of in vitro PPAR competitive binding and 3T3L1 preadipocytes assays further confirm their binding to PPAR-α & γ receptors and their PPAR-γ agonistic activities, respectively. The present study thus reveal diverse PPAR modulatory potential for (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidin-2,4-dione derivatives. These molecules, therefore, may have the required potential to overcome the existing problems with the glitazones in the management of T2DM.

5. Conclusion
In conclusion, the 11 novel (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione derivatives synthesized in the present study show dual binding potential with the PPAR-α and γ LBD in in silico docking and in vitro competitive binding studies. The results of 3T3L1 preadipocytes assays confirm their PPAR-γ agonistic activities. The post docking analysis results show diverse PPAR modulatory potential for these molecules. These molecules may, therefore, have the potential to overcome the problems with the existing glitazones in the management of T2DM.

Acknowledgements
We thank Prof. S. Chandrasekaran and Mr. Ganesh V., Dept. of Organic Chemistry, IISc., Bangalore, and Mr. Raghu R., Senior Director, Business Development, Schrodinger Ltd., Bangalore, for helpful discussions.

Reference List


Fig. (3). (a) Conformational comparison of rosiglitazone from the crystal structure of rosiglitazone–PPAR-γ complex (grey, ball and stick) and that predicted by XP Glide (green, tube) are shown within the active site of PPAR-γ (RMSD=0.4930 Å); (b) Conformational comparison of aleglitazar from the crystal structure of aleglitazar–PPAR-α complex (grey, tube) and that predicted by XP Glide (green, ball and stick) are shown within the active site of PPAR-α (RMSD=0.1735 Å).
Fig. (4). Hydrogen bond interactions of (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione derivatives (4a-k) and benzafibrate with the LBD of PPAR-α (PDB ID: 3G8I).
Fig. (5). Hydrogen bond interactions of (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione derivatives (4a-k) and rosiglitazone with the LBD of PPAR-γ (PDB ID: 2PRG).
Fig. (6). Effect of (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione derivatives (4a-k) on 3T3-L1 preadipocyte differentiation (Oil Red-O staining, 10X).
Fig. (7). Effect of (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione derivatives (4a-k) on fat accumulation in 3T3-L1 preadipocyte estimated in terms of Oil Red-O contents.

Fig. (8). (a) Crystal structure of apo PPAR-γ; (b) Ligand binding cavity; (c) Binding site entrance (Source: BBA, 2007; 1771: 915)
Table 1. The glide scores for the (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione derivatives (4a-k) along with their XP descriptor terms (PPAR-α).

<table>
<thead>
<tr>
<th>Compound</th>
<th>PPAR-α</th>
<th>PPAR-γ</th>
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<tr>
<td></td>
<td>GS</td>
<td>Lip</td>
</tr>
<tr>
<td>4a</td>
<td>-8.3</td>
<td>-6.5</td>
</tr>
<tr>
<td>4b</td>
<td>-11.9</td>
<td>-7.5</td>
</tr>
<tr>
<td>4c</td>
<td>-10.5</td>
<td>-6.1</td>
</tr>
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<td>-7</td>
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<tr>
<td>4e</td>
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<td>4h</td>
<td>-10.9</td>
<td>-6.1</td>
</tr>
<tr>
<td>4i</td>
<td>-10.6</td>
<td>-6.5</td>
</tr>
<tr>
<td>4j</td>
<td>-10.6</td>
<td>-6.2</td>
</tr>
<tr>
<td>4k</td>
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<td>-6.7</td>
</tr>
<tr>
<td>Rosiglitazone</td>
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<td>-5.8</td>
</tr>
<tr>
<td>Benzafibrate</td>
<td>-9.7</td>
<td>-6.5</td>
</tr>
</tbody>
</table>

GS: Glide score, which is some of rewards and penalties; Lip: Lipophilic reward; PE: Hydrophobic enclosure reward; PEPHB: Reward for hydrophobically packed H-bond; PEPHB: Reward for for hydrophobically packed correlated H-bond; HB: H-bonding reward; Ele: Electrostatic reward; SM: Site map, rewards for ligand/receptor non H-bonding polar/hydrophobic and hydrophobic/hydrophilic complementarity terms; LMW: Rewards for ligands with low molecular weight; RP: Rotatable bond penalty
Table 2. PPAR competitive binding assays of (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione derivatives (4a-k).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (nM)</th>
<th>PPAR-γ</th>
<th>PPAR-α</th>
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<tr>
<td>4a</td>
<td>460 ± 5.2</td>
<td>340 ± 2.7</td>
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<tr>
<td>4b</td>
<td>320 ± 3.9</td>
<td>234 ± 3.3</td>
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<tr>
<td>4c</td>
<td>380 ± 6.7</td>
<td>418 ± 8.5</td>
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</tr>
<tr>
<td>4d</td>
<td>465 ± 8.1</td>
<td>687 ± 7.4</td>
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</tr>
<tr>
<td>4e</td>
<td>483 ± 3.9</td>
<td>732 ± 9.5</td>
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<tr>
<td>4f</td>
<td>1056 ± 8.1</td>
<td>1979 ± 10.6</td>
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</tr>
<tr>
<td>4g</td>
<td>889 ± 5.0</td>
<td>1045 ± 11.7</td>
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<tr>
<td>4h</td>
<td>964 ± 6.2</td>
<td>1084 ± 9.0</td>
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<tr>
<td>4i</td>
<td>850 ± 10.1</td>
<td>933 ± 7.5</td>
<td></td>
</tr>
<tr>
<td>4j</td>
<td>704 ± 11.5</td>
<td>893 ± 11.7</td>
<td></td>
</tr>
<tr>
<td>4k</td>
<td>2540 ± 15.9</td>
<td>1745 ± 12.4</td>
<td></td>
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<tr>
<td>Rosiglitazone</td>
<td>140 ± 3.1</td>
<td>----</td>
<td></td>
</tr>
<tr>
<td>Benzaflbrate</td>
<td>----</td>
<td>52 ± 2.8</td>
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</table>

Values are mean ± SD, n=3.
Table 3. Cytotoxic effect of the (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione derivatives (4a-k) on 3T3-L1 preadipocytes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CTC 50 (μg/ml)</th>
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<tbody>
<tr>
<td>4a</td>
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</tr>
<tr>
<td>4b</td>
<td>390 ± 18.4</td>
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<tr>
<td>4c</td>
<td>320 ± 15.8</td>
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<td>4d</td>
<td>350 ± 16.9</td>
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<tr>
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<td>4g</td>
<td>340 ± 10.0</td>
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<tr>
<td>4h</td>
<td>300 ± 14.6</td>
</tr>
<tr>
<td>4i</td>
<td>360 ± 13.2</td>
</tr>
<tr>
<td>4j</td>
<td>475 ± 12.6</td>
</tr>
<tr>
<td>4k</td>
<td>490 ± 21.0</td>
</tr>
</tbody>
</table>

Values are mean ± SD, n=3.
Table 4. Hydrogen bond interaction of (5Z)-5-[4-(3-phenoxypropoxy)benzylidene]-1,3-thiazolidine-2,4-dione derivatives (4a-k) with the conserved residues of the PPAR-α & γ LBD.

|                | PPAR-γ |                | PPAR-α |                |
|----------------|--------|----------------|--------|
|                | Arm I  | Arm-II         | Arm I  | Arm-II         | Entrance |
|                | Ser 289| His 323        | Tyr 473| Gln 286        | Tyr 314  |
| 4a             | √      | √              | √      | √              | Tail     |
| 4b             | √      | √              | √      | √              | Tail     |
| 4c             | √      | √              | √      | √              | Tail     |
| 4d             | √      | √              | √      | √              | Tail     |
| 4e             | √      | √              | √      | √              | Tail     |
| 4f             | √      | √              | X      | √              | Tail     |
| 4g             | √      | √              | X      | √              | Tail     |
| 4h             | √      | √              | X      | √              | Tail     |
| 4i             | √      | √              | X      | √              | Tail     |
| 4j             | √      | √              | X      | √              | Tail     |
| 4k             | √      | √              | X      | √              | Tail     |
| Rosi           | √      | √              | X      | √              | Head     |
| Alegli         | √      | √              | X      | √              | Tail     |

√: H-bond interaction, X: No interaction, Rosi: Rosiglitazone, Alegli: Aleglitazar