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Design, Synthesis, and Evaluation of Potent Novel Peroxisome Proliferator-Activated Receptor γ Indole Partial Agonists

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Abstract

Peroxisome Proliferator-Activated Receptor γ (PPAR γ) is a nuclear receptor important for glucose homeostasis and insulin sensitivity. The anti-diabetic drugs thiazolidinediones improve insulin sensitivity by blocking PPAR γ phosphorylation at S273; however, their full agonism on PPAR γ also causes significant unwanted side effects. The indole derivative UHC1 displays insulin-sensitizing effect by acting as a partial agonist through the inhibition of PPAR γ S273 phosphorylation, but without full agonist-associated side effects; however, its potency leaves much to be desired. Herein we report the design and synthesis of potent indole analogs as partial PPAR γ agonists via the structure-activity relationship studies. Our studies revealed that vanillylamine and piperonyl benzylamine at Site 1 are favored to bind PPAR γ with either biphenyl or 3-trifluoromethyl benzyl group at Site 2. In particular, compound WO91A with vanillylamine at Site 1 displays highly potent PPAR γ binding affinity (IC_{50} = 16.7 nM), over 30-fold more potent than the parental compound UHC1, yet with less side effect-associated transactivation activity.

Graphical Abstract

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Type 2 Diabetes (T2D), a current pandemic, usually develops in obese and insulin-resistant subjects with the onset of insulin-producing β cell dysfunction and death. The thiazolidinedione (TZD) drugs such as rosiglitazone (Rosi) and pioglitazone are effective in improving insulin sensitivity and are used for the treatment of insulin resistance and T2D.¹ However, the current TZD drugs are associated with long-term serious side effects such as weight gain, fluid retention, loss of bone mass and increased risk of congestive heart failure.^{2, 3} As a result, prescription of such drugs has recently declined significantly.

TZDs are full agonists of peroxisome proliferator-activated receptor γ (PPAR γ).¹ PPAR γ is a member of the nuclear receptor family of transcription factors and plays an important role in the regulation of insulin sensitivity, adipogenesis, and glucose homeostasis by regulating the transcription of many genes involved in these processes.⁴ TZDs hence activates the PPAR γ -dependent expression of genes responsible for insulin sensitivity; however, their full agonism of PPAR γ also activates the expression of genes associated with unwanted side effects.

Recently, it has been reported that the insulin-sensitizing properties of TZDs are attributed to their ability to block the cyclin-dependent kinase 5 (CDK5) or extracellular signal-regulated kinase (ERK)-dependent PPAR γ phosphorylation at S273.^{5, 6} Blockage of PPAR γ S273 phosphorylation led to the restoration of obesity-induced dysregulation in insulin-sensitivity relevant genes including adiponectin and adipisin.⁶ Importantly, this effect is independent of the full agonism of TZDs that is associated with adverse effects.⁶ Compounds that block the PPAR γ S273 phosphorylation but without full PPAR γ agonism could therefore be promising direction for T2D drug development.

Several chemical structures have so far been identified to bind to PPAR γ and inhibit PPAR γ S273 phosphorylation but without fully stimulating the transcriptional activity of PPAR γ ; these compounds, including partial agonists⁶⁻⁹ or non-agonist PPAR γ ligands^{10, 11}, improve insulin sensitivity with diminished adverse effects associated with full agonist TZDs in vivo. The mechanisms by which these ligands inhibit PPAR γ S273 phosphorylation remains incompletely understood; however, one recent report indicates that ligand binding at the alternate binding site of PPAR γ directly block the S273 from phosphorylation by kinases.¹²

The indole-based derivatives SR1664 and UHC1 (in Figure 1) were reported as non-agonist synthetic ligands of PPAR γ as they inhibit phosphorylation of S273 and are anti-diabetic in vivo but do not exhibit full agonist activity.^{10, 11} These derivatives thus represent a new generation of improved anti-diabetic scaffold through PPAR γ . However, these compounds have less potency in binding affinity or poor pharmacokinetic properties.^{10, 11} In this study, we report the synthesis of a series of novel analogs of compounds based on the UHC1 scaffold that possesses highly potent PPAR γ binding affinity but with weak PPAR γ transcription activation. These compounds therefore represent more effective therapeutics over the current indole-derived PPAR γ ligands and provides a promising approach to treat T2D.

The syntheses of **2a-j** and **3a-e** are shown in Scheme 1 and substituents are listed in Table 1. Key substituted indole acid derivatives were prepared as previous reported.¹³ Compounds

2a-j (Scheme 1A) and **3a-e** (Scheme 1B) were prepared by coupling commercially available substituted benzylamines with indole acid derivative by using HATU coupling reagent in DCM at ambient temperature. Analogs **2a-j** were obtained after deprotection of *t*-butyl group with TFA. All these synthesized compounds were listed in Table 1 and characterized by physical and spectral analysis data that confirmed their assigned structures.

Compound **5** was synthesized using mono-alkylation as shown in Scheme 2. Intermediate **4** was prepared by alkylating of 4-aminobenzoate in DMF in presence of potassium carbonate followed by hydrolysis of ester to obtain acid intermediate **4**. Compound **5** was obtained by HATU coupling with corresponding amines in DCM at ambient temperatures.

To identify more potent analogs of UHC1, we synthesized a series of novel compounds with structure-activity-relationship (SAR) studies. The newly synthesized compounds were initially tested for their binding affinity to the ligand binding domain of PPAR γ protein using an in vitro competition binding assay. We first focused our efforts on the modification of the amide group in the left-hand side of the UHC1 compound. Elimination of the amide group that produces the key intermediate (**1**), as seen in Scheme 1, led to a significant loss in PPAR γ binding affinity, indicating that the amide function is compulsory for the binding (Table 1).

We next examined the electron withdrawing substituents on the benzyl group of amides (Scheme 1A) and found that the 4-sulfonamide substituted benzamide (**2a**) showed comparable biochemical affinity as the parent UHC1 compound (IC_{50} 0.312 μ M for **2a** vs. 0.536 μ M for UHC1). Encouraged by this outcome, we then introduced a pyridine ring at the amide side contributed to significant improvement in PPAR γ binding affinity (**2b**). These results indicate that the electron-withdrawing substituents are favored on the left-hand side. To investigate other electronic effects, we synthesized compound (**2c**) with a piperonylamine functional group. Compound **2c** demonstrated a 20-fold improvement in binding affinity compared to the parent compound UHC1. Replacement of the phenyl ring by non-aromatic piperidine moiety led to the resultant compound (**2d**) significantly losing its binding affinity, indicating that phenyl substituents are essential for the PPAR γ binding at that position. On the other hand, compound (**2e**, herein we refer it as WO91A), where the piperonyl splits into the OH and OMe groups as the vanillyl amine derivative, exhibited the most potent binding affinity to PPAR- γ with IC_{50} = 16.7 nM (Figure 3A). Such strong affinity may be attributed to the -OH forming hydrogen bond with the binding pocket of the PPAR γ ligand binding domain.

Next, we determined whether shortening of the length of benzyl to phenyl ring may contribute to improved binding affinity. In consideration of the instability of aniline derivatives, we prepared a piperidine analog (**2f**) to test the shortened analog, which turned out to exhibit poor binding affinity in compare with its counterpart **2d**. Subsequently, we extended the length of the analog by preparing a tyramine derivative (**2g**) that maintained significant binding affinity. Previous reports have shown that acidic groups increase ligand affinity to the PPAR- γ binding pocket.^{14, 15} We applied this concept to our analog preparation by synthesizing compound (**2h**), which possesses a phenoxy acetic acid in the R¹ site (see Scheme 1A). However, compared to **2e** (WO91A), **2h** exhibited significantly

less potent PPAR γ binding affinity, although it is comparable to that of compounds with no acidic group (e.g. **2g**).

In the next set of analogs, we explored the importance of biphenyl substituents at the indole site. We prepared compounds **3a-e** by switching the biphenyl substituent with a 3-trifluoromethyl benzyl group (Table 1, Scheme 1B). First, we chose the most potent compound WO91A obtained from the above SAR analysis, which possesses the vanillyl amine on the left side. We replaced the biphenyl group in WO91A with the 3-trifluoromethyl benzyl group and found that the resultant compound **3a** maintained significant PPAR γ binding affinity compared to its biphenyl counterpart WO91A. Moreover, **3b** also retained its same binding affinity as its biphenyl counterpart **2c**. Analogs **3c** and **3d** exhibited reasonable binding affinity too. Together, these results suggest that the trifluoromethyl benzyl moiety retains potent binding affinity to PPAR γ similar to their biphenyl counterpart. We next introduced an amino acid link on the left hand-side while keeping the trifluoromethyl benzyl group at the indole site so as to balance the ClogP for solubility purpose. Surprisingly, the tyrosine analog **3e** exhibited a binding affinity for PPAR γ significantly worse than WO91A. Lastly, we examined the importance of indole group per se. Breaking the indole ring into the non-cyclic aniline derivative (compound **5**, seen in Scheme 2) did not offer any advantage for PPAR γ binding affinity; in fact, **5** exhibited poorer PPAR γ binding affinity than most of indole-based 2- and 3-series analogs, indicating the importance of indole group in PPAR γ binding.

Together, these SAR studies indicate that both vanillylamine (WO91A and **3a**) and piperonyl benzylamine (**2c** and **3b**) at Site 1 convey favorable PPAR γ binding affinity regardless whether it is biphenyl or 3-trifluoromethyl benzyl group at Site 2. In particular, the SAR identified WO91A as the most potent derivative for PPAR γ binding and reveals that WO91A binds to PPAR γ with significantly more favorable chemical properties than UHC1. We next used the *in silico* docking modeling method (Autodock) to compare the docking simulations of WO91A and UHC1 with PPAR γ LBD. The docking scores for binding affinity (UHC1 -10.2 kcal/mol vs. WO91A -11.3 kcal/mol) suggests that WO91A fits better than UHC1 in the pocket within the LBD of PPAR γ (Figure 2A, 2B). Notably, in our simulation, both UHC1 and WO91A occupy the alternate ligand-binding site (Figure 2A, 2B), a site comprised of H2'-H3, β -sheet, and Ω loop regions, which was previously identified in the PPAR γ LBD structure complexed with a partial agonist MRL20.¹⁶ This docking mode is distinct from the canonical PPAR γ ligand binding pocket occupied by full agonists, which usually comprises H3, H3-4 loop, H11 and H12.¹⁷ Further, although SR1664 and its derivative UHC1 were previously reported to bind to the canonical ligand binding pocket similar to that of a full agonist^{10, 18}, our docking model is consistent with a recent X-ray crystal structure study that SR1664 binds to the alternate binding site of PPAR γ .¹² Our docking simulation further showed that WO91A formed hydrogen bonds with multiple amino acids in the alternate binding site (Figure 2C). The two carboxylate oxygens of WO91A formed hydrogen bonds with both Arg280 and Gln283 on helix H3 of PPAR γ LBD. The amide oxygen of WO91A also forms hydrogen bond with Ser342 on β -sheet. In contrast, UHC1 formed hydrogen bond with Ser342 only (Figure 2D). These results may explain the more potent binding affinity of PPAR γ with WO91A than UHC1.

The indole derivatives SR1664 and UHC1 were reported to act as non-agonist PPAR γ ligands in that similar to full agonist Rosi, they bind to PPAR γ and increase insulin sensitivity, but in contrast to Rosi, they do not or weakly activate the transactivation activity of PPAR γ , which probably explain their less or minimal side effects as observed for Rosi.^{10, 11} We therefore investigated the transactivation activity of the above newly synthesized compounds. PPAR γ serves as a transcription factor to activate the expression of a number of genes under the control of promoter carrying PPAR γ -responsive element (PRE). To determine the effect of these UHC analogs on PPAR γ transactivation activity, we transfected HEK293 cells with both PPAR γ -expressing plasmid and 3xPRE-Luciferase reporter plasmid and treated the cells with compounds in a dose-dependent manner (with increasing concentrations from 0.001 μ M to 10 μ M). As shown in Table 1 and Figure 3B, compared to the full PPAR γ agonist Rosi that markedly increased the reporter activity, the parental compound UHC1 activated the reporter as an approximately 45% of strength of Rosi. We noticed that although UHC1 was reported as non-agonist ligand¹⁰, the data obtained here indicates that UHC1 maintains significant activity on PPAR γ reporter activation. The majority of our newly synthesized compounds that exhibited high PPAR γ binding affinity activated the reporter in a manner similar to or less potently than UHC1, indicating that they are partial agonists of PPAR γ . WO91A is in particular of interest in that it exhibited not only the highest PPAR γ binding affinity among all active compounds (its IC₅₀ is over 30-fold lower than that of the parental compound UHC1) but also the lower transactivation activity of PPAR γ compared to UHC1 (15% for WO91A vs. 45% for UHC1).

We then chose WO91A for further biological evaluation. PPAR γ phosphorylation at S273 is reported to be responsible for obesity-related insulin resistance.⁶ Indole derivatives such as UHC1 inhibits PPAR γ phosphorylation at S273 to improve insulin sensitivity. It is reported that ligand binding to the PPAR γ alternate binding site directly blocks the S273 phosphorylation.¹² We therefore investigated whether WO91A suppresses the phosphorylation of PPAR γ at S273. As shown in Figure 4A, like Rosi and UHC1, WO91A significantly inhibited the S273 phosphorylation of PPAR γ in 3T3-L1 differentiated adipocytes in the presence of TNF α which is known to activate CDK5 α to phosphorylate PPAR γ at S273, by Western blotting using PPAR γ phospho-S273-specific antibody. Of note, WO91A inhibited the S273 phosphorylation to a greater degree than UHC1 (Figure 4A). PPAR γ S273 phosphorylation is associated with repression of a set of genes that are believed to be responsible for insulin sensitivity and dephosphorylation of PPAR γ S273 led to the derepression of these genes.⁶ We investigated the effect of WO91A on the expression of S273 phosphorylation -associated genes. We selected Adiponectin, Adipsin, and Car3, three genes that were reported to be associated with insulin sensitivity for this purpose. We observed that WO91A treatment caused the upregulation of mRNA levels of Adiponectin, Adipsin, and Car3 in 3T3-L1 differentiated adipocytes (Figure 4B–D). Together, these results indicate that WO91A inhibits PPAR γ phosphorylation.

Activation of PPAR γ by full agonists such as Rosi not only inhibits PPAR γ phosphorylation to improve insulin resistance but also regulates additional events including such side effects as fluid retention and fat and weight gain, events that partial or non-agonists exhibit weak or minimal impact. One such event is adipogenesis. We therefore investigated whether WO91A affects adipogenesis in a cell-based assay. As a master regulator of adipose differentiation,

the full agonism of PPAR γ by TZDs drives 3T3-L1 mouse embryonic fibroblast cells to differentiate into adipocyte. When 3T3-L1 cells were differentiated into adipocytes in the presence of differentiation media, Rosi treatment markedly induced the differentiation of adipocytes, as indicated by the widespread presence of lipid droplets with positive Oil Red O staining, whereas there was little or no Oil Red O staining when treated with DMSO (Figure 5A, 5B). In contrast to Rosi, both WO91A and UHC1 exhibited significantly reduced Oil Red O staining, but WO91A achieved so with a greater degree (Figure 5C, 5D), indicative of its little effect on adipogenesis. Next, we investigated the impact of WO91A on the expression of genes responsible for adipogenesis. We chose genes FABP4, LPL and Glut4 as representatives for this purpose. Full agonist Rosi is known to induce the expression of these adipogenic genes during adipogenesis. Indeed, Rosi treatment of differentiated 3T3-L1 pre-adipocytes significantly upregulated the mRNA levels of FABP4, Glut4, and LPL genes (Figure 5E–G). In contrast, WO91A only weakly increased the expression these genes at the contraction of 10 μ M (Figure 5E–G).

In this study, we have described here the design and synthesis of a series of derivatives of antidiabetic compound UHC1. These studies combine in vitro competition binding assay and transcriptional activity assay, allowing the identification of derivatives that exhibit potent PPAR γ binding affinity but yet minimal PPAR γ transactivation activity. These SAR studies indicate that both vanillylamine (WO91A and **3a**) and piperonyl benzylamine (**2c** and **3b**) at Site 1 are favored to bind PPAR γ in an indole scaffold with either biphenyl or 3-trifluoromethyl benzyl group at Site 2. In particular, compound WO91A with vanillylamine at Site 1 and a biphenyl group at Site 2 conveys highly potent PPAR γ binding affinity (IC_{50} = 16.7 nM, K_i = 13 nM), over 30-fold more potent than the parental compound UHC1, which may serve as a foundation for further drug development endeavor. Our further results indicate that WO91A inhibits PPAR γ S273 phosphorylation but plays little role in other full agonist-dependent but S273 phosphorylation-independent events such as adipogenesis. In summary, these results present a clearer picture of how future efforts for new therapeutics targeting PPAR γ can be directed for high binding potency but without eliciting transactivation activity.

Experimental Section

Chemistry

Materials and Methods.—Reagents and solvents were obtained from commercial suppliers and were used without further purification. Reactions using air- or moisture sensitive reagents were performed under an atmosphere of Argon or Nitrogen. Reactions were monitored by TLC. Flash chromatography was performed with 230–400 mesh silica gel, NMR spectra were measured on Bruker 400 MHz spectrometers. Chemical shifts are reported in ppm in the indicated solvent with TMS as an internal standard. Data are reported in the form: chemical shift (multiplicity, coupling constants, and integration). Multiplicities are recorded by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Analytical HPLC and ESI-MS analyses were performed using either Agilent or Krats MS 80 mass spectrometer. All tested compounds were evaluated on the Agilent HPLC systems using ACE-C18 column (250 $\times$ 4.6 mm) was used as the stationary

phase. HPLC conditions include a flow rate of 1.0 mL/min using water and acetonitrile as solvents and a detection wavelength of 254 nm and determined to be 95% pure.

General procedure: Amino acid coupling.—To a mixture of the acid (1eq) in DCM was added DIEA (10 equivalents) and HATU (1 equivalents). The mixture was stirred for 5 min, and then the appropriate amine (1 equivalents) was added. The reaction mixture was stirred at room temperature for 30 min. The completion of the reaction was monitored by TLC. The solvent was removed *in vacuo* to obtain the crude which was purified by flash chromatography to provide product.

Deprotection.—To a room temperature solution of above material in DCM was added TFA. The reaction was stirred at room temperature for 30 min. The completion of the reaction was monitored by TLC. The solvent was removed to obtain the crude which was purified by Flash chromatography was performed with 230–400 mesh silica gel to obtain final products.

1-((2'-Carboxy-[1,1'-biphenyl]-4-yl)methyl)-2,3-dimethyl-1H-indole-5-carboxylic acid (1): ¹H NMR (DMSO-d₆, 400 MHz) δ: 13.79 (s, 1H), 12.50 (s, 1H), 8.77 (d, *J* = 4.3 Hz, 1H), 8.53 (d, *J* = 8.4 Hz, 1H), 8.13 (s, 1H), 7.69 (d, *J* = 7.9 Hz, 2H), 7.53 (m, 1H), 7.42 (s, 1H), 7.32 (d, *J* = 7.5 Hz, 1H), 7.25 (d, *J* = 7.6 Hz, 2H), 7.01 (d, *J* = 7.7 Hz, 2H), 5.48 (s, 2H), 2.32 (s, 3H), 2.26 (s, 3H); ¹³C NMR (100MHz, DMSO-d₆) δ: 168.7, 166.6, 139.6, 138.8, 136.7, 136.4, 133.2, 131.4, 130.0, 129.5, 128.2, 127.7, 126.7, 126.4, 125.1, 124.3, 119.3, 116.7, 107.9, 106.3, 42.0, 10.2, 8.9. HPLC purity 98.31%

4'-((2,3-Dimethyl-5-((4-sulfamoylbenzyl)carbamoyl)-1H-indol-1-yl)methyl)-[1,1'-biphenyl]-2-carboxylic acid (2a): ¹H NMR (DMSO-d₆, 400 MHz) δ: 8.97 (t, *J* = 6.0 Hz, 1H), 8.11 (s, 1H), 7.77 (d, *J* = 7.9 Hz, 2H), 7.64 (m, 3H), 7.49 (d, *J* = 8.4 Hz, 2H), 7.45 (d, *J* = 8.1 Hz, 2H), 7.36 (d, *J* = 7.5 Hz, 1H), 7.29 (s, 2H), 7.19 (d, *J* = 7.7 Hz, 2H), 7.00 (d, *J* = 7.9 Hz, 2H), 5.48 (s, 2H), 4.55 (d, *J* = 5.6 Hz, 2H), 2.32 (s, 3H), 2.26 (s, 3H). ¹³C NMR (100MHz, DMSO-d₆) δ: 168.4, 167.8, 144.7, 142.9, 141.3, 140.0, 138.1, 137.8, 134.5, 131.8, 131.5, 130.8, 129.7, 128.9, 128.1, 127.9, 126.3, 126.1, 125.2, 120.5, 118.1, 109.2, 107.7, 61.0, 46.1, 10.4, 9.1. HPLC purity 96.12%

4'-((2,3-Dimethyl-5-((pyridin-2-ylmethyl)carbamoyl)-1H-indol-1-yl)methyl)-[1,1'-biphenyl]-2-carboxylic acid (2b): ¹H NMR (CDCl₃, 400 MHz) δ: 9.01 (s, 1H), 8.60 (s, 1H), 8.25 (m, 2H), 8.15 (s, 1H), 8.09 (m, 2H), 7.67 (m, 3H), 7.42 (m, 3H), 6.94 (d, *J* = 7.6 Hz, 2H), 5.33 (s, 2H), 4.91 (s, 2H), 2.31 (s, 3H), 2.24 (s, 3H); ¹³C NMR (100MHz, DMSO-d₆) δ: 168.5, 167.9, 144.6, 143.0, 141.3, 140.0, 138.1, 137.8, 134.5, 131.8, 131.5, 130.8, 129.7, 128.9, 128.1, 127.9, 126.3, 126.1, 125.2, 120.5, 118.1, 109.2, 107.7, 61.0, 46.1, 10.2, 9.1. HPLC Purity 95.01%

4'-((5-((Benzo[d][1,3]dioxol-5-ylmethyl)carbamoyl)-2,3-dimethyl-1H-indol-1-yl)methyl)-[1,1'-biphenyl]-2-carboxylic acid (2c): ¹H NMR (CDCl₃, 400 MHz) δ: 7.99 (s, 1H), 7.89 (d, *J* = 7.6 Hz, 1H), 7.50 (m, 2H), 7.39 (m, 1H), 7.29 (d, *J* = 7.6 Hz, 1H), 7.20 (d, *J* = 7.8 Hz, 2H), 7.15 (d, *J* = 8.5 Hz, 1H), 6.92 (d, *J* = 7.8 Hz, 2H), 6.84 (s, 1H), 6.80 (d, *J* = 8.0 Hz, 1H), 6.74 (d, *J* = 7.8 Hz, 1H), 6.46 (d, *J* = 5.5 Hz, 1H), 5.91 (s, 2H), 4.53 (d, *J* = 5.4

Hz, 2H), 2.28 (s, 3H), 2.26 (s, 3H); ^{13}C NMR (100MHz, CDCl_3) δ : 171.2, 168.8, 147.9, 146.9, 142.5, 140.2, 138.2, 136.6, 134.1, 132.5, 131.9, 131.0, 130.5, 129.4, 128.9, 127.3, 125.7, 124.9, 121.1, 119.8, 117.8, 108.7, 108.5, 108.4, 108.3, 101.0, 46.6, 43.9, 10.3, 8.8. HPLC purity 99.16%

4'-((2,3-Dimethyl-5-((piperidin-4-ylmethyl)carbamoyl)-1H-indol-1-yl)methyl)-[1,1'-biphenyl]-2-carboxylic acid (2d): ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 8.78 (bs, 1H), 8.44 (t, $J = 5.7$ Hz, 1H), 8.05 (s, 1H), 7.68 (d, $J = 7.3$ Hz, 1H), 7.61 (d, $J = 8.4$ Hz, 1H), 7.53 (d, $J = 7.3$ Hz, 1H), 7.44 (d, $J = 6.6$ Hz, 2H), 7.31 (d, $J = 7.4$ Hz, 2H), 7.24 (d, $J = 7.4$ Hz, 2H), 6.99 (d, $J = 7.7$ Hz, 2H), 5.47 (s, 2H), 3.22 (m, 4H), 2.86 (m, 2H), 2.31 (s, 3H), 2.27 (s, 3H), 1.83 (m, 3H), 1.40 (m, 2H); ^{13}C NMR (100MHz, CDCl_3) δ : 168.7, 166.6, 139.6, 138.8, 136.7, 136.4, 133.2, 131.4, 130.0, 129.5, 128.2, 127.7, 126.7, 126.4, 125.1, 124.3, 119.3, 116.7, 107.9, 106.3, 42.0, 25.5, 9.2, 7.9. HPLC purity 98.91%

4'-((5-((4-Hydroxy-3-methoxybenzyl)carbamoyl)-2,3-dimethyl-1H-indol-1-yl)methyl)-[1,1'-biphenyl]-2-carboxylic acid (2e): ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 9.27 (s, 1H), 8.81 (t, $J = 5.7$ Hz, 1H), 8.11 (s, 1H), 7.66 (m, 2H), 7.45 (m, 3H), 7.31 (d, $J = 7.5$ Hz, 1H), 7.24 (d, $J = 7.6$ Hz, 2H), 7.09 (d, $J = 7.8$ Hz, 1H), 2.04 (s, 2H), 6.60 (d, $J = 7.8$ Hz, 1H), 5.46 (s, 2H), 4.42 (d, $J = 5.6$ Hz, 2H), 2.31 (s, 3H), 2.26 (s, 3H); ^{13}C NMR (100MHz, $\text{DMSO}-d_6$) δ : 168.0, 145.9, 144.4, 135.6, 129.3, 129.1, 126.9, 124.3, 118.3, 110.2, 105.7, 97.9, 95.6, 53.9, 50.3, 8.46, 7.1. HPLC purity 99.83%

4'-((2,3-Dimethyl-5-(piperidin-4-ylcarbamoyl)-1H-indol-1-yl)methyl)-[1,1'-biphenyl]-2-carboxylic acid (2f): ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 12.69 (s, 1H), 8.79 (s, 1H), 8.71 (s, 1H), 8.30 (d, $J = 7.5$ Hz, 1H), 8.05 (s, 1H), 7.68 (d, $J = 7.4$ Hz, 1H), 7.62 (d, $J = 8.6$ Hz, 1H), 7.53 (m, 1H), 7.44 (t, $J = 7.1$ Hz, 2H), 7.34 (m, 1H), 7.23 (d, $J = 7.6$ Hz, 2H), 6.98 (d, $J = 7.8$ Hz, 2H), 5.47 (s, 2H), 4.04 (m, 1H), 3.32 (m, 2H), 3.01 (m, 2H), 2.31 (s, 3H), 2.27 (s, 3H), 1.97 (m, 2H), 1.80 (m, 2H); ^{13}C NMR (100MHz, $\text{DMSO}-d_6$) δ : 168.7, 139.7, 138.9, 136.9, 136.5, 131.5, 130.0, 129.6, 128.2, 127.8, 126.8, 126.5, 125.1, 124.2, 119.6, 117.0, 107.9, 106.5, 44.9, 43.6, 41.5, 27.6, 9.3, 8.0. HPLC purity 97.88%

4'-((5-((4-Hydroxyphenethyl)carbamoyl)-2,3-dimethyl-1H-indol-1-yl)methyl)-[1,1'-biphenyl]-2-carboxylic acid (2g): ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 9.18 (s, 1H), 8.36 (t, $J = 5.4$ Hz, 1H), 8.02 (s, 1H), 7.69 (d, $J = 7.5$ Hz, 1H), 7.54 (m, 2H), 7.43 (m, 2H), 7.32 (d, $J = 7.5$ Hz, 1H), 7.25 (d, $J = 7.8$ Hz, 1H), 7.01 (m, 4H), 6.69 (d, $J = 8.1$ Hz, 2H), 5.46 (s, 2H), 3.42 (2H, merged in water peak), 2.74 (t, $J = 7.4$ Hz, 2H), 2.32 (s, 3H), 2.27 (s, 3H); ^{13}C NMR (100MHz, $\text{DMSO}-d_6$) δ : 170.0, 167.6, 156.0, 140.9, 140.9, 140.1, 137.9, 137.7, 134.4, 132.7, 131.2, 130.8, 130.2, 129.9, 129.4, 129.0, 128.0, 127.7, 126.4, 125.8, 120.5, 117.9, 115.5, 109.1, 107.6, 99.9, 55.3, 41.7, 35.0, 10.5, 9.1. HPLC purity 100%

4'-((5-((3-(Carboxymethoxy)benzyl)carbamoyl)-2,3-dimethyl-1H-indol-1-yl)methyl)-[1,1'-biphenyl]-2-carboxylic acid (2h): ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) δ : 12.78 (s, 2H), 8.86 (t, $J = 5.9$ Hz, 1H), 8.10 (s, 1H), 7.66 (t, $J = 8.1$ Hz, 2H), 7.48 (m, 3H), 7.32 (d, $J = 7.5$ Hz, 1H), 7.23 (m, 2H), 7.00 (d, $J = 7.9$ Hz, 2H), 6.92 (d, $J = 7.7$ Hz, 1H), 6.89 (s, 1H), 6.77 (d, $J = 8.1$ Hz, 1H), 5.47 (s, 2H), 4.63 (s, 2H), 4.46 (d, $J = 5.7$ Hz, 2H), 2.31 (s, 3H), 2.27 (s, 3H); ^{13}C NMR (100MHz, $\text{DMSO}-d_6$) δ : 170.6, 170.0, 167.7, 158.2,

142.3, 140.9, 140.1, 138.1, 137.7, 134.5, 132.7, 131.2, 130.8, 129.0, 128.1, 127.7, 126.4, 125.4, 120.6, 120.3, 118.0, 64.8, 42.9, 10.5, 9.1. HPLC purity 95.93%

N-(4-Hydroxy-3-methoxybenzyl)-2,3-dimethyl-1-(3-(trifluoromethyl)benzyl)-1H-indole-5-carboxamide (3a): ¹H NMR (CDCl₃, 400 MHz) δ: 8.03 (s, 1H), 7.55 (d, *J* = 8.4 Hz, 1H), 7.49 (d, *J* = 7.4 Hz, 1H), 7.34 (m, 2H), 7.14 (d, *J* = 8.4 Hz, 1H), 6.91 (m, 4), 6.41 (bs, 1H), 5.66 (s, 1H), 5.34 (s, 2H), 4.60 (d, *J* = 5.4 Hz, 2H), 3.87 (s, 3H), 2.29 (s, 3H), 2.27 (s, 3H). ¹³C NMR (100MHz, CDCl₃) δ: 167.8, 147.3, 146.7, 140.6, 136.5, 132.5, 130.9, 128.9, 127.7, 126.1, 124.4, 123.2, 122.1, 119.4, 115.4, 111.1, 130.9, 106.7, 56.1, 51.9, 44.4, 11.1, 9.5. HPLC purity 96.03%

N-(Benzo[d][1,3]dioxol-5-ylmethyl)-2,3-dimethyl-1-(3-(trifluoromethyl)benzyl)-1H-indole-5-carboxamide (3b): ¹H NMR (DMSO-d₆, 400 MHz) δ: 8.02 (s, 1H), 7.54 (d, *J* = 8.5 Hz, 1H), 7.49 (d, *J* = 7.9 Hz, 1H), 7.36 (m, 2H), 7.14 (d, *J* = 5.9 Hz, 1H), 6.94 (d, *J* = 7.8 Hz, 1H), 6.89 (s, 1H), 6.84 (d, *J* = 8.0 Hz, 1H), 6.77 (d, *J* = 7.8 Hz, 1H), 6.41 (bs, 1H), 5.94 (s, 2H), 5.34 (s, 2H), 4.59 (d, *J* = 5.4 Hz, 2H), 2.30 (s, 3H), 2.27 (s, 3H); ¹³C NMR (100MHz, DMSO-d₆) δ: 168.5, 138.7, 138.0, 133.7, 132.5, 129.5, 129.1, 128.5, 125.4, 124.3, 122.7, 121.2, 120.0, 118.0, 108.9, 108.6, 108.4, 108.3, 101.0, 46.3, 44.0, 10.2, 8.9. HPLC purity 99.03%

N-(2,5-Dimethoxybenzyl)-2,3-dimethyl-1-(3-(trifluoromethyl)benzyl)-1H-indole-5-carboxamide (3d): ¹H NMR (CDCl₃, 400 MHz) δ: 8.02 (s, 1H), 7.50 (d, *J* = 8.5 Hz, 2H), 7.34 (d, *J* = 7.7 Hz, 2H), 7.13 (d, *J* = 8.4 Hz, 1H), 6.96 (m, 2H), 6.80 (m, 2H), 6.68 (t, *J* = 5.4 Hz, 1H), 5.34 (s, 2H), 4.65 (d, *J* = 5.6 Hz, 2H), 3.84 (s, 3H), 3.76 (s, 3H), 2.29 (s, 3H), 2.27 (s, 3H); ¹³C NMR (100MHz, CDCl₃) δ: 168.3, 153.6, 151.8, 138.8, 137.9, 133.5, 129.4, 129.1, 128.5, 127.7, 125.9, 124.3, 122.7, 119.9, 118.0, 115.8, 113.1, 111.4, 108.8, 108.3, 55.9, 55.7, 46.3, 39.9, 10.2, 8.8. HPLC purity 99.94%

(2,3-Dimethyl-1-(3-(trifluoromethyl)benzyl)-1H-indole-5-carbonyl)tyrosine (3e): ¹H NMR (DMSO-d₆, 400 MHz) δ: 12.57 (s, 1H), 9.17 (s, 1H), 8.44 (d, *J* = 8.0 Hz, 1H), 8.02 (s, 1H), 7.50 (m, 5H), 7.12 (m, 3H), 7.27 (m, 2H), 6.64 (d, *J* = 8.0 Hz, 2H), 5.54 (s, 2H), 4.55 (m, 1H), 4.02 (q, *J* = 7.1 Hz, 1H), 3.02 (m, 2H), 2.27 (s, 3H), 2.28 (s, 3H); ¹³C NMR (DMSO-d₆, 100 MHz) δ: 174.7, 167.5, 155.7, 140.6, 136.5, 132.5, 130.9, 129.2, 128.9, 126.7, 127.0, 126.1, 124.4, 122.1, 119.4, 115.8, 111.1, 106.7, 59.7, 51.9, 36.5, 11.1, 9.5. HPLC purity 96.24%

4'-(((4-((3-Hydroxybenzyl)carbamoyl)-2-methylphenyl)amino)methyl)-[1,1'-biphenyl]-2-carboxylic acid (5): ¹H NMR (DMSO-d₆, 400 MHz) δ: 12.68 (s, 1H), 9.25 (s, 1H), 8.50 (t, *J* = 5.8 Hz, 2H), 7.68 (d, *J* = 7.5 Hz, 1H), 7.51 (m, 3H), 7.38 (m, 4H), 7.27 (m, 2H), 7.06 (t, *J* = 7.9 Hz, 1H), 6.66 (s, 2H), 6.58 (d, *J* = 8.0 Hz, 1H), 6.44 (d, *J* = 8.4 Hz, 1H), 6.22 (d, *J* = 5.7 Hz, 1H), 4.46 (d, *J* = 5.4 Hz, 2H), 4.33 (d, *J* = 5.7 Hz, 2H), 2.21 (s, 3H); ¹³C NMR (100MHz, DMSO-d₆) δ: 170.1, 166.6, 157.7, 149.2, 142.2, 141.1, 139.5, 139.4, 132.8, 131.2, 130.8, 129.6, 129.5, 129.4, 128.7, 127.5, 127.0, 121.5, 121.3, 118.1, 114.3, 113.9, 108.8, 46.1, 42.6, 18.3. HPLC purity 98.56%

Chemicals, Cell Culture Reagents, and Plasmids

PolarScreen™ PPAR γ -Competitor Assay Kit, Green was purchased from Thermo Fisher Scientific (Waltham, US); Bright-Glo™ Luciferase Assay System was purchased from Promega (Madison, US). Rosiglitazone and Oil Red O dye were purchased from Sigma (St. Louis, US). The 3T3-L1 mouse fibroblasts and HEK-293 (ATCC, Manassas, VA, USA) were maintained at 37°C in a 5% CO₂, humidified atmosphere. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/liter glucose with 10% bovine calf serum (BCS; HyClone, Logan, UT, USA). DMSO was used as solvent and solvent vehicle controls were always included. For cell-based assays, the final concentration of DMSO is 0.1%.

In Vitro Competition Binding Assay

The in vitro competition binding assays was performed using PolarScreen™ PPAR γ -Competitor Assay Kit according to the manufacturer's instructions. Briefly, serial concentration of compounds was incubated with GST-tagged PPAR γ ligand-binding domain (PPAR γ LBD) and Fluormone PPAR Green tracer in a black 384-well assay plate (Corning Glass catalog no. 677). After a 4-h incubation in the dark at room temperature, fluorescence polarization value (mP) was measured at 485 nm (excitation) and at 535 nm (emission) using a 384-microplate reader. The inhibition constant (K_i) for UHC1 analogs (competitors) was calculated by applying the Cheng-Prusoff equation as following:

$$K_i = IC_{50}/(1 + [\text{tracer}]/K_d)$$

Where IC₅₀ is the concentration of competitor that produces 50% displacement of the tracer, [tracer] is the concentration of Fluormone PPAR Green tracer used in the assay (9 nM), and the K_d is the binding constant of the Fluormone PPAR Gamma Green to PPAR Gamma-LBD (GST) (32 nM).

In Silico Docking Simulations—The molecular docking simulation was performed using the AutoDock Vina program. The crystal structure of PPAR γ LBD (PDB ID: 5GTO) was used for the docking simulation and subsequent structural analysis. The grid dimensions for PPAR γ LBD protein was 44× 44 × 44 grid points with spacing 0.375 Å between the grid points and centered on the ligand for protein (−27.376, 18.813, 2.589 coordinates). A compound was docked in the binding pocket site using the highest accuracy mode of docking.

PPAR γ Transactivation Reporter Assay

HEK-293 cells were seeded and co-transfected with plasmids pBabe bleo human PPAR γ 2 and PPRE X3-TK-luc (Addgene) in 384-well plate and incubated for 16 h. Compounds (100, 10, 1, 0.1, 0.01, 0.001 μ M) were added and then incubated another 24h. Cell lysates were then analyzed for luciferase activity with Bright-Glo Luciferase kit (Promega). Luminescence was measured using a plate reader.

Adipocyte differentiation

3T3-L1 preadipocytes were differentiated as previously described¹⁹. In brief, cells were subcultured at around 60%–70% confluence. To induce differentiation, full confluent cell cultures were placed in medium containing 0.5 mmol/L 1-methyl-3-isobutylxanthine, 1 μ mol/L dexamethasone, and 10 μ g/mL insulin in 10% FBS/DMEM for 48 h with compounds. Next, the medium was changed, and 10 μ g/mL insulin was added for another 48 h. The cells are harvested for RNA extraction.

Oil Red O staining

Adipocyte differentiation of 3T3-L1 was induced with differentiation medium (DMEM containing 10%FBS, 1 μ g/ml insulin, 1 μ M dexamethasone, and 0.5 mM IBMX) containing compounds for 48 h. Cells were then incubated in maintaining medium (DMEM containing 10%FBS) containing compounds. The medium was renewed every 2 days until the cells became fully differentiated and lipid droplets were apparent. At day 7, fully differentiated 3T3-L1 cells were washed carefully with phosphate-buffered saline (PBS) and then fixed with 4% formaldehyde for 30 min. After removal of the formalin, the cells were washed with PBS two or three times. Subsequently, 0.3 % Oil Red O staining solution was added to each well for 1 h incubation at 37 °C. In the final step, the wells were washed 3 times with PBS or distilled water, and the cell morphology and staining of the lipid droplets were observed using an inverted microscope (ECLIPSE TS100-F; Nikon).

Gene Expression Analysis

Total RNA (2 μ g) was isolated from cells using TRIzol reagents (Life Technologies) and reverse transcribed using oligo d(T) primers (New England Biosystems) and SuperScript III reverse transcription kit (Applied Biosystems). qPCR was performed using SYBR Green mix (Applied Biosystems) with an Applied Biosystems 7500 real-time PCR system. The amplification program was as follows: initial denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 15 s, 60°C for 1 min, and 40°C for 30 s. Relative mRNA expression was determined by the $\Delta\Delta$ Ct method normalized to *TBP* mRNA. The sequences of primers used in this study are found in Supplemental Table 1.

Western blotting for PPAR γ phosphorylation—3T3-L1 adipocyte differentiated with the above-mentioned differentiation medium were pretreated with the treatment of compounds for 24 h followed by TNF α (50 ng/mg) for 3 h. Cells were then washed with PBS and lysed with lysis buffer (Cell Signaling Technology, Danvers, MA) containing EDTA (Thermo, IL) and phosphatase inhibitors (Thermo). Aliquots of 20 μ g total protein were separated on 7% SDS-PAGE gels (Life Technologies) and transferred to PVDF membranes (Life Technologies). The membranes were probed with primary antibodies followed by the appropriate HRP-conjugated secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG, 1:3000; Santa Cruz Biotechnology, CA, USA). Blots were then developed. The primary antibodies were: anti-Ser-273 PPAR γ (Bioss Antibodies) or anti-PPAR γ antibody (Santa Cruz Biotechnology).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Chemical structures of PPAR-g full agonist Rosiglitazone (Rosi), non- or partial agonists (SR1664 and UHC1), and current lead SAR design.

Figure 2.

WO91A binds to PPAR γ LBD in a distinct mode. Docking simulation was performed with crystal structure of PPAR γ LBD (Protein Data Bank PDB ID 5GTO) with Autodock Vina. A. Docking simulation of the PPAR γ LBD:WO91A complex. WO91A is in red. Docking score for affinity shown. B. Docking simulation of the PPAR γ LBD:UHC1 complex. UHC1 is in red. Docking score for affinity shown. C, D. Schematic representation of atomic interaction between PPAR γ LBD and WO91A (C) or UHC1 (D). hydrogen bonds were shown in brown dashed lines with donor-acceptance distances in angstroms.

Figure 3.

WO91A acts as partial PPAR γ agonist. A. The in vitro competition binding assays was performed as in Experimental sections. Fluorescence polarization value (mP) was measured at 485 nm (excitation) and at 535 nm (emission). B. Transactivation activity of compounds in HEK293 cells transfected with PPAR γ 2 and PPRE X3-TK-luc reporter. Transactivation activity was presented as fold change against DMSO vehicle.

Figure 4.

WO91A inhibits PPAR γ S273 phosphorylation and controls the expression of genes associated with S273 phosphorylation. A. Phosphorylation of PPAR γ S273 in 3T3-L1 differentiated preadipocytes treated with TNF α 10 ng/ml for 1 h in the presence of compounds at 10 μ M. pSer273 of PPAR γ was detected by Western blotting using PPAR γ pSer273-specific antibody. * undifferentiated parental 3T3-L1 cells. B-D, Relative mRNA expression levels of pSer273-associated genes Adiponectin (B), Adipsin (C), and Car3 (D) in 3T3-L1 differentiated with differentiation media in the presence of compounds at 10 μ M by qRT-PCT.

Figure 5.

WO91A does not induce adipogenesis. A-D, Oil Red O staining in 3T3-L1 cells differentiated with differentiation media in the presence of compounds at 10 μ M. E-G, Relative mRNA expression levels of adipogenic genes FABP4 (E), LPL (F), and Glut4 (G) in 3T3-L1 differentiated with differentiation media in the presence of compounds at 10 μ M by qRT-PCT.

Scheme 1.

Reagents and conditions: (a) HATU, DIPEA, DCM at rt; (b) TFA/DCM

Scheme 2.

Reagents and conditions: (a) K_2CO_3 , DMF; (b) KOH, EtOH; (c) HATU, DIPEA, 3-OH Benzylamine, DCM; (d) TFA/DCM

Table 1.

SAR evaluation of PPAR γ binding IC₅₀, inhibition constant, and transactivation activity values of synthesized compounds.

Comp. ID	R ¹	PPAR γ Binding (IC ₅₀ , μ M) ^a	Inhibition Constant (K _i , μ M) ^b	Transactivation activity (%) ^c
1	4'-methyl-[1,1'-biphenyl]-2-carboxylic acid	4.866	3.799	16
2a	4-SO ₂ NH ₂ -benzylamine	0.312	0.244	00
2b	Pyridin-2-ylmethylamine	0.034	0.027	15
2c	Piperonylamine	0.015	0.012	36
2d	piperidin-4-ylmethanamine	12.120	9.461	11
2e (W091A)	Vanillylamine	0.016	0.013	15
2f	Piperidine-4-amine	41.280	32.225	17
2g	Tyramine	0.048	0.037	31
2h	2-(3-(aminomethyl)phenoxy) acetic acid	0.066	0.051	56
3a	Vanillylamine	0.010	0.007	46
3b	Piperonyl benzylamine	0.016	0.012	32
3c	Pyridin-2-ylmethylamine	0.185	0.144	59
3d	2,5-di methoxy benzylamine	0.048	0.037	27
3e	Tyrosine	0.346	0.270	44
5	3-Hydroxy Benzylamine	0.325	0.254	42
UHC1	Pyridin-3-ylmethylamine	0.536	0.440	45
Rosiglitazone	-	0.076	0.059	100

^aIC₅₀ (the concentrations that reach half-maximal inhibition) for PPAR γ binding was calculated with GraphPad Prism from the data of ten 3-fold serial titration points.

^bInhibition constant (K_i) calculated as detailed in Experimental Section.

^cTransactivation activity is defined as the maximum activity that a compound achieves and reported as % compared to that of Rosi, which is designated as 100%. All experiments were performed in triplicate.