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Letter

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Novel Phenyldiazenyl Fibrate Analogues as PPAR $\alpha/\gamma/\delta$ Pan-Agonists for the Amelioration of Metabolic Syndrome

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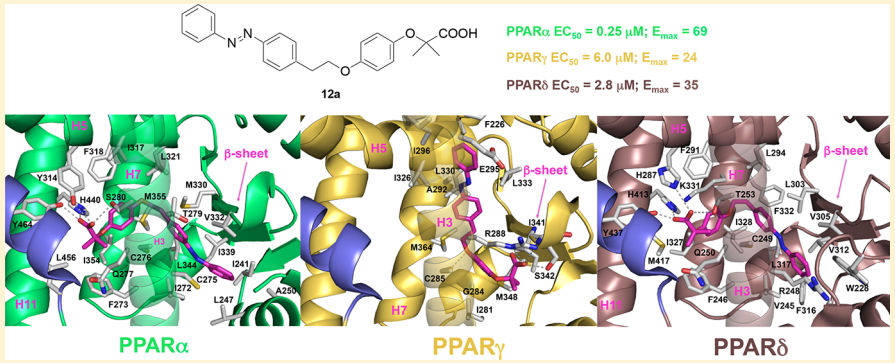
Supporting Information



ABSTRACT: The development of PPARα/γ dual or PPAR$\alpha/\gamma/\delta$ pan-agonists could represent an efficacious approach for a simultaneous pharmacological intervention on carbohydrate and lipid metabolism. Two series of new phenyldiazenyl fibrate derivatives of GL479, a previously reported PPARα/γ dual agonist, were synthesized and tested. Compound **12a** was identified as a PPAR pan-agonist with moderate and balanced activity on the three PPAR isoforms (α, γ, δ). Moreover, docking experiments showed that **12a** adopts a different binding mode in PPARγ compared to PPARα or PPARδ, providing a structural basis for further structure-guided design of PPAR pan-agonists. The beneficial effects of **12a** were evaluated both *in vitro*, on the expression of PPAR target key metabolic genes, and *ex vivo* in two rat tissue inflammatory models. The obtained results allow considering this compound as an interesting lead for the development of a new class of PPAR pan-agonists endowed with an activation profile exploitable for therapy of metabolic syndrome.

KEYWORDS: *PPARs, pan-agonist, docking, metabolic syndrome, type 2 diabetes mellitus, gene expression, PGE_2*

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and play a decisive role in the regulation of lipid metabolism and glucose homeostasis. PPARs exist as three subtypes with different localizations and physiological functions, designated as PPARα, PPARγ, and PPARδ.[1] These receptors are targets for the therapy of metabolic syndrome, a group of risk factors for cardiovascular disease and type 2 diabetes mellitus (T2DM) including insulin resistance, elevated fasting blood glucose, hypertension, obesity, and atherogenic dyslipidemia.[1] Furthermore, metabolic syndrome is accompanied by a low-grade inflammatory state in white adipose tissue and liver, which may exacerbate insulin resistance and diabetes.[2] Fibrates (e.g., fenofibrate and gemfibrozil), a class of lipid-lowering drugs, are PPARα ligands.[3] Thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are insulin-sensitizing agents that activate PPARγ.[4] PPARδ agonists have not yet reached the market, but some of them, for example, GW501516, GW0741, L165,041, GFT505, and MBX8025, are being investigated for a possible therapeutic usefulness in the treatment of metabolic

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disorders, inflammation, and angiogenesis (Figure S1).[5] However, due to the adverse effects associated with these drug treatments,[3,4] new research strategies have been undertaken with the aim to obtain new PPAR ligands with reduced side effects and improved beneficial effects. One of these strategies consists in the synthesis of PPARα/γ dual or PPAR$\alpha/\gamma/\delta$ pan-agonists, which beneficially alter carbohydrate and lipid metabolism in a coordinated manner.[6−8] In particular, pan-agonists would merge the agonist activities with regard to PPARα, -γ, and -δ in a unique ligand but with a balanced activation profile, which could be useful for treatment of metabolic syndrome and T2DM. In the search for new PPAR ligands with these properties, we have recently discovered a series of derivatives of the antilipidemic drugs gemfibrozil or clofibric acid, in which the clofibric acid skeleton was combined with the lipophilic scaffolds derived from natural products, such as α-asarone, stilbene, chalcone, and their bioisosteres.[9−12] In particular, the combination of the clofibric acid with a phenyldiazenyl function resulted in GL479 (R = H, X = O, Y = CH_2; Figure 1), a PPARα/γ dual agonist.[13]

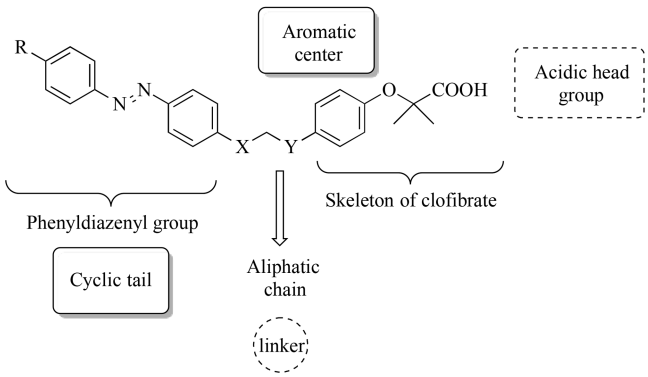

Figure 1. General features of synthetic PPAR agonists and structural development of the presented compounds.

Cocrystal structures of GL479 with PPARα and -γ revealed that the ligand adopts different binding modes at these receptor subtypes, reflecting the distinct activation profile observed for each receptor.[14]

Herein we wish to report the synthesis and biological evaluation of a series of derivatives of GL479 (compound **11a**, Table 1) in which (i) small substituents with different stereochemical properties were inserted at the *para* position of the distal aromatic ring of phenyldiazenyl moiety (compounds **11b−g**) and (ii) the oxygen atom of the linker was moved to the para position of the phenoxy-propanoic acid (compounds **12a−f**). Such design strategy was planned taking into account the crystallographic pose of GL479 into both PPARα and -γ: the hydrophobic tail binds upward into arm II of PPARα, where it explores a relatively large accessible volume. Instead, this group bends downward into arm III of PPARγ, surrounded by hydrophobic residues such as I388, I325, M329, and F266. Therefore, with the aim of probing further binding interactions in this region of the protein, we further explored the introduction of small substituents at the para position of the phenyldiazenyl group. Furthermore, C285 on H3, which assumes two possible conformations reflecting a certain degree of flexibility, and the oxygen in the linker of GL479 are at a favorable distance for H-bonding. This cysteine residue is also conserved in PPARα. Thus, in the attempt to promote possible interactions with the cysteine residues on H3, we varied the position of the oxygen atom on the linker.

Phenyldiazenyl derivatives **11a−g** and **12a−f** (Table 1) were easily obtained in good yields using simple synthetic procedures (see Supporting Information for the detailed procedures). The intermediates **2a−g** and **3a−f** were synthesized according to Scheme 1. Phenols **2a−g** were synthesized by diazotization of *para*-substituted anilines with $NaNO_2$ in 6 N HCl at 0 °C and copulation of the obtained diazonium salts with phenol in 4 N NaOH at 0 °C. The alcohols **3a−f** were synthesized by oxidation of *para*-substituted anilines by oxone in dichloromethane/water at r.t. and subsequent reaction with 2-(4-aminophenyl)ethanol in CH_3COOH at r.t. (Scheme 1).

Esters **7** and **8** were obtained by S_N2 reaction of phenols **4** or **5** with ethyl 2-bromo-2-methylpropanoate (**6**), in the presence of dry K_2CO_3 in DMF at reflux (**7**) or in the presence of KOH in EtOH at reflux (**8**) as reported in Scheme 2.

Esters **9a−g** and **10a−f** were synthesized by Mitsunobu reaction between **2a−g** or **3a−f** and the appropriate alcohol **7** or phenol **8**. These esters were hydrolyzed in basic condition with 1 N NaOH in EtOH at room temperature to obtain the target acids **11a−g** and **12a−f** (Scheme 2).

All synthesized compounds were evaluated for their agonist activity toward the three subtypes of human PPARs (hPPARs) by a cell-based transactivation assay, according to a previously reported procedure.[15] The results were compared with those obtained with clofibric acid (100 μM), rosiglitazone (2 μM), and L-165,041 (2 μM), used as reference compounds for PPARα, -γ, and -δ, respectively (Table 1). Maximum fold induction obtained with the reference agonists was set at 100%. The activity of all compounds was evaluated at 25 μM, and only those showing efficacy percentage (E_{max}) higher than 20% were selected for determination of EC_{50}. Because of the different cell line used in this assay, GL479 turned out to be a partial agonist instead of a full agonist, as reported earlier;[13] moreover, this ligand displayed a significant activation of PPARδ subtype that had not been previously investigated. Derivatives **11b−g** displayed partial agonist activity toward PPARα. The introduction of a chlorine or bromine resulted in about 3- and 5-fold increase of potency compared to lead compound GL479 (**11b**, EC_{50} = 0.38 ± 0.05 μM; **11c**, EC_{50} = 0.81 ± 0.06 μM; GL479, EC_{50} = 2.1 ± 0.6 μM) and a slight increase of efficacy, suggesting a favorable influence of the weak electron-withdrawing properties of these substituents on the activity. In contrast, the introduction of stronger electron-withdrawing substituents, such as trifluoromethyl and cyanide, afforded compounds with potency comparable to GL479 (**11d**, EC_{50} = 2.3 ± 1.3 μM; **11e**, EC_{50} = 2.6 ± 0.8 μM), but a concomitant loss of efficacy from 56% to about 30%. The presence of the highly hydrophilic nitro group caused an almost complete loss of PPARα activation (**11f**). Compound **11g**, bearing the electron-donating group methoxy, showed a similar activity to the derivative **11d**. Therefore, we argued that the electronic properties of the substituent in the para position of the distal aromatic ring of phenydiazenyl moiety do not seem to exert a crucial role on the activity. Surprisingly, compounds **11b−g** showed poor PPARγ activity as well as a weak activation of PPARδ. In particular, the substitution with trifluoromethyl or methoxy increased the potency toward this receptor subtype, with **11d** and **11g** being the most potent.

Derivatives **12a−f** displayed partial agonism on PPARα. Compound **12a**, a close analogue of GL479, showed about 8-

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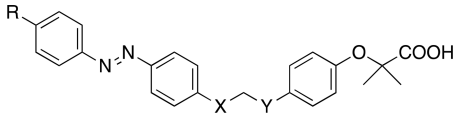
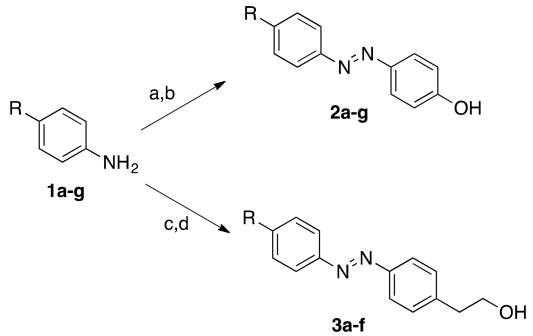
Table 1. In Vitro Transactivation Activity of Compounds with Various Lipophilic Tails and Linkers



				hPPARα	hPPARγ	hPPARδ
compd	R	X	Y	EC_{50} (μM)[a] (E_{max})[b]	EC_{50} (μM)[a] (E_{max})[b]	EC_{50} (μM)[a] (E_{max})[b]
11a (GL_{479})	H	O	CH_2	2.1 ± 0.6 (56±9)	n.d. (13 ± 1)	6.4 ± 1.2 (39 ± 4)
11b	Cl	O	CH_2	0.38 ± 0.05 (60 ± 6)	n.d. (12 ± 1)	3.1 ± 0.4 (23 ± 2)
11c	Br	O	CH_2	0.81 ± 0.06 (71 ± 10)	n.d. (12 ± 3)	3.9 ± 0.4 (29 ± 2)
11d	CF_3	O	CH_2	2.3 ± 1.3 (35 ± 8)	n.d. (8 ± 1)	1.8 ± 0.4 (24 ± 3)
11e	CN	O	CH_2	2.6 ± 0.8 (25 ± 7)	n.d. (12 ± 1)	6.5 ± 1.2 (20 ± 4)
11f	NO_2	O	CH_2	n.d. (11 ± 7)	n.d. (14 ± 1)	4.1 ± 1.0 (21 ± 3)
11g	OCH_3	O	CH_2	2.4 ± 0.8 (32 ± 2)	n.d. (10 ± 1)	1.4 ± 0.2 (23 ± 2)
12a	H	CH_2	O	0.25 ± 0.07 (69 ± 12)	6.0 ± 1.5 (24 ± 3)	2.8 ± 0.3 (35 ± 2)
12b	Cl	CH_2	O	0.51 ± 0.12 (52 ± 9)	n.d. (10 ± 1)	1.2 ± 0.3 (41 ± 6)
12c	Br	CH_2	O	1.9 ± 0.2 (35 ± 4)	n.d. (8 ± 1)	1.1 ± 0.2 (29 ± 3)
12d	CF_3	CH_2	O	2.2 ± 1.2 (37 ± 7)	n.d. (9 ± 1)	n.d. (12 ± 4)
12e	CN	CH_2	O	1.4 ± 0.1 (61 ± 11)	n.d. (12 ± 2)	2.8 ± 0.3 (50 ± 3)
12f	NO_2	CH_2	O	n.d. (19 ± 8)	n.d. (10 ± 1)	2.3 ± 0.4 (28 ± 3)
clofibric acid				50 ± 6 (100 ± 10)	-	-
rosiglitazone				-	0.04 ± 0.02 (100 ± 9)	-
L165,041				-	-	0.21 ± 0.04 (100 ± 4)

[a]EC_{50} values were determined by testing compounds in at least three separate experiments at five concentrations ranging from 1 to 50 or 100 μM. The results are expressed as the mean ± SEM. [b]Efficacy values were calculated as percentage of the maximum fold induction obtained with clofibric acid, rosiglitazone, and L165,041 as reference compounds for PPARα, PPARγ, and PPARδ, respectively. n.d.: EC_{50} not determined.

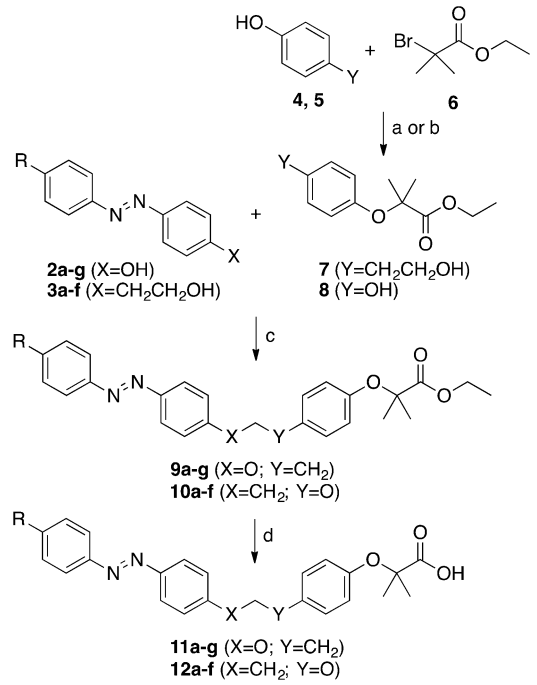
Scheme 1. Synthetic Pathways of Compounds 2a−g and 3a−f[a]



[a]Reagents and conditions: (a) $NaNO_2$, 6 N HCl, H_2O, 0−5 °C, 1 h; (b) phenol, 4 N NaOH, 0−5 °C, 1 h; (c) oxone, CH_2Cl_2, H_2O, r.t., 4 h; (d) 2-(4-aminophenyl)ethanol, CH_3COOH, r.t., 24−48 h.

fold potency increase and a concomitant slight increase of E_{max} (about 70%). Substitutions at the *para* position of the phenyldiazenyl moiety afforded compounds displaying lower potency and efficacy compared to **12a**. The chloro (**12b**) and cyano (**12e**) derivatives were the most active. Again, the presence of the hydrophilic and strong electron-withdrawing nitro group caused an almost complete loss of PPARα activation (**12f**). As the activity is not strikingly affected from the electronic properties of the *para*-substituent of the distal aromatic ring of phenyldiazenyl moiety and given that the preparation of the derivative bearing the methoxy was somewhat troublesome, this last compound was not synthesized. Derivatives **12b−f** displayed very weak activation of PPARγ; only the unsubstituted derivative **12a** showed higher potency and efficacy than the lead compound GL479.

Scheme 2. Synthetic Pathways of Compounds 4−6, 11a−g, and 12a−f[a]



[a]Reagents and conditions: (a) dry K_2CO_3, DMF, reflux, 4 h, (Y = CH_2CH_2OH) (4 → 7); (b) KOH, EtOH, reflux, N_2, 72 h, (Y = OH) (5 → 8); (c) PPh_3, DIAD, N_2, THF dry, r.t., 10−12 h; (d) 1 N NaOH, EtOH, r.t., 15−24 h.

Regarding PPARδ, all derivatives **12a−f** displayed a partial agonism profile toward this receptor subtype. Introduction of chlorine or bromine increased potency with respect to the unsubstituted derivative **12a**, whereas the introduction of

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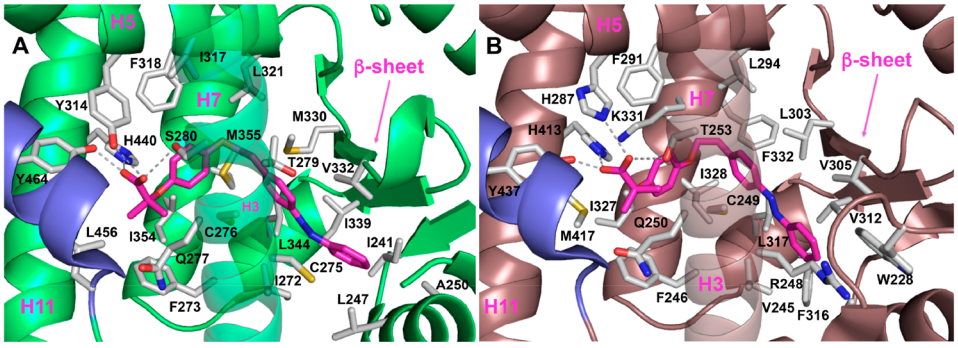

Figure 2. Binding mode of compound **12a** (magenta sticks) into the PPARα (A) and -δ (B) LBDs, represented as green and dirty violet ribbon models, respectively. Only amino acids located within 4.5 Å of the bound ligand are displayed (white sticks) and labeled. H-bonds discussed in the text are depicted as dashed gray lines. H12 is shown in slate.

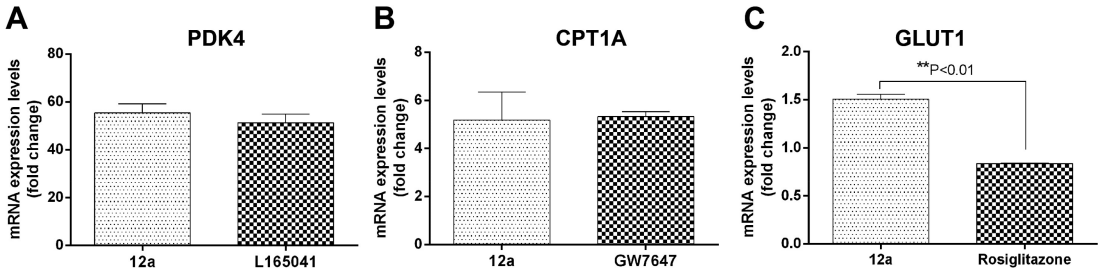

Figure 3. Expression of *PDK4* (A), *CPT1A* (B), and *GLUT1* (C) after treatment with PPAR agonist **12a** (100 μM). The enhanced activity on the gene expression is compared with the commercial compounds L165,041 (2 μM, A), GW7647 (2 μM, B), or rosiglitazone (10 μM, C) as reference positive controls.

trifluoromethyl group was detrimental. The cyano and nitro derivatives displayed a similar potency to **12a**. Overall, this second series of derivatives (**12a–f**) displayed higher potency and a slightly higher efficacy than the corresponding derivatives of the first series (**11a–f**) toward PPARδ. This suggests that the shift of the oxygen of the linker to the para position of the phenoxy group is more favorable to the interaction and resulting activation of PPARδ. Interestingly, **12a** resulted more potent than GL479 but with basically the same significant and well-balanced activity on all three PPAR subtypes. In fact, **12a** exhibits a weak/moderate efficacy, which is desirable given that moderately effective PPAR agonists demonstrate good tolerability and safety in a large population of patients.[16−18] In addition, the partial agonist activity of **12a** allows hypothesizing that this compound behaves like a selective PPAR modulator (SPPARM), which is a ligand that, compared with a full agonist, differentially induces specific receptor effects, ideally uncoupling the benefits of PPAR activation from the adverse side effects.[19] Until a few years ago, SPPARM activity was pursued only for PPARγ agonist, but nowadays, it is considered desirable also for the other two subtypes.[20] As far as we know, **12a** is one of the few pan-agonists showing moderate activity toward all three PPAR subtypes. This is noteworthy if one considers that most pan-agonists introduced and then discontinued in clinical trials (e.g., indeglitazar, etc.)[21] were full agonists at least toward one of the three subtypes.

To help interpretation of SAR data and to increase our understanding of the molecular basis for the observed partial agonism of **12a** toward PPARα, -γ, and -δ, we undertook docking simulations and cluster analysis (see details in the Supporting Information). Compound **12a** occupies arms I and II in both PPARα and -δ (Figure 2), whereas it interacts mainly with arms II and III in PPARγ (Figure S7). As illustrated in Figure 2A,B, the clofibric acid moiety of **12a** is located in the arm I, making conventional interactions with the polar side chains (S280, Y314, H440, and Y464 for PPARα and T253, H287, H413, and Y437 for PPARδ), the central phenoxy ring is placed in the center, and the hydrophobic tail part is buried in the arm II. In the PPARα LBD complex, the gem-dimethyl substituents are directed into the lipophilic "benzophenone pocket", lined by F273, Q277, V444, and L456.[22] However, in PPARδ V444 is substituted with a methionine, which has a bulkier side chain and is less prone to accommodate the fibrate headgroup.[23] This might account for the potency increase of **12a** toward PPARα and for the decrease of PPARδ potency. Noteworthy, the phenyldiazenyl moiety in PPARα forms sulfur−aromatic interactions[24] with C275, C276, M355, and M330. This remarkable environment might rationalize the augmented potency of **12a** in the PPARα pocket, which is more lipophilic and less solvent-exposed than the corresponding pocket of PPARδ.[25]

In fact, with the exception of C276, sulfur-containing residues are not conserved in PPARδ. The 10-fold potency decrease of **11a** compared to **12a** in PPARα can be explained based on the valence-bond (or resonance) theory (see details in the Supporting Information).

Compound **12a** binds to PPARγ LBD in a different binding mode as compared to that of either PPARα LBD or PPARδ LBD because it does not interact directly with H12 (Supporting Information, Figure S7). Therefore, the low affinity and the attenuated transcriptional response of **12a** and its derivatives toward PPARγ might be ascribed to the peculiar binding mode, which is not able to replicate the spectrum of contacts of full agonists.[26]

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Next, we evaluated whether **12a** could affect the expression of three PPAR target genes involved in the control of lipid and carbohydrate metabolism, namely, carnitine palmitoyltransferase 1A (*CPT1A*), pyruvate dehydrogenase kinase 4 (*PDK4*), and glucose transporter 1 (*GLUT1*).[27] *CPT1A* is a molecular component of the carnitine shuttle system that catalyzes the entry of fatty acids into the mitochondrial matrix and their subsequent enzymatic oxidation. Importantly, *CPT1A* gene expression is enhanced by PPARα agonists.[27] *PDK4* is a well-established PPARδ target gene and contributes to the regulation of glucose and fatty acid metabolism and homeostasis.[27] *GLUT1* is a constitutive glucose transporter whose expression is increased by PPARγ ligands, resulting in enhanced glucose uptake and utilization into adipocytes.[28] Therefore, a screening of gene activation by real-time quantitative PCR (RTqPCR) analysis was performed for compound **12a** in HepG2 human hepatocellular carcinoma cell line, using the PPARδ agonist L165,041, the PPARα agonist GW7647, and the PPARγ agonist rosiglitazone as reference positive controls. As illustrated in Figure 3, treatment with **12a** (100 μM) showed enhanced activity on the expression of *PDK4* and *CPT1A* similar to the agonists L165,041 (2 μM) and GW7647 (2 μM, Figure 3A,B). Moreover, **12a** significantly increased the mRNA expression level of *GLUT1* as compared to rosiglitazone (10 μM, $P < 0.01$; Figure 3C). The choice of performing the experiment at the fixed concentration of 100 μM was based on the lower potency and/or efficacy of **12a** in the transactivation assay compared to the reference compounds as reported in Table 1. However, these results indicate that **12a**, even though less potent than positive controls, through its simultaneous and balanced activation of the three PPAR subtypes, may have a beneficial outcome on carbohydrate and lipid metabolism. In light of these results, for a deeper investigation of the biological properties of **12a**, we decided to evaluate its anti-inflammatory activity. Numerous studies indicate that the role of PPARs in inflammation is particularly significant in the case of metabolic syndrome and atherosclerosis, which present an inflammatory component.[2] This allows hypothesizing that the beneficial effects of PPAR activation on insulin sensitivity are mediated, at least in part, by its anti-inflammatory activities. For this reason, we conducted *ex vivo* experiments for evaluating the ability of **12a** to reduce the LPS-induced PGE2 production in liver and cortex of male adult Sprague–Dawley rats. LPS is often used as a stimulus for the induction of inflammation-related disorders simulating metabolic diseases.[29]

To assess the degree of inflammation, we measured the levels of prostaglandin PGE2, which can contribute to diabetes by inhibiting insulin secretion, leading to impaired glucose tolerance and insulin sensitivity.[30] Rats were sacrificed and both liver and cortex specimens were immediately collected and maintained in a humidified incubator in RPMI (Roswell Park Memorial Institute) buffer with added bacterial LPS (10 μg/mL). During the incubation period, tissues were treated alternatively with 1 μM WY14643, pioglitazone, and L165,041 as selective PPARα, -γ, and -δ agonists, respectively, or with scalar concentrations of **12a** (0.1–10 μM). Compound **12a** was able to significantly inhibit LPS-induced PGE2 production in both rat liver (Figure 4A) and cortex (Figure 4B), two tissues that play a central role in the regulation of glucose homeostasis and metabolism, in a nondose-dependent manner. The efficacy of **12a** was comparable with that of WY14643 and pioglitazone, whereas the PPARδ agonist L165,041 did not cause any decrease of the LPS-induced PGE2 production in both tissues.

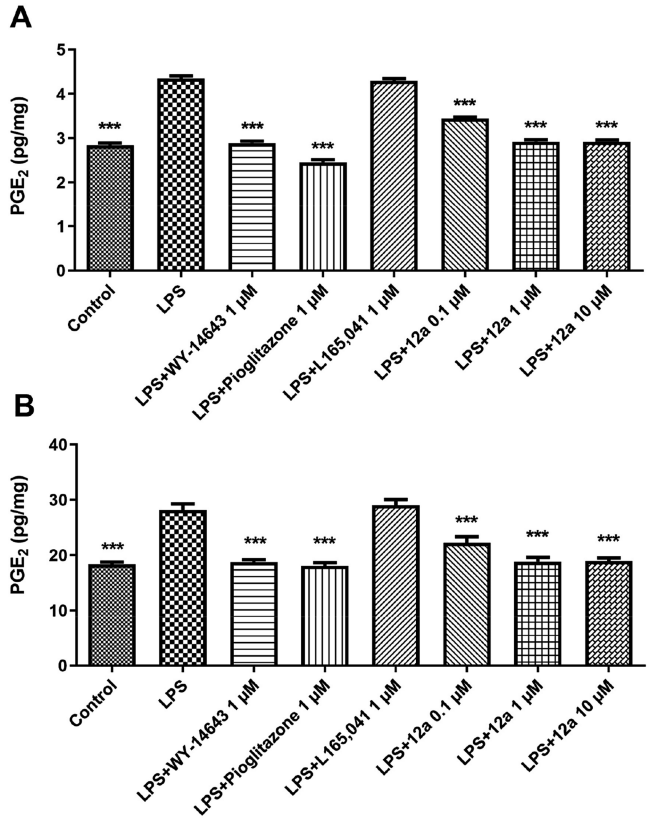

Figure 4. Effects of **12a** (0.1–10 μM) on LPS-induced production of PGE2 (pg/mg) in rat liver (A) and cortex (B), *ex vivo*. Data were reported as means ± SEM. Results were analyzed by analysis of variance (ANOVA) followed by Newman–Keuls posthoc test. ANOVA, $p < 0.0001$; posthoc ***$p < 0.001$ vs LPS-treated group.

In conclusion, this study led to the identification of **12a** endowed with moderate activity on the three PPAR subtypes (α, γ, δ), which might represent a potential lead for the development of a new class of PPAR pan-agonists for treating metabolic syndrome and T2DM.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00574.

Complete experimental procedures, characterization data for all compounds, biological procedures, docking and cluster analysis, as well as spectra (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PPARs, peroxisome proliferator-activated receptors; T2DM, type 2 diabetes mellitus; TZDs, thiazolidinediones; SAR, structure−activity relationship; CPT1A, carnitine palmitoyltransferase 1A; PDK4, pyruvate dehydrogenase kinase 4; GLUT1, glucose transporter 1; LPS, lipopolysaccharide; LBD, ligand binding domain

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