

A Novel N-Substituted Valine Derivative with Unique Peroxisome Proliferator-Activated Receptor γ Binding Properties and Biological Activities

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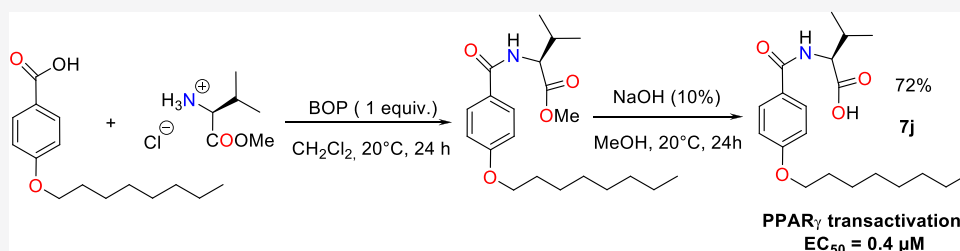
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ABSTRACT: A proprietary library of novel *N*-aryl-substituted amino acid derivatives bearing a hydroxamate head group allowed the identification of compound **3a** that possesses weak proadipogenic and peroxisome proliferator-activated receptor γ (PPAR γ) activating properties. The systematic optimization of **3a**, in order to improve its PPAR γ agonist activity, led to the synthesis of compound **7j** (*N*-aryl-substituted valine derivative) that possesses dual PPAR γ /PPAR α agonistic activity. Structural and kinetic analyses reveal that **7j** occupies the typical ligand binding domain of the PPAR γ agonists with, however, a unique high-affinity binding mode. Furthermore, **7j** is highly effective in preventing cyclin-dependent kinase 5-mediated phosphorylation of PPAR γ serine 273. Although less proadipogenic than rosiglitazone, **7j** significantly increases adipocyte insulin-stimulated glucose uptake and efficiently promotes white-to-brown adipocyte conversion. In addition, **7j** prevents oleic acid-induced lipid accumulation in hepatoma cells. The unique biochemical properties and biological activities of compound **7j** suggest that it would be a promising candidate for the development of compounds to reduce insulin resistance, obesity, and nonalcoholic fatty liver disease.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily and activated by ligands such as dietary fatty acids, particularly polyunsaturated fatty acids. The three PPAR subtypes: PPAR α , PPAR γ , and PPAR δ (β), have different, yet overlapping, tissue expression patterns¹ and exert major roles in the regulation of specific physiological functions including glucose and lipid metabolism and energy homeostasis.^{2–4} These features make PPARs important molecular targets for the development of drugs for metabolic diseases.

PPAR α is expressed in all metabolic tissues but predominantly in the liver where it is involved in the regulation of the uptake and oxidation of fatty acids and lipoprotein metabolism.⁵ The fibrate family of drugs (clofibrate and fenofibrate) is pharmacologically weak agonists of PPAR α that are used to treat dyslipidemia as they lower plasma triglycerides and increase high-density lipoprotein cholesterol levels.⁶ The PPAR α agonist fenofibrate has also a beneficial effect in patient with nonalcoholic fatty liver disease

(NAFLD) characterized by the accumulation of triglycerides in hepatocytes.⁷ However, fibrates increase markers of cardiovascular and renal disease and that of liver dysfunction, which underlines their ability to trigger adverse effects.⁸ Therefore, efforts are being made to develop PPAR α agonists with improved clinical efficacy, penafibrate being one of these new generation agonists.⁹

PPAR δ is involved in the regulation of fatty acid oxidation and mitochondrial respiration predominantly in skeletal muscle, liver, and adipose tissue.¹⁰ Therefore, agonists targeting PPAR δ may be considered as potential therapeutic agents for insulin resistance-related conditions. PPAR δ

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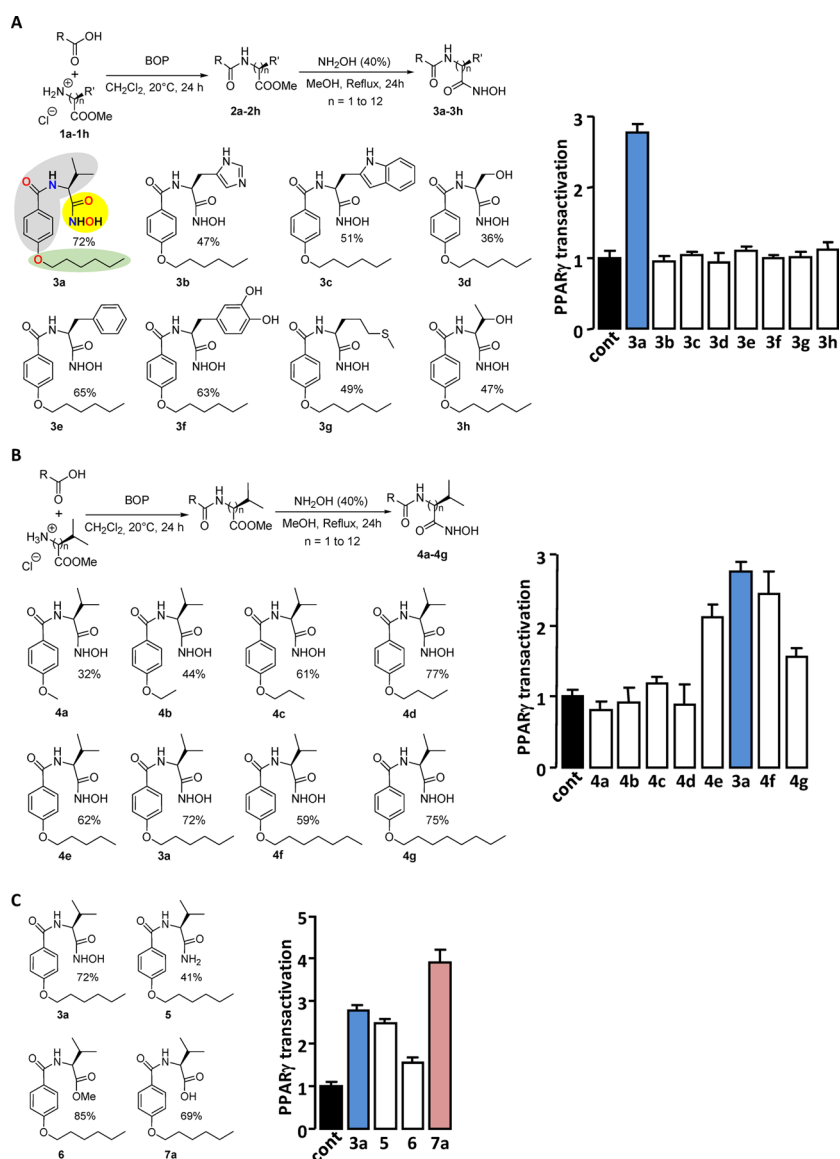


Figure 1. Chemical structures and PPAR γ agonist activity of 3a and its derivatives. (A) Amino acid core, (B) hydrophobic capping group, and (C) binding head group of 3a were modified (left panels), and PPAR γ transactivation activity of each molecule (5 μ M) was measured (right panels). Color fills accentuate the amino acid core (gray), hydrophobic capping group (green), and binding head group (yellow) of 3a. The percentage indicates the yield of the synthesis. PPAR γ transactivation values are means \pm SD expressed relative to the mean of control values, which was set to 1.

agonists have been developed and used in research,¹¹ but none of them are currently approved for clinical use.

PPAR γ is considered the master regulator of adipogenesis via its promotion of lipid production and storage. Thiazolidinediones, including rosiglitazone (Rosi) and pioglitazone, are the most effective PPAR γ activating drugs that were widely prescribed for the treatment of type 2 diabetes.¹² However, their strong agonist activities are partly responsible for unwanted harmful side effects such as weight gain, fluid retention, osteoporosis, heart failure, and cancer,^{13,14} which precipitated their withdrawal from the market.

The quest for antidiabetic compounds targeting PPAR with good therapeutic potential and reduced adverse effects has followed two main directions. The one based on the observation that a moderate, rather than full, activation of PPAR γ dissociates the deleterious from the therapeutic effects of the agonist has led to the generation of selective PPAR γ

modulators (SPPAR γ M) with higher therapeutic profiles than full agonists.^{15,16} The peculiar properties of SPPAR γ M are being explained by the ability of PPAR γ to adopt ligand-specific conformations with different transcriptional signatures. In addition, it has been shown that the clinical benefit of PPAR γ partial agonists and SPPAR γ M also involves their ability to inhibit the cyclin-dependent kinase 5 (CDK5)-mediated PPAR γ phosphorylation at serine 273.^{17,18} The other concept for developing safe antidiabetic drugs targeting PPAR considers that beneficial effects of their activation could counteract their harmful effects. PPAR α/γ dual agonists, so-called glitazars, that combine the insulin sensitizing effect of PPAR γ agonists with the beneficial effect of PPAR α agonists on the lipid profile are representative of this class of drugs.^{19,20} Saroglitazar is approved in India for the treatment of peculiar type of diabetic dyslipidemia and hypertriglyceridemia.^{21,22} Moreover, dual α/δ and γ/δ PPAR agonists as well as “pan”

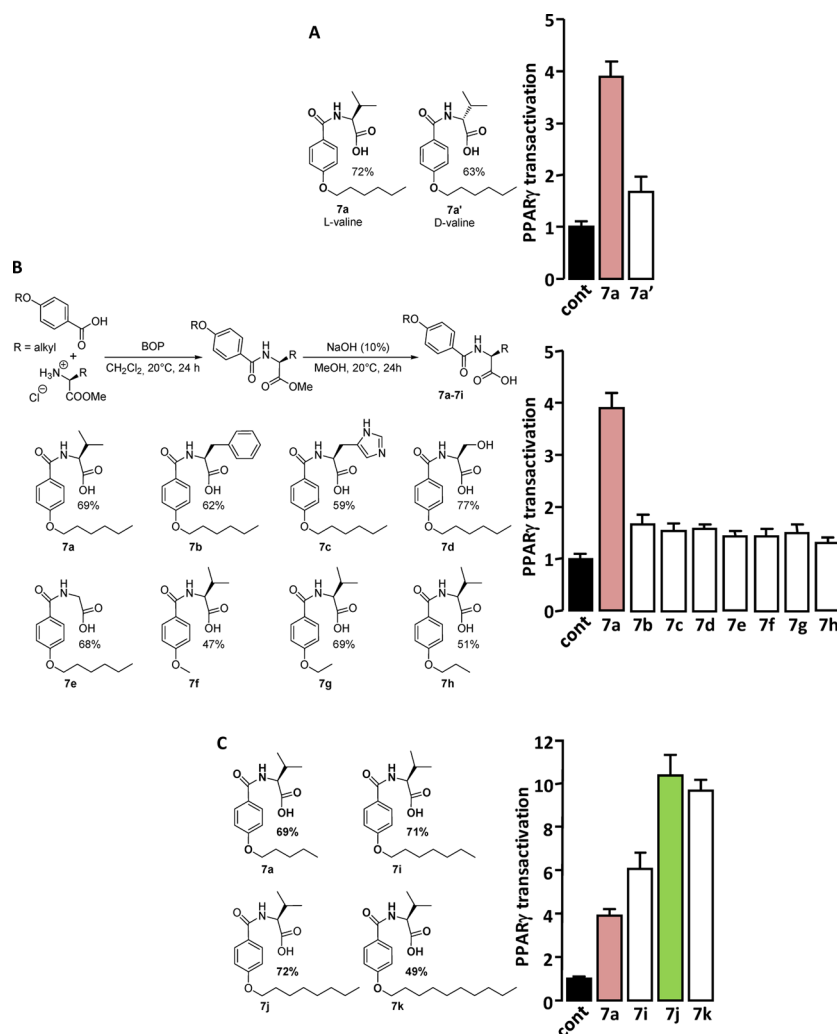


Figure 2. Chemical structures and PPAR γ agonist activity of 7a and its derivatives. (A–C) Absolute conformations of the valine core (A), the amino acid core (B), or the hydrophobic capping group (C) of 7a were modified (left panels), and PPAR γ transactivation activity of each molecule (5 μ M) was measured (right panels). The percentage indicates the yield of the synthesis. PPAR γ transactivations are expressed as shown in Figure 1.

agonists acting on all three isoforms are the subject of intense investigations^{23–25} that could lead to the generation of molecules with potential additional therapeutic indications.

This work reports a mild two-step synthesis of a library of new *N*-aryl-substituted amino acid derivatives from commercially easily available and inexpensive reagents. The effect of these compounds, in particular the influence of substitutions on their phenyl group, was evaluated on PPAR activity and led to the development of a new balanced and potent dual PPAR α/γ agonist with unique ligand binding properties and singular biological activities that would be expected for a potential therapeutic candidate to reduce insulin resistance, obesity, and NAFLD.

RESULTS AND DISCUSSION

New *N*-aryl-substituted amino acid derivatives bearing a hydroxamate head group (head), initially designed to identify MMP inhibitors, were screened on their ability to induce spontaneous (in the absence of any other inductor) adipocyte differentiation of 3T3-L1 cells by measuring intracellular lipid accumulation. The molecule named 3a (4-hexyloxy-*N*-((*S*)-1-hydroxycarbonyl-2-methyl-propyl)-benzamide), which con-

tains an L-valine (core) and a six-carbon atom chain (capping group) (Figure 1A and Figure S1), was identified as a weak activator of adipocyte differentiation as compared with Rosi (Figure S2). We hypothesized that 3a increased adipocyte differentiation by activating the master regulator of adipogenesis: PPAR γ . The PPAR γ agonistic activity of 3a was confirmed by the use of a luminescence-based cell-based PPAR γ transactivation assay in which a wild-type PPAR γ ligand binding domain (PPAR γ -LBD) is fused to the GAL4 DNA binding domain (PPAR γ -LBD-GAL4) and the Firefly luciferase reporter gene is under the control of GAL4 binding elements (Figure 1A). Importantly, 3a increased PPAR γ activity by a factor of 2.8, while 1 μ M Rosi (a full PPAR γ agonist) increased it by a factor of 25 \pm 7. A thorough comparison of the PPAR γ activation properties of the newly synthesized compounds with that of Rosi will be carried out at the later stage. We initiated a process to identify the domains of 3a involved in PPAR γ activation that would ideally lead to an optimization of its PPAR γ agonist activity.

Effect of the Amino Acid Core. Analogues of the 3a hit were designed and synthesized through a reaction involving a two-step synthesis procedure in dichloromethane from amino

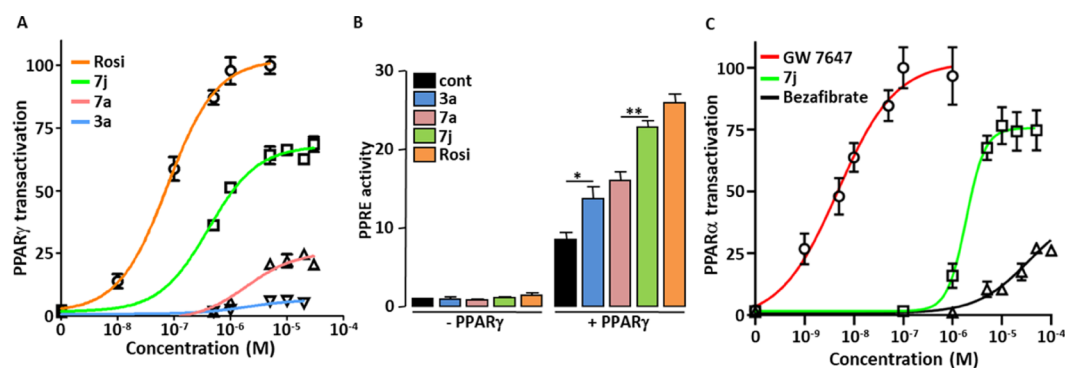


Figure 3. PPAR agonist activity of 3a, 7a, and 7j. (A) Concentration-dependent PPAR γ transactivation activities of 3a, 7a, and 7j were compared to that of Rosi using the PPAR γ -LBD-GAL4 chimera assay. Values are means \pm SD expressed as % of the maximal response measured with Rosi (5 μ M). (B) HEK293 cells were transfected with PPRE-driven Firefly luciferase and SV40-driven Renilla luciferase coding vectors together with an empty plasmid (–PPAR γ) or with the PPAR γ expression vector (+PPAR γ). After 36 h of post-transfection, cells were incubated for 17 h with 3a, 7a, 7j (1 μ M), or Rosi (0.1 μ M). PPRE promoter activity was calculated as the ratio of Firefly/Renilla luciferase. Values are means \pm SD expressed relative to the control situation (* p < 0.05 and ** p < 0.01 (one-way ANOVA followed by Bonferroni's post-hoc test). (C) Concentration-dependent PPAR α transactivation activities of 7j, GW 7647, and bezafibrate were measured using the PPAR α -LBD-GAL4 chimera assay. Values are means \pm SD expressed as % of the maximal response measured with GW 7647 (1 μ M).

acid methyl ester hydrochloride **1a–1h**, easily prepared from amino acids. By using a BOP reagent as an efficient and versatile reagent for the coupling of alkyloxybenzoic acid with **1a–1h**, the synthesis of various substituted amino acid ester derivatives **2a–2h** in high chemical yields of up to 90% was achieved. It is noteworthy that the transient ester species are successfully transformed into their corresponding hydroxamic acid parent derivatives **3a–3h** by using hydroxylamine (40% H₂O) in MeOH at reflux for 24 h in yields varying from 36 to 72% (Figure 1A). Changing the L-valine core by any other amino acid decreased the PPAR γ activation potency of the molecule (Figure 1A) perhaps due to the larger steric hindrance generated by the isopropyl moiety with respect to a benzyl or hydroxy methyl group.

Effect of the Carbon Atom Chain Capping Group.

The different derivatives **4a–4h** were synthesized according to a similar procedure than for compounds **3a–3h** in good-to-excellent yields, varying from 32 to 75% (Figure 1B). Altering the number of carbons in the polycarbon chain capping group of the aryl moiety showed that a linear six-carbon chain was optimal for PPAR γ activation (Figure 1B). This result suggests that the steric hindrance and the hydrophobic nature of the polycarbon chain are important factors to consider when designing most potent analogues.

Effect of the Head Group. Substitution of the hydroxamate head moiety in **3a** (Figure 1C) revealed that the preferred functions for PPAR γ activation are OH > NHOH \geq NH₂ > COOMe (Figure 1C). The analogue of **3a**, with a carboxylic acid moiety in place of the hydroxamate head group that optimally activated PPAR γ , was named **7a** (Figure S3).

Optimization of PPAR γ Agonist Activity of 7a.

Changing the absolute conformation of the core valine from L to D abolished the PPAR γ activation capacity of **7a** (Figure 2A). Furthermore, substitution of the amino acid core (L-valine) by other amino acid (L-form) and/or reducing the length of the polycarbon chain decreased the PPAR γ activation efficiency of **7a** (Figure 2B). The PPAR γ transactivation activity of **7a** was gradually enhanced by the extension of the polycarbon chain up to eight carbon atoms (Figure 2C). The analogue of **7a** with the eight-carbon atom chain in place of the six-carbon atom chain, which optimally

activates PPAR γ , was named **7j** (Figure S4). It should be noted that the presence of a chain with 10 carbon atoms in the molecule **7k** did not further enhance its ability to activate PPAR γ .

Typical PPAR agonists are known to consist of three parts: a polar head group (usually bearing a carboxylic acid functionality), a hydrophobic tail moiety, and a linker, which consists of flexible methylene units and an aromatic ring.²⁶ Interestingly, **7a** and **7j** closely meet these elementary criteria.

Potency and Efficacy of PPAR γ Activation. The concentration-dependent activation of PPAR γ by the “hit” compound **3a** and its two “lead” derivatives **7a** and **7j** was compared to that of the PPAR γ full agonist Rosi using the PPAR γ -LBD-GAL4 chimera assay (Figure 3A).

The potencies of the compounds were ranked as follows: **3a** \approx **7a** < **7j** < Rosi (Table 1). Regarding their efficacy (maximal PPAR γ transactivation), compounds were ranked as follows: **3a** < **7a** < **7j** \leq Rosi (Table 1).

Table 1. EC₅₀ and Maximal Activation of PPAR γ and PPAR α ^a

compound	PPAR	EC ₅₀ (nM)	activity (%)
Rosi	γ	76 \pm 30	100
7j	γ	400 \pm 150	66.36 \pm 3.41
7a	γ	1940 \pm 940	25.06 \pm 2.03
3a	γ	1840 \pm 1020	6.33 \pm 1.03
GW 7647	α	5.4 \pm 1	100
7j	α	1882 \pm 665	75.6 \pm 3.83
bezafibrate	α	31,700 \pm 5410	35.4 \pm 3.81

^aActivities are expressed as % of the maximal response measured with 5 μ M Rosi (for PPAR γ) or with 1 μ M GW 7647 (for PPAR α). Values are means \pm SD.

PPAR γ activation elicited by **7j** was around 66% of that obtained with Rosi. However, this value is certainly overestimated. Indeed, in this specific cell-based assay, we observed that the Renilla luciferase expression (Figure S5A), which is used to normalize reporter gene values for variations inherent to transfection efficiency and sample handling, was more reduced by **7j** concentrations that induce maximal

PPAR γ activity ($>10^{-6}$ M) than by Rosi. As a consequence, inclusion of Renilla luciferase expression values in the calculation of PPAR γ activation by 7j leads to an “artificial rise” of the maximum response (Figure S5B). Interestingly, the measurement of ATP cell content revealed that the number of viable HEK293 cells was no more reduced by 7j than by Rosi, ruling out that the 7j-dependent decrease in Renilla luciferase expression is due to a reduction in the number of viable cells (Figure S5C).

Taken together, these data allow us to define 3a and 7a as partial agonists of PPAR γ and 7j as a strong partial agonist of PPAR γ .

A PPAR responsive element (PPRE)-based luciferase assay was carried out to assess the ability of 3a, 7a, and 7j to transactivate genes controlled by the binding of PPAR γ to PPRE. The three molecules increased the expression of PPRE-driven luciferase only when full-length PPAR γ was expressed (Figure 3B), showing that these molecules stimulate the actual transcriptional activity of PPAR γ . Interestingly, the transactivation efficiencies were in agreement with those measured by the PPAR γ -LBD-GAL4 chimera assay, i.e., $3a \approx 7a < 7j$. In addition, and as expected, 1 μ M 7j and 0.1 μ M Rosi gave similar activation levels in both the PPAR γ -LBD-GAL4 chimera assay and PPRE-based assay.

To attest that 3a, 7a, and 7j are actual PPAR γ ligands, their binding affinity (K_d) and rate constants (k_{on} and k_{off}) for PPAR γ were compared to those of the reference ligand Rosi by surface plasmon resonance technology-based experiments (Table 2 and Figure S6).

Table 2. Affinity (K_d) and Rate Constants (k_{on} and k_{off}) for PPAR γ /Ligand and PPAR α /Ligand Interactions^a

interaction	k_{on} ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	K_d (nM)
PPAR γ /Rosi	$3.45 (0.02) \times 10^5$	0.0164 (0.0001)	47.6 (0.3)
PPAR γ /7j	$5.65 (0.06) \times 10^5$	0.0170 (0.0001)	30.1 (0.3)
PPAR γ /7a	$1.52 (0.02) \times 10^5$	0.0143 (0.0001)	94 (1)
PPAR γ /3a	$1.55 (0.02) \times 10^5$	0.0190 (0.0001)	123 (2)
PPAR α /7j	$1.01 (0.03) \times 10^4$	0.0312 (0.0005)	3100 (50)

^aExperimental error is reported in parentheses.

The affinities of 3a and 7a for PPAR γ were lower than those of 7j and Rosi, which is consistent with their low PPAR γ transactivation capacity measured in cell-based assays. Remarkably, the partial agonist 7j shows an affinity for PPAR γ similar or higher to that of the full agonist Rosi. It was shown that some compounds with moderate PPAR γ agonist activity but with high binding affinity to PPAR γ retain significant antidiabetic activity while having fewer and/or less severe adverse events than full PPAR γ agonists.²⁷ Therefore, 7j can reasonably be considered as the scaffold molecule to develop new antidiabetic drugs.

Specificity. The concentration-dependent activations of PPAR δ and PPAR α by 3a, 7a, and 7j were analyzed using the appropriate PPAR-LBD-GAL4 chimera assays. None of the molecules activated PPAR δ (Figure S7). Only 7j transactivated PPAR α (Figure 3C and Table 1), with potency between bezafibrate (a weak pan agonist for all three PPAR isoforms) and GW 7647 (a full PPAR α agonist). 7j appeared to be a good PPAR α agonist. In order to confirm that 7j is a PPAR α ligand, their direct physical binding was measured by surface plasmon resonance technology-based experiments. We

found that 7j concentration dependently bound to PPAR α with a K_d value of 3.1 μ M (Table 2).

PPAR forms permissive heterodimers with RXR, thus allowing both the ligands of PPAR and RXR to regulate the transcription of PPAR target genes. Using a mammalian two-hybrid system, we showed that 7j was less potent and less efficient than Rosi in inducing PPAR γ /RXR α heterodimerization (Figure S8A). Moreover, using the RXR α -LBD-GAL4 chimera assay (Figure S8B) and RXR responsive element (RXRE)-based luciferase assay (Figure S8C), we demonstrated that 7j is not a direct RXR agonist.

Therefore, 7j is a dual PPAR γ / α agonist (glitazar), and as such, it could theoretically have beneficial synergistic activities on glucose and lipid homeostasis.^{19,20}

Binding of 7j, 7a, and 3a to PPAR γ . The crystal structure of the complex of PPAR γ LBD with the ligand 7j was solved, collecting diffraction data from apo-crystals soaked for 3 days in the presence of the ligand at 0.5 mM. Initial difference Fourier maps revealed clear electron density for the ligand (Figure S9), showing that it occupies the typical LBD region of the PPAR γ agonists (Figure 4A and Figure S10), similar to that of the full agonist Rosi (Figure 4B and Figure S11), with its carboxylate group directly interacting through H bonds with Y473 of helix 12 (H12), at a short distance of 2.3 Å, the two histidines (H323 and H449) of the canonical triad, and S289 (Figure 4A). The NH of the 7j amide bond is also involved in the H bond with S289, and the CO makes a H bond with Y327. The terminal aliphatic chain of 7j is in equilibrium between two different conformations (occupation factors of 0.6 and 0.4) and makes vdW contacts with several residues of the internal strand of the β -sheet (Figure 4A and Figure S12) with a consequent effective stabilization of this domain. The isopropyl terminal group of 7j, makes vdW interactions in the hydrophobic pocket formed by the residues F282, C285, Q286, L453, and L469 (belonging to H3, H11, and H12), contributing, in this way, to a tighter binding of the ligand (Figure 4C and Figure S13).

The thorough analysis of the effective binding network of 7j into the PPAR γ LBD indicates that the ligand is very tightly locked into the LBD, with its carboxylate group strongly interacting with Y473 of H12 (2.3 Å), but in a slightly distorted mode, with respect to Rosi. As a consequence, there is an adjustment of the conformation of helix 12 (H12), the loop 11/12, and the beginning of helix 11 (H11) (Figure 4D and Figure S14). Importantly, H12 is a critical regulatory structural element in the activation function-2 (AF-2) coregulator binding surface, which determines the transcriptional output of PPAR γ through differential recruitment of coregulators.

Comparison of the crystal structures of PPAR γ in complex with 7j, 7a, or 3a²⁸ shows no difference in the binding mode of these ligands, regardless of the presence of a carboxylic or hydroxamic head (Figure S15). However, the shorter aliphatic chains of 7a and 3a accommodate in a different part of the PPAR γ LBD with respect to 7j, farther away from the β -sheet (Figure S15). Moreover, it is known that partial agonists of PPAR γ preferentially stabilize the β -sheet region of the LBD. In the PPAR γ /7j structure, the long aliphatic chain of the ligand strongly contributes to the stabilization of this region, through vdW interactions, with both its observed conformations facing residues of the innermost β -strand. The solvent entropic gain arising from a more efficacious displacement of water molecules, known to occupy the β -sheet pocket, could

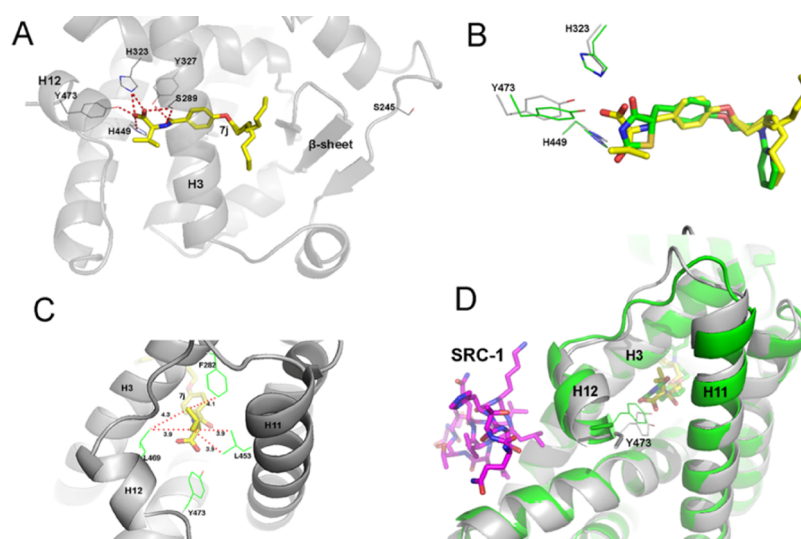


Figure 4. Binding of 7j to PPAR γ . (A) Hydrogen-bond network of 7j in the PPAR γ LBD. (B) Superimposition of 7j (yellow) and Rosi (green) structures. (C) vdW network of 7j with residues belonging to H3, H11, and H12 of the PPAR γ LBD. (D) Superimposition of 7j (yellow) and Rosi (green) with the coactivator SRC-1 (magenta); the PPAR γ /Rosi structure is colored in green, and the PPAR γ /7j structure is colored in gray (PDB codes: 6QJ5 for PPAR γ /7j and 2PRG for PPAR γ /Rosi).

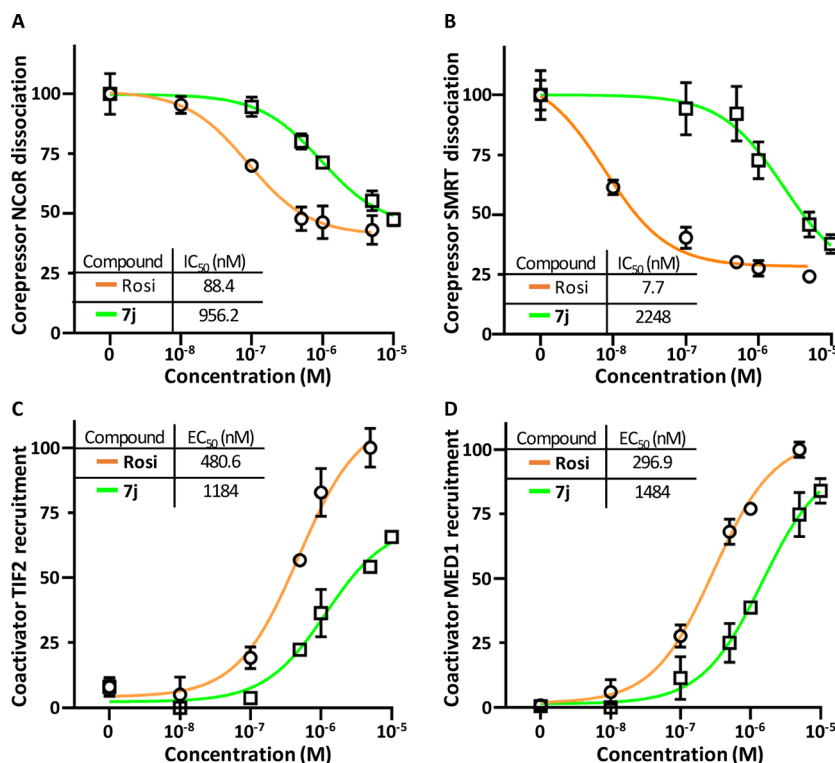


Figure 5. Ligand-specific coregulator binding profiles. (A–D) HEK293 cells were transfected with GAL4-NCOR (A), GAL4-SMRT (B), GAL4-TIF2 (C), GAL4-MED1 (D), and VP16-PPAR γ -LBD expression vectors in a mammalian two-hybrid setting and treated with vehicle or various concentrations of 7j or Rosi. Values are means \pm SD expressed as % of the maximal response measured.

play an important role in lowering the free energy of the binding. The higher affinity of 7j for PPAR γ with respect to those of 7a and 3a (Table 2) demonstrates that the affinity is largely affected by the length of the aliphatic chain, rather than the character of the acidic head.

Interestingly, a similar unique binding network was observed in the PPAR γ crystal structure with the partial agonist SR2067 (Figure S16).²⁹ Both ligands share a common amide group that forms two hydrogen bonds with Y327 and

S289, interactions that are not possible with Rosi. However, unlike 7j, SR2067 does not interact with Y473.

Interaction of Coregulators with PPAR γ . Since Rosi and 7j stabilize different conformations of the AF2 coregulator binding surface of PPAR γ , we analyzed how these molecules modulate the interaction of PPAR γ with some of its coregulators. In the mammalian two-hybrid system, 7j was less potent than Rosi in inducing displacement of the NCoR and SMRT corepressors from PPAR γ and in inducing

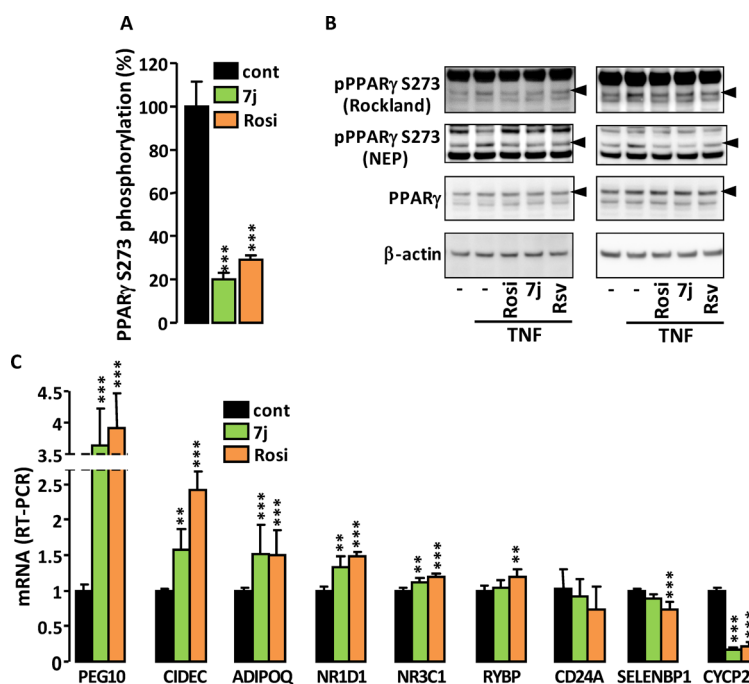


Figure 6. Effect of 7j on PPAR γ phosphorylation. (A) Percentage of *in vitro* PPAR γ Ser273 phosphorylation by CDK5 in the presence of 0.1 μ M of 7j or Rosi. (B) Phosphorylation of PPAR γ Ser273 in 3T3-L1 adipocyte incubated for 60 min with 5 μ M Rosi, 7j, or roscovitine (Rsv) before TNF α stimulation (50 ng mL $^{-1}$; 90 min). Two independent experiments are shown. Phosphospecific antibodies were from Rockland or New England Peptide (NEP). Arrow heads indicate phosphorylated PPAR γ and PPAR γ 2. (C) RT-PCR analysis of the expression levels of a selection of genes known to be regulated by CDK5-dependent phosphorylation of PPAR γ . Values are means \pm SD ($n = 5$) expressed relative to the mean of control. ** $p < 0.01$ and *** $p < 0.001$ vs control (one-way ANOVA followed by Dunnett's post-hoc test).

recruitment of TIF2 and MED1 coactivators to PPAR γ (Figure 5).

The differences between the profiles of the concentration–response curves of 7j and Rosi to modulate the interaction of PPAR γ with its partners (NCoR, SMRT, TIF2, MED1, mediator, and also RXR α) suggest that these two molecules induce distinct patterns of coregulatory protein recruitment.

CDK5-Mediated Phosphorylation of PPAR γ . Part of the antidiabetic effects of PPAR γ partial agonists has been associated with their ability to prevent the CDK5-mediated phosphorylation of the PPAR γ serine 273 residue.^{17,18} Therefore, we assessed the ability of 7j to inhibit such phosphorylation. An ELISA protocol was optimized to quantify the phosphorylation of PPAR γ triggered *in vitro* by recombinant CDK5. 7j prevented the phosphorylation of PPAR γ serine 273 at least as efficiently as Rosi (Figure 6A). Furthermore, pretreatment of 3T3-L1 adipocytes with 7j or Rosi reduced the TNF α -stimulated phosphorylation of PPAR γ serine 273 (Figure 6B). The involvement of CDK5 in this phosphorylation was attested by the fact that it was impeded by treatment of cells with roscovitine, a CDK5 inhibitor. Moreover, out of 10 genes known to be significantly controlled by CDK5-dependent PPAR γ phosphorylation in fully differentiated adipocytes,^{17,18} seven were regulated in the same way by 7j and Rosi (Figure 6C). Two genes were only regulated by Rosi, and the expression of one gene was not significantly modified by 7j and Rosi, although both molecules tended to alter this expression in the same direction.

These results underline the ability of 7j to reduce CDK5-mediated phosphorylation of PPAR γ serine 273 and to alter the expression of genes controlled by PPAR γ phosphorylation. This feature supports a potential antidiabetic effect of 7j.

Adipocyte Differentiation and Glucose Uptake.

Adipogenesis-mediated weight gain is a major side effect of PPAR γ full agonists.³⁰ Partial PPAR γ agonists are expected to have a reduced effect on lipid storage while maintaining a significant insulin sensitization effect. Therefore, the proadipogenic properties of 3a, 7a, and 7j were compared to that of Rosi. 3T3-L1 fibroblast cells were incubated in the presence of PPAR γ agonists as the sole inducer of adipocyte differentiation. Only 7j and Rosi (the strongest PPAR γ agonists) significantly increased the intracellular lipid content (Figure 7A and Figure S17A), showing that these two molecules stimulate adipocyte differentiation. However, for the same concentration, the proadipogenic property of 7j was significantly lower than that of Rosi. To ascertain that the proadipogenic effect of 7j involved PPAR γ activation, 3T3-L1 cells were coincubated with the specific PPAR γ antagonist GW 9662 and with 7j; then, intracellular lipid accumulation was measured. This approach has been successfully used to demonstrate the involvement of PPAR γ activation in the proadipogenic effect of Rosi.³¹ Because of its short half-life, GW 9662 was added every day, and to simplify the experiment, the duration of the treatment was reduced to 4 days instead of 6 days, thus limiting the intracellular lipid accumulation. Antagonizing PPAR γ activation significantly prevented 7j- and Rosi-induced adipogenesis (Figure 7B and Figure S17B), showing that PPAR γ activation is necessary for the proadipogenic effect of these molecules. When 3T3-L1 fibroblast cells were primed for adipocyte differentiation using the conventional adipogenic cocktail (insulin, dexamethasone, and IBMX) and then treated for 7 days with 3a, 7a, 7j or Rosi, mRNA levels of the markers of adipocyte differentiation were all increased in proportion to the ability of the molecules to activate PPAR γ (Figure 7C).

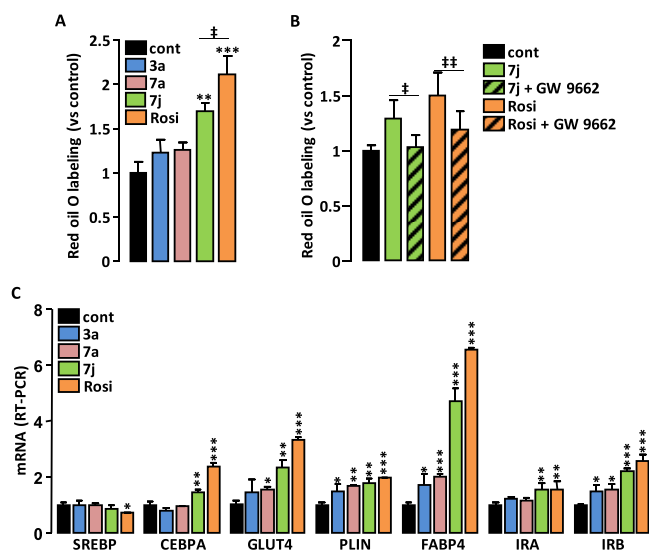


Figure 7. Adipogenic effect of the compounds. (A, B) 3T3-L1 fibroblasts were incubated for 6 days (A) or for 4 days (B) with insulin (350 nM), the indicated PPAR γ agonists (1 μ M), and the PPAR γ antagonist GW 9662 (5 μ M). Intracellular lipids were stained with Oil Red O, and then colored lipids were quantified. (C) 3T3-L1 adipocyte differentiation was triggered by the standard mixture of inductors, and then cells were incubated for 7 days with 3a, 7a, 7j (1 μ M), or Rosi (0.1 μ M). The expression levels of adipogenesis-related genes were measured by RT-PCR. Values are means \pm SD expressed as fold relative to untreated situation. * p < 0.05, ** p < 0.01, and *** p < 0.001 vs control (one-way ANOVA followed by Dunnett's post-hoc test); # p < 0.05 and ## p < 0.01 (t -test).

As expected for partial PPAR γ agonists, 3a, 7a, and 7j have reduced proadipogenic properties compared with the full agonist Rosi.

It has been shown that the ability of PPAR γ ligands to increase cellular glucose uptake is not necessarily related to their transactivation activity or proadipogenic potential.³² Therefore, glucose uptake was measured in fully differentiated 3T3-L1 adipocytes after an acute treatment with PPAR γ agonists. The insulin-dependent glucose uptake was increased by a short-term treatment with 7j or Rosi (Figure 8), denoting that part of their insulin sensitizing effect is independent of their ability to increase adipocyte differentiation.

Adipocyte Browning. Brown fat is a target for antiobesity and antidiabetes experimental therapies that aim to increase energy expenditure.³³ Interestingly, strong PPAR γ agonists activate the "browning" of white adipose tissues,^{34,35}

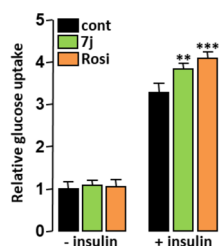


Figure 8. Adipocyte glucose uptake. 3T3-L1 adipocytes were treated with 7j (1 μ M) or Rosi (0.1 μ M) for 16 h prior to insulin stimulation (50 nM, 10 min), and cellular glucose uptake was determined. Values are mean \pm SD expressed as fold relative to the control situation. ** p < 0.01 and *** p < 0.001 vs control (one-way ANOVA followed by Dunnett's post-hoc test).

suggesting that partial PPAR γ agonists, which retain significant white fat browning ability, may also have therapeutic benefits in the treatment of obesity and diabetes. We therefore studied the ability of 3a, 7a, and 7j to induce brite/brown-like adipocytes in 3T3-L1 cells.

Only 7j (and Rosi) upregulated mRNA levels of genes considered as brite/brown adipocyte-selective transcript (Figure 9A,B).³⁶ Immunoblotting analysis confirmed that 7j and Rosi increased the expression of uncoupling protein 1 (UCP1), which is a functional marker of brown adipocytes (Figure 9B inset). Coherent with their adipocyte browning effect, Rosi and 7j increased mRNA levels of additional genes involved in (or associated with) mitochondrial biogenesis (Figure 9C)³⁷ and in accordance with these two molecules significantly increased the selective labeling of active mitochondria by the MitoTracker dye (Figure 9D). We have previously shown that Rosi induced the conversion of hMADS white adipocytes into brite adipocytes as evidenced by the strong expression of UCP1.³⁸ In order to test whether compounds 3a, 7a, and 7j were able to substitute for Rosi, hMADS cells first differentiated into white adipocytes were treated with these compounds between days 14 and 18. UCP1 expression was analyzed as an indicator of the degree of white-to-brown adipocyte conversion. UCP1 mRNA levels were increased in 3a, 7a and 7j-treated cells compared to untreated cells, and 7j was found to be the most potent compound (Figure 9E), although less potent than Rosi (Figure 9F). The PPAR γ agonists also increased the levels of UCP1 antigen, which is consistent with observations of its mRNA levels (Figure 9G).

The expression of the adipogenic marker perilipin was not modified by Rosi or 7j (Figure S18A), whereas the expression of adiponectin, a PPAR γ -responsive gene, was more efficiently increased by Rosi than by 7j (Figure S18B), which confirms that the PPAR γ agonist activity of 7j is lower than that of Rosi.

These data suggest that 7j can efficiently promote the conversion from white to brown adipocytes.

Lipid Accumulation in Hepatocytes. As PPAR α agonists decrease hepatic steatosis,^{5,7} we compared the effect of 7j (dual PPAR α/γ agonist) to those of GW 7647 (PPAR α agonist) and Rosi (PPAR γ agonist) on the lipid accumulation into hepatocytes. In primary rat hepatocytes, only GW 7647 prevented the basal intracellular lipid accumulation and oleic acid-induced lipid accumulation was prevented by Rosi, 7j, and GW 7647 (Figure 10 A). In human HuH7 hepatoma cells, 7j and GW 7647 similarly prevented the oleic acid-stimulated lipid accumulation (Figure 10B). These results suggest that 7j is a promising compound to prevent hepatic steatosis.

Cytotoxicity of 7j. We confirmed, as previously reported,^{39–41} that viability, membrane integrity, and apoptosis of primary hepatocytes were not significantly altered by concentrations of Rosi up to 10 μ M. These parameters were also not altered by the 7j compound (Figure S19).

CONCLUSIONS

We have described the synthesis and optimization of compound 7j: a new *N*-aryl-substituted valine derivative with a balanced agonist activity on PPAR α and PPAR γ . Compound 7j occupies the typical LBD region of the PPAR γ agonists with a unique high-affinity binding mode and efficiently prevents CDK5-mediated phosphorylation of

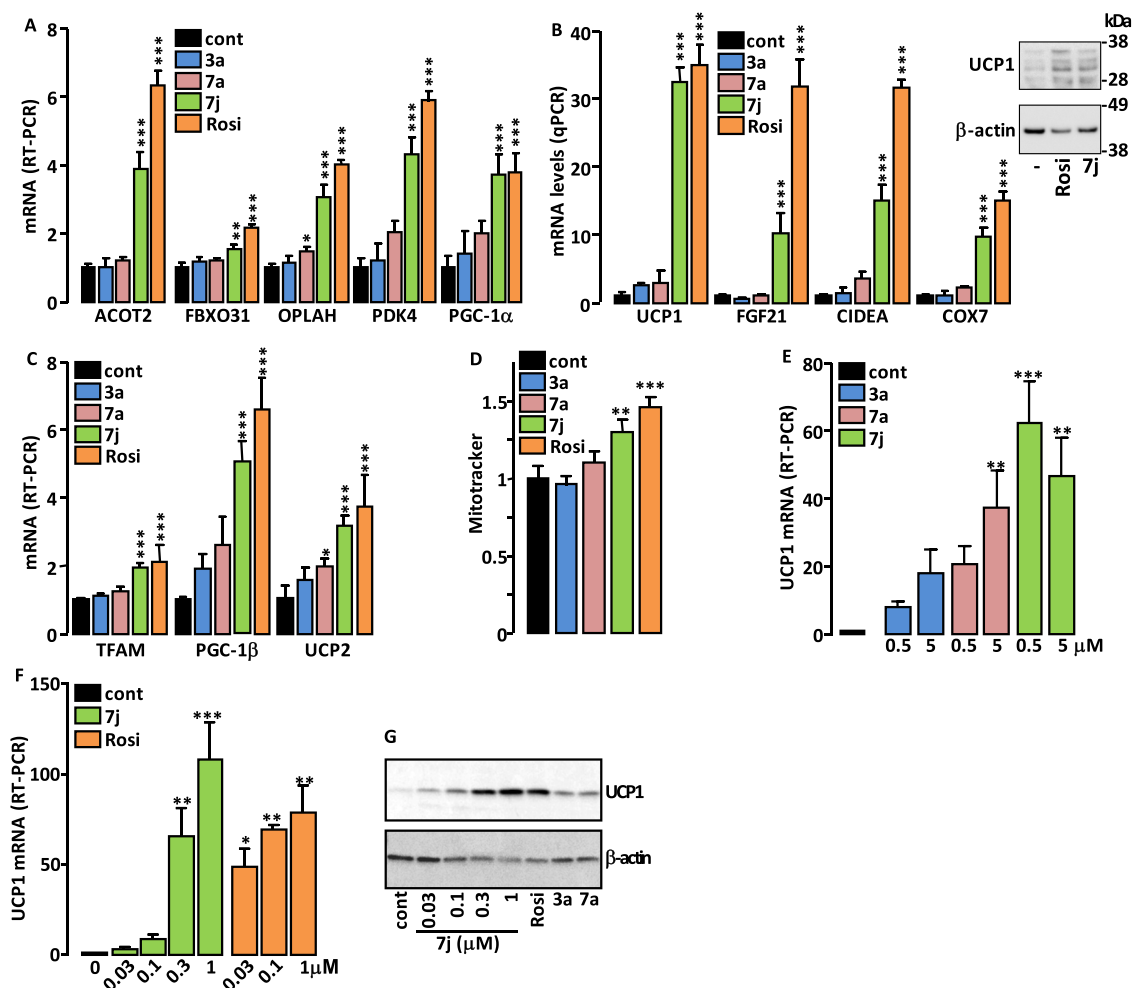


Figure 9. Adipocyte browning effect of the compounds. 3T3-L1 cells were treated as in Figure 7C. (A–C) Expression levels of beige (A) and brown (B) adipocyte markers as well as those of genes involved in mitochondrial biogenesis (C) were measured by RT-PCR. Inset in panel B: immunodetection of UCP1 and β -actin (loading control) in lysates of 3T3-L1 cells treated with 7j and Rosi. (D) Adipocyte mitochondria content was evaluated by flow cytometry after their selective labeling with MitoTracker dye. (E, F) hMADS white adipocytes were treated with the indicated compounds, and UCP1 mRNA levels were measured by RT-PCR. (G) Immunodetection of UCP1 and β -actin (loading control) in lysates of hMADS treated with the indicated concentrations of 7j, Rosi (0.1 μ M), 3a, and 7a (1 μ M). Values are mean \pm SD expressed as fold relative to the control (cont) situation. * p < 0.05, ** p < 0.01, and *** p < 0.001 vs control (one-way ANOVA followed by Dunnett's post-hoc test).

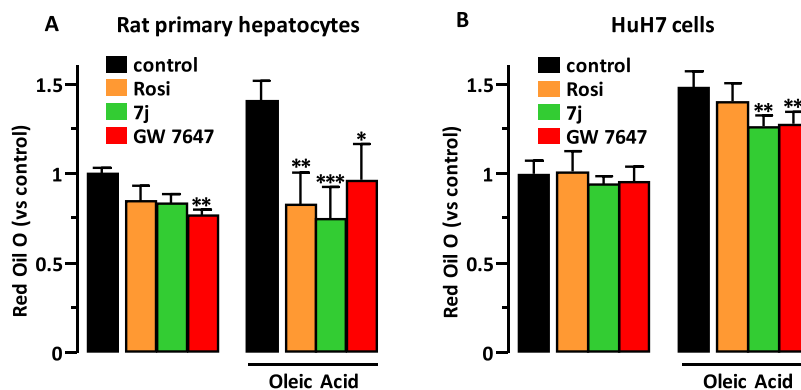


Figure 10. Lipid accumulation in hepatocytes. (A) Primary rat hepatocytes and (B) human HuH7 hepatoma cells were treated with 7j, Rosi, or GW 7647 (1 μ M) for 8 h before addition of oleic acid (0.25 mM). Twenty-four hours later, cellular lipid content was measured by Oil Red O staining. Values are mean \pm SD expressed as fold relative to the control. * p < 0.05, ** p < 0.01, and *** p < 0.001 vs control (Kruskal–Wallis followed by Dunn's post-hoc test).

PPAR γ . Meanwhile, poorly proadipogenic, compound 7j increases adipocyte insulin-stimulated glucose uptake and

efficiently promotes white-to-brown adipocyte conversion. In addition, compound 7j prevents the oleic acid-induced lipid

accumulation in hepatocytes. The unique biochemical properties of compound 7j, its specific biological activities, and its low toxicity make it a promising candidate for the development of compounds to reduce insulin resistance, obesity, and NAFLD.

EXPERIMENTAL SECTION

Chemistry. All solvents were purified according to reported procedures, and reagents were used as commercially available. Methanol, ethyl acetate, dichloromethane, ammonia, and petroleum ether (35–60 °C) were purchased from VWR and used without further purification. Column chromatography was performed on VWR silica gel (70–230 mesh). ¹H NMR and ¹³C NMR spectra were recorded in CDCl₃ or DMSO-*d*₆ on a Bruker AC 300 spectrometer working at 300 and 75 MHz, respectively (the usual abbreviations are used: s, singlet; d, doublet; t, triplet; q, quadruplet; and m, multiplet). Tetramethylsilane was used as the internal standard. All chemical shifts are given in ppm. Purity of all the new compounds evaluated by HPLC (Agilent 1100, C18) analysis was >95%.

Typical Procedure for the Synthesis of Amino Acid Methyl Ester Hydrochloride Derivatives. Synthesis of L-Valine Methyl Ester Hydrochloride. In a two-neck round flask equipped with a condenser was placed at room temperature 3 g of L-valine (2.56 × 10⁻² mol) in 20 mL of methanol. The mixture was placed under stirring at 0 °C, and 3.4 mL of thionyl chloride (4.7 × 10⁻³ mol) was slowly added. After removal of the solvents, diethyl ether was added and the product precipitates as a white solid. After filtration, the product was dried under vacuum to afford the expected L-valine methyl ester hydrochloride in 86% yield.

White solid. ¹H NMR (D₂O): δ = 4.14–4.17 (m, 1H), 3.80–3.85 (m, 3H), 2.50–2.56 (m, 1H), 1.12–1.15 (m, 6H). ¹³C NMR (D₂O): δ = 168.91, 58.57, 53.02, 29.95, 18.15.

(S)-2-(4-Hexyloxy-benzoylamino)-3-methyl-butyric Acid Methyl Ester (6). In a two-neck round flask equipped with a condenser were placed at room temperature 2.34 g of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP; 5.26 × 10⁻³ mol), 2.34 mL of diisopropylethylamine (1.8 × 10⁻² mol), 1.2 g of 4-(hexyloxy)benzoic acid (5.40 × 10⁻³ mol), and 0.88 g of L-valine methyl ester hydrochloride (5.25 × 10⁻³ mol) in 15 mL of CH₂Cl₂. The mixture was placed under stirring at room temperature for 24 h. Water was added to allow phase separation. The bottom phase layer was washed with NaHCO₃ (10%) solution, dried over Na₂SO₄, filtered, and concentrated in vacuo. After removal of the solvents, the crude residue was purified by chromatography on a silica gel column using CH₂Cl₂/ethyl acetate (1/1) as the eluent, affording the expected product 6 in 85% yield.

White solid. ¹H NMR (CDCl₃): δ = 8.06 (m, 2H), 7.15 (m, 2H), 4.15–4.22 (m, 4H), 3.71–3.75 (m, 3H), 1.81–2.05 (m, 3H), 1.21–1.37 (m, 6H), 0.69–0.92 (m, 9H). ¹³C NMR (CDCl₃): δ = 172.90, 166.54, 160.52, 133.08, 130.21, 130.01, 114.55, 68.22, 57.56, 52.34, 33.49, 31.32, 29.34, 25.67, 22.61, 18.48, 14.03. MS (ESI) *m/z*: calcd for C₁₉H₂₉NO₄, 336.2146 (100%, (M + H⁺)).

4-Hexyloxy-N-((S)-1-hydroxycarbonyl-2-methyl-propyl)-benzamide (3a). In a 25 mL round flask was placed at room temperature 0.6 g of 6 (1.78 × 10⁻³ mol) in 15 mL of ethanol. A hydroxylamine solution (40%, 2 mL) was subsequently added, and the mixture was allowed to stir at reflux for 24 h. After removal of the solvents, the crude residue was purified by chromatography on a silica gel column using petroleum ether/ethyl acetate (1/1) and methanol/ethyl acetate (1/1) as eluents, affording the expected product 3a in 72% yield.

White solid. ¹H NMR (DMSO-*d*₆): δ = 8.01 (m, 2H), 7.09 (m, 2H), 3.95–3.98 (m, 3H), 1.78–1.82 (m, 2H), 1.01–1.31 (m, 6H), 0.71–0.93 (m, 9H). ¹³C NMR (DMSO-*d*₆): δ = 167.20, 166.23, 161.1, 133.41, 129.80, 113.98, 69.41, 57.31, 31.03, 29.42, 25.76, 22.43, 18.62, 13.89. MS (ESI) *m/z*: calcd for C₁₈H₂₈N₂O₄, 337.2045 (100%, (M + H⁺)).

4-Hexyloxy-N-((S)-1-hydroxycarbonyl-2-(3H-imidazol-4-yl)-ethyl)-benzamide (3b). The procedure is similar to that applied for the preparation of 3a.

Pale yellow solid. ¹H NMR (DMSO-*d*₆): δ = 7.52–7.89 (m, 3H), 6.89–7.42 (m, 5H), 5.55 (s, 2H), 4.75–4.73 (m, 2H), 4.10–4.19 (m, 2H), 1.22–1.92 (m, 9H), 0.89–0.96 (m, 3H). ¹³C NMR (DMSO-*d*₆): δ = 174.19, 164.07, 162.03, 133.17, 129.03, 123.47, 118.22, 115.35, 67.56, 52.96, 30.99, 30.91, 29.05, 25.49, 22.54, 14.25. MS (ESI) *m/z*: calcd for C₁₉H₂₆N₄O₄, 375.2014 (100%, (M + H⁺)).

4-Hexyloxy-N-((S)-1-hydroxycarbonyl-2-(1H-indol-2-yl)-ethyl)-benzamide (3c). The procedure is similar to that applied for the preparation of 3a.

White solid. ¹H NMR (DMSO-*d*₆): δ = 7.32–7.62 (m, 5H), 6.29–7.25 (m, 7H), 3.92–4.03 (m, 2H), 2.89–3.34 (m, 4H), 1.20–1.79 (m, 6H), 0.88–0.92 (m, 3H). ¹³C NMR (DMSO-*d*₆): δ = 172.22, 168.98, 161.25, 139.48, 131.05, 129.14, 126.29, 120.15, 118.78, 113.69, 110.36, 104.25, 71.24, 53.32, 30.21, 28.28, 25.14, 22.04, 14.13. MS (ESI) *m/z*: calcd for C₂₄H₂₉N₃O₄, 424.2236 (100%, (M + H⁺)).

4-Hexyloxy-N-((S)-2-hydroxy-1-hydroxycarbonyl-ethyl)-benzamide (3d). The procedure is similar to that applied for the preparation of 3a.

White solid. ¹H NMR (DMSO-*d*₆): δ = 7.02–7.51 (m, 4H), 3.62–4.20 (m, 5H), 1.31–1.82 (m, 9H), 0.89–0.92 (m, 3H). ¹³C NMR (DMSO-*d*₆): δ = 170.36, 168.24, 160.89, 130.14, 125.34, 113.47, 68.78, 61.24, 53.89, 31.01, 28.14, 25.98, 21.33, 13.88. MS (ESI) *m/z*: calcd for C₁₆H₂₄N₂O₅, 325.1768 (100%, (M + H⁺)).

4-Hexyloxy-N-((S)-1-hydroxycarbonyl-2-phenyl-ethyl)-benzamide (3e). The procedure is similar to that applied for the preparation of 3a.

White solid. ¹H NMR (DMSO-*d*₆): δ = 7.66–7.68 (m, 2H), 6.47–7.16 (m, 2H), 3.55–3.97 (m, 2H), 2.89–3.23 (m, 2H), 1.35–1.74 (m, 8H), 0.90–0.91 (m, 3H). ¹³C NMR (DMSO-*d*₆): δ = 178.45, 168.32, 161.25, 137.47, 129.02, 125.68, 114.98, 60.01, 53.87, 40.36, 30.23, 28.14, 24.12, 22.98, 13.48. MS (ESI) *m/z*: calcd for C₂₂H₂₈N₂O₄, 385.2057 (100%, (M + H⁺)).

N-((S)-2-(3,4-Dihydroxy-phenyl)-1-hydroxycarbonyl-ethyl)-4-hexyloxy-benzamide (3f). The procedure is similar to that applied for the preparation of 3a.

White solid. ¹H NMR (DMSO-*d*₆): δ = 7.71–7.73 (m, 2H), 6.59–6.93 (m, 4H), 6.32 (s, 1H), 5.87 (s, 1H), 3.99–4.02 (m, 2H), 2.95–2.99 (m, 2H), 1.17–2.02 (m, 9H), 0.87–0.89 (m, 3H). ¹³C NMR (DMSO-*d*₆): δ = 171.25, 168.33, 160.58, 146.01, 143.69, 128.69, 126.47, 123.74, 116.36, 115.47, 69.71, 52.34, 38.38, 28.47, 22.14, 25.69, 22.47, 13.12. MS (ESI) *m/z*: calcd for C₂₂H₂₈N₂O₆, 417.1934 (100%, (M + H⁺)).

4-Hexyloxy-N-((S)-1-hydroxycarbonyl-4-methylsulfanyl-butyl)-benzamide (3g). The procedure is similar to that applied for the preparation of 3a.

White solid. ¹H NMR (DMSO-*d*₆): δ = 6.91–7.69 (m, 5H), 1.17–2.74 (m, 20H), 0.87–0.88 (m, 3H). ¹³C NMR (DMSO-*d*₆): δ = 170.78, 168.65, 162.21, 128.12, 125.14, 117.77, 69.66, 51.45, 34.12, 31.78, 28.02, 25.63, 22.33, 15.15, 13.19. MS (ESI) *m/z*: calcd for C₁₉H₃₀N₂O₄S, 383.1935 (100%, (M + H⁺)).

4-(Hexyloxy)-N-((2S,3S)-3-hydroxy-1-(hydroxyamino)-1-oxobutan-2-yl)benzamide (3h). The procedure is similar to that applied for the preparation of 3a.

White solid. ¹H NMR (DMSO-*d*₆): δ = 7.71–7.73 (m, 2H), 6.98–7.11 (m, 3H), 5.32 (s, 1H), 4.60–4.65 (m, 1H), 4.06–4.44 (m, 3H), 1.80–1.92 (m, 1H), 1.07–1.37 (m, 12H). ¹³C NMR (DMSO-*d*₆): δ = 169.75, 167.63, 162.54, 132.53, 125.82, 114.51, 68.78, 67.63, 59.34, 31.88, 28.02, 24.89, 22.33, 15.12, 13.89. MS (ESI) *m/z*: calcd for C₁₇H₂₆N₂O₅, 339.1879 (100%, (M + H⁺)).

(S)-N-(1-(Hydroxyamino)-3-methyl-1-oxobutan-2-yl)-4-methoxybenzamide (4a). White solid. ¹H NMR (DMSO-*d*₆): δ = 0.91–0.96 (m, 6H), 2.16–2.23 (m, 1H), 3.84 (s, 3H), 4.33–4.45 (m, 1H), 6.98–7.53 (m, 2H), 7.83–7.89 (m, 2H), 8.79 (s, 2H). ¹³C NMR (DMSO-*d*₆): δ = 174.44, 170.42, 164.51, 130.95, 127.71, 115.17, 56.40, 52.96, 32.19, 20.12, 19.65. MS (ESI) *m/z*: calcd for C₁₃H₁₈N₂O₄, 267.1386 (100%, (M + H⁺)).

(*S*)-4-Ethoxy-*N*-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)-benzamide (**4b**). White solid. ^1H NMR (DMSO- d_6): δ = 0.91–0.95 (m, 6H), 1.49 (t, J = 7.0 Hz, 3H), 1.68–1.75 (m, 1H), 4.01–4.11 (m, 2H), 4.31–4.41 (m, 1H), 6.94–6.99 (m, 2H), 7.80–7.85 (m, 2H), 8.59 (s, 2H). ^{13}C NMR (DMSO- d_6): δ = 171.10, 166.47, 166.91, 132.01, 129.30, 114.59, 63.42, 56.95, 30.10, 18.64, 14.69. MS (ESI) m/z : calcd for $\text{C}_{14}\text{H}_{20}\text{N}_2\text{O}_4$, 281.1412 (100%, (M + H $^+$)).

(*S*)-*N*-(1-(Hydroxyamino)-3-methyl-1-oxobutan-2-yl)-4-propoxybenzamide (**4c**). White solid. ^1H NMR (DMSO- d_6): δ = 0.92–1.12 (m, 9H), 1.69–1.89 (m, 2H), 3.27–3.38 (m, 2H), 3.90–4.02 (m, 2H), 6.90–7.07 (m, 2H), 7.76–7.89 (m, 2H). ^{13}C NMR (DMSO- d_6): δ = 171.14, 165.95, 162.22, 130.95, 128.03, 115.09, 70.35, 57.03, 30.75, 22.63, 18.64, 10.63. MS (ESI) m/z : calcd for $\text{C}_{15}\text{H}_{22}\text{N}_2\text{O}_4$, 294.1634 (100%, (M + H $^+$)).

(*S*)-4-Butoxy-*N*-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)-benzamide (**4d**). White solid. ^1H NMR (DMSO- d_6): δ = 0.90–0.98 (m, 9H), 1.52–1.62 (m, 2H), 1.77–1.83 (m, 2H), 2.16–2.22 (m, 2H), 3.97–4.02 (m, 2H), 4.37–4.42 (m, 1H), 7.75–7.77 (m, 2H), 7.98–8.02 (m, 2H), 8.53 (m, 1H). ^{13}C NMR (DMSO- d_6): δ = 171.49, 166.14, 166.16, 133.37, 130.18, 122.86, 69.16, 57.04, 30.74, 30.69, 18.48, 13.75. MS (ESI) m/z : calcd for $\text{C}_{16}\text{H}_{24}\text{N}_2\text{O}_4$, 309.1701 (100%, (M + H $^+$)).

(*S*)-*N*-(1-(Hydroxyamino)-3-methyl-1-oxobutan-2-yl)-4-pentyloxybenzamide (**4e**). White solid. ^1H NMR (DMSO- d_6): δ = 0.90–0.98 (m, 9H), 1.52–1.62 (m, 2H), 1.77–1.83 (m, 2H), 2.16–2.22 (m, 2H), 3.97–4.02 (m, 2H), 4.37–4.42 (m, 1H), 7.75–7.77 (m, 2H), 7.98–8.02 (m, 2H), 8.53 (m, 1H). ^{13}C NMR (DMSO- d_6): δ = 169.71, 167.51, 162.83, 132.86, 126.86, 119.98, 68.72, 58.13, 31.14, 30.98, 29.34, 28.14, 18.54, 14.16. MS (ESI) m/z : calcd for $\text{C}_{17}\text{H}_{26}\text{N}_2\text{O}_4$, 323.1934 (100%, (M + H $^+$)).

(*S*)-4-(Heptyloxy)-*N*-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)benzamide (**4f**). White solid. ^1H NMR (DMSO- d_6): δ = 0.90–0.96 (m, 9H), 1.28–1.74 (m, 10H), 2.02–2.16 (m, 1H), 3.97–4.07 (m, 2H), 4.37–4.42 (m, 1H), 7.03–7.08 (m, 2H), 8.02–8.09 (m, 2H), 8.53 (m, 1H). ^{13}C NMR (DMSO- d_6): δ = 171.09, 166.42, 162.02, 130.07, 129.03, 115.18, 68.15, 57.63, 31.80, 29.18, 26.04, 22.14, 18.74, 13.98. MS (ESI) m/z : calcd for $\text{C}_{19}\text{H}_{30}\text{N}_2\text{O}_4$, 351.2245 (100%, (M + H $^+$)).

(*S*)-*N*-(1-(Hydroxyamino)-3-methyl-1-oxobutan-2-yl)-4-octyloxybenzamide (**4g**). White solid. ^1H NMR (DMSO- d_6): δ = 0.90–0.97 (m, 9H), 1.29–1.82 (m, 12H), 2.06–2.15 (m, 1H), 3.95–4.07 (m, 2H), 4.32–4.41 (m, 1H), 7.00–7.08 (m, 2H), 8.01–8.10 (m, 2H), 8.51 (m, 1H). ^{13}C NMR (DMSO- d_6): δ = 171.10, 166.35, 162.35, 131.37, 129.90, 115.11, 68.24, 57.52, 31.79, 30.95, 29.31, 26.82, 22.47, 18.63, 14.21. MS (ESI) m/z : calcd for $\text{C}_{20}\text{H}_{32}\text{N}_2\text{O}_4$, 365.2423 (100%, (M + H $^+$)).

(*S*)-2-(4-Hexyloxy-benzoylamino)-3-methyl-butyric Acid (**7a**). In a 25 mL round flask was placed at room temperature 0.6 g of **6** (1.78×10^{-3} mol) in 15 mL of ethanol. A sodium hydroxide solution (10%, 2 mL) was subsequently added, and the mixture was allowed to stir at room temperature for 24 h. The bottom phase layer was discarded, and the aqueous phase was acidified with HCl (1 N). After extraction with ethyl acetate, the organic phase was dried over Na_2SO_4 , filtered, and concentrated in vacuo. The crude residue was purified by chromatography on a silica gel column using petroleum ether/ethyl acetate (1/1) as the eluent, affording the expected product **7a** in 69% yield.

White solid. ^1H NMR (CDCl_3): δ = 7.80–7.84 (m, 2H), 6.95–7.04 (m, 2H), 4.44–4.46 (m, 1H), 4.02–4.05 (m, 2H), 2.19–2.35 (m, 1H), 1.73–1.84 (m, 2H), 1.43–1.57 (m, 2H), 1.26–1.35 (m, 5H), 1.04 (s, 3H), 1.03 (s, 3H), 0.91–0.94 (m, 3H). ^{13}C NMR (CDCl_3): δ = 174.59, 168.63, 162.17, 128.97, 125.94, 113.84, 67.86, 58.88, 31.35, 30.54, 28.87, 25.42, 22.27, 18.43, 17.52, 12.98. MS (ESI) m/z : calcd for $\text{C}_{18}\text{H}_{27}\text{NO}_4$, 322.1932 (100%, (M + H $^+$)).

(*R*)-2-(4-Hexyloxy-benzoylamino)-3-methyl-butyric Acid (**7a'**). The procedure is similar to that applied for the preparation of **7a**.

White solid. ^1H NMR (CDCl_3): δ = 6.55–7.19 (m, 4H), 3.89–3.99 (m, 3H), 1.50–2.11 (m, 7H), 0.89–1.32 (m, 11H). ^{13}C NMR (CDCl_3): δ = 177.56, 166.34, 159.95, 132.36, 130.12, 114.08, 72.30,

64.36, 31.32, 31.04, 29.14, 25.31, 22.14, 18.47, 13.96. MS (ESI) m/z : calcd for $\text{C}_{18}\text{H}_{27}\text{NO}_4$, 322.1932 (100%, (M + H $^+$)).

(*S*)-2-(4-Hexyloxy-benzoylamino)-3-phenyl-propionic Acid (**7b**). The procedure is similar to that applied for the preparation of **7a**.

White solid. ^1H NMR (CDCl_3): δ = 10.98 (s, 1H), 7.89–8.01 (m, 2H), 6.95–7.21 (m, 7H), 4.83–4.85 (m, 11H), 3.89–3.92 (m, 2H), 3.01–3.03 (m, 2H), 0.95–1.61 (m, 12H). ^{13}C NMR (CDCl_3): δ = 176.15, 168.13, 161.14, 140.03, 129.18, 128.82, 123.52, 123.42, 115.22, 73.34, 61.56, 38.78, 33.14, 31.42, 26.14. MS (ESI) m/z : calcd for $\text{C}_{23}\text{H}_{30}\text{NO}_4$, 385.2221 (100%, (M + H $^+$)).

(*S*)-2-(4-Hexyloxy-benzoylamino)-3-(3H-imidazol-4-yl)-propionic Acid (**7c**). The procedure is similar to that applied for the preparation of **7a**.

Yellow solid. ^1H NMR (CDCl_3): δ = 7.93 (s, 1H), 7.63–7.65 (m, 2H), 6.75–6.92 (m, 4H), 4.69–4.72 (m, 2H), 4.12–3.92 (m, 2H), 2.67–2.78 (m, 2H), 0.76–1.52 (m, 11H). ^{13}C NMR (CDCl_3): δ = 174.75, 169.70, 161.08, 132.04, 131.03, 129.92, 125.75, 121.56, 111.04, 68.13, 52.67, 31.14, 29.57, 29.15, 25.56, 22.59, 13.82. MS (ESI) m/z : calcd for $\text{C}_{19}\text{H}_{25}\text{N}_3\text{O}_4$, 360.1834 (100%, (M + H $^+$)).

(*S*)-2-(4-Hexyloxy-benzoylamino)-3-hydroxy-propionic Acid (**7d**). The procedure is similar to that applied for the preparation of **7a**.

White solid. ^1H NMR (DMSO- d_6): δ = 7.68 (s, 2H), 6.92–6.96 (m, 2H), 3.92–4.54 (m, 6H), 1.20–1.95 (m, 9H), 0.89–0.92 (m, 3H). ^{13}C NMR (DMSO- d_6): δ = 173.32, 166.74, 161.44, 128.45, 125.18, 111.14, 68.84, 62.30, 56.14, 31.12, 29.11, 24.13, 22.59, 14.13. MS (ESI) m/z : calcd for $\text{C}_{16}\text{H}_{23}\text{NO}_5$, 310.1667 (100%, (M + H $^+$)).

(4-Hexyloxy-benzoylamino)-acetic Acid (**7e**). The procedure is similar to that applied for the preparation of **7a**.

White solid. ^1H NMR (DMSO- d_6): δ = 7.68 (s, 2H), 6.98 (s, 2H), 4.10–4.12 (m, 2H), 3.78 (m, 2H), 1.49–1.76 (m, 8H), 0.89–0.92 (m, 3H). ^{13}C NMR (DMSO- d_6): δ = 172.31, 168.28, 161.02, 130.06, 114.55, 114.53, 66.42, 42.85, 34.56, 32.12, 29.14, 23.12, 21.45, 14.14. MS (ESI) m/z : calcd for $\text{C}_{15}\text{H}_{21}\text{NO}_4$, 280.1511 (100%, (M + H $^+$)).

(*S*)-2-(4-Methoxy-benzoylamino)-3-methyl-butyric Acid (**7f**). The procedure is similar to that applied for the preparation of **7a**.

White solid. ^1H NMR (DMSO- d_6): δ = 7.53–7.55 (m, 2H), 6.95–6.97 (m, 2H), 6.05 (s, 1H), 4.75–4.77 (m, 1H), 3.72 (s, 3H), 1.75–1.87 (m, 1H), 1.04–1.06 (m, 6H). ^{13}C NMR (DMSO- d_6): δ = 172.33, 167.28, 160.14, 131.12, 127.30, 110.15, 58.66, 55.65, 30.93, 19.03. MS (ESI) m/z : calcd for $\text{C}_{13}\text{H}_{17}\text{NO}_4$, 251.1232 (100%, (M + H $^+$)).

(*S*)-2-(4-Ethoxy-benzoylamino)-3-methyl-butyric Acid (**7g**). The procedure is similar to that applied for the preparation of **7a**.

White solid. ^1H NMR (DMSO- d_6): δ = 7.59 (s, 2H), 7.02–7.05 (m, 2H), 4.16–4.40 (m, 3H), 1.87–1.92 (m, 1H), 1.32–1.36 (m, 3H), 0.98–1.03 (m, 6H). ^{13}C NMR (DMSO- d_6): δ = 173.15, 167.10, 160.83, 129.98, 127.04, 113.21, 61.89, 57.93, 29.98, 19.19, 14.31. MS (ESI) m/z : calcd for $\text{C}_{14}\text{H}_{19}\text{NO}_4$, 266.1342 (100%, (M + H $^+$)).

(*S*)-3-Methyl-2-(4-propoxy-benzoylamino)-butyric Acid (**7h**). The procedure is similar to that applied for the preparation of **7a**.

White solid. ^1H NMR (DMSO- d_6): δ = 6.95–7.32 (m, 4H), 6.75 (s, 1H), 3.98–4.05 (m, 3H), 1.54–1.89 (m, 3H), 1.12–1.17 (m, 3H), 0.97–1.02 (m, 6H). ^{13}C NMR (DMSO- d_6): δ = 172.34, 167.42, 160.15, 128.64, 127.42, 112.45, 69.37, 58.62, 29.89, 21.12, 18.82, 10.29. MS (ESI) m/z : calcd for $\text{C}_{15}\text{H}_{21}\text{NO}_4$, 280.1534 (100%, (M + H $^+$)).

(4-(Heptyloxy)benzoyl)-*L*-valine (**7i**). White solid. ^1H NMR (DMSO- d_6): δ = 0.93–1.41 (m, 19H), 1.41–1.46 (m, 2H), 2.23–2.35 (m, 1H), 3.87–3.92 (t, J = 5 Hz, 2H), 4.46–4.69 (m, 1H), 6.82–6.89 (m, 2H), 7.66–7.69 (m, 2H). ^{13}C NMR (DMSO- d_6): δ = 175.24, 167.12, 162.78, 129.02, 114.39, 68.28, 57.84, 31.80, 31.27, 29.14, 29.07, 25.98, 22.64, 19.15, 17.92, 14.12. MS (ESI) m/z : calcd for $\text{C}_{19}\text{H}_{29}\text{NO}_4$, 336.2165 (100%, (M + H $^+$)).

(4-(Octyloxy)benzoyl)-*L*-valine (**7j**). White solid. ^1H NMR (DMSO- d_6): δ = 0.91–1.34 (m, 10H), 1.38–1.54 (m, 11H), 1.76–1.87 (m, 2H), 2.23–2.37 (m, 1H), 4.03–4.08 (t, J = 5 Hz, 2H), 4.48–4.52 (dd, J = 5 Hz, 1H), 6.98–7.01 (m, 2H), 7.83–8.86 (m, 2H). ^{13}C NMR (DMSO- d_6): δ = 175.31, 170.16, 163.59, 130.46,

127.28, 115.21, 69.24, 59.88, 33.04, 31.82, 30.53, 30.46, 30.34, 27.19, 23.77, 19.79, 18.97, 14.49. MS (ESI) m/z : calcd for $C_{20}H_{31}NO_4$, 350.2215 (100%, (M + H⁺)).

(4-(Decyloxy)benzoyl)-L-valine (7k). White solid. ¹H NMR (DMSO-*d*₆): δ = 0.92–1.32 (m, 10H), 1.44–1.54 (m, 15H), 1.82–1.88 (m, 2H), 2.33–2.46 (m, 1H), 3.94–4.06 (m, 2H), 4.82–4.87 (m, 1H), 6.86–6.94 (m, 2H), 7.73–7.83 (m, 2H). ¹³C NMR (DMSO-*d*₆): δ = 175.36, 167.71, 162.20, 129.31, 129.10, 125.71, 114.33, 114.15, 68.25, 57.62, 31.94, 31.43, 29.61, 29.43, 29.37, 29.16, 26.03, 22.73, 19.09, 17.91, 14.18. MS (ESI) m/z : calcd for $C_{22}H_{35}NO_4$, 378.2645 (100%, (M + H⁺)).

Chemicals. Rosiglitazone, bezafibrate, and roscovitine were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). GW 7647 was from Axon Medchem (Groningen, The Netherlands). GW 9662 and SR 11235 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Antibodies. PPAR γ antibody (E-8) was from Santa Cruz Biotechnology. PPAR γ phospho Ser273 antibodies were from Rockland (Limerick, PA, USA) or custom produced by New England Peptide (Gardner, MA, USA). UCP1 antibodies (ab10983) were from Abcam (Cambridge, MA, USA). β -actin (13E5) was from Cell Signaling Technology (Danvers, MA, USA).

Cell Culture. Media and cell culture reagents were from Thermo Fisher Scientific (Illkirch-Grattenstaden, France). HEK293 cells (GripTite 293 MSR) were from Thermo Fisher Scientific. 3T3-L1 cells (from ATCC) were routinely cultured in DMEM with 4 mM L-glutamine, 4.5 g L⁻¹ glucose, and 0.11 g L⁻¹ sodium pyruvate and supplemented with 10% fetal bovine serum and antibiotics. Two days after confluence, adipocyte differentiation was triggered by changing the conventional induction mixture (0.1 μ M dexamethasone, 500 μ M 3-isobutyl-1-methylxanthine, and 174.5 nM insulin from Sigma-Aldrich, L'Isle d'Abeau Chesnes, France). After 48 h, the medium was removed and replaced by a fresh medium containing only 174.5 nM insulin. HuH7 hepatoma cells from the Japanese Cancer Research Resources Bank were cultured in DMEM containing 10% fetal bovine serum (FBS). At the confluence, cells were treated for 24 h with 0.25 mM oleic acid (Sigma-Aldrich) complexed with BSA. The establishment, characterization, and culture protocols of human multipotent adipose-derived stem (hMADS) cells have been described previously.⁴² Briefly, confluent cells were submitted to differentiation medium (DMEM/Ham's F12 media containing 10 μ g mL⁻¹ transferrin, 10 nM insulin, and 0.2 nM triiodothyronine from Sigma-Aldrich) supplemented with 1 μ M dexamethasone and 500 μ M isobutyl-methylxanthine. Two days later, the medium was changed, dexamethasone and isobutyl-methylxanthine were omitted, and 100 nM Rosi (Sigma-Aldrich) was added for the indicated periods. Cells were treated between days 2 and 9 with Rosi to enable white adipocyte differentiation to take place. After 5 days in the absence of Rosi, brite adipocyte conversion was induced by adding compounds to be tested (day 14). The medium was changed every other day, and cells were used at day 18. Pooled plateable cryopreserved primary rat hepatocytes (Male, Sprague–Dawley) from XenoTech (Kansas city, KS, USA) were plated and cultured as described by the manufacturer.

Cell Viability, Toxicity, and Apoptosis Assay. Cell viability was monitored using the CellTiter-Blue cell viability assay (Promega, Madison, WI) based on the ability of living cells to convert resazurin into the fluorescent end product resorufin and by measuring cellular ATP levels, which decline rapidly when cells undergo necrosis or apoptosis, using an ATP-Lite Istep luminescence assay system (PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions. Cytotoxicity was determined by measuring the lactate dehydrogenase (LDH) released into the culture medium upon plasma membrane damage using the LDH-Glo cytotoxicity assay (Promega). Apoptosis was determined by measuring caspase 3/7-dependent cleavage of a luminogenic substrate using the Caspase-Glo 3/7 Assay (Promega). Experiments were performed two to three times in triplicate.

Cellular Lipid Content Staining and Measurement. *Oil Red O staining.* 3T3-L1 cells, HuH7 hepatoma cells, or primary rat hepatocytes were washed with PBS and fixed with 4% formaldehyde

for 20 min, then washed again, and stained with 0.35% Oil Red O solution (Sigma-Aldrich) in 60% isopropanol for 20 min. Then, cells were washed with water, and photographs were taken. The stain from the cells was eluted using 100% isopropanol, and the absorbance of the eluted stain was read at 490 nm. Experiments were performed five times in duplicate for cell lines and twice in triplicate for primary cells.

AdipoRed assay. 3T3-L1 preadipocytes were cultured in a black 96-well plate. Two days post-confluency, cells were incubated with the test molecules (10 μ M) for 7 additional days. Cells were washed with PBS, and then a solution of AdipoRed reagent (1/40 in PBS) was added into each well. After 10 min at room temperature and in the dark, fluorescence was measured at 485 and 572 nm for excitation and emission, respectively, on an EnSight microplate reader (PerkinElmer) in the scanning mode. Since this technique was used for screening purposes, the experiment was performed once in duplicate.

2-Deoxy-D-glucose Uptake Assay. Glucose uptake activity of fully differentiated 3T3-L1 adipocytes was measured by the chemiluminescent assay⁴³ using a Glucofax kit as described by the manufacturer (Yelen, Ensues la Redonne, France). Briefly, 3T3-L1 adipocytes (80% of the cells displaying the characteristic lipid-filled phenotype) were gently detached from the plate by Accutase (Thermo Fisher Scientific) treatment, seeded (3.5×10^4 cells well⁻¹) at confluence in 96-well culture dishes, and cultured for 2 more days in DMEM supplemented with 10% FBS. Adipocytes were then incubated in glucose-free DMEM for 4 h, then washed twice with Krebs-Ringer-phosphate-Hepes (KRPH) buffer (20 mM Hepes, 5 mM KH₂PO₄, 1 mM MgSO₄, 1 mM CaCl₂, 136 mM NaCl, and 4.7 mM KCl at pH 7.4) containing 0.2% BSA, and incubated 30 min in 100 μ L of KRPH/BSA. KRPH buffer was removed, and cells were incubated for 20 min with 170 μ L of 100 nM insulin diluted in KRPH buffer; then, 19 μ L of 10 mM 2-deoxy-D-glucose (Sigma-Aldrich) was added and the cells were incubated for 20 min. Cells were then washed four times with cold PBS and lysed with 60 μ L of reagent I of the Glucofax kit. After 60 min of incubation at 37 °C, 20 μ L of cell lysates was collected and transferred into a white 96-well plate. Then, 100 μ L of reagent II was added, and after 10 min of incubation, the chemiluminescence was recorded on the EnSight multimode reader (PerkinElmer). Experiments were performed three times in sextuplet.

Real-Time PCR Analysis. Total RNA was extracted using a NucleoSpin RNA kit (Macherey-Nagel, Hoerdt, France), and cDNA was synthesized from 0.5 μ g of RNA using Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific) and used for PCR amplification. Real-time PCR (RT-PCR) was performed on the LightCycler 480 instrument (Roche Applied Science, Basel, Switzerland) using the EvaGreen master mix (Euromedex, Souffelweyersheim, France). The comparative Ct method ($2^{-(\Delta\Delta Ct)}$) was used to calculate the relative differences in mRNA expression. The acidic ribosomal phosphoprotein P0 (*Rplp0*) was used as the housekeeping gene. Changes were normalized to the mean of control values, which was set to 1. Primers were synthesized by Eurogentec (Seraing, Belgium), and their sequences were previously published.^{17,36,42} Experiments were performed five times in duplicate.

MitoTracker Staining. 3T3-L1 adipocytes were trypsinized and centrifuged at 300g at 4 °C for 5 min. Cells were suspended in KRPH containing 0.5% BSA and incubated with 0.1 μ M MitoTracker Green FM (Thermo Fisher Scientific) for 30 min at 37 °C. Cells were spun at 300g at 4 °C for 5 min and suspended in 400 μ L of fresh KRPH; then, 50,000 cells were analyzed using a BD Accuri C6 flow cytometer (BD Biosciences). Experiments were performed three times in triplicate.

Cell-Based PPAR and RXR α Transactivation Assay. The PPAR γ -LBD-*Gal4* or PPAR α -LBD-*Gal4* expression vector (given by Dr. Teruo Kawada, Kyoto University, Japan) was transfected along with the SV40-driven Renilla luciferase expression vector in HEK293 cells stably expressing the Gal4 response element-driven Firefly luciferase reporter (pGL4.35[luc2P/9XGAL4UAS/Hygro]) vector from Promega, Madison, WI, USA). Thirty-six hours after trans-

fection, cells were exposed to the tested compounds for additional 16 h; then, Firefly and Renilla luciferase activities were measured in the cell lysates using the reagents Genofax A and C (Yelen) in an EnSight multimode reader (PerkinElmer). PPAR transactivation activity of the compounds is calculated as the ratio of Firefly to Renilla luciferase activity. Experiments were performed at least three times in duplicate (allowing the calculation of EC_{50} and maximal activation, Table 1), and a representative experiment is shown in Figure 3 and Figure S5. For the measure of PPAR δ transactivation, HGSLN-GAL-PPAR δ reporter cell line was previously described.⁴⁴ Reporter cells were seeded at a density of 20,000 cells well⁻¹ in 96-well white opaque tissue culture plates and maintained in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% dextran-coated, charcoal-treated fetal calf serum. Twenty-four later, the culture medium was replaced with DMEM containing tested compounds. After 16 h, exposure media were replaced with media containing 0.3 mM luciferin. Luminescence was measured in intact living cells for 2 s in a MicroBeta Wallac luminometer (PerkinElmer). Experiments were performed twice in duplicate. The measurement of RXR α transactivation was performed as described for PPAR γ except that the RXR α -LBD-Gal4 expression vector was transfected.

Mammalian Two-Hybrid Assay. HEK293 cells stably expressing the GAL4 response element-driven Firefly luciferase reporter were transfected with the SV40-driven Renilla luciferase expression vector together with the expression vector of a fusion protein of the VP16 activation domain to PPAR γ -LBD and with the expression vector of a fusion protein of the GAL4-DNA binding domain to NCoR1, SMRT, TIF2, MED1, or RXR α . Then, cells were treated as described for the cell-based PPAR transactivation assay. Experiments were performed two to three times in duplicate.

Immunoblot. 3T3-L1 adipocytes were incubated 24 h with 1% SVF and then treated with PPAR γ agonists for 60 min followed by 90 min of stimulation with murine TNF (50 ng mL⁻¹). Cells were lysed in the presence of a cocktail of protease and phosphatase inhibitor. Identical amounts of total protein were heat-denatured and reduced (70 °C; 10 min), then submitted to SDS-PAGE separation on 4–12% gradient (Thermo Fisher Scientific), and transferred to polyvinylidene fluoride membranes. Membranes were blocked for 1 h in 5% BSA solution and incubated with the appropriate primary and HRP-conjugated secondary antibodies (1/1000 and 1/10,000 dilutions, respectively). Immunodetections were performed using the ECL reagent, and image acquisition was performed by using a chemiluminescent CCD imager ImageQuant LAS 4000 (GE Healthcare, Velizy-Villacoublay, France).

Measure of *In Vitro* PPAR γ Phosphorylation. *In vitro* phosphorylation was performed on wild-type PPAR γ in the apo form and in complex with 7j and Rosi. Stock solutions of ligands were prepared by diluting with 100% DMSO to a concentration of 50 mM. The stock solutions were further diluted with 50 mM Tris-HCl (pH 7.5) up to the final concentrations of 0.1, 1, and 10 μ M and pre-equilibrated overnight at 4 °C with the protein. Phosphorylation was carried out at 30 °C for 3.5 h in 300 μ L of buffer containing 50 mM Tris-HCl (pH 7.5), 7.2 μ g mL⁻¹ PPAR γ , 0.1 μ M, 1 μ M, 10 μ M ligand, 25 mM MgCl₂, 50 μ M DTT, 2 mM ATP, and 0.66 ng mL⁻¹ CDK5/p35 (Sigma-Aldrich code no. SRP5011). Then, polystyrene microwell plates were coated overnight at 4 °C with the reaction mixture, then washed three times with PBS + Tween 0.005%, and left to block in PBS containing 1% bovine serum for 90 min at 37 °C. The wells were washed three times and incubated for 60 min at 37 °C with 100 μ L of antiphospho-Ser/The-Pro antibody (SigmaAldrich code no. A05368) diluted 1/500 in PBS. After three washes, 100 μ L of antimouse IgG-peroxidase antibody produced in goat (Sigma Aldrich code no. A4416; 1/1000 in PBS) were added to the wells and incubated 60 min at 37 °C. The wells were washed, and 200 μ L of *o*-phenylenediamine dihydrochloride (SIGMAFAST OPD code no. P9187) dissolved in water was added to the wells. Optical density was measured at 450 nm using an ApplyScan Thermo Fisher reader, and the data were processed using Excel.

Experiments were performed three times in triplicates.

Protein Expression and Purification. PPAR γ LBD was expressed as N-terminal His-tagged proteins using a pET28 vector and purified as previously described.⁴⁵ Briefly, freshly transformed *Escherichia coli* BL21 DE3 were grown in the LB medium with 30 μ g of kanamycin mL⁻¹ at 310 K to an OD of 0.6. The culture was then induced with 0.2 mM isopropyl- β -D-thio-galactopyranoside and further incubated at 291 K for 20 h. Cells were harvested and resuspended in a 20 mL L⁻¹ culture of buffer A (20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1 mM Tris 2-carboxyethylphosphine HCL (TCEP), pH 8) in the presence of protease inhibitors (cComplete Mini EDTA-free; Roche Applied Science). Cells were sonicated, and the soluble fraction was isolated by centrifugation (35,000g for 45 min). The supernatant was loaded onto a Ni²⁺-nitrilotriacetic acid column (GE Healthcare) and eluted with a gradient of imidazole 0–500 mM in buffer A (20 mM Tris-HCl, 20 mM NaCl, 10% glycerol, 1 mM TCEP, pH 8) (batch method). The pure protein was identified by SDS PAGE. The protein was then dialyzed over buffer A to remove imidazole, and it was cleaved with thrombin protease (Sigma-Aldrich Life Science) (10 units mg⁻¹) at room temperature for 2 h. The digested mixture was reloaded onto a Ni²⁺-nitrilotriacetic acid column to remove the His tag and the undigested protein. The flow-through was dialyzed with buffer B (20 mM Tris-HCl, 10% glycerol, 1 mM TCEP, pH 8) to remove NaCl, loaded onto a Q Sepharose HP column (GE Healthcare), and eluted with a gradient of NaCl 0–500 mM in buffer B with a BioLogic DuoFlow FPLC system (Bio-Rad Laboratories, Italy). Finally, the protein was purified by gel filtration chromatography on a HiLoad Superdex 75 column (GE Healthcare) and eluted with buffer C (20 mM Tris-HCl, 1 mM TCEP, 0.5 mM EDTA, pH 8). The protein was then concentrated at 8 mg mL⁻¹ using Amicon centrifugal concentrators with a 10 kDa cutoff membrane (Millipore, USA).

Crystallization and Data Collection. Crystals of apo-PPAR γ were obtained by vapor diffusion at 18 °C using a sitting drop made by mixing 2 μ L of protein solution with 2 μ L of reservoir solution (0.8 M Na citrate, 0.15 M Tris, pH 8.0). The crystals were soaked for 3 days in a storage solution (1.2 M Na citrate, 0.15 M Tris, pH 8.0) containing the ligand 7j or 7a (0.5 mM). The ligand dissolved in DMSO (50 mM) was diluted in the storage solution so that the final concentration of DMSO was 1%. The storage solution with glycerol 20% (v/v) was used as the cryoprotectant. Crystals (0.15 \times 0.15 mm) of PPAR γ /7j and PPAR γ /7a belong to the space group C2 with cell parameters shown in Table S1.

Structure Determination and Refinement. X-ray data sets were collected at 100 K under a nitrogen stream using synchrotron radiation (beamline ID30B at ESRF, Grenoble, France). The collected data were processed using the programs Mosflm and Scala.⁴⁶ Structure solution was performed with AMoRe,⁴⁷ using the coordinates of PPAR γ /LT175R (27) (PDB code: 3D6D) as the starting model. The coordinates were then refined with CNS⁴⁸ and with PHENIX⁴⁹ including data between 58.2 and 2.0 Å for PPAR γ /7j (57.4–1.8 Å for PPAR γ /7a). The statistics of crystallographic data and refinement is summarized in Table S1. The coordinates and structure factors described here have been deposited in the PDB under accession numbers 6QJ5 and 6ZLY for PPAR γ /7j and PPAR γ /7a, respectively.

Surface Plasmon Resonance. Surface plasmon resonance analyses were performed by using a Pioneer AE optical biosensor equipped with COOH5 chips (SensiQ). PPAR γ surfaces were prepared by using standard amine-coupling procedures⁵⁰ and HBS (Hepes-buffered saline: 10 mM Hepes, 150 mM NaCl, 0.005% P20, DMSO 1%, pH 7.4) as the running buffer. Flow cells were activated for 7 min by injecting 140 μ L of 50 mM N-hydroxysuccinimide (NHS)/200 mM ethyl-3-(3-dimethylamino) propylcarbodiimide (EDC). A 0.25 mg mL⁻¹ PPAR γ solution (150 μ L, in 10 mM NaOAc, pH 5.0) was injected for 15 min at 10 μ L min⁻¹ on channels 1 and 3 (channel 2 was used as the reference, for a duplicate experiment) followed by a 70 μ L injection of ethanolamine to block any remaining activated groups on the surface. RU (11,320 and 11,220) of protein was immobilized on channels 1 and 3,

respectively. The screening of the analytes was performed using HBS, with 1% DMSO. To collect details, a dilution protocol was used, injecting different concentrations of the analytes at a flow rate of 50 $\mu\text{L min}^{-1}$ over the two channels at 20 °C (association phase of 60 s). A similar protocol was followed for the experiments with PPAR α , using a PCH chip (Pall ForteBio).

Four buffer blanks were injected for double referencing. The regeneration of the surfaces between binding cycles was not necessary because all the analytes dissociate quickly in the 120 s dissociation phase. A DMSO calibration plot was constructed (buffer sample containing 0–2% (vol/vol) DMSO) to correct for bulk refractive index shifts. All sensorgrams were processed by using double referencing. To obtain kinetic rate constants and affinity constants, the corrected response data were fit in the program QDAT. A kinetic analysis of each ligand/analyte interaction was obtained by fitting the response data to a 1/1 bimolecular interaction model. The equilibrium dissociation constant (K_d) was determined by the ratio of $k_{\text{off}}/k_{\text{on}}$.

Statistical Analyses. Statistical significance was estimated with one-way ANOVA followed by the Bonferroni or Dunnett post-hoc test, with the two-tailed *t*-test or with the *F*-test (statistical test is specified in figure legends) using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Differences with *p* values of less than 0.05 were considered statistically significant.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c01555>.

PDB data of the compounds (PDB)

NMR and HPLC spectra of **3a**, **7a**, and **7j**; X-ray crystal structures of **3a**, **7a**, **7j**, rosiglitazone and SR2067 in PPAR γ LBD; SPR sensorgrams of **3a**, **7a**, and **7j**; adipogenic effect of the compounds; PPAR γ , PPAR δ , and RXR α transactivation; PPAR γ /RXR α heterodimerization; toxicity of **7j**; statistics of crystallographic data and refinement (PDF)

Molecular formula string for **3a**, **7a**, and **7j** (CSV)

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Author Contributions

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Notes

The authors declare no competing financial interest.

Coordinates and structure factors of the PPAR γ complexes with the compounds **7j** and **7a** have been deposited in the Protein Data Bank under the accession codes 6QJ5 and 6ZLY, respectively. Authors will release the atomic coordinates and experimental data upon article publication.

Genes are identified by the symbols approved by the Human Genome Organization.

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■ ABBREVIATIONS

BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; CDK5, cyclin-dependent kinase 5; hMADS, human multipotent adipose-derived Stem; LBD, ligand binding domain; NAFLD, nonalcoholic fatty liver disease; PPRE, PPAR responsive element; Rosi, rosiglitazone; SPPAR γ M_s, selective PPAR γ modulators

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