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A novel N-substituted valine derivative with unique PPARγ binding properties and biological activities

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ABSTRACT

A proprietary library of novel N-aryl substituted amino acid derivatives bearing hydroxamate head group allowed the identification of compound 3a that possesses weak proadipogenic and PPARγ activating properties. The systematic optimization of 3a, in order to improve its PPARγ agonist activity, led to the synthesis of compound 7j (N-aryl substituted valine derivative) that possesses dual PPARγ / PPARα agonistic activity. Structural and kinetic analyses reveal that 7j occupies the typical ligand binding domain of the PPARγ agonists with, however, a unique high-affinity binding mode. Furthermore, 7j is highly effective in preventing CDK5-mediated phosphorylation of PPARγ 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 the palmitate-induced lipid accumulation in hepatoma cells. The unique biochemical properties and biological activities of compound 7j suggest its potential efficacy in reducing insulin resistance, obesity and non-alcoholic fatty liver disease.
**INTRODUCTION**

Peroxisome proliferator-activated receptors (PPAR) 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: PPARα, γ, and δ (β), have different, yet overlapping, tissue expression patterns \(^1\) 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.

PPARα 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 fibrates family of drugs (clofibrate and fenofibrate) are pharmacological weak agonists of PPARα that are used to treat dyslipidemia as they lower plasma triglycerides and increase HDL cholesterol levels \(^6\). The PPARα agonist fenofibrate has also beneficial effect in patient with non-alcoholic 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 PPARα agonists with improved clinical efficacy, Penafibrate being one of these new generation agonists \(^9\).

PPARδ is involved in the regulation of fatty acid oxidation and mitochondrial respiration predominantly in skeletal muscle, liver and adipose tissue \(^10\). Therefore, agonists targeting PPARδ may be considered as potential therapeutic agents for insulin-resistant related conditions. PPARδ agonists have been developed and used in research \(^11\) but none are currently approved for clinical use.
PPARγ 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 PPARγ 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 PPARγ dissociates the deleterious from the therapeutic effects of the agonist has led to the generation of selective PPARγ modulators (SPPARγMs) with higher therapeutic profiles than full agonists 15,16. The peculiar properties of SPPARγM being explained by the ability of PPARγ to adopt ligand-specific conformations with different transcriptional signatures. In addition, it has been shown that the clinical benefit of PPARγ partial agonists and SPPARγMs also involves their ability to inhibit the cyclin-dependent kinase 5 (CDK5)-mediated PPARγ 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. PPARα/γ dual agonists, so-called Glitazars, that combine the insulin sensitizing effect of PPARγ agonists with the beneficial effect of PPARα 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" 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 PPARs activity and led to the development of a new balanced and potent dual PPARα/γ agonist with unique ligand binding properties and singular biological activities that qualify it as a potential therapeutic candidate to reduce insulin resistance, obesity and NAFLD.
RESULTS AND DISCUSSION

New N-aryl substituted amino acid derivatives bearing 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-hydroxycarbamoyl-2-methyl-propyl)-benzamide), that contains a L-Valine (core) and a 6 carbon atoms chain (capping group) (Figure 1A and Supplemental Figure S1A and S1B), was identified as a weak activator of adipocyte differentiation as compared with Rosi (Supplemental Figure S2). We hypothesized that 3a increased adipocyte differentiation by activating the master regulator of adipogenesis: PPARγ. The PPARγ agonistic activity of 3a was confirmed by the use of a luminescence-based cell-based PPARγ transactivation assay, in which wild-type PPARγ ligand binding domain (PPARγ-LBD) is fused to the GAL4 DNA-binding domain (PPARγ-LBD-GAL4) and the Firefly luciferase reporter gene is under the control of GAL4 binding elements (Figure 1A). We initiated a process to identify the domains of this molecule involved in PPARγ activation that would ideally lead to an optimization of its PPARγ agonist activity.

Effect of the amino acid core. Analogues of the 3a hit were designed and synthesized through a reaction involving a two steps synthesis procedure in dichloromethane from amino acid methyl ester hydrochloride 1a-1h, easily prepared from amino acids. By using 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 hours in yields varying from 36 to
72% (Figure 1A). Changing the L-Valine core by any other amino acid, decreased the PPARγ activation potency of the molecule (Figure 1A) perhaps due to a steric hindrance more important generated by the isopropyl moiety with respect to a benzyl or a hydroxy methyl group, for example.

Figure 1. Chemical structures and PPARγ agonist activity of **3a** and its derivatives. The amino acid core (A), hydrophobic capping group (B) and binding head group (C) of **3a** were modified (left panels) and PPARγ transactivation activity of each molecule (5 μM) was measured (right panels). Color fills accentuates the amino acid core (gray), hydrophobic capping group (green) and binding head group (yellow) of **3a**. LogP of each molecule is shown in brackets below the chemical structure and is represented by a red dot in B. The percentage indicates the yield of
the synthesis. PPARγ transactivation values are means ± s.d (n = 3) expressed relative to the mean of control values, which was set to 1.

**Effect of the carbon atoms chain capping group.** The different derivatives 4a-4h were synthesized according 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 6 carbons chain was optimal for PPARγ activation (Figure 1B). This result suggests that the steric hindrance and/or the hydrophobic nature of the polycarbon chain are important factors to consider when designing most potent analogues. A correlation was observed between the LogP parameter of the molecules, which reflects the true behavior and bioavailability of an ionizable compound in a solution at a physiological pH, and their ability to transactivate PPARγ (Figure 1B). Indeed, derivatives displaying a LogP superior to 2.5 (i.e., the most hydrophobic derivatives 3a, 4e - 4g) also displayed higher PPARγ activation whereas low activities were encountered for compounds 4a - 4d presenting low LogP values varying from 1.21 to 2.45. However, it is unlikely that the increase in PPARγ transactivation related to the extension of the polycarbon chain results solely from the increased LogP of the molecules. Indeed, beyond 6 carbons atoms LogP continued to increase while the transactivation activity decreased.

**Effect of the head group.** Substitution of the hydroxamate head moiety in 3a (Figure 1C) revealed that the preferred functions for PPARγ activation are OH > NHOH ≥ NH₂ > COOMe (Figure 1C). The analog of 3a, with a carboxylic acid moiety in place of hydroxamate head group that optimally activated PPARγ was named 7a.

**Optimization of PPARγ agonist activity of 7a.** Changing the absolute conformation of the core Valine from L to D abolished the PPARγ 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 PPARγ activation efficiency of 7a (Figure 2B). The PPARγ transactivation activity of 7a was gradually enhanced by the extension of the polycarbon chain up to 8 carbon atoms (Figure 2C). The analog of 7a with the 8 carbon atoms chain in place of the 6 carbon atoms chain which optimally activates PPARγ was named 7j (Supplemental Figure S1C and S1D). Of note, the presence of a chain with 10 carbon atoms in the molecule 7k did not further enhance its ability to activate PPARγ while the LogP value is increased.

Figure 2. Chemical structures and PPARγ agonist activity of 7a and its derivatives. The absolute conformation of the valine core (A), the amino acid core (B) or the hydrophobic capping group (B and C) of 7a were modified (left panels) and PPARγ
transactivation activity of each molecule (5 μM) was measured (right panels). LogP is shown in brackets below the chemical structure and represented by a red dot in C. The percentage indicates the yield of the synthesis. PPARγ transactivations are expressed as in figure1. 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. Interestingly, 7a and 7j closely meet these elementary criteria.

**Potency and efficacy of PPARγ activation.** The dose-dependent activation of PPARγ by the "hit" compound 3a and its two "lead" derivatives 7a and 7j were compared to that of the PPARγ full agonist Rosi using PPARγ-LBD-GAL4 chimera assay (Figure 3A).

**Figure 3.** PPAR agonist activity of 3a, 7a and 7j. (A) Concentration-dependent PPARγ transactivation activities of 3a, 7a and 7j were compared to that of Rosi using PPARγ-LBD-GAL4 chimera assay. Values are expressed as % of the maximal response measured with Rosi (5 μM). (B) HEK293 cells were transfected with PPRE-driven Firefly luciferase and SV40-driven Renilla luciferase coding vectors together with an empty plasmid (-PPARγ) or with the PPARγ expression vector (+PPARγ). 36 hours post transfection, cells were incubated for 17 hours with 3a, 7a, 7j (1 μM) or Rosi (0.1 μM). PPRE promoter activity was calculated as the ratio Firefly/Renilla luciferase. Values are means ± s.d (n = 5) expressed relative to the control situation. *p < 0.05, **p < 0.01 (one-way ANOVA followed by Bonferroni’s post hoc test). (C) Concentration-dependent PPARγ transactivation activities of 7j, GW 7647 and Bezafibrate were measured using PPARγ-LBD-GAL4 chimera assay. Values are expressed as % of the maximal response measured with GW 7647 (1 μM).
The potencies of the compounds were ranked as follow: $3a \approx 7a < 7j < \text{Rosi}$ (Table 1). Regarding their efficacy (maximal PPAR$\gamma$ transactivation) compounds were ranked as follow: $3a < 7a < 7j \leq \text{Rosi}$ (Table 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>PPAR</th>
<th>EC$_{50}$ (nM)</th>
<th>Activity (%)</th>
</tr>
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<tbody>
<tr>
<td>Rosi</td>
<td>$\gamma$</td>
<td>$76 \pm 30$</td>
<td>100</td>
</tr>
<tr>
<td>7j</td>
<td>$\gamma$</td>
<td>$400 \pm 150$</td>
<td>$66.36 \pm 3.41$</td>
</tr>
<tr>
<td>7a</td>
<td>$\gamma$</td>
<td>$1940 \pm 940$</td>
<td>$25.06 \pm 2.03$</td>
</tr>
<tr>
<td>3a</td>
<td>$\gamma$</td>
<td>$1840 \pm 1020$</td>
<td>$6.33 \pm 1.03$</td>
</tr>
<tr>
<td>GW 7647</td>
<td>$\alpha$</td>
<td>$5.4 \pm 1$</td>
<td>100</td>
</tr>
<tr>
<td>7j</td>
<td>$\alpha$</td>
<td>$2910 \pm 920$</td>
<td>$98.2 \pm 5.53$</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>$\alpha$</td>
<td>$31700 \pm 5410$</td>
<td>$35.4 \pm 3.81$</td>
</tr>
</tbody>
</table>

**Table 1.** EC$_{50}$ and maximal activation of PPAR$\gamma$ and PPAR$\alpha$. Activities are expressed as % of the maximal response measured with 5 $\mu$M of Rosi (for PPAR$\gamma$) or with 1 $\mu$M of GW 7647 (for PPAR$\alpha$). Values are means $\pm$ s.d ($n = 3$).

PPAR$\gamma$ activation elicited by 7j was around 70% of that obtained with Rosi. However, 7j reduced the Renilla luciferase expression (Supplemental Figure S3A), used to normalize the values of the reporter gene for variations inherent to transfection efficiency and sample handling, making difficult to assess its true levels of PPAR$\gamma$ activation. Indeed, taking account of Renilla luciferase expression values to calculate PPAR$\gamma$ activation dose-response curves significantly increased the maximal response triggered by 7j (Supplemental Figure S3B). This side effect of 7j is not strictly related to a toxic effect as the reduction in Renilla luciferase expression occurred at lower concentrations than those causing reduction of cellular ATP content (Supplemental Figure S3C). Moreover, Rosi that poorly reduced Renilla luciferase expression was not less toxic than 7j (Supplemental Figure S3C). Taken together, these data
allow us to define 3a and 7a as partial agonists of PPARγ and 7j as a strong partial agonist of PPARγ.

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 PPARγ to PPRE. The three molecules increased the expression of PPRE-driven luciferase only when full-length PPARγ was expressed (Figure 3B), showing that these molecules stimulate the actual transcriptional activity of PPARγ. Interestingly, the transactivation efficiencies were in agreement with those measured by the PPARγ-LBD-GAL4 chimera assay, i.e. 3a ≤ 7a < 7j ≤ Rosi.

Some compounds with moderate PPARγ agonist activity (partial agonists or modulators) maintain significant anti-diabetic activity while having fewer and/or less severe adverse effects than full PPARγ agonists 27. Therefore, 3a, 7a and 7j can reasonably be considered as scaffold molecules to develop new anti-diabetic drugs.

**Specificity.** The dose-dependent activations of PPARδ and PPARα by 3a, 7a and 7j were analyzed using the appropriate PPAR-LBD-GAL4 chimere assays. None of the molecules activated PPARδ (Supplemental Figure S4). Only 7j transactivated PPARα (Figure 3C and Table 1), with potency between that of Bezafibrate (a weak pan agonist for all three PPAR isoforms) and GW 7647 (a full PPARα agonist). 7j appeared to be a full PPARα agonist. Therefore, 7j is a dual PPARγ/α agonist (Glitazar) and as such, it could theoretically have beneficial synergistic activities on glucose and lipid homeostasis 19,20.

**Binding of 7j to PPARγ.** The crystal structure of the complex of PPARγ LBD with the ligand 7j was solved collecting diffraction data from apo-crystals soaked for three days in the presence of the ligand at 0.5 mM. Initial difference Fourier maps revealed clear electron density for the ligand (Supplemental Figure S5) showing that it occupies the typical LBD region of the PPARγ agonists (Figure 4A), similar to that of the full agonist Rosi (Figure 4B), with its
carboxylate group directly interacting through H-bonds with Y473 of helix 12 (H12), at the short distance of 2.3 Å, the two histidines (H323 and H449) of the canonical triad, and S289, (Figure 4A). The NH of 7j amide bond is also involved in a 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, respectively) and makes vdW contacts with several residues of the internal strand of the β-sheet, as shown in Figure 4A, 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).

Figure 4. Binding of 7j to PPARγ. (A) Hydrogen-bond network of 7j in the PPARγ LBD. (B) Superimposition of 7j (yellow) and Rosi (green) structures. (C) vdW network of the 7j with residues belonging to H3, H11 and H12 of the PPARγ LBD. (D) Superimposition of 7j (yellow) and Rosi (green) with the co-activator SRC-1 (magenta); the PPARγ / Rosi structure is colored in green, PPARγ / 7j in gray.

The thorough analysis of the effective binding network of 7j into the PPARγ 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 H12, the loop 11/12 and the beginning of H11 (Figure 4D), with respect to Rosi, that could affect the binding of co-activator, possibly determining the partial agonism of 7j.

Interestingly, a similar unique binding network was observed in the PPARγ crystal structure with the partial agonist SR2067 (Supplemental Figure S6). 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.

It is commonly accepted that a PPARγ partial agonist is likely to have anti-diabetic provided that it combines low transactivation activity with high binding affinity. Therefore, the affinity (K_d) and rate constants (k_on, k_off) of PPARγ for the partial agonist 7j were compared with those of PPARγ for the reference ligand Rosi using surface plasmon resonance (Table 2 and Supplemental Figure S7).

<table>
<thead>
<tr>
<th>Interaction</th>
<th>k_on (M⁻¹s⁻¹)</th>
<th>k_off (s⁻¹)</th>
<th>K_d (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ/Rosi</td>
<td>4.1 (0.4) 10⁵</td>
<td>0.102 (0.005)</td>
<td>250 (30)</td>
</tr>
<tr>
<td>PPARγ/7j</td>
<td>2.9 (0.2) 10⁵</td>
<td>0.044 (0.001)</td>
<td>150 (10)</td>
</tr>
</tbody>
</table>

Table 2. Affinity (K_d) and rate constants (k_on, k_off) for PPARγ/ligand interactions. Experimental error is reported in brackets.

Remarkably, 7j shows an affinity towards PPARγ similar or higher than Rosi. These results confirm that the partial agonism of 7j can be the result of a unique high-affinity binding which, although similar to that of the full agonist Rosi, slightly alters the active conformation of AF2 surface, a necessary requirement for recruiting co-activators.

Moreover, it is known that partial agonists of PPARγ preferentially stabilize the β-sheet region of the LBD. In the PPARγ/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 play an important role in lowering the free energy of the binding.

**CDK5-mediated phosphorylation of PPARγ.** Part of the anti-diabetic effects of PPARγ partial agonists have been associated with their ability to prevent the CDK5-mediated phosphorylation of PPARγ serine 273 residue\(^{17,18}\). Therefore, we assessed the ability of \(7j\) to inhibit such a phosphorylation. An ELISA protocol was optimized to quantify the phosphorylation of PPARγ triggered *in vitro* by recombinant CDK5. \(7j\) prevented the phosphorylation of PPARγ serine 273 at least as efficiently as Rosi (Figure 5A).

**Figure 5.** Effect of \(7j\) on PPARγ phosphorylation. (A) Percentage of *in vitro* PPARγ Ser273 phosphorylation by CDK5 in the presence of 0.1 \(\mu\)M of \(7j\) or Rosi. (B) RT-PCR analysis of the expression levels of a selection of genes known to be regulated by CDK5-dependent phosphorylation of PPARγ. Values are means ± s.d (n = 5) expressed relative to the mean of control. **p < 0.01, ***p < 0.001 vs. control (one-way ANOVA followed by Dunnett’s *post hoc* test)

The ability of \(7j\) to prevent CDK5-mediated phosphorylation of PPARγ was also studied in 3T3-L1 adipocytes. Out of ten genes known to be significantly controlled by CDK5-dependent PPARγ phosphorylation in fully differentiated adipocytes\(^{17,18}\), seven were regulated in the same way by \(7j\) and Rosi (Figure 5B). 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 PPARγ serine 273 and to alter the expression of genes controlled by PPARγ phosphorylation. This feature supports a potential anti-diabetic effect of 7j.

**Adipocyte differentiation and glucose uptake.** Adipogenesis-mediated weight gain is a major side effect of PPARγ full agonists 30. Partial PPARγ 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 PPARγ agonists as the sole inducer of adipocyte differentiation. Only 7j and Rosi (the strongest PPARγ agonists) significantly increased the intracellular lipid content (Figure 6A and Supplemental Figure S8), 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. When 3T3-L1 fibroblast cells were primed for adipocyte differentiation using the conventional adipogenic cocktail (Insulin, Dexamethasone, IBMX) and then treated for 7 days with the 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 PPARγ (Figure 6B). As expected for partial PPARγ agonists, 3a, 7a and 7j have reduced proadipogenic properties compared with the full agonist Rosi.
Figure 6. Adipogenic effect of the compounds. (A) 3T3-L1 fibroblasts were incubated for 6 days with insulin (350 nM) and the indicated PPARγ agonists (1 μM). Intracellular lipids were stained with Red Oil O then colored lipids were quantified. (B) 3T3-L1 adipocyte differentiation was triggered by the standard mixture of inductors then cells were incubated for 7 days with 3a, 7a, 7j (1 μM) or Rosi (0.1 μM). The expression levels of adipogenesis-related genes were measured by RT-PCR. Values are means ± s.d. (n = 5) expressed as fold relative to untreated situation. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control (one-way ANOVA followed by Dunnett’s post hoc test).

It has been shown that the ability of PPARγ ligands to increase cellular glucose uptake is not necessarily related to their transactivation activity or proadipogenic potential 31. Therefore, glucose uptake was measured in fully differentiated 3T3-L1 adipocytes after an acute treatment with PPARγ agonists. The insulin-dependent glucose uptake was increased by a short-term treatment with 7j or Rosi (Figure 7), denoting that part of their insulin sensitizing effect is independent of their ability to increase adipocyte differentiation.

Figure 7. Adipocyte glucose uptake. 3T3-L1 adipocytes were treated with 7j (1 μM) or Rosi (0.1 μM) for 16 h prior insulin stimulation (50 nM; 10 min) and cellular glucose uptake was determined. Values are mean ± s.d. (n = 4) expressed as fold relative to the control situation. **p < 0.01, ***p < 0.001 vs. control (one-way ANOVA followed by Dunnett’s post hoc test).
**Adipocyte browning.** Brown fat is a target for anti-obesity and anti-diabetes experimental therapies that aim to increase energy expenditure \(^{32}\). Interestingly, strong PPAR\(\gamma\) agonists activate the “browning” of white adipose tissues \(^{33,34}\), suggesting that partial PPAR\(\gamma\) agonists that 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 \(7j\) increased mRNA levels of genes considered as brite/brown adipocyte markers (Figure 8A, B) \(^{35}\). Coherent with their adipocyte browning effect, Rosi and \(7j\) increased mRNA levels of additional genes involved in (or associated with) mitochondria biogenesis (Figure 8C) \(^{36}\) and in accordance these two molecules significantly increased the selective labeling of active mitochondria by the MitoTracker dye (Figure 8D).

We have previously shown that Rosi induced the conversion of hMADS white adipocytes into brite adipocytes as evidenced by the strong expression of UCP1 \(^{37}\). 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 mRNA 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 8E). Moreover, UCP1 mRNA levels were similarly increased by 300 nM of \(7j\) or 100 nM of Rosi (Figure 8F). The expression of the adipogenic marker Perilipin was not modified by Rosi or \(7j\) (Supplemental Figure S9A), whereas the expression of Adiponectin, a PPAR\(\gamma\)-responsive gene, was more efficiently increased by Rosi than by \(7j\) (Supplemental Figure S9B), which confirms the that the PPAR\(\gamma\) agonist activity of \(7j\) is lower than that of Rosi.
**Figure 8.** Adipocyte browning effect of the compounds. 3T3-L1 were treated as in Figure 6B then 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. (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. Values are mean ± s.d. (n = 5) expressed as fold relative to the control (cont) situation. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control (one-way ANOVA followed by Dunnett’s post hoc test).

These data show that **7j** represents a potential and novel therapy targeting adipose tissue based on its potency to activate PPAR pathways, in order to combat obesity and associated diseases via the recruitment and activation of thermogenic adipocytes.
**Hepatocytes lipid accumulation.** As PPARα agonists decrease hepatic steatosis, we compared the effect of 7j (a dual PPARα/γ agonist) to that of Rosi (PPARγ agonist) on the lipid accumulation into HuH7 hepatoma cells. Both compounds impeded the basal intracellular lipid accumulation, however only 7j efficiently prevented the palmitate-induced lipid accumulation (Figure 9), suggesting 7j as a promising compound in the treatment of NAFLD.

![Figure 9](image)

**CONCLUSION**

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 γ. Compound 7j occupies the typical LBD region of the PPARγ agonists with a unique high-affinity binding mode and efficiently prevents CDK5-mediated phosphorylation of PPARγ. While poorly proadipogenic, compound 7j increases adipocyte insulin-stimulated glucose uptake and efficiently promotes white-to-brown adipocyte conversion. In addition, compound 7j impedes the palmitate-induced lipid accumulation in hepatoma cells. The unique biochemical properties and in vitro biological activities of compound 7j suggest its potential effectiveness in reducing insulin resistance, obesity and NAFLD.
EXPERIMENTAL SECTION

1. 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). $^1$H NMR and $^{13}$C NMR spectra were recorded in CDCl$_3$ or DMSO-d6 on a Bruker AC 300 spectrometer working at 300 MHz and 75 MHz, respectively (the usual abbreviations are used: s: singlet, d: doublet, t: triplet, q: quadruplet, m: multiplet). Tetramethylsilane was used as internal standard. All chemical shifts are given in ppm. Purity of all the new compounds is up to 99% and has been evaluated by HPLC analysis (Agilent 1100, C18).

Typical procedure for the synthesis of amino acids methyl ester hydrochloride derivatives

**Synthesis of L-Valine methyl ester hydrochloride**

In a two necked round flask equipped with a condenser were 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 thionylchloride (4.7 $10^{-3}$ mol) were slowly added. After removal of the solvents, diethylether was added and the product precipitate 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; $^1$H NMR (D$_2$O): $\delta = 4.14$-$4.17$ (m, 1H), 3.80-$3.85$ (m, 3H), 2.50-$2.56$ (m, 1H), 1.12-$1.15$ (m, 6H). $^{13}$C (D$_2$O): $\delta = 168.91$, 58.57, 53.02, 29.95, 18.15.
Synthesis of (S)-2-(4-Hexyloxy-benzoylamino)-3-methyl-butyric acid methyl ester 6

In a two necked 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 CH$_2$Cl$_2$. The mixture was placed under stirring at room temperature for 24 hours. Water was added to allow phase separation. The bottom phase layer was washed with NaHCO$_3$ (10%) solution, dried over Na$_2$SO$_4$, filtered and concentrated in vacuo. After removal of the solvents, the crude residue was purified by chromatography on a silicagel column using CH$_2$Cl$_2$/Ethylacetate (1/1) eluent affording the expected product 6 in 85% yield.

White solid; $^1$H NMR (CDCl$_3$): $\delta$ = 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). $^{13}$C (CDCl$_3$): $\delta$ = 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) C$_{19}$H$_{29}$NO$_4$ m/z 336.2146 (100%, (M+H$^+$)).

Synthesis of 4-Hexyloxy-N-((S)-1-hydroxycarbamoyl-2-methyl-propyl)-benzamide 3a

In a 25 mL round flask were placed at room temperature 0.6 g of 6 (1.78 $10^{-3}$ mol) in 15 mL of ethanol. 2 mL of a hydroxylamine solution (40%) were subsequently added and the mixture was allowed to stir at reflux for 24 hours. After removal of the solvents, the crude residue was purified by chromatography on a silicagel column using petroleum ether/ethylacetate (1/1) then methanol/ethylacetate (1/1) as eluents affording the expected product 3a in 72% yield.

White solid; $^1$H NMR (DMSO d$_6$): $\delta$ = 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). $^{13}$C (DMSO d$_6$): $\delta$ = 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) C\textsubscript{18}H\textsubscript{28}N\textsubscript{2}O\textsubscript{4} m/z 337.2045 (100\%, (M+H\textsuperscript{+})).

4-Hexyloxy-N-[(5)-1-hydroxycarbamoyl-2-(3H-imidazol-4-yl)-ethyl]-benzamide \textbf{3b}

Procedure similar to that applied for the preparation of \textbf{3a}

Pale yellow solid; \textsuperscript{1}H NMR (DMSO d\textsubscript{6}): \(\delta = 7.52-7.89\) (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). \textsuperscript{13}C (DMSO d\textsubscript{6}): \(\delta = 174.19, 164.07, 162.03, 133.17, 129.03, 123.47, 118.22, 115.35, 67.56, 52.96, 30.99, 30.91, 29.05, 25.49, 22.54, 14.25\). MS (ESI) C\textsubscript{19}H\textsubscript{26}N\textsubscript{4}O\textsubscript{4} m/z 375.2014 (100\%, (M+H\textsuperscript{+})).

4-Hexyloxy-N-[(5)-1-hydroxycarbamoyl-2-{(1H-indol-2-yl)-ethyl}-benzamide \textbf{3c}

Procedure similar to that applied for the preparation of \textbf{3a}

White solid; \textsuperscript{1}H NMR (DMSO d\textsubscript{6}): \(\delta = 7.32-7.62\) (m, 3H), 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). \textsuperscript{13}C (DMSO d\textsubscript{6}): \(\delta = 172.22, 168.98, 161.25, 139.48, 131.05, 129.14, 126.29, 120.15, 118.78, 113.69, 110.36, 104.25, 71.24, 53.32, 30.21, 28.28, 25.14, 22.04, 14.13\). MS (ESI) C\textsubscript{24}H\textsubscript{29}N\textsubscript{3}O\textsubscript{4} m/z 424.2236 (100\%, (M+H\textsuperscript{+})).

4-Hexyloxy-N-((S)-2-hydroxy-1-hydroxycarbamoyl-ethyl)-benzamide \textbf{3d}

Procedure similar to that applied for the preparation of \textbf{3a}

White solid; \textsuperscript{1}H NMR (DMSO d\textsubscript{6}): \(\delta = 7.02-7.51\) (m, 4H), 3.62-4.20 (m, 5H), 1.31-1.82 (m, 9H), 0.89-0.92 (m, 3H). \textsuperscript{13}C (DMSO d\textsubscript{6}): \(\delta = 170.36, 168.24, 160.89, 130.14, 125.34, 113.47, 68.78, 61.24, 53.89, 31.01, 28.14, 25.98, 21.33, 13.88\). MS (ESI) C\textsubscript{16}H\textsubscript{24}N\textsubscript{2}O\textsubscript{5} m/z 325.1768 (100\%, (M+H\textsuperscript{+})).
4-Hexyloxy-N-((S)-1-hydroxycarbamoyl-2-phenyl-ethyl)-benzamide 3e

Procedure similar to that applied for the preparation of 3a

White solid; $^1$H NMR (DMSO d6): $\delta$ = 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). $^{13}$C (DMSO d6): $\delta$ = 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) C$_{22}$H$_{28}$N$_2$O$_4$ m/z 385.2057 (100%, (M+H$^+$)).

N-[(S)-2-{3,4-Dihydroxy-phenyl}-1-hydroxycarbamoyl-ethyl]-4-hexyloxy-benzamide 3f

Procedure similar to that applied for the preparation of 3a

White solid; $^1$H NMR (DMSO d6): $\delta$ = 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). $^{13}$C (DMSO d6): $\delta$ = 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) C$_{22}$H$_{28}$N$_2$O$_6$ m/z 417.1934 (100%, (M+H$^+$)).

4-Hexyloxy-N-((S)-1-hydroxycarbamoyl-4-methylsulfanyl-butyl)-benzamide 3g

Procedure similar to that applied for the preparation of 3a

White solid; $^1$H NMR (DMSO d6): $\delta$ = 6.91-7.69 (m, 5H), 1.17-2.74 (m, 20H), 0.87-0.88 (m, 3H). $^{13}$C (DMSO d6): $\delta$ = 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) C$_{19}$H$_{30}$N$_2$O$_4$S m/z 383.1935 (100%, (M+H$^+$)).

4-(hexyloxy)-N-((2S,3S)-3-hydroxy-1-(hydroxyamino)-1-oxobutan-2-yl)benzamide 3h

Procedure similar to that applied for the preparation of 3a
White solid; $^1$H NMR (DMSO d6): $\delta$ = 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); $^{13}$C (DMSO d6): $\delta$ = 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) C$_{17}$H$_{26}$N$_2$O$_5$ m/z 339.1879 (100%, (M+H$^+$)).

(S)-N-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)-4-methoxybenzamide 4a

White solid; $^1$H NMR (DMSO d6): $\delta$ = 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); $^{13}$C (DMSO d6): $\delta$ = 174.44, 170.42, 164.51, 130.95, 127.71, 115.17, 56.40, 52.96, 32.19, 20.12, 19.65. MS (ESI) C$_{13}$H$_{18}$N$_2$O$_4$ m/z 267.1386 (100%, (M+H$^+$)).

(S)-4-ethoxy-N-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)benzamide 4b

White solid; $^1$H NMR (DMSO d6): $\delta$ = 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 (DMSO d6): $\delta$ = 171.10, 166.47, 166.91, 132.01, 129.30, 114.59, 63.42, 56.95, 30.10, 18.64, 14.69. MS (ESI) C$_{14}$H$_{20}$N$_2$O$_4$ m/z 281.1412 (100%, (M+H$^+$)).

(S)-N-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)-4-propoxybenzamide 4c

White solid; $^1$H NMR (DMSO d6): $\delta$ = 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 (DMSO d6): $\delta$ = 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) C$_{15}$H$_{22}$N$_2$O$_4$ m/z 294.1634 (100%, (M+H$^+$)).

(S)-4-butoxy-N-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)benzamide 4d
White solid; $^1$H NMR (DMSO d6): $\delta = 0.90-0.98$ (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 (DMSO d6): $\delta = 171.49, 166.14, 166.16, 133.37, 130.18, 122.86, 69.16, 57.04, 30.74, 30.69, 18.48, 13.75$.

MS (ESI) C$_{16}$H$_{24}$N$_2$O$_4$ m/z 309.1701 (100%, (M+H$^+$)).

(S)-N-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)-4-(pentyloxy)benzamide 4e

White solid; $^1$H NMR (DMSO d6): $\delta = 0.90-0.98$ (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 (DMSO d6): $\delta = 169.71, 167.51, 162.83, 132.86, 126.86, 119.98, 68.72, 58.13, 31.14, 30.98, 29.34, 28.14, 18.54, 14.16$. MS (ESI) C$_{17}$H$_{26}$N$_2$O$_4$ m/z 323.1934 (100%, (M+H$^+$)).

(S)-4-(heptyloxy)-N-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)benzamide 4f

White solid; $^1$H NMR (DMSO d6): $\delta = 0.90-0.96$ (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 (DMSO d6): $\delta = 171.09, 166.42, 162.02, 130.07, 129.03, 115.18, 68.15, 57.63, 31.80, 29.18, 26.04, 22.14, 18.74, 13.98$. MS (ESI) C$_{19}$H$_{30}$N$_2$O$_4$ m/z 351.2245 (100%, (M+H$^+$)).

(S)-N-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)-4-(octyloxy)benzamide 4g

White solid; $^1$H NMR (DMSO d6): $\delta = 0.90-0.97$ (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 (DMSO d6): $\delta = 171.10, 166.35, 162.35, 131.37, 129.90, 115.11, 68.24, 57.52, 31.79, 30.95, 29.31, 26.82, 22.47, 18.63, 14.21$. MS (ESI) C$_{20}$H$_{32}$N$_2$O$_4$ m/z 365.2423 (100%, (M+H$^+$)).
Synthesis of (S)-2-(4-Hexyloxy-benzoylamino)-3-methyl-butyric acid 7a.

In a 25 mL round flask were placed at room temperature 0.6 g of 6 (1.78 $10^{-3}$ mol) in 15 mL of ethanol. 2 mL of a sodium hydroxide solution (10%) were subsequently added and the mixture was allowed to stir at room temperature for 24 hours. The bottom phase layer was discarded, and the aqueous phase was acidified with HCl 1N. After extraction with ethylacetate, the organic phase was dried over Na$_2$SO$_4$, filtered and concentrated in vacuo. The crude residue was purified by chromatography on a silicagel column using petroleum ether/ethylacetate (1/1) as eluent affording the expected product 7a in 69% yield.

White solid; $^1$H NMR (CDCl$_3$): $\delta$ = 6.54-7.14 (m, 4H), 3.89-3.98 (m, 3H), 1.51-2.14 (m, 7H), 0.89-1.34 (m, 11H). $^{13}$C (CDCl$_3$): $\delta$ = 177.57, 166.34, 159.95, 132.32, 130.12, 114.07, 72.30, 64.36, 31.32, 31.03, 29.14, 25.32, 22.14, 18.48, 13.95. MS (ESI) C$_{18}$H$_{27}$NO$_4$ m/z 322.1932 (100%, (M+H$^+$)).

(R)-2-(4-Hexyloxy-benzoylamino)-3-methyl-butyric acid 7a’

Procedure similar to that applied for the preparation of 7a

White solid; $^1$H NMR (CDCl$_3$): $\delta$ = 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 (CDCl$_3$): $\delta$ = 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) C$_{18}$H$_{27}$NO$_4$ m/z 322.1932 (100%, (M+H$^+$)).

(S)-2-(4-Hexyloxy-benzoylamino)-3-phenyl-propionic acid 7b

Procedure similar to that applied for the preparation of 7a

White solid; $^1$H NMR (CDCl$_3$): $\delta$ = 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 (CDCl$_3$): $\delta$ = 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) C_{23}H_{30}NO_4 m/z 385.2221 (100\%, (M+H^+)).

(S)-2-(4-Hexyloxy-benzoylamino)-3-(3H-imidazol-4-yl)-propionic acid 7c

Procedure similar to that applied for the preparation of 7a

Yellow solid; \(^1\)H NMR (CDCl\(_3\)): \(\delta = 7.93\) (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 (CDCl\(_3\)): \(\delta = 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) C_{19}H_{25}N_3O_4 m/z 360.1834 (100\%, (M+H^+)).

(S)-2-(4-Hexyloxy-benzoylamino)-3-hydroxy-propionic acid 7d

Procedure similar to that applied for the preparation of 7a

White solid; \(^1\)H NMR (DMSO d\(_6\)): \(\delta = 7.68\) (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 (DMSO d\(_6\)): \(\delta = 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) C\(_{16}\)H\(_{23}\)NO\(_5\) m/z 310.1667 (100\%, (M+H^+)).

(4-Hexyloxy-benzoylamino)-acetic acid 7e

Procedure similar to that applied for the preparation of 7a

White solid; \(^1\)H NMR (DMSO d\(_6\)): \(\delta = 7.68\) (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 (DMSO d\(_6\)): \(\delta = 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) C\(_{15}\)H\(_{21}\)NO\(_4\) m/z 280.1511 (100\%, (M+H^+)).
(S)-2-(4-Methoxy-benzoylamino)-3-methyl-butyric acid \( \text{f} \)

Procedure similar to that applied for the preparation of \( \text{7a} \)

White solid; \(^1\)H NMR (DMSO d6): \( \delta = 7.53-7.55 \) (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 (DMSO d6): \( \delta = 172.33, 167.28, 160.14, 131.12, 127.30, 110.15, 58.66, 55.65, 30.93, 19.03 \). MS (ESI) \( \text{C}_{13}\text{H}_{17}\text{NO}_4 \) m/z 251.1232 (100%, (M+H\(^+\))).

(S)-2-(4-Ethoxy-benzoylamino)-3-methyl-butyric acid \( \text{7g} \)

Procedure similar to that applied for the preparation of \( \text{7a} \)

White solid; \(^1\)H NMR (DMSO d6): \( \delta = 7.59 \) (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 (DMSO d6): \( \delta = 173.15, 167.10, 160.83, 129.98, 127.04, 113.21, 61.89, 57.93, 29.98, 19.19, 14.31 \). MS (ESI) \( \text{C}_{14}\text{H}_{19}\text{NO}_4 \) m/z 266.1342 (100%, (M+H\(^+\))).

(S)-3-Methyl-2-(4-propoxy-benzoylamino)-butyric acid \( \text{7h} \)

Procedure similar to that applied for the preparation of \( \text{7a} \)

White solid; \(^1\)H NMR (DMSO d6): \( \delta = 6.95-7.32 \) (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 (DMSO d6): \( \delta = 172.34, 167.42, 160.15, 128.64, 127.42, 112.45, 69.37, 58.62, 29.89, 21.12, 18.82, 10.29 \). MS (ESI) \( \text{C}_{15}\text{H}_{21}\text{NO}_4 \) m/z 280.1534 (100%, (M+H\(^+\))).

(4-(heptyloxy)benzoyl)-L-valine \( \text{7i} \)

White solid; \(^1\)H NMR (DMSO d6): \( \delta = 0.93-1.41 \) (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 (DMSO
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\[ \delta = 175.24, 167.12, 162.78, 129.02, 114.39, 68.28, 57.84, 31.80, 31.27, 29.14, 29.07, 25.98, 22.64, 19.15, 17.92, 14.12 \]

MS (ESI) \( C_{19}H_{29}NO_4 \) m/z 336.2165 (100\%, (M+H\(^+\))).

(4-(octyloxy)benzoyl)-L-valine 7j

White solid; \(^1\)H NMR (DMSO d6): \( \delta = 0.91-1.34 \) (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 (DMSO d6): \( \delta = 175.31, 170.16, 163.59, 130.46, 127.28, 115.21, 69.24, 59.88, 33.04, 31.82, 30.53, 30.46, 30.34, 27.19, 23.77, 19.79, 18.97, 14.49. \) MS (ESI) \( C_{20}H_{31}NO_4 \) m/z 350.2315 (100\%, (M+H\(^+\))).

(4-(decyloxy)benzoyl)-L-valine 7k

White solid; \(^1\)H NMR (DMSO d6): \( \delta = 0.92-1.32 \) (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)

\(^{13}\)C (DMSO d6): \( \delta = 175.36, 167.71, 162.20, 129.31, 129.10, 125.71, 114.33, 114.15, 68.25, 57.62, 31.94, 31.43, 29.61, 29.43, 29.37, 29.16, 26.03, 22.73, 19.09, 17.91, 14.18. \) MS (ESI) \( C_{22}H_{35}NO_4 \) m/z 378.2645 (100\%, (M+H\(^+\))).

**Cell culture.** 3T3-L1 cells (from ATCC) were routinely cultured in DMEM with 4 mM L-glutamine, 4.5 g/liter glucose, 0.11 g/liter sodium pyruvate, and supplemented with 10% fetal bovine serum plus antibiotics. Two days after confluence, adipocytes differentiation was triggered by changing the adding the conventional induction mixture (0.1 \( \mu \)M dexamethazone, 500 \( \mu \)M 3-Isobutyl-1-methylxanthine, and 174.5 nM insulin). 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. At the confluence, cells were treated for 24 hours with 0.5 mM palmitic acid complexed with BSA. The establishment, characterization and culture protocols of human Multipotent Adipose-Derived Stem (hMADS) cells have been described previously \cite{38}. Briefly, confluent cells were submitted to differentiation medium (DMEM/Ham’s F12 media containing 10 µg/ml transferrin, 10 nM insulin, and 0.2 nM triiodothyronine) supplemented with 1 µM dexamethasone and 500 µM isobutyl-methylxanthine. Two days later, the medium was changed, dexamethasone and isobutyl-methylxanthine were omitted and 100 nM Rosi were 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). Medium was changed every other day and cells were used at day 18.

**Oil Red O staining.** 3T3-L1 or HuH7 hepatoma cells were washed with PBS and fixed with 4% formaldehyde solution for 20 minutes, then washed again and stained with 0.35% Oil Red O solution in 60% isopropanol for 20 minutes. 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.

**2-deoxy-D-glucose uptake assay.** Glucose uptake activity of fully differentiated 3T3-L1 adipocytes was measured by the chemiluminescent assay \cite{39} using Glucofax kit as described by the manufacturer (Yelen, Ensuès la Redonne, France).
**Real Time PCR Analysis.** Total RNA was extracted using a Nucleospin RNA kit (Macherey-Nagel, Hoerdt, France), cDNA was synthesized from 0.5 μg of RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and used for PCR amplification. Real Time PCR (RT-PCR) were performed on the LightCycler 480 instrument (Roche Applied Science) using the Eva Green MasterMix (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 housekeeping gene. Changes were normalized to the mean of control values, which was set to 1. Primer sequences were previously published $^{17,35,38}$.

**MitoTracker staining.** 3T3-L1 adipocytes were trypsinized and centrifuged at 300 g at 4°C for 5 min. Cells were suspended in Kreb’s Ringer solution buffered with HEPES and 0.5% BSA and incubated with 0.1 μM MitoTracker Green FM for 30 min at 37°C. Cells were spun at 300 g at 4°C for 5 min and suspended in 400 μl of fresh KR BH then analyzed using a BD Accuri C6 flow cytometer (BD Biosciences).

**Cell-based PPAR transactivation assay.** $PPAR_\gamma$-LBD-Gal4 or $PPAR_\alpha$-LBD-Gal4 expression vector was transfected along with SV40-driven Renilla luciferase expression vector in HEK293 cells stably expressing the Gal4 response element driven Firefly luciferase reporter. 36 hours after transfection, cells were exposed to the tested compounds for additional 16 hours then Firefly and Renilla luciferase activities were measured in the cell lysates using the reagent Genofax A and C (Yelen) in an Ensight multimode reader (Perkin Elmer). PPAR transactivation activity of the compounds is calculated as ratio of Firefly to Renilla luciferase activity.
**In vitro kinase assay.** The assay was performed on WT-PPAR\(\gamma\) in the apo form and in the complex with 7j and Rosi. For the kinase assay, 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 \(\mu\)M, 1 \(\mu\)M and 10 \(\mu\)M respectively, and pre-equilibrated overnight at 4°C with the protein. Kinase assay was carried out at 30 °C for 3.5 hours in 300 \(\mu\)L of buffer containing 50 mM Tris HCl pH 7.5, 7.2 \(\mu\)g.mL\(^{-1}\) PPAR\(\gamma\), 0.1-1-10 \(\mu\)M ligand, 25 mM MgCl\(_2\), 50 \(\mu\)M DTT, 2 mM ATP, 0.66 ng mL\(^{-1}\) CDK5/p35 (Sigma Aldrich code n. SRP5011).

**ELISA of PPAR\(\gamma\) phosphorylation.** Polystyrene micro well 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 \(\mu\)L of anti-phospho-Ser/The-Pro antibody (Sigma Aldrich code n. A05368) diluted 1:500 in PBS. After three washes, 100 \(\mu\)L of Anti-Mouse IgG-Peroxidase antibody produced in goat (Sigma Aldrich code n. A4416; 1:1000 in PBS) were added to the wells and incubated 60 min at 37°C. The wells were washed and 200 \(\mu\)L of o-phenylenediamine dihydrochloride (Sigmaprest OPD code n. P9187) dissolved in water were added to the wells. Optical density was measured at 450nm using ApplyScan Thermofisher Reader and the data were processed using Excel.

**Crystallization and Data Collection.** PPAR\(\gamma\) LBD was expressed as N-terminal His-tagged proteins using a pET28 vector and purified as previously described \(^{40}\). Crystals of apo-PPAR\(\gamma\) were obtained by vapor diffusion at 18°C using a sitting drop made by mixing 2 \(\mu\)L of protein solution with 2 \(\mu\)L of reservoir solution (0.8 M Na Citrate, 0.15M Tris, pH 8.0). The crystals
were soaked for three days in a storage solution (1.2 M Na Citrate, 0.15 M Tris, pH 8.0) containing the ligand 7j (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 cryoprotectant. Crystals (0.15 x 0.15 mm) of PPARγ/7j belong to the space group C2 with cell parameters shown in Table 1 of Supporting Information.

**Structure Determination and Refinement.** X-ray data set 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. Structure solution was performed with AMoRe, using the coordinates of PPARγ/LT175R (27) (PDB code 3D6D) as the starting model. The coordinates were then refined with CNS and with PHENIX including data between 58.2 and 2.0 Å. The statistics of crystallographic data and refinement are summarized in Table 1 of Supporting Information. The coordinates and structure factors for the PPARγ/7j structure described here have been deposited in the PDB under accession number 6QJ5.

**Surface Plasmon Resonance.** Surface plasmon resonance analyses were performed by using Pioneer AE optical biosensor equipped with COOH5 chips (SensiQ). PPARγ surfaces were prepared by using standard amine-coupling procedures 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 μL of 50 mM N-hydroxysuccinimide (NHS):200 mM ethyl-3(3-dimethylamino) propylcarbodiimide (EDC). 150 μL of a 0.25 mg/mL PPARγ solution (in 10 mM NaOAc, pH 5.0) were injected for 15 min at 10 μL/min on channels 1 and 3 (channel 2 was used as reference, for a duplicate
experiment), followed by a 70 μL injection of ethanolamine to block any remaining activated groups on the surface. 12,220 and 7500 RU of protein were immobilized on channel 1 and 3, respectively. The screening of the analytes (7j and Rosi) was performed using HBS, with 1% DMSO. To collect detailed kinetic data the OneStep protocol was used, injecting the analytes at a flow rate of 50 μL/min and at the concentration of 1 μM over the two channels at 20 °C (association phase of 180 s). 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 k_{off}/k_{on}.

**Statistical Analyses.** Statistical significance was estimated with one-way ANOVA followed by Bonferroni or Dunnett post hoc test or with F-Test using Graph Pad Prism version 5.0 (GraphPad Software, San Diego, CA). Differences with p values of less than 0.05 were considered statistically significant.
SUPPORTING INFORMATION

Experimental Section

Table 1. Statistics of crystallographic data and refinement

Figure S1. Typical 1D 1H-NMR and 1D 13C-NMR spectra

Figure S2. Screening of the adipogenic effect of hydroxamic acid-based molecules

Figure S3. Effect of 7j on PPARγ transactivation and cell toxicity

Figure S4. PPARδ transactivation activities

Figure S5. Electron density map

Figure S6. Superimposition of the crystal structures PPARγ / 7j and PPARγ / SR2067

Figure S7. Interaction PPARγ / agonists

Figure S8. Adipogenic effect of the compounds

Figure S9. Effect of 7j and Rosi on hMADS

PDB ID

PDB 6Qj5

Authors will release the atomic coordinates and experimental data upon article publication.

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Notes

The authors declare no competing financial interest

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**ABBREVIATIONS USED**

benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)

human Multipotent Adipose-Derived Stem (hMADS)

non-alcoholic fatty liver disease (NAFLD)

PPAR Responsive Element (PPRE)

Rosiglitazone (Rosi)

selective PPARγ modulators (SPPARγMs)

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


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\[
\begin{align*}
\text{O} & \quad \text{CONH}_2 \\
\text{Cl} & \quad \text{CONH}_2 \\
\text{O} & \quad \text{CONH}_2 \\
\text{O} & \quad \text{CONH}_2 \\
\text{CH}_2\text{Cl}_2, 20^\circ\text{C}, 24\text{ h} & \quad \text{BOP (1 equiv.)} \\
\text{MeOH, 20^\circ\text{C}, 24h} & \quad \text{NaOH (10\%)} \\
\text{72\%} & \quad \text{7j} \\
\end{align*}
\]

PPAR\_\text{\textgamma} transactivation \EC_{50} = 0.4 \mu\text{M}