



Research paper

Benzopyran hydrazones with dual PPAR α / γ or PPAR α / δ agonism and an anti-inflammatory effect on human THP-1 macrophagesAinhoa García^{a,b}, Laura Vila^b, Isabelle Duplan^c, María Ayelén Schiel^d, Ricardo D. Enriz^d, Nathalie Hennuyer^{c,**}, Bart Staels^c, Nuria Cabedo^{a,b,*}, Diego Cortes^a^a Department of Pharmacology, University of Valencia, 46100, Burjassot, Valencia, Spain^b Institute of Health Research-INCLIVA, University Clinic Hospital of Valencia, 46010, Valencia, Spain^c Univ. Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U-1011-EGID, F-59000, Lille, France^d Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis-IMIBIO-SL-CONICET, Chacabuco, 917-5700, San Luis, Argentina

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) play a major role in regulating inflammatory processes, and dual or pan-PPAR agonists with PPAR γ partial activation have been recognised to be useful to manage both metabolic syndrome and metabolic dysfunction-associated fatty liver disease (MAFLD). Previous works have demonstrated the capacity of 2-prenylated benzopyrans as PPAR ligands. Herein, we have replaced the isoprenoid bond by hydrazone, a highly attractive functional group in medicinal chemistry. In an attempt to discover novel and safety PPAR activators, we efficiently prepared benzopyran hydrazone/hydrazine derivatives containing benzothiazole (*series 1*) or 5-chloro-3-(trifluoromethyl)-2-pyridine moiety (*series 2*) with a 3- or 7-carbon side chain at the 2-position of the benzopyran nucleus. Benzopyran hydrazones **4** and **5** showed dual hPPAR α / γ agonism, while hydrazone **14** exerted dual hPPAR α / δ agonism. These three hydrazones greatly attenuated inflammatory markers such as IL-6 and MCP-1 on the THP-1 macrophages via NF- κ B activation. Therefore, we have discovered novel hits (**4**, **5** and **14**), containing a hydrazone framework with dual PPAR α / γ or PPAR α / δ partial agonism, depending on the length of the side chain. Benzopyran hydrazones emerge as potential lead compounds which could be useful for treating metabolic diseases.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of transcription factors, comprising three isotypes [PPAR α (NR1C1), PPAR β / δ (NR1C2) and PPAR γ (NR1C3)] which have different tissue distribution, ligand specificities, and metabolic regulatory activities [1,2]. In structural terms, PPARs contain a DNA-binding domain (DBD) in the N-terminus and a ligand-binding domain (LBD) in the C-terminus. The DBD sets the binding with PPAR response elements (PPREs) in specific regions of the target genes and the LBD forms the pocket for direct interaction with specific ligands. After the interaction with ligands, PPARs are translocated to the nucleus and heterodimerise with the retinoid X receptor (RXR) to form the PPAR-RXR complex. PPAR-RXR binding by agonists induces the recruitment of coactivators (e.g., CBP or p300) and the release of corepressors (e.g., NCoR or SMRT) by stimulating the gene

transcription of the target genes [3–5]. The three PPAR isoforms are involved in fatty acid oxidation, glucose metabolism, and lipid metabolism. PPAR α is expressed mainly in the tissues involved in increased fatty acid oxidation, such as liver, skeletal muscle and heart, and controls lipid metabolism. PPAR α activation decreases triglyceride levels in plasma and increases HDL-c to play a significant role in the treatment of dyslipidaemia [6]. PPAR α is also implicated in glucose homeostasis and insulin resistance [7,8]. PPAR β / δ is ubiquitous, but is highly expressed in those tissues involved in fatty acid metabolism, such as skeletal and cardiac muscle, hepatocytes and adipocytes. Its activation improves insulin sensitivity and the plasma lipid profile, which may manage both dyslipidaemia and type 2 diabetes, although no PPAR β / δ agonist has yet been approved for clinical use [9]. PPAR γ is widely expressed in adipose tissue and its activation is related to adipogenesis, lipid storage, insulin sensitivity and glucose homeostasis. Its agonists have been widely used for treating type 2 diabetes. However, much evidence indicates that

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potent and full PPAR γ activators are related with serious adverse effects, and a partial agonism would lead to safer drugs [2]. The endogenous ligands for PPARs are fatty acids, cyclooxygenase-derived eicosanoids and prostaglandins, as well as their metabolites. The most known synthetic ligands are fibrates and WY-14,643, GW501516 or rosiglitazone (thiazolidinedione) (Fig. 1), which act as strong agonists for PPAR α , PPAR β/δ or PPAR γ , respectively [10]. In addition, the three isoforms (α , β/δ , γ) play a major role in regulating inflammatory processes, and have been recognised as the target for the management of metabolic syndrome, obesity, dyslipidaemia, type 2 diabetes, atherosclerosis [2,4], and metabolic dysfunction-associated fatty liver disease (MAFLD). They are all accompanying with a persistent chronic, low-grade inflammation state in several tissues, including adipose tissue, pancreatic islets and liver [11]. Macrophages are phagocytic innate immune cells involved in immunity, tissue remodelling and lipid homeostasis. These immune cells are present in all tissues and play a crucial role during the development of metabolic disorders [11–13] and associated inflammation. The three PPAR isoforms are highly expressed in human macrophages [13], whose function can be regulated by PPAR agonists to prevent metabolic diseases. Infiltrate macrophages in tissues also release pro-inflammatory

cytokines and chemokines, which contribute to an inflammation state. Of them, monocyte chemoattractant protein-1 (MCP-1/CCL2) is an important chemokine that is implicated in both the recruitment and activation of monocytes [14], and its down-regulation has been found to occur with improvement of metabolic disorders [15,16]. Interleukin 6 (IL-6) is an inflammatory cytokine IL-6 whose overproduction is directly related to obesity, diabetes and progression to metabolic and cardiovascular disorders [17]. PPAR agonists have been reported to suppress the immunoreactive state of macrophages by the suppression of immune reactive cytokine and chemokines markers, including MCP-1 and IL-6 [2,18].

In 1995, our research group isolated polycerasoidol from the stem bark of *Polyalthia cerasoides* (Annonaceae) [19] (Fig. 1). This 2-prenylated benzopyran contains the chroman-6-ol nucleus like the PPAR γ agonist troglitazone (Fig. 1), two isoprenoid units and a terminal carboxylic group on the side chain at 2-position. Polycerasoidol displayed potent dual PPAR α/γ agonism and anti-inflammatory effects by inhibiting mononuclear cell-endothelium interactions in a dysfunctional endothelium [20]. Polycerasoidol became the first-in-class with potential to manage several of the risk factors involved in the development of

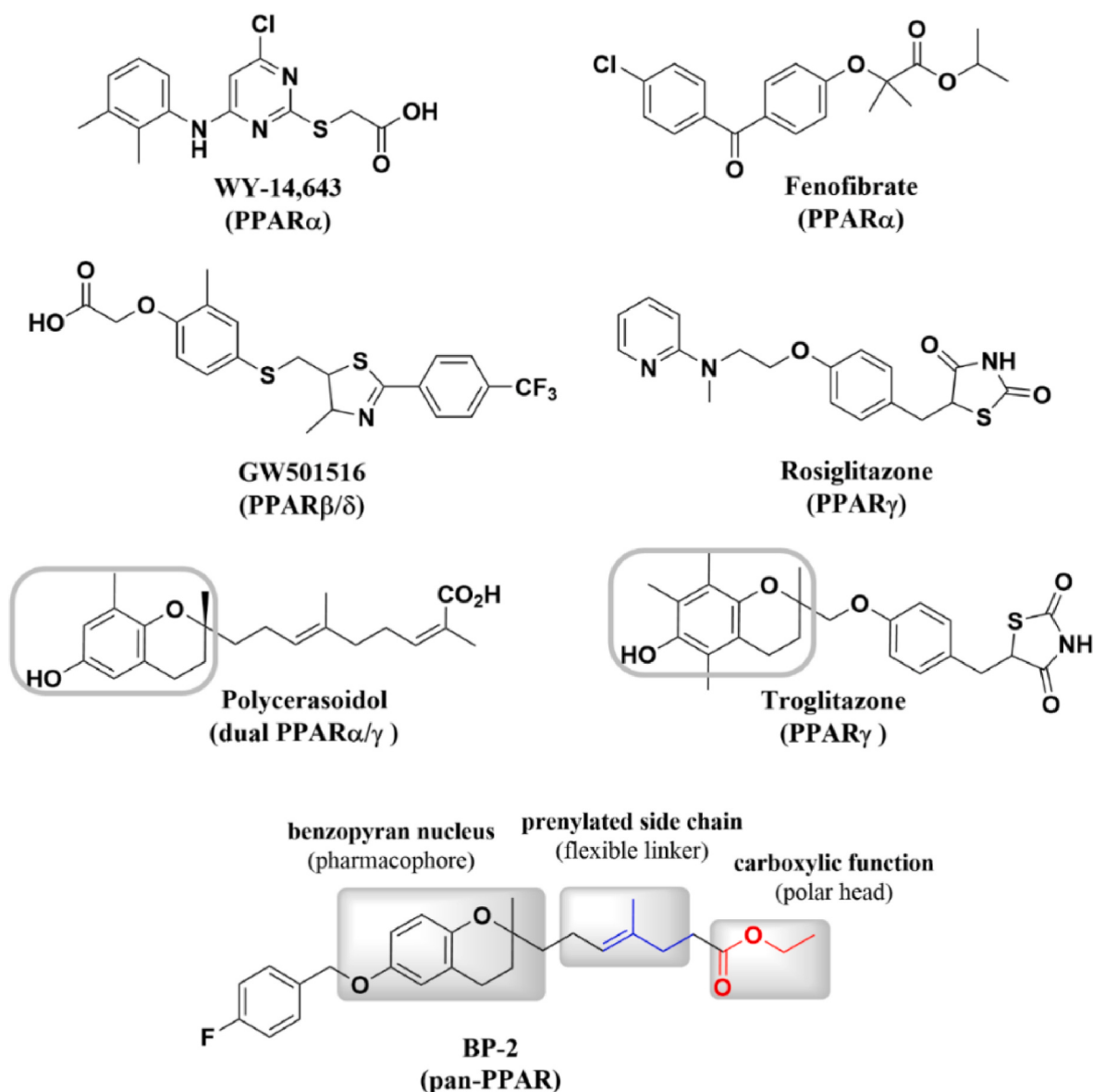


Fig. 1. Synthetic ligands, and natural or synthetic benzopyrans as PPAR agonists.

metabolic syndrome. Next, we carried out structure-activity relationship (SAR) studies of synthetic polycerasoidol analogues and molecular modelling study analyses to explore those key structural features implicated in the modulation of PPARs [21–23]. These studies have indicated that: i) the oxygen atom at 6-position in the benzopyran nucleus (pharmacophore); ii) the length of the prenylated side chain (flexible linker) from 5- to 11-carbons; iii) ester, amide and *O*-alkoxylated bioisosters in the carboxylic function (polar head) can improve hPPAR α and hPPAR γ interactions. A recent hit-to-lead strategy identified 2-(ethyl-4'-methylhept-4'-enoate)-6-(*p*-fluorobenzyloxy)-2-(methyl)-benzodihydropyran (BP-2) (Fig. 1) as a pan-PPAR with PPAR γ partial activation, and capable of ameliorating metabolic alterations in an obese and diabetic mouse model (*ob/ob* mice) [24].

On the other hand, hydrazone ($-\text{CH}=\text{N}-\text{NH}-$) and hydrazine ($-\text{CH}-\text{N}-\text{NH}-$) functions are present in many bioactive natural and synthetic compounds [25–29], attracting the interest as privilege moieties in medicinal chemistry. Among them, the antibiotic rifampicin and the antiparasitic nifurtimox (anti-parasitic drug for Chagas disease) contain the hydrazone moiety, while the hydrazine group is found in the anti-hypertensive hydralazine (vasodilator), the antidepressant phenelzine (monoamine oxidase inhibitor) and the anticancer procarbazine, all of which are clinically used drugs (Fig. 2). Synthesised hydrazones were also reported to display antidyslipidaemic and antidiabetic activities by different mechanisms [30,31]. In addition, heterocyclic systems are valuable scaffolds to design new therapeutic derivatives. Benzo[*d*]thiazole moiety is found in lanifibranor (Fig. 3), a pan-PPAR agonist and the first drug clinically approved for MAFLD, whereas pyridyl fragment is present in rosiglitazone (Fig. 1) and pioglitazone (Fig. 3), two thiazolidinediones with full PPAR γ agonism and used as anti-diabetic agents.

Based on our previous works on 2-prenylated benzopyrans, and in a view of discovering novel chemical entities with dual- or pan-PPAR agonism, but with PPAR γ partial agonism to avoid adverse effects associated to a full and selective PPAR γ activation, we have explored the possibility to replace both the isoprenyl (flexible linker) and the ethyl carboxylate (polar head) moieties by potential bioisosters. Herein, we have synthesised and evaluated the PPAR activity of novel benzopyrans (pharmacophore) containing in the hydrocarbon side chain at 2-position

a hydrazone or hydrazine (its reduced form) function linked to a heterocyclic system such as benzothiazole or 5-chloro-3-(trifluoromethyl)-2-pyridine, two attractive templates for PPAR target (Fig. 3). The anti-inflammatory potential of synthesised compounds was also evaluated *in vitro* in terms of modulating the expression of MCP-1 and IL-6 in human THP-1 macrophages under inflammatory conditions. Accordingly, we have identified novel hits as potential drugs to develop lead candidates which could be useful for metabolic syndrome, type 2 diabetes, dyslipidaemia, MAFLD or inflammation, which can further progress to microvascular problems and cardiovascular events, primary causes of morbidity and death worldwide [32].

2. Results and discussion

2.1. Synthesis of benzopyran hydrazones

We prepared two series of benzopyran hydrazones/hydrazines containing benzothiazole moiety (*series 1*) or 1-[5-chloro-3-(trifluoromethyl)-2-pyridyl] (*series 2*). The benzopyran nucleus was synthesised *via* a chroman-4-one scaffold, which was obtained by the aldol condensation between an *ortho*-hydroxyacetophenone and a ketone in the presence of a secondary amine by Michael addition as previously reported [21–23]. The reaction started with the condensation between 2,5-dihydroxyacetophenone and ethyl levulinate as the proper ketone in the presence of pyrrolidine (Scheme 1). Then, the γ -benzopyrone was reduced under Clemmensen conditions using dust zinc in acid medium to afford the benzodihydropyran 1, and its phenolic group was protected utilising *p*-fluorobenzyl chloride in the presence of potassium carbonate to give the *p*-fluorobenzyloxy benzodihydropyran 2. The controlled reduction of the ester function of benzopyran 2 using DIBAL-H reagent allowed us to obtain the aldehyde intermediate 3.

In a first approach, we prepared benzopyran hydrazones with a 3-carbon side chain. For this purpose, aldehyde intermediate 3 condensed with the available hydrazines such as 2-hydrazinobenzothiazole and 1-[5-chloro-3-(trifluoromethyl)-2-pyridyl]hydrazine in anhydrous dichloroethane gave imines (Schiff bases) with hydrazone moieties 4 (*series 1*) and 5 (*series 2*), respectively [33]. Deprotection of compounds 4 and 5 with

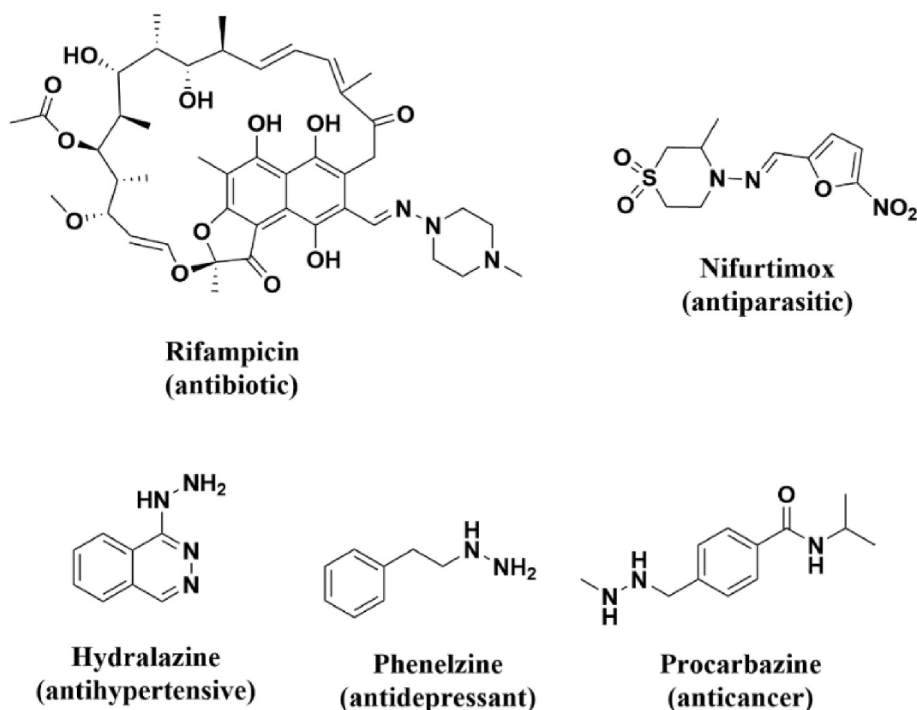


Fig. 2. Clinical drugs containing hydrazone ($\text{C}=\text{N}-\text{NH}$) or hydrazine ($\text{NH}-\text{NH}$) moiety.

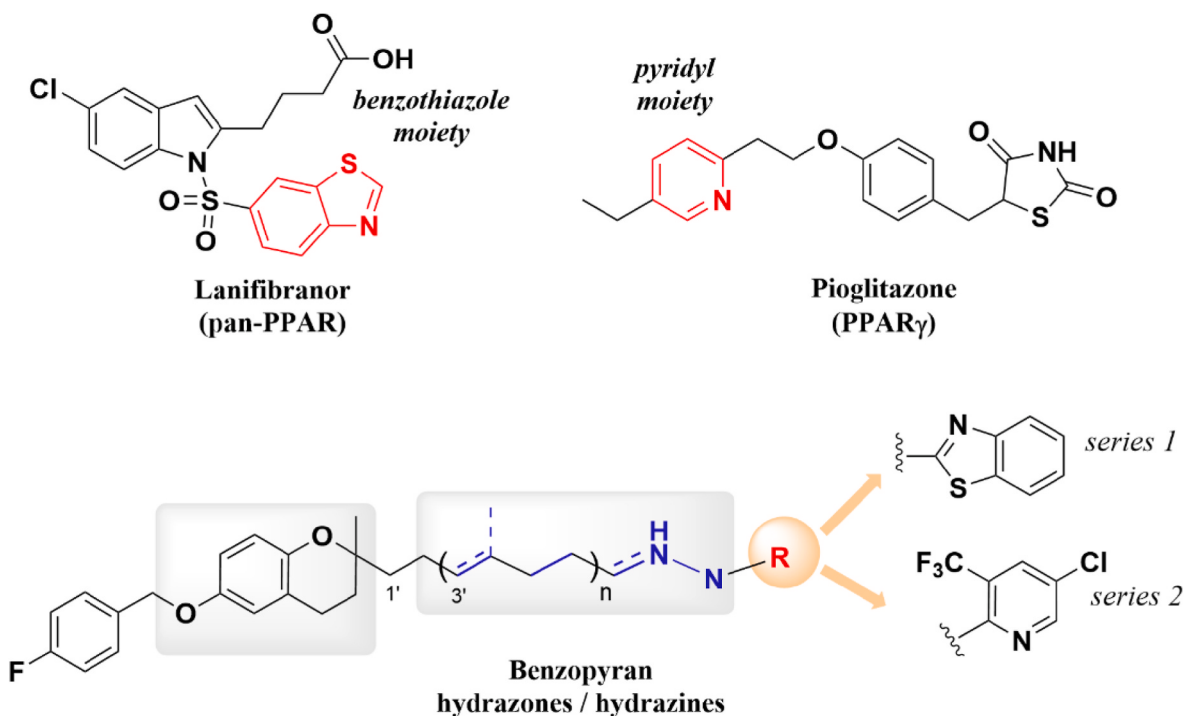
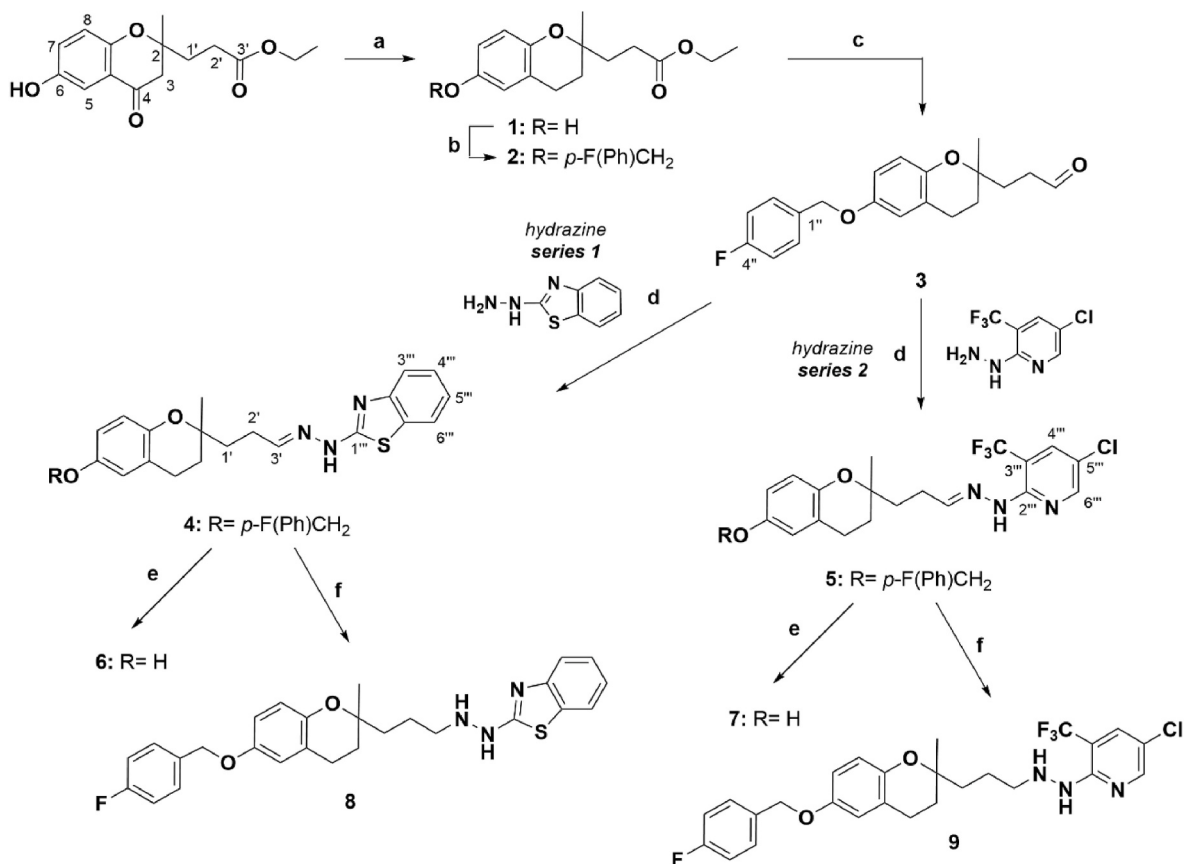


Fig. 3. Clinical drugs and designed benzopyran hydrazones/hydrazines containing benzothiazole or pyridinyl moiety.



Scheme 1. Synthesis of 2-substituted benzopyrans containing hydrazone/hydrazine moiety. *Reagents and conditions:* (a) Zn dust/conc HCl, AcOH-H₂O (2:1, v/v), r.t., 1 h 30 min (58%); (b) *p*-fluorobenzyl chloride, K₂CO₃, EtOH, reflux, 4 h (79%); (c) 1 M DIBAL-H in THF, CH₂Cl₂, -78 °C, N₂, 15 min (92%); (d) hydrazines: 2-hydrazinobenzothiazole or 1-[5-chloro-3-(CF₃-2-pyridyl)]NHNH₂, (CH₂)₂Cl₂, r.t., N₂, 1 h (80% for 4; 89% for 5); (e) BBr₃, CH₂Cl₂, r.t., N₂, 1 h (91% for 6; 92% for 7); (f) NaBH₄CN, BF₃OEt, MeOH, N₂, reflux, 1 h (96% for 8; 75% for 9).

boron tribromide afforded compounds **6** (series 1) and **7** (series 2), respectively, bearing both a hydrazone moiety and a free phenolic group (Scheme 1) [34]. The reduction of the imine double bond in hydrazones ($-C=N-NH-$) to attain hydrazines ($-CH_2-NH-NH-$) has been reported by catalytic hydrogenation under high pressure or hydride reductions, including lithium aluminium hydride [35], sodium borohydride in Raney nickel [36], sodium cyanoborohydride [37], the borane trimethylamine complex in hydrochloric acid [38] and magnesium in methanol [39], which are usually tedious or inefficient procedures. In our approach, hydrazones were reduced using a methodology previously reported to reduce quinolines to tetrahydroquinolines [40]. Thus, benzopyran hydrazines **8** (series 1) and **9** (series 2) were easily and efficiently prepared from their hydrazones **4** and **5**, respectively, by sodium cyanoborohydride and boron trifluoride etherate as catalyst to generate diborane *in situ* (Scheme 1).

In a second approach, we synthesised two series of benzopyran hydrazones with a 7-carbon side chain (C-1' to C-7') instead of 3-carbons (C-1' to C-3'). The elongation of the hydrocarbon side chain from aldehyde **3** was performed by an efficient Grignard reaction, followed by Johnson-Claisen rearrangement to obtain benzopyran ester **11** [21,22,24]. The Grignard reaction consisted in the nucleophilic attack of propenyl magnesium bromide to the electrophilic carbon of aldehyde **3** to form a carbon-carbon bond. The Johnson-Claisen rearrangement of allylic alcohol intermediate **10** was carried out using 1,1,1-triethoxyethane and catalytic amounts of isobutyric acid. Then benzopyran ester **11** was subjected to controlled reduction with DIBAL-H reagent to give aldehyde **12**. The condensation of aldehyde **12** with the corresponding hydrazines, such as 2-hydrazinobenzothiazole and 1-[5-chloro-3-(trifluoromethyl)-2-pyridyl]hydrazine in anhydrous dichloroethane gave benzopyran hydrazones **13** (series 1) and **14** (series 2), respectively (Scheme 2).

2.2. PPAR α , PPAR β/δ and PPAR γ agonist activity

All the synthesized compounds were assayed *in vitro* for hPPAR α , hPPAR β/δ or hPPAR γ transcriptional activity by a cell-based

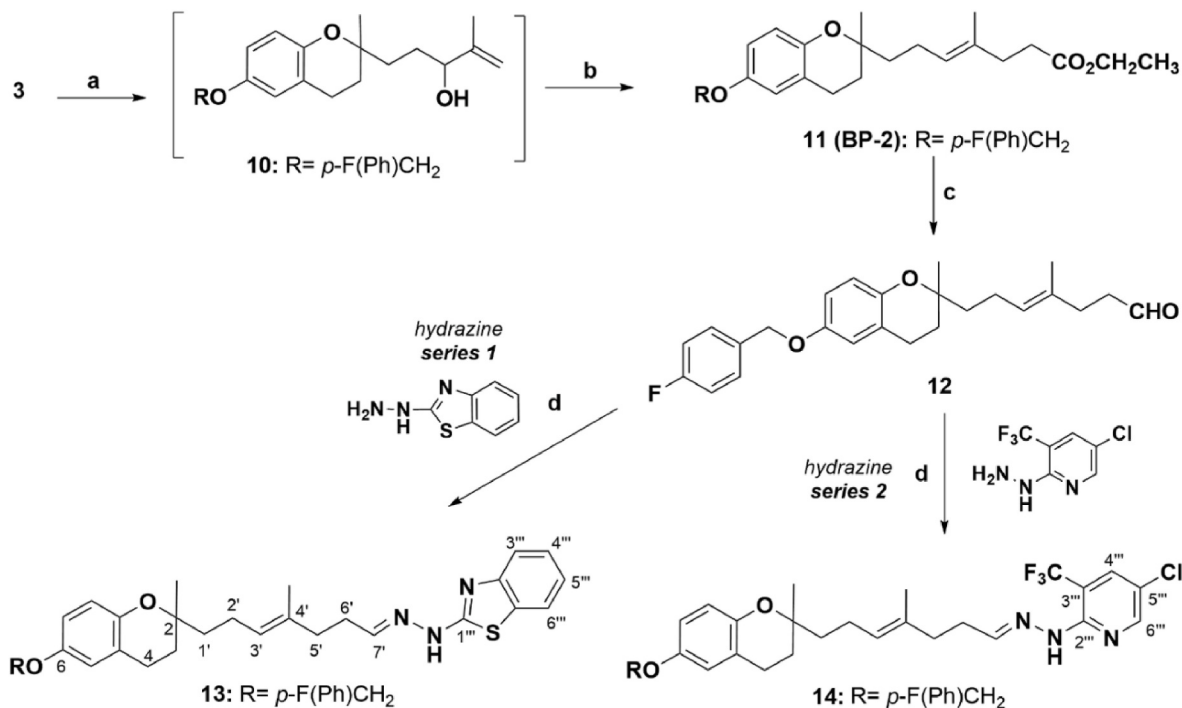
transactivation assay in Cos-7 cells properly transfected with a luciferase-reported plasmid in the presence of expression vectors pGAL4hPPAR α , pGAL4hPPAR β/δ and pGAL4hPPAR γ [24]. The activity of each compound was expressed as each compound' percentage of efficacy at 10 μ M by comparing to the maximal efficacy of the reference compounds: WY-14,643 (at 10 μ M), rosiglitazone (at 1 μ M) or GW501516 (at 1 μ M) as hPPAR α , hPPAR γ or hPPAR β/δ respectively. Half maximal effective concentration EC₅₀ was calculated by using the Prism software (Table 1). The results showed that benzopyran hydrazones **4** (series 1) and **5** (series 2) at 10 μ M displayed dual hPPAR α/γ partial activation. It was noteworthy that hydrazone framework, instead of the isoprenyl, also exerts a PPAR activity providing a new class of benzopyran agonists. Benzopyran hydrazone **14** (series 2), bearing an elongated 7-carbon side chain with an isoprenoid unit and

Table 1

Evaluation of agonist activity in a cell-based transactivation assays for human PPAR/Gal₄ receptors. EC₅₀ values against human PPAR α /Gal₄, PPAR β/δ /Gal₄ and PPAR γ /Gal₄ receptors.

	Efficacy (%) at 10 μ mol/L			EC ₅₀ (nmol/L)		
	PPAR α	PPAR β/δ	PPAR γ	PPAR α	PPAR β/δ	PPAR γ
4	47	NA	39	493	NA	3,270
5	48	NA	42	226	NA	5,540
6	22	NA	14	NA	NA	NA
7	1	NA	8	NA	NA	NA
8	24	NA	15	NA	NA	NA
9	11	NA	16	NA	NA	NA
13	28	NA	13	NA	NA	NA
14	121	43	11	3,255	1,475	NA
WY-14,643	100	NA	NA	4,193	NA	NA
GW501516	NA	100	NA	NA	4	NA
Rosiglitazone	NA	NA	100	NA	NA	60

Efficacy: Emax was the maximal PPAR fold-activation relative to maximum activation obtained with WY14,643 (10 μ M), GW501516 (1 μ M) and rosiglitazone (1 μ M) corresponded to 100% in GAL4 chimeric hPPAR α , hPPAR β/δ and hPPAR γ system. NA: not active.



Scheme 2. Synthesis of benzopyran hydrazones with a 7-carbon length side chain. *Reagents and conditions:* (a) $CH_3C(MgBr)=CH_2$, THF, $-78^\circ C$, N_2 , 3 h; (b) MeC(OEt)₃, isobutyric acid, $140^\circ C$, 2 h (48%); (c) 1 M DIBAL-H in THF, CH_2Cl_2 , $-78^\circ C$, 15 min (89%); (d) hydrazines: 2-hydrazinobenzothiazole or 1-[5-Cl-3-(CF₃-2-pyridyl)]NHNH₂, dry $(CH_2)_2Cl_2$, r.t, N_2 , 1 h (70% for **13**; 60% for **14**).

1-[5-chloro-3-(trifluoromethyl)-2-pyridyl]hydrazone moiety, displayed dual hPPAR α/δ activation with a full PPAR α agonism. SAR studies established that: i) the free phenol group was detrimental for PPAR activation and afforded non-active analogues **8** and **9**, compared to *p*-fluorobenzylbenzopyran hydrazones **4** and **5**, respectively; ii) the reduction of the imine bond of hydrazones **4** and **5** led to non-active hydrazines **6** and **7**, which indicated that the C(sp²) = N–NH– function that contains a C=N bond conjugated with a lone pair of electrons of the amine nitrogen atom (–NH–) plays a key role in the interaction with the binding pocket of hPPARs; iii) the elongation of the isoprenyl side chain at 2-position to 7-carbons to give benzopyran **14** (*series 2*) drastically increased the activation for hPPAR α , and moderately for hPPAR β/δ instead of hPPAR γ moiety, but not in benzopyran **13** (*series 1*). Therefore, we have discovered novel hits (**4**, **5** and **14**), containing a hydrazone framework which exhibited dual PPAR α/γ or PPAR α/δ partial agonism, depending on the length of the side chain. Hydrazones (C=N¹–N²) have been reported to possess great intrinsic hydrolytic stability due to the participation of N² in electron delocalization [41]. We previously demonstrated the importance of bioisosteric replacement in carboxylic function (polar head) of polycerasoidol by ester, amide and *O*-alkoxylated bioisosters to modulate hPPAR α and hPPAR γ interactions [20–23]. In addition, given the potential metabolic instability and toxicity associated to an acid functionality, the use of bioisosters could counter such issues and improve drug-like properties of the new PPAR agonists [42]. In this work, both benzothiazole and pyridyl groups have also shown ability to replace carboxylic moiety to activate hPPAR isoforms. Furthermore, these groups, which appear in many clinically approved drugs, have been reported to provide a good solubility range and stability in polar solvents and human plasma [43,44].

2.3. Molecular Modelling studies

At the molecular level, to understand the dual PPAR α/γ agonism for hydrazones **4** and **5**, as well as the dual PPAR α/δ agonism for hydrazone **14**, we carried out a combined analysis by means of a docking study and MD simulations. These studies predict how compounds **4**, **5** and **14** bind in the same region of the active site of PPAR α as that reported for WY-14,643 [45,46] (Fig. 4A–C). These results can be observed in the per residue analysis, which was performed on the different ligand-receptor complexes (Fig. S1, Supporting Information). MD simulations indicate that these molecules are arranged spatially in a slightly different way. In agreement with the results obtained for WY-14643, the simulations obtained for the complexes showed the relevance of His440, Leu321 and Ser280 for ligand binding [45,46]. The other residues with significant interactions to stabilise these complexes are Cys276, Thr464, Lys358 and Ile317. In accordance with our experimental data, the interactions obtained for compounds **4** and **5** were generally weaker than those observed for WY-14,643 (Fig. S1), while the interactions obtained for hydrazone **14** were closely related to those observed for the reference compound (Fig. S1). With the PPAR γ receptor, our simulations indicated that hydrazones **4** and **5** bound to the active site in a closely related way to that reported for rosiglitazone [47,48] (Fig. 3D and E). Indeed, hydrazones **4** and **5** interacted mainly with Cys285, Arg288, Tyr324, Leu330, Ile342, Phe366, Met364, His449 and Tyr473, although they generally displayed weaker interactions compared to rosiglitazone (Fig. S1). In contrast, the complex of hydrazone **14** with PPAR δ (Fig. 4F) showed this hydrazone with a 7-carbon side chain to be spatially arranged in a similar way to reference compound GW501516 [49]. The analysis per residues of **14** (Fig. S1) showed that its interactions with PPAR δ were significantly weaker to those formed by GW501516, which falls in line with our experimental results.

2.4. Anti-inflammatory effects of benzopyran hydrazones **4**, **5** and **14**

In order to evaluate the anti-inflammatory effect of the dual PPAR α/γ and PPAR α/δ agonists on macrophages, the PMA-differentiated THP-1

cells were treated with benzopyran hydrazones **4**, **5** and **14** at 10 μ M prior to the LPS stimulation. The mRNA expression and secretion of the pro-inflammatory markers, such as chemokine MCP-1 and cytokine IL-6, were determined in the LPS-stimulated THP-1 macrophages for 4 h and 24 h. The results at 4 h showed that benzopyran **4**, with a 3-carbon side chain and benzothiazole moiety, decreased the gene expression of MCP-1 and IL6 by 67% and 71%, respectively. Its pyridyl analogues **5** and **14**, respectively bearing a 3-carbon and 7-carbon side chain, showed a moderate activity. The results at 24 h revealed that benzopyran **4** inhibited the gene expression of MCP-1 and IL-6 by 71% and 93%, respectively, but also the chemo- and cytokine secretion by 85% and 79%, respectively. Its pyridyl analogue **5** moderately reduced the gene expression of MCP-1 (54%) and IL-6 (63%), and did not inhibit their secretion in the LPS-stimulated THP-1 macrophages. Finally, dual PPAR δ/γ agonist **14** lowered the IL-6 gene expression levels by 88% at 24 h and reduced the secretion of both MCP-1 and IL-6 by 70–80% in the LPS-stimulated THP-1 macrophages (Fig. 5). Therefore, **4**, **5** and **14** may ameliorate the inflammation state associated with the progression of metabolic disorders.

2.5. Anti-inflammatory effects of benzopyran hydrazones **4**, **5** and **14** via NF- κ B

In order to investigate the intracellular signalling pathways underlying the anti-inflammatory effect of hydrazones **4**, **5** and **14**, the THP-1 cells were stimulated with LPS for 1 h in the presence or absence of compounds (10 μ M, 24 h) prior to the pathway analysis. The results showed that the three hydrazones significantly blunted the TNF α -induced phosphorylation of NF- κ B by 34% for **4**, 35% for **5** and 36% for **14** (Figs. 6 and 7).

2.6. Cytotoxicity study of compounds **4**, **5** and **15**

Apoptosis cell death was studied by an Annexin V-FITC/PI dual staining assay on THP-1 cells. None of the three compounds showed toxicity at doses 10 μ M and 30 μ M, and only hydrazone **5** displayed slight toxicity at the highest concentration of 100 μ M (Figs. 8 and 9).

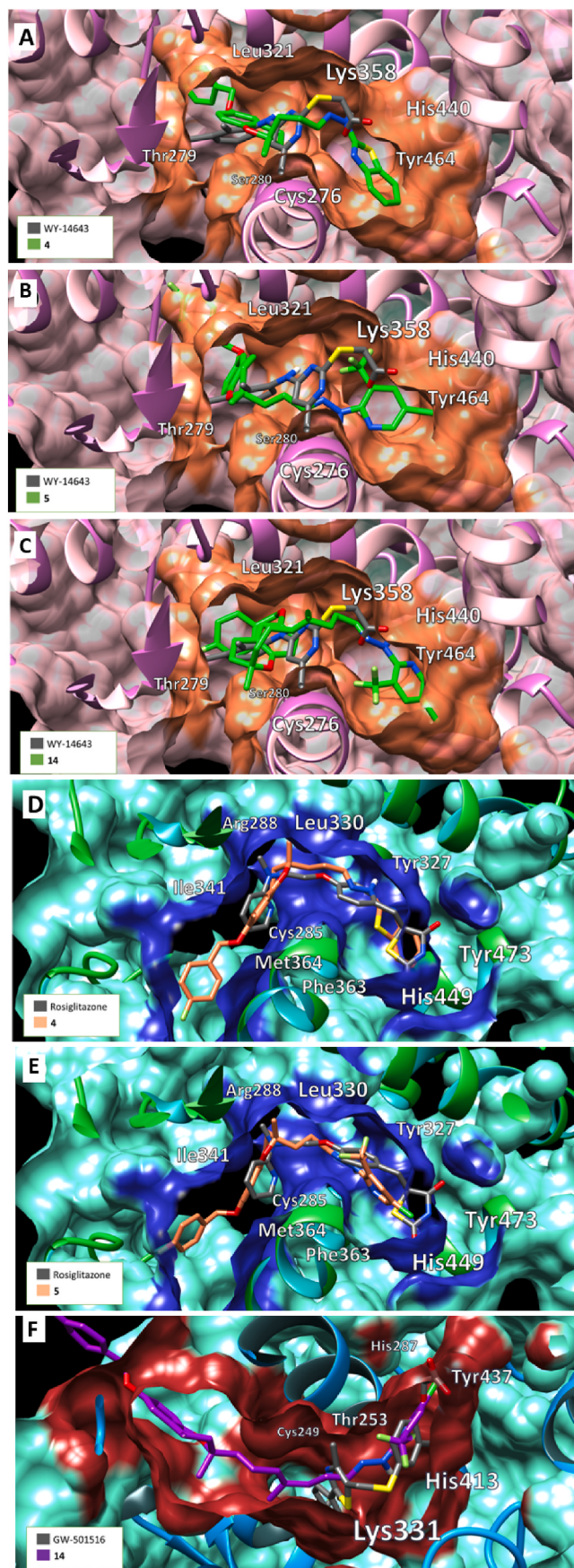
3. Conclusions

We herein efficiently prepared two series of benzopyran hydrazones and hydrazines as *polycerasoidol* analogues containing the hydrazone function in the 3-carbon or 7-carbon side chain at 2-position of the benzopyran nucleus. Benzopyran hydrazones **4** and **5** with a 3-carbon side chain, showed dual hPPAR α/γ partial agonism, and hydrazone **14**, with 1-[5-chloro-3-(trifluoromethyl)-2-pyridyl]hydrazone moiety on the prenylated 7-carbon side chain, exerted dual PPAR α/δ partial agonism. However, the direct reduction of hydrazones **4** and **5** effectively provided hydrazine derivatives **8** and **9**, respectively, which were inactive in any PPAR isoform. The SAR studies indicated that the side chain at the 2-position in the benzopyran nucleus required the C=N double bond in the hydrazone function to activate the PPAR isoforms. Structurally speaking, the hydrazone function alongside the presence or absence of a prenylated moiety on the elongated side chain can modulate the activation of the PPAR isoforms. In addition, hydrazones **4**, **5** and **14** greatly attenuated inflammatory markers such as IL-6 and MCP-1, on the THP-1 cells via NF- κ B activation, which are associated with obesity, type 2 diabetes and the progression of metabolic disorders. Therefore, benzopyran hydrazones emerge as novel dual PPAR α/γ or dual PPAR α/δ agonists with the potential for treat metabolic diseases.

4. Experimental section

4.1. Chemistry methods

High-resolution electrospray ionization mass spectrometry (HRMS



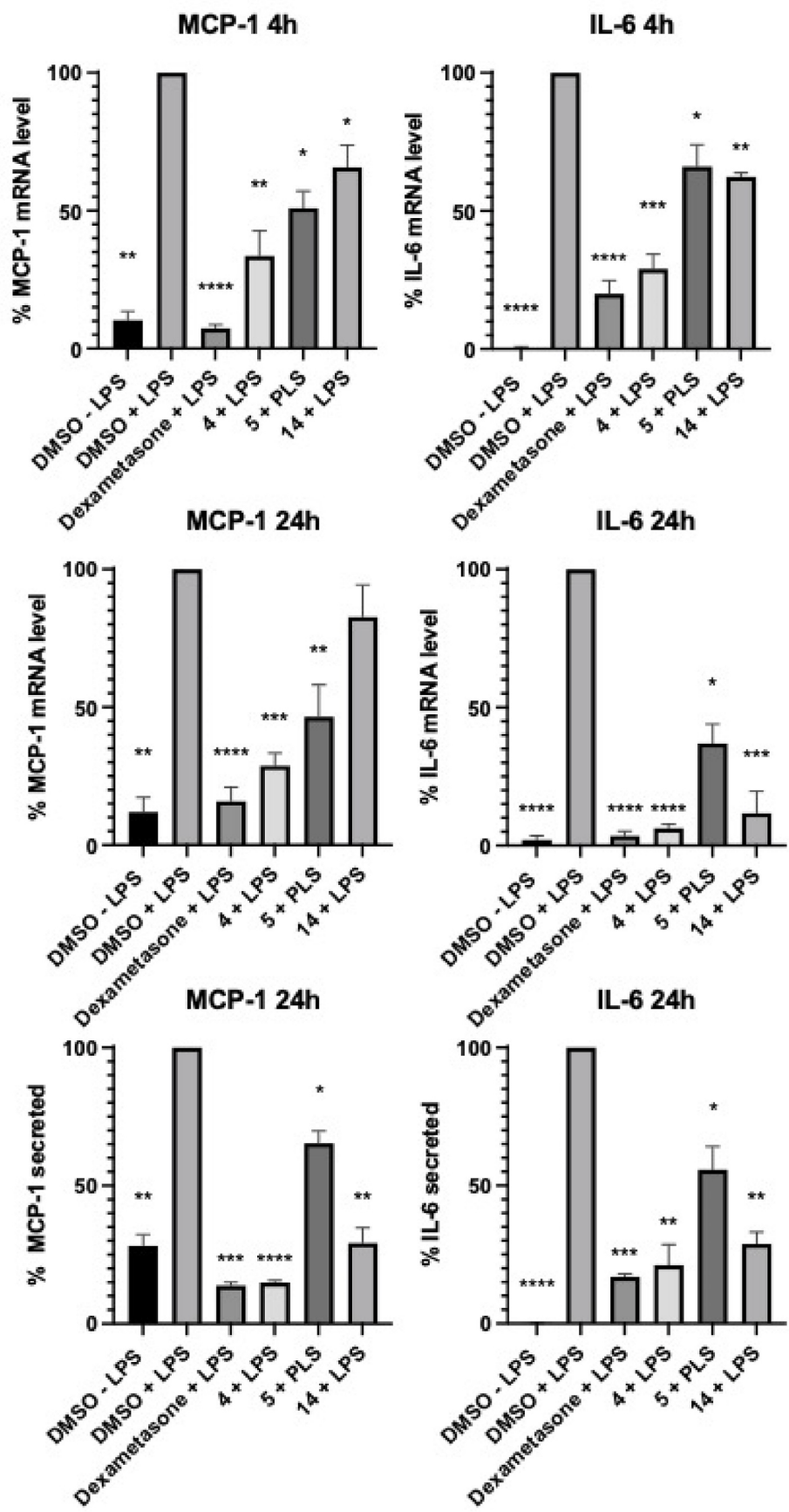
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Fig. 4. Spatial view of interactions for WY-14,643, rosiglitazone or GW501516, and active benzopyran hydrazones bonded in the binding pocket of PPAR α , PPAR γ or PPAR δ by docking experiments. Spatial view of (A) WY-14,643 (grey) and **4** (green)/PPAR α interaction, (B) WY-14,643 (grey) and **5** (green)/PPAR α , (C) WY-14,643 (grey) and **14** (green)/PPAR α , (D) rosiglitazone (grey) and **4** (pink)/PPAR γ interaction, (E) rosiglitazone (grey) and hydrazone **5** (purple)/PPAR γ , (F) GW501516 (grey) and **14** (pink)/PPAR δ . The orange, blue or burgundy zone represent the binding site for PPAR α , PPAR γ or PPAR δ , respectively, and the rest is shown in pink color or light green. The names of the residues stabilizing the complex are remarked in the figure.

(ESI)) was performed on a TripleTOFTM 5600 LC/MS/MS System (AB SCIEX) (Toronto, Canada). Liquid chromatography-mass spectrometry detection was performed on a liquid chromatography UHPLC apparatus (Shimadzu, LCMS-8040) coupled to a tandem mass spectrometry (MS/MS) triple quadrupole equipped with electrospray ionization (ESI) ion source (Shimadzu, Kyoto, Japan). ¹H and ¹³C NMR spectra were recorded on a Bruker AC-300 or AC-500 (Bruker Instruments, Kennewick, WA). The assignments in ¹H and ¹³C NMR were made by COSY 45, HSQC and HMBC correlations. Chemical shifts (δ) are reported in ppm relative to an internal deuterated solvent reference, with multiplicities indicated as s (singlet), br (broad singlet), d (doublet), t (triplet), q (quartet) m (multiplet) or dd (doublet). All reactions were monitored by analytical TLC with silica gel 60 F254 (Merck 5554). All reactions were monitored by analytical thin-layer chromatography with silica gel 60 F254 (Merck 5554; Merck Group, Darmstadt, Germany). Residues were purified by silica gel column chromatography (40–63 μ m, Merck Group). Solvents and reagents were purchased from the commercial sources Scharlab S.L. (Barcelona, Spain) and Sigma-Aldrich (St. Louis, MO), respectively, and used without further purification unless otherwise noted. Dry and freshly distilled solvents were used in those reactions performed under N₂. Quoted yields are of purified material. Final compounds were purified to $\geq 95\%$ as assessed by ¹H NMR and LC-MS/MS analysis.

4.2. Ethyl 3-(6-(p-fluorobenzyloxy)-2-methyldihydrobenzopyran-2-yl)propanoate (**2**)

Ethyl 3-(6-hydroxy-2-methylbenzopyran-4-one-2-yl)propanoate (1.2 g, 4.32 mmol) was dissolved in a mixture of AcOH-H₂O (2:1, v/v) (14 mL). Then, Zn dust (5.07 g, 76.45 mmol) followed by concentrated HCl (9 mL) was added slowly in small portions for 30 min. After stirring for an additional 1 h at room temperature, water (15 mL) was added and the mixture was extracted with AcOEt (3 $\times$ 15 mL). The organic layers were combined, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure. The residue was purified by column chromatography (hexane/EtOAc, 85:15) to yield the ethyl 3-(6-hydroxy-2-methyldihydrobenzopyran-2-yl)propanoate (**1**), 0.66 g, 2.50 mmol, 58%) as a colorless oil [24]. A solution of benzopyran ester **1** (500 g, 1.89 mmol), p-fluorobenzyl chloride (0.3 mL, 2.46 mmol), anhydrous K₂CO₃ (0.4 g, 2.83 mmol) in absolute EtOH (20 mL) was refluxed under N₂ for 4 h. The mixture was evaporated, water was added (20 mL) and the mixture was extracted with dichloromethane (3 $\times$ 15 mL). The organic layers were combined, washed with 1 M HCl and brine, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure. The residue was purified by column chromatography (hexane/EtOAc, 90:10) to yield the O-protected benzopyran ester (**2**) (556 mg, 1.49 mmol, 79%) as a colorless oil. ¹H NMR (300 MHz, CDCl₃) δ 7.41–7.36 (m, 2H, CH-2'', CH-6''), 7.09–7.03 (m, 2H, CH-3'', CH-5''), 6.72–6.67 (m, 3H, CH-5, CH-7, CH-8), 4.94 (s, 2H, OCH₂Ph-p-F), 4.13 (q, J = 7.1 Hz, 2H, CO₂CH₂CH₃), 2.76 (t, J = 6.7 Hz, 2H, CH₂-4), 2.48 (t, J = 7.7 Hz, 2H, CH₂-2'), 2.0–1.75 (m, 4H, CH₂-3, CH₂-1'), 1.24 (t, J = 7.2 Hz, 3H, CO₂CH₂CH₃), 1.23 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃) δ 173.6 (COOCH₂CH₃), 162.3 (d, J_{CF} = 244 Hz, C-4''), 152.0 (C-6), 147.9 (C-8a), 133.1 (d, J_{CF} = 3 Hz, C-1''), 129.1 (d, J_{CF} = 8 Hz, CH-2'', CH-6''), 121.4 (C-4a), 117.7 (CH-5), 115.2 (d, J_{CF} = 25 Hz, CH-3'', CH-5''), 115.1 (CH-7), 114.4 (CH-8), 74.6



(caption on next page)

Fig. 5. Anti-inflammatory effects of hydrazones **4**, **5**, **14**, and dexamethasone by inhibition of pro-inflammatory cytokine IL-6 and chemokine MCP-1 production in LPS-induced THP-1 cells. THP-1 cells were pretreated with benzopyran hydrazones **4**, **5** and **14** (10 μ M) or dexamethasone (Dexa) (positive control at 10^{-8} μ M) for 1 h before stimulation with LPS (100 ng/mL) for 4 h and 24 h. The levels of IL-6 and MCP-1 were measured using qPCR (A, B, C, D) or ELISA (E, F). The data are expressed as % of LPS stimulated condition and presented as means $\pm$ SD of three independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. LPS-induced group.

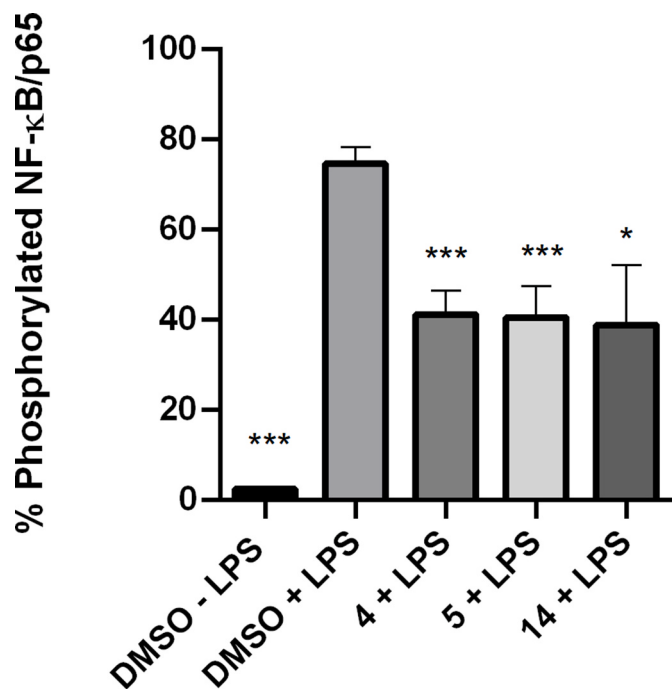


Fig. 6. Phosphorylated NF- κ B/p65% of hydrazones **4**, **5**, **14** at 10 μ M. on the THP-1 cells. The data are presented as means $\pm$ SD of three independent experiments performed in triplicate. * $P < 0.05$, *** $P < 0.001$ vs. LPS-induced group.

(C-2), 69.9 (OCH₂Ph-*p*-F), 60.3 (CO₂CH₂CH₃), 34.3 (CH₂-1'), 31.0 (CH₂-3), 28.7 (CH₂-2'), 23.6 (CH₃-2), 22.2 (CH₂-4), 14.1 (CO₂CH₂CH₃); HREIMS m/z calcd for C₂₂H₂₅O₄F [M]⁺ 372.1737, found: 372.1730.

4.3. 3-(6-((*p*-Fluorobenzyl)oxy)-2-methyldihydrobenzopyran-2-yl)propanal (**3**)

A solution of **2** (250 mg, 0.67 mmol) in anhydrous dichloromethane (10 mL) at -78°C under N₂ atmosphere was stirred for 10 min. To this solution was added dropwise 3 (203 mg, 0.62 mmol, 92%) as a colorless oil. ¹H NMR (300 MHz, CDCl₃) δ 9.78 (t, $J = 1.6$ Hz, 1H, CHO), 7.39–7.37 (m, 2H, CH-2'', CH-6''), 7.08–7.04 (m, 2H, CH-3'', CH-5''), 6.74–6.67 (m, 3H, CH-5, CH-7, CH-8), 4.94 (s, 2H, OCH₂Ph-*p*-F), 2.77–2.74 (m, 2H, CH₂-4), 2.62–2.59 (m, 2H, CH₂-2'), 2.03–1.75 (m, 4H, CH₂-3, CH₂-1'), 1.26 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃) δ 202.2 (CHO), 162.4 (d, $J_{\text{CF}} = 244$ Hz, C-4''), 152.1 (C-6), 147.7 (C-8a), 133.1 (d, $J_{\text{CF}} = 3$ Hz, C-1''), 129.3 (d, $J_{\text{CF}} = 8$ Hz, CH-2'', CH-6''), 121.4 (C-4a), 117.7 (CH-5), 115.2 (d, $J_{\text{CF}} = 25$ Hz, CH-3'', CH-5''), 115.2 (CH-7), 114.5 (CH-8), 74.6 (C-2), 69.9 (OCH₂Ph-*p*-F), 38.4 (CH₂-2'), 31.8 (CH₂-1'), 31.2 (CH₂-3), 23.7 (CH₃-2), 22.3 (CH₂-4); HRMS (ESI) m/z calcd for C₂₀H₂₁O₃F [M+H]⁺ 329.1547, found: 329.1552.

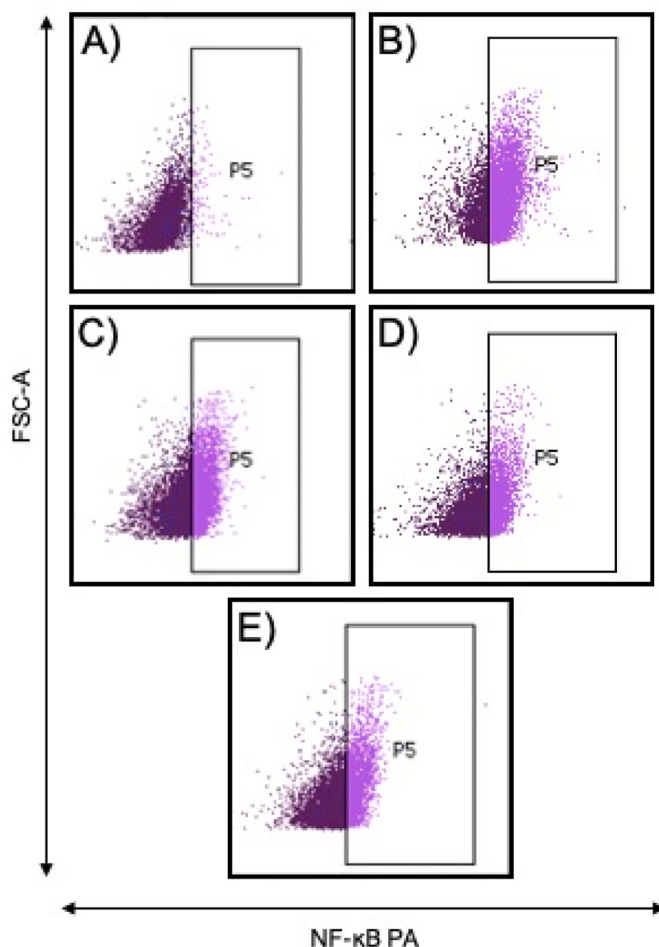


Fig. 7. Representative images of one of the flow cytometry analyses for **4**, **5** and **14** at 10 μ M of activated NF- κ B on the THP-1 macrophages. A) DMSO, B) **4**, C) **5**, D) **14**.

4.4. General procedure for synthesis of hydrazone benzopyrans (**4**, **5**)

A mixture of aldehyde **3** (58.1 mg, 0.18 mmol) and 2-hydrazinobenzothiazole (48.6 mg, 0.294 mmol) or 1-[5-chloro-3-(trifluoromethyl)-2-pyridyl]hydrazine (62.2 mg, 0.29 mmol) in anhydrous dichloroethane (3 mL) was stirred for 1 h under N₂. The resulting mixture was basified with 5% aq NH₃ and then, water was added and extracted EtOAc (3 x 5 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and evaporated to dryness. The residues obtained were purified by silica gel column chromatography (hexane/EtOAc, 80:20) to afford hydrazone **4** (68.6 mg, 0.14 mmol, 80 %) as a reddish oil or **5** (82.3 mg, 0.16 mmol, 89 %) as a yellowish oil.

4.4.1. 2-(2-(3-(6-((*p*-Fluorobenzyl)oxy)-2-methylbenzodihydropyran-2-yl)propylidene)hydrazineyl)benzo[d]thiazole (**4**)

¹H NMR (300 MHz, CDCl₃) δ 8.96 (1H, brs, NH), 7.62 (dd, $J = 7.8$ Hz, 1.3 Hz, 1H, CH-3'''), 7.42–7.35 (m, 3H, CH-2'', CH-6'', CH-6'''), 7.29–7.25 (m, 2H, CH-4''', CH-3'), 7.11–7.03 (m, 3H, CH-3'', CH-5'', CH-5'''), 6.72–6.67 (m, 3H, CH-5, CH-7, CH-8), 4.92 (s, 2H, OCH₂Ph-*p*-F), 2.77–2.75 (m, 2H, CH₂-4), 2.36–2.31 (m, 2H, CH₂-2'), 1.78–1.74 (m, 4H,

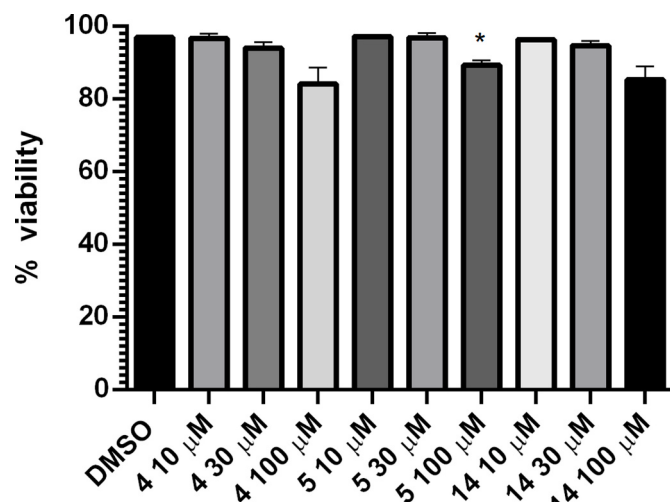


Fig. 8. Cytotoxic effects of hydrazones **4**, **5**, **14** on the THP-1 cells. The data are presented as means $\pm$ SD of three independent experiments performed in triplicate. *P < 0.05 vs. LPS-induced group.

CH₂-1', CH₂-3), 1.27 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃): δ 168.9 (C-1'''), 162.4 (d, J_{CF} = 244 Hz, C-4''), 152.0 (C-6), 149.9 (C-2a'''), 147.9 (CH-3', C-8a), 133.1 (d, J_{CF} = 3 Hz, C-1''), 129.8 (C-6a'''), 129.2 (d, J_{CF} = 8 Hz, CH-2', CH-6''), 125.9 (CH-4''), 121.7 (CH-5''), 121.5 (C-4a), 121.3 (CH-3''), 117.7 (CH-5), 117.6 (CH-6''), 115.3 (d, J_{CF} = 21 Hz, CH-3'', CH-5''), 115.2 (CH-7), 114.4 (CH-8), 75.1 (C-2), 69.9 (OCH₂Ph-*p*-F), 35.7 (CH₂-1'), 31.1 (CH₂-3), 26.7 (CH₂-2), 23.9 (CH₃-2), 22.3 (CH₂-4); HRMS (ESI) m/z calcd for C₂₇H₂₆FN₃O₂S [M+H] + 476.1803, found: 476.1808.

4.4.2. 5-Chloro-2-(2-(3-(6-((*p*-fluorobenzyl)oxy)-2-methylbenzodihydropyran-2-yl) propylidene)hydrazineyl)-3-(trifluoromethyl)pyridine (**5**)

¹H NMR (300 MHz, CDCl₃) δ 8.47–8.44 (m, 1H, CH-6''), 8.36–8.33 (brs, 1H, NH), 7.72 (d, J = 2 Hz, 1H, CH-4''), 7.42–7.36 (m, 3H, CH-2'', CH-6'', CH-3'), 7.07–7.01 (m, 2H, CH-3'', CH-5''), 6.75–6.64 (m, 3H, CH-5, CH-7, CH-8), 4.92 (s, 2H, OCH₂Ph-*p*-F), 2.78–2.71 (m, 2H, CH₂-4), 2.66–2.57 (m, 2H, CH₂-2'), 1.96–1.72 (m, 4H, CH₂-3, CH₂-1'), 1.29 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃): δ 162.4 (d, J_{CF} = 244 Hz, C-4''), 152.3 (q, J_{CF} = 4 Hz, C-2''), 152.0 (C-6), 149.6 (CH-3'), 147.8 (C-8a), 144.5 (CH-6''), 133.8 (q, J_{CF} = 3 Hz, CH-4''), 133.1 (d, J_{CF} = 3 Hz, C-1''), 129.2 (d, J_{CF} = 8 Hz, CH-2'', CH-6''), 123.2 (q, J_{CF} = 270 Hz, CF₃), 121.6 (C-4a), 118.5 (q, J_{CF} = 33 Hz, C-3''), 117.8 (CH-5), 115.3 (d, J_{CF} = 21 Hz, CH-3'', CH-5''), 114.6 (CH-7), 114.3 (C-5''), 113.8 (CH-8), 75.2 (C-2), 70.0 (OCH₂Ph-*p*-F), 36.4 (CH₂-1'), 31.2 (CH₂-3), 26.9 (CH₂-2'), 23.9 (CH₃-2), 22.3 (CH₂-4); HRMS (ESI) m/z calcd for C₂₆H₂₄ClF₄N₃O₂ [M+H] + 522.1566, found: 522.1567.

4.5. General procedure for O-deprotection to prepare compounds **6** and **7**

A solution of compound **4** (30 mg, 0.063 mmol) or **5** (33 mg, 0.063 mmol) in anhydrous dichloromethane (1 mL) was added BBr₃ (0.25 mL, 0.25 mmol) at -78°C , and stirred for 15 min under N₂ atmosphere. Then, the reaction mixture was stirred under N₂ and at room temperature for an additional 45 min. The resulting mixture was basified with 5% NH₃ and then, water was added and extracted with EtOAc (3 x 5 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue obtained was purified by silica gel column chromatography (CH₂Cl₂/MeOH, 98:2) to afford compound **6** (21 mg, 0.06 mmol, 91 %) as a greenish oil and compound **7** (24 mg, 0.06 mmol, 92 %) as a yellowish oil, respectively.

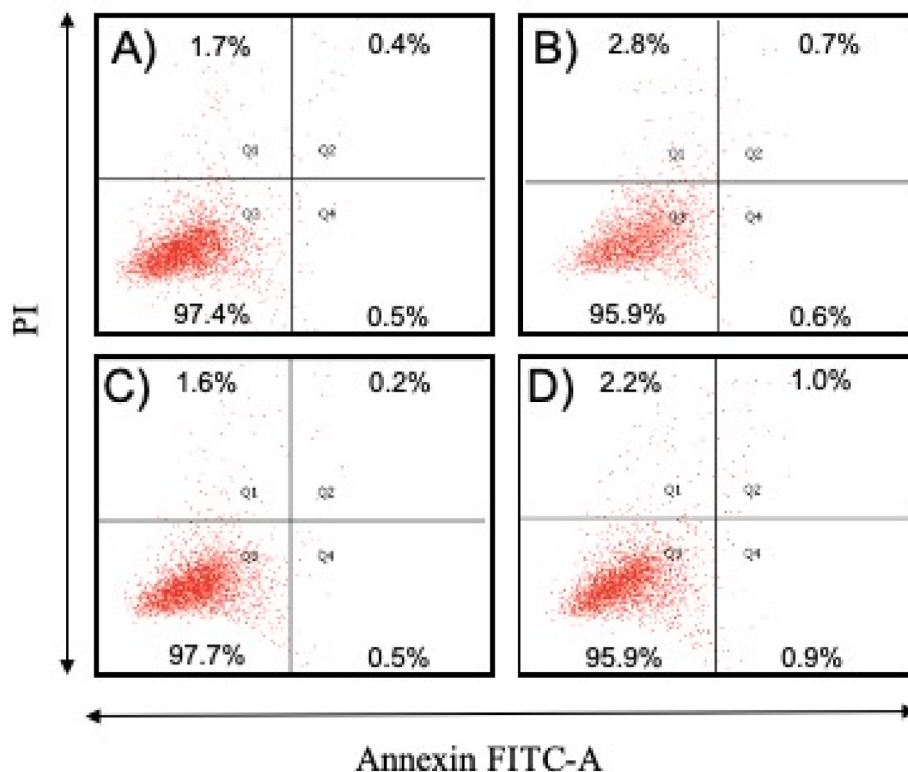


Fig. 9. Representative images of one of the apoptosis/necrosis analyses for **4**, **5** and **14** at 30 μM on the THP-1 macrophages. A) DMSO, B) **4**, C) **5**, D) **14**.

4.5.1. 2-(3-(2-(Benzo[d]thiazol-2-yl)hydrazineylidene)propyl)-2-methylbenzodihydropyran-6-ol (6)

¹H NMR (300 MHz, CDCl₃ + 1 drop CD₃OD) δ 7.59 (dd, J = 7.8 Hz, 1.3 Hz, 1H, CH-3''), 7.45–7.42 (m, 1H, CH-6''), 7.37–7.23 (m, 2H, CH-4'', CH-3'), 7.16–7.06 (m, 1H, CH-5''), 6.67–6.43 (m, 3H, CH-5, CH-7, CH-8), 2.75–2.65 (m, 2H, CH₂-4), 2.51–2.37 (m, 2H, CH₂-2'), 1.91–1.64 (m, 4H, CH₂-1', CH₂-3), 1.27 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃ + 1 drop CD₃OD): δ 168.0 (C-1''), 149.9 (C-2a''), 149.4 (C-6), 147.7 (C-8a), 146.9 (CH-3'), 128.9 (C-6a''), 126.3 (CH-4''), 122.4 (CH-5''), 121.6 (C-4a), 121.3 (CH-3''), 117.7 (CH-5), 117.4 (CH-6''), 115.3 (CH-7), 114.5 (CH-8), 74.9 (C-2), 35.5 (CH₂-1'), 31.2 (CH₂-3), 26.9 (CH₂-2'), 23.9 (CH₃-2), 22.1 (CH₂-4); HRMS (ESI) m/z calcd for C₂₀H₂₂N₃O₂S [M+H] + 368.1433, found: 368.1417.

4.5.2. 2-(3-(2-(5-chloro-3-(trifluoromethyl)pyridin-2-yl)hydrazineylidene)propyl)-2-methylbenzodihydropyran-6-ol (7)

¹H NMR (300 MHz, CDCl₃ + 1 drop CD₃OD) δ 8.39–8.38 (m, 1H, CH-6''), 7.90 (brs, 1H, NH), 7.74–7.73 (m, 1H, CH-4''), 7.31 (t, J = 3 Hz, 1H, CH-3'), 6.64–6.52 (m, 3H, CH-5, CH-7, CH-8), 2.77–2.69 (m, 2H, CH₂-4), 2.62–2.55 (m, 2H, CH₂-2'), 1.92–1.72 (m, 4H, CH₂-3, CH₂-1'), 1.28 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃ + 1 drop CD₃OD) δ 150.8 (CH-6''), 149.6 (q, J_{CF} = 4 Hz, C-2''), 148.8 (C-8a, C-6), 147.4 (CH-3'), 135.4 (q, J_{CF} = 4 Hz, CH-4''), 123.2 (q, J_{CF} = 270 Hz, CF₃), 121.7 (C-4a), 118.5 (q, J_{CF} = 33 Hz, C-3''), 117.7 (CH-5), 115.4 (CH-7), 114.6 (CH-8), 108.8 (C-5''), 75.0 (C-2), 36.2 (CH₂-1'), 31.1 (CH₂-3), 26.8 (CH₂-2'), 23.9 (CH₃-2), 22.1 (CH₂-4); HRMS (ESI) m/z calcd for C₁₉H₁₇ClF₃N₃O₂ [M+H] + 412.1034, found: 412.1028.

4.6. General procedure for synthesis of hydrazine benzopyrans (8, 9)

A solution of compound 4 (37.9 mg, 0.08 mmol) or 5 (41.4 mg, 0.08 mmol) in anhydrous MeOH (4 mL) was added NaBH₄CN (15 mg, 0.238 mmol) and two drops of BF₃OEt. The reaction was refluxed for 1 h under N₂ atmosphere. The resulting mixture was basified with 5% NH₃ and then, water was added and extracted ethyl acetate (3 x 5 mL). The combined organic layers were washed with water, dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue obtained was purified by silica gel column chromatography (hexane/EtOAc, 60:40 or hexane/EtOAc, 85:15) to afford compound 8 (37 mg, 0.08 mmol, 96%) as a bluish grey oil and compound 9 (31 mg, 0.06 mmol, 75%) as a reddish yellow oil.

4.6.1. 2-(3-(3-(6-((p-Fluorobenzyl)oxy)-2-methylbenzodihydropyran-2-yl)propyl)hydrazineyl) benzo[d]thiazole (8)

¹H NMR (300 MHz, CDCl₃) δ 7.64 (dd, J = 8 Hz, 1.2 Hz, 1H, CH-3''), 7.48 (dd, J = 8 Hz, 1.2 Hz, 1H, CH-6''), 7.43–7.37 (m, 2H, CH-2'', CH-6''), 7.29 (td, J = 8 Hz, 1.2 Hz, 1H, CH-4''), 7.13–7.03 (m, 3H, CH-3'', CH-5'', CH-5''), 6.75–6.65 (m, 3H, CH-5, CH-7, CH-8), 4.94 (s, 2H, OCH₂Ph-p-F), 3.07–2.92 (m, 2H, CH₂-3'), 2.71 (t, J = 7 Hz, 2H, CH₂-4), 1.82–1.61 (m, 6H, CH₂-1', CH₂-2', CH₂-3), 1.24 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃): δ 174.0 (C-1''), 162.3 (d, J_{CF} = 244 Hz, C-4''), 152.3 (C-6), 151.9 (C-2a''), 148.0 (C-8a), 133.1 (d, J_{CF} = 3 Hz, C-1''), 130.6 (C-6a''), 129.2 (d, J_{CF} = 8 Hz, CH-2'', CH-6''), 125.6 (CH-4''), 121.6 (C-4a), 121.2 (CH-3''), 121.0 (CH-5''), 118.3 (CH-6''), 117.7 (CH-5), 115.3 (d, J_{CF} = 21 Hz, CH-3'', CH-5''), 115.1 (CH-7), 114.3 (CH-8), 75.4 (C-2), 69.9 (OCH₂Ph-p-F), 52.6 (CH₂-3'), 36.9 (CH₂-1'), 30.8 (CH₂-3), 23.8 (CH₃-2), 22.3 (CH₂-2'), 22.0 (CH₂-4); HRMS (ESI) m/z calcd for C₂₇H₂₆FN₃O₂S [M+H] + 476.1803, found: 476.1802.

4.6.2. 5-Chloro-2-(3-(3-(6-((p-Fluorobenzyl)oxy)-2-methylbenzodihydropyran-2-yl)propyl)hydrazineyl)-3-(trifluoromethyl)pyridine (9)

¹H NMR (300 MHz, CDCl₃) δ 8.32–8.28 (m, 1H, CH-6''), 7.72 (d, J = 2.0 Hz, 1H, CH-4''), 7.42–7.33 (m, 2H, CH-2'', CH-6''), 7.11–7.01 (m, 2H, CH-3'', CH-5''), 6.77–6.61 (m, 3H, CH-5, CH-7, CH-8), 4.94 (s, 2H, OCH₂Ph-p-F), 2.98–2.89 (m, 2H, CH₂-3'), 2.80–2.71 (m, 2H, CH₂-4),

1.88–1.61 (m, 6H, CH₂-3, CH₂-1', CH₂-2'), 1.27 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃): δ 162.4 (d, J_{CF} = 244 Hz, C-4''), 153.3 (q, J_{CF} = 4 Hz, C-2''), 152.0 (C-6), 149.6 (CH-6''), 148.2 (C-8a), 135.2 (q, J_{CF} = 4 Hz, CH-4''), 133.3 (d, J_{CF} = 3 Hz, C-1''), 129.2 (d, J_{CF} = 8 Hz, CH-2'', CH-6''), 123.2 (q, J_{CF} = 270 Hz, CF₃), 121.7 (C-4a), 119.9 (q, J_{CF} = 33 Hz, C-3''), 117.8 (CH-5), 115.4 (d, J_{CF} = 21 Hz, CH-3'', CH-5''), 115.2 (CH-7), 114.4 (CH-8), 109.3 (C-5''), 75.6 (C-2), 70.0 (OCH₂Ph-p-F), 51.7 (CH₂-3'), 36.9 (CH₂-1'), 31.0 (CH₂-3), 24.0 (CH₂-2'), 22.4 (CH₃-2), 22.0 (CH₂-4); HRMS (ESI) m/z calcd for C₂₆H₂₇ClF₄N₃O₂ [M + H] + 524.1728, found: 524.1683.

4.7. General procedure for the synthesis of hydrazone benzopyrans (13, 14)

A solution of compound aldehyde (3) (130 mg, 0.40 mmol) in anhydrous THF (5 mL) was stirred at –78 °C under N₂ for 15 min and treated with 0.5 M isopropenylmagnesium bromide solution (4.8 mL, 1.35 mmol). The mixture was stirred at –78 °C for 3 h. The resulting mixture reaction was quenched by the addition of a half-saturated aqueous NH₄Cl solution. The reaction was stirred for 15 min at room temperature. Subsequently, water was added, and the mixture was extracted with ethyl acetate (3 x 15 mL). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and evaporated to dryness under reduced pressure. The residue obtained (162 mg) was treated without further purification with 10 mL of triethylorthoacetate and a catalytic amount of isobutyric acid (3 drops). The mixture was stirred at 140 °C for 2 h. After cooling, the mixture was concentrated under reduced pressure to remove the excess triethylorthoacetate. Then, water was added and extracted with dichloromethane (3 x 15 mL), dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc, 98:2) to yield benzopyran ester 11 (84 mg, 0.2 mmol, 48 %) as a white solid.

4.7.1. Ethyl 7-(6-((p-fluorobenzyl)oxy)-2-methyldihydrobenzopyran-2-yl)-4-methylhept-4-enoate (11)

¹H NMR (500 MHz, CDCl₃) δ 7.41–7.36 (m, 2H, CH-2'', CH-6''), 7.09–7.06 (m, 2H, CH-3'', CH-5''), 6.72–6.66 (m, 3H, CH-5, CH-7, CH-8), 5.14 (t, J = 7.0 Hz, 1H, CH-3'), 4.94 (s, 2H, OCH₂Ph-p-F), 4.11 (q, J = 7.4 Hz, 2H, CO₂CH₂CH₃), 2.72 (t, J = 6.8, 2H, CH₂-4), 2.41–2.35 (m, 2H, CH₂-6'), 2.29–2.27 (m, 2H, CH₂-5'), 2.09–2.05 (m, 2H, CH₂-2'), 1.84–1.72 (m, 2H, CH₂-3), 1.66–1.60 (m, 2H, CH₂-1'), 1.60 (s, 3H, CH₃-4'), 1.27 (s, 3H, CH₃-2), 1.23 (t, J = 7.3 Hz, 3H, CO₂CH₂CH₃); ¹³C NMR (125 MHz, CDCl₃) δ 173.4 (CO), 162.4 (d, J_{CF} = 244 Hz, C-4''), 151.9 (C-6), 148.2 (C-8a), 133.5 (C-4'), 133.3 (d, J_{CF} = 3 Hz, C-1''), 129.2 (d, J_{CF} = 8.3 Hz, CH-2'', CH-6''), 124.9 (C-3'), 121.7 (C-4a), 117.8 (CH-5), 115.3 (d, J_{CF} = 24.8 Hz, CH-3'', CH-5''), 115.2 (CH-7), 114.4 (CH-8), 75.6 (C-2), 70.1 (OCH₂Ph-p-F), 60.2 (CO₂CH₂CH₃), 39.2 (CH₂-6'), 34.6 (CH₂-1'), 33.2 (CH₂-5'), 30.9 (CH₂-3), 24.1 (CH₃-2), 22.4 (CH₂-2'), 22.2 (CH₂-4), 15.8 (CH₃-4'), 14.2 (CO₂CH₂CH₃); HREIMS m/z calcd for C₂₇H₃₃FO₄ [M]⁺ 441.2436, found: 441.2441.

4.7.2. 7-(6-((p-Fluorobenzyl)oxy)-2-methyldihydrobenzopyran-2-yl)-4-methylhept-4-enal (12)

The title compound was prepared from benzopyran ester 11 (80 mg, 0.18 mmol) following the general procedure for the synthesis of aldehyde 3 to afford compound 12 (63 mg, 0.16 mmol, 89%) as a colorless oil. ¹H NMR (300 MHz, CDCl₃) δ 9.74 (t, J = 2 Hz, 1H, CHO), 7.42–7.36 (m, 2H, CH-2'', CH-6''), 7.09–7.03 (m, 2H, CH-3'', CH-5''), 6.73–6.67 (m, 3H, CH-5, CH-7, CH-8), 5.14 (t, J = 7 Hz, 1H, CH-3'), 4.94 (s, 2H, OCH₂Ph-p-F), 2.74 (t, J = 7 Hz, 2H, CH₂-4), 2.50–2.47 (m, 2H, CH₂-6'), 2.33–2.30 (m, 2H, CH₂-5'), 2.15–2.08 (m, 2H, CH₂-2'), 1.86–1.73 (m, 2H, CH₂-3), 1.71–1.54 (m, 5H, CH₂-1', CH₃-4), 1.27 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃) δ 202.6 (CHO), 162.4 (d, J_{CF} = 244 Hz, C-4''), 151.9 (C-6), 148.2 (C-8a), 133.4 (C-4'), 133.3 (d, J_{CF} = 3 Hz, C-1''), 129.2 (d, J_{CF} = 8 Hz, CH-2'', CH-6''), 124.9 (C-3'), 121.7 (C-4a), 117.8 (CH-5),

115.3 (d, J_{CF} = 25 Hz, CH-3'', CH-5''), 115.2 (CH-7) 114.4 (CH-8), 75.6 (C-2), 70.1 (OCH₂Ph-*p*-F), 42.1 (CH₂-6'), 39.2 (CH₂-1'), 31.8 (CH₂-5'), 31.0 (CH₂-3), 24.1 (CH₃-2), 22.4 (CH₂-2'), 22.2 (CH₂-4), 16.0 (CH₃-4'); EIMS m/z (%) 396.50 [M]⁺.

4.7.3. 2-(2-(7-(6-((*p*-Fluorobenzyl)oxy)-2-methylbenzodihydropyran-2-yl)-4-methylhept-4-en-1-ylidene)hydrazineyl)benzo[d]thiazole (13)

The title compound was prepared from aldehyde **12** (26.50 mg, 0.07 mmol) and 2-hydrazinobenzothiazole (18.4 mg, 0.11 mmol) following the general procedure for the synthesis of compound **4**. The residue obtained was purified by silica gel column chromatography (CH₂Cl₂/MeOH, 98:2) to afford compound **13** (26.5 mg, 69.5%) as a reddish oil. ¹H NMR (300 MHz, CDCl₃) δ 7.64 (dd, J = 8 Hz, 1.2 Hz, 1H, CH-3''), 7.45 (dd, J = 8 Hz, 1.2 Hz, 1H, CH-6''), 7.42–7.35 (m, 2H, CH-2'', CH-6''), 7.34–7.24 (m, 2H, CH-7', CH-4''), 7.16–7.01 (m, 3H, CH-5'', CH-3'', CH-5''), 6.74–6.63 (m, 3H, CH-5, CH-7, CH-8), 5.22–5.08 (m, 1H, CH-3'), 4.93 (s, 2H, OCH₂Ph-*p*-F), 2.70 (t, 2H, J = 7.0 Hz, CH₂-4), 2.42–2.38 (m, 2H, CH₂-5'), 2.21 (t, 2H, J = 7.7 Hz, CH₂-6'), 2.17–2.05 (m, 2H, CH₂-2'), 1.89–1.67 (m, 2H, CH₂-3), 1.63 (s, 3H, CH₃-4'), 1.61–1.53 (m, 2H, CH₂-1'), 1.26 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃) δ 168.5 (C-1''), 162.4 (d, J_{CF} = 244 Hz, C-4''), 151.9 (C-6), 150.3 (C-2a''), 148.2 (CH-7'), 147.6 (C-8a), 133.6 (C-4'), 133.2 (d, J_{CF} = 3 Hz, C-1'), 130.1 (C-6a''), 129.3 (d, J_{CF} = 8 Hz, CH-2'', CH-6''), 125.9 (CH-4''), 125.4 (CH-3'), 121.8 (CH-5''), 121.7 (C-4a), 121.3 (CH-3''), 119.0 (CH-6''), 117.7 (CH-5), 115.3 (d, J_{CF} = 21 Hz, CH-5'', CH-3''), 115.2 (CH-7), 114.3 (CH-8), 75.6 (C-2), 70.0 (OCH₂Ph-*p*-F), 39.2 (CH₂-1'), 36.3 (CH₂-6'), 31.0 (CH₂-3), 30.7 (CH₂-5'), 24.1 (CH₃-2), 22.4 (CH₂-2'), 22.1 (CH₂-4), 15.9 (CH₃-4'); HRMS (ESI) m/z calcd for C₃₂H₃₅FN₃O₂S [M+H]⁺ + 544.2429, found: 544.2409.

4.7.4. 5-Chloro-2-(2-(7-(6-((*p*-fluorobenzyl)oxy)-2-methylbenzodihydropyran-2-yl)-4-methylhept-4-en-1-ylidene)hydrazineyl)-3-(trifluoromethyl)pyridine (14)

The title compound was prepared from aldehyde **12** (26.50 mg, 0.07 mmol) and 1-[5-chloro-3-(trifluoromethyl)-2-pyridyl]hydrazine (23.6 mg, 0.114 mmol) following the general procedure for the synthesis of compound **5**. The residue obtained was purified by silica gel column chromatography (CH₂Cl₂/MeOH, 98:2) to afford compound **14** (24.5 mg, 0.04 mmol, 59.5%) as a yellowish oil. ¹H NMR (300 MHz, CDCl₃) δ 8.42–8.40 (m, 1H, CH-6''), 7.96 (brs, 1H, NH), 7.74–7.72 (m, 1H, CH-4''), 7.40–7.36 (m, 2H, CH-2'', CH-6''), 7.28–7.26 (m, 1H, CH-7'), 7.08–7.02 (m, 2H, CH-3'', CH-5''), 6.72–6.65 (m, 3H, CH-5, CH-7, CH-8), 5.21–5.16 (m, 1H, CH-3'), 4.93 (s, 2H, OCH₂Ph-*p*-F), 2.74–2.70 (m, 2H, CH₂-4), 2.57–2.40 (m, 2H, CH₂-5'), 2.33–2.09 (m, 4H, CH₂-6', CH₂-2'), 1.84–1.76 (m, 2H, CH₂-3), 1.74 (s, 3H, CH₃-4'), 1.73–1.56 (m, 2H, CH₂-1'), 1.27 (s, 3H, CH₃-2); ¹³C NMR (75 MHz, CDCl₃) δ 162.4 (d, J_{CF} = 244 Hz, C-4''), 151.9 (C-6), 150.9 (q, J_{CF} = 4 Hz, CH-2''), 148.4 (CH-7'), 148.2 (C-8a), 146.9 (CH-6''), 135.2 (q, J = 4 Hz, CH-4''), 135.5 (C-4'), 133.2 (d, J_{CF} = 3 Hz, C-1'), 129.2 (d, J_{CF} = 8 Hz, CH-2'', CH-6''), 125.4 (CH-3'), 123.4 (q, J_{CF} = 270 Hz, CF₃), 121.6 (C-4a), 121.2 (C-5''), 117.7 (CH-5), 115.3 (d, J_{CF} = 21 Hz, CH-3'', CH-5''), 115.2 (CH-7), 114.3 (CH-8), 108.5 (q, J_{CF} = 33 Hz, C-3''), 75.5 (C-2), 70.0 (OCH₂Ph-*p*-F), 39.1 (CH₂-1'), 36.7 (CH₂-6'), 31.0 (CH₂-3), 30.6 (CH₂-5'), 24.0 (CH₃-2), 22.4 (CH₂-2'), 22.1 (CH₂-4), 15.8 (CH₃-4'); HRMS (ESI) m/z calcd for C₃₁H₃₁ClF₄N₃O₂ [M – H]⁺ + 588.2040, found: 588.2017.

4.8. Evaluation of PPAR activity by transactivation assays

PPAR transcriptional activity of synthesized compounds was performed using a human chimera PPAR/Gal4 gene reporter luciferase system for each compound and compared with WY-14,643 (pirinixic acid, Sigma-Aldrich, St. Louis, MO) at 10 μ M for PPAR α , rosiglitazone (Sigma-Aldrich) at 1 μ M for PPAR γ and GW501516 (Sigma-Aldrich) at 1 μ M for PPAR β/δ . Cos-7 cells (CRL-1651, ATCC, Manassas, VA) were maintained under standard culture conditions (Dulbecco's modified Eagle's minimal essential medium: DMEM supplemented with 10% fetal

calf serum [FCS], Thermo Fisher Scientific, Waltham, MA) at 37 °C in a humidified atmosphere of 5% CO₂. The medium was changed every 2 days. Cells (5.5 $\times$ 10⁵ cells/mL) were seeded in 60-mm dishes in DMEM supplemented with 10% FCS and incubated at 37 °C for 16 h prior to transfection. Cells were transfected in DMEM with the jetPEI transfection reagent (Polyplus-Transfection S.A., Strasbourg, France) using the reporter plasmid pG5-TK-pGL3 in combination with one of the expression plasmids, pGal4hPPAR α , pGal4hPPAR γ or pGal4hPPAR β/δ . The pCMV- β -galactosidase expression plasmid was included as a control of transfection efficiency. The transfection was stopped after 16 h by the addition of DMEM supplemented with 0.2% FCS, and cells were detached with trypsin and re-seeded in 96-well plates and incubated for 6 h in DMEM containing 0.2% FCS. Cells were then incubated for 24 h in DMEM containing 0.2% FCS and increasing concentrations of the compound tested or vehicle (DMSO, 0.1% final concentration). At the end of the experiment, cells were washed once with ice-cold phosphate buffered saline (PBS) and lysed, and luciferase and β -galactosidase activity were measured. Each experiment was achieved in triplicate, and mean $\pm$ standard error (SEM) values were calculated using GraphPad Prism 5 Software.

4.9. Molecular modelling

In molecular simulations for PPAR α , PPAR γ and PPAR δ receptors, we have used the same methodology as previously reported [20,24]. Docking calculations were carried out by using the Autodock 4.2 program [50] to localize the different ligands in the binding pocket; such complexes were used as starting structures for MD simulations. We used a molecular mechanics generalized born surface area (MM-GBSA) free energy decomposition analysis to determine the molecular interactions between the different ligands with PPAR α , PPAR γ and PPAR δ receptors. The 3D crystal structure of PPAR γ in complex with rosiglitazone (PDB code: 4eMA) of PPAR α in complex with WY-14643 (PDB code: 4BCR), and of PPAR δ in complex with GW501516 (PDB code: 5U46) were used for MD simulations. The missing loop (261–275) in the PPAR γ was modeled based on the 3D structure (PPAR γ 1PRG model) [51] using the Swiss-Model server [52]. Geometries of the complexes obtained from docking were soaked in boxes of explicit water using the TIP3P model and subjected to MD simulation. MD simulations were performed with the Amber 22 software package [52]. The geometry of the system went through a two-step energy minimization process: in the first step, the backbone atoms of the complex were constrained with 10.0 kcal/(mol Å²) force constants; in the second step, all solute and solvent atoms were allowed to move with no constraint to obtain the final relaxed geometry. The nonbonded interaction cutoff was kept at its default value, and the particle mesh Ewald method was also used. Simulations were performed with a total of 90 ns for each complex (three runs of 30 ns). The cluster process was carried out in the following manner: using the 90 ns obtained from three runs, 15 ns were discarded (the first 5 ns of each individual run). The remaining 75 ns were submitted to the cluster process, from which 10 different families of complexes were obtained. The free energy decomposition by residue was calculated using the *mm_pbsa* program in Amber22 [52]. Each ligand–residue pair includes four energy terms: van der Waals contribution (ΔE_{vdw}), electrostatic contribution (ΔE_{ele}), polar desolvation term (ΔG_{GB}), and nonpolar desolvation term (ΔG_{SA}), which are summarized in the following equation: $\Delta G_{ligand-residue} = \Delta E_{vdw} + \Delta E_{ele} + \Delta G_{GB} + \Delta G_{SA}$. MD trajectories and the explicit water molecules were removed from the snapshots.

4.10. Study of anti-inflammatory activity

THP-1 human monocytes (ATCC, TIB-202™, Manassas, VA) were cultured in RPMI-1640 medium containing gentamycin (40 mg/mL), 1% (v/v) glutamine and 10% (v/v) fetal calf serum and maintained in a 37 °C, 5% CO₂ incubator. Cells (2 $\times$ 10⁶ cells/mL) were seeded in 6-well

plates and were differentiated into macrophages by the addition of phorbol-12-myristate-13-acetate at 5 ng/mL (PMA, Promega). After 48 h, the cells were washed with PBS and replaced with 0% SVF medium for 1 h. After 1 h the depravation medium was replaced with stimulation medium containing hydrazones (**4**, **5** and **14** at 10 μ M) or vehicle (<0.02% DMSO). The following hour, THP-1 macrophages were stimulated with LPS (100 ng/mL) in presence or not of PPAR activators hydrazones **4**, **5** and **14** at 10 μ M, and secreted cytokine levels were measured 24 h later with ELISA kits according to the manufacturer's instructions (R&D System, Minneapolis, MN, USA). Cytokine mRNA levels were determined by quantitative PCR analysis.

4.11. Flow cytometry analysis of activated NF- κ B

The effect of hydrazones **4** and **5** on LPS-induced NF- κ B activation was determined in THP-1 cells by flow cytometry. THP-1 cells were incubated for 24 h with compounds (10 μ M) or vehicle (0.02% DMSO) and then stimulated for 1 h with LPS 100 ng/mL.

Cells were then fixed and permeabilized using a commercial kit (Transcription Factor Phospho Buffer Set, 563239, BD Biosciences). Cells were then incubated with saturated amounts of a PE-conjugated monoclonal antibody against human NF- κ B p65 (clone K10-895.12.50, IgG2B, BD Biosciences), for 45 min at 4 °C in the dark. Samples were run in a flow cytometer (BD LSRFortessa™ X-20, BD Biosciences). Results are presented as the mean fluorescence intensity of p65-NF- κ B-expressing (PE fluorescence) THP-1.

4.12. Study of cytotoxic activity

THP-1 human monocytes (ATCC, TIB-202™, Manassas, VA) were cultured in RPMI-1640 medium containing gentamycin (40 mg/mL), 1% (v/v) glutamine and 10% (v/v) fetal calf serum and maintained in a 37 °C, 5% CO₂ incubator. Cells (1×10^5 cells/mL) were seeded in 24-well plates and were differentiated into macrophages by the addition of phorbol-12-myristate-13-acetate at 5 ng/mL (PMA, Promega). After 48 h, the cells were washed with PBS and replaced with the different compounds at 10 μ M, 30 μ M and 100 μ M in complete medium or vehicle (<0.2% DMSO). Then, all cells were processes with Annexin V-assay kit (ANXCKF7, Immunoestep, Salamanca, Spain) according to the manufacturer's instructions. BD LSR Fortessa cytometer (BD Biosciences, New Jersey, USA) was used for samples and cell analyses were performed by using BD FACSDiva X20 Software).

4.13. Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical analyses were carried out by one-way or two-way ANOVA, followed by Tukey's or Dunnett's multiple comparisons test (GraphPad Prism 9). Paired analysis was performed by Wilcoxon matched-pairs signed rank test (GraphPad Prism 9). Differences with a p-value < 0.05 were considered statistically different.

CRediT authorship contribution statement

Ainhua García: Writing – original draft, Investigation, Formal analysis. **Laura Vila:** Methodology, Investigation, Formal analysis. **Isabelle Duplan:** Software, Methodology, Investigation, Formal analysis. **María Ayelén Schiel:** Software, Methodology, Investigation, Formal analysis. **Ricardo D. Enriz:** Validation, Supervision. **Nathalie Hennuyer:** Visualization, Validation, Supervision, Investigation. **Bart Staels:** Visualization, Supervision. **Nuria Cabedo:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Diego Cortes:** Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmech.2024.116125>.

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