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Frontal affinity chromatography with MS detection of the ligand binding domain of PPARγ receptor: Ligand affinity screening and stereoselective ligand–macromolecule interaction

E. Calleri[a], G. Fracchiolla[b], R. Montanari[c], G. Pochetti[c], A. Lavecchia[d], F. Loiodice[b], A. Laghezza[b], L. Piemontese[b], G. Massolini[a], C. Temporini[a,*]

[a] Dipartimento di Scienze del Farmaco, Università degli Studi di Pavia, 27100 Pavia, Italy
[b] Dipartimento Farmaco-Chimico, Università degli Studi di Bari "Aldo Moro", 70126 Bari, Italy
[c] Istituto di Cristallografia, Consiglio Nazionale delle Ricerche, Montelibretti, 00015 Monterotondo Stazione, Roma, Italy
[d] Dipartimento di Chimica Farmaceutica e Tossicologica, "Drug Discovery" Laboratory, Università degli Studi di Napoli "Federico II", 80131 Napoli, Italy

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ABSTRACT

In this study we report the development of new chromatographic tools for binding studies based on the gamma isoform ligand binding domain (LBD) of peroxisome proliferator-activated receptor (PPARγ) belonging to the nuclear receptor superfamily of ligand-activated transcription factors. PPARγ subtype plays important roles in the functions of adipocytes, muscles, and macrophages with a direct impact on type 2 diabetes, dyslipidemia, atherosclerosis, and cardiovascular disease. In order to set up a suitable immobilization chemistry, the LBD of PPARγ receptor was first covalently immobilized onto the surface of aminopropyl silica particles to create a PPARγ-Silica column for zonal elution experiments and then onto the surface of open tubular (OT) capillaries to create PPARγ-OT capillaries following different immobilization conditions. The capillaries were used in frontal affinity chromatography coupled to mass spectrometry (FAC–MS) experiments to determine the relative binding affinities of a series of chiral fibrates. The relative affinity orders obtained for these derivatives were consistent with the EC_{50} values reported in literature. The optimized PPARγ-OT capillary was validated by determining the K_d values of two selected compounds. Known the role of stereoselectivity in the binding of chiral fibrates, for the first time a detailed study was carried out by analysing two enantioselective couples on the LBD-PPARγ capillary by FAC and a characteristic two-stairs frontal profile was derived as the result of the two saturation events. All the obtained data indicate that the immobilized form of PPARγ-LBD retained the ability to specifically bind ligands.

1. Introduction

The peroxisome proliferator-activated receptors (PPARs), a group of transcription factors belonging to the nuclear receptor superfamily, have emerged as key players in the regulation of metabolic pathways and cellular functions extremely relevant in the pathophysiology of diabetes and obesity and in the connected cardiovascular and cerebrovascular complications [1,2]. As such, the three PPAR isoforms designated α, γ and δ, bind to fatty acids and their metabolites regulating the expression of genes involved in the transport, metabolism and buffering of these ligands within cells [1,3–6].

Because of the well-documented therapeutic actions of their synthetic agonists, PPARs have been the focus of intense academic and pharmaceutical research since their discovery in the early 1990s. The thiazolidinedione (TZD) anti-diabetic agents (e.g. rosiglitazone and pioglitazone) are PPARγ agonists whose insulin-sensitizing actions are mediated largely by pleiotropic effects in adipose tissue [5–8] while the fibrate anti-atherosclerotic, hypolipidemic agents (e.g. fenofibrate and gemfibrozil) are PPARα agonists [5,6,9,10]. Despite their wide prescription, PPAR-activating drugs revealed unwanted-effects that cannot be under-estimated [11,12]. To overcome these side-effects, novel PPARs ligands have been identified that are potentially superior therapeutic agents for metabolic disease. These include PPARα/γ dual agonists or PPARα/γ/δ pan-agonists, which beneficially alter carbohydrate and lipid metabolism in a coordinate manner, and selective PPARγ modulators (SPPARγMs) with robust anti-diabetic efficacy and fewer adverse effects than currently available agonists.

In our previous studies, we reported the synthesis and biological activity of some chiral carboxylic acid derivatives whose

* Corresponding author. Tel.: +39 0382 987383; fax: +39 0382 422975.
E-mail address: caterina.temporini@unipv.it (C. Temporini).

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structure is related to that of the active metabolites of the PPARα agonist clofibrate and the selective PPARγ modulator metaglidasen, respectively [13–19]. These compounds showed an interesting dual activity towards PPARα and PPARγ receptors with the stereochemistry playing a crucial role in the receptor activation.

To rapidly identify novel PPARs ligands with the aim of developing safer and more effective drugs, a robust ligand binding assay amenable to high-throughput screening towards all PPAR isoforms would be desirable. Numerous technologies, such as competition radioreceptor assay, protease protection assay, coactivator-dependent receptor ligand assay (CARLA), and scintillation proximity assay (SPA) have been used to measure the binding constants for ligand–PPARγ interactions and in the screening of ligands [20–25]. By employing these technologies, some important parameters evaluating the binding affinity for many ligands to PPARs, such as K_i (equilibrium dissociation constant of a ligand determined in inhibition studies), K_d (equilibrium dissociation constant), and IC_{50} (molar concentration of an antagonist that reduces the response to an agonist by 50%), have been obtained. However, these technologies either need specific radio-ligands for labeling or a reporter gene has to be transfected into the cell to be detected, both of which limit the screening speed for finding new ligands, especially at the primary screening step. Recently, new technologies as the surface plasmon resonance (SPR) biosensor technology, circular dichroism (CD) spectroscopy or fluorescence polarization (FP) technology and isothermal microcalorimetry (ITC) have been recognized as powerful tools in monitoring receptor–ligand interactions with advantages of no use of radioactive ligands [26–29]. Among the new technologies, ITC is one of the most rigorous methods for characterizing protein–ligand interactions which are detected from the intrinsic heat (binding enthalpy) change of the reaction. From an ITC experiment it is possible to directly obtain the molar binding ratio of the interaction and the affinity constant (K_d) of the protein–ligand complex.

In addition to these technologies, separative approaches, such as chromatographic and electromigration methods, have been significantly applied for studies on small molecule-biomacromolecule interactions [30]. In particular, over the past 15 years, biointeraction chromatography has emerged as useful and promising technique for studying drug–protein interactions and for determining dissociation constants [31]. In this technique the biological target is immobilized on a chromatographic support, and the retention of analytes is based on the same type of specific, reversible interactions that are found in biological systems, such as the binding of a drug to a receptor. Two general ways can be used in high-performance affinity chromatography experiments: zonal elution and frontal analysis. In both these formats, the protein of interest is used as the immobilized target and an injection (zonal) or application (frontal) of analyte is made onto the affinity column. With both techniques by examining the elution time (zonal elution chromatography) or volume (frontal affinity chromatography or FAC) of the analyte after it has passed through the column, it is possible to obtain information on the equilibrium constants that describe the binding of the analyte to the immobilized target. Zonal elution differs from frontal analysis in aspects that a small plug of sample (linear elution condition) rather than a continuous application is introduced into HPAC column. Chromatographic data obtained from zonal elution is generally characterized by retention factor (k) of injected solute and the value of k is related to how strongly a compound interacts with the immobilized target. Differently, in frontal affinity chromatography as ligands flow through the column and bind with the target, individual ligands are retained in the column on the basis of their affinity for the target and detected as characteristic breakthrough curves. The saturation of the target by the analyte produces a vertical rise in the chromatographic trace, which ends, or plateaus, when the target is saturated. The mean position (inflection point) of the breakthrough curve is the experimental parameter used to derive the breakthrough volume.

From a discovery standpoint, aside from the utility of the frontal analysis method to provide precise and accurate K_d measurements on single ligands, interfacing of FAC to mass spectrometry (MS) enables the screening of compounds mixtures and provides the opportunity to rank order binding strengths in a single experiment as each compound has a unique m/z value [32,33]. In principle, FAC can be also used to derive information on a stereoselective binding event if the immobilized receptor acts as a chiral selective receptor. This opportunity has been very recently underlined from Slon-Usakiewicz by a pioneering experiment in this regard using immobilized renin and infusing a racemic leucine containing peptide [34].

In spite of the enchanting new applications of this technique, still FAC–MS has been applied to a small number of targets of pharmaceutical interest. In the current study this approach has been extended to the ligand binding domain of PPARγ isoform to explore the use of FAC–MS as a method for investigating ligand–PPAR binding. The use of PPARγ-LBD for the experiments, instead of the full-length nuclear receptor, is justified by the fact that the presence of the other domains does not affect the binding of the ligands [35].

In order to set up a suitable immobilization chemistry, the LBD of PPARγ receptor was first covalently immobilized onto the surface of aminopropyl silica particles to create a PPARγ-Silica column and then onto the surface of open tubular capillaries to create PPARγ-OT columns following different immobilization conditions. The OT columns were used in FAC–MS experiments to determine the relative binding affinities of a series of chiral fibrates and the results were compared with previously reported data of activity.

In order to validate the optimized PPARγ-OT capillary, the K_d values of two selected compounds were calculated by frontal analysis experiments and the FAC–MS results were compared with the affinity constants obtained on purpose by ITC.

Known the role of stereoselectivity in the binding of chiral fibrates, the ability of the capillary system to discriminate between enantioselective interactions was fully investigated for two enantiomeric couples by frontal affinity chromatography and a characteristic two-stairs frontal profile was derived as the results of the two saturation events.

The data from this study confirm that the PPARγ-OT column can be successfully used to determine the binding affinity for a single compound/enantiomer and to screen a mixture of multiple compounds. Moreover, the microcalorimetry analysis performed on two ligands of the series provided binding affinity values which were found in a very good agreement with those measured by the method object of the present study.

All these results suggest that the developed FAC–MS system can be very useful in structure–activity relationship (SAR) studies on PPARγ ligands and may represent an important tool in modern medicinal chemistry studies devoted to the identification of new molecules endowed with activity towards this particular type of nuclear receptor.

2. Experimental

2.1. Materials

Aminopropylsilica (5 μm, 100 Å pores) was from Macherey-Nagel GmbH & Co. KG, (Düren, D), 3-aminopropyltriethoxysilane, glutaraldehyde, $NaBH_3CN$, monoethanolamine, CH_3COONH_4, KH_2PO_4, CH_3OH and DMSO were supplied form Sigma-Aldrich Chemical Co. (St. Louis, MO). Acetonitrile was from Carlo Erba

Reagenti (Rodano, MI, I). Stainless steel columns (2.1 mm × 30 mm) used for home-made packed silica columns preparation were from Alltech (Sedriano, MI, I). Silica-fused capillary (100 μm I.D. × 0.375 mm × 40 cm) used to immobilize PPARγ-LBD was from Thermo Fisher Scientific (San Jose, CA). The water used in this study was prepared using a Milli-Q Water purification system (Millipore Corporation, Bedford, MA). BRL 49653 (rosiglitazone) was purchased from Cayman Chemical (MI, USA).

Medium and other cell culture reagents were purchased from Sigma (Milan, Italy).

Human hepatoblastoma cell line HepG2 was purchased from Interlab Cell Line Collection (Genoa, Italy).

The expression vectors expressing the chimeric receptors containing the yeast GAL4-DNA binding domain fused to the human PPARγ ligand binding domain, and the reporter plasmid for these GAL4 chimeric receptors (pGAL5TKpGL3) containing five repeats of the GAL4 response elements upstream of a minimal thymidine kinase promoter that is adjacent to the luciferase gene were described previously [36]. The LBD of human PPARγ was expressed as N-terminal His-tagged protein using a pET28 vector and purified onto a Ni^{2+}-nitriloacetic acid column (GE Healthcare) [18].

2.2. Apparatus

Receptor immobilization on aminopropyl silica and zonal elution experiments were performed with an Agilent HP-1100 series modular system (Palo Alto, CA, USA) connected to an HPLC ChemStation (Revision A.04.01). The system was equipped with a manual Rheodyne sample valve (20 μL loop), an UV detector (223 nm) and thermostat oven (25 ± 0.5 °C). The mobile phase delivered at 300 μL min^{-1} was 70/30 phosphate buffer 50 mM, pH 4.0, 5.5 and 7.4/ACN.

Frontal analysis experiments were carried out in a chromatographic system consisting of a syringe pump (Thermo Fisher) delivering, at 2.5 μL min^{-1}, an isocratic mobile phase of 90% ammonium acetate 10 mM pH 7.4, 10% MeOH (500 μL-syringe). To improve sensitivity, the eluent from capillary column was mixed to an organic make-up flow (100% MeOH pumped at 5 μL min^{-1} with a syringe pump) *via* a T-connection before ESI-MS. The Mass spectrometer used was an LTQ linear ion trap MS with electrospray ionization (ESI) ion source controlled by Xcalibur software 1.4 (Thermo Finnigan, San Jose, CA). The syringe pump of the chromatographic system was also used for the receptor immobilization on the inner surface of the capillaries diverting to waste the solutions. Luciferase activity in cell extracts was determined by a luminometer (VICTOR^3 V Multilabel Plate Reader, PerkinElmer).

2.3. Methods

2.3.1. Cell culture and transfections

Human hepatoblastoma cell line HepG2 was cultured in minimum essential medium (MEM) containing 10% of heat-inactivated foetal bovine serum, 100 U penicillin G mL^{-1}, and streptomycin sulphate 100 μg mL^{-1} at 37 °C in a humidified atmosphere of 5% CO_2. For transactivation assays, 10^5 cells per well were seeded in a 24-well plate and transfections were performed after 24 h with CAPHOS, a calcium–phosphate method, according to the manufacturer's guidelines. Cells were transfected with expression plasmids encoding the fusion protein GAL4/PPARγ-LBD (30 ng), pGAL5TKpGL3 (100 ng), pCMVβgal (200 ng). Four hours after transfection, cells were treated for 20 h with the indicated ligands in triplicate. Luciferase activity in cell extracts was then determined by a luminometer. β-Galactosidase activity was determined using β-D-galactopyranoside as described previously [37]. All transfection experiments were repeated at least twice.

2.3.2. Immobilization of PPARγ on aminopropyl silica

Aminopropyl silica stationary phase previously packed in a stainless still column (2.1 I.D. × 30 mm) was inserted into the HPLC system and conditioned for 20 min with a mobile phase consisting of phosphate buffer (50 mM, pH 7.4) at 0.5 mL min^{-1}. Then, the column was activated with a 10% glutaraldehyde solution in phosphate buffer (50 mM, pH 7.4) for 5 h at 0.3 mL min^{-1}. The stationary phase was then washed with phosphate buffer (100 mM, pH 7.4) for 30 min at 0.5 mL min^{-1} to remove the excess of glutaraldehyde.

An aliquot of 350 μL of PPARγ solution in 20 mM Tris buffer pH 8, 150 mM NaCl, 10% glycerol, 1 mM TCEP (approximately 5 mg protein) was diluted to 10 mL with phosphate buffer (100 mM, pH 7.4), was added to the matrix and left to react overnight.

The Schiff bases were reduced by flowing with a 0.1 M $NaBH_3CN$ solution in phosphate buffer (100 mM, pH 7.4) for 1.5 h at 0.5 mL min^{-1}. The column was then washed with phosphate buffer (100 mM, pH 7.4) and for 3 h with 0.1 M monoethanolamine solution in phosphate buffer (100 mM, pH 7.4) Finally, the column was washed with a mobile phase consisting of phosphate buffer (100 mM, pH 7.4) Approximately 3 mg receptors were immobilized as estimated by the difference between the UV absorbance at the beginning and at the end of the immobilization step.

The PPAR-column obtained was stored at 4 °C in phosphate buffer (100 mM, pH 7.4) containing 0.1% sodium azide.

Non-specific interaction of ligands to silica support was measured using a control support which was prepared by following the same Shiff base method except that no receptor was added during the immobilization step. This control material was washed and stored in the same manner as the immobilized PPAR support.

2.3.3. Immobilization of PPARγ on open tubular capillaries

Human PPARγ was covalently immobilized on silica capillaries after optimization of a previously reported procedure [38,39]. The open tubular capillary (100 μm I.D. × 40 cm) was first activated with 2 mL 0.5 N NaOH at 100 μL min^{-1}, washed with 1 mL water at 100 μL min^{-1} and dried at 95 °C for 1 h. Then, 1 mL of 3-amminopropyltrietoxysilane solution (10%, v/v in water) was pumped by syringe pump at 100 μL min^{-1} and incubated at 95 °C for 30 min. This step was repeated twice and the capillary stored overnight at room temperature. A 1% glutaraldehyde solution (2 mL in phosphate buffer 50 mM, pH 7.0) was pumped at 100 μL min^{-1}, unreacted aldehyde removed with 1 mL pure buffer followed by the infusion of human PPARγ solution at 50 μL min^{-1} (250 μg protein in 500 μL phosphate buffer 50 mM, pH 7.0, corresponding to a final concentration of 0.5 μg mL^{-1}) and the unreacted groups inactivated with monoethanolamine for 1 h at 5 μL min^{-1} (capillary C1). Three other capillaries were prepared following the same procedure but with some modifications.

Capillary C2, the protein infusion was performed at lower flowrate (5 μL min^{-1}); capillary C3, the Shiff bases were reduced with $NaBH_3CN$ 5 mg mL^{-1} in phosphate buffer flushed over the capillary for 1 h at 100 μL min^{-1} before inactivation with ethanolamine; capillary C4, the protein was infused complexed with rosiglitazone 1 μM. Before performing the ranking experiment, rosiglitazone was removed by washing with the mobile phase used for the FAC experiments and monitoring its elution by MS detection.

2.3.4. Sample preparation

All ligands were dissolved in DMSO at concentration of 1 mM and stock solutions were stored at −20 °C. Further dilutions were carried out in mobile phase to obtain samples to be injected (zonal elution) or infused (frontal affinity chromatography).

2.3.5. Isothermal microcalorimetry

Isothermal calorimetric titration of selected ligands was performed using ITC_{200} calorimeter (MicroCal, Inc. Northampton, MA,

USA). Protein was extensively dialyzed against the buffer of choice (Hepes 20 mM, pH 8.0, TCEP 1 mM or Tris HCl 20 mM, pH 8.0, TCEP 1 mM) with Amicon Ultra filters and the dialysis buffer was used to dilute the ligand stock solutions (20 or 50 mM in DMSO). Titrations were performed at 25 °C. DMSO was added to the protein solution in the same percentage of the ligand solution (below 5%). Protein solution (50 μM) was added to the sample cell and the ligand solution (10 times more concentrated than the protein) was injected into the cell in 19 aliquots of 2 μL for 4 s (the first injection was 0.4 mL for 0.8 s) with delay intervals between injections of 180 s. Blank experiments were performed by titrating the ligand into buffer, added with the same percentage of DMSO as in the ligand solution, to take into account for dilution heats. The titration curves were analyzed using the Origin software provided by MicroCal. In the case of the ligand ***R*-8** the parameter K_d was kept fixed during the refinement to obtain a best fit, expecially at the beginning of the curve.

2.4. Chromatographic studies

2.4.1. Zonal elution experiments

The relative affinity order of studied compounds on PPARγ-column was measured by zonal elution chromatography, injecting individually enantiomers **1–7** at 0.1 mM (20 μL). Zonal elution experiments were performed at 0.3 mL min^{-1} and the elution of compounds was monitored at 225 nm. All chromatographic data were obtained at room temperature with 50 mM KPB at different pH values (7.4, 5.5 and 4.0) and 30% CH_3OH as mobile phase. The void time was determined using DMSO as a non-retained solute.

2.4.2. Frontal affinity chromatography–MS experiments

For ranking experiments, equimolar solutions (1 μM each dissolved in mobile phase) of enantiomers **1–7** reported in Table 1 were infused at 2.5 μL min^{-1}. 500 μL of mobile phase was infused between injections. During enantioselective frontal affinity chromatographic studies the concentration of the analytes infused over PPARγ-OT capillary was 50 nM for single enantiomer (***R*-8** and ***S*-8**) and racemic mixtures infusion (***R*-8**/***S*-8** and ***S*-5**/***R*-5**).

For the K_d determination of ***R*-8**, a series of concentrations of 0.2, 0.3, 0.4, 0.5 and 1.0 μM were applied to the capillary at 2.5 μL min^{-1}.

Dedicated MS methods were developed in the different studies. During FAC–MS ranking experiments, full scan mass spectra were generated in negative ion mode under constant instrumental conditions: source voltage 5.0 kV, capillary voltage 46 V, sheath gas flow 22 (arbitrary units), auxiliary gas flow 2 (arbitrary units), capillary temperature 250 °C, tube lens voltage −85.06 V. Scan range 175–500 *m*/*z*.

For enantioselective frontal affinity chromatography of enantiomers ***S*-8** and ***R*-8** and their racemic mixture, as well as for ***R*-8** K_d determination, the instrument was operated in the electrospray positive mode under MS/MS conditions. Fragmentation was achieved with nitrogen gas (Isolation width 1.0 *m*/*z* and Collision Energy 35.0) and ion transition monitored was *m*/*z* 453.6 → 353.3. ***R*-5** and ***S*-5** enantiomers, as well as the corresponding racemate, were monitored using single ion monitoring (M-1) at *m*/*z* value 312.6, with an isolation width of 2 Da. Source voltage 5.0 kV, capillary voltage 46 V, sheath gas flow 22 (arbitrary units), auxiliary gas flow 2 (arbitrary units), capillary temperature 250 °C, tube lens voltage −85.06 V.

2.5. Data analysis

2.5.1. Zonal elution experiments

Chromatographic data obtained from zonal elution were characterized by retention factor (*k*) of injected solute and the value of *k* is related to how strongly a compound interacts with immobilized receptor in column by Eq. (1)

$$k = \frac{t_r - t_m}{t_m} = \frac{(K_{a1}n_1 + \cdots + K_{an}n_n)m_{Ltot}}{V_m} = \frac{K'_a m_{Ltot}}{V_m} \quad (1)$$

where K_a' and m_{Ltot} are global association constant and the moles of binding sites for the analyte in the column while t_r, t_m, and V_m are retention time, void time and void volume of column, respectively.

The same samples were also injected on the control column under the same chromatographic conditions. The results obtained for the control column expressed as retention time (non-specific retention factor) were subtracted from those obtained for PPAR column to adjust for any non-specific interactions between the analytes and the support's surface. The specific retention factor was expressed by the difference between total and non-specific retention factors [40,41].

$$k_{specific} = k_{total} - k_{non\text{-}specific} \quad (2)$$

2.5.2. FAC–MS screening

The frontal affinity profiles were obtained from analysis of the mass spectra that give rise to the TIC. Selective ion chromatograms were reconstructed from the TIC for each analyte. Each extracted ion breakthrough curve was analyzed with a polynomial equation of degree 3 ($y = ax^3 + bx^2 + cx + d$) to fit the chromatographic data, and the inflection point, corresponding to the breakthrough time, was determined by the second derivative.

2.5.3. Dissociation constant determination

The dissociation constants (K_d) for ***R*-8** and ***S*-5** were calculated on PPARγ-OT column C4 and C5 respectively using the following equation:

$$[L] \times (V - V_0) = \frac{B_{max} \times [L]}{K_d + [L]} \quad (3)$$

where [*L*] is the concentration of the ligand, *V* (μL) is the retention volume of ligand calculated at the breakthrough time, V_0 is the retention volume of a non-retained ligand and B_{max} is the number of dynamic active binding sites for each ligand in the two capillaries. In this study ***S*-1** was considered as a non-retained compound being 9600 nM its EC_{50} value.

From the linearization of the plot of $1/[L]$ versus $1/(V - V_0)$, K_d was calculated ($K_d = m/q$ of the derived equation).

2.5.4. Determination of column capacity by FAC–MS

The number of active binding sites (B_{max}) in all the prepared capillaries was calculated using a second method previously described, which allows determination of B_{max} from Eq. (4) by infusion of a single ligand with known K_d [42]. By using ***S*-5** as reference compound, its binding time at 1 μM concentration was calculated on each capillary and the B_{max} values accordingly estimated using Eq. (4) where V_0 is the void volume of the system (10.4 μL) and K_d is the dissociation constant of ***S*-5** compound obtained by FAC–MS.

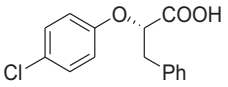
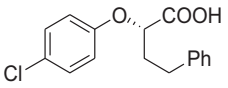
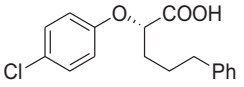
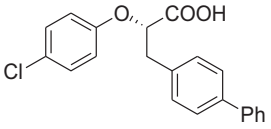
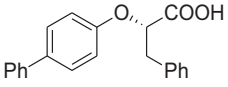
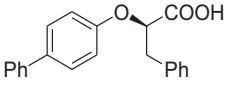
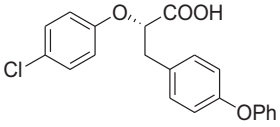
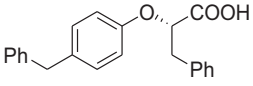
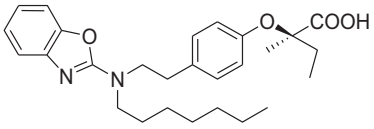
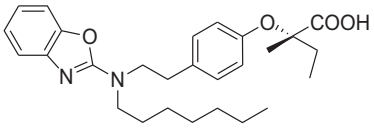
$$V - V_0 = \frac{B_{max}}{K_d + [L]} \quad (4)$$

2.5.5. Enantioselectivity studies

The enantioselective frontal affinity profiles were fitted using Matlab 7.5.0. A script was implemented to fit the data with a double-sigmoid function $g(x)$.

$g(x)$ was built through the Matlab function *fittype*. In *fittype*, nonlinear least squares method was set as fitting method, specifying the *Trust-Region* algorithm as solver of the optimization problem.

Table 1
Chemical Structure and biological data of PPARγ agonists selected for zonal elution and FAC–MS studies.

Compound	Structure	PPARγ activity	
		EC_{50}[a] (nM)	E_{max} (%)
***S*-1**		9600 ± 1500	61 ± 6
***S*-2**		7030 ± 4620	48 ± 17
***S*-3**		910 ± 330	46 ± 6
***S*-4**		49 ± 3	70 ± 6
***S*-5**		550 ± 120	60 ± 8
***R*-5**		5820 ± 3270	27 ± 4
***S*-6**		26 ± 4	68 ± 6
***S*-7**		570 ± 10	40 ± 4
***R*-8**		160 ± 45	90 ± 2
***S*-8**		860 ± 570	40 ± 7

[a] Determined by the transactivation assay.

The double-sigmoid $g(x)$ was:

$$g(x) = a_1 \frac{1}{1 + e^{-x-b_1/c_1}} + a_2 \frac{1}{1 + e^{-x-b_2/c_2}} \quad (5)$$

where a_1, a_2, b_1, b_2, c_1 and c_2 were the coefficients computed by *fittype* to fit the data. The data was then plotted using the resulting fitting model by the Matlab function *fit*. b_1 and b_2 correspond the two inflection.

The frontal analysis selectivity, when infusing a racemic mixture, was calculated using the equation,

$$\alpha_F = \frac{t_2}{t_1} \quad (6)$$

where t_1 and t_2 are breakthrough times for the first and the second enantiomers.

3. Results and discussion

3.1. Compounds selection

Compounds considered for the present study are shown in Table 1.

These chiral carboxylic acid derivatives, whose structure is related to that of the active metabolites of the PPARα agonist clofibrate and the selective PPARγ modulator metaglidasen, were selected on the basis of their different potency and efficacy towards PPARγ receptor.

All of them were prepared as pure enantiomers following the synthetic procedures reported in our previous works and were evaluated for their agonist activity on the human PPARγ (hPPARγ) subtype by the transactivation assay [13–19]. The S stereoisomers **1–8** exhibited a partial agonist behavior being their E_{max} between 40% and 60% with potencies ranging from 26 to 9600 nM. ***R*-5** was

the ligand with the lowest activity, whereas ***R*-8** displayed a full agonist profile.

Generally, there is a good correlation between the dissociation constant of the receptor–ligand complex (K_d) and the transactivation activity (EC_{50}), especially among ligands of the same series [43].

Therefore, given that K_i and K_d of all the compounds tested in the experiments were not available, we compared the retention factors to the known EC_{50} of the ligands.

3.2. Zonal elution experiments on silica column

The first point considered in the present study was the development of a suitable immobilization chemistry for the LBD of PPARγ. Two columns were prepared by using epoxy-monolithic silica and aminopropyl silica particles as supports; in addition two control columns (same chemistry without receptor) were prepared.

PPARγ was first immobilized on epoxy-modified monolithic silica following a previously reported procedure [44]. Scouting zonal elution experiments revealed that the receptor was immobilized with a non proper orientation of the binding site. Thus, the receptor was immobilized by the Schiff base method on aminopropyl silica particles activated with glutaraldehyde.

This column was used for zonal elution experiments to test the ability of the system to rank ligands according to their relative affinity and to highlight analyte–receptor interaction forces.

The first set of experiments were carried out at the physiological pH (7.4) but with a significant percentage of methanol (30%) in the mobile phase necessary to decrease the retention times of strongly bound compounds.

The level of concentration for injected compounds (0.1 mM) was found to be sufficiently low to avoid any significant changes in the retention time due to overloading effects, thus indicating that linear elution conditions were present. In general, less than a 10% variation in the retention with triplicate measurements of all injected compounds was noted indicating that a local equilibrium had been established on the receptor and control columns under these conditions.

The results of zonal elution experiments at pH 7.4 are reported in Table 2.

In these experiments the assumption is that the non-specific interactions between the support and the compound under study are the same for the control and receptor columns. If a retardation in the elution time on the experimental column is observed, the difference provides a measure of the extent of specific interactions between the injected compound and the immobilized receptor. In this way, an affinity order within a set of compounds can be obtained.

For the considered compounds, the non-specific interaction to silica support (i.e., the retention time on control column) was significant compared to the specific interaction to PPARγ. The non-specific retention time measured at pH 7.4 was found to be very high (mean non-specific retention time percentage of approximately 80%). This contribute can be corrected by simple subtraction of retention factors on the control column from those observed on the PPAR column under equivalent conditions. A protein binding calibration curve was therefore derived by plotting the specific retention factor values against functional data ($\log EC_{50}$), indicating that the column can be used to rank ligands for their relative affinity ($r = 0.9175$; $p < 0.01$ at pH 7.4).

Molecular modeling and crystallographic studies suggested that the carboxylate group of both (aryloxy)arylalkyl carboxylic acid enantiomers **1–7** and ureidofibrate enantiomers ***R*-8** and ***S*-8** forms a H-bonding network with the residues H323, H449, Y473 and S289 generally involved in the receptor activation [13–19]. For derivatives **1–7**, the aryloxy group is deeply inserted in the so-called "diphenyl pocket", forming several favorable hydrophobic interactions [14,17] while in case of ***R*-8** and ***S*-8**, the aliphatic chain and the benzoxazole ring occupy the lower and upper part of the distal cavity, there making hydrophobic contacts with the surrounding protein residues [18,19].

Thus, zonal elution studies by changing the pH of mobile phase (50 mM phosphate buffers with pH values of 7.4, 5.5 and 4.0) were carried out on both the PPARγ and control columns to examine the forces involved in ligand–receptor binding (see Table 2).

As expected, by reducing the pH, the electrostatic interaction decreases due to the reduction of carboxylic group dissociation, and the correlation with activity data becomes lower while the hydrophobic specific interactions become predominant. These results indicate that the receptor immobilized with the second procedure maintains its binding capacity. The increasing of non-specific interactions on the control column by reducing the pH suggests that these interactions are mainly electrostatic. They can be ascribed to the enhancing protonation of ethanolamine groups which interact with carboxylic groups of the analytes.

3.3. Capillary preparation and ranking experiments

Literature and author's experience suggest that, in high-performance affinity experiments, it is important to have a biochromatographic support that minimizes the aspecific interaction of the potential ligand with the chromatographic material and maximizes the specific interactions between the analyte and the target macromolecule [45,46]. Thus, the results obtained using the aminopropyl silica column were not considered optimal and an alternative technique for receptor immobilization was investigated. His-tagged LBD of PPARγ was therefore immobilized on the inner surface of a silica capillary following the same immobilization chemistry (Shiff-base method) and using a syringe pump to deliver the reagents. This strategy is particularly appealing in order to reduce the large amount of non-specific binding observed between the ligand and the solid support and also to decrease receptor consumption and analysis time [47].

To explore the ability of the system to rank mixtures containing multiple ligands, a first capillary was prepared (capillary C1, see Section 2 for details).

The capillary was tested in the ranking experiment of seven chiral structurally related fibrates (enantiomers **1–7**). Solutions of the ligands, each at 1 μM, were prepared in mobile phase buffer and continuously infused through the column. The effluent was analyzed in an ESI mass spectrometer to detect each component. The mass analysis of the library was performed in negative mode to ensure efficient ionization and detection of the compounds. Detailed chromatographic experimental conditions are reported in Section 2. Because the molecular weight for each compound is known, the individual breakthrough front of each compound was easily identified. The breakthrough times are the results of a single determination carried out on the freshly prepared capillary.

As stated before, chromatographic breakthrough times were correlated to EC_{50} values. The rank order determined by FAC–MS followed the general trend of EC_{50} values as demonstrated by the good correlation between the calculated breakthrough times and the functional data. However, a low resolution of the breakthrough curves was observed since only a 4 min resolution window was achieved (6.61–10.95 min).

Bioaffinity phases that provide breakthrough curves with increased resolution are highly desirable as these should allow small changes in elution times to be discriminated, thereby improving the reliability of the results. Accordingly, different immobilization conditions were considered such as the reduction of flow-rate for the infusion of the receptor from 50 $\mu L\,min^{-1}$ to 5 $\mu L\,min^{-1}$ in order to increase the contact time (capillary C2), the

Table 2
Zonal elution data performed at different pH values.

Compd	EC_{50}[a] (nM)	Total retention factor (k_{total})[b]			Non-specific retention factor ($k_{non\text{-}specific}$)[c]			Specific retention factor ($k_{specific}$)[d]		
		pH 7.4	pH 5.5	pH 4.0	pH 7.4	pH 5.5	pH 4.0	pH 7.4	pH 5.5	pH 4.0
***S*-6**	26	4.80	40.38	227.10	2.93	10.54	16.66	1.87	29.84	210.44
***S*-4**	45	7.75	46.20	188.32	6.20	18.73	30.43	1.55	27.47	157.89
***S*-5**	550	4.40	24.42	62.04	3.54	10.86	16.14	0.86	13.56	45.90
***S*-7**	570	3.49	24.57	124.46	2.50	7.64	13.08	0.99	16.92	111.39
***S*-3**	910	1.84	24.28	44.92	1.36	3.89	6.05	0.48	20.39	38.87
***S*-2**	7030	1.43	14.60	30.18	0.95	3.09	6.39	0.47	11.51	23.79
***S*-1**	9600	1.31	8.60	25.40	1.04	3.19	5.29	0.27	5.41	20.11

[a] Determined by the transactivation assay.
[b] The total retention factor is the retention factor measured for the different analytes on the PPARγ column.
[c] The non-specific retention factor is that obtained on the control column.
[d] The specific retention factor is the difference between total and non-specific retention factors.

Table 3
Comparison of the breakthrough times obtained by FAC–MS experiments on the five capillaries.[a]

Compound	EC_{50} (nM)	C1	C2	C3	C4	C5
B_{max}[b] (pmol)		50.98	41.25	117.32	135.95	190.22
		t[c] (min)				
***S*-1**	9600	6.61	7.18	4.46	9.28	15.27
***S*-2**	7030	6.67	7.37	6.67	9.78	15.89
***S*-3**	910	6.74	7.58	10.08	12.65	17.35
***S*-7**	570	7.61	7.42	13.73	15.24	19.94
***S*-5**	550	8.51	7.68	14.17	15.76	20.39
***S*-4**	45	9.77	10.64	24.36	30.49	28.40
***S*-6**	26	10.95	9.93	24.38	26.36	26.11

[a] The breakthrough times are from a single ranking experiment carried out on the freshly prepared capillary. See text for detailed experimental conditions.
[b] Column capacity expressed as number of active sites.
[c] t is the breakthrough time of the ligand with the immobilized LBD of PPARγ receptor.

Shiff base reduction with $NaBH_3CN$ (capillary C3), and the protection of the receptor binding pocket with a high affinity ligand during the immobilization step in order to preserve the binding pocket orientation (capillary C4) [47]. The three capillaries were tested in the same ranking experiment and the results are reported in Table 3.

It is interesting to observe that the same ranking order was obtained in the new capillaries. The reduction of the immobilization flow rate (C2) did not improve resolution of the breakthrough curve, while the reduction of the Shiff-base (C3) and the protection of the binding pocket with a high affinity ligand during the immobilization (C4) led to a significant increase of the resolution window (about 20 min). As an example Fig. 1 reports the extracted breakthrough curves for each analytes obtained with capillary C3.

The reproducibility of the method was assessed by preparing a new capillary (capillary C5) following the preparation of C4 and testing it in the screening of the same fibrates. The ranking data were highly consistent for the two capillaries with a comparable resolution window. The different run times between the capillaries can be ascribed to the use of two different protein batches. Globally, these results confirm that FAC–MS is a powerful tool that can be used as qualitative and reproducible method to discriminate between low, medium and high-affinity ligands.

3.4. Determination of ligand binding affinities

Once the procedure for the preparation of the receptor-capillary was established, the system was characterized determining the binding affinity (expressed as dissociation constant, K_d) and the number of active binding sites (B_{max}). This was accomplished by using frontal chromatography with ***R*-8** and ***S*-5** as reference ligands. For K_d determination, increasing concentrations of these analytes (1000–3000 nM for ***S*-5** and 300–1000 nM for ***R*-8**) were infused onto the capillary until a typical sigmoidal profile was reached. The injection of increasing concentration of these ligands resulted in frontal traces with reduced saturation times. Each chromatographic profile was analyzed with a polynomial equation to derive the inflection point corresponding to the breakthrough time. Known the flow-rate, the breakthrough volumes were derived and correlated to each ligand concentration using Eq. (3). K_d values were calculated by regression analysis with a R^2 of 0.9183 for ***R*-8** and 0.9628 for ***S*-5**. The calculated K_d values and the number of binding sites are presented in Table 4.

The affinity constants for the ligands ***R*-8** and ***S*-5** were also determined by ITC experiments (Table 4), using the "one binding site" model to obtain the best fitting to the experimental data. A good correspondence was obtained between the K_d values calculated with the chromatographic assay and those measured by ITC experiments, confirming that the receptor was correctly immobilized on the inner surface of the capillary. It could be noted that the K_i obtained for ***R*-8** by SPA (88 nM) is slightly different; however, the peculiarity of SPA, which is based on displacement experiments, can account for the different values observed [18].

3.5. Stereoselective frontal affinity chromatography

In principle, frontal affinity chromatography has the capability to discriminate between enantiomers if their binding affinities to

Table 4
Dissociation constant, K_d, and number of binding sites B_{max} determined by frontal affinity chromatography on immobilized PPARγ-LBD in comparison with ITC_{200} binding capacity results.

ligand	B_{max} (pmol)	K_d (nM)	ITC_{200} K_d (nM)
***R*-8**[a]	25.25	542	270
***S*-5**[b]	166.7	3688	3660

[a] Calculated on capillary C4.
[b] Calculated on capillary C5.

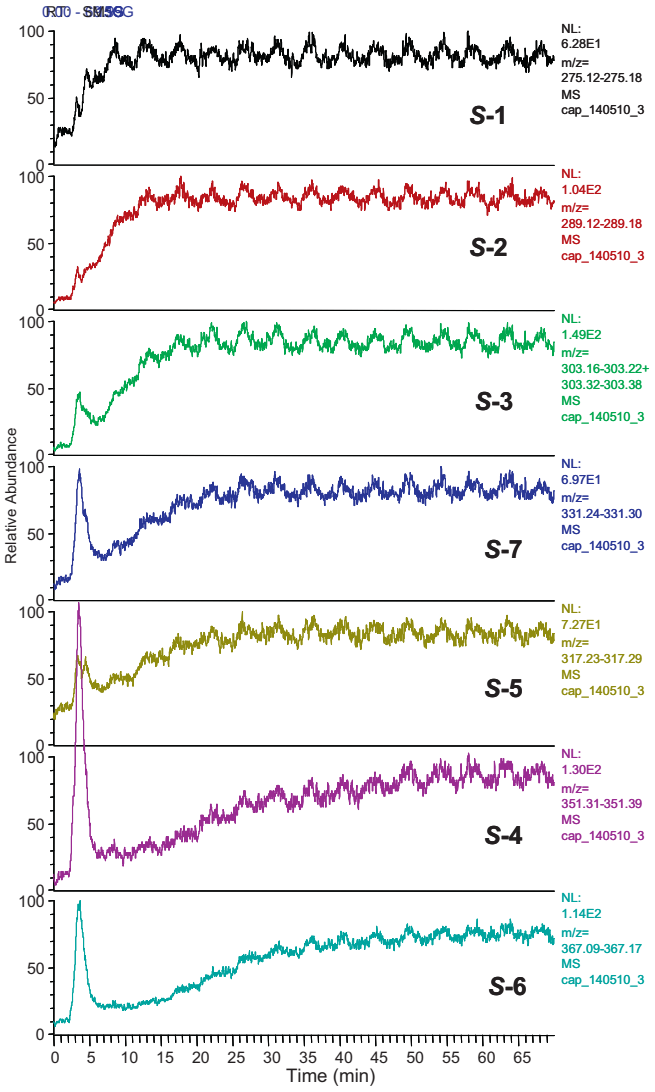


Fig. 1. Extracted breakthrough curves for enantiomers **1–7**. A mixture of ligands each at a concentration of 1 μM was infused through capillary C3 using the mass spectrometer in negative mode.

the biological target are different. However, the use of FAC–MS to characterize an enantioselective interaction for a biological target using racemic mixtures has never been deeply investigated.

The availability of the enantiomeric couples ***R*-8**/***S*-8** and ***R*-5**/***S*-5** and the knowledge of their stereoselective activity allowed us to investigate the capacity of the capillary system to discriminate between their enantioselective interactions with PPARγ.

First, isolated frontal analysis experiments on the single enantiomers ***S*-8** and ***R*-8** were performed. Each enantiomer was infused at a concentration of 50 nM in 10 mM ammonium acetate buffer pH 7.4/MeOH (90:10) at 2.5 μL min^{-1}. The corresponding chromatograms are reported in Fig. 2.

The calculated breakthrough times were 45.5 (±6.2) min and 80.8 (±5.9) min for ***S*-8** and ***R*-8**, respectively. As expected, the two curves were shifted in agreement with the difference in affinity which was measured by ITC obtaining K_d = 2000 and 270 nM for ***S*-8** and ***R*-8**, respectively. A similar behavior for the two ligands has been also observed in SPA experiments (K_i = 971 and 88 nM for ***S*-8** and ***R*-8**, respectively) [18]. The racemic mixture was then infused through the capillary at a concentration of 50 nM in 10 mM ammonium acetate buffer pH 7.4/MeOH (90:10) at 2.5 μL min^{-1}. As reported in Fig. 3 the infusion of the racemic mixture (25 mM

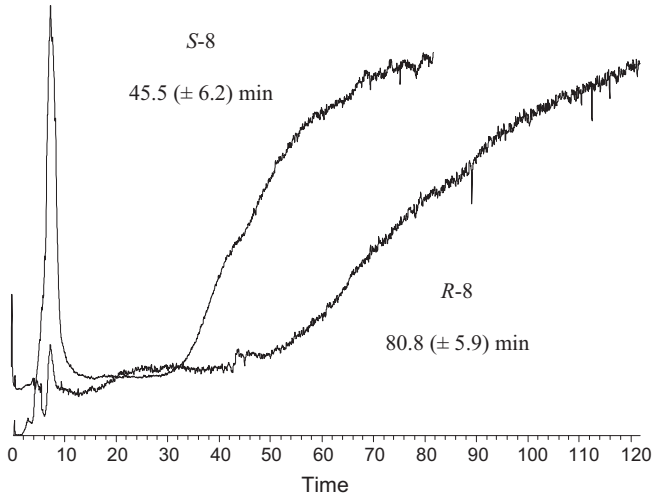


Fig. 2. Single infusion of enantiomers ***S*-8** and ***R*-8** at 50 nM in 90/10 CH_3COONH_4 10 mM, pH 7.4/MeOH, Flow rate 2.5 mL min^{-1}, Detection (+) ESI-MS/MS, daughter ion 351.3 *m/z*.

Table 5
Stereoselective frontal affinity chromatography results.

	EC_{50} (nM)	*t* (min)	α_F[a]	EC_{50} ratio[b]
***R*-5**	5820	8.15	9.15	10.58
***S*-5**	550	74.63		
***S*-8**	860	68.48	2.19	5.38
***R*-8**	160	150.0		

[a] α_F frontal selectivity was calculated as reported in Section 2.5.
[b] The EC_{50} ratio was calculated from the ratio between the EC_{50} values of the less active enantiomer and the more active one.

for each enantiomer) resulted in a characteristic biphasic frontal profile as the result of the two saturation events.

This double plateau chromatogram arises as a result of the *R*- and *S*-enantiomers having different binding affinities to PPARγ. The two inflection points were mathematically derived using a double sigmoidal function which describes the trend (68.48 min and 150.0 min for ***S*-8** and ***R*-8**, respectively).

The same experiments were performed with ***R*-5** and ***S*-5** enantiomers. The calculated inflection points were 8.151 and 74.63 for ***R*-5** and ***S*-5**, respectively. The results from the enantioselective frontal analysis experiments are reported in Table 5.

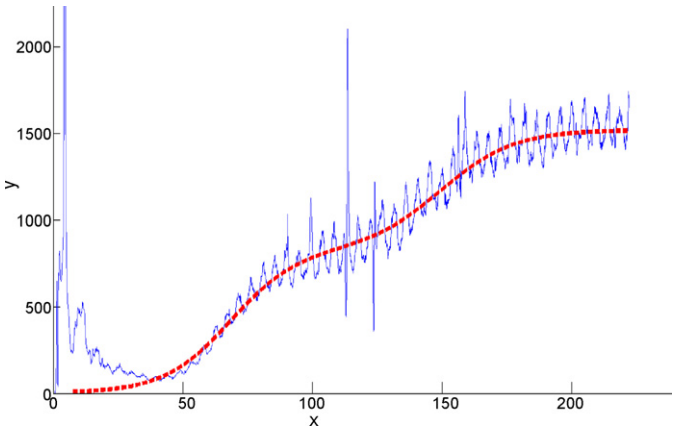


Fig. 3. FAC–MS chromatogram infusing the racemic mixture (***S*-8**/***R*-8**) at 50 nM in 90/10 CH_3COONH_4 10 mM, pH 7.4/MeOH, Flow rate 2.5 mL min^{-1}, Detection (+) ESI-MS/MS, daughter ion 351.3 *m/z*. The biphasic curve represents the binding of ***S*-8** and ***R*-8** enantiomers to the receptor.

It is interesting to note that the highest frontal selectivity (9.15) was observed injecting the enantiomeric pair ***R*-5**/***S*-5** characterized by the higher EC_{50} ratio (10.58). Moreover, the measured breakthrough times for all the four enantiomers were in agreement with the corresponding EC_{50} values.

The results of these enantioselective frontal affinity chromatographic experiments are very exciting for future applications of FAC–MS methodology for the study of the relative affinity of enantiomeric ligands.

The developed FAC–MS approach based on immobilized PPARγ allows the selection of high affinity PPARγ agonists and the assessment of receptor–chiral drug interaction.

4. Conclusions

The present work reports the development and the characterization of new chromatographic tools based on the LBD of PPARγ receptor for the affinity screening of drug candidates. The results demonstrate that PPARγ maintains its ability to bind known ligands when immobilized on silica particles and on the inner surface of silica capillaries.

The presented FAC–MS approach allows to generate accurate K_d measurements and demonstrates the ability to achieve such measurements with an environmentally friend procedure, as no radio-labeled compounds are required. Moreover, the proposed binding assay can be easily carried out with a common HPLC–MS system. The strength of the FAC–MS approach has been validated using a completely different methodology (ITC) and the calculated K_d values were found to have the same order of magnitude.

We have also shown that immobilized PPARγ acts as chiral selective receptor and for the first time a thorough study using FAC–MS has been carried out to investigate the enantioselective interactions between the ligands and the biological target. This is the first characterization of an enantioselective receptor by FAC–MS and the results demonstrate that the system can account for chiral specificity.

After the validation of the system, further investigations on new classes of small molecule ligands (agonists and antagonists) for PPARγ will be carried out with FAC–MS methodology in order to draw preliminary structure–activity relationships.

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References

[1] I. Issemann, S. Green, Nature 347 (1990) 645.
[2] R.M. Evans, G.D. Barish, Y.-X. Wang, Nat. Med. 10 (2004) 355.
[3] O. Braissant, F. Foufelle, C. Scotto, M. Dauca, W. Wahli, Endocrinology 137 (1996) 354.
[4] J. Berger, D.E. Moller, Annu. Rev. Med. 53 (2002) 409.
[5] B.M. Forman, J. Chen, R.M. Evans, Ann. N. Y. Acad. Sci. 804 (1996) 266.
[6] T.M. Willson, P.J. Brown, D.D. Sternbach, B.R. Henke, J. Med. Chem. 43 (2000) 527.
[7] J.M. Lehmann, L.B. Moore, T.A. Smith-Oliver, W.O. Wilkison, T.M. Willson, S.A. Kliewer, J. Biol. Chem. 270 (1995) 12953.
[8] T.M. Willson, J.E. Cobb, D.J. Cowan, R.W. Wiethe, I.D. Correa, S.R. Prakash, K.D. Beck, L.B. Moore, S.A. Kliewer, J.M. Lehmann, J. Med. Chem. 39 (1996) 665.
[9] B. Staels, J. Dallongeville, J. Auwerx, K. Schoonjans, E. Leitersdorf, J.-C. Fruchart, Circulation 98 (1998) 2088.
[10] J.-C. Fruchart, P. Duriez, B. Staels, Curr. Opin. Lipidol. 10 (1999) 245.
[11] R.W. Nesto, D. Bell, R.O. Bonow, V. Fonseca, S.M. Grundy, E.S. Horton, M. Le Winter, D. Porte, C.F. Semenkovich, S. Smith, L.H. Young, R. Kahn, Diabetes Care 27 (2004) 256.
[12] M. Bodmer, C. Meier, M.E. Kraenzlin, C.R. Meier, Drug Saf. 32 (2009) 539.
[13] A. Pinelli, C. Godio, A. Laghezza, N. Mitro, G. Fracchiolla, V. Tortorella, A. Lavecchia, E. Novellino, J.C. Fruchart, B. Staels, M. Crestani, F. Loiodice, J. Med. Chem. 48 (2005) 5509.
[14] R. Montanari, F. Saccoccia, E. Scotti, M. Crestani, C. Godio, F. Gilardi, F. Loiodice, G. Fracchiolla, A. Laghezza, P. Tortorella, A. Lavecchia, E. Novellino, F. Mazza, M. Aschi, G. Pochetti, J. Med. Chem. 51 (2008) 7768.
[15] G. Fracchiolla, A. Laghezza, L. Piemontese, G. Carbonara, A. Lavecchia, P. Tortorella, M. Crestani, E. Novellino, F. Loiodice, ChemMedChem 2 (2007) 641.
[16] G. Fracchiolla, A. Lavecchia, A. Laghezza, L. Piemontese, R. Trisolini, G. Carbonara, P. Tortorella, E. Novellino, F. Loiodice, Bioorg. Med. Chem. 16 (2008) 9498.
[17] G. Fracchiolla, A. Laghezza, L. Piemontese, P. Tortorella, F. Mazza, R. Montanari, G. Pochetti, A. Lavecchia, E. Novellino, S. Pierno, D. Conte-Camerino, F. Loiodice, J. Med. Chem. 52 (2009) 6382.
[18] G. Pochetti, C. Godio, N. Mitro, D. Caruso, A. Galmozzi, S. Scurati, F. Loiodice, G. Fracchiolla, P. Tortorella, A. Laghezza, A. Lavecchia, E. Novellino, F. Mazza, M. Crestani, J. Biol. Chem. 282 (2007) 17314.
[19] G. Pochetti, N. Mitro, A. Lavecchia, F. Gilardi, N. Besker, E. Scotti, M. Aschi, N. Re, G. Fracchiolla, A. Laghezza, P. Tortorella, R. Montanari, E. Novellino, F. Mazza, M. Crestani, F. Loiodice, J. Med. Chem. 53 (2010) 4354.
[20] J.E. Cobb, S.G. Blanchard, E.G. Boswell, K.K. Brown, P.S. Charifson, J.P. Cooper, J.L. Collins, M. Dezube, B.R. Henke, E.A. Hull-Ryde, D.H. Lake, J.M. Lenhard, W.O. Jeffery Oplinger Jr., M. Pentti, D.J. Parks, K.D. Plunket, W.-Q. Tong, J. Med. Chem. 41 (1998) 5055.
[21] P.W. Young, D.R. Buckle, B.C. Cantello, H. Chapman, J.C. Clapham, P.J. Coyle, D. Haigh, R.M. Hindley, J.C. Holder, H. Kallender, A.J. Latter, K.W. Lawrie, D. Mossakowska, G.J. Murphy, L. Roxbee Cox, S.A. Smith, J. Pharmacol. Exp. Ther. 284 (1998) 751.
[22] R.P. Rhoads, P.L. Greenwood, A.W. Bell, Y.R. Boisclair, Endocrinology 141 (2000) 1425.
[23] P. Dowell, J.E. Ishmael, D. Avram, V.J. Peterson, D.J. Nevrivy, M. Leid, J. Biol. Chem. 272 (1997) 2013.
[24] G. Krey, O. Braissant, F. L'Horset, E. Kalkhoven, M. Perroud, M.G. Parker, W. Wahli, Mol. Endocrinol. 11 (1997) 779.
[25] J.S. Nicholsa, D.J. Parks, T.G. Consler, S.G. Blanchard, Anal. Biochem. 257 (1998) 112.
[26] M.M. Pierce, C.S. Raman, B.T. Nall, Methods 19 (1999) 213.
[27] M.A. Cooper, Nat. Rev. Drug Discov. 1 (2002) 515.
[28] R. Seethala, R. Golla, Z. Ma, H. Zhang, K. O'Malley, J. Lippy, L. Cheng, K. Mookhtiar, D. Farrelly, L. Zhang, N. Hariharan, P.T. Cheng, Anal. Biochem. 363 (2007) 263.
[29] C. Yu, L. Chen, H. Luo, J. Chen, F. Cheng, C. Gui, R. Zhang, J. Shen, K. Chen, H. Jiang, X. Shen, Eur. J. Biochem. 271 (2004) 386.
[30] K. Vuignier, J. Schappler, J.-L. Veuthey, P.A. Carrupt, S. Martel, Anal. Bioanal. Chem. 398 (2010) 53.
[31] J.E. Schiel, K.S. Joseph, D.S. Hage, Adv. Chromatogr. 48 (2010) 145.
[32] E. Calleri, C. Temporini, G. Caccialanza, G. Massolini, ChemMedChem 4 (2009) 905.
[33] E. Calleri, C. Temporini, G. Caccialanza, G. Massolini, J. Pharm. Biomed. Anal. 54 (2011) 911.
[34] J.J. Slon-Usakiewicz, P. Redden, Methods Mol. Biol. 572 (2009) 219.
[35] V. Chandra, P. Huang, Y. Hamuro, S. Raghuram, Y. Wang, T.P. Burris, F. Rastinejad, Nature 456 (2008) 350.
[36] E. Raspé, L. Madsen, A.M. Lefebvre, I. Leitersdorf, L. Gelman, J. Peinado-Onsurbe, J. Dallongeville, J.C. Fruchart, R. Berge, B. Staels, Lipid Res. 40 (1999) 2099.
[37] T. Hollon, F.K. Yoshimura, Anal. Biochem. 182 (1989) 411.
[38] C. Cardoso, V. Lima, A. Zottis, G. Oliva, A.D. Andricopulo, I.W. Wainer, R. Moaddel, Q.B. Cass, J. Chromatogr. A 1120 (2006) 151.
[39] R. Moaddel, P.L. Bullock, I.W. Wainer, J. Chromatogr. B 799 (2004) 255.
[40] H.S. Kim, R. Mallik, D.S. Hage, J. Chromatogr. B 837 (2006) 138.
[41] H.S. Kim, I.W. Wainer, J. Chromatogr. B 870 (2008) 22.
[42] D.C. Schriemer, D.R. Bundle, L. Li, O. Hindsgaul, Angew. Chem. Int. Ed. 37 (1998) 3383.
[43] R. Moaddel, K. Jozwiak, R. Yamaguchi, C. Cobello, K. Whittington, T.K. Sarkar, S. Basak, I.W. Wainer, J. Chromatogr. B 813 (2004) 235.
[44] E. Calleri, G. Massolini, D. Lubda, C. Temporini, F. Loiodice, G. Caccialanza, J. Chromatogr. A 1031 (2004) 93.
[45] C. Temporini, S. Ceruti, E. Calleri, S. Ferrario, R. Moaddel, M.P. Abbracchio, G. Massolini, Anal. Biochem. 384 (2009) 123.
[46] E. Calleri, S. Ceruti, G. Cristalli, C. Martini, C. Temporini, C. Parravicini, R. Volpini, S. Daniele, G. Caccialanza, D. Lecca, C. Lambertucci, M.L. Trincavelli, G. Marucci, I.W. Wainer, G. Ranghino, P. Fantucci, M.P. Abbracchio, G. Massolini, J. Med. Chem. 53 (2010) 3489.
[47] R. Moaddel, I.W. Wainer, Nat. Protoc. 4 (2009) 197.