Open tubular columns containing the immobilized ligand binding domain of peroxisome proliferator-activated receptors α and γ for dual agonists characterization by frontal affinity chromatography with mass spectrometry detection


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The peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor superfamily. In the last years novel PPARs ligands have been identified and these include PPARα/γ dual agonists. To rapidly identify novel PPARs dual ligands, a robust binding assay amenable to high-throughput screening toward PPAR isomers would be desirable. In this work we describe a parallel assay based on the principles of frontal affinity chromatography coupled to mass spectrometry (FAC-MS) that can be used to characterize dual agonists. For this purpose the ligand binding domain of PPARα receptor was immobilized onto the surface of open tubular capillaries to create new PPAR-alpha-OT columns to be used in parallel with PPAR-gamma-OT columns. The two biochromatographic systems were used in both ranking and Kd experiments toward new ureidofibrate-like dual agonists for subtype selectivity ratio determination. In order to validate the system, the Kd values determined by frontal analysis chromatography were compared to the affinity constants obtained by ITC experiments. The results of this study strongly demonstrate the specific nature of the interaction of the ligands with the two immobilized receptor subtypes.

1. Introduction

In the research for new chemical entities that are able to interact with a relevant pharmacological target, a significant effort has been made in the development and application of innovative target-based screening assays [1]. In the wide scenario of assay technologies for the measurement of receptor–ligand interactions, protein affinity selection methods that utilize mass spectrometry (MS) have a significant advantage [2,3]. In particular, frontal affinity chromatography (FAC) coupled to mass spectrometry detector has emerged as a reliable method [4–6].

FAC-based methods have the advantage to be extremely reproducible and, unlike the biochemical or cellular assays commonly used, are able to identify strong (nM range) but also weak binding molecules (μM range). Importantly, FAC-based screening does not require ligand labeling to support the read-out of experimental results but only needs access to purified protein and to suitable compound collections. This method also has advantage over many solution-based affinity selection methods in that it can be used with both soluble and membrane-associated receptors [7].

Another invaluable tool for measuring the formation and dissociation of molecular complexes is isothermal titration calorimetry (ITC) in which an endothermic or exothermic change generated by a molecular interaction is detected [8].

In the last years, in our laboratory we have successfully developed reliable biochromatographic systems based on FAC-MS methods for some G-protein coupled receptors, P2Y1, A2A and GPR17 receptors [9–12].

More recently, we have described and validated a new biochromatographic system based on PPAR-gamma receptor immobilized on the inner surface of an open tubular (OT) silica capillary for screening a series of chiral fibrate analogues [13].

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily playing a crucial role in the regulation of metabolic homeostasis. In clinical practice, PPARα agonists such as fibrates improve dyslipidaemia, while
PPARγ agonists such as thiazolidinediones improve insulin resistance and diabetes control. However, these agents have different and sometimes conflicting clinical benefits with adverse event profiles,[14,15] accordingly new molecules able to activate more PPAR subtypes, dual agonists and pan-agonists have received considerable attention. In particular the development of dual PPARα/γ agonists may prove beneficial in effectively managing glycaemic control and improving dyslipidaemia in patients with type 2 diabetes [16].

One of the critical challenges for developing dual agonists is how to identify in a fast way the receptor subtype selectivity ratio. The conventional PPAR binding assays such as competition radioreceptor assay, protease protection assay, coactivator-dependent receptor ligand assay (CARLA), and scintillation proximity assay (SPA) 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 ITC have been recognized as powerful tools in monitoring receptor–ligand interactions with advantages of no use of radioactive ligands [17–20].

The interesting results obtained in the previous work report the development of PPAR-gamma-OT supports, lead us to widen the application of the FAC-MS approach also to PPAR-alpha. For this purpose we prepared and characterized a novel PPAR-alpha-OT column to be used in parallel with the PPAR-gamma-OT column for the determination of the Kd values and selectivity ratio of some new ureidofibrate-like dual agonists, only characterized in terms of affinity toward the PPAR-gamma isoform by ITC experiments [21].

In order to validate the system, the Kd values of the selected compounds were determined by frontal analysis experiments and by ITC experiments toward the alpha isoform. This work demonstrates that once again FAC-MS is a pragmatic tool for screening, investigating and understanding target–ligand interactions and a fast and powerful technique that is valuable in predicting the affinity of molecules on biological targets.

2. Experimental

2.1. Materials

The reagents for target immobilization on silica capillaries were NaOH, Carlo Erba Reagenti (Rodano, MI, l), 3-aminopropyltriethoxysilane, glutaraldehyde, KH₂PO₄, NaBH₄, CN, monoethanolamine, CH₃OH, DMSO, CH₃COONH₄, and GW1929 or N-(2-benzoylphenyl)-O-[2-(methyl-2-pyridylamino)ethyl]-l-tyrosine hydrate were from Sigma Aldrich Chemicals Co. (St. Louis, MO). The water was obtained by a Milli-Q water purification system (Millipore Corporation, Bedford, MA).

The open tubular silica capillaries (100 μm I.D. × 0.375 mm × 40 cm) are from Thermo Fisher Scientific (San Jose, CA). PPAR-alpha and gamma ligand binding domains (LBD) were obtained by a cell culture and transfection previously reported method [13].

The compounds used in this study are ureidofibrate-like derivatives and were prepared and assayed as described by Porcelli et al. [21].

2.2. Instrumentations

Receptor immobilization on the inner surface of the capillaries was carried out with a 500 μL syringe moved at different speeds by a syringe pump (Thermo Fisher Scientific, San Jose, CA) and diverting to waste the solutions.

Frontal analysis was carried out with a chromatographic system composed by a syringe pump delivering the mobile phase (90% ammonium acetate 10 mM pH 7.4, 10% MeOH) through the capillary at 2.5 μL min⁻¹. To improve the sensitivity, the eluent from the capillary was mixed with an organic make-up flow (100% methanol) pumped at 5 μL min⁻¹ with an HPLC pump (Surveyor, Thermo Finnigan, San Jose, CA) by a tee-union before ESI-MS. Detection of the ligands was made by a Linear Trap Quadrupole (LTQ) mass spectrometer with electro-spray ionization (ESI) as ion source (Thermo Finnigan, San Jose, CA). The system was controlled by Xcalibur software 1.4 (Thermo Finnigan, San Jose, CA).

The amount of immobilized receptor was measured by a spectrophotometric assay of the PPAR solutions collected before and after the infusion during the immobilization step (Lambda 25, Perkin Elmer, Walkham, MA, USA).

2.3. Immobilization of the PPARs receptors on the open tubular capillary

The LBD of PPAR-gamma receptor was immobilized on the inner surface of 40 cm open tubular capillary following a previously reported method [13]. The same procedure was applied to the alpha subtype. Briefly the capillary was first activated with 2 mL of 0.5 N NaOH at 100 μL min⁻¹ flow rate, washed with 1 mL of water at the same flow rate and dried at 95 °C for 1 h. Then, 1 mL of a solution of 3-aminopropyltriethoxysilane (10%, v/v in water) was infused in the capillary 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% (v/v in water) glutaraldehyde solution was pumped through the capillary at 50 μL min⁻¹ and the unreacted aldehydic groups were removed by washing with 1 mL of 50 mM phosphate buffer pH 7.0 at 50 μL min⁻¹. This step was followed by the infusion of 1 mL of PPAR solution (0.5 mg mL⁻¹ in 50 mM phosphate buffer pH 7.0) at 50 μL min⁻¹. After the rinsing with pure buffer, the Shiff bases were reduced by the infusion of a 5 mg mL⁻¹ solution of NaBH₄ in phosphate buffer. After this step, unreacted groups were inactivated with 0.1 M monoethanolamine at 5 μL min⁻¹ for 1 h. In this study, the procedure was slightly modified compared to that reported for PPAR-gamma as no rosiglitazone, a selective PPAR-gamma agonist, was added during the receptor infusion [13].

2.4. Determination of the amount of immobilized receptor

For each receptor subtypes two calibration curves in a range of receptor concentration from 0.020 mg mL⁻¹ to 0.100 mg mL⁻¹ with an UV wavelength of 214 nm were built. After this step, the absorbance of the PPARα or of the PPARγ solutions collected from the capillary during the immobilization protocol were determined. By measuring the concentration of these solutions and subtracting this value from that of the solution used to infuse through the capillary (0.5 mg mL⁻¹) it is possible to determine the amount of the receptor immobilized on the inner surface of the capillary.

2.5. Sample preparation

The ligands were dissolved in DMSO at concentration of 1 mM and stock solutions were stored at −20 °C. Further dilutions were prepared in mobile phase to obtain samples to be infused through the capillaries.
2.6. Chromatographic studies

2.6.1. FAC-MS

For ranking experiments of ureidofibrates compounds, an equimolar solution (500 nM) of each compound was prepared in mobile phase and it was infused at 2.5 μL min⁻¹.

The Kᵣ for each analyte was measured in a range of concentrations around the Kᵣ value determined by ITC, and each concentration was infused at 2.5 μL min⁻¹.

Dedicated MS methods were developed in the different steps of this study: for ranking experiments the instrument was operated in the electrospray positive mode under MS/MS conditions, and it was monitored the specific fragmentation of each compound of the mixture. For R-1 the ion transition was m/z 453.6 → 353.2 (collision energy 23.0, isolation width 1.0 m/z); for R-3 we have monitored m/z 425.5 → 193.0, 353.2 (collision energy 28.0, isolation width 1.0 m/z); compound 2 had a specific fragmentation at m/z 439.3 → 353.2 (collision energy 28.0, isolation width 1.0 m/z) and 4 was monitored at m/z 411.3 → 132.9, 179.0, 233.1 (collision energy 34.0, isolation width 1.0 m/z). Instrumental conditions were: source voltage 5.0 kV, capillary voltage 49.0 V, sheat gas flow 22.0 (arbitrary units), auxiliary gas flow 2.0 (arbitrary units), capillary temperature 250 °C and tube lens voltage 135.0 V.

For Kᵣ determination the instrument was operated in positive ion mode under MS/MS conditions. Fragmentation was achieved with nitrogen gas with a collision energy optimized for each analyte (previously reported in the ranking methods). The instrumental conditions were constant: source voltage 5.0 kV, sheat gas flow 2.0 (arbitrary units), auxiliary gas flow 16.0 (arbitrary units), capillary voltage 14.95 V, capillary temperature 250 °C and tube lens voltage 90 V. The ion transitions monitored were: for R-1 and S-1, m/z 453.6 → 353.2 (CID 23.0); for R-3, m/z 425.5 → 193.0, 353.2 (CID 28.0), for analyte 2, m/z 439.3 → 353.2 (CID 28.0) and for compound 4, m/z 411.3 → 132.9, 179.0, 233.1 (CID 34).

In frontal displacement studies, the instrumental conditions were the same and it was monitored the specific fragmentation of the marker R-3 at m/z 425.5 → 193.0, 353.2 (CID 28.0).

2.6.2. Ranking experiments

For ranking experiments, an equimolar solution of the analytes (500 nM of each dissolved in mobile phase) was infused in the capillary at 2.5 μL min⁻¹.

Selective ion chromatograms were reconstructed from the specific ion transition of each analyte and they were analyzed with a polynomial equation of degree 3 (y = ax³ + bx² + cx + d) to fit the chromatographic data. The inflection points of the breakthrough curves were determined by the second derivative of this equation.

2.6.3. Dissociation constant determination

Frontal chromatography. In this experimental approach, increasing analyte concentrations were infused through the capillary until characteristic sigmoidal curves were obtained. It was observed a shift of the sigmoidal curves to shorter times when the concentration increased.

The dissociation constants (Kᵣ) were calculated using the following equation:

\[ [L] \times (V - V₀)B_{\text{max}} \times \frac{[I]}{Kᵣ + [I]} \]  

where [L] is the concentration of the ligand, V (μL) is the retention volume of the ligand calculated at the breakthrough time, V₀ is the retention volume of a non-retained ligand and Bₘₐₓₐ is the number of dynamic binding sites for each ligand in the capillary.

Kᵣ was calculated by non-linear regression using Prism 4 software (Graph Pad Software Inc., San Diego, CA, USA).

Frontal displacement chromatography. Increasing analyte concentrations in the presence of a constant concentration of marker (R-3), were infused through the capillary until characteristic sigmoidal curves were obtained. The increase in analyte concentrations made the slope of sigmoidal curves change to shorter times. This method allows to distinguish between non-specific interactions that can occur with the receptor or the chromatographic backbone.

The shift of the breakthrough curves of the marker were determined and used to calculate the Kᵣ of the competing ligand by the following equation:

\[ (V - V_{\text{min}}) = P \times (Kᵣ + [A])^{-1} \]  

where V (μL) is the retention volume of the marker ligand calculated at the breakthrough time, Vₐₜₜ is the retention volume of the marker ligand when the specific interaction is completely suppressed, P is the product of the Bₘₐₓₐ (number of active binding sites) and Kᵣ/Kᵣₐₜₜ and [A] is the analyte concentration.

To calculate the dissociation constant values can be obtained for each analyte. The data were analyzed by non-linear regression using Prism 4 software (Graph Pad Software Inc., San Diego, CA, USA).

2.7. Isothermal titration calorimetry

ITC experiments on alpha subtype were performed at 25 °C using a MicroCal iTC₂₀₀ microcalorimeter (MicroCal Inc., Northampton, MA, USA). Protein was extensively dialyzed against the buffer of choice (Hepes 20 mM, pH 7.4, 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). DMSO was added to the protein solution in the same percentage of the ligand solution (below 3%). 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 μL for 0.8 s) with delay intervals between injections of 180 s. Reference titration of ligand into buffer was used to correct for heat of dilution. The syringe stirring speed was set at 1000 rpm. The thermodynamic data were processed with Origin 7.0 software provided by MicroCal. To correct for any discrepancies in the baseline outlined by the software, a manual adjustment was performed. In some cases the parameter ΔH was kept fixed during the refinement to obtain a best fit, especially at the beginning of the curve.

3. Results and discussion

3.1. Compounds tested

The compounds used in this study are ureidofibrate-like derivatives and the chemical structures are reported in Table 1. For ranking experiments compound GW1929, a potent and subtype-selective (>1000-fold) PPARγ agonist was chosen as marker ligand (Fig. 1).

The ureidofibrate-like derivatives were previously characterized for their agonist activity on PPAR-gamma and alpha-subtypes by a cell-based transactivation assay. However binding affinities were estimated only for the gamma subtype by ITC technique [21].
Table 1
Chemical structure of ureidofibrate-like derivatives considered in the present study.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R'</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1</td>
<td>CH₂</td>
<td>CH₂</td>
</tr>
<tr>
<td>S-1</td>
<td>CH₃</td>
<td>CH₂</td>
</tr>
<tr>
<td>2</td>
<td>CH₃</td>
<td>CH₂</td>
</tr>
<tr>
<td>R-3</td>
<td>H</td>
<td>CH₂</td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

Except for R-1, all of these ligands exhibited a partial agonist behavior on the gamma subtype with potencies ranging from 0.07 (R-1) to 0.80 µM (4) and affinities between 0.27 (R-1) and 7.7 µM (4). On the contrary, they acted as full agonists on the alpha subtype, with potencies ranging from 0.003 (R-1) to 0.122 µM (4) [21]. In particular enantiomers R-1 and S-1 were fully investigated for their enantioselective interaction with PPAR-gamma by both FAC-MS and ITC experiments [13].

3.2. Reproducibility of PPARγ-OT and PPARα-OT capillary preparation

The protocol, previously described for PPARγ LBD, was followed for PPARα LBD immobilization on the inner surface of silica capillaries [13]. As demonstrated for PPARγ the addition of a high affinity selective ligand to preserve the binding pocket was found to be not necessary for the immobilization of the LBD of PPARα receptor.

The reproducibility of the PPARα-OT and PPARγ-OT capillaries synthesis was assessed by preparing three different capillaries. The procedure for the preparation is described in details in Section 2.3. A spectrophotometric method was developed to determine the amount of the immobilized receptor and two calibration curves for the LBD of PPAR-gamma and PPAR-alpha subtypes were obtained (y = 8.6191x – 0.1497 and y = 9.0320x – 0.1135 for PPAR-alpha and PPAR-gamma respectively) with an acceptable linearity (R² = 0.9800 and R² = 0.9854 for PPAR alpha and PPAR-gamma respectively).

By the difference in UV absorbance before and after the infusion of the receptor solution into the capillary, the amount of bound PPAR-alpha receptor was calculated (0.258 ± 0.007 mg, 2.71% RSD) with an immobilization yield of 51.6 ± 1.44%. For PPAR-gamma receptor the amount of bound receptor was 0.429 ± 0.027 mg, 6.29% RSD (immobilization yield 85.8 ± 5.37%). These data demonstrate the good reproducibility of the immobilization method for both receptor subtypes.

3.3. Ranking experiments on PPARγ and PPARα OT capillaries

In the FAC-MS screening approach a mixture of potential ligands is continuously infused into an affinity column resulting in an equilibrium between the target and individual test compounds. In a ranking experiment the retention time on the receptor column is determined by the relative affinities of compounds, which elute in inverse order of binding affinity.

A first ranking experiment was carried out in parallel on PPARγ and PPARα capillaries. The capillaries were tested in the ranking of four structurally related ureidofibrates (compounds R-1, 2, R-3, 4).

The distomers S-1 and S-3 were not added to the mixture as they have the same m/z values of their corresponding eutomers R-1 and R-3. However, a high-affinity full agonist of PPARγ, the carboxylic derivative GW1929, was selected as reference compound and was added to the mixture as marker ligand.

Solutions of the ligands, each at 0.5 µM, were prepared in the mobile phase buffer and continuously infused through the capillaries. The effluent was analyzed in an ESI mass spectrometer to detect each component in positive mode to ensure efficient ionization and detection of the compounds. Because the molecular weight for each compound is known, the individual breakthrough front of each compound was easily identified. Detailed chromatographic and detection conditions are reported in Section 2.6.1. The breakthrough times, reported in Table 2 are the results of a single determination carried out on freshly prepared capillaries.

In the case of PPARγ-OT capillary when the chromatographic data were correlated to the Kᵣ values of the compounds obtained by ITC method [21], a good correspondence was observed indicating the capability of the FAC-MS assay to screen compounds with different affinities in a single run (rank 1). The highest breakthrough time (34.47 min) was obtained for GW1929, in agreement with its high affinity (Kᵣ = 1.4 nM) as reported in the literature [22]. Of interest for the PPARγ-OT capillary, is the increase in breakthrough time with the presence of GW1929, indicating a possible allosteric effect or conformational change induced by GW1929.

The same mixture, infused on PPARα-OT capillary resulted in a different ranking order. This order followed the general trend of EC₅₀ values [21]. We must underline that this correlation is reliable as these ligands behave as full agonists on alpha receptor subtype.

The result of this ranking experiment strongly demonstrates the specific nature of the interaction of the ligands with the two immobilized receptor subtypes. The same ranking experiment was

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Kᵣ γ (µM)²</th>
<th>Bt (min) PPARγ</th>
<th>Bt (min) PPARα</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1</td>
<td>0.27</td>
<td>33.61</td>
<td>29.50</td>
</tr>
<tr>
<td>2</td>
<td>4.5</td>
<td>25.74</td>
<td>19.54</td>
</tr>
<tr>
<td>R-3</td>
<td>1.5</td>
<td>27.21</td>
<td>24.69</td>
</tr>
<tr>
<td>4</td>
<td>7.7</td>
<td>24.25</td>
<td>13.02</td>
</tr>
<tr>
<td>GW1929</td>
<td>&lt;0.04°</td>
<td>34.47</td>
<td>18.78</td>
</tr>
</tbody>
</table>

* Kᵣ values as calculated by ITC experiments [21].

Table 2
Relative affinity data expressed as breakthrough times obtained by ranking experiment of the ureidofibrate-like derivatives on PPARγ receptor and PPARα receptor subtypes in comparison with ITC data.
carried out on both capillaries without adding GW1929 to the infused mixture and the order was respected (rank 2).

3.4 Determination of ligand binding affinities by FAC-MS

The two PPAR subtype-OT capillaries were also used for the accurate determination of the binding affinity (expressed as dissociation constant, $K_d$) of each analyte of the ureido-fibrate series.

In the case of PPARγ-OT capillary we apply two different experimental approaches in order to evaluate the aspecific interactions of the analytes with the support.

The first approach, frontal chromatography, is the simplest one and it is conducted by the infusion of increasing concentrations of the analyte through the capillary (Section 2.6.3). The concentration range investigated was different for each analyte (200–1000 nM for R-1; 500–3000 nM for S-1; 2000–5000 nM for 2; 100–10,000 nM for R-3; 4000–7000 nM for 4). As the analyte concentration increases, the frontal profile reaches reduced saturation times. Each chromatographic curve was analyzed with a polynomial equation to derive the inflection point corresponding to the breakthrough time. The breakthrough volume was calculated and correlated to each ligand concentration to obtain a plot that allows to calculate by non-linear regression the $K_d$ values. As an example in Fig. 2 the breakthrough curves of R-3 are shown.

In order to verify the reproducibility of the method, for ligand R-3 each concentration was infused in duplicate and the breakthrough times were considered reproducible (data not shown). The observed variation in breakthrough time is less than 10% and has been considered as not significant, since this change is linked to the experimental variability.

The second approach is frontal displacement chromatography. Experimental details are given in Section 2.6.3. Similarly, in frontal displacement experiments, various concentrations of a competitive binding ligand can be infused into a bioaffinity column along with an affinity indicator ligand, and displacement in the frontal elution times of an indicator ligand can be correlated to the binding constants of both the indicator and displacer ligands. In this experimental method we used ligand R-3 as a marker and we determined the dissociation constants ($K_d$) of the other compounds by the infusion of increasing analyte concentrations in the presence of constant marker concentration. The resulting chromatographic trace is due to the marker elution and contains initially a relatively flat portion, which represents the non-specific and specific binding of the marker ligand to the stationary phase, followed by a vertical breakthrough, which reflects the saturation of the specific binding sites on the immobilized protein, and ending in a plateau, which corresponds to a complete saturation of these sites [4]. Marker concentration was kept constant at 100 nM, while the competitive ligand concentration range varied (100–3000 nM for R-1; 500–6000 nM for S-1; 2000–10,000 nM for 2; 4000–12,000 nM for 4). In Fig. 3 are reported the chromatographic profiles obtained for the displacement experiment of compound 2.

The dissociation constants evaluated with the two techniques on the PPARγ-OT column are reported in Table 3. The data show that the affinity order is the same with the two techniques (chromatographic assay and ITC) and that the dissociation constants calculated with the two techniques show similar values in their

### Table 3

Comparison between the PPARγ affinity data obtained by ITC measurements and affinity data obtained by frontal chromatography and frontal displacement chromatography.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>$K_d$ (μM)\textsuperscript{a}</th>
<th>$K_d$ (μM)\textsuperscript{b}</th>
<th>$K_d$ (μM)\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1</td>
<td>0.27 ± 0.045</td>
<td>0.24 ± 0.045</td>
<td>0.94 ± 0.20</td>
</tr>
<tr>
<td>S-1</td>
<td>2.00 ± 0.37</td>
<td>3.54 ± 0.92</td>
<td>3.45 ± 0.98</td>
</tr>
<tr>
<td>2</td>
<td>4.54 ± 1.34</td>
<td>6.94 ± 3.86</td>
<td>5.63 ± 2.30</td>
</tr>
<tr>
<td>R-3</td>
<td>1.50 ± 0.45</td>
<td>2.00 ± 0.65</td>
<td>(used as marker)</td>
</tr>
<tr>
<td>4</td>
<td>7.69 ± 1.87</td>
<td>8.34 ± 0.08</td>
<td>13.43 ± 2.56</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Determined by ITC assay [21].
\textsuperscript{b} Data obtained by frontal chromatography.
\textsuperscript{c} Data obtained by frontal displacement chromatography.
\textsuperscript{d} The $K_d$ values were kept fixed during the refinement to allow a better fit of the experimental data.

![Fig. 2](image-url) Breakthrough curves for $K_d$ measurement of the ligand R-3 in frontal chromatographic experimental approach on PPARγ receptor.
order of magnitude. The differences may in part be ascribed to the different techniques and to the different media of the experiments in terms of buffer and pH. In particular $K_d$ values obtained by ITC were underestimated compared with the $K_d$ values calculated with the chromatographic assay. Notably, as reported in Table 3, the results obtained with the two chromatographic approaches (frontal chromatography and frontal displacement chromatography) are very close confirming the strength of the chromatographic $K_d$ determination. Moreover the good correspondence between the data obtained with the two

Fig. 3. Frontal chromatographic study of the displacement of R-3 by compound 2 on the PPARy-OT column using a mobile phase containing 100 nM R-3: (A) mobile phase; (B) after the addition 2000 nM ligand 2 to the mobile phase; (C) after the addition 4000 nM ligand 2 to the mobile phase; (D) after the addition 10,000 nM ligand 2 to the mobile phase.

Fig. 4. Breakthrough curves $K_d$ measurement of the ligand R-1 in frontal affinity chromatography experiments on PPARα receptor.
chromatographic methods suggests that non-specific interactions with the receptor or with the chromatographic back-bone are not significant. For these reasons, between the evaluated chromatographic methods, only frontal chromatography was applied for the further $K_d$ determination of the same ligands on PPARα-OT column.

In these experiments the concentration range was different for each analyte (200–1000 nM for R-1; 200–9000 nM for S-1; 2000–10,000 nM for 2; 300–10,000 nM for R-3). As an example in Fig. 4 are shown the breakthrough curves of ligand R-1.

Calculated $K_d$ values on PPARα subtype capillary were $0.22 \pm 0.08 \mu M$, $0.51 \pm 0.12 \mu M$, $2.50 \pm 1.12 \mu M$ and $4.89 \pm 0.83 \mu M$ for R-1, S-1, 2 and R-3 respectively while the number of binding sites $B_{max}$ were $49.53 \pm 6.07, 112.60 \pm 6.52, 355.40 \pm 49.30$ and $324.20 \pm 27.24$ pmol for R-1, S-1, 2 and R-3 respectively.

Fig. 5. Binding of (a) R-1, (b) S-1, (c) 2 and (d) R-3 to PPARα-LBD. The upper panels show the raw data, the lower panels show the corresponding binding isotherm fitted according to the “one binding site” model. The thermodynamic parameters ($K_d$, $\Delta H$ and $\Delta S$) are indicated in the boxes.
<table>
<thead>
<tr>
<th>Cpd</th>
<th>$K_d$ gamma (μM)</th>
<th>$K_d$ alpha (μM)</th>
<th>$K_d$ gamma/$K_d$ alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1</td>
<td>0.24 ± 0.045</td>
<td>0.22 ± 0.08</td>
<td>1.09</td>
</tr>
<tr>
<td>S-1</td>
<td>3.54 ± 0.92</td>
<td>0.51 ± 0.12</td>
<td>6.94</td>
</tr>
<tr>
<td>2</td>
<td>6.94 ± 3.86</td>
<td>2.50 ± 1.12</td>
<td>2.77</td>
</tr>
<tr>
<td>R-3</td>
<td>1.16 ± 0.55</td>
<td>4.89 ± 0.83</td>
<td>0.24</td>
</tr>
</tbody>
</table>

**Table 4**

Data obtained by frontal chromatography and ITC on PPARα and PPARγ subtype.

Moreover it was found that the two receptor subtype capillaries can be used to perform a quantitative estimation of binding affinity constant. The strength of the FAC-MS approach has been validated using a completely different methodology (ITC) and the calculated $K_d$ values were in good accordance with those of FAC-MS.

In conclusion we have presented an experimental proof-of-concept that FAC-MS approach can be used as a reliable quantitative method for the characterization of PPARα/γ dual ligands. This method can promote an efficient affinity selection of new potential lead compounds at an early stage in drug discovery.

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**References**