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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 MS detection

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Abstract

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 PPAR α / γ dual agonists. To rapidly identify novel PPARs dual ligands, a robust binding assay amenable to high-throughput screening towards PPAR isoforms 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 PPAR α receptor was immobilized onto the surface of open tubular capillaries to create new PPAR- α -OT columns to be used in parallel with PPAR- γ -OT columns. The two biochromatographic systems were used in both ranking and K_d experiments towards new ureidofibrate-like dual agonists for subtype selectivity ratio determination. In order to validate the system, the K_d 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.

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Keywords

High-Performance Affinity Chromatography; Frontal Affinity Chromatography coupled to Mass Spectrometry; Isothermal Titration Calorimetry; immobilized PPAR γ and PPAR α receptors; ligand affinity determination

1. INTRODUCTION

In the research for new chemical entities that are able to interact with a relevant pharmaceutical 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 (mM range). Importantly, FAC-based screening does not require ligand labelling 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- γ receptor immobilized on the inner surface of an open tubular (OT) silica capillary for screening a series of chiral fibrates 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, PPAR α agonists such as fibrates improve dyslipidaemia, while PPAR γ 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 PPAR α/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 radioligands 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 reporting 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 K_d values and selectivity ratio of some new ureidofibrate-like dual agonists, only characterized in terms of affinity towards the PPAR-gamma isoform by ITC experiments [21].

In order to validate the system, the K_d 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 SECTION

2.1 Materials

The reagents for target immobilization on silica capillaries were NaOH, Carlo Erba Reagenti, (Rodano, MI, I), 3-aminopropyltriethoxysilane, glutaraldehyde, KH_2PO_4 , NaBH_3CN , monoethanolamine, CH_3OH , DMSO, $\text{CH}_3\text{COONH}_4$, and GW1929 or N-(2-Benzoylphenyl)-O-[2-(methyl-2-pyridinylamino)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. x 0.375mm x 40cm) 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 L. [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 10mM pH 7.4, 10% MeOH) through the capillary at 2.5 $\mu\text{L min}^{-1}$. To improve the sensitivity, the eluent from the capillary was mixed with an organic make-up flow (100% methanol) pumped at 5 $\mu\text{L min}^{-1}$ 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, Massachusetts, 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 $\mu\text{L min}^{-1}$ flow rate, washed with 1 mL of water at the same flow rate and dried at 95°C for 1 hour. Then, 1 mL of a solution of 3-aminopropyltriethoxysilane (10% v/v in water) was infused in the capillary at 100 $\mu\text{L min}^{-1}$ and incubated at 95°C for 30 minutes. 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 $\mu\text{L min}^{-1}$ and the unreacted aldehydic groups were removed by washing with 1 mL of 50 mM phosphate buffer pH 7.0 at 50 $\mu\text{L min}^{-1}$. 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 $\mu\text{L min}^{-1}$. After the rinsing with pure buffer, the Schiff bases were reduced by the infusion of a 5 mg mL⁻¹ solution of NaBH₃CN in phosphate buffer. After this step, unreacted groups were inactivated with 0.1 M monoethanolamine at 5 $\mu\text{L min}^{-1}$ for 1 hour. 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 PPAR α or of the PPAR γ 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 $\mu\text{L min}^{-1}$.

The K_d for each analyte was measured in a range of concentrations around the K_d value determined by ITC, and each concentration was infused at 2.5 $\mu\text{L min}^{-1}$.

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 $\rightarrow$ 353.2 (Collision Energy 23.0, isolation width 1.0 m/z); for **R-3** we have monitored m/z 425.5 $\rightarrow$ 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 $\rightarrow$ 353.2 (Collision Energy 28.0, Isolation width 1.0 m/z) and **4** was monitored at m/z 411.3 $\rightarrow$ 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, sheath 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 K_d 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 method). The instrumental conditions were constant: source voltage 5.0 kV, sheath 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 $\rightarrow$ 353.2 (CID 23.0); for **R-3**, m/z 425.5 $\rightarrow$ 193.0, 353.2 (CID 28.0), for analyte **2**, m/z 439.3 $\rightarrow$ 353.2 (CID 28.0) and for compound **4**, m/z 411.3 $\rightarrow$ 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 $\rightarrow$ 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 $\mu\text{L min}^{-1}$.

Selective ion chromatograms were reconstructed from the specific ion transition of each analyte and they were analyzed with a polynomial equation of degree 3 ($y=ax^3 + bx^2 + cx + d$) 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 (K_d) were calculated using the following equation:

$$[L] \times (V - V_0) = B_{\max} \times [L] / K_d + [L] \quad (1)$$

Where $[L]$ is the concentration of the ligand, V (μL) is the retention volume of the 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 binding sites for each ligand in the capillary.

K_d 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 marker sigmoidal curves shift 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 K_i of the competing ligand by the following equation:

$$(V - V_{\min}) = P \times (K_i + [A])^{-1} \quad (2)$$

Where V (μL) is the retention volume of the marker ligand calculated at the breakthrough time, $V_{\min}$ is the retention volume of the marker ligand when the specific interaction is completely suppressed, P is the product of the $B_{\max}$ (number of active binding sites) and K_d/K_{dM} and $[A]$ is the analyte concentration.

From the plot of $(V - V_{\min})$ versus $[A]$, 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 (> 1,000-fold) PPAR γ agonist was chosen as marker ligand (Figure 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].

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**) to 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 PPAR γ -OT and PPAR α -OT capillary preparation

The protocol, previously described for PPAR γ LBD, was followed for PPAR α LBD immobilization on the inner surface of silica capillaries [13]. As demonstrated for PPAR γ 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 PPAR α receptor.

The reproducibility of the PPAR α -OT and PPAR γ -OT capillaries synthesis was assessed by preparing three different capillaries. The procedure for the preparation is described in details in paragraph 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 ($R^2 = 0.9800$ and $R^2 = 0.9854$ 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 PPAR γ and PPAR α 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 PPAR γ and PPAR α capillaries. The capillaries were tested in the ranking of four structurally related ureidofibrates (compounds **R-1**, **2**, **R-3**, **4**). The diastomers **S-1** and **S-3** were not added to the mixture as they have the same m/z values of their corresponding enantiomers **R-1** and **R-3**. However, a high-affinity full agonist of PPAR γ , the carboxylic derivative **GW1929**, was selected as reference compound and was added to the mixture as marker ligand.

Solutions of the ligands, each at $0.5 \mu\text{M}$, 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 PPAR γ OT capillary when the chromatographic data were correlated to the K_d 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 ($K_i = 1.4 \text{ nM}$) as reported in literature [22]. Of interest for the PPAR γ 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 PPAR α OT capillary resulted in a different ranking order. This order followed the general trend of EC_{50} 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 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 γ -OT capillaries were also used for the accurate determination of the binding affinity (expressed as dissociation constant, K_d) of each analyte of the ureidofibrate series.

In the case of PPAR γ -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 (paragraph 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**; 100–10000 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 Figure 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 paragraph 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 [24]. 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**; 2000–10000 nM for **2**; 4000–12000 nM for **4**). In Figure 3 are reported the chromatographic profiles obtained for the displacement experiment of compound **2**.

The dissociation constants evaluated with the two techniques on the PPAR γ -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 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 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 PPAR α column.

In these experiments the concentration range was different for each analyte (200–1000 nM for **R-1**; 200–9000 nM for **S-1**; 2000–10000 nM for **2**; 300–10000 nM for **R-3**). As an example in Figure 4 are shown the breakthrough curves of ligand **R-1**.

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

3.5 Determination of ligand binding affinities on PPAR α by ITC

In order to validate the chromatographic results obtained on the PPAR α subtype capillary ITC experiments were carried out on PPAR α at the same pH value.

The ITC technique allows to measure the thermodynamic parameters associated with the formation of the complex PPAR/ligand. Particularly, by measuring the heat absorbed or released by titrating the protein with a ligand at constant temperature it is possible to obtain the stoichiometry of the reaction, the binding enthalpy and the affinity constant. All the experiments were performed in buffer Hepes at pH 7.4. Figure 5a-d shows the calorimetric data (raw and integration data) obtained in the titrations of PPAR α with the four ureidofibrates **R-1**, **S-1**, **2** and **R-3**. After subtraction of the heat of dilution from the raw data and linear curve fitting under “one binding site” model the thermodynamic parameters shown in the Figure were obtained. As far as the binding affinity is concerned, the results (K_d) are in good accordance with those of

FAC-MS (Table 4) with the only exception of **S-1** whose K_d is 4 times higher than that measured by FAC-MS ($2.2 \mu\text{M}$ versus $0.5 \mu\text{M}$). The K_d gamma/ K_d alpha ratios (Table 4) show that, with the only exception of **R-1**, all compounds have good PPAR gamma/alpha subtype selectivity

Conclusions

In this work, a new open tubular capillary based on the LBD of PPAR-alpha subtype was prepared and the system was used in parallel with a previously developed system based on the gamma subtype.

Through the simple application of a parallel ranking experiment, a rapid evaluation of receptor subtype affinity in a series of strictly related analogs was carried out. The result of this ranking experiment strongly demonstrates the specific nature of the interaction of the ligands with the two immobilized receptor subtypes indicating that the immobilized ligand binding domains maintain their binding capacity.

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 PPAR α / 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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PPAR α receptor was immobilized onto the surface of open tubular capillaries.
The PPAR-alpha-OT column was used in parallel with a PPAR-gamma-OT column.
The selectivity ratio of new dual agonists was determined.
The affinity data were validated with ITC experiments.

GW1929

Figure 1.
Chemical structure of the PPAR-gamma agonist GW1929.

Figure 2.
Breakthrough curves for K_d measurement of the ligand **R-3** in frontal chromatographic experimental approach on PPAR γ receptor.

Figure 3.

Frontal chromatographic study of the displacement of **R-3** by compound **2** on the PPAR-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 10000 nM ligand **2** to the mobile phase.

Figure 4.
Breakthrough curves K_d measurement of the ligand **R-1** in frontal affinity chromatography experiments on PPAR α receptor.

Figure 5.

Binding of (a) **R-1**, (b) **S-1**, (c) **2** and (d) **R-3** to PPAR α -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 , ΔH and ΔS) are indicated in the boxes.

Table 1

Chemical structure of ureidofibrate-like derivatives considered in the present study.

Compound	R	R'
R-1	CH ₃	C ₂ H ₅
S-1	C ₂ H ₅	CH ₃
2	CH ₃	CH ₃
R-3	H	CH ₃
4	H	H

Table 2

Relative affinity data expressed as breakthrough times obtained by ranking experiment of the ureidofibrate-like derivatives on PPAR γ receptor and PPAR α receptor subtypes in comparison with ITC data.

Cpd	K_d (μ M) ^a	Bt (min) PPAR α		Bt (min) PPAR γ	
		Rank 1	Rank 2	Rank 1	Rank 2
R-1	0.27	33.61	29.50	25.7	27.66
2	4.5	25.74	19.54	18.66	26.49
R-3	1.5	27.21	24.69	18.33	24.95
4	7.7	24.25	13.02	16.51	23.53
GW1929	< 0.04 ^b	34.47	-	18.78	-

^a K_d values as calculated by ITC experiments [21]

^b Ref. [23]

Table 3

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

Cpd	K_d ITC (μ M) ^a	K_d (μ M) ^b	K_i (μ M) ^c
R-1	0.27 ^d	0.24 $\pm$ 0.045	0.94 $\pm$ 0.20
S-1	2.00 $\pm$ 0.37	3.54 $\pm$ 0.92	3.45 $\pm$ 0.98
2	4.54 $\pm$ 1.34	6.94 $\pm$ 3.86	5.63 $\pm$ 2.30
R-3	1.50 ^d	1.16 $\pm$ 0.55	(used as marker)
4	7.69 $\pm$ 1.87	8.34 $\pm$ 0.08	13.43 $\pm$ 2.56

^aDetermined by ITC assay [21].

^bData obtained by frontal chromatography

^cData obtained by frontal displacement chromatography

^dThe K_d values were kept fixed during the refinement to allow a better fit of the experimental data.

Table 4Data obtained by frontal chromatography and ITC on PPAR α and PPAR γ subtype.

Cpd	Frontal chromatography		K_d gamma/ K_d alpha
	K_d gamma (μ M)	K_d alpha (μ M)	
R-1	0.24 ± 0.045	0.22 ± 0.08	1.09
S-1	3.54 ± 0.92	0.51 ± 0.12	6.94
2	6.94 ± 3.86	2.50 ± 1.12	2.77
R-3	1.16 ± 0.55	4.89 ± 0.83	0.24
ITC			
Cpd	K_d gamma (μ M) ^a	K_d alpha (μ M)	K_d gamma/ K_d alpha
R-1	0.27^b	0.27 ± 0.06	1.0
S-1	2.00 ± 0.37	2.16 ± 0.91	0.92
2	4.54 ± 1.34	2.05 ± 0.25	2.21
R-3	1.50^b	4.24 ± 0.48	0.35

^aDetermined by ITC assay in Ref [21].^bThe K_d values were kept fixed during the refinement to allow a better fit of the experimental data.