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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

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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.

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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

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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

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