Journal of Steroid Biochemistry & Molecular Biology 152 (2015) 1–7

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Environmental pollutants directly affect the liver X receptor alpha activity: Kinetic and thermodynamic characterization of binding

Matteo Mozzicafreddo *, Massimiliano Cuccioloni, Laura Bonfili, Valentina Cecarini, Francesco Alessandro Palermo, Paolo Cocci, Gilberto Mosconi, Aida Capone, Irene Ricci, Anna Maria Eleuteri, Mauro Angeletti

School of Biosciences and Veterinary Medicine, University of Camerino, Camerino 62032 Italy

ARTICLE INFO

Article history:
Received 25 September 2014
Received in revised form 2 April 2015
Accepted 8 April 2015
Available online 11 April 2015

Keywords:
Liver X receptor
Plasticizers
Molecular docking
Binding study
q-RT-PCR
Cell culture

ABSTRACT

Liver X receptor is a ligand-activated transcription factor, which is mainly involved in cholesterol homeostasis, bile acid and triglycerides metabolism, and, as recently discovered, in the glucose metabolism by direct regulation of liver glucokinase. Its modulation by exogenous factors, such as drugs, industrial by-products, and chemicals is documented. Owing to the abundance of these synthetic molecules in the environment, and to the established target role of this receptor, a number of representative compounds of phthalate, organophosphate and fibrate classes were tested as ligands/modulators of human liver X receptor, using an integrated approach, combining an *in silico* molecular docking technique with an optical SPR biosensor binding study. The compounds of interest were predicted and proved to target the oxysterols-binding site of human LXRα with measurable binding kinetic constants and with affinities ranging between 4.3×10^{-7} and 4.3×10^{-8} M. Additionally, non-cytotoxic concentration of these chemicals induced relevant changes in the *LXRα* gene expression levels and other target genes (*SREBP-1c* and *LGK*) in human liver hepatocellular carcinoma cell line (HepG2), as demonstrated by q-RT-PCR.

1. Introduction

Liver X receptors (LXRs) are nuclear receptors (NRs) mainly involved in the gene transcription process of constituents of lipid and carbohydrate metabolism, including reverse cholesterol transport (RCT) [1–4]. Furthermore, they regulate inflammatory response in macrophages [5]. Two isoforms of this receptor are well characterized: LXRα (or Nr1h3), principally expressed in liver and, to a lesser extent, in adipose tissue, adrenal glands, kidneys, macrophages, lungs and intestine, and LXRβ (or Nr1h2), expressed ubiquitously [6]. LXR, like other members of NR1 family, works together with co-activators and co-repressors, forming for example heterodimers with the retinoid X receptor (RXR) [7]. Upon interaction with specific endogenous ligands (oxysterols like 22(*R*)-hydroxycholesterol, 24(*S*)-hydroxycholesterol and 24(*S*), 25-epoxycholesterol [8,9], oxysterol metabolites [10], and some bile acids [10]), it activates the transcription by binding LXR responsive elements (LXREs), a series of direct repeats (DRs) of the core sequence AGGTCA separated by four nucleotides (DR-4) [11]. In particular, LXRs have been characterized as main transcriptional regulators of lipid and sugar metabolism, acting on sterol regulatory element-binding protein-1c (*SREBP-1c*) gene and liver glucokinase (*LGK*) gene, respectively [12,13].

Other recent studies documented the modulation of NRs by a series of small molecules, such as common pharmaceutical drugs [14], and environmental pollutants, such as industrial by-products and widely used chemicals, which can eventually act also as endocrine disruptors [15–17]. Among these compounds, phthalates, organophosphate and fibrates are extensively studied because of their significant reactivity against NRs.

Phthalates or phthalic acid esters (PAEs) are synthetic organic compounds used as additives or plasticizers of polyvinyl chloride (PVC) in several consumer products [18,19]. They can hence be found in numerous products such as toys, childcare articles, food packaging materials, vinyl gloves, floor and wall coverings, and medical devices [20]. Since these molecules are not covalently bound to PVC, they could be easily released into the environment, and come into contact with humans via intravenous, oral, inhalation and dermal routes [21]. Among these, di-2-ethyl hexyl phthalate (DEHP) and mono-2-ethyl hexyl phthalate (MEHP) were demonstrated to interact with constitutive androstane receptor

* Corresponding author at: Gentile III da Varano, 62032, Camerino, Italy. Tel.: +39 737403247.
E-mail address: matteo.mozzicafreddo@unicam.it (M. Mozzicafreddo).

http://dx.doi.org/10.1016/j.jsbmb.2015.04.011
0960-0760/

(CAR) [22] and LXRα [23], respectively. These results were also confirmed by an *in silico* analysis of some phthalates with the three subtype of human peroxisome proliferator activated receptor (PPAR) and retinoid X receptor (RXR) [18].

Organophosphates (OPs), derivatives of phosphoric, phosphonic and phosphinic acids, are commonly used as some ophthalmic agents, antihelmintics, herbicides, insecticides, nerve chemical weapons, in addition to plasticizers, solvents, and extreme pressure additives for lubricants, having a high probability to interact with humans. In this respect, recently, a large number of studies have reported dangerous relationships between the extensive use of OPs and the increase of some diseases like Parkinson, Alzheimer, diabetes, obesity, and chronic obstructive respiratory syndrome [24]. In particular, farnesyl pyrophosphate (FPP) was reported to bind some NRs, thus activating the transcription [25].

Finally, fibrates are amphipathic carboxylic acids used for some time as lipid regulators, reducing plasma triglyceride and modulating the apolipoprotein content in low-density lipoproteins (LDL), acting on PPARs [26]. Specifically, a number of studies conducted in United States, United Kingdom and Germany reported the presence of bezafibrate mainly in aquatic environments, like streams, river and effluents, acting as endocrine disruptor by affecting the gonadal steroidogenesis and spermatogenesis and regulating the PPAR activity [16,27].

To obtain further information on the molecular basis of the observed effects caused by these chemicals, a representative selection of the phthalate, organophosphate and fibrate class of compounds was taken into consideration in this work; the kinetic and thermodynamic characterization of the binding to LXRα and the evaluation of the modulatory capability toward the receptor have been the main focus of the present paper. Starting from a preliminary *in silico* screening data on the interaction with LXRα, five out of seventeen potential pollutant molecules, namely bisphenol A (BPA), diisodecyl phthalate (DiDP), diisononyl phthalate (DiNP), bezafibrate (BZF) and tri-m-tolyl phosphate (TMTP) were selected on the basis of their predicted affinity for this receptor and their chemical class. These compounds were used for a molecular docking analysis, for the biosensor binding *in vitro* assay with LXRα, and tested as modulators of transcription of specific genes regulated by this receptor in a polarized human liver hepatocellular carcinoma cell line (HepG2).

2. Materials and methods

2.1. Molecular docking

A preliminary screening of a capability of some pollutants to bind the liver X receptor was performed using molecular docking, a structural bioinformatics tool that provides geometric and affinity information about the complex. In particular, Autodock Vina [28] (version 1.1.2), the automated molecular docking software, was used on an Intel Core i7/Mac OS X 10.9 – based platform. All pollutant molecules were retrieved from Pubchem database [29], minimized (with a universal force field, UFF, and a conjugate gradient algorithm until a ΔE lower than 0.001 kJ/mol) using Avogadro software (Version 1.1.0. http://avogadro.openmolecules.net/) [30] and saved as pdbqt files. The three-dimensional structure of LXRα (PDB ID:3IPQ [31]) was obtained from Protein Data Bank [32] and its pdbqt file was prepared removing water molecules, all ligands included in the crystal and considering polar hydrogen atoms. Then, LXRα molecule was set as receptor, pollutant molecules as ligands and docking grid box was created around the entire receptor and the oxysterols-binding site with a size of $60 \times 65 \times 55$ Å and $26 \times 27 \times 25$ Å, respectively.

Predicted binding affinities were expressed throughout as equilibrium dissociation constants, resulting from the equation:

$$K_d = e^{\Delta G}\text{bind}\frac{1000}{RT} \quad (1)$$

and obtained using both default settings and *ad-hoc* optimized scoring function weights. The latter were derived carrying on a multivariate liner regression of the predicted energy contributions (Supplementary Table 1) versus the experimental free energy of binding (ΔG) of 12 available LXRα/ligand complexes (see Fig. 3 legend), using the Matlab R2014b estimation algorithms (the ordinary multivariate normal maximum likelihood estimation, the maximum likelihood estimation via the expectation conditional maximization algorithm, and covariance-weighted least squares estimation) [33].

All models and images were rendered using Mac PyMOL software (Python Molecular Graphics – version 1.3), whereas the intermolecular interaction features of each complex were obtained using LigandScout software (Inte:Ligand version 3.12) [34].

2.2. Biosensor studies

The liver X receptor, BPA, DiDP, DiNP, BZF and TMTP, Na_2HPO_4, CH_3COONa, KCl, NaCl, Tween-20, were all purchased from Sigma–Aldrich (Milan, Italy). The carboxylate cuvette used for the interaction studies and the immobilization kit (NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide; ethanolamine) were obtained from Farfield Group (Cheshire, UK). All chemicals were of the highest grade available.

After PBS-T wash (Na_2HPO_4 10 mM, KCl 2.7 mM, NaCl 138 mM, Tween-20 0.05% $_{(v/v)}$, pH 7.4) and equilibration with Tween-20 free PBS for approximately 15 min, the surface of a IAsys biosensor carboxylate cuvette was covalently charged with a given concentration of liver X receptor (100 μg/mL) by a standard EDC/NHS coupling procedure [35]. The non-coupled receptor was removed by washing with PBS buffer and the remaining reactive groups of the surface were deactivated by a treatment with ethanolamine (1 M). Then, each pollutant was added to the LXR-functionalized surface at increasing concentrations in the range 1.6×10^{-6} -1.6×10^{-7} M, and association kinetics were routinely followed up to the equilibrium. Dissociation and surface regeneration steps were obtained by a PBS buffer addition.

The measured LOD values for the pollutants under study were in the range 7.02×10^{-9}–6.41×10^{-10} M. Kinetic raw data were analysed with Fast Fit software (Fison Applied Sensor Technology; Affinity Sensors) and globally fitted [36] with a standard monophasic time course equation:

$$R_t = R_{eq,[L]}\left(1 - e^{-(K_{ass[L]} + K_{diss})t}\right) \quad (2)$$

where the response at equilibrium (extent) is:

$$R_t = R_{eq,[L]} = \frac{R_{max}K_{ass[L]}}{K_{ass[L]} + K_{diss}} \quad (3)$$

and R_{max} is the extent at asymptotically high concentrations of a ligand *[L]*. Time courses measured at several ligand concentrations are simultaneously analysed using Eqs. (2) and (3), sharing k_{ass},k_{diss}, and R_{max} parameters.

2.3. Cell culture and viability assays

Cells were grown in a 5% CO_2 atmosphere at 37 °C in 100 mm tissue culture dishes. Growth media were MEM supplemented with 10% FBS, 1% sodium pyruvate, antibiotic, and antimycotic for HepG2. Media and reagents for cell culture were purchased from EuroClone S.p.A. (Milan, Italy).