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Radioligand binding assay for accurate determination of nuclear retinoid X receptors: A case of triorganotin endocrine disrupting ligands

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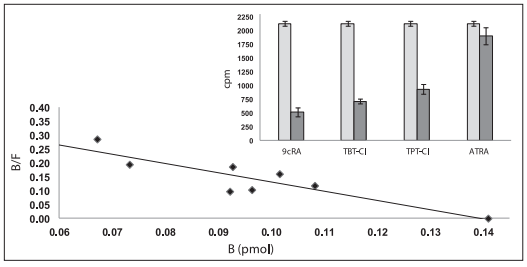
HIGHLIGHTS

- Novel radioligand binding assay for accurate determination of retinoid X receptors binding capabilities.
- Optimal conditions for 9-*cis* retinoic acid specific binding to its cognate nuclear retinoid X receptors.
- Endocrine disruptors trialkyltin and triphenyltin have been confirmed as RXR ligands by radioligand binding assay.

GRAPHICAL ABSTRACT



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ABSTRACT

Nuclear 9-*cis* retinoic acid receptors (retinoid X receptors, RXR) are promiscuous dimerization partners for a number of nuclear receptors. In the present study, we established a novel in vitro method for quantitative determination of the nuclear retinoid X receptors in rat liver. One type of high affinity and limited capacity RXR specific binding sites with the K_a value ranging from 1.011 to 1.727 $\times 10^9$ l/mol and the B_{max} value ranging from 0.346 to 0.567 pmol/mg, was demonstrated. Maximal 9-*cis* retinoic acid (9cRA) specific binding to nuclear retinoid X receptors was achieved at 20 °C, and the optimal incubation time for the 9cRA-RXR complex formation was 120 min. From a number of endocrine disruptors, tributyltins and triphenyltins are known as RXR ligands. Our data confirmed the property of tributyltin chloride or triphenyltin chloride to bind to a high affinity and limited capacity RXR binding sites. Described optimal conditions for ligand binding to RXR molecules enabled us to calculate maximal binding capacity (B_{max}) and affinity (K_a) values. This study provides an original RXR radioligand binding assay that can be employed for investigation of novel RXR ligands that comprise both drugs and endocrine disruptors.

Abbreviations: ATRA, all-*trans* retinoic acid; B_{max}, maximal binding capacity (pmol/mg); DNA, deoxyribonucleic acid; DR, direct repeat; DTT, 1,4-dithiothreitol; FXR, farnesoid X receptor; K_a, affinity (l/mol); LBD, ligand-binding domain; LXR, liver X receptor; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator activated receptor; PXR, pregnane X receptor; RAR, retinoid receptor; RARE, retinoid response element; RXR, retinoid X receptor (rexinoid receptor); RXRE, retinoid X response element; TBT-Cl, tributyltin chloride; TPT-Cl, triphenyltin chloride; TR, thyroid hormone receptor; VDR, dihydroxyvitamin D3 receptor; 9cRA, 9-*cis* retinoic acid.

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1. Introduction

Retinoic acids, all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9*c*RA) are essential components of numerous biological processes starting from cell division, differentiation, embryonal development, and maintenance of vertebrate homeostasis (Mangelsdorf, 1994; Brtko and Thalhamer, 2003; Brtko, 2007). ATRA acts as a natural ligand for appropriate ligand-activated transcription factors, nuclear retinoid receptors (RAR) and 9*c*RA represents a natural ligand for nuclear retinoid X (rexinoid, RXR) receptors (Brtko and Dvorak, 2011; Flodrova et al., 2015). RXR molecules are known to be promiscuous partners for a number of nuclear receptors that activate multiple signaling pathways (Lefebvre et al., 2010; Evans and Mangelsdorf, 2014). They form three types of heterodimers with several other nuclear receptors: RXR permissive heterodimers, RXR nonpermissive and also RXR conditional heterodimers (Yamada and Kakuta, 2014; Evans and Mangelsdorf, 2014; Di Masi et al., 2015). Activation of RXRs by their cognate ligands have potentially pleiotropic effects on numerous biological pathways, and a cross-talk between various RXR heterodimers and other signaling pathways seems to be crucial in understanding their molecular mechanisms of action (Jacobs and Paulsen, 2005; Ross-Innes et al., 2010; Brtko and Dvorak, 2011).

Receptor–ligand interactions play a crucial role in biological systems and their measurement forms an important part of molecular pharmacology, toxicology or endocrinology. Nowadays assays formats are available for screening and quantifying receptor ligands by non-radioactive assays based on optical methods like fluorescence polarization, fluorescence resonance energy transfer or surface plasmon resonance (De Jong et al., 2005).

Fluorescence polarization (FP) method is suited to observe the binding of small fluorescent molecules to proteins (Parker et al., 2000). This method has been applied to almost every protein class, including G-protein-coupled receptors, nuclear receptors, enzymes, and it has also been applied to the analysis of molecular interactions including protein-protein, protein-DNA or protein-ligand binding (Lea and Simeonov, 2011). Boichenko et al. (2016) demonstrated the high potential and general applicability of the fluorescence resonance energy transfer (FRET) based assay for the identification and characterization of the protein cereblon effector molecules. In a comparative substrate binding study, it has been demonstrated that representatives of all cereblon proteins showed the same differential affinities to the same substrates on a relative scale (Boichenko et al., 2016). Surface plasmon resonance (SPR) spectroscopy is a technique for the study of ligand binding interactions with proteins, which is label-free and capable of measuring real-time quantitative binding affinities and kinetics for proteins interacting with ligand molecules. This technique requires one binding component to be immobilized on a sensor chip whilst the other binding component in solution is flowed over the sensor surface. Binding interaction is detected using an optical method that measures small changes in refractive index at the sensor surface (Patching, 2014). The interacting molecules may be proteins, peptides, lipids, viruses, nucleic acids, or small organic molecules such as fragments or drug candidates (Frostell et al., 2013). Whited and Park (2014) reported an atomic force microscopy (AFM), as a technique evolved for visualizing single membrane protein–ligand complexes and conformational changes elicited through ligand binding. They also describe how AFM can be used to characterize interactions through dissociation of membrane protein–ligand complexes by force.

In the present study, we established the radioligand binding assay for accurate characterization of specific ligand-receptor complexes in order to determine RXR binding parameters. For this purpose, we have taken experience from our previously published radioligand binding assay for accurate determination of nuclear all-*trans* retinoic acid receptors (RARs) established in our laboratory more than two decades ago (Brtko, 1994). This assay deals with *in vitro* quantitative determination of RARs in rat liver nuclear proteins obtained from purified nuclei depleted of any contaminating cytoplasmic proteins. Since the equilibrium association constant (K_a) and the maximal binding capacity (B_{max}) have not been precisely designated for RXRs in mammals yet, we applied this modified technique for the determination of RXR binding characteristics.

Trialkyltins and triaryltins, potent endocrine disruptors, function as nuclear retinoid X receptors (RXR) agonists due to their capability to bind to the ligand-binding domain of RXR subtypes and function as transcriptional activators (Nakanishi et al., 2005; Brtko and Dvorak, 2015; Hunakova et al., 2015).

Since, tributyltin chloride and triphenyltin chloride are RXR ligands, they were used as proof-of-concept for the newly developed assay.

2. Materials and methods

2.1. Isolation of nuclei

Rat liver nuclear fraction was prepared from rat livers by the procedure established and optimalized in our laboratory, previously (Brtko, 1994). Nuclear fraction can be prepared either from fresh or deep frozen liver tissues (−80 °C), and all subsequent steps have to be carried out at 0–4 °C. The liver tissue was at first washed in SM solution (0.32 M sucrose, 1 mM $MgCl_2$, 0.1 mM PMSF and freshly prepared 1 mM DTT), consequently cut into smaller pieces and homogenized by the glass-teflon homogenizator (8–10 smooth strokes). Thus prepared homogenate was centrifuged at 1000*g* for 10 min. Crude pellet was again washed with SM solution and thus prepared pellet was mixed with 2.3 M sucrose solution, with 1 mM $MgCl_2$, 0.1 mM PMSF and 1 mM freshly prepared DTT, and treated by isopycnic ultracentrifugation at 220 000*g* for 40 min. Isolated nuclei was twice washed with SMCT solution (0.32 M sucrose, 10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF and freshly prepared 1 mM DTT at pH 7.4) once in the presence of 0.25% Triton X-100 and once in its absence. Nuclear protein fraction containing RXRs was obtained from purified nuclear pellet with a high ionic strength buffer containing 0.3 M KCl, with addition of 1 mM $MgCl_2$,10 mM Tris-HCl and freshly prepared 1 mM DTT at 0 °C for 60 min followed by ultracentrifugation at 135 000*g* for 60 min. Concentration of thus prepared nuclear fraction of proteins was estimated by Lowry's method using bovine serum albumin as a standard.

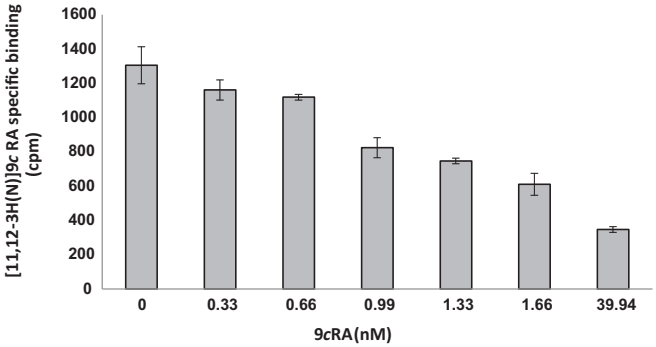

Fig. 1. Competition curve of [11,12-^{3}H(N)] 9cRA specific binding to rat liver nuclear receptors.