Published in final edited form as:

Adv Protein Chem Struct Biol. 2018 ; 110: 31–63. doi:10.1016/bs.apcsb.2017.06.001.

Using TR-FRET to investigate protein-protein interactions: a case study of PXR-coregulator interaction

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Abstract

Time-resolved fluorescence resonance energy transfer (TR-FRET) protein-protein interaction assays, especially in the format of receptor-coregulator (coactivator and corepressor) recruitment/repression assays, have been widely used in nuclear receptor research to characterize the modes of action, efficacies, and binding affinities of ligands (including their properties as agonists, antagonists, and inverse agonists). However, there has been only limited progress in using this assay format for pregnane X receptor (PXR). In this chapter, we discuss TR-FRET protein-protein interaction assays and focus on a novel PXR TR-FRET coactivator interaction assay that we have developed based on a PXR-coactivator co-crystal study. This new PXR TR-FRET coactivator interaction assay can characterize the binding affinities of PXR ligands and also differentiate antagonists from agonists. This assay is very robust, with the signal remaining stable over a long incubation time (up to 300 min has been tested). It can tolerate high concentrations of DMSO (up to 5%) and has a high signal-to-noise ratio (six under typical assay conditions). This newly developed PXR TR-FRET coactivator interaction assay has potential application in high-throughput screening (HTS) to identify and characterize novel PXR agonists and antagonists.

Keywords

nuclear receptor; pregnane X receptor; TR-FRET; coregulator; coactivator; corepressor; agonist; antagonist

1. INTRODUCTION

The nuclear receptor pregnane X receptor (PXR) is one of the master xenobiotic receptors that regulate the metabolism and excretion of xenobiotics, as well as endobiotics, by regulating the expression of drug-metabolizing enzymes and drug transporters (Gong et al., 2005; Kliewer et al., 1998; Y. M. Wang, Ong, Chai, & Chen, 2012; Yan & Xie, 2016). Because PXR can affect drug metabolism and distribution, changes in PXR activity can affect the therapeutic and toxicologic response to drugs and potentially cause adverse drug-drug interactions (Timsit & Negishi, 2007; Y. M. Wang et al., 2012).

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PXR activity is mainly modulated by direct ligand binding (Timsit & Negishi, 2007), and its large and flexible ligand-binding pocket allows it to bind a broad spectrum of structurally diverse ligands (Chai, Cherian, Wang, & Chen, 2016; Watkins et al., 2001). PXR activity can also be regulated by various cell signaling pathways (Oladimeji, Cui, Zhang, & Chen, 2016; Y. M. Wang et al., 2012).

As a member of the nuclear receptor (NR) superfamily, PXR has many structural features that are commonly found in other NRs, including a highly conserved N-terminal DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (Kliwer, Goodwin, & Willson, 2002; J. Zhang et al., 2001). The DBD allows PXR to bind to short stretches of DNA response elements in the regulatory regions of target genes. Besides its function in interacting with ligands, the LBD of PXR also contains a dimerization motif and an activation function 2 (AF-2) domain that are, respectively, critical for receptor dimerization and coregulator interactions.

The binding of a ligand to the PXR LBD changes the conformation of PXR (Ekins et al., 2007; Teotico et al., 2008; C. Y. Wang, Li, Chen, & Welsh, 2006; Watkins, Davis-Searles, Lambert, & Redinbo, 2003) and its interactions with its partner proteins, such as its dimerization partner retinoid X receptor alpha (RXR α), via its dimerization motif, and various co-activators/co-repressors, via its AF-2 domain. Depending on the nature of individual ligands, e.g., whether they are agonists or antagonists, the ligand-bound PXR will modulate target-gene expression in a ligand-specific manner. The ligand-mediated interactions between PXR and its partner proteins can be used to design a fluorescence resonance energy transfer (FRET) assay, especially one in the time-resolved FRET (TR-FRET) assay format.

In a TR-FRET protein-protein interaction assay, a rare-earth lanthanide metal with a long emission half-life, such as terbium (Tb) or europium (Eu), is typically used as the donor fluorophore to label one protein. A properly matched fluorophore, such as fluorescein for terbium or Cy5 for europium, can be used as the acceptor fluorophore to label the other interacting protein. The interaction of the two proteins brings the properly matched donor-acceptor fluorophore pair into close proximity, resulting in the generation of a time-resolved FRET signal, which can be recorded (Comley, 2006; Degorce et al., 2009). TR-FRET protein-protein interaction assays, especially in the form of NR-coregulator (coactivator and corepressor) recruitment/repression assays, have been widely used in NR research to characterize the modes of action, efficacies, and binding affinities of ligands (including their properties as agonists, antagonists, and inverse agonists) (Chen, Xie, Agler, & Banks, 2003). Most efforts have focused on NR-coactivator interaction assays, primarily in the format of one fluorescence donor (terbium or europium) paired with one fluorescence acceptor (a proper fluorophore-labeled coactivator peptide), as for androgen receptor (AR) (Ozers et al., 2007), constitutive androstane receptor (CAR) (Carazo & Pavcek, 2015; Carazo Fernández, Smutny, Hyrsová, Berka, & Pavcek, 2015; Cherian, Lin, Wu, & Chen, 2015; Cherian, Yang, Chai, Lin, & Chen, 2016; Lin, Yang, Chai, Lu, & Chen, 2016), estrogen receptor alpha and beta (ER α and ER β) (Gunther et al., 2009), estrogen-related receptor gamma (ERR γ) (Gowda, Marks, Zielinski, & Ozers, 2006), farnesoid X receptor (FXR) (G. Li et al., 2012), glucocorticoid receptor (GR) (Sharma et al., 2011), retinoid X receptor beta (RXR β)

(Stafslie, Vedvik, De Rosier, & Ozers, 2007), and thyroid receptor beta (TR β) (Jeyakumar, Webb, Baxter, Scanlan, & Katzenellenbogen, 2008). Other assays for estrogen receptor alpha (ER α) have been developed in the format of one fluorescence donor (terbium or europium) paired with two fluorescence acceptors (a proper fluorophore-labeled coactivator peptide and a proper fluorophore-labeled small molecular ligand) (Hilal, Puetter, Otto, Parczyk, & Bader, 2010) or three fluorescence acceptors (one proper fluorophore-labeled coactivator peptide and two proper fluorophore-labeled small molecular ligands) (Kim, Gunther, & Katzenellenbogen, 2010). However, there have been only a few reports of ligand characterization using TR-FRET NR-corepressor interaction assays. The assays used in those characterizations were in the format of one fluorescence donor (terbium or europium) paired with one fluorescence acceptor (a proper fluorophore-labeled corepressor peptide) for NR2E3 (nuclear receptor subfamily 2 group E member 3) (Qin et al., 2013) and one fluorescence donor (terbium or europium) paired with two fluorescence acceptors (a proper fluorophore-labeled corepressor peptide and a proper fluorophore-labeled coactivator peptide) for thyroid receptor beta (TR β) (Jeyakumar & Katzenellenbogen, 2009). In the latter case, only one peptide could bind to a single molecule of the target NR at a given time because the two peptides interact with the same binding site of the NR. Table 1 summarizes these representative TR-FRET NR coregulator interaction assays, including 2 PXR TR-FRET coactivator recruitment assays. In addition, Invitrogen (now a part of ThermoFisher Scientific) offers researchers a range of TR-FRET NR coactivator interaction assay kits, including ones for AR, CAR, ER α , ER β , ERR α , ERR β , ERR γ , FXR, GR, LXR α , LXR β , PPAR α , PPAR γ , PPAR δ , PR, RAR α , RAR β , RAR γ , ROR α , RXR α , RXR β , TR α , TR β , VDR, and approximately 31 fluorescently labeled nuclear receptor coregulator peptides. These commercially available TR-FRET NR coactivator interaction assays use a three-component, one donor—one acceptor TR-FRET format, with a terbium-labeled anti-GST antibody as the fluorescence donor, a fluorescein-labeled coactivator peptide as the fluorescence acceptor, and a GST–NR-LBD protein.

As shown in Table 1, the most commonly used NR TR-FRET coregulator interaction assay has a three-component format, with one fluorescence donor (typically a terbium- or europium-labeled anti-tag antibody) paired with one fluorescence acceptor (a proper fluorophore-labeled coregulator peptide) and a properly-tagged target NR (Table 1). In this chapter, we will focus mainly on a three-component TR-FRET assay format, with a Tb-anti-Tag as the donor fluorophore paired with a fluorescein-labeled coactivator or corepressor as the acceptor fluorophore and a properly tagged NR. The subsequent design, development, and optimization of the new PXR TR-FRET coactivator interaction assays will use this typical three-component format, with the Tb–anti-GST antibody as the donor fluorophore, the GST–PXR-LBD protein as the mediator, and the FAM-labeled representative steroid receptor coactivator 1 (SRC1) peptide as the acceptor fluorophore.

For a typical NR TR-FRET coregulator interaction assay in the three-component format, either a suitable coactivator or corepressor peptide could be selected as the NR interaction partner provided that the selected coactivator or corepressor peptide has the desired binding affinity to the target NR after being labeled with fluorescein. For most NRs, which have relatively low basal interactions with their coactivators or corepressors under unliganded apo conditions, the use of the TR-FRET interaction between these NRs and their respective

partner coregulator peptides to characterize ligand potency and modes of action is schematically outlined in Figure 1 (Figure 1A for coactivators and Figure 1B for corepressors).

For coactivators (Figure 1A), the TR-FRET interaction system could typically be constructed with a Tb-anti-tag antibody as the donor fluorophore and fluorescein-labeled coactivator as the acceptor fluorophore for a properly tagged NR. In the absence of a ligand (**Status 1**), this system has a low basal TR-FRET signal. Upon the binding of an agonist to the NR (**Route 1**), the NR changes its conformation to enhance the binding of its coactivator, resulting in a high TR-FRET signal (**Status 2**). The change from **Status 1** (low TR-FRET signal) to **Status 2** (high TR-FRET signal) by following **Route 1** in the presence of a coactivator is typically adopted for characterizing the potency and mode of action of an agonist. To characterize an antagonist, the TR-FRET system is typically started in **Status 2** in the presence of a suitable concentration of the agonist so that the system starts with a relatively high TR-FRET signal. The binding of an antagonist (**Route 3A**) to displace the agonist causes the NR to change its conformation and decrease coactivator binding. The dissociation of coactivator from the NR leads to **Status 4A**, which has a low TR-FRET signal. In addition to agonists and antagonists, which bind to the NR ligand-binding pocket, allosteric modulators interact with NRs outside of their ligand-binding pockets (Billas & Moras, 2013; Caboni & Lloyd, 2013; Christopoulos et al., 2014; Moore, Mayne, & Katzenellenbogen, 2010). Activating allosteric modulators could increase the basal TR-FRET signal of **Status 1** via **Route 2** to produce a high TR-FRET signal in **Status 3**, thus acting like agonists. Inhibitory allosteric modulators could decrease the agonist-induced high TR-FRET signal of **Status 2** via **Route 3B** to produce a low TR-FRET signal in **Status 4B**, thereby functioning as antagonists. There are also inhibitors of NR-coregulator interaction (Billas & Moras, 2013; Caboni & Lloyd, 2013; Christopoulos et al., 2014; Moore et al., 2010). Such inhibitors could bind to the NR-coactivator interaction interface via **Route 3C** and change the high TR-FRET signal of **Status 2** to a low TR-FRET signal in **Status 4C**. The presence of NR allosteric modulators and inhibitors of NR-coregulator interaction complicates the mode-of-action studies for various NR ligands. However, TR-FRET NR coregulator (coactivator or corepressor) interaction assays can be used to distinguish activating ligands from inhibitory ligands, in addition to characterizing ligand potency, thus offering an advantage over binding assays that measure only ligand-binding potencies.

Similar to the use of a coactivator, as illustrated in Figure 1A, TR-FRET NR-corepressor assays could be designed based on the interaction between an NR and its low basal interacting partner corepressor and constructed with a Tb-anti-tag antibody as the donor fluorophore and a fluorescein-labeled corepressor as the acceptor fluorophore for a properly tagged NR (Figure 1B). In the absence of a ligand (**Status 1**), this system has a low basal TR-FRET signal. Upon the binding of an antagonist to the NR (**Route 1**), the NR changes its conformation to enhance the binding of its corepressor, resulting in a high TR-FRET signal (**Status 2**). The change from **Status 1** (low TR-FRET signal) to **Status 2** (high TR-FRET signal) by following **Route 1** in the presence of a corepressor is typically used to characterize the potency and mode of action of an antagonist. To characterize agonists, the TR-FRET system is typically started in **Status 2** in the presence of a suitable concentration

of an antagonist so that the system starts with a relatively high TR-FRET signal. The binding of an agonist (**Route 3A**) to displace the antagonist causes the NR to change its conformation and decrease corepressor binding. The dissociation of bound corepressor from the NR generates **Status 4A**, which has a low TR-FRET signal. An inhibitory allosteric modulator would increase the basal TR-FRET interaction signal of **Status 1** via **Route 2** to produce a high TR-FRET signal in **Status 3**, thus acting like an antagonist. In contrast, an activating allosteric modulator would decrease the antagonist-induced high TR-FRET signal of **Status 2** via **Route 3B** to produce a low TR-FRET signal in **Status 4B**, thereby functioning like an agonist. NR-coregulator inhibitors would decrease the high TR-FRET signal of **Status 2** to a low TR-FRET signal in **Status 4C** via **Route 3C**.

A few NRs have a relatively high level of constitutive basal interaction with their partner coregulators, such as the constitutive androstane receptor (CAR), which has a relatively high level of basal interaction with its partner coactivator (Dussault et al., 2002; Windshügel, Jyrkkärinne, Poso, Honkakoski, & Sippl, 2005). Figure 2 schematically illustrates the characterization of ligands for this class of NRs by using the TR-FRET assays, using a coactivator as the representative protein interacting partner. The TR-FRET interaction system could be constructed with a Tb-anti-tag antibody as the donor fluorophore and a fluorescein-labeled coactivator as the acceptor fluorophore for a properly tagged NR. In the absence of a ligand (**Status 1**), this system has a medium basal TR-FRET signal as a result of the constitutive activity of the NR. The binding of an agonist to the NR (**Route 1**) further enhances coactivator binding and increases the TR-FRET signal (**Status 2**). The change from **Status 1** (medium TR-FRET signal) to **Status 2** (high TR-FRET signal) via **Route 1** in the presence of a coactivator is typically used to characterize the potency and mode of action of an agonist. The binding of an inverse agonist reduces the constitutive activity of a receptor. The binding of an inverse agonist to the NR changes its conformation to reduce the binding of its coactivator (**Route 2**), resulting in a low TR-FRET signal (**Status 3**). Thus, the change from **Status 1** (medium TR-FRET signal) to **Status 3** (low TR-FRET signal) via **Route 2** in the presence of a coactivator is typically used to characterize the potency and mode of action of an inverse agonist. In this assay system, inhibitors of NR-coactivator interaction act like inverse agonists by reducing the medium TR-FRET signal of **Status 1** to a low TR-FRET signal in **Status 4** via **Route 3**. Activating allosteric modulators act like agonists by enhancing coactivator recruitment to the NR (**Status 5A**) via **Route 4A**, whereas inhibitory allosteric modulators act like inverse agonists by dissociating the coactivator from the NR (**Status 5B**) via **Route 4B**. Similarly, for an NR with relatively high basal interaction with a corepressor, it is feasible to develop an NR-corepressor TR-FRET assay to characterize the potency and mode of action of the NR ligands.

To our knowledge, there is currently no commercially available assay for characterizing PXR ligand-binding potencies and modes of action in the format of PXR-coregulator TR-FRET interaction. The few reports of assays based on the interaction between PXR and biotinylated or fluorescently labeled SRC1 coactivator peptides provide no details of how the assays were established (Mitro, Vargas, Romeo, Koder, & Saez, 2007; Y. M. Wang et al., 2013). Several PXR TR-FRET competitive binding assays have been reported (Lin & Chen, 2013; Lin et al., 2014; Lin et al., 2008; Oladimeji, Lin, Brewer, & Chen, 2017; Shukla et al.,

2009), but they could reveal only the binding potencies of ligands, not their modes of action. Here, we detail the development of a PXR TR-FRET coactivator recruitment assay for human PXR (hPXR) in the format of one fluorescence donor (with terbium as the donor fluorophore) paired with one fluorescence acceptor (a FAM-labeled SRC1 coactivator peptide), based on the reported hPXR-SRC1 co-crystal structure (Watkins et al., 2003). This newly developed PXR TR-FRET coactivator recruitment assay is very robust, with the signal being stable over a long incubation time (times of up to 300 min have been tested), and it tolerates high concentrations of DMSO (up to 5%). The assay has a high signal-to-noise ratio (six-fold under typical assay conditions) and can differentiate hPXR antagonists from PXR agonists, in addition to characterizing the PXR-binding affinities of ligands. This newly developed PXR TR-FRET coactivator interaction assay has potential application in high-throughput screening (HTS) to identify and characterize novel PXR agonists and antagonists.

2. MATERIALS

The peptides FAM-SRC1-A (FAM-GG-CPSSHSSLTERHKILHRLLQEGSPS-NH₂), FAM-SRC1-B (FAM-(PEG)₅-CPSSHSSLTERHKILHRLLQEGSPS-NH₂) and FAM-SRC1-C (Ac-CPSSHSSLTERHKILHRLLQEGSPS-GG-NHCH₂CH₂NH-FAM) were synthesized by the Macromolecular Synthesis Section of the Hartwell Center for Bioinformatics and Biotechnology at St Jude Children's Research Hospital. GST-hPXR-LBD, LanthaScreen Tb-anti-GST antibody, 1 M Tris (pH 7.5), 1 M MgCl₂, 1 M DTT, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). Bovine serum albumin (BSA), clotrimazole, nicardipine, nimodipine, and ketoconazole were purchased from Sigma (St. Louis, MO). T0901317, PK11195, silybin, and trans-resveratrol were purchased from Cayman Chemical (Ann Arbor, MI). SR12813 was purchased from Enzo Life Sciences (Farmingdale, NY). Black 384-well low-volume plates were purchased from Corning Life Sciences (Tewksbury, MA). DMSO was used as a solvent for all compounds used in this study.

3. METHODS

All assays were performed in 20 μ L assay buffer (50 mM TRIS, pH 7.5, 20 mM MgCl₂, 0.1 mg/mL BSA, and 0.05 mM DTT) with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST at room temperature (approximately 25°C) in black 384-well low-volume plates. Unless specified otherwise, all assays were performed in triplicate. All chemicals were solubilized in DMSO. The final DMSO concentration was 1.1% or 1.2% in all assays, unless specified otherwise. In a typical assay development, individual fluorescently labeled SRC1 peptide or chemical (solubilized in DMSO) diluted in the assay buffer as a 2 $\times$ working solution (containing 2% DMSO) was first dispensed (at 10 μ L/well) into a 384-well plate. This was followed by the dispensing of the chemical (if the fluorescent SRC1 peptide was dispensed first) or the fluorescently labeled SRC1 solution (if the chemical was dispensed first) (as a 4 $\times$ working solution in the assay buffer with 0.4% DMSO; 5 μ L/well) and a mixture of 20 nM GST-hPXR-LBD and 20 nM Tb-anti-GST solution (prepared in the assay buffer; 5 μ L/well). In a typical ligand characterization assay, the chemical (solubilized in DMSO) diluted in the assay buffer as a 4 $\times$ working solution (containing 4% DMSO) was first dispensed (at 5

$\mu\text{L}/\text{well}$) into a 384-well plate. This was followed by the dispensing of DMSO (0.4%, 5 $\mu\text{L}/\text{well}$, for agonist characterization) or T0901317 (2 μM with 0.4% DMSO, 5 $\mu\text{L}/\text{well}$, for antagonist characterization), fluorescently labeled SRC1 solution (as a 4 $\times$ working solution in the assay buffer with 0.4% DMSO; 5 $\mu\text{L}/\text{well}$) and a mixture of 20 nM GST-hPXR-LBD and 20 nM Tb-anti-GST solution (prepared in the assay buffer; 5 $\mu\text{L}/\text{well}$). After all the assay components had been mixed by shaking for 1 min on an IKA MTS 2/4 digital microtiter plate shaker (IKA Works, Wilmington, NC), the plates were briefly centrifuged at $201 \times g$ (1000 rpm) for 30 s in an Eppendorf 5810 centrifuge with the A-4-62 swing-bucket rotor (Eppendorf AG, Hamburg, Germany). The typical assay incubation time was 120 min, with the exception of the longitudinal signal stability assays, for which the incubation times are specified. All assay data were generated using a PHERAstar FS plate reader (BMG Labtech, Durham, NC) to measure the fluorescence emission ratio (10,000 $\times$ 520 nm/490 nm) of each well, using a 340-nm excitation filter, a 100- μs delay, and a 200- μs integration time. Raw data from the plate reader were used directly for analysis. The curve-fitting software GraphPad Prism 7.00 (GraphPad Software, La Jolla, CA) was used to generate graphs and curves and to determine K_d , EC_{50} , and IC_{50} values. The signal fold change or signal-to-background ratio (Figures 5A, 5C, 6B, and 7B) was calculated by subtracting the signal of the negative-control group (the DMSO group) from the signal of the positive-control group (which had 10 μM T0901317) and dividing the result by the signal from the negative-control group (the DMSO group) in the presence of FAM-SRC1-B, Tb-anti-GST, and GST-hPXR-LBD for each experiment. The methods described here were applied to all the specific assays described below; where applicable, additional information is included in the description of a specific assay.

3.1 PXR TR-FRET Binding K_d Determinations for the Fluorescent SRC1 Peptides FAM-SRC1-A, FAM-SRC1-B, and FAM-SRC1-C

Serial dilutions of FAM-SRC1-A, FAM-SRC1-B, and FAM-SRC1-C (1:2 dilutions giving 13 concentration levels from 2500 nM to 0.6 nM) were incubated with Tb-anti-GST (5 nM), GST-hPXR-LBD (5 nM), and either DMSO or T0901317 (10 μM). The final DMSO concentration was 1.1% for all wells. As an additional background control group, the same serial dilutions of FAM-SRC1-A, FAM-SRC1-B, and FAM-SRC1-C were incubated with Tb-anti-GST (5 nM), but with 1.1% DMSO (vehicle) and without GST-hPXR-LBD. All assays were performed in the assay buffer (50 mM TRIS, pH7.5, 20 mM MgCl_2 , 0.1 mg/mL BSA, and 0.05 mM DTT). The TR-FRET signals of individual wells after 30, 60, 90, 120, and 180 min of incubation were measured with a PHERAstar FS plate reader. The collected data (10 000 $\times$ 520 nm/490 nm) were fitted into a one-site total binding equation for the DMSO vehicle group, 10 μM T0901317 group, and DMSO vehicle group (without GST-hPXR-LBD as the background control group) (Figure 4A–D). The individual dissociation constant (K_d), if applicable, was derived from the T0901317 (10 μM) group by using the one-site total binding equation in GraphPad Prism.

3.2 Optimization of the Concentration of the Fluorescent Coactivator FAM-SRC1-B in the PXR TR-FRET Coactivator Recruitment Assay

DMSO or 10 μM T0901317 was incubated with FAM-SRC1-B (50, 100, 250, or 500 nM), GST-hPXR-LBD (5 nM), and Tb-anti-GST (5 nM). The final DMSO concentration was

1.1% in all wells. The TR-FRET signals of individual wells after 120 min of incubation were measured with a PHERAstar FS plate reader. The signal-to-background ratios or signal ratios for the 10 μ M T0901317 group versus the DMSO group were calculated and plotted, along with the TR-FRET signals for the DMSO and 10 μ M T0901317 groups (Figure 5B–C).

3.3 Determination of Signal Stability in the FAM-SRC1-B–Mediated PXR TR-FRET Coactivator Recruitment Assay

Serial dilutions of T0901317 (1-to-3 dilutions giving 11 concentration levels from 10 μ M to 0.17 nM), DMSO, or 10 μ M T0901317 were incubated with FAM-SRC1-B (100 nM), GST–hPXR-LBD (5 nM), and Tb–anti-GST (5 nM). The final DMSO concentration in all samples was 1.1%. The TR-FRET signals of individual wells were measured after 30, 60, 90, 120, 150, 180, 240, and 300 min of incubation and summarized (Figure 6A). The corresponding signal-to-background ratios were then calculated (Figure 6B). In the Z' -factor signal stability test (Figure 6C), 16 data points were included for each high-signal group (10 μ M T0901317, a positive control to determine the total interaction) or low-signal group (DMSO, a negative vehicle control to determine the nonspecific interaction), and the Z' -factor value was calculated using Equation (1) (J. H. Zhang, Chung, & Oldenburg, 1999),

$$Z' = 1 - [(3\sigma^+ + 3\sigma^-) / (M^+ - M^-)], \quad (1)$$

where σ^+ is the standard deviation of the positive-control group (10 μ M T0901317), σ^- is the standard deviation of the negative-control group (DMSO), M^+ is the mean of the positive-control group (10 μ M T0901317), and M^- is the mean of the negative-control group (DMSO). The data from the T0901317 dilutions were fitted into a sigmoidal dose-response equation to derive the EC_{50} values (Figure 6D).

3.4 DMSO Tolerance Test for the FAM-SRC1-B–Mediated hPXR TR-FRET Coactivator Recruitment Assay

DMSO, T0901317 (10 μ M), or serial dilutions of T0901317 (1:3 dilutions giving 11 concentration levels from 10 μ M to 0.17 nM) were incubated with FAM-SRC1-B (100 nM), GST–hPXR-LBD (5 nM), and Tb–anti-GST (5 nM). The final DMSO concentrations were 0.2, 0.5, 1.0, 1.1, 2.0, 5.0, and 10.0% in the respective groups. After 120 min of incubation, the TR-FRET signals were measured and summarized (Figure 7A), and the corresponding signal-to-background ratios were then calculated (Figure 7B). The data from the T0901317 dilutions were fitted into a sigmoidal dose-response equation to determine the EC_{50} values (Figure 7C).

3.5 Determination of Binding Affinity and Mode of Action against a Panel of Putative hPXR Ligands in the FAM-SRC1-B–Mediated PXR TR-FRET Coactivator Recruitment Assay

Serial dilutions of test chemicals without T0901317 (for agonist characterization) or with 500 nM T0901317 (for antagonist characterization), along with DMSO, T0901317 (10 μ M), or T0901317 (500 nM), were incubated with FAM-SRC1-B (100 nM), GST–hPXR-LBD (5

nM), and Tb-anti-GST (5 nM). The final DMSO concentration in all samples was 1.2%. The TR-FRET signals for individual wells were measured after 120 min of incubation. Where applicable, the TR-FRET signals for individual chemicals at their respective concentrations were fitted into a sigmoidal dose-response equation to derive the respective EC₅₀ (for agonists, Figure 8A) or IC₅₀ (for antagonists, Figure 8B) values.

4. RESULTS AND DISCUSSION

4.1 Fluorescently Labeled SRC1 Peptide Design

A co-crystal study demonstrated that the binding of a coactivator SRC1 peptide (676-CPSSHSSLTERHKILHRLQLQEGSPS-700) (Figure 3A) can promote a specific interaction between the ligand SR12813 and hPXR protein (Watkins et al., 2003). Based on this reported coactivator SRC1 peptide (676-CPSSHSSLTERHKILHRLQLQEGSPS-700), three fluorescently labeled SRC1 peptides (Figure 3B) were designed and synthesized. These three newly designed SRC1 peptides (FAM-SRC1-A, FAM-SRC1-B, and FAM-SRC1-C) have the same core amino acid residue sequence (Figure 3, highlighted in bold and red) as the SRC1 peptide used in the co-crystal study, so that they retain binding affinity for hPXR, but they each have a slightly different modification at the N- or C-terminus to enable the identification of the one with the highest binding affinity for hPXR. Specifically, FAM-SRC1-A has a FAM at its N-terminus with two additional glycine (G) residues serving as a spacer between the FAM and the core amino acid sequence, as well as a C-terminal amide modification. FAM-SRC1-B has a FAM at its N-terminus and a (PEG)₅ linkage serving as a spacer between the FAM and the core amino acid sequence. FAM-SRC1-B also has a C-terminal amide modification. FAM-SRC1-C has an N-terminal acetylation modification of the core amino acid sequence, and the FAM was labeled at its C-terminus with two glycine residues and an NHCH₂CH₂NH group (-GG-NHCH₂CH₂NH-) as the spacer.

4.2 The FAM-SRC1-B Peptide Binds to PXR with the Highest Agonist-Mediated Specificity and High Affinity

The three newly designed and synthesized FAM-labeled SRC1 peptides (FAM-SRC1-A, FAM-SRC1-B, and FAM-SRC1-C) were tested for their hPXR binding affinities with or without the hPXR agonist T0901317 at a concentration of 10 μM in an hPXR-mediated TR-FRET coactivator recruitment assay. Representative results are summarized in Figure 4. In the TR-FRET PXR coactivator recruitment assay, dilutions of individual FAM-labeled SRC1 peptides were incubated with 5 nM Tb-anti-GST and 5 nM GST-hPXR-LBD in the presence of DMSO or T0901317 (10 μM) (Figure 4A–C; the upper and middle curves show the results at a representative 120-min incubation time). Dilutions of individual FAM-labeled SRC1 peptides incubated with 5 nM Tb-anti-GST and DMSO, but without GST-hPXR-LBD, were also included to serve as an absolute background interaction control (Figure 4A–C, lower curve). In the absence of GST-hPXR-LBD, these FAM-SRC1 peptides interacted with the Tb-anti-GST only very weakly, and these weak interactions correlated positively with the respective FAM-SRC1 peptide concentrations in a linear manner (Figure 4A–C, lower curves with data points shown as closed triangles). These weak and linear interactions indicated one type of nonspecific interaction, here referred to as absolute background binding. In the presence of GST-hPXR-LBD, the overall interactions between individual

FAM-SRC1 peptides and the complex of Tb-anti-GST and GST-hPXR-LBD increased (Figure 4A–C, middle curves with data points shown as closed circles) but still followed a linear pattern. The basal interactions between FAM-SRC1 peptides and the C-terminal AF2 domain of the GST-hPXR-LBD protein contributed to the signal increase over the absolute background interactions between FAM-SRC1 peptides and Tb-anti-GST. The overall interactions between individual FAM-SRC1 peptides and the complex of Tb-anti-GST and GST-hPXR-LBD are here referred to as background bindings. Of the three FAM-SRC1 peptides, FAM-SRC1-B had the least background interaction. With the addition of the hPXR agonist T0901317 (10 μ M), the total interactions (10 μ M T0901317-mediated total bindings) between individual FAM-SRC1 peptides and the complex of Tb-anti-GST and GST-hPXR-LBD increased further, first in an exponential manner when the FAM-SRC1 peptide concentrations were low and then in a linear manner when the FAM-SRC1 peptide concentrations increased (Figure 4A–C, upper curves with data points shown as closed squares). The signal increase is caused by the presence of the hPXR agonist T0901317, which induced hPXR conformation change and enhanced the interaction between the hPXR AF2 domain and SRC1 coactivators (Y. M. Wang et al., 2013). The difference in interaction with and without hPXR agonist T0901317 (10 μ M) in the presence of FAM-SRC1 peptides and the complex of Tb-anti-GST and GST-hPXR-LBD corresponds to the T0901317-induced specific interactions between FAM-SRC1 peptides and GST-hPXR-LBD. Of the three FAM-SRC1 peptides, FAM-SRC1-B had the largest T0901317-mediated SRC1 peptide-hPXR interaction increase (Figure 4A–C), demonstrating that of the three FAM-SRC1 peptides tested, FAM-SRC1-B is the most specific and inducible coactivator peptide in terms of binding to hPXR. These T0901317-induced specific interactions between FAM-SRC1 peptides and hPXR could be quantified by the relative areas (Figure 4A–C, gray areas, calculated by using the GraphPad software Prism) between the respective T0901317 curves (Figure 4A–C, upper curves) and DMSO curves (Figure 4A–C, middle curve) in the presence of Tb-anti-GST and GST-hPXR-LBD, with areas of 7,764,875, 16,729,790, and 10,406,140 for FAM-SRC1-A, FAM-SRC1-B, and FAM-SRC1-C, respectively, with FAM-SRC1-B giving the largest induction among the three peptides tested. Because it has the largest T0901317-induced specific interaction with hPXR, FAM-SRC1-B was chosen for use in the further development of the hPXR TR-FRET coactivator recruitment assay. The equilibrium dissociation constant (K_d) of FAM-SRC1-B with respect to hPXR in the TR-FRET coactivator recruitment assay was 115.3 nM in the presence of 10 μ M T0901317, as determined by using dilutions of FAM-SRC1-B in the presence of Tb-anti-GST and GST-hPXR-LBD (Figure 4B, upper curve). The interactions between FAM-SRC1 peptides and hPXR were very stable over a long incubation period in the hPXR TR-FRET coactivator recruitment assay, although the 120-min incubation was taken as representative. For example, over a 3-h incubation period, the binding curves of FAM-SRC1-B to hPXR at different time points showed little shift with or without 10 μ M T0901317 (Figure 4D). Correspondingly, the equilibrium dissociation constants (K_d s) of FAM-SRC1-B with respect to hPXR in the presence of 10 μ M T0901317 at different incubation times (Figure 4D, derived from the set of curves in the presence of 10 μ M T0901317) were also very stable, with K_d s of 172.7, 163.4, 118.9, 115.3, and 118.3 nM for incubation times of 30, 60, 90, 120, and 180 min, respectively. The signal was extremely stable from 90 to 180 min. An incubation time of 120 min was, therefore, selected as the typical assay incubation time for

the hPXR TR-FRET coactivator recruitment assay so that stable signals could be recorded within a relatively short period, because the 120-min incubation time was within the stable signal period and in its early stage. The signal stability was further confirmed in an additional assay development effort discussed below.

4.3 The Optimal Concentration of FAM-SRC1-B peptide in the PXR TR-FRET Coactivator Recruitment Assay is 100 nM

To determine the optimal FAM-SRC1-B concentration for the hPXR TR-FRET coactivator recruitment assay, we first calculated the signal fold changes or signal-to-background ratio (Figure 5A) between assays with 10 μ M T0901317 (the positive-control group) and without 10 μ M T0901317 (the DMSO group or negative-control group) when FAM-SRC1-B at the respective concentrations was incubated with Tb-anti-GST and GST-hPXR-LBD. (The values were calculated by dividing the data point on the upper [T0901317] curve in Figure 4B by the corresponding data point on the middle [DMSO] curve). Ideally, a fluorescent probe concentration close to its K_d concentration should be selected to enable the calculation of an accurate K_i value for a chemical evaluated with the fluorescent probe-mediated assay without deviating from the Cheng-Prusoff equation (Cheng & Prusoff, 1973). The calculated results revealed that a FAM-SRC1-B concentration of approximately 100 nM generated the highest signal change of nearly 7.5 fold with and without 10 μ M T0901317 in the TR-FRET coactivator recruitment assay (Figure 5A). This concentration is actually close to the FAM-SRC1-B K_d probe concentration of 115.3 nM. To further determine whether 100 nM would be the optimal FAM-SRC1-B concentration for the hPXR TR-FRET coactivator recruitment assay, we chose FAM-SRC1-B concentrations of 50, 100, 150, and 200 nM for further investigation. All four of the selected FAM-SRC1-B concentrations were close to the optimal concentration of 100 nM and the K_d concentration of 115.3 nM discovered in the previous study (Figure 4B). Two different assay conditions were tested for each selected FAM-SRC1-B concentration to gain insight into the total binding of FAM-SRC1-B to hPXR (in the 10 μ M T0901317 group or signal group) and the background binding of FAM-SRC1-B to hPXR (in the DMSO group). Consistent with the results shown in Figure 4B, both total binding (in the 10 μ M T0901317 group) and background binding (in the DMSO group) increased as the concentration of FAM-SRC1-B increased from 50 to 200 nM (Figure 5B). The ratios of total binding signal (in the 10 μ M T0901317 group) to background binding signal (in the DMSO group) for FAM-SRC1-B at concentrations of 50, 100, 150, and 250 nM were 7.3, 6.0, 4.9 and 4.3, respectively (Figure 5C), with higher probe concentrations of FAM-SRC1-B leading to lower signal fold change. We chose to use FAM-SRC1-B at 100 nM for further experimentation because this concentration was close to the K_d of 115.3 nM, and at this concentration, a six-fold signal difference between total binding (in the 10 μ M T0901317 group) and background binding (in the DMSO group) is high for ratiometric TR-FRET assays, by comparison with other reported results. In the literature, HTS of 8280 chemicals was successfully accomplished with the Invitrogen PXR TR-FRET binding kit, with signal-to-background ratios ranging from 2.5 to 3.5 and a Z' -factor greater than 0.5 (Shukla et al., 2009). A lower FAM-SRC1-B concentration, e.g., 50 nM, although resulting in a higher signal fold change between total binding and background binding, was not considered further because the lower overall signals for both the total binding and background binding groups might contribute to signal variation. FAM-SRC1-B

concentrations higher than 100 nM, such as 150 nM or 200 nM, were also not considered further because of the lower signal fold change between total binding and background binding and the deviation from the FAM-SRC1-B K_d of 115.3 nM, which might compromise the accurate determination of ligand K_i calculation.

4.4 The FAM-SRC1-B-Mediated PXR TR-FRET Coactivator Recruitment Assay Has High Signal Stability

To further assess the potential application of the FAM-SRC1-B-mediated hPXR TR-FRET coactivator recruitment assay in HTS to identify and characterize hPXR ligands, we further examined its signal stability, which is an important parameter of an HTS assay. To examine the signal stability of the assay, we measured the binding interactions of 100 nM FAM-SRC1-B with DMSO vehicle control or with various concentrations of T0901317 after incubation for 30, 60, 90, 120, 150, 180, 240, and 300 min with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST. Both the total TR-FRET binding (presented as fluorescence emission ratio) (with 10 μ M T0901317; 4203, 4442, 4644, 4677, 4659, 4688, 4697, and 4540) and the background TR-FRET binding (with DMSO; 685.9, 707.9, 750.5, 771.0, 779.8, 774.6, 801.0, and 817.0) were relatively stable (Figure 6A) at all incubation time points tested, with the signals being very stable for incubation periods of 90 min to 240 min. Correspondingly, the signal-to-background ratios (the ratios of the total binding signal with 10 μ M T0901317 to the background binding signal with DMSO) were also very stable, with values of 6.1, 6.3, 6.2, 6.1, 6.0, 6.1, 5.9, and 5.6 for incubation times of 30, 60, 90, 120, 150, 180, 240, and 300 min, respectively, and with only a very slight decreasing trend over a long incubation time of 300 min (Figure 6B). In the 100 nM FAM-SRC1-B-mediated hPXR TR-FRET coactivator recruitment assay, the Z' -factor remained constant over the entire test period, with Z' -factor values of 0.86, 0.91, 0.91, 0.89, 0.90, 0.92, 0.92, and 0.87 for the incubation times of 30, 60, 90, 120, 150, 180, 240, and 300 min, respectively (Figure 6C). The Z' -factor is an HTS window coefficient, and a Z' -factor value of more than 0.5 generally indicates that an assay is suitable for HTS (J. H. Zhang et al., 1999). This TR-FRET PXR coactivator recruitment assay has consistent Z' -factor values of more than 0.5 over long time periods (up to 300 min), demonstrating that the assay is very stable and is suitable for HTS. The EC_{50} values of T0901317 were relatively stable over the entire 300-min incubation, being 94.6, 109.7, 113.3, 110.7, 116.1, 118.1, 130.6, and 154.4 nM for incubations of 30, 60, 90, 120, 150, 180, 240, and 300 min, respectively, with slightly low signals at 30 and 300 min, which may be why the T0901317 EC_{50} values at 30 and 300 min deviated slightly from the values for all the other incubation times (Figure 6D). The consistent Z' -factor values demonstrate that the assay is very stable and suitable for HTS. Although all the incubation times could be used for HTS, the signals and signal-to-background ratios suggest that an optimal incubation time of 120 min or an optimal incubation window of 90 to 180 min should be implemented for the FAM-SRC1-B-mediated hPXR TR-FRET coactivator recruitment assay. The optimal 120-min incubation time was then selected for use in all further assays.

4.5 The FAM-SRC1-B-Mediated PXR TR-FRET Coactivator Recruitment Assay Tolerates a Wide Range of DMSO Concentrations

DMSO tolerance is another important factor to consider in assays, because DMSO is a solvent widely used for compounds in drug discovery, including the compounds used in this

study. In the DMSO tolerance test, the TR-FRET signals from the FAM-SRC1-B-mediated PXR coactivator recruitment assays were measured after 120 min of incubation with either DMSO vehicle control or various concentrations of T0901317 in final DMSO concentrations of 0.2%, 0.5%, 1%, 1.1%, 2%, 5%, or 10% plus 100 nM FAM-SRC1-B, 5 nM GST-hPXR-LBD, and 5 nM Tb-anti-GST. The total binding signal in the presence of 10 μ M T0901317 remained stable at 5017, 4977, 4907, 5101, 5059 and 4959 with DMSO concentrations of 0.2%, 0.5%, 1%, 1.1%, 2%, and 5%, respectively, and then decreased to 4241 when the DMSO concentration was increased to 10% (Figure 7A). The background binding signal (in the presence of the same concentration of DMSO) remained constant over the entire DMSO concentration range at 909, 917, 920, 950, 966, 972, and 901 with DMSO concentrations of 0.2%, 0.5%, 1%, 1.1%, 2%, 5%, and 10%, respectively (Figure 7A). The signal-to-background ratio (the ratio of the signal from the 10 μ M T0901317 group to that from the DMSO group) was also relatively stable, ranging from 5.5 to 5.1 at DMSO concentrations at or below 5% and falling to 4.7 when the DMSO concentration was 10% (Figure 7B). In the 100 nM FAM-SRC1-B-mediated hPXR TR-FRET coactivator recruitment assay, the EC₅₀ values of T0901317 at DMSO concentrations of 0.2%, 0.5%, 1%, 1.1%, and 2% remained constant at 114.8, 111.5, 115.4, 111.3, and 118.6 nM, respectively, and then increased to 146.3 and 431.0 nM at DMSO concentrations of 5% and 10%, respectively (Figure 7C). For the putative hPXR agonist T0901317, slight EC₅₀ deviation was observed with 5% DMSO (146.3 nM versus a typical 110–120 nM), and relatively substantial EC₅₀ deviation was observed with 10% DMSO (431.0 nM versus a typical 110–120 nM). Therefore, the FAM-SRC1-B-mediated PXR TR-FRET coactivator recruitment assay can tolerate a wide range of DMSO concentrations up to at least 2%. The DMSO concentration of 1.1% or 1.2% was used in further characterizing hPXR ligands for their agonistic or antagonistic activities.

4.6 The FAM-SRC1-B-Mediated PXR TR-FRET Coactivator Recruitment Assay Can Assess the PXR Binding Affinities of PXR Ligands and Differentiate Antagonists from Agonists

To evaluate whether the FAM-SRC1-B-mediated hPXR TR-FRET coactivator recruitment assay could assess the hPXR binding affinities of hPXR ligands and differentiate agonists from antagonists, we selected a panel of nine reported hPXR ligands, including six hPXR agonists, T0901317 (Mitro et al., 2007), SR12813 (Jones et al., 2000), nimodipine (Pan, Kim, Ekins, Wang, & Swaan, 2011), nicardipine (Drocourt et al., 2001), PK11195 (L. Li et al., 2008), and clotrimazole (Lehmann et al., 1998), and three hPXR antagonists, trans-resveratrol (Deng et al., 2014), silybin (Mooiman et al., 2013), and ketoconazole (Huang et al., 2007), to represent different types of hPXR ligands with high, medium, or low potency. No potent PXR antagonist has been reported. The reported IC₅₀ value is 135 μ M for silybin in a cell-based reporter assay, 74.4 μ M for ketoconazole in a binding assay, and has not been determined for trans-resveratrol.

All nine of these selected ligands were first tested in the FAM-SRC1-B-mediated hPXR agonistic assay, in which dilutions of chemicals were incubated with 100 nM FAM-SRC1-B, 5 nM GST-hPXR-LBD, and 5 nM Tb-anti-GST. In the agonistic assay, the agonists T0901317, SR12813, nimodipine, nicardipine, PK11195, and clotrimazole could be clearly separated from antagonists because agonists can enhance the TR-FRET signal by recruiting more of the coactivator peptide FAM-SRC1-B to hPXR (Figure 8A). However, the

antagonists ketoconazole, silybin, and trans-resveratrol could not enhance the TR-FRET signal because they could not recruit significantly more of the coactivator peptide FAM-SRC1-B to hPXR beyond the basal interaction level as a result of their antagonistic nature (Figure 8B, lower-panel curves without 500 nM T0901317). In fact, silybin and trans-resveratrol could further decrease the basal TR-FRET interaction between the coactivator peptide FAM-SRC1-B and hPXR at higher concentrations, although ketoconazole could marginally increase the basal TR-FRET interaction between the coactivator peptide FAM-SRC1-B and hPXR at higher concentrations. In the FAM-SRC1-B-mediated hPXR agonistic test, the agonists T0901317, SR12813, nimodipine, nicardipine, PK11195, and clotrimazole had respective EC_{50} s of 112.8 nM, 278.9 nM, 656.8 nM, 842.3 nM, 1.64 μ M, and 1.85 μ M. The EC_{50} values of T0901317 (112.8 nM), SR12813 (278.9 nM), and clotrimazole (1.85 μ M) determined by the FAM-SRC1-B-mediated hPXR coactivator recruitment assay are consistent with their respective hPXR binding inhibitory IC_{50} values of 101.6 nM, 166.7 nM, and 4.2 μ M, as reported in the literature (Lin et al., 2014), with the same order of high-to-low potency. No hPXR agonistic EC_{50} values have been reported for the other three hPXR agonists, nimodipine, nicardipine, and PK11195, although they have been well characterized as hPXR agonists (Drocourt et al., 2001; L. Li et al., 2008; Pan et al., 2011).

As for the three reported hPXR antagonists ketoconazole, silybin, and trans-resveratrol, besides the regular FAM-SRC1-B-mediated hPXR agonistic assay, in which dilutions of chemicals were incubated with 100 nM FAM-SRC1-B, 5 nM GST-hPXR-LBD, and 5 nM Tb-anti-GST, they were further subjected to the FAM-SRC1-B-mediated hPXR antagonistic assay, in which an additional 500 nM T0901317 (approximately equivalent to its EC_{80} concentration) was included in the regular agonistic assay system (Figure 8B, upper-panel curves with 500 nM T0901317). The presence of 500 nM T0901317 resulted in the recruitment of a substantial amount of the coactivator FAM-SRC1-B to hPXR, thereby increasing the basal interaction level between FAM-SRC1-B and hPXR and rendering this activated system more sensitive to inhibition by hPXR antagonists. In the presence of 500 nM T0901317, trans-resveratrol had an hPXR antagonistic IC_{50} of 1.08 μ M in the FAM-SRC1-B-mediated hPXR coactivator recruitment assay, although its hPXR antagonistic IC_{50} value has not been reported in the literature. Under the same hPXR antagonistic assay conditions, silybin had an hPXR antagonistic IC_{50} value of 55.1 μ M, which is close to the reported value of 135 μ M (Mooiman et al., 2013). However, an hPXR antagonistic IC_{50} value for ketoconazole could not be reliably estimated with this FAM-SRC1-B-mediated hPXR coactivator recruitment assay because of the low potency of the compound and its low solubility at high concentrations.

5. CONCLUSIONS

We have developed a TR-FRET PXR coactivator recruitment assay based on the reported hPXR crystal structure. This assay can characterize the potency of PXR ligands, as well as their mode of action; it is very robust, with the signal being stable over a long period of incubation (incubations up to 300 min have been tested); it can tolerate high concentrations of DMSO (up to 5%); and it has a good signal-to-noise ratio window (six-fold under selected assay conditions). This newly developed PXR TR-FRET coactivator interaction

assay has potential application in HTS to identify and characterize novel PXR agonists and antagonists.

Acknowledgments

We thank the Macromolecular Synthesis Section, Hartwell Center for Bioinformatics and Biotechnology, St Jude Children's Research Hospital for peptide synthesis. We are grateful to Dr. Keith A. Laycock (St. Jude Department of Scientific Editing) for editing the manuscript. This work was supported by ALSAC, St. Jude Children's Research Hospital, and grants from the National Institutes of Health (RO1-GM086415, RO1-GM110034, R35-GM118041, and P30-CA21765).

ABBREVIATIONS

TR-FRET	time-resolved fluorescence energy transfer
PXR	pregnane X receptor
NR	nuclear receptor
DNA	deoxyribonucleic acid
DBD	DNA-binding domain
LBD	ligand-binding domain
AF-2	activation function 2
RXRα	retinoid X receptor alpha
FRET	fluorescence resonance energy transfer
Tb	terbium
Eu	europium
Cy5	cyanine 5
CAR	constitutive androstane receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERRγ	estrogen-related receptor gamma
FXR	farnesoid X receptor
GR	glucocorticoid receptor
RXRβ	retinoid X receptor beta
TRβ	thyroid receptor beta
NR2E3	nuclear receptor subfamily 2 group E member 3
AR	androgen receptor

ERRα	estrogen-related receptor alpha
ERRβ	estrogen-related receptor beta
LXRα	liver X receptor alpha
LXRβ	liver X receptor beta
PPARα	peroxisome proliferator-activated receptor alpha
PPARγ	peroxisome proliferator-activated receptor gamma
PPARδ	peroxisome proliferator-activated receptor delta
PR	progesterone receptor
RARα	retinoic acid receptor alpha
RARβ	retinoic acid receptor beta
RARγ	retinoic acid receptor gamma
RORα	RAR-related orphan receptor alpha
RXRα	retinoid X receptor alpha
TRα	thyroid hormone receptor alpha
VDR	vitamin D receptor
TRE	thyroid hormone response element
NRD	nuclear receptor domain
FAM	fluorescein amidite
SRC1	steroid receptor coactivator 1
GST	Glutathione S-transferase
hPXR	human pregnane X receptor
LBD	ligand-binding domain
PEG	polyethylene glycol
DTT	dithiothreitol
DMSO	dimethyl sulfoxide
BSA	bovine serum albumin
TRIS	tris(hydroxymethyl)aminomethane
MgCl₂	magnesium chloride
K_d	dissociation constant

K_i	inhibition constant
EC₅₀	half maximal effective concentration
IC₅₀	half maximal inhibitory concentration
HTS	high-throughput screening

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Figure 1.

Schematic representation of nuclear receptor TR-FRET co-regulator interaction assays for characterizing ligand efficacy and potency in the presence or absence of various ligands for nuclear receptors that have low basal interaction with their coactivators or corepressors. (A). Representative TR-FRET interactions of nuclear receptors with their coactivator peptides. (B). Representative TR-FRET interactions of nuclear receptors with their corepressor peptides. NR, nuclear receptor.

Figure 2.

Schematic representation of nuclear receptor TR-FRET coactivator interaction assays for characterizing ligand efficacy and potency in the presence or absence various ligands for nuclear receptors that have medium basal interaction with their coactivators.

A

 $\text{NH}_2\text{-CPSSHSSLTERHKILHRLLQEGSPS-COOH}$

B

FAM-SRC1-A FAM-GG-CPSSHSSLTERHKILHRLLQEGSPS-NH₂FAM-SRC1-B FAM-(PEG)₅-CPSSHSSLTERHKILHRLLQEGSPS-NH₂FAM-SRC1-C Ac-CPSSHSSLTERHKILHRLLQEGSPS-GG-NHCH₂CH₂NH-FAM**Figure 3.**

SRC1 coactivator peptides designed for the development of the PXR TR-FRET coactivator interaction assay. (A). SRC1 coactivator peptide reported in the hPXR-SRC1 crystal study. (B). FAM-labeled SRC1 peptides designed for the development of the PXR TR-FRET coactivator interaction assay.

Figure 4.

Binding interactions of FAM-SRC1-A, FAM-SRC1-B, and FAM-SRC1-C with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST after 120 min or the specified time of incubation. (A) Binding of the indicated concentrations of FAM-SRC1-A to 5 nM Tb-anti-GST and 5 nM GST-hPXR-LBD in the presence of T0901317 (10 μ M, T0901317-mediated total binding) or DMSO (background binding) or to 5 nM Tb-anti-GST and DMSO (absolute background binding). (B) Binding of the indicated concentrations of FAM-SRC1-B to 5 nM Tb-anti-GST and 5 nM GST-hPXR-LBD in the presence of T0901317 (10 μ M, T0901317-mediated total binding) or DMSO (background binding) or to 5 nM Tb-anti-GST and DMSO (absolute background binding). (C) Binding of the indicated concentrations of FAM-SRC1-C to 5 nM Tb-anti-GST and 5 nM GST-hPXR-LBD in the presence of T0901317 (10 μ M,

T0901317-mediated total binding) or DMSO (background binding) or to 5 nM Tb-anti-GST and DMSO (absolute background binding). (D) Binding of the indicated concentrations of FAM-SRC1-B to 5 nM Tb-anti-GST and 5 nM GST-hPXR-LBD in the presence of T0901317 (10 μ M, T0901317-mediated total binding) or DMSO (background binding).

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Figure 5.

Binding activity of the indicated concentrations of FAM-SRC1-B in the hPXR TR-FRET coactivator recruitment assay after 120 min of incubation with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST. (A). Calculated signal-to-background ratio for FAM-SRC1-B at the indicated concentrations interacting with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST. The signal and background are defined as $10,000 \times$ the 520 nm/490 nm ratios obtained with T0901317 (10 μ M) and DMSO, respectively, in Figure 1B. (B) Interaction of FAM-SRC1-B at the indicated concentrations with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST in the presence of DMSO or T0901317 (10 μ M). (B) Signal-to-background ratio for FAM-SRC1-B at the indicated concentrations interacting with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-

GST, with the signal and background being defined as $10,000 \times$ the 520 nm/490 nm ratios obtained with T0901317 (10 μ M) and DMSO, respectively.

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Figure 6.

Longitudinal signal stability of the interaction of 100 nM FAM-SRC1-B with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST. (A) Interaction of 100 nM FAM-SRC1-B with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST at the indicated time points in the presence of DMSO or T0901317 (10 μM). (B) Signal-to-background ratios for the interaction of 100 nM FAM-SRC1-B with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST at the indicated time points, with the signal and background being defined as $10,000 \times$ the 520 nm/490 nm ratios obtained with T0901317 (10 μM) and DMSO, respectively. (C) Z' -factor values for the interaction of 100 nM FAM-SRC1-B with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST at the indicated time points. The Z' -factor was calculated from the total binding-signal group (10 μM T0901317) and background binding-signal group (DMSO) by using Equation 1. (D) T0901317 dose-response curves in the presence of 100 nM FAM-SRC1-B, 5 nM GST-hPXR-LBD, and 5 nM Tb-anti-GST at the indicated time points.

Figure 7.

DMSO tolerance in the interaction of 100 nM FAM-SRC1-B with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST after 120 min of incubation. (A) Interaction of 100 nM FAM-SRC1-B with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST in the presence of DMSO control or T0901317 (10 μM) at the indicated final DMSO concentrations. (B) Signal-to-background ratios for the interaction of 100 nM FAM-SRC1-B with 5 nM GST-hPXR-LBD and 5 nM Tb-anti-GST in the presence of the indicated DMSO concentrations, with the signal and background being defined as $10,000 \times$ the 520 nm/490 nm ratios obtained with T0901317 (10 μM) and DMSO, respectively. (C) T0901317 dose-response curves in the presence of 100 nM FAM-SRC1-B, 5 nM GST-hPXR-LBD, and 5 nM Tb-anti-GST at the indicated DMSO concentrations.

Figure 8.

Dose-response curves for a panel of hPXR ligands after 120 min of incubation in the 100 nM FAM-SRC1-B-mediated hPXR TR-FRET coactivator recruitment assay. (A) Dose-response curves for a panel of six putative hPXR agonists, T0901317, SR12813, nimodipine, nicardipine, PK11195, and clotrimazole, in the 100 nM FAM-SRC1-B-mediated hPXR TR-FRET coactivator recruitment assay. (B) Dose-response curves for a panel of three putative hPXR antagonists, ketoconazole, silybin, and trans-resveratrol, with T0901317 (antagonistic mode) or without 500 nM T0901317 (agonistic mode) in the 100 nM FAM-SRC1-B-mediated hPXR TR-FRET coactivator recruitment assay.

Table 1

Representative TR-FRET nuclear receptor coregulator interaction assays reported in the literature (with the target in **bold**)

Target	Donor	Acceptor	Note	Reference
GST- AR -LBD	Tb-anti-GST	Fluorescein-D11FxxLF	3-component single TR-FRET	Ozers et al., 2007
ERRγ -LBD-GST	Tb-anti-GST	Fluorescein-PGC1 α	One donor-one acceptor	Gowda et al., 2006
GST- RXRβ -LBD	Tb-anti-GST	Fluorescein-D22	Assay development	Stafšlien et al., 2007
Biotin- ERα -LBD	Streptavidin-Tb	SRC3-Cy5	Assay application*	Gunther et al., 2009
Biotin- ERβ -LBD	Streptavidin-Tb	SRC3-Cy5		Gunther et al., 2009
TRβ (with RXR α)	Streptavidin-Tb with biotin-TRE	Fluorescein-NCoR or fluorescein-SRC3	4-component single TR-FRET	Jeyakumar et al., 2008
GST- NR2E3	Eu-anti-GST	RetCOR-biotin Streptavidin-XL665	one donor-one acceptor Assay development Assay application	Qin et al., 2013
GST- ERα -LBD	Tb-anti-GST	Fluormone-ES2 TM green Dy-PGC1 α	4-component dual TR-FRET	Hilal et al., 2010
TRβ (with RXR α)	Streptavidin-Tb with biotin-TRE	Fluorescein-NCoR Cy5-SRC3	One donor-two acceptors Assay development Assay application	Jeyakumar et al., 2009
Biotin- ERα -LBD	Streptavidin-Tb	Cy5-SRC3 NRD Fluorescein-ethynylestradiol SNAPFL-antiestrogen	5-component triple TR-FRET	Kim et al., 2010
GST- CAR -LBD	Tb-anti-GST	Fluorescein-PGC1 α	One donor-three acceptors Assay development Assay application	Carazo & Pavlek, 2015 Carazo et al., 2015 Cherian et al., 2015 Cherian et al., 2016 Lin et al., 2016
GST- FXR -LBD	Tb-anti-GST	Fluorescein-SRC2-2	3-component single TR-FRET	G. Li et al., 2012
GST- GR -LBD	Tb-anti-GST	Fluorescein-SRC2-2	One donor-one acceptor	Sharma et al., 2011
GST- PXR -LBD	Tb-anti-GST	Fluorescein-SRC1-4	Assay application	Wang et al., 2013
HIS- PXR -LBD	Eu-anti-HIS	Biotin-SRC1 Allophycocyanin-streptavidin	4-component single TR-FRET	Mitro et al., 2007
			One donor-one acceptor Assay application	

* Assay application: characterization of ligand potency and mode of action.