Drug discovery technologies to identify and characterize modulators of the pregnane X receptor and the constitutive androstane receptor

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The pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) are ligand-activated nuclear receptors (NRs) that are notorious for their role in drug metabolism, causing unintended drug–drug interactions and decreasing drug efficacy. They control the xenobiotic detoxification system by regulating the expression of an array of drug-metabolizing enzymes and transporters that excrete exogenous chemicals and maintain homeostasis of endogenous metabolites. Much effort has been invested in recognizing potential drugs for clinical use that can activate PXR and CAR to enhance the expression of their target genes, and in identifying PXR and CAR inhibitors that can be used as co-therapeutics to prevent adverse effects. Here, we present current technologies and assays used in the quest to characterize PXR and CAR modulators, which range from biochemical to cell-based and animal models.

Introduction

PXR (*NR1I2*) and CAR (*NR1I3*) are ligand-activated transcription factors with major roles in the biotransformation, metabolism, and elimination of xenobiotics and endobiotics [1,2]. PXR and CAR are involved in several important physiological functions, including gluconeogenesis, lipid metabolism, and the homeostasis of bile acids and steroids [3]. However, much impetus is placed on their significant impact on drugs in clinical use, where they can cause adverse drug–drug interactions and reduced efficacy by regulating the expression of phase I and II drug-metabolizing enzymes (DMEs) and ATP-binding cassette drug transporters [4,5]. Among the proteins encoded by these target genes of PXR and CAR, the oxidative enzymes of the family of cytochrome p450 (CYPs) account for most of the drugs metabolized [6].

PXR and CAR control gene expression through the PXR-responsive element module (PXRRE) and the phenobarbital response enhancer module (PBREM), respectively, in the promoter region of target genes [7,8]. The conserved N-terminal DNA-binding domain (DBD) is responsible for engaging these responsive elements through zinc fingers [9]. A flexible hinge, with limited amino acid similarity between PXR and CAR, connects the DBD to the ligand-binding domain (LBD) [3]. The LBD is the most prominent feature and is characterized as a large and flexible module that accommodates a vast spectrum of chemicals in a highly hydrophobic ligand-binding pocket. Figure 1 illustrates a representative group of PXR agonists that encompass large natural products and smaller synthetic chemicals: rifampicin [10], rifaximin [11], amprenavir [12], lithocholic acid [13], solomonsterol A [14] and B [15], dexamethasone [16], nicardipine [17], SR12813 [18], and SJB7 [19]. Some of these compounds have very different scaffolds, which provide a glimpse into the potentially diverse chemical space of molecules that can bind to PXR.

Agonist binding to PXR and CAR triggers conformational changes leading these receptors to heterodimerize with retinoid X receptor (RXR) and to recruit or dissociate from coregulators. Steroid receptor coactivator 1 (SRC-1) and the transcriptional mediator/intermediary factor 2 (TIF2) are among the most common coactivators; NR corepressor (NCoR) and silencing mediator for retinoid or thyroid hormone receptors (SMRT) are typical corepressors that interact with PXR and CAR. The positioning of the C-terminal activation function 2 (AF-2) helix acts as a switch enabling discrimination between coactivator and corepressor recruitment [20–22]. The AF-2 helix of CAR is more rigid than that of PXR, which is believed to contribute to the constitutive activity of CAR. Antagonists for human PXR (hPXR), which antagonize the

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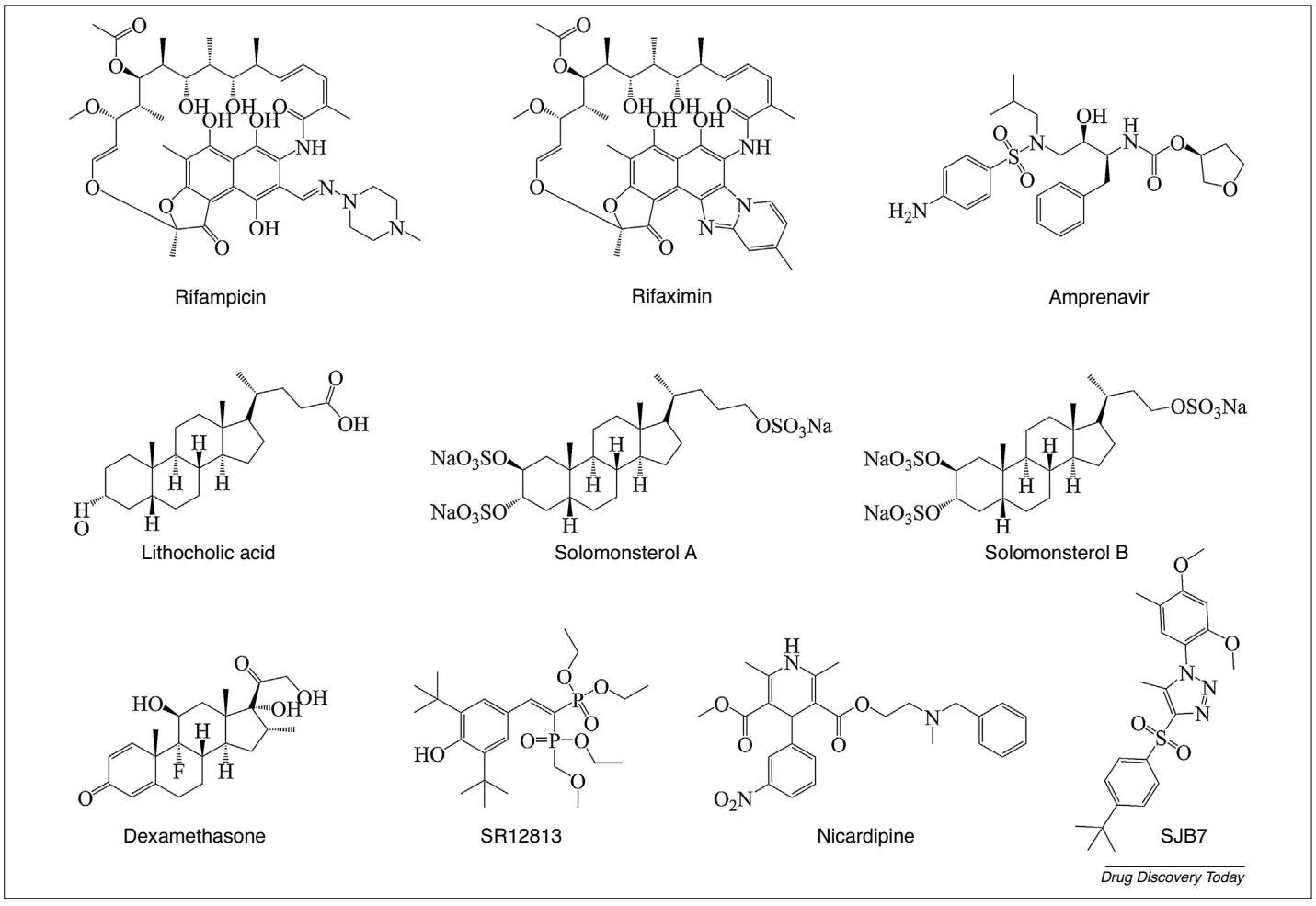

FIGURE 1

Representative pregnane X receptor agonists.

action of agonists, have been reported, such as ketoconazole [23], sulforaphane [24], and SPA70 [19] (Fig. 2). Inverse agonists, which reduce the constitutive activity and antagonize the action of agonists, have also been reported for mouse CAR (mCAR) (androstenol [22]) and hCAR (PK11195 [25] and CINPA1 [26]) (Fig. 2).

In this review, we outline existing technologies and assays that enable the identification and characterization of PXR and CAR modulators. Many of these assays are well established in the field and have been used routinely on a large scale. However, we also describe less prevalent methods that have potential to complement and, in some cases, provide unique and more accurate information than those in common use.

Approaches and technologies to identify and characterize PXR and CAR modulators

A vast repertoire of tools is available to discover and characterize ligands that interact with PXR and CAR, which encompass the entire spectrum of the drug discovery process. Several biochemical assays have been developed to monitor ligand binding, determine its direct interaction with PXR or CAR, reveal the ligand-induced changes in cofactor interactions with PXR and CAR, and evaluate its functional aspects. Cell-based assays provide additional information, such as compound cellular permeability, stability, and toxicity, and the cellular outcome of ligand binding to the receptors. Most of these assays can be miniaturized for high-throughput screening (HTS) [27,28]. Recent advances in humanized animal models enable more reliable validation of ligands that can be extrapolated to humans. Given the inherent advantages and disadvantages of individual assays and technologies, a combination of these is required to comprehensively characterize the ligands.

Scintillation proximity assays

Among the first biochemical assays to be implemented in the characterization of PXR and CAR ligands, the scintillation proximity assay (SPA) was widely used because of a lack of fluorescent probes that could be used in alternative fluorescence-based assays. SPA uses radioligands, such as [^{3}H]SR12813 [29], [^{3}H]T0901317 [30], and [^{3}H]NMTB [31] for assays involving PXR, and [^{3}H]clotrimazole for those to study CAR [2]. The target protein is captured on beads containing a scintillant and, upon ligand binding, the radiolabeled molecule is positioned in close proximity to efficiently transfer energy to the scintillant, resulting in light emission. The protein-bound radioligand can be displaced by an unlabeled (cold) compound, which reduces emitted light [27]. In contrast to conventional radioligand-binding methods, which require the separation of bound and unbound radioligands, SPA is a simple mix-

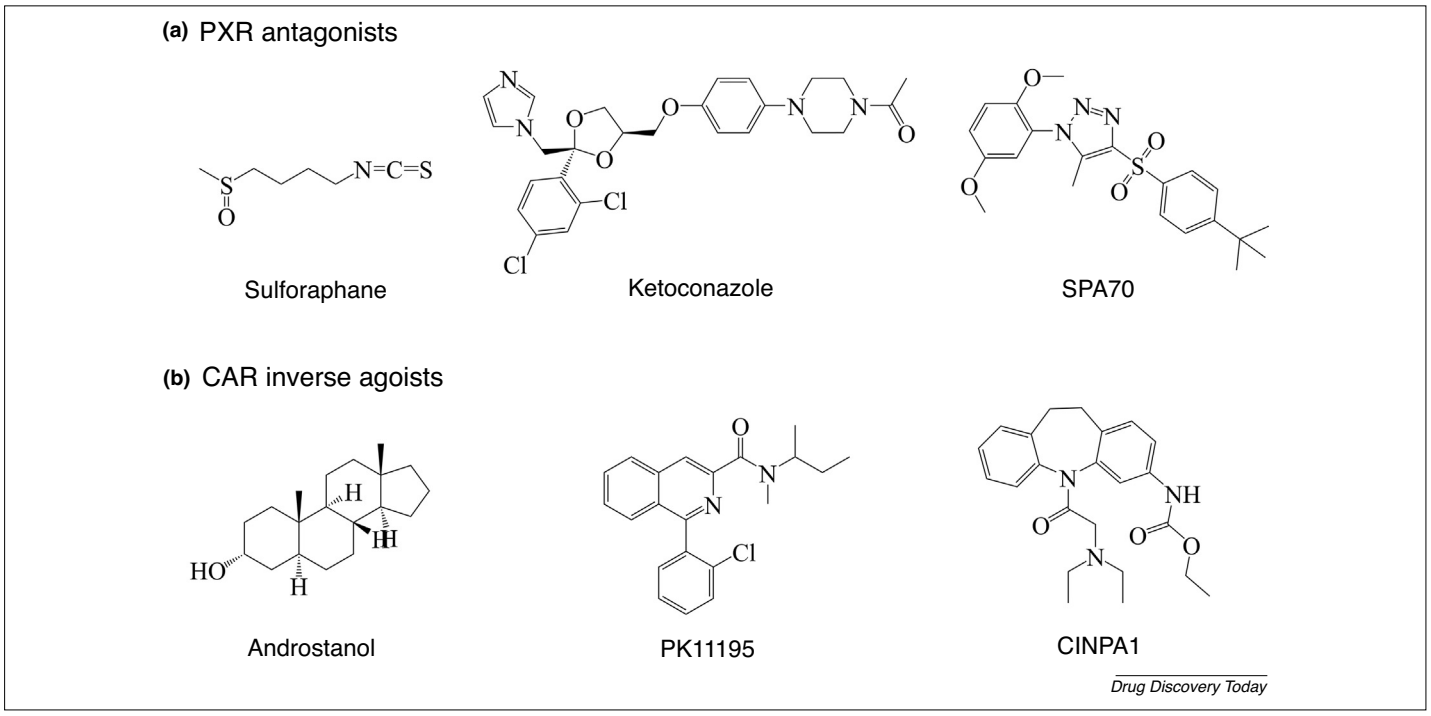

FIGURE 2

Representative pregnane X receptor antagonists **(a)** and constitutive androstane receptor inverse agonists **(b)**.

and-read approach amenable for large-scale compound screening [32]. By using [^{3}H]SR12813 as the radioligand, hits from a HTS campaign to identify PXR ligands were shown to correlate well with functional activities in a cell-based reporter assay [33]. However, SPA is not widely applied on a large scale because doing so generates large volumes of liquid radioactive waste [32].

Fluorescence polarization

Fluorescence polarization (FP; fluorescence anisotropy) measures the difference in molecular rotation between a protein-bound and an unbound fluorescently labeled ligand. When bound to the target protein, the probe rotates slower than when in its unbound form [27]. Although FP has become a common technique in HTS, this assay has not been widely used to characterize PXR ligands. The only reported FP assay uses a fluorescein-labeled SRC-1 coactivator peptide as the fluorescent probe that interacts with the PXR/RXR heterodimer [34], which measures ligand-mediated recruitment of the coactivator to PXR LBD instead of measuring displacement of a fluorescent-label bound ligand. FP assays typically require high concentrations of the target protein (in the μM range), which could limit its spread in studying PXR ligands, particularly when difficulties arise in purifying PXR at high concentrations. These shortcomings are likely to be ameliorated by the use of maltose-binding protein (MBP)-tagged PXR to facilitate solubility and purification [34].

Time-resolved fluorescence energy transfer

Time-resolved fluorescence energy transfer (TR-FRET), also known as homogeneous time-resolved fluorescence (HTRF), is based on the fluorescence energy transfer between a donor fluorophore (a lanthanide, such as terbium or europium) and a suitable acceptor fluorophore (e.g., fluorescein, BODIPY dye, Alexa Fluor dye, Cy5, or allophycocyanin) in a time-resolved manner [27,35,36]. The donor fluorophore is normally coupled to the target protein through covalent bonding, streptavidin–biotin complex formation, or binding of a labeled antibody that recognizes an affinity tag in the target protein (e.g., a polyhistidine tag or glutathione-*S*-transferase tag). The lanthanide metals have a long fluorescence lifetime, enabling delayed detection (time-resolved measurement), which makes TR-FRET less susceptible to interference by fluorescent compounds. Nevertheless, certain chemicals can still adversely affect the TR-FRET signal, particularly at high concentrations. TR-FRET has become a staple assay in large-scale screening because it is sensitive and robust, largely because it is a ratiometric method (the emission corresponding to the wavelengths of the donor and acceptor fluorophores are measured simultaneously, and a ratio calculated) and requires only low protein concentrations in the nM range.

There are two main approaches in using TR-FRET to investigate PXR-ligand interactions (Fig. 3). First, ligands that bind directly in the ligand-binding pocket have been labeled with a suitable acceptor fluorophore [37–39], which can be displaced by unlabeled test compounds (Fig. 3a). A LanthaScreen® competitive binding assay with a proprietary fluorescent ligand has been extensively used to characterize PXR ligands [34,37,40,41], investigating the effects of PXR mutants in ligand-binding [42] and HTS campaigns [43]. Given that ligands can interact with PXR's binding pocket in various ways, researchers have worked to expand the arsenal of fluorescent-labeled ligands, such as BODIPY FL vinblastine [38] and BODIPY FL vindoline [39].

The other approach to utilize TR-FRET in PXR and CAR ligand-binding studies provides deeper mechanistic information about the effect of ligand binding to PXR and CAR, such as differentiating agonists from antagonists that recruit their respective co-

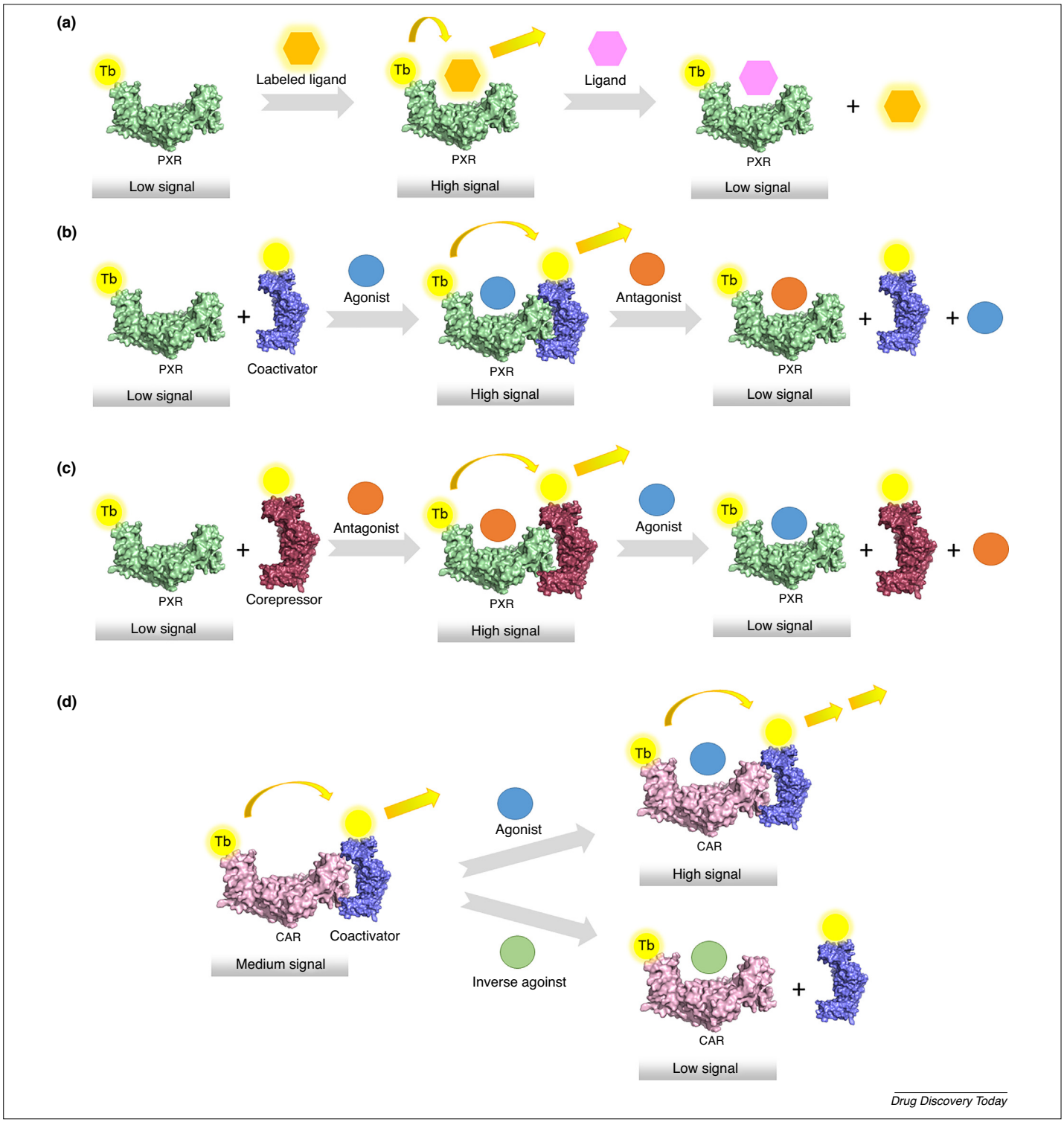

FIGURE 3

Schematic representation of time-resolved fluorescence energy transfer (TR-FRET) binding assays. **(a)** Displacement of labeled ligand by unlabeled ligand. Tb, terbium. **(b)** Principle of pregnane X receptor (PXR) TR-FRET coactivator recruitment with subsequent repression. **(c)** Principle of PXR TR-FRET corepressor recruitment with subsequent repression. **(d)** Principle of constitutive androstane receptor (CAR) TR-FRET coactivator recruitment and repression assay.

regulatory peptides (Fig. 3b,c). This approach makes use of labeled partner peptides (coactivator or corepressor peptides) tagged with the acceptor fluorophore, the recruitment of which to PXR or CAR is enhanced upon binding of the appropriate ligand (agonist or antagonist) [19,36]. In the presence of a suitable agonist at its optimal concentration (high TR-FRET signal), the coactivator interaction assay can be configured to characterize PXR antagonists (Fig. 3b), with antagonists repressing coactivator binding (causing a low TR-FRET signal). Similarly, the corepressor interaction assay can be configured to characterize PXR agonists (Fig. 3c),

TABLE 1

Coactivator and corepressor peptides reported in TR-FRET assays used to study PXR and CAR[a]

NR	Coregulator peptide	Amino acid sequence and tag
PXR	SRC-1	Biotin-CPSSHSSLTERHKILHRLLQEGSPSC-OH
		Fluorescein-GPQTPQAQQKSLLQQLLTE-OH
		FAM-$(PEG)_5$-CPSSHSSLTERHKILHRLLQEGSPS-NH2
	NCoR	Fluorescein-DPASNLGLEDIIRKALMGSFDDK-OH
CAR	SRC-1	Europium-CPSSHSSLTERHKILHRLLQEGSPS-OH
	PGC1α	Fluorescein-EAEEPSLLKKLLLAPANTQ-OH

[a] The LXXLL (coactivator) and the I/L-X-X-I/V-I (corepressor) motifs are highlighted in yellow, where X represents any other amino acid residue.

with agonists repressing corepressor binding (causing a low TR-FRET signal). Two versions of coactivator peptides derived from SRC-1 have been reported [19,30,36,41] and their sequences are detailed in Table 1. Both SRC-1 peptides have the conserved LXXLL motif (also known as 'NR box' motif; L = leucine, X = any amino acid), which is believed to be required for interactions between NRs and nuclear coactivators [44].

The PXR TR-FRET corepressor recruitment assay has also been used to investigate PXR antagonists [19], where the fluorescently labeled peptide (F-NCoRID2) was derived from NCoR with an I/L-X-X-I/V-I motif (also known as CoRNR box; I = isoleucine, L = leucine, X = any amino acid, V = valine) [44]. Given its high constitutive activity, CAR has high basal interactions with its coactivators [45], a fact that has been exploited to screen for inverse agonists (Fig. 3d). Two types of peptide have been reported: a europium-labeled SRC-1 peptide [2] and a fluorescein-labeled PGC1α peptide [26,46,47].

Crystallography

Since the crystal structure of hPXR LBD was elucidated [18], cocrystals of hPXR LBD for several ligands have become widely available (Fig. 4a,b). By contrast, only three structures of CAR LBD are available, including those for hCAR [48] and mCAR [22,49]. Crystallography has become a vital tool for determining the binding modalities of hPXR agonists of diverse chemical space [50], the molecular basis of mCAR superagonism by TCPOBOP [49], and inverse agonism by androstenol [22]. These insights form the basis for improving the potency and selectivity of chemical tools and potential therapeutics by modulating interactions with key residues. Tighter ligand binding can be achieved by manipulating interactions with a few residues capable of forming hydrogen bonds, such as S247, Q285, and H407 in hPXR, or H203 in hCAR. Aromatic side chains can also form additional attractive forces, such as π-stacking by W299 (hPXR). Protein crystallography has been adopted to obtain valuable feedback for structure–activity relationship (SAR) studies. An interesting finding has been reported about two compounds that work synergistically, and the crystal structure of the binary complex reveals the basis for the cooperativity [34]. This discovery can open possibilities for the prediction of potential synergism by compounds.

Crystallography has become useful not only in designing or improving ligands, but also in expanding our understanding of the effects that these ligands have on the structure of PXR and CAR, which is correlated with their function. Crystal structures have helped researchers to rationalize the constitutive activity of CAR

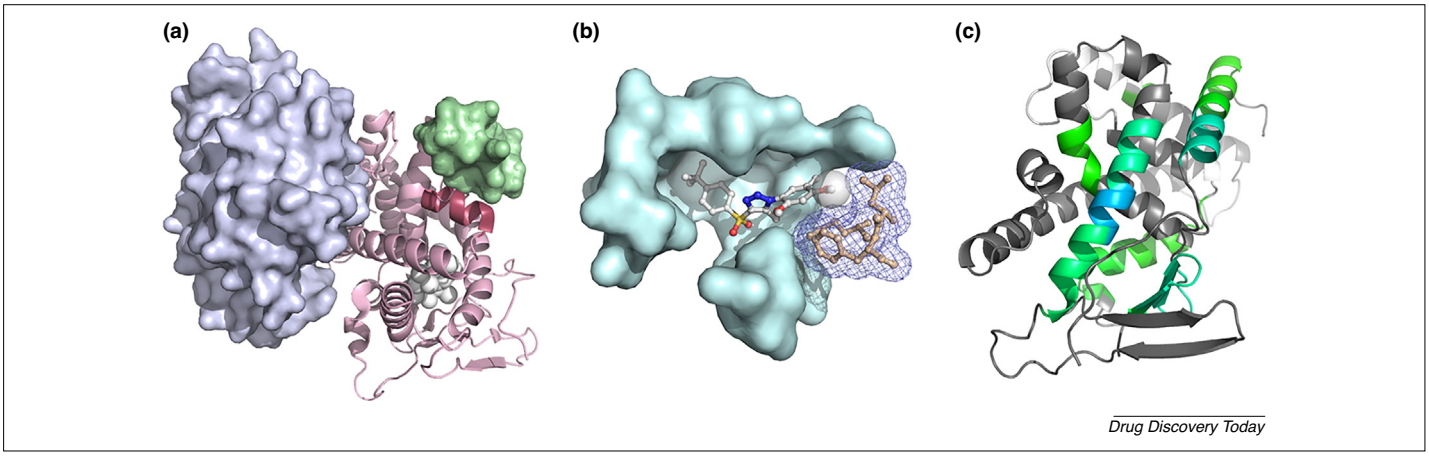

FIGURE 4

Approaches to investigate ligand binding to the pregnane X receptor (PXR) involving protein structural information. **(a)** Crystal structure of PXR ligand-binding domain (LBD) (pink) showing interactions with retinoid X receptor (RXR) LBD (light blue) and steroid receptor coactivator-1 (SRC-1) peptide (green). The AF2 helix is red, and the ligand is shown as white spheres. **(b)** Representation of the PXR agonist SJB7 in the PXR ligand-binding pocket, with crucial interactions with key AF2 helix residues. The chemical element present in SJB7 but absent in SPA70 is represented as a white sphere; comparison of the crystal structure of SJB7 with the results of docking studies using SPA70 suggest that the interaction represented by the sphere is the switch from agonism to antagonism. The software Autodock Vina was used to dock SPA70 to the crystal structure of PXR (Protein Data Bank code: 5X0R). **(c)** Hydrogen–deuterium exchange (HDX)-mass spectrometry (MS) data of PXR in the presence of SPA70 indicate structural dynamic changes because of ligand binding, as reflected by the color gradient. Unaffected regions are in gray. Figures were prepared in PyMOL.

[48], supported the notion that PXR could function as a homodimer [51], and identified the interface for heterodimerization with RXR [52] (Fig. 4a).

Mass spectrometry

Protein crystallography, with all its advantages, is limited to providing only snapshots of protein crystals that are obtained under certain conditions (e.g., type and quantity of buffer components, pH, and temperature). The models resulting from these experimental studies represent static situations. However, global dynamic changes that accompany ligand binding to PXR and CAR can be elucidated by using alternative techniques, such as those that monitor hydrogen–deuterium exchanges (HDX) occurring in protein backbone hydrogens. These changes can be detected by mass spectrometry (MS) or nuclear magnetic resonance (NMR).

The LBDs of PXR and CAR display structural features that are common in other NRs: they form a compact α-helix sandwich fold, with three β-strands present in CAR and a layer of five-stranded antiparallel β-sheets in PXR. The flexibility of some of these regions can change upon protein–ligand binding, which can be detected as variations in deuterium incorporation. The protein segments do not necessarily need to be in direct contact with the ligand. HDX coupled with MS has been utilized to provide evidence of direct interactions of the potent and selective hPXR antagonist SPA70 to the ligand binding pocket of the protein (Fig. 4c) [19]. Similarities were observed between the protein regions affected by SPA70 binding and those affected by the agonist SJB7, which is an analog of SPA70. The solvent-protection profiles in the presence of these ligands were further affected by the subsequent addition of a coactivator peptide (in the case of SJB7) or corepressor peptide (for SPA70), demonstrating enhanced interactions of the peptides to hPXR LBD because of binding of the respective ligands.

MS methods have also been used under native conditions to measure the noncovalent complex formation between PXR and two chemicals that act synergistically [34]. In this study, under nondenaturing conditions, MS distinguished the stoichiometry of the ligand:PXR ratio, providing evidence of the existence of a binary complex formed by two different compounds that reside in the PXR ligand-binding site. Furthermore, MS does not require labeling of any ligands and enables monitoring of the displacement of one ligand by another.

Computational studies

Before the existence of crystal structures, there were limited efforts to use computational tools to interrogate PXR and CAR. Homology models of CAR LBD predicted several of the important features later revealed by crystallography, including the role of the residues lining the binding pocket and the nature of the basal constitutive activity [53].

Largely hydrophobic residues line the binding pockets of PXR and CAR, which are larger and more flexible than those of other NRs. These attributes allow PXR and, to a lesser extent, CAR to bind to molecules with differing scaffold, size, and physicochemical properties [18,22,54]. To understand the promiscuity of PXR, computational solvent mapping was used to identify five well-defined hot spots on all sides of the binding pocket, with the most notable spot formed by the residues F288, W299, and Y306 [55].

One of the most prevalent applications of computational research is in identifying potential ligands, in large part driven by the drastic reduction in cost and labor compared with that of screening a large collection of compounds via biological assays. Several approaches have been considered, including *in silico* pharmacophore [56] and quantitative SARs (QSARs) [57], many of which try to determine features within the ligand or receptor that enable biomolecular recognition.

A more interesting application of computational tools is in providing potential mechanisms or explanations for the perceived biological effect or phenotype of particular ligands. A likely molecular mechanism for the inhibition of PXR by the antagonist SPA70 was reported based on the results of docking studies and comparative analysis with the cocrystal structure of the agonist SJB7 (Fig. 4b), a SPA70 analog [19]. Docking studies of the CAR inverse agonist CINPA1 and its metabolites provided a plausible explanation for their observed affinity differences [47].

Thermal shift assay

The thermal stability of a protein can be determined by subjecting the protein to a temperature gradient, and the presence of a bound ligand is expected to increase the thermal stability. The presence of a particular fluorescent dye aids in monitoring the denaturation of the protein by binding to hydrophobic regions as the protein begins to unfold at increasing temperatures. The dye–protein interaction increases fluorescence intensity, resulting in a melt profile, and the melting temperature at the inflection point (Tm) of the rising signal can then be determined by fitting the Boltzmann equation or using the derivative-curve method. An increase in melting temperature indicates enhanced protein thermal stability because of ligand binding, although it does not necessarily correlate with binding affinity.

Interactions of various ligands (the agonist CITCO, the potent inverse agonist PK11195, and the newly discovered inverse agonist CINPA1) with hCAR LBD were investigated by using SYPRO Orange as the fluorescent dye using real-time PCR to create a gradient at a rate of 1 °C/min from 25 °C to 95 °C [58]. Surprisingly, no discernible thermal curve for the hCAR LBD could be detected in the absence of ligand, but the prototypical curve became apparent once any of the tested ligands was included. These observations suggest that, at the start of the thermal gradient, hCAR LBD is transiently in an unstructured state with solvent-exposed hydrophobic regions to which SYPRO Orange can already bind. The presence of PK11195 and CINPA1 increased the melting temperature of hCAR-LBD by 6.7 °C and 8 °C, respectively; CITCO provided little thermal protection, increasing the melting point by only 1 °C over a concentration range of up to 200 mM.

Surface plasmon resonance and isothermal titration calorimetry

Direct binding events between the protein and ligand can be measured by using label-free biophysical techniques, such as surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). SPR monitors the association and dissociation of the ligand to and from a captured target protein in real time [59]. Therefore, it can determine binding kinetic parameters in addition to ligand-binding affinities. SPR has been used to quantitate the dissociation constant of pesticides to PXR [60] and demonstrate direct interactions of a newly discovered CAR inhibitor [58].

ITC detects heat changes upon ligand interactions with proteins, and it can be used to calculate thermodynamic parameters and stoichiometry [59]. ITC has been used to define direct interactions of a binary chemical complex to PXR and their synergistic effects [34]. The entropic and enthalpic changes of the coactivator SRC-1 peptide to the CAR/RXR complex in the presence or absence of its ligands have also been investigated [61]. In contrast to SPR, ITC requires larger amounts of sample, and the process cannot screen large sample sets.

Tryptophan fluorescence quenching

The intrinsic fluorescence emission profile of tryptophan residues within proteins can change when their local surrounding environment is altered because of protein–ligand interactions [62]. The tryptophan fluorescence quenching approach was applied to validate PXR modulators from cell-based assays [63]. Its utility resides in demonstrating direct interaction of compounds with the PXR LBD and, because the signal behaves in a dose-dependent manner, the dissociation constants can be calculated.

This approach was applied to the mCAR-RXR heterodimer to investigate perturbations in RXR conformation because of interactions of mCAR with its agonist TCPOBOP [64]. It was concluded that TCPOBOP-induced structural changes of mCAR altered the dimerization interface, which in turn affected the environment of RXR tryptophan residues. The study of allosteric transfer of information between these partner proteins could be undertaken because there is no tryptophan present in mCAR LBD. Interestingly, the mCAR agonist meclizine did not display the same effect, which indicates a different molecular mechanism of mCAR activation depending on individual agonist. Although intrinsic tryptophan quenching is a simple technique that can be used to identify ligands and determine their effects on structural dynamics, it can be susceptible to intrinsic compound fluorescence.

Cell-based assays

Cell-based assays test ligands for their activities under a more physiologically relevant condition than biochemical assays, but they could also introduce artifacts because of the complexity of the cellular systems [65]. Currently, luciferase-based transactivation assays are widely used to characterize PXR and CAR ligands, which generally include overexpressing PXR or CAR along with a luciferase reporter gene driven by the respective PXR or CAR target gene promoter, using either transient or stable transfection [19,26,37,40,41,63,66,67]. Transient transfections are practical for short-term experiments, but they can introduce large experiment-to-experiment variations. Although establishing stable cell lines is time consuming, they prove to be highly reproducible in the long term [68]. Small interfering RNA (siRNA) technology is used to knock down genes of interest, and this technology complements the use of a PXR or CAR inhibitor in a luciferase reporter assay [19,26,40,41,69]. One-hybrid, two-hybrid, and reverse yeast two-hybrid systems have been used to study the effect of compounds on PXR and CAR and their interactions with coregulators in a cellular environment; the reverse yeast two-hybrid system was deemed particularly useful in identifying allosteric PXR inhibitors that bind outside the ligand-binding pocket [19,26,41,63,70,71]. Primary hepatocytes, especially primary human hepatocytes, are considered to be the gold standard in cellular models for *in vitro* drug functional assessments [72]. Primary human hepatocytes are widely used to characterize the PXR or CAR functions of ligands [19,26,41,67,73,74].

Transactivation reporter-based assays are widely used methods for identifying compounds that modulate the activity of PXR and CAR. However, there are several other techniques that have been routinely used to further characterize the chemicals in a cellular context. Fluorescence microscopy has been applied to the study of PXR and CAR cellular localization, because they can translocate between the cytoplasm and nucleus [26,75]. Real-time live-cell imaging can be used to monitor cell morphology and proliferation, and it is particularly useful in the analysis of potentiation in drug sensitivity that is mediated by PXR and CAR target genes [76]. Many other techniques rely on additional experimental steps following cell lysis, including western blotting, protein co-immunoprecipitation, chromatin immunoprecipitation, real-time PCR, and electrophoretic mobility shift assays, among others [26,75,77].

Animal models

Transgenic animal models have become powerful systems to understand the underlying mechanisms of diseases and validate novel compounds that can modulate those biological processes [78]. Given that ligands can affect PXR in a species-specific manner, 'humanized' PXR or CAR mouse models have been developed to minimize species-selective responses. In these models, the endogenous *PXR* or *CAR* gene is replaced with the functional human counterpart [79,80]. Human *PXR* transgenic mice with luciferase reporter have been used for organism-level confirmation of the agonism of PXR by piperine and evidence that a PXR antagonist can be used to prevent PXR-mediated drug–drug interactions [19,41].

Concluding remarks

PXR and CAR are prominent NRs known for their promiscuous nature in ligand binding that are activated by compounds of diverse chemical structure and size. They have key roles in drug resistance and cause adverse effects via drug–drug interactions. Therefore, it is imperative to know whether drug candidates modulate PXR or CAR before clearing them for clinical studies and, eventually, patient use. A panel of various assay types to interrogate ligand–receptor interactions has become well-established (Table 2). Biochemical assays should be used in concert with cell-based assays to ensure that the effect of a ligand can be translated from cell-free to cellular and animal levels, taking into consideration parameters such as nonspecific protein binding, cell permeability, solubility, and off-target effects [33,37,40]. The study of PXR and CAR modulators can be particularly difficult in some cases, which emphasizes the need for multidisciplinary approaches. For example, phenobarbital activates CAR via an indirect mechanism by inhibiting epidermal growth factor receptor signaling [81], which can be detected in cell-based assays but not in biochemical assays.

From a practical point of view, initial screening for modulators of PXR and CAR are normally conducted using reporter

TABLE 2

Summary of different assay formats and their advantages and disadvantages

Assay	Advantages	Disadvantages
SPA	A simple mix-and-read approach suitable for HTS	Generates radioactive waste Ligand–protein interaction indirectly determined by labeled-probe displacement
FP	HTS amenable	Requires large amounts of proteins Prone to interference by fluorescent compounds Ligand–protein interaction indirectly determined by labeled-probe displacement
TR-FRET	HTS amenable Less susceptible to interference by fluorescent compounds Requires small amounts of protein	Limited donor/acceptor pair availability Higher reagent costs Ligand–protein interaction indirectly determined by labeled-probe displacement
Crystallography	Primary approach for obtaining structural information at high resolution	Requires large amounts of protein Difficulty obtaining cocrystals with any ligand Represents a static representation (not dynamic) Requires highly specialized user
MS	Requires small sample amount Provides structural dynamic information when coupled to HDX Label-free method	Requires highly specialized user
Computational studies	Inexpensive Suitable for large-scale analysis in automated manner	Theoretical: results need to be validated experimentally
Thermal shift assay	Provides thermal-stability information other than binding Label-free method Determination of direct ligand–protein interactions	Requires large amounts of protein Results do not necessarily correlate with ligand affinity Appropriate signal highly dependent on protein
SPR	Label-free method Real-time and direct monitoring of ligand–protein interactions Provides kinetics parameters	Protein needs to be immobilized
ITC	Label-free method Direct monitoring of ligand–protein interactions Provides thermodynamic parameters	Requires large amounts of sample Very low throughput
Tryptophan fluorescence quenching	Simplicity	Requires tryptophan, the fluorescence of which is affected by ligand binding Prone to interference by fluorescent compounds
Cell-based assays	More physiologically relevant than biochemical assays Provide information regarding compound cell permeability, stability, and toxicity	Potential off-target effects Typically higher batch-to-batch variations compared with biochemical assays
Animal models	Organism-level information Provide additional information, such as pharmacokinetics and pharmacodynamics	Time-consuming High cost

cell-based assays because of their robustness, low cost, and transferability to HTS format. Subsequent studies include biochemical assays to probe for direct interactions of the compounds with PXR and CAR. Among these, label-free methods, such as ITC and SPR, are the preferred choices. However, they are restricted in application because of limitations of acceptable buffer conditions, the need for large amounts of reagents, low throughput, and case-by-case compound behavior. Alternative biochemical approaches that can be broadly applied to diverse types of chemical include TR-FRET. Crystal structures provide a wealth of information regarding interactions of the ligand with the protein, facilitating the chemical optimization of the modulators. By contrast, ligand-induced PXR and CAR structure dynamics are typically analyzed using techniques that employ HDX or monitor perturbations in the environment of certain amino acid residues in PXR and CAR, such as the quenching of tryptophan residues. An extensive array of cell-based approaches is available to query the cellular outcomes of PXR and CAR modulators, which is necessary before *in vivo* studies. An important aspect of these cellular assays is in defining the mechanisms of actions by determining signaling pathways, post-translational modifications, subcellular localization, and associations with partner proteins.

The discovery of PXR- or CAR-selective antagonists has been challenging because of the flexible PXR ligand pocket that recognizes most molecules as agonists, even those that act as CAR inhibitors. With the recent reports of SPA70 and CINPA1 with selectivity for PXR and CAR that directly bind to the ligand-binding pocket, we expect that the technologies outlined in this review will provide means of expanding PXR- and CAR-selective inhibitors that could be useful therapeutics.

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