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Searching for Constitutive Androstane Receptor Modulators

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ABSTRACT

The constitutive androstane receptor (CAR; NR1I3) has been established as one of the main drug- and xenobiotic-responsive transcriptional regulators, collectively called xenosensors. CAR activates the expression of several oxidative, hydrolytic, and conjugative drug-metabolizing enzymes and drug transporters, and therefore, it contributes to drug and xenobiotic elimination, drug interactions, and toxicological processes. This minireview introduces mechanisms that modulate CAR activity and focuses on the recent approaches used to search and characterize CAR agonists, inverse agonists, and indirect activators. This minireview is dedicated to Dr. Masahiko Negishi to celebrate his scientific achievements during his long service at the National Institutes of Health.

SIGNIFICANCE STATEMENT

Discovery and characterization of human constitutive androstane receptor (CAR) modulators is important for drug development, toxicity studies, and in generation of chemical tools to dissect biological functions of CAR. This minireview focuses on the main methods used to search for these compounds and discusses their essential features.

Introduction

The constitutive androstane receptor (CAR; NR1I3) is one of the key drug- and xenobiotic-sensitive regulators of enzymes and transporters important for drug metabolism, disposition, interactions, and toxicological outcomes along with the related pregnane X receptor (PXR; NR1I2) and the aryl hydrocarbon receptor. Additionally, CAR participates in the metabolism of glucose, lipids, and bile acids and has a role in cell-cell communication, cell cycle, and chemical carcinogenesis. Searching the PubMed database in October 2021 with the phrase "constitutive androstane receptor OR nr1i3" yielded over 1560 publications, an increase of ∼700 papers after our earlier review in 2013 on CAR properties and actions (Molnár et al., 2013).

The readers are referred to recent reviews (Table 1) that cover many aspects of CAR properties and its involvement in biology. These reviews are highly recommended reading for those seeking specific information on the CAR protein and its functions. In this minireview, I will focus on the key aspects of human CAR structure that affect its activity, current approaches used to search and characterize human CAR modulators, provide some examples of novel compounds, and highlight important issues associated with these studies.

Brief Historical Perspective. Human and mouse CAR were initially described as constitutively active nuclear receptors (NRs) by David Moore's laboratory (Baes et al., 1994; Choi et al., 1997), but true target genes were unknown at that time. Work on the phenobarbital (PB) induction of mouse *Cyp2b10* gene expression by the Negishi group defined the PB-responsive enhancer module that was activated by multiple cytochrome P450 (P450) inducers and CAR/retinoid X receptor (RXR) as the crucial heterodimeric transcription factor translocating from the cytoplasm into the nucleus (Honkakoski and Negishi, 1997; Honkakoski et al., 1998a,b; Kawamoto et al., 1999). Anecdotally, we submitted our seminal publication (Honkakoski et al., 1998a) first to the *Nature* journal, which declined it on the grounds that we should have demonstrated the direct binding of PB to CAR to gain acceptance for this manuscript. In hindsight, the editors were asking for the moon, because this is not the actual mode of PB action that was finally elucidated some 15 years later (Mutoh et al., 2013).

Subsequent reports showed that mice lacking CAR could not induce P450s, increase liver size, nor enhance tumor promotion in response to PB exposure (Wei et al., 2000; Ueda et al., 2002; Yamamoto et al., 2004). Concurrent characterization of PXR as the main regulator for CYP3A expression (Kliewer et al., 1998) established these sister receptors as key controllers of drug metabolism and disposition (Willson and Kliewer, 2002; Yan and Xie, 2016).

Diverse chemical classes including pesticides, fire retardants, environmental contaminants, drugs, and industrial chemicals are now known to bind to or modulate human and animal CAR activity in cell-free

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ABBREVIATIONS: CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo(2,1-b)(1,3)thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; LBD, ligand-binding domain; LBP, ligand-binding pocket; NR, nuclear receptor; P450, cytochrome P450; PK11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide; PB, phenobarbital; PXR, pregnane X receptor; RXR, retinoid X receptor; TCPOBOP, 1,4-*bis*-[2-(3,5-dichloropyridyloxy)]benzene.

TABLE 1

Selection of recent review articles on characteristics and functions of CAR

Topic	References
CAR ligand-binding domain structures	Buchman et al., 2018
Computational modeling	Kato, 2020
Genetic variants	Mbatchi et al., 2018
CAR phosphorylation and dimerization	Negishi, 2017; Negishi et al., 2020
Direct and indirect activation mechanisms	Mackowiak and Wang, 2016
Small-molecule modulators	Chai et al., 2016
Main assay technologies	Chai et al., 2019
Developmental and tissue expression	Daujat-Chavanieu and Gerbal-Chaloin, 2020
Role in liver physiology and disease	Tanaka et al., 2017; Cai et al., 2021
Role in endocrine disruption	Küblbeck et al., 2020; Toporova and Balaguer, 2020
Role in liver tumor formation	Lake, 2018; Bae et al., 2021

and cell-based assays (Table 2; Molnár et al., 2013; Chai et al., 2016; Lynch et al., 2019; Küblbeck et al., 2020). However, some compounds such as PB, bilirubin, phenytoin, and teriflunomide (Mackowiak and Wang, 2016; Carazo et al., 2018) are P450 inducers without any apparent binding to or activation of CAR in cell-based reporter gene assays. These indirect activators can translocate CAR from the cytoplasm into the nucleus (Fig. 1). At least for PB, this involves inhibition of the epidermal growth factor (EGF) receptor, participation of mitogen-activated protein kinase and extracellular signal-regulated kinase (ERK) pathways, and cytoplasmic protein phosphatase 2A–mediated dephosphorylation of CAR at residue T38 in the cytoplasm prior to translocation and target gene activation (Mutoh et al., 2013; Negishi et al., 2020). However, this process is poorly understood for many compounds listed as indirect activators.

The overlapping ligand preferences (Chai et al., 2016; Lin et al., 2020) and shared P450 target genes (Li et al., 2015; Ochsner et al., 2016) of CAR and PXR make it difficult to ascertain which receptor is responsible for P450 induction in primary hepatocytes and in vivo. Moreover, CAR modulators have additional targets that may influence P450 induction and other drug disposition processes independently of CAR. For instance, recent reviews indicate that pesticides, phthalates, and flame retardants often activate CAR, PXR, and peroxisome proliferator–activated receptors, and bisphenols modulate CAR, PXR, and steroid hormone receptors (Küblbeck et al., 2020; Toporova and Balaguer, 2020). Flavonoids are reported as either indirect or direct CAR activators (Chai et al., 2016; Yao et al., 2010). They are also known inhibitors of the mitogen-activated protein kinase, ERK or EGF signaling and activators of the nuclear factor erythroid 2–related factor 2 (Yahfoufi et al., 2018; Clifford et al., 2021; Hazafa et al., 2022). These issues complicate the search strategies to identify true CAR modulators and require multiple different assays for the correct assignment of their mechanism of action.

Structural Features that Contribute to High Constitutive Activity and Agonism of CAR. So far, only three agonist-bound and one inverse agonist-bound CAR/RXR ligand-binding domain (LBD) crystal structures from early 2000s exist (Buchman et al., 2018). Despite advances in molecular modeling, this limits our understanding of mechanisms for CAR modulation. The high constitutive activity of CAR is due to stabilization of the helix H12 in the active position (Fig. 2) that allows coactivator binding by three mechanisms. First, an additional LBD helix X forces the short H12 toward the active position (Xu et al., 2004). Second, the residues F161, N165, F234, and Y326 shield the ligand-binding pocket (LBP), preventing in most cases the direct interaction between the agonist and H12 but providing further H12 stabilization at the same time (Xu et al., 2004; Molnár et al., 2013). Third, the interface for CAR/RXR dimerization (comprised of helices 7, 10, and 11 in Fig. 2) is larger than in most NRs, and RXR binding seems to stabilize CAR LBD in the active conformation (Suino et al., 2004). All three interactions increase the coactivator recruitment in the absence of ligands and thus create difficulties in detecting responses elicited by CAR agonists.

The observed CAR LBP volumes (525–675 $Å^3$) can accommodate various ligands that employ mostly hydrophobic and some hydrogen-bonding interactions with the LBP-lining residues. In human CAR structures, cocrystallized agonists do not interact with H12 like the mouse CAR does with its specific agonist 1,4-*bis*-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (Molnár et al., 2013). Due to the lack of other agonist-bound and ligand-free CAR structures, a comprehensive and mechanistic view of agonist-elicited changes in coactivator recruitment is still missing. The developments in computing power, molecular docking, and molecular dynamics simulations may help address these gaps (Kato, 2020). It is likely that these in silico methods will not be discriminating enough to quickly and reliably detect and distinguish between all CAR agonists and inverse agonists, but they are valuable as supportive tools either for preselection before or mechanistic

TABLE 2

Established indirect activators, direct agonists, and inverse agonists of human CAR

Modulator Classes	Chemicals	References
Indirect activators	PB, teriflunomide, phenytoin[a], some flavonoids[a], polychlorinated biphenyls[a]	Yao et al., 2010; Molnár et al., 2013; Mutoh et al., 2013; Fernández et al., 2015; Chai et al., 2016; Carazo et al., 2018; Carazo Hardesty et al., 2018
Selective agonists	DL5050, rimcazole, CITCO[b], clemizole[b]	Maglich et al., 2003; Keminer et al., 2019; Liang et al., 2019; Lynch et al., 2019;Lin et al., 2020
Inverse agonists	PK11195[b], S07662[b], CINPA1[b], clotrimazole[c]	Küblbeck et al., 2011b; Jeske et al., 2017; Cherian et al., 2018; Toporova et al., 2020

[a]Some evidence for both indirect and direct activation.
[b]Reports indicate additional activation of PXR.
[c]Reports indicate variable results from agonist to inactive to inverse agonist.

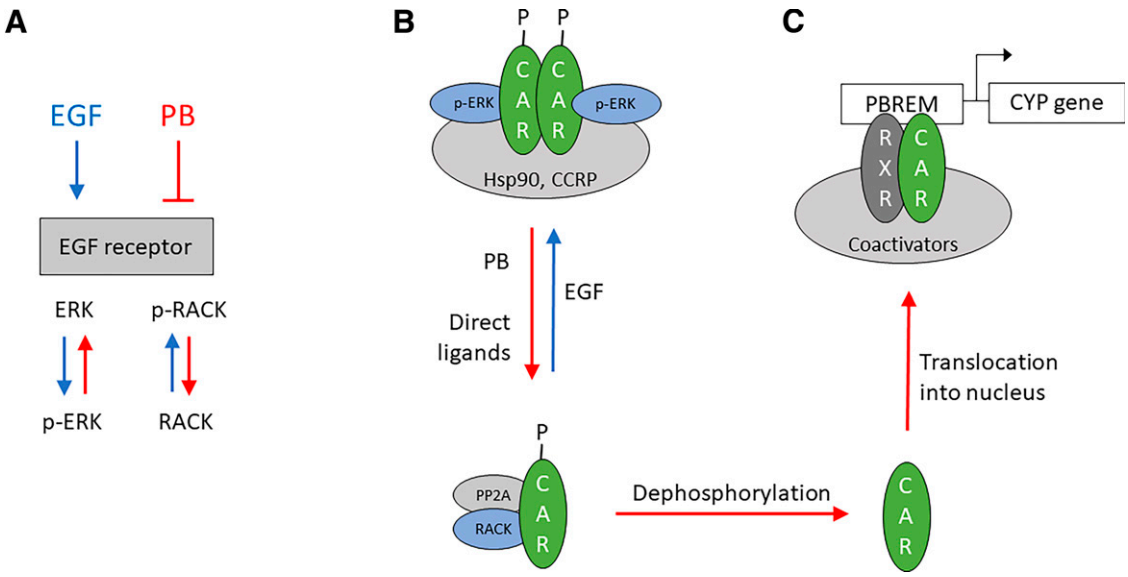

Fig. 1. Activation mechanisms of CAR. In the indirect activation, PB acts as an antagonist of EGF receptor, which results in dephosphorylation of phospho-ERK and dephosphorylation of RACK (A). Both PB and directly acting CAR ligands can dissociate the cytoplasmic complex that contains CAR as a phosphorylated homodimer. The monomeric CAR associates with RACK and PP2A, leading to dephosphorylation of CAR (B). Dephosphorylated CAR is translocated into the nucleus, which heterodimerization with RXR, binding to DNA response elements such as PBREM, and recruitment of NR coactivators culminates in increased expression of P450 and other genes (C). It should be noted that details of these mechanisms have formally been demonstrated only for some CAR activators. PBREM, PB-responsive enhancer module; PP2A, protein phosphatase 2A.

binding evaluations after the functional assays (e.g., Küblbeck et al., 2011a,b; Lynch et al., 2013; Keminer et al., 2019) that will be described below.

Activation Differences Among CAR Isoforms and Variants. Alternative splicing produces multiple human and primate CAR splicing isoforms (Lamba et al., 2004; Mbatchi et al., 2018), which are not present in rodents. An abundant human transcript (∼50%) encodes the wild-type CAR1 that displays high basal activity, whereas the minor isoform CAR2 (∼10%) and the abundant CAR3 (∼40%) have low constitutive activity, likely due to their reduced interaction with RXR and resulting weaker binding to DNA and coactivators (Auerbach et al., 2005, 2007). In addition, some CAR activators such as phthalates, antivirals, and artemisinin derivatives appear to display some isoform selectivity (Auerbach et al., 2005, 2007; DeKeyser et al., 2011; Burk et al., 2012; Paul et al., 2013; Sharma et al., 2015). These differences in CAR basal and ligand-dependent activity may be explained by the structural changes near the RXR heterodimerization surface (CAR3; insertion of APYLT, which likely destabilizes RXR binding) or the LBP (CAR2; insertion of SPTV), respectively. Both CAR2 and CAR3 are highly dependent, unlike CAR1, on inclusion of RXR in transactivation assays.

The gnomAD database (Karczewski et al., 2020) lists hundreds of rare single-nucleotide human CAR variants (<0.1% allele frequency) with unknown functionality. In vitro characterization has been done only for a few rare naturally occurring variants: LBD variants H246R and L308P lead to complete inactivation or reduced reporter activity, respectively (Ikeda et al., 2005). I281T, which is also near the RXR heterodimerization surface, reduces the interaction with coactivators and dampens the activation elicited by weaker agonists (Prantner et al., 2018). More common noncoding or silent CAR variants are associated with drug plasma concentrations and/or adverse effects (Mbatchi et al., 2018) by yet unknown mechanisms that may include changes in CAR expression.

Species Differences in CAR Activation. Mammalian CAR genes have undergone positive selection that has resulted in only 72% sequence similarity between the mouse and human CAR LBDs (Reschly and Krasowski, 2006). Such divergent evolution explains the wide variability in the chemicals' ability to activate CAR and induce P450s between species. For examples, 6-(4-chlorophenyl)imidazo-[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) and TCPOBOP are potent species-selective agonists for the human and mouse CAR, respectively. Androstenol is a stronger inverse agonist for mouse than human CAR (Molnár et al., 2013; Chai et al., 2016), rat CAR is activated by clotrimazole to greater extent than the dog CAR (Omiecinski et al., 2011), whereas the opposite is true for artemisinin (Pinne et al., 2016), and triphenyl phosphate derivatives show opposing characters between the mouse and human CAR (Honkakoski et al., 2004). Different sizes and contacts of the LBP-lining residues with ligands are the likely determinants for these species differences: human CAR residues that confer species-specific responses to TCPOBOP (M340) and to 17α-ethinylestradiol, an inverse agonist for human CAR and a partial agonist for mouse CAR (F243), have been identified (Jyrkkärinne et al., 2003, 2005). Bovine CAR has two mutations at critical residues for CAR function (N165I, Y326F) that may explain its low responsiveness to both human and mouse CAR ligands (Küblbeck et al., 2016). Similarly, rat CAR (F234E, F243I) and dog CAR (F161L) have mutations among these key residues. However, these or other amino acid differences have not been probed between multiple species nor verified by mutagenesis.

Strategies in Identifying CAR Modulators. Most commonly, the search for CAR modulators begins by using assays (Table 3) that measure ligand-dependent CAR-mediated activation of reporter gene in transiently or stably transfected cells (Raucy and Lasker, 2013). There is a continuing debate about the pros and cons of using either the full-length CAR or its LBD as a GAL4 fusion protein in the reporter assays. The former is more representative of the natural CAR/RXR heterodimer binding to its target DNA enhancer while the presence of other NRs may interfere with this process (Mäkinen et al., 2002). Using the CAR LBD as a fusion protein in the hunt for CAR agonists eliminates this problem and, in addition, may avoid phosphorylation-dependent effects due to omission of the residue T38 within the DNA-binding domain. Regardless of the assay type, increased reporter activity by test compounds is hard to detect due to the spontaneous translocation of CAR

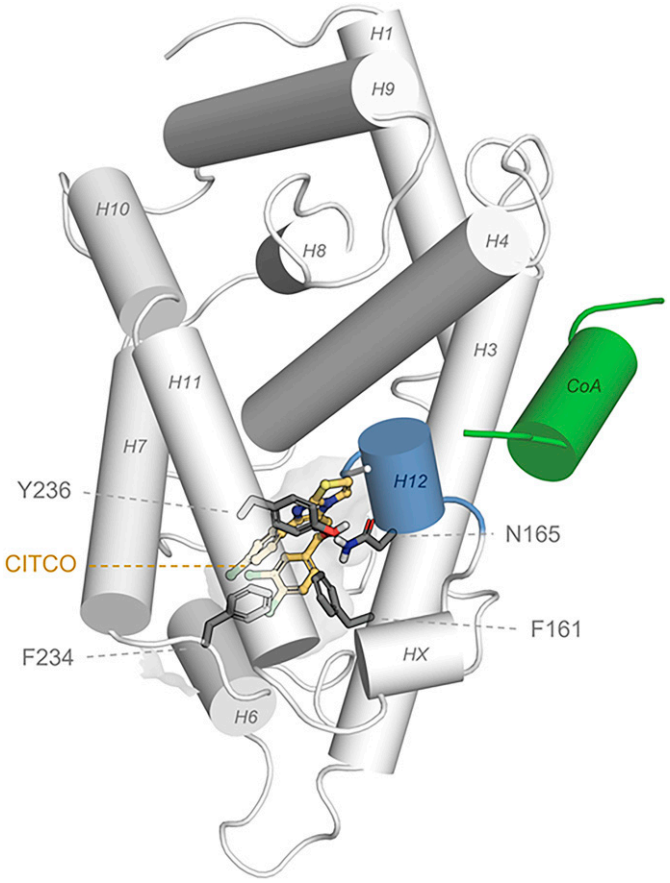

Fig. 2. A model of the agonist-bound CAR ligand-binding domain. Helices are displayed with labeled cylinders starting from the LBD N-terminus (H1 to H12). The activation helix (H12) is shown in blue and the NR-binding coactivator peptide (CoA) in green. The CAR agonist CITCO is shown in gold and the crucial amino acid residues in gray licorice (F161, N165, F234, Y326). The light-gray "cloud" in the background and near H6 is due to the β-sheet structure of the CAR LBD. The model was created with Discovery Studio (BIOVIA, Dassault Systèmes, San Diego, CA) using the reported crystal structure of CAR/RXR heterodimer bound with SRC1 peptide, fatty acid, and CITCO (1XVP; rcsb.org).

into nucleus and its high constitutive activity in continuous cell lines. Initially, inverse agonists were easily recognized but repression or no effect was observed for compounds that later turned out to be CAR agonists (Moore et al., 2002).

The problem of high constitutive activity has been dealt with several ways. First, the addition of a CAR inverse agonist such as 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195) (Lynch et al., 2019) or androstanol (Sueyoshi et al., 1999; Auerbach et al., 2007) decreases the basal reporter level. The observed increase in reporter activity by the test compound is interpreted as agonistic competition for binding to the LBP that results in gene activation. This set-up is reasonable for detection of stronger CAR agonists, but there is some risk of misclassification for compounds that bind weakly. Depending on the relative affinities to CAR between the inverse agonist and partial/weak agonists, the latter compounds may not be easily detected. In addition, true but less-potent inverse agonists can be defined as weak agonists.

The second approach relies on the use of natural (CAR3; Keminer et al., 2019; Skoda et al., 2020) or artificial CAR variants where the basal activity is reduced by addition of amino acids to the human (Chen et al., 2010; Kanno and Inouye, 2010; Imai et al., 2013) or animal CAR LBDs (Omiecinski et al., 2011; Pinne et al., 2016). This approach carries the risk of affecting selectivity of the LBP (CAR2 or insertions near H12) or the CAR/RXR binding (CAR3 or insertion of single alanine instead of APYLT) that may affect the ligand-dependent changes in coactivator recruitment.

Because ligand responses can vary depending on available cellular coactivators, the third approach is to find an appropriate cell line and culture conditions that allow direct measurement of CAR1 activity without the addition of inverse agonists or modification of its amino acid sequence. Our laboratory has consistently detected clotrimazole as a partial CAR1 agonist in validated assays in C3A cells (Küblbeck et al., 2008; 2011a), whereas other reports found it either inactive in COS-1 (Chen et al., 2010; Omiecinski et al., 2011) or an inverse agonist in CV-1 and HepG2 cells (Maglich et al., 2003; Auerbach et al., 2007). Similar assignment differences have been published for meclizine (Huang et al., 2004; Lau et al., 2011). Clotrimazole has repeatedly shown to be a CAR3 agonist (Chen et al., 2010; Omiecinski et al., 2011; Keminer et al., 2019; Toporova et al., 2020). Given the very high similarity of CAR1 and CAR3 LBPs and their similar activation profiles (Keminer et al., 2019), the above discrepancies are likely due to assay-related reasons.

The dynamic range of the employed reporter assays, as defined by the maximal response of established positive controls such as TCPOBOP or CITCO relative to the dimethylsulfoxide vehicle, varies significantly. These values range from about twofold (Yao et al., 2010; Wahlang et al., 2014; Hardesty et al., 2018) to ∼fivefold (Mäkinen et al., 2003; Lynch et al., 2019) and to 21-fold (Küblbeck et al., 2011a) with CAR1, and between 6- to 26-fold with CAR3 (Keminer et al., 2019; Skoda et al., 2020). A low dynamic range may limit the sensitivity to detect true activators, and more importantly, observed lack of reporter activity in a suboptimal assay does not indicate discovery of an indirect CAR activator.

Finally, because compounds may influence reporter gene activity independent of CAR, it is essential to conduct control studies in its absence. These control experiments can show if the reporter activity is modulated by e.g., physicochemical interference by the test chemical (Dahlin et al., 2015) or by inhibition of e.g., luciferase or other reporter activity (Ho et al., 2013; Poutiainen et al., 2013). Other potential interferences include inhibition of cellular protein kinases necessary for CAR activation or binding to the heterodimer partner RXR for which CAR2 and CAR3 are dependent on, as recently described for retinoids (Keminer et al., 2019). Naturally, any issues with cytotoxicity or solubility of the compounds should be taken into account with cell-based assays.

Even if the above criteria for the assay are met, the search may still fail. My first project after returning from the postdoctoral period at National Institute of Environmental Health Sciences was to isolate the endogenous small molecule thought at that time to keep CAR inactive, similarly to androstenol (Forman et al., 1998). We fractionated liver extracts from untreated mice and isolated fractions with potent suppressive activity. Much to our dismay, these compounds turned out to be man-made lubricants (Honkakoski et al., 2004), whereas androstenol and related steroids were present in only trace amounts.

After the primary screen with reporter gene assays, evidence for direct binding to CAR is required. Usually, this comes from various two-hybrid assays that measure the agonist- or inverse agonist–dependent interaction of CAR LBD with a selected coactivator or corepressor peptide, respectively (Chai et al., 2019). A typical format is the mammalian two-hybrid system in which the CAR LBD is fused with a strong transactivation domain such as VP16, the coregulator peptide is linked to the GAL4 DNA-binding domain (Küblbeck et al., 2011a,b), and their ligand-dependent interaction enables activation of reporter gene expression. This assay type is quite sensitive in identifying weak

TABLE 3

Proposed strategy in identifying CAR modulators
Changes in readout (↑): identification of direct binding to CAR; may notdistinguish between agonists and inverse agonists

Assay Type	Interpretation and Follow-Up Studies
Reporter gene assay[a,b] with full-length or LBD constructs with or without competitive inverse agonist	Changes in reporter activity (↑↓): agonists and inverse agonists identified; replication studies; verification by two-hybrid or binding assays; PXR counterscreen is extremely useful
Cell-based two-hybrid assays[a,b]	Changes in reporter activity (↑): identification of respective coactivator or corepressor; indication of ligand-CAR interaction; supported by binding assays
Cell-free two-hybrid or binding studies[b]	
Nuclear translocation assay	Changes in cytoplasmic/nuclear staining of tagged CAR: detection of CAR indirect activators; requires secondary analysis by reporter gene or two-hybrid systems; analysis of contributing signaling pathways useful
RNA expression studies in hepatic cells or in vivo	Increase in CAR target gene mRNAs (↑): verification in cells lacking CAR (knockout or siRNA knockdown in cells, knockout animals, humanized mice) analysis of PXR target genes useful

siRNA, small interfering RNA.
[a]Supported by competitive displacement studies (agonist versus inverse agonist) in the same system.
[b]Supported by molecular modeling studies for directly binding agonists/inverse agonists.

or partial agonists that may recruit either type of coregulator to the CAR LBD as shown for clotrimazole (Mäkinen et al., 2003; Jyrkkärinne et al., 2005). However, if a certain ligand shows preferential binding for distinct coregulators, then it may miss detection. Another variation, halfway between a reporter gene and a two-hybrid assay, is the so-called assembly assay that detects ligand-enhanced association between the CAR helix H1 and rest of the LBD (Hoffart et al., 2012; Carazo Fernández et al., 2015), but it cannot separate between agonists and inverse agonists.

The same two-hybrid approach is also popular with recombinant proteins produced in vitro and used on assays such as the fluorescence resonance energy transfer where the CAR LBD and the coregulator peptide are labeled with distinct fluorophores (Lau et al., 2011; Carazo Fernández et al., 2015) or the coregulator arrays (Murayama et al., 2014; Hsu et al., 2016). The latter system allows for parallel interrogation of ligand-elicited interactions of NRs with over 150 coregulator peptides at the same time. Other platforms include e.g., surface plasmon resonance (Režen et al., 2017; Cherian et al., 2018), ligand-induced limited proteolysis (Küblbeck et al., 2011a; Keminer et al., 2019), and thermal stability (Cherian et al., 2018; Kobashigawa et al., 2021) assays that are label-free and measure the interaction between the test chemical and CAR LBD as ligand-induced changes in optical properties of the immobilized receptor, in susceptibility to protease degradation, and in protein heat denaturation, respectively (Chai et al., 2019). Because these assays are cell-free, they do not suffer from cytotoxicity or major solubility issues. However, they do not easily distinguish between agonists and inverse agonists unless a coregulator peptide is linked to the CAR protein (Kobashigawa et al., 2021). All use one form of CAR LBD fusion proteins, and nonspecific binding by lipophilic test compounds tested at high concentrations may become a problem (Küblbeck et al., 2011a; Keminer et al., 2019). In addition, recombinant CAR protein tends to be quite unstable (Kobashigawa et al., 2021). This may decrease the lifetime and performance of the CAR protein preparation and require carefully controlled experiments.

Because CAR is retained in the cytoplasm of unexposed hepatocytes and accumulates into nucleus after treatment of both direct and indirect activators, researchers have used adenovirus-mediated transduction of fluorescently tagged CAR into primary hepatocytes as a tool to mimic this process (Li et al., 2009). This allows for quick visualization of potential CAR modulators regardless of the mechanism (Mackowiak et al., 2019). Because many inverse agonists seem to produce the same response, this assay should be complemented with other experiments to separate them from true agonists. In addition, subtle differences among CAR agonists are not easily discernible due to variation in both morphology and fluorescence intensity among hepatocytes, and comparisons between test compounds requires the use of automated imaging systems (Mackowiak et al., 2019).

As mentioned earlier, the coexpression, common target genes, and ligand sharing by CAR and PXR complicates identification of true human CAR modulators in primary hepatocytes or in vivo. Knockout and humanized CAR and PXR animals (Scheer and Wolf, 2014; Skoda et al., 2020) can assist in dissection and verification of P450 induction process. A similar and very promising approach is the generation of human HepaRG knockout cell lines which do not express CAR or PXR. Treating wild-type and knockout HepaRG cells with the test compounds enables large-scale identification of P450 inducers and NRs recognizing them in a more physiologic context than the reporter gene assays (Li et al., 2015; Preiss et al., 2021). Another option is the use of antisense oligonucleotides to down-regulate CAR expression in hepatocytes (Nudischer et al., 2020). Because all CAR inverse agonists tend to activate PXR (Table 2; Küblbeck et al., 2011b; Jeske et al., 2017; Mackowiak et al., 2017), their use is cautioned as they complicate the analysis of agonist-elicited increases in P450 expression. One potential strategy to find CAR ligands that takes into account many of the above issues has recently been published (Berthier et al., 2021).

Some Examples on Identification of Novel CAR Ligands. Combinations of assays have been applied to detect many novel CAR ligands and their mechanisms of action. The earlier reviews have already detailed many classes of CAR modulators (Table 2; Molnár et al., 2013; Chai et al., 2016; Küblbeck et al., 2020; Marx-Stoelting et al., 2020), so the remainder of this minireview will focus on presenting the main approaches, some novel compounds identified, and difficulties encountered in these studies.

Many drug candidates and insecticides show induction of CYP2B isoforms, liver hypertrophy, and tumor formation in rodents that are associated with CAR activation (Lake, 2018; Bae et al., 2021). To evaluate their relevance for humans, comparative P450 mRNA induction studies in wild-type, CAR-null, or humanized mice are first conducted. This is often followed either by reporter gene assays or RNA interference in rodent and primate/human hepatic cells to evaluate participation of CAR in this process, as recently shown for e.g., the synthetic pyrethroid momfluorothrin and the antidepressant drug candidate basimglurant (Okuda et al., 2017; Nudischer et al., 2020). Liver hyperplasia and increased DNA synthesis are not usually detected in human-derived systems while P450s are induced.

Recent studies have shown the power of high-throughput tiered assay platforms to identify novel CAR modulators. First, screening of

the Tox21 10K library in 1536-well format with a stable CYP2B6-human CAR HepG2 cell line and suppression of the basal CAR activity with 0.75 μM PK11195 yielded ∼15% active CAR activators, 8% inconclusive, and 78% inactive compounds (Lynch et al., 2019). Twenty-four most-potent compounds were tested for PXR activation, and eight CAR agonists were carried forward to studies of CYP2B6 mRNA inducibility in human primary hepatocytes. Among these, neticonazole, diphenamid, phenothrin, and rimcazole increased CYP2B6 expression and translocated tagged human CAR into the nuclear compartment. Although most of the selected CAR agonists were also PXR agonists, rimcazole appeared rather selective for human CAR (Lynch et al., 2019).

Another large screen was done in 293 cells transiently transfected with CAR3 and RXR expression vectors and a CYP3A4 reporter gene. This resulted in 66 hits with at least twofold activation from 2054 compounds (Keminer et al., 2019). Among 10 chosen chemicals, five were previously recognized CAR modulators. It is notable that two retinoids positive in this assay were activators also in the absence of CAR3. This suggests that their activity was dependent on RXR or cellular retinoid acid receptor, highlighting again the need for good controls. In follow-up studies, clemizole, mitotane, and sulconazole translocated CAR1 into nucleus and induced CYP2B6 mRNA in human primary hepatocytes. These compounds promoted SRC1, SRC3, or DRIP205 coactivator binding with CAR1 or CAR3 LBDs to very variable extents, which demonstrates the risks in relying on a single coactivator in two-hybrid assays. Clemizole tended to be the strongest recruiter of coactivators and the only compound that interacted with CAR1 protein in vitro. Again, all three compounds were PXR agonists, indicating the difficulty in finding specific CAR agonists. Moreover, apomorphine and phenelzine that were earlier classified as CAR1 actives (Lynch et al., 2015) turned out to be inactive (Keminer et al., 2019). These discrepancies were assigned to the use of different inverse agonists to suppress the basal activity between these two screening studies.

The nuclear translocation assay (Mackowiak et al., 2019) identified 86 translocation-positive compounds, whereas the overlap with modulators of CAR activity was less than perfect. Only 45% of identified CAR agonists showed translocation, and 58% of translocators were either inconclusive or suppressed the CAR1 activity. A retrospective analysis of literature for CYP2B6 or CYP3A4 mRNA induction indicated that among 34 translocation-positive compounds, 31 displayed P450 induction, whereas about 42% of the translocation-negative chemicals were P450 inducers. These comparisons show that none of the above assays is alone sufficient for reliable identification of CAR ligands, and therefore, robust strategies should be based on tiered reporter gene, nuclear translocation, and other assays.

Cross-activation of PXR is often seen among ligands of CAR discovered by screening programs (Keminer et al., 2019; Lynch et al., 2019), suggesting that PXR counterscreens should be included early in the discovery process. Even though many CAR-activating chemicals can activate multiple NRs (Küblbeck et al., 2020), it must be noted that the candidate CAR ligands have been tested for PXR and aryl hydrocarbon receptor only (e.g., Küblbeck et al., 2011a; Smutny et al., 2016; Liang et al., 2019; Marx-Stoelting et al., 2020) and only rarely for activation of other NRs (Maglich et al., 2003; Toporova et al., 2020) or with CAR from other species (Kublbeck et al., 2016; Pinne et al., 2016).

Metabolism of the test compounds in the cells can create assignment problems. For instance, the antibacterial triclosan was first reported as an inverse agonist of CAR1 and a weak agonist of CAR3 in full-length NR reporter gene assays (Paul et al., 2013). It proved to be an agonist in full-length mouse CAR, inactive in GAL4-mouse CAR LBD reporter gene assays but able to induce CYP2B10 mRNA in wild-type but not in CAR-null mouse livers (Yueh et al., 2014). Later studies showed that triclosan is actually metabolized in hepatocytes to a more lipophilic and potent CAR agonist than the parent compound (Ashrap et al., 2017). Another example is the finding that the CAR inverse agonist PK11195 induces CYP2B6 in primary human hepatocytes. This could be rationalized by the fact that many CAR inverse agonists including PK11195 are also PXR agonists (Küblbeck et al., 2011b). However, PK11195 can activate CYP2B6 expression in PXR-knockout HepaRG cells, which was explained by CYP3A4-catalyzed N-demethylation of PK1195 to a potent CAR agonist (Mackowiak et al., 2017). In summary, the lack or presence of metabolism may mask or change induction potential of the test compounds.

Finally, we do not yet have a test system that captures all or most aspects of indirect CAR activation. So far, a small number of compounds such as PB, flavonoids, chlordane, trans-nonachlor, and two polychlorinated biphenyls have been shown to act by competitive inhibitory binding of the EGF receptor (Table 2; Mutoh et al., 2013; Carazo Fernández et al., 2015; Mackowiak and Wang, 2016; Hardesty et al., 2018). In addition, inhibition of EGF signaling can also take place via inhibition of the EGF receptor kinase or its downstream mediators. Mechanisms additional to EGF receptor inhibition can also take place as shown by teriflunomide-elicited upregulation (Carazo et al., 2018) and EGF-mediated repression of CAR expression (de Boussac et al., 2018). There are reports in which both CAR and its P450 target expressions are increased (Pascussi et al., 2000; Ayed-Boussema et al., 2012; Toporova and Balaguer, 2020), or CAR activity can be decreased (Yang et al., 2014) in response to xenobiotic exposure. Recently, 3D cultures of HepG2 cells were reported to retain CAR in the cytoplasm and respond to PB as in primary hepatocytes (Yokobori et al., 2019), perhaps providing a useful system to investigate mechanisms of indirect

TABLE 4

Key methods, problems, and potential solutions in identification of CAR modulators

Typical Assay	Problems	Potential Solutions
CAR reporter gene assaysand two-hybrid assays	High basal activity	Useful for inverse agonists; agonist detection requires addition of an inverse agonist, use of a CAR variant, or a cell line with low basal activity
	Dynamic range	Selection of cell line; choice of positive controls; selection of coregulators in two-hybrid systems
	Off-target effects	Screen for inhibition of reporter enzyme, cell toxicity, counterscreen for PXR activation due to common target genes
RNA expression assays inhepatic cells or liver tissues	Off-target effects	Measure responses of CAR (CYP2B) and PXR (CYP3A) target genes; check for effects on CAR expression and phosphorylation; verification in wild-type and knock-out cells or humanized animals; use of CAR inverse agonists is problematic as they are often PXR agonists
All assays	Reproducibility	Positive and negative controls, repeated measurement; formal validation of the assay recommended
	Solubility	Solubility measurements in appropriate medium/buffer, increase vehicle content in cell-free systems

CAR activation. Finally, changes in CAR phosphorylation have not been often assessed. There is much to be learned about CAR phosphorylation at sites other than the residue T38 and especially about how CAR activity could be regulated by phosphorylation in the nucleus (Negishi et al., 2020).

In conclusion, searching for CAR ligands has become a highly complicated process extending beyond the simple agonist-elicited receptor activation. Multiple signaling pathways intersect at CAR. Therefore, their accurate evaluation requires several complementary assays and control experiments to exclude off-target effects, highlight ligand-specific coactivator recruitment, or detect changes in CAR phosphorylation status (Table 4).

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