Review

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An update on the constitutive androstane receptor (CAR)

Abstract: The constitutive androstane receptor (CAR; NR1I3) has emerged as one of the main drug- and xenobiotic-sensitive transcriptional regulators. It has a major effect on the expression of several oxidative and conjugative enzymes and transporters, and hence, CAR can contribute to drug/drug interactions. Novel functions for CAR are also emerging: it is able to modulate the metabolic fate of glucose, lipids, and bile acids, and it is also involved in cell-cell communication, regulation of the cell cycle, and chemical carcinogenesis. Here, we will review the recent information available on CAR and its target gene expression, its interactions with partner proteins and mechanisms of action, interindividual and species variation, and current advances in CAR ligand selectivity and methods used in interrogation of its ligands.

Keywords: constitutive androstane receptor (CAR); CYP expression; in vitro assays; ligand-binding domain; ligand specificity; nuclear receptor.

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Introduction

During the past 15 years, the constitutive androstane receptor (CAR; NR1I3) has been established as a key drug- and xenobiotic-sensitive regulator of oxidative and conjugative enzymes and transporters important for drug metabolism, disposition, and drug interactions. Searching the PubMed database in January 2013 with the phrase "constitutive androstane receptor OR nr1i3" yields over 860 publications, and a wealth of information on CAR and its sister, the pregnane X receptor (PXR; NR1I2), has been compiled in excellent reviews [1–24] listed in Table 1. We advise the readers to consult these reviews for details, and we will highlight only most relevant and recent findings here.

Brief history

CAR, PXR, and the vitamin D receptor (VDR; NR1I1) form the nuclear receptor (NR) subfamily 1, group I. In mid-1990s, human and mouse CAR were identified as constitutively active NRs potentially modulating retinoic acid signaling, but the actual target genes of CAR were unknown at that time [25, 26]. Studies on phenobarbital (PB)-inducible expression of rodent cytochrome P450 (CYP) *2B* genes [27, 28] led to the identification of PB-responsive DNA elements mediating the response to several classes of xenobiotics [29] and of CAR as the key factor interacting with these elements [30]. A string of studies in the early 2000s showed the following: *CYP2B* genes in *CAR* null mice were unresponsive to PB-type inducers; the formation of reactive metabolites from liver toxins was drastically modulated; and liver hypertrophy and tumor promotion linked with PB exposure were absent [31–34]. Efforts during the past decade have shown that diverse chemical classes such as pesticides, fire retardants, environmental contaminants, drugs, and industrial chemicals can activate mammalian CAR receptors, albeit with species-specific effects [2, 4, 35]. These findings reinforce the role of CAR as a crucial sensor for xenobiotics, and some insights into the molecular basis of xenobiotic recognition have been made [22]. CAR is also important for the endobiotic metabolism of steroids, bile acids, vitamin D, thyroid hormone, and bilirubin [21], and evidence shows [36, 37] a disruption of the cellular homeostasis by the inappropriate activation of CAR due to xenobiotic exposure. Experiments in the past 5 years have revealed that CAR is actively controlling hepatic glucose and lipid metabolism, with CAR agonism producing beneficial effects in animal models of obesity and insulin

Table 1 Selected review articles on CAR.

Focus area of the review	References
General reviews on CAR and its function	Honkakoski et al., 2003 [1] Stanley et al., 2006 [2] Timsit and Negishi, 2007 [3] di Masi et al., 2009 [4]
Evolution and species differences	Reschly and Krasowski, 2006 [5] Graham and Lake, 2008 [6]
Human pharmacogenetics	Lamba et al., 2005 [7] Lamba, 2008 [8]
Target genes in phase I and II drug metabolism and transport	Tirona and Kim, 2005 [9] Zhou et al., 2005 [10] Tolson and Wang, 2010 [11] Staudinger et al., 2010 [12] Higgins and Hayes, 2011 [13] Chai et al., 2013 [14]
Cross talk and mechanisms of action	Swales and Negishi, 2004 [15] Pascussi et al., 2008 [16] Li and Wang, 2010 [17] Chai et al., 2013 [14]
Role in energy (glucose and lipid) metabolism	Moreau et al., 2008 [18] Wada et al., 2009 [19] Gao and Xie, 2012 [20] Chai et al., 2013 [14]
Role in metabolism of bilirubin and bile acids	Wagner et al., 2010 [21]
CAR ligands, activators, and associated in silico and in vitro methodology	Poso and Honkakoski, 2006 [22] Raucy and Lasker, 2010 [23]
Hepatocarcinogenesis in human and animal models	Köhle et al., 2008 [24]

resistance [20]. The role of CAR in chemical carcinogenesis and hepatic proliferation in rodents is currently under intense research [24, 38, 39], but its significance for humans is uncertain. The discovery and subsequent characterization of PXR (as cited in a review by Chai et al. [14]) during the same time revealed that both receptors have a crucial role in regulation of drug metabolism and disposition. However, the elucidation of CAR- and PXR-mediated signaling is very complex due to overlapping CAR and PXR ligand specificities and target gene profiles and to the intricate cross talk with other transcription factors (TFs) such as hepatocyte nuclear factor (HNF) 4α, cAMP response element-binding protein, and the family of forkhead box (Fox) proteins [14, 40]. An additional complexity arises from the fact that CAR appears to be activated by some CYP inducers such as PB indirectly via a cytoplasmic dephosphorylation-dependent mechanism, culminating in nuclear translocation of CAR [15]. Exciting results on the physiological functions of CAR are expected because knowledge of CAR properties and its connections with other cellular processes is being accumulated.

Structural features of the NR CAR

Crystal structures of agonist-bound CAR

Three crystal structures of mouse or human CAR agonist-bound ligand-binding domains (LBDs) (Table 2) conform to the standard three-layer sandwich architecture seen in other NRs [44]. The CAR LBDs contain 11 α-helices and 3 short β-strands, and helices 2 and 2′ assume the 3_{10} conformation [41, 42] (Figure 1A). The unique structural features for CAR LBD include an additional helix called "X" between helices 11 and 12 and an unusually short helix 12 (Figure 1A). The helix X is also present in VDR [45], retinoid-related orphan receptor (ROR) β [46], and RORα [47], but the linker between helices X and 12 appears to be more rigid in constitutively active RORs and CAR. The short helix 12 is stabilized by interactions with a lysine residue in helix 4 (K195 in human CAR) and intrahelical H-bonds [42] (Figure 1B), contributing in part to the constitutive activity. The two short 3_{10} helices 2 and 2′ appear to form a ligand entry point as postulated for the peroxisome proliferator-activated receptor (PPAR) α [43, 48]. Similarly to other NRs, the CAR ligand-binding pocket (LBP) is made up by about 30 residues in helices 2–7 and 10 and in β-sheets 3 and 4 that form a mostly apolar lining of the

Table 2 The human and mouse CAR LBD crystal structures.

PDB ID	Protein molecules	Co-crystallized ligands	Co-regulator peptide	Resolution, Å	Completeness, %	References
1XVP	hCAR hRXRα	CITCO Pentadecanoic acid	SRC1	40.0–2.60	86.3	Xu et al., 2004 [41]
1XV9	hCAR hRXRα	5β-Pregnanedione C16–C18 fatty acids	SRC1	40.0–2.70	86.4	Xu et al., 2004 [41]
1XLS	mCAR hRXRα	TCPOBOP 9-*cis*-Retinoic acid	TIF2	20.0–2.95	93.2	Suino et al., 2004 [42]
1XNX	mCAR	Androsten-3α-ol	None	30.0–2.90	99.8	Shan et al., 2004 [43]

CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4 dichlorobenzyl)oxime; TCPOBOP, 1,4-bis-[(3,5-dichloropyridyl) oxy]benzene; SRC1, steroid receptor co-activator 1; TIF2, transcriptional intermediary factor 2.

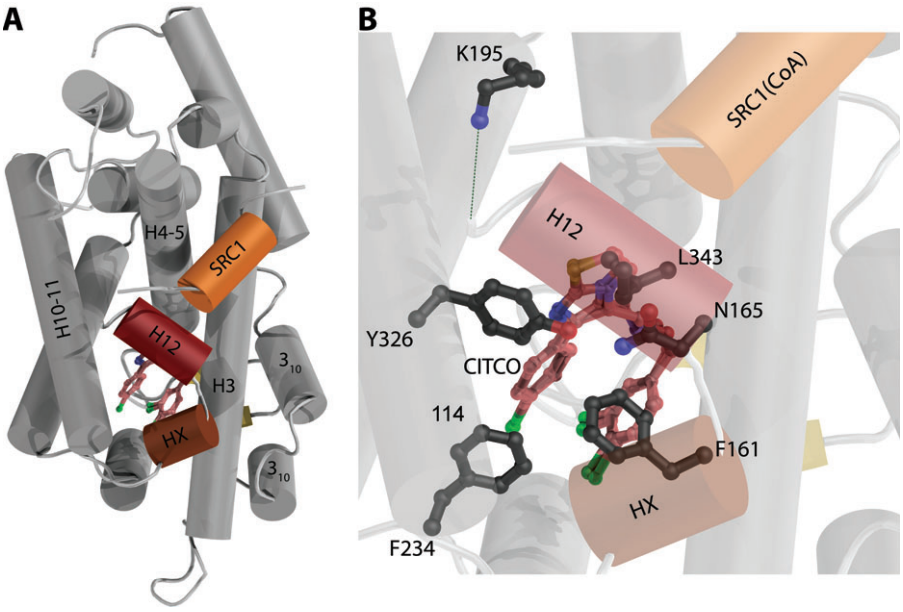

Figure 1 The crystal structure of the human CAR complexed to CITCO.
(A) Overall view on the whole ligand-protein complex with highlighted helices discussed in the text. (B) Detailed view on the LBP with some of the residues displayed that are discussed in the text. The interaction of the K195 with the terminal part of the H12 is schematically depicted with green dashed line. The important features are illustrated in color. The co-activator peptide bearing the LXXLL motif derived from steroid receptor co-activator 1 (SRC1/NCOA1) is in orange, helix 12 (H12) is in red, and helix X (HX) is in brown.

pocket, although two hydrophilic patches may allow the formation of hydrogen bonds with the ligands. The LBP volumes of CAR range from 525 to 675 Å^3, placing them in size between the classical steroid receptors and PXR. Although the co-crystallized ligands are structurally different, they use the hydrophobic character of the cavities and hydrogen bonds that are formed toward the polar residues to orient the ligand. In mouse CAR, none of the ligands makes a direct hydrogen bond contact with helix 12, but 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) forms a number of hydrophobic interactions with helix 12 (L353) and the linker helix (L346, T350). Because these interactions contribute toward the stabilization of helix 12, they may be responsible for the "superagonistic" properties of this ligand. In human CAR, co-crystallized ligands do not form direct contacts with helix 12. The closest residue is L343, which is positioned at a distance of 4.9 Å from the C21 of 5β-pregnane-3,20-dione and 3.9 Å from the thiazole ring of 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO). The barrier formed by residues of F161, N165, F234, and Y326 excludes the possibility of a direct interaction between the ligand and helix 12 [41, 42, 49] (Figure 1B). The structure of mouse CAR co-crystallized with the inverse agonist androstenol indicates a structural change in helices 10 and 11, which resembles the inactive *apo* forms of NRs [43]. However, the lack of corepressor peptide in this structure and the unavailability of the ligand-free CAR crystals preclude further speculation on the mechanisms of (inverse) agonism.

Molecular modeling studies

Before the crystal structures for CAR LBD became available in 2004, structural features were analyzed by creating homology models [22]. The selection of the template had a substantial effect on the modeled LBP volume and residue orientation, as exemplified by the early excessively large LBP volume estimates [50] and the relatively accurate prediction of the LBP performed later [49]. Prediction of the protein flexibility is based on molecular dynamics (MD) simulations [51]. Such studies have yielded information on the basis of constitutive and agonist-induced activity of CAR [51–53], and recently, on the probable mechanism of inverse agonist-induced binding of NR corepressors [54]. Due to the limited number of agonist-bound CAR crystal structures, information on binding of novel agonists must be acquired by docking studies and supported by, e.g., site-directed mutagenesis. The advances in speed and incorporation of protein flexibility in docking programs have enabled more detailed analysis of ligand binding [54–57]. Due to the promiscuity for diverse ligands and the inherent flexibility of CAR, the building of pharmacophore/

quantitative structure-activity relationship models remains problematic [22]. For a limited set of structurally similar ligands, the pharmacophore alignment has been possible [58–60], but for dissimilar ligands, an alignment based on docking is almost a necessity [61–63].

Interspecies and interindividual differences

Evolution and species differences

Invertebrates have a single protein orthologous to *NR1I* genes that does not seem to respond to known xenobiotics [64]. In birds, the sole xenosensor appears to share both CAR and PXR sequence similarity and ligand-binding properties [65], and similarly, fish and *Caenorhabditis elegans* possess a single *NR1I* gene [66, 67]. The previous notion that CAR evolved through gene duplication of a single *CAR*/*PXR* ancestral gene has been challenged by a new view that all *NR1I* genes result from whole genome duplication [68]. This theory is supported by recent analysis showing that *PXR/CAR* duplication took place after the split of tunicates and vertebrates but before that of fish and land vertebrates [69]. In contrast to *PXR*, *CAR* genes are not found in the fish lineages but are conserved in all land vertebrates, including amphibians. Functionally, mammalians use both PXR and CAR as xenosensors, whereas in nonmammalian land vertebrates, CAR may be the predominant xenosensing receptor [69].

The sequence comparisons among NR1I members indicate that both *CAR* and *PXR* genes have been under positive selection [70], presumably due to exposure to different diet-derived xenobiotics. This divergent evolution may explain the wide species differences in CYP induction and/or CAR activation profiles, even though the basic mechanism of receptor activation is well conserved. The sequence similarity between the mouse and human CAR LBDs is only 72%, in contrast to more than 90% similarity in steroid hormone receptors [5, 71]. Changes in the LBD residues contribute to the different sizes, contours, and contact points with the ligands between the mouse and human CAR LBPs. For examples, residues F171, N175, F244, and Y336 forming the "barrier" in mouse CAR do not appear to restrict the ligand projecting toward helix 12 as much as the corresponding residues in human CAR do, enabling a direct contact between the mouse-specific agonist TCPOBOP with helix 12 [41–43]. Second, mutagenesis studies have identified key residues that dictate the species-specific response to 17α-ethinylestradiol, an inverse agonist for human CAR and a partial agonist for mouse CAR (F243) and for TCPOBOP (M340). Third, species differences exist in residues at positions critical for human CAR function [49]. However, the role of these amino acid differences and extent for species-specific ligand-dependent activation remains enigmatic because CAR has not been cloned and/or systematically characterized from many other species relevant for drug development such as the rat or the dog [72, 73].

Genetic variation in the human CAR

Exons 2 and 3 and part of exon 4 encode the DNA-binding domain (DBD) and the hinge regions, whereas the LBD is encoded by the rest of exon 4 and exons 5–9. Alternative splicing has been shown to produce at least 26 splicing variants, many of which contain a premature stop codon or code for a variant protein [74, 75] and thus heavily influence expression of functional CAR [76]. The most important isoforms are termed CAR1 (wild type), CAR2 (insertion of SPTV, near LBP), and CAR3 (insertion of APYLT in the LBD/heterodimerization region) [7, 77, 78]. Although CAR1 has a high basal activity, splice variants CAR2 and CAR3 display low constitutive activity. Due to the changes in the LBD structures, it is not surprising that some differences in ligand activation have been reported between the wild-type and CAR2/CAR3 isoforms [60, 79]. Of note, similar splice variants are not present in experimental animals. At least 30 single-nucleotide polymorphisms (SNPs) have been identified [8, 80], albeit at a low frequency (<2%) in major populations. All five known nonsynonymous SNPs are located in the LBD, and two of them disrupt CAR function: H246A was inactive, whereas L380P had a reduced basal but normal CITCO-elicited *CYP3A4* reporter activity [81]. There is some recent evidence of CAR polymorphisms being associated with exposure to efavirenz, a selective substrate for human CYP2B6 [82, 83]. However, the effects of more frequent polymorphism in the CAR targets such as *CYP2B6* [84] may mask the relevance of CAR polymorphisms.

Regulation of CAR levels and activity

CAR expression

The *CAR* gene is expressed in tissues with high capacity for drug metabolism such as liver and intestine derived from the endoderm. The key regulator in such cells is

the HNF4α, which recognizes a conserved element in the proximal *CAR* gene promoter [85, 86]. Different isoforms of HNF4α appear to either activate (isoform 1) or suppress (isoform 7) the expression of CAR in a co-activator-dependent manner [86]. The integration of CAR to many physiological processes controlled by other NRs gains support from the findings that CAR expression and/or CYP inducibility is increased by the glucocorticoid receptor [87] and the retinoic acid receptor [88]. CAR expression is also activated by PXR agonists (e.g., PCN, dexamethasone [87, 89]), potentially by peroxisome proliferators (e.g., fibrates [88]) and is dependent on thyroid hormones [90]. The discovery of serum response elements in the CAR promoter [91] provides a link to stress-activated protein kinase pathways via the binding of the ETS domain-containing protein Elk-1. This finding may explain why many growth factors and the presence of serum inhibit PB-inducible CYP expression in several experimental settings [92] and why the dephosphorylation of CAR is associated with its nuclear translocation [93]. Finally, CAR is under the control of the circadian clock-related PAR-domain basic leucine zipper TFs such as albumin gene D-site-binding protein, thyrotroph embryonic factor, and hepatic leukemia factor [94].

Cytoplasmic CAR interactions

Groundbreaking work from the Negishi Laboratory showed that CAR is complexed with heat shock protein 90 and a retaining CCRP protein in the liver cytoplasm in unexposed animals [95] and that PB exposure leads to nuclear translocation of CAR and to target gene activation. The translocation process is influenced by phosphorylation status, with phosphorylation by extracellular signal-regulated kinase 1/2 and protein kinase C affecting the DBD (T38 in CAR) and retaining inactive CAR in the cytoplasm [93, 96], whereas dephosphorylation by a protein phosphatase 1β (PP1β) and protein phosphatase 2A (PP2A) [97] enhances nuclear translocation of active CAR [98]. Also, AMP-activated protein kinase (AMPK) has been shown to be involved in the induction of CYPs by PB [99]. Although CAR itself is not phosphorylated by AMPK, this kinase seems to affect p300 and PPARγ co-activator (PGC) 1α, suggesting a possible mechanism for the observed liver kinase B1/AMPK cascade activation by indirect inducers, such as PB. These interactions are important as they link CAR activation to other signal pathways activated by, e.g., stress and cell proliferation pathways. Indeed, cell cycle proteins have been identified as CAR targets [100, 101]. CAR is required for chemically induced liver growth [31] and signaling via phosphorylation has long been known to affect CYP inducibility [102, 103].

PPP1R16A, the membrane subunit of PP1β, facilitates the ligand-independent translocation of CAR into the nucleus, indicating a novel mechanism for the translocation of NRs in which ligands and other receptors are not involved [98]. However, the translocation effect is more enhanced in the presence of PB. Given the fact that exposure to PB decreases hepatic cell-cell communication by affecting the activity and levels of connexins [104, 105], it is likely that novel cytoplasmic interactions of CAR remain to be identified.

Interactions of CAR with DNA and nuclear partner proteins

Specificity of DNA binding

Many of the CAR target genes have been listed in earlier reviews (Table 1). They include the established genes of enzymes of phase I and II biotransformation, uptake, and efflux transporters (Table 3), but new targets continue to emerge in genes responsible for endobiotic metabolism and cell cycle control [9–13]. The initially identified binding site for CAR/RXR heterodimer was a direct repeat 5 (DR5; two AGGTCA-related hexamers separated by five nucleotides) in retinoic acid-sensitive gene promoters [25]. Later studies indicated that most efficacious PB-responsive enhancers consist of clusters of DR4 elements in vicinity of other TF-binding sites in, e.g., *CYP2B* and *UGT1A1* genes [30, 106]. In addition, CAR is also able to transactivate and/or bind the PXR-responsive DR3 and everted repeat (ER) 6 elements present in the proximal and distal regions of the *CYP3A* genes [107, 108] as well as the PPAR-responsive DR1 elements [109–111]. Experiments with in vitro-translated proteins have indicated that the CAR/RXR heterodimer prefers DR4 over DR5, whereas ER6–ER9 elements are recognized and DR1/3 show little binding [29, 112]. CAR has been shown to bind DNA as a monomer in human *UGT1A1* and *MDR1* promoter elements and to be activated by ligands, which may point to a physiological role also for CAR monomers [112, 113]. Intriguingly, two nucleotides at the 5′ flank of each hexamer motif appear to influence the binding of CAR/RXR or CAR monomer by up to 20-fold [112].

The lack of high-quality antibodies for CAR has precluded the assessment of true in vivo binding sites by chromatin immunoprecipitation, and selection of

Table 3 CAR target genes.

Target gene	Gene symbol[a]	Species
Phase I		
Aldehyde dehydrogenases	*Aldh1a1, 1a7*	*M. musculus*
Cytochrome P450s	*CYP2A6, 2B6, 2B10, 2C9, 2C19, 3A4, 3A11*	*H. sapiens*
	Cyp1a1, 2a4, 2b10, 3a11	*M. musculus*
	Cyp2b1, 2b2, 2c6, 2c7, 3a1	*R. norvegicus*
P450 (cytochrome) oxidoreductase	*Por*	*M. musculus*
Phase II		
Glutathione *S*-transferases	*Gsta1, a2, a3, m1, m2*	*M. musculus*
	Gsta1, a2, a3, m1	*R. norvegicus*
Sulfotransferases	*Sult1a1, 2a1, 2a9*	*M. musculus*
UDP-glucuronosyltransferases	*UGT1A1*	*H. sapiens*
	Ugt1a1	*M. musculus*
	Ugt1b2	*R. norvegicus*
Phase III		
ATP-binding cassette family	*ABCB1, C2, C3*	*H. sapiens*
	Abcb1a, c1, c2, c3, c4	*M. musculus*
	Abcc2	*R. norvegicus*
Solute carrier transporters	*Slco2a1*	*M. musculus*
		R. norvegicus

Data were compiled from di Masi et al. [4] and Tirona and Kim [9]. *M. musculus, Mus musculus; H. sapiens, Homo sapiens; R. norvegicus, Rattus norvegicus*. [a]Approved by the HUGO Gene Nomenclature Committee (http://www.genenames.org/).

random DNA sites by either in vitro amplification or yeast genetics to identify CAR-binding sites has not been performed. This suggests that we do not yet have a full view of DNA-binding specificity by CAR, whereas gene expression studies (with selective NR ligands, delivery of siRNA or knockout animals) cannot distinguish between direct DNA binding-mediated gene activation from responses that are either secondary or mediated by protein/protein interactions.

Interactions with the NR co-regulators

The interaction partners of CAR are summarized in Table 4. Most NR co-activators (CoAs) contain one or more NR interaction boxes, bearing a short peptide motif LXXLL, where L is leucine and X is any amino acid [114]. The anchoring salt bridges at the ends of this motif help orient it properly in the surface groove on the NR LBD, whereas the leucines provide numerous van der Waals interactions with the hydrophobic residues located in LBD helices 3, 4–5, and 12 [42]. The only structural information for CAR/CoA interactions is derived from the steroid receptor co-activator (SRC) family members NCOA1 (SRC1) and NCOA2 [transcriptional intermediary factor 2 (TIF2)] [41, 42]. Many CoAs share characteristic enzymatic activities such as histone acetyltransferase activity, which targets histones or other proteins at NR-regulated gene promoters for acetylation, which can enhance the transcriptional activity [115].

CAR has been shown to physically interact with all three members of the SRC family co-activators SRC1 [41, 49], TIF2 [34], and NCOA3 (receptor-associated cofactor 3, RAC3) [116] in vitro. Studies in cellular models indicate that all three co-activators are redundant with regard to enhancing CAR-mediated induction of CYP genes. However, only NCOA3 is able to enhance CAR transactivation in hepatic cells [38, 117]. Although CAR interacts with another NR co-activator NCOA6 [118], its liver-specific deletion does not interfere with the regulation of CAR target genes [119]. However, similar tissue-specific disruption of mediator of RNA polymerase II transcription subunit 1 (MED1) resulted in the near abrogation of TCPOBOP-activated gene expression and acetaminophen-induced hepatotoxicity [120]. It has also been shown that MED1 but not NCOA6 is required for nuclear translocation of CAR in mouse liver [121]. The critical effect of MED1 on CAR-mediated signaling could be anticipated from the fact that MED1 is a key component of the mediator complex, which essential for transcriptional activation via a variety of TFs [122].

The PPARα-interacting cofactor (PRIC) complex component, PRIC320, associates with CAR in ligand-independent and ligand-dependent manner in vitro [123], but the physiological consequences of this interaction have not been explored further. The discovery of interaction between

Table 4 List of CAR-interacting proteins.

CAR-interacting protein		Group/function
Full name	**Gene symbol[a]**	
Steroid receptor co-activator 1 (SRC1)	*NCOA1*	p160 Co-activator
Transcriptional intermediary factor 2 (TIF2)	*NCOA2*	p160 Co-activator
Receptor-associated co-activator 3 (RAC3)	*NCOA3*	p160 Co-activator
Activating signal co-integrator 2 (ASC2)	*NCOA6*	General NR co-activator
PPAR-binding protein (PBP)	*MED1*	Mediator TRIP/TRAP co-activator
PPARα-interacting cofactor 320 (PRIC320)	*CHD9*	General transcription machinery-interacting protein
PPARγ co-activator 1α (PGC-1α)	*PPARGC1A*	General NR co-activator
Forkhead box O1 (FoxO1)	*FOXO1*	Metabolic transcriptional factor
Growth arrest and DNA damage-inducible 45β (Gadd45β)	*GADD45B*	Cell cycle-regulating factor
Protein phosphatase 1 regulatory subunit 16A (PPP1R16A)	*PPP1R16A*	Regulator of signal transduction
Splicing factor 3a, subunit 3, 60 kDa (SF3a)	*SF3A3*	Splicing/inhibitor of CAR signaling
Nuclear receptor corepressor (NCoR)	*NCOR1*	General NR corepressor
Silencing mediator for retinoid or thyroid hormone receptors (SMRT)	*NCOR2*	General NR corepressor

For references, see the section Interactions of CAR with other nuclear proteins. [a]Approved by the HUGO Gene Nomenclature Committee (http://www.genenames.org/).

CAR and PGC-1α again highlights the connections among energy metabolism and detoxification [124]. Later in vivo studies using knockout animals demonstrated that fasting upregulates CAR expression and ligand-independent CAR activity that involves the interaction with PGC-1α [85].

The interaction of CAR with prototypic NR corepressors NCoR (NCOR1) and silencing mediator of retinoid or thyroid hormone receptors (SMRT; NCOR2) in vitro explains the mechanism of inverse agonist suppression of CAR activity [57, 125, 126]. Ex vivo, an association of CAR with SMRT on *CYP24A1* gene promoter has been reported, thus mediating cross talk with VDR signaling [127].

Interactions of CAR with other nuclear proteins

Additional interaction partners of CAR are listed in Table 4. In analogy to most NRs, CAR makes heterodimers with retinoid X receptor (RXR) isotypes [30, 112]; thus, the lack of RXRα reduces expression of CAR target genes. In addition to this natural partner, CAR has been reported to interact with by small heterodimer partner (SHP, NR0B1) and NR0B2, resulting in the suppression of CAR activity and target gene expression [128, 129]. SMRT and NCoR can inhibit CAR-mediated signaling independent of SHP, demonstrating that they may bind to distinct sites [128]. The recently identified SHP-interacting leucine zipper protein (SMILE) [130] is able to interact with CAR, competing with co-activators TIF2 and PGC-1α in vitro and in vivo [131].

In vitro and cell-based assays have shown that CAR interacts directly with FoxO1 and represses FoxO1-mediated transcription of the insulin-responsive phosphoenolpyruvate carboxykinase 1 (*PEPCK1*) and glucose 6-phosphatase (*G6Pase*) promoters [32, 40]. These findings provide a mechanistic basis to following observations: long-term treatment with PB is known to decrease plasma glucose levels, improve insulin sensitivity in diabetic patients [132], and repress rodent *PEPCK1* and *G6Pase* [32, 133]. In lipogenesis, CAR counters the effect of PXR by suppressing lipogenic genes such as sterol regulatory element-binding protein 1C and fatty acid synthase [134]. Therefore, CAR is able to modulate glucose and lipid metabolism, and its activators may be potential candidate drugs for hepatobiliary and metabolic diseases.

Gadd45β is a growth arrest- and DNA damage-inducible protein that interacts with CAR in a ligand-dependent way and enhances liver growth in mice. The administration of TCPOBOP in mice results in drug-induced hyperplasia, which is associated with dramatic and rapid hepatocyte growth [135]. Although the proliferation seems to be intact in *Gadd45b* null mice, the hepatic growth is delayed and the early transcriptional stimulation of CAR target genes is weaker [39]. Another CAR partner, a component of the splicing factor 3a, has been identified via yeast two-hybrid screening and confirmed in other interaction assays [136].

CAR ligands and associated methods

Variability of CAR ligands

Only few selective CAR agonists and inverse agonists are currently known because many reported ligands have turned out to modulate other NRs or TFs, hampering their use as tools to interrogate CAR biology. Examples of this low selectivity include many drugs, pesticides, and polychlorinated biphenyls (CAR and PXR), phthalates (CAR and PPAR), estrogens (CAR and estrogen receptor, albeit at different affinities), and oltipraz (CAR and nuclear factor erythroid 2-related factor).

Meanwhile, the list of CAR-activating chemicals is rapidly expanding (Table 5), including steroids [144], natural compounds [145], pesticides [139], industrial chemicals [146], drugs [62, 63], and various synthetic compounds including thiazolidin-4-ones, sulfoamides [59], and flexible diaryl compounds [54, 56]. The activity of CAR is also thought to be modulated by the so-called indirect activators (acetaminophen, bilirubin, 6,7-dimethylsculetin, PB, and phenytoin) that stimulate the nuclear translocation of CAR and the expression of its target genes but without binding directly to the LBD [15, 147]. However, at least for phenytoin and PB, this view has been challenged because assays with natural CAR or its variants have shown increased reporter activity by these compounds [56, 79, 148–150].

The inverse agonists bind the CAR LBD and cause a reduction in CAR transcriptional activity due to the recruitment of corepressors. These include different steroids, the isoquinoline carboxamide PK11195 [143] and the novel compound 1-[(2-methylbenzofuran-3-yl)methyl]-3-(thiophen-2-ylmethyl) urea (S07662) [54, 57]. In some cases, reports on ligand binding and ligand-elicited CAR activation are controversial such as for clotrimazole [58, 138] and meclizine [57, 140]. This might be due to different cell lines with variable co-regulator contents used in the studies. The activation of CAR can be also decreased or increased by retinoid-like substances, but the mechanisms remain unknown [151, 152].

Assays to discover novel ligands

One significant reason behind this expansion of CAR ligands has been the development of assays for the measurement of ligand-dependent CAR activation and/or interaction. Most commonly, various reporter gene assays measure the activation of human CAR and thus indirectly assess CAR/ligand interaction [56, 57, 59, 61, 141, 142, 145, 153]. Naturally occurring splice variants (CAR3) or mutated CAR LBDs are suggested to improve the assay sensitivity due to the lower basal activity of the modified receptor [73, 79, 146, 154]. Another approach to lower the basal activity has been the addition of a CAR inverse agonist [55, 60, 108]. Recently, a careful selection of the cell line used for transfection has made it possible to use the wild-type human CAR without any modification to the LBD structure or the addition of any inverse agonists [56, 57]. The mammalian two-hybrid assay measures the ligand-dependent interaction of CAR with a selected co-regulator peptide. This assay appears to be more sensitive in identifying weak or partial agonists that may elicit both co-activator and corepressor recruitment and very useful in dissecting the co-regulator profile of human CAR [57] and to gain support for human CAR/ligand interactions [35, 63].

Similar CAR/co-regulator assays, which resulted in the identification of the potent agonist CITCO, can be designed for in vitro screening [138]. An LBD assembly assay, originally described by Pissios et al. [155] for mouse CAR, is also useful in identifying novel human CAR ligands [62, 156, 157]. More recently, surface plasmon resonance has been utilized in the identification of novel ligands and species-specificity studies on human CAR [62, 137]. Here, a solution with CAR LBD protein and increasing concentrations of agonist is flushed over the surface bound by a co-activator peptide, and the resulting optical change of the surface is then monitored. Because the detection measures any binding reactions taking place on the surface, it must be carefully controlled for and verified for dependency on the CAR LBD using, e.g., a mutated CAR.

Because the CAR resides in the hepatocyte cytoplasm in the absence of its activators, the reporter gene measurements have sometimes been complemented with nuclear translocation assays. This requires the transfection of primary hepatocytes with, e.g., constructs encoding yellow fluorescent protein-tagged CAR. The translocation of CAR into the nucleus in response to compound exposure can be monitored by confocal microscopy [23, 158].

The direct assessment of CAR/ligand interactions in biochemical assays in vitro has lagged behind the reporter assays. There is limited evidence that the presence of an agonist increases DNA binding by human CAR/RXR heterodimers [108]. Both agonists and inverse agonists provide increased protection for human CAR

Table 5 Latest additions to human CAR ligands and/or activators.

Compounds	Effect on human CAR	References
Steroids		
Androstan-3α-ol and androsten-3α-ol	IA (h>m)	Dau et al., 2013 [137]
3,17β-Estradiol and 17α-ethinylestradiol	IA (h), A (m)	Dau et al., 2013 [137]
5β-Pregnanedione	A (h), IA (m)	Maglich et al., 2003 [138]
Pesticides		
Pyrethroids (e.g., permethrin, cypermethrin)	A	Küblbeck et al., 2011 [56]
Carbamates (e.g., benfuracarb)	A	Abass et al., 2012 [139]
Organochlorines (e.g., methoxychlor, PCB153, *o,p′*-DDT)	A	Küblbeck et al., 2011 [56]
Drugs		
Clotrimazole	IA or A	Jyrkkärinne et al., 2008 [61] Lynch et al. 2012 [63]
Meclizine	IA or inactive	Huang et al., 2004 [140]
Artemisinin and some derivatives	A	Burk et al., 2012 [62]
Carbamazepine	A	Faucette et al., 2007 [79]
Nevirapine	A	Faucette et al., 2007 [79]
Phenytoin	Activator or A	Küblbeck et al., 2011 [56]
Natural polyphenols		
Food-derived flavonoids (e.g., chrysin)	A	Yao et al., 2011 [141]
Alcohol-derived flavonoids (e.g., ellagic acid)	A	Yao et al., 2011 [141]
Plasticizers		
Triaryl phosphates	A	Jyrkkärinne et al., 2008 [61]
Di(2-ethylhexyl)phthalate	A for hCAR2	DeKeyser et al., 2009 [142]
Synthetic compounds		
CITCO	A	Maglich et al., 2003 [138]
Flexible diaryl compounds (FL81)	A	Küblbeck et al., 2011 [56]
Thiazolidin-4-ones	A	Küblbeck et al., 2008 [59]
Sulfonamides	A	Küblbeck et al., 2008 [59]
A series of chemotypes	A	Li et al., 2008 [143]
PK11195	IA	Küblbeck et al., 2011 [57] Lynch et al., 2012 [63]
S07662	IA	Küblbeck et al., 2011 [57]

A, agonist; IA, inverse agonist; activator, indirect activation, no evidence of direct binding; h, human CAR; m, mouse CAR.

LBD against proteolytic digestion [56, 57]. Displacement of labeled clotrimazole from the CAR LBD by test compounds [159] has the disadvantage that it cannot distinguish between agonists and inverse agonists. Due to the high basal activity and complex activation mechanisms of CAR as well as rather tedious protocols and/or technical issues, these assays are only low-throughput and/or prone to false positives [17].

Future directions

To elucidate the diverse biological functions of human CAR in more detail, we must first develop more potent and selective CAR agonists and inverse agonists. Nevertheless, the combination of molecular modeling and biological assays [57, 59, 63] has proven a very fruitful approach in raising the range and diversity of CAR ligands. It is expected that advances in structural biology, such as the determination of ligand-free and corepressor-bound CAR LBD structures, and in comparative molecular modeling will resolve the frequent problem of PXR activation by many of the currently available CAR ligands. Second, the identification of ligand-dependent CAR/co-regulator and cytoplasmic interactions constitutes an important avenue in deciphering the mechanisms of CAR activation and in helping the identification of novel, primary CAR target genes. This in turn should highlight the role of CAR in processes of liver growth, cell-cell communication, intermediate metabolism, and in discovering new absorption, distribution, metabolism, and excretion (ADME)-related CAR targets in addition to *CYP2B6*. Finally, the development of comprehensive assays for reliable screening of CAR activation will help in the prediction of its in vivo

relevance, in the study of its ramifications in ADME and drug safety research.

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