Identification of a Novel Human Constitutive Androstane Receptor (CAR) Agonist and Its Use in the Identification of CAR Target Genes

Jodi M. Maglich, Derek J. Parks, Linda B. Moore, Jon L. Collins, Bryan Goodwin, Andrew N. Billin, Catherine A. Stoltz, Steven A. Kliewer, Millard H. Lambert, Timothy M. Willson, and John T. Moore†

From Nuclear Receptor Discovery Research, GlaxoSmithKline, Research Triangle Park, North Carolina 27709

The orphan nuclear constitutive androstane receptor (CAR) is proposed to play a central role in the response to xenobiotic stress. Identification of CAR target genes in humans has been limited by the lack of a selective CAR agonist. We report the identification of 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) as a novel human CAR agonist with the following characteristics: (a) potent activity in an in vitro fluorescence-based CAR activation assay; (b) selectivity for CAR over other nuclear receptors, including the xenobiogen pregnane X receptor (PXR); (c) the ability to induce human CAR nuclear translocation; and (d) the ability to induce the prototypical CAR target gene CYP2B6 in primary human hepatocytes. Using primary cultures of human hepatocytes, the effects of CITCO on gene expression were compared with those of the PXR ligand rifampicin. The relative expression of a number of genes encoding proteins involved in various aspects of steroid and xenobiotic metabolism was analyzed. Notably, CAR and PXR activators differentially regulated the expression of several genes, demonstrating that these two nuclear receptors subserve overlapping but distinct biological functions in human hepatocytes.

The nuclear receptors CAR† (NR1I3) and PXR (NR1I2) play key roles in the response to chemical stress (1–4). Both CAR and PXR have been shown to bind to a wide range of structurally unrelated ligands (5–7) and to regulate genes involved in the humoral response to both endobiotic and xenobiotic stress (2–4). Global gene expression profiling has shown that PXR and CAR regulate an overlapping set of genes that encode proteins involved in the detoxification of potentially harmful xenobiogens and endobiogens (8, 9). For example, CAR- and PXR-dependent signaling pathways converge on common response elements in the regulatory regions of a number of genes, notably members of the CYP3A and CYP2B subfamilies of xenobiogenic inducible cytochromes P450 (10–12). Current studies are aimed at broadening our understanding of the biology of these receptors and the genes that they regulate. An important goal is to delineate CAR- and PXR-specific target genes to define their distinct physiological roles.

The identification of target genes for each receptor is facilitated by the availability of potent and selective ligands. PXR has been shown to be activated by a structurally and chemically diverse set of ligands (13). Examples of human PXR activators include the xenobiogens rifampicin and SR12813 (14–17), the endobiogens lithocholic acid (4, 18) and 5β-pregnane-3,20-dione (15), and the botanical hyperforin (19). In human studies, rifampicin has been shown to be a useful chemical tool to define PXR target genes in human hepatocytes (9). Expression studies using rifampicin have shown that PXR activates the expression of a battery of genes involved in the response to xenochemoic and endobiogenic stress, most notably those genes involved in oxidation (phase I enzymes), conjugation (phase II enzymes), and transport (9).

In contrast to PXR, a selective chemical tool has not been available to study the function of CAR in humans. The hepatotogen TCPOBOP is a potent murine CAR ligand that has been used to delineate CAR target genes in mice, but does not activate human CAR (5, 7, 9). The barbiturate phenobarbital activates both human and mouse CARs; however, it does so through an indirect mechanism (7, 20). Thus, although phenobarbital does not bind to the receptor (7), it causes CAR to be translocated from the cytoplasm to the nucleus (20–22). Because CAR exhibits an intrinsically high transcriptional activity, nuclear localization of the receptor results in the activation of target gene expression in the absence of ligand binding (20, 23). The induction of CAR translocation by phenobarbital can be blocked by the phosphatase inhibitor okadaic acid, suggesting that translocation involves a dephosphorylation event (21). The induction of CAR translocation by phenobarbital can be blocked by the phosphatase inhibitor okadaic acid, suggesting that translocation involves a dephosphorylation event (21).

Published, JBC Papers in Press, February 27, 2003, DOI 10.1074/jbc.M300138200

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: GlaxoSmithKline, 5 Moore Dr., V-116B, Research Triangle Park, NC 27709. Tel.: 919-483-3896; Fax: 919-315-6720; E-mail: john.t.moore@gsk.com.

‡ The abbreviations used are: CAR, constitutive androstane receptor; PXR, pregnane X receptor; LBD, ligand-binding domain; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; RTQ, real-time quantitative; VDR, vitamin D receptor; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene.

This paper is available online at http://www.jbc.org

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
tive CAR ligands for both the murine and human receptors will enable direct comparison of their species-specific roles. Through a combination of in vitro and cell-based screening, we have identified an imidazothiazole derivative that is a selective human CAR agonist. This chemical tool has allowed us to unambiguously define CAR target genes. This compound should be a powerful tool in differentiating the role of human CAR and PXR. We have demonstrated the utility of this compound in human hepatocyte studies.

**EXPERIMENTAL PROCEDURES**

*Chemicals—6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzoxime (CITCO) was purchased from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Unless otherwise stated, cell culture reagents were obtained from Invitrogen. Dexamethasone and rifampicin were acquired from Sigma.*

*Fluorescence Resonance Energy Transfer (FRET) Ligand Sensing Assay—The FRET ligand sensing assay was performed by modification of a previously published procedure (26) and is described in Ref. 7. Polyhistidine-tagged human CAR LBD was purified from Escherichia coli as previously described (7).*  

*Cotransfection Assays—CV-1 cells were cotransfected with a CAR expression plasmid in combination with the XREM-CYP3A4-LUC reporter (27) as described previously (7).*  

*Construction of a Green Fluorescent Protein (GFP)-CAR Expression Plasmid—Full-length human CAR cDNA (GenBank™/EBI accession number Z30425) was amplified by PCR using primers with flanking EcoRI and BamHI sites and subsequently inserted into the EcoRI and BamHI sites of pEGFP-C1 (Clontech, Palo Alto, CA), producing pGFP-hCAR. The sequence of the human CAR cDNA was confirmed by sequence analysis.*

*Nuclear Translocation Assay—Primary rat hepatocytes were prepared by perfusion as previously described (28) and plated at a density of ~3 × 10^6 cells/well of a six-well dish in Williams' E medium containing 10% fetal bovine serum, 100 nM dexamethasone, and 1% ITS-G (insulin/transferrin/selenium; Invitrogen). After overnight incubation, cells were transferred to 2 ml of Williams' E medium as described above, but without serum. For each well, 1.6 µg of pGFP-hCAR expression plasmid in 100 µl of Opti-MEM was mixed with 3 µl of LipofectAMINE 2000 (Invitrogen) in 100 µl of Opti-MEM according to the manufacturer's directions and subsequently added directly to the hepatocytes in serum-free medium. After a 4-h incubation, cells were incubated overnight in Williams' E medium containing 10% serum and incubated overnight prior to the addition of compounds in fresh medium. The intracellular localization of the GFP-CAR fusion protein was determined by fluorescence microscopy ~4 h after the addition of compound.*

*Treatment of Primary Human Hepatocytes—Primary human hepatocytes were obtained from BioWhittaker, Inc. (Walkersville, MD), and plated at an approximate density of 3 × 10^6 cells/well in a six-well plate. Cells were maintained in Williams' E medium supplemented with 100 nM dexamethasone, 2 mM ta-glutamine, and 1% ITS-G. Cells were treated with vehicle (0.1% MeSO), rifampicin (10 µM), or CITCO (100 nM). Fresh compound and medium were added after 24 h, and cells were harvested after 48 h.*

*RNA Preparation and Expression Analysis—Total RNA from human hepatocyte cultures was isolated using TRIazol reagent (Invitrogen) according to the manufacturer's instructions. Real-time quantitative (RTQ) PCR was performed using an ABI PRISM 7100 sequence detection system instrument and software (Applied Biosystems, Inc., Foster City, CA). RNA samples were prepared for RTQ-PCR as described (9). Gene-specific primers and probes were designed using Primer Express Version 2.0.0 (Applied Biosystems, Inc.) and synthesized by Keystone Laboratories (Camarillo, CA). All primers and probes were entered into the NCBI BLAST program to ensure specificity. Fold induction values were calculated by subtracting the mean threshold cycle number for each treatment group from the mean threshold cycle number from the vehicle group and raising 2 to the power of this difference.*

*For Northern blot analysis, total RNA (10 µg) was resolved on a 2.2% formaldehyde-containing 1% agarose denaturing gel and transferred to a nylon membrane (Hybond N™, Amersham Biosciences). Blots were hybridized to [32P]-labeled cDNAs corresponding to CYP2B6 (bases 3–3509; GenBank™/EBI accession number AF182277), CYP2A6 (bases 790–1322; accession number M18907), human UGT1A1 (bases 75–766; accession number NM_000463), CYP2A6 (bases 121–1200; accession number X13930), SULT1A1 (bases 561–1300; accession number NM_001055), and rat 18 S rRNA (bases 293–970; accession number X01117). Signal intensity was quantitated using ImageQuant software (Amersham Biosciences).*

**RESULTS**

*Identification of a Selective Human CAR Agonist*

Several criteria were used to identify a potent and selective CAR agonist. These included (a) activity in an in vitro FRET-based assay, (b) ~50-fold selectivity over PXR in a transient transfection assay, and (c) the ability to induce CAR translocation from the cytoplasm to the nucleus in primary hepatocytes. We then sought to use this chemical tool to identify genes that are regulated by CAR in primary cultures of human hepatocytes.

*Identification of a CAR Agonist in a FRET-based Screen—A nuclear receptor-biased chemical library was screened in a CAR FRET-based assay using human CAR LBD and a peptide containing the second LXXLL motif of SRC-1 (steroid receptor coactivator-1; amino acids 676–700). Compounds that induced increased interaction between these partners with potencies of >100 nM were chosen for further analysis. One of the compounds, the imidazothiazole derivative CITCO (Fig. 1A), displayed a half-maximal effective concentration (EC_{50}) of 49 nM in the CAR/SRC-1 FRET assay (Fig. 1B). CITCO was 50-fold more potent than the human CAR agonist 5β-pregnane-3,20-dione (EC_{50} = 3000 nM) (Fig. 1B). The human CAR antagonist clomiphene (7) was also evaluated in this assay and demonstrated an IC_{50} of 58 nM.*

*Selectivity for CAR Versus PXR of >50-fold—CAR agonists derived from the in vitro assay were tested for CAR selectivity. Using the CAR- and PXR-responsive XREM-CYP3A4-LUC reporter gene construct in CV-1 transient transfection assays, the selectivity of the compounds for human CAR over human PXR was assessed. The majority of compounds that were active...*
in CAR/SRC-1 FRET assays were <50-fold selective for CAR in comparison with human PXR (data not shown). CITCO was one of the few compounds that displayed >50-fold selectivity for CAR over PXR in the transient transfection assay (Fig. 2, A and B). CITCO displayed calculated EC$_{50}$ values of 25 nM in the CAR transient transfection assay and 3 µM in the PXR transient transfection assay (>100-fold selectivity for CAR). We also carried out transient transfection studies to show that CITCO and the human CAR antagonist clotrimazole are competitive in their effects on CAR transcriptional activity (Fig. 2A). In the presence of 1.5 µM clotrimazole (an approximate IC$_{50}$ in this assay), the EC$_{50}$ of CITCO increased by >10-fold (EC$_{50}$ = 304 nM), indicating competition between the two compounds. Data represent the mean of assays performed in duplicate. In A, data are expressed as luciferase activity normalized by subtraction of base-line luciferase activity (base-line values for CAR activity in the presence of clotrimazole were ~2 times the values in the absence of clotrimazole). In B, data are expressed as a percentage of luciferase activity obtained from transfected cells treated with 10 µM rifampicin.

We also tested CITCO for selectivity across a panel of 15 nuclear receptors for which we had available validated transient transfection activation assays, viz., estrogen receptor-α and -β; hepatocyte nuclear factor-4α; LRH-1 (liver receptor homolog-1); liver X receptor-α and -β; peroxisome proliferator-activated receptor-α, -δ, and -γ; retinoic acid receptor-α; farnesoid X receptor; SHP (small heterodimer partner); thyroid hormone receptor-α; vitamin D receptor (VDR); and glucocorticoid receptor. CITCO did not have any detectable activity on any of these additional receptors at a dose of 10 µM (data not shown).

**CAR Translocation Assay**—Although CITCO was a potent activator of human CAR, the efficacy of this compound and other CAR activators was relatively weak in assays performed in immortalized cell lines, possibly because CAR is constitutively present in the nucleus in these assays. Thus, the results of the transfection assays do not accurately predict the overall efficacy of the compound in hepatocytes because inactive CAR is restricted to the cytoplasm in these cells. Thus, to assess the ability of CAR ligands to induce translocation of CAR from the cytoplasm to the nucleus, we developed a translocation assay in primary cultures of hepatocytes. The full-length human CAR coding region was fused in-frame to the GFP coding region and transfected into rat hepatocytes. The effects of various compounds on CAR translocation were visualized by fluorescence microscopy (Fig. 3). Although endogenous CAR has previously been shown to be predominantly localized in the cytoplasm in the absence of stimulation, GFP-CAR was present in both the cytoplasm and nucleus (Fig. 3, panels 1 and 2). This is likely due to the relatively high concentration of the GFP-CAR chimera expressed under these assay conditions. In addition to a widespread GFP-CAR distribution, control cells showed a somewhat crenulated pattern of fluorescence, indicating that CAR may be attached to a subcellular structure. Further stud-
ies are needed to determine whether this is true for native CAR as well or whether this property is unique to the GFP-CAR chimera.

When hepatocytes expressing GFP-CAR were treated with phenobarbital, the GFP signal was localized predominantly in the nucleus (Fig. 3, panels 3 and 4), as expected. Notably, in cells treated with CITCO, the pattern of GFP localization was similar to that seen after phenobarbital treatment (Fig. 3, panels 5 and 6), indicating that CITCO causes efficient nuclear translocation of CAR in hepatocytes. In summary, CITCO fulfills our criteria for a useful human CAR chemical tool: it is a potent and selective human CAR ligand that activates the receptor in a transfection assay and promotes its translocation into the nucleus of hepatocytes.

Comparison of the Effects of CITCO and Rifampicin on Gene Expression in Primary Human Hepatocytes

In the absence of a selective human CAR agonist, it has been difficult to ascertain which genes are regulated by this receptor in human hepatocytes. We used CITCO to study the effects of CAR activation on gene expression in primary cultures of human hepatocytes. Hepatocytes derived from three separate donors were treated for 48 h with either 1 μM CITCO or 10 μM rifampicin, a selective PXR agonist. The comparative effects of the agonists on the expression of eight genes involved in a variety of aspects of xenobiotic metabolism were quantitated by RTQ-PCR (Table I). RNA in sufficient quantity was available from two donors (Donors 1 and 2) to further evaluate selected gene expression changes by Northern blot analysis (Fig. 4). The mRNAs evaluated included those encoding multiple cytochrome P450 enzymes (CYP2A6, CYP2B6, and CYP3A4); enzymes involved in supporting phase I metabolism (aldehyde dehydrogenase (ALDH1A4) and aminolevulinate synthase); and enzymes involved in phase II (conjugation) metabolism, including glutathione S-transferase A2 (GSTA2) and sulfotransferase (SULT1A1). The mRNA encoding the conjugation enzyme UDP-glucuronosyltransferase (UGT1A1) was examined exclusively by Northern analysis due to difficulties in generating functional RTQ-PCR primers for this mRNA. We also examined the effects of CITCO and rifampicin on the expression of the MDR1 (multidrug resistance-1) transporter mRNA.

Phase I Enzyme Genes—CAR regulates both Cyp2b10 and Cyp3a11 in mouse liver (8–10, 12) and has also been implicated in the regulation of CYP2B6 and CYP3A4 in human hepatocytes (11, 12, 29). However, the role of CAR in the regulation of these genes has not been examined rigorously in human hepatocytes using a selective CAR chemical tool. Both CITCO and rifampicin induced CYP2B6 and CYP3A4 in human hepatocytes from Donors 1 and 2 as measured by RTQ-PCR (Table I). Rifampicin was not effective at inducing CYP2B6 in Donor 3, whereas CITCO was not effective at inducing CYP3A4 in this donor. This likely reflects the relative efficacy of rifampicin and CITCO in induction of CYP3A4 and CYP2B6, respectively, coupled with higher basal levels of expression of these genes in Donor 3. When the effects of CITCO were evaluated by Northern blot analysis, CYP2B6 mRNA was found to be induced by 4.4- and 20-fold in Donors 1 and 2, respectively (Fig. 4). Rifampicin also induced CYP2B6 expression, although to a lesser extent than CITCO (Fig. 4 and Table I). In all three donors, CYP2B6 was induced more efficiently by CITCO than by rifampicin, indicating that this gene is more responsive to CAR than to PXR.

In contrast to CYP2B6, CYP3A4 mRNA displayed a more robust response to rifampicin than to CITCO. When assessed by RTQ-PCR, rifampicin induced CYP3A4 mRNA by 15-fold in Donor 1 and by 78-fold in Donor 2, whereas CITCO induced CYP3A4 mRNA by 7.0- and 46-fold in the same donors. When evaluated by Northern analysis, rifampicin induced CYP3A4 expression by 30- and 5.9-fold, respectively, whereas CITCO induced CYP3A4 by 11- and 6.2-fold, respectively (Fig. 4). These data indicate that both PXR and CAR regulate CYP3A4 in human hepatocytes.

Although technically not a phase I enzyme, the CYP2A6 gene was also examined for PXR and CAR regulation. The mouse homolog (Cyp2a4) has previously been shown to be regulated by mouse CAR (8, 9). Analysis of the three donors by RTQ-PCR showed that CYP2A6 mRNA was induced selectively by CITCO (Table I). Analysis of Donors 1 and 2 by Northern blotting was consistent with these results (Fig. 4). In this analysis, CYP2A6 mRNA was induced by 5.4- and 9.6-fold in Donors 1 and 2, respectively, whereas rifampicin did not cause changes in the expression of this mRNA. Thus, CYP2A6 is selectively induced by CAR, but not by PXR.

Other genes involved in phase I metabolism were also evaluated by RTQ-PCR. Aminolevulinate synthase mRNA was induced by both CITCO and rifampicin in all of the donors as assessed by RTQ-PCR (Table I). Notably, ALDH1A4 showed a highly variable response to CITCO and rifampicin. Induction of ALDH1A4 varied from 1.8-fold (Donor 1) to 60-fold (Donor 3) in response to CITCO and from no induction (Donor 1) to a 160-fold induction (Donor 3) in response to rifampicin. Thus, certain genes appear to display a high degree of inter-individual variability in terms of their response to selective CAR and PXR agonists.

Phase II Conjugation Genes—The effects of CITCO and rifampicin on the expression of the mRNAs encoding the conjugation enzymes GSTA2, SULT1A1, and UGT1A1 were also examined. Both CITCO and rifampicin were able to induce the expression of GSTA2 when assessed by RTQ-PCR and North-

| Gene | Donor 1 | Donor 2 | Donor 3 |
|------|---------|---------|---------|
|      | CITCO   | RIF     | CITCO   | RIF     | CITCO   | RIF     |
| ALAS | 1.7     | 4.9     | 6.9     | 4.9     | 2.2     | 2       |
| ALDH1A4 | 1.8 | NC      | 10      | 1.6     | 60      | 180     |
| CYP2A6 | 4.7     | NC      | 8.7     | NC      | 1.7     | NC      |
| CYP2B6 | 5.6     | 1.5     | 32      | 5.2     | 3.2     | NC      |
| CYP2A4 | 7       | 15      | 46      | 78      | NC      | 2       |
| GSTA2 | 11      | 12      | 8.1     | 1.9     | 1.5     | NC      |
| MDR1  | NC      | NC      | 8.8     | 2.2     | NC      | NC      |
| SULT1A1 | 1.7 | NC      | 7.5     | NC      | NC      | NC      |

Table I: Effects of CITCO and rifampicin on expression of genes in primary human hepatocytes

Human hepatocytes were treated with CITCO (100 nM) or rifampicin (RIF: 10 μM). Gene expression levels were calculated from raw RTQ-PCR data as described under “Experimental Procedures” and are expressed as fold change relative to cells treated with vehicle (0.1% Me2SO) alone. No change (NC) in relative mRNA expression was observed for some of the treatments. Data represent the average of duplicate determinations on each sample. ALAS, aminolevulinate synthase.

*Comparison of the Effects of CITCO and Rifampicin on Gene Expression in Primary Human Hepatocytes*

| Gene | Donor 1 | Donor 2 | Donor 3 |
|------|---------|---------|---------|
|      | CITCO   | RIF     | CITCO   | RIF     | CITCO   | RIF     |
| ALAS | 1.7     | 4.9     | 6.9     | 4.9     | 2.2     | 2       |
| ALDH1A4 | 1.8 | NC      | 10      | 1.6     | 60      | 180     |
| CYP2A6 | 4.7     | NC      | 8.7     | NC      | 1.7     | NC      |
| CYP2B6 | 5.6     | 1.5     | 32      | 5.2     | 3.2     | NC      |
| CYP2A4 | 7       | 15      | 46      | 78      | NC      | 2       |
| GSTA2 | 11      | 12      | 8.1     | 1.9     | 1.5     | NC      |
| MDR1  | NC      | NC      | 8.8     | 2.2     | NC      | NC      |
| SULT1A1 | 1.7 | NC      | 7.5     | NC      | NC      | NC      |
ern analysis (Fig. 4 and Table I). When mRNAs from Donors 1 and 2 were evaluated by Northern analysis, CITCO induced GSTA2 mRNA by 3.4-fold in Donor 1 and by 19-fold in Donor 2 (Fig. 4). Rifampicin induced GSTA2 mRNA by 3.8- and 3.5-fold in these same donors (Fig. 4).

SULT1A1 mRNA was robustly induced only by CITCO in one of the three donors. In Donor 1, induction by either compound was 2-fold as measured by Northern blotting or RTQ-PCR; and in Donor 3, no induction was seen by either compound by RTQ-PCR. In contrast, CITCO strongly induced SULT1A1 in Donor 2 (11-fold increase as measured by Northern blot analysis and 7.5-fold increase as measured by RTQ-PCR). The variation in donor response may again be attributable to inter-individual heterogeneity in basal levels of gene expression. Northern blot analysis showed that Donor 1 had relatively high basal levels of expression of SULT1A1 (Fig. 4).

Transporter Expression—The multidrug resistance genes, including MDRI, function as broad-specificity transporters in the liver. We examined the response of MDRI to CITCO and rifampicin by RTQ-PCR (Table I). MDRI expression was induced by both CITCO and rifampicin in Donor 2, but no induction by either compound was seen in Donors 1 and 3. Thus, for MDRI, a significant inter-individual response is seen. In certain individuals, induction of MDRI gene expression occurs in response to both PXR and CAR agonists.

Model of CITCO Binding in the Ligand-binding Pocket of CAR—Although no x-ray structure is available for CAR, x-ray structures have been done for the closely related receptors PXR (30) and VDR (31). The x-ray structure of PXR revealed a large and practically spherical ligand-binding pocket that can bind a wide range of lipophilic ligands, whereas VDR has a smaller pocket with polar side chains positioned to recognize specific ligands (31). In PXR, the pocket expansion is due primarily to a 50–60-residue insert between helixes 1 and 3. This helix 1–3 insert displaces helix 6, thereby opening the pocket. VDR also has a helix 1–3 insert; there is no evidence that it displaces helix 6. Although residues in the “core” of CAR LBD have greater identity to PXR (50%) than to VDR (40%), CAR lacks the helix 1–3 insert, and its helix 6 should have a geometry more similar to that in VDR than in PXR (6). Consequently, we chose to use VDR as the template in building a model for CAR. The MVP program (32) was used to build the model for CAR and to dock CITCO into the model. A number of different binding modes were obtained for CITCO, one of which is shown
in Fig. 5. Asn\textsuperscript{165} lies near the oxime linkage and might possibly donate a hydrogen bond to the oxime. This particular binding mode has the \textit{para}-chlorophenyl ring directed downwards and the imidazothiazole group directed upwards, but the calculations also gave binding modes where the positions of these groups were interchanged, with the oxime linker still located near Asn\textsuperscript{165}. The modeling and docking calculations are not accurate enough to distinguish among the possible binding orientations, but it is clear that the CAR binding pocket can accommodate CITCO. The model CAR pocket is smaller than that in PXR and somewhat more lipophilic than that in VDR, suggesting that CAR should be intermediate between VDR and PXR in terms of ligand promiscuity.

**DISCUSSION**

To date, little is known about the function of CAR in humans. Attempts to delineate CAR biology in man have been hindered by the significant overlap in the pharmacology of human CAR and PXR and the lack of a selective CAR activator. CITCO is a potent and, importantly, highly selective human CAR agonist that should prove to be a useful tool in dissecting the structure and function of this receptor.

Direct comparison of human CAR and PXR target genes is now possible, as is direct comparison of CAR target genes in mouse versus human cells. In mice, PXR and CAR differential gene expression studies have previously been carried out using the selective tools pregnenolone 16α-carbonitrile (selective PXR agonist in mouse) and TCPOBOP (9). Gene expression studies with these compounds provide the framework to begin comparative analysis. For the majority of the genes that overlapped between the mouse and human studies, similar profiles were seen using PXR- and CAR-specific compounds. For example, similar to mice, both selective CAR and PXR agonists regulated \textit{Cyp2b} and \textit{Cyp3a} expression, consistent with previous studies suggesting that PXR and CAR cross-regulate these genes (10–12, 22). Interestingly, CITCO had more robust effects on \textit{Cyp2b} expression, whereas rifampicin had more robust effects on \textit{Cyp2a4}, indicating that the receptors have different quantitative effects depending on the specific gene (7).

In our comparison of CITCO with the human PXR ligand rifampicin in three separate sets of human hepatocytes, we observed remarkable inter-donor heterogeneity. This observation is consistent with previous studies showing that cytochrome P450 expression is quite variable in primary human hepatocyte preparations (33–35). For example, inter-individual variations in \textit{Cyp3a4} protein levels ranging up to 40-fold have been reported (36, 37). Moreover, ethnic differences in \textit{Cyp3a4}-mediated drug metabolism have been reported (38), and it is estimated that ~90% of the inter-individual variability in \textit{Cyp3a4} expression is due to genetic factors (39). In the case of the hepatocytes used in these studies, the inter-individual variability would be expected to be even higher due to the fact that the donors were typically undergoing drug therapy just prior to the harvest of the hepatocytes. The relatively high basal levels of multiple genes seen in Donor 1 versus Donor 2 might reflect genetic differences, differences in drug exposure, or both.

When comparing CAR target genes in mice versus humans, we found that, generally, genes regulated by only selective CAR ligands in mice were also regulated by the selective human CAR ligand. Notably, the phase II conjugation enzyme sulfo-
K. I., LaTour, A., Liu, Y., Klaassen, C. D., Brown, K. K., Reinhard, J., Willson, T. M., Koller, B. H., and Kliewer, S. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3369–3374
19. Moore, L. B., Goodwin, B., Jones, S. A., Wisely, G. B., Serabjit-Singh, C. J., Willson, T. M., Collins, J. L., and Kliewer, S. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7500–7502
20. Honkakoski, P., Zelko, I., Sueyoshi, T., and Negishi, M. (1998) Mol. Cell. Biol. 18, 5652–5658
21. Kawamoto, T., Sueyoshi, T., Zelko, I., Moore, R., Washburn, K., and Negishi, M. (1999) Mol. Cell. Biol. 19, 6318–6322
22. Sueyoshi, T., Kawamoto, T., Zelko, I., Honkakoski, P., and Negishi, M. (1999) J. Biol. Chem. 274, 6043–6046
23. Forman, B. M., Tzameli, I., Choi, H. S., Chen, J., Simha, D., Seol, W., Evans, R. M., and Moore, D. D. (1998) Nature 395, 612–615
24. Ueda, A., Hamadeh, H. K., Webb, H. K., Yamamoto, Y., Sueyoshi, T., Afshari, C. A., Lehmann, J. M., and Negishi, M. (2002) Mol. Pharmacol. 61, 1–6
25. Iwata, H., Yoshinari, K., Negishi, M., and Stegeman, J. J. (2002) Pharmacogenetics 12, 121–132
26. Westlind, A., Lolberg, L., Tindberg, N., Andersson, T. B., and Ingelman-Sundberg, M. (1999) Biochem. Biophys. Res. Commun. 260, 201–205
27. Goodwin, B., Hodgson, E., and Liddle, C. (1999) Mol. Pharmacol. 56, 1329–1339
28. David, G. B., Galbraith, W., Geyer, S. B., Koether, A. M., Palmer, N. F., and Paxler, J. (1975) Prog. Histochem. Cytochem. 7, 1–49
29. Goodwin, B., Hodgson, E., D’Costa, D. J., Robertson, G. R., and Liddle, C. (2002) Mol. Pharmacol. 62, 359–365
30. Watkins, R. E., Wisely, G. B., Moore, L. B., Collins, J. L., Lambert, M. H., Williams, S. P., Willson, T. M., Kliewer, S. A., and Redinbo, M. R. (2001) Science 292, 2329–2333
31. Rochel, N., Wurtz, J. M., Mitschler, A., Klaholz, B., and Moras, D. (2000) Mol. Cell 5, 173–179
32. Lamba, J. K., Lin, Y. S., Thummel, K., Daly, A., Watkins, P. B., Strom, S., Zhang, J., and Schuetz, E. G. (2002) Xenobiotica 32, 165–206
33. Code, E. L., Crequi, C. L., Pennman, B. W., Gonzalez, F. J., Chang, T. K., and Waxman, D. J. (1997) Drug Metab. Dispos. 25, 985–993
34. Gibson, G. G., Plant, N. J., Swales, K. E., Ayrton, A., and El-Sankary, W. (2002) Xenobiotica 32, 165–206
35. Lambert, M. H. (1997) in Practical Application of Computer-Aided Drug Design (Charifson, P. S., ed) pp. 243–303, Marcel Dekker, Inc., New York
36. Xie, H. G., Kim, R. E., Wood, A. J., and Stein, C. M. (2001) Annu. Rev. Pharmacol. Toxicol. 41, 815–850
37. Kalow, W., Ozdemir, V., and Tothfalusi, L. (2001) Pharmacogenomics J. 1, 234–236
38. Tyndale, R. F., Pianezza, M. L., and Sellers, E. M. (1999) Nicotine Tob. Res. 1, 63–67
39. Sugatani, J., Kojima, H., Ueda, A., Kakizaki, S., Yoshinari, K., Geng, Q. H., Owens, I. S., Negishi, M., and Sueyoshi, T. (2001) Hepatology 33, 1232–1238
Identification of a Novel Human Constitutive Androstane Receptor (CAR) Agonist and Its Use in the Identification of CAR Target Genes

Jodi M. Maglich, Derek J. Parks, Linda B. Moore, Jon L. Collins, Bryan Goodwin, Andrew N. Billin, Catherine A. Stoltz, Steven A. Kliewer, Millard H. Lambert, Timothy M. Willson and John T. Moore

J. Biol. Chem. 2003, 278:17277-17283.
doi: 10.1074/jbc.M300138200 originally published online February 27, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300138200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 20 of which can be accessed free at http://www.jbc.org/content/278/19/17277.full.html#ref-list-1