Nuclear translocation of Ad/EYFP-hCAR: a novel tool for screening human CAR activators in human primary hepatocytes

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Abbreviations: 3MC, 3-methylcholanthrene; Ad/EYFP-hCAR, adenoviral-enhanced yellow fluorescent protein-human constitutive androstane receptor; ART, artemisinin; BHA, butylated hydroxyanisole; CAR, Constitutive androstane receptor; CDCA, chenodeoxycholic acid; CITCO, 6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl) oxime; CLZ, clotrimazole; CMZ, carbamezapine; CPZ, chlorpromazine; DMSO, dimethyl sulfoxide; DZP, diazepam; EFV, efavirenz; FLU, fluconazole; HOC, 22(R)-hydroxycholesterol; HPH, human primary hepatocytes; MCB, myclobutanil; MLZ, meclizine; NVP, nevirapine; OA, okadaic acid; PB, phenobarbital; PHN, phenytoin; PK11195, 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinoline-carboxamide; PXR, pregnane X receptor; RT-PCR, Reverse transcriptase - polymerase chain reaction; RIF, rifampicin; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridylox)benzene
Abstract

The constitutive androstane receptor (CAR; NR1I3) is a hepatic transcription factor that controls the expression of numerous drug-metabolizing enzymes and transporters in response to xenobiotic exposures. In primary hepatocytes and intact liver, CAR resides in the cytoplasm under basal condition and translocates to the nucleus upon exposure to inducers. However, CAR spontaneously accumulates in the nucleus of immortalized cell lines and exhibits constitutive activation in the absence of activators, which makes the identification of CAR activators extremely challenging. Here, we have established an efficient screening method for determining the nuclear translocation of human (h) CAR in human primary hepatocytes (HPH). Our results demonstrated that adenoviral-enhanced yellow fluorescent protein-tagged hCAR (Ad/EYFP-hCAR) infects HPH with high efficiency, and the majority of Ad/EYFP-hCAR (>80%) is expressed in the cytoplasm of non-induced HPH and is translocated to the nucleus in response to activators and antagonists of hCAR. Furthermore, 22 compounds including known hCAR activators, non-activators, CYP2B inducers, as well as deactivators were evaluated in this system. Our results indicated that chemical-mediated Ad/EYFP-hCAR translocation in HPH significantly correlated with hCAR activation and target gene induction. Compared with cell-based reporter assay in cell lines and in vitro ligand binding assays, the established Ad/EYFP-hCAR translocation assay in HPH exhibits apparent advantages such as sensitivity to chemical activators, and responding to both direct and indirect hCAR activators. Thus, nuclear translocation of Ad/EYFP-hCAR in HPH represents an efficient means for in vitro prediction of chemical-mediated hCAR nuclear accumulation.
Introduction

Predominantly expressed in the liver, the constitutive androstane receptor (CAR, NR1I3) is defined as an important xenobiotic-sensor that transfers endogenous and exogenous stimuli into cellular responses by regulating the expression of numerous hepatic genes. Upon xenobiotic stimulation, CAR and its closest relative pregnane X receptor (PXR, NR1I2) coordinate the cellular defensive response by enhancing the expression of a broad and overlapping set of drug-metabolizing enzymes (DME) and transporters (Honkakoski et al., 2003; Stanley et al., 2006). In addition to xenobiotic detoxification, activation of CAR is also involved in other hepatic functions, such as gluconeogenesis, fatty acid oxidation, biotransformation and clearance of steroid hormones and bilirubin (Sugatani et al., 2001; Ueda et al., 2002; Huang et al., 2003; Kodama et al., 2004; Tien and Negishi, 2006), as well as chemical-mediated tumor promotion (Yamamoto et al., 2004; Huang et al., 2005). Therefore, it is of great interest to develop an efficient screening method for identifying drug candidates as human (h) CAR activators at an early stage of drug development.

In contrast to PXR, CAR expresses a high level of constitutive transcriptional activity in immortalized cell lines and can be accumulated spontaneously into nuclei of these cells without the presence of xenobiotic activators (Baes et al., 1994; Wang and Negishi, 2003). Moreover, CAR could be activated through either direct ligand binding, such as the selective hCAR agonist 6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichloro-benzyl)oxime (CITCO) and the antimalarial artemisinin (ART), or ligand-independent (indirect) mechanisms, such as phenobarbital (PB)-type...
activators (Yamamoto et al., 2003; Simonsson et al., 2006; Merrell et al., 2008). These characteristics of CAR significantly decreased the value of the cell-based reporter assay and the in vitro ligand binding assays for assessing xenobiotic-mediated CAR activation. Nonetheless, CAR is sequestered primarily in the cytoplasm of non-induced hepatocytes in vivo and in primary culture conditions, and undergoes a two-step activation process after exposure to activators. The initial step in response to chemical activators is the translocation of CAR from the cytoplasm to the nucleus (Kawamoto et al., 1999).

Significant efforts have been centered on elucidating the molecular mechanisms underlying the chemical-mediated nuclear translocation of CAR. Recently, it has been proposed that the CAR cytoplasmic complex is composed of heat shock protein 90 (Hsp90), cytoplasmic CAR retention protein (CCRP), membrane-associated subunit of protein phosphatase 1 (PPP1R16A), and other unknown proteins (Kobayashi et al., 2003; Sueyoshi et al., 2008). Apparently, direct ligand binding seems not essential for a drug to stimulate CAR nuclear translocation. For example, PB does not bind to either mouse (m) or hCAR, but stimulates translocation of both receptors to the nucleus (Kawamoto et al., 1999; Moore et al., 2000; Tzameli et al., 2000). Although the exact mechanisms of nuclear translocation are yet elusive, cytoplasmic retention of CAR in the primary hepatocyte cultures and accumulation in the nucleus following CAR activation, provide a valuable in vitro system for investigating the signaling pathway involved in the activation of CAR.

To date, mounting evidence indicates that identification of hCAR activators has been extremely complicated due to the significant species differences in CAR activation.
For instance, 1,4-bis [2-(3,5-dichlorpyridyloxy)] benzene (TCPOBOP) activates mouse but not human CAR, while CITCO activates human but not mouse CAR. Several compounds including androstanol, progesterone, and testosterone showed potent repression of the constitutive activity of mCAR in cell lines. However, such chemical tools pertaining to hCAR are limited. Recently, we showed that 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinoline-carboxamide (PK11195), a known ligand for peripheral benzodiazepam receptor, exhibits potent and selective repression of hCAR activity in HepG2 cells, but the repressed hCAR activity was only reestablished in the presence of direct activators such as CITCO and ART not by indirect activators such as PB and phenytoin (PHN) (Li et al., 2008). Given that a large number of hCAR activators function through the ligand-independent mechanisms, antagonistic repressors of hCAR only provide limited value in screening hCAR activation.

In this report, we generated a functional adenoviral-enhanced yellow fluorescent protein-hCAR (Ad/EYFP-hCAR) construct. Taking the advantage of human primary hepatocytes (HPH) in which CAR resides in the cytoplasm before activation, we have established a method for assessing the activation of hCAR in adenoviral-transduced HPH. Our results showed that Ad/EYFP-hCAR is primarily expressed in the cytoplasm of HPH, and the chemical-mediated Ad/EYFP-hCAR nuclear accumulation correlated well with hCAR activation and target gene induction in HPH. This method exhibits clear advantages over the cell-based reporter assays and in vitro ligand-binding assays in determining hCAR activation.
Materials and Methods

Chemicals and biological reagents

PB, PK11195, TCPOBOP, PHN, rifampicin (RIF), ART, carbamezapine (CMZ), Wy-14643, chenodeoxycholic acid (CDCA), 22(R)-hydroxycholesterol (HOC), 3-methylcholanthrene (3MC), butylated hydroxyanisole (BHA), clotrimazole (CLZ), diazepam (DZP), meclizine (MLZ), and chlorpromazine (CPZ) were purchased from Sigma-Aldrich (St. Louis, MO). Okadaic acid (OA) was purchased from Calbiochem (Gibbstown, NJ). CITCO was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Efavirenz (EFV) was purchased from Toronto Research Chemicals (Toronto, ON, Canada), and nevirapine (NVP) was purchased from US Pharmacopeia (Rockville, MD). Fluconazole (FLU) and myclobutanil (MCB) were purchased from LKT Laboratories (St. Paul, MN). Oligonucleotide primers were synthesized by Integrated DNA technologies (Coralville, IA). The Dual-Luciferase Reporter Assay System was purchased through Promega (Madison, WI). FuGENE® 6 transfection reagent was obtained from Roche (Basel, Switzerland). Matrigel, insulin and ITS+ were obtained from BD Biosciences (Bedford, MA). Other cell culture reagents were purchased from Invitrogen (Calsbad, CA) or Sigma-Aldrich.

Plasmids and generation of adenovirus-EYFP tagged hCAR

The pCR3-hCAR and the enhanced yellow fluorescent protein tagged hCAR (EYFP-hCAR) expression plasmids were kindly provided by Dr. Masahiko Negish (National Institute of Environmental and Health Sciences, National Institutes of Health, Research Triangle Park, NC). As reported previously, the CYP2B6-2.2kb firefly
luciferase construct is composed of the PBREM containing 1.8 kb of the native promoter, and the 400 bp distal XREM region (Wang et al., 2003). The pRL-TK Renilla luciferase plasmids used to normalize firefly luciferase activities were from Promega. The EYFP tagged hCAR was sub-cloned into pShuttle-CMV expression vector at Sal I and Xba I sites. The linearized shuttle vector and AdEasy vector were then cotransformed into BJ5183 cells. Positive recombinant Ad/EYFP-hCAR plasmids were selected, and transduced into HEK293 cell cultures for virus packaging and amplification. Viruses were purified by using ViraBind™ Adenovirus Purification kit (Cell Biolabs, San Diego, CA).

**Human primary hepatocytes culture and treatments**

Human liver tissues were obtained following surgical resection by qualified pathology staff after diagnostic criteria were met and prior approval from the Institutional Review Board at the University of Maryland at Baltimore. Hepatocytes were isolated from human liver specimens by a modification of the two-step collagenase digestion method as described previously (LeCluyse et al., 2005). Hepatocytes were seeded at 3.75 × 10^5 cells/well in 24-well biocoat plates in DMEM supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 μg/ml insulin, and 1 μM dexamethasone, and cultured as described previously (Wang et al., 2003). Hepatocyte cultures were infected with 2 μl Ad/EYFP-hCAR for 12 hrs before the treatment with vehicle control (0.1% DMSO) or test compounds. After 24 hrs treatment, cells were washed twice with phosphate-buffered saline and fixed for 30 min in 4% buffered paraformaldehyde. The cells were then stained with 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) for 30 min. Twenty-two various compounds as classified in Table 1 were tested in Ad/EYFP-
hCAR infected HPH. In separate experiments, HPH seeded in 6-well biocoat plates were treated with BHA (100 μM), DZP (50 μM), FLU (50 μM), MCB (50 μM), RIF (10 μM), or 0.1% DMSO as vehicle control for real-time PCR and Western blotting analysis.

**Confocal laser scanning microscopy imaging**

Confocal laser scanning microscopy was performed with a Nikon C1-LU3 instrument based on an inverted Nikon Eclipse TE2000 microscope. The confocal system was equipped with three fluorescence detection channels (photomultipliers) and a nonconfocal transmitted light detector. One of the photomultipliers was used to collect fluorescence signals from the green and yellow region of the fluorescence emission, and the nonconfocal transmitted light detector was used to collect brightfield images.

**Real-time PCR analysis**

Total RNA was isolated from treated hepatocytes using the RNeasy Mini Kit (Qiagen) and reverse transcribed using High Capacity cDNA Archive kit (Applied Biosystems, Foster, CA) following the manufacturers’ instructions. CYP2B6 and CYP3A4 mRNA expression was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time PCR assays were performed in 96-well optical plates on an ABI Prism 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems). Primers for CYP2B6, CYP3A4, and GAPDH mRNA detection are as follows: CYP2B6 1215 to 1319 bp, 5’- AGACGCCTTCAATCCTGACC -3’ (forward), and 5’- CCTTCACCAAGACAAATCCGC - 3’ (reverse); CYP3A4 213 to 462 bp, 5’-GTGGGGCTTTTATGATGGTCA-3’ (forward), and 5’- GCCTCAGATTTCTCACCAACACA - 3’ (reverse); and GAPDH 217 to 501 bp, 5’- CCCATCACCATCTTCCAGGAG -3’ (forward), and 5’-
GTTGTCATGGATGACCTTGGC - 3’ (reverse). Fold induction values were calculated according to the equation $2^{\Delta \Delta Ct}$, where $\Delta Ct$ represents the differences in cycle threshold numbers between the target gene and GAPDH, and $\Delta \Delta Ct$ represents the relative change in these differences between control and treatment groups.

**Western Blot Analyses**

Homogenate proteins (20 µg) from treated HPH were resolved on SDS-polyacrylamide gels, and electrophoretically transferred onto Immobilon-P polyvinylidene difluoride membranes. Subsequently, membranes were incubated with specific antibodies against CYP2B6 or CYP3A4 (Millipore-Chemicon, CA) diluted 1:4000 and 1:5000, respectively. β-actin (Sigma-Aldrich, St. Louis, MO) was used as internal control. Blots were washed and incubated with horseradish peroxidase goat anti-rabbit IgG antibody diluted 1:4000. Blots were developed using ECL Western blotting detection reagent (GE Healthcare).

**Transfection assays in cell lines**

HepG2 cells in 24-well plates were transfected with hCAR expression vector, and CYP2B6-2.2kb reporter construct using Fugene 6 Transfection Kit following the manufacturer’s instruction. Twenty four hours after transfection, cells were treated with solvent (0.1% DMSO) or test compounds at indicated concentrations (Fig. 6D) in the absence or presence of PK11195 (5 µM). Twenty-four hours later, cell lysates were assayed for firefly activities normalized against the activities of cotransfected Renilla luciferase using Dual-Luciferase Kit (Promega, WI). Data were represented as mean±S.D. of three individual transfections.

**Statistical analysis**
Experimental data are presented as a mean of triplicate determinations ± S.D. unless otherwise noted. Statistical comparisons were made using one-way analysis of variance (ANOVA). The statistical significance was set at \( p \) values <0.05 (*), or <0.01 (**).
Results

Localization of exogenous hCAR in HepG2 cells and HPH

Spontaneous nuclear accumulation and activation of CAR has been one of the major obstacles in studying CAR activation in immortalized cell lines. As demonstrated in Fig. 1, our recombinant adenoviral EYFP-human CAR (Ad/EYFP-hCAR) infected both HepG2 cells and HPH with high efficiency (Fig. 1A). Consistent with previous observations (Kawamoto et al., 1999; Kanno et al., 2005), the expression of Ad/EYFP-hCAR was primarily observed in the nuclei of HepG2 cells (nucleus = 98%; mixed = 2%). In contrast, the Ad/EYFP-hCAR has been visualized predominantly in the cytoplasm of human primary hepatocytes prior to activation (nucleus = 4%; cytoplasm = 87%; mixed = 9%). Together, the unique characteristics of hCAR distribution, the direct visualization of EYFP-hCAR, plus the high efficiency of Ad/EYFP-hCAR infection of HPH offer an attractive avenue for in vitro identification of hCAR activators.

Ad/EYFP-hCAR response to known hCAR activators

A series of 22 compounds have been chosen to evaluate the correlation between chemical-mediated hCAR nuclear accumulation and target gene induction in current studies. These compounds include known hCAR activators, hCAR deactivators, selective mouse CAR activators, selective activators of other nuclear receptors, and known or suspected CYP2B inducers for which information regarding hCAR activation is not available (Table 1). With no activation, the profusely expressed Ad/EYFP-CAR was observed primarily in the cytoplasm of cultured hepatocytes (84.5%), while around 15.5% infected cells displayed nucleus or mixed (nucleus + cytoplasm) allocation (Fig. 2A, and 2B). On the contrary, all the eight known hCAR activators resulted in
remarkable nuclear accumulation after 24 hrs treatment, where nucleus and mixed hCAR distribution accounts for approximately 90% of the infected hepatocytes (Fig. 2C). Representative images of hCAR localization were demonstrated in Fig. 2A and 2B following the treatment of vehicle control (0.1% DMSO), the typical hCAR activator PB (1 mM) or CITCO (1 µM). Notably, the selective hCAR agonist, CITCO revealed a unique pattern of hCAR translocation in which up to 64.5% of infected cells exhibit mixed hCAR distribution (Fig. 2B).

**Ad/EYFP-hCAR response to selective activators of other nuclear receptors**

Liver is enriched with various transcription factors that govern the regulation of both constitutive and inducible DME expression (Tirona and Kim, 2005). To determine the specificity of chemical-mediated hCAR translocation, several typical activators of other nuclear receptors including RIF (10 µM) for PXR, CDCA (50 µM) for farnesoid X receptor (FXR), HOC (10 µM) for liver X receptor (LXR), 3MC (5 µM) for aryl hydrocarbon receptor (AhR), and Wy-14,643 (50 µM) for peroxisome proliferator-activated receptor α (PPARα) were characterized in HPH infected with Ad/EYFP-hCAR (Fig. 3). Representative images of hCAR localization were demonstrated in Fig. 3A and 3B following the treatment of vehicle control (0.1% DMSO), the typical hPXR activator RIF (10 µM) or AhR activator 3MC (5 µM). As demonstrated in Fig. 3C, none of the selective activators of these other nuclear receptors caused significant shift of Ad/EYFP-hCAR from the cytoplasm to the nucleus compared with the vehicle control, indicating the chemical-selectivity of Ad/EYFP-hCAR nuclear accumulation in HPH.

**Translocation of Ad/EYFP-hCAR by mCAR agonist and hCAR antagonists**

TCPOBOP is a known selective and potent mCAR agonist, and robustly induces
Cyp2b10 expression in mouse liver and cultured hepatocytes (Honkakoski et al., 2003). In order to assess the species-specificity of Ad/EYFP-hCAR translocation, infected HPH were treated with TCPOBOP (250 nM) for 24 hrs. As shown in Fig. 4, treatment of TCPOBOP was not associated with nuclear accumulation of Ad/EYFP-hCAR in human hepatocytes. Intriguingly, a recent study showed that the typical PPARα ligand, Wy-14,643 remarkably translocated mCAR from the cytoplasm to the nucleus in mouse liver (Guo et al., 2007), whereas our results revealed that Wy-14,643 neither enhanced the nuclear accumulation of hCAR in HPH (Fig. 3) nor induced the expression of CYP2B6 in HPH (data not shown). Together, these data underscore the species-selectivity of Ad/EYFP-hCAR translocation in HPH. Conversely, two recently reported hCAR antagonists, CLZ and PK11195 resulted in a significant nuclear accumulation of Ad/EYFP-hCAR in cultured human hepatocytes (Fig. 4C), signifying the complexity of the mechanisms underlying CAR activation (Li et al., 2008; Moore et al., 2000). In a parallel experiment, the indirect CAR deactivator, OA (10 nM) alone exhibited no effects on the translocation of Ad/EYFP-hCAR (Fig. 4A). Intriguingly, after 1 hr pretreatment of OA (10 nM), the cotreatment of OA + PB (0.5 or 1 mM) failed to inhibit PB-mediated hCAR nuclear translocation in HPH (Supplemental Fig. 1).

**Correlation between hCAR translocation and CYP2B6 induction**

The CYP2B genes are typical CAR targets in different species. Although other nuclear receptors such as PXR also mediate CYP2B induction, activation of CAR is closely associated with the induction of CYP2B in a species-specific manner. In the current study, a number of known or suspected CYP2B inducers without available hCAR activation data have been evaluated by the Ad/EYFP-hCAR translocation assays. These
compounds include four reported rodent CYP2B inducers MCB, BHA, DZP, and FLU (Sun and Fukuhara, 1997; Parkinson et al., 2006; Sun et al., 2006; Sun et al., 2007). As shown in Fig. 5, three out of four compounds (BHA, DZP, and MCB) exhibited remarkable capacity of translocating hCAR to the nucleus of HPH. However, one of the four rodent CYP2B inducers, FLU did not shift Ad/EYFP-hCAR towards the nucleus of treated HPH.

To determine whether the hCAR nuclear accumulation correlates with its target induction, HPH were treated with the four rodent CYP2B inducers as described in Material and Methods. Total RNA and proteins were prepared from the treated cells for determining the induction of CYP2B6 and CYP3A4 in human liver. The results demonstrated that DZP and MCB significantly enhanced the expression of CYP2B6 and CYP3A4 at both mRNA and protein levels, while BHA exhibited selective induction of CYP2B6 over CYP3A4 (Fig. 6A, 6B and 6C). In contrast, FLU (50 µM) had little, if any induction effects on either CYP2B6 or CYP3A4. Taken together, these data indicate that nuclear translocation of Ad/EYFP-hCAR correlates well with its target gene inducible expression.

Activation of hCAR in cell-based reporter assays

HepG2 cells transfected with hCAR expression and CYP2B6 reporter constructs exhibited high basal reporter activity and were not sensitive to chemical-mediated activation, as expected (Kawamoto et al., 1999). On the other hand, we previously observed that the potent hCAR deactivator, PK11195 significantly inhibited the constitutive activity of hCAR in HepG2 cells, and the inhibited hCAR activity could only be reactivated by direct activator CITCO but not by indirect activators such as PB (Li et
al., 2008). In the current reporter experiment, little or no reactivation was observed after the cotreatment with PK11195, and BHA, DZP, or MCB at 50 µM /each (Fig. 6D). Combined with the observed hCAR translocation data in HPH, these results indicate that BHA, DZP, and MCB most likely activate hCAR through PB-type indirect mechanisms.
Discussion

Although there is an emerging need for efficient screening of hCAR activators at the early stage of drug development, CAR is constitutively activated in all the immortalized cell lines independent of xenobiotic stimulation. In addition, CAR displays unique activation mechanisms compared with other nuclear receptors, where CAR could be activated through either direct ligand binding or indirect phosphorylation/dephosphorylation-related signaling pathways (Kawamoto et al., 1999; Qatanani and Moore, 2005). These CAR features significantly lowered the value of cell-based reporter and \textit{in vitro} ligand binding assays, making the investigation of CAR activation much more challenging. In contrast to the observations in immortalized cells, CAR is primarily compartmentalized in the cytoplasm of primary cultured hepatocytes and intact liver \textit{in vivo}, and only accumulates in the nucleus upon chemical-mediated activation. Nuclear translocation in hepatocytes appears to be the essential first step of xenobiotic-induced CAR activation, and may offer a novel avenue for predicting CAR activation. However, \textit{in vitro} detection of hCAR translocation has been difficult and time-consuming, partly due to the quiescent nature of human primary hepatocyte cultures. In this report, we have generated an Ad/EYFP-hCAR construct that infects HPH with high efficiency and maintains hCAR distribution characteristics in a physiologically relevant manner.

Several lines of evidence indicate that activation of CAR is a multi-step process, with nuclear accumulation as the essential first stride (Kawamoto et al., 1999; Sueyoshi et al., 2002). To determine the value of Ad/EYFP-hCAR transduced HPH as a tool for screening hCAR activators \textit{in vitro}, the current study evaluated 22 compounds including known hCAR activators, deactivators, typical activators of other nuclear receptors,
mCAR activators, and known or suspected CYP2B inducers without available CAR activation data for the correlation between hCAR nuclear translocation and target gene induction. Thirteen compounds resulted in significant nuclear accumulation of Ad/EYFP-hCAR in HPH. Among them, eight compounds (PB, CITCO, ART, PHN, EFV, NVP, CPZ, and CMZ) are known hCAR activators and CYP2B6 inducers (Sueyoshi et al., 1999; Maglich et al., 2003; Wang et al., 2004; Faucette et al., 2007), and three compounds (BHA, DZP, and MCB) are newly established human CYP2B6 inducers by the current studies, which display a total of 85% (11/13) correlation of Ad/EYFP-hCAR nuclear translocation with hCAR target gene induction (Sun and Fukuhara, 1997; Parkinson et al., 2006; Sun et al., 2007). In contrast, we also observed that two reported hCAR deactivators (CLZ and PK11195) remarkably translocated Ad-EYFP-hCAR to the nucleus of HPH, which are consistent with our previous reports (Li et al., 2008; Wang and Tompkins, 2008). Similarly, Guo et al (Guo et al., 2007) reported that the typical PPARα agonist Wy-14,643 acts as an antagonist of mCAR and stimulates mCAR nuclear translocation without accompanied transcriptional enhancement of CAR target gene in mouse liver. Conversely, the known indirect CAR deactivator, OA alone has no effects on Ad/EYFP-hCAR translocation, and moreover, cotreatment of OA with PB didn’t inhibit the PB-mediated hCAR nuclear accumulation in HPH (supplemental Fig. 1). This observation is in contrast with an early report from Dr. Negishii and colleagues, where OA treatment inhibited PB-mediated mCAR translocation in mouse primary hepatocytes (Kawamoto et al., 1999). Although the species-specificity of hCAR vs. mCAR might be one of the reasons for the controversial effects of OA between human and mouse, detailed explanations are beyond the scope of the current studies. Given the antagonistic
nature of CLZ and PK11195, along with the agonistic property of CITCO, it is reasonable to speculate that ligand binding itself may trigger CAR nuclear accumulation regardless of its agonistic or antagonistic nature.

Although hCAR shares some common characteristics with its rodent counterparts, apparent species-specific activation between human and rodent CARs render the necessity for evaluating hCAR activation in the physiologically relevant in vitro system-HPH. In the current study, TCPOBOP, the most effective mCAR agonist identified thus far, exhibited negative nuclear translocation of hCAR in HPH. Notably, our results also showed that Wy-14,643 failed to reallocate hCAR to the nucleus of HPH (Fig. 3B), which are in contrast to the observation made by Guo et al (Guo et al., 2007) in adenoviral-mCAR infected mouse liver, emphasizing the species-selectivity of hCAR activation. To date, accumulating evidence has revealed that liver is abundant with xenobiotic receptors that coordinately regulate the expression of their target genes through cross-talking. For instance, PB activates both hCAR and hPXR, while RIF selectively actives hPXR. Activation of hCAR and hPXR induces the expression of a broad spectrum of DMEs and transporters in the liver. To gain insight into the chemical-specificity of Ad/EYFP-hCAR nuclear translocation, five selective activators of other xenobiotic receptors have been evaluated in Ad/EYFP-hCAR infected HPH. Without exception, typical activators of PXR (RIF), AhR (3MC), LXR (HOC), FXR (CDCA), and PPARα (Wy-14, 643) all failed to significantly relocate Ad/EYFP-hCAR from the cytoplasm to the nucleus in HPH. Together, current evidence suggests that the Ad/EYFP-hCAR translocation assay in HPH exhibits both species-specific and chemical-specific selectivity in hCAR activation.
Due to the apparent difficulties associated with identifying hCAR activators, particularly in a higher throughput fashion, the numbers of known hCAR activators thus far, are relatively small. In the current study, we also evaluated the hCAR nuclear translocation by four reported rodent CYP2B (FLU, BHA, DZP, and MCB) inducers that have no available information regarding hCAR activation. Notably, three out of the four compounds exhibited significant nuclear translocation of the Ad/EYFP-hCAR in HPH. Through a combination of direct and indirect experimental approaches, our results showed that BHA, DZP, and MCB mediated hCAR nuclear translocation is closely associated with the actual induction of their target gene CYP2B6 and CYP3A4. To our knowledge, this is the first report utilizing hCAR translocation assay in HPH to successfully identify novel CYP2B6 and CYP3A4 inducers. Notably, although FLU is a potent inducer of CYP2B1 in rat (Sun et al., 2006), there is barely visible induction of either CYP2B6 or CYP3A4, and FLU did not enhance the nuclear translocation of Ad/EYFP-hCAR in HPH. These data further highlight the species-specificity and the value of the Ad/EYFP-hCAR translocation assay in HPH.

In summary, our data suggest that Ad/EYFP-hCAR infection of HPH provides a valuable tool for efficiently identifying hCAR activators in vitro. Because of the unique characteristics of hCAR activation, Ad/EYFP-hCAR translocation assay in HPH exhibits several apparent advantages over the cell-based reporter assays in cell lines and in vitro ligand binding assays, regarding the sensitivity to chemical stimulation and responsiveness to both direct and indirect activators. Meanwhile, we do realize that one limitation of this screening test is the incapability of discerning the agonist and antagonist of hCAR. Nonetheless, we have demonstrated recently that the constitutive activation of
hCAR in HepG2 cells was efficiently repressed by hCAR antagonist PK11195, and was reactivated by hCAR agonist CITCO but not by indirect activators such as PB and PHN (Li et al., 2008). Utilizing this reactivation assay, our results indicated that all three novel hCAR activators identified in the current study are most likely indirect activators (Fig. 6D). Overall, the combination of Ad/EYFP-hCAR translocation assay in HPH with the hCAR reactivation assay in HepG2 cells offers a valuable avenue for the identification of hCAR activators in vitro.
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oltipraz also activates the constitutive androstane receptor. *Drug Metab Dispos* **36**:1716-1721.


Footnotes

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Figure Legends

Fig. 1. Localization of Ad/EYFP-hCAR in HepG2 cells and human primary hepatocytes (HPH). HepG2 cells and HPH (HL-#009, HL-#014) were infected with Ad/EYFP-hCAR as described in Material and Methods. (A) Confocal images depict the localization of Ah/EYFP-hCAR in HepG2 and HPH. The left panels are Ad/EYFP-hCAR expression (green); the middle panels represent the nuclear staining (red); and the merged images are on the right. (B, C) 100 hCAR-expressing cells from each group were counted and classified by cytosolic, nuclear, or mixed (cytosolic + nuclear) hCAR cellular localizations.

Fig. 2. Known hCAR activators promote nuclear translocation of Ad/EYPF-hCAR in HPH. HPH (HL-#009, HL-#014) were infected with Ad/EYFP-hCAR described in Materials and Methods and treated with vehicle (0.1% DMSO), or eight known hCAR activators at the indicated concentrations. Following 24 hrs of treatment, hepatocytes were DAPI stained and subjected to confocal microscopy. (A) Representative Ad/EYFP-hCAR localizations from vehicle control, PB (1 mM) or CITCO (1 µM) treated HPH. Three panels are shown for each treatment, with the left, Ad/EYFP-hCAR (green); the middle, nuclear staining (red); and the right, the merged image. (B, C) For each treatment, 100 hCAR-expressing cells were counted and classified based on cytosolic, nuclear, or mixed (cytosolic + nuclear) hCAR cellular localizations.

Fig. 3. Ad/EYFP-hCAR was not translocated by typical activators of other nuclear receptors. HPH (HL-#009, HL-#014) were infected with Ad/EYFP-hCAR as described in Materials and Methods and treated with vehicle (0.1% DMSO), or five typical
activators of other nuclear receptors at the indicated concentrations. Following 24 hrs of
treatment, hepatocytes were subjected to confocal microscopy. (A) Representative
Ad/EYFP-hCAR localizations from vehicle control, RIF (10 µM) and 3MC (5 µM)
treated HPH. (B, C) For each treatment, 100 hCAR-expressing cells were counted and
classified based on cytosolic, nuclear, or mixed (cytosolic + nuclear) hCAR localizations.

**Fig. 4.** Translocation of Ad/EYFP-hCAR in HPH following the treatment with mCAR
activator and hCAR deactivators. HPH (HL-#009, HL-#014) were infected with
Ad/EYFP-hCAR as described in *Materials and Methods* and treated with vehicle (0.1%
DMSO), or tested compounds at indicated concentrations. Following 24 hrs of treatment,
hepatocytes were subjected to confocal microscopy analysis. (A) Representative
Ad/EYFP-hCAR localizations from vehicle control, PK1195 (10 µM), OA (0.01 µM), or
TCPOBOP (250 nM) treated HPH. (B, C) For each treatment, 100 hCAR-expressing
cells were counted and classified based on cytosolic, nuclear, or mixed (cytosolic +
nuclear) hCAR localizations.

**Fig. 5.** Ad/EYFP-hCAR localization in HPH following the treatment with CYP2B
inducers. HPH (HL-#009, HL-#014) were infected with Ad/EYFP-hCAR as described in
*Materials and Methods* and treated with vehicle (0.1% DMSO), or tested compounds at
indicated concentrations. Following 24 hrs of treatment, hepatocytes were subjected to
confocal microscopy analysis. (A) Representative images illustrate Ad/EYFP-hCAR
localizations in HPH treated with vehicle control, FLU (50 µM), BHA (100 µM), DZP
(50 µM), or MCB (50 µM). (B, C) For each treatment, 100 hCAR-expressing cells were
counted and classified based on cytosolic, nuclear, or mixed (cytosolic + nuclear) hCAR localizations.

**Fig. 6.** Effects of FLU, BHA, DZP, and MCB on CYP2B6/CYP3A4 expression in HPH, and hCAR reactivation in HepG2 cells. HPH (HL-#012, HL-#014) were treated with FLU (50 µM), BHA (100 µM), DZP (50 µM), MCB (50 µM), RIF (10 µM), CITCO (1 µM) or vehicle control (0.1% DMSO). Total RNA extracted from 24 hrs treatment was subjected to real-time PCR analysis (A, B). Homogenates (20 µg) harvested from 72 hrs treatments were prepared for CYP2B6 and CYP3A4 immunoblot analysis (C). In a separate experiment, HepG2 cells were transfected with CAR expression plasmid along with CYP2B6-2.2kb reporter plasmid as described under Materials and Methods. Cells were treated with vehicle (0.1% DMSO) and test compounds alone or in combination with PK11195 (5 µM). Luciferase activities were determined and expressed relevant to control (D). Data represent the mean ± SD of three independent transfections. (*p < 0.05; **p < 0.01).
### Table 1. Classification of compounds used in the screening test

<table>
<thead>
<tr>
<th>Classification</th>
<th>Compounds</th>
<th>References</th>
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<tr>
<td>known hCAR activators</td>
<td>CITCO; artemisinin; phenobarbital;</td>
<td>Wang et al., 2003;</td>
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<td></td>
<td>phenytoin; chlorpromazine;</td>
<td>Yamamoto et al., 2003;</td>
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<td></td>
<td>carbamazepine; efavirenz; nevirapine</td>
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<td>Faucette et al., 2007;</td>
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<td>selective activators of other nuclear receptors</td>
<td>rifampcin; 3-methylcholanthrene; Wy-14,643; chenodeoxycholic acid; 22(R)-hydroxycholesterol</td>
<td>Tirona and Kim, 2005;</td>
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<td>known hCAR deactivators</td>
<td>TCPOBOP and meclizine (mCAR activator); clotrimazole; PK11195; okadaic acid</td>
<td>Kawamoto et al., 1999; Honkakoski et al., 2003; Huang et al., 2004;</td>
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<tr>
<td>Rodent CYP2B inducers without available CAR information</td>
<td>fluconazole; diazepam; myclobutanil; butylated hydroxyanisole</td>
<td>Sun and Fukuhara, 1997; Parkinson et al., 2006; Sun et al., 2006; Sun et al., 2007</td>
</tr>
</tbody>
</table>
Figure 1

A

EYFP-hCAR  Nucleus  Merge

HepG2

HPH

B

Percentage

0 20 40 60 80 100 120

HepG2

HPH

Cytosolic

Mixed

Nuclear
Figure 2

A

EYFP-hCAR  Nucleus  Merge

0.1% DMSO

PB 1 mM

CITCO 1 µM

B

Percentage

0.1% DMSO  PB 1 mM  CITCO 1 µM

C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytosolic (%)</th>
<th>Nuclear (%)</th>
<th>Mixed (%)</th>
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<td>PB 1 mM</td>
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<td>CITCO 1 µM</td>
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Figure 3

A

Ad/EYFP-hCAR localization

0.1% DMSO  RIF 10µM  3MC 5µM

B

Percentage

Cytosolic (%) Nuclear (%) Mixed (%) Total

HL-#009  HL-#014  HL-#009  HL-#014  HL-#009  HL-#014  HL-#009  HL-#014

0.1% DMSO  87  82  4  3  9  15  100  100
RIF10µM  89  82  3  6  8  12  100  100
3MC 5µM  85  80  4  3  11  17  100  100
Wy-14643 50µM  77  82  5  3  18  15  100  100
CDCA 50µM  81  72  3  9  16  19  100  100
HOC 10µM  78  76  3  12  19  12  100  100
Figure 4

Ad/EYFP-hCAR localization

A

0.1% DMSO  PK11195 10μM  OA 10nM  TCPOBOP 250 nM

B

Percentage

Cytosolic (%) Nuclear (%) Mixed (%) Total

C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytosolic (%)</th>
<th>Nuclear (%)</th>
<th>Mixed (%)</th>
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<tr>
<td>PK11195 10μM</td>
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<td>81</td>
<td>11</td>
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<tr>
<td>CLZ 20μM</td>
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<tr>
<td>OA 10nM</td>
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<td>14</td>
<td>100</td>
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<tr>
<td>TCPOBOP 250nM</td>
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<tr>
<td>MLZ 20μM</td>
<td>85</td>
<td>5</td>
<td>10</td>
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</table>
Figure 5

Ad/EYFP-hCAR localization

A

0.1% DMSO  FLU 50µM  BHA 100µM  DZP 50µM  MCB 50µM

B

Percentage

0 10 20 30 40 50 60 70 80 90 100

0.1% DMSO  FLU 50µM  BHA 100µM  DZP 50µM  MCB 50µM

C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytosolic (%)</th>
<th>Nuclear (%)</th>
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<td>FLU 50µM</td>
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<td>BHA 100µM</td>
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<td>MCB 50µM</td>
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<td>DZP 50µM</td>
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</table>
Figure 6

A. CYP2B6

B. CYP3A4

C. Western Blot: CYP2B6, CYP3A4, β-Actin

D. Relative Luciferase Activity

0.1% DMSO FLU BHA DZP MCB RIF CITCO

DMSO 50µM 100µM 50µM 50µM 10µM 1µM

PK11195 5µM