Published in final edited form as:

Mol Pharmacol. 2008 August ; 74(2): 443–453. doi:10.1124/mol.108.046656.

The Peripheral Benzodiazepine Receptor Ligand 1-(2-Chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide Is a Novel Antagonist of Human Constitutive Androstane Receptor

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Abstract

As a promiscuous xenobiotic sensor, the constitutive androstane receptor (CAR; NR1I3) regulates the expression of multiple drug metabolizing enzymes and transporters in liver. The constitutively activated nature of CAR in the cell-based transfection assays has hindered its utilization as a predictor of metabolism-based drug-drug interactions. Here we have identified PK11195 [1-(2-Chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide], a typical peripheral benzodiazepine receptor (PBR) ligand, as a selective and potent inhibitor of human (h) CAR. In cell-based transfection assays, PK11195 inhibited the constitutive activity of hCAR more than 80% at the concentration of 10 μ M, and the PK11195-inhibited activity was efficiently reactivated by the direct CAR activator, CITCO but not by the indirect hCAR activator, phenobarbital. Mammalian two hybrid, and GST pull-down assays showed that PK11195 repressed the interactions of hCAR with the co-activators SRC1 and GRIP1 to inhibit hCAR activity. The inhibition by PK11195 specifically occurred to the hCAR: PK11195 strongly activated human PXR, while did not alter the activity of the mouse CAR and mouse PXR. In addition, PBR played no role in the PK11195 inhibition of hCAR since the inhibition fully occurred in the HeLa cells in which the PBR was knocked down by siRNA. In the *Car*^{-/-} mouse liver, PK11195 translocated EYFP-hCAR into the nucleus. These results are consistent with the conclusion that PK11195 is a novel hCAR specific antagonist that represses the CAR-co-activator interactions to inhibit the receptor activity inside the nucleus. Thus, PK11195 can be used as a chemical tool for studying the molecular basis of CAR function.

Introduction

The constitutive androstane receptor (CAR, NR1I3) is an important transcription factor that transfers endogenous and exogenous stimuli into cellular responses by regulating the expression of its target genes that code for cytochrome P450 (CYP) and transport proteins

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This research was supported by National Institute of Health Grant DK061652 (to H.W) and by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences.

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(Huang et al., 2004; Maglich et al., 2004; Sueyoshi et al., 1999; Tzameli et al., 2000; Ueda et al., 2002). Thus, CAR organizes a cellular defense system against xenobiotic insults by increasing hepatic capacity for metabolism and excretion. Besides its role in drug metabolism, CAR is also reported to have the ability to regulate various hepatic functions, including gluconeogenesis, fatty acid oxidation, the metabolism of steroid hormones and bilirubin (Huang et al., 2003; Kodama et al., 2004; Sugatani et al., 2001; Ueda et al., 2005). Moreover, CAR promotes the development of hepatocellular carcinoma in phenobarbital (PB)-treated mice (Huang et al., 2005; Kodama et al., 2004).

Although the importance of CAR in the regulation of liver function becomes clear, the molecular mechanism underlying CAR activation is still elusive. In contrast to most NRs, CAR displays high constitutive activity in the absence of agonistic ligand binding (Baes et al., 1994). CAR is also characterized as a NR that could be activated by either direct ligand binding, such as 6-(4-chlorophenyl)imidazo [2,1-*b*][1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl) oxime (CITCO) (Maglich et al., 2004) or indirect activation, such as phenobarbital (PB) (Honkakoski et al., 2003; Maglich et al., 2004). In intact liver, CAR is localized predominantly in the cytoplasm in the absence of CAR activators, but is accumulated in the nucleus after exposure to PB-type compounds (Kawamoto et al., 1999; Wang et al., 2004). Thus, nuclear translocation of CAR has been established as the initial step for CAR-mediated transcriptional activation. In contrast, this nuclear receptor exhibits a high level of constitutive transcriptional activity in immortalized cells and accumulates spontaneously in nuclei. This characteristic of CAR in cell lines has become one of the major difficulties in elucidating the mechanism underlying CAR activation. Alternatively, identification of highly effective CAR antagonists would provide novel tools to study the molecular basis of CAR function, and offer new molecules with potential clinical applications. Although hCAR exhibits several common characteristics with its rodent counterparts, there are distinct biochemical differences between rodent and hCAR. For example, TCPOBOP can bind and activate mouse but not human CAR, and the androstane metabolites androstanol and androstenol repress the constitutive activity of mouse CAR in HepG2 cells, but have only marginal effect on hCAR (Forman et al., 1998; Tzameli et al., 2000). Moreover, rodent but not hCAR is susceptible to calcium/calmodulin-dependent kinase (CAMK)-mediated inhibition of PB induction of CYP2B expression and CAR transactivation of phenobarbital-responsive enhancer module (PBREM) (Kawamoto et al., 2000; Yamamoto et al., 2003). Among the few known antagonistic modulators of hCAR, the effect of clotrimazole (CLZ) on the constitutive activity of hCAR is contradictory [ranging from repressing (Tzameli et al., 2000) to no effect (Toell et al., 2002) and to activating (Jyrkkari et al., 2005)] in different cell lines. Recently, meclizine was reported to act as a moderate inverse agonist of hCAR which inhibits the hCAR activity approximately 50% in cell-based transfection assays (Huang et al., 2004).

Here, we have found that PK11195 the well-known PBR ligand, is a specific and potent antagonist of hCAR in both HepG2 cells and human primary hepatocytes. Moreover, we employed siRNA knock-down, mammalian two-hybrid, GST pull-down, and *in situ* expression of hCAR in the *Car*^{-/-} mouse liver to further investigate the molecular mechanism by which PK11195 inhibits the hCAR activity. Overall, identification of PK11195 as a novel functional antagonist of human CAR provides a useful chemical tool for studying the molecular basis of hCAR function.

Material and Methods

Chemicals and Biological Reagents

PK11195, PB, rifampicin (RIF), Picrotoxin, Ro5-4864, FGIN-1-27, carbamezapine (CMZ), and sulforaphane (SFN) were purchased from Sigma-Aldrich (St. Louis, MO). CITCO was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Oligonucleotide

primers and TaqMan fluorescent probes were synthesized by Sigma Genosys (The Woodlands, TX) and Applied Biosystems (Foster, CA), respectively. The Dual-Luciferase Reporter Assay System was purchased through Promega (Madison, WI). siGENOME Duplex specific for PBR or Non-Target was synthesized by Dharmacon (siRNA-BPR cat# J-009559-05-0005; siRNA-NT cat# D-001210-01-05). Matrigel, insulin and ITS⁺ were obtained from BD Biosciences (Bedford, MA). Other cell culture reagents were purchased from Invitrogen (Calsbad, CA) or Sigma-Aldrich.

Plasmid constructions

The pCR3-hCAR, hCAR/pEYFP-c1, and (NR1)₅pGL3-tk were generated as described previously (Sueyoshi et al, 1999, Kawamoto et al., 1999; Zelko et al., 2001). Plasmids used for mammalian two hybrid assay, pG5-*Luc*, pACT, and pBind were obtained from Promega. pACT-hPXR, pM-SRC-1 (621–765), pBind-SMRT (981–1450) were described in previous reports (Chang et al., 1999; Ueda et al., 2005). Human CAR full length coding DNA was cloned into BamHI and XbaI sites of pACT (pACT-hCAR). Plasmids for GST pull-down assay, pcDNA3.1-hGRIP1 and pcDNA3.1-mSRC-1 were described previously (Hosseinpour et al., 2006; Ueda et al., 2002). The pSG5-hPXR expression vector and the CYP3A4-PXRresponse element (PXRE)/XREM luciferase reporter construct [p3A4-362(7836/7208ins)] were obtained from Drs. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX) and Bryan Goodwin (GlaxoSmithKline, Research Triangle Park, NC), respectively. The CYP2B6 reporter constructs, containing the proximal PBREM (CYP2B6-1.8kb) or both PBREM and the distal XREM (CYP2B6-2.2kb) were generated as described previously (Sueyoshi et al., 1999; Wang et al., 2003). The pRL-TK Renilla luciferase plasmid used to normalize firefly luciferase activities were from Promega.

Transfection Assays in Cell Lines

HepG2 or HeLa cells in 24-well plates were transfected with hCAR or hPXR expression vector, and CYP2B6-2.2kb, CYP2B6-1.8kb, (NR1)₅pGL3-tk, or CYP3A4-PXRE/XREM reporter constructs 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 the concentration of PK11195 (10 μM), PicROTOXIN (10 μM), Ro5-4868 (10 μM), FGIN-1-27 (10 μM), CMZ (30 μM), CITCO (1 μM), or RIF (10 μM) for 24 hrs. 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. To analyze PXR- and CAR-mediated CYP3A4 expression in HepG2 and Huh7 cells, cells seeded in 12-well plates were transfected with hCAR or hPXR expression vectors, then treated with PK11195 (10 μM), CITCO (1 μM), or RIF (10 μM) for 24 hr. Total RNA was isolated using RNeasy Mini Kit (Qiagen).

Human Primary Hepatocytes Cultures and Treatments

Liver tissues were obtained by qualified medical staff following donor consent 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 previously described (LeCluyse et al., 2005). Hepatocytes were seeded at 1.5×10^6 cells/well in six-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. After 4 to 6 h of attachment at 37°C in a humidified atmosphere of 5% CO₂, cells were overlaid with Matrigel (0.25 mg/ml) in William's Medium E (WME) supplemented with ITS⁺ (insulin, transferrin, and selenium), 0.1 μM dexamethasone, 100 U/ml penicillin and 100 μg/ml streptomycin. The hepatocytes were maintained for 36 hrs before the treatment with

PK11195 (10 μ M), SFN (25 μ M), RIF (10 μ M), CITCO (1 μ M) or co-treatment of SFN with PK11195, RIF, or CITCO, respectively, for another 24 hrs.

Determination of CYP3A4 and CYP2B6 mRNA Expressions by Real-Time PCR Analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using High Capacity cDNA Archive kit (Applied Biosystems, Foster, CA) following the manufacturers' instructions. Primers and probes for CYP2B6 and CYP3A4 mRNA detection are as follows, in the order of forward primer, probe, and reverse primer: CYP2B6 1299 to 1366 bp, 5-AAGCGGATTTGTCTTGGTGAA-3, 6-FAM-CATCGCCCGTGCGGAATTGTTC-TAMRA, and 5-TGGAGGATGGTGGTGAAGAAG-3; and CYP3A4 59 to 179 bp, 5-TCAGCCTGGTGTCTCTATCTAT-3, 6-FAM-TCCAGGGCCCACACCTCTGCCT-TAMRA, and 5-AAGCCCTTATGGTAGGACAAAATATTT-3. CYP2B6 or CYP3A4 mRNA expression was normalized against that of human β -actin, which was detected using a predeveloped primer/probe mixture (Applied Biosystems, Foster, CA). Multiplexed TaqMan PCR assays were performed in 96-well optical plates on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Fold induction values were calculated according to the equation $2^{\Delta\Delta C_t}$, where ΔC_t represents the differences in cycle threshold numbers between the target gene and β -actin, and $\Delta\Delta C_t$ represents the relative change in these differences between control and treatment groups.

siRNA Knockdown of the PBR in HeLa Cells

The siRNA specific for PBR and a Non-Targeting siRNA were obtained from Dharmacon. To detect knockdown of endogenous PBR, HeLa cells plated in 12-well plates were transfected with siRNA-PBR (40 pmol) or siRNA-NT (40 pmol), using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were harvested and total RNA was isolated and reverse-transcribed into cDNA as described above. PBR gene expression was measured using SYBR real-time PCR with specific primers for PBR (forward: 5-CTTCTTTGGTGCCCGACAAAT-3 and reverse: 5-GCCATACGCAGTAGTTGAGTG-3), and normalized against the housekeeping gene GAPDH. Detection of the PCR products was done with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster, CA). Gene knockdown were determined by calculating the $2^{\Delta\Delta C_t}$ values as described above. PCR products (15 μ l each), at 29 cycles for PBR and 26 cycles for GAPDH were also subjected to electrophoresis in 2% agarose gel. In cell-based reporter assays, following 24 hrs of siRNA transfection, hCAR expression vector and CYP2B6-2.2kb reporter vector were also transfected in HeLa cells. The double transfected cells were treated with PK11195 (10 μ M) or CITCO (1 μ M) for 24 hrs, then subjected to dual-luciferase assays as described above.

In Vivo Gene Transfection and Confocal Microscopy

Car^{-/-} mice, as described previously (Ueda et al., 2002), were housed in a pathogen-free animal facility with standard 12 h light/dark cycles and provided autoclaved rodent chow and drinking water ad libitum. Animals weighing 18–20 g were used for hCAR translocation studies. In hCAR localization experiments, *Car*^{-/-} mice were injected with 10 μ g of EYFP-hCAR expression plasmid using the tail vein injection method (Sueyoshi et al., 2002; Wang et al., 2004). Treatment occurred at 3 hrs after gene delivery by i.p. injection of vehicle (DMSO, CTL), 15 mg/kg PK11195, or 100 mg/kg PB. Mouse livers were collected 8 hrs after the plasmid injection, embedded into Tissue-Tek OTC, and immediately frozen. Microscopic analysis of frozen liver sections was performed as described previously (Zelko et al., 2001). EYFP-hCAR was visualized in hepatocytes using a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss GmbH, Jena, Germany) at an excitation wavelength of 514 nm. For each treatment group, approximately 100 mouse hepatocytes expressing EYFP-hCAR were

counted per mouse liver and classified according to cytosolic, nuclear, or mixed (cytosolic and nuclear) hCAR localization. Livers from two mice were evaluated by confocal analysis for each treatment.

Mammalian two-hybrid assays

COS1 cells seeded in 24-well plates were transfected with 110 ng of the reporter gene plasmid pG5luc, 40 ng of expression plasmids encoding GAL4-DBD/coregulators fusions, 80 ng of expression plasmids encoding the respective VP16-AD/hCAR fusions and 20 ng of reference plasmid pRL-TK each well using Eugene 6 (Roche). Twenty four hours after transfection, the cells were treated with CITCO (1 μ M), PK11195 (10 μ M), RIF (10 μ M) or vehicle control (0.1% DMSO) for 24 hrs. Luciferase activities were measured in cell lysates using the Dual-Luciferase kit (Promega, WI) as described above. Data represent the mean $\pm$ S.D. of three individual transfections.

GST Pull-down Assay

The GST-hCAR fusion protein was expressed in *Escherichia coli* strain BL21 cells and purified using glutathione-Sepharose 4B (Amersham Biosciences). 35 S-labeled SRC-1 and GRIP-1 were produced from pcDNA3.1-mSRC1 and pcDNA3.1-hGRIP1 using the TNT T7 quick coupled transcription/translation system (Promega) along with 35 S-labeled methionine. GST-hCAR or GST coupled to the glutathione-Sepharose 4B was incubated with the 35 S-labeled SRC-1 or GRIP-1 in 50 mM HEPES buffer, pH 7.5, containing 0.1 M NaCl and 0.1% Triton X-100 for 20 min at room temperature in the presence of DMSO (0.1%), PK11195 (100 μ M), CITCO (1 μ M) or CITCO (1 μ M) + PK11195 (100 μ M). The resin was then recovered by centrifugation and washed three times in the same buffer. Proteins were extracted from the resin by heating for 10 min at 70 $^{\circ}$ C in NuPAGE LDS sample buffer (Invitrogen) and separated on 4–10% BisTris gel in NuPAGE MOPS SDS running buffer for 1h at 150 volts. The gel was then dried under vacuum, and proteins detected by autoradiography.

Statistical Analysis

All data represent at three independent experiments and are expressed as the mean $\pm$ S.D., Statistical comparisons were made using Students' *t*-test. Statistical significance was set at *p* values <0.05 (*), or <0.01 (**).

Results

Inhibition of the hCAR's Constitutive Activity by PK11195

First, we detected the effect of PK11195 on the constitutive activity of hCAR by transfection assay in HepG2 cells. It was found that PK11195 could efficiently reduce hCAR activity in a dose-dependent manner. Compared with vehicle control, the luciferase activities of CYP2B6-2.2kb reporter construct decreased by 85% after PK11195 (10 μ M) treatment (Figure 1B). In a similar experiment, transfected HepG2 cells were cotreated with PK11195 and a variety of hCAR activators. Interestingly only the direct activator of hCAR, CITCO, reversed the inhibitory effect of PK11195, while PB, the typical indirect activator of hCAR, had no effect on the PK11195-mediated deactivation (Figure 1B). This result suggests that PK11195 might bind specifically to the ligand binding domain of hCAR.

The Effect of PK11195 on the Activities of Other Nuclear Receptors

Because significant species-specific difference exists between hCAR and its rodent counterparts, expression vectors of mCAR, hCAR, mPXR, and hPXR were used to determine the inhibitory specificity of PK11195. HepG2 cells were transfected with CYP2B6 luciferase reporter constructs containing PBREM (CYP2B6-1.8kb), five-repeat of the NR1 (NR1)₅pGL3-

tk, or CYP3A4-PXRE/XREM in the presence of different CAR and PXR expression vectors. As demonstrated in Figure 2, hCAR activity was significantly suppressed by PK11195 in assays with all three reporter constructs. In contrast, only negligible activity changes of mouse CAR was observed after treatment with PK11195. Notably, PK11195 exhibits potent activation of human PXR through all three reporter constructs, whereas moderate activation of mPXR was observed only with the CYP3A4 construct (Figure 2C).

PK11195 Induction of CYP2B6 and CYP3A4 Gene Expression in Human Primary Hepatocytes

Given the fact that human CAR and PXR share several common target genes such as CYP2B6 and CYP3A4, we further evaluated the effects of PK11195 on the expression of CYP2B6 and CYP3A4 genes in human primary hepatocyte cultures. Human hepatocytes from two different livers were treated with PK11195, CITCO, or RIF in the presence or absence of a newly identified hPXR deactivator SFN as described in “materials and methods”. In agreement with previous reports, RIF efficiently induced both CYP2B6 and CYP3A4, while CITCO exhibited marked preferential induction of CYP2B6 over CYP3A4 in both hepatocyte cultures (Figure 3, A and B) (Faucette et al., 2006; Faucette et al., 2007). Notably, 10 μ M of PK11195 strongly induced the mRNA expression of both CYP2B6 and CYP3A4 in treated hepatocytes, and these inductions were significantly repressed by the cotreatment of 25 μ M of SFN. Concomitant treatment with SFN (25 μ M) and RIF nearly abolished the induction of CYP3A4 and CYP2B6 by RIF alone (Figure 3, A and B), in consistent with previous report (Zhou et al., 2007). In contrast, induction of CYP2B6 by the selective hCAR activator CITCO was only moderately repressed by SFN treatment. Collectively, these observations suggest that PK11195 induces CYP expression in human primary hepatocytes via the activation of endogenous PXR.

PK11195 inhibits the hCAR-mediated endogenous CYP3A4 expression

Because growing evidence demonstrates that both CAR and PXR regulate CYP3A4 gene expression (Xie et al., 2000), we further tested the distinct roles of PK11195 on hCAR and hPXR in HepG2 and Huh7 cells. Cells were transfected with hCAR or hPXR expression vectors and as expected, transfection of hCAR in HepG2 and Huh7 cells enhanced the basal expression of CYP3A4 gene due to the constitutive activation of hCAR in immortalized cell lines. Treatment of PK11195 resulted in significant repression of both basal and CITCO induced CYP3A4 mRNA expression levels (Figure 4A). Consistent with the results from our cell-based reporter assays (Figure 2), PK11195 also induced CYP3A4 mRNA expressions in hPXR transfected HepG2 and Huh7 cells (Figure 4B).

The effects of PBR ligands on the activation of hCAR and hPXR

Other than PK11195, a number of PBR ligands with diverse chemical structures have been identified previously (Roberge et al., 2004). To examine if other PBR ligands exhibit similar effects on the activation/deactivation of hCAR and hPXR, 4 known PBR ligands, Picrotoxin, Ro5-4864, FGIN-1-27, and CMZ (Figure 5A) were tested in cell-based transfection experiments. As demonstrated in Figure 5B, among all the tested PBR ligands only PK11195 substantially and picrotoxin moderately repressed the constitutive activity of hCAR, whereas PK11195 and FGIN-1-27 showed potent activation of hPXR, which were comparable to that of RIF, in reporter assays (Figure 5C). Recently, a hCAR splicing variant (hCAR3) has been identified with low basal activity, but could be activated by CITCO in cell-based transfection assays (Auerbach et al., 2005; Faucette et al., 2007). Utilizing a hCAR3 expression vector, our cell-based transfection assay revealed no obvious activation of hCAR3 by any of the tested PBR ligands (Figure 5D).

PK11195 deactivates hCAR through a PBR-independent pathway

To further test whether the deactivation effect of PK11195 on hCAR is mediated through a PBR signaling pathway, small interfering RNA (siRNA) specific for PBR was used to knockdown PBR expression in HeLa cells, which express high levels of endogenous PBR (Gonzalez-Polo et al., 2005). Forty eight hours after transfection of siRNA specific for PBR, the expression of PBR gene in HeLa cells was significantly down regulated compared with the control group transfected with non-targeting siRNA (Figure 6A). However, the knockdown of PBR expression did not affect the deactivation effect of PK11195 on hCAR in parallel cotransfection assays (Figure 6B). Thus, these results support the notion that PK11195-mediated inhibition hCAR is PBR independent.

The effect of PK11195 on CAR translocation in *CAR*^{-/-} Mice

CAR activation is a multi-step process with nuclear translocation as the initial step. Our results thus far demonstrate that PK11195 deactivates hCAR in immortalized cell lines; therefore, evidence from a physiologically relevant system is required to establish whether this effect is exerted at CAR translocation or at CAR activation inside the nucleus. *Car*^{-/-} mice were injected with EYFP-tagged hCAR or mCAR and treated with PK11195 or vehicle control as outlined in “materials and methods”. Confocal microscopy analysis of the liver sections revealed that EYFP-hCAR and FYFP-mCAR were primarily localized in the cytoplasm without treatment. Upon treatment with PK11195 or PB, EYFP-hCAR was efficiently accumulated in the nucleus (Figure 7C and D). Analysis of one hundred hCAR-expressing cells in liver sections from each treatment group showed that hCAR nuclear localization increased from 13.6 % (control) to 46.3% and 76%, whereas cytoplasmic localization decreased from 80% (control) to 40% and 17% after PK11195 and PB treatment, respectively (Figure 7A). In contrast, the cellular distribution of EYFP-mCAR in the transfected liver has not been altered by the treatment of PK11195 (Figure 7B). Overall, these results indicated that the deactivation of hCAR by PK11195 primarily occurred inside of the nucleus.

Interaction of hCAR with coactivators was inhibited by PK11195

Since CAR was demonstrated to have constitutive interactions with several co-activators (Huang et al., 2004; Tzameli et al., 2000), mammalian two-hybrid, and GST pull-down assays were used to further explore the mechanisms underlying the PK11195-mediated hCAR deactivation. In agreement with previous reports, hCAR was able to directly interact with SRC-1 and GRIP1 in the absence of ligand, and the addition of hCAR agonist CITCO further enhanced the interaction (Figure 8A and B). Notably, both basal and CITCO enhanced interactions of hCAR with SRC-1 and GRIP1 were significantly repressed by 10 μ M of PK11195. In our GST pull-down experiments, inclusion of CITCO clearly enhanced the direct interaction between hCAR and SRC-1 or GRIP1, and these increased binding was partially disrupted in the presence of PK11195 (Figure 8E). Given that both recruitment of coactivators to, and disruption of corepressors from NRs are accepted mechanisms associated with activation, we further tested whether PK11195 could recruit corepressors to hCAR. As shown in Figure 8C and 8D, two co-repressors (NcoR and SMRT) only marginally bound to hCAR in the absence of ligand, and the addition of PK11195 did not increase these interactions. PXR constitutively interacted with NcoR and SMRT in the absence of ligand, and these interactions were partially disrupted by the presence of RIF.

Discussion

In this study, we have identified PK11195, a well-known peripheral benzodiazepine receptor ligand used for the treatment of cancer, auto-immune diseases, and neurodegenerative diseases (Galiegue et al., 2003), is a selective and potent inhibitor of hCAR, and effectively represses hCAR-mediated gene expression in different cell lines. Previously only two compounds, CLZ

and meclizine, have been reported as selective hCAR antagonists with moderate deactivation activities. In cell-based transfection assays, the maximal deactivation of hCAR achieved by both CLZ and meclizine were approximately 50% of the constitutive activity levels in CV-1 and HepG2 cells (Huang et al., 2004; Tzamelis et al., 2000). Moreover, CLZ's antagonistic effects on hCAR were also challenged by the observations that instead of repression, CLZ consistently activated hCAR in HEK293 cell transfection assays (Honkakoski et al., 2001), and activated a splicing variant of hCAR (hCAR3) in COS1 cells (Auerbach et al., 2005). In contrast, our results demonstrated that PK11195 (10 μ M) could inhibit the activity of hCAR by 85% in HepG2 cells (Figure 1B), and this potent inhibition of hCAR was also observed in cell-based reporter assays using several other cell lines including HEK293 (data not shown). To our knowledge, PK11195 represents the most potent hCAR deactivator identified thus far.

Recent studies established that differences between hCAR and their rodent counterparts dictate the species-specific expression of target genes. For example, TCPOBOP activates mouse but not human CAR, androstanol represses mouse but not human CAR, and meclizine represses human but activates mouse CAR. Here, we show that PK11195 exhibited negligible effects on mouse CAR activation in multiple transfection assays. Interestingly, PK11195 also displayed potent activation of hPXR, complicating its effect on several PXR and CAR shared target genes. Indeed, PK11195 induced the expression of both CYP2B6 and CYP3A4 in human primary hepatocytes in which endogenous expression of NRs are well maintained. Furthermore, in agreement with *in vitro* reporter assays, the observed inductions of CYP2B6 and CYP3A4 in human hepatocytes were significantly repressed by a selective hPXR deactivator SFN. Given that human CAR and PXR hold both common and distinct target genes, PK11195 would provide a valuable chemical tool to illustrate CAR specific roles in regulating gluconeogenesis, and fatty acid oxidation (Kodama et al., 2004; Sugatani et al., 2001; Ueda et al., 2005). In addition, *Car*^{-/-} mice displayed enhanced weight loss during caloric restriction suggesting PK11195 may represent a potentially novel drug target for uncoupling metabolic rate from food intake with implications to obesity and its associated disorders (Huang et al., 2005; Maglich et al., 2004).

CAR displays unique activation mechanisms compared with other orphan nuclear receptors, requiring both nuclear translocation and nuclear activation. Notably, nuclear translocation of CAR could be induced by both indirect activators such as PB-type compounds and agonistic ligands such as CITCO. Several known mCAR deactivators exhibit their effects through blocking the nuclear accumulation of mCAR. For example, pretreatment of primary mouse and rat hepatocytes with okadaic acid, a protein phosphatase 2A (PP2A) inhibitor, results in decreased CAR nuclear accumulation and CYP2B induction by PB and TCPOBOP (Honkakoski and Negishi, 1998; Kawamoto et al., 1999). The mouse antagonists, androstanes, also cause CAR cytoplasmic retention in mouse hepatocyte cultures. In contrast, our results in CAR-null mice showed that PK11195 efficiently induced nuclear translocation of transfected EYFP-hCAR, even though it deactivated the constitutive activity of hCAR in HepG2 cells where hCAR was spontaneously accumulated in nuclei. It was also reported previously that TCPOBOP induced translocation of human CAR in CAR-null mice but was incapable of activating a Cyp2b10-PBREM reporter gene (Honkakoski et al., 2003). Together, these results argue against constitutive activation of nuclear localized CAR, and indicate that nuclear activation is one of the two required steps for target gene activation.

Several coactivators and corepressors have been reported to modulate the function of nuclear localized CAR. Ligand-independent interaction of hCAR with SRC-1, PGC-1 and GRIP-1 has been purported to confer constitutive activity to CAR (Miao et al., 2006; Muangmoonchai et al., 2001; Shiraki et al., 2003). Present studies further demonstrated that PK11195 could effectively inhibit co-activators (SRC-1 and GRIP1) binding to hCAR in mammalian two-hybrid, and GST pull-down assays. In contrast, the low binding of hCAR with corepressors

(NcoR and SMRT) was not changed after the treatment of PK11195. Since coactivator assembly was necessary for hCAR function in the nucleus (Huang et al., 2004; Tzamelis et al., 2000), these observations suggest that PK11195 mediates the inhibition of hCAR activity through disrupting the interaction of coactivators with hCAR in nucleus.

PK11195 is a typical ligand of peripheral benzodiazepine receptor, which is located at the surface of the mitochondria and has been associated with numerous biological functions, such as regulation of cell proliferation, stimulation of steroidogenesis, immunomodulation, and regulation of mitochondrial functions (Galiegue et al., 2003). Several structurally unrelated ligands of PBR including Picrotoxin, Ro5-4864, FGIN-1-27, and CMZ have been tested in current studies to evaluate their potential roles on hCAR activity. Intriguingly, PK11195 is the only PBR ligands which strongly repressed hCAR activation in cell-based transfection assays. Further investigation showed that PK11195 deactivation of hCAR activity was not affected by knockdown expression of the endogenous PBR gene. Therefore, these results indicate that the antagonistic effect of PK11195 on hCAR is mediated through PBR independent signaling pathways. Since PK11195 exerts promising clinical applications including regulation of cholesterol transport, synthesis of steroid hormones, and potential cancer therapeutics by enhancing apoptosis (Gonzalez-Polo et al., 2005), our present study could potentially broaden the therapeutic scope of PK11195.

In conclusion, accumulated evidence suggests that CAR may have evolved in mammals to function as both a metabolic “stress” receptor and a xenobiotic receptor. Despite its involvement in several physiologically protective mechanisms, CAR also may contribute to liver toxicity by exerting the same effects usually considered beneficial. Moore and colleagues demonstrated that CAR enhances acetaminophen toxicity by inducing enzymes involved in toxic metabolite formation and glutathione depletion (Zhang et al., 2002). Furthermore, CAR activation by PB and TCPOBOP results in sensitization to cocaine hepatotoxicity (Wei et al., 2000). Finally, CAR activation has been shown to mediate PB-induced liver tumor promotion in mice (Huang et al., 2005; Kodama et al., 2004). Thus, there appears to be a tenuous balance between the positive and negative effects of CAR. Because these beneficial and detrimental physiological effects of CAR have been demonstrated primarily with rodent isoforms, identification of PK11195 as a selective and potent hCAR deactivator will provide a unique tool for obtaining additional insight into the mechanism underlying hCAR activation.

Acknowledgements

We thank Rick Moore (NIEHS, National Institutes of Health, Research Triangle Park, NC) for helping with the mouse tail vein injection assays, Drs. Steve Klierer (University of Texas, Southwestern Medical Center, Dallas, TX), David Moore (Baylor College of Medicine, Houston, TX), and Bryan Goodwin (GlaxoSmithKline, Research Triangle Park, NC) for kindly providing pSG5-hPXR, GAL4-NCoR expression vectors and CYP3A4-PXRE/XREM reporter vector, respectively. We also thank Dr. Leslie Tompkins for insightful comments and critical reading of the manuscript. Human liver tissues were procured with the assistance of John Cottrell from University of Maryland at Baltimore Medical Center, Baltimore, MD.

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Abbreviations

CAR, constitutive androstane receptor
PXR, pregnane X receptor
PBR, peripheral benzodiazepine receptor
PK11195, 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide
NR, nuclear receptor
PB, phenobarbital
CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl) oxime
RIF, rifampicin
CMZ, carbamazepine
CLZ, clotrimazole
SFN, sulforaphane
SRC-1, steroid receptor coactivator-1
GRIP1, glucocorticoid receptor-interacting protein 1
NcoR, nuclear receptor corepressor
SMRT, silencing mediator of retinoid and thyroid hormone receptor
EYFP, enhanced yellow fluorescent protein

Figure 1. PK11195 deactivates constitutive activity of hCAR in HepG2 cells

(A) Structure of PK11195. (B) HepG2 cells were transfected with CYP2B6-2.2 kb reporter, and hCAR expression vectors. Transfected cells were treated with PK11195, CITCO, or PB at indicated concentrations for 24 hrs. Luciferase activities were determined and expressed relative to vehicle control (CT). Data represent the mean $\pm$ S.D. ($n=3$) (**, $P<0.01$ denotes comparison with DMSO group; #, $P<0.05$, and ##, $P<0.01$ denote comparison with 10 μ M PK11195 group).

Figure 2. PK11195 selectively inhibited the constitutive activity of hCAR

HepG2 cells were transfected with CYP2B6-1.8kb (A), (NR1)₅pGL3-tk (B), or CYP3A4-PXRE/XREM (C) reporter construct in the presence of mCAR, hCAR, mPXR, or hPXR expression vectors. Transfected cells were then treated with PK11195 (10 μ M) or 0.1% DMSO as vehicle control (CT). Dual luciferase activities were determined and expressed relative to CT. All data are presented as mean $\pm$ S.D. ($n=3$) (**, $p<0.01$).

Figure 3. Induction of CYP2B6 and CYP3A4 expression in human primary hepatocyte cultures
Human hepatocytes (UM-HL-001 and UM-HL-002) cultured in WME were treated for 24 hrs with PK11195 10 μ M, SFN 25 μ M, Picrotoxin 10 μ M, Ro5-4868 10 μ M, FGIN-1-29 10 μ M and CMZ 30 μ M, as well as co-treatment of SFN with PK11195, RIF, and CITCO, respectively. Total RNA was collected, reverse transcribed, and subjected to TaqMan real-time PCR. CYP2B6 and CYP3A4 expression levels were normalized against β -actin. Induction of CYP2B6 and CYP3A4 relative to control was calculated as described under "Materials and Methods". All data are presented as mean $\pm$ S.D. (n=3), (*, p<0.05; **, p<0.01).

Figure 4. The effect of PK11195 on hCAR or hPXR induced expression of CYP3A4
HepG2 and Huh7 cells were transfected with hCAR (A), or hPXR (B) expression vectors, and treated with PK11195 (10 μ M), CITCO (1 μ M), or RIF (10 μ M) for 24 hrs. Total RNA was collected, reverse transcribed, and subjected to TaqMan real-time PCR as described in “Materials and Methods”. All data are expressed as mean $\pm$ S.D. (n=3), (*, $p<0.05$; **, $p<0.01$).

Figure 5. The effects of PBR Ligands on the activity of hCAR and hPXR

Four structurally different PBR ligands (A) were selected in this reporter assay. HepG2 cells were transfected with hCAR (B), hPXR (C), or hCAR3 (D) expression vectors in the presence of CYP2B6-2.2kb reporter construct. Transfected cells were then treated with PBR ligands (PK11195 10 μ M, Picrotoxin 10 μ M, Ro5-4868 10 μ M, FGIN-1-29 10 μ M, or CMZ 30 μ M) for 24 hrs. CITCO 1 μ M and RIF 10 μ M were used as positive control for hCAR and hPXR activation, respectively. Luciferase activities were determined and expressed relative to CT. Data represent the mean $\pm$ S.D. ($n=3$) (*, $p<0.05$; **, $p<0.01$).

Figure 6. PK11195 deactivation of hCAR is independent of PBR

HeLa cells were transfected with siRNA-NT or siRNA-PBR. Forty-eight hour after transfection, downregulation of PBR gene was detected by real-time PCR (A). In parallel experiment, HeLa cells were first subjected to the specific siRNA-PBR for 24 hrs, the cells were subsequently transfected with hCAR expression vector and CYP2B6-2.2 kb reporter vector (B). The transfected cells were then treated with PK11195 (10 μ M) or CITCO (1 μ M). Luciferase activities were determined and expressed relative to CT. Data represent the mean $\pm$ S.D. ($n=3$) (**, $p<0.01$).

Figure 7. PK11195 translocates hCAR in CAR^{-/-} mouse liver

CAR^{-/-} mice were injected with 10 µg of expression plasmid encoding EYFP-tagged hCAR or EYFP-mCAR as described under “Materials and Methods” and treated 3 hrs later with vehicle (DMSO, CTL), 100 mg/kg PB, or 10 mg/kg PK11195. Mice livers were harvested 8 hrs after tail vein delivery and slides of frozen liver sections were prepared for confocal microscopy. Approximately 100 EYFP expressed cells were classified according to hCAR (A) or mCAR (B) localization status. Representative images depict examples of hCAR localization as EYFP-hCAR in yellow and nuclear staining in blue (C and D).

Figure 8. PK11195 disrupts co-activators binding to hCAR

Mammalian two-hybrid, assays were performed in COS1 cells transiently transfected with expression plasmids encoding VP16-AD/hCAR fusion proteins and GAL4-DBD/coregulators fusion proteins, as indicated, together with the reporter gene plasmid pG5luc. Cells were treated with DMSO (0.1% v/v), CITCO (1 μ M), or PK11195 (10 μ M) for an additional 24 hrs before determination of luciferase activities (A, B, C, and D). Data represent mean $\pm$ S.D. of three independent transfections (**, $p < 0.01$). GST pull-down assay (E). In vitro-translated 35S-labeled SRC-1 and GRIP1 were incubated with bacterially expressed GST-hCAR fusion protein in the presence of 0.1% DMSO, 100 μ M PK11195, or 1 μ M CITCO as indicated. GST was used as a negative control for binding, *in vitro* incubation, gel electrophoresis and autoradiography were done as described in "Material and Methods".