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**CINPA1 Is an Inhibitor of Constitutive Androstane Receptor (CAR) that Does Not Activate
Pregnane X Receptor (PXR)**

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Running title: CINPA1 Is a Novel and Unique CAR Inhibitor

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ABBREVIATIONS: CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo [2,1-b][1,3] thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime; ChIP, chromatin immunoprecipitation; CLZ, clotrimazole; CYP, cytochrome P-450; DMSO, dimethylsulfoxide; HPH, human primary hepatocytes; MDR1, multi-drug resistance 1; PBREM, phenobarbital-responsive enhancer module; PK, PK11195; PXR, pregnane X receptor; Rif, rifampicin; RXR, retinoid X receptor; XREM, xenobiotic responsive enhancer module

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ABSTRACT

Constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are xenobiotic sensors that enhance detoxification and elimination of xenobiotics and endobiotics by modulating the expression of genes encoding drug-metabolizing enzymes and transporters. Elevated levels of drug-metabolizing enzymes and efflux transporters, resulting from CAR activation in various cancers, promote the elimination of chemotherapeutic agents, leading to reduced therapeutic effectiveness and acquired drug resistance. CAR inhibitors in combination with existing chemotherapeutics could therefore be used to attenuate multidrug resistance in cancers. Interestingly, all previously reported CAR inverse-agonists are also activators of PXR, rendering them mechanistically counterproductive in tissues where both these xenobiotic receptors are present and active. We used a directed high-throughput screening approach, followed by subsequent mechanistic studies, to identify novel, potent, and specific small-molecule CAR inhibitors that do not activate PXR. We describe here one such inhibitor, CINPA1, capable of reducing CAR-mediated transcription with an IC_{50} of ~70 nM. CINPA1 (a) is a specific xenobiotic receptor inhibitor and has no cytotoxic effects up to 30 μ M; (b) inhibits CAR-mediated gene expression in primary human hepatocytes, where CAR is endogenously expressed; (c) does not alter the protein levels or sub-cellular localization of CAR; (d) increases corepressor and reduces coactivator interaction with the CAR ligand-binding domain in mammalian two-hybrid assays; and (e) disrupts CAR binding to the promoter regions of target genes in chromatin immunoprecipitation assays. CINPA1 could be used as a novel molecular tool for understanding CAR function.

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INTRODUCTION

Constitutive androstane receptor (CAR, MB67, NR1I3 (Baes et al., 1994)) is a transcription factor that acts as a xenobiotic sensor, capable of regulating cellular function in response to xenobiotics and endobiotics (di Masi et al., 2009; Ma et al., 2008; Timsit and Negishi, 2007). Activated CAR in tissues heterodimerizes with retinoid X receptor (RXR) and translocates to the nucleus. CAR shares a large portion of its metabolic functions with pregnane X receptor (PXR, NR1I2). PXR has structural features in its ligand-binding domain (LBD) that allow it to bind a diverse set of chemical motifs. CAR's LBD is more compact and yet capable of binding varied structural entities (Wu et al., 2013). CAR and PXR bind similar response elements on chromatin and, hence, regulate an overlapping set of genes (Wei et al., 2002). CAR remains a major player in xenobiotic metabolism by controlling the transactivation of many P450 enzymes and transporters, particularly CYP2B6 and multi-drug resistance 1 (MDR1). Overexpression of CAR in hepatic-induced human embryonic stem cells enhanced expression of hepatic phenotype markers and has been hypothesized as having a key functional role in directing human hepatogenesis (Chen et al., 2013; DeKeyser et al., 2011; Zamule et al., 2011). CAR is the molecular target of phenobarbital (PB)-induced hepatocellular carcinoma, and activation of this receptor is an essential requirement for liver tumor development (Huang et al., 2005; Yamamoto et al., 2004). CAR overexpression in neuroblastoma drives doxorubicin resistance by increasing MDR1 levels (Takwi et al., 2014). In ovarian cancer cells, whereas pharmacological activation of CAR leads to upregulation of MDR1 and decreased effectiveness of anticancer drugs, downregulation of CAR by RNA interference enhances the cell growth inhibition and apoptosis mediated by these anticancer drugs (Wang et al., 2014). CAR activation also causes hepatic lipogenesis and insulin-insensitivity by upregulating the thyroid hormone-responsive *SPOT14* gene, which might promote fatty liver diseases and insulin resistance (Breuker et al., 2010). The involvement of CAR in various diseases emphasizes its clinical and pharmacological significance (Kachaylo et al., 2011).

Exogenously expressed human CAR1 in immortalized cell lines spontaneously accumulates in the cell nuclei and tends to be constitutively active in the absence of agonists (Baes et al., 1994). CAR also

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exhibits high basal but low agonist-induced activation in immortalized cells (Faucette et al., 2007). The splice variant hCAR3 is inducible by agonists when overexpressed in cells (Auerbach et al., 2003; Chen et al., 2010), but the agonist-inducible activity is not substantial without simultaneously overexpressing RXR α (Auerbach et al., 2005). In tissues expressing endogenous hCAR, the receptor is predominantly cytoplasmic unless activated by direct-binding ligands such as CITCO (Maglich et al., 2003) or indirect activators such as phenobarbital (Honkakoski et al., 1998; Mutoh et al., 2013). Among the reported inhibitors of hCAR, PK11195 is the most potent small molecule inhibitor (Li et al., 2008). Clotrimazole, meclizine, and androstanol are moderate inhibitors of CAR that function in both *in vitro* biochemical assays and cell-based transfection assays (Forman et al., 1998; Huang et al., 2004; Moore et al., 2002; Moore et al., 2000; Omiecinski et al., 2011; Tzamelis et al., 2000). However, we and others have observed that all these CAR inhibitors are also moderate to potent activators of PXR function (Jones et al., 2000; Lau et al., 2011; Li et al., 2008; Moore et al., 2002).

In addition to a smaller set of target genes unique for each receptor, CAR and PXR coregulate an overlapping set of metabolizing genes depending on the activation status of these receptors (Wei et al., 2002). The *CYP2B6* gene, although considered to be a predominantly CAR-regulated gene, is also induced equally well by activated PXR (Faucette et al., 2006; Faucette et al., 2007). In tissues such as liver or colon that express both PXR and CAR, treatment with CAR inhibitors that also activate PXR would result in a confused gene expression profile, and interpretation of receptor function is confounded by the opposing dual activity of such CAR inhibitors. Therefore, the goal of our directed screening approach was to discover CAR inhibitors that either antagonize PXR or, at least, do not activate PXR; such CAR inhibitors are expected to reduce the induction of target genes either uniquely regulated by CAR or coregulated by both PXR and CAR.

We identified CINPA1 (CAR Inhibitor Not PXR Activator 1) as a potent inhibitor of hCAR that does not activate PXR. CINPA1 represents a novel class of chemicals that can be used as investigative tools to study CAR biology without concern about PXR activation.

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MATERIALS AND METHODS

Materials – HEK293T, HepG2, LS174T, and U2OS cells were obtained from American Type Culture Collection (Manassas, VA, USA). GeneBLAzer® Validated Assays for Nuclear Receptors, Tb-anti-GST antibody, GST-hCAR-LBD, Fluorescein-PGC-1 α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) coactivator peptide, and TR-FRET coregulator buffer G were obtained from Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) and charcoal-dextran–treated FBS were obtained from HyClone (Logan, UT). Anti-mouse IRDye secondary antibody was purchased from LICOR Biosciences (Lincoln, NE, USA). Anti-CAR antibody (Clone N4111) was purchased from R&D Systems (Minneapolis, MN), and anti-RNA polymerase II, from EMD Millipore (USA). PK11195, rifampicin, clotrimazole, anti-FLAG M2 antibody, and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO). CITCO was obtained from Tocris Bioscience (Bristol, UK). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA, USA). CINPA1 was obtained from ChemDiv (San Diego, CA). Chemicals used in the GeneBLAzer assays: dexamethasone, GW9662, rosiglitazone, (E)-guggulsterone, 9-cis retinoic acid, RU-486 (mifepristone), and 22(S)-hydroxycholesterol from Sigma-Aldrich (St. Louis, MO); fenofibrate, TO901317, and vitamin D₃ from Cayman Chemicals (Ann Arbor, MI); and HX531 from Tocris Bioscience (Bristol, UK).

Cell culture, plasmids, and transfection – A humidified incubator at 37°C with 5% CO₂ was used to maintain all cell lines. HEK293T, HepG2, HepG2-PXR Clone 1 (Li et al., 2012; Lin et al., 2008), and LS174T cells were maintained in EMEM medium supplemented with 10% FBS and PenStrep (100 μ g/mL; Life Technologies). GeneBLAzer cell lines were maintained according to manufacturer's instructions. A clone of HepG2 cells stably over-expressing FLAG-hCAR1 (HepG2-hCAR1 Clone 17) was generated by antibiotic selection with G418 (Life Technologies), using a limited dilution method. HepG2-hCAR1 and HepG2-PXR Clone 1 cells were maintained in media containing G418 (500 μ g/mL). U2OS (human osteosarcoma derived) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Primary human hepatocytes (HPH) were obtained through the Liver Tissue Cell Distribution System

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(Donors 1-6 and 8, corresponding to case # 13-001 [M], 13-003 [F], 13-005 [M], 13-006 [M], 14-005 [F], 14-008 [M], and 14-016 [F] respectively; Pittsburgh, PA, USA) or from Triangle Research Labs (Donor 7, case # HUM4043 [F]; TRL, NC, USA) and maintained in Williams E medium containing Primary Hepatocyte Maintenance Supplement (Life Technologies).

The CAR expression vector (FLAG-hCAR1 in pcDNA3.1 vector) and CYP3A4-luciferase reporter (CYP3A4-luc; in pGL3 vector) were previously described (Li et al., 2012). A luciferase reporter gene under the control of the *CYP2B6* promoter region (phenobarbital-responsive enhancer module/xenobiotic responsive enhancer module, or PBREM/XREM), CYP2B6-luc, was kindly provided by Dr. Hongbing Wang (Wang et al., 2003). The pK-Renilla luciferase plasmid was purchased from Promega (Madison, WI, USA). Plasmids used for mammalian two-hybrid assays, pG5-Luc (a GAL4-luciferase reporter construct) and pACT, were obtained from Promega (CheckMate). The pBIND-SRC-1 (621–765) plasmid was previously described (Wang et al., 2013). The pBIND-mNCoR (1958-2401) and pBIND-SMRT α (2004-2517) plasmids were constructed as previously described (Wang et al., 2013). The pACT-hCAR1 plasmid was prepared by PCR amplification of hCAR1 by using oligonucleotides 5'- GTA CCG AGC TCG GAT CCA ACT AGT AA-3' and 5'- CAG GAT CCG CGG CCG CTC AGC TGC AGA T-3', digested using *Bam*HI (Promega) and *Not*I (Promega), and ligating the resulting fragment into *Bam*HI and *Not*I-cleaved pACT vector plasmid at a 1:7 molar ratio. The pBIND-TIF2 plasmid was similarly prepared by PCR amplification of TIF2 using oligonucleotides 5'- ATT CGG ATC CAT ACC ATG GAG AGA GCT-3' and 5'- ATA AGA TCT GGA TCC CTA GCT CTG TGA-3', digested using *Bam*HI (Promega), and ligating the resulting fragment into *Bam*HI-cleaved pBIND vector plasmid at a 1:10 molar ratio. All transfections were performed by using FuGENE 6 or FuGENE HD (Promega) according to the manufacturer's recommendations.

Gene expression assays, Luciferase assays – HepG2 cells grown in flasks were transfected with FLAG-hCAR1 and CYP2B6-luciferase reporter at a 1:3 ratio with FuGENE HD (Promega) and incubated for 24 h. Cells were trypsinized and plated in 384-well plates (CulturPlate-384, PerkinElmer) at 5000 cells/well for the primary or secondary screening assays. Cells were treated with chemicals transferred by

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using a pintool 24 h prior to measuring reporter luciferase activity with SteadyLite firefly luciferase reagent and an EnVision plate reader (PerkinElmer). The percentage of CAR inhibition was calculated by setting 50 μ M PK11195 (positive control) to 100% inhibition and DMSO (negative control) to 0%. Unless otherwise noted, total DMSO (vehicle) concentration in all assays was maintained at or below 0.56%. Compounds were screened in a dose-responsive format (56 μ M to 2.8 nM, 1-to-3 dilutions for 10 concentrations). PXR activity was measured in HepG2 cells stably transfected with hPXR and CYP3A4-luciferase reporter (previously described as PXR Clone1 cells (Li et al., 2012; Lin et al., 2008). Briefly, cells were treated with clotrimazole, PK11195 or CINPA1 at the indicated concentrations with or without 5 μ M of hPXR agonist rifampicin in phenol red-free DMEM supplemented with 5% charcoal-dextran-treated FBS and incubated for 24 h before Steadylite luciferase assays. The luminescence signal was detected by using an Envision plate reader (PerkinElmer) and the percentage of PXR activation was calculated.

Gene expression assays, GeneBLAzer assays – GeneBLAzer cells individually expressing the ligand-binding domains of either FXR, GR, LXR α , LXR β , PPAR γ , RXR α , RXR β , or VDR fused to the GAL4-DNA binding domain (GAL4-DBD) were obtained from Life Technologies (Li et al., 2012; Yu et al., 2013). Upon activation with the respective agonist, β -lactamase is expressed under the transcriptional control of an Upstream Activator Sequence (UAS), and a FRET-based substrate (CCF2-AM) is used to measure the enzyme activity of β -lactamase. The following agonists were used at an excitatory concentration that resulted in at least 75% receptor activation (EC_{75}): 375 nM GW4064 for FXR; 3 nM dexamethasone for GR; 50 nM TO901317 for LXR α ; 32 nM TO901317 for LXR β ; 52 nM Rosglitazone for PPAR γ ; 267 nM 9-cis retinoic acid (9-cisRA) for RXR α and β ; and 0.2 nM 1 α , 25-dihydroxyvitamin D3 for VDR. Control antagonists for each receptor were used as suggested by the manufacturer: 25 μ M eGuggulsterone for FXR, 100 nM RU-486 for GR, 50 μ M Fenofibrate for LXR α/β , 10 μ M GW9662 for PPAR γ , 10 μ M HX531 for RXR α/β , and 10 μ M 22(S)-Hydroxycholesterol for VDR.

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Gene expression assays, Quantitative real-time PCR – Cell lines or human hepatocytes were treated with chemicals for 24-48 hours before RNA was extracted and purified by using the Maxwell 16LEV simplyRNA tissue kit (Promega). Then, cDNA was prepared from 2 μ g of RNA, and diluted cDNA was used to perform quantitative RT-PCR assays by using TaqMan probes (ABI; 7500 Thermocycler) with 18S as the internal standard.

Mammalian two-hybrid assay – The pACT-hCAR1, pBIND-coregulator peptide and pG5-luc were cotransfected into HEK293T cells. Renilla luciferase is constitutively expressed from the pBIND plasmids. The Dual-Glo Luciferase Assay (Promega) was used to measure pG5-luc luciferase activity. The relative luciferase activity was determined by normalizing firefly luciferase activity with Renilla luciferase activity.

Time-resolved fluorescence resonance energy transfer (TR-FRET) coactivator recruitment assay – The effect of putative CAR ligands on the recruitment or repression of PGC-1 α binding to hCAR was evaluated by using a LanthaScreen TR-FRET assay according to the manufacturer's instructions. Briefly, GST-hCAR-LBD (5 nM) and a mixture of Tb-anti-GST antibody/FI-PGC-1 α peptide (5 nM/125 nM) was added to each well containing titrations of test compounds or DMSO solvent control. The final chemical concentrations were 70 μ M to 3.5 nM (1-to-3 dilutions for 10 concentration levels). DMSO and clotrimazole (42 μ M) were used as negative (0% inhibition) and positive (100% inhibition) controls, respectively. The final DMSO concentration was 0.7% in all assay wells. Assay plates were then briefly centrifuged and incubated at room temperature for 1 h, and TR-FRET emissions at 490 and 520 nm were measured following a 340-nm excitation. Emission signals collected on a PHERAstar plate reader (BMG Labtech, Durham, NC) were used to calculate the 520:490 TR-FRET ratio and normalized to positive and negative controls to derive individual % Inhibition values. The % Inhibition values were then plotted for individual chemicals. When applicable, the graphic software GraphPad Prism 5.04 (GraphPad Software, La Jolla, CA, USA) was used to fit the data into a one-site competitive-binding equation to derive IC₅₀ values.

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Immunofluorescence – U2OS cells were transiently transfected to express FLAG-hCAR1. After 24 h, cells were treated with DMSO (control), 1 μ M CITCO, 5 μ M CINPA1, or 5 μ M PK11195. Cells were fixed by using a 4% paraformaldehyde solution (Sigma), permeabilized by using 0.5% Triton X-100 in PBS, and incubated with anti-FLAG-antibody overnight at 4°C. Cells were washed three times with PBS following each step. Secondary antibody labeled with Alexa Fluor® 555 dye was used to visualize FLAG-tagged hCAR1 (red) using a NikonC1Si microscope. Nuclei were stained with DAPI (blue).

Chromatin immunoprecipitation (ChIP) assays – ChIP assays were performed as described previously (Cherian et al., 2012). Briefly, human hepatocytes in 6-well plates were maintained for 3-4 days in Williams E medium containing Primary Hepatocyte Maintenance Supplement (Life Technologies), with daily media changes. Nine wells were used for each treatment group. Hepatocytes in Figure 8 were treated overnight with DMSO, 1 μ M CITCO, 5 μ M CINPA1, or 5 μ M PK11195. In Figure 9, the hepatocytes from donor 7 were treated with DMSO, 0.1 μ M CITCO, 1 μ M CINPA1, or 0.1 μ M CITCO + 1 μ M CINPA1 for 45 min. Proteins were cross-linked with 1% formaldehyde for 10 min. Cell extracts were digested for 10 min with 50 units of micrococcal nuclease (New England Biolabs) at 37°C and further sonicated to yield sheared DNA fragments having an average length of 200–1000 base pairs. The sonicated samples were pelleted by centrifugation, and the supernatant was diluted 3- to 5-fold with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, and protease inhibitor cocktail). Then, 100 μ L of diluted supernatant was reserved as input (10%) for each treatment. The samples were precleared with Protein G-Sepharose 4 Fast Flow (GE Healthcare) in ChIP dilution buffer (1:1) preblocked with sheared herring sperm DNA (200 μ g/mL) and BSA (500 μ g/mL; Roche). The samples were then divided, and the remaining proteins were incubated with either anti-CAR (Clone N4111, R&D Systems), anti-RNA polymerase II (clone CTD4H8, Millipore), or control mouse IgG overnight at 4°C. The antibody-protein-DNA complex was precipitated by incubating the samples with Protein G-Sepharose beads for 2 h at 4°C. The protein-DNA complex was washed and eluted from the beads with elution buffer (1% SDS, 0.1 M NaHCO₃). Cross-links were reversed, and DNA was eluted from the protein-DNA complexes by adding 200 mM NaCl and incubating overnight at 65°C. Protein was

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digested by incubation with proteinase K at 45°C for 2 h. DNA was recovered and purified. Quantitative real-time PCR assays were performed to determine the change in CAR occupancy at various sites of CAR binding. The double-negative controls were a nonspecific antibody (normal mouse IgG) and primers coding for intergenic regions that do not bind CAR. Thermal cycling conditions were 95°C for 10 min followed by 40 cycles of 25 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The primers used were as follows: CYP2B6-PBREM forward 5'-AGG CCC TTG GTT CAG GAA AG-3', CYP2B6-PBREM reverse 5'-CTG CCT GTC TCA TCC TAC GC-3'; CYP2B6-XREM (dNR3) forward 5'-ATT GCA CAA CAC AGC AGG AG-3', CYP2B6-XREM (dNR3) reverse 5'-CAA CCC ACA CTT TCC TGA CC-3'; CAR-free region forward 5'-CAG CTG GAG GGG TCA TCA AA-3', CAR-free region reverse primer 5'-GCT AGC CAG AGA CCC TTC AC-3'; CYP3A4-XREM forward – 5'-AAG GTC ATA AAG CCC AGT TTG T-3', CYP3A4-XREM reverse – 5'-CAC CTG GGG TCA ACA CAG GAC-3', and GAPDH promoter (ChIP positive control with RPol) forward 5'-TAC TAG CGG TTT TAC GGG CG-3', reverse 5'-TCG AAC AGG AGG AGC AGA GAG CGA-3'.

Statistical Analysis – Results are expressed as the mean \pm SD of at least three independent experiments. Significance was established when $p < 0.05$. Student's *t* test was used for comparison of the means between two groups as specified. One-way ANOVA was used to analyze data sets with multiple comparisons between treatments and experiment-wide significance level α was set to 0.05.

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RESULTS

Identification of CINPA1 as an inhibitor of CAR-mediated transactivation. We focused our efforts on identifying CAR inhibitors that are not agonists of PXR. For this purpose, we performed our primary screen using a library of ~1000 chemicals previously identified as possible antagonists of PXR (Wenwei Lin et al., unpublished work) in a dose-responsive format. A flowchart of our screening strategy is illustrated in Fig. 1A. HepG2 cells were transiently transfected with pcDNA-FLAG-hCAR1 and CYP2B6-luciferase plasmids and treated with varying concentrations of the test compounds in 384-well plates for 24 h, and CAR-mediated luciferase activity was measured. Twenty-five hits, defined as those displaying dose-responsive inhibitory effect on the constitutively active hCAR1, were selected and tested in various secondary screening assays. CINPA1 (Fig. 1B) was selected based on its potent inhibitory effect on hCAR1 and lack of detectable agonistic activity against PXR. CINPA1 inhibited CAR-mediated CYP2B6-luciferase activity with an IC_{50} of 70 nM, which is slightly more potent than PK11195 (Fig. 2A). CINPA1 inhibition of CAR was not limited to the *CYP2B6* gene but was also seen in CAR-mediated CYP3A4-luciferase activity (Fig. 2B). In contrast to other known CAR inhibitors such as PK11195 (PK) and clotrimazole (CLZ), CINPA1, even at 40 μ M, did not exhibit any agonistic effect on PXR-regulated gene expression (Fig. 2C and Fig. 2D). CINPA1 is a weak antagonist of PXR with an estimated IC_{50} value of 6.6 μ M in a stable HepG2 cell line co-expressing PXR and a CYP3A4-luciferase plasmid (HepG2-PXR Clone 1, as previously described in (Li et al., 2012; Lin et al., 2008)(Fig. 2D). Interestingly, CINPA1 is also able to modestly inhibit the CITCO-induced activation of the hCAR3 isoform, with no partial agonistic activity (Supplemental Fig 1).

CINPA1 is a potent CAR-specific inhibitor. CAR and PXR have flexible ligand-binding pockets (Wu et al., 2013) that allow binding of many different chemical core structures. CAR inhibitors identified in the past had limitations because of their PXR agonistic effect, which made them less useful for dissecting CAR-specific gene manipulations, especially in the context of genes regulated by many nuclear receptors. We evaluated CINPA1 in a panel of GeneBLazer cells individually expressing the ligand-binding domains of selected nuclear receptors closely related to PXR or CAR: FXR, GR, LXR α , LXR β ,

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PPAR γ , RXR α , RXR β or VDR, fused to the GAL4-DNA binding domain (GAL4-DBD). CINPA1 did not activate any of the nuclear receptors tested (Figs. 3A-I). Whereas CINPA1 weakly inhibited PXR-mediated gene expression (Fig. 3A), it did not attenuate the agonist-induced activation of any of the receptors tested in the GeneBLAzer assays (Figs. 3B-I).

In a one-day cell viability assay 5 μ M CINPA1 did not exert any toxic effects on HepG2 cells prepared similarly as the luciferase screening assays. Within the concentration range effective in inhibiting CAR and PXR, CINPA1 does not exhibit general cytotoxicity in any of the cells lines evaluated in an extended 4-day cell viability assay (Figs. 4B-D). At higher concentrations (30 μ M and 60 μ M), all chemicals tested, CINPA1, clotrimazole, and PK11195 were cytotoxic to varying degrees in different cell lines.

CINPA1 inhibits expression of CAR-regulated genes in cell lines and human hepatocytes. CAR is a transcription factor that regulates the expression of various gene families, including those involved in drug metabolism and lipogenesis, and CYP2B6 is considered to be a principal CAR-regulated gene. We established a stable clone of HepG2 cells that overexpress hCAR1 (HepG2-hCAR1, Clone 17, Fig. 5A). We also examined various cell lines and identified LS174T as moderately expressing endogenous CAR and its downstream targets; LS174T has previously been reported to express endogenous PXR with inducible activity (Wang et al., 2013). In HepG2-hCAR1, where CAR but not PXR is overexpressed and known to be constitutively active, CINPA1 substantially and significantly attenuated the levels of both endogenous *CYP2B6* and *CYP3A4* genes although PK11195 attenuated the level of CYP2B6 without significantly affecting that of CYP3A4 (Fig. 5B). However, in LS174T cells that endogenously express both CAR and PXR, CINPA1 moderately reduced the levels of CYP2B6 without significantly affecting CYP3A4, whereas PK11195 reduced the levels of CYP2B6 but increased that of CYP3A4 (Fig. 5C), most likely working through PXR activation as previously reported (Li et al., 2008).

Primary human hepatocytes express varying levels of endogenous PXR and CAR, resulting in varying levels of P450 enzyme. CAR in primary hepatocytes is predominantly cytoplasmic therefore the basal levels of CAR target genes such as CYP2B6 is likely not CAR-mediated (Honkakoski et al., 1998;

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Maglich et al., 2003) but can be induced by CAR activators such as CITCO. We tested the effect of CINPA1 on PXR and CAR in primary human hepatocytes from 7 different donors and show the results of a representative set of 3 donors. Fresh or cryopreserved hepatocytes from each donor were obtained and treated with CINPA1 or PK11195 in the presence or absence of CAR activator CITCO. Because hepatocyte sample availability was limited, each donor shown was tested with decreasing concentrations of CINPA1 (5 μ M treatment for Donor 1, 1 μ M for Donor 2, and 0.3 μ M for Donor 3). In all three donors, CITCO induced CYP2B6 levels, indicating that CAR is functional. CINPA1 treatment effectively inhibited CITCO-induced CAR transactivation of the *CYP2B6* gene in all three donors (Figs. 5D-F). We noticed that in donor 2 (Fig. 5E) but not in donors 1 and 3 (Figs. 5D & 5F), CINPA1 alone significantly reduced basal CAR transactivation, reflecting the donor variations typically observed among primary human hepatocytes. In contrast to CINPA1, PK11195 alone increased CYP2B6 transcription in all three donors (Figs. 5D-F), consistent with a previous report demonstrating that PK11195 induces CYP2B6 through PXR activation (Li et al., 2008). Interestingly, both CINPA1 and PK11195 reduced MDR1 levels in LS174T cells (Fig. 5G). Taken together, these data show that unlike PK11195, CINPA1 specifically targets and reduces CAR-mediated gene expression without activating PXR. Figure 5H shows the CAR mRNA levels in the various cell models used to obtain these data.

CINPA1 disrupts CAR interaction with co-activators, enhances recruitment of co-repressors, but does not reduce CAR protein levels or nuclear translocation. We first used a LanthaScreen™ TR-FRET assay to determine the effect of CINPA1 on the constitutive interaction between CAR-LBD and a coactivator peptide. In this assay, GST-tagged CAR-LBD constitutively interacts with a fluorescently labeled PGC-1 α coactivator peptide and emits a strong FRET signal. CAR inverse agonist-binding to the CAR-LBD results in a reduced FRET signal. We compared CINPA1 to known CAR inverse agonists, clotrimazole and PK11195 in this assay. As shown in Fig. 6A, CINPA1 efficiently inhibits CAR-LBD interaction with the coactivator peptide, indirectly suggesting that CINPA1 is a ligand of CAR. CINPA1 had no effect on the protein levels of CAR in human hepatocytes as illustrated by Western blotting in Fig. 6B, which is representative of similar results from 2 other hepatocyte donors. CINPA1 did not affect the

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mRNA levels of either PXR or CAR in human hepatocytes from the same donor used for protein quantification (Donor 8) (Supplemental Fig. 2). Exogenous CAR is functional in U2OS cells (Fig. 6C) and these cells were chosen for immunofluorescence assays for their flat morphology and large cytoplasmic extensions (Fritz et al., 2009). In U2OS cells expressing FLAG-hCAR1 and immunostained with FLAG antibody, CAR localized to the nucleus irrespective of treatment conditions (DMSO, CITCO, CINPA1 or PK11195). This indicates that CINPA1 treatment does not alter the nuclear localization of CAR (Fig. 6D).

Remarkable species selectivity is exhibited by CAR in its ligand binding and activation profiles. Ligands that bind human CAR are largely unable to bind rodent CAR and *vice versa*. We analyzed CAR-mediated transcription at the Cyp2b10 gene in mouse primary hepatocytes and found that CINPA1 does not inhibit mouse CAR (mCAR) activity (Supplemental Fig. 3A). Furthermore CINPA1 did not inhibit luciferase reporter activity driven by transiently expressed mCAR in HepG2 cells (Supplemental Fig. 3B) (Li et al., 2008; Sueyoshi et al., 1999).

Activated CAR protein binds response elements on DNA and recruits coactivator proteins that are essential for transcriptional activation (Forman et al., 1998; Min et al., 2002) while inverse-agonist bound CAR binds corepressor proteins (Bae et al., 2004; Jyrkkarinne et al., 2003; Kublbeck et al., 2011). We further determined the effect of CINPA1 on the interaction between CAR-LBD and coregulators by using mammalian two-hybrid systems. As shown in Figure 7A, CINPA1 treatment resulted in reduced SRC-1 and TIF-2 coactivator binding to CAR-LBD in the presence or absence of agonist CITCO. This finding is in congruence with that in earlier gene expression assays, in which CINPA1 reduced basal and ligand-initiated CAR activity (Fig. 5B-F) and the TR-FRET assay (Fig. 6A). Conversely, CINPA1 treatment resulted in increased interaction of CAR-LBD with the corepressor proteins SMRT α and mNCoR. Thus, we show that CINPA1 binding to CAR-LBD results in reduced coactivator recruitment and increased corepressor interaction, which contributes to explaining the inhibitory mechanism of CINPA1.

CINPA1 inhibits CAR recruitment to the CYP2B6 gene promoter. Chromatin immunoprecipitation assays were used to evaluate the effect of CINPA1 on CAR recruitment to promoter regions of CAR-

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regulated genes in human hepatocytes. *CYP2B6* is a well-characterized CAR-regulated gene with a defined PBREM around –1.8kb upstream, and an XREM (which contains a DR4 motif also called an NR3 binding site) around –8.8kb upstream of its transcriptional start site, respectively (Fig. 8A) (Wang et al., 2003). Both PXR and CAR are capable of binding to these response elements depending on their differential activation states. As shown in Figs. 8B and 8C, CITCO treatment enhances recruitment of CAR to the XREM (dNR3) regions of the *CYP2B6* gene in all 3 donors (Fig. 8B) and to the PBREM in donors 4 and 5 but not donor 6 (Fig. 8C), consistent with the increased *CYP2B6* mRNA levels in response to CITCO shown in Figs. 5D-F. CINPA1 treatment prevents CAR recruitment to the dNR3 in all 3 donors (Fig. 8 B), but the effect on the PBREM is less consistent among different donors (Fig. 8C). Similar donor variation on the basal level of *CYP2B6* in response to CINPA1 was also observed in Figs. 5D-F. CAR protein was below detection levels (when compared to normal mouse IgG) in ChIP experiments at the *CYP2B6* promoter region in 2 other human hepatocyte donors (data not shown), further demonstrating the donor-to-donor variation in human hepatocytes. Figure 8D illustrates the relative absence of CAR compared to mouse IgG at a CAR-free intergenic region within the *CYP2B6* gene, a negative control for ChIP.

In hepatocytes, CITCO treatment consistently enhanced recruitment of CAR to the XREM (dNR3) region of the *CYP2B6* promoter (Fig. 8B) and induced the expression of *CYP2B6*, which is effectively inhibited by CINPA1 co-treatment (Figs. 5D-F). Consistent with these observations, CINPA1 effectively reduced the CITCO-mediated recruitment of CAR and RNA polymerase II to the XREM (dNR3) region of the *CYP2B6* promoter (Fig. 9A). As expected, CINPA1 also reduced the CITCO-mediated recruitment of CAR and RNA polymerase II to the XREM region of the *CYP3A4* promoter (Fig. 9B). As a negative control, CAR and RNA polymerase II were not recruited to the CAR-free region (Fig. 9C). The parallel recruitment of CAR and RNA polymerase II to the promoter regions of *CYP2B6* observed in the ChIP assays (Fig. 9) is consistent with the corresponding transcriptional activity shown in Fig. 5. Taken together, these data suggest that CINPA1 acts, in part, by reducing the association of CAR with its target promoter.

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DISCUSSION

CAR is an important xenobiotic sensor that controls the metabolism and elimination of many xenobiotics and endobiotics. Downstream target genes of CAR are involved in various important physiological processes such as energy homeostasis, cell cycle regulation, and cell-cell communication (Maglich et al., 2004; Molnar et al., 2013). Dysregulation of these genes often results in pathological conditions like insulin resistance, fatty liver disease, chemical carcinogenesis, and tumor promotion (Breuker et al., 2010; Huang et al., 2005; Takwi et al., 2014). There is a need to develop potent and specific chemical tools to uncover the molecular mechanisms governing CAR activation and its regulation of target gene expression.

CAR displays several distinctive characteristics in its activation. Endogenously expressed CAR, such as that in human hepatocyte cultures, is activated by both direct and indirect activators, resulting in nuclear accumulation of the receptor. But CAR exogenously expressed in immortalized cell lines, such as HepG2 cells, results in spontaneous nuclear accumulation and constitutive activation in the absence of an agonist (Baes et al., 1994; Honkakoski et al., 1998; Maglich et al., 2003). Many naturally occurring splice variants of CAR have also been identified, but their individual contributions to CAR physiology remain unclear.

A few inhibitors of hCAR with varying potencies have been previously reported. Clotrimazole, meclizine, and androstanol moderately repress hCAR function in various assays (Huang et al., 2004; Moore et al., 2002; Moore et al., 2000; Tzamelis et al., 2000). Interestingly, clotrimazole, in addition to being a good mouse CAR agonist, may act as an activator of human CAR depending upon cell-type-specific factors (Auerbach et al., 2005; Jyrkkarinne et al., 2003; Simonsson et al., 2006). Li et al. identified a well-known peripheral benzodiazepine receptor ligand, PK11195, as a CAR inhibitor—the most potent inhibitor of CAR identified thus far (Li et al., 2008). Interestingly, all the known CAR inhibitors are also moderate-to-potent activators of PXR function, complicating expression levels of their shared target genes (Huang et al., 2007; Jones et al., 2000; Lau et al., 2011; Li et al., 2008; Moore et al., 2002).

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With the aim of identifying specific CAR inhibitors that do not activate PXR function, we used a directed HTS strategy and identified CINPA1 as a potent inhibitor of hCAR activity. The flexible LBD of PXR and, to some extent, CAR allow them to bind a broad range of chemical structures, making it hard to design structure-based chemical libraries for screening. For our primary screen of CAR inhibition, we started with ~1000 compounds shortlisted as putative PXR antagonists from a previous screen. This allowed us to search for CAR inhibitors from a set of chemicals that would not activate PXR. CINPA1 was identified as the most potent among these in its ability to inhibit hCAR1. As expected since the screen was started with compounds that inhibit PXR, CINPA1 is a weak antagonist of PXR. Most importantly, CINPA1 does not activate PXR-mediated gene expression, distinguishing it from previously known CAR inhibitors. CINPA1 binding results in reduced coactivator interaction with CAR-LBD, suggesting that the site of CINPA1 binding is most likely the ligand-binding pocket of CAR.

Because CAR has distinctive subcellular localization and basal activity in primary hepatocytes and immortalized cell lines, we have used both primary hepatocytes and cell lines to evaluate the effect of CINPA1 on CAR. CYP2B6 is the best-characterized CAR-regulated P450 enzyme. *CYP2B6* expression can be upregulated via activation of either CAR or PXR, but it is primarily regulated by activated CAR. The *CYP2B6* gene promoter has multiple xenobiotic-response elements concentrated mainly at two loci. The PBREM region at ~1.8 kb upstream, harboring two DR4 motifs, and the 400-bp distal XREM region, containing one DR4 motif (and a few half sites), are the main binding sites for CAR (Wang et al., 2003). Human hepatocytes express endogenous CAR, which is not constitutively active but inducible by activators such as CITCO. There is considerable variation between donors, especially the basal CAR activity and its response to CINPA1 treatment. However, the induction of CAR target genes and CAR binding to the target promoter by CAR activator CITCO and the inhibitory effect of CINPA1 on such inductions by CITCO are more consistent among donors. Importantly, although CAR target genes such as *CYP2B6* are induced by the activation of CAR, the expression of these CAR target genes is also regulated by pathways independent of CAR and PXR. The activation states of other nuclear receptors such as glucocorticoid receptor (GR) (Lim and Huang, 2008; Timsit and Negishi, 2007) and hepatocyte nuclear

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factor 4alpha (HNF4 α) (Ding et al., 2006; Tirona et al., 2003) could also contribute to the regulation of PXR and CAR-mediated gene expression. Therefore, it is not surprising that the response of the basal CYB2B6 levels to CINPA1 varies among primary hepatocytes from different donors.

Treatment with CINPA1 does not alter the nuclear localization or reduce the levels of CAR in the cells. Consistent with previous reports (Baes et al., 1994), exogenously expressed CAR in immortalized cell lines accumulates in the nucleus, which was not altered by CINPA1 treatment as illustrated by the results of immunofluorescence assays. However, CAR interaction with coregulators is modulated by the presence of CINPA1. The results of mammalian two-hybrid assays revealed reduced CAR binding to the coactivators SRC-1 and TIF-2 in the presence of CINPA1. No substantial increases in CAR-coactivator interaction was seen with the CAR agonist CITCO, possibly due to the high constitutive activity of CAR. This is consistent with gene expression data from immortalized cells overexpressing hCAR1 (Fig. 5B) and has been previously reported by others (Chen et al., 2010). Conversely, increased interaction of CINPA1-associated CAR to the co-repressors SMRT α and mNCoR was evident and reinstates the inhibitory effect of CINPA1. It is possible for small molecule inhibitors that bind the LBD of a nuclear receptor to change receptor conformation such that co-activator binding and/or DNA-binding is compromised. For instance, MDV-3100 (Enzalutamide) is an androgen receptor (AR) antagonist that binds in the ligand binding pocket of the receptor but effectively reduces nuclear translocation, inhibits co-activator binding and prevents AR binding to DNA (Tran et al., 2009). Our data suggests that CINPA1 might have a similar mechanism of action for CAR inhibition wherein it binds the LBD of CAR but alters the co-activator recruitment (Fig. 6A) and DNA-binding properties of CAR.

CAR and PXR exhibit a high level of redundancy in their role as xenobiotic sensors. Their flexible ligand-binding domains make it hard to design chemical entities that bind either receptor specifically. Lack of specific chemical modulators makes dissecting the separate functions of these receptors a difficult task. All CAR inhibitors previously identified are also potent activators of PXR, rendering them ineffective in assay environments in which both receptors are present and active, such as hepatocytes or whole animal models. Our studies identify and characterize CINPA1 as a potent inhibitor of CAR that

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does not activate PXR. CINPA1 interacts with CAR-LBD and reduces coactivator recruitment. The reduction in CAR-mediated target gene expression with CINPA1 treatment is consistent with reduced CAR and RNA polymerase II recruitment to the promoter regions of those genes. CAR is responsible for phenobarbital-induced hepatocellular carcinoma (Huang et al., 2005; Yamamoto et al., 2004) and decreased effectiveness of anticancer drugs in ovarian cancer (Wang et al., 2014). A unique chemical entity like CINPA1 could be further developed for therapeutic purposes.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Cherian, Lin, and Chen.

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Wrote or contributed to the writing of the manuscript: Cherian, and Chen.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. CINPA1 identified as an inhibitor of CAR activity through a directed small-molecule screening.

A. Flowchart of screening strategy. B. Chemical structure of CINPA1.

Figure 2. CINPA1 inhibits CAR-mediated transactivation without activating PXR.

A. HepG2 cells were transfected with plasmids expressing hCAR1 and CYP2B6-luc reporter. After 24 h incubation, cells were treated with DMSO (negative control) or indicated concentrations of CINPA1, clotrimazole (CLZ), or PK11195 (positive control). Luciferase activity was measured 24 h after treatment by using SteadyLite reagent. Activity in DMSO-treated samples was set as 0% inhibition; activity in samples treated with 50 μ M PK11195 was set as 100% CAR inhibition. B. HepG2 cells were transiently transfected with plasmids expressing hCAR1, CYP3A4-luc reporter and control plasmid pTK-RL. After 24 h incubation, cells were treated with DMSO (control), 1 μ M CITCO, 5 μ M PK11195, 1 μ M or 5 μ M CINPA1, or 5 μ M rifampicin (Rif) for 24 hours. Dual luciferase activity was measured 24 h after treatment using Dual-Glo luciferase reagent. Firefly luciferase was normalized to renilla luciferase and % CAR activation was calculated by setting DMSO to 100%. ***, $p < 0.001$, when comparing treatments to DMSO. C. HepG2 cells stably expressing hPXR and CYP3A4-luciferase reporter (PXR Clone 1) were treated with indicated compounds for 24 h. Luciferase activity was measured 24 h after treatment by using SteadyLite reagent. ***, $p < 0.001$, when comparing treatments to DMSO. D. HepG2-PXR Clone 1 cells were treated with CINPA1 in a dose-responsive format (40 μ M to 2 nM, 1-to-2 dilutions for 10 concentrations) in the presence or absence of 5 μ M rifampicin (Rif) for 24 h before luciferase assay using Steadylite. % PXR activation was calculated by setting DMSO (negative. control) treated cells to 0% and 5 μ M rifampicin (positive. control) treated cells to 100%. GraphPad Prism was used to fit the data into a dose-response stimulation equation to derive IC_{50} values.

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Figure 3. CINPA1 is a weak antagonist of PXR but does not modulate the activity of other nuclear receptors.

A. HepG2-PXR Clone 1 cells were treated with DMSO or 18 μ M of CINPA1 in the presence or absence of 5 μ M rifampicin for 24 h before luciferase assay using SteadyLite. B-I. GeneBLAzer cells were plated according to the manufacturer's instructions and treated with DMSO (control), a predetermined concentration of the indicated NR antagonist (as detailed in Experimental Procedures), or 18 μ M of CINPA1. NR-mediated β -lactamase activity was measured as a FRET ratio after 24 h treatment. Data is presented as % Activity, determined by setting the maximum FRET signal obtained for each receptor (in the respective agonist + DMSO wells) to 100%. One-way ANOVA with Bonferroni corrections for multiple comparisons was used to analyze data (as noted in brackets), and statistical significant differences are indicated by *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, and ns (not significant; $p > 0.05$). eGugg = e Guggulsterone, Feno = Fenofibrate, GW9 = GW9662, 22HC = 22(S)-hydroxycholesterol, 9-cis RA = 9-cis retinoic acid.

Figure 4. CINPA1 is not cytotoxic within the concentration range effective for modulating CAR and PXR.

A. HepG2 cells were transfected with hCAR1 and CYP2B6-luc plasmid to mimic conditions used in the luciferase screening assays. Cells were treated with indicated compounds for 24 h, and viability was measured by using CellTiter Glo reagent. (Rif, rifampicin). No significant change was observed between treatments. B. HepG2, C. LS174T, or D. HEK293T cells were treated with DMSO (control), CINPA1, clotrimazole (CLZ) or PK11195 at the indicated concentrations. Cell viability was measured 4 days post treatment by using CellTiter Glo reagent. Viability of cells treated with DMSO (negative control) was set to 100% in all assays.

Figure 5. CINPA1 attenuates CAR-mediated gene expression in CAR-expressing cell lines and human primary hepatocytes.

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A. FLAG-CAR1 expression in the HepG2-hCAR1 stable clone was evaluated. CAR mRNA expression in HepG2-hCAR1 Clone 17 cells was compared to parental HepG2 cells using quantitative RT-PCR. 18S was used as the internal control and showed no change in threshold cycle (Ct) between the two cell lines. FLAG-tagged CAR protein expression levels in a few selected clones were analyzed by Western Blot using anti-FLAG antibody. Clone 17 had the highest expression levels of hCAR1 and was used in subsequent studies. B. HepG2-hCAR1 cells were treated with DMSO (control), 1 μ M CITCO, 1 μ M CINPA1, or 1 μ M PK11195 in triplicated wells for 24 h. RNA was extracted, cDNA synthesized, and gene expression analyzed by performing real-time PCR using Taqman probes for each gene. C. LS174T cells were maintained in 10% charcoal-dextran-treated, FBS media for 4 days before the experiment. Cells were treated with DMSO, 5 μ M CITCO, 1 μ M CINPA1, or 5 μ M PK11195 for 24 h before RNA extraction. In B and C, *p* values (*, **, or ***) were determined by comparing compound-treated samples to DMSO-treated samples in a Student's *t*-test. D-F. Human primary hepatocytes (HPH) were maintained in William's E media with supplements for 4 days before treatment. Cells were treated in the presence or absence of 1 μ M CITCO, and DMSO, 5 μ M PK11195, or CINPA1 (5 μ M treatment for Donor 1, 1 μ M for Donor 2, and 0.3 μ M for Donor 3) for 48 h. RNA extracted was used for cDNA synthesis and measured by performing quantitative real-time PCR assays with Taqman probes. In Panel D HPH-Donor 1, PK11195 alone was tested, but CITCO + PK11195 could not be tested due to limited hepatocyte availability. Statistical significance was determined using one-way ANOVA by comparing compound-treated samples to DMSO-treated samples (* or ***), or to CITCO treated samples (#, or ###). G. LS174T cells were maintained in 10% charcoal-dextran treated-FBS media for 4 days prior to the experiment. Cells were treated with DMSO, 1 μ M CINPA1 or 5 μ M PK11195 for 24 h. MDR1 mRNA was normalized to the internal control 18S and DMSO treated samples were set to 1. *, *p* < 0.05 when compared to DMSO samples in a Student's *t*-test. H. Comparison of CAR mRNA levels in the cells lines and human primary hepatocytes used for gene expression analysis. RNA from the DMSO treated cells (panels B-F) was used for cDNA synthesis and hCAR measured by quantitative real-time PCR. deltaCt

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(Ct= threshold cycle) values were calculated by normalizing to the internal control 18S. Lower deltaCt values indicate more CAR mRNA detected in the respective cell line/donor.

Figure 6. CINPA1 does not reduce protein levels or alter nuclear localization of CAR.

A. CAR binding to PGC-1 α coactivator peptide was determined by using the LanthaScreen fluorescent coregulator peptide assay. Fluorescein-labeled PGC-1 α peptide (125 nM) was complexed with GST-hCAR-LBD (5 nM) and Tb-anti-GST antibody (5 nM) as described in Experimental Procedures. Compounds (clotrimazole (CLZ), PK11195 or CINPA1) at concentrations ranging from 70 μ M to 3.5 nM (1-to-3 dilutions for 10 concentration levels) were used along with DMSO as a negative control, and clotrimazole (42 μ M) was used as a positive control. % Inhibition of binding was calculated for each treatment, and the data were normalized to the positive control (42 μ M clotrimazole, 100% inhibition) and negative control (DMSO, 0% inhibition). B. Human primary hepatocytes (Donor 8) were treated with DMSO, 1 μ M CITCO, 5 μ M CINPA1, or 5 μ M PK11195 for 48 h. Lysates were prepared, and CAR expression was analyzed by Western blot using anti-CAR monoclonal antibody (Clone N4111, R&D Systems). Tubulin was used as a loading control. Data shown is representative of at least three hepatocyte donors tested. C. U2OS cells were transiently transfected with plasmids expressing FLAG-hCAR1, CYP2B6-luc reporter and control plasmid pTK-RL. After 24 h incubation, cells were treated with DMSO (control), 1 μ M CITCO, 5 μ M CINPA1, 1 μ M CITCO + 5 μ M CINPA1 or 5 μ M PK11195. Dual luciferase activity was measured 24 h after treatment. Renilla luciferase was used to normalize firefly luciferase values and % CAR activation was calculated by setting DMSO to 100%. Statistical significance was determined using one-way ANOVA for comparisons between multiple treatment groups. ***, $p < 0.001$ compared to DMSO treatment and ####, $p < 0.001$ comparing CITCO + CINPA1 to CITCO treated cells. D. U2OS cells were transfected to express FLAG-hCAR1. Cells were treated with indicated chemicals for 2 h before fixing and staining with anti-FLAG-M2 antibody (Sigma). Secondary antibody labeled with Alexa Fluor® 555 dye was used to visualize FLAG-tagged hCAR1 (red) using a NikonC1Si microscope. Nuclei were stained with DAPI (blue).

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Figure 7. CINPA1 disrupts interaction of CAR with coactivators and enhances corepressor binding.

A, B. Mammalian two-hybrid assays were set up in HEK293T cells transfected with expression plasmids encoding VP16AD-hCAR1 fusion protein, GAL4DBD-coregulator fusion proteins, and the reporter plasmid pG5luc. Cells were treated with DMSO (control), 5 μ M CITCO, 5 μ M CINPA1, 10 μ M PK11195, or 1 μ M CITCO + 5 μ M CINPA1 for 24 h prior to measuring luciferase activities. Fold interaction represents pG5luc reporter activity normalized to the Renilla luciferase internal control. Data presented are the mean \pm SD of at least three independent transfections. ***, $p < 0.001$ compared to DMSO treatment within the same coregulator set.

Figure 8. CINPA1 treatment forbids CAR binding to DNA response elements at the CYP2B6 gene promoter in human hepatocytes.

A. PBREM and XREM (dNR3) in the CYP2B6 promoter region. B-D. Human primary hepatocytes from three separate donors were treated overnight with DMSO, 1 μ M CITCO, 5 μ M CINPA1, or 10 μ M PK11195. Protein complexes were cross-linked and chromatin was immunoprecipitated by using anti-CAR antibody or control IgG. CAR occupancy at two separate CYP2B6 promoter regions (dNR3 and PBREM) was determined by performing quantitative real time PCR assays. CAR occupancy at a CAR-free intergenic region around +14kb was also analyzed as a negative control for recruitment. Fold-enrichment normalized to IgG control was plotted. Data represent the mean \pm SD of 3 experiments. p values (denoted *, **, or ***) were determined by comparing compound treatments to DMSO-treated samples.

Figure 9. CINPA1 disrupts CITCO-activated CAR binding to DNA response elements at the CYP2B6 and CYP3A4 gene promoter regions.

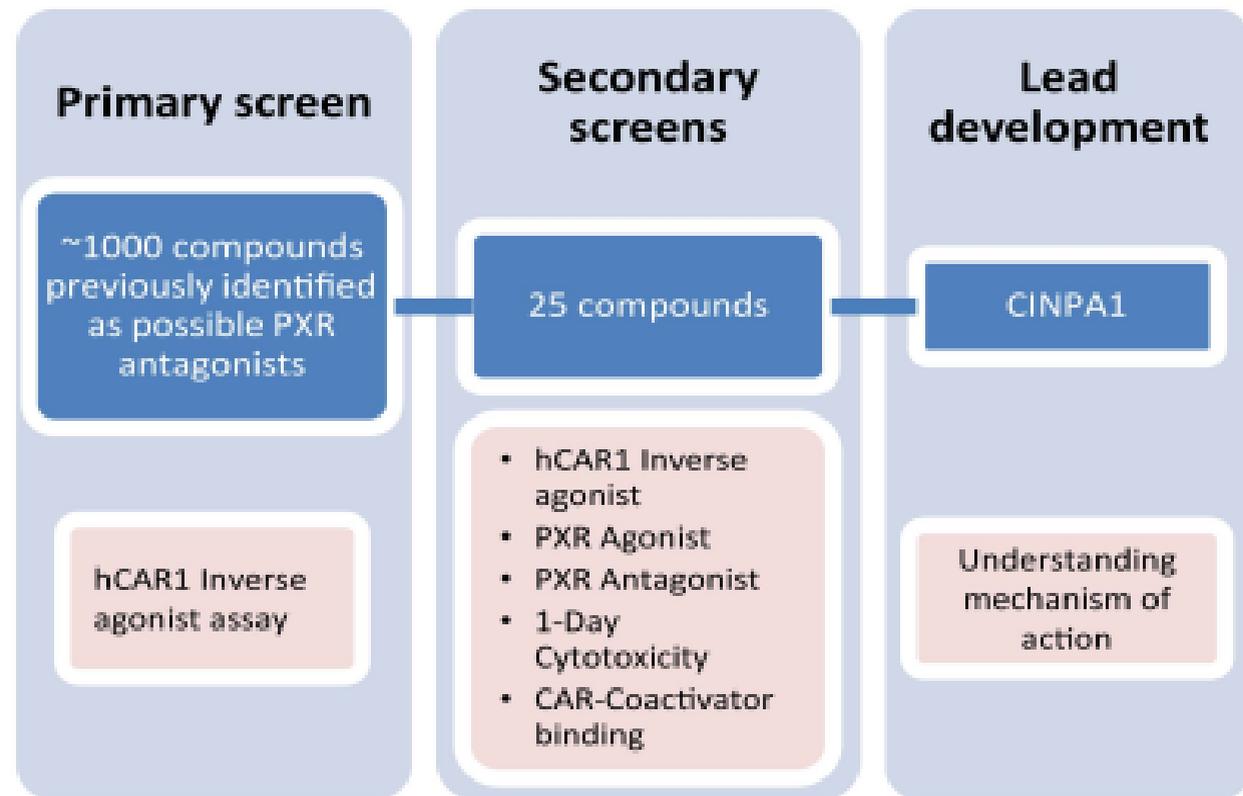
A-C. Freshly plated human hepatocytes were treated for 45 min with DMSO, 0.1 μ M CITCO, 1 μ M CINPA1, or 0.1 μ M CITCO + 1 μ M CINPA1. Protein complexes were cross-linked and chromatin was

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immunoprecipitated by using anti-CAR antibody, anti-RNA polymerase II (RPol) antibody, or control IgG. CAR or RPol occupancy at the CYP2B6-dNR3 region, CYP3A4-XREM region and a CAR-free intergenic region (ChIP negative control) was determined by performing quantitative real time PCR assays. Fold-enrichment was normalized to IgG control. Data represent the mean \pm SD of three PCRs and are representative of other experiments. One-way ANOVA with Fisher's LSD test was used to compare multiple treatment groups. **, $p < 0.01$ and ***, $p < 0.001$ compared to DMSO samples. ##, $p < 0.01$ and ###, $p < 0.001$ compared to CITCO samples.

Figure 1

A



B

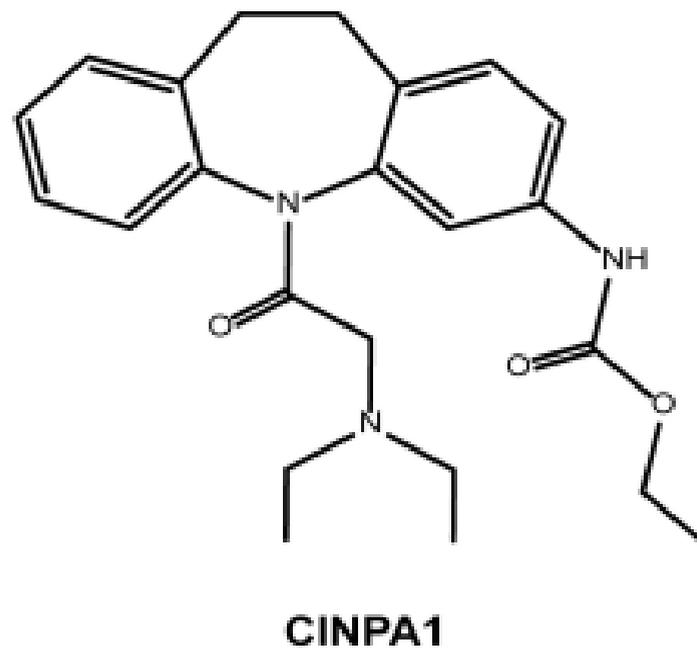
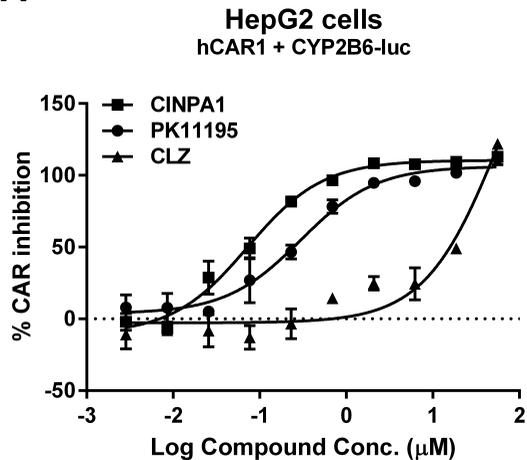
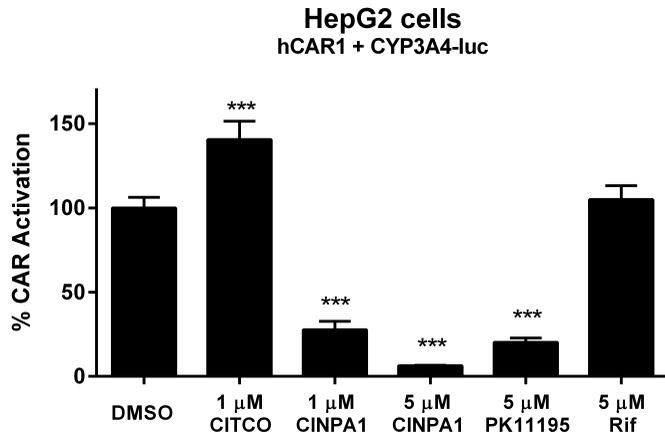


Figure 2

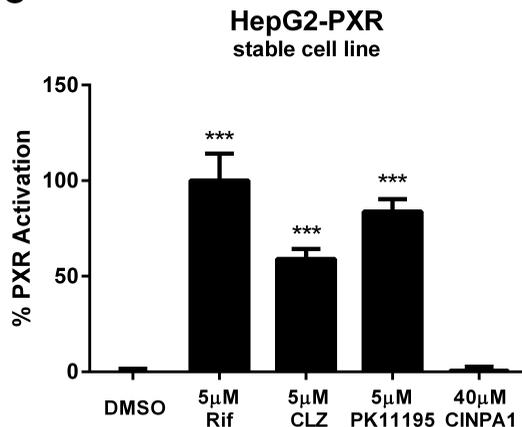
A



B



C



D

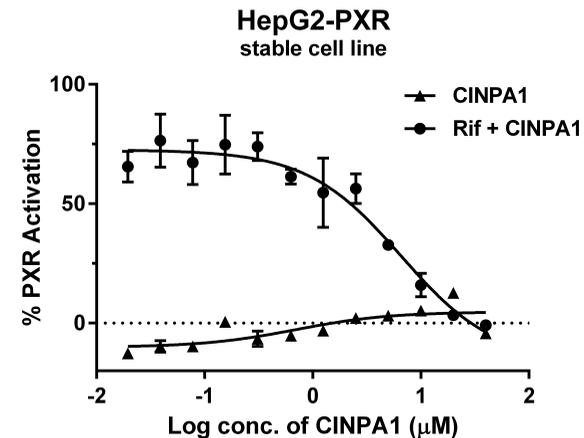


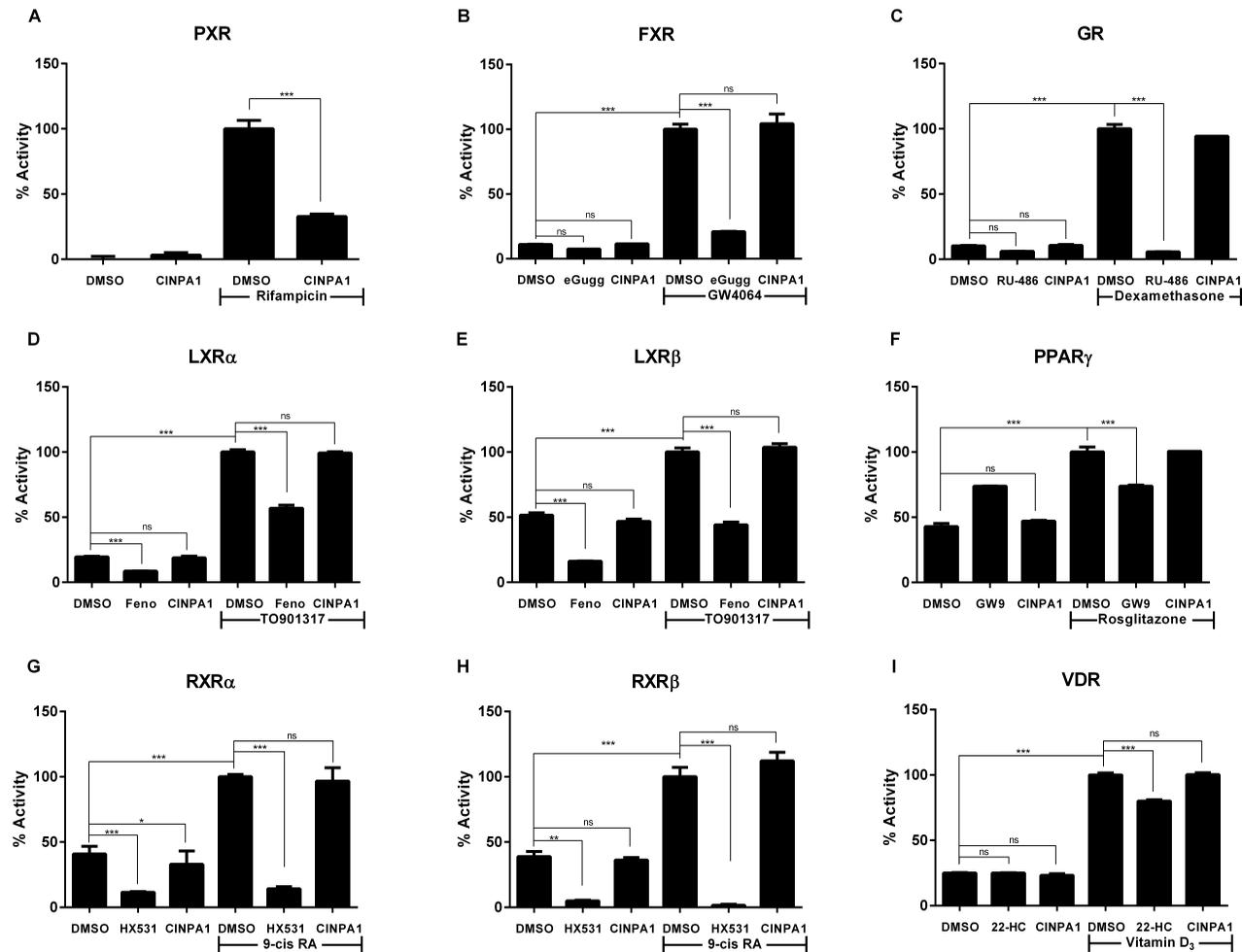
Figure 3

Figure 4

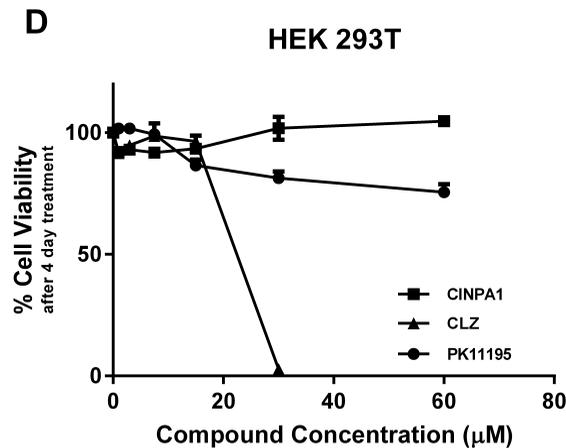
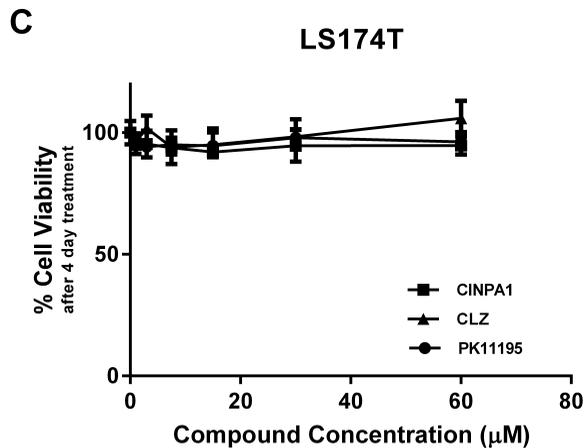
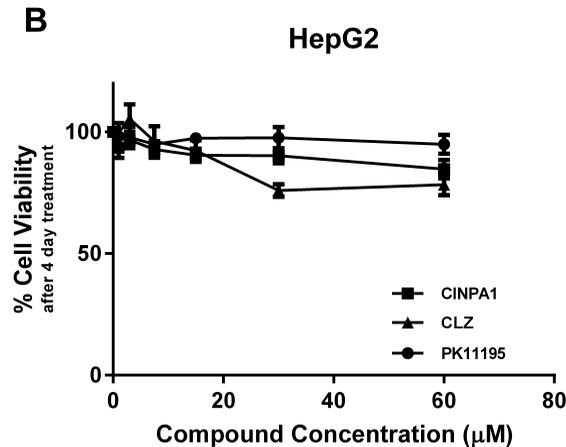
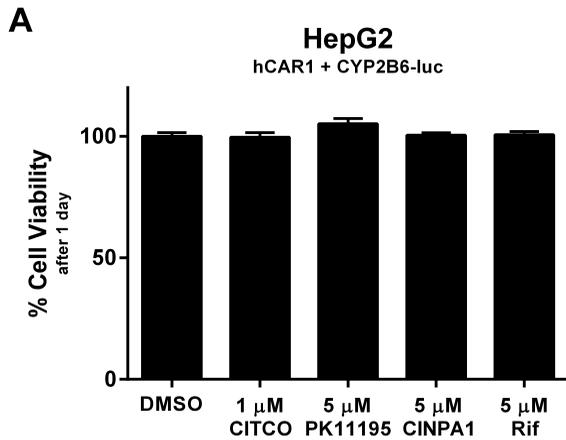
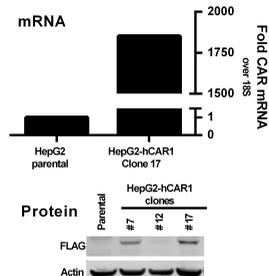
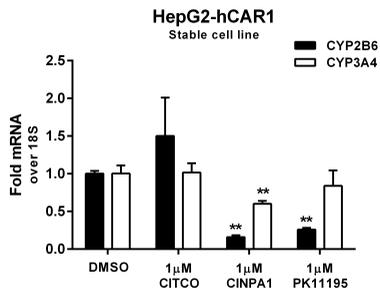


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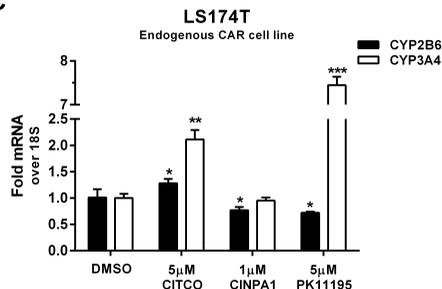
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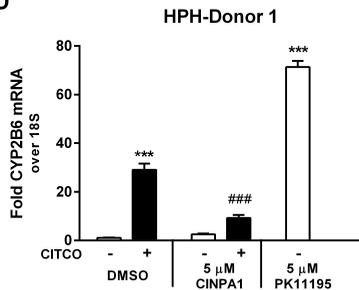
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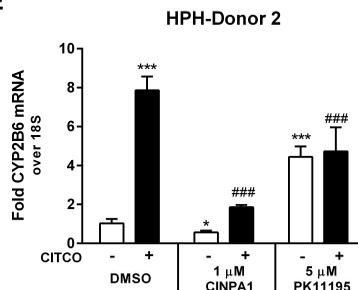
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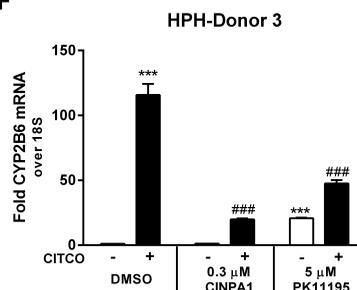
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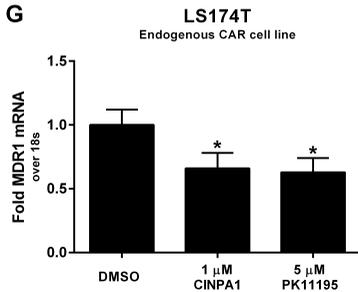
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F



G



H

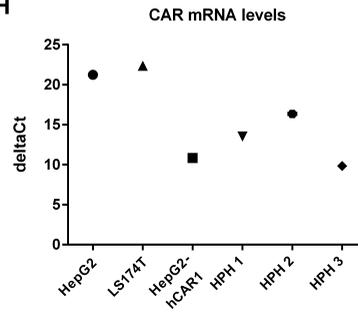
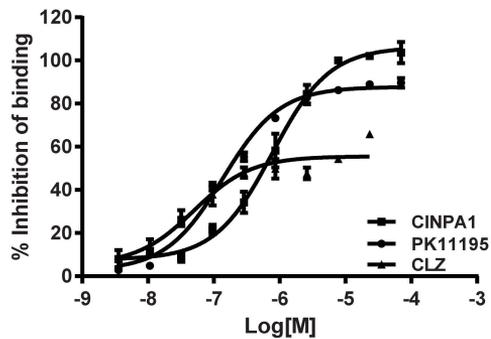
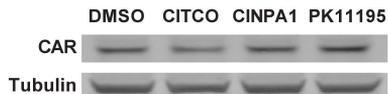


Figure 6

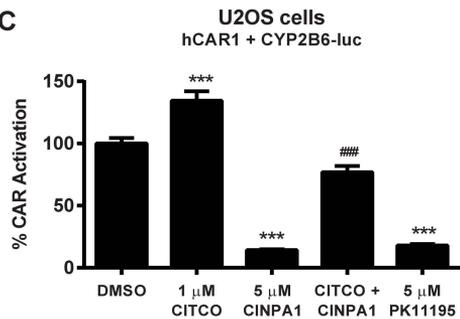
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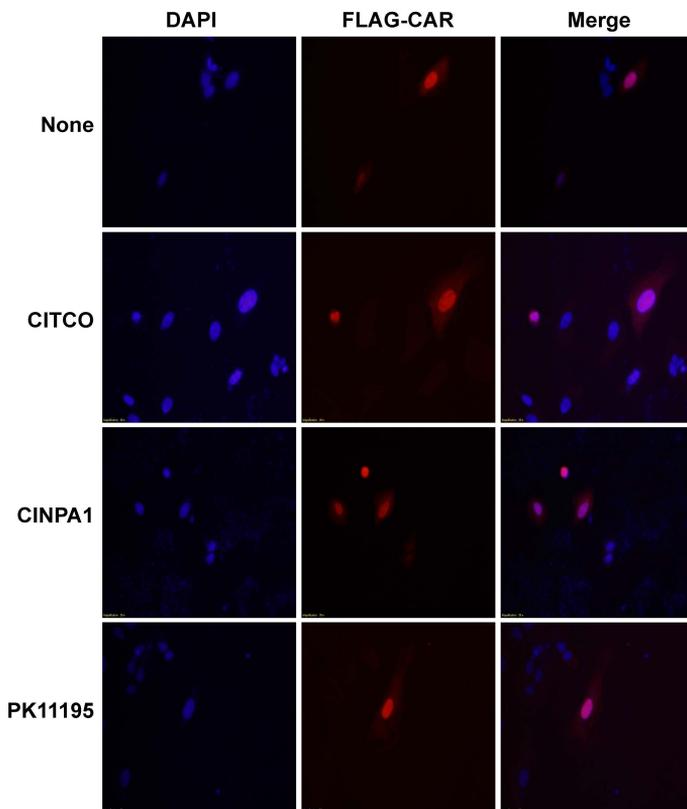


Figure 7

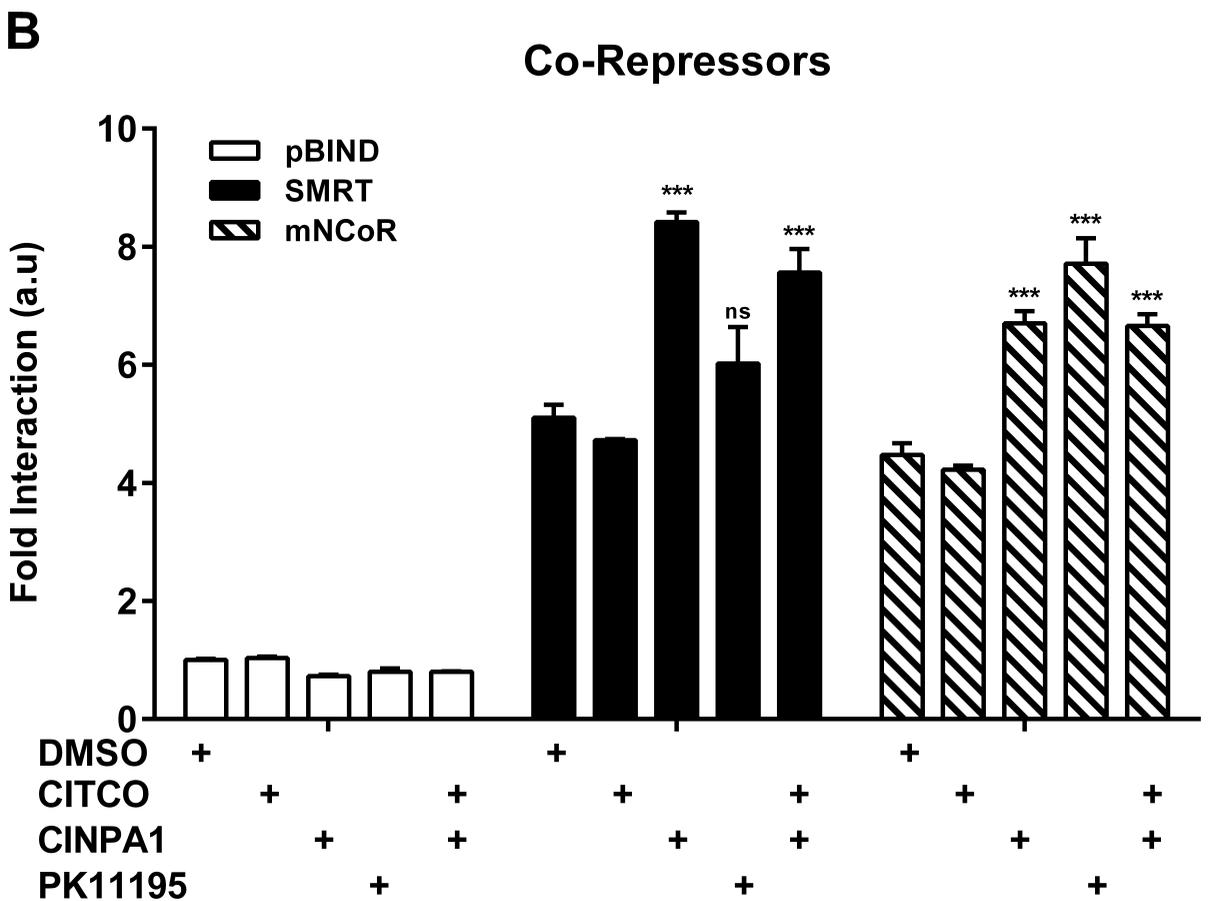
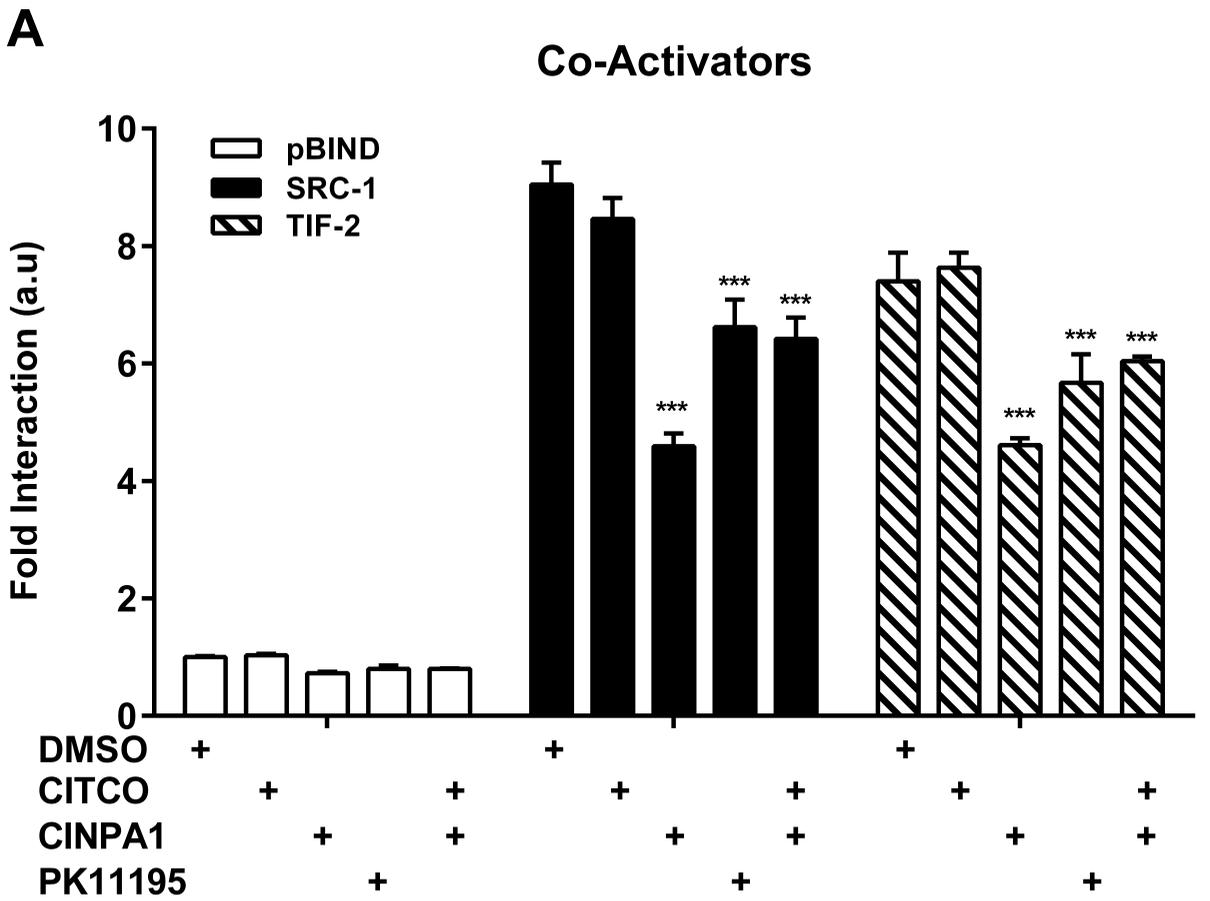


Figure 8

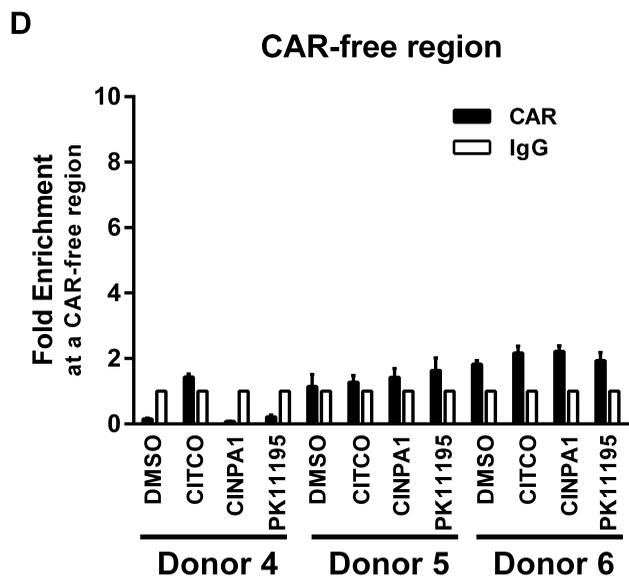
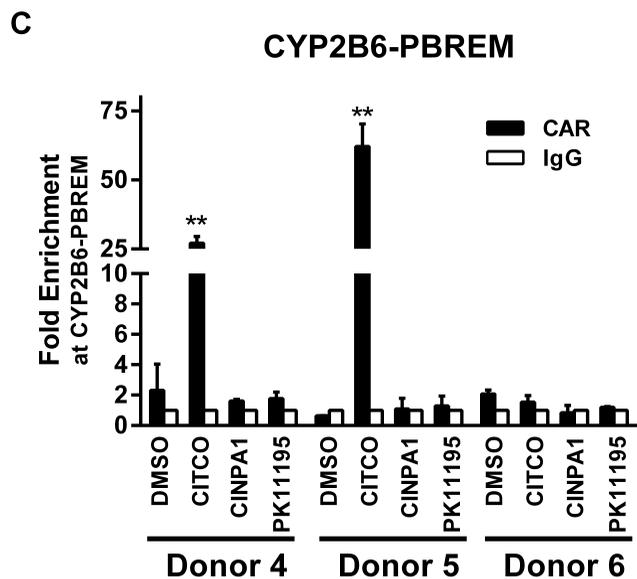
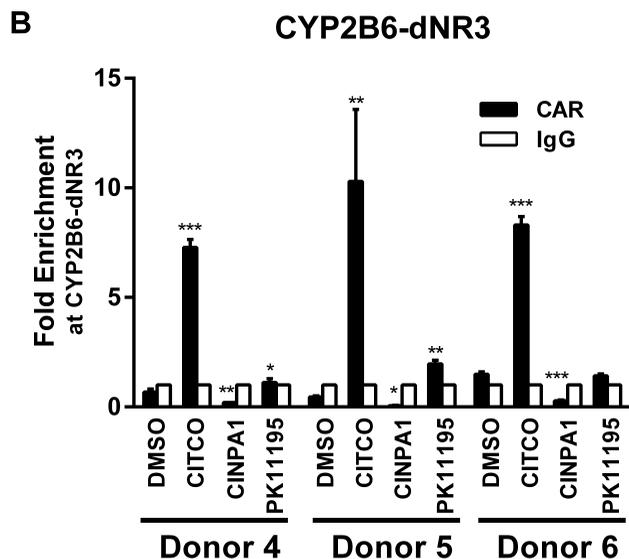
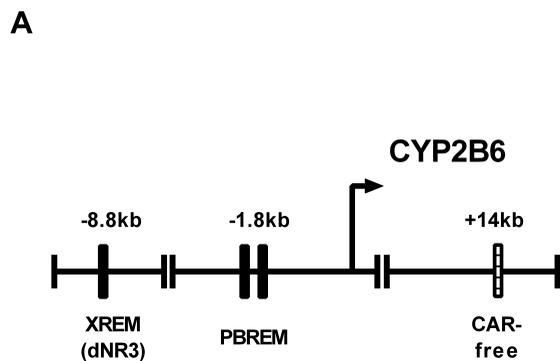
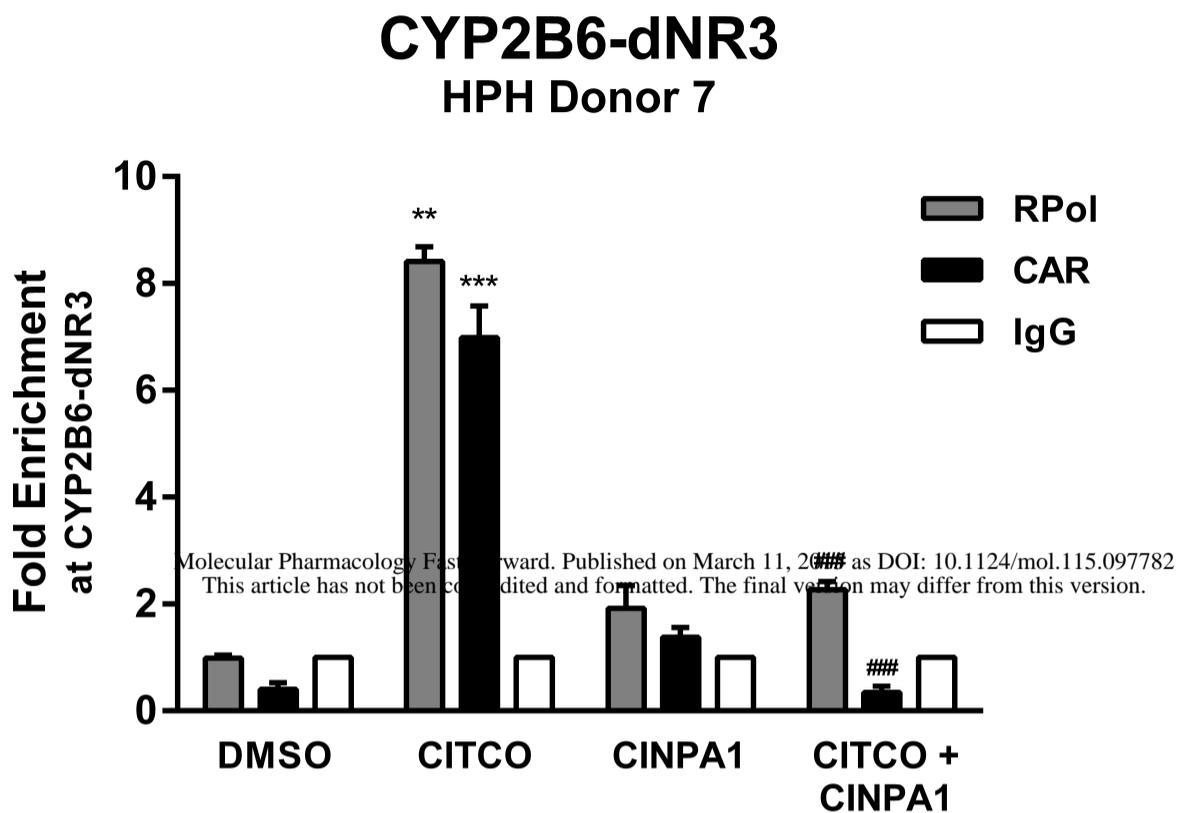
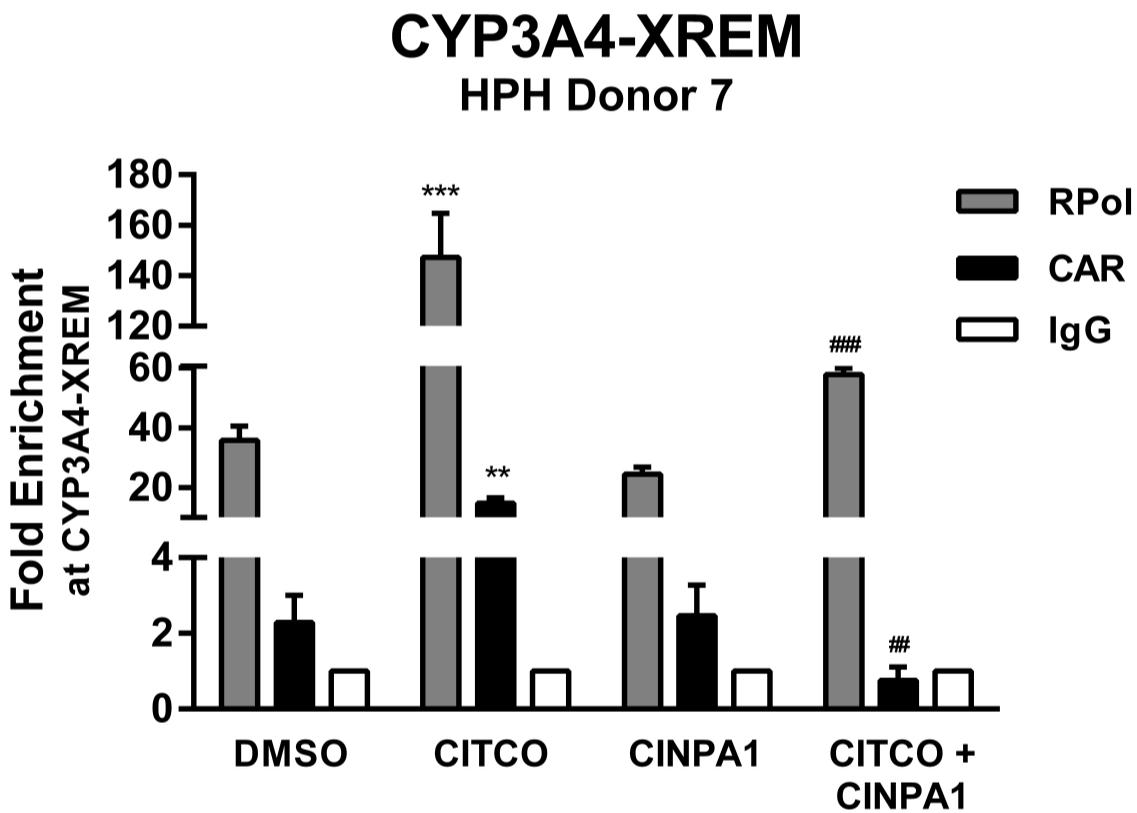


Figure 9

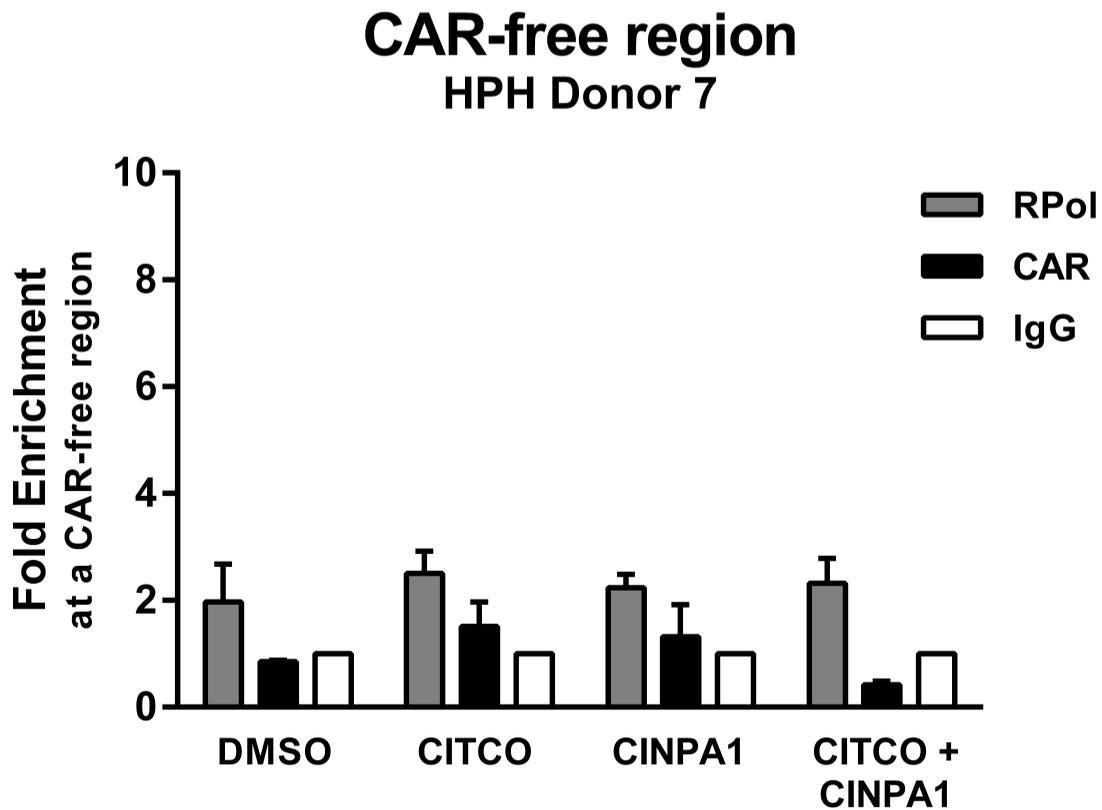
A



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C



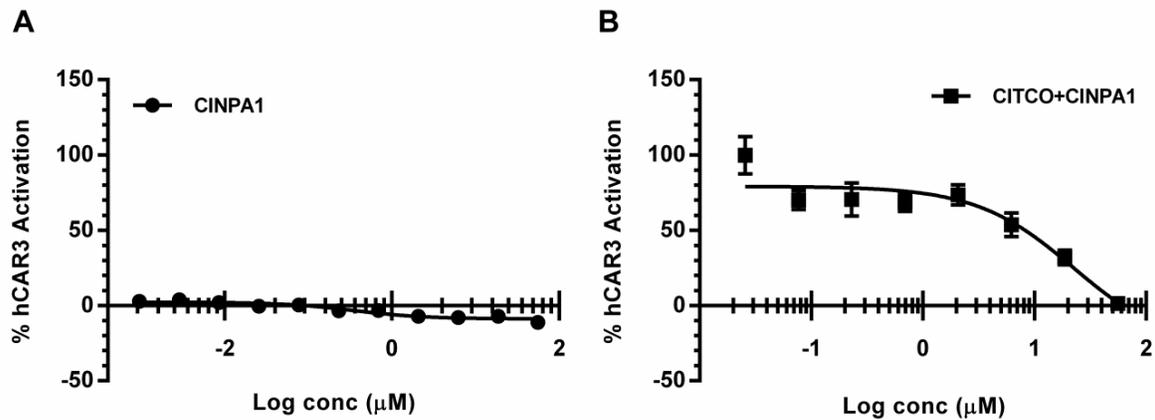
Molecular Pharmacology
Supplemental Information

**CINPA1 Is an Inhibitor of Constitutive Androstane Receptor (CAR) that Does Not
Activate Pregnane X Receptor (PXR)**

Milu T Cherian, Wenwei Lin, Jing Wu, and Taosheng Chen

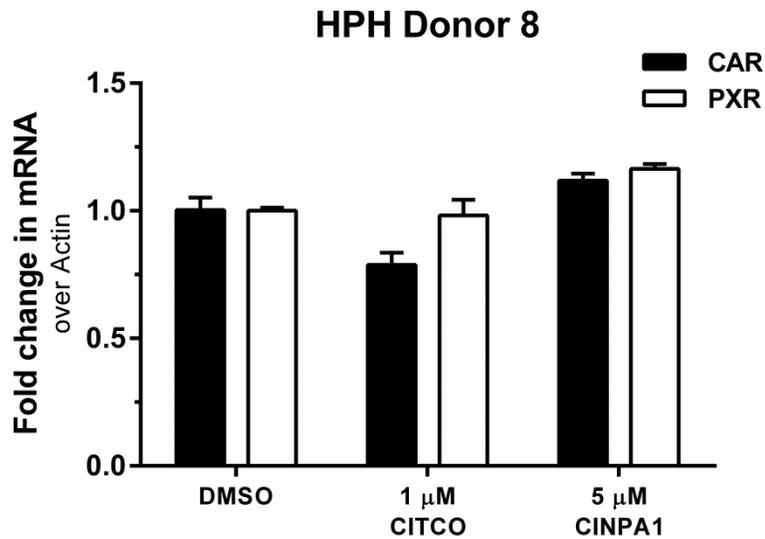
From the Department of Chemical Biology and Therapeutics, St. Jude Children's Research
Hospital, Memphis, Tennessee 38105

Supplemental Figure 1



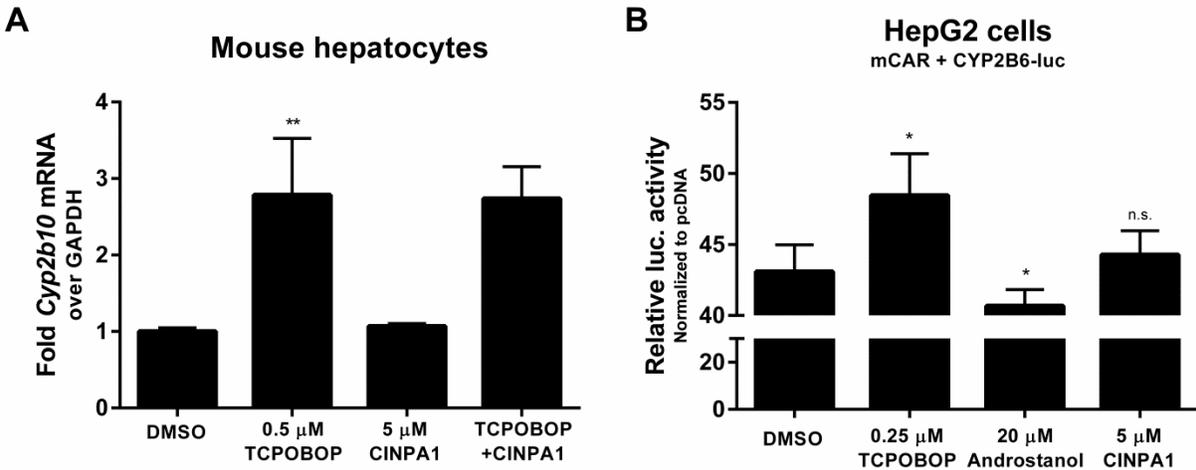
Supplemental Figure 1. CINPA1 acts as a weak antagonist of the hCAR3 isoform.

An hCAR3 expression construct was generated by inserting 5 amino acids (APYLT) at position 271 in the pcDNA-FLAG-hCAR1 plasmid. HepG2 cells were transfected with plasmids expressing hCAR3, RXR α and CYP2B6-luc reporter. After 24 h incubation, cells were treated with indicated concentrations of CINPA1, in the absence (A) or presence (B) of 2 μ M CITCO. DMSO, 50 μ M PK11195, or 2 μ M CITCO was used as controls. Luciferase activity was measured 24 h after treatment by using SteadyLite reagent (PerkinElmer). A. Activity in DMSO-treated samples was set as 0% activation; activity in samples treated with 2 μ M CITCO was set as 100% activation. B. Activity in samples treated with 50 μ M PK11195 was set as 0% CAR activation and samples treated with 2 μ M CITCO was set as 100% activation. GraphPad Prism was used to fit the data into a dose-response stimulation equation to derive IC₅₀ values. CINPA1 inhibits hCAR3 with an IC₅₀ of 18 μ M and shows no agonistic effect on hCAR3 activation.



Supplemental Figure 2. CINPA1 does not alter CAR or PXR mRNA levels in human hepatocytes.

Human primary hepatocytes (Donor 8) were treated with DMSO, 1 μM CITCO, or 5 μM CINPA1 for 48 h. RNA was extracted and used for cDNA synthesis, followed by quantitative real-time PCR assays with Taqman probes. β-actin was used as the internal control. No significant change was observed in CINPA1 treated hepatocytes when compared to DMSO treated samples.



Supplemental Figure 3. CINPA1 does not affect mouse CAR.

A. Mouse hepatocytes were isolated from C57Bl/6 mouse liver (TRL Cat # MBF06OL or MBFS1M; Triangle Research Labs, LLC, Research Triangle Park, NC) and plated in 6-well plates in Hepatocyte Maintenance Medium (TRL Cat # MM250). Cells were treated with DMSO, 0.5 μ M TCPOBOP (mouse CAR agonist), 5 μ M CINPA1, or 0.5 μ M TCPOBOP + 5 μ M CINPA1 for 24 h. RNA was extracted and used for cDNA synthesis, followed by performing quantitative real-time PCR assays with mouse-specific Taqman probes. GAPDH was used as the internal control. Statistical analysis was performed using one-way ANOVA for multiple comparisons and **, $p < 0.01$. While TCPOBOP significantly induced Cyp2b10 mRNA, no significant change was detected with 5 μ M CINPA1 treatment on basal (DMSO) or TCPOBOP-induced Cyp2b10. B. Plasmids expressing CYP2B6-luc reporter and pTK-Renilla-luciferase were cotransfected with pcDNA (control plasmid) or pCR3-mCAR [kind gift from Dr. Hongbing Wang, (Li et al., 2008)] in HepG2 cells. After 24 h incubation, cells were treated with DMSO, 0.25 μ M TCPOBOP (mCAR agonist), 20 μ M androstanol, or 5 μ M CINPA1. Luciferase activity was measured 24 h after treatment by using Dual-Glo luciferase reagents (Promega, Madison, WI). Relative luciferase activity was calculated as the firefly to renilla luciferase ratio for each treatment condition and normalized to pcDNA (control plasmid) transfected samples. *, $p < 0.05$. CINPA1 does not inhibit mCAR function in these assays.

Reference:

Li L, Chen T, Stanton JD, Sueyoshi T, Negishi M and Wang H (2008) The peripheral benzodiazepine receptor ligand 1-(2-chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide is a novel antagonist of human constitutive androstane receptor. *Mol Pharmacol* **74**(2): 443-453.