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Modulation of constitutive androstane receptor (CAR) and pregnane X receptor (PXR) by 6-arylpyrrolo[2,1-d] [1,5]benzothiazepine derivatives, ligands of peripheral benzodiazepine receptor (PBR)

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Abstract

Constitutive androstane receptor (CAR) and pregnane X receptor (PXR) regulate xenobiotic sensing and metabolism through interactions with multiple exogenous and endogenous chemicals. Compounds that activate CAR are often ligands of PXR; attention is therefore given to discovery of new, receptor-specific chemical entities that may be exploited for therapeutic and basic research purposes. Recently, ligands of the peripheral benzodiazepine receptor (PBR), PK11195 and FGIN-1-27, were shown to modulate both CAR and PXR. PBR is a mitochondrial transport protein responsible for multiple regulatory functions, including heme biosynthesis, a major component in cytochrome P450 (CYP) enzymes. To investigate possible new roles for PBR involvement in metabolic regulation, expression of the CAR and PXR target genes, CYP2B6 and CYP3A4, was measured in human hepatocytes following treatment with a targeted PBR ligand set. Luciferase reporter assays with transiently expressed wild-type CAR (CAR1), splice variant CAR3, or PXR in HuH-7 cells were used to further study activation of these receptors. Four structurally-related PBR ligands (benzothiazepines) differentially modulate CAR1, CAR3 and PXR activity. Benzothiazepine NF49 is an agonist ligand of CAR3, a partial agonist of PXR, exhibits greater inverse agonist activity on CAR1 than does PK11195, and is a new tool for studying these closely related nuclear receptors.

Keywords

CAR; PXR; hepatocyte; CYP2B6; CYP3A4; PBR

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CONFLICT OF INTEREST STATEMENT

THE AUTHORS DECLARE THAT THERE ARE NO CONFLICTS OF INTEREST.

1. INTRODUCTION

Hepatocytes express many nuclear receptor proteins that regulate the expression of drug metabolizing enzymes, including the cytochrome P450s (CYP), responsible for the metabolism of multiple endogenous and exogenous chemicals (Mottino and Catania, 2008). It is crucial to understand which drugs are inducers or inhibitors of CYP enzyme expression and activity so that adverse drug-drug interactions might be minimized (Amacher, 2010). Constitutive androstane receptor (CAR; NR1I3) and pregnane X receptor (PXR, also known as SXR; NR1I2), are involved in the regulation of all phases of xenobiotic metabolism and transport (Xu et al., 2005) including the transcriptional regulation of the phase I metabolizing enzymes, CYP2B6 and CYP3A4. PXR and CAR are expressed predominately in the liver; CAR has little expression in other tissues, while PXR is found also in intestine, stomach and kidney (Baes et al., 1994; Kliewer et al., 1998). Both receptors heterodimerize with retinoid X receptor-α (RXRα; NR2B1), which is important for target gene regulation (Blumberg et al., 1998; Goodwin et al., 1999; Honkakoski et al., 1998). Inactive CAR and PXR are tethered in the cytoplasm until chemical activation causes translocation to the nucleus (Qatanani and Moore, 2005; Squires et al., 2004; Yoshinari et al., 2003). CAR translocation can be activated directly by ligand binding or indirectly via cell transduction pathways (Rencurel et al., 2005; Yoshinari et al., 2003). Phenobarbital (PB) is an indirect CAR activator that does not bind directly to the receptor (Moore et al., 2000), yet treatment with PB facilitates translocation of CAR to the nucleus where it can regulate the expression of the CYP2B gene, for example, by binding specific promoter motifs (Kawamoto et al., 1999). An example of a direct activator of CAR is 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO), which is a human CAR agonist ligand that increases activation of CYP2B6 and coactivator recruitment (Maglich et al., 2003). It appears that only direct activators initiate PXR translocation; other mechanisms of activation have yet to be discovered (Squires et al., 2004). Once in the nucleus, both CAR and PXR can bind to a phenobarbital-responsive enhancer module (PBREM) site upstream of CYP2B6 (Goodwin et al., 2001; Honkakoski et al., 1998), and to a xenobiotic-responsive enhancer module (XREM) site upstream of CYP3A4 (Goodwin et al., 2002; Goodwin et al., 1999); CAR binds preferentially to PBREM over XREM whereas the reverse is true for PXR.

While mouse CAR activity has been shown to be regulated by two metabolites of androstane (Forman et al., 1998), human CAR remains an orphan member of the nuclear receptor family of transcription factors, with no endogenous ligand or non-ligand activators yet known (di Masi et al., 2009). Multiple exogenous compounds with direct or indirect effects on CAR-dependent gene regulation have been discovered, but few are selective for CAR specifically as most bind also to PXR (Chang and Waxman, 2006). CAR ligand binding is also highly species-dependent; for example, from a list of 13 CAR ligands detailed in a recent review, only six bind to human CAR versus mouse CAR (di Masi et al., 2009). Thus far, binding studies have revealed that most ligands of CAR also bind PXR and elicit significant transcriptional activation even with relatively low binding affinity. However, recently in a screen of 16 medications on the market, three compounds were identified as specific CAR activators: carbamazepine (CMZ), efavirenz (EFV), and nevirapine (NVP); all three compounds activated CYP2B6 expression to a much greater extent than they activated CYP3A4 expression (Faucette et al., 2007).

Wild type reference CAR (noted henceforth as CAR1) differs from typical nuclear receptors in that the amino terminal A/B and carboxy terminal F domains of the protein are absent, which together with unique intramolecular amino acid contacts, confer upon CAR1 a constitutive ligand-independent activity and nuclear expression in transiently transfected cells (Auerbach et al., 2003). There are at least 22 known splice variants of human CAR, 15

of which produce unique CAR proteins (di Masi et al., 2009). The most abundantly expressed CAR isoforms are CAR1 and the ligand-dependent variant CAR3, detected in roughly equal amounts in human liver (Jinno et al., 2004). CAR3 differs from CAR1 by an APYLT amino acid insertion outside of the ligand binding pocket, possibly causing steric hindrance interactions with RXR (Auerbach et al., 2003). CAR1 has high basal activity and most ligands of CAR1 deactivate its constitutive activity. CAR3 exhibits low basal activity, but is ligand-activated and so has been used as a surrogate to identify potential ligands of CAR1 with the expectation that ligands, which bind to CAR3, will similarly activate CAR1. However, a recent study from our lab revealed that multiple ligands that modulate CAR3 have no effect on CAR1 and vice versa (Dring et al., 2010). Similarly ligands that modulate activity of PXR can have different effects on CAR1 and CAR3.

In 2008, Li et al. found that 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195), a well-known peripheral benzodiazepine receptor (PBR) ligand, antagonized human CAR, while activating PXR (Li et al., 2008). Although the study showed CAR deactivation was independent of PBR, it is possible that PBR, CAR, PXR, and other receptors may have evolved as metabolic stress receptors to work in conjunction with one another (Gavish et al., 1999; Gong et al., 2006; Huang et al., 2005). We were interested to see if additional PBR ligands, not incorporated in the Li et al. study, would modulate CAR and/or PXR, whether selectively or promiscuously. Using *in vitro* approaches, we identified 6-arylpyrrolo[2,1-d][1,5]benzothiazepine derivatives (synthesized to be specific PBR ligands) (Fiorini et al., 1994) as novel human CAR and PXR ligands.

2. MATERIALS AND METHODS

2.1 Chemical treatments

Benzodiazepine receptor ligands used for the initial CYP2B6/CYP3A4 activation screen (Fig. 1), including DCPPBT, were sourced from within the Biomol GABA-ergic chemical library (version 3.6, lot #N1205; Enzo Life Sciences, Farmingdale, NY). NF compounds: NF49, NF51 and NF115 (≥98% purity) were from Alexis Biochemicals (part of Enzo Life Sciences). Positive control treatments rifampicin and CITCO were from Sigma-Aldrich (St. Louis, MO), 4-nonylphenol (as mixed isomers) was from Acros Organics (Morris Plains, NJ), clotrimazole was from MP Biomedicals (Solon, OH), PK11195 was from Tocris Bioscience (Ellisville, MO), and phenobarbital was generously provided by Dr. Bingfang Yan (University of Rhode Island, Kingston, RI). Solvent control for all treatments was dimethylsulfoxide (DMSO) (Fisher Scientific, Pittsburgh, PA). Stock solutions for all treatments were diluted in DMSO to 1000x final concentration to ensure final DMSO concentration would not exceed 0.2% (v/v).

2.2 Cell culture

HuH-7 cells (JTC-39) were a gift from Drs. Bingfang Yan and Ruitang Deng (University of Rhode Island, Kingston, RI) and were maintained at 37°C/5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% (v/v) fetal bovine serum, 100 U/mL penicillin G, 100 μg/mL streptomycin, 2 mM GlutaMAX, 0.15% (w/v) sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES buffer, and 100 μM non-essential amino acids. Primary human hepatocyte monolayers, obtained from the NIH-funded liver tissue cell distribution system (LTCDS) through Dr. Stephen Strom (University of Pittsburgh, Pittsburgh, PA), were maintained in supplemented Williams' E media, essentially as described (Zamule et al., 2008). Cell culture media and additives were from Invitrogen/GIBCO Corp. (Carlsbad, CA) or Lonza (Hopkinton, MA). Hepatocytes were maintained in 12-well plates for 4–8 days prior to chemical treatment to ensure CYP mRNA levels were at basal levels prior to treatment with test compounds.

2.3 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from primary human hepatocytes using TRIzol reagent (Invitrogen/GIBCO), following 24 h chemical treatments, and reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) according to manufacturers' instructions. Relative quantification of CYP2B6 and CYP3A4 mRNA expression was measured by SYBR Green using a 7500 Real-Time PCR System (Applied Biosystems). Two technical replicate wells were run for each cDNA sample. Data were analyzed in Applied Biosystems SDS v2.0 software using auto threshold and auto baseline settings. Technical replicates were combined to provide mean C_T values and mRNA expression levels relative to β-Actin were calculated using the $\Delta\Delta C_T$ method. SYBR Green primers were designed using QPrimerDepot (http://primerdepot.nci.nih.gov) (Cui et al., 2007). The β-Actin, CYP2B6, and CYP3A4 primers (Integrated DNA Technologies, Coralville, IA) were as follows: β-Actin, F: GTTGTCGACGACGAGCG, R: GCACAGAGCCTCGCCTT; CYP2B6, F: GTCCCAGGTGTACCGTGAAG, R: CCCTTTTGGGAAACCTTCTG; CYP3A4, F: CACAGGCTGTTGACCATCAT, R: TTTTGTCCTACCATAAGGGCTTT. Data in Figure 1 resulted from singlet treatment wells from two hepatocyte cases (HH1390 and HH1420). In Figure 2, data points are the average of two treatment replicates for each of two hepatocyte cases (HH1460 and HH1461). Error bars, where present, represent the standard deviation of treatment replicates.

2.4 Plasmid constructs

Full-length CAR1 and CAR3 sequences were ligated into the pM vector (Clontech, Mountain View, CA) expressing an amino terminal GAL4 DNA binding domain fusion to the nuclear receptor. The pM-CAR1 or pM-CAR3 reporter plasmid was pFR-Luc, containing five copies of the consensus GAL4 response element, upstream activation sequence (UAS), cloned 5′ of the firefly luciferase gene (Auerbach et al., 2005). The previously-described PXR reporter plasmid, p3A4-XREM-TK-Luc, contained the proximal ER-6 and distal XREM sequences of CYP3A4, blunt-end ligated into the *Sma I* site of luciferase vector pTK-Luc by PCR amplification from genomic DNA (Auerbach et al., 2005). Original pTK-Luc and p3A4-XREM-TK-Luc cloning was performed in the laboratory of Dr. Curtis Omiecinski (The Pennsylvania State University, State College, PA). p3xFLAG-PXR, and p3xFLAG-RXRα expression plasmids were produced in p3xFLAG-CMV10 vector (Sigma-Aldrich) using gene specific primers and PCR amplification from human liver cDNA.

2.5 Transient transfections and treatments

For the NF compound screen with CAR1 and CAR3 (Figures 3A, B and 4A, B), T-25 flasks of HuH-7 cells were transfected with 1.25 μg pM-CAR1 or pM-CAR3 and 5 μg pFR-Luc. Similarly for PXR assays (Fig. 3C and 4C), HuH-7 cells were transfected with 1.25 μg each of p3XFLAG-PXR and p3XFLAG-RXRα and 5 μg p3A4-XREM-TK-Luc. 1.25 μg of enhanced green fluorescent protein plasmid (pEGFP-C1) (Clontech) and 0.5 μg of *Renilla* luciferase plasmid, pRL-CMV (Promega, Madison, WI) were included in each transfection to monitor transfection efficiency and for assay normalization, respectively. Transfections were performed in UltraMEM (Lonza) for 24 h using 25 kDa polyethylenimine (PEI) (Polysciences, Inc, Warrington, PA) transfection reagent (4 μL of 1 mg/mL PEI per 1 μg DNA in 20 mM HEPES buffer). Following transfection, cells were trypsinized and re-seeded into clear-bottomed, white-walled 96-well plates. Chemical treatments were performed in replicate wells for 24 h; n= the number of wells for each chemical treatment. Both single and co-treatment controls contained equal amounts of DMSO. Luciferase activity was measured using Dual-Glo Luciferase Reporter kit (Promega) on a GloMax 96 Microplate Luminometer (Promega).

2.6 Statistical analysis

Unpaired Student's t-tests were used to evaluate the differences between treatment replicates where n ≥ 2 (Fig. 2, 3 and 4). Differences were deemed statistically significant where $p < 0.05$. Firefly/*Renilla* luciferase luminescence ratios and qRT-PCR expression change is reported as mean fold change relative to DMSO (control) ± standard deviation (SD).

3. RESULTS

3.1 Benzodiazepine receptor ligands modulate CYP2B6 and CYP3A4 expression

Several ligands of benzodiazepine receptors were screened for activation of CAR and PXR target genes in primary human hepatocytes from two separate individuals (HH1390 and HH1420) (Fig. 1). Following RNA extraction, expression of CYP2B6 and CYP3A4 mRNA was measured using quantitative real-time PCR (qRT-PCR). Results show that CAR activators, phenobarbital (PB) (500 μM) (Kawamoto et al., 1999) and CITCO (5 μM), and the PXR agonist, rifampicin (25 μM) (Blumberg *et al.*, 1998) induced CYP2B6 and CYP3A4 gene expression relative to DMSO (control) treated cells. CITCO and PB induction of CYP2B6 expression was more prominent than that seen with CYP3A4 expression, as expected for preferential CAR activators. Rifampicin treatment in HH1420 cells increased the expression of both CYP2B6 and CYP3A4. FG7142, PK11195, DCPPBT, and FGIN-1-27 treatments all increased CYP2B6 expression in HH1420, while DCPPBT was the only compound to increase CYP2B6 in HH1390. Multiple compounds increased CYP3A4 expression in HH1420, including PK11195, DCPPBT, and FGIN-1-27. Additionally, PK11195 and DCPPBT treatments increased CYP3A4 expression in HH1390. Although absolute levels of gene expression differed between the two hepatocyte cases (HH1420 showing greater magnitude of induction in response to treatments than HH1390), the trends were similar; induction of CYP2B6 and CYP3A4 by DCPPBT treatments is clearly seen in both individuals.

3.2 Benzothiazepine analogs of DCPPBT modulate CAR and PXR

3.2.1 CYP2B6 and CYP3A4 expression—Since DCPPBT was the only compound that increased CYP2B6 expression in both hepatocyte cases, attention was focused on this benzothiazepine and studies were extended to include a set of structurally related analogs (NF compounds) for their effects on CYP2B6 and CYP3A4 expression in human hepatocytes. Human hepatocytes cases HH1460 and HH1461 were treated with DCPPBT and related compounds: NF49, NF51 and NF115. CYP2B6 and CYP3A4 gene expression changes were assessed for these compounds using qRT-PCR analysis (Fig. 2). The absolute levels of induction seen in response to treatments vary between the hepatocyte cases, with HH1460 showing a greater magnitude of CYP2B6 expression changes in response to treatments, while HH1461 shows a more equal magnitude of both CYP2B6 and CYP3A4 expression changes. In HH1461 cells, CYP2B6 and CYP3A4 expression were increased equally following PB treatment, and responses to CITCO and rifampicin control treatments were as expected. A consistent expression pattern in response to treatment with NF49 and NF115 was seen in both sets of hepatocytes. NF49 and NF115 treatments increase CYP2B6 expression to a greater extent than NF51 and DCPPBT, and NF51 treatment increases CYP3A4 expression to a greater extent than NF49 and NF115. In summary, all benzothiazepines tested were modulators of CYP2B6 and CYP3A4 expression, and therefore are likely CAR and/or PXR regulators.

3.2.2 CAR1 basal repression—Hepatocytes express many nuclear receptor proteins that are responsible for both basal and inducible CYP2B6 and CYP3A4 gene expression. Since compounds that bind to CAR frequently also bind to PXR, receptor-specific reporter constructs were used to screen for specific activation of CAR1, CAR3 or PXR. In Figure 3A

and 4A, HuH-7 cells were transfected with pMCAR1 and reporter pFR-Luc to express exogenous human CAR1 and were used to measure the luciferase reporter activity in response to treatment with the benzothiazepines at 10 μM. Previous studies have shown 10 μM to be an effective concentration for CAR ligand compound screening, affording the best chance that any ligand effect can be detected without inducing cytotoxicity (data not shown). In addition to DMSO solvent control, cotreatments of 10 μM PK11195 and 3 μM 4-nonylphenol (Hernandez *et al.*, 2007) were used in order to widen the CAR1 assay window by respectively antagonizing or raising further the high basal activity (Fig. 3A). As expected, treatment with a CAR1 inverse agonist, clotrimazole (Moore *et al.*, 2000) resulted in decreased CAR1 basal activity. CAR1-transfected cells treated with benzothiazepines, both alone and in combination with PK11195 or 4-nonylphenol, yielded surprising results: DCPPBT treatment alone had no significant effect but treatments with NF49, NF51 and NF115 all suppressed CAR1-driven luciferase reporter activity. PK11195 cotreatment, as shown previously, repressed CAR1 activity, and this repression was partially reversed by co-treatment with 4-nonylphenol, DCPPBT, NF51 and NF115. Treatment with 4-nonylphenol alone nearly doubled the activity of CAR1, and co-treatments with clotrimazole, DCPPBT, NF49, NF51 and NF115 suppressed 4-nonylphenol induced luciferase reporter expression. Interestingly, NF49 further repressed CAR1 antagonism by PK11195 and suppressed 4-nonylphenol-induced luciferase reporter expression below basal. Since NF49 and NF51 produced the most pronounced effects on all three receptors, a dose-response study was carried out for these compounds (Fig. 4). Increasing concentrations of NF49 had increasingly inverse agonist effects on CAR1, while NF51 only repressed CAR1 mediated luciferase expression at 20 μM (Fig. 4A).

3.2.3 CAR3 activation—Very different results from benzothiazepine treatments (10 μM) were observed with HuH-7 cells transiently transfected with ligand-dependent pM-CAR3 and reporter pFR-Luc (Fig. 3B). As expected, treatment with the CAR3 agonist control clotrimazole (Auerbach et al., 2005) led to significant activation of CAR3-dependent luciferase expression. However, treatments with DCPPBT, NF49 and NF115 at 10 μM dramatically activated CAR3, even more so than did clotrimazole, the opposite effect to that seen with CAR1. Strikingly, NF51 (10 μM) did not activate CAR3 (Fig. 3B), however, the dilution series (Fig. 4B) shows an unexpected U-shaped dose-response relationship, where NF51 acts as a weak CAR3 agonist at concentrations ≥ 100 nM and >10 μM. NF49 has no discernable effect on CAR3 activation at and below 1 μM, but concentrations of 5 μM and above have significant agonist ligand effects of far greater magnitude than those observed for NF51. The pattern of CAR3 response to NF49, NF51 and NF115 mirrors that of CYP2B6 expression observed in the hepatocyte cases HH1460 and HH1461 treated with the same concentrations (Fig. 2A).

3.2.4 PXR activation—HuH-7 cells also were transfected with p3xFLAG-PXR and the reporter, p3A4-XREM-TK-Luc, in the presence of additional RXRα (p3xFLAG-RXRα), and treated with 10 μM benzothiazepines in the absence and presence of PXR agonist rifampicin (Fig. 3C). All compounds tested were strong agonists of PXR transcriptional activity, with the exception of NF49; NF49 alone was a weak agonist of PXR. DCPPBT further increased PXR activation in cotreatment with rifampicin. NF51 and NF115 did not antagonize rifampicin activation, but PK11195 showed slight antagonism in rifampicin co-treatment. NF49 strongly antagonized rifampicin-activated PXR. It was noted that with both CAR1 and PXR, neither antagonist nor agonist treatments altered the inverse agonist/antagonist effects of NF49. Both NF49 and NF51 at higher concentrations increasingly activated PXR (Fig. 4C); however, NF49-induced luciferase activity at 5–20 μM was lower than that induced by NF51 at 1 μM. NF49 and NF51 activation of PXR plateaus around 5–10 μM.

4. DISCUSSION

A role for PBR ligands in the maintenance of metabolic homeostasis was shown in that a set of benzothiazepine analogs differentially modulated two CAR splice variants, CAR1 and CAR3, and the related nuclear receptor PXR. Human hepatocytes from two individuals were treated with a panel of 16 known benzodiazepine receptor ligands in addition to PB, CITCO, rifampicin (HH1420 only), and the solvent control, DMSO. Changes in expression of the CAR and PXR target genes, CYP2B6 and CYP3A4, two CYP enzymes involved in the metabolism of many endogenous and exogenous compounds, were measured (Fig. 1). There were clear inter-individual differences between the hepatocyte cases with respect to responses to chemical treatments. It is evident in Figure 1 that HH1390 and HH1420 possess varying endogenous levels of nuclear receptors that affected the overall response to these compounds, however, despite the differences in magnitude of response, a trend is clearly apparent. DCPPBT was selected from this experiment as the stand-alone compound that increased CYP2B6 and CYP3A4 expression in both hepatocyte cases.

To investigate the structure-activity aspects of DCPPBT, we screened a set of closely structurally-related benzothiazepine analogs: NF49, NF51 and NF115 (Fig. 2B). The greatest divergence in activity was seen with NF49 and NF51, two compounds that share the same phenyl pyrrolo-benzothiazepine core structure; NF51 has a dimethylamine in place of the ester of NF49. The greatest difference in effect between these two compounds was seen on CAR3 activation (Fig. 3B and 4B); at 10 μM, NF49 activated CAR3, while NF51 showed no response. While demonstrating agonist activity with CAR3, NF49 also showed the greatest inverse agonist effect on CAR1. NF51 and NF115 were also inverse agonists of CAR1 when treated alone and in the presence of CAR the agonist 4-nonylphenol, though to a lesser degree than NF49. In contrast to NF49 however NF51 and NF115 had CAR1 agonist activity in the presence of PK11195 cotreatment (Fig. 3A). Thus small changes in the benzothiazepine substituents likely alter nuclear receptor binding to yield receptor-specific ligand effects.

Since CYP2B6 and CYP3A4 are regulated by both CAR and PXR, it was necessary to investigate whether regulation of these target genes could be occurring through CAR and/or PXR. We used transient over-expression of CAR1, CAR3 and PXR with receptor-specific reporter constructs in a human hepatoma cell line (HuH-7) to measure the interaction of each nuclear receptor with a specific DNA motif upstream of a luciferase gene (Fig. 3 and 4). Proliferating HuH-7 cells have low endogenous expression of many CYP enzyme genes as well as low levels of CAR and PXR (Choi et al., 2009) making them highly suitable for the study of specifically transfected CAR and PXR constructs without endogenous interference. An additional advantage of using HuH-7 cells is that they are relatively easy to transfect with high efficiency. Full-length CAR fusions to GAL4 DNA binding domain were used instead of GAL4-CAR LBD fusions, but GAL4 fusions to CAR LBD alone would likely have yielded similar results, since the plasmid reporter construct contains response elements for GAL4 and was used instead of traditional CAR response elements in order to eliminate possible interference from any DNA binding by endogenous nuclear receptors. Since the GAL4 protein is interacting with the DNA directly, instead of CAR and RXR together interacting with a DR-4 motif, the only response from chemical treatment should be due to interactions with the CAR ligand binding domain. The splice variation that results in the insertion of the amino acids APYLT in CAR3 is predicted to interfere with RXR heterodimerization, potentially altering the constitutive activity of CAR (Auerbach et al., 2003). It should be noted that the expression of transfected CAR1, CAR3, and PXR is already localized in the nucleus in the absence of any direct or indirect activators. Any modulation of these transfected receptors by the benzothiazepines is therefore the result of direct receptor binding and we cannot comment at this time as to whether or not the NF

compounds act also as indirect modulators. The use of 3xFLAG-PXR and 3xFLAG-RXRα expression plasmids in conjunction with the CYP3A4 specific reporter construct provided a larger assay window than similar experiments using GAL-4 PXR plasmids (data not shown).

Due to its ligand-dependent activity, it has previously been thought that CAR3 was useful for general screening of possible CAR ligands. However, in a recent study of 60 compounds, it was revealed that the same ligand can act very differently upon CAR1 and CAR3 (e.g. one compound that is an agonist of CAR1 may not have an effect on CAR3 or antagonize CAR3) (Dring et al., 2010). Also, studies have shown that in some cell lines, clotrimazole is an inverse agonist of CAR1 but is a strong agonist of CAR3 (Auerbach et al., 2005). In this study, we noticed that NF49 and NF51, differing only by a dimethylamine, exhibited very different agonist or antagonist characteristics when binding to CAR1, CAR3 and PXR.

Typically if a compound antagonizes CAR and PXR, CYP2B6 and CYP3A4 expression would show little, if any, increase; however, it is possible that other receptors regulate these two genes. With this in mind, it would be expected that treatment with NF49, a CAR1 and PXR antagonist, would not increase either CYP2B6 or CYP3A4 gene expression beyond basal levels. However, in Figure 2, NF49 significantly increases CYP2B6 and CYP3A4 expression above basal. Also, NF49 and NF115 increase CYP2B6 expression equally, a response similar to that seen with CAR3 activation. DCBBPT, NF51 and NF115 activate PXR similarly, while NF49 partially antagonizes PXR (Figure 3C); these three compounds also increase CYP3A4 expression similarly, while NF49 is less prominent. Since CYP2B6 is also regulated by PXR, a CAR antagonist/PXR agonist could still increase CYP2B6 expression. However, it would be expected that NF49, the weakest PXR agonist of the benzothiazepines and strongest CAR1 inverse agonist, should not increase CYP2B6 more than or equally compared to the other benzothiazepines. This observation leads us to believe there is a strong CAR3 influence on the regulation of CYP2B6.

In summary, we classify DCPPBT as a CAR1 partial agonist, as it de-represses PK11195-treated CAR1 but antagonizes 4-nonylphenol-treated CAR1. NF49 is a strong CAR1 inverse agonist because it strongly represses CAR1-dependent activity by itself, in the presence of PK11195 and in the presence of 4-nonylphenol. NF51 and NF115 are classified as partial CAR1 agonists, but with stronger inverse agonist activity than DCPPBT, because they antagonize CAR1 activity alone and in co-treatment with 4-nonylphenol, but they also de-repress PK11195 dependent CAR1 activity. DCPPBT, NF49, and NF115 are agonist activators of CAR3 while DCPPBT, NF51 and NF115 are PXR activators; NF49 is a partial agonist of PXR. Our combined data suggest a rather intriguing possibility, that CAR3 and PXR, and not CAR1 are more responsible and necessary for CYP2B6 expression in response to chemical ligands. At the very least, our description of a set of benzothiazepines as previously undescribed ligands of CAR1, CAR3 and PXR provides researchers with an expanded pharmacological tool chest for *in vitro* and *in vivo* studies of nuclear receptor activity.

Abbreviations

CAR	constitutive androstane receptor
CITCO	6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime
DCPPBT	7-(dimethylcarbamoyloxy)-6-phenylpyrrolo-[2,1-d][1,5]benzothiazepine
FGIN-1-27	*N*,*N*-Dihexyl-2-(4-fluorophenyl)indole-3-acetamide
FGIN-1-43	*N*,*N*-Dihexyl-2-(4-chlorophenyl)-5-chloroindole-3-acetamide

FG7142	N-methyl-9H-pyrido[5,4-b]indole-3-carboxamide
HH (number)	human hepatocyte (case number)
GBLD345	2-(4-Aminophenyl)-3-methoxy-6-(3-methoxyphenyl)-methylimidazo[1,2-*b*]pyridazine
NF49	7-(Acetoxy)-6-(p-methoxyphenyl) pyrrolo-[2,1-d][1,5]benzothiazepine
NF51	7-(dimethylcarbamoyloxy)-6-(p-methoxyphenyl)pyrrolo-[2,1-d][1,5]benzothiazepine
NF115	7-(Hexanoyloxy)-6-phenylpyrrolo-[2,1-d][1,5]benzothiazepine
PB	phenobarbital
PBR	peripheral benzodiazepine receptor (TSPO, translocator protein 18 kDa, modern designation of the PBR)
PK11195	1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide
PXR	pregnane X receptor
qRT-PCR	quantitative real-time PCR
RXRα	retinoid X receptor-α
XREM	xenobiotic-responsive enhancer module

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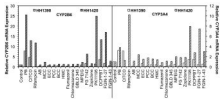

Figure 1.

qRT-PCR evaluation of CYP2B6 and CYP3A4 gene expression in response to benzodiazepine receptor ligand treatments in two human hepatocytes cases (HH1390 and HH1420). CYP2B6 and CYP3A4 gene expression was measured by qRT-PCR using gene specific primers and SYBR Green; expression data were normalized to β-Actin and expressed as fold change over control (DMSO). Cells were treated for 24 h. Treatment concentrations: PB (500 μM), rifampicin (25 μM), and CITCO (5 μM), all others 10 μM. Note, HH1390 has no rifampicin treatment. Additional abbreviations are as follows: AB, 1-amino-5-bromouracil; MCC, methyl-β-carboline-3-carboxylate; ECC, ethyl-β-carboline-3-carboxylate; PCC, propyl-β-carboline-3-carboxylate; BCC, butyl-β-carboline-3-carboxylate; HMC, 3-hydroxymethyl-β-carboline; MPEIG, N-[-(4-methoxyphenyl)ethyl]-3-indoleglyoxamide. All treatments, n=1.

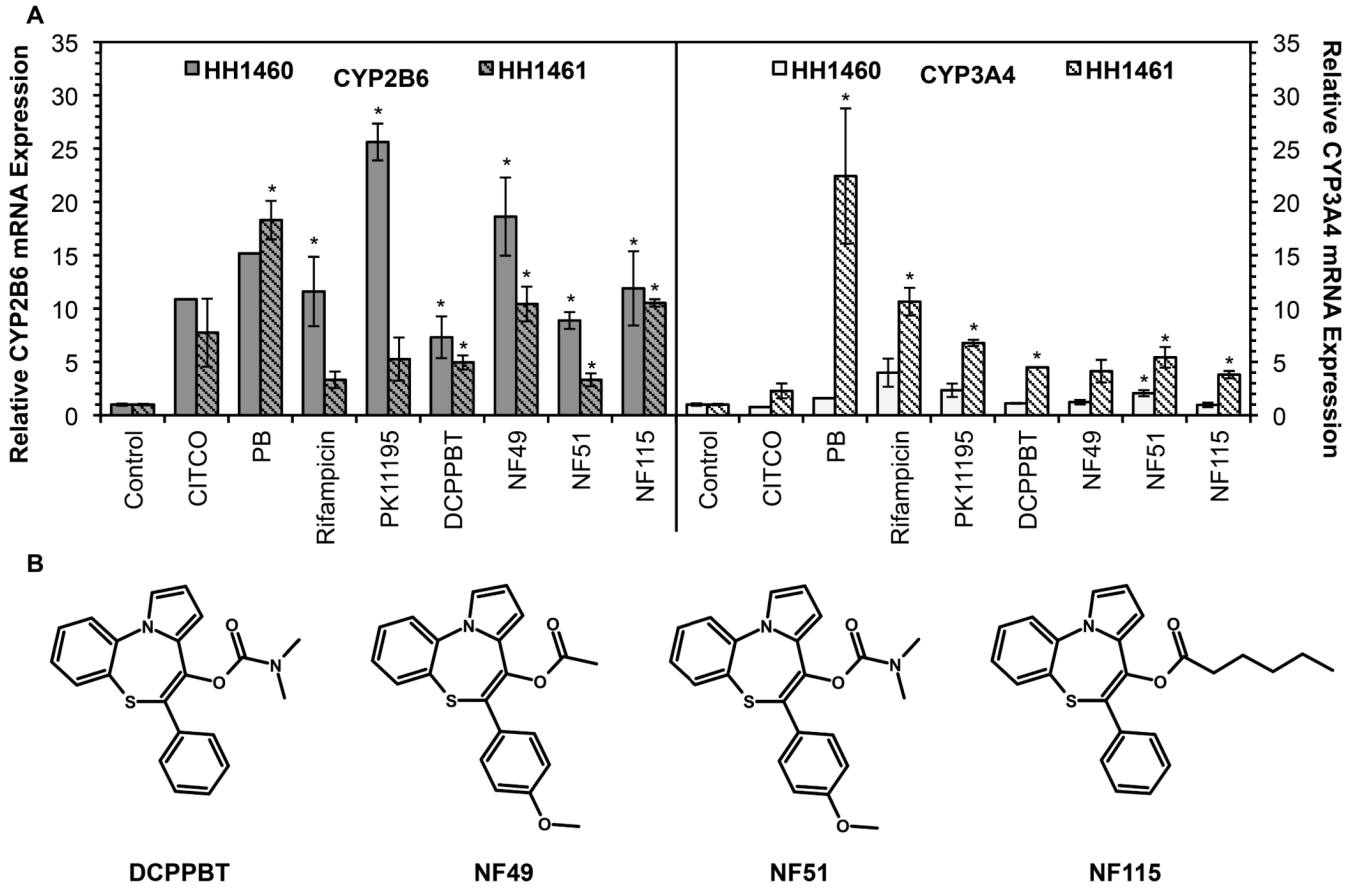

Figure 2.

(A) Modulation of CYP2B6 and CYP3A4 expression by DCPPBT and related benzothiazepine in human hepatocytes (HH1460 and HH1461). Gene expression was measured by qRT-PCR as in Figure 1. Data are expressed as fold change over control ± SD of treatment replicates. Treatment concentrations: PB (500 μM), rifampicin (25 μM), and CITCO (5 μM), all others 10 μM. Asterisk (*) indicates significantly different from control (DMSO), $p < 0.05$. All treatments, n=2 (except HH1460 CITCO and PB, n=1). **(B)** Chemical structures of DCPPBT, NF49, NF51 and NF115.

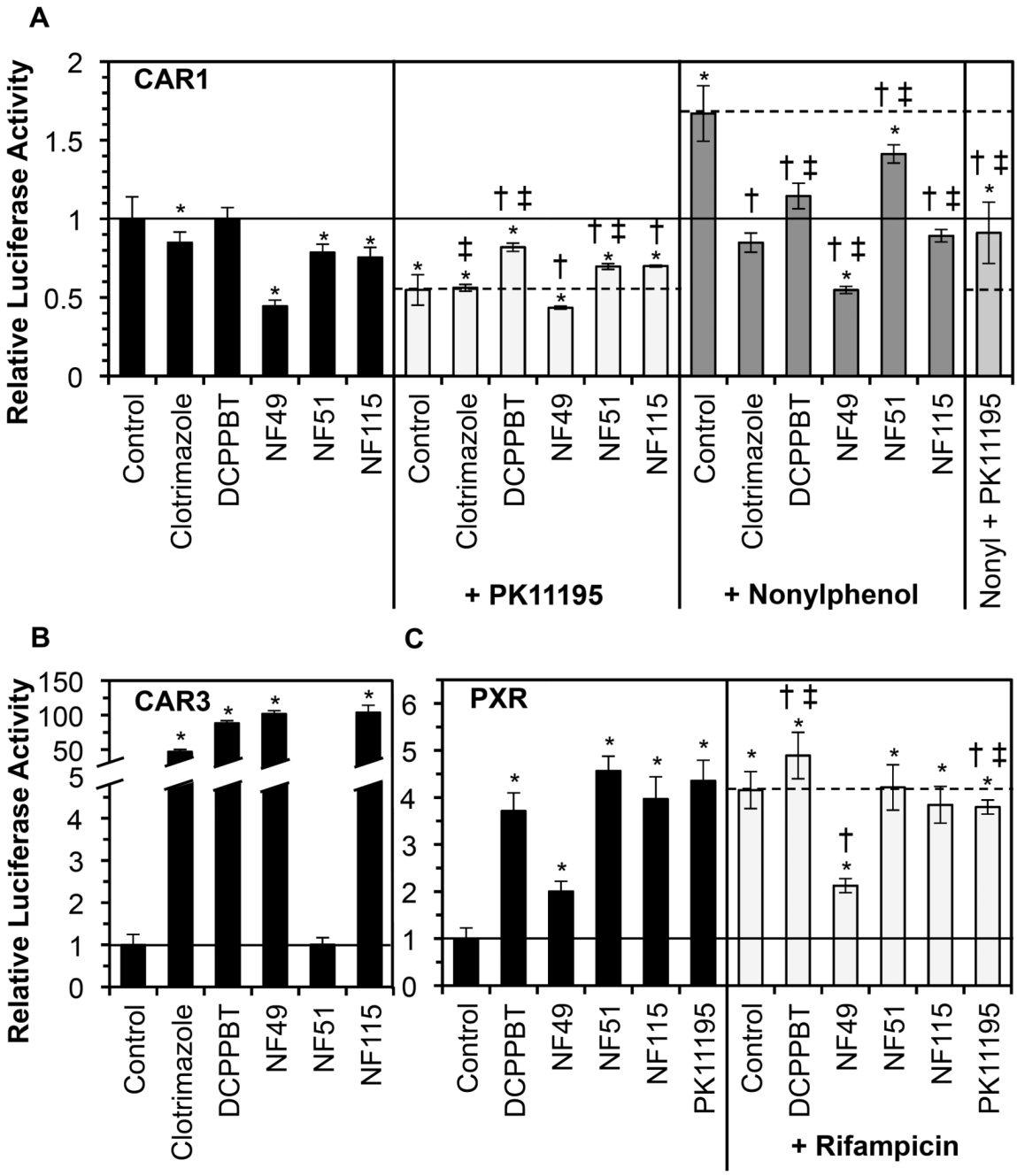

Figure 3.

Luciferase reporter assay evaluation of DCPPBT and benzothiazepine analogs for agonist/inverse agonist activity on CAR1, CAR3, and PXR. HuH-7 cells were transiently transfected with **(A)** expression plasmid pM-CAR1 and reporter pFR-Luc, **(B)** pM-CAR3 and pFR-Luc, or **(C)** p3xFLAG-PXR, p3xFLAG-RXR and p3A4-XREM-TK-Luc; all transfections included pEGFP-C1 (enhanced green fluorescent protein expression plasmid) to monitor transfection efficiency and pRL-CMV (*Renilla* luciferase normalization control plasmid). Cells were treated for 24 h. Treatments were as follows: **(A)** 10 μM each of clotrimazole, DCPPBT, NF49, NF51, NF115, co-treated with DMSO (control) n=8, PK11195 (10 μM, n=4), or 4-nonylphenol (3 μM, n=4). 4-Nonylphenol and PK11195 controls, n=12. **(B)** 10

µM each of clotrimazole, DCPPBT, NF49, NF51, NF115, n=6. **(C)** 10 µM DCPPBT, NF49, NF51, NF115 and co-treated with DMSO (control) or 25 µM rifampicin, n=8. Asterisk (*) indicates significant difference from control (solid line), $p < 0.05$; dagger (†) indicates significant difference from co-treatment (PK11195, 4-nonylphenol, or rifampicin) (dotted line), $p < 0.05$; double dagger (‡) indicates significant difference compared to respective control treatment (black bars), $p < 0.05$.

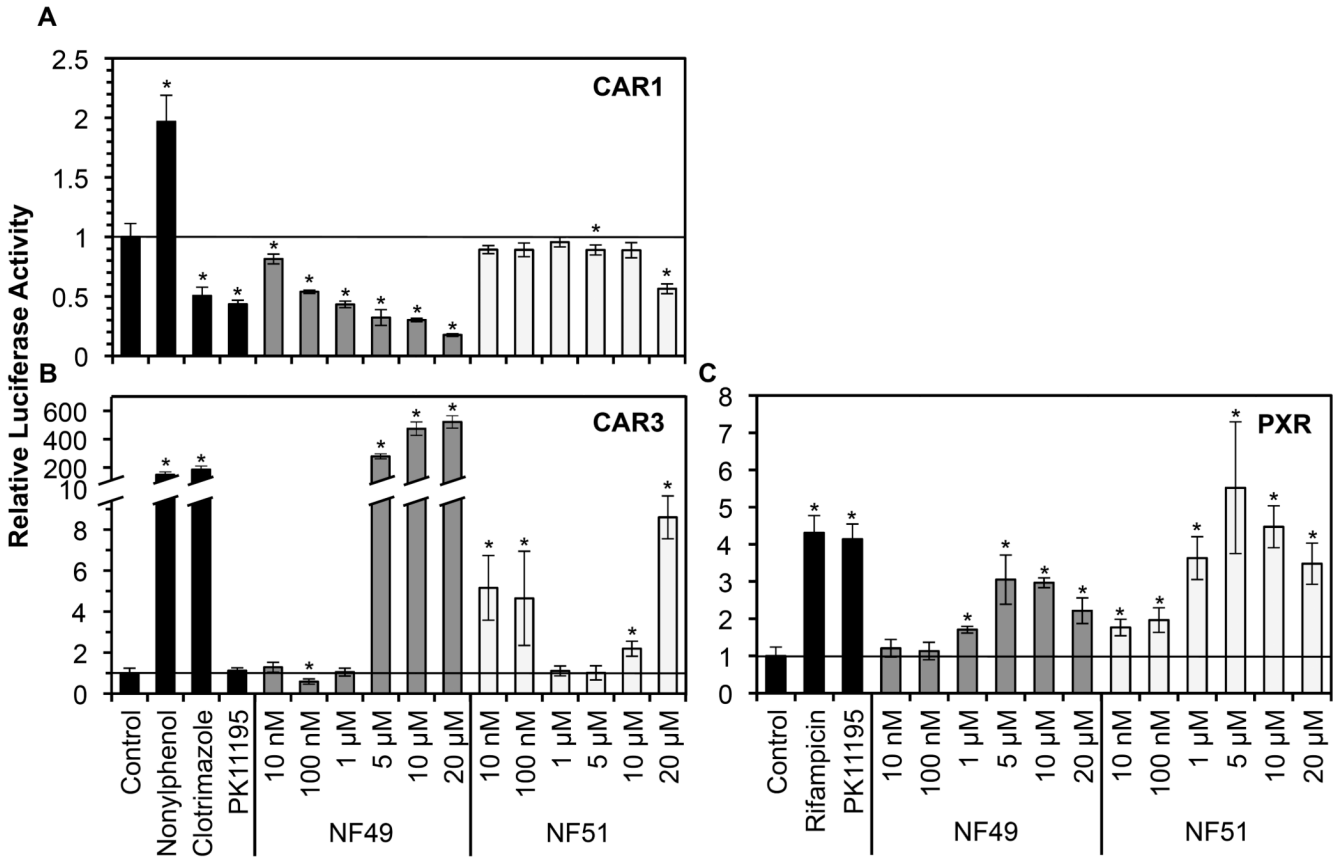

Figure 4.

Luciferase reporter assay evaluation of NF49 and NF51 dilution series effects on CAR1, CAR3 and PXR. HuH-7 cells were transiently transfected as in Figure 3: **(A)** expression plasmid pM-CAR1 and reporter pFR-Luc, **(B)** pM-CAR3 and pFR-Luc, **(C)** p3xFLAG-PXR, p3xFLAG-RXR and p3A4-XREM-TK-Luc. Cells were treated for 24 h with NF49 or NF51 at the following concentrations: 10 nM, 100 nM, 1 µM, 5 µM, 10 µM and 20 µM. Controls (solid black bars) were as follows: 4-nonylphenol (3 µM), clotrimazole and PK11195 (10 µM), and rifampicin (25 µM). Asterisk (*) indicates significant difference from control (solid line), $p < 0.05$, n=6.