Identification of Modulators That Activate the Constitutive Androstane Receptor From the Tox21 10K Compound Library

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ABSTRACT

The constitutive androstane receptor (CAR; NR1I3) is a nuclear receptor involved in all phases of drug metabolism and disposition. However, recently it's been implicated in energy metabolism, tumor progression, and cancer therapy as well. It is, therefore, important to identify compounds that induce human CAR (hCAR) activation to predict drug-drug interactions and potential therapeutic usage. In this study, we screen the Tox21 10,000 compound collection to characterize hCAR activators. A potential novel structural cluster of compounds was identified, which included nitazoxanide and tenonitroazole, whereas known structural clusters, such as flavones and prazoles, were also detected. Four compounds, neticonazole, diphenamid, phenothrin, and rimcazole, have been identified as novel hCAR activators, one of which, rimcazole, shows potential selectivity toward hCAR over its sister receptor, the pregnane X receptor (PXR). All 4 compounds translocated hCAR from the cytoplasm into the nucleus demonstrating the first step to CAR activation. Profiling these compounds as hCAR activators would enable an estimation of drug-drug interactions, as well as identify prospective therapeutically beneficial drugs.

Key words: constitutive androstane receptor; quantitative high-throughput screening; human primary hepatocytes; drug-drug interactions.

The constitutive androstane receptor (CAR, NR1I3) is a transcription factor well-known for modulating genes from all phases of drug metabolism (Qatanani and Moore, 2005). Drug metabolizing enzymes regulated by CAR, such as cytochrome P450 (CYP) 2B6 and CYP3A4, are involved in the metabolizing of nearly 25% and 50% of marketed drugs, respectively (Wang et al., 2003; Xie and Evans, 2001). Changes in the expression of these drug metabolizing enzymes can lead to drug-drug interactions, altering the therapeutic efficacy and/or toxicity of drugs (Gao and Xie, 2010; Pascucci et al., 2008). Although it is clearly important to study CAR activation from a chemical safety perspective, more recent studies have shown that CAR influences a

diverse array of cellular processes from energy homeostasis to tumor progression (Gao and Xie, 2010; Hedrich et al., 2016; Yamamoto et al., 2004). These nontraditional roles of CAR combined with its importance in drug-drug interactions make it an important and unique nuclear receptor which requires complete analyses of its potential modulators.

Expressed predominantly in the liver, CAR is localized in the cytoplasm of hepatocytes until exposure to a ligand causes its translocation into the nucleus. This translocation can be caused by either a directly bound ligand or an indirect ligand independent pathway (Kawamoto et al., 1999; Yang and Wang, 2014). However, unlike *in vivo* conditions and human primary

hepatocytes (HPH), CAR spontaneously accumulates inside the nucleus and exhibits constitutive activity in immortalized cell lines (Li et al., 2009). Due to this unique aspect of CAR, it is prudent to decrease this high basal activity as an initial step to any assay involving immortalized cells. Our previous study found that a low concentration of the known human CAR (hCAR) antagonist, 1-(2-chlorophenylmethylpropyl)-3-isoquinoline-carboxamide (PK11195), could be co-treated with the potential CAR agonists so as to alleviate the inherent constitutive activity and identify potential CAR activators (Lynch et al., 2015).

Another distinctive characteristic of CAR is cross-talk with its sister nuclear receptor, pregnane X receptor (PXR), resulting in co-regulation of shared target genes (Wang and Negishi, 2003). Therefore, to gain a better understanding of how a drug will act *in vivo*, it is critical to have a comprehensive knowledge of how xenobiotics interacts with both of these nuclear receptors. For instance, clotrimazole is an activator of PXR, but an inverse agonist of CAR, which suggests a complicated physiological impact (Pascussi et al., 2008). The closest physiologically relevant way to fully understand how a compound would react in the liver, aside from an *in vivo* assay, is to treat HPH and determine how a particular compound regulates a specific gene. Through this method, the compound's pathway (CAR vs PXR) can be inferred for use in further studies.

The expense and availability of HPH make it unreasonable to perform a high-throughput assay in this system. To overcome these obstacles, it has become more commonplace to use immortalized cell lines to perform an initial screen of a large group of compounds to determine nuclear receptor modulation (Hsu et al., 2014; Huang et al., 2014; Lynch et al., 2017, 2018). In this study, we screened the Tox21 10,000 (7,872 unique) compound collection to identify hCAR activators. The Tox21 program is a federal collaboration among the National Institutes of Health (National Center for Advancing Translational Sciences [NCATS] and the National Toxicology Program [NTP] at the National Institute of Environmental Health Sciences), the Environmental Protection Agency (EPA), and the Food and Drug Administration. We then selected potential CAR activators to examine in follow-up studies including a powder confirmation assay, PXR luciferase activity assay, as well as HPH experiments (mRNA induction, protein expression, and nuclear translocation), which were used to further validate hCAR activation.

MATERIALS AND METHODS

Tox21 chemical library. The approximately 10,000 (7,872 unique) small molecules of the Tox21 chemical library (NCATS, 2016; PubChem, 2013) consists of pesticides, drugs, industrial chemicals, and food additives commercially sourced by NCATS (Huang et al., 2011), NTP, and the EPA. Multiple criteria were used to select each small molecule, including properties allowing for high-throughput screens (HTS) (molecular weight, volatility, solubility, log P), commercial availability, possible and definite environmental hazards or exposure concerns, and cost. A diverse group of 88 compounds was selected to be used as an internal control and plated in duplicate on each library plate to perform reproducibility analysis (Huang, 2016) as well as to determine positional plate effects.

Cell culture. A stably transfected cell line, derived from HepG2 cells, was utilized for the duration of the CAR quantitative (q) HTS. The human hepatoma cell line was stably transfected with the pEF6/V5-hCAR1 expression plasmid alongside the pGL4.17[luc2/Neo]-CYP2B6-2.2kb construct containing both the

PBREM and XREM (Lynch et al., 2015; Wang et al., 2003). HepG2-CYP2B6-hCAR cells were cultured in DMEM (Invitrogen) supplemented with 10% Hyclone™ FBS (GE Healthcare Life Sciences, Logan, Utah), 5 µg/ml blasticidin (Invitrogen, Carlsbad, California), 0.5 mg/ml geneticin (Invitrogen), and 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). For quantifying the human PXR (hPXR)-mediated CYP3A4 activators/inhibitors, hPXR-Luc HepG2 cells were used. These cells were provided by Dr Taosheng Chen (Department of Chemical Biology and Therapeutics, St. Jude Children's Research Hospital) (Lin et al., 2008). These are HepG2 cells stably transfected with human PXR and CYP3A4-luc, in which the expression of luciferase was controlled by hPXR-regulated CYP3A4 promoter. Cells were cultured in EMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, and 500 µg/ml of geneticin. Both cell lines were maintained at 37°C under a humidified atmosphere and 5% CO₂.

CAR luciferase reporter gene assay. The HepG2-CYP2B6-hCAR cells (Lynch et al., 2016) were dispensed at 2500 cells/4 µl/well in tissue culture-treated 1536-well white assay plates (Greiner Bio-One North America, Monroe, North Carolina) using a Thermo Scientific Multidrop Combi (Thermo Fisher Scientific Inc., Waltham, Massachusetts) in the same culture media, without geneticin and blasticidin included. After the assay plates were incubated at 37°C/5% CO₂ for 5 h, 23 nl of compounds dissolved in dimethyl sulfoxide (DMSO), positive control (6-(4-Chlorophenyl)imidazo[2, 1-b][1, 3]thiazole-5-carbaldehyde O-(3, 4-dichlorobenzyl)oxime [CITCO], Sigma-Aldrich Corp., St. Louis, Missouri), or vehicle control (DMSO) were transferred to the assay plates by a Wako Pintool station (Wako Automation, San Diego, California). One µl of the known hCAR antagonist, PK11195 (Sigma-Aldrich Corp., Li et al., 2008), was then added to generate a final concentration of 0.75 µM PK11195 in each well using a Flying Reagent Dispenser (Aurora Discovery, Carlsbad, California). The final test compound concentrations in the 5 µl assay volume ranged from 1.2 nM to 92 µM in 15 different concentrations at a 1:5 dilution, whereas the final concentration of DMSO (used for the negative control) was 0.46%. The plate format of the positive control is as follows: Column 1: concentration-response titration of CITCO from 1.2 nM to 92 µM at a 1:2 dilution with DMSO; Column 2 top half: 60 µM of CITCO (used as the positive control); Column 2 bottom half: 48 µM of CITCO; Column 3 top half and Column 4: DMSO only; and Column 3 bottom half: 92 µM of tetraoctyl ammonium bromide. The plates were incubated at 37°C/5% CO₂ for 23 h. One µl of CellTiter-Fluor™ (Promega, Madison, Wisconsin) was added, using the Flying Reagent Dispenser, after which, all plates were put back into the incubator at 37°C/5% CO₂ for another hour. Fluorescence intensity was measured at 540 nm following excitation at 405 nm using a ViewLux plate reader (Perkin Elmer, Shelton, Connecticut) to determine cell viability. Directly after this fluorescence reading, 4 µl of ONE-Glo™ Luciferase reagent (Promega) was added to each well using the Flying Reagent Dispenser. After a 30 min incubation at room temperature, luminescence intensity was measured using the ViewLux plate reader. Data was then expressed in relative luminescence units.

PXR luciferase reporter gene assay. hPXR-Luc HepG2 cells were plated at 2000 cells/5 µl/well in phenol red-free DMEM containing 5% charcoal stripped FBS into white/solid 1536-well plates (Greiner Bio-One North America) using the Flying Reagent Dispenser. After 4–5 h of incubation at 37°C for cell attachment,

the positive control (rifampicin [RIF]) and follow-up test compounds were transferred at 23 nl to columns 1–4 and 5–48, respectively using the Pintool station. The positive control plate format is as follows: Column 1: Dose titration of RIF from 92.0 μ M to 2.8 nM at 1:2 dilutions with 16 duplicate points; top half of columns 2 and 3: 46 (used as the positive control) and 23 μ M RIF, respectively; bottom half of column 2: 92 μ M tetraoctyl ammonium bromide; and the bottom half of column 3 as well as column 4: DMSO only. The assay plates were incubated for 24 h at 37°C, after which 1 μ l/well of CellTiter-Fluor reagent was added into the assay plates using a Flying Reagent Dispenser and after a 30 min incubation at 37°C, the fluorescence intensity was measured using the ViewLux plate reader. Data were expressed as relative fluorescence units. Directly after this fluorescence reading, 4 μ l/well of ONE-Glo reagent was added into the assay plates using a Flying Reagent Dispenser. After a 30 min incubation at room temperature, the luminescence intensity was measured using the ViewLux plate reader. Data were expressed as relative luminescence units.

Data analysis for CAR and PXR luciferase reporter gene assays. Data analysis was performed according to a previous protocol (Huang, 2016). Briefly, raw plate reads for each titration point were first normalized relative to the positive control compound (agonist mode: CITCO = 100%) and DMSO-only wells [0%] as follows: % Activity = $[(V_{\text{compound}} - V_{\text{DMSO}})/(V_{\text{pos}} - V_{\text{DMSO}})] \times 100$, where V_{compound} denotes the compound well values, V_{pos} denotes the median value of the positive control wells, and V_{DMSO} denotes the median values of the DMSO-only wells. The dataset was then corrected using the DMSO-only compound plates at the beginning and end of the compound plate stack by applying an in-house pattern correction algorithm (Wang and Huang, 2016). The half maximum effective values (EC_{50}) and maximum response (efficacy) values were obtained by fitting the concentration-response curves of each compound to a four-parameter Hill equation. Compounds designated as Class 1–4 according to the type of concentration-response curve observed (1.1, 1.2, 1.3, 1.4, 2.1, 2.2, 2.3, 2.4, and 3 for activators; 4 for inactive) were converted to curve ranks (1 to 9 integers for increasing curve quality, potency and efficacy; 0 for inactive) according to the criteria previously described (Supplementary Table 1) (Huang et al., 2014; Huang, 2016). For each reading, the activity outcome of a test compound was categorized based on the average curve rank from the triplicate runs and the reproducibility calls. Data reproducibility was categorized as active match, inactive match, inconclusive, and mismatch according to the previously described criteria (Huang et al., 2014; Huang, 2016). The 10K library was clustered based on structural similarity (Leadscope® fingerprints; Leadscope, Inc., Columbus, Ohio) using the self-organizing map (SOM) algorithm (Kohonen, 2006). Each cluster was evaluated for its enrichment of active agonists and significance of enrichment as determined by *p*-values from the Fisher's exact test.

Human primary hepatocytes (HPH). HPH were obtained from BioIVT (Baltimore, Maryland) and seeded at 7.5×10^5 cells/well in 12-well collagen-coated plates using seeding medium (BioIVT) for 4–6 h at 37°C and 5% CO₂. Media was changed to serum-free William's E Medium supplemented with ITS⁺ (6.25 μ g/ml insulin, 6.25 μ g/ml transferrin, and 6.25 ng/ml selenium), 0.1 μ M dexamethasone, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and overlaid with Matrigel (0.25 mg/ml) for 24 h. HPH were then treated with the vehicle control (0.1% DMSO), CITCO (1 μ M), or test compounds for

another 24 or 72 h for detection of mRNA or protein, respectively, before harvesting.

Real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from treated hepatocytes using the TRIzol® reagent (Thermo Fisher Scientific Inc.) and reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California) following the manufacturers' instructions. CYP2B6 and CYP3A4 mRNA expression were normalized against the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Real-time PCR assays were performed on an ABI StepOnePlus real-time PCR system (Applied Biosystems) with SYBR Green PCR Master Mix. Primer sequences are as follows: CYP2B6, 5'-AGACGCCTTCAATCCTGACC-3' and 5'-CCTTCACCAAGACA AATCCGC-3'; CYP3A4, 5'-GTGGGGCTTTTATGATGGTCA-3' and 5'-GCCTCAGATTCTCACCAACACA-3'; and GAPDH, 5'-CCCATC ACCATCTTCCAGGAG-3' and 5'-GTTGTCATGGATGACCTTGGC-3'. Induction values were calculated using the equation: Fold = $2^{\Delta\Delta Ct}$, where ΔCt represents the differences in cycle threshold numbers between CYP2B6 or CYP3A4 and GAPDH, and $\Delta\Delta Ct$ represents the relative change in these differences between control and treatment groups.

Western blot analysis. Homogenate proteins (15 μ g) from treated HPH were resolved on SDS-PAGE (4–12%) gels and transferred to polyvinylidene difluoride membranes. The membrane was then blocked using 5% blotting-grade blocker at room temperature for 1 h. Membranes were then incubated with primary antibodies against CYP2B6 (1:1000; Abcam Inc., Cambridge, Massachusetts), CYP3A4 (1:5000; MilliporeSigma, Burlington, Massachusetts), or β -actin (1:30 000; Sigma-Aldrich Corp.) diluted in 1% blotting-grade blocker at 4°C overnight for CYP2B6, whereas CYP3A4 and β -actin were incubated at room temperature for 1 h. Subsequently, membranes were incubated with horseradish peroxidase secondary antibodies for 1 h at room temperature and developed using ECL Western blotting detection reagent (Thermo Fisher Scientific Inc.).

Nuclear hCAR translocation. HPH were plated in collagen-coated 96-well plates (6×10^6 cells/plate) and infected with adenovirus-expressing enhanced yellow fluorescent protein-tagged hCAR (Ad/EYFP-hCAR) as described previously (Li et al., 2009). Twenty-four hours after infection, HPH were treated with vehicle control (0.1% DMSO), CITCO (1 μ M), or the test compounds at their EC_{80} concentrations for another 8 h. After treatment, cells were fixed with 4% paraformaldehyde, stained with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 30 min, and EYFP-hCAR localization in hepatocytes was visualized on a Nikon Eclipse TI fluorescent microscope. Quantitative distribution of EYFP-hCAR was analyzed using General Analysis in the Nikon Elements AR High Content Analysis software package (Version 4.50.00). Nuclear localization was defined and quantified as the number of nuclei (denoted by DAPI) that exhibited more than 50% overlap with EYFP, normalized to the number of hepatocytes expressing EYFP. Data from a representative liver donor is shown and represent the mean $\pm$ SD of 5 individual images for each treatment.

Statistical analysis for RT-PCR, Western blot, and nuclear hCAR translocation. All experimental data are expressed as a mean of triplicate values $\pm$ standard deviation unless otherwise noted. Statistical comparisons were made by one-way analysis of

variance with post-hoc Dunnett's analysis. The statistical significance was set at p-values of <.05 (*), <.01 (**), or <.001 (***).

RESULTS

qHTS Performance and Reproducibility

The HepG2-CYP2B6-hCAR stable cell line (Lynch et al., 2015), was used to perform a primary screen of 10,000 chemical compounds from the Tox21 chemical library to identify hCAR activators. Due to the constitutive activation of hCAR in immortalized cell lines, 0.75 μ M of PK11195 was co-treated in each well to lower the basal activity of hCAR. CITCO, the positive control, displayed consistent activity throughout the primary screen, containing about 400 assay plates, with an EC₅₀ of 675 $\pm$ 143 nM. The screening performance statistics indicate a good quality of the primary screen with the following values: signal-to-background ratio (S/B), 4.92 $\pm$ 0.347; coefficient of variance, 6.04% $\pm$ 1.56; and Z' factor, 0.687. Generally, assay plates with S/B > 2, coefficient of variance <10%, and Z' factor >0.5 are considered good quality (Zhang et al., 1999).

We further evaluated the reproducibility of the primary screen by assessing the three separate runs of said screen in addition to the Tox21-88 array data, which includes each selected compound plated in duplicate on every compound plate. After the primary 10K compound screening, each compound was sorted into 1 of 4 categories based on reproducibility of activity: active match, inactive match, mismatch, or inconclusive (Huang, 2016). The mismatch rates were low, with values of 0.10% for the 10K screen and 0.57% for the Tox21-88 screen, indicating a robust assay performance (Table 1).

Identification of hCAR Activators

Following the primary screen, an SOM algorithm was used to group the entire Tox21 10K compound collection into 1014 clusters based on their structural similarities (Kohonen, 2006). A heatmap was generated to illustrate the amount of hCAR activators in each structural class (Figure 1). Sixty-two structural clusters, encompassing 1,831 samples, were significantly ($p < .01$) enriched with hCAR activators, including flavones which are a known CAR active group (Yao et al., 2010). A group of prazoles, including lansoprazole, omeprazole, and rabeprazole, were shown to have a significantly active cluster as well (Figure 1). These data also correlate with recent literature showing connections between prazoles and hCAR (Zhou et al., 2009). Additionally, a potential novel cluster of compounds, containing nitazoxanide and tenonitroazole, was identified as having a significant amount of hCAR activators (Figure 1). Both of these compounds had efficacies above 40% of CITCO's value as well as potencies of less than 1 μ M.

Out of the 7,872 unique compounds examined in the primary screen, 83 compounds were selected for further evaluation based on efficacy ($E > 30\%$). All 83 compounds were re-tested in the same hCAR cell line used in the primary screen and confirmed as hCAR activators, thereby giving a 100% confirmation

rate (data not shown). Based on potency (EC₅₀ < 15 μ M), efficacy ($E > 40\%$), commercial availability, QC analysis, cytotoxicity, and cost, 24 compounds were obtained from various vendors and another confirmation assay was performed on those powders (Supplementary Table 2). Five compounds (5, 6-benzoflavone, drometrizole, fanetizole, frentizole, and phenazopyridine hydrochloride) had efficacies higher than 100% CITCO's value when the powder confirmation was performed (Supplementary Table 2), with a full list of the potentially novel compounds in (Table 2). However, only 5, 6-benzoflavone exhibited a potency of less than 5 μ M, identifying it as the most efficacious and potent compound from the powder confirmation (Supplementary Table 2).

PXR Activity of Selected Compounds

A PXR luciferase reporter assay was performed on the 24 compounds identified above to determine selectivity of each compound. Three of these compounds (diphenamid, ethoxzolamide, and rimcazone dihydrochloride), generated an efficacy of less than 50% in PXR luciferase as opposed to the hCAR assay. However, there were six compounds (alpha-terthiophene, diphenamid, drometrizole, ethoxzolamide, genistein, and phenothrin) which had a lower potency (EC₅₀ > 15 μ M) compared with that in the hCAR assay (Supplementary Table 2). Based on these two factors, potency and efficacy in the CAR assay, as well as novelty of compound correlation with hCAR, eight compounds (nitazoxanide, axitinib, 2-aminoanthraquinone, neticonazole, frentizole, diphenamid, phenothrin, and rimcazone) were chosen for further examination in HPH.

mRNA Induction of CYP2B6 and CYP3A4

Although many hepatic genes are regulated by hCAR, CYP2B6 is its primary target gene (Wang and Negishi, 2003). CYP3A4 is also moderately induced by hCAR and therefore, the expression of these important drug metabolizing enzymes were investigated in HPH treated with the compounds of interest (Faucette et al., 2006). The eight compounds previously chosen for further study were treated in HPH from two different donors at their own EC₅₀ or EC₈₀ identified in the hCAR luciferase assay. RT-PCR analysis identified 4 out of the 8 compounds (neticonazole, diphenamid, phenothrin, and rimcazone) as demonstrating significant mRNA induction of CYP2B6 in at least one liver donor (Figure 2). Diphenamid and rimcazone exhibited a strong CYP2B6 induction ($p < .001$) in both livers implying they are strong agonists of hCAR. Diphenamid also significantly induced CYP3A4, in both livers, suggesting its promiscuity toward both CAR and PXR (Figure 2), whereas rimcazone significantly induced CYP3A4 in only one liver (Figure 2B). Although neticonazole and phenothrin had an increase in CYP2B6 induction in the first liver donor (Figure 2A), there was only a significant induction of CYP2B6 in the second liver donor (Figure 2B). In Figure 2B, neticonazole also significantly induced CYP3A4, whereas phenothrin does not.

Table 1. Assay Performance of the CAR Agonist Screen Using Tox21-88 Duplicates and 10K Triplicate Runs

	Active Match (%)	Inactive Match (%)	Inconclusive (%)	Mismatch (%)	EC ₅₀ Fold ^a Change
10K screen	14.78	77.84	7.27	0.10	1.33
Tox21-88 screen	38.12	49.48	11.84	0.57	1.24

^aFor each compound with at least one replicate that is not class 4, the standard deviation (SD) of the EC₅₀ values from all replicates was calculated. The SDs were then averaged across these compounds. The EC₅₀ fold was calculated as the inverse log of the average SD.

Figure 1. Structural class heatmap of hCAR agonist activity. The HepG2-CYP2B6-hCAR stable cell line was screened against the Tox21 10,000 compound collection and compounds were divided into structural clusters. Each hexagon represents a class of structurally similar compounds. The color gradient is indicative of the enrichment of CAR actives in that specific cluster (negative logarithmic scale of the p -value, $-\log[p\text{-value}]$). Each color represents a group of chemicals with similar scaffolds to activate CAR. Clusters with multiple actives in their class are closer to a maroon color, whereas clusters with no activity are a gray color. Empty clusters with no available (N/A) compounds in them are light gray in color. The structural backbone is shown next to the class name, as well as representative dose-response curves for the compounds within that structural cluster. Data are expressed as mean $\pm$ SD from three experiments.

Table 2. EC₅₀ and Efficacy of Potential Novel CAR Agonists Identified from qHTS Primary and Follow-Up Studies

Chemical Name (CAS #, Supplier)	Structure	CAR-luc		PXR-luc	
		Primary EC ₅₀ (μM) (Efficacy [%])	Powder confirmation EC ₅₀ (μM) (Efficacy [%])	Primary EC ₅₀ (μM) (Efficacy [%])	Powder confirmation EC ₅₀ (μM) (Efficacy [%])
2-Aminoanthraquinone (117-79-3, Sigma-Aldrich Corp.)		0.986 $\pm$ 0.226 [45.7 $\pm$ 5.72]	1.20 $\pm$ 0.0810 [51.6 $\pm$ 1.11]	0.500 $\pm$ 0.263 [51.7 $\pm$ 10.5]	1.29 $\pm$ 0.460 [85.1 $\pm$ 10.9]
Axitinib (319460-85-0, Sigma-Aldrich Corp.)		0.774 $\pm$ 0.249 [67.6 $\pm$ 33.7]	0.814 $\pm$ 0.00 [52.7 $\pm$ 3.20]	0.874 $\pm$ 0.437 [130 $\pm$ 24.0]	2.05 $\pm$ 0.236 [123 $\pm$ 26.8]

(continued)

Table 2. (continued)

Chemical Name (CAS #, Supplier)	Structure	CAR-luc		PXR-luc	
		Primary EC ₅₀ (μM) (Efficacy [%])	Powder confirmation EC ₅₀ (μM) (Efficacy [%])	Primary EC ₅₀ (μM) (Efficacy [%])	Powder confirmation EC ₅₀ (μM) (Efficacy [%])
Diphenamid (957-51-7, Santa Cruz Biotechnology [Dallas, Texas])		11.8 ± 2.02 [66.9 ± 4.85]	9.95 ± 3.94 [66.9 ± 16.0]	27.0 ± 7.52 [39.6 ± 15.1]	19.5 ± 14.7 [23.6 ± 4.58]
Frentizole (26130-02-9, AK Scientific, Inc. [Union City, California])		8.01 ± 2.36 [106 ± 29.2]	15.1 ± 1.02 [118 ± 13.3]	1.77 ± 0.663 [87.0 ± 49.7]	14.5 ± 0.00 [74.7 ± 6.00]
Neticonazole (130726-68-0, Alpha Chemistry [Ronkonkoma, New York])		6.71 ± 0.771 [75.9 ± 9.02]	19.6 ± 5.79 [61.1 ± 21.9]	5.48 ± 2.29 [91.4 ± 17.3]	7.98 ± 4.36 [27.8 ± 2.33]
Nitazoxanide (55981-09-4, Sigma-Aldrich Corp.)		0.630 ± 0.142 [47.8 ± 2.75]	0.516 ± 0.0593 [46.1 ± 1.88]	8.84 ± 6.44 [227 ± 16.7]	0.357 ± 0.134 [98.6 ± 21.9]
Phenothrin (26002-80-2, Chem Service, Inc. [West Chester, Pennsylvania])		8.99 ± 2.65 [64.5 ± 3.81]	9.43 ± 3.04 [47.1 ± 6.77]	15.4 ± 4.97 [93.8 ± 29.7]	23.9 ± 1.62 [54.4 ± 6.81]

(continued)

Table 2. (continued)

Chemical Name (CAS #, Supplier)	Structure	CAR-luc		PXR-luc	
		Primary EC ₅₀ (μM) (Efficacy [%])	Powder confirmation EC ₅₀ (μM) (Efficacy [%])	Primary EC ₅₀ (μM) (Efficacy [%])	Powder confirmation EC ₅₀ (μM) (Efficacy [%])
Rimcazole dihydrochloride (75859-03-9, Tocris [Minneapolis, Minnesota])		3.07 ± 0.856 [48.5 ± 4.84]	3.56 ± 1.45 [56.0 ± 13.1]	Inactive ^a	Inactive ^a

^aInactive = compounds with an efficacy less than 20% of the positive control's activity.

Figure 2. mRNA induction in HPH of CYP2B6 and CYP3A4. HPH from donor #1 (HPH133—Caucasian male, 41 years old, BMI = 24.7; A) and donor #2 (HPH139—Caucasian male, 46 years old, BMI = 31.9; B) were treated with potential CAR agonists selected from qHTS, CITCO (1 μM), RIF (10 μM), or the vehicle control (0.1% DMSO) as described in Materials and Methods. Real-time PCR was used to analyze the mRNA expression of CYP2B6 and CYP3A4. Each bar represents the mean ± SD in three experiments. *, $p < .05$; **, $p < .01$; ***, $p < .001$. NIT, nitazoxanide; AXI, axitinib; 2-AM, 2-aminoanthraquinone; NET, neticonazole; FRE, frentizole; DIP, diphenamid; PHE, phenothrin; RIM, rimcazole.

Protein Induction of CYP2B6 and CYP3A4

The mRNA data correlates well with the Western blot protein induction analysis, which shows neticonazole, diphenamid, and rimcazole having strong CYP2B6 protein induction in both liver donors (Figure 3). Phenothrin exhibited a very minor

increase in CYP2B6 protein in both livers identifying another clear correlated comparison between mRNA and protein (Figure 3). The CYP3A4 protein induction was consistent as well, displaying a strong increase from neticonazole and diphenamid, whereas generating a weaker increase when treated with phenothrin and rimcazole (Figure 3).

Nuclear Translocation of hCAR

Although CAR is localized to the nucleus and constitutively active in immortalized cell lines, CAR is sequestered in the cytoplasm and translocates to the nucleus upon activation in physiologically relevant systems such as HPH and *in vivo*. As CAR nuclear translocation is the first essential step in its activation, observing CAR translocation from the cytoplasm into the nucleus of HPH, with chemical treatments, is a translational assay for hCAR activation. Although hCAR is the most potent inducer of CYP2B6, other nuclear receptors and transcription factors can also play a role in CYP2B6 induction (Moscovitz *et al.*, 2018). Therefore, we validated the ability of our compounds to activate hCAR by infecting HPH with adenovirus expressing enhanced yellow fluorescent protein tagged hCAR (Ad/EYFP-hCAR) and examining the effects each of the 8 compounds had on its translocation. The positive control, CITCO, along with 4 of the test compounds, neticonazole, diphenamid, phenothrin, and rimcazole, clearly translocate hCAR into the nucleus, corroborating the previous induction studies (Figure 4A). Quantitation of the data was also performed, which showed that these four compounds significantly translocate hCAR (Figure 4B).

DISCUSSION

The classic role of CAR is that of a transcription factor which regulates drug metabolizing enzymes in all phases of metabolism (Qatanani and Moore, 2005). However, hCAR has many nontraditional roles besides modulation of drug metabolism, including the regulation of multiple physiological homeostatic pathways (di Masi *et al.*, 2009). Furthermore, a recent study in mice demonstrated an alleviation of renal ischemia-reperfusion-induced fatty liver and kidney injury when CAR

Figure 3. Protein induction in HPH of CYP2B6 and CYP3A4. In separate experiments, induction of CYP2B6 and CYP3A4 proteins were detected in an immunoblotting assay for HPH133 (A) and HPH139 (B). Membranes were soaked in primary antibodies at the following concentrations: 1:1000, 1:5000, and 1:30 000 for CYP2B6, CYP3A4, and β -actin, respectively. The secondary antibody was used at a concentration of 1:4000. NIT, nitazoxanide; AXI, axitinib; 2-AM, 2-aminoanthraquinone; NET, neticonazole; FRE, frentizole; DIP, diphenamid; PHE, phenothrin; RIM, rimcazole.

Figure 4. Translocation of hCAR. Cultured HPH were infected with Ad/EYFP-hCAR and treated with vehicle (0.1% DMSO), CITCO (1 μ M) or each of the 8 selected agonists from qHTS assays at the indicated concentrations. After 8 h of treatment, hepatocytes were fixed and subjected to confocal microscopy for visualization of EYFP-hCAR localization (A). Quantitative distribution of EYFP-hCAR was then analyzed (B) using General Analysis in the Nikon Elements AR High Content Analysis software package. Each bar represents the mean $\pm$ SD of five individual images for each treatment. *, $p < .05$; **, $p < .01$; ***, $p < .001$. NIT, nitazoxanide; AXI, axitinib; 2-AM, 2-aminoanthraquinone; NET, neticonazole; FRE, frentizole; DIP, diphenamid; PHE, phenothrin; RIM, rimcazole.

was activated pharmacologically (Choi et al., 2018). Combining all of these important implications illustrates that identifying drugs and other environmental chemicals which affect CAR should be a necessary practice.

HPH are an excellent physiologically relevant model with which to study nuclear receptors and drug metabolizing enzymes, due to their expression of a majority of liver transcription factors (LeCluyse, 2001). As previously discussed, low availability and high cost of HPH make this unique system undesirable for HTS. Nevertheless, the constitutive activity in

immortalized cell lines, generates another complex issue when seeking modulators of hCAR. To overcome this problem, this study used a previously generated method (Lynch et al., 2015) and screened the Tox21 10,000 compound collection to identify novel and known hCAR activators. Known structural clusters, such as flavones and prazosins, were identified through this screen, alongside a previously uncharacterized cluster, which includes certain antiprotozoals (nitazoxanide and tenonitrazole). Nitazoxanide is a broad spectrum anti-infective FDA-approved drug which has been used to treat infectious, rotavirus,

and cryptosporidium diarrhea among many other bacterial and viral diseases (Rossignol et al. 2006a,b, 2012). Once ingested, nitazoxanide, a prodrug, rapidly hydrolyzes into tizoxanide, its active metabolite which could accommodate for the lack of CAR activation in HPH, in this study (Korba et al., 2008). Potentially, nitazoxanide is a CAR activator, whereas its metabolites could have no effect on this nuclear receptor. Tenonitroazole is an anti-protozoal drug, approved in Europe, used to treat urogenital trichomoniasis (Komorowska, 1969). Due to tenonitroazole's structural similarity to nitazoxanide, it is reasonable to theorize its metabolic pathway is comparable to the description above. However, in this study, neticonazole did not induce CYP2B6 in HPH, implying the requirement of further testing and investigation on how this compound interacts in the body to identify the complete consequence of this drug.

In this study, through the use of HPH, neticonazole, diphenamid, phenothrin, and rimcazone were identified as novel activators of hCAR. Neticonazole, a topical antifungal drug, is currently used only in Japan (Tatsumi et al., 2002; Tsuboi et al., 1996). Diphenamid has long since been used as an herbicide on crop fields to control unwanted vegetation (Heidelberger and Iype, 1967). Phenothrin, a synthetic pyrethroid, is used as an insecticide against many public health-related insects, such as head lice and ticks (Yang et al., 2018). Although phenothrin has not previously been reported as modulating CAR, as per our knowledge, certain pyrethroids have been shown to activate human and mouse CAR (Abass et al., 2012). With the current usage of all three of these chemicals, it is necessary to perform further testing on these compounds to determine their full effect on the human system and potential toxic side effects caused by any drug-drug interactions.

As sister receptors, the ligand specificities of CAR and PXR exhibit significant overlap, with almost all CAR ligands interacting with PXR in some way. Although CAR activation seems to show promise for the treatment of metabolic disorders by inhibiting gluconeogenesis, PXR has recently been shown to repress the expression of glucose transporter 2 leading to glucose intolerance in mice (Gao et al., 2015; Hassani-Nezhad-Gashti et al., 2018; Lahtela et al., 1984; Yarushkin et al., 2013). Therefore, the cross-talk of CAR and PXR make dual activators difficult to study as potential therapeutics and identification of a selective hCAR activator would be important to validate as a therapeutic target. Therefore, we screened 24 confirmed CAR activators for PXR activation. Although almost all of the compounds potently activated PXR in luciferase assays, rimcazone did not activate PXR in this assay and may be a novel CAR-selective activator.

Previous studies have demonstrated that CAR preferentially regulates CYP2B6 over CYP3A4 in HPH, indicating that a more selective CAR activator would likely show similar preferential induction (Faucette et al., 2006). In HPH, CITCO displayed preferential induction of CYP2B6 expression over CYP3A4 at the mRNA and protein levels, demonstrating highly selective hCAR activation (Maglich et al., 2003). In the current study, rimcazone exhibited strong induction of CYP2B6 whereas only generating a weak induction of CYP3A4 in HPH, similar to CITCO, which support our luciferase results indicating rimcazone's selectivity toward hCAR over hPXR. It is important to note that rimcazone also translocated hCAR from the cytoplasm into the nucleus using HPH, validating its activation of CAR.

Rimcazone was originally discovered as a moderately potent σ -receptor (described as a nonopioid receptor [Mei and Pasternak, 2002]) antagonist used as an atypical antipsychotic (Ferris et al., 1982, 1986). However, recently this compound has also been implicated in cocaine addiction treatment (Hiranita

et al., 2011; Menkel et al., 1991). Identifying this compound as a novel hCAR activator brings up many interesting queries which will need to be addressed. Given the potent activation of hCAR and induction of drug metabolizing enzymes, rimcazone will need to be thoroughly studied *in vitro* and/or *in vivo* for drug-drug interactions. In a study by Schmidt et al., rimcazone had significant synergistic interactions between different compounds, where rimcazone plus sertraline was described as the most robust combination when studying co-treatments for therapeutic use against glioblastoma multiforme, the most aggressive tumor in an adult brain (Schmidt et al., 2013). Further follow-up studies should, therefore, also be performed in the future to determine rimcazone's therapeutic effect, if any, involving the hCAR pathway.

In summary, our screen of the Tox21 compound collection was able to identify novel and known hCAR activators. The reproducibility of the primary 10K screen was very good with a mismatch rate of only 0.10% due to, in part, full concentration response curves, from 15 concentrations in triplicate (Table 1). A potential novel group of compounds, antiprotozoals, was identified as hCAR activators implicating their potential role in drug-drug interactions. Likewise, rimcazone was classified as a potentially selective hCAR activator. Future studies will need to be done to truly categorize this compound which will potentially generate drug-drug interactions and/or be therapeutically active.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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