Identification of chemical modulators of the constitutive activated receptor (CAR) in a gene expression compendium

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Abbreviations: AhR, aryl hydrocarbon receptor; AOP, adverse outcome pathway; B[a]P, benzo[a]pyrene; CAR, constitutive activated receptor; Cypro, cyproconazole; DEHP, di-(2-ethylhexyl)phthalate; LPS, lipopolysaccharide; MIE, molecular initiating event; PCN, pregnenolone-16α-carbonitrile; PCR, polymerase chain reaction; PFHxS, perfluorohexanesulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCPOBOP, 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene; TNFα, tumor necrosis factor α; WY, WY-14,643.

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The nuclear receptor family member constitutive activated receptor (CAR) is activated by structurally diverse drugs and environmentally-relevant chemicals leading to transcriptional regulation of genes involved in xenobiotic metabolism and transport. Chronic activation of CAR increases liver cancer incidence in rodents, whereas suppression of CAR can lead to steatosis and insulin insensitivity. Here, analytical methods were developed to screen for chemical treatments in a gene expression compendium that lead to alteration of CAR activity. A gene expression biomarker signature of 83 CAR-dependent genes was identified using microarray profiles from the livers of wild-type and CAR-null mice after exposure to three structurally-diverse CAR activators (CITCO, phenobarbital, TCPOBOP). A rank-based algorithm (Running Fisher’s algorithm (p-value < 10-4)) was used to evaluate the similarity between the CAR biomarker signature and a test set of 28 and 32 comparisons positive or negative, respectively, for CAR activation; the test resulted in a balanced accuracy of 97%. The biomarker signature was used to identify chemicals that activate or suppress CAR in an annotated mouse liver/primary hepatocyte gene expression database of ~1850 comparisons. CAR was activated by 1) activators of the aryl hydrocarbon receptor (AhR) in wild-type but not AhR-null mice, 2) progesterone X receptor (PXR) activators in wild-type and to lesser extents in PXR-null mice, and 3) activators of PPARα in wild-type and PPARα-null mice. CAR was consistently activated by five conazole fungicides and four perfluorinated compounds. Comparison of effects in wild-type and CAR-null mice showed that the fungicide propiconazole increased liver weight and hepatocyte proliferation in a CAR-dependent manner, whereas the perfluorinated compound perfluorooctanoic acid (PFOA) increased these endpoints in a CAR-independent manner. A number of compounds suppressed CAR coincident with increases in markers of inflammation including acetaminophen, concanavalin A, lipopolysaccharide, and 300 nm silica particles. In conclusion, we have shown that a
CAR biomarker signature coupled with a rank-based similarity method accurately predicts CAR activation. This analytical approach, when applied to a gene expression compendium, increased the universe of known chemicals that directly or indirectly activate CAR, highlighting the promiscuous nature of CAR activation and signaling through activation of other xenobiotic-activated receptors.

**Introduction**

An adverse outcome pathway (AOP) is the process by which a chemical causes an adverse outcome in a tissue starting with an interaction with a molecular target (termed the molecular initiating event (MIE)), through a number of key molecular and cellular events (Ankley et al., 2010; Pery et al., 2013; Vinken, 2013). A subset of AOPs that lead to liver cancer involve the chronic activation of xenobiotic-activated receptors that regulate growth of the liver. The MIE of one of these AOPs is the activation of the nuclear receptor constitutive activated receptor (CAR, NR1I3) (Elcombe et al., 2014). CAR plays critical roles in regulating enzymes involved in xenobiotic metabolism, including members of the cytochrome P450 (CYP) family (Ueda et al. 2002), sulfotransferases, uridine diphospho-glucuronosyltransferases (Chen et al. 2007; Sugatani et al. 2001), as well as various transporters (Assem et al. 2004). Heterodimers of CAR and the retinoid X receptor (RXR, NR2B1) bind to phenobarbital-responsive elements in chromatin, resulting in gene activation. CAR can be activated through two distinct mechanisms. A number of compounds (e.g., 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), and 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO)) bind directly to CAR, leading to nuclear localization and transcriptional activation. In contrast, phenobarbital activates CAR by binding to and inactivating the epidermal growth factor receptor (EGFR) and an associated protein kinase cascade that in the absence of exposure, suppresses CAR nuclear translocation (Mutoh et al., 2013; Molnár et al., 2013). The ability of CAR to respond to environmentally-relevant chemicals allows CAR, in concert with other transcription factors (e.g., pregnane X receptor (PXR, NR1I2) and aryl hydrocarbon receptor (AhR)), to induce gene expression of enzymes and transporters that metabolize and remove potentially toxic xenobiotics from the liver.

Many activators of rodent CAR, including phenobarbital, are well known inducers of liver cancer in mice and rats. The CAR-dependent liver cancer AOP has been recently reevaluated (Elcombe et al., 2014), building on previous efforts (e.g., Holsapple et al., 2006). Sustained CAR activation (the MIE) is followed by a number of key events including alteration of the expression of genes involved in hepatocyte fate, increased hepatocyte proliferation, formation of altered hepatic foci and ultimately, the development of hepatocellular adenomas and carcinomas. Induction of hepatic CYP2B gene expression and enzymes has been used as a surrogate indicator of CAR activation (Elcombe et al., 2014). Many of the most important studies used to support the AOP stem from work comparing effects in wild-type and CAR-null mice in which both short- and long-term exposures to CAR activators were shown to be CAR-dependent, including phenobarbital- or TCPOBOP-induced liver cancer (Huang et al., 2005; Yamamoto et al., 2004). Although the CAR AOP is well established for phenobarbital and TCPOBOP, other CAR-activating chemicals that induce liver cancer have not been systematically evaluated by a weight of evidence approach for causing liver cancer through the CAR AOP (Elcombe et al., 2014).

Recent studies have expanded the biological and pathophysiological functions of CAR to include cross-talk with regulators of liver energy homeostasis that impact metabolic diseases (Konno et al., 2008; Gao and Xie, 2010). In models of diabetes (high fat diet or leptin-deficient mice (ob/ob)), activation of CAR significantly reduces serum glucose levels and improves glucose tolerance and insulin sensitivity through suppression of glucose production and stimulation of glucose uptake and metabolism in the liver (Dong et al., 2009; Gao et al., 2009). While activation of CAR results in a more favorable metabolic profile, suppression of CAR (i.e., in CAR-null mice) led to spontaneously-impaired insulin sensitivity that was not responsive to TCPOBOP (Gao et al., 2009). Loss of CAR resulted in increased hepatic triglyceride accumulation that was associated with increased expression of the lipogenic nuclear receptor liver X receptor (LXR) and target genes including the sterol regulatory element binding protein 1 (SREBP-1). Activation of CAR inhibited the expression of LXR target genes and LXR ligand-induced lipogenesis (Zhai et al., 2010). Therefore, a hypothesized AOP that leads to liver triglyceride accumulation associated with insulin insensitivity involves suppression of CAR as the MIE, loss of negative regulation of LXR, and indirect activation of LXR- and SREBP-1-dependent lipogenic genes (Jiang and Xie, 2013).

The ability to accurately predict CAR activation or suppression would help in evaluating the potential for chemicals and other factors to contribute to liver...
Figure 1. CAR biomarker signature development/characterization and screening of a mouse liver gene expression compendium. Left, biomarker signature development and characterization. Wild-type and CAR-null mice were treated with CITCO, phenobarbital (PB) or TCPOBOP (Chua and Moore, 2005) and microarray analysis was carried out on the livers. Rosetta Resolver was used to identify differentially expressed genes (DEGs), as indicated. Biomarker signature genes were identified from the DEGs after applying a number of filtering steps described in the Methods. Genes in the biomarker signature were evaluated by the Comparative Toxicogenomics Database (CTD) to evaluate literature evidence for consistent regulation of biomarker signature genes by CAR activators and by Ingenuity Pathway Analysis (IPA) for canonical pathway enrichment and potential transcription factor (TF) regulators. Right, biomarker signature testing and screening. The CAR biomarker signature was imported into the NextBio environment. Internal protocols rank ordered the genes based on their fold-change. A pair-wise rank-based enrichment analysis (the Running Fisher’s algorithm) was used to compare the CAR biomarker signature to each bioset in the NextBio database, resulting in the direction of correlation and p-value of the comparison for each bioset in the compendium. All comparison information was exported and used to populate a master table containing bioset experimental details. An accuracy test of the biomarker signature predictions was carried out with treatments that are known positives and negatives for CAR activation. A number of predictions were tested in independent studies based on screening “hits”. An external gene expression database of experiments using Affymetrix gene chips was used for the machine learning classification analysis by BRB Array Tools. The database was also used to assess the relationship between the Running Fisher’s algorithm p-value and behavior of the CAR biomarker signature genes. Parts of the figure were adapted from a figure in Kupershmidt et al. (2010) and Oshida et al. (2015).

cancer or metabolic derangements through this nuclear receptor. In the present study, a gene expression biomarker signature coupled with a rank-based similarity test was used to predict CAR activation or suppression. The biomarker signature was found to be very accurate in predicting the activation of CAR and was used to screen a compendium of gene expression profiles to find chemicals that modulate CAR.

Materials and Methods

Strategy for identification of chemicals that affect CAR

The methods used in the present study are outlined in Figure 1. The methods used are similar to those described previously (Oshida et al., 2015).
Animal studies

There were a total of 4 animal studies carried out as part of this investigation. The studies of PFNA and PFHxS in wild-type and PPARα-null mice, pregnenolone-16β-carbonitrile (PCN) in wild-type and Pxr-null mice and the 12-treatment study in male and female mice have been described previously (Oshida et al., 2015). Perfluorooctanoic acid, perfluorooctane sulfonate, propiconazole, and triadimefon in wild-type and CAR-null mice: this study was carried out at the University of Kansas Medical Center (Kansas City, KS) in a fully-accredited American Association for Accreditation of Laboratory Animal Care facility. The animal study was conducted under federal guidelines for the use and care of laboratory animals and was approved by KUMC Institutional Animal Care and Use Committees. Breeder pairs from the CAR-null mouse line on the C57BL/6 background were obtained from Dr. Ivan Rusyn (University of North Carolina, Chapel Hill, NC) that were engineered by Tularik, Inc. (South San Francisco, CA), as described previously (Ueda et al., 2002). Randomized animals were allowed to acclimate for a period of one week prior to conducting the study. Food (Purina Rodent Chow (Harlan Teklad 8604) and filtered distilled water were provided ad libitum. Animal facilities were controlled for temperature (20-24°C), relative humidity (40-60%), and kept under a 12 hr light-dark cycle. Wild-type and CAR-null mice were given perfluorooctanoic acid (3 mg/kg), perfluorooctane sulfonate (3 mg/kg), propiconazole (210 mg/kg), or triadimefon (165 mg/kg) each day by gavage for 7 days. Control mice received 7.5% alkamuls by gavage. Livers were removed 24-hrs after the last dose. Portions of the livers were rapidly snap-frozen in liquid nitrogen and stored at -70°C until analysis. All animal studies were conducted under federal guidelines for the use and care of laboratory animals and were approved by Institutional Animal Care and Use Committees.

Evaluation of cell proliferation

Cell proliferation by Ki67 immunohistochemical staining was determined in the livers from mice treated with PFOA and propiconazole (Study 4) by Experimental Pathology Laboratories, Inc., Durham, NC. Tissue samples in paraffin blocks were sectioned, deparaffinized and hydrated. Samples were incubated in 1:20 citrate buffer for 7 min under pressure (decoaking) and then cooled. Blocking steps included quenching of endogenous peroxides with 3% H2O2, an avidin block, a biotin block and incubation with blocking serum. Sections were labeled with rat anti-mouse Ki67 antibody (1:25 dilution) and a rabbit anti-rat IgG secondary antibody (1:300). Slides were developed using an avidin-biotin complex method following an application of 3,3′-diaminobenzidine as the chromogen (Muskhelishvili et al., 2003). The percent labeling indices (LI) were determined by counting the number of positively-stained Ki67 nuclei in 900-1400 hepatocyte nuclei/animal from photographic images taken at 40X. Each image was scored and analyzed for accuracy.

Classification analysis using machine learning methods

Classifiers were developed using machine learning methods to model predictions of CAR activation from the set of 7,860 probesets used in the classification study. The 7,860 probesets used in the classification study. The 7,860 probesets were selected based on their ability to predict CAR activation: the samples from wild-type and CAR-null mice from Chua and Moore (2005) and the same dataset lacking the control and treated CAR-null samples. The derived classifiers of 110 or 247 probesets were then used to predict CAR activation of the remaining samples. A test set of 80 and 239 samples known to be positive or negative, respectively, for CAR activation came from a number of studies in which mouse or mouse primary hepatocytes were exposed to CAR activators or
control substances (Geter et al., 2014; Schaap et al., 2012; Study 1 and Study 3 above).

**Construction of a CAR-dependent biomarker signature**

The general strategy for biomarker signature development is outlined in Figure 1, left. A list of probe sets that comprise the CAR biomarker signature was derived using livers of wild-type and CAR-null mice treated with CITCO, phenobarbital, or TCPOBOP for 3 days (Chua and Moore, 2005). Three statistical tests were used for each chemical: 1) chemical-treated wild-type vs. wild-type control; 2) chemical-treated CAR-null vs. CAR-null controls; and 3) chemical-treated wild-type vs. chemical-treated CAR-null. For each chemical, genes were identified which exhibited differences in expression in wild-type mice, but no statistically significant expression in the same direction in the CAR-null mice. This list of genes was then examined for statistical differences between the treated wild-type mice and the treated CAR-null mice. The three lists of genes (one for each chemical) were compared and probe sets were selected based on the following criteria: 1) probe sets were altered in at least 2 or 3 out of the 3 comparisons, 2) probe sets exhibited the same direction of change after exposure to all chemicals, 3) probe sets that encoded the same gene had identical direction of change after exposure, 4) the average fold change for each probe set was > 1.5-fold, and 5) the probe sets were not also altered in the same direction in gene expression biomarker signatures for AhR, PPARα, Nrf2, and STAT5b (Oshida et al., 2015 and in preparation). These criteria for selection ensured that the probe sets exhibited absolute dependence on CAR, a robust response, and consistent chemical-independent regulation. The final list of probesets in the CAR biomarker signature is found in Supplementary File 1.

**Additional methods**

All additional methods used in this study have been previously described in Oshida et al. (2015), including RNA isolation, microarray analyses, identification of differentially expressed genes in microarray datasets, functional analyses of the signature genes, assembly of an annotated mouse liver gene expression compendium, evaluation of activation using the Running Fisher's algorithm, tissues used for RT-PCR analysis and RT-PCR.

**Results**

**Development and analysis of the CAR biomarker signature**

CAR biomarker genes were identified as described in the Methods using profiles from the livers of wild-type and CAR-null mice treated with phenobarbital, TCPOBOP or CITCO for 3 days (Chua and Moore, 2005) (Figure 1). A total of 128 probe sets (120 with increased expression and 8 with decreased expression, collapsing to 83 genes) were identified which exhibited similar regulation by two or three out of the three compounds. The full list of genes is found in Supplementary File 1. Figure 2A shows the dependence of chemical-induced changes in expression on CAR when comparing treated wild-type and CAR-null mice for the three chemicals. The identified signature genes could be either direct transcriptional targets of CAR or indirect targets but still dependent on CAR for altered expression. Many of the genes in the CAR signature are known direct targets of CAR including Cyp2b10, Cyp2c55 and Gadd45b (Tolson and Wang, 2010; Columbano et al., 2005). To comprehensively assess whether the genes in the CAR signature were previously identified as being regulated by CAR activators, we used the Comparative Toxicogenomics Database (CTD; http://ctdbase.org/) to find published relationships between chemicals and the signature genes in mice. Figure 2B shows the gene-chemical interactions for 2 of the 3 CAR activators (phenobarbital, TCPOBOP) used to construct the signature. In addition, information on gene expression effects of 5 conazole fungicides previously thought to activate CAR were examined. The conazoles are further evaluated for CAR activation (see below). Most of the gene-chemical interactions for the signature genes have been annotated for phenobarbital, TCPOBOP, and propiconazole. Fewer, but generally consistent interactions have been annotated for the other chemicals. Overall, the majority of the CAR signature genes exhibited directional changes consistent with findings in the literature.

The CAR signature genes were evaluated for canonical pathway enrichment by Ingenuity Pathway Analysis (IPA) (Figure 2C). The top 10 pathways enriched with the signature genes included those previously associated with CAR regulation, e.g., Xenobiotic Metabolism Signaling (Chai et al., 2013), and included those regulated by AhR (Aryl Hydrocarbon Receptor Signaling) and PXR (PXR/RXR Activation). (It should be noted that the genes in the CAR signature do not include those in either the AhR or PXR signatures developed using similar methods (Oshida et al., in preparation)). Activation of CAR by AhR or PXR activators is discussed below. The upstream analysis function of IPA identified a number of transcription factors that were predicted to regulate the signature genes (Figure 2D). CAR was the top scoring transcription...
Figure 2. Characterization of the CAR biomarker signature. A. Expression behavior of genes in the signature. The heat map shows the expression of the 128 probe sets after exposure to CITCO, phenobarbital (PB), and TCPOBOP in wild-type and CAR-null mice compared to the final signature. B. Expression behavior of the CAR signature genes in the Comparative Toxicogenomics Database (CTD). The signature shows the fold-change of the genes. To the right of the signature, yellow and blue represents the number of publications which showed increased or decreased expression of the gene, with intensity representing the number of individual publications which showed the effect. Green represents genes where there is conflicting information regarding the expression of the gene by the chemical exposure, which could be due in part to lack of annotation of tissue of origin. PB, phenobarbital; Cypro, cyproconazole; Epoxi, epoxiconazole; Flu, fluconazole; Propi, propiconazole; Tri, triadimefon. C. Top canonical pathways enriched for the genes in the CAR biomarker signature. Genes were examined by Ingenuity Pathways Analysis. D. Top transcription factors predicted to regulate the genes in the CAR biomarker signature, as determined by Ingenuity Pathways Analysis.
Figure 3. The CAR biomarker signature accurately predicts CAR activation. A. Heat map showing the expression of genes in the CAR biomarker signature across 468 biosets. Genes in the biomarker signature were ordered based on their average fold-change. Biosets were ordered based on their similarity to the CAR biomarker signature using the p-value of the Running Fisher’s test. Biosets with positive correlation are on the left and biosets with negative correlation are on the right. The red vertical lines denote a p-value of 10^{-4}. B. Similarity of the CAR biomarker signature to biosets from the three chemicals used to derive the biomarker signature in wild-type but not CAR-null mice. Additional comparisons of mice expressing the human CAR gene (hCAR) expressed in the CAR-null background are shown. All p-values from comparisons of each bioset to the CAR biomarker signature were converted to –log_{10} values. Those comparisons which exhibited negative correlation to the biomarker signature were given a negative value. C. The CAR biomarker signature correctly identifies three known CAR activators (Aroclor-1260, PCB-153 and phenobarbital) in mice exposed to 12 diverse treatments. Open bars, females. Filled bars, males. Conditions of exposure are found in Supplementary File 2. D. Summary of the sensitivity and specificity of the CAR biomarker signature. The biomarker signature was compared to chemicals that were known positives or negatives for CAR activation.
factor (p-value = 1.09E-11). Other transcription factors included PXR (NR1I2), NFE2L2 (Nrf2), POU2F1, PPARα, retinoic acid orphan receptors (RORA, RORC), TP53, PPARGC1A, and the CAR heterodimeric partner, RXRA.

A rank-based strategy to predict CAR activation

The biomarker signature was compared to gene lists using the Running Fisher’s algorithm, resulting in a correlation direction (positive or negative) and an associated p-value of the similarity (Kupershmidt et al., 2010). Because the Running Fisher’s algorithm uses similarity as a metric, it could be hypothesized that biosets that have similarity to each other (based on a low p-value) would exhibit similar gene expression behavior. To visualize the relationships between the Running Fisher’s algorithm p-value and the expression of genes in the biomarker signature, 468 biosets of statistically-filtered genes were evaluated for similarity to the CAR biomarker signature and then sorted by p-value. The left of Figure 3A shows that for biosets which had a positive correlation to the biomarker signature, the lower the p-value, the more the bioset appears similar to the biomarker signature, due to similarities in the direction and the relative magnitude of the changes. These biosets include a number of known activators of CAR (phenobarbital and TCPOBOP), as well as perfluorinated compounds (discussed below). The right of the figure shows a smaller group of biosets that exhibited negative correlation to the biomarker signature; the biosets on the far right exhibited the lowest p-value for negative correlation. In general, these biosets exhibit a pattern of gene expression that was opposite to that of the biomarker signature, and included lipopolysaccharide (LPS) and TNFα exposure, as well as various infections. Given that CAR can be suppressed by a number of chemicals (see below), the biosets with negative correlation to the CAR biomarker signature may be hypothesized to reflect CAR suppression.

The ability of the biomarker signature to correctly identify known CAR activators was determined. The biosets from wild-type mice treated with the three compounds used to build the biomarker signature exhibited statistically-significant similarity to the biomarker signature (p-values < 10-17), whereas the biosets from the corresponding treated CAR-null mice were not significant (Figure 3B). The species-specificity of the biomarker signature was also examined in this dataset by assessing CAR activation in CAR-null mice expressing a human CAR gene (hCAR mice). Exposure to the human CAR activator CITCO led to the greatest significance compared to the other chemicals, and the significance was approximately equal to that in wild-type mice. Phenobarbital, also known to activate human CAR, resulted in significant CAR activation. In contrast, the mouse-specific activator TCPOBOP did not result in significant CAR activation. In an additional analysis, we show that the CAR biomarker signature can detect CAR activation even at low doses of an inducing compound (Supplementary File 2).

The ability of the biomarker signature to distinguish compounds that are known activators of CAR from those that activate other transcription factors was examined. Male and female mice were administered 12 different chemicals or biological agents. These included those that principally activate AhR (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), β-naphthoflavone), CAR (Aroclor-1260, PCB-153, phenobarbital), or peroxisome proliferator-activated receptor α (PPARα) (ciprofibrate, WY-14,643 (WY)). Other treatments induce inflammation (LPS, interleukin-6, tumor necrosis factor α (TNFα)) or hypoxia (cobalt, phenylhydrazine). As expected, Aroclor-1260 and phenobarbital activated CAR in males and females (Figure 3C). The third CAR activator, PCB-153, activated CAR in females only. CAR was also activated by AhR activators β-naphthoflavone and TCDD, the PPARα activator WY, and phenylhydrazine, all in females but not males. A number of treatments suppressed CAR including WY in male mice and LPS and TNFα in female mice. An inflammatory state has been shown to suppress CAR activity (Assenat et al., 2004 and discussed below). There is evidence that PPARα and CAR interact to suppress the activity of each other (Corton et al., 2014). The activation of CAR by AhR activators is explored further below. Therefore, the biomarker signature correctly identified compounds which activate CAR, as well as some intriguing regulation of CAR by prototypical activators of other transcription factors, sometimes in a sex-dependent manner.

A classification analysis using the Running Fisher’s algorithm was performed. The final number of biosets evaluated was 28 positives and 32 negatives. Using a p-value < 10-4 as the cutoff, the biomarker signature resulted in 96% sensitivity and a 97% specificity (Figure 3D). These methods were superior to a number of machine learning algorithms (described in Supplementary File 2).

Analysis of a mouse liver gene expression compendium

To find factors that affect activation of CAR, a mouse liver gene expression compendium was constructed and annotated, as detailed in the Methods. The compendium consists of biosets of gene expression changes in the livers of mice, mouse primary hepatocytes, or mouse liver-derived cell lines altered by diverse factors. The compendium contains ~1850 biosets of gene expression changes between control and experimental states including ~470 chemical, ~450 gene, ~220 diet, ~100 hormone or cytokine, ~90 life stage, ~90 stress and ~120 strain comparisons.

Using the Running Fisher’s algorithm, the CAR biomarker signature was used for classifying the
Figure 4. CAR activation or suppression in a mouse liver compendium. A. Assessment of CAR activation or suppression in a mouse liver compendium. A p-value of each of the ~1850 comparisons to the CAR biomarker signature was derived using the Running Fisher’s algorithm. All p-values were converted to –log₁₀ values as described in Figure 3B. The number of biosets with a p-value < 10⁻⁴ for either activation or suppression in the indicated categories are shown. B. Activation or suppression of CAR by chemical exposure. The cutoff values are shown for reference. C. Relationships between Car mRNA expression changes and predictions of CAR activation or suppression. The biosets were divided into those in which Car mRNA expression was increased, decreased or exhibited no change. Predictions of the number of biosets for CAR activation or suppression are shown for the three groups.

286 biosets were classified as affecting CAR, including 208 activating and 78 suppressing CAR (p-value < 10⁻⁴). A summary of the bioset factors in which CAR was altered are shown in Figure 4A. The distribution of the biosets indicates that out of all of the factors examined, chemicals and genetic models have the largest effects on CAR. The effects of chemicals on CAR are discussed below. Because of space limitations, the effects of other factors will be described in another publication (Vasani et al., in preparation).

The distribution of –log(p-values) across the 461 chemical comparisons representing ~150 chemicals is shown in Figure 4B and shows that 144 of the chemical treatments significantly activated CAR and 15 significantly suppressed CAR.

A number of mechanisms may determine how chemical exposure affects CAR-regulated biomarker signature genes. Because CAR exhibits constitutive activity, one mechanism of activation could involve increases in the expression of the Car gene and protein. The relationship between expression of the Car gene and the biomarker signature predictions was determined (Figure 4C). Car expression was derived using the statistically-filtered gene lists from the same microarray experiments used to determine CAR activation status. The biosets were divided into those in which Car gene expression was increased, decreased or exhibited no change. For those biosets in which Car gene expression significantly increased (fold change > 1.2), there were 129 biosets which exhibited...
**Figure 5.** Activation of CAR by AhR activators. A. Activation of CAR by TCDD. Activation of CAR by TCDD in two studies in which transcriptional effects were examined. (Left) Wild-type and AhR-null mice were exposed to 1 mg/kg of TCDD for 19 hrs (from study GSE10082). (Right) Three strains of mice were exposed to two dose levels of TCDD (from E-MEXP-1231). B. Activation of CAR by benzo[a]pyrene (B[a]P) in three studies in which transcriptional effects were examined. (Left) The Hepa1c1c7 and AhR-null Hepa1c1c7 cell lines were exposed to 5 µM B[a]P for 8 hrs (from study GSE11796). (Middle) Primary hepatocytes from wild-type or Xpa1/p53-null mice were exposed to four dose levels of B[a]P for 24 hrs (from study E-TABM-1139). (Right) Hepatocytes from wild-type mice were dosed with 30 µM of B[a]P (from E-MEXP-2209).

no significant CAR activation or suppression (p-value > 10^-4) (not shown), 49 biosets in which CAR was activated and only 6 biosets in which CAR was suppressed. For those biosets in which Car expression was decreased, there were 169 biosets which exhibited no significant CAR activation or suppression (p-value > 10^-4) (not shown), 11 biosets in which CAR was activated and 27 biosets in which CAR was suppressed. For those biosets in which there was no change in Car expression, there were...
157 and 47 biosets that exhibited activation or suppression of CAR, respectively. Therefore, Car gene expression does not appear to be tightly correlated with CAR activation or suppression and is not a reliable predictor of CAR activation status.

**Crosstalk between CAR and other signaling pathways: activation of CAR by AhR activators**

Given the extensive evidence of overlapping functions in regulating xenobiotic metabolism and transport by CAR and PXR (Chai et al., 2013), the effects of PXR activator exposure on CAR activation were examined. Wild-type and PXR-null mice were administered the PXR activator, pregnenolone-16-a-carbonitrile (PCN) for 4 days and global gene expression was examined (Methods). CAR was significantly activated by PCN in wild-type mice (Figure 6, left). In the PXR-null mice, the significance of the activation of CAR was diminished but not abolished compared to wild-type mice. In a similar study (GSE23780), wild-type and PXR-null mice were administered the β-secretase inhibitor “Compound 13” for 4 days. CAR was significantly activated in wild-type but not PXR-null mice. The expression of Cyp2b10 and Akr1b7 was examined in the livers of PCN-treated mice (Figure 6, right). PCN caused increases in both genes in wild-type mice. Although the activation was still significant from controls in PXR-null mice, the level of activation was significantly less than that in treated wild-type mice. Overall, these results indicate that activation of CAR by PXR activators was partially or completely PXR-dependent.

**Suppression of CAR activation by chemical exposure**

Compounds were identified that suppressed constitutive CAR activation. Acetaminophen (APAP) is known to be metabolized by CAR-regulated enzymes to toxic metabolites; CAR-null mice are resistant to APAP toxicity (Zhang et al., 2002). APAP suppressed CAR in a strain- and time-dependent manner, with the most significant suppression being in the C57 and DBA strains at 6 hrs of exposure (from Liu et al., 2010) (Figure 7A). The pattern of CAR suppression did not parallel strain susceptibility to APAP (C57 > SMJ > DBA > SJL). Two of the 9 biosets from mice exposed to lipopolysaccharide (LPS) showed suppression of CAR, with 3 of the other biosets approaching significance for suppression (Figure 7B). Concanavalin A, after a 6 hr exposure but not 1 or 3 hr exposure, led to suppression of CAR (Figure 7C). Lastly, a study in which mice were exposed to different sized silicon dioxide particles for 6 hrs showed that 300 nm particles caused suppression of CAR; exposure to the smaller-sized nanoparticles approached significance (Figure 7D).
Figure 7. Chemical suppression of CAR. Predictions of CAR activation/suppression were compared to the expression of the Car gene and expression of two markers of inflammatory responses, Rela and Jun (derived from the same microarray experiments). A. Suppression by acetaminophen. Effects of acetaminophen treatment were examined at either 3 or 6 hrs of exposure in four strains of mice from Liu et al. (2010) study. B. Suppression by lipopolysaccharide exposure. The study from which the bioset was derived is indicated by the GEO number. One study is not archived in GEO. C. Suppression of CAR by concanavalin A. Balb/c mice were injected with 20 mg/kg concanavalin A and sacrificed at the indicated times (from GSE17184). D. Suppression of CAR by 300 nm silicon dioxide nanoparticles. Mice were given intravenous injections of the indicated doses of the various sized silicon dioxide nanoparticles and then sacrificed at 6 hrs (from GSE30861).
All of the treatments that suppressed CAR resulted in increases in inflammatory mediators, indirectly assessed by examination of the expression of two genes, the AP-1 subunit, Jun and the NF-kB subunit, Rela, both known to be increased under inflammatory conditions (Figure 7A-D). A number of previous studies have shown that inflammatory signaling leads to suppression of CAR gene expression (Assenat et al., 2004; Beigneux et al., 2002). Except for LPS exposure, most of the conditions in which CAR was suppressed also exhibited decreases in Car gene expression (Figure 7A-D). Overall, the data add to the evidence that compounds that induce inflammatory responses in the liver negatively regulate CAR, consistent with the long-standing observation that inflammation suppresses xenobiotic metabolism (Morgan, 2009).

**Conazole pesticides activate CAR**

CAR activation by anti-fungal conazole pesticides has been hypothesized to be part of the mechanistic process leading to liver cancer (Nesnow, 2013). However, except for cyproconazole (Peffer et al., 2007), direct evidence that CAR mediates conazole effects is lacking. Biosets from wild-type mice treated with 5 conazoles were evaluated for CAR activation. Figure 8A shows that with increasing dose and time of exposure, there was usually increasing significance of the similarity to the CAR biomarker signature for cyproconazole, epoxiconazole, myclobutanil, propiconazole, and triadimefon biosets.

To directly determine whether CAR is involved in conazole-mediated effects, wild-type and CAR-null mice were exposed to propiconazole or triadimefon for 7 days by gavage, as described in the Methods. Liver to body weight ratios were increased in wild-type mice for each compound (Figure 8B). The increases were also observed in CAR-null mice exposed to triadimefon, but not in CAR-null mice exposed to propiconazole. The labeling index was increased in hepatocytes from wild-type but not CAR-null mice exposed to propiconazole (Figure 8C). (Triadimefon was not tested for cell proliferation effects.)

Marker genes were examined for expression changes after exposure (Figure 8D). Cyp2b10 was increased by propiconazole and triadimefon, and the increases were dependent on CAR, as they were no longer significantly altered in CAR-null mice. Other genes that were markers of CAR or PXR were examined. Birc5, found to be regulated by PXR and part of a PXR biomarker signature (Oshida et al., in preparation), as well as Cyp3a11, a prototypical marker gene for PXR, were activated by propiconazole and triadimefon in wild-type and CAR-null mice, consistent with a CAR-independent mechanism. The protein encoded by Cyp51 is inhibited by conazoles in fungi (Vanden Bossche et al., 1989). Cyp51 and Gstm3 were increased in both wild-type and CAR-null mice, with significantly higher increases for Gstm3 in Car-null mice after exposure to both compounds. Gsta2 was induced by propiconazole and triadimefon in wild-type mice and by propiconazole in CAR-null mice.

In summary, the biomarker signature identified multiple time-dose combinations that led to CAR activation by all 5 of the tested conazoles using microarray profiles. In our study of wild-type and CAR-null mice, propiconazole exhibited CAR-dependent effects including increases in liver to body weight, increases in hepatocyte proliferation and increases in Cyp2b10. CAR-dependent transcriptional effects were noted for triadimefon including induction of Cyp2b10 and Gsta2. As propiconazole and triadimefon increase liver tumors in mice (Allen et al., 2006 and references therein), the adverse outcome pathways that lead to liver cancer for propiconazole and triadimefon are likely different, and may include a CAR AOP for propiconazole.

Perfluorinated compounds activate CAR, but CAR is not required for growth effects by PFOA. The perfluorinated surfactant chemicals are environmentally-relevant compounds that appear to mediate most of their effects in the liver through the PPARα nuclear receptor (Corton et al., 2014). Previous studies with PFOA and PFOS identified a subset of genes regulated by these compounds that were PPARα-independent and hypothesized to be regulated by CAR activation (Rosen et al., 2008, 2010). However, direct evidence for CAR activation is lacking.

The ability of four perfluorinated compounds and other PPARα activators to activate CAR was examined in wild-type and PPARα-null mice. The hypolipidemic compounds, fenofibrate and WY, given for 6 hrs (GSE8396), activated CAR in wild-type but not PPARα-null mice (Figure 9A). Di-(2-ethylhexyl)phthalate (DEHP) given to wild-type mice for up to 72 hrs was also a CAR activator (from GSE55733, data not shown), supporting earlier studies in which DEHP effects were compared between wild-type and CAR-null mice (Ren et al., 2010). Not all compounds that activated PPARα were CAR activators, as three piperidine-derived PPARα activators did not activate CAR (from GSE12147, data not shown). The effects of exposure on CAR activation was examined for perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) from published studies (GSE22871 and GSE9786) and perfluorononanoic acid (PFNA) and perfluorohexanesulfonic acid (PFHxS), which were evaluated in additional experiments, as detailed in the Methods. All four compounds activated CAR in wild-type mice, and the activation of CAR was generally more significant in PPARα-null mice (Figure 9A), consistent with previous findings showing increased expression of CAR marker genes by PPARα activators in PPARα-null mice compared to wild-type mice (summarized in Corton et al., 2014).
Figure 8. Conazole fungicides activate CAR. A. Effects of exposure to 5 conazoles on CAR activation. Biosets were derived from Ward et al. (2006) (for Myclo, Propi, and Triad) or Hester et al. (2012) (for Cypro and Epoxi) studies. Abbreviations: Cypro, cyproconazole; Epoxi, epoxiconazole; Myclo, myclobutanil; Propi, propiconazole; Triad, triadimefon. B. Liver to body weight ratios of wild-type and CAR-null mice given propiconazole (210 mg/kg) or triadimefon (165 mg/kg) for 7 days. **Significant from corresponding control at p-value < 0.01. C. Hepatocyte proliferation in wild-type and CAR-null mice exposed to propiconazole for 7 days. Hepatocyte proliferation was evaluated as detailed in the Methods. *Significant from corresponding control at p-value < 0.05. D. Expression of marker genes for CAR and PXR in the livers of wild-type and CAR-null mice given propiconazole or triadimefon for 7 days. **significant from corresponding control at p-value < 0.01; ##significant between wild-type and nullizygous comparisons at p-value < 0.01.

Three structurally-diverse PPARα activators were examined for effects on CAR-regulated genes. There was no activation of Cyp2b10 or Akr1b7 in livers of mice treated with WY for 3 days (Figure 9B, left), consistent with the lack of significant CAR activation by WY given for 5 days in wild-type or PPARα-null mice (from GSE8295, data not shown). Examination of the expression of CAR target genes showed minimal increases in expression of Cyp2b10 and Akr1b7 in wild-type mice treated with PFNA (3 mg/kg), but greater increases in the treated PPARα-null mice (Figure 9B, middle). Gene expression was examined in wild-type and PPARα-null mice after exposure to the panRXR agonist (AGN194,204), which activates nuclear receptor-RXR heterodimers through RXR activation. Treatment with AGN resulted in activation of Akr1b7 but not Cyp2b10 in wild-type and PPARα-null mice (Figure 9B, right). The results are consistent with the predictions of CAR activation for PFNA and weak or no activation of CAR by WY.
Figure 9. Perfluorinated compounds activate CAR in a PPARα-independent manner. A. Effect of exposure to PPARα activators in wild-type and PPARα-null mice on CAR activation. The indicated dose levels are shown. For GSE8396, mice were given one 400μL injection of a 10mg/mL solution of either fenofibrate or WY in 0.5% carboxymethyl cellulose (CMC) or 400 μl of 0.5% CMC only. Abbreviations: Feno, fenofibrate; PFHxS, perfluorohexane sulfonate; PFNA, perfluoronanoic acid; PFOS, perfluorooctane sulfonate; PFOA, perfluorooctanoic acid. B. Effects of WY-14,643, PFNA and AGN194,204 on activation of CAR marker genes in wild-type and PPARα-null mice. *Significant from corresponding control at p-value < 0.05; **Significant from corresponding control at p-value < 0.01. C. Liver to body weights of wild-type and CAR-null mice given PFOA or PFOS for 7 days. **Significant from corresponding control at p-value < 0.01. D. Hepatocyte proliferation in wild-type and CAR-null mice exposed to PFOA for 7 days. **Significant from corresponding control at p-value < 0.01. E. Expression of marker genes for PPARα, CAR and PXR in the livers of wild-type and CAR-null mice given PFOA for 7 days. *Significant from corresponding control at p-value < 0.05; **Significant from corresponding control at p-value < 0.01; #significant between wild-type and nullizygous comparisons at p-value < 0.05; ##significant between wild-type and nullizygous comparisons at p-value < 0.01. F. Expression of marker genes for PPARα, CAR and PXR in the livers of wild-type and CAR-null mice given PFOS for 7 days. *Significant from corresponding control at p-value < 0.05; **Significant from corresponding control at p-value < 0.01. #Significant from corresponding control at p-value < 0.05; ##Significant from corresponding control at p-value < 0.01.

The results also indicate that AGN regulates only a subset of CAR-regulated genes in a PPARα-independent manner.

To directly determine the role of CAR in mediating the effects of PFOA and PFOS, these compounds were given by gavage to wild-type and CAR-null mice each day for 7 days, as detailed in the Methods. Increases in liver to body weights were observed in both wild-type and CAR-null mice that were not significantly different between strains (Figure 9C). PFOA increased hepatocyte proliferation that was also not significantly different between strains (Figure 9D). (PFOS was not evaluated for cell proliferation effects.)
A number of marker genes for PPARα, CAR and PXR were examined after PFOA exposure in wild-type and CAR-null mice (Figure 9E). Marker genes for PPARα (Acox1, Cyp4a10) were increased in both strains of mice. Increases in Cyp2b10 observed in wild-type mice were abolished in CAR-null mice. Increases in Birc5 were partially dependent on CAR. A number of genes were induced in both strains to various extents, with the increases being significant in wild-type mice only (Cyp3a13), CAR-null mice only (Gstm3, Cyp3a11), or both strains (Gsta2). Gene expression was also examined after PFOS exposure (Figure 9F). The induction of Acox1 and Cyp4a10 were muted compared to PFOA, with marginal induction of Acox1 only becoming significant in CAR-null mice and induction of Cyp4a10 only significant in wild-type mice. The induction of Cyp2b10 and Gstm3 were clearly CAR-dependent. Inductions of the other genes were significant in wild-type (Cyp3a11) or CAR-null (Cyp3a11, Gsta2) mice. Another perfluorinated compound, perfluorodecanoic acid exposure, was shown to require CAR but not PPARα for induction of Cyp2b10 (Cheng and Klaassen, 2008). Taken together with a large number of published studies (summarized in Corton et al., 2014), PPARα plays a dominant role in mediating the effects of PFOA and PFOS in the mouse liver, including effects on liver to body weights, hepatocyte proliferation, and gene expression. Although we present direct evidence that the perfluorinated compounds activate CAR, the activation of CAR likely plays a subordinate role to PPARα in mediating the adverse effects of these compounds, including the induction of liver cancer.

**Discussion**

A biomarker signature-based approach was used to identify chemicals that activate or suppress CAR. To create the CAR biomarker signature, a unique microarray study was utilized in which wild-type and CAR-null mice were exposed to three structurally-diverse CAR activators. Genes that were consistently activated or repressed in a CAR-dependent manner were identified using a stringent set of criteria, including 9 statistical tests and a number of filters, ultimately resulting in a final list of 128 probe sets representing 83 genes. To screen for factors that led to alterations of CAR, we compared the biomarker signature to a gene expression database of annotated biosets using the fold-change rank-based nonparametric Running Fisher’s algorithm (Kupershmidt et al., 2010), analogous to the Gene Set Enrichment Analysis (GSEA) method (Lamb et al., 2006; Subramanian et al., 2005). The biomarker signature reliably predicted CAR activation. Our test to assess predictive capability gave a balanced accuracy of 97% (Figure 9E), far superior to predictions using a number of well-known machine learning classification algorithms (Supplementary File 2). Additionally, the biomarker signature was able to distinguish between chemical activators of CAR and other xenobiotic-activated transcription factors (Figure 3D). Therefore, the CAR biomarker signature coupled with the Running Fisher’s algorithm will be a useful strategy for predicting CAR effects in future genomic studies. The set of genes in the biomarker signature is a useful starting point for identifying a smaller subset of genes that can reliably predict effects on CAR in high-throughput screens of environmentally-relevant chemicals or drugs.

Comparison of the CAR biomarker signature to biosets in a gene expression compendium identified diverse factors that when perturbed, resulted in effects on CAR (Figure 4A). Consistent with CAR serving as a promiscuous target for diverse drugs and chemicals, about two-thirds of the biosets in the compendium that activated CAR were from chemical exposure. Other factors, including knockout or overexpression of genes in the liver, did have effects on CAR, and will be described in future reports. Activation of CAR through direct binding, typified by CITCO and TCPOBOP, is one mechanism by which CAR is activated (Figure 10A). CAR is also activated indirectly by a number of compounds, with the best characterized being phenobarbital. In the absence of phenobarbital exposure, a key residue on CAR (Thr38) is phosphorylated, resulting in sequestration of CAR in the cytoplasm. Blocking EGFR activity by interaction with phenobarbital results in dephosphorylation of Thr38 by protein phosphatase 2A, nuclear translocalization of CAR and regulation of gene expression (Mutoh et al., 2013). Our methods for assessing chemical-induced CAR activation, like typical in vitro trans-activation techniques, cannot distinguish between direct or indirect activation of CAR. However, the downstream consequences appear to be similar, if not identical, including alteration of xenobiotic metabolism genes and increases in hepatocyte proliferation. Long term exposure to direct (TCPOBOP) or indirect (phenobarbital) CAR activators leads to increases in liver cancer in mice that are both dependent on CAR (discussed in Elcombe et al., 2014).

Our biomarker signature-based approach for chemical screening led to a number of novel observations. First, chemicals were identified that highlight the cross-talk with other signaling pathways regulated by xenobiotic-activated receptors. These included two activators of AhR (TCDD and B[a]P) that activate CAR in an AhR-dependent manner (Figure 5A-C). Compound 13 and PCN activate CAR at least partly...
through a PXR-dependent mechanism (Figure 6). In contrast, a number of PPARα activators activate CAR independent of PPARα (Figure 9A). The fact that AhR and PXR activators (but not PPARα activators) require their receptors for CAR activation, leads to the hypothesis that the activation of CAR requires a factor(s) that is dependent on prior activation of AhR or PXR.

Second, compounds were identified that negatively regulate CAR, including acetaminophen, LPS, concanavalin A, and 300 nm silicon dioxide particles (Figure 10B). These chemicals are all known to cause a cascade of effects to varying degrees, including increases in liver injury, infiltration of inflammatory cells, secretion of cytokines and induction of inflammatory mediators. Suppression of CAR may be through decreases in Car gene expression, as decreases in expression of the Car gene paralleled CAR suppression, even though the Car gene was not part of the CAR biomarker signature (Figure 7). Glucocorticoid receptor (GR) regulates the basal expression of the Car gene, and under conditions of inflammation, the NF-kB subunit RelA interacts with and prevents GR from activating Car (Assenat et al., 2004). Even though decreases in Car gene expression do not consistently lead to decreases in CAR activation (Figure 4C), exposure to LPS, ConA or 300 nm silicon dioxide particles showed a close association between induction of Rela and Jun, CAR suppression, and decreases in the expression of the Car gene.

Lastly, we followed up on our biomarker signature-based screening to determine dependency on CAR of effects of members of the environmentally-relevant chemical classes conazoles and perfluorinated compounds. The studies showed that effects of propiconazole linked to liver cancer (increases in liver weight and hepatocyte proliferation) are CAR-dependent (Figure 8), and support the hypothesis that propiconazole induces liver cancer by a complex mechanism that includes a CAR-dependent event (Nesnow, 2013). The other conazole examined, triadimefon, may cause liver effects via an alternative AOP(s). PFOA and PFOS, while clearly activating Cyp2b10 in a CAR-dependent manner, exhibited increases in cell proliferation and/or liver weight that
were CAR-independent (Figure 9). Linkage of CAR activation in these studies to CAR-dependent induction of liver tumors would require a comprehensive assessment of the long-term effects of chemical exposure in wild-type and CAR-null mice.

The methods described here for identifying factors that affect CAR will be a useful strategy for identification of CAR modulators in future genomic studies. Because there are likely a minor number of CAR gene targets that exhibit similar regulation across tissues and species (Molnár et al., 2013), reliable biomarker signatures that predict CAR activation might have to be built using treated and control samples from the tissue and species of interest. For example, a human CAR biomarker signature might be built using microarray data before and after exposure to CAR activators in wild-type cells which exhibit appropriate expression of hCAR compared to those in which hCAR has been knocked down using shRNA technologies.

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Supplementary Material

Supplementary Files 1 & 2. See the electronic version of this article for Supplementary Material at www.nrsignaling.org/nrs13002.

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