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Activation of the Constitutive Androstane Receptor Inhibits Gluconeogenesis without Affecting Lipogenesis or Fatty Acid Synthesis in Human Hepatocytes

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Abstract

Objective—Accumulating evidence suggests that activation of mouse constitutive androstane receptor (mCAR) alleviates type 2 diabetes and obesity by inhibiting hepatic gluconeogenesis, lipogenesis, and fatty acid synthesis. However, the role of human (h) CAR in energy metabolism is largely unknown. The present study aims to investigate the effects of selective hCAR activators on hepatic energy metabolism in human primary hepatocytes (HPH).

Methods—Ligand-based structure-activity models were used for virtual screening of the Specs database (www.specs.net) followed by biological validation in cell-based luciferase assays. The effects of two novel hCAR activators (UM104 and UM145) on hepatic energy metabolism were evaluated in HPH.

Results—Real-time PCR and Western blotting analyses reveal that activation of hCAR by UM104 and UM145 significantly repressed the expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, two pivotal gluconeogenic enzymes, while exerting negligible effects on the expression of genes associated with lipogenesis and fatty acid synthesis. Functional experiments show that UM104 and UM145 markedly inhibit hepatic synthesis of glucose but not triglycerides in HPH. In contrast, activation of mCAR by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, a selective mCAR activator, repressed the expression of genes associated with gluconeogenesis, lipogenesis, and fatty acid synthesis in mouse primary hepatocytes, which were consistent with previous observations in mouse model *in vivo*.

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Conflict of interest

The authors declare no conflicts of interest.

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Conclusion—Our findings uncover an important species difference between hCAR and mCAR in hepatic energy metabolism, where hCAR selectively inhibits gluconeogenesis without suppressing fatty acid synthesis.

Implications—Such species selectivity should be considered when exploring CAR as a potential therapeutic target for metabolic disorders.

Keywords

constitutive androstane receptor; energy metabolism; human primary hepatocytes; species difference

Introduction

Xenobiotic receptors, such as the constitutive androstane receptor (CAR, NR1I3), are responsible for both controlling the expression of genes associated with all phases of drug metabolism and transport, as well as defense against stress caused by xenobiotics. CAR is most notably known as the transcriptional regulator for a number of drug-metabolizing enzymes, such as cytochrome P450 (CYP) 2B6 and CYP3A4, UDP glucuronosyltransferase 1A1 and sulfotransferase 2A1, as well as drug transporters including organic anion transporting polypeptide 2 and multidrug resistance protein 1 (Qatanani and Moore, 2005; Timsit and Negishi, 2007; Tolson and Wang, 2010). Collectively, up-regulation of these metabolizing enzymes and membrane transport proteins, not only alters the clearance of xenobiotic compounds, such as prescription drugs and environmental chemicals, but also influences the excretion of endobiotic metabolites such as bilirubin and bile acids (Yamamoto *et al.*, 2003; Kakizaki *et al.*, 2009). Activation of CAR may represent a precursor for potential drug-drug interactions by accelerating the metabolism of co-administered medications, often leading to decreased therapeutic efficacy or enhanced toxicity (Wang and LeCluyse, 2003). In addition to these well-established roles of CAR in xenobiotic metabolism and clearance, accumulating evidence reveals that CAR has also emerged as a coordinate factor that modulates diverse liver functions under physiological and pathophysiological conditions including energy homeostasis, insulin signaling, cell proliferation and apoptosis, inflammation, and tumor development (Yamamoto *et al.*, 2004; Huang *et al.*, 2005; Moreau *et al.*, 2008; Gao and Xie, 2010). A need for further understanding the endobiotic roles of CAR is evident.

Hepatic energy homeostasis is maintained by comprehensive physiological mechanisms that balance the formation and oxidation of lipids, fatty acids, and glucose in the liver. The correlation between CAR activation and energy metabolism was initially observed by Negishi and colleagues in comparison of the global gene expression profiles between wild-type and CAR knockout mice (Ueda *et al.*, 2002). Notably, other than the induction of typical drug-metabolizing enzymes and transporters, treatment with phenobarbital (PB), a prototypical CAR activator, profoundly repressed the expression of a group of genes associated with energy metabolism in a CAR-dependent manner (Ueda *et al.*, 2002). Subsequent studies revealed that through cross-talk with forkhead box O1 or peroxisome proliferator activated receptor γ -coactivator 1 α , activation of CAR leads to attenuated gluconeogenesis or increased energy expenditure in mouse liver (Kodama *et al.*, 2004).

Activation of CAR also compromised the liver X receptor interaction with the promoter region of the sterol regulatory element binding protein 1 (SREBP-1), a master lipogenic regulator (Zhai *et al.*, 2010). Under high fat diet challenge or in leptin-deficient (ob/ob) mice, activation of CAR significantly ameliorated hyperglycemia and improved insulin sensitivity, while such beneficial effects were absent in CAR knockout mice (Dong *et al.*, 2009; Gao *et al.*, 2009). On the other hand, CAR-null mice appear to be defective in fasting adaptation and exhibit greater weight loss under calorie restriction (Maglich *et al.*, 2004). It is noteworthy, however, that current conception regarding the role of CAR in energy metabolism was drawn predominantly from studies using either animal models *in vivo* or cultured cells with ectopic expression of mouse (m) CAR. Although human (h) CAR and its rodent counterparts share several common characteristics, significant species differences between these receptors exist. For instance, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl)oxime (CITCO), the selective hCAR activator, cannot bind or activate mCAR (Maglich *et al.*, 2003), while 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), the most potent mCAR agonist, does not activate hCAR (Tzamei *et al.*, 2000). Moreover, while activation of mCAR was essential for TCPOBOP- and PB-mediated tumor promotion in mice, activation of hCAR by CITCO appears to be associated with cell cycle arrest and enhanced apoptosis in human brain tumor stem cells (Yamamoto *et al.*, 2004; Chakraborty *et al.*, 2011). As such, direct extrapolation of conclusions drawn from animal models to humans is unsafe. The role of hCAR in energy homeostasis is yet to be elucidated.

Recent studies have demonstrated that docking and pharmacophore-based virtual screening of chemical databases combined with cell-based biological assays are effective in identifying new compounds as modulators of nuclear receptors including hCAR (Pan *et al.*, 2011; Lynch *et al.*, 2013). Utilizing this combined approach, here, we have retrieved 144 compounds as potential hCAR activators by virtual screening of the Specs database (www.specs.net) based on their structure-activity features. Of the 144 lead compounds, ten hCAR activators that exhibited equal or greater potency than that of CITCO were identified by cell-based luciferase assays. Subsequently, the role of hCAR in hepatic energy metabolism was evaluated in cultured human primary hepatocytes (HPH) using CITCO and a number of newly identified hCAR activators. Importantly, we have uncovered a significant species difference between the effects of hCAR and mCAR on energy homeostasis. Unlike mCAR, activation of hCAR selectively inhibits hepatic gluconeogenesis by repressing the expression of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) without significantly affecting lipogenesis or the synthesis of fatty acids.

Materials and Methods

Materials

PB and CITCO were obtained from Sigma-Aldrich (St. Louis, MO). All 144 compounds (designated as UM050-UM200) were obtained from Specs (Delft, The Netherlands). The Dual-Luciferase Reporter Assay System was purchased through Promega (Madison, WI). Antibodies against CYP2B6, PEPCK, G6Pase, fatty acid synthase (FAS), and SREBP-1c were purchased from Santa Cruz (Dallas, TX). The acetyl-coA carboxylase 1 (ACC- α) and

stearoyl-coA desaturase-1 (SCD-1) antibodies were acquired from Cell Signaling (Danvers, MA). β -actin antibody was from Sigma-Aldrich. Matrigel, insulin, and ITS⁺ (insulin/transferrin/selenium) were from BD Biosciences (Bedford, MA). The glucose and triglyceride assay kits were from Sigma-Aldrich. All other cell culture reagents were purchased from Life Technologies (Grand Island, NY) or Sigma-Aldrich.

Computational modeling

The pharmacophore model of hCAR was generated as described previously (Lynch *et al.*, 2013). In brief, the pharmacophore protocol embedded in Discovery Studio (DS) software suite (version 3.0; Accelrys; San Diego, CA) was used to generate a qualitative CAR pharmacophore based on 17 previously reported hCAR modulators. A Bayesian model was generated based on 26-reported hCAR modulators and non-modulators (Supplementary Table S1) with protocols similar to previous publications (Pan *et al.*, 2011; Pan *et al.*, 2013). The model was generated with 70 physicochemical and structural descriptors including fingerprint ECFP-6 by using the “Create Bayesian Model” protocol embedded in DS 3.0 (Rogers *et al.*, 2005). The crystal structure of hCAR-ligand binding domain (LBD) (Protein DataBank ID: 1XV9) was used for flexible docking of ligands using the extensively validated algorithm Surflex (Tripos, St. Louis, MO). Protein atoms were kept rigid during the initial docking; however, flexible docking was performed to further narrow down the retrieved compounds as described previously (Lynch *et al.*, 2013). In addition, compounds with structural similarity to the known hCAR modulators CITCO and PK11195 were retrieved using the “Find Similar Molecules by Fingerprints” protocol in the library analysis module of DS 3.0. The MDL public keys and long range extended class fingerprint description 6 keys (referred to as ‘ECFP_6’) were used separately with the Tanimoto similarity coefficient and an input query molecule representing the target compound of the assay (Li *et al.*, 2008; Yu and Wild, 2012). It should be noted that MDL public keys and ECFP_6 do not recognize differences between racemic mixtures and stereoisomers.

Virtual screening of Specs compounds

Approximately 30,000 compounds from the Specs database were initially screened based on pharmacophore and Bayesian models of hCAR modulators, and docking and similarity searches followed by further focusing with flexible docking, clustering, and molecular property analysis, including LogP and number of rotatable bonds. Retrieved hits were also filtered by our previously published model of PXR to exclude potential PXR activators (Pan *et al.*, 2011). Bayesian-based screening was via the “Calculate Molecular Properties” protocol of DS 3.0. Pharmacophore-based virtual screening was employed through the “Ligand Profiler” protocol, with maximum conformations set to 55 and the fitting algorithm RIGID (Chang *et al.*, 2006). To further concentrate potential hCAR modulators, compounds were clustered by “MDLPublicKeys fingerprints” descriptors using the “Cluster Ligands” protocol of DS 3.0. The number of clusters for individual runs was set between 30 to 40.

Transient Transfection in HepG2 Cells

HepG2 cells cultured in a 75 cm² flask were co-transfected with the CYP2B6-2.2kb reporter and hCAR1+A expression vectors using a Lipofectamine® 2000 Transfection Kit (Life

Technologies) following the manufacturer's instruction. Eighteen hours after transfection, cells were trypsinized then re-plated in 96- or 48-well collagen coated plates. For luciferase reporter assays, transfected cells were treated with vehicle control (0.1% DMSO), CITCO (1 μ M) or the test compounds at concentrations as indicated in the figures for another 24 h. Cell lysates were assayed for firefly luciferase activities normalized against the activities of co-transfected renilla luciferase using a Dual-Luciferase Kit (Promega). Data were represented as mean $\pm$ SD of three individual transfections.

Human Primary Hepatocytes

Human liver tissues were obtained after surgical resection by pathology staffs, with consent forms signed by all participating patients, and prior approval from the Institutional Review Board at the University of Maryland, School of Medicine. HPH were isolated from human liver specimens by a modification of the two-step collagenase digestion method as described previously or obtained from Bioreclamation In Vitro Technologies (Baltimore, MD) (Li *et al.*, 1999). Fresh HPH were seeded at 1.5×10^6 , 7.5×10^5 , 3.75×10^5 , cells/well in 6-well, 12-well, or 24-well, collagen coated plates in seeding medium (DMEM supplemented with 5% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 4 μ g/ml insulin, and 1 μ M dexamethasone) for 4 to 6 h. Subsequently, hepatocytes were cultured for 36 h at 37°C in the complete medium [serum free Williams' E medium supplemented with ITS⁺ (6.25 μ g/ml insulin, 6.25 μ g/ml transferrin, and 6.25 ng/ml selenium) and 0.1 μ M dexamethasone] before treatment with specified compounds for another 24 or 72 h for detection of mRNA or protein expression, respectively.

Mouse Primary Hepatocytes

Male C57BL/6J mice (6–8 week old) were purchased from Charles River Laboratories, Inc. (Germantown, Maryland). Mice were kept in 12:12 h dark/light cycle with free access to water and food prior to surgery. All animal procedures complied with the guidelines of the Institutional Animal Care and Use Committee (University of Maryland Baltimore, MD). Mouse primary hepatocytes were isolated using a two-step collagenase perfusion method as described previously (Swift and Brouwer, 2010). Isolated hepatocytes were seeded in 12-well collagen coated plates in the same seeding DMEM containing 5% fetal bovine serum, insulin (4 μ g/ml), dexamethasone (0.1 μ M), and penicillin G (100 units/ml)/streptomycin (100 μ g/ml). One hour after seeding, hepatocytes were cultured in the same complete medium as described above for 36 h at 37°C before treatment with vehicle control (0.1% DMSO) or TCPOBOP (0.1, 0.25, and 0.5 μ M) for 24 h.

Real-time PCR Analysis

Total RNA was isolated from treated HPH using the TRIzol® reagent and reverse transcribed with a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturers' instructions. Expression of *CYP2B6*, *G6Pase*, *PEPCK*, *FAS*, *ACC- α* , *SREBP-1c*, *fructose 1, 6-bisphosphatase1 (FBPase1)*, and *SCD-1* genes was normalized against GAPDH. Real-time PCR assays were performed in 96-well optical plates on an ABI Prism 7000 Sequence Detection System with SYBR Green PCR Master Mix. Forward and reverse primers for these genes were summarized in Supplementary Table S2.

Induction values were calculated using the equation: $\text{Fold} = 2^{\Delta\Delta C_t}$, where C_t represents the differences in cycle threshold numbers between each of the target gene and GAPDH, and ΔC_t represents the relative change in these differences between control and treatment groups.

Nuclear Translocation of CAR in HPH

A recombinant adenovirus expressing enhanced yellow fluorescent protein-tagged hCAR (Ad/EYFP-hCAR) has been functionally evaluated as an efficient means for prediction of chemical-mediated hCAR nuclear translocation in HPH (Li *et al.*, 2009). Human hepatocytes cultured in 24-well collagen-coated plates were infected with Ad/EYFP-hCAR for 24 h followed by the treatment of vehicle control (0.1% DMSO), PB (1 mM), or selected compounds at 10 μM each for another 8 h. After treatment, the cellular localization of Ad/EYFP-hCAR in the hepatocytes was analyzed via confocal microscopy. Quantitative distribution of hCAR was classified as cytosolic, nuclear, or mixed (nuclear + cytosolic); and 100 Ad/EYFP-hCAR expressing hepatocytes were counted from each group.

Western Blot Analyses

Homogenate proteins (30 μg each) from treated HPH were resolved on SDS polyacrylamide gels and electrophoretically transferred onto Immobilon-P polyvinylidene difluoride membranes and blocked using 5% blotting-grade blocker (nonfat dry milk, Bio-RAD). Subsequently, membranes were incubated with specific antibodies against CYP2B6 and PEPCK diluted at 1:400, and G6Pase, FAS, SCD-1, ACC- α , and SREBP-1c diluted at 1:200 in 1% blotting-grade blocker, respectively. β -Actin was used to normalize protein loadings. Membranes were washed using 1 $\times$ Tri buffered saline-Tween 20 (Bio-RAD) and incubated with horseradish peroxidase goat anti-rabbit IgG antibody diluted at 1:5000, and developed using ECL Western blotting detection reagent (GE Healthcare, Chalfont St. Giles, UK). Densitometry was analyzed using NIH ImageJ software.

Glucose Assay

HPH seeded in 48-well collagen coated plates were treated for 48 h (in complete Williams' E media) with vehicle control DMSO (0.1%), CITCO (1 μM), or UM104 and UM145 at indicated concentrations. Cells were then washed using phosphate buffered saline and treated with the compounds at the same concentrations in a glucose-free media (DMEM:D5030 supplemented with sodium bicarbonate, sodium pyruvate, and sodium lactate) for 24 h. Media was then collected for glucose measurement using the Glucose (GO) Assay Kit (Sigma-Aldrich) per the manufacturer's protocol. Protein concentration measured by the Bradford protein assay from each well was used to normalize cell density.

Triglyceride Assay

HPH were cultured in 12-well collagen coated plates. Twenty-four hours after seeding, culture media was changed to insulin free Williams' E media (2% BSA was used in lieu of ITS⁺) for another 24 h. HPH were then cultured in the insulin free Williams' E media supplemented with glucose, in the presence of the experimental compounds or vehicle control (0.1% DMSO) for 24 h. Cell lysates were extracted for triglyceride measurement

using the Serum Triglyceride Determination Kit (Sigma-Aldrich) following the manufacturer's instruction. The protein concentration was used to normalize cell density.

Statistical Analysis

Experimental data are presented as a mean of triplicate determinations $\pm$ S.D. 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 <0.05 (*) and <0.01 (**). $E_{\max}$ and EC_{50} values for hCAR1+A activation were estimated using the Michaelis-Menten equation (GraphPad, Prism).

Results

Initial virtual screening

Around 30,000 structurally diverse compounds from the Specs database were initially screened based on the generated pharmacophore and Bayesian models, CAR-LBD based docking, and chemical-structural similarity (Fig. 1). The docking scores obtained from Surflex indicate that the parameter $-\log K_d$ is associated with more favorable binding (Jain, 1996). There were 242 compounds with docking scores above 9.0. The similarity search was based on MDLpublicKey fingerprints and Tanimoto similarity index, with 1.0 indicating a full match with a template compound. 110 compounds had similarity scores of above 0.638 (template: PK11195) or 0.642 (template: CITCO). In this study, all compounds were subjected to virtual screening with our previously established pharmacophore model which retrieved 106 compounds with pharmacophore scores above 0.9, suggesting a fit to all pharmacophore features with $>90\%$ CI (1.0 being a 'perfect' match). The Bayesian model was validated with leave-one-out cross-validation of training set compounds. The "receiver operator curve" area under the curve (ROC AUC) was 0.951; the true positive, false negative, false positive, and true negative were 17, 1, 0, and 8, respectively, indicating a robust model for predicting activation effects on hCAR. The virtual screening based on the generated Bayesian model resulted in 200 drugs with scores above 4.09. Retrieved hits from all three methodologies (docking, pharmacophore and Bayesian modeling) were subjected to consensus analysis and further focusing of lead compounds.

Further virtual screening of retrieved hits

Leads from initial screening were narrowed down by flexible docking, clustering, molecular properties, and a previously developed Bayesian model of hPXR. Retrieved molecules from similarity and pharmacophore-based virtual screening were docked with Surflex to the LBD of hCAR (PDB ID: 1XV9) while protein atoms within 4 Å of a ligand allowed to move during docking process. Compounds with docking scores above 4.0 remained, leading to 94 drugs out of 110 and 106 compounds from initial hits obtained by using similarity search and pharmacophore-based screening, respectively. Lead compounds were further narrowed down with molecular properties, i.e. LogP and number of rotatable bonds ($N_{\text{rot}} = 10$). To ensure the highest number of possible retrieved hits, the inclusion criterion for a compound's LogP value varied between 5 to 7. Subsequently, MDLPublicKeys-based clustering was used to ensure that retrieved hits included structurally diverse drugs from each pathway. To increase the selectivity of hCAR over hPXR activators, the identified

leads were further screened with our previously published Bayesian model of hPXR to remove compounds that could be potential hPXR activators. Compounds identified through docking (38), pharmacophore (27), and Bayesian-based screening (49), as well as the top 30 hits retrieved from similarity searching, were subjected to subsequent cell-based luciferase activity assays (Table 1).

Activation of hCAR in HepG2 cells

To define the biological activity of the lead compounds obtained via virtual screening, CAR-responsive reporter gene activation was examined in HepG2 cells. As reported previously, a chemical responsive surrogate of the wild-type hCAR, the hCAR1+A plasmid, was co-transfected with a CYP2B6-reporter vector in HepG2 cells (Chen *et al.*, 2010). Lead compounds (144) with optimal parameters of the computational models were initially evaluated in cell-based reporter assays at a single concentration of 10 μ M (Fig. 2A). Ten out of the 144 compounds exhibited hCAR1+A activation at a value equal to or greater than that of CITCO, the positive control (Fig. 2B). The cell-based hCAR activation was further confirmed at a full concentration-response spectrum in the same experiment. All ten compounds subjected to this experiment were found to have a maximal activation ($E_{\max}$) of hCAR greater than CITCO response that was set as 100% (Fig. 2C–2L). In addition, a parallel cytotoxicity assay was carried out to provide a preliminary cytotoxic scope for further experiments (Data not shown).

Induction of CYP2B6 and nuclear translocation of CAR in HPH

CYP2B6, a highly inducible P450 gene, represents the primary transcriptional target of hCAR in the liver (Wang and Negishi, 2003). To validate the association between cell-based hCAR1+A activation and the induction of CYP2B6 expression in humans, effects of the ten potential CAR activators on CYP2B6 mRNA expression was examined in HPH prepared from two liver donors (HL#45 and HL#46). As shown in Fig. 3A, four (UM068, UM104, UM138, and UM145) of the ten compounds induced the mRNA expression of CYP2B6 to a minimum of 40% of the level achieved by CITCO treatment, in at least one liver donor. In separate experiments, using HPH from liver donors (HL#49 and HL#50), concentration-dependent induction of CYP2B6 expression was confirmed in all four compounds (Fig. 3B). UM104, in particular, demonstrated robust increase of CYP2B6 mRNA that reaches the level equal to or greater than that of CITCO. Due to cytotoxicity considerations (data not shown), the maximal concentration used for UM145 was 25 μ M.

A hallmark of hCAR activation in HPH is its chemical-stimulated nuclear translocation from the cytoplasm; a process that was obviously missed in immortalized cells (Kawamoto *et al.*, 1999; Li *et al.*, 2009). Next, we examined the effects of these four compounds on hCAR intracellular localization in HPH. Hepatocytes were infected with the Ad/EYFP-hCAR followed by treatment with the four compounds at a concentration of 10 μ M. As demonstrated in Fig. 3C, representative images for the Ad/EYFP-hCAR localization showed that without activation (DMSO, 0.1%), hCAR is primarily retained in the cytoplasm of infected HPH, while it clearly accumulates in the nucleus after exposure to the typical CAR activator PB. Notably, all four compounds translocated hCAR to the nuclei of HPH at proportions that were comparable to that by PB (Fig. 3D). Collectively, these results suggest

that the four hCAR activators identified from a combination of virtual screening and luciferase-reporter assays can induce the expression of *CYP2B6* gene in HPH initiated by nuclear accumulation of hCAR.

Effects of hCAR on the expression of genes involved in energy metabolism in HPH

Recent studies using wild-type and CAR knockout mice revealed a novel endobiotic effect of CAR in energy homeostasis (Dong *et al.*, 2009; Gao *et al.*, 2009). Activation of mCAR is associated with anti-obesity, hepatosteatosis attenuation, and insulin sensitization in mice. To explore the specific role of hCAR activation in hepatic energy metabolism, HPH were treated with vehicle control, the known selective hCAR activator CITCO, and two novel hCAR activators (UM145 and UM104) selected from the current studies. As shown in Fig. 4A, the mRNA expression of *G6Pase* gene was significantly repressed in HPH obtained from three donors (HL#49, HL#50, and HL#64) upon treatment with CITCO (1 μ M) or UM104 and UM145 at the indicated concentrations. Protein levels of G6Pase were also reduced after the same treatments as shown in HPH from a representative liver donor (HL#54; Fig. 4C). Both mRNA and protein expression of PEPCK, another important enzyme in the gluconeogenesis pathway, were markedly reduced by the presence of CITCO, UM104, or UM145 (Fig. 4B, 4D). Additionally, the expression of FBPase1, the third regulatory gluconeogenic enzyme, was also repressed by both UM104 and UM145 (Supplementary Fig. S1). These results indicate that activation of hCAR can repress the expression of genes associated with hepatic gluconeogenesis in humans.

Next we tested the modulation of fatty acid synthesis by these hCAR activators in HPH. Two representative fatty acid synthesis genes, FAS and ACC- α , were selected in this experiment. As shown in Fig. 5A and 5B, neither CITCO nor UM104 and UM145 treatment affected the mRNA and protein expression of these two genes in HPH. In parallel experiments, expression of the lipogenic-associated transcription factor and enzyme SREBP-1c and SCD-1, was also evaluated in HPH. Notably, there was no repression of either SREBP-1c or SCD-1 at the mRNA and protein levels by the selected hCAR activators (Fig. 5C and 5D). In fact, the mRNA expression of SCD1 was moderately increased by UM145 at 25 μ M. Overall, these findings suggest that hCAR activation has no clear effect on fatty acid synthesis and lipogenesis, which contradicts results obtained from mice *in vivo*.

Modulation of hepatic glucose and triglyceride syntheses by hCAR

To understand whether perturbation of gene expression associated with energy homeostasis by hCAR activation would lead to functional alterations in gluconeogenesis and fatty acid synthesis, cell-based glucose and triglyceride syntheses were examined in HPH. As shown in Fig. 6A, synthesis of glucose was repressed by both UM104 and UM145 in a concentration-dependent manner but only moderately affected by CITCO in HPH obtained from three liver donors (HL#58, HL#59, and HL#69). Alternatively, activation of hCAR by the same compounds did not alter the hepatic synthesis of triglycerides (Fig. 6B). These data further corroborate the ability of hCAR to selectively inhibit gluconeogenesis but not fatty acid synthesis in human liver, which differ from the observations in mice.

Effects of mCAR on the expression of genes involved in energy metabolism in mouse primary hepatocytes

To date, the conclusions of mCAR in energy metabolism have been obtained predominantly from studies in whole animal models (Wada *et al.*, 2009). Therefore, it is critical to address whether the currently observed species-specific roles of CAR between humans and mice are due to the *in vitro-in vivo* system differences. Primary mouse hepatocytes prepared from liver perfusion of C57BL/6J mice were treated with vehicle control (0.1% DMSO) or the prototypical mCAR activator TCPOBOP at concentrations of 0.1, 0.25, and 0.5 μ M. As expected, activation of mCAR by TCPOBOP robustly induced the mRNA expression of Cyp2b10, the counterpart of the human CYP2B6 in mice (Fig. 7A). Importantly, TCPOBOP also significantly down-regulated the expression of genes associated with gluconeogenesis (G6pase and Pepck), fatty acid synthesis (Fas and Acc- α), and lipogenesis (Srebp-1c and Scd-1) (Fig. 7B–7G). These results, in agreement with mouse data *in vivo*, suggest that the differential roles of mouse and human CAR in hepatic energy homeostasis were a species-specific event.

Discussion

A growing body of evidence has broadened the physiological role of CAR from a xenobiotic receptor to an endobiotic regulator, by which it not only alters drug therapeutic efficacy and toxicity but also modulates energy homeostasis, cell proliferation, and inflammation. CAR regulation of hepatic energy metabolism has been well established in animal models. Activation of mCAR by TCPOBOP markedly alleviated obesity and diabetes in mice (Dong *et al.*, 2009; Gao *et al.*, 2009). In contrast, current knowledge regarding the role of hCAR in energy metabolism is limited. In this report, using HPH, we showed that activation of hCAR by the known selective activator, CITCO, or two newly identified hCAR activators, significantly repressed the expression of G6Pase and PEPCK, two important enzymes associated with the gluconeogenesis cascade, but elicited negligible effects on a number of genes involved in lipogenesis (SREBP1c, SCD-1) and fatty acid synthesis (FAS, ACC- α). Functional analyses further revealed that activation of hCAR significantly decreased hepatic production of glucose without affecting triglyceride synthesis. Our findings suggest an important species difference in the role of human and mouse CAR in energy metabolism (Fig. 8). Thus, it is reasonable to speculate that pharmacological activation of hCAR might be beneficial in managing hyperglycemia but not hyperlipidemia.

Compared with most other nuclear receptors, CAR demonstrates significant species selectivity in its ligand binding and activation profiles. Many rodent CAR activators do not activate hCAR, while others, such as meclizine, function as mCAR activators but hCAR deactivators (Huang *et al.*, 2004). Currently, elucidation of the biological function of hCAR relies predominantly on pharmacological manipulation of this receptor; chemical tools pertaining to hCAR activation, however, are limited. In the current study, ligand and structure-based virtual screening of a Specs database has resulted in the selection of 144 drug-like compounds with optimal computational parameters as potential hCAR agonists. Biological determination of hCAR activators *in vitro* has been challenged by the fact that wild-type hCAR is spontaneously accumulated in the nucleus and constitutively activated in

immortalized cell lines prior to xenobiotic stimulation (Li and Wang, 2010). In contrast, the hCAR splicing variant hCAR3 and a hCAR3-based chimeric mutant, hCAR1+A, displayed minimal basal activity and significant xenobiotic-response to known hCAR activators in hepatoma cell lines (Auerbach *et al.*, 2005; Chen *et al.*, 2010). Subsequent experiments utilizing the hCAR1+A-based luciferase assay in HepG2 cells revealed that ten of the 144 compounds exhibited robust activation of hCAR to an extent, challenging that of CITCO at certain concentrations.

It is noteworthy that although luciferase reporter assays in immortalized cell lines, including HepG2, have been used extensively for the identification of nuclear receptor activators and hold the potential to screen compounds in a high-throughput manner, these cells often lack physiologically relevant expression of major hepatic drug-metabolizing enzymes and transcription factors. Comparatively, HPH, which retain the major metabolic features of human liver, have been recognized as a powerful *in vitro* model for predicting metabolism-based drug-drug interactions, hepatotoxicities, as well as energy metabolism. Our current results demonstrate that in HPH, four of the ten compounds of interest are potent inducers of CYP2B6, the prototypical target gene of hCAR, despite all of them exhibiting potent activation of hCAR1+A in HepG2 cells. In agreement with this observation, all four compounds efficiently translocated Ad/EYFP-hCAR from cytoplasm to the nucleus of HPH, the initial and essential step of CAR activation (Kawamoto *et al.*, 1999). Although it is out of the scope of this manuscript, we speculate that extensive metabolism in HPH may contribute to the lack of CYP2B6 induction by the other six compounds.

Gluconeogenesis is an important biological process of glucose production from non-carbohydrate precursors. This process is tightly controlled in response to the change of energy status and glucose levels in the cell. PEPCK and G6Pase represent two key gluconeogenic enzymes that are regulated by transcriptional and non-transcriptional mechanisms. Inhibition of hepatic gluconeogenesis by repression of the expression of PEPCK and G6Pase is associated with many anti-diabetic drugs' pharmacological actions (Mues *et al.*, 2009). Previous studies have shown that activation of mCAR by TCPOBOP or PB markedly represses the expression of Pepck and G6pase in a CAR-dependent manner (Ueda *et al.*, 2002; Gao *et al.*, 2009). A decreased serum glucose level was also observed in TCPOBOP treated ob/ob but not ob/ob-CAR^{-/-} mice (Dong *et al.*, 2009). Our studies with HPH demonstrated that activation of hCAR also significantly repressed the expression of PEPCK and G6Pase, and inhibited hepatic synthesis of glucose. In particular, the new hCAR activators UM104 and UM145 showed promising inhibition of gluconeogenesis, which was stronger than that of CITCO (Fig. 6A). Together these data suggest that the role of CAR in gluconeogenesis is a highly conserved response across multiple species.

The role of CAR in obesity and hepatic steatosis is rather complicated in comparison to that in gluconeogenesis and has been contradictorily reported in animal models (Maglich *et al.*, 2004; Dong *et al.*, 2009). Studies using both high fat diet-induced obesity and ob/ob models revealed that treatment with TCPOBOP was effective in preventing obesity and hepatic steatosis in wild-type but not in CAR knockout mice. Mechanistically, it was believed that these beneficial effects were attributed, at least partly, by decreased expression of genes associated with fatty acid synthesis, such as Fas and Acc- α , as well as lipogenesis such as

Srebp-1c and Scd-1 (Gao *et al.*, 2009). Using the methionine choline-deficient (MCD) diet-induced nonalcoholic steatohepatitis mouse model, Baskin-Bey *et al.*, reported that TCPOBOP treatment significantly reduced hepatic steatosis and serum triglyceride levels (Baskin-Bey *et al.*, 2007), while such beneficial effects were not observed by Yamazaki *et al.*, using the same MCD-based model (Yamazaki *et al.*, 2007). In this report, we have not observed significant repression of either the expression of fatty acid synthesis- and lipogenic-associated genes (FAS, ACC- α , SREBP-1c and SCD-1) or the synthesis of triglycerides in HPH, upon the activation of hCAR by CITCO, UM104 or UM145. These findings are consistent with a recent report indicating that treatment with CITCO did not affect lipid accumulation in HPH and HepG2 cells (Moya *et al.*, 2010), but appear to be contradictory to the majority of observations in animal models. As the role of mCAR in energy metabolism has predominantly been investigated in mice *in vivo*, additional experiments in mouse primary hepatocytes were performed to address whether the *in vitro-in vivo* experimental conditions contribute to the observed species difference. Our results indicated that activation of mCAR by TCPOBOP significantly repressed the expression of the corresponding genes associated with gluconeogenesis, fatty acid synthesis and lipogenesis. Hence, it is most likely that mouse and human CAR mediate differential responses in lipogenesis but not in gluconeogenesis.

In summary, our results suggest that although human and mouse CAR share many similarities in drug metabolism and detoxification, they show significant differences in modulating energy metabolism. In particular, whereas activation of both human and mouse CAR remarkably inhibits hepatic gluconeogenesis, activation of hCAR may not render the liver resistant to steatosis. However, it is noteworthy that many additional metabolic pathways involving glycolysis, amino acid synthesis, beta-oxidation, and glycogen synthesis, can all affect the cellular levels of glucose and triglyceride in the liver. Future studies are warranted to fully elucidate the molecular mechanisms underlying CAR-mediated energy metabolism. Importantly, our current findings also warrant reasonable apprehension with respect to the extrapolation of data obtained from animal models to humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Auerbach SS, Stoner MA, Su S, Omiecinski CJ. Retinoid X Receptor- α -Dependent Transactivation by a Naturally Occurring Structural Variant of Human Constitutive Androstane Receptor (NR1I3). *Mol Pharmacol*. 2005; 68:1239–1253. [PubMed: 16099843]

- Baskin-Bey ES, Anan A, Isomoto H, Bronk SF, Gores GJ. Constitutive androstane receptor agonist, TCPOBOP, attenuates steatohepatitis in the methionine choline-deficient diet-fed mouse. *World J Gastroenterol.* 2007; 13:5635–5641. [PubMed: 17948939]
- Chakraborty S, Kanakasabai S, Bright JJ. Constitutive androstane receptor agonist CITCO inhibits growth and expansion of brain tumour stem cells. *Br J Cancer.* 2011; 104:448–459. [PubMed: 21224854]
- Chang C, Bahadduri PM, Polli JE, Swaan PW, Ekins S. Rapid Identification of P-glycoprotein Substrates and Inhibitors. *Drug Metabolism and Disposition.* 2006; 34:1976–1984. [PubMed: 16997908]
- Chen T, Tompkins LM, Li L, Li H, Kim G, Zheng Y, Wang H. A Single Amino Acid Controls the Functional Switch of Human Constitutive Androstane Receptor (CAR) 1 to the Xenobiotic-Sensitive Splicing Variant CAR3. *Journal of Pharmacology and Experimental Therapeutics.* 2010; 332:106–115. [PubMed: 19820207]
- Dong B, Saha PK, Huang W, Chen W, Abu-Elheiga LA, Wakil SJ, Stevens RD, Ilkayeva O, Newgard CB, Chan L, Moore DD. Activation of nuclear receptor CAR ameliorates diabetes and fatty liver disease. *Proc Natl Acad Sci U S A.* 2009; 106:18831–18836. [PubMed: 19850873]
- Gao J, He J, Zhai Y, Wada T, Xie W. The constitutive androstane receptor is an anti-obesity nuclear receptor that improves insulin sensitivity. *J Biol Chem.* 2009; 284:25984–25992. [PubMed: 19617349]
- Gao J, Xie W. Pregnane X receptor and constitutive androstane receptor at the crossroads of drug metabolism and energy metabolism. *Drug Metab Dispos.* 2010; 38:2091–2095. [PubMed: 20736325]
- Huang W, Zhang J, Washington M, Liu J, Parant JM, Lozano G, Moore DD. Xenobiotic stress induces hepatomegaly and liver tumors via the nuclear receptor constitutive androstane receptor. *Mol Endocrinol.* 2005; 19:1646–1653. [PubMed: 15831521]
- Huang W, Zhang J, Wei P, Schrader WT, Moore DD. Meclizine Is an Agonist Ligand for Mouse Constitutive Androstane Receptor (CAR) and an Inverse Agonist for Human CAR. *Molecular Endocrinology.* 2004; 18:2402–2408. [PubMed: 15272053]
- Jain AN. Scoring noncovalent protein-ligand interactions: a continuous differentiable function tuned to compute binding affinities. *J Comput Aided Mol Des.* 1996; 10:427–440. [PubMed: 8951652]
- Kakizaki S, Takizawa D, Tojima H, Yamazaki Y, Mori M. Xenobiotic-sensing nuclear receptors CAR and PXR as drug targets in cholestatic liver disease. *Curr Drug Targets.* 2009; 10:1156–1163. [PubMed: 19925451]
- Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, Negishi M. Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol.* 1999; 19:6318–6322. [PubMed: 10454578]
- Kodama S, Koike C, Negishi M, Yamamoto Y. Nuclear receptors CAR and PXR cross talk with FOXO1 to regulate genes that encode drug-metabolizing and gluconeogenic enzymes. *Mol Cell Biol.* 2004; 24:7931–7940. [PubMed: 15340055]
- Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, Silber PM. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. *Chem Biol Interact.* 1999; 121:17–35. [PubMed: 10418968]
- Li H, Chen T, Cottrell J, Wang H. Nuclear Translocation of Adenoviral-Enhanced Yellow Fluorescent Protein-Tagged-Human Constitutive Androstane Receptor (hCAR): A Novel Tool for Screening hCAR Activators in Human Primary Hepatocytes. *Drug Metabolism and Disposition.* 2009; 37:1098–1106. [PubMed: 19196842]
- Li H, Wang H. Activation of xenobiotic receptors: driving into the nucleus. 2010:409–426.
- Li L, Chen T, Stanton JD, Sueyoshi T, Negishi M, Wang H. The Peripheral Benzodiazepine Receptor Ligand 1-(2-Chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide Is a Novel Antagonist of Human Constitutive Androstane Receptor. *Mol Pharmacol.* 2008; 74:443–453. [PubMed: 18492798]

- Lynch C, Pan Y, Li L, Ferguson S, Xia M, Swaan P, Wang H. Identification of Novel Activators of Constitutive Androstane Receptor from FDA-Approved Drugs by Integrated Computational and Biological Approaches. *Pharmaceutical Research*. 2013; 30:489–501. [PubMed: 23090669]
- Maglich JM, Parks DJ, Moore LB, Collins JL, Goodwin B, Billin AN, Stoltz CA, Klier SA, Lambert MH, Willson TM, Moore JT. Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes. *J Biol Chem*. 2003; 278:17277–17283. [PubMed: 12611900]
- Maglich JM, Watson J, McMillen PJ, Goodwin B, Willson TM, Moore JT. The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction. *J Biol Chem*. 2004; 279:19832–19838. [PubMed: 15004031]
- Moreau A, Vilarem MJ, Maurel P, Pascussi JM. Xenoreceptors CAR and PXR activation and consequences on lipid metabolism, glucose homeostasis, and inflammatory response. *Mol Pharm*. 2008; 5:35–41. [PubMed: 18159929]
- Moya M, Gomez-Lechon MJ, Castell JV, Jover R. Enhanced steatosis by nuclear receptor ligands: a study in cultured human hepatocytes and hepatoma cells with a characterized nuclear receptor expression profile. *Chem Biol Interact*. 2010; 184:376–387. [PubMed: 20079722]
- Mues C, Zhou J, Manolopoulos KN, Korsten P, Schmoll D, Klotz LO, Bornstein SR, Klein HH, Barthel A. Regulation of glucose-6-phosphatase gene expression by insulin and metformin. *Horm Metab Res*. 2009; 41:730–735. [PubMed: 19579180]
- Pan Y, Chothe PP, Swaan PW. Identification of Novel Breast Cancer Resistance Protein (BCRP) Inhibitors by Virtual Screening. *Molecular Pharmaceutics*. 2013; 10:1236–1248. [PubMed: 23418667]
- Pan Y, Li L, Kim G, Ekins S, Wang H, Swaan PW. Identification and Validation of Novel Human Pregnane X Receptor Activators among Prescribed Drugs via Ligand-Based Virtual Screening. *Drug Metabolism and Disposition*. 2011; 39:337–344. [PubMed: 21068194]
- Qatanani M, Moore DD. CAR, the continuously advancing receptor, in drug metabolism and disease. *Curr Drug Metab*. 2005; 6:329–339. [PubMed: 16101572]
- Rogers D, Brown RD, Hahn M. Using Extended-Connectivity Fingerprints with Laplacian-Modified Bayesian Analysis in High-Throughput Screening Follow-Up. *Journal of Biomolecular Screening*. 2005; 10:682–686. [PubMed: 16170046]
- Swift B, Brouwer KL. Influence of seeding density and extracellular matrix on bile Acid transport and mrp4 expression in sandwich-cultured mouse hepatocytes. *Mol Pharm*. 2010; 7:491–500. [PubMed: 19968322]
- Timsit YE, Negishi M. CAR and PXR: the xenobiotic-sensing receptors. *Steroids*. 2007; 72:231–246. [PubMed: 17284330]
- Tolson AH, Wang H. Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Adv Drug Deliv Rev*. 2010; 62:1238–1249. [PubMed: 20727377]
- Tzamelis I, Pissios P, Schuetz EG, Moore DD. The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. *Mol Cell Biol*. 2000; 20:2951–2958. [PubMed: 10757780]
- Ueda A, Hamadeh HK, Webb HK, Yamamoto Y, Sueyoshi T, Afshari CA, Lehmann JM, Negishi M. Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. *Mol Pharmacol*. 2002; 61:1–6. [PubMed: 11752199]
- Wada T, Gao J, Xie W. PXR and CAR in energy metabolism. *Trends Endocrinol Metab*. 2009; 20:273–279. [PubMed: 19595610]
- Wang H, LeCluyse EL. Role of orphan nuclear receptors in the regulation of drug-metabolising enzymes. *Clin Pharmacokinet*. 2003; 42:1331–1357. [PubMed: 14674787]
- Wang H, Negishi M. Transcriptional regulation of cytochrome p450 2B genes by nuclear receptors. *Curr Drug Metab*. 2003; 4:515–525. [PubMed: 14683479]
- Yamamoto Y, Kawamoto T, Negishi M. The role of the nuclear receptor CAR as a coordinate regulator of hepatic gene expression in defense against chemical toxicity. *Arch Biochem Biophys*. 2003; 409:207–211. [PubMed: 12464260]

- Yamamoto Y, Moore R, Goldsworthy TL, Negishi M, Maronpot RR. The orphan nuclear receptor constitutive active/androstane receptor is essential for liver tumor promotion by phenobarbital in mice. *Cancer Res.* 2004; 64:7197–7200. [PubMed: 15492232]
- Yamazaki Y, Kakizaki S, Horiguchi N, Sohara N, Sato K, Takagi H, Mori M, Negishi M. The role of the nuclear receptor constitutive androstane receptor in the pathogenesis of non-alcoholic steatohepatitis. *Gut.* 2007; 56:565–574. [PubMed: 16950832]
- Yu P, Wild DJ. Discovering Associations in Biomedical Datasets by Link-based Associative Classifier (LAC). *PLoS ONE.* 2012; 7:e51018. [PubMed: 23227228]
- Zhai Y, Wada T, Zhang B, Khadem S, Ren S, Kuruba R, Li S, Xie W. A functional cross-talk between liver X receptor-alpha and constitutive androstane receptor links lipogenesis and xenobiotic responses. *Mol Pharmacol.* 2010; 78:666–674. [PubMed: 20592274]

Highlights

Novel hCAR activators were identified by computational and biological approaches.

The role of hCAR in hepatic energy metabolism was examined.

hCAR activators repress gluconeogenesis but not lipogenesis and fatty acid synthesis.

Human and mouse CAR exhibit differential effects on energy metabolism.

Fig. 1.

Feature of computational models for hCAR. A: Surflex docking conformation of UM196 in hCAR-LBD. The hydrogen bonding interaction is shown in white. The amino acid residues are shown in green. B: Superimpose between template (CITCO) and ligand obtained through similarity search (UM104). CITCO is indicated with capped sticks, while UM104 with ball-and-stick. C: Pharmacophore models generated with known hCAR modulators. The structure in the figure is UM084, which exhibits the highest fit values to the pharmacophore models. The cyan, magenta, green, and orange spheres indicate hydrophobic, hydrophobic-aromatic, hydrogen-bond acceptor, and ring-aromatic features.

Fig. 2.

Activation of hCAR1+A in HepG2 cells. CYP2B6 luciferase reporter plasmid was co-transfected with hCAR1+A expression vector in HepG2 cells. Twenty-four hours after transfection, cells were treated with vehicle control (0.1% DMSO), positive control CITCO (1 μ M), or test compounds as described in Materials and Methods. (A) The pie chart depicts activation of hCAR1+A by 144 compounds in comparison to that of CITCO. The ten compounds that demonstrated activity above 100% of CITCO were evaluated at a single concentration of 10 μ M (B) or in a concentration-dependent manner (C-L). Luciferase activities were determined according to the manufacturer's instruction. The E_{max} and EC_{50} values were estimated using the Michaelis-Menten equation. Data represent mean $\pm$ SD (n = 3).

Fig. 3.

Induction of CYP2B6 mRNA and nuclear translocation of hCAR in HPH. Human hepatocytes prepared from donors (HL#45, HL#46, or HL#49, HL#50) were treated with vehicle control (0.1% DMSO), CITCO (1 μ M), and test compounds at 10 μ M each (A) or increasing concentrations (B) for 24 h. CYP2B6 mRNA levels were determined relative to the levels of GAPDH and normalized to the levels of vehicle control. Cellular localization of hCAR was tested in HPH (HL#64) infected with Ad/EYFP-hCAR as outlined in *Materials and Methods*. Images of YFP-tagged hCAR after treatment with vehicle control, PB (1 mM) or test compounds were visualized with a confocal microscope (C). Over 100 cells expressing Ad/EYFP-hCAR from each group were categorized into three groups: cytoplasmic (green), nuclear (red), and both cytoplasmic and nuclear (yellow) (D).

Fig. 4.

Repression of G6Pase and PEPCK expression in HPH. HPH from three donors (HL#49, HL#50, and HL#64) were treated with vehicle control (0.1% DMSO), CITCO (1 μ M), or increasing concentrations of UM104 and UM145 as described in *Materials and Methods*. Real-time PCR was used to determine the mRNA expression of G6Pase (A) and PEPCK (B). Each bar represents the mean $\pm$ SD (n = 3). **, $p < 0.01$. Protein expression levels of G6Pase (C) and PEPCK (D) were analyzed using Western blotting. **Densitometry was determined using the NIH ImageJ software.**

Fig. 5.

Expression of genes related to fatty acid syntheses and lipogenesis in HPH. Human hepatocytes isolated from donors (HL#49, HL#50, and HL#64) were treated with vehicle control (0.1% DMSO), CITCO (1 μM), or UM104 and UM145 at indicated concentrations for 24 or 72 h for analyses of mRNA and protein expression, respectively. Real-time PCR and western blotting were used to measure the expression of FAS (A), ACC-α (B), SREBP-1c (C), and SCD-1 (D). Each bar represents the mean ± SD (n = 3). **Densitometry was determined using the NIH ImageJ software.**

Fig. 6.

Production of glucose and triglyceride in HPH. Human hepatocytes isolated from three donors (HL#58, HL#59, and HL#69) were treated with vehicle control (0.1% DMSO), CITCO (1 μ M), or increasing concentrations of UM104 and UM145 for 72 h. Hepatic syntheses of glucose (A) and triglycerides (B) were measured as detailed in *Materials and Methods*. Each bar represents mean $\pm$ SD (n = 3), *: $P < 0.05$; **: $P < 0.01$.

Fig. 7.

Repression of genes associated with energy metabolism by TCPOBOP in mouse primary hepatocytes. Mouse primary hepatocytes isolated from C57BL/6J male mice were treated with vehicle control (0.1% DMSO) or TCPOBOP (0.1, 0.25, and 0.5 μM) for 24 h. Real-time PCR was used to analyse the expression of Cyp2b10 (A), G6Pase (B), Pepck (C), Fas (D), Acc-α (E), Srebp-1c (F), and Scd-1 (G). Each bar represents mean $\pm$ SD ($n = 3$), *: $P < 0.05$; **: $P < 0.01$.

Fig. 8. Schematic illustration of the role of hCAR and mCAR in gluconeogenesis, fatty acid syntheses, and lipogenesis.

Table 1

Virtual screening of the Specs database for hCAR activators

CAR-LBD Docking	Structure Similarity	Pharmacophore	Bayesian Modeling
Criteria (# of compounds filling requirement)			
	Similarity Score .638 PK11195 . 642 CITCO (110)	Pharmacophore Score > 0.9 (106)	Bayesian Score 4.09 (200)
FlexX > 9 (242)	FlexX 4 (94)	FlexX Score 4 (106)	
LogP 5 (157)	LogP 6 (61)	LogP 5 (69)	LogP 7 (199)
N _{rot} 10 (120)	N _{rot} 10 (61)	N _{rot} 10 (66)	N _{rot} 10 (170)
Non-PXR Activator MDLPublic Keys (38)	Non-PXR Activator MDLPublic Keys (61)	Non-PXR Activator MDLPublic Keys (27)	Non-PXR Activator MDLPublic Keys (49)

N_{rot} = number of rotational bonds