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Stable Overexpression of the Constitutive Androstane Receptor Reduces the Requirement for Culture with Dimethyl Sulfoxide for High Drug Metabolism in HepaRG Cells[S]

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ABSTRACT

Dimethylsulfoxide (DMSO) induces cellular differentiation and expression of drug metabolic enzymes in the human liver cell line HepaRG; however, DMSO also induces cell death and interferes with cellular activities. The aim of this study was to examine whether overexpression of the constitutive androstane receptor (CAR, NR1I3), the nuclear receptor controlling various drug metabolism genes, would sufficiently promote differentiation and drug metabolism in HepaRG cells, optionally without using DMSO. By stable lentiviral overexpression of CAR, HepaRG cultures were less affected by DMSO in total protein content and obtained increased resistance to acetaminophen- and amiodarone-induced cell death. Transcript levels of CAR target genes were significantly increased in HepaRG-CAR cultures without DMSO, resulting in increased activities of cytochrome P450 (P450) enzymes and bilirubin conjugation to levels equal or surpassing those of HepaRG cells cultured with DMSO. Unexpectedly, CAR overexpression also increased the activities of non-CAR target P450s, as well as albumin production. In combination with DMSO treatment, CAR overexpression further increased transcript levels and activities of CAR targets. Induction of CYP1A2 and CYP2B6 remained unchanged, whereas CYP3A4 was reduced. Moreover, the metabolism of low-clearance compounds warfarin and prednisolone was increased. In conclusion, CAR overexpression creates a more physiologically relevant environment for studies on hepatic (drug) metabolism and differentiation in HepaRG cells without the utilization of DMSO. DMSO still may be applied to accomplish higher drug metabolism, required for sensitive assays, such as low-clearance studies and identification of (rare) metabolites, whereas reduced total protein content after DMSO culture is diminished by CAR overexpression.

Introduction

The hepatic progenitor cell line HepaRG is a promising alternative to primary human hepatocytes (PHHs) as a human liver model for in vitro applications. When fully differentiated, the culture consists of a mixture of hepatocyte islands and cholangiocyte-like cells (Gripon et al., 2002). HepaRG cells possess high hepatic functionalities compared with PHHs and have been characterized as a useful model for drug metabolism studies (Andersson et al., 2012; Hoekstra et al., 2013). HepaRG cells are also proposed for predicting the human intrinsic clearance (CL_{int}) of many different drugs (Zanelli et al., 2012). Their long-term culture makes it possible to predict intrinsic clearance of slowly metabolizable compounds; however, one study showed that the rate and prediction of low-clearance substances in HepaRG cells are still suboptimal (Bonn et al., 2016). This limited value may relate to the relatively low expression of the constitutive androstane receptor (CAR, NR1I3).

CAR is a classic xenobiotic nuclear receptor that is predominantly expressed in hepatocytes; however, CAR is involved not only in drug metabolism but in recent years has also been characterized as an important endobiotic player by regulating a vast array of genes involved in lipid homeostasis, cell proliferation, metabolism of bilirubin and thyroid hormone, and in energy metabolism (Yan et al., 2015). In PHHs, CAR is present in the cytoplasm, where it translocates to the nucleus after ligand-dependent or -independent activation (Li et al., 2009; Mutoh et al., 2013). Many endogenous and exogenous compounds, including steroid hormones, bile acids, and drugs, have been identified as activators of CAR (Hernandez et al., 2009); however, CAR also has high transcriptional activity in the absence of any externally added ligand (Baes et al., 1994; Choi et al., 1997).

CAR was found to be a key regulator for the hepatic differentiation and maturation of human embryonic stem cells (ESCs) (Chen et al., 2013), which seems to be in agreement with a recent report in which CAR ligand–activated HepaRG CAR knockout cells were lacking expression in gene sets for embryonic development, cell proliferation,

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ABBREVIATIONS: ACN, acetonitrile; AST, aspartate aminotransferase; CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; CL_{int}, intrinsic clearance; P450, cytochrome P450 enzyme; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ESC, embryonic stem cell; FBS, fetal bovine serum; HEK, human embryonic kidney, HPLC; high-performance liquid chromatography; PBS, phosphate-buffered saline; PHH, primary human hepatocyte; UDGPA, uridine 5′diphospho-glucuronic acid.

and tumor development (Li et al., 2015). Moreover, a detailed analysis of different hepatic liver-like cells induced from ESCs and induced pluripotent stem cells identified genes regulated by CAR as severely downregulated compared with PHHs (Godoy et al., 2015).

Differentiated HepaRG cells seem to have normal CAR signaling since they mimic ligand-stimulated CAR trafficking from the cytoplasm to the nucleus as observed in PHHs (Jackson et al., 2016); however, CAR expression in fully differentiated HepaRG cells is 5- to 10-fold lower compared with PHH and requires the addition of dimethylsulfoxide (DMSO) during differentiation (Aninat et al., 2006). In HepaRG cells, the high expression of drug- metabolizing enzymes and xenobiotic receptors other than CAR is also dependent on the addition of DMSO (Aninat et al., 2006; Kanebratt and Andersson, 2008). DMSO is commonly used in hepatic cell cultures to induce and maintain liver-specific differentiation by unknown mechanisms (Higgins and Borenfreund, 1980; Paran et al., 2001; Sainz and Chisari, 2006); however, DMSO concentrations as low as 0.1% can have a significant negative effect on the activity of phase 1 drug-metabolizing enzymes (Chauret et al., 1998; Busby et al., 1999; Gonzalez-Perez et al., 2012). It is well known that long-term dosing and high concentrations of DMSO reduce cell viability (Galvao et al., 2014). Standard 2-week DMSO treatment of HepaRG monolayers reduces total cellular protein content by more than 50% and increases cell leakage 3- to 4-fold (Hoekstra et al., 2011). Moreover, DMSO reduces most hepatic functions of HepaRG cells unrelated to drug metabolism, particularly when cultured under optimal conditions, as supplied by a three-dimensional, oxygenated, and medium-perfused environment in a bioartificial liver (Nibourg et al., 2012).

We hypothesized that stable overexpression of CAR in HepaRG cells would increase their rate of differentiation and abolish the requirement of DMSO for induction of drug metabolism, thereby creating a more physiologically relevant environment for studies on hepatic (drug) metabolism. Indeed, we find that overexpression of CAR by itself can compensate for the addition of DMSO with regard to the expression and activity of phase 1 and 2 drug metabolism. Moreover, our results indicate that overexpression of CAR in combination with the addition of DMSO induces an altered, but improved, differentiation of HepaRG cells into a more liver-like phenotype.

Materials and Methods

Chemicals, Drugs, and Antibodies

The following primary antibodies were used for immunofluorescence staining: mouse monoclonal anti multidrug resistance–associated protein 2 (MRP2/ABCC2) (M_2III6) (Paulusma et al., 1996) and goat polyclonal anti-human albumin (A80-229A; Bethyl Laboratories, Montgomery, TX). The following secondary antibodies were used: goat polyclonal anti-mouse IgG Alexa Fluor 488 (A-11001; Thermo Fisher, Waltham, MA) and donkey polyclonal anti-goat IgG Alexa Fluor 488 (A-11055; Thermo Fisher). The following drugs were used to study induction rates: omeprazole (Cayman Chemical, Ann Harbor, MI), 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) (Santa Cruz Biotechnology, Dallas, TX), rifampicin (Sigma-Aldrich, St. Louis, MO). The following drugs were used to study their metabolism: dextromethorphan hydrobromide monohydrate (Santa Cruz Biotechnology), bupropion hydrochloride (Cayman Chemical), chlorzoxazone (Sigma-Aldrich), testosterone (Sigma-Aldrich), and tolbutamide (Sigma-Aldrich). The following drugs were used to study their clearance: warfarin, theophylline anhydrous, prednisolone (all from Sigma-Aldrich). The following drugs were used to study their toxicity: acetaminophen, amiodarone hydrochloride, indomethacin (all Sigma-Aldrich). All other, nonspecified chemicals and reagents were purchased from Sigma-Aldrich.

Cell Culture

All cultures were kept at 37°C in a humidified 5% CO_2 atmosphere. The cell line HepaRG (Biopredic International, Rennes, France) (Gripon et al., 2002) was cultured in William's E medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS, Lonza), 100 U/ml penicillin (Lonza), 100 μg/ml streptomycin (Lonza), 2 mM L-glutamine (Lonza), 50 μM hydrocortisone hemisuccinate, and 5 μg/ml insulin. For maintenance, the HepaRG cells were passaged every 2 weeks. Passaging was done by washing the cells twice with phosphate-buffered saline (PBS) and incubating them with a mixture of Accutase (Innovative Cell Technologies, San Diego, CA), Accumax (Innovative Cell Technologies), and PBS (2:1:1) at 37°C until detachment. The cells were then centrifuged for 5 minutes at 50*g* and seeded at a 1:5 split ratio in new culture flasks. For testing, the cells were fully matured over 28 days in 12- or 24-well culture plates with 1 ml and 0.5 ml medium per well, respectively. These cells were cultured for 14 days in normal HepaRG medium, after which they were either switched to HepaRG medium containing 1.7% DMSO or maintained on normal HepaRG medium for an additional 14 days as indicated in the results. For testing the effect of CAR overexpression, HepaRG and HepaRG-CAR cultures of similar passage ($\pm$1 passage) were compared between passages 15 and19.

Human embryonic kidney (HEK) 293T cells were obtained from ATCC (Manassas) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Generation of HepaRG-CAR

Plasmid Construction. A cDNA sequence containing the murine phosphoglycerate kinase (Pgk-1) promoter driving puromycin N-acetyl-transferase [from plasmid pHA263Pur/PGK*pur*, a gift from Dr. C. C. Paulusma, Academic Medical Center, Amsterdam, The Netherlands) (Robanus-Maandag et al., 1998] was cloned into the PPTPGKPRE backbone plasmid (pRRLcpptPGKmcsPRESsin, a gift from Dr J. E. Seppen, Academic Medical Center, Amsterdam, The Netherlands (Seppen et al., 2002), yielding the plasmid pBAL117. In detail, pHA263Pur was digested with ClaI and XhoI, Klenow blunted, and the 1.8-kb fragment was subcloned into an *Eco*RV digest of PPTPGKPRE to generate pBAL117 and verified by sequencing (BigDye Terminator, Thermo Fisher).

A cDNA sequence containing NR1I3 (CAR) (transcript variant 3, NCBI reference sequence NM_005122.4) was cloned into the pBAL117 plasmid yielding the plasmid pBAL117xCAR. In detail, the plasmid pEF-hCAR [a gift from Prof Dr R. Kim, Vanderbilt University School of Medicine, Nashville, Tennessee (Tirona et al., 2003)] was digested with SpeI and XbaI, and the 1-kb fragment was subcloned into a XbaI digest of pBAL117 to generate pBAL117x-CAR, which was verified by sequencing.

Lentiviral Vector Production. HEK 293T cells were transiently transfected with pBAL117xCAR using polyethylenimine and a third-generation lentiviral vector system (Dull et al., 1998; Zufferey et al., 1998). The DMEM culture medium was refreshed 4 hours after transfection. Medium containing viral particles (i.e., the viral DMEM) was harvested 44 hours after transfection, filtered through 0.45-μm filters (Millipore, Billerica, MA), and stored at $-$80°C.

Transduction. Low-passage (P12) HepaRG cells were seeded and 24 hours later transduced for 8 hours in a 1:1 mixture of viral DMEM:HepaRG medium containing 10 μg/ml DEAE-dextran. The polyclonal and stable CAR overexpressing HepaRG line was obtained by selection for puromycin resistance for 8 days with 2.5 μg/ml puromycin, starting from 1 day after transduction. The CAR-overexpressing HepaRG cell line was cultured as described for its parental cell line.

Ammonia Metabolism, Albumin Production, Cell Leakage, and Total Protein

The cultures were washed twice with PBS and then exposed to phenol-red free HepaRG culture medium with 1.5 mM $^{15}NH_4Cl$ (Sigma), 2.27 mM *D*-galactose (Sigma), and 2 mM L-lactate (Sigma). Medium samples were taken after 45 minutes, 8 hours, and 24 hours of incubation. Cells were assessed for total protein content after 24 hours of incubation. Production or elimination rates were established by calculating the concentration changes of different compounds, as indicated as follows, in the test media in time and corrected for protein content per well.

Ammonia concentrations in the test media were determined with the Ammonia (Rapid) kit (Megazyme, Bray, Ireland) according to manufacturer's instructions. Albumin protein levels in the test media were assessed with the human serum albumin duoset enzyme-linked immunosorbent assay (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Cell leakage was established by spectrophotometrical determination of aspartate aminotransferase (AST) levels of test medium samples and diluted cell lysates using a P800 Roche Diagnostics analyzer (Roche, Basel, Switzerland). AST levels were expressed

relative to total cellular AST content (Nibourg et al., 2010). Protein levels of cell cultures were assessed with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions after lysis in 0.2 M NaOH for 1 hour at 37°C.

ATP

ATP levels were assessed with the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

WST-1 Assay

Relative cellular NADH levels were assessed with the WST-1 assay. The WST-1 assay is based on the extracellular reduction of a tetrazolium dye via transmembrane electron transport (Berridge et al., 2005). NADH, the electron donor, is produced mainly by the mitochondrial tricarboxylic acid cycle. The assay was performed by washing cells once with PBS and then incubating for 15 minutes in Cell Proliferation Reagent WST-1 (Roche) 20× diluted into phenol-red free HepaRG culture medium. Supernatants were transferred to a clear 96-well plate; absorbance at $\lambda = 450$nm, subtracted by absorbance at $\lambda = 620$ nm, was measured on a Synergy HT (BioTek, Winooski, VT) plate reader.

Immunofluorescence

Cells were washed 3× with cold PBS after they were fixed with 10% formalin (VWR, Radnor, PA) for 1 hour at 4°C. The cells were permeabilized with 0.3% Triton-X 100 (Bio-Rad) at 4°C for 15 minutes. Cells were then blocked with 10% FBS in PBS on ice for 1 hour and incubated overnight at 4°C with primary antibody diluted 1:200 in PBS. The cells were washed 3x with cold PBS, incubated 2 hours at 4°C with secondary antibody Alexa Fluor 488 (Thermo Fisher) diluted 1:1000 in PBS and washed again 3x with cold PBS before incubation with DAPI-containing Vectashield (Vector Laboratories, Burlingame, CA). Immunofluorescence images were taken on a DMi8 microscope (Leica Microsystems, Wetzlar, Germany), phase-contrast images were taken on a DMIL microscope (Leica Microsystems).

Transcript Levels

Total RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA using a mix of 18S rRNA reverse primer, gene-specific reverse primers, and SuperScript III reverse transcriptase (RT; Invitrogen, Waltham, MA) as described (Hoekstra et al., 2005), with the modification of using gene-specific antisense primers for the RT reaction that are also used in the real-time quantitative PCR (RT-qPCR) reaction instead of separate, downstream RT primers. RT-qPCR measurements were performed on a Lightcycler 480 (Roche) with Sensifast SYBR Green master mix (Bioline, London, UK), as described (Hoekstra et al., 2005). Expression levels of genes of interest were quantified using the LinregPCR program (Ruijter et al., 2009) and normalized for 18S rRNA levels determined on 1000× diluted templates. Normalized mRNA levels are expressed as a percentage of the mean mRNA levels of two human liver samples normalized to 18S rRNA. The human liver samples were obtained from two female patients aged 40 and 41 years with liver adenoma and no elevated liver damage. These patients were not receiving medication and had no history of drug or alcohol abuse. The samples were taken after obtaining written informed consent, and the procedure was approved by the Academic Medical Center's committee on human experimentation (protocol number 03/024). For a list of PCR-primers used, see Supplemental Table 1.

Bilirubin Glucuronidation

Bilirubin glucuronidation was determined in medium and cell samples. First, cells were incubated in FBS-free and phenol red-free HepaRG medium containing 10 μM bilirubin (mixed isomers, B-4126, Sigma) for 0, 1, or 4 hours. At the 0-hour time point, the cells were incubated for 5 seconds to correct for nonspecific binding of bilirubin to the cells and the culture plate. Medium samples were immediately stored at −80°C. For measurements of intracellular bilirubin glucuronidation, bilirubin-exposed cells were scraped into ice-cold PBS and pestle-homogenized on ice (30×, tight pestle). Samples of 25 μl containing 20 μg of protein were incubated for 1 hour at 37°C together with 75 μl of bilirubin incubation solution (50 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 1 mM D-saccharic acid 1,4-lactone, 50 μM bilirubin, 3.5 mM uridine 5′diphospho-glucuronic acid (UDGPA), 2.5 mg/ml 1,2-dioleoyl-sn-glycerol-3-phosphocholine). The reaction was stopped by incubating in 2 volumes of methanol for 10 minutes on ice. The cell homogenate samples were stored at −80°C.

Before analysis, the medium and cell homogenate samples were thawed on ice, deproteinized with 2 volumes of methanol, and centrifuged for 5 minutes at 20,000*g* at 4°C. Supernatants were analyzed for bilirubin and bilirubin-conjugates by high-performance liquid chromatography (HPLC). Reverse-phase HPLC detection of bilirubin and its conjugates was adapted from a method described previously (Spivak and Carey, 1985). Briefly, 100 μl of methanol deproteinized sample was applied to a Pursuit C18, 5 μm, 10-cm HPLC column (Varian, Palo Alto, CA). Starting eluent consisted of 50% methanol/50% ammonium acetate (1%, pH 4.5), followed by a linear gradient to 100% methanol in 20 minutes. Detection of bilirubin was performed at λ = 450 nm. Quantification of biconjugated, monoconjugated, and unconjugated bilirubin was done by using a calibration curve of unconjugated bilirubin.

Cytochrome P450 Induction

Transcript levels of cytochrome P450 (P450) genes were compared in cultures with and without induction. Cells were induced with omeprazole (40 μM, stock solution dissolved in DMSO), CITCO (1 μM, stock solution dissolved in DMSO) or rifampicin (4 μM, stock solution dissolved in DMSO) with a final concentration of 0.1% DMSO in FBS-free and phenol red–free HepaRG medium for 24 hours, after which total RNA was isolated immediately.

P450 Activity

Cells were incubated in FBS-free and phenol red–free HepaRG medium with testosterone for 1 hour (200 μM, 200 mM stock in ethanol) or the following drugs for 5 hours: bupropion (100 μM, 50 mM stock dissolved in ethanol), phenacetin (200 μM, 100 mM stock in ethanol), dextromethorphan (40 μM, 40 mM stock in water), chlorzoxazone (100 μM, 100 mM stock in DMSO), or tolbutamide (100 μM, 100 mM stock in ethanol). The medium was immediately harvested and stored at −80°C before analysis.

Frozen samples were thawed at room temperature, diluted with a solution containing metabolites with stable isotopes, or diluted with 0.1% formic acid in ultrapure water (for 6β-OH-testosterone and OH-chlorzoxazone). P450 metabolites were quantified by HPLC tandem mass spectrometry. The system consisted of an API3200 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) working in electrospray ionization mode, interfaced with a 1200SL HPLC (Agilent, Santa Clara, CA). Chromatography was performed at 70°C with 10 μl injected into a Zorbax Eclipse XDB C18 column (Agilent, 50 mm × 4.6 mm, 1.8 μm particle size), at a flow rate of 1.5 ml/min. The mobile phase was 0.1% formic acid in ultrapure water (A) and 0.3% formic acid in a mixture of 50% methanol and 50% acetonitrile (ACN) (B). The proportion of the mobile phase B was increased linearly from 0% to 98% in 3 minutes, and then the column was allowed to re-equilibrate at the initial conditions. The total run time was 5 minutes. For 6β-OH-testosterone, the mobile phase was ammonium acetate 5 mM in ultrapure water (A) and 0.3% formic acid in a mixture of 50% methanol and 50% ACN (B). The proportion of the mobile phase B was increased linearly from 30% to 37% in 2.8 minutes, and then, after 1 minute at 99% of B, the column was allowed to re-equilibrate at the initial conditions. The total run time was 5 minutes. For OH-chlorzoxazone, the mobile phase was 0.01% formic acid in ultrapure water (A) and ACN (B). The proportion of the mobile phase B was increased linearly from 10% to 50% in 1.2 minutes, and then the column was flushed with 95% of the mobile phase B and allowed to re-equilibrate at the initial conditions. The total run time was 3.0 minutes. The column eluent was split to an electrospray ionization interface, operating at 650°C in both modes operating in multiple reaction monitoring mode.

Drug-Induced Toxicity

Cells were incubated in phenol red–free HepaRG medium with amiodarone (stock solution dissolved in DMSO, total concentration of 0.2% DMSO during incubation), acetaminophen (dissolved directly into culture medium), indomethacin (stock solution dissolved in DMSO, total concentration of 1% DMSO during incubation), or dextromethorphan (stock solution dissolved in DMSO, total concentration of 0.1% DMSO during incubation) with the indicated fold C_{max} (Xu et al., 2008) for 24 hours, after which ATP levels were assessed with the

CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. C_{max} is defined as the therapeutically active average plasma maximum concentration. TC50 is defined as the toxic concentration for ATP levels for 50% of the population and was calculated using Prism 6.07 (GraphPad Software, San Diego, CA) after performing a nonlinear regression fit of the data points using the log (inhibitor) versus response algorithm.

Elimination of Low-Clearance Compounds

Cells were incubated in a 24-well plate in 500 μl/well FBS-free and phenol red–free HepaRG medium with 1 μM warfarin (stock solution dissolved in DMSO), theophylline (stock solution dissolved in DMSO), or prednisolone (stock solution dissolved in DMSO), with a final concentration of 0.01% DMSO for 1, 4, 8, or 24 hours. At the 0-hour time point, the cells were incubated for 5 seconds to correct for nonspecific binding of the added compounds to the cells and the culture plate. Medium samples of the indicated time points were immediately frozen at −80°C. Before HPLC analysis, the samples were thawed on ice and deproteinized with the addition of 4 volumes of ACN (Biosolve, Valkenswaard, The Netherlands) (for prednisolone and theophylline), vacuum-evaporated, and dissolved in water. Warfarin samples were instead deproteinized with 2 volumes of methanol and spun down for 5 minutes at 20,000*g* at 4°C. Supernatants were used for HPLC analysis.

Reverse-phase HPLC detection was done as follows. Deproteinized samples (100 μl for prednisolone and theophylline, 30 μl for warfarin) were applied to a Hypersil C18, 3 μm, 15 cm HPLC column (Thermo Scientific). Starting eluent consisted of 6.8 mM ammoniumformate (pH 3.9), followed by several steps of linear gradients to different concentrations of ACN. For prednisolone, 0 minute 0% ACN, 1 minute 0% ACN, 7 minutes 30% ACN, 17 minutes 36% ACN, 18 minutes 60% ACN, 19 minutes 60% ACN, 19.5 minutes 0% ACN, and 25 minutes 0% ACN. For theophylline and warfarin, 0 minute 0% ACN, 1 minute 0% ACN, 15 minutes 60% ACN, 19 minutes 60% ACN, 19.5 minutes 0% ACN, and 25 minutes 0% ACN. Detection of prednisolone was performed at λ_{abs} = 254 nm, theophylline at λ_{abs} = 270 nm, warfarin at λ_{exc} = 310 nm/λ_{em} = 390 nm. Quantification was done by using calibration curves of prednisolone, theophylline, or warfarin.

In vitro CL_{int} was calculated from compound loss according to using eq. 1 and 0.45×10^6 cells/well:

$$CL_{int}\left(\mu l \cdot min^{-1} \cdot 10^6 cells^{-1}\right) = \frac{V \times 0.693}{t_{1/2}} \quad (1)$$

where

$$V\left(\mu l \cdot 10^6 cells\right) = \frac{\text{incubation volume}(\mu l)}{\text{number of cells in incubation}(\times 10^6)}$$

$$\text{Half life}\left(t_{1/2}\right)(\text{min}) = \frac{0.693}{k}$$

$$\text{Elimination rate constant}(k) = -\text{slope of ln}(\%\ \text{drug remaining vs time})$$

Statistics

Data are expressed as mean ± S.D. and were calculated with Prism 6.07. Statistical significance was determined by performing a Student's *t* test (when comparing two sets of data) or two-way analysis of variance with Tukey's post hoc correction for multiple testing (when comparing more than two sets of data). Graphs were plotted with Prism 6.07.

Results

Overexpression of CAR in HepaRG Cells Alters Morphology during Culture with DMSO. To investigate the role of CAR in the function and differentiation of HepaRG cells, we generated a stable cell line overexpressing CAR in HepaRG cells via lentiviral transduction,

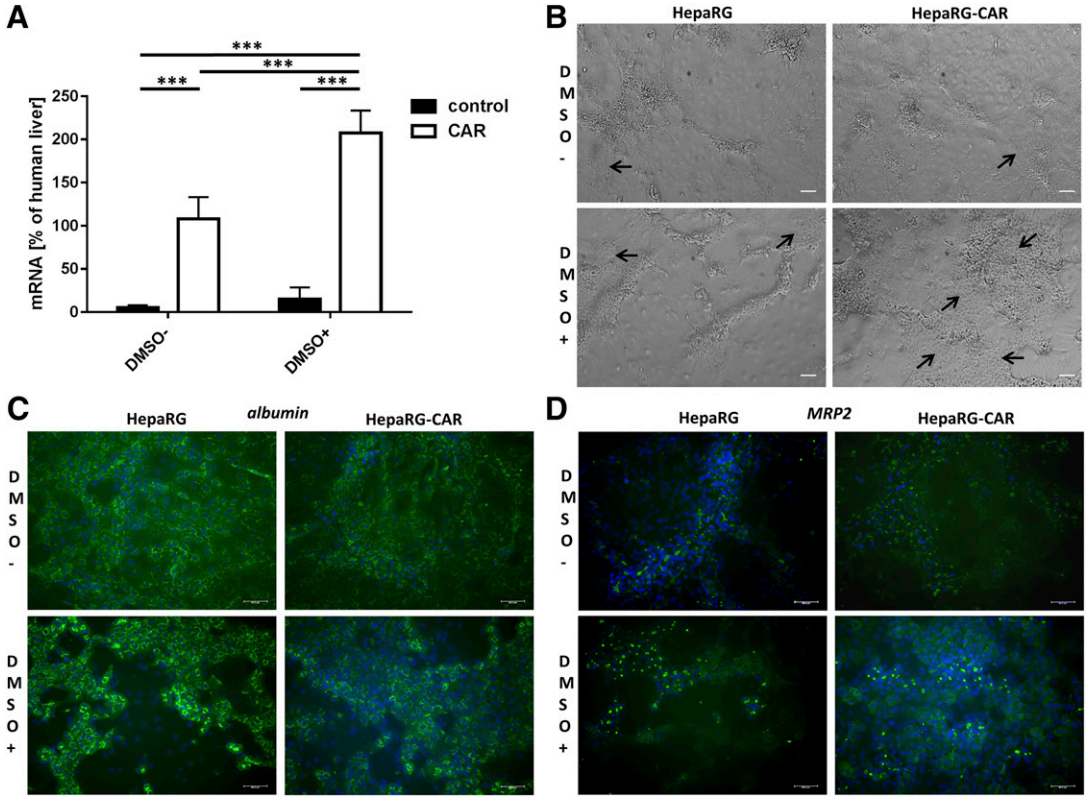

Fig. 1. CAR overexpression and morphology of DMSO-treated and -untreated HepaRG cultures. (A) Relative *CAR* transcript levels (average of six independent experiments of at least three separate samples in each group). ***$P < 0.001$. (B) Phase-contrast images. Black arrows indicate inter-island hepatocyte-like cells. Bar = 60 μm. (C and D) Immunofluorescence images. Cells were stained for albumin (C, green) or MRP2 (D, green), and nuclei (blue). Bar = 60 μm.

which we named HepaRG-CAR. Compared with the average of two human liver samples, CAR mRNA levels were increased from 5.3% in control HepaRG cells to 108% in HepaRG-CAR cells, both cultured without DMSO (Fig. 1A). Increased expression of CAR was stable over at least seven passages (not shown). Culture with DMSO increased CAR mRNA levels to 15% of human liver in control HepaRG cells and to 208% in HepaRG-CAR cells (Fig. 1A). HepaRG cells normally differentiate into a 1:1 ratio of hepatocyte islands and cholangiocyte-like cells (Gripon et al., 2002). Surprisingly, when cultured with DMSO, HepaRG-CAR cultures showed an altered morphology in which most of the cholangiocyte-like cells were absent and an increase in a subset of hepatocyte-like cells between the hepatocyte islands (inter-island hepatocyte-like cells) (Fig. 1B). These inter-island hepatocyte-like cells are also sparsely present around hepatocyte islands in normal HepaRG cells and are likely not fully differentiated HepaRG hepatocytes. Culture of HepaRG-CAR cells without DMSO resulted in no visible morphologic alterations (Fig. 1B) compared with the parental HepaRG cells.

To determine the level of differentiation of this new subset of hepatocyte-like cells in HepaRG-CAR, we examined the expression of albumin and the apical multidrug transporter MRP2 (ABCC2). Albumin was intensely stained in the cytoplasm and particularly peri-nuclear in both hepatocyte islands and cholangiocytes in control and HepaRG-CAR cells cultured without DMSO (Fig. 1C). In DMSO-treated control and HepaRG-CAR cultures hepatocyte islands were positive; however, cholangiocyte-like cells were negative. In addition, inter-island hepatocytes in the HepaRG-CAR cultures expressed albumin (Fig. 1C). MRP2, a marker for hepatic polarization, was strongly stained in canalicular structures and channels in hepatocyte islands of both cell lines cultured with or without DMSO, although more intense staining could be observed in DMSO cultures (Fig. 1D). The inter-island hepatocyte-like cells in the DMSO-treated HepaRG-CAR cultures did not form MRP2-stained canaliculi. Diffuse intracellular staining of MRP2 was also clearly visible in DMSO-treated cells and in HepaRG-CAR cells cultured without DMSO (Fig. 1D).

Increased Expression of CAR Target Genes in HepaRG-CAR Cells. We determined the transcript levels of a panel of genes including CAR target genes in CAR-overexpressing HepaRG and control cells (Table 1). Transcript levels of typical CAR targets were increased by CAR overexpression to different degrees, with most pronounced effects after treatment with DMSO: *CYP2B6* (26×), *CYP2C8* (2.8×), *CYP2C9* (2.3×), *CYP2C19* (3.0×), *CYP3A4* (2.7×), *UGT1A1* (6.2×), *MRP2* (1.5−), *ALAS1* (2.4×), and *cytochrome P450 reductase* (*POR*) (2.2×), however, *CYP1A2* transcript level was unchanged. Transcript levels of non-CAR target cytochromes were unaltered (*CYP2D6*) or decreased (*CYP2E1*: 9.2×). Other tested hepatic genes were unaffected by CAR overexpression, including *OATP1B1*, *OATP2B1*, *NTCP*, *MRP3*, *AOX1*, *HNF4α*, *FXR*, *AHR*, and *PXR*.

Induction Rates of CYP1A2 and CYP2B6, but not CYP3A4, Are Unaffected by Overexpression of CAR in HepaRG Cells. To determine whether the induction of target CYPs of the major xenobiotic

TABLE 1

Effect of CAR overexpression and DMSO treatment on transcript levels of HepaRG cells

Transcript levels of HepaRG ± CAR cells cultured with or without DMSO as the percentage of the mean transcript levels of two human liver samples. Transcript levels were assessed in one to six independent experiments of at least three separate samples in each group. The number of experiments is indicated in parentheses after the gene name.

Gene	Description	HepaRG (DMSO−)	HepaRG-CAR (DMSO−)	HepaRG (DMSO+)	HepaRG-CAR (DMSO+)
	Transcription factor				
CAR (6)	Constitutive androstane receptor	5.3 (±2.7)	108 (±25)***	15 (±14)******	208 (±26)***.******.###
PXR (6)	Pregnane X receptor	20 (±10)	18 (±11)	37 (±23)*.*****	25 (±16)
AHR (4)	Aryl hydrocarbon receptor	161 (±37)	133 (±23)	287 (±112)*.*****	308 (±123)**.******
HNF4α (3)	Hepatocyte nuclear factor 4 α	176 (±70)	140 (±76)	172 (±62)	209 (±80)
FXR (3)	Farnesoid X receptor	123 (±27)	89 (±5.0)*	88 (±10)**	94 (±21)*
	Phase 1 drug metabolism				
CYP1A2 (5)	Cytochrome P450 1A2	0.04 (±0.02)	0.04 (±0.05)	0.59 (±0.35)***. ******	0.51 (±0.43)**.*****
CYP2A6 (2)	Cytochrome P450 2A6	4.5 (±1.7)	10 (±4.3)	5.9 (±2.1)	7.1 (±2.5)
CYP2B6 (5)	Cytochrome P450 2B6	2.5 (±1.3)	136 (±93)	33 (±20)	874 (±548)***.******.###
CYP2C8 (2)	Cytochrome P450 2C8	12 (±0.9)	22 (±1.2)***	9.8 (±2.9)******	28 (±2.7)***.****.###
CYP2C9 (3)	Cytochrome P450 2C9	8.9 (±2.3)	18 (±8.9)	35 (±10)***	80 (±19)***.###
CYP2C19 (1)	Cytochrome P450 2C19	21 (±1.6)	46 (±6.2)**	32 (±5.4)	96 (±9.1)***.******.###
CYP2D6 (2)	Cytochrome P450 2D6	1.9 (±0.3)	1.8 (±0.3)	4.4 (±3.3)	4.3 (±3.4)
CYP2E1 (3)	Cytochrome P450 2E1	0.12 (±0.04)	0.06 (±0.03)	0.83 (±0.31) ***.******	0.09 (±0.02)###
CYP3A4 (5)	Cytochrome P450 3A4	3.7 (±4.4)	16 (±24)	58 (±46)	160 (±101) ***.******.###
AOX1 (4)	Aldehyde oxidase 1	63 (±13)	58 (±8.0)	111 (±15)***.******	128 (±28) ***.******
POR (6)	Cytochrome P450 reductase	54 (±13)	78 (±17)	81 (±23)*	180 (±38) ***.******.###
	Phase 2 drug metabolism				
UGT1A1 (3)	Uridine diphosphate glucuronosyltransferase 1 family. polypeptide A1	37 (±14)	135 (±76)	259 (±135)	1595 (±866) ***.******.###
	Membrane transporter				
NTCP (4)	Na^+-taurocholate cotransporting polypeptide	6.6 (±3.0)	5.5 (±3.4)	12 (±5.4)****	13 (±7.2)*.*****
OATP1B1 (3)	Organic anion-transporting polypeptide 1B1	5.3 (±1.9)	4.0 (±2.1)	4.8 (±1.9)	4.7 (±3.0)
OATP2B1 (2)	Organic anion-transporting polypeptide 2B1	83 (±15)	71 (±10)	59 (±13)*	65 (±14)
MRP2 (2)	Multidrug resistance-associated protein 2	77 (±21)	117 (±42)	145 (±61)*	216 (±34)***.*****.#
MRP3 (1)	Multidrug resistance-associated protein 3	335 (±54)	328 (±58)	323 (±56)	268 (±33)
	Blood protein				
ALB (1)	Albumin	47 (±6.7)	63 (±8.2)	41 (±7.7)****	31 (±2.9)*****
	Nitrogen metabolism				
ARG1 (1)	Arginase 1	4.9 (±0.4)	4.4 (±1.0)	4.9 (±0.9)	0.8 (±0.0) ***.******.###
ARG2 (1)	Arginase 2	385 (±79)	172 (±26)*	654 (±62)**.******	495 (±90)*****
CPS1 (1)	Carbamoyl-phosphate synthase 1	9.0 (±0.9)	11 (±0.7)**	2.7 (±0.4) ***.******	0.7 (±0.1)***.******.#
	Heme synthesis				
ALAS1 (2)	5′-Aminolevulinate synthase 1	23 (±5.0)	34 (±5.9)*	30 (±4.2)	73 (±7.8)***.******.###

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus HepaRG (DMSO−); ****$P < 0.05$; *****$P < 0.01$; ******$P < 0.001$ versus HepaRG-CAR (DMSO−); #$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$ versus HepaRG (DMSO+).

nuclear receptors would be preserved by overexpression of CAR, we investigated the transcript levels of typical target P450s of *AHR*, *CAR*, and *PXR* after a 24-hour incubation with specific inducers: omeprazole for *AHR*, CITCO for *CAR*, and rifampicin for *PXR*. Addition of omeprazole and CITCO resulted in a similar fold induction of AHR target gene *CYP1A2* (93-134×) and CAR target gene *CYP2B6* (4.2–6.1×), respectively, in control and HepaRG-CAR cells, independent of DMSO treatment (Fig. 2A). Interestingly, the addition of rifampicin resulted in a reduced fold induction of *CYP3A4* in HepaRG-CAR cells after DMSO treatment (3.6×) compared with control cells (10×) (Fig. 2A). After induction, total transcript levels of *CYP1A2* and *CYP2B6* remained higher in HepaRG-CAR cells compared with controls, up to 23% (*CYP1A2*) and 430% (*CYP2B6*) of human liver, whereas *CYP3A4* expression was equal in all groups (Fig. 2B). Because of the omission of FBS from the culture medium during the induction experiments, there is a discrepancy in P450 transcript levels with those presented in Table 1.

Increased Activity of CAR- and Non-CAR target P450s in HepaRG-CAR Cells. Next, we assessed whether increased transcript levels of canonical CAR target P450s also resulted in increased enzyme activity in HepaRG-CAR cells versus control cells. Indeed, for all four tested CAR P450 targets, we observed increased activity in HepaRG-CAR compared with control: CYP1A2 (5.3× without DMSO, 4.2× with DMSO), CYP2B6 (54× without DMSO, 21× with DMSO), CYP2C9 (3.4× without DMSO, 2.2× with DMSO), and CYP3A4 (7.7× without DMSO, 2.6× with DMSO) (Fig. 3). Unexpectedly, enzyme activities of non-CAR target P450s were also increased by CAR overexpression, despite unchanged or reduced transcript levels. Activities of CYP2D6 (4.1× without DMSO, 4.1× with DMSO) and CYP2E1 (3.8× without DMSO, 5.7× with DMSO) were increased in HepaRG-CAR cells compared with control cells (Fig. 3).

Increased UGT1A1 Activity in HepaRG-CAR Cells. To determine whether phase 2 drug metabolism was also increased, we examined the activity of UGT1A1, the enzyme for bilirubin glucuronidation, by the accumulation of bilirubin glucuronides in the culture medium after bilirubin loading. Despite increased transcript levels of *UGT1A1* upon CAR overexpression in HepaRG cells, we did not observe an increased accumulation of bilirubin glucuronides in the culture medium (Fig. 4A). Unfortunately, we were unable to measure bilirubin in the cellular fractions; however, when determined in cell homogenates, to which a nonlimiting amount of UDGPA cofactor was added, bilirubin monoglucoronidation and diglucuronidation levels were increased in homogenates of HepaRG-CAR cells compared with homogenates of control cells, respectively, by 5.3× and 8.3× in cells cultured without DMSO and by, respectively, 7.7× and 12.8× in cells cultured with DMSO (Fig. 4B). This finding indicates that CAR overexpression increases UGT1A1 activity; however, UDPGA levels or transport of bilirubin over the plasma membrane seem to limit the accumulation of bilirubin conjugates in the culture medium.

Decreased Toxicity of Acetaminophen and Amiodarone in HepaRG-CAR Cells. The increased expression and activity of CYPs in HepaRG-CAR cells should lead to faster metabolism and clearance of toxic concentrations of drugs. We treated DMSO-cultured HepaRG-CAR cells and controls with several hepatotoxic drugs—acetaminophen, amiodarone, and indomethacin—and dextromethorphan was included as a nonhepatotoxic control (Ramaiahgari et al., 2014). Indeed, TC50 values were significantly higher in HepaRG-CAR cells treated with

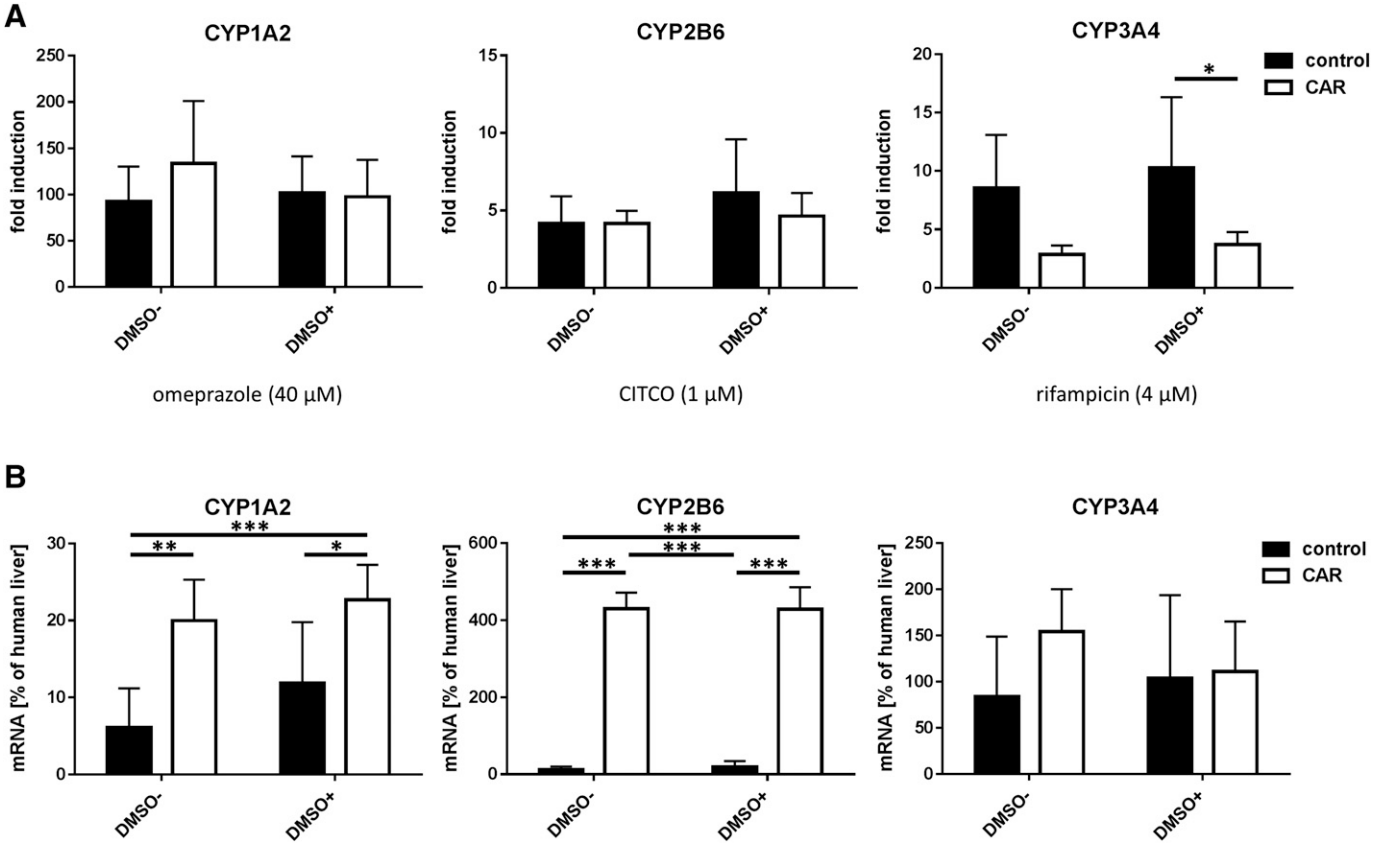

Fig. 2. The effect of CAR overexpression and DMSO treatment on the induction of *CYP1A2, CYP2B6,* and *CYP3A4* in HepaRG cells. (A) Fold increase in *CYP1A2, CYP2B6,* or *CYP3A4* transcript levels in HepaRG ± CAR cells after treatment with omeprazole, CITCO, or rifampicin, respectively. (B) Relative *CYP1A2*, *CYP2B6*, or *CYP3A4* transcript levels in HepaRG ± CAR cells after treatment with omeprazole, CITCO, or rifampicin, respectively. All figures represent the average of two independent experiments of at least three separate samples in each group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

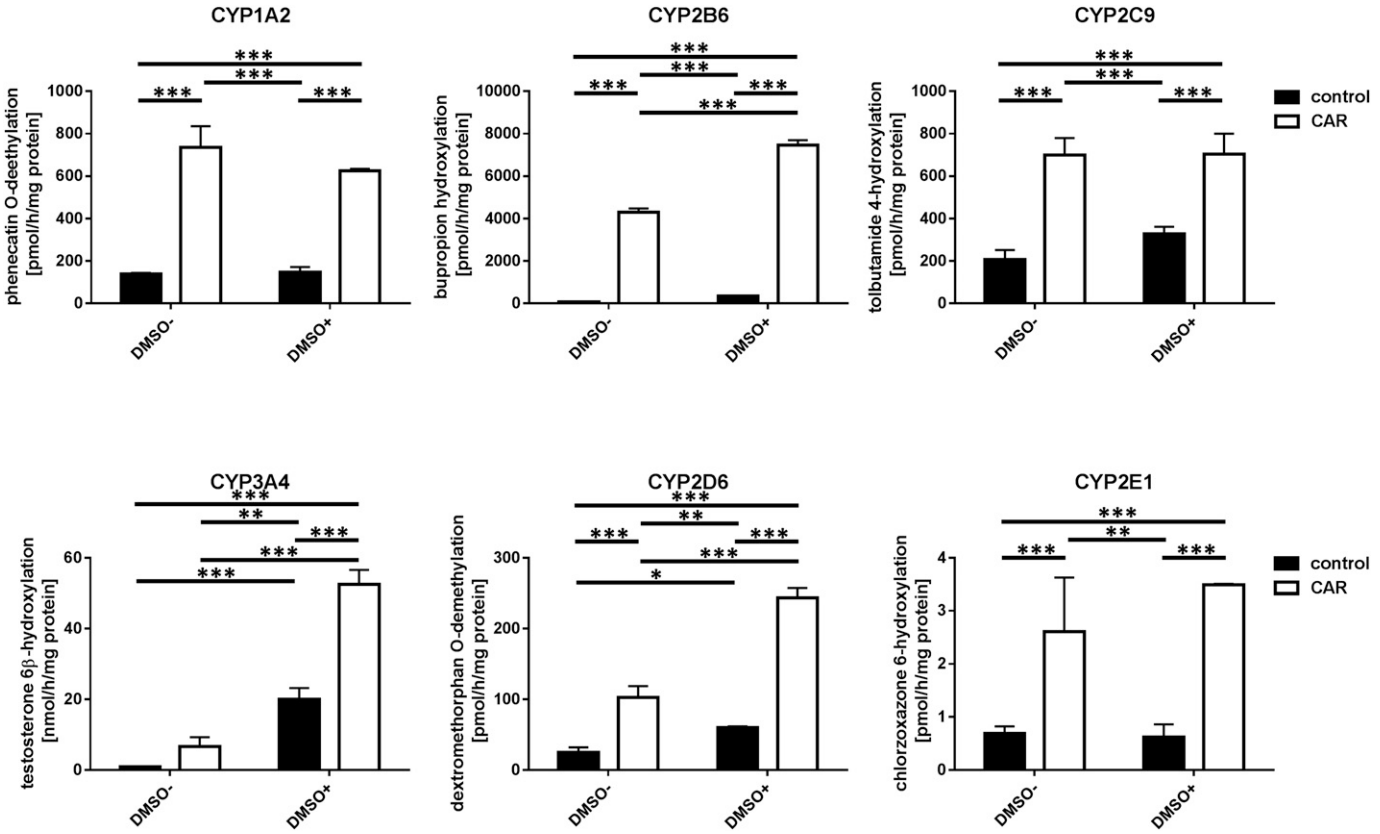

Fig. 3. The effect of CAR overexpression and DMSO treatment on CYP activity in HepaRG cells. CYP1A2, CYP2B6, CYP2C9, CYP3A4, CYP2D6, and CYP2E1 activities in HepaRG ± CAR cells by determination of P450-specific metabolite production. All figures represent one experiment of at least three separate samples in each group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

acetaminophen or amiodarone compared with control cells (Fig. 5; Table 2). The TC50 values for indomethacin were not significantly different between groups.

Increased Clearance of Warfarin and Prednisolone in HepaRG-CAR Cells. Since HepaRG-CAR cells have an increased rate of drug metabolism, we assessed their capability to clear three slowly metabolizable compounds: warfarin, theophylline, and prednisolone. Both HepaRG and HepaRG-CAR cells showed a linear clearance of warfarin during the first 24–48 hours; this rate declined in a nonlinear fashion afterward (Fig. 6A). Therefore, we assessed clearance of all three

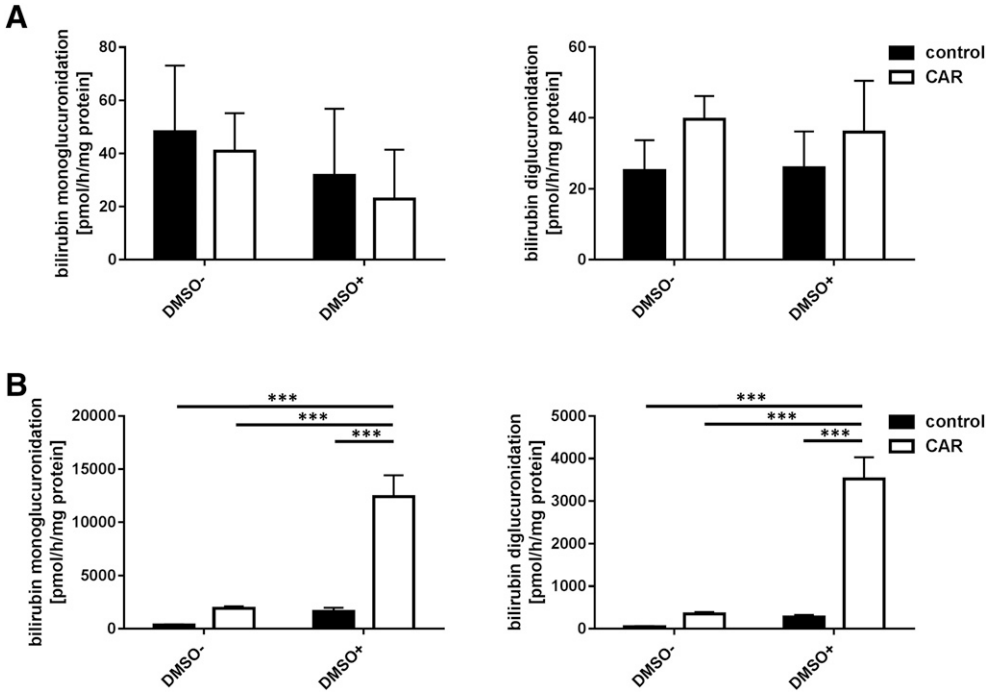

Fig. 4. The effect of CAR overexpression and DMSO treatment on bilirubin glucuronidation activity of HepaRG cells. (A) Bilirubin monoglucoronidation and diglucuronidation activity of intact HepaRG ± CAR cells, measured in culture medium. (B) Bilirubin monoglucuronidation and diglucuronidation activity in cell homogenates of HepaRG ± CAR cells. All figures represent the average of two independent experiments of at least three separate samples in each group. $***P < 0.001$.

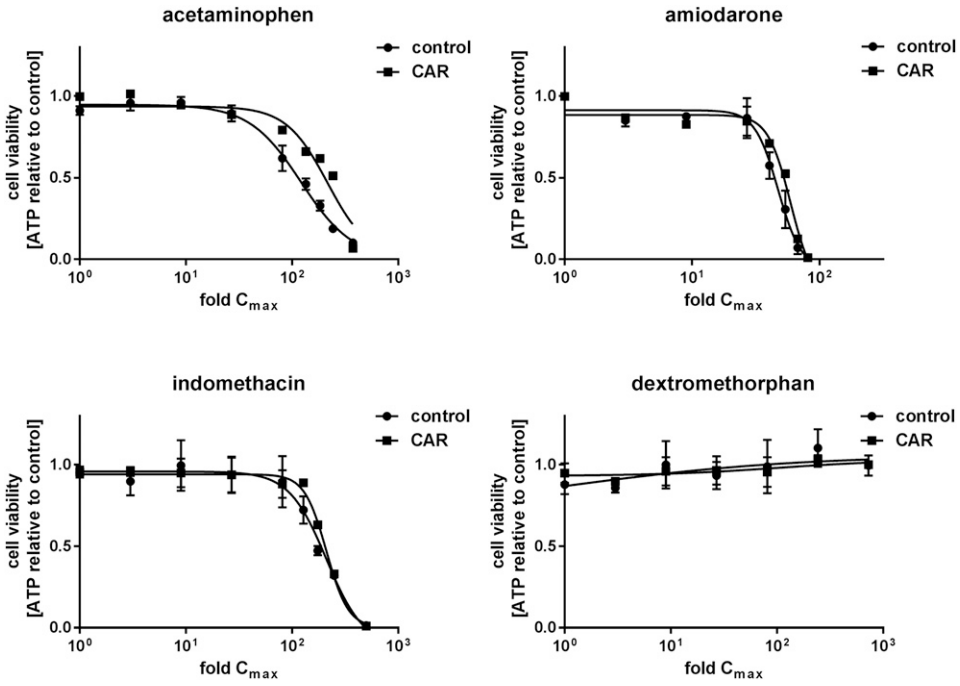

Fig. 5. The effect of CAR overexpression on acetaminophen-, amiodarone-, indomethacin-, and dextromethorphan-induced toxicity in HepaRG cells cultured with DMSO. Total relative ATP levels of HepaRG ± CAR cells after 24 hours' treatment with increasing concentrations of the indicated compounds. All figures represent the average of two or three independent experiments of three separate samples in each group.

compounds from $t = 0$–24 hours. Both warfarin and prednisolone were cleared at an increased rate in HepaRG-CAR cultured with DMSO compared with all other conditions (Fig. 6B; Table 3). Similar to other reports in the literature, we could not reliably observe any clearance of theophylline (Bonn et al., 2016).

Limited or No Effect on Albumin Synthesis and Ammonia Elimination. To determine the effect of CAR overexpression on hepatic activities expected to be unrelated to CAR, we assessed albumin production and ammonia elimination for 24 hours in DMSO-free medium. Surprisingly, CAR overexpression in HepaRG cultures increased albumin synthesis by 49% in the absence of DMSO; however, in DMSO-treated cultures, CAR overexpression did not change albumin synthesis compared with DMSO-treated controls (Fig. 7A). Ammonia elimination was unchanged after CAR overexpression in both DMSO-untreated and -treated HepaRG cultures (Fig. 7B).

Increased Viability of HepaRG-CAR Cells during Culture with DMSO. The addition of DMSO induced cell death in control HepaRG cells, as judged from the 40% reduction of protein content, similar to previously reported (Hoekstra et al., 2011) (Fig. 8A). HepaRG-CAR cells were less affected by DMSO and showed only a 19% reduction in total protein content compared with untreated cells (Fig. 8A). Cellular integrity, assessed by AST leakage over 24-hour culture in DMSO-free medium, and total ATP content was similar in control HepaRG and HepaRG-CAR cells (Fig. 8, B and C). Interestingly, WST-1 activity, as a marker for cellular NAD(P)H levels was 40% increased by CAR overexpression (Fig. 8D).

Discussion

In this article, we show the pleiotropic effects of CAR overexpression in HepaRG cells. CAR overexpression increases activities of CAR and non-CAR targets in drug metabolism and consequently increases the sensitivity of toxicity assays and metabolism of low-clearance drugs. In addition, in DMSO-free cultures, CAR increases albumin production. Similar to normal HepaRG cells, culture with DMSO increases hepatic drug metabolism of HepaRG-CAR cells, indicating that the effect of DMSO is not directly or indirectly mediated by CAR. Moreover, DMSO culture of HepaRG-CAR cells induces the formation of a large subset of a morphologically distinct undifferentiated hepatocyte-like cell type. Culture of HepaRG-CAR without DMSO results in increased drug metabolic capacity beyond that of normal HepaRG cells cultured with DMSO while maintaining the improved hepatic synthetic capabilities associated with DMSO-free culture of HepaRG cells (Hoekstra et al., 2011).

Our HepaRG-CAR cells are generated by lentiviral transduction. Lentiviral treatment of cells carries a risk of insertional mutagenesis because of potential insertion of the lentiviral payload into active genes. Although we obtained a heterogeneous population after antibiotic

TABLE 2

TC50 × C_{max} values of amiodarone, acetaminophen, indomethacin, and dextromethorphan

Major metabolic routes, human C_{max} values and TC50 values of amiodarone, acetaminophen, indomethacin, and dextromethorphan in HepaRG and HepaRG-CAR cells.

Drug	Metabolism	C_{max} (μM)	HepaRG TC50 (×C_{max})	HepaRG-CAR TC50 (×C_{max})
Amiodarone	CYP3A4, CYP2C8	0.807	47.5	60.0*
Acetaminophen	Glucuronidation, sulfation > CYP2E1, CYP3A4	139	122	216**
Indomethacin	CYP2C9	5.59	191	216
Dextromethorphan	CYP2D6 > CYP3A4	0.021	ND	ND

ND, not determined.
*$P < 0.05$; **$P < 0.001$ versus HepaRG.

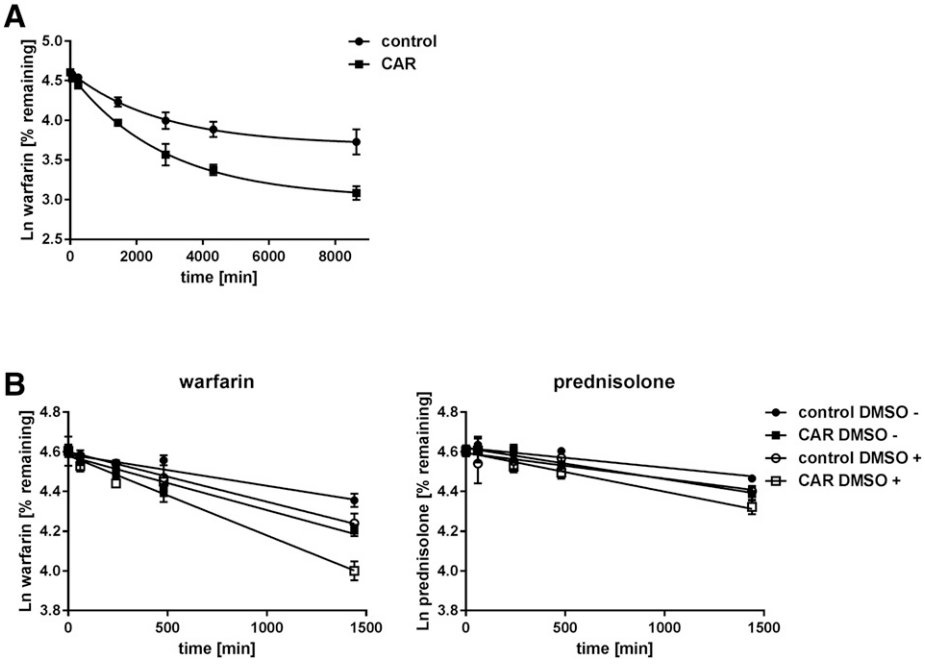

Fig. 6. The effect of CAR overexpression and DMSO treatment on the clearance of warfarin, theophylline, and prednisolone of HepaRG cells. (A) Levels of warfarin in culture medium of DMSO-cultured HepaRG ± CAR cells over 6 days. One experiment of three separate samples for each point. (B) Levels of warfarin and prednisolone in culture medium of HepaRG ± CAR cells ober 1 day. Figures in (B) represent the average of three or four independent experiments of three separate samples in each group.

selection, we cannot exclude that a subpopulation of HepaRG-CAR cells has an altered differentiation profile owing to random integration of the CAR cassette (e.g., differentiation toward inter-island hepatocytes after culture with DMSO). This seems unlikely, however, because inter-island hepatocytes are also present to a lesser degree in normal HepaRG cell cultures. In addition, CAR plays an important role in inducing or maintaining the differentiation of other hepatocyte cell lines. For example, overexpression of *CAR* in human ESC induces differentiation toward a hepatocyte phenotype, whereas knockdown of *CAR* attenuates this differentiation (Chen et al., 2013). *CAR*, together with nuclear receptors *HNF1, FXR*, and *PXR*, was recently identified to be essential in maintaining high liver gene expression, as their target genes are highly expressed in fresh PHH, but lose expression rapidly (Godoy et al., 2015). HepaRG *CAR*-knockout cells have increased expression of genes related to cell proliferation and tumorigenesis, whereas those related to energy and drug metabolism are decreased (Li et al., 2015). Interestingly, this is in contrast to studies in mice where liver tumorigenesis is induced by diethylnitrosamine upon sustained activation of *CAR* by phenobarbital (Wei et al., 2000; Yamamoto et al., 2004). HepaRG-CAR cells show a tendency to increased differentiation toward hepatocytes based on the increased albumin production and activity of non-CAR target CYPs; however, ammonia elimination is unaltered, as well as the transcript levels of hepatic genes unrelated to CAR. In the light of our data and the data presented herein, CAR seems to be a player in hepatocyte differentiation, but it probably also requires interaction with sufficiently expressed other nuclear receptors and hepatic factors for a sustained and robust differentiation.

Despite increased levels of CAR in HepaRG-CAR, induction rates are mostly unaltered, enabling studies on drug induction in HepaRG-CAR as in normal HepaRG cells. Only with CYP3A4 did we observe a lack of induction in HepaRG-CAR cells by rifampicin, which may indicate that CYP3A4 had reached maximal expression. Although mRNA levels of nontarget P450s in HepaRG-CAR cells are not increased, their activities do show improvement through a not-yet revealed mechanism. CAR upregulates the expression of *POR* in HepaRG-CAR cells. Previously, it was reported that the indirect CAR activator phenobarbital induces expression of *POR* in mice and PHH (Maglich et al., 2002; Ueda et al., 2002). POR is a membrane-bound protein in the endoplasmic reticulum that feeds P450 activity by mediating electron transfer to P450s (Gutierrez et al., 2003). A correlation between *POR* mRNA expression and activity of CYP2A6, CYP2B6, CYP2C9, and CYP2E1 in human liver microsomes has been reported (Wortham et al., 2007). Therefore, the upregulation of POR may contribute to the increase in activity of non-CAR target P450s in HepaRG-CAR.

In contrast to CAR overexpression, DMSO culture causes increased mRNA levels of P450s and POR without a concomitant increase in P450 activity. This suggests that P450 activity is limited by lack of induction by DMSO of another factor necessary for activity. One possibility for indirect activation might be via induction of the rate limiting and tightly controlled heme synthesis gene *ALAS1* (May et al., 1995; Ponka, 1999).

TABLE 3

CL_{int} of prednisolone, warfarin, and theophylline in HepaRG cells

Major metabolic routes and CL_{int} (μl/min/10^6 cells) ± S.D. of prednisolone, warfarin, and theophylline in HepaRG and HepaRG-CAR cells cultured without or with DMSO. No reliable clearance of theophylline could be detected. Prednisolone, warfarin, and theophylline were determined respectively in three, four, and one independent experiment with three separate samples each.

Drug	Metabolism	HepaRG (DMSO−)	HepaRG-CAR (DMSO-)	HepaRG (DMSO+)	HepaRG-CAR (DMSO+)
Prednisolone	CYP3A4	0.12 ± 0.08	0.12 ± 0.06	0.20 ± 0.13	0.33 ± 0.11*. ***.****
Warfarin	CYP2C9 > CYP3A4	0.32 ± 0.17	0.39 ± 0.14	0.28 ± 0.08	0.59 ± 0.14 *. **.*****
Theophylline	CYP1A2	ND	ND	ND	ND

ND, not determined.
*$P < 0.001$ versus HepaRG (DMSO-); **$P < 0.01$; ***$P < 0.001$ versus HepaRG-CAR (DMSO-); ****$P < 0.05$; *****$P < 0.001$ versus HepaRG (DMSO+).

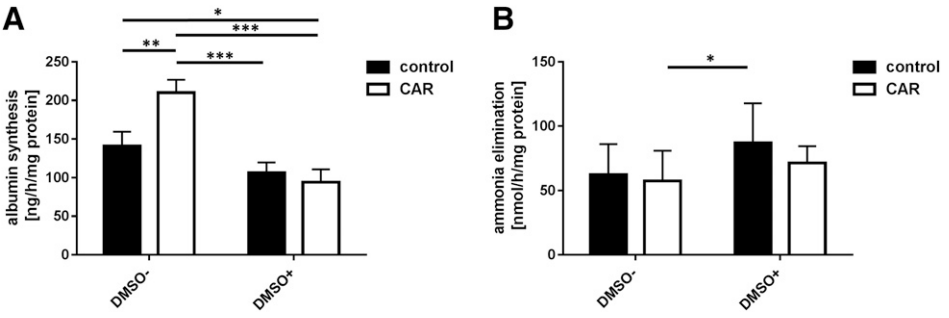

Fig. 7. The effect of CAR overexpression and DMSO treatment on albumin synthesis and ammonia elimination in HepaRG cells. (A) Albumin synthesis in HepaRG ± CAR cells. One experiment of three separate samples in each group (B) Ammonia elimination in HepaRG ± CAR cells. Average of four independent experiments of at least three separate samples in each group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Heme is incorporated into P450s and is therefore critical for functional expression (reviewed in (Correia et al., 2011)). CAR can induce expression of *ALAS1* in PHH and HepG2 cells (Maglich et al., 2003; Podvinec et al., 2004). We show that overexpression of *CAR* increases *ALAS1* expression in HepaRG cells, possibly resulting in increased heme production and subsequent incorporation into CYP enzymes, rendering functional P450 activity.

CAR overexpression does not alter bilirubin glucuronidation when tested in culture medium of intact cells, but it greatly increases bilirubin glucuronidation in cell homogenates. Basolateral uptake of unconjugated bilirubin occurs via OATP1B1 and OATP1B3 (Cui et al., 2001; Briz et al., 2003), although it has been suggested that passive diffusion is also possible (Zucker et al., 1999). We and others have shown that OATP1B1 expression is low in HepaRG cells (~5% of human liver), whereas OATP1B3 is almost absent (nondetectable in our hands) (Kotani et al., 2012), and therefore import of bilirubin may have been a limiting factor in our experimental setup. Although we did not investigate the export of conjugated bilirubin via MRP2, it seems unlikely that this is a limiting factor considering its high expression and functional activity in HepaRG cells (Bachour-El Azzi et al., 2015). Unfortunately, we are unable to detect bilirubin or its conjugates in cell lysates after incubation with intact cells, making it difficult to reach a decisive conclusion concerning bilirubin transport. In addition, we cannot exclude an effect of a limiting concentration of intracellular UDGPA cofactor in the metabolism of bilirubin by UGT1A1.

We find that HepaRG-CAR cells are less sensitive to the toxicity of amiodarone and acetaminophen. Amiodarone is metabolized mainly by CYP2C8 and CYP3A4 to its major pharmacologically active metabolite mono-*N*-desethylamiodarone (Ohyama et al., 2000). Overexpression or induction of CYP3A4 in HepG2 cells causes amiodarone-induced toxicity (Zahno et al., 2011); however, amiodarone and its metabolites are also extensively metabolized before excretion into bile, as observed by the large amount of different metabolites in human bile (Deng et al., 2011). Because of increased P450 activity in HepaRG-CAR cells, it is expected that MDEA concentrations are temporarily higher, but increased metabolism of MDEA via other CYPs or UGTs may account for the reduction in toxicity that we observed. Acetaminophen is metabolized mainly by glucuronidation and sulfation, although at high concentrations, the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) will be formed by oxidation via CYP2E1 (Mazaleuskaya et al., 2015). At therapeutic concentrations of acetaminophen, NAPQI is detoxified by conjugation to glutathione. At toxic concentrations, glucuronidation by UGT1A1 and UGT1A9 and oxidation by CYP2E1 are increased, but a limited supply of glutathione may prevent further elimination of NAPQI (Mazaleuskaya et al., 2015). Since CYP2E1 activity is increased in HepaRG-CAR cells, but toxicity of acetaminophen is decreased, glucuronidation, sulfation, or gluthathionylation of acetaminophen and its metabolites is likely increased in HepaRG-CAR cells. Thus, elevated glucuronidation in HepaRG-CAR cells, as we show by bilirubin glucuronidation via UGT1A1, may contribute to reduced

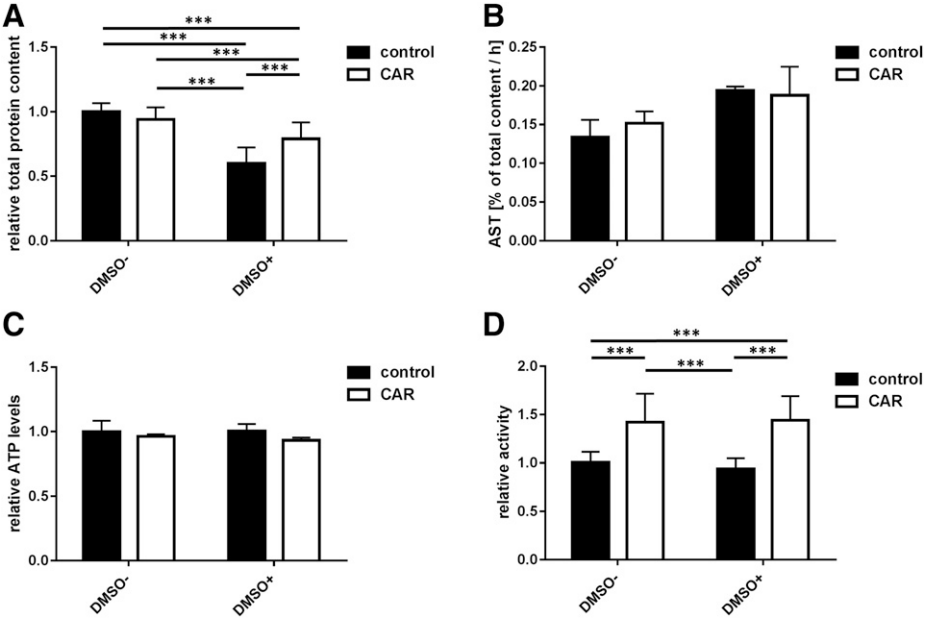

Fig. 8. The effect of CAR overexpression and DMSO treatment on total protein, viability, and cellular integrity of HepaRG cells. (A) Total protein of HepaRG ± CAR cells (average of 11 independent experiments of at least three separate samples in each group). (B) Cellular AST leakage of HepaRG ± CAR cells (one experiment with three separate samples in each group). (C) Cellular ATP levels of HepaRG ± CAR cells (one experiment with three separate samples in each group). (D) Cellular WST-1 activity corrected for protein of HepaRG ± CAR cells (average of four independent experiments of at least three separate samples in each group). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

toxicity of amiodarone and acetaminophen. This effect makes HepaRG-CAR cells useful for studies on the role of CAR during hepatotoxicity, for example, in combination with HepaRG-CAR knockout cells.

DMSO-cultured HepaRG-CAR cells exhibit a robust increase in clearance of two low-turnover compounds compared with parental HepaRG cells: prednisolone and warfarin. This indicates that HepaRG-CAR cells could be used as a predictive model for the clearance of slowly metabolized compounds. In vitro prediction of clearance and metabolites of small molecules that are slowly metabolized remains a challenge (reviewed in Hutzler et al., 2015), despite some promising developments, for example, the hepatocyte relay method and the Hepatopac and Hμrel hepatocyte coculture models (Chan et al., 2013; Di et al., 2013; Bonn et al., 2016). Testing with unmodified HepaRG cells tends to underpredict the in vivo clearance of several low-clearance compounds (Bonn et al., 2016). Increased clearance of prednisolone and warfarin in HepaRG-CAR indicates that drug metabolic enzyme conversion, and not cellular uptake, is a limiting factor for their clearance. It would be interesting to determine whether other drugs are similarly affected. A more extensive study of clearance of slowly metabolizable compounds by HepaRG-CAR cells is needed for a proper validation.

Culture with DMSO reduces the synthetic capacity of HepaRG cells, with or without overexpression of CAR (this article and Hoekstra et al., 2011); however, CAR overexpression in HepaRG cells without DMSO resulted in activities of UGT1A1 and most tested P450s equal or surpassing those of HepaRG cells cultured with DMSO and therefore may be a promising model for simultaneous studies on hepatocyte synthetic and drug metabolic functions. The additional increase in expression and activities of phase 1 and phase 2 drug metabolic enzymes by DMSO treatment of HepaRG-CAR cells could enable more sensitive studies of low-clearance compounds, (rare) metabolite formation, and detoxification mechanisms in HepaRG cells without the need to exogenously overexpress multiple drug metabolic enzymes. Taken together, overexpression of CAR in HepaRG cells provides a model for further elucidating the role of CAR and its target genes in hepatic differentiation and metabolism of endogenous and exogenous compounds.

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Authorship Contributions

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Conducted experiments: Van der Mark, De Waart, Shevchenko.

Performed data analysis: Van der Mark, De Waart, Shevchenko, Oude-Elferink, Chamuleau, Hoekstra.

Wrote or contributed to the writing of manuscript: Van der Mark, De Waart, Shevchenko, Oude-Elferink, Chamuleau, Hoekstra.

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