Comparative Analysis and Functional Characterization of HC-AFW1 Hepatocarcinoma Cells: Cytochrome P450 Expression and Induction by Nuclear Receptor Agonists

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ABSTRACT

Enzymatic conversion of most xenobiotic compounds is accomplished by hepatocytes in the liver, which are also an important target for the manifestation of the toxic effects of foreign compounds. Most cell lines derived from hepatocytes lack important toxifying or detoxifying enzymes or are defective in signaling pathways that regulate expression and activity of these enzymes. On the other hand, the use of primary human hepatocytes is complicated by scarce availability of cells and high interdonor variability. Thus, analyses of drug metabolism and hepatotoxicity in vitro are a difficult task. The cell line HC-AFW1 was isolated from a pediatric hepatocellular carcinoma and so far has been used for tumorigenicity and chemotherapy resistance studies. Here, a comprehensive characterization of xenobiotic metabolism in HC-AFW1 cells is presented along with studies on the functionality of the most important transcriptional regulators of drug-metabolizing enzymes. Results from HC-AFW1 cells were compared with commercially available HepaRG cells and cultured primary human hepatocytes. Data show that the nuclear receptors and xenosensors AHR (aryl hydrocarbon receptor), CAR (constitutive androstane receptor), PXR (pregnane-X-receptor), NRF2 [nuclear factor (erythroid-derived 2)–like 2], and PPARα (peroxisome proliferator–activated receptor α) are functional in HC-AFW1 cells, comparable to HepaRG and primary cells. HC-AFW1 cells possess considerable activities of different cytochrome P450 enzymes, which, however, are lower than corresponding enzyme activities in HepaRG cells or primary hepatocytes. In summary, HC-AFW1 are a new promising tool for studying the mechanisms of the regulation of drug metabolism in human liver cells in vitro.

Introduction

The majority of exogenous substances are metabolized in the liver, where hepatocytes possess the highest levels of most drug- and xenobiotic-metabolizing enzymes to catalyze detoxification or bioactivation. In pharmacology and toxicology it is therefore essential to understand the hepatic metabolism of substances as well as the molecular mechanisms of the regulation of drug-metabolizing enzymes. Studying these phenomena in human cells in vitro is challenging owing to the limited availability and high interdonor variability of primary human hepatocytes (PHH). Primary liver cells also tend to lose hepatocyte-specific gene expression profiles when cultivated outside their physiologic environment. Large efforts have been made to overcome these drawbacks, leading to the introduction of highly sophisticated three-dimensional cultivation techniques or artificial bioreactor models. Recent advances in hepatocyte cultivation have been comprehensively reviewed (Godoy et al., 2013).

Immortalized cell lines are not prone to shortcomings such as availability and missing standardization procedures. However, most cell lines derived from liver tumors lack the expression of many important drug-metabolizing enzymes and are insensitive to the regulation of these enzymes by xenobiotics. This is primarily attributable to low expression and/or activity of important liver-specific transcription factors. This includes, for example, the different hepatocyte nuclear factors and the group of drug-sensing xenoreceptors, e.g., the aryl hydrocarbon receptor (AHR), the constitutive androstane receptor (CAR), the pregnane-X-receptor (PXR), and nuclear factor (erythroid-derived 2)–like 2 (NRF2), a cellular sensor for oxidative stress. Currently, the human hepatocarcinoma cell line HepaRG represents a widely used standard for in vitro hepatocyte models, since this cell line exhibits well preserved activity of many drug-metabolizing enzymes.

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enzymes together with the functionality of many important mechanisms that regulate their expression (Guillouzo et al., 2007; Antherieu et al., 2012; Klein et al., 2015). Major disadvantages of this commercially available cell line, however, are the high costs and the complex, time-consuming differentiation procedure.

The HC-AFW1 cell line was derived from a pediatric hepatocellular carcinoma of a 4-year-old boy a few years ago (Armeanu-Ebinger et al., 2012) and since then has been explored as a novel human in vitro model for hepatocellular carcinoma. So far, the cell line has been used in studies focused mainly on tumorigenicity, xenograft models, and cytostatic tumor cell treatment (Armeanu-Ebinger et al., 2012; Hoh et al., 2013; Chiu et al., 2014; Tao et al., 2014). Of note, HC-AFW1 cells possess active β-catenin (Armeanu-Ebinger et al., 2012; Chiu et al., 2014; Tao et al., 2014), a transcription factor that is critically involved in the regulation of drug-metabolizing enzymes in mouse liver (Braeuning et al., 2009; 2011; Giera et al., 2010; Schreiber et al., 2011; Ganzenberg et al., 2013; Gougelet et al., 2014), human hepatoblastoma (Schmidt et al., 2011), PHH (Gerbal-Chaloin et al., 2014), and HepaRG (Thomas et al., 2015). Nevertheless, no information about the drug-metabolizing properties of this cell line is available from the literature.

In the present study, we analyzed a broad spectrum of drug metabolism-related functions and underlying regulatory mechanisms in HC-AFW1 cells to characterize this cell line with respect to its applicability as a new model for the study of human drug metabolism in vitro.

Materials and Methods

Cell Culture. Human hepatocarcinoma cells from line HC-AFW1 (Armeanu-Ebinger et al., 2012) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) and antibiotics (all purchased from Invitrogen/ThermoFisher Scientific, Karlsruhe, Germany). In some experiments, cells were incubated with different concentrations of FBS, with adult bovine serum, horse serum, or goat serum (all purchased from Invitrogen/ThermoFisher Scientific, Regensburg, PHH were isolated from partial liver resections by collagenase digestion with replacement of medium every 2 days. All cells were maintained in HepaRG differentiation medium for the duration of the experiments with replication of medium every 2 days. All cells were maintained at 37°C and 5% CO₂ in a humidified atmosphere throughout the experiment.

Transfections and Luciferase Reporter Analyses. Cells were transfected with the firefly luciferase reporter constructs detailed below, using standard methods as recently described (Braeuning and Vetter, 2012). The plasmid pRL-CMV, encoding Renilla luciferase under the control of a constitutively active viral promotor, was cotransfected for normalization. Twenty-four hours after seeding of the cells, 800 ng of plasmid DNA (750 ng of the respective firefly luciferase reporter plasmid, 50 ng pRL-CMV) were transfected per cavity of a 24-well plate using Lipofectamine 2000 (Invitrogen/ThermoFisher Scientific).

Firefly luciferase reporter plasmids used in the study were: a pT81Luc-based 3xDRE-driven reporter for luciferase expression under the control of three dioxin response elements responsive to activation by the AHR (Schreiber et al., 2006), and a pGL3-based reporter system driven by approximately 2000 bp of the human CYP2B6 promoter responsive to activation by CAR (Zukunft et al., 2005). Transfection experiments with the respective empty vectors were conducted as controls. Cells were incubated with inducers for 24 or 48 hours prior to lysis with 1× Passive Lysis Buffer (Promega, Mannheim, Germany) and luciferase activity determination as previously described (Braeuning and Vetter, 2012).

Assessment of P450 Metabolic Activities. Cytochrome P450 enzyme activities were determined in HC-AFW1, PHH, and HepaRG cell culture supernatants using a liquid chromatography–tandem mass spectrometry–based substrate cocktail assay, as previously described (Feidt et al., 2010). The P450 substrate mix was added to cell cultures after 21 or 45 hours of incubation with the enzyme inducers as detailed above. The following substrates were used: 50 μM phenacetin (CYP1A2), 25 μM bupropion (CYP2B6), 5 μM amodiaquine (CYP2C8), 100 μM tolbutamide (CYP2C9), 5 μM propafenone (CYP2D6), 100 μM atorvastatin (CYP3A4). Aliquots of the supernatant were taken after 3 hours of incubation at 37°C. Metabolite formation was normalized to cellular protein content.

Gene Expression Analysis. Total RNA was isolated from HC-AFW1, PHH, and HepaRG cells using the RNaseasy Mini Kit, including on-column genomic DNA digestion with RNase-Free DNase Set (Qiagen, Hilden, Germany). RNA was reverse transcribed to cDNA with TaqMan Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany) using random hexamers as primers listed in Supplemental Table 1 and the FastStart DNA MasterPLUS SYBR Green Mix (Roche). Genotype status of the remaining enzymes as determined by AmpliChip CYP450 Test (Roche) was performed using the AmpliChip CYP450 Test (Roche). Genotype status of the remaining enzymes was determined using cycle sequencing for the following alleles: CYP2C9*2 (430C>T, rs1799853), CYP2C9*3 (1075A>C, rs1057910), CYP3A4*1C (698A>G, rs776746), CYP3A4*22 (15289C>T, rs35599367 C>T), CYP2B6*6 (1515G>T, rs3745274), cytochrome oxidase–proactivator–activated receptor α (PPARA) rs4253728 G>A. Allele designation of the selected P450 polymorphism and their functional
effects are according to the Human Cytochrome P450 (CYP) Allele Nomenclature Database (www.cypalleles.ki.se). The PPARα rs4253728 polymorphism has been described as a determinant of CYP3A4 activity (Klein et al., 2012). Primers for PCR and cycle sequencing are available on request.

Statistical Analyses. Statistical significance was determined by performing Student’s t test analysis comparing solvent control and treatment groups using GraphPad Prism 5.0.4 software (GraphPad Software, Inc., La Jolla, CA). The asterisks indicate statistical significance at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***) Correction for multiple testing was performed using Benjamini-Hochberg correction.

Results

Comparative Analysis of Basal Gene Expression and Metabolic Capacity of HC-AFW1, HepaRG, and Primary Human Hepatocytes. To examine the applicability of the cell line HC-AFW1 as an in vitro model for human liver gene expression and metabolism studies, the mRNA expression and metabolic activity profiles of these cells were compared with that of the frequently used commercial cell line HepaRG as well as PHH. Expression levels of a panel of genes encoding important drug-metabolizing enzymes, transporters, and nuclear receptors/transcription factors were determined using quantitative PCR (Fig. 1). In the absence of xenobiotic inducers of drug metabolism, PHH were generally superior to both cell lines with respect to the mRNA expression of most phase I (Fig. 1A) and phase II/III (Fig. 1B) enzymes. Especially mRNA expression of the different cytochrome P450 (P450) isoforms was almost consistently lower in the two immortalized cell lines with relative expression levels of mostly <20% of the primary cells. HepaRG cells expressed higher levels of many P450 family 2 and 3 members, compared with HC-AFW1, while levels of CYP1A2, CYP2A6, and CYP3A7 were similar in both cell lines (Fig. 1A). With respect to important nuclear receptors and transcriptional regulators of hepatic drug metabolism, both cell lines displayed moderately higher expression at the mRNA level compared with PHH. Conversely, a slight downregulation of CAR and PXR mRNAs was observed in HepaRG and HC-AFW1 (Fig. 1C). In line with the findings at the mRNA expression level, the metabolism of model substrates by six different P450 enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2D6, and CYP3A4) differed substantially between PHH and the two cell lines, with the latter displaying a consistently lower level of model substrate metabolism (Fig. 1D). HepaRG possessed a substantially higher metabolizing capacity of CYP1A2, CYP2C8, CYP2C9,
and CYP3A4 than did HC-AFW1, while CYP2D6 activity was extremely low in both cell lines (Fig. 1D).

To elucidate the basis of the apparent lack of CYP2D6 enzymatic activity in HC-AFW1, genotyping of the CYP2D6 gene locus was performed along with a genetic analysis of other polymorphic gene loci. A heterozygous gene deletion of CYP2D6 (*1/*5) indicating decreased enzyme activity was found (Table 1). Alleles corresponding to normal enzyme activities were observed for both CYP2C9 and CYP2C19. For CYP2B6, the heterozygous *1/*6 allele status corresponded to a partially decreased protein expression and activity (Desta et al., 2007). The most frequent CYP3A5 allele in Europeans, CYP3A5*3, was detected in a homozygous state, which predicts a severely decreased enzyme expression and activity. Genetics for CYP3A4 (Elenes et al., 2013) and PPARα rs4253728 (Klein et al., 2012) suggested a normal phenotype.

Comparative Analysis of Drug-Induced Gene Expression of HC-AFW1, HepaRG, and Primary Hepatocytes. Next, the different cell types, i.e., HC-AFW1, HepaRG, and PHH, were exposed to a selection of nuclear receptors agonists known for their ability to induce drug metabolism in the liver in vivo: BNF (target receptors: AHR and NRF2), CITCO (CAR), PB (CAR and PXR), RIF (PXR), tBHQ (NRF2 and AHR), TCDD (AHR), and WY14,643 (PPARα). Visualizations of these data are presented in Fig. 2.

When treated with the indirect CAR inducer PB, the expected pattern of upregulation of known target P450s from families 2 and 3 was clearly visible in PHH after 24 hours (Fig. 2A) and 48 hours (Fig. 2B).
corresponding patterns observed for HC-AFW1 and HepaRG were similar following 24 hours of treatment with the inducer, indicating functional signaling through the CAR pathway in both cell lines (Fig. 2A). Interestingly, the induction persisted in PHH, as documented by the continued upregulation of CAR target genes after 48 hours. By contrast, the response of most genes to CAR activation in HC-AFW1 and HepaRG, with the exception of the model target P450s showing the most pronounced degree of regulation, was limited to 24 hours (Fig. 2B). The response to another CAR agonist, CITCO, included mainly known CAR target genes but also an unexpected but robust and consistent induction of the AHR target CYP1A1 (Fig. 2). In the case of CITCO, the response of PHH seemed to be more pronounced, compared with the immortalized cell lines. Activation of PXR by RIF resulted in a marked induction of CYP3A genes in all three cell types after 24 and 48 hours, while most other genes analyzed were less and/or not consistently affected (Fig. 2). Again, the responses of PHH, HepaRG, and HC-AFW1 were comparable. A very strong induction of P450s from family 1A was seen following activation of the AHR by TCDD or BNF, as expected. The responses of HC-AFW1, HepaRG, and PHH were similar at the 24-hour time point, with some differences between the cellular induction patterns between the individual cell types (Fig. 2). The transcriptional responses to PPARα activation by WY14,643 and to the combined NRF2/AHR activation by tBHQ were again similar in PHH and both cell lines. In summary, transcriptional profiling of the three cell lines indicated that signaling through the respective nuclear receptors and the induction of their target genes is similar. The induction of important P450 isoforms in HC-AFW1 was verified by real-time reverse transcription-polymerase chain reaction analysis using independent samples (Fig. 3A). Transcriptional induction of genes downstream of xenobiotic-activated nuclear receptors in HC-AFW1 cells was further verified by the use of luciferase reporter assays driven by activated AHR (3xDRE reporter system) and CAR (CYP2B6 promoter reporter system). As depicted in Fig. 3B, the reporter genes were activated by xenobiotic treatment of the cells. No induction of luciferase activities from the corresponding empty control vectors was observed (Fig. 3B). The transcriptional changes were well reflected by concomitant alterations in the metabolic capacity of all three cell types (Fig. 4). Induction of CYP3A4 activity was observed already after 24 hours in PHH, whereas the response seemed to be delayed in HC-AFW1 and HepaRG, where more pronounced effects

![Fig. 4. Comparison of xenobiotic-inducible P450 metabolic activity in HC-AFW1, HepaRG, and PHH. Cells were treated with the inducers PB, RIF, TCDD, BNF, CITCO, WY14,643, or tBHQ as indicated in Materials and Methods and incubated for 21 hours (A) or 45 hours (B) prior to 3 hours of incubation with a P450 substrate mix. P450 activities were determined by LC-MS. Data are given as the mean fold regulation of three independent experiments relative to the respective untreated cells (set to 1 separately for each time point). Relative expression levels were color-coded. Statistical significance is indicated by asterisks. Please note that the apparent reduction of CYP2D6 activity in HepaRG cells might be artifactual owing to extremely low values near the detection limit of the method. Abbreviation: n.a., not analyzed.](https://dmd.aspetjournals.org/content/23/12/1785)
were observed at the 48 hours time point. Continuous analyses over several passages demonstrated the robustness of the system and reproducibility of transcriptional P450 induction in HC-AFW1 cells, as demonstrated by the data presented in Table 2.

**Modulation of Cell Culture Conditions for HC-AFW1.** Variations in culture conditions, such as serum content, confluence, and/or the presence of DMSO, are frequently implicated in the modulation of drug metabolism in liver-derived cells in vitro. Therefore, the effects of serum concentration, serum origin, confluence, and incubation with DMSO were analyzed in HC-AFW1 cells. As shown in Fig. 5, incubation of cells with a wide range of FBS concentrations did not markedly influence the expression of most P450s (Fig. 5A). Similarly, the use of different sera, i.e., FBS, adult bovine serum, horse serum, and goat serum, did not result in profound differences with regard to P450 mRNA expression (Fig. 5B), nor did the modulation of cell density (Fig. 5C). DMSO, effective in the maintenance of differentiation of primary rat hepatocytes (Cable and Isom, 1997) and important for the 2-week differentiation protocol of HepaRG (Guillouzo et al., 2007), also did not exert pronounced effects on P450 expression in HC-AFW1, regardless of its concentration in the culture medium and the duration of exposure, except for CYP2B6, which was strongly upregulated in the presence of DMSO (Fig. 5D).

**Discussion**

The present study provides a comprehensive overview of the activity and regulation of enzymes related to drug metabolism in HC-AFW1 human pediatric hepatocarcinoma cells and a comparison with two well-established hepatic in vitro systems, namely PHH and HepaRG cells. In summary, the present data suggest that the relevant major mechanisms of induction of hepatic drug metabolism, i.e., signaling through AHR, CAR, PXR, and NRF2, are functional in the cell line HC-AFW1. This is an important feature because signaling through CAR and PXR is defective in most standard hepatoma cell lines. Activation of the various nuclear receptors triggers a transcriptional response similar to primary cells, which has been demonstrated by a selection of representative model inducers of hepatic drug metabolism–related gene expression, followed by transcriptional profiling and metabolic analyses. Moreover, the observed fold induction levels in the cell line HC-AFW1 are not only qualitatively but also quantitatively comparable to the fold induction of the respective genes observed in equally treated PHH or HepaRG, with respect to the majority of target genes analyzed. This renders HC-AFW1 cells a promising model for the study of drug metabolism–related gene regulation by nuclear receptors in vitro. The HC-AFW1 cell line is especially suited for mechanistic studies involving transfection experiments, since this cell line can be easily...

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**TABLE 2**

Reproducibility of CYP3A4 induction in HC-AFW1 cells

Mean of two replicates per time point is presented relative to controls (set to 1) for four independent experiments with consecutive passages of the cells. Cells were incubated with the inducers for 48 hours.

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>CYP3A4 mRNA</th>
<th>CYP3A4 Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PB RIF TCDD</td>
<td>PB RIF TCDD</td>
</tr>
<tr>
<td>1</td>
<td>12.44 4.39 0.30</td>
<td>6.81 4.37 0.35</td>
</tr>
<tr>
<td>2</td>
<td>10.88 3.16 0.07</td>
<td>6.75 1.96 0.22</td>
</tr>
<tr>
<td>3</td>
<td>6.86 3.42 0.40</td>
<td>6.71 2.62 0.25</td>
</tr>
<tr>
<td>4</td>
<td>8.57 3.46 0.60</td>
<td>7.01 3.28 0.37</td>
</tr>
</tbody>
</table>

**Fig. 5.** Influence of variations in cell culture conditions on P450 mRNA expression in HC-AFW1 cells. (A) Cultivation of HC-AFW1 in the presence of different amounts of FBS for 1, 2, or 3 days. (B) Cultivation of HC-AFW1 in the presence of different sera. Abbreviations: ABS, adult bovine serum; HS, horse serum; GS, goat serum. (C) Comparison of P450 expression at 10, 75, and 100% confluence. (D) Cultivation of HC-AFW1 in the presence of different concentrations of DMSO for 1, 2, 3, or 14 days. Data are given as the mean fold regulation of three independent experiments (each in triplicate determinations) relative to cells grown in the presence of 1% FBS (A), 10% FBS (B), 10% confluent cell cultures (C), or DMSO-free cultures (D); the controls were set to 1 separately for each time point. Relative expression levels were color-coded. Statistical significance is indicated by asterisks.

Abbreviation: n.a., not analyzed.
transfected with plasmids at high efficiency. The latter constitutes a rather difficult task in HepaRG and PHH, especially when dealing with larger expression plasmid constructs.

Another advantage of HC-AFW1 cells is the ease of handling; they do not require a complex differentiation protocol as is mandatory for HepaRG. Moreover, their use is not hampered by scarce availability or donor-dependent variations, common problems in the case of PHiH. In contrast to the HepaRG hepatocarcinoma cell line (Guillouzo et al., 2007) and to primary rat hepatocytes (Cable and Isom, 1997), we observed that DMSO treatment did not remarkably influence the expression of most P450s in HC-AFW1 cells nor induced a general differentiation process in this cell line, a phenomenon that would be reflected by expression changes in a broad range of P450s. This view is supported by the fact that DMSO treatment did not influence the expression of hepatectomy differentiation-related genes such as albumin or the hepatocyte nuclear factors (own unpublished data). The rather constant P450 expression data obtained from HC-AFW1 cells following variation of serum type, serum content, and confluence show that the basal P450 expression of HC-AFW1 is rather tolerant to alterations in experimental conditions, again underlining the suitability of HC-AFW1 as a widely applicable in vitro model for hepatocytes. Cultivation for up to 14 days was well tolerated by the cells, thus allowing for the analysis of long-term effects in HC-AFW1 cell cultures.

With regard to the metabolic activity of all P450 isoforms investigated, however, HC-AFW1 cells are inferior to PHiH, which displayed the highest P450 activities in our study. HepaRG cells also were metabolically less competent than PHiH, yet displayed higher P450 activities compared with HC-AFW1 for most enzymes tested. An exception was CYP3A4, where both cell lines, HepaRG and HC-AFW1, displayed the highest P450 activities in our study. HepaRG cells also were metabolically more competent than PHH, which displayed the longest-term stability of all cell lines investigated.

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Authorship Contributions

Participated in research design: Braeuning, Thomas, Schwarz.

Conducted experiments: Thomas, Hofmann, Vetter, Zeller, Petzuch, Johanning, Schrot.

Contributed new reagents or analytic tools: Weiss.

Performed data analysis: Braeuning, Thomas, Johanning, Schrot, Schwarz.

Wrote or contributed to the writing of the manuscript: Braeuning, Thomas, Zanger, Johanning, Schwarz.

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