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**Comparative analysis and functional characterization of HC-AFW1 hepatocarcinoma  
cells: CYP expression and induction by nuclear receptor agonists**

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DMD # 64667

## Running title page

**Running title:** induction of drug metabolism in HC-AFW1 cells

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**List of abbreviations:** ABS, adult bovine serum; AHR, aryl hydrocarbon receptor; BNF,  $\beta$ -naphthoflavone CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)-oxime; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; DRE, dioxin response element; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, goat serum; HCC, hepatocellular carcinoma; HS, horse serum; NRF2, nuclear factor (erythroid-derived 2)-like 2, PB, phenobarbital; PXR, pregnane-X-receptor; PHH, primary human hepatocytes; PPAR, peroxisome proliferator-activated receptor; RIF, rifampicin; tBHQ, tert.-butylhydroquinone; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin

## 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 which 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 inter-donor 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 to commercially available HepaRG cells and to 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 PPAR $\alpha$  (peroxisome proliferator-activated receptor  $\alpha$ ) are functional in HC-AFW1 cells, comparable to HepaRG and primary cells. HC-AFW1 cells possess considerable activities of different cytochrome P450 (CYP) 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 their detoxification or bio-activation. 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 due to the limited availability and high inter-donor variability of primary human hepatocytes (PHH). Primary liver cells also tend to lose hepatocyte-specific gene expression profiles when cultivated outside their physiological environment. Large efforts have been made in order 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, to a major part, due 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 xeno-receptors, 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 together with the functionality of many important mechanisms which regulate their expression (Antherieu et al., 2012; Guillouzo et al., 2007; 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 has been derived from a pediatric hepatocellular carcinoma (HCC) of a 4 year old boy a few years ago (Armeanu-Ebinger et al., 2012) and since then been explored as a novel human *in vitro* model for HCC. So far, the cell line has been used in studies which were mainly focused on tumorigenicity, xenograft models and cytostatic tumor cell treatment (Armeanu-Ebinger et al., 2012; Chiu et al., 2014; Hoh et al., 2013; Tao et al., 2014). Of note, HC-AFW1 cells possess active  $\beta$ -catenin (Armeanu-Ebinger et al., 2012; Chiu et al., 2014; Tao et al., 2014), a transcription factor which is critically involved in the regulation of drug-metabolizing enzymes in mouse liver (Braeuning et al., 2011; Braeuning et al., 2009; Ganzenberg et al., 2013; Giera et al., 2010; Gougelet et al., 2014; Schreiber et al., 2011), human hepatoblastoma (Schmidt et al., 2011), and PHH (Gerbál-Chaloin et al., 2014; 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 D-MEM medium containing 10% fetal bovine serum (FBS) and antibiotics (all purchased from Invitrogen, Karlsruhe, Germany). In some experiments, cells were incubated with different concentrations of FBS, with adult bovine serum (ABS), horse serum (HS), or goat serum (GS; all purchased from Invitrogen). Cells were treated with the following inducers of xenobiotic metabolism: 3mM phenobarbital (PB; Sigma, Taufkirchen, Germany; dissolved in H<sub>2</sub>O), 10nM 2,3,7,8-tetrachlorodibenzop-[p]-dioxin (TCDD; Ökometric, Bayreuth, Germany; dissolved in DMSO), 10μM Rifampicin (RIF; Sigma; dissolved in DMSO), 5μM 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)-oxime (CITCO; Enzo Life Sciences, Loerrach, Germany; dissolved in DMSO), 50μM β-naphthoflavone (BNF; Sigma; dissolved in DMSO), 30μM tert.-butylhydroquinone (tBHQ; Sigma; dissolved in DMSO), or 100μM pirinixic acid (WY14,643; gift from Dr. C. Gembardt, Ludwigshafen, Germany; dissolved in DMSO) at the indicated time points for 24h or 48h prior to cell harvest. Cells were seeded at a density of 40,000 cells/cm<sup>2</sup> on 6-well (for RNA expression, protein expression and metabolism studies) or on 24-well plates (for reporter gene assays). Absence of treatment-related toxicity was checked by means of the resazurin reduction and neutral red uptake assays as described (Braeuning et al., 2012).

Detailed description of culturing primary human hepatocytes (PHH) and HepaRG cells can be found elsewhere (Klein et al., 2015). Briefly, with written informed consent from donors (2 male, 1 female) and approvals by the local ethics committee in Regensburg, PHH were isolated from partial liver resections by collagenase digest as described previously (Godoy et al., 2013). Isolated cells were plated at a density of 4 x 10<sup>5</sup> viable cells/well onto BioCoat Collagen I Cellware 12-well culture plates (Becton Dickinson, Bedford, USA) in William's E Medium, supplemented with 10% FBS, 2mM L-glutamine, 32mU/ml human insulin, 1mM sodium pyruvate, 1X non-essential amino acids, 15mM Hepes, 0.8μg/ml hydrocortisone and

antibiotics. After 24h, cells were equilibrated for another 24h in cultivation medium, containing William's E Medium, supplemented with 10% FBS, 2mM L-glutamine, 32mU/ml human insulin, 0.1% DMSO, 0.1  $\mu$ M dexamethasone, and antibiotics.

HepaRG cells (batch HPR101007) were obtained from Biopredic International (Rennes, France) and expanded according to the provider's instructions. The cells were cultivated for the first 14d in HepaRG growth medium based on William's E Medium, supplemented with 10% FBS, 2mM L-glutamine, 32mU/ml human insulin, 20 $\mu$ g/ml hydrocortisone, and antibiotics. Medium was exchanged every two to three days. Cells were passaged and transferred to MULTIWELL 24-well plates (Becton Dickinson, Bedford, USA) at a density of 50,000 cells/well and cultivated for two more weeks. Medium was replaced by HepaRG growth medium containing 1% DMSO for two days. Starting from the third day, cells were cultivated in HepaRG growth medium containing 2% DMSO (HepaRG differentiation medium) for another 12 days. At that stage, HepaRG cells reached a differentiated hepatocyte-like morphology and showed liver-specific functions. The cells were further maintained in HepaRG differentiation medium for the duration of the experiments with replacement of medium every two days. All cells were maintained at 37°C and 5% CO<sub>2</sub> 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 promoter, was co-transfected for normalization. 24h after seeding of the cells, 800ng of plasmid DNA (750ng of the respective Firefly luciferase reporter plasmid, 50ng pRL-CMV) were transfected per cavity of a 24-well plate using Lipofectamine 2000 (Invitrogen). Firefly luciferase reporter plasmids used in the study were: a pT81luc-based 3xTRE-driven reporter for luciferase expression under the control of 3 dioxin response elements (DREs) 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 24h or 48h prior to lysis with 1x Passive Lysis Buffer (Promega, Mannheim, Germany) and luciferase activity determination as previously described (Braeuning and Vetter, 2012).

### **Assessment of CYP 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 CYP substrate mix was added to cell cultures after 21h or 45h of incubation with the enzyme inducers as detailed above. The following substrates were used: 50µM phenacetin (CYP1A2), 25µM bupropion (CYP2B6), 5µM amodiaquine (CYP2C8), 100µM tolbutamide (CYP2C9); 5µM propafenone (CYP2D6), 100µM atorvastatin (CYP3A4). Aliquots of the supernatant were taken after 3h 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 RNeasy 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). Quantification of expression of 86 genes was performed using Fluidigm's BioMark HD high-throughput quantitative chip platform (Fluidigm Corporation, San Francisco, USA), following the manufacturer's instructions. The mRNA expression levels were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. Relative gene expression changes were calculated using the delta delta Ct ( $\Delta\Delta Ct$ )-method (Livak and Schmittgen, 2001). Additional gene expression analyses (data in Figs. 3, 5 and 6) were performed using a capillary-based



LightCycler system (Roche) and 18s rRNA as a housekeeping gene. Here, reverse transcription was carried out by avian myeloblastosis virus reverse transcriptase (Promega) using oligo(dT)<sub>20</sub> and random (dN)<sub>6</sub> primers. Relative quantification of target gene expression was performed using the primers listed in Supplemental Table 1 and the FastStart DNA Master<sup>PLUS</sup> SYBR Green I kit (Roche). The BLAST algorithm and the NCBI data base were used to ensure specificity of the primers. PCR products were verified by melting point analyses and gel electrophoresis.

### Genotyping

HC-AFW1 genomic DNA was isolated from 10<sup>6</sup> freshly harvested cells (ZR Genomic DNA, Zymo Research) and genotyped for common polymorphisms known to affect phase I enzyme activities. CYP2D6 and CYP2C19 genotypes and corresponding phenotypes were determined using 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), CYP3A5\*3 (6986A>G, rs776746), CYP3A4\*22 (15289C>T, rs35599367 C>T), CYP2B6\*6 (515G>T, rs3745274), PPARa rs4253728 G>A. Allele designation of the selected CYP polymorphism and their functional effects are according to the Human Cytochrome P450 (CYP) Allele Nomenclature Database ([www.cypalleles.ki.se](http://www.cypalleles.ki.se)). The PPARa 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 t-test analysis comparing solvent control and treatment groups using GraphPad Prism 5.0.4 software (GraphPad Software, Inc., La Jolla, USA). The asterisks indicate statistical significance at p<0.05 (\*) or p<0.01 (\*\*). 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 to that of the frequently used commercial cell line HepaRG as well as to primary human hepatocytes (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 (CYP) 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 CYP family 2 and 3 members, as compared to 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, as compared to PHH. Conversely, a slight down-regulation 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 6 different CYP 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 as 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*, respectively. 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* (Elens et al., 2013) and *PPARα* rs4253728 (Klein et al., 2012) suggested a normal phenotype.

### **Comparative analysis of drug-induced gene expression 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 Figure 2.

When treated with the indirect CAR inducer PB, the expected pattern of up-regulation of known target CYPs from families 2 and 3 was clearly visible in PHH after 24h (Fig.2A) and 48h (Fig.2B). The corresponding patterns observed for HC-AFW1 and HepaRG were similar following 24h 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 up-regulation of CAR target genes after 48h. By contrast, the response of most genes to CAR activation in HC-AFW1 and HepaRG, with the exception of the model target CYPs showing the most pronounced degree of regulation, was rather limited to 24h (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, as compared to the immortalized cell lines. Activation of PXR by RIF resulted in

a marked induction of CYP3A genes in all three cell types after 24h and 48h, 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 CYPs 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 24h time point, with some differences between the cellular induction patterns between the individual cell types (Fig.2). The transcriptional responses to PPAR $\alpha$  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 CYP isoforms in HC-AFW1 was verified by real-time RT-PCR 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 (3xTRE reporter system) and CAR (*CYP2B6* promoter reporter system). As depicted in Figure 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 24h in PHH, whereas the response seemed to be delayed in HC-AFW1 and HepaRG, where more pronounced effects were observed at the 48h time point. Continuous analyses over several passages demonstrated the robustness of the system and reproducibility of transcriptional CYP 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

DMD # 64667

with DMSO were analyzed in HC-AFW1 cells. As shown in Figure 5, incubation of cells with a wide range of FBS concentrations did not markedly influence the expression of most CYPs (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 CYP 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-weeks differentiation protocol of HepaRG (Guillouzo et al., 2007), did also not exert pronounced effects on CYP expression in HC-AFW1, regardless of its concentration in the culture medium and the duration of exposure, except for CYP2B6, which was strongly up-regulated 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 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 which does not require a complex differentiation protocol as mandatory for HepaRG. Moreover, their use is not hampered by scarce availability or donor-dependent variations, which are common problems in the case of PHH. 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 CYPs in HC-AFW1 cells nor induced a general differentiation process in this cell line, a phenomenon which would be reflected by expression changes in a broad range of CYPs. This view is supported by the fact that DMSO treatment did not influence the expression of hepatocyte differentiation-related

genes such as albumin or the hepatocyte nuclear factors (own unpublished data). The rather constant CYP expression data obtained from HC-AFW1 cells following variation of serum type, serum content, and confluence show that the basal CYP 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 CYP isoforms investigated, however, HC-AFW1 cells are inferior to PHH, which displayed the highest CYP activities in our study. HepaRG cells also were metabolically less competent than PHH, yet displayed higher CYP activities compared to HC-AFW1 for most enzymes tested. An exception was CYP2D6, where both cell lines, HepaRG and HC-AFW1, displayed a poor metabolizer phenotype. Given the fact that the majority of drugs is converted by CYPs which are more abundant and active in PHH or HepaRG (Zanger and Schwab, 2013), primary cells or HepaRG cells still remain the model systems of choice for studying metabolite formation *in vitro*.

In summary, we have characterized drug-metabolizing activity and transcriptional regulation of a broad spectrum of drug-metabolizing enzymes in a novel human hepatocarcinoma cell line, HC-AFW1. These cells show less metabolic activity of CYP enzymes compared to PHH or HepaRG. However, they constitute a suitable *in vitro* system for analyses of the mechanisms of regulation of hepatic drug metabolism, due to the presence of functional nuclear receptor signaling and enzyme induction and the absence of disadvantages of HepaRG or PHH, such as complex cultivation procedures or inter-donor variability.

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## **Authorship contributions:**

Participated in research design: Braeuning, Thomas, Schwarz

Conducted experiments: Thomas, Hofmann, Vetter, Zeller, Petzuch, Johänning, Schroth

Contributed new reagents or analytic tools: Weiss

Performed data analysis: Braeuning, Thomas, Johänning, Schroth, Schwarz

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



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DMD # 64667

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DMD # 64667

## Footnotes

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## Figure legends

**Figure 1.** Comparison of basal gene expression and CYP activity in HC-AFW1, HepaRG, and PHH. Fluidigm PCR arrays were used to determine the expression of mRNAs related to phase I **(A)** or phase II/III **(B)** of drug metabolism at 48h after seeding. **(C)** Expression of nuclear receptors and transcription factors involved in the regulation of hepatic drug metabolism. **(D)** Differences in metabolic activity of different CYPs in HC-AFW1 and PHH at 48h or 72h after seeding. HepaRG were cultivated according to the manufacturer's protocol. Data are given as fold regulation as the mean of 3 independent experiments relative to PHH (set to 1; in **(D)** all data are given relative to PHH at 72h after seeding). Relative expression levels were color-coded according to the provided color scheme. Abbreviation: n.a., not analyzed. Statistical significance is indicated by asterisks.

**Figure 2.** Comparison of xenobiotic-inducible gene expression in HC-AFW1, HepaRG, and PHH. Cells were treated with the inducers PB, RIF, TCDD, BNF, CITCO, WA14,643, or tBHQ as indicated in the Materials and Methods section and incubated for 24h **(A)** or 48h **(B)** prior to RNA isolation and gene expression analysis. Data are given as the mean fold regulation of 3 independent experiments relative to the respective untreated cells (set to 1 separately for each time point). Relative expression levels were color-coded according to the provided color scheme. Statistical significance is indicated by asterisks.

**Figure 3.** Validation of CYP induction in HC-AFW1. Cells were incubated with the inducers PB, RIF, TCDD, BNF, CITCO, WA14,643, or tBHQ as indicated in the Materials and Methods section and incubated for 24h or 48h. **(A)** Validation of mRNA induction by real-time RT-PCR using independent biological replicates, i.e. other than those used for the analyses shown in Figure 1 and Figure 2. Data are given as the mean fold regulation of 3 independent experiments (each in triplicate determinations) relative to untreated HC-AFW1 cells (set to 1). **(B)** Induction of AHR (3xTRE)- and CAR (*CYP2B6* promoter)-dependent luciferase reporter

activity. Data are given as the mean fold regulation of at least 5 independent experiments (each in triplicate determinations) relative to untreated HC-AFW1 cells (set to 1 separately for each time point). Relative expression levels were color-coded according to the provided color scheme. Statistical significance is indicated by asterisks.

**Figure 4.** Comparison of xenobiotic-inducible CYP metabolic activity in HC-AFW1, HepaRG, and PHH. Cells were treated with the inducers PB, RIF, TCDD, BNF, CITCO, WA14,643, or tBHQ as indicated in the Materials and Methods section and incubated for 21h **(A)** or 45h **(B)** prior to 3h of incubation with a CYP substrate mix. CYP activities were determined by LC-MS. Data are given as the mean fold regulation of 3 independent experiments relative to the respective untreated cells (set to 1 separately for each time point). Relative expression levels were color-coded according to the provided color scheme. Statistical significance is indicated by asterisks. Please note that the apparent reduction of CYP2D6 activity in HepaRG cells might be artifactual due to extremely low values near the detection limit of the method.

**Figure 5.** Influence of variations in cell culture conditions on CYP 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 CYP expression at 10%, 75%, and 100% confluency. **(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 3 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)**, respectively; the controls were set to 1 separately for each time point. Relative expression levels were color-coded according to the provided color scheme. Statistical significance is indicated by asterisks.



DMD # 64667

## Tables

**Table 1.** Genotyping of HC-AFW1 cells.

Gene	Tested allele/polymorphism	Genotype	Phenotype/function
<i>CYP2B6</i>	*6	*1/*6	Decreased
<i>CYP2C9</i>	*2, *3	*1/*1	Normal
<i>CYP2C19</i>	*2, *3	*1/*1	Normal
<i>CYP2D6</i>	29 common alleles	*1/*5	Decreased
<i>CYP3A4</i>	*22	*1/*1	Normal
<i>CYP3A5</i>	*3	*3/*3	Severely decreased
<i>PPARa</i>	rs4253728	G/G	Normal

DMD # 64667

**Table 2.** Reproducibility of CYP3A4 induction in HC-AFW1 cells.

experiment no.	CYP3A4 mRNA			CYP3A4 enzyme activity		
	PB	RIF	TCDD	PB	RIF	TCDD
1	12.44	4.39	0.30	6.81	4.37	0.35
2	10.88	3.16	0.07	6.75	1.96	0.22
3	6.86	3.42	0.40	6.71	2.62	0.25
4	8.57	3.46	0.60	7.01	3.28	0.37

Mean of 2 replicates per time point is presented relative to controls (set to 1) for 4 independent experiments with consecutive passages of the cells. Cells were incubated with the inducers for 48h.

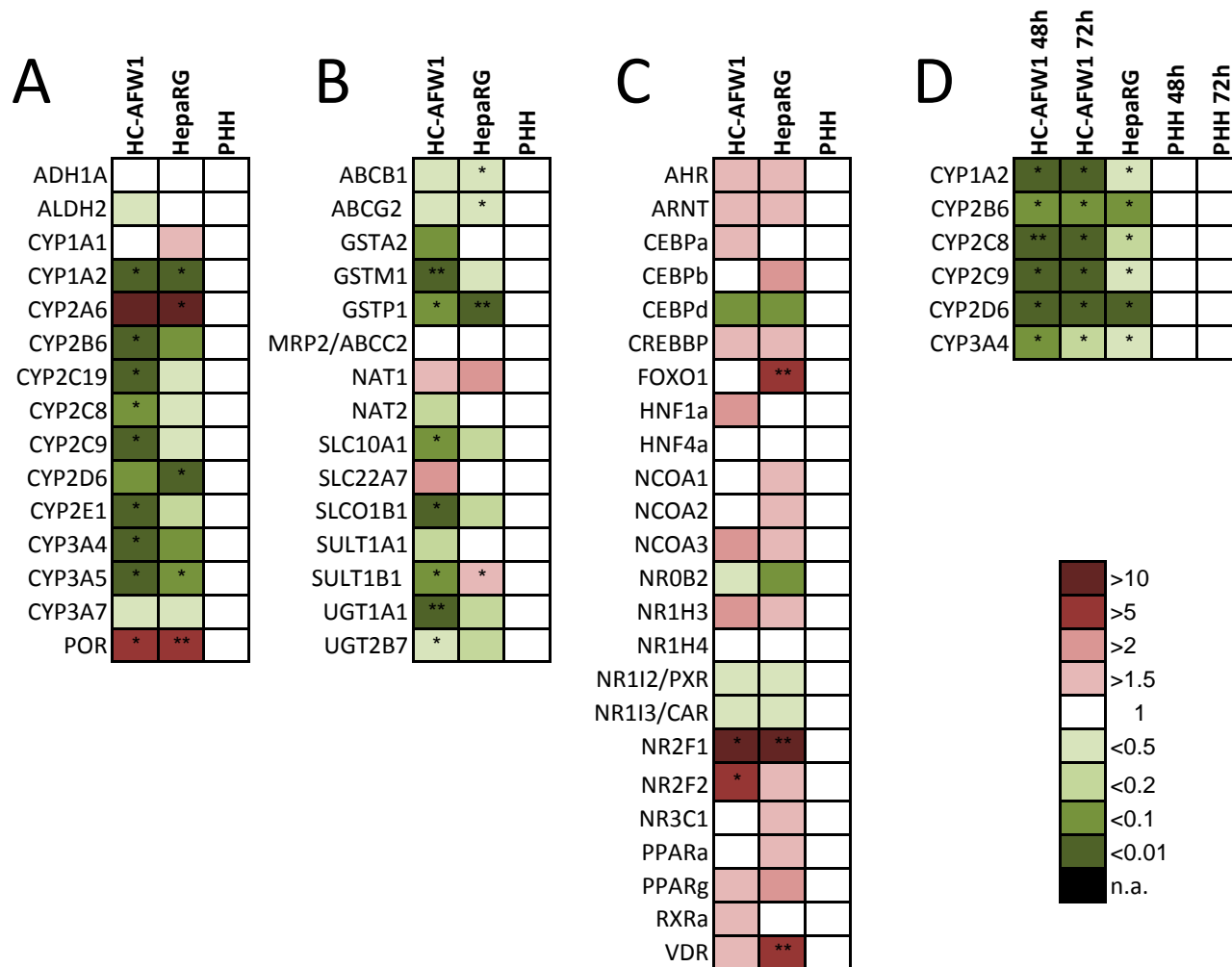


Figure 1

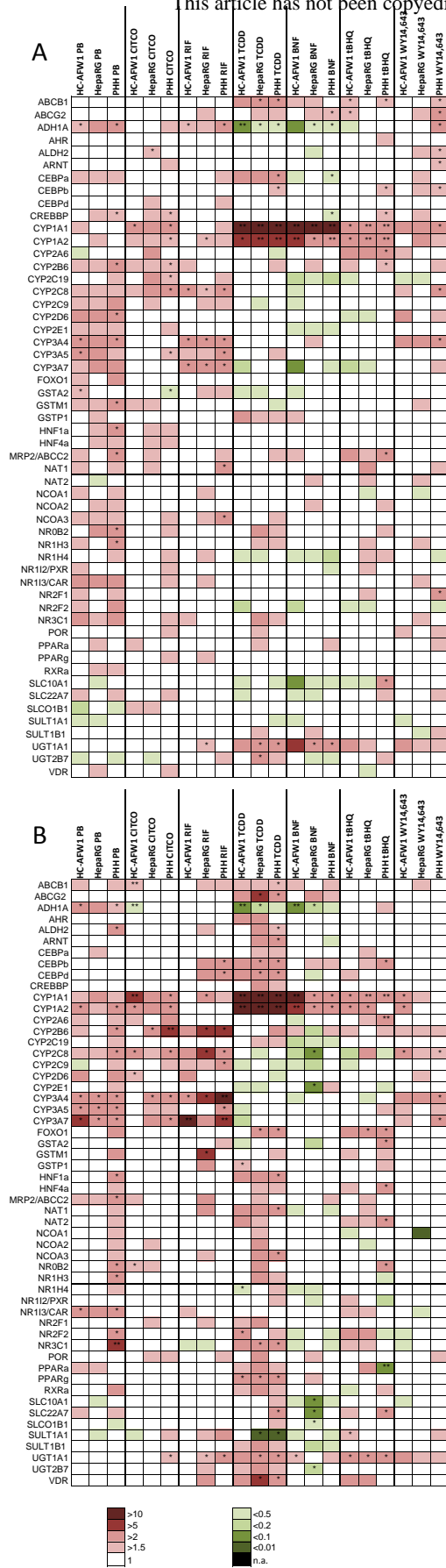


Figure 2

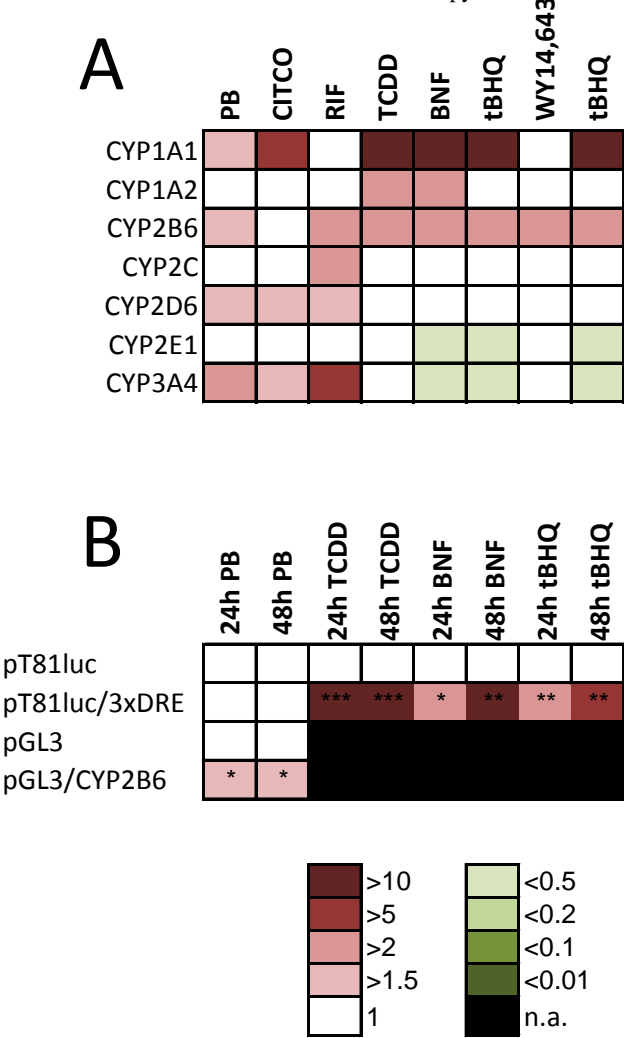


Figure 3

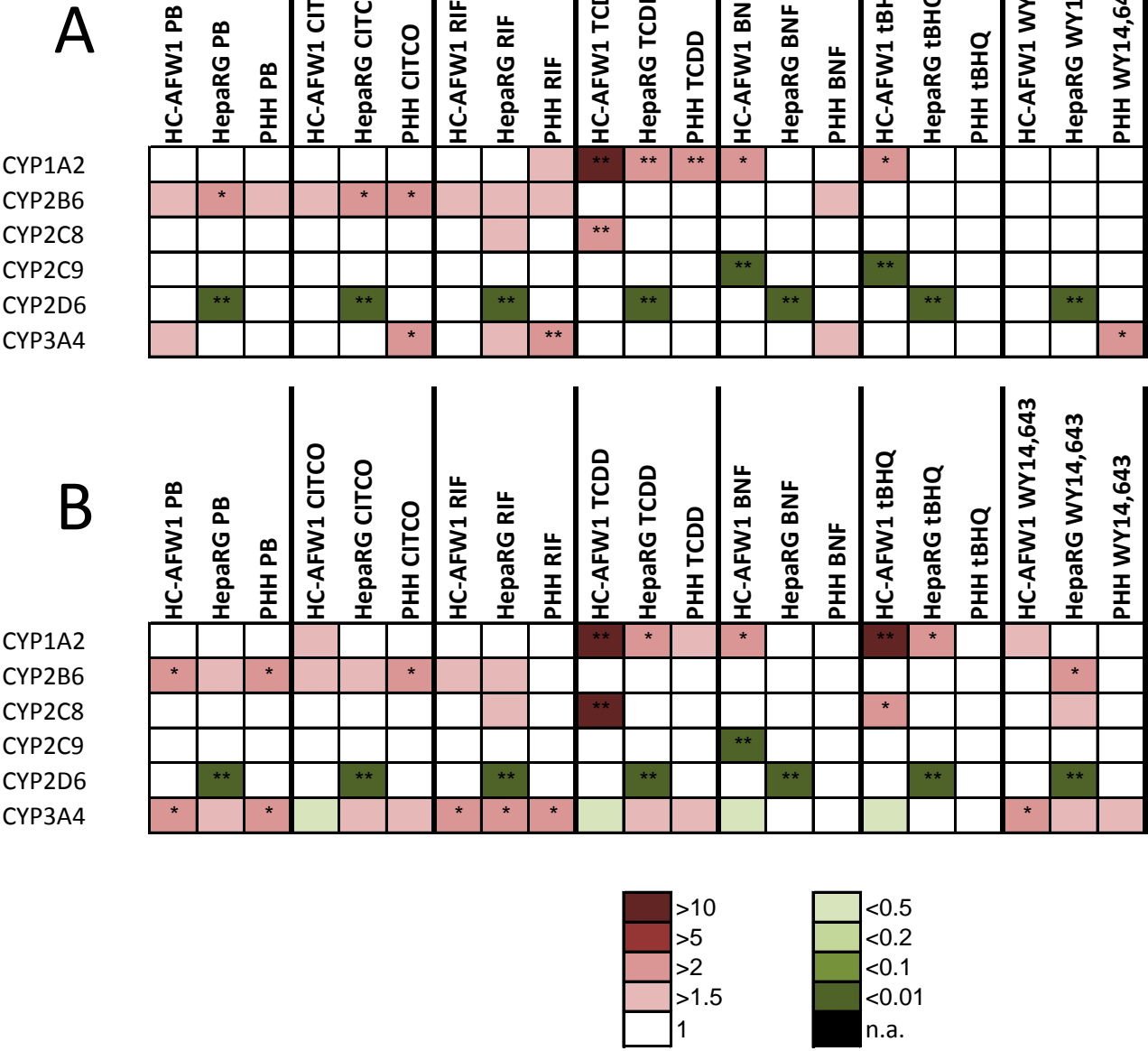


Figure 4

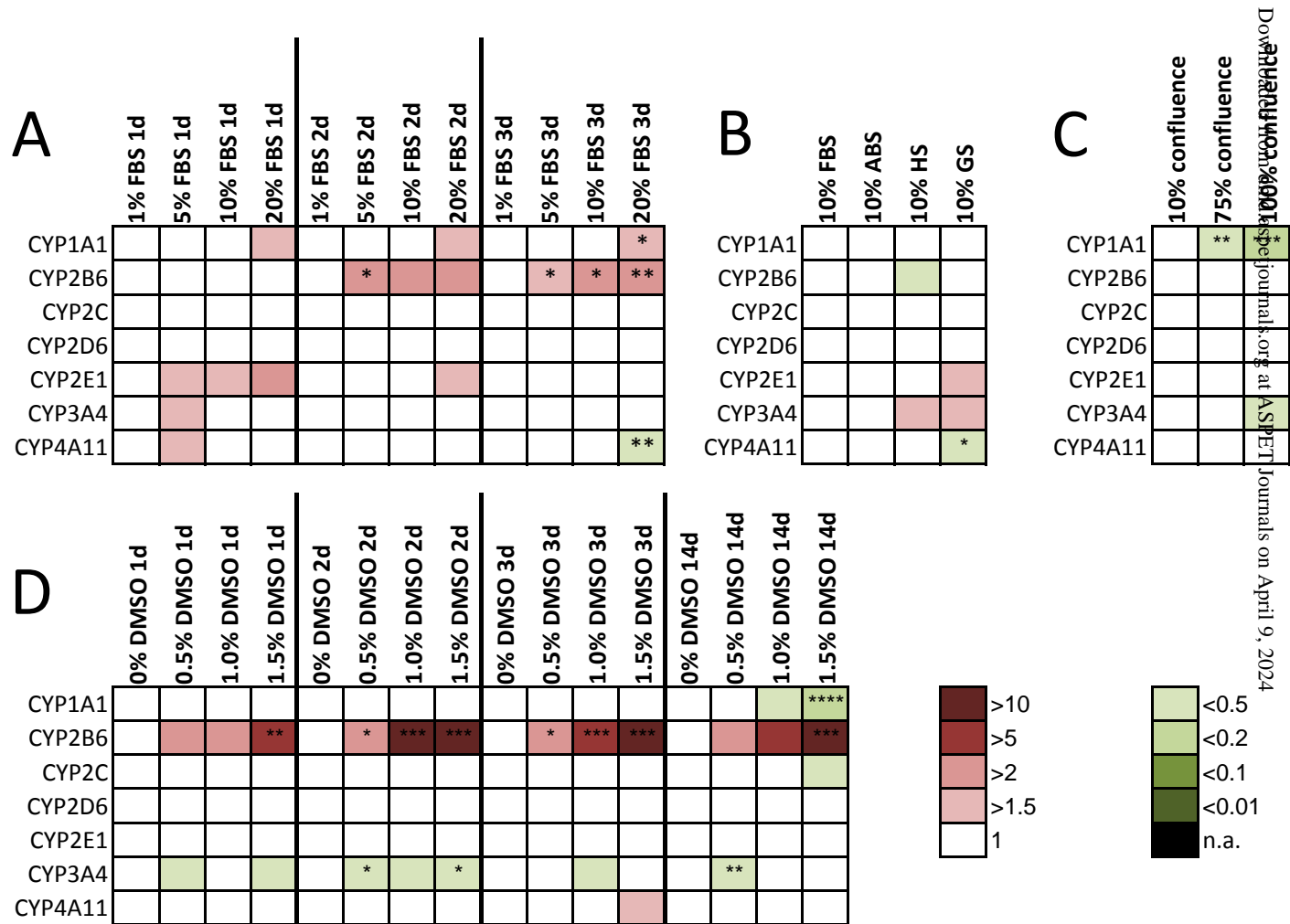


Figure 5

## SUPPLEMENTAL MATERIAL

### **Comparative analysis and functional characterization of HC-AFW1 hepatocarcinoma cells: CYP expression and induction by nuclear receptor agonists**

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Journal: Drug metabolism and disposition

Supplemental Table 1. Primer sequences for real-time RT-PCR.

<b>Gene</b>	<b>Forward (5'-3')</b>	<b>Reverse (5'-3')</b>
CYP1A1	GTCTTTCTCTTCCTGGCTATC	TACCTGTTGTCTCTGGAGGGT
CYP1A2	GGGCACTTCGACCCTTACAAT	TGTGTCAAATCCTGCTCCAAA
CYP2B6	GGTTCAGCGCCACCCTAACA	CTTTCCCATCCCGAAGTCCC
CYP2C	CTCCCTCCTGGCCCCAG	GGAGCACAGCTCAGGATGAA
CYP2D6	GACATCCCGTGACATCGAAGTAC	CAGCAGGGAGGTGAAGAAGAGG
CYP2E1	CAACGTCATAGCCGACATCCT	CTTCTCCATTTCCACGAGCAG
CYP3A4	GATGAAAGAAAGTCGCCTCGA	GGTGGTGCCTTATTGGGTAAA
CYP4A11	TTGCCCAAAGGTATCATGGTC	TTTCCCGATGCAGTTCCTTGAT
18s rRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT